LYMPHOCYTE DYNAMICS DURING ACUTE EXERCISE IN TYPE ONE DIABETES

By

Michelle Curran

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Abstract

Type 1 diabetes (T1D) is a T cell mediated autoimmune disease that targets and destroys insulin-secreting pancreatic beta cells. Exercise induced lymphocytosis facilitates immune surveillance and regulation. This study aimed to explore whether exercise could modulate the T1D autoimmune process through regulation of lymphocyte mobilisation and trafficking. N=12 T1D (mean age 33.2yrs, predicted VO_2 max 32.2 mL/(kg·min), BMI 25.3Kg/m2) and N=12 control (mean age 29.4yrs, predicted VO_2 max 38.5mL(kg.min), BMI 23.7Kg/m2) male participants completed 30-minutes cycling at both 40% (moderate) and 80% predicted VO_2 max (vigorous) on separate days and in a fasting state. Peripheral blood was collected at baseline, immediately post-exercise, and 1 hour post-exercise. Intensity dependent lymphocytosis was observed in both groups; specifically, $CD8^+$ effector memory $CD45RA^+$ (EMRA) T cells and $CD56^{dim}CD16^{bright}$ NK cells. However, the percentage increase of these subsets was blunted in T1D following vigorous intensity exercise. The reasons for this may include differences in blood glucose levels, adrenaline responses, and sequestration of these subsets in the pancreas of people with T1D. Lymphocyte trafficking was also regulated by vigorous exercise in T1D. Transmigration of lymphocytes was significantly suppressed during the recovery period together with a reduction in adhesion molecules on T cells following vigorous exercise.

“I am not what happened to me,
I am what I choose to become”

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ABBREVIATIONS

aBM-MNC	autologous Bone Marrow Mononuclear Cell
ADH	Adherence
AIRE	Autoimmune Regulator
ANCOVA	Analysis of Covariance
APC	Antigen Presenting Cell
ATG	Anti-thymocyte Globulin
Bcl-2	B-cell lymphoma -2
Bcl-x	B-cell lymphoma x
BMI	Body Mass Index
BP	Blood Pressure
BSA	Bovine Serum Albumin
CA^{2+}	Calcium
CCL	Chemokine Ligand
CCR	Chemokine Receptor
CD	Cluster of Differentiation
CD62L	CD62 Ligand
cDC	conventional Dendritic Cell
CDH-15	Cadherin 15
CMV	Cytomegalovirus
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T lymphocyte associated-4
CVB4	Coxsackievirus B4
CXCR	C-X-C chemokine receptor
DC	Dendritic Cell
ddH_2O	Double-distilled water
DNA	Deoxyribonucleic acid
DPPIV	Dipeptidyl peptidase IV
ED	Early Differentiated
EDTA	Ethylenediaminetetraacetic Acid
ELD	Early-like Differentiated
EM	Effector Memory
EMRA	Effector Memory re-expressing CD45RA
EXTOD	Exercise for Type One Diabetes
FBS	Fetal Bovine Serum
FcR	Fc Receptor
FCRL3	Fc receptor-like protein 3
FCS	Fetal Calf Serum
Fgl2	Fibrinogen-like protein 2
FlT3l	FmS-like tyrosine kinase 3 ligand

FMO	Fluorescence Minus One
FoxP3	Forkhead winged helix transcription factor
FSC-A	Forward Scatter-Area
FSC-H	Forward Scatter-Height
GAD	Glutamic Acid Decarboxylase
GADA	GAD autoantibodies
GCP	Good Clinical Practice
GLP-1	Glucose-dependent insulinotropic Peptide 1
GLUT-2	Glucose transporter 2
GWAS	Genome Wide Association Studies
HbA1c	Haemoglobin A1c
HDBEC	Human Dermal Blood Endothelial Cells
HELIOS	Zinc finger protein encoded by the IKZF2 gene
HLA	Human Leukocyte Antigen
HLA-DR	Human Leukocyte Antigen – antigen D Related
HR	Heart Rate
HSC	Hematopoietic Stem Cells
HSPC	Hematopoietic Stem and Progenitor Cells
IA-2	Protein tyrosine phosphatase-like protein
IAA	Inuslin autoantibodies
ICAM-1	Intercellular Adhesion Molecule 1
ID	Intermediately Differentiated
IDF	Internation Diabetes Federaion
IFN-γ	Interferon gamma
IL	Interluekin
IL-1RA	IL-1R agonist
IL2RA	IL-2 Receptor alpha
IL-6R	IL-6 Receptor
iNOS	inducible Nitric Oxide
IPAQ	Internation Physical Activity Questionnaire
KIR	Killer cell Immunoglobulin-like Receptors
KLRG1	Killer cell Lectin-like Receptor subfamily G member 1
KO	Knock-out
L3T4	Mouse CD4
LFA-1	Lymphocyte Function-associated Antigen 1
LN	Lymph Node
mAb	Monoclonal antibody
Mac-1	Macrophage-1 antigen
MadCAM-1	Mucosal vascular addressin Cell Adhesion Molecule 1
MAPK	Mitogen-Activated Protein Kinase
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Haemoglobin
MDA	malondialdehyde
MFI	Median Fluorescent Intensity

MHC	Major Histocompatibility Complex
MIN6	Mouse Insulinoma cell line
MPV	Mean Platelet Volume
MS	Multiple Sclerosis
MSC	mesenchymal stem cells
mTEC	medullary Thymic Epithelial Cells
mTReg	memory T-Regulatory
NA	Naïve
naTReg	Naïve T-Regulatory
NFAT	Nuclear Factor of Activated T cells
NFκB	Nuclear Factor-kappaB
NHS	National Health Service
NIHR	National Institute for Health Research
NK	Natural Killer
NKG2D	Natural-Killer Group 2-member D
NO	Nitric Oxide
NOD	Non-Obese Diabetic
SCID	Severe combined immunodeficiency
NOR	Non-Obese diabetes Resistant
O2	Oxygen
PBL	Peripheral Blood Lymphocyte
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Saline
PCT	plateletcrit
PD-1	programmed cell death protein 1
pDC	plasmacytoid Dendritic Cell
PDW	Platelet Distribution Width
PEPITEM	Peptide Inhibitor of Trans-Endothelial Migration
PGD2	Prostaglandin D2
PIS	Participant Information Sheet
PLN	Pancreatic Lymph Node
PNAd	Peripheral Lymph Node Addressins
PolyIC	Polyinosinic-polycytidylic acid
pTReg	periphery T-Regulatory cell
RBC	Red Blood Cell
RDW	Red Cell Distribution Width
REC	Research Ethics Committee
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPE	Rate of Perceived Exertion
RPM	Revolutions Per Minute
RV	Rotavirus
S1PR	Sphingosine-1-Phosphate Receptor
SIP	Sphingosine-1-Phosphate

sIL2RA	soluble IL2RA
sIL-6R	soluble IL-6R
SNP	Single Nucleotide Polymorphism
SOCS	Suppressor of cytokine signalling
SSC-A	Side Scatter- Area
SSC-H	Side Scatter- Height
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TCR	T cell Receptor
TEM	Transendothelial Migration
Tg	Transgenic
TGF-β	Transforming growth factor beta
Th1	Type 1 helper
Th2	Type 2 helper
Th17	Type 17 helper
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TLR	Toll-like Receptor
TReg	T-Regulatory
TReg17	regulatory T-helper type 17
tTReg	thymus T-Regulatory cell
T_{SCM}	Stem cell like memory T cells
UC-MSC	Umbilical Cord blood Mesenchymal Stem Cells
UHBFT	University Hospitals Birmingham NHS Foundation Trust
VCAM-1	Vascular cell adhesion protein 1
VLA-4	Very Late Antigen-4
VNTR	Variable Number Tandem Repeat
WBC	White Blood Cell
WHO	World Health Organization
WTCRF	Wellcome Trust Clinical Research Facility
ZnT8	Zinc Transporter
ZnT8A	Zinc Transporter Autoantibodies

CHAPTER 1. GENERAL INTRODUCTION

1.1 Type 1 Diabetes

Type 1 Diabetes (T1D) is a chronic inflammatory disease characterised by autoimmune destruction of insulin secreting beta cells. This results in the loss of insulin production and subsequent hyperglycaemia [1].

1.1.1 Epidemiology

Diabetes mellitus has a worldwide prevalence of 451 million, and its incidence is rising. It is estimated to rise to 693 million by 2045 [2]. Ten percent of all National Health Service (NHS) costs go towards diabetes treatment, of which 10% accounts for T1D [3]. Scandinavia has the highest T1D prevalence in Europe [4]. The global incidence of T1D is rising by 2-5% annually [5]. The DIAMOND project (World Health Organization Multinational Project for Childhood Diabetes) reported that the average global incidence between 1990-1999 rose by 2.8% [6]. Similar observations were shown in Europe between 1989-1994 [7]. The EURODIAB study showed that incidence of juvenile T1D (under 15 years) in Europe increased annually by 3.9% from 1989-2003 [4]. Currently, the estimates of children and adolescents below the age of 20 with T1D has risen to over a million (IDF Diabetes Atlas, 8th Edition). The incidence of adult-onset T1D has also increased [8-10]. Some reports show that there is a higher incidence of T1D in males [11]. Others show that more cases of T1D are diagnosed in winter [12].

1.1.2 Clinical presentation and diagnosis

Polydipsia and polyuria, along with abnormally elevated glucose (above 11.1 mmol/L), are diagnostic features of T1D [1]. Further examination of people presenting with these classical symptoms can be carried out to confirm T1D diagnosis. People with T1D will often present with significant weight loss and complain of tiredness. A diagnosis of T1D can, if required, also be confirmed using antibodies (as described below) and C-peptide tests. People with T1D can also present with diabetic ketoacidosis [13]. Poorly managed T1D over the long-term

results in complications including retinopathy, nephropathy, and cardiovascular disease. The primary treatment for T1D is insulin replacement through multiple daily injections [1] or insulin pumps [14].

Antibody testing allows for the prediction and diagnosis of T1D [15]. Antibodies against islet autoantigens can be detected before beta cell destruction occurs. The presence of more than one autoantigen indicates risk for T1D development [16]. Islet autoantigens are proteins within the beta cell and include insulin, proinsulin, protein tyrosine phosphatase-like protein (IA-2), glutamic acid decarboxylase (GAD), and the zinc transporter (ZnT8) [17-20]. Autoantibodies can be detected at diagnosis at any age [18]. The combined measurement of insulin autoantibodies (IAAs), GAD autoantibodies (GADA), and ZnT8 autoantibodies (ZnT8A) provides >90% detection rates of T1D disease onset [21, 22]. IAAs are believed to be the first to appear in early childhood [23, 24].

1.1.3 Natural History of T1D

Beta cell loss occurs in a step-wise fashion with periods of beta cell recovery (Figure 1) [25]. At least 10% of beta cells are believed to remain at the time of diagnosis [26, 27]. C-peptide, released at the same time as insulin, is a measure of beta cell function. Evidence of residual beta cell function, indicated by plasma C-peptide levels, has been shown within the first months of T1D diagnosis [26, 27]. Beta cell turnover and residual insulin production can be maintained at very low levels for up to 50 years post diagnosis [28].

The existing beta cells following diagnosis contribute to controlling blood glucose levels. After beginning insulin therapy, insulin requirements often fall. This is referred to as the honeymoon phase, which occurs in both children [29] and adults [30, 31]. The length of the honeymoon phase is influenced by numerous factors. Higher blood glucose levels, incidence of diabetic ketoacidosis, and longer duration of symptoms prior to diagnosis inversely correlate with

honeymoon duration [29]. Conversely, C-peptide levels positively correlate with honeymoon duration [31].

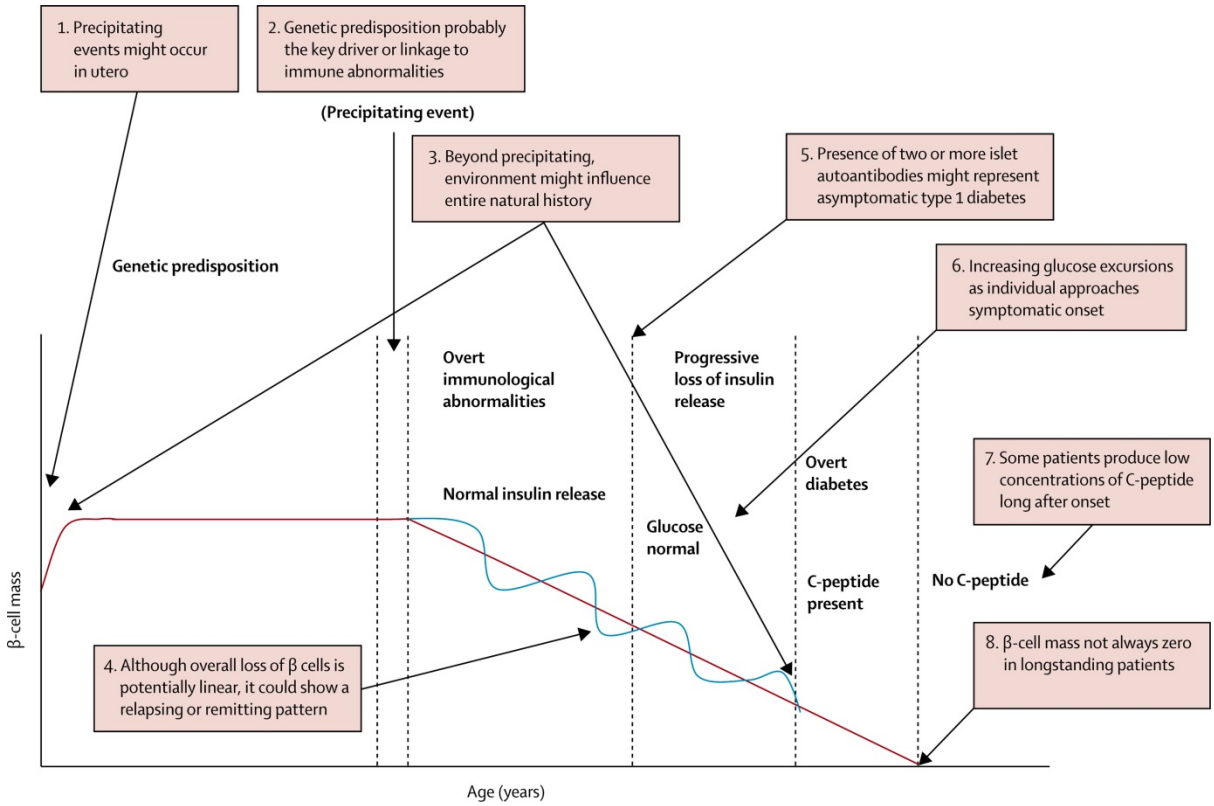

Figure 1 The Natural History of Type 1 Diabetes

The natural history of T1D model adapted from the original model proposed by Eisenbarth (1986) to show that beta cell loss occurs in a step-wise fashion [1].

1.1.4 Aetiology

Many causes of T1D have been explored. Within these, links to both genetic and environmental factors have been identified.

1.1.4.1 Genetic factors

Genotype plays a role in the risk for T1D [32]. A study conducted in monozygotic and dizygotic twin pairs demonstrated that there was up to 50% concordance of T1D between monozygotic twins, but only 10% between dizygotic twins [33]. Another study reported that monozygotic

twin siblings had a higher risk of progression to T1D than dizygotic twins indicated by a 20% higher expression of autoantibodies [34].

Genome wide associated studies (GWAS) have identified over 40 loci associated with T1D [35, 36]. The Human Leukocyte Antigen (HLA) loci, located on chromosome 6p21, makes up 50% of this genetic susceptibility [36-38]. T1D risk is linked to an increase in HLA-DR3 and DR4, and a deficiency in HLA-DR2 haplotypes [39-41]. Certain HLA-DR3 (DRB1-0301, DQA1-0501, DQB1-0201) and HLA-DR4 (DRB1-04, DQA1-0301, DQB1-0302) haplotypes are positively associated with the disease and represent the key predisposing genetic determinants [40, 41]. HLA-DR2 haplotypes (DRB1-1501, DQA1-0102, DQB1-0602) are negatively associated with T1D [39, 40]. The above genotype is strongly associated with T1D onset in children under 5 years and the prevalence of carrying this genotype declines dramatically beyond this age [41].

The other loci associated with T1D risk are those related to insulin and immune regulation [36, 42]. A variable number tandem repeat (VNTR) in the promoter of the insulin gene on chromosome 11p15 is linked to T1D risk [36]. Differential expression of the insulin protein in the thymus has been shown to lead to defective central tolerance towards insulin [43, 44]. Polymorphisms in genes relating to immune regulation have been identified in T1D [45-47]. Cytotoxic T lymphocyte associated-4 (CTLA-4) on chromosome 2q33 is involved in T cell regulation [48]. This was identified as a risk locus for T1D [45, 49]. The IL-2Rα gene (IL2RA) on chromosome 10p14 is involved in IL-2 signalling on T cells [50]. GWAS identified allelic heterogeneity at the IL2RA region to be associated with susceptibility to T1D [46]. This was caused by 2 single nucleotide polymorphisms (SNP) in IL2RA gene (the intron 1 and the 5' regions). This genetic variation correlated with lower circulating levels of soluble IL2RA

(sIL2RA) in T1D [47]. Furthermore, lower expression of IL-2RA on $CD4^+$ naïve and memory T cells strongly correlates with the haplotypes in the IL2RA gene [51].

1.1.4.2 Environmental factors

Although genotype plays a major role in the predisposition to T1D, it does not account for all of the susceptibility. The risk of T1D is 50% between monozygotic twins [33], therefore environmental factors must also play a role [32, 52]. Environmental factors such as vitamin D deficiency [53, 54], diet [32, 55-57], gut dysfunction [58-60], and viral infections [61-63] have been implemented in the cause of T1D.

The impact of diet at a young age has been associated with increased risk of T1D. Cow's milk and gluten in the first few months of life is linked to islet autoantibody and T1D development [32]. In one study, spontaneous T1D development in rats was reduced by exclusion of cow's milk [55]. It was also shown that increased antibodies (anti-beta-lactoglobulin and anti-bovine serum albumin) to milk proteins were higher in diabetic rats and children. These antibodies could cross react with beta cell membrane proteins, and had similar homology to HLA-DR and DQ [55]. Gluten and early infant feeding (before 3 months) is associated with increased islet autoantibody risk but not with coeliac disease [56]. Genetically pre-disposed children exposed to cereals before 3 months or after 7 months for the first time have increased hazard of islet autoimmunity compared to those who were exposed between 4-6 months therefore, there may be a window for gluten exposure [57].

Gut dysfunction has also been associated with T1D [58]. Lower functional diversity of the gut microbiome in T1D was reported by Gut Microbiome Metagenomics Analysis [59]. Children with HLA conferred T1D (Finland – TRIGR and FINDIA pilot studies) had different fecal microbiota [60]. The abundance of several lactate- and butyrate-producing bacteria was inversely related to the number of islet autoantibodies in the T1D cohort. A low abundance

(<12%) of the two most common bifidobacteria, B. adolescentis and B. pseudocatenulatum, was associated with T1D in the aforementioned study [60]. Gut dysfunction is supported by studies in non-obese diabetic (NOD) mice lacking the MyD88 protein, necessary for recognition of microbial stimuli by toll like receptors (TLRs). Germ-free MyD88 knock-out (KO) NOD mice develop T1D. This was due to a lack of microbiota as colonization of these germ-free NOD mice with a microbial flora attenuated T1D [64].

Viral infections have been linked to the development of T1D. This may be caused by molecular mimicry of viral antigens with beta cell antigens. Enterovirus infections are associated with a higher risk of T1D. Enterovirus infections were higher in children with T1D and were more frequent in children during the 6-month period preceding the appearance of autoantibodies in the Finnish DIPP study [62]. Rotavirus (RV) contains peptide sequences highly similar to GAD and IA-2 T cell epitopes. A strong association between RV and increases in GAD and IA-2 autoantibodies has been reported. Therefore, it is postulated that RV infection may trigger islet autoimmunity in genetically susceptible individuals [63]. Additionally, polyinosinic-polycytidylic acid (PolyIC), a mimic of double-stranded viral RNA, can induce diabetes when administered to rats that have been previously immunized with the insulin (i.e. susceptible to T1D development) [61].

1.2 Autoimmune Pathogenesis of T1D

T1D is a complex autoimmune disorder with multiple components to its development. The pathogenesis of T1D involves a combination of a breakdown in immune tolerance, major immune cell cross-talk, and beta cell vulnerability. Ultimately, this leads to the infiltration of self-reactive T cells into the pancreas where beta cell destruction occurs.

1.2.1 Breakdown in Tolerance in T1D

Immune tolerance is divided into central and peripheral tolerance. Both central and peripheral tolerance defects have been found in T1D.

Central tolerance is mediated in part by the autoimmune regulator (AIRE) in the thymus. AIRE is primarily expressed by medullary thymic epithelial cells (mTECs). It functions to detect self-reactive T cells by exposing thymocytes (pre-T cells) to a broad repertoire of self-antigens and facilitate the deletion of self-reactive thymocytes [65]. Differential expression of insulin in the thymus has previously been described for the insulin VNTR gene. This is linked to defects in central tolerance in T1D [43, 44]. AIRE presents self-insulin in the thymus to developing T cells [66-68]. This expression induces tolerance to insulin. Deletion of insulin from the thymus can cause T1D therefore, exposure of developing T cells to insulin is important for developing immune tolerance [69]. Diabetes is exacerbated in AIRE deficient NOD mice where there is also a lack of T-Regulatory cells (TRegs) [70]. This indicates that defects in both central and peripheral tolerance can be involved in the autoimmune pathogenesis of T1D.

Self-reactive, or also referred to as autoreactive, T cells that escape central tolerance are subsequently controlled through peripheral tolerance. Peripheral tolerance aims to block activation of self-reactive effector T cells. It functions through mechanisms that include co-inhibitory signals (programmed cell death protein 1 (PD-1), CTLA-4) as well as regulation by TRegs [71, 72].

Breakdown of peripheral tolerance is involved in the pathogenesis of T1D [73]. This is supported by a number of pieces of evidence. Firstly, polymorphisms in the co-inhibitory receptors PD-1 [74] and CTLA-4 [45] are associated with T1D. Secondly, TReg dysfunction has been reported in T1D [75-78]. TRegs from T1D donors co-cultured with effector T cells

failed to suppress IFN-γ production and the proliferation capacity of effector T cells [75, 77, 79]. CTLA-4 polymorphisms are linked to this reduced TReg suppressive capacity [80, 81].

T1D onset can be delayed by TRegs. Whilst pre-diabetic and overtly diabetic NOD mice have similar numbers of autoreactive diabetogenic T cells, the difference in disease status relates to the number of TRegs in both models. [82]. However, diabetogenic T cells can also develop resistance to suppression by TRegs. Diabetogenic T cells show resistance to transforming growth factor beta (TGF-β) dependent mediated inhibition by TRegs [82, 83]. Furthermore, TRegs could not supress diabetogenic $CD8^+$ T cells bearing a dominant negative TGF-β receptor type II [83]. This highlights a mechanism by which some diabetogenic $CD8^+$ T cells develop resistance to peripheral tolerance i.e. regulation by TRegs.

1.2.2 Immune cell involvement in T1D

Part 1. Insulitis

T1D beta cell destruction occurs following lymphocyte infiltration into the pancreas. This results in a hallmark inflammation within the pancreas referred to as insulitis [84]. In the initial stages of insulitis, a mixed leukocyte population including $CD4^+$ T cells, $CD8^+$ T cells, natural killer (NK) cells, Dendritic cells (DC), B cells, and macrophages infiltrates the pancreas [85-88]. Lymphocytes expressing CD45 (the common leukocyte antigen) were identified at week 4 of insulitis in NOD mice, and had expanded by week 14 [85]. $CD4^+$ T cells, $CD8^+$ T cells, B cells, and macrophages were also present by week 14 [85]. Trafficking of $CD45^+$ cells from lymphoid organs to the pancreas, specifically to the region of insulitis, has been demonstrated by cell tracking in mice [85]. In the aforementioned study, the cells which migrated into the pancreas were identified as $CD4^+$ T cells, $CD8^+$ T cells, and B cells. The majority of the recent immigrant T cells exhibited a naïve phenotype which then became activated ($CD69^+$) [85].

Furthermore, there were no recent immigrants into the pancreas in the absence of insulitis [85]. Therefore, insulitis may drive lymphocyte infiltration in to the pancreas.

$CD3^+$ T cells are the dominant population in the pancreatic infiltrate [87]. High numbers of $CD4^+$ and $CD8^+$ T cells were found in the pancreas of diabetic donors, with $CD8^+$ T cells dominating the infiltration [87]. Another study found that eighty percent of lymphocytes present in the pancreas were $CD3^+$ T cells, and these were, again, mainly $CD8^+$ T cells [86]. Pancreatic $CD4^+$ and $CD8^+$ T cells were comprised of central memory and effector memory T cell subsets which expressed CD69 and CD103, markers of activation and tissue resident memory subsets [86]. Additionally, intra-pancreatic T cells had increased proliferation compared to those isolated from peripheral lymph nodes and spleen of mice [85].

The remaining leukocyte subsets found within the pancreas include NK cells, B cells, DC, and macrophages. NK cells and B cells make up much lower proportions of pancreatic lymphocyte populations [86]. DC and macrophages can be detected within the pancreas during insulitis in diabetic donors [87, 89]. Macrophage infiltration is mediated by C-C Motif Chemokine Ligand 2 (CCL2) expression on beta cells. CCL2 promotes recruitment of macrophages from the bone marrow to the islets. Furthermore, CCL2 receptor inactivation prevented macrophage recruitment in the aforementioned study [89].

Although insulitis is a hallmark of T1D onset, and is key to the pathogenesis of T1D, it does not solely promote T1D autoimmunity. $CD8^+$ T cell infiltration into the pancreas was also found in T1D in the absence of insulitis in other studies [87, 90]. T1D donors without insulin containing islets and those with long disease duration had elevated $CD8^+$ T cell infiltration into the pancreas. $CD4^+$ T cells and $CD11c^+$ cells were also found at high levels [87]. Furthermore, adoptive transfer of insulin-specific $CD8^+$ T cells into NOD and control mice induced diabetes

in both models indicating that pre-existing inflammation is not a necessity in T1D induction [90].

Part 2. Immune cells involved in T1D pathogenesis

As demonstrated by the mixed immune populations infiltrating the pancreas in T1D, a range of immune cell subsets are involved in the pathogenesis of T1D [91]. Following insulitis, autoreactive T cells are primed in the circulation by beta cell antigens. DCs present autoantigens to beta cell specific T cells, which are in turn activated and migrate to the pancreas where they attack beta cells [92]. This process requires substantial coordination and cross-talk between innate and adaptive immune cell subsets (Figure 2) [91].

Innate immunity

Differentiation of islet-specific T cells depends on innate immune cells. Innate cells produce proinflammatory cytokines or suppressive cytokines which creates an environment that influences the islet-specific T cells [91]. In particular, macrophages secrete high levels of IL-12 which promotes Th1 rather than Th2 differentiation.

Dendritic cells

Dendritic cells are present in the islets of diabetic donors [87, 88]. Depletion of DCs in transplanted pancreatic islets ensures graft survival in mice [93]. Total DC frequency is decreased in the peripheral blood of children with T1D [94]. However, there are mixed reports on the frequency of DC subpopulations in T1D. A lower proportion of circulating plasmacytoid DC (pDCs) was reported in people with T1D [95]. Conversely, the proportion of pDCs was reported to be elevated in another study [96]. In this study [96], pDCs were measured in early-diagnosed T1D patients. pDCs were shown to present immune complexes to $CD4^+$ T cells more efficiently than conventional DCs (cDCs), suggesting a possible pathogenic role of pDCs in T1D onset [96].

Self-antigens released after beta cell death can be taken up by infiltrating DCs in the pancreatic islets and presented to T cells in the pancreatic lymph nodes (PLN). The infiltration of $CD11c^{+}CD11b^{+}CD8\alpha^{-}$ DC into the pancreas at 2 weeks of age in NOD mice precedes insulitis [92]. In NOD mice, DCs are efficient antigen presenting cells (APC). DCs effectively stimulated GAD-reactive T cell proliferation in *in vivo* co-cultures [97].

An imbalance favouring development of DC from myeloid-committed progenitors predisposes to autoimmune disease in NOD mice. Increased DC generation from myeloid cells in NOD mice compared to diabetes free Non-obese Diabetes-Resistant (NOR), Balb/C and C57BL/6 mice has been shown [98]. NOD and NOR mice had increased numbers of DC in blood and thymus. However, NOD mice had an increased proportion of myeloid DC ($CD11c^{+}$, $CD11b^{+}$) subsets within the spleen. Prediabetic NOD mice exhibit a myeloid lineage-specific increase in DC generative capacity relative to diabetes-resistant NOR mice [98].

cDCs play a crucial role in activating cells and driving lymphocyte infiltration into the pancreas. cDCs isolated from NOD mice had increased ability to activate T cells through higher IL-12 production and co-stimulatory molecule expression [99]. DCs from NOD mice had elevated nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) activation upon stimulation. Neutralisation of NFκB and IL-12 reduced the capacity of NOD DC to stimulate T cells [99]. Lymphocytic choriomeningitis virus (LCMV) glycoprotein under the rat insulin promoter (RIP) causes diabetes as a result of infiltrating $CD8^{+}$ T cells, forming a transgenic (Tg) rodent model of T1D. Repeated injection of cDCs expressing the immunodominant LCMV epitope into LCMV-RIP mice resulted in severe lymphocyte infiltration into the pancreas and development of T1D [100].

pDCs have also been shown to play a role in diabetes development. pDCs, which detect viral RNA or DNA, can also induce T1D via type I interferons (IFNs). Type 1 IFNs include IFN-α

and IFN-β. Blocking IFN-α prevented inflammation and T1D development in a Tg-mouse model [101]. There is elevated production of type I IFNs by pDCs and $CD4^+$ T cells in the PLN [102]. Blockade of IFN-α decreased diabetes incidence and delayed onset, whilst simultaneously increased immature DC in pancreatic lymph node (PLN) and IL-10/IL-4 producing $CD4^+$ T cells in the spleen [98]. Furthermore, FmS-like tyrosine kinase 3 ligand (FlT3l) treatment, which expands both cDC and pDC populations, accelerated T1D in 15-week-old NOD mice when islet antigen-specific cytotoxic T lymphocytes (CTLs) were detectable in blood [102]. Another study reported that FlT3l treatment expanded DC and delayed diabetes in NOD mice but antigen-specific CTLs were not measured [103]. Therefore, both the presence of diabetogenic T cells and DC is necessary for T1D development.

Macrophages

Another innate cell population, macrophages, have also been implemented in the pathogenesis of T1D. Macrophages are recruited to the pancreas by $CD4^+$ T cells [104]. One week after adoptive transfer of $CD4^+$ T cell into NOD.SCID mice, macrophages were detected in pancreatic infiltrates. Additionally, inflammatory cytokines from macrophages were increased in the pancreas. Recruited macrophages were activated by $CD4^+$ T cells and subsequently secreted proinflammatory cytokines IL-1β, TNF-α, and nitric oxide (NO). When non-pathogenic T cells were transferred, macrophages secreted less IL-1β [104]. Diabetogenic $CD4^+$ T cells secrete CCL1 in the pancreas. This interacts with and recruits activated $CCR8^+$ macrophages [104]. Furthermore, IL-1β secretion by activated macrophages induces NO by beta cells and subsequent beta cell death [105].

Macrophages influence T cell differentiation in T1D. Blockade of macrophages in NOD mice (using a monoclonal antibody (mAb) for myelomonocytic adhesion-promoting type-3 complement receptor specific for macrophages, but doesn't bind T cells) prevents macrophage

and T cell infiltration into the islet, and subsequently inhibits T1D [106]. T cells in a macrophage deficient environment did not differentiate into diabetogenic T cells, therefore prevented T1D [107]. In this environment, Th1 immunity decreased, whereas Th2 immunity increased due to decreased IL-12 secretion by macrophages. T cell activation, denoted by Fas ligand and perforin expression, was also reduced. IL-12 administration caused T1D in NOD mice in the macrophage deficient environment, indicating that IL-12, predominantly secreted from macrophages, is necessary for T1D development [107]. In support of this, macrophages display dysregulated cytokine production in NOD mice; elevated IL-12 production combined with reduced IL-10 and TNF-α [108].

Natural Killer cells

NK cells can be found within the pancreas, even before T cell infiltration occurs [109-112]. NK cells were detected from day 1 in the pancreatic infiltrate, before established inflammatory conditions in NOD mice [109]. NK specific genes were also upregulated in the pancreatic infiltrate (Ly49A/P, Ly49C, Ly49D, Ly49I/F/H, NKG2A, and 2B4 changes on cell surface). NK cell frequency correlated with pancreatic infiltrate aggressivity in anti-CTLA-4 treated mice, with no change in splenic NK cell frequency [109]. Increased intra-pancreatic NK cells accelerated the onset of diabetes in NOD mice. Furthermore, NK cell associated cytokines were hyper-expressed in the pancreas from mice with accelerated diabetes onset [110]. Activated NK cells within the pancreas of NOD mice produced large amounts of IFN-γ , promoting effector function of diabetogenic $CD4^+$ T cells [111]. A high percentage of NK cells were detected at weeks 4-5 in the NOD pancreas, i.e. the prediabetic stage. Both NK cells and T cells were then detected in the pancreas at weeks 9-10 in NOD mice [112]. Furthermore, NK cells were present in the pancreas of NOD-Rag (no B or T cells). Together this indicates that NK cells infiltrate the pancreas independent of T cells and established inflammation, and is believed to be the first event in insulitis [112].

NK cells within the islet have a distinct phenotype that differs to splenic NK cells [113]. Pancreatic NK cells are in an activated state as indicted by increased expression of CD25, CD69, and PD-1, coupled with down-regulation of CD62 ligand (CD62L). Pancreatic NK cells also expressed higher killer cell lectin-like receptor subfamily G member 1 (KLRG1) than splenic NK cells, indicating their high proliferation state [113]. In the aforementioned study, natural-killer group 2-member D (NKG2D), a natural cytotoxicity receptor, was also reported to be down-regulated on pancreatic NK cells. Pancreatic NK cells were hyporesponsive in NOD mice but spontaneously produced high levels of IFN-γ and were highly cytotoxic i.e. $CD107a^+$, a marker of granule exocytosis [113].

Depletion of NK cells has prevented T1D in animal models [109, 110, 114]. Depletion of NK cells using anti-Asialo GM1 polyclonal antibody and administration of anti-CTLA4 at the same time lead to a significant decrease in diabetes incidence and insulitis in treated NOD mice. Activated T cell infiltration was not affected, but more than 85% NK cells were eliminated [109]. NK cells depleted with anti-asialo GM1 antibodies in NOD mice with accelerated disease onset also abolished accelerated diabetes [110]. Suppressor of cytokine signalling (SOCS)-1-Tg NOD mice infected with coxsackievirus B4 (CVB4) are used as another T1D rodent model (develop early hyperglycaemia, acute diabetes, and show loss of insulin positive beta cell staining). In this model, NK cells but not $CD8^+$ T cells were necessary for acute diabetes development. Again, depletion of NK cells in SOCS-1-Tg NOD mice prevented the loss of insulin staining beta cells [114].

Adaptive immunity

Adaptive immunity, or also known as acquired immunity, plays a significant role in T1D pathogenesis. Adaptive immune cells are highly specialized antigen specific cells, and therefore can be highly specific for beta cell antigens.

B cells

B cells are another lymphocyte subset which play a role in the pathogenesis of T1D. It is believed that presentation of beta cell antigens by B cells to T cells is important for T1D development. B cell depletion in NOD mice significantly reduces T1D development. B cell deficient NOD mice (NOD.Ig mu null mice) had normal T cell frequencies but were insulitis resistant and did not develop T1D [115]. Anti-CD20 is used to deplete $CD20^+$ B cells. Anti-CD20 treatment in NOD mice expressing human CD20 on B cells delayed or reversed T1D [116]. Anti-CD20 treatment in 5 week old NOD mice reduced B cells by 95%, reduced insulitis, and prevented T1D in 60% NOD mice. T cell frequencies were not altered. However, impaired $CD4^+$ and $CD8^+$ T cell activation in lymph nodes was noted indicating a lack of antigen presentation by B cells to T cells [117]. Rituximab (anti-CD20 mAb) administration in people newly diagnosed with T1D preserved beta cell function as evidenced by preserved C-peptide [118]. Again, this indicates a role for B cells in promoting autoreactive T cells and subsequent beta cell destruction.

T cells

Although many immune populations are involved the pathogenesis of T1D, it is considered to be a T cell mediated disorder. T cells are the predominant cell type found within the pancreas of T1D models and donors [85-88]. Disease can be transferred by splenic T cells from diabetic donors [119, 120]. Adoptive transfer of splenic T cells from diabetic donors into irradiated NOD mice, i.e. lacking immunity, induces T1D. Both T-helper and CTLs are involved; neither can induce diabetes independently [119]. T1D is prevented by T cell depletion. Depletion of T-helper cells (mAb for L3T4 determinant on surface of T-helper cells) in NOD mice prevented lymphocyte infiltration into the islet and mice became normoglycaemic [120]. Th1 transferred into NOD.SCID mice caused insulitis and T1D development, but not in those which received Th2 cells [121]. Islet-specific $CD4^+$ T cell clones derived from overtly diabetic NOD mice

injected into young NOD mice accelerated disease progression. Control mice did not become diabetic and had no significant pancreatic infiltration [122]. $CCR4^+$ T cells, mainly $CD4^+$, were detected within islets of prediabetic NOD mice. Depletion of $CCR4^+$ T cells inhibited the development of insulitis and diabetes [123]. Cytokine secretion (IFN-γ, IL-1β, TNF-α) and major histocompatibility complex (MHC) class I mediated cytotoxicity are mechanisms involved in T cell-meditated beta cell death [91, 124].

T cells migrate specifically to the pancreas in T1D. Transfer of diabetogenic T cells from T1D and nondiabetic control donors into NOD mice revealed that diabetogenic T cell from T1D donors only accumulated in the pancreas of NOD mice. This suggests that homing to the pancreas is dominant in T1D [125]. Autoreactive T cells express elevated levels of homing receptors. Lymphocytes found within the NOD pancreas expressed adhesion molecules Lymphocyte Function-associated Antigen 1 (LFA-1), Very Late Antigen-4 (VLA-4), CD11a, CD11b, and CCR7 [90, 126, 127]. CXCR3 on T cells also mediates their migration into pancreas [128].

Homing of diabetogenic T cells to the pancreas does not require pre-existing inflammation. Adoptive transfer of insulin-specific $CD8^+$ T cells into NOD and control mice (BALB/c and DBA/2J) caused rapid diabetes in all strains. Tracking of insulin-specific $CD8^+$ T cells showed that they accumulated in the islets 24 hours after transfer in all strains [90]. Transfer of labelled insulin-specific $CD8^+$ T cells into NOD.Rag1-KO mice, which lack T and B cells, and are inflammation-free shows the accumulation of insulin-specific $CD8^+$ T cells in the islets, therefore, pre-existing inflammation is not essential [90]. Furthermore, diabetogenic $CD8^+$ T cells express a multitude of adhesion molecules including integrins and selectins. This allows them to engage with receptors constitutively expressed by the pancreatic endothelium in the absence of inflammation. This contributes to the initial stages of T cell infiltration and insulitis

[90]. Homing of diabetogenic T cells to the pancreas is MHC class I dependent. Diabetogenic T cells injected into NOD mice lacking MHC class I molecules (NOD.β2m-KO mice) do not accumulate in the pancreas [90]. Diabetogenic T cells can recognise islet endothelial cells. Islets co-cultured with diabetogenic $CD8^+$ T cells were destroyed. Here, $CD8^+$ T cells were specific for Glucose transporter 2 (GLUT-2) and CD105 (endothelial cell marker) on beta cells. Beta cells were intact in NOD.β2m-KO islet cultures [90].

T1D displays ongoing islet autoimmunity and $CD8^+$ T cell infiltration into the islet [28, 129, 130]. Islet reactive $CD8^+$ T cells have been found within insulin lesions of recent-onset and long-standing T1D, sustaining autoimmune destruction of beta cells post the initial presentation of T1D, and into the larger natural history of T1D [130]. This shows that T cells play a role in the onset and maintenance of T1D.

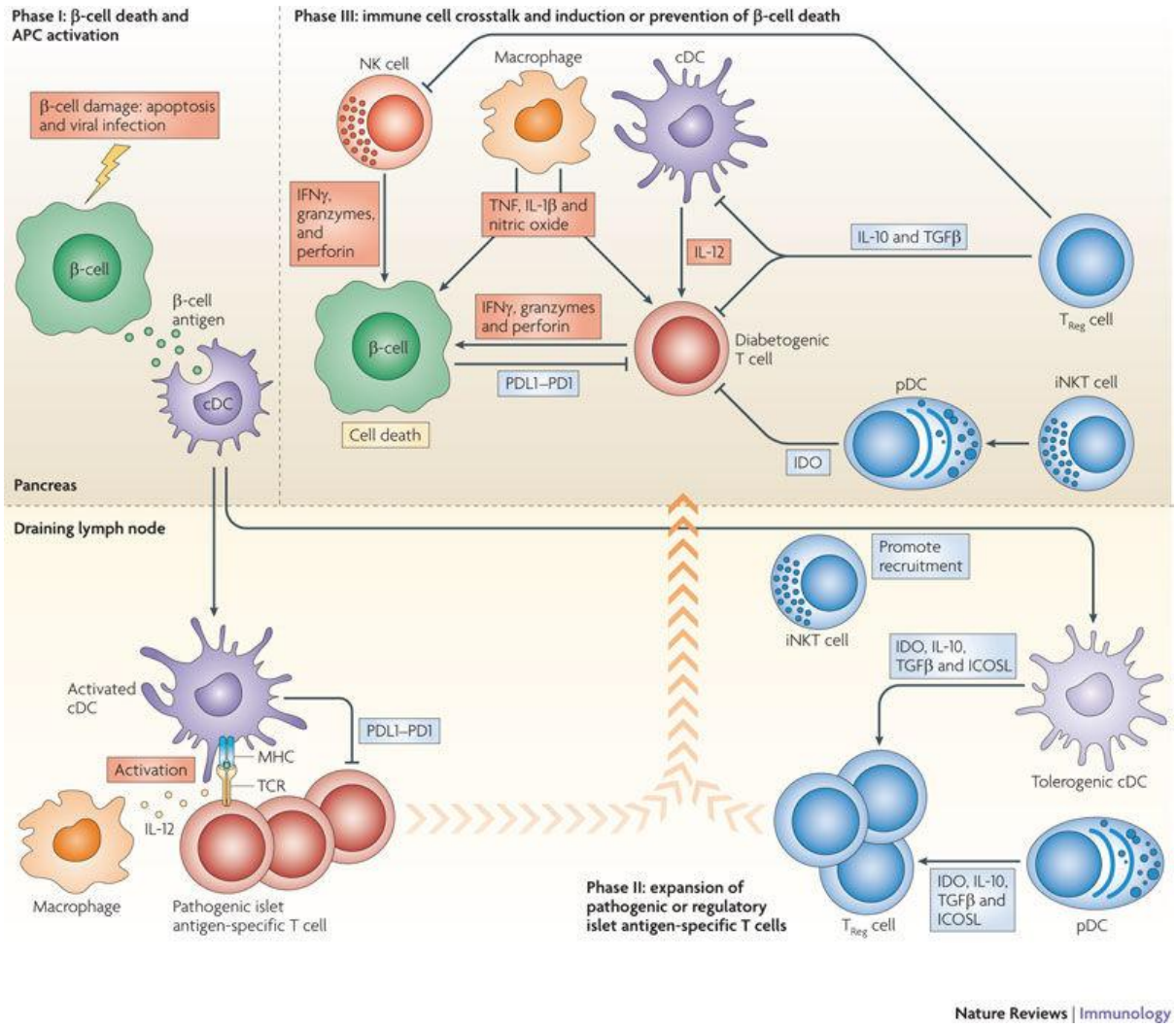

Figure 2 Immune cell cross-talk in T1D

The coordination and cross-talk between innate and adaptive immune cell subsets involved in the pathogenesis of T1D [91].

1.2.3 Beta cell vulnerability in T1D

Diabetogenic T cells can be found in the peripheral blood of healthy individuals, in which T1D does not progress [131]. Therefore, beta cell vulnerability may also be important in the natural history of T1D development.

1.2.3i Co-stimulatory molecules

Co-stimulatory molecules expressed by beta cells increase their vulnerability to inflammatory destruction. CD80 expression is increased by beta cells during inflammation and this is targeted

by $CD8^+$ T cells [132]. Normally, the co-stimulatory molecule CD28 preferentially binds CTLA-4 on T-Regulatory cells, and therefore beta cell vulnerability is reduced through peripheral tolerance. However, in T1D CTLA-4 expression on T-Regulatory cells is reduced and therefore competition for binding is reduced [80, 81]. As a result, CD28 on memory $CD8^+$ T cells binds effectively to CD80 on the beta cell. Additionally, reduced co-inhibitory molecule expression on beta cells, such as PD-L which binds to PD-1, also increases susceptibility to $CD8^+$ T cell target [133].

1.2.3ii Chemokines

The expression of chemokines on beta cells increases beta cell vulnerability in T1D. The pancreatic endothelium in T1D is chronically activated [124]. During inflammation, high levels of IFN-γ are produced and this induces chemokine receptors on beta cells [91]. Adhesion molecules such as Intercellular Adhesion Molecule 1 (ICAM-1), VLA-4, CD44, peripheral lymph node addressins (PNAd), and mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) are upregulated by inflamed islets in T1D, therefore increasing lymphocyte homing to the islets [90, 126, 134, 135].

In addition to the expression of adhesion molecules, beta cells secrete chemokines in response to inflammatory stimuli in T1D [128, 136, 137]. Chemokine production by islets from NOD.SCID, NOR. and BALB/c mice is 50% lower than NOD mice [137]. In one study, beta cells were exposed to inflammatory cytokines IL-1β, IFN-γ, and TNF-α for 24 hours. Beta cells secreted the chemokines CXCL9 and CXCL10 in response to the inflammatory stimuli [128]. This specifically attracted $CXCR3^+$ T cells. In $CXCR3^{-/-}$ NOD mice the onset of T1D is significantly delayed due to reduced T cell recruitment [128]. The chemokines CCL2, CCL3, CCL17, CCL21, and CCL22 are also produced by NOD beta cells, but to a lesser extent than CXCL10 [137-139]. CCL3 elevated in the pancreas of NOD mice correlated with insulitis.

Neutralisation of CCL3 following transfer of diabetogenic T cells into NOD mice with severe combined immunodeficiency (SCID) delayed onset of diabetes [139]. Furthermore, altered chemokine levels have been detected in individuals at risk of T1D development. CCL3 and CCL4 were increased while CCL2 was decreased in the serum of at risk individuals. This strongly correlated with auto-antibody status [140]. Increased CCL3 and CCL4 also correlated with proinsulin, a marker of beta-cell stress and negatively associated with C-peptide in T1D donors [141].

1.2.3iii Toll-like receptors

Expression of TLRs increases beta cell vulnerability in T1D. Pancreatic islets in mice and humans express TLR1-TLR9 [137, 142-144]. TLR3 is most strongly expressed in humans [144]. TLR expression renders beta cells susceptible to bacterial and viral derived molecules [142-144]. Stimulation of islets with specific TLR ligands (such as peptidoglycan for TLR2, poly(I:C) for TLR3, LPS for TLR4, flagellin for TLR5, R848 for TLR7, and CpG for TLR9) results in marked up-regulation of CXCL10, CCL5, CCL2, and CCL3 secretion which increases chemotaxis and trafficking of T cells to the islet [137]. Treatment of LCMV.RIP mice (T1D model) with the TLR3 ligand, poly(I:C), causes upregulation of MHC class I on beta cells. This does not occur in TLR3 -/- mice [143]. Injection of the TLR3 ligand poly(I:C), or the TLR7 ligand R-848 upregulated MHC I on beta cells and induced overt diabetes in LCMV.RIP mice [143]. In TLR-2 and TLR-4 deficient mice, diabetes development is attenuated [64].

1.3 T1D treatment and Immunotherapies

The primary treatment for T1D is insulin replacement [1, 14]. However, patients remain at risk of diabetic complications [145-147]. Therefore, there has been significant interest in approaches to prevent T1D, predominantly by modulating the immune system. These have

included cell depletion, peptide-based, anti-inflammatory, cell therapy, and stem cell strategies [148].

1.3.1 Cell depleting strategies

Given their role in T1D pathogenesis, a number of T cell depletion strategies have been tested. OKT3, a CD3 mAb, treatment in T1D maintained insulin production for 1 year [149]. In the DEFEND-1 study, OKT3 treatment in new onset T1D reduced C-peptide loss by up to 25% [150]. Teplizumab, another anti-CD3 mAb, preserved C-peptide and beta cell mass for 1-2 years [149-153]. Teplizumab treatment resulted in maintained or improved beta cell function for at least 2 years post treatment in recent-onset T1D patients [149-152]. Evidence for beta cell preservation was also reported in long standing T1D patients for up to 1 year [154, 155]. Otelixizumab, also an anti-CD3 mAb, did not however prevent C-peptide decline as shown in the DEFEND-2 study [156]. Thymoglobulin, an anti-thymocyte globulin (ATG), administration showed no difference between the treated and placebo group in one study [157]. However, another study later found that intravenous ATG combined with subcutaneous pegylated granulocyte colony-stimulating factor (G-CSF) resulted in significantly reduced C-peptide loss compared to the placebo group [158].

B cell depletion has also been explored with some beneficial outcomes. Firstly, anti-CD22 mAb treatment in NOD mice delayed disease and hyperglycaemia in two studies [116, 159]. Following this, Rituximab treatment, an anti-CD20 mAb, in people with T1D infused for 22 days preserved the beta cell function as indicated by maintenance of C-peptide levels, but did not prevent T1D [118].

1.3.2 Antigen specific strategies

Strategies using insulin, GAD, and peptide islet antigens have also been explored. In the Diabetes Prevention Trial type-1 (DPT-1), insulin was administered to at risk individuals

containing islet autoantigens. Unfortunately, this was ineffective at preventing diabetes onset [160]. Some effect was however observed in a sub group of high IAAs [161]. A phase 3 study currently ongoing, aims to induce protective immunity in the sub group with high IAAs through administration of oral insulin (NCT00419562). GAD-based immunisation has also been explored. GAD-alum injections in recent onset T1D did not affect insulin secretion decay over 1 year [162]. Another study employing lymph node injections of GAD-alum (Dynamid) once per month in combination with vitamin D supplementation is now underway (NCT02352974). A recent study administering a single proinsulin peptide in a newly diagnosed T1D cohort reported C-peptide preservation and increased regulation of immunity through IL-10 and TRegs (increased FoxP3 expression) [163]. Currently ongoing, MultiPepT1De, is a phase 1 trial combining peptide islet autoantigens to induce beta cell tolerance in recent onset T1D. This involves 6 injections over 20 weeks at low, medium, and high dose, compared to a placebo group (NCT02620332).

1.3.3 Anti-inflammatory strategies

Inflammation plays a large role in T1D disease progression, therefore anti-inflammatory therapies have been explored. Nicotinamide alone or in combination with Vitamin E, both exhibiting anti-inflammatory and anti-oxidant properties, were shown to preserve basal C-peptide secretion for up to 2 years i.e. preserved beta cell function [164]. However a follow up study, ENDIT, showed no difference [165]. Cyclosporin A associated with Methothrexate, immunosuppressant and anti-inflammatory drugs, reduced insulin requirements, lowered haemoglobin A1c (HbA1c), and induced remission of T1D [166]. Anakinra, IL-1R antagonist (IL-1RA), had no effect on C-peptide [167]. However, some improvement in glycaemic control was found in another study [168]. Canakimumab, an IL-1β mAb, had no effect on C-peptide [169]. Mycophenolate mofetil alone, an immunosuppressant drug, and associated with

Daclizumab, an anti-CD25 mAb, had no effect in T1D [170]. Golimumab (SIMPONI), an anti-TNF-α mAb-based formulation, will be assessed in T1D (NCT02846545).

1.3.4 Cell therapy strategies

The dysregulation in regulatory cell types in T1D has led to efforts to boost immune regulation and tolerance in T1D. TRegs isolated and expanded from T1D patients maintained exhibited suppressive capacity equal to the control cohort [171]. Adoptive transfer of the expanded TRegs into a T1D maintained C-peptide levels for 2 years. Furthermore, up to 25% of TRegs were still detectable in the bloodstream after 1 year [172]. A new trial is exploring the effects of a single infusion of autologous TRegs combined with subcutaneous IL-2 for 5 days (NCT02772679). Administering a single infusion of CLBS03, a formulation containing autologous TRegs, in a low or high dose to T1D patients is also being explored (NCT02691247). A single infusion of umbilical cord blood TRegs combined with Liraglutide, an incretin-mimetic molecule, 3 days after the TRegs infusion and daily for 6 months is another TReg therapy currently ongoing (NCT03011021).

Dendritic cell therapy strategies have also been explored. Autologous dendritic cells were administered every 2 weeks over 2 months to a T1D cohort. An increase in peripheral $B220^{+}$ $CD11c^{-}$ B cells populations, a suppressive B cell population, was reported. However, no significant effect on glycaemia was shown [173].

1.3.5 Stem cell strategies

Further to cell based strategies, stem cells have also been examined. Autologous mesenchymal stem cells (MSCs) transplanted in T1D patients show a reduced decay in C-peptide levels after treatment [174]. Umbilical cord blood MSCs (UC-MSCs) were tested in association with autologous bone marrow mononuclear cells (aBM-MNC) infused through the pancreatic artery. Here, C-Peptide increased, coupled with reduced insulin requirements 1 year following

treatment [175]. In another trial, people with newly diagnosed T1D received ATG followed by autologous hematopoietic stem cells (HSCs). After treatment, all patients but 1 became insulin independent for at least 6 months, with increased C-peptide levels and decreased anti-GAD autoantibodies [176]. In another study, a combination of cyclophosphamide, ATG and G-CSF to induce immunosuppression was given to people with recent onset T1D, followed by the infusion of cryopreserved $CD34^+$ HSCs as a single infusion. Among the treated patients, 59% reached insulin-independence and 32% remained insulin independent at the last follow-up (48 months) [177].

1.4 Exercise for beta cell preservation

Previous research in the field of diabetes has revealed that exercise has beta cell preserving effects in type 2 diabetes (T2D) [178-183]. I have undertaken a systematic review detailing the effects of exercise on beta cell health parameters such as beta cell viability, proliferation, and insulin secretion, in which 56 relevant studies were found (manuscript in review). However, only 3 of these studies were in T1D animal models, and all were focused on regular exercise [184-186]. These studies showed that exercise training in diabetic rodents improved beta cell mass, beta cell viability, insulin content, insulin secretion, insulin signalling, and overall glucose homeostasis.

In light of this lack of evidence for exercise in T1D, the exercise for type 1 diabetes (EXTOD) pilot study was set up to investigate exercise in people newly diagnosed with T1D (<3 months) [187]. In the EXTOD study, participants completed 12 months of unsupervised moderate intensity exercise (150-240 minutes per week). Clinical reviews took place at 3, 6, and 12 months measuring blood glucose, meal stimulated C-peptide, and fitness levels. The EXTOD pilot trial showed that regular exercise was safe and maintained by T1D participants. Improvements in insulin sensitivity and reduced insulin requirements were reported. Beta cell

function, measured by C-peptide, did not change between groups but the increase in insulin sensitivity in the exercise group likely under-estimated beta cell function. Beta cell function corrected for insulin sensitivity, as done routinely for T2D but not for T1D yet, was however preserved by exercise compared to the non-exercise arm, where there was loss of beta cell function. This provides preliminary human data to support the role of exercise for beta cell preservation in T1D [187].

Conducting an exercise trial in a T1D cohort may face difficulties due to the barriers to exercise for people with T1D [188]. In a qualitative analysis carried out by Lascar et al (2014), T1Dspecific barriers to exercise included low levels of knowledge about managing diabetes and its complications around exercise. Currently underway, the EXTOD education study aims to reduce barriers to exercise in T1D and support effective exercise in people with T1D.

Two studies have examined exercise training in T1D mouse models. Huang et., al (2011) first showed that 6 weeks of exercise training in streptozotocin-induced T1D mice significantly improved insulin content and insulin secretion in islets compared to sedentary mice suggesting an increase in beta cell preservation [184]. A more recent study has examined the effects of exercise training on immune parameters in T1D [189]. Twenty weeks of training in NOD mice resulted in reduced immune cell infiltration into the pancreas and subsequently the insulitis index. This is the only exercise study in a model of T1D to demonstrate the modulatory effects of exercise on islet immunity [189].

The above studies have examined some effects of regular exercise in T1D only. One study has measured haematological changes in people with T1D during acute exercise. Here, they found that lymphocytes, monocytes, granulocytes, and platelets all increase following an exhaustive step test and endurance exercise in people with T1D [190]. However, only total blood counts were measured and not changes in specific immune cell subsets.

Whilst the effects of exercise on immunity has been characterised in healthy cohorts, there is a clear lack of research into the effects of acute exercise on immune parameters in people with T1D. Therefore, this study aimed to explore the effects of exercise on immune cells in T1D. Acute exercise has an abundance of beneficial effects on the immune system which could translate into diseases such as T1D. Firstly, exercise has the potential to modulate immune cells involved in the pathogenesis of T1D i.e. antigen experienced, memory T cells. Secondly, the aims of potential immunotherapies are to reduce aggressive memory phenotypes and inflammation, and promote the generation of new naïve cells. All of which have been shown during acute exercise and exercise training in healthy cohorts. The effects of exercise on immunity in T1D may be therefore used as an adjunct for other immunotherapeutic strategies. For example, agents that target $CD8^+$ T cells may be more effective if administered following a bout of exercise when $CD8^+$ T cells are mobilised into the peripheral circulation.

1.5 Exercise Immunology

A significant amount of evidence on the effects of exercise on immunity has been established outside T1D. Acute exercise causes immune cell mobilisation. The significant exercise intensity-dependant increase in the frequency of lymphocytes following acute exercise is one of the most replicated findings in exercise immunology. Lymphocytosis can then be followed by lymphopenia in healthy cohorts following vigorous intensity exercise [191-196].

There is differential mobilisation within the lymphocyte subsets during acute exercise. Firstly, NK cells are the most responsive lymphocyte subset to acute exercise. Highly differentiated NK cells ($CD56^{dim}CD16^{bright}CD57^+KLRG1^+$) demonstrate the largest percentage increase following exercise [197, 198]. T cells show the next largest percentage increase during exercise, with fully differentiated $CD8^+$ T cells (EMRA) being the most sensitive to mobilisation [199, 200]. Lastly, there is a small increase in $CD4^+$ T cells and B cells [193, 199,

201]. Beta-adrenergic receptor expression is higher on NK and $CD8^+$ T cells than other immune subsets, resulting in their preferential intensity-dependant mobilisation in response to adrenaline during acute exercise [202-205]. Further detail on the mobilisation of lymphocyte subsets during acute exercise is given in subsequent chapters.

Other mononuclear cells also respond to exercise. Monocytes mobilise in an exercise intensity-dependent manner, with mature monocytes ($CD14^{low}$) increasing the most [206-208]. Granulocytes also increase, with the majority of these being neutrophils. Neutrophils increase immediately post exercise and fall below baseline during the 1 hour post exercise recovery period, this is followed by an increase 2 hours post exercise referred to as the "second wave" [209-211]. Vigorous acute exercise also increases hematopoietic stem and progenitor cells (HSPC) post exercise [212, 213].

Dysregulated immunity underlies the pathogenesis and natural history of T1D. Acute exercise modulates immunity through the mobilisation of immune cells. Lymphocyte trafficking and tissue redistribution is essential for immunosurveillance and regulation [191]. The use of animal models demonstrates that T cells are released from the spleen and accumulate in the lungs, bone marrow, and Peyer's patches of mice following acute exercise [214]. The dramatic increase in the frequency of highly differentiated memory T cells following exercise is followed by a larger egress of these subsets from the peripheral blood during the recovery period [200, 215]. Lymphopenia following vigorous exercise is thought to partly occur from T cell apoptosis [216-218]. Vigorous intensity exercise (i.e. above 70% VO_2 max) mobilises memory T cells expressing CD95, a marker of apoptosis [216, 219]. Therefore deletion of memory T cells following acute exercise may create "immunological space" [216-221]. Vigorous intensity exercise also increases hematopoietic stem and progenitor cells (HSPC) and stimulates haematopoiesis [212, 213]. Therefore, vacant immune space following exercise can

be taken up by newly generated immature cells, creating the opportunity to reprogram our immune memory. T1D and beta cell loss is sustained by aggressive memory T cell phenotypes. Acute exercise has the potential to reduce aggressive memory T cell phenotypes in T1D and thus reduce disease severity and beta cell loss.

Amongst the abundance of cellular changes, acute exercise also causes other systemic changes. During acute exercise cytokines, adipokines, myokines, and various hormones are released into the bloodstream which reduce inflammation [222]. Muscle-derived IL-6 is a central myokine secreted by skeletal muscle during and after exercise. IL-6 peaks immediately following exercise [208, 211, 223, 224]. Conventionally, IL-6 is better known for its pro-inflammatory effects; released from pro-inflammatory immune cells and dependant on NFκB pathways. However, intramuscular IL-6 is regulated by pathways such as Ca^{2+}/nuclear factor of activated T cells (NFAT) and glycogen/p38 mitogen-activated protein kinase (MAPK) pathways resulting in the release of IL-6 without the activation of pro-inflammatory pathways [225]. Furthermore, IL-6 release is followed by an increase in regulatory cytokines, IL-10 and IL-1RA, during the recovery period (1-2 hours post-exercise) [223, 224, 226, 227]. This increase is greater than that with IL-1RA treatment used in trials for beta cell preservation in people with T1D. One week treatment with IL-1RA treatment in T1D caused a two-fold increase in adipose tissue IL-1RA. Whereas acute exercise can result in up to 128-fold increase in plasma IL-1RA [227]. However, as this was measured in different tissues, it is difficult to directly compare. IL-6 also stimulates glucose-dependent insulinotropic peptide 1 (GLP-1) secretion from intestinal cells. GLP-1 in turn potentiates insulin secretion [228].

In addition to the above anti-inflammatory effects of exercise, pro-inflammatory adipokine, resistin is reduced [229] and anti-inflammatory adipokine, adiponectin is increased following acute exercise [230-232]. Acute exercise also reduces oxidative stress by lowering reactive

oxygen species (ROS), nitric oxide (NO), and inducible nitric oxide synthase (iNOS) production. It also reduces the markers of oxidative stress, malondialdehyde (MDA) and aggresome-like induced structures [233-235]. Chronic inflammation in T1D promotes beta cell loss of function and destruction; therefore exercise has the potential to preserve beta cell health through modulating beta cell function, reducing inflammation, and reducing oxidative stress. For this reason, it is important that residual beta cell function, measured by C-peptide, still exists in T1D patients in order to increase the initial honeymoon phase following diagnosis through exercise [236].

The effects of acute exercise are well established in healthy and athletic cohorts. However, the effects of acute exercise on immune parameters in a disease state have only recently gained attention. Multiple sclerosis (MS), another T cell mediated autoimmune disorder, displayed normal cytokine responses to acute exercise. Plasma IL-6, TNF-α, and IFN-γ increased following exercise and decreased during the recovery period to a similar extent to control participants [237]. Other autoimmune disorders however, such as rheumatoid arthritis (RA) and systemic lupus (SLE), have abnormal responses to acute exercise. Here, $CD8^+$ T cells are reduced following exercise, and $CD4^+$ T cells are reduced at peak exercise but increased after cessation [238]. In a chronic disorder such as chronic kidney disease (CKD), normal leucocytosis and cytokine responses (IL-6, IL-10 increase) were found following acute exercise [226]. To date, no study has examined the effects of exercise on immune cell subset mobilisation in T1D.

1.6 Hypothesis and Aims

Here, I have set up an acute exercise trial in people with T1D in order to characterise the mobilisation of immune subsets following a moderate (40% VO_2 max) and vigorous (80% VO_2 max) exercise bout. This will be compared to control participants and previous literature in the field of exercise immunology. Evidence from this study will provide insight for future exercise training studies and prospective immunotherapy targets in T1D.

1.6.1 Overarching Hypothesis

Acute exercise will induce intensity-dependent lymphocytosis thereby improving immune regulation in T1D

1.6.2 Overarching Aims

- Examine the safety and clinical effects of acute exercise in people with T1D.
- Characterise the effects of acute exercise on lymphocyte trafficking in T1D.
- Phenotype immune populations mobilised by acute exercise in T1D.

CHAPTER 2. GENERAL METHODS

2.1 EXTOD Mechanisms clinical trial set up

2.1.1 Ethics

Ethical approval was granted by the Preston Research Ethics Committee (REC) (supplementary). The study was sponsored by the University of Birmingham. The final protocol herein for the *EXTOD Mechanisms* study describes the design and organisation of the study. The protocol was maintained by the *EXTOD* Study team at the University Hospitals Birmingham NHS Foundation Trust (UHBFT) and the University of Birmingham (supplementary).

2.1.2 Study Participants

The study aimed to recruit 20 participants with T1D and 20 participants without T1D. No formal upper limit to participant recruitment was specified in this study. This was an exploratory study and formal power calculations were not possible. However, recruitment numbers were based on preliminary work which demonstrated that exercise serum improves proliferation and reduces apoptosis of a mouse insulinoma cell line (MIN6), and that 10 samples were sufficient to demonstrate statistical significance. Screening and enrolment of participants with T1D was supported by medical and research staff, who had a Good Clinical Practice (GCP) certificate and experience in recruiting to studies. All patients with T1D who fulfilled the selection inclusion criteria were invited to participate in the research study, unless they were known to fulfil one of the exclusion criteria. Participants continued to receive their usual diabetes NHS care provided by their hospital consultant or GP during their participation in this study.

2.1.3 Inclusion & Exclusion Criteria

Members of the research team ensured that potential study participants fulfilled the inclusion and exclusion criteria prior to proceeding to obtaining full informed consent.

2.1.3i Inclusion Criteria

1. Adult over 16 and below the age of 65 years of age at the start of the study.
2. Male.
3. Were not achieving the recommended physical exercise guidelines of 150mins moderate intensity exercise plus 2 days of strengthening exercise per week during the study.
4. Both participant and physician felt that they were able to exercise safely.
5. In addition if participants were in the T1D group, they were;
    1. given a clinical diagnosis of T1D
    2. on basal bolus insulin regime or insulin pump therapy
    3. ability to estimate carbohydrate content of meals
    4. willing to test glucose through capillary testing and adjust insulin and carbohydrate doses accordingly
    5. able to recognise hypoglycaemic symptoms before capillary blood glucose falls to 3.9mmol/L

2.1.3ii Exclusion Criteria

1. Unable to provide fully informed consent.
2. A history of cardiac disease.
3. Significant illness or condition (including psychological, psychiatric, developmental or physical disease) that prevented attendance at a study site clinical centre.
4. If it was felt by the principal investigator that participation in the study would not be in the individual's best interest.

5. In addition, if subjects were in the T1D group:
    1. active proliferative diabetic retinopathy
    2. clinically evident autonomic neuropathy
    3. history of severe hypoglycaemia requiring third party assistance within the last 3 months

2.1.4 Consent

All individuals who expressed an interest in participating in the study must have received the current version of the participant information sheet (PIS) (supplementary). At the point that consent was obtained the participant must have had enough time to have read the PIS and to discuss any concerns they may have with family, friends and medical staff. Also, participants were provided with the opportunity to discuss any remaining questions the participant may have had with a member of the research team prior to agreeing to provide their consent. Consent was taken by the approved members of the research team who had a certificate of GCP in line with current international research guidelines. All participants had the right to withdraw their consent at any time and without penalty. Once the form was signed, dated and counter-signed, the baseline assessment proceeded.

2.1.5 Participant Withdrawal

If at any time during the study, a participant fulfilled any of the withdrawal criteria stated below, his participation was discontinued. Wherever possible these reasons were discussed with the principal investigator prior to withdrawing the participant. All participants withdrawn due to an adverse effect were to be followed up until resolution of the event or until it was stable. Withdrawal from the trial did not affect the future provision of diabetes care provided by the primary or secondary care providers. All data collected up until the point the participant

withdrew from the study was kept as part of the study, unless the participant provided written instructions for it to be removed.

Withdrawal Criteria:

1. The participant was unwilling or unable to continue.
2. The participant was lost during follow up or became uncontactable.
3. Participant no longer fulfilled the eligibility criteria (see inclusion and exclusion)

2.1.6 Study Plan for Investigation

The study was divided into 3 phases:

- Screening
- Enrolment and baseline visit
- Exercise study visits

All visits occurred in the National Institute for Health Research (NIHR)/Wellcome Trust Clinical Research Facility (WTCRF) at UHBFT.

2.1.6i Screening and Recruitment

Screening and recruitment involved the identification of individuals who fulfilled the inclusion/exclusion criteria and who may be interested in participating in the study. For the T1D group, this involved approaching clinicians with responsibility of managing patients with T1D and fully informing them about the study. Then seeking a formal agreement for the research team to approach their patients with T1D, either directly in clinic or indirectly through a letter of invitation, to participate in the study. All letters of invitation whether posted or handed directly to the patient in clinic included a PIS. Patients were then asked to contact the research team should they be interested in participating in the study. A reminder follow-up phone call or SMS text message (*"We are recruiting individuals who have diabetes to a clinical*

research study at The University of Birmingham. To find out more call [insert name] on [insert contact number]") was made to patients.

2.1.6ii Enrolment & Baseline visit

This involved the collection of informed consent and baseline information, including participant's demographics and anthropometric assessment. For patients with T1D, HbA1c was checked if not done within the previous 6 weeks.

Food diaries were provided for all participants to record all meals in the 24 hours prior to each exercise visit. Participants were required to disclose any medication (other than insulin for T1D) they were taking during the period of the exercise study. Participants were required to disclose other medical history (other than T1D). Participants were also required to disclose any symptoms of infection or illness in the last 7 days prior to each exercise visit. If infection or illness was present the visit was rescheduled. In addition, blood glucose diaries were provided for participants with T1D. T1D participants were asked to measure their glucose at least 4 times a day during the study (before each meal, before bed, and any time they were feeling unwell or it was appropriate to do so).

All participants completed the Astrand-Rhyming predicted VO_2 max test on the cycle ergometer [239]. This submaximal test was chosen as it is unknown if maximal tests are safe for T1D participants at risk of exercise-induced hypoglycaemia. This was undertaken in the exercise rooms of the NIHR/WTCRF. These values were used to guide the setting of the subsequent exercise study visits where the participant underwent moderate (40% VO_2 max) and vigorous (80% VO_2 max) exercise intensities.

Participants were asked to complete an IPAQ [240] and perceived stress questionnaires; the life scale events questionnaire [241], perceived stress scale [242], the undergraduate stress

questionnaire [243], self-perceived health status [244], and the Pittsburgh sleep quality index [245].

2.1.6iii Exercise study visits

Participants underwent two exercise study visits, each separated by between 7-21 days to allow for recovery (immune changes evident up to 72 hours following exercise [246]) and minimise variation between visits. Participants attended the study site at 8.30 am without having consumed food (water allowed) since midnight the night before. For all participants, any meals consumed within the previous 24 hours should have been repeated for the 24 hours preceding each of the other two study visits to control for variation in the effects of diet between exercise visits. These were recorded in a food diary by all participants. Participants were encouraged to undertake their usual levels of physical activity between study visits, but refrain from exercise for a 24-hour period before an exercise study visit.

Participants with T1D were allowed and encouraged to take their basal insulin (with either pens or insulin pump) as usual in the morning, but not to take any bolus quick acting insulin prior to the visit.

The exercise visits consisted of 30 minutes cycling at either moderate intensity exercise (40% VO_2 max) or vigorous intensity exercise (80% VO_2 max). Individuals cycled at pre-defined workload (wattage) and heart rate for each exercise intensity. Heart rate and RPE [247] were monitored throughout the exercise bout (measurements recovered at 5 minute intervals) and workload was adjusted to keep the heart rate within the pre-defined range (± 2bpm). All exercise was performed at the same speed (50 revolutions per minute (RPM)), with only workload adjusted to correct exercise intensity. The exercise intensity was blinded until the participant arrived at the WT CRF facility on the day of the visit.

The procedure when they arrived at the WT CRF for each study visit involved:

1. If they had T1D, ensure they are safe to exercise according to *EXTOD* exercise guidelines.
2. All participants completed a perceived stress questionnaire for the time between visits.
3. Cannula was inserted into ante cubital fossa and secured by research nurses.
4. Resting blood sample was taken once cannula was inserted.
5. Heart Rate, blood glucose levels and blood pressure were recorded at each full blood sampling time point.
6. Waited for 20 minutes whilst participant was resting.
7. A full blood sample drawn immediately before 30-minute exercise intervention commenced.
8. Participants completed a 5-minute warm up (50 RPM between 25-50 watts) on the cycle ergometer.
9. Blood samples for glucose measurement was taken 10 mins and 20 mins post-exercise commencement.
10. A full blood sample was drawn immediately after exercise cessation.
11. Heart rate and blood pressure was recorded every 15 minutes during the recovery period for 1 hour. If T1D, blood glucose levels were also checked*.
12. A full blood sample was drawn 60 minutes after exercise cessation
13. Breakfast was provided. If T1D, checked blood glucose was stable.

    If T1D, advise patient to test blood glucose 6-8 hours post exercise / consume carbohydrates to avoid post-exercise hypoglycaemia.
14. For the control group, a 4-hour post exercise fasted blood sample was taken if participant agreed to do so.
15. Arranged next visit and paid for travel.

*If a participant's blood sugar dropped to 4mM before 60 minutes post exercise time-point, a blood sample was taken and the participants were fed immediately.

2.1.6iv. Sample collection

A full set of blood samples were taken immediately before exercise, immediately post-exercise, and 1 hour post-exercise. All blood samples were kept on a roller until all samples were ready for simultaneous processing (did not exceed 2 hours). Figure 3 displays the sample collection at each time point. Table 1 displays the outcome measures for the samples collected.

Blood pressure, heart rate, and blood glucose was measured at each time point. A full set of blood samples included 5ml for serum separation, 5ml for plasma separation, 2 ml for plasma with DPPIV-inhibitor, and 20ml for immune based assays. All plasma samples were taken in Ethylenediaminetetraacetic Acid (EDTA) vacuette tubes (454087, Greiner Bio-one GmbH, Frickenhausen, Germany) and stored on ice until processing to prevent cytokine degradation. A separate 2ml blood sample was taken in EDTA tubes with Dipeptidyl Peptidase IV (DPPIV) inhibitor (DPP4, EMD Millipore Corporation, Billerica, MA, USA) added freshly prior to the visit and stored on ice until processing (20µl DPPIV inhibitor and 20µl of 100mM Pefabloc® SC (76307, Sigma-Aldrich, Dorset, UK) added to EDTA vacuette tubes). All serum samples were taken in clotted tubes (456048 VACUETTE® TUBE 6 ml Z Serum Clot Activator, Greiner Bio-one GmbH, Frickenhausen, Germany) and stored upright at room temperature until processing. Blood samples for immune function and phenotyping analysis were taken in lithium heparin vacuette tubes (95057-405, Greiner Bio-one GmbH, Frickenhausen, Germany) and placed on roller at room temperature to ensure constant mixing of the blood sample and processing within 2 hours of blood-draw.

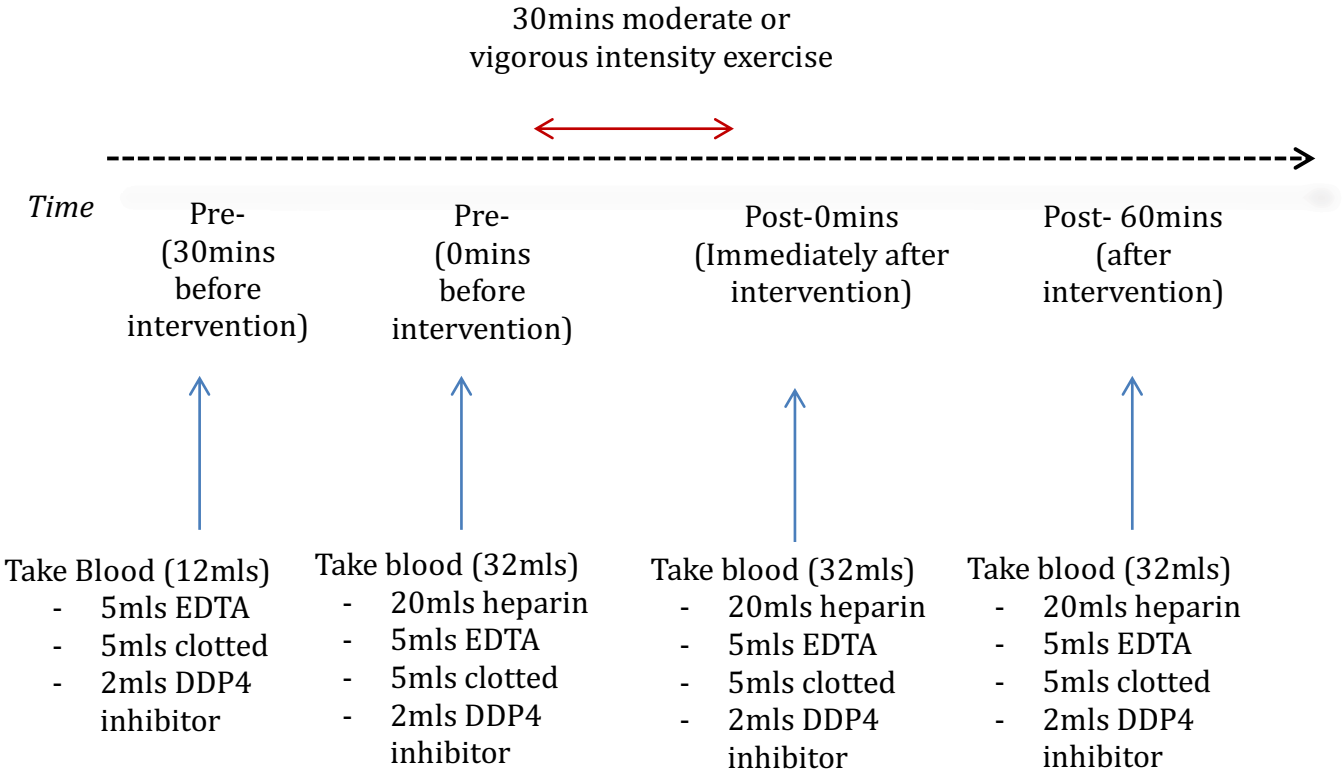

Figure 3 Sample Collection during exercise visits

Sample collection during moderate or vigorous intensity exercise visits.

Volume of blood sample	Fresh samples	Frozen samples
Take 25µl from whole blood assigned for serum separation	Haematocrit- Measurement of Plasma Volume	---
5mls (clotted) 5mls (EDTA) 2mls (EDTA + DDP4 inhibitor)	Plasma and Serum Separation	Cryopreserve plasma and serum for Metabolomics (2 x 500µl aliquots), Luminex (5 x 500µl aliquots) and CMV (200µl aliquot) testing
20mls (Heparin)	FACS (whole blood)- 5 mls Fresh PBMC separation for Transmigration assay ($1x10^6$ lymphocytes) – 15mls	Cryopreserve left over PBMC

Table 1 Sample processing

Sample volume, processing, and storage for moderate or vigorous intensity exercise visits.

2.1.6v Storage and use of samples

Participant samples were collected in the NIHR/WT CRF and transported at the required storage temperatures (heparin and lithium tubes at room temperature, EDTA tubes on ice) to the laboratories of the University of Birmingham where they were processed and analysed. Samples were stored in the laboratories of the University of Birmingham.

2.2. General haematological methods

2.2.1 PBMC isolation

Blood was collected in lithium heparin vacuette tubes (95057-405, Greiner Bio-one GmbH, Frickenhausen, Germany). A density gradient was formed using 5ml of Histopaque-1199 (11191, Sigma-Aldrich, Dorset, UK) and 5ml of Histopaque-1077 (10771, Sigma-Aldrich, Dorset, UK). Ten ml of blood was layered onto the histopaque gradient and centrifuged at 2500RPM for 25 minutes. The PBMC layer (i.e. the buffy coat) was removed carefully using a Pasteur pipette. PBMCs were washed with 12mls of media (M199-2%BSA) and centrifuged for 10 minutes at 1500RPM. Supernatant was removed and cells were washed a second time. Supernatant was removed and cells were be resuspended in 1 ml of media for counting.

2.2.2 Haematological Measures

Haematological measures were conducted on 25µl of whole blood using an automated coulter counter (ABX Micros ES 60, HORIBA Medical). Blood samples were kept on a roller at room temperature until cell counts were performed. An aliquot of 25µl of blood was transferred to a 1.5ml micro-centrifuge tube. Once the coulter counter had warmed up, the micro-centrifuge tube was placed under the needle and the samples were extracted for analysis. This measures the following haematological parameters: total white blood cell (WBC) count, lymphocytes, monocytes, granulocytes, total red blood cell (RBC) count, haemoglobin (HBG), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW) (variation and size of red blood cells), total platelet count, plateletcrit (PCT), mean platelet volume (MPV) , and platelet distribution width (PDW).

2.3 Designing an optimal multi-colour flow cytometry panel

Five panels of 9-12 fluorophore-conjugated antibodies were designed to identify immune populations of interest (Table 2). The fluorophores for each marker were matched based on their level of expression or cell subset size i.e. dimly expressed/small cell subset surface markers were matched with bright fluorophores and highly expressed/large cell subset markers were matched with dim fluorophores. To minimise spectral overlap of the fluorophores they were spread across all possible filters, avoiding close emission wavelengths on the same laser where possible for surface markers expressed together on immune cell subsets (see Figure 4). This was approved by the BD biosciences technical support team (BD Biosciences Scientific Support Europe) as an optimal immunophenotyping panel.

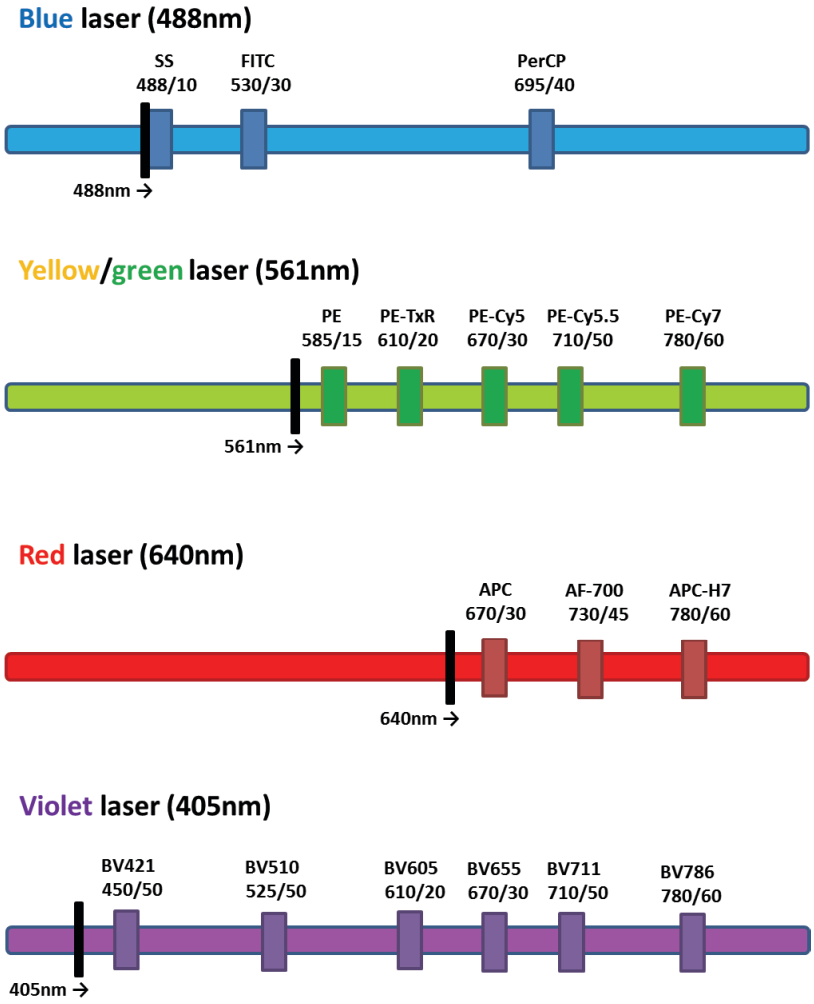

Figure 4 BD LSR Fortessa X-20 Lasers and Filters

Laser and filters available on the BD LSR Fortessa X-20 which fluorophores were spread across to clearly determine immune cell populations.

Panels	Phenotyping markers
Tube 1 - Lineage T cells subsets	CD3, CD8, CD4, CCR7, CD45RA, CD95, CD127, CD11b, CD27, CD28, CD69, Viability
Tube 2 - T-Regulatory and T-helper subsets	CD3, CD4, CCR4, CD45RO, CD127, CXCR3, CCR6, CD25, IL-6R, TIGIT, Viability
Tube 3 - B cells	CD19, CD3, CD10, CD34, CD62L, CD27, CD38, AdipoR1, AdipoR2, Viability
Tube 4 - NK cells	CD56, CD16, CD57, CD3, NKG2A, KIR (CD158a), CD25, CD122, LFA-1 (CD18), Viability

Table 2 Four panels developed for phenotyping immune populations of interest

2.3.1 Antibody conjugation

Lightning-link PerCP-Cy5.5 (763-0005, Innova Biosciences, Cambridge, UK) and Pe-Cy7 tandem conjugation kits (762-0005, Innova Biosciences, Cambridge, UK) were used for anti-AdipoR1 and R2 fluorochrome conjugation.

2.3.2 Whole Blood Staining

Blood was collected in 13 x 75 mm green cap-black ring vacuette heparin tubes (454087, Greiner Bio-one GmbH, Frickenhausen, Germany). Red blood cells were lysed by preparing whole blood in 4ml aliquots and washed with 16mls Ammonium Chloride lysis buffer (16g Ammonium Chloride (326372, Sigma-Aldrich, Dorset, UK), 2g sodium hydrogen carbonate (S/4240/60, Fisher scientific Ltd, Loughborough, UK), 0.2g EDTA (E5134, Sigma-Aldrich, Dorset, UK), and 2L ddH_2O) by centrifuging at 1000g for 5 minutes. Pelleted cells were resuspended in 10mls RPMI-1640 (R0833, Sigma-Aldrich, Dorset, UK) (supplemented with 2% FBS) and washed again. Cells were then counted and resuspended to a concentration of $1x10^6$ cells/ml. Cells were stained with appropriate antibodies according to the panels shown

in Table 1 (antibodies shown in Table 2) and incubated in dark at 4°C for 15-20 mins. Stained cells were lysed and fixed with 500µl 1X BD FACS lysing solution (containing 14% formaldehyde) (349202, BD Biosciences, Wokingham, UK) and incubated in dark at 4°C for 15minutes. Fixed cells were washed in 2mls phosphate-buffered saline (PBS). Pelleted cells were resuspended in 500µl PBS and stored at 4°C until analysis. The stability of fixed stains was assessed and confirmed that cells could be stored up to 24hours at 4°C during the optimisation of the staining conditions. All samples were analysed using flow cytometry (BD LSR Fortessa X-20). Parent populations (i.e. lymphocytes) were gated on based on their size on FSC/SSC. Doublets were omitted by gating on the linear population shown on FSC-A/FSC-H prior to recording. Events to record were set to 100,000 within the parent population gate. Newest compensation set up (see Section 2.3.4) was linked to each experiment. A negative control (unstained whole blood) was run for each experiment.

Notes: all flow cytometry antibodies used were from BD Biosciences (Wokingham, UK) excluding NKG2A (RNDSystems, Oxford, UK), AdipoR1 and AdipoR2 (Phoenix Europe GmbH, Viktoriastraße, Karlsruhe, Germany).

Cat. #	Description	Clone	Reaction	Format
563423	CD3	UCHT1	Hu	PE-Cy7
560179	CD8	SK1	Hu	APC-H7
564975	CD4	RPA-T4	Hu	APC-R700
552176	CD197 (CCR7)	3D12	Hu	PE
563870	CD45RA	HI100	Hu	BV786
562616	CD95	DX2	Hu	BV421
558598	CD127 (IL-7 Receptor α chain)	HIL-7R-M21	Hu	Alexa Fluor 647
562399	CD11b/Mac-1 (CR3)	ICRF44	Hu	PE-CF594
564642	CD27	M-T271	Hu	BB515
563075	CD28 (TLR2)	CD28.2	Hu	BV510
563835	CD69 (Very Early Activation Antigen)	FN50 (also known as FN 50)	Bab, Cyno, Hu, Rhe	BV650
563041	CD56 (N-CAM)	NCAM16.2	Hu	BV510
562293	CD16	3G8	Hu	PE-CF594
565285	CD57	NK-1	Hu	BB515

563183	CD158a	HP-3E4	Hu	BV711
560989	CD25 (IL-2 Receptor α chain)	M-A251	Hu	PE
562887	CD122 (IL-2 Receptor β chain)	Mik-β3	Hu	BV421
551060	CD18 (Integrin b2 chain, CR3/CR4)	6.7	Cyno, Hu, Pig, Rab, Rhe	APC
562579	CD194 (CCR4)	1G1	Hu	BV421
564290	CD45RO	UCHL1	Hu	BV786
562451	CD183 (CXCR3)	1C6/CXCR3	Hu	PE-CF594
563923	CD196 (CCR6)	11A9	Bab, Cyno, Hu, Rhe	BV711
340526	IL-6R	AS12	Hu	FITC
46-9500-41	TIGIT	MBSA43	Hu	PerCP-eFluor710
565388	FVS780	-	-	APC-Cy7
643397	Lineage cocktail 2 (lin 2) CD3, CD14, CD19, CD20, CD56	SK7, MΦP9, SJ25C1, L27, NCAM16.2	Hu	FITC
563362	CD123 (IL-3 Receptor α chain)	7G3	Hu	BV421
563026	CD11c (Integrin αX chain)	B-ly6	Hu	BV510
563883	CD103 (Integrin αE chain)	Ber-ACT8	Hu	APC
563372	CD14	MPhiP9	Hu	BV711
564041	HLA-DR	G46-6	Hu	BV786
564727	CD207 (Langerin)	2G3	Hu	PE
562294	CD19	HIB19	Hu	PE-CF594
563219	CD3 (Leu4)	SK7 (also known as Leu-4)	Hu	BV605
562902	CD10	HI10a	Hu	BV421
555822	CD34 (gp 105-120)	581	Hu	PE
559772	CD62L (L-selectin)	DREG-56	Hu	APC
563965	CD38	HIT2	Hu	BV711
FAB1059C	NKG2A	131411	Hu	PerCP
G-001-44	Adipo R1 (357-375)		Hu	*unconjugated
G-001-23	Adipo R2 (374-386)		Hu	*unconjugated

Table 3 List of fluorophore conjugated antibodies used in immunophenotyping panels (Table 2)

2.3.3 Antibody Titrations

All antibodies were titrated using single stained lysed whole blood ($1x10^6$ cells per stain). The optimal titre of each antibody was chosen where there were distinct negative and positive

peaks. Examples antibody titrations for CD8, CD45RA, CCR6, and CD11b are shown below (Figure 5). In Figure 5 (a), all stains show a separate positive peak, therefore the lower titration i.e 1µl was chose. In Figure 5 (b), 2 µl and 5 µl show a separate positive peak, but the 2µl titration is closer to the negative peak. Here, 5 µl was chosen to ensure the best positive staining.

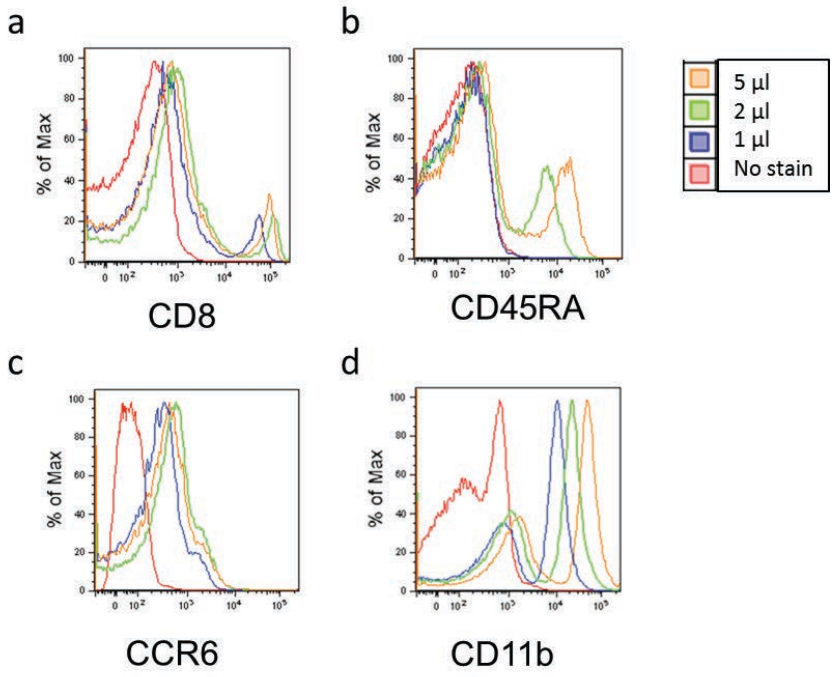

Figure 5 Antibody titration for flow cytometry optimisation

Antibody titrations shown for (a) CD8 (b) CD45RA (c) CCR6 and (d) CD11b.

2.3.4 Compensation

Compensation beads (BD CompBead Set Anti-Ms Ig k, 552843, BD Biosciences, Wokingham, UK) were used to calculate automatic compensation. A negative bead control was used to gate on the bead population using the same FSC/SSC voltages applied for whole blood samples. Voltages for each filter were adjusted on the unstained whole blood and negative beads to ensure all filters were set to negative. Positive beads were stained with an antibody representative of each filter (antibodies for highly expressed markers were chosen for this). Once compensation was calculated, single stained whole blood for each filter were used to

manually test compensation. Spectral overlap and median fluorescence intensity (MFI) were adjusted to correct for under or over e-compensation. The MFI for each fluorophore (excluding the single stain) must be equal to its corresponding MFI for the unstained cells. Adjusting the spectral overlap will increase or decrease the percentage of the fluorophore being taken away from overlapping spectrums to equalise the MFI to that of the unstained. Compensation was performed at least monthly to accommodate for changes in filter excitation and emission.

2.3.5 Fluorescence Minus One controls

Florescence minus one (FMO) controls were used to define gating strategies for the absent antibody population, indicating where the negative and positive populations for this marker should be found. For each panel, one antibody was omitted from the staining procedure, whilst the rest of the antibodies were still included in the panel. This was done for all the antibodies apart from distinct surface markers such as CD3, CD4, CD8, CD19, and CD56. This is because these markers always have a strong positive signal due to their high cell surface expression. Examples for CD95, CD127, CD28, and CD27 are shown in Figure 6. Doublets were omitted by gating on the linear population shown on FSC-A/FSC-H prior to recording. In Figure 6 (d), the unstained peak (red) and the negative peak for the CD27 are different due to the presence of other stains in the CD27 FMO tube. This indicates how an FMO can be used to define gating strategies by knowing where the true negative populations are located. Isotype controls can also be used for the same purpose as FMO controls.

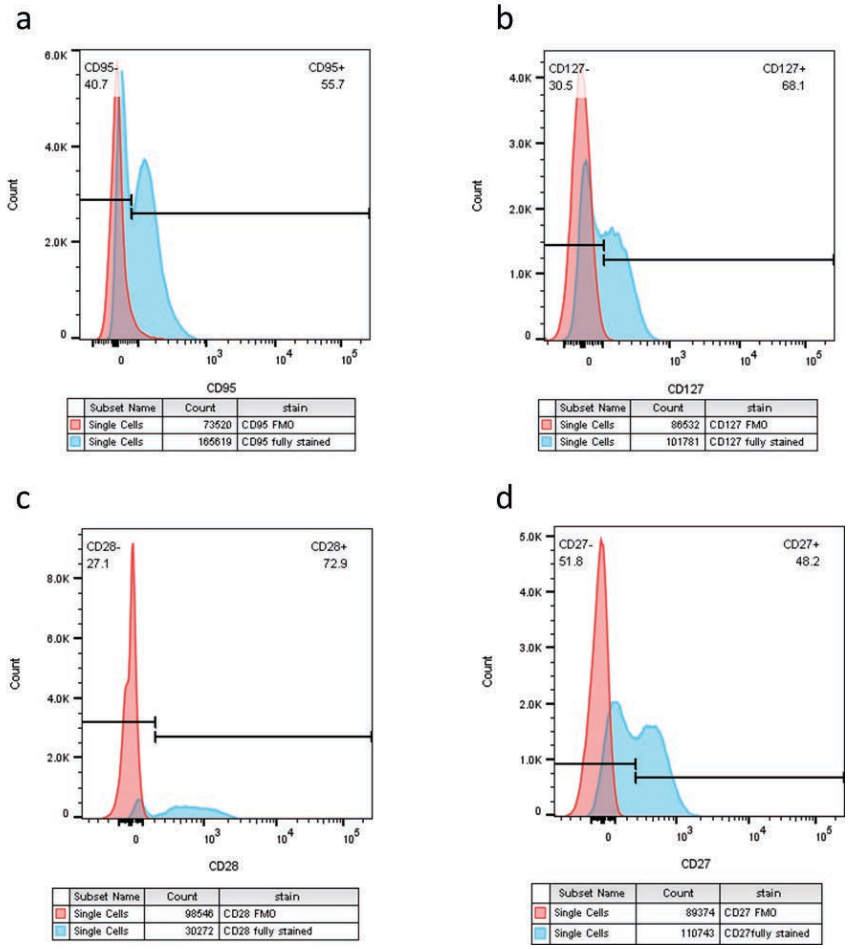

Figure 6 Fluorescent Minus One controls

Florescent minus one controls shown for (a) CD95 (b) CD127 (c) CD28 and (d) CD27.

2.3.6 Whole blood counts

Whole blood counts (cells/µl) to calculate the total cell frequency of subsets measured by flow cytometry were performed as described in Section 2.2.2.

2.3.7 Data analysis

All analysis was performed using FlowJo version 10.2 (FlowJo LLC, Oregon. Gating strategies were optimised through the use of unstained controls and FMO controls (Section 2.3.5). Gating strategies for all populations of interest are represented in subsequent results chapters. Further compensation could be applied on FlowJo to acquire the cleanest data. Population proportions (%) given in flowjo were exported to excel where the relative cell frequencies were calculated

for each sample using coulter counter cell count data. This was done by multiplying the percentage proportions (found through specific population gating) by the total cell count for each main population (e.g. lymphocytes, monocytes etc.). Once total cell frequencies (cells/μl) for each main population were calculated, these were used as the parent population to calculate frequencies of daughter cell subsets.

2.4 Statistical analysis

Statistical analysis was performed using SPSS version 24 (IBM, Chicago) and GraphPad Prism version 7 (GraphPad Software, California). Firstly, normality tests were performed on all data using Q-Q plots in SPSS. Multiple regression analysis was used to analyse within subjects effect (time) and between subjects effects overtime (time*group). Changes over time compared to baseline were also reported in tables for each group under the heading “contrast”. P values were reported as sphericity assumed however where mauchly’s test of sphericity was violated i.e. $p \leq 0.05$, Greenhouse-Geisser corrected value was used. The p values, F values and degrees of freedom (df) are reported in tables as [F = (df, df error) value, p-value]. Variation in n frequencies is a result of a participant having no data for one time-point or no data for the second visit. Baseline data compared between groups was analysed using parametric students T-test. All results are presented as means and SD unless otherwise stated. P values ≤ 0.05 were considered significant.

CHAPTER 3. THE EFFECTS OF ACUTE EXERCISE ON CLINICAL AND HAEMATOLOGICAL OUTCOMES IN TYPE 1 DIABETES

3.1 Introduction

3.1.1 Clinical and physiological outcomes

Clinical and physiological outcomes such as VO_2 max, cytomegalovirus (CMV) status, physical activity levels, and psychological stress levels were measured in the *EXTOD Mechanisms* study. This was conducted in order to validate and stratify our findings during the acute exercise intervention.

VO_2 max, a measurement of individual fitness, is the maximum amount of oxygen that an individual can use during maximal exercise [248-250]. VO_2 max can be measured in a number of ways including treadmill tests, step tests, and the Astrand-Rhyming sub-maximal predicted VO_2 max test [239]. In this study herein, the Astrand-Rhyming sub-maximal cycling performance test was used to predict VO_2 max. A sub-maximal test was chosen as this was deemed safer for T1D participants who were at risk of exercise-induced hypoglycaemia. The Astrand-Rhyming test is consistent and significantly correlates with VO_2 max [251-253]. In comparison to a maximal treadmill test and a step test, the Astrand- Rhyming method was reported as reliable, deviating only by 6% from the maximal treadmill test [253].

CMV infection is highly prevalent amongst the general population [254-256] and affects the mobilisation of lymphocyte subsets during acute exercise [200, 257, 258]. The prevalence of CMV is approximately 60% in the US and up to 80% in the UK, increasing with age [254-256]. CMV prevalence measured between 1991 to 2002 in England and Wales was 15% in those aged 1-4 and 80% in those aged 65 and above [255]. Prior CMV infection increases the mobilisation of lymphocyte subsets and therefore it is important to measure the prevalence amongst participants when comparing between independent groups [258]. Specifically, CMV^+ $CD8^+$ memory T cells are preferentially mobilised during acute exercise, with a two-fold greater increase compared to CMV^- $CD8^+$ memory T cells [200]. Because memory T cell

populations are analysed in the *EXTOD Mechanisms* study it is important to control for CMV status between the groups. In addition to CMV infection, other underlying infections will increase white blood cell counts at rest and therefore skew the baseline lymphocyte numbers. It is important to record recent infection history before conducting the exercise intervention so that this can be considered for data and statistical analysis.

Other factors affecting lymphocyte frequencies include physical activity levels and psychological stress. Physical activity levels can be measured using the International Physical Activity Questionnaire (IPAQ) [240]. Higher baseline lymphocyte frequencies are reported during stress, predominantly in memory $CD4^+$ and $CD8^+$ T cell subsets [259, 260]. As is standard practice in exercise immunology studies and in order to have a true baseline sample, pre-existing life stress can be accounted for using a combination of validated stress questionnaires; the life scale events questionnaire [241], perceived stress scale [242], the undergraduate stress questionnaire [243], self-perceived health status [244], and the Pittsburgh sleep quality index [245]. These questionnaires can be used to compare stress levels between groups, and to enable interpretation of any potential differences in lymphocyte mobilisation. In addition to lifestyle stress, other lifestyle factors such as dietary habits and intolerances may affect immunity. Dietary components can also affect the proportion of lymphocytes at rest. In one study, mice fed carbohydrate-rich and fat-rich diets had higher $CD3^+$ lymphocytes than those mice fed a standard diet [261]. In this study, higher systemic adiponectin in mice fed a fat-rich diet compared to carbohydrate-rich diet was also observed [261]. Adiponectin has anti-inflammatory and anti-diabetic properties, and this further supports the impact of dietary components on immunity. Furthermore, increasing probiotic intake and improving gut health impacts immunity through boosting TReg function [262]. Although dietary habits can be difficult to control in an intervention, food diaries can be supplied to record this information and used for future data and statistical analysis.

3.1.3 Haematological parameters in T1D

Some differences in haematological parameters compared to healthy cohorts have been reported in T1D. Whilst there are no differences in RBC between T1D and healthy cohorts, differences in white blood cells have been described [263-271]. Perturbations in WBC potentially reflect chronic inflammation and disease pathogenesis in T1D. Some studies have reported a decrease in lymphocytes in T1D [264, 265]. Whereas, others have reported no change in lymphocytes in T1D [263]. Differences in monocytes and granulocytes have also been found in T1D. Increased frequency and activation of monocytes has been shown in people with T1D [266-268]. Additionally, peripheral neutropenia has been reported to precede and accompany the onset of T1D [263]. However, increased neutrophil frequencies and neutrophil activity have also been found [269-271]. This increase in neutrophils correlated with risk of vascular disease in T1D [270].

Studies of platelet count in T1D have also been inconsistent [263, 272-274]. Platelet counts were found to be marginally lower in T1D [263]. However, in another study platelets were found to be increased in T1D with poor metabolic controls and this is linked to the risk of cardiovascular events [272]. Platelets from children with T1D show morphological evidence of hyper-reactivity, particularly, higher MPV [272-274]. Therefore, platelet parameters can vary in T1D depending on age, duration of disease, and metabolic control.

3.1.4 Haematological parameters during exercise

The frequency of WBC, lymphocytes, monocytes, and granulocytes all increase during acute exercise in healthy cohorts [275, 276]. Acute exercise causes a significant increase in the frequency of lymphocytes in the peripheral blood, and can be followed by lymphopenia, in healthy cohorts. This increase is intensity-dependent and can double in response to vigorous intensity exercise [191-196]. Monocytes also respond to acute exercise in an intensity-

dependent manner [206, 207]. Mature monocytes ($CD14^{low}$) increased more than regular monocytes ($CD14^{bright}$) during exercise [206, 207]. Monocytes fall below baseline during the recovery period measured at up to 3 hours after exercise cessation [206]. Granulocytes, predominantly neutrophils, increase post exercise. A decrease in neutrophils immediately post exercise followed by a "second wave" increase 2 hours post exercise has been found [209, 210].

Blood flow and RBC measures change during exercise. [277-279]. Physical exercise is characterized by an increase in oxygen consumption by the whole body. During acute exercise, blood is circulated to supply oxygen to the working muscles. RBC carry O^2, bound to haemoglobin, from lungs to tissues, and transport CO_2 back to the lungs for expiration [277, 278]. Haematocrit, the ratio of RBC to total blood volume, increases during exercise as a result of fluid loss from sweating and accumulation of metabolites during exercise, all leading to a decrease in plasma volume [279].

Platelets also mobilise during acute exercise [280-284]. All platelet parameters increased following short exhaustive running exercise (PCT, MPV, Pct, PDW) [280]. The transient increase in platelets is due to platelets released into circulation predominantly from the spleen, and some from the liver and lungs [281-283]. Splenic platelets have a larger MPV and contribute to the increased MPV noted following exercise [281, 283]. Platelets express α2-adrenergic receptors, therefore mobilise in response to adrenaline during exercise [283, 284].

Only one study has measured haematological outcomes in T1D during acute exercise [190]. In the aforementioned study, 16 T1D and 16 control male participants performed two exercise tests; an exhaustive incremental VO_2 max test on a cycle ergometer and an endurance test at 90% of VO_2 max lasting for 45 minutes. It was found that lymphocytes, monocytes,

granulocytes, and platelets all increased following both the exhaustive and the endurance exercise.

Acute exercise causes alterations in haematological measures in healthy individuals, with some evidence of haematological alterations in people with T1D. This study explores changes in clinical and haematological parameters during moderate and vigorous intensity acute exercise in T1D.

3.1.5 Aims

The overarching aim of the *EXTOD Mechanisms* study was to explore physiological and immune changes arising in response to acute exercise in people with T1D.

3.1.6 Specific Aims

In this Chapter, the specific aims were to explore:

1. Explore the feasibility of an acute exercise intervention in T1D.
2. Differences in clinical and haematological parameters at rest in T1D and control participants.
3. Changes in clinical and haematological parameters during and in response to acute exercise in T1D and control participants.

3.2 Methods

3.2.1 Experimental design

Twelve controls and twelve T1D male participants were recruited into the study (as described in Chapter 3). Briefly, participants had one enrolment visit, where an incremental sub-maximal cycle ergometer test was performed to calculate their predicted VO_2 max. This was used to calculate workload and heart rate for subsequent exercise visits adjusted to individual fitness [285]. These two visits consisted of a thirty-minute bout of cycling at either 40% VO_2 max (moderate) or 80% VO_2 max (vigorous). Blood was collected intravenously at baseline, immediately post exercise, and 1 hour post exercise in vacuette heparin tubes (95057-405, Greiner Bio-one GmbH, Frickenhausen, Germany).

3.2.2 Cytomegalovirus ELISA

Anti-CMV IgG was measured in baseline serum (serum separation, section 2.2.1) samples using the Cytomegalovirus (CMV) IgG Enzyme Immunoassay Test Kit (GWB-892399, Genway, 2BScientific, Upper Heyford, UK).

3.2.3 Haematological Measurements

As described in Chapter 2 (Section 2.3.2). Briefly, this measures WBC parameters (lymphocytes, monocytes, granulocytes), RBC parameters (RBC count, HBG, HCT, MCV, MCH, MCHC, RDW), and platelet parameters (platelet count, PCT, MPV, PDW).

3.3 Results

3.3.1 Recruitment

The *EXTOD Mechanisms* study aimed to recruit 20 Caucasian male participants into both the T1D and control group. However, due to time constraints recruitment was limited to 12 participants in each group. Control participants were identified and recruited directly through work relations which may have led to a bias in the control cohort as those most likely to participate were interested in exercise and therefore likely to be fitter than the T1D group.

Although a high number of people with T1D were contacted through clinics, there was a low rate of agreement to enter the study. A total of 63 potential T1D participants were contacted at UHB, of these 48 were contacted through clinics at the Diabetes Research Unit and a further 15 of these were participants original participants in the original EXTOD study in Birmingham. Diabetes clinics at Heartlands and Dudley Trusts were also contacted, however no participants were referred to the study from these sites. To boost recruitment in future it may be worth offering a monetary incentive to cover the cost of time off work which participants needed to attend the study visits.

Efforts were made to increase recruitment. This included requesting contacts through clinical lists at Heartlands and Dudley to recruit T1D participants. To recruit control participants, other departments within the University of Birmingham were approached. Amendments to the inclusion/exclusion criteria were also made to boost recruitment. For example, the age range was increased from 20-40 years to 16-65 years. The ethnicity was changed from white Caucasian only to include all ethnicities. An increase in the recruitment, particularly for T1D participants, is noted following these amendments from January 2017 indicated by the green box in Figure 7 (Figure 7b). Completion of study visits also took longer than predicted as a

result of delayed recruitment. The study was due to finish in April 2017 but was completed in June 2017 with 12 participants in each group (Figure 7c).

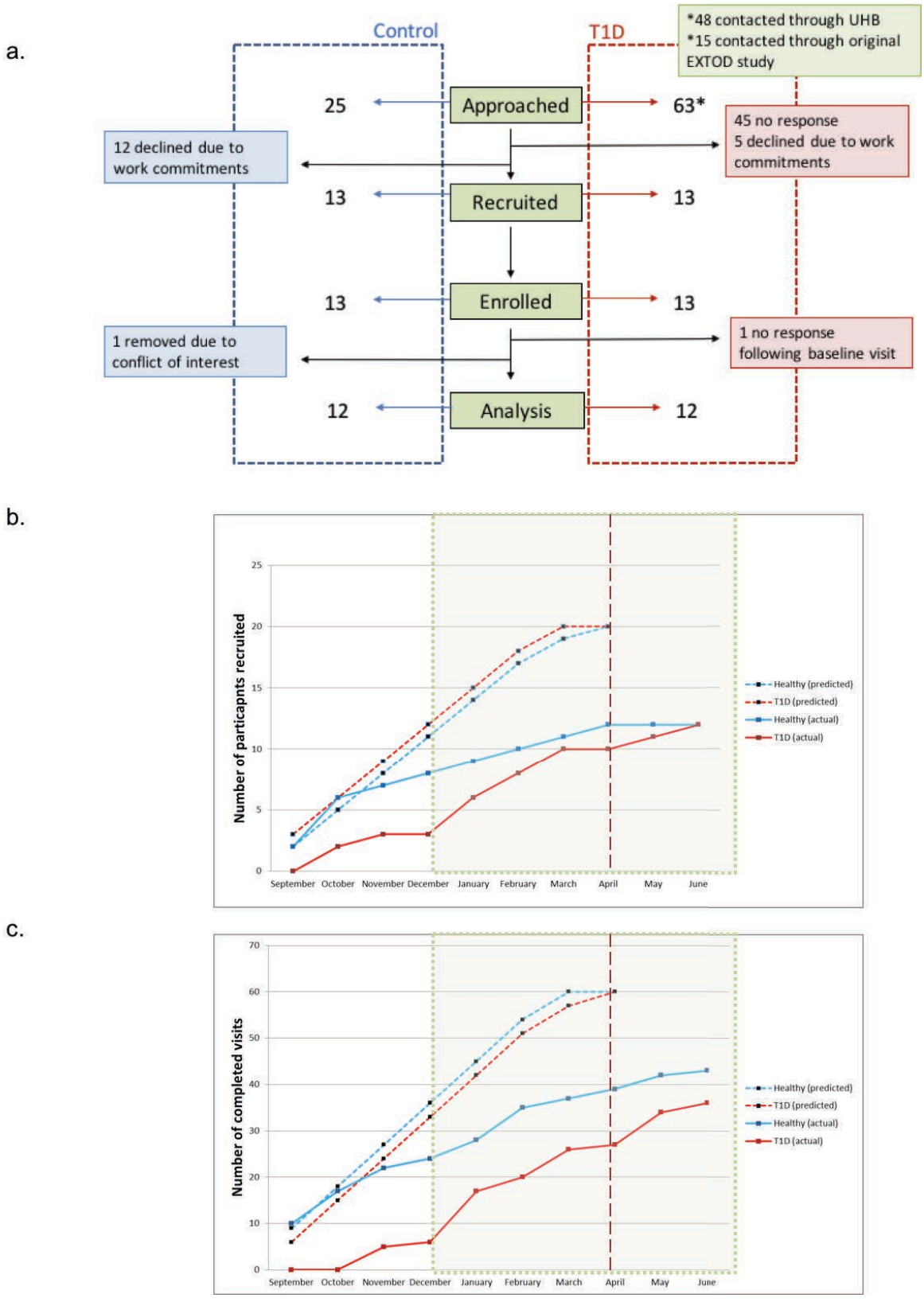

Figure 7 EXTOD mechanisms participant recruitment and visit timeline

(a) CONSORT flow diagram of participant recruitment, enrolment, and analysis (b) Predicted (blue) and actual (red) participant recruitment timeline (c) Predicted (blue) and actual (red) participant visit timeline. Increase in participant recruitment and visits in January 2017 indicated by the green box.

3.3.2 Baseline Characteristics for T1D and control cohorts

Baseline characteristics for both groups are displayed in Table 4. There were no statistically significant differences in demographics or clinical characteristics (age, weight, height, BMI, waist circumference, hip circumference, chest circumference, waist-hip ratio, body fat, and VO_2 max) between T1D and control participants. However, there was a trend for VO_2 max to be lower in the T1D group ($p=0.059$). Although there were no statistically significant differences between groups for age, weight, height, BMI and body fat percentage, the differences observed between groups are clinically significant and this may impact differences in response to exercise between groups.

Within the T1D group there was evident variation in HbA1c levels amongst the participants (65±12.4mmol). There was also evident variation in the disease duration (13.5±8.9 years) (Table 4). These are important factors which may impact on the disparity of responses observed during exercise in the T1D participants and should be taken into account for statistical analysis where possible.

There was no significant difference in CMV index between groups. Both T1D and control groups had an equal proportion of participants that were CMV seropositive (Table 4).

The average number of cigarettes and alcohol units per week for each group was recorded. There was no significant difference between groups (Table 4).

Physical activity (min/wk) was recorded for each participant according to the International Physical Activity Questionnaire (IPAQ) [240]. Job related physical activity was higher in the T1D group (this is mainly due to one participant who cycled as part of his work role). Physical activity which was undertaken by participants as part of transportation was higher in the control group than the T1D group. Physical activity which was undertaken by participants as part of

house maintenance was similar between the two groups. The sport and leisure physical activity was divided into light, moderate, and vigorous physical activity. The T1D group participated in more light and moderate physical activity than the control group. The control group participated in more vigorous physical activity than the T1D group. Overall, the total physical activity was higher in the T1D group. This was driven by one participant who cycled to and from work, as well as during working hours. Furthermore, the time spent sitting (hours/wk) was marginally lower in the T1D group compared to the control group.

Psychological stress scores were recorded for the past year, the past month and for the time passed in between each visit for each participant [241-244]. There were no differences in the stress scores in the past year or the past month between T1D and control groups. There were no differences in the stress scores recorded for the time that passed before each visit between T1D and control groups (Table 4).

Sleep quality was recorded for each participant using the Pittsburgh sleep quality index [245]. No participants from the control group reported abnormal sleeping patterns. However, two participants from the T1D reported an average of less than 4-5 hours sleep per night.

Dietary preferences were recorded for each participant. One participant from the T1D group and one participant from the control group followed a vegetarian diet. One participant from the control group reported lactose and soya intolerance.

Influenza vaccine history was recorded for each participant. Eight participants from the T1D group and four participants from the control group had received an influenza vaccine.

	[1]Control	[2]T1D
	mean± SD	mean± SD
Age (years)	28.8±4.6	33.2±9.7
Weight (kg)	74.5±8.7	80.8±15.6
Height (cm)	156±55	178±7
BMI (kg/m2)	24±2	25±4
Waist circumference (cm)	86±7	90±12
Hip circumference (cm)	90±7	95±8.6
Chest Circumference (cm)	94±4	99±12
Waist-hip ratio	0.95±0.03	0.94±0.06
Body fat (%)	16.6±4.8	21.1±6.2
VO_2 max (mL/(kg·min))	38.5±5.4	32.2±9.3
CMV index	0.42±0.47	0.51±0.5
Glucose (mmol)	5.24±0.48	8.91±3.15
HbA1c (mmol)*		65±12.4
Disease duration (years)*		13.5±8.9
Heart Rate (bpm)	68±8	74±14
Systolic BP (mmHg)	123±8	129±19
Dystolic BP (mmHg)	72±11	79±11
Number of cigarettes (per wk)[#]		
0	10	11
1-5	2	1
Alcohol intake (units per wk)[#]		
0	2	3
1-5	3	4
6-10	4	1
11-20	1	3
21-40	2	0
Job related PA (min/wk)	325±470	557±1221
Transportation PA (min/wk)	313±237	240±190
House Maintenance (min/wk)	129±122	136±129
Sport and Leisure PA (min/wk)	140±86	286±282
Light PA (min/wk)	43±45	81±91
Moderate PA (min/wk)	7.5±19	65±132
Vigorous PA (min/wk)	90±97	140±12

Time sitting (hrs/wk)	48±18	40±12
Stress score (1 year)	4±3	5±4
Stress score (1 month)	10±8	12±10
Stress score (visit 1)	7±10	6±9
Stress score (visit 2)	4±3	4±5

Table 4 Baseline Characteristics of T1D and control participants

Mean and standard deviation values for baseline characteristics in control and T1D participants.
[#] number of participants
[*]T1D only
[1]controls n=12
[2]T1D n=12

3.3.3 Clinical parameters in T1D and control participants

3.3.3.1 Clinical parameters in T1D and control participants at rest

Clinical parameters including heart rate, blood pressure, fasting blood glucose, and VO_2 max were measured at rest in T1D and control participants. The mean and standard deviation are displayed in Table 4. Fasting blood glucose was significantly higher in the T1D group compared to the control group (p=0.001). There was no significant difference in heart rate and blood pressure between groups.

3.3.3.2 Clinical measures in T1D and control participants during a submaximal fitness test

Clinical measures including heart rate and rate of perceived exertion (RPE) [247] were measured during a submaximal fitness test in T1D and control participants. The mean and standard deviation are displayed in Table 5. There was no significant difference in heart rate (bpm), RPE (Borg), or time (minutes) to reach sub maximal target heart rate between groups during the sub-maximal fitness test.

	Time (mins)	Heart Rate (bpm)	RPE (Borg)
	mean±SD	mean±SD	mean±SD
[1]Control	14.5±3.2	162±5	16±1.6
[2]T1D	15.5±2.6	159±8	15.6±2.5

Table 5 Measurements taken during a sub-maximal fitness test in T1D and control participants

Mean and standard deviation values for submaximal fitness test in control and T1D participants.
[1]controls n=12
[2]T1D n=12

3.3.3.3 Clinical parameters in T1D and control participants in response to acute exercise

Clinical parameters including heart rate, blood pressure, fasting blood glucose, and RPE were measured during moderate (40% VO_2) and vigorous (80% VO_2) intensity exercise in T1D and control participants. Clinical measures were recorded pre, post, and 1 hour post-exercise; this is described as the change overtime. The change over time was analysed in groups combined under the heading "time (overall)" and in each group independently (time) displayed in all results tables. The interaction over time between groups was compared and results are displayed under the heading "time*group". Post and 1 hour post-exercise measurements were compared to baseline levels as shown under the heading "contrasts" in the results tables. The mean, standard deviation, and statistical analyses are displayed in Table 6.

As expected, heart rate significantly changed over time during moderate intensity exercise overall ($p<0.001$), and in both the T1D ($p<0.001$) and control group ($p=0.011$) independently. Heart rate was significantly increased post moderate exercise in both the T1D and control group ($p=0.0001$, $p<0.001$ respectively). Heart rate returned to resting levels 1 hour post exercise, with no significant difference compared to baseline in heart rate in either group. Heart rate significantly changed over time during vigorous intensity exercise overall ($p<0.001$), and in both the T1D ($p<0.001$) and control group ($p<0.001$). Heart rate was significantly increased

post vigorous exercise in both the T1D and control group ($p<0.001$, $p<0.001$ respectively). Heart rate returned to baseline 1 hour post vigorous intensity exercise in the T1D with no significant differences compared to baseline in heart rate. However, heart rate in the control group remained above resting levels 1 hour post vigorous intensity exercise ($p=0.005$) (Table 6).

Systolic blood pressure significantly changed over time during moderate intensity exercise overall ($p<0.001$), and in both the T1D ($p=0.007$) and control group ($p=0.038$) independently. Systolic blood pressure was significantly increased post moderate exercise in the T1D ($p=0.029$) but not in the control group. Systolic blood pressure levels returned to baseline 1 hour post moderate intensity exercise, with no significant differences compared to baseline measurements in either group. Systolic blood pressure also significantly changed over time during vigorous intensity exercise overall ($p<0.001$), and in the control group ($p=0.001$), but only a trend to change was seen in the T1D group ($p=0.052$). Systolic blood pressure was significantly increased post vigorous exercise in the control group ($p=0.033$) but not in the T1D group (Table 6).

There was an overall change in diastolic blood pressure during moderate intensity exercise ($p=0.043$), but not in either the T1D or control group independently. No changes in diastolic blood pressure were found during vigorous intensity exercise in either group. This is considered a normal response during acute exercise; systolic BP increases post exercise and decreases following recovery, with no significant changes in diastolic BP [286-288]. Systolic and diastolic blood pressure changes from pre-exercise (0 minutes) to 1 hour post exercise (+60 minutes) in T1D and control participants during moderate and vigorous intensity exercise are shown in Figure 8a (Table 6).

Glucose significantly changed over time in the control group (p=0.002) but not the T1D group during moderate intensity exercise. There was no significant change immediately post moderate exercise, however there was a significant decrease 1 hour post moderate exercise (p=0.009) in the control group only. There was also a significant change over time during vigorous intensity exercise in the control group, but not the T1D group. The response immediately post vigorous exercise was significantly different between groups (p=0.037) (Table 6).

Glucose was also measured during exercise at 10 and 20 minutes after the exercise commenced (Table 7). There was a change in glucose during moderate intensity exercise overall (p=0.045), but this was not seen in the T1D or control group independently. Glucose showed a trend to change at 10 minutes in the T1D group (p=0.052) compared to pre-exercise measurements. No changes were seen at this time point in the control group. Glucose had significantly reduced at 20 minutes into the moderate exercise in both T1D and control groups (p=0.046, p=0.034 respectively). There was a change in glucose during vigorous intensity exercise overall (p=0.046), and in both the T1D (p=0.017) and control group (p=0.004) independently. Glucose was significantly lower at 20 mins into the vigorous exercise in the T1D only (p=0.027). The glucose changes over time were significantly different between groups (time*group) (p=0.002). This is due to the fact that glucose continued to fall in the T1D group throughout the exercise and was lower post exercise compared to pre-exercise levels. However, glucose levels began to rise above baseline in the control group post exercise. Glucose changes from baseline (-20 minutes) to 1 hour post exercise (+60 minutes) in T1D and control participants during moderate and vigorous intensity exercise are shown in Figure 8b.

As expected, the rate of perceived exertion (RPE) significantly changed during moderate intensity exercise overall (p<0.001), and in both the T1D (p<0.001) and control groups

($p<0.001$) independently. RPE was significantly increased immediately post moderate intensity exercise in both T1D and control groups ($p=0.001$, $p<0.001$ respectively). RPE significantly changed during vigorous intensity exercise overall ($p<0.001$), and in both the T1D ($p<0.001$) and control groups ($p<0.001$) independently. RPE was significantly increased immediately post vigorous intensity exercise in both T1D and control groups ($p<0.001$, $p<0.001$ respectively). The above changes in RPE validate that both exercise protocols were completed at the correct intensity. A higher RPE is seen following both moderate and vigorous intensity exercise compared to pre-exercise, with a higher RPE recorded following vigorous intensity exercise. There was no difference in the changes in RPE between groups indicating that both T1D and control participants were exercising at a comparable workload (Table 6).

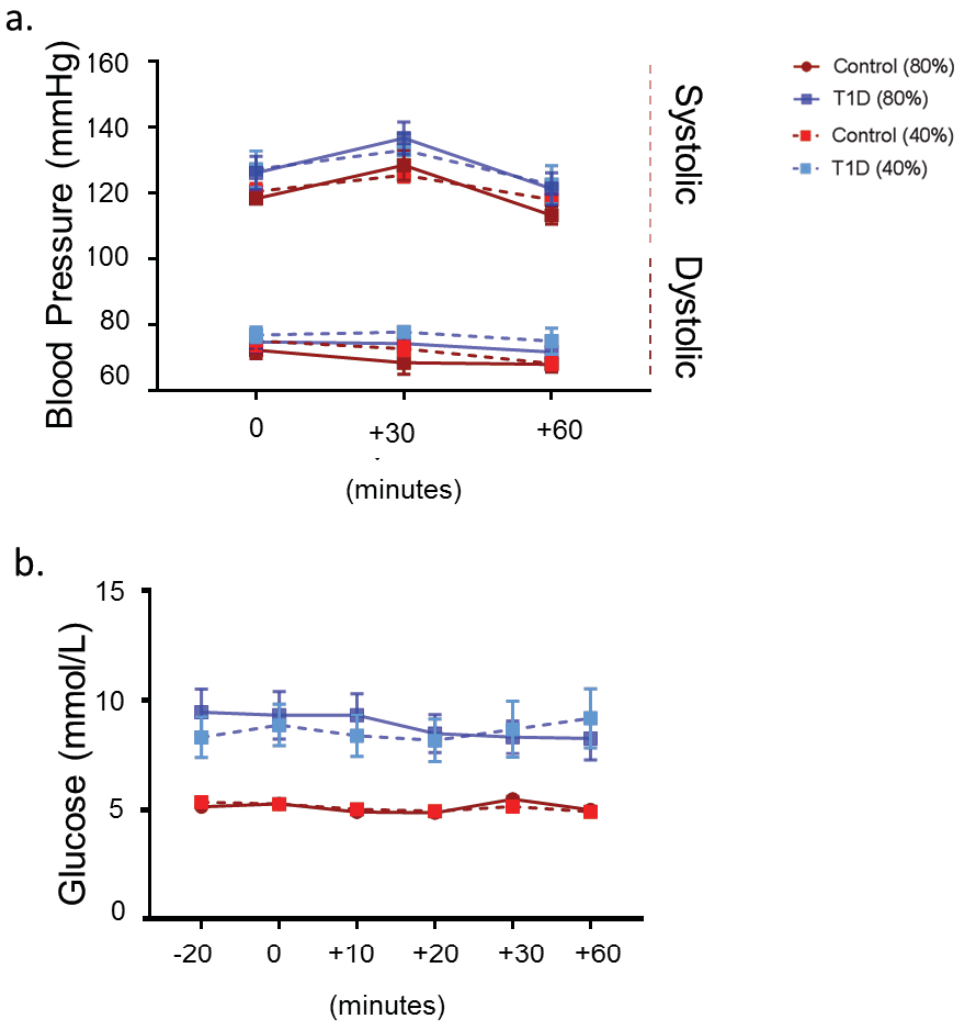

Figure 8 Clinical parameters during moderate and vigorous intensity exercise in T1D and control participants.

Clinical parameters for T1D (blue) and control (red) participants during moderate and vigorous intensity exercise (a) Systolic and Diastolic blood pressure and (b) fasting blood glucose levels. Error bars represent SEM.

Parameter	Intensity	[1]Controls										[2]T1D										[d]Time (overall)	[d]Time*Group
		T1		T2		T3		[b]Time		[c]contrast	[a]Δ%	T1		T2		T3		[b]Time		[c]contrast	[a]Δ%		
		mean	SD	mean	SD	mean	SD					mean	SD	mean	SD	mean	SD						
Glucose (mmol)	**moderate**	5.21	0.38	5.09	0.26	4.86	0.29	**$F_{(2, 12)}$= 10.774, p=0.002**	*T2*	$F_{(1, 6)}$= 1.235, p=0.309	2.27	8.82	2.83	8.62	3.62	9.13	3.56	$F_{(2, 12)}$= 0.192, p=0.828	*T2*	$F_{(1, 6)}$= 0.267, p=0.624	2.24	$F_{(2, 24)}$= 0.429, p=0.656	$F_{(2, 24)}$= 0.656, p=0.528
									T3	**$F_{(1, 6)}$= 14.580, p=0.009**	6.64									*T3*	$F_{(1, 6)}$= 0.001, p=0.980	3.46		
	vigorous	5.32	0.64	5.54	0.36	5.05	0.45	**$F_{(2, 18)}$= 5.271, p=0.016**	*T2*	$F_{(1, 9)}$= 1.923 p=0.199	4.13	9.36	3.43	8.36	2.36	8.30	2.94	$F_{(2, 18)}$= 2.818, p=0.086	*T2*	$F_{(1, 9)}$= 3.599, p=0.090	10.68	$F_{(1.5, 27.1)}$= 3.543, p=0.055	$F_{(1.5, 27.1)}$= 2.470, p=0.116
									T3	$F_{(1, 9)}$= 3.243, p=0.105	5.10									*T3*	$F_{(1, 9)}$= 3.220, p=0.106	11.36		
Heart Rate (bpm)	**moderate**	68.45	9.57	90.58	11.98	70.90	11.23	**$F_{(1.2, 9.5)}$= 9.260, p=0.011**	*T2*	**F(1, 8)= 33.208, p<0.001**	32.33	74.00	12.83	94.10	14.26	74.38	15.29	**F(2, 14)= 21.737, p<0.001**	*T2*	**$F_{(1, 7)}$= 36.458, p=0.001**	27.16	**F(1.3, 19)= 26.945, p<0.001**	$F_{(1.3, 19)}$= 0.269, p=0.766
									T3	$F_{(1, 8)}$= 0.157, p=0.703	3.57									*T3*	$F_{(1, 7)}$= 0.206, p=0.664	0.51		
	vigorous	68.08	8.65	123.00	21.99	73.58	9.02	**F(1.1, 11.9)= 64.206, p<0.001**	*T2*	**F(1, 11)= 79.508 p<0.001**	80.66	78.45	12.21	126.00	16.78	79.60	12.19	**F(2, 18)= 70.657, p<0.001**	*T2*	**F(1, 9)= 125.187, p<0.001**	60.60	**F(1.2, 23.8)= 126.532, p<0.001**	$F_{(1.2, 23.8)}$= 0.697, p=0.436
									T3	**$F_{(1, 11)}$= 12.137, p=0.005**	8.08									*T3*	$F_{(1, 9)}$= 0.085, p=0.777	1.46		
Systolic BP (mmHg)	**moderate**	120.55	7.74	125.50	6.95	118.00	9.54	**$F_{(2, 16)}$= 4.025, p=0.038**	*T2*	$F_{(1, 8)}$= 2.830, p=0.131	4.11	127.30	16.79	133.10	15.33	122.63	15.19	**$F_{(2, 14)}$= 7.188, p=0.007**	*T2*	**$F_{(1, 7)}$= 7.461, p=0.029**	4.56	**F(2, 30)= 11.466, p<0.001**	$F_{(2, 30)}$= 0.790, p=0.463
									T3	$F_{(1, 8)}$= 1.827, p=0.213	2.11									*T3*	$F_{(1, 7)}$= 2.224, p=0.179	3.67		
	vigorous	118.33	6.30	128.50	15.07	113.25	8.63	**$F_{(2, 22)}$= 9.143, p=0.001**	*T2*	**$F_{(1, 11)}$= 5.907 p=0.033**	8.59	126.18	15.87	136.82	15.56	121.40	14.55	$F_{(2, 18)}$= 3.504 p=0.052	*T2*	$F_{(1, 9)}$= 1.748, p=0.219	8.43	**F(2, 40)= 11.230, p<0.001**	$F_{(2, 40)}$= 0.070, p=0.932
									T3	$F_{(1, 11)}$= 4.448, p=0.059	4.30									*T3*	$F_{(1, 9)}$= 1.510, p=0.250	3.79		
Dystolic BP (mmHg)	moderate	75.09	9.80	72.73	7.02	64.27	45.56	$F_{(2, 16)}$= 3.188, p=0.068	*T2*	$F_{(1, 8)}$= 0.124, p=0.734	3.15	76.90	7.11	77.80	5.00	75.00	10.56	$F_{(2, 14)}$= 0.962, p=0.406	*T2*	$F_{(1, 7)}$= 0.285, p=0.610	1.17	**$F_{(2, 30)}$= 3.492, p=0.043**	$F_{(2, 30)}$= 0.332, p=0.720
									T3	$F_{(1, 8)}$= 4.059, p=0.079	14.41									*T3*	$F_{(1, 7)}$= 1.306, p=0.291	2.47		
	vigorous	72.25	8.30	68.42	11.54	67.92	7.68	$F_{(2, 22)}$= 0.710, p=0.502	*T2*	$F_{(1, 11)}$= 0.685, p=0.425	5.31	74.82	9.44	74.27	9.44	71.70	8.12	$F_{(2, 18)}$= 0.890 p=0.428	*T2*	$F_{(1, 9)}$= 0.080, p=0.783	0.73	$F_{(2, 40)}$= 1.261, p=0.294	$F_{(2, 40)}$= 0.171, p=0.844
									T3	$F_{(1, 11)}$= 1.641, p=0.227	6.00									*T3*	$F_{(1, 9)}$= 1.641, p=0.227	4.17		
RPE (Borg)	**moderate**	8.00	1.91	12.17	2.44			**F(2, 22)= 51.780, p<0.001**	*T2*	**F(1, 11)= 123.809, p<0.001**	47.92	7.60	1.56	11.40	2.20			**F(1.3, 11.8)= 22.974, p<0.00**	*T2*	**$F_{(1, 9)}$= 27.563, p=0.001**	36.84	**F(1.4, 28.1)= 68.810, p<0.001**	$F_{(1.4, 28.1)}$= 1.037, p=0.364
									T3	**F(1, 11)= 52.481, p<0.001**	52.08									*T3*	**$F_{(1, 9)}$= 25.186, p=0.001**	50.00		
	vigorous	7.67	1.65	17.42	2.81			**F(2, 22)= 91.914, p<0.001**	*T2*	**F(1, 11)= 127.890, p<0.001**	107.61	7.60	1.11	16.82	2.17			**F(2, 18)= 102.461 p<0.001**	*T2*	**F(1, 9)= 189.897, p<0.001**	101.32	**F(2, 40)= 186.618, p<0.001**	$F_{(2, 40)}$= 0.134, p=0.875
									T3										*T3*				

Table 6 Clinical parameters during moderate and vigorous intensity exercise in control and T1D groups

Mean, standard deviation, and statistical analysis of clinical parameters for control and T1D participants during moderate and vigorous intensity exercise. Significant results highlighted in bold where p values <0.05 were considered significant.

Δ% Percentage change from baseline (T1) to immediately post exercise (T2).

[b] Results were analysed using multiple regression analysis in control and T1D groups independently.

[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).

[d] Results were analysed using multiple regression analysis in control and T1D groups combined.

[1]controls n= 11-12

[2]T1D n= 10-11

Parameter	Intensity	[1]Controls												[2]T1D												[d]Time (overall)	[d]Time*Group
		T1		T1+10mins		T1+20mins		T2		[b]Time		[c]contrast	[a]Δ%	T1		T1+10mins		T1+20mins		T2		[b]Time		[c]contrast	[a]Δ%		
		mean	SD	mean	SD	mean	SD	mean	SD					mean	SD	mean	SD	mean	SD	mean	SD						
Glucose (mmol)	moderate	5.21	0.38	4.97	0.53	4.88	0.35	5.09	0.26	$F_{(3, 27)}$= 2.347 p=0.095	*10*	$F_{(1, 9)}$= 2.022, p=0.189	4.64	8.82	2.83	8.33	2.86	8.12	2.92	8.62	3.62	$F_{(3, 24)}$= 1.718, p=0.190	*10*	$F_{(1, 8)}$= 5.192, p=0.052	5.56	**$F_{(1.9, 33.1)}$= 3.441, p=0.045**	$F_{(1.9, 33.1)}$= 0.460, p=0.630
											30	**$F_{(1, 9)}$= 6.279, p=0.034**	6.40										*30*	**$F_{(1, 8)}$= 5.547, p=0.046**	7.94		
											T2	$F_{(1, 9)}$= 5.063, p=0.051	2.27										*T2*	$F_{(1, 8)}$= 0.695, p=0.429	2.24		
	vigorous	5.32	0.64	4.94	0.30	4.91	0.54	5.54	0.36	**$F_{(3, 30)}$= 5.428, p=0.004**	*10*	$F_{(1, 10)}$= 2.596, p=0.138	7.05	9.36	3.43	9.37	3.07	8.53	2.73	8.36	2.36	**$F_{(1.7, 17.3)}$= 5.549, p=0.017**	*10*	$F_{(1, 10)}$= 0.001, p=0.977	0.10	**$F_{(1.9, 39.4)}$= 3.360, p=0.046**	**$F_{(1.9, 39.4)}$= 7.682, p=0.002**
											30	$F_{(1, 10)}$= 1.313, p=0.278	7.68										*30*	**$F_{(1, 10)}$= 6.688, p=0.027**	8.93		
											T2	$F_{(1, 10)}$= 3.350, p=0.097	4.13										*T2*	$F_{(1, 10)}$= 4.572, p=0.058	10.68		

Table 7 Blood glucose levels during moderate and vigorous intensity exercise in control and T1D groups

Mean, standard deviation, and statistical analysis of blood glucose levels for control and T1D participants during moderate and vigorous intensity exercise. Significant results highlighted in bold where p values <0.05 were considered significant.
Δ% Percentage change from baseline (T1) to immediately post exercise (T2).
[b] Results were analysed using multiple regression analysis in control and T1D groups independently.
[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).
[d] Results were analysed using multiple regression analysis in control and T1D groups combined.
[1]controls n= 11-12
[2]T1D n= 10-11

3.3.3.4 Clinical parameters in T1D and control participants during recovery from acute exercise

Clinical parameters including heart rate and blood pressure were measured during the recovery period in T1D and control participants. The recovery period was defined as the time between the end of the acute exercise bout and 1 hour post cessation of exercise. Measurements were taken every 15 minutes within this period. The mean, standard deviation, and statistical analyses are displayed in Table 8.

As anticipated, heart rate significantly changed during the recovery period following moderate intensity exercise overall ($p<0.001$), and in both the T1D ($p<0.001$) and control group ($p<0.001$) independently. Heart rate was significantly lower compared to post moderate exercise at 15, 30, and 45 minutes post moderate exercise in the T1D ($p=0.011$, $p=0.001$, $p=0.002$ respectively) and control group ($p<0.001$, $p<0.001$, $p<0.001$ respectively). Heart rate significantly changed during the recovery period following vigorous intensity exercise overall ($p<0.001$), and in both the T1D ($p<0.001$) and control group ($p<0.001$) independently. Heart rate was significantly lower compared to immediately post vigorous exercise at 15, 30, and 45 minutes post vigorous exercise in the T1D ($p=0.000$, $p<0.001$, $p<0.001$ respectively) and control group ($p=0.001$, $p<0.001$, $p<0.001$ respectively) (Table 8).

Systolic blood pressure significantly changed during the recovery period following moderate intensity exercise overall ($p=0.001$), and in both the T1D ($p=0.005$) and control group ($p=0.001$) independently. Systolic blood pressure was significantly lower compared to immediately post moderate exercise at 15, 30, and 45 minutes post moderate exercise in the T1D group ($p=0.000$, $p=0.032$, $p=0.002$ respectively). Systolic blood pressure was significantly lower compared to immediately post moderate exercise at 30 and 45 minutes post moderate exercise the control group ($p=0.002$, $p=0.003$ respectively). The change in systolic

blood pressure during the recovery period was significantly different between groups (time*group) (p=0.042). Systolic blood pressure is lower in the control group than the T1D group throughout the recovery period. Systolic blood pressure significantly changed during the recovery period following vigorous intensity exercise overall (p=0.003), and in the T1D group (p=0.002), but this was only seen as a trend to change in the control group (p=0.070). Systolic blood pressure was significantly lower compared to immeidately post vigorous exercise at 15 and 30 post vigorous exercise in the T1D group (p=0.000, p=0.032 respectively) and a trend to be lower at 45 minutes post vigorous exercise (p=0.059). Systolic blood pressure was significantly lower compared to immediately post moderate exercise at 15, 30, and 45 minutes post moderate exercise the control group (p=0.015, p=0.033, p=0.003 respectively) (Table 8).

No changes in diastolic blood pressure were seen during the recovery period following either exercise intensity, in either group (Table 8).

Both T1D and control groups recovered similarly during the recovery period. The above results suggest that T1D and control participants were fully recovered following moderate and vigorous intensity exercise; heart rate and blood pressure returned to resting levels (shown in Table 8) by 1 hour post exercise.

Parameter	Intensity	[1]Controls												[2]T1D												[d]Time (overall)	[d]Time*Group
		T2		T2+15mins		T2+30mins		T2+45mins		[b]Time		[c]contrast	[a]Δ%	T2		T2+15mins		T2+30mins		T2+45mins		[b]Time		[c]contrast	[a]Δ%		
		mean	SD	mean	SD	mean	SD	mean	SD					mean	SD	mean	SD	mean	SD	mean	SD						
Heart Rate (bpm)	**moderate**	90.58	11.98	66.40	8.14	64.40	5.75	64.10	5.97	**F(1.3, 12.1)= 35.250, p<0.001**	*15*	**F(1, 9)= 55.917, p<0.001**	26.70	94.10	14.26	77.38	18.54	72.38	13.36	73.50	14.77	**F(3, 21)= 15.396, p<0.001**	*15*	**$F_{(1, 7)}$= 11.542, p=0.011**	17.77	**F(1.8, 28.5)= 46.672, p<0.001**	$F_{(1.8, 28.5)}$= 0.351, p=0.682
											30	**F(1, 9)= 36.241, p<0.001**	28.91										*30*	**$F_{(1, 7)}$= 32.591, p=0.001**	23.09		
											45	**F(1, 9)= 39.777, p<0.001**	29.24										*45*	**$F_{(1, 7)}$= 22.495, p=0.002**	21.89		
	vigorous	123.00	21.99	82.00	13.57	78.00	8.76	74.40	10.72	**F(1.7, 13.3)= 19.721, p<0.001**	*15*	**$F_{(1, 8)}$= 28.952, p=0.001**	33.33	126.00	16.78	98.27	17.83	86.70	11.38	82.10	12.79	**F(1.9, 17.1)= 51.308, p<0.001**	*15*	**F(1, 9)= 46.451, p<0.001**	22.01	**F(2.2, 37.4)= 77.078, p<0.001**	$F_{(2.2, 37.4)}$= 1.282, p=0.291
											30	**F(1, 8)= 33.309, p<0.001**	36.59										*30*	**F(1, 9)= 104.343, p<0.001**	31.19		
											45	**F(1, 8)= 49.082, p<0.001**	39.51										*45*	**F(1, 9)= 164.406, p<0.001**	34.84		
Systolic BP (mmHg)	**moderate**	125.50	6.95	121.64	10.89	113.64	12.48	116.64	9.65	**$F_{(1.5, 15.1)}$= 14.565, p=0.001**	*15*	$F_{(1, 10)}$= 3.835, p=0.079	3.08	133.10	15.33	124.75	13.87	127.25	14.28	124.88	15.81	**$F_{(3, 21)}$= 5.847, p=0.005**	*15*	**$F_{(1, 7)}$= 7.177, p=0.032**	6.27	**F(3, 51)= 15.544, p<0.001**	**$F_{(3, 51)}$= 2.945, p=0.042**
											30	**$F_{(1, 10)}$= 17.950, p=0.002**	9.45										*30*	**$F_{(1, 7)}$= 22.389, p=0.002**	4.40		
											45	**$F_{(1, 10)}$= 14.581, p=0.003**	7.06										*45*	**$F_{(1, 7)}$= 8.741, p=0.021**	6.18		
	vigorous	128.50	15.07	115.50	11.03	107.75	23.90	113.42	10.19	$F_{(1.5, 16.1)}$= 3.408, p=0.070	*15*	**$F_{(1, 11)}$= 8.281, p=0.015**	10.12	136.82	15.56	120.64	12.87	120.00	10.73	122.30	13.29	**$F_{(3, 27)}$= 6.171, p=0.002**	*15*	**$F_{(1, 9)}$= 10.519, p=0.010**	11.83	**$F_{(1.9, 37.1)}$= 7.135, p=0.003**	$F_{(1.9, 37.1)}$= 0.229, p=0.780
											30	**$F_{(1, 11)}$= 5.900, p=0.033**	16.15										*30*	**$F_{(1, 9)}$= 11.901, p=0.007**	12.29		
											45	**$F_{(1, 11)}$= 13.897, p=0.003**	11.74										*45*	$F_{(1, 9)}$= 4.687, p=0.059	10.61		
Dystolic BP (mmHg)	moderate	72.73	7.02	70.00	8.82	69.45	10.69	72.27	9.28	$F_{(3, 27)}$= 0.888, p=0.460	*15*	$F_{(1, 9)}$= 2.484, p=0.149	3.75	77.80	5.00	76.38	10.42	73.13	8.84	76.38	11.69	$F_{(3, 21)}$= 1.086, p=0.377	*15*	$F_{(1, 7)}$= 0.608, p=0.461	1.83	$F_{(3, 48)}$= 1.772, p=0.165	$F_{(3, 48)}$= 0.352, p=0.788
											30	$F_{(1, 9)}$= 0.919, p=0.363	4.50										*30*	$F_{(1, 7)}$= 0.919, p=0.363	6.01		
											45	$F_{(1, 9)}$= 0.134, p=0.722	0.63										*45*	$F_{(1, 7)}$= 0.134, p=0.722	1.83		
	vigorous	68.42	11.54	69.67	9.37	67.00	8.07	68.92	8.22	$F_{(1.9, 20.6)}$= 0.352, p=0.694	*15*	$F_{(1, 11)}$= 0.158, p=0.698	1.83	74.27	9.44	72.18	10.72	71.70	8.23	71.60	8.20	$F_{(3, 27)}$= 0.755, p=0.529	*15*	$F_{(1, 9)}$= 0.986, p=0.347	2.82	$F_{(2.1, 41.9)}$= 0.561, p=0.583	$F_{(2.1, 41.9)}$= 0.392, p=0.688
											30	$F_{(1, 11)}$= 0.191, p=0.671	2.07										*30*	$F_{(1, 9)}$= 1.119, p=0.318	3.46		
											45	$F_{(1, 11)}$= 0.021, p=0.886	0.73										*45*	$F_{(1, 9)}$= 1.256, p=0.291	3.60		

Table 8 Heart rate and blood pressure during the recovery period following moderate and vigorous intensity exercise in control and T1D groups

Mean, standard deviation, and statistical analysis of heart rate, systolic blood pressure, and diastolic blood pressure for control and T1D participants during moderate and vigorous intensity exercise. Significant results highlighted in bold where p values <0.05 were considered significant.
Δ% Percentage change from baseline (T1) to immediately post exercise (T2).
[b] Results were analysed using multiple regression analysis in control and T1D groups independently.
[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).
[d] Results were analysed using multiple regression analysis in control and T1D groups combined.
[1]controls n= 11-12
[2]T1D n= 10-11

3.3.4 Haematological measures in T1D and control participants

3.3.4.1 Haematological parameters in T1D and control participants at rest

Haematological parameters including white blood cell parameters, red blood cell parameters, and platelet parameters were measured at rest in T1D and control participants. The mean and standard deviation are displayed in Table 9.

3.3.4.1i White blood cells

There was a significantly higher WBC count in the T1D group compared to the control group (p=0.008) (Figure 9a). Within WBC, differences in lymphocytes, monocytes, and granulocytes were noted (Table 9). The percentage of lymphocytes was significantly lower in T1D participants (p=0.002) compared to control participants, with no difference in the frequency observed. The frequency, but not the percentage, of monocytes was significantly higher in the T1D group (p=0.007) compared to control participants. However, the percentage and frequency of granulocytes were significantly higher in the T1D group (p=0.017, p=0.004 respectively) compared to control participants. Therefore, the elevation in WBC and altered WBC proportions in people in our study with T1D was driven by an increase in granulocytes.

3.3.4.1ii Red blood cells

As expected. there was no difference in red blood cell count between groups (Figure 9b). There was no difference in any red blood cell parameters between groups; HGB, HCT, MCV, MCH, MCHC, or RDW (Table 9).

3.3.4.1iii Platelets

There was a significantly higher platelet count in the T1D group (p=0.004) (Figure 9c). The PCT was also significantly higher in the T1D group (p=0.0011). There was no difference in MPV or PDW between groups (Table 9).

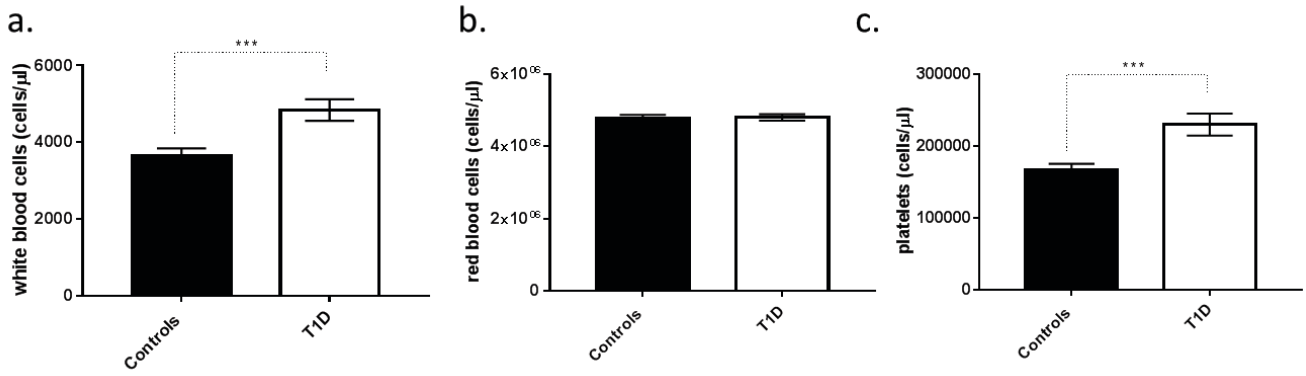

Figure 9 Haematological measures at rest in T1D and control participants

Baseline haematological counts in control and T1D participants (a) frequency of white blood cells (b) frequency of red blood cells(c) frequency of platelets Error bars represent SEM.

Parameter	[1]Controls		[2]T1D	
White Blood Cells	mean	SD	mean	SD
WBC count (cells/µl)	3650	922	4844	1156
Lymphocyte (%)	39.22	5.93	30.09	8.11
Monocyte (%)	6.29	1.44	7.13	2.06
Granulocyte (%)	54.49	6.69	62.80	8.90
Lymphocyte (cells/µl)	1354.17	381.86	1333.33	249.44
Monocyte (cells/µl)	175.00	59.51	294.44	139.33
Granulocyte (cells/µl)	2120.83	611.68	3216.67	1168.21
Red Blood Cells	mean	SD	mean	SD
RBC count (cells/µl)	4793750	407081	4807222	376664
HGB (g/dL)	15.48	1.48	15.15	1.13
HCT (%)	41.85	3.78	41.70	3.34
MCV (L/cell)	87.88	4.38	86.41	3.22
MCH (g/cell)	32.48	1.93	31.65	1.73
MCHC (g/L)	36.99	1.28	36.31	0.71
RDW (%)	13.23	0.72	13.31	0.49
Platelets	mean	SD	mean	SD
PLT count (cells/µl)	167042	38261	229765	61562
MPV (fL)	8.61	0.90	8.12	0.62
PCT (%)	0.14	0.03	0.18	0.04
PDW (%)	15.98	1.46	15.62	1.08

Table 9 Haematological parameters at rest in T1D and control participants

Mean and standard deviation values for haematological parameters at rest in control and T1D participants.
[1]controls n=12
[2]T1D n=10

3.3.4.2 Haematological measures in T1D and control participants in response to acute exercise

Haematological parameters including WBC, RBC, and platelets were measured during moderate (40% VO_2 max) and vigorous (80% VO_2 max) intensity exercise in T1D and control participants.

3.3.4.2i White blood cells

The mean, standard deviation, and statistical analyses for white blood cell parameters are displayed in Table 10.

The WBC count significantly changed over time during vigorous intensity exercise overall ($p<0.001$), and in both the T1D and control group ($p<0.001$, $p<0.001$ respectively). There was a significant increase immediately post vigorous exercise in both T1D and control groups ($p<0.001$, $p<0.001$ respectively). No significant changes were observed 1 hour post vigorous exercise in either group. Moderate intensity exercise also caused a significant change overtime overall ($p<0.001$), and in both the T1D and control groups independently ($p=0.026$, $p=0.023$ respectively). There was a significant increase post moderate exercise in both T1D and control groups ($p=0.019$, $p=0.013$ respectively), and remained significantly elevated above baseline 1 hour post moderate exercise in the control group only ($p=0.007$) (Table 10).

Lymphocytes significantly mobilised during vigorous intensity exercise overall ($p<0.001$), and in both T1D and control groups independently ($p=0.003$, $p=0.000$ respectively). There was a significant increase post vigorous exercise in both T1D and control groups ($p=0.004$, $p<0.001$ respectively), and a significant decrease below baseline 1 hour post exercise in the control group only ($p=0.020$). However, the percentage increase post vigorous exercise was larger in the control group (T1D: 55.5%, control: 71.4%). Moderate intensity exercise caused a significant change over time overall ($p=0.013$) and in the control group ($p=0.019$), but only a

trend to mobilise was seen in the T1D group (p=0.068). There was a significant increase post moderate exercise in the control group only (p=0.034). This is supported by a larger percentage increase post moderate exercise in the controls (T1D: 25.62%, control: 43.9%). No significant changes were found 1 hour post moderate exercise in either group (Table 10).

Monocytes significantly mobilised during vigorous intensity exercise overall (p<0.001), and in both T1D and control groups independently (p=0.004, p<0.001 respectively). There was a significant increase post vigorous exercise in both T1D and control groups (p=0.038, p=0.002 respectively). However, the percentage increase post vigorous exercise was larger in the control group (T1D: 41.38%, control: 63.6%). No significant changes were found 1 hour post vigorous exercise in either group. Moderate intensity exercise caused a significant change over time overall (p=0.005), but this was only seen as a trend to mobilise in the T1D group (p=0.063). No trends to significantly mobilise were seen in the control group. There was a significant increase post moderate exercise in the T1D group (p=0.030), but this was only seen as a trend to increase in the control group (p=0.054). The percentage increase post moderate exercise was similar between groups (T1D: 41.7%, control: 40.9%). No significant changes were found 1 hour post exercise in either group (Table 10).

Granulocytes significantly mobilised during vigorous intensity exercise overall (p<0.001), and in both T1D and control groups independently (p<0.001, p<0.001 respectively). There was a significant increase post vigorous exercise in both T1D and control groups (p<0.001, p<0.001 respectively). However, the percentage increase post vigorous exercise was larger in the control group (T1D: 33.2%, control: 51.6%). Granulocytes remained significantly elevated 1 hour post vigorous exercise in the control group only (p=0.012), returning to baseline in the T1D group. This change overtime during vigorous exercise was significantly different between groups (time*group) (p=0.046). Moderate intensity exercise caused a significant change over time

overall ($p<0.001$) and in the control group ($p=0.009$), but only a trend to mobilise was seen in the T1D group ($p=0.079$). There was a significant increase post moderate exercise in both T1D and control groups ($p=0.013$, $p=0.005$ respectively). The percentage increase post moderate exercise was similar between groups (T1D: 24.4%, control: 32%). The frequency of granulocytes remained significantly elevated 1 hour post moderate exercise in the control group ($p=0.003$). They also remained significantly elevated in the T1D group, but this did not reach statistical significance (Table 10).

Subset (cells/μl)	Intensity	[1]Controls T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast		[a]Δ%	[2]T1D T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast		[a]Δ%	[d]Time (overall)	[d]Time*Group
WBC	**moderate**	3725.00	967.92	5091.67	1849.98	3890.91	1058.69	**$F_{(1, 10.3)}$= 7.076, p=0.023**	*T2*	**$F_{(1, 10)}$= 9.086, p=0.013**	36.69	4566.67	991.07	5744.44	1726.34	5228.57	1890.36	**$F_{(2, 12)}$= 5.035, p=0.026**	*T2*	**$F_{(1, 6)}$= 10.045, p=0.019**	25.79	**F(1.4, 23.1)= 10.803, p<0.001**	$F_{(1.4, 23.1)}$= 0.471, p=0.628
									T3	**$F_{(1, 10)}$= 11.489, p=0.007**	4.45								*T3*	$F_{(1, 6)}$= 2.849, p=0.142	14.49		
	vigorous	3575.00	868.07	5718.18	1054.70	3941.67	946.45	**F(2, 20)= 65.274, p<0.001**	*T2*	**F(1, 10)= 91.528, p<0.001**	59.95	5122.22	1238.98	7144.44	1529.06	5125.00	1186.12	**F(2, 14)= 36.449, p<0.001**	*T2*	**F(1, 7)= 39.947, p<0.001**	39.48	**F(1.5, 25.5)= 97.397, p<0.001**	$F_{(1.5, 25.5)}$= 0.675, p=0.477
									T3	$F_{(1, 10)}$= 4.011, p=0.073	10.26								*T3*	$F_{(1, 7)}$= 0.036, p=0.855	0.05		
Lymphocytes	**moderate**	1308.33	325.21	1883.33	814.28	1336.36	393.75	**$F_{(1.2, 11.2)}$= 4.845, p=0.019**	*T2*	**$F_{(1, 10)}$= 6.029, p=0.034**	43.95	1344.44	275.32	1688.89	766.59	1314.29	203.04	$F_{(1.1, 6.3)}$= 3.390, p=0.068	*T2*	$F_{(1, 6)}$= 2.777, p=0.147	25.62	**$F_{(1.1, 17.6)}$= 7.228, p=0.013**	$F_{(1.1, 17.6)}$= 0.195, p=0.824
									T3	$F_{(1, 10)}$= 0.124, p=0.732	2.14								*T3*	$F_{(1, 6)}$= 5.170, p=0.063	2.24		
	vigorous	1400.00	426.22	2400.00	453.27	1233.33	322.32	**F(2, 20)= 70.685, p<0.001**	*T2*	**F(1, 10)= 83.814, p<0.001**	71.43	1322.22	219.99	2055.56	643.10	1262.50	172.75	**$F_{(1.1, 7.4)}$= 17.502, p=0.003**	*T2*	**$F_{(1, 7)}$= 18.584, p=0.004**	55.46	**F(1.2, 20.1)= 71.426, p<0.001**	$F_{(1.2, 20.1)}$= 1.359, p=0.265
									T3	**$F_{(1, 10)}$= 7.577, p=0.020**	11.90								*T3*	$F_{(1, 7)}$= 2.739, p=0.142	4.52		
Monocytes	**moderate**	183.33	55.28	258.33	118.73	218.18	93.60	$F_{(2, 20)}$= 2.692, p=0.092	*T2*	$F_{(1, 10)}$= 4.776, p=0.054	40.91	266.67	141.42	377.78	209.64	328.57	148.46	$F_{(2, 12)}$= 3.500, p=0.063	*T2*	**$F_{(1, 6)}$= 8.000, p=0.030**	41.67	**$F_{(2, 32)}$= 6.420, p=0.005**	$F_{(2, 32)}$= 0.317, p=0.731
									T3	$F_{(1, 10)}$= 1.000, p=0.341	19.01								*T3*	$F_{(1, 6)}$= 2.077, p=0.200	23.21		
	vigorous	166.67	62.36	272.73	86.24	158.33	49.30	**F(2, 20)= 20.137, p<0.001**	*T2*	**$F_{(1, 10)}$= 18.333, p=0.002**	63.64	322.22	131.47	455.56	149.90	300.00	122.47	**$F_{(2, 14)}$= 8.680, p=0.004**	*T2*	**$F_{(1, 7)}$= 6.481, p=0.038**	41.38	**F(1.4, 23.8)= 25.595, p<0.001**	$F_{(1.4, 23.8)}$= 0.346, p=0.635
									T3	$F_{(1, 10)}$= 2.222, p=0.167	5.00								*T3*	$F_{(1, 7)}$= 1.000, p=0.351	6.90		
Granulocytes	**moderate**	2233.33	720.34	2950.00	1035.62	2336.36	673.22	**$F_{(1.1, 11.1)}$= 9.631, p=0.009**	*T2*	**$F_{(1, 10)}$= 12.982, p=0.005**	32.09	2955.56	878.27	3677.78	1168.83	3585.71	1703.30	$F_{(1.2, 7)}$= 4.091, p=0.079	*T2*	**$F_{(1, 6)}$= 12.294, p=0.013**	24.44	**F(2, 32)= 10.909, p<0.001**	$F_{(2, 32)}$= 1.906, p=0.165
									T3	**$F_{(1, 10)}$= 14.613, p=0.003**	4.61								*T3*	$F_{(1, 6)}$= 3.284, p=0.120	21.32		
	vigorous	2008.33	451.77	3045.45	678.72	2550.00	720.53	**F(2, 20)= 21.282, p<0.001**	*T2*	**F(1, 10)= 58.573, p<0.001**	51.64	3477.78	1349.71	4633.33	1534.06	3562.50	1186.32	**F(2, 14)= 26.387, p<0.001**	*T2*	**F(1, 7)= 61.606, p<0.001**	33.23	**F(2, 34)= 44.692, p<0.001**	**$F_{(2, 34)}$= 3.372, p=0.046**
									T3	**$F_{(1, 10)}$= 9.434, p=0.012**	26.97								*T3*	$F_{(1, 7)}$= 0.229, p=0.647	2.44		

Table 10 White blood cells mobilised during moderate and vigorous intensity exercise in control and T1D groups

Mean, standard deviation, and statistical analysis of white blood cell populations for control and T1D participants during moderate and vigorous intensity exercise.
Significant results highlighted in bold where p values <0.05 were considered significant.
Δ% Percentage change from baseline (T1) to immediately post exercise (T2).
[b] Results were analysed using multiple regression analysis in control and T1D groups independently.
[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).
[d] Results were analysed using multiple regression analysis in control and T1D groups combined.
[1]controls n= 11-12
[2]T1D n= 8-10

3.3.4.2ii Red blood cells

The mean, standard deviation, and statistical analyses for red blood cell parameters are displayed in Table 11.

The RBC count significantly changed over time during vigorous intensity exercise overall (p=0.001), and in the control group (p=0.004). There was a small significant increase post vigorous exercise (11.9%) in the control group (p=0.014). No mobilisation was seen in the T1D group. There were no significant changes during moderate intensity exercise in either the T1D or control group (Table 11).

HGB significantly changed over time during vigorous intensity exercise overall (p=0.001), and in the control group (p=0.012). There was a small significant increase post vigorous exercise (10.2%) in the control group (p=0.016). There were no changes during vigorous intensity exercise in the T1D group. There were no significant changes during moderate intensity exercise in either the T1D or control group (Table 11).

HCT significantly changed over time during vigorous intensity exercise overall (p=0.002), and in the control group (p=0.010). There was a small significant increase post vigorous exercise (12.17%) in the control group (p=0.013). There were no changes during vigorous intensity exercise in the T1D group. During moderate intensity exercise, HCT significantly increased in the T1D group only post moderate exercise (p=0.008). No changes over time or 1 hour post moderate exercise were seen in either group (Table 11).

MCH significantly changed over time during vigorous intensity exercise in the control group (p=0.034), with a small significant decrease (0.55%) post exercise (p=0.007). There were no significant changes in the T1D group. MCH significantly changed over time during moderate intensity exercise in the control group (p=0.019), with a small significant increase immediately

post moderate exercise and 1 hour post moderate exercise (0.74%, 0.99% respectively) (p=0.033, p=0.007 respectively). No changes were seen in the T1D group. This difference in response was significant between groups overall (time*group) (p=0.014), and at both the post-exercise and 1 hour post-exercise time points (p=0.013, p=0.025 respectively) (Table 11).

MCHC significantly changed over time during vigorous intensity exercise overall (p=0.002) and in the control group (p=0.034), but only a trend to change was seen in the T1D group (p=0.062). A small significant decrease (1.07%) post vigorous exercise was seen in the control group (p=0.014). However, small significant increase (1.05%) post vigorous exercise was seen in the T1D group (p=0.046). This difference in response post vigorous exercise was significant between groups (time*group) (p=0.002). There were no significant changes during moderate intensity exercise in either the T1D or control group (Table 11).

RDW significantly changed over time during vigorous intensity exercise in the control group (p=0.010), but only a trend to change was seen overall (p=0.061) and no changes in the T1D group. A small significant increase (2.06%) post vigorous exercise (p=0.004), and a trend to remain elevated 1 hour post vigorous exercise was seen in the control group (p=0.057). There were no changes in RDW in the T1D group during vigorous intensity exercise. There were no significant changes during moderate intensity exercise in either the T1D or control group (Table 11).

Parameter	Intensity	[1]Controls T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast		[a]Δ%	[2]T1D T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast		[a]Δ%	[d]Time (overall)	[d]Time*Group
RBC (cells/µl)	moderate	4935000	309771	4671500	1324942	4887273	487183	$F_{(1.1,\ 10.9)}$= 0.425, p=0.545	*T2*	$F_{(1,\ 10)}$= 0.553, p=0.474	5.34	4745556	335596	3481000	2153043	4985714	538539	$F_{(1.1,\ 6.5)}$= 1.875, p=0.218	*T2*	$F_{(1,\ 6)}$= 1.540, p=0.261	26.65	$F_{(1.1,\ 17.4)}$= 2.736, p=0.114	$F_{(1.1,\ 17.4)}$= 1.092, p=0.317
									T3	$F_{(1,\ 10)}$= 0.573, p=0.467	0.97								*T3*	$F_{(1,\ 6)}$= 0.752, p=0.419	5.06		
	vigorous	4652500	442232	5209091	744757	4667500	504631	**$F_{(2,\ 20)}$= 7.577, p=0.004**	*T2*	**$F_{(1,\ 10)}$= 8.956, p=0.014**	11.96	4868889	404377	4997778	358136	4578750	421113	$F_{(1.1,\ 7.9)}$= 3.242, p=0.108	*T2*	$F_{(1,\ 7)}$= 0.407, p=0.544	2.65	**$F_{(2,\ 34)}$= 8.342, p=0.001**	$F_{(2,\ 34)}$=1.983, p=0.153
									T3	$F_{(1,\ 10)}$= 0.037, p=0.851	0.32								*T3*	$F_{(1,\ 7)}$= 1.905, p=0.210	5.96		
HGB (g/dL)	moderate	15.85	1.65	16.31	1.59	16.00	2.36	$F_{(1.3,\ 13.2)}$= 0.413, p=0.587	*T2*	$F_{(1,\ 10)}$= 0.729, p=0.413	2.89	14.99	0.94	15.43	1.02	15.42	1.45	$F_{(1.1,\ 5.2)}$= 0.496, p=0.519	*T2*	**$F_{(1,\ 6)}$= 19.612, p=0.007**	2.92	$F_{(2,\ 30)}$= 0.684, p=0.474	$F_{(2,\ 30)}$= 0.103, p=0.847
									T3	$F_{(1,\ 10)}$= 0.038, p=0.849	0.95								*T3*	$F_{(1,\ 6)}$= 0.366, p=0.572	2.86		
	vigorous	15.11	1.18	16.73	1.69	15.07	1.32	**$F_{(1.3,\ 13.4)}$= 7.390, p=0.012**	*T2*	**$F_{(1,\ 10)}$= 8.325, p=0.016**	10.72	15.29	1.26	15.76	0.79	14.48	1.17	$F_{(1,\ 7.3)}$= 3.401, p=0.105	*T2*	$F_{(1,\ 7)}$= 0.593, p=0.466	3.05	**$F_{(2,\ 34)}$= 8.792, p=0.001**	$F_{(2,\ 34)}$= 1.427, p=0.254
									T3	$F_{(1,\ 10)}$= 0.032, p=0.863	0.28								*T3*	$F_{(1,\ 7)}$= 1.715, p=0.232	5.32		
HCT (%)	moderate	42.90	4.13	43.96	3.01	43.04	5.92	$F_{(1.3,\ 13.9)}$= 0.231, p=0.699	*T2*	$F_{(1,\ 10)}$= 0.364, p=0.560	2.47	41.00	2.53	42.50	2.94	42.57	4.12	$F_{(1.1,\ 5.3)}$= 0.747, p=0.433	*T2*	**$F_{(1,\ 5)}$= 17.868, p=0.008**	3.66	$F_{(1.5,\ 21.9)}$= 0.644, p=0.488	$F_{(1.5,\ 21.9)}$= 0.209, p=0.744
									T3	$F_{(1,\ 10)}$= 0.001, p=0.970	0.32								*T3*	$F_{(1,\ 5)}$= 0.549, p=0.492	3.82		
	vigorous	40.79	3.05	45.75	5.41	40.79	3.75	**$F_{(1.3,\ 13.1)}$= 8.038, p=0.010**	*T2*	**$F_{(1,\ 10)}$= 9.125, p=0.013**	12.17	42.32	3.82	43.19	2.66	39.86	3.53	$F_{(1.1,\ 7.6)}$= 2.618, p=0.146	*T2*	$F_{(1,\ 7)}$= 0.173, p=0.690	2.05	**$F_{(2,\ 34)}$= 7.901, p=0.002**	$F_{(2,\ 34)}$= 2.178, p=0.129
									T3	$F_{(1,\ 10)}$= 0.003, p=0.961	0.00								*T3*	$F_{(1,\ 7)}$= 1.725, p=0.230	5.81		
MCV (L/cell)	moderate	88.00	4.60	88.25	4.30	88.27	4.59	$F_{(2,\ 20)}$= 1.681, p=0.211	*T2*	$F_{(1,\ 10)}$= 1.000, p=0.341	0.28	86.00	3.24	86.25	3.23	84.67	2.49	$F_{(2,\ 10)}$= 0.294, p=0.687	*T2*	$F_{(1,\ 5)}$= 0.294, p=0.611	0.29	$F_{(2,\ 30)}$= 0.888, p=0.422	$F_{(2,\ 30)}$= 0.754, p=0.479
									T3	$F_{(1,\ 10)}$= 3.378, p=0.096	0.31								*T3*	$F_{(1,\ 5)}$= 0.000, p=1.000	1.55		
	vigorous	87.75	4.15	88.27	4.47	87.67	4.23	$F_{(2,\ 20)}$= 1.957, p=0.167	*T2*	$F_{(1,\ 10)}$= 1.957, p=0.192	0.60	86.78	3.15	86.44	3.34	87.13	3.22	$F_{(2,\ 14)}$= 1.145, p=0.346	*T2*	$F_{(1,\ 7)}$= 2.032, p=0.197	0.38	$F_{(2,\ 34)}$= 0.074, p=0.928	$F_{(2,\ 34)}$= 2.988, p=0.064
									T3	$F_{(1,\ 10)}$= 0.000, p=1.000	0.09								*T3*	$F_{(1,\ 7)}$= 0.000, p=1.000	0.40		
MCH (g/cell)	**moderate**	32.44	1.98	32.68	2.07	32.76	1.93	**$F_{(2,\ 20)}$= 4.889, p=0.019**	*T2*	**$F_{(1,\ 10)}$= 8.692, p=0.015**	0.74	31.39	1.17	31.30	1.33	30.65	0.83	$F_{(2,\ 10)}$= 1.486, p=0.272	*T2*	$F_{(1,\ 5)}$= 1.839, p=0.233	0.28	$F_{(2,\ 30)}$= 0.362, p=0.699	**$F_{(2,\ 30)}$= 4.950, p=0.014**
									T3	**$F_{(1,\ 10)}$= 6.131, p=0.033**	0.99								*T3*	$F_{(1,\ 5)}$= 1.984, p=0.218	2.35		
	vigorous	32.53	1.88	32.35	1.95	32.35	1.72	**$F_{(2,\ 20)}$= 4.014, p=0.034**	*T2*	**$F_{(1,\ 10)}$= 11.645, p=0.007**	0.55	31.89	2.08	31.59	1.31	31.65	1.04	$F_{(2,\ 14)}$= 0.587, p=0.569	*T2*	$F_{(1,\ 7)}$= 0.374, p=0.560	0.94	$F_{(1.3,\ 22.3)}$= 1.762, p=0.199	$F_{(1.3,\ 22.3)}$= 0.366, p=0.608
									T3	$F_{(1,\ 10)}$= 3.159, p=0.106	0.54								*T3*	$F_{(1,\ 7)}$= 0.744, p=0.417	0.75		
MCHC (g/L)	moderate	36.93	1.50	37.03	1.42	37.14	1.33	$F_{(2,\ 20)}$= 0.611, p=0.553	*T2*	$F_{(1,\ 10)}$= 1.006, p=0.340	0.27	36.50	0.86	36.31	0.64	36.27	0.59	$F_{(2,\ 10)}$= 0.760, p=0.493	*T2*	$F_{(1,\ 5)}$= 1.106, p=0.341	0.51	$F_{(2,\ 30)}$= 0.030, p=0.970	$F_{(2,\ 30)}$= 1.271, p=0.295
									T3	$F_{(1,\ 10)}$= 0.564, p=0.470	0.55								*T3*	$F_{(1,\ 5)}$= 0.455, p=0.530	0.64		
	vigorous	37.04	1.01	36.65	1.23	36.93	1.09	**$F_{(2,\ 20)}$= 4.886, p=0.019**	*T2*	**$F_{(1,\ 10)}$= 8.878, p=0.014**	1.07	36.13	0.48	36.51	0.55	36.35	0.43	$F_{(2,\ 14)}$= 3.404, p=0.062	*T2*	**$F_{(1,\ 7)}$= 5.898, p=0.046**	1.05	$F_{(2,\ 34)}$= 0.035, p=0.963	**$F_{(2,\ 34)}$= 7.889, p=0.002**
									T3	$F_{(1,\ 10)}$= 1.171, p=0.305	0.29								*T3*	$F_{(1,\ 7)}$= 0.697, p=0.431	0.60		
RDW (%)	moderate	13.28	0.76	13.43	0.61	13.44	0.59	$F_{(2,\ 20)}$= 0.385, p=0.685	*T2*	$F_{(1,\ 10)}$= 0.636, p=0.444	1.13	13.25	0.46	13.43	0.65	13.35	0.48	$F_{(1.1,\ 5.4)}$= 0.412, p=0.561	*T2*	$F_{(1,\ 5)}$= 0.558, p=0.489	1.32	$F_{(2,\ 30)}$= 0.777, p=0.469	$F_{(2,\ 30)}$= 0.021, p=0.979
									T3	$F_{(1,\ 10)}$= 0.728, p=0.414	1.22								*T3*	$F_{(1,\ 5)}$= 0.251, p=0.638	0.75		
	vigorous	13.19	0.68	13.46	0.66	13.41	0.61	**$F_{(2,\ 20)}$= 5.916, p=0.010**	*T2*	**$F_{(1,\ 10)}$= 14.157, p=0.004**	2.06	13.37	0.51	13.38	0.48	13.25	0.32	$F_{(2,\ 14)}$= 0.185, p=0.833	*T2*	$F_{(1,\ 7)}$= 0.121, p=0.738	0.08	$F_{(2,\ 34)}$= 3.051, p=0.061	$F_{(2,\ 34)}$= 1.940, p=0.159
									T3	$F_{(1,\ 10)}$= 4.608, p=0.057	1.64								*T3*	$F_{(1,\ 7)}$= 0.074, p=0.794	0.87		

Table 11 Red blood cells mobilised during moderate and vigorous intensity exercise in control and T1D groups

Mean, standard deviation, and statistical analysis of red blood cell measures for control and T1D participants during moderate and vigorous intensity exercise. Significant results highlighted in bold where p values <0.05 were considered significant.
Δ% Percentage change from baseline (T1) to immediately post exercise (T2).
[b] Results were analysed using multiple regression analysis in control and T1D groups independently.
[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).
[d] Results were analysed using multiple regression analysis in control and T1D groups combined.
[1]controls n= 11-12
[2]T1D n= 8-10

3.3.4.2iii Platelets

The mean, standard deviation, and statistical analyses for platelet parameters are displayed in Table 12.

Vigorous intensity exercise caused a significant change over time overall ($p<0.001$), and in both the control and T1D groups independently ($p=0.000$, $p=0.001$ respectively). There was a small significant increase post vigorous exercise in both T1D and control groups ($p=0.008$, $p=0.016$ respectively). Both groups showed a similar percentage increase post vigorous exercise (T1D: 16.4%, control: 20.8%). No significant changes were seen 1 hour post vigorous exercise in either group. The platelet count significantly changed over time during moderate intensity exercise overall ($p<0.001$), and in both the control and T1D groups independently ($p=0.006$, $p=0.009$ respectively). There was a small significant increase post moderate exercise in both the T1D and control groups ($p=0.016$, $p=0.039$ respectively). Both groups showed a similar percentage increase post moderate exercise (T1D: 11.2%, control: 13.6%). No significant changes were seen 1 hour post exercise in either group (Table 12).

MPV significantly changed over time during vigorous intensity exercise overall ($p<0.001$), and in both the control and T1D groups independently ($p=0.004$, $p=0.048$ respectively). There was no significant increase post vigorous exercise in either the T1D or control group, however there was a significant drop below baseline 1 hour post vigorous exercise in the T1D group ($p=0.009$). Moderate intensity exercise caused a significant change over time overall ($p=0.017$), and in the control group ($p=0.013$), but not the T1D group. MPV fell (by 2%) significantly below baseline post moderate exercise in the T1D group ($p=0.033$), no changes immediately post moderate exercise were seen in the control group. This difference in response post moderate exercise showed a trend to be different between groups (time*group) ($p=0.059$). However, MPV fell (by 2.38%) significantly below baseline 1 hour post moderate exercise in

the control group only (p=0.002). No significant changes 1 hour post moderate intensity exercise were seen in the T1D group (Table 12).

PCT significantly changed over time during vigorous intensity exercise overall (p<0.001), and in both the control and T1D groups independently (p<0.001, p<0.001 respectively). There was a significant increase post vigorous exercise in both T1D and control groups (p=0.003, p=0.009 respectively). The percentage increase post vigorous exercise was similar between groups (T1D: 18.5%, control: 23.2%). There was a significant fall below baseline in both the T1D group (p=0.049) and a trend to decrease in the control group 1 hour post vigorous exercise (p=0.068). Moderate intensity exercise caused a significant change over time overall (p<0.001), and in both the control and T1D groups independently (p=0.009, p=0.004 respectively). There was a significant increase post moderate exercise in both T1D and control groups (p=0.043, p=0.034 respectively). The percentage increase post moderate exercise was similar between groups (T1D: 9.3%, control: 14%). PCT significantly fell below baseline 1 hour post moderate exercise in the T1D group (p=0.037), but not in the control group (Table 12).

PDW significantly changed over time during vigorous intensity exercise overall (p=0.012), but this was not seen in either group independently. There was a significant increase (2.94%) post vigorous exercise in the control group (p=0.027), but not in the T1D group. No significant changes were seen 1 hour post vigorous exercise in either group. There were no significant changes during moderate intensity exercise in either the T1D or control group (Table 12).

Parameter	Intensity	[1]Controls T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast		[a]Δ%	[2]T1D T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast		[a]Δ%	[d]Time (overall)	[d]Time*Group
Platelet count (cells/µl)	moderate	162667	39942	184833	39057	150364	35405	**$F_{(2, 20)}$= 6.087, p=0.009**	*T2*	**$F_{(1, 10)}$= 5.630, p=0.039**	13.63	229250	44654	255000	47671	202333	18776	**$F_{(2, 10)}$= 8.724, p=0.006**	*T2*	**$F_{(1, 5)}$= 12.945, p=0.016**	11.23	**F(2, 30)= 13.788, p<0.001**	$F_{(2, 30)}$= 0.555, p=0.580
									T3	$F_{(1, 10)}$= 0.963, p=0.350	7.56									*T3*	$F_{(1, 5)}$= 3.693, p=0.113	11.74		
	vigorous	171417	35973	207091	39223	158917	29652	**$F_{(2, 20)}$= 10.526, p=0.001**	*T2*	**$F_{(1, 10)}$= 8.369, p=0.016**	20.81	230222	73388	267889	83479	221125	73062	**F(2, 14)= 15.338, p<0.001**	*T2*	**$F_{(1, 7)}$= 13.489, p=0.008**	16.36	**F(2, 34)= 22.073, p<0.001**	$F_{(2, 34)}$= 0.137, p=0.872
									T3	$F_{(1, 10)}$= 2.543, p=0.142	7.29									*T3*	$F_{(1, 7)}$= 0.882, p=0.379	3.95		
MPV (fL)	moderate	8.64	0.84	8.69	0.97	8.44	0.97	**$F_{(1.3, 12.8)}$= 7.493, p=0.013**	*T2*	$F_{(1, 10)}$= 0.427, p=0.528	0.58	8.13	0.60	7.96	0.57	7.92	0.71	$F_{(1.1, 5.5)}$= 1.361, p=0.298	*T2*	**$F_{(1, 5)}$= 8.571, p=0.033**	2.00	**$F_{(2, 30)}$= 4.696, p=0.017**	$F_{(2, 30)}$= 1.507, p=0.238
									T3	**$F_{(1, 10)}$= 18.519, p=0.002**	2.38									*T3*	$F_{(1, 5)}$= 2.238, p=0.195	2.56		
	vigorous	8.58	0.95	8.63	0.88	8.43	0.93	**$F_{(2, 20)}$= 3.551, p=0.048**	*T2*	$F_{(1, 10)}$= 1.202, p=0.299	0.51	8.12	0.64	8.23	0.69	7.88	0.73	**$F_{(2, 14)}$= 8.171, p=0.004**	*T2*	$F_{(1, 7)}$= 1.579, p=0.249	1.37	**F(2, 34)= 11.270, p<0.001**	$F_{(2, 34)}$= 0.867, p=0.429
									T3	$F_{(1, 10)}$= 2.608, p=0.137	1.84									*T3*	**$F_{(1, 7)}$= 13.000, p=0.009**	3.04		
PCT (%)	moderate	0.14	0.04	0.16	0.04	0.13	0.04	**$F_{(2, 20)}$= 7.225, p=0.004**	*T2*	**$F_{(1, 10)}$= 6.067, p=0.034**	14.03	0.18	0.02	0.20	0.02	0.16	0.01	**$F_{(2, 10)}$= 10.866, p=0.009**	*T2*	**$F_{(1, 5)}$= 7.225, p=0.043**	9.25	**F(2, 30)= 15.892, p<0.001**	$F_{(2, 30)}$= 0.873, p=0.428
									T3	$F_{(1, 10)}$= 1.361, p=0.270	9.06									*T3*	**$F_{(1, 5)}$= 7.982, p=0.037**	15.10		
	vigorous	0.15	0.03	0.18	0.04	0.13	0.02	**F(2, 20)= 13.922, p<0.001**	*T2*	**$F_{(1, 10)}$= 10.250, p=0.009**	23.22	0.18	0.05	0.22	0.06	0.17	0.05	**F(2, 14)= 24.594, p<0.001**	*T2*	**$F_{(1, 7)}$= 19.842, p=0.003**	18.49	**F(2, 34)= 31.271, p<0.001**	$F_{(2, 34)}$= 0.009, p=0.991
									T3	$F_{(1, 10)}$= 4.186, p=0.068	8.77									*T3*	**$F_{(1, 7)}$= 5.631, p=0.049**	7.37		
PDW (%)	moderate	16.13	1.27	15.53	1.32	15.01	1.94	$F_{(2, 20)}$= 2.584, p=0.100	*T2*	$F_{(1, 10)}$= 3.899, p=0.077	3.72	15.79	0.92	16.13	1.57	16.15	1.25	$F_{(2, 10)}$= 0.728, p=0.507	*T2*	$F_{(1, 5)}$= 0.953, p=0.374	2.14	$F_{(2, 30)}$= 0.340, p=0.714	$F_{(2, 30)}$= 2.421, p=0.106
									T3	$F_{(1, 10)}$= 4.564, p=0.058	6.97									*T3*	$F_{(1, 5)}$= 0.821, p=0.406	2.30		
	vigorous	15.82	1.61	16.28	1.76	15.55	1.52	$F_{(2, 20)}$= 2.521, p=0.106	*T2*	**$F_{(1, 10)}$= 6.702, p=0.027**	2.94	15.48	1.18	15.56	0.88	14.81	1.10	$F_{(2, 14)}$= 3.190, p=0.072	*T2*	$F_{(1, 7)}$= 0.014, p=0.910	0.50	**$F_{(2, 34)}$= 5.021, p=0.012**	$F_{(2, 34)}$= 0.776, p=0.468
									T3	$F_{(1, 10)}$= 0.274, p=0.612	1.69									*T3*	$F_{(1, 7)}$= 3.127, p=0.120	4.30		

Table 12 Platelets mobilised during moderate and vigorous intensity exercise in control and T1D groups

Mean, standard deviation, and statistical analysis of platelet measures for control and T1D participants during moderate and vigorous intensity exercise. Significant results highlighted in bold where p values <0.05 were considered significant.
Δ% Percentage change from baseline (T1) to immediately post exercise (T2).
[b] Results were analysed using multiple regression analysis in control and T1D groups independently.
[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).
[d] Results were analysed using multiple regression analysis in control and T1D groups combined.
[1]controls n= 11-12
[2]T1D n= 8-10

3.4 Discussion

The first aim of this study was to measure clinical and haematological parameters at rest in T1D and control participants. Participants in each group were closely matched for gender, age, and BMI to minimise differences in these parameters. The clinical measures showed that fasting blood glucose levels were significantly higher in the T1D group compared to the control group. There was also a trend for VO_2 max to be lower in the T1D group even though their total physical activity levels were higher. The haematological measurements showed a higher WBC count in the T1D group compared to the control group. Within the WBC, the proportions were skewed by a substantial increase in the proportion and frequency of granulocytes in the T1D group. A higher platelet count was also found in the T1D group.

This study also aimed to investigate the effects of moderate and vigorous intensity exercise on clinical and haematological parameters in T1D and control participants. During exercise, heart rate and RPE increased with exercise intensity as expected. Higher heart rates and RPE were detected in the vigorous compared to the moderate intensity exercise bout as anticipated. These observations validate the intensity of both acute exercise protocols. Systolic BP increases during exercise as expected however it is only significant in the control group. During the recovery period, all parameters returned to basal levels by 1 hour post exercise. This indicates that participants from both the T1D and control group fully recovered to a similar degree by the 1 hour time point. Glucose responses were different in T1D and control participants. Glucose levels decreased initially in the control group, but then began to increase before exercise cessation. However in the T1D group, glucose levels continued to fall during the exercise. This provides insight into the clinical factors such as heart rate, blood pressure, and fasting blood glucose responses that should be considered during vigorous exercise in people

with T1D. These observations indicate that acute vigorous intensity exercise is safe in people with T1D as recorded for the first few hours following the exercise bout.

There were a number of limitations in this study. Although data was collected for lifestyle factors by providing glucose and food diaries, these only covered a short time period of 24 hours prior to the exercise visits and did not provide data for these factors in between visits. Additionally, due to time constraints, the data collected was not analysed and therefore was not used to account for any differences between participants or groups. Measuring glucose in the 24 hours following the exercise visit would provide insight into the safety of vigorous exercise in T1D participants.

A number of haematological changes occurred during exercise. Firstly, during vigorous intensity exercise all WBC populations increased in frequency immediately following exercise in both groups. However, the percentage increase of lymphocytes following vigorous exercise was lower in the T1D group. Yet, monocytes and granulocytes increased following exercise to a similar degree in both groups. Therefore, this blunted response is specific to lymphocytes in T1D participants. Secondly, all RBC parameters were affected by vigorous exercise in the control group, but not in the T1D group. However, the percentage increases for all RBC parameters are small and unlikely to contribute to a difference in effects of exercise between groups. Lastly, there was an increase in platelet count during moderate and vigorous exercise in both groups. Altogether, the WBC response during exercise was much higher than that of RBC and platelet measures.

Previous studies have also demonstrated elevated granulocytes and platelets in T1D. Elevated neutrophils in T1D have been reported, and this is linked to an increased risk of vascular disease [269-271]. An elevated platelet count in T1D is linked to poor metabolic control and increase

in the risk of cardiovascular events [272]. Higher MPV has also been found in children with T1D [273, 274].

During acute exercise, a number of cellular changes occur within WBC, RBC, and platelet subsets in healthy cohorts. It is established that the frequency of WBC, lymphocytes, monocytes, and granulocytes all increase during acute exercise in healthy cohorts [206, 207, 209, 210, 275, 276]. In this study, leucocytosis was demonstrated in both the T1D and control group. Specifically, acute exercise caused a significant intensity-dependent increase in the frequency lymphocytes in the peripheral blood, followed by lymphopenia, in healthy cohorts [191-196]. Here, lymphocytosis following vigorous intensity exercise occured in both the T1D and control group. However lymphocytosis was marginally blunted in the T1D group. One other study has measured haematological outcomes in T1D during acute exercise [190]. An increase in lymphocytes, monocytes, and granulocytes was found following exhaustive and endurance exercise. In the aforementioned study, lymphocytosis was found to a similar extent in both groups (T1D: 120%, controls: 133%). However, the exercise bout was performed at a higher intensity than the exercise conducted in the *EXTOD Mechanisms* study and therefore cannot be directly compared.

Increases in RBC parameters during exercise in healthy cohorts have also been previously demonstrated [277-279, 289]. Likewise, platelets increase following exercise in heathy cohorts. All platelet parameters increase (PCT, MPV, Pct, PDW), mainly accounted for by platelets released from the spleen during exercise [280-284]. One other study has shown increased platelets following acute exercise in T1D [190].

The findings from this analysis provide evidence of a typical immune response to acute exercise in people with T1D. The data from this chapter also provides validation of the exercise protocol used in the study, as physiological and clinical parameters responded as expected

during both moderate and vigorous intensity exercise bouts. A detailed analysis of the effects of acute exercise on lymphocyte subsets are explored in Chapters 4-6.

CHAPTER 4. THE EFFECTS OF ACUTE EXERCISE ON LYMPHOCYTE MOBILISATION AND TRAFFICKING IN TYPE 1 DIABETES

4.1 Introduction

Lymphocyte trafficking and mobilisation is involved in immune surveillance and regulation. Acute exercise induces changes in lymphocyte trafficking and mobilisation. Lymphocyte subsets, NK cells, and $CD8^+$ T cells exhibit the largest mobilisation during acute exercise in healthy individuals. However, the effects of moderate or vigorous acute exercise on the mobilisation of lymphocyte subsets in T1D is not fully known.

4.1.1 Lymphocytes

Human lymphocytes are comprised of T cells, B cells, and NK cells. T cells are formed and mature in the thymus. Phenotypically identified by co-expression of T cell receptor (TCR) and CD3, T cells are the most abundant lymphocyte population [290, 291]. $CD3^+$ T cells are made up of highly heterogeneous $CD4^+$ and $CD8^+$ populations. Through their diverse TCR they recognise foreign antigen. B cells, derived from pluripotent haematopoietic stems cells, are formed and mature in the bone marrow. They are the smallest lymphocyte population and are phenotypically defined as $CD3^-CD19^+$ [291]. B cells mediate humoral immunity through the secretion of antigen specific antibodies [292].

NK cells, making up 15% of lymphocytes, are derived from common lymphoid progenitor populations and are formed in the bone marrow [293, 294]. NK cells act as a first line of defence and are critical for immune surveillance. Unlike, T and B cells, they rapidly respond to foreign antigen without the need for priming. NK cells are phenotypically identified as $CD3^-CD56^+$ [291, 295]. NK cells can be further divided based on their CD16 expression into functionally different subsets; $CD56^{dim}CD16^{bright}$, $CD56^{bright}CD16^{dim}$, $CD56^{dim}CD16^{dim}$ $CD56^{bright}CD16^-$, and $CD56^-CD16^+$ [296]. $CD56^{dim}CD16^{bright}$ are the most abundant NK cell subset in the peripheral blood, making up to 90% of the NK population [294]. $CD56^{dim}CD16^{bright}$ NK cells are derived from $CD56^{bright}$ NK cells but are much more phenotypically heterogeneous.

$CD56^{dim}CD16^{bright}$ NK cells express high levels of killer cell immunoglobulin-like receptors (KIR) and perforin granules, rendering them the superior cytotoxic subset [296, 297]. In contrast, $CD56^{bright}CD16^{dim}$ are predominantly found in secondary lymphoid tissues and do not express KIR [294]. $CD56^{bright}CD16^{dim}$ NK cells express higher CD62L for migration to secondary lymph nodes [197]. $CD56^{bright}CD16^{dim}$ NK cells secrete a diverse repertoire of proinflammatory cytokines such as TNF-α and IFN-γ to counteract their poor cytotoxic capabilities [297]. More recently identified, $CD56^{dim}CD16^{dim}$ represent a high percentage of immature cells denoted by lack of CD57 expression and are $NKG2A^{+}$ [298]. Less is known about the other two minor NK cells subsets: $CD56^{bright}CD16^{-}$ and $CD56^{-}CD16^{+}$. $CD56^{bright}CD16^{-}$ NK cells have been found in psoriasis and express high levels of chemokine receptors [299]. However, another study suggested a regulatory role for this subset [300]. Very little is known about the function of the $CD56^{-}CD16^{+}$ NK cell subset. However, it has been found to be expanded in human immunodeficiency virus (HIV) infection with lower cytotoxic activity and cytokine secretion compared to $CD56^{+}$ NK cell subsets [301].

4.1.2 Lymphocyte Trafficking

Lymphocyte trafficking is essential for immunosurveillance and efficient immune responses. Peripheral blood lymphocytes are recruited to sites of inflammation during an immune response by chemoattraction [302, 303]. During lymphocyte recruitment, also described as lymphocyte homing, inflamed tissues also release cytokines and chemokines which guide lymphocytes to the target site [304, 305]. Lymphocytes express homing receptors that mediate lymphocyte trafficking such as LFA-1, CXCR3, members of the integrin family (CD11a, CD11b, CD11c), and an array of chemokine receptors such as CCR4, CCR6, CCR7 [305-307]. Expression of homing receptors allows lymphocytes to respond to cytokines and chemokines in circulation [308, 309]. Whereas blocking of homing receptors blunts lymphocyte recruitment [310-312]. Once recruited, trans-endothelial migration (TEM) can then occur in four

coordinated steps; rolling, activation, arrest and diapedesis (Figure 10) [313, 314]. Endothelial cells (EC) on the inflamed tissue, also referred to as the target tissue, upregulate cellular adhesion receptors such as MAdCAM-1, ICAM-1, vascular cell adhesion protein 1 (VCAM-1), P-Selectin, and E-Selectin [304, 315, 316]. Lymphocytes slow down and roll while lymphocyte adhesion molecules bind to the above corresponding receptors on endothelial cells. P-selectin mediates initial contact and rolling of lymphocytes. Beta-2 integrins on lymphocytes interact with VCAM-1 on the EC which results in rolling and tethering of the lymphocyte [317]. Sphingosine-1-phosphate receptor 1/4 (S1PR1/4), necessary for tissue retention, is now upregulated on the lymphocyte surface [318-320]. Together, this results in stable lymphocyte arrest, allowing for lymphocyte cytoskeleton rearrangement and spreading across the endothelium mediated by prostaglandin D2 (PGD2) [310, 314, 321]. Diapedesis, also referred to as extravasion or TEM, into the target tissue can then occur. Preferentially memory T cells are recruited through endothelium [310, 315, 322, 323]. It is predominantly the chemokine receptor CXCR3 on memory T cells that mediates TEM [322]. This is supported by evidence of blockade of CXCR3, which results in reduced TEM of total $CD3^+$ T cells [324]. CD18, which can form complexes with CD11a and CD11b to form LFA-1 and Macrophage-1 antigen (Mac-1) respectively, also mediates transmigration [325, 326].

Lymphocyte migration can be studied using static TEM assays. This is done by layering peripheral blood lymphocytes (PBL) over activated endothelium monolayers [327-329]. PBLs can migrate across endothelium monolayers in minutes, migrating back and forward continuously [330]. Activation of human endothelium monolayers with inflammatory stimuli induces upregulation of ICAM-1, VCAM-1, P-selectin, and E-selectin, as well as chemokines CXCL9, CXCL10, and CXCL11 [315, 316, 331, 332]. P-selectin and E-selectin are essential for the initial lymphocyte arrest, evidenced by inhibited adhesion through blocking of these receptors [332]. Mac-1 and LFA-1 on lymphocytes bind to ICAM-1, and $CXCR3^+$ lymphocytes

bind to CXCL9, CXCL10, and CXCL11 on endothelial cells in these static TEM models [322, 325, 326].

Using the static TEM assay, a novel adiponectin-dependent mechanism of TEM regulation was identified by Myriam Chimen et al., (2015) (Figure 10). Adiponectin is an adipokine with anti-inflammatory, anti-diabetic, and insulin sensitizing properties [333, 334]. It signals through its receptors, adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2) [335, 336]. AdipoR1 and AdipoR2 are expressed by monocytes, B cells, and NK cells, but not T cells [337]. B cells respond to adiponectin in circulation through these receptors resulting in secretion of a homeostatic molecule, PEPITEM (peptide inhibitor of trans-endothelial migration). PEPITEM binds to cadherin-15 (CDH15) expressed on stimulated endothelium resulting in the release of sphingosine-1-phosphate (SIP) from endothelial cells. SIP binds to its receptors, S1PR1/4, on lymphocytes to down-regulate LFA-1 expression, preventing lymphocyte binding to endothelial adhesion molecules and therefore preventing TEM. Lymphocyte homing to the endothelial is however unaffected as chemotactic responses to CXCL10, and PGD2 were unaltered by adiponectin treatment [315].

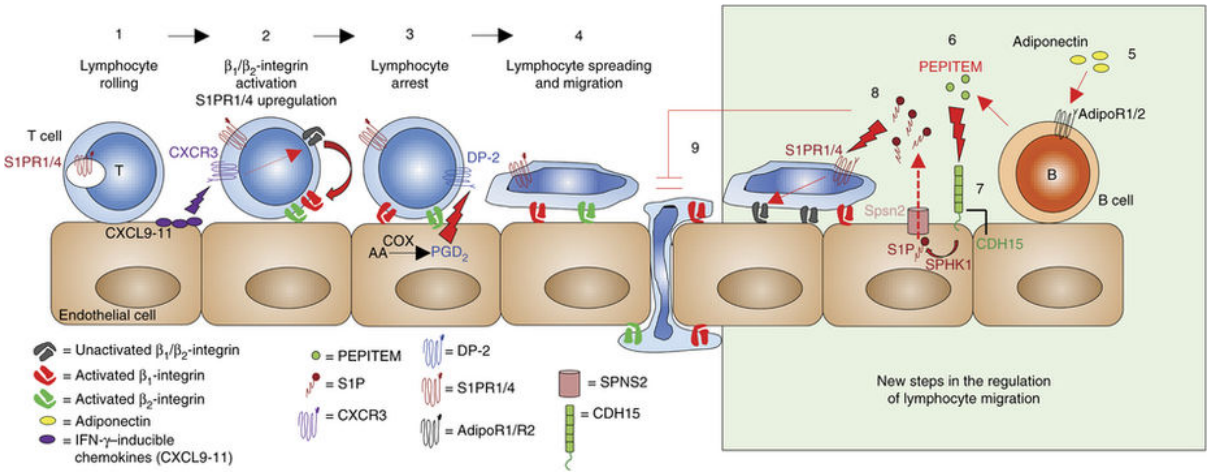

Figure 10 Transendothelial migration and regulation via PEPITEM

Transendothelial migration and regulation by the B cell-adiponectin-PEPITEM axis as shown by Myriam Chimen et al., (2015) [315].

4.1.3 Lymphocyte trafficking in T1D

The pancreatic endothelium in T1D is chronically activated due to ongoing beta cell damage and inflammation. Adhesion molecules ICAM-1, VLA-4, CD44, PNAd, and MAdCAM-1 are upregulated by inflamed islets in T1D, increasing pancreatic lymphocyte trafficking [126, 134, 338]. Beta cells also secrete the chemokine ligands CXCL9, CXCL10, CCL2, CCL3, CCL17, CCL21, and CCL22 in NOD mice [128, 137-139]. Corresponding adhesion molecules and homing receptors expressed by T cells allow for tissue homing and TEM to the islet. Lymphocytes found within NOD pancreas were $CD69^+$ and $CD103^+$, markers of tissue resident memory subsets, and they expressed markers of adhesion; LFA-1, VLA-4, CD11b, and CCR7 [86, 126, 127, 191]. $CD11b^+$ $CD4^+$ and $CD8^+$ T cells have also been found within the NOD islet [127, 134]. Beta cell specific T cells display a stem cell like memory (T_{SCM}) phenotype [339]. T_{SCM} express CCR7 and may contribute to the $CCR7^+$ islet population [340].

Alterations in NK cell frequency in the peripheral blood has not been well characterised in T1D. A higher proportion of peripheral blood $CD56^{dim}CD16^+$ NK cells were identified in

recent-onset and long-standing T1D in one study [341]. There have however been a number of studies showing a high frequency of NK cells infiltrating the pancreas and contributing to the onset and pathogenesis of T1D [109-112]. NK cells have been found to surround and infiltrate the pancreas of NOD mice [111, 112]. NK cells within the islet have a distinct phenotype, differing from splenic NK cells. Pancreatic NK cells demonstrated an increased expression of CD25, CD69 and PD-1, and a down-regulation of CD62L. Pancreatic NK cells also expressed higher KLRG1 than splenic NK cells, indicating a high proliferation state. In this study, NKG2D was also shown to be down-regulated on pancreatic NK cells [111, 112]. This is due to the expression of the rae-1 ligand. Rae-1 normally binds NK cells to stimulate their activation, however, as rae-1 ligand is increased this preferentially binds to rae-1 over NKG2D. This also indicates that NK cells are not activated readily in T1D [342, 343]. On the other hand, beta cells express rae-1 which may be involved in the initial homing of NK cells into the pancreas prior to established insulitis. Other homing mechanisms include the binding of NK cells to CXCL10 through CXCR3 and LFA-1 expression [113].

As discussed, the B cell-adiponectin-PEPITEM axis has been shown to regulate TEM in healthy individuals. This TEM regulation is perturbed in inflammatory disorders such as T1D [315]. Although, adiponectin levels are unchanged in T1D [344] the expression of AdipoR1 and AdipoR2 by monocytes and B cells is reduced compared to healthy individuals [315, 344]. Therefore, adiponectin signalling is disrupted and consequently, so is PEPITEM release. This results in a “leaky” endothelium allowing autoreactive T cells into the islet and causing further beta cell destruction [315, 344].

4.1.4 Lymphocyte trafficking and mobilisation during exercise

Acute exercise causes a significant intensity-dependent increase in the number of lymphocytes in the peripheral blood, and can be followed by lymphopenia, in healthy cohorts [191-195].

NK cells are the most responsive lymphocyte subset to acute exercise [345-347]. Of these, $CD56^{dim}CD16^{bright}$ and those with a highly differentiated phenotype ($CD57^{+}$) are preferentially redeployed, demonstrating the largest increase following exercise [197, 198]. This is followed by a larger decrease below baseline during the recovery period. Following exercise, $CD56^{bright}$ subsets return to baseline levels, whereas $CD56^{dim}$ decrease below baseline levels [197]. Preferentially mobilised NK cell subsets are highly differentiated, $CD57^{+}$ KIR^{+} $NKG2A^{-}$ [198]. Furthermore, IL-2Rβ ($CD122^{+}$) and IL-2Rα ($CD25^{+}$) NK cells increase following exercise. CD25 is expressed on $CD56^{bright}$ NK cells in comparison to CD122 which is constitutively expressed on all naïve NK cells [346, 347]. Secondary to NK cells, fully differentiated $CD8^{+}$ T cells are significantly mobilised in an exercise intensity-dependant manner [199, 200], with lower levels of mobilisation amongst $CD4^{+}$ T cells and B cells [193, 199, 201].

Lymphocyte trafficking and mobilisation is regulated by adrenergic responses [214, 348]. Beta-adrenergic receptor expression is highest on NK and $CD8^{+}$ T cells therefore are preferentially mobilised in an intensity-dependant manner during acute exercise [202-205]. Adrenergic stimulation causes detachment of NK cells from endothelium, releasing NK cells for mobilisation [349]. Blocking of the adrenaline response prevents an increase in NK cells, reiterating that lymphocytosis is controlled by adrenergic mechanisms [350].

Lymphopenia following exercise may be explained by changes in lymphocyte trafficking due to altered adhesion molecule expression and TEM [191, 348]. Studies have shown that lymphocytes expressing adhesion molecules are mobilised during exercise. Increases in $CD11b^{+}$, $CD11c^{+}$, and $LFA\text{-}1^{+}$ lymphocytes are reported following exercise [195, 351-353]. $CD11b^{+}$ and $CD11c^{+}$ lymphocytes increased following exercise are largely due to $CD16^{+}$ cells, likely to be NK cells. On the other hand, $CD62L^{+}$ lymphocytes, also known as L-selectin, were

not shown to mobilise with exercise [354]. However, another study showed that $CD11a^+$ and $CD62L^+$ T cells increased post exercise, and this increase was blunted in fit

individuals [355].

TEM is increased with shear force, suggesting that acute exercise would increase TEM [331]. However, increased adrenaline reduces CD11b/CD18 expression on PBMC [356, 357]. This means that during an acute exercise bout, whilst adrenaline levels are elevated, lymphocyte TEM would be impaired until exercise cessation, when adrenaline levels drop again. Furthermore, exercise training may reduce TEM. One study found that fit individuals show reduced PMBC adhesion to endothelial cells following acute exercise compared to a non-fit cohort which showed no change [358]. This may be explained by a reduction in adhesion molecules on lymphocytes in fit individuals. Furthermore, in vitro migration of $CD4^+$ and $CD8^+$ T cells decreased following exercise [359]. Therefore, exercise may inhibit TEM of lymphocytes. Clarification of the effects of acute exercise on lymphocyte TEM is needed.

Acute exercise may modulate TEM through the adiponectin-PEPITEM axis. Acute exercise increased circulating adiponectin following low and vigorous intensity, and remained above baseline levels 30 minutes following exercise [360]. In a separate study, when lymphocytes were treated with adiponectin, a positive correlation was seen between the expression of AdipoR1 and AdipoR2 on B cells with the quantity of PEPITEM released [315]. Therefore, the effects of exercise on B cells expressing AdipoR1 and AdipoR2, and subsequent PEPITEM release, needs to be explored.

Lymphocyte trafficking and TEM is essential for homeostasis of immune function. However, immune function is dysregulated in T1D [73]. Constant recruitment and TEM of autoreactive T cells and cytotoxic NK cells to the pancreas sustains this chronic inflammatory state [28, 109-113, 129, 130]. Exercise induced demargination of lymphocytes increases efficiency of

immunosurveillance and regulation [199, 214, 220, 345, 361, 362]. It is not known if acute exercise elicits the same response in T1D. Immunomodulation by exercise has the potential to resolve perturbations to immune regulation and lymphocyte trafficking in T1D, thus reducing beta cell destruction.

4.1.4 Hypothesis

Acute exercise will improve lymphocyte trafficking and transmigration in T1D.

4.1.5 Aims

To characterise adhesion molecule expression by lymphocytes using multi-parameter flow cytometry:

1. In T1D and control participants at rest.
2. In T1D and control participants following an acute bout of moderate intensity exercise.
3. In T1D and control participants following an acute bout of vigorous intensity exercise.

To measure transendothelial migration of lymphocytes:

1. In T1D and control participants at rest.
2. In T1D and control participants following an acute bout of moderate intensity exercise.
3. In T1D and control participants following an acute bout of vigorous intensity exercise.

4.2 Methods

4.2.1 Experimental design

Twelve controls and twelve T1D male participants were recruited into the study (as described in Chapter 3). Briefly, participants had one enrolment visit, where an incremental sub-maximal cycle ergometer test was performed to calculate their predicted VO_2 max. This was used to calculate workload and heart rate for subsequent exercise visits adjusted to individual fitness [285]. These two visits consisted of a thirty-minute bout of cycling at either 40% VO_2 max (moderate) or 80% VO_2 max (vigorous). Blood was collected intravenously at baseline, immediately post exercise, and 1 hour post exercise in vacuette heparin tubes (95057-405, Greiner Bio-one GmbH, Frickenhausen, Germany).

4.2.2 Endothelial cell culture

Human Dermal Blood Endothelial Cells (HDBEC) were cultured for 4 passages before aliquoting and storing in liquid nitrogen (500,000 cryopreserved: Female Caucasian, aged 22, tissue origin: Labia) (C-12225, Lot#398Z028.2, Promocell, Heidelberg, Germany).

4.2.2.1 Passaging HDBEC

All reagents were pre-warmed to 37^{o}C prior to use. Cells were detached using 2mls of 0.25% trypsin-EDTA (C-41000, Promocell, Heidelberg, Germany) per T25 flask, or 3mls for T75 flasks. Cells became phase-bright once they had detached after approximately 2 minutes. Then, 3ml M199 (M4530, Sigma-Aldrich, Dorset, UK) supplemented with 2% bovine serum albumin (BSA) (A2153, Sigma-Aldrich, Dorset, UK) (referred to as M199-2%BSA from here on) (6ml for T75) was added to detached cells and centrifuged for 5 minutes at 1500 RPM. Supernatant was removed and cells were split accordingly. HDBEC were cultured in endothelial cell growth medium (C-22020, Promocell, Heidelberg, Germany) (including supplement mix 2% Fetal Calf Serum, 0.004 ml/ml Endothelial Cell Growth Supplement, 0.1 ng/ml Epidermal Growth

Factor (recombinant human), 1 ng/ml Basic Fibroblast Growth Factor (recombinant human), 90 µg/ml Heparin, 1 µg/ml Hydrocortisone) supplemented with 350µl gentamycin (50mg/ml) (SLBJ8029V, Sigma-Aldrich, Dorset, UK) (referred to as BEC media from here on). For the first passage (P1), cells were split 1 in 2, into T25 flasks. Passages P2-P4 were split 1 in 3 into T75 flasks. Cells were then frozen at P4 for cryo-storage (see section 4.2.2.2).

4.2.2.2 Freezing HDBEC

Cryovials were pre-labelled and pre-chilled on ice. One T75 flask of HDBEC was aliquoted into 3 cryovials. Cells were detached (trypsin-EDTA) and pelleted as previously described for passaging (Section 2.3.1). Pelleted cells were resuspended in 1500µl of cryoSFM freezing media (C-29910, Promocell, Heidelberg, Germany, Heidelberg, Germany) and 500 µl of this was transferred to each cryovial. All cryovials were placed in a Mr Frosty freezing chamber and left -80 ^{o}C overnight. Cryovials were then transferred to liquid nitrogen for long-term storage.

4.2.3 Preparation of HDBEC layer

4.2.3.1 Defrosting HDBEC

Firstly, 14ml of BEC media (C-22020, Promocell, Heidelberg, Germany) was incubated at 37^{o}C in a T25 flask. One cryovial of HDBEC was collected from liquid nitrogen on ice in the meantime. To defrost, HDBEC cells were warmed in a water bath for up to 2 minutes, until there was only a small ice pellet left in the vial. HDBEC were collected using a 5ml pipette and transferred to the pre-warmed BEC media. To ensure a monolayer is formed, the flask was circulated in a side to side motion, avoiding the lid to prevent contamination, to spread the cells. Cells were incubated at 37°C for 48hours.

4.2.3.2 Plating cells

Following 2 days of culture, HDBEC were detached (trypsin-EDTA) and pelleted as previously described in passaging protocol (Section 2.3.1). Pelleted cells were resuspended in 15mls of pre-warmed BEC media (C-22020, Promocell, Heidelberg, Germany). One vial of HDBEC was enough for 10 wells of a 12 well plate, 1.5ml was added to each well. The plate was circulated in a side to side motion to spread HDBEC in the well. They were incubated at 37°C for 24hours.

4.2.3.3 Stimulation of HDBEC

HDBEC were activated with inflammatory cytokines; 100 U/ml TNF-α (210-TA-010, RNDsystems, Abingdon, UK) and 10ng/ml recombinant human IFN-γ (300-02, Peprotech, London, UK) was added to 5ml of pre-warmed BEC media. Media was removed from each well and replaced with 1ml of stimulation media. Cells were incubated at 37°C for 24hours.

4.2.4 PBL isolation and adiponectin treatment

Freshly isolated PBMCs (>5 million) (see Section 2.2.3) were placed into a T25 with 5mls of M199-2%BSA. Cells were incubated at 37°C for 30 minutes. PBLs were collected by intense washing of the flasks. Monocytes will stick to the bottom of the flask whilst PBLs will come off into solution [363]. Collected PBLs were transferred to a 15ml falcon and centrifuged for 7 minutes at 1500RPM. Pelleted cells were resuspended in 1ml of MI99-2%BSA. PBLs were resuspended to a concentration of $1x10^6$/ml. One million of these cells were taken for adiponectin treatment; PBLs were centrifuged at 1500RPM for 7 minutes and resuspended in 100µl of sterile PBS. Adiponectin (10 µg/ml) (ALX-522-063-C050, Enzo Life Sciences, Exeter, UK) was added to these cells and incubated for 1 hour on a rocker. Post adiponectin

treatment, cells were centrifuged for 7minutes at 1500RPM, then resuspended in 1ml M199-2%BSA.

4.2.5 Transmigration Assay

Media was removed from HDBEC wells. Cells were washed twice with 1.5ml M199-2%BSA. One condition was added to each well (i.e adiponectin treated PBL, pre-exercise PBL, post-exercise PBL, 1 hour post exercise PBL). The plate was incubated for 6.5 minutes at 37°C. Media was removed from wells. Cells were washed twice with 0.5ml M199-2%BSA. Cells were fixed with 1 ml of 2% paraformaldehyde and incubated for 10minutes at 37°C. Supernatant was removed from wells and cells were washed twice with 0.5ml M199-2%BSA. To analyse wells, 1ml of M199-2%BSA was added to each well. Using Image ProPlus 6.0 software, 7 random images were taken for each well. Phase bright and phase dark cells were counted for each image (Figure 11b). Percentage transmigration was the calculated for each condition (average transmigration/average cell adhesion).

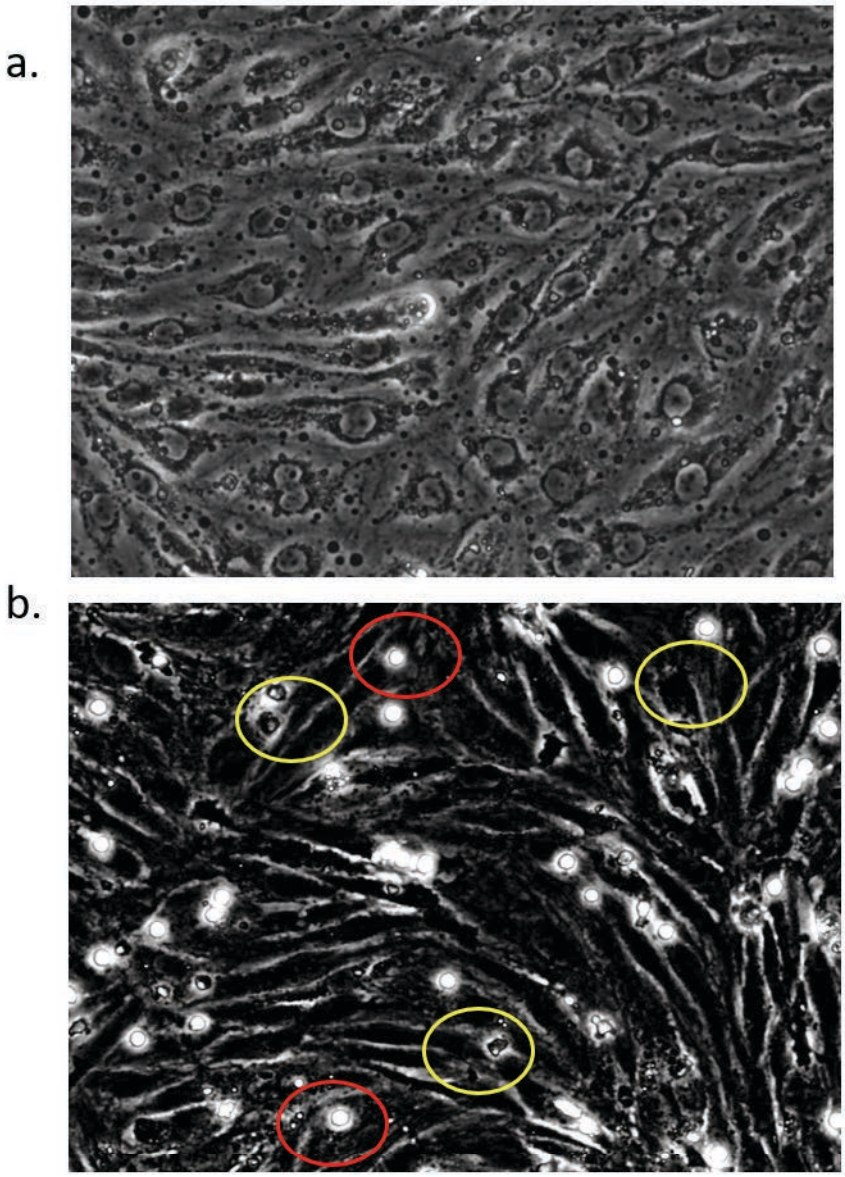

Figure 11 Transendothelial migration of lymphocytes shown by representative phase contrast images

Representative phase contrast images showing (a) unstimulated HDBEC control (b) Stimulated HDBEC; adherent phase bright lymphocyte (red circles), transmigrated phase dark lymphocyte (yellow circles).

4.2.6 Lymphocyte subset analysis

Four multicolour flow cytometry panels were designed to enable phenotypic analysis of lymphocyte subsets. The following mAbs (see Table 3, Section 2.3.2 for mAb details); **T cells tube 1**: anti-CD3 PE-Cy7, anti-CD4 APC-R700, anti-CD8 APC-H7, anti-CD11b PE-CF594, anti-7-AAD PerCP-Cy5.5. **T cells tube 2**: anti-CD3 PE-Cy7, anti-CD4 APC-R700 ,anti-CXCR3 PE-CF594, anti-CCR4 BV421, anti-CCR6 BV711, Live/Dead fixable viability stain 780. **B cells**: anti-CD3 BV605, anti-CD19 PE-CF594, anti-CD62L APC, anti-AdipoR1 PerCP-Cy5.5, anti-AdipoR2 Pe-Cy7, Live/Dead fixable viability stain 780. **NK cells**: anti-CD3 Pe-Cy7, anti-CD16 PE-CF594, anti-CD18 (LFA-1) APC, anti-CD56 BV510, Live/Dead fixable viability stain 780. Whole blood was stained as per Chapter 2 (Section 2.3.2). Whole blood counts were completed using a coulter haematology analyser (Section 2.2.2) to calculate relative cell frequency (cells/µl) of lymphocyte subsets.

4.2.7 Data analysis

Flowjo v.10 was used to analyse flow cytometry data. Doublets were removed using FSC-A against FSH-H. Dead cells positive for viability stain were removed, and lymphocytes were gated based on size on SSC-A versus FSC-A dot plot (Figure 12). T cells were selected as $CD3^+$, and further selected on $CD4^+$ and $CD8^+$ expression (Figure 13.2). NK cells were selected as $CD3^-$ and further selected on $CD56^+/CD16^{+/-}$ expression as follows: $CD56^{dim}CD16^{bright}$, $CD56^{bright}CD16^{dim}$, $CD56^{bright}$CD16-, $CD56^{dim}CD16^{dim}$, and CD56-$CD16^+$ (Figure 13.3). B cells were selected as $CD3^-CD19^+$ (Figure 13.4). Adhesion molecules and chemokine receptors CD11b, LFA-1, CXCR3, CCR6, CCR4, and CD62L expression were selected on $CD3^+$ and $CD3^-$ in their corresponding panels (Figure 14). Adiponectin receptor expression, AdipoR1 and AdipoR2, was selected on $CD3^-CD19^+$ B cells (Figure 15).

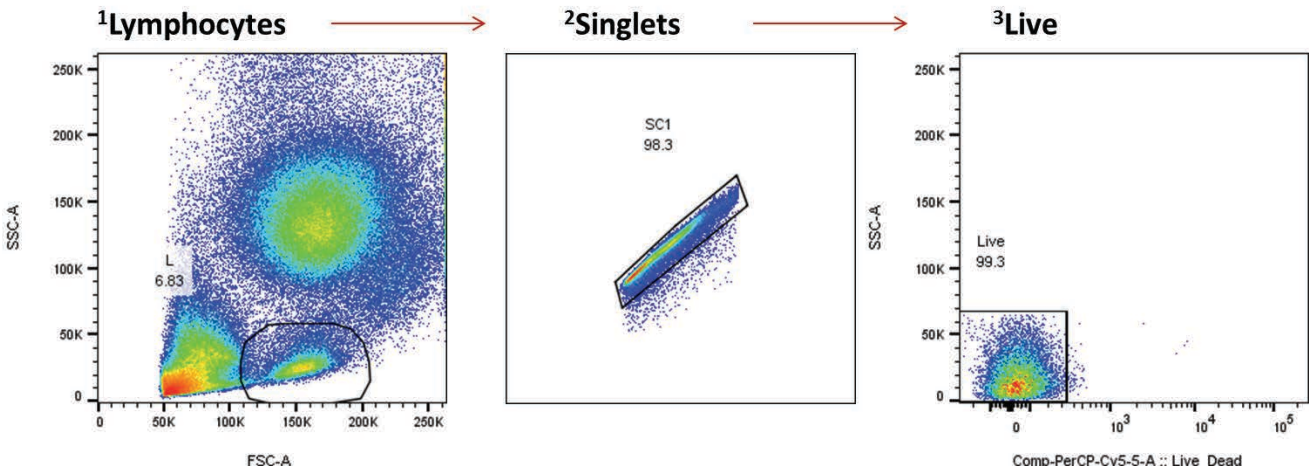

Figure 12 Selection of live lymphocytes shown by representative flow plots

Representative flow cytometry plots showing (1) Lymphocyte selection based on size (SSC-A vs FSC-A) (2) Doublets exclusion (FSC-A vs FSC-H) (3) Dead cell removal based on positivity for viability stain (7-AAD PerCP-Cy5.5).

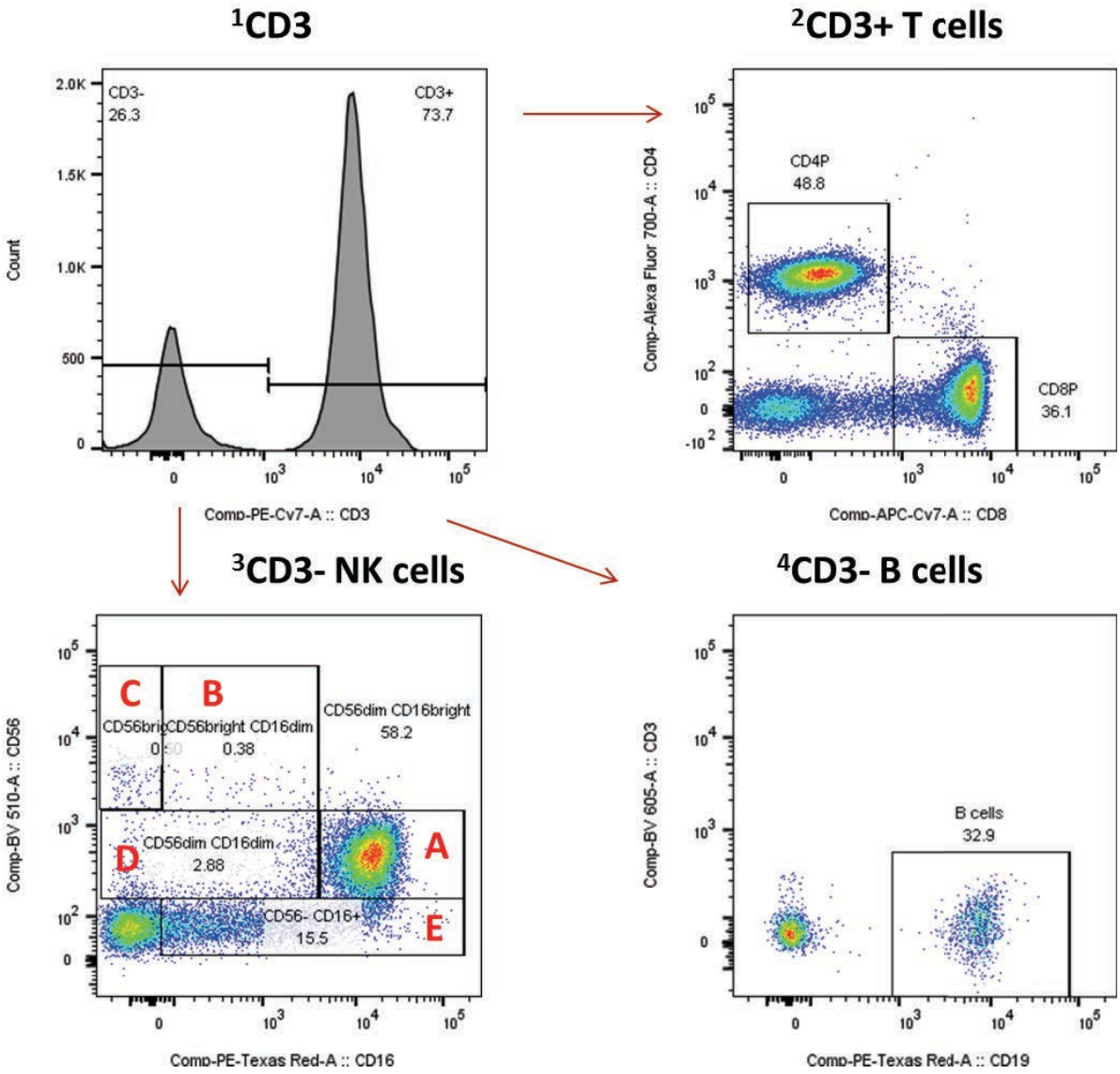

Figure 13 Lymphocyte subset selection; T cells, NK cells, and B cells gating strategy shown by representative flow plots.

Representative flow cytometry plots following parent gate for lymphocytes (shown in figure 3) (1) CD3 histogram (2) CD3+ gate showing CD4+ vs CD8+ T cells gates (3) NK cell subsets following CD3- population selection; **A.** CD56dimCD16bright, **B.** CD56brightCD16dim, **C.**CD56brightCD16-, **D.** CD56dimCD16dim, **E.** CD56-CD16+ (4) B cell selection from CD3-CD19+ gate.

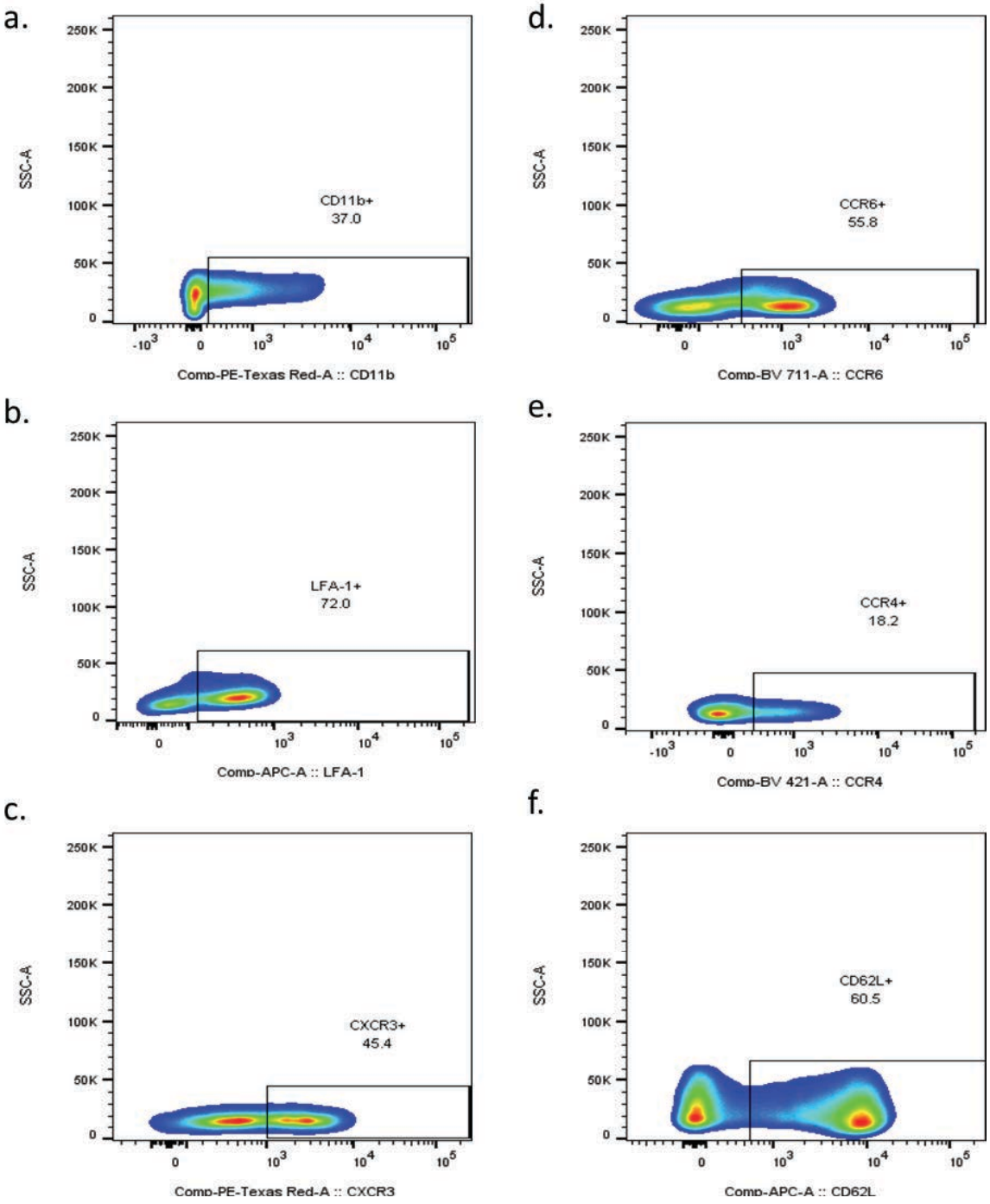

Figure 14 Adhesion molecule and chemokine receptor gating shown by representative flow plots.

Representative flow cytometry plots showing following parent gate for CD3+/- populations; (a) CD11b+ (b) LFA-1+ (c) CXCR3+ (d) CCR6+ (e) CCR4+ (f) CD62L+.

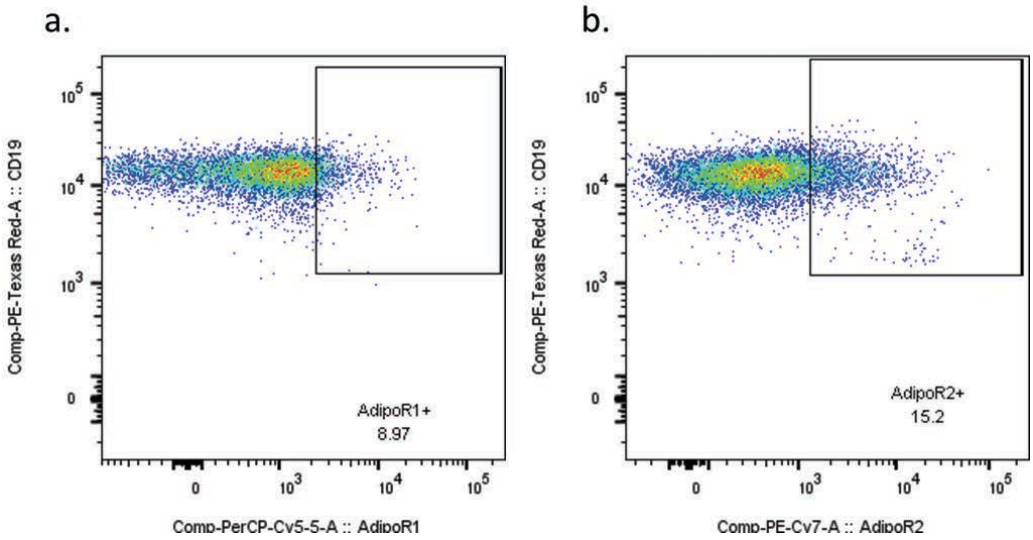

Figure 15 Adiponectin receptor gating strategy shown by representative flow plots.

Representative flow cytometry plots showing adiponectin receptors following parent gate for CD3-CD19+ B cells (as shown in figure 4.4) (a) AdipoR1+ (b) AdipoR2+.

4.3 Results

4.3.1 Peripheral blood lymphocyte populations in T1D and control participants

4.3.1.1 Lymphocyte populations at rest

The proportion (%) and frequency (cells/μl) of peripheral blood lymphocytes, $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, NK cells, and B cells were measured at rest in T1D and control participants.

The proportion and frequency of NK cells were significantly higher in T1D than control participants (p=0.041, p=0.011 respectively) (Figure 16a and b). The proportion of $CD56^{bright}CD16^-$ NK cells were significantly increased in T1D (p=0.025), but this was not seen in the frequency of this subset. There were no significant differences between groups in the other NK cell subsets; $CD56^{dim}CD16^{bright}$, $CD56^{bright}CD16^{dim}$, $CD56^{dim}CD16^{dim}$ (Table 16) (Figure 16c and d).

There were no significant differences in the proportion or frequency of total peripheral lymphocytes, $CD3^+$, $CD4^+$, $CD8^+$, $CD3^-$, and B cell populations between groups at baseline (Table 13) (Figure 16).

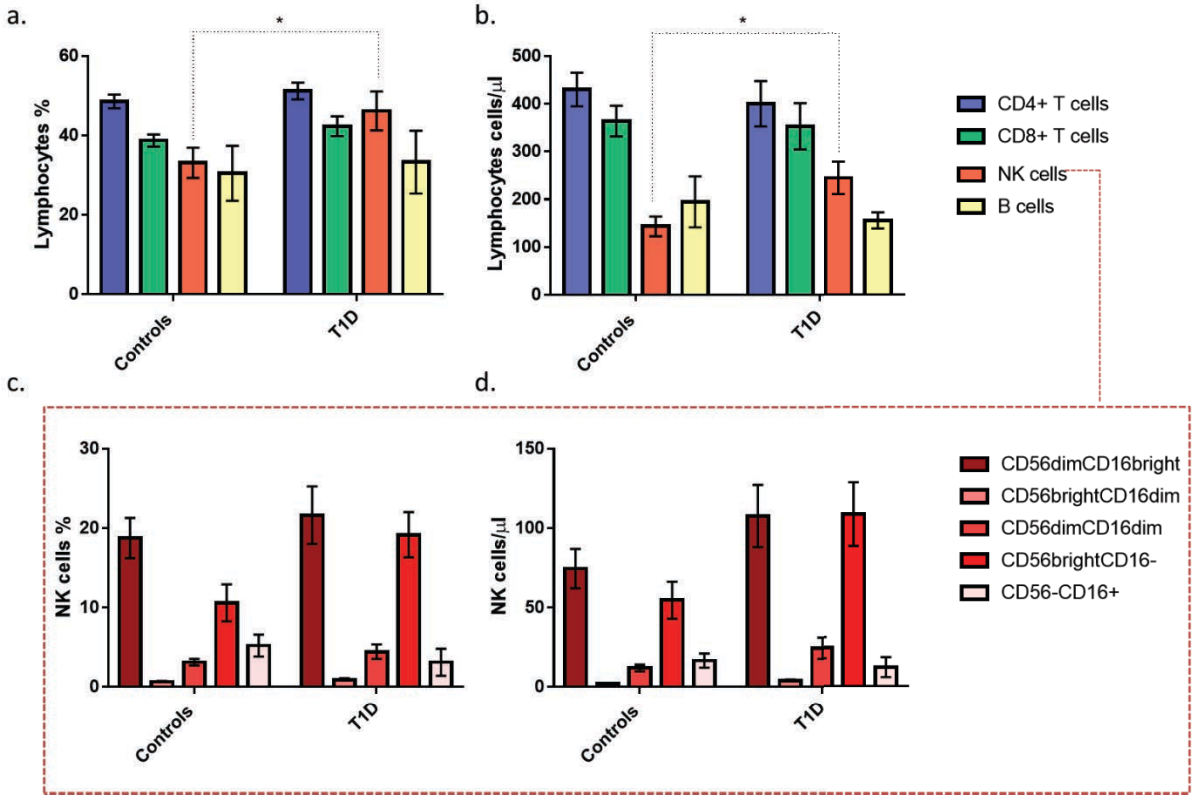

Figure 16 The proportion and frequency of lymphocyte subsets at baseline in T1D and control groups.

Flow cytometry and whole blood cell counts were used to determine the frequency of CD4+T cells, CD8+ T cells, NK cell and B cell lymphocyte subsets in control and T1D participants at baseline. (a) Proportion of lymphocyte populations in control and T1D groups (b) Frequency of lymphocyte populations in control and T1D groups (c) Proportion of NK cell subsets in control and T1D groups (d) Frequency of NK cell subsets in control and T1D groups. Error bars represent SEM. Statistical significance represented by * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

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Subset		[1]Controls		[2]T1D	
		mean	SD	mean	SD
Lymphocytes	(cells/µl)	1348	398	1375	262
$CD3^+$	(%)	61.75	19.61	55.57	25.64
	(cells/µl)	833.8	414.4	776.3	350.2
$CD4^+$ T cells	(%)	48.56	11.01	51.17	11.57
	(cells/µl)	430.0	222.9	400.2	236.5
$CD8^+$ T cells	(%)	38.70	7.32	42.31	10.50
	(cells/µl)	364.0	150.2	352.9	188.4
$CD3^-$	(%)	38.34	19.69	44.11	25.59
	(cells/µl)	554.7	337.7	585.9	392.9
NK cells ($CD56^+$)	(%)	33.11	17.06	46.17	19.04
	(cells/µl)	143.4	91.8	244.6	127.3
B cells ($CD19^+$)	(%)	30.45	21.91	33.27	19.31
	(cells/µl)	194.7	150.9	155.4	45.1

Table 13 Lymphocyte populations at baseline in T1D and controls.

Mean and standard deviation values for the proportion and frequency of lymphocytes populations in controls and T1D participants.
Note: % of total lymphocytes
[1]controls CD3+/- n=76, CD4+ n=40, CD8+ n=22, NK cells n=20, B cells n=8.
[2]T1D CD3+/- n=51, CD4+ n= 2, CD8+ n=15, NK cells n=14, B cells n=7

Subset		[1]Controls		[2]T1D	
		mean	SD	mean	SD
$CD56^{dim}CD16^{bright}$	(%)	18.76	11.35	21.63	14.05
	(cells/µl)	74.58	55.41	107.64	73.07
$CD56^{bright}CD16^{dim}$	(%)	0.63	0.46	0.92	0.73
	(cells/µl)	2.23	1.56	4.05	2.48
$CD56^{dim}CD16^{dim}$	(%)	3.12	1.83	4.44	3.52
	(cells/µl)	11.98	9.46	24.36	25.11
$CD56^{+}CD16^{-}$	(%)	10.60	10.40	19.17	10.98
	(cells/µl)	54.66	52.33	108.49	75.12
$CD56^{-}CD16^{+}$	(%)	5.21	6.14	3.10	6.57
	(cells/µl)	16.57	19.89	12.04	23.51

Table 14 NK cell subsets at baseline in T1D and controls.

Mean and standard deviation values for the proportion and frequency of NK cell subsets in controls and T1D participants.
Note: % of total NK cells
[1]controls n=20, [2]T1D n=14

4.3.1.2 Lymphocyte subsets in response to acute exercise in T1D and control participants

The effects of moderate (40% VO_2 max) and vigorous (80% VO_2 max) intensity exercise were investigated in T1D and control participants on $CD3^+$ lymphocyte populations ($CD4^+$ and $CD8^+$ T cells) and $CD3^-$ lymphocyte populations (NK cells and B cells). Lymphocyte subpopulations were measured pre, post, and 1 hour post exercise. The change over time was analysed in groups combined under the heading "time (overall)" and in each group independently under the heading "time" displayed in all results tables. The interaction over time between groups was compared and results are displayed under the heading "time*group" displayed in all results tables. Post and 1 hour post exercise measurements were compared to baseline levels as shown under the heading "contrasts" in the results tables. The mean, standard deviation, and statistical analyses are displayed in Table 15.

As described in Chapter 3, lymphocytes mobilise during moderate and vigorous intensity exercise. There was an overall change over time in total lymphocytes during moderate intensity exercise (p=0.021), a trend was seen in controls (p=0.058) but not in T1D (Figure 6a). Lymphocytes were significantly increased post moderate exercise in the control group (p=0.046). Lymphocytes were significantly mobilised by vigorous intensity exercise in T1D (p=0.002) and control (p<0.001) groups, with an overall significant change over time (groups combined) (p<0.001). Lymphocytes were significantly increased immediately post vigorous exercise in T1D (p=0.003) and controls (p<0.001), followed by a significant fall below baseline in controls only (p=0.032) 1 hour post vigorous exercise, with a trend seen in the T1D group at this time point (p=0.067) (Figure 17a).

4.3.1.3 $CD3^+$ subsets in response to acute exercise in T1D and control participants

There was an overall change over time in total $CD3^+$ lymphocytes during moderate intensity exercise (p<0.001), and this was seen in control (p=0.003) and T1D (p=0.025) groups independently. Total $CD3^+$ lymphocytes were significantly increased post moderate exercise overall (p<0.001) (groups combined) and in the control group (p=0.003), but not in the T1D group. However, there was a significant decrease below baseline at 1 hour post moderate exercise in the T1D group (p=0.026). The observed change in response 1 hour post moderate exercise was significantly different between groups (p=0.005) (time*group) because the frequency of $CD3^+$ lymphocytes remained above (7.1%) baseline in the control group at this time point, but dropped below baseline (14.13%) in the T1D group. Total $CD3^+$ lymphocytes significantly changed during vigorous intensity exercise overall (p<0.001) and in both the T1D (p<0.001) and control (p<0.001) groups independently. There was an overall significant increase post vigorous exercise (p<0.001) and significant decrease below baseline 1 hour post vigorous exercise (p<0.001). There was a significant increase post vigorous exercise (T1D: p=0.001, control: p<0.001) followed by a significant decrease 1 hour post vigorous exercise

(T1D: p=0.007, control: p<0.001) in both groups. However, the difference in response 1 hour post exercise was significantly different between groups (p=0.013) (time*group) because there was a larger decline below baseline in the control group (T1D: 0.14%, control: 15.95%) (Table 15).

There was a significant overall change in $CD4^+$ T cells during vigorous intensity exercise (p<0.001), this was seen in the control (p=0.001), but a only trend was seen in the T1D (p=0.052) group. $CD4^+$ T cells significantly increased post exercise overall (p=0.011), with a trend to increase in T1D (p=0.060), and then $CD4^+$ T cells significantly decreased 1 hour post exercise overall (p=0.005) and in the control group (p=0.008). The observed contrast in response 1 hour post exercise between groups showed a trend to be different (p=0.052) (time*group) because there was a decline below baseline in the control and not the T1D group (control: 18.74%, T1D: 0%). $CD4^+$ T cells did not significantly mobilise during moderate intensity exercise in either group, but a trend to mobilise was seen overall (p=0.051) (Figure 17b) (Table 15).

There was a significant overall change in $CD8^+$ T cells during vigorous intensity exercise (p<0.001), and this was seen in the T1D (p=0.016) and control (p=0.001) groups independently. $CD8^+$ T cells significantly increased post vigorous exercise in T1D (p=0.040) and control (p=0.033) groups. This was followed by a significant decrease 1 hour post vigorous exercise overall and in the control group (p=0.021), but only a trend to decrease in the T1D group (p=0.057). $CD8^+$ T cells did not significantly mobilise during moderate intensity exercise in either group, but a trend to mobilise was seen in the control group (p=0.061) (Figure 17c) (Table 15).

4.3.1.4 $CD3^-$ subsets in response to acute exercise in T1D and control participants

There was an overall change over time in total $CD3^-$ lymphocytes during moderate intensity exercise (p=0.002), and this was seen in the control group only (p=0.013). Total $CD3^-$ lymphocytes significantly increased post exercise overall (p=0.003) and in the control group (p=0.13). Total $CD3^-$ lymphocytes significantly changed during vigorous intensity exercise overall (p<0.001), and in both the control (p<0.001) and T1D (p<0.001) groups. There was an overall significant increase post exercise (p<0.001), and this was seen in T1D (p<0.001) and control (p<0.001) groups independently. However, the magnitude of the response post exercise showed a trend to be different between groups (p=0.066) (time*group) as there is a larger increase post exercise in the control group (T1D: 66.7%, control: 114.4%). A trend in difference between groups (time*group) was also seen in the change overtime (p=0.061) (Table 15).

There was an overall change over time in NK cells during moderate intensity exercise (p=0.002), and this was seen in the T1D group only (p=0.010). There was a significant increase post moderate exercise overall (p=0.001), and in the T1D group (p=0.035) with a trend to increase post moderate exercise in the control group (p=0.052). The observed contrast in response to moderate exercise was significantly different between groups (p=0.036) (time*group). The magnitude of the response post moderate exercise was significantly different between groups (p=0.032) because there was a larger increase in NK cells in T1D participants at this time point (T1D: 125.4%, control: 96.65%). NK cells significantly mobilised during vigorous intensity exercise overall (p=0.002), and this was seen in control (p=0.021) and T1D (p=0.005) groups independently. NK cells were significantly increased post vigorous exercise overall (p=0.003) and in both groups independently (T1D: p=0.044, control: p=0.021). The magnitude of the response post exercise was higher in control participants (T1D: 100.49%,

control: 174.13%) but this was not significantly different between groups. There was a significant fall below baseline overall (p=0.010), and a trend to decrease in the control (p=0.051) at this time point but not the T1D group (Figure 17d) (Table 15).

There was an overall change in B cells during vigorous intensity exercise (p=0.045), but this was only seen in the control group (p=0.008). B cells did not significantly increase post vigorous exercise in either group. However, there was a significant decrease 1 hour post exercise in the control group only (p=0.012). There was no significant mobilisation of B cells during moderate intensity exercise, however an overall trend to mobilise was seen (p=0.063). The mobilisation of B cells also showed a trend to be different between groups (p=0.063) (time*group) (Figure 17e) (Table 15).

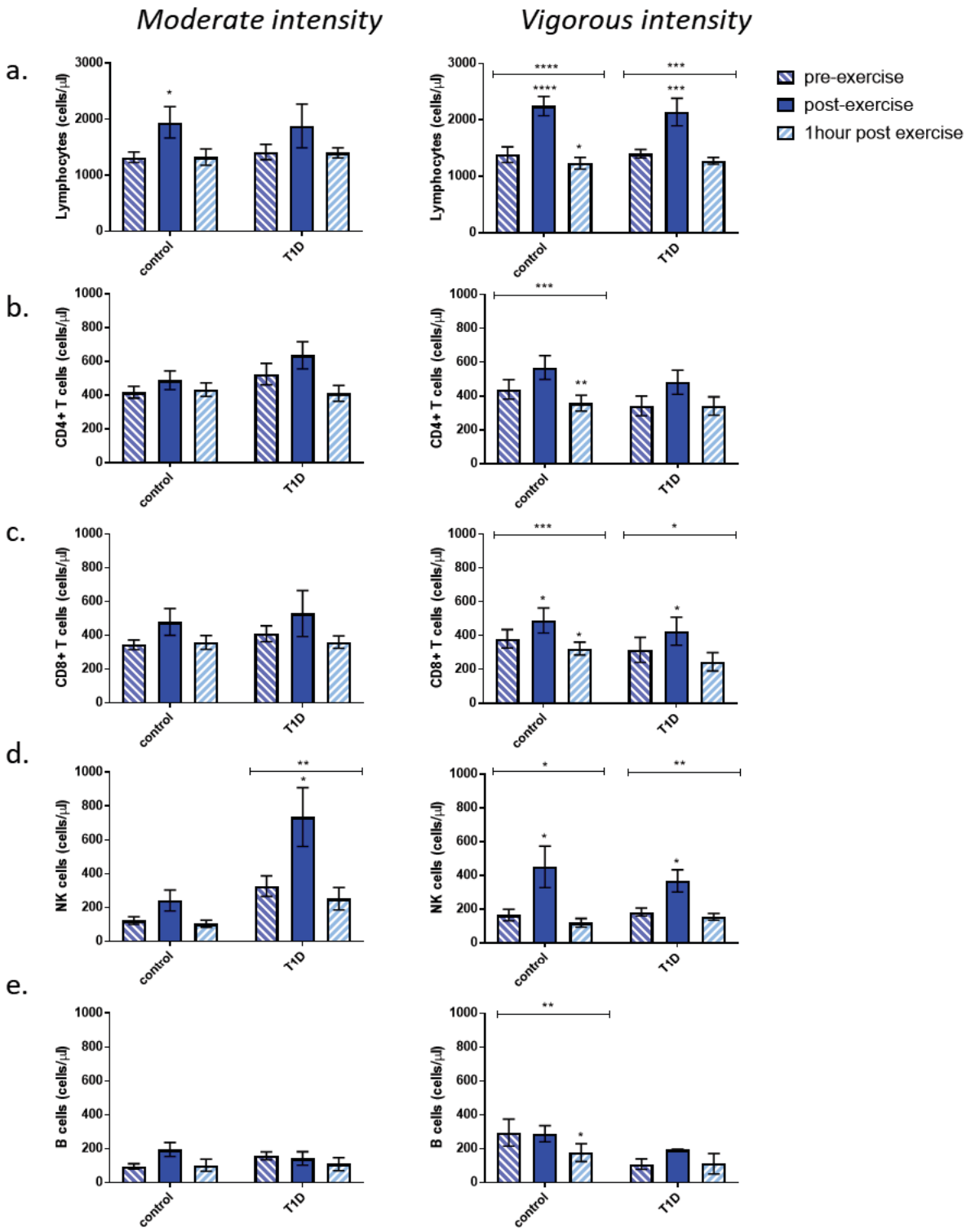

Figure 17 Lymphocyte populations during moderate and vigorous exercise in control and T1D groups

Flow cytometry and whole blood cell counts were used to determine the frequency of lymphocyte populations in control and T1D participants during moderate and vigorous intensity exercise. (a) Frequency of lymphocytes (b) frequency of CD4+ T cells (c) frequency of CD8+ T cells (d) frequency of NK cells and (e) the frequency of B cells during moderate and vigorous intensity exercise. Error bars represent SEM. Statistical significance represented by * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

Subset (cells/μl)	Intensity	[1]Controls T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast		[a]Δ%	[2]T1D T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast		[a]Δ%	[d]Time (overall)	[d]Time*Group
Lymphocytes	**moderate**	1290.91	350.58	1754.55	759.43	1340.00	435.12	$F_{(1.2, 12.1)}$= 4.177, p=0.058	*T2*	**$F_{(1, 10)}$= 5.202, p=0.046**	35.92	1400.00	310.91	1814.29	893.36	1350.00	216.79	$F_{(1.05, 5.3)}$= 3.405, p=0.074	*T2*	$F_{(1, 5)}$= 2.925, p=0.148	29.59	**$F_{(1.2, 15.8)}$= 6.209, p=0.021**	$F_{(1.2, 15.8)}$= 0.230, p=0.667
									T3	$F_{(1, 10)}$= 0.013, p=0.912	3.80								*T3*	$F_{(1, 5)}$= 4.000, p=0.102	3.57		
	vigorous	1400.00	445.18	2400.00	475.39	1233.33	336.65	**F(1.26, 13.9)= 46.127, p<0.001**	*T2*	**$F_{(1, 11)}$= 55.113, p<0.001**	71.43	1355.56	235.11	2088.89	658.49	1255.56	174.01	**$F_{(1.1, 8.8)}$= 319.030, p=0.002**	*T2*	**$F_{(1, 8)}$= 18.615, p=0.003**	54.10	**$F_{(1.24, 22.4)}$= 74.637, p<0.001**	$F_{(1.24, 22.4)}$= 1.867, p=0.185
									T3	**$F_{(1, 11)}$= 6.044, p=0.032**	11.90								*T3*	$F_{(1, 8)}$= 4.500, p=0.067	7.38		
CD3+	moderate	739.78	337.59	1000.60	452.79	792.22	364.65	**$F_{(1.5, 46.4)}$= 7.594, p=0.003**	*T2*	**$F_{(1, 31)}$= 10.426, p=0.003**	35.26	886.87	326.51	1112.88	466.55	752.82	238.49	**$F_{(1.4, 18.1)}$= 5.206, p=0.025**	*T2*	$F_{(1, 13)}$= 2.211, p=0.161	25.48	**$F_{(1.5, 63.9)}$= 12.194, p<0.001**	$F_{(1.5, 63.9)}$= 2.235, p=0.129
									T3	$F_{(1, 31)}$= 1.211, p=0.280	7.09								*T3*	**$F_{(1, 13)}$= 6.277, p=0.026**	15.12		
	vigorous	922.91	453.67	1311.97	483.18	775.68	343.18	**F(1.6, 48.2)= 37.853, p<0.001**	*T2*	**$F_{(1, 31)}$= 25.158, p<0.001**	42.16	698.97	339.38	1051.24	440.65	600.20	332.16	**F(1.1, 28.6)= 17.892, p<0.001**	*T2*	**$F_{(1, 26)}$= 15.612, p=0.001**	50.40	**$F_{(1.3, 74.4)}$= 51.340, p<0.001**	**$F_{(1.3, 74.4)}$= 0.950, p=0.357**
									T3	**$F_{(1, 31)}$= 22.832, p<0.001**	15.95								*T3*	**$F_{(1, 26)}$= 8.609, p=0.007**	14.13		
-- CD4+	moderate	417.47	145.29	488.44	229.49	432.59	157.11	$F_{(2, 30)}$= 0.873, p=0.428	*T2*	$F_{(1, 15)}$= 1.484, p=0.242	14.53	525.02	168.51	636.07	213.75	410.10	114.35	$F_{(1.1, 6.6)}$= 3.276, p=0.114	*T2*	$F_{(1, 6)}$= 4.168, p=0.087	21.19	$F_{(2, 42)}$= 3.201, p=0.051	$F_{(2, 42)}$= 0.861, p=0.413
									T3	$F_{(1, 15)}$= 0.169, p=0.687	3.62								*T3*	$F_{(1,6)}$= 1.696, p=0.241	21.89		
	vigorous	440.21	265.70	569.00	306.55	357.71	204.76	**$F_{(2, 34)}$= 9.354, p=0.001**	*T2*	$F_{(1, 17)}$= 3.019, p=0.100	29.26	341.50	234.21	481.75	284.29	341.14	209.30	$F_{(1.8, 16.6)}$= 4.267, p=0.052	*T2*	$F_{(1, 15)}$= 4.149, p=0.060	41.07	**$F_{(1.4, 45)}$= 11.752, p<0.001**	$F_{(1.4, 45)}$= 0.763, p=0.429
									T3	**$F_{(1, 17)}$= 8.877, p=0.008**	18.74								*T3*	$F_{(1,15)}$= 1.216, p=0.287	0.10		
-- CD8+	moderate	448.95	149.11	626.88	335.59	480.60	162.84	$F_{(2, 20)}$= 3.224, p=0.061	*T2*	$F_{(1, 10)}$= 3.357, p=0.097	39.63	478.30	157.11	677.33	430.18	491.82	80.48	$F_{(2,10)}$= 2.186, p=0.163	*T2*	$F_{(1, 5)}$= 1.317, p=0.303	41.61	F $_{(1, 17.1)}$= 0.315, p=0.597	F $_{(1, 17.1)}$= 0.474, p=0.513
									T3	$F_{(1, 10)}$= 0.352, p=0.566	7.05								*T3*	$F_{(1, 5)}$= 3.020, p=0.143	2.83		
	vigorous	329.85	165.90	460.90	178.81	229.11	98.35	**$F_{(2, 22)}$= 10.326, p=0.001**	*T2*	**$F_{(1, 11)}$= 5.961, p=0.033**	39.73	248.34	158.17	354.41	225.31	192.54	122.80	**$F_{(1.2, 9.7)}$= 7.791, p=0.016**	*T2*	**$F_{(1, 8)}$= 6.021, p=0.040**	42.71	**F $_{(2, 42)}$= 3.295, p=0.047**	F $_{(2, 42)}$= 0.126, p=0.882
									T3	**$F_{(1, 11)}$= 7.253, p=0.021**	30.54								*T3*	$F_{(1, 8)}$= 4.942, p=0.057	22.47		
CD3-	moderate	616.22	416.03	799.23	535.95	599.74	408.74	**$F_{(1.6, 51.4)}$= 5.170, p=0.013**	*T2*	**$F_{(1, 31)}$= 6.898, p=0.013**	29.70	544.10	346.54	751.03	631.26	617.57	339.57	$F_{(1.1, 14.9)}$= 3.269, p=0.087	*T2*	$F_{(1, 13)}$= 3.354, p=0.090	38.03	**$F_{(1.6, 69.6)}$= 7.963, p=0.002**	$F_{(1.6, 69.6)}$= 0.383, p=0.634
									T3	$F_{(1, 31)}$= 0.172, p=0.681	2.68								*T3*	$F_{(1, 13)}$= 1.022, p=0.330	13.50		
	vigorous	496.38	219.47	1064.24	519.78	453.08	272.30	**F(1.4, 41.8)= 43.458, p<0.001**	*T2*	**$F_{(1, 30)}$= 55.897, p<0.001**	114.40	614.27	413.10	1023.82	531.45	645.71	451.19	**F(1.6, 44.2)= 41.125, p<0.001**	*T2*	**F(1, 28)= 61.552, p<0.001**	66.67	**$F_{(1.5, 83.9)}$= 79.996, p<0.001**	$F_{(1.5, 83.9)}$= 3.215, p=0.061
									T3	$F_{(1, 30)}$= 0.457, p=0.504	8.72								*T3*	$F_{(1, 28)}$= 0.054, p=0.819	5.12		
-- NK cells (CD56+)	**moderate**	122.67	69.29	241.24	187.31	103.63	59.06	$F_{(1.1, 9)}$= 4.917, p=0.051	*T2*	$F_{(1, 8)}$= 5.195, p=0.052	96.65	326.23	135.16	735.27	388.70	251.50	162.63	**$F_{(1.1, 5.1)}$= 7.441, p=0.010**	*T2*	**$F_{(1, 5)}$= 8.185, p=0.035**	125.38	**$F_{(1, 13.4)}$= 14.777, p=0.002**	**$F_{(1, 13.4)}$= 5.366, p=0.036**
									T3	$F_{(1, 8)}$= 0.225, p=0.648	15.53								*T3*	$F_{(1, 5)}$= 1.965, p=0.220	22.91		
	vigorous	164.22	101.76	450.18	345.08	119.25	72.19	**$F_{(1.1, 7.1)}$= 8.584, p=0.021**	*T2*	**$F_{(1, 7)}$= 8.710, p=0.021**	174.13	183.29	62.26	367.47	173.00	154.55	40.17	**$F_{(2, 8)}$= 11.212, p=0.005**	*T2*	**$F_{(1, 4)}$= 8.402, p=0.044**	100.49	**$F_{(1, 11.4)}$= 14.696, p=0.002**	$F_{(1, 11.4)}$= 0.118, p=0.748
									T3	$F_{(1, 7)}$= 5.549, p=0.051	27.38								*T3*	$F_{(1, 4)}$= 5.026, p=0.088	15.68		
-- B cells (CD19+)	moderate	114.38	51.46	153.51	65.02	109.92	76.97	$F_{(2, 12)}$= 3.634, p=0.058	*T2*	**$F_{(1, 6)}$= 7.323, p=0.035**	34.21	105.60	56.64	97.22	68.90	87.72	87.80	$F_{(2, 2)}$= 2.950, p=0.253	*T2*	$F_{(1, 1)}$= 1.305, p=0.458	7.94	$F_{(2, 14)}$= 1.458, p=0.266	$F_{(2, 14)}$= 1.010, p=0.389
									T3	$F_{(1, 6)}$= 1.047, p=0.346	3.90								*T3*	$F_{(1, 1)}$= 6.643, p=0.236	16.94		
	vigorous	147.66	93.61	236.23	68.43	112.99	73.44	**$F_{(2, 10)}$= 7.468, p=0.010**	*T2*	$F_{(1, 5)}$= 2.992, p=0.144	59.98	80.36	36.61	180.64	153.89	68.38	3.68	$F_{(1, 2)}$= 1.219, p=0.469	*T2*	$F_{(1, 1)}$= 1.462, p=0.440	124.78	**$F_{(1.2, 7.1)}$= 6.398, p=0.036**	$F_{(1.2, 7.1)}$= 0.139, p=0.760
									T3	**$F_{(1, 5)}$= 7.662, p=0.039**	23.48								*T3*	$F_{(1, 1)}$= 0.265, p=0.698	14.91		

Table 15 Lymphocytes populations mobilised during moderate and vigorous intensity exercise in control and T1D groups

Mean, standard deviation, and statistical analysis of lymphocytes populations for control and T1D participants during moderate and vigorous intensity exercise Significant results highlighted in bold where p values <0.05 were considered significant.
Δ% Percentage change from baseline (T1) to immediately post exercise (T2).
[b] Results were analysed using multiple regression analysis in control and T1D groups independently.
[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).
[d] Results were analysed using multiple regression analysis in control and T1D groups combined.
[1]controls CD3+/- n=38, CD4+ n=18-22, CD8+ n=10-12, NK cells n=10, B cells n=4.
[2]T1D CD3+/- n=20-30, CD4+ n= 8-17, CD8+ n=6-9, NK cells n=6-8, B cells n=2.

4.3.1.5 NK cell subsets in response to acute exercise in T1D and control participants

NK cell subsets were defined using the markers CD56 and CD16; $CD56^{dim}CD16^{bright}$, $CD56^{bright}CD16^{dim}$, $CD56^{dim}CD16^{dim}$, $CD56^{bright}CD16^{-}$, and $CD56^{-}CD16^{+}$. The mean, standard deviation, and statistical analyses are displayed in Table 16.

There was an overall change over time in $CD56^{dim}CD16^{bright}$ NK cells during moderate intensity exercise (p=0.030), and this was seen in the T1D (p=0.009) group only. There was a significant increase post moderate exercise overall (p=0.028) and in the T1D group (p=0.041). $CD56^{dim}CD16^{bright}$ NK cells mobilised significantly during vigorous intensity exercise overall (p<0.001) and in both groups independently (T1D: p=0.049, controls: p=0.001). $CD56^{dim}CD16^{bright}$ NK cells were significantly increased post vigorous exercise overall (p<0.001) and in the control group only (p=0.001). The magnitude of response post exercise was much larger in control participants (T1D: 97.06%, control: 185.12%) but this difference was not significant between groups (time*group) (Figure 18a) (Table 16).

There was an overall change over time in $CD56^{bright}CD16^{dim}$ during moderate intensity exercise (p=0.030), and this was seen in the T1D (p=0.008) group only. There was a significant increase post moderate exercise in the T1D group (p=0.043). $CD56^{bright}CD16^{dim}$ NK cells mobilised significantly during vigorous intensity exercise overall (p=0.001) and in the control group (p=0.002), but only a trend to change over time was seen in the T1D group (p=0.07). There was a significant increase post vigorous exercise overall (p=0.002) and in the control group (p=0.006) (Figure 18b) (Table 16).

There was an overall change over time in $CD56^{dim}CD16^{dim}$ NK cells during moderate intensity exercise (p<0.001) and in the T1D group (p=0.018), with a trend to mobilise seen in control group (p=0.055). There was a significant increase post moderate exercise overall (p=0.001), but this was seen in the T1D group only (p=0.025). The magnitude of response post exercise

was much larger in T1D participants (T1D: 168.5%, control: 22.74%) and this was significantly different group at this time point (p=0.025) (time*group) and overall (p=0.024). $CD56^{dim}CD16^{dim}$ NK cells significantly mobilised during vigorous intensity exercise overall (p=0.009), but this was seen only in the control group (p=0.015). $CD56^{dim}CD16^{dim}$ NK cells were significantly increased post vigorous exercise overall (p=0.007) and in the control group (p=0.025), but not the T1D group (Figure 18c) (Table 16).

There was an overall change over time in $CD56^{bright}CD16^{-}$ NK cells during moderate intensity exercise (p=0.005) and in the T1D group (p=0.037), with a trend to mobilise seen in control group (p=0.059). There was a significant increase post moderate exercise in the T1D group only (p=0.032). However, there was a significant decrease below baseline 1 hour post exercise in the control group only (p=0.038). $CD56^{bright}CD16^{-}$ NK cells significantly mobilised during vigorous intensity exercise overall (p<0.001) and in both groups independently (T1D: p=0.012, control: p=0.001). There was a significant increase post vigorous exercise overall (p<0.001) and in both groups (T1D: p=0.029, control: p=0.004) (Figure 18d) (Table 16).

There was an overall change over time in $CD56^{-}CD16^{+}$ cells during moderate intensity exercise (p=0.005) but this was seen in the control group only (p=0.047). There was a significant increase post exercise overall (p=0.006) and in the control group only (p=0.026). $CD56^{-}CD16^{+}$ NK cells significantly mobilised during vigorous intensity exercise overall (p=0.020), but this was seen in the control group only (p=0.005). There was a significant increase post exercise overall (p=0.001) and in the control group only (p=0.004) (Table 16).

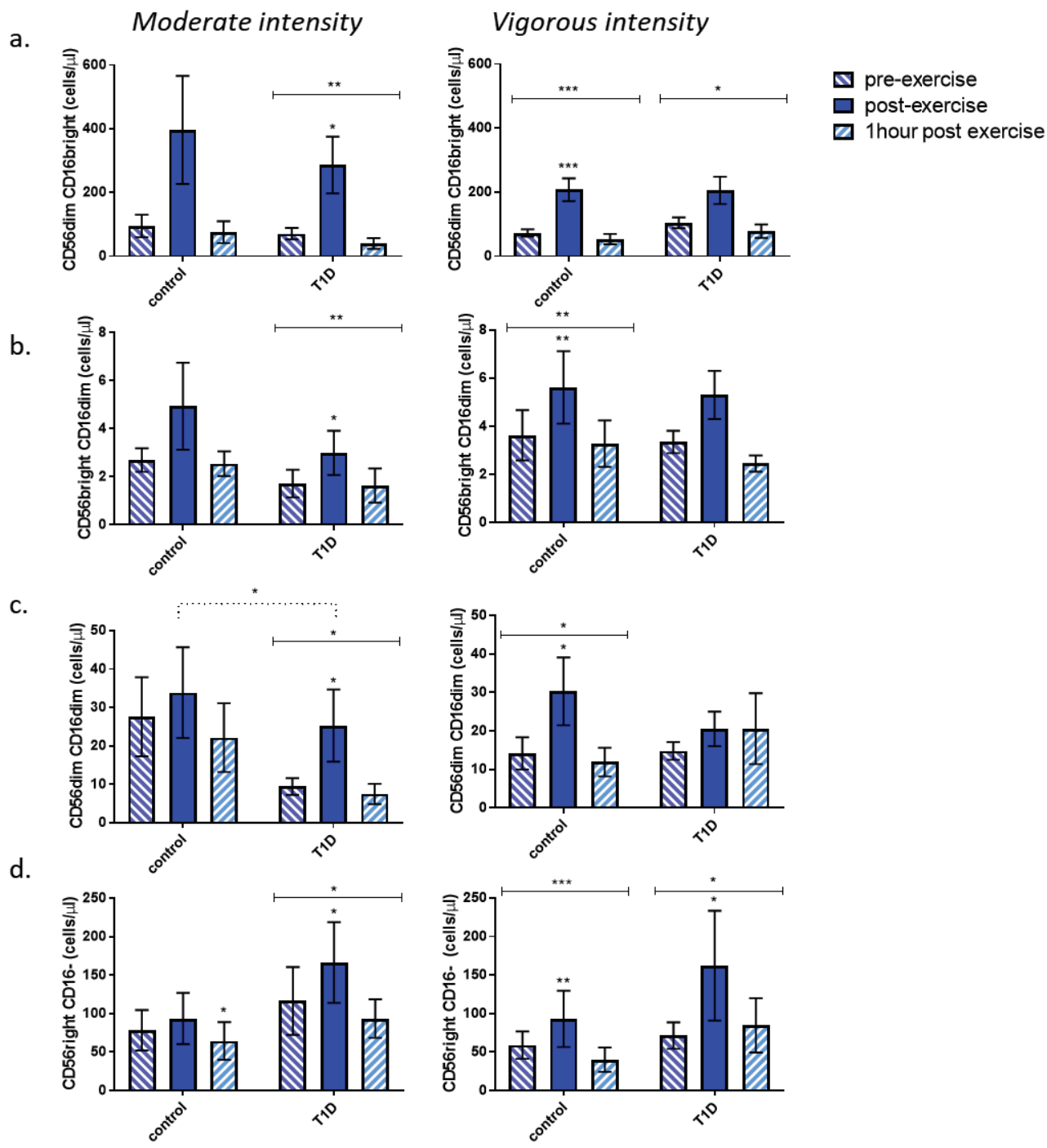

Figure 18 NK cell subsets during moderate and vigorous exercise in control and T1D groups

Flow cytometry and whole blood cell counts were used to determine the frequency of NK cell subsets in control and T1D participants during moderate and vigorous intensity exercise. (a) Frequency of CD56dimCD16bright (b) frequency of CD56brightCD16dim (c) frequency of CD56dimCD16dim (d) frequency of CD56brightCD16- NK cells during moderate and vigorous intensity exercise. Error bars represent SEM. Statistical significance represented by * $p<0.05$, ** $p<0.01$, ***$p<0.001$. Note: broken line (----) represents time*group differences.

Subset (cells/μl)	Intensity	[1]Controls T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast	[a]Δ%	[2]T1D T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast	[a]Δ%	[d]Time (overall)	[d]Time*Group
$CD56^{dim}CD16^{bright}$	moderate	94.88	100.01	396.61	449.08	75.54	96.61	$F_{(1, 7)}$= 3.807, p=0.092	*T2* $F_{(1, 7)}$= 4.003, p=0.086	318.01	70.91	36.01	286.25	178.41	39.87	33.71	**$F_{(2, 6)}$= 11.175, p=0.009**	*T2* **$F_{(1, 3)}$= 11.909, p=0.041**	303.66	**$F_{(1, 10.1)}$= 6.379, p=0.030**	$F_{(1, 10.1)}$= 0.008, p=0.992
									T3 $F_{(1, 7)}$= 1.279, p=0.295	20.38								*T3* $F_{(1, 3)}$= 2.501, p=0.212	43.77		
	vigorous	72.85	34.95	207.70	106.46	53.35	43.13	**$F_{(1.1, 7.5)}$= 27.545, p=0.001**	*T2* **$F_{(1, 7)}$= 29.471, p=0.001**	185.12	104.38	47.47	205.69	120.49	78.18	51.31	**$F_{(2, 12)}$= 3.929, p=0.049**	*T2* $F_{(1, 6)}$= 3.345, p=0.117	97.06	**$F_{(1.4, 18.2)}$= 22.237, p<0.001**	$F_{(1.4, 18.2)}$= 2.113, p=0.159
									T3 $F_{(1, 7)}$= 2.637, p=0.148	26.77								*T3* $F_{(1, 6)}$= 0.702, p=0.434	25.11		
$CD56^{bright}CD16^{dim}$	moderate	2.69	1.38	4.93	4.79	2.54	1.45	$F_{(1, 7.2)}$= 2.798, p=0.137	*T2* $F_{(1, 7)}$= 2.391, p=0.166	83.14	1.72	1.14	2.99	1.84	1.63	1.42	**$F_{(2, 6)}$= 12.130, p=0.008**	*T2* **$F_{(1, 3)}$= 11.372, p=0.043**	74.34	**$F_{(2, 20)}$= 4.184, p=0.030**	$F_{(2, 20)}$= 0.062, p=0.940
									T3 $F_{(1, 7)}$= 1.810, p=0.220	5.51								*T3* $F_{(1, 3)}$= 0.013, p=0.916	5.00		
	vigorous	3.63	3.14	5.62	4.52	3.28	2.55	**$F_{(2, 14)}$= 10.682, p=0.002**	*T2* **$F_{(1, 7)}$= 15.235, p=0.006**	54.79	3.35	1.32	5.31	2.83	2.45	0.82	$F_{(2, 12)}$= 3.340, p=0.070	*T2* $F_{(1, 6)}$= 2.911, p=0.139	58.34	**$F_{(1.4, 17.9)}$= 12.477, p=0.001**	$F_{(1.4, 17.9)}$= 0.662, p=0.473
									T3 $F_{(1, 7)}$= 2.137, p=0.187	9.70								*T3* $F_{(1, 6)}$= 1.580, p=0.256	26.82		
$CD56^{dim}CD16^{dim}$	moderate	27.60	29.14	33.87	31.24	22.15	25.28	$F_{(2, 14)}$= 3.602, p=0.055	*T2* $F_{(1, 7)}$= 2.676, p=0.146	22.74	9.42	4.33	25.30	18.80	7.50	5.32	**$F_{(2, 6)}$= 8.413, p=0.018**	*T2* **$F_{(1, 3)}$= 17.656, p=0.025**	168.50	**$F_{(2, 20)}$= 15.736, p<0.001**	**$F_{(2, 20)}$= 4.502, p=0.024**
									T3 $F_{(1, 7)}$= 1.321, p=0.288	19.72								*T3* $F_{(1, 3)}$= 2.169, p=0.237	20.36		
	vigorous	14.17	12.58	30.29	26.43	11.93	9.88	**$F_{(1.2, 8.2)}$= 8.822, p=0.015**	*T2* **$F_{(1, 7)}$= 8.095, p=0.025**	113.78	14.81	6.47	20.51	12.72	20.60	22.54	$F_{(1.1, 6.3)}$= 0.385, p=0.689	*T2* $F_{(1, 6)}$= 3.877, p=0.096	38.54	**$F_{(2, 26)}$= 5.679, p=0.009**	$F_{(2, 26)}$= 2.657, p=0.089
									T3 $F_{(1, 7)}$= 0.648, p=0.447	15.82								*T3* $F_{(1, 6)}$= 0.107, p=0.755	39.09		
$CD56^{bright}CD16^{-}$	moderate	78.39	74.13	93.73	87.71	64.55	69.07	$F_{(2, 14)}$= 3.484, p=0.059	*T2* $F_{(1, 7)}$= 0.075, p=0.792	19.57	116.46	88.20	166.46	104.64	93.67	50.05	**$F_{(2, 6)}$= 5.976, p=0.037**	*T2* **$F_{(1, 3)}$= 14.489, p=0.032**	42.93	**$F_{(2, 20)}$= 7.083, p=0.005**	$F_{(2, 20)}$= 2.565, p=0.102
									T3 **$F_{(1, 7)}$= 6.548, p=0.038**	17.65								*T3* $F_{(1, 3)}$= 0.232, p=0.663	19.57		
	vigorous	59.03	52.93	93.16	109.78	40.12	41.74	**$F_{(2, 14)}$= 13.011, p=0.001**	*T2* **$F_{(1, 7)}$= 17.043, p=0.004**	57.81	71.46	48.75	162.20	201.27	84.84	86.38	**$F_{(2, 12)}$= 6.493, p=0.012**	*T2* **$F_{(1, 6)}$= 8.078, p=0.029**	126.98	**$F_{(2, 26)}$= 17.607, p<0.001**	$F_{(2, 26)}$= 0.471, p=0.630
									T3 $F_{(1, 7)}$= 2.099, p=0.191	32.03								*T3* $F_{(1, 6)}$= 0.053, p=0.825	18.72		
$CD56^{-}CD16^{+}$	moderate	16.35	20.51	25.15	30.78	23.47	30.95	**$F_{(2, 12)}$= 4.004, p=0.047**	*T2* **$F_{(1, 6)}$= 8.607, p=0.026**	53.82	10.52	18.13	15.71	25.65	12.10	20.93	$F_{(2, 6)}$= 2.974, p=0.127	*T2* $F_{(1, 3)}$= 4.344, p=0.128	49.42	**$F_{(2, 18)}$= 7.362, p=0.005**	$F_{(2, 18)}$= 1.875, p=0.182
									T3 $F_{(1, 6)}$= 0.808, p=0.403	43.51								*T3* $F_{(1, 3)}$= 0.941, p=0.404	15.02		
	vigorous	22.29	27.52	36.13	43.37	28.28	28.36	**$F_{(2, 14)}$= 7.781, p=0.005**	*T2* **$F_{(1, 7)}$= 18.022, p=0.004**	62.10	8.35	8.58	12.80	13.36	11.98	16.08	$F_{(2, 12)}$= 2.076, p=0.168	*T2* $F_{(1, 6)}$= 4.559, p=0.077	53.38	**$F_{(1.4, 18.4)}$= 5.590, p=0.020**	$F_{(1.4, 18.4)}$= 0.778, p=0.432
									T3 $F_{(1, 7)}$= 1.955, p=0.205	26.87								*T3* $F_{(1, 6)}$= 0.368, p=0.566	43.50		

Table 16 NK cell subsets mobilised during moderate and vigorous intensity exercise in control and T1D groups

Mean, standard deviation, and statistical analysis of NK cell subsets for control and T1D participants during moderate and vigorous intensity exercise. Significant results highlighted in bold where p values <0.05 were considered significant.

[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2) or 1 hour post exercise (T3).

[b] Results were analysed using multiple regression analysis in control and T1D groups independently.

[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).

[d] Results were analysed using multiple regression analysis in control and T1D groups combined.

[1]Controls n=9-10, [2]T1D n =5-9

4.3.2 Migratory capacity of peripheral blood lymphocyte populations in T1D and control participants

3.2.1 Lymphocyte transendothelial migration and migratory capacity at rest

Transendothelial migration (TEM) of lymphocytes was measured at rest in T1D and control participants. The percentage TEM of lymphocytes showed a trend to be higher in the T1D group than control group (p=0.061) (Figure 19a). The total adherence of lymphocytes was not significantly different between groups (Figure 19b) (Table 17).

Subset	[1]Controls		[2]T1D	
	mean	SD	mean	SD
Percentage TEM	26.77	11.36	33.70	7.83
Total adherence	300.29	194.23	245.63	100.30

Table 17 Transendothelial migration of lymphocytes at baseline in T1D and controls.

Mean and standard deviation values for the percentage behaviour of transendothelial migration and total adherence of lymphocytes in controls and T1D participants.
[1]controls n=16, [2]T1D n=16

The homing propensity and migratory capacity of total $CD3^+$ and $CD3^-$ lymphocyte subpopulations were measured using the expression of the following adhesion molecules and chemokine receptors; CD11b, LFA-1, CXCR3, CCR6, CCR4, and CD62L. The mean and standard deviations values are displayed in Table 18.

The frequency of $CD3^+CXCR3^+$ cells was significantly lower in T1D (p=0.033), but there was no significant difference in the proportion of this subset between groups (Figure 19a and b). However, the proportion of $CD3^-CXCR3^+$ cells were significantly higher in T1D (p=0.043), but there was no significant difference in the frequencies of this subset between groups (Figure 19c and d). The frequency of $CD3^-LFA\text{-}1^+$ cells showed a trend to decrease in the T1D group (p=0.064). However, the frequency of $CD3^+LFA\text{-}1^+$ cells showed a trend to increase in the T1D group (p=0.064), and a small trend in the proportion of this subset (p=0.085). There were no

differences between groups in CD11b, CCR6, CCR4 or CD62L expression by $CD3^+$ and $CD3^-$ lymphocytes (Table 18). This data provides evidence of an imbalance in adhesion molecules on lymphocyte subpopulations in T1D.

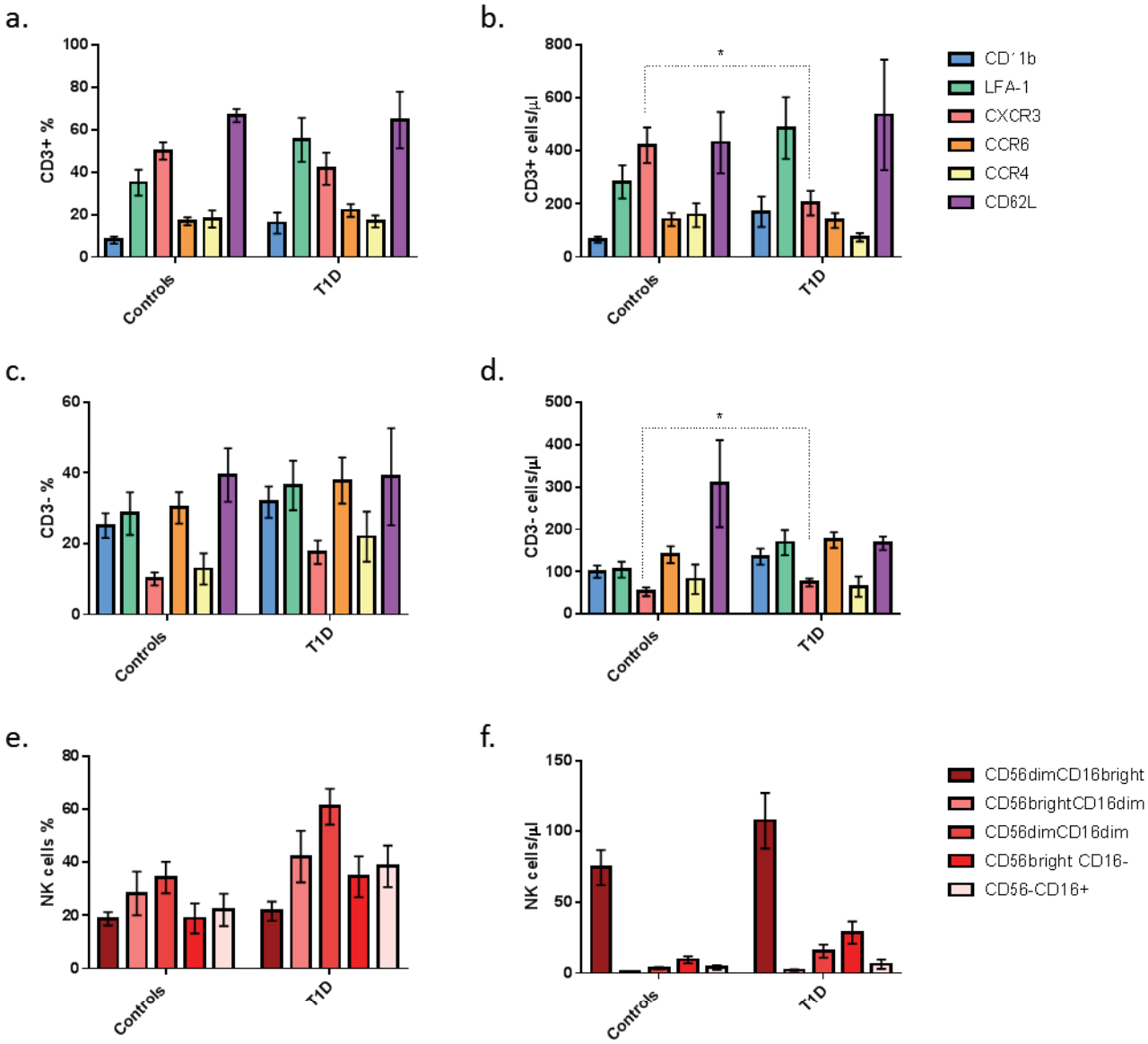

Figure 19 The proportion and frequency adhesion molecules expressed by lymphocyte subsets at baseline in T1D and control groups.

Flow cytometry and whole blood cell counts were used to determine the frequency of CD11b, LFA-1, CXCR3, CCR6, CCR4, and CD62L expression by lymphocyte subsets in control and T1D participants at baseline. (a) Proportion of adhesion molecules expressed by CD3+ cells (b) Frequency of adhesion molecules expressed by CD3+ cells (c) Proportion of adhesion molecules expressed by CD3- cells (d) Frequency of adhesion molecules expressed by CD3- cells. (e) Proportion of LFA-1 expressed by NK cell subsets (f) Frequency of LFA-1 expressed by NK cell subsets. Error bars represent SEM. Statistical significance represented by * $p<0.05$, ** $p<0.01$, ***$p<0.001$. Note: broken line (----) represents time*group differences.

	[1]Controls											
	CD11b		**LFA-1**		**CXCR3**		**CCR6**		**CCR4**		**CD62L**	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
$CD3^+$ (%)	8.02	7.97	35.04	27.16	50.01	17.11	16.88	8.49	17.96	17.38	66.68	9.42
(cells/µl)	65.76	54.58	283.38	278.45	421.36	284.61	141.56	110.29	158.02	195.07	431.35	347.67
$CD3^-$ (%)	25.11	16.41	28.51	27.01	10.04	7.72	30.11	19.88	12.89	19.18	39.43	22.59
(cells/µl)	100.44	68.26	105.17	83.56	53.09	43.91	140.40	87.95	82.45	151.32	307.84	308.50
	[2]T1D											
$CD3^+$ (%)	16.01	19.27	55.19	40.08	41.65	27.32	21.98	12.34	16.83	9.59	64.53	23.08
(cells/µl)	170.81	204.96	486.01	435.55	203.26	147.23	137.96	104.70	74.41	48.02	535.51	360.50
$CD3^-$ (%)	31.76	17.10	36.48	27.18	17.59	12.36	37.85	27.69	21.98	24.53	38.93	23.71
(cells/µl)	135.67	68.41	168.95	110.69	74.76	30.32	175.10	71.74	64.83	71.86	167.32	27.13

Table 18 Adhesion molecule and chemokine receptor expression by CD3+ and CD3- lymphocytes at baseline in T1D and control participants

Mean and standard deviation values for the proportion and frequency of adhesion molecules expressed by CD3+ and CD3- lymphocytes populations in control and T1D participants.
[1]controls CD11b n=22, LFA-1 n=20, CXCR3 n=18, CCR6 n= 20, CCR4 n=19, CD62L n= 9.
[2]T1D CD11b n=13, LFA-1 n=14, CXCR3 n=10, CCR6 n=14, CCR4 n=9, CD62L n= 3.

The homing propensity and migratory capacity of NK cell subpopulations was measured using the surface expression of the LFA-1 on NK cell subsets; $CD56^{bright}CD16^{dim}$, $CD56^{dim}CD16^{dim}$, $CD56^{dim}CD16^{dim}$, $CD56^{bright}CD16^{-}$, and $CD56^{-}CD16^{+}$ (Table 19) (Figure 19e and f).

The proportion of $CD56^{dim}CD16^{dim}$ NK cells expressing LFA-1 was significantly elevated in the T1D group (p=0.006), but no significant difference in the frequencies of this subset were observed between groups (Figure 19a). There were no differences in LFA-1 expression between groups on $CD56^{bright}CD56^{dim}$, $CD56^{dim}CD16^{dim}$, $CD56^{bright}CD16^{-}$, and $CD56^{-}CD16^{+}$ NK cell subsets (Figure 19e and f). The mean and standard deviations values are displayed in Table 19.

Subset	[1]Controls		[2]T1D	
	mean	SD	mean	SD
$CD56^{dim}CD16^{bright}$ (%)	18.76	11.35	21.63	14.05
(cells/µl)	74.58	55.41	107.64	73.07
$CD56^{bright}CD16^{dim}$ (%)	28.33	36.72	42.24	37.78
(cells/µl)	0.51	0.67	2.06	2.63
$CD56^{dim}CD16^{dim}$ (%)	34.31	26.63	61.04	26.06
(cells/µl)	3.51	2.94	15.50	17.37
$CD56^{+}CD16^{-}$ (%)	18.89	25.36	34.60	29.92
(cells/µl)	9.37	10.54	28.53	29.58
$CD56^{-}CD16^{+}$ (%)	22.13	27.19	38.51	30.35
(cells/µl)	3.84	6.88	6.22	12.28

Table 19 LFA-1 expression by NK cell subsets at baseline in T1D and controls.

Mean and standard deviation values for the proportion and frequency of LFA-1+ NK cell subsets in control and T1D participants.
[1]controls n=20, [2]T1D n=14

4.3.2.2 Lymphocyte transendothelial migration in response to acute exercise in T1D and control participants

Transendothelial migration (TEM) and total adherence (ADH) of lymphocytes was measured during moderate (40% VO_2 max) and vigorous (80% VO_2 max) acute exercise in T1D and control participants.

There was a significant change over time in the TEM of lymphocytes during vigorous intensity exercise overall (p=0.001), but this was observed for the T1D group only (p=0.001) (Figure 20e). TEM was significantly reduced 1 hour post vigorous exercise overall (p=0.007) and in the T1D group (p=0.018). There was a significant decrease with adiponectin treatment overall (p=0.035), but this was seen only as a trend to decrease in the T1D group (p=0.060). There was no change in TEM during moderate intensity exercise for either group (Table 20) (Figure 20c).

There was a significant change over time in the total ADH of lymphocytes to endothelial cells during vigorous intensity exercise overall (p=0.020), but this was seen in the T1D group only (p=0.027). There was a significant decrease in total ADH with adiponectin treatment overall (p=0.004), but this was seen only in the T1D group (p=0.003) (Figure 20f). There was no change in TEM during moderate intensity exercise. There was a significant decrease in total ADH with adiponectin treatment overall (p=0.001), but this was seen only in the control group (p=0.004) (Table 20) (Figure 20d).

In summary, TEM and total ADH of lymphocytes is affected by vigorous intensity exercise in the T1D group only.

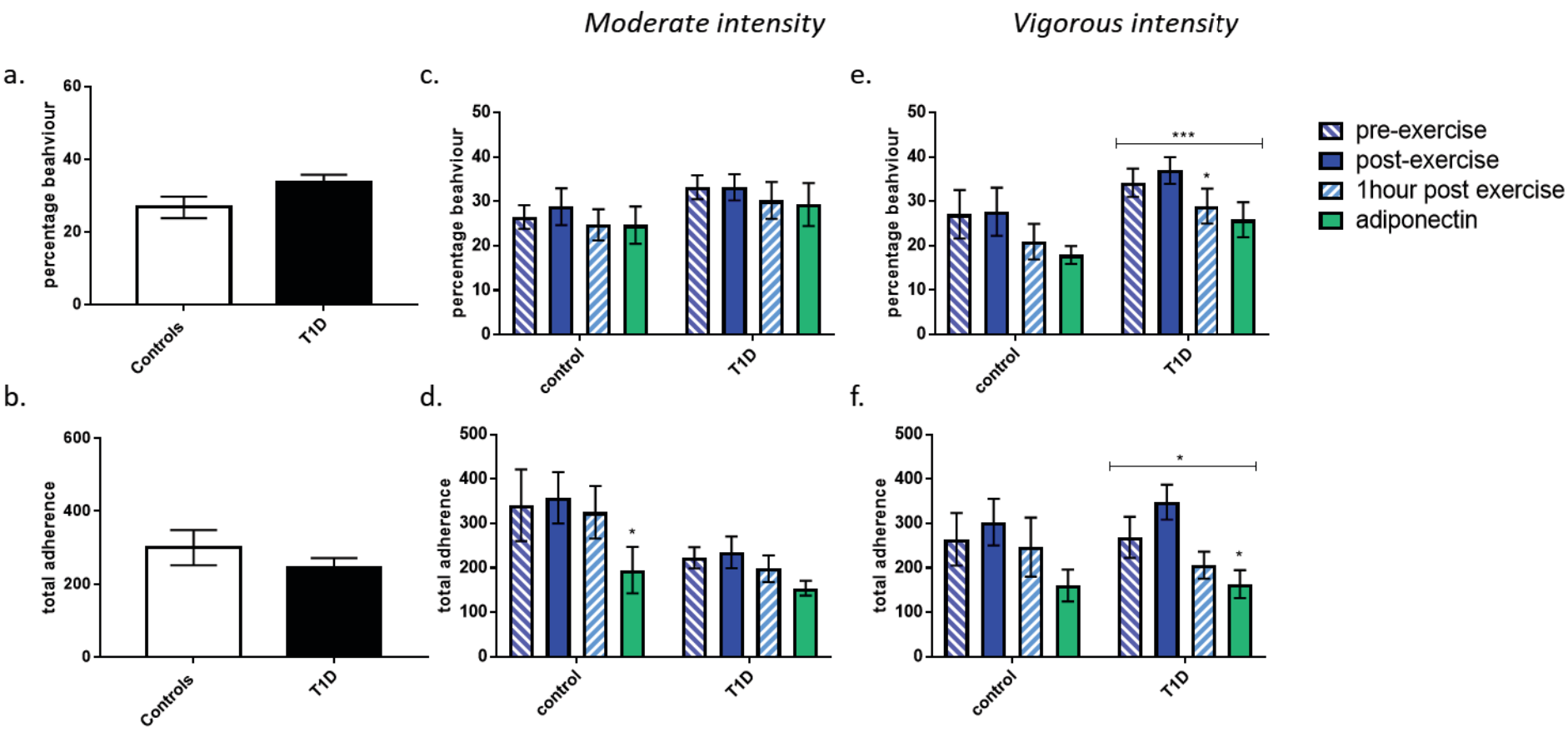

Figure 20 The percentage transmigration and total adherence by lymphocytes at baseline, and during moderate and vigorous intensity exercise in T1D and control participants.

Transendothelial migration of lymphocytes was in control and T1D participants at baseline, and during moderate and vigorous intensity exercise. (a) Percentage transmigration of lymphocytes at baseline (b) Total adherence of lymphocytes at baseline (c) Percentage transmigration of lymphocytes at during moderate intensity (d) Total adherence of lymphocytes at during moderate intensity (e) Percentage transmigration of lymphocytes at during vigorous intensity (f) Total adherence of lymphocytes at during vigorous intensity. Error bars represent SEM. Statistical significance represented by * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

	Intensity	[1]Controls T1		T2		T3		Adipo		[b]Time	[c]contrast		[a]Δ%	[2]T1D T1		T2		T3		Adipo		[b]Time	[c]contrast		[a]Δ%	[d]Time (overall)	[d]Time*Group
		mean	SD	mean	SD	mean	SD	mean	SD					mean	SD	mean	SD	mean	SD	mean	SD						
Percentage TEM	moderate	26.48	7.059	28.84	10.98	24.72	9.2202	24.67	11.12	$F_{(2, 14)}$= 0.778, p=0.478	*T2*	$F_{(1, 7)}$= 0.384, p=0.555	8.91	33.22	7.11	33.20	7.85	30.25	9.28	29.30	12.77	$F_{(2, 10)}$= 0.635, p=0.550	*T2*	$F_{(1, 5)}$= 0.361, p=0.574	0.08	$F_{(2, 24)}$= 0.932, p=0.408	$F_{(2, 24)}$= 0.412, p=0.667
											T3	$F_{(1, 7)}$= 0.593, p=0.467	6.63										*T3*	$F_{(1, 5)}$= 0.847, p=0.400	8.95		
											adipo	$F_{(1, 7)}$= 0.775, p=0.408	6.83										*adipo*	$F_{(1, 5)}$= 0.948, p=0.375	11.81		
	vigorous	27.07	14.42	27.65	14.29	20.89	10.56	17.91	4.887	$F_{(2, 14)}$= 3.004, p=0.082	*T2*	$F_{(1, 6)}$= 0.056, p=0.821	2.15	34.18	8.46	36.93	7.97	28.92	10.41	25.86	10.45	**$F_{(2, 14)}$= 6.826, p=0.009**	*T2*	$F_{(1, 7)}$= 1.706, p=0.233	8.06	**$F_{(2, 28)}$= 8.452, p=0.001**	$F_{(2, 28)}$= 0.167, p=0.847
											T3	$F_{(1, 6)}$= 2.833, p=0.143	22.83										*T3*	**$F_{(1, 7)}$= 9.493, p=0.018**	15.38		
											adipo	$F_{(1, 6)}$= 1.401, p=0.281	33.83										*adipo*	$F_{(1, 7)}$= 5.019, p=0.060	24.34		
Total adherence	moderate	340.75	213.15	357.63	153.23	325.13	156.46	195.13	138.16	$F_{(2, 14)}$= 0.310, p=0.738	*T2*	$F_{(1, 7)}$= 0.139, p=0.721	4.95	222.75	64.36	235.00	94.00	198.00	67.12	154.25	44.22	$F_{(2, 10)}$= 0.434, p=0.660	*T2*	$F_{(1, 5)}$= 0.144, p=0.720	5.50	$F_{(2, 24)}$= 0.760, p=0.479	$F_{(2, 24)}$= 0.031, p=0.970
											T3	$F_{(1, 7)}$= 0.104, p=0.757	4.59										*T3*	$F_{(1, 5)}$= 0.298, p=0.609	11.11		
											adipo	**$F_{(1, 7)}$= 17.902, p=0.004**	42.74										*adipo*	$F_{(1, 5)}$= 4.272, p=0.094	30.75		
	vigorous	264.33	167.72	303.22	148.68	246.78	187.88	160.11	101.06	$F_{(2, 14)}$= 0.534, p=0.598	*T2*	$F_{(1, 7)}$= 0.767, p=0.410	14.71	268.50	122	347.88	103.6	206.00	80.1	163.25	83.5	**$F_{(2, 10)}$= 4.705, p=0.027**	*T2*	$F_{(1, 7)}$= 2.391, p=0.166	29.56	**$F_{(2, 28)}$= 4.494, p=0.020**	$F_{(2, 28)}$= 1.623, p=0.215
											T3	$F_{(1, 7)}$= 0.005, p=0.946	6.64										*T3*	$F_{(1, 7)}$= 2.489, p=0.159	23.28		
											adipo	$F_{(1, 7)}$= 3.460, p=0.105	39.43										*adipo*	**$F_{(1, 7)}$= 19.807, p=0.003**	39.20		

Table 20 Transendothelial migration and total adherence of lymphocytes during moderate and vigorous intensity exercise in control and T1D participants

Mean, standard deviation, and statistical analysis of NK cell subsets for control and T1D participants during moderate and vigorous intensity exercise. Significant results highlighted in bold where p values <0.05 were considered significant.

[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2) or 1 hour post exercise (T3).

[b] Results were analysed using multiple regression analysis in control and T1D groups independently.

[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).

[d] Results were analysed using multiple regression analysis in control and T1D groups combined.

[1]Controls n=8, [2]T1D n =8

4.3.2.3 $CD3^+$ subsets migratory capacity in response to acute exercise in T1D and control participants

The homing propensity and migratory capacity of total $CD3^+$ lymphocytes was measured during moderate (40% VO_2 max) and vigorous (80% VO_2 max) acute exercise using the expression of the following adhesion molecules and chemokine receptors; CD11b, LFA-1, CXCR3, CCR6, CCR4, and CD62L. The mean, standard deviation and statistical analysis are displayed in Table 21.

CD11b

$CD3^+CD11b^+$ cells significantly mobilised during vigorous intensity exercise overall ($p=0.002$), and in both groups independently (T1D: $p=0.046$, control: $p<0.001$). There was a significant increase post vigorous exercise in the control group only ($p<0.001$). A significant decrease below baseline 1 hour post vigorous exercise was seen overall ($p=0.001$), but this was seen in the T1D only ($p=0.011$). This response 1 hour post vigorous exercise was significantly different between groups ($p=0.009$) (time*group) because there was a much larger decrease below baseline in the T1D group at this time point (T1D: 42.3%, control: 9.09%) (Figure 21a). There was no significant mobilisation of $CD3^+CD11b^+$ cells during moderate intensity exercise.

LFA-1

There was no significant mobilisation of $CD3^+LFA\text{-}1^+$ cells during moderate or vigorous intensity exercise in either group (Table 21).

CXCR3

$CD3^+CXCR3^+$ cells significantly mobilised during vigorous intensity exercise overall ($p=0.003$), but this was seen in the control group only ($p=0.006$). There was a significant increase post vigorous exercise overall ($p=0.018$), with a trend to increase seen in the control

group (p=0.058). This was followed by a significant decrease below baseline 1 hour post vigorous exercise (p=0.035) overall, with a trend to decrease seen in the control group (p=0.071) (Figure 21b). There was no significant mobilisation of $CD3^+CXCR3^+$ cells during moderate intensity exercise.

CCR6

$CD3^+CCR6^+$ cells significantly mobilised during moderate intensity exercise overall (p=0.032), and in both groups (T1D: p=0.023, control: p=0.049). There was a significant increase post moderate exercise in the T1D group only (p=0.008), with a small trend to increase seen in the control group (p=0.075). There was a significant decrease below baseline 1 hour post moderate exercise overall (p=0.023), but this was only seen in the control group (p=0.038) (Table 21). $CD3^+CCR6^+$ cells significantly changed over time during vigorous intensity exercise overall (p<0.001), and in the control group (p<0.001), with a trend to mobilise seen in the T1D group (p=0.056). There was a significant increase post vigorous exercise overall (p<0.001), and in the control group (p= 0.000), with a trend to increase observed in the T1D group (p=0.056). This was followed by a significant decrease below baseline 1 hour post exercise overall (p<0.001), and in the control (p=0.002) and T1D (p=0.044) groups independently (Figure 21c).

CCR4

$CD3^+CCR4^+$ cells significantly mobilised during vigorous intensity exercise overall (p=0.002), and in the T1D (p=0.004) and control (p=0.014) groups independently. There was a significant increase post vigorous exercise overall (p=0.002), and in both the T1D (p=0.003) and control (p=0.014) groups. There was a significant decrease below baseline 1 hour post vigorous exercise overall (p=0.002), and in the T1D (p=0.004) and control (p=0.014) groups (Figure 21d). There was no significant mobilisation of $CD3^+CCR4^+$ cells during moderate intensity exercise (Table 21).

CD62L

Data for $CD3^{+}CD62L^{+}$ cells is shown for the control group only due to missing data for the T1D group (Table 21). There was no significant change over time during vigorous intensity exercise. A decrease below baseline 1 hour post vigorous exercise was observed (p=0.046). There was no significant mobilisation of $CD3^{+}CD62L^{+}$ cells during moderate intensity exercise.

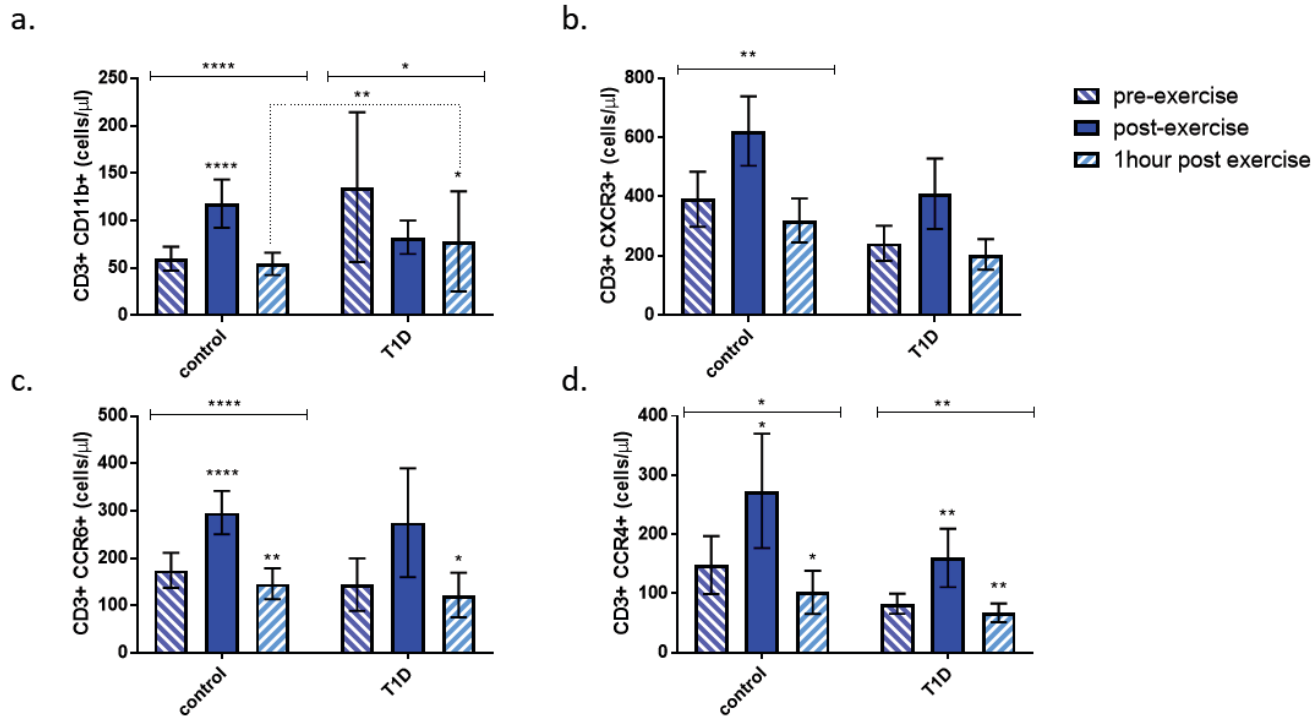

Figure 21 Adhesion molecules expressed by CD3+ cells mobilised during vigorous exercise in control and T1D participants

Flow cytometry and whole blood cell counts were used to determine the frequency of CD11b, CXCR3, CCR6, and CCR4 expression on CD3+ cells in control and T1D participants during vigorous intensity exercise. (a) Frequency of CD3+CD11b+ cells (b) frequency of CD3+CXCR3+ cells (c) frequency of CD3+CCR6+ cells (d) frequency of CD3+CCR4+ cells during vigorous intensity exercise. Error bars represent SEM. Statistical significance represented by * p<0.05, ** p<0.01, ***p<0.001. Note: broken line (----) represents time*group differences.

Subset (cells/μl)	Intensity	[1]Controls T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast	[a]Δ%	[2]T1D T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast	[a]Δ%	[d]Time (overall)	[d]Time*Group
$CD3^+CD11b^+$	moderate	69.74	62.58	95.77	100.50	62.14	77.36	$F_{(1.3, 11.153)}$= 3.301, p=0.090	*T2* $F_{(1, 9)}$= 0.013, p=0.911	37.32	274.97	144.10	335.53	147.25	249.91	149.96	$F_{(2, 6)}$= 1.373, p=0.323	*T2* $F_{(1, 3)}$= 3.190, p=0.172	22.03	$F_{(1.3, 16)}$= 2.132, p=0.161	$F_{(1.3, 16)}$= 0.933, p=0.377
									T3 $F_{(1, 9)}$= 3.466, p=0.096	10.90								*T3* $F_{(1, 3)}$= 0.000, p=0.985	9.11		
	vigorous	59.80	42.10	117.95	80.44	54.36	37.35	**F(2, 18)= 17.796, p<0.001**	*T2* **$F_{(1, 9)}$= 29.527, p<0.001**	97.23	135.33	208.68	82.47	46.41	78.08	129.36	**$F_{(2, 12)}$= 4.007, p=0.046**	*T2* $F_{(1, 6)}$= 0.183, p=0.684	39.06	**$F_{(1.3, 19.3)}$= 22.464, p=0.002**	$F_{(1.3, 19.3)}$= 2.096, p=0.161
									T3 $F_{(1, 9)}$= 2.153, p=0.176	9.09								*T3* **$F_{(1, 6)}$= 13.373, p=0.011**	42.30		
$CD3^+LFA1^+$	moderate	252.37	221.36	375.33	333.87	240.18	220.35	$F_{(1.1, 7.8)}$= 2.983, p=0.122	*T2* $F_{(1, 7)}$= 0.694, p=0.432	48.72	370.77	354.31	404.64	280.41	387.61	361.16	$F_{(1, 3)}$= 3.014, p=0.181	*T2* $F_{(1, 3)}$= 8.561, p=0.061	9.14	**$F_{(2, 20)}$= 6.781, p=0.006**	$F_{(2, 20)}$= 0.715, p=0.501
									T3 $F_{(1, 7)}$= 1.382, p=0.278	4.83								*T3* $F_{(1, 3)}$= 1.732, p=0.280	4.54		
	vigorous	293.66	329.12	474.92	426.27	294.71	215.22	$F_{(2, 14)}$= 1.739, p=0.212	*T2* $F_{(1, 7)}$= 2.796, p=0.138	61.73	580.84	409.29	565.19	338.03	529.31	465.31	$F_{(2, 12)}$= 2.564, p=0.118	*T2* $F_{(1, 6)}$= 0.001, p=0.974	2.69	$F_{(2, 26)}$= 2.877, p=0.074	$F_{(2, 26)}$= 1.181, p=0.323
									T3 $F_{(1, 7)}$= 0.43, p=0.841	0.36								*T3* $F_{(1, 6)}$= 2.151, p=0.193	8.87		
$CD3^+CXCR3^+$	moderate	468.22	239.25	509.08	279.29	464.01	221.56	$F_{(2, 10)}$= 0.082, p=0.922	*T2* $F_{(1, 5)}$= 0.052, p=0.829	8.73	112.03	57.99	174.07	118.37	98.61	21.42	$F_{(2, 4)}$= 1.077, p=0.422	*T2* $F_{(1, 2)}$= 2.082, p=0.286	55.38	$F_{(2, 14)}$= 0.583, p=0.571	$F_{(2, 14)}$= 0.171, p=0.845
									T3 $F_{(1, 5)}$= 0.690, p=0.804	0.90								*T3* $F_{(1, 2)}$= 0.150, p=0.736	11.98		
	vigorous	391.54	294.07	621.30	351.46	319.44	222.92	**$F_{(2, 16)}$= 7.304, p=0.006**	*T2* $F_{(1, 8)}$= 4.880, p=0.058	58.68	242.35	146.07	409.65	290.22	204.96	127.25	$F_{(1.1, 6.6)}$= 3.420, p=0.108	*T2* $F_{(1, 6)}$= 2.667, p=0.154	69.03	**$F_{(1.3, 18)}$= 10.007, p=0.003**	$F_{(1.3, 18)}$= 0.234, p=0.695
									T3 $F_{(1, 8)}$= 4.325, p=0.071	18.42								*T3* $F_{(1, 6)}$= 2.185, p=0.190	15.43		
$CD3^+CCR6^+$	**moderate**	101.31	76.47	161.51	174.84	112.88	103.57	**$F_{(2, 12)}$= 3.921, p=0.049**	*T2* $F_{(1, 6)}$= 4.609, p=0.075	59.42	131.50	42.33	262.98	296.39	128.82	60.01	**$F_{(2, 4)}$= 11.065, p=0.023**	*T2* **$F_{(1, 2)}$= 125.133, p=0.008**	99.98	**$F_{(2, 16)}$= 4.306, p=0.032**	$F_{(2, 16)}$= 0.164, p=0.850
									T3 **$F_{(1, 6)}$= 6.986, p=0.038**	11.41								*T3* $F_{(1, 2)}$= 10.489, p=0.084	2.04		
	vigorous	174.49	117.55	296.13	136.97	146.22	98.07	**F(2, 16)= 25.744, p<0.001**	*T2* **$F_{(1, 8)}$= 32.771, p<0.001**	69.71	144.42	135.95	275.09	281.24	122.45	115.29	$F_{(1.1, 6.4)}$= 5.364, p=0.056	*T2* $F_{(1, 6)}$= 5.616, p=0.056	90.49	**$F_{(1.2, 16.6)}$= 21.344, p<0.001**	$F_{(1.2, 16.6)}$= 0.090, p=0.809
									T3 **$F_{(1, 8)}$= 22.150, p=0.002**	16.20								*T3* **$F_{(1, 6)}$= 6.491, p=0.044**	15.21		
$CD3^+CCR4^+$	moderate	171.51	229.00	191.77	233.51	215.74	258.05	$F_{(1, 7)}$= 3.765, p=0.093	*T2* $F_{(1, 7)}$= 3.795, p=0.092	11.81	45.05	44.20	26.82	36.47	27.73	37.09	$F_{(2, 2)}$= 1.041, p=0.490	*T2* $F_{(1, 1)}$= 1.049, p=0.492	40.47	$F_{(1, 8)}$= 1.347, p=0.288	$F_{(1, 8)}$= 0.472, p=0.632
									T3 $F_{(1, 7)}$= 3.736, p=0.095	25.79								*T3* $F_{(1, 1)}$= 1.033, p=0.495	38.44		
	vigorous	148.20	154.59	273.48	289.59	102.11	109.62	**$F_{(1, 9)}$= 9.249, p=0.014**	*T2* **$F_{(1, 9)}$= 9.294, p=0.014**	84.53	82.80	41.96	160.21	120.61	67.30	38.76	**$F_{(1, 6)}$= 21.571, p=0.004**	*T2* **$F_{(1, 6)}$= 21.876, p=0.003**	93.49	**$F_{(1, 15)}$= 13.590, p=0.002**	$F_{(1, 15)}$= 1.516, p=0.237
									T3 **$F_{(1, 9)}$= 9.204, p=0.014**	31.10								*T3* **$F_{(1, 6)}$= 21.281, p=0.004**	18.72		
$CD3^+CD62L^+$	moderate	274.11	130.57	491.99	241.95	262.02	151.61	$F_{(2, 6)}$= 1.120, p=0.528	*T2* $F_{(1, 3)}$= 1.409, p=0.321	79.49										$F_{(2, 6)}$= 2.952, p=0.128	
									T3 $F_{(1, 3)}$= 0.035, p=0.861	4.41											
	vigorous	627.92	388.45	912.97	492.10	478.26	347.20	$F_{(2, 4)}$= 2.311, p=0.215	*T2* $F_{(1, 2)}$= 1.013, p=0.420	45.40										$F_{(2, 4)}$= 2.949, p=0.163	
									T3 **$F_{(1, 2)}$= 20.076, p=0.046**	23.83											

Table 21 CD3+ lymphocytes mobilised during moderate and vigorous intensity exercise in control and T1D participants

Mean, standard deviation, and statistical analysis of CD3+ lymphocytes for control and T1D participants during moderate and vigorous intensity exercise. Significant results highlighted in bold where p values <0.05 were considered significant.

[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2) or 1 hour post exercise (T3).

[b] Results were analysed using multiple regression analysis in control and T1D groups independently.

[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).

[d] Results were analysed using multiple regression analysis in control and T1D groups combined.

[1]controls CD11b n=11, LFA-1 n=10, CXCR3 n=7-11, CCR6 n= 9-11, CCR4 n=19, CD62L n= 4-5.

[2]T1D CD11b n= 4-8, LFA-1 n=5-9, CXCR3 n=4-7, CCR6 n=7, CCR4 n=7, CD62L n= 1-2.

4.3.2.4 $CD3^-$ subsets migratory capacity in response to acute exercise in T1D and control participants

The homing propensity and migratory capacity of total $CD3^-$ lymphocytes was measured during moderate (40% VO_2 max) and vigorous (80% VO_2 max) acute exercise using the expression of the following adhesion molecules and chemokine receptors; CD11b, LFA-1, CXCR3, CCR6, CCR4, and CD62L. The mean, standard deviation and statistical analysis are displayed in Table 22.

CD11b

$CD3^-CD11b^+$ cells significantly mobilised during moderate intensity exercise overall ($p=0.013$), and in the T1D group ($p=0.034$), with a trend to mobilise seen in the control group ($p=0.060$). There was a significant increase post moderate exercise overall ($p=0.016$), with a trend to increase seen in the T1D ($p=0.064$) and control group ($p=0.071$). $CD3^-CD11b^+$ significantly changed over time during vigorous intensity exercise overall ($p=0.012$), but this was only significant in the control group ($p=0.038$). $CD3^-CD11b^+$ cells significantly increased post vigorous exercise overall ($p=0.015$), and in the control group ($p=0.040$). This was followed by a significant decrease below baseline 1 hour post vigorous exercise overall ($p<0.001$), and in the T1D ($p<0.001$) and control ($p=0.030$) groups (Figure 22a).

LFA-1

$CD3^-LFA\text{-}1^+$ cells significantly mobilised during moderate intensity exercise overall ($p=0.014$), but this was only significant in the control group ($p=0.026$). There was a trend to increase post moderate exercise in the control group ($p=0.053$). There was a significant difference 1 hour post moderate exercise overall ($p=0.007$), and this response was significantly different between groups ($p=0.002$) (time*group) because there was a decrease below baseline in the T1D group, whereas $CD3^-LFA\text{-}1^+$ cells remained above baseline at this time point in the

control group. $CD3^{+}LFA\text{-}1^{+}$ cells significantly mobilised during moderate intensity exercise overall (p=0.002), and in both groups independently (T1D: p=0.001, control: p=0.048). There was a significant increase post moderate exercise overall (p=0.002), and in both groups (T1D: p=0.016, control: p=0.035) (Figure 22b).

CXCR3

$CD3^{-}CXCR3^{+}$ cells significantly mobilised during vigorous intensity exercise overall (p=0.001), and in the control group only (p=0.008), with a trend to mobilise in the T1D group (p=0.061). There was a significant increase post vigorous exercise overall (p=0.003), and in the control group (p=0.012), with a trend to increase observed in the T1D group (p=0.076). This was followed by a significant decrease below baseline 1 hour post vigorous exercise in the control group only (p=0.041). There was no significant mobilisation of $CD3^{-}CXCR3^{+}$ cells during moderate intensity exercise (Table 22).

CCR6

There was a significant change over time in $CD3^{-}CCR6^{+}$ cells during vigorous intensity exercise overall (p=0.001), and in the control group (p<0.001), with a trend to mobilise seen in the T1D group (p=0.058). $CD3^{-}CCR6^{+}$ cells significantly increased post vigorous exercise overall (p=0.003), and in the control group (p= 0.003). This was followed by a significant fall below baseline 1 hour post vigorous exercise overall (p=0.0016), and in the control (p=0.030) group. $CD3^{-}CCR6^{+}$ cells significantly mobilised during moderate intensity exercise overall (p=0.014), but this was not seen in either the T1D or control group independently. A trend to mobilise was seen in the T1D group (p=0.071) (Figure 22c).

CCR4

Data for $CD3^{-}CCR4^{+}$ cells during moderate intensity exercise is shown for the control group only. There was no significant mobilisation of $CD3^{-}CCR4^{+}$ cells during moderate intensity

exercise in the control group. During vigorous intensity exercise, there was significant mobilisation of $CD3^{-}CCR4^{+}$ cells overall ($p<0.001$), but this was only significant in the control group ($p=0.002$). There was a significant increase post vigorous exercise overall ($p=0.001$), and in the control ($p=0.005$) group. This response post exercise was significantly different between groups ($p=0.047$) (time*group) because there was a much larger increase at this time point in the control group (T1D: 94.16%, control: 162.48%). This was supported by a trend in difference between groups overall ($p=0.060$) (time*group) (Table 22).

CD62L

Data for $CD3^{-}CD62L^{+}$ cells is shown for the control group only during moderate intensity exercise due to missing data for the T1D group (Table 22). There was no significant change over time during moderate intensity exercise in the control group.

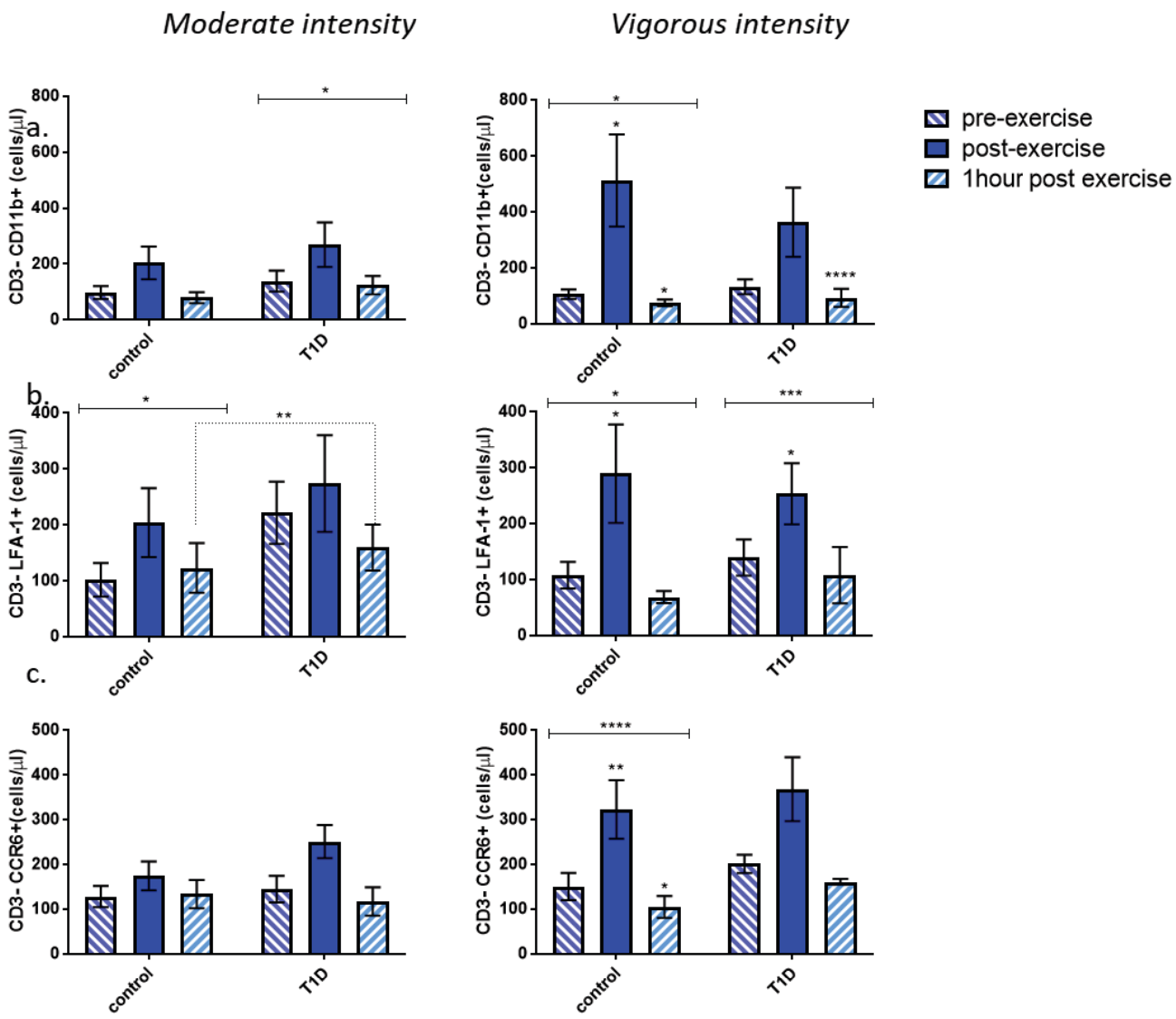

Figure 22 Adhesion molecules expressed by CD3- cells mobilised during moderate and vigorous exercise in control and T1D participants

Flow cytometry and whole blood cell counts were used to determine the frequency of CD11b, LFA-1, and CCR6 expression on CD3- cells in control and T1D participants during moderate and vigorous intensity exercise. (a) Frequency of CD3-CD11b+ cells (b) frequency of CD3-LFA-1+ cells (c) frequency of CD3-CCR6+ cells during moderate and vigorous intensity exercise. Error bars represent SEM. Statistical significance represented by * $p<0.05$, ** $p<0.01$, ***$p<0.001$. Note: broken line (----) represents time*group differences.

Subset (cells/µl)	Intensity	[1]Controls T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast	[a]Δ%	[2]T1D T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast	[a]Δ%	[d]Time (overall)	[d]Time*Group
CD3⁻CD11b⁺	**moderate**	98.12	73.31	204.36	186.41	79.77	59.02	$F_{(1.03, 9.3)}$= 4.541, p=0.060	*T2* $F_{(1, 9)}$= 4.184, p=0.071	108.28	138.94	64.27	268.95	138.87	124.08	56.64	**$F_{(2, 6)}$= 6.226, p=0.034**	*T2* $F_{(1, 3)}$= 8.257, p=0.064	93.57	**$F_{(1.1, 12.7)}$= 8.249, p=0.013**	$F_{(1.1, 12.7)}$= 0.211, p=0.667
									T3 $F_{(1, 9)}$= 0.853, p=0.380	18.70								*T3* $F_{(1, 3)}$= 0.608, p=0.492	10.70		
	vigorous	106.13	57.23	513.04	521.27	74.95	37.86	**$F_{(1, 9.1)}$= 5.864, p=0.038**	*T2* **$F_{(1, 9)}$= 5.741, p=0.040**	383.42	133.10	70.95	363.47	327.03	92.90	78.02	$F_{(1, 6)}$= 3.526, p=0.109	*T2* $F_{(1, 6)}$= 2.862, p=0.142	173.09	**$F_{(1, 15.1)}$= 8.193, p=0.012**	$F_{(1, 15.1)}$= 0.412, p=0.532
									T3 **$F_{(1, 9)}$= 6.606, p=0.030**	29.38								*T3* **F(1, 6)= 51.224, p<0.001**	30.20		
CD3⁻LFA1⁺	**moderate**	102.07	90.47	204.10	185.10	123.13	125.55	**$F_{(1.2, 9.7)}$= 4.636, p=0.026**	*T2* $F_{(1, 8)}$= 5.147, p=0.053	99.95	221.96	111.37	274.31	172.80	159.45	71.13	$F_{(2, 4)}$= 4.309, p=0.100	*T2* $F_{(1, 2)}$= 0.297, p=0.641	23.58	**$F_{(1.3, 12.6)}$= 7.339, p=0.014**	$F_{(1.3, 12.6)}$= 2.350, p=0.147
									T3 $F_{(1, 8)}$= 0.640, p=0.447	20.63								*T3* $F_{(1, 2)}$= 8.430, p=0.101	28.16		
	vigorous	108.27	71.14	289.79	248.39	68.94	30.45	**$F_{(1, 7)}$= 5.730, p=0.048**	*T2* **$F_{(1, 7)}$= 6.756, p=0.035**	167.67	139.50	91.53	253.87	154.86	108.02	133.45	**$F_{(2, 14)}$= 11.840, p=0.001**	*T2* **$F_{(1, 7)}$= 9.886, p=0.016**	81.99	**$F_{(1.2, 16.3)}$= 13.283, p=0.002**	$F_{(1.2, 16.3)}$= 0.454, p=0.539
									T3 $F_{(1, 7)}$= 2.187, p=0.183	36.33								*T3* $F_{(1, 6)}$= 0.905, p=0.373	22.56		
CD3⁻CXCR3⁺	moderate	52.94	30.96	89.94	69.39	62.63	41.20	$F_{(1.1, 5.3)}$= 1.424, p=0.287	*T2* $F_{(1, 5)}$= 1.404, p=0.289	69.90	75.08	25.71	135.13	62.82	48.49	7.09	$F_{(2, 2)}$= 2.027, p=0.330	*T2* $F_{(1, 1)}$= 2.054, p=0.388	80.00	$F_{(2, 12)}$= 3.193, p=0.077	$F_{(2, 12)}$= 0.660, p=0.534
									T3 $F_{(1, 5)}$= 0.604, p=0.472	18.31								*T3* $F_{(1, 1)}$= 1.931, p=0.397	35.41		
	vigorous	53.18	48.68	183.44	148.15	30.11	28.30	**$F_{(1, 8.2)}$= 11.968, p=0.008**	*T2* **$F_{(1, 8)}$= 10.369, p=0.012**	244.92	74.58	30.59	199.05	159.60	72.86	49.12	$F_{(1.1, 6.4)}$= 5.133, p=0.061	*T2* $F_{(1, 6)}$= 4.603, p=0.076	166.89	**$F_{(1.1, 14.7)}$= 15.177, p=0.001**	$F_{(1.1, 14.7)}$= 0.076, p=0.799
									T3 **$F_{(1, 8)}$= 5.918, p=0.041**	43.38								*T3* $F_{(1, 6)}$= 0.018, p=0.899	2.31		
CD3⁻CCR6⁺	**moderate**	128.32	68.29	174.27	91.36	133.56	83.73	$F_{(2, 14)}$= 1.045, p=0.377	*T2* $F_{(1, 7)}$= 1.933, p=0.207	35.81	144.90	72.69	251.16	90.93	117.30	55.07	$F_{(2, 6)}$= 4.255, p=0.071	*T2* $F_{(1, 3)}$= 4.621, p=0.121	73.33	**$F_{(2, 20)}$= 5.351, p=0.014**	$F_{(2, 20)}$= 1.866, p=0.181
									T3 $F_{(1, 7)}$= 0.989, p=0.353	4.08								*T3* $F_{(1, 3)}$= 0.262, p=0.644	19.05		
	vigorous	150.28	96.58	322.72	196.77	94.58	72.80	**F(2, 16)= 18.688, p<0.001**	*T2* **$F_{(1, 8)}$= 17.205, p=0.003**	114.75	201.52	53.72	368.38	189.37	161.09	15.89	$F_{(1.1, 6.7)}$= 5.085, p=0.058	*T2* $F_{(1, 6)}$= 4.212, p=0.086	82.80	**$F_{(1.2, 16.5)}$= 16.631, p=0.001**	$F_{(1.2, 16.5)}$= 0.250, p=0.663
									T3 **$F_{(1, 8)}$= 6.888, p=0.030**	37.07								*T3* $F_{(1, 6)}$= 1.947, p=0.212	20.06		
CD3⁻CCR4⁺	moderate	125.75	202.78	208.03	228.19	98.28	113.06	$F_{(1.2, 6.95)}$= 3.073, p=0.122	*T2* $F_{(1, 6)}$= 3.235, p=0.122	65.43										$F_{(1.2, 6.95)}$= 3.073, p=0.122	
									T3 $F_{(1, 6)}$= 0.003, p=0.958	21.84											
	vigorous	50.96	72.18	133.77	114.85	30.09	48.24	**$F_{(2, 14)}$= 10.337, p=0.002**	*T2* **$F_{(1, 7)}$= 16.644, p=0.005**	162.48	76.19	72.93	147.92	158.28	56.48	41.57	$F_{(2, 12)}$= 3.215, p=0.076	*T2* $F_{(1, 6)}$= 2.855, p=0.142	94.16	**$F_{(2, 26)}$= 12.547, p<0.001**	$F_{(2, 26)}$= 3.150, p=0.060
									T3 $F_{(1, 7)}$= 0.810, p=0.398	40.95								*T3* $F_{(1, 6)}$= 1.899, p=0.217	25.86		
CD3⁻CD62L⁺	moderate	259.25	325.30	387.20	354.01	271.48	380.72	$F_{(2, 6)}$= 1.002, p=0.421	*T2* $F_{(1, 3)}$= 0.246, p=0.654	49.36										$F_{(2, 6)}$= 0.845, p=0.475	
									T3 $F_{(1, 3)}$= 0.469, p=0.543	4.72											
	vigorous																				

Table 22 CD3- lymphocytes mobilised during moderate and vigorous intensity exercise in control and T1D participants

Mean, standard deviation, and statistical analysis of CD3- lymphocytes for control and T1D participants during moderate and vigorous intensity exercise. Significant results highlighted in bold where p values <0.05 were considered significant.

[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2) or 1 hour post exercise (T3).

[b] Results were analysed using multiple regression analysis in control and T1D groups independently.

[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).

[d] Results were analysed using multiple regression analysis in control and T1D groups combined.

[1]controls CD11b n=11, LFA-1 n=10, CXCR3 n=7-11, CCR6 n= 9-11, CCR4 n=19, CD62L n= 4-5.

[2]T1D CD11b n= 4-8, LFA-1 n=5-9, CXCR3 n=4-7, CCR6 n=7, CCR4 n=7, CD62L n= 1-2.

4.3.2.5 NK cell subsets migratory capacity in response to acute exercise in T1D and control participants

The homing propensity and migratory capacity of NK cell subsets was measured during moderate (40% VO_2 max) and vigorous (80% VO_2 max) acute exercise using cell surface expression of the adhesion molecule LFA-1 on NK cell subsets; $CD56^{bright}CD16^{dim}$, $CD56^{dim}CD16^{dim}$, $CD56^{dim}CD16^{dim}$, $CD56^{bright}CD16^{-}$, and $CD56^{-}CD16^{+}$. The mean, standard deviation and statistical analysis are displayed in Table 23.

$CD56^{dim}CD16^{bright}$

There was an overall change over time in LFA-1^{+} $CD56^{dim}CD16^{bright}$ NK cells during moderate intensity exercise ($p<0.001$), and in both T1D ($p=0.009$) and control ($p=0.007$) groups independently. There was an increase post moderate exercise overall ($p=0.001$), and in both T1D ($p=0.041$) and control ($p=0.034$) groups. However, there was an overall trend to respond differently between groups ($p=0.062$) (time*group) because there was a much larger increase post moderate exercise in the T1D group (T1D: 243.55%, control: 139.35%). $CD56^{dim}CD16^{bright}$ NK cells significantly mobilised during vigorous intensity exercise overall ($p<0.001$), and in control group ($p=0.007$), but not in the T1D group. There was an overall trend between groups to respond differently to vigorous intensity exercise ($p=0.074$). An increase post vigorous exercise was seen overall ($p=0.003$) and in the control group only ($p=0.006$) (Figure 23a).

$CD56^{bright}CD16^{dim}$

There was an overall change over time in LFA-1^{+} $CD56^{bright}CD16^{dim}$ NK cells during moderate intensity exercise ($p=0.028$), but this was not seen in either group independently. There was an increase post moderate exercise in the control group only ($p=0.016$). LFA-1^{+} $CD56^{bright}CD16^{dim}$ NK cells increased post vigorous intensity exercise in the control group only

(p=0.035), but no effect over time was seen. This response was significantly different between groups at this time point (p=0.046) (time*group) (Table 23).

CD56^{dim}CD16^{dim}

There was an overall change over time in LFA-1^{+} CD56^{dim}CD16^{dim} NK cells during moderate intensity exercise overall (p=0.023) and in the T1D group (p=0.029), but this was not seen in the control group. There was a trend to increase post moderate exercise in the T1D group only (p=0.069). No mobilisation of LFA-1^{+} CD56^{dim}CD16^{dim} NK cells was seen during vigorous intensity exercise (Figure 23b).

CD56^{bright}CD16^{-}

There was an overall change over time in LFA-1^{+} CD56^{bright}CD16^{-} NK cells during moderate intensity exercise overall (p=0.002), with a trend to mobilise in both T1D (p=0.068) and control (p=0.061) groups. There was an overall decrease below baseline 1 hour post moderate exercise (p=0.041), but this was not seen in either T1D or control groups independently. LFA-1^{+} CD56^{dim}CD16^{-} NK cells significantly mobilised during vigorous intensity exercise overall (p=0.001), and in both T1D (p=0.039) and control (p=0.01) groups. There was an increase post vigorous exercise overall (p=0.018) and in the control group (p=0.025), but this was not significant in the T1D group (Table 23).

CD56^{-}CD16^{+}

There was an overall change over time in LFA-1^{+} CD56^{-}CD16^{+} NK cells during moderate intensity exercise (p=0.012) and in the control group (p=0.006), but this was not seen in the T1D group. There was a significant increase post moderate exercise overall (p=0.011) and in the control group only (p=0.005). LFA-1^{+} CD56^{-}CD16^{+} NK cells significantly mobilised during vigorous intensity exercise overall (p=0.009) and in the control group (p=0.015), but this was not seen in the T1D group. There was a significant increase post vigorous exercise

overall (p=0.013) and in the control group only (p=0.008). No mobilisation of LFA-1^{+} CD56^{-} CD16^{+} NK cells was seen during either exercise intensity in the T1D group (Figure 23c).

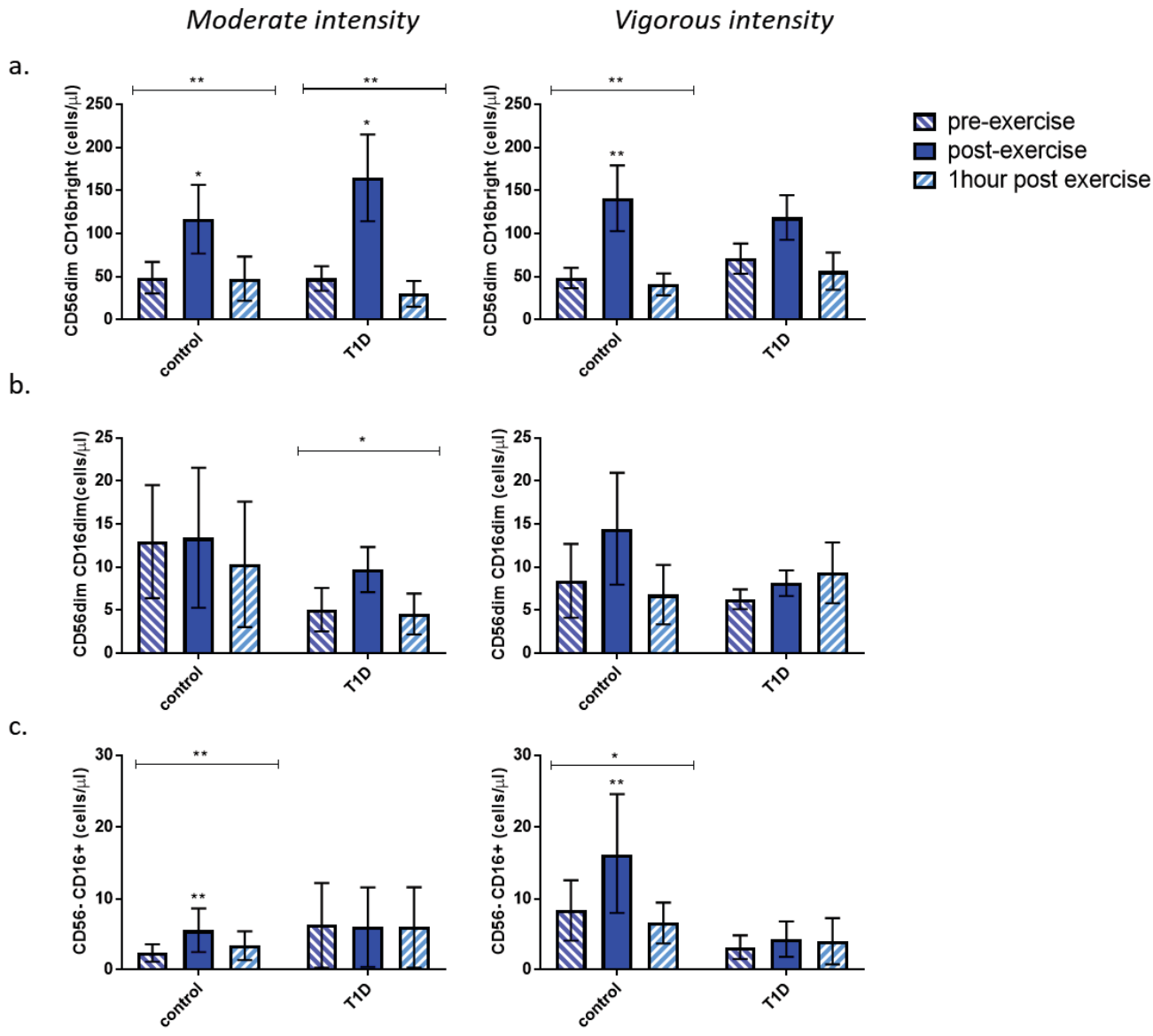

Figure 23 LFA-1 expressed by CD56+ and CD56- cells mobilised during moderate and vigorous exercise in control and T1D participants

Flow cytometry and whole blood cell counts were used to determine the frequency of LFA-1 expression on NK cells in control and T1D participants during moderate and vigorous intensity exercise. (a) Frequency of LFA-1+ CD56dimCD16bright NK cells (b) frequency of LFA-1+ CD56brightCD16dim (c) frequency of LFA-1+ CD56-CD16+ cells during moderate and vigorous intensity exercise. Error bars represent SEM. Statistical significance represented by * p<0.05, ** p<0.01, ***p<0.001.

Subset (cells/µl)	Intensity	[1]Controls T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast	[a]Δ%	[2]T1D T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast	[a]Δ%	[d]Time (overall)	[d]Time*Group
CD56^{dim}CD16^{bright} LFA-1+	moderate	48.81	51.80	116.82	105.40	47.80	72.75	**$F_{(2, 14)}$= 7.156, p=0.007**	*T2* **$F_{(1, 7)}$= 6.909, p=0.034**	139.35	47.95	28.63	164.73	100.77	30.23	29.75	**$F_{(2, 6)}$= 11.236, p=0.009**	*T2* **$F_{(1, 3)}$= 11.921, p=0.041**	243.55	**$F_{(1.3, 12.9)}$= 22.201, p<0.001**	$F_{(1.3, 12.9)}$= 3.887, p=0.062
									T3 $F_{(1, 7)}$= 0.009, p=0.928	2.06								*T3* $F_{(1, 3)}$= 1.151, p=0.362	36.96		
	vigorous	48.56	35.22	141.14	114.30	41.14	33.83	**$F_{(1, 7.3)}$= 13.541, p=0.007**	*T2* **$F_{(1, 7)}$= 13.633, p=0.008**	190.63	70.92	49.46	118.81	73.34	56.46	52.87	$F_{(2, 12)}$= 2.674, p=0.110	*T2* $F_{(1, 6)}$= 2.081, p=0.199	67.53	**$F_{(1.5, 20.1)}$= 14.133, p<0.001**	$F_{(1.5, 20.1)}$= 2.880, p=0.074
									T3 $F_{(1, 7)}$= 3.680, p=0.097	15.29								*T3* $F_{(1, 6)}$= 0.551, p=0.486	20.39		
CD56^{bright}CD16^{dim} LFA-1+	moderate	0.44	0.63	0.70	0.90	0.41	0.63	$F_{(1, 4.2)}$= 5.239, p=0.081	*T2* **$F_{(1, 4)}$= 15.867, p=0.016**	59.40	0.64	0.71	0.66	0.68	0.50	0.49	$F_{(2, 2)}$= 4.688, p=0.275	*T2* $F_{(1, 1)}$= 0.207, p=0.728	3.80	**$F_{(1.1, 5.3)}$= 8.888, p=0.028**	$F_{(1.1, 5.3)}$= 3.937, p=0.100
									T3 $F_{(1, 4)}$= 0.167, p=0.703	5.88								*T3* $F_{(1, 1)}$= 3.115, p=0.328	22.17		
	vigorous	1.88	2.83	2.88	3.92	1.30	1.49	$F_{(2, 12)}$= 2.383, p=0.134	*T2* **$F_{(1, 6)}$= 7.342, p=0.035**	52.99	1.73	1.88	1.97	2.74	1.23	1.16	$F_{(2, 10)}$= 0.616, p=0.559	*T2* $F_{(1, 5)}$= 0.643, p=0.459	13.80	$F_{(2, 22)}$= 1.110, p=0.347	$F_{(2, 22)}$= 1.850, p=0.181
									T3 $F_{(1, 6)}$= 0.000, p=0.987	31.00								*T3* $F_{(1, 5)}$= 1.586, p=0.264	29.01		
CD56^{dim}CD16^{dim} LFA-1+	moderate	12.96	18.56	13.41	21.48	10.32	20.57	$F_{(2, 12)}$= 0.674, p=0.528	*T2* $F_{(1, 6)}$= 0.075, p=0.793	3.44	5.06	5.04	9.72	5.23	4.57	4.76	**$F_{(2, 6)}$= 6.745, p=0.029**	*T2* $F_{(1, 3)}$= 7.700, p=0.069	91.88	**$F_{(2, 18)}$= 4.684, p=0.023**	$F_{(2, 18)}$= 1.444, p=0.262
									T3 $F_{(1, 6)}$= 0.500, p=0.506	20.36								*T3* $F_{(1, 3)}$= 2.407, p=0.219	9.72		
	vigorous	8.41	12.84	14.47	19.42	6.81	9.12	$F_{(2, 14)}$= 1.381, p=0.284	*T2* $F_{(1, 7)}$= 0.922, p=0.369	72.04	6.28	3.25	8.15	4.20	9.34	8.62	$F_{(1.4, 7)}$= 0.671, p=0.463	*T2* $F_{(1, 6)}$= 2.188, p=0.190	29.85	$F_{(2, 26)}$= 1.589, p=0.223	$F_{(2, 26)}$= 0.514, p=0.604
									T3 $F_{(1, 7)}$= 0.762, p=0.412	19.04								*T3* $F_{(1, 6)}$= 0.266, p=0.624	48.74		
CD56^{bright}CD16- LFA-1+	moderate	15.13	18.83	18.50	26.63	12.50	24.55	$F_{(1.2, 8.1)}$= 4.541, p=0.061	*T2* $F_{(1, 7)}$= 0.160, p=0.701	22.24	6.96	6.71	16.46	14.61	6.69	7.72	$F_{(2, 6)}$= 4.338, p=0.068	*T2* $F_{(1, 3)}$= 5.559, p=0.100	136.56	**$F_{(2, 20)}$= 8.761, p=0.002**	$F_{(2, 20)}$= 1.644, p=0.218
									T3 $F_{(1, 7)}$= 4.298, p=0.077	17.38								*T3* $F_{(1, 3)}$= 2.017, p=0.251	3.92		
	vigorous	13.58	11.85	25.58	31.93	12.13	14.23	**$F_{(2, 12)}$= 6.959, p=0.010**	*T2* **$F_{(1, 6)}$= 3.032, p=0.025**	88.34	29.04	32.95	37.62	47.60	21.68	18.94	**$F_{(2, 10)}$= 4.542, p=0.039**	*T2* $F_{(1, 5)}$= 0.826, p=0.405	29.57	**$F_{(2, 22)}$= 9.611, p=0.001**	$F_{(2, 22)}$= 1.696, p=0.207
									T3 $F_{(1, 6)}$= 0.001, p=0.972	10.71								*T3* $F_{(1, 5)}$= 3.052, p=0.141	25.34		
CD56-CD16+ LFA-1+	moderate	2.32	3.42	5.46	8.00	3.33	5.64	**$F_{(2, 6)}$= 12.145, p=0.006**	*T2* **$F_{(1, 3)}$= 55.173, p=0.005**	134.69	6.27	11.94	6.01	11.23	5.97	11.35	$F_{(2, 2)}$= 1.040, p=0.490	*T2* $F_{(1, 1)}$= 1.051, p=0.492	4.10	**$F_{(2, 8)}$= 8.047, p=0.012**	$F_{(2, 8)}$= 1.496, p=0.281
									T3 $F_{(1, 3)}$=4.180, p=0.133	43.42								*T3* $F_{(1, 1)}$= 1.297, p=0.459	4.75		
	vigorous	8.32	12.89	16.24	25.08	6.57	7.77	**$F_{(2, 12)}$= 6.135, p=0.015**	*T2* **$F_{(1, 6)}$= 15.628, p=0.008**	95.13	3.13	4.63	4.23	6.94	3.94	7.82	$F_{(2, 12)}$= 1.843, p=0.200	*T2* $F_{(1, 6)}$= 1.387, p=0.283	35.36	**$F_{(2, 24)}$= 5.825, p=0.009**	$F_{(2, 24)}$= 0.190, p=0.829
									T3 $F_{(1, 6)}$= 0.630, p=0.457	21.13								*T3* $F_{(1, 6)}$= 0.636, p=0.455	26.02		

Table 23 LFA-1+ NK cell subsets during moderate and vigorous intensity exercise in control and T1D participants

Mean, standard deviation, and statistical analysis of LFA-1+ NK cell subsets for control and T1D subjects during moderate and vigorous intensity exercise. Significant results highlighted in bold where p values <0.05 were considered significant.

[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2) or 1 hour post exercise (T3).

[b] Results were analysed using multiple regression analysis in control and T1D groups independently.

[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).

[d] Results were analysed using multiple regression analysis in control and T1D groups combined.

[1]Controls n=9-10, [2]T1D n =5-9

4.3.2.6 AdipoR1 and AdipoR2 B cell expression in T1D and control participants

AdipoR1^{+} and AdipoR2^{+} B cells were measured at rest in T1D and control participants. The frequency of AdipoR1^{+} and AdipoR2^{+} B cells showed a trend to be reduced in T1D compared to control participants (Figure 24c and d). However, there was no significant difference was observed in the proportion of thee subsets between groups (Figure 24a and b) (Table 24).

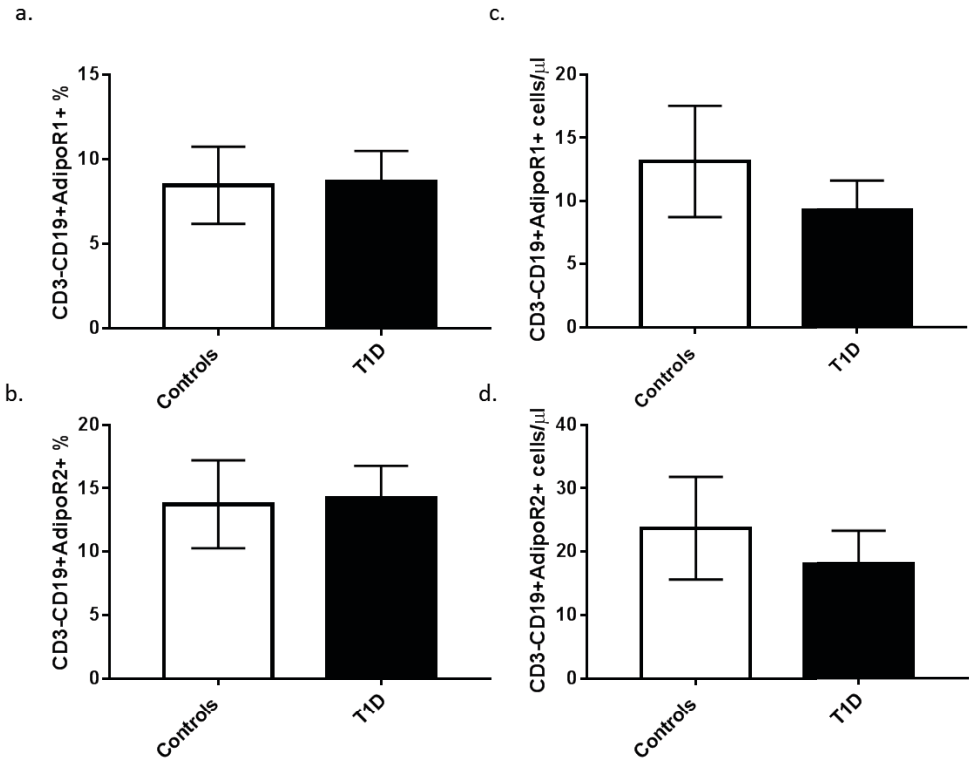

Figure 24 The proportion and frequency adiponectin receptors expressed by B cells at baseline in T1D and control groups.

Flow cytometry and whole blood cell counts were used to determine the frequency of AdipoR1 and AdipoR2 expression by B cells in control and T1D participants at baseline. (a) Proportion AdipoR1+ B cells (b) Proportion of AdipoR2+ B cells (c) Frequency AdipoR1+ B cells (d) Frequency of AdipoR2+ B cells. Error bars represent SEM. No statistical significance observed.

Subset	[1]Controls		[2]T1D	
	mean	SD	mean	SD
AdipoR1$^+$ (%)	7.07	3.09	8.71	3.59
(cells/µl)	17.10	9.44	9.26	4.69
AdipoR2$^+$ (%)	15.03	8.71	14.24	4.38
(cells/µl)	32.94	14.70	18.07	8.96

Table 24 Adiponectin receptor expression on B cells at baseline in T1D and control groups.

Mean and standard deviation values for the proportion and frequency of AdipoR1+ and AdipoR2+ B cells in controls and T1D participants.
[1]controls n=5, [2]T1D n=5

The frequency of AdipoR1$^+$ and AdipoR2$^+$ B cells were measured in response to moderate (40% VO_2 max) and vigorous (80% VO_2 max) acute exercise. Data is missing for AdipoR1 during vigorous intensity exercise in the T1D group. Data is missing for AdipoR2 during vigorous intensity exercise in both the T1D and control group due to missing time-points for each group. The mean, standard deviation and statistical analysis are displayed in Table 25. However, no changes were observed in the frequency of AdipoR1$^+$ and AdipoR2$^+$ B cells in either group during moderate or vigorous intensity exercise.

Subset (cells/µl)	Intensity	[1]Controls										[2]T1D										[d]Time (overall)	[d]Time*Group
		T1		T2		T3		[b]Time	[c]contrast		[a]Δ%	T1		T2		T3		[b]Time	[c]contrast		[a]Δ%		
		mean	SD	mean	SD	mean	SD					mean	SD	mean	SD	mean	SD						
B cells AdipoR1+	moderate	32.06	13.31	39.89	12.75	11.59	13.82	$F_{(1,2)}$= 4.651, p=0.276	*T2*	$F_{(1,1)}$= 1.371, p=0.450	24.42	53.78	58.01	53.70	76.83	48.54	58.89	$F_{(1,2)}$= 1.281, p=0.461	*T2*	$F_{(1,1)}$= 0.010, p=0.450	0.16	$F_{(2,4)}$= 4.463, p=0.096	$F_{(2,4)}$= 0.056, p=0.946
									T3	$F_{(1,1)}$= 6.255, p=0.242	63.85								*T3*	$F_{(1,1)}$= 23.037, p=0.131	9.75		
	vigorous	63.10	56.92	57.68	48.83	35.19	32.61	$F_{(1,2)}$= 2.858, p=0.340	*T2*	$F_{(1,1)}$= 0.896, p=0.517	8.59								*T2*			$F_{(1,2)}$= 1.000, p=0.500	$F_{(1,2)}$= 1.101, p=0.485
									T3	$F_{(1,1)}$= 2.635, p=0.351	44.24								*T3*				
B cells AdipoR2+	moderate	23.12	30.12	49.99	54.77	4.68	0.10	$F_{(1,2)}$= 21.465, p=0.135	*T2*	$F_{(1,1)}$= 15.804, p=0.157	116.21	86.11	71.55	74.75	86.70	51.49	58.52	$F_{(1,2)}$= 3.131, p=0.327	*T2*	$F_{(1,1)}$= 1.123, p=0.482	13.19	$F_{(2,14)}$= 1.458, p=0.266	
									T3	$F_{(1,1)}$= 1.518, p=0.434	79.76								*T3*	$F_{(1,1)}$= 14.125, p=0.166	40.20		
	vigorous								*T2*										*T2*				
									T3										*T3*				

Table 25 B cells expressing adiponectin receptors mobilised during moderate and vigorous intensity exercise in control and T1D participants

Mean, standard deviation, and statistical analysis AdipoR1+ and AdipoR2+ B cells for control and T1D subjects during moderate and vigorous intensity exercise. Significant results highlighted in bold where p values <0.05 were considered significant.

[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2) or 1 hour post exercise (T3).

[b] Results were analysed using multiple regression analysis in control and T1D groups independently.

[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).

[d] Results were analysed using multiple regression analysis in control and T1D groups combined.

[1]Controls n=2-3, [2]T1D n =4-1

4.4 Discussion

The first aim of this study was to measure peripheral blood lymphocyte populations and their migratory capacity in T1D and control participants at rest. NK cells were elevated in T1D, but no other baseline differences in lymphocyte populations were found. Lymphocytes from T1D participants showed a trend to have higher proportion of subsets expressing adhesion molecules. Although lymphocyte adherence to endothelial cells was not higher in T1D, TEM of lymphocytes showed a trend to increase in T1D at rest indicating an increased migratory capacity of the present lymphocytes. Additionally, the mean frequency of AdipoR1^{+} and AdipoR2^{+} B cells show a trend to be higher in control participants compared to T1D contributing to TEM regulation in control participants. However, the numbers in this analysis were low, and therefore it is difficult to conclude these findings.

This study also aimed to investigate the effects of moderate and vigorous intensity exercise on peripheral blood lymphocyte populations and their migratory capacity in T1D and control participants. In summary, vigorous intensity exercise elicited a strong increase in lymphocytes in both T1D and control participants. This increase in lymphocytes was mainly comprised of CD8^{+} T cells and NK cells. Minimal mobilisation of CD4^{+} T cells and B cells was observed in controls, with no significant mobilisation of these lymphocyte subsets observed in T1D. Exercise-induced mobilisation of particular lymphocyte subsets appeared to be blunted in T1D compared to control participants. In T1D, there was a reduced percentage increase of lymphocytes following vigorous exercise. This was driven by a blunted increase in total NK cells during vigorous exercise in T1D. Both of the CD56^{dim} NK cells subsets, CD56^{dim}CD16^{bright} and CD56^{dim}CD16^{dim}, were blunted following vigorous exercise in the T1D group. However, moderate exercise mobilised NK cells dramatically in the T1D group, which was not observed in control group. Further phenotyping showed that all NK cell subsets were

mobilised in the T1D cohort during moderate exercise. Amongst these subsets $CD56^{dim}CD16^{bright}$ displayed the largest increase post exercise.

Vigorous intensity exercise only affected total lymphocyte adherence and TEM, and this was seen only in the T1D group. No change in TEM immediately following vigorous exercise was found, however TEM was significantly suppressed 1 hour following vigorous exercise cessation in the T1D group. This is supported by minimal changes in adhesion molecules on lymphocytes immediately following vigorous exercise and a larger significant decrease in lymphocytes expressing adhesion molecules 1 hour following vigorous exercise in T1D participants. In the TEM model used in this study, LFA-1^+ NK cells have the potential to adhere and transmigrate. However, no changes in LFA-1^+ NK cell subsets were found during exercise. Therefore, it can be postulated that changes in TEM are likely due to alterations in peripheral blood $CD3^+$ T cells. The adhesion molecules CD11b, CCR4, and CCR6 on $CD3^+$ T cells were significantly reduced in T1D participants 1 hour following vigorous exercise.

In the TEM model used in this study, activated endothelial cells express ICAM-1 and chemokines CXCL9, CXCL10, and CXCL11 [315, 316, 331, 332]. This facilitates the migration of LFA-1^+ (ICAM-1) and $CXCR3^+$ (CXCL9, CXCL10, and CXCL11) lymphocytes across HDBEC [322, 325]. It is possible that $CD11b^+$ lymphocytes may also transmigrate because when bound to CD18 this forms Mac-1 which can also bind to ICAM-1 [326]. However, this model does not express the chemokine receptors for CCR4 (CCL2, 4, 5, 17, and 22) or CCR6 (CCL20) [305, 307]. Therefore, the changes in adhesion molecules during exercise in this study herein cannot be directly linked to TEM in the model used. They can however be used to understand physiological changes in TEM that may occur during acute exercise in humans. In the context of T1D, islets express numerous adhesion molecules and chemokine ligands to which the homing markers on lymphocytes used in this study can respond

to. $CD3^+$ T cells expressing CD11b, CCR4, and CCR6 can respond to ICAM-1 and the chemokine ligands CXCL9, CXCL10, CCL2, CCL3, CCL17, CCL21, and CCL22 expressed by islets in T1D [126, 128, 134, 137-139, 338].Therefore, TEM into the pancreas may be suppressed during the recovery period following exercise.

The strengths of this study are that it is the first study of the effects of steady-state acute exercise on lymphocyte subpopulations in T1D. This study gives an in-depth analysis of lymphocyte subsets mobilised by two different exercise intensities, in two cohorts. This is also the first study looking at lymphocyte trafficking and TEM during exercise in T1D. However, the number of recruited participants were low therefore comparisons between groups are difficult. In particular, missing data for AdipoR1 and AdipoR2 makes it difficult to elucidate the mechanisms involved in TEM changes during vigorous exercise in T1D. As discussed, one disadvantage is that the model used to measure TEM cannot be directly related to the changes in adhesion molecules observed following vigorous exercise in T1D participants. Furthermore, it is not defined if exercise directly affects diabetogenic T cell migration. In order to preserve beta cell health, it is imperative that migration of diabetogenic T cells to the pancreas is impaired by exercise. In future work, the migrated lymphocytes could be collected from the bottom of the well and characterised by flow cytometry. It would be useful to include a tetramer for diabetogenic T cells and quantify the number of diabetogenic T cells adhered and migrating across the endothelium at each time point. This would clarify whether diabetogenic T cells specifically migrate during vigorous exercise and if this is reduced 1 hour following exercise cessation as observed for total lymphocytes in this study. Furthermore, soluble chemokine receptors and ligands could be measured in cryo-preserved plasma samples taken for the purpose of this study. This would elucidate trafficking signals during and after vigorous exercise.

In agreement with previous findings, this study demonstrates that vigorous exercise causes a significant increase in peripheral blood lymphocytes, followed by lymphopenia, in healthy cohorts [191-195]. This is the first time this has been shown during steady-state acute exercise in T1D. More specifically, the majority of redeployed lymphocytes were NK cells and $CD8^+$ T cell, with minimal mobilisation of $CD4^+$ T cells and B cells in healthy cohorts [191, 197-200, 215, 257, 364, 365]. Furthermore, the effects of exercise on $CD56^{dim}CD16^{bright}$ and $CD56^{bright}CD16^{dim}$ NK subsets are well established [197, 198]. However, the effects of exercise on $CD56^{dim}CD16^{dim}$, $CD56^{bright}CD16^-$, and $CD56^-CD16^+$ NK cells have not been explored. This study also provides evidence of exercise-induced mobilisation of the less characterised NK cell subsets; $CD56^{dim}CD16^{dim}$, $CD56^{bright}CD16^-$, and $CD56^-CD16^+$.

Although adhesion molecules on lymphocytes increase following vigorous exercise in control participants, no changes in TEM behaviour occurred, suggesting a mechanistic regulation of lymphocyte trafficking during exercise in control participants. Reduced migration of $CD4^+$ and $CD8^+$ T cells following exercise has been previously observed [359]. Firstly, this may be due to the regulation of TEM by the adiponectin-PEPITEM axis reported at rest previously in healthy individuals [315]. However, PEPITEM has not yet been measured following acute exercise. Secondly, crosstalk between stem cells and endothelial cells has been reported to reduce the activation of endothelium [366]. IL-6 produced by stem cells down-regulated adhesion of lymphocytes to endothelial cells. Both IL-6 and HSPC are increased during acute exercise, therefore could modulate TEM regulation following exercise [212, 213, 367].

The effects of acute exercise on lymphocyte trafficking have important implications in T1D. The mobilisation of NK cells and T cells increases immune surveillance through tissue redistribution [191, 214]. This may be important to decrease the high susceptibility to infection observed in people with T1D [368, 369]. Furthermore, suppression of TEM in the recovery

period may inhibit ongoing islet infiltration seen in long-standing T1D [28, 129, 339, 359, 370-372]. This is imperative for beta cell preservation thereby reducing insulin requirements, disease severity, and T1D associated complications.

Preliminary data presented here provides evidence to investigate exercise training in T1D. Exercise-induced improvements in T1D following exercise training has been shown in NOD mice [189]. NOD mice that were trained for 20 weeks demonstrated reduced immune cell infiltration into the pancreas and therefore a reduction in insulitis [189]. Exercise training also acts as a preventative measure for atherosclerosis in people at risk [373, 374]. Exercise training in people with T2D has been shown to improve atherosclerosis risk through reduction in endothelial activation parameters. These studies reported that decreased HbA1c following exercise training correlated with reduced ICAM-1 and endothelial activation [375, 376]. Furthermore, exercise training in cancer models reduced tumour growth and increased immune surveillance [362]. NK cell infiltration into tumours was increased in trained mice. It was reported that epinephrine and IL-6 were imperative for NK cell mobilisation, redistribution, and activation to control tumour growth [362].

In conclusion, exercise-induced lymphocytosis of particular subsets during vigorous intensity vigorous exercise is blunted in T1D compared to control participants. However, the increase in lymphocytes following exercise may still contribute to increased immune surveillance in T1D. Furthermore, suppression of TEM in the recovery period following vigorous exercise may offer protection from T cell infiltration into the islet in T1D.

CHAPTER 5. THE EFFECTS OF ACUTE EXERCISE ON T CELL LINEAGE SUBSETS IN THE PERIPHERAL BLOOD OF TYPE 1 DIABETES

5.1 Introduction

T cells are mobilised by acute exercise in healthy individuals. In particular, memory T cell subsets which are highly differentiated are the most responsive to acute exercise [199, 200, 215, 257, 364, 365]. However, the effects of acute exercise on T cell subsets, including those implicated in the pathogenesis of T1D is not yet known.

5.1.1 T cell differentiation stages

The linear differentiation pathway that gives rise to a range of phenotypically and functionally diverse T cell subsets is described below (Figure 25). T cells are characterised by their co-expression of CD3 and the T cell receptor (TCR) [290]. T cells mature in the thymus into two distinct subsets; $CD4^+$ and $CD8^+$ T cells. Both $CD4^+$ and $CD8^+$ T cells are released into the circulation as naïve T cells which are characterised by their cell surface co-expression of CD45RA, CCR7, CD27 and CD28 [291, 377-381]. CD45, the leukocyte common antigen, has multiple isoforms which are differentially expressed based on maturation status [382]. Naïve T cells express the high molecular weight CD45RA isoform [383, 384]. CCR7 is a chemokine which mediates lymph node (LN) homing, and as a result $CCR7^+$ cells are continuously redeployed into the blood stream [380, 385, 386]. CD27 and CD28 are costimulatory molecules involved in T cell activation and formation of antigen-primed cells [387-389].

Central memory (CM) cells are formed ($CD45RA^-CCR7^+$) following antigen experience and loss of CD45RA [378, 381]. Memory cells lose the expression of the high molecular weight CD45RA isoform and gain expression of the low molecular CD45RO isoform following activation [383]. $CD45RA^-$ memory T cells can respond to recall antigens whereas $CD45RA^+$ naïve T cells cannot [383]. Both naïve and CM T cells are typically $CD27^+CD28^+$ [390]. This is because co-stimulation from antigen presenting cells is required for the activation of these early stage T cells during antigen presentation and TCR engagement [391-393].

Following antigen presentation, CM T cells can rapidly differentiate into T cells with effector functions [380]. CCR7 is down-regulated as CM T cells become specialised, antigen specific T cells or now known as effector memory (EM) T cells. Subsequently, EM T cells upregulate adhesion molecules to facilitate their migration to peripheral non-lymphoid tissues [380, 394]. EM T cells acquire increased cytolytic activity and capacity for cytokine release to exert their effector functions [395-397]. EM T cells can be divided into four subsets based on their expression of CD27 and CD28: early (ED) ($CD27^+CD28^+$), early-like (ELD) ($CD27^-CD28^+$), intermediate (ID) ($CD27^+CD28^-$), and fully differentiated EM T cells ($CD27^-CD28^-$) [398, 399]. $CD27^+CD28^+$ EM exhibit limited cytotoxic functions whereas $CD27^+CD28^-$ and $CD27^-CD28^-$ EM display higher cytotoxic killing and cytokine secretion capabilities [399]. CD27 and CD28 diminish in a stepwise fashion following successive rounds of differentiation in response to antigen stimulus [399-401]. CD27 expression is lost after prolonged antigen exposure such as that received by EM T cells [402]. Downregulation of CD28 on EM T cells is associated with loss of proliferative capacity and telomere length [403, 404], but increased cytolytic activity and susceptibility to apoptosis following an immune response [401, 405]. Therefore, loss of CD27 and CD28 expression by late differentiated EM T cells indicates progression towards senescence.

Re-expression of CD45RA on fully differentiated EM T cells (EMRA) is the final stage of T cell differentiation [384]. EMRA express both CD45RA and CD45RO isoforms ($CD45RA^+CCR7^-$) [406]. This leads to a more stable resting memory T cell pool that responds to recall antigen [407]. EMRA express lower levels of apoptotic proteins B-cell lymphoma -2 (Bcl-2) and B-cell lymphoma x (Bcl-x), in comparison to EM T cells which express higher levels because they undergo programmed cell death after resolution of infection [407]. EMRA exhibit high migratory capacity, cytotoxic functions and cytokine secretion in response to stimulus, analogous to EM T cells [407]. Conventionally EMRA are $CD27^-CD28^-$ as they have

undergone successive rounds of differentiation [400, 401]. However there is evidence of further subdivisions of differentiation based on the differential expression of these markers [408]. Pedro Romero et al., (2007) identified small subpopulations of $CD27^{-}CD28^{+}$ and $CD27^{+}CD28^{-}$ EMRA [408]. Nonetheless, $CD27^{+}CD28^{+}$ and $CD27^{-}CD28^{-}$ EMRA make up the larger proportion of EMRA subpopulations [408, 409].

The phenotypic outline of T cell differentiation (Figure 25) is well defined in $CD8^{+}$ T cells, with evidence of similar heterogeneity in $CD4^{+}$ T cells [390, 410]. Naïve and early differentiated subsets are the most abundant $CD4^{+}$ and $CD8^{+}$ T cells in peripheral blood in the steady state. Naïve and CM cells are the most abundant within the LN, with EM and EMRA residing in peripheral tissues [411, 412]. $CD4^{+}$ T cells comprise the major part of CM cells, whereas $CD8^{+}$ T cells comprise the major part of EM and EMRA [291].

More recently, a memory T cell population has been identified with hematopoietic stem cell-like properties, stem-cell like memory T cells (T_{SCM}) [340, 413-415]. T_{SCM} express naïve markers such as CD45RA, CCR7, CD27, and CD28 and co-localise in the lymph nodes [415, 416]. T_{SCM} are the least differentiated memory subset, following naïve T cells in the linear differentiation pathway [416]. However, T_{SCM} have the ability to rapidly acquire effector functions upon stimulation and respond to recall antigens, analogous to memory subsets [416]. T_{SCM} express CD95 which is also found on conventional memory subsets [416]. T_{SCM} have shortened telomeres compared to naïve T cells, indicative of their sustained proliferation cycles [416, 417]. T_{SCM} are capable of extensive proliferation and self-renewal, similar to hematopoietic stem cells [340, 416, 417]. Wnt signalling, which is involved in the self-renewal capacity of hematopoietic stem cells, was found to inhibit T cell differentiation and promote generation of T_{SCM} [414]. T_{SCM} were first identified in the $CD8^{+}$ compartment [340] and subsequently in the $CD4^{+}$ compartment [418].

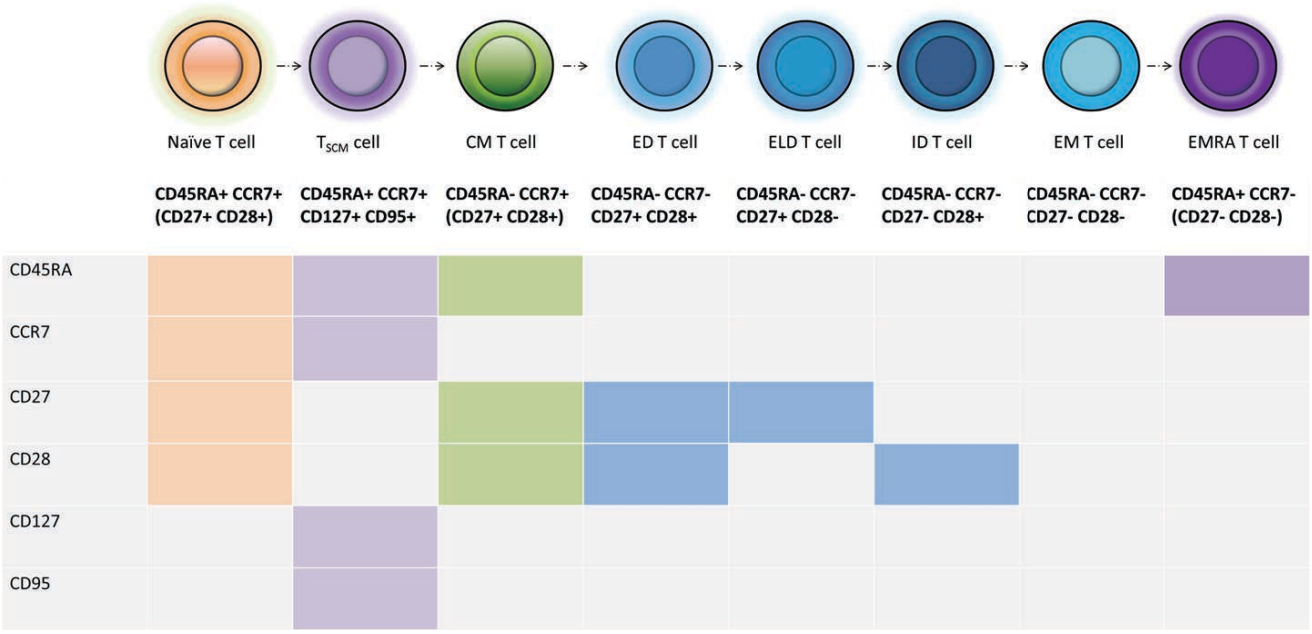

Figure 25 Linear T cell differentiation pathway

The progressive linear differentiation pathway of CD4+ and CD8+ T cell subsets; naïve T cells --> Stem cell like memory T cells (T_{SCM}) --> Central Memory (CM) --> Effector Memory (EM) --> Early Differentiated (ED) --> Early-like Differentiated (ELD) --> Intermediately differentiated (ID) --> Effector Memory Re-expressing CD45RA (EMRA).

5.1.2 T cells in T1D

A few studies have reported differences in peripheral blood $CD4^+$ T cell subsets in T1D compared to healthy cohorts. One study showed increased $CD4^+$ T cell proportions, which was comprised of increased $CD4^+$ $CD45RA^+$ T cells in T1D donors [419]. Another study found no differences in $CD4^+$ T cell proportions or absolute numbers, but did report a lower absolute count of $CD4^+$ CM T cells [420]. A more comprehensive phenotyping study reported that antigen exposed memory T cell subsets were elevated in T1D [339, 371, 421]. Absolute numbers of naïve and CM T cells in both the $CD4^+$ and $CD8^+$ compartment were reported to be lower in T1D, whereas both $CD4^+$ and $CD8^+$ EMRA were dramatically higher [371]. Elevated EMRA subsets in T1D were highly differentiated as denoted by CD26 expression [371]. CD26 is a costimulatory molecule expressed late in the differentiation of T cells, therefore indicating a mature phenotypic state [371].

No differences in total peripheral blood $CD8^+$ T cell counts have been described in T1D. Furthermore, circulating islet specific $CD8^+$ T cells have been found at similar frequencies in both T1D and non-diabetic cohorts [131]. In the aforementioned study, circulating islet specific $CD8^+$ T cells displayed a naïve phenotype ($CD45RO^+CD45RA^-CD62L^{hi}$) and equal functionality in both cohorts [131]. The antigen-experienced islet reactive $CD8^+$ T cells ($ZnT8_{186–194}$ $multimer^+$) were found sequestered in the pancreas of T1D donors, but not non-diabetic donors [131]. A decrease in peripheral $CD8^+CD11b^+$ subsets in T1D compared to healthy donors has been described [419]. This is likely due to sequestration of pan $CD8^+CD11b^+$ T cells within the islets of T1D donors, similar to that seen for islet reactive $CD8^+$ T cells [131]. This is supported by evidence of $CD8^+CD11b^+$ T cells within NOD islets [127, 134]. Islet resident $CD8^+$ T cells in the human pancreas also express CD69 and CD103 which are markers of activated tissue resident memory cells [86].

Antigen exposed memory T cell subsets elevated in the peripheral blood of T1D such as that found by Matteucci et al [371], and more recently identified populations by Skowera et al [339], are thought to sustain autoimmune destruction throughout T1D lifetime. Skowera et al also showed that beta cell specific $CD8^+$ T cells exhibit a highly differentiated memory phenotype that reflects chronic antigen exposure [339]. These cells express CD57, a marker of differentiation, and have shorter telomeres, indicative of their successive rounds of cell division [339, 422]. Furthermore, it was also demonstrated that a population of beta cell specific $CD8^+$ T cells exhibit a T_{SCM}-like phenotype ($CD27^+CD45RO^-CD95^+CCR7^+$) [339]. A recent study detected beta cell specific autoreactive $CD8^+$ T_{SCM} in people with T1D, indicating a role for T_{SCM} in the pathogenesis of T1D [421].

5.1.3 T cells and acute exercise

Acute exercise causes a significant increase in lymphocyte frequency, proportional to exercise intensity, in the peripheral blood immediately following exercise. This rise can be followed by intensity-dependent lymphopenia during the recovery period. [192, 193, 195, 196]. The observed lymphopenia is primarily due to the redistribution of cells to peripheral tissues [191, 214] and T cells constitute a large proportion of these redeployed lymphocytes [193, 201].

The largest mobilisation within T cells is seen by $CD8^+$ T cells, with minimal $CD4^+$ T cell mobilisation. Increases in both $CD4^+$ and $CD8^+$ T cells following 120 minutes cycle ergometer test at 65% VO_2 max is described in one study [201]. $CD4^+$ T cells increased by only 30% compared to $CD8^+$ T cells which increased by 105%. [201]. Another study reported increases in both $CD4^+$ and $CD8^+$ T cells following a 120 minutes treadmill test at 65% VO_2 max. Again, a smaller increase in $CD4^+$ T cells was found and this resulted in a decrease in the CD4/CD8 ratio [193]. $CD3^+$ T cells fell below baseline during the recovery period measured at 2 hours post exercise, with an increase in the CD4/CD8 ratio due to decreased $CD8^+$ T cells at this time point [193]. The CD4/CD8 ratio was also significantly reduced following 60 minutes running at 70% VO_2 max [196] and 30 minute steady state cycling at 15% above participant's blood lactate threshold [257].

There has been a greater focus on $CD8^+$ T cells than $CD4^+$ T cells due to their preferential mobilisation. $CD8^+$ T cells with a highly differentiated memory phenotype demonstrate the largest percentage increase following exercise [199, 200, 215, 257, 364, 365]. In one study, $CD8^+$ EM and EMRA increased three-fold following vigorous exercise (30 minute steady state cycling at 15% above blood lactate threshold) [257]. Another study found that $CD8^+$ EMRA were the most sensitive to exercise induced mobilisation increasing following 20 minutes of both low (35% $Watt_{max}$) and vigorous (85% $Watt_{max}$) exercise. $CD8^+$ EMRA showed the

greatest percentage increase (450%) following vigorous exercise compared to naïve $CD8^+$ T cells which increased by 84% [199]. Further studies showed that $CD8^+$ EM and EMRA that exhibited the largest increase following exercise (60 minute treadmill running at 60% VO_2 max) were fully differentiated ($CD27^-CD28^-$). This effect was almost twice as large in antigen experienced CMV^+CD8^+ memory T cells [200]. Other studies have investigated markers of terminal differentiation and senescence such as CD57 and $KLRG1^+$ on $CD8^+$ T cells following acute exercise. Exhaustive exercise (80% VO_2 max for 1 hour) resulted in an increase in the proportion of $KLRG1^+CD57^+$ (56%) and $KLRG1^+CD28^-$ (61%) $CD8^+$ T cells [215]. These findings were also shown following a maximal treadmill protocol [364]. A maximal bicycle exercise test resulted in an increase in $CD8^+CD28^-$ memory T cells with shorter telomeres, another marker of senescence [365].

Following the dramatic increase in frequency after exercise, it is the exercise sensitive $CD8^+$ EM and EMRA subsets which exhibit the largest egress from the peripheral blood during the recovery period [200, 215]. Multiple causes of exercise induced lymphopenia such as tissue redistribution and lymphocyte apoptosis have been explored, and are described next.

Tissue redistribution of lymphocytes is one cause of exercise induced lymphopenia [191]. However, this mechanism has not been well studied because of difficulties in measuring lymphocyte relocation to tissues. Krüger et al. 2007 used fluorescent cell tracking in mice to examine T cell redistribution after exercise. This study showed that T cells were released from the spleen and accumulated in the lungs, bone marrow and Peyer's patches of the mice following acute exercise [214]. More depth regarding the homing propensity and function of the redeployed T cell populations is needed. CD69 is a marker of recent activation and tissue resident T cell subsets [423]. To date, only two studies have shown higher levels of $CD69^+$ $CD4^+$ and $CD8^+$ T cells following endurance exercise [424, 425]. Measuring CD69 expression

on specific subsets would identify activated T cell subsets that have migrated out from secondary lymphoid tissues during acute exercise. CD11b is an adhesion molecule involved in T cell migration, and potentially identifies activated T cell subsets migrating to secondary lymphoid tissues following mobilisation during exercise [426-429]. Increased $CD11b^+$ lymphocytes following exercise has been shown previously; however $CD11b^+$ T cell subsets specifically have not yet been examined [195, 351-353].

Lymphopenia is also thought to partially occur from lymphocyte apoptosis [216-218]. Acute exercise, particularly of vigorous intensity (i.e. above 70% VO_2 max), mobilises $CD95^+$ memory T cells [216, 219]. It has been postulated that CD95 expression may indicate an apoptotic fate and create "immunological space" [220, 221]. This theory has been examined in multiple studies [216-220]. The percentage of apoptotic lymphocytes increased following exhaustive exercise (80% VO_2 max) in healthy participants. This increase was accompanied by an increase in $CD95^{hi}$ lymphocytes [216]. A study including paediatric participants cycling at 70% VO_2 max for 60 minutes found an increase in $CD95^{hi}$ T cells in females and $CD95^{lo}$ T cells in all groups with exercise [219]. Following a treadmill test to exhaustion, apoptosis measured by TUNEL was increased in 63% of lymphocytes immediately following exercise and in 86.2% of lymphocytes 24 hours later [218]. Furthermore, mice subjected to a strenuous 90-minute treadmill running protocol showed a decrease in $CD8^+$ T cells 24 hours following exercise. However, apoptosis of intestinal $CD8^+$ T cells was higher 24 hours following exercise [217].

It is hypothesised that this vacant immune space following exercise can be taken up by newly generated immature cells, creating the opportunity to reprogram our immune memory. Vigorous acute exercise also increases hematopoietic stem and progenitor cells (HSPC) post exercise [212, 213]. Furthermore, acute exercise stimulates haematopoiesis [213]. HSPC

proliferation in the bone marrow was increased by 40% in mice following an intense acute exercise bout [213].

The mobilisation of T cells in a non-disease state is well reported. However, the effects of acute exercise on T cell subsets in T1D has not been studied. Given that acute exercise mobilises $CD8^+$ T cells with a highly differentiated phenotype that express markers of tissue residency, and that $CD8^+$ T cells with this phenotype are thought to sustain beta cell destruction in T1D, it is important to explore the impact of acute exercise on these T cell subsets in T1D. It may be that benefits observed in T1D are in part attributable to changes arising to the immune system during single, repeated bouts of exercise. This study aims to provide a comprehensive phenotypic characterisation of exercise induced T cell mobilisation in healthy and T1D cohorts.

5.1.4 Hypothesis

Acute exercise preferentially mobilises progressively differentiated T cell subsets in T1D participants.

5.1.5 Aims

To characterise progressively differentiated T cell subsets in the peripheral blood using multi-parameter flow cytometry:

1. In T1D and control participants at rest.
2. In T1D and control participants following an acute bout of moderate intensity exercise.
3. In T1D and control participants following an acute bout of vigorous intensity exercise.

5.2 Methods

5.2.1 Experimental design

Twelve controls and twelve T1D male participants were recruited into the study (as described in Chapter 3). Briefly, participants had one enrolment visit, where an incremental sub-maximal cycle ergometer test was performed to calculate their predicted VO_2 max. This was used to calculate workload and heart rate for subsequent exercise visits adjusted to individual fitness [285]. These two visits consisted of a thirty-minute bout of cycling at either 40% VO_2 max (moderate) or 80% VO_2 max (vigorous). Blood was collected intravenously at baseline, immediately post exercise, and 1 hour post exercise in vacuette heparin tubes (95057-405, Greiner Bio-one GmbH, Frickenhausen, Germany).

5.2.2 T cell subset analysis

A twelve-colour flow cytometry panel was designed to phenotype T cells subsets using the following mAbs (see Table 3, Section 2.3.2 for mAb details); anti-CD3 PE-Cy7, anti-CD4 APC-R700, anti-CD8 APC-H7, anti-CD11b PE-CF594, anti-CD27 BB515, anti-CD28 BV510, anti-CD45RA BV786, anti-CD69 BV650, anti-CD95 BV421, anti-CD127 AF647, anti-CCR7 PE, anti-7-AAD PerCP-Cy5.5. Whole blood was stained as per Chapter 2 (Section 2.3.2). Whole blood counts were completed using a coulter haematology analyser (Section 2.2.2) to calculate relative cell frequency (cells/μl) of T cell subsets.

5.2.3 Data analysis

Flowjo v.10 (FlowJo LLC, Oregon) was used to analyse flow cytometry data. Doublets were removed using FSC-A versus FSH-H. Lymphocytes were gated based on size on SSC-A versus FSC-A dot plot (Figure 26.1), single cells were selected as the linear populations on FSC-H versus FSC-A dot plot (Figure 26.2), and dead cells positive for 7-AAD viability stain were removed (Figure 26.3). T cells were selected as $CD3^+$ (Figure 26.4), and further selected as

separate $CD4^+$ and $CD8^+$ populations. Spider gates were used within $CD4^+$ and $CD8^+$ populations to define distinct progressively differentiated subsets as outlined in Figure 27; naïve ($CD45RA^+CCR7^+$), T_{SCM} ($CD45RA^+CCR7^+CD95^+CD127^+$), central memory (CM: $CD45RA^-CCR7^+$), effector memory (EM: $CD45RA^-CCR7^-$), early differentiated (ED; $CD45RA^-CCR7^-CD27^+CD28^+$), early-like differentiated (ELD: $CD45RA^-$ $CCR7^-$ $CD27^-$ $CD28^+$) and intermediately differentiated (ID; $CD45RA^-$ $CCR7^-CD27^+CD28^-$), and effector memory re-expressing CD45RA (EMRA: $CD45RA^+CCR7^-$). Single cell surface expression of CD69, CD11b, CD127, and CD95 was gated on CD4 and CD8 naïve, CM, EM, and EMRA T cell subsets. See Figure 27 displaying T cell gating strategy and Figure 28 displaying T_{SCM} gating strategy.

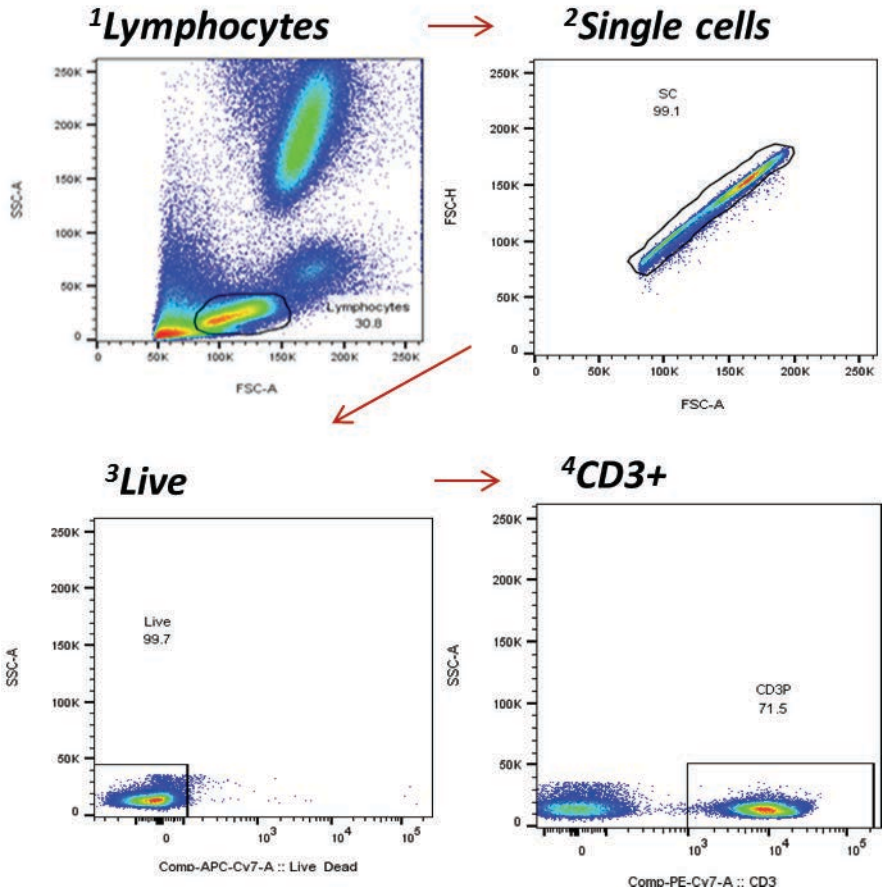

Figure 26 Selection of CD3+ lymphocytes shown by representative flow plots

Representative flow cytometry plots showing *(1)* Lymphocyte selection based on size (SSC-A vs FSC-A) *(2)* Doublets exclusion (FSC-A vs FSC-H) *(3)* Dead cell removal based on positivity for viability stain (7-AAD PerCP-Cy5.5) *(4)* CD3+ lymphocytes gate.

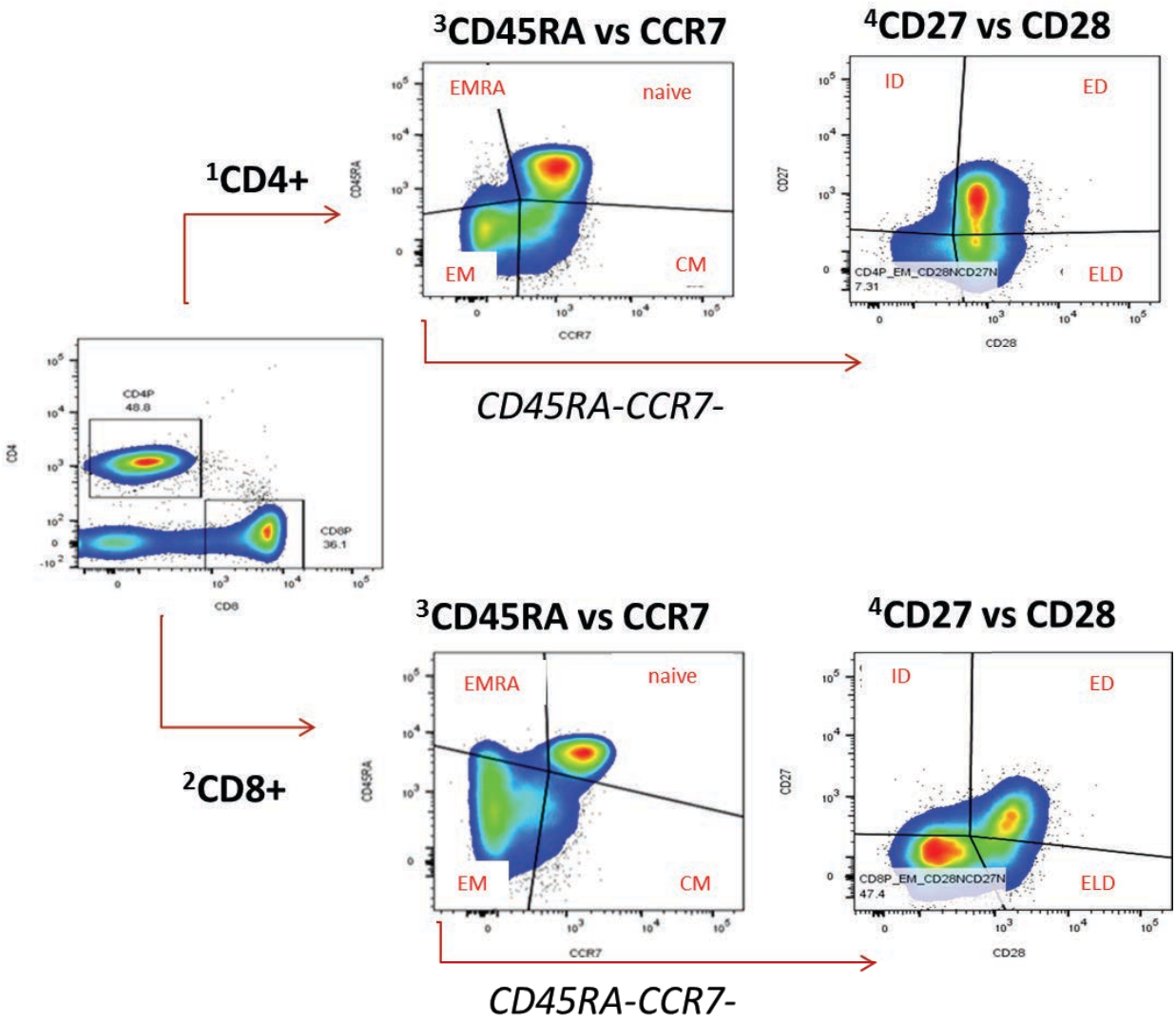

Figure 27 CD4 and CD8 T cell sub populations gating strategy shown by representative flow plots.

Representative flow cytometry plots showing following parent gate for CD3+ T cell populations (shown in figure 2) *(1)* CD3+CD4+ T cells gate *(2)* CD3+CD8+ T cells gate *(3)* Quadrant gating for naïve (CD45RA+ CCR7+), central memory (CM: CD45RA- CCR7+), effector memory (EM: CD45RA- CCR7-), and EMRA (CD45RA- CCR7-) *(4)* CD45RA- CCR7- selected to gate on early differentiated (ED; CD45RA- CCR7- CD27+CD28+), early-like differentiated (ELD: CD45RA- CCR7- CD27-CD28+) and intermediately differentiated (ID; CD45RA- CCR7-CD27+CD28-) populations.

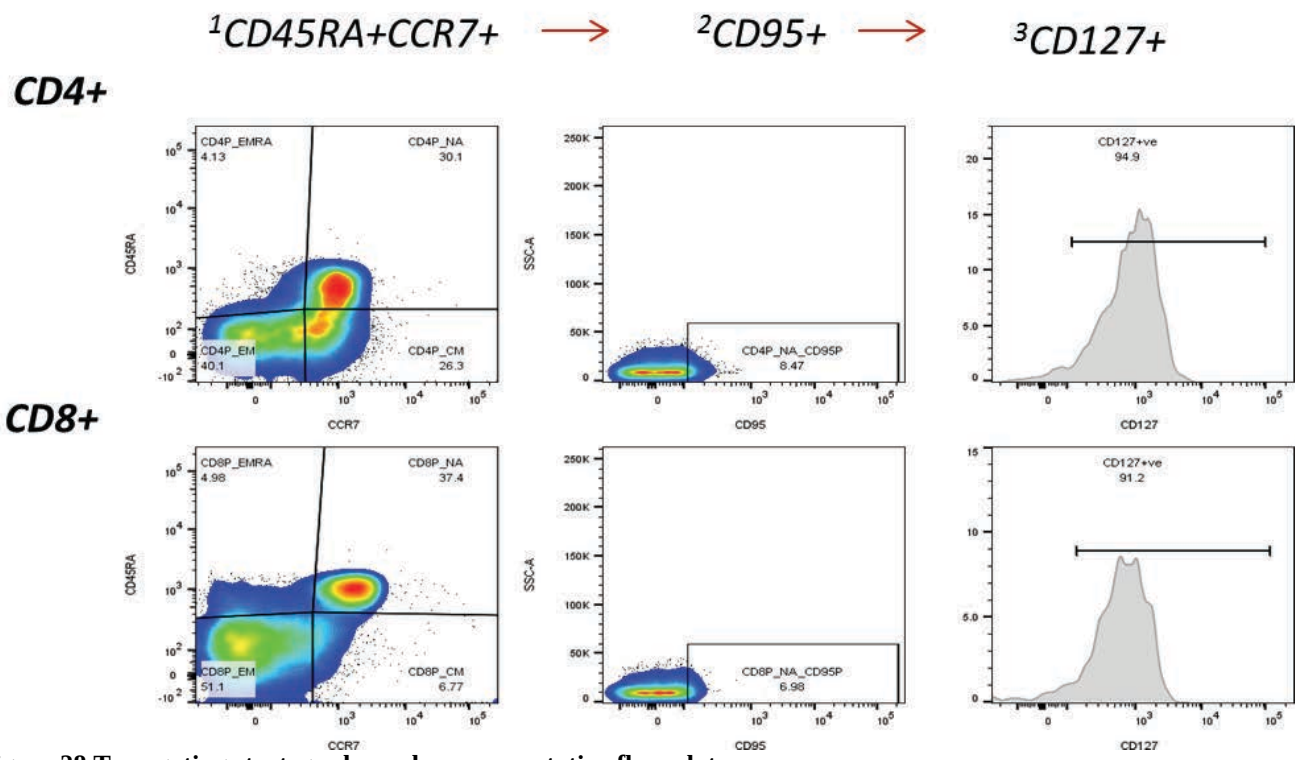

Figure 28 T_{SCM} gating strategy shown by representative flow plots

Representative flow cytometry plots showing following parent gate for CD3+, CD4+ and CD8+ T cell populations (shown in figure 2 and 3) *(1)* CD45RA+ CCR7+ T cell gate *(2)* CD45RA+ CCR7+CD95+ T cell gate *(3)* T_{SCM} selection (CD45RA+ CCR7+ CD95+ CD127+).

5.3 Results

5.3.1 Peripheral blood lymphocyte and T cell populations in T1D and control participants

The proportion and frequency of peripheral blood lymphocytes, $CD3^+$, $CD4^+$ and $CD8^+$ populations were measured at rest in T1D and control participants. There were no significant differences in the proportion (%) or frequency (cells/µl) of peripheral lymphocytes, $CD3^+$, $CD4^+$, and $CD8^+$ populations between T1D and control groups at baseline (Table 26).

	[1]Controls		[2]T1D	
	mean	SD	mean	SD
Lymphocyte (cells/µl)	1348	398	1375	262
CD3 (%)	58.73	18.98	49.86	28.06
CD3 (cells/µl)	805.6	381.7	695.0	340.1
CD4 (%)	53.52	6.70	57.03	9.24
CD4 (cells/µl)	610.0	253.5	580.9	316.8
CD8 (%)	35.18	5.32	35.92	7.35
CD8 (cells/µl)	386.8	166.0	348.9	192.6

Table 26 Lymphocyte and T cell populations at baseline in T1D and controls.

Mean and standard deviation values for the proportion and frequency of lymphocytes, CD3+, CD4+, and CD8+ populations in controls and T1D subjects.
[1]controls n=23, [2]T1D n=15

The effects of moderate (40% VO_2 max) and vigorous (80% VO_2 max) intensity exercise on the frequency of lymphocyte subpopulations (cells/µl) were investigated in T1D and control participants.

Lymphocyte subpopulations were measured pre, post, and 1 hour post exercise. The change over time was analysed in groups combined under the heading “time (overall)” and in each group independently (time) displayed in all results tables. The interaction over time between

groups was compared and results are displayed under the heading “time*group”. Post and 1 hour post exercise measurements were compared to baseline levels as shown under the heading “contrasts” displayed in the results tables. The mean, standard deviation, and statistical analyses are displayed in Table 27.

There was an overall change over time in lymphocytes during moderate intensity exercise ($p=0.021$), but this was not seen in either the T1D or control group independently. Lymphocytes were increased immediately post moderate intensity exercise in controls ($p=0.046$) but not in the T1D group (Figure 29a). Lymphocytes were significantly mobilised by vigorous intensity exercise overall ($p<0.001$), and in both T1D ($p=0.002$) and control ($p<0.001$) groups independently. They were significantly increased immediately post vigorous exercise in T1D ($p=0.003$) and controls ($p<0.001$), but significantly decreased below baseline in controls only 1 hour post vigorous exercise ($p=0.032$) (Figure 29b).

There was an overall change over time in the frequency of $CD3^+$ cells during moderate intensity exercise ($p=0.029$), but this was not seen in either group independently (Figure 29c). $CD3^+$ cells were significantly mobilised by vigorous intensity exercise overall ($p<0.001$), and in T1D ($p=0.008$) and control ($p=0.001$) groups independently. $CD3^+$ cells were significantly increased immediately post vigorous exercise in T1D ($p=0.048$) and controls ($p=0.030$), but significantly decreased below baseline in T1D participants only 1 hour post vigorous exercise ($p=0.035$) (Figure 29d).

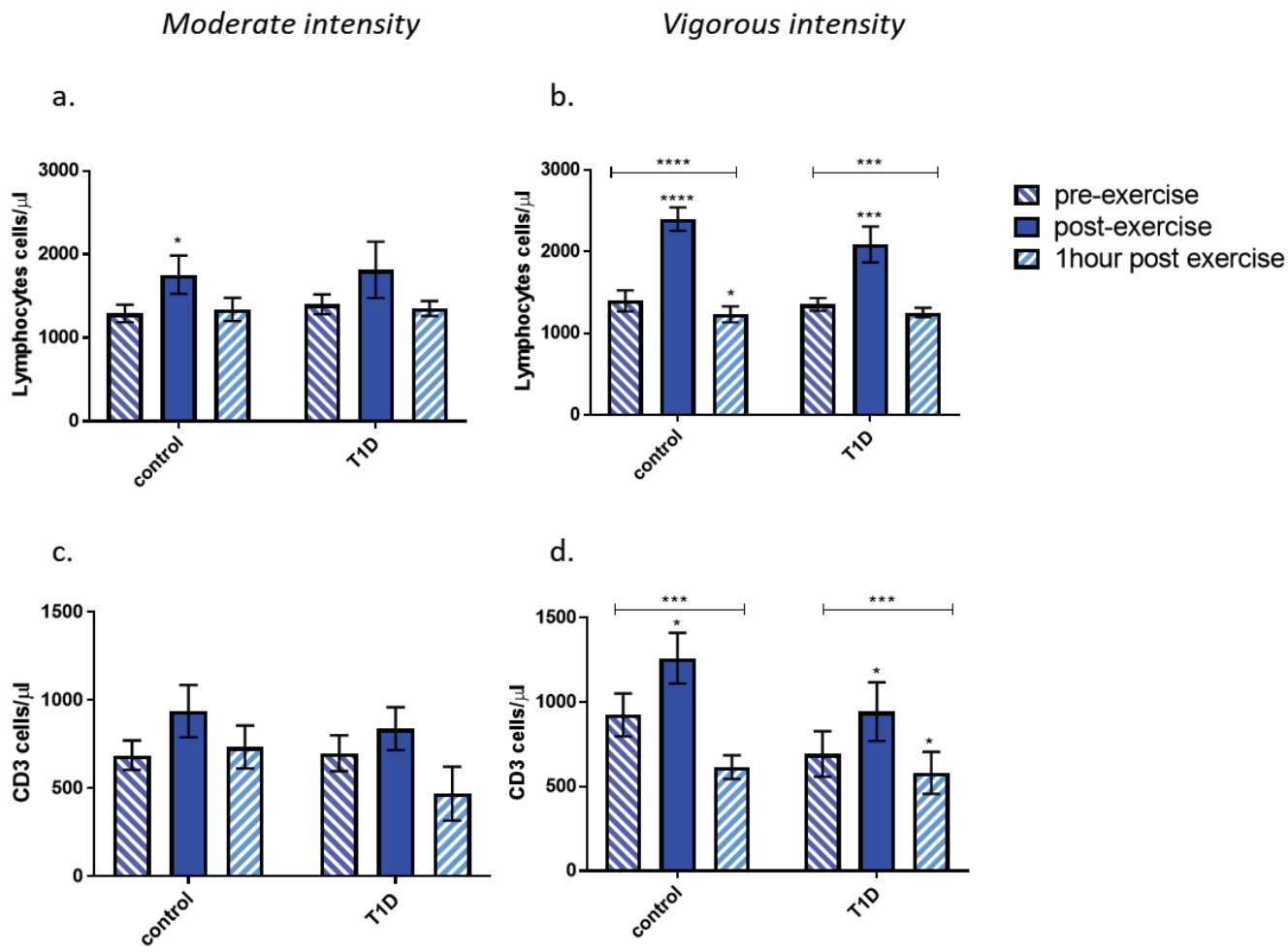

Figure 29 Lymphocyte and CD3+ cell frequencies during moderate and vigorous exercise in control and T1D groups

Flow cytometry and whole blood cell counts were used to determine the frequency of lymphocytes and CD3+ populations in control and T1D participants during moderate and vigorous intensity exercise. (a) Frequency of lymphocytes during moderate intensity (b) Frequency of lymphocytes during vigorous intensity exercise (c) The frequency of CD3+ cells during low intensity (d) The frequency of CD3+ cells during vigorous intensity. Error bars represent SEM. Statistical significance represented by * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

Subset (cells/µl)	Intensity	[1]Controls T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time		[c]contrast	[a]Δ%	[2]T1D T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time		[c]contrast	[a]Δ%	[d]Time (overall)	[d]Time*Group
Lymphocytes	**moderate**	1290.91	350.58	1754.55	759.43	1340.00	435.12	$F_{(1.2, 12.1)}$= 4.177, p=0.058	*T2*	**$F_{(1, 10)}$= 5.202, p=0.046**	35.92	1400.00	310.91	1814.29	893.36	1350.00	216.79	$F_{(1.05, 5.3)}$= 3.405, p=0.074	*T2*	$F_{(1, 5)}$= 2.925, p=0.148	29.59	**$F_{(1.2, 15.8)}$= 6.209, p=0.021**	$F_{(1.2, 15.8)}$= 0.230, p=0.667
									T3	$F_{(1, 10)}$= 0.013, p=0.912	3.80								*T3*	$F_{(1, 5)}$= 4.000, p=0.102	3.57		
	vigorous	1400.00	445.18	2400.00	475.39	1233.33	336.65	**F(1.26, 13.9)= 46.127, p<0.001**	*T2*	**$F_{(1, 11)}$= 55.113, p<0.001**	71.43	1355.56	235.11	2088.89	658.49	1255.56	174.01	**$F_{(1.1, 8.8)}$= 319.030, p=0.002**	*T2*	**$F_{(1, 8)}$= 18.615, p=0.003**	54.10	**$F_{(1.24, 22.4)}$= 74.637, p<0.001**	$F_{(1.24, 22.4)}$= 1.867, p=0.185
									T3	**$F_{(1, 11)}$= 6.044, p=0.032**	11.90								*T3*	$F_{(1, 8)}$= 4.500, p=0.067	7.38		
CD3	**moderate**	687.29	286.59	938.15	514.82	733.70	403.06	$F_{(2, 20)}$= 3.397, p=0.054	*T2*	$F_{(1, 10)}$= 2.862, p=0.122	36.50	698.67	269.53	838.30	320.03	469.46	340.67	$F_{(1.1, 5.5)}$= 2.315, p=0.184	*T2*	$F_{(1, 5)}$= 1.923, p=0.224	19.98	**F $_{(2, 26)}$= 4.069, p=0.029**	F $_{(2, 26)}$= 0.483, p=0.572
									T3	$F_{(1, 10)}$= 0.417, p=0.533	6.75								*T3*	$F_{(1, 5)}$= 2.41, p=0.181	32.81		
	vigorous	923.84	438.08	1259.75	494.55	614.76	222.14	**$F_{(2, 22)}$= 10.144, p=0.001**	*T2*	**$F_{(1,11)}$= 6.217, p=0.030**	36.36	692.14	402.88	942.91	522.38	581.22	373.82	**$F_{(2, 16)}$= 6.612, p=0.008**	*T2*	**$F_{(1, 8)}$= 5.417, p=0.048**	36.23	**$F_{(1.33, 22.6)}$= 14.429, p<0.001**	$F_{(1.33, 22.6)}$= 0.345, p=0.625
									T3	$F_{(1, 11)}$= 7.463, p=0.020	33.46								*T3*	**$F_{(1, 8)}$= 6.428, p=0.035**	16.03		

Table 27 Lymphocytes and CD3+ populations mobilised during moderate and vigorous intensity exercise in control and T1D groups

Mean, standard deviation and statistical analysis of lymphocytes and CD3+ populations for control and T1D subjects during moderate and vigorous intensity exercise.
[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2) or 1 hour post exercise (T3).
[b] Results were analysed using multiple regression analysis in control and T1D groups independently.
[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).
[d] Results were analysed using multiple regression analysis in control and T1D groups combined.
[1]Controls n=11, [2]T1D n =6-9

5.3.2 $CD4^+$ T cells

5.3.2.1 CD4+ T cells at baseline in T1D and control participants

$CD4^+$ T cells subsets were analysed in T1D and control participants at baseline, before proceeding to assessing the effect of exercise on the following populations: naïve ($CD45RA^+CCR7^+$), CM ($CD45RA^-CCR7^+$), EM ($CD45RA^-CCR7^-$) ED ($CD45RA^-CCR7^-CD27^+CD28^+$), ELD ($CD45RA^-CCR7^-CD27^-CD28^+$), ID ($CD45RA^-CCR7^-CD27^+CD28^-$) and EMRA ($CD45RA^+CCR7^-$) [430].

The proportion and frequency of $CD4^+$ EMRA cells were reduced in T1D compared to the control group (p=0.015, p=<0.0001 respectively) (Figure 30). However, there were no differences in the proportion or frequency of $CD4^+$ CM, EM, ED, ELD or ID $CD4^+$ T cells between groups. The mean and SD values for these subsets are displayed in Table 28.

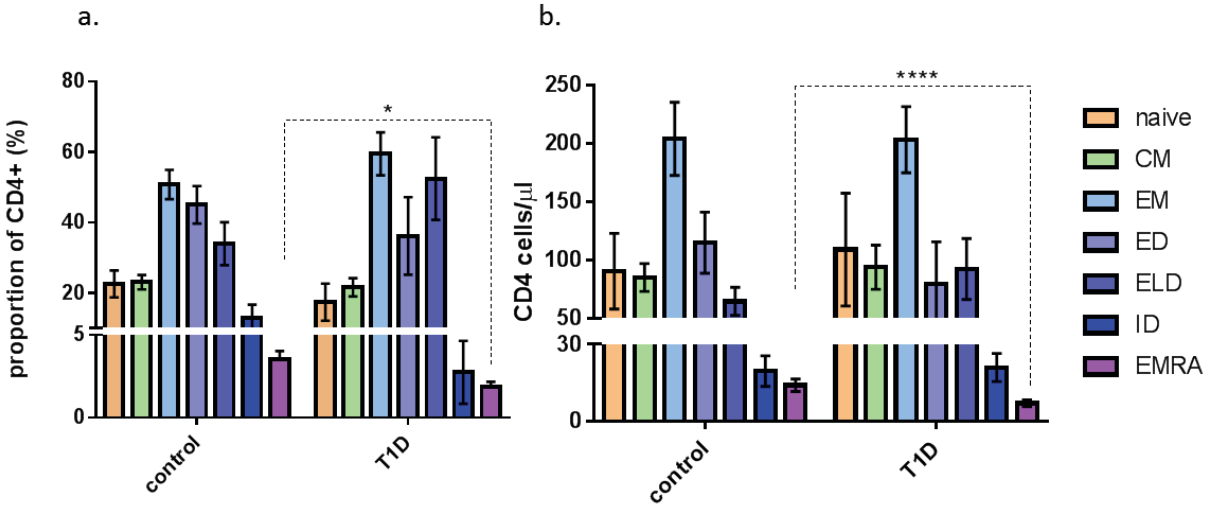

Figure 30 The proportion and frequency of CD4+ T cell subsets at baseline in T1D and control groups.

Flow cytometry and whole blood cell counts were used to determine the frequency of CD4+ sub populations in control and T1D participants at baseline. (a) Proportion of CD4+ subpopulations in control and T1D groups (b) Frequency of CD4+ subpopulations in control and T1D groups. Error bars represent SEM. Statistical significance represented by * p<0.05, ** p<0.01, ***p<0.001. Note: broken line (----) represents time*group differences.

	[1]Controls													
	naïve		CM		EM		ED		ELD		ID		EMRA	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
CD4 (%)	22.53	18.16	23.01	10.11	50.79	19.92	45.02	23.83	33.97	27.37	12.92	16.61	3.57	2.23
CD4 (cells/µl)	90.45	145.04	85.12	57.42	204.06	150.11	114.92	117.47	64.53	54.17	19.55	26.71	13.98	11.72
	[2]T1D													
CD4 (%)	17.36	20.52	21.55	9.98	59.48	23.71	36.15	34.77	52.44	37.07	2.74	6.03	1.88	1.09
CD4 (cells/µl)	109.00	187.15	93.84	73.37	203.31	110.06	79.74	113.33	92.37	82.76	20.98	21.26	6.96	5.06

Table 28 CD4+ T cell sub populations at baseline in T1D and controls.

Mean and standard deviation values for the proportion and frequency of CD4+ subpopulations in control and T1D groups.
[1] controls n=23, [2] T1D n=15

5.3.2.2 $CD4^+$ T cell phenotype at baseline in T1D and control participants

A number of cell surface markers were measured on $CD4^+$ T cells to define the homing propensity and function of circulating $CD4^+$ populations in T1D and control participants. These were examined on naïve, CM, EM, and EMRA $CD4^+$ T cell subsets. CD69 was used as a marker of tissue-resident $CD4^+$ T cells to understand where they had migrated from. This is in contrast to $CCR7^+$ cells which migrate from lymph nodes rather than tissue [431, 432]. CD11b, is an adhesion molecule which identifies $CD4^+$ T cells subsets with tissue homing propensity [433]. CD127, otherwise known as IL-7Rα, is necessary for memory $CD4^+$ T cell maintenance [434]. Lastly, CD95 is highly expressed on memory populations. CD95 is also a marker for apoptosis and may underlie memory T cell lymphopenia during the recovery period post exercise [216, 218-220]. Baseline levels for the proportion and frequency of these markers on $CD4^+$ subsets are shown in Table 29.

5.3.2.2i CD69

There were no significant differences between groups in $CD69^+$ expression on $CD4^+$ naïve, CM, EM or EMRA populations (Table 29). This indicates that both groups had an equal amount of $CD4^+$ T cells that were recently activated and migrated from tissue into the circulation at rest.

5.3.2.2ii CD11b

The proportion $CD4^+CD11b^+$ CM cells was significantly higher in T1D compared to control participants ($p=0.027$). There were no significant differences in $CD11b^+$ expression on $CD4^+$ naïve, EM or EMRA populations (Table 29). This suggests that $CD4^+$ CM T cells have a higher homing propensity in T1D participants than controls.

5.3.2.2iii CD127

The proportion of $CD4^+CD127^+$ naïve and $CD4^+CD127^+$ EMRA cells were significantly higher in T1D compared to control participants (p=0.047, p=0.023 respectively). There were no significant differences in $CD127^+$ expression on $CD4^+$ CM or EM populations (Table 29). This suggests that $CD4^+$ naïve and $CD4^+$ EMRA cells expressing higher IL-7Rα (CD127) are preferentially maintained in T1D.

5.3.2.2iv CD95

There were no significant differences between groups in $CD95^+$ expression on $CD4^+$ naïve, CM, EM or EMRA populations (Table 29). This suggests that both groups had an equal amount of $CD4^+$ T cells expressing CD95 on lineage subsets.

5.3.2.2v EMRA differentiation (CD27/CD28)

It has been assumed that EMRA are $CD27^-CD28^-$ (fully differentiated) however recent studies have shown the presence of mixed differentiated subsets of $CD4^+$ EMRA in healthy and ageing cohorts [409]. In this study herein, differentiated subsets of $CD4^+$ EMRA were identified in T1D. There was a significant difference in the proportion and frequency of $CD4^+$ $CD27^+CD28^-$ EMRA (early-like differentiation stage) between groups (p=0.014, p=0.001 respectively) (Figure 31a and b). A significant difference in the frequency of $CD4^+CD27^+CD28^+$ EMRA (early differentiation stage) was also found (p=0.031) (Figure 31b). Table 30 displays the mean baseline data for the proportion and frequency differentiated EMRA subsets.

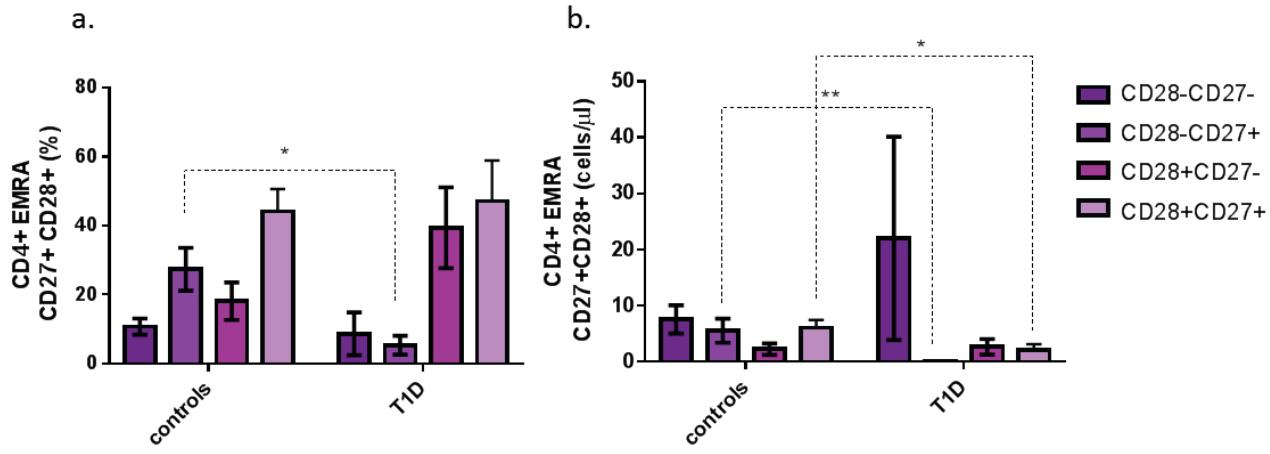

Figure 31 The proportion and frequency of differentiated CD4+ EMRA T cell subsets at baseline in T1D and control groups.

Flow cytometry and whole blood counts were used to determine the differentiation CD4+ EMRA populations using CD27 and CD28 in control and T1D subjects *(a)* The proportion of differentiated CD4+ EMRA subsets *(b)* The frequency of differentiated CD4+ EMRA subsets. Error bars represent SEM. Statistical significance represented by * $p<0.05$, ** $p<0.01$, ***$p<0.001$. Note: broken line (----) represents time*group differences.

		[1]Controls							
		CD69		CD11b		CD127		CD95	
		mean	SD	mean	SD	mean	SD	mean	SD
(%)	CD4 naïve	2.67	2.60	2.31	3.41	89.31	9.62	7.19	21.72
	CD4 CM	3.12	2.22	2.34	2.86	84.86	8.50	19.40	25.61
	CD4 EM	3.32	2.06	2.17	1.99	84.89	6.00	23.25	26.17
	CD4 EMRA	4.57	4.63	5.48	7.38	77.87	11.23	9.42	24.46
(cells/µl)	CD4 naïve	5.02	6.02	1.56	2.73	86.61	90.04	0.97	1.04
	CD4 CM	2.45	2.24	2.17	2.40	76.14	52.01	0.25	0.29
	CD4 EM	6.00	4.75	4.23	4.41	190.76	138.35	42.20	57.04
	CD4 EMRA	0.56	0.46	0.73	1.01	10.78	9.20	1.46	2.63
		[2]T1D							
(%)	CD4 naïve	2.06	3.04	0.94	1.94	95.01	4.04	13.61	25.84
	CD4 CM	2.33	1.59	0.57	0.95	85.71	9.20	36.71	30.92
	CD4 EM	3.44	1.95	9.03	23.19	87.04	6.21	35.63	34.26
	CD4 EMRA	7.79	7.17	6.53	12.01	86.15	7.43	19.36	31.54
(cells/µl)	CD4 naïve	4.44	9.68	0.88	1.96	110.03	190.47	7.34	16.88
	CD4 CM	2.01	1.87	0.94	1.40	82.97	71.19	0.62	1.32
	CD4 EM	6.68	4.64	14.89	38.55	176.69	98.06	76.34	102.00
	CD4 EMRA	0.46	0.38	0.36	0.73	6.07	4.73	7.42	17.15

Table 29 Surface marker expression on CD4+ naïve, CM, EM, and EMRA populations at baseline in T1D and controls

Mean and standard deviation values for the proportion and frequency of CD69+, CD11b+, CD127+, and CD95+ CD4+ naïve, CM, EM, and EMRA populations in controls and T1D subjects.
[1]controls n=23, [2]T1D n=15

	[1]Controls							
	$CD28^-CD27^-$		$CD28^-CD27^+$		$CD28^+CD27^-$		$CD28^+CD27^+$	
	mean	SD	mean	SD	mean	SD	mean	SD
CD4 (%)	10.69	10.38	27.33	27.93	18.08	24.21	44.07	29.10
CD4 (cells/µl)	7.57	11.53	5.55	9.32	2.25	4.50	6.06	6.33
	[2]T1D							
CD4 (%)	8.64	19.65	5.31	8.66	39.37	37.18	47.01	37.39
CD4 (cells/µl)	22.02	57.34	0.10	0.18	2.66	4.37	2.13	3.15

Table 30 Differentiated CD4+ EMRA T cell subsets at baseline in T1D and control groups.

Mean and standard deviation values for the proportion and frequency of differentiated CD4+ EMRA subsets in controls and T1D subjects.
[1]controls n=22, [2]T1D n=15

5.3.2.3 $CD4^+$ T cell subsets in response to acute exercise in T1D and control participants

The effects of moderate (40% VO_2 max) and vigorous (80% VO_2 max) intensity exercise on $CD4^+$ subsets were examined (Table 31). The results are given in progressive order of differentiation in Table 31.

$CD4^+$ T cells were mobilised by vigorous intensity exercise in the control group (p=0.028) but not in the T1D group. Within the $CD4^+$ population, three subsets showed significant mobilisation during moderate and/or vigorous intensity exercise; CM, ED, and EMRA.

$CD4^+$ CM significantly mobilised overall during moderate (p=0.029) and vigorous (p=0.040) intensity but this was not seen in either T1D or control groups independently. There were no significant changes seen immediately post or 1 hour post moderate or vigorous exercise (Table 31).

$CD4^+$ ED significantly mobilised during moderate intensity exercise in the control group only (p=0.029). There was a significant decrease below baseline at 1 hour post moderate exercise in this subset (p=0.018). $CD4^+$ ED changes over time were significantly different between groups (p=0.010) (time*group). Both groups showed a similar percentage increase post moderate exercise (T1D:14.87%, control: 12.53%). However, the fall below baseline 1 hour post moderate exercise was much larger in the T1D group compared to controls. There were fewer participants in the T1D group for this time-point which may contribute to a skewed difference in response. There were no significant changes in $CD4^+$ ED during vigorous intensity exercise in either group (Table 31).

$CD4^+$ EMRA significantly mobilised (p=0.039) with moderate intensity exercise in the control group only. There was a trend to fall below baseline at 1 hour post moderate exercise in this subset (p=0.067). $CD4^+$ EMRA did not show significant mobilisation during vigorous intensity exercise. However, $CD4^+$ EMRA significantly fell below baseline at 1 hour post vigorous exercise in the control group following vigorous intensity exercise (p=0.029) (Table 31).

There were no significant changes in $CD4^+$ naïve, EM, ELD or ID during moderate or vigorous intensity in either group (Table 31).

Subset (cells/µl)	Intensity	[1]Controls T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast	[a]Δ%	[2]T1D T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast	[a]Δ%	[d]Time (overall)	[d]Time*Group
Total CD4	moderate	707.02	204.45	939.65	438.80	715.06	280.96	$F_{(2, 20)}$= 2.617, p=0.098	*T2* $F_{(1, 10)}$= 1.467, p=0.254	32.90	815.54	220.42	1014.44	366.69	772.06	226.57	$F_{(2, 10)}$= 2.472, p=0.134	*T2* $F_{(1, 5)}$= 3.230, p=0.132	24.39	$F_{(2, 26)}$= 3.306, p=0.053	$F_{(2, 26)}$= 0.252, p=0.779
									T3 $F_{(1, 10)}$= 1.142, p=0.310	1.14								*T3* $F_{(1, 5)}$= 1.703, p=0.249	5.33		
	vigorous	512.94	269.18	628.42	291.04	319.58	121.06	**$F_{(2, 22)}$= 4.290, p=0.028**	*T2* $F_{(1, 10)}$= 2.087, p=0.179	22.51	398.44	257.07	511.93	302.77	356.99	251.93	$F_{(1.1, 8.82)}$= 3.506, p=0.092	*T2* $F_{(1, 8)}$= 3.596, p=0.094	28.48	**$F_{(1.31, 20.9)}$= 5.859, p=0.018**	$F_{(1.31, 20.9)}$= 0.311, p=0.644
									T3 $F_{(1, 10)}$= 4.815, p=0.053	37.70								*T3* $F_{(1, 8)}$= 0.910, p=0.368	10.40		
CD4 naïve	moderate	93.73	119.16	112.04	131.14	84.75	100.64	$F_{(2, 20)}$= 1.624, p=0.222	*T2* $F_{(1, 10)}$= 0.398, p=0.542	19.53	142.84	210.45	169.83	224.61	48.81	48.33	$F_{(2,10)}$= 0.366, p=0.702	*T2* $F_{(1, 5)}$= 0.340, p=0.585	18.90	$F_{(1.1, 13.2)}$= 2.398, p=0.144	$F_{(1.1, 13.2)}$= 2.536, p=0.133
									T3 $F_{(1, 10)}$= 1.126, p=0.314	9.58								*T3* $F_{(1, 5)}$= 0.454, p=0.530	65.83		
	vigorous	107.02	86.43	122.49	130.99	74.07	42.46	$F_{(1.25, 12.5)}$= 0.541, p=0.590	*T2* $F_{(1, 10)}$= 0.643, p=0.441	14.45	86.44	179.40	112.23	187.25	77.05	149.60	$F_{(2,16)}$= 1.074, p=0.365	*T2* $F_{(1, 6)}$= 1.779, p=0.219	29.84	$F_{(1.12, 17.9)}$= 0.092, p=0.793	$F_{(1.12, 17.9)}$= 1.300, p=0.275
									T3 $F_{(1, 10)}$= 1.522, p=0.246	30.79								*T3* $F_{(1, 6)}$= 0.052, p=0.825	10.87		
CD4 CM	**moderate**	75.95	50.19	89.93	57.88	71.43	36.39	$F_{(2, 20)}$= 2.185, p=0.139	*T2* $F_{(1, 10)}$= 1.009, p=0.339	18.41	139.08	70.99	171.38	114.70	99.26	65.61	$F_{(2, 8)}$= 3.055, p=0.103	*T2* $F_{(1, 4)}$= 2.066, p=0.224	23.23	**$F_{(2, 24)}$=4.123, p=0.029**	$F_{(2, 24)}$= 1.252, p=0.126
									T3 $F_{(1, 10)}$= 0.964, p=0.349	5.94								*T3* $F_{(1, 4)}$= 2.634, p=0.180	28.63		
	vigorous	93.53	64.36	107.32	68.03	81.44	44.53	$F_{(2, 20)}$= 2.125, p=0.146	*T2* $F_{(1, 10)}$= 0.709, p=0.419	14.75	63.69	60.94	102.84	90.52	60.25	60.93	$F_{(1.06, 8.45)}$= 3.096, p=0.114	*T2* $F_{(1, 8)}$= 3.318, p=0.106	61.47	**$F_{(2, 32)}$=3.552, p=0.040**	$F_{(2, 32)}$=0.917, p=0.410
									T3 $F_{(1, 10)}$= 01.842, p=0.205	12.92								*T3* $F_{(1, 8)}$= 0.418, p=0.536	5.40		
CD4 EM	moderate	183.52	116.82	210.82	137.22	166.70	100.61	$F_{(1.2, 12.4)}$= 1.249, p=0.297	*T2* $F_{(1, 10)}$= 0.069, p=0.799	14.88	276.04	87.90	313.00	105.71	284.55	137.07	$F_{(2,10)}$= 0.738, p=0.503	*T2* $F_{(1, 5)}$= 0.094, p=0.771	13.39	$F_{(2, 24)}$= 1.076, p=0.357	$F_{(2, 24)}$= 0.240, p=0.789
									T3 $F_{(1, 10)}$= 1.214, p=0.296	9.17								*T3* $F_{(1, 5)}$= 1.170, p=0.329	3.08		
	vigorous	222.89	178.47	294.17	253.01	188.04	144.15	$F_{(1.2, 12.3)}$= 2.051, p=0.177	*T2* $F_{(1, 10)}$= 0.261, p=0.620	31.98	154.82	98.78	221.62	134.35	145.01	111.24	$F_{(1.18, 9.5)}$= 1.335, p=0.285	*T2* $F_{(1, 8)}$= 0.402, p=0.544	43.14	$F_{(1.3, 19.9)}$= 2.262, p=0.145	$F_{(1.3, 19.9)}$= 0.274, p=0.658
									T3 $F_{(1, 10)}$= 2.301, p=0.160	15.64								*T3* $F_{(1, 8)}$= 3.629, p=0.093	6.34		
CD4 ED	**moderate**	80.89	95.74	91.03	117.69	50.68	63.05	**$F_{(2, 18)}$= 4.341, p=0.029**	*T2* $F_{(1, 9)}$= 0.233, p=0.640	12.53	116.47	181.71	133.79	192.19	22.27	0.00	$F_{(2,8)}$= 0.478, p=0.636	*T2* $F_{(1, 4)}$= 0.569, p=0.493	14.87	**$F_{(2, 16)}$= 5.943, p=0.012**	**$F_{(2, 16)}$= 6.308 p=0.010**
									T3 $F_{(1, 9)}$= 8.289, p=0.018	37.35								*T3* $F_{(1, 4)}$= 0.572, p=0.491	80.88		
	vigorous	148.96	131.88	196.75	174.51	133.70	105.19	$F_{(1.1, 9.7)}$= 0.758, p=0.415	*T2* $F_{(1, 9)}$= 0.847, p=0.381	32.09	64.00	85.42	82.53	85.71	58.79	85.44	$F_{(1.1, 6.5)}$= 0.720, p=0.438	*T2* $F_{(1, 6)}$= 0.191, p=0.677	28.95	$F_{(1.2, 15.5)}$= 3.739, p=0.066.	$F_{(1.2, 15.5)}$= 0.036, p=0.889
									T3 $F_{(1, 9)}$= 0.524, p=0.488	10.24								*T3* $F_{(1, 6)}$= 2.206, p=0.188	8.14		
CD4 ELD	moderate	53.10	43.78	69.22	61.57	48.29	47.86	$F_{(2, 18)}$= 2.899, p=0.081	*T2* $F_{(1, 9)}$= 0.847, p=0.381	30.35	143.09	91.03	197.87	131.04	134.58	0.00	$F_{(2,8)}$= 1.155, p=0.363	*T2* $F_{(1, 4)}$= 0.358, p=0.582	38.29	$F_{(2, 16)}$= 2.506, p=0.113	$F_{(2, 16)}$= 1.798, p=0.198
									T3 $F_{(1, 9)}$= 2.087, p=0.182	9.07								*T3* $F_{(1, 4)}$= 1.866, p=0.244	5.95		
	vigorous	75.96	63.15	117.72	85.08	67.49	48.91	$F_{(1.2, 10.8)}$= 1.980, p=0.189	*T2* $F_{(1, 9)}$= 1.597, p=0.238	54.97	70.64	75.33	86.24	86.95	64.15	75.73	$F_{(1.172, 7)}$= 0.909, p=0.390	*T2* $F_{(1, 6)}$= 0.089, p=0.775	22.09	$F_{(1.1, 14.1)}$= 1.149, p=0.308	$F_{(1.1, 14.1)}$= 0.283, p=0.622.
									T3 $F_{(1, 9)}$= 1.766, p=0.217	11.16								*T3* $F_{(1, 6)}$= 5.243, p=0.062	9.18		
CD4 ID	moderate	24.87	34.80	20.92	25.29	34.48	41.89	$F_{(2, 18)}$= 0.313, p=0.735	*T2* $F_{(1, 9)}$= 0.886, p=0.371	15.89	1.16	2.01	0.99	1.71	0.00	0.00	$F_{(2,8)}$= 0.106, p=0.901	*T2* $F_{(1, 4)}$= 0.026, p=0.880	14.89	$F_{(1.2, 9.5)}$= 4.733, p=0.051	$F_{(1.2, 9.5)}$= 1.484, p=0.260
									T3 $F_{(1, 9)}$= 0.000, p=0.991	38.64								*T3* $F_{(1, 4)}$= 0.434, p=0.546	100.00		
	vigorous	14.22	15.22	12.74	13.78	12.25	13.53	$F_{(1, 9.4)}$= 0.524, p=0.494	*T2* $F_{(1, 9)}$= 0.210, p=0.658	10.45	25.93	20.99	35.35	26.47	19.22	11.57	$F_{(1.1, 6.4)}$= 0.180, p=0.700	*T2* $F_{(1, 6)}$= 0.179, p=0.687	36.29	$F_{(1.2, 15.3)}$= 1.696, p=0.215	$F_{(1.2, 15.3)}$= 0.285, p=0.638.
									T3 $F_{(1, 9)}$= 2.684, p=0.136	13.87								*T3* $F_{(1, 6)}$= 0.143, p=0.719	25.90		
CD4 EMRA	**moderate**	14.59	14.52	16.01	13.44	12.73	13.30	**$F_{(2, 20)}$= 3.823, p=0.039**	*T2* $F_{(1, 10)}$= 0.361, p=0.561	9.75	8.20	3.19	11.32	3.41	15.36	18.04	$F_{(2,10)}$= 0.961, p=0.415	*T2* $F_{(1, 5)}$= 2.932, p=0.148	37.97	$F_{(2, 24)}$= 0.126, p=0.853	$F_{(2, 24)}$= 1.716, p=0.496
									T3 $F_{(1, 10)}$= 4.228, p=0.067	12.75								*T3* $F_{(1, 5)}$= 0.373, p=0.568	87.21		
	vigorous	13.42	9.07	16.54	14.76	9.46	7.71	$F_{(1.2, 12)}$= 2.851, p=0.113	*T2* $F_{(1, 10)}$= 0.405, p=0.539	23.26	6.13	6.04	12.00	16.36	6.22	5.65	$F_{(1.26, 10)}$= 2.032, p=0.185	*T2* $F_{(1, 8)}$= 2.161, p=0.180	95.70	$F_{(1.4, 23.8)}$= 2.117, p=0.154	$F_{(1.4, 23.8)}$=0.748, p=0.439
									T3 $F_{(1, 10)}$= 6.467, p=0.029	29.49								*T3* $F_{(1, 8)}$= 0.004, p=0.951	1.42		

Table 31 CD4+ T cell sub populations mobilised by moderate and vigorous intensity exercise in control and T1D groups

Mean, standard deviation, and statistical analysis of CD4+ sub populations for control and T1D subjects during moderate and vigorous intensity exercise. Significant results highlighted in bold where p values <0.05 were considered significant.

[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2) or 1 hour post exercise (T3).

[b] Results were analysed using multiple regression analysis in control and T1D groups independently.

[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).

[d] Results were analysed using multiple regression analysis in control and T1D groups combined.

[1]Controls n=11, [2]T1D n =6-9

5.3.2.4 $CD4^+$ T cell subset phenotypes in response to acute exercise in T1D and control participants

As shown for $CD4^+$ T cell subsets at baseline, surfaces markers were used to examine the homing propensity and function of $CD4^+$ T cell subsets mobilised during moderate (40% VO_2 max) and vigorous (80% VO_2 max) intensity exercise. The mean, standard deviation, and statistical analyses are displayed in Table 32.

5.3.2.4i CD69

There were no significant changes in $CD4^+$ $CD69^+$ naïve subsets during moderate or vigorous exercise.

The frequency of $CD4^+CD69^+$ CM cells were significantly mobilised by vigorous intensity in the control group (p=0.049). There was a significant increase post vigorous exercise in the control group (p=0.018). There was an overall significant change in $CD4^+CD69^+$ CM subsets during moderate intensity (p=0.044) but not seen in either T1D or control groups independently.

The frequency of $CD4^+CD69^+$ EM cells were significantly mobilised during moderate intensity in T1D but not control groups (p=0.043). $CD4^+CD69^+$ EM subsets were significantly mobilised during vigorous intensity in the control group (p=0.007), but not in the T1D group, increasing significantly post vigorous exercise in controls only (p=0.012).

The frequency of $CD4^+CD69^+$ EMRA T cells showed a trend to change over time during moderate intensity exercise (p=0.051). However, there was a significant change in frequency over time post vigorous intensity exercise overall (p=0.029) but this is not seen in either T1D or control groups separately (Table 32).

5.3.2.4ii CD11b

There were no significant changes in the frequency of $CD4^+CD11b^+$ naïve cells during moderate or vigorous exercise.

The frequency of $CD4^+CD11b^+$ CM cells significantly fell below baseline following vigorous intensity in T1D group only (p=0.038). There were no significant changes in this subset during moderate intensity exercise.

The frequency of $CD4^+CD11b^+$ EM cells significantly mobilised during vigorous intensity in controls but not T1D (p=0.013), increasing significantly post vigorous exercise (p=0.020). There were no significant changes in this subset during moderate intensity exercise.

The frequency of $CD4^+CD11b^+$ EMRA cells significantly changed during moderate intensity exercise in the control group only (p=0.028), decreasing below baseline at 1 hour post moderate exercise (p=0.032). There were no significant changes during vigorous intensity exercise (Table 32).

5.3.2.4iii CD127

There were no significant changes in $CD4^+$ naïve, CM or EM $CD127^+$ subsets during moderate or vigorous exercise.

The frequency of $CD4^+CD127^+$ EMRA cells were significantly mobilised during moderate intensity exercise in controls only (p=0.035). $CD4^+CD127^+$ EMRA were significantly increased immediately post vigorous exercise in the control group only (p=0.028) (Table 32).

5.3.2.4iv CD95

There were no changes in $CD4^+$ CM, EM or EMRA $CD95^+$ subsets during moderate or vigorous exercise.

The frequency $CD4^+CD95^+$ naïve cells significantly changed over time during vigorous intensity exercise overall but not in either T1D or control group independently (p=0.003). There were no significant changes in this subset during moderate intensity exercise (Table 32).

5.3.2.4v EMRA differentiation (CD27/CD28)

Data for vigorous intensity exercise only in T1D and both exercise intensities in control participants is shown (Table 32). There were no changes in $CD4^+$ EMRA $CD27^+CD28^+$ (early differentiation stage), CD4+ EMRA $CD27^+CD28^-$ (early-like differentiation stage) or $CD4^+$ EMRA $CD27^-CD28^-$ (fully differentiated) subsets during moderate or vigorous exercise.

The frequency of $CD4^+CD27^-CD28^+$ EMRA (intermediate differentiation stage) significantly mobilised during vigorous intensity exercise in controls only (p=0.025). There were no significant changes in this subset during moderate intensity exercise.

Table 32 Surface marker expression on CD4+ naïve, CM, EM, and EMRA populations mobilised by moderate and vigorous intensity exercise in control and T1D groups

Mean, standard deviation, and statistical analysis of CD69+, CD11b+, CD127+, and CD95+ CD4+ naïve, CM, EM, and EMRA populations for control and T1D subjects during moderate and vigorous intensity exercise. Significant results highlighted in bold where p values <0.05 were considered significant.
[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2) or 1 hour post exercise (T3).
[b] Results were analysed using multiple regression analysis in control and T1D groups independently.
[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).
[d] Results were analysed using multiple regression analysis in control and T1D groups combined.
[1]Controls n=11, [2]T1D n =6-9

Subset (cells/µl)	Intensity	[1]Controls										[2]T1D										[d]Time (overall)	[d]Time*Group
		T1		T2		T3		[b]Time		[c]contrast	[a]Δ%	T1		T2		T3		[b]Time		[c]contrast	[a]Δ%		
		mean	SD	mean	SD	mean	SD					mean	SD	mean	SD	mean	SD						
CD4 Na CD69+	moderate	6.85	7.83	4.85	7.51	6.18	7.45	$F_{(2, 20)}$= 0.678, p=0.519	*T2*	$F_{(1, 10)}$= 0.554, p=0.074	29.20	9.08	14.74	9.11	11.46	11.71	13.33	$F_{(2,10)}$= 0.099, p=0.907	*T2*	$F_{(1, 5)}$= 0.068, p=0.804	0.28	$F_{(1.1, 12.8)}$= 3.186 p=0.096	$F_{(1.1, 12.8)}$= 2.485, p=0.105
									T3	$F_{(1, 10)}$= 0.119, p=0.737	9.76								*T3*	$F_{(1, 5)}$= 0.123, p=0.740	28.94		
	vigorous	3.01	1.98	6.14	8.09	4.19	2.59	$F_{(1.3, 11.75)}$= 42.393, p=0.12	*T2*	$F_{(1, 9)}$= 4.185, p=0.071	103.82	3.59	6.77	3.48	7.05	4.17	5.71	$F_{(2,16)}$= 0.716, p=0.504	*T2*	$F_{(1, 8)}$= 1.851, p=0.211	3.19	$F_{(1.1, 16.8)}$= 0.083, p=0.790	$F_{(1.1, 16.8)}$= 1.671, p=0.215
									T3	$F_{(1, 9)}$= 3.966, p=0.078	39.07								*T3*	$F_{(1, 8)}$= 0.001, p=0.971	16.28		
CD4 CM CD69+	**moderate**	2.77	2.65	3.07	2.93	2.82	2.54	$F_{(2, 20)}$= 0.343, p=0.714	*T2*	$F_{(1, 10)}$= 0.054, p=0.821	10.73	3.12	2.07	3.94	2.29	2.19	2.12	$F_{(2, 10)}$= 0.261, p=0.775	*T2*	$F_{(1, 5)}$= 0.041, p=0.848	26.35	**$F_{(2, 26)}$= 3.529 p=0.044**	$F_{(2, 26)}$= 1.584, p=0.224
									T3	$F_{(1, 10)}$= 0.325, p=0.581	1.60								*T3*	$F_{(1, 5)}$= 0.523, p=0.502	29.83		
	vigorous	2.08	1.76	4.69	3.74	2.58	2.40	**$F_{(2, 18)}$= 3.585, p=0.049**	*T2*	**$F_{(1, 9)}$= 8.299, p=0.018**	124.81	1.27	1.37	1.74	2.04	1.18	1.40	$F_{(2, 16)}$= 1.317, p=0.296	*T2*	$F_{(1, 8)}$= 0.962, p=0.355	36.57	$F_{(1.1, 27.7)}$= 3.805 p=0.056	$F_{(1.1, 27.7)}$= 1.192, p=0.302
									T3	$F_{(1, 9)}$= 0.462, p=0.514	13.21								*T3*	$F_{(1, 8)}$= 0.954, p=0.357	7.41		
CD4 EM CD69+	**moderate**	6.29	5.02	6.94	7.02	7.54	8.14	$F_{(1.3, 12.9)}$= 0.525, p=0.526	*T2*	$F_{(1, 10)}$= 0.017, p=0.899	10.35	7.78	2.94	8.01	3.10	6.91	2.45	**$F_{(2,10)}$= 4.376, p=0.043**	*T2*	$F_{(1, 5)}$= 1.555, p=0.268	2.90	$F_{(2, 24)}$= 0.376, p=0.691	$F_{(2, 24)}$= 0.125, p=0.883
									T3	$F_{(1, 10)}$= 0.526, p=0.485	19.89								*T3*	$F_{(1, 5)}$= 1.978, p=0.219	11.29		
	vigorous	5.67	4.69	16.94	23.81	6.80	5.12	**$F_{(2, 20)}$= 6.647, p=0.007**	*T2*	**$F_{(1, 9)}$= 9.924, p=0.012**	198.59	5.94	5.54	9.78	9.92	5.85	6.42	$F_{(2, 16)}$= 2.104, p=0.154	*T2*	$F_{(1, 8)}$= 1.675, p=0.232	64.50	**$F_{(1.4, 20.8)}$= 6.303, p=0.013**	$F_{(1.4, 20.8)}$= 0.188, p=0.748
									T3	$F_{(1, 9)}$= 2.764, p=0.131	19.89								*T3*	$F_{(1, 8)}$= 0.034, p=0.858	1.56		
CD4 EMRA CD69+	moderate	0.64	0.51	0.73	0.61	0.62	0.51	$F_{(2, 20)}$= 1.321, p=0.289	*T2*	$F_{(1, 10)}$= 0.395, p=0.544	14.18	0.65	0.46	0.74	0.95	0.88	1.08	$F_{(2, 12)}$= 0.965, p=0.409	*T2*	$F_{(1, 6)}$= 1.761, p=0.233	12.43	$F_{(2, 24)}$= 3.366 p=0.051	$F_{(2, 24)}$= 0.452, p=0.641
									T3	$F_{(1, 10)}$= 1.281, p=0.284	3.32								*T3*	$F_{(1, 6)}$= 0.095, p=0.769	34.84		
	vigorous	0.48	0.42	1.15	1.03	0.60	0.82	$F_{(2, 22)}$= 0.918, p=0.414	*T2*	$F_{(1, 11)}$= 0.037, p=0.851	139.75	0.33	0.25	0.43	0.47	0.24	0.21	$F_{(2,20)}$= 2.965, p=0.075	*T2*	$F_{(1, 10)}$= 0.686, p=0.427	28.65	**$F_{(2, 32)}$= 3.963, p=0.029**	$F_{(2, 32)}$= 1.463, p=0.247
									T3	$F_{(1, 11)}$= 0.852, p=0.376	25.21								*T3*	$F_{(1, 10)}$= 3.083, p=0.110	28.47		

Subset (cells/µl)	Intensity	[1]Controls T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast	[a]Δ%	[2]T1D T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast	[a]Δ%	[d]Time (overall)	[d]Time*Group
CD4 Na CD11b+	moderate	2.78	3.49	2.44	2.84	1.85	1.77	$F_{(2, 20)}$= 1.856, p=0.182	*T2* $F_{(1, 10)}$= 0.071, p=0.796	12.19	2.04	2.84	2.86	4.35	4.40	5.10	$F_{(2,10)}$= 0.495, p=0.624	*T2* $F_{(1, 5)}$= 0.840, p=0.401	40.74	$F_{(2, 24)}$=2.413 p=0.111	$F_{(2, 24)}$= 0.262, p=0.772
									T3 $F_{(1, 10)}$= 2.208, p=0.168	33.51								*T3* $F_{(1, 5)}$= 2.208, p=0.168	116.26		
	vigorous	1.84	3.43	6.58	17.27	1.47	1.77	$F_{(1.3, 13.2)}$= 0.919, p=0.384	*T2* $F_{(1, 10)}$= 2.732, p=0.129	257.97	0.21	0.28	0.43	0.66	0.22	0.25	$F_{(2,16)}$= 1.673, p=0.219	*T2* $F_{(1, 8)}$= 2.887, p=0.128	99.25	$F_{(1.3, 20.8)}$= 0.057, p=0.872.	$F_{(1.3, 20.8)}$= 1.462, p=0.248
									T3 $F_{(1, 10)}$= 0.376, p=0.553	20.03								*T3* $F_{(1, 8)}$= 0.074, p=0.793	5.03		
CD4 CM CD11b+	moderate	2.35	2.84	2.18	2.66	1.58	1.77	$F_{(2, 20)}$= 2.296, p=0.127	*T2* $F_{(1, 10)}$= 0.024, p=0.880	7.10	1.42	2.08	1.97	2.39	2.48	3.03	$F_{(2, 10)}$= 1.460, p=0.278	*T2* $F_{(1, 5)}$= 0.126, p=0.737	39.08	$F_{(2, 24)}$=1.553 p=0.232	$F_{(2, 24)}$=1.553, p=0.446
									T3 $F_{(1, 10)}$= 3.124, p=0.108	32.71								*T3* $F_{(1, 5)}$= 3.543, p=0.119	75.34		
	vigorous	1.98	1.94	4.26	6.49	1.90	2.09	$F_{(2, 18)}$= 1.288, p=0.300	*T2* $F_{(1, 9)}$= 53.697, p=0.087	114.75	0.61	0.65	0.61	0.53	0.39	0.42	$F_{(1.16, 9.37)}$= 1.696, p=0.228	*T2* $F_{(1, 8)}$= 0.012, p=0.916	1.05	$F_{(1.3, 22.2)}$= 1.646 p=0.218	$F_{(1.3, 22.2)}$= 0.465, p=0.552
									T3 $F_{(1, 9)}$= 0.060, p=0.813	4.19								***T3* $F_{(1, 8)}$= 6.165, p=0.038**	35.81		
CD4 EM CD11b+	moderate	5.67	5.43	4.03	5.05	3.73	4.26	$F_{(2, 20)}$= 0.259, p=0.774	*T2* $F_{(1, 10)}$= 2.937, p=0.117	28.99	6.36	8.52	5.89	7.89	5.74	6.47	$F_{(2,10)}$= 3.201, p=0.084	*T2* $F_{(1, 5)}$= 0.043, p=0.844	7.36	$F_{(1.16, 22)}$= 3.749 p=0.070	$F_{(1.16, 22)}$= 0.063, p=0.841
									T3 $F_{(1, 10)}$= 5.916, p=0.035	34.16								*T3* $F_{(1, 5)}$= 2.668, p=0.163	9.75		
	vigorous	2.80	2.60	7.12	8.41	3.14	3.38	**$F_{(2, 20)}$= 5.498, p=0.013**	*T2* **$F_{(1, 10)}$= 7.649, p=0.020**	154.34	20.01	48.57	4.15	5.31	2.12	3.38	$F_{(1.2, 9.8)}$= 2.022, p=0.187	*T2* $F_{(1, 8)}$= 0.682, p=0.433	79.27	$F_{(1.3, 21.5)}$= 2.012 p=0.169	$F_{(1.3, 21.5)}$= 1.908, p=0.181
									T3 $F_{(1, 10)}$= 0.338, p=0.574	12.11								*T3* $F_{(1, 8)}$= 3.305, p=0.107	89.40		
CD4 EMRA CD11b+	**moderate**	1.11	1.29	0.82	1.07	0.59	0.67	**$F_{(2, 20)}$= 4.315, p=0.028**	*T2* $F_{(1, 10)}$= 1.801, p=0.209	26.08	0.72	1.07	1.04	1.51	1.26	1.57	$F_{(1.1, 6.4)}$= 0.553, p=0.548	*T2* $F_{(1, 6)}$= 1.384, p=0.284	44.03	**$F_{(2, 24)}$= 4.080 p=0.030**	$F_{(2, 24)}$= 0.381, p=0.687
									T3 **$F_{(1, 10)}$= 6.170, p=0.032**	47.11								*T3* $F_{(1, 6)}$= 0.028, p=0.873	74.88		
	vigorous	0.34	0.40	1.13	2.63	0.37	0.60	$F_{(2, 22)}$= 1.982, p=0.162	*T2* $F_{(1, 11)}$= 4.052, p=0.069	229.13	0.12	0.20	0.09	0.12	0.05	0.08	$F_{(2,20)}$= 1.170, p=0.331	*T2* $F_{(1, 10)}$= 0.213, p=0.654	24.69	$F_{(1.3,23.2)}$= 0.947 p=0.364	$F_{(1.3,23.2)}$= 0.854, p=0.393
									T3 $F_{(1, 11)}$= 0.284, p=0.604	7.22								*T3* $F_{(1, 10)}$= 2.339, p=0.157	55.46		
CD4 Na CD127+	moderate	92.83	116.60	112.98	127.51	87.14	99.01	$F_{(2, 18)}$= 1.370, p=0.279	*T2* $F_{(1, 9)}$= 0.531, p=0.485	21.71	139.79	208.63	166.55	222.33	58.79	44.98	$F_{(2,10)}$= 0.523, p=0.608	*T2* $F_{(1, 5)}$= 0.271, p=0.625	19.15	$F_{(2, 24)}$=2.413 0.588 p=0.561	$F_{(2, 24)}$=2.4131.382, p=0.270
									T3 $F_{(1, 9)}$= 0.271, p=0.625	6.13								*T3* $F_{(1, 5)}$= 0.637, p=0.461	57.94		
	vigorous	80.38	59.53	115.80	124.97	64.80	39.53	$F_{(1.2, 10.6)}$= 0.399, p=0.575	*T2* $F_{(1, 9)}$= 0.480, p=0.506	44.06	87.71	186.96	112.90	194.25	77.56	156.12	$F_{(2,16)}$= 0.968, p=0.384	*T2* $F_{(1, 8)}$= 1.707, p=0.228	28.71	$F_{(2, 36)}$= F 3.151, p=0.075	$F_{(2, 36)}$= 0.487, p=0.556.
									T3 $F_{(1, 9)}$= 0.980, p=0.348	19.39								*T3* $F_{(1, 8)}$= 0.095, p=0.766	11.58		
CD4 CM CD127+	moderate	67.03	39.69	80.82	47.62	64.32	24.34	$F_{(2, 18)}$= 0.259, p=0.774	*T2* $F_{(1, 9)}$= 0.080, p=0.784	20.58	126.75	69.04	156.74	110.70	113.73	34.22	$F_{(2,10)}$= 0.308, p=0.742	*T2* $F_{(1, 5)}$= 0.75, p=0.796	23.66	$F_{(2, 22)}$=1.010 p=0.380	$F_{(2, 22)}$=1.010, p=0.661
									T3 $F_{(1, 9)}$= 0.400, p=0.543	4.04								*T3* $F_{(1, 5)}$= 0.395, p=0.557	10.27		
	vigorous	85.26	63.13	106.67	59.77	78.29	36.32	$F_{(2, 16)}$= 0.147, p=0.865	*T2* $F_{(1, 8)}$= 0.361, p=0.564	25.11	50.14	55.97	84.39	84.34	46.33	53.74	$F_{(1.1, 9.1)}$= 1.385, p=0.279	*T2* $F_{(1, 8)}$= 0.918, p=0.366	68.30	$F_{(1.2, 16.6)}$= 0.823 p=0.397	$F_{(1.2, 16.6)}$= 0.695, p=0.440.
									T3 $F_{(1, 8)}$= 0.337, p=0.578	8.17								*T3* $F_{(1, 8)}$= 2.057, p=0.189	7.61		
CD4 EM CD127+	moderate	164.92	99.48	191.89	120.46	141.94	62.14	$F_{(1.3, 11.5)}$= 1.248, p=0.311	*T2* $F_{(1, 9)}$= 0.112, p=0.746	16.36	241.41	67.74	276.38	87.85	205.23	51.45	$F_{(2,10)}$= 0.362, p=0.705	*T2* $F_{(1, 5)}$= 0.045, p=0.840	14.49	$F_{(2, 22)}$=1.063 p=0.359	$F_{(2, 22)}$=0.211, p=0.812
									T3 $F_{(1, 9)}$= 1.182, p=0.305	13.93								*T3* $F_{(1, 5)}$= 0.543, p=0.494	14.99		
	vigorous	216.60	171.10	311.57	238.44	194.39	131.40	$F_{(1.25, 11.3)}$= 1.760, p=0.215	*T2* $F_{(1, 9)}$= 0.453, p=0.518	43.85	128.15	91.08	184.03	124.82	114.92	96.80	$F_{(1.2, 9.67)}$= 1.172, p=0.319	*T2* $F_{(1, 8)}$= 0.506, p=0.497	43.60	$F_{(1.2, 18.14)}$= 1.663, p=0.217	$F_{(1.2, 18.14)}$= 0.267, p=0.656
									T3 $F_{(1, 9)}$= 2.142, p=0.177	10.25								*T3* $F_{(1, 8)}$= 2.398, p=0.160	10.32		
CD4 EMRA CD127+	**moderate**	11.21	11.83	11.94	8.18	8.36	7.03	**$F_{(2, 20)}$= 3.988, p=0.035**	*T2* $F_{(1, 10)}$= 0.853, p=0.377	6.57	7.24	2.79	9.93	3.15	6.68	4.85	$F_{(2, 12)}$= 0.251, p=0.782	*T2* $F_{(1, 6)}$= 0.340, p=0.581	37.23	**$F_{(2, 24)}$= 3.837 p=0.036**	$F_{(2, 24)}$= 0.631, p=0.531
									T3 $F_{(1, 10)}$= 3.573, p=0.088	25.43								*T3* $F_{(1, 6)}$= 1.175, p=0.320	7.73		
	vigorous	10.36	6.31	13.46	10.82	9.29	4.86	$F_{(1.1, 11.9)}$= 4.282, p=0.058	*T2* **$F_{(1, 11)}$= 6.430, p=0.028**	29.92	5.20	5.82	10.83	16.40	5.00	5.18	$F_{(1.2, 12.3)}$= 1.905, p=0.194	*T2* $F_{(1, 10)}$= 1.946, p=0.193	108.22	**$F_{(2, 32)}$=5.414, p=0.009**	$F_{(2, 32)}$= 0.101, p=0.904
									T3 $F_{(1, 11)}$= 3.199, p=0.101	10.31								*T3* $F_{(1, 10)}$= 0.045, p=0.836	3.88		

Subset (cells/µl)	Intensity	[1]Controls T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast	[a]Δ%	[2]T1D T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast	[a]Δ%	[d]Time (overall)	[d]Time*Group
CD4 Na CD95+	moderate	1.05	0.92	1.52	1.61	1.14	1.42	$F_{(1.1, 9.6)}$= 0.362, p=0.575	*T2* $F_{(1, 9)}$= 2.466, p=0.151	44.92	4.71	7.13	19.65	34.71	12.03	10.51	$F_{(1, 4)}$= 0.855, p=0.461	*T2* $F_{(1, 4)}$= 0.848, p=0.409	317.54	$F_{(1.2, 16)}$= 3.779, p=0.063	$F_{(1.2, 16)}$= 0.618, p=0.476
									T3 $F_{(1, 9)}$= 0.519, p=0.490	8.77								*T3* $F_{(1, 4)}$= 0.887, p=0.400	155.58		
	vigorous	0.90	1.18	2.20	4.66	0.72	0.95	$F_{(2, 18)}$= 0.518, p=0.603	*T2* $F_{(1, 10)}$= 0.986, p=0.344	144.96	9.32	21.96	2.30	6.02	18.25	48.82	$F_{(1, 7)}$= 0.562, p=0.489	*T2* $F_{(1, 7)}$= 0.705, p=0.429	75.34	**$F_{(1.3, 23.2)}$= 9.840, p=0.003**	$F_{(1.3, 23.2)}$= 0.325, p=0.631
									T3 $F_{(1, 10)}$= 0.999, p=0.341	20.20								*T3* $F_{(1, 7)}$= 0.008, p=0.932	95.86		
CD4 CM CD95+	moderate	0.33	0.35	0.40	0.48	0.28	0.40	$F_{(1.02, 10.18)}$= 0.605, p=0.457	*T2* $F_{(1, 10)}$= 0.470, p=0.509	19.55	1.18	2.00	1.55	2.16	2.10	2.55	$F_{(2,10)}$= 2.712, p=0.115	*T2* $F_{(1, 5)}$= 0.407, p=0.551	31.98	$F_{(2, 24)}$= 3.281, p=0.055	$F_{(2, 24)}$= 1.666, p=0.210.
									T3 $F_{(1, 10)}$= 0.670, p=0.432	14.88								*T3* $F_{(1, 5)}$= 4.429, p=0.089	78.10		
	vigorous	0.17	0.19	0.59	0.85	0.23	0.39	$F_{(1.1, 10)}$= 1.056, p=0.368	*T2* $F_{(1, 9)}$= 4.454, p=0.064	238.13	0.25	0.43	0.18	0.23	0.13	0.22	$F_{(1.2, 9.64)}$= 1.421, p=0.270	*T2* $F_{(1, 8)}$= 0.309, p=0.593	27.85	$F_{(2, 34)}$= 2.112, p=0.137	$F_{(2, 34)}$= 2.061, p=0.153
									T3 $F_{(1, 9)}$= 1.410, p=0.265	33.30								*T3* $F_{(1, 8)}$= 4.301, p=0.072	47.08		
CD4 EM CD95+	moderate	51.72	75.11	63.20	88.81	58.19	84.12	$F_{(1.3, 13.1)}$= 0.166, p=0.756	*T2* $F_{(1, 10)}$= 1.149, p=0.309	22.18	138.57	141.03	179.27	174.23	170.38	108.69	$F_{(2,10)}$= 0.514, p=0.613	*T2* $F_{(1, 5)}$= 0.792, p=0.414	29.37	$F_{(2, 24)}$=2.484, p=0.105	$F_{(2, 24)}$= 0.994, p=0.385
									T3 $F_{(1, 10)}$= 0.080, p=0.782	12.51								*T3* $F_{(1, 5)}$= 0.956, p=0.373	22.96		
	vigorous	32.68	31.49	87.47	199.46	28.31	23.21	$F_{(2, 20)}$= 0.093, p=0.912	*T2* $F_{(1, 10)}$= 0.107, p=0.750	167.70	34.86	30.61	61.88	95.04	43.94	37.24	$F_{(2, 16)}$= 0.223, p=0.802	*T2* $F_{(1, 8)}$= 0.261, p=0.623	77.55	$F_{(2, 32)}$= 0.712, p=0.499	$F_{(2, 32)}$= 0.187, p=0.831
									T3 $F_{(1, 10)}$= 0.026, p=0.875	13.36								*T3* $F_{(1, 8)}$= 0.006, p=0.941	26.06		
CD4 EMRA CD95+	moderate	1.15	1.96	1.23	1.95	0.89	1.31	$F_{(2, 20)}$= 1.830, p=0.186	*T2* $F_{(1, 10)}$= 0.388, p=0.547	6.92	2.63	3.47	2.95	3.01	3.19	3.71	$F_{(2,12)}$= 0.107, p=0.899	*T2* $F_{(1, 6)}$= 0.002, p=0.968	12.14	$F_{(2, 24)}$= 0.650, p=0.531	$F_{(2, 24)}$= 0.083, p=0.921
									T3 $F_{(1, 10)}$= 0.163, p=0.311	23.09								*T3* $F_{(1, 8)}$= 3.616, p=0.094	21.08		
	vigorous	0.63	0.37	1.32	2.16	0.61	0.89	$F_{(1.32, 14.5)}$= 2.060, p=0.171	*T2* $F_{(1, 11)}$= 0.443, p=0.519	110.38	1.67	2.56	2.30	5.45	1.60	2.42	$F_{(1.1, 11.1)}$= 0.061, p=0.833	*T2* $F_{(1, 10)}$= 0.087, p=0.774	37.75	$F_{(1.3, 20.2)}$= 0.314, p=0.633	$F_{(1.3, 20.2)}$=0.578, p=0.494
									T3 $F_{(1, 11)}$= 4.267, p=0.063	2.73								*T3* $F_{(1, 10)}$= 0.101, p=0.757	4.07		
CD4 EMRA CD28$^-$CD27$^-$	**moderate**	1.62	2.54	1.95	3.15	1.44	2.30	$F_{(2, 18)}$= 0.591, p=0.564	*T2* $F_{(1, 9)}$= 0.568, p=0.470	20.13								*T2*			**$F_{(2, 16)}$= 12.080, p=0.001**	
									T3 $F_{(1, 9)}$= 0.154, p=0.704	11.06								*T3*			
	vigorous	1.52	2.00	2.92	3.82	0.85	0.81	$F_{(2, 18)}$= 3.189, p=0.065	*T2* $F_{(1, 9)}$= 4.579, p=0.061	91.93	0.27	0.55	0.39	0.39	0.27	0.31	$F_{(2,12)}$= 0.508, p=0.614	*T2* $F_{(1, 6)}$= 0.617, p=0.462	42.19	$F_{(1.14, 15.9)}$= 0.269, p=0.641	$F_{(1.14, 15.9)}$= 0.727, p=0.424
									T3 $F_{(1, 9)}$= 0.021, p=0.887	43.89								*T3* $F_{(1, 6)}$= 0.181, p=0.685	0.13		
CD4 EMRA CD28$^-$CD27$^+$	moderate	6.49	11.68	4.41	6.57	4.70	5.93	$F_{(2, 16)}$= 1.024, p=0.381	*T2* $F_{(1, 8)}$= 1.875, p=0.208	32.05								*T2*			$F_{(2, 24)}$= 2.124, p=0.142	
									T3 $F_{(1, 9)}$= 1.160, p=0.313	27.56								*T3*			
	vigorous	4.70	7.13	3.39	4.38	3.96	6.99	$F_{(1.2, 10.6)}$= 0.502, p=0.524	*T2* $F_{(1, 9)}$= 0.016, p=0.901	27.90	0.11	0.21	0.18	0.35	0.14	0.14	$F_{(2,12)}$= 0.153, p=0.860	*T2* $F_{(1, 6)}$= 0.265, p=0.625	68.22	$F_{(1.4, 21.9)}$= 0.105, p=0.826	$F_{(1.4, 21.9)}$=1.527, p=0.237
									T3 $F_{(1, 9)}$= 2.865, p=0.125	15.64								*T3* $F_{(1, 6)}$= 0.307, p=0.599	29.18		
CD4 EMRA CD28$^+$CD27$^-$	moderate	2.84	6.36	4.48	8.79	3.08	6.30	$F_{(2, 18)}$= 0.620, p=0.549	*T2* $F_{(1, 9)}$= 1.404, p=0.266	58.05								*T2*			$F_{(2, 24)}$= 0.244, p=0.712	
									T3 $F_{(1, 9)}$= 0.651, p=0.440	8.49								*T3*			
	vigorous	1.67	1.23	2.72	2.11	1.45	0.98	**$F_{(2, 18)}$= 4.557, p=0.025**	*T2* $F_{(1, 9)}$= 4.925, p=0.054	62.64	2.51	4.98	3.42	6.72	2.24	3.80	$F_{(2,12)}$= 1.406, p=0.283	*T2* $F_{(1, 6)}$= 2.182, p=0.190	36.62	$F_{(1.2, 19.8)}$= 0.536, p=0.499	$F_{(1.2, 19.8)}$= 1.051, p=0.330
									T3 $F_{(1, 9)}$= 0.006, p=0.942	13.36								*T3* $F_{(1, 6)}$= 0.000, p=0.991	10.57		
CD4 EMRA CD28$^+$CD27$^+$	moderate	4.92	7.19	6.12	9.54	4.46	8.13	$F_{(2, 18)}$= 0.071, p=0.932	*T2* $F_{(1, 9)}$= 0.270, p=0.616	24.44								*T2*			$F_{(2, 24)}$= 2.545, p=0.099	
									T3 $F_{(1, 9)}$= 0.000, p=0.986	9.26								*T3*			
	vigorous	7.19	5.49	8.81	7.59	5.89	3.08	$F_{(1.1, 10)}$= 0.456, p=0.535	*T2* $F_{(1, 9)}$= 1.852, p=0.207	22.45	1.36	1.81	2.33	2.65	1.44	1.68	$F(1.1_{,6.7})$= 0.816, p=0.412	*T2* $F_{(1, 6)}$= 1.036, p=0.348	70.80	$F_{(1.2, 18.6)}$= 0.466, p=0.532	$F_{(1.2, 18.6)}$= 0.655, p=0.451
									T3 $F_{(1, 9)}$= 0.557, p=0.474	18.15								*T3* $F_{(1, 6)}$= 0.282, p=0.614	5.62		

5.3.3 $CD8^+$ T cells

5.3.3.1 $CD8^+$ T cells at baseline in T1D and control participants

$CD8^+$ T cells subset were analysed in T1D and control groups at baseline, before proceeding to assessing the effects of exercise on these populations. Naïve ($CD45RA^+CCR7^+$), CM ($CD45RA^-CCR7^+$), EM ($CD45RA^-CCR7^-$) ED ($CD45RA^-CCR7^-CD27^+CD28^+$), ELD ($CD45RA^-CCR7^-CD27^-CD28^+$), ID ($CD45RA^-CCR7^-CD27^+CD28^-$) and EMRA ($CD45RA^+CCR7^-$) were examined [430]. Table 33 displays the mean baseline values of the proportion (%) and frequency (cells/μl) CD8+ T cell populations in T1D and control groups.

There was a significant difference in the proportion of early differentiated (ED: $CD27^+CD28^+$), early-like differentiated (ELD: $CD27^-CD28^+$) and intermediately differentiated (ID: $CD27^+CD28^-$) subsets between groups (Figure 32a). The proportion of $CD8^+$ ED were significantly lower in T1D subjects (p=0.023) (Figure 32a) with a supporting trend in the relative cell frequency (p=0.062) (Figure 32b). The proportion of $CD8^+$ ELD were significantly higher in T1D subjects (p=0.026) (Figure 32a). The proportion of $CD8^+$ ID (p=0.019) (Figure 32a) and frequency (p=0.005) (Figure 32b) were significantly reduced in T1D subjects.

In contrast to $CD4^+$ EMRA (Section 3.2.1), the proportion of $CD8^+$EMRA were significantly higher in T1D subjects (p<0.0011) (Figure 32a), with no increase in relative cell frequencies found (Figure 32b).

This data shows that the distribution of $CD8^+$ ED, ELD, ID and EMRA T cells, i.e. manly later differentiated subsets, differs between T1D and control groups at baseline. There were no differences in the proportion or frequency of $CD8^+$ naïve, CM, or EM T cells between groups.

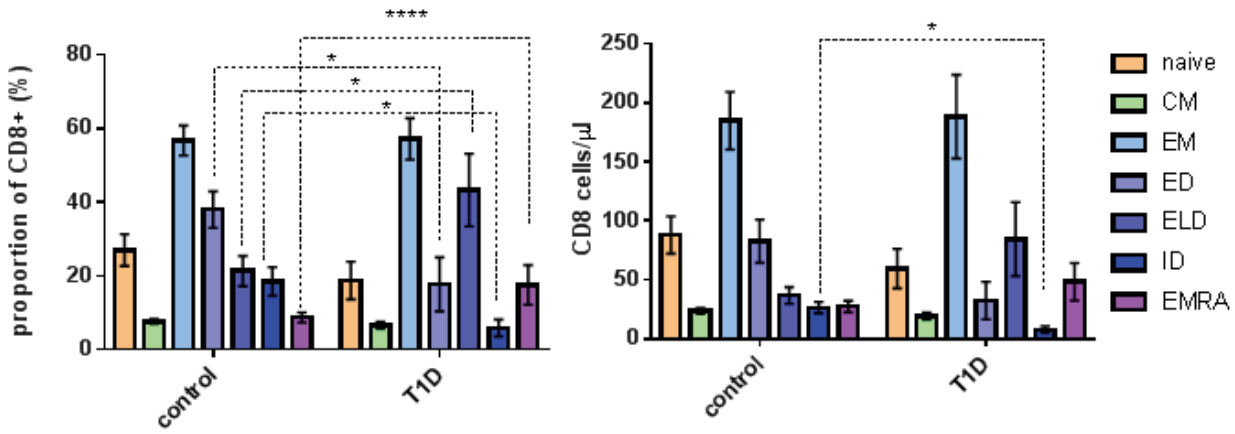

Figure 32 The proportion and frequency of CD8+ T cell subsets at baseline in T1D and control groups.

Flow cytometry and whole blood cell counts were used to determine the frequency of CD8+ sub populations in control and T1D participants at baseline. (a) Proportion of CD8+ subpopulations in control and T1D groups (b) Frequency of CD8+ subpopulations in control and T1D groups. Error bars represent SEM. Statistical significance represented by * p<0.05, ** p<0.01, ***p<0.001. Note: broken line (----) represents time*group differences.

	[1]Controls													
	naïve		CM		EM		ED		ELD		ID		EMRA	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
CD8 (%)	26.99	20.69	7.65	2.98	56.70	19.54	37.94	23.68	21.35	19.63	18.53	18.57	8.74	6.61
CD8 (cells/µl)	88.09	75.76	23.67	12.11	184.88	116.78	82.77	88.13	36.75	33.58	26.46	22.49	27.38	23.01
	[2]T1D													
CD8 (%)	18.77	19.99	6.64	3.58	57.17	21.73	17.75	28.28	43.28	38.25	5.87	8.81	17.59	20.79
CD8 (cells/µl)	59.46	64.99	19.29	11.47	188.37	137.56	32.42	61.14	84.47	120.76	7.82	11.19	48.31	61.66

Table 33 CD8+ T cell sub populations at baseline in T1D and controls.

Mean and standard deviation values for the proportion and frequency of CD8+ subpopulations in controls and T1D groups.
[1] controls n=23, [2] T1D n=15

5.3.3.2 $CD8^+$ T cell subset phenotypes at baseline in T1D and control participants

A number of cell surface markers were measured on $CD8^+$ T cell subsets to define the homing propensity and function of circulating $CD8^+$ populations in T1D and control participants. These markers included CD69, CD11b, CD127, and CD95 (as described for $CD4^+$ subsets in Section 3.2.2) and were measured on naïve, CM, EM and EMRA $CD8^+$ T cells (Table 34).

5.3.3.2i CD69

$CD8^+$ T cell subsets that were activated and had migrated from tissues, defined by CD69 expression, were measured in T1D and control groups. There were no differences between groups in CD69 expression on $CD8^+$ naive and CM subsets.

A higher proportion of $CD8^+CD69^+$ EM ($p=0.025$), with a trend to increase in frequencies ($p=0.069$), was found in T1D than controls (Table 34).

The frequency, but not the proportion, of $CD8^+CD69^+$ EMRA were significantly increased in T1D subjects ($p=0.037$) (Figure 33c) (Table 34).

This suggests that a higher frequency of $CD8^+$ EM and EMRA in T1D were activated and had recently migrated from tissues into circulation compared to controls.

5.3.3.2ii CD11b

$CD8^+$ T cell subsets with a migratory capacity, defined by CD11b expression, were measured in T1D and control groups. There were no differences between groups in CD11b expression on $CD8^+$ naïve, CM or EM subsets.

The frequency of $CD8^+CD11b^+$ EMRA were significantly elevated in the T1D group ($p=0.038$) (Figure 33c) (Table 34). This indicates that there was a higher frequency of $CD8^+$ EMRA with a homing propensity in the T1D group compared to controls.

5.3.3.2iii CD127

CD127, necessary for T cell maintenance, expression across $CD8^+$ T cell subsets was measured in T1D and control subjects. No significant differences are reported in CD127 expression on $CD8^+$ T cell subsets (Table 34).

5.3.3.2iv CD95

$CD95^+$, marker of apoptosis, expression on $CD8^+$ T cell subsets was measured in both groups. No significant differences are reported in CD95 expression on $CD8^+$ T cell subsets (Table 34). There was a trend to increase in the frequency $CD8^+CD95^+$ EMRA in T1D subject (Figure 33c).

5.3.3.2v EMRA Differentiation (CD27/CD28)

As discussed in section $CD4^+$ baseline, it has been assumed EMRA are $CD27^-CD28^-$ i.e. fully differentiated, however recent studies have shown the presence of mixed differentiated subsets of EMRA in healthy and ageing populations [408, 409]. This has not been measured in T1D previously.

Table 35 displays the mean baseline data for the proportion and frequency of differentiated EMRA subsets. $CD8^+$ EMRA subsets showed a significant difference in the proportion of $CD27^+$ $CD28^-$ subsets between groups (p=0.021) (Figure 33a), but this was not significant in frequencies (Figure 33b). There were no differences between groups in the other EMRA subsets (Table 35). This shows that there were less intermediately differentiated $CD8^+$ EMRA in the T1D group.

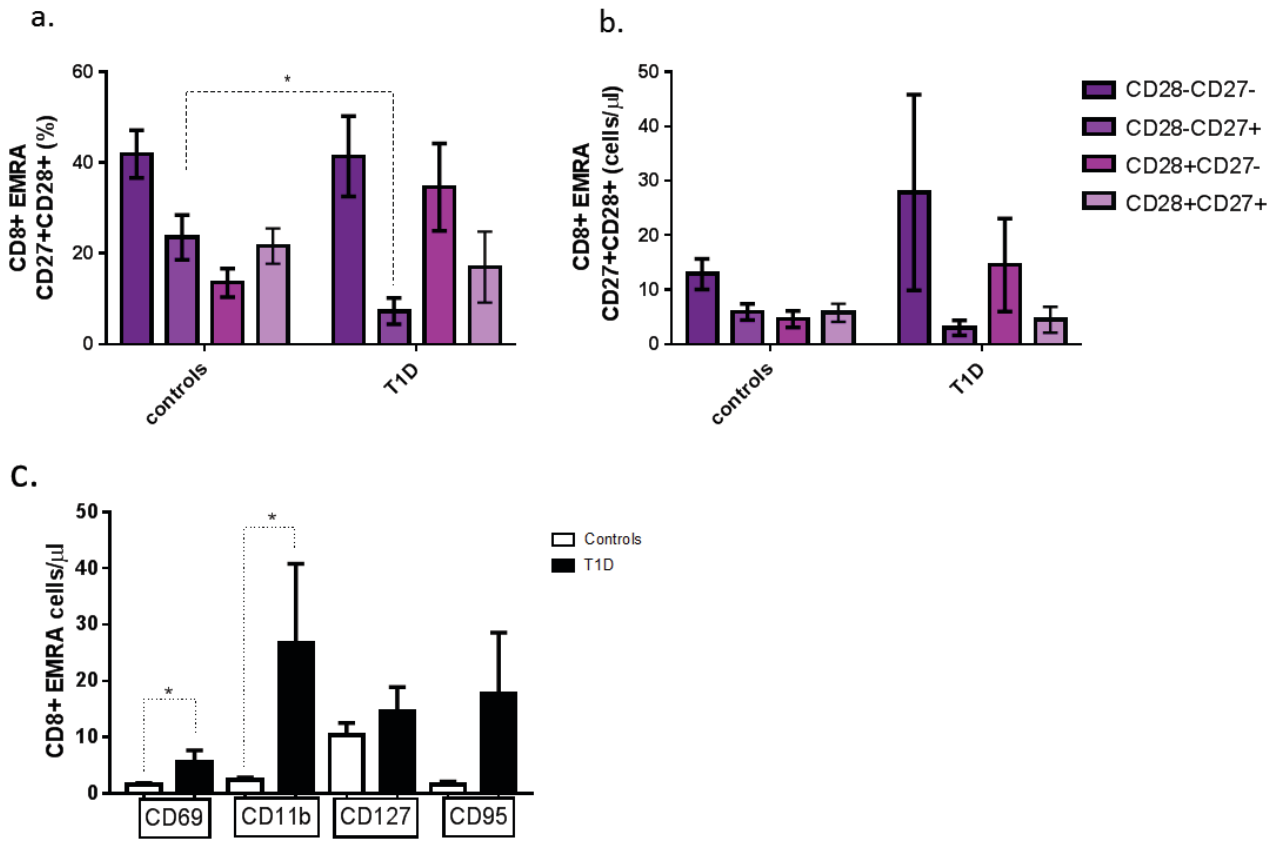

Figure 33 The frequency of surface marker expression on CD8+ naïve, EM, CM, and EMRA. The proportion and frequency of differentiated CD8+ EMRA T cell subsets at baseline in T1D and control groups.

Flow cytometry and whole blood counts were used to determine the phenotype CD8+ sub populations in control and T1D subjects *(a)* The proportion of differentiated CD8+ EMRA subsets *(b)* The frequency of differentiated CD8+ EMRA subsets *(c)* The frequency of CD69+, CD11b+, CD127+, and CD95+ CD8+ subsets. Error bars represent SEM. Statistical significance represented by * $p<0.05$, ** $p<0.01$, ***$p<0.001$. Note: broken line (----) represents time*group differences.

		[1]Controls							
		CD69		CD11b		CD127		CD95	
		mean	SD	mean	SD	mean	SD	mean	SD
(%)	CD8 naïve	4.57	3.89	10.25	23.93	75.59	20.26	6.67	18.88
	CD8 CM	4.65	3.74	8.23	13.42	77.19	15.06	11.84	20.46
	CD8 EM	4.83	2.04	10.33	17.18	64.80	15.58	12.15	23.33
	CD8 EMRA	6.77	7.62	15.43	21.91	45.59	21.80	6.67	18.88
(cells/µl)	CD8 naïve	2.86	2.61	3.88	3.51	62.90	63.81	1.60	2.55
	CD8 CM	1.14	1.20	13.22	34.96	18.42	10.48	2.39	4.03
	CD8 EM	7.85	6.04	20.23	35.70	124.23	100.64	18.70	33.73
	CD8 EMRA	1.56	1.16	2.37	2.20	10.37	9.65	1.57	2.61
		[2]T1D							
(%)	CD8 naïve	5.59	4.09	18.40	35.32	86.72	24.50	15.07	24.85
	CD8 CM	3.65	2.27	11.58	18.32	83.99	24.66	26.57	28.91
	CD8 EM	6.91	3.39	20.25	24.12	70.35	18.14	25.99	32.05
	CD8 EMRA	11.04	5.46	29.41	34.38	42.21	23.79	15.07	24.85
(cells/µl)	CD8 naïve	1.61	1.19	8.43	24.99	57.25	66.17	11.17	24.41
	CD8 CM	0.74	0.59	28.31	66.10	16.54	10.85	10.21	21.53
	CD8 EM	14.50	15.04	50.69	82.59	140.78	101.37	41.46	66.68
	CD8 EMRA	5.47	8.41	26.71	54.64	14.53	16.29	17.82	41.52

Table 34 Surface marker expression on CD8+ naïve, CM, EM, and EMRA populations at baseline in T1D and controls

Mean and standard deviation values for the proportion and frequency of CD69+, CD11b+, CD127+, and CD95+ CD8+ naïve, CM, EM, and EMRA populations in controls and T1D participants.
[1]controls n=23, [2]T1D n=15

	[1]Controls							
	$CD28^-CD27^-$		$CD28^-CD27^+$		$CD28^+CD27^-$		$CD28^+CD27^+$	
	mean	SD	mean	SD	mean	SD	mean	SD
CD8 (%)	41.89	24.04	23.51	22.71	13.53	14.49	21.62	17.82
CD8 (cells/µl)	12.89	12.89	5.92	6.83	4.57	6.95	5.76	7.59
	[2]T1D							
CD8 (%)	41.38	27.97	7.30	9.14	34.61	30.46	16.96	24.63
CD8 (cells/µl)	27.87	56.81	2.98	4.35	14.51	27.02	4.48	7.94

Table 35 Differentiated CD8+ EMRA T cell subsets at baseline in T1D and control groups.

Mean and standard deviation values for the proportion and frequency of differentiated CD8+ EMRA subsets in controls and T1D participants.
[1]controls n=22, [2]T1D n=15

5.3.3.3 $CD8^+$ T cell subsets in response to acute exercise in T1D and control participants

The effects of moderate (40% VO_2 max) and vigorous (80% VO_2 max) intensity exercise on $CD8^+$ T cell subsets were examined. The results are provided in progressive order of differentiation in Table 36. Table 36 displays the mean, standard deviation, and statistical values for $CD8^+$ T cell subsets.

$CD8^+$ T cells were significantly mobilised by vigorous intensity exercise in T1D (p=0.016) and control groups (p=0.001). Both groups showed a significant increase immediately post vigorous exercise (p=0.40, p=0.033 respectively). The percentage increase post vigorous exercise between groups was similar. Within the $CD8^+$ T cell population, significant changes were seen in $CD8^+$ EM, ED, ELD, ID, EM, and EMRA during moderate and/or vigorous exercise. It is evident from this data that the increase in $CD8^+$ T cells is driven by mobilisation of later differentiated $CD8^+$ T cell subsets and these changes were most consistent between groups in the $CD8^+$ EMRA subset. Naïve subsets do not significantly change with exercise in either group, however there was a marginal difference in the response between groups during moderate intensity exercise (p=0.048). There was no significant change in $CD8^+$ CM T cells in either group independently (Table 36).

$CD8^+$ EM were significantly mobilised by moderate intensity exercise in the T1D only (p=0.018). $CD8^+$ EM showed a trend to mobilise during vigorous exercise (p=0.067), with a fall below baseline at 1 hour post vigorous exercise (p=0.040) in the T1D group. No significant changes were seen in the control group (Table 36).

$CD8^+$ ED changed with vigorous intensity exercise overall only (p=0.040), but this is not seen in either T1D or control groups independently (Table 36).

$CD8^+$ ELD were significantly mobilised by moderate intensity exercise in T1D (p=0.025) and controls (p=0.029). This was supported by an overall change in this subset (p=0.001). There was an overall change in $CD8^+$ ELD during vigorous intensity exercise (p<0.001), with a trend to change in the T1D group (p=0.054) but not the control group. There was a significant fall below baseline 1 hour post vigorous exercise in the T1D group only (p=0.029) (Table 36).

There was a significant change during moderate intensity exercise in $CD8^+$ ID in the T1D group (p=0.022) and overall (p=0.001), but not in the control group. $CD8^+$ ID significantly increased immediately post moderate exercise in the T1D group (p=0.046). There was a significant overall change in $CD8^+$ ID with vigorous exercise (p<0.001) but not in either T1D or control groups independently. $CD8^+$ ID significantly fell below baseline 1 hour post vigorous exercise in the control group only (p=0.027) (Table 36).

Figure 34 displays that both intensities resulted in a significant change in $CD8^+$ EMRA cell frequencies overall (low: p=0.005, vigorous: p=0.001). $CD8^+$ EMRA cells were significantly mobilised by moderate intensity exercise in T1D (p=0.002) and control groups (p=0.002). They were significantly increased immediately post moderate exercise in the T1D group only (p=0.013), but decreased significantly below baseline 1 hour post exercise in the control group only (p=0.022) (Figure 34a). $CD8^+$ EMRA were significantly mobilised by vigorous intensity exercise in T1D (p=0.004) and control groups (p=0.010). There was a significant decrease 1 hour post vigorous exercise in both T1D (p=0.019) and control groups (p=0.004) (Figure 34b). This concludes that $CD8^+$ EMRA subsets are mobilised by both moderate and vigorous intensity exercise in both T1D and control groups. However, the percentage increase post vigorous intensity exercise is blunted in the T1D group (T1D: 26.45%, control: 66.13%) (Table 36).

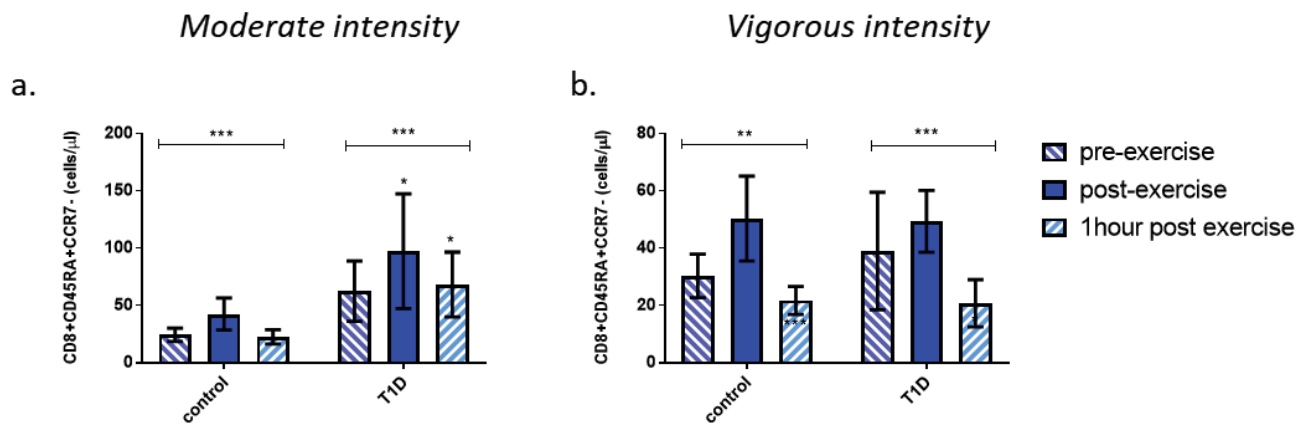

Figure 34 The frequencies of CD8+ EMRA subsets mobilised during moderate and vigorous intensity exercise in control and T1D participants

Flow cytometry and whole blood counts were used to determine the frequency of CD8+ EMRA populations in control and T1D subjects during moderate and vigorous intensity exercise. *(a)* The frequency of CD8+ EMRA during moderate intensity exercise *(b)* The frequency of CD8+ EMRA during vigorous intensity exercise. Error bars represent SEM. Statistical significance represented by * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

Subset (cells/µl)	Intensity	[1]Controls T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast		[a]Δ%	[2]T1D T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast		[a]Δ%	[d]Time (overall)	[d]Time*Group
Total CD8	moderate	448.95	149.11	626.88	335.59	480.60	162.84	$F_{(2, 20)}$= 3.224, p=0.061	*T2*	$F_{(1, 10)}$= 3.357, p=0.097	39.63	478.30	157.11	677.33	430.18	491.82	80.48	$F_{(2,10)}$= 2.186, p=0.163	*T2*	$F_{(1, 5)}$= 1.317, p=0.303	41.61	$F_{(1, 17.1)}$= 0.315, p=0.597	$F_{(1, 17.1)}$= 0.474, p=0.513
									T3	$F_{(1, 10)}$= 0.352, p=0.566	7.05								*T3*	$F_{(1, 5)}$= 3.020, p=0.143	2.83		
	vigorous	329.85	165.90	460.90	178.81	229.11	98.35	**$F_{(2, 22)}$= 10.326, p=0.001**	*T2*	**$F_{(1, 11)}$= 5.961, p=0.033**	39.73	248.34	158.17	354.41	225.31	192.54	122.80	**$F_{(1.2, 9.7)}$= 7.791, p=0.016**	*T2*	**$F_{(1, 8)}$= 6.021, p=0.040**	42.71	**$F_{(2, 42)}$= 3.295, p=0.047**	$F_{(2, 42)}$= 0.126, p=0.882
									T3	**$F_{(1, 11)}$= 7.253, p=0.021**	30.54								*T3*	$F_{(1, 8)}$= 4.942, p=0.057	22.47		
CD8 naïve	**moderate**	74.25	82.28	79.03	76.02	64.54	64.17	$F_{(2, 20)}$= 2.770, p=0.087	*T2*	$F_{(1, 10)}$= 0.693, p=0.425	6.44	76.41	70.98	88.04	76.07	59.95	61.22	$F_{(2, 10)}$= 0.185, p=0.834	*T2*	$F_{(1, 5)}$= 0.261, p=0.631	15.22	$F_{(2, 24)}$= 1.743, p=0.196	**$F_{(2, 24)}$= 3.465, p=0.048**
									T3	$F_{(1, 10)}$= 2.095, p=0.178	13.08								*T3*	$F_{(1, 5)}$= 0.217, p=0.661	21.55		
	vigorous	100.77	70.41	108.43	84.76	81.54	57.25	$F_{(1.3, 14.5)}$= 0.751, p=0.436	*T2*	$F_{(1, 10)}$= 0.368, p=0.556	7.60	48.16	62.31	61.36	66.97	48.68	53.50	$F_{(2,16)}$= 1.159, p=0.339	*T2*	$F_{(1, 8)}$= 1.654, p=0.234	27.41	$F_{(2, 34)}$=1.834, p=0.175	$F_{(2, 34)}$=0.797, p=0.459
									T3	$F_{(1, 10)}$= 2.051, p=0.180	19.08								*T3*	$F_{(1, 8)}$= 0.156, p=0.704	1.07		
CD8 CM	moderate	19.94	9.38	25.15	16.89	26.73	16.75	$F_{(2, 20)}$= 0.434, p=0.654	*T2*	$F_{(1, 10)}$= 0.497, p=0.497	26.16	25.82	10.51	29.66	17.33	17.95	10.19	$F_{(2,10)}$= 1.274, p=0.322	*T2*	$F_{(1, 5)}$= 0.441, p=0.536	14.90	$F_{(2, 24)}$= 2.374, p=0.115	$F_{(2, 24)}$= 1.872, p=0.770
									T3	$F_{(1, 10)}$= 0.168, p=0.690	34.07								*T3*	$F_{(1, 5)}$= 0.904, p=0.385	30.48		
	vigorous	27.09	13.66	27.70	16.48	25.42	13.49	$F_{(2, 22)}$= 1.343, p=0.282	*T2*	$F_{(1, 11)}$= 0.012. p=0.914	2.25	14.94	9.79	24.13	15.94	13.85	11.05	$F_{(1.35, 4.9)}$= 2.209, p=0.173	*T2*	$F_{(1, 8)}$= 2.048, p=0.190	61.51	$F_{(1.3, 21.9)}$= 0.546, p=0.511	$F_{(1.3, 21.9)}$= 0.255, p=0.678
									T3	$F_{(1, 8)}$= 2.059, p=0.179	6.15								*T3*	$F_{(1, 8)}$= 1.961, p=0.199	7.30		
CD8 EM	**moderate**	153.96	97.90	210.92	145.86	162.59	108.94	$F_{(2, 20)}$= 0.907, p=0.420	*T2*	$F_{(1, 10)}$= 0.259, p=0.622	37.00	235.32	108.88	300.97	233.38	209.06	46.63	**$F_{(2,10)}$= 6.119, p=0.018**	*T2*	$F_{(1, 5)}$= 2.058, p=0.211	27.90	$F_{(2, 24)}$= 1.031, p=0.130	$F_{(2, 24)}$= 0.698, p=0.507
									T3	$F_{(1, 10)}$= 0.720, p=0.416	5.61								*T3*	$F_{(1, 5)}$= 2.594, p=0.168	11.16		
	vigorous	213.22	129.35	314.69	218.55	179.30	102.03	$F_{(2, 22)}$= 1.486, p=0.248	*T2*	$F_{(1, 11)}$= 0.06, p=0.763	47.59	157.07	151.50	269.78	224.42	124.62	115.28	$F_{(1.2, 9.8)}$= 4.060, p=0.067	*T2*	$F_{(1, 8)}$= 2.124, p=0.183	71.76	$F_{(1.257, 21.4)}$= 0.822, p=0.401	$F_{(1.257, 21.4)}$=0.286, p=0.651
									T3	$F_{(1, 11)}$= 3.356, p=0.094	15.91								*T3*	**$F_{(1, 8)}$= 5.978, p=0.040**	20.66		
CD8 ED	moderate	61.46	74.81	66.14	78.03	57.25	73.21	$F_{(2, 20)}$= 1.656, p=0.216	*T2*	$F_{(1, 10)}$= 0.041, p=0.844	7.61	25.14	61.10	31.14	74.81	0.28	0.56	$F_{(1.07, 5.34)}$= 2.934, p=0.074	*T2*	$F_{(1, 5)}$= 1.501, p=0.275	23.86	$F_{(1.3, 16.3)}$= 1.103, p=0.326	$F_{(1.3, 16.3)}$= 1.214, p=0.300
									T3	$F_{(1, 10)}$= 2.146, p=0.174	6.86								*T3*	$F_{(1, 5)}$= 2.772, p=0.157	98.90		
	vigorous	102.30	97.85	123.23	116.89	65.15	52.83	$F_{(2, 22)}$= 0.882, p=0.428	*T2*	$F_{(1, 11)}$= 0.011, p=0.918	20.46	37.27	64.35	53.51	85.79	8.50	11.81	$F_{(1.2, 9.3)}$= 3.599, p=0.085	*T2*	$F_{(1, 8)}$= 1.289, p=0.289	43.55	**$F_{(1.2, 20.3)}$= 4.520, p=0.040**	$F_{(1.2, 20.3)}$= 0.051, p=0.864
									T3	$F_{(1, 11)}$= 1.498, p=0.247	36.32								*T3*	$F_{(1, 8)}$= 4.830, p=0.059	77.20		
CD8 ELD	**moderate**	34.40	28.22	49.32	52.42	33.25	32.30	**$F_{(2, 20)}$= 4.266, p=0.029**	*T2*	$F_{(1, 10)}$= 0.517, p=0.489	43.37	85.10	96.31	108.69	114.59	78.54	82.81	**$F_{(2, 10)}$= 5.477, p=0.025**	*T2*	$F_{(1, 5)}$= 0.876, p=0.392	27.72	**$F_{(2, 24)}$= 9.012, p=0.001**	$F_{(2, 24)}$= 0.194, p=0.825
									T3	$F_{(1, 10)}$= 3.849, p=0.078	3.34								*T3*	$F_{(1, 5)}$= 3.722, p=0.112	7.70		
	vigorous	38.91	39.00	63.49	62.18	33.49	30.31	$F_{(1.1, 12.1)}$= 1.390, p=0.266	*T2*	$F_{(1, 11)}$= 0.906, p=0.362	63.17	84.05	140.43	144.09	222.84	54.26	81.30	$F_{(1.2, 9.9)}$= 4.492, p=0.054	*T2*	$F_{(1, 8)}$= 2.044, p=0.191	71.44	**$F_{(1.4, 24.5)}$= 14.969, p<0.001**	$F_{(1.4, 24.5)}$= 1.501, p=0.240
									T3	$F_{(1, 11)}$= 2.436, p=0.147	13.94								*T3*	**$F_{(1, 8)}$= 7.006, p=0.029**	35.44		
CD8 ID	**moderate**	27.03	25.04	36.94	45.26	43.66	50.73	$F_{(2, 20)}$= 0.490, p=0.620	*T2*	$F_{(1, 10)}$= 1.194, p=0.300	36.66	5.79	9.06	6.08	9.42	5.69	11.39	**$F_{(2, 10)}$= 5.765, p=0.022**	*T2*	**$F_{(1, 5)}$= 6.933, p=0.046**	5.05	**$F_{(2, 24)}$= 9.558, p=0.001**	$F_{(2, 24)}$= 0.151, p=0.860
									T3	$F_{(1, 10)}$= 0.002, p=0.961	61.54								*T3*	$F_{(1, 5)}$= 0.534, p=0.498	1.63		
	vigorous	25.93	20.99	35.35	26.47	19.22	11.57	$F_{(1.3, 13.9)}$= 3.252, p=0.086	*T2*	$F_{(1, 11)}$= 1.143, p=0.308	36.29	9.18	12.76	14.78	22.44	8.52	13.08	$F_{(1.1, 8.75)}$= 2.317, p=0.163	*T2*	$F_{(1, 8)}$= 1.819, p=0.214	60.94	**$F_{(1.4, 24)}$= 15.543, p<0.001**	$F_{(1.4, 24)}$= 0.660, p=0.476
									T3	**$F_{(1, 11)}$= 6.518, p=0.027**	25.90								*T3*	$F_{(1, 8)}$= 2.922, p=0.126	7.22		
CD8 EMRA	**moderate**	24.21	19.60	42.31	46.36	22.33	19.83	**$F_{(2, 20)}$= 9.075, p=0.002**	*T2*	$F_{(1, 10)}$= 0.517, p=0.489	74.79	62.28	64.68	97.35	122.66	68.15	63.20	**$F_{(2, 10)}$= 12.227, p=0.002**	*T2*	**$F_{(1, 5)}$= 14.035, p=0.013**	56.31	**$F_{(2, 24)}$= 6.582, p=0.005**	$F_{(2, 24)}$= 0.049, p=0.952
									T3	**$F_{(1, 10)}$= 7.390, p=0.022**	7.75								*T3*	$F_{(1, 5)}$= 3.106, p=0.138	9.42		
	vigorous	30.29	26.28	50.32	49.30	21.67	16.27	**$F_{(1.37, 15.1)}$= 7.458, p=0.010**	*T2*	$F_{(1, 11)}$= 2.5052, p=0.180	66.13	39.00	61.60	49.31	32.36	20.75	24.74	**$F_{(2,16)}$= 8.053, p=0.004**	*T2*	$F_{(1, 8)}$= 3.981, p=0.081	26.45	**$F_{(1.5, 25)}$= 11.823, p=0.001**	$F_{(1.5, 25)}$= 0.472, p=0.571
									T3	**$F_{(1, 11)}$= 13.361, p=0.004**	28.45								*T3*	**$F_{(1, 8)}$= 8.487, p=0.019**	46.80		

Table 36 CD8+ T cell sub populations mobilised by moderate and vigorous intensity exercise in control and T1D groups

Mean, standard deviation, and statistical analysis of CD8+ sub populations for control and T1D subjects during moderate and vigorous intensity exercise. Significant results highlighted in bold where p values <0.05 were considered significant.

[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2) or 1 hour post exercise (T3).

[b] Results were analysed using multiple regression analysis in control and T1D groups independently.

[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).

[d] Results were analysed using multiple regression analysis in control and T1D groups combined.

[1]Controls n=11, [2]T1D n =6-9

5.3.3.4 $CD8^+$ T cell phenotype in response to acute exercise in T1D and control participants

As shown for $CD8^+$ T cell subsets at baseline, cell surface markers were used to examine the homing propensity and function of $CD8^+$ T cell subsets mobilised during moderate and vigorous intensity exercise. Table 37 displays the frequency (cells/μl) of each marker on $CD8^+$ subsets during moderate and vigorous intensity exercise.

5.3.3.4i CD69

$CD8^+CD69^+$ naïve T cells showed a significant change over time during moderate intensity exercise in controls only (p=0.029), falling below baseline 1 hour post moderate exercise (p=0.042). $CD8^+CD69^+$ naïve were significantly increased immediately post vigorous exercise in T1D (p=0.021) and control groups (p=0.029), with a significant change over time in the T1D group only (p=0.010). This percentage increase was marginally blunted in the T1D group (T1D: 43.69%, Control: 79.90%) (Table 37).

$CD8^+CD69^+$ CM T cells were mobilised during moderate intensity exercise in the T1D group only (p=0.037). Vigorous intensity exercise mobilised this subset in T1D (p=0.020) and control groups (p=0.037), with a significant increase immediately post vigorous exercise in both groups (p=0.021, p=0.010 respectively). This percentage increase was marginally blunted in the T1D group (T1D: 59.98%, Control: 85.57%) (Table 37).

$CD8^+CD69^+$ EM T cells changed during moderate intensity exercise overall (p=0.044), but not in either T1D or control groups separately. Vigorous intensity exercise resulted in the mobilisation of this subset in the T1D group (p=0.012), but not in controls (Table 37).

$CD8^+CD69^+$ EMRA T cells significantly changed during moderate intensity exercise in T1D (p=0.003) and control groups (p=0.022), with a significant increase seen immediately post

moderate exercise seen in the T1D only (p=0.012). Vigorous intensity exercise resulted in the mobilisation of this subset in T1D (p=0.002) and control groups (p=0.003), with a significant increase seen immediately post vigorous exercise observed in the control group only (p=0.010) (Figure 35a). The percentage increase was blunted in the T1D group following moderate (T1D: 44.05%, Control: 92.33%) and considerably blunted following vigorous (T1D: 29.87%, Control: 161.43%) intensity exercise (Table 37).

5.3.3.4ii CD11b

$CD8^+CD11b^+$ EMRA subsets significantly changed during moderate intensity exercise in T1D (p=0.014) and control groups (p=0.001). They were further elevated 1 hour post moderate exercise in the T1D group (p=0.047), but were significantly below baseline at this time point in the control group (p=0.017). However, this difference in response was not significant. $CD8^+CD11b^+$ EMRA subsets were mobilised by vigorous intensity exercise in T1D (p=0.007) and control groups (p=0.001). There was a significant increase immediately post vigorous exercise in the control group only (p=0.010). In the T1D group only, this subset significantly fell below baseline 1 hour post vigorous exercise (p=0.006) (Figure 35b). The percentage increase was blunted in the T1D group following moderate (T1D: 14.82%, Control: 38.61%) and vigorous (T1D: 37.70% decrease, Control: 91.48%) intensity exercise (Table 37).

There were no significant changes in $CD8^+CD11b^+$ naïve, CM or EM subsets with either exercise intensity (Table 37).

5.3.3.4iii CD127

$CD8^+CD127^+$ CM subsets significantly changed overall with moderate (p=0.040) and vigorous (p=0.038) intensity exercise but this is not seen in either T1D or control groups independently (Table 37).

$CD8^+CD127^+$ EM significantly changed vigorous (p=0.035) intensity exercise overall but this was not seen in either T1D or control groups independently. There was a significant fall below baseline 1 hour post vigorous exercise in the T1D group (p=0.037) (Table 37).

$CD8^+CD127^+$ EMRA significantly changed overall during moderate (p=0.026) and vigorous (p=0.037) exercise. This was observed in both T1D (p=0.038) and control groups (p=0.033) during moderate but not vigorous intensity exercise, with a significant increase immediately post moderate exercise (p=0.046) in the T1D group (Figure 35c). A blunted increase post moderate exercise in the T1D is not seen with $CD8^+CD127^+$ EMRA. Furthermore, the percentage increase post vigorous exercise was higher in the T1D group (T1D: 61.49%, Control: 36.16%) (Table 37).

There were no significant changes in $CD8^+CD127^+$ naïve with either exercise intensity (Table 37).

5.3.3.4iv CD95

$CD8^+CD95^+$ CM significantly changed overall with moderate (p=0.024) intensity exercise but this was not seen in either T1D or control groups independently. There were no significant changes during vigorous intensity exercise in this subset (Table 37).

$CD8^+CD95^+$ EMRA were significantly mobilised by moderate (p=0.004) and vigorous (p=0.003) overall, but this was not seen in either T1D or control groups independently (Figure 35d) (Table 37).

There were no significant changes in $CD8^+$ naïve and EM expressing CD95 with either exercise intensity (Table 37).

The percentage increase post exercise of all $CD8^+CD95^+$ subsets was higher in the T1D group following moderate intensity exercise (Table 37). However, the percentage increase post

exercise of all $CD8^+CD95^+$ subsets was blunted in the T1D group following vigorous intensity exercise (Table 37).

5.3.3.4v Differentiation of EMRA (CD27/CD28)

Using the same approach as in $CD4^+$ subsets (Section 3.2.4), $CD8^+$ EMRA subsets at the 4 differentiation stages were assessed during exercise in T1D and control participants. Data for vigorous intensity exercise only in T1D and both exercise intensities in control participants is shown (Table 37).

The frequency of $CD8^+CD27^-CD28^-$ EMRA significantly changed during vigorous intensity exercise overall (p=0.005) and was seen in both the T1D (p=0.050) and control group (p=0.037) independently. The percentage increase post vigorous exercise was considerably blunted in the T1D group (T1D: 7.02% decrease, control: 113.76%). There was a significant fall below baseline 1 hour post vigorous intensity exercise in the T1D group only (p=0.005) (Figure 35e) (Table 37).

There were significant changes over time in the $CD8^+CD27^-CD28^+$ EMRA (intermediately differentiated) during vigorous (p=0.022) intensity exercise in the T1D group only (Table 37).

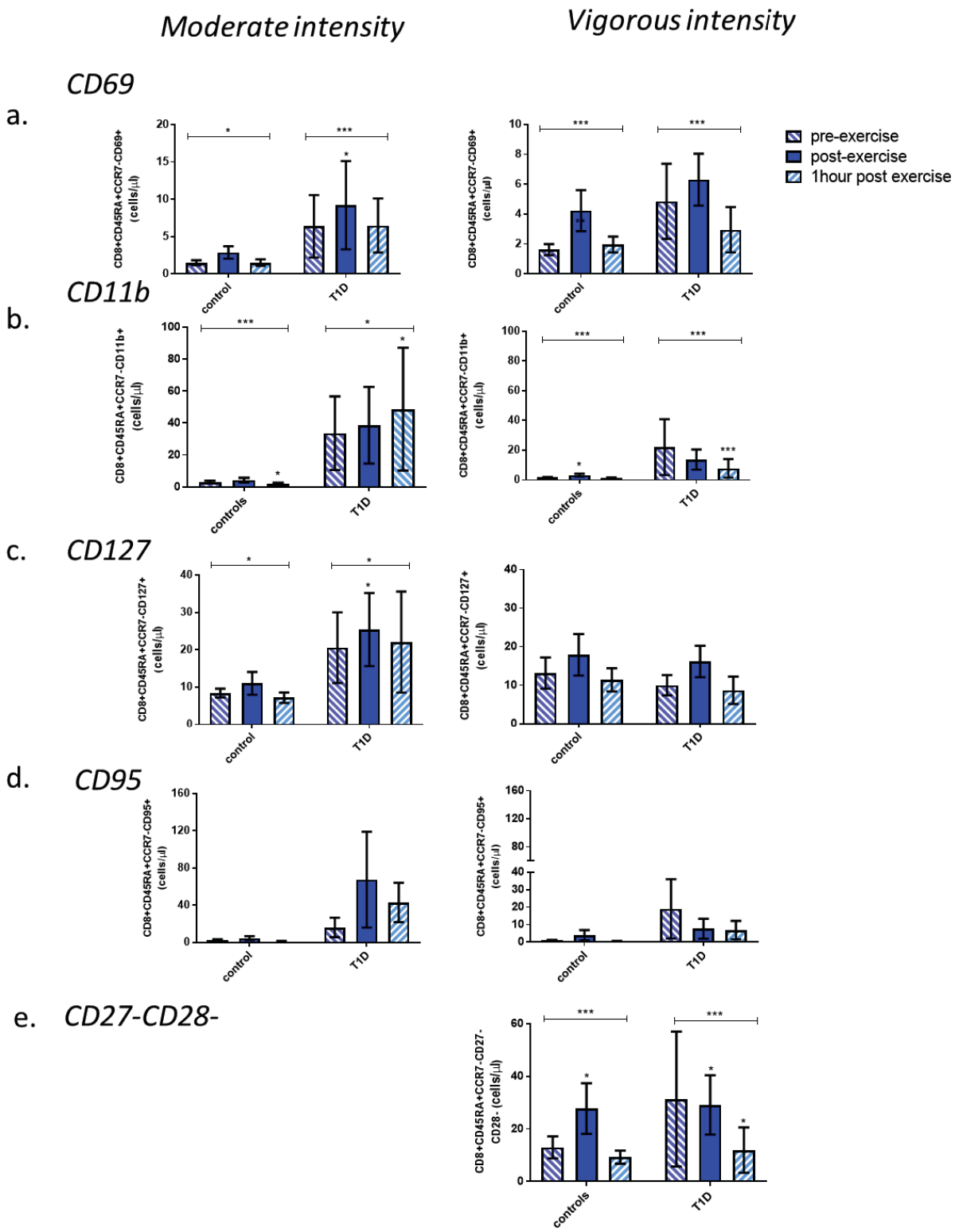

Figure 35 Expression of surface markers CD69, CD11b, CD127, CD95, CD27 and CD28 on CD8+ EMRA subsets during low and vigorous intensity exercise in control and T1D subjects

Flow cytometry and whole blood counts were used to determine the frequency of CD8+ subsets expressing CD69, CD11b, CD127, and CD95 in control and T1D participants *(a)* The frequency of CD8+CD69+ EMRA *(b)* CD8+CD11b+ EMRA *(c)* CD8+CD127+ EMRA and *(d)* CD8+CD95+ EMRA during low and vigorous intensity exercise *(e)* The frequency of CD8+CD27-CD28- EMRA during vigorous intensity exercise. Error bars represent SEM. Statistical significance represented by * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

Table 37 Surface marker expression on CD8+ naïve, CM, EM, and EMRA populations mobilised by moderate and vigorous intensity exercise in control and T1D groups

Mean, standard deviation and statistical analysis of CD69+, CD11b+, CD127+, and CD95+ on CD8+ naïve, CM, EM, and EMRA populations for control and T1D subjects during moderate and vigorous intensity exercise. Significant results highlighted in bold where p values <0.05 were considered significant.
[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2) or 1 hour post exercise (T3).
[b] Results were analysed using multiple regression analysis in control and T1D groups independently.
[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).
[d] Results were analysed using multiple regression analysis in control and T1D groups combined.
[1]Controls n=11, [2]T1D n =6-9

Subset (cells/µl)	Intensity	[1]Controls										[2]T1D										[d]Time (overall)	[d]Time*Group
		T1		T2		T3		[b]Time	[c]contrast		[a]Δ%	T1		T2		T3		[b]Time	[c]contrast		[a]Δ%		
		mean	SD	mean	SD	mean	SD					mean	SD	mean	SD	mean	SD						
CD8 Na CD69+	moderate	2.94	2.76	2.79	2.99	2.13	2.20	**$F_{(2, 20)}$= 4.230, p=0.029**	*T2*	$F_{(1, 10)}$= 0.266, p=0.617	5.17	2.02	1.36	2.33	0.24	2.31	1.21	$F_{(2, 10)}$= 1.980, p=0.189	*T2*	$F_{(1, 5)}$= 4.563, p=0.086	15.70	$F_{(2, 24)}$= 1.962 p=0.163	$F_{(2, 24)}$= 0.797, p=0.462
									T3	**$F_{(1, 10)}$= 5.400, p=0.042**	27.49								*T3*	$F_{(1, 5)}$= 0.020, p=0.894	14.29		
	vigorous	2.78	2.58	5.00	5.82	3.52	3.41	$F_{(1.2, 12.1)}$= 2.761, p=0.118	*T2*	**$F_{(1, 10)}$= 6.517, p=0.029**	79.90	1.34	1.06	1.93	1.44	1.18	1.26	**$F_{(2,16)}$= 6.162, p=0.010**	*T2*	**$F_{(1, 8)}$= 8.294, p=0.021**	43.69	$F_{(1.26, 22.8)}$= 0.907p=0.374	$F_{(1.26, 22.8)}$=0.370, p=0.598
									T3	$F_{(1, 10)}$= 4.602, p=0.058	26.61								*T3*	$F_{(1, 8)}$= 1.414, p=0.269	12.12		
CD8 CM CD69+	moderate	1.24	1.10	1.46	1.16	1.21	0.87	$F_{(2, 20)}$= 0.996, p=0.387	*T2*	$F_{(1, 10)}$= 1.598, p=0.235	17.87	1.05	0.49	1.94	2.13	0.37	0.26	**$F_{(2,10)}$= 4.685, p=0.037**	*T2*	$F_{(1, 5)}$= 3.865, p=0.106	84.49	$F_{(2, 24)}$= 1.384 p=0.270	$F_{(2, 24)}$= 0.624, p=0.545
									T3	$F_{(1, 10)}$= 0.006, p=0.941	1.88								*T3*	$F_{(1, 5)}$= 4.453, p=0.089	64.79		
	vigorous	1.04	1.33	1.92	2.17	1.32	1.41	**$F_{(1.33, 13.25)}$= 4.884, p=0.037**	*T2*	**$F_{(1, 10)}$= 10.016, p=0.010**	85.57	0.53	0.59	0.85	1.11	0.38	0.40	**$F_{(1.2, 9.7)}$= 7.190, p=0.020**	*T2*	**$F_{(1, 8)}$= 8.133, p=0.021**	59.98	$F_{(1.11, 20)}$= 2.978, p=0.362	$F_{(1.11, 20)}$= 1.905, p=0.163.
									T3	$F_{(1, 10)}$= 1.160, p=0.307	27.61								*T3*	$F_{(1, 8)}$= 3.895, p=0.084	28.86		
CD8 EM CD69+	moderate	7.79	6.72	10.01	9.20	11.01	10.02	$F_{(1.3, 13)}$= 0.510, p=0.535	*T2*	$F_{(1, 10)}$= 0.382, p=0.551	28.44	15.02	9.75	16.04	10.76	7.11	2.99	$F_{(2, 10)}$= 2.886, p=0.102	*T2*	$F_{(1, 5)}$= 4.134, p=0.098	6.80	**$F_{(2, 24)}$= 3.582 p=0.044**	$F_{(2, 24)}$= 1.403, p=0.265.
									T3	$F_{(1, 10)}$= 0.834, p=0.383	41.28								*T3*	$F_{(1, 5)}$= 1.143, p=0.334	52.67		
	vigorous	7.91	5.60	21.44	25.80	9.28	8.97	$F_{(2, 20)}$= 2.070, p=0.152	*T2*	$F_{(1, 10)}$= 3.141, p=0.107	170.99	14.15	18.34	28.62	42.23	19.90	43.81	**$F_{(2,16)}$= 5.970, p=0.012**	*T2*	$F_{(1, 8)}$= 5.052, p=0.055	102.26	$F_{(2, 34)}$= 1.046 p=0.362	$F_{(2, 34)}$= 0.527, p=0.595
									T3	$F_{(1, 10)}$= 0.095, p=0.764	17.25								*T3*	$F_{(1, 8)}$= 2.708, p=0.138	40.62		
CD8 EMRA CD69+	moderate	1.50	1.12	2.89	2.76	1.52	1.43	**$F_{(2, 20)}$= 4.678, p=0.022**	*T2*	$F_{(1, 10)}$= 4.267, p=0.066	92.33	6.39	10.26	9.21	14.43	6.50	7.24	**$F_{(2, 10)}$= 10.886, p=0.003**	*T2*	**$F_{(1, 5)}$= 14.783, p=0.012**	44.05	**$F_{(2, 24)}$= 6.517 p=0.005**	$F_{(2, 24)}$= 0.234, p=0.793
									T3	$F_{(1, 10)}$= 0.730, p=0.413	1.10								*T3*	$F_{(1, 5)}$= 0.852, p=0.398	1.64		
	vigorous	1.62	1.25	4.24	4.34	1.96	1.70	**$F_{(2, 20)}$= 8.049, p=0.003**	*T2*	**$F_{(1, 10)}$= 10.152, p=0.010**	161.43	4.86	7.55	6.31	5.23	2.96	4.56	**$F_{(2,16)}$= 9.665, p=0.002**	*T2*	$F_{(1, 8)}$= 4.554, p=0.065	29.87	$F_{(1.2, 21.2)}$= 1.884 p=0.184	$F_{(1.2, 21.2)}$= 1.638, p=0.217
									T3	$F_{(1, 10)}$= 0.087, p=0.774	21.13								*T3*	$F_{(1, 8)}$= 3.616, p=0.094	39.21		

Subset (cells/µl)	Intensity	[1]Controls T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast		[a]Δ%	[2]T1D T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast		[a]Δ%	[d]Time (overall)	[d]Time*Group
CD8 Na CD11b$^+$	moderate	4.96	4.33	6.42	8.78	4.83	7.37	$F_{(2, 20)}$= 2.873, p=0.080	*T2*	$F_{(1, 10)}$=0.289, p=0.602	29.64	18.86	39.01	23.88	46.58	41.47	58.91	$F_{(2, 10)}$= 0.191, p=0.829	*T2*	$F_{(1, 5)}$= 0.625, p=0.465	26.64	$F_{(2, 24)}$=2.983 p=0.070.	$F_{(2, 24)}$= 3.079, p=0.065.
									T3	$F_{(1, 10)}$= 2.442, p=0.149	2.45									*T3*	$F_{(1, 5)}$= 0.015, p=0.906	119.93		
	vigorous	2.90	2.31	4.74	6.16	3.20	2.72	$F_{(2, 22)}$= 0.418, p=0.663	*T2*	$F_{(1, 11)}$= 0.977, p=0.344	63.15	1.48	2.39	1.56	1.73	1.15	1.48	$F_{(2,16)}$= 1.274, p=0.307	*T2*	$F_{(1, 8)}$= 1.196, p=0.306	5.80	$F_{(1.1,18.7)}$= 0.086 p=0.797	$F_{(1.1,18.7)}$= 1.378, p=0.259
									T3	$F_{(1, 11)}$= 0.067, p=0.801	10.30									*T3*	$F_{(1, 8)}$= 0.285, p=0.608	22.46		
CD8 CM CD11b$^+$	moderate	2.43	3.72	3.95	8.09	5.83	11.38	$F_{(1.3, 13.06)}$= 0.783, p=0.470	*T2*	$F_{(1, 10)}$= 1.563, p=0.240	62.86	4.28	5.96	4.19	5.41	5.67	6.35	$F_{(1.05, 5.3)}$= 0.230, p=0.798	*T2*	$F_{(1, 5)}$= 0.127, p=0.736	2.23	$F_{(2, 24)}$= 0.665 p=0.523	$F_{(2, 24)}$=1.201, p=0.318
									T3	$F_{(1, 10)}$= 0.778, p=0.398	140.03									*T3*	$F_{(1, 5)}$= 0.321, p=0.596	32.51		
	vigorous	1.10	1.32	1.52	1.42	0.96	0.99	$F_{(2, 22)}$= 2.455, p=0.109	*T2*	$F_{(1, 11)}$= 2.497, p=0.114	37.25	0.61	0.60	0.62	0.67	0.50	0.67	$F_{(2,16)}$= 1.225, p=0.313	*T2*	$F_{(1, 8)}$= 0.013, p=0.911	1.09	$F_{(1.22, 20.7)}$= 1.404, p=0.252	$F_{(1.22, 20.7)}$= 1.055, p=0.319
									T3	$F_{(1, 11)}$= 0.111, p=0.745	12.81									*T3*	$F_{(1, 8)}$= 1.825, p=0.214	18.59		
CD8 EM CD11b$^+$	moderate	26.43	48.16	39.54	76.72	29.01	57.67	$F_{(1.2, 11.8)}$= 1.830, p=0.204	*T2*	$F_{(1, 10)}$= 2.233, p=0.166	49.60	69.86	93.70	68.61	88.75	82.18	78.01	$F_{(2, 10)}$= 0.321, p=0.733	*T2*	$F_{(1, 5)}$= 1.925, p=0.224	1.79	$F_{(2, 24)}$=1.077 p=0.357	$F_{(2, 24)}$= 0.192, p=0.826
									T3	$F_{(1, 10)}$= 1.129, p=0.313	9.76									*T3*	$F_{(1, 5)}$= 0.033, p=0.862	17.64		
	vigorous	14.55	19.17	32.14	46.56	14.35	16.81	$F_{(2, 22)}$= 1.186, p=0.324	*T2*	$F_{(1, 11)}$= 3.799, p=0.077	120.96	37.91	77.40	58.88	120.62	13.86	18.71	$F_{(2,16)}$= 1.858, p=0.188	*T2*	$F_{(1, 8)}$= 2.295, p=0.168	55.33	$F_{(1.1, 18.6)}$= 2.040, p=0.169	$F_{(1.1, 18.6)}$= 0.742, p=0.412
									T3	$F_{(1, 11)}$= 0.168, p=0.689	1.37									*T3*	$F_{(1, 8)}$= 0.692, p=0.430	63.44		
CD8 EMRA CD11b$^+$	**moderate**	3.07	2.84	4.26	4.74	1.99	2.01	**$F_{(2, 20)}$= 10.454, p=0.001**	*T2*	$F_{(1, 10)}$= 2.000, p=0.188	38.61	33.63	56.42	38.62	58.86	48.70	76.74	**$F_{(2, 10)}$= 6.752, p=0.014**	*T2*	$F_{(1, 5)}$= 4.405, p=0.090	14.82	$F_{(2, 24)}$=3.134 p=0.062	$F_{(2, 24)}$=1.514, p=0.240
									T3	**$F_{(1, 10)}$= 8.225, p=0.017**	35.28									*T3*	**$F_{(1, 5)}$= 6.881, p=0.047**	44.81		
	vigorous	1.72	1.17	3.30	3.05	1.29	1.39	**$F_{(2, 22)}$= 10.424, p=0.001**	*T2*	**$F_{(1, 11)}$= 9.678, p=0.010**	91.48	22.09	56.35	13.76	20.34	7.86	18.66	**$F_{(1, 16)}$= 6.807, p=0.007**	*T2*	$F_{(1, 8)}$= 2.296, p=0.168	37.70	$F_{(1.34, 22.8)}$= 1.483 p=0.243	$F_{(1.34, 22.8)}$=1.428, p=0.254
									T3	$F_{(1, 11)}$= 3.828, p=0.076	24.82									*T3*	**$F_{(1, 8)}$= 14.156, p=0.006**	64.43		
CD8 Na CD127+	moderate	57.31	75.58	60.46	62.52	48.90	54.33	$F_{(2, 18)}$= 2.262, p=0.133	*T2*	$F_{(1, 9)}$= 0.585, p=0.464	5.50	71.89	69.24	82.69	74.09	66.03	52.29	$F_{(2, 10)}$= 0.218, p=0.808	*T2*	$F_{(1, 5)}$= 0.170, p=0.697	15.01	$F_{(1.3, 19.5)}$= 3.216 p=0.087	$F_{(1.3, 19.}$5)= 3.126, p=0.092
									T3	$F_{(1, 9)}$= 1.782, p=0.215	14.66									*T3*	$F_{(1, 5)}$= 0.243, p=0.643	8.16		
	vigorous	67.94	54.84	84.76	66.64	58.84	43.04	$F_{(1.4, 11.17)}$= 0.498, p=0.530	*T2*	$F_{(1, 10)}$= 0.076, p=0.788	24.76	46.26	66.22	58.96	70.71	46.59	56.68	$F_{(2,16)}$= 0.895, p=0.428	*T2*	$F_{(1, 8)}$= 1.642, p=0.236	27.45	$F_{(1.24, 19.8)}$= 0.162 p=0.744	$F_{(1.24, 19.8)}$= 0.832, p=0.397.
									T3	$F_{(1, 10)}$= 1.140, p=0.311	13.39									*T3*	$F_{(1, 8)}$= 0.012, p=0.915	0.70		
CD8 CM CD127+	**moderate**	15.29	2.68	19.87	13.56	20.21	10.65	$F_{(2, 20)}$= 1.131, p=0.345	*T2*	$F_{(1, 9)}$= 0.287, p=0.605	29.93	22.53	10.47	26.09	18.04	19.02	4.38	$F_{(2,10)}$= 0.819, p=0.468	*T2*	$F_{(1, 5)}$= 0.011, p=0.922	15.79	**$F_{(2, 24)}$= 3.681 p=0.040**	$F_{(2, 24)}$= 2.809, p=0.080
									T3	$F_{(1, 9)}$= 1.542, p=0.246	32.14									*T3*	$F_{(1, 5)}$= 1.098, p=0.343	15.58		
	vigorous	21.24	13.95	23.70	14.20	20.85	10.42	$F_{(2, 20)}$= 0.288, p=0.753	*T2*	$F_{(1, 10)}$= 0.256, p=0.624	11.61	12.04	9.30	19.84	15.56	10.64	10.10	$F_{(1.1, 8.78)}$= 1.437, p=0.267	*T2*	$F_{(1, 8)}$= 1.292, p=0.289	64.86	**$F_{(2, 36)}$= 3.583, p=0.038**	$F_{(2, 36)}$= 0.810, p=0.453.
									T3	$F_{(1, 10)}$= 0.468, p=0.510	1.83									*T3*	$F_{(1, 8)}$= 1.330, p=0.282	11.62		
CD8 EM CD127+	moderate	92.83	42.12	120.96	65.88	98.29	46.14	$F_{(2, 18)}$= 0.893, p=0.427	*T2*	$F_{(1, 9)}$= 0.197, p=0.668	30.30	167.63	82.21	190.21	98.76	144.76	50.20	$F_{(2, 10)}$= 3.125, p=0.088	*T2*	$F_{(1, 5)}$= 0.193, p=0.679	13.48	$F_{(2, 24)}$= 2.620 p=0.100	$F_{(2, 24)}$= 0.13, p=0.987
									T3	$F_{(1, 9)}$= 0.193, p=0.679	5.89									*T3*	$F_{(1, 5)}$= 3.796, p=0.109	13.64		
	vigorous	152.49	129.64	223.97	166.96	136.06	90.49	$F_{(1.2, 12.1)}$= 0.470, p=0.632	*T2*	$F_{(1, 10)}$= 0.094, p=0.766	46.87	120.65	114.79	195.35	168.84	97.52	97.81	$F_{(1.1, 9.1)}$= 2.603, p=0.105	*T2*	$F_{(1, 8)}$= 1.068, p=0.332	61.91	**$F_{(1.2, 19.9)}$= 1.274 p=0.035**	$F_{(1.2, 19.9)}$= 0.855, p=0.385.
									T3	$F_{(1, 10)}$= 1.319, p=0.277	10.78									*T3*	**$F_{(1, 8)}$= 6.288, p=0.037**	19.17		
CD8 EMRA CD127+	**moderate**	8.41	3.51	11.04	9.16	7.18	3.93	**$F_{(2, 18)}$= 4.143, p=0.033**	*T2*	$F_{(1, 10)}$= 0.328, p=0.581	31.26	20.57	23.13	25.43	23.96	22.08	27.10	**$F_{(2, 10)}$= 4.625, p=0.038**	*T2*	**$F_{(1, 5)}$= 7.008, p=0.046**	23.61	**$F_{(2, 24)}$= 4.271 p=0.026**	$F_{(2, 24)}$= 0.044, p=0.957.
									T3	$F_{(1, 10)}$= 4.719, p=0.058	14.59									*T3*	$F_{(1, 5)}$= 1.544, p=0.269	7.34		
	vigorous	13.15	12.71	17.90	16.17	11.41	9.04	$F_{(1.3, 12.5)}$= 1.793, p=0.192	*T2*	$F_{(1, 10)}$= 1.541, p=0.243	36.16	10.00	7.50	16.15	11.51	8.70	10.00	$F_{(1.2, 9.7)}$= 3.852, p=0.074	*T2*	$F_{(1, 8)}$= 2.333, p=0.165	61.49	**$F_{(1.24, 21)}$= 4.606 p=0.037**	$F_{(1.24, 21)}$=0.200, p=0.711
									T3	$F_{(1, 10)}$= 1.398, p=0.264	13.24									*T3*	$F_{(1, 8)}$= 5.058, p=0.055	12.98		

Subset (cells/µl)	Intensity	[1]Controls T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast	[a]Δ%	[2]T1D T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast	[a]Δ%	[d]Time (overall)	[d]Time*Group
CD8 Na CD95$^+$	moderate	2.24	3.55	3.12	5.37	1.28	2.48	$F_{(1.06, 9.58)}$= 0.089, p=0.787	*T2* $F_{(1, 9)}$= 2.619, p=0.140	39.13	10.12	20.35	15.40	22.21	23.95	32.94	$F_{(1, 4)}$= 0.885, p=0.400	*T2* $F_{(1, 4)}$= 6.447, p=0.064	52.20	$F_{(1.3, 13.9)}$= 3.126, p=0.087	$F_{(1.3, 13.9)}$=3.126, p=0.092
									T3 $F_{(1, 9)}$= 0.017, p=0.900	42.78								*T3* $F_{(1, 4)}$= 0.961, p=382	136.66		
	vigorous	1.06	1.20	11.50	36.36	0.71	1.45	$F_{(2, 22)}$= 0.218, p=0.806	*T2* $F_{(1, 11)}$= 0.460, p=0.512	983.77	11.97	28.45	1.69	2.88	14.88	38.89	$F_{(1.5, 7.3)}$= 0.642, p=0.454	*T2* $F_{(1, 7)}$= 0.201, p=0.667	85.84	$F_{(1, 16.5)}$= 0.009, p=0.991	$F_{(1, 16.5)}$=1.588, p=0.220
									T3 $F_{(1, 11)}$= 0.193, p=0.669	33.41								*T3* $F_{(1, 7)}$= 0.536, p=0.488	24.35		
CD8 CM CD95$^+$	**moderate**	3.84	5.45	5.44	8.01	4.22	7.42	$F_{(1.07, 10.71)}$= 0.210, p=0.673	*T2* $F_{(1, 10)}$= 3.810, p=0.079	41.58	7.87	7.78	15.12	23.28	9.99	9.12	$F_{(2,10)}$= 0.448, p=0.651	*T2* $F_{(1, 5)}$= 0.557, p=0.489	92.00	**$F_{(2, 26)}$= 4.316, p=0.024**	$F_{(2, 26)}$=1.800, p=0.185
									T3 $F_{(1, 10)}$= 0.080, p=0.783	9.81								*T3* $F_{(1, 5)}$= 0.025, p=0.880	26.84		
	vigorous	1.42	1.17	3.94	9.92	1.54	1.98	$F_{(2, 22)}$= 0.196, p=0.823	*T2* $F_{(1, 11)}$= 0.434, p=0.523	176.70	2.68	3.26	2.33	2.93	2.35	3.38	$F_{(1.14, 9.1)}$= 0.056, p=0.847	*T2* $F_{(1, 8)}$= 0.004, p=0.950	12.77	$F_{(1.5, 25.2)}$= 2.081, p=0.155	$F_{(1.5, 25.2)}$= 0.585 p=0.515
									T3 $F_{(1, 11)}$= 0.117, p=0.739	8.09								*T3* $F_{(1, 8)}$= 0.704, p=0.426	12.23		
CD8 EM CD95$^+$	moderate	26.53	47.35	38.74	66.38	28.97	51.70	$F_{(1.17, 11.65)}$= 0.332, p=0.609	*T2* $F_{(1, 10)}$= 2.445, p=0.149	46.03	73.02	97.65	179.36	288.58	126.12	86.95	$F_{(1.1, 5.5)}$= 0.458, p=0.544	*T2* $F_{(1, 5)}$= 0.521, p=0.503	145.65	$F_{(1.1, 13.7)}$= 0.063, p=0.818	$F_{(1.1, 13.7)}$= 0.036, p=0.863
									T3 $F_{(1, 10)}$= 0.016, p=0.903	9.21								*T3* $F_{(1, 5)}$= 0.192, p=0.679	72.72		
	vigorous	11.53	10.99	58.31	170.75	8.27	9.83	$F_{(2, 22)}$= 0.104, p=0.901	*T2* $F_{(1, 11)}$= 0.006, p=0.941	405.65	20.42	24.03	23.64	44.90	16.44	18.71	$F_{(1.1, 8.7)}$= 0.103, p=0.777	*T2* $F_{(1, 8)}$= 0.051, p=0.827	15.79	$F_{(1.4, 23)}$= 2.993, p=0.087	$F_{(1.4, 23)}$= 0.383, p=0.605
									T3 $F_{(1, 11)}$= 0.258, p=0.622	28.27								*T3* $F_{(1, 8)}$= 2.424, p=0.158	19.50		
CD8 EMRA CD95$^+$	**moderate**	2.30	3.58	4.56	7.06	0.96	1.64	$F_{(1.48, 12.93)}$= 2.026, p=0.158	*T2* $F_{(1, 10)}$= 3.880, p=0.077	98.49	16.03	25.78	67.45	126.20	42.96	42.21	$F_{(2, 10)}$= 0.489, p=0.627	*T2* $F_{(1, 5)}$= 0.443, p=0.535	320.67	**$F_{(2, 24)}$= 7.135, p=0.004**	$F_{(2, 24)}$= 0.762, p=0.478
									T3 $F_{(1, 10)}$= 0.622, p=0.448	58.46								*T3* $F_{(1, 5)}$= 0.007, p=0.938	167.92		
	vigorous	0.91	0.98	3.91	9.85	0.44	0.74	$F_{(1.35, 14.8)}$= 0.909, p=0.386	*T2* $F_{(1, 11)}$= 1.003, p=0.338	330.92	19.02	50.96	7.65	17.02	6.85	15.61	$F_{(1.4, 11.143)}$= 0.289, p=0.753	*T2* $F_{(1, 8)}$= 0.292, p=0.604	59.76	**$F_{(1.24, 21)}$= 10.025, p=0.003**	$F_{(1.24, 21)}$= 1.106, p=0.320
									T3 $F_{(1, 11)}$= 0.380, p=0.550	51.84								*T3* $F_{(1, 8)}$= 1.016, p=0.343	63.96		
CD8 EMRA CD28$^-$CD27$^-$	**moderate**	12.78	12.47	28.41	35.96	12.42	11.96	**$F_{(2, 18)}$= 10.310, p=0.001**	*T2* **$F_{(1, 9)}$= 5.977, p=0.037**	122.33								*T2*			**$F_{(2, 16)}$= 18.722, p<0.001**	
									T3 $F_{(1, 9)}$= 4.485, p=0.063	2.79								*T3*				
	vigorous	12.98	13.87	27.76	30.42	9.24	7.78	**$F_{(2, 20)}$= 3.912, p=0.037**	*T2* $F_{(1, 10)}$= 3.426, p=0.094	113.76	31.36	67.78	29.13	29.74	11.94	22.78	**$F_{(1.13, 6.8)}$= 5.464, p=0.050**	*T2* $F_{(1, 6)}$= 2.061, p=0.201	7.12	**$F_{(1.4, 20.5)}$= 8.955, p=0.004**	$F_{(1.4, 20.5)}$= 1.967, p=0.174
									T3 $F_{(1, 10)}$= 1.074, p=0.324	28.82								*T3* **$F_{(1, 6)}$= 19.107, p=0.005**	61.91		
CD8 EMRA CD28$^-$CD27$^+$	moderate	4.79	4.85	4.87	6.20	4.39	5.66	$F_{(2, 18)}$= 2.543, p=0.106	*T2* $F_{(1, 9)}$= 0.039, p=0.849	1.66								*T2*			$F_{(2, 16)}$= 0.132, p=0.877	
									T3 $F_{(1, 9)}$= 3.517, p=0.093	8.36								*T3*				
	vigorous	6.94	8.34	9.46	12.59	5.70	7.68	$F_{(1.3, 13.1)}$= 3.765, p=0.066	*T2* $F_{(1, 10)}$= 3.041, p=0.112	36.30	3.16	4.73	4.34	5.09	2.52	3.73	$F_{(2,12)}$= 1.174, p=0.342	*T2* $F_{(1, 6)}$= 0.829, p=0.398	37.12	$F_{(1.35, 18.9)}$= 2.559, p=0.119.	$F_{(1.35, 18.9)}$= 0.808, p=0.456
									T3 $F_{(1, 10)}$= 2.860, p=0.122	17.94								*T3* $F_{(1, 6)}$= 1.295, p=0.298	20.45		
CD8 EMRA CD28$^+$CD27$^-$	**moderate**	3.21	4.14	6.63	12.85	2.83	3.78	$F_{(2, 18)}$= 2.870, p=0.083	*T2* $F_{(1, 9)}$= 2.841, p=0.126	106.79								*T2*			$F_{(2, 24)}$= 1.583, p=0.226	
									T3 $F_{(1, 9)}$= 1.026, p=0.338	11.79								*T3*				
	vigorous	5.81	8.82	10.34	19.88	4.43	8.52	$F_{(2, 20)}$= 3.214, p=0.062	*T2* $F_{(1, 10)}$= 1.430, p=0.259	78.06	7.68	9.94	12.83	17.45	3.32	4.43	**$F_{(2,12)}$= 5.293, p=0.022**	*T2* $F_{(1, 6)}$= 1.951, p=0.212	67.07	$F_{(1.4, 23)}$= 0.409, p=0.590.	$F_{(1.4, 23)}$= 1.092, p=0.328
									T3 $F_{(1, 10)}$= 2.133, p=0.175	23.74								*T3* $F_{(1, 6)}$= 6.245, p=0.047	56.78		
CD8 EMRA CD28$^+$CD27$^+$	moderate	4.89	7.84	4.96	8.34	4.38	8.09	$F_{(2, 18)}$= 0.037, p=0.964	*T2* $F_{(1, 9)}$= 0.393, p=0.547	1.39								*T2*			$F_{(2, 24)}$= 0.890, p=0.424	
									T3 $F_{(1, 9)}$= 0.000, p=0.986	10.52								*T3*				
	vigorous	6.55	7.64	6.41	7.14	3.84	3.74	$F_{(1.1, 10.1)}$= 0.114, p=0.757	*T2* $F_{(1, 10)}$= 0.009, p=0.926	2.26	4.41	8.14	7.25	13.92	6.01	12.95	$F_{(2,12)}$= 1.185, p=0.339	*T2* $F_{(1, 6)}$= 1.989, p=0.208	64.38	$F_{(1.3, 22.7)}$= 1.132, p=0.314	$F_{(1.3, 22.7)}$= 0.083, p=0.831
									T3 $F_{(1, 10)}$= 0.097, p=0.761	41.33								*T3* $F_{(1, 6)}$= 0.339, p=0.582	36.47		

5.3.4 Stem cell like memory (T_{SCM}) T cell subsets

$CD4^+$ and $CD8^+$ T_{SCM} ($CD45RA^+CCR7^+CD95^+CD127^+$) populations were measured in T1D and control participants at baseline, and following moderate and vigorous intensity exercise. Table 38 displays the mean data for $CD4^+$ and $CD8^+$ T_{SCM} subsets during exercise in control and T1D groups.

5.3.4.1 $CD4^+$ T_{SCM} in T1D and control participants

$CD4^+$ T_{SCM} ($CD4^+CD45RA^+CCR7^+CD95^+CD127^+$) populations were measured in T1D and control participants at rest (Table 38). There was a trend to increase in the frequency of $CD4^+$ T_{SCM} populations in the T1D group (Figure 36a).

There were no significant changes overtime in $CD4^+$ T_{SCM} during moderate or vigorous intensity exercise in either group. Although not significant, increases in $CD4^+$ T_{SCM} frequency immediately post exercise are evident in the control group. The percentage increase following moderate exercise is 364.39% in the T1D group and 48.34% in the control group (Figure 36c). However, during vigorous intensity exercise a blunted response is noted in the T1D group. $CD4^+$ T_{SCM} decrease following vigorous exercise by 76.44% in the T1D group but increase by 331.06% in the control group (Figure 36d).

5.3.4.2 $CD8^+$ T_{SCM} in T1D and control participants

$CD8^+$ T_{SCM} ($CD8^+CD45RA^+CCR7^+CD95^+CD127^+$) populations were measured in T1D and control participants at rest (Table 38). There was a trend to increase in the frequency of $CD8^+$ T_{SCM} populations in the T1D group (Figure 36b).

$CD8^+$ T_{SCM} significantly changed overtime during moderate intensity exercise in control participants only. There were significant no change overtime in $CD8^+$ T_{SCM} during vigorous intensity exercise in either group. Although not significant, increases in $CD8^+$ T_{SCM} frequency

immediately post exercise are evident. The percentage increase following moderate exercise is 57.27% in the T1D group and 49.09% in the control group (Figure 36e). However, during vigorous intensity exercise a blunted response is noted in the T1D group. $CD8^+$ T_{SCM} decrease following vigorous exercise by 90.97% in the T1D group but increase dramatically by 1898.69% in the control group (Figure 36f).

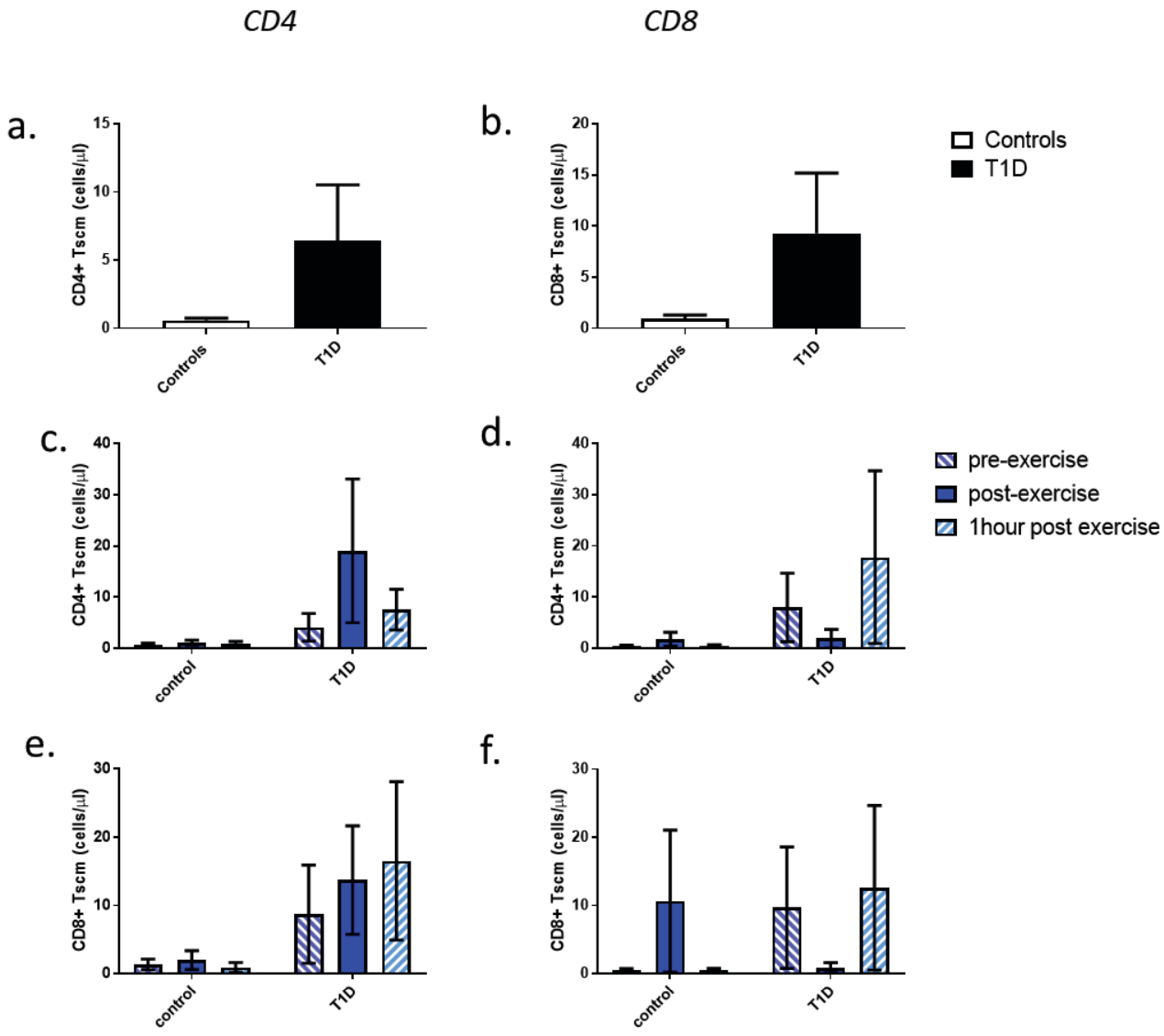

Figure 36 T cells with beta cell specific phenotype during low and vigorous intensity exercise in control and T1D subjects

Flow cytometry and whole blood counts were used to determine the T_{SCM} populations in control and T1D subjects. (a) The frequency of CD4+ and (b) CD8+ T_{SCM} (CD45RA+ CCR7+ CD95+ CD127+) at baseline (c) The frequency of CD4+ T_{SCM} during low and (d) vigorous intensity exercise (e) The frequency of CD8+ T_{SCM} during moderate and (f) vigorous intensity exercise. Error bars represent SEM. No statistical significance observed.

Subset (cells/µl)	Intensity	[1]Controls T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time		[c]contrast	[a]Δ%	[2]T1D T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time		[c]contrast	[a]Δ%	[d]Time (overall)	[d]Time*Group
CD4 T_{SCM}	moderate	0.77	0.83	1.14	1.53	0.91	1.31	$F_{(1, 9)}$= 0.993, p=0.345	*T2*	$F_{(1, 9)}$= 1.217, p=0.299	48.34	4.10	6.63	19.06	34.39	7.57	9.74	$F_{(1, 4)}$= 1.017, p=0.370	*T2*	$F_{(1, 4)}$= 0.992, p=0.376	364.39	$F_{(1.35, 17.54)}$= 0.029, p=0.924	$F_{(1.35, 17.54)}$= 2.172, p=0.134
									T3	$F_{(1, 9)}$= 0.997, p=0.344	17.54								*T3*	$F_{(1, 4)}$= 1.005, p=0.373	84.37		
	vigorous	0.41	0.69	1.78	4.38	0.42	0.76	$F_{(1, 10)}$= 0.991, p=0.343	*T2*	$F_{(1, 10)}$= 1.833, p=0.206	331.06	7.97	20.13	1.88	5.47	17.79	47.77	$F_{(1, 7)}$= 1.008, p=0.349	*T2*	$F_{(1, 7)}$= 0.816, p=0.396	76.44	$F_{(1.5, 25.5)}$= 2.061, p=0.157	$F_{(1.5, 25.5)}$= 0.647, p=0.489
									T3	$F_{(1, 10)}$= 1.007, p=0.339	0.42								*T3*	$F_{(1, 7)}$= 1.001, p=0.350	123.31		
CD8 T_{SCM}	**moderate**	1.35	2.50	2.01	4.39	0.93	2.10	**$F_{(2, 14)}$= 4.419, p=0.033**	*T2*	$F_{(1, 7)}$= 1.802, p=0.221	49.09	8.73	17.59	13.73	19.52	16.53	25.92	$F_{(1, 4)}$= 0.875, p=0.403	*T2*	$F_{(1, 4)}$= 0.797, p=0.423	57.27	$F_{(1.27, 12.7)}$= 2.853, p=0.110	$F_{(1.27, 12.7)}$= 2.740, p=0.117
									T3	$F_{(1, 7)}$= 2.344, p=0.170	31.21								*T3*	$F_{(1, 4)}$= 4.118, p=0.112	89.43		
	vigorous	0.53	0.69	10.65	34.51	0.47	1.01	$F_{(1.1, 9.6)}$= 1.036, p=0.340	*T2*	$F_{(1, 9)}$= 1.084, p=0.325	1898.68	9.71	26.74	0.88	2.19	12.62	36.22	$F_{(1.02, 7.12)}$= 0.701, p=0.432	*T2*	$F_{(1, 7)}$= 0.730, p=0.421	90.97	$F_{(1.04, 16.6)}$= 0.052, p=0.832	$F_{(1.04, 16.6)}$= 1.140, p=0.304
									T3	$F_{(1, 9)}$= 0.008, p=0.931	12.62								*T3*	$F_{(1, 7)}$= 0.081, p=0.784	30.02		

Table 38 T_{SCM} populations mobilised during moderate and vigorous intensity exercise in control and T1D groups

Mean, standard deviation, and statistical analysis of T_{SCM} populations for control and T1D subjects at baseline, during moderate and vigorous intensity exercise. Significant results highlighted in bold where p values <0.05 were considered significant.

[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2) or 1 hour post exercise (T3).

[b] Results were analysed using multiple regression analysis in control and T1D groups independently.

[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).

[d] Results were analysed using multiple regression analysis in control and T1D groups combined.

[1]Controls n=10-11, [2]T1D n =6-9

5.4 Discussion

The first aim of this study was to measure progressively differentiated T cell subsets at baseline in T1D and control participants. A shift in EMRA proportions at baseline was found in T1D compared to the control group; $CD4^+$ EMRA were reduced whereas $CD8^+$ EMRA were elevated in the T1D group. The elevated $CD8^+$ EMRA population in T1D comprised of subsets with tissue residency and homing propensity i.e. $CD69^+$ and $CD11b^+$.

This study also aimed to investigate the effects of moderate and vigorous intensity exercise on the mobilisation of T cell subsets in T1D and control participants. As expected, vigorous intensity exercise induced a significant mobilisation of $CD3^+$ T cells in both T1D and control participants, with no significant mobilisation during moderate intensity exercise. $CD4^+$ T cells mobilised during vigorous exercise in the control group, but not the T1D group. This had a downstream effect on $CD4^+$ T cell subsets in T1D participants because no significant mobilisation of differentiated $CD4^+$ T cell subsets was seen during either exercise intensity in the T1D group. The changes seen during exercise within the $CD4^+$ compartment were minimal compared to changes seen within the $CD8^+$ compartment. Vigorous intensity exercise significantly mobilised $CD8^+$ T cells in both T1D and control participants. $CD8^+$ T cells increased to the same extent after vigorous intensity exercise in both the T1D and control group. Within $CD8^+$ T cells, $CD8^+$ EMRA cells were the most responsive to exercise. $CD8^+$ EMRA mobilised during both moderate and vigorous intensity exercise in the T1D and control groups. However, the percentage increase of $CD8^+$ EMRA in the T1D group following vigorous intensity exercise was blunted.

Further phenotyping of $CD8^+$ EMRA populations revealed that the mobilised subsets were mainly comprised of fully differentiated ($CD27^-CD28^-$), recently activated tissue-resident ($CD69^+$) EMRA with migratory capacity ($CD11b^+$). However, the percentage increase of the

above EMRA subsets following vigorous exercise was blunted in the T1D group. Additionally, $CD8^+CD127^+$ EMRA and $CD8^+CD95^+$ EMRA significantly changed overall during vigorous intensity exercise, but this was not seen in either T1D or control groups independently.

This study also identified that the rise in peripheral blood lymphocytes during exercise is a result of recently activated $CD8^+$ T cell egress from tissues as shown by CD69 expression. Naïve, CM, and EMRA $CD8^+$ T cell subsets that increased immediately following vigorous exercise were $CD69^+$.

The strengths of this study are that it is the first study of steady state acute exercise and T cell subset mobilisation in T1D. This study gives an in-depth analysis of T cell subsets mobilised by two different exercise intensities, in two cohorts. The use of surface markers to define T cell function and fate improves understanding of the specific subsets mobilised. Increases in both CD69 and CD11b on $CD4^+$ and $CD8^+$ lymphocytes have been reported following exercise previously, but not on specific T cell subsets as demonstrated in this study herein [195, 351-353, 424-429]. The effects of exercise have not been looked at previously on $CD127^+$ T cells and T_{SCM} subsets. This is the first time this has been shown in T1D and healthy cohorts. Furthermore, the differentiation status of EMRA based on differential expression of CD27 and CD28 is reported here in T1D participants at rest and following exercise.

However, the limitations of the study are that the number of recruited participants were low therefore comparisons between groups are difficult. Secondly, the majority of the experimental group were long-standing T1D. However, baseline differences highlighted in this study support findings from recently diagnosed and long standing T1D [339, 370, 371]. Long-standing T1D also show persistence of islet autoimmunity and $CD8^+$ T cell infiltration [28, 129, 372]. Nonetheless, a large proportion of the autoimmune destruction and immune alterations occur before diagnosis and in the stages proceeding diagnoses. Therefore, a cohort including those

who have been recently diagnosed may provide more insight into early disease prevention. Additionally, with a larger cohort, data could be adjusted for T1D duration.

Previously, studies have demonstrated that vigorous exercise causes a significant rise in peripheral blood lymphocytes, followed by lymphopenia, in healthy cohorts [191-195]. This is also the case in this study and the first time this has been shown in T1D. The data shown agrees with previous studies because only minor exercise induced mobilisation of $CD4^+$ T cells has been reported [199, 257]. The majority of redeployed lymphocytes are late differentiated $CD8^+$ T cell subsets, with a decrease in naïve and CM populations [191, 200, 215, 257, 364, 365]. $CD8^+$ EMRA are the most sensitive to mobilisation, and are shown to mobilise by both moderate and vigorous exercise in this study herein and others [199, 200, 257]. However, it has not been previously reported that $CD8^+$ EMRA subsets that express adhesion and activation markers are mobilised by exercise [199, 200, 215]. $CD95^+$ subsets were mobilised by exercise, yet not to the same extent as seen in previous studies [216, 218, 220]. This is possibly due to the limitations of a small study cohort.

This is the first study of T cell subset mobilisation in T1D and the blunted T cell egress during vigorous intensity exercise has not been demonstrated before. It is possible that the blunting is due to sequestration of T cell subsets in the pancreas of people with T1D. T cell subsets exhibiting a blunted response share similarities with those seen in the pancreas of T1D models (NOD mice) [126, 127, 134, 435]. The proportion of $CD8^+CD69^+$ T cells was higher in the pancreas than salivary glands taken from NOD mice [126]. $CD8^+CD11b^+$ T cells have also been found within NOD islets [127, 134] and found to be higher in the islets of NOD mice compared to peripheral blood [435]. Furthermore, antigen-experienced islet reactive $CD8^+$ T cells were found sequestered in the pancreas of T1D donors [131]. Islet reactive $CD8^+$ T cells have been shown to exhibit a highly differentiated memory phenotype [339, 422]. This is

analogous to the $CD8^+$ EMRA T cell phenotype. More recently, autoreactive $CD8^+$ T cells were shown to display a T_{SCM} phenotype [421]. In this study herein, there is an evident blunted percentage increase of $CD8^+$ EMRA that express CD69, CD11b, and were highly differentiated, immediately following vigorous exercise in the T1D group. Both $CD4^+$ and $CD8^+$ T_{SCM} also displayed a blunted egress following vigorous exercise compared to the control group. It therefore is possible to postulate that the blunted increase following exercise in these $CD8^+$ T cell subsets is due to their sequestration in the pancreas in T1D.

However, the mechanisms for this blunted T cell egress in T1D warrants further investigation. The down regulation of S1PR, necessary for T cell exit from tissue into the blood stream, is one potential mechanism. Islet reactive T cells accumulate in the pancreas during T1D onset [436] and are found to be enriched within the pancreas of T1D donors [131]. Yet, the mechanisms by which islet reactive T cells are retained in the pancreas are unknown. It is however known that tissue resident memory T cells down-regulate S1PR [437]. Pancreatic resident T cells exhibit a tissue resident memory phenotype; therefore the lack of S1PR expression may inhibit their egress from the pancreas during acute exercise.

This study provides insight into the implications of acute exercise in T1D. Access to the pancreas is limited and therefore human research on pancreatic T cell infiltration is challenging. However, peripheral blood is to some extent a representative source of diabetogenic T cells [438, 439]. Identical beta cell specific $CD8^+$ T cell subsets were found in the pancreas and peripheral blood in NOD mice [438, 439]. Exercise induced lymphocytosis in T1D participants may improve accessibility to diabetogenic T cells in peripheral blood.

On the contrary to the observed blunted increase, lymphocytosis followed by lymphopenia is still evident in T1D. However, a major limitation of this study is that the cause of lymphopenia was not explored. It is imperative that the mobilised T cells do not migrate solely to the

pancreas to cause further beta cell destruction. As discussed in Chapter 4, measuring diabetogenic T cell migration specifically would offer insight into their redeployment patterns during vigorous exercise. If the migrated lymphocytes had been collected at the end of the TEM assay and characterised by flow cytometry, quantification of diabetogenic T cells during and following exercise would clarify whether migration of diabetogenic T cells is impaired by vigorous exercise, thereby preserving beta cell health. In addition to including this in future studies, tracking of T cells in NOD mice during and following vigorous exercise could be done using bioluminescent tagging of the T cells, live imaging of T cell location before and after acute exercise, as well as harvesting organs to measure T cell infiltration into multiple organs including the pancreas. Although the impact of exercise-induced lymphopenia in T1D is not defined, the potential benefits of lymphopenia following exercise is discuss below.

The mobilisation of terminally differentiated memory subsets by acute exercise has been hypothesised to create immune space in healthy participants [220, 221]. Lymphocytes isolated from healthy participants following vigorous exercise were more susceptible to apoptosis induction and expressed higher levels of CD95 [216, 218, 219]. Total $CD8^+$ T cells positive for annexin V, a marker for apoptosis, were increased in the gut 24 hours after the cessation of treadmill running in mice [217]. There is a strong link between the gut and the pancreas, leading to the premise that this organ specific deletion of memory T cells could occur in T1D [440, 441]. Given that a comparable response to acute exercise is seen in T1D participants, immune space could also be created in T1D. Vigorous acute exercise increases the frequency of hematopoietic stem and progenitor cells (HSPC) in the peripheral blood and haematopoiesis in the bone marrow [212, 213]. This would allow for more naïve and newly differentiated subsets with a less aggressive autoimmune phenotype to take up the vacant immune space thereby reducing beta cell destruction by T cells in T1D.

Current immunotherapies target pathogenic T cells to reduce autoreactive subsets in T1D [442]. One such immunotherapy, teplizumab, a nonactivating anti-CD3 monoclonal antibody, aims to reduce effector memory subsets, and increase naïve and early memory subsets; comparable to an acute exercise bout. Anti-CD3 mAbs, that are non-Fc receptor (FcR) binding, selectively induce apoptosis of antigen-activated T cell phenotypes such as those with memory/pathogenic phenotypes but not naïve T cells [153]. Teplizumab treatment resulted in maintained or improved beta cell function for at least 2 years post treatment in recent-onset T1D patients [149-152]. Evidence for beta cell preservation was also reported in long standing T1D patients up to 1 year post clinical diagnosis [154]. One study identified a group of responders who had higher activated $CD8^+$ terminally differentiated effector and $CD8^+$ EM in T cells at baseline [155]. This is consistent with increased $CD8^+$ terminally differentiated EM T cells following acute exercise, therefore exercise could improve access to target T cell populations. Pairing an acute exercise bout with treatment administration could potentially increase response to treatment. Responders also had different metabolic features such as lower levels of HbA1c and insulin use, features observed with frequent exercise in T2D [182, 443, 444]. Therefore, frequent bouts of acute exercise could create an environment in which T1D participants respond better to treatment.

Preliminary data from this study herein provides a basis for a future chronic exercise trial, leading to the hypothesis that current findings in healthy participants can be implemented in T1D, as reported here with acute exercise. Chronic exercise reduces senescent T cells in the blood [445]. In an ageing study, physically fit age matched controls had a lower proportion of peripheral blood memory T cells ($KLRG1^+CD57^+$, $KLRG1^+CD28^-$) compared to those with a lower VO_2 max [221, 446]. Naïve-memory T cell balance is disrupted in the ageing population and a similar observation has been found in T1D [419]. Therefore, reduced senescent memory T cell populations may also occur from exercise training, by increasing VO_2 max, in T1D.

The mechanisms by which exercise training could preserve beta cells in T1D have yet to be explored. Exercise training modulates immunity in other T cell mediated autoimmune disorders [447]. Mice with EAE, a routinely used model for multiple sclerosis (MS), completed 6 weeks exercise training. T cells taken from lymph nodes had an inhibited immune response to autoantigen whilst sustaining an increased immune response to non-specific stimulus such as concanavalin A [447]. This suggests the generation of new naïve and early memory T cells with exercise training as the recall response to autoantigen is reduced. Another study showed improved immune modulation by exercise in EAE models resulting in delayed onset of disease and increased T cells with a regulatory phenotype [448]. However, other autoimmune disorders such as rheumatoid arthritis (RA) and systemic lupus (SLE) have different responses to exercise, where $CD8^+$ T cells are reduced following exercise and $CD4^+$ T cells are reduced at peak exercise but increased after cessation [238]. It is unclear if exercise training would result in the same improvements in these disorders such as those seen in MS and T1D, where responses to exercise are comparable to healthy controls i.e. lymphocytosis occurs in a similar pattern.

In conclusion, acute exercise preferentially mobilised differentiated and antigen experienced $CD8^+$ T cells in T1D, but to a lesser extent than healthy individuals. A contributing factor to the relatively reduced mobilisation pattern in T1D was attributable to a blunted response amongst highly differentiated $CD8^+$ T cells, which may indicate sequestering of $CD8^+$ T cells in the pancreas. Further evaluation of the immunomodulatory effects of acute exercise on autoreactive memory T cells in T1D is warranted to ascertain impact on disease prognosis. These findings need to be validated in a chronic exercise study.

CHAPTER 6.THE EFFECTS OF ACUTE EXERCISE ON PERIPHERAL BLOOD T-HELPER AND T-REGULATORY CELL SUBSETS IN TYPE 1 DIABETES

6.1 Introduction

6.1.1 $CD4^+$ T cell differentiation

The effects of exercise on $CD4^+$ and $CD8^+$ T cells at different stages of their respective differentiation have been described in Chapter 5. In addition to the analytical approach adopted in Chapter 5, $CD4^+$ T cells can also be subdivided into T-helper (Th) and T-Regulatory (TReg) subsets [291, 449]. There is strong evidence to suggest that the balance between T-helper and TRegs is disrupted by T1D. $CD4^+$ Th differentiation is skewed towards increased pro-inflammatory Th1 and Th17 cells in T1D [450, 451]. Whereas $CD4^+$ TReg function, and potentially TReg frequency, are compromised in T1D [452]. It is proposed that regular exercise may facilitate a shift in this balance back towards homeostasis [453-457]. Accordingly, this study explored the effects of acute exercise on T-helper and TReg populations in T1D.

6.1.1.1 $CD4^+$ T-helper cells

The T-helper arm of $CD4^+$ T cells are defined by $CD45RO^+CD127^{hi}CD25^{low}$ cell surface expression. Subsets are further distinguished by chemokine receptor expression and cytokine secretion patterns [291, 458-460].

Type 1 helper (Th1) cells are identified by CXCR3 expression. They are critical for cell-mediated immunity and secrete large amounts of IFN-γ [291, 461, 462]. Type 2 helper (Th2) cells are $CXCR3^-CCR4^+$ and can also co-express the chemokines CCR3, CCR6, CCR8, and CCR10. They support activation of other leukocytes through production of cytokines IL-4, IL-5, and IL-13 to do so [291, 461]. Th1 and Th2 were initially thought to work in the classical biphasic model. Additional subsets Th9, Th17, and Th22 cells have also been discovered, and are characterised by IL-9, IL-17 and IL-22 secretion respectively [463]. Type 17 helper (Th17) cells are $CXCR3^-CCR4^-CCR6^+$ and are known for their potent IL-17 secretion [291, 464-466]. CCR6 regulates migration of Th17 cells to inflammatory tissues. CCL20, the only ligand for

CCR6, is expressed on Th17 cells. This specifically mediates Th17 cell recruitment via CCR6, but not Th1 or Th2 cells [467].

6.1.1.2 $CD4^+$ T-Regulatory cells

$CD4^+$ T- Regulatory cells (TRegs) play a critical role in the maintenance of self-tolerance and protection against autoimmunity [468-472]. $CD4^+$ TRegs are generated in the thymus (tTReg) or in the periphery (pTReg) in the presence of TGF-β [473, 474]. TRegs make up 2-4% of total $CD4^+$ T cells [475]. They are conventionally defined by surface marker expression of $CD4^+CD127^{low}CD25^+$ and their transcription factor expression of forkhead winged helix transcription factor (FoxP3) [378, 476, 477]. Additionally, HELIOS is a transcription factor which can distinguish tTReg ($HELIOS^+$) from pTReg ($HELIOS^-$) [478, 479]. Ubiquitous expression of FoxP3 is required for $CD4^+$ TReg maintenance and function [477]. IL-2 secreted from effector T cells is critical for $CD4^+$ TReg maintenance as it regulates FoxP3 expression [480-482]. $CD4^+$ TRegs are immunomodulatory and conduct their suppressive function via cell-cell (e.g. CTLA-4, granzyme B) and soluble factor (e.g. TGF-β, IL-10, IL-35) mediated mechanisms [483, 484].

$CD4^+$ TRegs are routinely identified by flow cytometry using their surface marker expression alone ($CD4^+CD127^{low}CD25^+$) [378]. Co- expression of T cell immunoreceptor with Ig and ITIM domains (TIGIT) and Fgl2 (FCRL3) on $CD4^+$ TReg cell surface can identify $HELIOS^+$ TRegs (i.e. tTReg) [485]. TIGIT is a coinhibitory molecule expressed by activated $CD4^+$ TRegs [486]. TIGIT was shown to supress Th1 and Th17 cell differentiation and responses, but promoted Th2 cell responses [486] . Upon TIGIT engagement, IL-10 and Fibrinogen-like protein 2 (Fgl2) are secreted which may supress IL-12 secretion by Th1 cells [486]. $CD4^+$ TRegs also express CCR6 which is responsible for their recruitment into inflammatory sites [467].

$CD4^+$ TReg subsets consist of naïve (naTRegs) and memory TRegs (mTRegs). As described for progressive differentiation of T cells in Chapter 5, naTRegs express CD45RA whereas mTRegs lose CD45RA and acquire CD45RO upon antigen exposure [378, 487]. mTRegs are an activated antigen primed subset of $CD4^+$ TRegs and make up the larger proportion of $CD4^+$ TRegs [488, 489]. CTLA-4, a coinhibitory molecule, is preferentially expressed by mTRegs to exert their suppressive functions [490]. Nonetheless, naTRegs and mTRegs have equal suppressive capacity, but carry out their regulatory functions at different sites. mTRegs are predominantly tissue resident whereas naTRegs home to the bone marrow [487]. Turnover of existing TRegs maintains the $CD4^+$ TReg pool as indicated by the highly proliferative capacity of mTRegs [487]. mTRegs show similar gene expression patterns to effector T cells and are highly differentiated. This is characterized by short telomeres, inability to upregulate telomerase, and susceptibility to apoptosis [487, 491].

A phenotypically distinct $CD4^+$ TReg subset with increased suppressive, tissue-infiltrating capacity was identified recently [492]. They are $TIGIT^-IL\text{-}6R^{hi}$ mTReg cells that lack HELIOS expression (i.e. formed in the periphery). They also exhibit a Th17 signature ($ROR\gamma t^+CD161^+CCR6^+$). Increased expression of CTLA-4 and CCR4 indicate that they are highly suppressive, tissue-infiltrating, effector TRegs. $TIGIT^-IL\text{-}6R^{hi}$ mTReg cells are also highly sensitive to IL-2, increasing dramatically with a single dose [492].

6.1.2 $CD4^+$ T-helper and T-Regulatory cells in T1D

Th1/Th2 imbalance has been reported in autoimmunity [493] and in T1D [494]. This is evidenced by diabetogenic T cells isolated from NOD mice shown to differentiate into Th1, not Th2, subsets [450, 494]. In support of this, a Th1 signature has been described in T1D, indicated by elevated soluble IFN-γ [495, 496]. Moreover, disease progression can be prevented by anti-IFN-γ treatment in NOD mice [497]. Instead, efforts to promote Th2

polarization demonstrate beneficial effects in T1D models. IL-4 is a Th2 polarising cytokine. When IL-4 was administered to NOD mice, diabetes was inhibited [498]. Furthermore, a Th2 response (as seen with helminth infection) protected NOD mice from diabetes [499].

Th17 cells are another subset of $CD4^+$ T-helper cells involved in the pathogenesis of T1D [78, 451, 500-503]. Studies have shown up-regulated Th17 immunity in T1D [78, 503]. Circulating IL-17^+ islet reactive $CD4^+$ T cells have been found at diagnosis of T1D, and are a potential biomarker of the disease [502]. Th17 cells are potent secretors of IFN-γ [504, 505]. Increased IFN-γ mRNA [506] and ratio of IFN-γ/IL-17 expression [451] in Th17 cells has also been shown in T1D. IFN-γ secreting, islet reactive Th17 cells were shown to drive T1D in NOD mice [501]. Furthermore, Th17 cells were highly plastic in T1D and had the potential to acquire an IFN-γ secreting Th1 like profile [507]. In addition to Th17 cells, it has been shown that islet-reactive T cells share characteristics with follicular helper T cells, such as expressing high levels of CXCR5 and IL-21 [508].

Peripheral blood frequencies of $CD4^+$ TRegs in T1D have been examined, yielding mixed results. Some studies have reported lower peripheral blood $CD4^+$ TRegs frequencies in young patients with T1D [509]. Others have reported higher activated $CD4^+$ TReg frequencies in T1D [77]. Other studies have shown no difference in peripheral blood frequencies of $CD4^+$ TReg between T1D and control groups [75, 76, 510].

There has however been consistency in studies of $CD4^+$ TReg function in T1D. Here, defects in $CD4^+$ TReg suppressive capacity/function have been described in T1D [75-78]. Co-culture of TRegs from people with T1D with effector T cells failed to suppress the proliferation capacity of the effector T cells in a number of studies [75, 77, 79]. Furthermore, IFN-γ production was increased from these co-cultures, whiles IL-10 was decreased [75]. These defects may have a number of underlying causes. Firstly, the T1D-associated variant of IL-

2RA correlates with reduced IL-2R (CD25) expression and therefore decreased TReg IL-2 sensitivity [452, 480, 511]. Secondly, HELIOS deficiency results in an unstable $CD4^+$ TReg phenotype and is associated with developing autoimmunity. HELIOS may be important for IL-2 signalling, therefore FoxP3 maintenance and $CD4^+$ TReg function [512]. Due to their plasticity, $CD4^+$ TRegs lose FoxP3 in chronic inflammatory conditions [513]. This may also occur in T1D. Lastly, CTLA-4 polymorphisms are associated with T1D due to decreased $CD4^+$ TReg suppressive capacity [80, 81]. Treatment with anti CTLA-4 (abatacept) suppresses effector T cell activity thereby delaying progression of T1D [514].

A population of IL-17 secreting regulatory T-helper type 17 (TReg17) cells have been previously described to have a preventative role in T1D. Adoptive transfer of TReg17 cells in NOD mice delayed disease onset [515-517]. This population shares similarities to the $TIGIT^-$ $IL\text{-}6R^{hi}$ TReg suppressive subset identified by Ferreira, R.C., et al. as they also are IL-17 secreting TReg cells [492].

6.1.3 $CD4^+$ T cells during acute exercise

Exercise evokes a shift from Th1 to Th2 polarisation [453, 454, 518-520] . Gene expression of PBMCs isolated following a marathon displayed upregulation of Th2 related genes, and down regulation of Th1 related genes, persisting for up to 1 week [518]. Other studies have shown that vigorous exercise reduces Th1 cells, with no change in Th2 cells, and thus the Th1/Th2 ratio declined [453, 454]. A shift in the Th1/Th2 ratio following exercise is also supported through studies measuring the IFN-γ/IL-4 ratio [519, 520].

There is a clear lack of evidence of the effects of acute exercise on Th17 cells. One study demonstrated an increase in the percentage of Th17 cells following endurance exercise (marathon and half-ironman triathlon) [521]. However, IL-17 following acute exercise has been measured and this may indicate changes in Th17 immunity following exercise [522, 523].

One study reported an increase in IL-17 following strenuous exercise in trained rats [522]. Another study showed no changes in IL-17 following an acute exercise bout in a group of male athletes [523].

Acute exercise increases the frequency and proportion of peripheral blood $CD4^+$ TRegs in healthy participants [455-457]. Increased absolute $CD4^+$ TReg frequencies following an intense acute exercise bout in trained and untrained participants has been reported [455, 456]. An increase in the percentage of peripheral $CD4^+$ TReg following moderate exercise has also been demonstrated [457]. Within $CD4^+$ TReg subsets, increases in naïve TReg frequencies, and not memory TReg frequencies, following low intensity endurance exercise was shown in one study [524]. Further characterisation of TReg mobilisation following acute exercise is needed.

No studies have measured the effects of acute exercise on $CD4^+$ T-helper and TReg populations in T1D. As seen previously (Chapter 5), fully differentiated effector memory T cells are preferentially redeployed during exercise. Therefore, it can be speculated that mTReg subsets will be responsive to exercise as they also exhibit a highly differentiated phenotype [491]. Furthermore, no studies have measured the effect of acute exercise on IL-$6R^{hi}$ TReg subsets in either T1D or control participants. Given that IL-6 is increased dramatically during exercise it could be postulated that suppressive IL-$6R^{hi}$ subsets will mobilise during exercise also [367].

$CD4^+$ TReg insufficiency drives T1D therefore expansion of TRegs with suppressive function via exercise mobilisation could overcome this deficiency. Cell based therapies increasing $CD4^+$ TRegs in NOD mice and children have been demonstrated to delay the onset of T1D [525, 526]. Indeed, increased C-peptide (measure of beta-cell function) and reduced insulin use 1-2 years post-trial was reported [526-528]. Accordingly, this study herein explored the effects of exercise induced $CD4^+$ T-helper and TReg subset mobilisation in healthy and T1D cohorts.

6.1.4 Hypothesis

Acute exercise will shift the $CD4^+$ T-helper balance towards Th2 immunity and will mobilise memory TReg subsets in T1D participants.

6.1.5 Aims

To characterise peripheral blood T-helper and T-Regulatory cell subsets using multi-parameter flow cytometry;

1. In T1D and control participants at rest.
2. In T1D and control participants following an acute bout of moderate intensity exercise.
3. In T1D and control participants following an acute bout of vigorous intensity exercise.

6.2 Methods

6.2.1 Experimental design

Twelve controls and twelve T1D male participants were recruited into the study (as described in Chapter 3). Briefly, participants had one enrolment visit, where an incremental sub-maximal cycle ergometer test was performed to calculate their predicted VO_2 max. This was used to calculate workload and heart rate for subsequent exercise visits adjusted to individual fitness [285]. These two visits consisted of a thirty-minute bout of cycling at either 40% VO_2 max (moderate) or 80% VO_2 max (vigorous). Blood was collected intravenously at baseline, immediately post exercise, and 1 hour post exercise in vacuette heparin tubes (95057-405, Greiner Bio-one GmbH, Frickenhausen, Germany).

6.2.2 T-helper and T-Regulatory cell subset analysis

A twelve-colour flow cytometry panel was designed to phenotype T-Regulatory and T-helper cell subsets using the following mAbs (see Table 3, Section 2.3.2 for mAb details); anti-CD3 PE-Cy7, anti-CD4 APC-R700, anti-CD25 PE, anti-CD45RO BV786, anti-CD127 AF647, anti-CXCR3 PE-CF594, anti-CCR4 BV421, anti-CCR6 BV711, anti-TIGIT PerCP-Cy5.5, anti-IL-6R FITC, and Live/Dead-Fixable Viability Stain 780. Whole blood was stained as per Chapter 2 (Section 2.3.2). Whole blood counts were done using a coulter haematology analyser (Section 2.2.2) to calculate relative cell frequency (cells/µl) of T-helper and T-regulatory cell subsets.

6.2.3 Data analysis

Flowjo v.10 (FlowJo LLC, Oregon) was used to analyse flow cytometry data. Doublets were removed using FSC-A versus FSH-H. Lymphocytes were gated based on size on SSC-A versus FSC-A dot plot (Figure 37.1), singlets were detected as the linear population on FSC-H versus FSC-A dot plot (Figure 37.2), and dead cells positive for 7-AAD viability stain were removed (Figure 37.3). T cells were selected as $CD3^+$ (Figure 37.4), and further gated on $CD4^+$

populations (Figure 37.5). Spider gates were used within these populations to define non-overlapping subsets; $CD4^+$ T-Regulatory (T-Reg:$CD127^{lo}$ $CD25^{hi}$), naïve T-Reg ($CD45RO^-$ $CD127^{lo}CD25^{hi}$), memory T-Reg ($CD45RO^-CD127^{lo}$ $CD25^{hi}$), non-TReg ($CD127^+CD25^-$), T-helper (Th: $CD127^+CD25^-CD45RO^+$), Th1 ($CXCR3^+CCR6^+CCR4^-$), Th2 ($CXCR3^-CCR6^-$ $CCR4^+$), and Th17 ($CXCR3^-CCR6^+CCR4^+$). TIGIT and IL-6R were analysed on TReg and non-TReg populations. Figure 37 displays gating strategy for $CD3^+CD4^+$ T cells. See Figure 38 and 39 for T-helper and TReg gating strategy following definition of $CD4^+$ T cells. Figure 40 displays gating strategy for $IL\text{-}6R^{hi}$ subsets as defined by Ricardo et al (2017) [492].

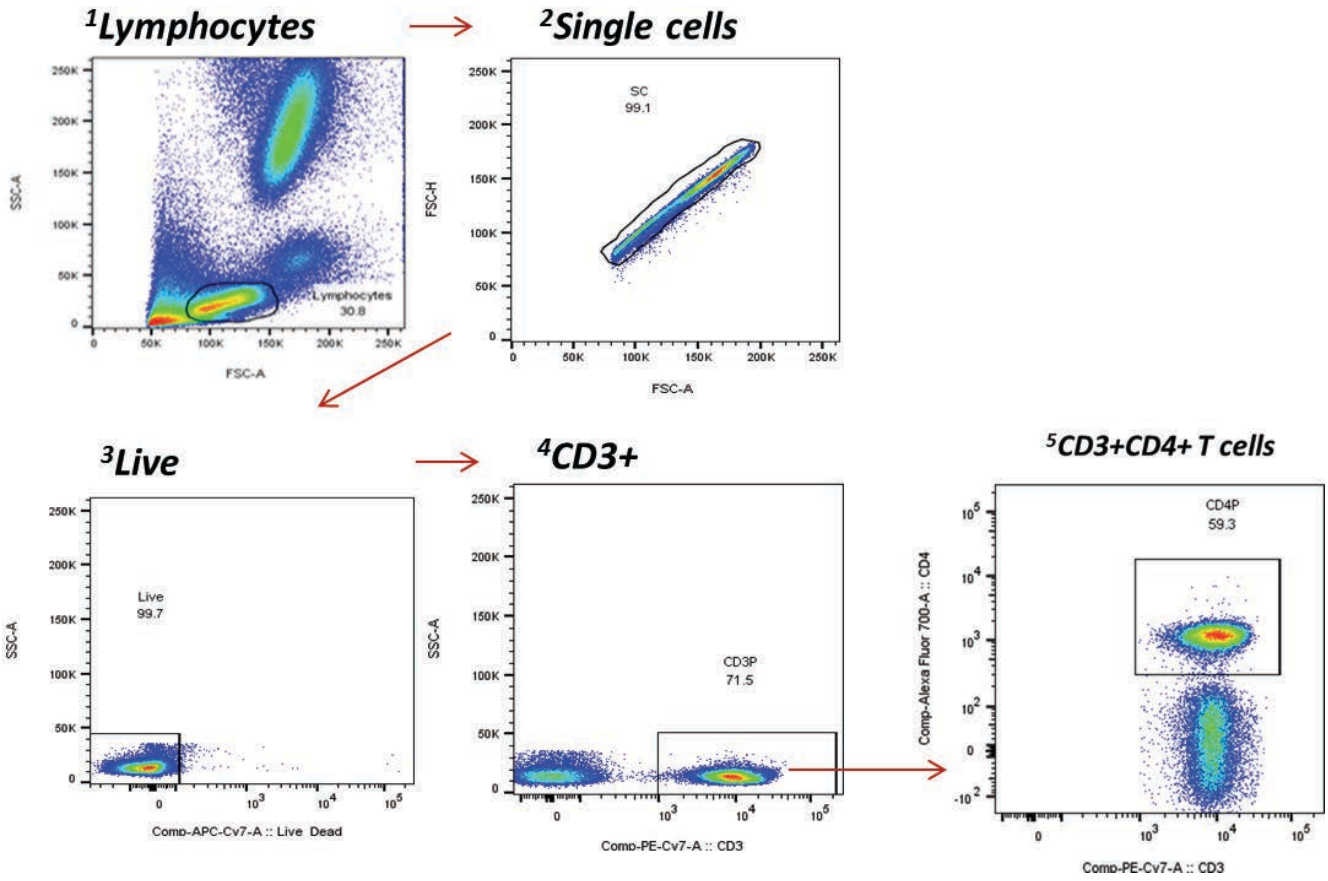

Figure 37 Selection of CD3+ lymphocytes shown by representative flow plots

Representative flow cytometry plots showing *(1)* Lymphocyte selection based on size (SSC-A vs FSC-A) *(2)* Doublets exclusion (FSC-A vs FSC-H) *(3)* Dead cell removal based on positivity for viability stain (7-AAD PerCP-Cy5.5) *(4)* CD3+ lymphocytes gate (*5*) CD3+ CD4+ T cell gate.

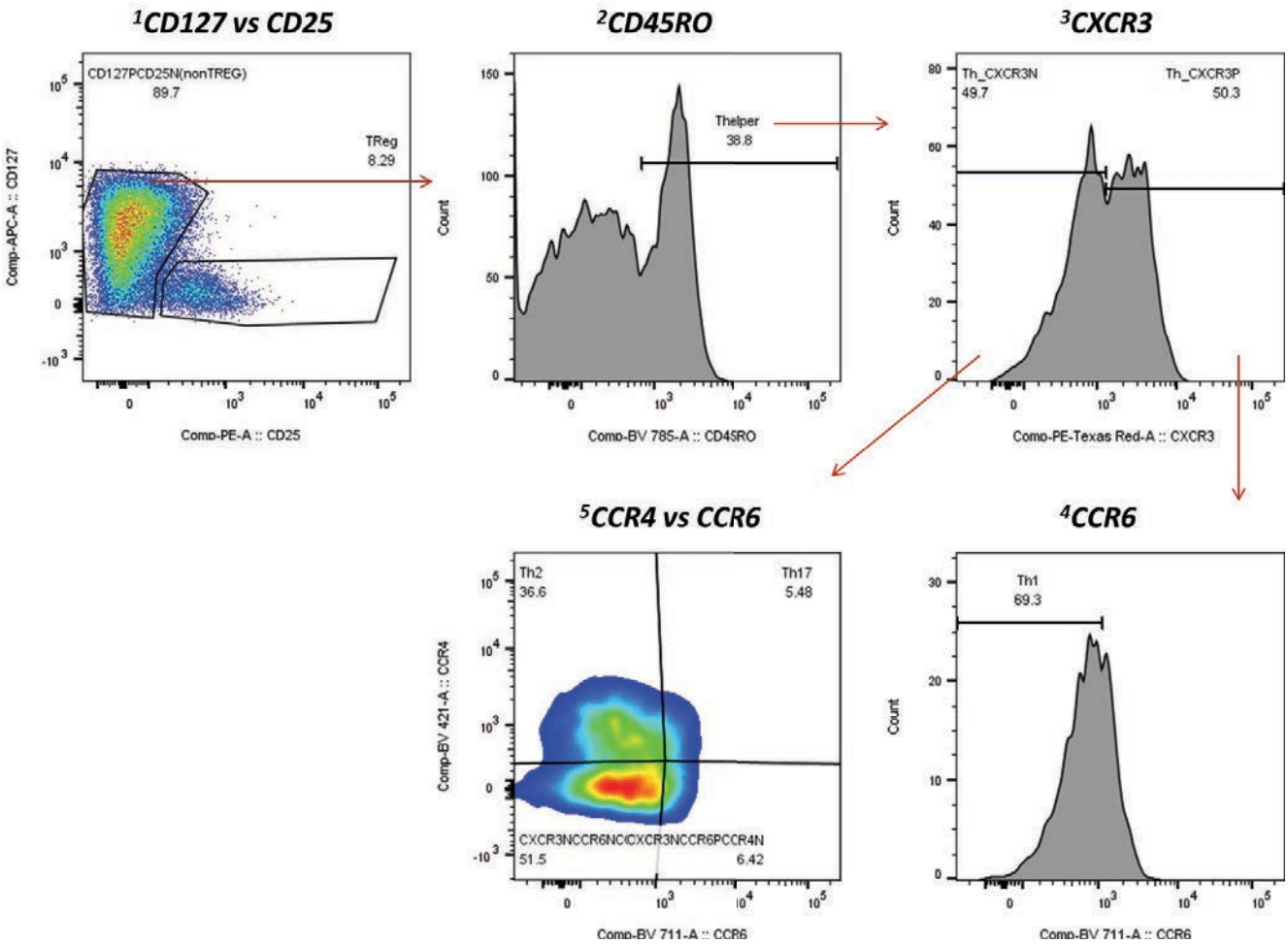

Figure 38 CD4 T-helper cell gating strategy shown by representative flow plots.

Representative flow cytometry plots showing following parent gate for CD3+CD4+ T-helper populations (shown in figure 1) *(1)* CD127+CD25- non-TReg gate *(2)* CD45RO+ T-helper cell gate *(3)* CXCR3+ gate for Th1 subsets, CXCR3- gate for Th2/Th17 subsets *(4)* CXCR3+CCR6- Th1 cells (*5*) Quadrant gate used to define CXCR3-CCR4+CCR6-Th2 cells and CXCR3-CCR4+CCR6+ Th17 cells.

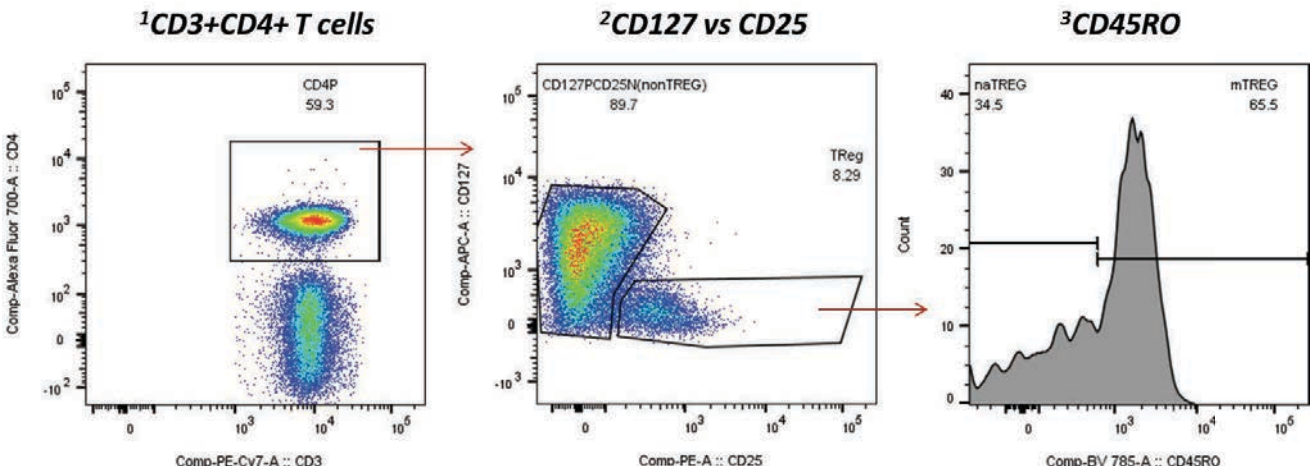

Figure 39 T-Regulatory cell gating strategy shown by representative flow plots

Representative flow cytometry plots showing following parent gate for CD3+CD4+ T-Regulatory cell populations *(1)* CD3+CD4+ TReg cells gate *(2)* $CD127^{low}CD25^{hi}$ TReg cell gate *(3)* nTReg (CD45RO-) and mTReg (CD45RO+) distinction.

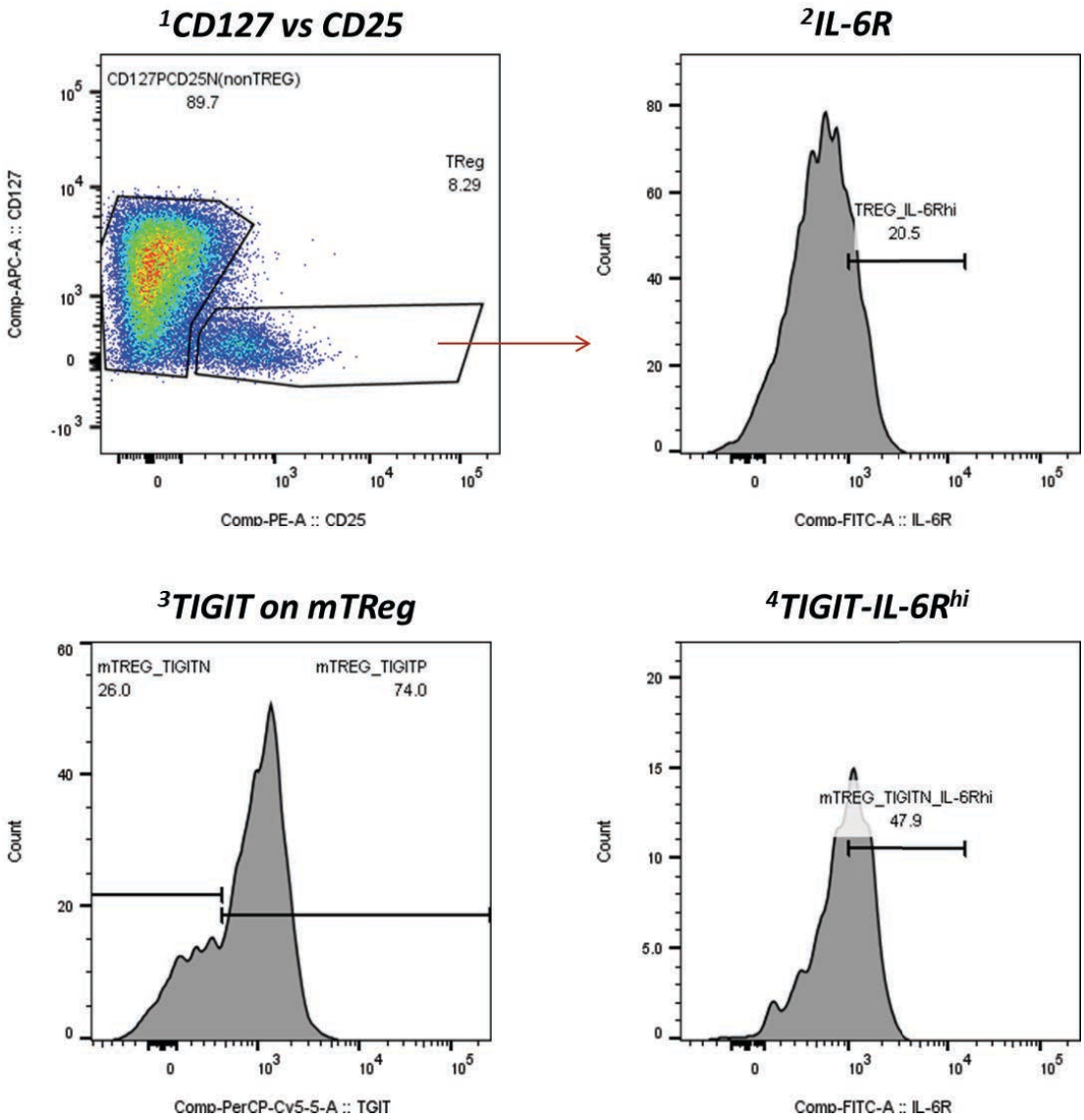

Figure 40 IL-6Rhi subsets gating strategy shown by representative flow plots.

Representative flow cytometry plots showing following parent gate for CD3+CD4+ T-helper populations (shown in figure 1) *(1)* $CD127^{low}CD25^{hi}$ TReg cell gate *(2)* IL-6Rhi defined as top 20^{th} percentile on TReg subsets *(3)* TIGIT on mTReg (CD45RO+ defined in figure 3.3.) *(4)* IL-6Rhi gate on all TRegs applied to TIGIT- mTReg gate.

6.3 Results

6.3.1 Peripheral blood lymphocyte and T cell populations in T1D and control participants

The proportion (%) and frequency (cells/µl) of peripheral blood lymphocytes, $CD3^+$, and $CD4^+$ cells were measured at rest in T1D and control participants. There were no significant differences in peripheral lymphocytes, $CD3^+$, and $CD4^+$ populations between groups at baseline (Table 39).

Subset		[1]Controls		[2]T1D	
		mean	SD	mean	SD
Lymphocytes	(cells/µl)	1357	391	1407	249
CD3	(%)	57.20	20.85	46.84	25.17
	(cells/µl)	750.2	417.6	678.4	317.8
CD4	(%)	51.51	9.29	53.76	7.70
	(cells/µl)	423.4	232.2	370.6	188.1

Table 39 Lymphocyte and T cell populations at baseline in T1D and controls.

Mean and standard deviation values for the proportion and frequency of lymphocytes, CD3+, and CD4+ populations in controls and T1D subjects.
[1]controls n=21, [2]T1D n=14

The effect of moderate (40% VO_2 max) and vigorous (80% VO_2 max) intensity exercise on lymphocytes was investigated in T1D and control participants. Lymphocyte subpopulations were measured pre, post, and 1 hour post exercise. The change over time was analysed in groups combined under the heading “time (overall)” and in each group independently (time) displayed in all results tables. The interaction over time between groups was compared and results are displayed under the heading “time*group” in all results tables. Post and 1 hour post exercise measurements were compared to baseline levels as shown under the heading “contrasts” displayed in the results tables. The mean, standard deviation, and statistical analyses are displayed in Table 40.

There was an overall change over time in lymphocytes during moderate intensity exercise ($p=0.025$), this was seen in controls ($p=0.046$) but not in T1D (Figure 41a). Lymphocytes significantly mobilised during vigorous intensity exercise overall ($p<0.001$), and in T1D ($p=0.004$) and control ($p<0.001$) groups independently. Lymphocytes were significantly increased immediately post vigorous exercise in T1D ($p=0.006$) and controls ($p<0.001$), followed by a significant fall below baseline in T1D ($p=0.028$) and controls ($p=0.026$) 1 hour post vigorous exercise (Figure 41b) (Table 40).

$CD3^+$ T cells were significantly mobilised by vigorous intensity exercise overall ($p=0.001$) and in controls ($p=0.002$), but not in the T1D group. $CD3^+$ T cells were significantly increased immediately post vigorous exercise in controls ($p=0.004$), and significantly fell below baseline 1 hour post vigorous exercise in control participants only ($p=0.041$) (Figure 5d). There was no change over time in $CD3^+$ populations during moderate intensity exercise (Figure 41c) (Table 40).

$CD4^+$ T cells were significantly mobilised by vigorous intensity exercise overall ($p=0.017$) and in controls ($p=0.009$), but not in the T1D group. $CD4^+$ T cells were significantly increased immediately post vigorous exercise in control participants only ($p=0.039$) (Figure 41f). However, the percentage increase immediately post vigorous exercise was similar in the T1D compared to the control group (T1D: 39.71%, control: 43.94%). There was no change over time in $CD4^+$ populations during moderate intensity exercise (Figure 41e) (Table 40).

Figure 42 displays raw data for $CD3^+$ and $CD4^+$ T cells during vigorous intensity exercise in T1D and controls. It is evident from this that one T1D participant responded negatively to vigorous exercise indicated by a decrease in $CD3^+$ and $CD4^+$ T cells immediately post vigorous exercise.

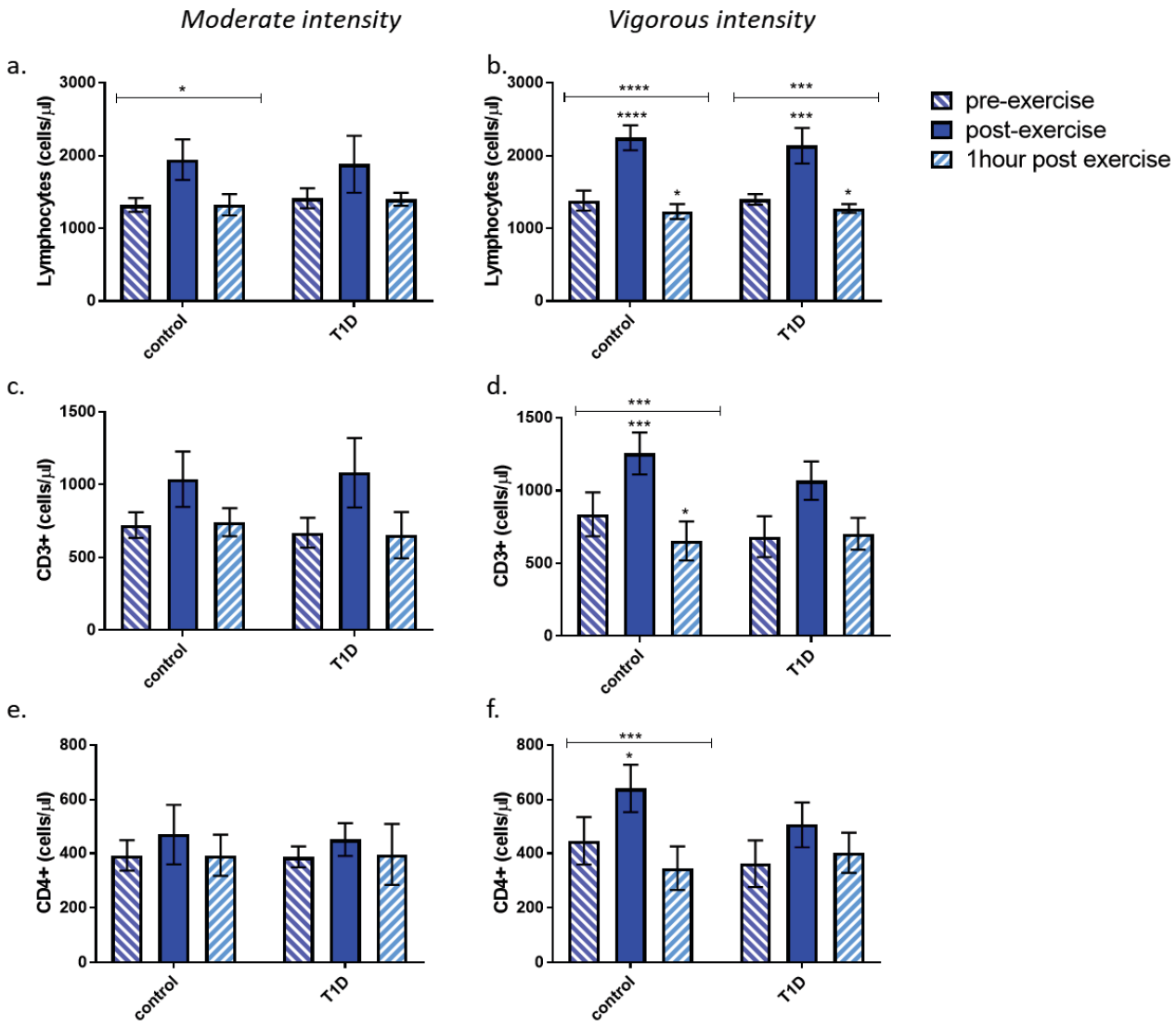

Figure 41 Lymphocyte, CD3+ and CD4+ populations during moderate and vigorous exercise in control and T1D groups

Flow cytometry and whole blood cell counts were used to determine the frequency of lymphocytes, CD3+, and CD4+ populations in control and T1D participants during moderate and vigorous intensity exercise. (a) Frequency of lymphocytes during moderate intensity (b) Frequency of lymphocytes during vigorous intensity exercise (c) The frequency of CD3+ cells during moderate intensity (d) The frequency of CD3+ cells during vigorous intensity (e) The frequency of CD4+ cells during low intensity (f) The frequency of CD4+ cells during vigorous intensity. Error bars represent SEM. Statistical significance represented by * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

Subset (cells/μl)	Intensity	[1]Controls T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time		[c]contrast	[a]Δ%	[2]T1D T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time		[c]contrast	[a]Δ%	[d]Time (overall)	[d]Time*Group
Lymphocytes	**moderate**	1322.22	269.89	1944.44	787.56	1325.00	386.49	**$F_{(2, 14)}$= 3.865, p=0.046**	*T2*	$F_{(1, 7)}$= 4.743, p=0.066	47.06	1416.67	307.77	1883.33	874.48	1400.00	178.89	$F_{(2, 8)}$= 2.983, p=0.108	*T2*	$F_{(1, 4)}$= 2.815, p=0.169	32.94	**$F_{(1.2, 12.4)}$= 6.233, p=0.025**	$F_{(1.2, 12.4)}$= 0.049, p=0.855
									T3	$F_{(1, 7)}$= 0.000, p=0.100	0.21									$F_{(1, 4)}$= 2.250, p=0.208	1.18		
	vigorous	1383.33	459.77	2245.46	539.97	1233.33	344.80	**F(1.22, 12.2)= 36.572, p<0.001**	*T2*	**$F_{(1, 10)}$= 40.810, p<0.001**	62.32	1400.00	193.65	2137.50	642.14	1275.00	163.94	**$F_{(1.1, 7.5)}$= 15.8904, p=0.004**	*T2*	**$F_{(1, 7)}$= 14.652, p=0.006**	52.68	**$F_{(1.2, 19.7)}$= 48.515, p<0.001**	$F_{(1.2, 19.7)}$= 0.268, p=0.766
									T3	**$F_{(1, 10)}$= 6.811, p=0.026**	10.84									**$F_{(1, 7)}$= 7.609, p=0.028**	8.93		
CD3	moderate	721.07	250.34	1037.12	539.31	742.42	256.25	$F_{(2, 14)}$= 2.086, p=0.161	*T2*	$F_{(1, 7)}$= 3.051, p=0.124	43.83	670.25	230.66	1082.79	532.05	653.44	274.55	$F_{(2, 14)}$= 2.086, p=0.161	*T2*	$F_{(1, 3)}$= 1.777, p=0.275	61.55	$F_{(1.2, 12.3)}$= 4.039, p=0.061	$F_{(1.2, 12.3)}$= 0.293, p=0.644
									T3	$F_{(1, 7)}$= 0.016, p=0.903	2.96									$F_{(1, 3)}$= 0.369, p=0.587	2.51		
	vigorous	838.18	503.21	1256.01	456.25	654.31	444.43	**$F_{(1.34, 13.4)}$= 12.486, p=0.002**	*T2*	**$F_{(1, 10)}$= 13.473, p=0.004**	49.85	684.52	369.74	1068.35	349.45	703.77	268.37	$F_{(1.05, 6.3)}$= 3.440, p=0.110	*T2*	$F_{(1, 6)}$= 2.492, p=0.165	56.07	**$F_{(1.3, 21.929)}$= 13.250, p=0.001**	$F_{(1.3, 21.929)}$= 0.550, p=0.582
									T2	**$F_{(1, 10)}$= 5.502, p=0.041**	21.94									$F_{(1, 6)}$= 2.469, p=0.167	2.81		
CD4	moderate	392.72	149.33	470.47	292.14	392.96	184.67	$F_{(2, 12)}$= 0.319, p=0.733	*T2*	$F_{(1, 6)}$= 0.636, p=0.456	19.80	387.12	66.34	450.90	104.44	396.48	158.19	$F_{(2, 4)}$= 0.558, p=0.611	*T2*	$F_{(1, 2)}$= 1.169, p=0.393	16.48	$F_{(1.3, 9.9)}$= 0.580, p=0.501	$F_{(1.3, 9.9)}$= 0.008, p=0.960
									T3	$F_{(1, 6)}$= 0.004, p=0.951	0.06									$F_{(1, 2)}$= 0.005, p=0.952	2.42		
	vigorous	445.68	275.15	641.52	261.10	346.20	251.58	**$F_{(2, 18)}$= 6.100, p=0.009**	*T2*	**$F_{(1, 9)}$= 5.809, p=0.039**	43.94	362.32	225.10	506.20	221.66	402.52	179.59	$F_{(1.4, 6.9)}$= 1.210, p=0.318	*T2*	$F_{(1, 6)}$= 1.119, p=0.331	39.71	**$F_{(1.4, 21)}$= 5.785, p=0.017**	$F_{(1.4, 21)}$= 1.402, p=0.261
									T3	$F_{(1, 9)}$= 3.426, p=0.097	22.32									$F_{(1, 6)}$= 0.032, p=0.864	11.10		

Table 40 Lymphocytes, CD3+ and CD4+ populations mobilised during moderate and vigorous intensity exercise in control and T1D groups

Mean, standard deviation, and statistical analysis of lymphocytes, CD3+ and CD4+ populations for control and T1D subjects during moderate and vigorous intensity exercise. Significant results highlighted in bold where p values <0.05 were considered significant.

[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2).

[b] Results were analysed using multiple regression analysis in control and T1D groups independently.

[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).

[d] Results were analysed using multiple regression analysis in control and T1D groups combined.

[1]Controls n=9-11, [2]T1D n =6-8

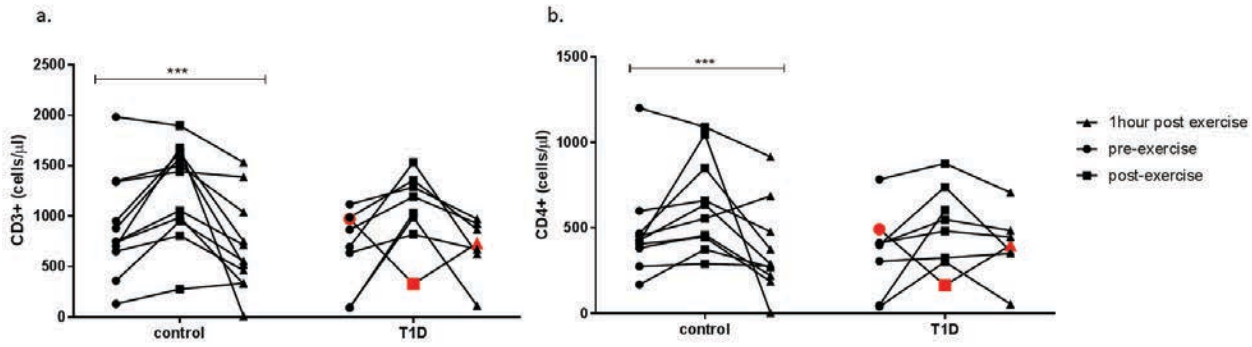

Figure 42 Raw data for CD3+ and CD4+ populations mobilised during moderate and vigorous intensity exercise in control and T1D groups

Flow cytometry and whole blood cell counts were used to determine the frequency of CD3+ and CD4+ populations in control and T1D participants during moderate and vigorous intensity exercise. (a) The frequency of CD3+ cells during vigorous intensity (b) The frequency of CD4+ cells during moderate intensity. Statistical significance represented by * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

6.3.2 $CD4^+$ T-helper subsets

6.3.2.1 $CD4^+$ T-helper subsets at baseline in T1D and control participants

The proportion (%) and frequency of peripheral $CD4^+$ T-helper cells (cells/µl) was measured at rest in T1D and control participants. There were no significant differences in T helper subsets (Th1, Th2, and Th17) between groups at baseline (Figure 43). The mean and standard deviation are displayed in Table 41.

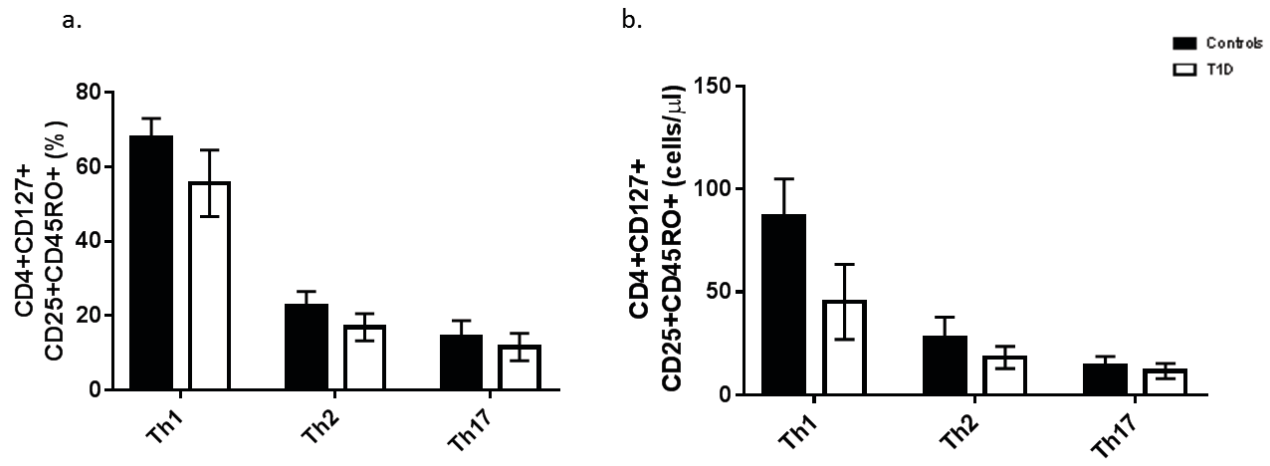

Figure 43 The proportion and frequency of CD4+ T-helper cell subsets at baseline in T1D and control groups.

Flow cytometry and whole blood cell counts were used to determine the frequency of T-helper populations Th1, Th2, and Th17 in control and T1D participants at baseline. (a) Proportion of Th1, Th2, and Th17 in control and T1D groups (b) Frequency of Th1, Th2, and Th17 in control and T1D groups. Error bars represent SEM. No statistical significance observed.

Subset		[1]Controls		[2]T1D	
		mean	SD	mean	SD
Th1	(%)	14.35	16.18	11.63	10.33
	(cells/μl)	86.55	69.03	45.21	54.79
Th2	(%)	22.48	15.31	16.93	10.85
	(cells/μl)	27.53	38.29	18.19	15.32
Th17	(%)	14.35	16.18	11.63	10.33
	(cells/μl)	14.35	16.18	11.63	10.33

Table 41 CD4+ T-helper subsets at baseline in T1D and controls.

Mean and standard deviation values for the proportion and frequency of CD4+ T-helper subsets in controls and T1D groups.
[1] controls n=14, [2] T1D n=8

6.3.2.2 $CD4^+$ T-helper subsets in response to acute exercise in T1D and control participants

The effect of moderate (40% VO_2 max) and vigorous (80% VO_2 max) intensity exercise on $CD4^+$ T-helper subsets was examined. Data for moderate intensity exercise is shown for the control group only due to missing data for the T1D group. Vigorous intensity exercise data for

both T1D and controls is displayed in Table 43. Figure 44 displays Th1, Th2, and Th17 cells during vigorous exercise in T1D and control groups.

Total non-TReg $CD4^+$ T cells significantly mobilised with vigorous intensity exercise overall (p=0.014), but this was only seen in control (p=0.008) and not T1D participants. There was a significant increase immediately post vigorous exercise overall (p=0.027) and in the control group (p=0.033) (Table 43).

Total $CD4^+$ T-helper populations significantly mobilised with vigorous intensity exercise overall (p=0.003), but this was only seen in control (p=0.017) and not T1D participants. There was an overall significant increase immediately post vigorous exercise (p=0.048), but this was not seen in either T1D or control group independently (Table 43).

Th1 subsets are significantly mobilised by vigorous intensity exercise overall (p=0.035), with a trend seen in control (p=0.061) but not T1D participants. There was a significant difference between groups at 1 hour post vigorous exercise (p=0.044). This is because a larger decrease below baseline was shown in controls. Th1 subsets did not significantly mobilise with moderate intensity exercise in control participants (Table 43).

Th2 subsets significantly mobilised during vigorous intensity exercise in control (p=0.042) but not T1D participants, with a significant increase post vigorous exercise in the control group (p=0.039). Th2 subsets did not significantly change during moderate intensity exercise in the control group, however there was a significant drop below baseline 1 hour post moderate exercise (p=0.019). The Th1/Th2 ratio was reduced post vigorous exercise in the control, but not the T1D group, indicating that Th2 cells increased in comparison to Th1 cells (Table 42).

There was a significant change in Th17 cells over time during vigorous intensity exercise overall (p=0.006), but this was not seen in either T1D or control group independently. There

was a significant increase post vigorous exercise overall (p=0.040), but this was not seen in either T1D or control group independently. Th17 did not mobilise with moderate intensity exercise in T1D or control participants (Table 43).

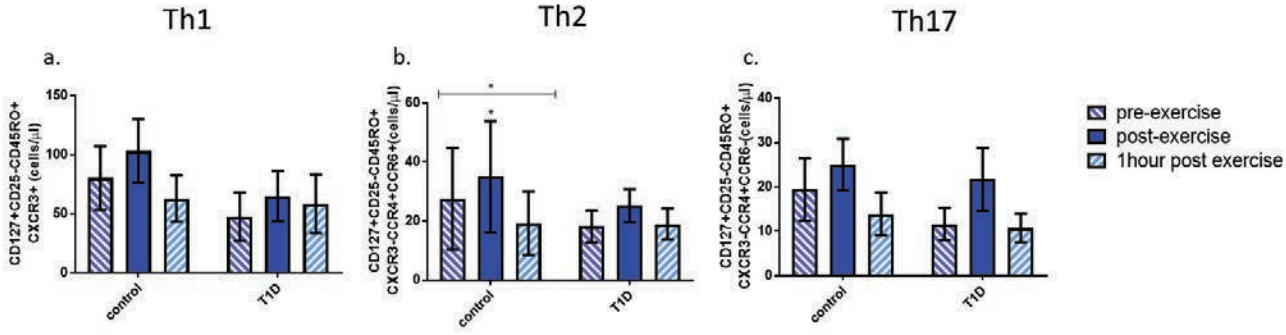

Figure 44 The frequencies of CD4+ Th1, Th2, and Th17 subsets mobilised during vigorous intensity exercise in control and T1D participants

Flow cytometry and whole blood counts were used to determine the frequency of CD4+ Th1, Th2, and Th17 subsets in control and T1D subjects during vigorous intensity exercise. *(a)* The frequency of CD4+ Th1 cells during vigorous intensity exercise *(b)* The frequency of CD4+ Th2 cells during vigorous intensity exercise *(c)* The frequency of CD4+ Th17 cells during vigorous intensity exercise. Error bars represent SEM. Statistical significance represented by * p<0.05, ** p<0.01, ***p<0.001.

	[1]Controls		[2]T1D	
	T1	T2	T1	T2
proportion	5.4	4.4	3.6	3.6
frequency	6.2	5.7	2.6	2.6

Table 42 Th1/Th2 ratio post vigorous exercise in T1D and controls.

Th1/Th2 ratio in controls and T1D groups.
[1] controls n=7-8, [2] T1D n=8-9

Subset (cells/µl)	Intensity	[1]Controls T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast		[a]Δ%	[2]T1D T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[a]Δ%	[c]contrast		[d]Time (overall)	[d]Time*Group
non-TReg (CD127+CD25-)	moderate	355.53	128.56	423.97	266.99	354.00	164.78	$F_{(2, 12)}$= 0.288, p=0.755	*T2*	$F_{(1, 6)}$= 0.571, p=0.479	19.25	322.66	103.68	389.27	80.98	325.72	205.29	$F_{(2, 2)}$= 0.792, p=0.558	20.64	*T2*	$F_{(1, 1)}$= 3.377, p=0.317	$F_{(2, 14)}$= 0.501, p=0.617	$F_{(2, 14)}$= 0.031, p=0.969
									T3	$F_{(1, 6)}$= 0.005, p=0.945	0.43								0.95	*T3*	$F_{(1, 1)}$= 0.000, p=0.989		
	vigorous	406.35	245.97	578.27	223.63	319.57	230.11	**$F_{(2, 18)}$= 6.322, p=0.008**	*T2*	**$F_{(1, 9)}$= 6.308, p=0.033**	42.31	321.62	201.91	456.01	198.40	360.77	162.32	$F_{(1.2, 6.9)}$= 1.252, p=0.311	41.79	*T2*	$F_{(1, 6)}$= 1.218, p=0.312	**$F_{(1.5, 21.8)}$= 5.968, p=0.014**	$F_{(1.5, 21.8)}$= 1.381, p=0.266
									T3	$F_{(1, 9)}$= 3.244, p=0.105	21.36								12.17	*T3*	$F_{(1, 6)}$= 0.001, p=0.975		
Thelper (CD127+CD25-CD45RO+)	moderate	182.25	104.84	227.25	178.44	130.97	95.93	$F_{(1.04, 5.2)}$= 1.070, p=0.351	*T2*	$F_{(1, 5)}$= 0.400, p=0.555	24.70									*T2*		$F_{(1.04, 5.2)}$= 1.070, p=0.351	
									T3	$F_{(1, 5)}$= 5.785, p=0.061	28.13									*T3*			
	vigorous	245.37	216.53	319.73	183.60	194.80	160.39	**$F_{(2, 12)}$= 5.890, p=0.017**	*T2*	$F_{(1, 6)}$= 2.736, p=0.149	30.31	173.48	93.79	277.47	128.04	192.20	94.74	$F_{(1.1, 6.4)}$= 2.626, p=0.153	59.94	*T2*	$F_{(1, 6)}$= 2.437, p=0.169	**$F_{(2, 24)}$= 7.316, p=0.003**	$F_{(2, 24)}$= 0.805, p=0.459
									T3	$F_{(1, 6)}$= 3.300, p=0.119	20.61								10.79	*T3*	$F_{(1, 6)}$= 0.015, p=0.907		
└ Th1 (CXCR3+CCR6+CCR4-)	moderate	94.80	58.98	107.10	66.11	69.90	62.26	$F_{(1.04, 4.2)}$= 0.734, p=0.444	*T2*	$F_{(1, 4)}$= 0.044, p=0.844	12.98									*T2*		$F_{(1.04, 4.2)}$= 0.734, p=0.444	
									T3	$F_{(1, 4)}$= 5.203, p=0.085	26.27									*T3*			
	vigorous	80.36	71.03	103.28	66.30	62.93	51.74	$F_{(2, 12)}$= 3.533, p=0.062	*T2*	$F_{(1, 6)}$= 1.122, p=0.330	28.51	47.51	54.36	65.08	56.56	58.42	61.04	$F_{(2, 12)}$= 1.232, p=0.326	36.97	*T2*	$F_{(1, 6)}$= 1.485 p=0.269	**$F_{(2, 24)}$= 3.865, p=0.035**	$F_{(2, 24)}$= 1.482, p=0.246
									T3	$F_{(1, 6)}$= 4.053, p=0.091	21.69								22.95	*T3*	$F_{(1, 6)}$= 1.027, p=0.350		
└ Th2 (CXCR3-CCR6-CCR4+)	**moderate**	27.51	20.20	33.08	23.35	21.91	21.71	$F_{(2, 8)}$= 3.320, p=0.089	*T2*	$F_{(1, 4)}$= 0.325, p=0.599	20.28									*T2*		$F_{(2, 8)}$= 3.320, p=0.089	
									T3	**$F_{(1, 4)}$= 14.648, p=0.019**	20.33									*T3*			
	vigorous	27.54	45.57	34.90	46.26	19.23	28.38	**$F_{(2, 12)}$= 4.192, p=0.042**	*T2*	**$F_{(1, 6)}$= 6.964, p=0.039**	26.72	18.19	14.33	25.20	14.77	18.96	12.83	$F_{(1.1, 6.7)}$= 0.836, p=0.406	38.52	*T2*	$F_{(1, 6)}$= 0.467, p=0.520	**$F_{(2, 24)}$= 3.634, p=0.042**	$F_{(2, 24)}$= 0.156, p=0.857
									T3	$F_{(1, 6)}$= 0.575, p=0.477	30.18								4.21	*T3*	$F_{(1, 6)}$= 1.341, p=0.291		
└ Th17 (CXCR3-CCR6+CCR4+)	moderate	7.57	4.98	11.68	13.09	5.78	3.77	$F_{(2, 8)}$= 1.588, p=0.263	*T2*	$F_{(1, 4)}$= 0.257, p=0.639	54.20									*T2*		$F_{(2, 8)}$= 1.588, p=0.263	
									T3	$F_{(1, 4)}$= 5.001, p=0.089	23.73									*T3*			
	vigorous	19.44	18.61	25.02	14.28	13.89	12.85	$F_{(2, 12)}$= 3.075, p=0.084	*T2*	$F_{(1, 6)}$= 3.561, p=0.108	28.72	11.63	9.66	21.71	18.75	10.75	7.80	$F_{(1.1, 6.8)}$= 3.475, p=0.104	86.68	*T2*	$F_{(1, 6)}$= 1.916, p=0.216	**$F_{(2, 24)}$= 6.363, p=0.006**	$F_{(2, 24)}$= 0.077, p=0.926
									T3	$F_{(1, 6)}$= 0.548, p=0.487	28.55								7.58	*T3*	$F_{(1, 6)}$= 8.154, p=0.029		
└ CXCR3-CCR6-CCR4-	moderate	38.92	23.44	48.39	29.00	30.43	13.06	$F_{(2, 8)}$= 1.133, p=0.369	*T2*	$F_{(1, 4)}$= 0.299, p=0.613	24.32									*T2*		$F_{(2, 8)}$= 1.133, p=0.369	
									T3	$F_{(1, 4)}$= 1.186, p=0.337	21.82									*T3*			
	vigorous	36.90	34.34	49.32	40.44	31.45	25.29	$F_{(1.2, 6.9)}$= 3.927 p=0.085	*T2*	**$F_{(1, 6)}$= 7.080, p=0.037**	33.67	45.64	46.78	70.70	68.32	51.45	49.66	$F_{(1, 6.3)}$= 0.374, p=0.571	54.90	*T2*	$F_{(1, 6)}$= 0.397, p=0.552	$F_{(1.4, 16.2)}$= 1.617, p=0.219	$F_{(1.4, 16.2)}$= 0.221, p=0.803
									T3	$F_{(1, 6)}$= 1.191, p=0.317	14.77								12.73	*T3*	$F_{(1, 6)}$= 0.179, p=0.687		
└ CXCR3-CCR6+CCR4-	moderate	10.17	7.85	15.22	19.96	6.61	6.40	$F_{(2, 8)}$= 0.641, p=0.552	*T2*	$F_{(1, 4)}$= 0.103, p=0.765	49.72									*T2*		$F_{(2, 8)}$= 0.641, p=0.552	
									T3	$F_{(1, 4)}$= 1.248, p=0.326	35.02									*T3*			
	vigorous	23.96	13.40	31.35	11.98	21.05	14.42	$F_{(1.1, 6.8)}$= 2.971 p=0.129	*T2*	**$F_{(1, 6)}$= 6.022, p=0.050**	30.83	42.49	65.50	57.39	72.46	45.45	68.52	$F_{(1.1, 6.6)}$= 0.741, p=0.432	35.05	*T2*	$F_{(1, 6)}$= 0.622, p=0.460	$F_{(2, 24)}$= 2.471, p=0.106	$F_{(2, 24)}$= 0.220, p=0.804
									T3	$F_{(1, 6)}$= 1.093, p=0.336	12.18								6.95	*T3*	$F_{(1, 6)}$= 0.021, p=0.890		

Table 43 T-helper populations mobilised during moderate and vigorous intensity exercise in control and T1D groups

Mean, standard deviation, and statistical analysis of T-helper populations for control and T1D subjects during moderate and vigorous intensity exercise. Significant results highlighted in bold where p values <0.05 were considered significant.

[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2).

[b] Results were analysed using multiple regression analysis in control and T1D groups independently.

[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).

[d] Results were analysed using multiple regression analysis in control and T1D groups combined.

[1] controls n=7-8, [2] T1D n=8-9

6.3.3 CD4$^+$ T-Regulatory subsets

6.3.3.1 CD4$^+$ T-Regulatory subsets at baseline in T1D and control participants

The proportion (%) and frequency of peripheral CD4$^+$ T-Regulatory subsets (cells/µl) were measured at rest in T1D and control participants. The proportion of TReg cells were significantly higher in T1D compared to controls at baseline (p=0.002). Within the TReg population, T1D had significantly higher memory TReg subsets (p=0.003) and significantly lower naïve TReg subsets (p=0.003) (Figure 45). No significant differences in TIGIT$^+$ or TIGIT$^-$ mTRegs between groups was found. The proportion of mTReg TIGIT$^+$ was higher in T1D (72%) than control participants (60%) but it did not reach significance. The mean and standard deviation are displayed in Table 44.

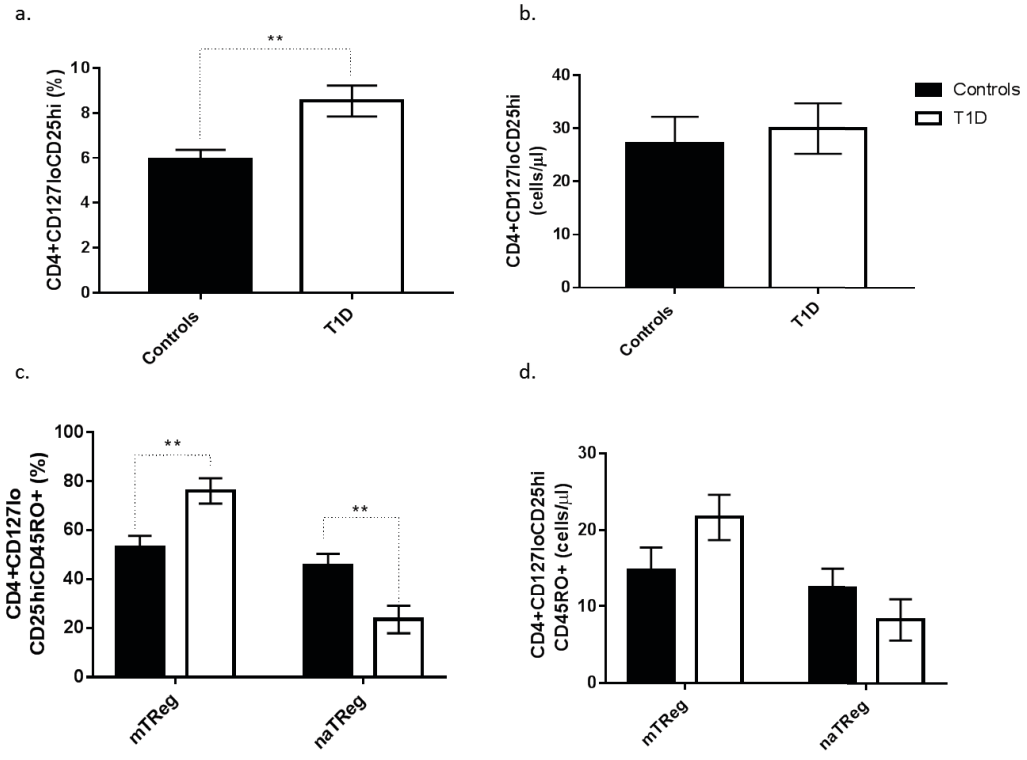

Figure 45 The proportion and frequency of CD4+ T-Regulatory cell subsets at baseline in T1D and control groups.

Flow cytometry and whole blood cell counts were used to determine the frequency of T-Regulatory populations in control and T1D participants at baseline. (a) Proportion of TRegs (b) Frequency of TRegs (c) Proportion of mTRegs and naTRegs (d) Frequency of mTRegs and nTRegs. Error bars represent SEM. Statistical significance represented by * p<0.05, ** p<0.01, ***p<0.001. Note: broken line (----) represents time*group differences.

Subset		[1]Controls		[2]T1D	
		mean	SD	mean	SD
TReg	(%)	5.94	1.85	8.53	2.48
	(cells/µl)	27.12	22.15	29.97	15.71
naTReg	(%)	45.60	19.81	23.38	18.57
	(cells/µl)	12.43	10.94	8.31	8.96
mTReg	(%)	52.99	20.21	75.98	17.85
	(cells/µl)	14.69	13.22	21.65	9.85
mTReg TIGIT+	(%)	59.98	30.03	72.15	23.73
	(cells/µl)	12.72	12.23	16.98	8.09
mTReg TIGIT-	(%)	40.03	30.04	27.85	23.73
	(cells/µl)	5.18	4.64	4.67	2.99

Table 44 CD4+ T-Regulatory cell subsets at baseline in T1D and control groups

Mean and standard deviation values for the proportion and frequency of CD4+ T-Regulatory subsets in controls and T1D groups.
[1] controls n=19, [2] T1D n=11

6.3.3.2 $CD4^+$ T-Regulatory subsets in response to acute exercise in T1D and control participants

The effect of moderate (40% VO_2 max) and vigorous (80% VO_2 max) intensity exercise on $CD4^+$ TReg subsets was investigated in T1D and control participants. The mean, standard deviation, and statistical analyses are displayed in Table 45.

$CD4^+$ TReg subsets were significantly mobilised by vigorous intensity exercise overall ($p=0.010$) and in control ($p=0.009$) but not T1D participants. There was a significant fall below baseline 1 hour post vigorous exercise overall ($p=0.038$), but this was not seen in either T1D or control group independently (Figure 46). The percentage change following vigorous exercise was much lower in the T1D compared to the control group (T1D: 23.74%, Control: 47.65%). $CD4^+$ TReg subsets did not significantly mobilise with moderate intensity exercise in either group (Table 45).

Further delineation of $CD4^+$ TRegs revealed that mTReg subsets were significantly mobilised by vigorous intensity exercise overall (p=0.004) and in the control group (p=0.006). There was a significant fall below baseline overall 1 hour post vigorous exercise (p=0.042), but this was not seen in either T1D or control group independently. Naïve TReg subsets did not significantly mobilise with either exercise intensity (Figure 46) (Table 45).

$TIGIT^+$ mTReg subsets were significantly mobilised by vigorous exercise overall (p=0.030) and in the control group (p=0.017). There was a significant drop below baseline 1 hour post vigorous exercise overall (p=0.003) and in the control group (p=0.032). There was a significant difference between T1D and controls 1 hour post vigorous exercise (p=0.023) because $TIGIT^+$ mTReg fell below baseline in controls, but not in T1D participants. (Table 45). No statistically significant mobilisation of $TIGIT^+$ mTReg subsets was observed following moderate intensity exercise in either group.

$TIGIT^-$ mTReg subsets were significantly mobilised by vigorous exercise in the control (p=0.049) but not T1D group. There was a significant drop below baseline 1 hour post vigorous exercise overall (p=0.005) and in the control group (p=0.048). There was a significant difference between T1D and controls 1 hour post vigorous exercise (p=0.044) as $TIGIT^-$ mTReg fell below baseline in controls, but not in T1D participants (Table 45). No statistically significant mobilisation of $TIGIT^-$ mTReg subsets was observed following moderate intensity exercise in either group.

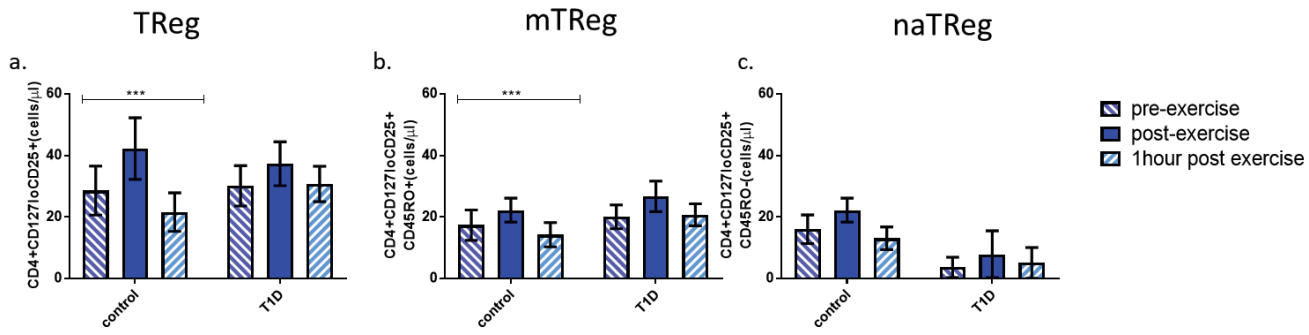

Figure 46 The frequencies of CD4+ TReg, mTReg, and nTReg subsets mobilised during vigorous intensity exercise in control and T1D participants

Flow cytometry and whole blood counts were used to determine the frequency of CD4+ TReg, mTReg, and naTReg subsets in control and T1D subjects during vigorous intensity exercise. *(a)* The frequency of CD4+ TReg cells during vigorous intensity exercise *(b)* The frequency of CD4+ mTReg cells during vigorous intensity exercise *(c)* The frequency of CD4+ naTReg cells during vigorous intensity exercise. Error bars represent SEM. Statistical significance represented by * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

Subset (cells/μl)	Intensity	T1		T2		T3		[b]Time	[c]contrast		[a]Δ%	T1		T2		T3		[b]Time	[c]contrast		[a]Δ%		
		mean	SD	mean	SD	mean	SD					mean	SD	mean	SD	mean	SD						
TReg (CD127loCD25hi)	moderate	25.08	14.85	30.62	22.21	23.43	15.03	$F_{(1.14, 6.8)}$= 0.851, p=0.403	*T2*	$F_{(1, 6)}$= 0.938, p=0.370	22.07	29.53	4.65	40.23	17.67	28.38	6.50	$F_{(2, 2)}$= 1.371, p=0.422	*T2*	$F_{(1, 1)}$= 2.091, p=0.385	36.23	$F_{(1.1, 7.8)}$= 2.551, p=0.149	$F_{(1.1, 7.8)}$= 0.569, p=0.491
									T3	$F_{(1, 6)}$= 0.434, p=0.534	6.59									*T3*	$F_{(1, 1)}$= 0.344, p=0.662	3.90		
	vigorous	28.60	25.24	42.23	30.07	21.56	18.79	**$F_{(2, 16)}$= 6.376, p=0.009**	*T2*	$F_{(1, 8)}$= 2.324, p=0.166	47.65	30.13	17.33	37.28	18.85	30.75	14.09	$F_{(1.1, 6.5)}$= 0.948, p=0.374	*T2*	$F_{(1, 6)}$= 0.418, p=0.542	23.74	**$F_{(2, 28)}$= 5.401, p=0.010**	$F_{(2, 28)}$= 0.519, p=0.600
									T3	$F_{(1, 8)}$= 3.658, p=0.092	24.60									*T3*	$F_{(1, 6)}$= 4.203, p=0.086	2.05		
naïve TReg (CD45RO-)	moderate	12.21	7.82	13.53	8.55	13.99	10.12	$F_{(2, 12)}$= 0.260, p=0.775	*T2*	$F_{(1, 6)}$= 1.140, p=0.327	10.79	3.61	4.66	7.88	10.71	5.07	5.00	$F_{(2, 2)}$= 0.981, p=0.505	*T2*	$F_{(1, 1)}$= 0.981, p=0.503	118.45	$F_{(2, 14)}$= 1.860, p=0.192	$F_{(2, 14)}$= 0.932, p=0.402
									T3	$F_{(1, 6)}$= 0.098, p=0.765	14.53									*T3*	$F_{(1, 1)}$= 0.833, p=0.529	40.43		
	vigorous	12.59	12.30	19.98	22.39	7.72	8.25	$F_{(1.1, 9.6)}$= 2.182, p=0.172	*T2*	$F_{(1, 9)}$= 1.692, p=0.226	58.69	10.08	8.99	10.59	8.63	10.03	7.60	$F_{(2, 12)}$= 0.382, p=0.690	*T2*	$F_{(1, 6)}$= 0.124, p=0.737	5.06	$F_{(1.1, 16.1)}$= 1.631, p=0.221	$F_{(1.1, 16.1)}$= 1.299, p=0.275
									T3	$F_{(1, 9)}$= 2.712, p=0.134	38.68									*T3*	$F_{(1, 6)}$= 4.382, p=0.081	0.48		
memory TReg(CD45RO+)	moderate	12.87	9.47	17.09	17.71	9.44	7.10	$F_{(1.04, 6.3)}$= 1.221, p=0.313	*T2*	$F_{(1, 6)}$= 0.798, p=0.409	32.77	25.92	4.13	32.36	9.05	23.31	11.50	$F_{(2, 2)}$= 1.474, p=0.404	*T2*	$F_{(1, 1)}$= 3.880, p=0.299	24.82	$F_{(1.1, 7.5)}$= 2.647, p=0.145	$F_{(1.1, 7.5)}$= 0.370, p=0.574
									T3	$F_{(1, 6)}$= 2.815, p=0.144	26.63									*T3*	$F_{(1, 1)}$= 0.341, p=0.664	10.06		
	vigorous	16.01	14.72	22.25	11.69	13.07	11.16	**$F_{(2, 16)}$= 7.222, p=0.006**	*T2*	$F_{(1, 8)}$= 2.785, p=0.134	38.98	20.05	10.27	26.70	13.15	20.72	8.76	$F_{(1.1, 6.6)}$= 1.606, p=0.252	*T2*	$F_{(1, 6)}$= 1.232, p=0.310	33.12	**$F_{(2, 28)}$= 6.632, p=0.004**	$F_{(2, 28)}$= 0.291, p=0.750
									T3	$F_{(1, 8)}$= 4.198, p=0.075	18.34									*T3*	$F_{(1, 6)}$= 1.8974, p=0.220	3.30		
└ **TIGIT-**	moderate	5.24	4.27	6.30	4.28	4.87	4.53	$F_{(2, 6)}$= 0.780, p=0.500	*T2*	$F_{(1, 3)}$= 0.345, p=0.598	20.34	4.26	2.56	5.54	4.53	3.32	0.57	$F_{(2, 2)}$= 1.141, p=0.467	*T2*	$F_{(1, 1)}$= 1.374, p=0.450	30.06	$F_{(2, 8)}$= 2.040, p=0.223	$F_{(2, 8)}$= 0.186, p=0.833
									T3	$F_{(1, 3)}$= 5.587, p=0.099	7.01									*T3*	$F_{(1, 1)}$= 0.945, p=0.509	22.23		
	vigorous	5.14	4.55	5.44	3.62	3.86	3.59	**$F_{(2, 8)}$= 4.503, p=0.049**	*T2*	$F_{(1, 4)}$= 0.146, p=0.722	5.83	4.83	2.94	6.54	4.13	5.05	2.60	$F_{(1.9, 6.4)}$= 1.376, p=0.286	*T2*	$F_{(1, 6)}$= 1.022, p=0.351	35.50	$F_{(1.2, 12)}$= 2.717, p=0.121	$F_{(1.2, 12)}$= 0.778, p=0.418
									T3	**$F_{(1, 4)}$= 7.930, p=0.048**	24.85									*T3*	$F_{(1, 6)}$= 2.060 p=0.201	4.51		
└ **TIGIT+**	moderate	9.93	8.34	15.36	17.56	6.39	5.28	$F_{(1, 3)}$= 0.934, p=0.405	*T2*	$F_{(1, 3)}$= 0.859, p=0.422	54.69	21.66	5.54	26.81	6.70	20.00	12.07	$F_{(2, 2)}$= 1.661, p=0.376	*T2*	$F_{(1, 1)}$= 5.606, p=0.254	23.80	$F_{(1, 4.2)}$= 1.915, p=0.238	$F_{(1, 4.2)}$= 0.044, p=0.851
									T3	$F_{(1, 3)}$= 1.180 p=0.357	35.69									*T3*	$F_{(1, 1)}$= 0.192 p=0.737	7.68		
	vigorous	15.04	13.39	18.49	10.48	10.63	10.30	**$F_{(2, 8)}$= 7.107, p=0.017**	*T2*	$F_{(1, 4)}$= 0.082, p=0.788	22.96	15.22	7.69	20.15	9.51	15.67	6.76	$F_{(1.9, 6.7)}$= 1.641, p=0.247	*T2*	$F_{(1, 6)}$= 1.280, p=0.301	32.37	**$F_{(1.3, 13.3)}$= 5.340, p=0.030**	$F_{(1.3, 13.3)}$= 1.069, p=0.342
									T3	**$F_{(1, 4)}$= 10.520, p=0.032**	29.32									*T3*	$F_{(1, 6)}$= 1.605p=0.252	2.92		

Table 45 T-Regulatory populations mobilised during moderate and vigorous intensity exercise in control and T1D groups

Mean, standard deviation, and statistical analysis of T-Regulatory populations for control and T1D subjects during moderate and vigorous intensity exercise. Significant results highlighted in bold where p values <0.05 were considered significant.

[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2).

[b] Results were analysed using multiple regression analysis in control and T1D groups independently.

[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).

[d] Results were analysed using multiple regression analysis in control and T1D groups combined.

[1]Controls n=10-11, [2]T1D n =8

6.3.4 IL-6R^{hi} subsets

A phenotypically distinct TReg subset with increased suppressive, tissue-infiltrating capacity was identified recently. They are identified as TIGIT^{-}IL-6R^{hi} mTReg cells that exhibit a Th17 signature (RORγt^{+}CD161^{+}CCR6^{+}) [492]. Here, IL-6R^{hi} expression, defined as the top 20th percentile of IL-6R expression, is measured on naïve and memory TReg subsets in T1D and control participants.

6.3.4.1 IL-6R^{hi} subsets at baseline in T1D and control participants

IL-6R^{hi} expression on naïve and memory TReg subsets was measured at rest in T1D and control participants. There were no significant differences in IL-6R^{hi} naTReg or mTReg subsets between groups at baseline (Figure 47). However, TIGIT^{-}IL-6R^{hi} mTRegs showed a trend to be lower in T1D. The mean and standard deviation are displayed in Table 46.

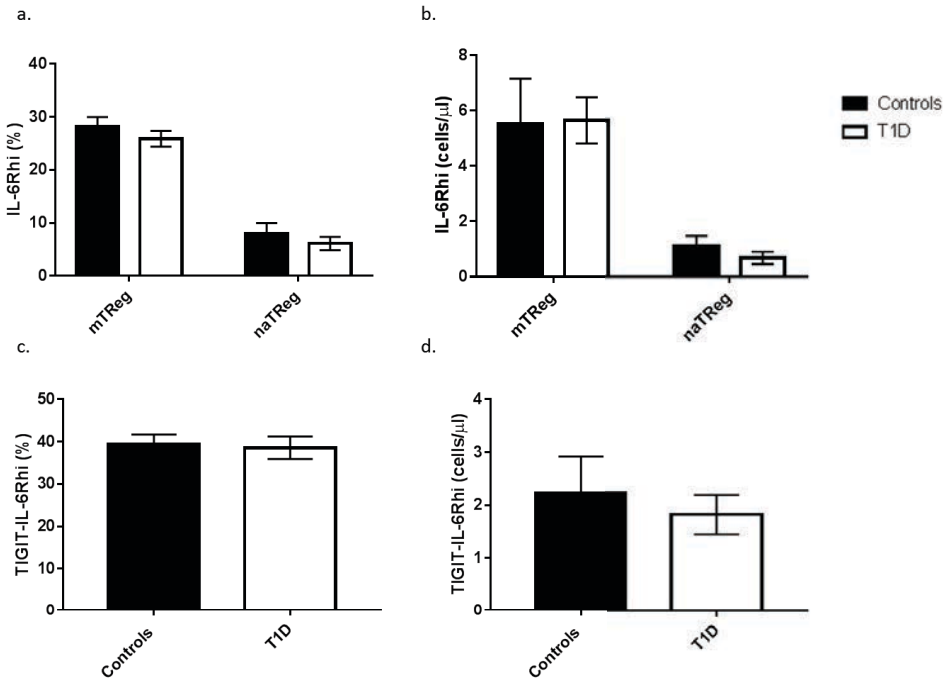

Figure 47 The proportion and frequency of CD4+ IL-6Rhi T-Regulatory cell subsets at baseline in T1D and control groups.

Flow cytometry and whole blood cell counts were used to determine the frequency of CD4+ IL-6Rhi T-Regulatory populations in control and T1D participants at baseline. (a) Proportion of IL-6Rhi mTRegs and nTRegs (b)

Frequency of IL-6Rhi mTRegs and nTRegs (c) Proportion of TIGIT-IL-6Rhi mTRegs (d) Frequency of TIGIT-IL-6Rhi mTRegs. Error bars represent SEM. No statistical significance observed.

Subset		[1]Controls		[2]T1D	
		mean	SD	mean	SD
IL-6Rhi TReg	(%)	20.34	0.94	21.03	1.07
	(cells/µl)	1.11	1.16	0.68	0.72
IL-6Rhi mTReg	(%)	28.16	5.79	25.89	4.95
	(cells/µl)	5.54	5.11	5.65	2.77
IL-6Rhi naTReg	(%)	8.05	6.09	6.14	3.94
	(cells/µl)	1.11	1.16	0.68	0.72
TIGIT+IL-6Rhi	(%)	22.97	4.42	22.80	4.04
	(cells/µl)	3.58	3.26	3.83	1.77
TIGIT-IL-6Rhi	(%)	39.40	7.19	38.55	8.85
	(cells/µl)	2.23	2.19	1.81	1.23
TIGIT-IL-6Rhi CCR6+	(%)	34.85	20.09	41.59	24.64
	(cells/µl)	0.87	1.07	0.84	0.83
TIGIT-IL-6Rhi CCR6-	(%)	65.16	20.10	58.41	24.65
	(cells/µl)	1.45	1.35	0.91	0.76

Table 46 CD4+ IL-6Rhi T-Regulatory cell subsets at baseline in T1D and control groups

Mean and standard deviation values for the proportion and frequency of CD4+ IL-6Rhi T-Regulatory subsets in controls and T1D groups.
[1] controls n=10, [2] T1D n=11

6.3.4.2 IL-6R^{hi} subsets in response to acute exercise in T1D and control participants

IL-6R^{hi} expression on naïve and memory TReg subsets was measured during moderate (40% VO_2 max) and vigorous (80% VO_2 max) intensity exercise in T1D and control participants. The mean, standard deviation, and statistical analyses are displayed in Table 47.

There was a significant difference between groups 1 hour post vigorous intensity exercise in IL-6R^{hi} non-TReg and T-helper subsets (p=0.049, p=0.043 respectively). This is because IL-6R^{hi} non-TReg and T-helper subsets fell below baseline in control, but not T1D participants. However, no overall significant mobilisation of these subsets was found following moderate of vigorous intensity exercise (Table 47).

Whilst not significant, IL-6R^{hi} TReg subsets showed a trend to increase immediately post moderate intensity exercise in the T1D (p=0.060), but not the control group (Table 47). A small but statistically insignificant increase in median fluorescent intensity (MFI) was found post moderate exercise in the T1D group (T1D; 1478 to 1547 MFI, controls; 1197 to 1238 MFI). Vigorous exercise exhibited no significant mobilisation or change in IL-6R^{hi} TReg MFI.

TIGIT^{-}IL-6R^{hi} mTReg significantly fell below baseline 1 hour post vigorous exercise in the control group only (p=0.033). However, no change over time during vigorous intensity exercise in T1D or control groups was found (Table 47). A small but statistically insignificant change in TIGIT-IL-6Rhi MFI post vigorous exercise in both groups was noted (T1D; 1348 to 1414 MFI, controls; 1502 to 1596 MFI). TIGIT^{-}IL-6^{Rhi} mTReg subsets did not significantly mobilise with moderate intensity exercise in either group.

TIGIT^{-}IL-6R^{hi}CCR6^{-} cells were significantly different 1 hour post vigorous exercise between groups (p=0.012) because TIGIT^{-}IL-6R^{hi}CCR6^{-} cells fell below baseline in control, and not T1D participants. However, when change over time during the exercise bout was analysed, TIGIT^{-}IL-6R^{hi}CCR6^{+} and TIGIT^{-}IL-6R^{hi} CCR6^{-} cells did not change significantly over time during moderate or vigorous intensity exercise in either group (Table 47).

There were no significant changes over time during moderate or vigorous intensity exercise in any IL-6R^{hi} subsets (T-helper, non-TReg, TReg, naïve TReg or memory TReg) (Table 47).

Subset (cells/µl)	Intensity	[1]Controls T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[c]contrast	[a]Δ%	[2]T1D T1 mean	T1 SD	T2 mean	T2 SD	T3 mean	T3 SD	[b]Time	[a]Δ%	[c]contrast	[d]Time (overall)	[d]Time*Group
non-TReg IL-6Rhi	moderate	101.73	32.35	137.63	76.84	92.18	62.16	$F_{(2,4)}$= 0.387, p=0.702	*T2* $F_{(1,2)}$= 0.329, p=0.624	35.29	72.45	25.27	102.63	33.45	92.44	64.74	$F_{(2,2)}$= 0.968, p=0.508	41.65	*T2* $F_{(1,1)}$= 30.037, p=0.115	$F_{(2,6)}$= 0.553, p=0.602	$F_{(2,6)}$= 0.104, p=0.903
									T3 $F_{(1,2)}$= 0.489, p=0.557	9.39								27.58	*T3* $F_{(1,1)}$= 0.194, p=0.736		
	vigorous	128.33	105.00	129.61	69.65	94.88	83.69	$F_{(2,8)}$= 2.021, p=0.195	*T2* $F_{(1,4)}$= 0.003, p=0.961	1.00	79.26	57.03	126.51	81.85	93.07	49.14	$F_{(2,12)}$= 1.984, p=0.180	59.61	*T2* $F_{(1,6)}$= 2.272, p=0.182	$F_{(2,20)}$= 2.701, p=0.092	$F_{(2,20)}$= 1.020, p=0.379
									T3 $F_{(1,4)}$= 6.853, p=0.059	26.07								17.43	*T3* $F_{(1,6)}$= 0.111, p=0.750		
Thelper IL-6Rhi	moderate	66.18	25.78	93.12	58.20	54.48	36.78	$F_{(2,4)}$= 0.427, p=0.679	*T2* $F_{(1,2)}$= 0.355, p=0.612	40.70	43.64	15.95	58.54	15.24	14.66	0.00		34.12	*T2*	$F_{(2,4)}$= 0.427, p=0.679	
									T3 $F_{(1,2)}$= 0.821, p=0.461	17.68								66.40	*T3*		
	vigorous	95.44	94.76	93.63	65.45	70.05	72.44	$F_{(2,8)}$= 1.638, p=0.253	*T2* $F_{(1,4)}$= 0.009, p=0.931	1.89	53.30	31.73	89.44	54.53	63.94	30.81	$F_{(2,12)}$= 1.976, p=0.181	67.81	*T2* $F_{(1,6)}$= 2.169, p=0.191	$F_{(1.3,13.2)}$= 2.283, p=0.151	$F_{(1.3,13.2)}$= 1.179, p=0.315
									T3 $F_{(1,4)}$= 4.349, p=0.105	26.60								19.97	*T3* $F_{(1,6)}$= 0.378, p=0.561		
TReg IL-6Rhi	moderate	5.93	3.38	7.77	5.60	6.38	5.18	$F_{(1,3.1)}$= 0.260, p=0.650	*T2* $F_{(1,3)}$= 0.444, p=0.553	31.18	6.41	0.76	8.85	2.67	7.42	3.18	$F_{(2,2)}$= 1.098, p=0.477	38.00	*T2* $F_{(1,1)}$= 113.438, p=0.060	$F_{(2,8)}$= 0.849, p=0.463	$F_{(2,8)}$= 0.088, p=0.917
									T3 $F_{(1,3)}$= 0.002, p=0.971	7.60								15.76	*T3* $F_{(1,1)}$= 0.022 p=0.906		
	vigorous	6.31	6.45	7.20	3.57	4.84	5.13	$F_{(2,8)}$= 1.086, p=0.383	*T2* $F_{(1,4)}$= 0.009, p=0.930	14.15	6.22	3.58	9.16	5.81	7.39	3.98	$F_{(2,12)}$= 1.531, p=0.256	47.40	*T2* $F_{(1,6)}$= 1.677, p=0.243	$F_{(1.3,12.6)}$= 1.504, p=0.250	$F_{(1.3,12.6)}$= 0.929, p=0.377
									T3 $F_{(1,4)}$= 5.358, p=0.082	23.34								18.79	*T3* $F_{(1,6)}$= 0.359, p=0.571		
naïve TReg IL-6Rhi	moderate	1.58	1.26	1.61	1.03	2.58	2.34	$F_{(1,3)}$= 0.544, p=0.515	*T2* $F_{(1,3)}$= 0.054, p=0.831	2.37	0.43	0.41	0.76	0.76	0.96	0.00		78.00	*T2*	$F_{(1,3)}$= 0.159, p=0.718	
									T3 $F_{(1,3)}$= 0.807, p=0.435	64.12								124.98	*T3*		
	vigorous	0.64	0.65	0.63	0.54	0.55	0.62	$F_{(1.1,4.3)}$= 0.130, p=0.751	*T2* $F_{(1,4)}$= 0.004, p=0.955	2.26	0.75	0.72	0.91	0.84	0.79	0.58	$F_{(2,12)}$= 0.849, p=0.452	22.15	*T2* $F_{(1,6)}$= 0.716, p=0.430	$F_{(2,20)}$= 0.640, p=0.538	$F_{(2,20)}$= 0.266, p=0.706
									T3 $F_{(1,4)}$= 2.978, p=0.159	14.18								6.08	*T3* $F_{(1,6)}$= 0.185, p=0.682		
memory TReg IL-6Rhi	moderate	4.35	2.88	6.17	5.78	3.79	3.17	$F_{(1,3)}$= 0.513, p=0.526	*T2* $F_{(1,3)}$= 0.487, p=0.536	41.65	6.12	0.48	8.35	2.38	6.93	3.65	$F_{(2,2)}$= 0.963, p=0.509	36.37	*T2* $F_{(1,1)}$= 24.788, p=0.126	$F_{(2,8)}$= 1.119, p=0.358	$F_{(2,8)}$= 0.086, p=0.830
									T3 $F_{(1,3)}$= 0.608, p=0.492	12.83								13.30	*T3* $F_{(1,1)}$= 0.017, p=0.918		
	vigorous	6.72	6.00	6.57	3.37	5.01	4.78	$F_{(2,8)}$= 1.297, p=0.325	*T2* $F_{(1,4)}$= 0.009, p=0.931	2.27	5.47	3.06	8.26	5.21	6.60	3.52	$F_{(2,12)}$= 1.504, p=0.261	50.89	*T2* $F_{(1,6)}$= 2.169, p=0.191	$F_{(2,20)}$= 1.524, p=0.242	$F_{(2,20)}$= 1.024, p=0.377
									T3 $F_{(1,4)}$= 4.349, p=0.105	25.44								20.53	*T3* $F_{(1,6)}$= 0.378, p=0.561		
└ TIGIT+IL-6Rhi	moderate	2.72	1.96	4.63	4.60	1.94	1.32	$F_{(1,2)}$= 0.764, p=0.474	*T2* $F_{(1,2)}$= 0.745, p=0.479	69.88	4.48	0.78	6.46	2.24	5.53	3.64	$F_{(2,2)}$= 1.231, p=0.467	44.16	*T2* $F_{(1,1)}$= 7.116, p=0.228	$F_{(2,6)}$= 1.421, p=0.312	$F_{(2,6)}$= 0.140, p=0.797
									T3 $F_{(1,2)}$= 0.830, p=0.458	28.65								23.36	*T3* $F_{(1,1)}$= 0.128 p=0.781		
	vigorous	4.26	3.59	4.43	1.92	3.27	2.81	$F_{(2,8)}$= 1.195 p=0.351	*T2* $F_{(1,4)}$= 0.026, p=0.879	3.91	3.59	1.86	5.49	3.27	4.37	2.32	$F_{(2,12)}$= 1.555, p=0.251	52.98	*T2* $F_{(1,6)}$= 1.941, p=0.213	$F_{(2,20)}$= 1.724, p=0.204	$F_{(2,20)}$= 0.835, p=0.448
									T3 $F_{(1,4)}$= 3.681, p=0.127	23.29								21.76	*T3* $F_{(1,6)}$= 0.642, p=0.454		
└ TIGIT-IL-6Rhi	moderate	2.00	1.63	2.36	1.64	2.23	2.22	$F_{(1.01,3.04)}$= 0.145, p=0.732	*T2* $F_{(1,3)}$= 0.217, p=0.673	18.20	1.63	0.87	1.88	1.39	1.41	0.02	$F_{(2,2)}$= 0.551, p=0.645	14.97	*T2* $F_{(1,1)}$= 2.020, p=0.390	$F_{(2,8)}$= 0.525, p=0.611	$F_{(2,8)}$= 0.105, p=0.902
									T3 $F_{(1,3)}$= 0.000, p=0.995	11.44								13.76	*T3* $F_{(1,1)}$= 0.242, p=0.709		
	vigorous	2.45	2.43	2.15	1.56	1.74	2.02	$F_{(2,8)}$= 1.922, p=0.208	*T2* $F_{(1,4)}$= 0.394, p=0.564	12.32	1.88	1.26	2.77	2.00	2.23	1.39	$F_{(2,12)}$= 1.378, p=0.289	47.06	*T2* $F_{(1,6)}$= 1.492, p=0.268	$F_{(2,20)}$= 1.263, p=0.304	$F_{(2,20)}$= 1.418, p=0.266
									***T3* $F_{(1,4)}$= 10.282, p=0.033**	28.95								18.37	*T3* $F_{(1,6)}$= 0.130, p=0.731		
└TIGIT-IL-6Rhi CCR6+	moderate	0.67	0.41	0.80	0.47	0.83	0.78	$F_{(2,6)}$= 0.087, p=0.917	*T2* $F_{(1,3)}$= 0.130, p=0.742	20.11	0.55	0.58	0.43	0.30	0.50	0.03	$F_{(2,2)}$= 0.323, p=0.756	21.40	*T2* $F_{(1,1)}$= 0.194, p=0.736	$F_{(2,8)}$= 0.035, p=0.965	$F_{(2,8)}$= 0.288, p=0.757
									T3 $F_{(1,3)}$= 0.521, p=0.523	24.18								7.97	*T3* $F_{(1,1)}$= 0.360, p=0.656		
	vigorous	1.13	1.39	0.85	0.95	0.87	1.28	$F_{(2,6)}$= 1.078, p=0.398	*T2* $F_{(1,3)}$= 1.052, p=0.380	24.34	0.96	0.83	1.51	1.63	1.17	1.14	$F_{(2,12)}$= 1.731, p=0.218	56.81	*T2* $F_{(1,6)}$= 2.032, p=0.204	$F_{(1.3,11.2)}$= 0.326, p=0.628	$F_{(1.3,11.2)}$= 1.841, p=0.204
									T3 $F_{(1,3)}$= 5.336, p=0.104	22.77								21.50	*T3* $F_{(1,6)}$= 1.502, p=0.266		
└ TIGIT-IL-6Rhi CCR6-	moderate	1.33	1.28	1.56	1.30	1.40	1.47	$F_{(1,3)}$= 0.362, p=0.590	*T2* $F_{(1,3)}$= 0.281, p=0.633	17.23	1.08	0.34	1.45	1.15	0.90	0.05	$F_{(2,2)}$= 0.577, p=0.634	33.36	*T2* $F_{(1,1)}$= 0.852, p=0.525	$F_{(1,4)}$= 1.096, p=0.355	$F_{(1,4)}$= 0.166, p=0.707
									T3 $F_{(1,3)}$= 0.829, p=0.430	5.03								16.70	*T3* $F_{(1,1)}$= 0.138 p=0.773		
	vigorous	1.59	1.26	1.60	0.69	1.06	0.95	$F_{(2,6)}$= 2.714, p=0.145	*T2* $F_{(1,3)}$= 0.000, p=0.996	0.13	0.84	0.82	1.27	0.90	1.04	0.77	$F_{(1.9,5.3)}$= 0.893, p=0.393	51.85	*T2* $F_{(1,5)}$= 0.943, p=0.376	$F_{(1.1,8.8)}$= 2.219, p=0.172	$F_{(1.1,8.8)}$= 1.539, p=0.251
									T3 $F_{(1,3)}$= 8.159, p=0.065	33.55								24.68	*T3* $F_{(1,5)}$= 0.844 p=0.400		

Table 47 CD4+ IL-6Rhi T-Regulatory subsets mobilised during moderate and vigorous intensity exercise in control and T1D groups

Mean, standard deviation, and statistical analysis of CD4+ IL-6Rhi T-Regulatory for control and T1D subjects during moderate and vigorous intensity exercise. Significant results highlighted in bold where p values <0.05 were considered significant.

[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2).

[b] Results were analysed using multiple regression analysis in control and T1D groups independently.

[c] Contrast displays changes from baseline (T1) to immediately post exercise (T2) and 1 hour post exercise (T3).

[d] Results were analysed using multiple regression analysis in control and T1D groups combined.

[1]Controls n=5-6, [2]T1D n =3-8

6.4 Discussion

The first aim of this study was to measure peripheral blood $CD4^+$ T-helper (Th) and T-Regulatory (TReg) subsets at rest in T1D and control cohorts. The proportion of $CD4^+$ TRegs was elevated in T1D compared to control participants. This is driven by an increase in mTREG subsets. The naïve-memory TReg ratio was shifted in T1D, with higher proportions of memory TRegs and lower proportions of naïve TRegs compared to control participants. A significant increase in $TIGIT^+$ mTRegs in T1D would support these findings because TIGIT is highly expressed by mTRegs [486]. However, only a non-statistical significant increase in $TIGIT^+$ mTRegs was found in this study herein. Additionally, $TIGIT^-IL\text{-}6R^{hi}$ mTRegs showed a trend to be lower in T1D. This agrees with reduced TReg suppressive capacity reported in T1D in other studies. No differences between groups in $CD4^+$ T-helper subsets were found.

This study also aimed to investigate the effects of moderate and vigorous intensity exercise on the mobilisation of $CD4^+$ T-helper and TReg subsets in T1D and control participants. As per the previous findings of this study (Chapter 4 and 5), vigorous intensity exercise caused a significant mobilisation of lymphocytes in both T1D and control participants, with minimal mobilisation during moderate intensity exercise. $CD3^+$ and $CD4^+$ T cells mobilised during vigorous intensity exercise only in the control group. There was no mobilisation of $CD4^+$ T cells or individual $CD4^+$ T cell subsets vigorous intensity exercise in T1D participants. Vigorous intensity exercise mobilised Th2 subsets, without changes in Th1 subsets, and this resulted in a shift in the Th1/Th2 ratio following vigorous exercise in the control group. No significant mobilisation of Th17 cells was found in T1D or control groups independently. However, there was significant mobilisation overall (i.e. groups combined) following vigorous exercise. There was a larger percentage increase in the frequency of Th17 cells following vigorous exercise in T1D compared to the control group, but this was not significant. Vigorous

intensity exercise mobilised $CD4^+$ TReg subsets in control participants, and these comprised of mainly memory TRegs, including both $TIGIT^+$ and $TIGIT^-$ mTReg subsets. Neither moderate or vigorous intensity exercise significantly mobilised any $IL\text{-}6R^{hi}$ $CD4^+$ subsets in T1D or control groups.

The strengths of this study are that it is the first study of acute exercise and $CD4^+$ T cell subset mobilisation in T1D. This study gives a comprehensive analysis of $CD4^+$ T-helper and TReg cell subsets mobilised by two different exercise intensities, in two cohorts. However, the limitations of this study were that the numbers of recruited participants were low therefore comparisons between groups are difficult. In Chapter 4 and 5, $CD3^+$ T cell mobilisation is shown in both T1D and control groups. However, in this analysis $CD3^+$ T cells were mobilised by vigorous exercise in the control group only. One T1D participant in particular responded negatively to vigorous exercise indicated by a decrease in $CD3^+$ and $CD4^+$ T cells immediately following exercise. This participant was not excluded because normal lymphocytosis occurred post-exercise and the CD3/CD4 whole blood staining appeared normal in this sample. This participant exhibits the same response in the data shown in Chapter 5; however there is less data for $CD4^+$ T-helper/TRegs subsets due to missing stains for this analysis and so statistical significance is affected greatly by this variation. Excluding this participant from analysis shows that $CD3^+$ T cells mobilised significantly during vigorous exercise in T1D, whereas $CD4^+$ T cells remained insignificant, agreeing with data shown in Chapter 5. This demonstrates the issues with small sample size affecting significance levels. It is likely that this decrease in $CD3^+$ T cells is due to a significant increase in the frequency of $CD3^-$ NK cells [198, 199]. Further analysis of subsets clarified that this is the case for this participant.

$IL\text{-}6R^{hi}$ $CD4^+$ TRegs showed a trend towards an increase during vigorous exercise in T1D but not in the control group. A larger sample size may have provided statistical significance. $CD4^+$

TReg subsets mobilised significantly following vigorous intensity exercise in control but not T1D participants. However, the difference in mobilisation was not significant between groups. The percentage increase of $CD4^+$ T cells following vigorous exercise was similar in T1D compared to the control group. It is possible with a larger cohort that exercise would have induced $CD4^+$ T cell mobilisation in T1D participants as shown for control participants. On the other hand, the difference in mobilisation may be significant between groups in a larger cohort leading to further questions about why $CD4^+$ T cells mobilisation is hindered in T1D.

Previously, studies have demonstrated that vigorous endurance exercise causes a significant increase in the frequency of peripheral blood lymphocytes, followed by lymphopenia, in healthy cohorts [191-195]. These findings are supported by this study herein and observed for the first time in T1D. The data shown also agrees with previous studies because only minor exercise-induced mobilisation of $CD4^+$ T cells in control cohorts has been reported [199, 257]. As discussed in Chapter 5, a blunted antigen-experienced $CD8^+$ T cell egress in T1D may be indicative of sequestration in the pancreas during vigorous exercise. This may also be true for $CD4^+$ T cells which show no significant mobilisation following vigorous exercise in T1D participants. As well as antigen-experienced $CD8^+$ T cells, $CD4^+$ T cells are also involved in the pathogenesis of T1D and therefore a proportion of the $CD4^+$ T cells, which would be expected to mobilise in healthy participants, may be withheld in the pancreas during vigorous exercise in T1D [122, 123].

A shift in the Th1/Th2 ratio indicated by increased Th2 cytokine levels has been demonstrated following an acute exercise bout in healthy cohorts. This is typically a result of lower Th1 T cells and Th1 cytokines, paired with an increase in Th2 cytokines only, not Th2 T cells [453, 454, 518, 519]. In this study herein, the Th1/Th2 ratio is also shifted towards Th2. However, in contrast to the above studies, no overall change in Th1 cells during exercise was found in

this study herein. Mobilisation of Th2 cells however is shown in this study. This resulted in a shift in the Th1/Th2 ratio. Plasma samples from this study have been cryo-preserved to measure T-helper cytokine patterns following moderate and vigorous exercise. This would provide insight into the Th1/Th2 shift following exercise in T1D.

Few studies have measured Th17 cell mobilisation during acute exercise. Evidence of the effects of acute exercise on Th17 immunity has centred around IL-17 measurement [522, 523]. One study measuring Th17 cells reported a decrease in the percentage of Th17 after endurance exercise [521]. However, endurance exercise involving marathon running and triathlons as shown in the aforementioned study has to some extent different effects on lymphocytosis compared to a short acute vigorous exercise bout due to differences in adrenaline responses [521]. Th17 cell frequencies significantly changed overall during vigorous exercise in this study, but not in either T1D or control groups independently. The cryopreserved plasma samples from this study can also be used to measure Th17 related cytokines, including IL-17, following moderate and vigorous exercise. This would provide further evidence of the effects of acute exercise on Th17 immunity in both healthy and T1D cohorts.

Previous studies report conflicting results on the effects of exercise on $CD4^+$ TReg subsets in control cohorts [455-457, 521, 524]. Some suggest that the percentage of $CD4^+$ TRegs decrease after endurance exercise [521]. As discussed, endurance exercise and short acute vigorous exercise bouts can cause different levels of lymphocytosis dependent on intensity and duration. However, acute exercise increased absolute $CD4^+$ TReg frequencies post exercise in trained athletes and control participants [455-457]. In this study herein, $CD4^+$ TRegs mobilised in control but not T1D participants, and these were mainly comprised of memory TRegs. One study previously reported increases in naïve TReg frequencies, but not memory TReg frequencies, following low intensity endurance exercise. However, baseline samples were

taken 12-36 hours before exercise began and the exercise consisted of 30km walking per day for 4 consecutive days. This would not be considered an acute exercise bout or comparable to the exercise protocol used in this study herein [524].

The effects of acute exercise on IL-6R^{hi} TReg subsets have not previously been examined. In this study herein, exercise did not significantly mobilise IL-6R^{hi} subsets in either T1D or control cohorts. As IL-6R is highly expressed on mTReg, which mobilised with vigorous exercise, mobilisation of IL-6Rhi subsets may be expected. However, IL-6R is shed by activated CD4^{+} T cells during shear stress; therefore CD4^{+} mTRegs may shed IL-6R during vigorous exercise. This is supported by elevated sIL-6R levels found following exercise [529, 530]. Elevated IL-6 levels previously shown during exercise could potentially interact with sIL-6R rather than IL-6Rhi subsets. This may explain why hypothesised changes in IL-6Rhi subsets are not detected during exercise [530, 531].

The implications of acute exercise on CD4^{+} T-helper and TReg subsets in T1D are not clear from this study because no significant mobilisation of CD4^{+} subsets was observed. However, evidence of elevated memory TRegs, and a subsequent decrease in naïve TRegs, reinforces TReg dysfunction/imbalance in T1D. Naïve-memory T cell balance is also disrupted in the ageing population and a similar observation has been found in T1D [419]. The baseline differences shown in this study herein support these findings. The proportion of naTRegs (CD45RA^{+}) decrease, whereas mTRegs (CD45RO^{+}) increase significantly with age [487]. Elevated mTRegs have a reduced proliferative capacity in the ageing group, indicating limited turnover of the TReg pool [487]. Assuming similarities, boosting naïve TRegs in T1D could restore naïve/memory TReg balance and improve turnover of the TReg pool. One such therapy, aldesleukin (Proleukin; recombinant human IL-2), increases suppressive TReg populations in melanoma [532]. Administration of IL-2 promoted TReg survival and protected NOD mice

from development of diabetes [533, 534]. The effect of IL-2 on TReg frequencies is likely due to increased conversion of effector T cells into TRegs [535]. Todd et al demonstrated that aldesleukin boosts TReg proportions by up to 20% in T1D cohorts. In the aforementioned study, they showed that mTRegs with efficient suppressive capacity (CTLA-4^+ $FoxP3^+$) were more responsive to IL-2 treatment due to higher CD25 expression than naTRegs [536]. Aldesleukin also upregulates CXCR3 and CCR6 on regulatory T cells, necessary for recruitment into inflammatory sites to exert their immunomodulatory effects [536].

The data presented here supports a case for exploration of $CD4^+$ T-helper and TReg subsets following exercise training in T1D. Clarification of the effects of exercise on $CD4^+$ T cell subsets in a larger T1D cohort is necessary. In this study, no significant mobilisation of $CD4^+$ T cells subsets during vigorous intensity exercise is shown in T1D participants. Regular exercise may however have a different effect. Additionally, TReg suppressive function should be assessed following regular exercise as a marker of change in immunomodulatory capacity of TReg subsets which would have positive implications in T1D. Increased TReg suppressive capacity in T1D would regulate effector diabetogenic T cell responses and therefore reduce beta cell destruction. TReg suppression can be measured in vitro by co-culturing autologous TRegs with effector T cells stained with CFSE. TReg suppression is defined as a reduction in the growth of T effector cells and therefore CFSE staining remains high.

Exercise training promotes Th2 polarization and increases in $CD4^+$ TReg frequencies [448, 456, 537, 538]. Elevated $CD4^+$ TReg frequencies have been found in trained humans and mice [456, 537]. A decrease in Th1 polarization indicated by lower IFN-γ levels was also found in one study [537]. An increase in the frequency of TRegs in the lungs and LN, but not the spleen, with improved suppressive function was reported in mice following 4 weeks of moderate intensity exercise training [538]. An increase in a Th2 cytokine profile was also noted [538].

Another study showed improved immune modulation by exercise in a mouse model of multiple sclerosis (MS), EAE, resulting in delayed onset of disease and increased T cell proportions with a regulatory phenotype [448]. These studies demonstrate that exercise training has the potential to modulate $CD4^+$ T cell immunity and increase tolerance in T1D.

In conclusion, T1D exhibits a skewed memory TReg phenotype i.e. elevated memory TReg and decreased naïve TReg subsets. Acute exercise does not mobilise $CD4^+$ T-helper or TReg subsets in T1D, but does so in control participants. A regular exercise study with a larger study cohort may provide insight into the reason for differential mobilisation of these subsets in T1D compared to control participants.

CHAPTER 7. GENERAL DISCUSSION

This study was undertaken to explore the effects of acute exercise on immunity in people with T1D. In particular, this study aimed to phenotype and characterise the effects of acute exercise on lymphocyte trafficking and lymphocyte subset mobilisation in T1D.

The most significant results were that acute exercise caused an intensity-dependent lymphocytosis in people with T1D. This is the first time this has been demonstrated in T1D. The subsets most sensitive to exercise-induced lymphocytosis in T1D were fully differentiated $CD8^+$ EMRA T cells and $CD56^{dim}CD16^{bright}$ NK cells. These subsets have been previously shown to preferentially mobilise in healthy cohorts. However, the percentage increase of these subsets was blunted in the T1D group compared to the control group following vigorous intensity exercise. There are a number of potential reasons for this blunted lymphocytosis response and are discussed below.

Firstly, blood glucose levels are elevated in people with T1D. High glucose conditions affect the mobilisation of several cell types and therefore may affect lymphocytosis. Elevated plasma glucose in T1D and T2D mouse models has been reported to impair $CD34^+$ hematopoietic stem progenitor cell (HSPC) and $CD45^+$ cell migration [539]. Fibroblast migration is also reduced in high glucose conditions resulting in impaired wound healing in diabetic rats [540]. Furthermore, fewer lymphocytes were found in the wound site of streptozotocin-induced diabetic mice compared to control mice, indicating lower migration of lymphocytes in T1D [541]. Therefore, high glucose levels in T1D may have an effect on lymphocyte migration during exercise. In contrast, however, similar lymphocytosis following exercise has been described to be similar in people with T2D compared to a control cohort following HIIT exercise [542]. In this study, baseline glycaemic control and HbA1c could be compared to the percentage increase of lymphocyte subpopulations following exercise. Correlations between HbA1c and percentage increase of lymphocyte subpopulations of interest may reveal the effect

of glucose levels and glycaemic control of lymphocytosis. However, this is difficult as the sample size is small and dividing the T1D cohort into groups would reduce the power greatly.

Secondly, the immune cell subsets which exhibit blunted lymphocytosis during vigorous exercise in T1D are those which typically exhibit a high level of beta-adrenergic receptor expression and thus a profound sensitivity to exercise-induced perturbations to catecholamines. Therefore, it is possible that the adrenaline response is blunted in T1D. NK cells and cytotoxic T cells express much higher levels of beta-adrenergic receptors than other mononuclear cells, causing their dramatic mobilisation during exercise [202-205]. Alterations in these receptors in T1D would alter the stress response to exercise. Reduced beta-adrenergic sensitivity of lymphocytes in T1D has been reported, resulting in a dampened adrenaline response during hypoglycaemic events [543-545]. During acute exercise, increased beta-adrenoceptor density and sensitivity of lymphocytes is noted in healthy participants. However, patients with congestive heart failure (CHF) exhibited a blunted increase in beta-adrenoceptor density and no increase in sensitivity [546]. A similar effect may be seen in T1D and may impact exercise-induced lymphocytosis. However, platelets increase during moderate and vigorous exercise in both control and T1D groups. Platelets also respond to adrenaline but are not blunted in this study herein [283, 284]. Therefore, the reduced adrenaline response may be specific to reduced beta-adrenoceptors on lymphocytes. In this study, beta-adrenoreceptor density on lymphocytes could be measured on cryo-preserved PBMC samples and systemic adrenaline could be measured in cryo-preserved plasma samples to address this mechanism.

Lastly, and most interestingly, the cell subsets exhibiting blunted lymphocytosis during vigorous exercise share characteristics with those sequestered in the pancreas during T1D [126, 127, 134, 435]. These include $CD8^+CD69^+$ T cells [85, 126], $CD8^+CD11b^+$ T cells [127, 134], highly differentiated memory T cells [131, 339, 422], and highly differentiated NK cells [112].

In this study herein, lymphocytosis of highly differentiated $CD8^+$ EMRA that express CD69 and CD11b was blunted immediately following vigorous exercise in T1D. NK cells, particularly $CD56^{dim}$ NK cell subsets, were also blunted immediately following vigorous exercise in T1D. Normally, tissue resident cells down-regulate S1PR which is necessary for egress from tissue into the blood stream [437]. It is therefore possible to postulate that the blunted increase following exercise in these subsets is due to their sequestration in the pancreas via down-regulation of S1PR. However, in humans this is difficult to measure. Diabetogenic T cells can be measured using tetramer staining by flow cytometry. This could be carried out on cryo-preserved PBMC samples to address this mechanism and assess the effect of acute exercise on the number of diabetogenic T cells in the peripheral blood. Furthermore, this could be studied using NOD mice. Diabetogenic T cells can be fluorescently tagged and tracked during acute vigorous exercise in NOD mice. In particular, the number of diabetogenic T cells in the pancreas of NOD mice before and after acute exercise could be quantified and therefore highlight whether they are sequestered in the pancreas during the acute exercise bout.

In addition to the mobilisation of NK cells and $CD8^+$ EMRA T cells, acute exercise also affected other immune parameters. Although $CD4^+$ T cell subset mobilisation was blunted in T1D, vigorous intensity exercise mobilised $CD4^+$ TReg subsets in control participants. Mobilised $CD4^+$ TRegs comprised mainly of memory TRegs. In addition to changes in lymphocytes, an increase in monocytes, granulocytes, and platelets was also found following vigorous exercise in both T1D and control groups. However, lymphocyte mobilisation during exercise exhibited the most pronounced response. Lastly, TEM and total adherence of lymphocytes to endothelium was affected by vigorous intensity exercise in T1D. Specifically, TEM was significantly suppressed during the recovery period following vigorous exercise in T1D. This is likely due to changes in the composition of the T cell repertoire and a reduction in adhesion molecules on T cells following vigorous exercise.

The strengths of this study are that it is the first comprehensive immunophenotyping analysis of lymphocyte mobilisation during acute exercise and provides imperative evidence of the effects of acute exercise on immunity in people with T1D. This study provides evidence of the immune response in T1D during two exercise intensities. Furthermore, measuring TEM of lymphocytes provides functional evidence of immunomodulation by exercise in T1D. This is complimented by quantifying lymphocyte trafficking signals such as chemokine receptors and adhesion molecules to identify the mechanisms by which transmigration of lymphocytes is modulated in T1D. Importantly, this provides a platform for future investigations of exercise in T1D, allowing for new avenues to be explored to increase the initial honeymoon phase following diagnosis [236], reduce disease severity, and ultimately for the treatment of T1D.

The limitations of this study are that the study groups were small, and low participant numbers increased the error of the data. Populations reported to be significantly mobilised overall, but not in either group independently, suggests that with a larger study group, significant mobilisation may be observed for these subsets in both groups individually. Moreover, due to the large number of parameters measured in a small study cohort, multiple testing may increase the chance of statistically significant changes. Therefore, to address this in future, significant changes should be defined by a lower p value (e.g. $p<0.001$). Another limitation due to the small sample size is the lack of adjustment for baseline values. Due to higher baseline values evident in T1D, this may impact the lower percentage change observed following exercise (referred to as regression to the mean). This could be due to disease duration or glycaemic control. In future this can be corrected for using analysis of covariance (ANCOVA), which adjusts for imbalances, such as disease duration or HbA1c levels, in baseline parameters between groups.

Furthermore, the analysis was limited by the number of parameters measurable by flow cytometry. Day to day variation of flow cytometry increases error and reduces sensitivity of detecting small subsets. However, in order to process fresh patient samples over the course of the study, this variation was inevitable. This variation made it difficult to compare data from moderate and vigorous intensity visits as they were conducted on separate days. New platforms, such as CyTOF, allow for a much larger scale single cell analysis of immune populations [547]. CyTOF is not limited by fluorophore-based detection; for this reason, there is increased sensitivity and reduced variability in longitudinal sample acquisition. Therefore, cryopreservation of samples may be more stable with CyTOF analysis, leading to the option of bulk sample analysis following the trial's cessation [548].

Another potential limitation of this study is that C-peptide was not measured. C-peptide is a measure of beta cell function and therefore indicates the degree of function in the remaining beta cells in T1D. If beta cell function is low in T1D, then improvements following exercise may not be evident. This may also increase variation in responses due to non-responders within the T1D group.

Mechanisms through which islet immunity can be modulated in people with T1D have been explored in this study. These mechanisms include mobilisation of highly differentiated T cells and reduced lymphocyte TEM. As previously discussed, lymphocyte trafficking and tissue redistribution is essential for immunosurveillance and regulation [191, 214]. Furthermore, the deletion of exercise sensitive EM and EMRA $CD8^+$ T cells following acute exercise has the potential to create "immune space" in people with T1D [216-221]. This immune space following exercise can be taken up by newly generated HSPC and reprogram immune memory in T1D [212, 213], reducing aggressive memory T cell phenotypes and therefore preserving residual beta cell function in T1D. Furthermore, the reduced TEM following exercise has the

potential to modulate egress of islet reactive T cells out of the circulation and into the islet [315].

Mechanisms through which acute exercise could be used in combination with current trial immunotherapies have also been explored. Some immunotherapeutic approaches to preserve beta cell function in T1D aim to reduce aggressive memory phenotypes, and promote the generation of new naïve cells in T1D. This has been achieved with acute exercise and exercise training in healthy cohorts. Therefore, exercise in T1D may be used as an adjunct for other immunotherapeutic agents. As discussed, agents that target $CD8^+$ T cells may be more effective if administered following a bout of vigorous intensity exercise when $CD8^+$ T cells are mobilised [149-153]. Furthermore, exercise also increases regulatory cytokines such as IL-1RA. The increases in IL-1RA reported following acute exercise are comparable, and may be higher, than those seen in trials of immunomodulation in T1D with synthetic IL-1RA therapy [167, 227].

This study provides for the first time a clear and detailed analysis of the effects of an acute bout of exercise at two different intensities in people with T1D. The findings support the potential for exercise to modulate the autoimmune response against the islet in T1D. However, a number of important questions remain. Chief amongst these questions is clarifying the effects of exercise on T cells specific to the beta cell, because these cells have a direct bearing on islet immunity. Other questions include those relating to the mechanisms underlying the blunted lymphocytosis following vigorous intensity exercise in T1D. Lastly, the question of the effects of regular exercise on immunity and beta cell health in people with T1D needs to be explored. These questions can be addressed in the future work listed below.

<u>Identify mechanisms of blunted lymphocytosis in T1D:</u>

- **Is the blunted lymphocytosis in T1D glucose or immune dependent?** This can be explored using T2D as a control group during acute exercise for elevated glucose levels.

- **Is the blunted lymphocytosis in T1D due to a blunted adrenaline response?** Plans to measure soluble catecholamines were in original experimental design. Plasma and PBMC samples were taken for the measurement of cortisol and adrenaline. For future work, this can be done by measuring systemic adrenaline (plasma catecholamine measurement by ELISA or Luminex) and beta-adrenergic receptor density measurement on lymphocytes (flow cytometry) using the stored plasma and PBMC samples.
- **Is the blunted lymphocytosis in T1D due to sequestration for islet specific T cells in the pancreas in T1D?** This could be explored by measuring islet specific T cells following acute exercise using tetramer staining on stored frozen PBMC samples (flow cytometry). This may highlight whether islet specific T cells increase following exercise in T1D, and if not this may indicate that islet specific T cells are sequestered in the pancreas during acute exercise in the T1D cohort. Furthermore, this could specifically be examined in murine T1D models using fluorescent dyes to track cell mobilisation. In future work, diabetogenic T cells from NOD mice could be tagged with a bioluminescent dye. Live imaging could be used to identify diabetogenic T cell within the pancreas before and after acute exercise. At the end of the experiment, organs should be harvested to quantify diabetogenic T cell within the pancreas and measure T cell infiltration into multiple organs including the pancreas.

<u>Identify immunomodulatory mechanisms to preserve beta cell health in T1D:</u>

- **Does acute exercise modulate systemic inflammatory responses in T1D?** Plans to measure inflammatory markers and hormones were in the original experimental design. Plasma samples were taken for the measurement of IL-6, IL-6R, GLP1, FGF21, Betatrophin, Irisin, Osteocalcin, BDNF, Adiponectin, Adiponectin Receptors, Metrnl,

IL-1Rα, IL-10, TNFα, Leptin, IL7, Cortisol, Glucagon, Resistin, Visfatin, Growth Hormone, IGF, IL-4, IL-13, and IL-17. The above soluble cytokines, adipokines, myokines, and hormones can be measured in the stored plasma samples by Luminex. This would provide more depth to the anti-inflammatory and immunomodulatory changes acute during exercise that may affect beta cell health in T1D.

Furthermore, serum samples were taken and stored to assess the impact of exercise on beta cell models in vitro. Beta cell models such as MIN6 and INS-1E cell lines can be grown in rest or exercise-conditioned media. Beta cell health outcome measures such as insulin secretion, viability, and proliferation can be assessed and then compared based on culture conditions to elucidate mechanisms by which exercise can improve beta cell health. This data could be used to plan future experiments to block specific cytokines or hormones identified by Luminex to elucidate mechanisms by which exercise could improve beta cell health.

- **Can acute exercise be used as an adjunct for T1D immunotherapy?** This could be explored through a trial combining vigorous acute exercise with immunotherapies targeting $CD8^+$ T cells, providing useful implications for advances in immunotherapy in T1D.

<u>Identify mechanisms of immunomodulation and beta cell preservation by regular exercise training in T1D:</u>

- **Can regular exercise training modulate immunity and preserve residual beta cell function in T1D?** This could be explored in NOD mice and T1D cohorts. These studies should include characterisation of changes to immune cell subsets and systemic inflammation, paired with C-peptide (beta cell function) measurements. Functional measurements to identify mechanisms of immunomodulation should also be explored.

These include lymphocyte trafficking and TEM, apoptosis of memory T cells following exercise, stem cell generation, and changes in islet specific T cell frequencies. This would follow up immunomodulation and immune space theories in T1D models and highlight the impact of exercise on regulating immunity and beta cell health in T1D.

Publications:

Published

- Narendran P, Jackson N, Daley A, Thompson D, Stokes K, Greenfield S, Charlton M, Curran M, Solomon TPJ, Nouwen A, Lee SI, Cooper AR, Mostazir M, Taylor RS, Kennedy A, Andrews RC. **Exercise to preserve beta-cell function in recent-onset Type 1 diabetes mellitus (EXTOD) - a randomized controlled pilot trial**. *Diabet Med*, 2017. **34**(11): p. 1521-1531. DOI: 10.1111/dme.13439

- Curran M and Narendran P, **Medical Benefits of Physical Fitness: Exercise and Diabetes,** *Medical Science for the Curious: Why Study Medical Science?* Chapter 5, 2015.

In Progress

- Curran M, Campbell JP, Narendran P **Type 1 Diabetes impairs CD8 T cell mobilisation during vigorous exercise**. Submitted: *Exercise Immunology Review*, September 2018.

- Curran M, Campbell JP, Narendran P **The mobilisation of highly differentiated NK cells during vigorous exercise is blunted in Type 1 Diabetes**. Planned submission: *Medicine and Science in Sport and Exercise*, December 2018.

- Curran M, Campbell JP, Chimen M, Narendran P **Vigorous exercise modulates transmigration of T cells in Type 1 Diabetes through a reduction in adhesion molecules**. Planned submission: *TBC*, 2018.

- Curran M, Campbell JP, Drayson MT, Andrews RC, Barlow JP, Solomon TPJ, Narendran P **Exercise for beta cell health: a systematic review of the literature and underlying mechanisms**. Submitted: *Frontier in Endocrinology*, September 2018.

Conference Proceedings:

Oral presentations

- **Does organ specific T cell autoimmunity impair the redistribution of T cells following exercise? The effects of acute exercise on peripheral blood T cells in patients with type 1 diabetes (T1D)** Curran M, Campbell JP, Narendran P. UK Society for Exercise Immunology 2018, Loughborough, UK – oral presentation.

Poster presentations

- **Exploring the mechanisms through which acute exercise affects transendothelial migration of lymphocytes in healthy and type 1 diabetes (T1D)** Curran M, Campbell JP, Drayson MT, Narendran P. Cell Symposium Exercise Metabolism 2017, Gothenburg, Sweden – poster presentation.

- **Exploring the mechanisms through which acute exercise affects transendothelial migration of lymphocytes in healthy and type 1 diabetes (T1D)** Curran M, Campbell JP, Drayson MT, Narendran P. Festival of Graduate Research, 2017, Birmingham, UK – poster presentation. **2017 Best Poster Presentation award.**

- **Exploring the mechanisms through which acute exercise affects transendothelial migration of lymphocytes in healthy and type 1 diabetes (T1D)** Curran M, Campbell JP, Drayson MT, Narendran P. Diabetes UK 2017, Manchester, UK – poster presentation.

- **Exploring the mechanisms through which exercise influences beta cell health in Type 1 diabetes (T1D)** Curran M, Campbell JP, Drayson MT, Gleeson M, Narendran P. Diabetes UK 2016, Glasgow, UK – poster presentation.

Prizes

- **Best Poster Presentation award,** Festival of Graduate Research, 2017.

Additional Certified Training:

- Introduction to Metabolomics for the Clinical Scientist, Birmingham Metabolomics Training Centre, 2017.

- Introduction to Good Clinical Practice (Primary Care), 2016.

- Phlebotomy training, University Hospital Birmingham, 2015.

CHAPTER 8. REFERENCES

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SUPPLEMENTARY

1. **R&D REC ethics approval**
2. **EXTOD Mechanisms protocol**
3. **Participant information sheet (T1D)**
4. **Participant information sheet (Healthy)**
5. **Consent Form**
6. **GP Letter**
7. **Case Report Form (baseline)**
8. **Case Report Form (exercise visits)**
9. **Psychological Stress Questionnaires**
10. **International Physical Activity Questionnaire**

NHS
Health Research Authority

[redacted]

14 March 2016

[redacted]

Dear [redacted]

Study title:	**Exploring the effect of exercise on immune function and beta cell health in patients with type 1 diabetes mellitus**
REC reference:	**16/NW/0051**
IRAS project ID:	**189283**

Thank you for your letter of 14 March 2016, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager, [redacted], [redacted]

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact , the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering letter on headed paper [Cover letter for ethics re-submission]	1	26 February 2016
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [insurance confirmation]		07 January 2016

GP/consultant information sheets or letters [GP letter]	1	01 December 2015
IRAS Checklist XML [Checklist_04032016]		04 March 2016
Letter from sponsor [sponsorship letter]		07 January 2016
Other [clarification on recruitment]		
Participant consent form [Consent Form]	2	16 February 2016
Participant information sheet (PIS)	3	11 March 2016
Participant information sheet (PIS) [Healthy Subjects]	1	16 February 2016
REC Application Form [REC_Form_08012016]		08 January 2016
Research protocol or project proposal [Protocol]	3	16 February 2016
Summary CV for Chief Investigator (CI) [CV CI]	1	19 January 2015
Summary CV for student [CV student researcher]	1	01 December 2015

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely

Signed on behalf of:

Chair

Email:

Enclosures: "After ethical review – guidance for researchers"

Copy to:

University Hospitals Birmingham NHS Foundation Trust

Exploring the effect of exercise on immune function and beta cell health in patients with type 1 diabetes mellitus

Participant information sheet for T1D participants

Version 3
11/3/2016

Location(s): Wellcome Trust Clinical Research Facility, University Hospital Birmingham.

Investigators: Michelle Curran, Dr. Parth Narendran, Dr. John Campbell, Professor Mark Drayson.

Study aims: This study aims to investigate how exercise can improve the health of the insulin producing beta-cells in healthy people and people with type 1 diabetes (T1D). This may help improve their diabetes control and reduce the risk of long-term complications.

Study Background:

There are two types of diabetes mellitus; type 1 diabetes (T1D) and type 2 diabetes (T2D). Both types of diabetes have different causes but similar symptoms.

The pancreas contains cells, known as beta-cells, which ensure blood glucose levels are maintained at a normal level through the secretion of insulin. Insulin transports glucose out from the bloodstream into tissues. If glucose is not removed from the bloodstream, it becomes toxic, a state referred to as hyperglycaemia. Hyperglycaemia results in the hallmark symptoms of diabetes including increased thirst, fatigue, and frequent urination. Chronic hyperglycaemia causes complications such as death, eye, kidney, and nerve damage.

T1D solely results from a lack of insulin. It is an autoimmune disorder, meaning that it is caused by our immune system destroying native tissue, in this case, beta cells. Consequently, there is no insulin production.

Exercise has marked improvements in fitness, insulin requirements, mortality and well-being as well as a reduction in fat, insulin resistance, and cardiovascular disease in T1D. Whilst there is some understanding that exercise improves the health of beta cells in patients with type 2 diabetes, little is known of the benefits in T1D. We would like to explore how exercise improves beta cell health in patients with T1D and healthy people.

At the time of diagnosis, a T1D patient will have some of their beta cells intact, but these remaining beta cells will also be destroyed gradually in the years that follow the diagnosis of T1D being make. Uptake of exercise early after diagnosis with T1D could therefore help preserve these beta cells, and we know that preserving beta cell function will help the management of diabetes.

During exercise a variety of substances and hormones are produced by the liver, muscles, and fat, which signal to beta cells to produce insulin and stop the autoimmune process. In this study we would like to identify these substances to better understand the mechanisms through which exercise could benefit patients with T1D. We will be undertaking this research in patients with new as well as long standing diabetes, as well as in patients without diabetes as a comparison.

Benefits:

A free fitness test is included in the first visit. We all also measure general health, for example blood pressure and heart rate. Once the study has finished, we will provide advice on options to continue to exercise and advised quantity of exercise to benefit your health and T1D.

Participating in the study will only cost you some of your time, **all travel costs upto the value of £10** for each visit will be compensated for at the end of the visit.

Study design:

If you agree to take part in the study and meet the inclusion criteria, you will be required to attend the study centre on three separate occasions. All health and fitness assessments will be performed at the Wellcome Trust Clinical Research Facility at University Hospital Birmingham.

Visit 1: Enrolment & Baseline visit

In this visit we will collect your informed consent to participate in the study. For patients with T1D, HbA1C will be checked if not done within the previous 6 weeks. We will also ask you to wear an activity meter to measure how much activity you are doing.

We will provide food diaries for you to record your meals and also as a reference to the meal you had before exercise study visits. We would like you to have the same meal each of the exercise visits to control for any dietary influences. This means the same quantity of the meal also.

Blood glucose diaries will also be provided. You will be asked to measure your glucose at least 4 times a day during the study (before each meal, before bed, and any time when you are feeling unwell or it is appropriate to do so).

You will undergo a fitness test on a stationary exercise bike in the exercise rooms of the NIHR/WT CRF at The Queen Elizabeth Hospital in Birmingham. This will be used as a guide for the required exercise intensity for the subsequent exercise study visits where you will partake in light and high exercise intensities.

Visit 2-3: Exercise Study Visits

These visits will lasts approximately 6 hours. You will be involved in three exercise study visits, each separated by between 7-21 days. During these visits we will take a small amount of blood at four different times (out-lined in the time plan). The exercise visits will consist of

30 minutes of either light or heavy exercise. The order of these visits will be randomised so you will only know what intervention you are doing on the day of the visit.

For all visits, you must be fasted (without having consumed food (water allowed) since midnight the night before). The meals consumed within the previous 24 hours (recorded in your food diary) should be repeated for the 24 hours preceding each of the other two study visits. You will be encouraged to undertake your usual levels of physical activity between study visits (measured by the actigraph), but you must refrain from exercise for a 24-hour period before an exercise study visit. For patients with T1D, basal insulin (with either pens or insulin pump) is encouraged to be taken as usual. ‘’

After the intervention glucose will be monitored post-exercise every 20 minutes until the hour blood sample. If your blood sugar drops to 5mM before the 60 minutes post exercise time-point, a blood sample will be and the subject will be fed.

Example time-plan of the day of exercise study visit:

8.00am – Arrive at WT CRF Reception

8.15am - Insert cannulae to take blood samples, wait 30 mins while you relax.

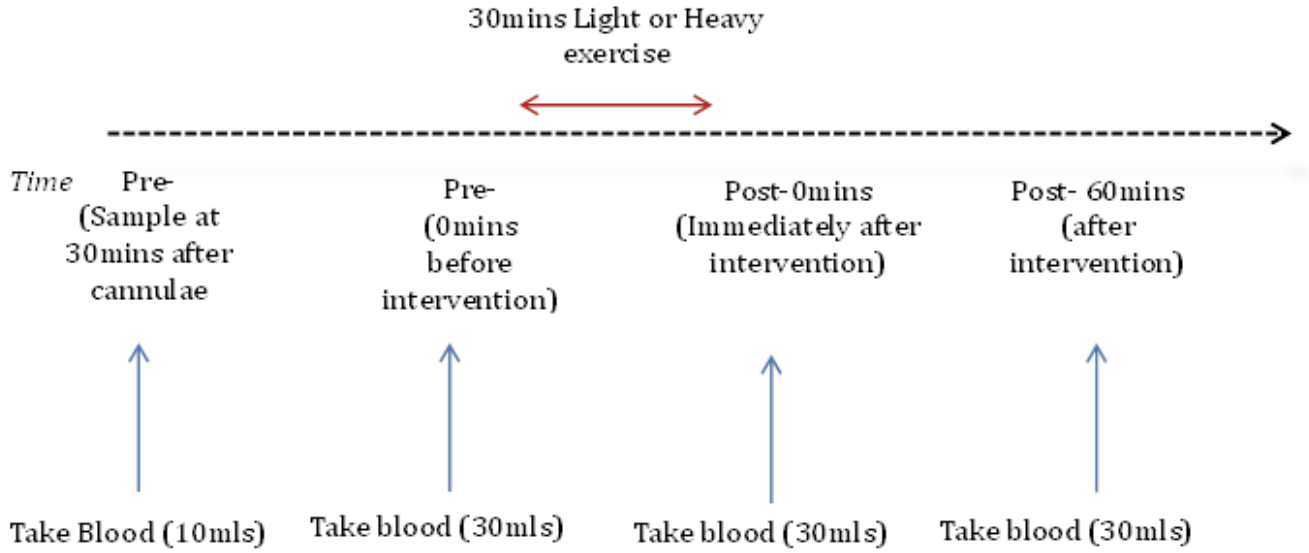
8.45am - Take resting blood sample.

9.05am - Take blood sample and start 30 min exercise intervention (light exercise or heavy exercise).

9.35am - Take blood sample at end of exercise.

10.35am - Take blood sample 60 minutes after end of exercise.

10.45am - Provide breakfast/lunch. For patients with T1D, we will check your blood glucose is stable. We also will advise you to test blood glucose 6-8 hours post exercise and consume carbohydrates to avoid post-exercise hypoglycaemia. Arrange next visit and pay for travel.



Total amount of blood taken per visit: 100ml. 100ml is about 6-7 tablespoons.

For your information:

Do I have to take part in the study?

No. Taking part in this study is entirely voluntary. If you would like to participate, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason.

May I be excluded from the study?

Yes. You can be excluded from the study at any time point if something happens that affects your eligibility for the study or you are unable to complete the study.

Confidentiality:

All data and samples will be labeled with a code, not with your name. Only the research team which is in contact with you has access to the link between code and name of the subject. The results of this study are expected to be published in a scientific journal, but names of participants will never be published.

Rights:

It is your choice whether or not you wish to take part in this study. If you wish to take part in this study, you will be given this information sheet to read and be asked to sign a consent form. You are reminded that if you decide to take part in the study, you are still free to withdraw from the study at any time without giving a reason. If you withdraw from the study, the data and blood samples already collected will be retained, unless you request for them to be removed within 12 weeks of the first visit.

What happens if something goes wrong on the day of the trials?

All procedures have been included within University Hospital Birmingham Liability insurances and if you are harmed in any way by taking part in this research project your normal rights apply and you may have ground for legal action.

Complaints

If you have any problems or complaints during the study procedure, you are welcome to contact either the investigator, or independently the hospital PALS (patient advice and liaison service - email PALS@uhb.nhs.uk, tel. 0121 371 3280)

What happens now?

You will be asked to complete an informed consent form to confirm that you are happy to participate in this study. You will be asked to keep a copy of this information sheet and the signed consent forms. We will then inform your GP that you have decided to take part in this study.

Thank you for your time, and if you want to participate in the study or have any further questions, please feel free to contact any of the investigators listed below. If you wish to discuss this with others, contact your GP.

Exploring the effect of exercise on immune function and beta cell health in patients with type 1 diabetes mellitus

Participant Information Sheet for Healthy Participants

Version 2
24/08/2016

Location(s): Wellcome Trust Clinical Research Facility, University Hospital Birmingham.

Investigators: Michelle Curran, Dr. Parth Narendran, Dr. John Campbell, Professor Mark Drayson.

Study aims: This study aims to investigate how exercise can improve the health of the insulin producing beta-cells in healthy people and people with type 1 diabetes (T1D). This may help improve their diabetes control and reduce the risk of long-term complications.

Study Background:

There are two types of diabetes mellitus; type 1 diabetes (T1D) and type 2 diabetes (T2D). Both types of diabetes have different pathogenesis but similar symptoms.

The pancreas contains cells, known as beta-cells, which ensure blood glucose levels are maintained at a normal level through the secretion of insulin. Insulin transports glucose out from the bloodstream into tissues. If glucose is not removed from the bloodstream, it becomes toxic, a state referred to as hyperglycaemia. Hyperglycaemia results in the hallmark symptoms of diabetes including increased thirst, fatigue, and frequent urination. Chronic hyperglycaemia causes complications such as death, eye, kidney, and nerve damage.

T1D solely results from a lack of insulin. It is an autoimmune disorder, meaning that it is caused by our immune system destroying native tissue, in this case, beta cells. Consequently, there is no insulin production.

Exercise has marked improvements in fitness, insulin requirements, mortality and well-being as well as a reduction in fat, insulin resistance, and cardiovascular disease in T1D. Whilst there is some understanding that exercise improves the health of beta cells in patients with type 2 diabetes, little is known of the benefits in T1D. We would like to explore how exercise improves beta cell health in patients with T1D and healthy people.

At the time of diagnosis, a T1D patient will have some of their beta cells intact, but these remaining beta cells will also be destroyed gradually in the years that follow the diagnosis of T1D being make. Uptake of exercise early after diagnosis with T1D could therefore help preserve these beta cells, and we know that preserving beta cell function will help the management of diabetes.

During exercise a variety of substances and hormones are produced by the liver, muscles, and fat, which signal to beta cells to produce insulin and stop the autoimmune process. In this study we would like to identify these substances to better understand the mechanisms through which exercise could benefit patients with T1D. We will be undertaking this research in patients with new as well as long standing diabetes, as well as in patients without diabetes as a comparison.

Benefits:

A free fitness test is included in the first visit. After following your routine of activity through a device called actigraph, we will be able to advise you on changes you can make to increase your participation in exercise as part of your daily life. We all also measure general health, for example blood pressure and heart rate.

Once the study has finished, we will provide advice on options to continue to exercise and advised quantity of exercise to benefit your health and T1D.

Participating in the study will only cost you some of your time, **all travel costs** for each visit will be compensated for at the end of the visit.

Study design:

If you agree to take part in the study and meet the inclusion criteria, you will be required to attend the study centre on four separate occasions. All health and fitness assessments will be performed at the Wellcome Trust Clinical Research Facility at University Hospital Birmingham.

Visit 1: Enrolment & Baseline visit

In this visit we will collect your informed consent to participate in the study. HbA1C will be checked in all participants, healthy and T1D. This is to exclude any underlying T2D that may not have been diagnosed.

We will provide food diaries for you to record your meals and also as a reference to the meal you had before exercise study visits. We would like you to have the same meal each of the exercise visits to control for any dietary influences.

You will undergo a fitness test on a stationary exercise bike in the exercise rooms of the NIHR/WT CRF. This will be used as a guide for the required exercise intensity for the subsequent exercise study visits where you will partake in light and high exercise intensities.

Visit 2 + 3: Exercise Study Visits

These visits will lasts approximately 6 hours. You will be involved in three exercise study visits, each separated by between 7-21 days. During these visits we will take a small amount of blood at five different times (out-lined in the time plan). The exercise visits will consist of 30 minutes of either light or heavy exercise. The order of these visits will be randomised so you will only know what intervention you are doing on the day of the visit.

For all visits, you must be fasted (without having consumed food (water allowed) since midnight the night before). The meals consumed within the previous 24 hours (recorded in

your food diary) should be repeated for the 24 hours preceding each of the other two study visits. This means same quantity of this meal. You will be encouraged to undertake your usual levels of physical activity between study visits (measured by the actigraph), but you must refrain from exercise for a 24-hour period before an exercise study visit.

Example time-plan of the day of exercise study visit:

8.30am - Arrive at WT CRF Reception

8.45am - Insert cannulae to take blood samples, wait 30 mins while you relax.

9.00am - Take resting blood sample.

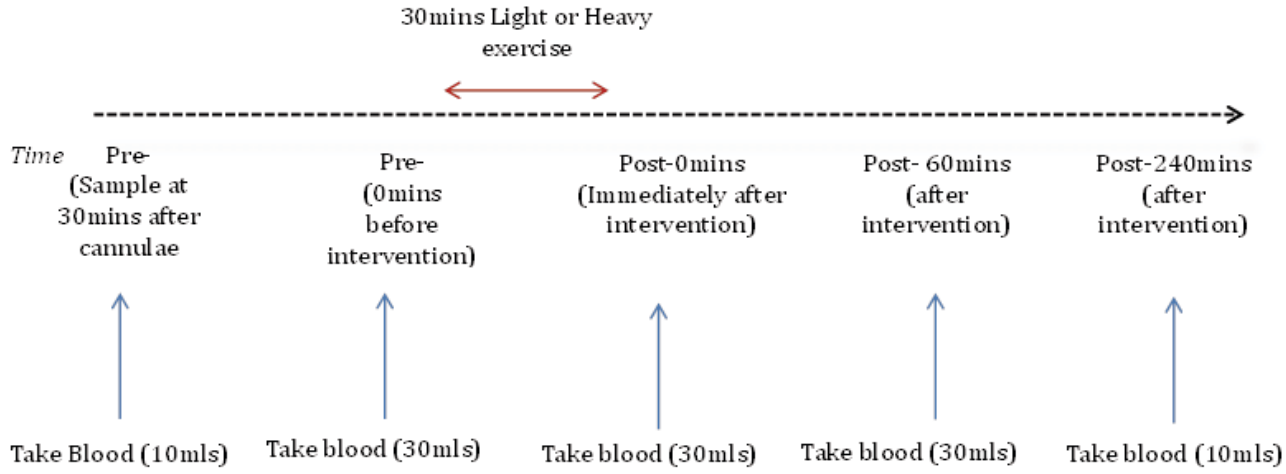
9.20am - Following 20minute rest, take blood sample and start 5 min warm up and 30 min exercise intervention (light exercise or heavy exercise).

9.55am - Take blood sample at end of exercise.

10.55am - Take blood sample 60 minutes after end of exercise.

13.55pm - Take blood sample 240 minutes post exercise.

14.00pm - Provide breakfast/lunch. Arrange next visit and pay for travel.



Total Blood Volume per visit: 110mls, about 6-7 tablespoons.

For your information:

Do I have to take part in the study?

No. Taking part in this study is entirely voluntary. If you would like to participate, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason.

May I be excluded from the study?

Yes. You can be excluded from the study at any time point if something happens that affects your eligibility for the study or you are unable to complete the study.

Confidentiality:

All data and samples will be labeled with a code, not with your name. Only the research team which is in contact with you has access to the link between code and name of the subject. The results of this study are expected to be published in a scientific journal, but names of participants will never be published.

Rights:

It is your choice whether or not you wish to take part in this study. If you wish to take part in this study, you will be given this information sheet to read and be asked to sign a consent form. You are reminded that if you decide to take part in the study, you are still free to withdraw from the study at any time without giving a reason. If you withdraw from the study, the data and blood samples already collected will be retained, unless you request for them to be removed within 12 weeks of the first visit.

What happens if something goes wrong on the day of the trials?

All procedures have been included within University Hospital Birmingham Liability insurances and if you are harmed in any way by taking part in this research project your normal rights apply and you may have ground for legal action.

Complaints

If you have any problems or complaints during the study procedure, you are welcome to contact the investigators.

What happens now?

You will be asked to complete an informed consent form to confirm that you are happy to participate in this study. You will be asked to keep a copy of this information sheet and the signed consent forms. We will then inform your GP that you have decided to take part in this study.

Thank you for your time, and if you want to participate in the study or have any further questions, please feel free to contact any of the investigators listed below. If you wish to discuss this with others, contact your GP.

CONSENT FORM
Version 2.1 (18/8/2016)

Title of Project: Exploring the effect of exercise on immune function and beta cell health in patients with type 1 diabetes mellitus

The EXTOD Mechanisms study

Name of Researcher: Dr Parth Narendran

Please initial box

1.	I confirm that I have read and understand the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. Version: ____ Date: _____ Healthy Volunteer/ Type 1 diabetes (delete as appropriate)	☐
2.	I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	☐
3.	I understand that individuals may look at relevant sections of my medical notes and data collected during the study, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	☐
4.	I agree to the taking and storage of blood samples for the purposes of this study	☐
5.	I agree to the taking and storage of DNA for the purposes of this and future studies	☐
6.	I agree to my GP being informed of my participation in the study	☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes.

GP Letter
Version 1 (18/6/2016)

Dear (insert doctor's name)

I am writing to advise you that (insert patient name) has agreed to participate in our EXTOD mechanisms exercise study. Please find attached the patient information sheet for details on the procedure. If you have any concerns for the health or wellbeing of your patient, please do not hesitate to contact the chief investigator.

Kind Regards,

_____

(Chief Investigator)

Clinical Senior Lecturer and Consultant in Medicine

Email:

The Institute of Biomedical Research, The Medical School, University of Birmingham, Edgbaston, B15 2TT, UK

Case Report Form (Baseline)

Participant ID: _____ Visit no._____ Date: _____

Inclusion Criteria

Tick the box to indicate YES this does apply

6. 16-65 years of age ☐
7. Male ☐
8. ≤150mins moderate intensity exercise plus 2 days of strengthening exercise per week. ☐
9. Safe to exercise ☐
10. In addition if subjects are in the T1D group, they will
    1. have been given a clinical diagnosis of T1D
    2. be on basal bolus insulin regime or insulin pump therapy ☐
    3. be able to estimate carbohydrate content of meals ☐
    4. be willing to test glucose through capillary testing and adjust insulin and carbohydrate doses accordingly ☐
    5. be able to recognise hypoglycaemic symptoms before capillary blood glucose falls to 3.0mmol/L ☐

Exclusion Criteria

Tick the box to indicate NO this does not apply.

6. Unable to provide fully informed consent. ☐
7. History of cardiac disease. ☐
8. Significant illness or condition that prevents attendance at a study site clinical centre.
9. PI feels that participation in the study would not be in the individual's best interest. ☐
10. In addition, if subjects are in the T1D group: ☐
    1. active proliferative diabetic retinopathy ☐
    2. autonomic neuropathy ☐
    3. history of severe hypoglycaemia requiring third party assistance within the last 3 months ☐

Received signed Consent ☐
Has the participant had caffeine in the last 12 hours? Yes/No
Has the participant had alcohol in the last 12 hours? Yes/No
Has the participant had breakfast this morning? Yes/No

Record participants anthropometric assessment:

Height: _____cm
Weight: _____kg
BMI:_____kg/m2
Waist: _____cm
Hip: _____cm
Chest: _____cm
Waist-to-hip ratio: (waist cm/hip cm) _____
Body Fat %: _____

Test for HbA1c in all participants: **3ml EDTA (purple top)** ☐

Fitness Test- Sub-maximal VO2 assessment

- Put polar watch and strap on participant
- Adjust seat and handle bar height (record for future visits)
  Seat: _____cm Handle bars: _____cm

  Target HR: (220-age) x 0.85=_____ _____bpm

- Begin fitness test:

Part 1:

- Set the workload at 50 – 100W (based on activity levels). Participant cycling @ 50rpm.
  WL _____ watts
  Resting HR _____ bpm (participant sitting on bike)
- Check HR and RPE at 2 mins *(if HR is <120bpm consider increasing work load and restarting six minute test).*

  1.00_____bpm
  2.00_____bpm RPE (Borg)_____
  3.00_____bpm
  4.00_____bpm
  4.15_____bpm
  4.30_____bpm
  4.45_____bpm
  5.00_____bpm
  5.15_____bpm
  5.30_____bpm
  5.45_____bpm
  6.00_____bpm RPE (Borg)_____

- End of 6 minutes: HR should be between 120 and 170bpm.

Then if HR:

< 120bpm: repeat part 1 at a higher workload
120 -170bpm, continue to part 2.
>170bpm, finish the test and allow the participant to cool down.

Part 2:

Increase workload incrementally over 3 stages.

- *Stage 1:* Increase the workload by 25-50W. Participant cycling @ 50rpm.
  WL _____

  1.00_____bpm
  2.00_____bpm
  2.30_____bpm
  2.45_____bpm
  3.00_____bpm RPE (Borg)_____
  Average HR: _____bpm

- *Stage 2:* (repeat stage 1)
  WL _____

  1.00_____bpm
  2.00_____bpm
  2.30_____bpm
  2.45_____bpm
  3.00_____bpm RPE (Borg)_____

  Average HR: _____bpm

- *Stage 3:* (repeat stage 2)
  WL _____

  1.00_____bpm
  2.00_____bpm
  2.30_____bpm
  2.45_____bpm
  3.00_____bpm RPE (Borg)_____

  Average HR: _____bpm

- If after the 3 incline stages the desired heart rate is not achieved, repeat this process:
  1.00_____bpm
  2.00_____bpm
  2.30_____bpm
  2.45_____bpm
  3.00_____bpm RPE (Borg)_____

- Target HR achieved: Yes/No
- Cool Down- 5 minutes cycling at a low resistance

- Give Participant questionnaire pack. Completed: _____

- Give Participant 2 food diaries + explain same evening dinner 24hours before next visit

Arrange next visit: Date:_____ Time:_____

Signed: _____ Date: _____

Name: _____

Case Report Form (visit 1/2)

Participant ID: _____ Visit no._____ Date: _____

Safety checks:

- Hypoglycaemia kit in the room ☐
- Bed available in the room ☐
- Polar watches working ☐
- Bucket of ice ready for samples ☐
- Glucose monitor ☐
- Blood pressure monitor ☐

Participant arrives @ WT CRF reception- check in at reception and bring to exercise room.

- collect informed consent
- check participant is **fasted** Yes/No
- Has the participant had alcohol or coffee in the last 24 hours? Yes/No
- Has the participant had any hypos? Yes/No

- check blood glucose: _____mmol/L
- Record time of waking-up: _____am

- Put HR monitor on participant ☐
- Participant resting- insert cannulae (blue)- ☐
- HR: _____bpm
- BP:_____mmHg

Wait upto 20mins

- Give them questionnaires and some light reading ☐

First resting blood sample

- **5ml clotted** (red top) ☐
- **5ml EDTA** (purple top) *Put straight onto ice* ☐

**Aliquot 1ml blood from plasma EDTA tubes into EDTA tube (for whole blood cell counts) and leave on roller*

- Check glucose: _____mmol/L

Wait upto 20 mins

- Adjust seat height and handle bars on bike (refer to baseline visit) ☐

Pre-exercise blood sample (person is seated on the bike)

- **20mls Lithium Heparin** (green top) *Put onto roller* ☐
- **5mls EDTA** (purple top) *Put straight onto ice* ☐
- **5mls clotted** (red top) ☐
- **2mls DDP4 inhibitor** (red top) *Put straight onto ice* ☐
  **Aliquot 1ml blood from plasma EDTA tubes into EDTA tube (for whole blood cell counts) and leave on roller.* ☐

- **Record HR**
  HR _____bpm
  BP _____mmHg

Warm-up: 5 minutes building up pace, and then start 30 minutes intervention. ☐

Exercise intervention: 40% / 80%

40% Intervention protocol: Desired HR
- 30mins @ 40% _____

80% Intervention protocol: Desired HR
- 0-10mins Get up 70% _____
- 10-20mins: maintain 75% _____
- 20-30mins: maintain 80% _____

- Record **HR** after 5 minute warm-up.
  HR _____bpm

- Record **glucose** and flush @ 0,10,20, 30minutes
  Pre _____mmol/L
  10mins _____mmol/L
  20mins _____mmol/L
  Post _____mmol/L

- Record **Borg scale** @ 0,10,15,25,30minutes
  0mins _____
  10mins _____
  15mins _____
  20mins _____
  25mins _____
  30mins _____

Post-exercise blood sample (immediately -while participant is sitting on the bike)

- **20mls Lithium Heparin** (green top) *Put onto roller*
- **5mls EDTA** (purple top) *Put straight onto ice*
- **5mls clotted** (red top)
- **2mls DDP4 inhibitor** (red top) *Put straight onto ice*

**Aliquot 1ml blood from plasma EDTA tube into EDTA tube (for whole blood cell counts) and leave on roller.*

- **Record blood pressure and HR**

  HR _____bpm

  BP _____mmHg

Cool down: 5 minutes post-blood sample.

Then have them seated for 60 minutes

T1D participants-check blood glucose levels every 30mins.

Must be above **5mM**!! (If it drops below this, take blood sample and feed)
Sample taken @ _____minutes post-exercise.

1 hour post-exercise blood sample

- **20mls Lithium Heparin** (green top) *Put onto roller*
- **5mls EDTA** (purple top) *Put straight onto ice*
- **5mls clotted** (red top)
- **2mls DDP4 inhibitor** (red top) *Put straight onto ice*

**Aliquot 1ml blood from plasma EDTA into EDTA tube (for whole blood cell counts) and leave on roller.*

Check glucose_____ mmol

- **Record BP** every 15minutes in the 1hour recovery.

  0mins BP _____mmHg HR_____ bpm
  15mins BP_____ mmHg HR_____ bpm
  30mins BP_____ mmHg HR_____ bpm
  45mins BP _____mmHg HR_____ bpm
  60mins BP _____mmHg HR_____ bpm

**Cannulae can be taken out at 1hour post-exercise. Healthy participants will then be asked to remain resting until 4hours post-exercise.

Heparin tubes brought to the IBR by Michelle

4 hour post-exercise blood sample
(Sample taken with butterfly needle)

- **5mls EDTA** (purple top) *Put straight onto ice*
- **5mls clotted** (red top)
- **2ml DDP4 inhibitor tube** (red top) *Put straight onto ice*

- **1ml EDTA** (purple top) - leave on roller
  Record blood pressure and HR
- HR _____bpm
- BP_____mmHg
- Glucose:_____mmol

Remaining EDTA and Serum tubes taken to IBR for serum and plasma isolation (Anmol or Michelle)

*Pay for travel and arrange next visit ☐
Date of next visit: _____

Signed: _____ Date:_____

Name: _____

Psychological Stress Questionnaires

Has this event happened to you in the **TWO WEEKS?**				**If yes, please indicate how stressful you found the event**			
				Not at all	**A little**	**Moderately**	**Very**
1	Death (family member or friend)	**Yes**	**No**	0	1	2	3
2	Had a lot of tests	**Yes**	**No**	0	1	2	3
3	It's finals week	**Yes**	**No**	0	1	2	3
4	Applying to postgraduate education	**Yes**	**No**	0	1	2	3
5	Victim of a crime	**Yes**	**No**	0	1	2	3
6	Coursework in all modules/courses due the same day	**Yes**	**No**	0	1	2	3
7	Breaking up with boy-/girlfriend	**Yes**	**No**	0	1	2	3
8	Found out boy-/girlfriend cheated on you	**Yes**	**No**	0	1	2	3
9	Lots of deadlines to meet	**Yes**	**No**	0	1	2	3
10	Property stolen	**Yes**	**No**	0	1	2	3
11	You have a hard upcoming week	**Yes**	**No**	0	1	2	3
12	Went into a test/exam unprepared	**Yes**	**No**	0	1	2	3
13	Lost something (especially wallet)	**Yes**	**No**	0	1	2	3
14	Death of a pet	**Yes**	**No**	0	1	2	3
15	Did worse than expected on test/exam	**Yes**	**No**	0	1	2	3
16	Had an interview	**Yes**	**No**	0	1	2	3
17	Had projects, essays due	**Yes**	**No**	0	1	2	3
18	Did badly on a test/exam	**Yes**	**No**	0	1	2	3
19	Parents getting divorce	**Yes**	**No**	0	1	2	3
20	Dependent on other people	**Yes**	**No**	0	1	2	3
21	Having housemate conflicts	**Yes**	**No**	0	1	2	3
22	Car/bike broke down, flat tyre, etc.	**Yes**	**No**	0	1	2	3
23	Got a traffic ticket	**Yes**	**No**	0	1	2	3
24	Missed your period and waiting	**Yes**	**No**	0	1	2	3
25	Coping with addictions	**Yes**	**No**	0	1	2	3
26	Thoughts about future	**Yes**	**No**	0	1	2	3
27	Lack of money	**Yes**	**No**	0	1	2	3
28	Dealt with incompetence in university administration	**Yes**	**No**	0	1	2	3
29	Thought about unfinished work	**Yes**	**No**	0	1	2	3
30	No sleep	**Yes**	**No**	0	1	2	3
31	Sick, injury	**Yes**	**No**	0	1	2	3
32	Had a class presentation	**Yes**	**No**	0	1	2	3
33	Applying for a job	**Yes**	**No**	0	1	2	3
34	Fought with boy-/girlfriend	**Yes**	**No**	0	1	2	3
35	Working (e.g. part-time job) while at university	**Yes**	**No**	0	1	2	3
36	Arguments, conflict of values with friends	**Yes**	**No**	0	1	2	3

37	Bothered by having no social support of family	**Yes**	**No**	0	1	2	3
38	Performed poorly at a task	**Yes**	**No**	0	1	2	3
39	Can't finish everything you needed to do	**Yes**	**No**	0	1	2	3

In the two weeks, has this event happened to you?				**If yes, please indicate how stressful you found the event**			
				Not at all	**A little**	**Moderately**	**Very**
40	Heard bad news	**Yes**	**No**	0	1	2	3
41	Had confrontation with an authority figure	**Yes**	**No**	0	1	2	3
42	Maintaining a long-distance boy-/girlfriend	**Yes**	**No**	0	1	2	3
43	Crammed for a test/exam	**Yes**	**No**	0	1	2	3
44	Feel unorganized	**Yes**	**No**	0	1	2	3
45	Trying to decide on module choices	**Yes**	**No**	0	1	2	3
46	Feel isolated	**Yes**	**No**	0	1	2	3
47	Parents controlling with money	**Yes**	**No**	0	1	2	3
48	Couldn't find a parking space	**Yes**	**No**	0	1	2	3
49	Noise disturbed you while trying to study	**Yes**	**No**	0	1	2	3
50	Someone borrowed something without permission	**Yes**	**No**	0	1	2	3
51	Had to ask for money	**Yes**	**No**	0	1	2	3
52	Ran out of printer ink for computer	**Yes**	**No**	0	1	2	3
53	Erratic schedule	**Yes**	**No**	0	1	2	3
54	Can't understand your lecturer	**Yes**	**No**	0	1	2	3
55	Trying to get onto a specific course/module	**Yes**	**No**	0	1	2	3
56	Problems applying for student loan	**Yes**	**No**	0	1	2	3
57	Stayed up late writing an essay	**Yes**	**No**	0	1	2	3
58	Someone you expected to call did not	**Yes**	**No**	0	1	2	3
59	Someone broke a promise	**Yes**	**No**	0	1	2	3
60	Can't concentrate	**Yes**	**No**	0	1	2	3
61	Someone did something that annoyed you	**Yes**	**No**	0	1	2	3
62	Living with boy-/girlfriend	**Yes**	**No**	0	1	2	3
63	Felt need for own car	**Yes**	**No**	0	1	2	3
64	Bad haircut today	**Yes**	**No**	0	1	2	3
65	Job requirements changed	**Yes**	**No**	0	1	2	3
66	No time to eat	**Yes**	**No**	0	1	2	3
67	Felt some peer pressure	**Yes**	**No**	0	1	2	3
68	You have a hangover	**Yes**	**No**	0	1	2	3
69	Problems with your computer	**Yes**	**No**	0	1	2	3
70	Problems getting home from bar when drunk	**Yes**	**No**	0	1	2	3

71	Stopped from entering nightclub	**Yes**	**No**	0	1	2	3
72	No sex in a while	**Yes**	**No**	0	1	2	3
73	Someone pushed ahead of you in a queue	**Yes**	**No**	0	1	2	3
74	Chequebook didn't balance	**Yes**	**No**	0	1	2	3
75	Visit from a relative and entertaining them	**Yes**	**No**	0	1	2	3
76	Worrying about whether to have sex with someone	**Yes**	**No**	0	1	2	3
77	Talked with a tutor/lecturer	**Yes**	**No**	0	1	2	3
78	Change of environment (new doctor, dentist, etc.)	**Yes**	**No**	0	1	2	3

In the two weeks, has this event happened to you?				**If yes, please indicate how stressful you found the event**			
				Not at all	**A little**	**Moderately**	**Very**
79	Exposed to upsetting TV programme, book, or film	**Yes**	**No**	0	1	2	3
80	Got to lecture/seminar late	**Yes**	**No**	0	1	2	3
81	Holiday	**Yes**	**No**	0	1	2	3
82	Sat through a boring lecture/seminar	**Yes**	**No**	0	1	2	3
83	Favorite sporting team lost	**Yes**	**No**	0	1	2	3

The questions in this scale ask you about your feelings and thoughts during **THE TWO WEEKS**. In each case, you will be asked to indicate how often you felt or thought a certain way. Indicate the alternative that seems like a reasonable estimate.

In the last month, how often have you been upset because of something that happened unexpectedly?	Never	Almost never	Some-times	Fairly often	Very often
In the last month, how often have you felt that you were unable to control the important things in your life?	Never	Almost never	Some-times	Fairly often	Very often
In the last month, how often have you felt nervous and stressed?	Never	Almost never	Some-times	Fairly often	Very often
In the last month, how often have you dealt with irritating life hassles?	Never	Almost never	Some-times	Fairly often	Very often
In the last month, how often have you felt that you were effectively coping with important changes that were occurring in your life?	Never	Almost never	Some-times	Fairly often	Very often
In the last month, how often have you felt confident about your ability to handle your personal problems?	Never	Almost never	Some-times	Fairly often	Very often
In the last month, how often have you felt that things were going your way?	Never	Almost never	Some-times	Fairly often	Very often
In the last month, how often have you found that you could not cope with all the things you had to do?	Never	Almost never	Some-times	Fairly often	Very often
In the last month, how often have you been able to control irritations in your life?	Never	Almost never	Some-times	Fairly often	Very often
In the last month, how often have you felt that you were on top of things?	Never	Almost never	Some-times	Fairly often	Very often
In the last month, how often have you been angered because of things that happened that were outside of your control?	Never	Almost never	Some-times	Fairly often	Very often
In the last month, how often have you found yourself thinking about things that you have to accomplish?	Never	Almost never	Some-times	Fairly often	Very often
In the last month, how often have you been able to control the way you spend your time?	Never	Almost never	Some-times	Fairly often	Very often
In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?	Never	Almost never	Some-times	Fairly often	Very often

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE (October 2002)

LONG LAST 7 DAYS SELF-ADMINISTERED FORMAT

FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains asked independently) and short (4 generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on health–related physical activity.

Background on IPAQ

The development of an international measure for physical activity commenced in Geneva in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites) during 2000. The final results suggest that these measures have acceptable measurement properties for use in many settings and in different languages, and are suitable for national population-based prevalence studies of participation in physical activity.

Using IPAQ

Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.

Translation from English and Cultural Adaptation

Translation from English is encouraged to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at www.ipaq.ki.se. If a new translation is undertaken we highly recommend using the prescribed back translation methods available on the IPAQ website. If possible please consider making your translated version of IPAQ available to others by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

Further Developments of IPAQ

International collaboration on IPAQ is on-going and an ***International Physical Activity Prevalence Study*** is in progress. For further information see the IPAQ website.

More Information

More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at www.ipaq.ki.se and Booth, M.L. (2000). *Assessment of Physical Activity: An International Perspective.* Research Quarterly for Exercise and Sport, 71 (2): s114-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** and **moderate** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?

   ☐ Yes

   ☐ No → ***Skip to PART 2: TRANSPORTATION***

The next questions are about all the physical activity you did in the **last 7 days** as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, heavy construction, or climbing up stairs **as part of your work**? Think about only those physical activities that you did for at least 10 minutes at a time.

   _____ **days per week**

   ☐ No vigorous job-related physical activity → ***Skip to question 4***

3. How much time did you usually spend on one of those days doing **vigorous** physical activities as part of your work?

   _____ **hours per day**
   _____ **minutes per day**

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads **as part of your work**? Please do not include walking.

   _____ **days per week**

   ☐ No moderate job-related physical activity → ***Skip to question 6***

5. How much time did you usually spend on one of those days doing **moderate** physical activities as part of your work?

_____ **hours per day**
_____ **minutes per day**

6. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **as part of your work**? Please do not count any walking you did to travel to or from work.

_____ **days per week**

☐ No job-related walking → ***Skip to PART 2: TRANSPORTATION***

7. How much time did you usually spend on one of those days **walking** as part of your work?

_____ **hours per day**
_____ **minutes per day**

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the **last 7 days**, on how many days did you **travel in a motor vehicle** like a train, bus, car, or tram?

_____ **days per week**

☐ No traveling in a motor vehicle → ***Skip to question 10***

9. How much time did you usually spend on one of those days **traveling** in a train, bus, car, tram, or other kind of motor vehicle?

_____ **hours per day**
_____ **minutes per day**

Now think only about the **bicycling** and **walking** you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the **last 7 days**, on how many days did you **bicycle** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐ No bicycling from place to place → ***Skip to question 12***

11. How much time did you usually spend on one of those days to **bicycle** from place to place?

_____ **hours per day**
_____ **minutes per day**

12. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐ No walking from place to place → ***Skip to PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY***

13. How much time did you usually spend on one of those days **walking** from place to place?

_____ **hours per day**
_____ **minutes per day**

PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the **last 7 days** in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, chopping wood, shoveling snow, or digging **in the garden or yard**?

_____ **days per week**

☐ No vigorous activity in garden or yard → ***Skip to question 16***

15. How much time did you usually spend on one of those days doing **vigorous** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, sweeping, washing windows, and raking **in the garden or yard**?

_____ **days per week**

☐ No moderate activity in garden or yard → ***Skip to question 18***

17. How much time did you usually spend on one of those days doing **moderate** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, washing windows, scrubbing floors and sweeping **inside your home**?

_____ **days per week**

☐ No moderate activity inside home → ***Skip to PART 4: RECREATION, SPORT AND LEISURE-TIME PHYSICAL ACTIVITY***

19. How much time did you usually spend on one of those days doing **moderate** physical activities inside your home?

_____ **hours per day**
_____ **minutes per day**

PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the **last 7 days** solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **in your leisure time**?

_____ **days per week**

☐ No walking in leisure time → ***Skip to question 22***

21. How much time did you usually spend on one of those days **walking** in your leisure time?

_____ **hours per day**
_____ **minutes per day**

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like aerobics, running, fast bicycling, or fast swimming **in your leisure time**?

_____ **days per week**

☐ No vigorous activity in leisure time → ***Skip to question 24***

23. How much time did you usually spend on one of those days doing **vigorous** physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis **in your leisure time**?

_____ **days per week**

☐ No moderate activity in leisure time → ***Skip to PART 5: TIME SPENT SITTING***

25. How much time did you usually spend on one of those days doing **moderate** physical activities in your leisure time?
_____ **hours per day**
_____ **minutes per day**

PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekday**?

_____ **hours per day**
_____ **minutes per day**

27. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekend day**?

_____ **hours per day**
_____ **minutes per day**

This is the end of the questionnaire, thank you for participating.