ADIPONECTIN AND IMMUNE
TOLERANCE
IN TYPE 1 DIABETES

By

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ABSTRACT

Type 1 diabetes (T1D) is characterised by pancreatic β cell autoimmunity and inflammation, resulting in β cell islet destruction and insulin deficiency. Prospective studies from different continents have shown that insulin resistance is independently associated with risk for the development of T1D. We wanted to investigate the role of adiponectin in mediating this link. Adiponectin is a circulating adipokine whose anti-inflammatory and insulin sensitising actions appear to be mediated via two related receptors, AdipoR1 and AdipoR2. We began by characterising adiponectin receptor expression on PBMC by flow cytometry. We showed that monocytes express both receptors abundantly, that this expression correlates with insulin sensitivity in both health and diabetes. Furthermore, expression can be increased with lifestyle intervention. Adiponectin receptor expression on monocytes is reduced in T1D, and we demonstrate this leads to an apparent resistance in the ability of adiponectin to inhibit the stimulatory capacity of antigen presenting cells (APC). Specifically, we show that adiponectin inhibits the stimulatory capacity of APCs through down-regulation of CD86 expression, and that this effect is decreased in T1D. In this way, the release from the regulatory effects of adiponectin is one potential mechanism by which immune tolerance is lost in T1D.

(197 words)

For Elisha and Joshua,

"I saw her. Your mother!

Your mother is near!

So, as fast as you can,

Think of something to do!

You will have to get rid of

Thing One and Thing Two!"

Acknowledgments

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=Abbreviations

18s	Ribosomal 18S RNA
ACEI	Angiotensin converting enzyme inhibitor
AdipoQ	Adiponectin Gene
AdipoR	Adiponectin Receptor
AdipoR	Adiponectin receptors
AdipoR1	Adiponectin Receptor 1
AdipoR2	Adiponectin Receptor 2
AF488	Alexa Fluor 488
AMP kinase	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
APC	Allophycocyanin
ARB	Angiotensin II receptor blocker
AT	Adipose tissue
ATF3	Activating transcription factor 3
BMI	Body mass index
Cat #	Catalog number
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CI	Confidence interval
CON	Conventional arm (Early-ACTID)
Ct	Threshold cycle
CREB	cAMP response element-binding
CTLA-4	Cytotoxic T-lymphocyte Antigen 4
CV	Coefficient of variation
DC	Dendritic cells
DCCT	Diabetes Control and Complications Trial
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non- integrin (CD 209)
DI	Division index
DPT-1	Diabetes prevention trial-1
EDIC	Epidemiology of Diabetes Interventions and Complications Study
eGDR	Estimated glucose disposal rate
ELISA	Enzyme linked immunosorbent assay
ENDIT	European Nicotinamide Diabetes Intervention Trial
ERK	Extracellular Signal-Regulated Kinase
EXE	Exercise Arm (Exercise & Overeating Study)

FCS	Foetal calf serum
FiTC	Fluorescein isothiocyanate
FPIR	First phase insulin release
gAd	Globular adiponectin
GAD65	Glutamic acid decarboxylase isoform 65kDa
GADA	Anti-GAD antibodies
GITR	Glucocorticoid-induced TNF receptor related protein
GM CSF	Granulocyte macrophage colony-stimulating factor
HbA1C	glycated haemoglobin
HC	Healthy Control
HLA	Human leucocyte antigen
HMW	High molecular weight
HOMA-B	Homeostatic model assessment of β cell function
HOMA-IR	Homeostatic model assessment of insulin resistance
HR	Hazard ratio
HRP	horseradish peroxidase
IA-2	Tyrosine phophatase
Ig	Immunoglobulin
IGRP	Islet-specific glucose-6-phosphatase catalytic subunit related protein
IL	Interleukin
IL-2R	Interleukin 2 receptor
INT	Intensive arm (Early ACTID)
IP	Immunoprecipitation
IR	Insulin resistance
IVGTT	Intravenous glucose tolerance test
JNK	c-Jun-N-terminal kinase
LMW	Low molecular weight
LPS	Lipopolysaccharide
MABP	Mean artierial blood pressure
mDC	Monocyte derived dendritic cells
MFI	Mean flurescence index
MHC	Major histocompatbility complex
MODY	Maturity-onset diabetes of the young (monogenic diabetes)
NF kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	National Health Service
NYHA	New York Heart Association
OGTT	Oral glucose tolerance test
PBDC	Peripheral blood dendritic cells

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	R-phycoerythrin
PHA	Phytohaemagglutinin
PI	Propidium iodide
PPAR	Peroxisome proliferator-activated receptor
PTPN 22	Protein tyrosine phosphatase, non-receptor 22 (lymphoid)
PWM	Pokeweed mitogen
Q {number}	Adiponectin at dose ug/ml
Q DC	Adiponectin treated monocyte derived dendritic cells
rt qPCR	Reverse transcriptional quantitative polymerase chain reaction
rt-ve	Reverse transcriptase negative control
SD	Standard deviation
SED	Sedantary Arm (Exercise & Overeating Study)
SF	Serum free
SI	Stimulation index
SM	Moderate intensity arm (Exercise Intensity Study)
SV	Vigorous Intensity Arm (Exercise Intensity Study)
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TNF	Tumour necrosis factor
Treg	Regulatory T cells
TTC	Tetanus toxoid
VO2 max	Maximal oxygen consumption
WHR	Waist hip ratio

1. INTRODUCTION

1.1 The costs of T1D

Type 1 diabetes (T1D) is a chronic disease of hyperglycaemia caused by insulin deficiency. Typical onset is in childhood and adolescence, at which point, the initiation of insulin replacement by injection is continued for life. The aims of insulin treatment are not only to prevent potentially fatal ketoacidosis and dehydration, but to also achieve near normal glycaemia. The clinical benefits were proven in the Diabetes Control and Complications Trial (DCCT). In this landmark multi-centre randomised controlled trial, intensive insulin treatment to maintain pre-meal glucose at 70-120 mg/dl (3.9 – 6.7 mmol l^{-1}) and <180mg/dl (10 mmol l^{-1}) postprandially decreased microvascular complications by as much as 76% (The Diabetes Control and Complications Trial Research Group, 1993). This reduction, including those in cardiovascular disease, persisted in the 10 year post-trial observational period (The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study Research,Group 2005).

However, achieving tight glycaemic control is complex for patients. Precise insulin dosing for normoglycaemia needs to account for meals, lifestyle and pharmacokinetics of the different insulin preparations. Furthermore, tight glucose control is associated with risk of hypoglycaemia, some of which may be sufficiently serious to warrant third party assistance (The Diabetes Control and Complications Trial Research Group, 1993). Fear of hypoglycaemia is common among patients and their carers, and the associated avoidance behaviour can hinder the optimisation of metabolic control (Wild, von Maltzahn et al. 2007).

T1D also impacts on our national health. The latest estimates suggest that it is associated with a 23 year reduction in life expectancy (Department of Health 2007), and mortality rates of up to 11 fold in certain populations (Soedamah-Muthu, Fuller et al. 2006). In addition, it is a major drain on healthcare costs to the NHS. The latest estimate stands at £2.5 billion, which accounts for 4% of its annual budget (Juvenile Diabetes Research Foundation. 2004). The costs of disease are projected to escalate further, as a direct consequence of the increasing incidence of T1D. In 2003, standardised incidence in the UK was 22-30 per 100 000, and the projected rise year-on-year is 3-4% (Patterson, Dahlquist et al. 2009). These problems have added further impetus to understanding the natural history of T1D, in order to facilitate new approaches in preventing or ameliorating this disease.

1.2 The pathogenesis of T1D
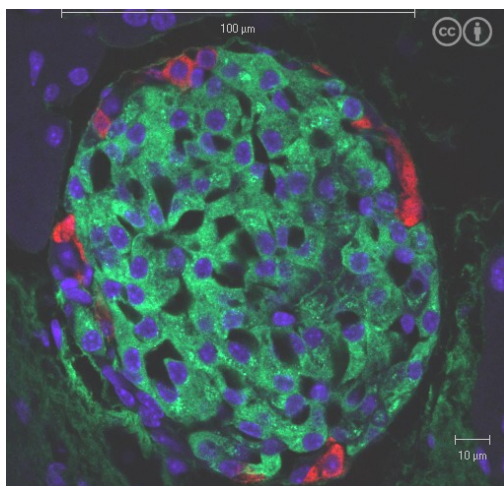
Figure 1.1. Normal pancreatic islet. Insulin is labelled in green, glucagons in red and the nuclei in blue. Freely licensed media, courtesy of Süß C et al, University of Technology, Dresden, Germany.

Insulin is produced by the β cells in the islet of Langerhans in the endocrine pancreas (figure 1.1). It is formed from post-translational cleavage of the prohormone proinsulin

(Sanger 1959), which is encoded by the INS gene at the position 11p15.5 (Genbank NM_000207). The destruction of β cells is the hallmark of T1D. In type 1B diabetes, the cause is idiopathic. A variant characterised by fuliminant onset and subacute pancreatitis has been described in Japanese patients (Imagawa, Hanafusa et al. 2000). In this thesis we will focus our discussion on type 1A diabetes. The immune mediated nature of type 1A diabetes is evidenced by its following characteristics, expanded further below:

1) Association of immune genes with risk for T1D
2) Chronic inflammation seen on islet histology in affected individuals
3) Serum autoantibodies predate and predict disease
4) Presence of auto-reactive T cells in affected individual
5) Transference of disease by the transfer of diabetogenic immune cells in humans and in animal models.
6) Preservation of β cell function by immunomodulatory therapies

T1D is heritable, and susceptibility for disease is associated with genes encoding proteins with immunoregulatory functions. The largest contribution comes from MHC class II haplotypes *HLA-DR* and *DQ* (Concannon, Erlich et al. 2005). Recent studies have identified other immune genes in HLA class 1 and non-HLA regions conferring additional risk (figure 1.2). These include *CTLA4*, *PTPN22* and *IL2RA* (Nejentsev, Howson et al. 2007, Concannon, Rich et al. 2009) (figure 1.2). This complex polygenicity indicates that multiple immune mechanisms lead to β cell destruction.

Histologically, the pathognomonic lesion in T1D is insulitis (figure 1.3), which can be seen before clinical onset and consistently in established disease (In't Veld, Lievens et al. 2007, Foulis, Liddle et al. 1986) . The islets are infiltrated by immune cells,
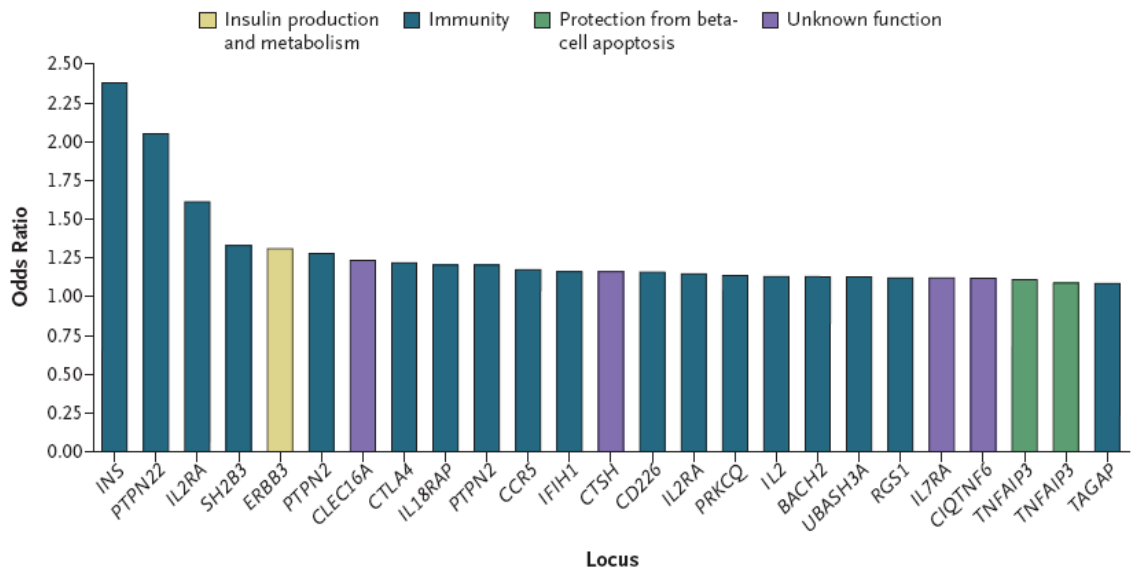
Figure 1.2: Putative functions of non-HLA genes that contribute to genetic risk of type 1A diabetes. Adapted from Concannon *et al* (Concannon, Rich et al. 2009). In contrast the odds ratio for the HLA region 6p21 is ~15. Reproduced with permission from the Massachusetts Medical Society. (ref PS-2011-0393).

composing largely of CD8+ T cells and CD68+ macrophages, with a minority of CD4+ T cells and CD20+ B cells (Willcox, Richardson et al. 2009, Itoh, Hanafusa et al. 1993, Uno, Imagawa et al. 2007). Their cytotoxicity appears to be confined to β cells only (Rohane, Shimada et al. 1995), as neighbouring α and δ cells are preserved (Foulis, Liddle et al. 1986, Hanafusa, Miyazaki et al. 1990). As disease progresses, insulin staining in the islets is replaced by an increasing infiltrate of of CD8+ T cells and CD20+ B cells (Rowe, Campbell-Thompson et al. 2010). Across the whole pancreas however, insulitis in patchy (Rowe, Campbell-Thompson et al. 2010). This hetereogeneity may be explained by pancreatic anatomy and differences in insulin content of the islets. Islets in the pancreatic head and body were more likely to be affected relative to the tail (Bottazzo, Dean et al. 1985), and this may relate to their more extensive lymphatic drainage(Donatini, Hidden et al. 1992). Also in support of β-

cell specificity, insulitis is seen much more frequently in islets rich in insulin content compared to relatively insulin-deficient ones (Foulis, Liddle et al. 1986).

Further demonstration of the immune specificity is the presence of autoantibodies to β cell proteins. Targeting the glutamic acid decarboxylase isoform of molecular weight 65 kDa (GAD65) (Hagopian, Michelsen et al. 1993), tyrosine phosphatase (IA-2) (Lampasona, Bearzatto et al. 1996), proinsulin/insulin (Yu, Robles et al. 2000), these major autoantibodies are present in serum before and at the time of diagnosis (Bingley, Christie et al. 1994, Wenzlau, Juhl et al. 2007, Achenbach, Bonifacio et al. 2005). The assays for these autoantibodies are standardised in the Diabetes Antibody Standardisation Programme (DASP) (Torn, Mueller et al. 2008) and are used to stratify risk for T1D in genetically susceptible individuals (Sherry, Tsai et al. 2005). Other β cell autoantibodies can also be detected (Lieberman, DiLorenzo 2003), including the recently identified zinc transporter ZnT8 (Slc30A8) (Wenzlau, Juhl et al. 2007), which appears to further improve risk estimation.
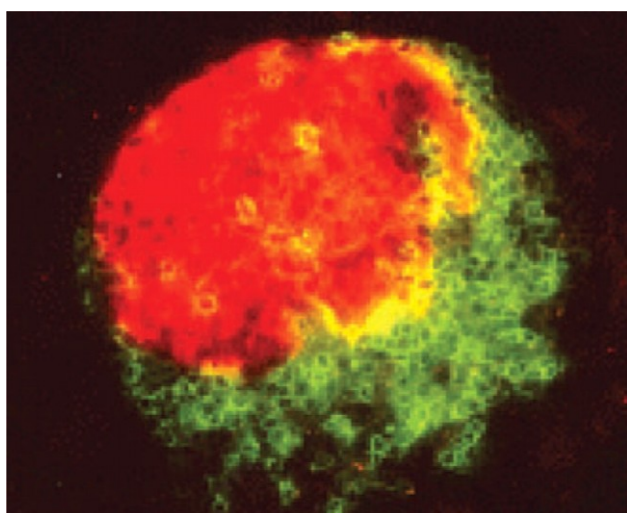
Figure 1.3 Insulitis. The normal distribution of β cells (insulin in red) is lost and replaced by an infiltrate of T cells (green). Courtesy of Anne Cooke, University of Cambridge. Reproduced from,(Narendran, Estella et al. 2005) with permission of the author.

Circulating T lymphocytes reactive to the autoantigens GAD65, IA-2, insulin, as well as to islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP), can be detected in patients with human T1D (Roep, Arden et al. 1990, Atkinson, Kaufman et al. 1992, Honeyman, Stone et al. 1997, Harrison, Chu et al. 1992, Hawkes, Schloot et al. 2000, Yang, Danke et al. 2006). Whilst each clone may have limited pathogenicity on its own (Burton, Vincent et al. 2008), collectively, these autoreactive cells are thought to be responsible for transferring diabetes in animal models (Wicker, Miller et al. 1986, Hanafusa, Sugihara et al. 1988, Bendelac, Carnaud et al. 1987). Analogously in humans, there are case reports of T1D transmission by bone marrow allograft (Lampeter, Homberg et al. 1993, Lampeter, McCann et al. 1998). The recipients, who were negative for islet autoantibodies, developed T1D two to four years following the transplant from HLA-matched donors with T1D. The duration is in keeping with the insidious course of islet autoimmunity, and there was no clinical evidence for generalised graft versus host disease. A contention is that the recipients could have developed T1D because of exposure to an environmental trigger, given that they possessed high risk HLA haplotypes.

Lastly, the immunomodulatory therapies can counter the natural decline in β cell function. Treatments tried include immunosuppressants, stem cell transplant, biological immunomodulators and vaccines against islet autoantigens. Cyclosporine, a calcineurin inhibitor, has been shown in two multicentre RCT to induce remission in newly diagnosed T1D (Assan, Feutren et al. 1990, The Canadian-European Randomized Control Trial Group 1988). The treatment was associated with nephrotoxicity, although the increase in serum creatinine was reversible upon lowering of dosage and did not result in participant withdrawal. Encouragingly, there was a

24.1% complete remission rate in the treatment group at nine months, compared with 5.8% in the placebo group. However, insulin independence rarely lasted beyond 12 months (Bougneres, Landais et al. 1990). Azathioprine, the pro-drug of 5-mercaptopurine which inhibits DNA synthesis, has also been tested in newly diagnosed patients in RCT setting. In adults, azathioprine at a dose of 2mg kg^{-1} d^{-1} was associated with increased insulin-free remission after 12 months of treatment (azathioprine: 7 out of 13 subjects, versus 1 of 11 placebo), but 5 subjects in the active arm withdrew and two because of side effects (Harrison, Colman et al. 1985). Bone marrow suppression, a recognised severe adverse event, was not seen. However, the remission was not sustained in the post-treatment follow-up period. In contrast, azathioprine in children had no significant impact on complete remission rates, metabolic control or β cell function (Cook, Hudson et al. 1989) , although adjuvant prednisolone in the early phase may be of benefit, with 3 out of 20 treated patients achieving insulin independence versus none in the control group (Silverstein, Maclaren et al. 1988).

Remission of up to 58 months has been reported with autologous non-myeloablative haemopoietic stem cell transplant (Voltarelli, Couri et al. 2007, Couri, Oliveira et al. 2009). 20 out 23 study patients became insulin free and 12 of these maintained remission for a mean of 31 months. Eight patients relapsed but maintained endogenous β cell function at 48 months. However this required high dose immunosuppression which carried serious risks of infection (two developed bilateral nosocomial pneumonia) and endocrine gland dysfunction (9 patients developed oligospermia).

More recent clinical trials have employed interventions with much fewer adverse effects. A short treatment course of humanised anti-CD3 monoclonal antibody,

modified to prevent Fc receptor binding, preserved β cell function and reduce insulin dosage in newly diagnosed patients, for up to 18 months (Keymeulen, Vandemeulebroucke et al. 2005, Herold, Hagopian et al. 2002). During treatment, 75% of patients experienced a transient infectious mononucleosis-like syndrome and increased parameters of Epstein-Barr virus immunity, as well as a reduction of circulating lymphocytes. Complete recovery was seen two weeks after the last infusion. Pre-clinical studies suggest the mechanism of the anti-CD3 treatment relates to a shift in pathogenic Th1 responses (interferon-γ and IL-2) in favour of Th2 (IL-10) (Smith, Tang et al. 1998). However, in the larger clinical trial, the temporary changes in cytokine levels and lymphocyte counts did not predict primary outcome (Keymeulen, Vandemeulebroucke et al. 2005). Additionally, the rise in titres for anti-idiotype antibodies during treatment phase may counteract its efficacy.

Rituximab is an anti-CD20 monoclonal antibody. It cross links cell surface CD20 and selectively depletes B lymphocytes through antibody-mediated and complement-dependent cytotoxicity (Bour-Jordan, Bluestone 2007). A four-dose course of the antibody in patients within 3 months of diagnosis ameliorated the decline in β cell function at 1 year (Pescovitz, Greenbaum et al. 2009). The intervention group had lower levels of HbA1C and required less insulin. The depletion of B cells persisted at 12 months (69% of baseline) but was not associated with increased infections. These results confirm the relevance of B cells in T1D, which can modify local production of cytokines at sites of autoimmunity and function as specialised antigen presenting cells for T lymphocyte action. Their roles are further discussed in Section 1.4.1.

Treatments discussed so far are not antigen specific, and thus can result in immunocompromise with a potential for opportunistic infections and neoplasia.

Vaccination using autoantigens is a realistic alternative for inducing specific immunologic tolerance. The effects of 65 kDa isoform of glutamic acid decarboxylase (GAD), a recognised autoantigen in T1D, have been reported in a phase 2 study (Ludvigsson, Faresjö et al. 2008). Combined with the adjvuvant alum, GAD vaccination in children within 18 months of diagnosis resulted in a slower decline in β cell function against placebo, for up to 30 months. There were no differences in frequency of adverse events between the two groups. Active treatment was associated with higher GAD antibodies, GAD-induced cytokines (interferon-γ, TNFα, interleukins 5, 10, 13 17) and expression of FoxP3 and TGF-β in whole PBMC. Larger confirmatory trials are in progress (NCT00723411 & NCT00751842).

1.3   Natural history of T1D

Follow-up studies of at-risk subjects show the loss of β cell function is insidious (figure 1.4). Family history and HLA class II genes determine genetic risk (Ziegler, Nepom 2010). Children with an affected first degree relative will have a 10 times increased risk compared to those without family history (Table 1.1). HLA class II genes have a comparable impact. The genotypes HLA DRB1*03, *04 and DQB1*0302 confer the highest risk (odds ratio for T1D = 17). In contrast, HLA-DQB alleles are protective. For example, the HLA-DQB1*0602 haplotype reduces lifetime risk in those with first degree family history from 5-8% to ~1% (Baisch, Weeks et al. 1990).

Table 1.1 Type 1 Diabetes Risk Stratification by T1D Family History and HLA Genotyping

Population	Type 1 Diabetes Risk (%)
Low Risk	
No affected FDR plus HLA protective genes	0.01
No affected FDR	0.4
Affected FDR plus HLA protective genes	0.3
Intermediate Risk	
No affected FDR plus HLA risk genes	4
One affected FDR	5
Mother with T1D	3
Father with T1D	5
Sibling with T1D	8
High Risk	
One affected FDR plus HLA high risk genes	10–20
Multiple affected FDRs	20–25
Very High Risk	
Identical twin affected	30–70
Multiple affected FDRs plus HLA risk genes	50
Sibling affected plus HLA risk genes, identical by descent	30–70

FDR, first-degree relative; HLA risk genes, HLA DRB1*03,*04;DQB1*0302; HLA protective genes, HLA DQB1*0602. Type 1 diabetes risk (%) refers to lifetime risk of develop disease. Table reproduced from (Ziegler, Nepom 2010) with permission from the publisher (Elsevier License number 2550221424517).

The development of autoantibodies compounds the baseline genetic risk. Rarely present before the age of 6 months, their detection signals initiation of islet autoimmunity (Bingley, Christie et al. 1994, Achenbach, Warncke et al. 2006). Autoantibodies used for risk prediction are insulin/proinsulin, GAD65/GAD67, IA-2 and the recently identified ZnT8 (Wenzlau, Juhl et al. 2007). Incremental risk is seen with multiple positive antibodies, as well as the presence of higher titre and greater affinity antibodies.

Thus, genetic and immunological factors can stratify an individual's risk for T1D. This strategy has been successfully used to identify high risk cohorts, with a predicted incidence of >25% in 5 years, for prevention studies (Diabetes Prevention Trial–Type 1

Diabetes Study Group 2002, Gale 2004). In these subjects with pre-clinical T1D, the rate of decline in β cell function in the pre-T1D period can be tracked by glucose challenge and mixed meal tolerance tests. The loss of first phase insulin secretion (FPIR) and impaired glucose tolerance identify subjects with accelerated progression to diabetes (Barker, McFann et al. 2007). Approximately 10-20% residual β cell function remains at diagnosis (Komulainen, Knip et al. 1997). In a proportion of patients, there is partial or complete clinical remission, resulting in insulin independence. This period, called a 'honeymoon' period, can last a number of years. However, all patients will inevitably relapse to requiring insulin therapy as the continuing autoimmune process proceeds to destroy surviving β cells. Therefore, after a number of years of diabetes there is minimal amount of β cell function, and this has traditionally been estimated using the technique of C-peptide measurement following a hyperglycaemic stimulus (Sherry, Tsai et al. 2005). Analysis of the DCCT shows that even at the time of presentation with diabetes, the small amounts of endogenous insulin secretion reduces the risk of vascular complications and severe hypoglycaemia (Steffes, Sibley et al. 2003). Hence, measures that dampen islet autoimmunity and sustain β cell function will be of clinical benefit to patients at the time of, as well as before, diagnosis with T1D.
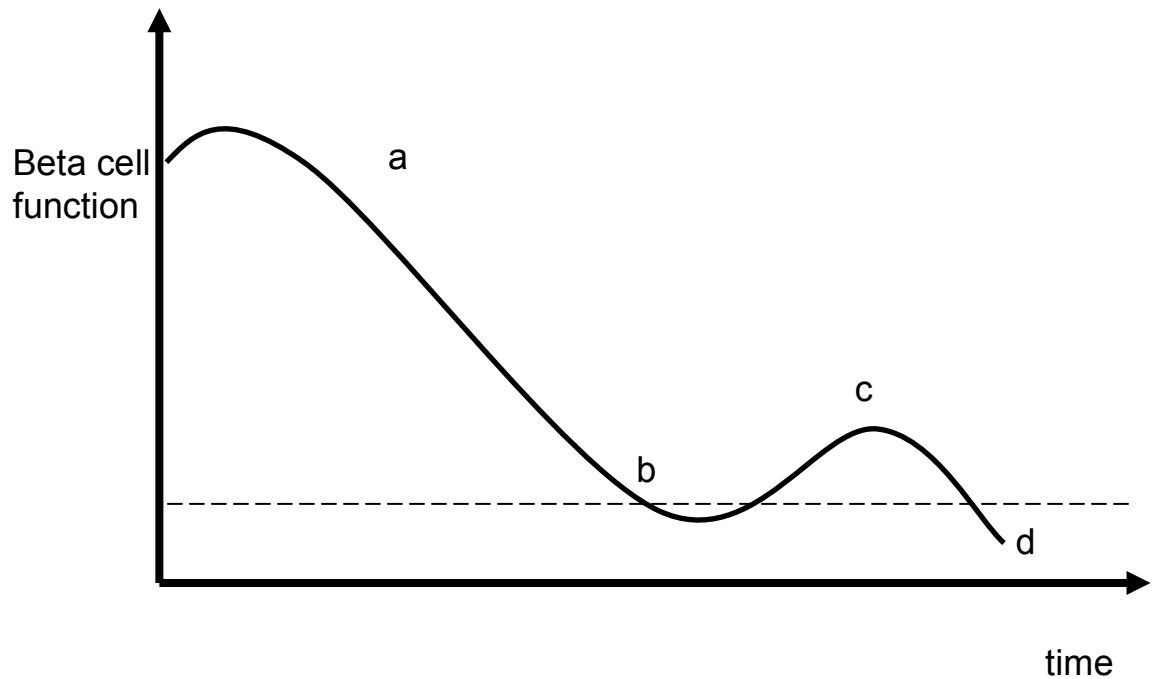
Figure 1.4: Natural history of type 1 diabetes. After birth, β cell mass may still increase with linear growth and increased metabolic demand from obesity or pregnancy (Sherry, Tsai et al. 2005). In pre-T1D (a), β cell mass declines because of autoimmune attrition. Glucose intolerance can be identified on dynamic testing. Symptomatic presentation (b) of T1D occurs when β cell mass falls below a threshold for required glucose homeostasis (dotted line). Honeymoon remission (c), characterised by a period of insulin independence, may occur in some patients following initial insulin treatment. However, in all patients this does not last and β cell mass declines and again falls below the threshold (d). Nevertheless, functional β cells can be isolated in patients with longstanding T1D (>10 years) (Walker, Johnson et al. 2010). β cell function is measured by a C-peptide response to a hyperglycaemic stimulus such as a mixed meal challenge.

1.4   Overview of current concepts in the immunopathogenesis of T1D

1.4.1 The non-obese diabetic (NOD) mouse

Understanding the immune dysregulation in human T1D is limited by the availability of tissue, and the slow progression of disease. Much of our knowledge is attributed to the non-obese diabetic (NOD) mouse model, an inbred strain that exhibits spontaneous autoimmune diabetes, usually within 12-16 weeks of birth (Adorini, Gregori et al. 2002, Anderson, Bluestone 2005). It mimics some features of human T1D. The genetic susceptibility for disease in NOD mice is largely determined by the single functionally homologous MHC class II allele H2g7. A similar panel of autoantigens, GAD, proinsulin, IA-2 and IGRP is targeted in both. NOD diabetes is preceded by a short prodrome of increasing glucose intolerance with parallel insulitis. The major advantage of the NOD mouse is that it allows the controlled interrogation of an immune mechanism. Techniques such as adoptive transfer, where cells from diseased mouse are injected into a healthy coisogenic and immunodeficient host, allow apportion of each cellular component in the autoimmune response. Genetic manipulations through transgenes and selective gene targeting can dissect the relevance of specific molecular pathways.

CD4+ and CD8+ T cells are obligate effectors in the aetiology of T1D (Wicker, Miller et al. 1986, Hanafusa, Sugihara et al. 1988, Bendelac, Carnaud et al. 1987, Miller, Appel et al. 1988, Christianson, Shultz et al. 1993). Their individual MHC-class restricted clones, generated from leucocytic infiltrates of insulitic lesions, transfer disease (Wong, Visintin et al. 1996, Graser, DiLorenzo et al. 2000, Haskins, Wegmann 1996). In addition, anti-CD3, CD4, and CD8 monoclonal antibodies confer protection and can induce disease remission (Chatenoud, Thervet et al. 1994, Koike, Itoh et al. 1987,

Herold, Montag et al. 1987, Makhlouf, Grey et al. 2004, Shizuru, Taylor-Edwards et al. 1988, Herold, Bluestone et al. 1992, Wang, Gonzalez et al. 1996, Parish, Cooke 2005). For activation, naive autoreactive T cells require the presentation of a processed cognate antigenic peptide complexed to the MHC class II molecule by an antigen-presenting cell (APC) (Itano, Jenkins 2003, Hoglund, Mintern et al. 1999, Gagnerault, Luan et al. 2002). This process initially takes place at the pancreatic lymph node (Gagnerault, Luan et al. 2002). Dendritic cells (DC) are a group of diverse and specialised APC found in tissues, blood and secondary lymphoid organs (Banchereau, Briere et al. 2000). They can be expanded *ex vivo* from myeloid or lymphoid progenitors, in peripheral blood and bone marrow. They are classified according to their maturation, phenotype and secretory profile (Penna, Vulcano et al. 2002). DC and also macrophages and monocytes, can perform the antigen presentation role and their introduction to the diabetes-prone NOD mouse can initiate disease (Saxena, Ondr et al. 2007, Jun, Yoon et al. 1999, Serreze, Fleming et al. 1998).

The B lymphocyte can also function as APC. Autoreactive B cells have constitutive HLA class II expression and surface-bound autoantibodies efficiently capture self-antigens (Falcone, Lee et al. 1998). In the NOD mouse, B cell deficiency ameliorated spontaneous diabetes and adoptive transfer of B cells from diabetic mice restored islet autoimmunity (Serreze, Fleming et al. 1998, Hämäläinen, Savola et al. 2001).

The pathogenicity of the B lymphocytes was not dependent on their antibody secretory capacity, as seen in an IgM transgene model which retained antibodies on the cell surface (Wong FS, Wen et al. 2004). Rather it is their antigen presenting function that is crucial, as selective inhibition of HLA expression on B cells prevented diabetes (Noorchashm, Lieu et al. 1999). There is also evidence to suggest B lymphocytes can modify autoimmunity in the NOD mouse in a paracrine fashion. B lymphocytes

activated by anti-IgM F(ab')2 antibodies could skew cytokine expression in T cells towards Th$_2$ phenotype, attenuate insulitis and delay diabetes (Hussain, Delovitch 2007), and this regulatory effect was fully dependent upon IL-10 expression. In contrast, islet autoantibodies are not believed to be pathogenic, as they do not bind to the cell surface or matrix antigens, or form immune complexes (Wong, Wen 2005). Furthermore, their transplacental transfer in humans did not initiate β cell autoimmunity (Serreze, Fleming et al. 1998, Hämäläinen, Savola et al. 2001).

In humans, B lymphocytes are present in human insulitic lesions and their numbers increase with progression (Willcox, Richardson et al. 2009). Their depletion by the anti-CD20 monoclonal antibody rituximab resulted in preservation of β cell function in clinical trials (Pescovitz, Greenbaum et al. 2009), This positive result confirms the importance of B lymphocytes in human T1D, although their role is not absolutely critical, as disease can occur in X-linked agammaglobulinemia, where tyrosine kinase mutation results in B cell deficiency (Martin, Wolf-Eichbaum et al. 2001).

NK lymphocytes are another cell type that can influence disease course. They are cytotoxic to β cells in co-cultures, and are a rich source of the pro-inflammatory cytokine IFN-γ (Nakamura, Woda et al. 1990). In corroboration they promote insulitis and diabetes in transgenic models (Poirot, Benoist et al. 2004, Alba, Planas et al. 2008). However, the picture in human T1D is less clear cut. NK cell counts are reduced at the time of disease onset and their induced IFN-γ expression does not correlate with more aggressive disease (Rodacki, Svoren et al. 2007).

1.4.2 The MHC and cytokines

Direct cytotoxicity by autoreactive CD8+ T cells is an important cause of β cell loss in T1D (Mathis, Vence et al. 2001). MHC-class 1 expression on the β cell target is critical, as molecular knockout prevents diabetes in NOD mouse (Serreze, Chapman et al. 1997). CD8+ T cells, recognising antigen-MHC class 1 complex, kill β cells via perforin, granzyme B and Fas ligand mechanisms (Mathis, Vence et al. 2001). This process may be enhanced by elevated glucose, which can increase proinsulin presentation (Skowera, Ellis et al. 2009). Inflammatory cytokines such as IL-1β and IFN-γ, secreted by immune cells, act in synergism in effecting β cell death (Cnop, Welsh et al. 2005). However, no single cytokine is an absolute requirement for autoimmune diabetes, as sole knockout strains remain susceptible to disease, albeit with delayed onset (Hultgren, Huang et al. 1996, Kagi, Ho et al. 1999, Thomas, Irawaty et al. 2004). This may simply highlight the redundancy common in cytokine networks. Collectively they switch on pathways that lead to increased presentation of antigens (↑MHC class 1 and proteasome components), chemokine expression (IL-15, IP-10, MCP-1 and ICAM-1) and apoptosis, which perpetuate immune process and β cell loss (Zhang, O'Brien et al. 2002).

1.4.3 Central and peripheral tolerance

Important questions in understanding T1D pathogenesis therefore include how islet reactive T cells develop and are later primed to action. Self-reactive T cells are thought to undergo negative selection during thymic development because of their strong affinity to self antigens (Hogquist, Baldwin et al. 2005), a process called central tolerance. Again the NOD mouse provides clues on how this process can be

compromised (Lesage, Hartley et al. 2002). Firstly, differences in the expression of MHC-class 2 molecules are important (Kanagawa, Martin et al. 1998). Attempts to alter MHC class II expression by transgenes are successful in preventing insulitis (Nishimoto, Kikutani et al. 1987, Lund, O'Reilly et al. 1990). It has been suggested that the single NOD I-Ag7 has an unstable tertiary structure, resulting in poor peptide binding (Kanagawa, Martin et al. 1998). However, this may be oversimplified, as the clonal deletion of diabetogenic thymocytes in transgenic class-II NOD mice appears to be autoantigen independent (Bohme, Schuhbaur et al. 1990, Schmidt, Amrani et al. 1999). Secondly, the ectopic expression of self-antigens may also account for disease susceptibility. The importance of this is shown by defects in the *Aire* gene (Liston, Lesage et al. 2003). *Aire* controls the expression of a number of self proteins in thymic medullary epithelial cells and its silencing creates the clinical syndrome APECED (autoimmune polyendocrinopathy, candidiasis and ectodermal dysplasia) characterised by spontaneous endocrine autommune diseases, including T1D. Whilst the *Aire* gene is not defective in NOD mice, measures that alter autoantigen expression in the thymus, such as proinsulin and GAD65, can modify disease (Tisch, Yang et al. 1993, Thébault-Baumont, Dubois-Laforgue et al. 2003). In corroboration, decreased thymic expression of insulin in humans, associated with alleles in the VNTR element of the insulin gene promoter, negatively correlates with T1D risk (Pugliese, Zeller et al. 1997). Lastly, diabetogenic thymocytes in the NOD mouse may be more resistant to apoptotic elimination, possibly through the upregulation of anti-apoptotic proteins (Lamhamedi-Cherradi, Luan et al. 1998, O'Brien, Geng et al. 2006).

The process of central tolerance is incomplete and circulating autoreactive T cells are detectable in health. Peripheral tolerogenic mchanisms keep these cells in check (Lohmann, Leslie et al. 1996, Semana, Gausling et al. 1999, Arif, Tree et al. 2004,

Mannering, Morris et al. 2004). The best characterised of these center on DC and regulatory T cells (Tregs).

1.4.4 Dendritic cells and regulatory T cells

Myeloid DC, defined by CD11c expression, are some of the first cells to infiltrate islets in the autoimmune diabetes in the NOD mouse, and are involved in the priming of autoreactive T cells (Rosmalen, Leenen et al. 1997, Turley, Poirot et al. 2003, Jansen, Homo-Delarche et al. 1994). In addition, they secrete abundant levels of IL-12 which skew effector T cells towards a Th1 phenotype (Trembleau, Penna et al. 1995, Weaver, Poligone et al. 2001, Trembleau, Penna et al. 2003). This response appears to be critical in the development of insulitis in the NOD mouse (Nitta, Kawamoto et al. 2001). Th1 type cytokines include IFN-γ, IL-2, and IL-21, promote inflammatory responses of effector cells (Hultgren, Huang et al. 1996, Allison, McClive et al. 1994, Sutherland, Van Belle et al. 2009). In particular, IL-21 may be especially potent, as the transgenic overexpression of IL-21 in islets can trigger diabetes in a normally diabetes resistant strain (Sutherland, Van Belle et al. 2009). This preferential expression of Th1 cytokines by T cells on encountering autoantigens appears to also apply in human T1D (Arif, Tree et al. 2004). In contrast, immature DCs are adept in antigen presentation and have reduced allo-stimulatory capacity (Lutz, Schuler 2002). They are identified by their low expression levels of MHC Class 2 and co-stimulatory molecules. Propagated from bone marrow cells and pulsed with autoantigenic peptides, these cells can delay disease onset in the NOD mouse (Feili-Hariri, Dong et al. 1999, Clare-Salzler, Brooks et al. 1992), and are the subject of current immunotherapy trials in human T1D (Lo, Clare-Salzler 2006).

Naïve T cells require a second signal in addition to the TCR-MHC-Class II-antigen complex for activation. This is provided by the binding of CD80 or CD86 on APC to CD28 on T cells (Sperling, Auger et al. 1996). In addition, CTLA-4 on the surface of T cells is an alternative ligand for CD80/86 (Walunas, Lenschow et al. 1994). This engagement produces an opposing inhibitory signal. Interventions on this pathway have complex effects on diabetes incidence on the NOD mouse. In keeping with its priming role, anti-CD86 antibodies can prevent diabetes onset only if administered before 10 weeks of age (Lenschow, Ho et al. 1995). However, antagonism of CD80/86 with a soluble CTLA-4 antibody, as well as gene knockouts of CD28, CD80 and CD86, all produced paradoxical acceleration of diabetes (Anderson, Bluestone 2005). This finding may potentially be explained by the finding that these models have significantly reduced population of cells called Treg. CD80/86 – CD28 stimulation is required for the maintenance of Treg (Salomon, Lenschow et al. 2000, Tang, Henriksen et al. 2003).

Treg police diabetogenic T cell responses in the NOD mouse by cell-contact and TGF-β dependent mechanisms (Bluestone, Tang et al. 2008). They possess CD62L, CTLA-4, GITR on their surface, and can be also be identified also by its low CD127 expression (Hartigan-O'Connor, Poon et al. 2007). Crucial to Treg function are transcription of the nuclear factor FoxP3 (Fontenot, Gavin et al. 2003), expression of CTLA-4 (Schmidt, Wang et al. 2009), and the cytokines IL-2 and TGFβ (Setoguchi, Hori et al. 2005, Peng, Laouar et al. 2004) . Their numbers are reduced in NOD mice, and inversely correlate with rate of β cell destruction (Salomon, Lenschow et al. 2000, Chen, Herman et al. 2005). Whilst anergic to antigenic and polyclonal stimulation (Setoguchi, Hori et al. 2005, Gavin, Clarke et al. 2002), Treg numbers can be expanded in vitro with anti-CD3, anti-CD28 and IL-2 (Tang, Henriksen et al. 2004) or using DC exposed to IL-10 and TGF-β (Rutella, Danese et al. 2006). Their subsequent

transfer to NOD mouse can curtail insulitis and reverse disease (Tang, Henriksen et al. 2004, Lepault, Gagnerault 2000, Stephens, Mason 2000, Bresson, Togher et al. 2006).

Whether defects in tolerance described in the NOD mouse are relevant to human T1D is open to debate (Bresson, von Herrath 2009, Atkinson, Leiter 1999). For example, a meta-analysis of Treg in human T1D found variable reports of functional impairments (Tree, Roep et al. 2006), and this is partly attributable to differences in stimulatory conditions. In positive studies, Tregs were tested against the stimulatory effects of anti-CD3 (Brusko, Wasserfall et al. 2005, Lindley, Dayan et al. 2005). Adaptive Tregs are a subset of antigen-specific Treg that had developed from mature T cells under certain conditions of antigen stimulation (Bluestone, Abbas 2003). Using an in vitro culture system with APC and peptides, GAD- and IGRP- specific Treg in T1D were found to have similar function compared to other adaptive Treg in the same subject (Long, Walker et al. 2009).

Moreover, DC from T1D patients and those at risk do not have an inflammatory phenotype (Takahashi, Honeyman et al. 1998, Zacher, Knerr et al. 2002, Angelini, Duca et al. 2005, Summers, Marleau et al. 2006). Adding complexity to the issue is that diabetogenic T cells may in fact be resistant to Treg suppression (D'Alise, Auyeung et al. 2008, Schneider, Rieck et al. 2008). This may be attributable to cytokines such as IL-21 and activation signals from the Toll-like receptor and the TNF receptor families (Walker 2009).

A working model of the immunopathogenesis of T1D is presented in figure 1.5.
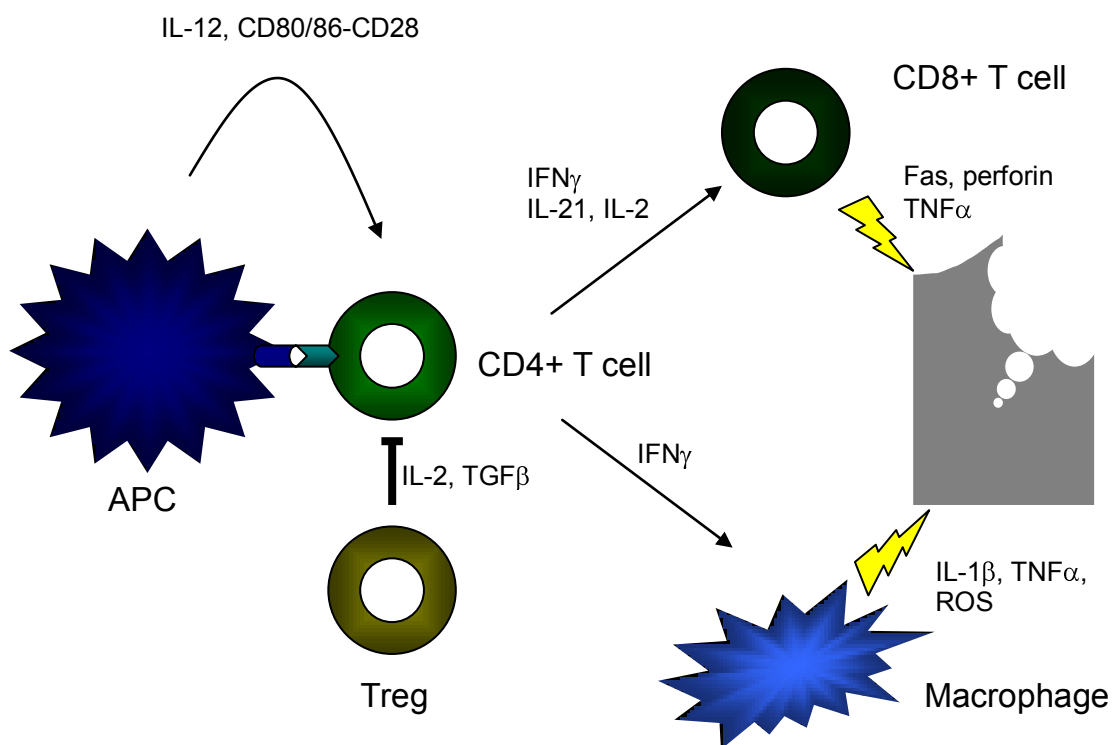
Figure 1.5: Working model of T1D autoimmunity. Antigen presenting cells (APC), in this case CD11c+ dendritic cells, process and traffick β cell antigens in the pancreatic lymph node. They activate naïve diabetogenic CD4+ T cells via MHC Class 2-antigen-T cell receptor synapse. Important co-stimulatory signals come from CD80/86-CD28 pathway and IL-12 secreted by DC. The CD4 T cells secrete predominantly Th1 type cytokines (IFNγ, IL-2 and IL21) in their recruitment of islet reactive CD8+ cytotoxic T cells and local macrophages. These cells effect β cell killing through Fas ligand, TNF, IL-1β and reactive oxygen species (ROS). Treg, defined by the phenotype markers CD4+CD25+FoxP3+, is able to suppress diabetogenic T cells via cell contact and TGF-β dependent mechanisms.

1.5 Insulin resistance is a propellant of T1D autoimmunity in susceptible individuals

The discordance for disease in monozygotic twins has previously been cited as evidence for the environment in the pathogenesis of T1D (Redondo, Yu et al. 2001). Studies showing the negative impact in genetic risk in causing the worldwide increase

in T1D incidence (Gillespie, Bain et al., Fourlanos, Varney et al. 2008) have drawn further interest into the area.

Insulin resistance (IR) refers to a fall in the biological activity of insulin at a given dose (Pang, Narendran 2008a). It manifests clinically as the metabolic syndrome (Reaven 1988). Its prevalence is rising because of our obesity pandemic (Ford, Giles et al. 2002, Mattsson, Ronnemaa et al. 2007). The gold standard in measurement of IR is the euglycaemic hyperinsulinaemic clamp (DeFronzo, Tobin et al. 1979). However this is time consuming and not suited to large scale clinical studies. Alternative validated measures include the HOMA-IR index (Wallace, Levy et al. 2004), which utilises paired fasting glucose and insulin, and the minimal model, based on insulin response at the IVGTT (Saad, Anderson et al. 1994).

In the accelerator hypothesis, Wilkin proposed diabetes is the result of three processes contributing to β cell apoptosis (Wilkin 2001). These are constitution (genetic factors), autoimmunity and IR. Based on the increasing obesity rate, IR has been proposed as a likely environmental factor in driving the increasing incidence of T1D. If this is true, then a cohort of recent new cases would have a higher pre-morbid body mass index compared to an older group. Also adjusting for other known risk factors, the more aggressive islet autoimmunity would lead to an earlier age of diagnosis. Several groups have now reported these associations (Betts, Mulligan et al. 2005, Kibirige, Metcalf et al. 2003, Knerr, Wolf et al. 2005, Libman, Pietropaolo et al. 2003), but there are disagreements (Porter, Barrett 2004, Dabelea, B. et al. 2006). Others have attempted to examine the relationship between weight gain and positivity for autoantibodies, but again there is conflicting data (Couper, Beresford et al. 2009, Cambuli, Incani et al. 2010). Despite this, stronger evidence can be found from prospective studies of

subjects with high-risk pre-T1D (Table 1) (Greenbaum, Sears et al. 1999, Fourlanos, Narendran et al. 2004, Mrena, Virtanen et al. 2006, Xu, Cuthbertson et al. 2007, Bingley, Mahon et al. 2008).

The Melbourne Prediabetes Study first reported on the deleterious effect of insulin resistance on the development of T1D (Fourlanos, Narendran et al. 2004). The authors had employed an earlier version of HOMA-IR, standardised against FPIR, a surrogate of insulin secretary function. This adjustment compensates for the systematic underestimate by the natural decline in β cell function. Adjusting for known immunogenetic risk factors, HOMA-IR:FPIR at baseline was a powerful and independent predictor of diabetes (hazard ratio (HR) 2.14, 95% CI 1.39 – 3.29). Conversely, subjects who were relatively insulin sensitive were protected from disease. These findings were consistent with later studies conducted on different continents (Mrena, Virtanen et al. 2006, Xu, Cuthbertson et al. 2007, Bingley, Mahon et al. 2008). In particular, investigators for the DPT-1 found this relationship to apply to high and intermediate risk groups (Xu, Cuthbertson et al. 2007). Additionally they reported further increases of IR relative to baseline 6-12 months before diabetes onset. In contrast, two other studies recorded neutral effects (Greenbaum, Sears et al. 1999, Bingley, Mahon et al. 2008). However, posthoc analysis of ENDIT suggested the effect is dependent on baseline β cell reserve. The strong predictive power of IR remains in those with low FPIR and thus at high risk of progression (HR for low FPIR 3.67, p<0.05 versus 1.27 ns for preserved insulin secretion) (Bingley, Mahon et al. 2008). Thus, the negative findings from the earlier Seattle Family Study could be explained by its lower risk study population (Greenbaum, Sears et al. 1999). Overall, the main effect of IR on diabetes onset is on those at high genetic risk (constitution) and those already with low

insulin reserve, because of autoimmunity. This is in keeping with its proposed role as an environmental modifier of disease risk in the accelerator hypothesis.

Confirming the environmental origin of the IR, genome wide scan studies of T1D have not shown any loci known to modify insulin sensitivity to be associated with disease (Barrett, Clayton et al. 2009). Further supporting evidence comes from the British Diabetic Twins Study, which prospectively followed non-diabetic identical twins whose co-twin has T1D (Hawa, Bonfanti et al. 2005). Despite sharing the same genetic risk, twins who later developed diabetes were more insulin resistant than their control twins who maintained normal glucose tolerance.

Table 1.2: Prospective studies evaluating the effect of IR in development of T1D

(in order of the year of publication)

Study	N	Median duration of follow-up (years)	Progression to clinical diabetes (%)	Measure of IR	Adjusted hazard ratio (95% CI)
Seattle Family Study (1999) (Greenbaum, Sears et al. 1999)	85	2.8	11.8	Minimal model of glucose kinetics from IVGTT	1.0 (NS)ζ
Melbourne Pre-Diabetes Family Study (2004) (Fourlanos, Narendran et al. 2004)	104	4.0	41.3	HOMA1-IR:FPIR	2.14 (1.39 – 3.29)
Childhood Diabetes in Finland study (2006) (Mrena, Virtanen et al. 2006)	77	15.0	40.3	HOMA1-IR:FRIR	2.4 (1.2 – 5.0)
Diabetes Prevention Trial- Type 1 (moderate risk group) (2007) (Xu, Cuthbertson et al. 2007)	186	4.3	28.5	HOMA1-IR	2.70 (1.45 – 5.06)
Diabetes Prevention Trial- Type 1 (high risk group) (2007) (Xu, Cuthbertson et al. 2007)	170	3.7	41.2	HOMA1-IR	1.83 (1.19 -2.82)
European Nicotinamide Diabetes Intervetion Trial (2008) (Bingley, Mahon et al. 2008)	213	4.2	49.3	HOMA2-IR	1.43 (0.99 – 2.06)

DPT-1 Moderate risk group – 25-50% 5 year risk of developing T1D
DPT-1 High Risk group - ≥ 50% 5 year risk of developing T1D
HOMA1-IR – measure of IR at steady state based on the formula: fasting insulin (mU/L) × fasting glucose (mmol/l) / 22.5
HOMA2-IR – refined model of HOMA1-IR, taking into account steady state changes in glucose and insulin at higher levels of fasting glucose and interference of insulin assays by proinsulin
FPIR – sum of serum insulin concentration at 1 and 3 minutes after glucose injection in the IVGTT
ζ - calculation based on insulin sensitivity index (× 10^{-5} min^{-1}/[pmol/l]) from frequently sampled IVGTT between progressors (4.47 ± 0.584) and non-progressors (4.48 ± 0.443)

1.6   Adipose tissue and immunity

How can we explain the association between IR and the accelerated development of T1D? Diabetes may be revealed earlier as the higher IR overloads the compromised β cell (Dahlquist 2006). But data exists to suggest IR may also directly modulate islet autoimmunity. In the Melbourne Prediabetes Study, the effect of increasing IR on diabetes incidence was analysed in a post hoc Kaplan Meier analysis (figure 1.6). Subjects, controlled for family history, HLA haplotype and autoantibody profile, were divided into four quartiles based on their IR on entry. Of interest is the divergence in the Kaplan Meier curves and the relative protection from disease in subjects who were insulin sensitive. If IR is to only have an overload effect, then the pattern seen would be that of a divergent-convergent curve, ie disease onset is delayed in the insulin sensitive quartile but eventually all four groups will develop disease because of the same rate in β cell mass loss. This analysis suggests IR is a modifier of β cell autoimmunity, and there is evidence to support such a link is biologically plausible.
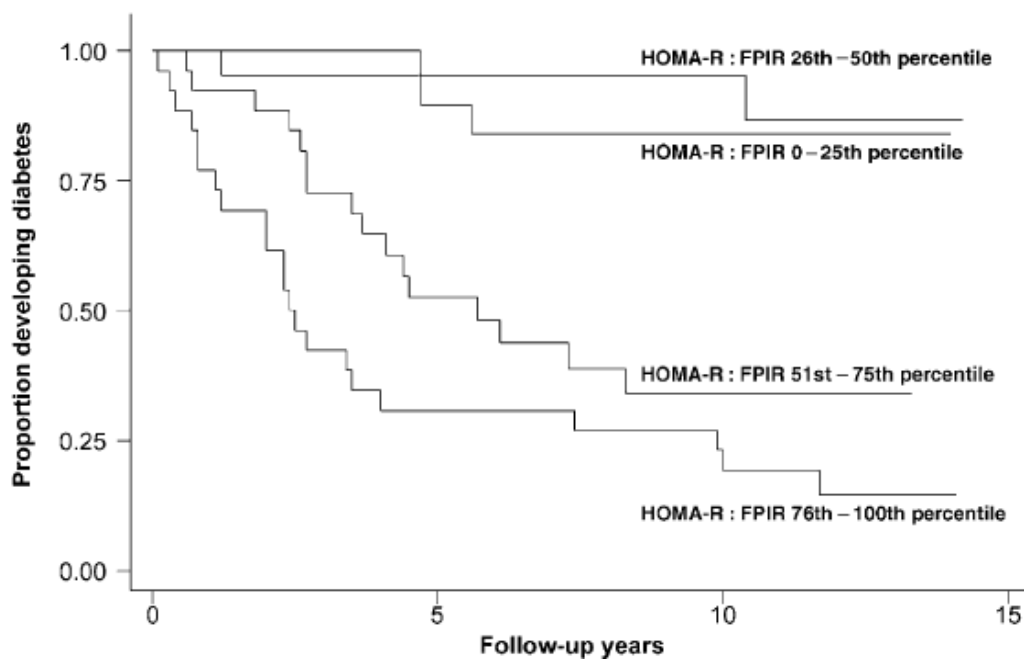
Figure 1.6 Kaplan-Meier analysis of increasing quartiles of IR on incidence of diabetes in the Melbourne Prediabetes Study (Fourlanos, Narendran et al. 2004). Reproduced with permission (Springer licence number: 2551431389203). HOMA-R denotes HOMA IR. Subjects controlled for known risk factors of T1D were stratified into quartiles according to their HOMA-IR, standardised to FPIR, on entry. The divergence in Kaplan-Meier curves and low diabetes incidence in the insulin sensitive quartiles (0-25th and 26th-50th percentiles) indicate IR is a modifier in the pathophysiology of T1D.

In animal models, β cells triggered to apoptose because of gluco- and lipotoxicity display increased autoantigens (Bjork, Kampe et al. 1992), and this in turn can activate DC and CD8+ T cells (Bjork, Kampe et al. 1992, Trudeau, Dutz et al. 2000). Alternatively, the association may be attributed to effects of adipose tissue (AT). AT decreases on whole body insulin sensitivity (Kahn, Hull et al. 2006), and recent studies show that it has the capability to direct T cell immunity.

Fat accretion shifts the balance of resident T cells in AT (Nishimura, Manabe et al. 2009, Winer, Chan et al. 2009, Feuerer, Herrero et al. 2009). Wild type mice on high chow diet had an overall higher number of T cells in their visceral adipose tissue. T cell numbers in cutaneous fat, which has a much lower proportion of white adipose tissue implicated in the metabolic syndrome, were unchanged (Winer, Chan et al. 2009). The infiltrate was largely CD8+ (Nishimura, Manabe et al. 2009) and IFN-γ expressing (Winer, Chan et al. 2009), with diminished numbers of CD4+FoxP3+ cells (Feuerer, Herrero et al. 2009). The mechanism for T cell influx is not clear, although intriguingly, analysis of TCR rearrangements of these shows the T cells were of limited repertoire, suggesting antigenic stimulation plays a role (Winer, Chan et al. 2009, Feuerer, Herrero et al. 2009, Feuerer, Herrero et al. 2009). Similar changes in the balance in favour of pro-inflammatory effector T cells over FoxP3+ cells are seen in ob/ob mice (Feuerer, Herrero et al. 2009), a model of genetic IR and also in obese human subjects (Winer, Chan et al. 2009, Feuerer, Herrero et al. 2009). These T cells appear to be important in modulating AT inflammation and IR. In mice fed with high fat diet, expanding FoxP3 numbers using recombinant IL-2 and a particular–IL-2–specific monoclonal antibody (Feuerer, Herrero et al. 2009), or mitogenic CD3 antibody (Winer, Zern et al. 2006) ameliorated IR. The selective removal of CD8+ T cells using neutralising CD8 antibodies mitigated the recruitment of macrophage into AT and downregulated IL-6 and TNFα expression in AT.

Moreover, the immunomodulatory potential of perinodal fat is recognised (Knight 2008) where adipocytes are in close proximity to DC. Here, crosstalk is likely to involve adipokines. Discovered by gene expression profiling of adipocytes, adipokines have paracrine and endocrine functions in energy metabolism and inflammation (Zhang, Proenca et al. 1994, Maeda, Okubo et al. 1996). Examples of adipokines include the

archetypal leptin, resistin, TNFα, as well as the adipokine that is the subject of this thesis, adiponectin. It is by far the highest expressed adipokine and has attracted the most research interest as a putative modulator of obesity-associated disease.

1.7 Adiponectin biology

1.7.1 The adiponectin gene (ADIPOQ) and protein structure

Adiponectin is encoded by the *ADIPOQ* gene on chromosome 3q21 (Kissebah, Sonnenberg et al. 2000, Takahashi, Arita et al. 2000), which spans 17kb. It consists of three exons and two introns (figure 1.7A). Its mRNA transcripts were previously believed to be restricted to AT and in mature adipocytes (Maeda, Okubo et al. 1996, Scherer, Williams et al. 1995, Maeda, Okubo et al. 1996). In the 3T3-L1 cell line, a widely used model in studies of adipocyte biology, *ADIPOQ* transcripts were absent in pre-adipocyte cultures, but is induced 100-fold upon maturation (Scherer, Williams et al. 1995). Recent studies have found *ADIPOQ* expression in extra-adipose tissues can be induced in inflammatory states, in exocrine glands (Katsiougiannis, Kapsogeorgou et al. 2006), heart muscle (Guo, Xia et al. 2007) and respiratory epithelium (Miller, Cho et al. 2009).

It is a 30 kDa monomeric protein, consisting of 244 amino acid residues (figure 1.7B). It has a N-terminal signal sequence, a domain containing 22 Gly-X-Pro repeats which forms the straight collagen stalk, and a globular domain at the carboxyl terminal that has sequence homology to the complement factor C1q (Scherer, Williams et al. 1995). The tertiary structure of the protein is similar to TNFα (Kadowaki, Yamauchi 2005).

A

1	MLLLGAVLLL LALPGHDQET TTQGPGVLLP LPKGACTGWM
41	AGIPGHPGHN GAPGRDGRDG TPGEKGEKGD PGLIGPKGDI
81	GETGVPGAEG PRGFPGIQGR KGEPGEGAYV YRSAFSVGLE
121	TYVTIPNMPI RFTKIFYNQQ NHYDGSTGKF HCNIPGLYYF
161	AYHI TVYMKD VKVSLFKKDK AMLFTYDQYQ ENNVDQASGS
201	VLLHLEVGDQ VWLQVYGEGE RNGLYADNDN DSTFTGFLLY
241	HDTN

B
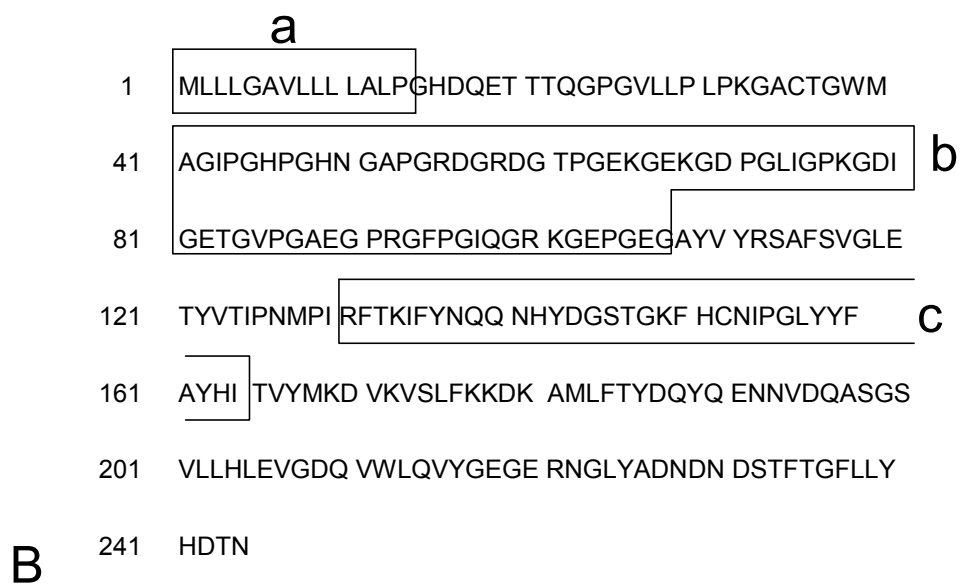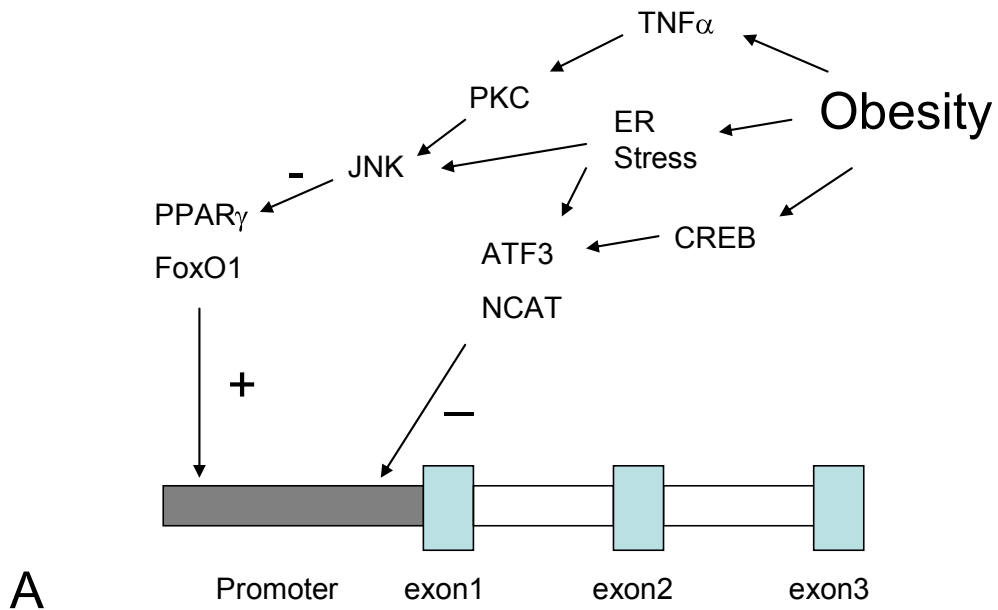
Figure 1.7 ADIPOQ gene (A) and primary protein structure of adiponectin (B)

A. Obesity decreases expression of adiponectin RNA. Increased TNFα, ER stress and associated increase in CREB levels act in concert to alter balance of promoter (PPARγ and FoxO1) and repressor (ATF3 and NCAT) activity on ADIPOQ transcription. B. Primary structure of adiponectin (244 aa). Box a indicates the signal sequence. Box b is a region containing short collagen like motif Gly-X-Y triplets. Box c shows sequence homology to C1q.

Through non-covalent associations between the collagenous domains, adiponectin can form dimers and trimers. These can further polymerise via disulphide bonds and other post-translational modifications to form low and high molecular weight species in circulation (Waki, Yamauchi et al. 2003, Tsao, Tomas et al. 2003, Richards, Stephens et al. 2006). The ability to form multimeric adiponectin appears to be confined to mammalian cells and is dependent on the glycosylation of lysine residues in the collagenous domain (Richards, Stephens et al. 2006). Together, adiponectin multimers circulate at concentrations of 10 ug ml^{-1} in plasma. Globular adiponectin (gAd), a fragment of full length adiponectin following cleavage of the collagenous tail by leucocyte elastase (Waki, Yamauchi et al. 2005), is present in small amounts but has recognised biological functions (Fruebis, Tsao et al. 2001).

1.7.2 The adiponectin Receptors AdipoR1 and AdipoR2

Using expression cloning, where binding of recombinant adiponectin was screened against proteins made from a human skeletal muscle cDNA library, Yamauchi *et al* were the first to describe specific surface receptors for adiponectin, termed AdipoR1 and AdipoR2 (Yamauchi, Kamon et al. 2003). The receptors belong to the G-protein related receptor superfamily and have an inverted topology, such that the C-terminus is on the external surface. They have similarities on crystallography to the TNFα receptor (Yamauchi, Kamon et al. 2003, Kupchak, Garitaonandia et al. 2009). Whereas AdipoR1 is exclusively expressed on mice skeletal muscle, both receptors are ubiquitously expressed in human tissues (Kadowaki, Yamauchi 2005).

Despite sharing 67% protein sequence homology, the two isoforms are distinguished by their genetic origin, affinity profile for adiponectin multimers and signal transduction.

The AdipoR1 gene lies on chromosome 1 (Genbank NM_015999) and the AdipoR2 gene is on chromosome 12 (Genbank NM_024551). AdipoR1 is a high affinity receptor for gAD and a low affinity receptor for full length adiponectin, whereas AdipoR2 exhibits intermediate avidity for all forms.

Although the full signal transduction pathway following agonist-receptor binding is unknown, both receptors activate PPARγ and ERK 1/2, key intermediaries in lipid metabolism and cell growth respectively (Yamauchi, Nio et al. 2007, Lee, Klein et al. 2008). However there are also key differences (fig 1.8). In the liver, selective manipulation of each receptor subtype resulted in a different phenotype (Yamauchi, Nio et al. 2007). Upregulation of AdipoR1 by adenovirus vector activated AMP kinase and reduced endogenous glucose production. The converse was seen with AdipoR1 knockout, but these changes were not seen with genetic manipulation of AdipoR2 expression. In contrast, AdipoR2 expression was found to have exclusive effects on PPARα targeted genes and glucose uptake by the liver. In human synovial fibroblasts which also express both receptors, it is the selective siRNA knockdown of AdipoR1, not AdipoR2, that resulted in the dissociation of AMP kinase, p38 MAPK and NFκB signalling with adiponectin (Tang, Chiu et al. 2007). In support of these downstream differences, upstream events critical to AdipoR1 signalling, such as clarthrin- and Rab 5- dependent endocytosis of agonist receptor complex, and interaction with signalling adaptor proteins APPL1 and ERp46, have not been shown to be of functional relevance for AdipoR2 (Charlton, Webster et al. 2010, Mao, Kikani et al. 2006, Ding, Wang et al. 2009). Thus, although both AdipoR1 and AdipoR2 can bind full length adiponectin, the differences in signalling argue against redundancy commonly seen in cytokine pathways. Hence, deficiency of one receptor isotype would not be compensated by the other. However, in combination, AdipoR1 & AdipoR2 signalling

accounted for the metabolic actions of adiponectin. Double receptor knockdown recapitulated the metabolic phenotype of the adiponectin-deficient mice (Yamauchi, Nio et al. 2007, Yamauchi, Kamon et al. 2001). When fed on a high fat diet, these animals develop diabetes with marked insulin resistance. Changes at the tissue level include increased triglyceride content and expression markers of inflammation and oxidative stress.

Mammalian high molecular weight adiponectin has also been reported to bind T-cadherin in vitro, a glycosylphosphatidylinositol-anchored extracellular protein expressed on endothelial and smooth muscle cells (Hug, Wang et al. 2004). The biological significance of this had been questioned, as T-cadherin lacks a cytoplasmic domain for intracellular signal transduction (Takeuchi, Adachi et al. 2007). However, recent studies have demonstrated co-localisation of adiponectin and T-cadherin in tissue and via the T-cadherin deficient model, the interaction is important in mediating adaptive changes of cardiac muscle to insult (Denzel, Scimia et al. 2010).

Interactions of adiponectin with LPS (Peake, Shen et al. 2006), C1q (Peake, Shen et al. 2008), C1q receptor (Yokota, Oritani et al. 2000), chemokines such as monocyte chemoattractant protein-1 and macrophage-inflammatory-protein 1α (Masaie, Oritani et al. 2007), and calreticulin receptor (Takemura, Ouchi et al. 2007) have also been described in vitro. However, criticisms against these studies, which include use of non-mammalian sourced adiponectin (Peake, Shen et al. 2006, Peake, Shen et al. 2008), pharmacological concentration of reagents (Yokota, Oritani et al. 2000, Takemura, Ouchi et al. 2007) and lack of supporting functional data (Peake, Shen et al. 2006, Masaie, Oritani et al. 2007), cast doubt on their physiological relevance.
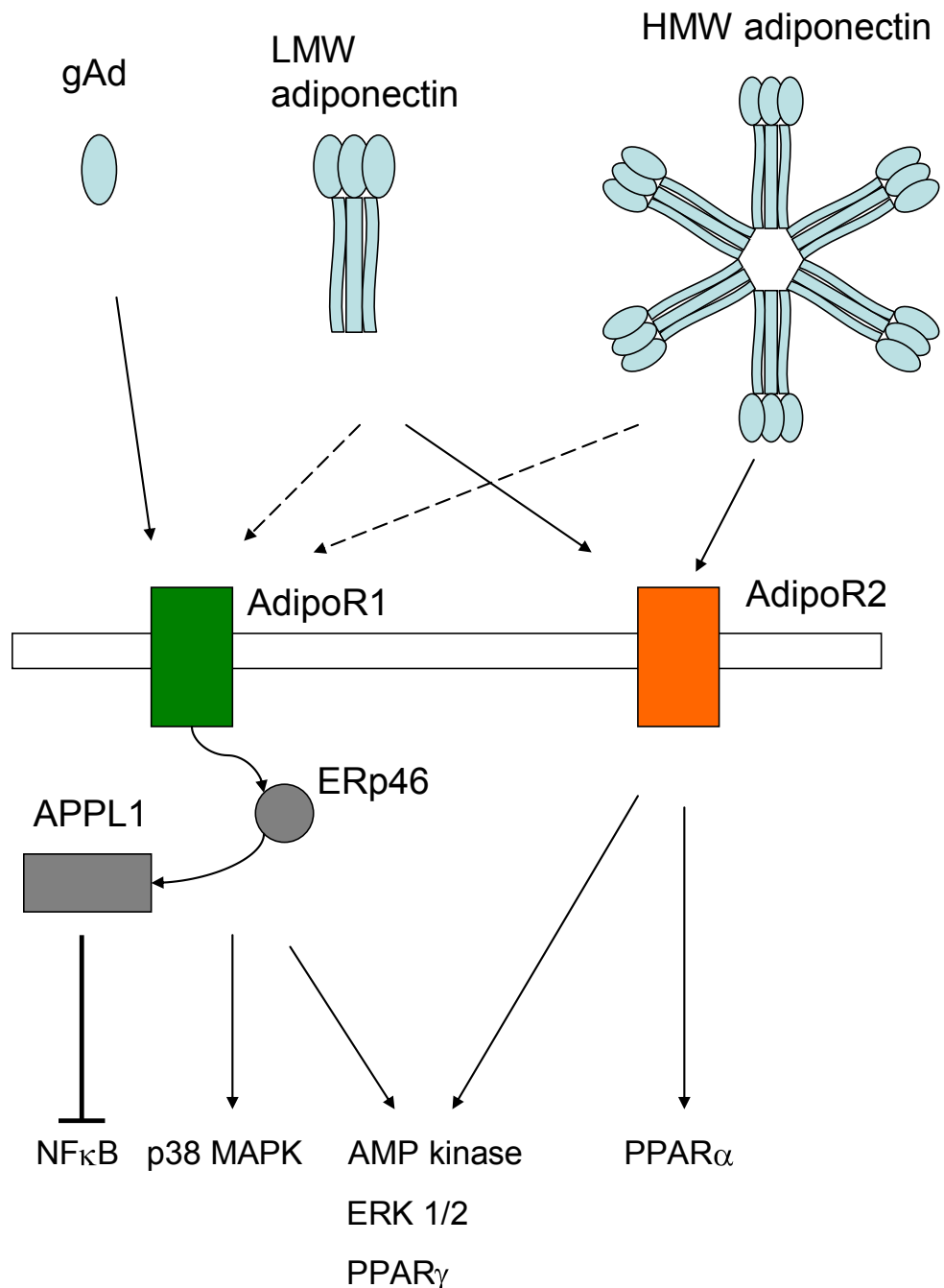
Figure 1.8 Adiponectin and its receptors AdipoR1 & AdipoR2. Monomeric adiponectin has a globular head and a collagenous tail. Circulating adiponectin is multimeric. Low molecular weight (LMW) and HMW adiponectin are formed through non-covalent bonds and disulphide linkage between the tails. The collagenous tail is cleaved in globular adiponectin (gAd). AdipoR1 binds gAd preferentially and has low affinity to LMW and HMW adiponectin (dotted arrow), whilst AdipoR2 binds all species equally. AdipoR1-agonist complex is endocytosed via a clathrin- and Rab5-dependent mechanism, and interacts with the chaperone protein ERp46 and the signal transducer APPL1 (adaptor protein containing pleckstrin homology domain, phosphotyrosine binding (PTB) domain and leucine zipper motif) {{692 Mao,Xuming 2006}}. Various downstream intracellular kinases and nuclear transcription factors are activated (→) or inhibited (⊣)

1.7.3. Regulation of adiponectin production

1.7.3.1 Negative regulators

Serum and mRNA adiponectin levels are reduced in obesity (Kern, Di Gregorio et al. 2003, Weyer, Funahashi et al. 2001, Lihn, Bruun et al. 2004, Hu, Liang et al. 1996). With weight gain, the hypoxic microenvironment and increased production pro-inflammatory cytokines are thought to be the main factors that drive the suppression of adiponectin expression (Ye 2009, Hotamisligil 2006, Bruun, Lihn et al. 2003, Hosogai, Fukuhara et al. 2007). Obesity leads to ER stress (Gregor, Hotamisligil 2007) and consequent activation of JNK (c-Jun-N-terminal kinase) and ATF3 (activating transcription factor 3), two repressors of ADIPOQ transcription (Koh, Park et al. 2007). Other repressor proteins induced in obesity include CREB and NFAT (nuclear factor of activated T-cells) (Liu, Liu 2009). Fat accretion also results in the increased production of TNFα from adipocytes (Kern, Ranganathan et al. 2001) and also the influx of activated lymphocytes and macrophages (Nishimura, Manabe et al. 2009, Winer, Chan et al. 2009). One mechanism by which TNFα suppresses adiponectin expression is through PPARγ. TNFα inhibits PPARγ expression on adipocytes by a protein kinase C dependent mechanism (Lim, Kim et al. 2008). The promoter activity of PPARγ on ADIPOQ transcription (Yu, Javorschi et al. 2002, Kanatani, Usui et al. 2007) is lost upon phosphorylation (Zhang, Berger et al. 1996). In addition, synthesis of ER chaperones involved in secretion of multimeric adiponectin, namely DsbA-L (disulfide-bond A oxidoreductase-like protein) and Ero1 (ER membrane-associated oxidoreductase)-Lα (Wang, Schraw et al. 2007, Liu, Zhou et al. 2008), is reduced by the inactivation of PPARγ by TNFα (Liu, Liu 2009).

1.7.3.2 Positive regulators

As discussed, PPARγ is a known inducer of adiponectin expression and secretion. The promoter region of ADIPOQ was also found to contain response elements to FoxO1, a member of the forkhead box O transcription factor family involved in cell differentiation. Changes in adiponectin production following FoxO1 over-expression and knockdown are consistent with FoxO1 being a positive transcriptional regulator (Nakae, Cao et al. 2008). FoxO1 mRNA can be increased in human inflammatory states (Valenti, Rametta et al. 2008). Also, post-translational modifications of FoxO1 can also influence its cellular localisation and activity. Sirt1, a NAD-dependent deacetylase promotes its nuclear translocation (Qiao, Shao 2006), and Sirt1 protein levels are increased with caloric restriction . This may be a mechanism for accounting the rise in serum adiponectin following lifestyle intervention (Mather, Funahashi et al. 2008, Cambuli, Musiu et al. 2008, Esposito, Pontillo et al. 2003, Monzillo, Hamdy et al. 2003).

Increased adiponectin expression is seen in some inflammatory conditions (Šenolt, Pavelka et al. 2006, Rovin, Song et al. 2005, Yamamoto, Kiyohara et al. 2005, Leth, Andersen et al. 2008); (Tsatsanis, Zacharioudaki et al. 2005, Ehling, Schaffler et al. 2006, Ehling, Schaffler et al. 2006, Haugen, Drevon 2007). This could relate to the induction of adiponectin in extra-adipose tissue (Katsiougiannis, Kapsogeorgou et al. 2006, Guo, Xia et al. 2007, Miller, Cho et al. 2009), and include skeletal muscle (Krause, Liu et al. 2008). Injection of LPS and TNF α increased expression of adiponectin in skeletal muscle by 10-fold (Delaigle, Jonas et al. 2004). The combination of TNFα and interferon-γ was also effective in inducing skeletal muscle adiponectin RNA and protein in vitro, in a dose dependent manner. This was dependent on stimulated nitric oxide production, as expression of inducibe nitric oxide (NO) synthase

preceded the rise in adiponectin mRNA, and that inhibitors of NO synthesis, L-NNMA, suppressed the inducive effect. The mechanism of how NO affect ADIPOQ transcription is not clear.

1.7.4 Physiological functions of adiponectin

1.7.4.1 Glucose and lipid metabolism

The functional relevance of adiponectin in energy metabolism is best demonstrated in the *ADIPOQ* knockout mice. These mice had normal features on normal caloric intake. However, when place on a high fat diet, the animals developed marked insulin resistance, despite comparable weight gain in wild type controls (Maeda, Shimomura et al. 2002, Kubota, Terauchi et al. 2002). Clamp studies showed increased hepatic glucose output and reduced peripheral glucose uptake. Tracer studies demonstrated impaired β oxidation, and in keeping with this, increased tissue triglyceride and appearance of ectopic intramyocellular fat. Moreover, insulin receptor substrate-1 activity was reduced and TNFα levels were markedly increased. Adiponectin replacement reversed this phenotype, through increased activation of AMP kinase, PPARα and p38 MAPK. This was followed by increased phosphorylation of acetyl coenzyme A carboxylase and β oxidation at both the liver and skeletal muscle. At the liver, there was also decreased expression of enzymes involved in gluconeogenesis, namely glucose-6-phosphatase and phosphoenolpyruvate carboxylkinase, with resultant decrease in hepatic glucose output. In skeletal muscle, increased glucose uptake was facilitated by increased expression of glucose transporter GLUT4. Plasma TNFα was reduced. Adiponectin treatment of the ob/ob mice, whose genetic leptin deficiency was associated with hyperphagia and exaggerated weight gain, was effective in ameliorating IR (Berg, Combs et al. 2001). In vitro testing showed

adiponectin allowed suppression of glucose output by hepatocytes at sub-physiological doses. These activities were dependent on AdipoR1 and AdipoR2 signalling (Yamauchi, Nio et al. 2007), through which AMP kinase and PPAR α/γ are activated.

In agreement with a role in human physiology, polymorphisms in ADIPOQ (Gong, Long et al. 2010.Hivert, Sullivan et al. 2008, Menzaghi, Trischitta et al. 2007), as well as reduced serum adiponectin levels (Li, Shin et al. 2009) are independent predictors of glucose intolerance and type 2 diabetes (Table . The genomic region in which ADIPOQ lies was found to be strongly associated with insulin resistance (Kissebah, Sonnenberg et al. 2000). The effect of two single nucleotide polymorphisms in the ADIPOQ promoter region (rs17360539 & rs266729) has been evaluated in case-control studies across the world. In a fixed effects modelling meta-analysis of these studies, G at position 11391 in place of A was associated with an odds ratio (OR) of 1.09 (p = 0.046) for T2D (Gong, Long et al. 2010). G in place of C at the 11377 position was associated with an OR of 1.08 (p = 0.03). There was some heterogeneity in the studies, and the authors cited ethnicity of the study group as a contributor. Neither SNP were found to be associated with T2D in a pooled analysis of studies of East Asians. The significance in the overall analysis was largely attributed to the estimates from studies of Caucasian subjects. In contrast, meta-analysis of the effect of adiponectin on T2D risk in prospective observational trial found a consistent protective effect of adiponectin, regardless of sex, age, BMI and ethnicity. A one log ug ml^{-1} increase was associated with an OR of 0.72 (95% CI 0.67-0.78), ie a 28% reduction in disease incidence (Li, Shin et al. 2009).

	No of studies	OR for T2D (95% CI)
ADIPOQ rs17360539	6	1.09 (1.00 – 1.19)
ADIPOQ rs266729	13	1.08 (1.01 – 1.15)
Caucasian	6	1.06 (1.00-1.12)
East Asians	7	1.09 (0.95 – 1.24)
Log serum adiponectin increase (overall)	14	0.72 (0.67 – 0.78)
Caucasian	6	0.71 (0.65 – 0.78)
East Asians	3	0.70 (0.61 – 0.80)
Asian Indians	1	0.74 (0.59 – 0.93)
Age < 50	3	0.51 (0.28 – 0.94)
Age > 60	6	0.77 (0.70 – 0.84)
BMI < 25	3	0.70 (0.61 – 0.80)
BMI > 25	11	0.74 (0.67 – 0.81)

Table 1.3 Effects of polymorphisms of ADIPOQ and serum adiponectin levels on T2D risk, from meta-analysis (Gong, Long et al. 2010, Li, Shin et al. 2009). OR derived from fixed effects modelling, which assumes the effect of the dependent variable is the same across studies. As a result, the weighting for each study in the pooled analysis is based on sample size and the SD of the estimate. A large study with a small SD of the point estimate will thus have a greater weighting.

Consistent with findings in the ADIPOQ knockout mice, normoglycaemic subjects with ADIPOQ polymorphisms associated with decreased adiponectin expression were of normal BMI (Kondo, Shimomura et al. 2002). This suggests that adiponectin does not determine constitutional fat mass, but rather it is an adaptive mechanism of restoring insulin sensitivity in an otherwise insulin resistant state. In support, adiponectin expression is upregulated in animals made insulin resistant by silencing insulin receptor expression (Lin, Kim et al. 2007).

1.7.4.2 Inflammation

Adiponectin has been widely reported to be anti-inflammatory. Population studies show an inverse correlation of serum adiponectin against TNFα (Hivert, Sullivan et al. 2008, Krakoff, Funahashi et al. 2003), IL-6 (Engeli, Feldpausch et al. 2003, Hung, McQuillan et al. 2008) and CRP (Winer, Zern et al. 2006, Monzillo, Hamdy et al. 2003, Ouchi, Kihara et al. 2003, Mantzoros, Li et al. 2005, Schulze, Rimm et al. 2004). Consistent with a causative association is the reduction of these inflammatory cytokines in insulin resistant animals with adiponectin treatment (Maeda, Shimomura et al. 2002, Uji, Yamamoto et al. 2009, Xu, Wang et al. 2003). In support, adiponectin has the ability to suppress expression of NF-κB, an essential transcription factor for inflammation-related proteins (Haugen, Drevon 2007, Maeda, Shimomura et al. 2002, Ouchi, Kihara et al. 2000, Ajuwon, Spurlock 2005). Recent studies suggest this inhibitory effect is specific to AdipoR1 signalling, and is cyclic AMP dependent (Tang, Chiu et al. 2007, Ouchi, Kihara et al. 2000).

At the cellular level, adiponectin has been shown to have anti-inflammatory effects on monocyte-macrophages, NK lymphocytes and neutrophils (Yokota, Oritani et al. 2000, Tsatsanis, Zacharioudaki et al. 2005, Chinetti, Zawadski et al. 2004, Wolf, Wolf et al. 2004, Wulster-Radcliffe, Ajuwon et al. 2004, Saijo, Nagata et al. 2005, Kim, Kim et al. 2006, Magalang, Rajappan et al. 2006, Neumeier, Weigert et al. 2006, Okamoto, Folco et al. 2008, Weigert, Neumeier et al. 2008, Turner, Smolinska et al. 2009, Kumada, Kihara et al. 2004, Wulster-Radcliffe, Ajuwon et al. 2004) (Table 1.2). Yokota et al were the first to describe anti-inflammatory effects of adiponectin on myeloid macrophages, grown ex vivo from primary monocytes in RPMI supplemented with 10% human type AB serum. The addition of recombinant adiponectin (10 ug ml^{-1}) at the beginning of

culture did not impair viability and differentiation of the monocytes. The macrophages were re-fed adiponectin for 24 hours before functional assays. Adiponectin treated macrophages had reduced phagocytic activity when incubated with fluorescent microspheres. Following brief LPS stimulation, these cells also had reduced TNFα mRNA and protein in the supernatant, compared human serum albumin control. A limitation of the paper is the bacterial source of recombinant adiponectin used. Recombinant adiponectin from non-mammalian sources are purely monomeric, as they lack post-translational modifications required for oligomerisation (Turner, Smolinska et al. 2009). Thus their action here may not be representative of human physiology. Wolf *et al* used a similar protocol to generate macrophages, but 10% FCS and a higher dose of adiponectin (20ug ml^{-1}) from a non-specified source were used instead. They found adiponectin treated macrophages expressed increased levels of IL-10 and IL-1RA in the culture supernatant, compared to a solvent control treatment. When stimulated with LPS, these cells secreted significantly less interferon-γ. The authors also demonstrated the adiponectin treated macrophages had reduced stimulatory capacity when tested with [^{3}H] thymidine labelled allogeneic T cells. They ascribed this to reduced phagocytic activity using a FiTC-dextran assay, although the raw data for this was not presented in the paper. No changes in costimulatory molecules on adiponectin treated macrophages were detected by flow cytometry. A strength of the paper is the investigation into the mechanism for anti-inflammatory cytokine production. The authors tested a range of kinase inhibitors against the effect of adiponectin stimulated IL-10 release. They found a selective inhibitor of PI3-kinase abrogated the effect. PI3 kinase is known to be modulated by APPL-1 dependent AMP kinase activity (Chandrasekar, Boylston et al. 2008) , which in turn, is an important downstream transducer of AdipoR1 (Mao, Kikani et al. 2006). AdipoR1 RNA expression has been described on macrophages (Chinetti, Zawadski et al. 2004), thus we speculate that macrophage functions are mediated by

the adiponectin receptors. Testing this requires the application of specific receptor agonist/antagonist, or means of genetic knockout, of the adiponectin receptor on the functional assay.

Adiponectin appears to inhibit leucocyte migration. In the atheroma model, adiponectin treatment inhibits inflammatory infiltrates of vascular plaques. The decreased recruitment of immune cells from the circulation is thought to be due to reduced expression of integrins on endothelial cells (Ouchi, Kihara et al. 1999), and attenuated function of chemokines (Masaie, Oritani et al. 2007, Okamoto, Folco et al. 2008). At sites of inflammation, cells undergoing apoptosis can be pro-inflammatory and trigger autoimmunity (Savill, Dransfield et al. 2002). Here adiponectin is thought to facilitate the phagocytosis of early apoptotic bodies (Takemura, Ouchi et al. 2007). Takemura et al found marked persistence of apoptotic bodies in thymi of adiponectin knockout mice, upon challenge with dexamethasone. Treatment with adiponectin reversed this phenotype. As further confirmation, the authors found adiponectin pre-incubation coated the surface of apoptotic Jurkat T cells and this facilitated their uptake by macrophages. This effect was thought to occur independently of the adiponectin receptors AdipoR1, AdipoR2 and T-cadherin, as their siRNA knockdown on macrophages did not affect phagocytosis. Instead the effect was attributed to the calreticulin receptor, as its knockdown and neutralisation by specific antibody (anti CD91), abrogated the enhanced phagocytosis. The case for the calreticulin receptor could be further strengthened if the effect of the anti-CD91 is studied in the in vivo model.

Furthermore, cytotoxicity of neutrophils and NK lymphocytes is inhibited (Kim, Kim et al. 2006, Magalang, Rajappan et al. 2006). Kim et al found adiponectin to inhibit IL-2 mediated activation of NF-κB and consequent cytotoxicity, tested in vitro using the

[51]Chromium release assay with labelled YAC-1 lymphoma cells. The supra-physiological dose (30ug ml^{-1}) and source of NK cells (mouse primary) are limitations to the data's applicability to human subjects. Adiponectin also appears to be a potent inhibitor of hydrogen peroxide generation by neutrophils (Magalang, Rajappan et al. 2006). However this effect did not apply across all pharmacological stimuli and the paper did not append any mechanistic data to explain the discrepancy.

Collectively, these functions are protective in inflammatory diseases associated with insulin resistance, such as atherosclerosis and steatohepatitis (Shimada, Miyazaki et al. 2004, Schaffler, Scholmerich et al. 2005). Thus, it has been proposed that the reduced synthesis of adiponectin with weight gain has a permissive effect on inflammation in obesity (Fantuzzi 2008).

However, the paradigm that adiponectin is anti-inflammatory has come under scrutiny. Controlling for body mass index, raised local and serum levels of adiponectin has been reported in chronic inflammatory and autoimmune diseases, including SLE, rheumatoid arthritis, Crohns disease and type 1 diabetes (Šenolt, Pavelka et al. 2006, Rovin, Song et al. 2005, Yamamoto, Kiyohara et al. 2005, Leth, Andersen et al. 2008). In support, adiponectin can be pro-inflammatory (Tsatsanis, Zacharioudaki et al. 2005, Ehling, Schaffler et al. 2006, Ehling, Schaffler et al. 2006, Haugen, Drevon 2007) (Table 1.4), and activate classical complement cascade (Peake, Shen et al. 2008) (Table 1.3).

Table 1.4 immune effects of adiponectin

Function	Source of adiponectin tested	Culture system	Reference
Anti-inflammatory			
(-) TNFα	Full length, bacterial	LPS-stimulated, primary macrophage	(Wulster-Radcliffe, Ajuwon et al. 2004)
	Not stated	LPS-stimulated porcine macrophage	(Neumeier, Weigert et al. 2006)
(-) IL-6	LMW, insect	Monocytic cell line THP-1	(Wulster-Radcliffe, Ajuwon et al. 2004)
	Not stated	LPS-stimulated porcine macrophage	
			(Saijo, Nagata et al. 2005)
(-) IL-8	HMW, plasma derived	Primary macrophage	(Wolf, Wolf et al. 2004)
(-) IFNγ	Not stated	LPS-activated primary macrophage	(Wolf, Wolf et al. 2004)
(+) IL-10	Not stated	LPS-activated human monocytes	(Kumada, Kihara et al. 2004)
	Full length, bacterial	Primary macrophage	(Wulster-Radcliffe, Ajuwon et al. 2004)
	Not stated	LPS-stimulated porcine macrophage	(Wolf, Wolf et al. 2004)
(+) IL-1RA	Not stated	LPS-activated human monocytes	(Yokota, Oritani et al. 2000, Chinetti, Zawadski et al. 2004)
(-) phagocytosis by macrophage	Full length, bacterial	Primary macrophage	(Wolf, Wolf et al. 2004)
	Full length, bacterial	Primary macrophage	(Saijo, Nagata et al. 2005)
	Not stated	Primary macrophage	(Wolf, Wolf et al. 2004)
	HMW adiponectin plasma derived	Primary macrophage	
(-) T cell stimulatory capacity of macrophage	Not stated	Primary macrophage	(Okamoto, Folco et al. 2008)
(-) CXCR3 on macrophage & T cell chemotaxis	Full length, insect cell	Primary macrophage	(Kim, Kim et al. 2006)

(-) NK cell cytotoxicity	Full length, bacterial	Mice C57BL/6 NK cells	(Magalang, Rajappan et al. 2006)
(-) neutrophil superoxide generation	Full length, bacterial	Primary neutrophils	(Peake, Shen et al. 2008)
Pro-inflammatory			
Complement activation Classical pathway	Full length, bacterial	Human serum	(Peake, Shen et al.)
(+) TNFα	gAd, bacterial	Macrophages derived from THP-1 monocytic cell line	(Tsatsanis, Zacharioudaki et al. 2005)
(+) IL-6	gAd, bacterial	Macrophages derived from THP-1 monocytic cell line	(Neumeier, Weigert et al. 2006)
	HMW, mouse	Primary monocytes	(Weigert, Neumeier et al. 2008)
	Full length, mammalian	Primary monocytes	(Ehling, Schaffler et al. 2006) (Weigert, Neumeier et al. 2008)
	Full length, bacterial	Primary synovial fibroblasts	
(+) IL-8	Full length, mammalian	Primary monocytes	(Haugen, Drevon 2007)
(+) NF- kB	Full length, mammalian	Monocytic cells lines THP-1 and U937	{{397 Haugen,Fred 2007}}

Notwithstanding, I believe adiponectin to be chiefly anti-inflammatory. The

heterogeneity from the in vitro studies (Table 1.3) can be explained by differences in

experimental conditions. How the cells were prepared is an important variable.

Adiponectin added to macrophages differentiated in its absence induced IL-6 and

TNFα in a dose dependent manner (Tsao, Tomas et al. 2003, Haugen, Drevon 2007,

Neumeier, Weigert et al. 2006, Palmer, Hampartzoumian et al. 2008). However, after

24 hours of adiponectin exposure, pro-inflammatory cytokine output following LPS or

further adiponectin dosage did not increase further, compared to placebo control. Also,

pre-treatment of macrophages with LPS can affect the readout. Adiponectin treated

macrophages nascent to LPS had reduced IL-8 expression and phagocytic ability

(Saijo, Nagata et al. 2005), but the converse was seen when the macrophages were

exposed to LPS before adiponectin treatment.

The source used may also be relevant, as there appears to be functional differences between multimers. The most direct demonstration of this comes from the paper by Neumeier *et al* (Neumeier, Weigert et al. 2006), where the actions of LMW and HMW adipnectin were compared in the same culture system with primary monocytes. The authors had used the same cDNA sequence and vector, but two different host cell lines to express recombinant adiponectin. But adiponectin from the insect cell source were of LMW multimers, as non-mammalians cells are not capable of the post-translational modifications required for HMW adiponectin formation (Richards, Stephens et al. 2006). HMW adiponectin, but not LMW species, induced IL-6 release from primary monocytes and the THP-1 cell line. In contrast LMW adiponectin selectively suppressed LPS-induced IL-6 production, and enhanced IL-10 release. In support of this, there were differences in intracellular signalling between the multimers. LMW adiponectin reduced, whereas HMW adiponectin increased nuclear NF-κB protein levels. This difference has been confirmed independently by another group, using monocytic cell lines and NF-κB activity was measured by a luciferase reporter system (Haugen, Drevon 2007).

Further confounding may be attributed to LPS contamination in commercially available adiponectin (Aprahamian, Bonegio et al. 2009). Adiponectin was used in doses of at least 10ug ml^{-1} in cell culture systems. The minimum amount of LPS contamination was reported to be 1-5%, and hence this translated to a potential exposure of 0.1-0.5ug ml^{-1} of LPS to macrophages rich in Toll-like receptor 4 expression. Only one study (Tsatsanis, Zacharioudaki et al. 2005) reporting had attempted to counteract this potential source of confounder by the use of polymyxin B, a LPS antagonist .

On the other hand, evidence from in vivo studies is overwhelmingly supportive of the anti-inflammatory role for adiponectin. Using gene silencing and then induced gene upregulation, adiponectin expression was shown to ameliorate pathology in a disease model of lupoid glomerulonephritis (Aprahamian, Bonegio et al. 2009). Similarly, overexpression of adiponectin by gene transfer can attenuate autoimmune arthritis in another in vivo model (Ebina, Oshima et al. 2009). Also, the increase in extra-adipose adiponectin expression following inflammatory stress is potentially protective (Jortay, Senou et al. 2010). LPS injection into skeletal muscle of the adiponectin knockout mice caused exaggerated inflammatory response, with marked NF κB and TNFα expression. This effect was attenuated with electrotransfer of the ADIPOQ gene to the study muscle. It is plausible therefore that increased levels of adiponectin seen in autoimmune diseases reflect an attempt by the body to mitigate the inflammatory response. The mechanism of this may involve NO, FoxO1 and PPARγ (Section 1.7.4). The increased adiponectin may also be a consequence of more advanced disease. In T1D, glycaemia, residual β cell function, microangiopathy and renal reserve are determinants of serum adiponectin levels (Maahs, Ogden et al. 2007). The mechanisms of how these factors drive up adiponectin levels and in turn, how the raised adiponectin influence disease course, are not clear.

1.8   Adiponectin in pre-T1D and autoimmunity

Evidence supporting a pathogenic role for adiponectin in T1D can be found in the Belgian Diabetes Registry (Truyen, De Grijse et al. 2007). Relatives of patients with T1D were followed up for a median duration of 81 months. The overall T1D risk for the antibody positive cohort is low at 18%, compared to the other studies of pre-T1D and insulin resistance. But in those subjects who developed T1D, serum adiponectin levels

for each subject fell significantly in the two years prior to onset, compared to matched controls whose levels at -1 and -2 years were similar to the last sample drawn at the close of follow-up (fig 1.9). Possibly because of the wide confidence interval (5-25 ug), there were no significant differences in adiponectin levels between the groups at each timepoint. In keeping with epidemiological studies, adiponectin had a significant inverse correlation with adiposity, and in a smaller group, with HOMA-IR. Overall, this study suggests in the later stages of pre-T1D, falling adiponectin levels in an individual is a risk factor for T1D onset.

The immunomodulatory potential of adiponectin has not been tested in the NOD mouse, although there are encouraging results from another murine model of autoimmunity. The *lpr* mouse has a single gene defect in *Fas*, resulting in impaired clearance of cell debris. This gives rise to development of multiple autoantibodies and lymphoproliferation, and leads to a clinical phenotype similar to human systemic lupus erythematosis. With the additional knockout of *ADIPOQ*, these mice displayed more pronounced autoimmunity, along with marked joint inflammation and glomerulonephritis (Takemura, Ouchi et al. 2007). These features were ameliorated upon genetic overexpression of *ADIPOQ*. The authors attributed this to the facilitation of apoptotic body clearance by adiponectin.
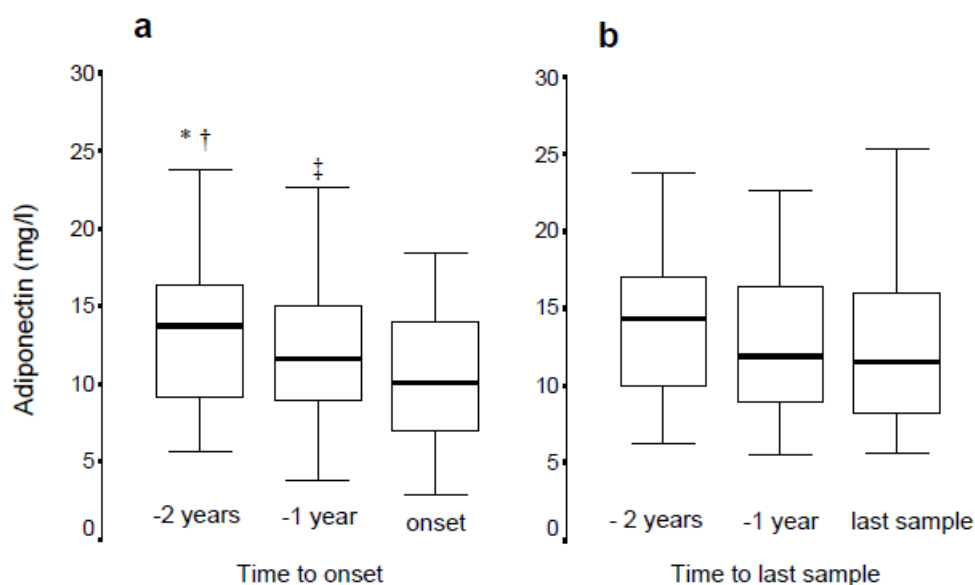
Figure 1.9. Adiponectin levels in pre-type 1 diabetes. (a) Serum levels in subjects (n=28) who developed diabetes (overall *p*<0.001; *p=0.029 vs onset –1 year; ‡p=0.028 and †p<0.001 vs clinical onset), (b) age and sex-matched subjects (n=28) at high risk (autoantibody positive) but remained non-diabetic in the follow-up period (overall *p*=0.113). Each box shows the median (solid black line) and IQR (box length) of adiponectin levels at a given time-point; the whiskers indicate extreme values within 1.5 box lengths from the upper and lower edge of the box. Adiponectin levels at onset of disease (a) were not significantly different from the last sampling drawn in high risk subjects who remained non-diabetic (b).Adapted from (Truyen, De Grijse et al. 2007) electronic supplementary material, with permission from Springer (license 2482151204744)

1.9   Summary

T1D is a costly disease with complex management issues. The destruction of the insulin producing β cell is the culmination of multiple breaches in immune tolerance. Immunogenetic factors contribute to risk. From prospective studies, IR is an additional predictor for progression in the most susceptible pre-T1D subjects. Obesity is the commonest cause of IR and its increased prevalence may explain the current rise in T1D incidence worldwide. The biological basis for this contention may lie with adipose

tissue, and in particular the adipokines it secretes. Adiponectin is an anti-inflammatory and insulin-sensitising adipokine whose functions are mediated by the specific receptors AdipoR1 and AdipoR2. Its levels appear to be reduced before development of T1D. This may be of significance in the maintenance of tolerance, as adiponectin appears to have the capability to downregulate autoimmunity in other disease models.

2. HYPOTHESIS AND AIMS

Given that adiponectin has anti-inflammatory and insulin-sensitising actions, we hypothesise that changes in the adiponectin signalling explains the association between insulin resistance and progression to T1D. If proven, there are clear benefits in augmenting adiponectin action in delaying onset of this disease.

Our aims are:

1) To characterise adiponectin and its receptor expression on PBMC.

2) To investigate effects of insulin resistance and T1D on adiponectin signalling to PBMC.

3) To investigate effects of modifying insulin resistance through exercise on adiponecin receptor expression.

4) To demonstrate effects of adiponectin on antigen-driven T cell responses

This experimental approach was chosen to explicitly test predictions based on our hypothesis. The adiponectin receptor should be expressed on immune cells for adiponectin to have immunomodulatory functions. Secondly, the adiponectin signalling to immune cells should respond to changes in insulin sensitivity, so to account for the accelerated disease onset with increasing insulin resistance. Crucially, adiponectin should have the capacity to direct antigen-driven T cell responses, as they are central to the immunopathogenesis of T1D.

3.MATERIALS AND METHODS

3.1 Subjects

3.1.1 Cross sectional clinical study

Male Caucasian subjects with T1D and T2D were recruited from diabetic outpatient clinics following informed consent. Patients with T1D had been diagnosed on clinical grounds that indicate β-cell destruction, as per 1999 World Health Organisation classification. These include presentation with osmotic symptoms, weight loss, ketosis and requirement for insulin within 3 months of diagnosis, or by the presence of the above with positive islet antibodies. Diabetic subjects were assumed to have T2D if they did not have the above features, together with the absence of a family history of maturity onset diabetes of the young (MODY), or a diagnosis of diabetes before the age of 30. All T2D subjects were insulin treated. Islet autoantibodies were not measured in this cohort for the definitive exclusion of LADA (Fourlanos, Dotta et al. 2005). Nevertheless, we believed this was unlikely, given the absence of associated clinical features in our study patients. They were diagnosed at an older age (>40 years), and did not have autoimmune diseases in their past medical history (Fourlanos, Dotta et al. 2005). Furthermore, insulin treatment was not initiated within six years of diagnosis (Turner, Stratton et al. 1997).

Exclusion criteria were:

1) acute illness and infection
2) poorly controlled diabetes (HbA1C >10)

3) presence of cardiac (NYHA grade 3 or above), liver (raised liver enzymes > 3 times of upper normal limit) or renal disease (estimated GFR from MDRD formula < 30 ml/min (Levey, Bosch et al. 1999))

4) use of thiazolidinediones or fibrates, whose PPARα/γ agonistic activity is known to increase AdipoR1 & AdipoR2 expression on monocytes (Chinetti, Zawadski et al. 2004)

5) use of exenatide or gliptins, as their effect on AdipoR1 and AdipoR2 expression on tissues is unknown.

Body mass index (BMI), blood pressure and point of care testing Hb_{A1C} were taken from clinic records on the day of recruitment. Waist circumference was taken as the transverse diameter at the level of the midpoint between costal cartiliage and iliac crest, after maximal exhalation. The hip circumference is the maximal transverse diameter across the buttocks. These measurements were done either by myself, or Dr Ed Goble, academic FY1 medical house officer. For T1D subjects, the estimated glucose disposal rate (eGDR), in mg kg^{-1} min^{-1}, was calculated using the formula: 24.31- (12.22 $\times$ WHR) – (3.29 $\times$ HT) – (0.57 $\times$ Hb_{A1C}) (Williams, Erbey et al. 2000). HT is a binary variable and refers to the hypertension status (140/90 mm Hg or on anti-hypertensives). eGDR was derived from linear regression using components of the metabolic syndrome, with the glucose disposal rate from the last 30 minutes of the euglycaemic clamp, as the dependent variable. It remains the only clinical estimate of IR to be validated against the gold standard of the euglycaemic clamp in T1D, and is an accepted surrogate measure in epidemiological studies (Chillaron, Goday et al. 2009, Kilpatrick, Rigby et al. 2007, Pambianco, Costacou et al. 2007, Orchard, Olson et al. 2003, Olson, Erbey et al. 2002). However, limitations of eGDR include the relatively

small validation set (n=24) used in the original study from which the model was derived (Williams, Erbey et al. 2000), and the lack of external replication.

Healthy volunteers were age and BMI matched to the T1D cohort. Their normal glucose tolerance was confirmed by random capillary glucose values < 6.0 mmol l^{-1}. Full ethical approval was obtained for this study (University Hospitals Birmingham NHS Foundation Trust research ethics committee, reference number 06/Q2703/47).

3.1.2   Lifestyle Intervention studies

3.1.2.1 Early ACTID –in collaboration with University of Bristol

Sixty-six consecutive participants enrolled in the Early ACTID trial (www.bristol.ac.uk/earlyactid) were studied. Subjects recruited into Early ACTID had been diagnosed with T2D within 6 months of entry. They were subsequently randomised to lifestyle interventions consisting of diet and exercise advice through monthly appointments with diabetic specialist nurses and dieticians, or to standard care. Venous blood samples in EDTA, taken at entry and at 6 months were stored at -80°C. Samples were transported on dry ice to the laboratories of the University of Birmingham, and RNA extracted for study. The recovery of RNA in frozen blood stored in EDTA has been previously described, although lower yields were to be expected because of degradation during storage (Theophilus 1998, Rainen, Oelmueller et al. 2002). We verified the quality the RNA using the Nanodrop spectrophotometer (Section 3.9) before rt-qPCR. In addition, we analysed the correlation between the date of sampling and gene expression values as a means of uncovering the potential confounding of frozen storage (fig 6.3). All samples were fully analysed, by intention-to-treat, before unblinding of trial data. We also examined nine subjects in the exercise

arm of the trial who had latent autoimmune diabetes of adulthood (LADA), a subtype of diabetes characterised by insidious progression to insulin dependency and the presence of antibodies to GAD65 at levels >14 WHO IU/ml (Tuomi, Groop et al. 1993). The DASP-validated radioimmunoassay for GAD65 was performed by Mr AJK Williams at the Medical School Unit, Southmead Hospitial, Bristol. Clinical parameters including estimated VO_2 max based on six-minute walk, and measurements of fasting insulin, glucose values, and HbA1C were kindly provided by Dr R Andrews, Principal Investigator of the Early ACTID trial, University of Bristol. Basal insulin resistance was estimated by HOMA-IR modeling (Wallace, Levy et al. 2004).

3.1.2.2 Exercise intensity study – in collaboration with University of Bath

Study participants were 45-64 years old, non-smokers, had a BMI of 25-35 kg m^{-2}, and not on medication. Female subjects were all post-menopausal. Prior to the study, they performed no regular structured exercise, defined as 30 minutes of activity for more than 5 days a week. All subjects followed a hypocaloric diet (5000 kCal/week) and had five supervised treadmill sessions per week for three weeks. The two arms were differentiated by exercise intensity, whilst the amount of energy expended per session (600kCa) was controlled. In the moderate intensity group (MOD), subjects were asked to perform at 50% VO_2 max, estimated based on target heart rate. In the vigorous intensity arm (VIG), prescribed exercise was executed at 70% VO_2 max. Expressed in terms of time for exercise completion, subjects in the MOD cohort would stop after 75 minutes versus 45 minutes in the VIG group. Subjects were allocated to one of the two groups using a sealed envelope. The envelopes were numbered with the sequence generated and known only by a third party.

The treadmill studies were supervised by Miss N Dixon, at the University of Bath.

Venous peripheral blood samples were collected with full informed consent, in the fasting state, on the morning of day 1 (start) and day 22 (completion). They were posted for over-night delivery to the laboratories of the University of Birmingham. The samples were processed on the day of arrival – no more than 24 hours after venesection. Thus, possible effects of diurnal variation, fed/fast state and transit on PBMC counts and distribution, as well as adiponectin receptor expression were controlled.

This study was conducted in collaboration with Dr D Thompson, Health and Science School for Health, University of Bath.

3.1.2.3 Exercise and overfeeding study – in collaboration with University of Bath

Lean (BMI < 25) male recruits of age 21-37 years were randomised to 1 of 2 arms. In the control arm (SED), subjects were asked to remain sedentary and increase caloric intake by 50%. In the interventional group (EXE), subjects consumed the equivalent hypercaloric diet in addition to supervised running sessions at 70% VO_2 max for 45 minutes everyday for 1 week. Again, randomisation was done using sealed envelopes and venous blood drawn in the fasting state on the morning of day 1 and day 8 of the study. The running sessions were supervised by Mr JP Wahlin, at the University of Bath.

In both studies, anthropometric data and laboratory values regarding serum adiponectin, fasting insulin and glucose were kindly provided by Miss N Dixon and Mr JP Wahlin, research fellows to Dr D Thompson.

3.2 Materials

3.2.1   Culture reagents, antigens and mitogens

Human recombinant adiponectin (Cat #: ALX-522-063-C050) was purchased from Alexis Biochemicals (Exeter, UK). It is produced from the human embryonic kidney 293 cell line. The manufacturer reports endotoxin content of < 0.1EU/ug of the purified protein by the LAL test. In addition the product data sheet states that the reconstituted preparation contains multimeric adiponectin. It mimics activation of AMP activated protein kinase in skeletal muscle (Suzuki, Okamoto et al. 2007) and stimulates islet insulin secretion (Okamoto, Ohara-Imaizumi et al. 2008).

Cellgro SCGM (product no: 2001) and Cellgro DC (product no: 2005) were obtained from Cellgenix (Freiburg, Germany), who discloses plasma derived human albumin, recombinant human insulin and transferrin as the only protein content in the media. They confirm specifically the absence of adiponectin in the media. GM-CSF (Cat # 300-03) and IL-4 (Cat # 200-04) were from Peprotech (Hamburg, Germany). Tetanus toxoid (Cat # 582231) was purchased from Calbiochem (San Diego, USA).

PHA (Cat # L8902), LPS (Cat # L2654) and PWM (Cat # L8777) were from Sigma (Dorset, UK). Dynabeads CD3/CD28 for T cell activation (Cat # 111-31D) were from Invitrogen (Paisley, UK). The stimulatory monoclonal OKT3 antibody was a kind gift from Dr David Sansom, University of Birmingham.

3.2.2 Antibodies

The details of primary anti-adiponectin, AdipoR1 and AdipoR2 and their secondary antibodies are presented in Table 3.1. Antibodies used in flow cytometry are listed in 3.2. Blocking goat and rabbit sera were from Sigma, and Fc receptor blocker purchased from Miltenyi (Church Lane Bisley, UK).

Antibody	Source	Supplier	Cat #	Use
Anti-human adiponectin IgG	Mouse monoclonal 19F1	Abcam	ab22554	WB
HRP-conjugated anti-mouse IgG	Rabbit polyclonal	Dako	P 0161	WB
Anti-human adiponectin IgG	Rabbit polyclonal	Abcam	ab18839	IP
Anti-AdipoR1 IgG	Rabbit polyclonal	Phoenix Peptides	G-001-044	FACS, sorting
Anti-AdipoR2 IgG	Rabbit polyclonal	Phoenix Peptides	G-001-023	FACS, sorting
AdipoR1 immunising peptide (357-375)	Chemical synthesis	Phoenix Peptides	001-044	Blocking
AdipoR2 immunising peptide (374-386)	Chemical synthesis	Phoenix Peptides	001-023	Blocking
Anti-rabbit IgG (H + L), conjugated to Alexa Fluor 488	Goat polyclonal	Invitrogen	A11034	FACS

Table 3.1 Anti-Adiponectin and anti-AdipoR1/AdipoR2 antibodies.

WB – Western blotting; IP – immunoprecipitation; FACS- flow cytometry

Abcam (Cambridge, UK); Phoenix Peptides (Karlsruhe, Germany); Dako (Ely, UK); Invitrogen (Paisley, UK)

Antigen – fluorochrome conjugate	Clone	Isotype	Supplier	Cat.No.	Optimised volume /10^6 cells (ul)
CD1a-PE	HI149	IgG1	eBiosciences	12-0019	5
CD3-APC	UCHT1	IgG1	eBiosciences	17-0038	2.5
CD4-FITC	OKT-4	IgG2b	eBiosciences	11-0048	4
CD4-PE	RPA-T4	IgG1	BD Pharmingen	555347	3
CD4-APC	OKT-4	IgG2b	eBiosciences	17-0048	4
CD4-PerCP	SK3	IgG1	BD Pharmingen	345770	4
CD11c-PE	B-ly6	IgG1	BD Pharmingen	555392	5
CD11c-APC	B-ly6	IgG1	BD Pharmingen	559877	8
CD14-PE	61D3	IgG1	eBiosciences	12-0149	2.5
CD14-APC	61D3	IgG1	eBiosciences	17-0149	2.5
CD19-PE	HIB19	IgG1	BD Pharmingen	555413	5
CD25-PE	4E3	IgG2b	Miltenyi	130-091-024	5
CD25-APC	M-A251	IgG1	BD Pharmingen	555434	5
CD56-PE	B159	IgG1	BD Pharmingen	555516	5
CD80-FitC	L307.4	IgG1	BD Pharmingen	557226	3
CD86-PE	FUN-1	IgG1	BD Pharmingen	555657	3
CD86-PE/ CD209-PERCP-Cy5.5/CD83-APC	FUN-1/ DCN46/ HB15e	IgG1/IgG2b	BD Pharmingen	334098	6
HLA-DR FitC	L243	IgG2a	BD Pharmingen	555811	3
FoxP3-APC	3G3	IgG1	Miltenyi	130-093-013	20
IFNγ-PE	26723.11	IgG2b	BD Biosciences	554707	3
IL10-APC	JES3-19F1	IgG2a	BD Pharmingen	340452	5
Isotype					
Mouse IgG1-FITC	MOPC-21	IgG1	BD Pharmingen	555748	3
Mouse IgG1-PE		IgG1	eBiosciences	12-4714	2
Mouse IgG1-APC		IgG1	eBiosciences	17-4714	3
Mouse IgG2a-PE		IgG2a	eBiosciences	12-4724	3
Mouse IgG2b-PE	27-35	IgG2b	BD Pharmingen	555743	3

Table 3.2: Antibodies used in flow cytometry.

3.3 Buffers, gels, and media

All chemicals were obtained from Sigma unless otherwise stated. The ingredients of

buffers and gels used in the experiments are listed in alphabetical order. All buffers and

media were stored at 4°C.

Agarose gel 2%: 1g of agarose (Bioline) was dissolved in 50ml of heated Bionic buffer 1×. Ethidium bromide 5ul was then added to the cooled solution in a fume cupboard.

Complete culture media: RPMI supplemented with 10% foetal calf serum, 2mM glutamine, penicillin 100 units/ml and streptomycin 100ug/ml.

HNET lysis buffer: HEPES 25mM, Sodium Chloride 150mM, EDTA 5mM, Triton X 1% pH 8.0, with ice cold protease inhibitor cocktail 1× (Sigma, Cat No: P8340) added immediately before use.

MACS buffer: PBS, 0.5% BSA and 2mM EDTA

PBS Tween (0.1%): 5 PBS tablets and 1ml of Tween 20% were mixed with 1L of deionised water using a magnetic stirrer

Reagent A (2L): under magnetic stirrer, 275g sucrose, 2.5ml 1.0M Tris HCl pH8.0, 2.55ml 4.9M MgCl, 25ml Triton X added to 2L distilled water.

RIPA wash buffer (50ml): Sodium deoxycholate 0.25g, NP40 0.5ml, SDS 10% 0.5ml, Sodium chloride 5M 1.5ml, Tris HCl 1M pH8.0 2.5ml, deionised water 45ml.

Serum free culture media: Cellgro SCGM supplemented with 2mM glutamine, penicillin 100 units/ml and streptomycin 100ug/ml.

SDS-PAGE resolving gel 12%: Protogel (30:0.8) 2.4ml, Tris Hcl 1.5M pH 8.8, Deionised water 2.0ml, SDS 10% 60ul, APS 10% 21ul, TEMED 9ul

Stacking gel 6%: Protogel (30:0.8) 0.4ml, Tris HCl 0.5M pH 6.8 0.75ml, deionised water 1.85ml, SDS 10% 30ul, APS 10% 15ul.

3.4 Extraction of peripheral blood mononuclear cells (PBMC)

Venous blood collected in EDTA was diluted 1:1 with RPMI at room temperature. Up to 25ml of the diluted blood was layered on 15ml Lympholyte-H (Cederlane, Burlington, NC, USA, Cat # CL-5016) at room temperature. After centrifugation at 800G for 25 minutes (brake off) PBMC were removed from the interface using pastettes and transferred into a new 50ml Falcon tube. The cell pellet was washed twice in RPMI, and then centrifuged at 800G for 10 minutes. A similar procedure was used for processing buffy coats but this was diluted 1:2 with RPMI, and the PBMC were washed in at least four 600G cycles to remove platelets. The cell pellets were then suspended in 10ml of RPMI and counted by light microscopy.

Cryopreserved blood samples from the Early ACTID trial were thawed on ice, transferred into a 50ml falcon tube, into which Reagent A (recipe in 3.3) was added to achieve a total volume of 20ml. The tube was shaken manually for 15 seconds and then on an orbital shaker for 5 minutes at maximum speed. The pellet containing leucocytes, acquired after a 15 minute 1500g centrifuge, was washed two more times with 10ml of reagent A before RNA extraction.

3.5 Magnetic cell sorting

PBMC were passed over a 30um nylon mesh to remove clumps. All washing steps were done in MACS buffer followed by centrifugation at 600G for 10 minutes.

PBMC subsets for AdipoR1 and AdipoR2 mRNA expression were obtained by positive selection. 10^7 PBMC were incubated in 100ul of MACS buffer with 10ul PE-conjugated anti-CD3, CD14, CD19 or CD56 antibodies (BD Biosciences) for 15 minutes in the dark at 4°C. After washing, the cells were resuspended in 80ul per 10^7 cells and incubated for 15 minutes at 4°C with 20ul of anti-PE microbeads (Cat # 120-000-294, Miltenyi), before a further wash and re-suspension in 500ul of MACS buffer. The cells were then passed over a magnetic field in a MS column (Miltenyi Cat # 130-042-201).

For isolation of cells expressing AdipoR1 and AdipoR2 surface receptors,10^7 PBMC in 100ul MACS buffer were incubated with 10ug (at 1ug/ul) of receptor antibody for 30 minutes at 4°C in the dark. After washing, 20ul of anti-rabbit antibody microbeads (Miltenyi, Cat # 120-000-292) were added to cells in 80ul of MACS buffer and incubated for a further 15 minutes at 4°C. After a 10 minute 400G wash in MACS buffer, the beads and cells mixture in 500ul of MACS buffer, was passed through the MS column once. The pre-passage mixture was labelled as "pre-sort". The flow through was labelled as the "receptor negative" fraction. The fraction retained in the column was subsequently flushed out by a sterile plunger and labelled as "receptor positive". We did not find that a second passage of the "receptor positive" fraction through the MS column, as recommended by the manufacturer, improved purity when AdipoR staining was checked on flow cytometry. CD14 microbeads (Miltenyi, Cat # 120-000-305) were used to extract monocytes for DC generation. CD4+CD25- effector cells were obtained from whole PBMC by initial negative selection of CD4+ cells (Human

CD4+ T cell enrichment kit, Cat # 19052, Stemcell Technologies SARL, Grenoble, France). This was followed by CD25+ depletion using anti-CD25 microbeads (Miltenyi Cat # 130-092-983) and LD columns (Miltenyi Cat # 130-042-901). Sort purity was assessed by flow cytometry.

3.6 Flow cytometry

Between 5×10^5 to 10^6 cells were suspended in 100ul PBS in a 5ml polypropylene tube. They were incubated with antibodies of choice (see Table 3.2) for 30 minutes on ice and in the dark, then washed in 4ml of PBS and pelleted at 400G for 6 mins. The cells were re-suspended in a final volume of at least 400ul PBS. Isotype control antibodies matched in species, isotype, flurochrome and protein quantity to the staining antibody were used for negative control. Gain adjustments and compensation settings of the flow cytometer were made using unstained cells, cells stained in single colour CD4 FitC, PE, PerCP and APC. With the exception of cells stained for intracellular antigens, PI at 3 ul (0.5ug/ml working solution) was added after the final wash to exclude dead cells. The manufactuer's protocol for the FoxP3 staining buffer kit (Cat No: 130-093-042, Miltenyi) was followed for intracellular FoxP3 labelling.

The FACSCalibur 4-colour flow cytometer was used, with Cellquest Pro version 4.0 (BD Biosciences) as the acquisition software. On average, 20000 events were recorded in the gate for viable cells, defined by forward/side scatter and negative PI staining. Analysis was performed using Flowjo version 8.8.6 (Treestar, Ashland, OR, USA).

3.6.1   AdipoR1 and AdipoR2 staining

We prepared 7 tubes for each clinical sample as follows.

Tube	Primary antibodies	Phenotyping antibodies
1	Pre-immune Rabbit serum	APC mouse IgG1 isotype
2	AdipoR1	CD11c APC, CD14 PE
3	AdipoR1	CD3 APC, CD19PE
4	AdipoR1	CD4 APC, CD56 PE
5	AdipoR2	CD11c APC, CD14 PE
6	AdipoR2	CD3 APC, CD19PE
7	AdipoR2	CD4 APC, CD56 PE

Table 3.3 Tubes set for quantification of AdipoR1 & AdipoR2 expression by flow cytometry

Tube 1 served as the internal negative control for each subject. It was the standard for background fluorescence from which net MFI values for AdipoR1 and AdipoR2 staining were calculated. For the exercise and cross-sectional clinical studies, we ensured that the batches of adiponectin receptor antibodies shared the same lot number (Lot no: 02301 & 04005 respectively). As a means of internally controlling for variations in experiments done on different days in the cross-sectional study, at least two each of healthy controls and diabetic subjects were analysed on the same run. This however, was not possible for the exercise studies done in collaboration with the University of Bath, where samples arrived singularly. Cells (10^6 in 100ul PBS) were pre-incubated with 3ul of FcR blocker for 10 minutes on ice. They were then exposed to AdipoR1/AdipoR2 antibodies at 1ug in 100ul of PBS for 30 minutes on ice and in the dark. After washing in PBS the cells were blocked with 5ul of goat serum for 15 minutes on ice and then incubated with secondary goat anti-rabbit antibody conjugated

to AF488 (100ul of a 1:250 dilution) for a further 30 minutes. The negative control (tube 1) was treated with isotype APC-conjugated mouse IgG1 antibodies (control for CD11c APC), goat and rabbit sera (5ul each), followed by secondary antibody.

Gates set for all viable PBMC, lymphocytes, monocytes (by scatter and CD14 labelling) and positive AdipoR1/AdipoR2 cells were set using the negative control tube (tube 1). They were then applied to the tubes 2-7 from the same donor. The net percentage of positive cells and the net MFI (geometric mean fluorescence of all cells in the FL-1 channel) for AdipoR1 (tubes 2-4) and AdipoR2 (tubes 5-7) was obtained by deducting the background staining of tube 1 (negative control).

3.7 Cell culture

All cultures were performed under aseptic conditions with sterile filtering of all media. The incubation conditions were controlled at 37°C and 5% humidified CO_2. In studies of AdipoR1 and AdipoR2 expression on PBMC, unsorted PBMC were grown in RPMI-1640 complete media on 24-well plates for 72 hours, at 10^6 cells ml^{-1}. Culture grade human insulin (Cat # I9278) and dextrose 10% solutions (Cat # G8644) were from Sigma and added at the beginning of culture.

For antigen and mitogen stimulation, 2×10^5 CFSE-labelled whole PBMC in 100ul of complete Cellgro SCGM media were cultured on 96-well round bottom plates for 5-8 days. Tetanus toxoid at 3ug ml^{-1} or PHA at 5ug ml^{-1} was added at the start of culture. The cells were fed a further 50ul of complete media on day 4.

3.7.1 DC generation

Monocytes were positively selected using CD14+ microbeads from PBMC extracted from venous blood of healthy and T1D subjects. They were of 85-93% purity on flow cytometry. Two million CD14+ monocytes were suspended in 2ml of Cellgro DC and seeded onto 6-well plates. They were stimulated with GM-CSF and IL-4 (both at 1ug ml^{-1}). The cells were re-fed GM-CSF and IL-4 (final concentration 1ug ml^{-1}) in complete media on day 3.

The cells were harvested on day 6 by cooling the culture plate on ice and using a cell scapper. After confirming surface phenotype on flow cytometry, the immature DCs were co-cultured, at ratio of 1:10, with 2×10^5 CFSE-labelled CD4+ CD25- responder cells in a total volume of 200ul of complete Cellgro SCGM on 96-well round bottomed plates for up to 7 days. Responder cells were retrieved on the day of the co-culture. Autogeneic T cells were used when tetanus toxoid (3 ug ml^{-1}) was the stimulus, and allogeneic T cells in co-cultures with OKT3 (0.5 ug ml^{-1}). Both stimuli were diluted in the suspension of the effector cells and then immediately added to the DC. Negative controls of responder cells without DC and stimuli were set up in parallel. To minimise variation, samples from at least three donors were processed in each run.

To attain mature DC, LPS at 1ug ml^{-1} was added to immature DC on day 6 and harvested after 24 hours.

3.8 Measurement of cell proliferation by CFSE

CFSE (carboxyfluorescein succinimidyl ester) dilution is a technique for measuring lymphocyte proliferation *in vitro*. The non-fluorescent and freely permeable precursor CFDA-SE (carboxyfluorescein diacetate succinimidyl ester) is cleaved by intracellular esterases to form the active moiety. This is then retained within cells and stablised by covalent linkage with protein thiols. Upon cell division, the dye is shared equally between the two daughter cells, such that successive generations of proliferating cells can be tracked by the exponential decrease in fluorescence. Advantages over the traditional radioactive labeling methodology are the increased sensitivity and capability of immuno-phenotyping responding cells.

Recognised parameters for quantifying proliferation are the division index (DI), and the stimulation index (SI) (Fulcher, Wong 1999, Mannering, Morris et al. 2003). When the majority of cells proliferate with stimulation, DI is believed to be the more representative measure and conceptually this is the average number of cell divisions undergone by the responding cell. It is calculated from curve fitting of data assuming CFSE dilutional kinetics. For rare proliferating events, such as the response of unsorted PBMC to antigen stimulation, SI is more sensitive. SI is calculated by the formula:

$$SI = \% \text{ CFSE}_{low} \text{ antigen } / \% \text{ CFSE}_{low} \text{ no antigen}$$

CFSE_{low} cells were defined as viable cells (scatter and PI negative) that have lower fluorescence in relation to the parent cells. This was determined from wells in the plate

containing unstimulated CFSE labelled cells, on the day of reading. The gating for this population was drawn based on the unstimulated control (figs 7.2 & 7.4).

Significant proliferation above background is taken when SI is ≥ 2.

Method

Cells to be labeled are washed in PBS to remove traces of protein and resuspended at 2×10^7 cells ml $^{-1}$ of PBS. CFDA-SE (cat # 1157, Invitrogen) was diluted with DMSO to give a stock solution at 10mM and stored at -20°C. The working solution was prepared by dissolving a 2ul aliquot in 8ml of PBS. An equal volume of the working solution was added to the cell suspension and mixed by pulse vortex. After incubation for 10 minutes at room temperature, under agitation and light protection, FCS at half total volume was added to quench the reaction. After one minute of standing, the cells were washed for three times in complete media, counted and resuspended at 2×10^6 cells ml^{-1} for stimulation. I found the final concentration of CFDA-SE at 1.25 uM adequately labeled daughter generations above autofluorescence without inhibiting blastogenesis.

3.9   Reverse transcriptional polymerase chain reaction (rt-PCR)

Total RNA was extracted from a maximum of 10^7 cells using the RNeasy Mini Kit (Cat #: 74404, Qiagen, Hilden, Germany) as per manufacturer's instructions, using 350ul of RLT lysis buffer and the 30ul of nuclease free water for the final elution. Precautions for RNAse free conditions were the use of vinyl gloves, nuclease free tips and collection tubes, as well as the use of RNaseZap Decontamination solution (Cat # AM 9780, Applied Biosystems, Warrington, UK) on the work bench and pipettes. Purity and

concentration of the sample was assessed by the Nanodrop spectrophotometer (Model ND-1000, Thermo Scientific, Wilmington DL USA). All samples had λ260/280 >1.8. One microgram of total RNA was reverse transcribed into cDNA by first annealing to 1.5ul of random primers at 500ug/ul (Cat # C118A, Promega, Southampton, UK) in a total volume of 16.5ul, at 95°C for 5 minutes. After cooling to room temperature and centrifuging at 8000G for 10s centrifuge, a mastermix was added. This contained 1ul of SuperScript II reverse transcriptase (Invitrogen), 1.5ul of RNAout RNAase inhibitor (Invitrogen), 6ul of 5× first strand buffer, 3ul of 10mM dNTP and 2ul nuclease free water. Using a thermal cycler (Tetrad PTC-225, MJ Research, Waltham, Massachusetts, USA), the total mix was incubated at 37°C for 1 hour and the reaction terminated by boiling at 95°C for 5 minutes. A reverse transcriptase negative control was made using 1ug of RNA diluted to the same total volume of 30ul with nuclease free water. It was subjected to the same heating conditions.

NTERA cells, a human cell line with phenotype similar to committed neuronal cells (Pleasure, Lee 1993), were used as a negative control for adiponectin rt-PCR (Spranger, Verma et al. 2006). These were donated by Dr S Chan, University of Birmingham. RNA from differentiated ChubS7 cells, which have characteristics similar to primary human adipocytes, was used as positive control (Gathercole, Bujalska et al. 2007). These cells were a kind gift from Dr J Tomlinson, University of Birmingham. Human pancreatic RNA, cloned from autopsy specimen, was purchased from AMS Biotechnology (Europe), Abingdon, UK.

For conventional PCR, 2ul of the cDNA sample and their negative control was amplified using BioTaq 0.2ul (Promega), 4mM of $MgCl_2$, dNTP (0.4ul of 10mM stock) and 0.125 uM of sequence specific, intron-spanning primers in a 20ul reaction.

Sequences of the primers are listed in Table 3.4. Optimised PCR conditions are listed in Table 3.5. BLAST and GenBank were used to check the primers were gene specific and intron spanning. Standard PCR conditions were 95°C for 5 minutes, followed by 30-35 cycles at 94°C for 1 minute, annealing for 1 minute and 72°C 1 minute and then a termination step of 72°C for 2 minutes. For amplification of the adiponectin gene, the conditions were modified to 96°C for 6 minutes, followed by 35 cycles of 94°C, 64°C and 72°C at 45s each. Amplicons were resolved on a 3% agarose gel, stained with ethidium bromide.

Real-time quantitative PCR of the samples was performed, in triplicate, on the Strategene thermocycler with Taqman 2× Universal PCR Mastermix (Cat #: 58003365-1, Applied Biosystems) on nuclease free 96-well plates, sealed with optically translucent covers. The cycling conditions were 50°C for 2 minutes, then 95°C for 10 minutes followed by 40 cycles of 95°C for 15s and 60°C for 1 minute.

Relative quantification of adiponectin and AdipoR1/R2 gene expression (Table 3.6), was calculated by the comparative $\Delta\Delta$Ct method (Livak, Schmittgen 2001). 18S was the internal control. The relative efficiencies of amplification in the duplex reaction was validated in a standard curve. Samples were repeated if the ΔCt SD of the triplicate is > 0.5. Samples with a target gene Ct value of >37 were excluded because it laid outside the dynamic range of our calibrator standard curve. Significant genomic DNA contamination was assessed by target gene amplification in the rt-ve control. cDNA samples were excluded if the ΔCt of their rt-ve control was <5.5, as this implied >2% of the PCR products in log phase originated from a genomic DNA template.

Table 3.4: Primers used in conventional PCR

	Forward (5'-3')	Reverse (5'-3')	Predicted bp
β-actin	GCTCCGGCATGTGCAA	AGGATCTTCATGAGGTAGT	541
Adiponectin (ADIPOQ)	AGGGTGAGAAAGGAGATCCAG	GGGCATGTTGGGGATAGTAA	194
Adiponectin receptor 1 (AdipoR1)	TGCCCTCCTTTCGGGCTTGC	GCCTTGACAAAGCCCTCAGCGATAG	526
Adiponectin receptor 2 (AdipoR2)	GGAGCCATTCTCTGCCTTTC	ACCAGATGTCACATTTGCCA	467

Table 3.5: Conditions for conventional PCR

Primers	Magnesium Volume of 50mM stock in a 20ul reaction (ul)	Annealing temperature °C
β-actin	1.6	58
Adiponectin (ADIPOQ)	1.4	64
Adiponectin receptor 1 (AdipoR1)	1.8	58
Adiponectin receptor 2 (AdipoR2)	1.8	60

Table 3.6: Taqman probes and primers used in real time qPCR

	Forward	Reverse	Probe
AdipoR1	TTCTTCCTCATGGCTGTGATGT	AAGAAGCGCTCAGGAATTCG	TCACTGGAGCTGGCCTTTATGCTGC
AdipoR2	ATAGGGCAGATAGGCTGGTTGA	GGATCCGGGCAGCATACA	CTGATGGCCAGCCTCTACATCACAGGA
ADIPOQ	CTATGATGGCTCCACTGGTA	GAGCATAGCCTTGTCCTTCT	CTGTACTACTTTGCCTACCACATCACAGTCT

3.10 Immunoblotting and immunoprecipitation

Up to 2×10^7 PBMC were lysed in 500ul ice cold RIPA lysis buffer containing $1\times$ protease inhibitor cocktail (Sigma, Cat# P2714) for 15 minutes. The mixture was subjected to maximum speed centrifuge for 10 minutes at 4°C. Protein content of the supernatant was quantified using Bradford reagent (Sigma, Cat# B6916). Briefly, 5ul of the supernatant was incubated with 250ul of Bradford reagent for 30 minutes and absorbance shift from 460nm to 595nm was measured by photometer. These values were referenced against a standard curve constructed from serial dilutions of 1ug ml^{-1} of BSA. Twenty micrograms of protein was added to Novex Tris-Glycine sample buffer (Invitrogen Cat# LC2676) and Nupage Sample Reducing Agent (Invitrogen Cat# NP0009), as per manufacturer's instructions. The samples were then heated to 70°C for 10 minutes before loading onto wells on the 12% SDS Bis-Tris gel, alongside SeeBlue Plus 2 pre-stained standard ladder (7ul, Cat# LC5925, Invitrogen). Human serum, precleared with protein A-Sepharose beads, and diluted at 1 in 20 with PBS was used as the positive control in immunoblots for adiponectin. The gel was resolved with the Xcell Surelock Electrophoresis Cell (Invitrogen) at 160°C and the proteins transferred onto nitrocellulose using the iBlot system (Invitrogen), for 7 minutes. The membranes were blocked with 5% Marvel in PBS Tween at room temperature for 1 hour, before incubation overnight with anti-adiponectin antibody (Abcam Cat# ab22554), diluted at 1:1000 with blocking solution, at 4°C under agitation. After three washes with PBS Tween for 10 minutes on an orbital shaker, the membranes were exposed to secondary anti-mouse antibody conjugated to HRP (Dako Cat# P0161) for 1 hour at RT on a rocker. The secondary antibody was diluted 1:2500 in blocking solution.

The membranes were washed three times before chemiflurorescent detection with ECL Plus and autoradiography with Amersham Hyperfilm ECL (GE Healthcare Cat# RN2132 & 28906832, respectively). With ECL Plus, 2ml of solution A was mixed with 50ul of solution B (40:1). The mix was applied drop-wise to a membrane of $2cm^2$ and left to stand for 3 minutes. To confirm specificity of the bands, immunoblotting was performed on a duplicate membrane with a) secondary antibody alone b) primary antibody blocked overnight with recombinant adiponectin, at a ratio of 1:4 by mass, before application to the transfer membrane.

For immunoprecipitation, rabbit polyclonal antibodies to adiponectin (Abcam, Cat# ab18851) were used as the capture antibody, and protein A sepharose beads (GE Healthcare Cat# 17-0780-01) were used to isolate the immune complexes. The lyophilised beads were prepared by swelling in MilliQ water (0.4g in 8ml) for 30 minutes and collected by centrifugation (2000 RPM 7 minutes at 4C). They were then equilibrated in successive 12ml of Tris-HCl buffers at concentrations 1M, 0.1M and 0.05M (all pH 8) for 30 minutes before storage in an equal volume of 0.01M Tris pH 7.5 to form a 50% slurry.

Lysates were pre-cleared by incubating with pre-immune rabbit serum for 2 hours and then adding protein A (50ul 50% slurry to 500ul of lysate). The mixture was left to stand for another hour at 4°C. The lysates were quantified by Bradford reagent, and at least 500ug of total protein was incubated with the capture antibody, at 10ug ml^{-1}, overnight at 4°C under gentle agitation. Protein A slurry (50ul 50% slurry to 500ul) was then added and the mix was incubated for a further 2 hours on a rotary mixer. The beads were then collected by centrifuge (10000g for 30s), washed in RIPA twice and proteins

were eluted by addition of 50ul SDS sample buffer 2× and boiling at 95C for 5 minutes. The supernatant from a 16000g 5 minute spin was then loaded onto resolving gel.

3.10.1 Covalent coupling of antibody to protein A Sepharose

Fifty micrograms of the IP capture antibody was incubated with 500ul of 50% protein A sepharose slurry for 1 hour with gentle mixing at room temperature. The beads were washed 3 times with 0.1M sodium borate pH9.0, and collected after a 30 second 10000g spin. Re-suspended in 1ml of sodium borate, 5.184mg of dimethylpimelimidate (M_r 259.2, final concentration 20mM) was added to the mixture and incubated for a further 40 minutes at room temperature. The beads were then washed twice with 0.2M ethanolamine pH 8.0 and left to stand overnight in this solution at 4°C. For storage at 4°C, the beads were washed twice with PBS and sodium azide added at 0.02%.

3.10.2 Coomassie staining and in gel digestion

The SDS-polyacrylamide gel containing resolved proteins was rinsed with MilliQ water before immersion in 50ml of SimplyBlue Safestain (Cat# LC6060, Invitrogen), a Coomassie G250 based stain, for 1 hour. The gel was destained overnight in MilliQ water.

Steps for in gel digestion were carried out in a negative pressure hood to avoid keratin contamination. The band corresponding to molecular weight of 30-36 kDa was excised with a sterile scalpel and cut into smaller pieces. It was then washed twice in 100ul of 50% acetonitrile in 50mM ammonium bicarbonate for 45 minutes at 37°C. After drying the band in a dessicator, the sample was incubated in 50ul of 50mM dithiothreitol,

made up in 10% acetonitrile/50mM ammonium bicarbonate at 56°C for 1 hour. After spinning, the pellet was then incubated with 50ul of 100mM Iodoacetamide (in 10% acetonitrile/50mM ammonium bicarbonate) at room temperature in the dark for 30 minutes. Again the sample was spun and supernatant discarded, followed by 3 washes in 10% acetonitrile in 50mM ammonium bicarbonate for 15 minutes under agitation. After drying for 1 hour, the sample was rehydrated in 20ul of 12.5ug ml^{-1} sequence grade modified trypsin (Cat# V5111, Promega) in 10% acetonitrile/ 50mM ammonium nitrate, for 1 hour at room temperature. Twenty microlitres of 10% acetonitrile/50mM ammonium nitrate was added and the mixture was incubated at 37°C overnight.

On the next day the sample was spun. This supernatant was pooled with that obtained from two further washes with 30ul 3% formic acid in water, each lasting 1 hour. The final sample containing trypsinised peptides, of volume 70-90ul, was submitted for mass spectrometry. The resultant spectra was searched against the National Center for Biotechnology non-redundant database (released April 2004) using the TurboSequest (3.1) search algorithm (ThermoFinnigan).

3.11 Serum adiponectin measurement by ELISA

Serum adiponectin levels were measured using the Quantikine Human Total Adiponectin Immunoassay (Cat # DRP300, R & D, Abingdon, UK). Standard curves and pre-dilution of serum samples were done according to manufacturer's protocol (appendix 7). Maximum CV for total adiponectin is reported at 6.9% and detection limit is 0.246 ng ml^{-1}. Absorbance was measured at 492nm for 0.1s by the Wallac Victor 4 plate reader.

3.12 Statistics

Statistical analysis and graphical composition were carried out using Excel 2003 (Microsoft), SPSS 15.0 (SPSS Inc, Chicago, USA) and Graphpad Prism 5.0 for Windows (GraphPad Software, San Diego, USA). Continuous variables were expressed as mean +/- SD and categorical data as percentage of cases. The Kolmogorov–Smirnov test was used to test for normal distribution of raw data. Residuals plots were used to see if logarithmic transformation yielded a Gaussian population.

Log transformed gene expression values, net percentage positive cells and proliferation indices from flow cytometry were compared using the two tailed student t-test or ANOVA with Bonferroni post-test when there are more than two groups. When distribution of test data did not conform to normal distribution, the non-parametric Wilcoxon signed rank test was used to test significance when there were two groups, and the Kruskal-Wallis statistic with Dunn's correction for multiple testing was applied for comparisons between three groups. The Fisher exact test was used for categorical data. p values of less than 0.05 were considered significant.

Two-tailed Pearson correlation analysis and scatterplots were used to determine relationships between serum adiponectin, AdipoR1 and AdipoR2 expression, as well as clinical parameters. The significant variables were entered into a forward stepwise linear regression model to assess their independent influence on AdipoR1 & AdipoR2 expression.

4. EXPRESSION ANALYSIS OF ADIPONECTIN RECEPTORS AND ADIPONECTIN ON PERIPHERAL BLOOD MONONUCLEAR CELLS

4.1 Introduction

The adiponectin receptors, AdipoR1 & AdipoR2, modulate at least in part, the insulin sensitising and anti-inflammatory functions of adiponectin (Kadowaki, Yamauchi 2005). The isoforms share 66.7% primary sequence homology and are differentiated by their affinities to adiponectin multimers. AdipoR1 has preferential avidity for globular adiponectin, whereas AdipoR2 is promiscuous for all multimers (Yamauchi, Kamon et al. 2003). Recent studies suggest that there are also differences in signal transduction following agonist binding (Xu, Wang et al. 2009. Charlton, Webster et al. 2010).

Evidence for AdipoR1 & AdipoR2 expression on cell types known to mediate tolerance in T1D would support our hypothesis. Their gene expression has been described on PBMC (Weigert, Neumeier et al. 2008, Palmer, Hampartzoumian et al. 2008, Sohn, Kwak et al. 2010, Shen, Charlesworth et al. 2007, Alberti, Gilardini et al. 2007, Tan, Wang et al. 2009). In this series of studies, we aimed to extend this current knowledge by first demonstrating their expression at the protein level by flow cytometry. We then used this method to characterise their relative expression on PBMC subsets. Lastly, we investigated whether PBMC themselves express adiponectin, as the secretion of other adipokines by PBMC can have important autocrine/paracrine immunoregulatory effects (De Rosa, Procaccini et al. 2007).

4.2 Expression of adiponectin receptor mRNA on PBMC

We first confirmed the presence of AdipoR1 & AdipoR2 mRNA (fig 4.1) on PBMC by conventional PCR, using intron-spanning primers. Amplicon size was consistent with the predicted base pair length on electrophoresis. Next, we validated our rt-qPCR assay for relative quantification of AdipoR1 & AdipoR2 mRNA by the $\Delta\Delta$Ct method (Livak, Schmittgen 2001) (fig 4.2). We derived standard curves of serial template dilutions against ΔCt (Ct target gene – Ct endogenous control) in duplex reactions. The reliability of 18S as a normalising control is demonstrated by the standard curve gradients of <0.1 (Applied Biosystems 2004)

Figure 4.1: AdipoR1 & AdipoR2 mRNA on PBMC . Lanes A-D: AdipoR1 32 cycles. Lanes E-G AdipoR2 35 cycles. Predicted amplicon size AdipoR1 526bp; AdipoR2 467bp.
A & E- Whole human pancreas RNA; B & F- rt-ve pancreas
C & G- PBMC; D & H- rt-ve PBMC.
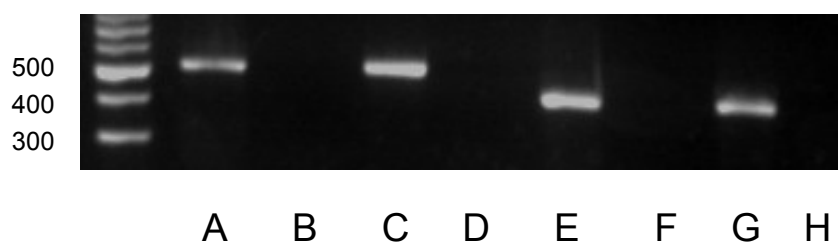
Figure 4.2: Validation of Taqman duplex rt-qPCR . Ct values and ΔCt values (Ct target gene – Ct 18S) are shown following serial template dilutions for AdipoR1 (A&B) & AdipoR2 (C&D). Best fit lines based on the linear regression model y=mx+C are shown. Error bars indicate SD.

A.

$y = -3.0443x + 29.678$
$R \text{ square} = 0.9955$

$y = -2.9349x + 15.768$
$R \text{ square} = 0.9961$

B.

$y = -0.0374x + 13.866$

C

18S ◆　AdipoR2 ■

$y = -2.9939x + 34.899$
$R^2 = 0.9982$

$y = -3.0834x + 22.127$
$R^2 = 0.9926$

D

$y = 0.0895x + 12.773$
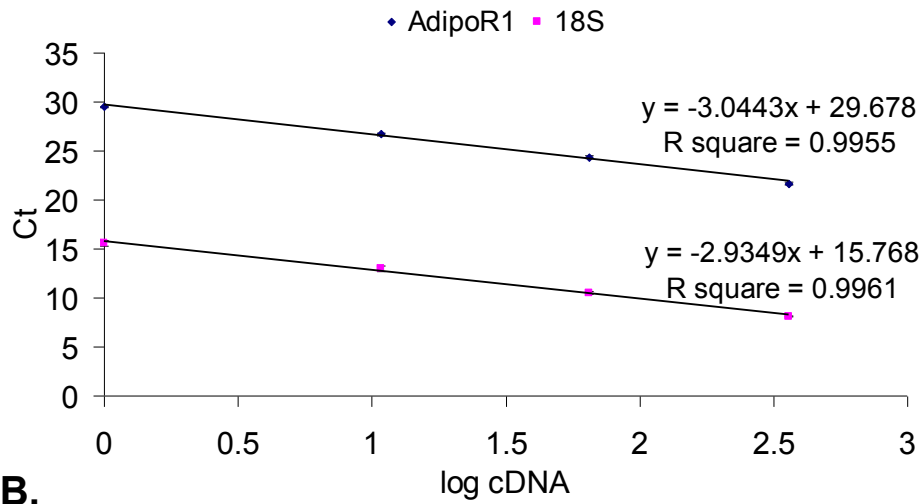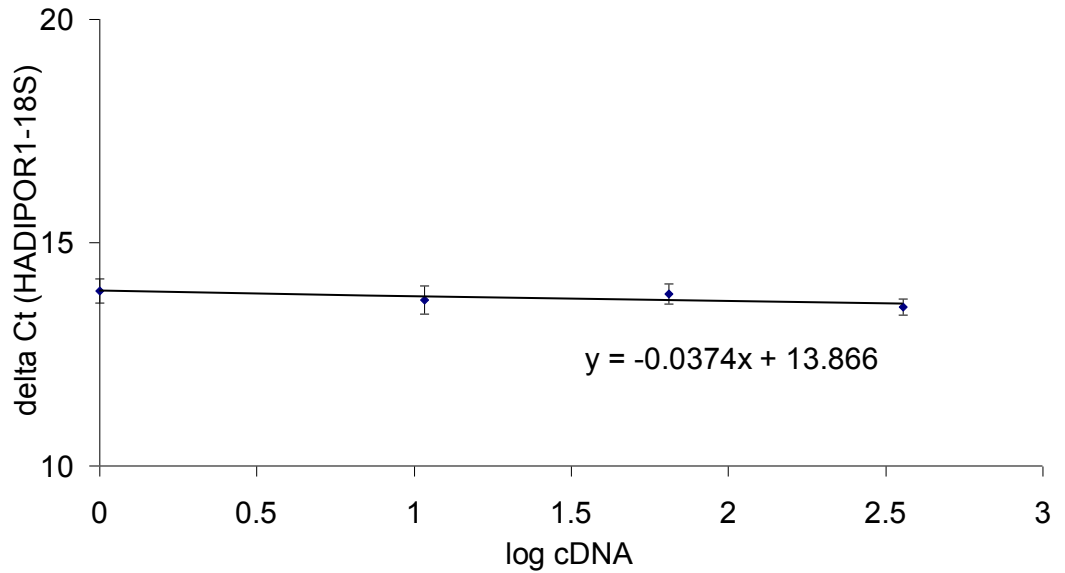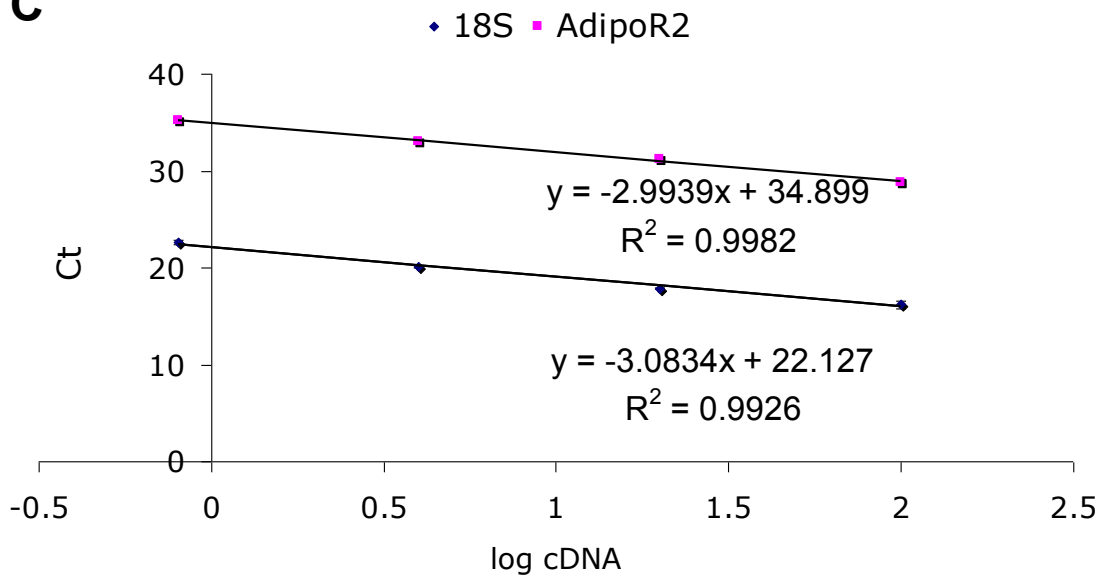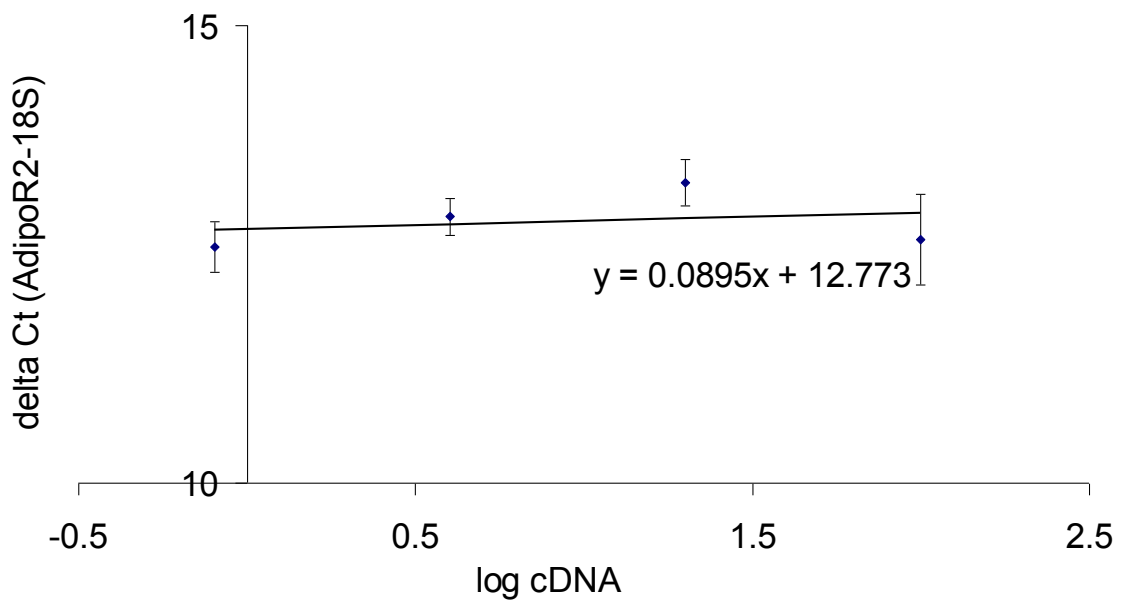
4.3 Characterising the surface expression of adiponectin receptor on PBMC by flow cytometry

Having optimised the concentration of primary and secondary antibodies (fig 4.3), we checked for the specificity of AdipoR1 & AdipoR2 labelling on PBMC. The staining for both AdipoR1 & AdipoR2 was inhibited when the primary antibody, raised from a rabbit host, was pre-incubated with the specific immunising peptides (fig 4.4). Because of the primary sequence homology between the two receptors, we then asked whether the antibody labelling was specific to the receptor isotype. The blocking peptide of AdipoR1 did not have significant inhibitory effect of labelling by the AdipoR2 antibody, and vice versa (fig 4.5). In addition, we looked for differences in gene expression levels in PBMC sorted according to their AdipoR1 & AdipoR2 labelling. Using anti-rabbit microbeads, the yield of positive cells was increased by ~2.5 fold (fig 4.6). Second passage over the column reduced the yield without significantly improving purity. By rt-qPCR, AdipoR1 & AdipoR2 gene expression was significantly higher in the respective positive fractions, relative to pre-sorted PBMC (Fold change AdipoR1+: 4.02 95%CI 2.39 – 6.76; AdipoR2+: 2.08, 95% CI 1.44 - 7.23) (fig 4.7). Gene expression in the negative fraction was similar to pre-sort. Conversely, AdipoR1 expression in the AdipoR2+ fraction was not significantly increased compared to the pre-sort (fold change = 1.64, 95% CI 0.97-2.75), as was AdipoR2 expression in AdipoR1+ fraction (fold change = 2.08, 95% CI 0.93 – 4.67). Thus, although limited by possible receptor co-expression and sort purity, these gene expression findings nonetheless support our antibody labelling as being relatively isotype specific.

Figure 4.3: Optimisation of antibodies for the labelling of AdipoR1 & AdipoR2 (A) Secondary antibody only. The smaller peak on the right of the histogram is due to higher auto-fluorescence of CD14+ monocytes (B). The primary antibody was suspended in 0.01ug ml^{-1} PBS. Differences in fluorescent labelling following addition of the primary antibody to AdipoR1 (C) & AdipoR2 (D) at 0, 20, 50 & 100ul. The amount of secondary antibody was fixed at 100ul of a 0.008ug ml^{-1} suspension. (E) Titrating secondary (2y) antibody. Primary AdipoR1 antibody was fixed at 100ul.
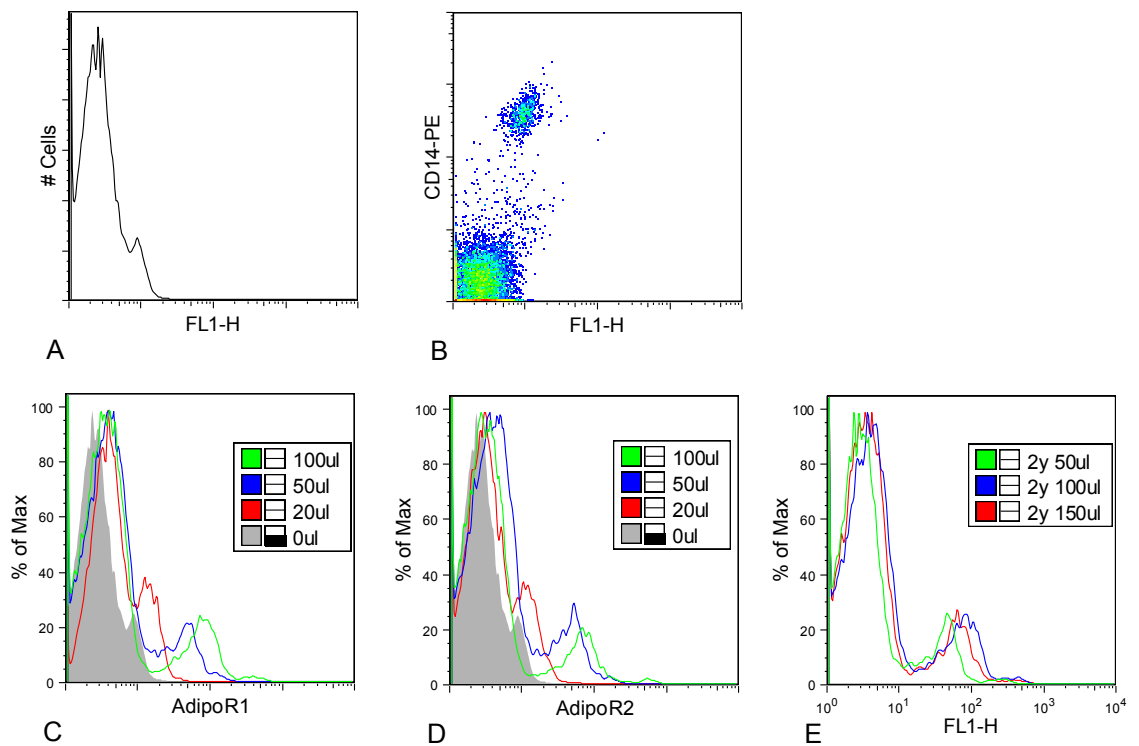
Figure 4.4: Effect on AdipoR1 (A) & AdipoR2 (B) labelling when the primary antibody is co-incubated with its immunising peptide, at a ratio of 1:5.
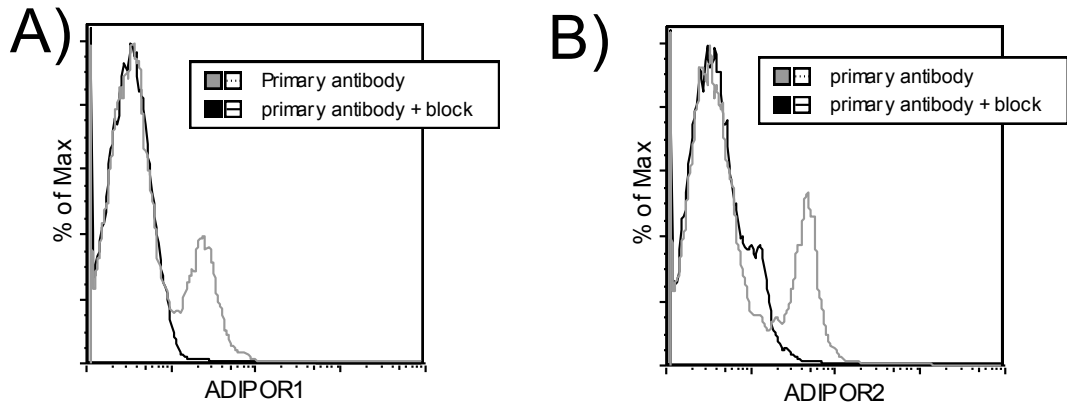
Figure 4.5: Inhibition of staining is receptor subtype specific. Numbers in legend denote MFI. AdipoR1 immunising peptide (AdipoR1p) inhibited AdipoR1 staining (a) but not AdipoR2 (b). The vice versa was seen with AdipoR2p. 50ul of the primary antibody at 0.01 ugml^{-1} was used.
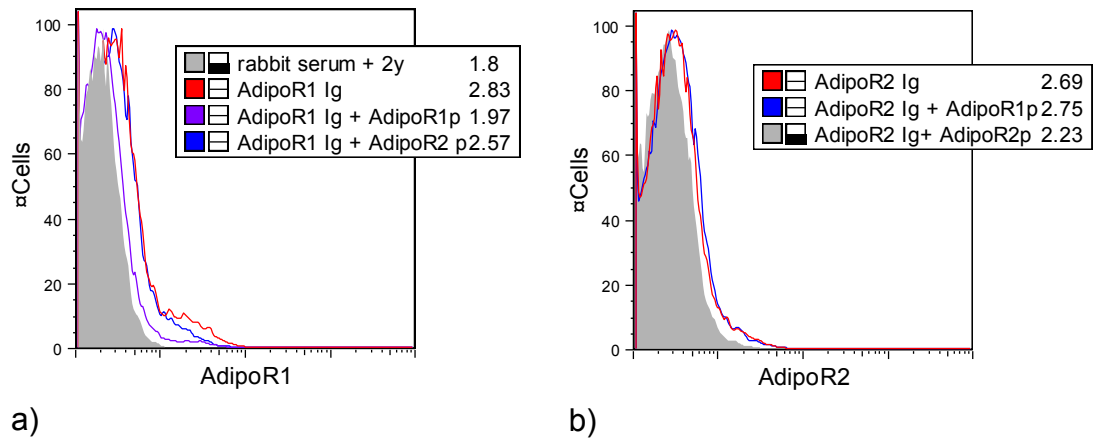
Figure 4.6: Sorting PBMC according to AdipoR1 & AdipoR2 expression. Gates are set against isotype. Numbers denote percentage frequency of positive cells among all viable PBMC. Presort expression levels of AdipoR1 (a) & AdipoR2 (c) are shown. Note that the majority of monocytes (higher forward scatter) stain positive for AdipoR1 & AdipoR2. b & d- staining of cells positively selected for AdipoR1 & AdipoR2.
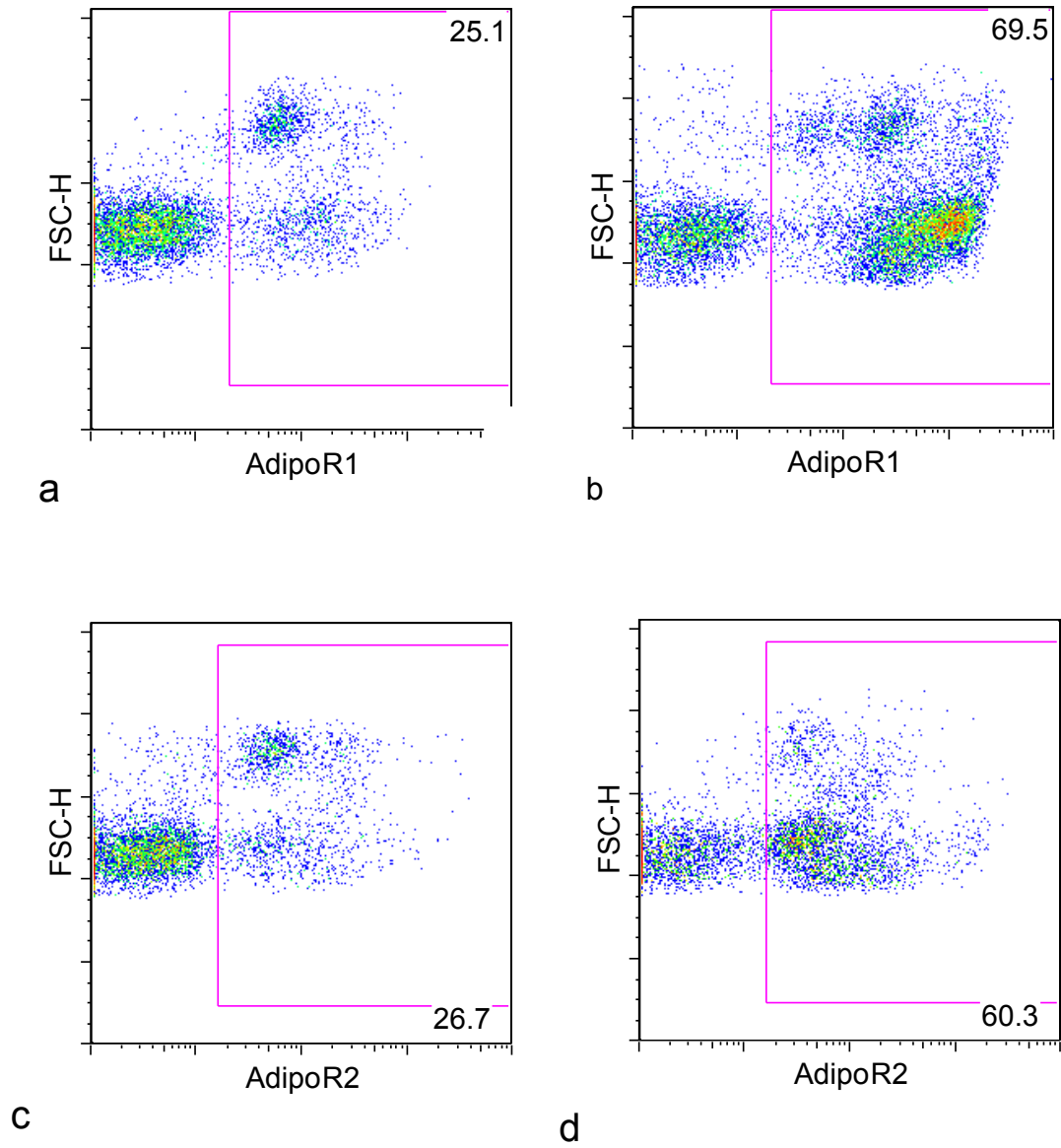
Figure 4.7: Gene expression of cells sorted according to AdipoR expression. Each sort was performed twice and the mean ΔCt values were calculated from the 2 triplicates. Error bars denote SD.

*** p<0.001; **p<0.01 ANOVA with Bonferroni correction
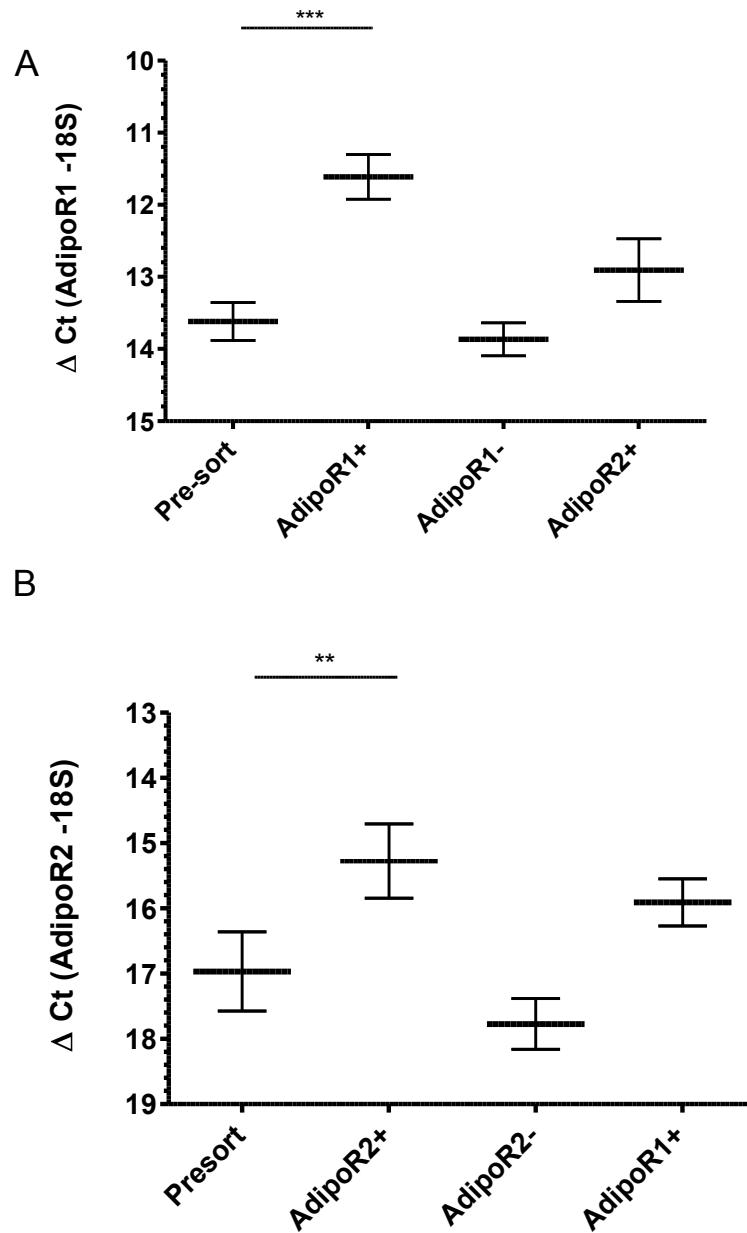
4.4 Quantification of AdipoR1 & AdipoR2 expression on FACS and determining intra-subject variability

The geometric mean fluorescence intensity (MFI) and the percentage of positively labelled cells (%pos), relative to isotype, are two conventional measures for quantifying surface receptor expression by FACS. The gating strategy for determining AdipoR1 & AdipoR2 is outlined in figure 4.8. Briefly, viable PBMC were defined according to scatter and propidium iodide staining. We found that monocytes have consistently higher auto-fluorescence compared to lymphocytes, hence 2 sets of gates for each cell type, accounting for 99% of events, were drawn to delineate negative staining in the isotype control.

Figure 4.8: Example gating for the determination of AdipoR expression on PBMC subsets. Viable lymphocytes were defined by their scatter characteristics (A) and propidium iodide staining (B). Quadrant gates were determined against isotype (C), such that at least 99% of cells are in the lower left quadrant. In this example, the percentage of CD3+ T cells expressing AdipoR1 (D) is 5.5% (4.01/(69.3+4.01)).
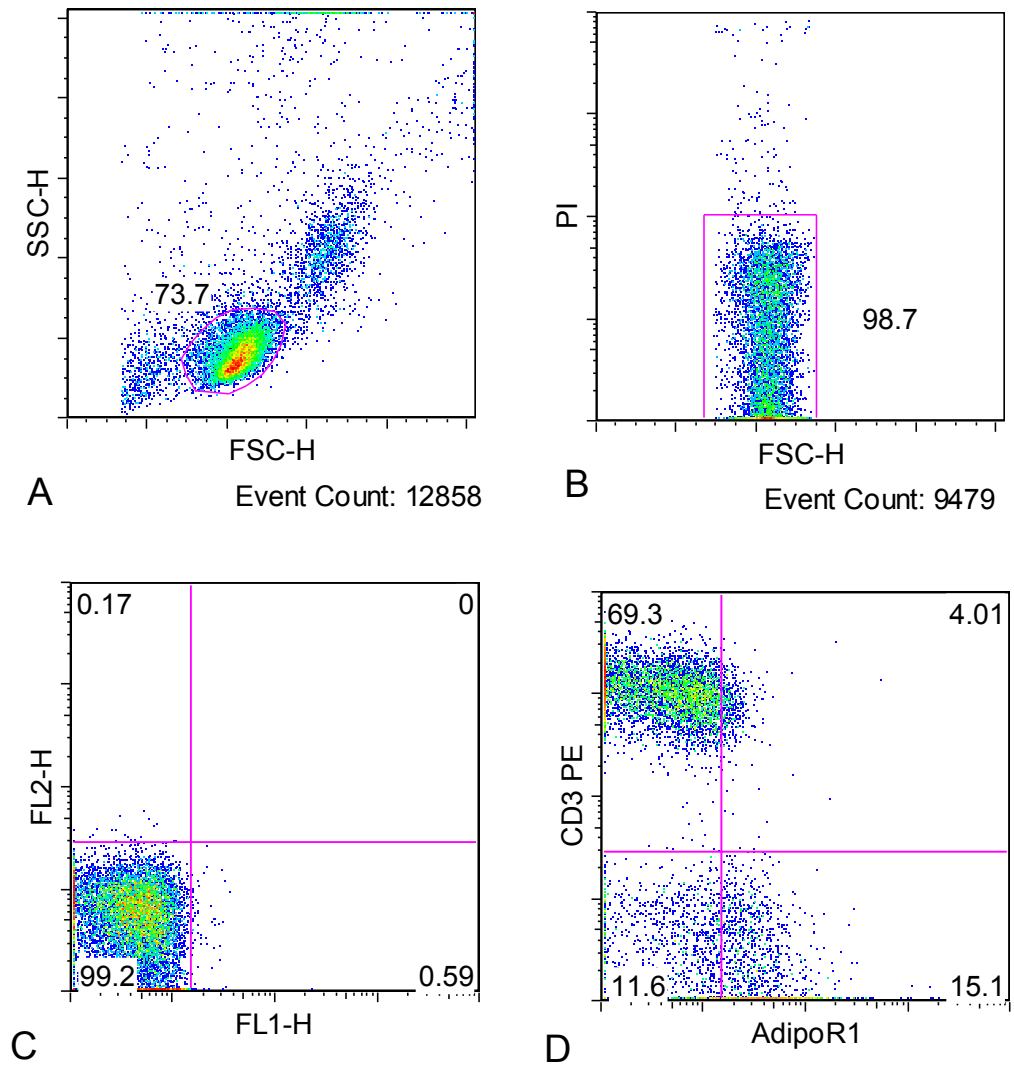
Next, we qualified the intra-subject variability of our assay. We calculated the CV based on independently stained replicates from 3 healthy subjects (Table 4.1 & 4.2). Lymphocytes and monocytes were defined according to scatter. The maximum CV for the assay was 13.2%.

Table 4.1: Intra-assay variability for AdipoR1 expression measurement

	Lymphocytes						Monocytes					
Subject	1		2		3		1		2		3	
	MFI	%pos	MFI	%pos	MFI	%pos	MFI	%pos	MFI	%pos	MFI	%pos
N	6	6	4	4	3	3	6	6	4	4	3	3
Mean	5.30	38.2	3.45	15.95	6.19	22.44	129.8	90.8	84.45	85.8	98.2	78.8
SD	0.51	2.22	0.46	0.58	0.68	0.85	12.2	3.64	8.69	8.28	7.40	4.62
CV (%)	9.71	7.87	13.23	3.64	10.99	3.79	9.40	4.02	10.2	9.65	7.54	5.86

Table 4.2: Coefficient of variation for AdipoR2 expression measurement of PBMC by FACS

	Lymphocytes						Monocytes					
Subject	1		2		3		1		2		3	
	MFI	%pos	MFI	%pos	MFI	%pos	MFI	%pos	MFI	%pos	MFI	%pos
N	6	6	4	4	3	3	6	6	4	4	3	3
Mean	5.82	19.45	3.54	12.3	6.24	18.32	148.8	53.8	87.4	79.4	98.6	65.5
SD	0.43	1.88	0.38	1.19	0.56	0.67	16.22	6.29	10.44	9.69	7.10	4.21
CV (%)	7.48	9.7	10.68	9.7	8.97	3.7	10.90	11.7	11.96	12.2	7.20	6.4

4.5 Distribution of adiponectin receptor expression on PBMC subsets

As the first step in elucidating the immunoregulatory potential of adiponectin, we examined the relative expression of its specific receptors on PBMC subsets, at both the protein and mRNA level. PBMC were classified as monocytes, T cells, B cells and NK lymphocytes, using the phenotypic markers, CD14, CD3, CD19 and CD56 respectively. We also labelled PBMC for AdipoR1 or AdipoR2 (fig 4.9), and derived our estimates for relative protein expression of the receptors from the frequency of the "double positive" population. Over 10 independent experiments (fig 4.10), the percentage of cells

staining positive (%pos) for AdipoR1 are as follow (mean ± SD): CD3+: 4.7 ± 0.9;

CD14+: 84.6± 2.0; CD19+: 55.6 ± 5.4; CD56+: 34.7 ± 3.4. For AdipoR2, %pos values

were: CD3+: 6.2 ± 1.4; CD14+: 80.2 ± 3.4; CD19+: 48.6 ± 8.6; CD56+: 36.8 ±

4.2.

Figure 4.9: Distribution of AdipoR1 & AdipoR2 surface expression on PBMC. Representative example. Numbers denote percentage frequencies of gates. A & E) All PBMC analysed. Higher autofluorescence accounts for the difference in thresholds set for positive receptor staining in CD14 positive cells. For B-D, F-H, analyses are confined to lymphocytes only. In this example, the percentage of CD14+ cells expressing AdipoR1 is 77% (7.57/9.74) (A) and 67% (6.67/10) for AdipoR2(E).For CD3+ cells, AdipoR1: 10.3% (B), AdipoR2: 6.1% (F). For CD19+ cells, AdipoR1: 71%; AdipoR2: 45%. For CD56+, AdipoR1: 48%; AdipoR2: 35%.
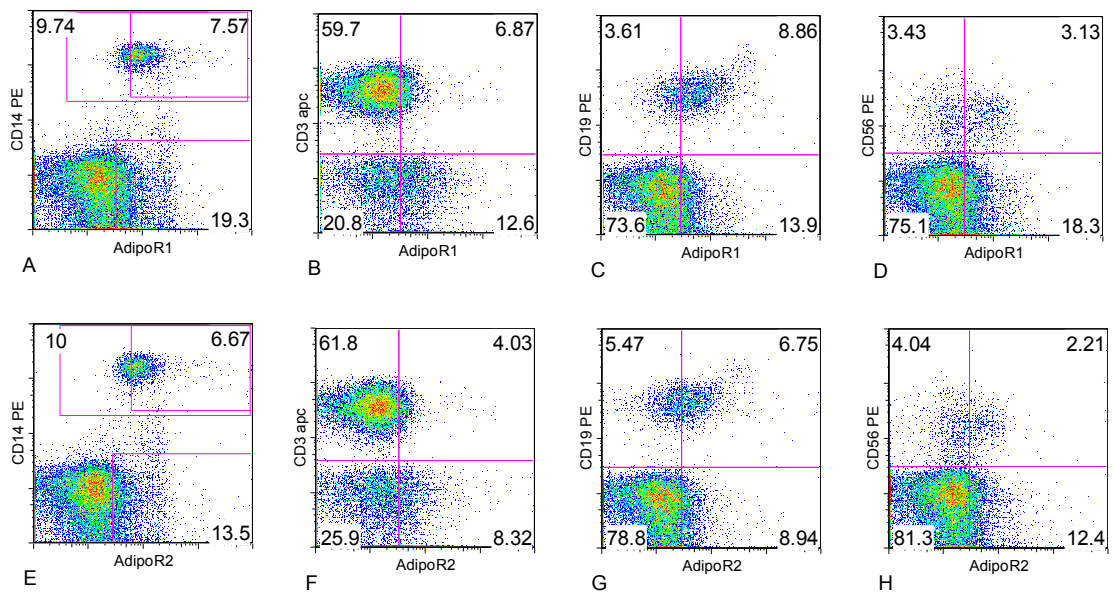
Figure 4.10: Distribution of AdipoR1 & AdipoR2 expression on PBMC by flow cytometry. (n=8) Error bars indicate SD.
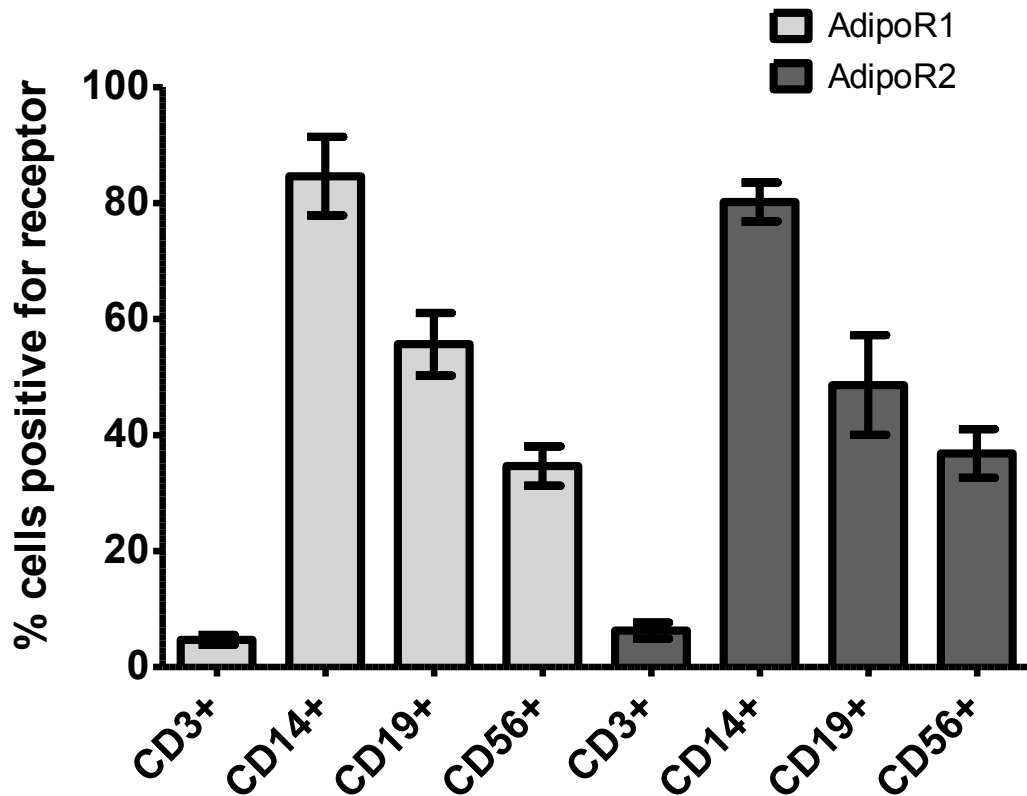
For the characterisation of AdipoR1 & AdipoR2 gene expression, we extracted RNA from positively selected CD3+, CD14+, CD19+ and CD56+ cells. The MACS sorts (n=4) were performed simultaneously from the same donor buffy coat, and had purities approaching 90% or over (figure 4.11). On conventional rt-PCR, AdipoR1 & AdipoR2 gene expression was confirmed on all 4 subsets. By densitometry of the amplicon bands, expression of both receptors appeared to be highest on monocytes (figure 4.12). We then proceeded to accurately quantify relative gene expressions by rt-qPCR. Relative to autologous CD3+ T cells (figure 4.13), AdipoR1 expression on CD14+ monocytes was 9.1 (95% CI: 8.0-10.2) times higher. The ratio for CD19+ was 6.9 (6.0-7.9) and for CD56+, 5.8 (5.0-6.8). AdipoR2 gene expression was also the highest on

monocytes (fold change: 4.5, 95% CI: 4.5-5.6) and CD19+ B cells (fold change: 4.6

95% CI: 3.8-5.4). This pattern of predominant expression on CD14+ monocytes and

CD19+ B cells was in agreement with protein expression data from flow cytometry.

Figure 4.11: Purity of PBMC subset sorts. Numbers denote gate frequencies (%). A) Presort B) Following positive selection for CD3 C) Presort. D) Positive selection for CD14. E) Presort F) CD19 sort G) Presort H) CD56 sort.
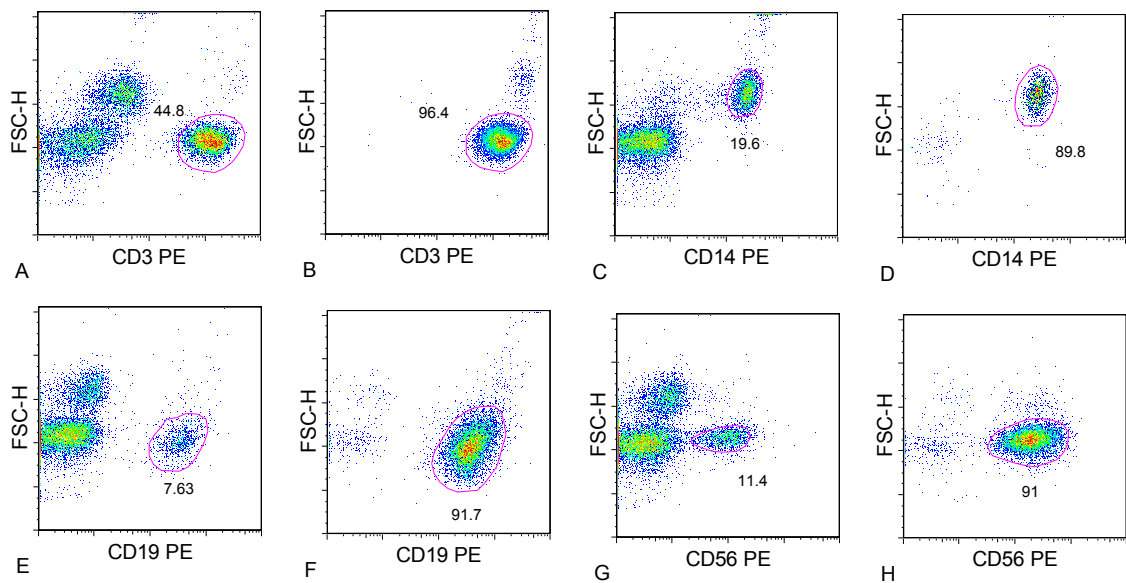
Figure 4.12: AdipoR1 & AdipoR2 mRNA expression on PBMC subsets.

(top) AdipoR1 (predicted amplicon size 526bp)

L- ladder

A-human pancreas RNA. B&C: CD3+ cells & rt-ve control.

D&E: CD14+ & rt-ve.

F&G: CD19+ & rt-ve.

H&I: CD56+ & rt-ve.
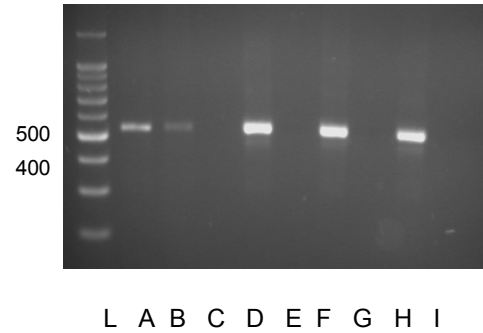
(middle) AdipoR2 (predicted size 467): A: water blank.

A-D: CD3+, CD14+ CD19+ & CD56+.

F-I: respective rt-ve control.
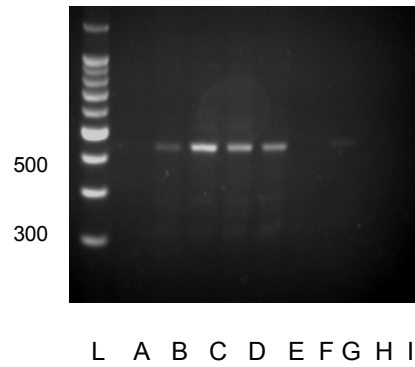
(bottom) beta-actin (predicted bp: 541bp). L-ladder.

A, C, E, H- CD3+, CD14+, CD19+ and CD56+ cells with rt-ve controls in lanes to the righ (B, D, F, I).
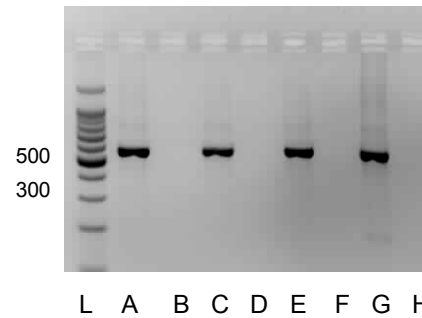
Figure 4.13: Relative gene expression of AdipoR1 & AdipoR2 in sorted PBMC, by rt-qPCR.
n =4. Expression levels in CD3 cells from each sort was used as reference in the calculation of relative gene expression level of the sorted population. Error bars indicate SD.
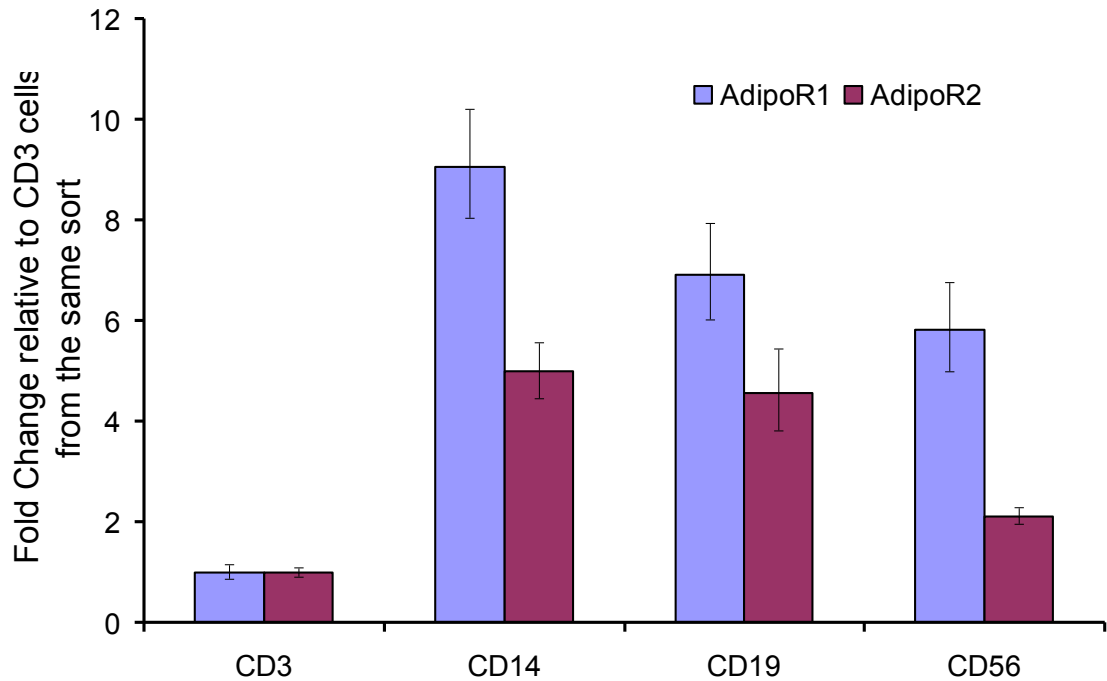
4.6 Expression of adiponectin receptors on Tregs and non-monocytic CD11c+ cells

Myeloid dendritic cells and Tregs are important in conferring tolerance in animal models of β cell autoimmunity (Bluestone, Tang et al. 2008, Saxena, Ondr et al. 2007). In particular CD11c+ antigen presenting cells are dominant in infiltrates of early insulitis (Uno, Imagawa et al. 2007, Shinomiya, Nadano et al. 2000). To address whether adiponectin signalling could influence function of these cells, we sought evidence of AdipoR1 & AdipoR2 expression, again at both mRNA and protein levels.

We identified naturally occurring Tregs in circulation using CD4$^+$CD25^{high}FoxP3$^+$ phenotype (Fontenot, Gavin et al. 2003, Hori, Nomura et al. 2003). Recent studies have demonstrated that low IL-7 receptor expression (CD127) could be used as a discriminator for Tregs (Seddiki, Santner-Nanan et al. 2006, Liu, Putnam et al. 2006, Hartigan-O'Connor, Poon et al. 2007). This has the advantage of allowing live sorting (Hartigan-O'Connor, Poon et al. 2007). Using both these criteria on flow cytometry, we found 5-10% of Tregs express AdipoR1 & AdipoR2 (figure 4.14 & 4.15). This is of similar magnitude with expression levels on all resting CD3+ cells. Following the CD4$^+$CD25$^+$CD127^{low} sort, we attained a Treg fraction of at least 80% purity (fig 4.16). AdipoR1 & AdipoR2 gene expression was demonstrated in these cells, and on rt-qPCR, their levels were similar to whole PBMC (fig 4.16E).

CD1a bears homology to MHC and is involved in the regulation of T cell responses to glycolipid antigens (Banchereau, Briere et al. 2000). It is expressed on myeloid DC (Ito, Inaba et al. 1999), and also on monocytes of healthy individuals (Gregory, Zilber et al. 2000). We used CD1a and high forward scatter to enumerate circulating DC, with CD14 as the exclusion marker. Using these criteria, we found that peripheral blood DC amount to ~0.5% of PBMC (fig 4.17) and that 50% of these cells express AdipoR1 & AdipoR2. DC can also be generated by differentiating peripheral blood monocytes using GM-CSF and IL-4 (fig 4.18). These monocyte derived DC (mDC) have increased costimulatory molecules (CD80, CD86, CD1a, DC SIGN) but have lost CD14 expression. They express both AdipoR1 & AdipoR2, at both the protein (fig 4.19) and mRNA level (fig 4.16E), but both methods of quantification point to a reduced level of expression in comparison to peripheral blood monocytes.

Figure 4.14: Expression of AdipoR on FoxP3+ T cells. Representative example. CD4+CD25high cells (A) express high levels of Fox P3 (B) relative to CD4 CD25- cells (C). AdipoR1 (D) and AdipoR2 (E) is expressed on ~6% of CD4+FoxP3+ cells. Expression of AdipoR1& AdipoR2 on CD4+FoxP3- cells is 7.4% and 8.4% respectively. The low numbers of co-expressers for AdipoR and FoxP3 is in keeping with the overall sparse expression of AdipoR on resting T cells, at both the protein (fig 4.10), and mRNA level (fig 4.13).
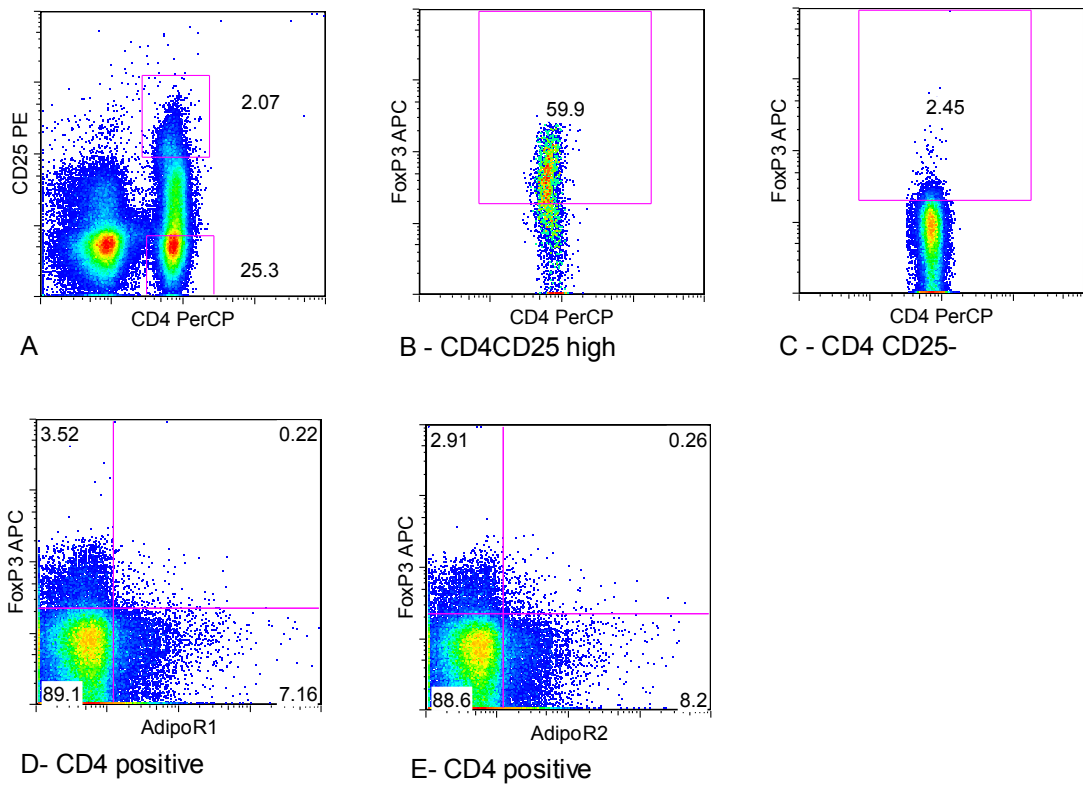
Figure 4.15: AdipoR on CD4+CD25highCD127low Tregs. A & B. Representative gating of CD4+CD25^{high}CD127^{low} cells. C & D. Numbers denote percentage of cells exhibiting positive staining for the receptor, against isotype.
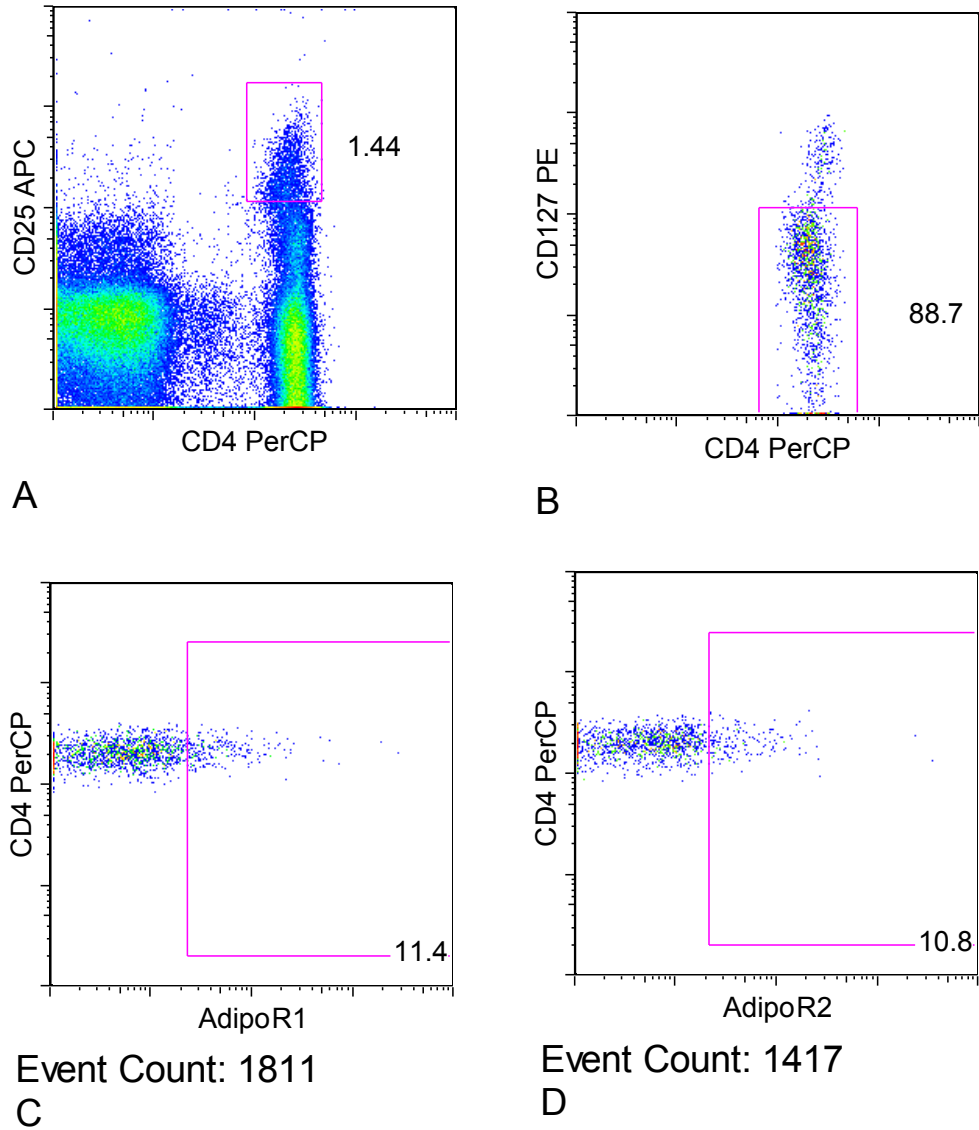
Fig 4.16: Gene expression of AdipoR on CD4+CD25^{high}CD127^{low} Tregs. *(A-C).* Purity of positively selected fraction. ~85% of cells were positive for CD4 & CD25, of which >90% were also CD127 low. Overall purity was 80%. In cells expressing the highest level CD25,(A) there was markedly reduced expression of CD127(C) .
D. Conventional rt-PCR of RNA from CD4+CD25^{high}CD127^{low} cells and monocyte derived dendritic cells.
1&6-PBMC; 2&3, 7&9 - CD4+CD25^{high}CD127^{low} and rt-ve; 4&5, 8&10- mDC & rt-ve.
E. Quantification by rt-qPCR. Fold change relative to pre-sorted PBMC. Error bars indicate SD.
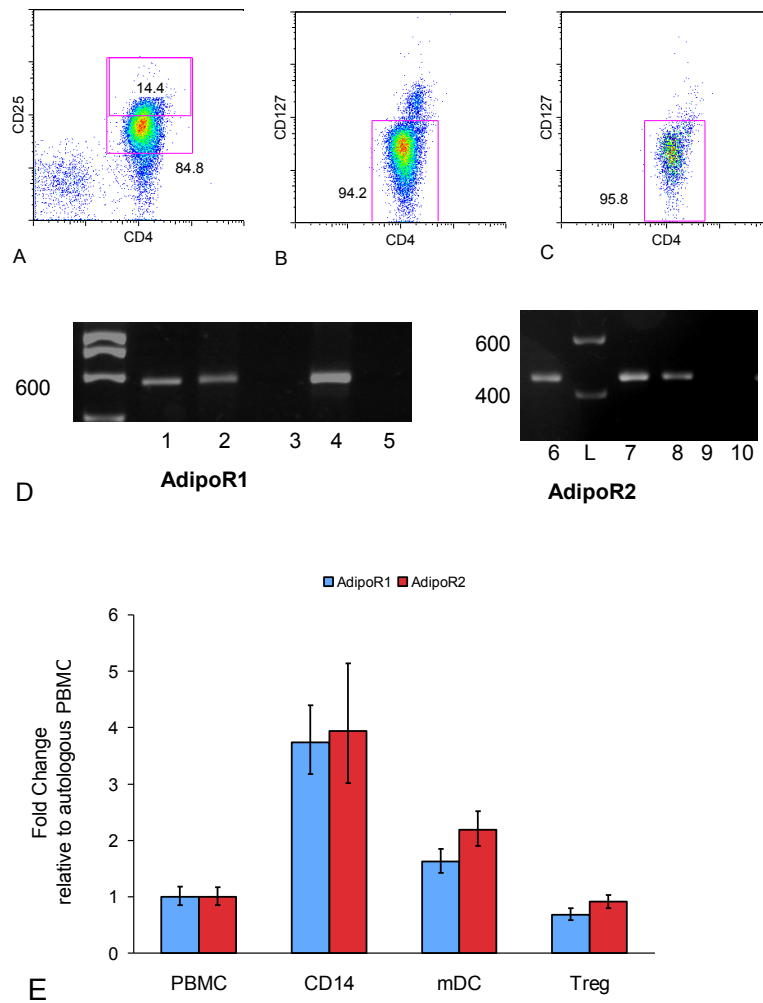
Figure 4.17: AdipoR on peripheral blood dendritic cells (PBDC). Representative example. Viable PBDC were identified by (A) lack of propidium iodide staining, (B) high forward scatter, as well as positive CD1a and negative CD14 labelling. We used CD1a expressing monocytes as an internal comparison for AdipoR1 & AdipoR2 staining (D&E). Numbers denote gate frequencies. (D&E). Cell counts for each subpopulation are appended in the legends.
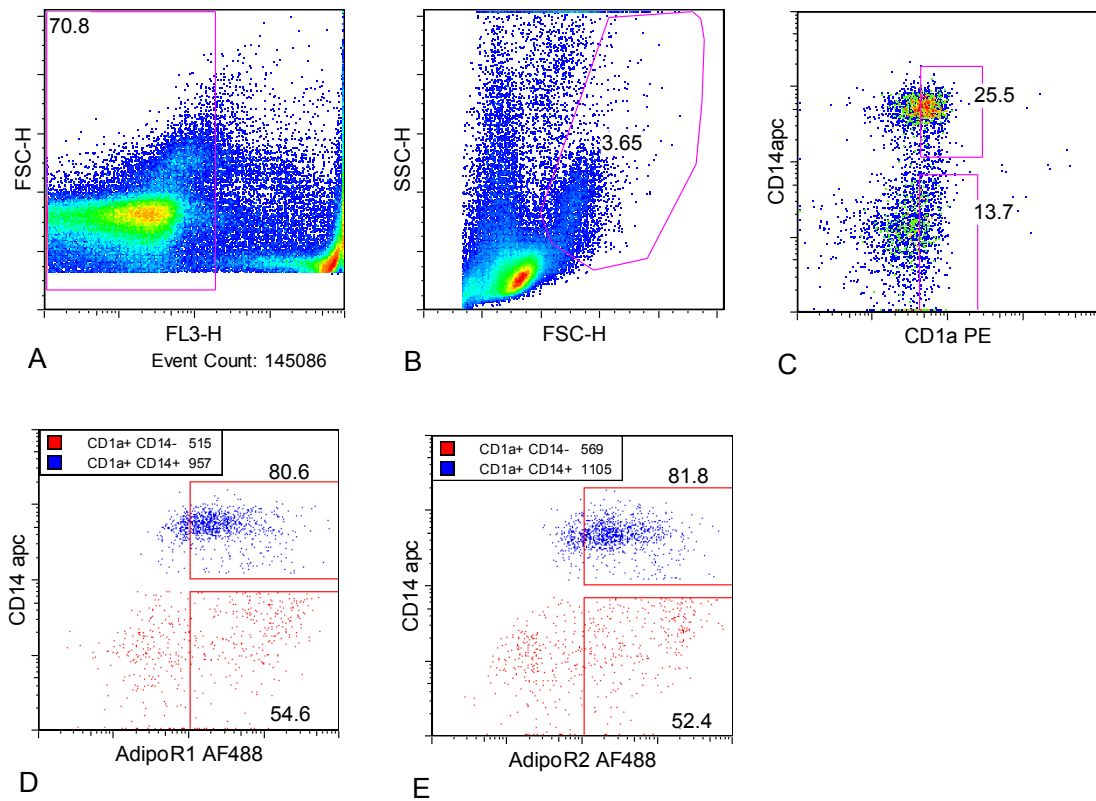
Figure 4.18: Phenotype of monocyte derived dendritic cells (mDC). The method of their generation is decribed in 3.7.1. CD14+ precurosrs were negative in their expression of CD80 (A), CD1a (C) and DC-SIGN (E). Following ex vivo expansion, mDC had increased expression of these markers (B, C, F). They had an immature phenotype (CD83-ve) and had lost CD14 expression (B).

CD14+ monocytes

mDC
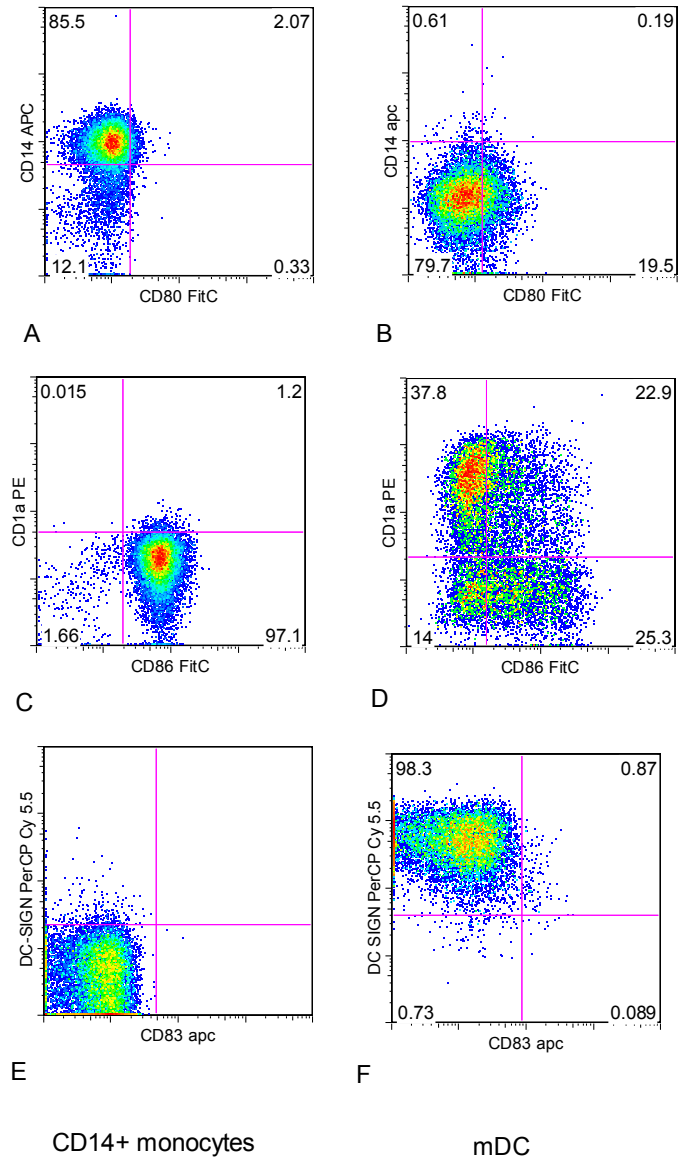
Figure 4.19: AdipoR on mDC. Surface staining for AdipoR1 & AdipoR2 was quantified on mDC gated on high CD1a (A) as well as double positive staining for DC-SIGN and CD86.
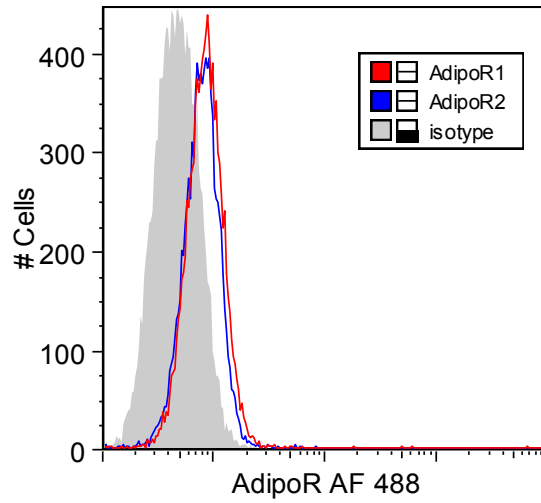
A. CD1a+ mDC
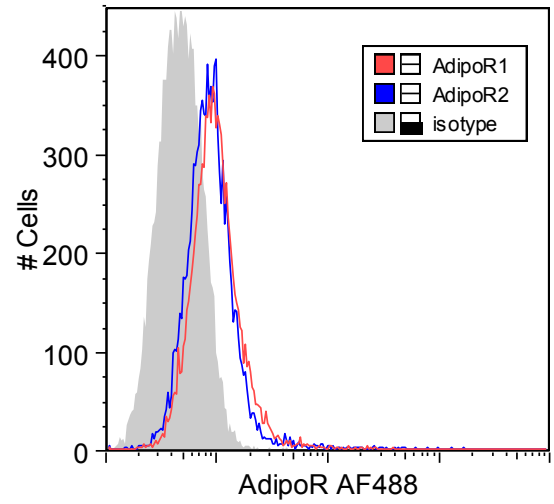
B. DC SIGN+ CD86+ mDC

4.7 Expression of adiponectin receptors on activated T cells

We then asked whether the relatively low expression of AdipoR1 & AdipoR2 on T cells could be altered by activation. Positively selected CD3+ cells (>97% purity) were activated using CD3+CD28+ Dynabeads or PHA, for 48-72 hours. Both means of activation resulted in a trend for AdipoR1 & AdipoR2 surface expression to decrease (fig 4.20 & 4.21). In particular, the fall in AdipoR2 expression following PHA was significant (fig 4.22). This change was also observed at the gene expression level (fig 4,22D). In addition, we found that the upregulation of CD25 on PHA activation strongly and significantly correlated with the reduction in both AdipoR1 & AdipoR2 expression (fig 4.22C) (AdipoR1 $r = -0.92$ $p<0.01$, AdipoR2 $r=-0.76$ $p<0.05$).

Figure 4.20:AdipoR on T cells activated by PHA. MACS sorted CD3+ cells were treated with PHA. After 72 hours, harvested cells were costained with CD25 and AdipoR1 or AdipoR2. Representative example. Numbers denote quadrant frequencies. Gates were set based on isotype, such that 99% of cells were bracketed in the left lower double negative quadrant. Differences in isotype gate setting between resting and activated T cells were due to autofluorescence (appendix 1).
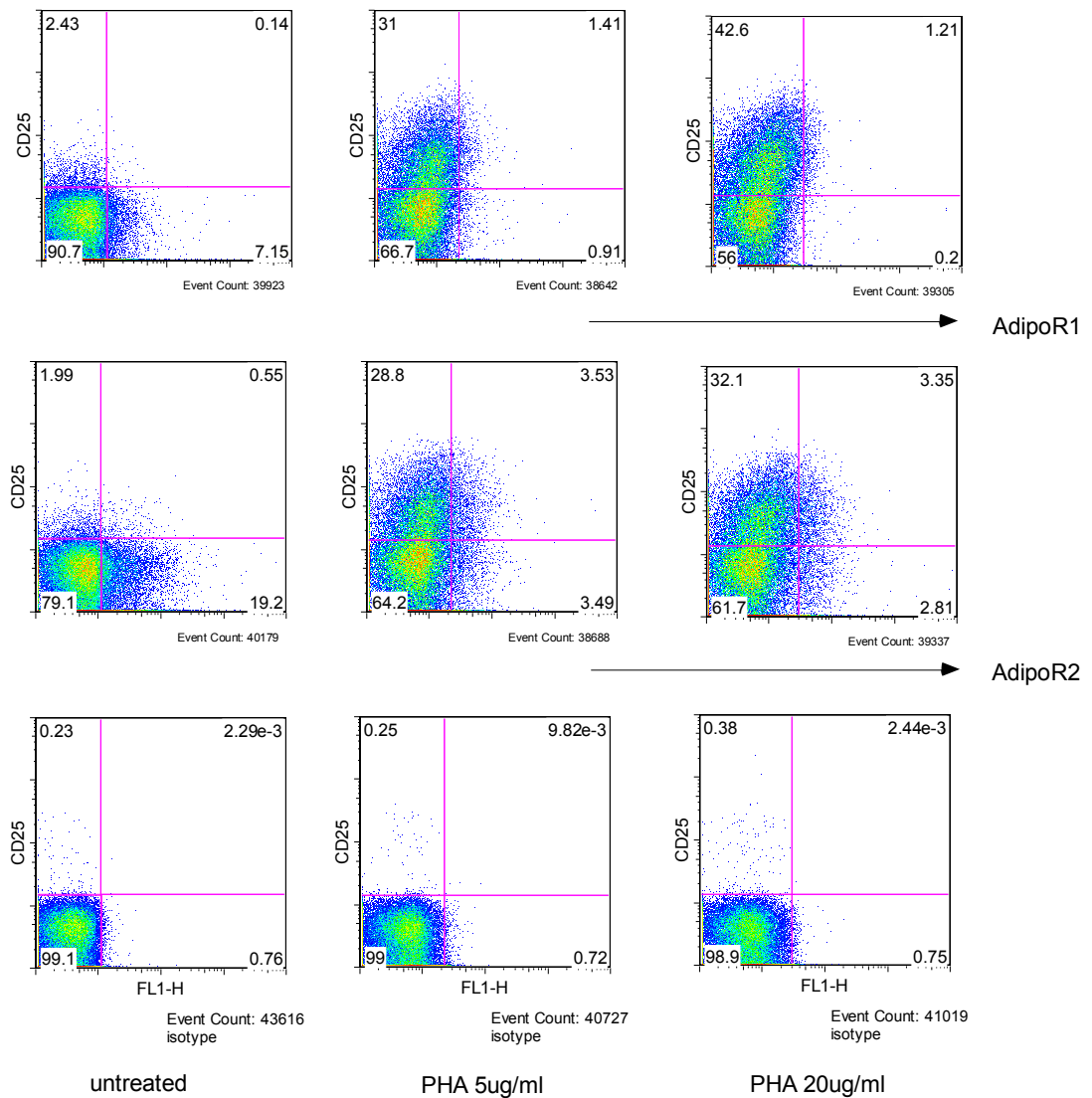
Figure 4.21: AdipoR on T cells activated by CD3+CD28+ Dynabeads. A & C: negative controls. B & D: treated. Expression of AdipoR1 (B) and AdipoR2 (D) were quantified alongside CD25 staining. Numbers denote gate frequencies. E & F. Comparison of AdipoR1 & AdipoR2 expression between Dynabeads treated T cells and control (n=4).
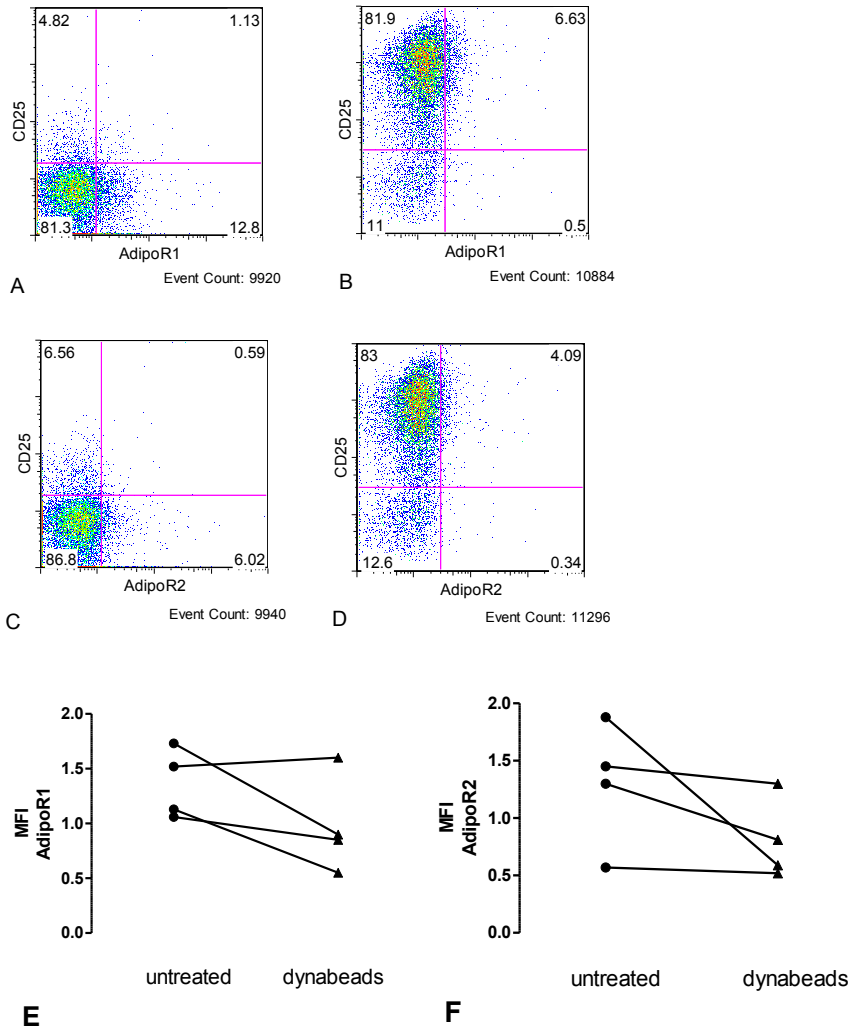
Figure 4.22: Effect of T cell activation on AdipoR expression. (A & B) Changes in AdipoR1 & AdipoR2 MFI with PHA stimulation for up to 72 hours. The paired experiments were from the same donor. (C) T cells from a single subject was exposed to increasing doses of PHA (0,1,2,5,10,20, 25 ug/ml). A significant inverse correlation is demonstrated for both AdipoR1 & AdipoR2 against the activation marker CD25 (IL-2 receptor). (D) Gene expression of T cells for AdipoR1 & AdipoR2 following activation
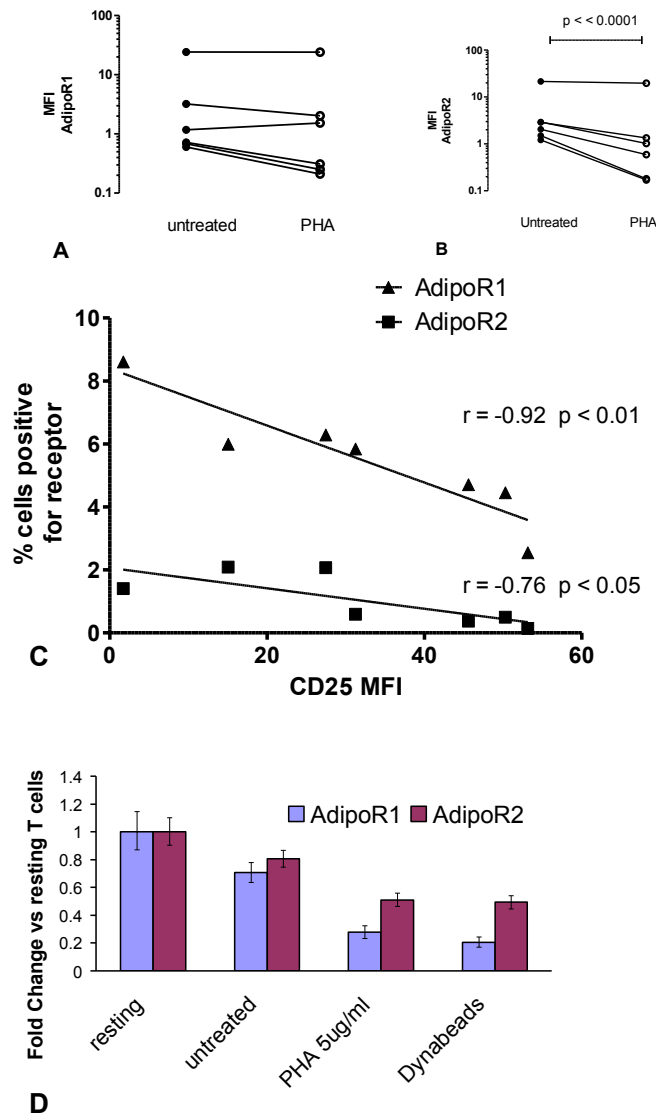
4.8 Expression of adiponectin by PBMC

Next, we asked whether PBMC express adiponectin, as its secretion has been documented in non-adipose tissue (Katsiougiannis, Kapsogeorgou et al. 2006, Chen, Tan et al. 2006, Ding, Qin et al. 2007), as well as in primary osteocytes which share a common ontogeny with myeloid cells (Berner, Lyngstadaas et al. 2004, Shinoda, Yamaguchi et al. 2006). We discovered that PBMC do express adiponectin mRNA (fig 4.23A), albeit at low levels when compared to mature adipocytes (figure 4.23B). Its gene expression was comparatively higher in CD19+ and CD56+ lymphocytes (fig 4.23C) among the PBMC subpopulations.

We then sought evidence of adiponectin protein expression on immunoblotting of PBMC lysates (Methods section 3.10), from buffy coats of healthy blood donors (fig 4.24). Following immunoprecipitation (IP) with a rabbit anti-adiponectin antibody, immunoblotting revealed a band corresponding to the reported molecular weight of adiponectin (28kDa). This was also present in our human serum positive control and in the pre-IP PBMC lysate. However, its situation also approximated to the molecular mass of the IgG light chain, cleaved from the whole antibody during the final elution steps. Indeed, a fainter but similar sized band was seen in the supernatant sample following IP with pre-immune rabbit serum. We addressed the possibility of a tandem band by covalently coupling the IP antibody (Methods section 3.10.1), as well as the irrelevant antibody control. Indeed, the contaminating light chain signal was now lost in the negative control (lane 7), whilst the band of interest was preserved (lane 8). A further complication in the interpretation of blots was the consistent appearance of bands at 34-36kDa. We confirmed its non-specificity by demonstrating its persistence when the membrane was only exposed to the secondary HRP-conjugated antibody.

The band at 28kDa was absent in the secondary antibody only blots (figure 4.25), and its luminescence abrogated when the probing primary antibody was pre-incubated with recombinant adiponectin (fig 4.26).

We attempted to isolate this band on Coomassie staining for further qualification on mass spectrometry (Methods section 3.10.2). However, only one band was visible in the 22-36 kDa range and this was excised and subjected to in-gel trypsinisation. The resultant spectra were a match for the IgG light chain.

We had also cultured whole PBMC in complete media, supplemented by 10% FCS. By ELISA, the level of adiponectin in supernatants after 3 days of culture was below the detection limit.

Figure 4.23: gene expression of adiponectin on PBMC. A. conventional rt-PCR (37 cycles). a. whole pancreas RNA b. Ntera cells c & d. differentiated Chub S7 adipocytes and rt-ve control e & f. PBMC RNA & rt-ve control. **B.** rt qPCR of adiponectin mRNA in whole PBMC, from 3 independent samples for each cell type/tissue tested. **C.** MACS-sorted subpopulations of PBMC. Standard curve for the adiponectin/18S duplex rt-qPCR is shown in appendix 2.
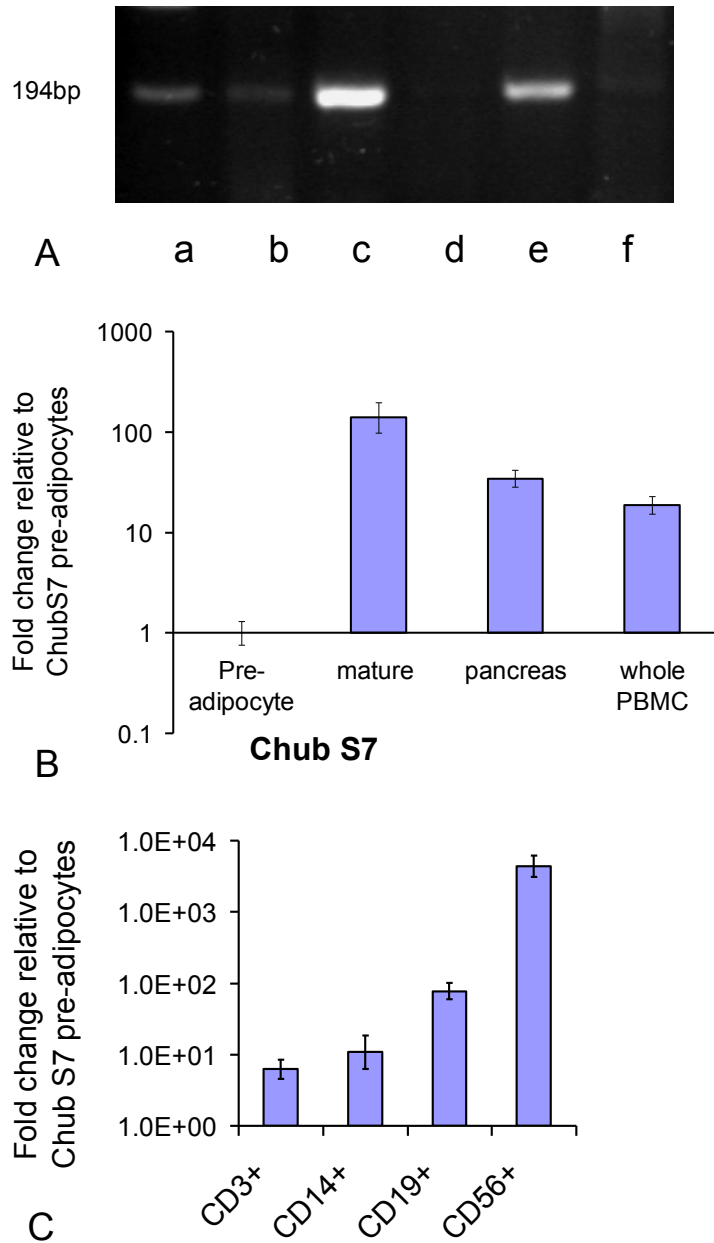
Figure 4.24 Immunoblot for human adiponectin (28kDa) in PBMC lysates.
Recombinant adiponectin (with FLAG tag) and human serum were used as positive controls (lanes 1 & 2). A band at 28kDa can be seen in pre-IP PBMC lysate (lane 4) and is intensified following IP using anti-adiponectin antibodies (lane 5). However, this band is also seen in the negative IP control where rabbit serum was used (lane 6). We postulated this was due to antibody light chain contamination (~25kDa). The 28kDa band of interest persists after covalent coupling of the anti-adiponectin IP antibody (lane 8), but is absent in the control, where an irrelevant antibody was covalently coupled to Sepharose beads. The non-specificity of the larger 35 kDa band in lanes 3-8 is investigated in figure 4.25.
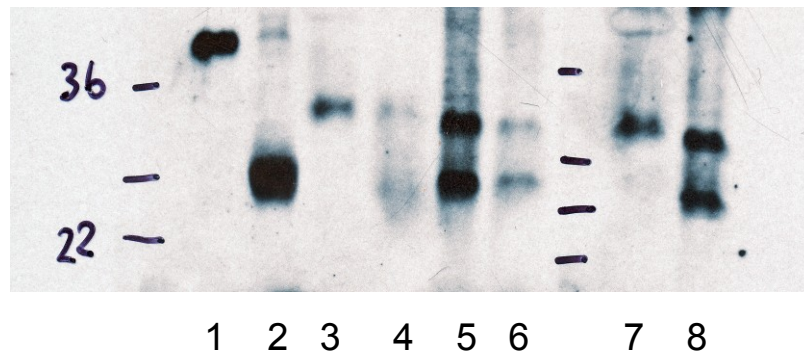
1. Recombinant adiponectin (FLAG tag)
2. Human serum
3. Rabbit serum
4. PBMC lysate

5-8 supernatant post IP of PBMC lysate

5. anti-adiponectin Ig
6. Rabbit serum
7. Covalent coupled rabbit anti-AdipoR2 Ig
8. Covalent coupled rabbit anti-adiponectin Ig

Figure 4.25 Non-specificity of the 35kDa band on immunoblots for adiponectin.
Shown on the left of the blot (lanes 1-6) are two more replicate experiments for adiponectin following IP with covalently coupled antibodies. Again, human serum and recombinant adiponectin were positive controls (lanes 1 & 2). Bands at ~30kDa, corresponding to the M_r of adiponectin, are seen in supernatants from PBMC lysates following IP (lanes 3 & 5). These are absent in the negative controls (4 & 6). The bands at 35kDa are seen when the membrane is exposed only to the secondary antibody (to the right), whilst the intensity of bands at 30kDa are visibly reduced.

1 2 – 3 4 – 5 6 ¦ 3 5

Probing antibodies:
Primary & secondary   ¦ secondary only
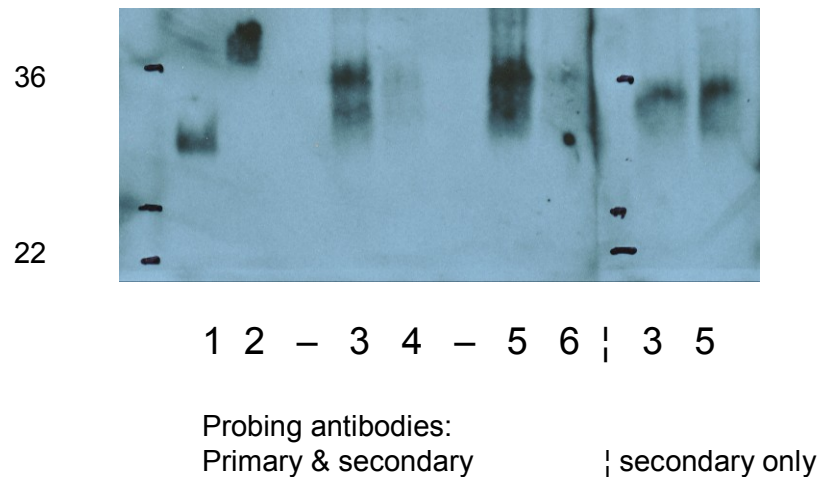
1. human serum

2. Recombinant adiponectin (FLAG tag)

3-6 supernatants following IP of PBMC lysates

3. Protein G covalently coupled rabbit anti-adiponectin IgG antibody [run 1]

4. Protein G covalently coupled rabbit anti-AdipoR2 IgG antibody

5. Protein G covalently coupled rabbit anti-adiponectin IgG antibody [run 2]

6. Protein G covalent coupled to rabbit pre-immune serum

Figure 4.26 Specificity of the 30kDa band in PBMC lysates following IP for adiponectin. The intensity of the band, following IP for adiponectin of PBMC lysates (lane C), is attenuated when the primary probing antibody is pre-adsorbed with recombinant adiponectin (lane E). Here, the primary probing antibody (1ug) was incubated with 5ug of recombinant human adiponectin (Cat # ALX-522-063-C050, Alexis) overnight at 4°C before application to the membrane. Again, the 30kDa band is just barely visible with the negative control IP of the PBMC lysate (lane D).
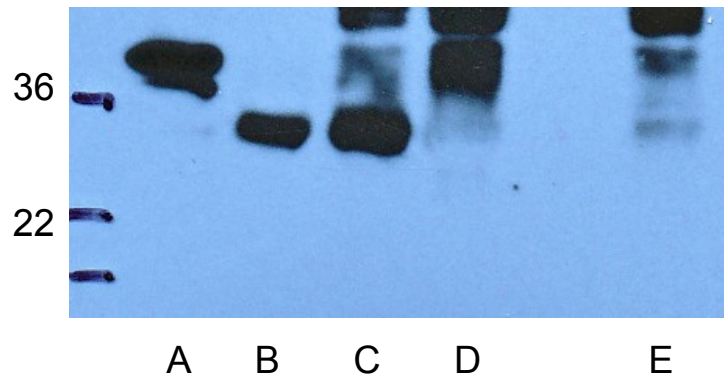
A – recombinant adiponectin (FLAG tag)

B- human serum

C & E- supernatant post-IP using anti-adiponectin Ig

D- supernatant post IP using rabbit serum

4.9 Discussion

Previous studies have shown AdipoR1 & AdipoR2 gene expression on PBMC (Weigert, Neumeier et al. 2008, Palmer, Hampartzoumian et al. 2008, Sohn, Kwak et al. 2010, Shen, Charlesworth et al. 2007, Alberti, Gilardini et al. 2007, Tan, Wang et al. 2009). In a novel finding, we identified AdipoR1 & AdipoR2 expression on PBMC at the protein level by flow cytometry. We have taken precautions in our methodology to avoid non-specific labelling, including the use of blocking serums, FcR blockade, as well as the optimisation of primary and secondary antibody concentrations. We have validated the specificity of receptor labelling by demonstrating its inhibition with a specific antibody blocking peptide. In addition, the increased gene expression for AdipoR1 & AdipoR2 in the positive sort using the labelling antibody was in proportion to increased purity of the fraction. Also in later experiments, there was general concordance in the level of gene and protein expression by the independent techniques. Furthermore, FACS labelling of AdipoR1 & AdipoR2 appeared to be exclusive, as the blocking peptide for one receptor isotype had negligible inhibition on the other. In support, the positively sorted cells for one isotype did not have increased gene expression for the other. This was an important question to address, given the structural homology of the receptors and their similar distributions on PBMC subsets.

The advantage of our flow cytometric technique is the ability for quantification of receptor expression at both the protein and cellular levels. For quality control we have developed an explicit experimental protocol, as well as a preset method of data analysis. Over 3 subjects, our assays have a CV ranging from 3-13%. This compares favourably to other methods of quantification based on FACS (Gratama, D'hautcourt et al. 1998, Faint, Pilling et al. 1999), despite the inherent variability that comes from the use of polyclonal antibodies. At the time of writing, no monoclonal antibodies to

AdipoR1 or AdipoR2 were available commercially. We recognised that a further contributor to the variability is our analogue flow cytometer, as its optical and compensation settings have to be calibrated manually before each run. Whilst this is not a factor in single day experiments, this variability could significantly undermine validity in longitudinal studies. To control for this, we would standardise MFI values by subtracting against that in the isotype sample. In addition, we would seek corroborative evidence at the mRNA level in all future experiments.

AdipoR1 & AdipoR2 was expressed in all PBMC, but there were significant differences in their distribution among subsets. We found the highest expression on monocytes, followed by B cells and then NK cells, with sparse expression seen on T cells. There was agreement of relative expression data acquired on FACS and rt-qPCR. In an earlier publication, we had found the high receptor expression on monocytes measured on FACS was not replicated at the gene expression level (Pang, Narendran 2008b). This could be reconciled by the increased purity of the monocyte sorts. For the presented set of experiments, we switched to using anti-CD14 microbeads, as opposed to the sequential application of PE-conjugated CD14 and anti-PE microbeads. The resultant purity of the positive fractions was consistently at 90%, as opposed to the 85% previously.

AdipoR1 & AdipoR2 mRNA in monocytes had been described by other groups (Chinetti, Zawadski et al. 2004, Weigert, Neumeier et al. 2008). We have extended this knowledge by demonstrating their expression at the protein level. We found reduced receptor expression on DC compared to their monocyte precursors, through direct comparison in peripheral blood and by the ex-vivo differentiation of primary monocytes. This is consistent with findings from Chinetti *et al* (Chinetti, Zawadski et al. 2004), who described reduced AdipoR1 & AdipoR2 in monocyte derived macrophages that

represent terminal differentiation of a myeloid antigen presenting cell. The time course for this decrease had occurred as early as the third day of culture, when cells are expected to acquire a DC phenotype.

A shortcoming of our characterisation work on DC is that we have used relatively crude markers (high FSC, CD1a+CD14-) for their definition in peripheral blood. Older studies had used a more extensive panel of costimulatory molecules (Zacher, Knerr et al. 2002, Vuckovic, Withers et al. 2007). More recent studies focused on the BDCA group of markers for delineating DC subtypes in circulation (Dzionek, Fuchs et al. 2000). Our approach had the benefit of co-identifying CD14+ monocytes, that served as an internal positive control for adiponectin receptor quantification on DC. However, not all human DC with antigen presenting capacity express CD1a (Chang, Wright et al. 2000, Gogolak, Rethi et al. 2007). Furthermore, we could not comment on the distribution of AdipoR1 & AdipoR2 on DC subsets. This may be relevant in context of in the early stages of islet autoimmunity, where a relatively expanded population of BDCA 2+ (CD303) plasmacytoid DC may have a pathogenic role (Allen, Pang et al. 2009).

The expression of AdipoR1 & AdipoR2 on T cells has been reported by others (Palmer, Hampartzoumian et al. 2008). Our quantitative experiments found this to be at relatively low levels, with no preferential expression on Tregs. This suggests that for adiponectin to modulate T cell responses, accessory cells with high adiponectin receptor expression such as monocytes, B cells and NK cells are required. This is supported by an in vitro model of hepatitis C immunity, where the abrogation of interferon-γ by T cells by adiponectin is dependent on the presence of NK cells (Palmer, Hampartzoumian et al. 2008). Our postulation does not contradict recent findings of adiponectin downregulation T cell responses to anti-CD3 antibodies, as

whole mice splenocytes were used in the initial stimulation (Okamoto, Christen et al. 2009).

We found that T cells upon activation had reduced AdipoR1 & AdipoR2 expression. The functional significance of this is limited by the relatively low expression at the outset. Notwithstanding, this observation is in agreement with findings of reduced adiponectin receptor expression on cells exposed to inflammatory stimuli (Rahman, Qadri et al. 2009, Ajuwon, Banz et al. 2009), and the increased expression with anti-inflammatory treatments (Chinetti, Zawadski et al. 2004, Ding, Qin et al. 2007, Sun, Han et al. 2006). However, we recognised that this is an area of controversy, with reports of reduced adiponectin receptors with inflammation in some primary human tissues (Kaser, Moschen et al. 2005, Nannipieri, Bonotti et al. 2007), but not seen at other sites (Miller, Cho et al. 2009, Shen, Charlesworth et al. 2007, Tan, Wang et al. 2009).

We accept two major limitations to this series of studies. Our data regarding the expression of adiponectin on PBMC requires validation. We had aimed to confirm our rt-qPCR and immunoblotting data via qualitative analysis by mass spectrometry. Our failure was due to inadequate yield of the purified protein following immunoblotting. A future strategy could involve immunomagnetic pooling of CD19+ and CD56+ cells before lysis, as these cells had higher adiponectin gene expression. Another switch would be the use of protein G affinity interaction chromatography columns that allow a more efficient scale-up. Other groups have used alternative means of demonstrating the non-adipose expression of adiponectin, by immunohistology (Katsiougiannis, Kapsogeorgou et al. 2006, Miller, Cho et al. 2009, Tan, Wang et al. 2009) and by ELISA of culture supernatants from tissue explants (Chen, Tan et al. 2006). Nevertheless, as the relative gene expression of adiponectin was low and the secretion

of adiponectin by PBMC was undetectable, we concluded any autocrine/paracrine effects in an in vitro system would be negligible.

Secondly, our investigation into the immune repertoire of adiponectin assumes AdipoR1 & AdipoR2 are the major mediators of its immune function. This may not be the case, as at least some of these effects are attributed to alternative pathways (Masaie, Oritani et al. 2007, Takemura, Ouchi et al. 2007, Peake, Shen et al. 2006). Furthermore, a third receptor of adiponectin, T-cadherin, has been described and is thought to have biological activity despite the lack of an intracellular domain (Hug, Wang et al. 2004, Takeuchi, Adachi et al. 2007).

4.10 Summary of findings

We have optimised and validated a novel method for the flow cytometric quantification of AdipoR1 & AdipoR2 surface expression on PBMC. We found on rt-qPCR and flow cytometry, that cells which have antigen presenting function for T cell priming and activation, such as DC, monocytes and B cells, have the highest expression of AdipoR1 & AdipoR2. The levels of expression on T cells, both resting and activated, are low in comparison. Adiponectin mRNA can be found in PBMC, however, its significance needs to be confirmed at the protein level.

5. THE EFFECT OF T1D ON CIRCULATING ADIPONECTIN AND ITS RECEPTOR EXPRESSION ON PBMC

5.1 Introduction

We proceeded to assess the effect of T1D on circulating adiponectin and its receptor (AdipoR) expression of PBMC. This is an important question to address in the testing of our hypothesis. For adiponectin to contribute to mediating in the relationship between insulin resistance and β cell autoimmunity, we would expect adiponectin signalling to immune cells to correlate with insulin sensitivity. The association between insulin sensitivity and serum adiponectin is seen in pre-T1D and in adolescents with early disease (Truyen, De Grijse et al. 2007, Diabetes Research in Children Network (DirecNet) Study Group 2008) , However, with age and progression, other factors including glycaemic control, renal function and presence of complications are more important determinants of serum adiponectin (Maahs, Ogden et al. 2007, Pfleger, Mortensen et al. 2008, Saraheimo, Forsblom et al. 2008, Frystyk, Tarnow et al. 2005, Barnes, Curran-Everett et al. 2008). There is evidence to support the association of reduced AdipoR1 & AdipoR2 expression on immune cells with obesity (Weigert, Neumeier et al. 2008, Sohn, Kwak et al. 2010, Alberti, Gilardini et al. 2007), but whether this extrapolates to T1D, where insulin resistance is also a feature (Pang, Narendran 2008), is not known.

In this chapter, we report on the results of a cross-sectional study looking at PBMC adiponectin receptor expression in T1D, with healthy and T2D subjects serving as controls.

5.2 Assessing variability of AdipoR1 & AdipoR2 expression with circadian clock and feeding state

Gene expression for AdipoR1 & AdipoR2 on visceral fat follows a diurnal variation with a nocturnal trough, and is increased with fasting (Tsuchida, Yamauchi et al. 2004, Gomez-Abellan, Gomez-Santos et al. 2010, Blüher, Fasshauer et al. 2005)Therefore, we asked whether feeding state and the time of venepuncture would alter adiponectin receptor expression on PBMC. Our approach was to measure AdipoR1 & AdipoR2 expression by rt-qPCR, using fasting blood samples at 0900 hours, and at 1530 (2 hours postprandial). These times would also approximate with the sampling times for subjects recruited from outpatient clinics. The samples were processed within an hour of venepuncture. There were no significant differences in AdipoR1 & AdipoR2 gene expression in PBMC from the 2 sampling times (figure 5.1) (n=6). We conclude that differences in sampling times between morning and afternoon subject catchments would not confound our study.
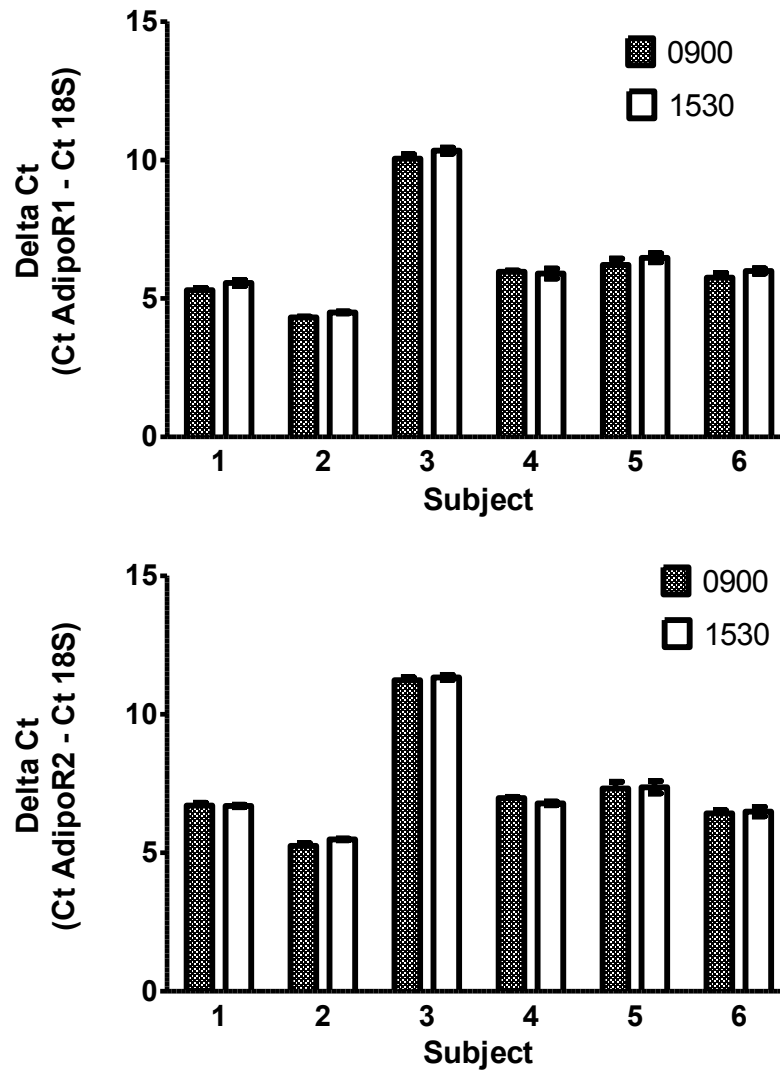
Figure 5.1: Effect of fasting and sampling time on AdipoR gene expression. 6 healthy volunteers were studied. By 2-way ANOVA and Bonferroni post-tests, there were no significant differences in normalised gene expression of AdipoR1 (top panel) and AdipoR2 (bottom) between the 2 sampling times: 0900 hours (fasting), 1530 (2hours post meal).

5.3 Clinical characteristics of our study group

Seventy six subjects were recruited. Only male Caucasian volunteers were enrolled to control for the possible effects of sex and ethnicity on adiponectin receptor expression (Barnes, Curran-Everett et al. 2008, Mente, Razak et al.). Their clinical parameters are described in Table 5.1. There were no significant differences in age, BMI and WHR between healthy (HC) and T1D subjects. We recruited a second comparator cohort with insulin treated T2D. Although they were older and more obese relative to subjects with T1D, they had similar HbA1C and duration of diabetes. Statin and ACE inhibitor/ARB use was higher in the T2D group.

	Healthy Control *n* = 22	T1D *n* = 29	T2D *N* = 25
Age (yrs)	32.6 ± 5.7	35.2 ± 10.7	54.8 ± 11.5***
BMI (kg m^{-2})	25.2 ± 2.8	25.1 ± 4.1	35.3 ± 7.3***
WHR	0.90 ± 0.06	0.91 ± 0.09	1.00 ± 0.08**
Systolic BP (mmHg)	112 ± 11	137 ± 17	141 ± 15
Diastolic BP	75 ± 12	85 ± 10	85 ± 9
Duration of Diabetes (yrs)	n/a	15 ± 10	10 ± 7
HbA1C (%)	n/a	9.0 ± 1.4	8.8 ± 1.9
Total cholesterol (mmol)	n/a	4.4 ± 0.7	4.1 ± 0.9
Triglycerides (mmol)	n/a	1.6 ± 1.0	2.3 ± 1.4
Insulin dose (total daily units/ kg)	n/a	0.85 ± 0.24	1.08 ± 0.15
Statin Use (%)	n/a	31	100*
ACE inhibitor or ARB use (%)	n/a	52	80*
Fibrate use (%)	n/a	0	4
Vascular complications (%)	n/a	52	50

Table 5.1: Clinical characteristics of study group. Mean ± SD values are represented for continuous variables. By t-test, there were no significant differences in age, BMI and WHR between T1D and HC groups. HbA1C, duration of diabetes, lipids and rates of vascular complications were similar between T1D and T2D groups. The T2D subjects were older and had greater BMI and WHR compared to both HC and T1D subjects (*** $p <0.0001$, ** $p<0.01$). By chi-square test, ACEI/ARB and statin use was significantly higher ($p < 0.05$*) in the T2D group, compared to T1D subjects.

5.4 Serum adiponectin levels in T1D

Serum total adiponectin between T1D subjects and matched HC was not significantly different (T1D: 9.6 ± 6.3 ug ml^{-1}; HC: 7.6 ± 2.3 ug ml^{-1} p = 0.17) (figure 5.2) . In contrast, levels in T2D were significantly reduced (T2D: 4.2 ± 2.8 ug ml^{-1}, p = 0.0003). Across all subjects, there were expected correlations between serum adiponectin and age (r = -0.333 p = 0.005), BMI (r = -0.461, p = 0.0005) and WHR (r =-0.273, p =0.026). In T1D, the inverse association with BMI was preserved (r = -0.397 p = 0.033), and in addition, HbA1C was a significant correlate (r = 0.442, p =0.016). There were no significant correlations were observed between WHR or eGDR with serum adiponectin. Levels in those with vascular complications compared to those without were not different (appendix 6).

Figure 5.2: Serum total adiponectin. All sample values are depicted by aligned dot plots. Mean values are represented by bar.
*** p < 0.001; * p<0.05 by Bonferroni post test
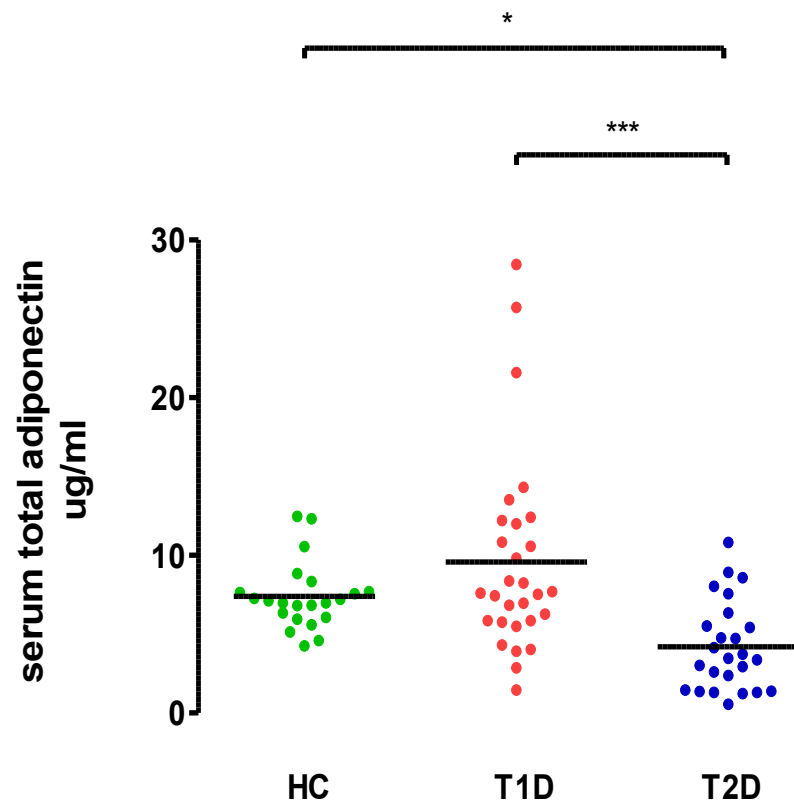
5.5 Measurement of AdipoR expression by FACS and qPCR

We used MFI as a measure of AdipoR1 & AdipoR2 surface expression on PBMC (fig 5.3). We then confirmed normal distribution of our qPCR and FACS data, as well as serum adiponectin measurements, using the one-sample Kolmogorov-Smirnov test (appendix 3). As further validation for our FACS and qPCR AdipoR expression assays, we found that MFI for both AdipoR1 (r = -0.84 p<0.0001) and AdipoR2 (r = -0.79 p<0.0001) significantly and closely correlated with ΔCt (fig 5.4).In addition, the

Figure 5.3: Gating analysis of FACS data from clinical study. Viable cells were identified based on propidium iodide staining (A) and scatter characteristics (B). PBMC were then further classified into lymphocytes and monocytes (C), which were co-stained with CD14 (D). Geometric mean fluorescence intensity (MFI) was calculated by subtraction against negative control (E). In this example (F), AdipoR1 MFI for monocytes is 107.8 (143-35.2). Numbers denote percentage frequencies of gates.
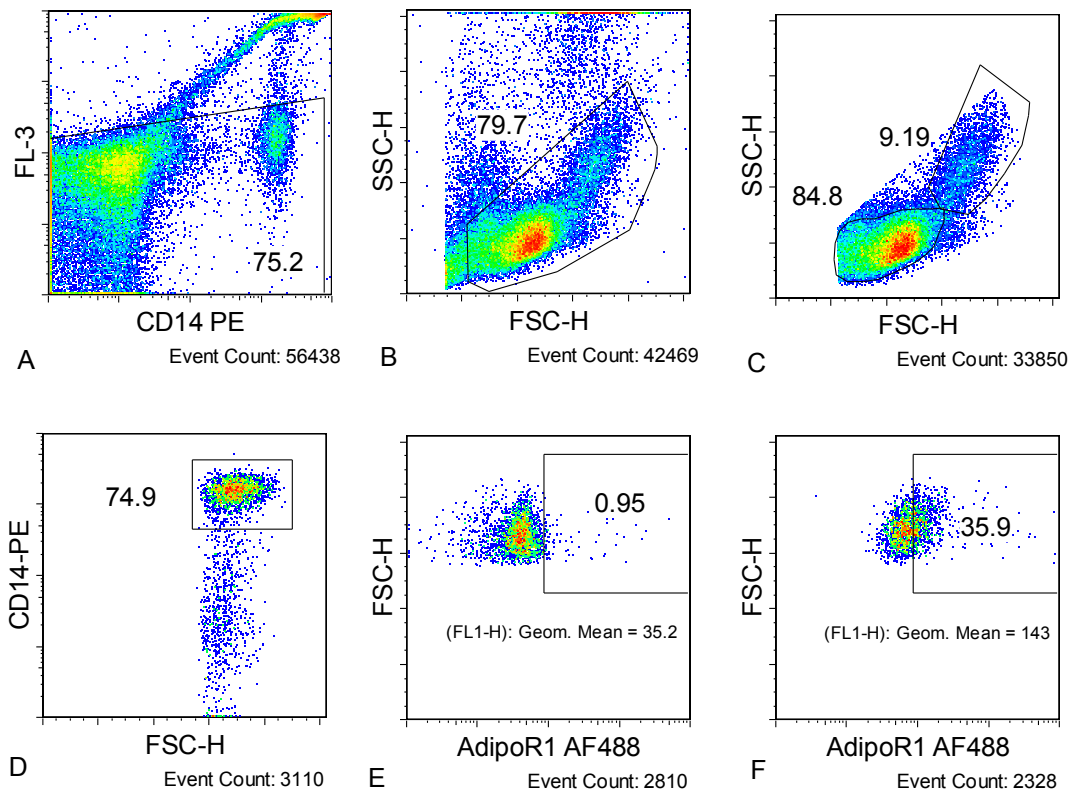
expression of both receptor isotypes significantly correlated with each other (r = 0.86 p<0.0001). Variations in monocyte count did not significantly affect MFI of both receptors in all PBMC or the CD14+ fraction (appendix 4).

5.6 Correlation of AdipoR expression with indices of insulin resistance

We then investigated whether insulin resistance is related to AdipoR expression in the three groups by correlation analysis (Tables 5.2-5.4). We used BMI and WHR as clinical surrogates of insulin resistance. For subjects with T1D, we also employed eGDR, a validated measure of insulin sensitivity calculated using HbA1C, blood pressure and WHR (Williams, Erbey et al. 2000).

In healthy subjects we found that AdipoR1 expression on all cells correlated inversely with BMI (r = -0.70 p<0.01) and WHR (r = -0.53 p<0.01) (figs 5.5A & 5.5B). There were no significant clinical correlates to AdipoR2 expression (figs 5.5C & 5.5D). BMI also followed a significant inverse correlation with both AdipoR1 and AdipoR2 expression on monocytes in T2D, but this relationship was not present for 'all cells' (AdipoR1: r = -0.38; AdipoR2: r=-0.38 p<0.05).

In T1D, eGDR significantly correlated with monocytic AdipoR expression (AdipoR1: r = 0.42 p<0.05; AdipoR2: r = 0.49 p<0.01) (figs 5.5E & 5.5F). However, the clinical parameters used to derive eGDR, namely HbA1C, BP and WHR, did not share significant correlation with AdipoR expression.

Figure 5.4: Surface expression of AdipoR1 & AdipoR2 is closely correlated with gene expression. We investigated this relationship by Pearson correlation analysis. MFI of AdipoR on all cells were analysed against normalised Ct values of AdipoR from qPCR. *A.* AdipoR1 *B.* AdipoR2. *C.* The level of AdipoR1 & AdipoR2 expression were also closely correlated with each other.

A

Pearson r	-0.8373
95% confidence interval	-0.8964 to -0.7491
P value (two-tailed)	< 0.0001

B

Pearson r	-0.7878
95% confidence interval	-0.8635 to -0.6774
P value (two-tailed)	< 0.0001

C

Pearson r	0.8538
95% confidence interval	0.7735 to 0.9072
P value (two-tailed)	< 0.0001
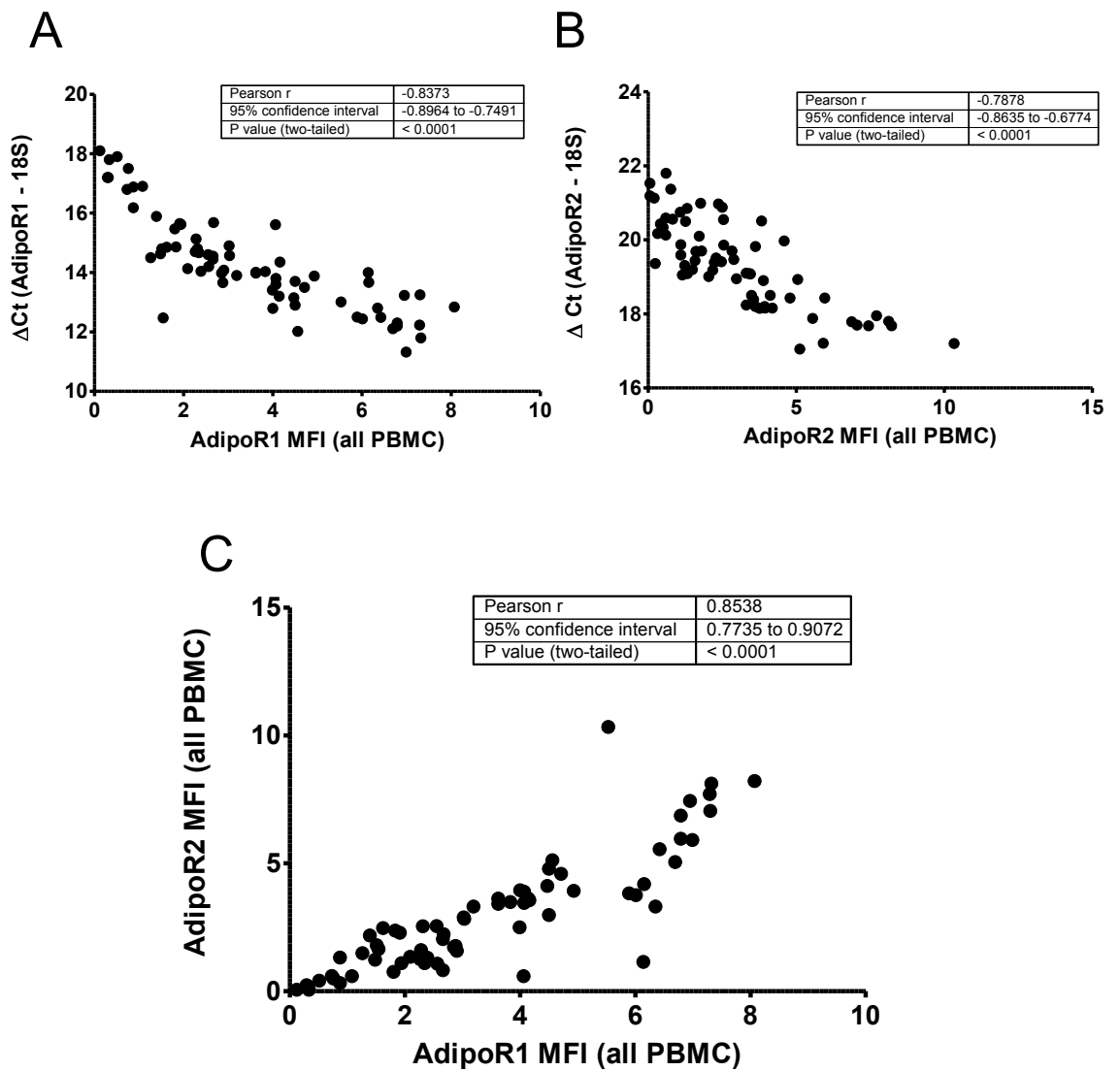
Table 5.2: Pearson correlation coefficients of AdipoR expression with surrogate measures of insulin resistance in healthy subjects

Healthy controls		Age	BMI	WHR
AdipoR1	Total	-.565	-.702**	-.526*
	P	.103	.003	.005
	Lymphocytes	-.251	-.270	-.114
	P	.226	.192	.586
	Monocytes	.021	.088	.015
	P	.922	.675	.942
AdipoR2	Total	-.356	-.059	.171
	P	.081	.779	.413
	Lymphocytes	-.284	.135	.376
	P	.169	.520	.064
	Monocytes	-.149	-.245	-.258
	P	.477	.238	.212

Table 5.3: Pearson correlation coefficients of AdipoR expression with surrogate measures of insulin resistance in T1D subjects

T1D		Age	BMI	WHR	eGDR
AdipoR1	Total	.402	-.177	.096	.250
	P	.102	.240	.472	.068
	Lymphocytes	.417	-.104	.114	.207
	P	.201	.493	.394	.132
	Monocytes	.247	-.149	.111	.418*
	P	.161	.322	.409	.024
AdipoR2	Total	.387	-.145	.041	.388**
	P	.203	.337	.759	.004
	Lymphocytes	.403	-.090	.078	.293*
	P	.102	.554	.563	.031
	Monocytes	.175	-.125	.033	.494**
	P	.189	.407	.806	.006

Table 5.4: Pearson correlation coefficients of AdipoR expression with surrogate measures of insulin resistance in subjects with T2D

T2D		Age	BMI	WHR
AdipoR1	Total	-.052	.061	-.187
	P	.804	.773	.371
	Lymphocytes	-.007	.074	-.179
	P	.974	.724	.392
	Monocytes	.050	-.375*	-.309
	P	.812	.045	.133
AdipoR2	Total	-.060	.068	-.166
	P	.777	.747	.427
	Lymphocytes	-.019	.086	-.176
	P	.930	.684	.400
	Monocytes	.236	-.373*	-.173
	P	.257	.019	.409

Figure 5.5: Correlation of AdipoR1 & AdipoR2 expression with indices of insulin resistance in health and T1D. In healthy individuals, BMI (A) and WHR (B) inversely correlated with AdipoR1 expression, but not AdipoR2 (C & D) on all PBMC. In T1D, AdipoR1 & AdipoR2 expression of monocytes significantly correlated with eGDR (E &F).
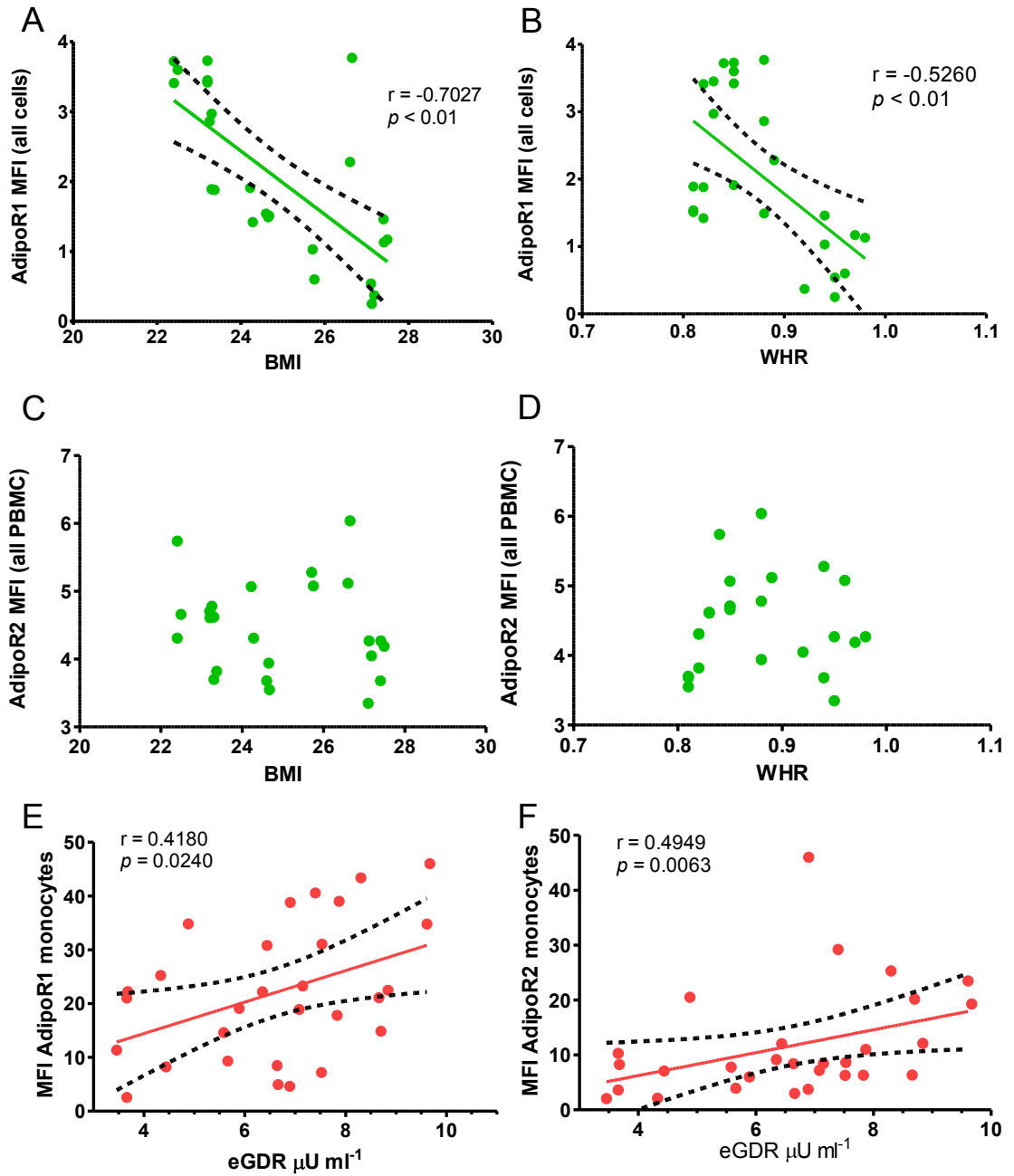
5.7 Effect of T1D on AdipoR expression

The most striking finding is the lower protein expression of both AdipoR1 & AdipoR2 in T1D (fig 5.6), by approximately 40% when compared to matched healthy controls (fig 5.7). This reduction is observed on both lymphocytes (MFI AdipoR1, HC= 3.42 ± 1.40; T1D=1.80 ± 1.70 p <0.01; AdipoR2, HC=3.04 ± 1.47; T1D= 1.34 ± 1.30 p<0.01) and monocytes (MFI AdipoR1, HC= 34.58 ± 18.47, T1D 19.46 ± 11.16 p<0.01; AdipoR2, HC= 23.14 ± 10.63, T1D= 11.12 ± 9.36 p<0.01). These changes at a protein level were confirmed at an RNA level through rt-qPCR (AdipoR1 relative gene expression =0.27, p <0.01; AdipoR2 = 0.55, p<0.05) (fig 5.8).

Subgroup analysis showed the reduction in AdipoR expression in T1D was not biased towards those with longer duration of diabetes (appendix 5) or vascular complications (appendix 6) as has previously been reported (Soccio, Zhang et al. 2006). In addition, neither insulin dose per weight in kg nor HbA1C correlated with AdipoR expression (appendix 5).

To explain the apparent reduction in AdipoR expression in T1D, we identified unadjusted parameters which have been reported to be associated with their expression. These include: hyperglycaemia (Fang, Palanivel et al. 2005, Guo, Xia et al. 2007), exogenous insulin (Tsuchida, Yamauchi et al. 2004), statins (Saito, Fujioka et al. 2007), lipids (Nannipieri, Bonotti et al. 2007, Staiger, Kaltenbach et al. 2004), serum adiponectin (Alberti, Gilardini et al. 2007, Civitarese, Jenkinson et al. 2004, Bauche, Ait El Mkadem et al. 2006) and vasculopathy (Soccio, Zhang et al. 2006). In addition, ACE inhibitors and ARB may directly modify AdipoR expression (Zhang, Li et al. 2009), or indirectly via the modulation of adiponectin signalling (Makita, Abiko et al. 2008, Clasen, Schupp et al. 2005, Pscherer, Heemann et al. 2010). Our cohort of subjects

with insulin treated T2D were controlled for some of these factors (Table 5.1), although significant differences were observed with age, BMI, serum adiponectin, statin and ACEI use. Notwithstanding, AdipoR expression in T1D was also lower compared to T2D, at both protein and mRNA levels (figs 5.7 & 5.8). Post-hoc analysis showed the level of AdipoR expression in T2D for each PBMC subpopulation to be similar to HC. As discussed previously, serum adiponectin in T2D was significantly lower, compared to both T1D and HC (fig 5.2). Incorporating all subjects, we found a modest but significant inverse relationship between serum adiponectin and AdipoR1 (r = -0.29 p<0.05), as well as AdipoR2 expression on all PBMC (r=-0.34 p<0.01) (fig 5.9). This association however did not hold within the three subgroups.

Figure 5.6 : Representative dot plots of PBMC from T1D subjects (A-C) against age & BMI-matched healthy controls (D-F). PBMC were co-stained for AdipoR1 and CD14-PE. Numbers represent percentage of cells positive for receptor staining.

T1D subjects

Age- BMI- matched healthy controls

129
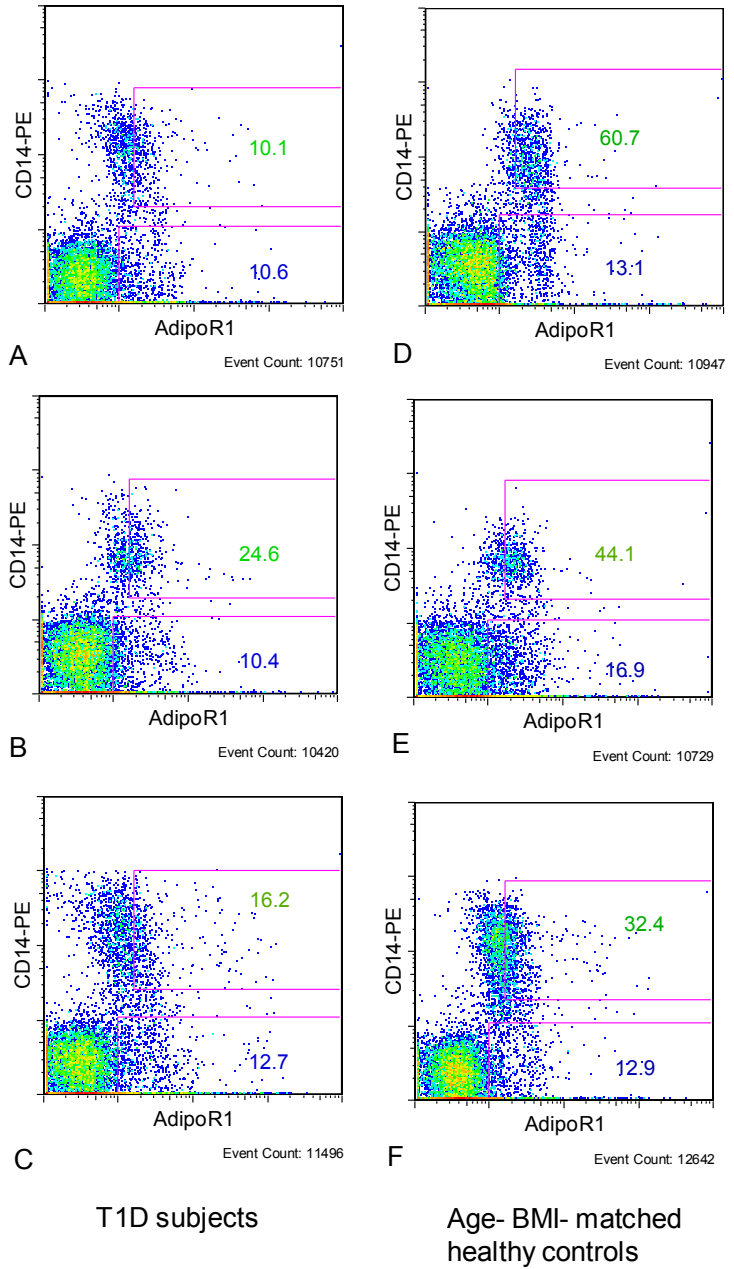
Figure 5.7: AdipoR1 & AdipoR2 surface expression on (A) all PBMC (B) lymphocytes (C) CD14+ monocytes

* p<0.05, ** p<0.01, *** p<0.005 by Bonferroni post test.
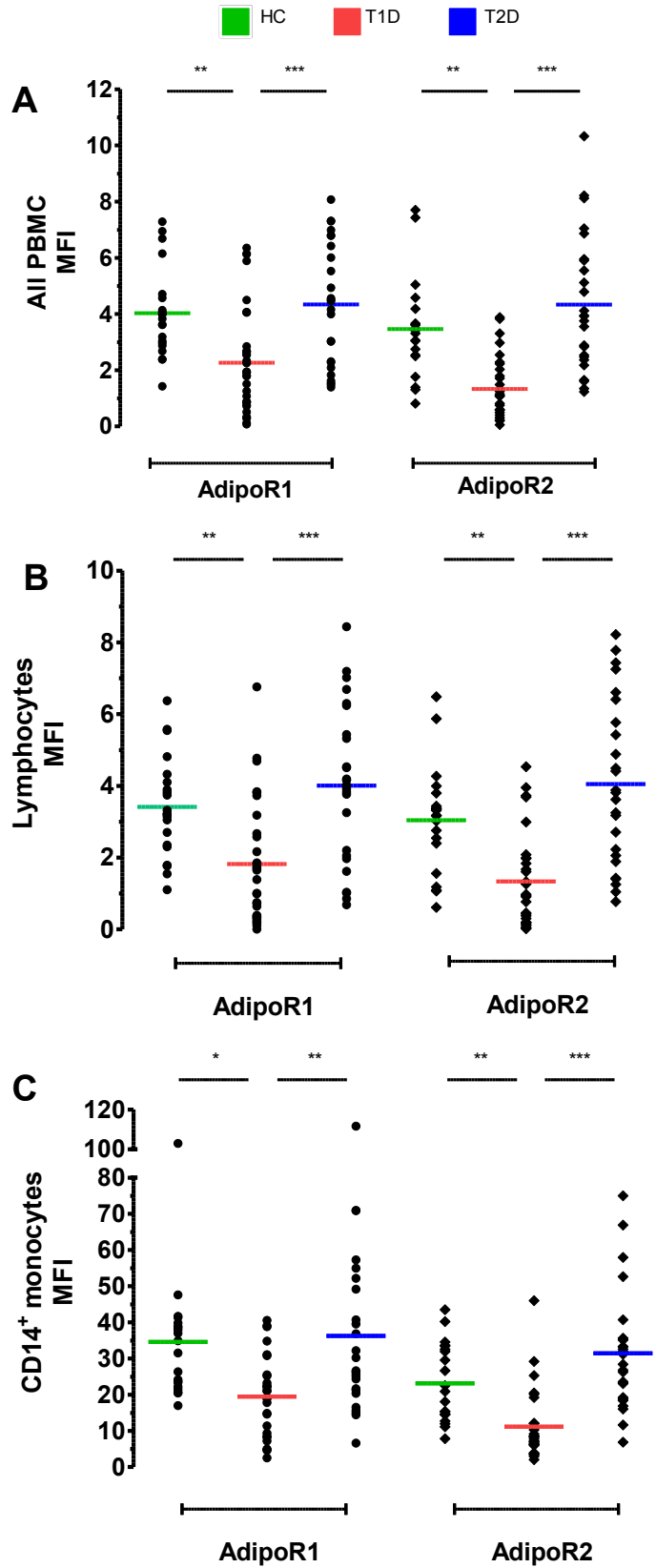
Figure 5.8: Gene expression of AdipoR1 (top) & AdipoR2 (bottom) of the three study groups. ANOVA testing was done on ΔCt values, before transformation to give fold change values. * $p<0.05$, *** $p<0.001$ by Bonferroni post test.
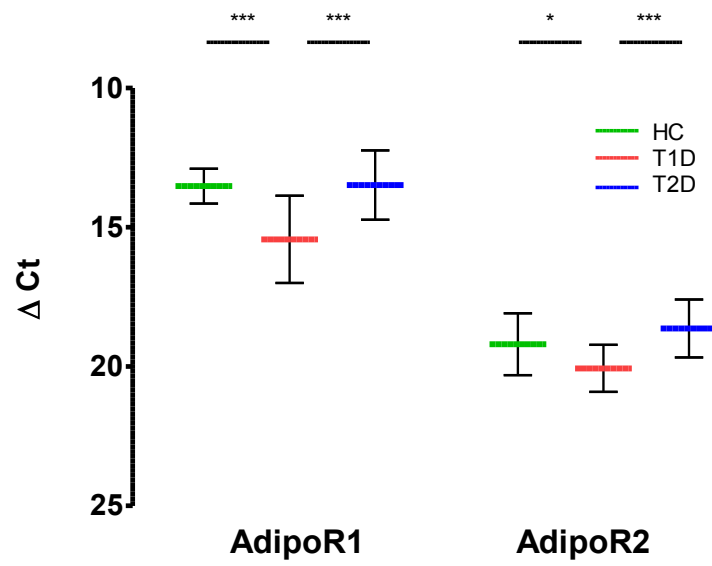
Figure 5.9: Inverse correlation of serum adiponectin with total PBMC expression of AdipoR1 (top panel) and AdipoR2 (bottom). All subjects were included for this analysis.

Pearson r	-0.2931
95% confidence interval	-0.4954 to -0.06055
P value (two-tailed)	0.0145

Pearson r	-0.3413
95% confidence interval	-0.5348 to -0.1138
P value (two-tailed)	0.0041
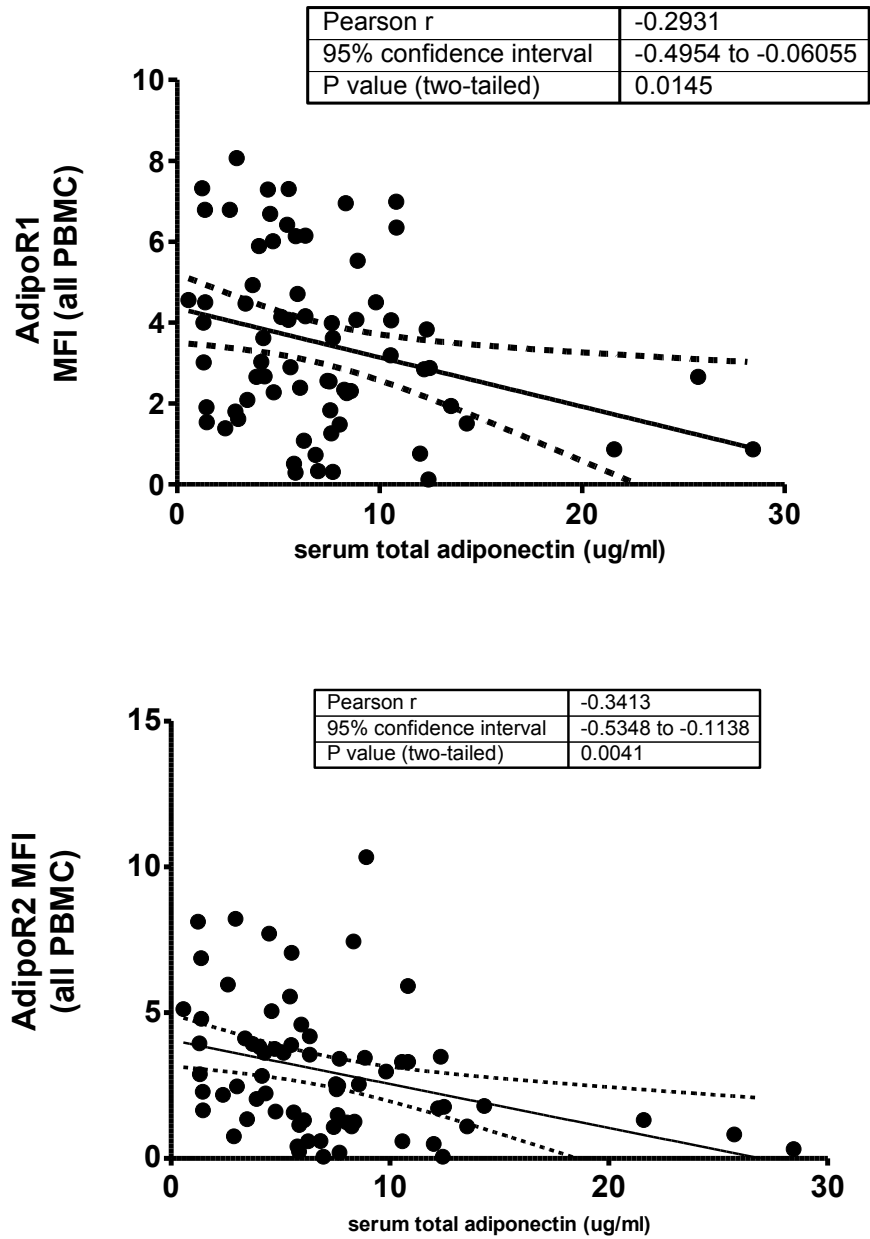
We employed multivariate linear regression to adjust for confounding between our diabetic subjects. AdipoR MFI on all PBMC, lymphocytes and monocytes were the dependent variables. We entered the following independent variables in a stepwise forward manner: age, BMI, WHR, HbA1C, total cholesterol, triglycerides, adiponectin, percentage of monocytes. The binary parameters were: presence of T1D, vascular complications, use of ACEI and statins. Variables with a *p* value of > 10% were removed from the model. Table 5.5 summarises results of the computation. We found that for AdipoR1 expression on all PBMC, presence of T1D and ACEI use were independent predictors. Age and presence of vascular complications were independently correlated with AdipoR1 expression for monocytes. For other measures of AdipoR expression, the presence of T1D was the only independent variable. Thus insofar as this analysis, the reduced expression of AdipoR in T1D appears to be disease specific, although the younger age, prevalence of vascular complications and absence of ACEI use in the group are also accountable.

Table 5.5: Linear regression modelling for AdipoR expression in diabetic subjects (T1D + T2D). R squared measures the proportion of variability of the dependent variable accounted by the model. For example, our model for AdipoR1 MFI on all PBMC accounts for 26% of the observed variation. Standardised coefficients measure the relative importance of the independent variable in predicting the dependent outcome.

Dependent variable	R square	Independent variable	Standardised coefficients	P
1. AdipoR1 (all PBMC)	0.255	T1D	-0.329	0.024
		ACEI	0.304	0.036
2. AdipoR1 (lymphocytes)	0.215	T1D	-0.463	0.002
3. AdipoR1 (monocytes)	0.253	Age	0.447	0.003
		Vasculopathy	- 0.397	0.008
4. AdipoR2 (all PBMC)	0.355	T1D	-0.596	0.000
5. AdipoR2 (lymphocytes)	0.337	T1D	-0.580	0.000
6. AdipoR2 (monocytes)	0.325	T1D	-0.570	0.000

5.8 In vitro effects of glucose and insulin on PBMC AdipoR expression

To further evaluate the effect of glucose and insulin on AdipoR expression, and to determine if this may contribute to the lower expression seen in T1D, we exposed whole PBMC to a gradient of glucose and insulin concentrations. These experiments were conducted in serum free media, and AdipoR expression was measured at 48-72 hours by FACS because our preliminary experiments had shown readouts at later time points were affected by monocyte loss. For the glucose experiments, we recruited 6 healthy subjects at different times. In each subject, AdipoR MFI was expressed as a ratio against untreated control for standardisation (fig 5.10). PBMC from 3 healthy subjects were used for the insulin dose-response experiments (fig 5.11), which were conducted simultaneously, and so unadjusted MFI values were used for comparison. Pearson correlation analyses of AdipoR1 & AdipoR2 MFI for both lymphocytes and monocytes, against logarithm of insulin and glucose did not uncover significant relationships.

5.9 In vitro effects of adiponectin on PBMC AdipoR expression

We then asked whether adponectin can modulate its receptor expression on PBMC, because of the significant albeit modest association between them in our clinical study. To test this, we performed dose response curves (n = 8) by adding back recombinant adiponectin to serum free media at concentrations of 0, 1, 2, 5 and 10 ug ml^{-1}. We did not find significant changes in AdipoR expression on monocytes or lymphocytes after 48 hours of culture (fig 5.12).
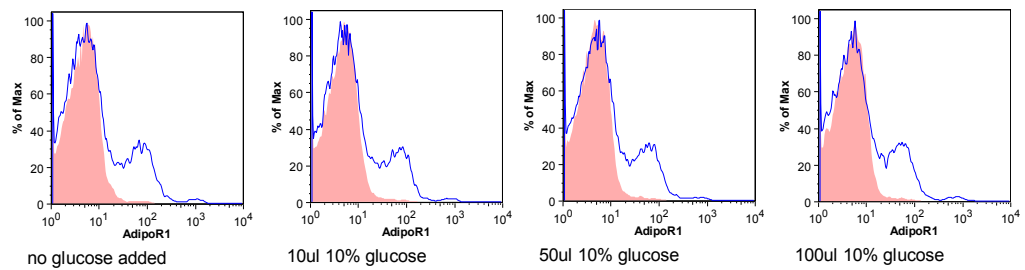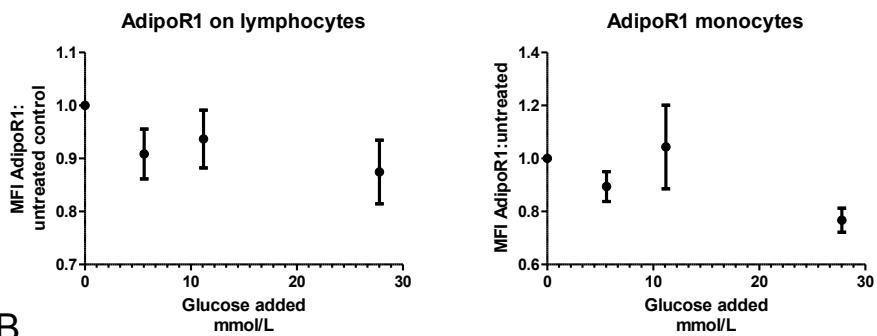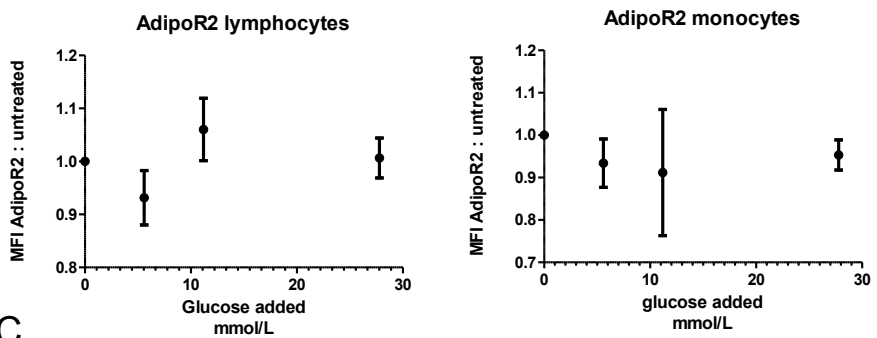
Figure 5.10: The effect of glucose in vitro. MFI are measured on day 2 following addition of glucose on day 0, with starting concentrations 5.6, 11.2 and 27.8 mmol/L (A). These values are then standardised as a ratio against the measures on cells incubated in media only (untreated). By Pearson correlation(n=6), MFI of AdipoR1 and AdipoR2 on lymphocytes (B) and monocytes (C) were not significantly different following exposure to increasing glucose.
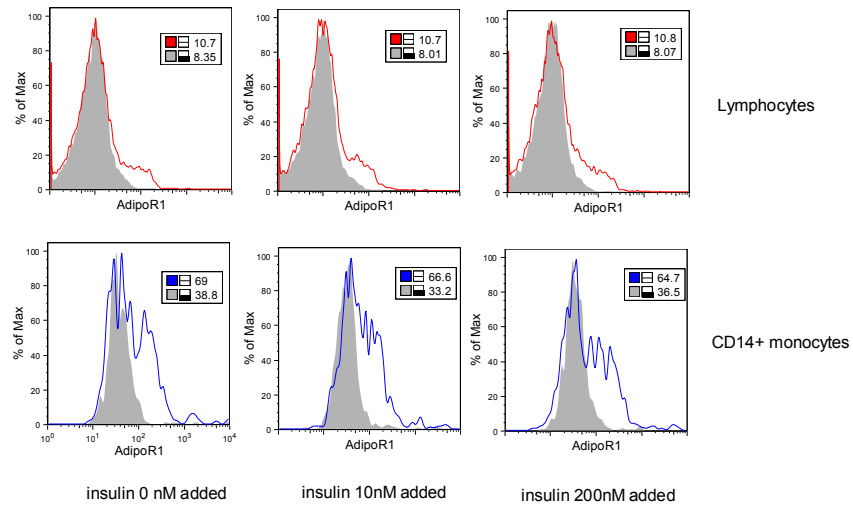
Figure 5.11: The effect of insulin in vitro (Above) Representative example. PBMC were incubated in media containing increasing amount of recombinant human insulin and expression levels were measured by FACS after 48 hours (coloured histogram). Grey histograms indicate fluorescence of negative control. Numbers in legend depict MFI values. (Below) By Pearson correlation, expression levels of both receptors on monocytes and lymphocytes were not significantly related to insulin concentration.
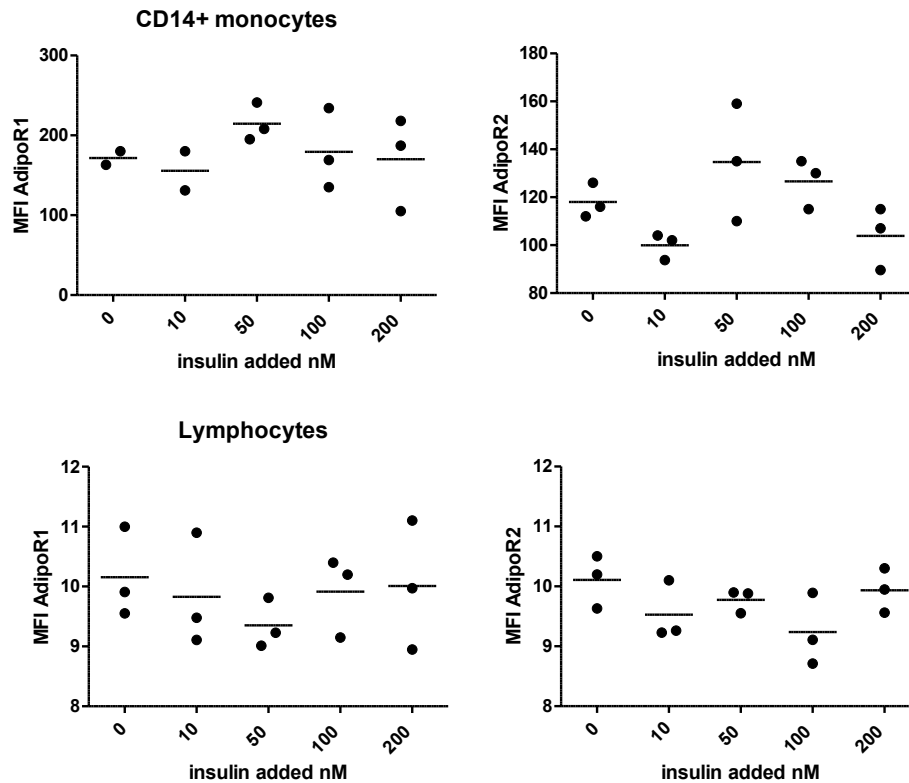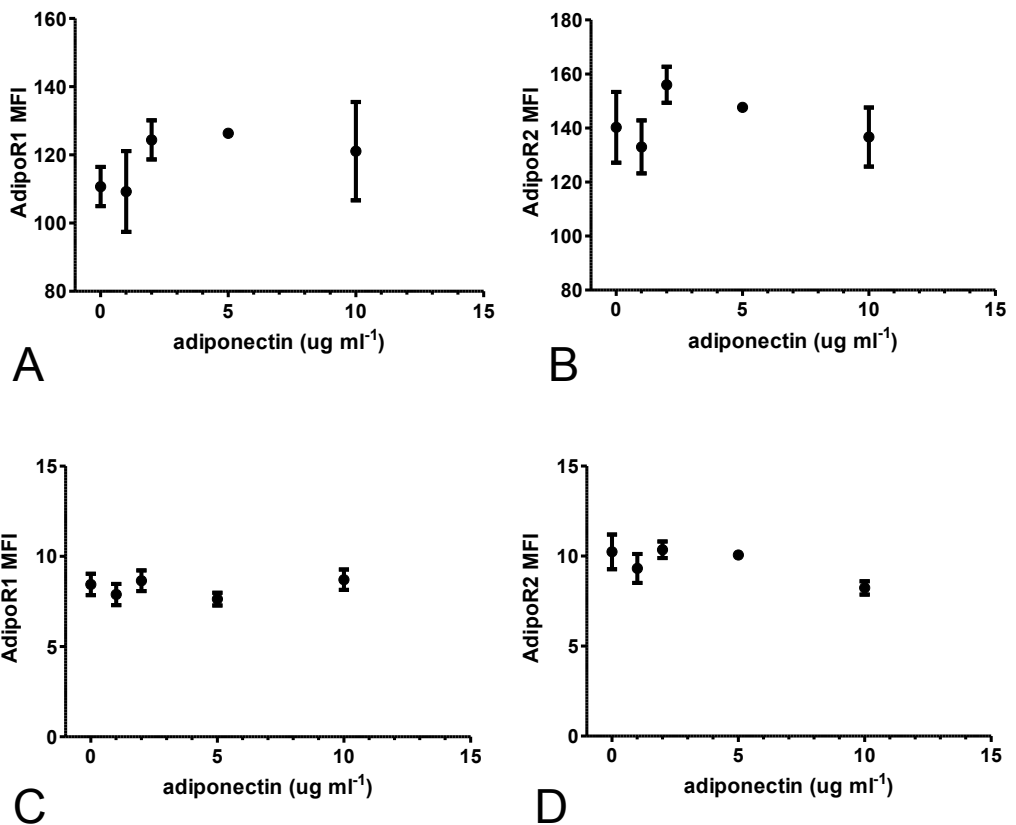
Figure 5.12: Effect of adiponectin on AdipoR expression on PBMC (n=8).
(Above) Whole PBMC were incubated for 48 hours in serum free media with
adiponectin added back at 0, 1, 2, 5 and 10ug/ml. There were no significant
correlations between adiponectin and AdipoR1 (A & C) and AdipoR2 (B & D)
expression on lymphocytes (A&B) and monocytes (C&D).

(Below): Overlaid histograms of AdipoR1 (A) & AdipoR2 (B) staining on
lymphocytes at the different concentrations, from one subject.
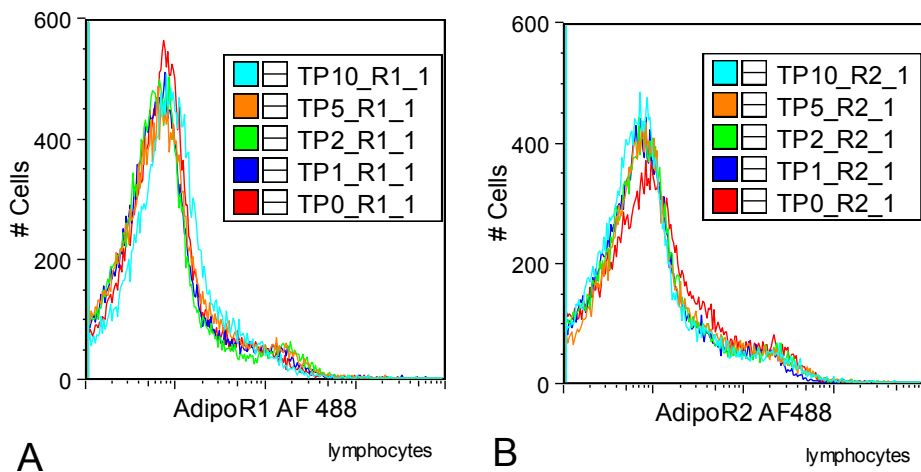
5.10 Discussion

To our knowledge, this is the first study to assess AdipoR protein expression on PBMC in T1D and T2D. Our sample size (n= 76) is comparable to that of previous studies (range 33-82) that investigated AdipoR gene expression of PBMC in insulin resistant states (Weigert, Neumeier et al. 2008, Sohn, Kwak et al. 2010, Shen, Charlesworth et al. 2007, Alberti, Gilardini et al. 2007). We achieved the desired matching of the pre-determined parameters in the two control groups. These were age, BMI and WHR for HC; HbA1C, duration of diabetes, insulin treatment, lipid profile and prevalence of vascular complications. A further strength is the significant intra-subject agreement of AdipoR protein and gene expression levels. This allowed the independent validation of group trends seen from the FACS measures against data acquired from qPCR. In agreement with other studies of AdipoR expression in primary tissues (Weigert, Neumeier et al. 2008, Sohn, Kwak et al. 2010, Shen, Charlesworth et al. 2007, Nannipieri, Bonotti et al. 2007, Alberti, Gilardini et al. 2007, Civitarese, Jenkinson et al. 2004, Bluher, Williams et al. 2007), we found that a strong interrelationship between the levels of expression of the two receptor isotypes.

Our first important finding is evidence for an inverse relationship between AdipoR expression on PBMC and insulin resistance in health, T1D and T2D (fig 5.5). The strongest evidence for this appears to be on the monocyte subpopulation (tables 5.3 & 5.4), cells which express the highest level of adiponectin receptors. BMI is a simple clinical surrogate of insulin sensitivity for the general population (MacKay, Haffner et al. 2009, Vazquez, Duval et al. 2007, Stern, Williams et al. 2005), as well as T2D (Inchiostro 2005). eGDR has been validated against the euglycaemic clamp in T1D (Williams, Erbey et al. 2000), and has been widely used in epidemiological studies as a measure of insulin sensitivity (Chillaron, Goday et al. 2009, Kilpatrick, Rigby et al.

2007, Pambianco, Costacou et al. 2007, Orchard, Olson et al. 2003). Other groups have reported on the negative correlation of BMI or HOMA-IR with AdipoR1 & AdipoR2 gene expression on PBMC (Weigert, Neumeier et al. 2008, Sohn, Kwak et al. 2010, Alberti, Gilardini et al. 2007), skeletal muscle (Civitarese, Jenkinson et al. 2004) and adipose tissue (Nannipieri, Bonotti et al. 2007, Bluher, Williams et al. 2007, Rasmussen, Lihn et al. 2006) of mostly non-diabetic individuals. Our data extends this literature by demonstrating the correlation at the level of protein expression, and also in diabetes. In the respective papers by Weigert and Alberti (Weigert, Neumeier et al. 2008, Alberti, Gilardini et al. 2007), only 11 (33%) and 12 (15%) of the study subjects had T2D. We attribute our finding of a significant correlation between monocytic expression of AdipoR1 & AdipoR2 to eGDR in T1D and BMI in T2D as the consequence of a larger sample size. We did not detect significant correlations between lymphocytic expression of AdipoR with indices of IR. Lymphocytes are low expressors of AdipoR1 and AdipoR2 (figures 4.10 & 4.13). Against the CV of the assay (3-9%, Tables 4.1 & 4.2), our study was underpowered to detect differences against the small range of IR in our subjects at the 5% significance level. In healthy subjects, we did not find AdipoR2 expression to correlate with BMI or WHR (Table 5.2). This has been described previously (Staiger, Kaltenbach et al 2004, Zhang, Holt et al. 2005,) and reflects the existence of factors other than IR, such as β cell function, lipids and truncal fat as independent modulators of expression.

Is the reduction of AdipoR expression on PBMC a cause or effect of insulin resistance? In the landmark paper by Yamauchi et al (Yamauchi, Kamon et al. 2003), the silencing of AdipoR1 & AdipoR2 gene expression suppressed insulin-stimulated glucose uptake on C2C12 myocytes. The same group has recently extended their findings to in vivo, using skeletal muscle specific AdipoR1 knockout mice (Iwabu, Yamauchi et al. 2010).

The corollary is the finding of reduced AdipoR expression in animals of insulin resistance, such as the ob/ob strain and the insulin receptor knockout mouse (Tsuchida, Yamauchi et al. 2004, Lin, Kim et al. 2007). Further support for the counterargument is the increased AdipoR expression in human peripheral tissues that follows the amelioration of insulin resistance (Bluher, Williams et al. 2007, Vu, Riddell et al. 2007). It is therefore possible for expression of AdipoR to be regulated in a bidirectional feedback manner according to the prevalent level of insulin sensitivity. Such a mechanism has been proposed for the modulation of adiponectin production (Cook, Semple 2010).

Our other significant and novel finding is the reduced expression of AdipoR by PBMC in T1D (fig 5.7). Indirect evidence to support this comes from Abke *et al* (Abke, Neumeier et al. 2006), who showed adiponectin stimulated release of cytokines on monocytes is impaired in T1D. We excluded feeding state and diurnal rhythm as confounders by demonstrating the absence of gene expression changes between morning fasting and afternoon postprandial sampling times (fig 5.1). Insulinisation had been shown to reduce AdipoR expression in the liver and skeletal muscle (Tsuchida, Yamauchi et al. 2004). However this was an unlikely explanation for our observations for a number or reasons. Firstly, there was no clinical evidence for over-insulinisation in our T1D cohort. They were not hypoglycaemic at the time of blood sampling, and the group's HbA1C (mean 9.0%, SD 1.4) was well above the level (<7.0%) where frequent hypoglycaemia would be suspected with intensive insulin therapy (The Diabetes Control and Complications Trial Research Group, 1993). As further support, insulin dose per unit weight did not correlate with AdipoR1 or AdipoR2 expression in T1D subgroup analysis (appendix 5). The observed reduction persisted when contrasting against our T2D cohort, where hyperglycaemia, duration of disease and exogenous insulin use were controlled. Lastly, our in vitro data showed changes in insulin did not

alter AdipoR expression on PBMC (fig 5.10) , and this is consistent with findings from skeletal muscle explants (Staiger, Kaltenbach et al. 2004). Using the same triple assessment (T1D subgroup correlation analysis, comparison with T2D and in vitro testing), we concluded that hyperglycaemia did not play a critical role in the modulation of AdipoR expression on human PBMC. We believe that species differences in receptor distribution (Kadowaki, Yamauchi 2005) and variations in expression regulation between tissues (Blüher, Fasshauer et al. 2005)

to account for the apparent contradiction against a previous report (Fang, Palanivel et al. 2005), which studied rat muscle cells where AdipoR1 is the predominant isotype.

Our regression analysis showed the reduction in AdipoR expression in T1D is disease specific (Table 5.5), although the relatively modest R^2 values of the models suggest a large proportion of the variability remains unaccounted for. The independent predictive value of serum adiponectin, suggested by its inverse association on univariate analysis, was lost. In any case, it was not sufficient to explain the reduced AdipoR expression in T1D, given the similar levels with HC group. We further confirmed its neutral effects in vitro. We recognise the results are at odds with reports from transgenic models where adiponectin negatively regulates expression of AdipoR2 on adipose tissue (Bauche, Ait El Mkadem et al. 2006). However, their findings did not apply to AdipoR1 expression.

Nonetheless, our regression modeling identified vascular complications as an independent predictor of reduced AdipoR1 expression on monocytes. This has previously been described (Soccio, Zhang et al. 2006). Although not significant, T1D subjects with vascular complications tended to have lower expression of AdipoR1 & AdipoR2 on monocytes. Vasculopathy in T1D is associated with subclinical inflammation (Schram, Chaturvedi et al. 2005, Lin, Glynn et al. 2008, Devaraj, Cheung

et al. 2007, van Hecke, Dekker et al. 2005). We propose therefore that the reduced AdipoR expression in T1D reflects the broad activation of innate immunity associated with this disease (Schaumberg, Glynn et al. 2005). Such a state of adiponectin resistance has been described in experimental models of diabetes, and in the setting of systemic inflammation that stems from chronic heart failure (Guo, Xia et al. 2007, Van Berendoncks, Garnier et al. 2010). Further measurements of serum inflammatory markers, such as IL-6, hsCRP and TNFα, can address this issue.

There were two unexpected findings from the clinical study. Firstly, older obese T2D subjects had similar AdipoR1 & AdipoR2 expression levels compared to young lean healthy controls (fig 5.7). This is however in agreement with two previous studies (Weigert, Neumeier et al. 2008, Debard, Laville et al. 2004). In contrast to its title, Weigert *et al* showed AdipoR gene expression levels on monocytes were not significantly different between T2D subjects and their obese non-diabetic controls. From our regression analysis, the older age and blockade of the renin-angiotensin system in T2D subjects may offset the reduced AdipoR expression associated with greater insulin resistance.

Secondly, we did not find the increased serum adiponectin widely reported for T1D (fig 5.2), when compared against age and BMI matched non-diabetic controls (Leth, Andersen et al. 2008, Maahs, Ogden et al. 2007, Barnes, Curran-Everett et al. 2008, Celi, Bini et al. 2006, Imagawa, Funahashi et al. 2002). These studies had employed a commericial radioimmunoassay for adiponectin measurement, but the ELISA method used is a validated alternative (Suominen 2004). In agreement with these studies, we found an inverse correlation of serum adiponectin with BMI and a positive association with HbA1C in T1D (Diabetes Research in Children Network (DirecNet) Study Group

2008, Barnes, Curran-Everett et al. 2008, Celi, Bini et al. 2006). Thus, we believe our study was underpowered to detect the difference between HC and T1D (posthoc power calculation 33.3%). Furthermore, the lower than expected absolute difference between the groups may be accounted for by the majority of T1D subjects having normal albumin excretion rate, normal renal function and a favourable lipid profile (Maahs, Ogden et al. 2007, Schalkwijk, Chaturvedi et al. 2006).

The higher adiponectin associated with poor glycaemic control in T1D may represent metabolic adaptation, given that adiponectin levels fall after the initiation of insulin treatment at diagnosis (Martos-Moreno, Barrios et al. 2006). We, and others, did not find total serum adiponectin levels in T1D to be associated with eGDR (Costacou, Zgibor et al. 2005), or IR as determined by the euglycaemic clamp (Perseghin, Lattuada et al. 2003). Nevertheless, HMW adiponectin may be more reflective for changes in insulin sensitivity (Pajvani, Hawkins et al. 2004). There had been one study reporting its increased levels in T1D (Leth, Andersen et al. 2008). We had attempted to measure HMW adiponectin in our subjects using a validated ELISA (Aso, Yamamoto et al. 2006), but in our hands, the intra-subject CV was unacceptably high. We had identified the initial protease digestion of the LMW adiponectin to be the source of the poor reproduciblity between duplicates.

We accept the following limitations. The case for insulin sensitivity correlating with AdipoR expression across health and diabetes would be strengthened if a universal laboratory measure of insulin sensitivity was applied in all 3 groups. HOMA-IR requires endogenous insulin secretion, and so is not applicable for subjects with established T1D. We believe our strategy of outpatient recruitment would have a low subject consent rate if the insulin tolerance test or the euglycaemic clamp were adopted. Secondly, both insulin and glucose are obligate ingredients of culture media. Further

addition of glucose and insulin could conceivably have only a marginal effect on AdipoR expression, if the control experiment was conducted at near the plateau of the dose-response range. In our defence, we applied wide ranging concentrations of insulin and glucose to PBMC from different subjects, and the results were consistent across replicates.

In conclusion, AdipoR expression on PBMC is reduced in T1D, with the lowest expression seen in the most insulin resistant. This data supports our working hypothesis that reduced adiponectin signaling to immune cells is a mechanism by which insulin resistance could drive islet autoimmunity.

5.11 Summary of findings

This is the first study to demonstrate differences in adiponectin receptor expression at the protein level in a pathophysiological state. AdipoR1 & AdipoR2 expression on PBMC significantly correlated with indices of insulin sensitivity in health, T1D and T2D. In addition, we found a global reduction of AdipoR expression on PBMC from T1D subjects, relative to matched controls, and this was most marked in the CD14+ monocyte population. We explored reasons for this by studying the effects of glycaemia, insulin and serum adiponectin, in vitro and by comparison with a cohort of HbA1C-matched subjects with insulin-treated T2D. However, these established regulators of AdipoR expression did not appear to play a critical role.

6. EFFECT OF LIFESTYLE INTERVENTION ON ADIPONECTIN RECEPTOR EXPRESSION BY PBMC

6.1   Introduction

In the previous chapter, we demonstrated the correlation of AdipoR expression on PBMC with insulin sensitivity in an observational study. To establish causality, we assessed the relationship between these variables in interventional trials. Lifestyle interventions, which include structured exercise and dietary counselling, are safe and have proven efficacy in ameliorating insulin resistance (Diabetes Prevention Program Research 2002, Tuomilehto, Lindstrom et al. 2001, Pan, Li et al. 1997). In the following, we assessed the effect of lifestyle interventions on the expression of AdipoR on PBMC in three independent, investigator-blinded, randomised controlled studies.

6.2   The Early ACTID

The Early ACTID study is a 12 month study based at the University of Bristol (principal investigator - Dr Rob Andrews) which examined the effects of diet and exercise in patients newly diagnosed with T2D.

6.2.1   Clinical parameters at baseline and at 6 months

In a collaborative sub-study with Dr Andrews, we tested whether the delivery of an educational lifestyle package would increase AdipoR expression over and above that of conventional T2D care. Subjects with newly diagnosed T2D were randomised to conventional care or intensive lifestyle interventions for 6 months. At the time of the

study, 349 subjects had been enrolled. We studied samples from 66 consecutively recruited subjects from the Early ACTID, as well as the 12 subjects who were positive for anti-GAD antibody (titres > 14 units/ml, WHO 97.5 centile) (Bingley, Christie et al. 1994). The total sample size of 78 subjects had previously been shown to be sufficient in demonstrating a significant increase of at least 50% in AdipoR expression of skeletal muscle (Bluher, Williams et al. 2007, Christiansen, Paulsen et al. 2010). All subjects were within 6 months of their diagnosis with T2D. Before unblinding of clinical data, we had excluded 21 subjects for further analysis, because at least 1 of 2 paired samples contained significant genomic DNA contamination. After unblinding, a further 4 subjects were excluded because of thiazolidinedione use, whose PPARγ agonistic activity independently increases AdipoR expression on monocytes (Chinetti, Zawadski et al. 2004). The final number of subjects included in the analysis was 53, with 19 in the conventional care arm (CON) and 34 in the diet and exercise interventional arm (INT). Baseline and 6 month data are shown in Table 1.

At baseline, subjects from both arms were matched for age, sex, BMI, WHR, VO_2 max, MABP, glycaemia and fasting insulin. In the setting of a new diagnosis of non-insulin dependent diabetes over age of 35, anti-GAD antibodies (GADA) identifies patients with latent autoimmune diabetes of adulthood (Tuomi, Groop et al. 1993). The proportion of subjects positive for GADA was similar in both arms (CON: 16%, INT 18%).

Table 6.1: Baseline and 6 month results of Early ACTID. Conventional (CON) and intensive (INT) arms are shown. Data expressed as mean ± SEM. Two-tailed unpaired t-test was applied for continuous data between the arms at baseline and 6 months, paired t-test for intra-group comparsions, and Fisher's exact test for categorical data.

** p <0.01 intragroup * p <0.05 intragroup

	CON (n=19)		INT (n=34)	
	Baseline	6 months	Baseline	6 months
Age	57.1 ± 3.5		60.8 ± 1.7	
Male sex (n)	13 (68%)		20 (59%)	
BMI (kg m^{-2})	32.6 ± 1.2		31.1 ± 0.9	
WHR	0.95 ± 0.01		0.95 ± 0.01	
Weight (kg)	89.2 ± 3.4	89.5 ± 3.4	87.8 ± 2.4	87.4 ± 2.5
WC (cm)	104.3 ± 2.4	103.4 ± 2.1	105.3 ± 2.2	104.7 ± 1.6
VO$_2$ max (L min^{-1})	26.5 ± 2.6	27.2 ± 2.5	25.1 ± 1.8	27.3 ± 1.7*
HbA1C (%)	6.67 ± 0.18	6.95 ± 0.20	6.61 ± 0.20	6.60 ± 0.14
MABP (mmHg)	101.6 ± 2.0	90.9 ± 2.9*	97.7 ± 1.6	92.9 ± 1.7**
Fasting glucose (mmol^{-1})	7.9 ± 0.3	7.2 ± 0.3*	7.9 ± 0.3	7.2 ± 0.2*
Fasting insulin (mU L^{-1})	15.6 ± 1.9	18.1 ± 3.0	15.8 ± 2.0	17.8 ± 2.8
HOMA-B (%)	72.1 ± 8.1	98.6 ± 15.6	74.0 ± 9.3	100.1 ± 15.7
HOMA IR	5.66 ± 0.81	4.95 ± 1.16	5.69 ± 0.85	4.93 ± 1.07
Medications at 6 months (n, %)				
Metformin		15 (79%)		21 (62%)
ACEi/ARB		7 (37%)		13 (38%)
Statin		11 (58%)		18 (53%)
GADA + (n)	3		6	

Use of metformin, drugs which block renin-angiotensin system and statins were not different between the arms at 6 months. On paired t-testing, no significant weight loss was detected with either treatments. However in both arms, MABP fell at 6 months, but the difference in MABP decrease between the arms were similar. There was also a small but significant intra-group increase in VO2 max in the INT arm (baseline: 25.1 ± 1.8, 6 months: 27.3 ± 1.7 paired t-test = 0.011). In both arms, there was a significant fall of the 6-month fasting glucose from baseline (CON: baseline 7.9 ± 0.3, 6 month 7.2 ± 0.3 p = 0.050; INT: baseline 7.9 ± 0.3, 6 month 7.2 ± 0.2, p = 0.048), but the difference in values between groups was similar. There was a non-significant trend for

subjects assigned to conventional care to have a higher HbA1C at 6 months (CON: 6.95 ± 0.20; INT: 6.60 ± 0.14; p = 0.14). Otherwise, no other differences in the metabolic parameters at 6 months, within and between arms were detected.

6.2.2 Changes in adiponectin receptor gene expression on PBMC

Consistent with our cross-sectional study, we found baseline AdipoR1 & AdipoR2 gene expression to be significantly inter-correlated (r = 0.687 p<0.001) and with HOMA-IR respectively (AdipoR1: r = -0.308 p = 0.025; AdipoR2: r = -0.363 p = 0.008) (fig 6.1). In addition, AdipoR2 gene expression was inversely related to fasting insulin levels (r = -0.322 p = 0.019). There were no significant correlations with other clinical parameters.

At 6 months, subjects randomised to the INT arm had a significant increase of AdipoR1 & AdipoR2 expression, compared to those in CON (fig 6.2). Expressed as fold change relative to baseline values, the average increase in the INT arm was 1.73 ± 0.42 for AdipoR1, and 5.57 ± 2.77 for AdipoR2. In subjects assigned to conventional care, AdipoR1 expression decreased, whereas AdipoR2 levels did not alter from baseline (AdipoR1: 0.64 ± 0.17; AdipoR2: 1.04 ± 0.37). We excluded the possibility of mRNA degradation during storage as a confounder by demonstrating the absence of a significant correlation between the normalised gene expression values against the date of sample collection (fig 6.3).

Figure 6.1: Correlation analysis of AdipoR1 & AdipoR2 gene expression at baseline. AdipoR expression was normalised against r18S by the ΔCt method, such that a low numerical value reflects high expression. (Top) AdipoR1 & AdipoR2 gene expression levels significantly inter-correlated and with HOMA-IR (middle and bottom panels). The correlations of AdipoR1 & AdipoR2 with HOMA-IR retained significance despite removal of the three outliers (°) to the right.

*$p < 0.05$

**$p < 0.01$
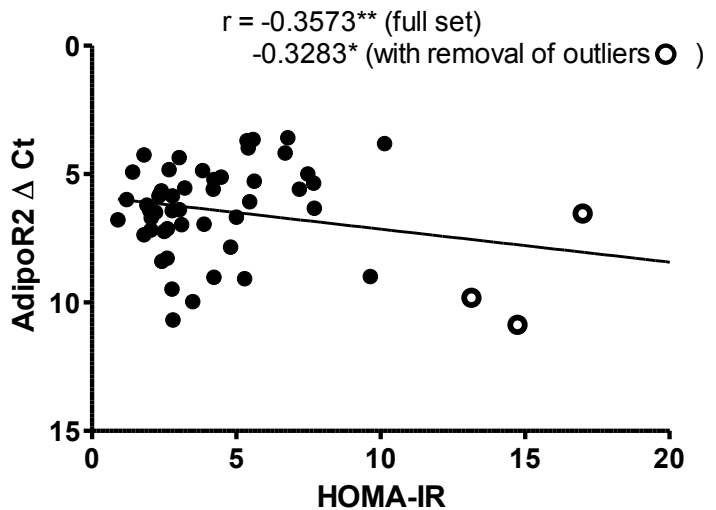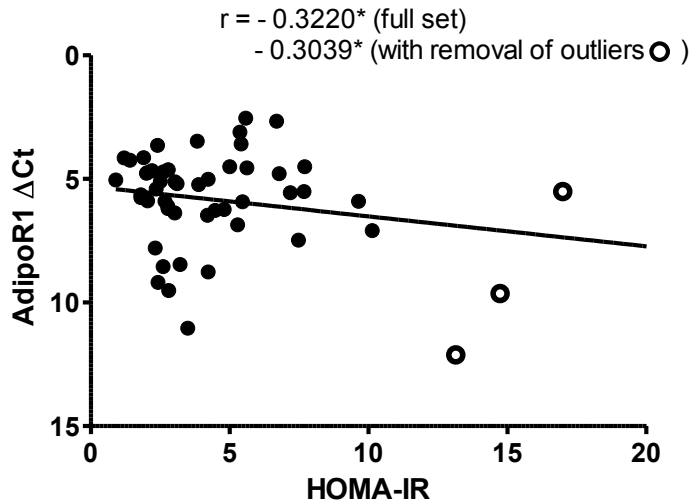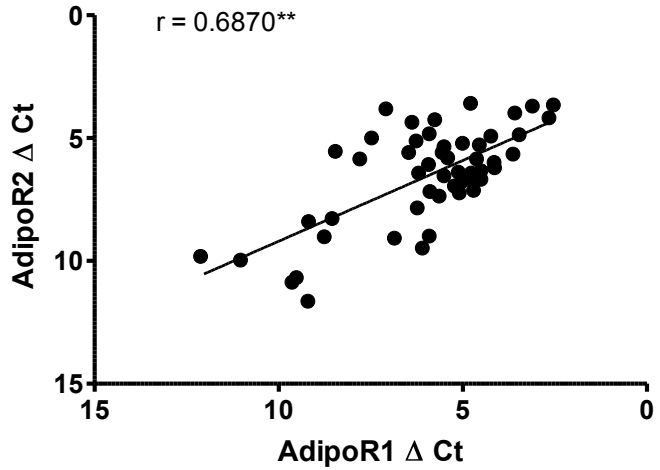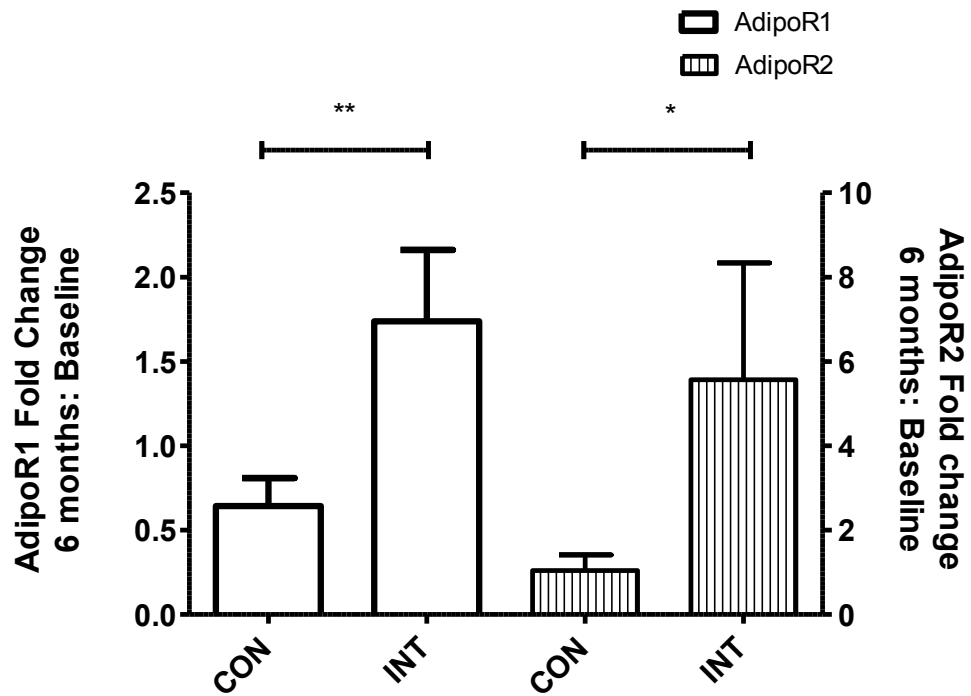
Figure 6.2: Gene expression changes of AdipoR1 and AdipoR2 on PBMC in the Early ACTID. Relative change at 6 months to baseline was calculated for each subject using the -ΔΔCt method. t-testing between the arms was done following logarithmic transformation.

The apparent reduction in HOMA-IR between 6 months and baseline for both groups did not reach statistical significance. Thus given that in both arms an appreciable proportion of subjects experienced improved insulin sensitivity, we performed a posthoc correlation analysis accounting all subjects, to investigate the association of HOMA-IR with AdipoR expression. We found the change in AdipoR1 & AdipoR2 expression, transformed to logarithm and normality, correlated inversely with absolute changes in HOMA-IR and weight (Table 6.2 & fig 6.4). Also, changes in AdipoR1 and AdipoR2 expression were inter-correlated (r = 0.707, p <0.0001).
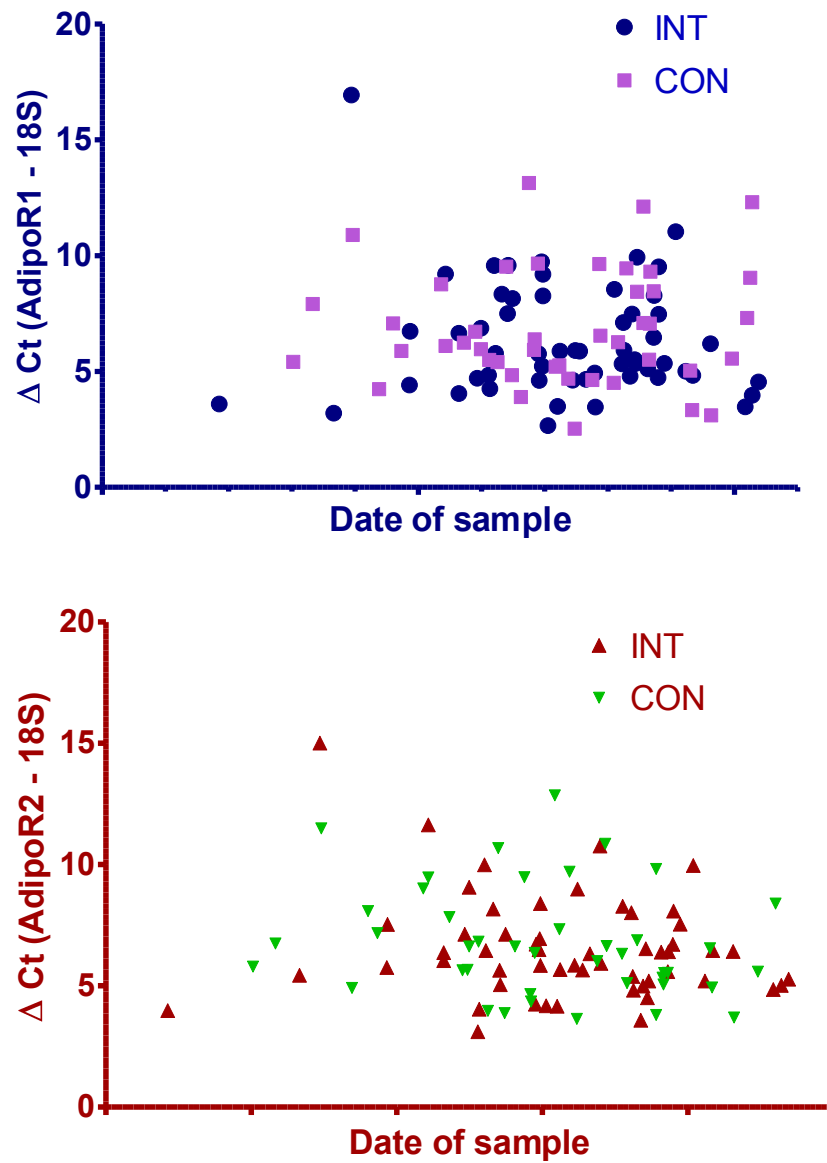
Figure 6. 3: Evaluating mRNA degradation of archived samples from Early ACTID.
There was no significant correlation between ΔCt AdipoR1 (top) & ΔAdipoR2 (bottom)
from both arms with the date of sample.
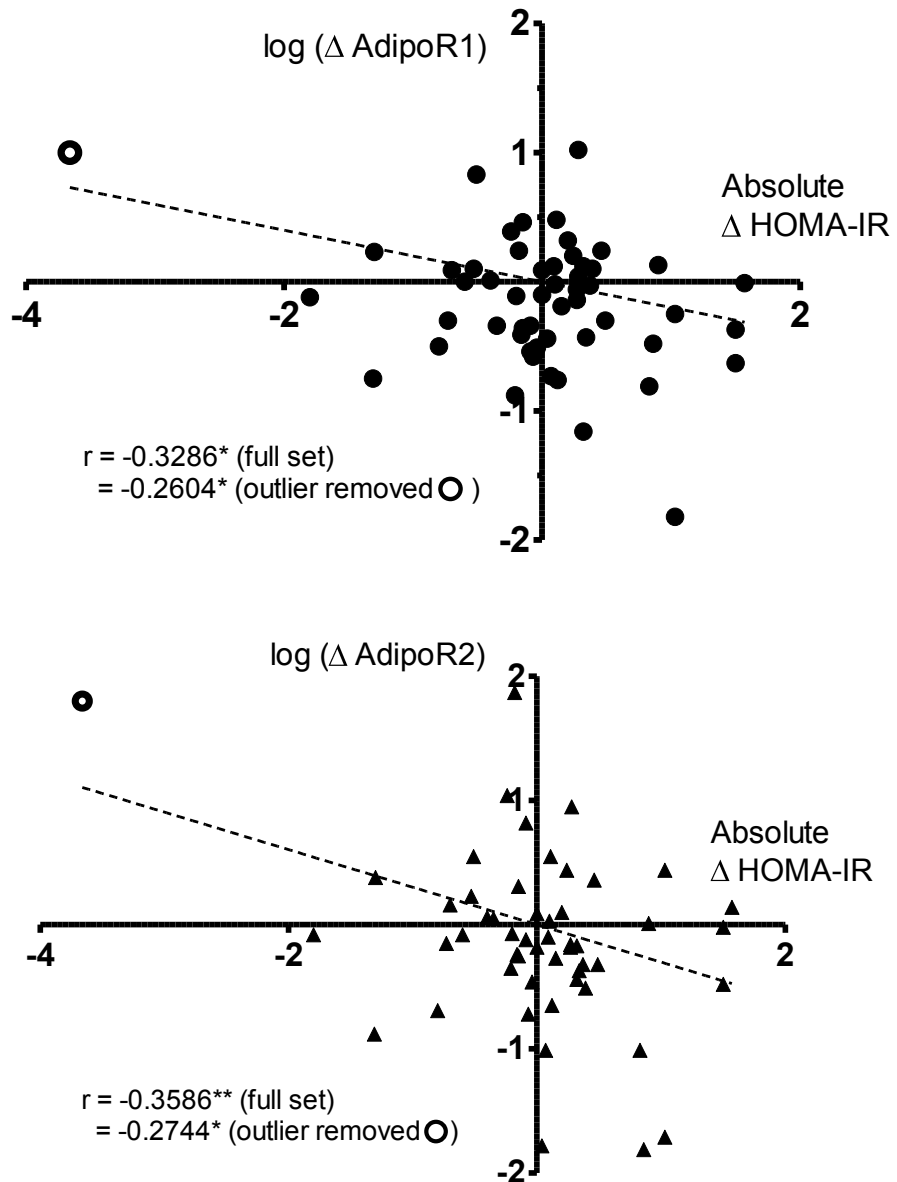
Figure 6.4: correlation of AdipoR1 & AdipoR2 gene expression change with insulin resistance across whole cohort (n = 53). Absolute HOMA-IR change was derived by subtracting HOMA IR at 6 months to baseline value. Gene expression change at 6 months relative to baseline for each subject was calculated by the $\Delta\Delta$Ct method. Logarithmic transformation was done so to allow for Pearson correlation analysis between the variables. The significance of the correlations persists despite removal of the outlier (°) from the analysis.
* p < 0.05, **p<0.01

Table 6.2: Pearson correlation of AdipoR1 & AdipoR2 gene expression changes at 6 month with absolute changes in weight and HOMA-IR (n=53). For this analysis, Δ AdipoR1 was the logarithm of fold change in gene expression at 6 months, compared to baseline. The transformed distribution approximates to normal, by KS testing.

		Δ **AdipoR1**	Δ **AdipoR2**
Weight	**r**	-0.114	-0.345
	p	0.416	0.011
HOMA-IR	**r**	-0.328	-0.356
	p	0.016	0.009

6.2.3 Subjects with latent autoimmune diabetes of adulthood (LADA)

We were interested in whether the changes in AdipoR expression with exercise, weight loss or improved insulin sensitivity in T2D could extend to autoimmune diabetes. LADA is a slowly progressive form of autoimmune diabetes (Fourlanos, Dotta et al. 2005). It is distinguished from T1D by the absence of insulin dependence at initial presentation. In the 9 subjects with LADA, there were significant decreases in weight, fasting glucose, together with a rise in VO_2 max at 6 months (Table 6.3). As demonstrated for patients with T2D, these metabolic changes were accompanied by a significant upregulation in AdipoR1 gene expression (2.63 fold, p = 0.017), with borderline increase in AdipoR2 expression (1.50, p = 0.07).

Table 6.3: Changes at 6 months for subjects positive for GADA.
Data presented as mean ± SEM. Fold change at months for AdipoR1 (Δ AdipoR1) & AdipoR2 (Δ AdipoR2) expressed as 95%CI.

	Baseline	6 months
Age (yrs)	60.3 ± 3.0	
Male sex (n)	7	
Weight (kg)	81.5 ± 3.9	78.3 ± 3.7**
WC (cm)	99.1 ± 3.2	99.4 ± 3.4
VO$_2$ max (L min^{-1})	30.2 ± 2.7	32.1 ± 2.4*
MABP (mmHg)	96.1 ± 2.5	96.2 ± 3.2
HbA1C (%)	6.3 ± 0.3	7.2 ± 0.7
Fasting glucose (mmol^{-1})	7.5 ± 0.6	7.3 ± 0.5*
Fasting insulin (mU L^{-1})	10.6 ± 2.1	10.3 ± 1.8
HOMA-B (%)	61.0 ± 17.3	62.4 ± 16.3
HOMA-IR	3.70 ± 0.78	3.22 ± 0.62
Δ AdipoR1	1.0	2.63ξ (1.00 – 6.89)
Δ AdipoR2	1.0	1.50ς (0.84 – 2.70)

** p <0.01   *p <0.05 paired t-test

ξ p <0.05 single two-tailed t-test against inferential value of 0 (no change), following logarithmic transformation

ς p =0.07

6.3 The relative importance of exercise as a component of lifestyle interventions

As lifestyle change consists of both exercise and diet, we then asked whether the component of exercise alone is sufficient in modulating changes in AdipoR expression. The Exercise and Over-eating Study from the University of Bath is a study of one week duration and included 11 healthy non-diabetic subjects (Principal Investigator: Dr Dylan Thompson). In this collaborative sub-study, we compared the effect of intensive exercise (EXE) (45 minutes of supervised exercise at 70% of maximum heart rate) versus activity restriction (SED) (<4000 steps/d). In both arms, subjects followed a hypercaloric diet, with energy intake increased by 50%. With the acquisition of live PBMC, we were able to quantify expression at both the mRNA and protein levels.

Table 6.4 Clinical data from the Exercise and Over-eating Study. Mean ± SD values shown.

	SED		EXE	
	Day 0	Day 7	Day 0	Day 7
n	4		7	
Age (yrs)	25.5 ± 5.4		27.9 ± 7.7	
BMI (kg m^{-2})	22.9 ± 1.7		24.7 ± 2.9	
Weight (kg)	77.1 ± 2.9	79.5 ± 3.7*	77.8 ± 11.4	79.2 ± 12.1*
Fasting glucose (mmol^{-1})	4.80 ± 0.28	4.81 ± 0.33	5.03 ± 0.33	4.95 ± 0.18
Fasting insulin (mU l^{-1})	4.03 ± 3.09	6.13 ± 3.68*	4.83 ± 2.97	6.37 ± 4.09
HOMA-IR	0.87 ± 0.70	1.29 ± 0.74*	1.09 ± 0.68	1.41 ± 0.92
Serum adiponectin (ug ml^{-1})	5.5 ± 1.6	7.5 ± 2.3*	7.0 ± 4.7	8.2 ± 4.9

* $p < 0.05$ paired t-test (within group d7 vs d0)

The metabolic changes of the subjects are described in Table 6.4. There were no significant differences between the groups at baseline or on day 7. In the SED arm, there were significant intra-subject increases in weight (1.9% p = 0.017), fasting insulin (52% p = 0.037) and HOMA-IR (48% p = 0.024) at the end of the study. Unexpectedly, serum adiponectin also increased (p = 0.034). In the EXE arm, there was significant weight gain (p=0.018). Interestingly, two of the seven subjects did not follow the increase in insulin resistance from weight gain seen in the rest of the group (fig 6.5).

Figure 6.5. Changes in HOMA-IR in the Exercise & Over-eating Study.

* P < 0.05 paired t-test
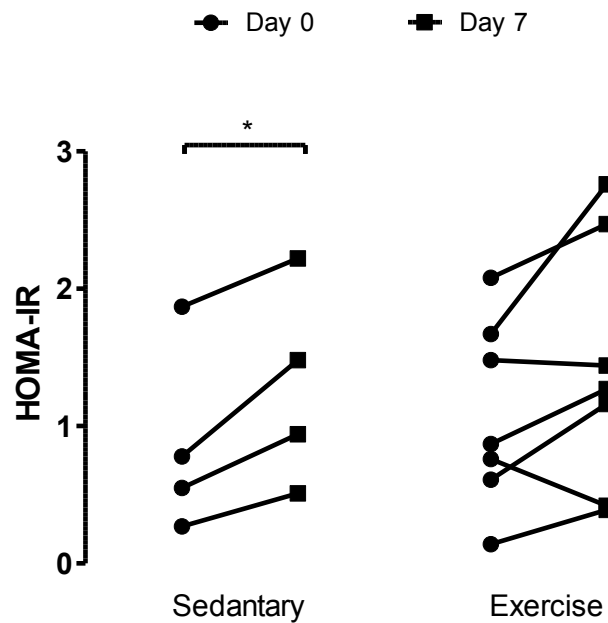
Monocyte numbers, as a percentage of total PBMC count, were similar between groups, at baseline and at the end of study. AdipoR1 mRNA levels in the SED group were significantly reduced from baseline (fold change: 0.6, p = 0.03), but not in the EXE group. There were no significant changes for AdipoR2 mRNA in either group on completion of the trial. Change in protein expression was standardised using the ratio of MFI on day 7 relative to day 0. The transformed measure passed the KS normality test. In both arms, there was heterogeneity in response between subjects, such that the confidence of the group mean estimates was wide. Consequently, a consistent change, in the direction of reduction, was observed only for AdipoR1 expression in both lymphocytes and monocytes for SED (95% CI, lymphocytes: 0.02-0.74; monocytes: 0.02-0.88). This is in keeping with the uniform reduction in gene expression for all subjects in SED. Of interest is the significant inter-group difference in monocytic

expression for AdipoR1 (fig 6.6), but not for lymphocytes (fig 6.7). Our data here suggests overeating alone can significantly suppress AdipoR1 expression across all PBMC in just 1 week. However, concurrent intensive exercise can negate part of this reduction, especially on monocytes. No significant changes in AdipoR2 expression in both arms were detected at entry or exit of the trial.

Table 6.5: Changes in AdipoR expression between the exercise (EXE) and sedentary (SED) arms of the trial. All subjects were on a hypercaloric diet (150% of normal intake) for 7 days.

	SED		EXE	
	Day 0	Day 7	Day 0	Day 7
Monocyte (%)	2.0 ± 0.8	3.4 ± 0.9	3.1 ± 1.4	3.2 ± 0.9
mRNA				
Δ AdipoR1		$0.6 \pm 0.1\zeta$		1.2 ± 0.6
Δ AdipoR2		0.7 ± 0.3		1.6 ± 0.5
Protein				
MFI Δ				
AdipoR1(D7 :D0)				
lymphocytes		$0.4 \pm 0.1\xi$		0.9 ± 0.4
Monocytes		$0.4 \pm 0.1\zeta$*		1.0 ± 0.2*
MFI Δ				
AdipoR2(D7:D0)				
lymphocytes		0.7 ± 0.5		1.1 ± 0.6
Monocytes		0.6 ± 0.2		1.6 ± 0.6

ξ - $p < 0.01$ ζ - $p < 0.05$ by one sample t-test against inferential value of 1.0 (no change)

* - $p < 0.05$ by t-test between groups
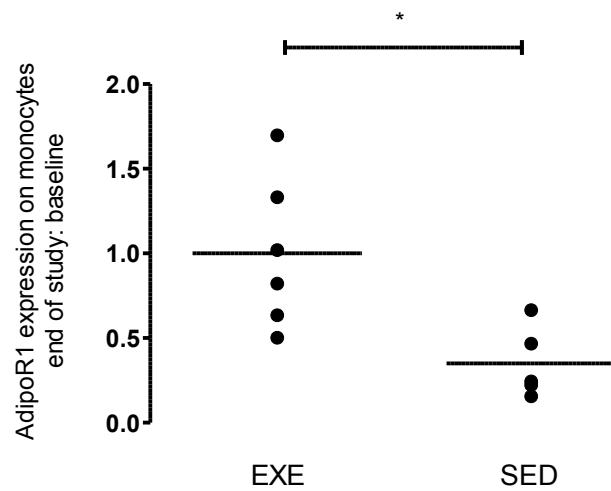
Figure 6.6: (top) AdipoR1 expression on monocytes in a 1 week study where subjects were randomised either to intensive exercise (INT: n=6) or sedentary activity (CON; n=5) against a hypercaloric diet. AdipoR1 MFI on CD14+ monocytes were measured at 1 week and standardised against values obtained at entry. (bottom) Representative calculation from dot plots. For subject 22 (CON), MFI Δ AdipoR1 is 62.8/94.5 = 0.66; for subject 25 (EXE), this is 1.0. Gates shown define positive cells and numbers denote percentage frequencies * p = 0.016, 2 tailed t-test.

A. Subject 22 (SED) 0d

B. Subject 22 7d

C. Subject 25 (EXE) 0d.

D. Subject 25 7d
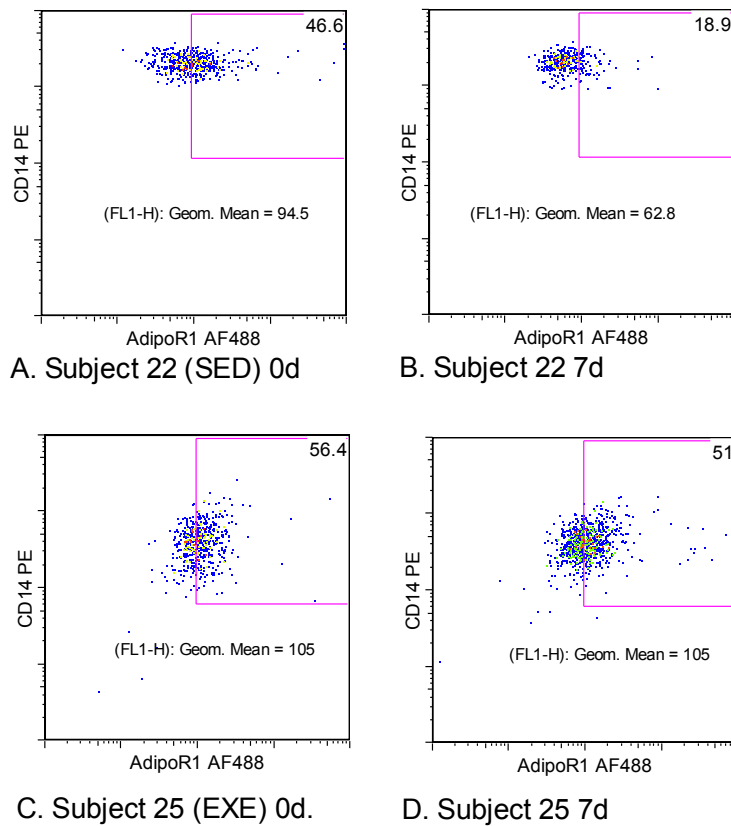
Figure 6.7: No significant difference in the mean change of AdipoR1 on lymphocytes between intensive exercise (INT) and sedentary activity (SED). (top) p = 0.33 between groups. (bottom) representative dot plots from 2 matched subjects where there were significant differences in monocytic expression of AdipoR1 (fig 6.6). Quadrant gates were drawn based on isotype control and numbers denote percentage frequencies.
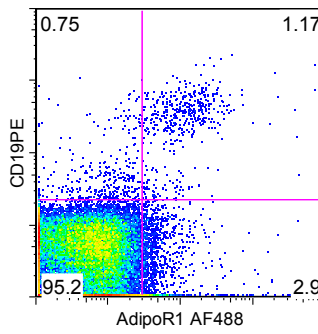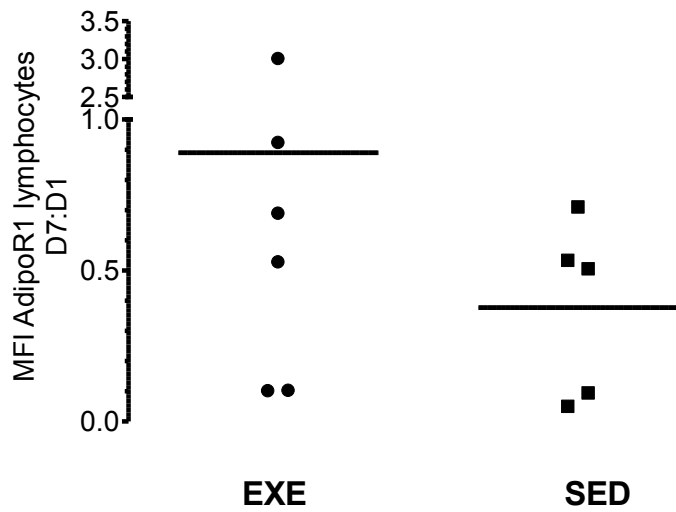
A. Subject 22 (SED) 0d
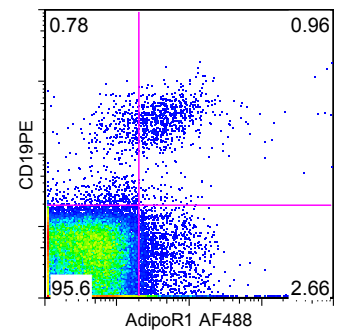
B. Subject 22 7d
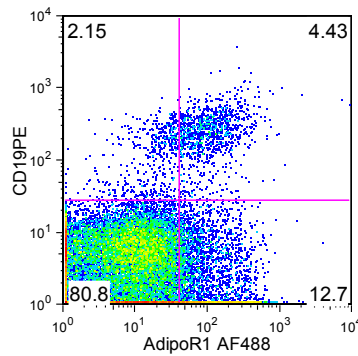
C. Subject 25 (EXE) 0d
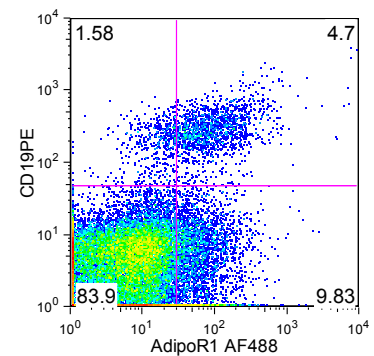
D. Subject 25 7d

6.4 The effect of moderate vs vigorous exercise on receptor expression

Next, we asked whether exercise intensity has differential effects on AdipoR expression. The Exercise Intensity Study is a three week study conducted at the University of Bath with Dr Dylan Thompson as the principal investigator. In this trial with a total of 13 healthy non-diabetic but overweight subjects, we compared the effects of vigorous (SV) against moderate (SM) exercise. In the SV group, subjects exercised on a treadmill at 70% VO_2 max, as opposed to 50% VO_2 max in the SM group. The total energy expenditure (600kCal), the frequency of exercise (5 times a week for 3 weeks), and dietary intake (714 kCal) were controlled.

Table 6.6: Clinical parameters at entry and on completion of the Exercise Intensity Study.

	SM (n=6)		SV (n=7)	
	Baseline	3 weeks	Baseline	3 weeks
Age (yrs)	49.7 ± 1.3		54.7 ± 2.1	
Male sex (n)	4		4	
BMI kg m^{-2}	29.0 ± 1.0		32.3 ± 0.9*	
Weight (kg)	89.0 ± 6.1	86.8 ± 6.1*	94.9 ± 4.8	92.3 ± 4.7**
WC (cm)	100.7 ± 4.0	97.4 ± 2.6*	105.0 ± 2.6	103.0 ± 3.0
Fasting glucose (mmol^{-1})	6.0 ± 0.8	5.7 ± 0.1	5.9 ± 0.6	5.98 ± 0.3
Fasting insulin (mU L^{-1})	9.7 ± 2.9	7.2 ± 1.4*	8.1 ± 2.3	6.5 ± 1.3
HOMA-IR	3.1 ± 1.5	2.1 ± 0.4	2.1 ± 1.0	1.6 ± 0.4
Adiponectin μg ml^{-1}	9.6 ± 2.5	9.0 ± 1.7	9.4 ± 2.6	8.6 ± 1.7

** $p < 0.01$ * $p < 0.05$

At baseline, age, sex, fasting glucose and insulin were equally distributed between the groups. Subjects from the SV arm had a significantly higher BMI, but starting weight

and WC were similar. At 3 weeks, subjects in both groups achieved significant weight loss (95% CI: SM: 0.7-3.6kg; SV: 1.3-3.9kg) but final weight was not different. In the SM arm there was also a significant decline in WC and fasting insulin. No significant changes in serum adiponectin or HOMA-IR were observed in either interventional arms.

At the mRNA level there was no significant change with AdipoR1 & AdipoR2 from baseline for either group (fig 6.8). At the protein level (fig 6.9), there was marked heterogeneity in response within each group and Δ AdipoR1 & Δ AdipoR2 values did not conform to normality. Using the non-parametric Wilcoxon signed rank test, there was significant change from unity for AdipoR1 & 2 expression on monocytes for subjects in the SM arm (median: ΔAdipoR1 2.2; ΔAdipoR2 2.3) and AdipoR2 on lymphocytes in the SV arm (median ΔAdipoR2 1.9). The application of Kruskal-Wallis test with Dunn's correction for multiple testing showed significant change in AdipoR1 protein expression with monocytes only (median ΔAdipoR1 SM 2.2, SV 0.7).
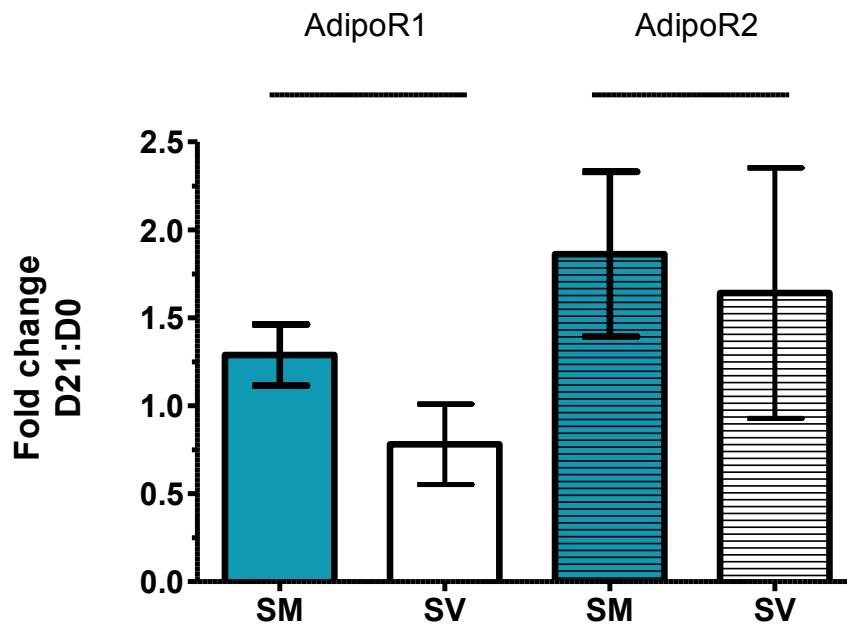
Figure 6.8: Changes in mRNA levels of AdipoR1 & AdipoR2 at completion of the trial. Fold change was calculated by the $\Delta\Delta$Ct method using ΔCt at d21 - ΔCt at d0. Following logarithmic transmformation, no significant group changes were observed for the gene expression of both receptors, by two-tailed one sample t-test against the inferential value of 0.

Figure 6.9: (top) representative examples of AdipoR1 & AdipoR2 protein expression changes n the exercise intensity study. For comparision, gates were drawn against isotype control. For subject SM17 at d0 (A), MFI of AdipoR1 for lymphocytes (CD14- cells) is 5.13 and for monocytes (CD14+) 114. At day 21 (B), the correponding values are 6.27 and 195 respectively. Hence the ratio of ∆AdipoR1 for lymphocytes is 1.22 and 1.71 for monocytes. For SV14, ∆AdipoR1 for monocytes is 1.08 and 1.27 for monocytes.

(bottom) Changes in AdipoR expression in the exercise intensity study. Median and range plotted. ζ $p < 0.05$ by Wilcoxon signed rank test, against inferential median value of 1.0 (no change).
* $p < 0.05$ by Dunns post test

A. SM17 d0

B. SM17 d21

C. SV14 d0

D. SV14 d21
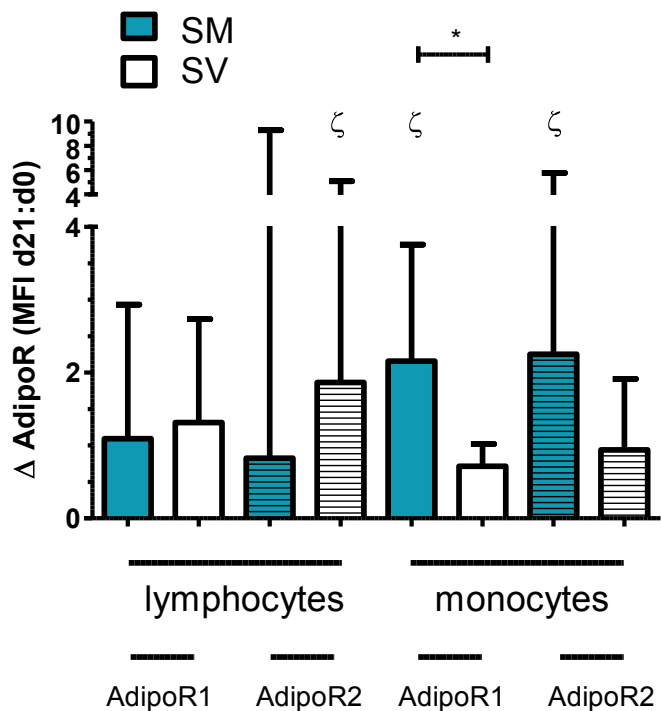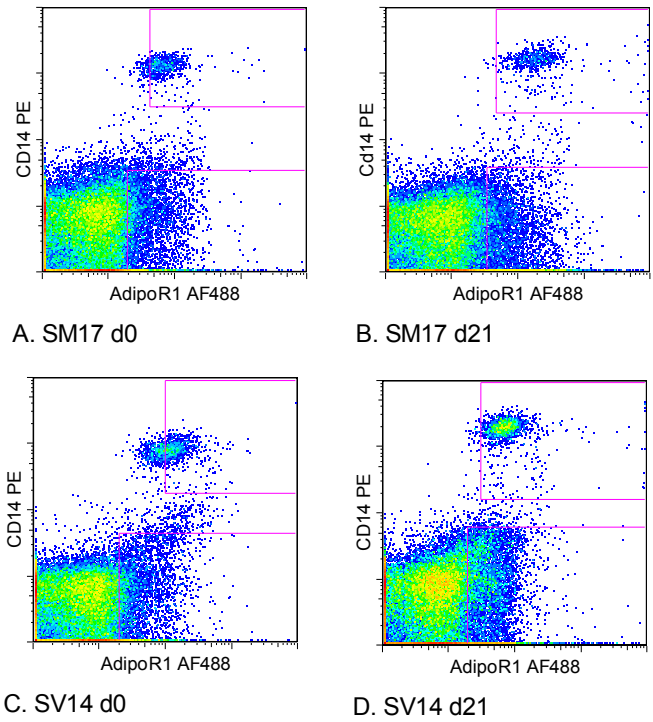
6.5 Discussion

Three large multi-centred trials have demonstrated that lifestyle interventions reduce insulin resistance and can prevent T2D in high risk groups (Diabetes Prevention Program Research 2002, Tuomilehto, Lindstrom et al. 2001, Pan, Li et al. 1997). Impressively, despite the relatively short duration of intervention in these studies, the metabolic benefits are maintained for at least 10 years (Lindström, Ilanne-Parikka et al. 2006, Diabetes Prevention Program Research Group 2009, Li, Zhang et al. 2008) The combination of exercise and diet is also effective in raising serum adiponectin levels in insulin resistant individuals (Mather, Funahashi et al. 2008, Cambuli, Musiu et al. 2008, Esposito, Pontillo et al. 2003, Monzillo, Hamdy et al. 2003). In other studies, this is also accompanied by increased adiponectin receptor gene expression on adipose tissue and skeletal muscle (Bluher, Williams et al. 2007, Christiansen, Paulsen et al. 2010, Kim, Maachi et al. 2006, O'Leary, Jorett et al. 2007). However, two systematic reviews of exercise studies have found an overall inconsistent effect on adiponectin signalling (Vu, Riddell et al. 2007, Simpson, Singh 2008). In addition, the authors noted that many studies in the area were observational only, and also the majority of interventional trials were uncontrolled.

In this series of controlled studies, we showed the upregulation of AdipoR expression by exercise can be achieved on immune cells in normal weight, obese and diabetic subjects. In the Early ACTID, both AdipoR1 & AdipoR2 gene expression is increased in the intensive interventional arm in early T2D and LADA. In the second study of exercise vs sedentary activity in young healthy subjects, we found that intensive exercise can compensate for the fall in AdipoR1 expression on monocytes brought on by overeating. In the final study of exercise intensity in overweight individuals, we found

regular moderate exercise intensity to be effective in augmenting AdipoR1 expression of monocytes. We attribute the variations in the response of AdipoR expression to lifestyle measures in these trials to be the result of differences in study group, design and duration, as well as number of patients studied. Such discrepancies have previously been reported. Subjects who are glucose intolerant appear to be more responsive when given the same treatment, and increase in AdipoR on adipose tissue was independent of the reduction in body weight (Bluher, Williams et al. 2007, Kim, Maachi et al. 2006). Furthermore, short term exercise interventions (< 1 week) have not been successful in upregulating AdipoR (Zeng, Isobe et al. 2007, Punyadeera, Zorenc et al. 2005, Zeng, Fu et al. 2007). Lastly, differences in the amount of prescribed exercise can affect the degree of AdipoR1 change (Zeng, Isobe et al. 2007).

Nevertheless, the three studies are unanimous in demonstrating the importance of exercise in the regulation of AdipoR on PBMC. In the Early ACTID, where the intervention was motivational only, the increased VO_2 max in the INT arm was consistent with increased exercise objective for the group. The difference in AdipoR expression change between groups had occurred despite the absence of significant change in weight, HOMA-IR and similar falls in fasting glucose. We justified our posthoc analysis because of probable dilutional effect in the benefits of intensive exercise between the groups. Subjects in CON arm were aiming for the same metabolic targets and were not restricted in the pursuit of lifestyle changes. Here, the finding of weight loss as a significant independent predictor of AdipoR change is consistent with the upregulation on at least part of the PBMC subset in the exercise intensity study. It appears that reduction of central obesity, measured by the fall in WC in the SM arm, is a potent stimulus for AdipoR expression change on monocytes.

Our second study illustrates the profound effect of diet, that one week of hypercaloric intake can suppress AdipoR1 expression, at both the gene and protein expression levels. This is a novel finding, and is consistent with the converse evidence where the sole institution of a very low caloric diet for 3-8 weeks promotes AdipoR expression on adipose tissue and skeletal muscle (Christiansen, Paulsen et al. 2010, Kim, Maachi et al. 2006). However, the significance of the reduced expression may be diminished by the increase in serum adiponectin with overeating. This rise is paradoxical to the change in insulin resistance, but has been observed in binge eating disorders (Monteleone, Fabrazzo et al. 2003). Given its insulin sensitising action, the increased adiponectin here may represent compensation against hyperinsulinaemia from a hypercaloric meal (Taylor, Hubbard et al. 1999).

One way of elucidating a mechanism for the exercise induced AdipoR upregulation is through correlation analysis with changes in other variables. Some studies have shown changes in serum adiponectin levels, AdipoR1 and AdipoR2 expression levels to coordinate (Bluher, Williams et al. 2007, Kim, Maachi et al. 2006, O'Leary, Jorett et al. 2007, Punyadeera, Zorenc et al. 2005), but this is not a universal finding (Christiansen, Paulsen et al. 2010, Kim, Maachi et al. 2006, Punyadeera, Zorenc et al. 2005). More consistent across these studies is the link with changes in insulin sensitivity and we have demonstrated this using posthoc analysis of our Early ACTID data. Myokines, of which a prototypical example is IL-6, are cytokines released by contracting skeletal muscle fibres and thus increased with exercise (Petersen, Pedersen 2005). They have endocrine effects on fuel metabolism and inflammatory cytokine profile and thus offer a possible molecular explanation.

The strengths of these studies are their controlled design and blinding during acquisition of AdipoR expression data. However there are a number of weaknesses.

The differences in BMI between SM and SV arms in the exercise intensity study could be a source of bias in the interpretation of our results. Statistical adjustments could not be done because of the relatively small sample size. Secondly, a significant number of subjects were primarily excluded in the Early ACTID because of genomic DNA contamination of the samples. However the baseline characteristics of subjects from both groups remained similar. Also, serum adiponectin levels from the Early ACTID were unavailable for analysis at the time of writing. I will arrange an addendum analysis as soon as I am in receipt of these from our collaborators. Lastly, we did not perform an internal validation assay of 18S as a housekeeping gene on PBMC for exercise studies, nor did we use multiple internal controls for adjustment. Whilst ribosomal RNA is an accepted normalising gene for rt-qPCR of PBMC (Bas, Forsberg et al. 2004) and has been used in some exercise studies (Richardson, Wagner et al. 2000, Kim, Cross et al. 2005), its expression on skeletal muscle fibre following exercise can increase (Jemiolo, Trappe 2004), thereby introduce a bias toward elevation when used as loading control for AdipoR expression level. In defence, there was relative concordance in the fold change values between mRNA of whole PBMC and lymphocytic (accounting for >95% of PBMC) AdipoR expression by FACS from the second study. We recognise the reported changes for monocytes could be strengthened by confirmation at the mRNA level following CD14+ sorting.

In conclusion, these studies confirm that exercise upregulates AdipoR expression on PBMC. The positive effects seen in patients with LADA suggest applicability to the setting of pre-T1D. Here, increased signalling may augment the putative anti-inflammatory functions of adiponectin. Our working hypothesis is that this is beneficial in the containment of islet autoimmunity and prevention of disease.

6.6 Summary of findings

We studied the effect of lifestyle intervention on AdipoR expression of PBMC via three independent, investigator-blinded controlled trials. In the Early ACTID, diabetic subjects in the intensive arm had a significant upregulation of both AdipoR1 & AdipoR2 gene expression, and these were accompanied by increases in VO_2 max, fasting glucose, as well as improvements in insulin sensitivity. Both exercise and dietary restriction are likely to be important components in mediating this change. Overeating for just 1 week can significantly downregulate AdipoR1 expression at both gene expression and protein levels, whilst intensive aerobic exercise in parallel can offset, at least in part, the decreased expression on monocytes. In the final study, a structured program employing moderate intensity exercise was more effective in reducing central obesity and fasting insulin, and increasing AdipoR1 expression on monocytes. Taken together, these studies demonstrate an increase in AdipoR expression can be achieved through lifestyle intervention, although its impact varies according to treatment group, duration, exercise intensity and degree of weight loss.

7. EFFECT OF ADIPONECTIN ON PBMC PROLIFERATION AND DC FUNCTION

7.1 Introduction

As proof of concept that adiponectin can modulate autoimmune diabetes, in this chapter we present studies describing effects of adiponectin on the function of cells involved in T-cell mediated immunity.

7.2 Optimisation of serum free (SF) conditions for testing effect of adiponectin on immune function

We developed a serum free (SF) culture system for assessing T cell proliferation to mitogens and antigens, as well as the transformation of DC from monocytes. FCS contains biologically active adiponectin (Wang, Lu et al. 2004). Given its high concentration in serum, the standard practice of 10% supplementation would result in the presence of adiponectin at 1-2 ug ml^{-1} in the complete media of control experiments. In addition, its constituents are largely unknown and the presentation of bystander antigens in FCS during DC maturation may affect interpretation of their antigen-specific effects on T cells (Lutz, Rößner 2008). There can also be batch-to-batch differences in FCS contributing to significant interculture variations (Witte, Kincade et al. 1986). Our methodology therefore has benefits in standardising culture conditions, and allows testing of exogenous adiponectin at physiological doses.

After verifying that the SF media contain no significant adiponectin as per manufacturer's disclosure (fig 7.1), we compared its efficacy in supporting proliferation of PBMC with serum based complete media. To ensure specificity in the enumeration of proliferating T cells to tetanus toxoid (TTC), we gated for cells that were PI negative, CD3 positive, CFSE dim, and increasing in CD25 expression (IL-2 receptor) and forward scatter in successive generations (fig 7.2). Derived from ratio CFSE dim cells under stimulation against unstimulated control, the stimulation indices (SI) of PBMC cultured in the two media with PHA (fig 7.3), PPD and TTC (fig 7.4) were similar. We confirmed that the level of proliferation in SF media was optimal by demonstrating no further increase in SI upon addition of 1% FCS (fig 7.5).

A B C D E F G H I J K
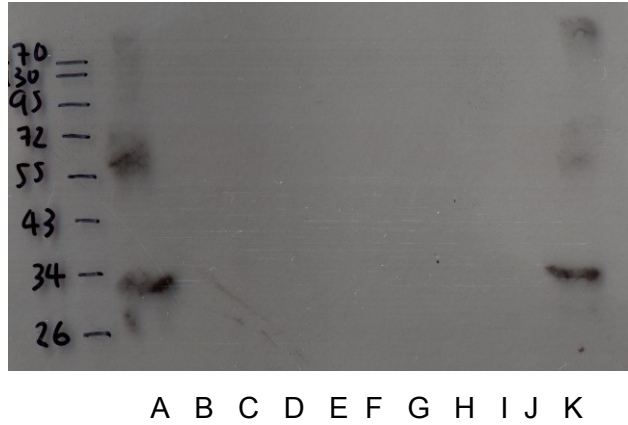
Immunblot for adiponectin in cellgro SCGM (predicted Mr = 30kDa) in cellgro SCGM media. A: recombinant adiponectin at 0.01ug. B to J, 1 in 2 serial dilutions of cellgro media. K: human serum (diluted 1 in 100 in PBS).
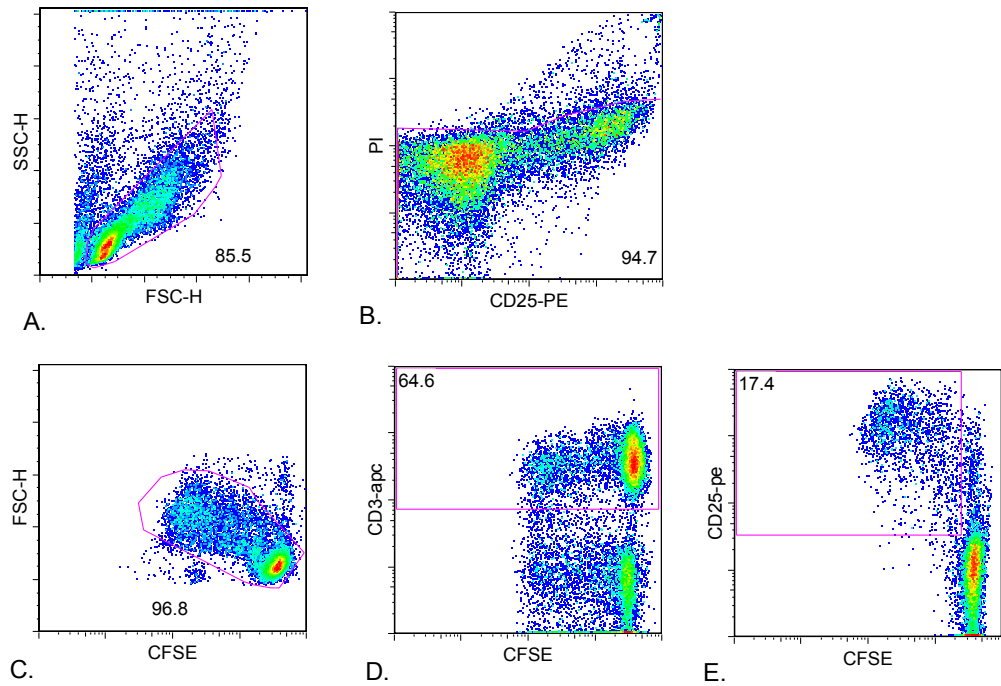
Figure 7.2: Example gating for enumerating proliferating T cells to TTC. This population is first gated according to scatter (A) and propidium iodide staining (B), followed by the simultaneous gain in forward scatter and loss in CFSE fluorescence (C). Proliferating T cells are then identified by CD3 and CD25 (IL-2 receptor) staining. Numbers denote percentage frequencies of gated cells.
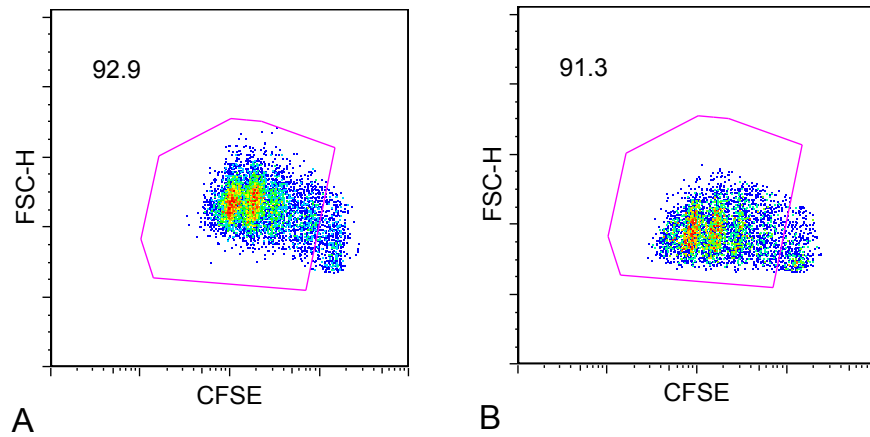
Figure 7.3: comparison of cellgro SCGM (A) vs serum based system (B) (RPMI + 10% human AB- serum) in whole PBMC culture stimulated by PHA. Gate denotes percentage of proliferating cells.
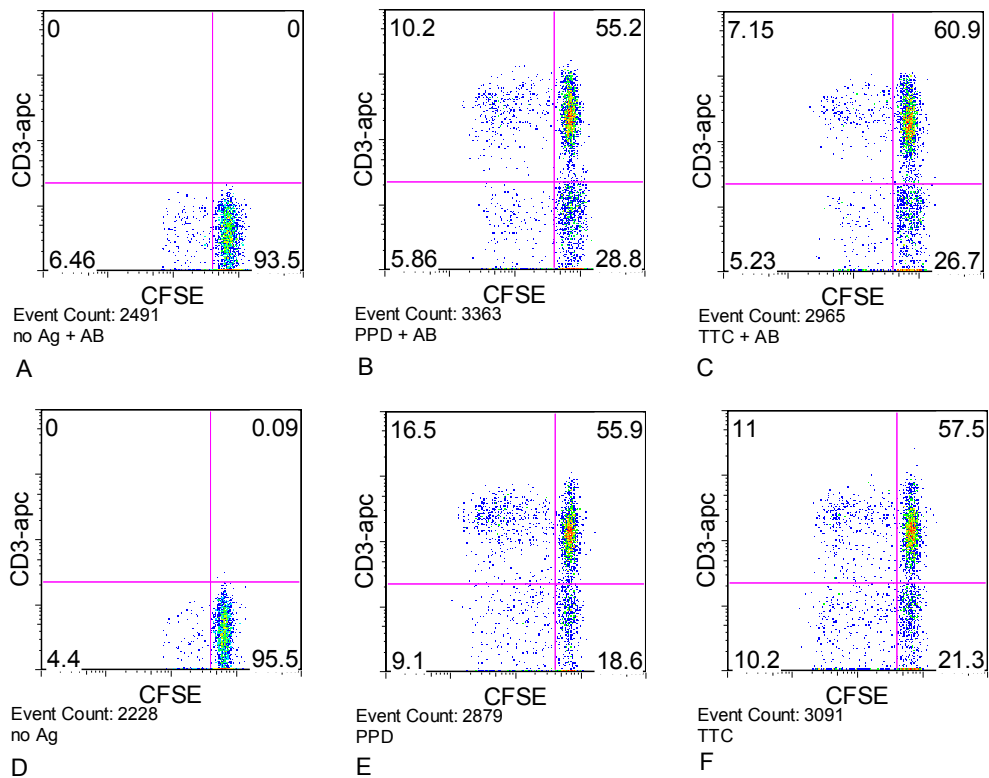
Figure 7.4: comparison of cellgro SCGM (D,E,F) vs serum based media (A,B,C) in whole PBMC cultures stimulated by the antigens purified protein derivative (PPD) (B&E) and TTC (C&E). Quadrant gates were set against undivided cells and APC isotype control (A&D), such that cells in top left quadrant are proliferating T cells proliferating. SI of PBMC for PPD and TT in serum based media are 2.5 ((10.2 + 5.86)/6.46) and 1.9; corresponding values in SF media are 5.8 and 4.8.
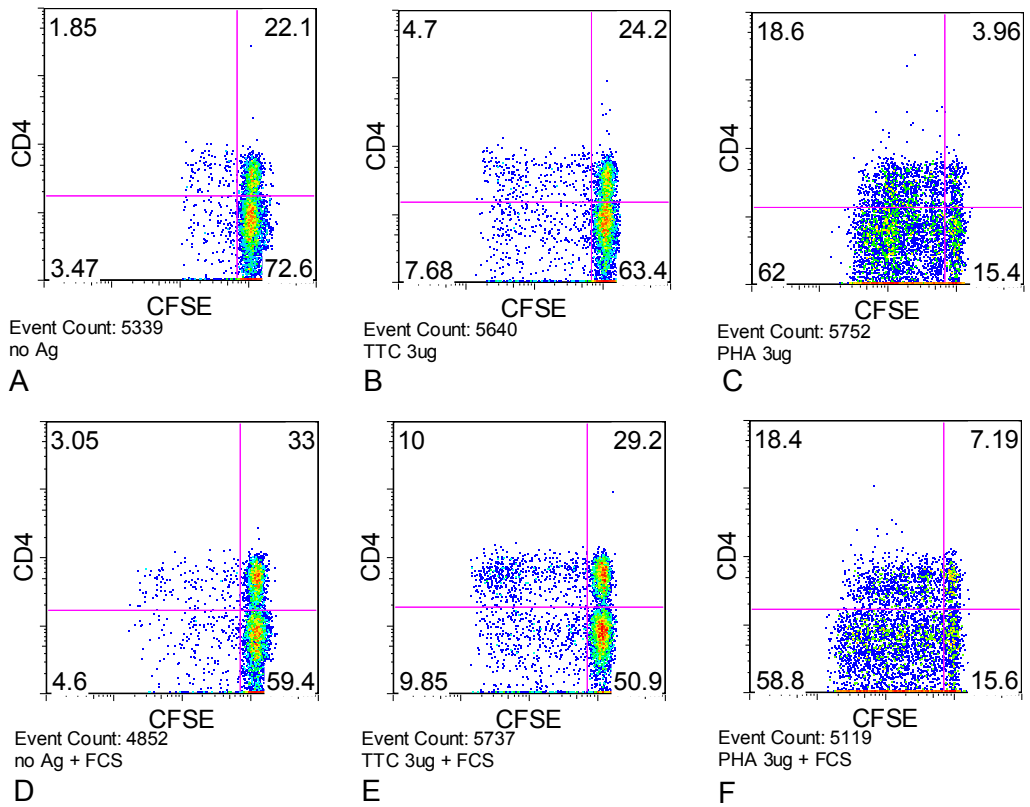
Figure 7.5: Effect of adding 1% FCS to cellgro SCGM in whole PBMC cultures. (A-C) cellgro media alone (D-F) addition of 1% FCS. (A,D) unstimulated (B,E) tetanus toxoid at 3ug/ml (C&F) PHA at 3 ug/ml. SI in cellgro SCGM alone, TTC 2.32 ((4.7+7.68)/(1.85+3.47)), PHA 15.2; addition of 1% FCS, TTC 2.6, PHA 10.1.
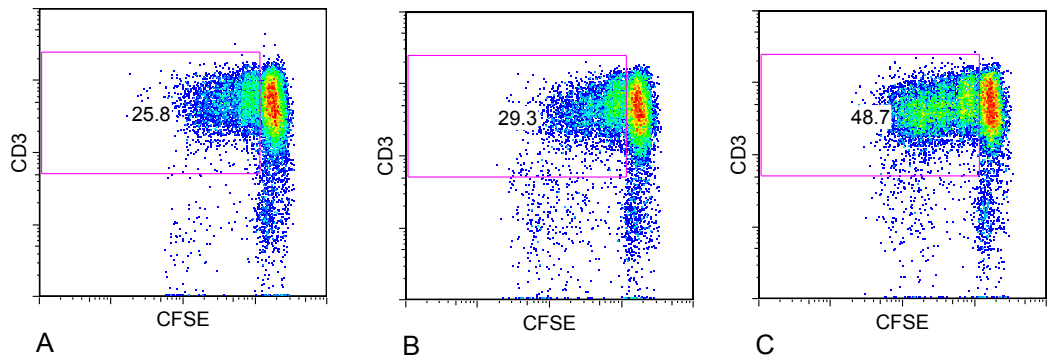
Figure 7.6: Titrating PHA dose in for PBMC stimulation in cellgro SCGM. (A) 3ug/ml (B) 5ug/ml (C) 10ug/ml. Gate denotes percentage of CD3+ cells that are CFSEdim after 3 days of culture.

Further optimisations were performed regarding the dose of mitogen stimulation and the duration of TTC culture. We found PHA at 3ug ml^{-1} to be the lowest dose producing consistent T cell proliferation at 3 days (fig 7.6). It had previously been reported that SI to TTC in a CFSE proliferation assay in serum based culture was maximal on day 10 (Mannering, Morris et al. 2003). In our system we found that SI were comparable between days 7 & 10 (fig 7.7).

Commercially available adiponectin had been reported to contain significant endotoxin contamination (Turner, Smolinska et al. 2009), in the order of 2-5%. This may be attributable to its binding avidity to LPS (Peake, Shen et al. 2006). We tested for this possibility with our batch of adiponectin by culturing with CFSE labelled PBMC (fig 7.8) and found no significant increase in SI compared to background. Spiking SF cultures with LPS at 0.1ug ml^{-1} (equivalent to a 1% contamination of 10ug adiponectin dose), produced marked proliferation of PBMC at 3 days and this response was ameliorated by the LPS antagonist polymyxin B.

Lastly, we confirmed monocyte derived DC (mDC) could be effectively generated from CD14+ monocytes in SF media under the influence of GM-CSF and IL-4 (fig 7.9). The cells after 5 days of culture had an immature DC phenotype, with a modest increase in CD80 and CD86, and were positive for DC-SIGN but negative for the mature DC marker CD83. As will be demonstrated in a subsequent section, they are functionally effective in stimulating T cells in the presence of antigen (TTC) and anti-CD3 antibody (OKT3) (figs 7.21 & 7.22). Compared to DC generated in serum-based media, expression of the co-stimulatory ligands CD80, CD86, DC-SIGN and the antigen presenting molecule HLA-DR were similar (fig 7.10). However, the yield of CD1a positive DC in SF culture was lower (92 vs 65% n=3 p<0.05).

Figure 7.7: Changes in SI of whole PBMC to TTC with time. Gates represent percentage of CFSE -dim proliferating cells on day 7 (A) and day 10 (E). Respective background counts are shown in B & F. Stimulation index is the ratio of proliferating cells exposed by antigen relative to the unstimulated control. On day 7 this is 8.7 (11.7/1.35) and on day 10 this is 6.0 (21.8/3.61). On day 7 (C), approximately 70% (16.1/(16.1+6.88)) of proliferating cells are CD3+ T cells. D is the no antigen control for C.
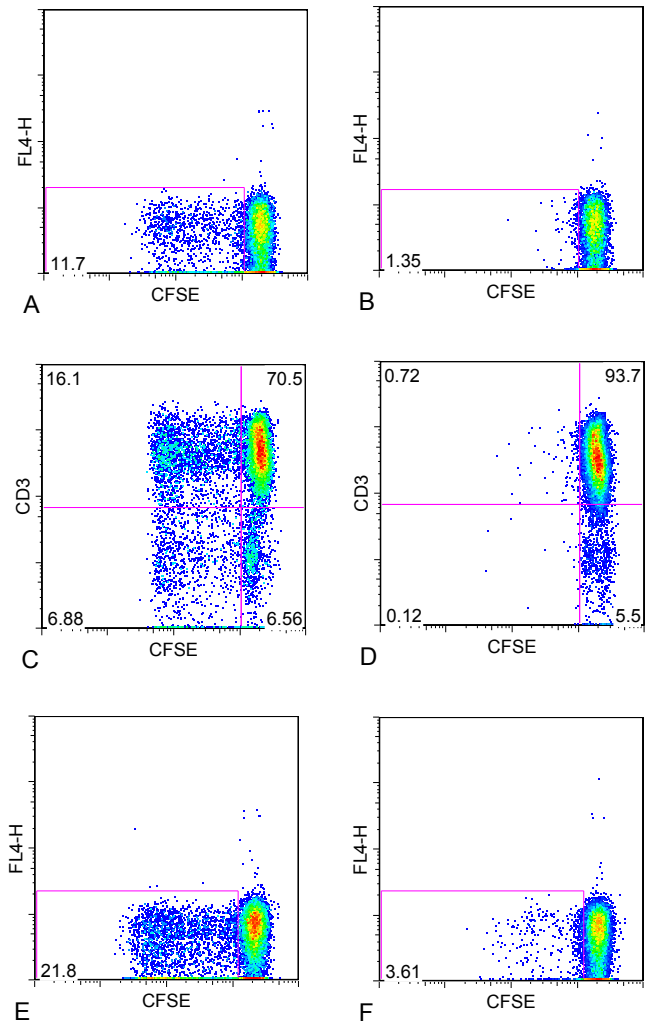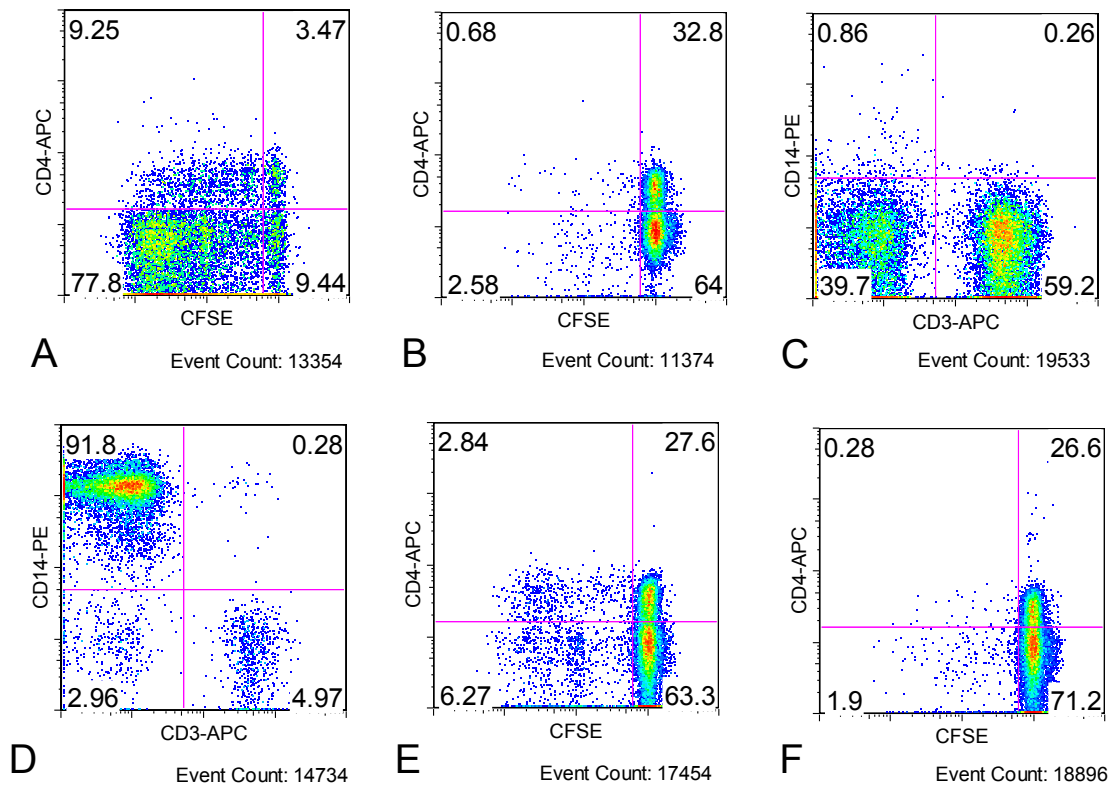
Figure 7.8 Testing for LPS contamination in the recombinant adiponectin used. A. To mimic 1% contamination of recombinant adiponectin, LPS at 0.1 ug ml^{-1} was added to CFSE labelled whole PBMC. On day 7, 88% of cells had undergone division (CFSE $_{low}$). In particular, up to 75% of CD4^{+} T cells had proliferated. **B.** CD4^{+} T cells did not proliferate in response to LPS if CD14+ monocytes were magnetically depleted (**C**) using anti-CD14 microbeads and MS columns. **D.** The positive fraction from the same sort after a single passage, showing purity of CD14^{+} cells to be at 91.8%. **E.** The proliferation of CD4^{+} T cells to LPS was antagonised by the addition of polymyxin B (10 ug ml^{-1}). **F.** Adiponectin at 10 ug ml^{-1} did not stimulate whole PBMC above background. Alternatives to a cell based system for LPS contamination include the in vitro Limulus amoebocyte lysate (LAL) assay and the in vivo rabbit pyrogen test. The LAL test is based on the coagulation of blood cells from the horseshoe crab upon LPS contact.

A

B

C

D

E

F
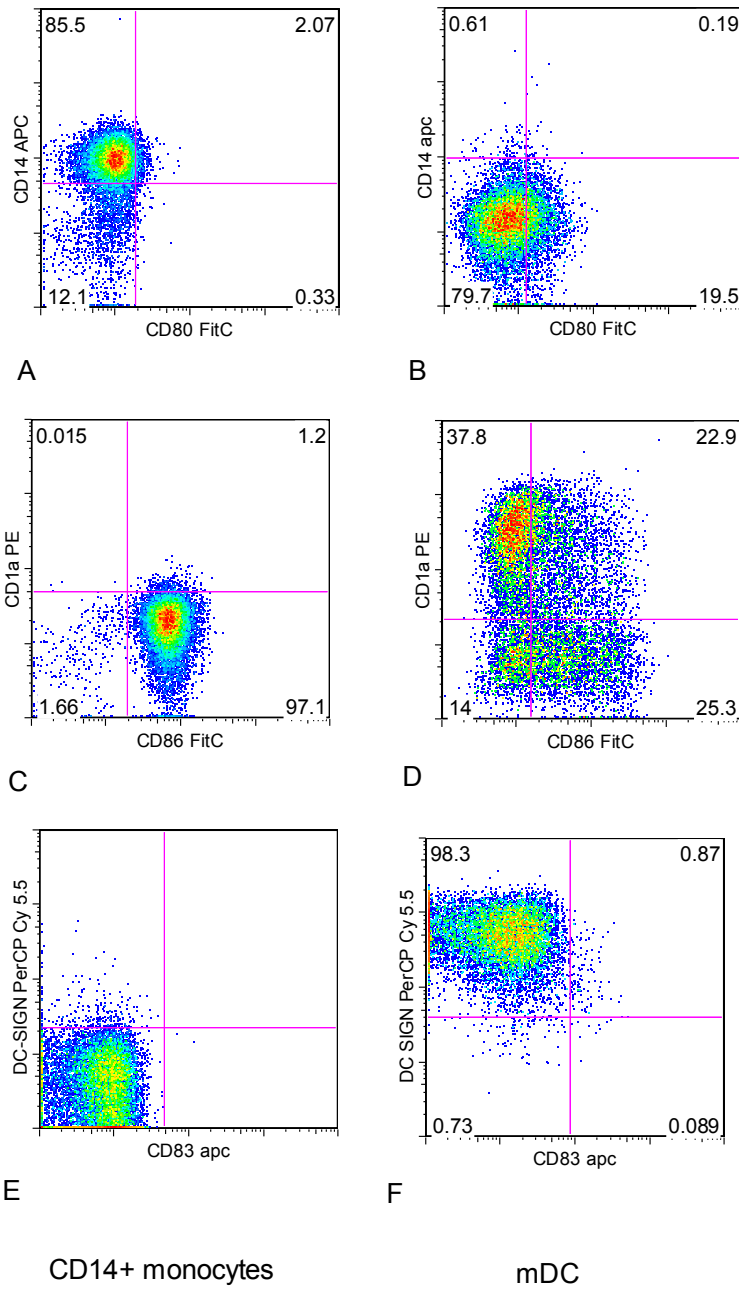
CD14+ monocytes   mDC

Figure 7.9: Transformation of CD14+ monocytes to mDC in Cellgro DC media. Positively selected CD14+ cells (A) with low CD1a (C) and DC SIGN expression (E) were cultured in the presence of GM-CSF and IL-4 for 5 days. The yield of mDC are deficient in CD14 expression (A). In keeping with their immature phenotype, they are also low in CD80 (A) and CD83 (F) expression, whilst acquiring DC-SIGN (F) and CD86 (D). About 60% of these are also CD1a positive.
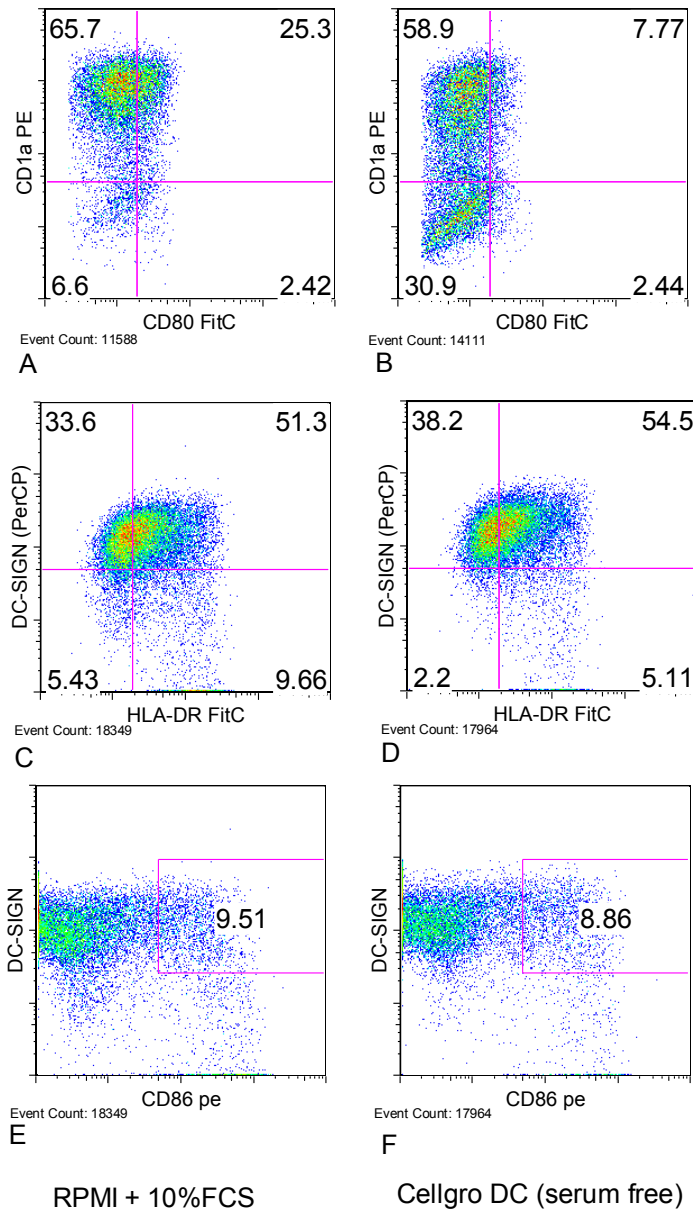
Figure 7.10. Comparison of mDC (day 6) grown in serum based media (A,C,E) (RPMI + 10% FCS) vs Cellgro DC (B,D,F).
Numbers denote percentage frequency of gated cells and quadrants defined against isotype control. mDC grown in cellgro DC are lower in their expression of CD80 and CD1a (A & B), whilst levels of DC SIGN, HLA-DR and CD86 are similar (C – F).

7.3 Adiponectin decreases T cell proliferation in healthy subjects

We modelled T cell immunity using PBMC responses to PHA and tetanus toxoid (TTC). TTC is a recall antigen to most young adults because of the UK immunisation schedule. We tested the effect of adiponectin by comparing SI with and without its addition (figs 7.11a & 7.11b) from the same subject. Over 8 healthy subjects, we found a physiological dose of adiponectin of 10ug ml^{-1} has a modest but significant effect in dampening PHA-stimulated T cell proliferation (mean change in SI = -9.5%, p < 0.05). More impressive is the reduction in SI by adiponectin in the TTC assay (figs 7.12a & 7.12b; mean suppression in SI = 46.9%, p < 0.01).

We next investigated the cellular mechanism of this reduced T cell stimulation. Adiponectin has been described to cause growth inhibition by promoting apoptosis in human cell lines (Yokota, Oritani et al. 2000, Dieudonne, Bussiere et al. 2006, Cong, Gasser et al. 2007, Konturek, Burnat et al. 2008, Bråkenhielm, Veitonmäki et al. 2004) We found that the proportion of cells staining for annexin V, a marker for early apoptosis, was significantly increased with adiponectin replacement (figs 7.13 & 7.14) in TTC and PHA stimulated cultures.

Our expression analysis of AdipoR had shown sparse distribution on resting and activated T cells. On this basis, we predicted the effect of adiponectin on dampening T cell immunity to be mediated via accessory cells. Tregs are potent suppressors of T cell proliferation. We found that the proportion of CD4^{+}CD25^{high} cells, which include the Treg subset, was increased with adiponectin replacement over 48 hours (fig 7.15), in a dose-dependent manner.
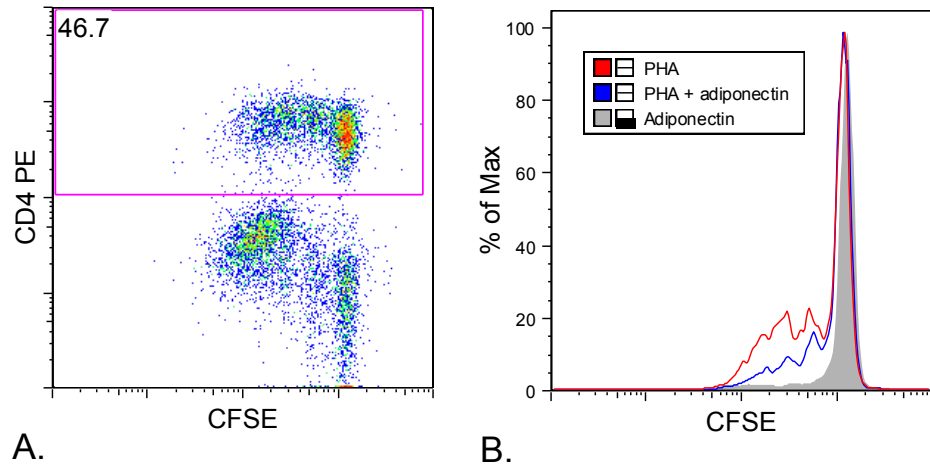
Figure 7.11a: Representative experiment showing reduction in proliferation of CD4 T cells (A) by adiponectin when whole PBMC are stimulated by PHA (B).
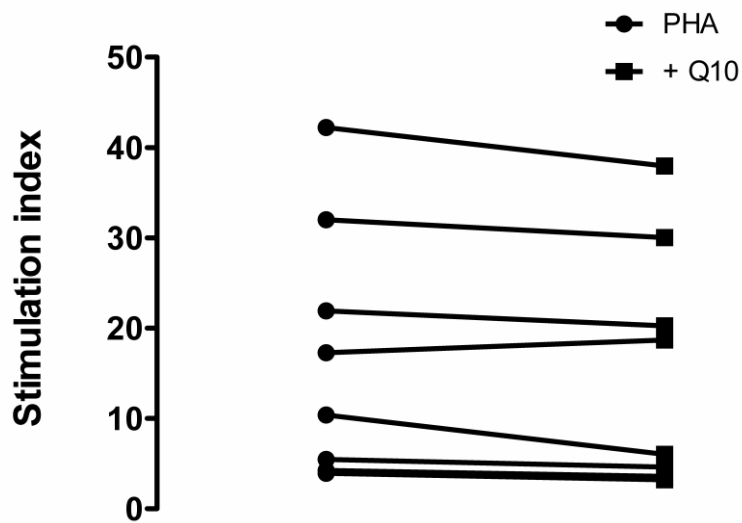
Figure 7.11b: Reduction in stimulation index of CD4+ T cells by adiponectin when whole PBMC are stimulated by PHA (3ug/ml) (n=8). Mean reduction = 9.5% p = 0.0476 by paired t-test.

Event Count: 7609
A. TTC

Event Count: 6949
B. TTC + Adiponectin
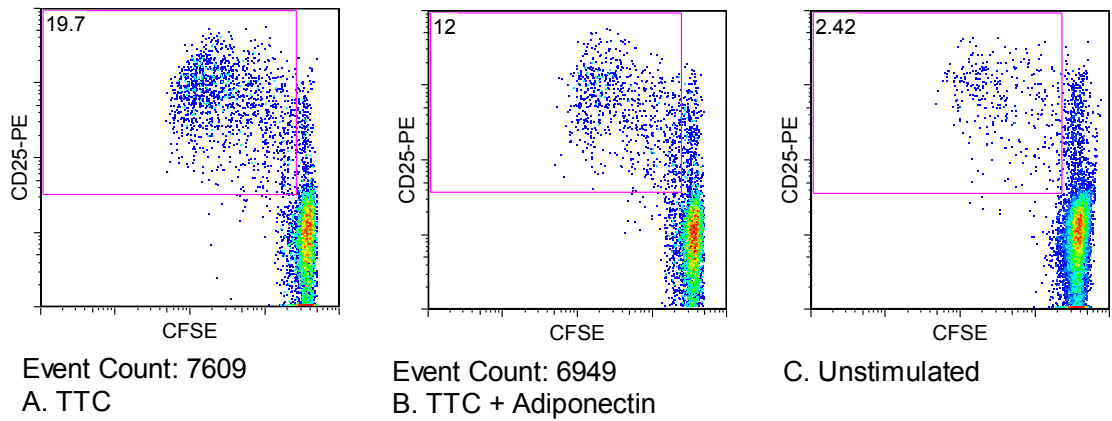
C. Unstimulated

Figure 7.12a: Representative experiment showing reduction in proliferation of CD3+ T cells (A) by adiponectin when whole PBMC are stimulated by tetanus toxoid (B). SI without adiponectin is 19.7/2.42 = 8.14; SI with adiponectin = 12/2.42 = 4.96.

Healthy Controls
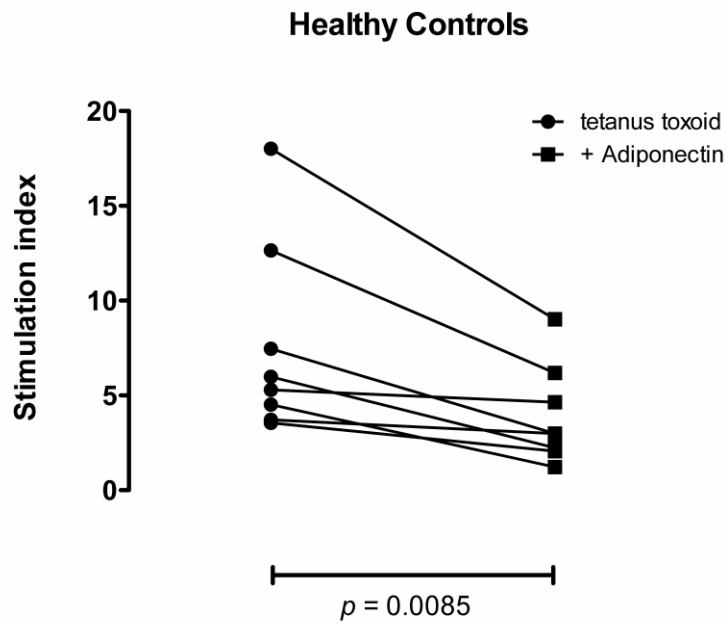
$p = 0.0085$

Figure 7.12b: Adiponectin suppresses proliferation of TTC stimulated CD3 T cells (n=8) in healthy controls . Mean suppression is 46.9% p = 0.0085, by two tailed paired t-test.
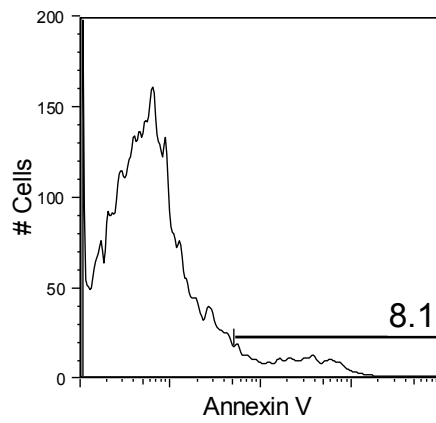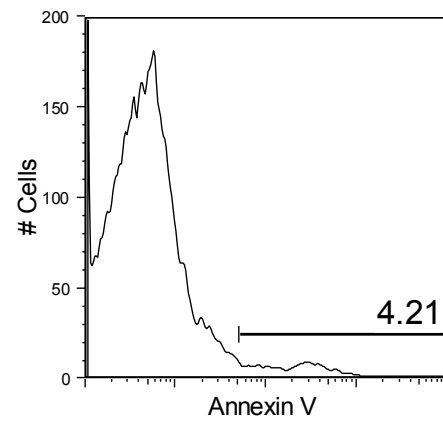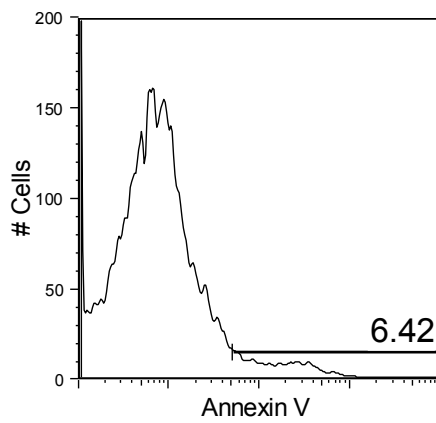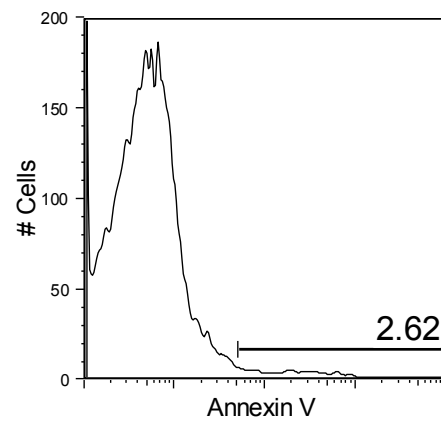
Figure 7.13: Adiponectin promotes apoptosis of PBMC during culture with TTC and PHA. Bars represent percentage cells staining positive for annexin V, relative to isotype. Cells staining positive for propidium iodide were excluded. Representative samples.
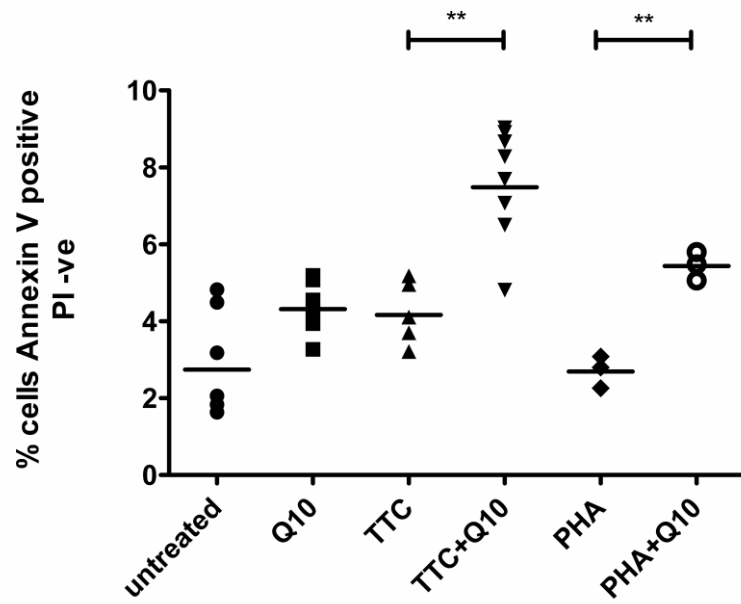
Figure 7.14: Adiponectin is pro-apoptotic. Whole PBMC stimulated by TTC and PHA had a higher proportion of annexin V positive, PI-ve cells when adiponectin in present.

 ** p <0.01 by Bonferroni's post-test correction for multiple testing.
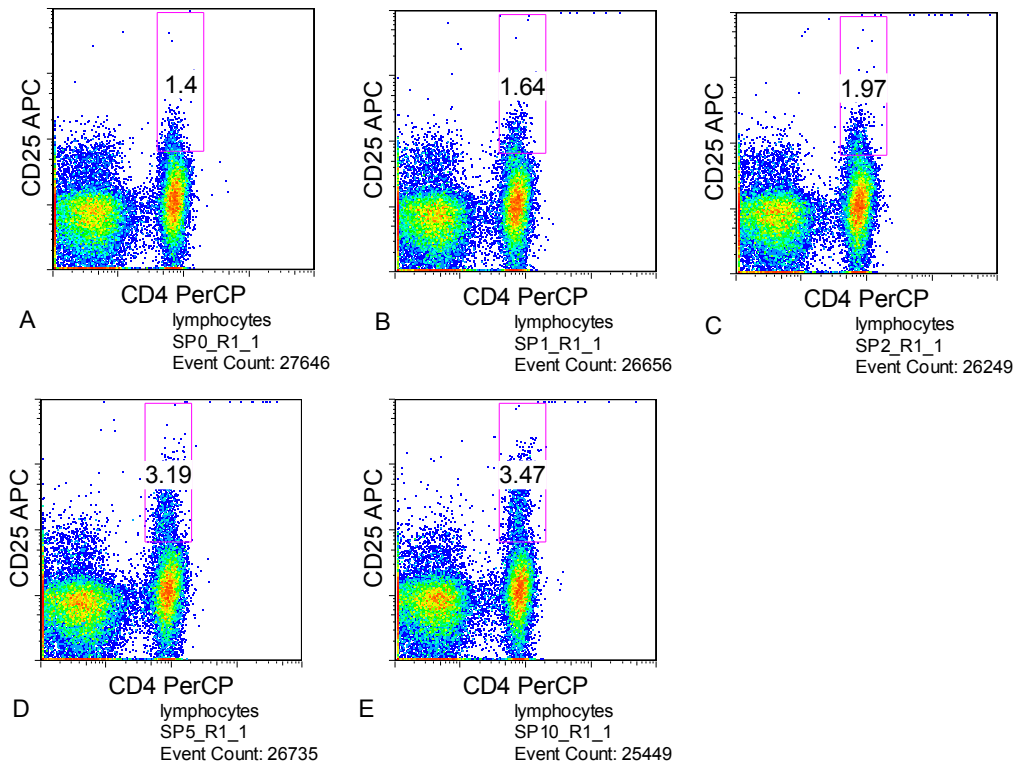
Figure 7.15: Adiponectin increases CD4⁺CD25ʰⁱᵍʰ cells. Whole PBMC were cultured in serum free media with adiponectin added back at 0 (A), 1(B), 2(C), 5(D), and 10ug/ml(E). CD4+CD25+ counts as a percentage of lymphocytes were assessed on day 2. (below) summary of experiments (8) with PBMC from 3 donors showing a significant positive correlation.

Pearson r	0.9681
95% confidence interval	0.5877 to 0.9980
P value (two-tailed)	0.0068
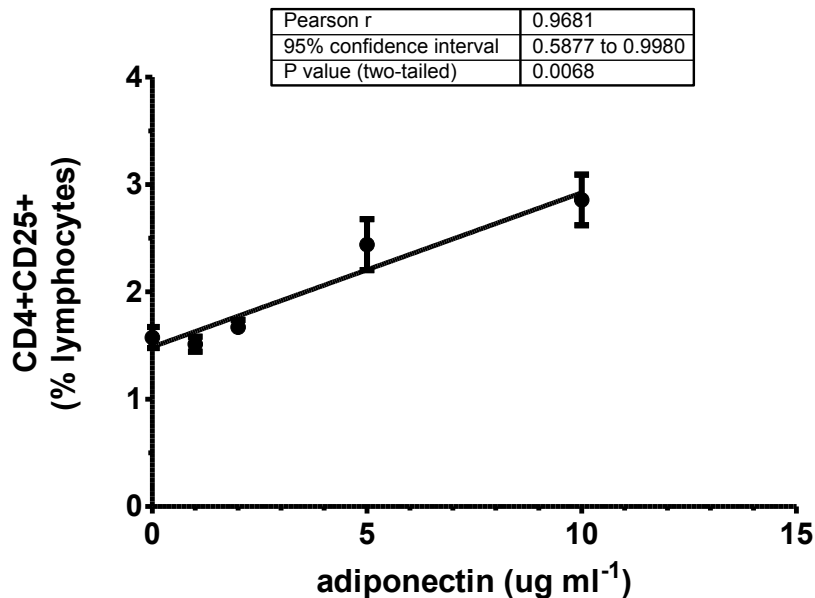
7.4   Adiponectin decreases dendritic cell function

Monocytes are also important in regulating the T cell responses studied. The induction of T cell proliferation by PHA in whole PBMC is monocyte-dependent. They also serve as APC for the antigen mediated T cell responses (Randolph, Jakubzick et al. 2008). Because monocytes express the highest level of AdipoR, we wanted to determine the effect of adiponectin on APC function. We chose the most potent monocyte derived APC, the DC.

7.4.1 Adiponectin decreases expression of CD86 on mDC

CD86 on APC is a ligand to CD28 on T cells and provides the critical secondary signal for T cell activation (Salomon, Bluestone 2001, Greenwald, Freeman et al. 2005). Adiponectin replacement at 10ug ml^{-1} to the SF generation of mDC resulted in a significant reduction of CD86 at both protein and mRNA levels by approximately 50-70% (fig 7.16). On FACS, the reduction of CD86 occurred in both CD1a positive and CD1a negative DC. Expression of HLA-DR, CD80, and DC-SIGN was similar between adiponectin treated DC and their control (Table 7.1 and figs 7.17, 7.26). The proportion of CD1a positive DC was also unchanged.

	Ratio MFI **Adiponectin DC: control DC**
CD1a	1.02 ± 0.21
CD86	0.40 ± 0.11**
CD80	1.00 ± 0.20
HLA-DR	1.09 ± 0.20

Table 7.1: MFI of DC surface markers of adiponectin treated DC relative to control in 10 normal weight healthy subjects. Ratio expressed as mean ± SD.
** $p < 0.0001$ by one sample two-tailed t-test against inferential mean of 1.0 (no change)

Event Count: 17950
Control

Event Count: 15455
+ adiponectin 10ug/ml
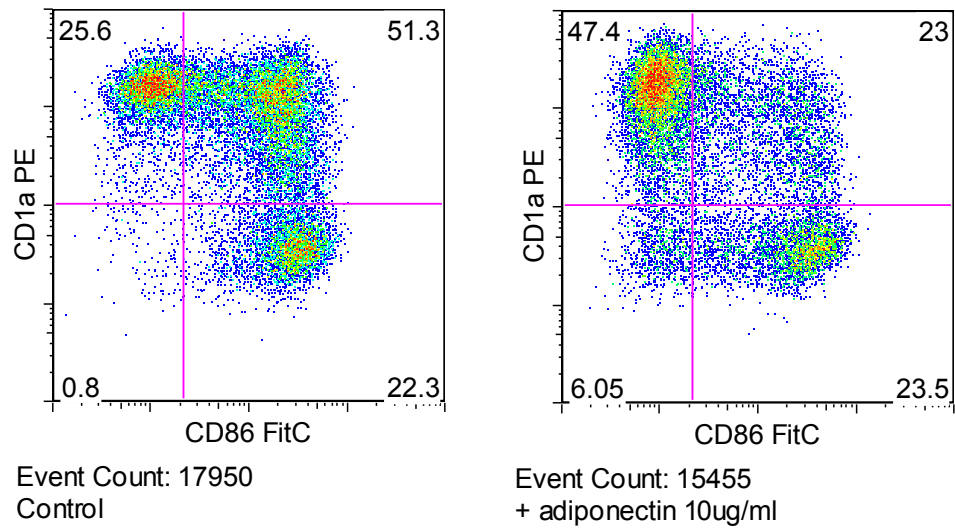
Figure 7.16: Adiponectin suppresses the upregulation of CD86 during transformation of monocytes to DC. This is demonostrated at the protein level by FACS (above) and at the RNA level by qPCR (below). Significant reduction in CD86 RNA levels in adiponectin treated mDC (n=5). Error bars represent SD of fold change, which is calculated by $\Delta\Delta$Ct method.
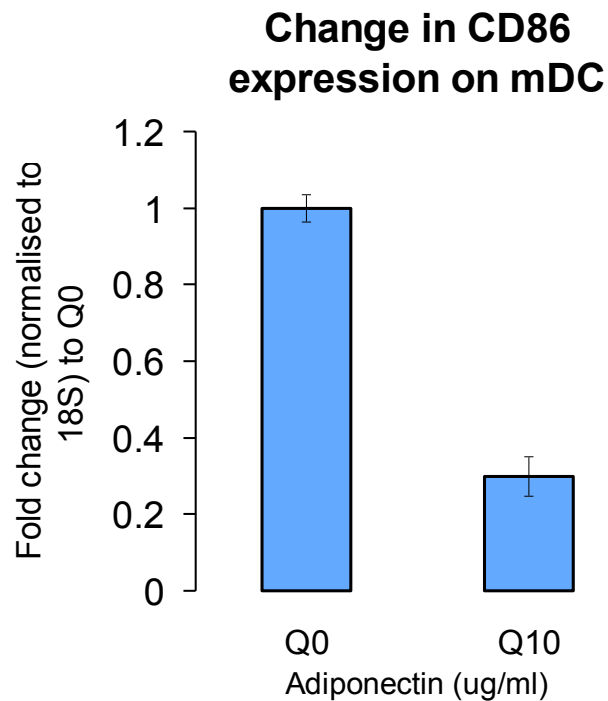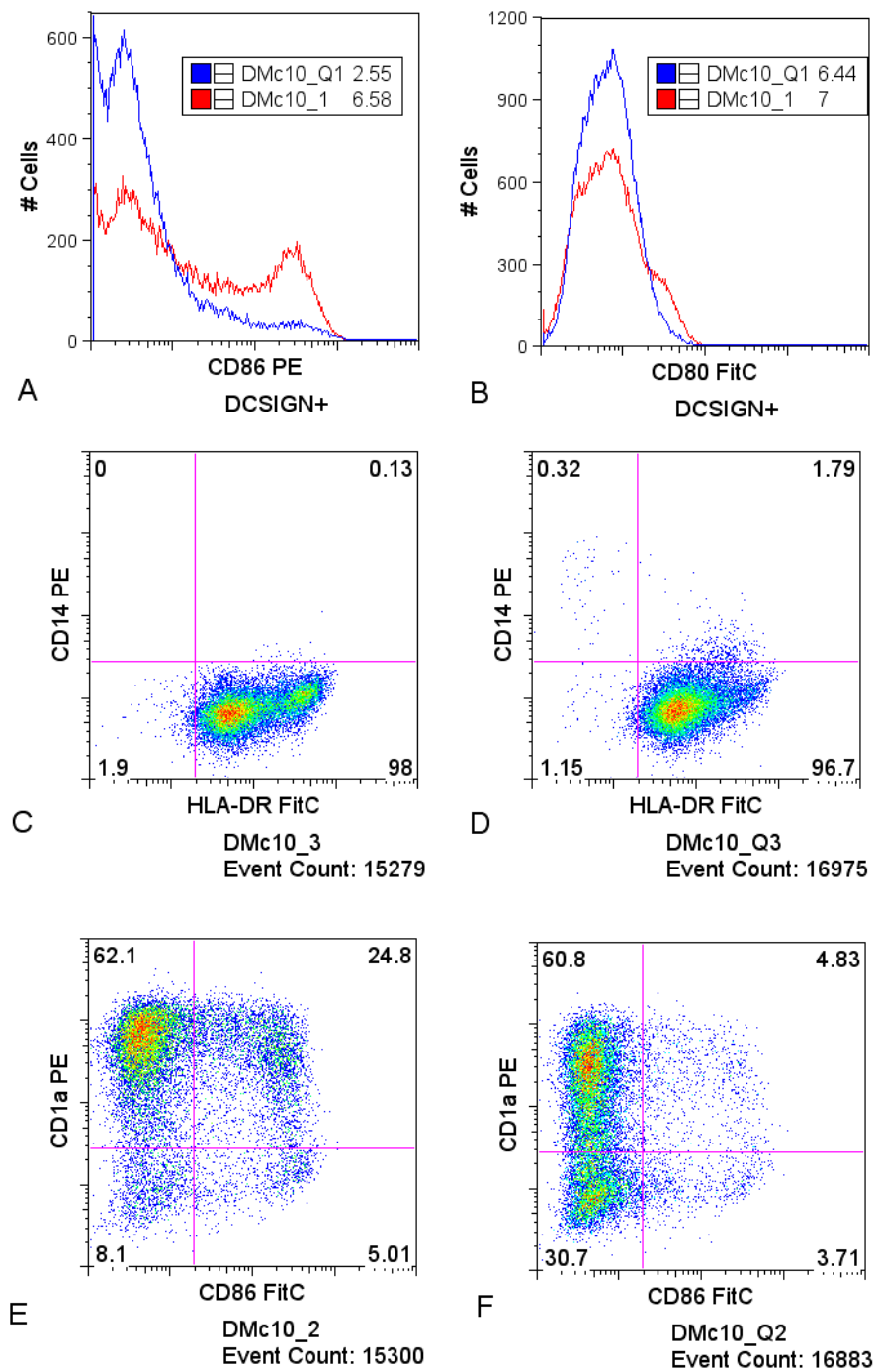
Figure 7.17: Effect of adiponectin on costimulatory molecules of DC. (A) A significant reduction in CD86 is seen DC SIGN positive DC (red – adiponectin treated DC, blue – control; numbers in legend denote CD86 MFI. In comparison, the change in CD80 (B) and HLA-DR (C-control; D- adiponectin-treated DC) is marginal. The reduction in CD86 expression is seen on both CD1a+ and CD1a- DC (E- control, F-adiponectin treated DC).

We followed the kinetics of the CD86 downregulation by adiponectin in time course experiments (fig 7.18, n=5). The ratio of CD86 MFI on CD1a positive cells of adiponectin treated DC to its negative control was 0.45 ± 0.09 at 0 hours. This was not significantly different to 6 hours (0.69 ± 0.06). However, this difference was completely abolished when adiponectin was added at 18 and 24 hours.

We discovered also that the inhibition of CD86 expression upon adiponectin replacement was dose-dependent and followed sigmoidal dose-response modelling (fig 7.19). It was overcome by the addition of LPS to DC, following transformation to mature DC phenotype (fig 7.20)

7.4.2   Adiponectin reduces mDC stimulated T cell proliferation

We next asked whether the inhibition of CD86 on DC by adiponectin translated to changes in stimulatory capacity. The synapsing of CD86 to CD28 and CTLA-4 on $CD4^+CD25^{high}$ Tregs is essential in the initiation maintenance of their immunoregulatory function (Salomon, Lenschow et al. 2000, Zheng, Manzotti et al. 2004). In our system however, this would complicate the interpretation of overall T cell proliferation as a measure of DC stimulation capacity. For this reason, we sorted responder cells according to the phenotype $CD4^+CD25^-$. We compared stimulatory capacity in two CFSE-based assays, using (1) autogeneic responder cells with TTC, and (2) allogeneic responder cells with OKT3 antibody. We found that adiponectin treatment of mDC reduced T cell proliferation by an average of 43.2% with TTC (three donors, see fig 7.21), and 39.4% with OKT3 (5 donors, fig 7.22).
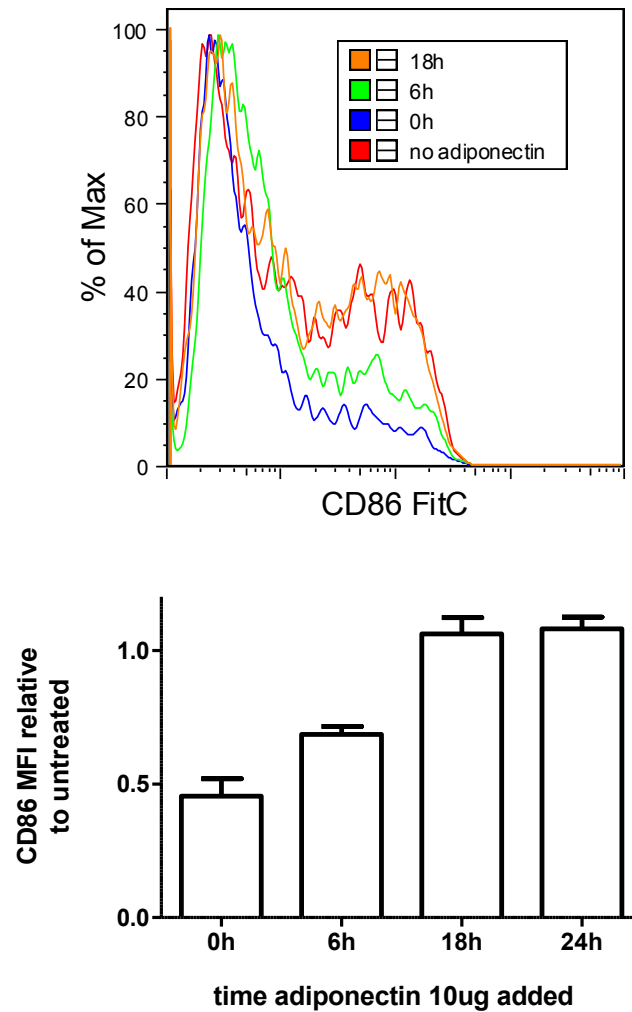
Figure 7.18: Time course of the CD86 suppression on DC by adiponectin. Adiponectin at 10ug/ml were added to cultures at 0, 6, 18 and 24h. (top) Histograms of CD86 expression on CD1a+ DC at the different timepoints. (bottom) Summary of 5 experiments demonstrating the effect of CD86 suppression occurred within 18h. CD86 MFI was standardised against untreated DC from the the same subject.
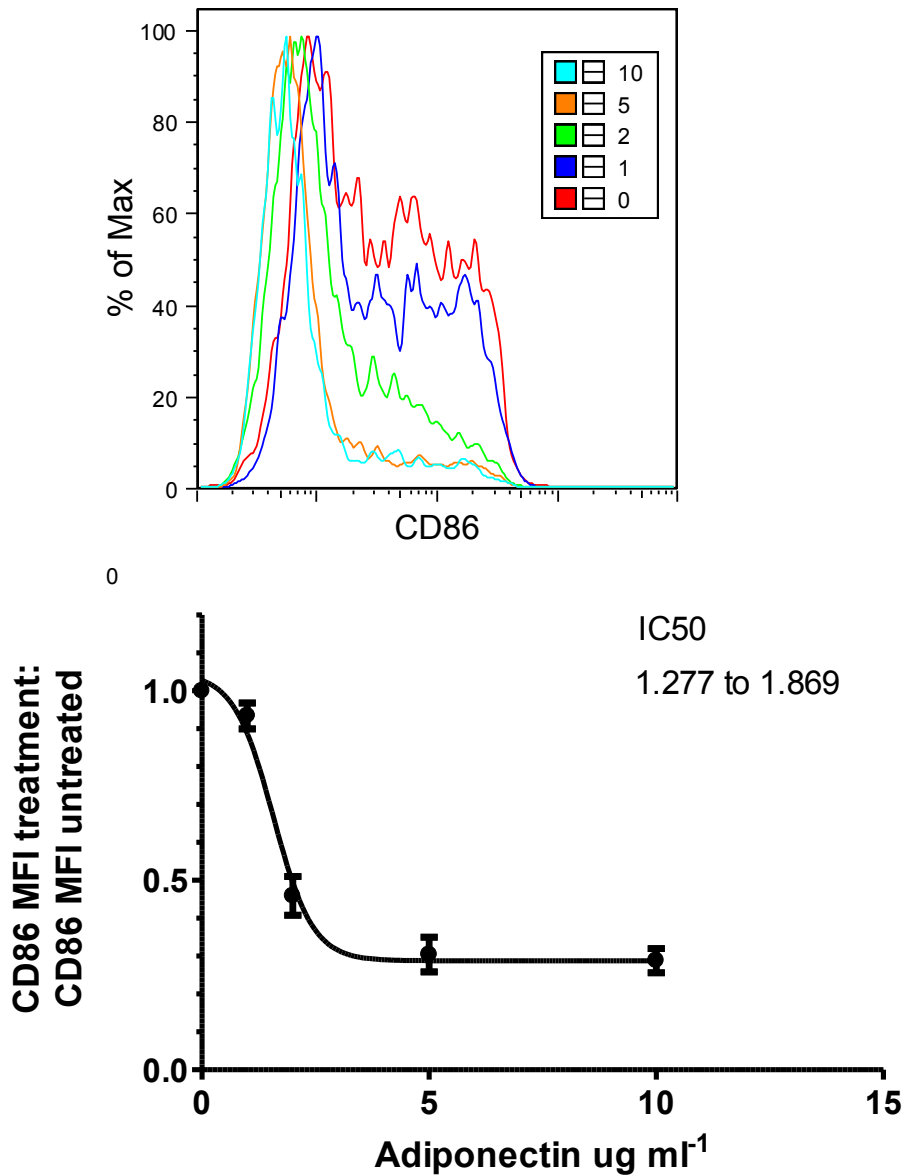
Figure 7.19: Reduction of CD86 expression on mDC by adiponectin is dose dependent. (top) Adiponectin at 0,1,2,5 & 10ug/ml was added at the beginning of culture. (bottom) Dose response curve derived from 3 independent experiments and fitted using standard log(inhibitor) vs response model*. IC50 (dose of adiponectin eliciting 50% inhibition) is shown at 95% CI.

*Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC50))) .
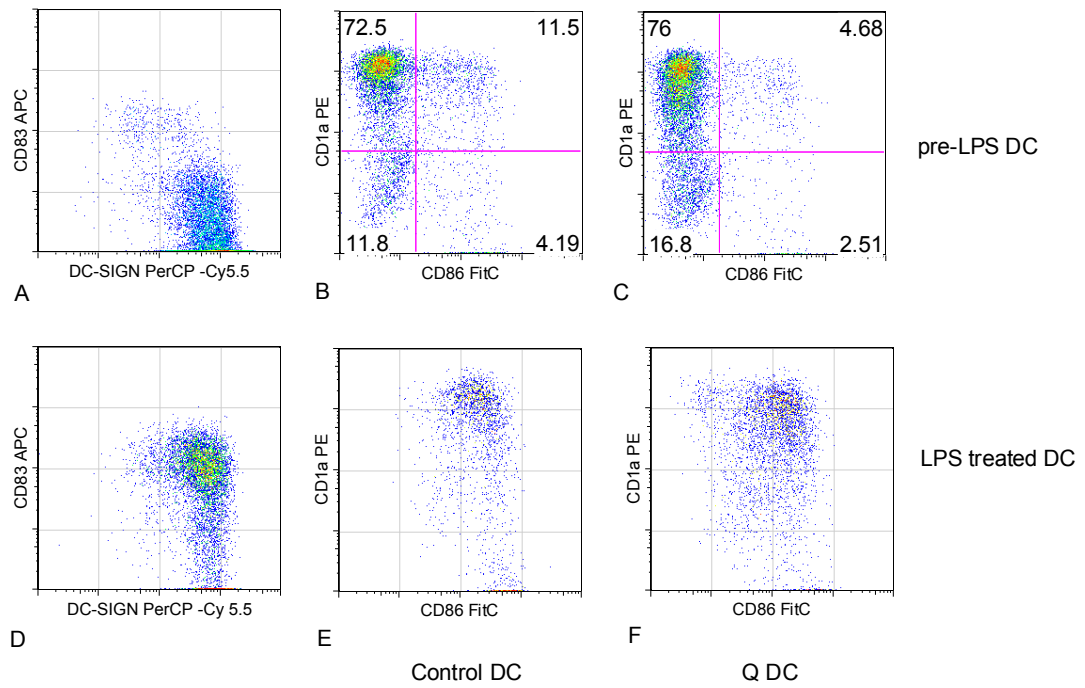
Figure 7.20: LPS overcomes inhibition of CD86 expression on DC by adiponectin.
CD83 is a marker of mature DC. mDC harvested on day 5 have an immature phenotype
(A). Q DC (C) have significantly lower CD86 expression compared to control DC (B). DC
harvested one day after addition of LPS are high on CD83 expression (D). Both control
DC (E) and Q DC (F) have increased and comparable expression of CD86.
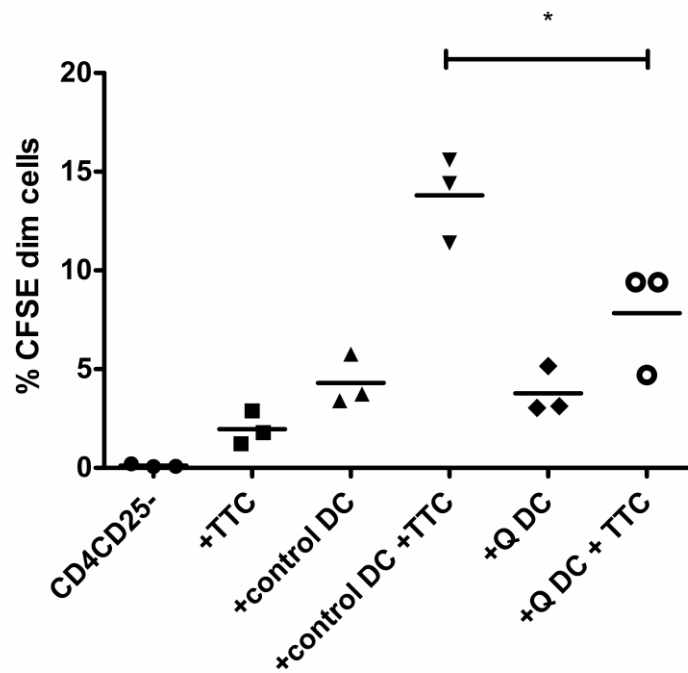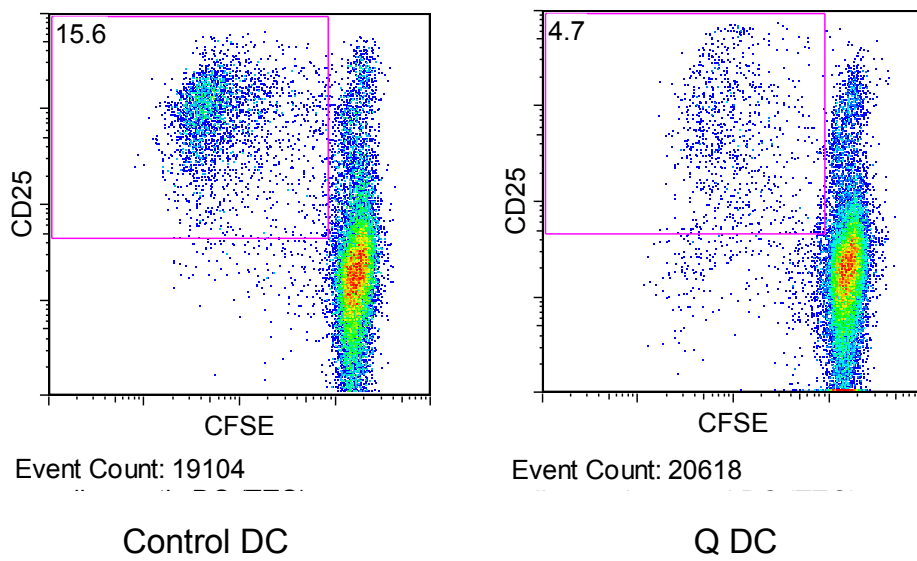
Figure 7.21: Adiponectin treated DC (Q DC) have reduced stimulatory capacity compared to DC grown in its absence (control DC). Both Q DC and control DC were washed twice in cold PBS after harvest and then co-cultured with effector CFSE+CD4CD25- T cells and tetanus toxoid (TTC). (top) representative example, gate represents frequency of CFSE dim, CD25+ cells.

* p < 0.05 by Bonferroni's Multiple Comparison Test.

7.5 Suppression of DC function by adiponectin is released in T1D

We then asked whether the effects of adiponectin on PBMC responses and DC function would be altered in T1D because of the reduction in AdipoR expression. We show this to be the case in a number of ways.

Firstly and most directly, there is a trend for reduced mDC stimulation of T cell proliferation in T1D (n=5), compared to non-diabetic controls (n=5) (figs 7.22 & 7.23) (HC = 39.4%, T1D = 9.6% p = 0.0556).

Secondly the reduction of PBMC proliferation to TTC by adiponectin is significantly reduced in T1D (fig 7.24) compared to HC (mean reduction in SI, HC = 47 (n=8) vs T1D = 26% (n=4) p<0.05).

Furthermore, the dose response curve of CD86 inhibition on mDC with adiponectin replacement in T1D is consistent with the effect of reduced AdipoR expression and not changes in agonist-receptor kinetics (fig 7.25). Maximal inhibition of CD86 was reduced (T1D: -61% HC: -71% p<0.05), whilst no significant change in IC_{50} was seen (95% CI: T1D = 0.6 - 2.1; HC =1.3-1.9).

In attributing the release of adiponectin-mediated suppression of APC driven T cell proliferation in T1D as the result of reduced AdipoR, we compared the degree of CD86 inhibition on mDC by adiponectin to the expression of AdipoR on precursor monocytes in healthy and T1D individuals. The same dose of adiponectin replacement at 10ug/ml was used at the start of culture. We justified not adding adiponectin in the re-feeding media on day 2 from findings in our time course experiment. We recruited T1D subjects (n=11) whose clinical features predicted low monocytic AdipoR expression.

Consequently, they had features of insulin resistance (mean HbA1C 8.5 ± 0.8, presence of hypertension and WHR 0.85 ± 0.08), presence of vascular complications (8 / 11), and were older than our healthy controls (HC – mean age: 28 ± 4; T1D: 35 ± 7).

Our T1D subjects had lower AdipoR1 expression approaching significance (MFI AdipoR1 T1D: 35.5 ± 5.3; HC 62.8 ± 13.0, p = 0.0579) but AdipoR2 expression levels were not significantly different (T1D = 33.4 ± 4.0, HC 51.7 ± 12.3 p = 0.190) (fig 7.26). Similar yields of CD1a positive DC were achieved at the end of culture in both groups, with or without adiponectin (HC 39.3 ± 5.0%, T1D 43.8 ± 5.5%). Importantly, we found that the degree of CD86 inhibition on mDC in T1D was significantly reduced (HC = -60.0 ± 3.0%; T1D = -40.8 ± 5.8%, p = 0.011), whilst expression of HLA-DR and CD80 was unchanged (figs 7.27a & 7.27b).

Consistent with our hypothesis that AdipoR signalling mediates CD86 expression on mDC (fig 7.28), AdipoR1 expression on CD14+ precursors negatively correlated with the degree of CD86 inhibition with adiponectin replacement (r = -0.53, p = 0.013). The correlation between AdipoR2 expression with adiponectin-mediated CD86 inhibition was not significant (r=-0.411 p=0.064). Changes in HLA-DR and CD80 expression did not appear to be associated with AdipoR expression (figure 7.29). We had previously shown AdipoR1 & AdipoR2 expression was unaffected by adiponectin replacement in SF media, thus excluding its possible confounding influence on our results (fig 5.11).
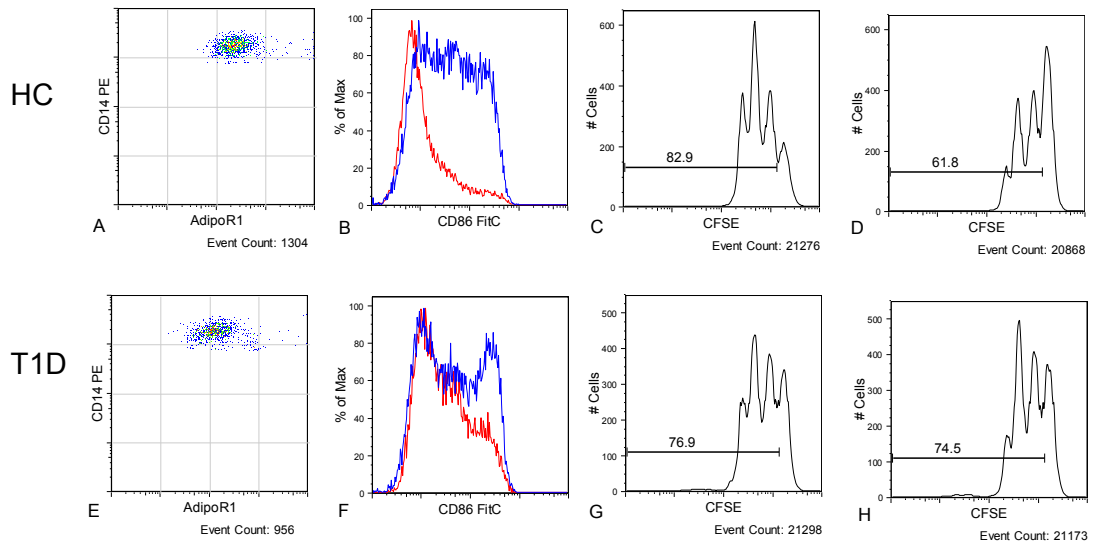
Figure 7.22: The reduced stimulatory capacity of Q DC is related to the degree of CD86 suppression. Subject 1 (A) had higher AdipoR1 expression on CD14 precursor cells compared to subject 2 (E), who has T1D. The suppression of CD86 by the same 10ug ml^{-1} adiponectin is greater (Q DC –red histogram, control DC- blue) on subject 1 (C) compared to 2 (F). The stimulatory capacity of the DC were tested in co-cultures with CFSE+CD4+CD25- effector cells. Bar indicates % of divided cells, In HC, Q DC stimulated fewer cells to proliferate (D) compared to control DC (C). In contrast, the difference between Q DC (H) versus control DC (G) in the T1D subject is marginal. (Bottom) summary of replicates for these two subjects. The difference in stimulatory capacity between control DC and Q DC, as a percentage, is 29.8 for subject 1, and 3.8% for subject 2.

* p = 0.0105 by Bonferroni's post-test
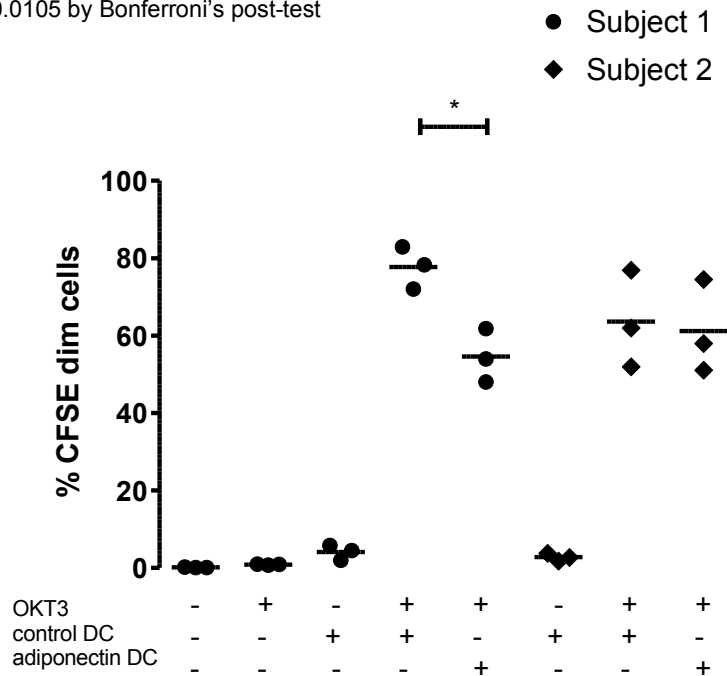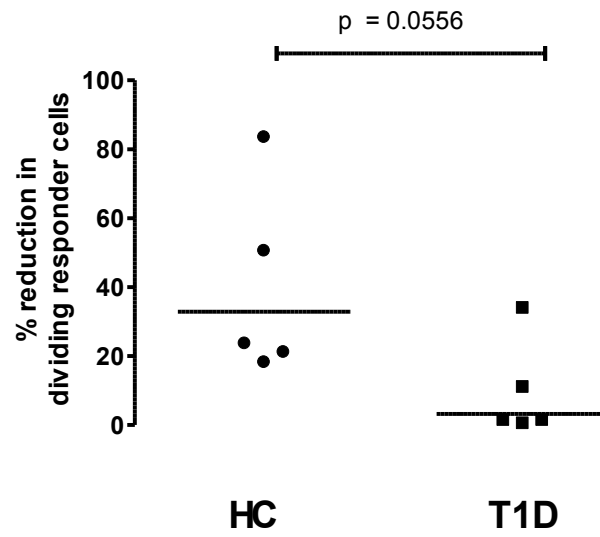
Figure 7.23: Stimulatory capacity of Q DC in T1D (Above) The difference in number of CFSEdim cells stimulated by Q DC and their paired unntreated control is expressed as percentage reduction. The non-parametric Mann Whitney U test was used for significance testing (Below) Example CFSE dilution plots from 4 more subjects. Blue histogram denotes control DC, grey filled histogram denotes Q DC. Event counts shown

A

B

C
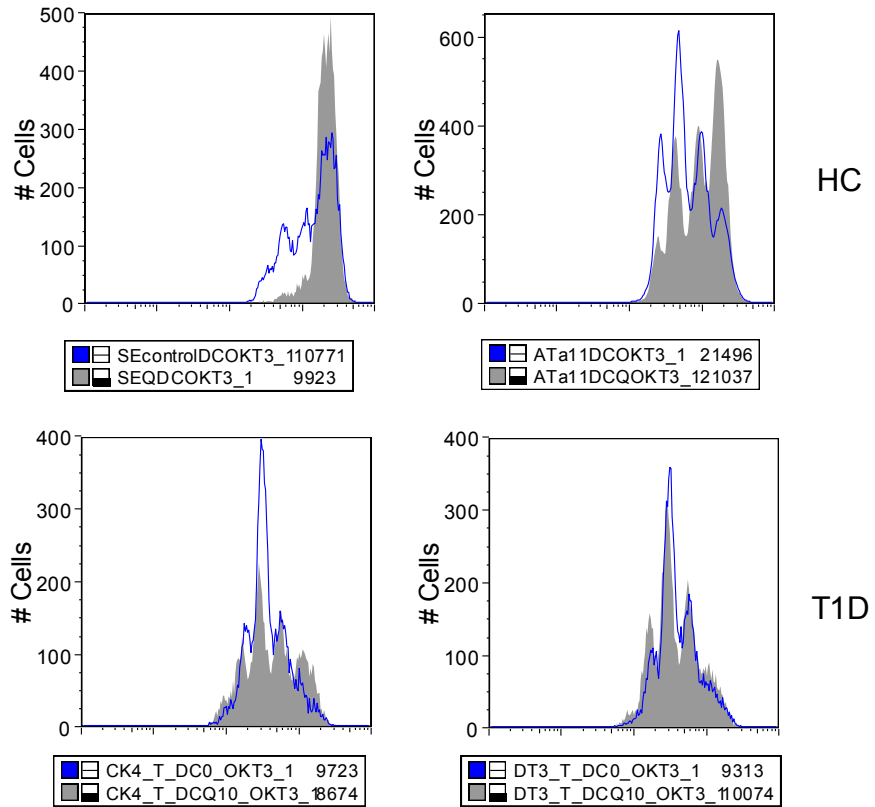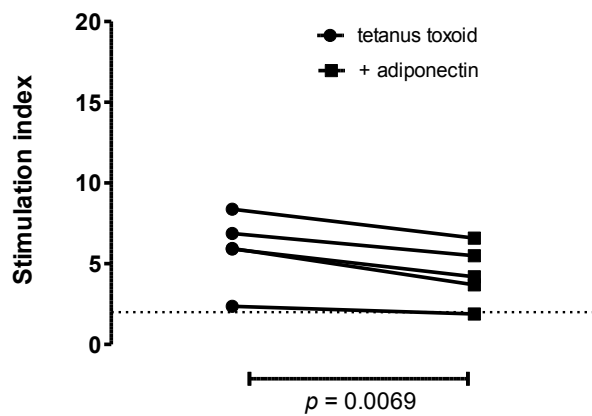
Figure 7.24: Reduced suppression of TTC driven T cell proliferation by adiponectin in T1D. (A) Similar to HC, there is reduced SI when adiponectin is replaced in serum free media at 10 ug/ml (Q10). (B) Th mean reduction in SI in T1D 26%, compared to 47% in HC p = 0.0464. (C) Data from a T1D subject (depicted in the bottom line in A). An example from a HC is shown in 7.12a
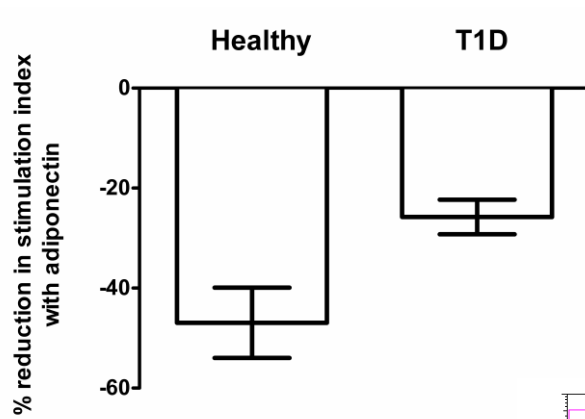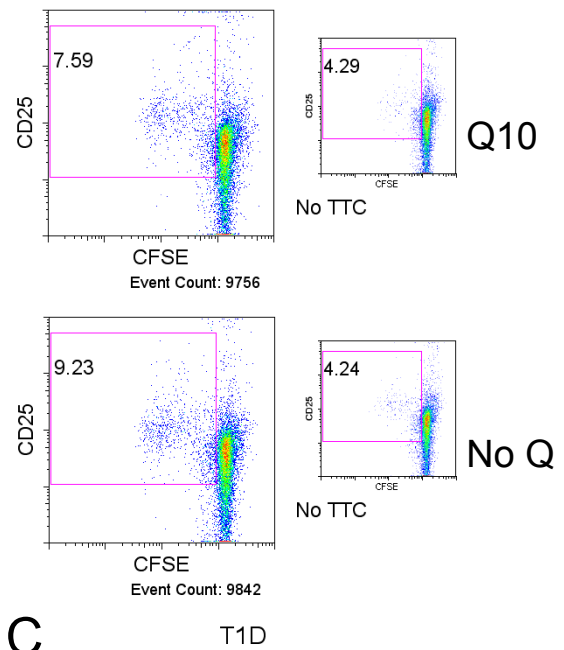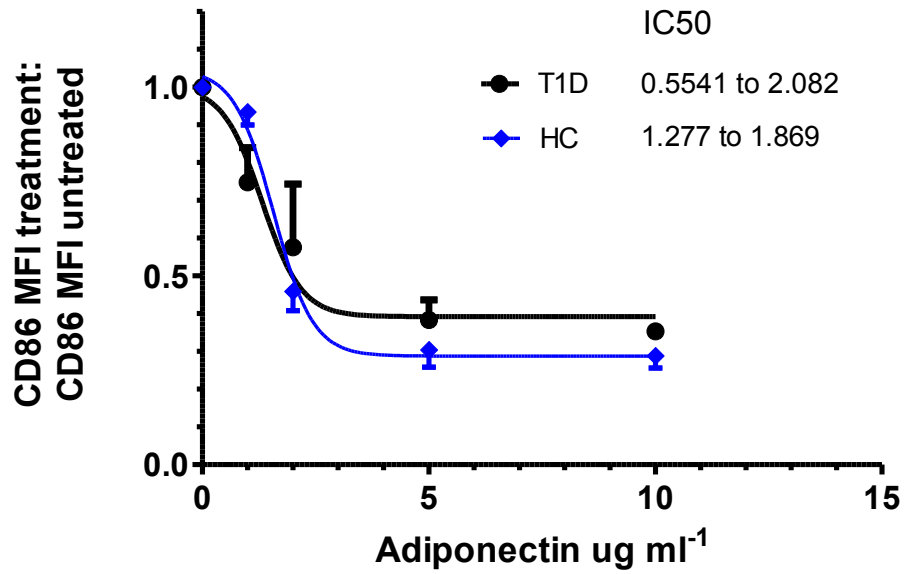
Figure 7.25: dose response curve of CD86 suppression on mDC in T1D (n = 3) (above). Error bars denote SD. IC_{50} was not significantly different compared to HC, although the level maximum inhibition was less than that seen with HC. (below) representative example from one donor. CD14+ monocytes were incubated with 0 (A), 1(B), 2(C) and 10ug/ml (D). Numbers denote percentage frequencies of quadrant gates, defined by isotype.
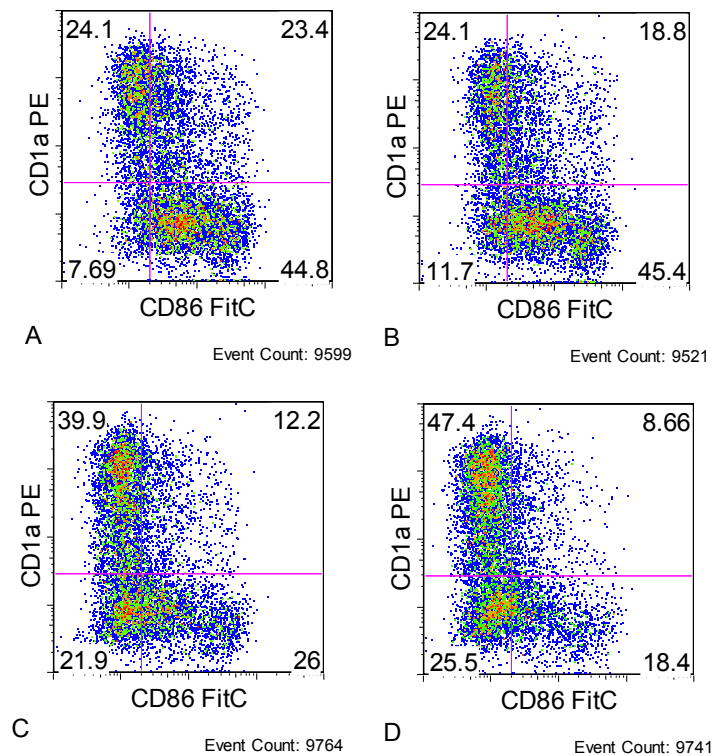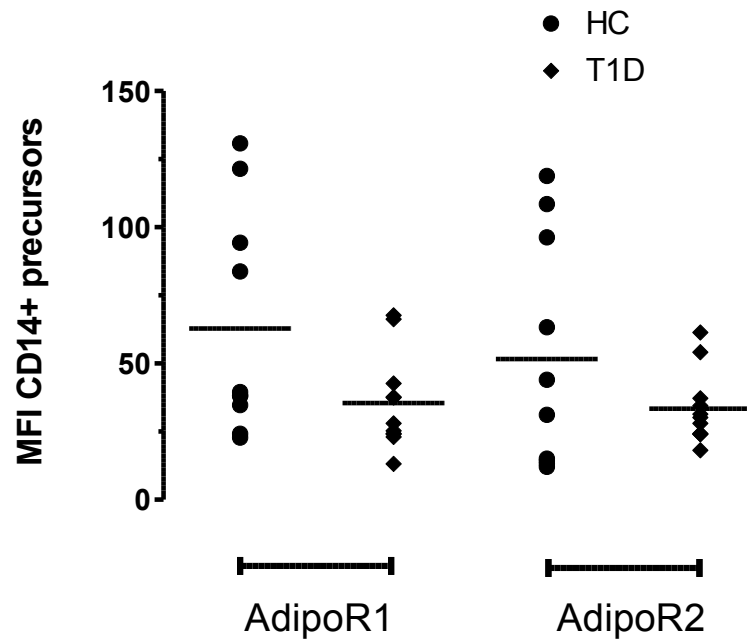
Figure 7.26: AdipoR expression of CD14+ precursors from HC (n=10) and T1D subjects (n=11). Subjects with T1D were chosen based on the prediction that their monocytes would have reduced AdipoR expression (older age and presence of vascular complications). There was a non-significant trend of reduced AdipoR1 expression (p = 0.06), whilst expression of AdipoR2 was not significantly different (p = 0.19).
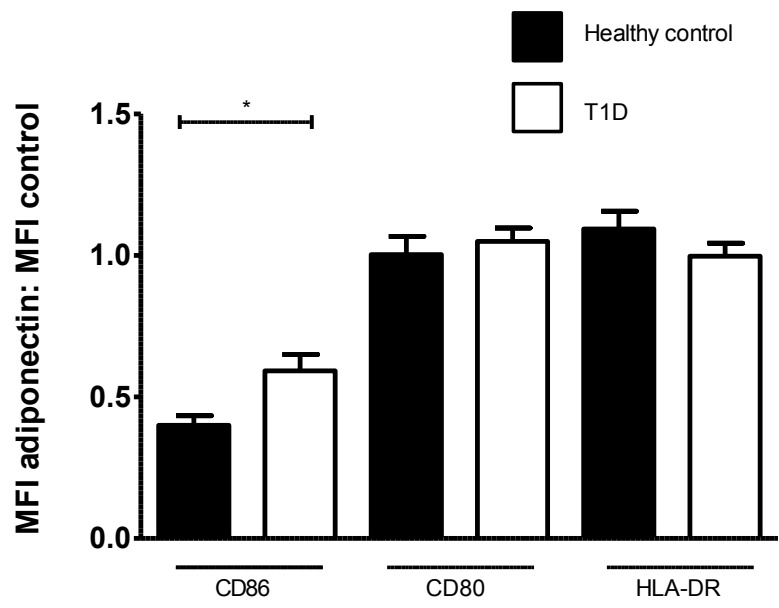
Figure 7.27a. Effect of adiponectin on the expression of co-stimulatory and antigen presenting molecules on mDC in T1D. MFI of CD80, CD86 and HLA-DR on mDC following adiponectin treatment is expressed as a ratio of MFI on control mDC from the same subject. Error bars denote SEM.

* p = 0.011
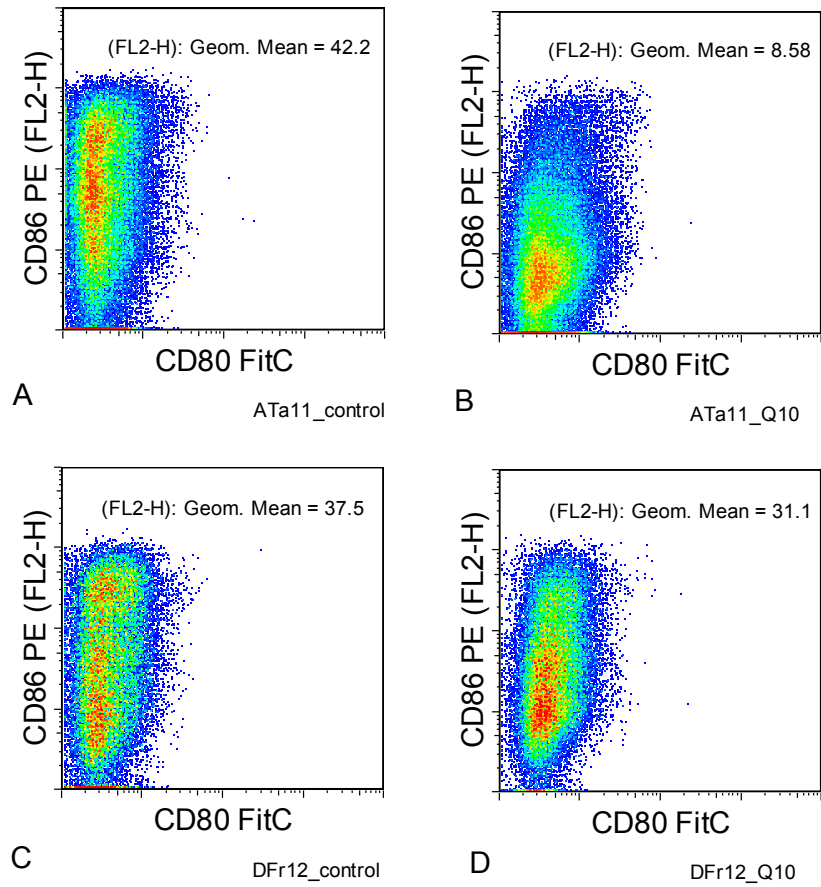
Figure 7.27b. Example raw data. ATa11 (A & B) is a HC and DFr12 is a T1D subject (C & D). The ratio of CD86 expression on Q DC relative to its control is 8.58/42.2 = 0.20 in ATa11 and 0.83 in DFr12.
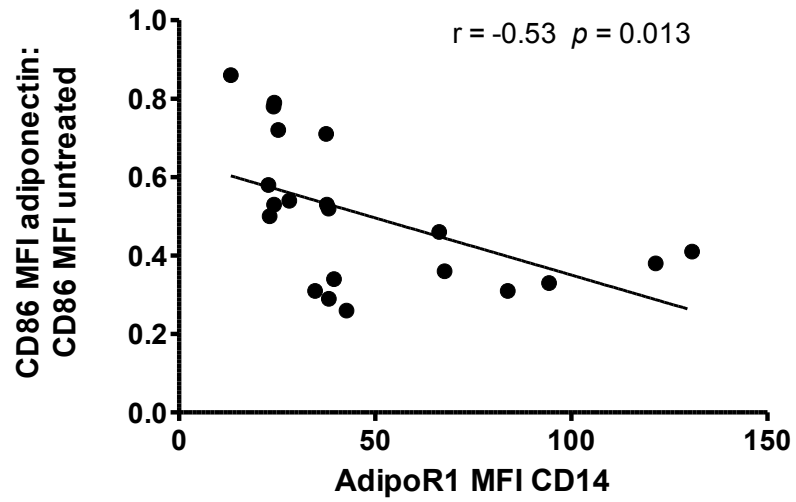
Figure 7.28: correlation of AdipoR1 expression with the reduction of CD86 expression of mDC following adiponectin treatment. All subjects (HC and T1D) were included for this analysis.
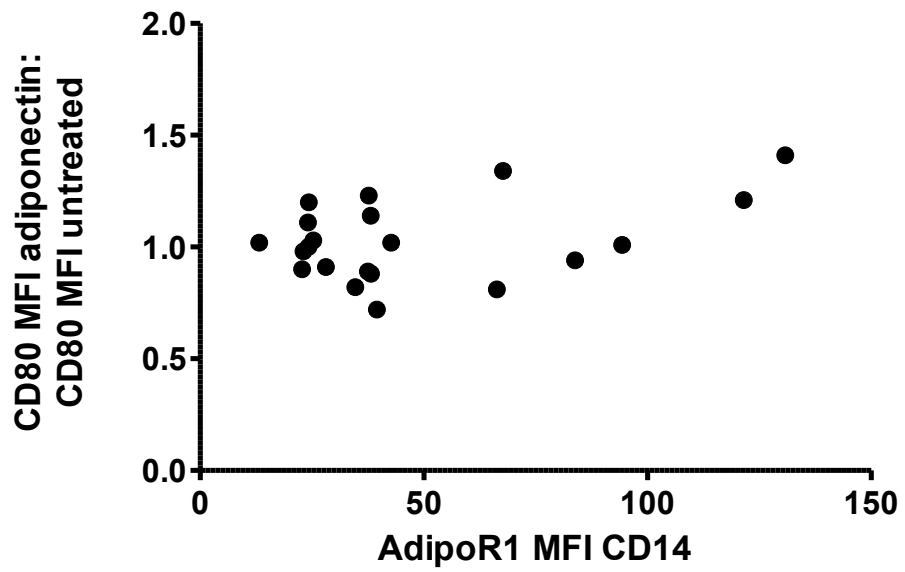
Figure 7.29: Absence of significant correlation between AdipoR1 expression of CD14+ precursors and expression of HLA-DR (bottom) and CD80 (top) following adiponectin treatment.
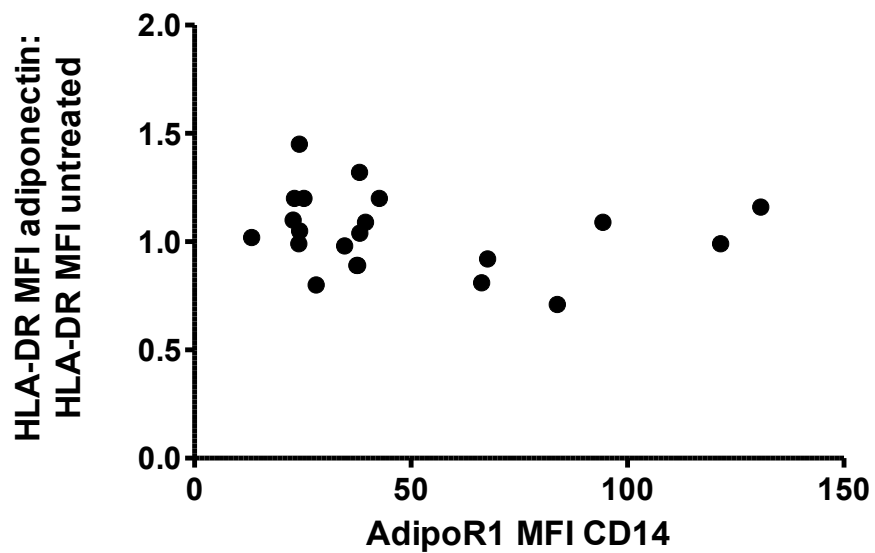
7.6   Discussion

Adiponectin has previously been shown to modulate cytokine secretion of macrophages, T cells and their recruitment to inflammatory loci (Wolf, Wolf et al. 2004, Okamoto, Folco et al. 2008, Palmer, Hampartzoumian et al. 2008). In this chapter, we present novel data describing its dampening effects on APC-dependent T cell immunity. Adiponectin treated DC had reduced CD86 expression and stimulatory capacity on T cells. This function appears to be diminished in T1D, resulting in relatively preserved CD86 expression despite equivalent adiponectin replacement. Reduced AdipoR expression of precursor monocytes in T1D may explain the difference, given the significant negative correlation between AdipoR1 expression and the degree of CD86 suppression on mDC.

Our *in vitro* tests were performed in SF conditions and with recombinant human adiponectin that can mimic physiological oligomeric distribution. We believe these factors explain the apparent disagreement between our findings and that of Wolf *et al*, who reported that despite treatment with adiponectin at 20ug/ml, no changes of monocyte derived DC phenotype or function were detected (Wolf, Wolf et al. 2004). In that paper, FCS was added at 10%, without prior removal of bovine adiponectin by affinity columns (Luo, Guo et al. 2005). As a result, we believed that the "negative control" experiments for mDC phenotype were conducted with active adiponectin levels close to the IC_{50} values of 1-2 ug/ml we had determined in SF conditions. Clearly, this would obscure any potential changes, whereas the improved sensitivity in our SF system allowed significant differences to be detected with physiological adiponectin replacement. Furthermore, the source of adiponectin used by Wolf et al was not disclosed in their paper. Variations in biological function of recombinant adiponectin have been reported because of differences in host species, ability to form high

molecular weight oligomers and LPS contamination (Richards, Stephens et al. 2006, Neumeier, Weigert et al. 2006, Turner, Smolinska et al. 2009). The adiponectin sourced (Alexis, Cat #: ALX-522-063) was derived from mammalian HEK cells and can form high molecular oligomers analogous to the circulating distribution (manufacturer's product information sheet). An independent group had reported that adiponectin can contain significant LPS contamination if used at ug ml^{-1}, contrary to manufacturer's internal validations (Turner, Smolinska et al. 2009). However, we believe that this was not a factor in influencing our findings given that cultures spiked with LPS had opposing effects to that of adiponectin.

We found the suppression of T cell proliferation by adiponectin in whole PBMC assays to be moderate with TTC (~50%) and modest with PHA (~10%). This is in keeping with the degree of monocyte participation in each reaction. We expected monocytes to be the main effector of adiponectin-mediated immunomodulation, given that they have highest receptor expression among all PBMC. Monocyte helper activity is critical for the initial activation of T cells by PHA stimulation (de Vries Caviles et al. 1979). Both cell contact and monocytic secretion of cytokines such as IL-1 and IL-6 are thought to be important in the induction of IL-2 and IL-2 receptor expression (Ceuppens, Baroja et al. 1988, Wakasugi, Bertoglio et al. 1985, Williams, Ransil et al. 1984, Manger, Weiss et al. 1985). After which, propagation of activated T cells escapes monocytic modulation (Manger, Weiss et al. 1985) and here we had expected adiponectin to have only modest effect given their relative paucity of AdipoR expression. In TTC stimulation, monocytes function as APC.

Our data indicate the effect of adiponectin on APC function, lymphocyte apoptosis and Treg induction may contribute to the overall dampening of antigen driven T cell immunity. Our finding that adiponectin increases lymphocyte apoptosis is consistent

with its effects on cell lines (Yokota, Oritani et al. 2000, Dieudonne, Bussiere et al. 2006, Cong, Gasser et al. 2007, Konturek, Burnat et al. 2008, Bråkenhielm, Veitonmäki et al. 2004). There is however lack of consensus in these studies regarding a common molecular mechanism. The demonstration of increased apoptosis of activated T cells with adiponectin in experiments of PHA stimulation suggests AdipoR signalling is not a requirement.

In vitro and ex vivo expansion of the $CD4^+CD25^{high}$ Treg has been proposed as means of immunotherapy (Tang, Henriksen et al. 2004, Peters, Hilbrands et al. 2008). We have shown adiponectin increases their proportion in whole PBMC culture in a dose dependent manner. This is consistent with recent studies demonstrating an in vivo role of adipose tissue in negatively regulating resident Treg numbers (Feuerer, Herrero et al. 2009, Winer, Chan et al. 2009). Furthermore, other adipokines, such as leptin, have also been shown to have similarly potent, albeit opposite effects (De Rosa, Procaccini et al. 2007).

We studied the effect of adiponectin on APC function using the mDC. Impressively, relatively physiological doses of adiponectin replacement brought a significant reduction in CD86 expression. This effect was dose-dependent, occurred within 18 hours of culture, reversible with LPS, and correlated with AdipoR1 expression on precursor monocytes. This data is consistent with a model of adiponectin, possibly via AdipoR1, in modulating CD86 expression. We believe the absence of significant change in other co-stimulatory markers excludes the effect on CD86 expression as a consequence of a global inhibition on DC maturation, in contrast to another example of a humoral factor, vitamin D (Griffin, Lutz et al. 2001, Penna, Adorini 2000). Indeed adiponectin treated mDC were able to antigen present TTC to stimulate responder cells, at a level well above that of the no antigen control. In the same co-culture

experiments we demonstrated lone CD86 suppression on DC resulted in reduced stimulatory capacity. This is in agreement with other in vitro studies (Lanier, O'Fallon et al. 1995, McLellan, Starling et al. 1995).

In contrast, AdipoR2 expression on monocytes did not follow the inverse correlation AdipoR1 had with CD86 inhibition on mDC. Our previous data had shown the expression of the two receptor isotypes on PBMC to be interrelated and co-regulated by insulin sensitivity. However, AdipoR1 and AdipoR2 may have distinct functions. Consistent with this are the differences in downstream signalling pathways. For example in the liver, AdipoR1 activates AMP kinase whilst AdipoR2 activates PPARα (Yamauchi, Nio et al. 2007). In immune cells, signal transduction via AMP kinase and p38 MAP kinase is important in mediating function (Tang, Chiu et al. 2007, Wolf, Wolf et al. 2004, Crawford, Peake et al. 2010), and the triggering of these secondary messengers is attributable to AdipoR1, and not AdipoR2 (Tang, Chiu et al. 2007). The molecular basis for specificity lies in interaction between AdipoR1 with the endoplasmic reticular protein ERp46 (Charlton, Webster et al., 2010). Thus, it is biologically plausible that AdipoR1 is more important in suppressing CD86 expression on mDC and hence the stronger association.

Following our clinical study, we wanted to define the functional significance of the reduced AdipoR expression in T1D. Accepting the borderline significant reduction in AdipoR1 expression for this new cohort, our data imply that in T1D, there is a partial release from the inhibitory effects of adiponectin. In other words, there is greater T cell proliferation than expected for the same dose of adiponectin compared to healthy individuals. Further studies are needed to define the pathologic importance of these in vitro effects in T1D.

Collectively, these studies demonstrate the potential for adiponectin to modulate T cell immunity. We recognised the case for causation and AdipoR mediation would be strengthened if the observed responses were abrogated by inhibitors of adiponectin signalling. We had attempted the adiponectin and mDC phenotype experiments using our existing stock of anti-adiponectin, and anti-AdipoR1/R2 antibodies, but saw no significant change. We believed further optimisations would be required regarding the choice of neutralising antibodies. The indicated use for our anti-adiponectin antibody was immunoblotting, as such it would be raised to recognise solubilised proteins. AdipoR1 & AdipoR2 receptors have recently been shown to undergo endocytic cycling between cell surface and endoplasmic reticulum by clathrin and Rab-5 dependent pathways (Ding, Wang et al. 2009). Blocking antibodies may thus only have short-lived effects during cell culture.

Ideally, we would also test the function of mDC from T1D in a TTC assay to confirm the changes seen with OKT3. However, antigen based mDC tests would require autogeneic responder cells and our attempts to re-stimulate after 5 days in culture were not successful. In the OKT3 assay, fresh allogeneic responder cells were used.

We accept also that as memory T cells were not depleted from our responders, some of their proliferation in the mDC stimulation assay with TTC would have occurred independently of APC. In defence, the percentage of divided cells from the responder cells and TTC only experiment was well below that of mDC and responder cells with no antigen. Hence we concluded that APC-independent activation was not a significant contributor to background proliferation levels used to derive SI.

7.7 Summary of results

We demonstrate adiponectin dampened T cell proliferative response to mitogens and antigens in an optimised serum free CFSE-based assay. The expansion of the CD4$^+$CD25^{high} subset and the promotion of apoptosis upon adiponectin replacement may contribute. In addition, adiponectin can modulate the phenotype and stimulatory function of monocyte derived DC, by downregulating expression of CD86. This inhibitory effect on CD86 was dose dependent, occurred within 18 hours of culture and correlated with AdipoR1 expression on monocyte precursors. The expression of other costimulatory molecules (HLA-DR, DC SIGN, CD80 and CD1a) were unaffected. The reduced expression of AdipoR in T1D may be functionally relevant, given that immune cells were released from the inhibitory effects of adiponectin replacement.

8. DISCUSSION AND CONCLUSIONS

8.1 Summary of key findings and a model for adiponectin in T1D

I have shown that AdipoR1 and AdipoR2 are expressed by peripheral blood myeloid cells, and that the level of expression of these receptors modulates T cell proliferation through adiponectin mediated inhibition of CD86 expression. There is release of this suppression in T1D attributable to the reduced AdipoR expression. We found that receptor expression was inversely related to insulin resistance, and that intervention could upregulate expression.

Figure 8.1 depicts these findings applied to our hypothesis. It defines a mechanism of how insulin sensitivity can modulate autoimmunity of T1D through adiponectin signalling in individuals with a predicted disease incidence of at least 20% in 10 years. Adiponectin synthesis by adipocytes is reduced by weight gain, and both these factors act in tandem to increase IR. The associated reduction of AdipoR on monocytes attenuates the inhibition of CD86 expression on DC exposed to adiponectin.The increased costimulation of CD4+ T cells favours the recruitment and maintenance of diabetogenic T cells. We speculate that this escape of T cell immunoregulation, in conjunction with other immune aberrations (Lesage, Hartley et al. 2002, Brusko, Wasserfall et al. 2005, Schneider, Rieck et al. 2008, Allen, Pang et al. 2009, Kukreja, Cost et al. 2002), predisposes the breakdown of peripheral tolerance in T1D and hastens the development of diabetes. CD86 expression on APC has a critical role in priming of diabetogenic CD4+ T cells and its knockout in the NOD mouse is effective in preventing diabetes (Yadav, Judkowski et al. 2004). However, the effect of CD86 , and hence that of adiponectin, is not antigen specific. Nevertheless, as LPS can override

the inhibitory effects of adiponectin, I believe this system would not lead to a state of

immunocompromise.

High risk for T1D

```
                              ┌──────────────┐
                              │  weight gain │
                              └──────────────┘
                                  │      │
                            (-) Lifestyle
                             intervention
                                  │      │
                                  ▼      ▼
                              ┌─────┐ ┌──────────┐
                              │ ↑IR │◄─│ ↓AdipoQ  │
                              └─────┘ └──────────┘
                                 │         │
                                 ▼         │
                            ┌──────────┐   │
                            │ ↓AdipoR  │   │
                            │ monocytes│◄──┘
                            └──────────┘
                                 │
                                 ▼
                            ┌──────────────┐
                            │ ↑CD86 on DC  │
                            └──────────────┘
                                 │
                                 ▼
                            ┌──────────────┐
                            │ CD4 T cell(+)│
                            └──────────────┘
                                 │
                                 ▼
                            ┌──────────────┐
                            │ (+) Autoreactive│
                            │ islet clone  │
                            └──────────────┘
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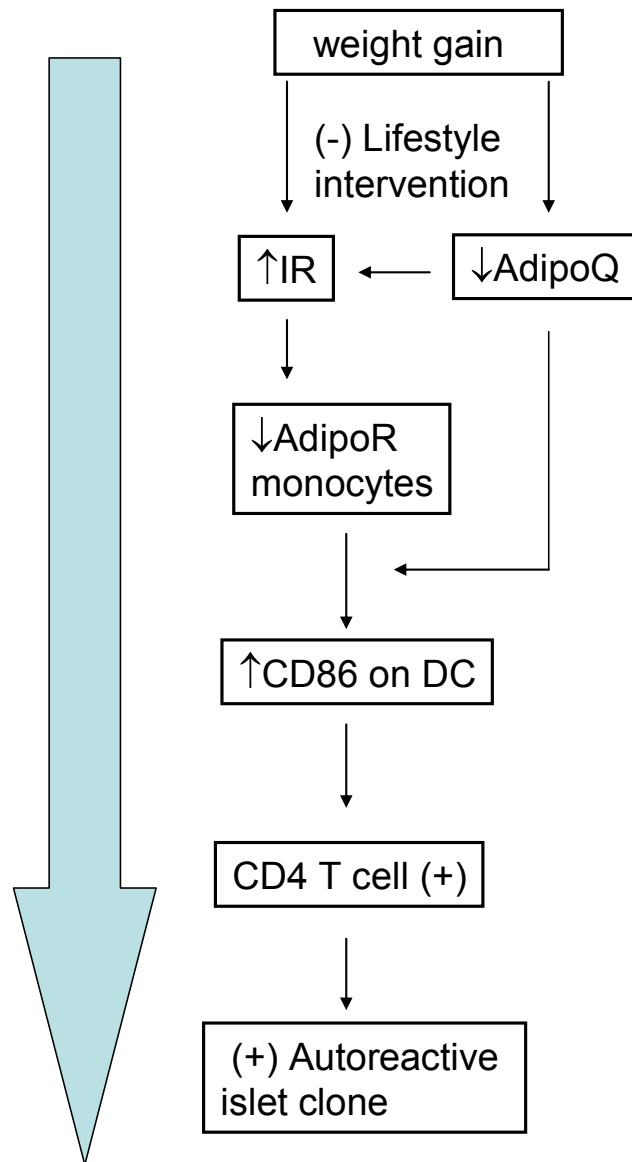
Acceleration of
islet autoimmunity
and development
of T1D

Figure 8.1 Summary diagram for the putative role of adiponectin signalling in immune tolerance of T1D. See sections 8.1 & 8.3 for commentary.

8.2 Critique of key findings

There are several weaknesses to our studies. Firstly, the quantification of AdipoR expression on PBMC by flow cytometry had a high intra-assay CV (up to 14%). As such, it could be prone to type 2 (false negative) errors. Therefore the findings of neutral effect of glucose, insulin and adiponectin on AdipoR expression *in vitro* need further validation, particularly since conflicting animal model data exists (Tsuchida, Yamauchi et al. 2004, Bauche, Ait El Mkadem et al. 2006). Furthermore from our own clinical study, adiponectin had a significant but modest inverse correlation with AdipoR1 and AdipoR2 expression when examined across all subjects (T1D and controls). Secondly, the strongest evidence for insulin resistance as a modulator of AdipoR expression on PBMC came from data in the early ACTID. However, as monocytes have the highest expression of AdipoR among PBMC, a simple increase in relative monocyte count from lifestyle intervention could explain the apparent increase in AdipoR gene expression of whole PBMC in the interventional arm. Nevertheless, a specific effect on monocyte counts by lifestyle changes had not been reported (Timmerman, Flynn et al. 2008, Shephard, Shek 1994), nor observed in our two smaller exercise studies. Lastly, unequivocal evidence for the mediatory role of AdipoR on the inhibition of CD86 expression on APC is lacking. As previously outlined, adiponectin can exert its immune effects independently of AdipoR (Takeuchi, Adachi et al. 2007, Peake, Shen et al. 2006, Masaie, Oritani et al. 2007, Takemura, Ouchi et al. 2007,). To clarify this issue, recent papers citing novel biological functions of adiponectin have included data describing the abrogation of effect through siRNA knockdown of AdipoR gene expression (Miller, Cho et al. 2009, Yamauchi, Kamon et al. 2003, Tang, Chiu et al. 2007, Iwabu, Yamauchi et al. 2010). In our case and as a

further project, similar AdipoR1 RNA interference can be achieved on CD34+ primary haemopoietic stem cells before their transformation to DC (Martino, di Girolamo et al. 2009).

Nevertheless, our data is consistent with what is already known about regulation of adiponectin receptor expression and the anti-inflammatory effects of adiponectin. We validated previous studies that reported mRNA of AdipoR on PBMC by showing expression at the protein level (Palmer, Hampartzoumian et al. 2008, Sohn, Kwak et al. 2010, Shen, Charlesworth et al. 2007, Alberti, Gilardini et al. 2007). Similar to skeletal muscle and adipose tissue, the expression on PBMC is also correlated to insulin sensitivity and can be increased with exercise (Nannipieri, Bonotti et al. 2007, Bluher, Williams et al. 2007, Christiansen, Paulsen et al. 2010, O'Leary, Jorett et al. 2007). The reduced APC-dependent T cell proliferation fits with the reported anti-inflammatory effects of adiponectin in models of T cell driven hepatitis (Palmer, Hampartzoumian et al. 2008, Kaser, Moschen et al. 2005, Sennello, Fayad et al. 2005). As previously elaborated, we attributed the discrepancy between our study and that of Wolf *et al* regarding co-stimulatory molecules on APC by the differences in culture conditions (Wolf, Wolf et al. 2004).

The functional significance of the high AdipoR expression on NK and B cells deserves investigation. Adiponectin may yet have further influence on disease pathogenesis, as these cells are known to have important accessory roles (Alba, Planas et al. 2008, Rodacki, Svoren et al. 2007, Brodie, Wallberg et al. 2008). In particular, B cell depletion by the anti-CD20 monoclonal antibody rituxamab preserves β cell function in patients with newly diagnosed T1D (Pescovitz, Greenbaum et al. 2009)

We have only studied a small number of functional readouts. The full immunoregulatory potential of adiponectin on APC function would be comprehensively documented using gene expression microarray approach. It would be intriguing to see if adiponectin activates the trancriptional repressor cyclic AMP response element modulator α, which has been recently been shown to be an important and specific negative regulator of CD86 expression in APC (Ahlmann, Varga et al. 2009). Apart from proliferation, other effects of APC-CD4 interaction arising from adiponectin treatment, such as the development of effector phenotype and cytokine production, would also be of interest. The data showing an increase of CD4CD25+ proportion following adiponectin replacement is preliminary, and further Treg phenotype characterisation, using markers such as FoxP3 CTLA-4 and CD127 low which confer protection from autoimmune diabetes (Schmidt, Wang et al. 2009, Liu, Putnam et al. 2006, Lundsgaard, Holm et al. 2005), should be performed.

8.3 Further refinements to proposed model

There are gaps in the model that require further elucidation. I have not accounted for how weight gain can inhibit AdipoR expression on PBMC. The level of control is likely to be at gene transcription, given the changes in AdipoR protein expression are also seen with mRNA. The AdipoR1 gene promoter region contains binding sites for ATF3 (activating transcription factor 3) (Park, Kang et al. 2010), a negative transcriptional factor that is upregulated with obesity-associated ER stress. Using a mouse myotube model, Park *et a*l have shown that ATF3 is a physiologically relevant regulator.

The mechanism of how lifestyle interventions can modulate AdipoR expression is not clear. I have shown that achieving weight loss and improvements in IR are necessary

for AdipoR expression to increase. The ATF3-AdipoR1 gene promoter interaction is potentially relevant as weight loss is associated with reduced ER stress and ATF3 expression levels (Gregor, Yang et al 2009).

Next, the cause for the reduced AdipoR expression in T1D, relative to HC and T2D, is not known. Genetic factors were unlikely to be responsible, as polymorphisms in both AdipoR1 and AdipoR2 genes were not found to be associated with increased incidence of T1D (Barrett, Clayton et al 2009). Neither was IR or hyperglycaemia, two known modulators of AdipoR expression matched for in the clinical study. Notwithstanding, IR accounted for some of the variation in AdipoR expression (figure 5.5).

We propose two alternative explanations for the difference between the groups. The reduced AdipoR expression in T1D could be the result of inflammatory stress. We had found AdipoR expression to reduce with T cell activation and was inversely related to that of CD25 (figure 4.22). In testing this, investigating for possible correlations between AdipoR expression with serum levels of inflammatory cytokines, such as TNFα, IL-6 and hsCRP, would be a useful first step.

The differences in β cell function between the groups may be important. Skeletal muscle AdipoR1 mRNA, whose levels are correlated with those on PBMC (Shen, Charlesworth et al. 2007), is independently associated with first phase insulin secretion (Staiger, Kaltenbach et al. 2004). However, despite AdipoR expression on β cells (Kharroubi, Rasschaert et al. 2003), a direct facilitative role for adiponectin signalling on insulin secretion is controversial (Okamoto, Ohara-Imaizumi et al. 2008, Staiger, Stefan et al. 2005, Winzell, Nogueiras et al. 2004). Nevertheless, the corollary is that low AdipoR expression in our cohort of longstanding T1D would relate to the presumed

absence of residual endogenous insulin secretion, and vice versa in healthy controls. A similar inverse correlation has been described for serum adiponectin levels with fasting C-peptide across the spectrum of non-obese healthy controls, T2D and T1D (Furuta, Tamai et al. 2006, Behre, Brohall et al. 2006). Thus, to further examine the temporal relationship and control for genetic confounders, a study of AdipoR expression on stored PBMC from pre-diabetic subjects developing disease could be done. An analogous study from animal models of autoimmune diabetes would also be supportive.

Whether lifestyle change, by increasing AdipoR expression, would ameliorate resistance to adiponectin inhibition on APC function is another area for study. CD86 expression on mDC following adiponectin replacement would be a suitable primary readout. If proven, then there would a possibility that the immune sequelae of reduced AdipoR expression in T1D is subject to therapy.

Importantly, we would need to confirm the tolerogenic effects of adiponectin at the level of islet autoimmunity. Our experiments of APC cocultured with effector CD4+ T cells could be repeated using clones reactive to β cell autoantigens (Mannering, Morris et al. 2004). Another approach would be to look for adiponectin receptor expression by immunohistochemistry in pancreatic nodes of patients with T1D by immunohistochemistry, with non-pancreatic lymph nodes from the same subject as control. Suitable tissue sample could be attained from the Network for Pancreatic Organ Donors with diabetes (www.jdrfnpod.org), a project funded by JDRF where pancreatic tissue is harvested from cadaveric organ donors with T1D.

Although circulating adiponectin can bind to both AdipoR1 and AdipoR2, there are differences in signal transduction downstream to both receptors (Section 1.7.2). We have identified that AdipoR1 expression on precursor monocytes may be more important in determinng the attenuating effect on CD86 of DC (figure 7.2.8). Thus a deficiency of AdipoR1 may not be simply compensated for by AdipoR2 on the same cell. Furthermore, we (figure 5.4) and others have found that the expression of both receptors appear to be under co-regulation (Staiger Kaltenbach et al 2004. Bluher, Williams et al. 2007. Shen, Charlesworth et al. 2007). Thus, it is unlikely under physiological conditions that a human subject would have reduced AdipoR1, but normal expression levels of AdipoR2.

In summary, our data supports adiponectin to be a molecular mediator of the link between insulin resistance and immunity in T1D. Further investigation is merited given the potential application in disease prevention, preservation of β cell function in LADA, islet allografts and early T1D.

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10. SUPPLEMENTARY DATA

Appendix 1: Autofluorescence of activated T cells. Positively selected CD3+ cells were activated with (A) PHA or (B) CD3+CD28+ Dynabeads. After 48-72 hours, they were then stained using the secondary AF-488 conjugated goat antib-rabbit antibody only, read on the FL-1 channel. Activated cells are larger (by forward scatter) and are higher in expression for CD25 (C &D). They also have higher background fluoescence, before addition of the primary labelling adiponectin receptor antibody.

A
Event Count: 51207

B
Event Count: 10694

C

D
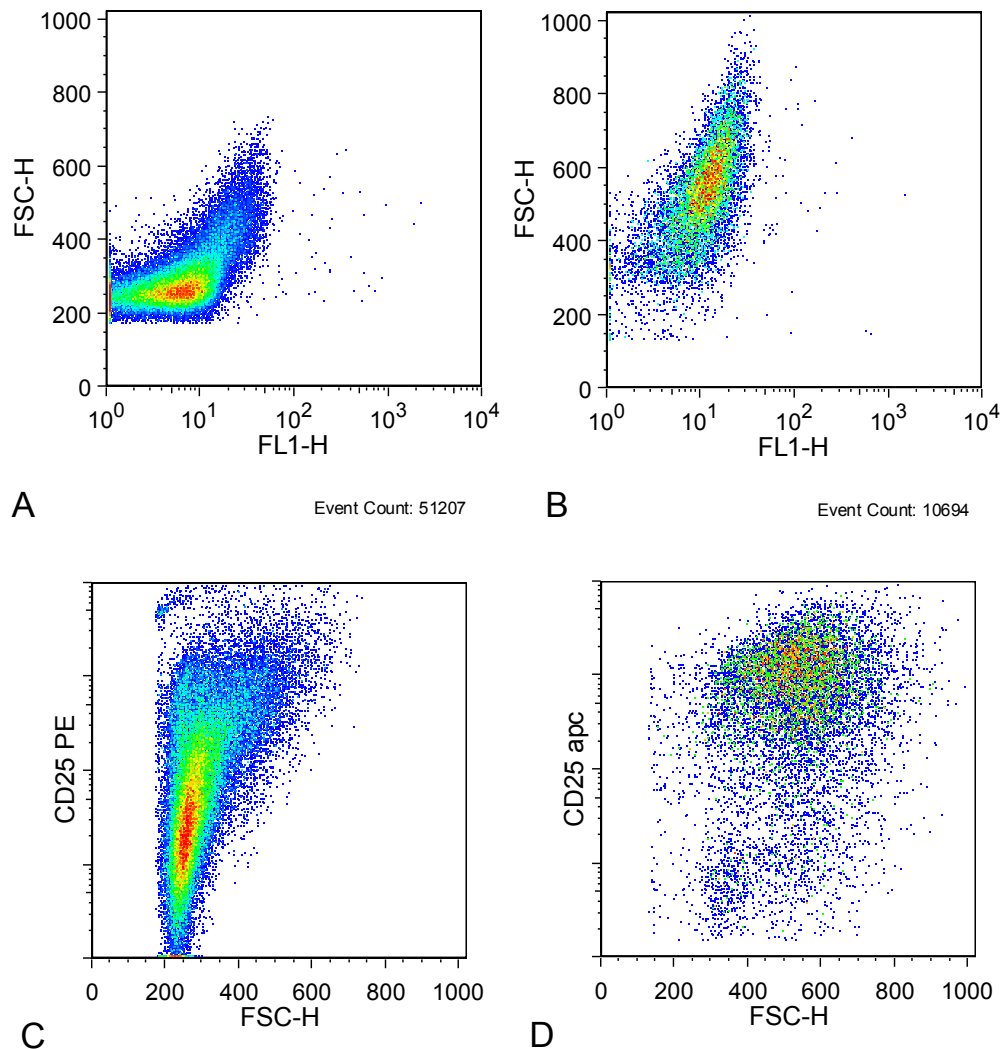
Appendix 2: standard curve for Taqman ADIPOQ rt-qPCR assay.
ΔCt (Ct ADIPOQ – Ct 18S) is plotted for serial dilutions of whole pancreas RNA template. The plot shows that ΔCt for the sample is unaffected by dilution, thus validating the application of the $\Delta\Delta$Ct method in the calculation of relative gene expressions.

y = -0.0464x + 16.576
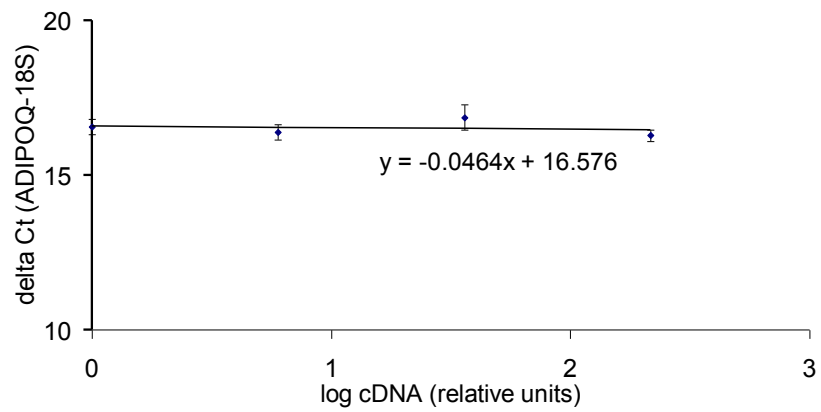
Appendix 3: Testing normal distribution of data. We applied the one-sample Kolmogorov-Smirnov test procedure to assess whether our FACS data, as well as serum adiponectin values, conform to Gaussian distribution. In this test, a *p* value of <0.05 indicates the observed distribution does not follow normality. Our data, allR1 (MFI AdipoR1 on all cells), lymphR1 (MFI AdipoR1 on lymphocytes), monoR1 (MFI AdipoR1 on monocytes), allR2, lymphR2, monoR2 and adiponectin (serum adiponectin by ELISA) passed this test, thus validating the subsequent application of ANOVA testing for group differences.

One-Sample Kolmogorov-Smirnov Test

		allR1	lymphR1	monoR1	allR2	lymphR2	monoR2	adiponectin
N		76	76	76	76	76	76	76
Normal Parameters [a,b]	Mean	3.4877	3.0402	28.6487	2.9791	2.7492	21.6690	7.1469
	Std. Deviation	2.14489	2.08204	18.66972	2.27769	2.14650	18.37408	5.16318
Most Extreme Differences	Absolute	.106	.105	.152	.111	.129	.149	.148
	Positive	.106	.105	.152	.111	.129	.149	.148
	Negative	-.086	-.080	-.084	-.100	-.101	-.143	-.111
Kolmogorov-Smirnov Z		.877	.876	1.266	.922	1.069	1.234	1.229
Asymp. Sig. (2-tailed)		.425	.427	.081	.364	.203	.095	.098

a. Test distribution is Normal.

b. Calculated from data.

Appendix 4: Correlation matrix of FACS data and monocyte count. AllR1 (MFI AdipoR1 on all PBMC). LymphR1 (MFI of AdipoR1 on lymphocytes). MonoR1 (MFI of AdipoR1 staining on CD14+monocytes). "Monocytes" denotes the percentage of viable monocytes in the sample.

Correlations

		allR1	lymphR1	monoR1	allR2	lymphR2	monoR2	monocyte
allR1	Pearson Correlation	1	.863**	.224	.854**	.787**	.258*	.201
	Sig. (2-tailed)		.000	.065	.000	.000	.033	.098
lymphR1	Pearson Correlation	.863**	1	.116	.796**	.924**	.314**	.059
	Sig. (2-tailed)	.000		.341	.000	.000	.009	.630
monoR1	Pearson Correlation	.224	.116	1	.140	.110	.638**	.112
	Sig. (2-tailed)	.065	.341		.252	.370	.000	.359
allR2	Pearson Correlation	.854**	.796**	.140	1	.907**	.436**	.032
	Sig. (2-tailed)	.000	.000	.252		.000	.000	.797
lymphR2	Pearson Correlation	.787**	.924**	.110	.907**	1	.457**	-.012
	Sig. (2-tailed)	.000	.000	.370	.000		.000	.925
monoR2	Pearson Correlation	.258*	.314**	.638**	.436**	.457**	1	-.011
	Sig. (2-tailed)	.033	.009	.000	.000	.000		.931
monocyte	Pearson Correlation	.201	.059	.112	.032	-.012	-.011	1
	Sig. (2-tailed)	.098	.630	.359	.797	.925	.931	

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Appendix 5: correlation matrix of glycaemic parameters against AdipoR MFI values for subjects with T1D. Insulin dose (total daily dose), insulinperwgt (total daily dose per kg), HbA1C and duration of disease did not bear significant correlations with AdipoR MFI values on all PBMC, lymphocytes or monocytes.

Correlations

		allR1	lymphR1	monoR1	allR2	lymphR2	monoR2
insulindose	Pearson Correlation	-0.221	-0.191	-0.158	-0.065	-0.047	-0.044
	Sig. (2-tailed)	0.249	0.322	0.413	0.737	0.810	0.820
	N	29	29	29	29	29	29
insulinperwgt	Pearson Correlation	-0.243	-0.108	-0.241	0.034	0.064	-0.130
	Sig. (2-tailed)	0.204	0.576	0.209	0.861	0.740	0.503
	N	29	29	29	29	29	29
duration	Pearson Correlation	0.180	0.232	0.010	0.226	0.213	0.258
	Sig. (2-tailed)	0.410	0.286	0.963	0.299	0.329	0.234
	N	29	29	29	29	29	29
HbA1C	Pearson Correlation	0.255	0.159	0.240	0.318	0.082	0.211
	Sig. (2-tailed)	0.182	0.411	0.209	0.093	0.674	0.272
	N	29	29	29	29	29	29

Appendix 6: effect of diabetic complications on AdipoR expression in T1D. We found no significant differences in AdipoR MFI values of subjects with (1) and without micro- and macrovascular complications (0) by the paired t-test.

	complications	N	Mean	Std. Deviation
allR1	0.00	18	2.4386	1.79971
	1.00	11	2.2123	1.96287
lymphR1	0.00	18	1.9301	1.88356
	1.00	11	2.0768	1.63882
monoR1	0.00	18	25.9180	17.68222
	1.00	11	16.0700	11.15844
allR2	0.00	18	1.4867	1.11495
	1.00	11	1.3253	1.21294
lymphR2	0.00	18	1.4482	1.33418
	1.00	11	1.4977	1.41485
monoR2	0.00	18	12.8549	11.82894
	1.00	11	7.5727	2.75591
adiponectin	0.00	18	10.1510	8.32817
	1.00	11	9.1383	3.13849

Appendix 7: Standard curve of ELISA for total serum adiponectin (Cat # DRP 300, R&D Systems, Abingdon, UK). We made serial dilutions of the adiponectin standard solution (250ng ml^{-1}). The lowest dilution was at 3.9 ng ml^{-1}. Each concentration was assayed in duplicate wells. Absorbance values for each well was recorded at 450nm and then at 540nm. The reading at the 540nm wavelength corrects for optical imperfections in the plate. The linear regression plot between the logarthithms of adiponectin concentration and corrected absorbance values is shown.

$$y = 0.9296x - 1.8554$$
$$R^2 = 0.9967$$

x-axis: **log adiponectin (ng/ml)**

y-axis: **log corrected absorbance**
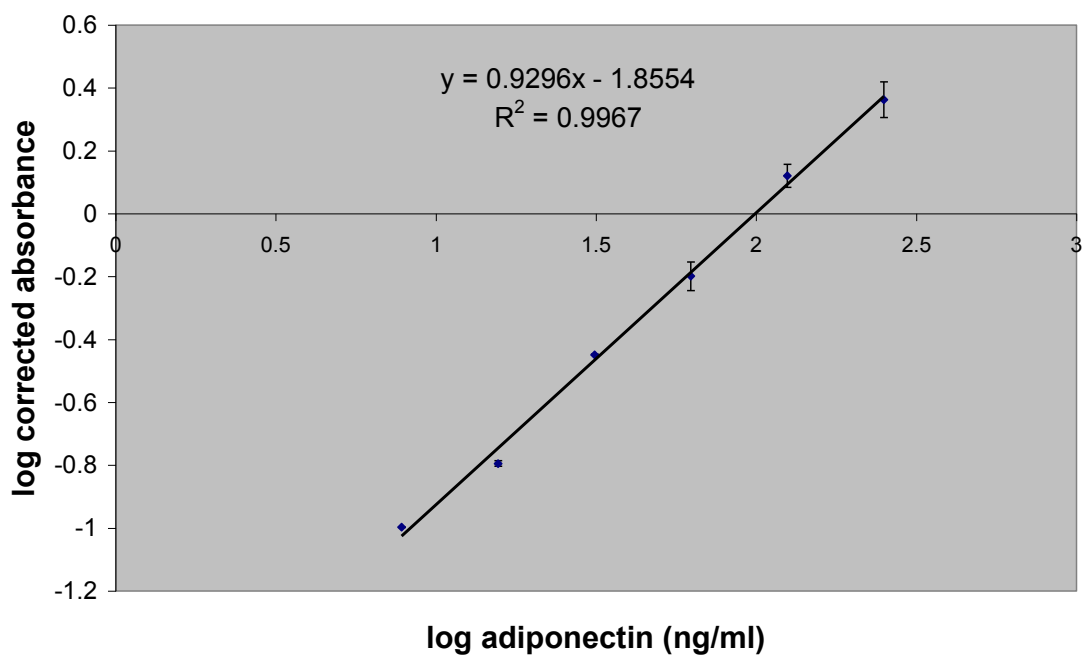
11. PUBLICATIONS AND ABSTRACTS AT NATIONAL AND INTERNATIONAL MEETINGS

Publications

Addressing insulin resistance in Type 1 diabetes.

Pang TT, Narendran P.

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Abstract

Type 1 diabetes is recognised to include an element of insulin resistance. Insulin resistance is an independent risk factor for the development of macro- and microvascular complications of Type 1 diabetes and may also contribute to the development of the disease. This understanding comes at a time when the incidence of Type 1 diabetes appears to be rising and the public health burden from its vascular complications is high. A variety of safe and efficacious manoeuvres are available to redress insulin resistance in Type 2 diabetes. So far however, clinical trials addressing insulin resistance in Type 1 diabetes have been small with only short periods of follow-up. Regardless, these trials have yielded promising results. This review examines the evidence for insulin resistance in the pathophysiology of Type 1 diabetes and its complications, the problems associated with its measurement, and summarizes the trials aimed at reducing insulin resistance in Type 1 diabetes. This includes a meta-analysis of controlled trials of adjuvant metformin in Type 1 diabetes.

The distribution of adiponectin receptors on human peripheral blood mononuclear cells.

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Abstract

Adiponectin, an adipocytokine with anti-inflammatory and insulin-sensitizing properties, may provide a mechanism by which insulin resistance accelerates autoimmunity in type 1 diabetes (T1D). Its actions are mediated by two receptors, adiponectin receptors 1 (ADIPOR1) and 2 (ADIPOR2). In this study, we measured their distribution on human peripheral mononuclear cells by flow cytometry. ADIPOR1 is present approximately on 1% of T cells, 93% of monocytes, 47% of B cells, and 21% of NK cells ($P < 0.01$ for difference between subsets). The distribution of ADIPOR2 was found to be similar (r= 0.992, $P < 0.01$), and staining could be blocked in an antigen-specific manner. We were also able to confirm our finding at an RNA level by PCR using sequence-specific primers. Our data are consistent with an immunoregulatory role for adiponectin in T1D.

Presentation abstracts

Adiponectin gene expression in peripheral blood mononuclear cells is modulated by activation, and by diet and exercise in patients with type 2 diabetes

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[2] Early ACTID office, Joint Clinical Research Unit, University of Bristol

Background and aims: Circulating adiponectin and skeletal-tissue expression of its receptors is reduced in obesity and increases following lifestyle intervention. We asked whether adiponectin and its receptors were also expressed by peripheral blood mononuclear cells (PBMC) and if so, whether expression was modulated by cell activation or lifestyle changes.

Method: Adiponectin expression was studied by real-time PCR in freshly isolated PBMC from healthy volunteers, and following activation with phytohaemagglutinin or lipopolysaccharide. Expression was also measured at baseline and following lifestyle intervention in 10 subjects from the Early ACTID trial, a randomised controlled study of exercise in newly-diagnosed type 2 diabetes. Adiponectin receptor 1 & 2 (ADIPOR1 & 2) expression was measured by flow cytometry.

Results: The adiponectin gene was expressed by PBMC in all healthy and diabetic subjects, at a level comparable to the pancreas. Following in-vitro stimulation, it decreased by 90.9 - 92.7% (95% CI), whilst the surface expression of its receptor was upregulated (1.8-2.7× 95% CI). In Early ACTID, adiponectin expression was increased at 6 months in subjects achieving moderate weight loss (mean= -2% of baseline), compared to those who did not (+52% vs -47%, $p<0.05$%). There was also a trend for increased receptor gene expression (ADIPOR1: +13% vs -16% p=, ADIPOR2 +15% vs -8% p=0.2)

Conclusions: Adiponectin and adiponectin receptor expression by PBMCs may be influenced by obesity, analogous to that described for circulating and peripheral tissue receptor levels. This may be an important mechanism by which lifestyle intervention modulates the inflammatory response associated with insulin-insensitivity.

Lifestyle intervention increases adiponectin receptor expression by peripheral blood cells (PBC) in type-2-diabetes (T2D)

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Background: PBC express the adiponectin receptors AdipoR1 and AdipoR2. In T2D, low levels of receptor expression on PBC are associated with obesity, insulin resistance and coronary artery disease. We have previously shown that weight loss increases the PBC expression of AdipoR1 and AdipoR2 in a small cohort of patients with T2D.

Aims: To assess the effect of lifestyle interventions, over and above that of weight loss, on the expression of adiponectin receptors by PBCs in T2D

Method: 71 consecutive subjects were recruited from early-ACTID, a blinded randomised controlled trial of lifestyle interventions in newly diagnosed T2D. AdipoR1 & AdipoR2 expression was measured by real-time quantitative PCR at baseline and at 6 months following intervention. Insulin resistance was measured by HOMA-IR using 2 pairs of fasting glucose and insulin.

Results: At baseline, subjects had a mean BMI of $31.6 +/- 1.3 kgm^{-2}$ and a HbA1C of 6.6+/-0.2%. By intention-to-treat analysis, AdipoR1 was significantly upregulated in the lifestyle intervention arm (fold change 2.02 vs 0.64 in the standard care arm, p=0.02). There was a trend for AdipoR2 to increase (fold change: 7.44 vs 1.08 p=0.06). Post-hoc analysis including all subjects showed a fall in HOMA-IR (mean+/-SEM = -26+/-4% vs +32%+/-7%) was associated with a significant increase (p<0.05) in AdipoR1 and log(AdipoR2), as well as improvements in HbA1C (-0.3% vs +0.5% p<0.05).

Conclusion: Lifestyle interventions and improved insulin sensitivity increase adiponectin receptor expression on PBC, and this could be a mechanism by which the glycaemic and cardiovascular benefits of diet and exercise are achieved.

Adiponectin receptor expression on peripheral blood mononuclear cells is reduced in autoimmune diabetes and can be upregulated with lifestyle intervention

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Background and aims: Studies of patients with genetic predisposition for T1D show that insulin resistance (IR) is an independent risk factor for T1D. We are interested in defining the mechanisms that link IR with progression to T1D, and in particular the potential role of adipocytokines such as adiponectin. Adiponectin is an anti-inflammatory and insulin sensitising cytokine whose levels are inversely associated with IR. It signals through receptors AdipoR1 & AdipoR2, whose expression levels are increased by manoevres that reduce IR. These receptors have previously been found on peripheral blood mononuclear cells (PBMC) where stimulation reduces their proliferation and cytotoxicity, and increases their secretion of anti-inflammatory cytokines. We hypothesise that adiponectin has an anti-inflammatory effect on human islet autoimmunity. Our initial aim was to assess the expression of adiponectin receptors on PBMC in healthy and T1D subjects, and whether these expression levels could be modulated by exercise.

Materials and methods: PBMC expression of AdipoR1 & AdipoR2 was quantified by flow cytometry and qPCR on 26 T1D subjects and 14 age and anthropometrically matched healthy male Caucasian volunteers. The proportion of cells expressing each receptor, and the mean fluorescence intensity (MFI) were calculated by subtracting from subject-specific isotype control staining. The estimated glucose disposal rate (eGDR) of T1D subjects was calculated using waist hip ratio, blood pressure and HbA1C as previously described. In addition, we measured the effect of 6 months of diet and exercise training on adiponectin receptor expression by PBMC using qPCR, in 9 subjects with autoimmune diabetes.

Results: AdipoR1 & AdipoR2 are expressed on monocytes and lymphocytes in both healthy and T1D subjects. On average, 47% of monocytes and 11% of lymphocytes expressed AdipoR1 and AdipoR2. AdipoR1 & AdipoR2 expression is decreased on monocytes (with a tendency to decrease in lymphocytes) in T1D compared to healthy subjects (figure). AdipoR1 & AdipoR2 expression by both monocytes and lymphcytes, measured by MFI, correlated significantly with eGDR in subjects with T1D (monocytes AdipoR1 $r = 0.475$, AdipoR2 $r = 0.512$; lymphocytes AdipoR1 $r = 0.292$, AdipoR2 $r = 0.420$). Lifestyle intervention increases AdipoR1 & AdipoR2 gene expression at 6 months (Fold change: AdipoR1 2.6 $p = 0.02$, AdipoR2 1.5, $p = 0.07$).

Conclusion: Adiponectin receptor expression is decreased on PBMC from T1D subjects. These findings are consistent with adiponectin playing an anti-inflammatory role on islet autoimmunity, and may explain the association between insulin resistance and the development of T1D. Furthermore, therapies that reduce IR may also modulate the development of this disease.

T cell function is released from the anti-inflammatory effects of adiponectin (apM1) in type 1 diabetes (T1D): a potential link between insulin resistance (IR) and T1D.

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IR is independently associated with the development of T1D, though the mechanism for this is not clear. ApM1 is an insulin sensitizing and anti-inflammatory adipokine whose serum levels are reduced with IR. We hypothesized that changes in apM1 and its receptors may mediate the link between IR and T1D. We have previously shown that receptors for apM1, AdipoR1 & AdipoR2, are expressed by peripheral blood mononuclear cells (PBMC), and in particular by monocytes and CD14-, CD11c+ CD1a+ dendritic cells (DC). In a cross-sectional study (n=86), we also found that patients with T1D have reduced AdipoR1 & AdipoR2 expression on monocytes, compared to matched healthy controls as well as subjects with insulin treated type 2 diabetes.

DC are key regulators T cell immunity in T1D. To determine the effect of apM1 on DC function, we optimized the serum-free generation of DC from CD14+ monocytes. We found that the addition of apM1 decreased the expression of the co-stimulatory molecule CD86 on DC at both RNA and protein level by 39-64%. This inhibitory effect was dose dependent (IC50 = 1.3-1.9 ug/ml) and was maintained despite LPS stimulation. The expression of HLA-DR & CD80 did not alter. DC generated in the presence of apM1 showed reduced stimulatory capacity when tested on CD4CD25- effector T cells in CFSE proliferation assays. To determine the significance of the lower PBMC AdipoR expression in T1D, we compared the effect of addition of fixed doses of 10ug/ml apM1 to monocytes from T1D and healthy subjects in the DC generation culture (n=12). ApM1 induced suppression of CD86 expression was significantly decreased in T1D by up to 31%, and the degree of inhibition significantly correlated with AdipoR1 protein expression of the monocyte precursors (r = -0.69 p <0.05). Moreover, the suppression of PBMC proliferation to the common antigen tetanus toxoid by the 10ug/ml apM1 dose was also significantly reduced in T1D.

In conclusion, T cells are released from the anti-inflammatory effects of apM1 in patients with T1D. This mechanism may contribute to the association between IR and the development of T1D.