Studies on the perturbations of CD4+T cells function by cigarette smoke.

by

Abdullah Alghamdi

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Abstract

Background: A number of genes including HLA-DRB1 and variants of PTPN22 (which codes for Lyp phosphatase) are associated with the development of rheumatoid arthritis (RA). Environmental factors, particularly cigarette smoking also promote RA and preliminary data suggested that cigarette smoke could impact Lyp phosphatase function in immune cells. Moreover, unpublished data suggested that Lyp may be involved in regulating metabolism in T cells. This project sought to discover then mechanisms by which PTPN22 and cigarette smoke may interact to alter immune cell metabolism and function.

Methods: Isolated naïve and memory T cells from human blood cones were treated with cigarette smoke extract CSE or Lyp inhibitor for 24h and then stimulated with anti CD3/CD28 for 72h. Protein expression and phosphorylation were assessed using immunoblotting, changes in energy metabolism were assessed using the Seahorse instrument and cytokines expression was assessed using Luminex assay. All the experiments and the analysis were done after 24h of CSE or Lyp inhibitor treatment and 72h of stimulation.

Results: CSE increased Lyp phosphatase activity in naïve, whereas Lyp inhibitor increased Lyp activity in memory only. Both treatments increased the phosphorylation of ZAP-70 in naïve and memory but not Lck. Lyp inhibitor increased Vav1 and Akt phosphorylation in naïve but not in memory, whereas CSE did not show any. CSE increased mTORC1 in naïve but not in memory, whereas CSE increased AMPK only in memory. Both treatments increased calcium (Ca^{2+}) flux made in response to TCR stimulation. Moreover, CSE and Lyp inhibitor

increased glycolysis and oxidative phosphorylation of naïve and memory T cells. Generally, CSE increased inflammatory cytokines such as IL6, IL-1β, IFNγ and TNFα, IL8, but not IL10.

Conclusions: Overall, these data suggest that CSE may promote altered TCR function through interaction with Lyp. One consequence is an alteration in energy metabolism through changes in signalling pathway and cytokines production. This may explain part of the underlying mechanisms by which cigarette smoke promote RA development.

Table of contents

List of figures

List of tables

Abbreviations

ACPA	Anti-Citrullinated Protein Antibody
APC	Antigen Presenting Cell
AMPK	AMP-activated protein kinase
AP	Alkaline phosphatase
ADAP	Adaptor protein
ATP	Adenosine triphosphate
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
BCL-2	B cell lymphoma 2
BAD	BCL2 associated agonist of cell death
CCP	Cyclic Citrullinated Peptide
CSE	Cigarette smoke extract
CS	Cigarette smoke
CD	Cluster of differentiation
Ca^{2+}	Calcium
CREB	cAMP-Response Element Binding protein
DIFUMP	6,8-Difluoro-4-Methylumbelliferyl Phosphate
DAG	Diacylglycerol
DTT	Dithiothreitol
DMSO	Dimethyl Sulfoxide
ECAR	Extracellular Acidification Rate
EDTA	Ethylenediaminetetraacetic Acid
ECL	Enhanced Chemoiluminescence

ETC	Electron Transport Chain
FACS	Fluorescent Assisted Cell Sorting
FCCP	Carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone
GPS	Glutamine Penicillin Streptomycin
GSK	Glycogen Synthase Kinase
GLUT1	Glucose Transporter 1
HBSS	Hanks Buffered Salt Solution
HLA	Human Leukocyte Antigen
IFN	Interferon
IL	Interleukin
ICAM	Intercellular Adhesion Molecule-1
IP3	Inositol 1,4,5-trisphosphate
ITAM	Immunoreceptor Tyrosine-based Activating Motifs
LYP	Lymphoid tyrosine phosphatase
LCK	Lymphocyte-Specific protein tyrosine Kinase
LFA-1	Lymphocyte function-associated antigen 1
MHC	Major Histocompatibility Complex
MAPK	Mitogen Activated Protein Kinase
mTORC	Mammalian Target of Rapamycin Complex
MFI	Mean Fluorescence Intensity
NFκB	Nuclear Factor Kappa B
NFAT	Nuclear Factor of Activated T cells
OCR	Oxygen Consumption Rate
PTPN22	Protein Tyrosine Phosphatase Non-receptor type 22
PBS	Phosphate Buffered Saline
PBMC	Peripheral Blood Mononuclear Cell
PADI	Peptidylarginine Deiminase
PLC	Phospholipase C

PKC	Protein Kinase C
PMA	Phorbol 12-myristate 13-acetate
PTP	Protein Tyrosine Phosphatase
PVDF	Polyvinylidene Fluoride
RA	Rheumatoid Arthritis
ROS	Reactive Oxygen Species
RF	Rheumatoid Factor
SEM	Standard Error Mean
SDS	Sodium Sodecyl Sulfate
SNP	Single Nucleotide Polymorphism
SLE	Systemic Lupus Erythematosus
SFK	Src-Family Kinase
TCR	T cell Receptor
TBST	Tris Buffered Saline with Tween
TNF-α	Tumour Necrosis Factor Alpha
WB	Western Blot
ZAP-70	Zeta-Chain-Associated Protein Kinase 70

Chapter 1: Background

1. General introduction

Rheumatoid arthritis (RA) is a chronic inflammatory condition that has a prevalence of up to 1% in the industrialized world [1]. It affects bone and cartilage leading to disability and deformities, and the systemic effects of the associated inflammation accelerate the progression of cardiovascular disease [2]. Destruction and inflammation of the synovial joint is the most common sign of RA [3]. While the initial trigger for RA is not clear, there is some evidence to suggest that abnormalities of the immune system may lead to abnormal inflammatory reactions, mainly in the joints, and the production of circulating auto-antibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibody (ACPA) [4]. RF recognises the IgG-Fc region and forms complement fixing immune complexes which induce the production of cytokines which contribute in driving the disease process [5]. ACPA is more specific to RA than RF and they are produced against citrullinated peptides, and may be involved in RA development at an early stage [6]. All these immune/inflammatory changes result in an increase in the amount of synovial fluid and cause swelling due to increase in proliferation of the synovial membrane and increase in blood flow in the joints, resulting in redness and hotness. If this inflammation is not inhibited, it can lead to an increase in the amount of protein-degrading enzymes, cytokines and other factors secreted by inflammatory cells and subsequently results in the transformation of the synovial membrane cells to an inflammatory phenotype that can eventually invade the joints [7].

It has become clear that the cytokine network, which includes anti-inflammatory and pro-inflammatory cytokines derived from immune cells such as macrophages, plays an important role in the pathogenesis of RA. In particular, the pro-inflammatory cytokines TNF-α and IL1 are important in the development of chronic joint inflammation [8]. In addition, recent

evidence has shown that the pro-inflammatory cytokine IL-18 is involved in the destruction of cartilage and bone through activation of macrophages [9]. TNF-α, which is released primarily by activated macrophages in patients with inflamed synovial membrane tissue, can induce the production of other pro-inflammatory cytokines such as IL6 and IL1 as well as inducing the release of chemokines, molecules that attract white blood cells to the site of inflammation [10].

Human population studies have shown that a combination of environmental (e.g. smoking and infection) and genetic (e.g. protein tyrosine phosphatase non-receptor 22 [PTPN22] and HLA-DRB1) factors promote the risk of developing RA. One of the most significant genetic risk factors for RA is variation in human leukocyte antigen (HLA) genes, particularly *HLA-DRB1*. The product of this gene presents short antigenic peptides to helper T cells and so helps the immune system recognise proteins produced by foreign invaders such as viruses and it is also involved in the generation of tolerance to prevent autoimmunity [11]. In addition, variants of the non-HLA gene *PTPN22* have been linked to a variety of autoimmune conditions including RA, systemic lupus erythematosus (SLE) and type 1 diabetes, but the underlying mechanisms are not fully understood [12].

The *PTPN22* locus encodes the lymphoid protein tyrosine phosphatase (Lyp), which is found in immune cells and acts as a negative regulator of B cell receptor and T-cell receptor signalling with this regulation being dependent on its dephosphorylation of several mediators of the TCR signalling pathway [13]. Thus, while a broad range of genetic loci may have some influence in promoting RA, *PTPN22* and *HLA-DRB1* seem to be the most important genes in the pathogenesis of RA.

Current treatment for RA focuses on pain relief, reduction of the inflammation and, more importantly, protection of the joint structure. This can be achieved by a range of disease-modifying anti-rheumatic drugs (DMARDS) such as methotrexate and sulfasalazine which act on a number of targets including cytokines, immune cells and by inhibiting matrix degrading factors and blocking inflammatory molecule receptors or their transcription factors [14]. However, it has been reported that smoking may not only increase the risk of RA development, it may also induce resistance to TNF alpha antagonists and thus poor therapeutic responses [15]. Therefore, it is crucial to understand the molecular effects of cigarette smoke, in particular where those impinge upon signalling pathways such as those initiated by HLA-DR and regulated by PTPN22, in order to better understand the pathology and to help design novel strategies that may provide effective treatments for RA patients.

1. Causes of RA development

1.1 Genetic factors

1.1.1 RA and HLADRB1

Genetic factors contribute 60% of the risk of development of RA [16]. HLA class II which encodes HLA DRB1 alleles that contain the shared epitope (SE) is the largest genetic risk factor for RA [17]. HLA class II is involved in the presentation to T cells of processed antigen during thymic selection and in the periphery [18]. The so-called ”shared epitope” (SE) is a five amino acid sequence motif at positions 70–74 in the region of the HLA-DRβ chain that is carried by alleles associated with RA and thus the majority of RA patients [19]. HLADRB1 genes encoding the SE increase the severity of RA and recently the SE has been linked with production of anti-citrullinated protein antibodies (ACPAs) [20].

Kerlan-Candon *et al.* showed that RA patients had variations in the genetic expression of *DRB1*01* and *DRB1*04*, and that healthy RA patients had reduced expression of *DRB1*X* genes. Patients with double shared RA-associated alleles expressed significantly higher DRB1 transcripts, while patients without these genes or with single shared RA-associated genes expressed lower levels of the transcript [21]. A recent meta-analysis and systemic review was conducted by Liu *et al.* to explore which *HLADRB1* alleles are associated with RA: a review of 40 studies and 331 articles (with 5837 control patients and 5470 RA patients) revealed that *HLADRB1*14* and *HLADRB1*04* were associated with RA and expressed at higher levels in the RA group than in the controls [22]. Moreover, a study found that *HLA-DRB1*01*, *HLA-DRB1*04* and *HLA-DRB1*14* were strongly associated with RA susceptibility and showed a significant increase in frequency in RA patients [23]. However, the associations

between *HLADRB1* genotypes and RA and other factors that influence *HLADRB1* gene expression remain unclear.

It has been shown that the association of *HLA-DRB1* SE with RA is stronger in ACPA positive RA than in ACPA negative RA, suggesting that HLA might increase the presentation of citrullinated autoantigens [24]. The mechanism of citrullination is a conversion of positive charged arginine into a no charge citrulline through an enzyme called Peptidylarginine deiminases (PADs). Therefore, the amino acids with positive charge in the *HLA-DRB1* could prevent the arginine-containing peptides being presented by the RA-associated MHC alleles and facilitate citrullinated peptides presentation leading to the ACPA development and subsequently increase the risk of RA [25]. ACPA can be involved in RA pathogenesis following previous infections. For instance, *Aggregatibacter Actinomycetemcomitans* (Aa) produced leukotoxin A which forms pores in the membrane of neutrophils which led to hypercitrullination in host neutrophils, resulting in citrullinated autoantigen release in the gum, suggesting that this kind of bacterium may trigger RA [26]. ACPA also binds to surface-expressed citrullinated Grp78 on monocytes and macrophages and so enhances the activity of NF-kB and TNF-α production which may also help drive RA development [27].

1.1.2 RA and PTPN22

The PTPN22 gene encodes lymphoid-tyrosine phosphatase (Lyp) and its mouse homologue proline enriched phosphatase (PEP). Lyp is expressed in hematopoietic cells and negatively modulates TCR signalling [28] through the dephosphorylation of several substrates, including Syk family Zap70, and the Src kinase FynT and Lck (Figure 1.1) [29]. It also plays a number of other roles in the cell and so genetic or chemically-induced changes in its activity could have profound effects on cell function. A study conducted on 475 patients with RA and 475

matched controls in North America found that a variant of PTPN22, R620W (W620 allele), was strongly associated with RA [30]. In addition, polymorphism of the PTPN22 allele C1858T, rs2476601 was associated with RA in the Italian population, but the frequency of the T1858 allele in the Italian population was lower than that reported for Northern Europe [31]. In contrast, such an association has been reported to be completely absent or weaker in some Southern European populations such as in Turkey [32] and Spain [33]. Moreover, this association was reported to be absent in Greek [34] and Tunisian populations [35]. Thus, it is necessary to take into consideration the geographical distribution of single-nucleotide polymorphisms (SNPs) when studying the genetic associations of this disease.

Even though PTPN22 was found to be associated with a wide range of autoimmune conditions, particularly RA and SLE, several studies have shown weak or no association of other autoimmune conditions with PTPN22. For instance, Begovich *et al*. showed that there was no association between multiple sclerosis (MS) and the *PTPN22 T1858* allele [36]. This lack of association was also supported by a genome-wide association study [37]. Furthermore, with regard to celiac diseases that affect the gastrointestinal tract, two studies found no association between celiac disease and *PTPN22 T1858* [38, 39], and a very weak association between psoriasis and the *PTPN22 T1858* allele [40]. The lack of association of some diseases with PTPN22 may be because the skin and gastrointestinal tract (in celiac diseases), which are the most common disease targets, have exposed surfaces, and are therefore more likely to be affected by environmental factors, for example, smoking and infection. Thus, the findings so far show that PTPN22 is particularly associated with RA, but not with several other autoimmune diseases.

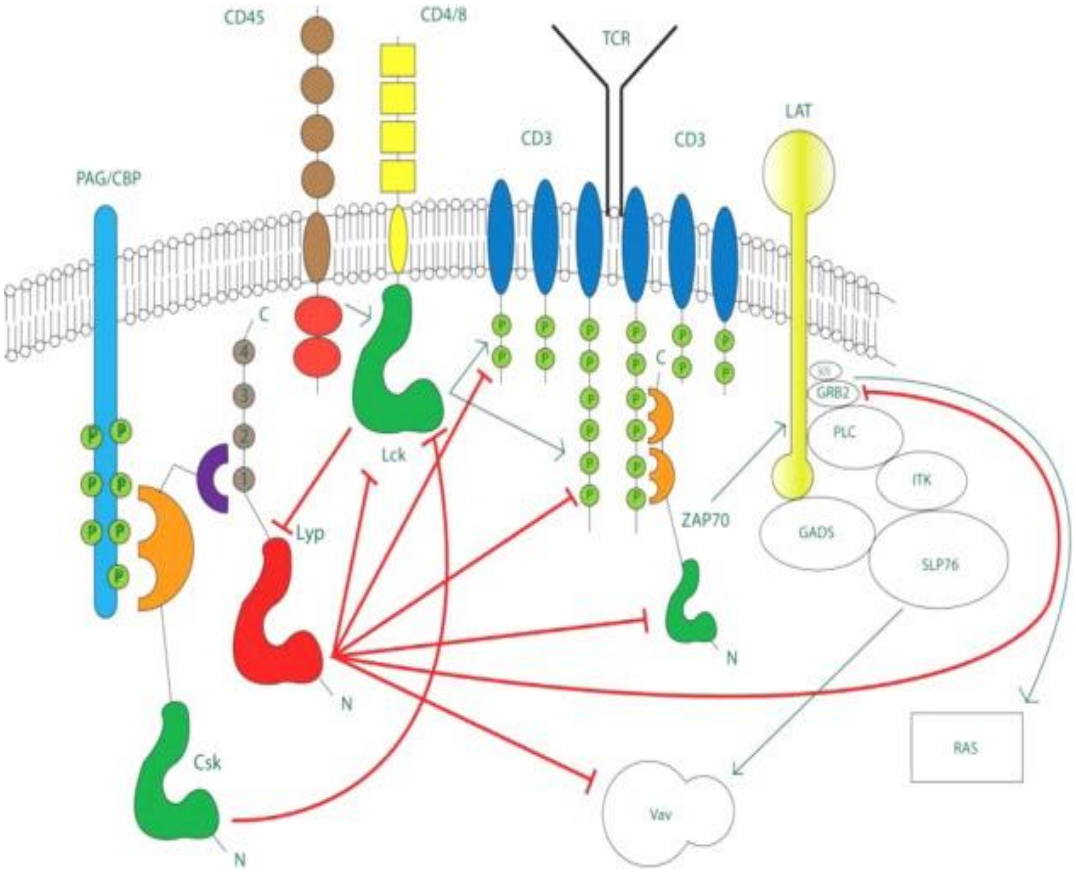


Figure 1. 1 The critical role of Lyp in regulating TCR signalling.

Lyp prevents T cell hyperactivation by dephosphorylating several substrates, including Syk family Zap70, and the Src kinase FynT and Lck. Lack or deletion of Lyp may cause autoimmune diseases. This was adapted from Burn et al.

1.1.3 RA and other susceptibility genes

Apart from HLA and PTPN22, several other genes (up to 100) have been associated with risk of RA many of which are involved in immune function. For instance, STAT4 (signal transducers and activators of transcription) [41], a transcriptional factor which mediates the intracellular signal activation in response to a number of different cytokines such as IL12 and IL23 in T cells and monocytes [42]. STAT4 rs7574865 T allele was connected with RA in Asian and white or European populations, and also associated with RA in the presence of anti-CCP [43]. Also, this polymorphism increases the risk of both RA and systemic lupus erythematosus (SLE) in Colombian populations [44]. However, the exact mechanism of how the polymorphism contributes to disease pathogenesis is still unknown.

TNFAIP3 is another gene which has been associated with RA and other autoimmune diseases. It encodes ubiquitin-editing protein A20, which plays a negative role in T cell activation and inflammatory signalling by inhibiting nuclear factor κB (NF-κB) [45]. TNFAIP3 knockout mice showed severe destructive polyarthritis with many features of rheumatoid arthritis, indicating the regulating role of TNFAIP3 in inflammation [46]. Polymorphisms in this gene increase the susceptibility and risk of RA in different ethnic group, in Asians rs10499194 and in Europeans rs6920220 [47]. In contrast, it has been observed that decreased MiR-128-3p might supress RA progression by upregulating the expression of TNFAIP3 [48].

TNF receptor-associated factor 1 (TRAF1) gene is located in chromosome 9 and encodes TRAF1 protein. TRAF1 has been implicated in susceptibility of RA by mediating the signal transduction through TNF1/TNF2 receptors, and forms a heterodimeric complex with TRAF2, which is required for the activation of NF-κB and MAPK8/JNK [49]. C5 is a vital complement

component involved in cell killing processes and inflammation, which has been associated with development of RA. Therefore, TRAF1/C5 could possibly contribute to the pathogenesis of autoimmune diseases RA [49]. It has been reported that the genetic variants within the *TRAF1/C5* region increases RA severity by influencing structure and thus function of both TRAF1 and C5 [50]. Single-nucleotide polymorphisms (SNP) in TRAF1 and C5 might contribute in the RA pathogenesis by altering inflammatory pathways. For instance, by increasing C5a and C5b generation and increasing TNF-mediated activation of NF-κB [51]. A large number of genome wide association studies (GWAS) have led to the implication of as many as 101 genetic loci in RA and a broad range of expressed genes have been added to the list of genetic variants most relevant to RA (summarised in table 1.1) [52].

Candidate genes	Encoded protein	Locus	SNP ID	Relative risk	Relative risk range
HLA-DRB1	Class 2 human leukocyte antigen	6p21	rs6910071	2.8	(2.73-3.03)
PTPN22	Protein tyrosine phosphatase, non-receptor type 2	1p13	rs2476601	1.9	(1.81-2.08)
STAT4	Signal transducer and activator of transcription 4	2q32	rs7574865	1.1	(1.10-1.23)
TNFAIP3	Tumour necrosis factor, alpha-induced protein 3	6q23	rs6920220	0.9	(0.87-0.96)
TRAF1/C5	Tumour necrosis factor receptor-associated factor 1	9q33	rs3761847	1.1	(1.08-1.18)
IL2RA	Interleukin-2 receptor alpha	10p15	rs2104286	0.9	(0.87-0.97)
CCR6	CC chemokine receptor 6	6q27	rs3093023	1.1	(1.06-1.16)

Table 1. 1 The most relevant susceptibility alleles in rheumatoid arthritis of European individuals.

It shows the SNP polymorphism of each locus of each gene. HLA-DRB1 is largest genetic risk factor for RA with high relative risk 2.8. PTPN22 is the second risk factor with relative risk 1.9. STAT4, TRAF1 and CCR6 have the same relative risk 1.1, whereas TNFAIP3 and IL2RA have 0.9 relative risk

1.2 Environmental factors

1.2.1 Smoking and RA

As stated previously, genetic factors account for approximately 60% of the risk of developing RA and the other 40% is attributed to environmental factors. Many environmental factors such as cigarette smoke, diet, ageing, previous infection and road traffic particulates have been linked to the risk of RA pathogenesis [53]. These factors could alter the immune system response and modulate signalling through immune cell receptors.

One of the most striking of these environmental factors is cigarette smoking which has been known for many years significantly increase the risk factor for RA [54]. Cigarette smoke is an extremely complex mixture of chemicals and so it could be thought that it was inevitable that regular dosing with such a toxic mixture would lead to some level of biochemical or genetic damage thus promoting the disease. However, the picture may be more complex since there seems to be no link between cigarette smoking and another widespread inflammatory joint disease, that is gout [55].

In fact, there seems to be protective effect against this condition which also seems to be the case for Behcet's disease [56, 57]. That there may be a special effect of smoking in RA was suggested by a Swedish study which found an environment–gene interaction between the HLA-DBR1 shared epitope (SE) genotype and smoking, with the risk of RA being higher in smokers carrying double shared epitope alleles or single shared epitope alleles [58]. This work also found a link to another risk allele, protein tyrosine phosphatase non-receptor 22 [PTPN22] gene. Given that the HLA-DRB1 product, the MHC class II protein determines responses through T cell antigen receptors and PTPN22 through signalling by these

receptors. This suggests that T cells responses may well be the critical target for perturbation by cigarette smoke in promoting RA.

1.2.1.1 Smoking and oxidative stress

Smoking is known to increase oxidative stress, and it has been shown that oxidative stress could increase the risk of RA by reducing the amount of antioxidants, such as catalase and glutathione reductase, through the production of reactive oxygen species (ROS) and free radicals [59]. In an animal model, it was found that cigarette smoke, which is known to contain several chemical compounds including oxidants, free radicals and particularly nicotine, induced the production of high levels of ROS, which activated the transcription factor NF-κB and led to an inflammatory response [60]. The toxicity of cigarette smoke could be due to a combination of these chemical compounds, which may induce cellular processes mediated by ROS. Epidemiological studies have shown that heavy smokers, males and females, have an increased risk of RA development after long-term smoking [61, 62], whereas recent evidence shows a connection between the development of RA and smokers with low lifelong exposure (light smoking) [63].

1.2.1.2 Smoking and ACPA

Smoking increases the production ACPA, which are highly specific for RA. A case-control study found that cigarette smoke increased the risk of ACPA positive RA [64]. Furthermore, consecutive sera samples from patients with RA have shown that cigarette smoke increased the anti-CCP titres in patient with RA [65]. Another study that included 20 non-smokers, 11 heavy smokers and 14 mild-to-moderate smokers showed that the anti-CCP antibody levels were significantly higher in heavy smokers than in mild-to-moderate smokers and were

lower in non-smokers [66]. However, the mechanism via which smoking drives effector cells, particularly B cells, to produce antibodies against CCP is still unclear.

1.2.1.3 Smoking and infection

Although cigarette smoke has a potential role as an immunostimulant for the development of many conditions, it also plays immunosuppressive roles through its chemical constituents, particularly nicotine, which enter the blood stream and travel to different parts of the body. For instance, when the lungs absorbed nicotine, the concentration of nicotine in the blood will be increased and can reach the brain very fast (10-20 second) [67]. Based on human autopsy from smokers, the nicotine highest affinity was in the spleen, liver, kidney and lung, suggesting that nicotine can reach to different parts via the blood stream [68]. The immunosuppressive effect may increase susceptibility to viral (e.g. EBV) or bacterial (e.g. *Streptococcus spp*.) infection and may thereby contribute to the pathogenesis of RA. Pratesi *et al.* reported that anti-CCP antibodies that are highly specific for RA patients could interact with citrullinated EBV nuclear antigen, suggesting that chronic smoking might induced previous EBV infection (superantigen) [69]. RA is also linked to *Porphyromonas gingivalis,* which can cause periodontitis, the incidence of which is known to increase as a result of cigarette smoking [70]. *P. gingivalis* contains an enzyme that is similar to the peptidyl-arginine deiminase enzyme in humans, as a result of which the infection might result in the production of citrullinated proteins, the activation of autoimmunity and consequently RA [71]. Thus, cigarette smoking could aid the infection to promote RA, but the current evidence focused on very few organisms. More studies need to be done on other microorganisms and their relationship with RA pathogenesis.

1.2.1.4 Smoking and anti-rheumatic drugs

Since high levels of cytokines, particularly TNF alpha and IL1, play a critical role in RA development, it is possible that cigarette smoke increases the ratio of TNF alpha /soluble TNF receptors in smokers. This high ratio may be linked with resistance to treatment with TNF alpha antagonists [72]. In addition, the soluble IL2 receptor level is higher in smokers; this may influence the response of RA patients to treatment with TNF alpha antagonists such as infliximab. This is because the IL-2-sIL-2 receptor and smoking have been correlated with higher activity of the disease which could impact the drug efficiency [73]. Hyrich *et al.* found poor response to TNF alpha antagonists, especially infliximab, in RA patients who smoke [74].

Following this study, Mattey *et al.* reported that RA patients with a history of smoking showed a reduced clinical response to TNF therapy with infliximab [75]. However, no study has examined the effect of cigarette smoking on rituximab or tocilizumab, although it seems that RA patients who smoke could develop resistance or poor response against anti-rheumatic drugs. This might be due to the higher basal metabolic rate in patients who smoke that is, the metabolism of the TNF antagonists may be accelerated in a state of high metabolic activity, and therefore, a high dose of anti-rheumatic drugs is required.

1.2.1.5 Smoking and genetic factors

In a study on the environment-gene interaction between HLA-DR SE alleles and smoking, Klareskog *et al*. found that smoking could trigger and increase immune reactions against citrullinated protein in the presence of the HLA-DRB1 SE allele [76]. Further, a case-control study proved that cigarette smoke increases the risk of ACPA in patients who are homozygous for the SE alleles [77]. Furthermore, interaction between smoking and the HLA-

DRB SE subtypes, including HLA DRB1 0102, HLA DRB1 0102 and HLA DRB1 1001, was found to be correlated with a higher level of ACPA in RA patients and with disease severity [78]. Another study conducted in a French population showed that there was an association between cigarette smoke and high levels of ACPA in the presence of HLA- DRB1 SE alleles, particularly HLA-DRB1 0401 [79]. These data indicate that cigarette smoke increases the production of anti-CCP antibodies only in SE allele-positive patients; this means that SE is not a risk factor for ACPA negative patients. However, further gene-environment studies are required to confirm the outcomes of these studies.

With regard to the environment-gene interaction between PTPN22 and smoking, a study on two cohorts of women conducted by Costenbader *et al*. reported a significant multiplicative interaction between the polymorphism of PTPN22 R620W and heavy cigarette smoking [80]. However, Kallberg *et al*. investigated gene-gene and gene-environment interactions in the development of RA by combining three large case-control studies–one North American, one Dutch and one Swedish study. The study found no significant association between cigarette smoking and the PTPN22 polymorphism R620W in the absence of HLA-DRB1 SE. However, combination interactions between PTPN22 R620W and HLA-DRB1 SE and smoking could drive RA with a very high odds ratio between 20 and 25 was observed [Figure 1.2] [81]. Thus, while PTPN22 is a risk factor for RA, the mechanisms via which cigarette smoking derive PTPN22 gene to lead to the development RA are unclear, as very few studies have investigated this interaction.

To conclude, there are several genetic and environmental risk factors that play a crucial role in RA pathogenesis, and these factors seem to have an interactive effect in this disease. In particular, smoking is one of the most common extrinsic factors that affects genetically

susceptible individuals carrying *HLA-DRB1* SE alleles. It has been reported to increase the levels of pro-inflammatory cytokines such as TNF-α and IL1, oxidative stress and autoantibody production, leading to some changes in the immune system and subsequently causing autoimmune diseases, including RA and MS. Furthermore, cigarette smoke has also been shown to have an immunosuppressive role, which may increase susceptibility to bacterial and viral infections and thereby contribute to RA pathogenesis. In addition, PTPN22 polymorphisms are significantly associated with a wide range of autoimmune conditions, including type 1 diabetes, SLE and RA. However, the association between PTPN22 and smoking is unclear, and further studies are required to investigate this relationship.

The currently available anti-rheumatic drugs are considerably effective in the treatment of RA, reduction of pain and swelling, and improvement quality of life, but as discussed, many studies have found that the activity of these drugs might be affected by smoking and lead to poor response. Further studies are needed to investigate the underlying mechanisms of RA to develop more effective drugs.

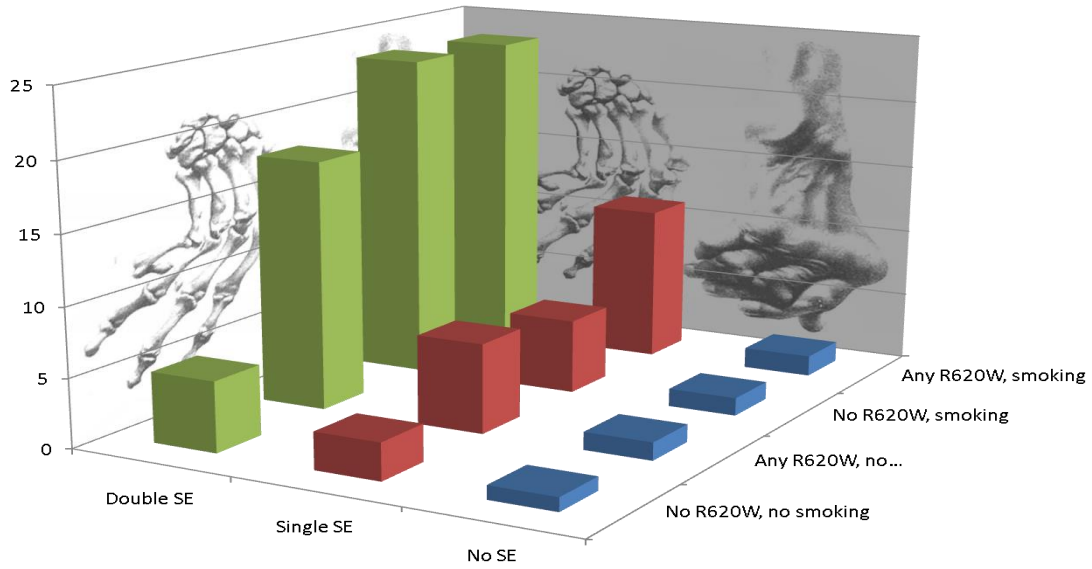


Figure 1. 2 The relations between smoking and genetic polymorphisms (PTPN22 R60W, HLA-RB1) to drive rheumatoid arthritis.

A combination interaction between PTPN22 R620W and HLA-DRB1 SE and smoking could drive RA with a very high odds ratio between 20 and 25 was observed. Adapted from Kallberg et al

1.3 Mechanisms involved in cigarette smoke triggering RA.

1.3.1 Chemical reactions of cigarette smoke

Cigarette smoke contains a mixture of more than 5000 compounds including carcinogens, gaseous substances (carbon monoxide), free radicals and predominantly nicotine. Aldehydes, oxidants, alpha and beta carbonyl including crotonaldehyde and acrolein are primary components in gaseous phase of tobacco smoking, are mediating oxidative stress that has been contributing in the development of many conditions such as inflammation and COPD [82]. It has been reported that crotonaldehyde and acrolein could react with glutathione (GSH), through Michael-type addition reaction, leading to formation of non-reducible GSH- aldehyde derivatives and depleting GSH pool [83]. This depletion of GSH results in cigarette smoke-induced cytotoxicity due to the fact that GSH plays an important role in cellular antioxidant defence [84]. Methyl vinyl ketone (MVK) also might react with GSH via Michael-type addition. The MVK-induced cell apoptosis was associated with increased generation of reactive oxygen species, mitochondrial transmembrane potential and GSH depletion [85].

1.3.2 Carbon monoxide

In addition, carbon monoxide (CO) is one of the most important components of cigarette smoke which could increase the incidence of cardiovascular and inflammatory diseases. It causes oxygen reduction in the tissue through interaction with hemoglobin to form carboxyhemoglobin (COHb) [86]. CO also binds to cytochrome and myoglobin, leading to impairment of their ability to utilize the oxygen [86]. Acrolein is a toxic aldehyde compound containing carbon-carbon double bond [87]. It causes perpetuated oxidative stress and

oxygen free radical formation by reacting with sulfhydryl groups, resulting in depletion of protein sulfhydryl concentrations and ascorbic acid levels [88].

1.3.3 Benzol[a]pyrene

Benzol[a]pyrene (B[a]P) is a major toxic component of cigarette smoke. It has been shown that B[a]P can stimulate the expression of Slug (transcription factor which plays a role in impairing apoptosis of RA FLS) in rheumatoid arthritis fibroblast-like synoviocytes (RA FLS) through the P13K/AKT /mTOR pathway [89]. Furthermore, it has been shown that B[a]P increased the level of p53 mRNA and could activate the p53 promoter in NIH 3T3 and lung epithelial A549 cell lines through induction of NF-κB [90]. Thus, p53 might be an important mediator for the effect of B[a]P on Slug expression in rheumatoid arthritis fibroblast-like synoviocytes. However, further investigation is needed to confirm this hypothesis.

1.3.4 TCDD

Another toxic component in cigarette smoke is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which has been found to upregulate the production of pro-inflammatory cytokines (IL-8, IL-6 and IL-1-beta) through binding to the aryl hydrocarbon receptor (AHR), which leads to stimulation of the NF-κB and ERK signalling cascades. Therefore, exposure to the cigarette component TCDD exacerbates RA pathophysiology (Figure 1.3) [91]. Smoking activates the AHR pathway in synovial tissue by elevating (Aryl-Hydrocarbon Receptor Repressor) AHRR and CYP1A1, which consequently impacts upon Th17 differentiation and alteration of synovial dendritic cell responses in the joint. These changes in DC responses might indicate a potentially relevant mechanism between the early and late phases of RA disease [92].

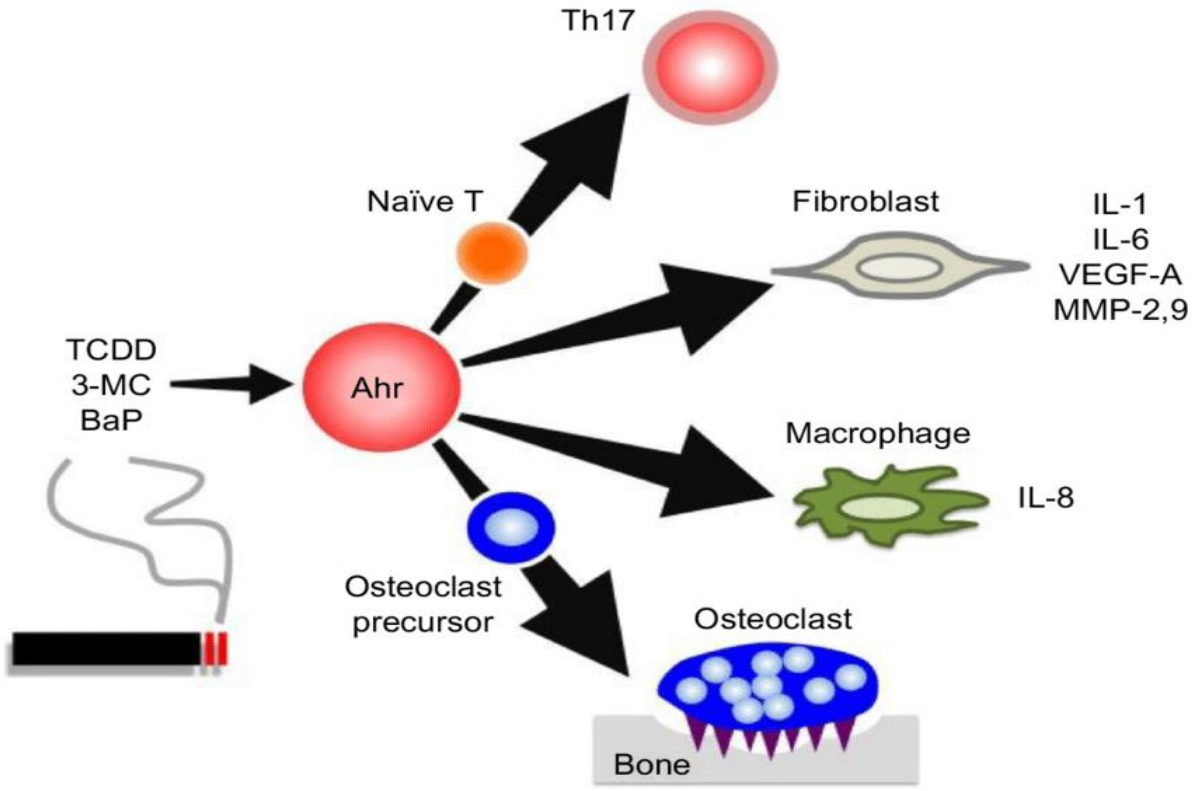


Figure 1. 3 The interactions between cigarette smoking components and AHR to drive RA.

TCDD could bind to the Ahr leading to T cell differentiation and secretion of pro-inflammatory cytokines such as IL-6, IL-1. It also could affect other cells such as macrophages to produce more IL-8, and osteoclast. This figure was adapted from Kobayashi et al.

1.3.5 Hydroquinone

Hydroquinone (HQ) is compound of the particle phase of cigarette smoke and a benzene metabolite. Rats in which CIA had been induced were exposed to HQ which resulted in enhanced serum ACPA, TNF-α and IL-1β levels, augmented influx of AHR positive cell and synoviocytes proliferation into synovial membrane, suggesting that HQ could participate on worsening RA in smoking patients [93]. Following this study, the same group found that HQ in vivo increased the levels of IL6 and cell infiltration in the synovial fluid, resulting a higher frequency of IL17+ and AHR+ neutrophils in the joint, suggesting that HQ may aggravate RA during early phase of CIA through AHR+/ IL17+ neutrophils pathway [94]. This change in the neutrophil phenotype after HQ exposure leads to increase the expression of platelet-endothelial cell adhesion molecule-1 (PECAM-1) and β2 and β3 integrins in circulating neutrophils which promote MMP13 production that has been implicated in RA pathogenesis [95, 96].

1.4 Cellular effects of cigarette smoke related to RA

1.4.1 Cellular mechanisms of cigarette smoke on T cell function

A recent study demonstrated that CSE downregulated Rho-associated coiled-coil containing protein kinase 2 (ROCK2) that phosphorylates IRF4 [97]. This effect on the ROCK2-IRF4 axis in T cells was associated with increased production of IL-22 and a decrease in the activation of Rho guanine nucleotide exchange factor 1 (ARHGEF1), an upstream regulator of RhoA [97]. Consequently, CSE could modulate T cell production of IL-22 by inhibition of ROCK2-IRF4, leading to development of RA. IL22 produced by CD4+ T cells under Th17-skewing conditions increased joint destruction in mice, whereas neutralising IL22 production reduced disease incidence, revealing the important role of IL22 in RA pathogenesis [98]. It has been shown

that cytotoxic CD8+ T cells might contribute to the development of autoimmune conditions such as autoimmune arthritis [99]. Therefore, a study was done to investigate the link between smoking and non-exhausted CD8+ T cells, defined as cells lacking the programmed cell death 1 (PD1) protein. The study found that smoking or its component nicotine induce accumulation of non-exhausted state of CD8+ T in the bone marrow, resulting in higher levels of serum survivin in smokers. [100]. The primary function of survivin is to inhibit apoptosis by inhibiting the caspase pathway leading to an increase in T cells infiltration and cytokine overproduction [101]. Elevation of serum survivin in RA patients is associated with poor anti-rheumatic response, and premature severe joint damage [102].

Several studies have indicated that inflammatory and autoimmunity reactions can occur systemically in patients with APCA-positive rheumatoid arthritis (RA) before developing any clinical signs of joint disease [103, 104]. Vassallo et al used transgenic mice expressing RA susceptibility genes including HLA-DQ8 and HLA-DR4 to demonstrate that cigarette smoke exacerbated collagen-induced arthritis (CIA) in mice carrying the DQ8 molecule through increasing antigen-specific T cell responses to citrullinated and native proteins. Cigarette smoke increased the expression of co-stimulatory molecules on lung dendritic cells and increased IL-17 and IL-10 gene expression in the lungs of DQ8 mice as well as other inflammatory cytokines such as IL-13 and TSLP. Interestingly, cigarette smoke suppressed CIA in DR4 mice, implying that gene-environmental factors might vary for DQ and DR molecules [105].

1.4.2 Cellular mechanisms of cigarette smoke on lung cells

Smoking increases the expression of PAD 2 enzymes in the alveolar and bronchial mucosal compartments in smokers leading to the generation of citrullinated proteins (arginine to

citrulline), and inducing citrullination in bronchoalveolar lavage (BAL) fluid in smokers as a potential first pathogenic step in the development of RA [106]. It has been found that smoking augmented the production of CXCL8 and induced the generation of prostaglandin-E2 and cyclooxygenase 2 (COX 2) in human maturing DC, suggesting that stimulated DC in the lung drives the induction of pro-inflammatory reaction which promote chronic inflammation in smokers [107]. These chronic inflammatory processes occurred in the lung of smokers promoting PAD2 and PAD4 to produce citrullinated protein and then production of ACPA which, in turn, increase the possibility of RA development [108].

In addition, an increase in the concentration of intracellular calcium could influence this citrullination reaction [109]. Smoking induces apoptosis of alveolar macrophages and a resultant elevation of intracellular calcium, suggesting a potential association between smoking and citrullination induction [110]. The lungs of RA patients who smoke exhibit pathogenic features such as follicular B-cell hyperplasia, which might contribute to the production of ACPA. Citrullinated peptides bind to antibody complexes through Fc receptors, which may increase production of pro-inflammatory cytokines such as IL-6 and TNF-alpha [111]. Furthermore, in the lung, T cells are recruited to support B cell differentiation and activation following antigen exposure [111].

While smoking is a major factor for RA, lung mucosal disequilibrium with inflammation and immune activation has been suggested as playing a role in the pathogenesis of RA. Mucosal disequilibrium is promoted by smoking through multiple mechanisms including chemoattraction of neutrophils, and activation of macrophages and DCs leading to production of citrullinated proteins and inflammation initiation [112]. Modulation of the

activity of Toll-like receptors (TLRs) and upregulation of CXCL8 (IL8) [113], with apoptotic cell impairment by macrophages are also important mechanisms [114].

1.4.3 Cellular effects of cigarette smoke on synovial fibroblasts

A recent study was conducted by Engler et al showing that cigarette smoke extract (CSE) increased the expression of sirtuins 6 (SIRT6) protein in rheumatoid arthritis synovial fibroblasts (RASFs). Subsequently, this resulted in enhanced production of the matrix-destructive and pro-inflammatory cytokines of RASF through an increase in the production of MMP1 and IL-8 but not MMP3 and IL-6. This suggests that SIRT6 produced by RASF through exposure to CSE regulates the production of MMP1 [115]. Furthermore, CSE decreased histone deacetylase 2 (HDAC2) in RASF, associated with hyper-acetylation and therefore enhanced expression of the gene regulated by HDAC2 [116]. Adachi et al showed that cigarette smoke condensate stimulates production of IL-1β mRNA from RA patient-derived synoviocytes and from the MH7A cell line, through novel NF-κB sites and the AHR-dependent NF-kB activation in the IL-1β promoter region [117].

To conclude, there are several genetic and environmental risk factors that play a crucial role in RA pathogenesis, and these factors seem to have an interactive effect in this disease. In particular cigarette smoke and its components (in particular nicotine) is an important risk factor for many diseases including RA. More recently, several studies have proposed different mechanisms by which smoking may contribute to the development of RA. The evidence showed that smoking increases the risk of ACPA production against citrullinated protein produced by PAD 2 enzyme in SE RA-positive patients. Furthermore, it induces production of many pro-inflammatory cytokines such as TNF-alpha, IL-6, and IL-8 as well as

inducing activation of macrophages and DCs in the lungs of RA patients. Moreover, it induces IL-22 by inhibition of ROCK2-IRF4, and induces NF-κB and ERK signalling through binding to the AHR to stimulate several cytokines such as IL-1β, which is involved in RA pathogenesis.

Several studies have linked the process within the lung to the production of ACPA several years before any sign of joint damage, suggesting that the development of autoimmunity might occur outside the joints. Although there are numerous studies, which demonstrate how cigarette smoke contributes to RA pathogenesis, the exact mechanisms have not been fully elucidated. These mechanisms may be influenced by many different pathways and factors. Therefore, it is important to understand the processes involved in these pathways in order to design novel strategies that provide effective treatments for RA patients. This is especially important for those patients who have typically poor responses to existing treatment regimens.

1.5- Immune cells involved in RA

The immune system employs a collection of molecules, cells and chemicals that have the function to destroy foreign pathogens such as bacteria and viruses. The immune system has the ability to avoid damaging self-tissues under a process called self-tolerance, so failure to maintain self-tolerance often leads to a broad range of autoimmune diseases [118]. Innate immunity is the first line defence and plays an immediate role against pathogens by recruiting immune cells such as macrophages and neutrophils to sites of inflammation through chemokines and cytokines production. Many of the cells involved in innate immunity are phagocytes which ingest foreign particles and inactivate them. Therefore, dysregulation in innate immune system is often associated with autoimmune and inflammatory conditions [119].

Unlike the innate immunity mechanisms defence, the adaptive immunity defence is able to recognise and distinguish between non self and self-antigens, and has memory which allows quicker responses to any subsequent repeated infections. The adaptive response is primarily based on antigen-specific T cells (T lymphocytes) which expressed specific T cell receptors (TCRs), and B cells (B lymphocytes) which expressed specific B cell receptors (BCRs) which can differentiate into plasma cells to produce antibodies [120]. Following TCR engagement with APC, T cells proliferate to expand their activity and differentiate into various subtypes including T helper $CD4^+$ cells, memory, cytotoxic $CD8^+$ T cells and regulatory T cells [121]. B cells can differentiate into a plasma cells secreting antibody or memory B cells which can directly recognise the antigen without the need for parallel recognition by a TCR. In addition, B cells can act as APCs under certain circumstances [122].

1.5.1 T Lymphocytes

1.5.1.1 T Cell biology

1.5.1.1.1 Naïve T cells

The naïve T cell, which represents a new emigrant from the thymus, has been considered as developmentally fairly quiescent and homogeneous cell population. However, an increasing number of studies have revealed that naïve T cells are heterogeneous in terms of function, phenotype and differentiation status. Many factors may influence this heterogeneity including total number of T cells, age and thymic function [123]. T cells enter to the peripheral naive T cell compartment following the process of positive and negative selection in the thymus. In the periphery, these cells recirculate between blood and lymph node a process dependent on their expression of surface receptors CD62 ligand (CD62L; also known as L-selectin) and CC-chemokine receptor 7 (CCR7) [124]. In response to newly encountered

pathogens in peripheral lymph nodes, naïve T cells will start to differentiate and proliferate into effector cells. The proliferation and the survival of naïve T cells are enhanced by homeostatic factors such as self-peptide MHC complexes and IL-7. These factors turnover the naïve T cells pool, and do not promote naïve T cells to differentiate into effector cells [125].

Human naïve T cells are identified by their expression of the surface marker CD45RA, but not CD45RO which is expressed in memory T cells. In addition, naïve T cells are required to express at least one extra marker e.g. CD27, CCR7 and CD62L [126]. After egressing from thymus, they are described as recent thymic emigrants (RTEs) which express high levels of CD31 and protein-tyrosine kinase 7 (PTK7). RTEs are different in function from mature naïve cells because they show reduced cytokine secretion and proliferation following stimulation. However, RTEs which are stimulated by cytokines lose the expression of PTK7, but will maintain the expression of CD31 [127].

Mature naïve T cells exhibit higher cytokine secretion, proliferation and CD25 expression following cognate antigen encounter than RTEs (6). Any trigger of naïve T cells proliferation or activation might lead to epigenetic changes and differentiate into several subtypes, including Th1, Th2, Th9 and Th17. Thus, diseases and environments might set up naive T cells for lineage differentiation [128]. In adults, the $CD4^+$ naïve cells proportion decreases with age in spleen, lymph nodes and blood [129]. However, there is some evidence showing that the naïve T cells life span increases with age in mice, and there is increased division of naïve T cells in human at an older age [130].

1.5.1.1.1.1 Th1 cells

In response to an intracellular pathogen, (IFN)-α/β and IL-12 induce the expression of the transcription factor T-bet (T-box expressed in T cells) which promotes polarisation of T cells towards the Th1 subset [131]. Th1 tend to secrete pro-inflammatory cytokines which regulate the host defence against infection by initiating the inflammatory response [132]. However, excessive production of inflammatory cytokines may contribute to uncontrolled tissue damage and autoimmunity. Th1 characteristically produce IFN-γ and TNF-α which activate macrophages to increase their phagocytic capacity, and also feed back into the general cytokine milieu [133]. STAT molecules could play a role in Th1 polarisation since IL12 activated STAT4 which mediates the upregulation of IFN-γ, resulted in Th1 polarisation. However, impaired STAT4 or IL12 responses in mice resulted in a similar phenotypes [134].

1.5.1.1.1.2 Th2 cells

IL4 plays a critical role in T cell differentiation into the Th2 subset. Th2 cells secret large amounts of IL4, IL13 and IL5 which activate eosinophils, basophils, and mast cells which target parasites. These cytokines produced by Th2, are responsible for inhibition of macrophages functions, and promote antibody production, including isotypes IgA and IgE which neutralise current and future parasitic and bacterial infections [135]. Th2 are also involved in atopic disorders, progressive systemic sclerosis and idiopathic pulmonary fibrosis [136]. A study showed that the deletion of transcription factor T- bet led to high IL4 levels and asthma-like airway changes. Thus, losing T-bet which is committed to Th1 may induce inflammatory features of asthma associated with Th2. [137]. It has been shown that the transcription factor GATA-3 induced Th2 development and gene transcription of Th2 cytokines [138], and suppressed the expression of IFN-γ and IL-12Rβ2, resulted in decreased

Th1 development, implying that GATA-3 may act as suppressor and activator of signalling molecules and cytokines that are involved in the regulation of Th1 and Th2 differentiation [139].

1.5.1.1.1.3 Th17 cells

Th17 is a unique subset of T helper cells with a distinct production of IL-17 which is a hallmark cytokine, and other cytokines such as IL21 and IL22 [140]. Th17 plays a crucial role in pathogen clearance at mucosal surfaces and maintaining mucosal barriers. However, it can be pathogenic by altering their differentiation program which eventually develop into autoimmune diseases [141]. It has been shown that Th17 plays a role at the early stage of RA development, and it was highly expressed in the patient synovial fluid [142, 143]. This highly expressed Th17 increased the expression of IL17A and IL22, which induced macrophages and synovial fibroblasts to secrete more TNF-α, IL6 and IL1, which increased synovial inflammation by upregulating type 1 collagen and matrix metalloproteinase (MMP) [144, 145]. This suggests that Th17 is an important player not only in immune homeostasis, but also in RA and inflammation initiation.

1.5.1.1.2 Memory cells

Following the natural collapse of a primary response, the surviving T cells with a "memory" for the pathogen are altered in their functional abilities, have less of a requirement for activation and a more rapid effector response [146]. Memory T cells have two subtypes: central memory T (Tcm) cells and effector memory T cells (Tem). Central memory T cells express CD45RO, CD62L and CCR7 and are commonly found in periphery and lymph nodes. Although Tcm cells are rare subset, they exhibit high self-renewal and proliferative capacity,

but no effector function. Tem cells produce more effector cytokines and express CD45RO, but not CD62L and CCR7 lymph node homing receptors and are commonly found in peripheral circulation and tissues where they are known as tissue resident memory cells (Trm) [147]. These subsets can survive for a long life of more than 15 years, providing stronger and faster immune response [148]. Memory cells also express certain levels of Tbet. For instance, low expression of T bet generates long-lived memory cells, whereas high expression generates short-lived memory cells. The expression of T bet can be induced by IFN-γ, leading to enhancement of memory $CD4^+$ T cells development generated from effector cells and naïve cells. Also, IFN-γ could give rise to long-lived effector and central memory T cells [149].

1.5.2 B lymphocytes

1.5.2.1 B cell biology

B cells are characterised by the production of antibodies which protect the body against infections, activate macrophages and complement system. However, these cells also play a critical role in autoimmunity and transplant rejection by presenting antigens to T cells [150]. Since the main function of B cells is to differentiate into plasma cells to produce antibodies, it is important to mention their different isotypes or classes. These isotypes are (IgA, IgD, IgM, IgG, IgE) and they are distinguished based on the C-terminus regions of the heavy chains [151]. As mentioned above, B cells can serve as APCs to activate T cell. B cells + RF can respond to IgG2a immune complexes, leading to taking up this complex through their Ig membrane receptor. This allows B cells to process the peptide generated from the antigen and present it to T cells which may be critical in initiating autoimmune responses [152]. Moreover, mature B cells in both RA and healthy individuals can recognise citrullinated

antigen and present it to T cells. This may lead B cells to generate autoantibodies against citrullinated peptide from antigen and thereby ACPAs may generated which is a crucial in the pathogenesis of RA. The formation of ACPA may be dependent on T cell help during the expansion phase of ACPA [153].

1.5.3 Neutrophils

1.5.3.1 Neutrophil biology

Neutrophils are polymorphonuclear leukocytes which have been considered as first line in the innate immune system defence mechanisms. They invade and destroy the microbial infection through degradation and phagocytosis, and formation of neutrophil extracellular traps (NETs) following pathogens detection [154]. Neutrophils generate large amount of ROS by activating their NADPH oxidase. This ROS is a powerful antimicrobial weapon and is important to eliminate bacterial pathogens. Therefore, deficiency of ROS function results in sever and recurrent infection [155]. This kind of ROS generated by neutrophils can induce the generation of granules and NETs which have antimicrobial and inflammatory properties [156, 157]. After engulfing the bacteria by neutrophils into vessels called phagosome, members of ROS, superoxide and hydrogen peroxide are released and promote the death of the microbes [158].since neutrophils play a role in synovial cavity infiltration, a study showed that high level of PAD is expressed in neutrophils and accumulate in the synovial fluid in RA. The study found that PAD was released into synovial fluid by neutrophils after undergoing NETosis in the joints, leading to the generation of extracellular citrullinated autoantigens in RA patients and increase the severity of the symptoms [159].

1.5.4 Role of smoking and PTPN22 in immune cells

1.5.4.1 Effect of cigarette smoke on T cell populations

It has been shown that the naïve T cells may contribute to the development of some diseases. For instance, a study found that the naïve T cells could contribute in the development of cancer by expressing high levels of CXCL8 (also known as IL8) which facilitates the migration and infiltration of neutrophils which support tumour formation through cytokines secretion [160]. A recent study showed that the number of circulating naïve T cells in smokers with lung cancer was low compared to healthy patients, suggesting that smoking may affect the number of T cells in cancer patients [161]. Furthermore, smoking disrupted the circulating naïve cells in chronic COPD patients compared to non-smokers [162]. However, passive smoking could augment circulating naïve T cells in human [163]. Taken together, active and passive smoking may correlate with the prevalence of $CD4^+$ T cell.

Although cigarette smoking is well known factor which increases immune response, the mechanisms by which $CD4^+$ T cells are affected are not fully understood. An in vivo study demonstrated that CSE increased the T bet expression and pushed $CD4^+$ T cells toward Th1 polarisation and to produce high levels of IFN-γ [164] which contributes in RA development [165]. Also, smoking could augment the inflammatory features of DCs with a consequent increase in Th1 and Th17 polarisation in patients with RA which may increase the severity of this disease. [166].

Smoking not only could enhance Th1 activity, but also could enhance Th2 and so promote Th2-mediated inflammation. It was reported that cigarette smoking enhanced IL13 and decreased IFN-γ expression in periodontitis patients. This suggests that smoking may elevate

a Th2 profile in patients and may induce development of other progressive diseases [167]. In an in vivo experiment, it was observed that passive smoking significantly increased Th2 cytokines, including IL13 and IL4 which induced pulmonary inflammation [168]. However, cigarette smoking can be diverse in its effect on immune system. For instance, nicotine suppressed chemokine and cytokine expression and eosinophil trafficking in mice sensitized with allergens, suggesting that nicotine may modulate Th2 responses [169].

Numbers of studies showed that memory T cells $CD3^+CD45RO^+$ and $CD4^+CD45RO^+$ in response to tobacco exposure were elevated in peripheral blood, which may increase the progression of atherosclerosis because most of $CD4^+$ T cells at atherosclerotic lesions expressed the $CD45RO^+$ antigen [170, 171]. However, subsequent findings reported the opposite effect in that cigarette smoking reduced frequencies of memory T cells $CD4^+$CD45RO in children's blood which was due to passive smoking [163]. This contradictory role of smoking in memory cells may depend on the environment and genetic background of individuals.

1.5.4.2 Effect of PTPN22 variant on T cells

PTPN22 1858T polymorphism was correlated with higher percentage of activated naïve T cells in RA patients, which may increase pro-inflammatory cytokines and establish autoimmune process in RA [172]. A study on memory cells reported that the $CD4^+$ memory T cells in type 1 diabetes patients with genetic variant *PTPN22 1858T* were significantly increased compared to healthy individuals, suggesting a role of this variant in increasing resistance to TCR-mediated apoptosis in $CD4^+$ memory T cells [173]. PEP is a mouse homologue of human PTPN22, so a study showed that $pep^{-/-}$ mice enhanced Lck activity. In turn, this promoted the function and expansion of memory T cell pool [174]. PTPN22

showed to regulate LFA-ICAM1 which play a role in Th1 activation. This study revealed that *Ptpn22*$^{-/-}$ *and Ptpn22*^{R619W} mice enhanced LFA which in turn induced Th1 and IFN-γ responses which may contribute in multiple autoimmune diseases [175].

1.5.4.3 Effect of PTPN22 variant on other immune cells

Not only T cells can express PTPN22, neutrophils also expressed high levels of PTPN22, but its function in these cells remains unclear. It has been observed that *PTPN22* polymorphisms increased the risk of RA by enhancing NETs formation and citrullination [176]. Another study showed that expression of PTPN22 (R620W) increased the functions of neutrophil in healthy and RA patients, including Ca^{2+} release, migration and ROS production which may directly contribute to the joint damage [177].

PTPN22 is also expressed in macrophages. It supressed the polarisation of M1 genes and increase the expression of M2 genes. Therefore, a study showed that PTPN22-deficient mice generated severe colitis due to high levels expression of macrophages M1 genes and lower levels of M2 genes, indicating the important role of PTPN22 in regulating the polarisation of macrophages [178]. Another in vivo study demonstrated that *PTPN22* Trp (620) allele variant enhanced the function and phagocytic ability of macrophages which may increase the risk of autoimmunity disease [179]. Interestingly, PTPN22 was shown to regulate Akt activity [180], which plays a role in regulating the polarisation of macrophages [181]. This means PTPN22 may modulate the polarisation of macrophages indirectly through Akt.

1.5.4.4 Immune cells and RA development

$CD4^+$ T cell infiltration into synovial joints has been reported for decades. The $CD4^+$ T cells in the RA synovium showed greater expression of the Th1 cytokine IFN-γ from those of $CD4^+$ T cells in the peripheral blood [182]. This may activate synoviocytes or macrophages to

produce joint destructive cytokines. Interestingly, blood samples from RA patients showed that IL4 level in RA patients was higher than in controls, whereas IFN-γ was lower, indicating that high Th2 expression may found in blood and the potency of Th2 over Th1 activity is higher in the blood of RA, but not in synovium which shows predominant Th1 polarisation [183, 184]. Moreover, Th2 cytokines from T cells and macrophages e.g. IL2, IL4, IL13, but not IFN-γ were significantly expressed in the very early RA patients (3 months after symptoms onset) [143] Th17 cells express high level of CCR4 and CCR6 which facilitate the migration of Th17 cells to the inflamed joints [185], and produce IL17A which stimulates the production of IL-6 and IL-8 in synovial fibroblasts [186], IL1 and TNF-α from macrophages and recruits neutrophils which is a hallmark of RA synovial fluid [187, 188]. This supports the notion that Th17 cells are strongly correlated with the development of RA and other autoimmune diseases.

A link between memory T cells and RA has been identified. It was found that the differentiated memory T cells in early RA patients may contribute to the characteristic Th1-dominated rheumatoid inflammation, leading to RA development [189]. Memory T cells in response to IL12 was redirected away from lymph node toward joints and therefore an increase in inflammatory activity [190]. Another study showed that the number of mature memory T cells in the synovial tissues and synovial fluids was increased in RA patients, indicating that memory T cells may migrate to inflamed tissue [191].

B cells participate in the disease and may propagate the autoimmune process. A study showed that the pathogenic role of IgG autoantibodies to glucose-6-phosphate isomerase (GPI) in healthy mice led to develop synovitis which is the common feature of RA [192]. A clinical study was conducted on patients with RA having joint synovitis to determine the

clinical significance of associated autoantibodies. The RF showed a specificity of 66%, whereas anti-CCP showed more than 90%, suggesting that anti-CCP is more specific for RA and may contribute sustained inflammatory response [193].

The neutrophil is an important candidate in helping to develop various systemic autoimmune diseases including rheumatoid arthritis, systemic lupus erythematosus and small vessel vasculitis [194]. Activated neutrophils in RA patients were found in large numbers within pannus and synovial fluid, and have the potential to drive much damage within RA joints [195]. In addition, blockade of CXCR1/CXCR2 signalling receptors led to inhibition of neutrophil recruitment and therefore prevented neutrophil-dependent joint injury [196].

1.6 T cell activation and signalling transduction pathways

T cells play important key roles in cell-mediated immunity: they recognise foreign antigens through a highly variable, surface expressed T cell receptor (TCR) which is critical for T cell activation, development and tolerance [197]. Engagement of TCRs with the major histocompatibility complex (MHC) which binds foreign antigen peptides, presented by antigen presenting cells (APC), such as dendritic cells trigger downstream pleiotropic effects, such as translation, transcription, increased cell size and production of chemokines, cytokines and effector molecules [198]. However, dysregulation of TCR signalling increases T cell activity (hyperactivation), leading to several autoimmune conditions [198]. The TCR consists of variable alpha and beta chains and a set of CD3 protein subunits [199].

Unlike alpha and beta TCR, CD3 proteins have long cytoplasmic tails which include immunoreceptor tyrosine-based activation motifs (ITAMs), changes to which result in T cell activation. The ITAM protein sequence includes a pair of tyrosine residues separated from a

leucine/isoleucine by six to eight amino acids [200]. ITAMs are phosphorylated by the SRC-family of protein tyrosine kinases (PTKs), following engagement of the TCR, leading to the activation of downstream signalling cascades [201]. Negative regulatory molecules are important in controlling TCR signalling. Impairment of any signalling pathways, including proximal and distal, can cause severe infections and immunodeficiency [202]. In addition, complementary receptors augment and control the signals through the TCR and the subsequent cellular responses.

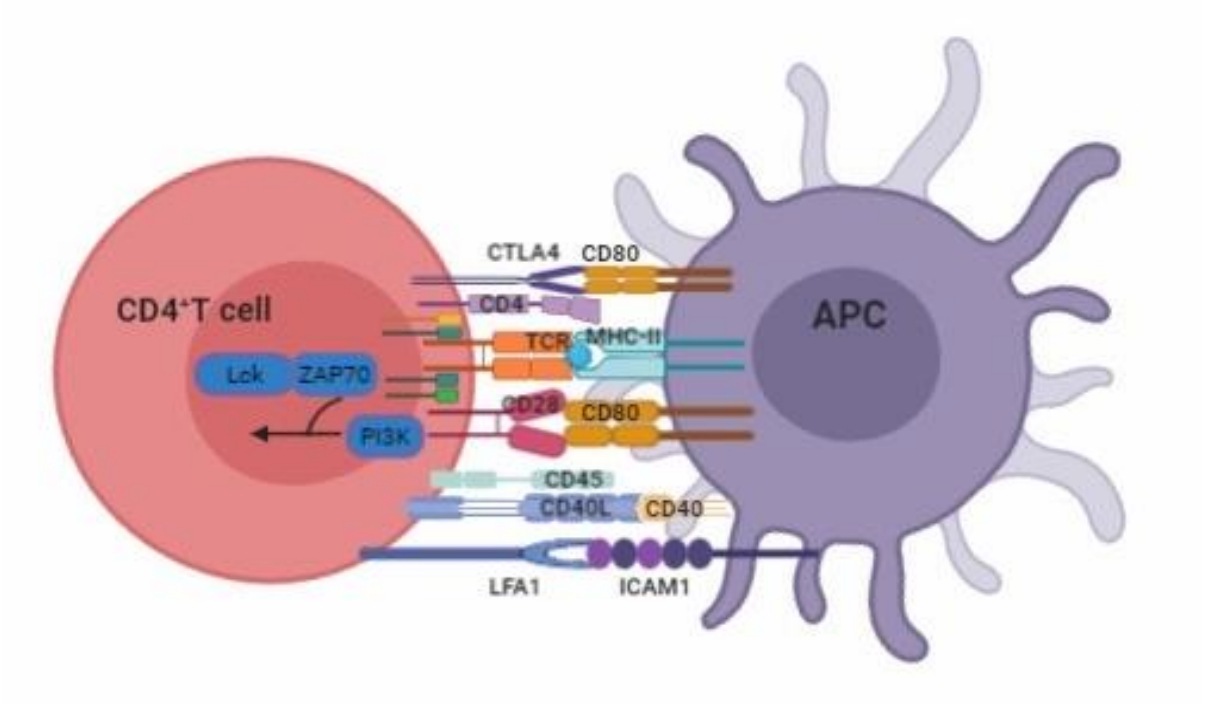
1.6.1 Immunological synapse

To initiate an antigen specific response the APC degrades the antigen into small peptides which bind to MHC class II to form a complex. This complex (peptide-MHC II) is transported to the cell surface for presentation to naïve $CD4^+$ cells to initiate responses [203]. Naïve T cell needs two signals for activation, termed signal-1 and signal-2. Signal-1 is equivalent to the interaction between peptide-MHC II and TCR. This signal is essential for signal transduction pathway activation and can be mimicked by antibodies and superantigens. Signal-2 requires one or more costimulatory molecules which are expressed on T cells and APCs (CD2/LFA-3, LFA-1/ICAM-1 and ICAM2, B7/CD28). The interaction between CD28, CD80 (B7-1) and CD86 (B7-2) leads to the activation of T cell to express other costimulatory molecules to regulate the stability of internal environment and the balance of immune response (Figure 1.4 A). Usually this step is not required for effector and memory T cell responses [204]. However, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) also known as (CD152) is a homologous to CD28. It plays a role in immune checkpoint and downregulates immune responses and has an immunoreceptor tyrosine-based inhibitory motif (ITIM). Therefore, CTLA-4 binds to CD80 with a greater avidity and affinity that is 20

times higher than interaction between CD28 and CD80, thus enabling it to outcompete CD28**.** The inhibitory signals by CTLA-4 can be sent through its ITIM motif to restore the immune response balance [205].

The complex interactions described above need to be carefully organized to be effective and the Immunological synapse (IS) describes the supra-molecular interaction between antigen presenting cells (APCs) and thymus-derived lymphocyte (T cell). This interaction initiates the antigen-specific immune response which is based on interaction between MHC and TCRs. The IS structure as shown in (Figure 1.4 B) contains a central region called central supramolecular activation cluster (cSMAC) which is considered a site of signal termination and receptor recycling and is composed of the Lck, ZAP-70 and Fyn [206]. The c-SMAC is surrounded by the peripheral SMAC (pSMAC) which contains adhesion molecules such as leukocyte function-associated antigen-1 (LFA-1) and Intercellular Adhesion Molecule 1 (ICAM-1), and the cytoskeletal protein talin. These molecules connect the cytoskeletons and plasma membranes of the APC and T cell together and provide a mechanical scaffold for the immunological synapse [207]. The distal SMAC (dSMAC) surrounds the pSMAC and contains immune-inhibitory receptors such as CD45 and CD43 [208, 209]. The dSMAC is linked with signalling molecules such as LAT, ZAP-70 and Lck, implying that receptor signalling primarily occurs in dSMAC [210, 211]. The segregation of LFA-1-ICAM-1 interaction from TCR-MHC interaction may be the initial trigger of signalling and receptor clustering in the nascent IS.

A



B

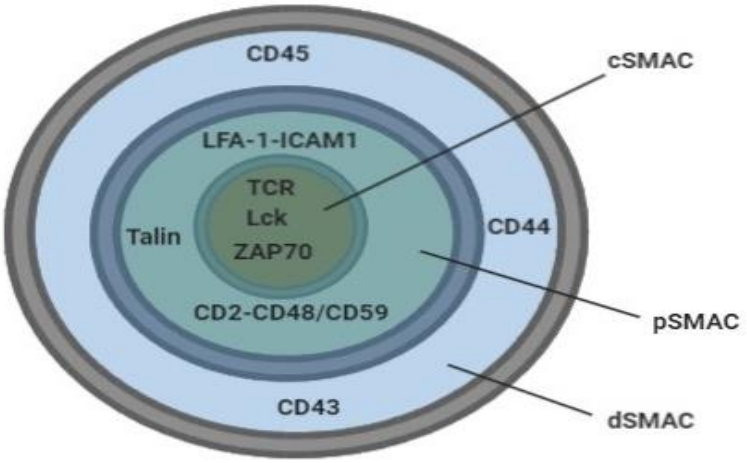


Figure 1. 4 Schematic representation of mature T cell synapse.

A) An interaction between APC and T cell, showing a selection of the key ligand pairs and signalling molecules. B) An immune synapse structure including the central region of the supra-molecular. Drawn in BioRender.

1.6.2 T cell signalling pathways

1.6.2.1 CD28 signalling

CD28 is a co-stimulatory molecule expressed on approximately 80% of $CD4^+$ and 50% of $CD8^+$ T cells [212]. Although CD28 has been detected in other cell lineages such as neutrophils, plasma cells and bone marrow stroma cells, the function of CD28 on these cells is not completely understood [213]. Following the recognition of peptides presented by MHC molecules, CD28 binds to CD80 and CD86 ligands on the surface of APC and generates signals required for T cell activation and survival [214]. CD28 has a cytoplasmic tail which contains proline-rich motifs that are phosphorylated in response to CD28 stimulation, and binds to SH3 domain containing proteins [215].

CD28 binds to the adaptor proteins Growth factor receptor-bound protein 2 (GRB2) and Grb2-related adapter protein downstream of Shc (GADS) through their SH2 domain at a PYAP motif. GRB2 binds Vav1 and Sos through its SH3 domain [216]. In turn, Vav1 and Sos activate CDC42, Rac1 and Ras, resulting in the activation of ERK and JNK and the formation of the AP-1 transcriptional complex [217]. GRBS2 and GADS form CARMA1-Bcl-10-Malt1 complexes, which play crucial roles in the activation of NF-κB [218]. As described, CD28 increases T cell proliferation and production of IL-2. However, this signal can be negatively regulated by immune checkpoints, such as cytotoxic T-lymphocyte–associated antigen 4 (CTLA4) [219]. CTLA4 binds B7 with much higher affinity than CD28, enabling CTLA4 competition with CD28 preventing co-stimulatory signalling and limiting the survival of T cells and IL-2 production [220].

1.6.2.2 Lck and ZAP-70 signalling

PTKs such as Lck, Fyn and ZAP-70 are activated by the ligation of TCRs. Following this, the activation of Lck (lymphocyte-specific protein tyrosine kinase) leads to the phosphorylation of ITAMs in CD3 proteins. Lck is the most important molecule in proximal signalling to be activated following TCR engagement. it regulates a number of signalling pathways, influencing the mobilisation of Ca^{2+} via activation of protein kinase C (PKC), phospholipase C-gamma and the mitogen-activated protein kinase (MAPK) pathway [221]. Moreover, Lck efficiently recruits Ras GRP1 to the cell membrane by stimulating the production of diacylglycerol (DAG), which plays a role in T cell and NF-κB activation [221] (Figure 1.5).

Zeta-chain-associated protein kinase 70 (ZAP-70), a tyrosine kinase that also plays a role in T cell activation, is recruited to phosphorylated ITAM. It is composed of two SRC homology 2 (SH2) domains that bind to double phosphorylated ITAMs, releasing ZAP-70 from its autoinhibited conformation, which eventually phosphorylates linker for the activation of T cells (LAT) and SH2 domain containing leukocyte phosphoprotein of 76 kDa (SLP-76) [222]. One of the most important roles of phosphorylated ZAP-70 is the regulation of cytosolic Ca^{+2} influx, phospholipase C y1 (PLCy1) and activation of distal signalling pathways such as NF-κB and activator protein 1 (AP-1) [223]. Phosphorylated LAT recruits the SH2 domain of PLCy1, growth factor receptor-bound protein 2 (GRB2) and GRB2-related adapter downstream of SHC (Gads) [224]. SLP-76 combines this complex through binding to PLCy1 and Gads through a proline-rich region (Figure 1.5). However, the absence of SLP-76 or LAT results in loss of the TCR signalling cascade [225].

1.6.2.3 Vav1 signalling.

The phosphorylation of SLP-76 by ZAP-70 leads to the recruitment of IL-2-induced tyrosine kinase (ITK), Vav1, a member of the guanine nucleotide exchange factors (GEF) family, and other adaptor proteins such as adhesions and degranulation promoting adapter protein (ADAP) and non-catalytic tyrosine kinase (NCK) [226]. Vav1 recruitment to SLP-76 via its SH2 domain leads to the activation of the Rho family of GTPases (e.g. RAC1) to enhance Actin reorganisation, whereas ADAP binds to the C- terminal of SLP-76 to activate integrin signalling [227, 228]. Activation of PLCy1 is key in connecting proximal to distal signalling of the TCR signalling cascade [229]. For the optimal activation of PLCy1, it must bind SLP-70, LAT, VAV1 and ITK [230]. It also hydrolyses membrane-bound phosphatidylinositol 4, 5-bisphosphate (PIP2), producing DAG and inositol-3-phosphate (IP3), which are essential for T cell function [231] (Figure 1.5). Activated IP3 increases intracellular Ca^{+2} to regulate cell proliferation, whereas DAG activates essential signalling pathways including, protein kinase C (PKC) and pyruvate dehydrogenase kinase 1 (PDK1) [231-233].

A study on Vav1-deficient T cells provided an important role for Vav1 in regulating ERK-MAP kinase, calcium and NF- κB pathways [234]. However, a study has shown that Vav1 was not required for TCR-induced ERK calcium flux and protein kinase D1 (PKD1), suggesting that Vav1 is essential for some, but not all of its functions [235]. Failure to activate PLCy1, due to Vav1 deficient double positive thymocytes, caused a defective TCR-induced calcium flux [236]. Vav1 is required for Itk and Tec activation, which could directly phosphorylate PLCy1. Therefore, the impairment of Tec and Itk could lead to secondary defective activation of PI3K, as both Tec and Itk have PH domains that can bind to PIP3, the second messenger generated by PI3K [237].

1.6.2.4 Calcium signalling

IP3 binds to its receptor IP3R on the endoplasmic reticulum (ER) membrane, releasing intracellular Ca^{+2} into the cytoplasm. Depletion of Ca^{+2} stores at the ER increases Ca^{+2} extracellular influx to T cells through a calcium release-activated calcium (CRAC) channel under a process called store-operated Ca^{+2} entry (SOCE) [238]. The ER expresses a sensor for depleting ER Ca^{+2} stores, the transmembrane stromal interaction molecule (STIM), which allows Ca^{+2} influx from ORAI pore subunits of CRAC channels [239]. As a result, Ca^{+2} influx activates signalling proteins and their transcriptional targets. For instance, the phosphatase calcineurin (activated by the binding of Ca^{+2} and calmodulin), dephosphorylates nuclear factor of activated T cells (NFAT), which translocated into the nucleus, and Ca^{+2}- calmodulin-dependent kinase (CaMK) dephosphorylates cyclic-AMP-responsive-element-binding protein (CREB) and myocyte enhancer factor 2 (MEF2) thereby mediating T cell activation [240].

Interaction between NFAT and the AP-1 transcription factors (Fos /Jun) induces interleukin 2 (IL2) gene expression, which plays a crucial role in T cell activation, maturation and proliferation [241] (Figure 1.5). Also, it has been shown that the forkhead transcription factor FOXP3, regulatory T cells lineage specification factor cooperates with NFAT [242].

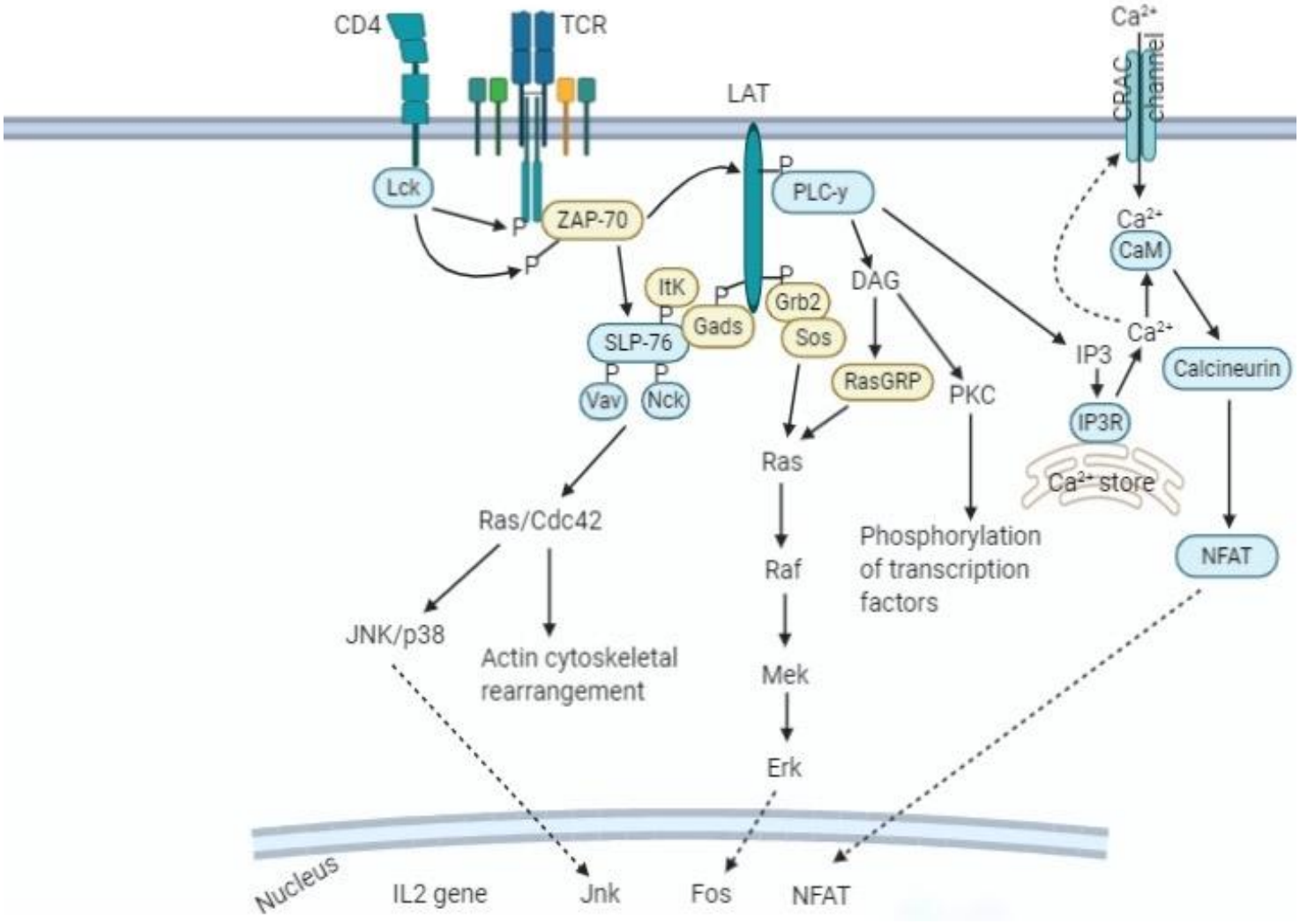


Figure 1. 5 Schematic representation of PTKs, Vav1, calcium signalling pathways.

Lck phosphorylate ITAMs in CD3 proteins, which in turn recruit ZAP-70. ZAP-70 phosphorylates LAT and SLP-76 which play a role in PLCy1 and Ca2+ activation. Vav1 is required for Itk and Tec activation, which could directly phosphorylate PLCy1. PLCy1 hydrolyses membrane-bound phosphatidylinositol 4, 5-bisphosphate (PIP2), producing DAG and inositol-3-phosphate (IP3), which are essential for T cell function. Drawn in BioRender.

1.6.2.5 PI3K/Akt/mTOR signalling

Following phosphorylation of the tyrosine residue in the CD28 cytoplasmic domain, the Tyr in the YMNM motif binds the p85 subunit of phosphoinositide 3-kinase (PI3K), via an SH2 interaction activating the P13K pathway [243]. P13K is involved in cellular functions, and it increases the production of PIP2, PIP3 and D3 lipids and recruits other proteins including phosphoinositide-dependent kinase 1 (PDK1) and PKB/Akt via their pleckstrin homology (PH) domains [244]. Akt is a master regulator of metabolism and proliferation. It is activated through phosphorylation of two residues: serine 473 (Ser473) in the C-terminal hydrophobic motif and threonine 308 (Thr308) in the activation loop by PDK1 [245]. However, this activation of PI3K/Akt is inhibited by phosphatase and tensin homolog (PTEN) which regulates protein synthesis involved in survival and proliferation [246]. PDK1 phosphorylates and activates PKB, which in turn targets and phosphorylates glycogen synthase kinase 3 (GSK3), mammalian target of rapamycin (mTOR), IκB and Bcl-2 antagonist of cell death (BAD) [247] (Figure 1.6). The phosphorylated IκB and mTOR induce transcriptional activity of NF-κB, whereas GSK3 and BAD induce NFAT transcriptional regulation. The activation of NFAT and NF-κB increases the transcription of IL-2, an essential cytokine in the proliferation and activation of T cells and Bcl-XL [247].

The mTOR kinase is a serine-threonine kinase of the PI3K family, which can be activated during insulin resistance, tumour formation and adipogenesis. mTOR serves as a central regulator of cell proliferation, growth and cell metabolism through the integration of both intracellular and extracellular signals [248]. This activity of mTOR is inhibited by tuberous sclerosis 1 and 2 (TSC1-TSC2) through conversion of the Ras homolog enriched in brain (RHEB) into its inactive GDP-bound state [249]. mTOR phosphorylates translation regulator

4E-BP1 and S6K1, to promote protein synthesis, and increases metabolic influx through the activation of translation and transcription of hypoxia inducible factor 1α (HIF1α), but not HIF2α [250] (Figure 1.6). Furthermore, it has been reported that mTORC1 increases the expression of genes involved in oxidative metabolism and mitochondrial DNA content, by mediating the nuclear association between the transcription factor Ying-Yang 1 (YY1) and the PPAR-γ co-activator 1α (PGC1α), which regulates mitochondrial biogenesis [251]. Therefore, mutant YY1 may cause significant decreases in mitochondrial gene expression and oxygen consumption.

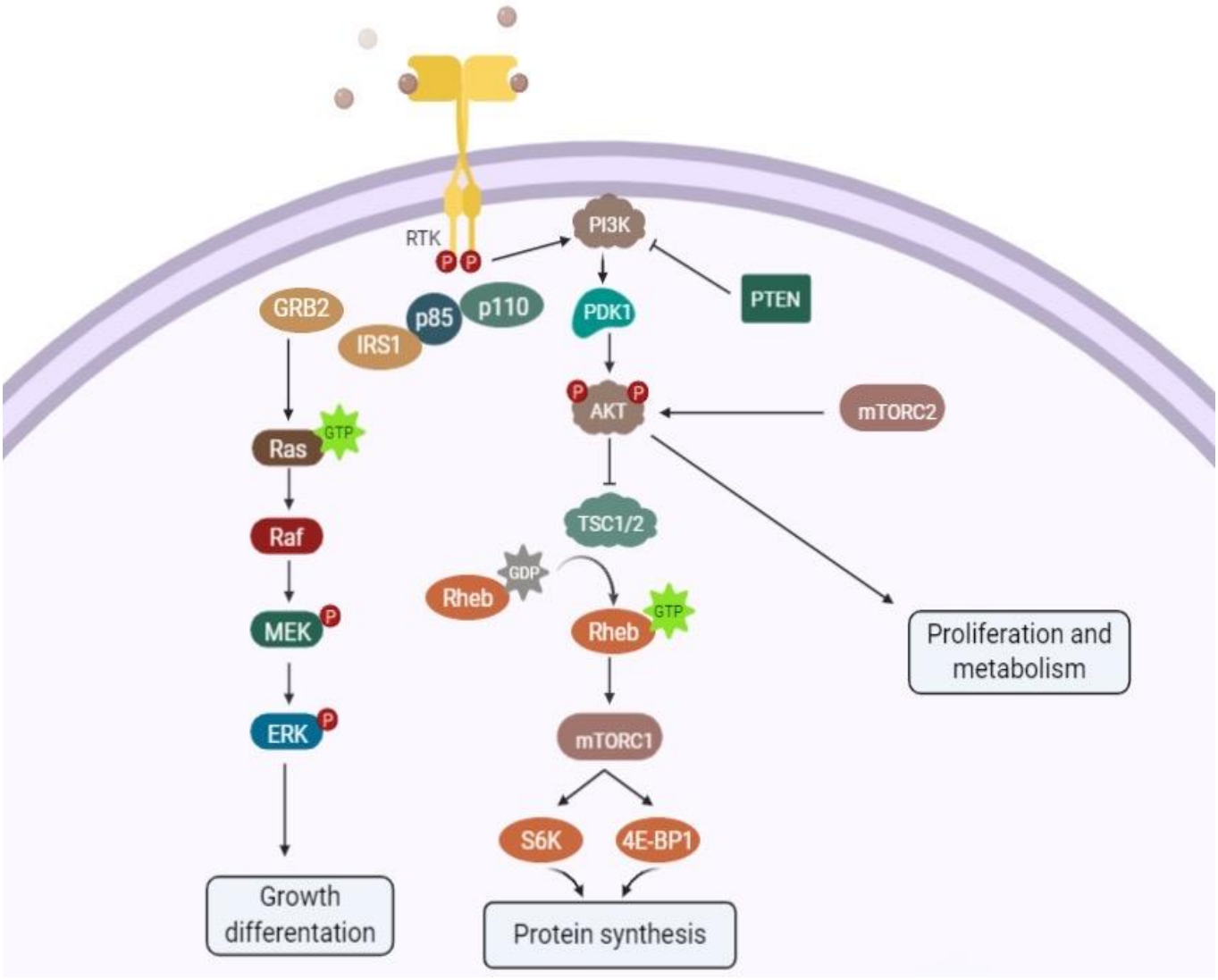


Figure 1. 6 Schematic representation of PI3K/Akt/mTOR signalling pathway.

Following phosphorylation of the tyrosine residue in the CD28 cytoplasmic domain PI3K increases the production of PIP2 and PIP3, and phosphorylates PDK1, which in turn phosphorylates Akt. Akt phosphorylates mTORC1 which regulates cellular metabolism and protein synthesis. This pathway can be inhibited by PTEN which regulates protein synthesis involved in survival and proliferation. Drawn in BioRender.

1.6.2.6 AMPK signalling

AMP-activated protein kinase (AMPK) belongs to serine/threonine kinases which is an important player in regulating cellular metabolism. It is activated when adenosine triphosphate (ATP) production is low, and by intracellular calcium through phosphorylation of Calcium/Calmodulin Dependent Protein Kinase (CaMKK) [252, 253]. AMPK controls cellular growth by inhibiting mTORC1 through phosphorylation of TSC2 to deregulate the Rheb GTPase required for mTORC1 activity [254]. Acetyl-coA carboxylase 1 (ACC1) which is converting acetyl-coA to malonyl-coA, and lipogenic transcription factor Sterol regulatory element-binding protein 1 (SREBP1) play a central role in stimulating fatty acid synthesis in cells [255]. To control lipogenesis, AMPK phosphorylates ACC1 at Ser79 and ACC2 at Ser212, and supresses the activity of SREBP1 by phosphorylation the conserved serine site Ser372 within SREBP1 [256, 257]. This demonstrates prolonged and acute AMPK nature in controlling biology (Figure 1.7).

Glucose uptake can be stimulated by AMPK through translocating GLUT4-containing intracellular vesicles. Rab family G proteins in their active GTP-bound state are required by these vesicles to fuse with plasma membrane**.** So, AMPK inhibits and phosphorylates TBC1D1, a member of the TBC1 Rab-GTPase family, leading to the activation of Rab family G proteins and increases plasma membrane fusion with GLUT4 vesicles [258] [figure 7]. Following glucose transport into the cells, it is consumed in different metabolic pathways including glycogen synthesis and glycolysis. The glycogen synthesis pathway is regulated by AMPK through inhibition of transcription factors such as CREB regulated transcription coactivator 2 (CRTC2) and hepatocyte nuclear factor 4 (HNF4), which increase gluconeogenic enzymes expression [259, 260].

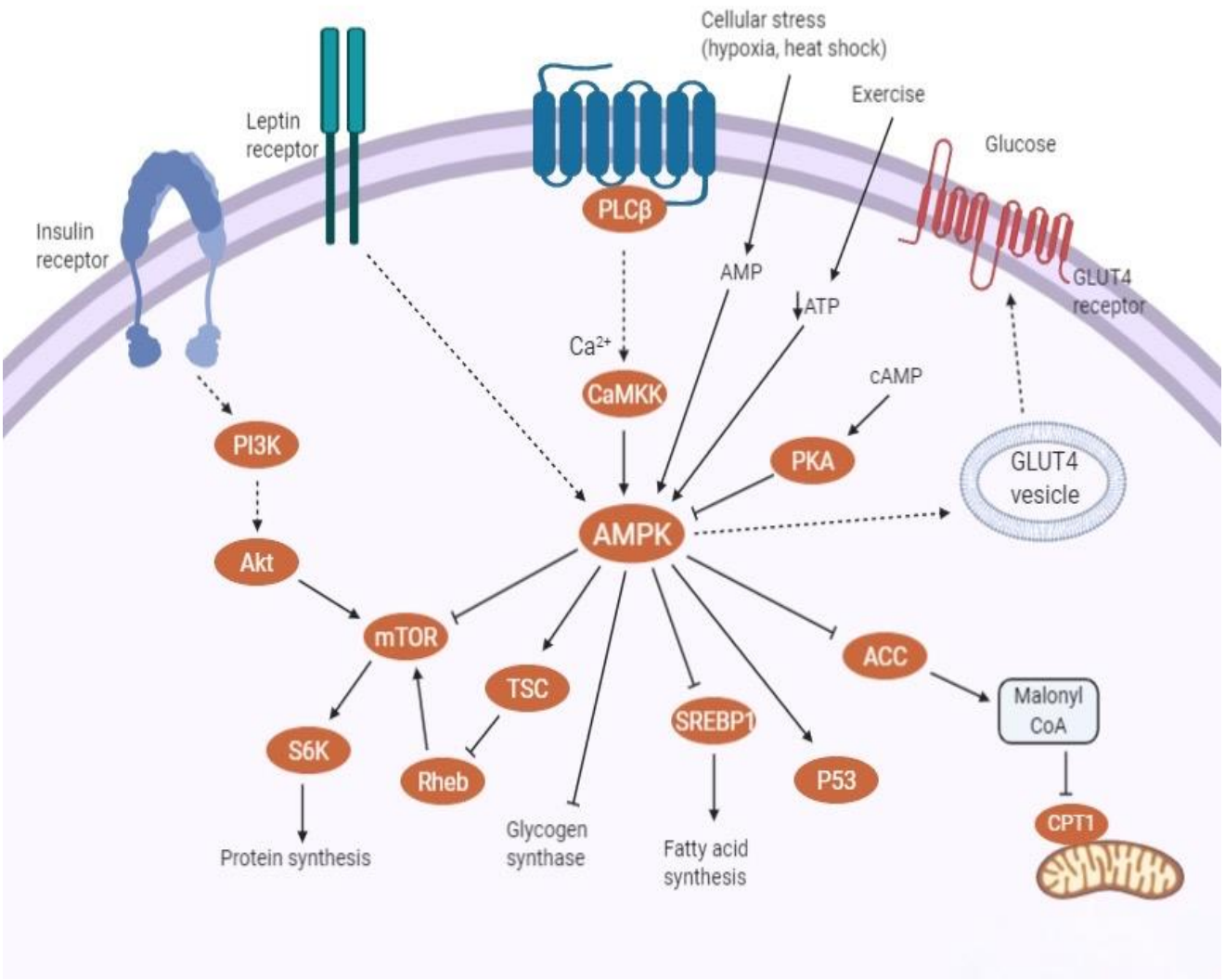


Figure 1. 7 Schematic representation of AMPK signalling pathway.

Similar to Akt and mTORC, AMPK plays a central role in cellular metabolism. CaMKK, low ATP and AMP activate AMPK signal. AMPK inhibits fatty acid synthesis by suppressing SREBP1, ACC, and mTOR through TSC phosphorylation. it also regulates glycogen synthesis by inhibiting HNF4 and CRTC2. Drawn in BioRender.

To conclude, TCRs interact with foreign antigens and activate several signalling transduction events, determining cell fate through regulating cell survival differentiation, proliferation and cytokine production. Activation of Lck results in phosphorylation of ITAM on CD3 tail, recruiting and activating ZAP-70, which phosphorylates LAT and SLP-76. Phosphorylated PLCy1 cleaves PIP2 to produce IP3 that triggers Ca^{+2} release from the ER through CRAC channels, and DAG that activates both MAPK and PKCθ pathways, which promote NF-κB activation, leading to DNA transcription and cytokine production. These signalling cascades are controlled by negative regulatory molecules such as ligases, ubiquitin and phosphatases to ensure proper TCR signalling and T cell activation (Figure 1.8).

Despite the fact that genetic and immunoblotting technologies have increased our understanding of TCR signalling, new approaches such as imaging may explain dynamic signal transduction and how different subcellular compartments are regulated. For example, mass spectrometry-based technologies and DNA sequencing together with CRISPR and RNA interference will help our understanding of the dynamic features of signalling from linear pathways to integrated networks. In order to visualise and analyse information flow and dynamics of cell signalling in living cells and animals with unprecedented spatiotemporal resolution, fluorescent reporters have been developed which allow visualisation of the activity of enzymes in the signalling pathways, such as proteases and kinases. Further new reporters have been used to describe and localise the protein-protein interactions in these pathways. These developments can help us understand these important regulators of immune activity. Extensive studies on TCR signalling may improve our understanding of intracellular pathogenesis, and help to design novel strategies to develop effective molecular therapies for treating various autoimmune diseases and cancers.

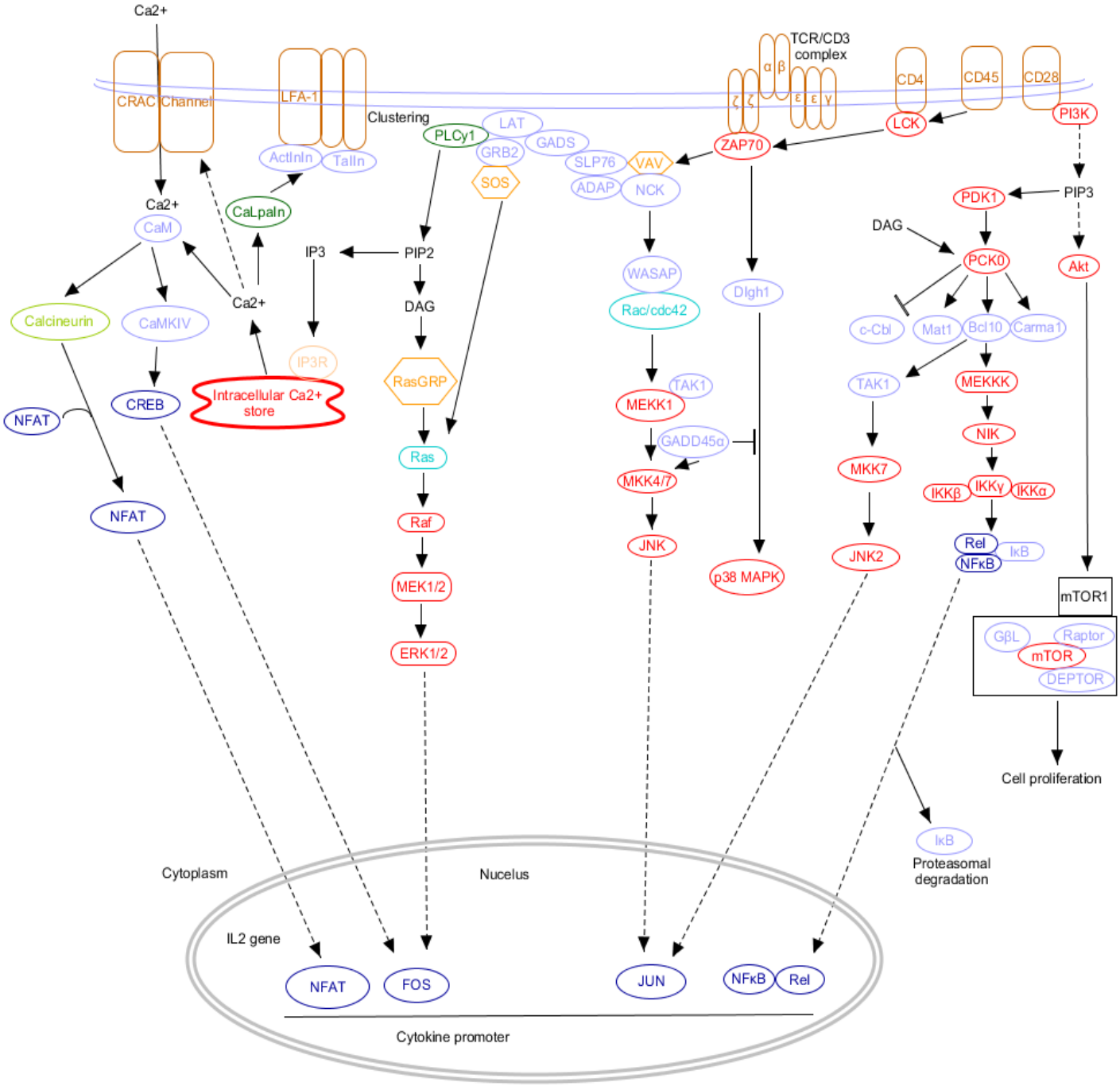


Figure 1. 8 Summary of the T cell signalling pathway.

Following TCR ligation, activated LCK phosphorylates ITAMs of the TCR/CD3, which recruits ZAP-70. ZAP-70 in turn phosphorylates SLP76 and LAT. PLCγ1 binds to LAT, SLP-70, ITK and VAV1, and hydrolyses PIP2, producing DAG and IP3, which initiates activating the calcium ion storage. Recruited VAV1 to SLP-76 leads to activation of Rac1 to enhance Actin recognition. Akt phosphorylates mTORC1 and regulates cellular metabolism. DAG activates PKCθ and MAPKs which eventually phosphorylate more kinases and activate Jun, Fos and NF-κB in the nucleus. Drawn in PathVisio.

1.7 Protein Tyrosine Phosphatases

Protein tyrosine phosphatases (PTPs) are essential enzymes for controlling and limiting signal transduction pathways, such as the MAPK pathway. Together with PTKs, PTPs maintain the cellular equilibrium of protein tyrosine phosphorylation and the phosphorylation states of many signalling proteins [261].

1.7.1 CD45

CD45 is a PTP expressed in all nucleated cells of the hematopoietic pathway. It plays regulatory roles in lymphocyte signalling pathways, controlling the Src family kinases (SFKs) such as Lck and Fyn [262]. It is highly glycosylated and is expressed as several isoforms including CD45RA (located in naïve T cells), CD45RO (located in memory T cells), and CD45RB and CD45RAB [263]. Therefore, it leads to the dephosphorylation of Lck at its negative regulatory carboxy-terminal tyrosine Y505, resulting in Lck activation [264]. Additional dephosphorylation of Lck is also performed by CD45 at the transphosphorylation site, Y394, leading to reduced Lck activity [265]. Therefore, CD45 is an important positive and negative regulator of Lck, which is essential for T cell activation. CD45 also negatively regulates neutrophil migration [266], integrins [267] and the Janus Kinase (JAK) family of tyrosine kinases, which negatively regulate cytokine receptor signalling [268]. It has been shown that CD45-mediated upregulation of P13K/Akt and Fas ligand pathways, which are crucial for CD45-mediated apoptosis [269]. Besides CD45 controlling Src, CD45 also plays an important role in Ras and Vav1 activation, PIP2 hydrolysis and Ca^{2+} mobilisation [270]. CD45 deficient T cells showed an inability to produce cytokines and proliferate in response to TCR or CD3 stimulation [271]. Furthermore, CD45 deficient T cells are defective in many signalling pathways, such as PKC, IL-2 production and phosphoinositide metabolism [272].

1.7.2 CD148

CD148 is another PTP which belongs to the type 3 family of PTPs. It is expressed in various cell types, including fibroblasts, endothelial cells and most hematopoietic cells [273]. The overexpression of CD148 in Jurkat T cells dephosphorylates LAT and PLCy-1, leading to TCR down-regulation [274]. Also, crosslinking CD148 with CD3 inhibits Ca^{2+} mobilisation. This inhibition may be associated with a decrease in PLCy-1 phosphorylation [275]. The expression of CD148 inhibits tumour growth by increasing p27 stability and supressing MAPK activation [276]. These findings suggest that CD148 is important for cell growth and differentiation. CD148 has similar function to that of CD45; they both enhance the signalling of T cells by dephosphorylating The Src family of protein tyrosine kinases (SFKs) [277], and both appear to have overlapping functions in terms of activating SFKs in monocytes [278].

1.7.3 Lyp/Pep

Lyp and its murine PEP homologue are encoded by *PTPN22* and they are important in controlling T cell signalling and maintain the cellular equilibrium. However, genetics polymorphism in *PTPN22* increase the risk of autoimmune diseases such as rheumatoid arthritis, type 1 diabetes and systemic lupus erythematosus [13, 279]. Neutrophils and natural killer cells express higher levels of Lyp, whereas monocytes and $CD4^+$ T cells express lower levels [13]. Although understanding the function of Lyp in T cells has expanded since the link of autoimmune conditions and *PTPN22 R620W*, there is still insufficient evidence describing signalling pathways. An evidence showed that Lyp inhibited Vav1, Lck at tyrosine residue Tyr394, ZAP-70 at tyrosine residue Tyr493, but not at the regulatory tyrosines Tyr319 (ZAP-70) or Tyr505 (Lck), resulted in decreased TCR signalling [280], whereas PEP targeted Fyn kinase [281]. A study on the *PTPN22* knock-out mouse resulted in increased expansion

and function of T cell memory compartments, and increased TCR signalling in effector T cells, suggesting that Lyp and Pep are potent regulators of TCR signalling [174]. Lyp/Pep can be regulated through the phosphorylation of Ser-35 by PKC, which impairs Lyp down-regulation of TCR signalling [282].

1.8 T cell metabolism

The flexible regulation of metabolism is directed by proliferation, growth and extrinsic signals that drive T-cell survival. However, failure of metabolism to fulfil the cell's demands may impair the cell functions or the cell could undergo apoptosis. Increased activity of cell metabolism may exacerbate the function of the cells, and possibility lead to inflammatory diseases [283]. Following the interaction between APC and T cell, the T cells experience different sets of metabolic demands which are regulated by a signalling cascade marked by phosphorylation of a number of tyrosine residues [284]. The resulting metabolic changes are essential for lymphocyte activation and survival. For instance, the metabolism of glucose as an energy source stimulates glycolysis to provide the pentose phosphate pathways with fuels, and contributes to lipid synthesis [285, 286] which is needed for cell growth. The pentose phosphate pathway is a component of cellular metabolism parallel to glycolysis. It produces NADPH by directing glucose to its oxidative branch [287]. Following the clearance of pathogen, the immune system returns to haemostasis and cells cease to proliferate and re-enter quiescence**.** So, to accommodate these processes, the glucose metabolism of T cells reduces and returns to a state in which oxidative metabolism dominates with to produce more ATP at the expense of biosynthetic precursors [288]**.**

1.8.1 Glucose metabolism

1.8.1.1 Glucose metabolism in naïve T cell

Metabolically, naïve T cells consume essential nutrients such as glutamine and glucose to fuel oxidative phosphorylation (OXPHOS) to generate ATP and maintain normal housekeeping functions. Therefore, to maintain this metabolic phenotype, naïve T cells rely on extrinsic signals [289]. As stated previously in T cell biology section, IL7 is required for survival of naive T-cell populations and has a well-defined role in metabolic regulation and as a homeostatic factor [290]. To support basal cell metabolism and naive T-cell survival, IL-7 can also promote glucose uptake and glycolysis. However, in the absence of IL7, naïve T cells will be unable to maintain glycolysis and prevent atrophy [291]. IL7-Akt signals induce glucose transporter 1 (GLUT1) expression which maintains basal uptake of glucose and amino acids which are used to fuel OXPHOS, which maintains T cell quiescence [292]. This means that glucose and mitochondrial metabolism are the key for regulating T cell quiescence. To maintain the metabolism during quiescence of naïve T cell, mTORC1 is inhibited by TSC1 which is more active in naïve than activated T cells [293]. However, TSC T cell deficiency will increase mTORC1 activity and glycolytic reprogramming [294].

1.8.1.2 Glucose metabolism in activated T cell

In the activated T cell metabolic reprogramming is induced which upregulates both oxidative phosphorylation and glycolysis (Figure 1.9). Although OXPHOS is more efficient than glycolysis at generating ATP, glycolysis is important in maintaining the redox balance (NAD^+/NADH) in the cell [295]. However, the mechanisms by which glucose is metabolised in activated T cell is still not certain. A T cell activated through TCR and CD28 signals can induce rapid and robust activation of GLUT1 and glucose uptake [296]**.** Several signalling pathways

support these metabolic changes in activated T cell. For instance, activated Akt increases the GLUT1 cell surface trafficking and prevents GLUT1 internalisation and targeting to lysosomes for degradation, thus enhancing glucose uptake [297]. However, inhibition of PI3K through inhibitory receptor CTLA-4 can reduce GLUT1 level [220]. Although GLUT1 plays a critical role to support the glucose metabolism of T cell, it is not sufficient to fully drive glycolysis. Therefore, to regulate metabolism, additional signals downstream are required to initiate glucose uptake. For example, Akt promotes the activity of hexokinase, leading to phosphorylation of glucose to support flux through both the pentose phosphate pathway and glycolysis [298]. Genetic deletion of mTORC increases regulatory T (Treg) production, but not Th1, Th2 and Th17 cells [299]. While Treg depends more on the lipid oxidation, Th1, Th2 and Th17 trigger glycolysis through mTORC signalling [300]. HIF-1α which is regulated by mTORC1 promotes glucose uptake and catabolism through glycolysis. However, inhibition of HIF-1α leads to blocking glycolysis and increased generation of Treg cells [301].

1.8.2 Mitochondrial metabolism in T cells

Pyruvate from glycolysis, and other substrates, such as Acetyl-CoA from fatty acids and glutamine via α-ketoglutarate (AKG) can be transported to the mitochondria and enter the tricarboxylic acid (TCA) cycle [302, 303]. This cycle reduces NAD^+ to NADH, which donate electrons to the electron transport chain (ETC) and leads to generation ATP in a process called OXPHOS [304] (Figure 1.9). Mitochondrial ATP is essential for proliferation and activation of effector $CD4^+$ and $CD8^+$ T cells. Signals through TCR and costimulatory CD28 lead to the fusion of outer and inner mitochondrial membranes in T cells, which increases the assembly of the electron transport chain to enhance OXPHOS [305]. This fusion with other organelles such as lysosomes and endoplasmic reticulum, regulates the activation and

haemostasis of T cells by modulating autophagy [306]. Mitochondrial fission driven by dynamin-related protein 1 (DRP1) which is activated by Ca^{2+}-activated kinase, calcineurin enhances anabolic metabolism. The phosphoenolpyruvate (PEP), a glycolytic metabolite inhibits endoplasmic reticulum calcium ATPases, leading to increased calcineurin activity, cytoplasmic Ca^{2+} concentration and potentially increased mitochondrial fission and DRP1 which inhibits OXPHOS and mitochondrial fusion [307].

1.8.2.1 Mitochondrial enzymes activated by Ca^{2+}

Mitochondrial Ca^{2+} uptake has important functions in controlling ATP production, mitochondrial enzymes and mitochondrial metabolism. There are some enzymes in mitochondria which are activated by Ca^{2+} to generate ATP by reducing NAD^+ to NADH. For example, pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase and isocitrate dehydrogenase [308]. PDH is one of the key mitochondrial enzymes which converts pyruvate and CoA to acetyl-CoA and CO_2, causing an increase in TCA cycle flux which generates $FADH_2$ and NADH, which are used in ETC as electron donors. Consequently, this increases ATP production and mitochondrial respiration occurs by increasing metabolite oxidation in the TCA cycle [309].

1.8.2.2 Mitochondrial ROS

Although only a few studies have looked at mitochondrial function in early naïve T cell activation, it has been found that mitochondrial ROS generated by ETC is essential for proper activation of T cells [310]. A study showed that complex I ROS could activate NF-κB and AP-1 which facilitates cytokine production [311]. Another study has shown that mitochondrial ROS generated in complex III in T cells is required for IL2 production and T cell expansion. This study also showed that mitochondrial ROS deficiency in T cells was unable to induce IL2

production due to failure to induce NFAT1 translocation to the nucleus, suggesting the role of mitochondrial ROS in controlling T cell activation and metabolism [310]. AMPK which is a master regulator of metabolism can be activated by mitochondrial ROS, leading to preserved mitochondrial function, indicating that complex III ROS may be an important signal by which AMPK could be activated downstream [312].

1.8.2.3 Fatty acid oxidation

As any other cells, T cells can also degrade fatty acids through a process called fatty acid oxidation (FAO) to produce lipids as a source of energy. Therefore, fatty acid metabolism is essential in T cell activation and their differentiation programme [313]. Usually fatty acid synthesis (FAS) occurs in the cytosol of the T cell and an enzyme called acetyl-CoA carboxylase (ACC) is the first step involved in this process by catalysing the carboxylation of acetyl-CoA to malonyl-CoA. Following that, fatty acid synthase (FASN) catalyses acetyl-CoA and malonyl-CoA to produce long-chain fatty acids (LCFAs) which form complex lipids [314]. These fatty acids need to be transported to mitochondria first for oxidation and ATP generation. In the mitochondria, fatty acids are oxidised by removing two carbon atoms from acyl-CoA chain to produce acetyl-CoA which shuttles into the TCA cycle to produce ATP through OXPHOS [315] (Figure 1.9).

It has been reported that AMPK is important in FAO induction in $CD8^+$ T cells. A study found that $CD8^+$ T cells- specific deletion of TNF receptor associated factor 6 (TRAF6) displayed defective mitochondrial FAO and AMPK activation, but when AMPK activated by metformin (a diabetic drug) FAO was restored in the absence of TRAF6 [316]. Furthermore, AMPK can upregulate FAO directly through phosphorylation of acetyl-CoA carboxylase 2 (ACC2) or indirectly through upregulation of mitochondrial enzyme carnitine palmitoyl transferase 1

(CPT1) [317, 318]. However, activation of PI3K/Akt/mTORC could downregulate CPT1 and therefore limit FAO in the mitochondria [319]. Thus, the FAO upregulation by AMPK depends on the inhibition or activation of cellular pathways such as the mTOR pathway.

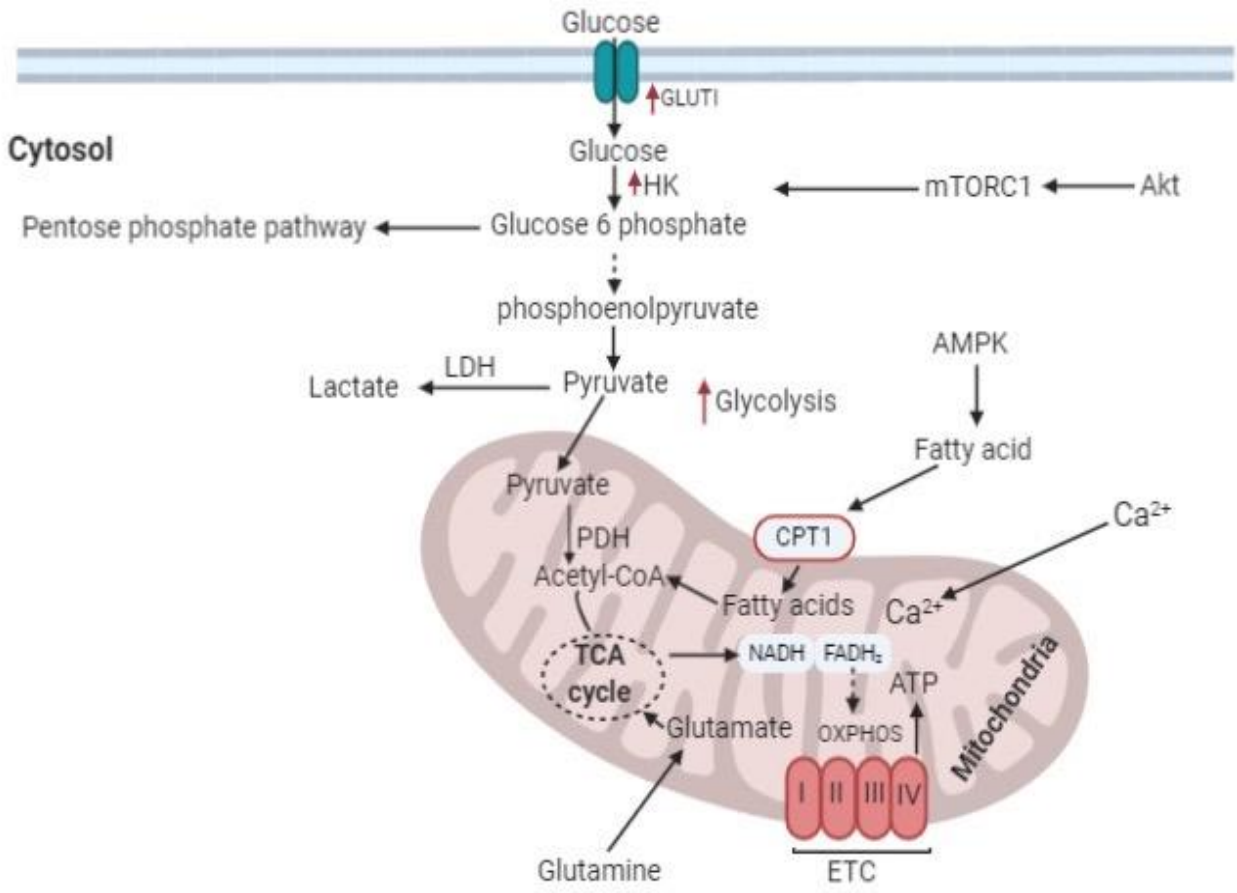


Figure 1. 9 Schematic representation of glucose and mitochondrial metabolism in T cell.

Activated T cell increases GLUT1 activation, thus enhancing glucose uptake and glycolysis. Pyruvate (a final product of glycolysis) and activated fatty acids by AMPK are transported into mitochondria and enter TCA cycle which reduces NAD+ to NADH, which donate electrons to the electron transport chain (ETC) and leads to generate ATP in a process called OXPHOS. Transported fatty acids in mitochondria can b oxidised under a process called fatty acid oxidation (FAO) to produce more ATP. Drawn in BioRender.

Summary

RA is a progressive systematic inflammatory disorder which can cause damage to the joints and damage throughout the body. Genetics factors have been associated with RA development. HLA class II which encodes HLA DRB1 is the strongest risk factor for RA. A non-HLA PTPN22 which encodes Lyp has been associated with the risk of RA. The polymorphism of the PTPN22 allele C1858T, rs2476601 is associated with RA. Environmentally, cigarette smoking is the most well-known risk factor for RA development. People who smoke are 40% higher risk of RA than non-smokers, suggesting that smoking could even exacerbate this disease. One mechanism is that smoking can affect the antioxidant system by inducing oxidative stress and free radicals which contribute to many chronic conditions such as RA.

Since PTPN22 could contribute to RA pathogenesis, our hypothesis was that:

Smoking interferes with Lyp function and thus promotes RA development through alteration in T cell signalling and metabolism.

The primary aims of this thesis were:

- To Investigate Lyp phosphatase activity and changes in energy metabolism following CSE and Lyp inhibitor treatment.
- To investigate signalling changes following CSE and Lyp inhibitor treatment.
- To investigate cytokines expressions following CSE treatment.

Chapter 2:
Materials and Methods

2.1 Introduction

This chapter includes all the reagents, chemicals and antibodies used throughout the project, and explains in detail each experimental method used. For the majority of the work, $CD4^+$ T cells naïve and memory were isolated from blood of healthy donors provided by NHS and transplant centre. These cells were treated with different concentrations of cigarette smoking extract (CSE) and PTP Lyp inhibitor and then subsequent stimulation with anti CD3/CD28.

Toxicity testing was done before carrying on to experiments to assess the effect of CSE on cell function. The function of immune cells is highly dependent on energy metabolism, so to investigate the changes in signalling pathway and alterations in energy metabolism, naïve and memory were treated with CSE and PTP Lyp inhibitor followed by CD3/CD28. Changes in energy metabolism was assessed using the Agilent Seahorse XF analyser instrument with ECAR (extracellular acidification rate – related to glycolysis) and OCR (oxygen consumption rate – related to oxidative phosphorylation. Immunoblotting was used to probe phosphorylation status of the known targets of PTPN 22 along with Akt, ZAP-70, Lck, Vav1, AMPK, S6 and mTOR.

Calcium signals was assessed cells exposed to CSE and Lyp inhibitor to provide an overall assessment of the strength of signal through the TCR. The expression of some key markers for T cells activation were investigated, including CD28, CD25, CD69 and CD62L following CSE treatment and activation using anti CD3/CD28. Modifications in the strength of signal and in energy metabolism in the cells may have significant consequences for differentiation and cytokine secretion of the T cells, so a range of cytokines produced by both memory and naïve T cells was assessed using Luminex system following exposure to CSE and activation

using anti-CD3/CD28, with a focus on the production of IL-6, IFN-γ, TNF-α, IL-1β and interleukin-10 with the aim of determining if the CSE promotes inflammatory cytokine production through changes in PTPN 22.

2.2 Materials

Table 2.1 Antibodies and drugs

Name	Stock concentration	Working concentration/dilution	Purchased from	Catalog number
Anti-human CD3	1mg/ml	3µg /ml	Immunotools, Friesoythe, Germany	21850030
Anti-human CD28	1mg/ml	1µg /ml	Immunotools, Germany	21270281
Anti-human Lck	Not known	1:1000	Cell signalling, Danvers, USA	27525
Anti-human pLck (Tyr 394)	1mg/ml	1:500-1:1000	Thermo Fisher scientific, Loughborough, UK	PA5-37628
Anti-human pLck (Tyr 505)	Not known	1:1000	Cell signalling, USA	2751S
Anti-human ZAP70	Not known	1:1000	Cell signalling, USA	27055
Anti-human ZAP70 (Tyr 493)	Not known	1:1000	Cell signalling, USA	2704S
Anti-human ZAP70 (Tyr 319)	Not known	1:1000	Cell signalling, USA	2701S
Anti-human VAV1	1mg/ml	1:250	Thermo Fisher scientific, UK	MA5-17198
Anti-human VAV1 (Tyr174)	1mg/ml	1:500-1:1000	Thermo Fisher scientific, UK	PA5-36699
Anti-human Akt	250µg/ml	1:1000	BD Biosciences, California, USA	610836
Anti-human Akt (T308)	1mg/ml	1:500-1/2000	Bioassay Technology Laboratory, Shanghai, China	BT-PHS00590
Anti-human pAMPK α (Thr172)	Not known	1:1000	Cell signalling, USA	2531S
Anti-human AMPK α	Not known	1:1000	Cell signalling, USA	2532S
Anti-human pS6 ribosomal protein (ser235/236)	Not known	1:1000	Cell signalling, USA	4858S
Anti-human S6 ribosomal protein	Not known	1:1000	Cell signalling, USA	2217S
Anti-human beta-Actin	Not known	1:5000-1:20000	Sigma-Aldrich, Burlington, USA	A2228
Goat Anti-Mouse IgG Antibody	1mg/ml	7.5µg/ml	Sigma-Aldrich, USA	M8642
Anti-rabbit IgG, HRP-linked antibody	Not known	1:1000–1:3000	Cell signalling, USA	7074S
Anti-mouse IgG, HRP-linked antibody	Not known	1:1000–1:3000	Cell signalling, USA	7076S

Brilliant violet 711 anti-human CD4 antibody	Not known	1:200	Biolegend, London, UK	300577
PE anti-human CD62L antibody	Not known	1:200	Biolegend, UK	304805
PE/cyanine5 anti-human CD59 antibody	Not known	1:200	Biolegend, UK	303005
APC anti-human CD28 antibody	Not known	1:200	Biolegend, UK	302911
Brilliant violet605 anti-human CD25 antibody	Not known	1:200	Biolegend, UK	302631
D-(+)-Glucose	2.5M	10mM	Sigma-Aldrich, USA	G8270
FCCP	10mM	1µM	Cayman chemical, Michigan, USA	15218
2-Deoxy-D-glucose (2-DG)	2.5M	50mM	Sigma-Aldrich, USA	D8375
Oligomycin	10mM	1µM	Cayman chemical, USA	11342
Rotenone	20mM	0.5µM	Cayman chemical, USA	13995
Antimycin A	2.5mM	1µM	Sigma-Aldrich, USA	A8674
PTP LYP inhibitor	15mM	150µM	Sigma-Aldrich, USA	540217
Human Lyp antibody	100µg	5µg	R&D systems Oxford UK	MAB348
Protein A	1mg	10µg	Sigma-Aldrich, USA	P6031
DIFUMP	5mg	0.2µM	Thermo Fisher scientific, UK	D6567

Table 2.2 Other drugs, reagents and chemicals

Name	Purchased from	Catalog number
L-Glutamine–Penicillin–Streptomycin (GPS)	Sigma-Aldrich, USA	G6784
Zombie Aqua™ Fixable Viability Kit	Biolegend, UK	423101
Annexin V	Abcam, Cambridge, UK	ab14085
LIVE/DEAD™ Fixable Near-IR Dead Cell Stain	Invitrogen, Paisley UK	L34975
RPMI-1640 medium	Sigma-Aldrich, USA	R0883
Seahorse XF Media	Agilent Technologies, California, USA	103680100
Bio-Plex Pro human cytokines group 1 14-plex assay	Bio-Rad, Watford, UK	M500ICUFXX
Page Ruler Pre-Stained Protein Ladder	Thermo Fisher scientific, UK	26616
Heat inactivated human serum	Sigma-Aldrich, USA	H3667

Gibco® Hank's Balanced Salt Solution (HBSS) with calcium	**Thermo Fisher scientific, UK**	**14025092**
RosetteSep™ Human T Cell Enrichment Cocktail	**Stem Cell technology, Cambridge, UK**	**07801**
CD45RO Microbeads	**Miltenyi Biotec technology, Surrey, UK**	**130046001**
SARS-CoV Rapid Colorimetric LAMP Assay kit	**New England Biolabs, Hitchin, UK**	**E2019S**
Monarch total RNA miniprep kit	**New England Biolabs, UK**	**T2010S**
Protease/phosphatase inhibitor cocktail	**Sigma-Aldrich, USA**	**PPC1010**
Ficoll-Paque Plus	**Sigma-Aldrich, USA**	**GE17144002**
Indo-1 AM ester	**Biotium, California, USA**	**50044**
Trypan blue	**Sigma-Aldrich, USA**	**T8154**
Phosphate-buffered saline (PBS)	**Thermo Fisher scientific, UK**	**003002**
EDTA	**Sigma-Aldrich, USA**	**E7889**
Bovine serum albumin *(BSA)*	**Sigma-Aldrich, USA**	**A2934**
Pierce BCA Protein Assay Kit	**Thermo Fisher scientific, UK**	**23225**
Clarity ECL western blot substrates kit	**Bio-Rad, UK**	**1705062**
ReBlot plus mild antibody stripping solution, 10x	**Sigma-Aldrich, USA**	**42029053**
Precast midi protein gel	**Bio-Rad, UK**	**5671084**
Trans-Blot Turbo Midi PVDF transfer packs	**Bio-Rad, UK**	**1704156**
6 well cell culture plate, non-pyrogenic, polystyrene	**Corning incorporated, New York, USA**	**3516**
12 well cell culture plate, non-pyrogenic, polystyrene	**Corning incorporated, USA**	**3513**
96 well cell culture plate flat bottom, non-pyrogenic, polystyrene	**Corning incorporated, USA**	**3598**

2.3 Methods

2.3.1 Generation of cigarette smoke extraction (CSE)

We used an established approach to generating a standard cigarette smoke extract (CSE) with slight modifications [320]. Cigarette smoke extract was prepared by bubbling smoke from a lighted red Marlboro cigarette into 40ml of PBS. Each cigarette was burned over a period of 1minute. The PBS containing CSE was then filtered using a 0.22µm filter, aliquoted into capped plastic tubes (1.5ml) and stored frozen at -80^{o}C until use. A fresh batch of CSE

was prepared just before isolating T cells and used for a series of experiments to avoid any variation in batches of CSE. This solution is referred to as “100% CSE” (Figure 2.1)

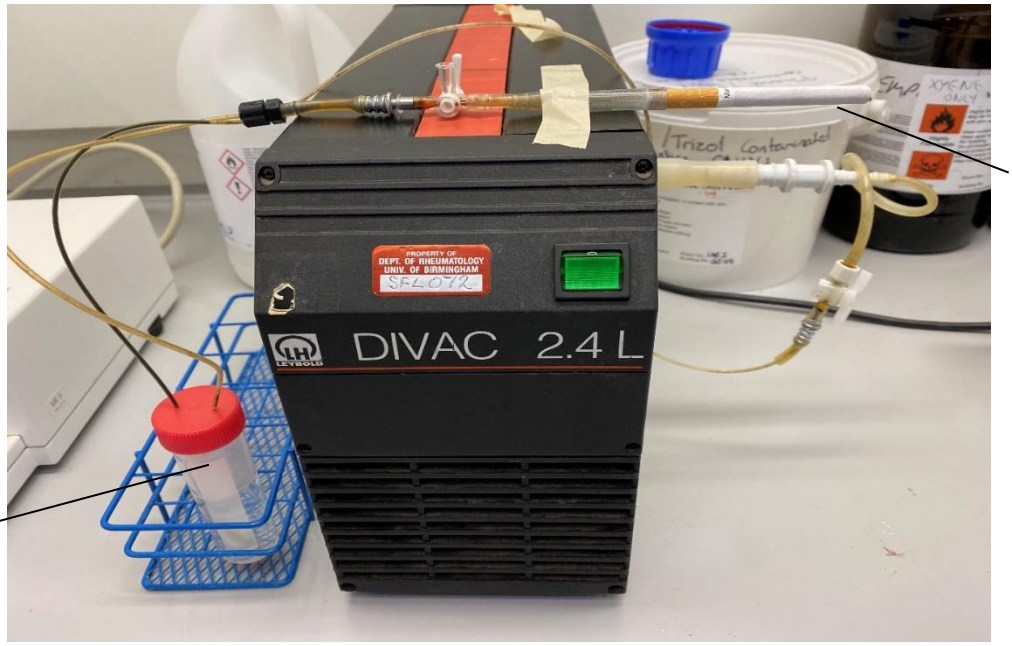


Figure 2. 1 Preparation of cigarette smoke extraction (CSE)

Smoking was prepared by bubbling 2 cigarettes of red Marlboro into 40ml of PBS using vacuum pump and then filtered to remove other small particles. A fresh aliquot was used before T cells isolation.

2.3.2 RNA extraction for testing SARS-CoV-2 virus

Since there has been enormous disruption to the lab work because of the COVID-19 pandemic and since the lab work required fresh human blood samples provided by the NHS Blood Service, the samples had to be screened using a custom PCR assay for the presence of the SARS-CoV-2 virus. Any positive samples obviously have to be discarded. Before starting RNA isolation, some reagents need to be prepared. DNase 1 was reconstituted by adding 275µl nuclease free water. Proteinase K (Prot K) by adding 1040µl of proteinase K buffer. 100ml of ethanol was added to 25ml of RNA wash buffer.

After preparing the regents, an equal volume of DNA/RNA protection reagent was added to aliquot of whole blood and vortexed (eg 200µl of blood + 200µl of DNA/RNA protection). 10µl of proteinase K was added to every 400µl of blood and DNA/RNA protection mixture. The mixture was vortexed and incubated for 30min at room temperature. After 30min, isopropanol with an equal volume was added and vortexed. The mixture was transferred into RNA purification column fitted with a collection tube and centrifuged for 30 second. 500ul of RNA wash buffer was added and centrifuged for 30 second. In a small tube, 75µl of DNase 1 reaction buffer was combined to 5µl of DNase 1 and then transferred to the top of the matrix and incubated for 15min at room temperature. After 15min, 500µl of RNA priming buffer was added and centrifuged for 30 second. Following that, 500µl of RNA wash buffer was added and centrifuged for 30 second. Another 500µl of RNA wash buffer was added and centrifuged for 2min. Finally, the RNA purification column was fitted in new collection tube and 50µl of nuclease free water was added to the centre of column matrix and centrifuged for 30 second to obtain RNA.

2.3.3 SARS-CoV-2 Rapid Colorimetric LAMP Assay

The SARS-CoV-2 Rapid Colorimetric Loop-mediated isothermal amplification (LAMP) Assay kit was used to detect SARS-CoV-2 RNA. The LAMP kit contains six components which are: WarmStart Colorimetric LAMP 2X Master Mix with UDG, Nuclease-free Water, SARS-CoV-2 LAMP Primer Mix (N/E), Guanidine Hydrochloride, SARS-CoV-2 Positive Control (N gene) and Internal Control LAMP Primer Mix (rActin). Two master mixes were prepared for this assay. The first master mix tube contained 41.25µl of WarmStart Colorimetric LAMP 2X Master Mix with UDG, 8.25µl of SARS-CoV-2 LAMP Primer Mix (N/E), 8.25µl of Guanidine Hydrochloride and 18.15µl of Nuclease-free Water (Table 2.1). The second master mix tube contained 13.75µl of WarmStart Colorimetric LAMP 2X Master Mix with UDG, 6.05µl of Nuclease-free Water, 2.75µl of Internal Control LAMP Primer Mix (rActin) and 2.75µl of Guanidine Hydrochloride (Table 2.2).

23µl of LAMP reaction mix (from the first master mix tube) and Internal Control LAMP Reaction Mix (from the second master mix tube) were added into PCR strip. 2.0µl of sample nucleic acid, SARS-CoV-2 Positive Control and Nuclease-free Water were added into the reaction according to the table below (Table 2.3). All the colors in each tube must be pink before running. The reactions were placed into pre-heated thermocycler at 65^{o}C for 30min. The result was reported based on the color obtained from the reaction which illustrated in the figure below (Figure 2.2).

Components	Volume for one sample	Volume for *n* samples
WarmStart Colorimetric LAMP 2X Master Mix with UDG	**41.25 µl**	**41.25 µl x *n***
Nuclease-free Water	**18.15 µl**	**18.15 µl x *n***
SARS-CoV-2 LAMP Primer Mix (N/E)	**8.25 µl**	**8.25 µl x *n***
Guanidine Hydrochloride	**8.25 µl**	**8.25 µl x *n***

Table 2. 3 SARS-CoV-2 LAMP Reaction Mix.

The first master mix tube contained 41.25µl of WarmStart Colorimetric LAMP 2X Master Mix with UDG, 8.25µl of SARS-CoV-2 LAMP Primer Mix (N/E), 8.25µl of Guanidine Hydrochloride and 18.15µl of Nuclease-free Water.

Components	Volume for one sample	Volume for *n* samples
WarmStart Colorimetric LAMP 2X Master Mix with UDG	**41.25 µl**	**41.25 µl x *n***
Nuclease-free Water	**18.15 µl**	**18.15 µl x *n***
Internal Control LAMP Primer Mix (rActin)	**8.25 µl**	**8.25 µl x *n***
Guanidine Hydrochloride	**8.25 µl**	**8.25 µl x *n***

Table 2.4 Internal Control LAMP Reaction Mix.

The second master mix tube contained 13.75µl of WarmStart Colorimetric LAMP 2X Master Mix with UDG, 6.05µl of Nuclease-free Water, 2.75µl of Internal Control LAMP Primer Mix (rActin) and 2.75µl of Guanidine Hydrochloride

Components	1. no template control	2. positive control	3. Internal control	4. SARS-CoV-2 test sample
SARS-CoV-2 LAMP Reaction Mix (prepared above)	**23µl**	**23µl**	**-**	**23µl**
Internal Control LAMP Reaction Mix (prepared above)	**-**	**-**	**23µl**	**-**
Nuclease-free Water	**2µl**	**-**	**-**	**-**
SARS-CoV-2 Positive Control (N gene)		**2µl**		
Sample nucleic acid	**-**	**-**	**2µl**	**2µl**

Table 2. 5 LAMP Reaction Mix.

23µl of LAMP reaction mix (from the first master mix tube) and Internal Control LAMP Reaction Mix (from the second master mix tube) were added into PCR strip. 2.0µl of sample nucleic acid, SARS-CoV-2 Positive Control and Nuclease-free Water were added into the reaction

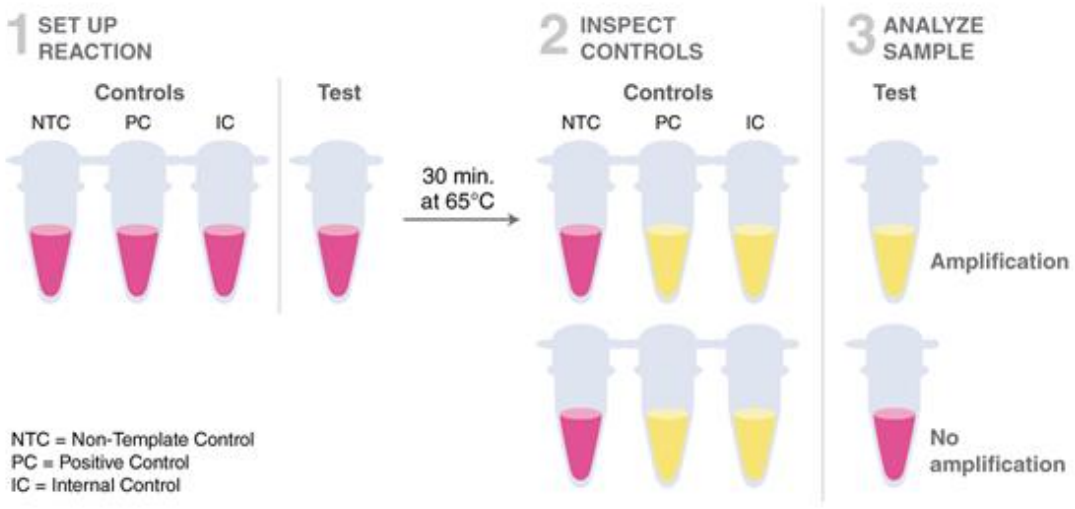


Figure 2. 2 LAMP reaction colour interpretation.

The reactions were placed into pre-heated thermocycler at 65°C for 30min. All the tubes should be pink in colour before running. For positive result, the NTC should be pink, whereas PC, IC and test should be yellow in colour. For negative result, NTC and test sample should be pink, whereas PC and IC should be yellow. This figure was adapted from New England Biolabs www.International.neb.com

2.3.4 Cell culture

To study the effects of CSE on human blood cells we needed substantial numbers of cells and so we made use of cells available from healthy donor via the NHS BTS. The blood cone was obtained under ethical approval from the NHS West Midlands Research Ethics Committee (12/WM/0077) and the University of Birmingham Life and Health Sciences Ethical Review Committee (ERN_10-1246). The blood cones containing the leukocytes derived from a blood donation (500ml) from a healthy donor was received from NHS blood and transplant centre. 75µl/ml of RosetteSep cocktail (containing antibodies for negative selection of $CD4^+$ T cells) were added to the blood, mixed gently and then incubated for 20 minutes at room temperature. The blood was diluted with phosphate-buffered saline (PBS) and mixed gently and carefully layered onto 20ml of Ficoll-Paque and centrifuged for 31 minutes at 1100g with setting one for slow acceleration and setting zero for slow braking. The T cells in the middle of the tube were aspirated, placed in a new tube and washed three times with PBS at 300g for 8 minutes. After the second wash, the cells were counted in a haemocytometer following trypan blue staining (dilution factor 1:10). After the third wash, the supernatant liquid was discarded. MACS buffer was added to the cell suspension (80 µl of MACS per 10^7 cells) followed by CD45RO microbeads for the positive selection of the $CD4^+CD45RO^+$ effector and memory T helper cells (20 µl of CD45RO microbeads per 10^7 cells) and incubated at 4^oC for 15-20 minutes.

MACS buffer (10ml) was added to the cells and the sample was centrifuged at 300g for 8 minutes. The LS column which is designed for positive selection was placed on a magnetic MACS separator and washed three times with MACS buffer (3ml each time). Following the washing step, the cell suspension was added to the LS column followed by three times

washing using MACS buffer to obtain naïve T cells. To obtain memory T cells, 5ml of MACS buffer was added to the LS column and the plunger was used to squeeze the memory T cells out of the column. RPMI media with human serum was added to both naïve and memory cells and centrifuged at 300g for 8 minutes and the cells were counted by haemocytometer using trypan blue (dilution factor 1:3) and seeded in plates based on the experiment and incubate for 24h (Figure 2.3).

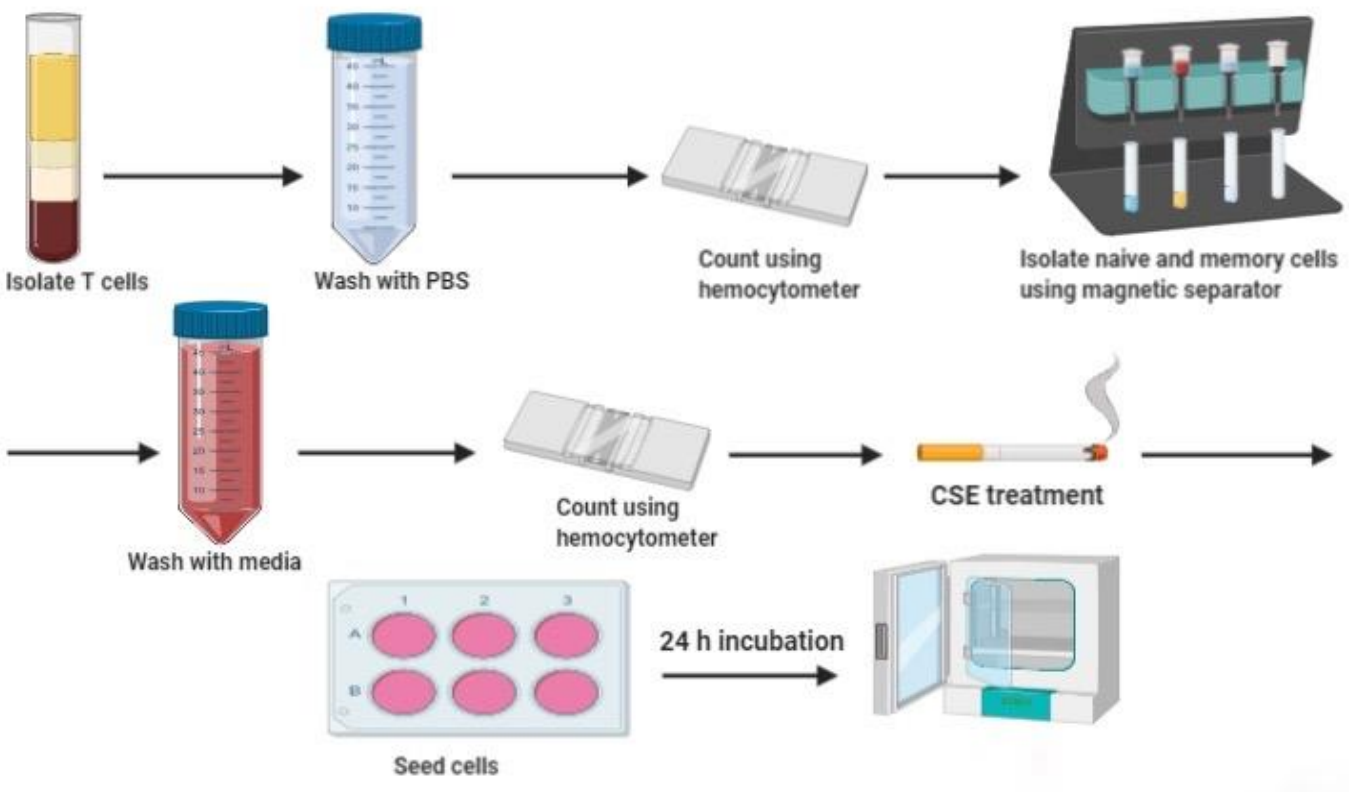


Figure 2. 3 T cells isolation and treatment in cell culture.

$CD4^+$ T cells were isolated using negative selection then washed two time with PBS and then counted. After that, naïve and memory cells were isolated using positive selection and washed two times with media and then counted. Finally, the cells were seeded in a plate and treated with CSE and incubated for 24 hours. Drawn in BioRender.

2.3.5 Cells treatment and stimulation

Cells were exposed to cigarette smoke extract (CSE) at eight concentrations: 0% (untreated cells), 0.5%, 1%, 2%, 5%, 10%, 15% and 30%. We also exposed cells to a cell-permeable, Au(I)-phosphine complex that acts as selective inhibitor of LYP (IC50= 1.5 µM) which has a ~10-fold selectivity for LYP over PTP-PEST, HePTP, and CD45 in both Jurkat T Ag cells and primary mouse thymocytes (Calbiochem Datasheet). In preliminary experiments (Stephen Young, Kalvin Sahota and Olivia Ng) we assessed the activity of this Lyp inhibitor in human cells, using a range of concentrations. We selected three concentrations, all below the published IC50, since these showed significant changes in the phosphorylation of the Lyp targets ZAP-70 and Lck (data shown in chapter 4). Given the 10-fold selectivity of the inhibitor we were confident that off-target effects would be minimised by using these sub-IC50 concentrations. The selected concentrations were: untreated cells, 0.2µM, 0.5µM and 1µM inhibitor. We started testing cells with eight concentrations of CSE to see which concentration has more effect on T cells. So, we then reduced the number of concentrations to four. Following cells isolation, cells were seeded in 6 or 12 well plate and treated with CSE and incubated for 24 hours at 37ºC incubator. A 96 well plate flat bottom was coated with 2.5µg/ml of anti-CD3 (10µl of anti-CD3 in 4ml of PBS and 50µl was transferred in each well) and incubated overnight at 4^{o}C.

After 24 hours, the cells were collected in polystyrene tubes (30ml) and centrifuged at 300xg(av) for 8 minutes. The cells were washed with media and counted using trypan blue (dilution factor 1:2) and centrifuged again at 300g for 8 minutes. The number of cells required for each condition was 2x10^{5}/well. The coated plate with anti-CD3 was washed two times with PBS and 100µl of cells suspension of each condition was added in each well

followed by 50µl of media and 50µl of anti-CD28 diluted in media (1µg/ml) and incubated for 72 hours to allow cells to proliferate (Figure 2.4). PMA (10ng/ml) and ionomycin (250ng/ml) were used also to stimulate T cells by bypassing TCR. PMA/ionomycin treatment was used in one experiment to show that if Lyp inhibitor treatment can directly affect Akt activity.

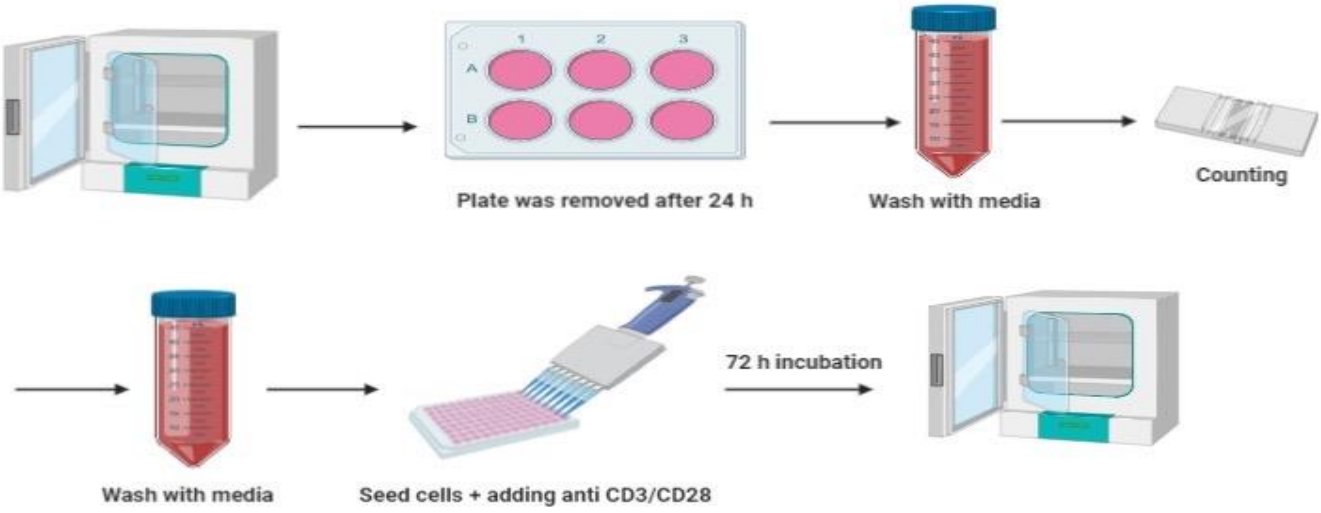


Figure 2. 4 T cells stimulation in cell culture

Naïve and memory cells were washed two times with PBS and counted using trypan blue. $2x10^5$/well were seeded in 96 well flat bottom plate and then stimulated with anti CD3/CD28 for 72 hours to allow cells to proliferate. Drawn in BioRender.

2.3.6 Viability test

Zombie Aqua fixable viability dye is a reactive dye that can pass through cells with damaged membranes but not through living cells. This fluorescent dye was used to assess live and dead cells (Figure 2.5). Cells were collected from the culture after 24 hours incubation and centrifuged at 300g for 8 minutes. 200µl of PBS was added to the cells and 100µl of cell suspension of each condition was added to the wells of a 96 well microplate. The plate was centrifuged, and the supernatant removed. The Zombie Aqua dye was diluted in PBS (1:1000) and 100µl was transferred to each well and the plate incubated for 20 minutes in the fridge. After 20 minutes, the plate was centrifuged and pellets were resuspended in 100µl of PBS. 100µl was transferred to flowcytometry tubes for analysis.

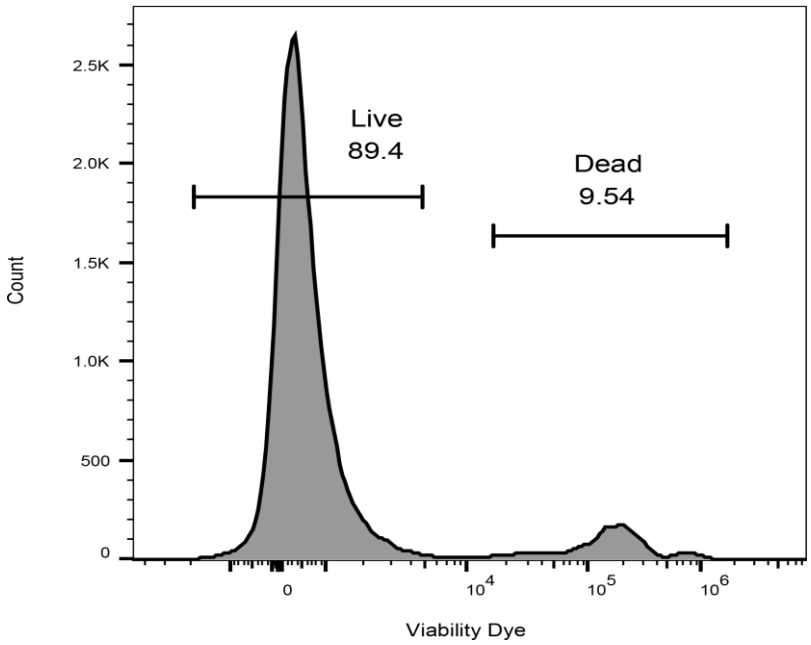


Figure 2. 5 Representative histogram of live and dead population following zombie aqua staining.

Cells were washed with PBS and stained with zombie aqua dye to assess live and dead cells in the flowcytometry machine. This graph was adapted from Biolegend website www.Biolegend.com.

2.3.7 Cell viability using Annexin V- Propidium iodide (PI)

Annexin V is useful method to detect apoptotic cells by binding to phosphatidylserine (PS) in a calcium-dependent manner, whereas a PI binds to DNA and is using for detecting necrosis and late necrotic cells. Cells were collected from culture and placed in FACS tubes and then centrifuged at 300g for 8 minutes. Cells then washed two times with PBS by centrifugation at 300g for 8 minutes. Cells were resuspended in 500µl of 1X Annexin V binding buffer. 5µl of Annexin V FITC and PI was added to the cells and incubated at room temperature for 15 minutes in the dark. Flowcytometry was used to analyse the cells.

2.3.8 Seahorse XF analyzer

To assess energy metabolism changes in $CD4^+$ T cells we made use of the Seahorse XF analyser which allowed accurate assessment of oxygen consumption and glycolysis in cells in a 96-well plate format for high throughput. 200µl of calibrant solution was added to each well for hydrating and calibrating the XF^e96 Flux sensor cartridge microplate and incubated for 24 hours in a non-CO_2 incubator at 37°C (Figure 2.6). The following day, the XF^e96 Flux cell culture microplate was coated with 30µl of poly-L-lysine for 5 minutes to allow cells to adhere properly (Figure 2.6). The poly-L-lysine was aspirated, and the plate was washed with sterile water and left to dry. While waiting for the plate to dry, stimulated cells with anti-CD3 and anti-CD28 in the culture were collected in Eppendorf tubes and centrifuged at 300xg(av) for 8 minutes. Cells were washed in XF media (pH=7.4), counted and resuspended in XF media. The number of cells required for each condition was $2x10^5$/well. 30µl of cell suspension of each condition was transferred to each well except wells in corners A1, A12,

H1, and H12 which must be XF media for background correction. Next, the plate was centrifuged at 200g for 4 minutes with 1 acceleration and zero deacceleration to allow the cells to stick at the plate bottom. 150µl of XF media was added to each well and the cell plate was incubated at 37°C in a non-CO_2 incubator for 30-60 minutes.

To assess mitochondrial function, mitochondria-interrogating drugs concentrations were optimised in our lab by Kalvin Sahota (unpublished data), which was guided by published work 'Measuring bioenergetic T cells using a seahorse extracellular flux analyser' [321]. The final concentration after injections was: 10mM glucose, 1µM oligomycin, 1µM FCCP, 50mM 2-DG, 0.5µM rotenone and 1µM antimycin A. Each port on the sensor cartridge needs to be loaded with the drugs diluted in XF media as following: port A) 20µl glucose; port B) 22µl oligomycin; port C) 24µl FCCP; port D) 26µl 2DG, rotenone and antimycin A. The calibrant plate was loaded into XF^e96 extracellular flux analyser for 20 minutes. After 20 minutes, the calibrant plate was replaced by the cell plate. Other parameters of OCR (oxygen consumption rate) and ECAR (extracellular acidification rate) were also calculated which are glycolysis, glycolytic capacity, glycolytic reserve, ATP-linked, maximal respiration and reserve capacity (Figure 2.7). These parameters can be calculated using the following equations:

Glycolysis = ECAR after addition of glucose − ECAR after 2DG treatment
Glycolytic capacity = ECAR after addition of oligomycin − ECAR after 2DG treatment
Glycolytic reserve = ECAR after addition of oligomycin − ECAR after addition of glucose
ATP-linked respiration = basal OCR – after addition of oligomycin
Maximal respiration = OCR after FCCP addition - after addition of antimycin A
Reserve capacity = OCR after FCCP addition – basal OCR.

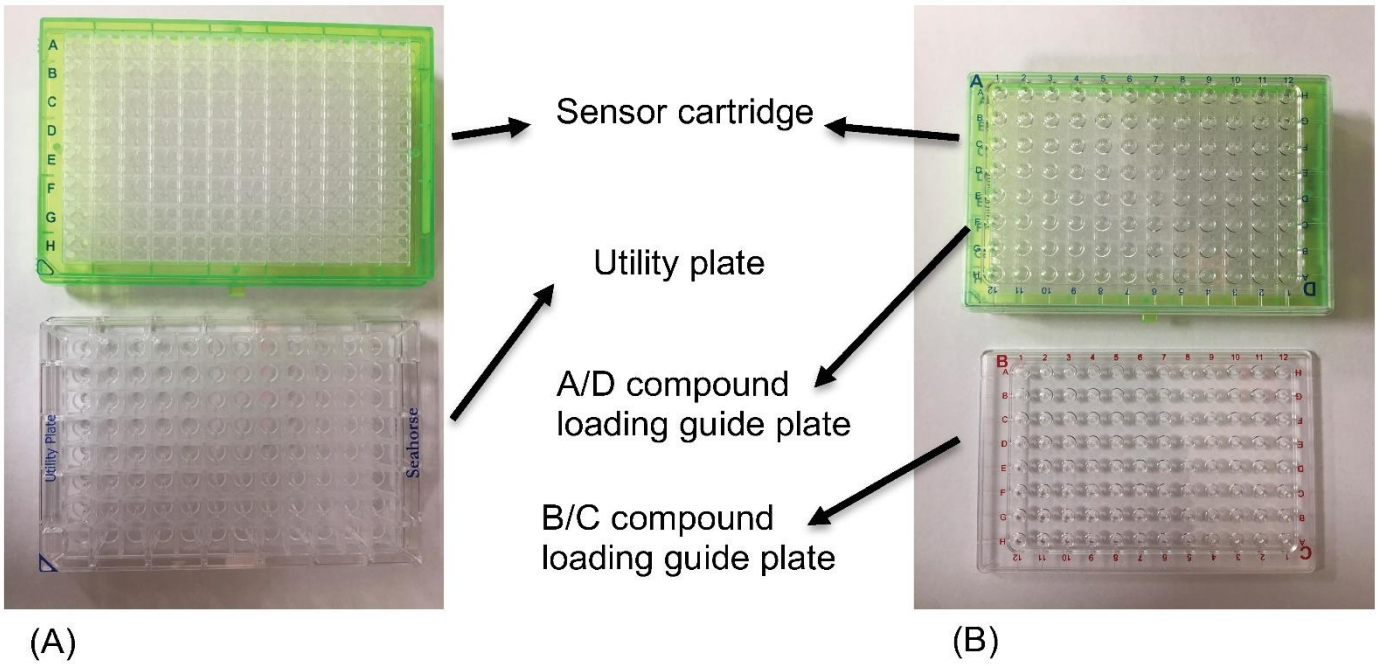


Figure 2. 6 Illustration of seahorse tissue culture microplate and sensor cartridge with ports.

Sensor cartridge was hydrated and loaded with mitochondrial drugs. Cells were seeded in the tissue culture plate and loaded into the machine. this figure was adapted from Agilent Technologies website www.aglient.com.

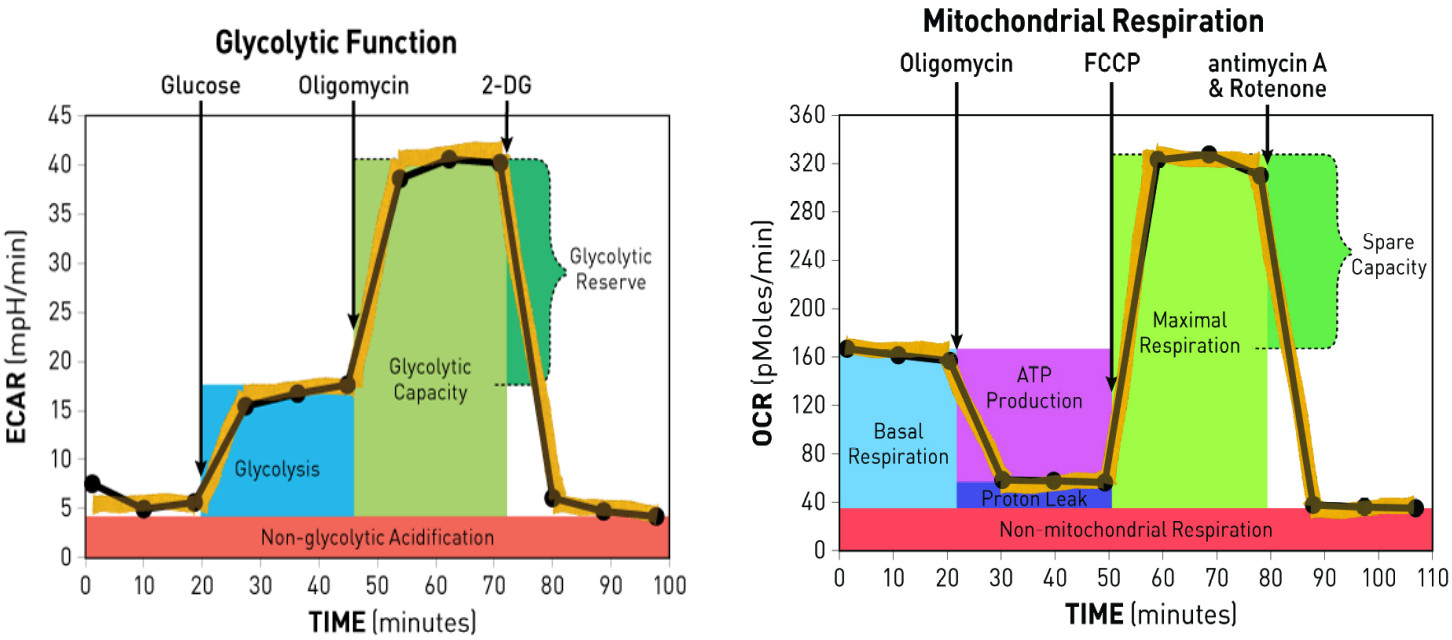


Figure 2. 7 Representative graphs of OCR and ECAR and their parameters provided by seahorse XF.

Sequential inhibitors were added for measuring basal ECAR, glycolysis, glycolytic capacity, glycolytic reserve, basal OCR, ATP production, maximal respiration and spare capacity. This figure was adapted from Agilent Technologies website www.aglient.com.

2.3.9 Immunoblotting

2.3.9.1 Sample preparation.

Cells were collected and washed with PBS and lysed with a mixture of RIPA buffer and inhibitors (phosphatase and protease inhibitors 1:100 dilution). The lysate was centrifuged at 4^{o}C for 10 minutes to remove cell pellet. Following that, the lysate transferred into Eppendorf tubes and stored in -80°C.

2.3.9.2 BCA protein assay for protein quantification.

The standard was prepared using serial dilution and kept at -20°C. Using 96 well plate flat bottom, 10µl of standard and samples were added in duplicate and a 200µl mixture of reagent A and B (1:50 dilution) from the kit obtained from Thermo Fisher was added to each well and incubated for 30 minutes at room temperature. The plate was scanned in the plate reader- BioTek at 595nm. Between 20ug-30ug of protein was used for loading in the gel (Figure 2.8).

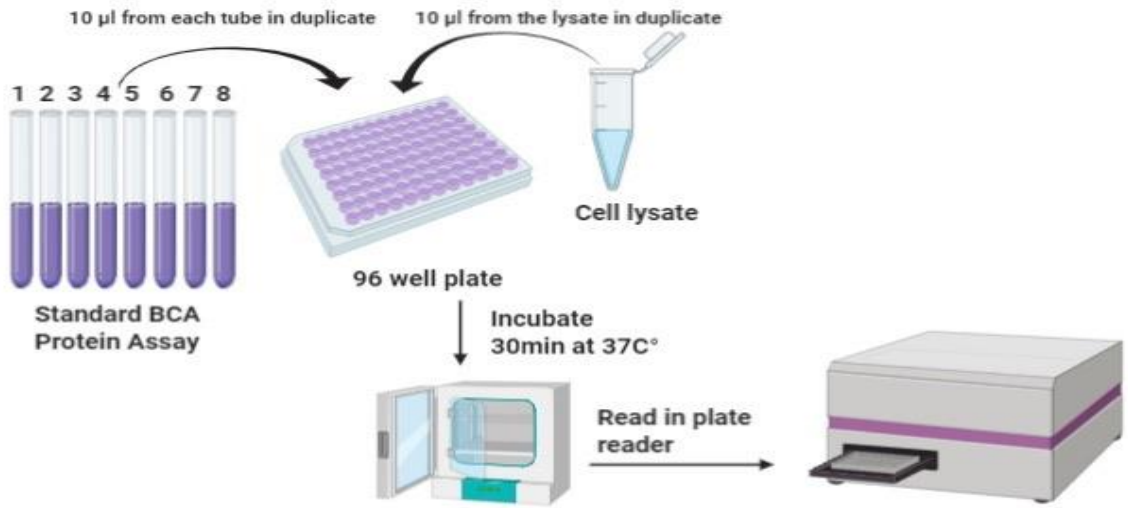


Figure 2. 8 Cell lysate and BCA assay.

The lysed cells and serial dilution of the standard were added in duplicate in 96 well plate. Using the kit from Thermo Fisher, a mixture of reagent A and B were added, and the plate was incubated for 30 min. The plate was scanned in plate reader at 595nm. Drawn in BioRender.

2.3.9.3 Running the gel and analysing the protein.

Samples were mixed with loading buffer (1:4 dilution) and heated at 95°C on a heating block for 5 minutes. Using Pre-cast gel, samples mixed with loading buffer, and marker were loaded into the wells and run at 100 voltages for 1 hour and 20 minutes. The gel was transferred to the membrane and placed in transblot machine for 7 minutes to allow protein transfer. Following protein transfer, the membrane was blocked in 5% milk in TBST solution for 1 hour on rocker to prevent nonspecific binding of antibodies. After 1 hour, the membrane was washed three times in TBST and primary antibody was added (the optimum dilution for primary antibody is illustrated in the data sheet for each antibody) and incubated overnight at 4°C.

After overnight incubation, the membrane was washed three times in TBST and secondary antibody was added (normally 1:2000 dilution) and incubated for 1 hour. The membrane was washed three times and equal amount of reagent A and B ECL was added to the membrane for 2 minutes. The membrane was then inserted into the Chemi-Doc imaging system to capture the chemiluminescent image and so detect the protein bands.

2.3.10 Activation marker staining

Staining surface markers is an important step to investigate whether the treatment can induce cell activation and proliferation. Cells were stained to assess if CSE would induce T cells activation following CD3/CD28. Cells were collected from the incubator and stained with live and dead stain (1:1000) and incubated at 4^{o}C for 30 minutes. Cells were washed twice with PBS and stained and then incubated at 4^{o}C for 30 minutes for extracellular markers: CD4 (BV711), CD25 (BV605), CD69 (PE-Cy5), CD28 (APC) and CD62L (PE). Following staining, cells were washed twice to prevent any unspecific binding. Cells then washed with

PBS + 2% FCS and transferred to FACS tube. Samples were run on flowcytometry and analysed using FlowJo.

2.3.11 Mitotracker staining

Mitotracker red is used to label mitochondria. It accumulates in the active mitochondria by passively diffusing across the plasma membrane to assess mitochondrial mass. Mitotracker was diluted in appropriate buffer or growth media to achieve 1µM final concentration. The working concentration was between 100-500nM. Cells were removed from tissue culture and centrifuged at 300xg(av) for 8 minutes. Following that, Cells were resuspended in the solution containing mitotracker probe and incubated for 45 minutes. Cells were centrifuged again and resuspended in prewarmed buffer or growth media. Finally, cells suspension was transferred to FACS tube and run on flowcytometry.

2.3.12 Luminex assay

The standard was prepared using serial dilution and incubated on ice for 30 minutes. 50µl of the diluted beads was added to each well of the assay plate. The plate was washed two times by adding 100µl of washing buffer. 50µl of samples, standard and blank were added to the wells and the plate was covered and incubated on shaker at 850 rpm at room temperature. The plate was washed three times and 25µl of the diluted detection antibody was added followed by incubation on shaker again for 30 minutes. The plate was washed three times and 50µl of the diluted SA-PE was added followed by incubation on shaker for 10 minutes. The plate was washed three times and 125µl of assay buffer was added and incubated on the shaker for 30 second. Finally, the reading was taken using Luminex machine.

2.3.13 Ca^{2+} flux measurement

Cells were collected from incubator and placed in falcon tubes and centrifuged at 300xg(av) for 8 minutes. Indo-1 AM ester was prepared by reconstituting in DMSO. Indo-1 AM ester was then added to the cells to achieve a final concentration of 1µM. Cells were incubated at 37°C in the dark for 40min. Following incubation, cells were washed three times in Ca^{2+} containing HEPES buffered saline solution (HBSS) and resuspended at 1 x 10^6/ml (to achieve a smoother curve, it is best to have more cells).

1.5ml of cells suspension were added to a plastic cuvette and placed in water bath for 8 minutes to warm up. Following incubation, the cuvette was placed into fluorimeter holder to start the measurement (allow 100 seconds to establish a baseline after running the sample in the machine). Cells were stimulated with 7.5µg/ml anti CD3 to induce Ca^{2+} release and then followed by 7.5µg/ml of crosslinking goat anti mouse antibody. Finally, 5.6µM ionomycin was added as positive control to establish the maximum ratio of calcium influx.

2.3.13 Measuring the activity of Lyp phosphatase

5µl of protein A (stock 10µg/ml) was added to carbonate bicarbonate. 100µl of Protein A/carbonate bicarbonate mixture was transferred to each well and incubated for overnight at 4°C. After overnight incubation, the wells were washed three times with 0.05% tween 20/PBS. 5µg of anti-human Lyp (mouse) was added to 1% BSA /0.05% tween 20/PBS and 50µl was transferred to each well and incubated for 2 hours at 37°C to allow antibody to bind to protein A. After 2 hours, the wells were washed three times with 0.05% tween 20/PBS and blocked with 2% BSA/0.05% tween 20/PBS for 1 hour at 37°C. CD4+ T cells naïve and memory were removed from culture and transferred into 15ml falcon tubes (4 million cells per condition) and centrifuged at 300xg(av) for 8 minutes. Cells were washed twice in

Ca2+ hanks buffer salt (HBSS) and transferred into 1.5ml tubes and centrifuged at 300xg(av) for 8 minutes. Cells were transferred into 1.5ml tubes and lysed in lysis buffer (20mM Tris, 150mM NaCl, 1mM EDTA, pH 7.4) which was supplemented with protease inhibitor. Lysis was incubated in ice for 30 minutes with regular agitation and then centrifuged at 13000xg (av) for 10 minutes at 4°C to remove cell debris. The blocking solution was removed, and lysate was added to the wells and incubated for 3 hours at 37°C. After 3 hours incubation, wells were washed three times with 0.05% tween 20/PBS and the Lyp activity was detected by adding 0.2mM of DiFUMP in phosphate reaction solution with 50mM DTT (Figure 2.9). The activity of Lyp phosphatase was measured using Fluoroskan Ascent plate reader with the 355/460nm reading. The reading was taken over 2 hours by measuring the accumulation of the fluorescent product.

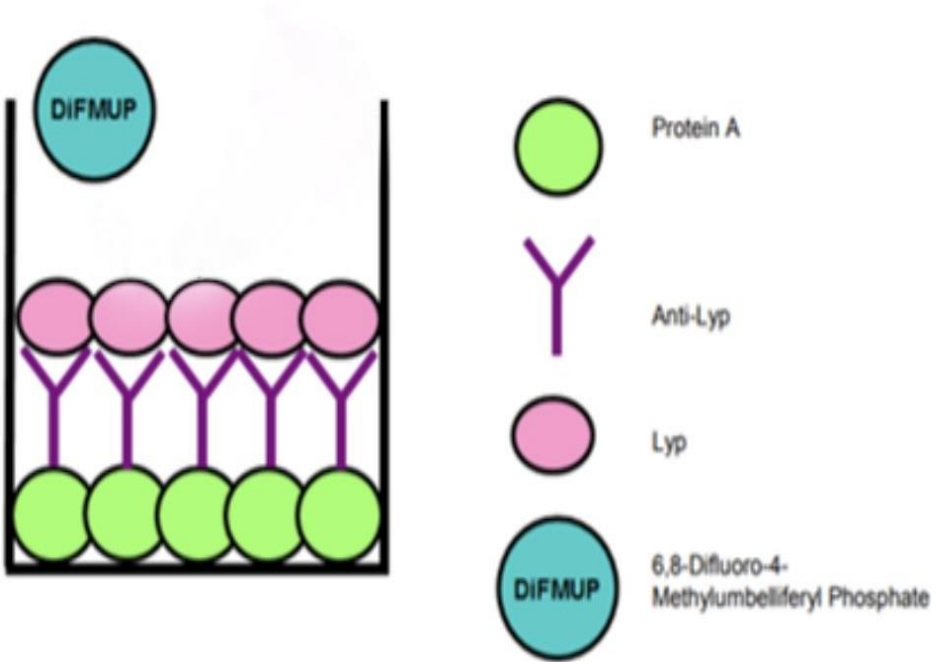


Figure 2. 9 measuring the specific activity of Lyp phosphatase.

The plate was coated with protein A and incubated overnight at 4°C. Plate was washed three times then anti-human Lyp was added and then followed by cell lysates. After further washing the activity was measured by adding DiFUMP to detect Lyp activity.

2.3.14 Statistical analysis.

Prism (version 7; GraphPad Software, Inc., San Diego, CA) was used to perform statistical analysis. One-Way ANOVA was used to compare the changes in naïve and memory cells following exposure to CSE and Lyp inhibitor. Significance was tested using ANOVA with Friedman's correction (*=$p<0.05$, **=$p<0.01$). ImageJ was used to assess and quantify western blot result through a comparison between band in different lane. BioRender was used to draw the figures.

Chapter 3:

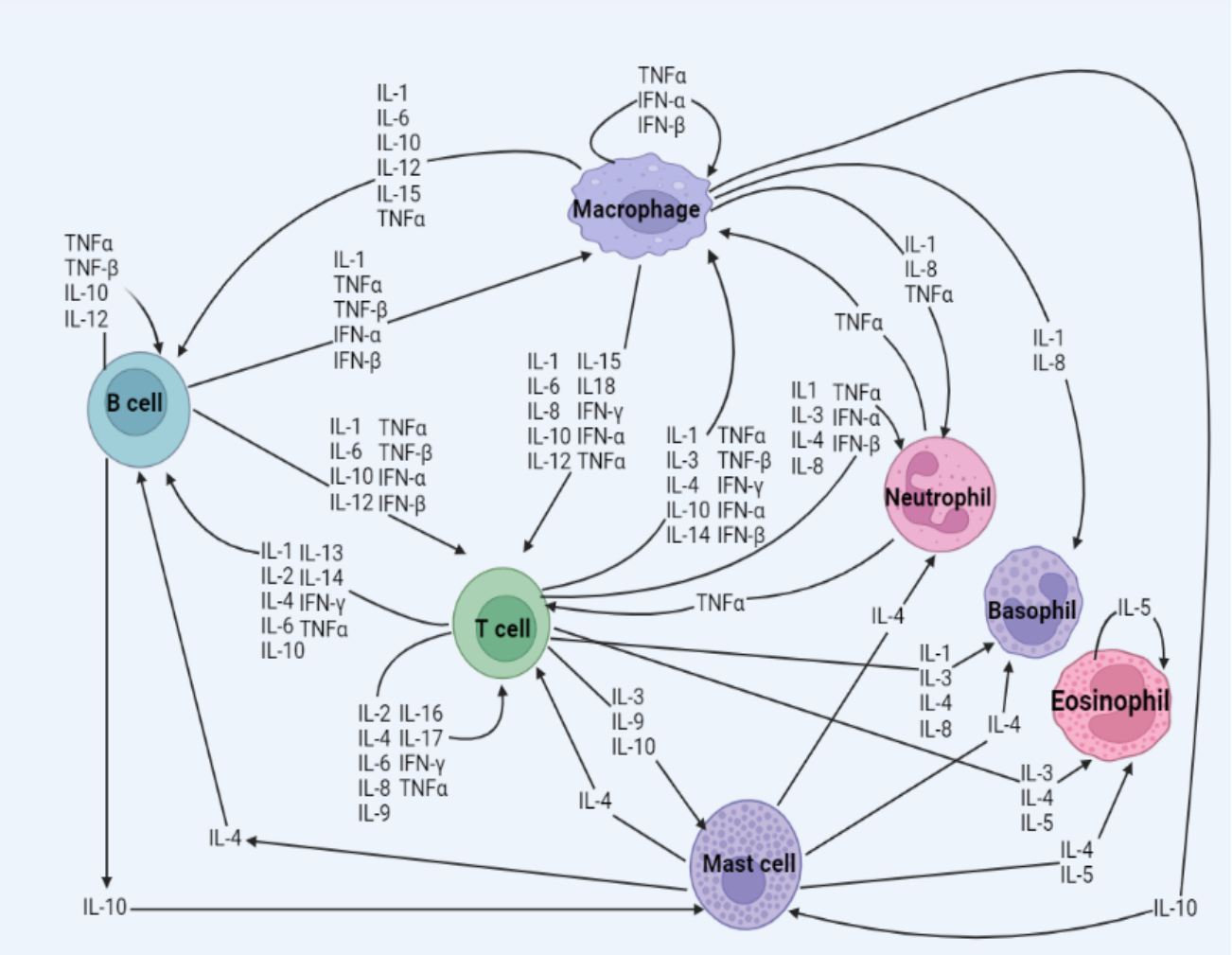
The effects of cigarette smoke extract and Lyp inhibition on the activation of, and cytokine production by, primary human CD4+ naïve and memory T cells.

3.1 Introduction

Changes in specific surface proteins on immune cells are important markers for the activation and proliferation of the cells, and can also distinguish different subsets which play roles in secreting different cytokines [322]. The most typical activation markers for T cells are CD25 (IL-2 receptor) and CD69, which are both associated with lymphocyte proliferation [323]. Although CD25 and CD69 are significant markers for T cell activation, there are others such as CD28, CD70 and CD154 [324]. The cytokines secreted following immune cell activation have specific roles in helping the immune system to fight foreign pathogens and regulate the subsequent immune response. However, excessive or aberrant production can be dangerous since they may promote autoimmune responses by activating other immune cells and stimulating the production of a cascade of other cytokines [325].

Proinflammatory cytokines (Figure 3.1) play a crucial role in upregulating inflammatory reactions and initiating inflammation. The main cytokines in this group are IL-6, IFN-γ and TNF-α. On the other hand, anti-inflammatory cytokines play a role in controlling inflammatory cytokine responses and preventing inflammation and autoimmunity. These include IL-10, IL4, IL-13 and IL-11, but among those, IL-10 is the most potent cytokine with anti-inflammatory properties [326] [327]. These studies reported that these Th2 cytokines can act to moderate inflammation. In this chapter, the viability of naïve and memory cells was investigated using both zombie aqua and annexin V/PI staining following CSE and Lyp inhibitor treatment. The expression of some key markers for T cells activation were investigated, including CD28, CD25, CD69 and CD62L following CSE treatment and activation of T cells using anti CD3/CD28. Modifications in the strength of signal and in energy metabolism in the cells may have significant consequences for differentiation and cytokine

secretion of the T cells. Therefore, a range of cytokines produced by both memory and naïve T cells was assessed following exposure to CSE and activation using anti CD3/CD28, with a focus on the production of IL-6, IFN-γ, TNF-α and IL-10 with the aim of determining if the CSE promotes inflammatory cytokine production through changes in signalling pathway and metabolism. In this chapter we showed that CSE enhanced T cells activity by activating some key surface markers such as CD25 and CD69 along with the expression of CD62L which is mainly expressed in naïve T cells and play a role in inflammation development. Also, CSE enhanced some key proinflammatory cytokines such as IL-6, IFN-γ and TNF-α, while IL-10 was decreased or show no effect following CSE treatment, implying that cigarette smoking can act as a proinflammatory mediator.



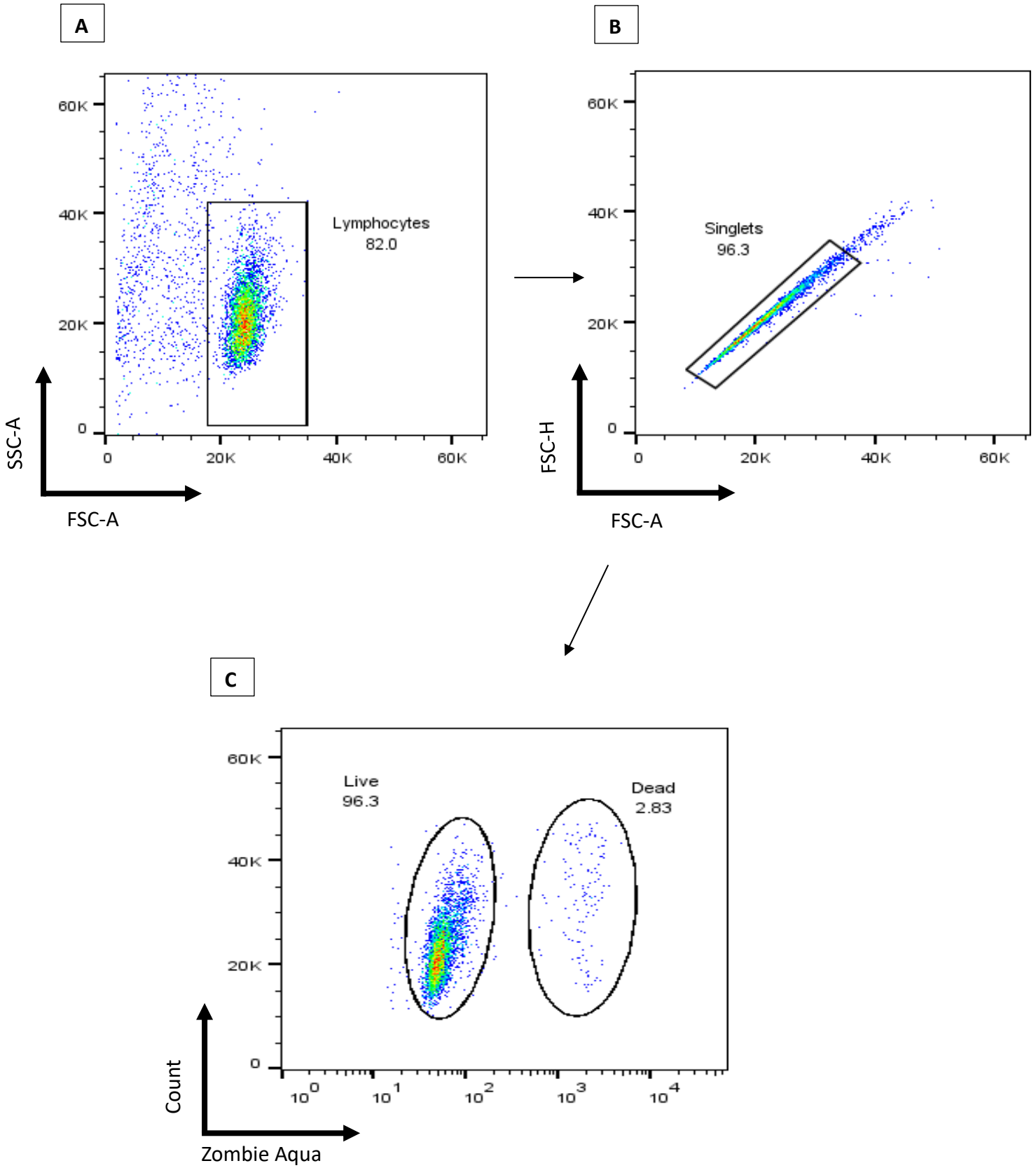
3. 1 An overview of cytokines network produced by different immune cells.

Several cells such as T cells, B cells, Neutrophils, macrophages, eosinophils and basophils coordinate their effort as a part of immune system. Each cell plays a specific role in immune system, and secrets cytokines to communicate with other immune cells. for example, T cells can secret cytokines to stimulate other cells such as B cells and macrophages in order to respond to specific antigen. Eosinophils, basophils and neutrophils can also respond to cytokines.

3.2 Results

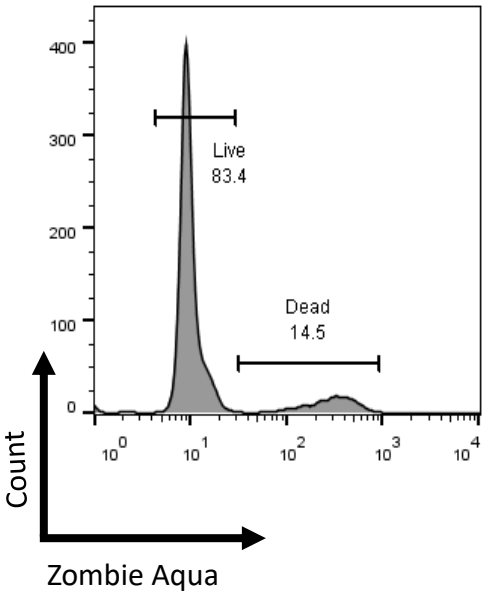
3.2.1 CSE showed no effect on the viability of both naïve and memory T cells following zombie aqua staining.

Cell viability is used to measure the health and response of cells following various stimuli. In vitro, drugs and chemicals have different effect on cell structure and function and even compromise cell viability. So, there was a need for cell viability and short-term cytotoxicity assays to determine that any effects seen were not due to the induction of cell death. Cigarette smoke contains many toxic substances which could affect the viability of cells and so prior to experiments to assess the effects of CSE on cell function, toxicity testing was implemented using the Zombie Aqua Viability test. This uses flow cytometry to assess the exclusion of a fluorescent dye from viable cells. Fresh human naïve and memory $CD4^+$ T cells were treated with different concentrations of CSE (0, 0.5%, 1%, 2%, 5%, 10% and 30%) and incubated for 24 hours with no anti-CD3/CD28. This was repeated in three independent experiments using different blood donors. Figure 3.2 A-C is a representative gating strategy of three independent experiments. Under most of the conditions used the cell viability remained above 83% (Figure 3.2 D-J), whereas dead cells represented low numbers up to 14%. Importantly, cell death was not greatly different from controls even at the highest concentrations of CSE. At intermediate concentrations dead cells represented small numbers from 10-16% which again was not significantly higher than the controls, at 14.5%. Results from exposing memory T cells showed very similar results with the viability remaining above 84% with most CSE concentrations which again was not significantly different from the controls (83.8%) (Figure 3.2 K-Q). (Figure 3.2 R+S) clearly showed no difference between the control and CSE treatment. From these results it was concluded that any effects of CSE in subsequent experiments would not be as a result of a loss of cell viability.


A
B
C
60K
40K
20K
0
Lymphocytes
82.0
SSC-A
FSC-A
Singlets
96.3
FSC-H
Live
96.3
Dead
2.83
Count
Zombie Aqua
10^0
10^1
10^2
10^3
10^4

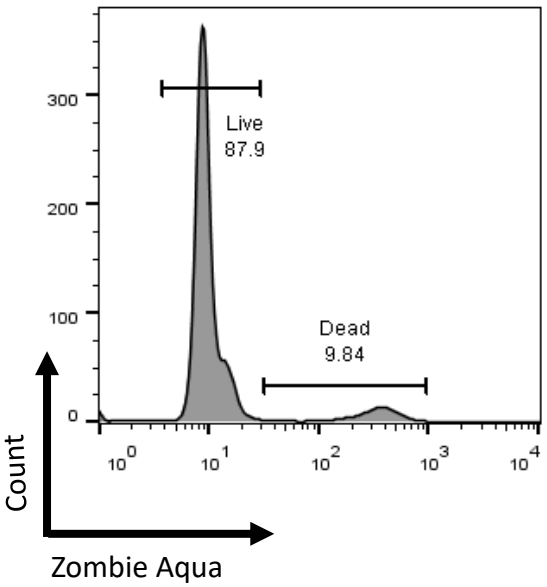
D

Untreated naïve cells



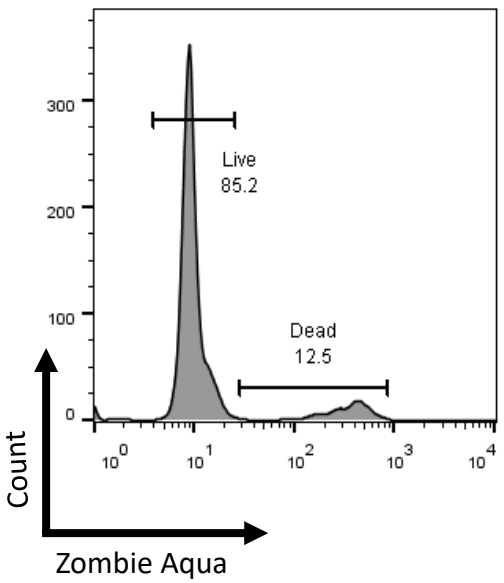
E

Naïve 0.5% CSE



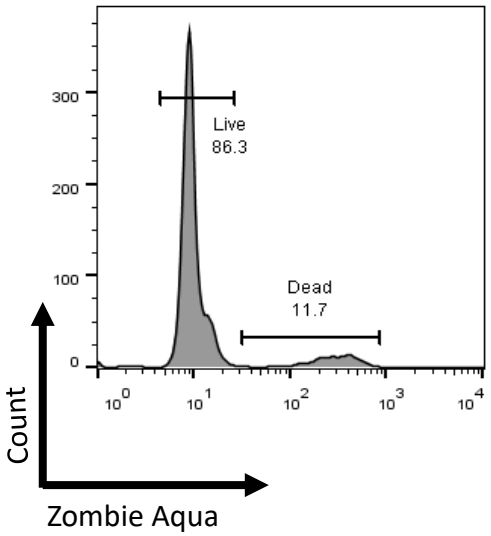
F

Naïve 1% CSE



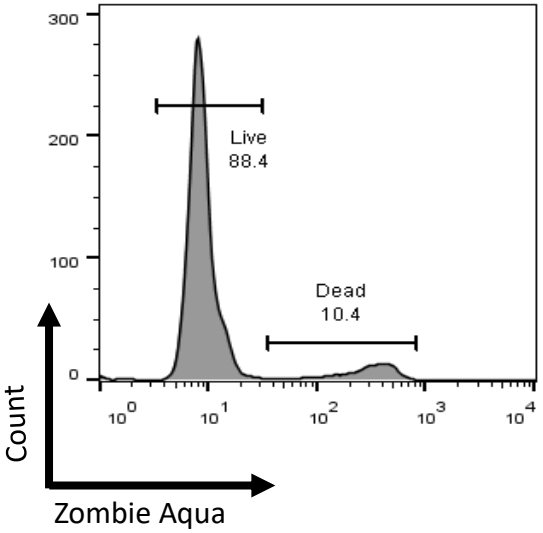
G

Naïve 2% CSE



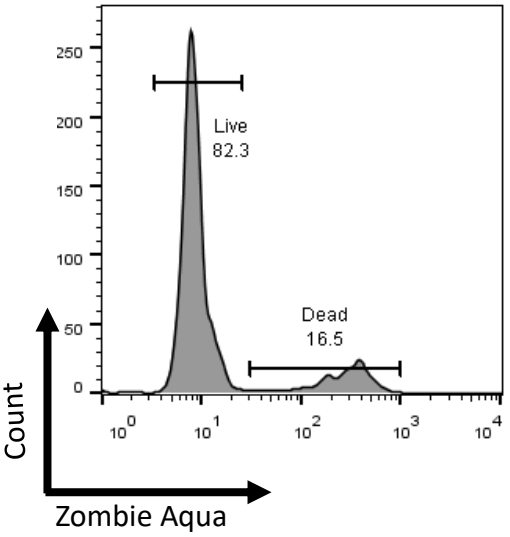
H

Naïve 5% CSE



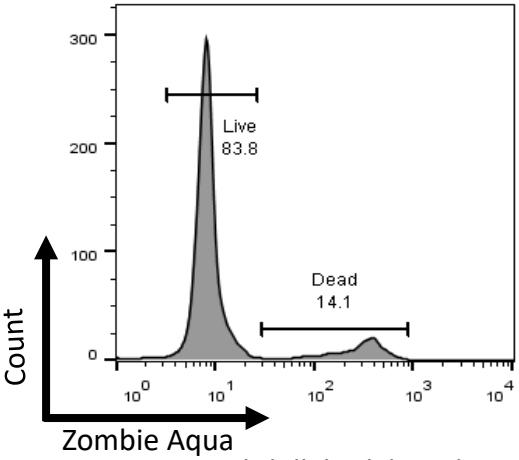
I

Naïve 10% CSE



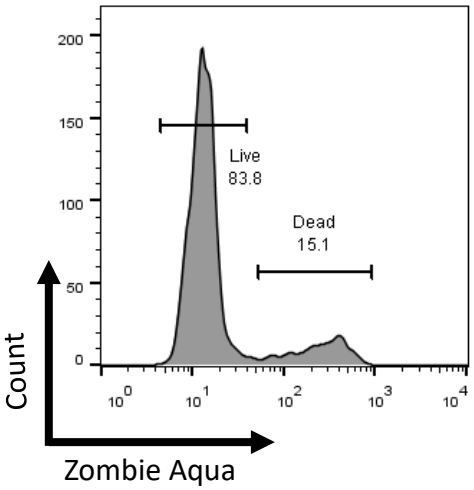
J

Naïve 30% CSE



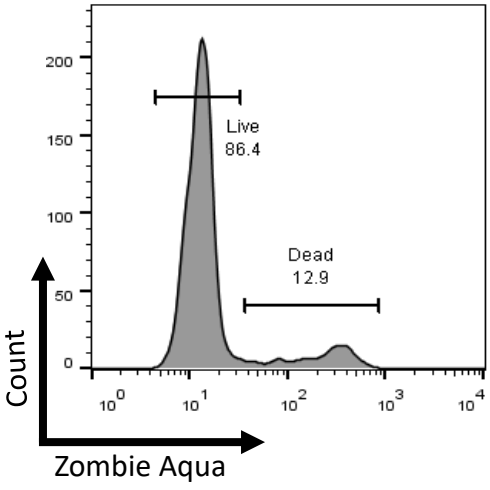
K

Untreated memory cells



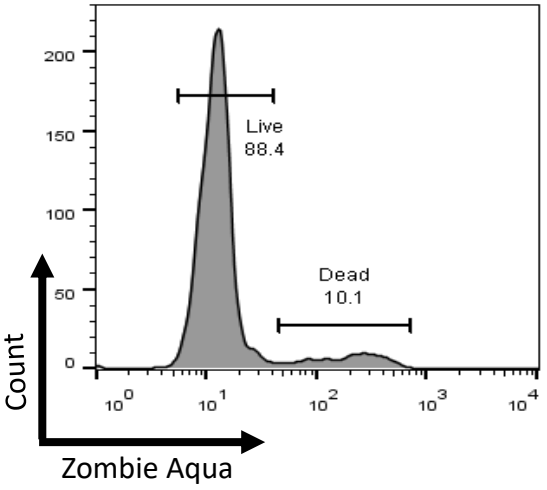
L

Memory 0.5% CSE



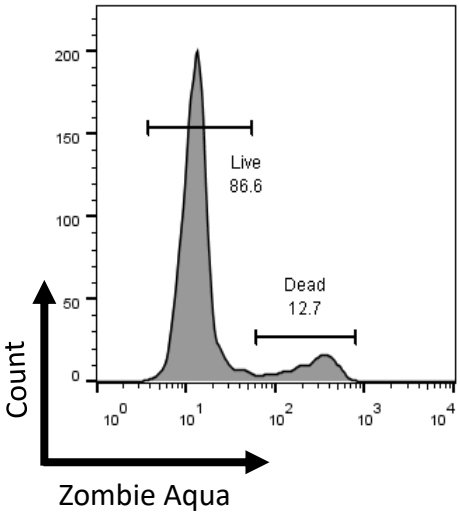
M

Memory 1% CSE



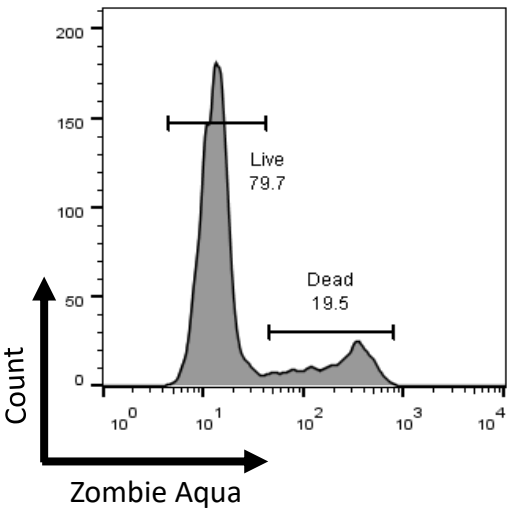
N

Memory 2% CSE



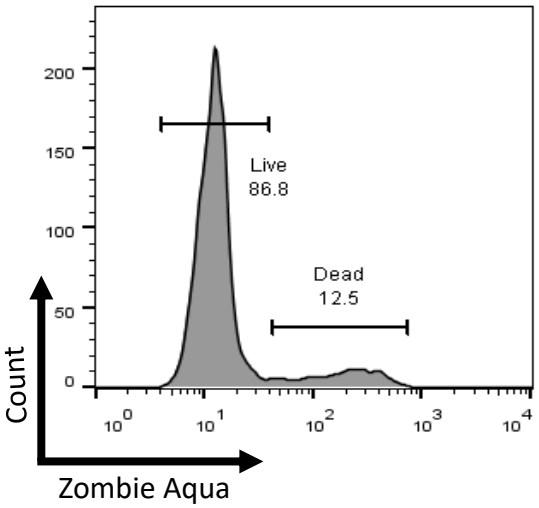
O

Memory 5% CSE



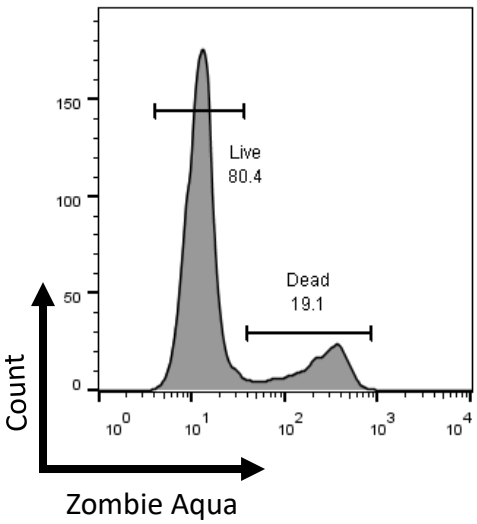
P

Memory 10% CSE

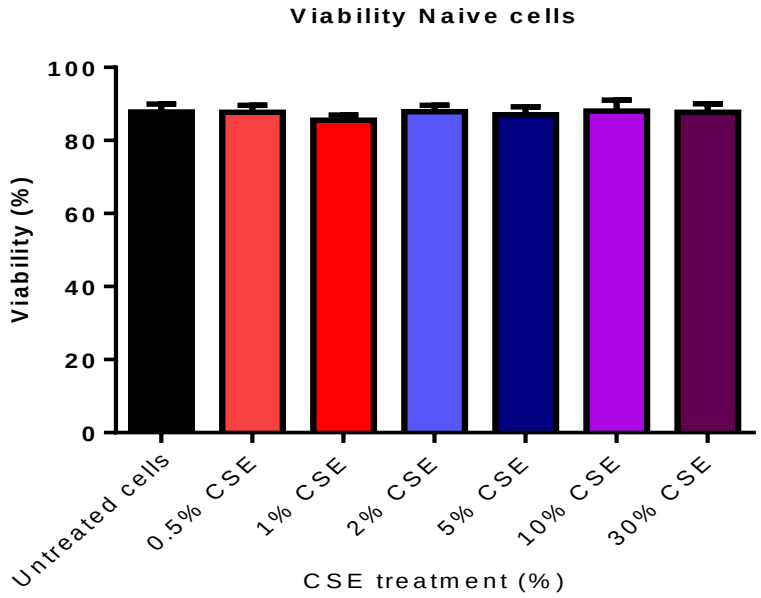


Q

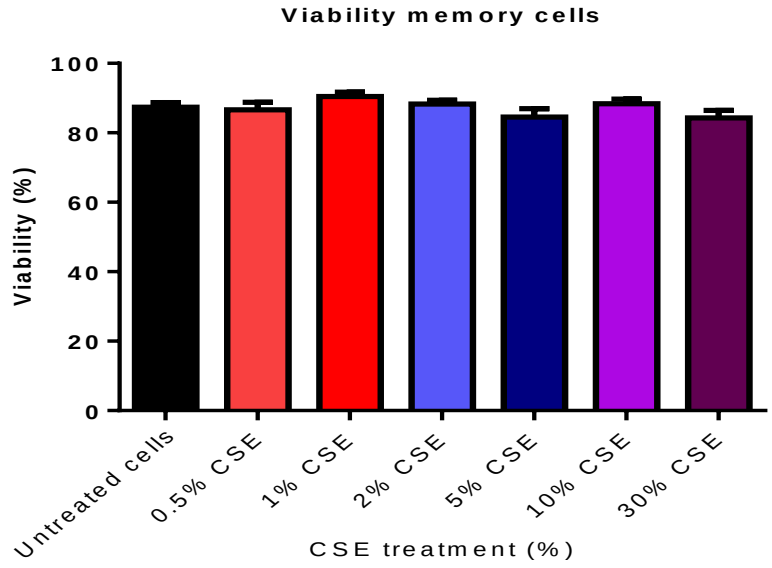
Memory 30% CSE



R



S

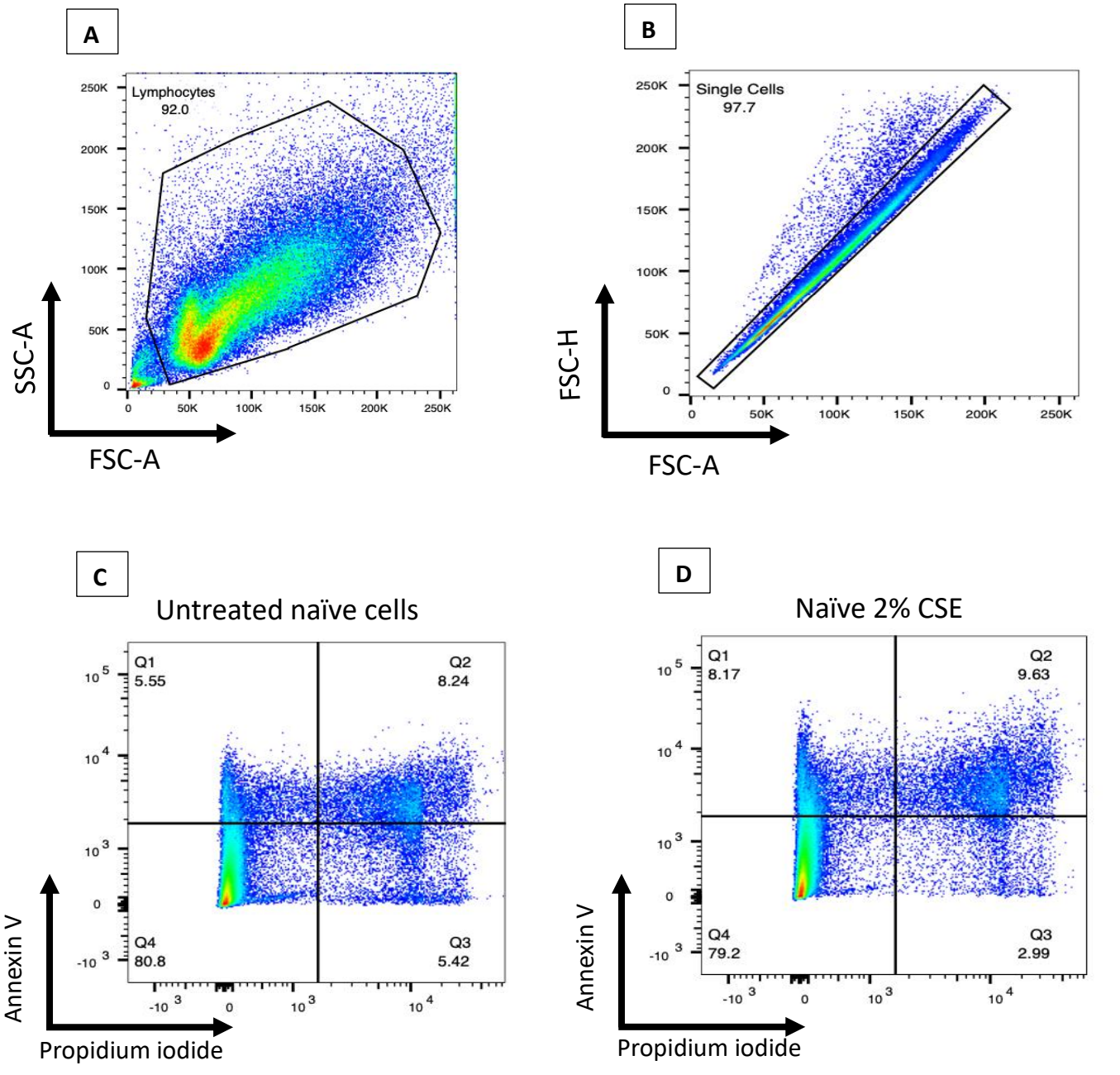


3. 2 Naïve and memory T cells viability were not affected by CSE treatment following zombie aqua staining.

Naïve and memory cells were treated with different concentrations of CSE for 24 hours but not stimulated with anti CD3/CD28 and stained with zombie aqua. A-C is a representative gating strategy for the experiment. Data from D-J and K-Q showed that the cell viability remained above 83%, whereas dead cells represented low numbers up to 14%. K-S clearly showed no difference between the control and CSE. Importantly, cell death was not greatly different from controls even at the highest concentrations of CSE. This suggests that after a 24hr incubation with CSE cells are not toxic to both naïve or memory T cells, so other experiments can be carried out and results not being affected by loss of cell viability. Representative graphs are shown from one of the three independent experiment. (n=3)

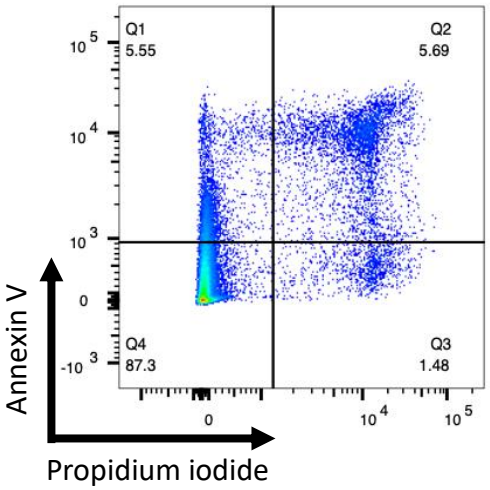
3.2.2 CSE and Lyp inhibitor showed no effect on the viability of both naïve and memory T cells following annexin V/PI staining.

In the previous result we investigated the cell death using zombie aqua. However, this type of result can give only live and dead without any further details. Therefore, we investigated the cell death using annexin V/PI which provides a sensitive approach to study apoptosis, necrosis and early or late apoptotic cells. This method would give more details and add more solid data to the result stated above. Naïve and memory cells were treated with CSE and Lyp inhibitor then stimulated with anti CD3/CD28. Cells then stained with annexin V/PI and analysed using flowcytometry. Figure 3.3 A+B is a representative gating strategy of three independent experiments. For accurate gating, fluorescence minus one (FMO) was used as a control. It was found that the viability of the cells (Figure 3.3 C-j) was not affected either by CSE (Naïve untreated = 80.8% live cells, Naive 2% CSE = 79.2% live cells, Naïve 15% CSE = 76.8% live cells, Naïve 30% CSE = 72% live cells, Memory untreated = 87.3% live cells, Memory 2% CSE = 76.5% live cells, 15% CSE = 84.3% live cells, 30% CSE = 80.2% live cells) or (Figure 3.2 K-P) Lyp inhibitor (Naïve untreated = 81.3% live cells, Naive 0.2µM = 83.1% live cells, Naïve 0.5µM = 79.1% live cells, Naïve 1µM = 80.4% live cells, Memory untreated = 79.1% live cells, Memory 0.2µM = 72.3% live cells, 0.5µM = 77.9% live cells, 1µM = 73.3% live cells). Although there were some cell death compared to control following CSE and Lyp inhibitor treatment, it was not a significant cells death. Also, it was expected to observe cell death at high concentrations because of toxic nature of high doses. Figure Q+R are clearly showed no difference between the control and CSE and Lyp inhibitor. From these results it was concluded that any effects of CSE and Lyp inhibitor in subsequent experiments would not be as a result of a loss of cell viability. The results showed the mean (±SEM) of three separate experiments.


A
Lymphocytes
92.0
SSC-A
FSC-A
B
Single Cells
97.7
FSC-H
FSC-A
C
Untreated naïve cells
Q1
5.55
Q2
8.24
Q4
80.8
Q3
5.42
Annexin V
Propidium iodide
D
Naïve 2% CSE
Q1
8.17
Q2
9.63
Q4
79.2
Q3
2.99
Annexin V
Propidium iodide
E
Naïve 15% CSE
Q1
6.89
Q2
13.3
Q4
76.8
Q3
2.97
Annexin V
Propidium iodide
F
Naïve 30% CSE
Q1
9.78
Q2
15.8
Q4
72.0
Q3
2.45
Annexin V
Propidium iodide

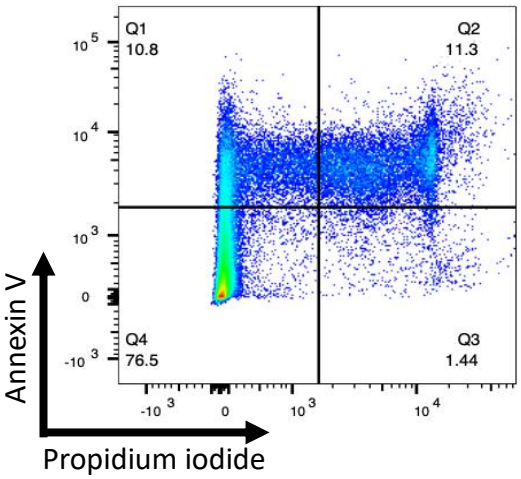
G

Untreated memory cells



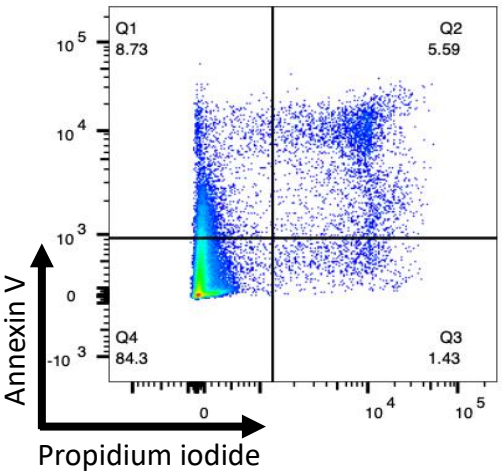
H

Memory 2% CSE



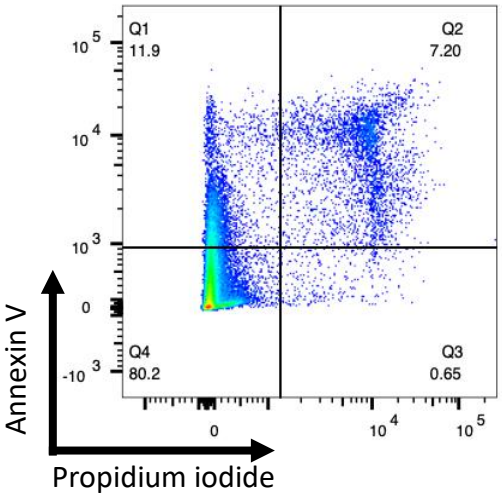
I

Memory 15% CSE



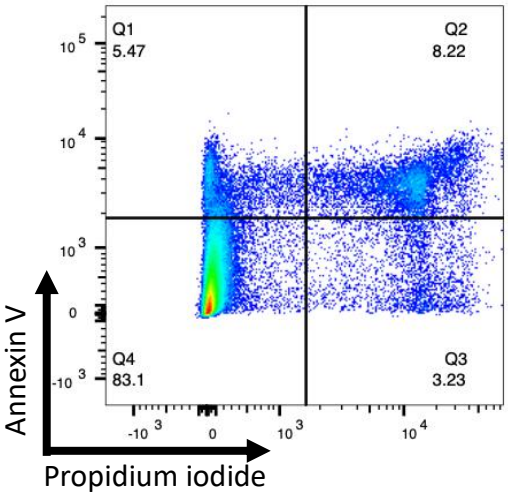
J

Memory 30% CSE



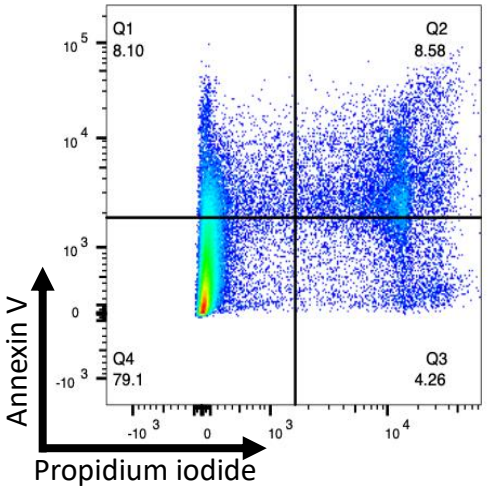
K

Naïve 0.2µM Lyp



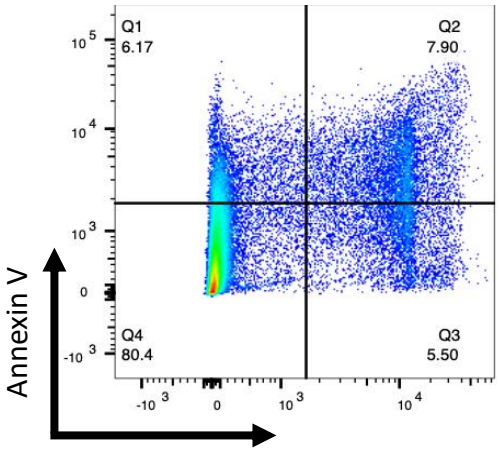
L

Naïve 0.5µM Lyp



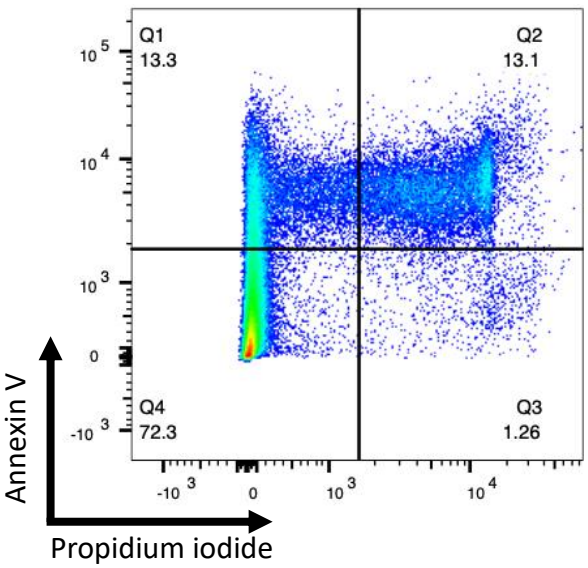
M

Naïve 1µM Lyp inhibitor



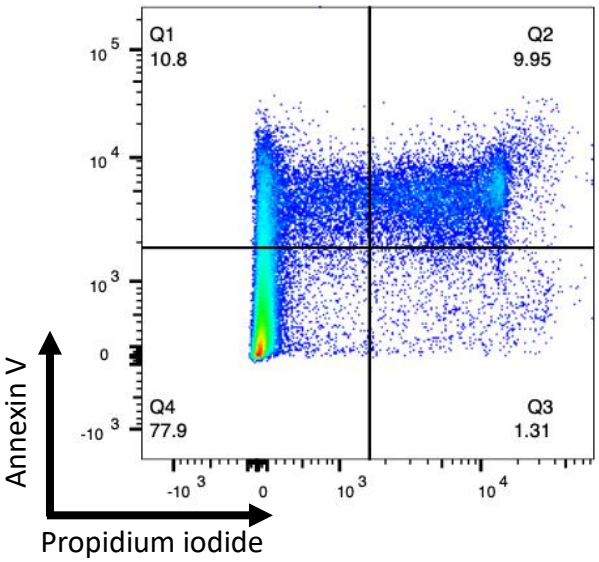
N

Memory 0.2µM Lyp inhibitor



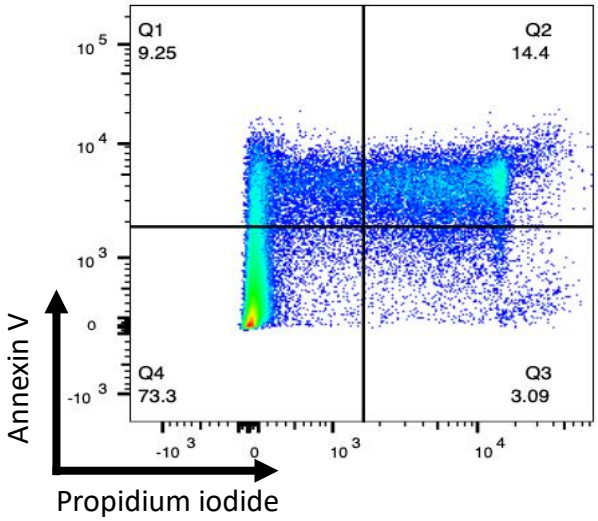
O

Memory 0.5µM Lyp inhibitor

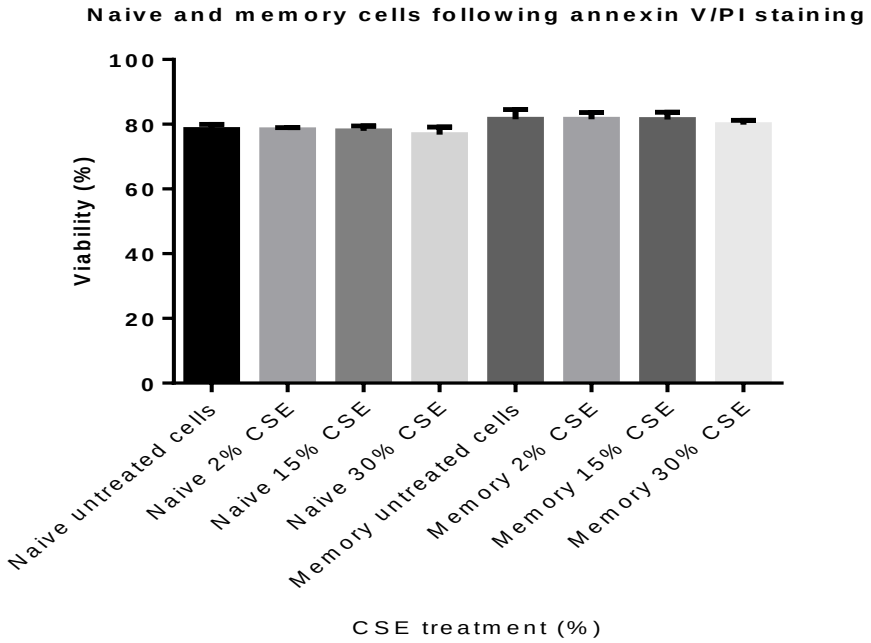


P

Memory 1µM Lyp inhibitor



Q



R

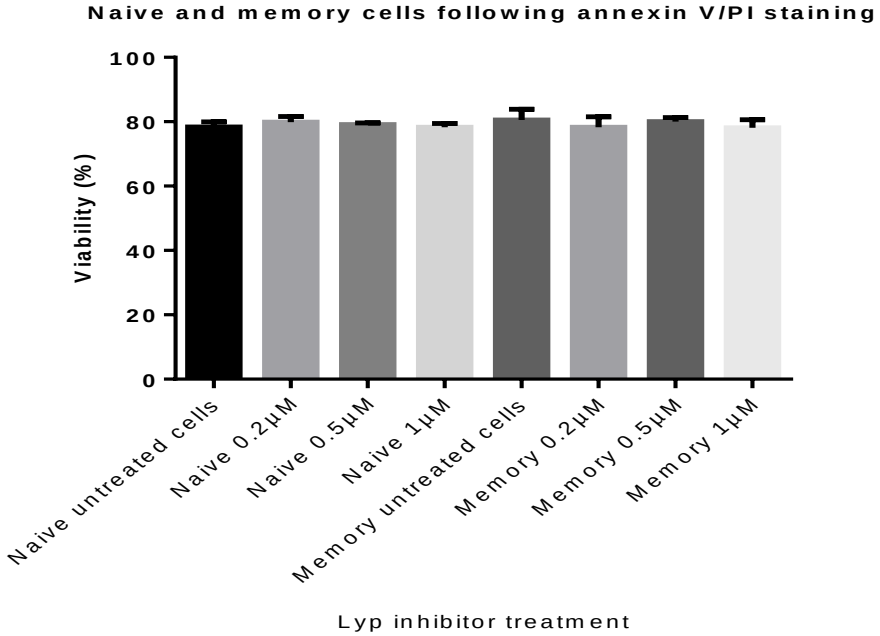


Figure 3. 3 Naïve and memory T cells viability were not affected by CSE and Lyp inhibitor treatment following annexin V/PI staining.

Naïve and memory cells were treated with different concentrations of CSE for 24 hours and stained with annexin V/PI. From C-F and G-J also from K-M and N-P most of cells were viable above 72%, whereas few cells were dead around 27% with high concentrations. Q+R are clearly showed no difference between the control and CSE and Lyp inhibitor. This suggests that after a 24hr incubation with CSE and Lyp inhibitor cells are not toxic to both naïve or memory T cells, so other experiments can be carried out and results not being affected by loss of cell viability. The results showed the mean (±SEM) of three separate experiments. (n=3)

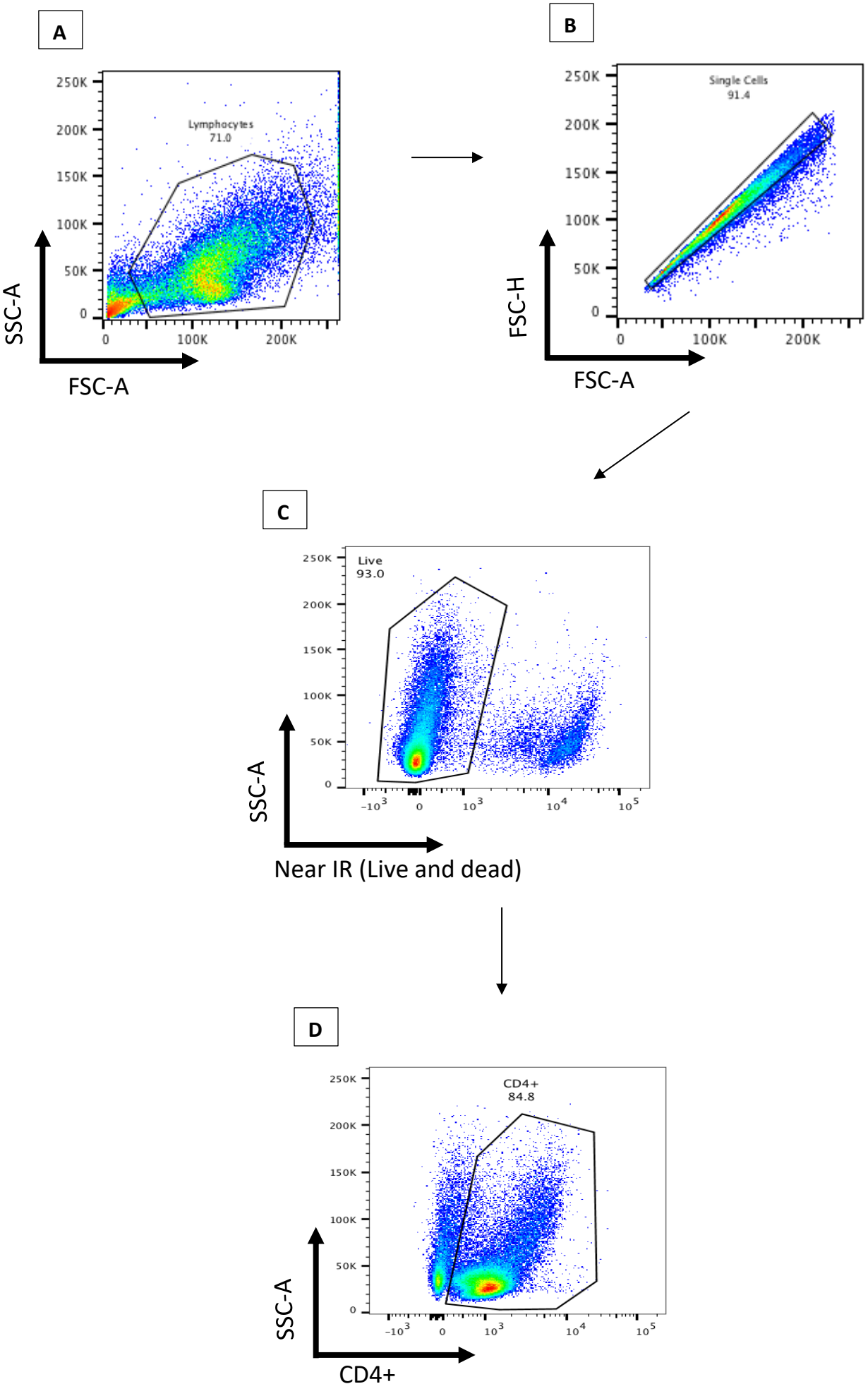
3.2.3 CSE increased the expression of activation markers of naïve and memory cells including CD25, CD62L and CD28

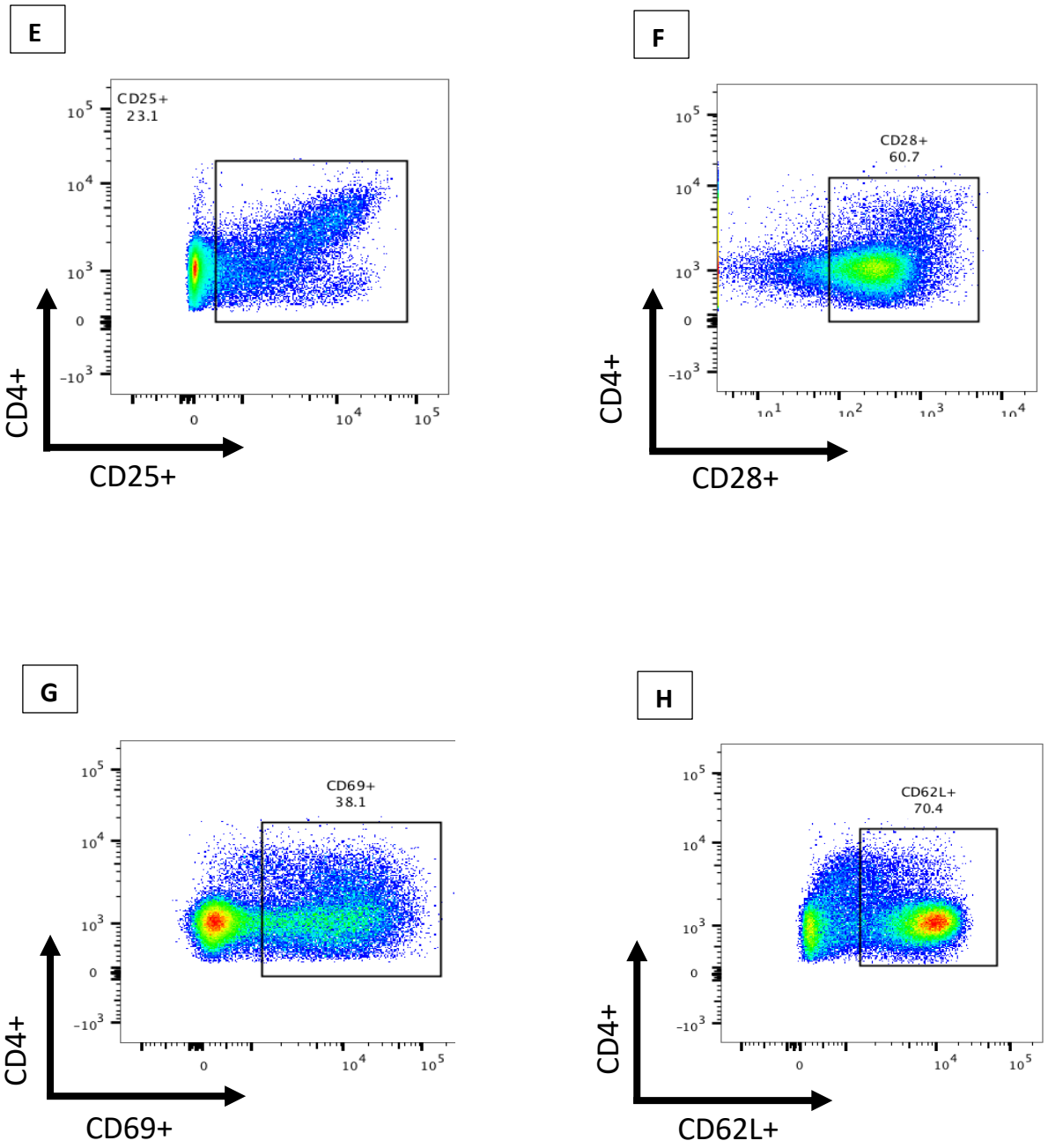
Staining for surface markers is an important approach to subset identification as well as assessment of activation of different sets of human lymphocytes. Surface markers of human T cells can be divided into different groups, namely those expressed on T cell subsets and those recognizing the total T cell population as a consequence of activation [328]. To investigate whether CSE could activate the surface markers of T cells and whether it has an impact on cytokine production, four markers were investigated $CD28^+$, $CD25^+$, $CD69^+$ and $CD62L^+$. Naïve T cells were treated with CSE for 24 hours then stimulated with CD3/CD28 for 72 hours. Flowcytometry was used to assess the expression of each marker. Representative gating strategies were performed to analyse each marker (Figure 3.4 A-H). For accurate gating, fluorescence minus one (FMO) was used as a control. The MFI was gated on the total population of CD4+, and the results showed the mean (±SEM) of three separate experiments.

It was found that the percentage of $CD28^+$ was not changed at 2%, 15% and 30% CSE (Figure 3.4 I), while MFI expression of $CD28^+$ was increased at 2% but not at 15% and 30% (Figure 3.4 J). The percentage of $CD69^+$ was significantly higher at 2% and 15% but not 30% (Figure 3.4 K), while MFI at 2% also increased but 15% was higher and significant. Also, the expression at 30% was increased (Figure 3.4 L). The percentage and MFI of $CD25^+$ were significantly increased at 2% and 15%. However, 30% did not show any effect (Figure 3.4 M+N). The percentage of $CD62L^+$ was not changed at 2%, 15% and 30% (Figure 3.4 O). The MFI showed that the expression of $CD62L^+$ did not show any effect at 2%. However, the expression was enhanced at 15%, and was higher at 30% (Figure 3.4 P). This suggests that CSE could enhance the activation markers of naïve cells specifically CD25 and CD69, which

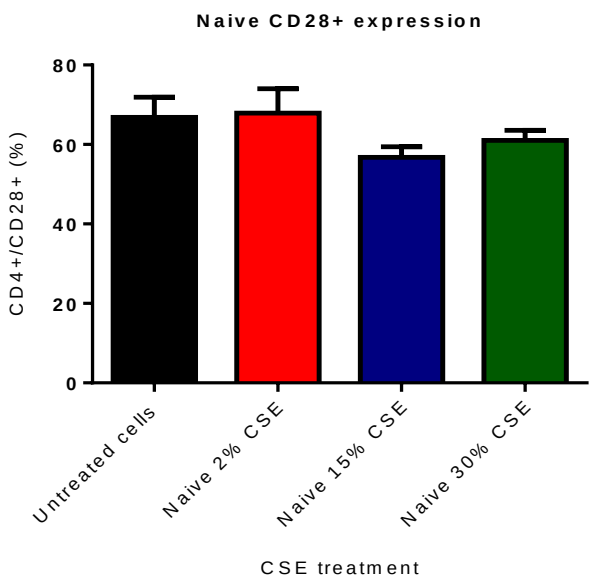
could lead to enhancement of naïve T cell activation and proliferation as well as changes in cytokine production.

Following the assessment of surface markers expression on naïve T cells, we examined the expression of the same markers on memory T cells following CSE treatment and CD3/CD28 activation. Similar to naïve, the percentage and MFI of $CD28^+$ of memory was not changed at 2%, 15% and 30% CSE (Figure 3.4 Q+R). However, both percentage and MFI of $CD69^+$ were higher and significant at 2%, 15% and 30% (Figure 3.4 S+T). The percentage and MFI of $CD25^+$ were significantly increased at 2%. The 15% showed an increase but not 30% (Figure 3.4 U+V). On the other hand, the percentage of $CD62L^+$ was not changed at 2%, 15% and 30% (Figure 3.4 W). However, the MFI showed that the expression of CD62L was enhanced at 15% and 30% but not at 2% (Figure 3.4 X). This suggests that CSE could enhance the activation of key markers of memory cells, leading to enhancement of memory T cells activation and proliferation as well as cytokines production.

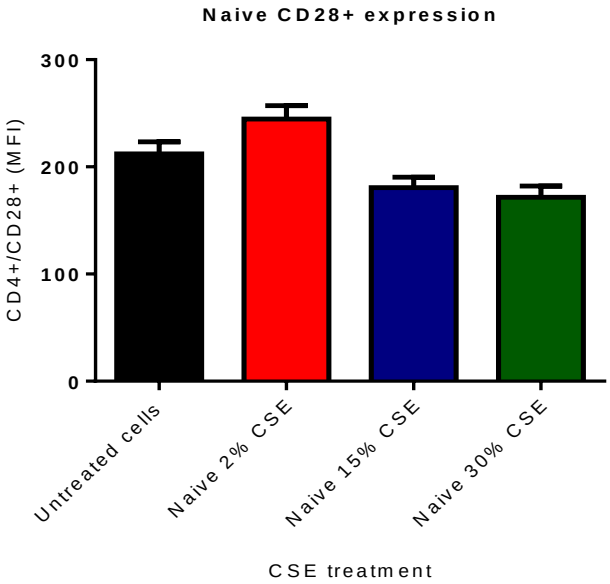

A
Lymphocytes
71.0
SSC-A
FSC-A
B
Single Cells
91.4
FSC-H
FSC-A
C
Live
93.0
SSC-A
Near IR (Live and dead)
D
CD4+
84.8
SSC-A
CD4+


E
CD25+
23.1
CD4+
CD25+
F
CD28+
60.7
CD4+
CD28+
G
CD69+
38.1
CD4+
CD69+
H
CD62L+
70.4
CD4+
CD62L+

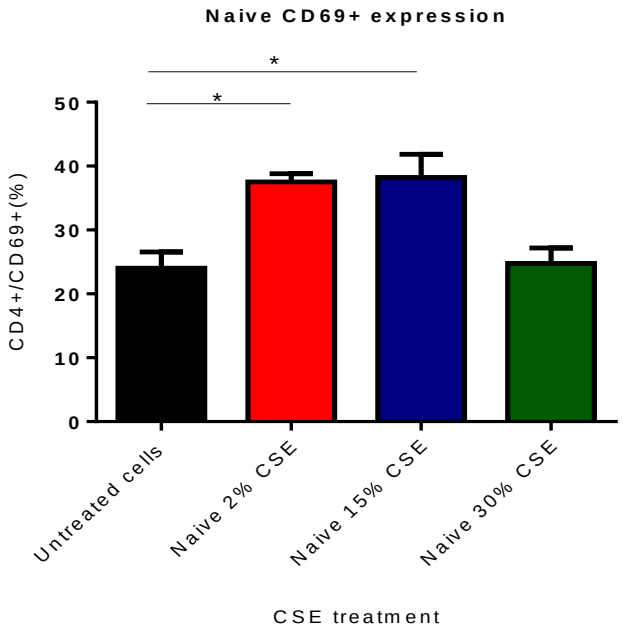
I


Naive CD28+ expression
CD4+/CD28+ (%)
80
60
40
20
0
Untreated cells
Naive 2% CSE
Naive 15% CSE
Naive 30% CSE
CSE treatment

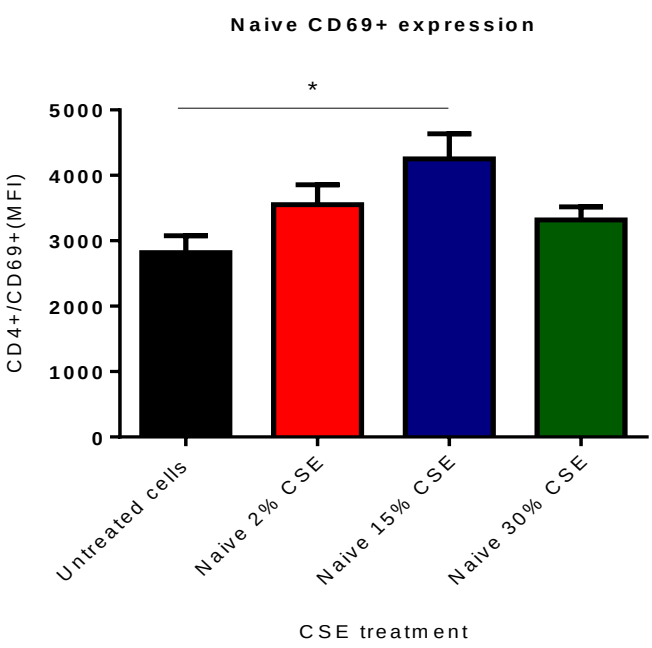
J

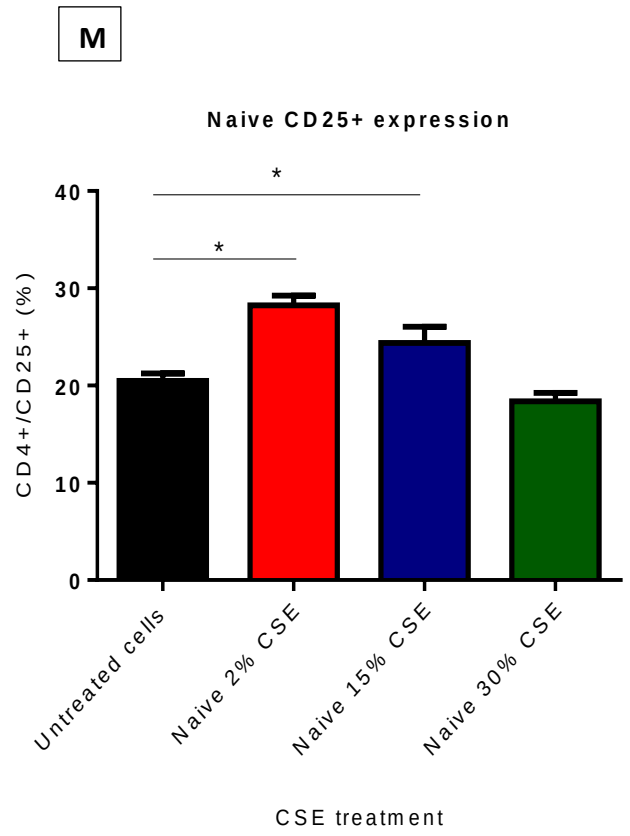

Naive CD28+ expression
CD4+/CD28+ (MFI)
300
200
100
0
Untreated cells
Naive 2% CSE
Naive 15% CSE
Naive 30% CSE
CSE treatment

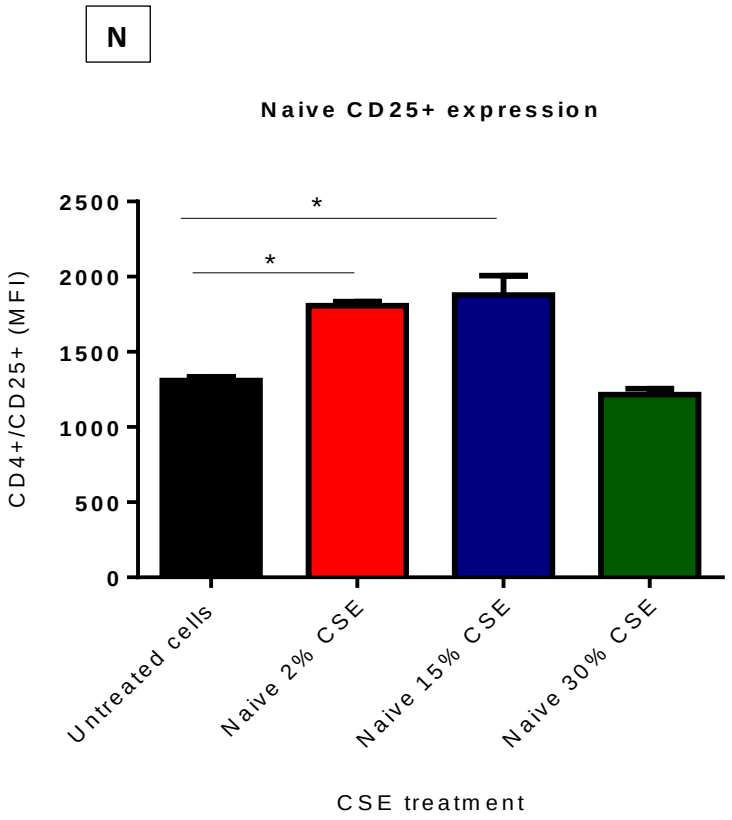
K

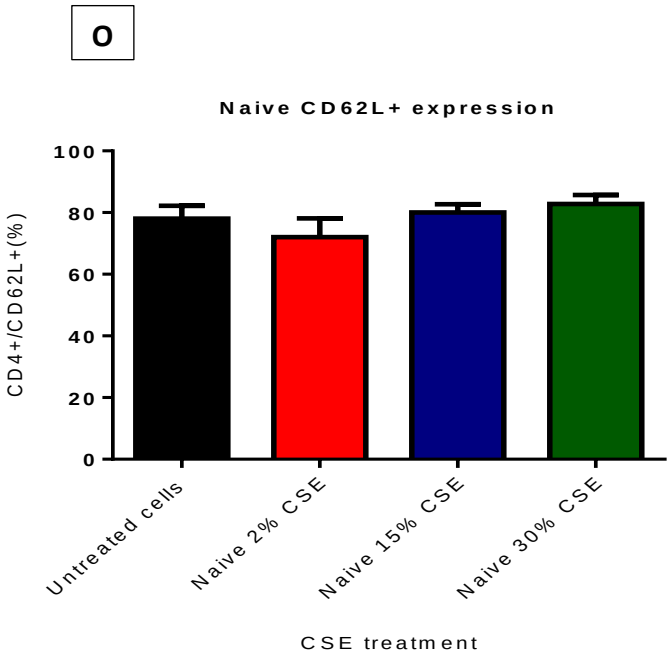

Naive CD69+ expression
*
*
CD4+/CD69+(%)
50
40
30
20
10
0
Untreated cells
Naive 2% CSE
Naive 15% CSE
Naive 30% CSE
CSE treatment

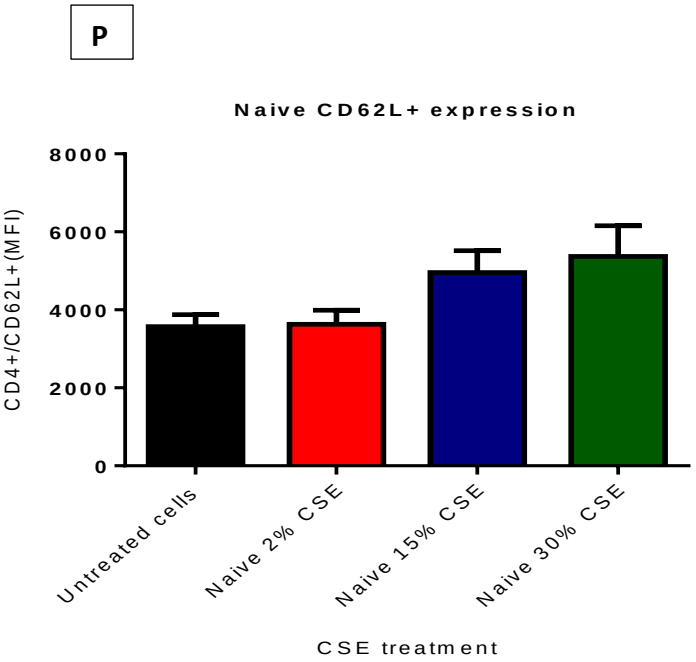
L


Naive CD69+ expression
*
CD4+/CD69+(MFI)
5000
4000
3000
2000
1000
0
Untreated cells
Naive 2% CSE
Naive 15% CSE
Naive 30% CSE
CSE treatment

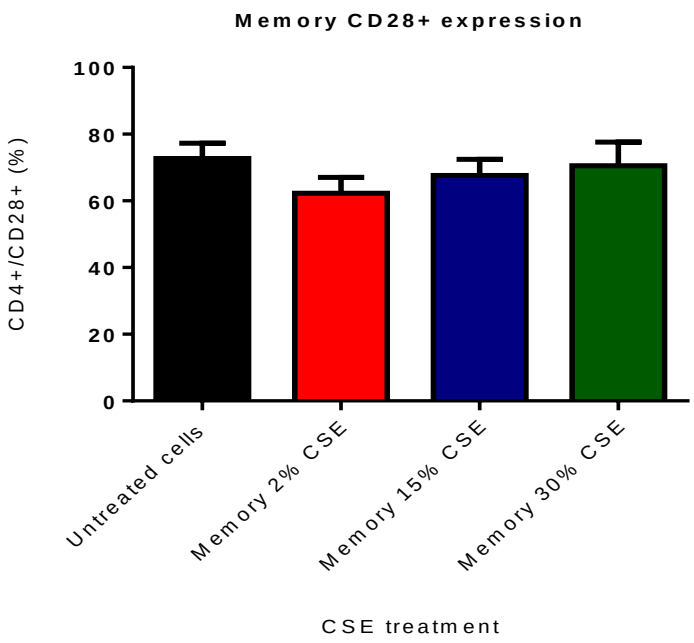

M
Naive CD25+ expression
CD4+/CD25+ (%)
40
30
20
10
0
*
*
Untreated cells
Naive 2% CSE
Naive 15% CSE
Naive 30% CSE
CSE treatment


N
Naive CD25+ expression
CD4+/CD25+ (MFI)
2500
2000
1500
1000
500
0
*
*
Untreated cells
Naive 2% CSE
Naive 15% CSE
Naive 30% CSE
CSE treatment

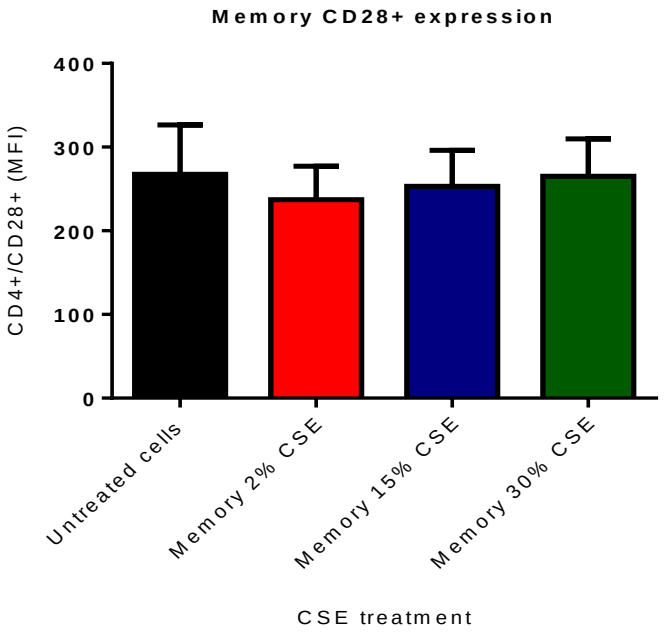

O
Naive CD62L+ expression
CD4+/CD62L+(%)
100
80
60
40
20
0
Untreated cells
Naive 2% CSE
Naive 15% CSE
Naive 30% CSE
CSE treatment


P
Naive CD62L+ expression
CD4+/CD62L+(MFI)
8000
6000
4000
2000
0
Untreated cells
Naive 2% CSE
Naive 15% CSE
Naive 30% CSE
CSE treatment

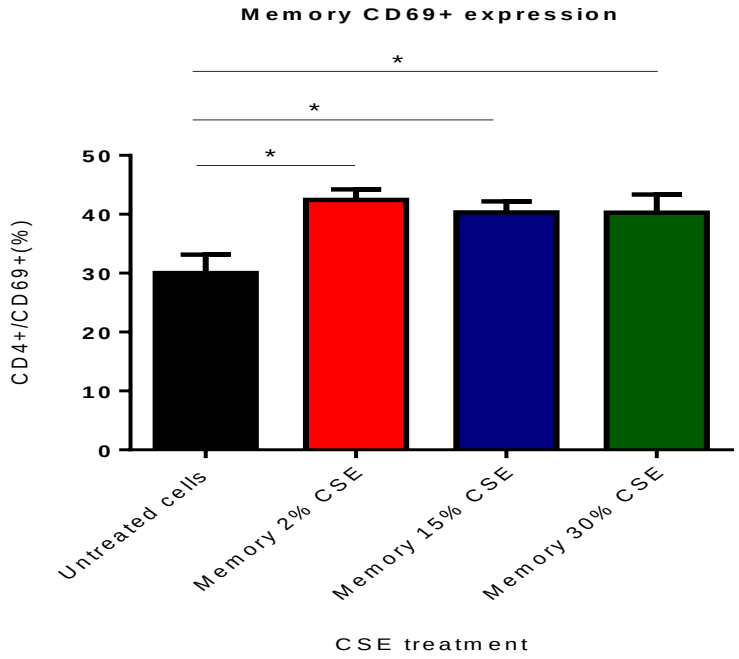
Q


Memory CD28+ expression
CD4+/CD28+ (%)
100
80
60
40
20
0
Untreated cells
Memory 2% CSE
Memory 15% CSE
Memory 30% CSE
CSE treatment

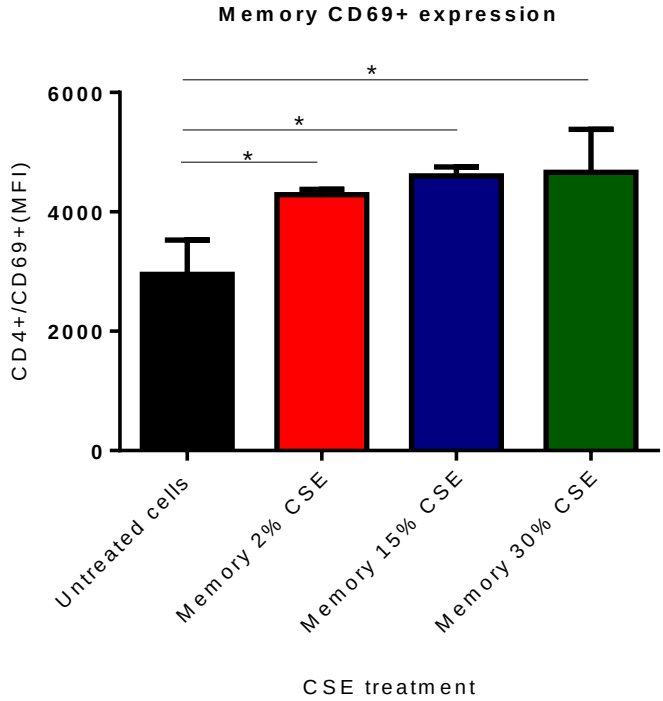
R


Memory CD28+ expression
CD4+/CD28+ (MFI)
400
300
200
100
0
Untreated cells
Memory 2% CSE
Memory 15% CSE
Memory 30% CSE
CSE treatment

S


Memory CD69+ expression
*
*
*
CD4+/CD69+(%)
50
40
30
20
10
0
Untreated cells
Memory 2% CSE
Memory 15% CSE
Memory 30% CSE
CSE treatment

T


Memory CD69+ expression
*
*
*
CD4+/CD69+(MFI)
6000
4000
2000
0
Untreated cells
Memory 2% CSE
Memory 15% CSE
Memory 30% CSE
CSE treatment

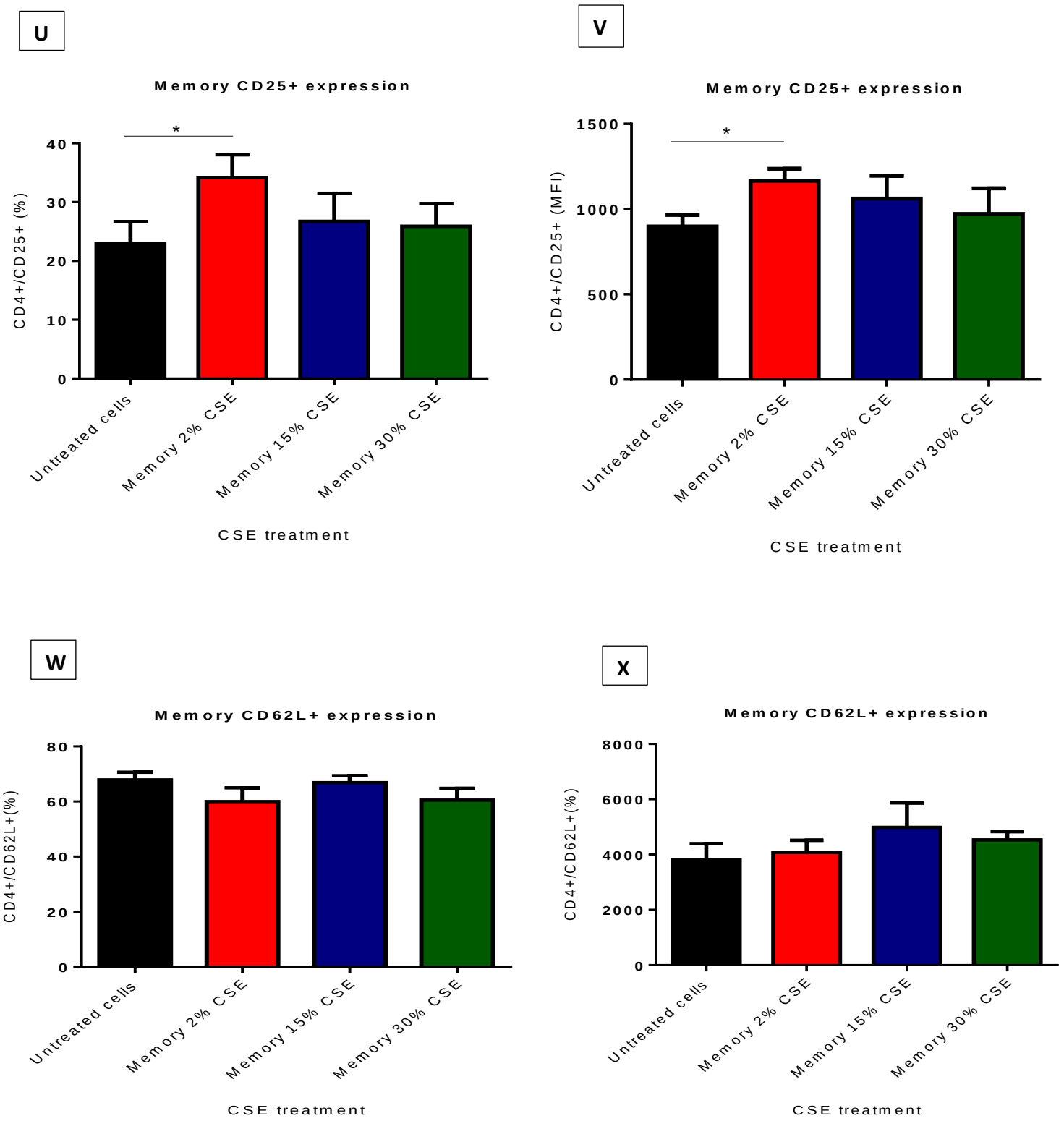


Figure 3.4 CSE increased the expression activation markers of naïve and memory cells.

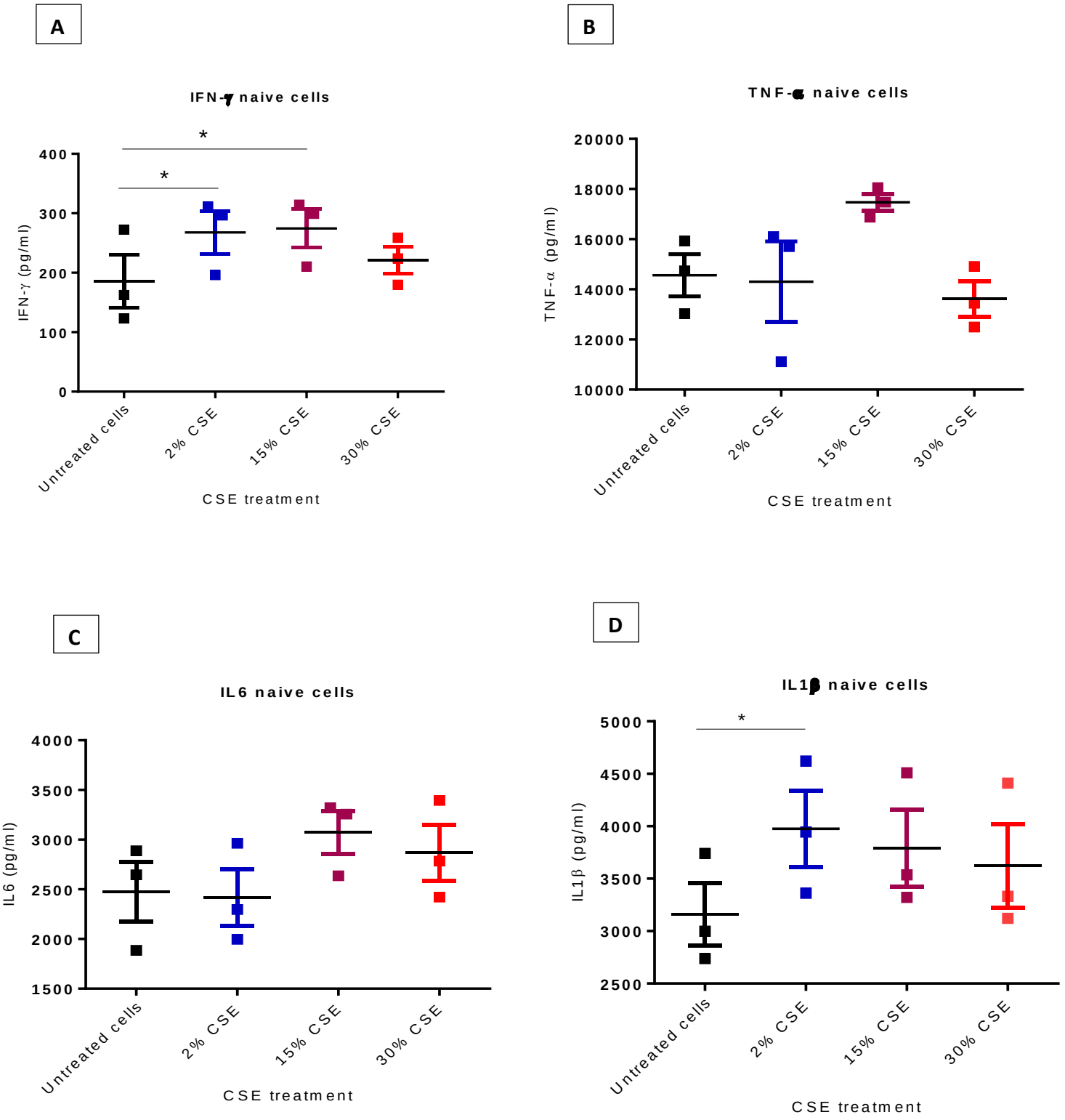
Naïve and memory cells were treated with CSE for 24 hours and then activated with anti-CD3/CD28 for 72 hours. **(A-H)** A representative blot of gating strategy which showed the mean (±SEM) of the three independent experiment. **(I-P)** The percentages and MFI Graphs of surface markers expression (CD25, CD69, CD28 and CD62L) of naïve cells. **(Q-X**) The percentages and MFI Graphs of surface markers expression (CD25, CD69, CD28 and CD62L) of memory cells. Flowcytometry was performed to assess each marker. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=3)

3.2.4 CSE increased the expression of inflammatory cytokines in naïve T cells.

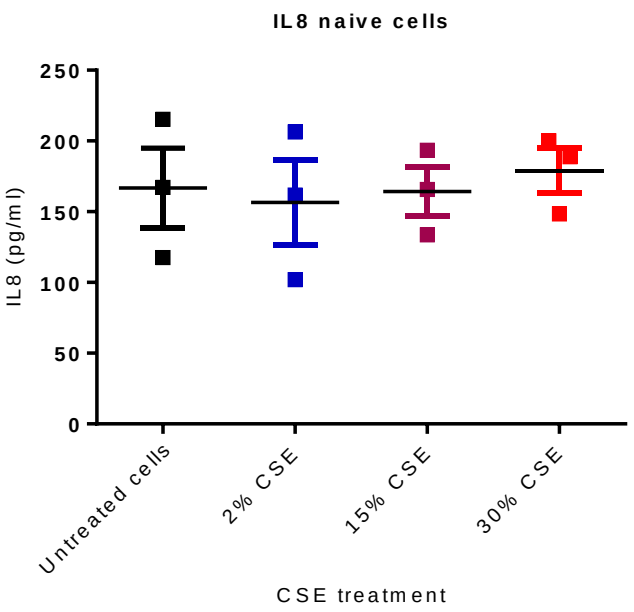
Cytokines are produced by many cells, but the main secretors are macrophages and T cells. These mediators are involved in persistence and initiation of inflammation and pathological pain. However, they can act as anti-inflammatory to regulate the immune system by controlling the proinflammatory cytokines response [329]. In context of the effect of smoking on cytokines production, there are many studies in literature have investigated this interaction. For instance, a study reported that cigarette smoking elevated some proinflammatory cytokines such as IL-6 and decreased the antioxidant status in smokers, indicating that smoking could influence cytokines production and antioxidant defences [330]. To determine if the CSE promotes inflammatory cytokines production, we analysed a range of proinflammatory cytokines produced by both memory and naïve T cells following exposure to CSE and activation using anti CD3/CD28. The Luminex technology was used to assess the level of each cytokine.

It was found that the IFN-γ was significantly upregulated following 2% and 15% CSE. The level of IFN-γ was increased also at 30% but it was not significant (Figure 3.5 A). The expression of TNF-α did not change at 2% and 30%, while the expression was higher following 15% CSE and this was not significant (Figure 3.5 B). Similar to TNF-α, the expression of IL-6 was not changed at 2% but was higher at 15% and 30% (Figure 3.5 C). The level of IL-1β was significantly augmented at 2%, while 15% and 30% increased the expression, they failed to reach significance (Figure 3.5 D). Neither IL-8 nor IL-12 showed any obvious change following 2%, 15% and 30% (Figure 3.5 E+F). Similarly, anti-inflammatory IL-10 which functions as immunomodulator of inflammation did not show any change after CSE treatment (Figure 3.5 G). IL-1ra, IP-10 and RANTES levels showed no effect following CSE

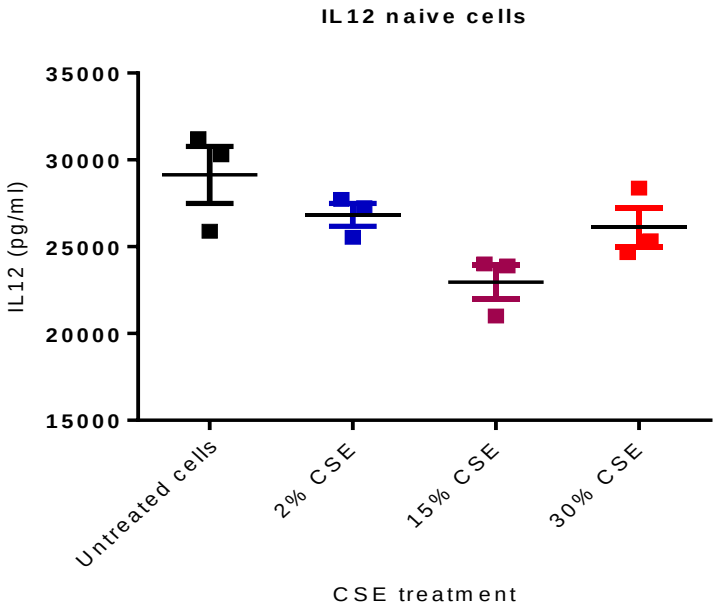
treatment (Figure 3.5 H-J). VEGF was increased following 2%, 15% and 30% but again did not reach significance (Figure 3.5 K). Taken together the results show an upregulation of proinflammatory cytokines, so this may suggest that CSE could drive Th1 induction and therefore may contribute to the development of autoimmune conditions. Although not significant, the increase in VEGF could lead to vasculitis and neovascularisation.


A
IFN-γ naive cells
IFN-γ (pg/ml)
400
300
200
100
0
*
*
Untreated cells
2% CSE
15% CSE
30% CSE
CSE treatment
B
TNF-α naive cells
TNF-α (pg/ml)
20000
18000
16000
14000
12000
10000
Untreated cells
2% CSE
15% CSE
30% CSE
CSE treatment
C
IL6 naive cells
IL6 (pg/ml)
4000
3500
3000
2500
2000
1500
Untreated cells
2% CSE
15% CSE
30% CSE
CSE treatment
D
IL1β naive cells
IL1β (pg/ml)
5000
4500
4000
3500
3000
2500
*
Untreated cells
2% CSE
15% CSE
30% CSE
CSE treatment

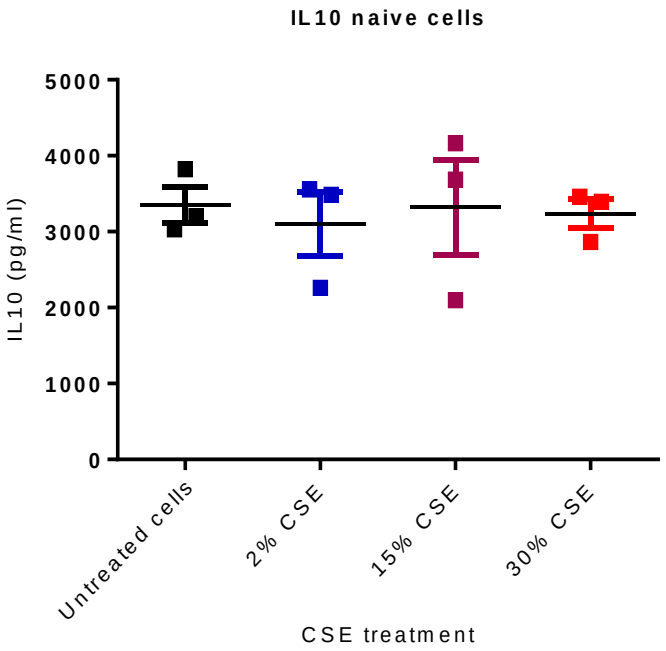
E


IL8 naive cells
IL8 (pg/ml)
250
200
150
100
50
0
Untreated cells
2% CSE
15% CSE
30% CSE
CSE treatment

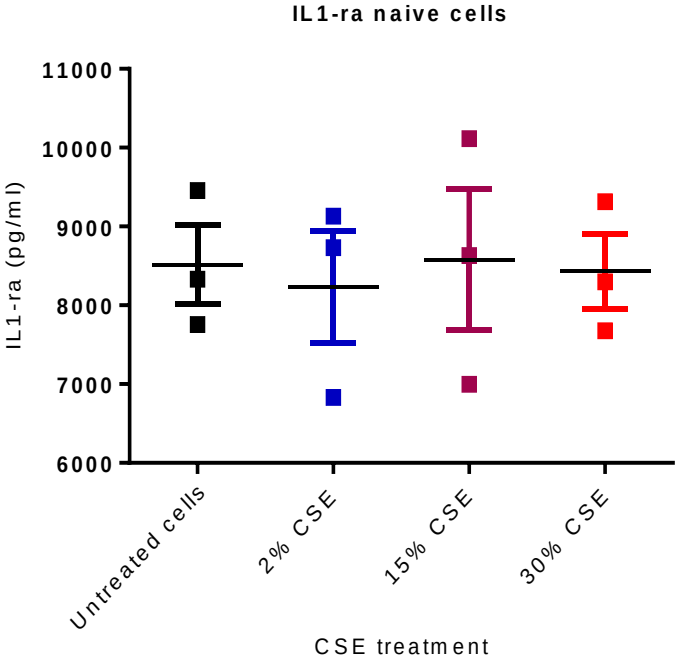
F


IL12 naive cells
IL12 (pg/ml)
35000
30000
25000
20000
15000
Untreated cells
2% CSE
15% CSE
30% CSE
CSE treatment

G


IL10 naive cells
IL10 (pg/ml)
5000
4000
3000
2000
1000
0
Untreated cells
2% CSE
15% CSE
30% CSE
CSE treatment

H


IL1-ra naive cells
IL1-ra (pg/ml)
11000
10000
9000
8000
7000
6000
Untreated cells
2% CSE
15% CSE
30% CSE
CSE treatment

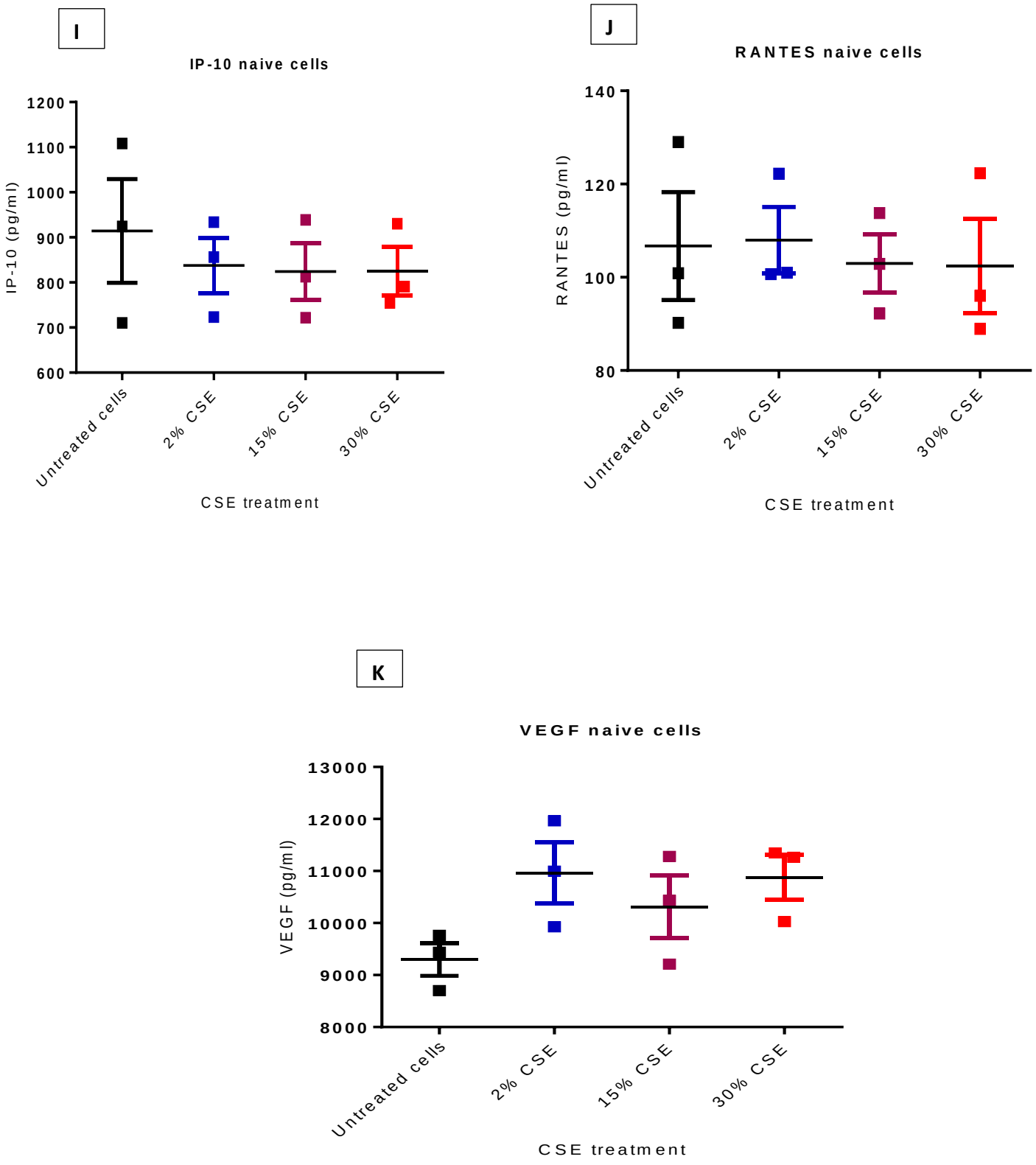
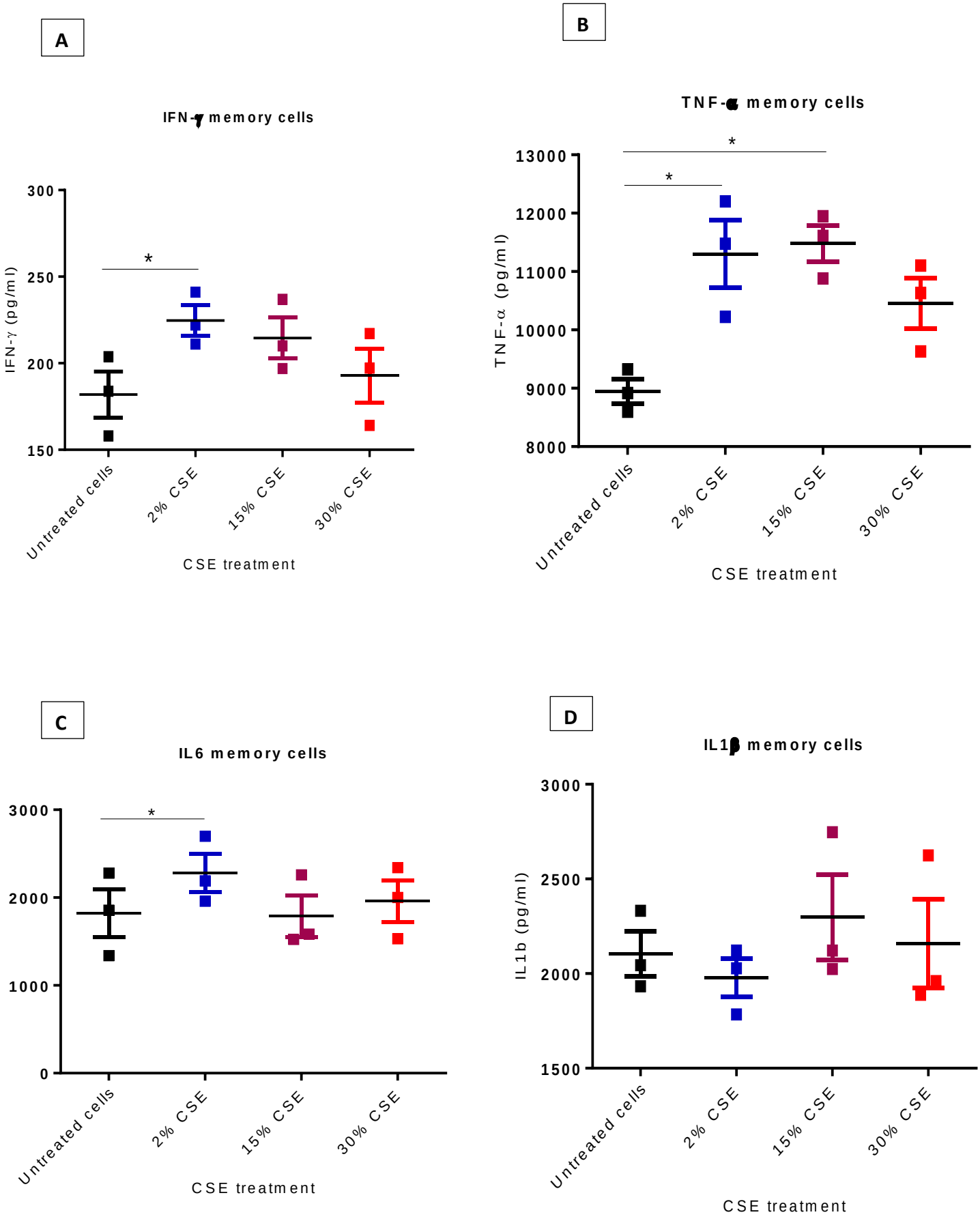


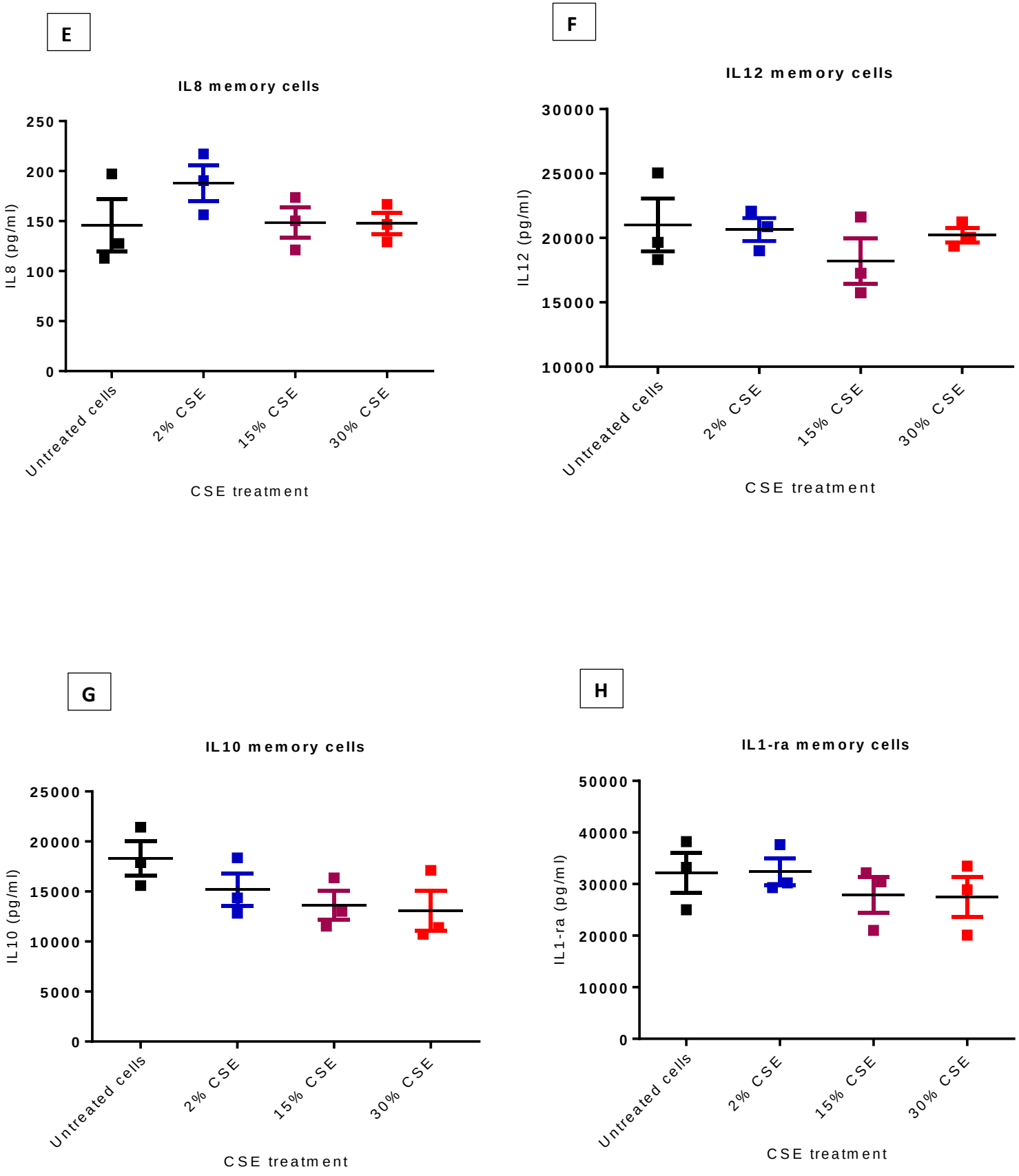
Figure 3. 5 CSE increased the expression of inflammatory cytokines in naïve T cells.

Naïve cells were treated with CSE for 24 hours and then activated with anti-CD3/CD28 for 72 hours. **(A-k)** Figures of inflammatory cytokines which showed the mean (±SEM) of three independent experiment. This includes IFN-γ TNF-α, IL6, IL-1β**,** IL8, IL12, IL1ra, IL10, IP10, RANTES and VEGF**.** Luminex technology was performed to assess each cytokine**.** Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=3)

3.2.5 CSE increased the expression of inflammatory cytokines in memory T cells.

Following the assessment of proinflammatory cytokines on naïve T cells, we examined the levels of proinflammatory cytokines on memory T cells. Similar to naïve T cells, the expression of IFN-γ in memory cells was significantly increased following 2%. Also, the expression was increased following 15% and 30% but it was not significant (Figure 3.6 A). In contrary to naïve T cells, the level of TNF-α was much higher and significant in memory cells following 2% and 15% CSE treatment. 30% showed an increase of TNF-α but it was not significant (Figure 3.6 B). As mentioned above naïve cells showed no effect of IL-6 at 2% CSE treatment, however in memory cells, the expression of IL-6 was high and significant at 2% CSE treatment, but the expression did not change following 15% and 30% (Figure 3.6 C). The level of IL-1β was not changed at 2%. However, the level was much higher at 15% and 30% with no significant changes (Figure 3.6 D). IL-8 was augmented at 2%, but there were not any changes in the expression of IL-8 following 15% and 30% (Figure 3.6 E). Regarding IL-12, the level in memory was not changed following 2%, 15% and 30% (Figure 3.6 F). The level of anti-inflammatory IL-10 was decreased following 2%, 15% and 30% (Figure 3.6 G). IL-1ra, IP-10 and RANTES did not show any effect following CSE treatment (Figure 3.6 H-J). However, VEGF was increased following 2% and 30% but not at 15% (Figure 3.6 K).


A
IFN-γ memory cells
IFN-γ (pg/ml)
300
250
200
150
*
Untreated cells
2% CSE
15% CSE
30% CSE
CSE treatment
B
TNF-α memory cells
TNF-α (pg/ml)
13000
12000
11000
10000
9000
8000
*
*
Untreated cells
2% CSE
15% CSE
30% CSE
CSE treatment
C
IL6 memory cells
IL6 (pg/ml)
3000
2000
1000
0
*
Untreated cells
2% CSE
15% CSE
30% CSE
CSE treatment
D
IL1β memory cells
IL1b (pg/ml)
3000
2500
2000
1500
Untreated cells
2% CSE
15% CSE
30% CSE
CSE treatment


E
IL8 memory cells
IL8 (pg/ml)
250
200
150
100
50
0
Untreated cells
2% CSE
15% CSE
30% CSE
CSE treatment
F
IL12 memory cells
IL12 (pg/ml)
30000
25000
20000
15000
10000
Untreated cells
2% CSE
15% CSE
30% CSE
CSE treatment
G
IL10 memory cells
IL10 (pg/ml)
25000
20000
15000
10000
5000
0
Untreated cells
2% CSE
15% CSE
30% CSE
CSE treatment
H
IL1-ra memory cells
IL1-ra (pg/ml)
50000
40000
30000
20000
10000
0
Untreated cells
2% CSE
15% CSE
30% CSE
CSE treatment

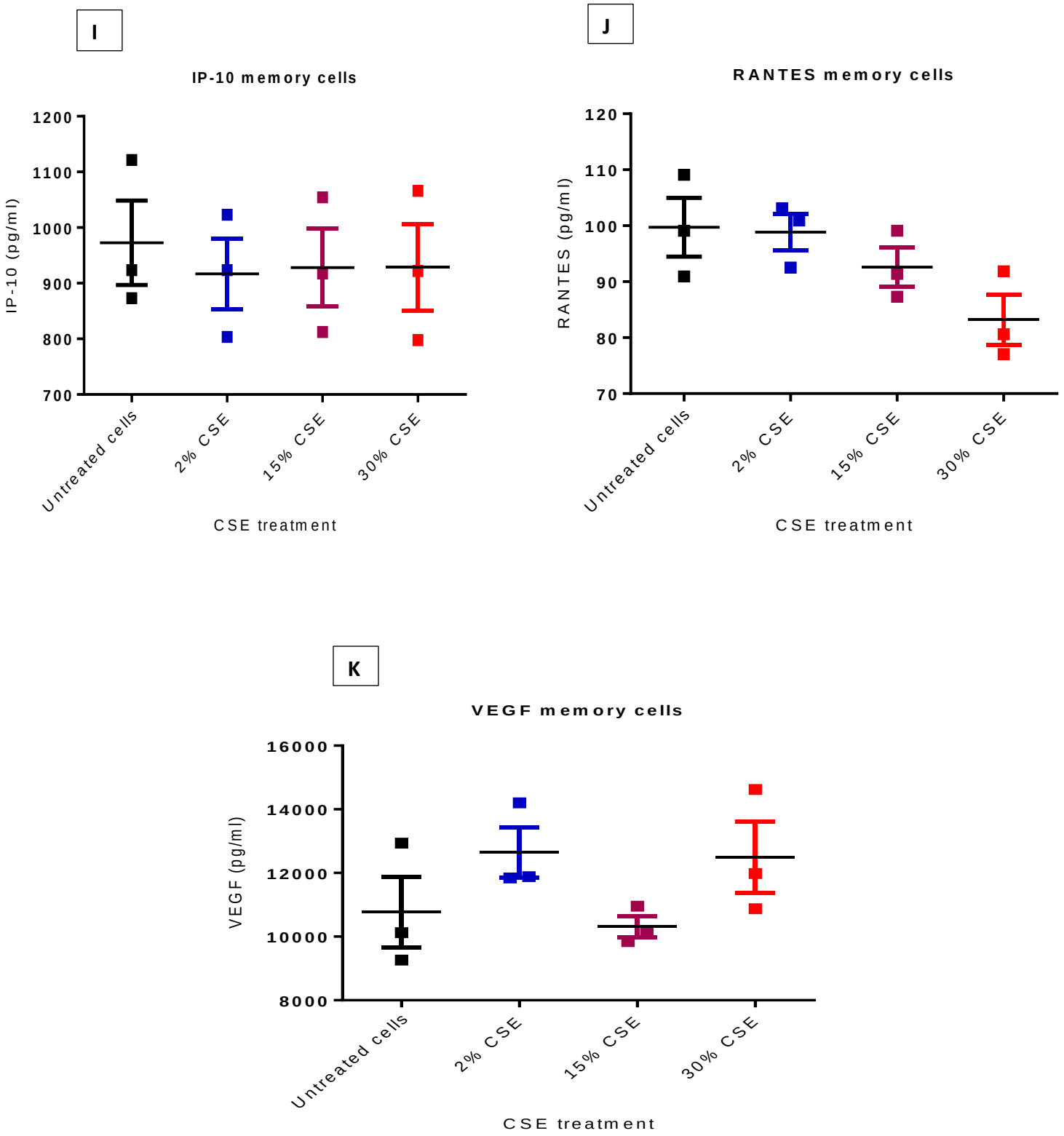


Figure 3. 6 CSE increased the expression of inflammatory cytokines in memory cells.

Memory cells were treated with CSE for 24 hours and then activated with anti-CD3/CD28 for 72 hours. **(A-k)** Graphs of inflammatory cytokines which showed the mean (±SEM) of three independent experiment. This includes IFN-γ TNF-α, IL6, IL-1β**,** IL8, IL12, IL1ra, IL10, IP10, RANTES and VEGF**.** Luminex technology was performed to assess each cytokine**.** Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=3)

3.3 Discussion

Cigarette smoking has been shown to be involved in the induction of several cytokines produced by T cells and macrophages, including anti-inflammatory and pro-inflammatory cytokines. Cigarette smoking can be diverse in its effect on the immune system; it can be immunosuppressive or pro-inflammatory and so contradictory results have been reported. [331]. We showed that CSE significantly enhanced the expression of IFNγ, IL6, TNF-α and IL-1β. By comparison, IL10 and some chemokines such as IP-10 and RANTES showed no effect following CSE treatment. We showed that CSE significantly increased the activity of some key activation markers of T cells such as CD69 and CD25 along with CD62L.

Overactivation of T cells may lead to produce more cytokines which increase the risk of autoimmunity. It has been shown that treating T cells with low concentration of nicotine increased CD69 expression, indicating that smoking could activate T cells CD69 marker [332]. Also, cigarette smoking induced inflammation, and this accompanied by CD69 and CD25 activation, expansion of dendritic cells and cytokines production, whereas the regulatory T cells markers FOXP3 and CD127 were decreased, suggesting that smoking could induce expansion of helper T cells [333-335]. Further to this, samples from smokers showed a significant increase in T cells proliferation compared to control. The same patients showed a significant increase in both CD25 and CD69 markers as well as a co-stimulatory marker CD28, suggesting that smoking could alter the immune function in smokers [336]. Our result showed that CSE increased CD25 and CD69 which is consistent to what reported in previous studies.

To discuss more about the role of cigarette smoking on cytokines production, a study on mice demonstrated that cigarette smoking induced the expression of Th1 cytokines IL17, IL6

and IFNγ in the peripheral blood [337, 338]. Furthermore, in human it has been reported that cigarette smoking increased IL6 and IFNγ in human $CD4^+$ T cell in smokers [339]. Thus, both human and mice experiments suggested that CS may alter the balance between Th1 and Th2; leading to elevate Th1 cells and produce more pro-inflammatory cytokines which promote inflammation. An interesting recent study showed that cigarette smoking enhanced colitis through induction of IFNγ. The study found that CD4+ T cells IFNγ deficiency protected colitis development but transfer of IFNγ CD4+ T cells into CS-exposed WT mice, the expression of IFNγ was upregulated and led to colitis. This suggests that IFNγ can be induced by cigarette smoking and cause inflammation [340]. In our results we showed that CSE increased IFNγ expression, suggesting that CSE may play a role in initiating inflammation by activating IFNγ which is involved in RA development.

Several epidemiological findings suggested a significant association between psoriasis and cigarette smoking. A study reported that CSE induced the expression of early growth response-1 (Egr-1), leading to activation of MAPK pathways including JNK and ERK. This activation led to a significant enhancement of TNF-α expression and secretion which in turn contribute to psoriasis which could progress to psoriatic arthritis [341]. A second study found that TNF-α receptors knockout mice did not show any changes in TNF-α expression following two hours exposure to CSE. compared to wild type mice where TNF-α expression was significantly enhanced and resulted connective tissue breakdown [342]. One of the most cytokines related to metabolism is IL6 which could play a role in regulating metabolism through changes in signalling pathway. In vitro Studies suggested that IL6 increased glucose and fatty acid metabolism as well as lactate production through phosphorylation of AMPK and PI3K [343-345]. We showed also that CSE enhanced IL-1β expression which plays a role

as key mediator in RA development. Overexpression of IL-1β caused an arthritis to rabbit joints with a histological and clinical feature of RA, while inhibition of IL-1β decreased damage of the joint. [346]. An interesting finding which may related to what we showed found that CSE at 2%, 5% and 10% significantly upregulated IL-1β secretion after 24 hours of exposure, suggesting that CSE could increase IL-1β level which in turn promote inflammation and RA development [347]. We showed in our data that treating cells with 2% increased IL-1β expression after 24 hours which is consistent to what reported in the literature. On the other hand, CS can induce Th2 cytokines and supress Th1 cytokines production. It has been reported that smokers had higher levels of Th2 IL-13 and it was significant, whereas the level of IFNγ was not significant [167]. A second study showed that smoking inhibited TNF-α, IL-1β and IL-6 upon stimulation, whereas anti-inflammatory such as IL10 and IL-1 receptor antagonist were not affected [348]. This suggests that both suppressive and proinflammatory effect can be induced by CS which indicates that cigarette smoking can be diverse in its effect on immune system due to the fact that cigarette smoking contains more than 5000 components which may play a role with that.

In conclusion, we showed that CSE successfully increased the expression of T cell activation markers CD25+ and CD69+ along with CD62L+. Also, it enhanced most of the proinflammatory cytokines such as IFNγ, IL6, TNF-α and IL-1β. However, IL10, IP-10 and RANTES showed no effect following CSE treatment. This finding suggests that smoking could activate T cells and promote inflammatory cytokines secretion which consequently could lead to RA development. There are some limitations in this chapter. For example, we did not investigate the production of pro inflammatory cytokines following Lyp inhibitor treatment as this would give more solid data to the chapter. This was mainly due to the high cost of

Luminex kits. Another limitation is that we did not investigate the surface marker expression before anti CD3/CD28 stimulation. This would show if the increase we observed in the surface marker expression was because of the treatment or anti CD3/CD28. Finally, we did not investigate the time points of cytokines production following CSE. This would show if cytokines can be upregulated in early stage or not. One of the future works for this chapter is to investigate the Th2 cytokines. This will show if CSE could have an effect on Th2 cytokines or not because some evidence we showed in the discussion above suggested that CSE could impact Th2. Also, one of the future works is to investigate the effect of CSE on regulatory T cell (Treg) because we showed that CSE significantly increased the activation markers such as CD25 and CD69, so it would be better if we investigated the response of Treg following CSE to determine if CSE would decrease Treg activity or not. Cytokines have consequences on signalling pathway activation, so in the next chapter the mechanisms by which some of the changes in immune cell function might occur and the consequences for cellular function will be investigated following CSE and Lyp inhibitor and whether this would affect the immediate TCR signalling.

Chapter 4:

Effects of cigarette smoke extract and Lyp inhibitor on Lyp phosphatase activity and signalling pathways of primary CD4+ naïve and memory T cells

4.1 Introduction

Signalling pathways are complex process which are involved in maintaining the function of cells and their responses to external stimuli. Dysregulation of signalling pathways in T cells such as protein tyrosine kinases (PTKs), protein tyrosine phosphatases (PTPs) and calcium signaling resulting from environmental and genetic factors has been shown to be responsible for increasing the risk of aberrant immune-cell survival and the development of autoimmune conditions [349]. PTKs are a major class of enzymes in signalling pathway which regulate differentiation, cell growth and biochemical processes. Evidence has shown that several PTKs are dysregulated in RA, so that the overexpression or altered activity of PTKs or their genes could, for example, increase resistance to apoptosis and promote exuberant cell survival. Another major class of signalling enzymes are the PTPs which also have a critical role in cell growth and biochemical processes, and several research studies continue to focus on the field PTPs in order to define their molecular mechanisms of function and develop therapeutic target. PTPN22, which encodes Lyp, is an important candidate in regulating T-cell activation and subsequent autoimmune conditions such as RA.

In this chapter we investigated the mechanisms by which some of the changes in immune cell function might occur and the consequences for cellular function. These studies were guided by our hypothesis that cigarette smoke extract and inhibition of PTPN22 phosphatase activity could give rise to signalling changes in CD4+ T cells together with alterations in energy metabolism through modulation of Akt. Since the latter is a master regulator of downstream processes including the switch between glycolysis and oxidative phosphorylation, confirmation of this effect on Akt was an important goal. Some preliminary data from our research group suggested there could be direct effects of cigarette smoke on

PTP function. When lysates from Jurkat T cells which had been exposed to CSE were assessed using immunoblotting it was found that changes in the phosphorylated forms of Lck, Zap70 and Akt could be induced by CSE, in a similar way to hydrogen peroxide, which suggests an oxidation process. Furthermore, a specific inhibitor of Lyp PTP similar induced an increase in Lck phosphorylation, which provides some evidence that Lyp could be the target of the CSE (Figure 4.1). Immunoblotting was used to probe the phosphorylation status of the known targets of PTPN22 such as ZAP-70, Lck and Vav1 following CSE and Lyp inhibitor. Lyp phosphatase activity was investigated to assess the catalytic activity of Lyp. CD4+ T cell calcium signalling in response to TCR ligation was also assessed in cells exposed to CSE and Lyp inhibitor treatment to provide an overall assessment of any changes in the strength of signal through the TCR. Summary of signalling pathway investigated in this chapter is shown in Figure 4.2.

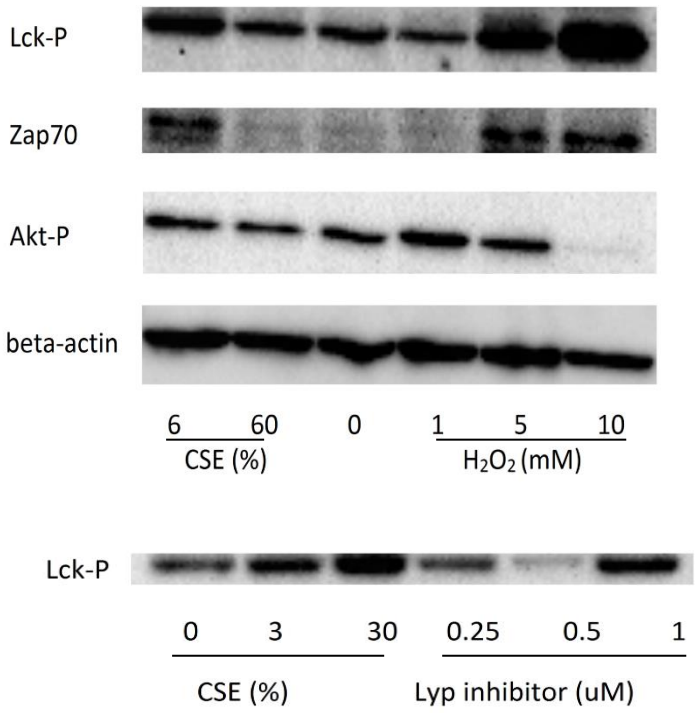


Figure 4. 1 Jurkat T cells increased Lck, Akt and ZAP-70 phosphorylation following CSE and Lyp inhibitor treatment.
Cells exposed to CSE or Lyp inhibitor at various concentrations for 1hr before lysis and separation on SDS PAGE and immunoblotting. (Olivia Ng, Kalvin Sahota, Stephen Young, unpublished).

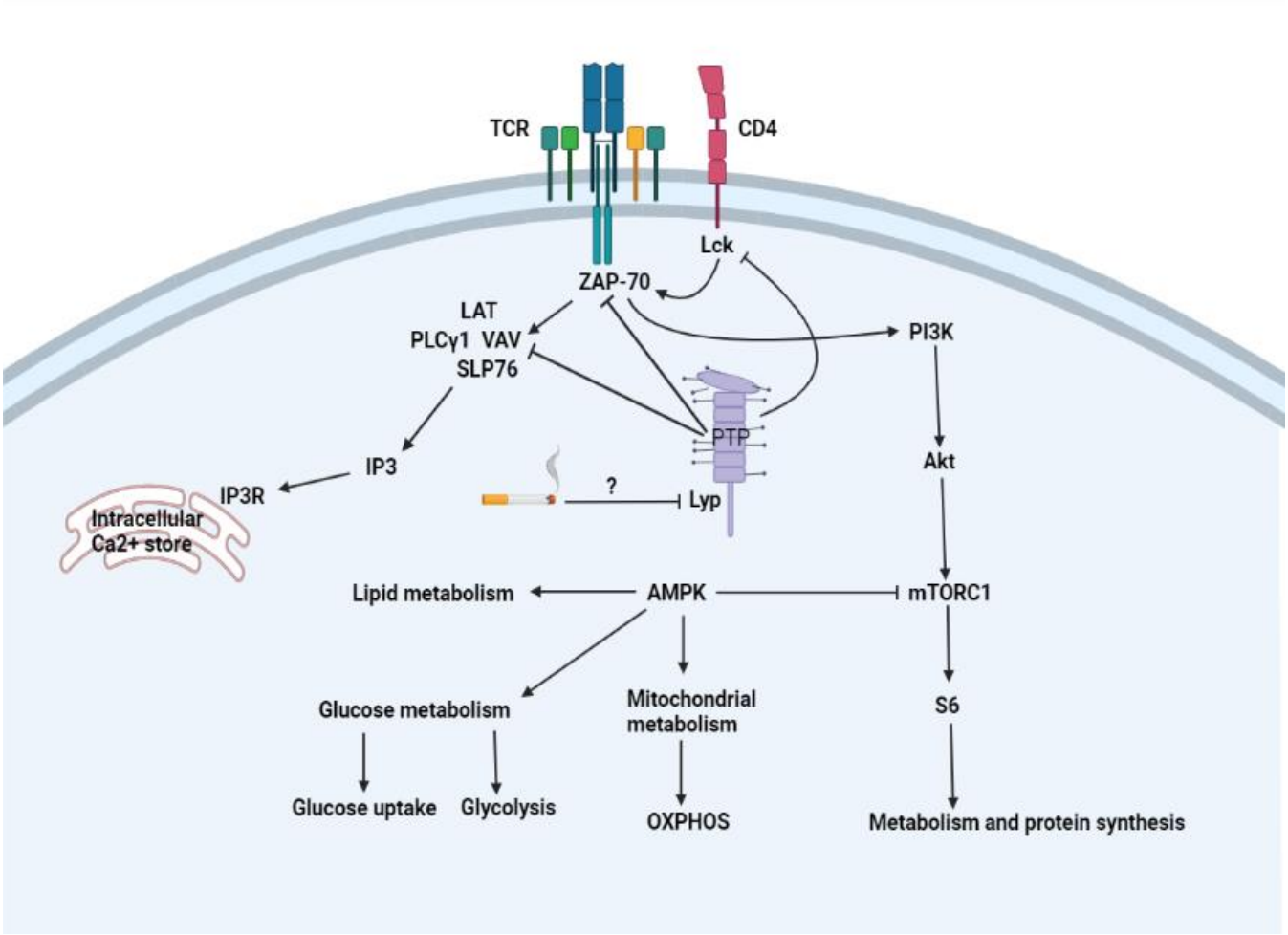


Figure 4. 2 The investigated pathways including Lck, Zap70, Vav1, Ca^{2+} signalling, AMPK and PI3K/Akt/mTORC.

Following TCR ligation, activated LCK phosphorylates ITAMs of the TCR/CD3, which recruits ZAP-70. ZAP-70 in turn phosphorylates SLP76 and LAT. PLCγ1 binds to LAT, SLP-70 and VAV1, and hydrolyses PIP2, producing DAG and IP3, which initiates activating the calcium ion storage. Akt phosphorylates mTORC1 and regulates cellular metabolism. AMPK activates mitochondrial metabolism, lipid metabolism and glucose metabolism

4.2 Results

4.2.1 CSE reduced the activity of Lyp phosphatase after a fluorometric measurement of Lyp phosphatase activity in memory but not naïve cells.

Protein tyrosine phosphatases (PTPs) such as Lyp which encoded by PTPN22 have been involved in the development of many autoimmune diseases. Therefore, to understand the biology of Lyp, an accurate measurement of its function is required. Our research group developed a fluorescence method approach to measure the activity of CD45 phosphatase [350]. This method was modified by using different fluorescent substrate DIFUMP to be able to detect the catalytic activity of Lyp phosphatase. Naïve and memory T cells were treated with CSE and incubated for 24 hours. Cells lysate supplemented with protease inhibitor was used to carry on with this assay. In this assay two readings were taken, the first reading at 0 hour and the second reading at 2 hours. The calculation of the fluorescence intensity (FI) was calculated by subtraction of 0 hour reading from 2 hours reading.

It was found that CSE did not show any change in the Lyp activity in naïve cells at 2%, 15% and 30%. Memory cells showed more reduction of Lyp activity following 2% and 15% up to more than 60% compared to control, but it was not significant. 30% showed a significant reduction of Lyp activity up to 80% compared to control (Figure 4.3 A). This result suggests that CSE could decrease the activity of Lyp phosphatase which is consistent to our hypothesis which hypothesised that CSE may interfere with Lyp function.

A

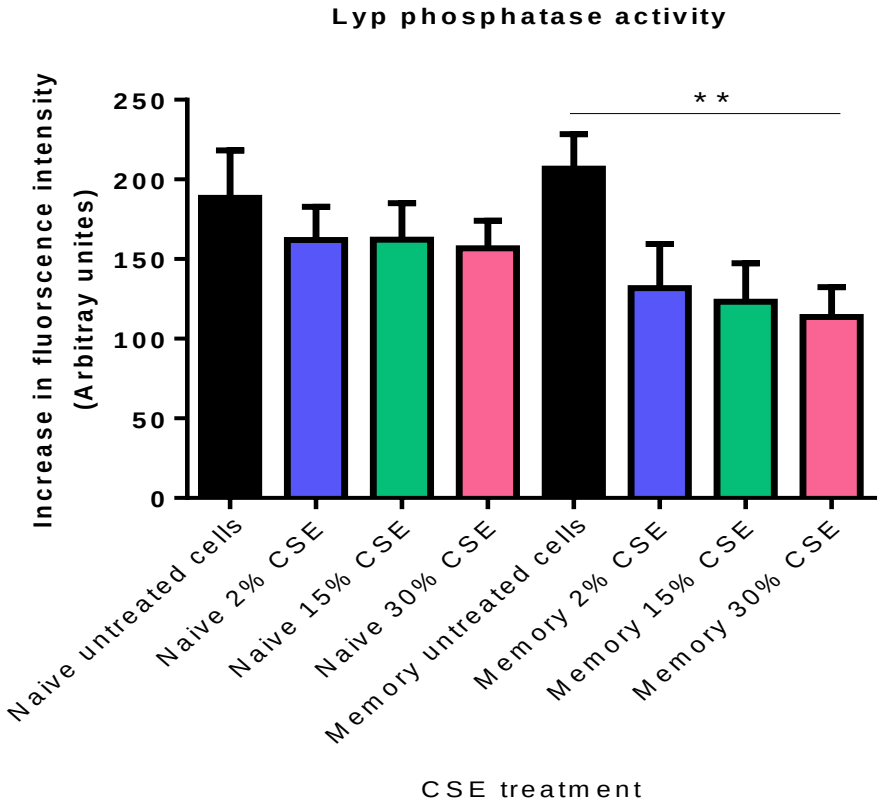


Figure 4. 3 CSE did not show any change in Lyp phosphatase activity in naïve but showed a reduction in memory cells.

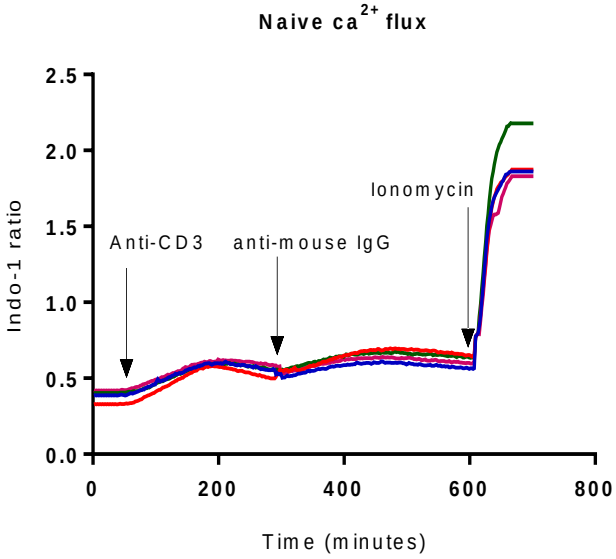
Naïve and memory cells were treated with CSE and incubated for 24. Cells were lysed and transferred to 96 well plate in triplicate coated with anti Lyp. The measurement activity of Lyp was described in methods section. (A) A figure showed the Lyp phosphatase activity in both naïve and memory cells following CSE. In this assay two readings were taken, the first reading at 0 hour and the second reading at 2 hours. The calculation of the fluorescence intensity (FI) was done by subtraction of 0 hour reading from 2 hours reading. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=5)

4.2.2 CSE increased calcium signalling flux in naïve and memory cells

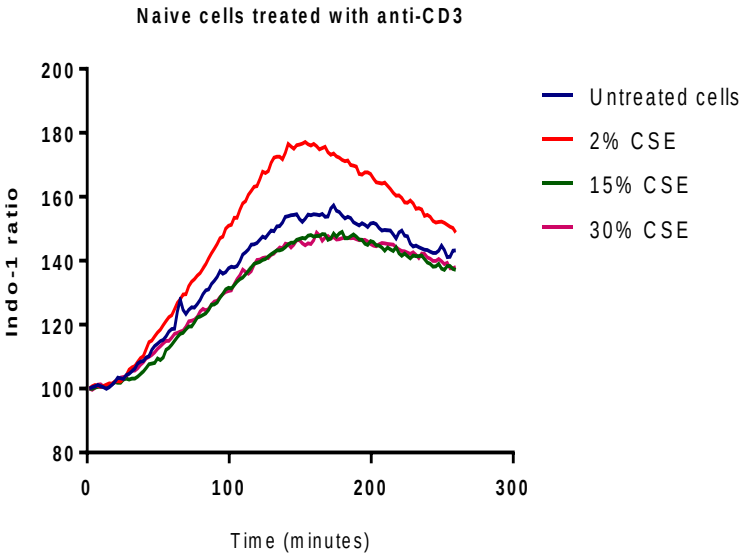
The effect of CSE on Ca^{2+} signalling was investigated in naïve and memory cells to determine if the change in ZAP-70 tyr319 was paralleled by overall signalling through TCR. Naïve and memory cells were treated with CSE for 24 hours, Indo-1 AM ester was loaded and the cells were stimulated with anti CD3. Following CD3, goat anti mouse was added which crosslinked the anti CD3 to induce the maximum TCR signal and the intracellular Ca^{2+} release was assessed by changes in indo-1 fluorescence. At the end of the experiment ionomycin was added as positive control to determine if Ca^{2+} sensitive dye Indo-1 AM was correctly loaded into cells.

The pattern of responses can be seen in figure 4.4 A, where an initial Ca flux was seen after the addition of anti-CD3 followed by a further signal after the addition of the goat ant-mouse IgG crosslinker. These two components were plotted separately and normalised to 100 to allow a better comparison. Ca^{2+} signalling in naïve cells was significantly increased after 2% CSE in response to anti CD3 and goat anti mouse IgG, whereas 15% and 30% did not show any effect following anti CD3 but there was a slight increase following goat anti mouse. (Figure 4.4 B-E). On the other hand, memory cells did not show any effect following anti CD3 treatment. However, following the addition of goat anti mouse IgG, the signal was much higher and there was a significant increase at 2%, 15% and 30%. (Figure 4.4 G-K). This may suggest that by increasing the activity of ZAP-70 tyr319, it may lead to an increase in Ca^{2+} signalling.

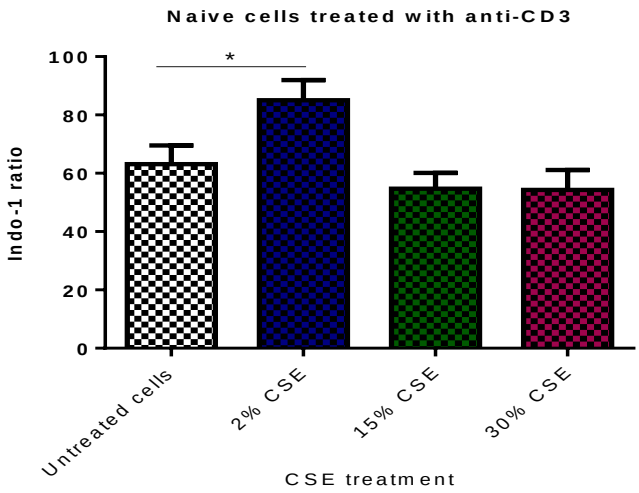
A


Naive ca2+ flux
Indo-1 ratio
Anti-CD3
anti-mouse IgG
Ionomycin
Time (minutes)

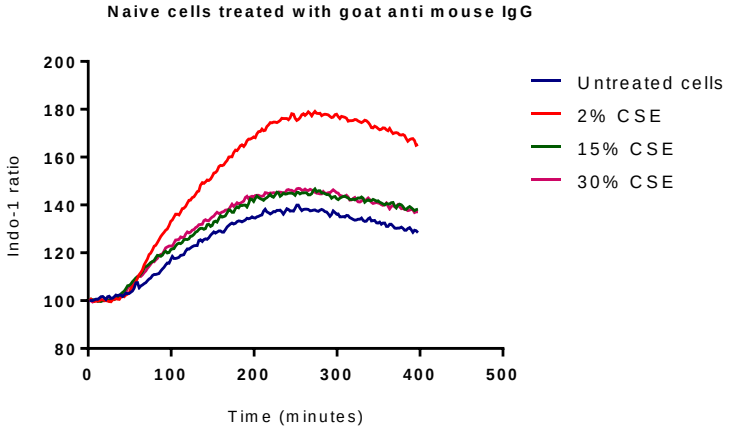
B


Naive cells treated with anti-CD3
Indo-1 ratio
Time (minutes)
Untreated cells
2% CSE
15% CSE
30% CSE

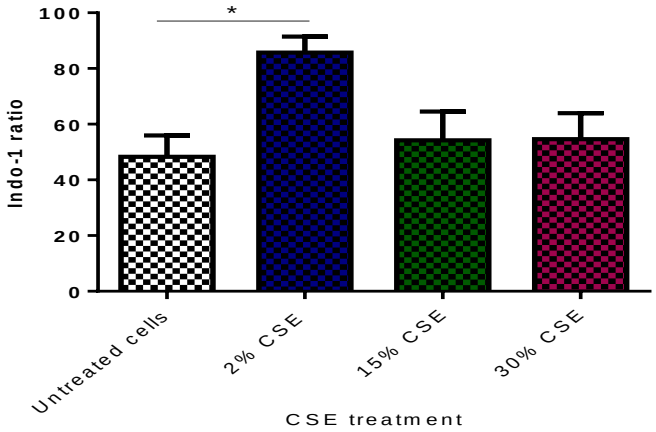
C


Naive cells treated with anti-CD3
Indo-1 ratio
*
Untreated cells
2% CSE
15% CSE
30% CSE
CSE treatment

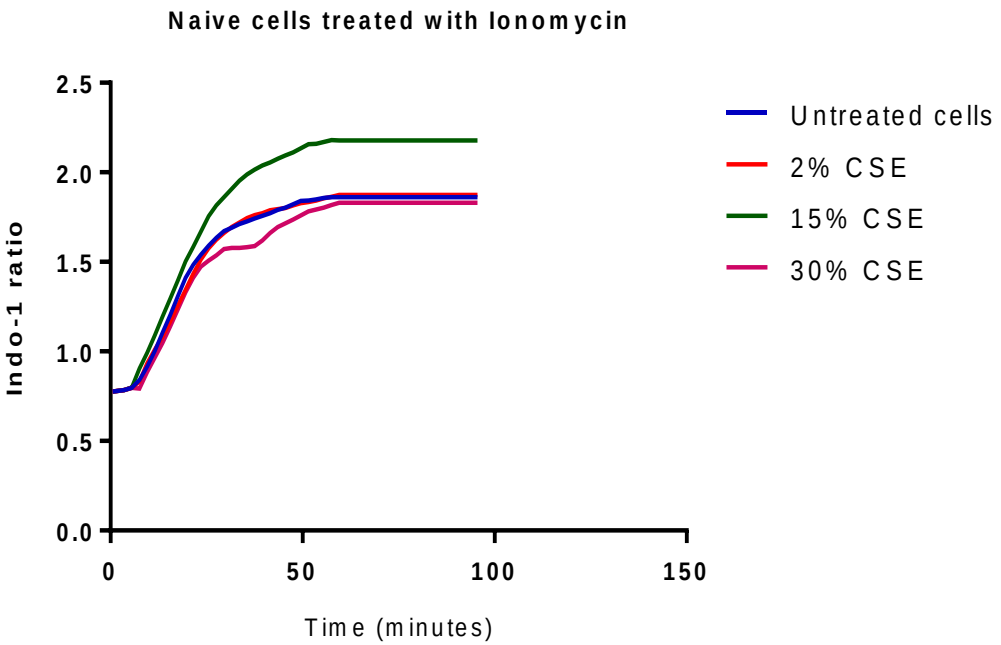
D


Naive cells treated with goat anti mouse IgG
Indo-1 ratio
Time (minutes)
Untreated cells
2% CSE
15% CSE
30% CSE

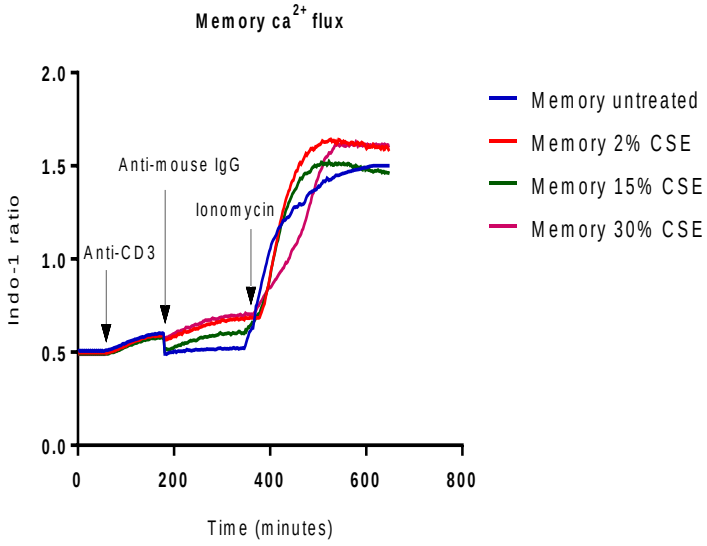
E


Naive cells treated with goat anti mouse IgG
Indo-1 ratio
*
Untreated cells
2% CSE
15% CSE
30% CSE
CSE treatment

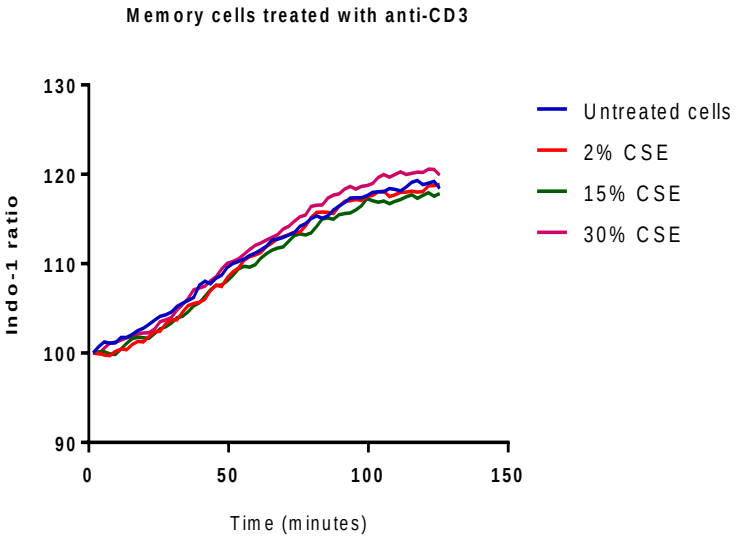
F


Naive cells treated with Ionomycin
Indo-1 ratio
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
Time (minutes)
Untreated cells
2% CSE
15% CSE
30% CSE

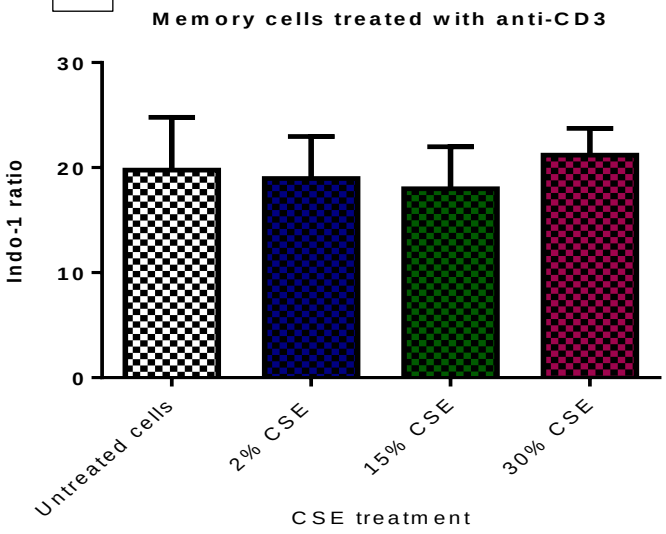
G


Memory ca2+ flux
Indo-1 ratio
2.0
1.5
1.0
0.5
0.0
Anti-CD3
Anti-mouse IgG
Ionomycin
0
200
400
600
800
Time (minutes)
Memory untreated
Memory 2% CSE
Memory 15% CSE
Memory 30% CSE

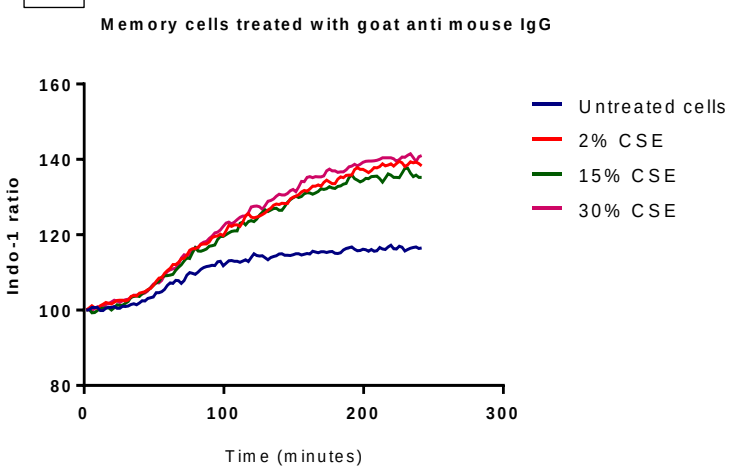
H


Memory cells treated with anti-CD3
Indo-1 ratio
130
120
110
100
90
0
50
100
150
Time (minutes)
Untreated cells
2% CSE
15% CSE
30% CSE

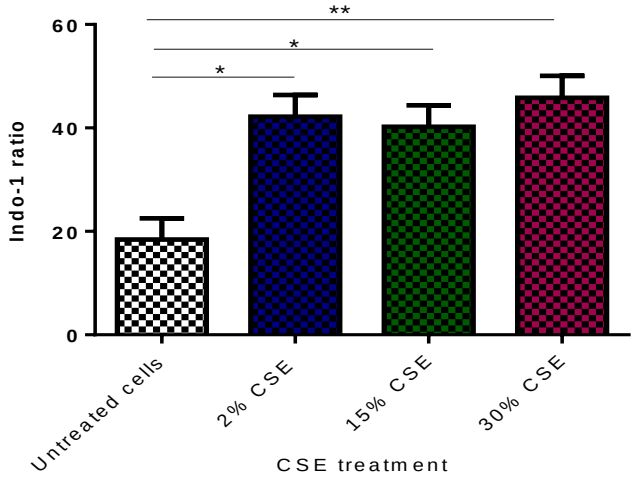
I


Memory cells treated with anti-CD3
Indo-1 ratio
30
20
10
0
Untreated cells
2% CSE
15% CSE
30% CSE
CSE treatment

J


Memory cells treated with goat anti mouse IgG
Indo-1 ratio
160
140
120
100
80
0
100
200
300
Time (minutes)
Untreated cells
2% CSE
15% CSE
30% CSE

K


Memory cells treated with goat anti mouse IgG
Indo-1 ratio
60
40
20
0
**
*
*
Untreated cells
2% CSE
15% CSE
30% CSE
CSE treatment

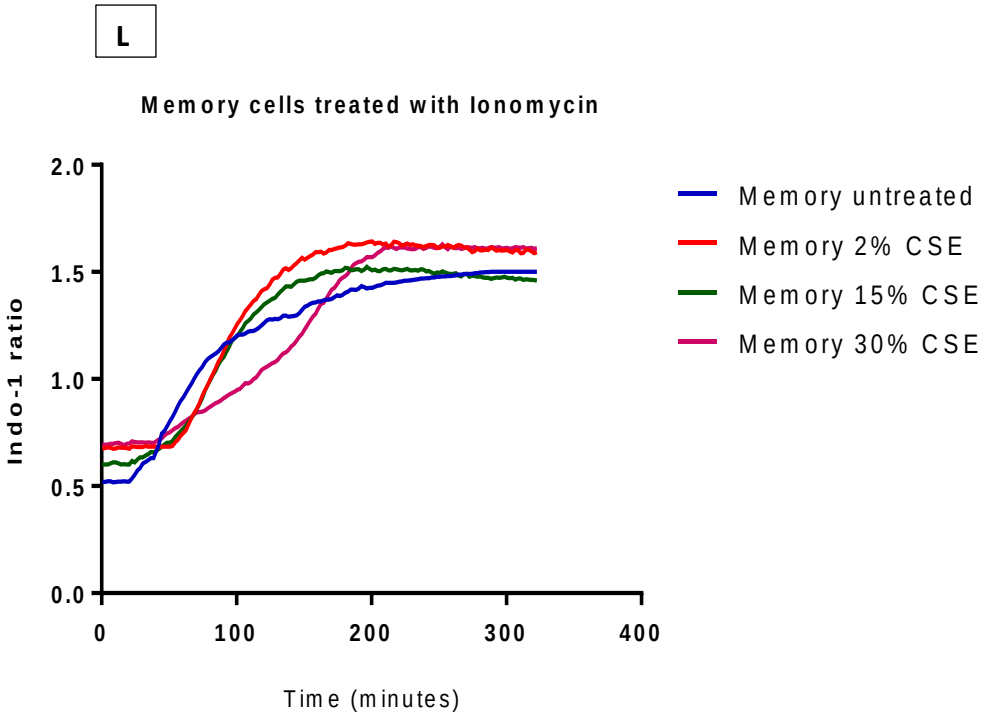


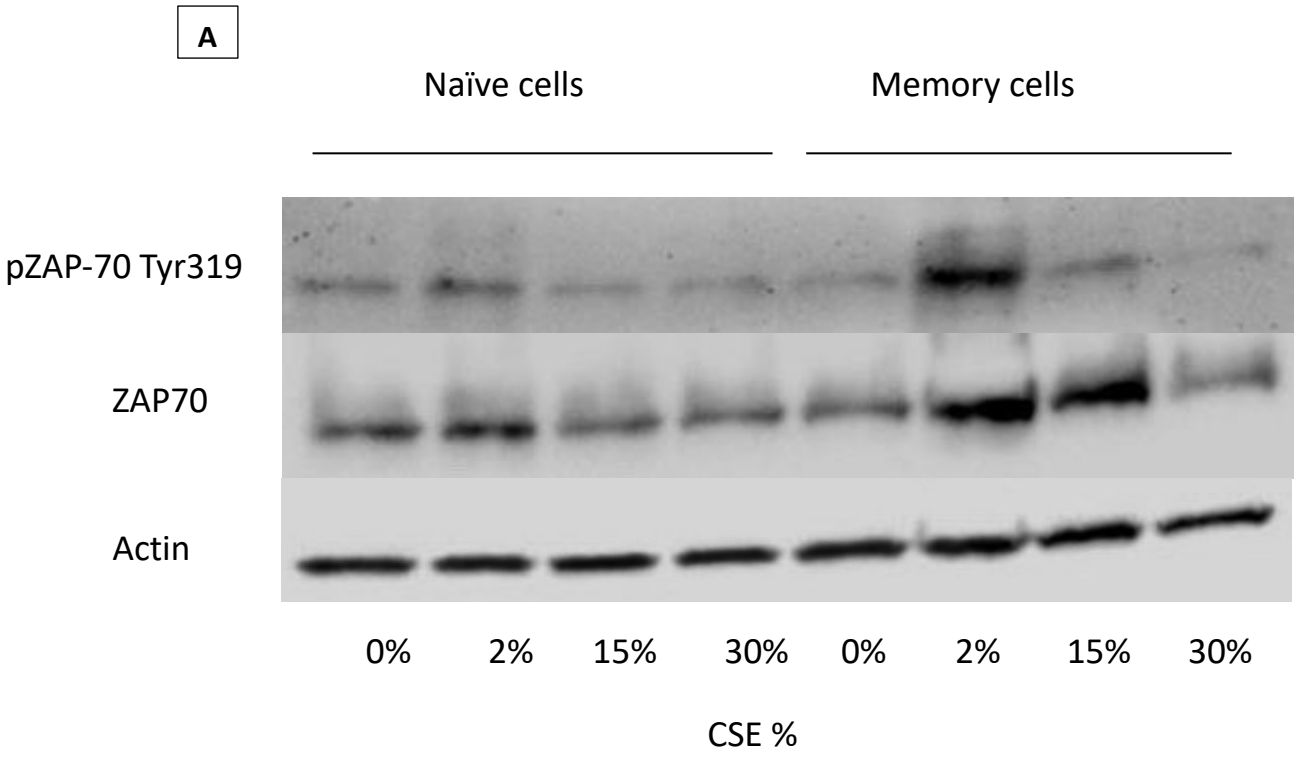
Figure 4. 4 CSE treatment increased the calcium signalling in naïve and memory cells at low and high concentration.

Naïve and memory cells were treated with CSE for 24 hours and then Indo-1 AM ester was added and then cells were treated with both anti CD3 and goat anti mouse IgG. To determine if Ca^{2+} sensitive dye Indo-1 AM was correctly loaded into cells, ionomycin was added as a positive control. (A) A representative graph of four independent experiments of naïve cells. (B+C) A representative graph after stimulation with anti CD3 from four independent experiments of naïve cells. (D+E) A representative graph after stimulation with goat anti mouse IgG from four independent experiments. (F) A representative graph after stimulation with ionomycin from four independent experiments of naïve cells. (G) A representative graph of four independent experiments of Memory cells. (H+I) A representative graph after stimulation with anti CD3 from four independent experiments of memory cells. (J+K) A representative graph after stimulation with goat anti mouse IgG from four independent experiments memory cells. (L) A representative graph after stimulation with ionomycin from four independent experiments memory cells. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=4)

4.2.3 CSE at low concentration increased the phosphorylation of ZAP-70 at Tyr319 residue in memory cells but not in naïve cells.

Accumulated evidence has shown that ZAP-70 plays an essential role in the activation and stimulation of signalling pathways downstream of the TCR. ZAP-70 (tyr319) is the major Lck binding site, and its phosphorylation is important for ZAP-70-containing signalling complex assembly which leads to PLC-gamma1 activation and then to Ca2+ signalling. The importance of this phosphorylation was well demonstrated in one study which showed that a ZAP-70 (tyr319) mutant failed to reconstitute CD69 expression, Ras activation and TCR-dependent Ca2+ mobilization. This defect reduced the activity of two key signalling molecules, PLC-gamma1 and Lck in stimulated T cells [351]. Furthermore, there is evidence that ZAP-70 mutations are associated with some cases of rheumatoid arthritis and with animal models of the disease. There have been no studies of the effect of CSE on ZAP-70 activity. Therefore, we investigated whether CSE would alter activation of ZAP-70 (tyr319), and whether this activation would initiate upstream pathways through TCR receptor.

To investigate this, both naïve and memory T cells were exposed to CSE for 24 hours and stimulated with anti CD3/CD28 for 72 hours. The immunoblotting technique was used to assess the phosphorylation status of ZAP-70 (tyr319) and total ZAP-70 protein. It was found that the phosphorylation of ZAP-70 (tyr319) following 2% CSE was increased in both naïve and memory cells, but the level was much higher and more significant in memory cells compared to control. However, 15% showed no effect in naïve but there was a slight increase in memory cells compared to control. The 30% showed no effect in both naïve and memory cells (Figure 4.5 A+B).



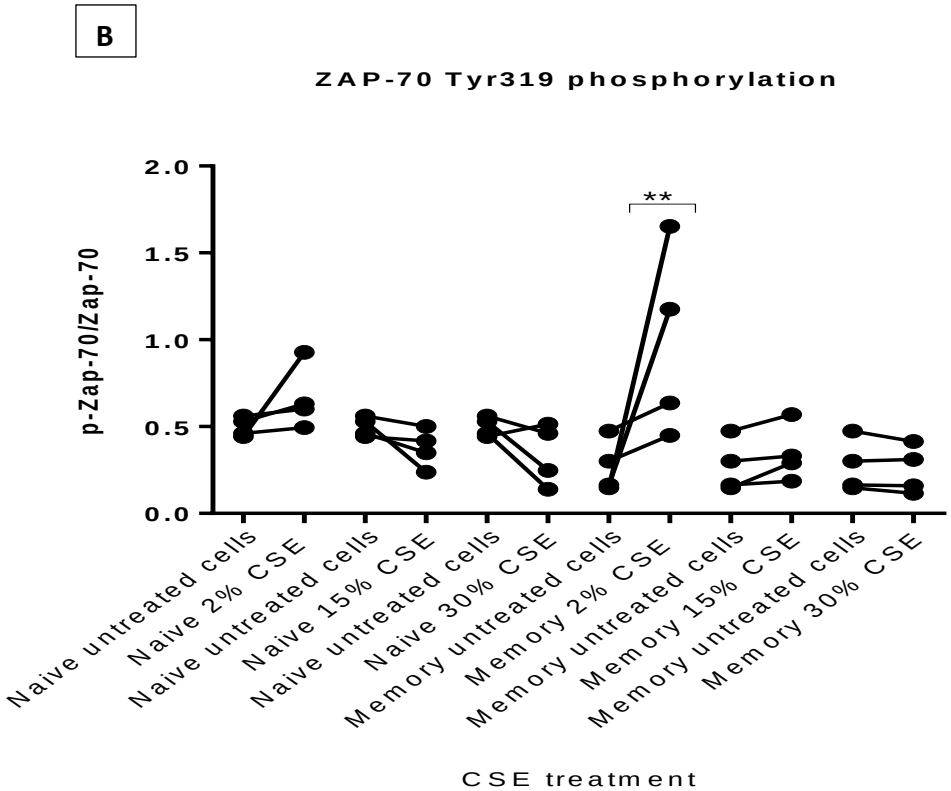


Figure 4. 5 CSE increased the phosphorylation of ZAP-70 Tyr319 in memory cells but not in naïve cells at low concentration, whereas high concentration showed no effect.

Naïve and memory cells were treated with CSE for 24 hours and then activated with anti-CD3/CD28 for 72 hours. (A+B) A representative blot and graph of the four independent experiment. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=4)

4.2.4 CSE increased the phosphorylation of ZAP-70 at Tyr493 residue in naïve and memory cells

After investigating the phosphorylation of ZAP-70 (tyr319), the phosphorylation of ZAP-70 (tyr493) was investigated to further clarify the underlying mechanisms by which ZAP-70 regulates the signalling pathways and whether CSE would increase the autophosphorylation of ZAP-70 and binding to Lck, which in turn activates tyr493 in the putative regulatory loop of its kinase domain. This phosphorylation of ZAP-70 at residue 493 which is found in the activation loop results in enzymatic activation of ZAP-70 and initiates the early steps of the TCR signalling cascade [352].

It was found that the phosphorylation of ZAP-70 (tyr493) in both naïve and memory cells was increased following CSE. The naïve cells showed a graded increase between 0% and 15%. However, 15% showed a significant increase, and memory cells showed also a significant increase at 2% CSE in the replicated experiments (n=4). This comparability was also seen in the decrease below controls of the level of the phosphorylation of ZAP-70 (tyr493) was following 30% CSE in both naïve and memory cells (Figure 4.6 A+B). This result showed similarity to the data we showed from ZAP-70 (tyr319) where the low concentration increased the activity of ZAP-70, whereas high concentration decreased or showed no effect, suggesting that CSE may increase the phosphorylation status of enzymatic activity of ZAP-70 which in turn initiates the downstream TCR signalling cascade.

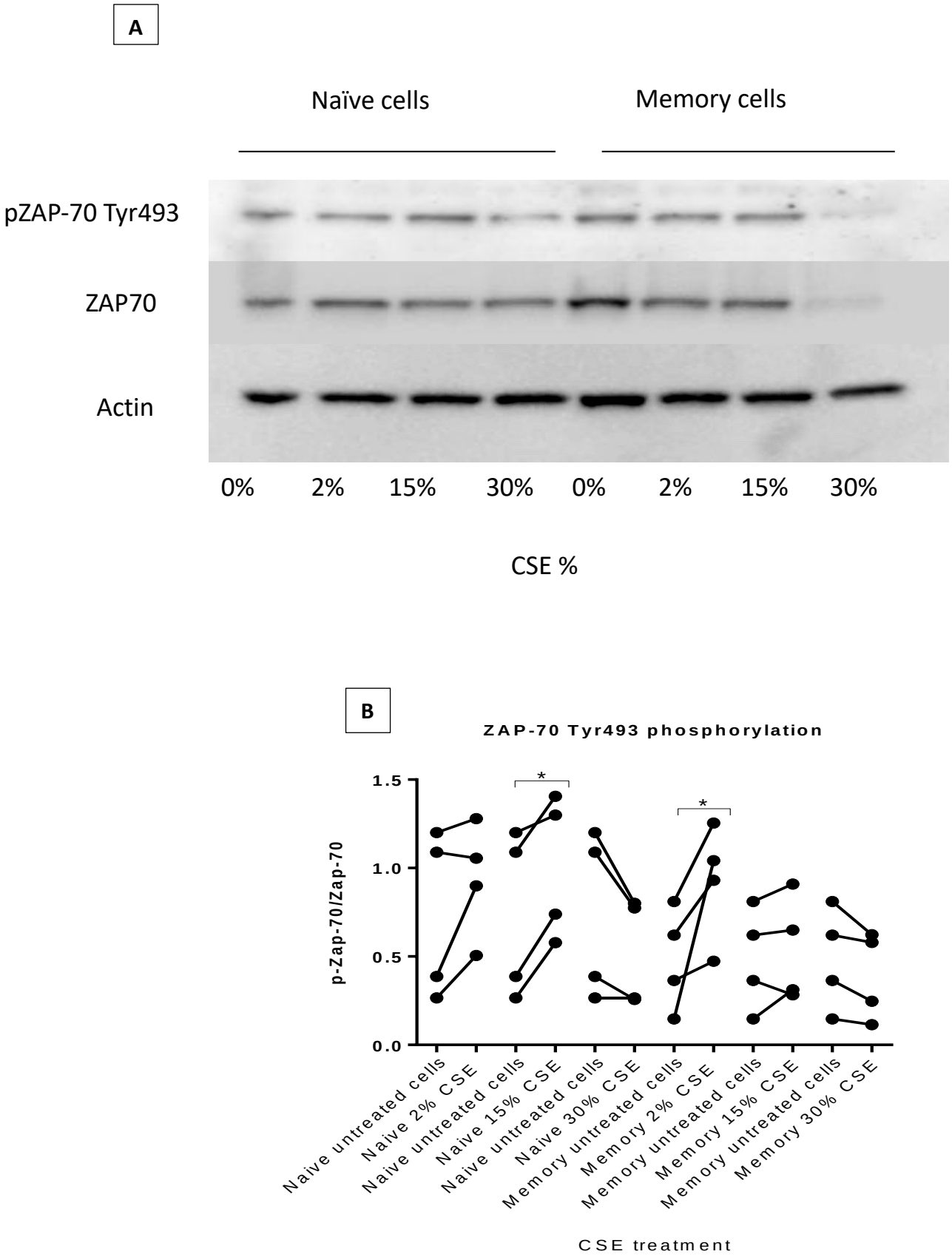


Figure 4. 6 CSE enhanced the phosphorylation of ZAP-70 Tyr493 in naive and memory cells at low concentration, whereas high concentration showed no effect.

Naïve and memory cells were treated with CSE for 24 hours and then activated with anti-CD3/CD28 for 72 hours. (A+B) representative blot of the four independent experiment. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=4)

4.2.5 CSE did not show any change in the phosphorylation of Lck Tyr394 in naïve and memory cells.

Lck is an essential molecule for signalling through the TCR complex and it is a part of the Src-tyrosine kinase family. It phosphorylates ZAP-70 and activates subsequent signalling platforms and involves in regulating apoptosis. However, dysregulation of Lck induces CD45 deficiency and immunodeficiency 22, a combined immunodeficiency of T and B cells. Lck tyr394 is the activation site of the catalytic domain, so phosphorylation of Y394 within the activation loop induces the enzymatic activity of Lck and therefore play a role in substrate specificity [353]. However, this activation can be regulated by a negative regulatory domain ending with a C terminal called Lck tyr505 and other phosphatases, including Lyp and CD45 [354, 355]. In the previous results we investigated the effect of CSE on ZAP-70. We found that CSE increased ZAP-70 activity at tyr493 and tyr319 residue and therefore initiates the signalling. Since the Lck can phosphorylate ITAMs and then activates ZAP-70, we tried to elucidate whether CSE has an effect on phosphorylation status of Lck tyr394 through TCR of activated naïve and memory T cells and whether this would show similar effect to that on ZAP-70. It was found that Lck was expressed and phosphorylated in naïve and memory cells following 2% CSE treatment compared to control. Therefore, the pLck/Lck ratio was done to assess the phosphorylation status of Lck, and it was found that Lck was more phosphorylated at 2%, but not at 15% and 30% (Figure 4.7 A+B). The result was similar to ZAP-70 data where the low concentration of CSE increased the phosphorylation, whereas the high concentration decreased this. Although the changes were not statistically significant, this may suggest that CSE could activate Lck which in turn phosphorylates ZAP-70 and subsequently initiates the downstream signalling.

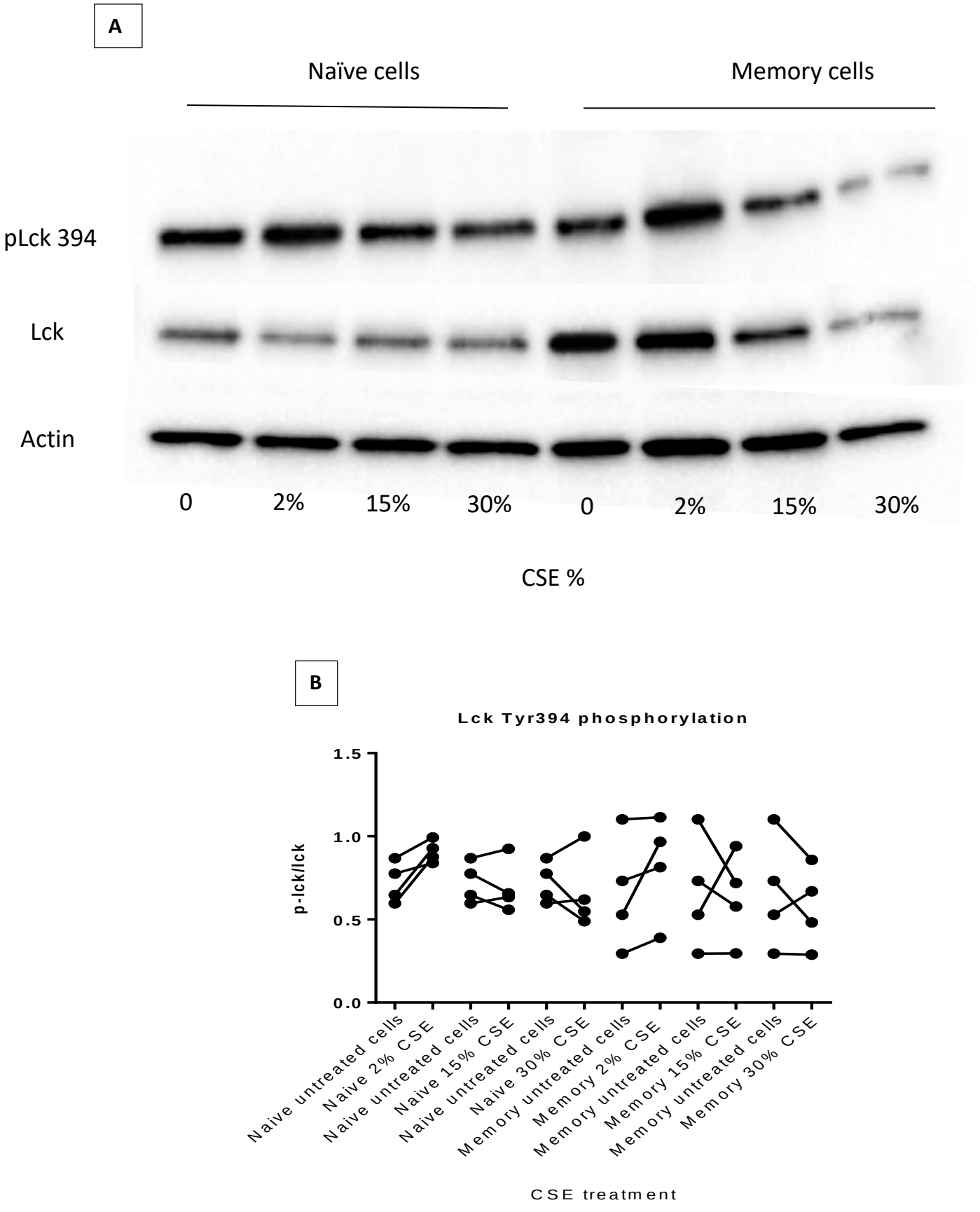


Figure 4. 7 CSE did not show any changes in the phosphorylation of Lck Tyr394 in naïve and memory cells.

Naïve and memory cells were treated with CSE for 24 hours and then activated with anti-CD3/CD28 for 72 hours. (A+B) A representative blot and graph of the four independent experiment. (n=4)

4.2.6 CSE did not show any effect on Lck phosphorylation at Tyr505 in naïve and memory cells

Lck is crucial in activating many targets and initiating signalling and it can be regulated by phosphorylation of tyr505 in the carboxylic terminal tail and which then limits TCR function. In the previous result it was found that CSE increased Lck activity by phosphorylating tyr394. Here we tried to elucidate whether CSE has an effect on phosphorylation status of Lck tyr505 through TCR of activated naïve and memory T cells. Neither naïve nor memory cells showed any effect at 2% CSE. However, 15% and 30% in both naïve and memory decreased the phosphorylation of Lck tyr505 (Figure 4.8 A+B). This suggests that CSE could decrease negative regulation of Lck through a decrease in tyr505 phosphorylation. This could act together with the previously demonstrated increase in the phosphorylation of tyr394 within the activation loop and induced the activity of Lck to enhance the downstream TCR signalling events.

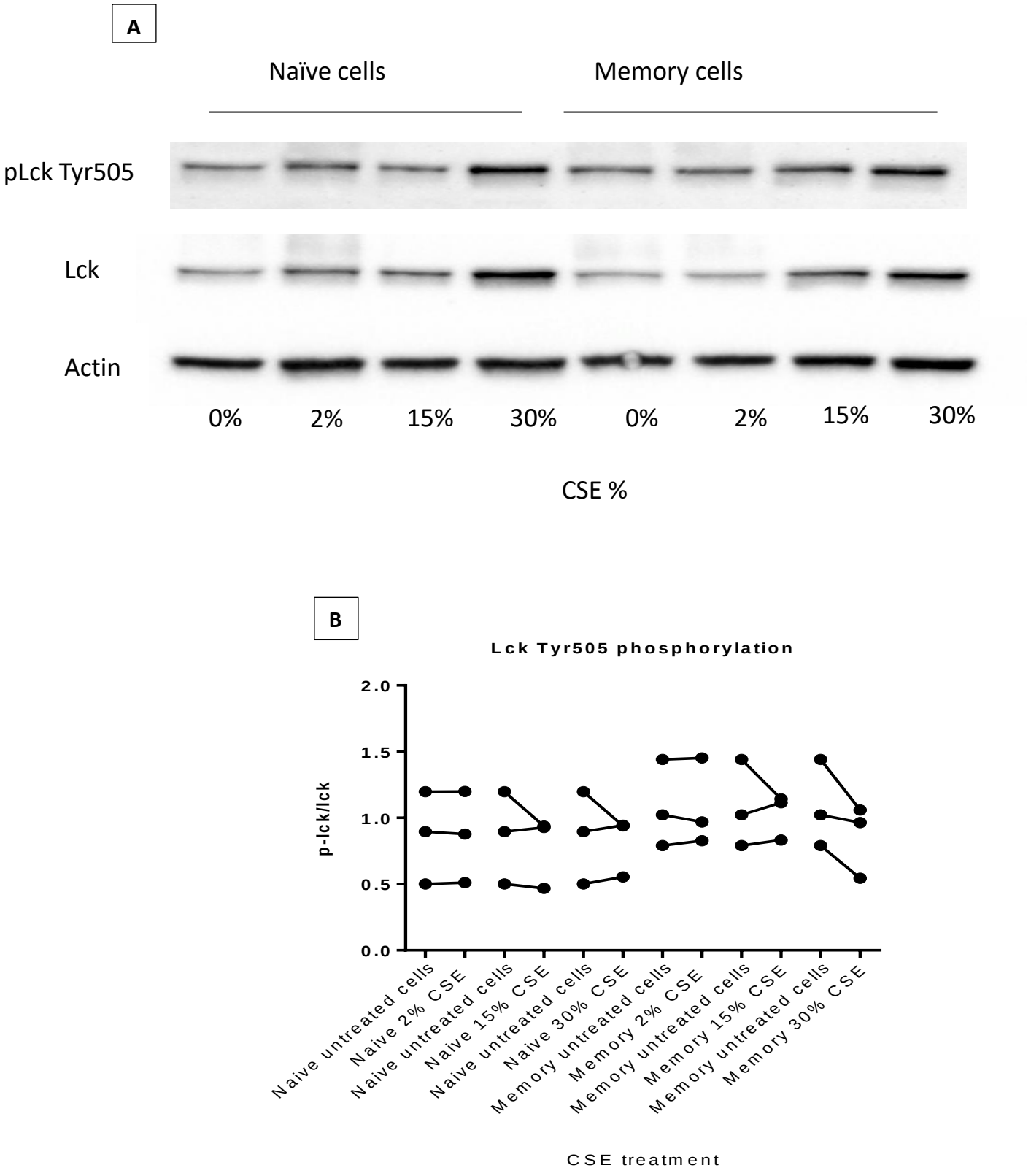


Figure 4. 8 CSE did show any effect on Lck Tyr505 in naïve and memory cells.

Naïve and memory cells were treated with CSE for 24 hours and then activated with anti-CD3/CD28 for 72 hours. (A+B) A representative blot and graph of the three independent experiment. (n=3)

4.2.7 CSE did not show any effect in Vav1 phosphorylation at Tyr174residue in naïve and memory cells.

Vav1 is a member of guanine nucleotide exchange factor (GEF) for Rho-family GTPases which is activated by Src family kinase, Lck. It is required for TCR interaction with APCs, activation of the LFA1 and signalling pathways such as NF-κB, ERK and calcium flux [356]. It was found that Vav1 could regulate the activation of PLCγ1 through independent and dependent PI3K pathways [357]. It has been shown that activation Vav1 at residue tyr174 increase the activity of Vav1 leading to enhancement of cytoskeletal reorganisation and nuclear factor of activated T cells [358]. Since the activation of Lck leads to phosphorylation of Vav1 and activates downstream targets, and since a quite few studies suggested a link between Lyp and Vav1, with Vav1 being a substrate for Lyp, and others showing that Vav1 may be required to induce Akt phosphorylation through TCR; we investigated the effect of CSE on Vav1 at activation loop Tyr174 to elucidate whether CSE could have an effect on Vav1 activity or not. Generally, CSE did not show a significant increase of Vav1 phosphorylation at 2% in both naïve and memory cells. However, the phosphorylation at 15% and 30% was decreased in both naïve and memory cells. (Figure 4.9 A+B).

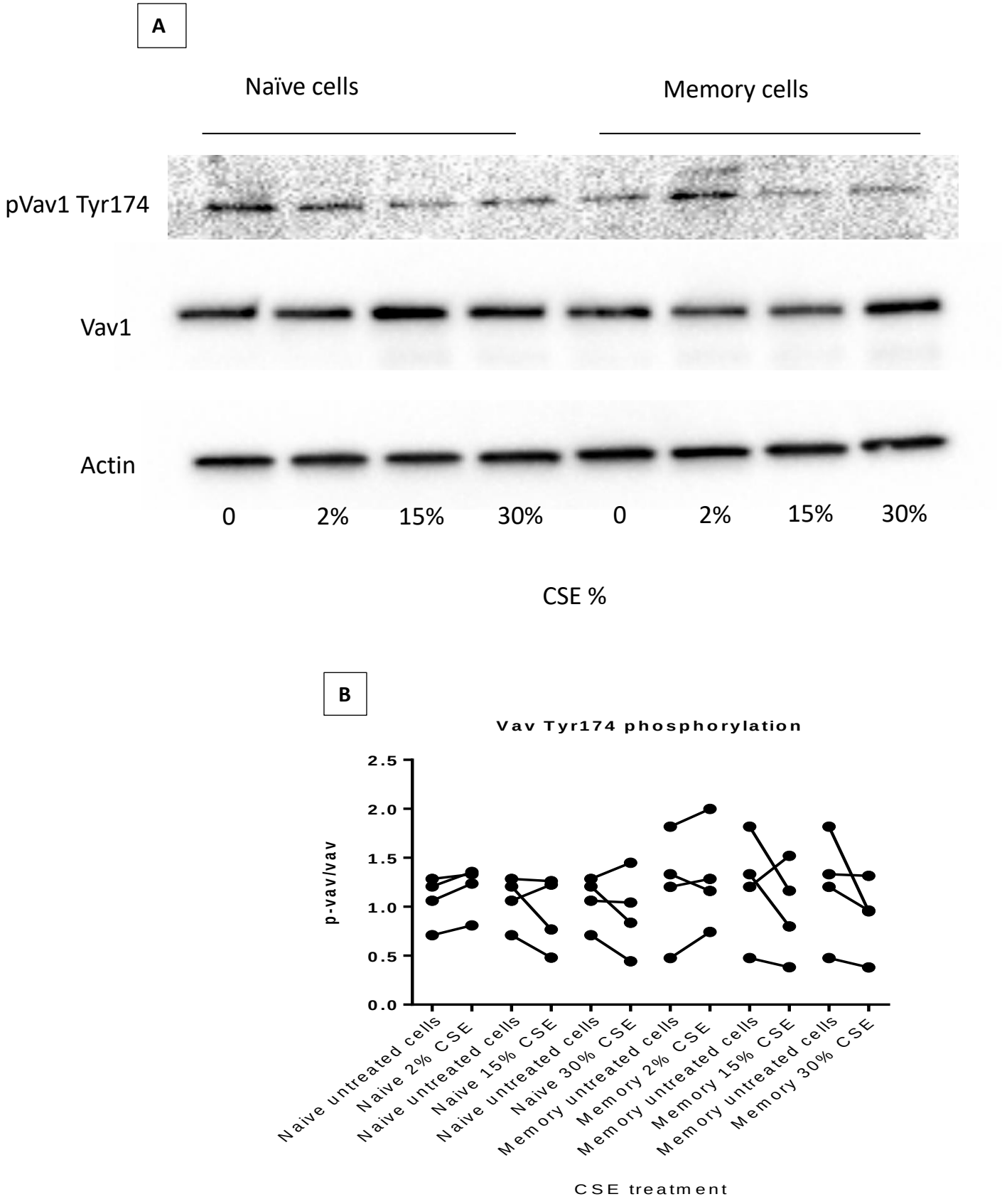


Figure 4. 9 CSE did not show any change in the phosphorylation of Vav1 tyr174 in naïve and memory cells.

Naïve and memory cells were treated with CSE for 24 hours and then activated with anti-CD3/CD28 for 72 hours. (A+B) A representative blot and graph of the four independent experiment. (n=4)

4.2.8 Lyp inhibitor reduced the activity of Lyp phosphatase after a fluorometric measurement of Lyp phosphatase activity in naïve but not in memory cells.

Since our hypothesis is focusing on Lyp function and how losing its function could drive rheumatoid arthritis, we assessed the Lyp phosphatase activity using chemical inhibitor (PTP LYP inhibitor) which inhibits the biological activity of Lyp. Naïve and memory cells were treated with Lyp inhibitor (untreated cells, 0.2µM, 0.5µM and 1µM) and incubated for 24 hours. It was found that the Lyp phosphatase activity in naïve cells was decreased at 2µM and 0.5µM up to 50% but it was not significant. The activity of Lyp phosphatase was susceptible and significantly reduced at 1µM treatment up to 70%. Memory cells showed an overall reduction in Lyp phosphatase activity following 0.2µM, 0.5µM and 1µM but it was not significant (Figure 4.10 A). This result suggests that by inhibiting Lyp function using Lyp inhibitor, the activity of Lyp decreased which is consistent to the result we showed previously where CSE decreased the Lyp activity. Based on this, CSE may interfere with Lyp function and therefore increases the metabolism and signalling pathway.

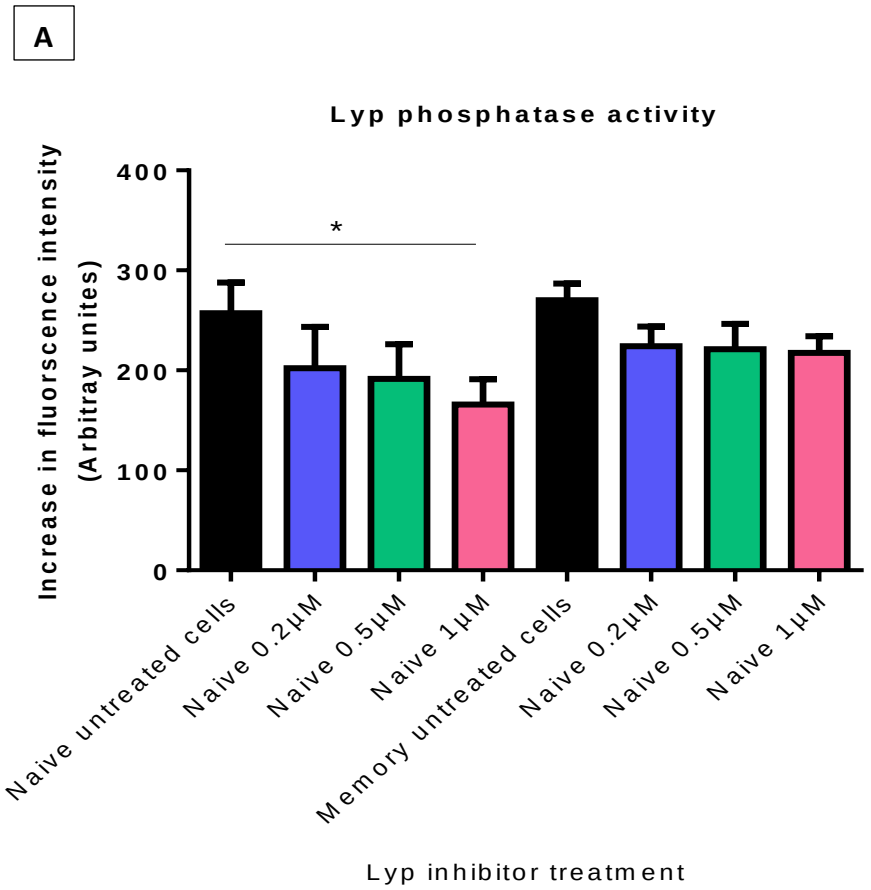


Figure 4. 10 Lyp inhibitor decreased Lyp phosphatase activity in naïve but not in memory cells.

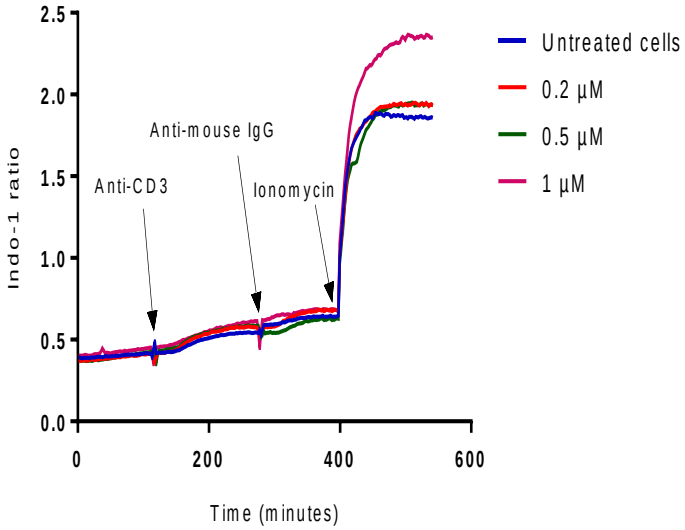
Naïve and memory cells were treated with Lyp inhibitor and incubated for 24. Cells were lysed and transferred to 96 well plate in triplicate coated with anti Lyp. The measurement activity of Lyp was described in methods section. (A) A figure showed the Lyp phosphatase activity in naïve and memory cells following Lyp inhibitor treatment. In this assay two readings were taken, the first reading at 0 hour and the second reading at 2 hours. The calculation of the fluorescence intensity (FI) was done by subtraction of 0 hour reading from 2 hours reading. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=5)

4.2.9 Lyp inhibitor increased calcium signalling flux in naïve and memory cells.

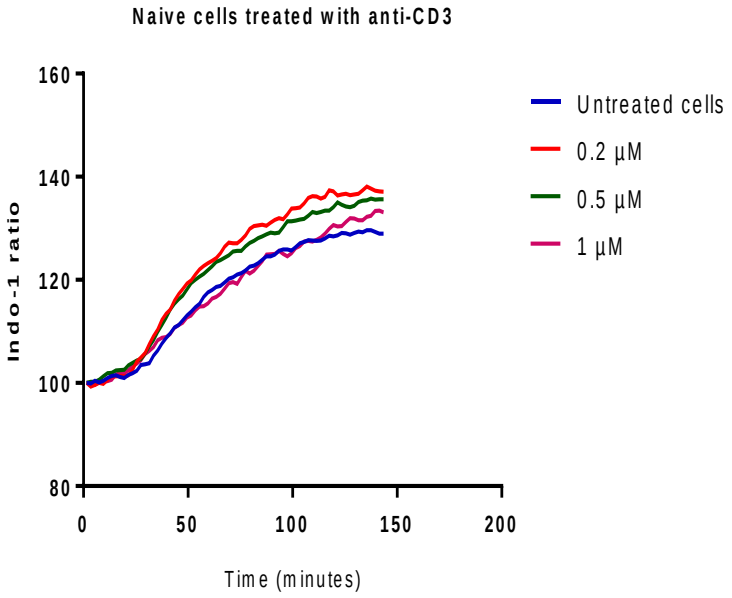
Following the investigation of Ca^{2+} signalling after CSE treatment, Ca^{2+} signalling was also investigated following Lyp inhibitor treatment to determine if Lyp inhibition would affect the overall signalling through TCR. Naïve and memory cells were treated with Lyp inhibitor (0, 2µM, 0.5µM and 1µM) for 24 hours and Indo-1 AM ester was added and then cells were stimulated with anti CD3 and followed by goat anti mouse The pattern of responses can be seen in figure 4.11 A, where an initial Ca flux was seen after the addition of anti-CD3 followed by a further signal after the addition of the goat ant-mouse IgG crosslinker. These two components were plotted separately and normalised to 100 to allow a better comparison. There was a significant increase of the signalling in naïve cells at 2µM in response to anti CD3. Also, there was an increase of Ca^{2+} signalling at 0.5µM and 1µM. Similar response was obtained after the addition of goat anti mouse. The 2µM, 0.5µM and 1µM increased the signalling but only 2µM was significant. (Figure 4.11 B-E).

Memory cells did not show any effect at 2µM, but there was an increase at 0.5µM and 1µM in response to anti CD3. However, following the addition of goat anti mouse, the signalling at 2µM, 0.5µM and 1µM was much higher than anti CD3 treatment, but only the 2µM showed a significant increase (Figure 4.11 G-K). So, these data may suggest that Lyp inhibitor treatment could have an effect on ZAP-70 which in turn enhances intracellular calcium flux through TCR activation. Since this data is very similar to the data from CSE, perhaps there may be a link between CSE effect and Lyp activity.

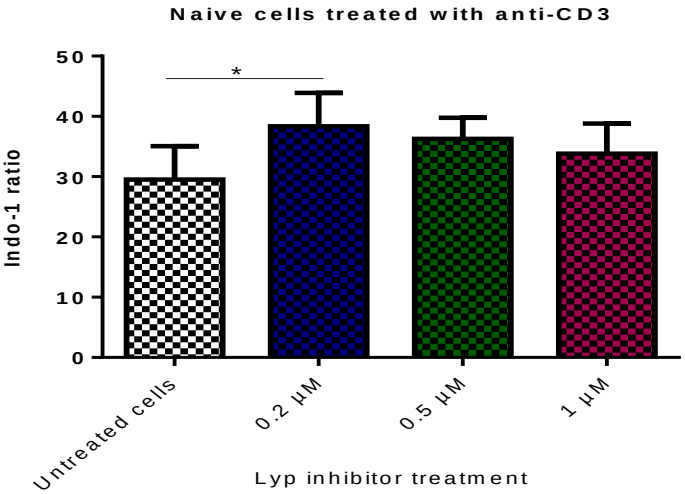
A


Naive ca2+ flux
Indo-1 ratio
Anti-CD3
Anti-mouse IgG
Ionomycin
Untreated cells
0.2 µM
0.5 µM
1 µM
Time (minutes)

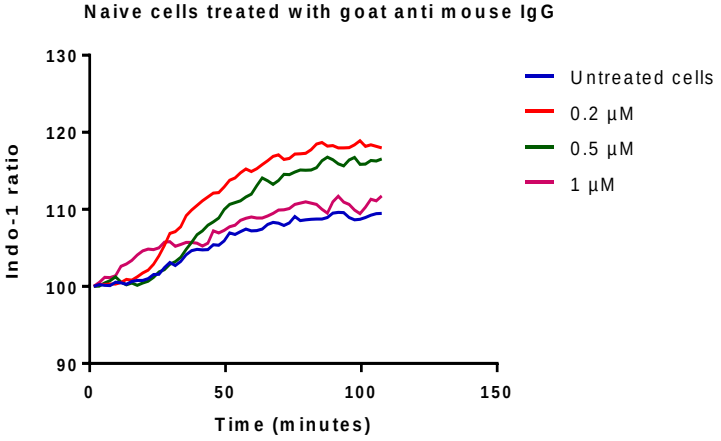
B


Naive cells treated with anti-CD3
Indo-1 ratio
Untreated cells
0.2 µM
0.5 µM
1 µM
Time (minutes)

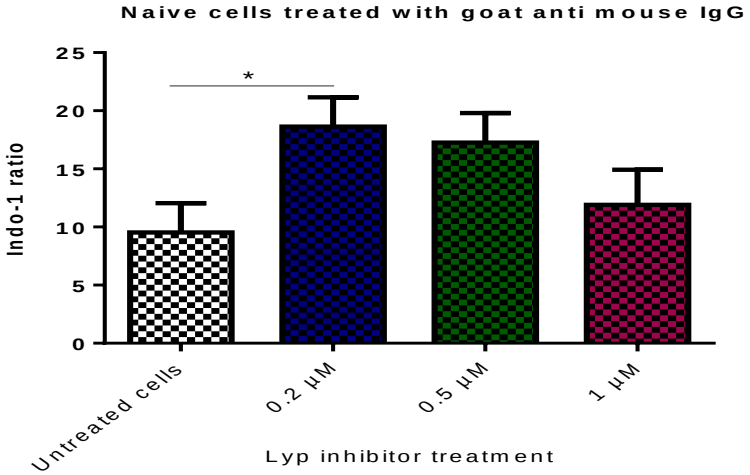
C


Naive cells treated with anti-CD3
Indo-1 ratio
*
Untreated cells
0.2 µM
0.5 µM
1 µM
Lyp inhibitor treatment

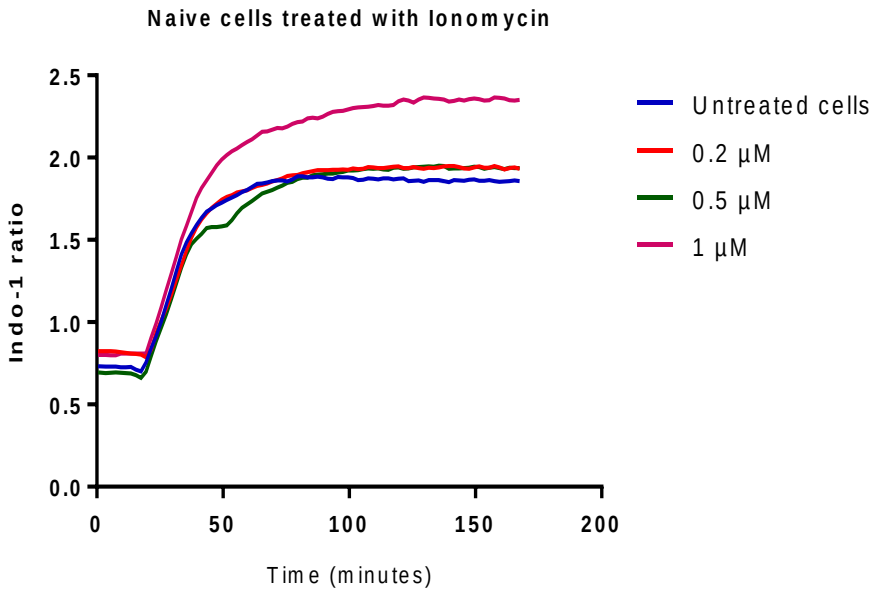
D


Naive cells treated with goat anti mouse IgG
Indo-1 ratio
Untreated cells
0.2 µM
0.5 µM
1 µM
Time (minutes)

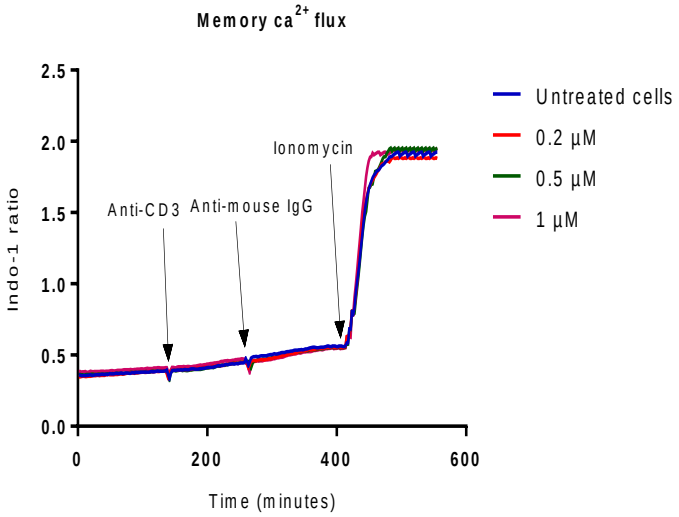
E


Naive cells treated with goat anti mouse IgG
Indo-1 ratio
*
Untreated cells
0.2 µM
0.5 µM
1 µM
Lyp inhibitor treatment

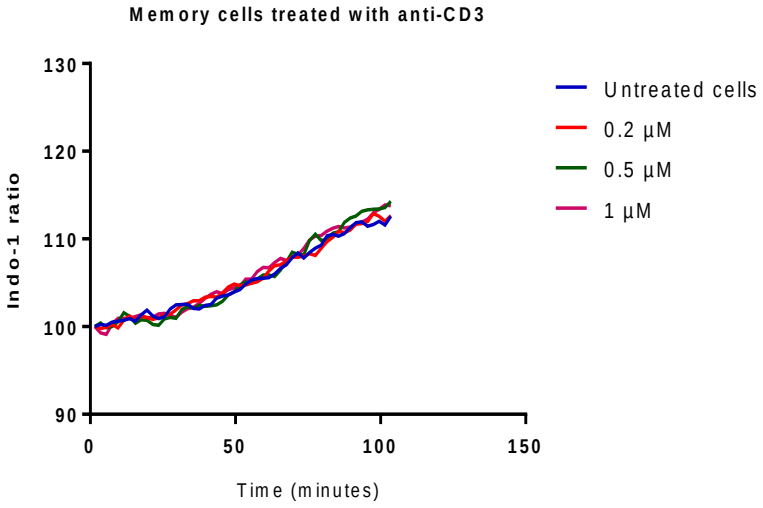
F


Naive cells treated with Ionomycin
Indo-1 ratio
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
Time (minutes)
Untreated cells
0.2 µM
0.5 µM
1 µM

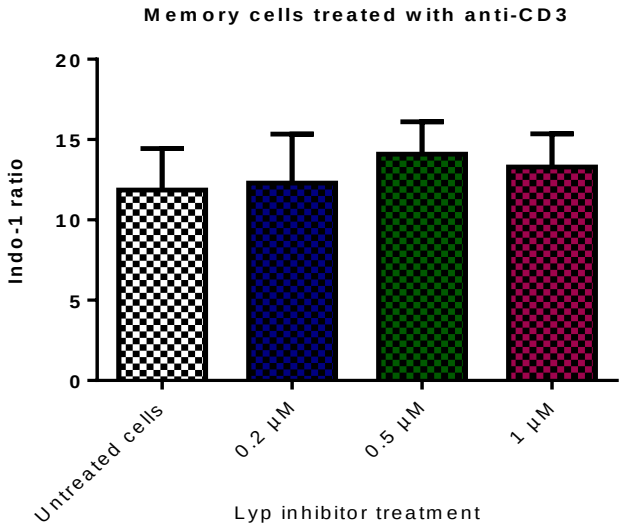
G


Memory ca2+ flux
Indo-1 ratio
2.5
2.0
1.5
1.0
0.5
0.0
0
200
400
600
Time (minutes)
Anti-CD3
Anti-mouse IgG
Ionomycin
Untreated cells
0.2 µM
0.5 µM
1 µM

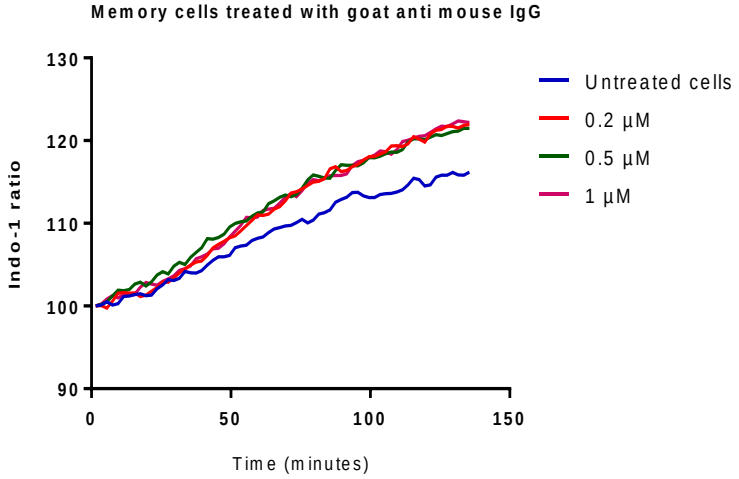
H


Memory cells treated with anti-CD3
Indo-1 ratio
130
120
110
100
90
0
50
100
150
Time (minutes)
Untreated cells
0.2 µM
0.5 µM
1 µM

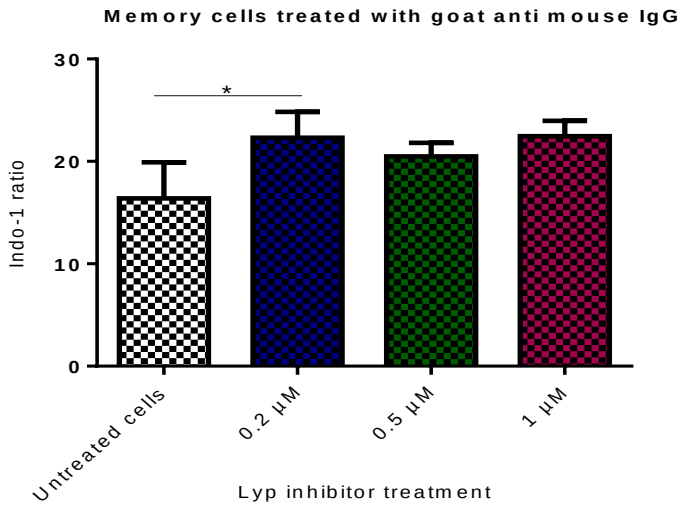
I


Memory cells treated with anti-CD3
Indo-1 ratio
20
15
10
5
0
Untreated cells
0.2 µM
0.5 µM
1 µM
Lyp inhibitor treatment

J


Memory cells treated with goat anti mouse IgG
Indo-1 ratio
130
120
110
100
90
0
50
100
150
Time (minutes)
Untreated cells
0.2 µM
0.5 µM
1 µM

K


Memory cells treated with goat anti mouse IgG
Indo-1 ratio
30
20
10
0
*
Untreated cells
0.2 µM
0.5 µM
1 µM
Lyp inhibitor treatment

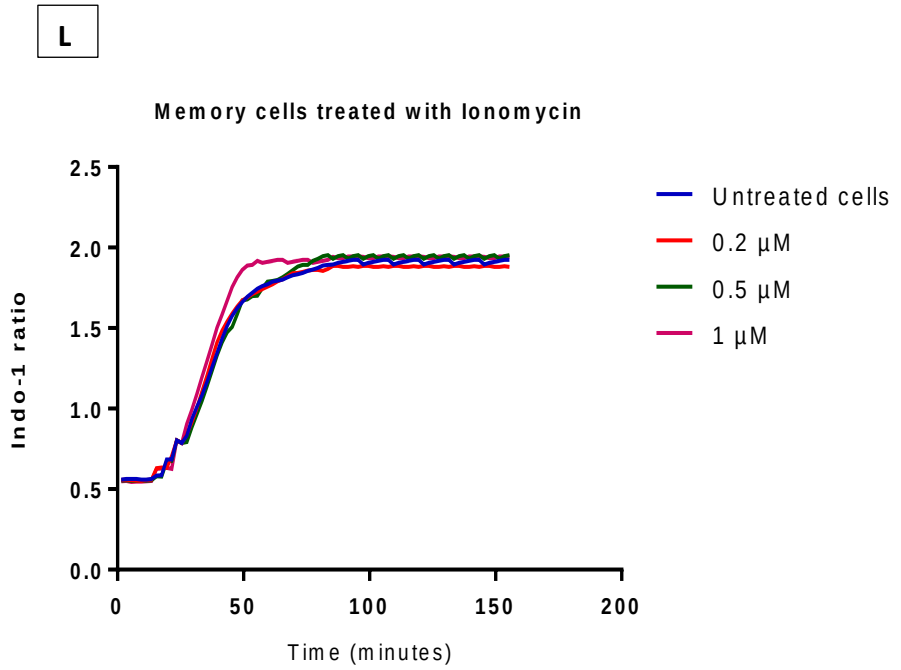


Figure 4. 11 Lyp inhibitor treatment increased the calcium signalling in naïve and memory cells at low and high concentration.

Naïve and memory cells were treated with Lyp inhibitor for 24 hours and then Indo-1 AM ester was added and then cells were treated with both anti CD3 and goat anti mouse. To determine if Ca^{2+} sensitive dye Indo-1 AM was correctly loaded into cells, ionomycin was added as a positive control. (A) A representative graph of four independent experiments of naïve cells. (B+C) A representative graph after stimulation with anti CD3 from four independent experiments of naïve cells. (D+E) A representative graph after stimulation with goat anti mouse IgG from four independent experiments. (F) A representative graph after stimulation with ionomycin from four independent experiments of naïve cells. (G) A representative graph of four independent experiments of Memory cells. (H+I) A representative graph after stimulation with anti CD3 from four independent experiments of memory cells. (J+K) A representative graph after stimulation with goat anti mouse IgG from four independent experiments memory cells. (L) A representative graph after stimulation with ionomycin from four independent experiments memory cells. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=4)

4.2.10 Lyp inhibitor did not show phosphorylation of ZAP-70 at Tyr319 in naïve and memory cells

In parallel with this approach, we hypothesised that CSE might interfere with PTPN22 which encodes Lyp protein and gives rise to energy metabolism and alteration of signalling pathways. Inhibition of Lyp is therapeutically attractive target to treat autoimmune conditions in human. A study showed that Lyp is expressed in mast cells and involved in mast cells signalling, so it has been shown that Lyp inhibitor could reduce this response. However, it has not yet made it to the clinic [359]. Because we hypothesised that CSE may interfere with Lyp, we used a chemical inhibitor (PTP LYP inhibitor) which inhibits the biological activity of Lyp to investigate whether the change in ZAP-70 activity following Lyp inhibitor treatment would be similar to the CSE effect or not. Isolated naïve and memory T cells from healthy donors were treated with different concentrations of Lyp inhibitor (untreated cells, 0.2µM, 0.5µM and 1µM) for 24 hours and then stimulated with anti CD3/CD28 for 72 hours. The phosphorylation of ZAP-70 (tyr319) was investigated using immunoblotting technique.

0.2µM and 1µM did not show any phosphorylation of ZAP-70 in naïve cells. However, this phosphorylation was supressed at 0.5µM. Memory cells did not show any phosphorylation of ZAP-70 at 0.2µM, 0.5µM and 1µM (Figure 4.12 A+B). The results do suggest that memory cells may respond more to the Lyp inhibitor treatment than naïve cells and therefore increase the activation of ZAP-70. Overall, this result showed that treating naïve and memory cells with Lyp inhibitor could increase the ZAP activity at tyr319 residue. This result also showed similarity to the result from CSE and may suggest that there is a link between CSE treatment and Lyp activity.

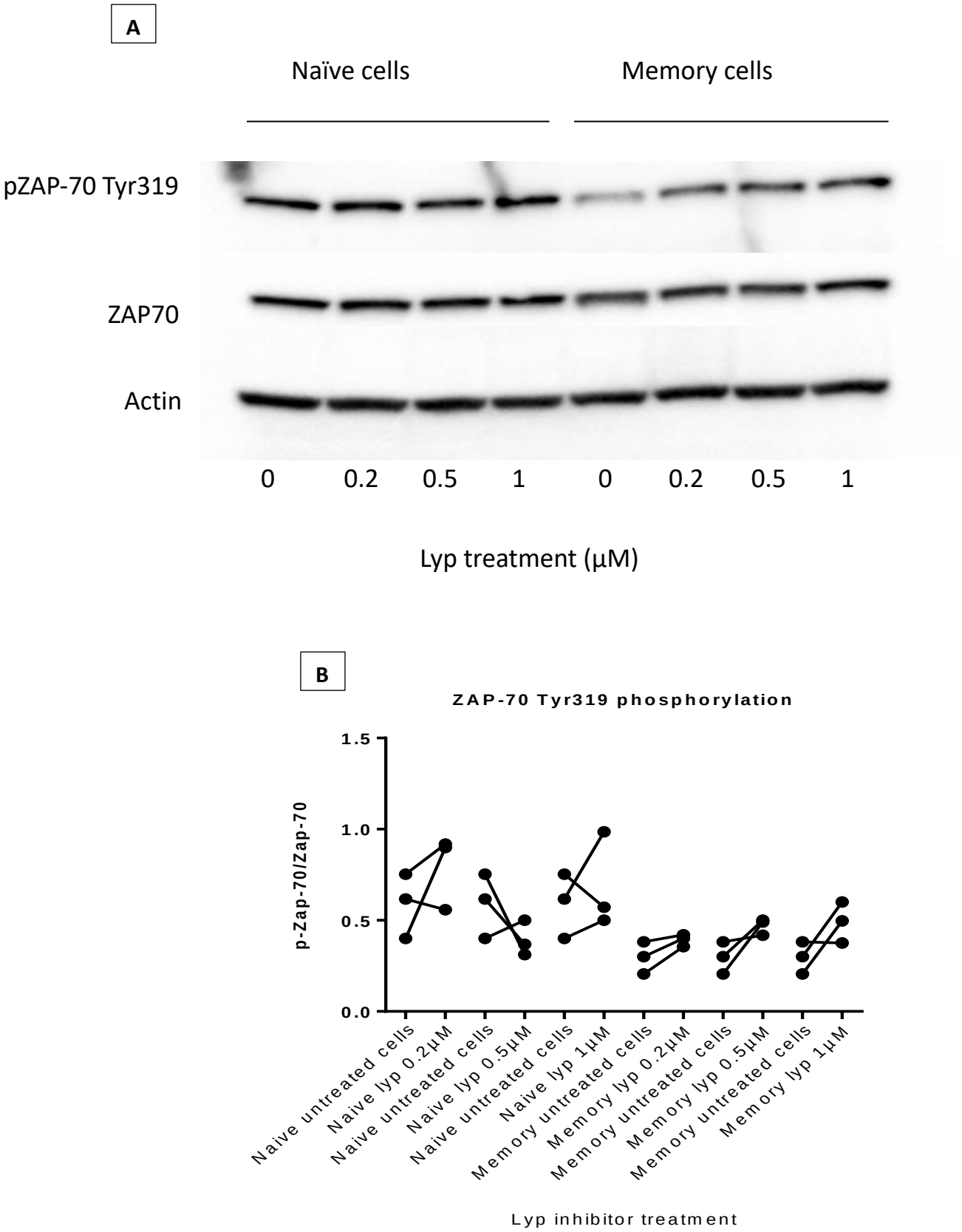


Figure 4. 12 Lyp inhibitor did not increased the phosphorylation of ZAP-70 Tyr319 in naïve and memory cells at low concentration and high concentration.

Naïve and memory cells were treated with Lyp inhibitor for 24 hours and then activated with anti-CD3/CD28 for 72 hours. (A+B) A representative blot and graph of the three independent experiment. (n-3)

4.2.11 Lyp inhibitor did not show phosphorylation of ZAP-70 at Tyr493 in naïve and memory cells

Given the effects of CSE on the phosphorylation of ZAP-70 at tyr493 residue we sought to study whether inhibition of Lyp would show similar effects. It was found that the phosphorylation of ZAP-70 (tyr493) in naïve cells was increased at low concentration 0.2µM of inhibitor, whereas it was decreased at 0.5µM and 1µM. Similarly, it was increased in memory cells at 0.2µM but not at 0.5µM and 1µM (Figure 4.13 A+B). Interestingly, the level of ZAP-70 (tyr493) showed similar pattern to the level of ZAP-70 (tyr319) after Lyp inhibitor treatment, suggesting that inhibition of Lyp could increase the phosphorylation of ZAP-70 and initiate signalling cascades. However, it must be noted that none of the results as expressed reached statistical significance. Nevertheless, the trends in the data from ZAP-70 suggests that there could be a link between CSE and Lyp activity and therefore enhanced the downstream signalling.

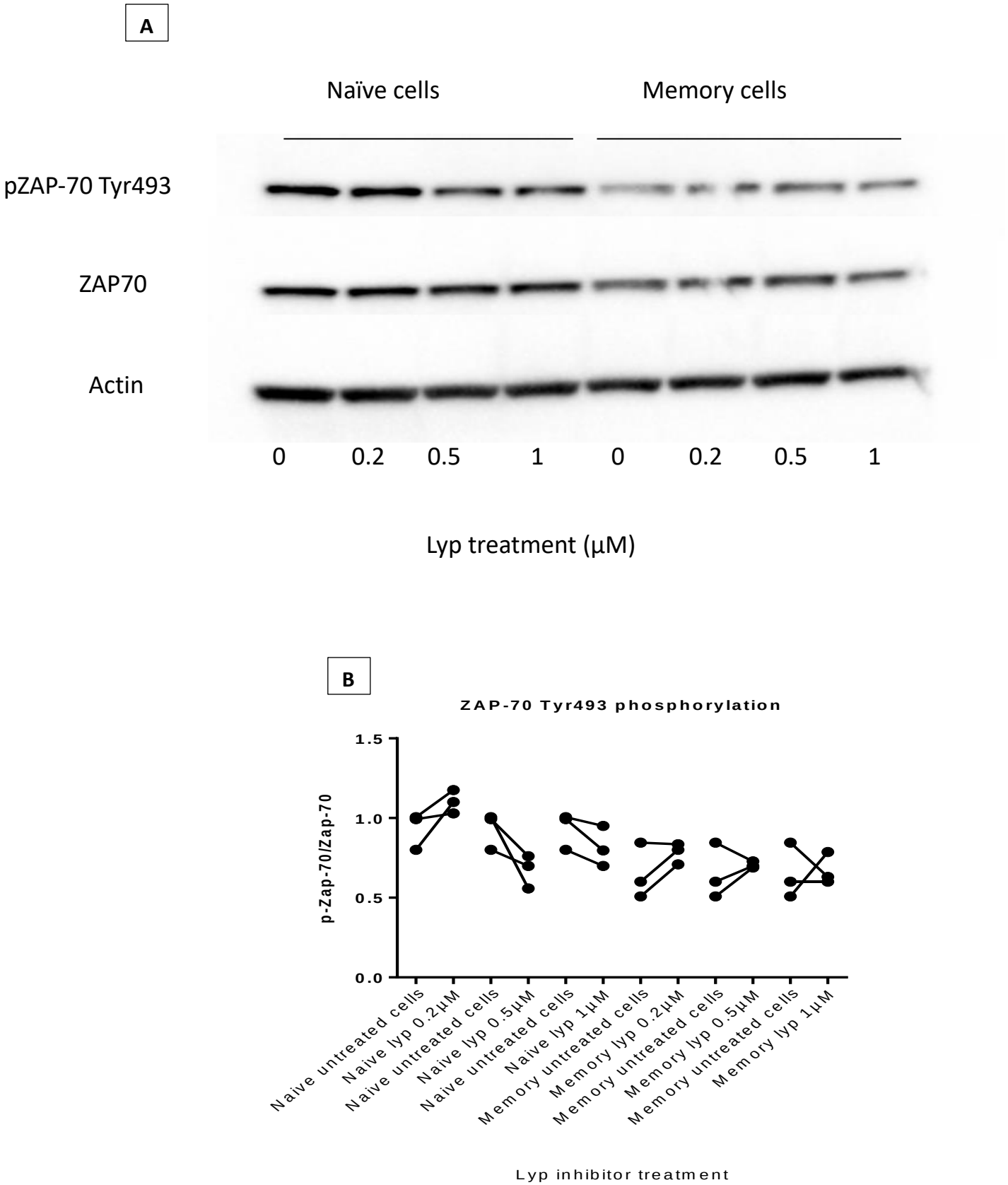


Figure 4. 13 Lyp inhibitor treatment did not increased the phosphorylation of ZAP-70 Tyr493 in naïve and memory cells.

Naïve and memory cells were treated with Lyp inhibitor for 24 hours and then activated with anti-CD3/CD28 for 72 hours. (A+B) representative blot and graph of the three independent experiment. (n=3)

4.2.12 Lyp inhibitor increased Lck phosphorylation at Tyr394 in naïve but not in memory cells

It has been shown that Lck is one of key substrates for Lyp phosphatase. The study showed that there was a direct interaction between them, suggesting that Lyp can control the activation of T cells by controlling Lck activity and other substrates such as Vav1 and ZAP-70 [360]. Based on that and the evidence in literature, we investigated the phosphorylation of Lck at activation site tyr394 to examine whether Lyp inhibitor would have an effect on Lck activity or not. It was found that there was a high expression and phosphorylation of Lck394 in naïve cells at low concentration 2µM of Lyp inhibitor. The phosphorylation was higher and statistically significant at 1µM. However, the activity was less at 0.5µM. Regarding memory cells, there was not any activity at low concentration 0.2µM which is opposite to what observed in naïve. The expression was much higher at 0.5µM but it was decreased at 1µM (Figure 4.14 A+B). This result is consistent with data in the literature which showed that by inhibiting Lyp the Lck activity would increase. Also, it consistent to the data we showed where Lyp inhibitor treatment enhanced ZAP-70 activity and calcium signalling, suggesting that upstream signalling can be also activated following inhibition of Lyp. Since CSE showed similar results, there may be a link between CSE and Lyp perhaps there may be an effect of CSE on Lyp which could lead to T cells hyperactivity.

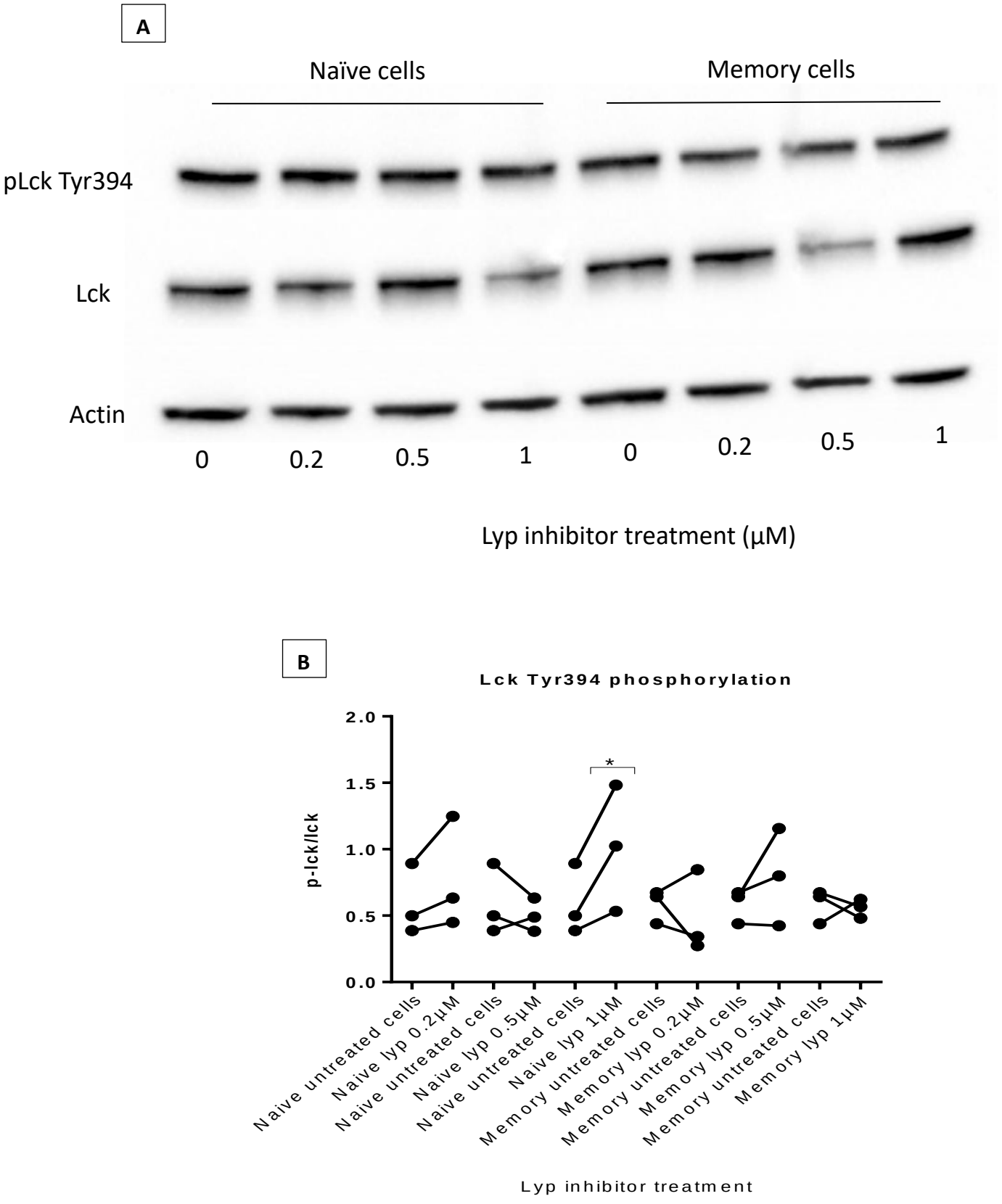


Figure 4. 14 Lyp inhibitor treatment enhanced the phosphorylation of Lck Tyr394 in naïve and memory cells at low and high concentration.

Naïve and memory cells were treated with Lyp inhibitor for 24 hours and then activated with anti-CD3/CD28 for 72 hours. (A+B) A representative blot and graph of the three independent experiment. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=3)

4.2.13 Lyp inhibitor did not show any effect on Lck phosphorylation at Tyr505 in naïve and memory cells

After investigating the effect of Lyp inhibitor treatment on the activation loop of Lck, we investigated the effect of Lyp inhibitor on the phosphorylation of negative regulatory loop tyr505 of Lck. In contrast to our observation that Lck394 phosphorylation was increased following Lyp inhibitor treatment, it was found that the phosphorylation of Lck505 in both naïve and memory was not changed (Figure 4.15 A+B). This result is consistent with the literature where Lyp could have a direct effect on Lck. Also, this result is similar to the result we showed where CSE showed no changes in the phosphorylation of Lck505, and enhanced Lck394.

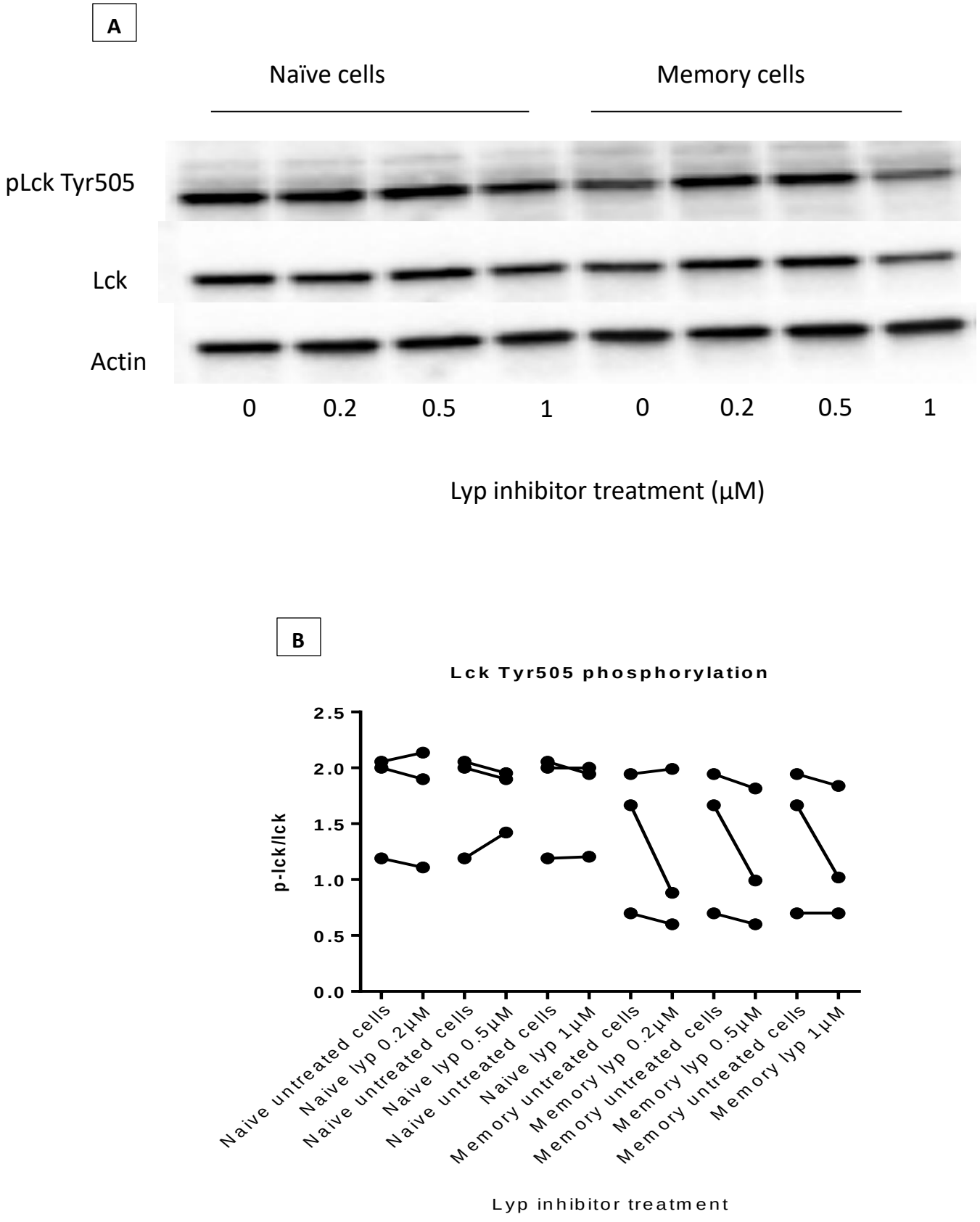


Figure 4. 15 Lyp inhibitor treatment did show any effect on Lck Tyr505 in naïve and memory cells.

Naïve and memory cells were treated with Lyp inhibitor for 24 hours and then activated with anti-CD3/CD28 for 72 hours. (A+B) A representative blot and graph of the three independent experiment. (n=3)

4.2.14 Lyp inhibitor increased Vav1 phosphorylation at Tyr174 in naïve and memory cells.

Since Vav1 being a substrate for Lyp, and required to induce Akt phosphorylation through TCR; we investigated the effect of Lyp inhibitor on Vav1 at activation loop 174. It was found that Vav1 phosphorylation in naive was increased at 2µM, but the expression was much higher at 0.5µM and 1µM with a significant increase at 1µM. The similar trend was found in memory. The Vav1 expression was increased at 2µM, but the expression was much higher at 0.5µM and 1µM, although it was not significant (Figure 4.16 A+B). In the previous results, we showed that Lyp inhibitor increased the activity of Lck and ZAP-70 at positive loop as well as calcium signalling. Now we showed that by inhibiting Lyp, the phosphorylation of Vav1 increased which gives a strong link that Lyp could control the activity of these substrates. This suggests that Vav1 phosphorylation could activate upstream signalling leading to activation of downstream signalling.

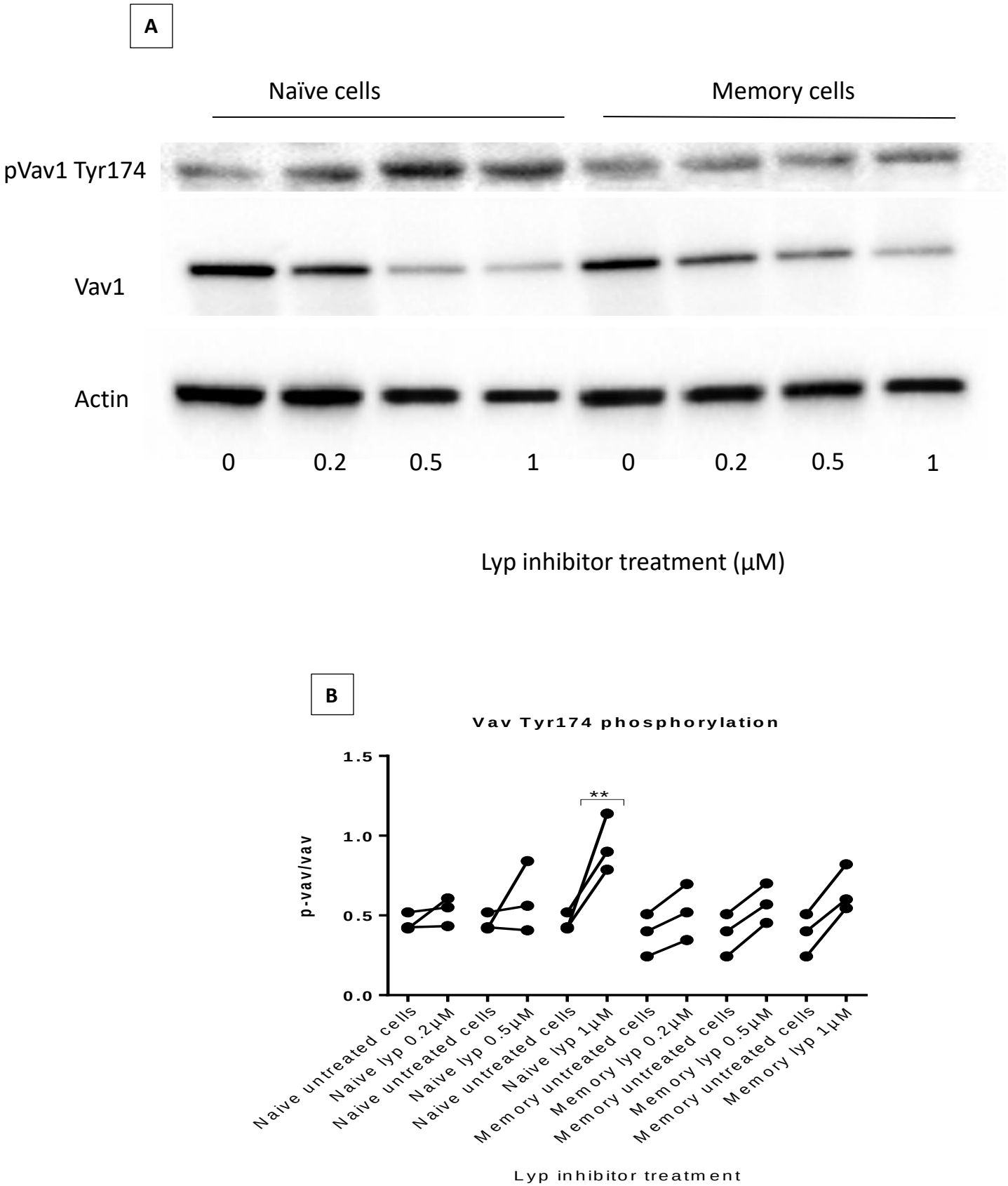


Figure 4. 16 Lyp inhibitor enhanced the phosphorylation of Vav1 Tyr174 in naïve and memory cells.

Naïve and memory cells were treated with Lyp inhibitor for 24 hours and then activated with anti-CD3/CD28 for 72 hours. (A+B) A representative blot and graph of the three independent experiment. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=3)

4.3 Discussion

Cigarette smoking is a major risk factor for developing several serious diseases and one of them is RA. Using in vitro experiment, we exposed naïve and memory T human $CD4^+$ cells to CSE and Lyp inhibitor then stimulated them with anti CD3/CD28. We found that both CSE and Lyp inhibitor induced the phosphorylation status of ZAP-70, Lck, Vav1 and calcium signalling. Also, CSE and Lyp inhibitor treatment successfully decreased the activity of Lyp phosphatase activity which may suggest that CSE could interfere with Lyp function and therefore promote metabolism and signalling pathway.

The underlying mechanisms by which cigarette smoking perturb immune functions and so promote RA development is not fully elucidated. Therefore, several targets were investigated and the PI3K/AKT/mTOR pathway is the main pathway which promotes metabolism in response to extracellular signals. One of the most important upstream targets is Lck which plays an essential role in signal transduction from TCR. It has been shown that alteration of Lck expression could promote several conditions including RA and diabetes [361]. Since we showed that CSE increased the activity of Lck at 394 residue, an interesting study showed that there may be a direct link between Lck and Akt. The study found that Lck in response to hydrogen peroxide induced the phosphorylation of Akt at T308, suggesting that Lck might have a direct effect on Akt which play a role in metabolism [362]. Apart from CSE, we showed also that Lyp inhibitor enhanced the phosphorylation of Lck, ZAP-70 and Vav1 at activation loops due to the inhibition of Lyp function. To support this claim, a study on human T cells found that Lyp and CSK are forming a complex to negatively regulate TCR signalling, and by testing a potent specific chemical Lyp inhibitor, the study found that the activity of Lyp was inhibited. This followed by an increase in the phosphorylation of Lck394,

ZAP70, calcium signalling and activation marker CD25, suggesting that Lyp could be a therapeutical target to treat autoimmunity [363]. Interestingly, another study showed similar result and was consistent to our results. using mass spectrometry, it was found that PTPN22 has novel potential substrates, including ZAP-70, Lck and Vav1. Also, the study found that PTPN22 showed a specific direct interaction with Lck and ZAP-70, suggesting that mutant PTPN22 could increase the activity of these substrates [280]. The increase in these substrates may lead to the activation of downstream signalling.

Although some studies indicated that cigarette smoking could increase inflammatory response by increasing the signalling of intracellular calcium, the exact mechanism remains unclear. Recent study pointed out that nicotine or total particular matter (TPM) from cigarette, significantly enhanced Ca^{2+} dependent signalling pathway in HL60 cell line, leading to the alteration of leukocyte inflammatory response [364]. Furthermore, a study reported that CSE had a direct effect on store operated Ca^{2+} entry (SOCE) and cell proliferation by enhancing Ca^{2+} responses to SOCE. This was mediated by increased the expression of *ORAI1* (ORAI Calcium Release-Activated Calcium Modulator 1) and STIM1 (Stromal Interaction Molecule 1), a Ca^{2+} sensor that activates CRAC channels and migrates from the Ca^{2+} store to the plasma membrane. However, when siRNAs were used against these proteins, the effect of CSE was attenuated [365]. Another finding found that CSE induced cell damage by activating protein kinase C (PKC) and NADPH oxidase (NOX), suggesting that CSE may trigger cell damage through Ca^{2+} dependent intracellular signalling pathway by activating NOX and PKC [366]. The chronic exposure of rats to CSE showed that T cells exhibited an activation of PLC-gamma1 which activated by ZAP-70, and involved in Ca^{2+}

signalling activation, suggesting that CSE may play a role in Ca^{2+} activation through activation of ZAP-70 and PLC-gamma1 [367].

In conclusion. We found that CSE increased the Ca^{2+} release through stimulation of TCR in both naïve and memory cells, and the phosphorylation of ZAP-70, Lck, Vav1. Furthermore, ZAP-70, Lck, Vav1 and calcium signalling were also increased following Lyp inhibitor treatment. Moreover, CSE and Lyp inhibitor decreased the activity of Lyp phosphates activity. This means CSE and Lyp inhibitor can affect Lyp function and consequently affect the known targets of Lyp such as ZAP-70, Lck, Vav1.

The main limitation in this chapter is that some data showed no significance, although there were trends. This because the number of the experiments performed was low not more than three or four independent experiments which would not allow subtle effects to reach significance. Also, the variability of blood cones, being from unknown donors, is an issue, so it can affect the consistency of the result. So, it would better if we could increase the number of the experiments which might reach significance even with variable donors. Another limitation is that we did not investigate other phosphatases such as CD45 which also regulates Lck. This could reveal whether the Lyp inhibitor has off target effects or not, and whether CSE could affect CD45 and show similar results to that we showed when we measured Lyp activity. If so, it means CSE could affect both Lyp and CD45 at the same time.

In this chapter we investigated the effect of CSE and Lyp inhibitor on immediate TCR signalling, so in the next chapter, the energy metabolism including glycolysis and oxidative phosphorylation will be assessed following CSE and Lyp inhibitor treatment to investigate whether these treatments would change the energy metabolism and related signalling pathway including Akt, AMPK and mTORC.

Chapter 5:

Effects of cigarette smoke extract and Lyp inhibitor on energy metabolism of primary CD4+ naïve and memory T cells

5.1 introduction

Environmental factors can alter the function of the immune system and have also been strongly implicated in the development of RA and other autoimmune conditions. As laid out in the introduction, some of these factors associated with RA are lifestyle, ageing, previous infection and cigarette smoke (CS) [53]. CS contains harmful components such as carbon monoxide, nicotine and ROS which activate inflammatory responses and damage the DNA and proteins in cells [368]. CS also consumes or suppresses antioxidant activity which may allow significant impairment of cellular function and induction of inflammation.

Glycolysis and oxidative phosphorylation are critical in supporting immune responses. They both generate ATP which supports cellular functions allowing cells to proliferate and differentiate. It has long been known that PTP are critical in regulating the signalling pathways involved in immune cell responses, but more recent studies showed that PTPs could also play a role in metabolism by regulating food intake and homeostasis [369, 370]. For example, phosphatase and tensin homolog (PTEN) is a phosphatase that plays a role in regulating PI3K/Akt signalling (a signalling that regulates metabolism and biological processes) [371]. PTEN dephosphorylates PIP3, leading to regulate insulin and leptin signalling which control energy homeostasis and energy intake [372-374]. Therefore, inactivation of PTPs could increase metabolism and also impair the regulation of signalling in the cell as well as increase insulin sensitivity. Thus, if CS has an effect on PTP function, it could as a result exacerbate changes in metabolism which could lead to development of autoimmune diseases. There are numerous studies which have investigated the effect of cigarette smoking on the function of T cells. These studies were done using either in vitro experiments by exposing T cells to CSE or after the isolation the cells from smokers. In vitro

studies are more appropriate to study the changes of underlying mechanisms that may occur in cells. Based on that, we use the in vitro experiments to investigate the changes of energy metabolism in immune cells isolated from healthy human donors.

Activated T cells rely on energy metabolism to orchestrate the action of other immune cells and to protect against pathogens. In response to activation, T cells switch their metabolism from oxidative phosphorylation to glycolysis to support cytokine production, proliferation and cell growth. It has been shown that the T cells subtypes $CD4^+$ T in blood were higher in number in smokers than non-smokers. Also, memory cells were positively and significantly higher in number in smokers, suggesting that lymphocytes might be sensitive to smoking cumulative effect [170]. We hypothesised that exposure of $CD4^+$ T cells to cigarette smoke would alter their metabolism to support this increase in numbers and function and ultimately the development of autoimmunity.

Preliminary data from our research group suggested that inhibition of Lyp enhanced extracellular acidification rate (ECAR) in human peripheral blood T cells (PBT). Since this is a measure of glycolysis this suggests that Lyp and thus cigarette smoke may have effects on cellular metabolism. T cells have been shown to be highly dependent on glycolysis for their functions and so these preliminary results and the background data on smoking and PTPN22 support the hypothesis that smoking might act directly on Lyp to affect signalling and metabolism and thus immune cell function (Figure 5.1). In this chapter we investigated the role of CSE and Lyp inhibitor in inducing perturbation in energy metabolism in immune cells to promote RA. To do this, $CD4^+$ naïve and memory T cells were exposed to CSE and Lyp inhibitor treatment and stimulated with anti CD3/CD28 in tissue culture and their responses investigated. In the previous chapter we showed that CSE and Lyp inhibitor treatment

changed and increased the calcium signal as this shows an overall increase in TCR signal, and the phosphorylation status of the known targets of PTPN22 such as ZAP-70, Lck and Vav1 in human $CD4^+$ T cells (naïve and memory), suggesting that smoking might change immediate TCR signalling and thus promoting T cell activation. In this chapter we build on this to investigate signalling pathways related to metabolism following CSE exposure. The phosphorylation status of the known targets of metabolism such as Akt, AMPK and S6 (mTORC1 substrate) were investigated to further examine whether CSE and Lyp inhibitor would induce changes in metabolism. Following that, energy metabolism including glycolysis and oxidative phosphorylation were investigated to assess the changes in metabolism in immune cells and how this may contribute to RA development. Mitochondrial mass was assessed using mitotracker staining to investigate any such changes in mitochondrial function following CSE treatment.

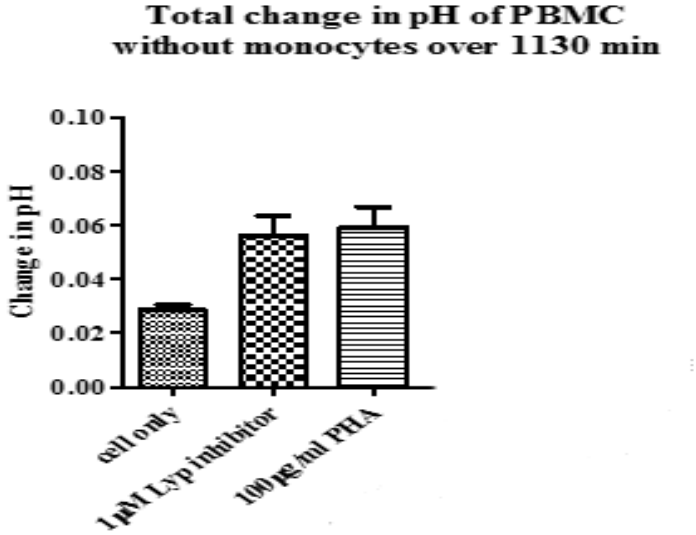


Figure 5. 1 Inhibition of Lyp enhanced extracellular acidification rate (ECAR) in human PBT cells.

Cells were incubated with Lyp inhibitor or PHA and then BCECF pH sensitive dye was added and the change in fluorescence assessed over time. The pH was taken from a standard curve of BCECF in different pH buffers (X. Xuexin and SP Young, unpublished)

5.2 Results

5.2.1 CSE did not showed an expression of Akt phosphorylation at Thr308 in naïve and memory cells

Akt is a master regulator of cell metabolism, and it plays a significant role in survival and proliferation. It phosphorylates and activates mTORC1 and its substrates such as ribosomal protein S6 kinase which is also play a crucial role in energy metabolism. Activation of Lck and ZAP-70 lead to initiate upstream signalling which triggers activation of PDK1 which in turn phosphorylates Akt at threonine (T308), the activation loop of Akt which leads to a full or partial activation of Akt. However, this activation can be controlled by a phosphatase called PTEN and prevent human diseases [375]. It has been reported that smoking could significantly phosphorylate Akt at T308 which is important step to phosphorylate downstream targets, leading to cancer formation [376]. A study showed that T308 phosphorylation is more reliable biomarker for assessing the activity of Akt than Serine (Ser473) which is also play a role in Akt activity [377]. Thus, based on the evidence in literature, we investigated the effect of CSE on Akt activity using T308 antibody to elucidate whether CSE would increase the activity of Akt and gives rise of downstream targets.

Following anti CD3/CD28 stimulation and CSE treatment, pAkt T308 showed an expression at low concentration 2% in both naïve and memory, but there was no significant increase. The Akt did not show a phosphorylation at 15% but the level was decreased at 30% in both naïve and memory T cell. 15% and 30% CSE did not show any change (Figure 5.2 A+B). This may suggest that the upstream signalling that was activated by CSE may led to the activation of downstream signalling. This may help to understand the underlying mechanisms by which CSE could play a role in inducing perturbation in signalling pathways and energy metabolism in primary human T cells.

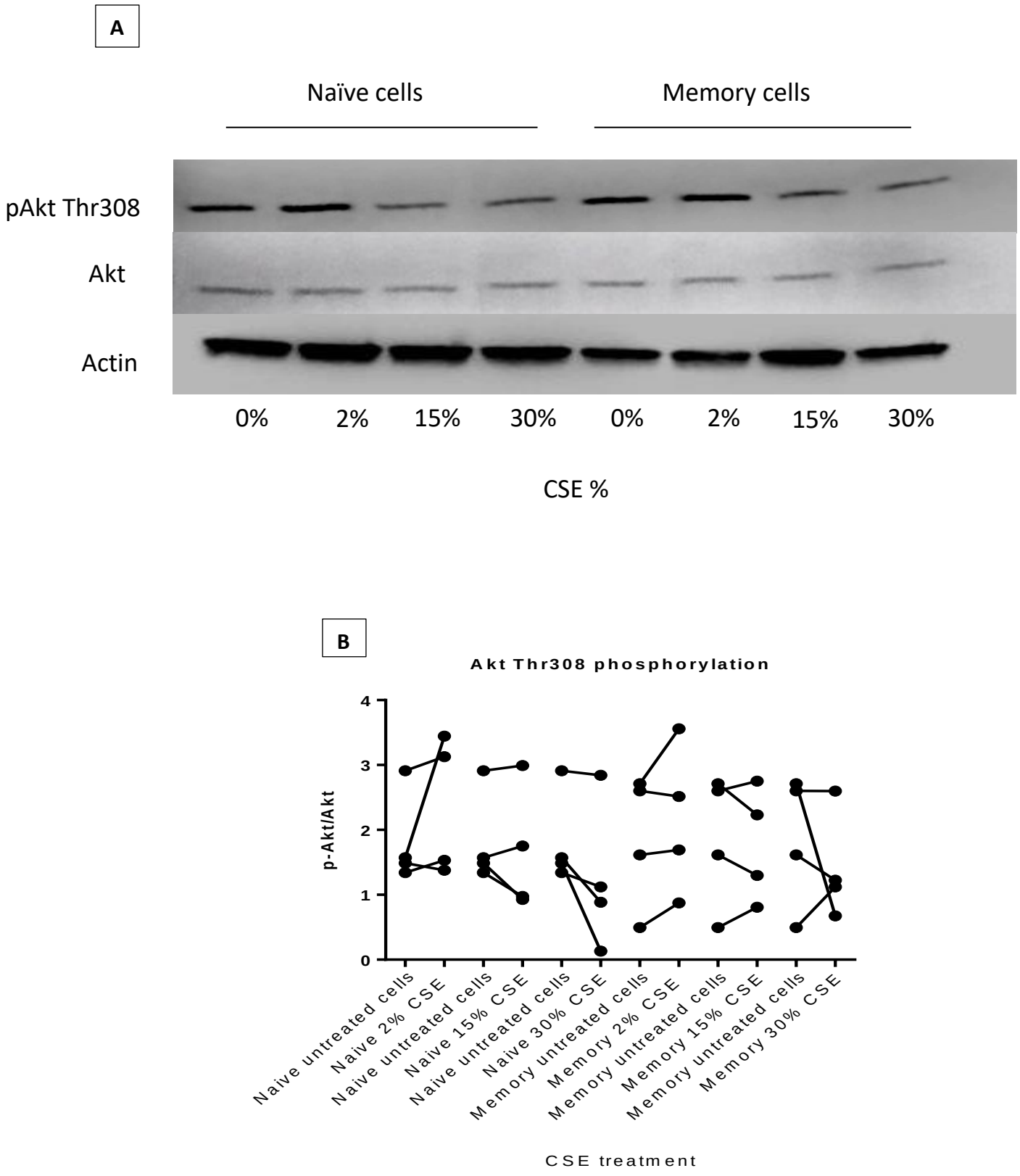


Figure 5. 2 CSE did not show an expression in Akt Thr308 in naïve and memory cells.

Naïve and memory cells were treated with CSE for 24 hours and then activated with anti-CD3/CD28 for 72 hours. (A+B) A representative blot and graph of the four independent experiment. (n=4)

5.2.2 CSE increased S6 phosphorylation at Ser235/236 in naïve but not in memory cells.

Since the effect of CSE on signalling pathways and metabolism is not fully understood, we continued to investigate the effect of CSE on some important downstream targets such as the marker for mTORC1 activity, ribosomal protein S6 kinase. S6 is mTORC1 substrate which involves in cell proliferation and glucose homeostasis. It has many activation sites in C terminus, Ser235/236 and Ser240/244 which are play a role in mTORC1 activation. It has been shown that phosphorylation of S6 at activation residue Ser235/236 enhanced the activity of ATP derivative cAMP , which is involved in metabolism [378]. So, we used a Ser235/236 antibody to assess the phosphorylation status of S6 following CSE treatment and anti CD3/CD28 stimulation.

It was found that there was an expression of S6 at Ser235/236 in naïve cells following 2% and 15%, but the phosphorylation at 30% was much higher and significant. On the other hand, the phosphorylation of S6 in memory cells was decreased following 2% but there was an expression at 15% and 30% but was not significant. (Figure 5.3 A+B). Surprisingly, the effect of low concentration 2% was limited, whereas the high concentration 30% showed an effect on S6 which is opposite to what we showed in previous results from ZAP-70, Lck, Vav1 and Akt where 2% was the highest. This may give an indication that the low concentration of CSE could have an effect on upstream targets, whereas high concentration could have an effect on downstream targets.

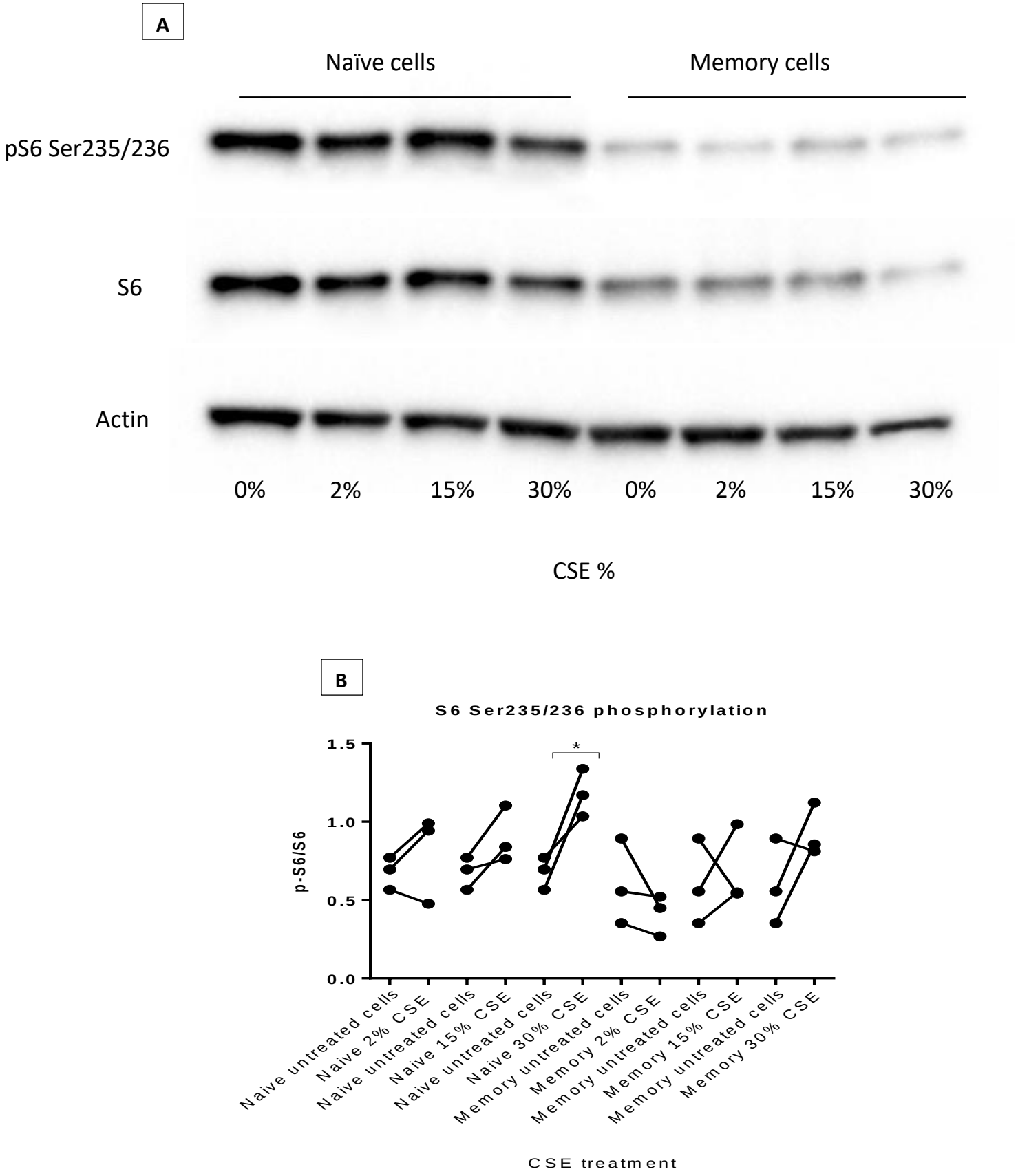


Figure 5. 3 CSE enhanced the phosphorylation of S6 Ser235/236 in naïve but not in memory cells.

Naïve and memory cells were treated with CSE for 24 hours and then activated with anti-CD3/CD28 for 72 hours. (A+B) A representative blot and graph of the three independent experiment. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=3)

5.2.3 CSE increased AMPK phosphorylation at Thr172 in memory cells but not in naïve cells.

AMPK is one of the most important molecules in metabolic signalling. It modulates the metabolism based on nutrient availability by increasing ATP generation and decrease ATP consumption [379]. AMPK has an activation loop at Thr172, so phosphorylation at this site is required for AMPK activation. To support this claim, a study reported that the phosphorylation of AMPK at Thr172 induced the overall activity of AMPK. However, in cell lacking Thr172, the activity of AMPK was undetectable [380]. Therefore, based on the evidence and since the AMPK is a key player in metabolism, we investigated the effect of CSE on AMPK activity using Thr172 antibody to elucidate whether CSE has an effect on AMPK activity or not.

It was found that the there was no expression of AMPK phospho-Thr172 in naïve following 2%, 15%, 30% CSE exposure. The memory cells showed an expression of AMPK phospho-Thr172 following 2% and 15% but it was not significant. However, the expression was much higher and significant following 30% (Figure 5.4 A+B). Similar to S6, AMPK showed high phosphorylation at higher concentrations. This may support the claim that the high concentration of CSE could increase the activity of downstream signalling, whereas the low concentration could have an effect on upstream targets.

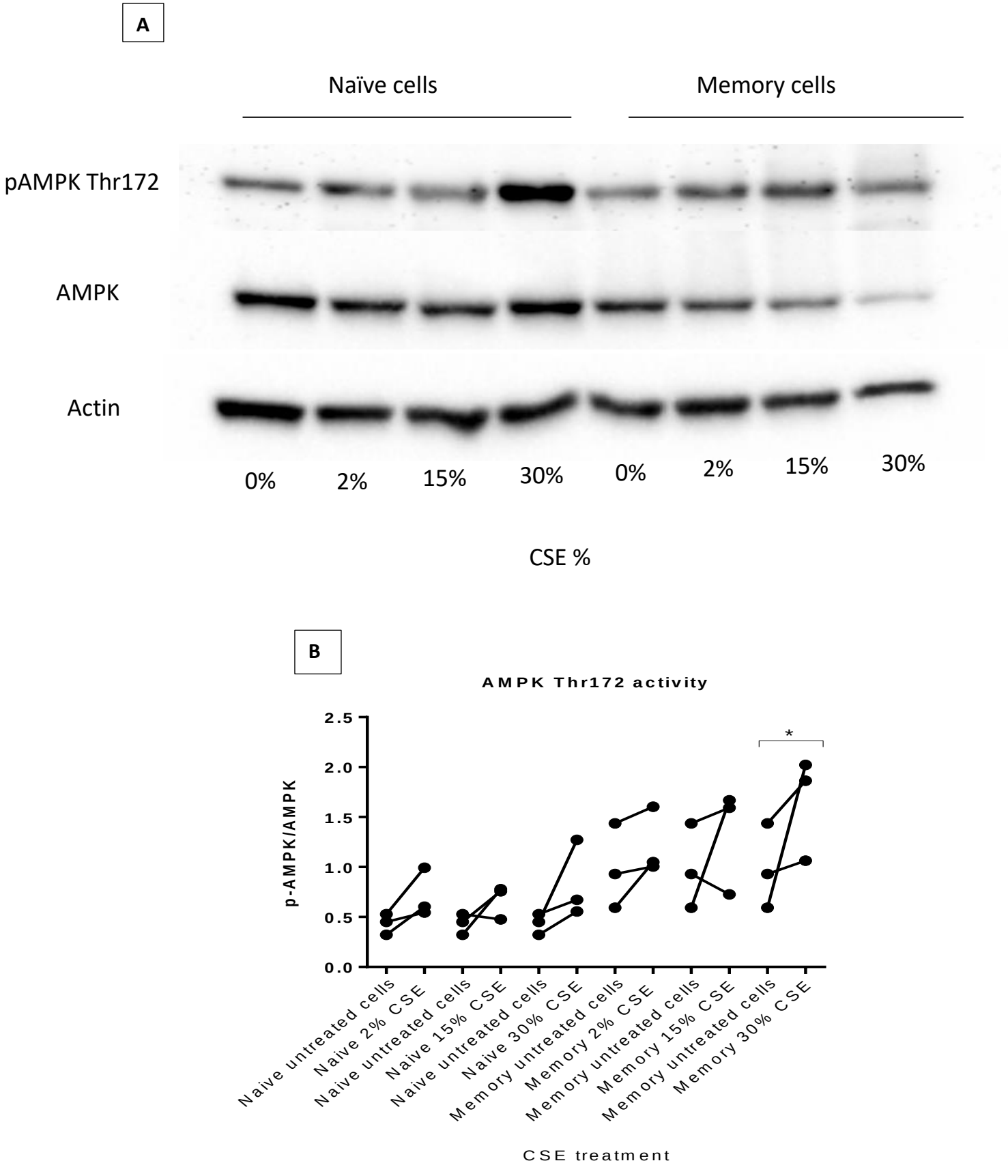


Figure 5. 4 CSE enhanced the phosphorylation of AMPK Thr172 in memory cells at high concentration but not in naïve cells.

Naïve and memory cells were treated with CSE for 24 hours and then activated with anti-CD3/CD28 for 72 hours. (A+B) A representative blot and graph of the three independent experiment. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=3)

5.2.4 Lyp inhibitor increased Akt phosphorylation at Thr308 at low concentration in naïve but not in memory cells

Since our hypothesis focuses also on PTPN22 which encodes Lyp, we investigated how does interfere with PTPN22 could give rise to energy metabolism alteration through modulation of Akt. In the previous result, we investigated the effect of CSE on Akt phosphorylation and we found that CSE induced Akt phosphorylation. Therefore, it is worthwhile investigating the effect of Lyp inhibitor on Akt activity at T308 residue. Naïve and memory cells were treated with different concentrations of PTP LYP inhibitor (untreated cells, 0.2µM, 0.5µM and 1µM) to investigate whether inhibiting Lyp would increase Akt activity and show similar effect to CSE. It was found that there was an expression of Akt in naïve cells following 0.2µM, but the expression was much higher and significant at 0.5µM. However, there was no effect at 1µM. On the other hand, memory cells did not show altered expression of Akt at 0.2µM, 0.5µM and 1µM inhibitor. (Figure 5.5 A+B). This shows similarity to the result we showed in CSE, which may suggest that there might be an effect of CSE on Lyp activity which led to increase in the phosphorylation of Akt.

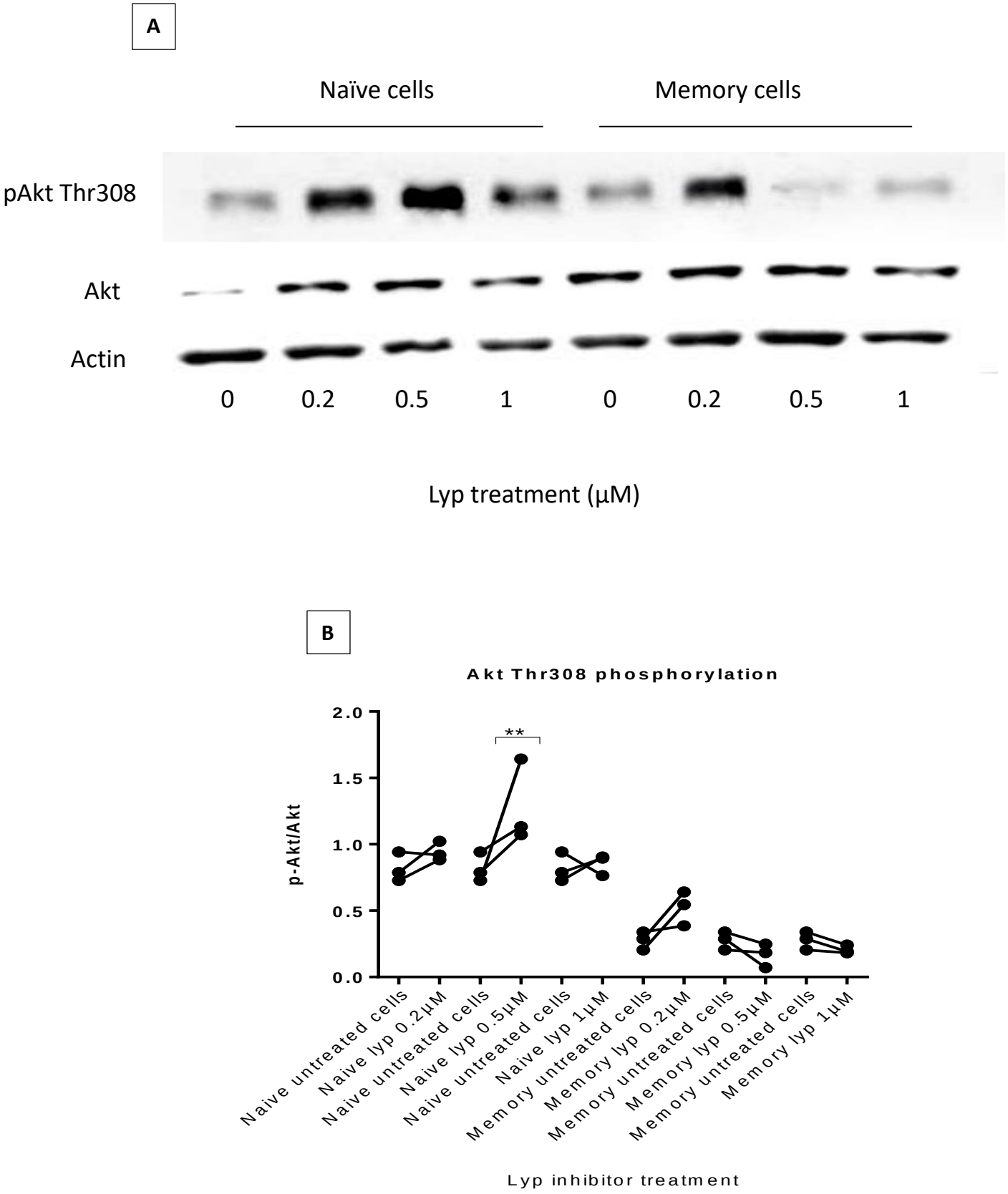
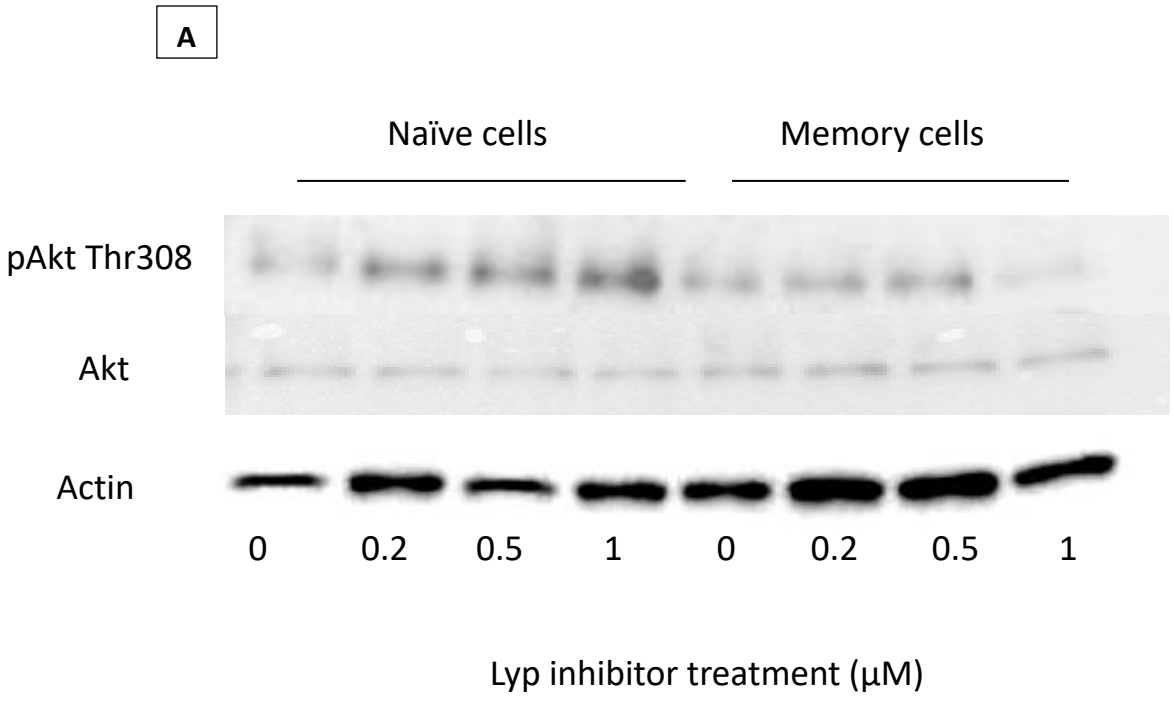


Figure 5. 5 Lyp inhibitor enhanced the phosphorylation of Akt Thr308 in naïve but not in memory cells

Naïve and memory cells were treated with Lyp inhibitor for 24 hours and then activated with anti-CD3/CD28 for 72 hours. (A+B) A representative blot and graph of the three independent experiment. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=3)

5.2.5 Lyp inhibitor increased Akt phosphorylation at Thr308 in naïve but not in memory cells following stimulation with PMA/Ionomycin

To investigate if there is a direct effect of Lyp on Akt and not only through TCR signal, we explored the effect of Lyp inhibitor on Akt by stimulating cells with PMA/Ionomycin which provides potent stimulation in order to bypass TCR following T cells activation. This will allow us to clarify if Lyp can have both direct and indirect effect on Akt. Naïve and memory were treated with Lyp inhibitor and stimulated with PMA/Ionomycin. It was found that the phosphorylation of Akt was not changed at 0.2µM and 0.5µM. However, the phosphorylation of Akt was increased and the expression was much higher and significant at 1µM compared to control. On the other hand, memory cells did not show any effect at 0.2µM, 0.5µM and 1µM. (Figure 5.6 A+B). The phosphorylation of Akt following Lyp inhibitor was increased after stimulation with both anti CD3/CD28 and or PMA/Ionomycin, suggesting that Lyp could have a direct effect also on Akt which may lead to increase in metabolism bypassing TCR.



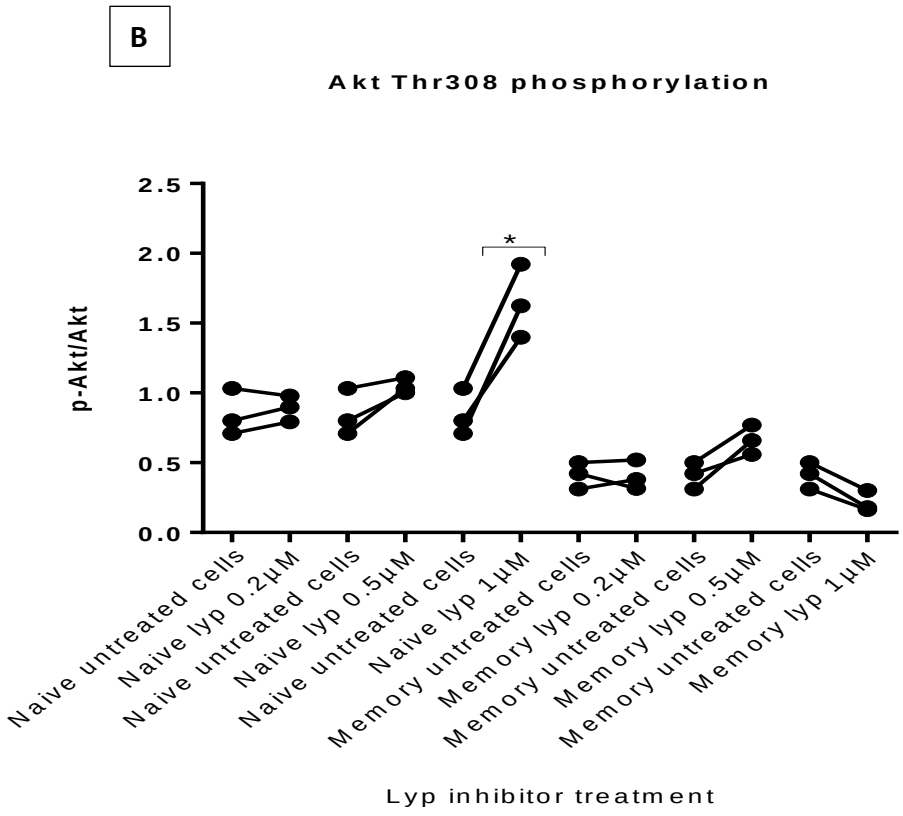


Figure 5. 6 Lyp inhibitor enhanced the phosphorylation of Akt Thr308 in naïve but not in memory cells.

Naïve and memory cells were treated with Lyp inhibitor for 24 hours and then activated with PMA/ionomycin. (A+B) A representative blot and graph of the three independent experiment. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=3)

5.2.6 Naïve cells treated with PBS did not show any changes or effects on basal ECAR, glycolysis, glycolytic capacity and glycolytic reserve.

To ensure that the changes in metabolism or the signalling pathway are not due to the dilution of the medium or PBS, naïve was treated with PBS as a control at different concentrations of PBS (0, 0.5%, 1%, 2%, 5%, 10% and 30%) and then stimulated with anti CD3/CD28 for 72 hours. Seahorse was used to assess the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) and their related parameters. This was repeated in three independent experiments using different blood donors.

The representative graph (Figure 5.7 A) is showing the changes in all parameters after adding inhibitors. It was found that the level of basal ECAR in naïve cells dropped at 0.5%, 1%, 2%, 5%, 10% and 30% compared to untreated cells after PBS treatment (Figure 5.7 B). Glycolysis was increased compared to the basal ECAR, but the levels were still below untreated control cells which means there is no significant effect shown (Figure 5.7 B+C). Both glycolytic capacity and glycolytic reserve showed similar effect to basal ECAR and glycolysis in that there were no changes in the levels following PBS treatment (Figure 5.7 D+E). This showed that there were not any significant changes in ECAR parameters following PBS treatment in naïve cells and the levels were not affected by medium or PBS concentrations.

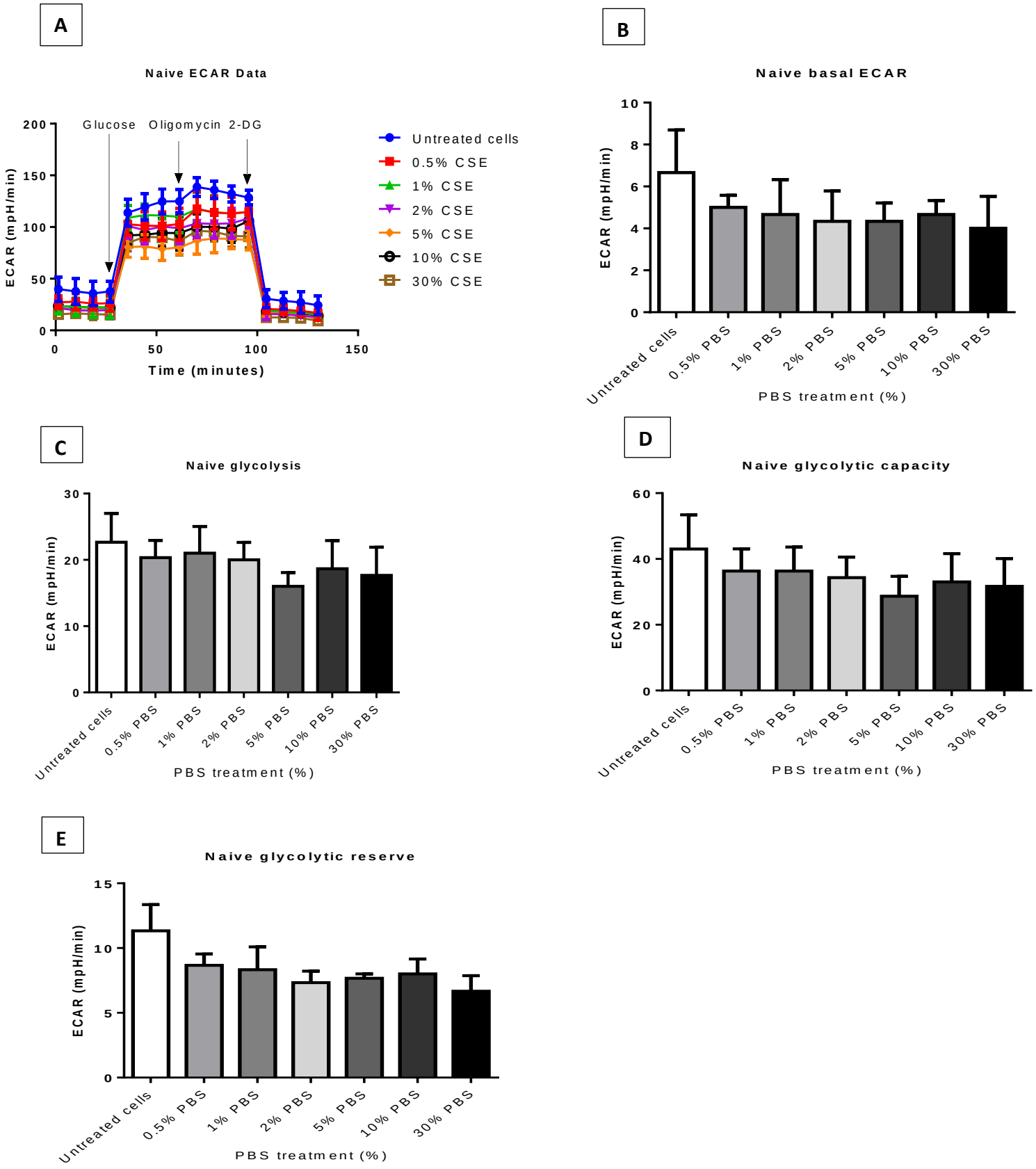


Figure 5. 7 PBS treatment did not show any effect on ECAR and other parameters such as glycolysis, glycolytic capacity and glycolytic reserve in naïve cells.

Naïve cells pre-stimulated with different concentrations of PBS for 24 hours with subsequent activation using anti-CD3/CD28 for 72 hours. A representative graph is shown from one of the three independent experiment. (n=3)

5.2.7 Naïve cells treated with PBS treatment did not show any changes or effect on basal OCR, ATP-linked respiration, maximal respiration and spare capacity.

The representative graph (Figure 5.8 A) is showing the changes in all parameters after adding inhibitors. Basal OCR showed similar result to ECAR in that PBS treatment did not show any effect and there were not any significant changes, except at 0.5% and 2% where we saw a big reduction (Figure 5.8 B). ATP-linked respiration, maximal respiration and spare capacity also showed a reduction in their levels with no significant changes compared to control cells (Figure 5.8 C+D+E), suggesting that there were not any significant changes in OCR parameters following PBS treatment and the levels were not affected by medium or PBS concentrations.

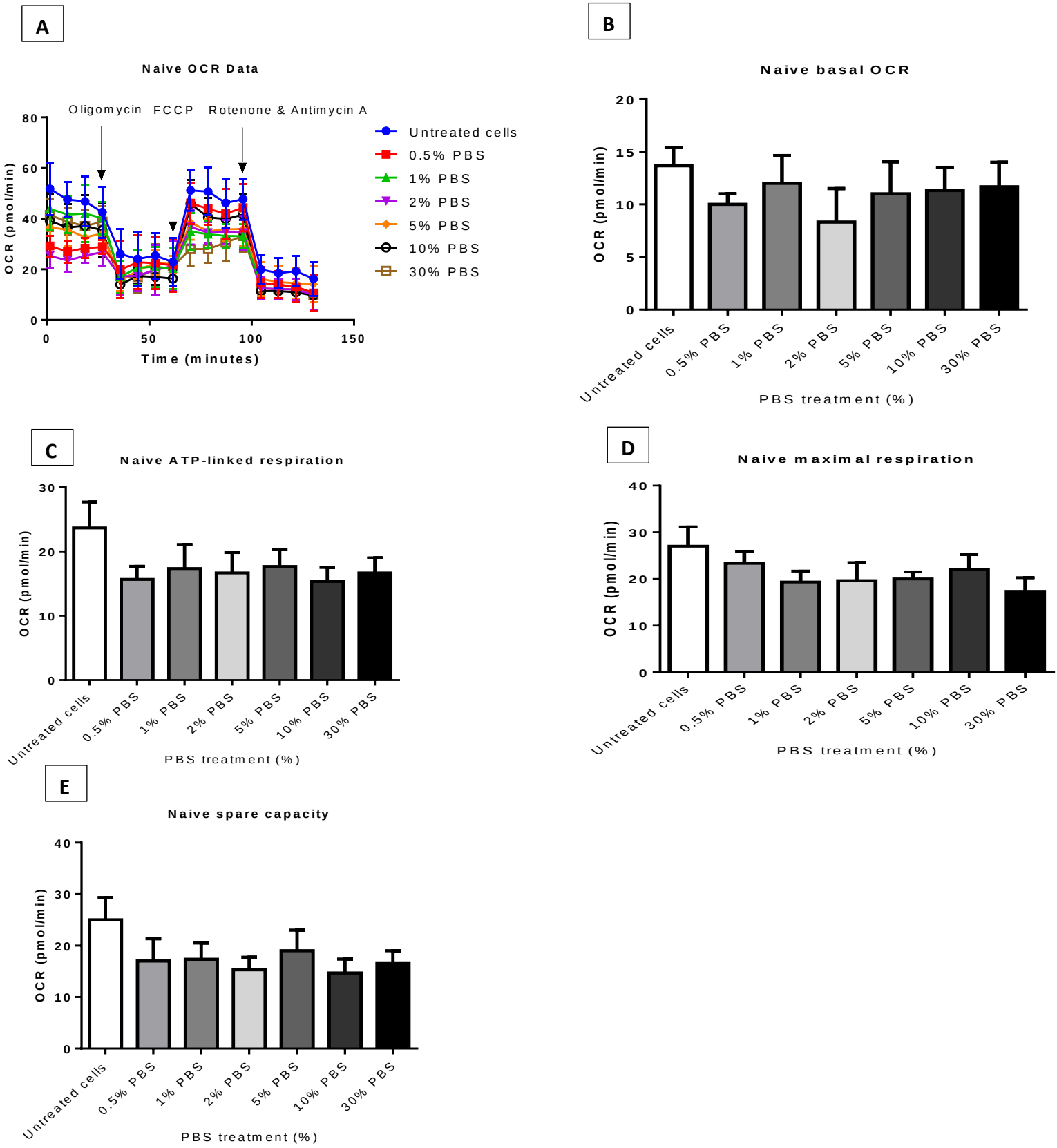


Figure 5. 8 PBS treatment did not show any effect on OCR and other parameters such as ATP-linked respiration, maximal respiration and spare capacity in naïve cells.

Naïve cells pre-stimulated with different concentrations of PBS for 24 hours with subsequent activation using anti-CD3/CD28 for 72 hours. A representative graph is shown from one of the three independent experiment. (n=3)

5.2.8 Memory cells treated with PBS treatment did not show any changes or effects on ECAR, glycolysis, glycolytic capacity and glycolytic reserve.

The representative graph (Figure 5.9 A) is showing the changes in all parameters after adding inhibitors. Similar to naïve cells, memory cells were treated with PBS as a control with the same concentrations we used for CSE (0, 0.5%, 1%, 2%, 5%, 10% and 30%) and then stimulated cells with anti CD3/CD28 for 72 hours. Seahorse was used to assess the ECAR and OCR and related parameters. This was repeated in three independent experiments using different blood donors. It was found that the basal ECAR was decreased after PBS treatment (Figure 5.9 B). The glycolysis, glycolytic capacity and glycolytic reserve showed no effect as well compared to control. All these levels were below the control and no significant changes were observed (Figure 5.9 C+D+E). This result indicated that there were not any significant changes in ECAR parameters in memory cells following PBS treatment and the levels were not affected by medium or PBS concentrations.

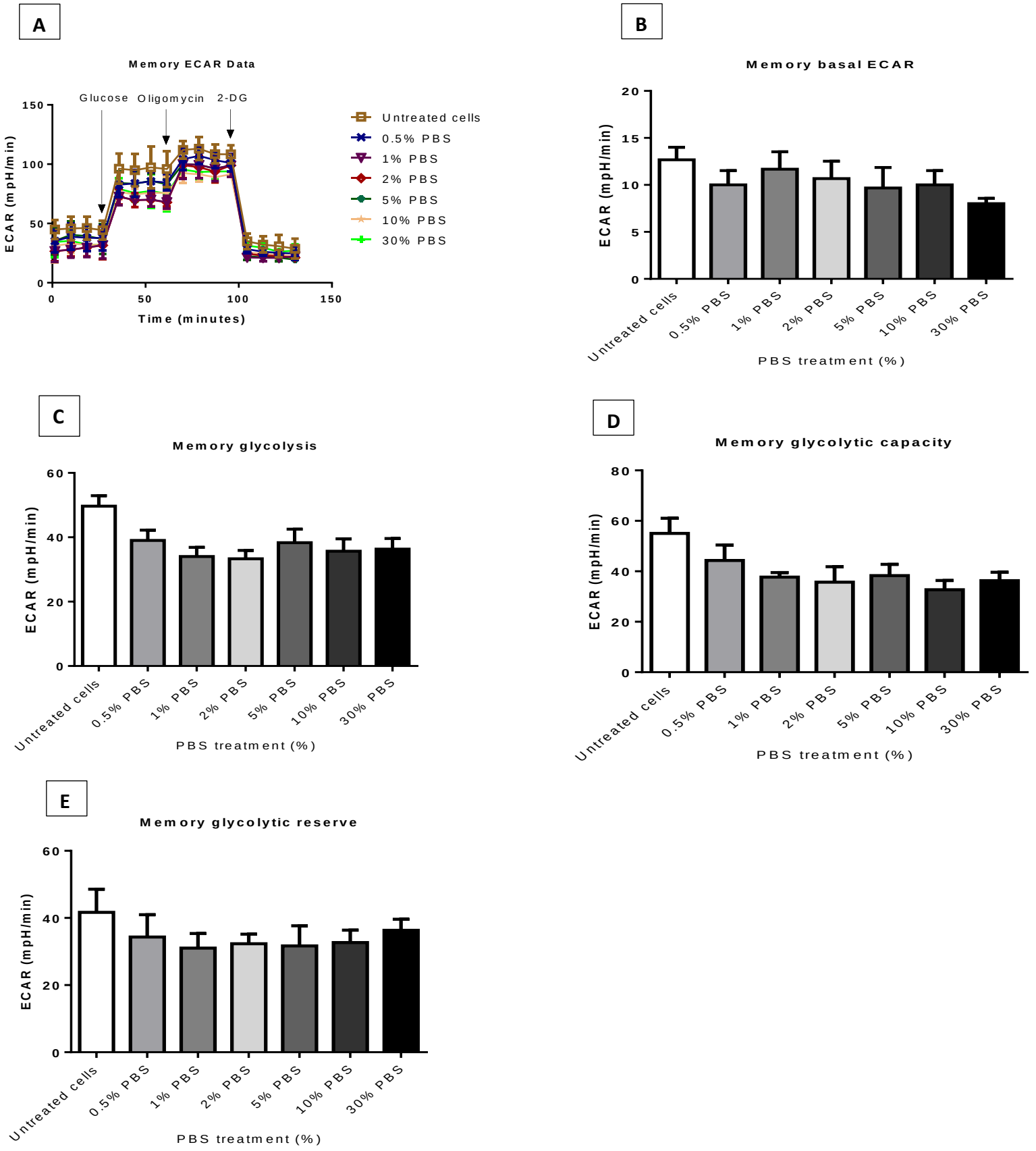


Figure 5. 9 PBS treatment did not show any effect on ECAR and other parameters such as glycolysis, glycolytic capacity and glycolytic reserve in memory cells.

Memory cells pre-stimulated with different concentrations of PBS for 24 hours with subsequent activation using anti-CD3/CD28 for 72 hours. A representative graph is shown from one of the three independent experiment. (n=3)

5.2.9 Memory cells treated with PBS treatment did not show any changes or effects on basal OCR, ATP-linked respiration, maximal respiration and spare capacity.

The representative graph (Figure 5.10 A) is showing the changes in all parameters after adding inhibitors. Basal OCR showed no effect following PBS treatment, but there was a big reduction at 0.5% and 30% but was not significant (Figure 5.10 B). ATP-linked respiration, maximal respiration and spare capacity showed a very similar levels with no significant changes compared to control (Figure 5.10 C+D+E). This suggest that there were not any significant changes in OCR parameters following PBS treatment and the levels were not affected by medium or PBS concentrations.

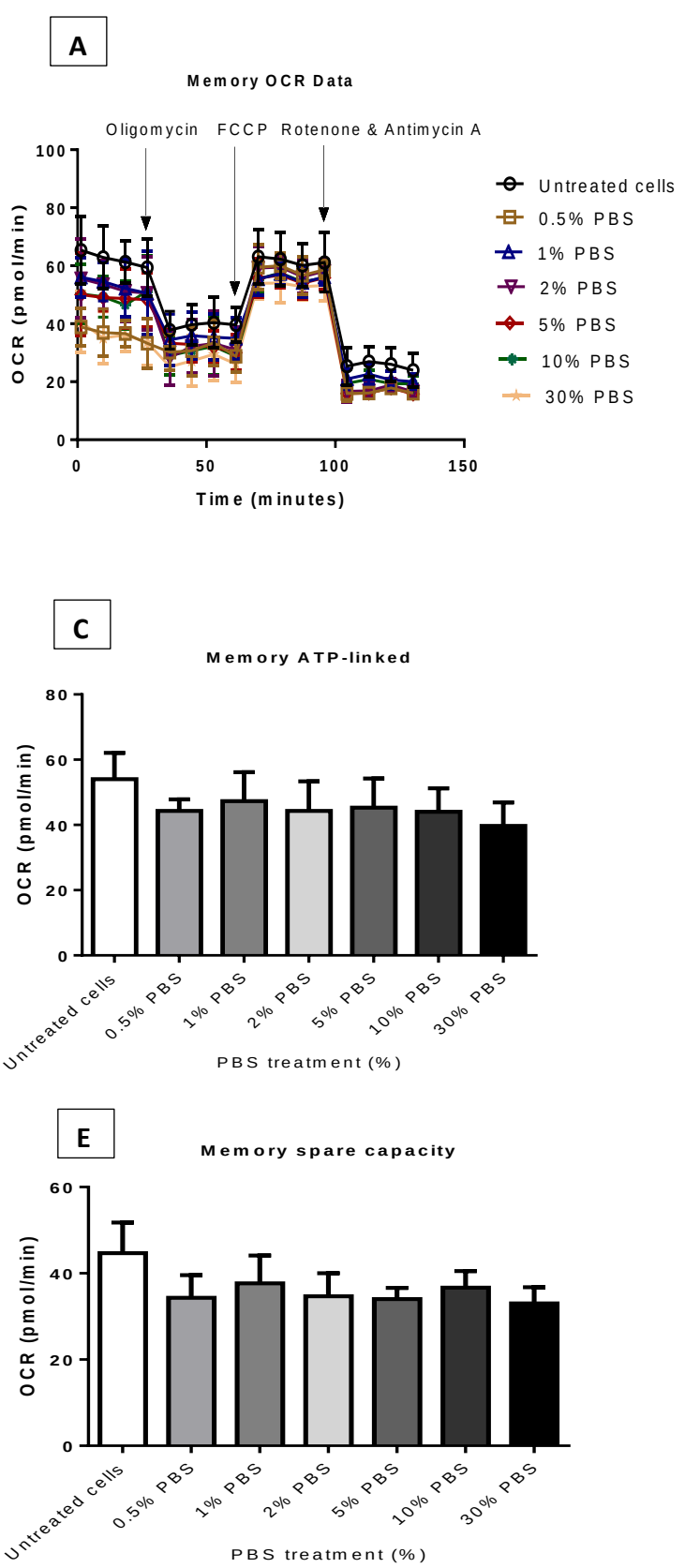


Figure 5. 10 PBS treatment did not show any effect on OCR and other parameters such as ATP-linked respiration, maximal respiration and spare capacity in memory cells.

Memory cells pre-stimulated with different concentrations of PBS for 24 hours with subsequent activation using anti-CD3/CD28 for 72 hours. A representative graph is shown from one of the three independent experiment. (n=3)

5.2.10 Low concentrations of CSE enhanced basal ECAR, but not glycolysis, glycolytic capacity and glycolytic reserve in naïve cells.

The function of immune cells is highly dependent on energy metabolism and we were interested to determine if exposure to CSE altered the energy generating pathways within the T cell. Human naïve $CD4^+$ T cells were treated with different concentrations of CSE (0, 0.5%, 1%, 2%, 5%, 10% and 30%) for 24 hours and then stimulated with anti CD3/CD28 for 72 hours. Seahorse was used to assess the extracellular acidification rate (ECAR) which is an indicator of glycolysis, and oxygen consumption rate (OCR) which is an indicator of oxidative phosphorylation. This was repeated in five independent experiments using different blood donors.

The representative graph (Figure 5.11 A) is showing the changes in all parameters after adding inhibitors. Basal ECAR following low concentrations of CSE such as 0.5%, 1% did not show any effect compared to controls and this trend was continued with 2% CSE which produced the highest, statistically significant increase compared to control. Although there were trends at the lower concentrations, the level was not statistically significant compared to controls, but there was an obvious concentration progression. Interestingly the increase in basal ECAR was not seen at higher CSE concentrations and in fact basal ECAR decreased at 5%, 10% and 30% compared to control (Figure 5.11 B). Following the assessment of basal ECAR, glycolysis was also investigated. Similar to the basal ECAR, the 0.5%, 1% and 2% showed a trend level of glycolysis, but this was not statistically significant. However, 5%, 10% and 30% showed no effect (Figure 5.11 C). Glycolytic capacity did not show any significant change following 0.5%, 1%, 2%. 5%, 10% and 30% CSE compared to control (Figure 5.11 D). Regarding glycolytic reserve, there was not any changes following 0.5, 1%, 2%, 5%, 10% and 30% (Figure 5.11 E).

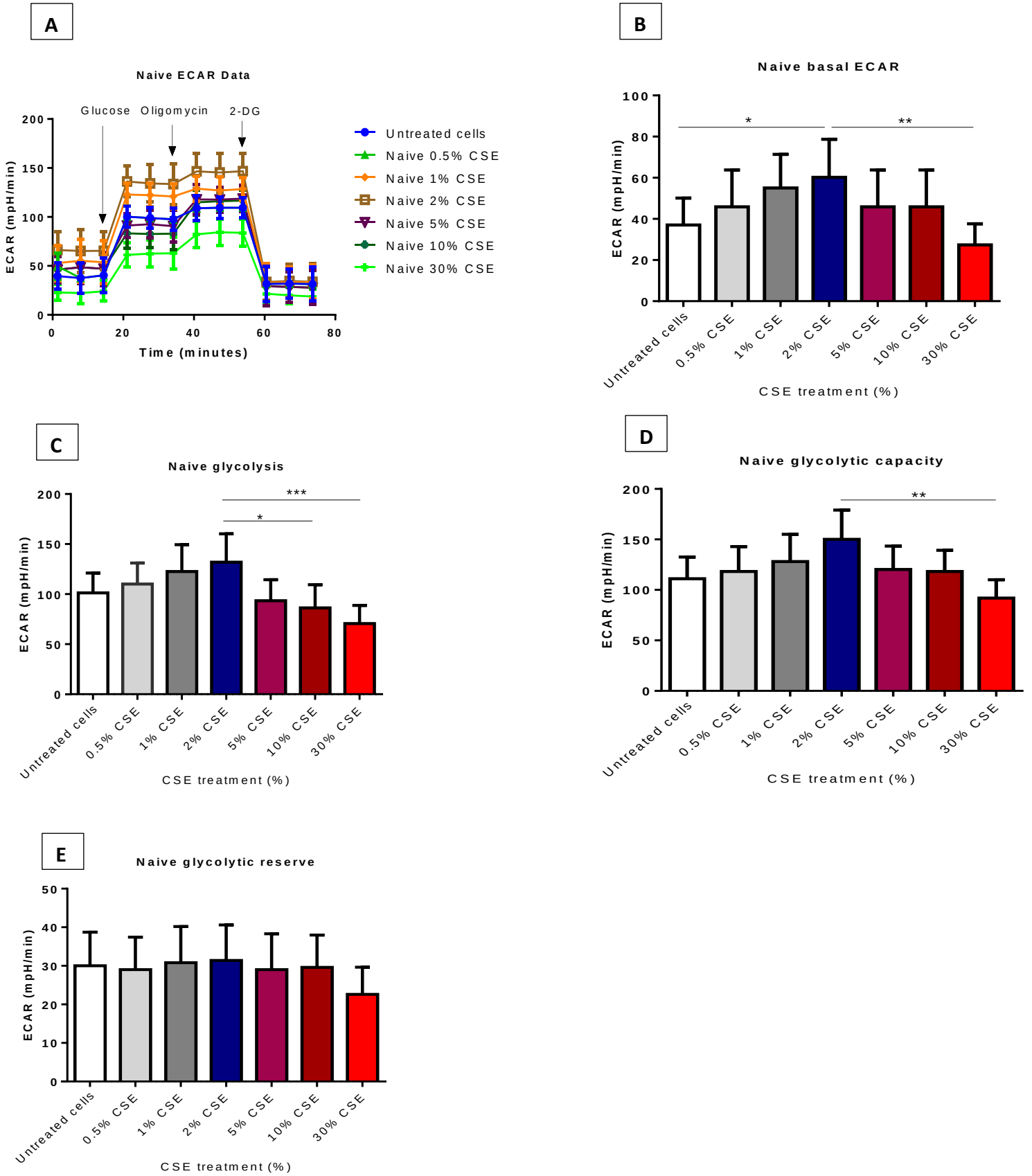


Figure 5. 11 CSE enhanced basal ECAR but not the other parameters such as glycolysis, glycolytic capacity and glycolytic reserve in naïve cells.

Naïve cells pre-stimulated with different concentrations of CSE for 24 hours with subsequent activation using anti-CD3/CD28 for 72 hours. A representative graph is shown from one of the five independent experiment. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=5)

5.2.11 CSE did not enhance the basal OCR, ATP-linked respiration and spare capacity, but maximal respiration was enhanced in naïve cells at low concentration.

Naïve cells were treated with different percentages of CSE (0, 0.5%, 1%, 2%, 5%, 10% and 30%) for 24 hours and stimulated with anti CD3/CD28 for 72 hours. Seahorse analysis was performed five times to assess the OCR which is an indicator of oxidative phosphorylation. The representative graph of five independent experiments (Figure 5.12 A) is showing the changes in all parameters after adding inhibitors. It was found that there was a trend at low concentrations of 0.5%, 1% and 2% of basal ECAR compared to controls and this trend was not statistically significant compared to control, but there was an obvious concentration progression. Interestingly the basal OCR was not seen at higher CSE concentrations and in fact basal OCR decreased at 5% and 10% and 30% compared to control (Figure 5.12 B).

ATP-linked respiration and maximal respiration also investigated. It was found that there was a trend at low concentrations of 0.5%, 1% and 2% in the level of ATP-linked respiration. Maximal respiration showed similar result but only 2% was statistically significant increase. However, 5%, 10% and 30% did not show any effect in both ATP-linked respiration and maximal respiration (Figure 5.12 C+D). Spare capacity did not show any change compared to control (Figure 5.12 E).

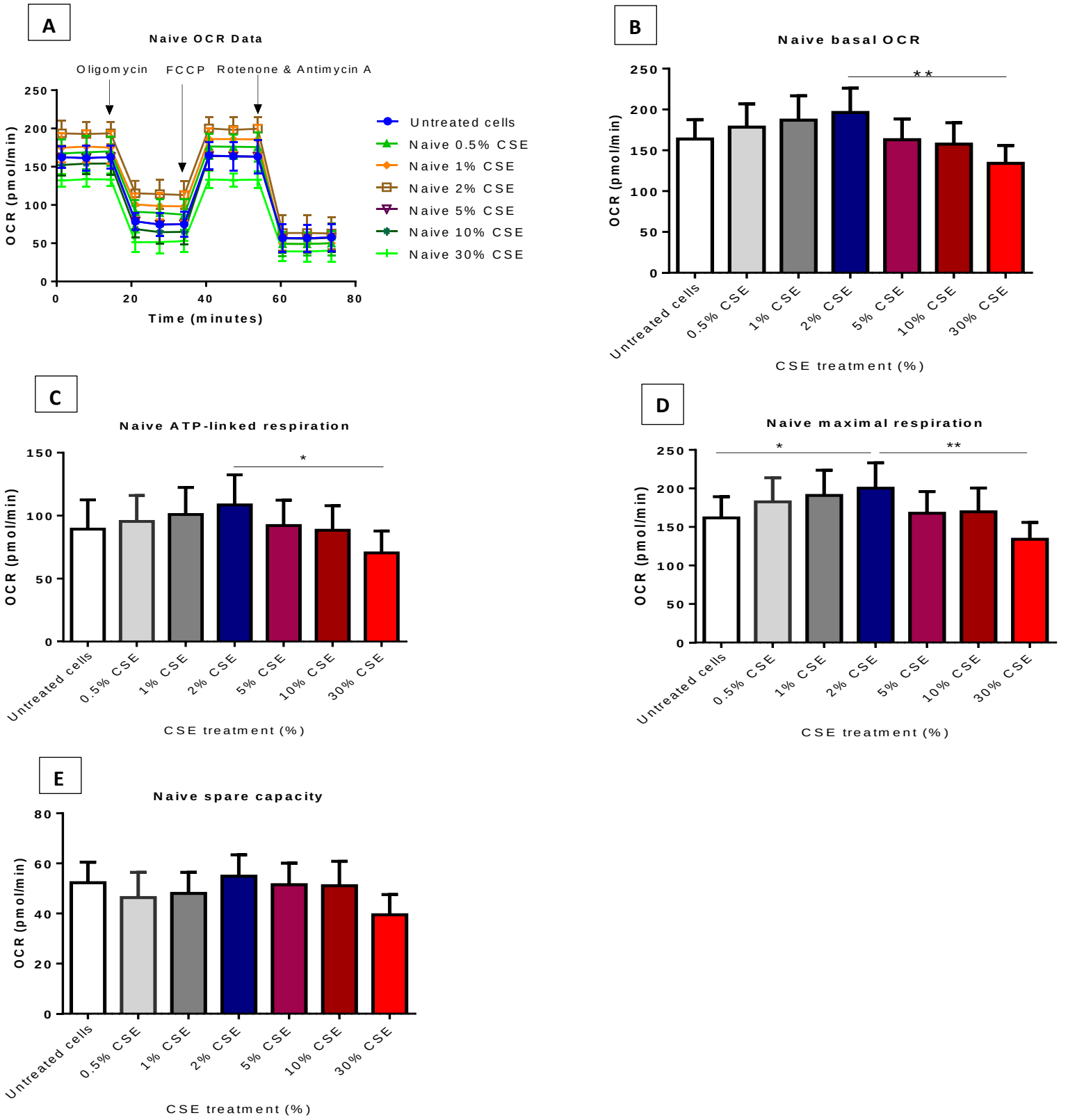


Figure 5. 12 CSE did not enhance basal OCR and the other parameters such as ATP-linked respiration, and spare capacity, but maximal respiration was increased at low concentration in naïve cells.

Naïve cells pre-stimulated with different concentrations of CSE for 24 hours with subsequent activation using anti-CD3/CD28 for 72 hours. A representative graph is shown from one of the five independent experiment. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=5)

5.2.12 Low concentrations of CSE enhanced the basal ECAR but not glycolysis, glycolytic capacity and glycolytic reserve in memory cells.

Memory cells treated with the same concentrations and stimulated with anti CD3/CD28. The representative graph of five independent experiments (Figure 5.13 A) is showing the changes in all parameters after adding inhibitors. Memory cells showed similar results to naïve cells in that low concentrations 0.5% and 1% showed a trend of the basal ECAR, but it was not significant. The 2% showed a significant increase compared to control. The 5%,10% and 30% did not show any change in the level of basal ECAR (Figure 5.13 B). Regarding glycolysis and glycolytic capacity, 0.5%, 1%, 2%, 5%, 10% and 30% did not show any effect (Figure 5.13 C+D). Glycolytic reserve did not show any change following CSE treatment (Figure 5.13 E).

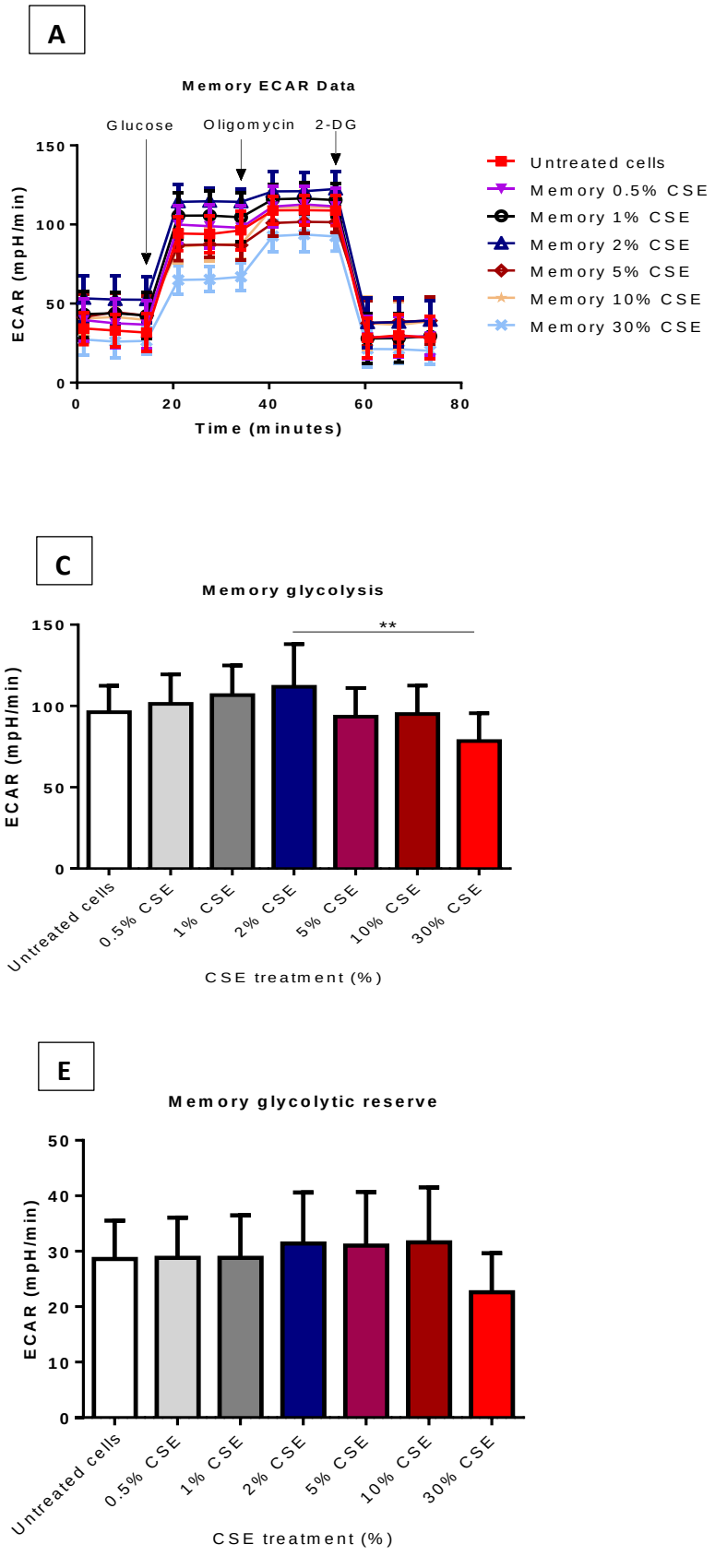


Figure 5. 13 CSE enhanced basal ECAR but not the other parameters such as glycolysis, glycolytic capacity and glycolytic reserve in memory cells.

Memory cells pre-stimulated with different concentrations of CSE for 24 hours with subsequent activation using anti-CD3/CD28 for 72 hours. A representative graph is shown from one of the five independent experiment. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=5)

5.2.13 Low concentrations of CSE enhanced the basal OCR, but not ATP-linked respiration, maximal respiration and not spare capacity in memory cells

The representative graph of five independent experiments (Figure 5.14 A) is showing the changes in all parameters after adding inhibitors. Memory cells did not show any change in the basal OCR level at 0.5%, 1%, 5%, 10% and 30%. However, 2% showed a significant increase compared to control (Figure 5.14 B). The 0.5%, 1% and 2% CSE showed a trend of ATP-linked respiration and maximal respiration, but they were not significant compared to control. The 5% and 10% and 30% showed no effect compared to control (Figure 5.14 C+D). Spare capacity did not show any change following CSE treatment (Figure 5.14 E).

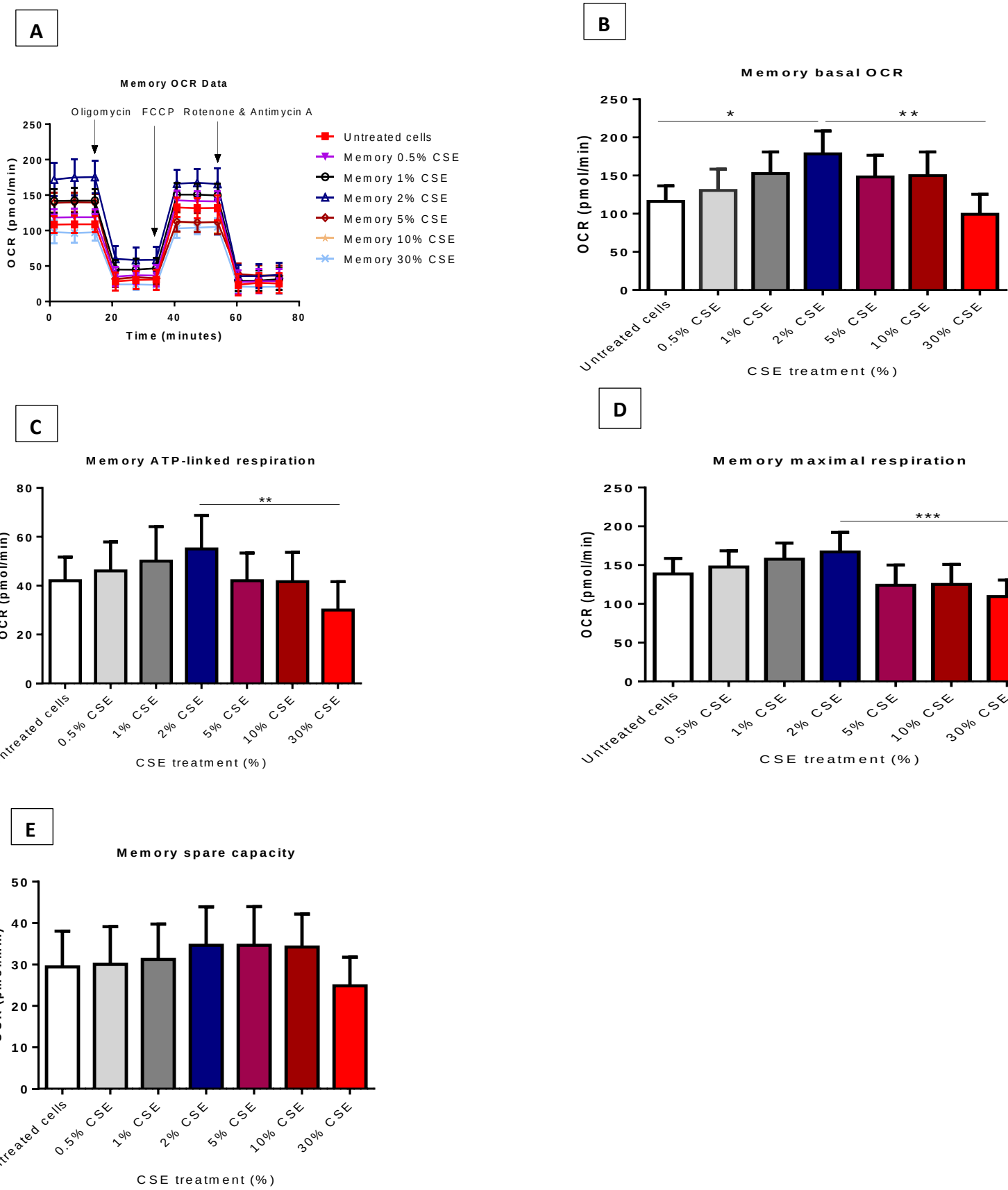


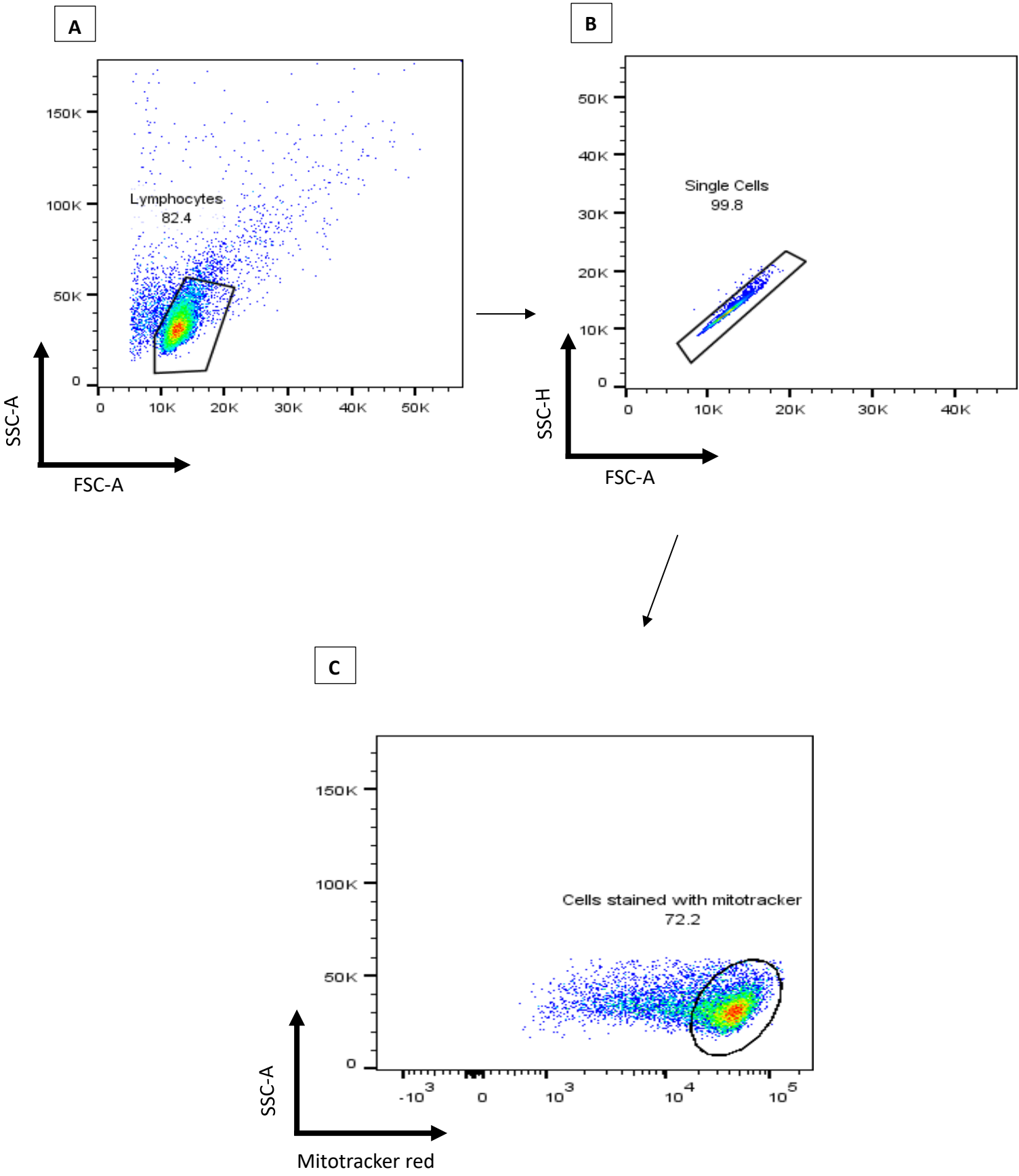
Figure 5. 14 CSE enhanced basal OCR but not the other parameters such as ATP-linked respiration, maximal respiration and spare capacity in memory cells.

Memory cells pre-stimulated with different concentrations of CSE for 24 hours with subsequent activation using anti-CD3/CD28 for 72 hours. A representative graph is shown from one of the five independent experiment. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=5)

5.2.14 CSE did not increase mitochondrial mass in naïve and memory cells

Mitochondria plays a pivotal role of the metabolic activity of the cell. So, one method to assess the function of mitochondrial is by assessing the mitochondrial mass. A study showed that increased in mitochondrial mass and network have been linked to increased OXPHOS efficacy [381]. Also, it has been shown that AMPK could play a role in activating mitochondrial mass. For instance, a study showed that AMPK could enhance mitochondrial mass by activating components of signalling pathway such as PGC-1α [382]. Interestingly, we showed that CSE increased AMPK phosphorylation and OXPHOS. Thus, based on the results obtained from energy metabolism and AMPK as well as the data from literature, we investigated the effect of CSE on mitochondrial mass using mitotracker dye.

Naïve and memory were treated with CSE and stimulated with anti CD3/CD28. Mito tracker red was used to assess mitochondrial mass and flowcytometry was performed. For accurate gating, FMO was used as a control. (Figure 5.15 A-C). By calculating the MFI, there was a trend of the mitochondrial mass at 2%, 15% and even at 30% in naïve cells, although it was not a significant increase (Figure 5.15 D+E). Memory cells did not show any changes at 2%, 15% and 30% (Figure 5.15 F+G). Since AMPK is key player in activating PGC-1α which is a marker for mitochondrial mass, there may be indirect effect of CSE on mitochondrial mass by increasing AMPK activity which in turn enhances mitochondrial mass.


A
150K
100K
50K
0
Lymphocytes
82.4
0
10K
20K
30K
40K
50K
SSC-A
FSC-A
B
50K
40K
30K
20K
10K
0
Single Cells
99.8
0
10K
20K
30K
40K
SSC-H
FSC-A
C
150K
100K
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72.2
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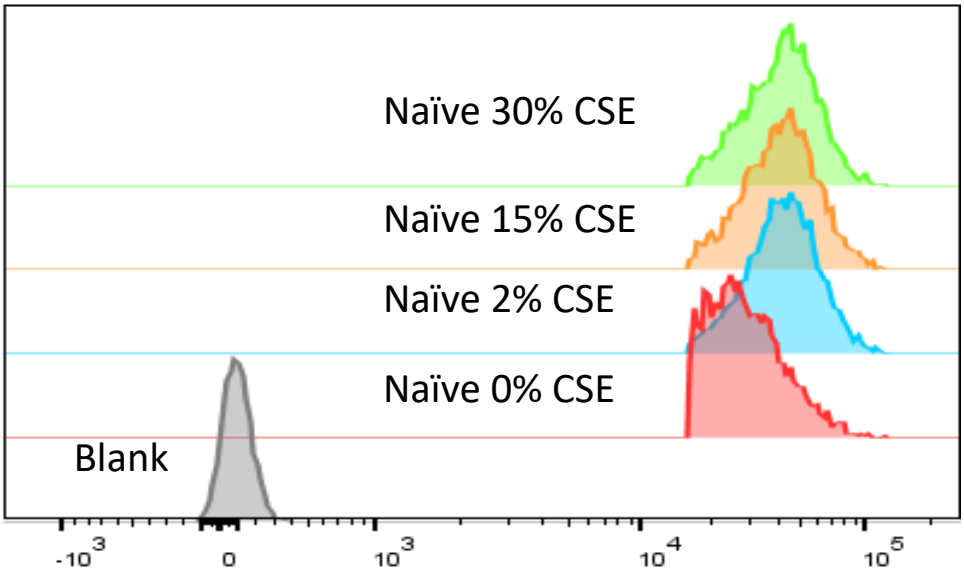
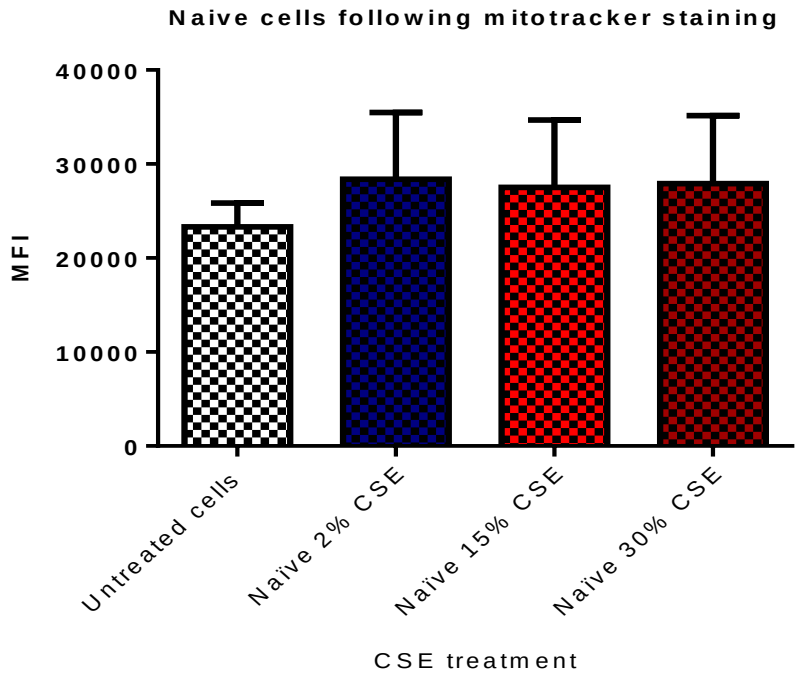
SSC-A
Mitotracker red

D

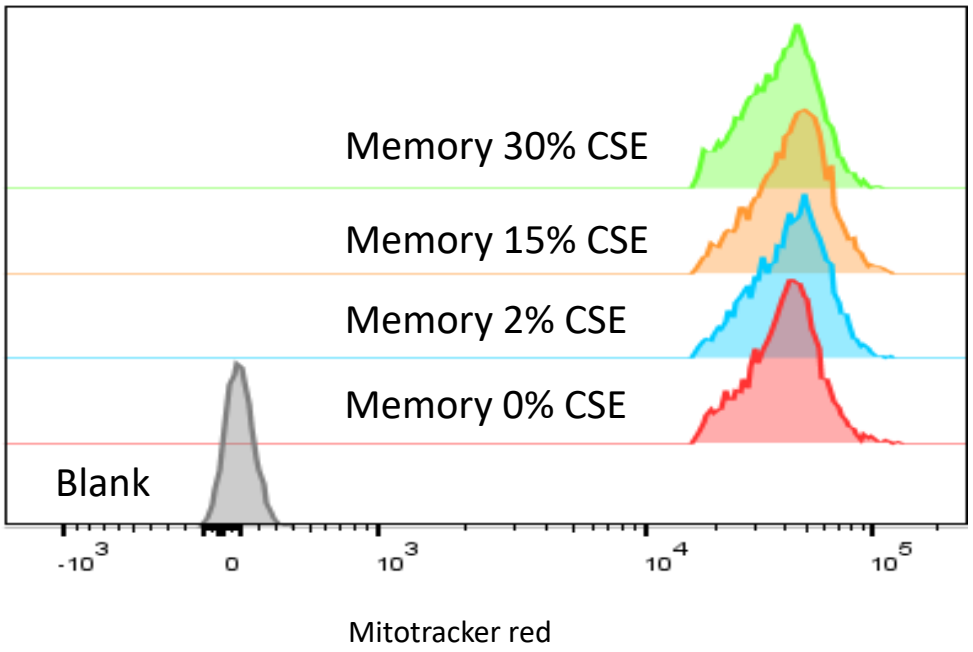


Mitotracker red

E



F



G

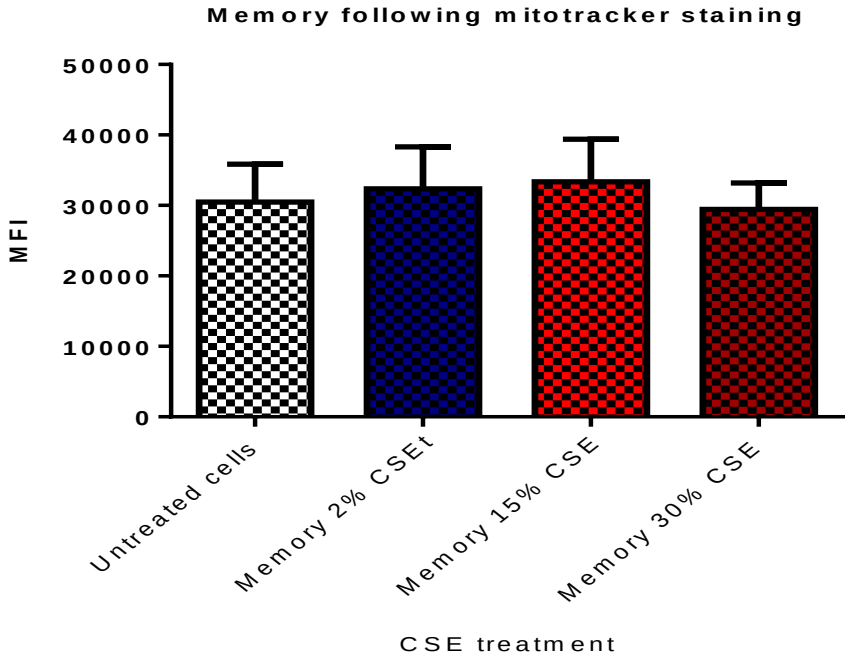


Figure 5. 15 CSE enhanced the mitochondrial mass in naïve and memory cells.

Naïve and memory cells were treated with CSE for 24 hours and then activated with anti-CD3/CD28 for 72 hours. (A-C) A representative gating strategy of the three independent experiment. (D+E) A representative graphs of naïve cells after staining with mitotracker red. (F+G) A representative graphs of memory cells after staining with mitotracker red. (n=3)

5.2.15 Lyp inhibitor did not enhance basal OCR, ATP-linked respiration and spare capacity levels, but maximal respiration was increased in naïve cells.

Previously we investigated the effect of CSE on glycolysis and OXPHOS. Therefore, we built on this to investigate the effect of Lyp inhibitor on metabolism and whether this would show any effect on glycolysis and OXPHOS. Naïve cells were treated with different concentrations of PTP LYP inhibitor (untreated cells, 0.2µM, 0.5µM and 1µM) and then stimulated with anti CD3/CD28. Changes in energy metabolism were assessed using seahorse instrument. The representative graph of four independent experiments (Figure 5.16 A) is showing the changes in all parameters after adding inhibitors. It was found that the level of the basal OCR in naïve did not change much following low concentration of Lyp inhibitor treatment 0.2µM and there was not any statistically significant difference compared to control. The 0.5µM and 1µM did not show any effect on basal OCR level (Figure 5.16 B). Following the assessment of basal OCR, ATP-linked respiration was investigated. Similar to the basal OCR, ATP-linked respiration did not show much at 0.2µM, and there was not any effect at 0.5µM and 1Mm (Figure 5.16 C). However, the level of maximal respiration was significantly increased at 0.2µM compared to control. The 1µM did not show much of maximal respiration (Figure 5.16 D). The spare capacity showed similar result to both basal OCR and ATP-linked respiration where 0.2µM did not show much of spare capacity, and 0.5µM and 1µM did not show any obvious effect (Figure 5.16 E). This may suggest that the low concentration of Lyp inhibitor treatment could augment the metabolism as we noticed in maximal respiration.

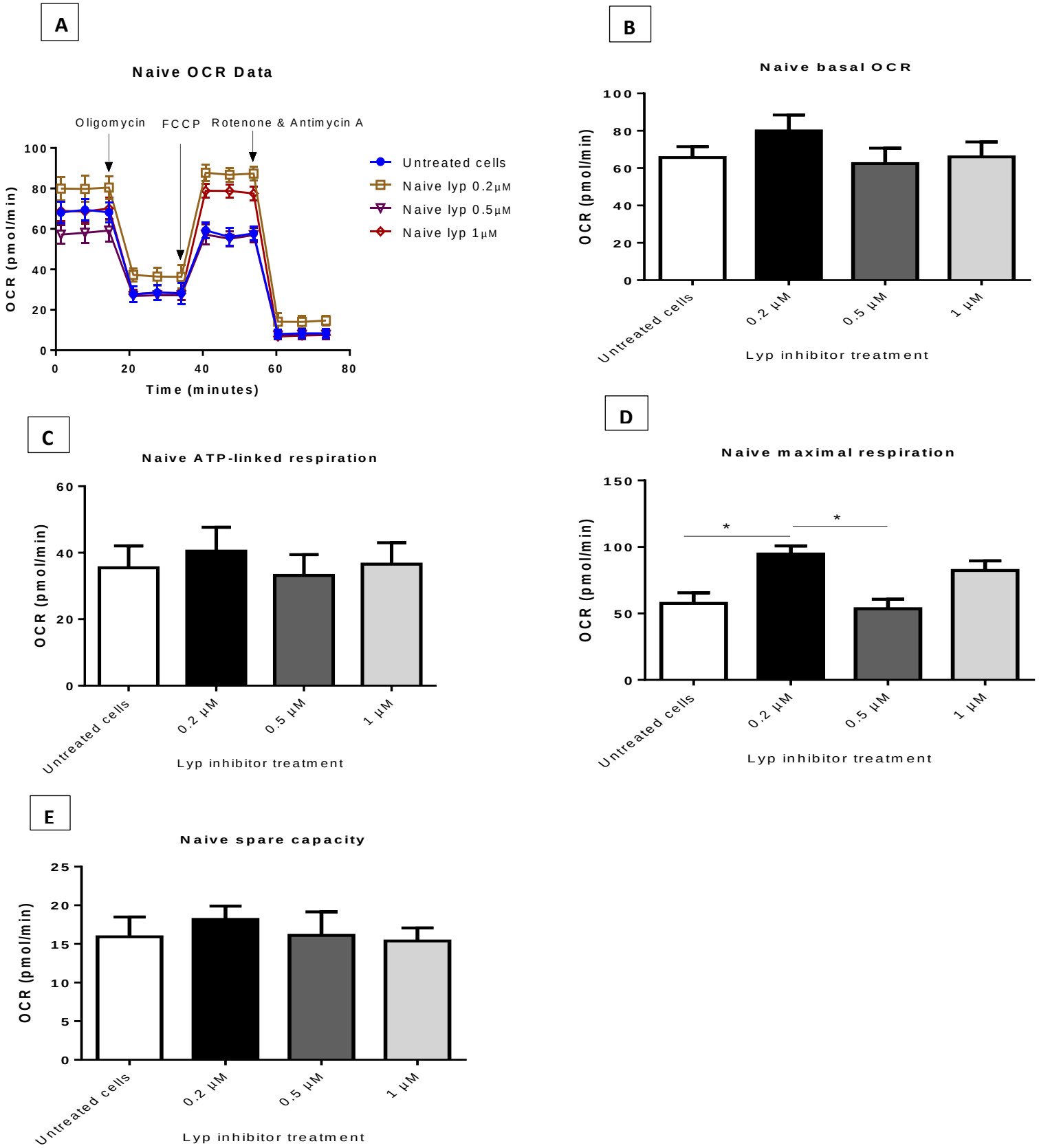


Figure 5. 16 Lyp inhibitor did not enhance basal OCR and the other parameters such as ATP-linked respiration and spare capacity, but maximal respiration was increased at low concentration in naïve cells.

Naïve cells pre-stimulated with different concentrations of Lyp inhibitor for 24 hours with subsequent activation using anti-CD3/CD28 for 72 hours. (A-D) A representative graphs of basal OCR, ATP linked respiration, maximal respiration and spare capacity. (E) A representative graph is shown from one of the four independent experiment. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=4)

5.2.16 Lyp inhibitor did not enhance ECAR, glycolysis and glycolytic reserve, but glycolytic capacity increased at low concentration in naïve cells.

After investigating the effect of Lyp inhibitor treatment on oxidative phosphorylation, we investigated the effect of Lyp inhibitor on glycolysis. The representative graph of four independent experiments (Figure 5.17 A) is showing the changes in all parameters after adding inhibitors. It was found that the level of basal ECAR did not change much at 0.2µM and 0.5µM compared to control. The level of basal OCR was decreased at 1µM compared to control (Figure 5.17 B). The glycolysis also did not show much following 0.2µM and it was not significant. Both 0.5µM and 1µM decreased the level of glycolysis compared to control (Figure 5.17 C). Regarding glycolytic capacity, the 0.2µM significantly augmented the level of glycolytic capacity in comparison to control. Both 0.5µM and 1µM showed no effect (Figure 5.17 D). The glycolytic reserve did not show any change at 0.2µM, 0.5µM and 01µM (Figure 5.17 E). This may suggest that low concentration of Lyp inhibitor could have an effect on Lyp and perhaps lead to increase the metabolism in naïve cells as we observed in glycolytic capacity.

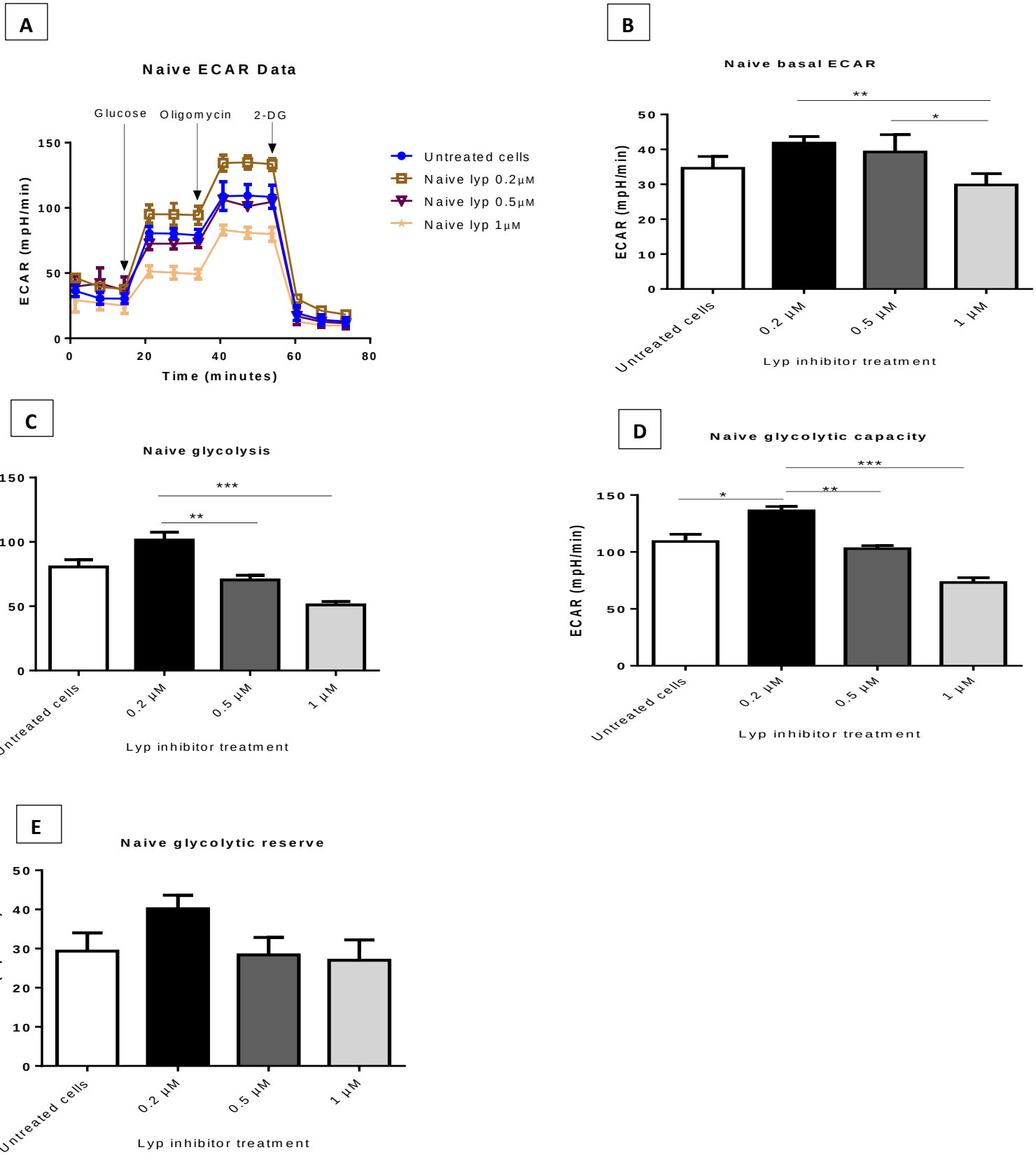


Figure 5. 17 Lyp inhibitor did not enhance ECAR and the other parameters such as glycolysis and glycolytic reserve, but glycolytic capacity increased at low concentration in naïve cells.

Naïve cells pre-stimulated with different concentrations of Lyp inhibitor for 24 hours with subsequent activation using anti-CD3/CD28 for 72 hours. (A-D) A representative graphs of basal ECAR, glycolysis, glycolytic capacity and glycolytic reserve. (E) A representative graph is shown from one of the four independent experiment. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=4)

5.2.17 Lyp inhibitor did not enhance basal OCR, ATP-linked respiration, maximal respiration and spare capacity levels in memory cells.

Following the assessment of metabolism in naïve cells, we also investigated the energy metabolism in memory cells after Lyp inhibitor treatment to assess whether memory cells would be affected by this treatment or not. The representative graph of four independent experiments (Figure 5.18 A) is showing the changes in all parameters after adding inhibitors. It was found that the low concentration 0.2µM, 0.5µM and 1µM did not change in the basal OCR level compared to control (Figure 5.18 B). ATP-linked respiration and maximal respiration showed a very similar result to basal OCR. The level did not change at 0.2µM, 0.5µM and 1µM compared to control (Figure 5.18 C+D). Regarding the spare capacity, there was not any change following Lyp inhibitor treatment (Figure 5.18 E).

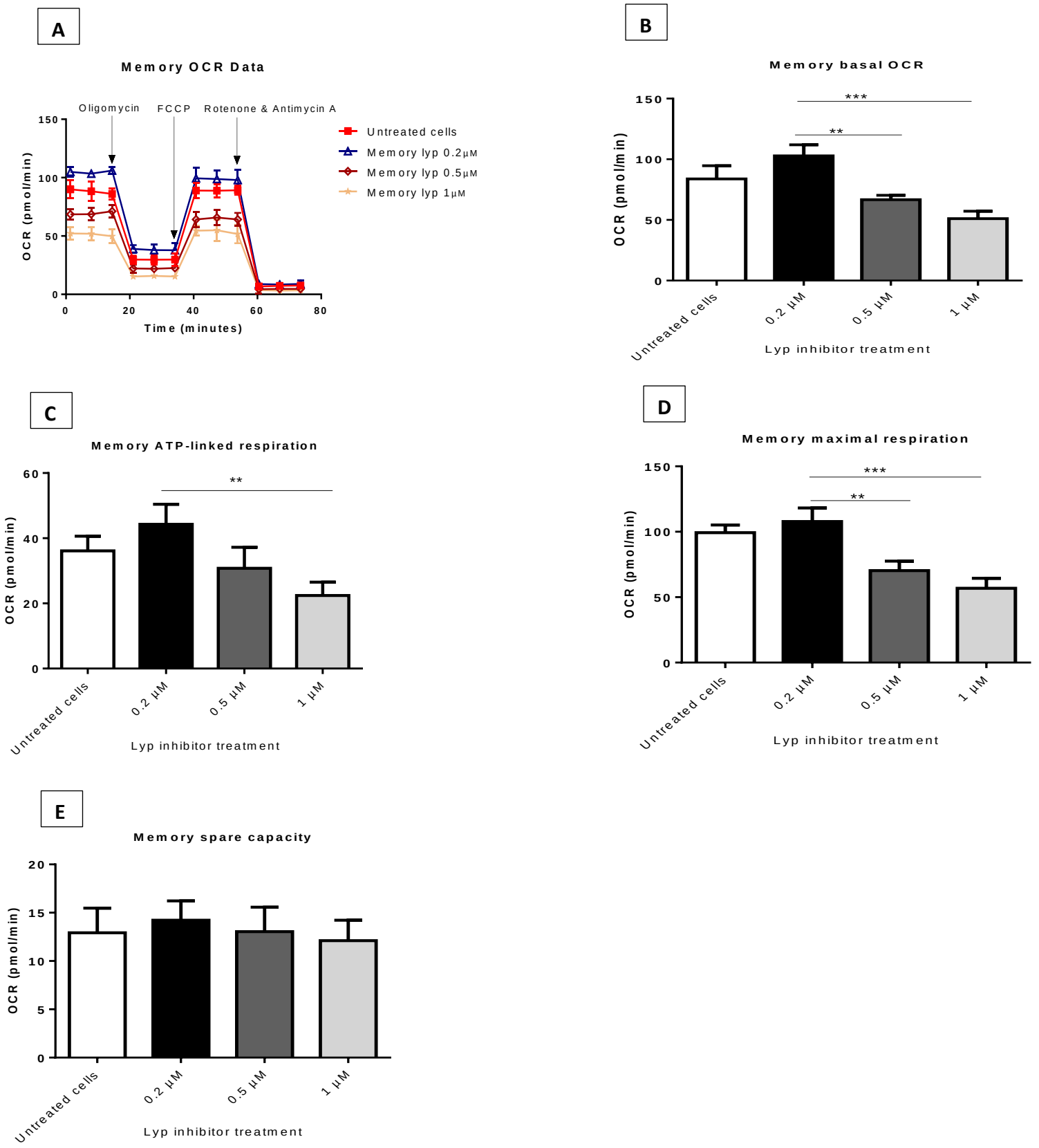


Figure 5. 18 Lyp inhibitor did not enhance OCR and the other parameters such as ATP-linked respiration, maximal respiration and spare capacity in memory cells

Memory cells pre-stimulated with different concentrations of Lyp inhibitor for 24 hours with subsequent activation using anti-CD3/CD28 for 72 hours. (A-D) A representative graphs of basal OCR, ATP linked respiration, maximal respiration and spare capacity. (E) A representative graph is shown from one of the four independent experiment. (n=4)

5.2.18 Low concentration of Lyp inhibitor enhanced basal ECAR, glycolysis and glycolytic capacity but not glycolytic reserve in memory cells

Next, we investigated the effect of Lyp inhibitor on glycolysis level in memory cells. The representative graph of four independent experiments (Figure 5.19 A) is showing the changes in all parameters after adding inhibitors. The basal ECAR, glycolysis and glycolytic capacity in memory showed a significant increase at 0.2µM compared to control. However, both 0.5µM and 1µM showed no effect compared to control (Figure 5.19 B+C+D). In addition, the glycolytic reserve did not change after 0.2µM, 0.5µM and 1µM compared to control. (Figure 5.19 E). This result showed that the Lyp inhibitor treatment could have an effect on memory cells and therefore can lead to increase in the energy metabolism.

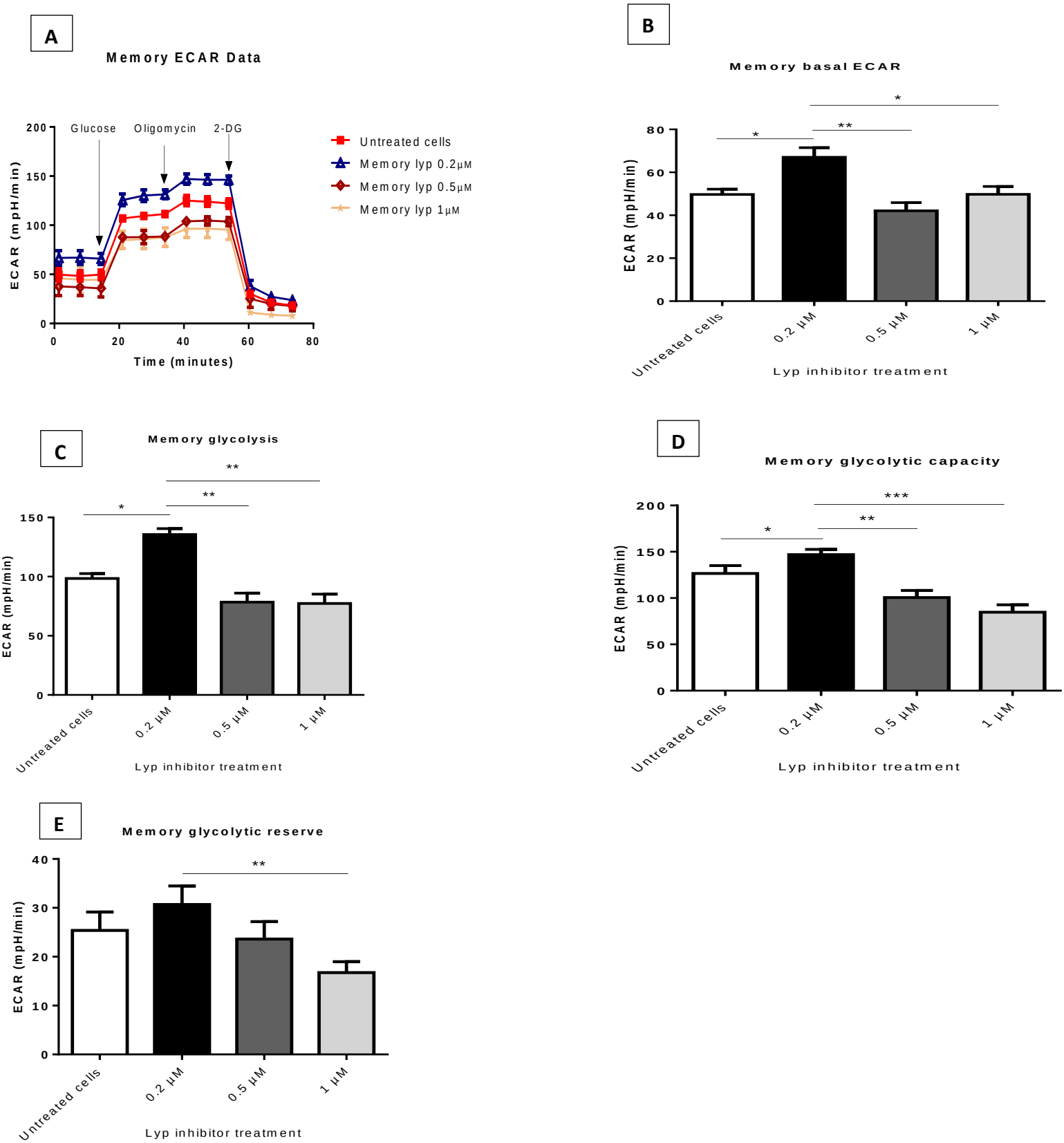


Figure 5. 19 Lyp inhibitor enhanced ECAR and the other parameters such as glycolysis, glycolytic capacity but not glycolytic reserve in memory cells.

Memory cells pre-stimulated with different concentrations of Lyp inhibitor for 24 hours with subsequent activation using anti-CD3/CD28 for 72 hours. (A-D) A representative graphs of basal ECAR, glycolysis, glycolytic capacity and glycolytic reserve. (E) A representative graph is shown from one of the four independent experiment. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=4)

5.3 Discussion

In this chapter it was hypothesised that CSE would alter energy metabolism in immune cells, and this might be involved in the processes by which smoking promotes RA development. Using in vitro experiments, we found that exposure of naïve and memory T human $CD4^+$ cells to CSE and Lyp inhibitor increased the phosphorylation of Akt, AMPK and S6 which are crucial in energy metabolism. Furthermore, CSE and Lyp inhibitor enhanced energy metabolic pathways such as glycolysis and oxidative phosphorylation at low concentrations, whereas high concentration reduced the metabolism or showed no effect. There was a significant increase at 2% of ECAR and OCR compared to control and a reduction at 30, but glycolytic reserve showed no change in either naïve or memory cells. Mitochondrial mass did not show any change in memory following CSE treatment.

The effect of cigarette smoking on metabolism and the underlying mechanisms are not fully understood. A study was conducted on smokers found that after 24 hours their energy expenditure was increased by 140-200 kcal/day compared to a day without smoking, suggesting that energy can be increased after 24 hours among smokers and would be expected to decrease when people stop to smoking [383]. An *in vivo* study reported that hamsters exposed to cigarette smoke increased blood glucose levels and decreased body weight [384]. Thus, cigarette smoking can affect energy utilization and food intake which contribute to overall body mass. Moreover, CSE changed mitochondrial structure and induced the expression of oxidative phosphorylation proteins (Complex II, III and V) which accompanied by elevation of proinflammatory cytokines levels [385]. CSE could contribute to cancer formation by changing metabolism. A study showed that CSE exposed non-malignant oesophageal epithelial cells induced glycolysis and dysfunction of mitochondria through

down-regulation of mitochondrial genes and upregulation of glycolysis enzymes. This suggests that chronic exposure to CSE may alter mitochondrial biogenesis and glycolysis which may contribute to malignant transformation of oesophageal epithelial cells [386].

The evidence in this hypothesis varies and some results reported different findings. For instance, evidence found that nicotine administered to healthy people attenuated energy use (lower calories) and positively associated with satiety [387, 388]. A recent study showed that CSE treated alveolar macrophages from smokers decreased metabolic activities such as glycolytic reserve and spare respiratory capacity and these metabolic activities were significantly reduced by CSE in response to infection [389].The samples were from the lung and this suggests it would be worthwhile to investigate the effect of CSE on synovial fluid cells from rheumatoid arthritis patients and measure the metabolic activities. In contrast to the previous results , a study on rats fed by nicotine for 24 hours reported different finding and showed an increase in the respiration up to 30% in hepatocytes isolated from rats and this accompanied by inhibition of glycolysis, suggesting that nicotine could increase oxygen uptake and therefore increase mitochondrial respiration due to increase in ADP-independent [390]. Also, a chronic exposure of nicotine to rats augmented number of genes expression in OXPHOS such as *Mt-nd1* (NADH dehydrogenase subunit I), *Mt-nd1* (NADH dehydrogenase subunit I) and *Atp5b* (ATP synthase, F1 complex, β subunit) of each complex in the electron transport chain leading to disrupt the intracellular signalling of oxidative free radicals and energy metabolism [391]. It seems nicotine has dual effects on metabolism as a study showed that the high doses may induce oxidative stress by increasing oxidation and develop metabolic disorders, whereas the low doses act as antioxidant [392].

With regard to oxidation, the effect of cigarette smoking on oxidation was investigated and the evidence showed that cigarette smoking significantly increased the amount of nitric oxide (NO) causing changes in oxidation, but this was prevented by activating the antioxidant [393]. Another study investigated the effect of cigarette smoking on mitochondrial respiration chain in circulating lymphocytes found that the oxidative rate was increased. Also, the lipid peroxidase was increased, but the mitochondrial complex VI was inhibited, suggesting that the mitochondrial respiration chain was disrupted by smoking which was correlated with the increase of oxidative damage of membranes [394]. This increase of oxidation rate may increase the production of endogenous of ROSs which involved in many metabolic conditions. Not only cigarette smoking could affect energy metabolism, drugs also could play a role on metabolism. For instance, olanzapine could alter energy metabolism via the mTOR pathway by increasing hepatic concentrations of amino acids leading to the disturbances in glucose and lipid metabolism in the liver [395].

Since the Akt is one of the most important master regulators of metabolism, we thought that there might be a strong link between cigarette smoking and Akt and the enhancement of energy metabolism. Smoking appeared to activate Akt through production of ROS, leading to inhibition of autophagy and increase energy and therefore promote cancer [396]. This inhibition could increase the energy metabolism, whereas decrease the metabolism leads to autophagy induction [397]. Tobacco and nicotine showed a potential and rapid activation of Akt which in turn activated downstream substrates which play a central role in metabolism, including mTORC, S6 and Glycogen synthase kinase 3 (*GSK-3*). This activation of Akt was due to the utilisation of nicotinic acetylcholine receptors (nAchR) subunits alpha3/alpha4 or alpha7 by tobacco or its component, suggesting that tobacco components could stimulate

Akt-dependent proliferation and promote cancer development [398]. Since Akt is the positive upstream regulator of mTOR, a study on lung epithelial tissue attempted to target Akt using CSE found that CSE activated Akt which in turn increased phosphorylation of downstream events such as mTOR1 [399]. Thus, cigarette smoking could target Akt and subsequently activates mTOR which in turn might increase cellular metabolism which my lead to alteration in immune cells function and thus promoting the development of various conditions. We also showed that Lyp treatment significantly enhanced Akt activity. To support this result, a study conducted on $CD4^+$ T cells found that Lyp variant (LYP*R620) was strongly interacted with p85 subunits of PI3K and led to Akt activation. Furthermore, the study found that at cellular level, this led to exacerbation of Th1 responses (e.g. IFNγ production) which involves in autoimmunity [400]

Another important master regulator of metabolism is mTORC which is downstream of Akt. It has a critical role in metabolism and cell growth. [401]. In vivo study showed that S6 which is a substrate of mTORC1 was important to control glucose metabolism and also could involve in controlling lipid metabolism [402]. In addition, In vitro study showed that S6 led to changes in mitochondria biogenesis and AMPK activity, suggesting the important role of S6 in energy metabolism [403] Nicotine which is one of the main components of cigarette smoking activated mTORC1 through increase the phosphorylation of 4E-BP1 and S6K [399]. As we have shown that CSE increased glycolysis, a study showed that chronic exposure to CSE enhanced glycolysis, pyruvate and lactate by upregulating PDK2 and HIFα expression, suggesting that CSE could have an effect on HIF-1α and therefore increase glycolysis [404]. This activation of HIFα was driven by mTORC1 in response to chronic smoking which increased HIF-1α synthesis through S6K1, 4E-BP1 and STAT3. However, inhibition of S6K1

decreased HIF-1α synthesis, suggesting that mTORC1 and its substrate S6 play a central role in metabolism by regulating HIF-1α through increased HIF-1α mRNA transcription [405]. AMPK is another master regulator of metabolism which plays a critical role in energy homeostasis and modulate inflammation. A study found that chronic smoking could activate AMPK signalling but this can be suppressed following smoking withdrawal, suggesting that AMPK might be a target for smoking [406]. An in vitro and in vivo experiments reported that chronic exposure to cigarette smoking increased AMPK, ROS and IL-8 induction. However, removal of ROS by N-acetylcysteine, an inhibitor of ROS decreased the activation of AMPK signalling, suggesting that cigarette smoking may be important for ROS production which activates AMPK signalling and promotes inflammation [407]. Furthermore, to support this study, another study found that nicotine increased the phosphorylation of AMPK and this mediated by ROS. The nicotine significantly increased superoxide which mainly contribute to AMPK activation [408]. In this chapter we reported that CSE increased the mitochondrial mass which reflects the mitochondrial biogenesis which increases metabolic enzymes for OXPHOS and glycolysis. The mitochondrial dynamics field is still evolving, but many studies showed that CSE can affect the mitochondrial morphology and network in human. For instance, a recent study showed that chronic exposure of human epithelial cells to CSE increased cytosolic Ca^{2+} levels and activated mitochondrial biogenesis process. [409]. Another study showed that prolonged exposure to CSE led to enhancement of ROS activity which increased mitochondrial mass and DNA content and subsequent alteration of mitochondrial metabolic activities [410]. It has been shown that AMPK could increase mitochondrial biogenesis and mitochondrial enzymes [411]. The mechanisms by which AMPK induces mitochondrial biogenesis are not clear yet. However, a study found that AMPK directly phosphorylates the peroxisome-proliferator-activated receptor gamma

coactivator 1alpha (PGC-1alpha) which act as a regulator of mitochondrial biogenesis both in vitro and in vivo. The study found that PGC-1alpha-deficient mice, the AMPK had no effect on mitochondrial genes and expression of glucose transport 4 (GLUT4), suggesting that the activation of PGC-1alpha by AMPK could increase oxidative metabolism and mitochondrial mass [412]. Since we showed that CSE increased the activity of AMPK in memory cells, it may be possible that CSE could play a role by activating AMPK which in turn induces mitochondrial biogenesis through expression of PGC-1alpha. However, CSE could cause mitochondrial dysfunction by decreasing mitochondrial rho GTPase 1 (miro 1) leading to reduced mitochondrial membrane potential and therefore reduced energy metabolism [413]. So, as mentioned the evidence in the literature vary and contradictory studies may be reported.

In this chapter we addressed that CSE and Lyp inhibitor could contribute to inducing energy metabolism in immune cells to promote RA. This was carried out by measuring ECAR and OCR parameters in naïve and memory T cells treated with CSE and Lyp inhibitor and stimulated with anti CD3/CD28. We found that low concentrations could alter the energy metabolism including glycolysis and OXPHOS, whereas high concentration suppressed or showed no effect compared to control. Although, we showed promising data that low concentrations of CSE increased the energy and showed trend, some of the results showed non-significant changes. Also, we found that CSE and Lyp inhibitor induced the phosphorylation of the known targets of metabolism such as Akt, AMPK and S6 (mTORC substrate), suggesting that CSE and Lyp inhibitor could increase signalling related to metabolism and subsequently enhance energy metabolism.

The main limitation in this chapter is that we did not add untreated cells without anti-CD3/CD28 in each experiment to determine whether changes with CSE are large compared to those seen with cell activation. Another limitation is that we did not investigate the effect of Lyp inhibitor on S6 and AMPK which are crucial in energy metabolism, but we could not due to COVID restrictions and then we did not have the time to do it. If we had the time, it would be better to investigate the effect of Lyp inhibitor on these two targets as they are crucial in energy metabolism. We did not investigate other pathways involve in energy metabolism such as fatty acid oxidation (FAO) and glutamine metabolism which are crucial in ATP production. It would be better to validate Seahorse data by studying the effect of CSE on FAQ and glutamine metabolism.

Chapter 6:
General discussion

6.1 Introduction

Cigarette smoking and PTPN22 variant are risk factors for developing several autoimmune diseases. Many studies have focused on the effect of cigarette smoking and PTPN22 variant on T cells in the context of RA [414, 415]. These studies suggested that PTPN22 variant could alter Lyp function and structure, which would then affect the regulation of TCR signalling. On the other hand, smoking may play a role in RA by activating NF-κB through increased oxidative stress which also impairs the antioxidant system [60]. Cigarette smoking could have an effect on Lyp which could increase the risk of rheumatoid arthritis development through unknown mechanisms. This study investigated how cigarette smoking and Lyp inhibition affected the overall Lyp activity and CD4+ T cells function and activation [416]. Several studies have suggested a role for smoking in RA pathogenesis through alteration of signalling pathways. In our work we showed that cigarette smoking actually enhanced signalling pathways in CD4+ T cells, which was linked to the alteration of the signalling pathway related to metabolism. This chapter discusses the possible mechanisms by which CSE and PTPN22 could enhance signalling and metabolism as well as how this could promote RA development.

6.2 Summary of results

CSE and Lyp inhibitor treatment had an effect on Lyp phosphates activity, TCR signalling and metabolism as well as cytokine production. Because cigarette smoke contains many toxic substances which could affect the viability of cells, toxicity testing was done before carrying on to experiments to assess the effects on cell function. CSE and Lyp inhibitor treatment showed no effect on viability of T cells, indicating that experiments could be carried out with results being unaffected by loss of cell viability. It was found that CSE significantly increased

the activity of naïve and memory cells by activating some key activation markers such as CD25, CD28, CD69 and CD62L. This activation of T cells had significant consequences for differentiation and cytokine secretion of the T cells. Therefore, a range of cytokines produced by both memory and naïve T cells was assessed following exposure to CSE and activation using anti CD3/CD28. It was found that CSE significantly augmented some key proinflammatory cytokines including IL-6, IFN-γ, TNF-α, IL-1β in both naïve and memory. In contrast, IL10 expression was decreased following CSE treatment, suggesting that CSE could drive TH1 induction and therefore increase some of proinflammatory cytokines which may lead to develop autoimmune conditions such as RA.

CSE decreased Lyp activity only in memory but not in naïve cells, whereas Lyp inhibitor decreased the Lyp activity only in naïve but not in memory cells. This suggests a mechanism by which both treatments could play a role in augmenting T cells signalling. Both treatments, at lower concentrations, increased glycolysis and oxidative phosphorylation in both naïve and memory T cells. Both CSE and Lyp inhibitor treatment increased the activity status of Lyp substrates, including the positive regulators Lck tyr394 in naïve cells, ZAP-70 tyr493 in naïve and memory cells, and Vav1 tyr174 in naïve cells only. On the other hand, CSE and Lyp inhibitor treatment successfully decreased the phosphorylation status of negative regulator Lck tyr505 in both naïve and memory cells. Consequently, calcium signalling was augmented in both naïve and memory cells which provided an overall assessment of the strength of signal through the TCR. This means that both CSE and Lyp inhibitor treatment increased the upstream signalling which had an effect on downstream signalling. Following the assessment of upstream signalling, CSE and Lyp inhibitor treatment showed to increase downstream signal through Akt which is one of the master regulators of metabolism. Akt phosphorylation

was also significantly enhanced following treatment with Lyp inhibitor in naïve cells but not memory, and activation with PMA/Ionomycin in naïve only, suggesting that Lyp could have a direct effect on Akt, independent of the signal through the TCR. Furthermore, CSE enhanced the other two master regulators of metabolism mTORC in naïve cells only, whereas AMPK in memory cells only, suggesting that CSE and Lyp inhibitor treatment can activate metabolism which could play a role in RA development and other autoimmune conditions. Since CSE enhanced oxidative phosphorylation, the mitochondrial mass was investigated to further investigate mitochondrial function. It was found that CSE enhanced mitochondrial mass in naïve cells only but not in memory, suggesting that CSE could change mitochondrial function which may lead to an increase in metabolism.

6.3 cigarette smoke and TCR signalling

We found that naïve and memory T cells in response to CSE increased TCR- dependent signalling. This was characterised by increased phosphorylation of some important substrates involved in signalling pathways, including ZAP-70, Lck, Vav1, Akt, mTORC and AMPK. Also, it was observed that CSE increased Ca^{2+} release consequent to the stimulation of the TCR, increased production of several cytokines and interestingly increased energy metabolism in both glycolytic and oxidative phosphorylation pathways. Increased expression of the key markers of T cell activation CD25 and CD69 were also seen. These findings show that cigarette smoke gave rise to enhanced signalling via TCR which may be because of the inhibition or a reduction in the phosphatase activity of Lyp.

In support of this, a study in mice showed that nicotine, an important component of cigarette smoke, significantly increased the expression of dendritic cell (DC) costimulatory molecules (CD40 and CD80), adhesion molecules (CD54 and LFA-1) and MHC class II. This

activation of DCs augmented and stimulated T cell proliferation and cytokine production as well as the phosphorylation of PI3K and Akt, suggesting that nicotine may contribute to T cell proliferation and initiating signalling through TCR [417]. Furthermore, in vitro study on human reported that smoking induced the expression of the activation marker CD69 on $CD4^+$ T cells, suggesting that smoking may contribute to T cell activation through TCR [418]. Our work showed that CSE enhanced CD69 expression which is consistent to the reported findings in literature. This may suggest that cigarette smoking could be an immunostimulatory as we also have shown, but the evidence in literature in this topic is still controversial and some studies different findings [419, 420].

Hyperactivation of T cells due to an increase in the signalling through the TCR could produce high amounts of aberrant inflammatory cytokines which drive a number of autoimmune disorders, including rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis [421]. Since we showed that CSE increased key inflammatory cytokines IL6, IFNγ and TNFα, this might explain the capability of smoking to enhance to role of T cells in inflammatory diseases. To investigate further of T cells contribution to disease process, a recent study reported that cigarette smoking associated with systemic inflammation by inducing $CD4^+$ Th17 lymphocytes. This was related to oxidative stress and cytokines production. However, the inhibition of ROS prevented CSE-induced Th17, suggesting that CSE may directly contribute and promote pathogenic T cells [422]. Moreover, a study revealed that when $CD3^+$ T cells from mice exposed to cigarette smoking, were transferred to recipients lacking T cells this led to the pathogenic T cells generation which caused significant changes including increased expression of chemokines and cytokines, accumulation of neutrophils and monocyte/macrophage [423]. Another in vivo study showed that CSE increased the

proliferation and influx of $CD8^+$ T cells which was mediated by CCL3, suggesting that CSE could increase the pathogenic effect of $CD8^+$ T cell [424].

In summary, the CSE induced over-activation of T cells resulted in the generation of potentially autoreactive T cells which may then recruit other immune cells to sites of inflammation through chemokines. The studies described above showed that both $CD4^+$ T cells and $CD8^+$ T were activated and stimulated following CSE treatment. Our work has shown that naïve and memory cells in response to CSE increased cell signalling and cytokines expression through TCR activation which is consistent with the studies discussed. It may be possible that CSE has diverse impact on different subsets of T cell. For instance, if the total $CD4^+$ T cell population which contains a combination of T regulatory, memory and naïve cells exposed to CSE, it may severely depress the signalling of T cells and shows different effects.

6.4 Cigarette smoke and metabolism

This work showed that CSE significantly enhanced energy metabolism by increasing glycolysis and oxidative phosphorylation in both naïve and memory T cells. The effect of cigarette smoking on metabolism of the immune cells is complex because cigarette smoking contains many toxic substances which could have some immunosuppressive and immunostimulant roles. For instance, it has been shown that smoking increased oxidative stress which showed a direct suppression of mitochondrial respiratory chain enzymes, DNA damage and loss of mitochondrial ROS which contribute to pathogenesis of some disorders. However, the precise mechanisms are not fully understood [425]. Moreover, a study found that CSE induced changes in mitochondrial function and morphology in human airway smooth muscle, leading to alteration in proliferation and energy metabolism [426]. On the other hand, cigarette smoking was directly implicated in disease pathogenesis such as cancer

by increasing oxidative phosphorylation in stromal fibroblasts, suggesting that cigarette smoking could increase OXPHOS and thereby driving tumour initiation [427]. Furthermore, a recent study found that cigarette smoking overexpressed enzymes involved in lactate synthesis and glutamine metabolism, suggesting that chronic exposure to cigarette smoking could play a role in metabolism alteration[428]. Even short-term exposure to cigarette smoking could lead to alteration in metabolism. For example, it was reported that cigarette smoking increased glycolysis and palmitate utilisation (fatty acid synthesis production) which was accompanied by expression of carnitine-palmitoyl transferase 1 (CPT1, an enzyme found in the outer membrane of mitochondria) in alveolar cells, suggesting that short term led to metabolic alteration in lung alveolar cells [429].

These studies described above showed how cigarette smoking could have an inhibition or stimulus effect on energy metabolism. The obvious explanation of this reduction in metabolism might be attributed to the toxic nature of cigarette smoke. Indeed, cigarette smoke has more than 5000 components which could change the pattern of gene expression and protein production, damage DNA, altering cellular behaviour and decrease cell proliferation [430]. However, other evidence reported that smoke or nicotine at low concentration increased proliferation in lymphocytes, fibroblasts and epidermal keratinocytes [431, 432]. Our results showed that CSE increased energy metabolism at low concentration and decreased or showed no effect at high concentration. The high concentration could have a strong toxic effect which may promote cell damage and inhibit metabolism. However, we showed that the number of dead cells at high concentration was very low which means any effects of CSE in subsequent experiments would not be as a result of a loss of cell viability. Our work showed that even at high concentration of CSE, the

number of cytokines were augmented and some of substrates in signalling pathway were activated. Therefore, this may indicate that high concentration of CSE would not affect the overall viability of cells.

In summary, it appears that cigarette smoking could increase energy metabolism and promote inflammation. However, due to the fact that cigarette smoke contains many toxic substances, it may play a different role and decrease the rate of metabolism to a certain degree, but the underlying mechanisms are still poorly understood. The main target for the smoking is the lung and so the main cell exposed to smoking is the alveolar macrophage. These alveolar macrophages occur in high numbers in the lung and produce many ROS which may be involved in metabolism [433]. Therefore, it may be easiest to study the effect of CSE on macrophages metabolism as there are several pieces of evidence described which have investigated the interplay between lung cells and metabolism. Our result showed that CSE increased metabolism via activation of PI3K/Akt/mTOR pathway which contributes to cellular metabolism. This could help to explain the underlying mechanisms by which cigarette smoking enhances energy metabolism which could implicate this process in autoimmune disorders such as RA.

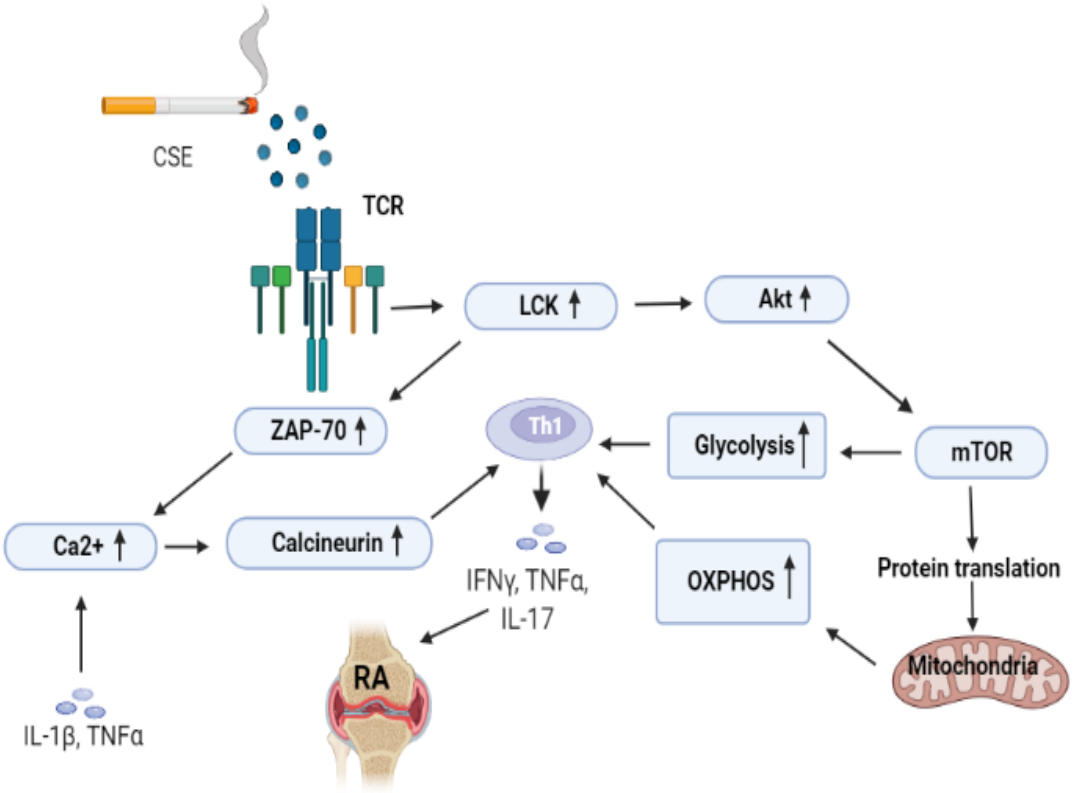


Figure 6. 1 A schematic representation of mechanism by which CSE could enhance signalling and metabolism in T cells.

CSE enhanced energy metabolism, including glycolysis and oxidative phosphorylation via PI3K/Akt/mTOR pathway. Also, CSE enhanced calcium signalling by increasing Lck and ZAP-70 activities. This may lead to Th1 induction and cytokines production which involve in joint inflammation. Drawn in BioRender.

6.5 PTPN22 and inflammatory T cells regulation

The presented work in this thesis has demonstrated that inhibition of Lyp which is encoded by the PTPN22 gene has a significant effect on T cell signalling. This work focuses on how losing Lyp function could contribute to signalling pathway activation and initiation of autoimmunity. We showed that CSE and Lyp inhibitor decreased Lyp phosphatase activity. Indeed, controlling the activation and signalling of the TCR is important in maintaining immunological tolerance and homeostasis. However, dysregulation of signalling networks controlled by Lyp could lead to unwanted consequences, such as autoimmunity and unregulated inflammation. One of the major risk factors for autoimmune development is the HLA class II allele which presents antigen to activate the T cell and which may allow auto-reactive immune responses. PTPN22, which encodes Lyp, has also been shown to be involved in the development of RA, and a study on T cells found that PTPN22 dephosphorylated the active site of Lck Y394 to regulate T cell responses and limit TCR proximal signalling. However, PTPN22-deficient mice developed widespread autoimmunity and inflammation which was associated with T cell infiltrates in non-lymphoid tissues and elevated levels of proinflammatory cytokines such as IL6 and TNF-α. Furthermore, the same study showed that deletion of PTPN22 enhanced the proliferation and activation of T cells in vitro by increasing the surface expression levels of CD69, CD44 and IL2 receptors (CD25/CD122). This study suggested that PTPN22 is a crucial negative regulator of TCR signalling to prevent inflammatory disorders caused by inflammatory T cells [434].

Mouse studies that have used the PTPN22 deficiency model have increased our understanding of how PTPN22 could play a role in maintaining T cell homeostasis and limit activation of T cells. An in vivo study showed that PTPN22 prevented the activation and the

proliferation of both effector and naïve T cells in response to low level antigen, suggesting that PTPN22 could be a therapeutic target for limiting inflammatory T cells [435]. A study on PTPN22 R620W variant showed that the inflammatory $CD4^+$ T cells was increased due to the expression of R620W, and this was associated with increased in the levels of IFN-γ and TNF-α [436]. Additionally, it has been shown that the isolated regulatory T cells from healthy individuals homozygous (AA) were not able to inhibit T helper 1 from producing IFN-γ [400]. These studies showed how PTPN22 R620W might increase inflammation and autoimmune diseases, by promoting inflammatory T cells to produce more inflammatory cytokines, and also a lack of regulatory T cells function. In contrast to the inhibition role of PTPN22 on Lck to limit the activation, our results showed that Lyp inhibitor treatment increased the phosphorylation of Lck tyr394, and decrease Lck tyr505 phosphorylation. Therefore, there was a decrease in the activity of Lyp phosphatase which led to the direct activation of subsequent targets such as ZAP-70, Vav1 and Akt which could play a role in activating inflammatory T cells which produce inflammatory cytokines such as IFN-γ and trigger inflammation.

In summary, CSE may interfere with many genes and proteins but based on our results, which showed that both CSE and Lyp inhibitor decreased the Lyp phosphatase activity and increased the phosphorylation of some known target of Lyp such as ZAP-70, Vav1, Lck and Akt, it seems likely that CSE can target Lyp. As a result CSE could interfere with Lyp in vivo and trigger inflammation. However, further experiments need to be done to validate this.

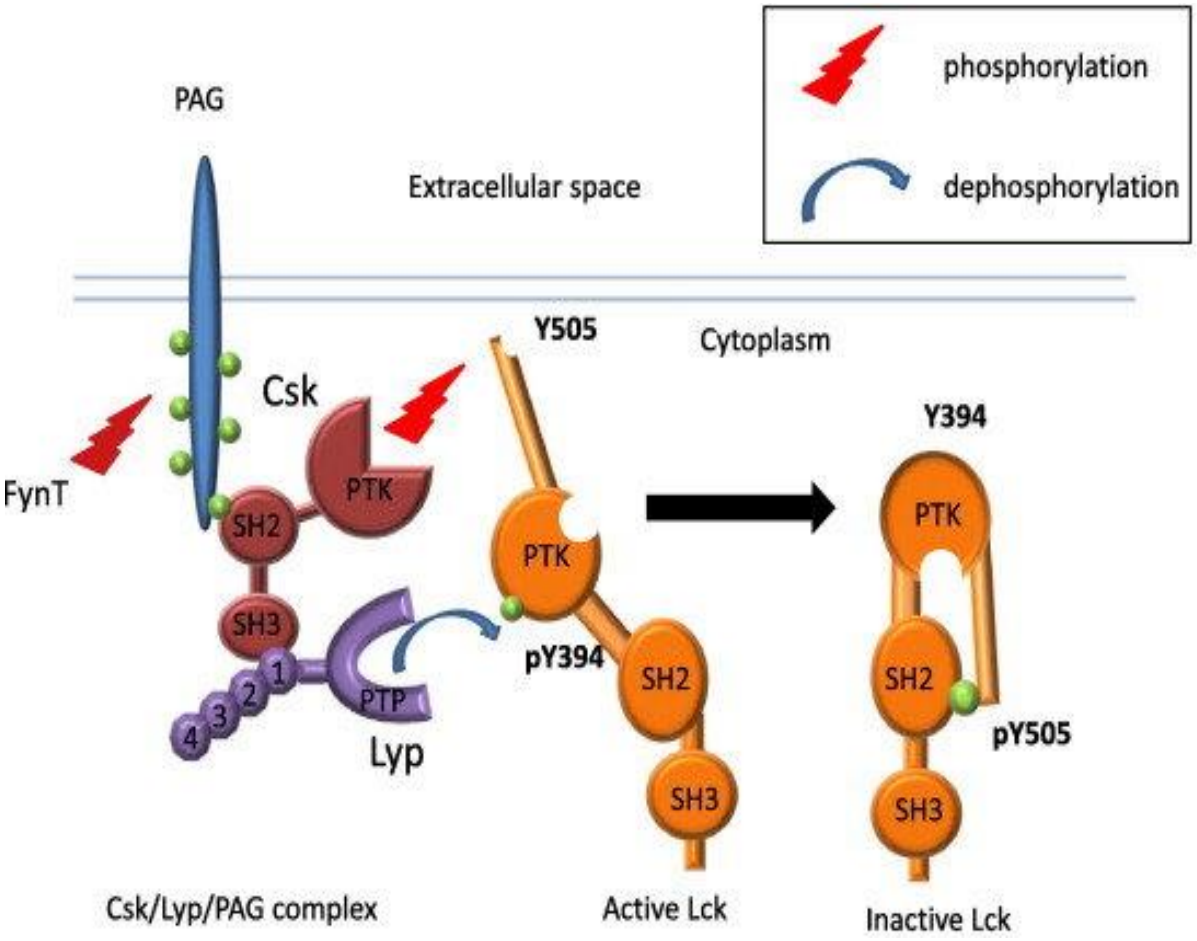


Figure 6. 2 A schematic representation of mechanism by which Lyp could regulate signalling and inflammation through Lck.

Lyp interacts with Csk and targeting the proximal TCR signalling and dephosphorylates the active site tyr394 within the kinase domain for Lck. To maintain the immunological tolerance, Lyp must phosphorylate the negative site tyr505 that results in an internal fold. Figure taken from (Burn et al. 2011)

6.6 How could PTPN22 or Lyp variant promote rheumatoid arthritis development in T cells

This work has focussed on investigating the underlying mechanisms by which smoking and Lyp dysfunction could promote RA development through changes in signalling pathways and metabolism. As stated previously the evidence which investigated the mechanisms by which Lyp operates in RA in particular and autoimmunity in general are still not yet known. One of the first studies reporting a link to PTPN22 showed a significant association of the RA with a PTPN22 variant with a high odds ratio of 2.63 for homozygous TT genotype. Moreover, the odds ratio also was higher in RF positive RA patients compared to RF negative RA patients [13]. Several studies have validated these findings and confirmed a link between smoking and ACPA and RA development. Most researchers have focused on T cells, and they suggested that PTPN22 variant could promote the survival of autoreactive T cells by altering the signalling of TCR during thymic selection. These T cells are then entered into circulation where can be activated by unknown trigger and develop RA and other autoimmune diseases [437, 438]. Given PTPN22 or Lyp variant can alter TCR signalling thresholds as described in this thesis, it may help T cells to trigger RA development.

Most reports in the experimental literature focussing on the PTPN22 variant and RA development have used mouse models. For instance, a study on mice with PTPN22 1858T variant found that Akt and ERK pathways were enhanced following TCR activation [439]. Another study found that IFNγ and IL-2 were enhanced, whereas IL-10 was inhibited, suggesting the crucial role of PTPN22 variant in RA development [440]. These studies described above showed that ERK which is activated by Ca^{2+} signalling, and Akt are involved in RA pathogenesis following loss of PTPN22 function which is consistent with our work, which also showed that loss of Lyp function led to a significant enhancement of Ca^{2+} and Akt

signalling either by stimulating cells with CD3/CD28 or PMA/Ionomycin. An interesting study investigated whether the PTPN22 variant is associated with RA in the UK. The study replicated the previous findings by Begovich and colleagues, who found a strong association of RA with the PTPN22 variant, specifically in people who were homozygous for T cell alleles [40]. This finding was confirmed by another study which showed that PTPN22 W620 in individuals who are homozygous for the T cell allele reduced binding of Lyp with tyrosine kinase Csk, thus decreasing the ability of immune system to regulate or downregulate T cells activation. In turn, these people would be more likely to develop RA due to overall increased reactivity of the immune system [441]. Another possible mechanism involved in RA development is that a potential association between PTPN22 or Lyp variant and autoantibody. A study reported that PTPN22 or Lyp negatively regulated the immune complexes which are formed of Fc receptors recognise autoantibodies binding to self-antigens. The study revealed that the *Ptpn22*$^{-/-}$ mice augmented CD4+T cell proliferation, increased presentation of immune complex and cytokine secretion due to Fc receptor signal via Syk and Src kinases, providing an association between PTPN22 or Lyp variant and autoantibodies and RA development [442].

In summary, the mechanisms by which PTPN22 or Lyp variant could promote RA are still not fully understood. However, some studies pointed out that dysregulation of kinase pathways which are regulated by Lyp could be involve not only in RA development but in many autoimmune diseases. Interestingly, in our work we showed that dysregulation of Lyp function led to enhancement of some kinases which are involved in RA. Our result may help to understand part of mechanisms by which PTPN22 or Lyp variant could promote RA in T cells. indeed, further work is needed to validate these results for example using patient

samples and mice models. Also, using different techniques such as genotyping and knocking out genes could help.

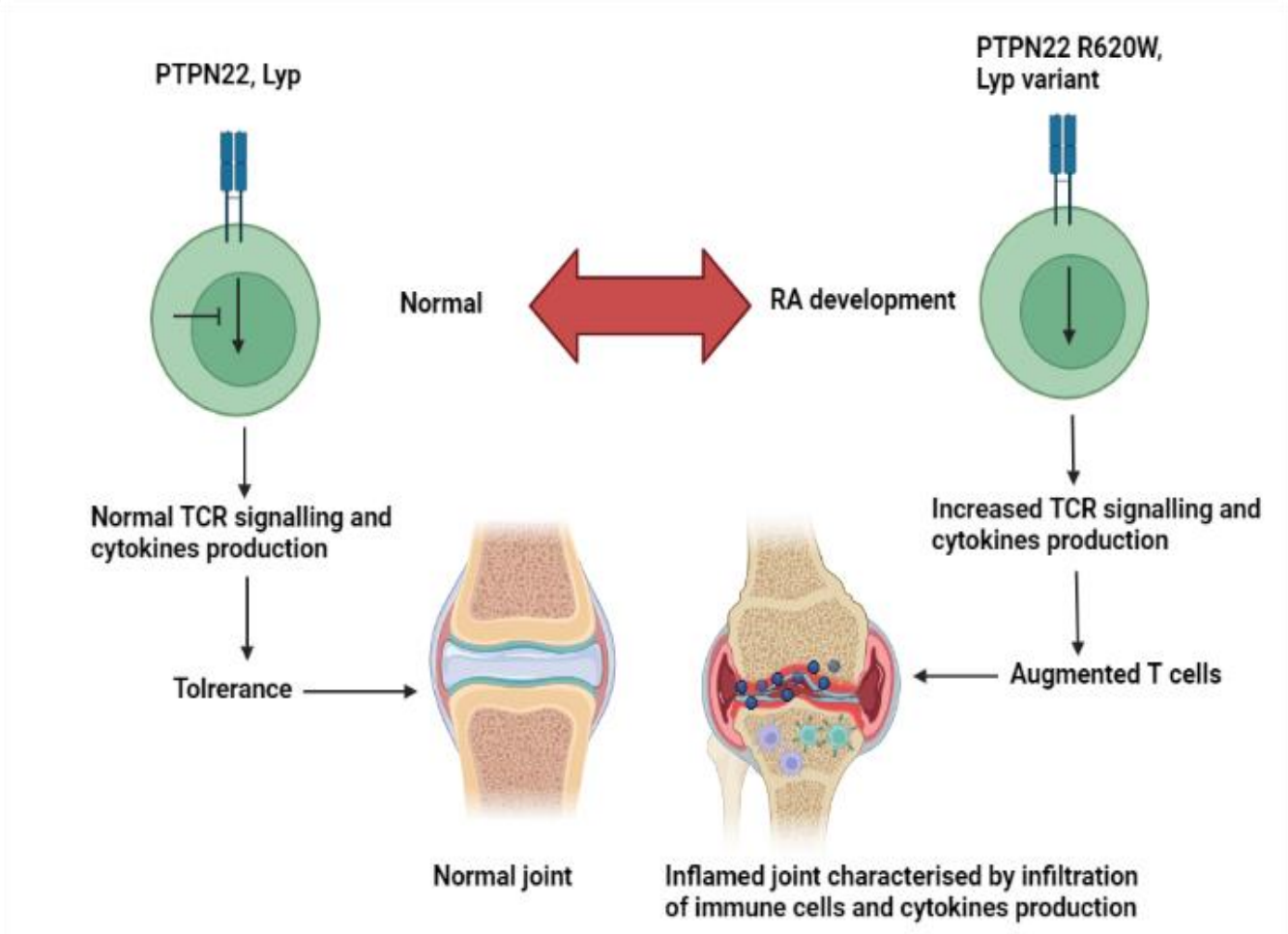


Figure 6. 3 A schematic representation of possible mechanism by which PTPN22 R620W or Lyp variant could promote RA development in T cells.

Normally, PTPN22 which encodes Lyp inhibit kinases activity and negatively regulates TCR signalling and cytokines production, leading to T cells tolerance. However, in the presence of PTPN22 variant, TCR signalling enhanced as well as cytokines production, leading to augmented T cells and infiltration of immune cells and pro inflammatory cytokines in the joints. Drawn in BioRender.

6.7 Further studies and limitations

The investigation in this thesis examined the effect of cigarette smoking and inhibition of Lyp function on energy metabolism and signalling pathways of human CD4+ T cells (naïve and memory) in healthy human donors. This investigation was done to enhance our understanding of how smoking increases the risk of inflammation and RA development. Results have discussed the effects of CSE on metabolism and signalling with reference to current literature, and presented the role of T cells dysregulation in autoimmune disease development. This project showed that CSE increased the activation of T cell and detailed the effect of CSE and Lyp inhibitor treatment on energy metabolism and signalling pathway of T cells, which have not been documented before, and indicated that CSE and inhibition of Lyp augmented T cells activation in healthy. Nonetheless, results obtained from this work have raised a question about how CSE and PTPN22 could interact to promote RA development.

6.7.1 Blood samples

The blood samples used in this work were provided by healthy donor via the NHSBTS. The main limitation of working on blood samples from healthy donor is the high level of variability because each blood cone comes from a different donor. These donors were of unknown age and sex, could have had underlying medical conditions or may have been smokers, all factors that could affect the data. However, in an attempt to minimise the donor influence, the experiments were repeated on as many samples as was feasible. To improve these results and outcomes, experiments could be carried out using T cells isolated from patients with RA because it would be better to investigate the effect of cigarette smoking on T cells which already autoreactive to observe if smoking could exacerbate the

disease. Also, working on patient samples could minimise the variability of the results, since more clinical details such as sex and age would have been available.

Finally, it would be useful to isolate and study naïve and memory T cells from synovial fluid of RA patients to investigate any difference in energy metabolism and signalling pathway between those infiltrating synovial fluid and peripheral blood from healthy donors, since these are likely to be the cells driving the major pathological processes.

6.7.2 Covid and some experiments limitation

There has been enormous disruption to the lab work because of the COVID pandemic. Since the start of pandemic, the lab work was limited, and some experiments were excluded due to lockdowns and restrictions. Safety and risk assessment process took almost a year to be approved. Also, since we are working on the blood cones provided by NHSBT, the process of approval due to COVID took extra time to be approved by university. Following the approval, the fresh human blood samples provided by the NHS Blood Service had to be screened using a custom PCR assay for the presence of the SARS-CoV-2 virus. Any positive samples obviously had to be discarded. Indeed, some planned experiments were affected. For example, since we investigated the effect of CSE and Lyp inhibitor on energy metabolism and signalling pathway, one of the planned experiments was to examine the synergistic combination of CSE and Lyp inhibitor to investigate whether both treatments can synergy and induce metabolism. This could help us to observe if there is any effect of CSE on Lyp following inhibition of its function. Another experiment planned was a migration assay to investigate whether CSE would increase the cell movement to the site of inflammation. It would be interesting to measure the ability of T cells to migrate to the site of inflammation. Because we showed that CSE increased cytokine production, this experiment could give us solid data

to show another aspect of the inflammatory role of CSE. Lyp inhibitor treatment was used to assess the function of T cells following inhibition of Lyp function. However, using inhibitors could also affect some results due to the off-target effects, meaning that they may not only work on intended target but also could work on different targets leading to inhibition of different pathways. To improve this work, it would be interesting to study the direct effect of CSE on PTPN22 or Lyp. This can be done by using siRNA knockdown genes to reduce the off-target effects or can be done using cells from patients with R620W variant. Furthermore, this work could be improved by measuring the activity of other phosphatases such as CD45 and PTEN which are also important in limiting T cell activity. This would allow us to determine whether CSE has an effect on Lyp or on other phosphatases.

6.7.3 Experiments design

Cigarette smoke extract was prepared by bubbling smoke from a lighted cigarette into PBS. However, some studies point out that normal media was used to prepare CSE to maintain the stability. Also, each study in the literature used different approach to prepare CSE, and used different brands of cigarette, so variability might be observed in these studies. Therefore, it would be interesting to improve our method and use different approach to investigate if there are any variations between these methods. Moreover, some studies suggested that to ensure consistency in the CSE preparation, the optical density measurement can be taken at 430nm wavelengths of 0.65 using spectrophotometer. Based on that, our method can be improved by measuring the CSE using the same method to achieve maximum consistency between experiments.

One of the limitations in our experiment design is that we did not add DMSO as a control when cells treated with Lyp inhibitor. In our experiments we setup different concentration of

Lyp inhibitor which reconstituted in DMSO, so it would be better if we added DMSO alone with cells to investigated whether DMSO would affect the function and the stability of the cells.

6.7.4 In vitro shortcomings and In vivo experiments

In this work we used in vitro approach to study the effect of CSE on energy metabolism and signalling pathway. In vitro experiment has many advantages and more effective to establish new protocols in the lab. In vitro studies are more appropriate to study the molecular mechanisms of action of specific treatment, and cells are easy to culture in the lab because the cells are derived from cell lines and animals. However, the main issues of using in vitro approach are that the variability between experiments can be seen which suggests that the data obtained might not be applicable or reliable to a wider population and it does not give an observation to the overall effects on all aspects. Also, the failure to convey the underlying complexity of organ systems is a key flaw. In vitro models, for example, may fail to account for cell-cell interactions and biochemical processes that occur during turnover and metabolism. Consequently, in vitro models have earned the reputation of being less harmonizable. Indeed, these issues can be overcome and increased the reproducibility of the data. This can be achieved by using in vivo approach as it is more appropriate to study the effect of a specific treatment in a complex model and help to minimise the variability between experiments. Our data showed that CSE altered the energy metabolism and signalling pathway in vitro. Therefore, it might be useful to translate this to in vivo by using mice tissues which are genetically identical to human and expose them to CSE to investigate whether the effect in vivo would be similar to the observed effect in vitro. This will allow us to track a variety of biological effects in complex organisms.

6.7.5 Future work

This project investigated the role of CSE and Lyp inhibitor in inducing perturbation in signalling pathways and energy metabolism in primary human CD4+ T cells in vitro. Our work was novel, and we found interesting data that could help our understanding of the mechanisms of RA development. However, every project has limitations, and we addressed these above but future work is needed to continue towards the ultimate goal which is finding an effective treatment for RA patients. One important approach will be to combine both the treatments with CSE and Lyp inhibitor because we found that both decreased Lyp phosphatase activity and increased signalling and metabolism. So, by combining them we might find a stronger synergistic effect on metabolism and Lyp activity. Another important approach could involve RNA sequencing which would allow us to measure the expression across the transcriptome of the cell which may be altered significantly by cigarette smoke. Once we established this, then we can confirm the results through PCR which will give us novel data that we can use to further explore the mechanisms by which CSE promotes RA development. Another important thing we can do as part of future work is to use individual components of cigarette smoke. For example, nicotine, acrolein or carbon monoxide are biologically active and might be contributing to the effects of the whole CSE. This can be combined with the application of our current approaches to investigate effects on signalling and metabolism but also using RNA-sequencing to detect single nucleotide variant, and mass spectroscopy to detect proteins related to metabolism and novel targets and features for those components. This would help us to identify any effect of those components on T cell signalling and metabolism and altered gene expression.

In our experiments we chose to incubate the cells for 24 hours following treatment. However, although this was based on published work and on previous work in the group, we may have missed more acute effects of the treatments on cell function. For example, some proteins and cytokines can be expressed early from minutes to a few hours. Furthermore, the activity of kinases and phosphatases may have changed immediately after CSE or inhibitor treatment, and may have recovered by 24hrs which means that what we observed may not have represented the maximum effects. It would be necessary to setup different time points to observe how the cells would react and which time point would give the maximum phosphorylation of each protein.

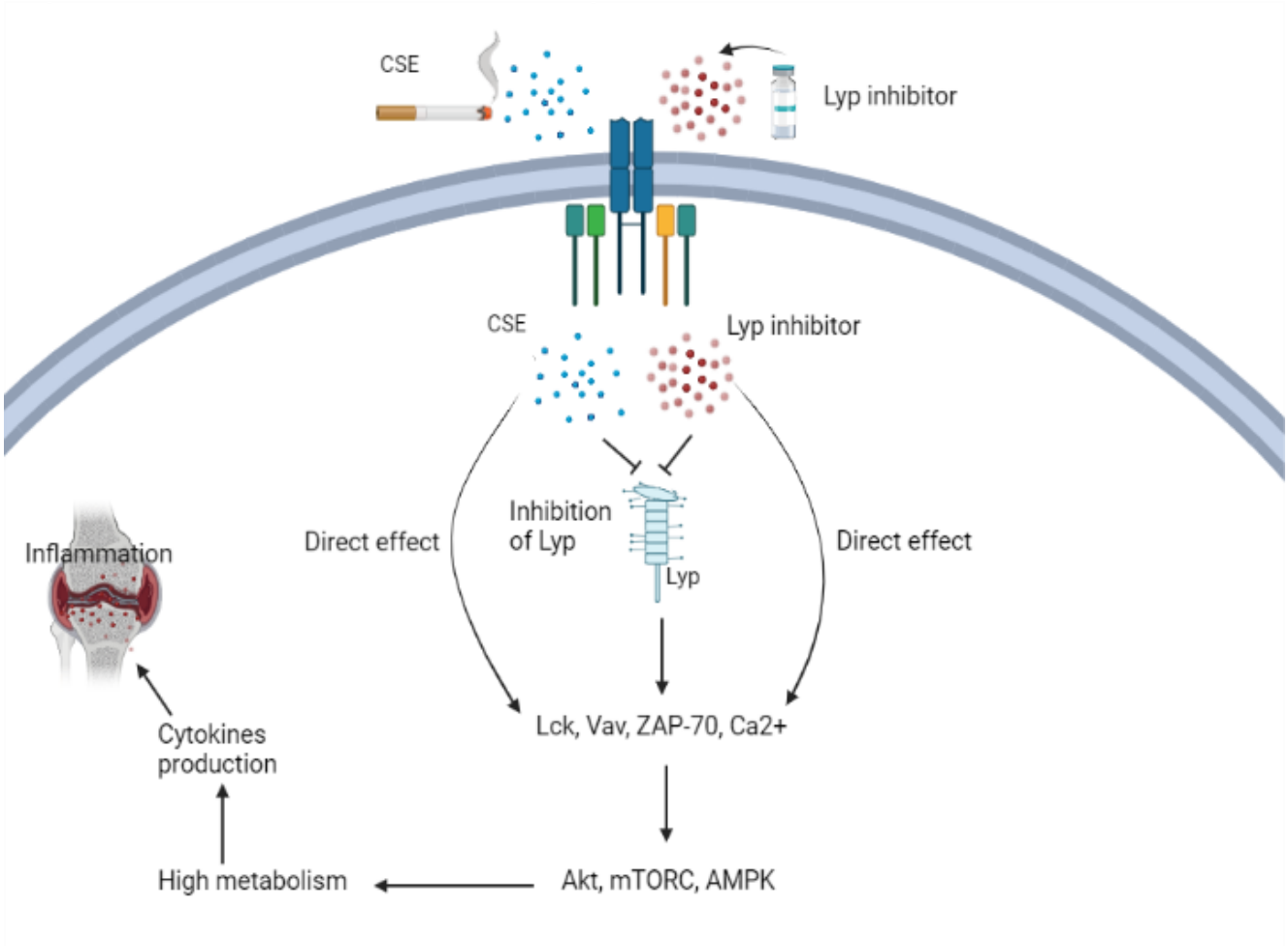


Figure 6. 4 A Summary of the project

CSE and Lyp inhibitor treatment decreased Lyp activity which led to an increase in signalling pathways such as Lck, Vav1, Ca2+ and PI3K/Akt/mTORC. This can be also a direct effect of CSE an Lyp inhibitor on signalling pathway. This led to an increase in cytokines production which promote inflammation. Drawn in BioRender.

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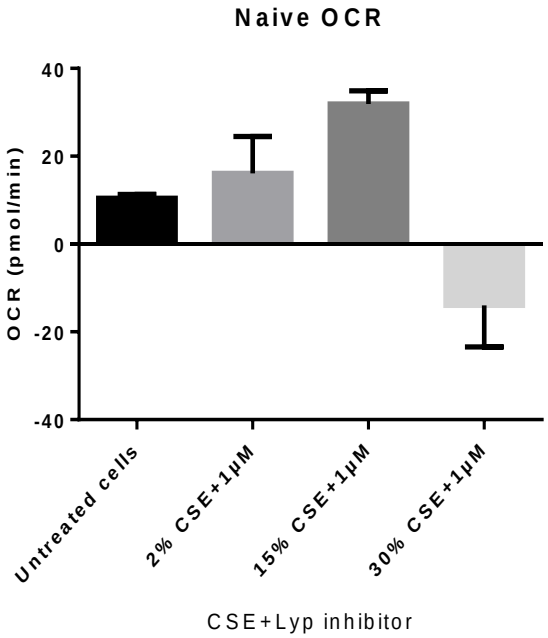
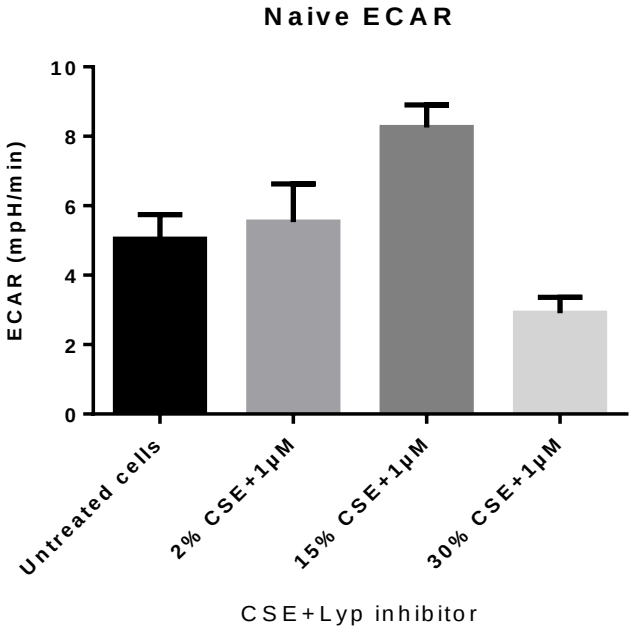
Publications

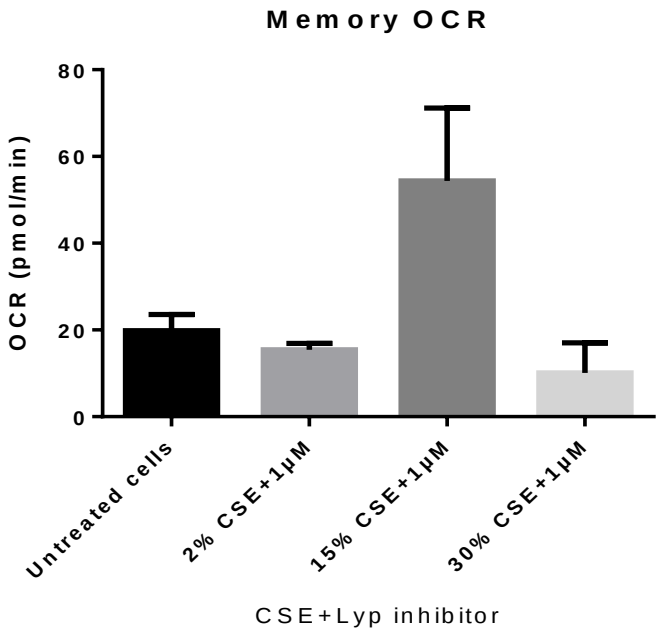
Differential Metabotypes in Synovial Fibroblasts and Synovial Fluid in Hip Osteoarthritis Patients Support Inflammatory Responses. Farah H, Wijesinghe SN, Nicholson T, Alnajjar F, Certo M, Alghamdi A, Davis ET, Young SP, Mauro C, Jones SW. Int J Mol Sci. 2022 Mar 17;23(6):3266.

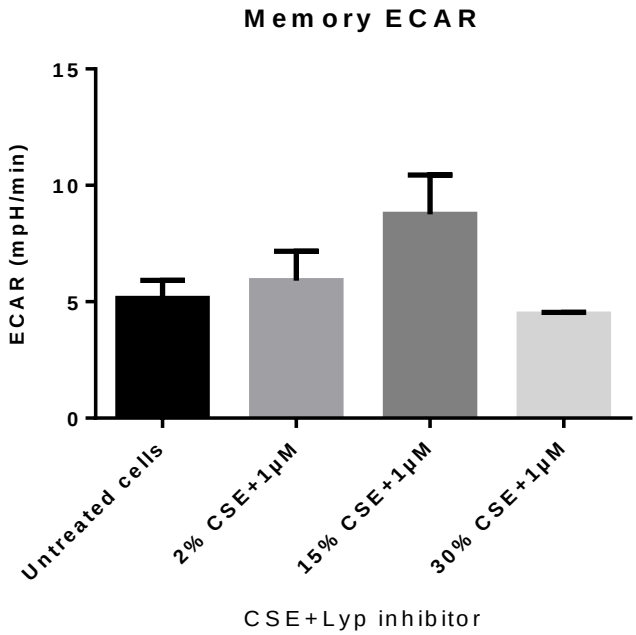
Appendices

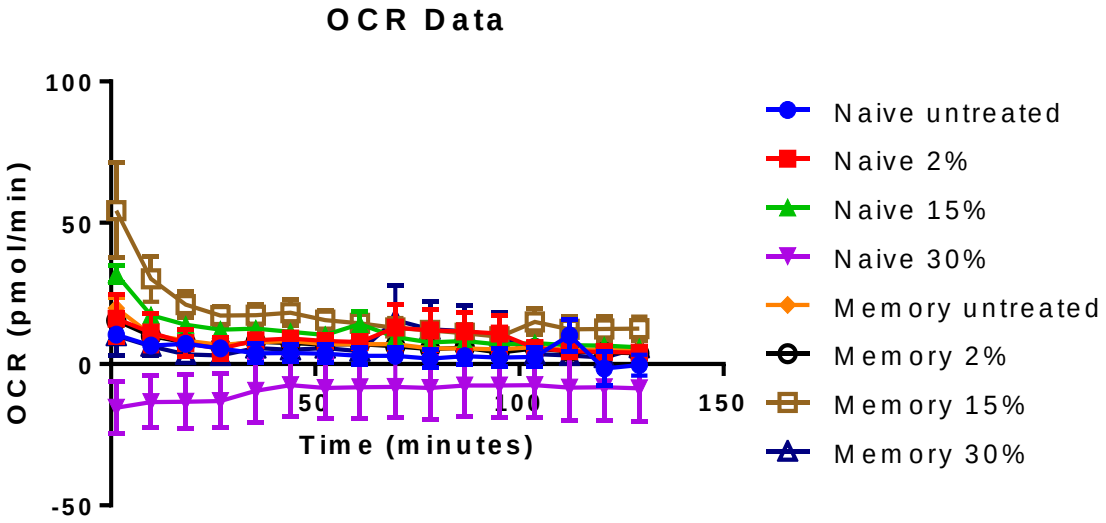
Appendix 1- CSE and Lyp inhibitor combined showed an increase in energy metabolism in naïve and memory T cells

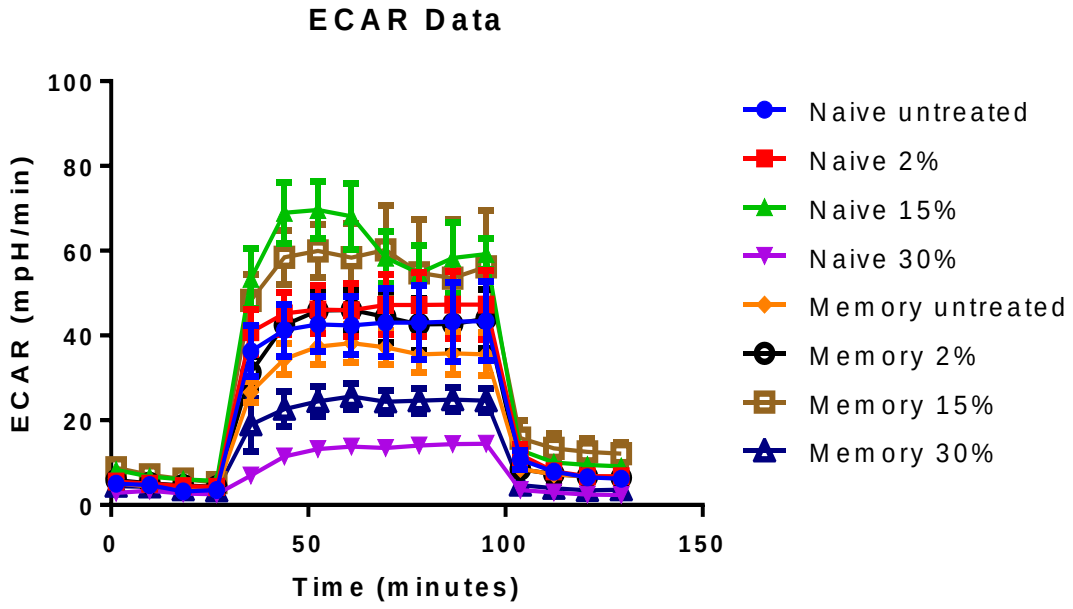












CSE and Lyp inhibitor enhanced combined enhanced ECAR and OCR in naïve and memory.

Naïve and memory cells were treated with CSE and Lyp inhibitor together for 24 hours and then activated with anti-CD3/CD28 for 72 hours. The level of ECAR (which represent glycolysis) and OCR (which represents oxidative phosphorylation) were increased following treatment which suggest that both treatments can work together and induce metabolism. Statistical analysis was carried out using GraphPad prism and significance was tested using ANOVA with Friedman's correction (*=p<0.05, **=p<0.01). (n=2)

Appendix 2- Blood cone ethical statement

Blood derived leukocyte cones from anonymous healthy adult blood donors were obtained from the NHS Blood and Transplant Service under ethical approval from the NHS West Midlands Research Ethics Committee (12/WM/0077) and the University of Birmingham Life and Health Sciences Ethical Review Committee (ERN_10-1246).

Appendix 3- Lysis buffer and Phosphatase reaction recipes

Lysis buffer (100ml)
20mM Tris, (xmg)
150mM NaCl, (xmg)
1mM EDTA, pH 7.4 (xmg)
1% NP-40 (1ml)
Take to pH 7.0
Add 100µl of Roche protease inhibitor cocktail to 1ml of TNE before use

Phosphatase reaction mixture (100ml)
0.1M sodium acetate (xmg)
1mM EDTA (xmg)
0.2% Triton X-100, (0.2ml)
Take to pH 6.0

Make 50mM with DTT before use (add 50ul 1M DTT per ml)

DiFMUP
Stock solution is 50mM
Dilute to 0.2mM for use
20ul aliquot to 5mls in phosphate reaction mixture with DTT

Carbonate Buffer pH 9.6
Make up capsule
Store at 4^{o}C