# Reactive oxygen species in protein sulphenic acid formation during T cell activation; implications for rheumatoid arthritis

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# Abstract

In recent years, the effect of reactive oxygen species (ROS) on T cell function in an immune response has gained increasing interest. T cell mediated chronic inflammation is influenced by changes in the redox environment. The cellular surface redox state plays an important role in receptor mediated T cell stimulation. Among many modifications, the reversible nature of sulphenic acid modified proteins act as redox switches to control protein function. T cell membrane proteins contain many cysteine residues that are susceptible to this modification in the presence of ROS. The source of ROS could be paracrine or autocrine by the NADPH complex (NOX) on T cell membrane in response to stimuli. Hence, the presence of free thiols or antioxidants such as thioredoxin (Trx1) and peroxiredoxin (Prx2) on the surface may influence interaction between the T cell receptor (TCR) and antigen presenting cells (APC). The main aims of this study were to analyse the surface redox state during T cell activation and to identify potential membrane proteins that undergo sulphenic acid modification in health and may be relevant to Rheumatoid Arthritis (RA). This thesis has used several approaches to achieve these aims: (1) A cell culture model of T cell-like Jurkat cells was challenged with hydrogen peroxide ($H_2O_2$) and novel ROS inducing compounds termed as ROX 1 and ROX 2. (2) Primary $CD4^+$ T cells isolated from healthy individuals were challenged with $H_2O_2$ during activation with anti-CD3 and anti-CD28 antibodies. Membrane sulphenic acid modifications were analysed by mass spectrometry approaches. (3) Collagen induced arthritis (CIA) mice model was used to analyse whether ROX plays a role in disease severity. Several sulphenated proteins were identified on the membrane of both Jurkat and primary $CD4^+$ T cells after activation. Activated T cells show increased expression of Trx1 and Prx2 on the surface along with reduced and oxidised thiols *in vitro* and *ex vivo*. ROX significantly inhibited T cell activation in human primary $CD4^+$ T cells and WT splenocytes. Compound ROX 2 that targets the NOX2 complex was shown to cause increased release of superoxide anions from WT mouse splenocytes in comparison to Ncf1-/- mice as measured by isoluminol. Results from this study indicated that ROS may not only be responsible for tissue damage but rather a means of modulating the immune response depending on the amount and location produced. This observation was shown confirmed by routine treatment of CIA induced mice with ROX 2. A decreased trend in the severity of arthritis was observed at later onset of the disease in WT mice with ROX 2 and there was no observable difference in Ncf1-/- mice. Therefore, targeting ROS represents a promising therapeutic strategy in dampening antigen-specific T cell responses and T cell-mediated autoimmune diseases. Further studies are required to examine the possible association of Trx1 and Prx2 on the surface proteins during T cell activation and rheumatoid arthritis.

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# List of abbreviations

ABC- ammonium bicarbonate

ACN- Acetonitrile

ACPA- Anti-citrunillated antibodies

ADAP- Adhesion and degranulation promoting adaptor protein

ANOVA- Analysis of variance

AP1- Activator protein 1

APC- antigen presenting cells

APS- Ammonium persulphate

ARF- auranofin

ASK1- Apoptosis signalling kinase 1

BCA- bicinchoninic assay

BSA- Bovine serum albumin

BSO- buthionine sulfoximine

CAIA- Collagen antibody induced arthritis

CBB- coomassie brilliant blue

CD25- cluster of differentiation 25

CD28- Cluster of differentiation 28

CD3- Cluster of differentiation 3

CD44- Cluster of differentiation 44

CD80- cluster of differentiation 80

CFA- Complete freund adjuvant

CIA- Collagen induced arthritis

CII- Collagen type II

CTLA-4- Cytotoxic T lymphocyte associated antigen-4

DA- Dark agouti

DC-Dendritic cells

DCFDA- 2',7'-dichlorodihydrofluorescein diacetate

DCP-Bio1- 3-(2,4-dioxocyclohexyl)propyl 5-((3aR,6S,6aS)-hexahydro-2-oxo-1H-thieno[3,4-d]imidazol-6-yl)pentanoate

DN- Double Negative

DNA- Deoxyribonucleic acid

DNPH- 2,4-Dinitrophenylhydrazine

DP- Double positive

DTNB- 5,5'-dithio-bis-[2-nitrobenzoic acid]

DTT- Dithiothreitol

EAE- Encephalomyelitis

ECL- Enhanced chemiluminescence

EDTA- Ethylenediaminetetraacetic acid

ELISA- Enzyme linked immunosorbent assay

EB- Elementary body

ER- endoplasmic reticulum

FBS- Foetal bovine serum

FC- Flow cytometry

FITC- Fluorescein isothiocyanate

GADS (GRB2 related adaptor protein

GAPDH- Glyceraldehyde 3-phosphate dehydrogenase

GCL- gamma-glutamyl cysteinyl ligase

GRB2- Growth factor receptor bound protein

Grx- Glutaredoxin

GSH- Glutathione

GSSG- Oxidised glutathione

$H_2O_2$- Hydrogen peroxide

$H_2SO_4$- Sulphuric acid

HCl- Hydrochloric acid

HLA- Human leukocyte antigen

HOCl- Hypochlorous acid

IAA- Iodoacetamide

IC- Isotype control

IgG- Immunoglobin G

IL-1β- Interleukin 1β

IL-2- Interleukin 2

IL-6- Interleukin 6

INF-ɣ- Interferon- ɣ

ITAM- Immunoreceptor tyrosine based activation motif

ITK (interleukin-2-inducible T cell kinase

LAT- Linker of activated T cells

LPS- Lipopolysaccharide

MAPK (mitogen activated protein kinase)

MFI- Mean/Median fluorescence intensity

MHC- Major histocompatibility complex

MIF- Migratory inhibitory factor

MS- Multiple sclerosis

MW- Molecular weight

NAC- N-Acetylcysteine

NADPH- Nicotinamide adenine dinucleotide phosphate

Ncf1- Neutrophil cytosolic factor 1

NFAT- Nuclear factor of activated T cells

NF-κB- Nuclear factor-κB

NK- Natural killer cells

NMDA- N-methyl-D-aspartate

NO- Nitric oxide

NOX2- NADPH Oxidase 2

OPD- *o*-phenylenediamine

PAD4- Peptidyl arginine deiminase type IV

PAMPs- Pathogen associated molecular pattern

PB- Pacific Blue

PBMC- Peripheral blood mononuclear cells

PDI- Protein dislulphide isomerase

PE- Phycoerythrin

PHA- Phytohaemagglutinin

PIA- Pristane induced arthritis

PLCɣ1- Phospholipase Cɣ1

PMA- Phorbol 12-myristate 13-acetate

PMCA- plasma membrane Ca2+ ATPase

Orai1- Calcium release-activated calcium channel protein 1

STIM1- Stromal interaction molecule 1

PI3K- Phosphatidylinositol-4,5-bisphosphate 3-kinase

PIL- Pristane induced lupus

Prx- Peroxiredoxin

PTEN- phosphatase and tensin homologue deleted on chromosome 10

PTK- Protein tyrosine kinase

PTM-Post translational modification

PTP- Protein tyrosine phosphatases

PVDF- Polyvinylidene difluoride membrane

RF- Rheumatic factors

RIPA- Radio immunoprecipitation assay

RNS-Reactive nitrogen species

ROS- Reactive oxygen species

RPMI- Rosewell Park Memorial Institute

SSA- Sulphosalicylic acid

SDS-PAGE- Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM- Standard error of the mean

-SH- Reduced thiols

SH2- Src homology 2

SLE- Systemic lupus erythematosus

SLP-76- SH” domain containing lymphocyte protein 76kDa

-SOH- Cysteine sulphenic acid

SP- Single positive

TCR – T cell Receptor

TEMED- N,N,N’,N’- tetramethylethyleneamine

Th1- Helper T cells 1

Th17- Helper T cells 17

Th2- Helper T cells 2

TNF-α Tumour necrosis factor α

Treg- Regulatory T cells

Trx1- Thioredoxin

TTBS- Tris buffered saline buffer

TXNIP- Thioredoxin interacting protein

WT- Wild type

ZAP70- Zeta chain associated protein kinase 70

# Chapter 1. General introduction

## 1.1. Immune system and inflammation

Inflammation is induced by invading pathogens or harmful stimuli. It is characterised by pain, heat, swelling and redness resulting in loss function of the inflamed tissue. These symptoms are due to host response towards antigens or toxins arising from invading pathogens such as pathogen-associated molecular pattern (PAMPs), lipopolysaccharide (LPS), and endotoxin (Mogensen 2009). The immune system that comprise of cells and anti-inflammatory/ resolution mediators, is responsible for activating and reducing this inflammatory response. The immune response is divided into innate and adaptive immunity. These two systems work together for the development of an effective immune response to eradicate harmful stimuli (Janeway et al. 2001; Lindstrom & Robinson 2010; Janeway et al. 2005) as shown in Figure 1.1.

### 1.1.1. Innate immune system

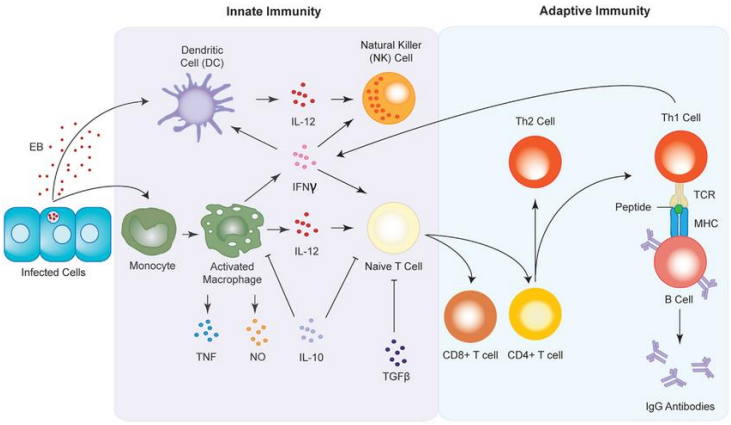
The innate Immune system comprises of natural killer (NK) cells, macrophages, dendritic cells (DC), neutrophils, basophils and eosinophils. These cells act as a first line of defence to initiate an immune response during invasion of foreign bodies including bacteria or pathogens. The activation of phagocytic cells leads to production of reactive oxygen species (ROS), and the release of cytokines and chemokines; the soluble mediators which mediate signalling pathways and recruit more neutrophils and monocytes to the inflammatory site. These soluble mediators include tumour necrosis factor- α (TNF-α), interleukins- IL-1β, IL-6, IL-10, IL-4, IL-12, IL-18 and interferon- ɣ (IFN-ɣ) (Lacy & Stow 2011). Neutrophils bind to invading pathogen and ingest at the site of infection, a process named phagocytosis. Microbial PAMPs interact with toll-like receptors (TLRs) on the surface of neutrophils, which initiates a signalling cascade allowing phagocytosis, release of cytokines, promotes degranulation and ROS release (Hayashi et al. 2003; Kobayashi & DeLeo 2009). The innate immune response by neutrophils is divided into two phases: 1) the generation of superoxide and other ROS by

nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex on the membrane, and 2) the release of granules which consists of proteins and degradative enzymes (Quinn 2004).

### 1.1.2. Adaptive immune system

The adaptive immune system is more efficient due to its targeted approach and immunological memory and comes into play with prolonged inflammation. The main cellular players in adaptive immune system are T and B lymphocytes. In order to initiate a cell mediated immune response by T cells, the recruitment and presentation of antigens is necessary and this is possible by the cells of the innate immune system including dendritic cells (DC) and macrophages (Janeway et al. 2001). The T cell receptor (TCR) on the cell surface is responsible for the interaction of a specific antigenic peptide on antigen presenting cells (APC) leading to their differentiation into effector T cells. These APC internalise pathogens via phagocytosis and present peptides to naïve T cells via the major histocompatibility complexes (MHC) (more detailed in section 1.4.2). The effector cells will then lead to the antibody production by B cells by delivering activating signals which targets specific antigens. Antibodies subsequently opsonise the pathogen, hence eliminating it after recognition by Fc receptors on innate immune cells (Janeway et al. 2005; Alberts et al. 2002). Following the elimination of pathogen, the activated T and B cells undergo apoptosis (Janeway et al. 2001). However, a few of these cells (both T and B) may differentiate into memory cells which trigger a quicker and robust immune response during second exposure to the antigen, also known as immunological memory (Janeway et al. 2005; Alberts et al. 2002).

a)




b)

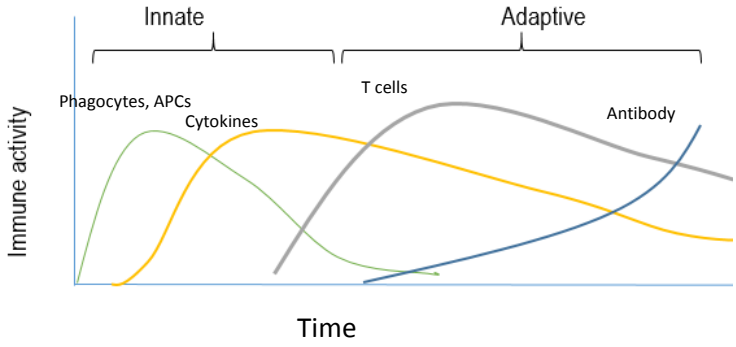



Figure 1.1: Innate and adaptive immune response. Upon infection, phagocytic cells are recruited as a first line of defence. Secreted cytokines and chemokines initiate a recruitment of immune cells to the site of infection. Prolonged infections leads to the recruitment of APC to the site of infection and present antigens and secrete pro-inflammatory cytokines and chemokines which will then induce the maturation of T cells into $CD4^+$ or $CD8^+$ T cells. The cellular pool of $CD4^+$ either differentiate into Th1 or Th2 T cells and produce antibodies by interacting with B cells (humoral response) against infection Adapted from (Redgrove & McLaughlin 2014), EB- elementary bodies. b) Time course showing the immune response following invasion of foreign pathogen.

### 1.1.3. T cell biology and maturation

T cells derived from hematopoietic stem cells migrate into the thymus from the bone marrow. The T cells in the thymus carry out several maturation steps that allow differentiation into alternative T cell lineages. This include the choice of committing to T cell lineage over B cell lineage and the choice of $CD4^+$ and $CD8^+$ lineages and between αβ to γδ T cell lineages. Developing T cells in the thymus also known as thymocytes then go through a maturation process which involves surface molecule expression including cluster, CD44 an adhesion molecule, and CD25, α-chain of the IL-2 receptor (IL-2R) and c-Kit, stem cell growth factor (Starr et al. 2003). Following this phase the TCR genes RAG-1 and RAG-2 expression is turned on facilitating rearrangement of the TCR genes leading to the origination of αβ to γδ T cell lineages and a lack $CD4^+$ and $CD8^+$ expression; these are considered as double negative (DN) (Dudley et al. 1995). Around 5-10% of the T cell subsets represent γδ thymocytes according to the expression of γδ TCR on the cell surface. The thymocytes expressing αβ TCR on the cell surface differentiate into double positive (DP) expressing $CD4^+$ and $CD8^+$ at the cell surface. Following this phase, negative and positive selection takes place whereby the thymocytes are further differentiated into either expressing $CD4^+$ or $CD8^+$ receptor at the cell surface, and are regarded as single positive cells (SP). Negative selection is the elimination of the DP thymocytes which strongly interact with the self-peptides. On the other hand, positive selection eliminates DP thymocytes that react with the TCR. The positively selected DP thymocytes then begin effective maturation depending on the interaction between the correct major histocompatibility complexes (MHC), into SP T cell lineage. Positively selected thymocytes become either $CD8^+$ cytotoxic T cell which recognises MHC class I complex self-peptides or $CD4^+$ also known as T helper cells (Th) which recognises MHC class II peptides (Kindt et al. 2007; Murphy et al. 2008; Starr et al. 2003) . Mature T cells which are positively selected leave the thymus and enter the periphery as mature naïve T cells.

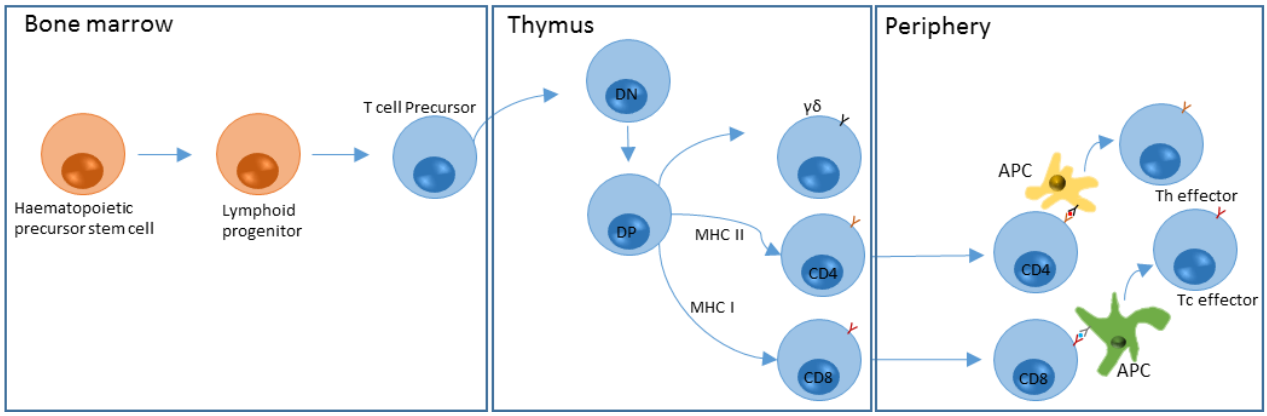



Figure 1.2: Schematic representation of T cell development and differentiation. T cell precursors migrate from the bone marrow into the thymus where they undergo positive and negative selection. Following migration of the positively selected cells into periphery, they can undergo antigen-induced activation by MHC II interacting with $CD4^+$ T cells and MHC I interacting with $CD8^+$ T cells, which results in proliferation and differentiation into T cell subsets (Helper T cells and cytotoxic T cells).

### 1.1.4. T cell activation and TCR signalling

Naïve T cells are present in the periphery until they encounter an antigen on antigen presenting cells (APC) for the activation. The nature of this interaction is highly specific as it leads to further differentiation and proliferation of naïve T cells into several T cell subsets. The T cell receptor (TCR) at the surface of naïve T cells recognises MHC peptides at the surface of APC, this interface is referred to as an immunological synapse (Joffre et al. 2009; Grakoui et al. 1999). Additionally, efficient T cell activation also depends on co-stimulatory molecules and chemical mediators expressed on the surface of APC. These comprise of CD80 or CD86 receptor on APC which interacts with either CD28 or CTLA4 expressed on naïve T cell and transmit necessary signal for proliferation and survival. The chemical mediators, such as interleukins (IFNα, IL-12, IL-4) , promote T cell differentiation into effector cells (Joffre et al. 2009; Pennock et al. 2013).

The TCR is a transmembrane protein which consists of peptide-MHC recognition complex (α and β chains) and intracellular signalling subunits (ɣ, δ, ε, and ζ chains or known as CD3 complex) and is collectively is referred to as TCR-CD3 complex (Birnbaum et al. 2014; Cantrell 2002). This can be also considered as a four-dimer complex. Once the MHC is presented with an antigen and binds to the TCR, the cytoplasmic domain of the CD3 chains consisting of immunoreceptor tyrosine-based activation motif (ITAM) interacts with protein tyrosine kinases (PTK) of Syk, Src and Tec families consequently initiating a signalling cascade (Brownlie & Zamoyska 2013).

A Src PTK member, lymphocyte-specific protein tyrosine kinase (LCK), phosphorylates ITAM allowing a docking site for a Syk PTK member, zeta chain-associated protein kinase 70 (ZAP-70) via Src-homology 2(SH2) domains. ZAP70 bound ITAM in turn phosphorylates the antigen receptor adaptors; liker of activated T cells (LAT) and SH2 domain containing lymphocyte protein of 76kDa (SLP-76). LAT acts as an anchor to various signalling proteins including phospholipase Cγ1 (PLCγ1), GRB2-related adaptor protein (GADS), adhesion and degranulation-promoting adaptor protein (ADAP) and growth factor receptor bound protein

(GRB2). Collectively, phosphorylated LAT and SLP-76 forms a platform for signal transduction molecules to be recruited and assembled, forming a cascade of signalling pathways during T cell activation. These include the Ras signalling pathways, mitogen activated protein kinase (MAPK) and nuclear factor-κB (NF-κB), mainly involved in calcium signalling, cytoskeletal reorganisation and regulation of transcription factors. The translocation of NF-κB and nuclear factor of activated T cells (NFAT), which is dephosphorylated by calcineurin in the presence of intracellular calcium ($Ca^{2+}$), into the nucleus allows transcription of cytokine related genes including IL-2, GM-CSF, IL-4 and TNF-α. These are essential for T cell differentiation and proliferation (Kuo & Leiden 1999; Lin & Weiss 2001).

The activation of T cells by peptides on APC induces T cell proliferation and differentiation into T cell subsets, and this process is also controlled by locally available cytokines. Primarily, cytokines are produced from APC but some cytokines produced from the differentiating T cells enhances proliferation in a positive feedback loop thereby amplifying differentiation reaction. For example, $CD4^+$ T cells when activated secrete IL-2 which induces T cell proliferation and differentiation. IL-2 is also secreted by $CD8^+$ and NK cells (Malek & Castro 2010). Besides its main role in T cell proliferation, it is also involved in activation induced cell death and Treg and $CD8^+$ T cell development. This process was shown to be regulated by IL-2 by maintaining the differentiated state of T cells. To allow this, secreted IL-2 in the extracellular environment binds to CD25 (IL-2 R) on the surface of T cells allowing further expression of the receptor by a paracrine effect. This is summarised in figure 1.3.

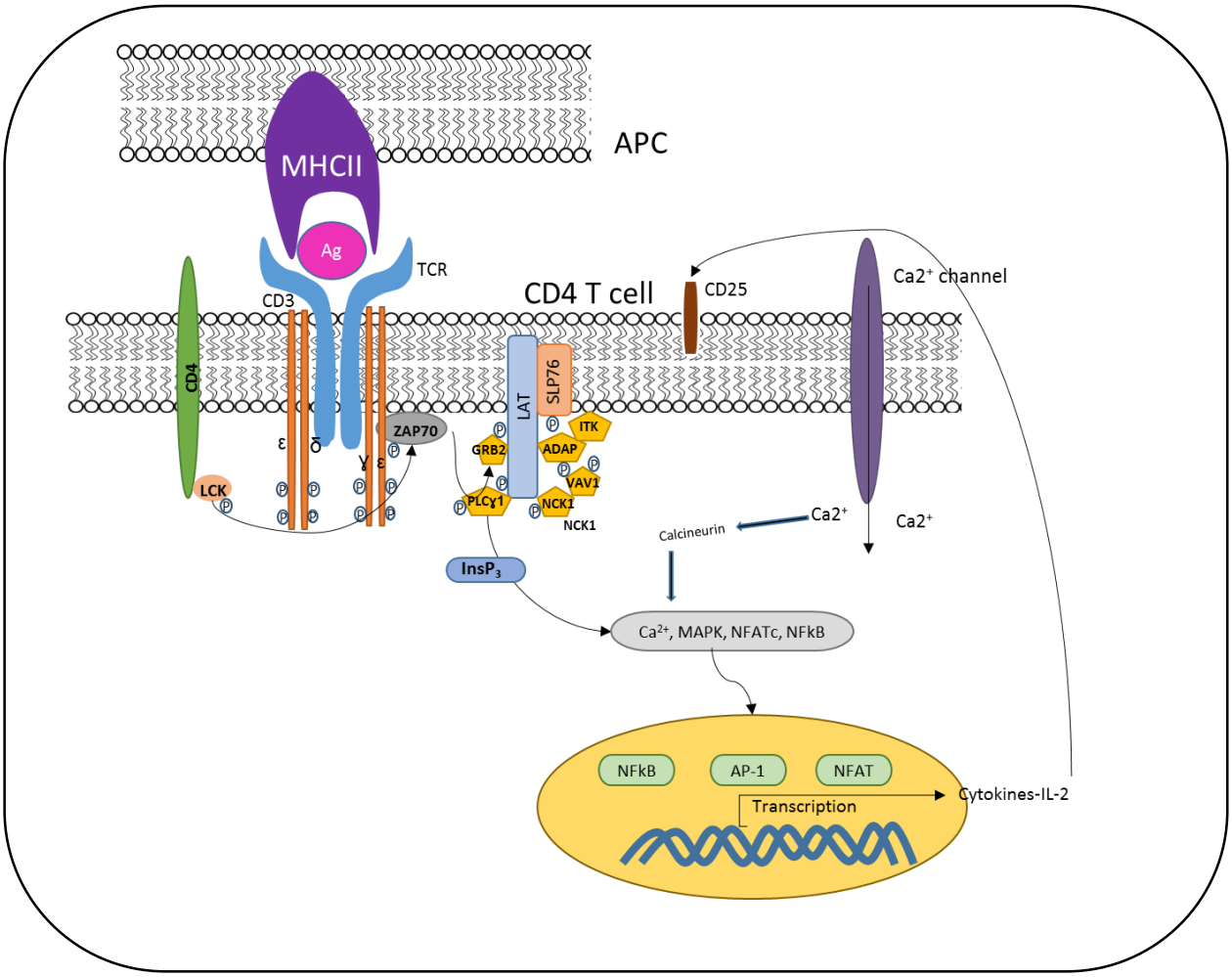



Figure 1.3: Schematic representation of TCR signalling. MHCII antigen presentation to TCR which initiates signalling cascade. The intramembrane domains are phosphorylated leading to phosphorylation of ZAP70 (zeta chain-associated protein kinase 70), LCK (Lymphocyte-specific protein tyrosine kinase), SLP-76 (SH-2 domain containing lymphocyte protein of 76 kDa molecules). LAT (Linker of activated T cells) behaves as an anchor to signalling proteins including GRB2 (growth factor receptor bound protein 2), GADS (GRB2 related adaptor protein), PCLγ1 (phospholipase Cγ1), NCK1 (non-catalytic region of tyrosine kinase adaptor protein 1), ADAP (adhesion and degranulation promoting adaptor protein) and ITK (interleukin-2-inducible T cell kinase). The signalling pathways initiated are MAPK (mitogen activated protein kinase), NFAT (Nuclear factor of activated T cells) and NF-κB (Nuclear factor-κB). This results translocation of transcription factors and synthesis of cytokines such as IL-2 (interleukin-2) which binds to CD25 (IL-2 receptor) for further proliferation and differentiation.

### 1.1.5. T cell differentiation

$CD4^+$ T cells which become activated via antigens on MHC II may differentiate into many helper subsets including Th1, Th2, Th17 and regulatory T cells (Treg). As mentioned above, specific cytokine signalling plays a major role in T cell differentiation into a particular subtype. Other factors involved are the concentration of antigen presented (Luckheeram et al. 2012).

The differentiation of $CD4^+$ T cells into Th1 is initiated by IL-12, which is secreted by APC. This causes NK cells to secrete IFNɣ suppressing Th2 differentiation. The production of these cytokines leads to activation of STAT1, STAT4 and Tbet transcription factors. Th1 cells secrete IL-2, LT-α, and IFN-γ and mainly involved in cell mediated immune response. IL-4 and IL-2 secretion are considered to be critical in Th2 differentiation. The transcription factor which regulates Th2 development is STAT6 which upregulates GATA3, c-MAF and NFATp. Th2 cells mainly produce IL-4 which regulates transcription factors suppressing Th1 differentiation. They are also involved in promoting B cell growth factors hence mediating allergic and humoral immune response (O'Shea et al. 2013; Zhu & Paul 2008; Feili-Hariri 2005; Luckheeram et al. 2012). Additionally, IL-6 has also been shown to play a role in Th2 differentiation. IL-6 promotes IL-4 production by naïve $CD4^+$ T cells via NFAT upregulation and inhibiting Th1 lineage (Diehl et al. 2000).

Further subtypes of $CD4^+$ T cells are Th9, Th22, Th17, follicular T cells (Tfh) and (Tregs). Th9 are characterised as subset of Th2 T cell and transforming growth factor-β (TGF-β) is identified as the responsible cytokine for Th9 differentiation. Th9 are mainly involved in defence against helminths and in allergy and they secrete higher levels of IL-9 (Veldhoen et al. 2008; Anuradha et al. 2016; Tan & Gery 2012).

The TGF-β and IL-2 cytokines are responsible for the differentiation of Treg from naïve $CD4^+$ T cells. The downstream signalling following TCR interaction and TGF-β ligand receptor binding induces FOXP3 expression which is uniquely expressed in $CD4^+CD25^+$ Treg cells. Also, the secretion of IL-2 promotes expression of STAT5 which enhances the expression of

FOXP3. The main role of Tregs is to modulate self-tolerance by secreting TGF-β, IL-10 and IL-35 (Brandenburg et al. 2008; Chen et al. 2003; Luckheeram et al. 2012).

Th17 subset is mainly differentiated in the presence of TGF- β and IL-6 cytokines. It also involves different steps of maturation which depend on IL-21 and IL-23. These cytokines act on the transcription of RORɣt, which is responsible for the production of IL-17. These cells are mainly observed in autoimmune conditions such as rheumatoid arthritis and multiple sclerosis. They are also involved in an immune response caused by invading bacteria (Marwaha et al. 2012).

### 1.1.6. T cells and ROS

Reactive oxygen species levels are known to play a part in T cell reactivity. ROS that are released from surrounding cells or endogenously produced from activated T cells, could regulate the outcome of an immune response (Kesarwani et al. 2013a; Chen et al. 2016). NADPH oxidase on the membrane is responsible for releasing ROS during interaction with T cells. The NADPH complex consists of several NOX subunits which are located both intracellularly and transmembrane. NOX2/gp91phox is located on the membranes and dimerises with p22phox to form flavocytochrome b558 complex. Other subunits located are in the cytosol (Ncf1 (p47phox), Ncf2 (p67phox) and Ncf4 (p40phox)) in resting state (Babior 2004; Babior 1999). Sufficient levels of ROS in T cells are produced by NOX2, mitochondria and DUOX1 (A. V Belikov et al. 2015).

ROS production occurs during TCR stimulation from NOX2 and mitochondria which are regarded as the main sources of ROS as shown by 2',7' –dichlorofluorescin diacetate (DCF-DA)(Phillips & Griffiths 2003) and MitoSox oxidation in primary $CD4^+$ T cells (Tse et al. 2010; Laura A Sena et al. 2013). $H_2O_2$ is a commonly ROS species to assess the effect of oxidative stress on T cell activation, mainly because it is diffusible through the plasma membrane and prominent source of ROS within cells (Winterbourn 2013).

The presence of intracellular ROS in T cells is thought to be the third signal following the antigen presentation and cytokine release towards the enhancement of T cell proliferative response (Tse et al. 2007). This was shown by Los and colleagues, whereby low levels of $H_2O_2$ induced the transcription factor NF-κB and IL-2 gene expression along with IL-2α receptor (Los et al. 1995). Also, ROS act as an essential mediator in T cell function where certain antioxidants such as cysteamine inhibit proliferation and IL-2 production when administered during the early stages of T cell activation (Hultqvist et al. 2006; Goldstone et al., 1995). In recent years, the effect of ROS and the role of T cells in an immune response has gained increasing interest as T cells are influenced by changes in the redox environment (Simeoni & Bogeski 2015a). When T cells reside in highly inflammatory milieu, in the presence

of ROS releasing phagocytic cells, their cellular redox state has shown to be altered (Angelini et al. 2002).

This was evident by increased release of antioxidants and upregulated intake of amino acid, cysteine, for GSH synthesis (Levring et al. 2015). To combat high level of oxidative stress within inflammatory site, T cells are equipped with several antioxidant defence mechanisms including peroxiredoxins, superoxide dismutases and glutaredoxins (Hanschmann et al. 2013a).

T cell activity is based upon expression and interaction of many surface receptors and proteins with APCs. Among the amino acids, cysteine residues are prone to be modified by oxidants such as $H_2O_2$. This is because they may be ionised at physiological pH, are stable in a number of oxidation states, are least abundant and hence have a high specificity for oxidative posttranslational modifications (Marino & Gladyshev 2010). Therefore, T cell proteins are highly susceptible for oxidative modifications. There is an increased amount of evidence suggesting an increase in cell surface thiol (-SH) levels in T cells when stimulated (Gelderman et al. 2006). Others have also demonstrated that activation leads to an increase in sulphenic acid (-SOH), the first reversible oxidation product of cysteine (Pellom et al. 2013; Michalek et al. 2007)).

In normal physiological conditions, the balance between ROS and antioxidant systems is maintained, and is required for the appropriate functioning of T cells. However, any disruption to this equilibrium by changing the levels of ROS or antioxidants can result in T cell hypo- or hyper-responsiveness, which, in turn, may lead to the development of various pathologies including T cell-mediated Rheumatoid Arthritis (RA); a chronic autoimmune disease caused by reacting to self-antigens (Gelderman et al. 2006a; Phillips et al. 2010; Hultqvist et al. 2009a)

## 1.2. Oxidative stress

Oxidative stress is an imbalance between reactive oxygen species (ROS) and antioxidant levels (Kregel & Zhang 2007). When ROS production overcomes the cellular defence systems, there is an alteration in redox homeostasis (Eruslanov & Kusmartsev 2010) cellular functions are changed.

### 1.2.1. Reactive oxygen species

ROS are chemically reactive molecules known to cause reversible and irreversible damage to biomolecules including lipids, proteins, and DNA (Freeman & Crapo 1982) leading to change of function, or tissue injury. Levels of ROS and the microenvironment where ROS have been produced are important factors which determine the extent of the damage. Excess amounts of ROS give rise to oxidative stress which results in molecular damage such as DNA-protein crosslinks leading to cell death and apoptosis (Nordberg & Arnér 2001; Kamata & Hirata 1999), Lipid peroxidation (Ylä-Herttuala 1999) and amino acid oxidation generating modified non-functioning protein (Stadtman & Berlett 1998; Griffiths 2000).

However, it has been widely shown that a low level of ROS play a critical role in controlling infection and modulating T cell responses (Kesarwani et al. 2013a; Griffiths 2005; Chen et al. 2016). ROS produced by various cell types in the immune system include: superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide ($H_2O_2$), singlet oxygen ($^1O_2$), and hydroxyl radical ($^{\bullet}OH$) and anion ($OH^-$).

Accumulating evidence suggests that ROS are not only harmful to cells and tissues but also behave as signalling molecules such as $H_2O_2$ (Rhee 1999).The presence of ROS at minimal concentrations (0.02–0.13 μM $H_2O_2$) can have beneficial effects, such as cell cycle (Sarsour et al. 2009) and resolving inflammation (Mittal et al. 2014). Also, moderate levels of ROS have been recognised to participate in intracellular signalling pathways (Finkel 2011; Apel & Hirt 2004; Thannickal & Fanburg 2000).

Therefore, ROS act as mediators to change protein structure.

### 1.2.2. Oxidative post translation modification

The reactions of proteins with increasing amounts of free radicals lead to protein oxidation. As explained in section 1.2.1, the thiol groups are the major target for reversible redox modifications. They can be oxidised in various ways: two cysteine residues can form a disulphide forming an inter- or intra molecular disulphide bridge. (Forman et al. 2010; Foster et al. 2009; Hamnell-Pamment et al. 2005; Hanschmann et al. 2013b). However, the functional consequence of protein oxidation depends on the particular modification and the modified amino acid. This may further lead to inhibition or activation of a protein involved in signalling pathway. Other types of PTMs are oxidative modifications, including carbonylation, glutathionylation and disulphide bridge formation.

Many thiol oxidative PTMs are considered as reversible and control metabolic pathways (Ghezzi et al. 2005; Ratnayake et al. 2013). However, a number of studies show the harmful effects of irreversible oxidative PTMs as a result of increasing ROS. Also, oxidative modified proteins have been linked to a number of diseases and disorders including ageing, degenerative disease, atherosclerosis and arthritis (Gianazza et al. 2007; Ryan et al. 2014).

### 1.2.3. Other PTMs and role of ROS

Other types of post translational modifications include, phosphorylation, ubiquitination, glycosylation, and S-nitrosylation. In some cases, these modification are influenced by the increase in ROS. For example increased ROS inhibits phosphorylation of tyrosine (Flescher et al. 1994) and PLC ɣ-1 (Cemerski et al. 2002). Additionally, glycosylation of CD45 on the T cell membrane is a dynamic process that modifies T-cell survival, activation and immune function (Earl & Baum 2008) and ubiquitination of LCK (Jury et al. 2003). ROS can also lead to modifications of proteins irreversibly. Reversible cysteine modifications can play in important role in cell signalling and function. Transcription factors including NF-κB and AP-1 undergo reversible thiol oxidative modifications that regulate their functions (Abate et al. 1990;

Nishi et al. 2002). Also phosphatases such as PTEN (phosphatase and tensin homologue deleted on chromosome 10) and SHP-2 (Scr homology 2 domain-containing phosphatase) are inhibited by thiol oxidation (Kwon et al. 2004; Michalek et al. 2007; Kwon et al. 2005).

### 1.2.4. Cysteine sulphenic acids

Cysteine residues are uncommon in proteins in comparison to other amino acids, but have an important functional role (regulatory, catalytic) (Qu et al. 2014). This is mainly due to high reactive nature of cysteine hence low abundance of cysteine on protein surface (Marino & Gladyshev 2010). The importance of this residue is due to its unique sulphur containing functional thiol group (-SH) (Leninger et al. 2005; Chung et al. 2013), and plays a ubiquitous role in allowing proteins to react with ROS or RNS giving rise to various oxidative PTM (Rudyk & Eaton 2014). The redox sensitivity of the cysteine residue plays a role in ROS mediated signalling and function as redox switch as it was originally identified as intermediates during a biochemical reaction. (Barford 2004; Chung et al. 2013). However because of the unstable nature and difficulty in capturing, the free thiol may be oxidised to form a disulphide (intra or inter-molecular) bond with another available –SH (Miseta & Csutora 2000). An intermolecular disulphide bond occurs when two cysteine of different proteins react with each other. As the role of ROS in intracellular signalling become further clear, the importance and identification of oxidised proteins as molecular switch can be looked into.

Likewise, reduced cysteine maybe oxidised to form cysteine sulphenic acid (RSOH) derivatives; intermediates to higher oxidation groups such as sulphinic ($RSO_2H$) and sulphonic acid ($RSO_3H$) derivatives which are considered to be irreversible losing protein structure and function. The availability of a terminal free thiol also allows formation of other (oxidative PTMs) Ox-PTMs including S-nitrosylation (SNO), sulfydration (SSH) and S-glutathionylation (SSG) (Chung et al. 2013; Leslie B Poole et al. 2004b; Poole & Nelson 2008; Ryan et al. 2014). Sulphenic acid and disulphide bond formation is important to maintain a reducing environment

as they are reversible and form a major component of cell mediated signalling pathways (Poole & Nelson 2008). As shown in Fig 3.1

### 1.2.5. Detection of sulphenic acid modification

Since cysteine sulphenic acid is unstable in several proteins, a number of studies have been carried out to monitor and identify proteins and sites that have redox-sensitive cysteines but with limited success. However, the selective targeting and designing of a probe to detect a desired functional group can be challenging. One of the first probes to be designed was presented 1974 by Benitez and Allison as 5, 5-dimethy-1, 3-cyclohexadione (dimedone). These studies have used various methods including dimedone, which traps cysteine sulphenic acid (Leonard et al. 2009; Reddie et al. 2008; Poole et al. 2007; Poole & Nelson 2008). The use of dimedone has been proven to be a useful tool to tag sulphenated proteins and further identification of particular protein requires advanced techniques such as mass spectrometry. Lately, the dimedone based technique was further optimised and tagged with fluorescence or biotinylated tag which allows affinity capture using streptavidin and SDS-PAGE. Biotin linked dimedone allows enrichment of proteins forming sulphenic acids using streptavidin HRP. The first biotinylated probe was 3-(2,4-dioxocyclohexyl)propyl-5-((3aR,6S,6aS)-hexahydro-2-oxo-1H-thieno[3,4-d]imidazole-6-yl)pentanoate (DCP-Bio1) (Poole et al. 2007). This was also used in this study to capture proteins undergoing sulphenic acid modification. DCP-Bio1 was first used to detect sulphenated proteins in $CD8^+$ T cell lysates and identified several including protein tyrosine phosphates (PTPs), SHP1 and SHP2 (Michalek et al. 2007).

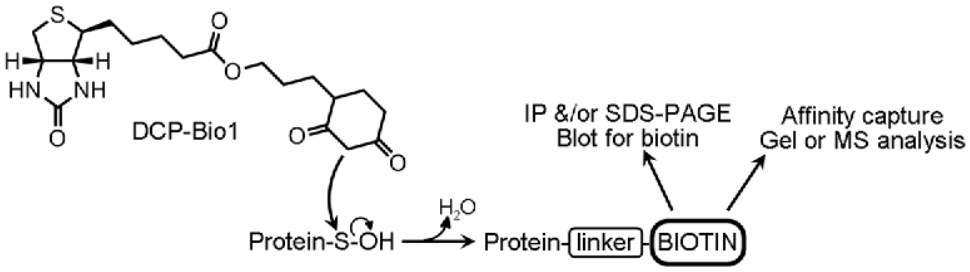



Figure 1.4. Dimedone reaction with sulphenic acid modification within proteins. Increased oxidative stress leads to localized cysteine sulfenic acid formation on proteins which can be detected using DCP-Bio1 (biotin linked dimeodone). (Adapted from Klomsiri et al 2014)

So far, direct and indirect approaches have been used to detect sulphenic acid in proteins mainly relying on the selective targeting probes (Leonard & Carroll 2011).

Mass spectrometry has evolved over the years as a critical tool in biological analysis. Since protein post translational modifications are recognised as essential for cellular signalling which includes the localisation and regulation of proteins, MS provides high throughput and in depth investigations of protein oxidative modifications. Studies with dimedone followed by MS have identified various sulphenated proteins and proteins which were sensitive to oxidation. However, the current MS technology of detecting affinity captured CySOH proteins or peptides still requires further optimisation for analysis of complex biological mixtures (Nelson et al. 2010).

Indirect approaches to detect sulphenic acid modification include the use of alkylating agents such as N-ethylmaleimide (NEM) and iodoacetamide (IAA) which react readily with thiolate anions (Ying et al. 2007). This method involves blocking all thiols with NEM then reducing –SOH with reducing agents such as DTT. The reduced thiols can be labelled with biotin maleimide and identified with SDS-PAGE and western blotting and also MS analysis. This is described as the ‘biotin switch’ approach (Saurin et al. 2004; Furdui & Poole 2014). However, proteins which have no cysteine residue were identified and hence the sensitivity of the approach was regarded as inefficient (Saurin et al. 2004).

## 1.3. Redox regulation by antioxidants

Several antioxidant enzymes are mainly involved in the detoxification of ROS such as catalase, superoxide dismutases (SOD), peroxiredoxins and also, low molecular weight peptides and proteins including GSH and thioredoxins which are present in abundant concentrations will react with oxidised thiols and hydrogen peroxide (Holmdahl et al. 2016).

SOD protects cells from oxidative stress by catalysing the dismutation of superoxide radical anions to water and $H_2O_2$. Three isoforms of SOD are present within cells; SOD1, SOD2 and SOD3. SOD1 is primarily located in the cytoplasm, SOD2 is present in the mitochondria (also known as manganese SOD, MnSOD) and SOD3 in the extracellular matrix (Gao et al. 2003). SOD2 in the mitochondrion protects from normal by products of cellular processes. This is vital as it is the first line of defence against superoxide produced from the mitochondria, also regulating apoptosis (Pardo et al. 2006)

$$2O_2^{\cdot -} + 2H^+ \xrightarrow{(SOD)} H_2O_2 + O_2$$

Additionally, the importance of SOD2 in T cells was shown in a study with SOD2 conditional knockout mouse. Lack of functional SOD2 lead to an increase in superoxide which eventually caused apoptosis and inefficient T cell development (Case et al. 2011). This effect was later recovered using drugs targeting mitochondrial superoxide. This suggests that lack of functional scavenging of mitochondrial superoxide production can lead to abnormal T cell functions.

### 1.3.1. GSH

Glutathione (GSH) is the most abundant tri-peptide antioxidant present in the cells, consisting of L-glutamate, L-cysteine and glycine. Under normal physiological conditions, GSH exists either free or bound to proteins. The free cysteine acts as a target for ROS hence oxidising

the reduced GSH to oxidised GSSG disulphide. This can further be reduced to GSH in the presence of glutathione reductase and NADPH (Ghezzi 2011) leading to free reduced glutathione. The ratio of GSH/GSSG serves as a marker of oxidative stress and is vital for the cells survival and response to oxidative damage (Zitka et al. 2012; Grant & Griffiths 2007; Carilho Torrao et al. 2013; Phillips et al. 2010). A decrease in this ratio; reduced to oxidised GSH might indicate the presence of oxidative stress at cellular or tissue level (Rahal et al. 2014). Similarly, around 1-5% of total GSH is present in its oxidised form GSSG suggesting a reducing milieu within the cell and so any change in this ratio can indicate the presence of oxidative stress (Winyard et al. 2005) The main role of GSH is studied by depletion of reduced glutathione using inhibitors such as buthionine sulfoximine (BSO) and has been associated with several diseases including rheumatoid arthritis and human immunodeficiency virus (HIV) (Ghezzi 2011; Sahaf et al. 2005; Gelderman et al. 2006a; Hildeman et al. 1999). Mammalian GSH is essential within cells as it is involved in cell proliferation and differentiation, prevention of apoptosis and protection against protein aggregation (Go et al. 2013a; Armstrong et al. 2002). The synthesis of GSH depends on available cysteine in the cytoplasm and the activity of γ-glutamylcysteinyl ligase (GCL) enzyme. Additionally, the presence of free thiol group in cysteine provides GSH its antioxidant activity of ROS scavenging. However, the levels of cysteine in T cells are low and given the fact that T cells lack oxidised form of cysteine (cystine) transporter, they rely on dendritic cells for cysteine availability. In contrast APC have the appropriate cystine transporter and its reduced in the cytoplasm hence secreted in the extracellular space (Yan & Banerjee 2010). Several studies have described the important role of GSH in T cell function by depleting GSH using BSO. For example, depleting GSH using BSO impairs T cell function as measured by IL-2 secretion and this was rescued by the addition of endogenous IL-2 (Hadzic et al. 2005).

### 1.3.2. Trx1

The Trx1 system is an essential system for protection against oxidative stress and plays a critical role in redox regulation. It consists of NADPH as a source of electrons, an enzyme thioredoxin reductase (TrxR), which catalyses the reduction of oxidised Trx1 (Trx1-S-S) to reduced Trx1-SH (Figure 4.2). Trx1, a 12kDa protein, has a highly conserved active site motif with two cysteine residues at position 32 and 35, allowing transfer of electrons to the substrate protein (Lee et al. 2013; Arnér & Holmgren 2000). It is primarily found in the cytosol but also translocated in the nucleus and on the cell surface. The expression of Trx1 can be upregulated in the presence of variety of stress stimuli including oxidative stress, hypoxia, lipopolysaccharide (LPS) and more. In addition to its main function as redox regulating enzyme (reducing disulphide bonds and sulphenic acid), Trx1 also acts as an anti-inflammatory agent and involved in apoptosis. It modulates immune response via regulating chemokine and cytokine activity and lipopolysaccharide (LPS) induced oxidative damage (Hanschmann et al. 2013a; Carilho Torrao et al. 2013; Nordberg & Arnér 2001; Nakamura et al. 2005). Trx1 may also be secreted via a leaderless secretary pathway exerting effects on adjacent T and B cells. Secretion of Trx1 is increased under oxidative stress conditions and inflammation (Riccardo Bertini et al. 1999; Mougiakakos et al. 2010b; Rubartelli et al. 1992). Hence, extracellular Trx1 may influence ligands and receptors in the surface such as IL-4 and CD4 (Moolla et al. 2016a; Curbo et al. 2009). Due to its various functions, Trx1 has many interaction partners such as apoptosis signalling kinase 1 (ASK1) and actin, where Trx1 bound ASK1 regulates apoptosis and preserving Trx1 degradation (Lillig & Holmgren 2007a; Zschauer et al. 2011). In the nucleus, it plays a role in modulating the DNA binding activity of transcription factors such as activator protein 1 (AP1), p53 and nuclear factor-κB (NF-κB) (Hirota et al. 1997; Kabe et al. 2005). Thioredoxin 2 (Trx2) is another isoform of Trx which is described in the literature. Trx2 is a mitochondrial redox protein sharing the common active site and plays an important role in embryonic development by allowing resistance to ROS-

induced apoptosis (Nonn et al., 2003). Similar to Trx1, Trx2 knockout is embryonic lethal and increases TNF-α expression which leads to cell death (Zhang et al., 2004).

Post translational modification to the free cysteine on Trx1 may influence its function and localisation within the cell. Glutathionylation of Trx1 leads to reduction in the enzymatic activity of Trx1 under oxidative stress but it is shown to regain its activity again by means of auto activation (Haendeler, 2006).

Trx1 has been linked to range of diseases in which redox imbalance has been associated, including HIV, cardiovascular disease, neurodegenerative disease and cancer (Collet & Messens 2010).

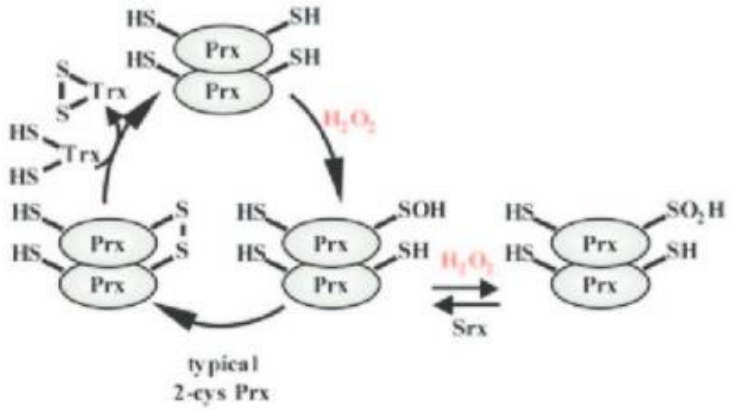
### 1.3.3. Prx

Peroxiredoxins (Prxs) are a group of thiol exchange proteins which are mainly involved in breaking down hydrogen peroxide, peroxynitrite and organic hydroperoxides. They are also recognised to be the prominent members of the antioxidant defence system (Rhee & Woo 2011; Poynton & Hampton 2014). They are susceptible to oxidation and act as redox sensors regulating cell signalling pathways (Fourquet et al. 2008). Mammalian Prxs are categorised into six different isoforms; Prx1, and 2 which are located primarily in the cytosol, Prx3 and 5 in the mitochondria and Prx4 in the ER. 1-cys Prx6 which is located in the cytosol, lysosomes and vesicles (Sorokina et al. 2011; Sorokina et al. 2009). Prx1 and 2 are also associated with the membrane of lymphocytes (Szabó-Taylor et al. 2012a; Hanschmann et al. 2013b). Their biochemical function is categorised based on the active cysteine that is oxidised to sulphenic acid during detoxification of $H_2O_2$ and peroxynitrite. In a typical 2-Cys Prxs the sulphenic acid may further react with adjacent cysteine to form a disulphide whereas in 1Cys Prx, –SOH is reduced by low molecular weight thiol such as GSH (Poynton & Hampton 2014; Peskin et al. 2007). 2-Cys Prx can also form decamers and the conformation regulates molecular function (Barranco-Medina et al. 2009; Turner et al. 2013) The active cysteine which is sulphenated can also react further with $H_2O_2$ to form sulphinic acid. This has been shown to be reduced by

sulfiredoxin (Srx) specific to 2-Cys Prxs (Woo et al. 2005). However, this reaction is slow hence oxidised Prx can exist in the cells after exposure (Stacey et al. 2012). The maintenance of reduced Prx pool is essential as 90% of $H_2O_2$ produced in the mitochondria reacts with Prx3 (Cox et al. 2010). Prx oxidation has been of keen interest and hence recognised as redox sensitive proteins and markers of oxidative stress. Prx2 was oxidised in Jurkat cells when treated with 20µM $H_2O_2$ (Baty et al. 2005). Therefore, Prxs are major scavengers of signalling ROS such as $H_2O_2$, playing a critical role in T cell redox signalling. Kwon et al, showed that overexpression of Prx2 increased activation of MAPK and ERK kinase pathway upon TCR stimulation and crosslinking by mitogens (Kwon et al. 2003).

Posttranslational modification influences the catalytic activity of Prx and susceptibility to hyperoxidation. These modification include glutathionylation, phosphorylation and s-nitrosylation, all are said to influence either the structure or the catalytic activity of Prxs (Park et al. 2011; Engelman et al. 2013; Chang et al. 2002)

A




B

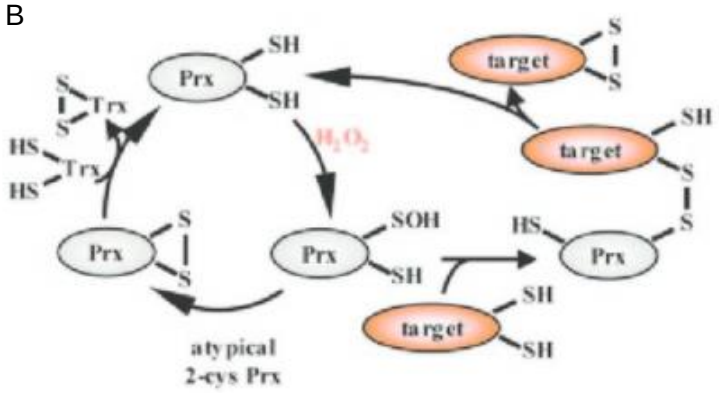



Figure 1.5. Peroxiredoxin enzymatic cycle. A and B in the presence of $H_2O_2$, cysteine oxidation leads to cysteine sulphenic acid which reacts with adjacent cysteine to form disluphine bind. The peroxidatic cycle ends with the reduction of the disulfide bond by thioredoxin and regeneration of reduced enzyme. (A) In typical 2-Cys peroxiredoxins (Prxs), this disulfide forms between two monomers, whereas (B) in atypical 2-Cys Prxs and CysG- Pxs, it is intramolecular. (Adapted from Forquet et al. 2008)

## 1.4. Autoimmunity and RA

The main function of the immune system is to initiate an immune response to harmful stimuli. This response needs to be discriminated between self and foreign antigens. The immune system is developed to prevent an inflammatory response against host structures or cells, (self-antigens) commonly referred as self-tolerance (Alberts et al. 2002). This mechanism is developed during T cell selection, whereby T cells which recognise MHC antigens too strongly and self-reactive are eliminated (Lindstrom & Robinson 2010). However, a proportion of T cells may be released from the thymus into the blood stream thus reacting to self-antigens

leading to an autoimmune response and Tregs play a critical role in eliminating self-reactive T cells (Wing & Sakaguchi 2010).

Autoimmune disease arises when immune cells are reacting to antigen expressed in tissues and cells resulting in an autoimmune response. These diseases include; rheumatoid arthritis (RA), multiple sclerosis (MS), systemic lupus erythematosus (SLE) and psoriasis.

RA is characterised by accumulation of inflammatory cells in the synovial membrane, these include lymphocytes (T and B cells), neutrophils and monocytes. The disease onset is characterised by swelling leading to inflammation, pain and lack of function in the joints (Scott et al. 2010; Bartold et al. 2010). Patients with RA show anti-citrullinated protein antibodies (ACPA) which are autoantibodies directed against citrullinated peptides, and rheumatoid factors (RF), which correlate with disease severity (Masi et al. 1976; De Rycke et al. 2004). RFs include antibodies against the Fc region of IgG. In most cases patients with RA show RF in association with ACPA, however, ACPA are more specific and used as a diagnostic marker such that it can even be found before typical onset of clinical symptoms (Van Der Linden et al. 2009; Rantapää-Dahlqvist et al. 2003; van Venrooij et al. 2002).

RA affects approximately 0.5-1% of the world population (Silman & Pearson 2002; Smolen et al. 2016). The inflammatory response is characterised by the activation of T cells which also activates monocytes, macrophages and synovial-like fibroblasts to secrete pro-inflammatory cytokines including IL-6, IL-1β and TNF-α. This is also associated with infiltration of inflammatory cells producing ROS and RNS ultimately leading to bone and tissue destruction (Bennett & Griffiths 2013). The underlying cause of RA is not known yet but it is believed that environmental and genetic factors are involved in the pathogenesis of the disease (Symmons et al. 1997; MacGregor et al. 2000), proposing that genetic factors make up to 60% contribution. Since RA is an aggressive chronic inflammatory disease, early diagnosis and effective treatment is essential. However, there is no cure for the disease yet but the treatment available may relieve the symptoms. these include Disease Modifying Anti-Rheumatic Drugs

(DMARDs) which are targeted toward specific abnormalities of the immune system such as TNF-α blockers infliximab, adulimumab (Kahlenberg & Fox 2011).

### 1.4.1. The immunogenetics of RA

Since RA is a complex disease, the genetic element is also complex thus depending on several genes and interactions. The first gene associated with RA was human leukocyte antigen (HLA), this was found in 1976 (Stastny 1976). The molecular diversity of HLA-DR1 still remains dominant genetic factor which contributes to RA (Olsson et al. 2016). HLA-DRB104 has been consistently found to confer increased risk of RA development and susceptibility (Gregersen et al. 1986; Weyand et al. 1992). This leads to approximately 30% of RA risk due to HLA genes (Deighton et al. 1989) making HLA the critical genetic factor in RA. However, some non-HLA genes have also been suggested to be associate with RA, such as protein tyrosine phosphatase non-receptor 22 (Gregersen 2005). PTPN22 is critical in the regulation of immune response and cell signalling. A single nucleotide polymorphisms in PTPN22 (Begovich et al. 2004), leads to weaker TCR signalling hence failure to delete self-reactive T cells in the thymus and decreased Treg function (Vang et al. 2005). In addition, cytotoxic T lymphocyte associated antigen-4 (CTLA-4), peptidyl arginine deiminase, type IV (PAD4) genes antihave been associated with RA (Orozco et al. 2006; Suzuki et al. 2003; Foulquier et al. 2007).

### 1.4.2. Models of arthritis

Various animal models have been developed to better understand the disease aetiology and pathways involved. The risk factors can be controlled including genetic manipulation and environmental conditions can be maintained. However, the limitation of animal models may be that the factor that initiates the disease in humans is unknown whereas in animals there is a detailed cause of the disease which is more or less reproducible. These include; pristane

induced arthritis, arthritis induced by bacteria, collagen induced arthritis and collagen antibody induced arthritis.

1.4.2.1. Pristane induced Arthritis (PIA)

Pristane induced arthritis includes the use of mineral oil pristine leading to severe arthritis in dark agouti (DA) rats after two weeks (Vingsbo et al. 1996). This model provides high resemblance to human RA as it shows symmetrical disease development and serum RF (Wernhoff et al. 2003). Despite, the absence of antigens in this model, PIA was shown to be T cell driven and MHC II dependent (Holmdahl et al. 1992; Holmberg et al. 2006). However, this was only observed in the chronic phase of the disease. PIA is specific to rats and not inducible in species other than rats, though some studies show PIA in mice (Hopkins et al. 1984), but the disease severity and phenotype is different.

1.4.2.2. Collagen induced arthritis (CIA)

This model is normally induced in rats and mice; mice CIA is a widely used model for CIA. It involves immunisation with type II collagen, a major collagen found in cartilage. Together with the adjuvant, clinical symptoms of arthritis develop after 2-3 weeks of immunisation. The disease is similar to PIA and human RA, however it can be easily be differentiated as CIA involves an immune response towards a known antigen as opposed to PIA (Holmdahl et al. 1992). CIA is dependent on both T and B cells together with pathogenic antibodies which attacks the joints (Corthay 1999; Svensson et al. 1998). *In vivo* activation of T cells require glycosylated CII molecules to be presented as peptides by APC. The particular APC is not identified yet but is likely to be macrophages compared to DCs as the latter present collagen antigen poorly (Holmdahl et al. 2002). The immune synapse formed by the interaction of MHC II and T cells doesn't not provide sufficient information on the pathogenesis of CIA, although provides a better scope for further analysis.

1.4.2.3. Collagen antibody induced arthritis (CAIA)

CIA is associated with the autoantibody production against type II collagen, ACPA and IgG. The anti-type II collagen is detectable a few weeks after immunisation and hence if the serum from a CIA immunised mouse is transferred to non-immunised mice, they may develop arthritis (Stuart & Dixon 1983; Holmdahl et al. 1990). This demonstrates the basic mechanism of CAIA, that disease transfer is achieved as the mice are injected with CII antibodies. Following this model, various cocktails have been developed allowing identification of autoantibody epitopes and the role of humoral autoimmunity in RA (Asquith et al. 2009). The clinical symptoms of CAIA is similar to CIA, but the onset of CAIA is immediate within 1-2 days of injection. The elimination of this model based on adaptive immunity is that it is one of few models or arthritis that is not dependent on T and B cells, however, CII reactive T cells have been shown to increase the severity (Nandakumar et al. 2004). The innate immune system is involved after collagen antibody binding to cartilage recruits neutrophils and activates macrophages (Nandakumar et al. 2003b).

1.4.2.4. Arthritis induced by bacteria

A number of bacteria are known to cause arthritis in animal models suggesting that infection may play a role in the induction of an autoimmune disease. One example of this may be Lyme arthritis whereby bacteria such as *Borrelia burgdorferi* can accumulate in joints leading to swelling and arthritis. This form of arthritis show a similar clinical symptoms to humans and essentially the bacteria is also found in synovial fluid of humans with RA (Nocton et al. 1994). Additionally, mice infected with this bacteria develop RA (Masuzawa et al. 1992). Furthermore, *Staphylococcus aureus* is the main cause of septic arthritis which can persist in the joints for a long time. It also develops in rats and mice when injected with the bacteria (Bremell 1999; Bremell et al. 1991; Bremell et al. 1994). Other structures from the bacteria including cell wall, DNA and proteins can also lead to arthritis in animals. For example, mycobacteria arthritis in rats, whereby rats develop arthritis when induced with *Mycobacterium tuberculosis* in specified adjuvant (Pearson 1956). This further lead to the use of complete Freund adjuvants (CFA) in current arthritis models, which consists of mineral oils, emulsifier and bacteria (Freund 1947).

The use of autoantigens together with CFA completely diminished tolerance in rats resulting in an autoimmune disease (Lipton & Freund 1953).

#### 1.4.2.5. Other models of RA

In addition to inducible models of arthritis, there are other spontaneous models of RA such as transgenic genetic modification technology which develop arthritis. These include the overexpression of TNF-α or IL-1 (Keffer et al. 1991; Niki et al. 2001). Genetic modification in TNF-α leads to the development of chronic inflammatory polyarthritis and mice treated with antibodies against TNF-α showed prevention against the disease. This is useful in terms of recognising the pathology of the effector cytokines in the progression of the disease and the inflammation involved in cartilage and bone destruction. Furthermore, a single gene mutation arthritis models such as the SKG mouse, single mutation is found e.g. in ZAP-70 which is a signal transduction molecule in T cells shown in Fig 1.3 (Sakaguchi et al. 2003; Chan et al. 1992). The SKG model represents RA by showing high levels of RF and anti-CII autoantibodies resembling clinical RA (Sakaguchi et al. 2003). One possibility of the pathological mechanism leading to RA due to mutation in ZAP70 can be the alteration in thymic selection. However, despite the presence of autoreactive T cells in the periphery, arthritis development is mainly dependent on foreign infectious bacteria (Yoshitomi et al. 2005).

### 1.4.3. ROS in inflammation and RA

A vast amount of evidence suggests the association of oxidative stress in RA. The role of ROS in chronic inflammation is presently changing from being toxic agents, which causes damage, to being considered as a crucial part of signalling pathways and therefore as a regulators. This was shown in mice, where neutrophil cytosolic factor 1 (Ncf1) or $p47^{phox}$ mutation leading to decreased NADPH oxidase 2 (NOX2) complex-derived ROS. $Ncf1^{-/-}$ mice show severe symptoms of arthritis compared to WT mice and increased susceptibility to the disease in

pristine induced arthritis in rats (K. A. Gelderman et al. 2006; Hultqvist & Holmdahl 2005). The levels of oxidative stress and cells unable to undergo apoptosis correlates strongly with hyporesponsiveness of T cells in the synovial joints. T cells from the synovial fluid have been shown to produce high levels of ROS compared to T cells in the periphery (S I Gringhuis et al. 2000). However, the levels of ROS in rheumatoid joints is controversial as (Pantano et al. 2006) have shown that cytosolic levels of ROS were inversely proportional to disease severity. Also in the presence or absence of mitogenic stimuli, T cells from rheumatic patients do not show an increase in ROS levels compared to healthy patients (Phillips et al. 2010).

T cells in the joints are hyper-activated but show poor TCR response leading to hypo-responsiveness (Maurice et al. 1997). This may be due to the PTNP22 gene associated with RA, which has a cysteine residue in the protein that may be prone to oxidation. Furthermore, other proteins involved in TCR signalling have also been suggested in the development of RA. This include LAT protein, which is phosphorylated by the phosphorylation of ZAP70. Oxidation of LAT may lead to dissociation from the membrane and eventually poor TCR mediated signalling (S I Gringhuis et al. 2000). Furthermore, ROS may also have a direct effect on the assembly of T cell activation complex in membrane localisation (Lu et al. 2007) as a result, regulation by the redox status of the intracellular thiol pool on TCR signalling (Hehner et al. 2000). Several reviews in the literature have summarised activation induced oxidative modification which has an influence on receptor-coupled signalling pathway in T cells (Simeoni & Bogeski 2015a), further linking increased ROS, loss of GSH, protein oxidation and NF-κB activation. Cysteine residues as described earlier, are the most conserved and specific to oxidative post translational modifications. $H_2O_2$ first oxidises cysteine thiols to form sulphenic acid, which is reversed by Trx1 and Grx (Hanschmann et al. 2013b)

Moreover, formation of sulphenic acid may lead to other post-translational modifications, for example; disulphide formation, glutathionylation and nitrosylation which may impair T cell function by inactivation of proteins. Several proteins have been shown to be regulated by sulphenation; these include kinases, phosphatases, proteases, ion channels, adaptor proteins

and cytoskeletal components. (Nathan & Cunningham-Bussel 2013; Holmström & Finkel 2014; Leslie B Poole et al. 2004a; Dickinson & Chang 2011; Ratnayake et al. 2013). Furthermore, depletion of GSH leads to cellular localisation of LAT, which is phosphorylated by tyrosine kinases altering its conformation (Gringhuis et al. 2000; Gelderman et al. 2006b). This further supports oxidation of an essential cysteine residue may result in loss of protein function and TCR signalling (Griffiths 2004; Cemerski et al. 2003).

## 1.5. Hypothesis and aims

An increase in ROS results protein modifications including protein sulphenic acid formation, where -SOH has been considered to play an important role in protein function under both normal physiological and stressful conditions. T cell activation is associated with reversible cysteine sulphenic acid formation which is an important mechanism by which T cells modulates signalling and proliferation (Michalek et al. 2007). Furthermore, several proteins have been identified to undergo sulphenic acid modification following TCR stimulation including actin and protein tyrosine phosphatases and that sulphenic acid was essential for phosphorylation, calcium flux and proliferation of $CD4^+$ and $CD8^+$ T cells. Previous studies show that increased Trx1 in plasma from RA patients together with higher number of IL-17 secreting cells expressing Prx2 on the surface. The prominent role of Trx1 and Prx2 on the surface of these T cells is unknown. Therefore, redox alteration and oxidative PTMs may be common biomarkers associated with increased risk of autoimmune diseases. This raises an interesting question regarding the role ROS may be playing in RA disease activity and severity. Overall, this suggests that loss of intracellular ROS in RA disease may promote T cell mediated autoimmunity.

Taking the previous evidence into consideration, this thesis aimed to identfy the localisation of membrane-bound reducing proteins and redox markers during T cell activation. The work extended to a mouse model of RA. Also, to identify possible membrane proteins which undergo sulphenic acid modification under stress condition and activation of T cells.

To address this question, the main objectives were:

- To optimise and develop a method to capture sulphenic acid modification in Jurkat T cells using commercially available probe DCP-Bio1.
- To mimic oxidative stress in T cells using buthionine sulfoximine and Auranofin and analyse the distribution of Trx1 and Prx2 and intracellular GSH levels.
- Characterise the effects of T cell activation on Trx/Prx distribution in the presence of $H_2O_2$ and to identify possible binding targets as proteins sulphenated on the membrane of activated $CD4^+$ T cells
- To understand the surface redox state and redoxins on T cells in CIA mouse model and how ROS affect the response to TCR stimulation.

# Chapter 2. Materials and Methods

## 2.1. Materials

All chemicals were purchased from Sigma (Poole, UK) or Fisher Scientific (Loughborough, UK) unless stated otherwise. Cell culture and general plastic ware were obtained from Thermo Fisher Scientific (Loughborough, UK). Needles and syringes for blood collection were purchased from BD (Becton, Dickinson and company, Oxford, UK). VACCUETE® ethylenediaminetetraacetic acid (EDTA) and VACCUETE® heparin tubes were purchased from Griener Bio-One Ltd. (Stonehouse, UK). Recombinant Trx1 was purchased from R&D (UK).

## 2.2 Methods

### 2.2.1. Cell culture and reagents

Jurkat T cells, derived from a 14 year boy with T cell acute lymphoblast leukaemia, were purchased from Sigma Aldrich (UK) proved by European Collection of Authenticated Cell Cultures (ECACC). They were cultured in Rosewell Park Memorial Institute (RPMI) 1640 with L-glutamine, 10% foetal bovine serum (FBS), penicillin (1000 $Uml^{-1}$) and streptomycin (10,000 $\mu g/ml^{-1}$) purchased from Gibco Life Technologies (Paisley, UK).

Jurkat T cells were passaged until reached ~ 80% confluency. On the day of treatment, the cells were washed with complete RPMI and counted using a haemocytometer and plated in a 12 well plate at an assay-dependent concentration.

## 2.2.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

### 2.2.2.1. Reagents

| Reagent | Description |
|---|---|
| Buffer 1 for the resolving gel | 1.5M Tris base and 0.4% w/v SDS at pH 8.4 |
| Buffer 2 for the stacking gel | 1.5M Tris base, 0.4% w/v SDS at pH 6.8 |
| Ammonium persulphate (APS) 10%w/v | 1g APS in 10ml of distilled water |
| Running Buffer | 3g Tris base, 14.4g Glycine and 1g SDS in 1L of distilled water |
| Transfer buffer | 4.5g of Tris base, 21.6g glycine, 300ml methanol in 1200ml of distilled water |
| Tris buffered saline buffer (TTBS) | 12g NaCl, 6g Tris base in 1L distilled water at pH 7.4 containing 0.05%v/v Tween |
| TEMED | N,N,N',N'- tetramethylethyleneamine |
| Acrylamide | Acrylamide/bis acrylamide 30% solution |
| Non-reducing sample buffer | Tris (1M, pH 6.8), glycerol (50% v/v), SDS (10% w/v), bromophenol blue (0.5% w/v) |
| Blocking Buffer | 3% w/v semi-skimmed milk in TTBS |
| Ponceau Red stain | 0.1 % (w/v) in 5% acetic acid |
| Coomassie brilliant blue (CBB) G250 | 0.1% w/v CBB G250, 10% v/v acetic acid, 50% v/v Methanol |
| Destain Solution | 40% v/v Methanol, 10% v/v, acetic acid and 50% v/v distilled water |

Table 2.1. Solutions for SDS-PAGE and Western blotting

| Reagent | 10% resolving | 15% resolving | 3% stacking |
|---|---|---|---|
| Distilled water | 5 ml | 3ml | 4.87ml |
| Buffer 1 | 3 ml | 3ml | |
| Buffer 2 | | | 1.87ml |
| Acrylamide | 4ml | 6ml | 0.75ml |
| TEMED | 100µl | 100µl | 100 µl |
| APS | 10µl | 10 µl | 10 µl |

Table 2.2. Reagents used for composition of SDS-PAGE resolving and stacking gel.

2.2.2.2. SDS gel electrophoresis and western blot

The protein samples were separated using polyacrylamide gel electrophoresis. The glass plates were assembled according to the manufacturer's instructions and resolving and stacking gels were prepared (BioRad, UK). Methanol (Fisher, UK) was overlayed on the surface of the gel to protect from air and the gels were allowed to set for 15min. After the resolving gel was set, methanol was drained and 3% stacking gel was pipetted in. A comb was then inserted into the stacking gel and it was left to set for 15min. The protein samples were prepared by mixing 1:1 with Laemmli sample buffer (Sigma, UK) for reducing conditions and 1:2 with 2X non-reducing buffer for non-reducing conditions and heated at 95°C in a heat block for 10min. The gel apparatus was set up and filled with running buffer (followed by loading of the samples and markers (Kaleidoscope, Precision plus protein, Bio Rad, UK). The gel was run at 115V for 1hour 45min.

The gel was then transferred to 0.2µm polyvinylidene difluoride membrane (PVDF) membrane (GE Healthcare, Amersham, UK) that was soaked in 100% methanol for 5min and transfer buffer prior to use. The transfer was run at 240mA for 1h 45min and the membrane was blocked overnight at 4°C with 3%w/v BSA in Tris buffered saline (TTBS). Primary antibody for Trx1 expression was mouse monoclonal [3A1] anti-Trx1 (Abcam, 1:2000) incubated for 2hours

on the rocker at room temperature and the membrane was washed 6 times every 5min. The secondary antibody used was goat anti-mouse polyclonal IgG Fc specific peroxidase (A1068, Sigma) 1:10,000 in TBST (0.1% w/v BSA) incubated for 2h at room temperature and washed 6 times every 5min and developed using ECL technique (GE Healthcare, Amersham, UK). A 1:1 mixture of reagents from the ECL kit was added to the membrane and left for 3min in absence of light. The membrane was developed using the G-Box chemi (Syngene, Geneflow, UK). The loading control antibodies including rabbit polyclonal anti-CD3 (ab16044, Abcam), rabbit polyclonal to NF-kB p65 (ab 16502, Abcam) and anti-β-actin produced in mouse (A5316, Sigma).

2.2.2.3. Coomassie staining

SDS-Page gels were stained with Coommassie blue G250 (0.1%w/v) for 1h on the shaker. Gels were then destained overnight using destaining solution with frequent change of the destain solution. Once the gel background was clear, the gels were analysed by G:BOX Chemi HR 1.4 imaging system and GeneTools 4.03 image analysis software (Syngene, Cambridge, UK).

### 2.2.3. Protein quantification by bicinchoninic assay (BCA)

The BCA assay was performed to determine the total protein concentration in the cell lysates or the plasma for further experiments. Unknown protein concentration was determined by adding 200µl $CuSO_4$ and BCA solution (1:50- v/v) to 10µl of either the unknown sample or BSA standards ranging from 0-1mg/ml.

| Protein concentration (mg/ml) | BSA (µl) | Water (µl) | Final volume (µl) |
|---|---|---|---|
| 0.0 | 0 | 10 | 10 |
| 0.2 | 2 | 8 | 10 |
| 0.4 | 4 | 6 | 10 |
| 0.6 | 6 | 4 | 10 |
| 0.8 | 8 | 2 | 10 |
| 1.0 | 10 | 0 | 10 |

Table 2.3. Reagents volume required to prepare the standard curve to determine protein concentrations.

A standard curve was generated plating BSA standards in triplicates on a 96 well plate with final volume of 10µl. The 96 well plate was incubated at 37°C for 30min and the absorbance was measured at 570nm (BioTek Instruments Inc., Swindon, UK)

## 2.2.4. Membrane protein extraction

### 2.2.4.1. Reagents

Mem-PER Membrane protein extraction kit was obtained from Pierce (Thermo-Fisher, UK)

### 2.2.4.2. Protocol

The Mem-PER kit uses a mild detergent which allows the solubility of membrane proteins. Jurkat T cells ($5x10^6$) or CD4 T cells ($1x10^6$) were harvested and protein fractions were isolated using the MEM-PR membrane protein isolation kit (Thermo Fisher). Briefly, cells were washed twice in cell wash buffer included in the kit. This was followed by centrifugation in 750µl permeabilisation buffer (Thermo Fisher) with protease inhibitor cocktail (1:100) (P8340, Sigma, UK) at 16,000g for 15min. Cytosolic protein fractions were collected and the pellet was re-suspended in 500µl solubilisation buffer (Thermo Fisher) and incubated for 1hour at 4°C with constant mixing. Following the incubation, the extracts was centrifuged at 16,000g for 15min at 4°C and the supernatant containing membrane proteins was collected and stored in -20°C for further experiments.

Membrane protein fractions were dialysed using Slide-A-Lyzer dialysis cassettes (0.5ml, Thermo scientific). Briefly, 500µl of the protein sample was inserted into the cassette using a syringe and the cassette was dialysed in a dialysis buffer (75mM aminocaproic acid, 2mM Bis-Tris), 200 times the volume of the protein sample. Firstly, dialysis was or 2h at 4°C and then the dialysis buffer was changed, followed by dialysis for another 2h. The dialysis buffer was changed again and dialysed overnight at 4°C. Protein samples were collected from the cassette the following day and protein concentration was measured again using BCA assay.

#### 2.2.4.3. Membrane protein validation

To validate the efficacy of membrane protein extraction, $5x10^6$ Jurkat T cells were collected and cytosolic proteins (CP) and membrane proteins (MP) fraction isolated according to the protocol described above. MP were dialysed and analysed by western blot with antibodies against CD3 and NF-kB p65 (Abcam, UK). Briefly 20µg of CP or MP were separated on 15% gel and transferred to a PVDF membrane. The membrane was blocked with 3% BSA in TTBS at 4°C and probed for rabbit polyclonal IgG anti-CD3 (ab16044, Abcam, UK) and Rabbit polyclonal IgG anti-NF-kB p65 (ab7970, Abcam, UK) (1:1000) in TTBS 0.02% BSA w/v for 2h at RT. Followed by an incubation with secondary goat anti-rabbit (1:10000) for 2h at RT.

### 2.2.5. Protein identification by mass spectrometry

#### 2.2.5.1. Reagents

Acetic acid, Acetonitrile (ACN) and methanol were purchased from Fisher Scientific (Thermo Fisher Scientific). Trypsin gold was purchased from Promega, UK.

#### 2.2.5.2. Protocol

Proteins separated on gels were briefly stained with Coomassie brilliant blue (CBB) G250 as previously described in section 2.2.2 and either the selected protein bands or sections of lanes were excised and destaining with (10% v/v acetic acid, 40% v/v methanol and 50% v/v distilled water). Briefly, the gel pieces were washed in 50% v/v ACN 50mM ammonium bicarbonate (ABC) and incubated at 37°C on a thermoshaker (Thermo Fisher Scientific, UK) at 550rpm until the pieces were clear. Washed gel pieces were resuspended in 150µl of 100% v/v ACN and incubated for 10min at RT. The samples were dried until the ACN was removed. The gel pieces were reduced with the reducing solution (10mM DTT in 50mM ABC (Ammonium bicarbonate)) at 56°C with shaking for 30-45min and allowed to cool down at RT.

Subsequently, the samples were alkylated with iodoacetamide (IAA) (55mM in 50mM ABC) and incubated at RT in the dark for 30min. The excess solution was discarded and the gel pieces were resuspended in 200µl 100% v/v ACN. Reduced and alkylated gel pieces were enzymatically digested with trypsin solution (25x) (Trypsin gold, sequencing grade, Promega, UK) solubilised in digestion solution (3mM ABC) overnight at 37°C. The peptides were collected using 50uL ACN and sonicated for 15min (this step was repeated twice). The supernatants were dried in a centrifugal concentrator for 1-2h and resuspended in 15µL of 3% v/v acetonitrile in 0.1% v/v formic acid (Fisher, UK) in distilled water.

2.2.5.3. Protocol: Mass spectrometry

Extracted dried peptides dissolved in ACN/formic acid were transferred into a borosilicate vial and placed into the automated sampler at 10°C (Dionex 3000, Thermoscientific, UK). Approximately half of the sample was injected onto the trap column (PepMapTM, C18, 5 µm, 100 Å, 300 µm x 1 mm, ThermoScientific, UK). Trapped peptides were washed with 2% acetonitrile in aqueous 0.1 % formic acid for 3minutes at a flow rate of 30µl/min. Peptides were separated on the analytical column (AcclaimTM, PepMapTM C18, 3 µm, 100 Å, 75 µm x 150 mm, ThermoScientific, UK). Electrospray was formed by spraying the nanoLC eluate at 2500 V using the PicoTipTM emitter (New Objective, Germany). Data was obtained on the 5600 TripleTof (AB Sciex, UK) Mass Spectrometer in information dependent mode.

2.2.5.4. Data analysis

The obtained files were subjected to Mascot Daemon search (v2.5) against the Swissprot database to identify the peptides with the following parameters: mass tolerance of 0.1 Da for MS and 0.6 Da for MS/MS spectra, maximum of 2 trypsin missed cleavages, human taxonomy, and variable modifications of methionine oxidation and cysteine carbamidomethylation.

## 2.2.6. Cell viability

### 2.2.6.1. Determination of cell viability using trypan blue exclusion

Trypan blue is a stain which dyes the cells with damaged cell membranes. Cells were collected and mixed with equal volumes of trypan blue solution. This was loaded onto a haemocytometer for the determination of viable and non-viable cells.

Viability %= number of viable cells/total number of cells (live and dead) x 100

### 2.2.6.2. Cell viability using Annexin V by flow cytometry

Briefly, $1x10^5$ cells were washed in PBS/BSA (1% w/v) and resuspended in 500µl binding buffer (150mM NaCl, 10mM HEPES, 2.5mM $CaCl_2$) and incubated with 5µl of anti-human Annexin V FITC (EBioscience, Hatfield, UK) for 15min and analysed by flow cytometry acquiring 10,000events.

## 2.2.7. Primary $CD4^+$ T cell activation

### 2.2.7.1. Reagents

Lymphoprep was purchased from Axis-Shield (Dundee, Scotland). Bovine serum albumin (BSA) was purchased from Thermo Fisher (Loughborough, UK). Untouched $CD4^+$ T cell isolation kit was from Invitrogen. Anti-CD3 and anti-CD28 antibodies were purchased from ebioscience (Hatfield, UK)

2.2.7.2. Volunteers

Blood was collected from healthy donors between 25-35 years old (n=5). Ethical approval was granted by Aston University Ethics Committee (Ref: 802)

Fresh blood (20ml) was collected from healthy consenting volunteers by venepuncture into VACCUETE® EDTA tubes. Briefly, 20ml of fresh blood was diluted with PBS with (0.1%w/v BSA) at 1:2.5 and layered over 11ml of Lymphoprep. 25ml of the layered gradient was centrifuged at 160g for 15min without deceleration followed by further centrifugation at 350g for 20min after discarding the top layer of plasma to wash off the platelets. After centrifugation, the layer between the plasma and the Lymphoprep containing the mononuclear cells, was collected and washed twice with PBS (0.1% w/v BSA) to remove excess Lymphoprep. The cells were counted and re-suspended in complete RPMI 1640.

2.2.7.3. $CD4^+$ T cell Isolation by negative isolation and activation

Isolated PBMCs were re-suspended in 100µl isolation buffer ($Ca^{2+}Mg^{2+}$ free PBS with 0.1% w/v BSA and 2mM EDTA) and 20µl antibody mix (Dynabeads untouched human $CD4^+$ T cell kit, Invitrogen) was added then incubated for 30min at 4°C. The cells were washed with isolation buffer (2ml) and centrifuged at 350g for 8min at 4°C. Meanwhile 100µl of MyOne Dynabeads (Dyanbeads untouched human $CD4^+$ T cells, Invitrogen) was washed in 1ml of isolation buffer. The supernatant was discarded after placing the beads in a magnet. The beads were re-suspended in 100µl isolation buffer and added to the cells and incubated for 30min at room temperature with gentle mixing and rotation. 2ml of isolation buffer was added and the bead bound cells was mixed by pipetting up and down roughly 10 times. After placing the tube in the magnet for 2min the supernatant containing untouched human $CD4^+$ T cells were isolated. This step was repeated and combined with previously isolated cells. Finally the cells were counted on the haemocytometer prior to further experiments.

Isolated $CD4^+$ T cells were resuspended in RPMI 10% FBS and 1% penicillin streptomycin for stimulation. $1x10^6$ cells/ml were stimulated with anti-CD3 (OKT3 clone, (1µg/ml) and anti-CD28.2 (2µg/ml) for 24, 48 and 72h. Following optimisation, the cells were stimulated in the presence NOX-2 compounds (Redoxis AB, Lund, Sweden) at 12.5µM and or $H_2O_2$ at 20µM. The cells were collected after 30min and 24h for appropriate analysis. After 24h at 37°C, the supernatants were collected and stored in -20°C until further analysis.

## 2.2.8. Analysis of surface proteins by flow cytometry

### 2.2.8.1. Reagents

Fc antibodies were obtained from Abcam (Cambridge, UK), eBioscience (Hatfield, UK), BD Biosciences and Bio legend (UK) as described in the table below.

| Antigen | Description | Supplier | Product code |
|---|---|---|---|
| Trx1 | Mouse monoclonal [3A1] to Thioredoxin | Abcam | ab16965 |
| Prx2 | Rabbit monoclonal [EPR5154] to Peroxiredoxin 2 | Abcam | ab109367 |
| CD25 | Mouse monoclonal [BC96] to IL2 Receptor with APC | Abcam | ab134477 |
| CD4 human | Mouse monoclonal anti-CD4 PE | Abcam | ab1155 |
| CD4 mouse | Rat anti-mouse CD4-PE, Rat anti anti-mouse -FITC | BD Biosciences | 553048/553651 |
| Secondary antibody to Trx1 | goat anti-mouse polyclonal conjugated dylight-488 | Abcam | ab96879 |
| Secondary to Prx2 | Goat anti-Rabbit IgG Secondary Antibody, Alexa Fluor 488 | Invitrogen | A-11034 |

| | | | |
|---|---|---|---|
| IC to Trx1 | IgG2b isotype control antibody | Abcam | ab 91366 |
| | | | |

Table 2.4. Antibodies used for analysing exofacial markers and proteins

2.2.8.2. Protocol

After the treatments, the cells were washed and resuspended in PBS/BSA (1% w/v) followed by incubation with the appropriate antibodies and the corresponding isotype control on ice for 20min and analysed using the flow cytometer FC500 (Beckman-Coulter, High Wycombe, UK) and or Attune NxT Flow cytometer (Thermoscientific, Sweden). Data was analysed by flowing software v2.5.

## 2.2.9. Cytokine quantification by sandwich enzyme linked immunosorbent assay (ELISA)

2.2.9.1. Reagents

BSA was purchased form Thermo Fisher Scientific. Human IL-2 and TNF-α ELISA development kit were purchased from ebioscience affymetrix.

2.2.9.2. Protocol

The secretion of IL-2 and TNF-α in cell culture supernatants was quantified using the manufacturer's instructions. Briefly, 96 well plates (Nunc, Maxisorp) were coated with a capture antibody and incubated overnight at 4°C. Duplicates of standards or samples were added to a monoclonal antibody specific for IL-2 or TNF-α, respectively, pre-coated 96-well plate and incubated for 2 hours at RT. After, the plate was incubated with human IL-2 or TNF-α conjugate, respectively, for 1 hour at RT. Four washes with wash buffer were performed between incubation steps. The plate was then incubated with substrate solution for 30 minutes at RT and protected from light and the colorimetric reaction stopped with stop solution.

Absorbance of each well at 450 nm was measured in a plate spectrophotometer (BioTek Instruments Inc., Swindon, UK) and concentration of the cytokines interpolated from a standard curve (IL-2 and TNF-α: 3.1 to 1000 pg/mL). Correction of wavelength was performed at 570 nm.

## 2.2.10. Competition Trx1 ELISA

### 2.2.10.1. Reagents

Recombinant Trx1 was purchased from R&D (UK), skimmed milk was purchased from Tesco, SIGMAFAST™ OPD was from (Sigma, UK)

### 2.2.10.2. Protocol

Trx-1 (0.2µg/ml) 50µl/well was applied to a Nunc microtiter 96 well plates (Nunc, Maxisorb) in 50mM carbonate buffer (1.59 g/L sodium carbonate ($Na_2CO_3$) 2.93 g/L sodium hydrogen carbonate ($NaHCO_3$) pH9.6 in distilled water) and incubated for 1 hour at 37°C. The plate was washed 3 times with 250µl of PBS containing 0.05% v/v Tween-20 (PBST) and were gently tapped on absorbent tissue paper. Following washing, nonspecific sites were blocked by adding skimmed milk 4% w/v in PBS, 200µl/well for overnight at 4°C. Standard curve (2.5µg/ml-0µg/ml) was prepared using recombinant human Trx1 protein (25µl/well). The supernatants (25µl/well) were plated in triplicates. Mouse monoclonalanti-Trx1 (25µl of 1:2000 in PBS, ab16965; Abcam) was added to all standards and samples and was incubated at 37°C for 2 h. Following incubation, wells were washed with PBST (250µl) 3 times and 200µl of peroxidase conjugated anti-mouse antibody (A1068, Sigma, UK) (1:5000) was added to each well. The plate was incubated at 37°C for 1h. Following washing 3 times with PBST (250µl), and 50µl of substrate solution containing *o*-phenylenediamine (Sigma, UK) and hydrogen peroxide in 0.15 M citrate-phosphate buffer was added. During incubation at room

temperature, colour development was observed from 2 to 10 min. The colour reaction was stopped with the addition of 2M sulphuric acid ($H_2SO_4$) (50µl/well). Absorbance was measured at 490 nm in a microplate reader (BioTek, UK).

## 2.2.11. Glutathione assay (GSH)

GSH is the major antioxidant system in the cells and its present in a reduced form. Total GSH and GSSG was determined using the recycling assay which involves the oxidation of GSH to GSSG in the presence of DTNB (Gherghel et al. 2005). The colour change can be detected by spectrophotometry. Oxidised GSSG can be regenerated to GSH in the presence of GSR and NADPH and measures the total GSH available.

### 2.2.11.1. Reagents

| Reagents | Description |
|---|---|
| Stock buffer | 125mM Sodium phosphate, 6.3mM disodium EDTA (monobasic anhydrous), pH 7.5 |
| Daily buffer | 3mg NADPH in 10ml stock buffer |
| SSA | 1g of SSA acid in 1ml distilled water |
| Reduced GSH | 10mM of GSH in 5ml of distilled water |
| Glutathione reductase | 4U/ml in stock buffer |
| DTNB | 6mM 5', 5' – Dithio-bis (2-nitrobenzoic acid) in stock buffer |
| Oxidised Glutathione (GSSG) | 5mM GSSG in distilled water |
| Vinylpyridine | 20µl per standards well; 2 µl per sample |
| Triethanolamine | 20µl per standards well; 2 µl per sample |

Table 2.5. Reagents required for GSH assay

2.2.11.2. Protocol for reduced GSH

| Concentration | GSH (µl) | SSA (µl) | Water (µl) |
|---|---|---|---|
| 0µM | 0 | 33.3 | 966 |
| 20µM | 2 | 33.3 | 964 |
| 40µM | 4 | 33.3 | 962 |
| 60µM | 6 | 33.3 | 960 |
| 80µM | 8 | 33.3 | 958 |

Table 2.6. Reagent combination required for the standard curve for total GSH determination

Cells were collected in 1.5ml micro centrifuge tubes and centrifuged at 6600 x g (Eppendorf centrifuge 5415D) for 1.5min. The supernatants were removed and PBS (0.5ml) was added to re-suspend the cell pellet. The tubes were centrifuged at 6600 x g for 2.5min and the supernatant was discarded. 3.3µl of SSA was added, vortexed and centrifuged immediately at 13000g for 1.5min then 96.6µl of stock buffer was added. When preparing plasma, 1% of SSA was directly added to 100 µl of plasma and 25µl of sample/standards (0-80µM) were loaded into a 96well plate. 150µl of daily buffer and 50µl of DTNB was added and incubated for 5min at 37°C. 25µl of the enzyme glutathione-S-reductase was added and the plate was read at 410nm after 0, 1, 2, 5 and 10min on a plate reader (BioTek instruments, Swindon, UK). GSH concentration was interpolated from the standard curve and data was analysed using GraphPad Prism 6/7.

2.2.11.3. Protocol for oxidised GSH (GSSG)

| Concentration | GSSG(µl) | SSA (µl) | Water(µl) |
|---|---|---|---|
| 0µM | 0 | 33.3 | 966 |
| 2µM | 0.4 | 33.3 | 965.6 |
| 4µM | 0.8 | 33.3 | 965.2 |
| 6µM | 1.2 | 33.3 | 964.8 |
| 8µM | 1.6 | 33.3 | 964.4 |

Table 2.7. Reagent combination required for standard curve for GSSG determination

Briefly, cells/plasma were collected in 1.5ml micro centrifuge tubes and centrifuged at 6600 x g (Eppendorf centrifuge 5415D) for 1.5min. The supernatants was removed and PBS (0.5ml) was added to re-suspend the cell pellet. The tubes were centrifuged at 6600 x g for 2.5min and the supernatant was discarded. 3.3µl of SSA was added, vortexed and centrifuged immediately at 13000g for 1.5min then 96.6µl of stock buffer was added. 25µl of sample/standards (0-8µM) were loaded into a 96well plate. 20µl of Vinylpyridine and Triethanolamine was added to the standards and 2µl of both was added to the samples to stabilise the glutathione (Mcgill & Jaeschke 2015). 150µl of daily buffer and 50µl of DTNB was added and incubated for 5min at 37°C. 25µl of the enzyme glutathione-S-reductase was added and the plate was read at 410nm after 0, 1, 2, 5 and 10min and the data was analysed using GraphPad Prism 6/7

## 2.2.12. Data analysis

Unless specified, all data obtained was analysed using GraphPad Prism v6/7 as mean and standard error of mean (SEM). Experiments were performed in at least three independent replicates. Comparison between more than two columns was performed using one way analysis of variance (ANOVA) followed by Tukey's/Sidaks multiple comparison test. Statistical significance was defined as * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001 and **** p-value < 0.0001.

# Chapter 3. Proteins which may undergo sulphenic acid modification on the membrane and cytosol in Jurkat T cells

## Preface

Protein oxidation is an unavoidable process that can be reversible and irreversible. Protein sulphenic acids are reactive by-products of cell signalling. Hence this chapter focused on understanding protein oxidation in Jurkat T cells including sulphenic acid, disulphide and carbonyl formation with the goal of identifying novel proteins which may undergo sulphenic acid modification on the membrane of T cells under conditions of oxidative stress. Novel proteins on the membrane of Jurkat T cells were identified to undergo sulphenic acid modification. Some of the proteins identified to undergo sulphenic acid modification are in line with those reported in the literature.

## 3.1. Introduction

Oxidation of proteins within cells can be due to a normal enzymatic reactions, and from ROS released from innate cells including $H_2O_2$, hypochlorous acid (HOCl) and nitric oxide (NO), and the regulated release of cellular oxidants for signalling purposes (Poole 2015). ROS are now recognised as signalling molecules which influence a range of biological activities (Bennett and Griffiths 2015). T cell protein thiol oxidation can arise due to either; an increase in ROS/RNS, lack of free thiols or antioxidants due to oxidation (Ratnayake et al. 2013). The cellular redox state in cells is maintained through three main redox couples CySS/Cys, GSH/GSSG and Trx1-SS/ Trx1-SH which reduce oxidised proteins in the presence of free radicals (Jones 2004). In any case when the difference between production of ROS and removal is disrupted, cells are said to be under oxidative stress, which eventually leads to irreversible modifications of proteins, lipids and nucleic acids (Kesarwani et al. 2013b). Reversible thiol modifications are a mediator of cell signalling caused by ROS; the initial product of cysteine oxidation by $H_2O_2$ or peroxynitrite is cysteine sulphenic acid (Nelson et al. 2010; Poole et al. 2004). These sulphenic acid modifications can be either directly reduced by thiol by antioxidants or form a disulphide bond with cysteine residues in the same or another protein. Sulphenic acid can also undergo irreversible modification including sulphinic and sulphonic acid.

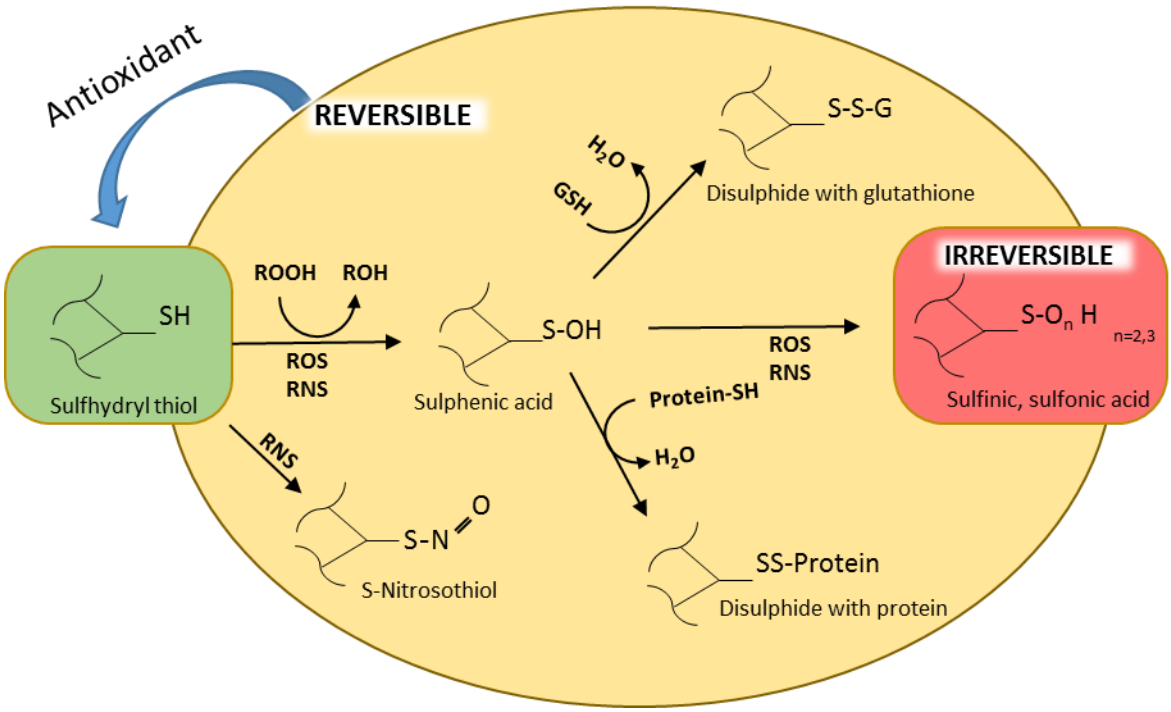



Figure 3.1 Oxidative modification of cysteine thiols. Reactive thiols (green) in their stable form are oxidised by reactive nitrogen species (RNS) and reactive oxygen species (ROS) including $H_2O_2$ and peroxynitrite to form a sulphenic acid product which is reversibly oxidised and may be reduced in the presence of reductants or antioxidants. Irreversibly modified (sulphonic or sulphonic acid, red) species. Adapted from (Poole & Nelson 2008)

## Membrane proteins and susceptibility to oxidation

Cell membranes are involved in maintaining cellular homeostasis providing a semi-permeable barrier between the intra- and the extracellular environment. Cell membranes consist of a lipid bilayer and approximately 50% is comprised of membrane proteins which include receptors, channels, enzymes and structural proteins that are responsible for interaction between adjacent cells, cytoskeleton contact, signalling and surface recognition (Cooper & Hausman 2007). T cell membrane proteins (MP) are involved in CD3/TCR engagement with MHC-I or II by antigen presenting cells. This is the initial step of T cell activation which subsequently activates downstream signalling pathways that are essential for an immune response. It is widely known that MP contain many cysteine residues and are susceptible to oxidative

modifications during T cell activation and increasing ROS (Redegeld et al. 1997; A. V. Belikov et al. 2015). Therefore changes in the redox state of extracellular proteins may result in changes in the redox sensors and cell activation by ligands (Reyes et al. 2005). However, there is not much information in the literature regarding which of the MP undergo sulphenic acid modification. $Ca^{2+}$ entry by $Ca^{2+}$ ATPase is essential for T cell activation, and the function of this redox-sensitive protein is markedly affected by any alteration in the redox state (Lewis 2001). Several biochemical studies indicate the sensitivity of proteins to oxidation, some of which are responsible for calcium trafficking intra- and extracellularly (Simeoni & Bogeski 2015b; Belikov et al. 2015). For example, Orai1 which is a calcium transporter, is believed to be inhibited in the presence of $H_2O_2$ and Orai3 activity is oxidation-dependent (Bogeski et al. 2010). Also, a redox sensitive cysteine thiol on STIM1 has been reported to lower its affinity for $Ca^{2+}$ (Hawkins et al., 2010).

Therefore, this chapter aims to explore protein oxidation and identify proteins which may undergo sulphenic acid modification on the membrane and cytosol of T cells under normal and oxidised conditions.

The objectives of this chapter are as follows:

1. To isolate cytosolic and membrane proteins
2. To optimise oxidation conditions for Jurkat T cell membrane proteins
3. To investigate the effect of $H_2O_2$ on the distribution of protein sulphenic acid modifications
4. To identify proteins which are sulphenated on the T cell membrane and cytosol
5. To further explore the effect of $H_2O_2$ on T cell function

## 3.2. Methods

### 3.2.1 Membrane protein isolation

Cytosolic and membrane proteins were isolated from Jurkat T cells as described in the section 2.2.4 above and analysed under either reducing or non-reducing conditions by SDS-PAGE.

### 3.2.2. Analysis of protein oxidation and or aggregation

CP and MP (20µg) were briefly treated with different concentrations of $H_2O_2$ (50µM-2mM) for 30min on ice followed by addition of IAA (10mM) and catalase to block the free thiols and stop the reaction by decomposing $H_2O_2$ to water ($H_2O$) and oxygen ($O_2$). The samples were concentrated using a vacuum concentrator and separated by reducing and non-reducing SDS-PAGE as described above. The proteins on the gel were transferred onto a PVDF membrane. Following transfer, the membrane was incubated with 1mM DNPH in 2M HCL in the dark for 1h. The membrane was washed 6times for 30min with TTBS (0.05% v/v Tween) and blocked overnight with TTBS (0.1%v/v Tween) at 4°C.

### 3.2.3. Calcium flux assay

Calcium influx was detected by Fluo-3AM (Invitrogen). Briefly, $1x10^6$ Jurkat T cells were incubated with 4µM of Fluo-3AM at room temperature in the dark. The cells were washed with PBS and resuspended with PBS pH 7.2 supplemented with 1mM calcium chloride ($CaCl_2$) and 20mM HEPES. Cells were allowed to rest for 15min for de-esterification of the dye before measuring calcium flux on a fluorescence plate reader at 10 sec intervals for 30min, Excitation 506nm: Emission 526nm.

### 3.2.4. Protein sulphenic acid detection in Jurkat T cells

Jurkat T cells were lysed and CP and MPs were isolated respectively. 50µg of CP and MP were exposed to different concentrations of $H_2O_2$ for 30 minutes in the presence of 0.5mM DCP-Bio1 which binds to any –SOH formed due to oxidation. 10mM of IAA was added. The samples were resuspended in Laemmli buffer and analysed by SDS PAGE and western blotting.

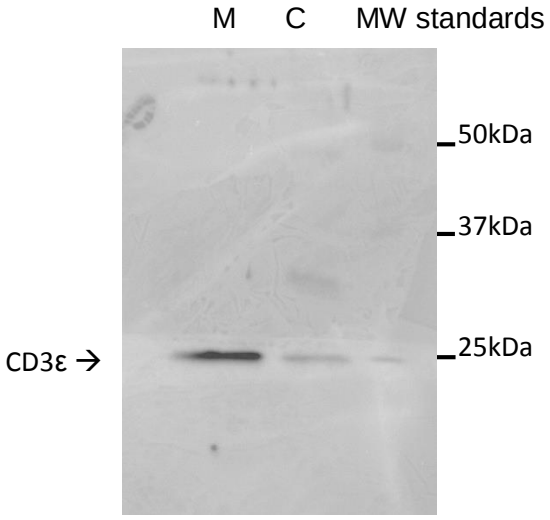
## 3.3. Results

### 3.3.1. Protein purity after membrane isolation

To identify those proteins which can be oxidised on the membrane and in the cytosol, membrane and cytosolic protein fractions were isolated from Jurkat T cells after treatment with $H_2O_2$. Their purity was assessed by analysing proteins specific to the membrane and cytoplasm including CD3Ɛ chain which forms part of the TCR receptor complex on the T cell membrane and the p65 subunit of the cytosolic protein NF-κB. The protein content of each fraction was measured by BCA assay. The samples were dialysed into dialysis buffer (75mM amino-n-caproic acid which improves membrane protein solubility and 5mM Bis-Tris pH7.0) to allow desalting and concentrating. After blotting for the CD3Ɛ chain, Figure 3.2a shows that a band of approximately 23kDa, and the intensity of CD3ε in the membrane (M) fraction appears to be higher compared to the cytosolic (C) lane. This suggests that good enrichment has taken place.

Also to further confirm the purity, protein samples were analysed for a cytosolic specific protein, the p65 subunit of NF-κB which is present abundantly inside the cell and mediates transcription and cell survival. After blotting for p65 in Figure 3.2b, both protein fractions yielded a band of approximately 65kDa, however the band intensity was different. The cytosolic protein fraction showed a greater intensity of p65 compared to membrane proteins lane (M). The low intensity band present in the M lane may possibly be due to possible contamination.

a) Western blot analysing CD3 Ɛ




b) Western blot analysing NF-κB p65

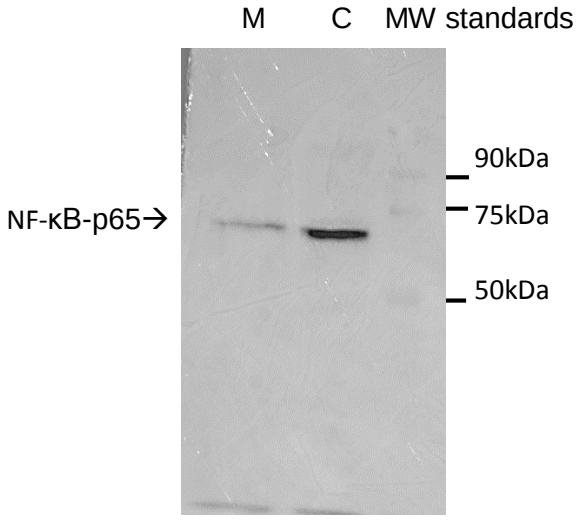


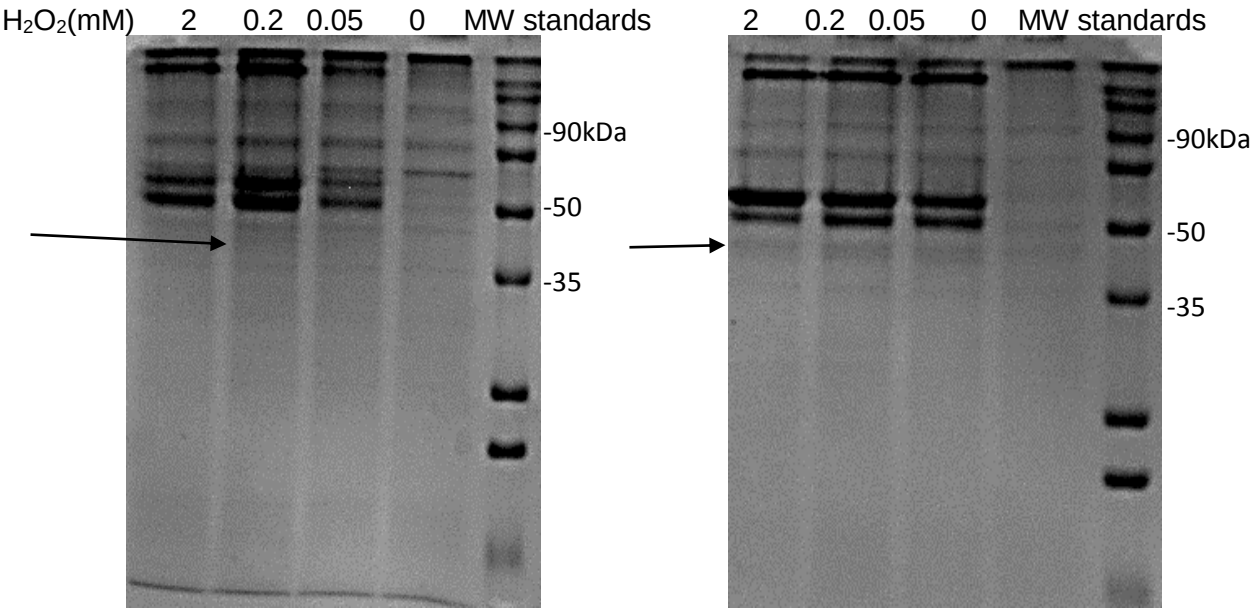

Figure 3.2: Western blot analysis of membrane and cytosolic marker CD3Ɛ and NF-κB. $4x10^6$ Jurkat T cells were fractionated into membrane and cytosol by using MEMPR kit (Thermo scientific); 20µg protein was separated using 15% SDS PAGE and transferred to PVDF membrane, blocked and probed with a) primary rabbit anti-CD3Ɛ (1:1000, 2h); secondary goat anti-rabbit (1:10,000) b) Primary rabbit anti- NF-κB p65 (1:1000, 2h); secondary goat anti-rabbit HRP (1:10,000, 2h). This data is representative of two independent experiments. M= Membrane fraction, C= Cystosolic fraction

## 3.3.2. Protein lysates treated with different concentration of hydrogen peroxide

CPs and MPs were isolated and treated with different concentrations of hydrogen peroxide to allow oxidation and formation of aggregates. Figure 3.3a shows that separation of proteins by SDS-PAGE in the absence of reducing agents such as DTT and β-mercaptoethanol demonstrates formation of aggregates and disulphides. The intensity of a band at approximately 60kDa increases with increase in concentration of $H_2O_2$, this may be the catalase which is a tetramer consisting of four equal subunits with a MW of ~60kDa which was only added in the treated samples. Additionally a band of higher MW approximately 240kDa was observed under non reducing conditions, possibly a catalase tetramer. The bands indicated with a black arrow are visible in treated CP or MP while lacking in untreated lane.

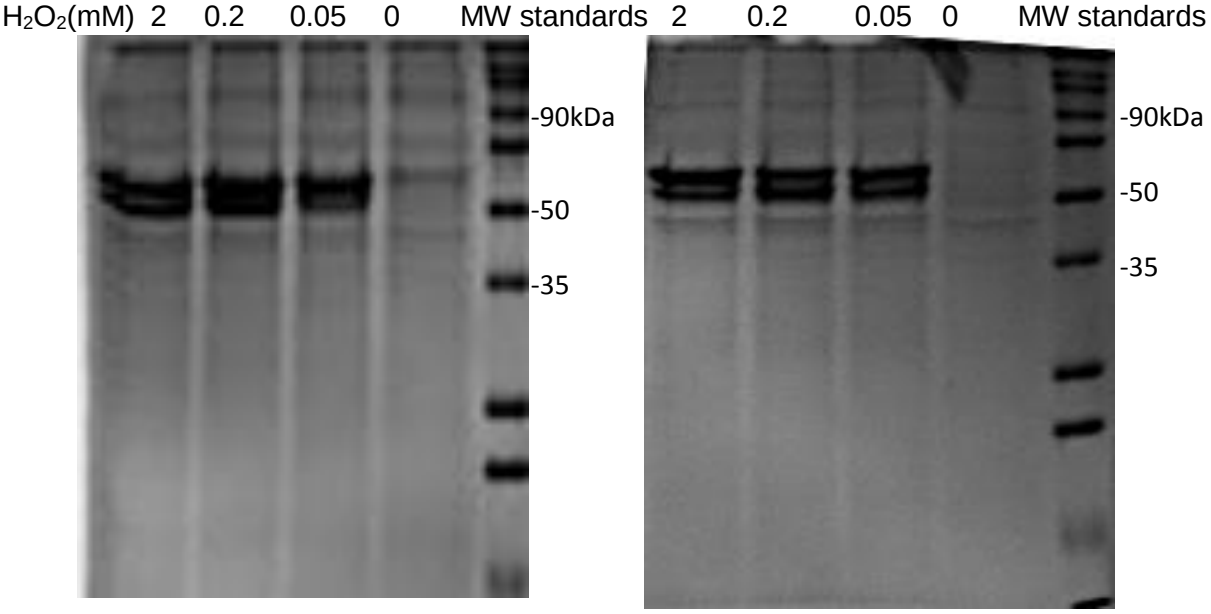
This suggests that higher concentrations of peroxide lead to formation of aggregates and or disulphides. On the other hand CP and MP proteins when separated in the presence of β-mercaptoethanol, are reduced and thus no disulphides or significant bands are visible.

a)




Membrane Cytosol

b)




Membrane Cytosol

Figure 3.3: SDS PAGE showing separation of cystosolic and membrane protein from $H_2O_2$ treated Jurkat T cells. 20µg of cytosolic and membrane proteins were treated with increasing concentrations of $H_2O_2$ for 30min on ice. 10mM IAA and catalase was added to stop the reaction. Protein fraction were concentrated and resuspended in 2x sample buffer with or without reducing agents and separated on 12% SDS PAGE. The gel was stained with Coomassie blue for visualisation of bands. a) non-reducing SDS. Black arrows at ~40 kD indicates protein aggregates in both CP and MP blots and b) reducing SDS PAGE.

## 3.3.3. Hydrogen peroxide leads to carbonyl modification

In order to assess protein oxidation, the determination of protein bound carbonyls (aldehydes and ketones) has been used as the most common method (Frijhoff et al. 2015). Protein carbonyls can be detected using various methods based on derivatisation of the carbonyl group. Figure 3.4a below shows the effect of hydrogen peroxide on CP and MP. Lysates treated with 200µM $H_2O_2$ show multiple bands, with the majority below 50kDa, compared to untreated lysates when derivatised with DNPH.

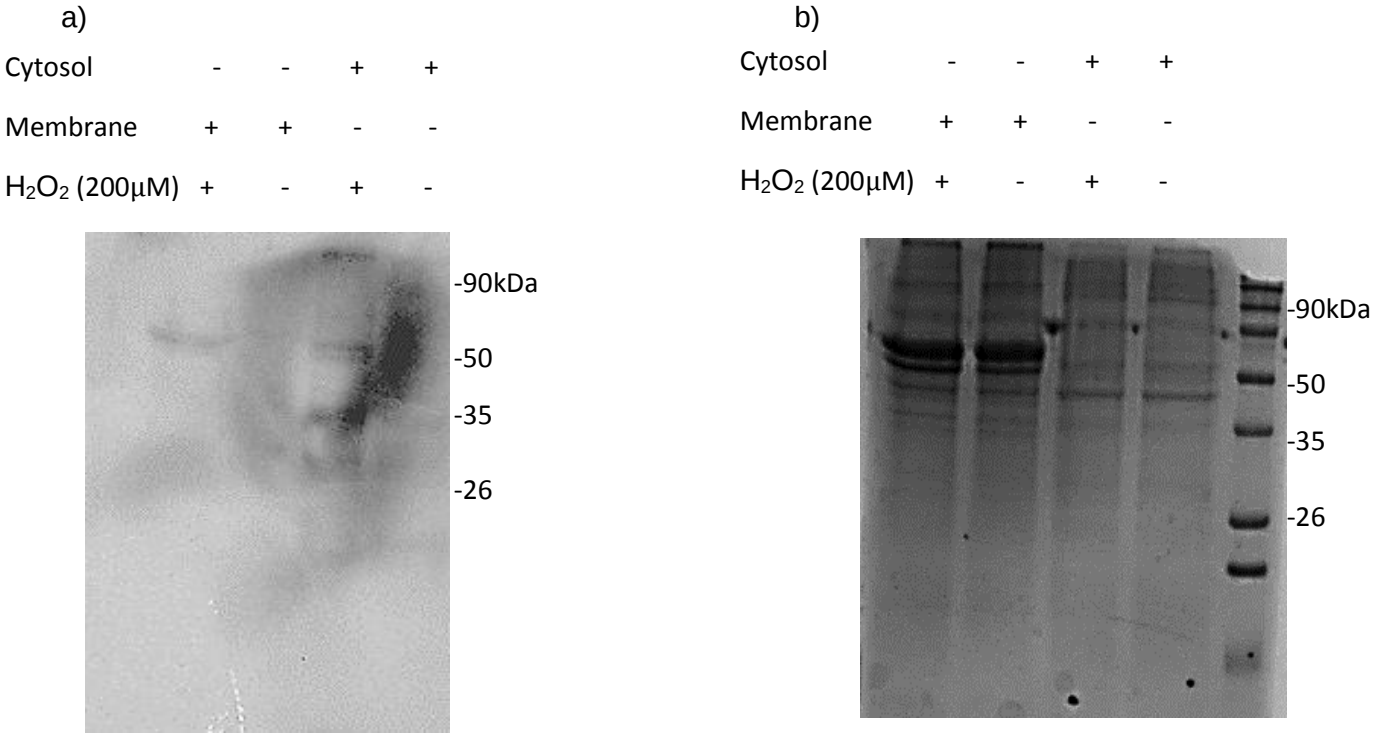



Figure 3.4: SDS-PAGE showing protein carbonyl modification when treated with 200µM $H_2O_2$. 20µg of CP and MP were treated with 200µM $H_2O_2$ for 30min on ice followed by 10mM IAA and catalase in the treated samples. The protein fraction were separated on 15% SDS PAGE and derivatised using DNPH. a) Carbonyl western blot. b) Gel showing loading control. This blot is representative of three independent experiments.

### 3.3.4. Protein sulphenic acid modification in Jurkat T cells

In order to detect protein sulphenic acid modification in T cells, the biotinylated dimedone based probe (DCP-Bio1) was used to capture sulphenic acid modified proteins. Cytosolic and membrane proteins were isolated and 20µg of CPs and MPs were treated with increasing concentration of $H_2O_2$ in the presence of DCP Bio1 (0.5mM). Proteins incubated with 0.5mM $H_2O_2$ show significant bands when probed with streptavidin HRP compared absence of DCP-Bio1 as shown in Figure 3.5 below suggesting that the proteins have suphenic acid modification. Oxidants including $H_2O_2$, peroxynitrite, or lipid hydroperoxides are capable of forming cysteine sulphenic acids by oxidizing cysteines (Nelson et al. 2010). Also, the treatment with hydrogen peroxide leads to increasing band intensity of MPs, suggesting that $H_2O_2$ leads to an increase in sulphenic acid modification in the membrane proteins of jurkat T cells. However, treatment of $H_2O_2$ leads to decreased intenstity of sulphenic acid modification in cytosolic protein fraction.

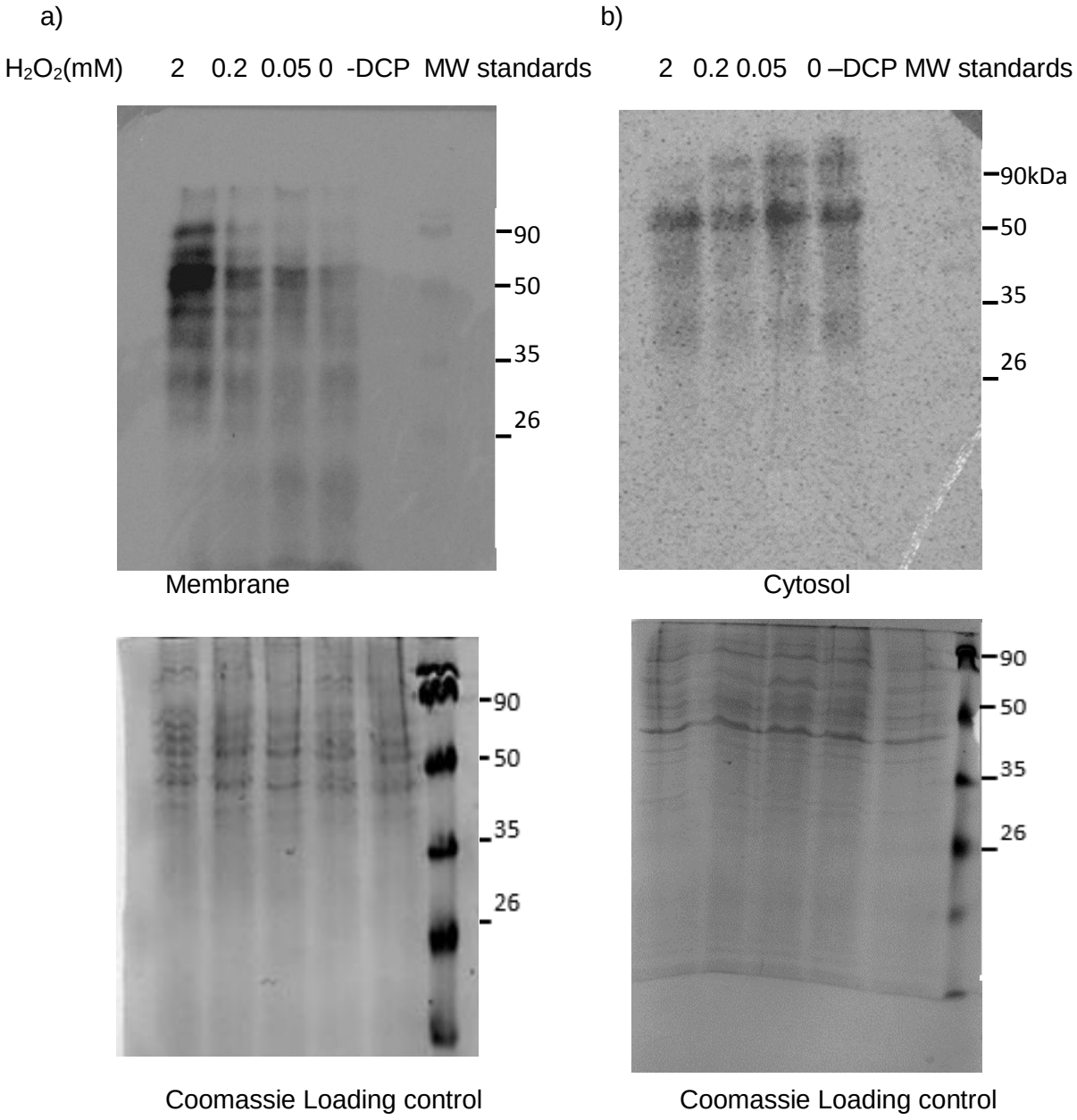



Figure 3.5: SDS-PAGE showing protein sulphenic acid modification in Jurkat T cells in the presence of increasing concentration of $H_2O_2$. CPs and MPs were treated with increasing concentration of $H_2O_2$ in the presence of 0.5mM DCP-Bio1 and 10mM IAA for 30min. The protein fractions were resuspended in Laemmli buffer and separated on 15% SDS PAGE and analysed by Western blotting using HRP conjugated streptavidin for biotinylated probe detection; (1:1000) for 2h and developed using ECL. a) Membrane proteins (MP) and loading control below and b) Cytosolic proteins (CPs) and loading control below. This is a representative of 3 independent replicates. n=3

### 3.3.5. Streptavidin beads capture to purify modified proteins

In order to capture sulphenic acid modified proteins for further identification, streptavidin magnetic beads were used to capture biotinylated proteins in the absence or presence of $H_2O_2$ (200µM). CPs were separated by SDS-PAGE and Figure 3.6a shows distinct bands around 30 and 15kDa and low intensity bands around 50kDa in both treated and untreated lanes showing that a number of proteins within the cytosol are sulphenated. Figure 3.6b shows similar bands representing sulphenated proteins in MP as observed in the cytosolic fraction. However, the intensity of the bands increases with the treatment of $H_2O_2$ suggesting that $H_2O_2$ leads to further sulphenic acid formation. A lower MW high intensity band was observed in the presence of DCP-Bio1 which may indicate the streptavidin monomer.

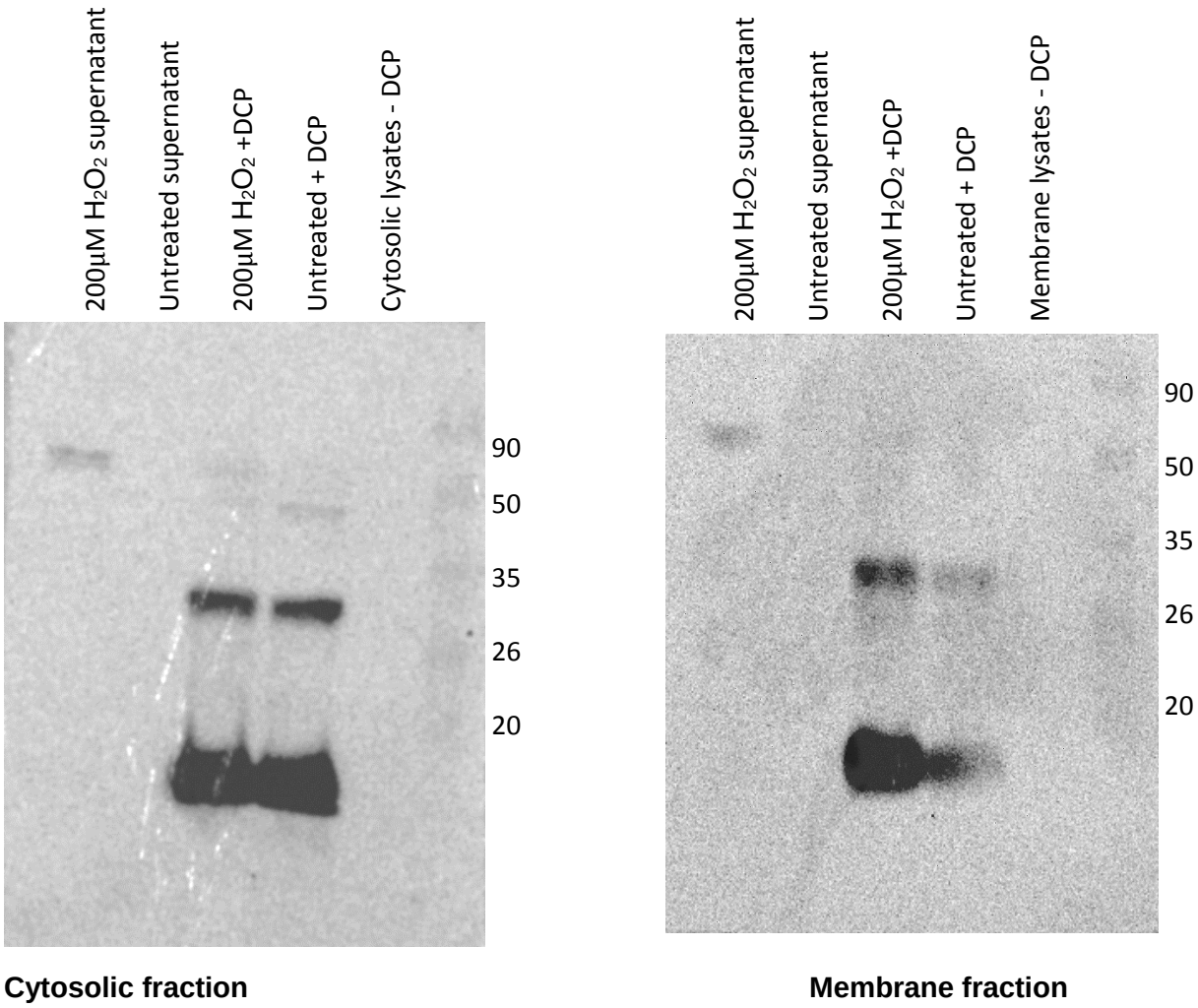



Figure 3.6: SDS-PAGE and western blot showing proteins undergoing sulphenic acid modification.20 µg of membrane and cytosolic proteins were treated with 200uM $H_2O_2$ alongside DCP-Bio1 at 0.5mM for 30min. Catalase and IAA (10mM) was added in the treated samples and modified proteins were captured with Magnabind streptavidin beads (200µL) and incubated for 30min at room temperature with rotation. The supernatant was collected after placing the samples on a magnet. The beads were boiled at 95C for five min with Laemmli buffer (40uL) and 20uL was separated on 15% SDS. Transferred and blocked with 3% BSA overnight. The membrane was probed with streptavidin-HRP (1:1000) for 1:30min and developed using ECL. This is a representative of three independent replicates, n=3.

### 3.3.6 Mass spectrometry identification of membrane proteins containing sulphenic acid modification.

In order to identify proteins that contain sulphenic acid modifications, the gel lanes in figure 3.7 were cut in three sections (white boxes) and digested by trypsin as described in the methods sections above 2.2.5. Trypsin digestion allows the identification of several proteins considered to have sulphenic acid modification. To note, the hits with the highest scoring were serum albumin and keratin and these were not considered as keratin contamination is likely during trypsin digestion and albumin is the most abundant protein within cell culture media.

In order to narrow down the dataset results, MW of proteins which match up to the position of migration on the gel were divided into sections 1,2 and 3 as presented in tables below (table 3.1-3.4). In addition, the proteins were grouped based on their molecular function using PANTHER database.

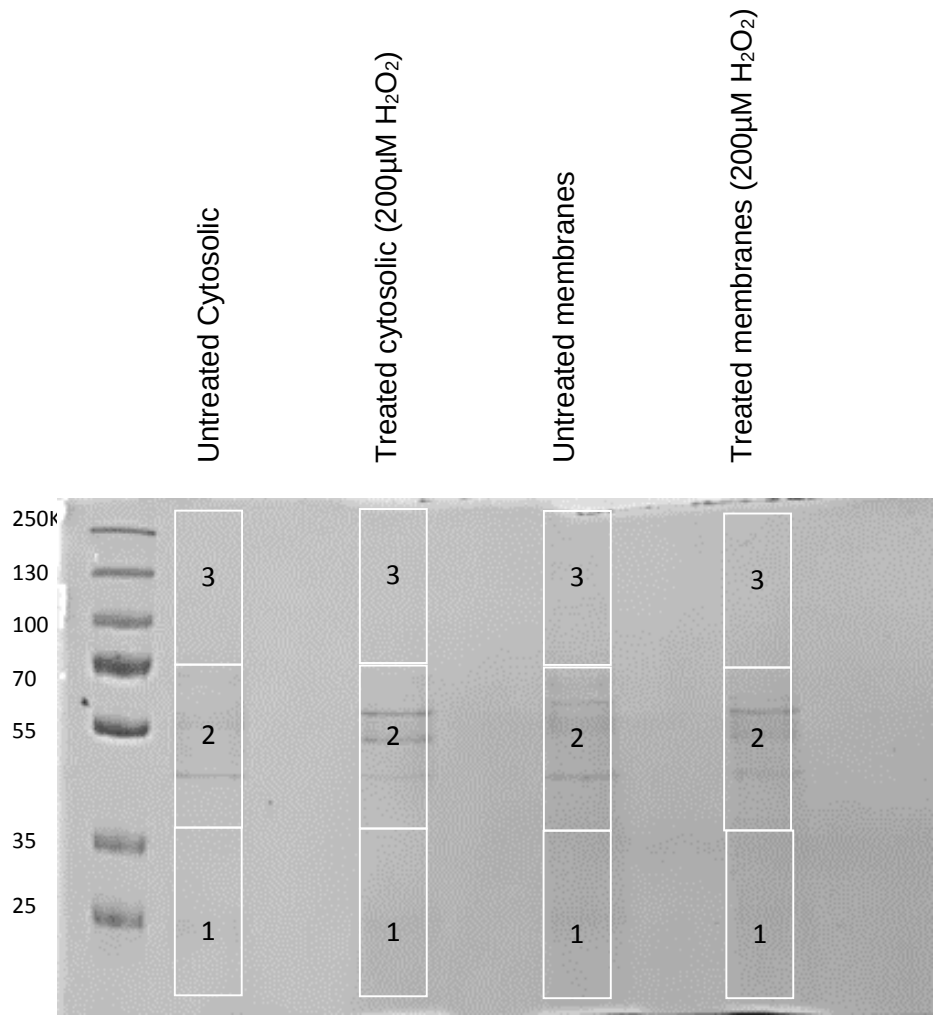



Figure 3.7: SDS-PAGE showing sulphenated proteins after streptavidin capture. 20 µg of membrane and cytosolic proteins were treated with 200µM $H_2O_2$ in the presence of 0.5mM DCP-Bio1 for 30min. Catalase and IAA (10mM) was added and modified proteins were captured with magnabind streptavidin beads (200µL) and incubated for 30min at room temperature with rotation. The supernatant was collected after placing the samples on a magnet. The beads were boiled at 95°C for five min with Laemmli buffer (40µL) and 20µL was separated on 10% SDS PAGE followed by Coomassie staining. this is representative of one experiment. 1,2, 3 show different sections based on molecular weight in the tables

**Section 3**

| ACCESSION | SCORE | MASS | NUM. OF SEQUENCES | DESCRIPTION |
|---|---|---|---|---|
| **HSP7C_HUMAN** | **468** | **70854** | **9** | **Heat shock cognate 71 kDa protein** |
| **TANC1_HUMAN** | 39 | 202093 | 6 | Protein TANC1 |
| **ZC3HD_HUMAN** | 38 | 196519 | 5 | Zinc finger CCCH domain-containing protein 13 |
| **FLNB_HUMAN** | 28 | 277990 | 5 | Filamin-B |
| **NMD3A_HUMAN** | 42 | 125385 | 4 | Glutamate receptor ionotropic, NMDA 3A |
| **KIF2C_HUMAN** | 21 | 81261 | 3 | Kinesin-like protein KIF2C |
| **UBP45_HUMAN** | 39 | 91675 | 3 | Ubiquitin carboxyl-terminal hydrolase 45 |

**Section 2**

| ACCESSION | SCORE | MASS | NUM. OF SEQUENCES | DESCRIPTION |
|---|---|---|---|---|
| **ACTG_HUMAN** | 4015 | 41766 | 15 | Actin, cytoplasmic 2 |
| **TBB5_HUMAN** | 6285 | 49639 | 11 | Tubulin beta chain |
| **TCPQ_HUMAN** | 1027 | 59583 | 11 | T-complex protein 1 subunit theta |
| **TBB4B_HUMAN** | 4709 | 49799 | 10 | Tubulin beta-4B chain |
| **KPYM_HUMAN** | 763 | 57900 | 10 | Pyruvate kinase PKM |
| **EF1A1_HUMAN** | 792 | 50109 | 9 | Elongation factor 1-alpha 1 |
| **TCPB_HUMAN** | 601 | 57452 | 9 | T-complex protein 1 subunit beta |
| **TBA1A_HUMAN** | 2550 | 50104 | 8 | Tubulin alpha-1A chain |
| **SERA_HUMAN** | 895 | 56614 | 8 | D-3-phosphoglycerate dehydrogenase |
| **TCPA_HUMAN** | 280 | 60306 | 8 | T-complex protein 1 subunit alpha |
| **ALBU_HUMAN** | 2866 | 69321 | 8 | Serum albumin |
| **TCPH_HUMAN** | 259 | 59329 | 7 | T-complex protein 1 subunit eta |
| **EWS_HUMAN** | 66 | 68436 | 7 | RNA-binding protein EWS |
| **PLSL_HUMAN** | 626 | 70244 | 7 | Plastin-2 |
| **TCPD_HUMAN** | 312 | 57888 | 6 | T-complex protein 1 subunit delta |
| **TCPE_HUMAN** | 446 | 59633 | 6 | T-complex protein 1 subunit epsilon |
| **TCPG_HUMAN** | 305 | 60495 | 6 | T-complex protein 1 subunit gamma |
| **PGK1_HUMAN** | 32 | 44586 | 5 | Phosphoglycerate kinase 1 |
| **EF1G_HUMAN** | 221 | 50087 | 5 | Elongation factor 1-gamma |
| **HNRPK_HUMAN** | 437 | 50944 | 5 | Heterogeneous nuclear ribonucleoprotein K |
| **COR1A_HUMAN** | 65 | 50994 | 4 | Coronin-1A |
| **RUVB2_HUMAN** | 45 | 51125 | 4 | RuvB-like 2 |
| **TCPZ_HUMAN** | 88 | 57988 | 4 | T-complex protein 1 subunit zeta |
| **PA2G4_HUMAN** | 42 | 43759 | 3 | Proliferation-associated protein 2G4 |
| **PUR6_HUMAN** | 209 | 47049 | 3 | Multifunctional protein |
| **RUVB1_HUMAN** | 78 | 50196 | 3 | RuvB-like 1 |
| **ANT3_HUMAN** | 225 | 52569 | 3 | Antithrombin-III |
| **SYFA_HUMAN** | 117 | 57528 | 3 | Phenylalanine--tRNA ligase alpha subunit |
| **IRGQ_HUMAN** | 22 | 62678 | 3 | Immunity-related GTPase family Q protein |
| **HSP7C_HUMAN** | 36 | 70854 | 3 | Heat shock cognate 71 kDa protein |
| **RAE1_HUMAN** | 39 | 73429 | 3 | Rabproteins geranylgeranyltransferasecomponent A1 |

**Section 1**

| ACCESSION | SCORE | MASS | NUM. OF SEQUENCES | DESCRIPTION |
|---|---|---|---|---|
| **PRDX1_HUMAN** | 593 | 22096 | 8 | Peroxiredoxin-1 |
| **RS3_HUMAN** | 363 | 26671 | 8 | 40S ribosomal protein S3 |
| **RAN_HUMAN** | 134 | 24408 | 6 | GTP-binding nuclear protein Ran |
| **RL12_HUMAN** | 230 | 17808 | 5 | 60S ribosomal protein L12 |
| **RLA0_HUMAN** | 268 | 34252 | 5 | 60S acidic ribosomal protein P0 |
| **H2B1H_HUMAN** | 135 | 13884 | 4 | Histone H2B type 1-H |
| **RS16_HUMAN** | 51 | 16435 | 4 | 40S ribosomal protein S16 |
| **PRDX2_HUMAN** | 255 | 21878 | 4 | Peroxiredoxin-2 |
| **RAB1A_HUMAN** | 30 | 22663 | 4 | Ras-related protein Rab-1A |
| **CYBP_HUMAN** | 38 | 26194 | 4 | Calcyclin-binding protein |
| **RS3A_HUMAN** | 74 | 29926 | 4 | 40S ribosomal protein S3a |
| **H2AV_HUMAN** | 37 | 13501 | 3 | Histone H2A.V |
| **NDKB_HUMAN** | 45 | 17287 | 3 | Nucleoside diphosphate kinase B |
| **COF1_HUMAN** | 84 | 18491 | 3 | Cofilin-1 |
| **RS7_HUMAN** | 70 | 22113 | 3 | 40S ribosomal protein S7 |
| **RS5_HUMAN** | 188 | 22862 | 3 | 40S ribosomal protein S5 |

Table 3.1: Proteins identified for lane 1 in Fig 3.7; untreated CP

**Section 3**

| ACCESSION | SCORE | MASS | NUM. OF SEQUENCES | DESCRIPTION |
|---|---|---|---|---|
| **ANR12_HUMAN** | 26 | 235507 | 5 | Ankyrin repeat domain-containing protein 12 |
| **ABCA3_HUMAN** | 20 | 191239 | 5 | ATP-binding cassette sub-family A member 3 |
| **SMC4_HUMAN** | 19 | 147091 | 3 | Structural maintenance of chromosomes protein 4 |

**Section 2**

| ACCESSION | SCORE | MASS | NUM. OF SEQUENCES | DESCRIPTION |
|---|---|---|---|---|
| **DHE3_HUMAN** | 11211 | 61359 | 17 | Glutamate dehydrogenase 1, mitochondrial |
| **ACTG_HUMAN** | 5835 | 41766 | 14 | Actin, cytoplasmic 2 |
| **TCPH_HUMAN** | 26 | 59329 | 9 | T-complex protein 1 subunit eta |
| **TBB5_HUMAN** | 4055 | 49639 | 9 | Tubulin beta chain |
| **TBB4B_HUMAN** | 1921 | 49799 | 8 | Tubulin beta-4B chain |
| **CATA_HUMAN** | 1259 | 59719 | 7 | Catalase |
| **ALBU_HUMAN** | 1953 | 69321 | 6 | Serum albumin |
| **TCPG_HUMAN** | 51 | 60495 | 6 | T-complex protein 1 subunit gamma |
| **TBA1A_HUMAN** | 1916 | 50104 | 6 | Tubulin alpha-1A chain |
| **HSP7C_HUMAN** | **358** | **70854** | **5** | Heat shock cognate 71 kDa protein |
| **TCPZ_HUMAN** | 65 | 57988 | 5 | T-complex protein 1 subunit zeta |
| **TCPB_HUMAN** | 233 | 57452 | 5 | T-complex protein 1 subunit beta |
| **EF1A1_HUMAN** | 1666 | 50109 | 4 | Elongation factor 1-alpha 1 |

| | | | | |
|---|---|---|---|---|
| **PGK1_HUMAN** | 26 | 44586 | 4 | Phosphoglycerate kinase 1 |
| **PLST_HUMAN** | 41 | 70766 | 3 | Plastin-3 |
| **ENTP7_HUMAN** | 22 | 68916 | 3 | Ectonucleoside triphosphate diphosphohydrolase 7 |
| **HNRH1_HUMAN** | 58 | 49198 | 3 | Heterogeneous nuclear ribonucleoprotein H |
| **PUR6_HUMAN** | 31 | 47049 | 3 | Multifunctional protein ADE2 |

**Section 1**

| ACCESSION | SCORE | MASS | NUM. OF SEQUENCES | DESCRIPTION |
|---|---|---|---|---|
| **SMKR1_HUMAN** | 17 | 7087 | 1 | Small lysine-rich protein 1 |
| **PRDX1_HUMAN** | 726 | 22096 | 10 | Peroxiredoxin-1 |
| **RL12_HUMAN** | 233 | 17808 | 6 | 60S ribosomal protein L12 |
| **RS3_HUMAN** | 225 | 26671 | 5 | 40S ribosomal protein S3 |
| **COF1_HUMAN** | 82 | 18491 | 4 | Cofilin-1 |
| **RL11_HUMAN** | 75 | 20240 | 4 | 60S ribosomal protein L11 |
| **TEX30_HUMAN** | 33 | 25568 | 4 | Testis-expressed protein 30 |
| **H2B1L_HUMAN** | 69 | 13944 | 3 | Histone H2B type 1-L |
| **RS10_HUMAN** | 49 | 18886 | 3 | 40S ribosomal protein S10 |
| **PRDX2_HUMAN** | 267 | 21878 | 3 | Peroxiredoxin-2 |
| **CC134_HUMAN** | 40 | 26544 | 3 | Coiled-coil domain-containing protein 134 |
| **PGAM2_HUMAN** | 17 | 28748 | 3 | Phosphoglycerate mutase 2 |
| **RLA0L_HUMAN** | 28 | 34343 | 3 | 60S acidic ribosomal protein P0-like |
| **GSTP1_HUMAN** | 60 | 23341 | 3 | Glutathione S-transferase P |

Table 3.2: Proteins identified for lane 2 in Fig 3.7; CP treated with 200µM $H_2O_2$

**Section 3**

| ACCESSION | SCORE | MASS | NUM. OF SEQUENCES | DESCRIPTION |
|---|---|---|---|---|
| **HYOU1_HUMAN** | 632 | 111266 | 21 | Hypoxia up-regulated protein 1 |
| **TLN1_HUMAN** | 76 | 269599 | 17 | Talin-1 |
| **ENPL_HUMAN** | 936 | 92411 | 15 | Endoplasmin |
| **EF2_HUMAN** | 787 | 95277 | 14 | Elongation factor 2 |
| **C1TC_HUMAN** | 143 | 101495 | 11 | C-1-tetrahydrofolate synthase, cytoplasmic |
| **GANAB_HUMAN** | 226 | 106807 | 10 | Neutral alpha-glucosidase AB |
| **SND1_HUMAN** | 135 | 101934 | 10 | Staphylococcal nuclease domain-containing protein 1 |
| **FLNA_HUMAN** | 100 | 280564 | 9 | Filamin-A |
| **PYR1_HUMAN** | 39 | 242829 | 7 | CAD protein |
| **TERA_HUMAN** | 29 | 89266 | 7 | Transitional endoplasmic reticulum ATPase |
| **P5CS_HUMAN** | 61 | 87248 | 7 | Delta-1-pyrroline-5-carboxylate synthase |
| **ACON_HUMAN** | 228 | 85372 | 6 | Aconitate hydratase, mitochondrial |
| **FLNB_HUMAN** | 27 | 277990 | 5 | Filamin-B |
| **ZC3HD_HUMAN** | 33 | 196519 | 5 | Zinc finger CCCH domain-containing protein 13 |
| **AFF3_HUMAN** | 39 | 133394 | 5 | AF4/FMR2 family member 3 |
| **SC31B_HUMAN** | 39 | 128615 | 5 | Protein transport protein Sec31B |
| **IPO5_HUMAN** | 37 | 123550 | 5 | Importin-5 |
| **UBA1_HUMAN** | 155 | 117774 | 5 | Ubiquitin-like modifier-activating enzyme 1 |
| **ERAP2_HUMAN** | 37 | 110391 | 5 | Endoplasmic reticulum aminopeptidase 2 |

| | | | | |
|---|---|---|---|---|
| **TRPC6_HUMAN** | 23 | 106258 | 5 | Short transient receptor potential channel 6 |
| **MCM4_HUMAN** | 21 | 96498 | 5 | DNA replication licensing factor MCM4 |
| **HNRPU_HUMAN** | 104 | 90528 | 5 | Heterogeneous nuclear ribonucleoprotein U |
| **TFR1_HUMAN** | 233 | 84818 | 5 | Transferrin receptor protein 1 |
| **XRCC5_HUMAN** | 22 | 82652 | 5 | X-ray repair cross-complementing protein 5 |
| **MCM7_HUMAN** | 45 | 81257 | 5 | DNA replication licensing factor MCM7 |
| **CFA57_HUMAN** | 35 | 144870 | 4 | Cilia- and flagella-associated protein 57 |
| **SYAC_HUMAN** | 37 | 106743 | 4 | Alanine--tRNA ligase, cytoplasmic |
| **IMB1_HUMAN** | 334 | 97108 | 4 | Importin subunit beta-1 |
| **SC23A_HUMAN** | 38 | 86105 | 4 | Protein transport protein Sec23A |
| **TRPM7_HUMAN** | 21 | 212561 | 3 | Transient receptor potential cation channel subfamily M member 7 |
| **ERAP1_HUMAN** | 135 | 107166 | 3 | Endoplasmic reticulum aminopeptidase 1 |
| **PDPR_HUMAN** | 25 | 99301 | 3 | Pyruvate dehydrogenase phosphatase regulatory subunit, mitochondrial |

**Section 2**

| ACCESSION | SCORE | MASS | NUM. OF SEQUENCES | DESCRIPTION |
|---|---|---|---|---|
| **CH60_HUMAN** | 6685 | 61016 | 28 | 60 kDa heat shock protein, mitochondrial |
| **ACTG_HUMAN** | 3190 | 41766 | 15 | Actin, cytoplasmic 2 |
| **ATPA_HUMAN** | 994 | 59714 | 15 | ATP synthase subunit alpha, mitochondrial |
| **PDIA3_HUMAN** | 951 | 56747 | 12 | Protein disulfide-isomerase A3 |
| **KPYM_HUMAN** | 435 | 57900 | 12 | Pyruvate kinase PKM |
| **PGK1_HUMAN** | 387 | 44586 | 9 | Phosphoglycerate kinase 1 |
| **SERA_HUMAN** | 629 | 56614 | 9 | D-3-phosphoglycerate dehydrogenase |
| **TBA1B_HUMAN** | 2394 | 50120 | 8 | Tubulin alpha-1B chain |
| **IDHP_HUMAN** | 816 | 50877 | 8 | Isocitrate dehydrogenase [NADP], mitochondrial |
| **GLYM_HUMAN** | 397 | 55958 | 8 | Serine hydroxymethyltransferase, mitochondrial |
| **TBB5_HUMAN** | 3466 | 49639 | 7 | Tubulin beta chain |
| **TBB4B_HUMAN** | 2596 | 49799 | 7 | Tubulin beta-4B chain |
| **HNRPK_HUMAN** | 689 | 50944 | 7 | Heterogeneous nuclear ribonucleoprotein K |
| **ALBU_HUMAN** | 3436 | 69321 | 7 | Serum albumin |
| **PTBP1_HUMAN** | 386 | 57186 | 6 | Polypyrimidine tract-binding protein 1 |
| **VAT1_HUMAN** | 151 | 41893 | 5 | Synaptic vesicle membrane protein VAT-1 homolog |
| **EF1A1_HUMAN** | 498 | 50109 | 5 | Elongation factor 1-alpha 1 |
| **TCPB_HUMAN** | 76 | 57452 | 5 | T-complex protein 1 subunit beta |
| **TCPG_HUMAN** | 35 | 60495 | 5 | T-complex protein 1 subunit gamma |
| **RBP56_HUMAN** | 64 | 61793 | 5 | TATA-binding protein-associated factor 2N |
| **TCPD_HUMAN** | 115 | 57888 | 4 | T-complex protein 1 subunit delta |
| **TCPQ_HUMAN** | 403 | 59583 | 4 | T-complex protein 1 subunit theta |
| **TCPE_HUMAN** | 72 | 59633 | 4 | T-complex protein 1 subunit epsilon |
| **DHE4_HUMAN** | 174 | 61395 | 4 | Glutamate dehydrogenase 2, mitochondrial |
| **AIFM1_HUMAN** | 308 | 66859 | 4 | Apoptosis-inducing factor 1, mitochondrial |
| **ODP2_HUMAN** | 54 | 68953 | 4 | Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial |
| **CODA1_HUMAN** | 25 | 69907 | 4 | Collagen alpha-1(XIII) chain |
| **IDH3B_HUMAN** | 374 | 42157 | 3 | Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial |
| **THIL_HUMAN** | 149 | 45171 | 3 | Acetyl-CoA acetyltransferase, mitochondrial |

| | | | | |
|---|---|---|---|---|
| **ACADM_HUMAN** | 65 | 46559 | 3 | Medium-chain specific acyl-CoA dehydrogenase, mitochondrial |
| **AATM_HUMAN** | 34 | 47487 | 3 | Aspartate aminotransferase, mitochondrial |
| **SAHH_HUMAN** | 55 | 47685 | 3 | Adenosylhomocysteinase |
| **HNRH1_HUMAN** | 234 | 49198 | 3 | Heterogeneous nuclear ribonucleoprotein H |
| **SUCB1_HUMAN** | 31 | 50285 | 3 | Succinate--CoA ligase [ADP-forming] subunit beta, mitochondrial |
| **ANT3_HUMAN** | 175 | 52569 | 3 | Antithrombin-III |
| **QCR1_HUMAN** | 52 | 52612 | 3 | Cytochrome b-c1 complex subunit 1, mitochondrial |
| **ATPB_HUMAN** | 343 | 56525 | 3 | ATP synthase subunit beta, mitochondrial |
| **TCPZ_HUMAN** | 52 | 57988 | 3 | T-complex protein 1 subunit zeta |
| **TCPH_HUMAN** | 45 | 59329 | 3 | T-complex protein 1 subunit eta |
| **CATA_HUMAN** | 71 | 59719 | 3 | Catalase |
| **HNRPL_HUMAN** | 68 | 64092 | 3 | Heterogeneous nuclear ribonucleoprotein L |

**Section 1**

| ACCESSION | SCORE | MASS | NUM. OF SEQUENCES | DESCRIPTION |
|---|---|---|---|---|
| **PRDX1_HUMAN** | 255 | 22096 | 7 | Peroxiredoxin-1 |
| **ADT2_HUMAN** | 27 | 32831 | 6 | ADP/ATP translocase 2 |
| **STMN1_HUMAN** | 89 | 17292 | 4 | Stathmin |
| **RAB1B_HUMAN** | 89 | 22157 | 4 | Ras-related protein Rab-1B |
| **RAN_HUMAN** | 447 | 24408 | 4 | GTP-binding nuclear protein Ran |
| **RS7_HUMAN** | 26 | 22113 | 3 | 40S ribosomal protein S7 |
| **RAB7A_HUMAN** | 110 | 23475 | 3 | Ras-related protein Rab-7a |
| **HCD2_HUMAN** | 93 | 26906 | 3 | 3-hydroxyacyl-CoA dehydrogenase type-2 |
| **ERP29_HUMAN** | 32 | 28975 | 3 | Endoplasmic reticulum resident protein 29 |
| **ELAV1_HUMAN** | 22 | 36069 | 3 | ELAV-like protein 1 |

Table 3.3: Proteins identified for lane 3 in Fig 3.7; untreated MP

**Section 3**

| ACCESSION | SCORE | MASS | NUM. OF SEQUENCES | DESCRIPTION |
|---|---|---|---|---|
| **VWF_HUMAN** | 29 | 309058 | 7 | von Willebrand factor |
| **CO6A6_HUMAN** | 22 | 247019 | 7 | Collagen alpha-6(VI) chain |
| **GRP78_HUMAN** | 537 | 72288 | 7 | 78 kDa glucose-regulated protein |
| **TANC1_HUMAN** | 36 | 202093 | 5 | Protein TANC1 |
| **ZC3HD_HUMAN** | 33 | 196519 | 5 | Zinc finger CCCH domain-containing protein 13 |
| **HYOU1_HUMAN** | 282 | 111266 | 5 | Hypoxia up-regulated protein 1 |
| **AGRB2_HUMAN** | 29 | 172544 | 4 | Adhesion G protein-coupled receptor B2 |
| **MINT_HUMAN** | 33 | 402004 | 3 | Msx2-interacting protein |
| **NMD3A_HUMAN** | 36 | 125385 | 3 | Glutamate receptor ionotropic, NMDA 3A |
| **GANAB_HUMAN** | 31 | 106807 | 3 | Neutral alpha-glucosidase AB |

**Section 2**

| ACCESSION | SCORE | MASS | NUM. OF SEQUENCES | DESCRIPTION |
|---|---|---|---|---|
| **CH60_HUMAN** | 8447 | 61016 | 35 | 60 kDa heat shock protein, mitochondrial |
| **ACTG_HUMAN** | 6348 | 41766 | 15 | Actin, cytoplasmic 2 |
| **DHE3_HUMAN** | 5598 | 61359 | 15 | Glutamate dehydrogenase 1, mitochondrial |
| **GRP75_HUMAN** | 740 | 73635 | 12 | Stress-70 protein, mitochondrial |
| **TBB5_HUMAN** | 3029 | 49639 | 10 | Tubulin beta chain |
| **KPYM_HUMAN** | 102 | 57900 | 10 | Pyruvate kinase PKM |
| **TBB4A_HUMAN** | 1926 | 49554 | 8 | Tubulin beta-4A chain |
| **PGK1_HUMAN** | 129 | 44586 | 7 | Phosphoglycerate kinase 1 |
| **TBA1A_HUMAN** | 1474 | 50104 | 7 | Tubulin alpha-1A chain |
| **CATA_HUMAN** | 2248 | 59719 | 7 | Catalase |
| **K1C14_HUMAN** | 521 | 51529 | 6 | Keratin, type I cytoskeletal 14 |
| **ATPB_HUMAN** | 384 | 56525 | 6 | ATP synthase subunit beta, mitochondrial |
| **PTBP1_HUMAN** | 173 | 57186 | 6 | Polypyrimidine tract-binding protein 1 |
| **ALBU_HUMAN** | 858 | 69321 | 6 | Serum albumin |
| **GRP78_HUMAN** | 431 | 72288 | 6 | 78 kDa glucose-regulated protein |
| **ANKR6_HUMAN** | 17 | 79922 | 6 | Ankyrin repeat domain-containing protein 6 |
| **GNAS2_HUMAN** | 233 | 45636 | 5 | Guanine nucleotide-binding protein G(s) subunit alpha isoforms short |
| **PDIA6_HUMAN** | 489 | 48091 | 5 | Protein disulfide-isomerase A6 |
| **HNRH1_HUMAN** | 297 | 49198 | 5 | Heterogeneous nuclear ribonucleoprotein H |
| **ENOA_HUMAN** | 76 | 47139 | 5 | Alpha-Enolase |
| **GLYM_HUMAN** | 135 | 55958 | 5 | Serine hydroxymethyltransferase, mitochondrial |
| **PDIA3_HUMAN** | 99 | 56747 | 5 | Protein disulfide-isomerase A3 |
| **TCPB_HUMAN** | 48 | 57452 | 5 | T-complex protein 1 subunit beta |
| **ATPA_HUMAN** | 287 | 59714 | 5 | ATP synthase subunit alpha, mitochondrial |
| **TCPG_HUMAN** | 59 | 60495 | 5 | T-complex protein 1 subunit gamma |
| **EFTU_HUMAN** | 315 | 49510 | 4 | Elongation factor Tu, mitochondrial |
| **EF1A1_HUMAN** | 98 | 50109 | 4 | Elongation factor 1-alpha 1 |
| **COR1A_HUMAN** | 50 | 50994 | 4 | Coronin-1A |
| **SERA_HUMAN** | 41 | 56614 | 4 | D-3-phosphoglycerate dehydrogenase |
| **PLSL_HUMAN** | 213 | 70244 | 4 | Plastin-2 |
| **HSP7C_HUMAN** | 215 | 70854 | 4 | Heat shock cognate 71 kDa protein |
| **IDH3B_HUMAN** | 37 | 42157 | 3 | Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial |
| **ILF2_HUMAN** | 165 | 43035 | 3 | Interleukin enhancer-binding factor 2 |
| **HNRPK_HUMAN** | 364 | 50944 | 3 | Heterogeneous nuclear ribonucleoprotein K |
| **AIFM1_HUMAN** | 31 | 66859 | 3 | Apoptosis-inducing factor 1, mitochondrial |
| **G3P_HUMAN** | 358 | 36030 | 2 | Glyceraldehyde-3-phosphate dehydrogenase |

**Section 1**

| ACCESSION | SCORE | MASS | NUM. OF SEQUENCES | DESCRIPTION |
|---|---|---|---|---|
| **RS16_HUMAN** | 112 | 16435 | 4 | 40S ribosomal protein S16 |
| **HCD2_HUMAN** | 91 | 26906 | 4 | 3-hydroxyacyl-CoA dehydrogenase type-2 |
| **ADT2_HUMAN** | 49 | 32831 | 4 | ADP/ATP translocase 2 |
| **PROF1_HUMAN** | 41 | 15045 | 3 | Profilin-1 |
| **NDK8_HUMAN** | 27 | 15519 | 3 | Putative nucleoside diphosphate kinase |
| **COF1_HUMAN** | 176 | 18491 | 3 | Cofilin-1 |
| **RAB1A_HUMAN** | 82 | 22663 | 3 | Ras-related protein Rab-1A |
| **RS5_HUMAN** | 199 | 22862 | 3 | 40S ribosomal protein S5 |
| **RAB7A_HUMAN** | 113 | 23475 | 3 | Ras-related protein Rab-7a |
| **RAN_HUMAN** | 195 | 24408 | 3 | GTP-binding nuclear protein Ran |
| **SMD3_HUMAN** | 21 | 13907 | 2 | Small nuclear ribonucleoprotein Sm D3 |
| **PPIA_HUMAN** | 30 | 18001 | 2 | Peptidyl-prolyl cis-trans isomerase A |
| **PRDX2_HUMAN** | 398 | 21878 | 2 | Peroxiredoxin-2 |
| **BID_HUMAN** | 16 | 21981 | 2 | BH3-interacting domain death agonist |
| **MOB1A_HUMAN** | 20 | 25064 | 2 | MOB kinase activator 1A |
| **NDUV2_HUMAN** | 43 | 27374 | 2 | NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial |
| **PGAM2_HUMAN** | 46 | 28748 | 2 | Phosphoglycerate mutase 2 |
| **ERP29_HUMAN** | 69 | 28975 | 2 | Endoplasmic reticulum resident protein 29 |
| **VDAC3_HUMAN** | 104 | 30639 | 2 | Voltage-dependent anion-selective channel protein 3 |
| **ECHM_HUMAN** | 25 | 31367 | 2 | Enoyl-CoA hydratase, mitochondrial |
| **SRP14_HUMAN** | 47 | 14561 | 1 | Signal recognition particle 14 kDa protein |
| **PA1B3_HUMAN** | 24 | 25718 | 1 | Platelet-activating factor acetylhydrolase IB subunit gamma |
| **PRDX3_HUMAN** | 46 | 27675 | 1 | Thioredoxin-dependent peroxide reductase, mitochondrial |
| **NUD22_HUMAN** | 29 | 32560 | 1 | Nucleoside diphosphate-linked moiety X motif 22 |
| **HCDH_HUMAN** | 99 | 34272 | 1 | Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial |
| **ETFA_HUMAN** | 47 | 35058 | 1 | Electron transfer flavoprotein subunit alpha, mitochondrial |
| **O52B2_HUMAN** | 33 | 36160 | 1 | Olfactory receptor 52B2 |

Table 3.4: Proteins identified for lane 4 in Fig 3.7; MP treated with 200µM $H_2O_2$

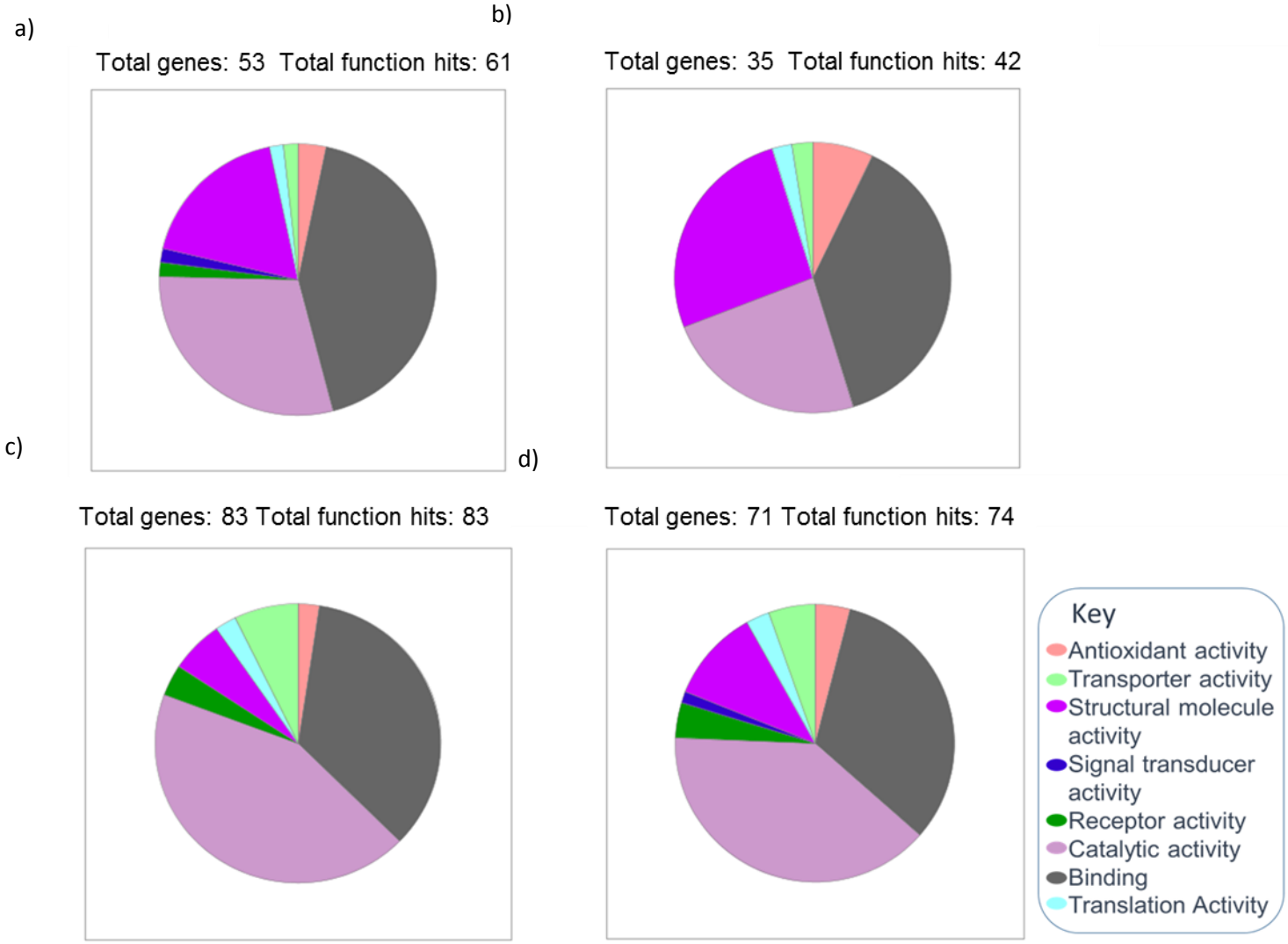
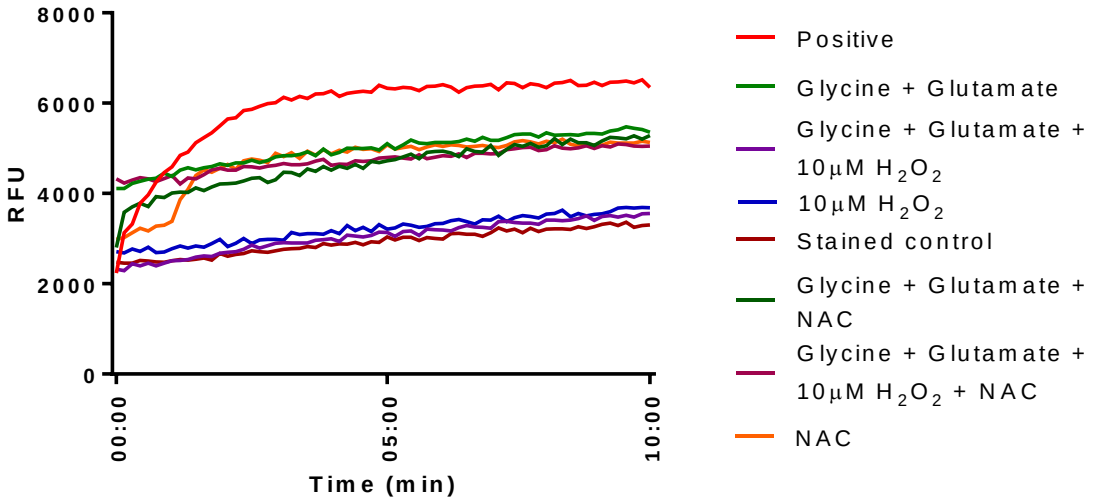



Figure 3.8: Proteins identified which undergo sulphenic acid modification grouped based on their molecular function. a) Untreated cytosolic proteins, b) Treated cytosolic proteins (200μM $H_2O_2$). c) Untreated membrane proteins, d) Treated membrane proteins (200μM $H_2O_2$). Proteins identified by Mascot daemon database and grouped based on their molecular function by PANTHER gene ontology database.

### 3.3.7 Hydrogen peroxide inhibits NMDA calcium influx in Jurkat T cells.

T cells express NMDA receptors as shown by previous studies (Miglio et al. 2005) and LC MS/MS results in section 3.3.6 showed that NMDA receptor is sulphenated in Jurkat T cell cytosolic fraction and this sulphenation is lost after treating with $H_2O_2$ (Table 3.1 section 3 compared to table 3.2 section 3). Also, NMDA is sulphenated in the membrane after $H_2O_2$ treatment. In order evaluate the effects and importance of NMDA receptor on T cell function and calcium influx under oxidising conditions, T cell NMDA receptor was stimulated with the agonist glycine/glutamate in the presence or absence of $H_2O_2$. Glycine and glutamate stimulates the receptor and increases calcium in T cells compared to stained control (Figure 3.9a). However, in the presence of 10µM $H_2O_2$ there was a decreased flux of calcium via the NMDA receptor suggesting that excess $H_2O_2$ leads to inactivation of the receptor thus decreased calcium influx (Figure3.9b) which might affect further T cell function.

a)




b)

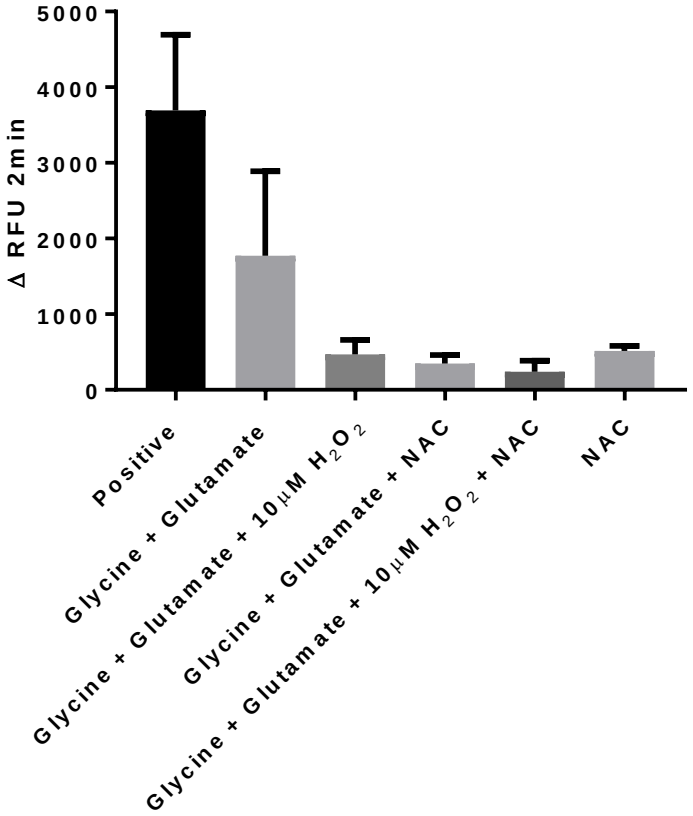



Figure 3.9: The effect of $H_2O_2$ on calcium influx by NMDA receptor. Jurkat T cells were incubated with Fluo3AM for 1h at room temperature in the dark. After washing, cells were resuspended in PBS supplemented with HEPES and $CaCl_2$. NMDA receptor was stimulated with 100µM glycine/glutamate (positive control) and or $H_2O_2$ (10µM) in the presence or absence of NAC (10mM) and the calcium flux was measured using a fluorescence plate reader for 30min at intervals of 10s. The data was normalised to unstained control. a) RFU from 0-10min, b) Δ RFU at 2min and 0min, Data represents the mean and SEM of three independent experiments, n=3. ANOVA. Excitation 506nm: Emission 526nm.

## 3.4. Discussion

Cysteine oxidative modification of proteins by ROS has gained increasing interest leading to the development of chemical reagents to detect cysteine sulphenic acid. Redox biology focuses on three major areas; identification of the nature of oxidation and protein targets, quantification of the extent of the modification and precise sites, the functional consequences on the individual protein activity due to the extent of the modification (Furdui & Poole 2014). Oxidative modification of cysteine to sulphenic acid has been regarded as an significant post-translational modifications in proteins and protein function under oxidative stress and physiological conditions (Poole & Nelson 2008; Leonard & Carroll 2011; Roos & Messens 2011).

The aim of this chapter was to develop and identify the proteins that may be sulphenated on the T cell membrane and cytosol. This chapter has looked into protein aggregation and modifications including carbonyl modification and sulphenic acid in both the cytosol and membrane proteins. Furthermore, the effect of $H_2O_2$ was also assessed on sulphenic acid formation in specific CP and MP.

A range of enzymes are responsible for production of $H_2O_2$, which is necessary for receptor mediated cellular activities including DNA synthesis, chemotaxis, and cell cycle progression (Page et al. 2000; Lee et al. 2002)

Under normal conditions, cells maintain a reducing environment in the presence of reducing agents. However, an increase in ROS concentration which exceeds the cellular capability to remove them, termed oxidative stress, can result in protein aggregation, disulphide formation and fragmentation (Squier 2001; Tabner et al. 2005). This was briefly observed in CP and MP treated with increasing concentration of $H_2O_2$ and separated under non-reducing conditions. A few bands which were observed in lanes with $H_2O_2$ indicated with an arrow which were not visible in the untreated samples. However, this experiment was to give an understanding on the extent of protein oxidation. A study by (Zhang et al. 2015) showed that the oxidation of

proteins in the presence of $H_2O_2$ led to protein aggregation but this was not observed when proteins were separated in the presence of DTT. Protein aggregation is likely, but not limited due to cysteine oxidation and disulphide bond formation in the presence of $H_2O_2$ (Zhang et al. 2015; Grune et al. 1997). Furthermore, protein disulphides are known to be the most common covalent link between amino acids in proteins formed in oxidising environment (Yi & Khosla 2016). Variety of these motifs are involved in redox reactions and act as switches in response to oxidative stress. The treatment with $H_2O_2$ shows some bands which are not present in the absence of low concentrations of $H_2O_2$. This may be due to the disulphide link formed in excess of peroxide. This is further reduced by the dithiol-disulphide exchange reaction, which is catalysed by oxidoreductases including members of the PDI and Trx1 family which are also present extracellularly (Söderberg et al. 2013). However, there is no known enzyme responsible for cysteine disulphide reduction. Reduction of CySS can also be achieved by GSH but is limited by GSH availability (Jones 2004). In the presence of an excess oxidative stress, protein thiols may react with adjacent cysteine thiols to form a disulphide bond. However, the formation of disulphide bonds between cysteine of the same proteins may be essential for protein folding in the endoplasmic reticulum (ER) and protein-protein interaction and to enable secondary and higher structures of proteins such as antibodies. Thus the reduction may be essential for the protein function. However, sulphenation may not be a result of an oxidative environment.

Carbonylation is the most common marker to assess protein oxidation (Weber et al. 2015; Trachootham et al. 2008; Frijhoff et al. 2015). In this chapter, it was observed that $H_2O_2$ leads to protein carbonyl formation which is an irreversible form of protein modification. Protein carbonyls are not formed as a result of a specific oxidant and are formed early during oxidative stress, and are thus regarded as a marker of general protein oxidation. Additionally, protein carbonyls are often used as an indicator of protein oxidation as they are easily detectable and present in high levels *in vivo* compared to other modifications (Dalle-Donne et al. 2006).

Previously, it has been shown by Ratnayake 2015 in our lab (unpublished data) that resting T cells undergo sulphenic modification and when treated with BSO, a number of proteins were identified to form the sulphenic acid PTM. The work in this chapter has followed up this work and showed proteins sulphenated on the membrane of T cells when treated with $H_2O_2$.

A Jurkat cell model was optimised to capture proteins with sulphenic acid on the membrane and cytosol which further allowed identification proteins which undergo during T cell activation in the later chapter. Firstly, the cell fractions including CP and MP were treated at different concentrations of $H_2O_2$. This showed a number of bands of increasing intensity with increasing concentrations of $H_2O_2$ in the CP and MP fraction. However, when CP were treated with $H_2O_2$, decreasing band intensity was observed. This might be due to the irreversible modification due to excessive oxidative stress caused by increasing $H_2O_2$ concentration. A study by Charles et al, showed cells treated with high concentrations of $H_2O_2$ did not show a significant sulphenation and suggested that proteins may be undergoing irreversible oxidation such as sulphinic or sulphenic acid, at a rate faster then the reaction of biotin-linked dimedone to sulphenic acid (Charles et al. 2007)

Several proteins have been identified as sulphenated including proteins that are responsible for T cell function and redox regulation. From the proteins identified from MS, a small number of them are involved in oxidation-reduction process as shown in the chart characterising proteins based on their molecular function, including Prxs, alpha enolase and protein disulphide isomerases. In support of this, several cytosolic proteins identified in this chapter have also been shown in the literature to undergo reversible cysteine oxidation (Baty et al. 2005).

At least six different isoforms of Prx have been identified in mammalian cells (Wood, Poole, et al. 2003; Hofmann et al. 2002). Prxs are important in keeping the toxic $H_2O_2$ and peroxynitrite in low levels and also involved in cellular processes such as proliferation, differentiation and apoptosis (Leslie B. Poole et al. 2004). Prx-1 is classified to have two conserved cysteines, out of which one is oxidised to cysteine sulphenic acid and then forms a disulphide with the

other. Under normal physiological conditions, this is reduced by NADPH dependent Trx1 and TrxR to regenerate reduced cysteines for Prx 1-5, while Prx 6 requires other antioxidants such as GSH (Watanabe et al. 2017; Smith-Pearson et al. 2008; Kang et al. 1998; Fisher et al. 1999).

In addition, the role of Prxs as regulators of $H_2O_2$ mediated cell signalling may be implicated in human diseases including neurodegeneration and cancer (Wood, Poole, et al. 2003; Georgiou & Masip 2003). Given their main role of monitoring $H_2O_2$ levels, any modifications to Prxs activity can alter redox dependent signalling pathways. Under high levels of oxidative stress, the cysteine residues can further be oxidised to sulphinic or to sulphonic acid, both of which can inactivate the protein (Wood, Poole, et al. 2003; Wood, Schröder, et al. 2003). Prx1 and Prx2 were identified to be sulphenated on the membrane. Also, peroxiredoxin 1 and 2 are sulphenated in the cytosol irrespective of $H_2O_2$ treatment suggesting Prxs can be oxidised to form sulphenic acid under normal conditions. Recent findings also show Prx1 is sulphenated under normal physiological conditions (Hall et al. 2009; Devarie-Baez et al. 2016)

However, recently the hyper oxidation of Prxs is shown to be reversible by sulfiredoxin, thereby reactivating the protein activity (Chang et al. 2004). The mechanism of Prxs activity is quite interesting such that these abundant antioxidant can be inactivated by its own substrate. This in fact may be due to the sensitivity to the peroxide levels and thus protect the normal cellular metabolism by preventing $H_2O_2$ accumulation.

α-enolase was also one of the proteins identified when T cell membranes were treated with $H_2O_2$. Intracellularly, α-enolase is responsible for dehydration of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis. It is also highly present on the membranes of various cell types including B cells, macrophages, and T cells (Plow et al. 1991) it's main role is to interact with plasminogen receptors on the membrane which allows extracellular proteolysis (Díaz-Ramos et al. 2012). α-enolase is known to undergo several PTM. α-enolase undergo phosphorylation and has been associated with pancreatic cancer (Tomaino et al. 2011), diabetic rat hearts showed nitration of tyrosine residues in α-enolase (Lu et al. 2010) and

carbonylation of α-enolase was shown in human myoblasts under oxidative stress (Baraibar et al. 2011). This chapter has showed that α-enolase undergoes sulphenic acid modification on the membrane of T cells. This was also shown by affinity capture in HEK 293 cells (Nelson et al. 2010). The inactivation or loss of α-enolase may lead to a decrease in localised plasminogen activation which may perhaps affect cell migration (Bennett et al. 2015; Majumdar et al. 2004). However, it still remains to fully understand how these PTM can affect α-enolase catalytic activity, protein stability and localisation. This will further provide an insight of how they play an important role in pathological conditions. Interestingly, Enolase α, peroxiredoxin 1, T complex protein 1 are found to undergo sulphenic acid modification in this study and according to the literature, these proteins were glutathionylated in T cells (Fratelli et al. 2002b). This observation may be explained that the above proteins undergo sulphenic acid modification followed by glutathionlyation.

Sulphenic acid affects protein function as shown by Benitez and Allison, whereby they presented the first evidence of sulphenic acid by using dimeodone (5.5-dimethyl-1,3-cyclohexadione), suggesting it can be used as a diagnostic tool for identification of sulphenated proteins. They identified GAPDH, which has an active cysteine at Cys149 to be sulphenated (Cowan-Jacob et al. 2003; Benitez & Allison 1974). The results from this study also identifies GAPDH as one of the protein undergoing sulphenylation. GAPDH is widely known to be sulphenated and its oxidation is linked to protein function (Maller et al. 2011; Peralta et al. 2015; Baty et al. 2005).

Additionally, protein disulphide isomerase was also identified to be sulphenated in MP of Jurkat cells. PDI is mainly located in the endoplasmic reticulum but studies show it is secreted and associated with the cell surface (Jiang et al. 1999). Cell surface PDI is mainly involved in thiol disulphide exchange reaction, whereby it catalyses the reduction of protein disulphide bonds (Jiang et al. 1999). For example, PDI forms a disulphide and with glycoprotein 120 on the surface of cells promoting HIV entry in vivo (Wang et al. 2010). Through this process, PDI may be oxidised hence forming a sulphenic acid.

Cell-cell adhesion is important in inflammation and wound healing. Actin has one cys residue exposed to the molecular surface which is likely to undergo oxidation. Results from this study show actin is also sulphenated. As PDI is shown to form a disulphide bridge during cell adhesion rearranging the cytoskeleton, it may be possible that this disulphide bridge is formed via sulphenic acid formation (Sobierajska et al. 2014). Also, oxidation of actin cysteine residue to sulphenic acid may lead to glutathionylation (mixed disulphide formed when reacted with GSH) (Johansson & Lundberg 2007).

Cofilin is an actin binding protein which plays a role in T cell migration and activation (Samstag et al. 2013). Cofilin in human T cells has cysteine residues (at positions 39, 80, 140, and 148), which makes it more susceptible to oxidation leading to cofilin regulation via thiol modifications (Klemke et al. 2008). Cysteine oxidation in cofilin leads to T cell hypo-responsiveness and cell death by necrosis. This impairs actin dynamics during antigen presentation and synapse formation. Studies show treatment with $H_2O_2$ causes inter or intra molecular disulphide formation, inter molecular disulphide bridge between cysteines leads to cofilin dimers (Pfannstiel et al. 2001; Klemke et al. 2008). However, this study shows for the first time that cofilin is sulphenated in T cells further supporting that the idea that cofilin is susceptible to get oxidised, which may further impair T cell migration. -SOH is an inevitable intermediate for disulphide bond formation (Rehder & Borges 2010) and may promote protein-protein interaction.

Various methods to detect sulphenic acid modification have been described (Poole et al. n.d.; Furdui & Poole 2014). Detection of sulphenic acid using DCP-Bio1 identifying via MS/MS analyses has provided promising outcomes in the past but with limitations. Mainly because the sulphenated sites were not identified. Here, this study has used similar technology hence limited to the peptides which are identified after enrichment following the use of streptavidin beads. Perhaps, different ionisation method may detect peptides with oxidised cysteines or labelled proteins analysed on LC-MS/MS. Another approach would be to trypsin digest the

peptides followed by biotin affinity capture of labelled peptides analysed by LC-MS/MS (Nelson et al. 2010). Although washing and streptavidin beads are used to capture biotinylated proteins, there may be some false positives. To directly identify oxidised cysteine, affinity capture of biotinylated peptides can be performed after trypsin digestion of proteins (Shin et al. 2007; Dennehy et al. 2006). The result of false positive without cysteine oxidation sites may be due to protein-protein interactions. For example, the labelled protein interacts with a protein which maybe pulled down and identified as sulphenated. Also, higher concentrations of the dimedone reagent can lead to non-specific binding to proteins.

Additionally, since the first reversible oxidation of cysteine is sulphenic acid formation and was shown to be important in response to ROS, the specificity of dimedone based capturing of –SOH has been looked into recently in the sense of specificity to Cys-SOH (Forman et al. 2017). This is because the thiols can also exist in the form of polysulphides (Cys-$SS_nH$) or hydropersulphide (Cys-SSH). This has been shown to undergo sulphenic acid modification. DCP-Bio1 is capable of detecting protein with the presence of Cys-$SS_nH$ and an example was shown in a study by Heppner et al, whereby they confirmed the presence of Cys-SSH in EGF or ATP stimulated H292 cell lysates, particularly in protein tyrosine kinases which are susceptible to $H_2O_2$ (Heppner et al. 2018). This indicate the presence of possible further intermediate of reversible cysteine oxidation, which is yet to be characterised.

The treatment with $H_2O_2$, did not yield a significant identification of new proteins. This may be due to the concentration used was not enough to oxidise majority of proteins within the lysate mixture. Also, it may suggest that the proteins are oxidised even further to higher oxidation states including disulphide and sulphinic acid formation (Brennan et al. 2004). Hence, these oxidation do not bind to DCP-Bio1 further showing the specificity of DCP-Bio1 to sulphenic acid modification only (Charles et al. 2007). However, several other proteins have been identified in the presence of $H_2O_2$, these include glutathione transferase P, a protein with

glutathione transferase activity. This can possibly be inactivated by sulphenation followed by glutathinoylation (Shen et al. 1991; Baty et al. 2005).

Isocitrate dehydrogenase [NAD] undergoes sulphenic acid modification and others have shown that it is susceptible to increased ROS and oxidative modifications in turn damaging protein activity (Lee et al. 2001). However, the MP sample has pulled out mitochondrial proteins. This may be due to the approach taken to isolate membrane proteins which uses detergents to extract and solubilise total membrane proteins.

Likewise, cysteine sulphenic acid modification is important as it may behave as a molecular switch to either activate or deactivate protein function. It was reasonable to follow up from the identified sulphenated proteins and how it may affect T cell function following exposure to increased oxidative stress. Ionotropic glutamate receptors are believed to be involved in regulating activation and proliferation in T cells, (Boldyrev et al. 2005; Ganor et al. 2003) including some evidence suggesting the role of N-methyl-D-aspartate receptor (NMDA) in $Ca^{2+}$ signalling (Lombardi et al. 2001; Miglio et al. 2005; Affaticati et al. 2011). Also, an increase in intracellular $Ca^{2+}$ is one of the key signals in T cell activation after TCR engagement. To investigate the effects of possible modifications to membrane receptors responsible for T cell function, the cells were exposed to glycine and glutamate in the presence or absence of $H_2O_2$ and calcium influx was measured. The results obtained clearly showed that $H_2O_2$ decreased calcium influx and the treatment with NAC restored the calcium levels as 100µM of glycine and glutamate and increases calcium influx in a study by (Guo et al. 2017). However, NMDA is not a key receptor responsible for $Ca^{2+}$ influx and has low permeability for calcium. Similarly, the T cell membrane contains of plasma membrane $Ca^{2+}$ ATPase (PMCA) which is mainly involved in $Ca^{2+}$ signalling across the membrane (Lewis 2001). Furthermore, to further elaborate the effect of low calcium levels is due to NMDA activity, competitive and non-competitive NMDA receptor agonists would further clarify the results observed. These may include MK-801 and ketamine or amantadine as they have been shown to inhibit PHA induced T cell proliferation (Miglio et al. 2005). Additionally, this has been shown in a study by

the same group; whereby the NMDA channel blocker MK-801, inhibited PHA induced T cell proliferation but not IL-2 secretion suggesting that NMDA activity may modulate early phases of T cell activation and this was confirmed by surface CD25 expression. RA T cells have consistently shown reduced intracellular GSH levels, signifying presence of stress within cells. Treatment of T cells with NAC restores GSH levels and phosphorylation and localisation of LAT and hence promoting activation of T cells (Simeoni & Bogeski 2015b; S I Gringhuis et al. 2000).

This chapter has identified potential sulphenated proteins in T cell cytosol and membranes, a number of proteins consistent with earlier work done by either the literature or Ratnayake 2015 (unpublished data). Additionally, an experiment was designed to look at oxidation of NMDA receptor function showing an effect on NMDA receptor-mediated $Ca^{2+}$ influx. It is sensible to speculate that –SOH may behave as a redox switch and post translational regulator and increased oxidation may inhibit or activate protein function.

# Chapter 4. GSH depletion influences intracellular Trx1 and Prx2 levels in T cells

## Preface

Redox alteration is associated with a progressive decline in immune function that contributes to age-related diseases, including altered T cell function and proliferation and differentiation to different lineages. Key antioxidants are involved in removing ROS and maintaining the cellular redox state of the cell; these include GSH, Prx and Trx systems. However, the extent and role of Trx1 and Prx2 on the surface of T cells is poorly understood. Hence this chapter looked at the relationship of these antioxidants and membrane association in the presence of oxidative stress. These data suggest that the T cell responds to oxidative stress conditions via Trx, Prx and GSH system. Also, increased expression of Trx1 on the surface of Jurkat T cells may suggest that Trx1 distributes to the membrane when in oxidised form.

## 4.1 Introduction

Growing evidence suggests that the cellular redox state affects various aspects of cellular function and since T cells are critical part of an immune response, any imbalance in the cellular redox state may affect or modulate the T cell reactivity (Kesarwani et al. 2013a). Recent evidence suggests the interaction between intracellular T cell redox environment and membrane proteins which can ultimately influence downstream T cell function (Mougiakakos et al. 2010b; Gelderman et al. 2006b; Carilho Torrao et al. 2013).

A network of antioxidant systems that are distributed in the cytoplasm and various organelles in the cells are described to exist in humans. (Rahal et al., 2014). Many key regulators of redox signalling have been known and expressed in all organisms namely the glutathione, thioredoxin and peroxiredoxin (Lillig & Holmgren 2007a; Lillig et al. 2008; Hanschmann et al. 2013a). The glutathione (GSH) redox system is described to be the most abundant antioxidant system in the cells (Hanschmann et al. 2013a; Peskin et al. 2016). Cellular GSH concentration is dependent on gamma-glutamyl cysteinyl ligase (GCL), a heterodimer of modifier (GCLM) and catalytic (GCLC) ligase enzymes for its synthesis (Harris et al., 2015). During oxidative stress such as a decrease in protein thiols, the synthesis of GSH is increased to restore the redox state of the cell. The ratio of oxidised and reduced GSH, GSH to GSSG ratio is one of the most abundant signals of the redox status in mammals (Zitka et al. 2012). A decrease in this ratio; reduced to oxidised GSH might indicate the presence of oxidative stress at cellular or tissue level (Rahal et al., 2007).

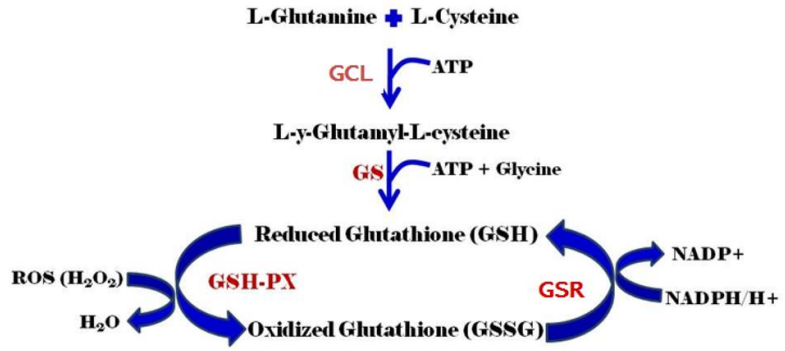



Figure 4.1: GSH synthesis and recycling. The combination of L-Glutamate and L-cysteine is catalysed by glutamate cysteine ligase (GCL) in the presence of adenosine triphosphate (ATP) to form L-ɣGlutamyl-L-cysteine. Glycine is added in the presence of glutathione synthase (GS). The enzyme glutathione reductase (GSR) catalyses the reduction of oxidised glutathione (GSSG) back to reduced GSH in the presence of nicotinamide adenine dinucleotide phosphate (NADP). And the enzyme glutathione peroxidase (GSH-PX) catalyses GSH to GSSG in the presence of reactive oxygen species (ROS) (Guildford and Hope 2014).

Trx1 is a conserved, small protein (MW) of 12kDa with several redox active cysteine residues, found primarily in the cytosol and is translocated in the nucleus and associated in the cell membrane (Holmgren, A. 1985; Collet & Messens 2010; Carilho Torrao et al. 2013; Holmgren & Lu 2010). Recent evidence suggests that Trx1 is also secreted into the extracellular environment (Hanschmann et al. 2013a). The most favourable reaction for Trx1 is to reduce oxidised Prxs within a redox system. This suggests Prx as a favourable interaction partner of Trx1 during combating stressful conditions (Winterbourn & Hampton 2008).

The Trx1 system is crucial and essential for protection against oxidative stress and plays a critical role in redox regulation of cells. This system consists of NADPH as a source of electrons, an enzyme thioredoxin reductase (TrxR) which catalyses the reduction of oxidised Trx1 (Figure 4.2) and indirectly, peroxiredoxin (Prx). Through its redox active cysteine residues, Trx1 maintains the cellular environment in a reduced state by receiving electrons from NADPH. In the presence of TrxR, reduced Trx1 transfers electrons thus reducing oxidised proteins including Prx with disulphide bonds (Arnér & Holmgren 2000). Depending on its cellular localisation Trx1 is involved in variety of physiological functions within the cell (Arner and Holmgren 2000) including; cell proliferation, cell growth and survival, apoptosis and

regulation of transcription (Lillig and Holmgren 2007). Trx1 catalyses reduction of disulphide bonds and quenches reactive oxygen species (ROS) by coupling with Trx-dependent peroxidases, or peroxiredoxins.

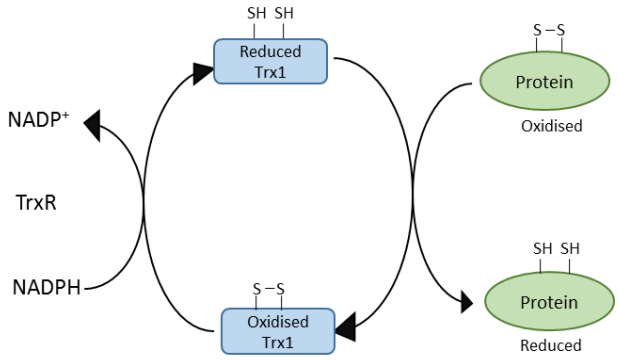



Figure 4.2: The thioredoxin antioxidant system. Trx1 interacts with other proteins by donating electrons leading to oxidation of Trx1. Oxidised Trx1 is then reduced in the presence of TrxR enzyme and NADPH. (Adapted from Matsuo et al. 2002)

Peroxiredoxins (Prxs) are highly conserved proteins that act as redox sensors and monitor signs of oxidative stress. They are divided into 6 isoforms (Prx1-6). 2-Cys Prx (Prx 1-4) have an additional conserved cysteine residue responsible for resolving the oxidised cysteine (Smith-Pearson et al. 2008). Prxs have been found to be present in different locations within the cells; Prx 1, 2 and 4 have been predominantly found in the cytoplasm and also have been found in the nucleus (Oberley et al. 2001; Seo et al. 2000; Kang et al. 1998). Prx2 has been previously described as being associated with the membrane of lymphocytes (Szabó-Taylor et al. 2012a) and Trx1 is one of the reducing partners of Prx2 (Szabó et al. 2009). They are mostly known as the antioxidants which remove excessive amounts of $H_2O_2$ and peroxynitrite (Peskin et al. 2016; Winterbourn & Hampton 2015; Randall et al. 2013; Jarvis et al. 2012).

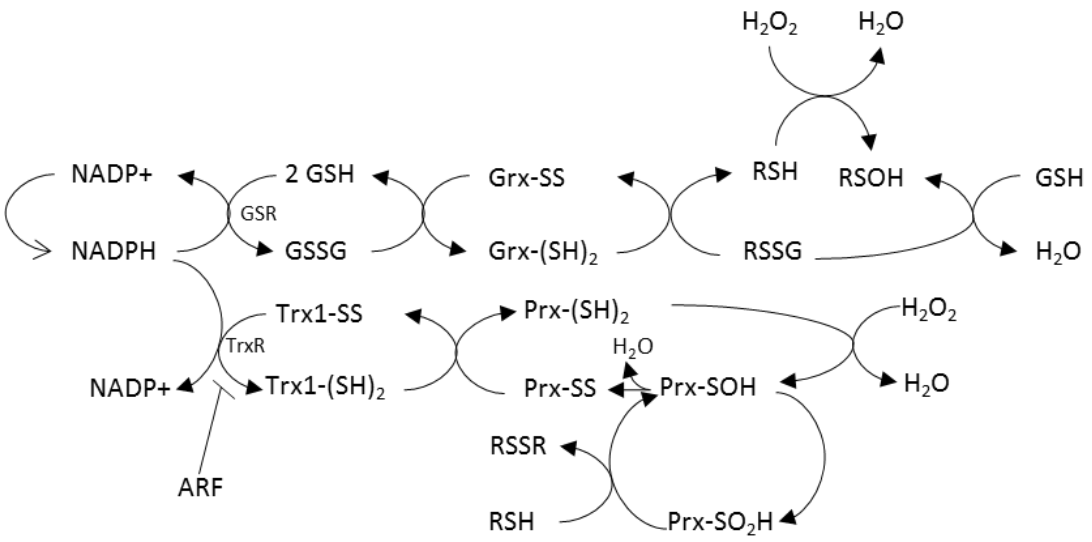



Figure 4.3: The link between antioxidants and the maintenance of cellular redox state through reduction of $H_2O_2$. Reduced molecules are proteins with hydrogen and disulphide and or sulphenation shows oxidation. Trx1-SS oxidised thioredoxin, Trx1-$(SH)_2$- reduced thioredxoin, Prx-SS oxidised peroxiredoxin, Prx-$(SH)_2$- reduced peroxiredoxin, Prx-SOH- sulphenated peroxiredoxin, Grx- glutaredoxin, Grx $(SH)_2$ – reduced glutaredoxin, GSR - glutathione reductase, GSH - reduced glutathione, GSSG - oxidised glutathione, RSSG- glutathionylated protein, $NADP^+$ - oxidised NADP, NADPH - reduced NADPH, RSSR - protein disulphide, RSOH -sulphenated protein, RSH - reduced protein, ARF - auranofin,

Even though, Trx1 and Prx2 together with GSH constitute a key system in redox balance, the relationship between them and the significance of redox enzymes as redox modifiers has not been completely understood in the context of their membrane association and distribution. Hence, this chapter aims to increase the understanding of how Trx1 and Prx2 are affected by the absence of GSH. Improved understanding of any changes in expression or distribution of Trx1 and Prx2 might improve our understanding of redox regulation in T cell responses. The objectives of this chapter are as follows:

- To optimise non-toxic BSO concentration to inhibit GSH synthesis
- To investigate Trx1 oxidation in absence of TrxR
- To examine the effect of BSO and ARF on Trx1 and Prx2 distribution using western blot analysis and flow cytometry

## 4.2. Methods

### 4.2.1. Jurkat T cell culture

Jurkat T cells were cultured according to the methods section 2.2.1.

### 4.2.2. Cell treatments

Jurkat T cells were passaged until reached certain confluency ~3-5x$10^5$/ml in a T75 tissue culture flask. On the day of treatment the cells were washed with complete RPMI and counted using a haemocytometer and plated in a 12 well plate at an assay-dependent concentration.

Cells were lysed with RIPA buffer, briefly, harvested cells were centrifuged at 4°C and wash with ice-cold PBS. Ice cold RIPA buffer was added and agitated for 30mins at 4°C. The sample was resuspended with 25 guage needle and centrifuged at 16,000 x g for 20 minutes in a 4°C pre-cooled centrifuge. After centrifugation, the supernatant was collected and analysed for further experiments. Protein concentration was determined by BCA assay as described in 2.2.3.

### 4.3.3. Surface and total staining for Trx1 and Prx2

1x$10^6$ of BSO and ARF-treated (0 and 25μM; 24h, 0 and 25μM, 2h) T cells were collected and washed twice with PBS by centrifugation at 300g for 5 minutes. The cells were resuspended in PBS (1%FBS), aliquoted into separate tubes for individual staining (5x$10^5$/per surface stain). To label, they were incubated with primary anti-Trx1 (ab16965, 1μl/$10^6$cells)) and or rabbit anti-Prx2 and mouse IgG2b isotype control (ab91366 1μl/$10^6$cells) for 30min in the dark. Following one wash, the cells were incubated with goat anti-mouse IgG H&L (DyLight® 488) (ab96879 1μl/$10^6$ cells) for 30min in the dark. The cells were analysed on cytomics FC 500 (Beckman Coulter).

For total Trx1 and Prx2 staining the cells were permeabilised with 0.1% w/v saponin. The permeabilised samples were incubated with primary antibody primary anti-Trx1 (ab16965, 1µl/$10^6$cells) and or rabbit anti-Prx2 and mouse IgG2b isotype control (ab91366 1µl/$10^6$cells) for 30min in the dark. Following one wash, the cells were incubated with goat anti-mouse IgG H&L (DyLight® 488) (ab96879 1µl/$10^6$ cells) for 30min in the dark. The cells were analysed on cytomics FC 500 (Beckman Coulter).

### 4.2.4. GSH Assay

Intracellular GSH and GSSG was determined by GSH recycling assay as described in the methods section 2.2.11.

### 4.2.5. SDS PAGE and WB

Membrane and cytosolic proteins were isolated as described in the methods section 2.2.4. Isolated CP and MP were separated under reducing and non-reducing conditions according to the protocol described in 2.2.2 above.

## 4.3. Results

### 4.3.1. BSO depletes GSH levels in Jurkat T cells

In order to investigate whether an alteration in cellular redox state leads to redistribution on Trx1 within the cells and develop a suitable model for inducing oxidative stress, Jurkat T cells were treated with buthionine sulphoximine (BSO), which inhibits the enzymatic activity of ɣ-glutamylcysteine synthetase (ɣ-GCS). After 24h incubation, BSO depleted intracellular reduced GSH (untreated 85.74±20.12; 25µM 26.12±1.95 and 50µM 20.86±5.683 nmol/mg of protein) levels significantly in Jurkat T cells (Figure 4.4a). To examine the toxicity of BSO, the Jurkat T cell viability was measured using trypan blue exclusion. The cell viability was not affected at concentrations up to 50µM BSO over 24 hours showing that BSO was not toxic to Jurkat T cells (Figure 4.4b).

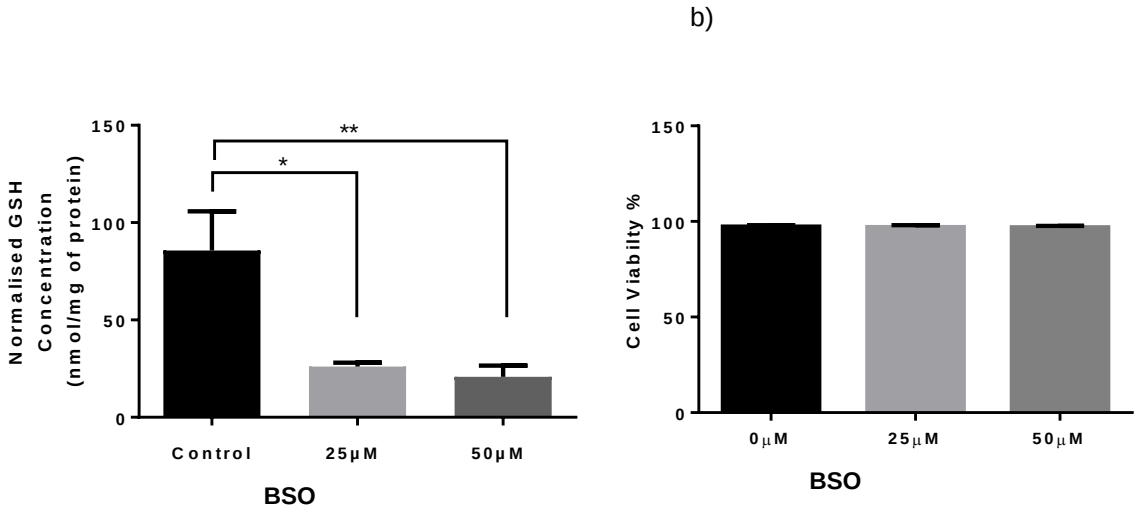



Figure 4.4: BSO depletes GSH levels in Jurkat T cells without affecting cell viability. Jurkat T cells ($1x10^6$/ml) were treated with 25 µM or 50 µM BSO for 24h. a) Intracellular GSH levels were determined by GSH recycling assay. b) Cell viability was measured the following day by trypan blue exclusion. Data represents the mean and standard error of the mean of three experiments, n=3 **p <0.0075, ANOVA with Dunnett's test

### 4.3.2. ARF increases monomeric and dimeric Trx1 in Jurkat T cells

In order to manipulate Trx1 redox state, Jurkat T cells were treated with auranofin (ARF). The toxicity of ARF was first examined by treating Jurkat T cells with 25µM or 50µM concentrations of ARF and Jurkat cell viability was assessed by trypan blue exclusion method (Figure 4.5a). Treatment with ARF at concentrations at 25µM or 50µM for 2h did not affect cell viability. Monomeric form of Trx1 was detected at 12kDa and dimeric form detected at 25kDa after separating on a non-reducing SDS PAGE (Figure 4.5b) The presence of 25kDa band on a non-reducing gel suggests that the treatment of Jurkat T cells with ARF leads to formation of dimers by a disulphide bridge and the intensity of the dimer increases with increasing concentration of ARF.

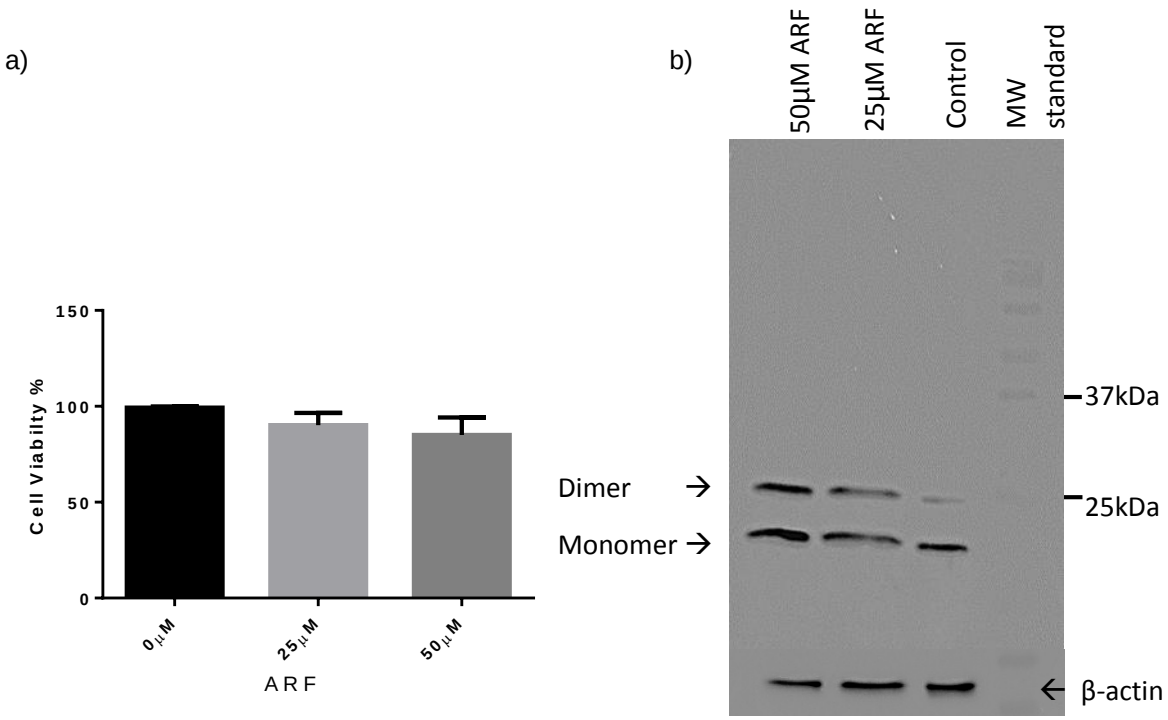



Figure 4.5: Effect of increasing concentrations of ARF on cell viability and Trx1 oxidation. $1x10^6$ Jurkat cells were treated with 0, 25 and 50µM of ARF for 2h. a) Cell viability was measured after 2h by trypan blue exclusion. Data shown is the mean and standard error of the mean n=3. b) Non-reducing SDS-PAGE-western blot for Trx1 and β-actin. Blots were probed with monoclonal mouse anti-β actin (1:30,000 for 2h) and mouse anti-human Trx1 (1:1000, 2h ab16965) before detection with goat anti-mouse IgG HRP (1:10,000 for 2h, A1068) developed using ECL detection kit. This blot is representative of three independent experiments.

## 4.3.3. ARF depletes GSH levels in Jurkat T cells

In order to examine the effect of ARF on intracellular GSH, Jurkat T cells were treated with ARF (25µM, 2h) and total cellular GSH levels were measured after treatments. The data (Figure 4.6) confirms observations in Figure 4.4a, that 25µM BSO depleted GSH levels compared to control (untreated 144.5±22.77 and BSO 25µM 11.08±5.49) whereas Jurkat T cells treated with ARF also show a significant decrease in GSH levels when normalised to the total protein concentration in the cell (ARF 25µM; 67.64±11.23; $p<0.006$). Both BSO and ARF treatments also reduced the level of GSSG in Jurkat cells (Figure 4.6b), however no statistical significance was observed.

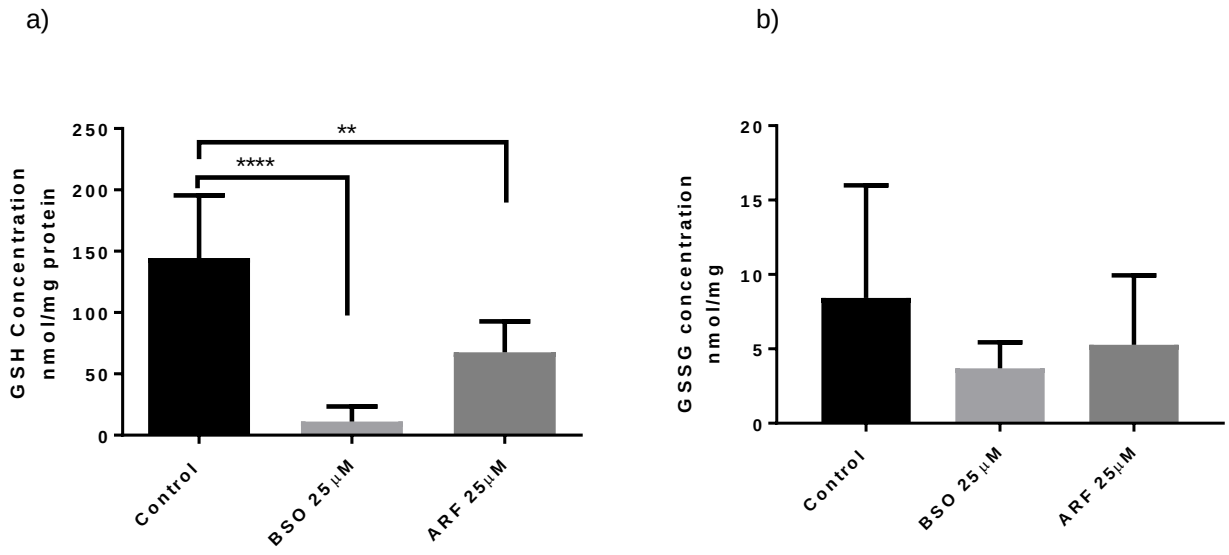



Figure 4.6: Effect of BSO and ARF on GSH and GSSG concentration. $1x10^6$ Jurkat cells were treated with 25µM of BSO for 24h, GSH level was measured by the DTNB recycling assay using glutathione reductase. Data represents mean and SEM, one way ANOVA, Dunnett's multiple comparisons test ****$p<0.0001$, **$p<0.006$.

### 4.3.4. The redox state of Trx1 in the presence of BSO and ARF

In order to evaluate Trx1 redox state and to investigate whether any mixed disulphides of Trx1 are observed in Jurkat T cells under redox stress where the cells were treated with BSO (25µM, 24h) and ARF (25µM; 2h). Cells were lysed with RIPA buffer and the lysates were separated in 15% SDS PAGE gel under reducing (β-mercaptoethanol) and non-reducing conditions by eliminating β-mercaptoethanol. Non-reducing conditions prevent the breakdown of disulphide bridges and aid the identification of proteins that are associated with Trx1 under oxidative stress. A 12kDa band was detected in reducing condition as seen from Figure 4.7a. Additionally, reduced Trx1 expression was increased when the cells were treated with BSO after 24h.

In addition, Figure 4.7b show a number of protein bands in non-reducing SDS-PAGE-WB conditions. High molecular weight bands of approximately 37kDa were observed when protein samples were separated under non-reducing conditions, irrespective of BSO or ARF treatment.

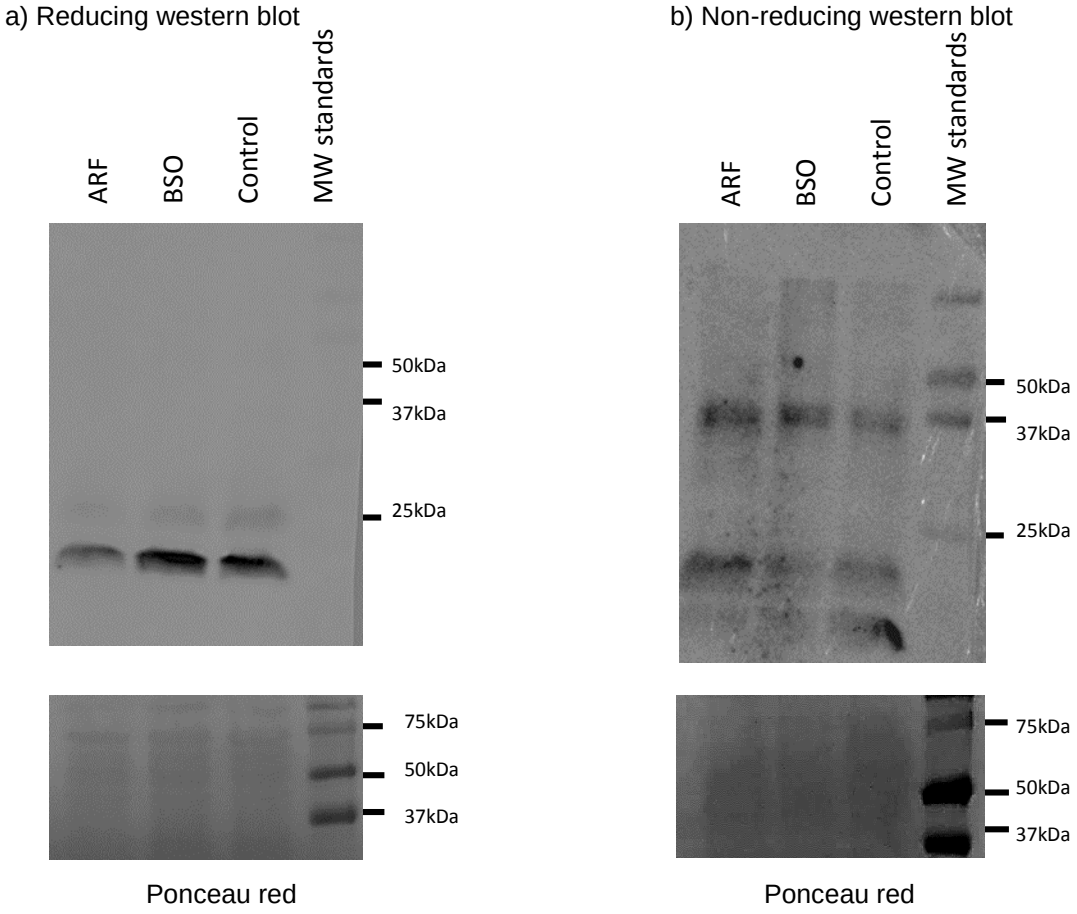



Figure 4.7: Western blot analysis of Trx1 expression after BSO and ARF treatment. $1x10^6$/ml Jurkat T cells were treated with BSO 25µM; 24h and ARF 25µM; 2h in complete RPMI and lysed using RIPA buffer. Proteins (20µg) were separated using 15% SDS PAGE in reducing (a) and non-reducing (b) conditions and transferred to PVDF membrane, blocked with 3% BSA and probed with primary mouse anti-human Trx1 ((1:1000) for 2h) and goat anti-mouse IgG HRP (1:10,000 for 2h, A1068). (1:20,000) for 2h and developed using ECL detection kit. This blot is representative of three independent experiments. b) Non-reducing blot in 4X non-reducing buffer.

### 4.3.5. Membrane and cytosolic distribution of Trx1

To explore whether the distribution of Trx1 between membrane and cytosol is different under oxidative stress, the membrane and cytosolic proteins were extracted after Jurkat T cells were treated with BSO and ARF. Trx1 is accumulated in the cytosol when cells were treated with BSO as shown in Figure 4.8a and Figure 4.8c) which shows an increased band intensity in ARF-treated cells compared to untreated in the cytosol relative to protein loaded. this was shown by probing the cytosolic proteins with NF-κB, which is the most abundant protein within the cytosol. On the other hand, Figure 4.8b shows a band of approximately 12kDa confirming the presence of Trx1 in the membrane, but no change to the intensity was observed between the untreated and BSO treated cells (Figure 4.8c). However, Trx1 is distributed to the membrane when the cells were treated with ARF compared to untreated cells (Figure 4.8b and 4.8d). The membrane was later re-probed for CD3ε as loading control for membrane proteins to verify the protein loaded.

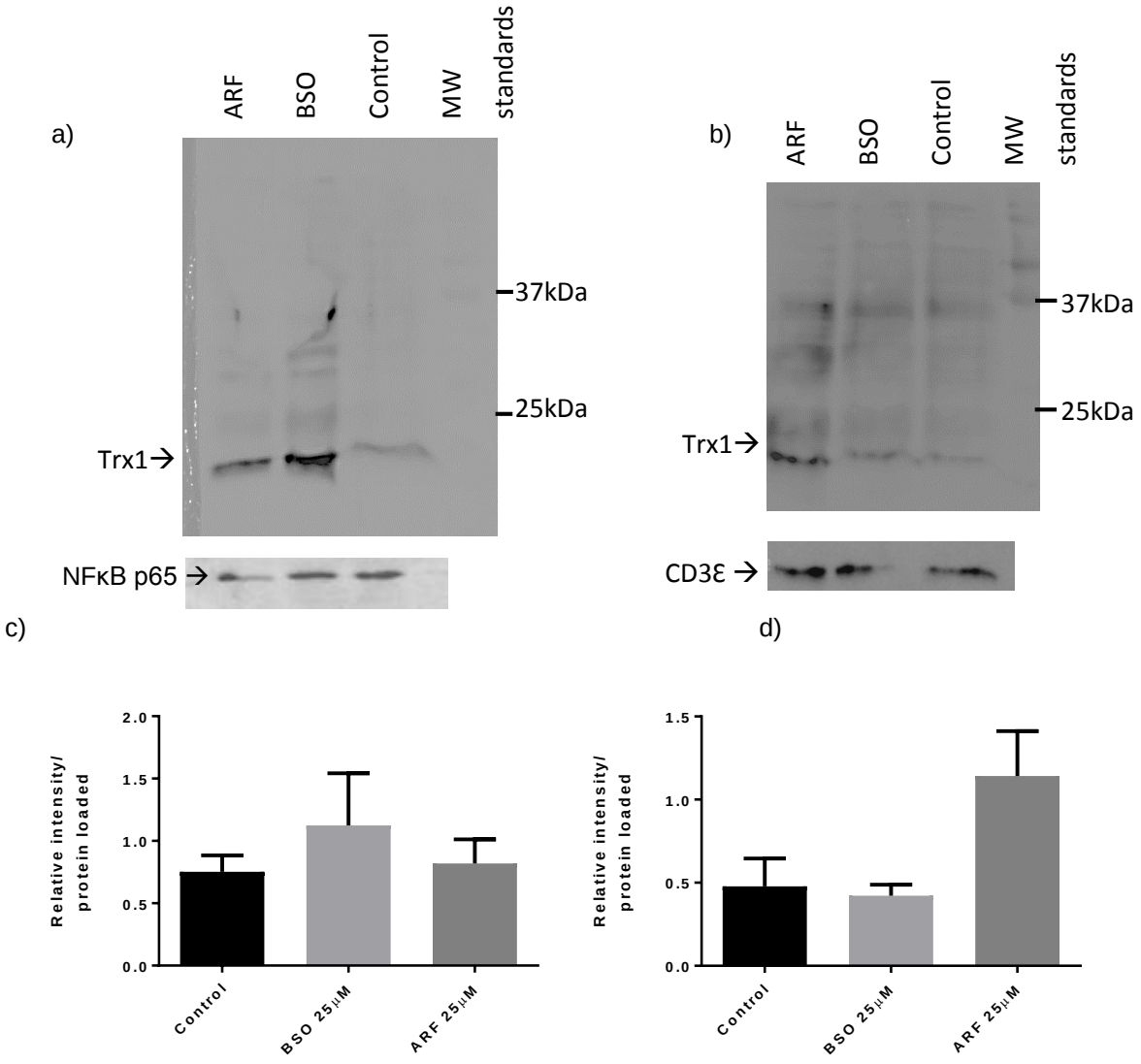



Figure 4.8: Reducing SDS PAGE WB showing that Trx1 expression in the cytosol and membrane of Jurkat T cells under oxidative stress. $4x10^6$ jurkat T cells treated with BSO 25μM; 24h and ARF 25μM; 2h in complete RPMI. Membrane and cytoslic protein fractions were isolated and protein concentration measured using BCA assay. 20μg protein was separated using 15% SDS PAGE and transferred to PVDF membrane, blocked with 3% BSA overnight and probed with mouse anti-human Trx1 (1:1000);2h) and goat anti-mouse IgG HRP (1:10,000 for 2h, A1068). Rabbit anti-CD3Ɛ antibody (1:1000; 2h) and rabbit anti-NF-κB p65 (1:1000, 2h) and secondary goat anti-rabbit (1:10000; 2h) developed with ECL reagent. a) cytosolic and b) membrane proteins. These blots are representative of three independent experiments. c) and d) Semi quantitative analysis of Trx1 distribution of cytosolic and membrane proteins analysed by ImageJ software. Data represents three independent experiments n=3, on way ANOVA, Dunnetts multiple comparison test.

### 4.3.6. Trx1 distribution using flow cytometry

To further explore membrane distribution of Trx1 under oxidative stress conditions, the expression of Trx1 was analysed on the surface by flow cytometry.

Jurkat cells were treated with 25 µM BSO and 25µM ARF and analysed using flow cytometry (Figure 4.9a). Trx1 expression in the presence of 25 µM BSO was decreased on the surface compared to untreated control. The MFI value which shows surface Trx1 expression per cell was 77.49±4 and 81.72±5.5 for BSO treated and untreated Jurkat cells. However, 25µM ARF treatment significantly increased (MFI; 370.9±120.8) surface Trx1 expression.

To further investigate whether the redistribution of Trx1 is specific to ARF treatment and to rule out the possibility other stress conditions such as serum starvation, Jurkat T cells were treated with indicated concentrations of serum in the media for 24h to induce serum-starvation stress and the distribution of Trx1 was analysed. Figure 4.9d shows no change in total Trx1 according to nutrient availability and surface Trx1 appeared to be lower in serum starved cells (Figure 4.9c).

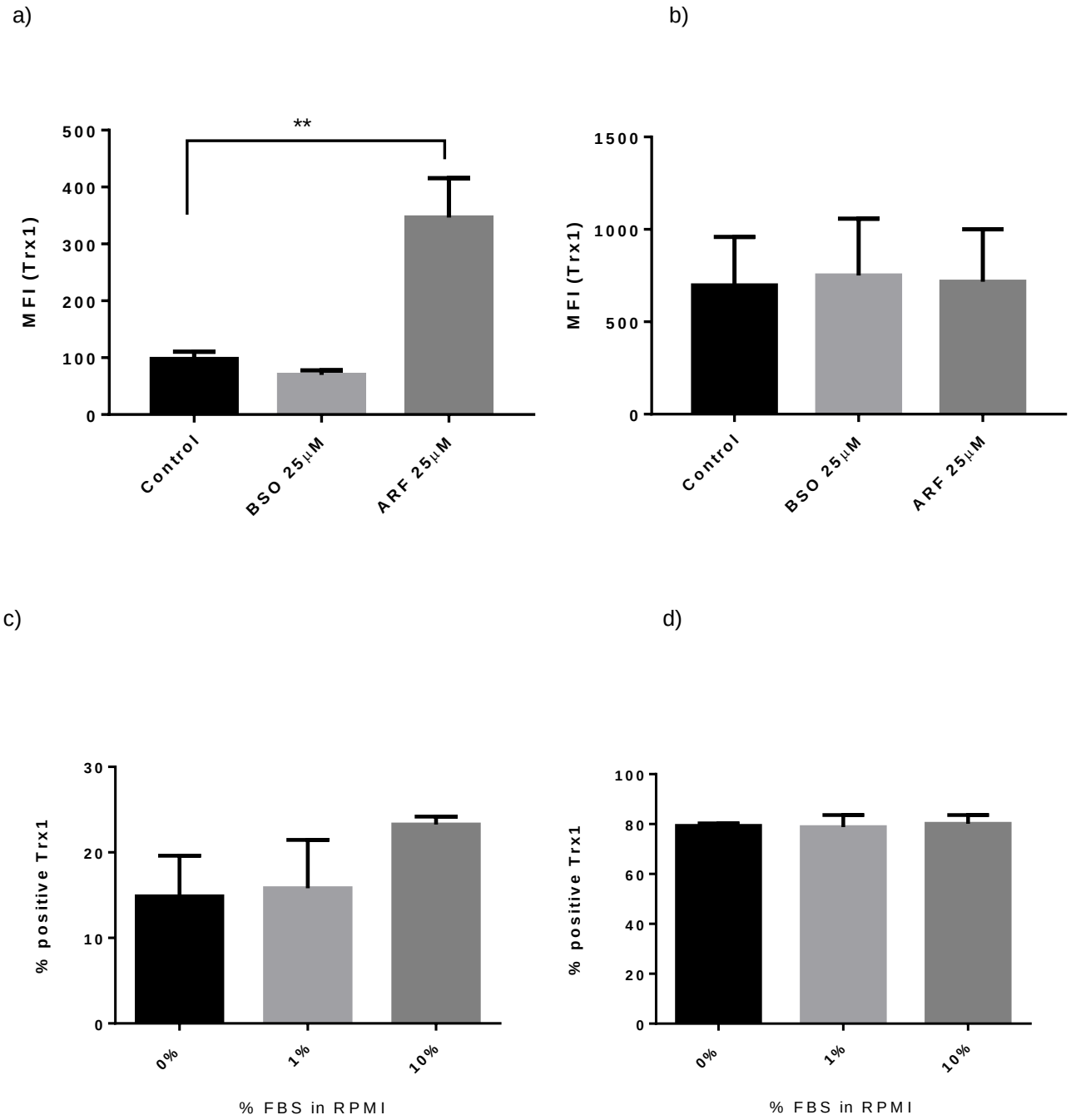



Figure 4.9: Distribution of Trx1 onto the surface of Jurkat cells. 1x$10^6$/ml Jurkat T cells were treated with BSO 25μM and ARF 25μM for 24h and 2h respectively. The cells were collected and analysed for a) MFI of cells expressing surface Trx1 and b) cells permeabilised using 0.1% w/v saponin to analyse total Trx1. Data represents the mean and standard error of the mean of three independent experiments n=3. **p< 0.0012. Additionally, 1x $10^6$ Jurkat cells treated with 0%, 1% and 10% FBS RPMI media for 24h and Trx1 distribution was analysed c) on the surface and d) total trx1. Data represents one independent experiment. Mouse anti-human Trx1 IgG2b (ab16965; 1μl/106 cells; 20min) goat anti-mouse IgG H&L (DyLight® 488) (ab96879 1μl/106 cells; 20min) and mouse IgG2b isotype control (ab91366 1μl/106cells). Trx1 positive cells were gated at 1% of the isotype control and the data was collected for 10,000 events and analysed using Flowing software v 2.5.1.

### 4.3.7. Prx2 levels is increased on the membrane when treated with ARF.

In order to evaluate the association of Prx2 with T cell membrane, the level of Prx2 expression was also investigated in the membrane and cytosol fractions in the presence or absence of BSO and ARF (Figure 4.10). Increased band intensity of Prx2 was observed when Jurkat cells were treated with BSO compared to the untreated control. Jurkat cells treated with ARF show a significant increase in membrane Prx2 according to the densitometry (Figure 4.10d). This suggests that Prx2 is likely to be distributed in the membrane at low intracellular GSH and Trx1 levels. However, no visible difference in the band intensity was observed in the cytosol.

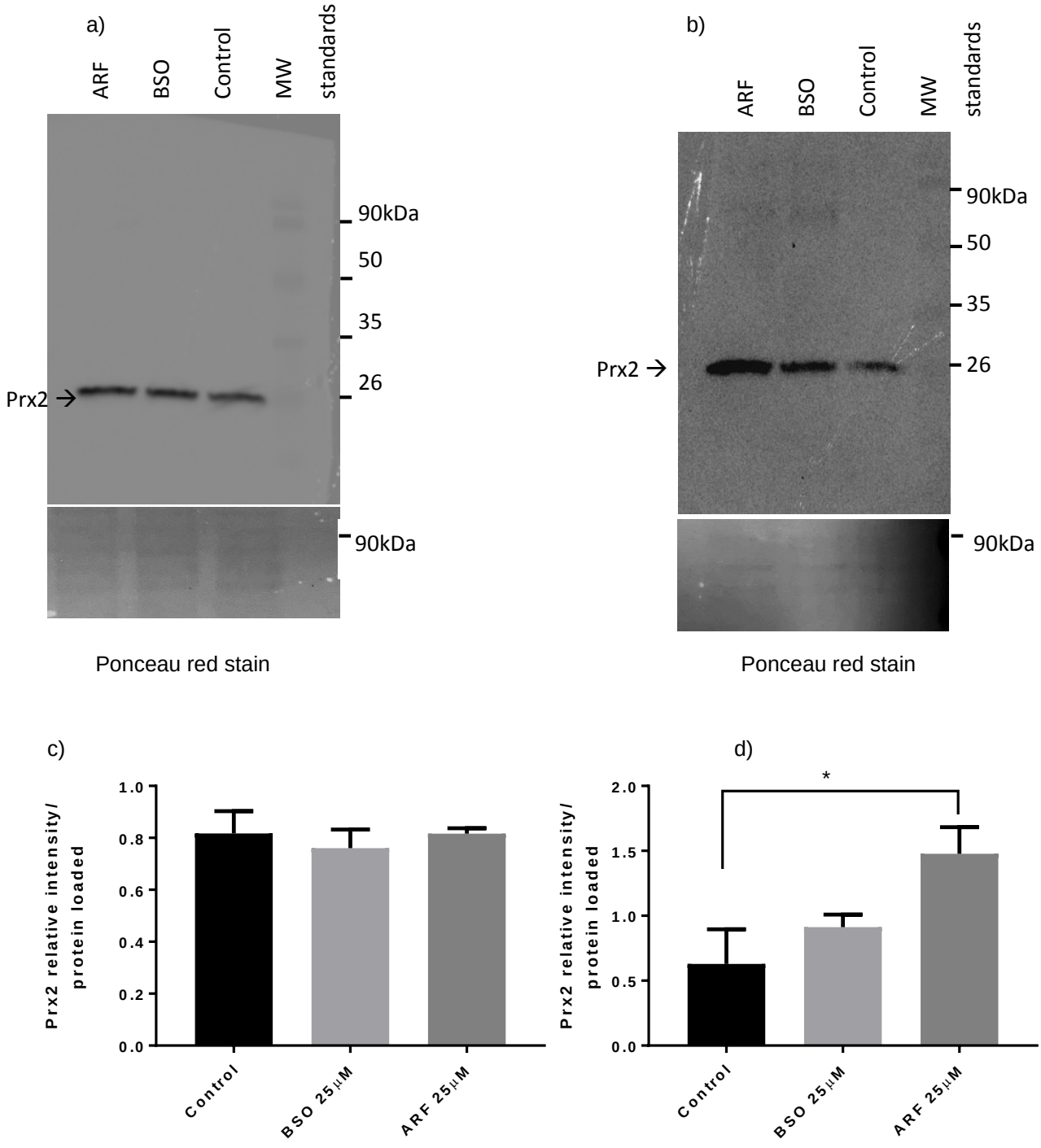
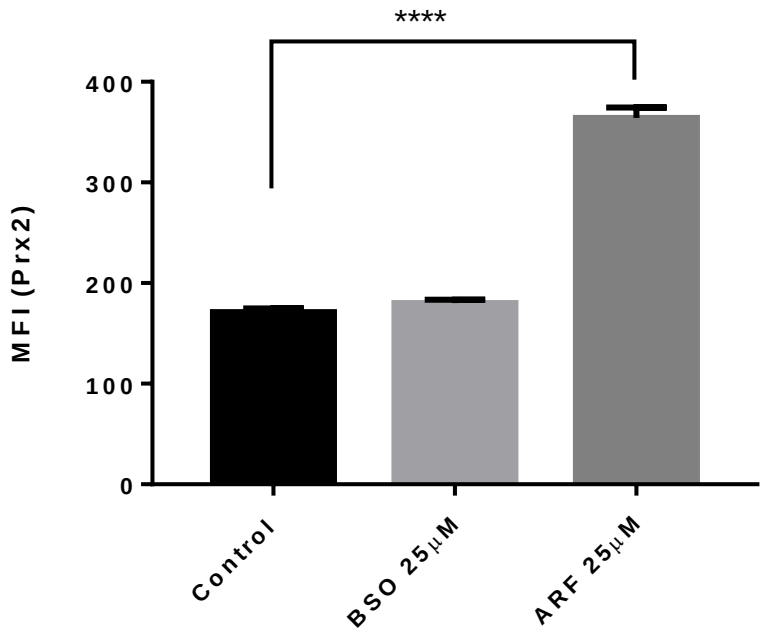



Figure 4.10: SDS-PAGE showing cytosolic and membrane distribution of Prx2 when Jurkat T cells are treated with BSO and ARF. $1x10^6$ Jurkat T cells treated with BSO 25µM;24h and ARF 25µM; 2h in complete RPMI. Membrane and cytoslic protein fractions were isolated and protein concentration measured using BCA assay. 20µg protein was separated using 15% SDS PAGE and transferred to PVDF membrane, blocked with 3% BSA overnight and probed with rabbit anti-Prx2 (ab109367; 1:1000);2h) and goat anti-rabbit HRP (1:5000 for 2h). developed with ECL reagent. a) cytosolic and b) membrane proteins. These blots are representative of three independent experiments. Semi quantitative analysis of Prx2 in the c) cytosol and d) membrane. Data represents three independent experiments n=3, analysed by ImageJ software. One way ANOVA, Dunnetts multiple comparison test, *p<0.04

### 4.3.8. Prx2 distribution using flow cytometry

In order to support the result obtained in Figure 4.10, surface Prx2 levels were also determined using flow cytometry. T cells show a significant increase in surface Prx2 when treated with ARF (MFI; 364.2±10.18) as shown in Figure 4.11 as compared to untreated (MFI; 171.2±3.55). However, BSO treatment did not change Trx1 distribution on to the membrane (MFI; 180.1±3.43). This suggests that membrane-bound Prx2 level is greater when cells treated with ARF. Additionally, this supports the result obtained using western blot analysis, whereby Prx2 level is increased when treated with ARF consistent with WB. Furthermore, no difference was observed in the total Prx2 levels when treated with BSO and ARF, similar result obtained as previously on western blot.

a)




b)

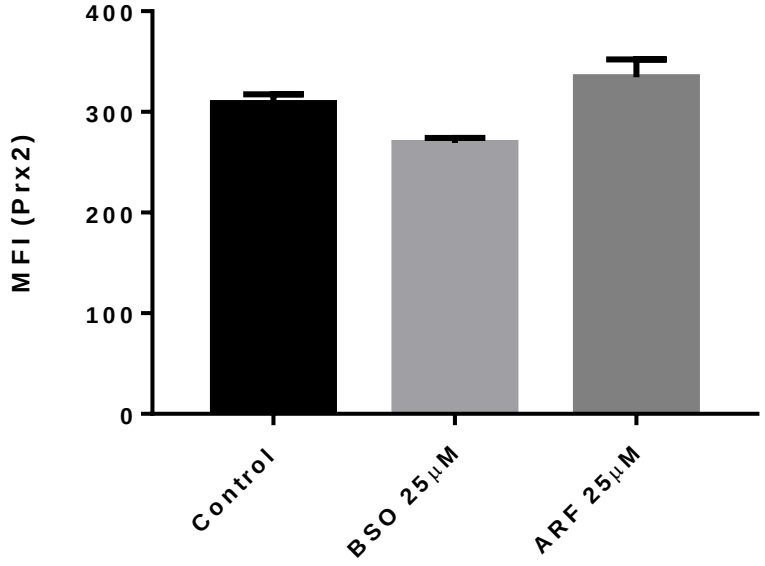



Figure 4.11. Distribution of Prx2 onto the surface of Jurkat cells. Jurkat T cells ($1x10^6$/ml) were treated with BSO 25µM for 24h and ARF for 2h respectively. The cells were collected and analysed for a) MFI of surface Prx2 and b) MFI values for total Prx2 in the cells. Rabbit anti-Prx2 (ab109367; 1µl/$10^6$ cells; 20min) goat anti-rabbit IgG H&L (DyLight® 488) (A-11034; 1µl/$10^6$ cells; 20min). Prx2 positive cells were gated at 1% of the unstained control and the data was collected for 10,000 events and analysed using Flowing software v 2.5.1. Data represents mean and SEM of three independent experiments. n=3, one way ANOVA Tukey's multiple comparison test ****p<0.0001.

## 4.3.9. Trx1 secretion when treated with BSO and ARF

In order to further understand whether the increase in surface Trx1 associates with changes to extracellular secretion, the cells were treated with BSO and ARF and the secreted Trx1 was determined using competition ELISA in the supernatants (Figure 4.12). There is no significant difference in the extracellular secreted Trx1 levels when treated with BSO or ARF.

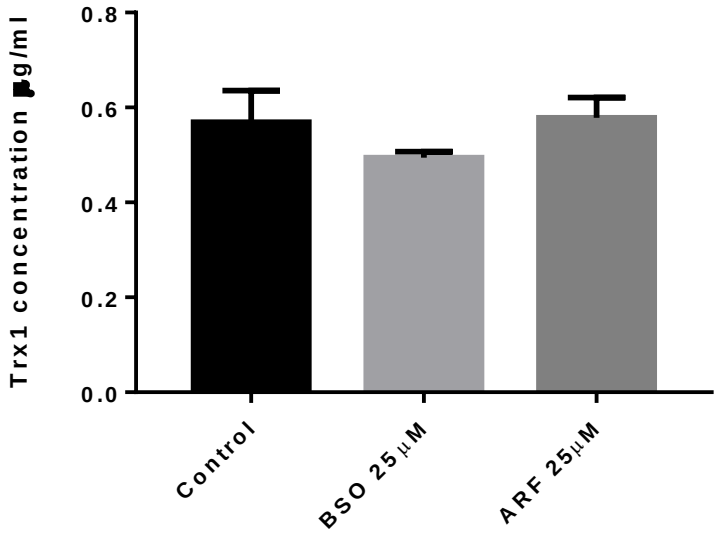



Figure 4.12. Level of secreted Trx1 when treated with BSO and ARF. Jurkat T cells were treated with BSO 25µM for 24h and ARF for 2h respectively. The supernatants were collected after centrifugation and analysed for Trx1 by competition ELISA. Data represents three independent experiments, n=3.

## 4.4. Discussion

The work in this chapter shows that non-toxic concentrations of BSO lead to increased Trx1 in the cytosol of Jurkat T cells. For the first time, this study has shown ARF leads to an increase in surface Trx1 and an accumulation of Trx1 intracellularly in aggregated forms. Moreover, surface Prx2 increases in the presence of ARF however, no difference was observed when the T cells were treated with BSO.

It is established that there is a significant decrease of immune cell function during ageing and age related diseases, which leads to immune dysfunction and reduced antigen specific immune response. One of the main characteristic of ageing T cells is accumulative oxidative stress (Espino et al., 2012), resulting protein- thiol oxidation and eventually changing protein function. The presence of oxidative stress with age is widely accepted and T cell studies reveal a direct association between altered T cell function and increased oxidative stress (reviewed by Bennett and Griffiths 2012). The presence of thiol (-SH) group allows GSH to initiate its scavenging effects (Jones et al. 2002). It also acts as the most abundant antioxidant within cells. Increased oxidative stress may arise due to lack of free thiols on protein surface or intracellularly and oxidation of thioredoxin and GSH leading to accumulation of ROS/RNS. Based on these observations this study was focused on investigating the effect of key antioxidant systems; the GSH, Trx and Prx system. These antioxidant systems were manipulated by adding BSO and ARF, which inhibited the synthesis of GSH and TrxR activity respectively. To confirm the activity of BSO on GSH levels, Jurkat T cells were treated with different concentration of BSO. BSO is widely used in vivo studies to deplete GSH levels by inhibiting ɣ-GCS synthesis and disrupt cellular GSH redox system. This affects a range of cellular mechanisms including cell proliferation (Go et al., 2013). This allowed the selection of optimum concentration of BSO (25μM) to inhibit GSH synthesis in the cells without being toxic to cells. Next, Jurkat T cells were treated with ARF to inhibit TrxR activity at different concentrations to obtain the optimum concentration without affecting the cell viability. ARF is

a gold compound that inhibits the catalytic activity of thioredoxin reductase (TrxR). It is also used to treat rheumatoid arthritis and as an anticancer drug (Go et al., 2013). WB suggests that ARF leads to oxidation of Trx1 by inhibiting TrxR activity although, ARF causes cell death and necrosis if treated for longer; 6h at 5µM (higher doses). Cox et al. 2008 showed that ARF causes cell death in Jurkat lymphomas and that it disrupts the mitochondrial redox homeostasis thus leading to apoptotic signalling events. Apoptosis signalling kinase 1 plays a major role in cytokine and stress-induced apoptosis including TNF (Saitoh et al. 1998; Liu et al. 2000). Since ASK1, which regulates apoptosis, is reduced by Trx1, Trx1 oxidation will lead to activation of forms of ASK1 found in the mitochondria and cytoplasm which are respectively inhibited by reduced form of Trx1. Even though ARF mediated TrxR inhibition promotes apoptotic or necrotic pathways, this action is depends on ARF concentration and cell type. For example; Jurkat cells treated with ARF for 24h significantly reduced number of viable cells. In contrast, apoptosis resistant B9 cell line did not show a decrease in viability but no increase in total number of cells was observed suggesting that ARF at 2µM for 24h inhibits proliferation (Cox et al., 2008). Oxidation of Trx1 leads to accumulation of intracellular ROS and cell death (Du et al. 2013). In this study ARF at 25µM results in accumulation of oxidised Trx1 after 2h. This is due to the inhibition of TrxR enzyme which is responsible to keep Trx1 in a reduced state. The mechanism of how ARF inhibits TrxR is not clear. However, according to pharmacological studies, ARF reacts with Selenocysteine 496 of TrxR by forming a diselenide bridges with the residue (Becker et al. 2000). Additionally, a study suggests that ARF leads to oxidation of all Prxs including Prx2, and that Prx2 was more sensitive to oxidation then Prx1 in the cytoplasm (Cox et al. 2008). This explains the relationship between Trx1 and Prx2 and that oxidation of Trx1 when treated with ARF may also lead to Prx2 oxidation.

Mammalian cells have variety of antioxidant mechanisms which allow to maintain cellular redox state. However, decreased GSH levels in ARF treated cells may show that the way cell responds to oxidative stress is linked such that GSH and Trx system might play a role together

to eliminate ROS by using NADPH as a source of electrons (Sobhakumari et al., 2012). Increased band intensity of Trx1 in the presence of BSO suggests accumulation of Trx1 in the cytosol in the absence of GSH. Also, reduced intracellular GSH level is also decreased in the absence of reduced Trx1 as shown in results. Under physiological conditions, the maintenance of redox states of Trx1 and GSH depend on each other and respond similarly to acute oxidative stress (Watson and Jones 2003). Additionally, a study showing the increase in Trx1 expression genes in GCLM knock out models and suggests that the Trx system can efficiently reduce ROS levels and can be regenerated by TrxR in a GSH independent manner (Harris et al., 2015), and upregulation of Trx activity is observed in low levels of GSH in acute liver failure (ALF) mice (Ruszkiewicz and Albrecht 2015). Additionally, a study by Go et el showed that Trx1 redox state was not affected by BSO treatments up to 200µM, but was significantly oxidised at 500µM in HT29 cells (Go et al. 2013b). This suggests that lower concentration of BSO is not sufficient to alter the redox state of Trx1.

Furthermore, to maintain a reducing environment, the expression of Trx1 is increased interacting with a number of binding partners. Increased expression of Trx1 in the cell has been shown in variety of stress conditions including oxidative stress, cellular stress and viral infections (Nakamura et al., 1999). On the other hand TrxR can reduce other proteins (Go et al 2013), which can affect several cellular functions such as detoxification of ROS and actin cytoskeletal control in neutrophils (Fang et al. 2005; Thom et al. 2012) .

The results obtained from western blotting of the protein samples in the absence of β-mercaptoethanol show a broad smearing of high molecular weight bands suggesting that possible interaction partners bound to Trx1 are linked by a disulphide bridge under non-reducing conditions. There are a number of binding partners which bind to Trx1 in order to restore their redox state within the cells in addition to controlling the protein activity and biological function as reported in literature. These include Prx, and intracellular proteins e.g. ASK-1, Txnip, transcription factors such as NF-kB, AP-1 and actin (Hirota et al. 1999; Kabe et al. 2005; Zschauer et al., 2010;Lillig & Holmgren 2007b);). In order to detect Trx1 binding

protein partners as reported in literature, western blotting of the targeted protein might be useful in the future.

Additionally, co-immunoprecipitation techniques will be a more useful approach to target protein binding partners of Trx1. By targeting the Trx1 using anti-Trx1 it may be possible to pull down the entire protein complex and thus identify the unknown partners of the complex. Additionally, other native techniques including blue native polyacrylamide gel electrophoresis (Blue native PAGE), which involves the separation of protein complexes according to their size is a valuable technique to study protein-protein interaction. Another approach to isolate membrane proteins which involves streptavidin beads, this captures surface proteins before addition of any detergent and washes which lowers the chances of losing low affinity proteins (Carilho Torrao et al. 2013). By using these techniques and other tools available we can possibly identify the binding partners of Trx1 on the membrane in the future.

Trx1 is widely distributed across the cell including the cytosol, nucleus and the membrane. This chapter primarily focuses on the changes to Trx1 and Prx2 distribution between membrane and cytosol under oxidative stress conditions.

Western blot experiments indicated that the distribution of Trx1 in the membrane was low with barely detectable band intensity. However, Trx1 levels in the cytosol is greater when the cells were treated with BSO. This may explain the retaining of Trx1 in the absence of GSH and that Trx1 may compensate for cytosolic reducing activity in the absence of GSH. Also, the possibility of Trx1 to redistribute from the membranes of Jurkat T cells as shown by Carilho Torrao et al. 2013 is shown in this chapter.

Even though others have reported membrane localisation of Trx1, how the protein is anchored onto the membrane and why inhibiting the activity of TrxR would lead to redistribution of Trx1 onto the membrane and oxidation of Trx1 leads to accumulation in the membrane, is still unclear. In addition, another form of TrxR inhibitor such as Lanthanum chloride ($LaCl_3$) was shown to prevent accumulation of Trx1 in the nucleus hence excluding oxidised Trx1 from the nucleus in neutrophils (Trevelin et al. 2016). However, it is believed the cysteine residue allows

anchorage to the membrane and tend to dissociate when exposed to detergents including DTT and methyl-β-cyclodextrin (Kondo et al. 2007b). Under oxidising conditions the disulphide which crosslinks Trx1 onto the membrane proteins is likely attach onto the membrane lipid rafts and not be released. Thus it is unclear of how the cellular and membrane distribution of Trx1 is maintained. It is assumed that, the thiols present in which make up Trx1 may be involved in the migration of the protein to the surface. However, there are no membrane export or membrane localisation sequences present on Trx1 gene (Wollman et al., 1988). Previous work from our lab demonstrated that Trx1 expression on the T cell surface is less in older adults compared to younger adults and may reflect the fact that the cells are more oxidised with age (Carilho Torrao et al. 2013).

Regarding the role it plays on the surface, some studies indicate that the Trx1 present on the surface of T cells may reduce certain receptors which are crucial in T cell activation as T cell activation requires a reduced environment. This includes the membrane domain localisation of CD4 which is regulated by Trx1, providing a hypothesis that T cell activation could be directly related to Trx1 dependent CD4 translocation (Moolla et al. 2016b). Also, it induces redox changes in glycoprotein 120 (gp120) on the surface (Azimi et al. 2010). Additionally, Trx1 is known to regulate cytokine activity such as IL-2 by reduction of intramolecular disulphides (Curbo et al. 2009). Besides, proteins on the surface may normally be oxidised and prevent T cell activation thus the –SH groups on the surface are maintained in a reduced state by redox active residues (Gelderman et al., 2006). Trx1 might modulate –SH of membrane target proteins to transduce its signal during cell-cell interaction. However, specific proteins or receptor which directly interact with Trx1 on the surface have not been discovered yet. It is also unknown to what state the Trx1 is on the surface; reduced or oxidised as ARF leads to accumulation of oxidised Trx1 in the cytosol. BSO and ARF have been shown to modify reduced free thiols on the membrane (Skalska et al. 2012). Further looking into thiol groups –SH using optimised conditions of maleimide will provide the number of free thiols on the surface in the next chapter.

This chapter also looked at the distribution of Prx2 levels and how it is affected when Jurkat cells are exposed to BSO and ARF. The results obtained suggests that the levels of Prx2 does not change in the absence of GSH or reduced Trx1. There is very little evidence suggesting the presence of Prx2 on the membrane. However, Szabó-Taylor et al 2012 show significant higher levels of Prx2 on the membrane of B lymphocytes in RA and a greater frequency of cells with surface Prx2 (Szabó-Taylor et al. 2012a). Prx2 is widely known as redox partners of Trx1 (Wood, et al. 2003), thereby studying the role of Prx2 might develop an understanding how they regulate the ROS levels within cells or enable reduction of membrane proteins by acting in partnership. From the results obtained in this chapter, ARF leads to increased levels of Prx2 on the surface, consistent with Trx1. This might propose Prx as being Trx1 dependent antioxidant system. For example, $H_2O_2$ reacts directly with Prx which leads to Prx oxidation, this then is reduced by its interacting partner Trx1 (Netto & Antunes 2016). It is not necessary that oxidised Prx is due to increasing amounts of $H_2O_2$ but inhibition of Trx1 system by auranofin will have the same effect (Gromer et al. 1998). Hence all redox sensitive proteins monitored closely and inhibition of interacting protein may lead to oxidation as well rather than increased ROS generation. Another approach would be to analyse the redox state of Prx2 in the presence of auranofin by eliminating reducing agents including DTT.

Healthy lymphocytes are reported to secrete Trx1 including T cells, macrophages and neutrophils which follows a non-classical pathway (Rubartelli et al. 1992; S. Salzano et al. 2014). It has cytokine like function and acts as a chemoattractant in the extracellular environment, recruiting cells during infection and inflammation (R Bertini et al. 1999) Finally, this chapter looked at whether the increase in surface Trx1 is also associated with increased secretion. However, no significant difference of the Trx1 levels was observed in the cell supernatants under normal or treated conditions suggesting that the increased levels of surface Trx1 observed previously is not associated with Trx1 secretion into the extracellular environment.

Since no change was seen in the secretion of Trx1 by ARF-treated cells, secretion may be independent of the redox state of the cell. Tanudji et al, showed that the redox state of the cell or Trx1 itself does not impact on its secretion, and the secretion of Trx1 is a slow and efficient process (Tanudji et al. 2003). Alternatively, analysis of secreted Trx1 after 2h incubation may not be effective and non-classical secretion pathway may be time dependent.

Therefore, this chapter has shown how each GSH and Trx1 redox system play a role in oxidative stress conditions within the cell in addition to Prx2 distribution. BSO depletes GSH and translocation of Trx1 on the surface. However, in the absence of reduced Trx1, increased levels of Trx1 and Prx2 were observed on the surface of T cells. Jurkat T cell line was used to establish a model to detect redox regulating proteins on the surface which can further be carried on primary $CD4^+$ T cell activation and collagen induced arthritis mice with or without Ncf1 gene expression.

# Chapter 5. Identification of sulphenated membrane proteins during T cell activation in the presence and absence of $H_2O_2$.

## Preface

T cell receptor signalling requires a reduced cell surface protein environment and increased reactive oxygen species (ROS) inhibit T cell activation. The main objective of this chapter was to identify sulphenated proteins on the membrane of T cells at rest, during activation and under oxidising conditions. Results show that increased peroxide concentration lead to a significant decrease in IL-2 secretion and intracellular GSH. Furthermore, activation alone did not affect the number of sulphenated membrane proteins. Activation in the presence of $H_2O_2$, which is associated with lower intracellular calcium flux, show a reduction in number of sulphenated proteins possibly due to further oxidation including disulphide bond formation

## 5.1. Introduction

T cells are critical for developing a specific immune response to a foreign pathogen. Activation of T cells, including $CD4^+$ and $CD8^+$ T cells, occurs when antigenic peptide is presented by MHC I or II on the surface of antigen presenting cells (APC). This activates a signalling cascade which results in the secretion of IL-2 and increase in expression of IL-2 receptor (CD25) on the surface in $CD4^+$ T cells. The binding of IL-2 to its receptors stimulates the $CD4^+$ T cells to proliferate and differentiate into effector cells and different T cell subtypes. $CD8^+$ T cells are mainly involved in killing virally infected cells with similar cytokine secreted as Th1 and Th2 cells. The rapid induction of ROS by T cells upon TCR stimulation depends on NADPH oxidases (NOX) (Jackson et al. 2004; Belikov et al. 2014). Once the T cells are activated, the constituents of NOX-2 assemble at the plasma membrane and generate $O_2^{\cdot-}$ by transferring electrons from the cytosol to the extracellular space. Subsequently, $O_2^{\cdot-}$ dismutates into $H_2O_2$ (Bedard and Krause, 2007). NOX-2 is an important source of ROS in T cells as NOX-2-deficient T cells display strongly reduced $O_2^{\cdot-}$ and $H_2O_2$ production (Jackson et al. 2004; Belikov et al. 2014).

ROS can cause oxidative damage to biomolecules and alter protein activity, regulate enzyme and transcription factor activity (Gelderman et al., 2006). Although ROS can modify macromolecules thus altering the signalling pathways and functions, the reversible oxidation of cysteine residues is necessary and is an important pathway by which protein function is regulated. There is evidence which suggests an increase in cell surface thiol (-SH) levels of stimulated T cells (K. A. Gelderman et al. 2006; Tse et al. 2017) while others have also demonstrated that activation leads to an increase in sulphenic acid (-SOH), the first reversible oxidation product of cysteine (Pellom et al. 2013). Historically, ROS have been considered as essential mediators in T cell function (Kesarwani et al. 2013b).

Some reports indicated that certain antioxidants inside the cell such as cysteine would inhibit proliferation and IL-2 production when administered during the early stages of T cell activation

at dose dependent manner (Goldstone et al. 1995) and treatment of mice with the antioxidant Mn(III)tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP) (metalloporphyrin-mimetic compound with superoxide dismutase activity) decreased proliferation and cytokine production in T cells in both autoimmune and infectious models (Piganelli et al. 2002; Laniewski & Grayson 2004). However, superoxide dismutases, catalase and ascorbic acid do not affect CD25 expression in human T cells (Belikov et al. 2014). This suggest that intracellular ROS released in response to TCR stimulation may act as mediators in T cell activation. However, sub lethal peroxide also caused, loss of transcription factor activity and reduced cytokine production in memory cells when stimulated by an antigen (Flescher et al. 1998; Lahdenpohja et al. 1998 Rodica Lenkei et al. 2001). Also, scavenging of ROS by manganese metalloporphyrin leads to reduced $CD4^+$ T cell responses (Previte et al. 2017).

Although Prx 1 and 2 are highly abundant and required for its role of detoxifying $H_2O_2$ in the cytosol, cysteine residues of Prx are prone to be oxidised at low levels of $H_2O_2$ (Woo et al. 2005; Wood et al. 2003). This may lead to accumulation of $H_2O_2$ within the cells inducing cellular damage or dampened T cell activation. Their presence on the membrane may be reducing disulphides (Carilho Torrao et al. 2013; Mullen et al. 2015a). However, some antioxidants may behave as pro-oxidants at higher concentrations such as vitamin C (Uetaki et al. 2015; Mendes-da-Silva et al. 2014; Griffiths & Lunec 2001). In contrast, oxidised Trx1 forms a disulphide hence inactive and disrupted interaction between Trx1 and target protein (Du et al. 2013)

Therefore, increased amounts on ROS can lead to potential oxidation of proteins. Thus this chapter looks at the effect of $H_2O_2$ during T cell activation on the surface redox state including IL-2 receptor expression and surface Trx1 and Prx2. Also, to identify the possible proteins which undergo sulphenic acid modification on the membrane of T cells during activation and in the presence of peroxide, to identify susceptible sulphenated proteins or receptors.

The main objectives of this chapter on primary human T cells are as follows:

- To optimise $CD4^+$ T cell activation using anti-CD3 and anti-CD28
- To analyse the surface redox state and analyse surface Prx2 and Trx1 including secreted Trx1
- To increase ROS levels during activation by using $H_2O_2$
- To identify sulphenated membrane proteins with and without activation in the presence and absence of peroxide

## 5.2. Methods

### 5.2.1. Primary $CD4^+$ T cell isolation and activation

Fresh blood (20 ml) was collected from healthy donors with consent and $CD4^+$ T cells were isolated as described and activated as described in the methods section 2.2.7.

### 5.2.2. Competition Trx1 ELISA

Trx-1 (0.2µg/ml) 50µl/well was applied to a Nunc microtiter 96 well plates (Nunc) in carbonate buffer pH9.6 and incubated for 1 hour at 37°C. The plate was washed 3 times with 250µl of phosphate buffered saline containing 0.05% Tween-20, w/v (PBST) and were gently tapped on absorbent tissue paper. Following washing, nonspecific sites were blocked by adding skimmed milk 4% w/v in PBS, 200µl/well overnight at 4°C. Standard curve (2.5µg/ml-0µg/ml) was prepared using recombinant human Trx1 protein (R&D); 25µl/well). The supernatants (25µl/well) were plated in triplicates. Mouse monoclonal anti-Trx1 (25µl of 1:2000 in PBS, ab16965; Abcam) was added to all standards and samples and was incubated at 37°C for 2 h. Following incubation, wells were washed with PBST (250µl) 3 times and 200µl of peroxidase conjugated anti-mouse antibody (1:5000) was added to each well. The plate was incubated at 37°C for 1h. Following washing 3 times with PBST (250µl), and 200µl of substrate solution containing *o*-phenylenediamine and hydrogen peroxide in 0.15 M citrate-phosphate buffer, was added. During incubation at room temperature, colour development was observed from 2 to 10 min. The colour reaction was stopped with the addition of 2 M sulphuric acid (50µl/well). Absorbance was measured at 490 nm in a microplate reader (Bio Tek, UK).

### 5.2.3. Flow cytometric analysis of Trx1 and Prx2

For flow cytometric analysis of Trx1, $5x10^5$ cells were collected and washed two times in 500µl cold wash buffer (PBS supplemented with 1% w/v BSA). Following two washes in cold wash buffer, cells were resuspended in cold wash buffer and incubated with mouse monoclonal anti-Trx1 (ab16965; Abcam, 1µl/$10^6$ cells) or IgG2b isotype control antibody (ab91366; Abcam, 1µl/$10^6$ cells) on ice for 30 minutes. After a further two washes, cells were incubated with goat anti-mouse polyclonal conjugated dylight-488 (96879; Abcam, 0.5µl/$10^6$ cells) antibody on ice for 30minutes in cold wash buffer. Finally, cells were analysed on a Cytomics FC 500 flow cytometer (Beckman Coulter, Wycombe, UK).

### 5.2.4. Cell viability

Cell viability was determined with Nucleocounter NC 3000 (ChemoMetec) imaging cytometer according to the manufacturer's instructions. Briefly $1x10^5$ cells were collected after indicated treatments and inserted from the tip of the Via1-Cassette™ (ChemoMetec). The inside of the Via1-Cassette™ is coated with two different dyes, Acridine Orange staining the entire population of cells and DAPI staining the non-viable cells, respectively.

### 5.2.5. Apoptosis Assay

Briefly the $1x10^5$ cells were resuspended in 500µl 'binding buffer' (150mM NaCl, 10mM HEPES and 2.5mM $CaCl_2$) (Castro et al. 2017) and incubated with anti-human Annexin V/FITC (eBioscience, 1µl per $2x10^5$ cells) or unstained control on ice for 10min and analysed by flow cytometer (Beckman coulter FC500, High Wycombe, UK).

### 5.2.6. Protein sulphenic acid detection on the membrane of human $CD4^+$ T cells

$CD4^+$ T cells ($1x10^6$cells/ml) were isolated and activated as described in methods section above 2.2.7. The cells were activated with anti-CD3 and anti-CD28 in the presence or absence of $H_2O_2$ (20µM) and 0.5mM DCP-Bio1 (Merck, Millipore, UK) and incubated for 30mins. Activated cells for 24h were incubated with DCP-Bio1 30min before collection of cells. Following treatment, the cells were washed and cytosolic proteins (CP) and membrane proteins (MP) were isolated (section 2.2.4) then biotinylated proteins were captured using streptavidin magnetic beads (Thermo-Fisher, UK). Captured biotinylated proteins were analysed by LC-MS/MS and the list of sulphenated proteins was generated using Mascot Daemon database.

## 5.3. Results

### 5.3.1. Purity of negatively isolated human CD4$^+$ T cells using flow cytometry

To determine the purity of negatively isolated CD4$^+$ T cells before further experiments, cells were either stained with allophycocyanin/cy7 labelled anti-CD4$^+$ antibody in the dark on ice for 20min or its relevant isotype control IgG1 allophycocyanin/cy7 labelled antibody. The purity of CD4$^+$ T cell gated population in Figure 5.1 was 99% (red).

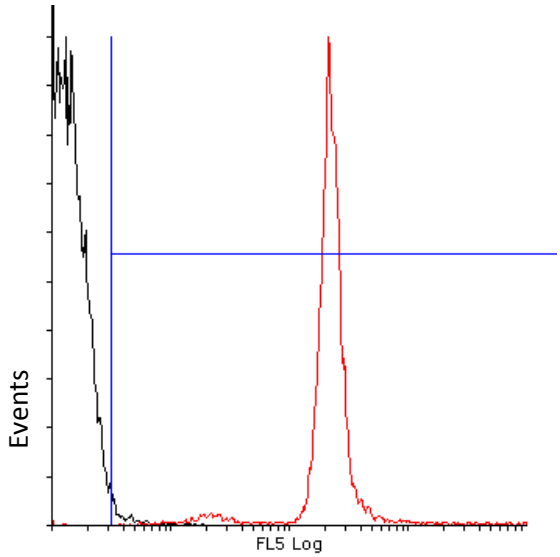



Figure 5.1: Purity of the negatively isolated T cells: CD4$^+$ T cells isolated from PBMCs were stained with anti-CD4 antibody or with Cy7 labelled isotype control and analysed by flow cytometry. CD4$^+$ positive cells were gated (blue line) at 1% of isotype control (black) and the data was collected for 10000 events (red).

## 5.3.2. CD4$^+$ T cells secrete IL-2 within 24h of activation

To determine the conditions required for T cell activation, human primary CD4$^+$ T cells were activated with anti-CD3 and anti-CD28 for 24, 48 and 72h (Figure 5.2). Activated T cells show a significant increase in IL-2 secretion after 24h, 48h, and 72h (0.5±0.1, 0.7±0.2, 0.7±0.1) compared to no activation (0.01±0.02) (Figure 5.2a) and increase in cells expressing surface CD25 (% cells; no activation: 3.71±1.33, 24h: 44.46±1.084, 48h: 50.01±1.681 and 72h: 67.3±1.587 respectively (Fig 5.2b). Hence, further activation experiments were performed at the 24h time point.

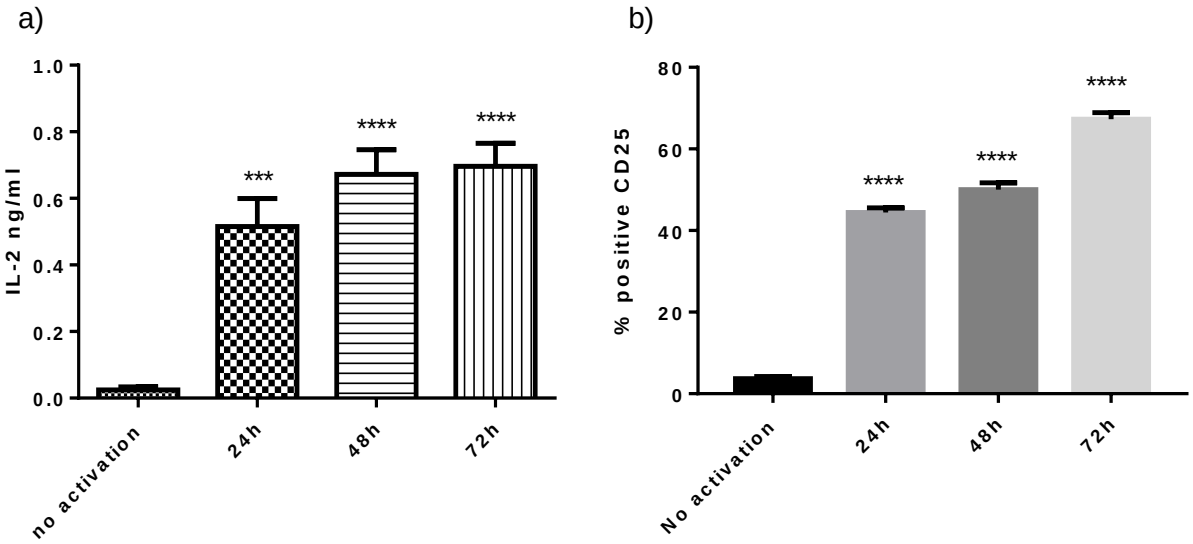



Figure 5.2: Increase in IL-2 secretion and CD25 expression after CD4$^+$ T cell activation. CD4$^+$ T cells were isolated and activated with 1µg/ml anti-CD3 (OKT3) and 2µg/ml anti-CD28.2 for 24, 48 and 72h. a) IL-2 secretion measured by sandwich ELISA. b) IL-2 receptor (CD25) expression on the surface, cells were incubated with anti-CD25 for 20mins and analysed by flow cytometry. Data expressed as the difference in cells expressing CD25 and isotype control signal at 1% over 5000 events n=3 mean and SEM, ANOVA ****p<0.0006, ****p<0.0001

### 5.3.3. Trx1 secretion levels after T cell activation

Trx1 on the surface of cells may act as an immunomodulatory agent by regulating certain cytokine and chemokine activity via modulating receptor redox state (Nordberg and Arner 2001). During inflammation and ageing, it is believed that secretion of cytoplasmic proteins is altered, including Trx1, into extracellular environment that is independent of the Golgi pathway (Rubartelli et al. 1992). In order to observe whether a change in Trx1 distribution is associated with T cell activation, $CD4^+$ were stimulated with anti-CD3/CD28 antibodies for 24h, 48 and 72h. A significant 3-fold increase in surface Trx1 was observed in activated T cells after 24h (Figure 5.3a), which suggests that activation leads to increased translocation of Trx1 to the membrane. Additionally, $CD4^+$ T cells show an increased trend in Trx1 secretion in the supernatants after activation for 24h (1.5±0.4) and 48h (1.6±0.6) as compared to unactivated (1.3±0.2), but a decrease after 72h (1.2±0.5) of activation. To investigate whether the decrease after 72 hours is associated with apoptosis, Annexin V staining was carried out (Figure 5.3c); this suggests that the activated cells at 72h might be undergoing apoptosis.

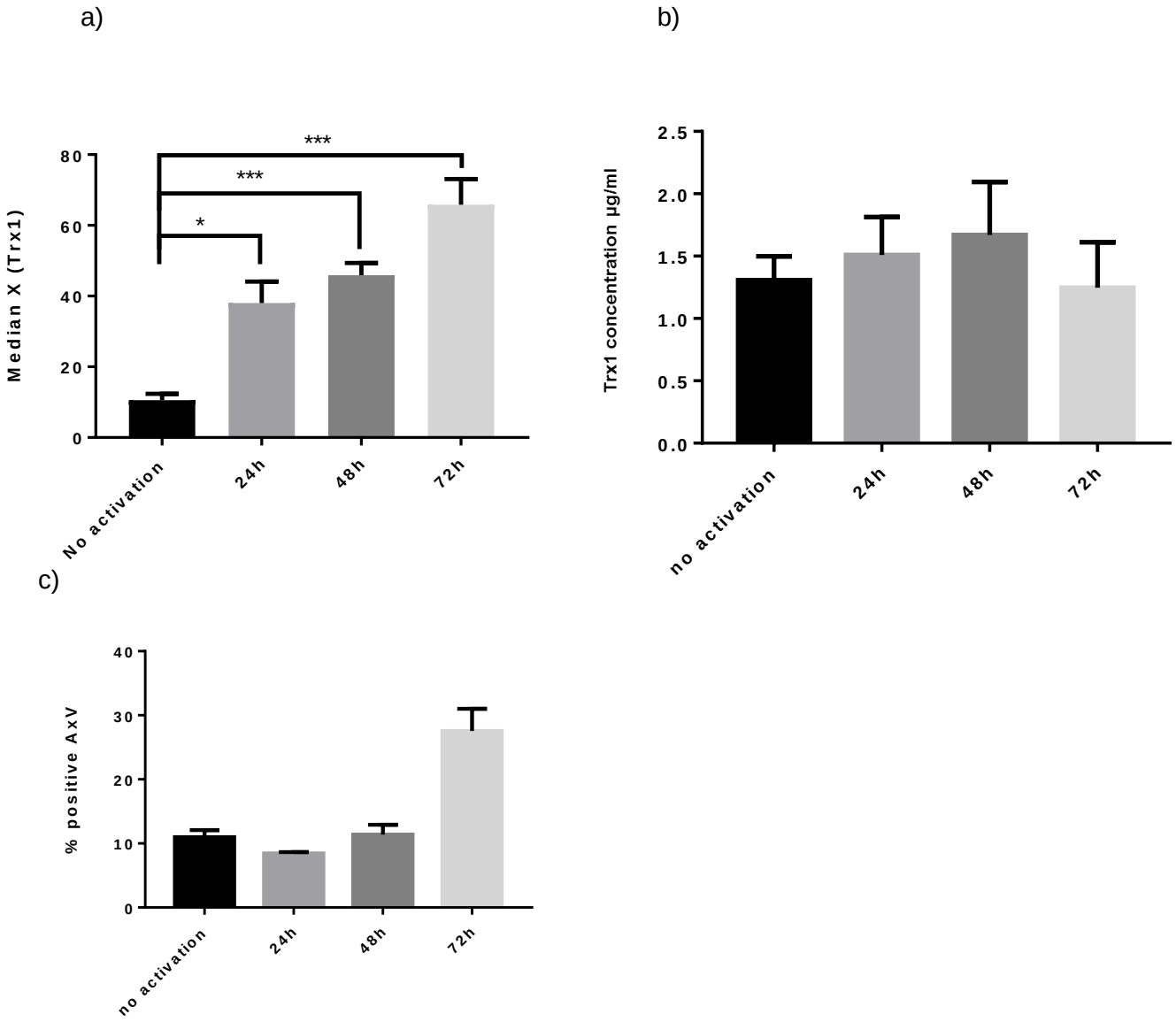



Figure 5.3: Trx1 redistributes to the membrane after activation. $CD4^+$ T cells were isolated and activated with 1µl/ml anti-CD3 (OKT3) and 2µg/ml CD28.2 for 24, 48 and 72h. The cells were isolated and washed with PBS 1% BSA and incubated with primary anti-Trx1 (ab16965) or isotype control (ab91366) for 30min. The cells were washed and secondary goat anti-mouse IgG H&L (ab96879) was added and incubated for 30min. RM ANOVA, Dunnett's multiple comparison test. Mean and SEM *$p<0.01$, ***$p<0.0006$. b) Trx1 secretion measured in the culture supernatants by competition ELISA. c) Cells expressing AxV on the surface after activation (n=3 independent experiments).

## 5.3.4. $CD4^+$ T cell activation leads to increase in surface thiols (-SH)

In order to understand how the surface redox state is affected during activation, $CD4^+$ T cells were activated and reduced surface thiols were determined by flow cytometry at early time points after activation (5 and 30minutes). Figure 5.4 shows an increased trend in –SH in activated T cells (X-mean; 633.6±96.86) after 5min compared to unactivated T cells (533±59.09). Additionally, a significant increase in –SH levels on the surface of activated $CD4^+$ T cells (569.9±109.4) was observed after 30min of activation compared to unactivated T cells (301.6±60.35).

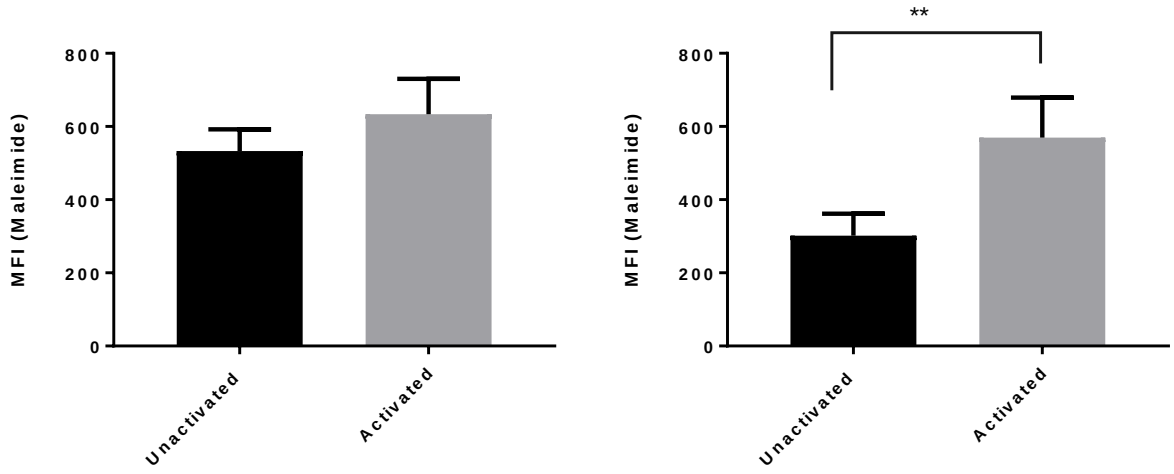



Figure 5.4: Surface thiol levels increases after T cell activation. $CD4^+$ T cells were isolated from blood and activated with 1µg/ml anti-CD3 (OKT3) and 2µg/ml anti-CD28.2 for 24h. Cells were collected after 5 and 30mins of activation and incubated with FITC-conjugated maleimide for 30min and analysed by flow cytometry. a) –SH after 5min, b) surface -SH after 30min activation. Data represents Mean peak staining intensity for thiols with standard error of the mean from 3 independent experiments. Statistical analysis were calculated using two tailed, paired student t test ***p<0.004.*

## 5.3.5. Activation of T cells causes an increase in sulphenic acid formation on the surface of T cells

To further understand the role of sulphenic acid in T cell activation and the surface redox state, this chapter looked at surface oxidised thiols (sulphenic acid) on the surface of T cells. Activated T cells show a significant increase in –SOH (X-Median 8.65±0.41) after 5min of activation compared to unactivated T cells (6.07±0.17) (Figure 5.5). Furthermore, activated T cells show an increased trend in –SOH on the surface (9.7±1.45) as compared to unactivated T cells (8.5±1.3) after 30min.

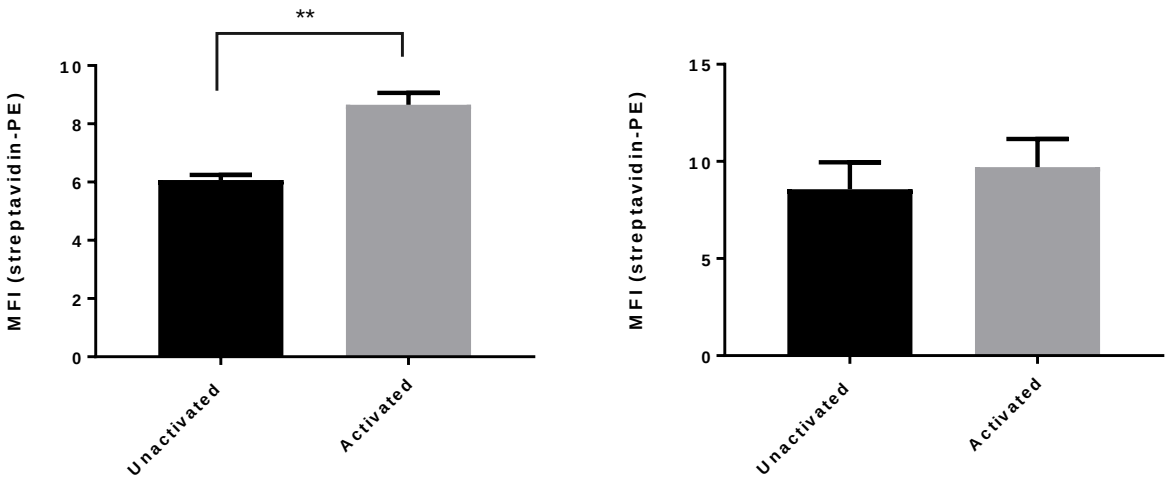



Figure 5.5: Surface -SOH increases after T cell activation. $CD4^+$ T cells were isolated from blood and activated with 1µg/ml anti-CD3 (OKT3) and 2µg/ml anti-CD28. Cells were collected after 5 and 30mins of activation and incubated with 0.1mM DCP-Bio1 and 10mM iodoacetamide (IAA) for 30min added at time zero. The cells were further washed and incubated with streptavidin-PE (1:100) for 30min on ice and analysed by flow cytometry. a) –SOH after 5min, b) surface –SOH after 30min activation, n=3. Data represents mean of X-median and standard error of mean (SEM) and statistical analysis were calculated using two tailed, paired student t test ***p<0.001.*

### 5.3.6. Increased oxidative stress inhibits T cell activation

In order to mimic the physiological phagocyte derived ROS, $CD4^+$ T cells were activated in the presence or absence of exogenous $H_2O_2$. Figure 5.6 shows that expression of CD25 on the surface was significantly decreased in the presence of $H_2O_2$ suggesting excess oxidative stress inhibits T cell activation. Furthermore, treatment with NAC together with anti-CD3/CD28 activation did not restore the activation response. Similarly, the cells show a significant decrease in IL-2 secretion when treated with $H_2O_2$ and NAC does not restore the effect (Figure 5.6d) under the conditions used here. Moreover, T cells were treated with $H_2O_2$ at non-cytotoxic concentration and thus the decrease in expression was not due to loss of viability, since there was no significant difference in viability in the presence and absence of $H_2O_2$ (Figure 5.6c)

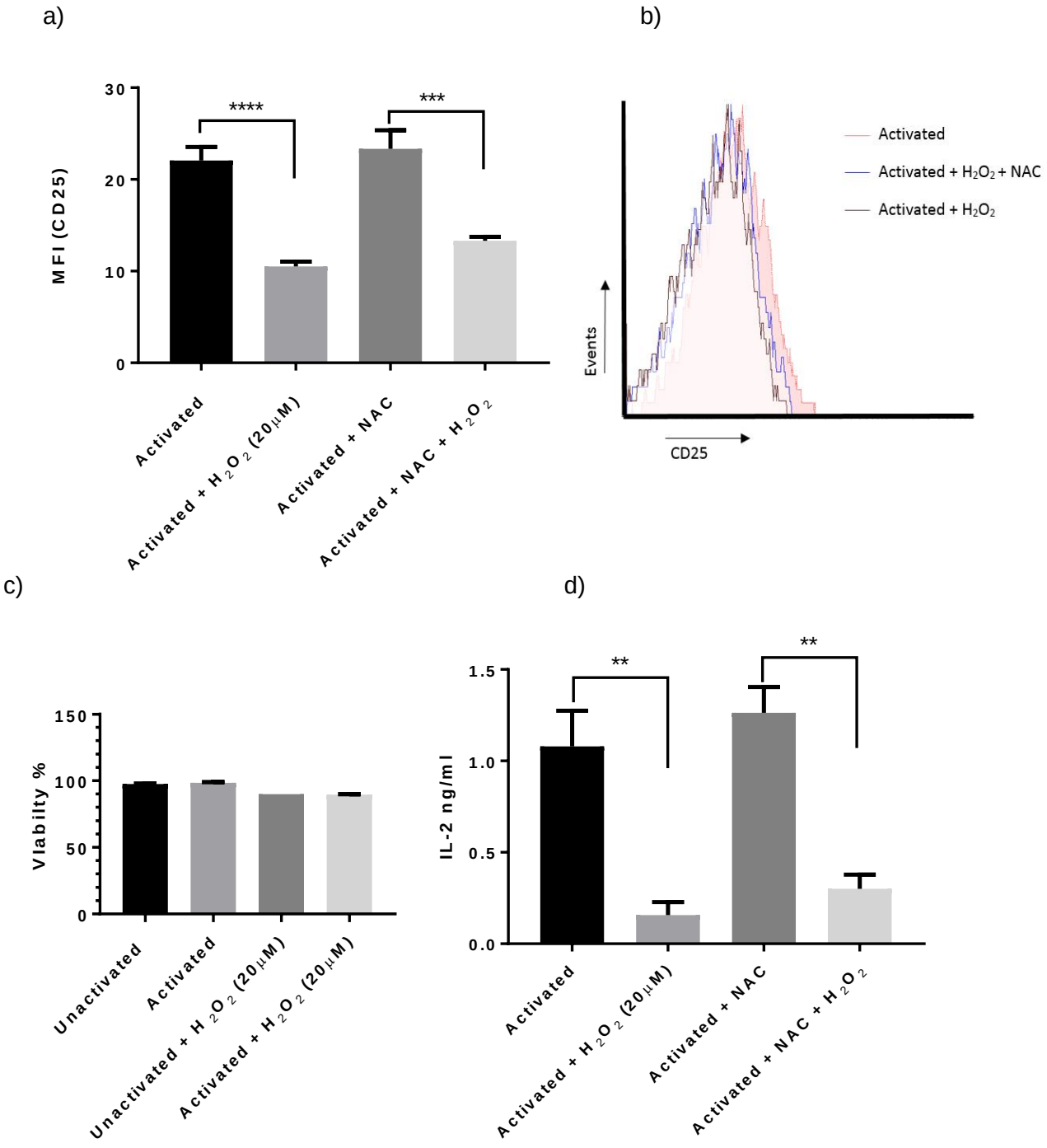



Figure 5.6. NAC does not restore CD25 expression on the surface of peroxide-treated T cells. $CD4^+$ T cells were isolated and activated with 1µg/ml anti-CD3 (OKT3) and 2µg/ml CD28.2 for 24h in the presence of $H_2O_2$ (20µM) and NAC (10mM). Cells were collected and stained for anti-CD25 for 20min on ice and analysed by FC. Supernatants were collected for IL-2 analysis a) X-Median CD25 expression on the surface of T cells, b) Log scale showing changes in CD25 expression with $H_2O_2$. c) Viability of activated cells after 24h. d) IL-2 secretion in the supernatants. Data expressed mean and SEM, n=3 ANOVA, Tukey's multiple comparison test. ****p<0.0006 **p<0.003.*

## 5.3.7. Activation in the presence of $H_2O_2$ has no effect on surface Trx1 and Prx2

In order to observe the distribution of Trx1 and Prx2 onto the surface when activated in the presence of oxidative stress, $CD4^+$ T cells were activated in the presence of $H_2O_2$ and surface Trx1 and Prx2 were analysed by flow cytometry after 24h. Figure 5.7 shows a significant increase in surface Trx1 after activation but no difference was observed when activated in the presence of $H_2O_2$. Also, a similar trend was observed for Prx2 on the surface.

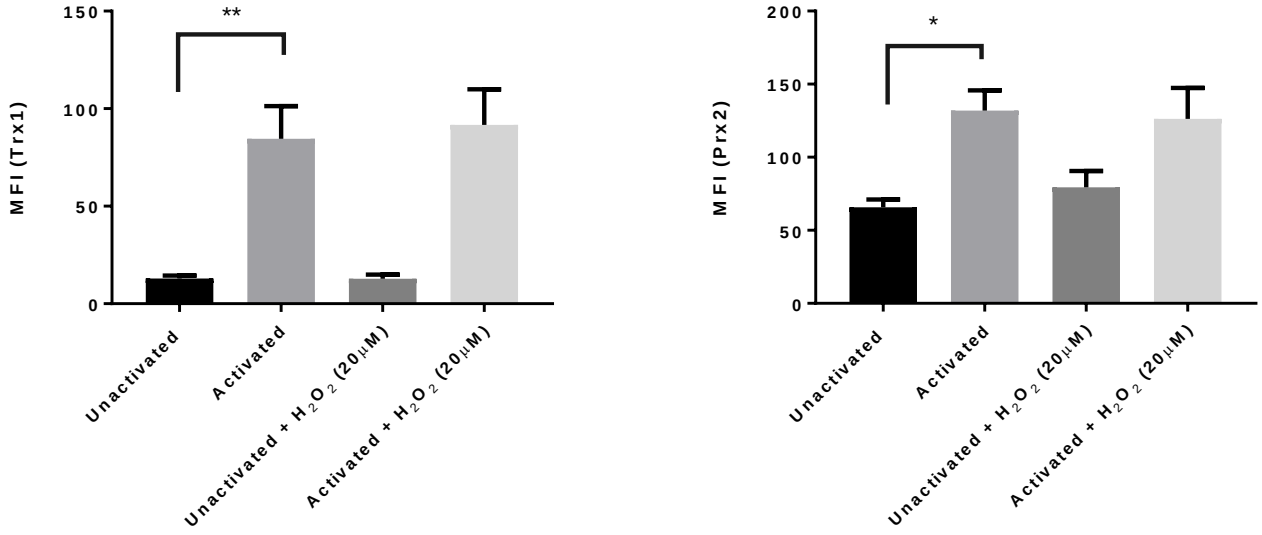



Figure 5.7. Exogenous $H_2O_2$ has no effect on surface Trx1 and Prx2 expression induced by activation of T cells. $1x10^6$/ml $CD4^+$ T cells were isolated and activated with 1µl/ml anti-CD3 (OKT3) and 2µg/ml anti-CD28.2 for 24h in the presence or absence of $H_2O_2$ (20µM). The cells were isolated and washed with PBS/BSA 1%w/v and incubated with primary anti-Trx1 (ab16965) or isotype control (ab91366) for 30min. The cells were washed and secondary goat anti-Mouse IgG H&L (ab96879) was added and incubated for 30min and analysed by flow cytometry, MFI-X-median. a) Surface Trx1 expression b) washed cells incubated with primary anti-Prx2. The cells were washed and secondary goat anti-rabbit IgG was added and incubated for 30min and analysed by flow cytometry. Data represents Mean and SEM of three independent experiments, n=3. Ordinary one way ANOVA, Tukey's Multiple comparison test, *p<0.019, **p<0.003.

## 5.3.8. $H_2O_2$ depletes intracellular GSH levels

Glutathione is required for DNA synthesis in human T cells and plays an important role in T cell function and proliferation (Levring et al. 2015; Smyth 1991). In order to determine the effect of $H_2O_2$ on GSH levels during activation, $CD4^+$ T cells were activated in the presence of $H_2O_2$ and NAC. Figure 5.8 shows a significant increase in GSH when the T cells were activated in the absence of $H_2O_2$ or NAC. However, $H_2O_2$ completely diminishes GSH levels, whereas T cells activated in the presence of $H_2O_2$ together with NAC slightly recovers GSH levels by twofold. This suggests that NAC protects against the effect of $H_2O_2$ on loss of intracellular GSH during T cell activation.

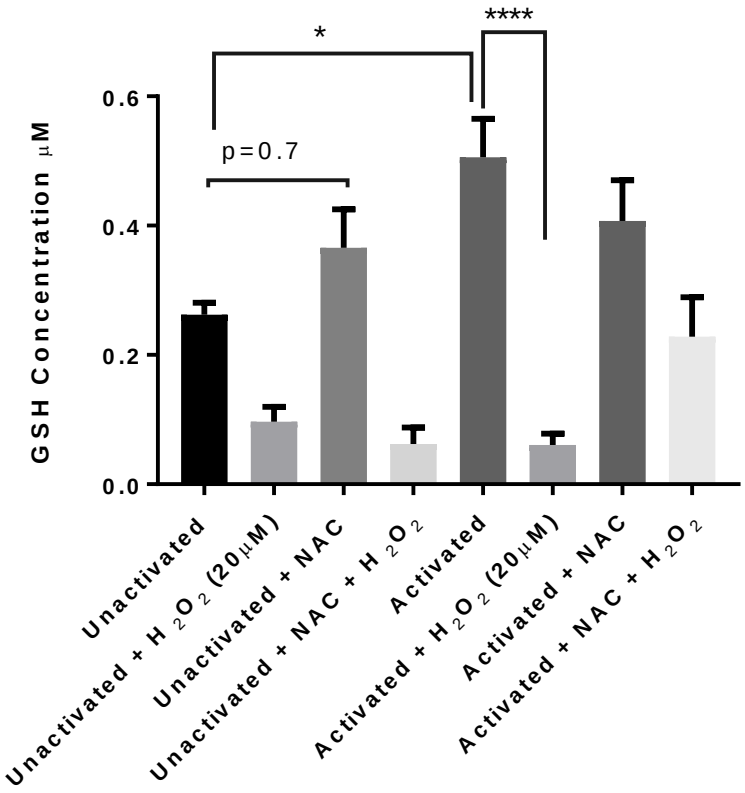



Figure 5.8. Effect of $H_2O_2$ on intracellular GSH after 24h. $CD4^+$ T cells were isolated and activated with 1µl/ml anti-CD3 (OKT3) and 2µg/ml anti-CD28.2 for 24h in the presence of $H_2O_2$ (20µM). $1x10^5$ cells were isolated and plated on a 96 well plate in duplicates, the GSH concentration was determined using GSH-Glo™ glutathione assay (Promega) using a standard curve. Data represents three independent experiments, mean and SEM, n=3. Ordinary one way ANOVA, Tukey's Multiple comparison test, *p<0.01, ****p<0.0001

## 5.3.9. Sulphenated proteins identified by MS/MS

In order to identify proteins that are susceptible to sulphenic acid modification on the membranes of $CD4^+$ T cells after activation and in the presence of $H_2O_2$, $CD4^+$ T cells were activated in the presence or absence of $H_2O_2$ for 30mins and 24h and modified thiols were captured using DCP-bio1. Several proteins were identified to be sulphenated as shown in the tables below at different time and treatment conditions.

| ACCESSION | SCORE | MASS | NUM. OF SEQUENCES | DESCRIPTION |
|---|---|---|---|---|
| **ALBU_HUMAN** | 4287 | 69321 | 394 | Serum albumin |
| **IQCA1_HUMAN** | 507 | 95281 | 191 | IQ and AAA domain-containing protein 1 |
| **ACTB_HUMAN** | 2100 | 41710 | 72 | Actin, cytoplasmic 1 |
| **HNRPC_HUMAN** | 317 | 33650 | 25 | Heterogeneous nuclear ribonucleoproteins C1/C2 |
| **AFF3_HUMAN** | 76 | 133394 | 21 | AF4/FMR2 family member 3 |
| **CC134_HUMAN** | 98 | 26544 | 17 | Coiled-coil domain-containing protein 134 |
| **HNRPK_HUMAN** | 207 | 50944 | 11 | Heterogeneous nuclear ribonucleoprotein K |
| **NMD3A_HUMAN** | 36 | 125385 | 11 | Glutamate receptor ionotropic, NMDA 3A |
| **CH60_HUMAN** | 158 | 61016 | 10 | 60 kDa heat shock protein, mitochondrial |
| **HNRPD_HUMAN** | 209 | 38410 | 8 | Heterogeneous nuclear ribonucleoprotein D0 |
| **HNRH1_HUMAN** | 232 | 49198 | 7 | Heterogeneous nuclear ribonucleoprotein H |
| **ROA2_HUMAN** | 143 | 37407 | 7 | Heterogeneous nuclear ribonucleoproteins A2/B1 |
| **PRDX1_HUMAN** | 117 | 22096 | 7 | Peroxiredoxin-1 |
| **GNAT3_HUMAN** | 108 | 40331 | 7 | Guanine nucleotide-binding protein G(t) subunit alpha-3 |
| **COF1_HUMAN** | 156 | 18491 | 6 | Cofilin-1 |
| **PTBP1_HUMAN** | 97 | 57186 | 6 | Polypyrimidine tract-binding protein 1 |
| **ROA1_HUMAN** | 171 | 38723 | 5 | Heterogeneous nuclear ribonucleoprotein A1 |
| **ZC3HD_HUMAN** | 45 | 196519 | 5 | Zinc finger CCCH domain-containing protein 13 |
| **COR1A_HUMAN** | 69 | 50994 | 4 | Coronin-1A |
| **PDIA3_HUMAN** | 81 | 56747 | 3 | Protein disulfide-isomerase A3 |

Table 5.1: Membrane proteins which are sulphenated in unactivated $CD4^+$ T cells after 30min.

| ACCESSION | SCORE | MASS | NUM. OF SEQUENCES | DESCRIPTION |
|---|---|---|---|---|
| **HNRPM_HUMAN** | 445 | 77464 | 20 | Heterogeneous nuclear ribonucleoprotein M |
| **ZC3HD_HUMAN** | 48 | 196519 | 12 | Zinc finger CCCH domain-containing protein 13 |
| **CH60_HUMAN** | 114 | 61016 | 11 | 60 kDa heat shock protein, mitochondrial |
| **MYH11_HUMAN** | 26 | 227199 | 11 | Myosin-11 |
| **ACTB_HUMAN** | 1085 | 41710 | 10 | Actin, cytoplasmic 1 |
| **CRCC2_HUMAN** | 32 | 185760 | 10 | Putative ciliary rootlet coiled-coil protein 2 |
| **TLL1_HUMAN** | 15 | 114635 | 10 | Tolloid-like protein 1 |

| | | | | |
|---|---|---|---|---|
| **ACINU_HUMAN** | 24 | 151771 | 9 | Apoptotic chromatin condensation inducer in the nucleus |
| **TANC1_HUMAN** | 50 | 202093 | 8 | Protein TANC1 |
| **COR1A_HUMAN** | 41 | 50994 | 8 | Coronin-1A |
| **IQCAL_HUMAN** | 496 | 94871 | 7 | IQ and AAA domain-containing protein 1-like |
| **UACA_HUMAN** | 27 | 162404 | 7 | Uveal autoantigen with coiled-coil domains and ankyrin repeats |
| **K0355_HUMAN** | 22 | 115946 | 7 | Uncharacterized protein KIAA0355 |
| **ABCA3_HUMAN** | 19 | 191239 | 7 | ATP-binding cassette sub-family A member 3 |
| **ALBU_HUMAN** | 5501 | 69321 | 6 | Serum albumin |
| **HNRPC_HUMAN** | 307 | 33650 | 6 | Heterogeneous nuclear ribonucleoproteins C1/C2 |
| **GRP78_HUMAN** | 434 | 72288 | 6 | 78 kDa glucose-regulated protein |
| **SC31B_HUMAN** | 50 | 128615 | 6 | Protein transport protein Sec31B |
| **PDIA3_HUMAN** | 126 | 56747 | 5 | Protein disulfide-isomerase A3 |
| **PTBP1_HUMAN** | 60 | 57186 | 5 | Polypyrimidine tract-binding protein 1 |
| **ATPB_HUMAN** | 38 | 56525 | 5 | ATP synthase subunit beta, mitochondrial |
| **KIFC3_HUMAN** | 33 | 92718 | 5 | Kinesin-like protein KIFC3 |
| **LTBP1_HUMAN** | 23 | 186673 | 5 | Latent-transforming growth factor beta-binding protein 1 |
| **DLG1_HUMAN** | 22 | 100393 | 5 | Disks large homolog 1 |
| **AFF3_HUMAN** | 88 | 133394 | 4 | AF4/FMR2 family member 3 |
| **ROA2_HUMAN** | 420 | 37407 | 4 | Heterogeneous nuclear ribonucleoproteins A2/B1 |
| **ZFP37_HUMAN** | 25 | 71164 | 4 | Zinc finger protein 37 homolog |
| **INT13_HUMAN** | 23 | 80174 | 4 | Integrator complex subunit 13 |
| **NALP2_HUMAN** | 22 | 120437 | 4 | NACHT, LRR and PYD domains-containing protein 2 |
| **GTPBA_HUMAN** | 20 | 42906 | 4 | GTP-binding protein 10 |
| **NMD3A_HUMAN** | 39 | 125385 | 3 | Glutamate receptor ionotropic, NMDA 3A |
| **HNRH1_HUMAN** | 279 | 49198 | 3 | Heterogeneous nuclear ribonucleoprotein H |
| **GNA12_HUMAN** | 164 | 44251 | 3 | Guanine nucleotide-binding protein subunit alpha-12 |
| **IDHP_HUMAN** | 81 | 50877 | 3 | Isocitrate dehydrogenase [NADP], mitochondrial |
| **VAT1_HUMAN** | 25 | 41893 | 3 | Synaptic vesicle membrane protein VAT-1 homolog |
| **SPA12_HUMAN** | 33 | 47145 | 3 | Serpin A12 |
| **CC152_HUMAN** | 27 | 29960 | 3 | Coiled-coil domain-containing protein 152 |
| **BAP29_HUMAN** | 26 | 28302 | 3 | B-cell receptor-associated protein |
| **CS047_HUMAN** | 25 | 44718 | 3 | Uncharacterized protein C19orf47 |
| **APOL2_HUMAN** | 22 | 37069 | 3 | Apolipoprotein L2 |
| **TAGL2_HUMAN** | 18 | 22377 | 3 | Transgelin-2 |
| **MVD1_HUMAN** | 17 | 43377 | 3 | Diphosphomevalonate decarboxylase |
| **WNT3_HUMAN** | 14 | 39619 | 3 | Proto-oncogene Wnt-3 |

Table 5.2: Membrane proteins which are sulphenated in activated $CD4^+$ T cells after 30mins

| ACCESSION | SCORE | MASS | NUM. OF SEQUENCES | DESCRIPTION |
|---|---|---|---|---|
| **BPTF_HUMAN** | 38 | 338054 | 12 | Nucleosome-remodeling factor subunit BPTF |
| **AKA11_HUMAN** | 29 | 210380 | 10 | A-kinase anchor protein 11 |
| **ACTB_HUMAN** | 2754 | 41710 | 9 | Actin, cytoplasmic 1 |
| **CH60_HUMAN** | 123 | 61016 | 9 | 60 kDa heat shock protein, mitochondrial |
| **HNRPM_HUMAN** | 88 | 77464 | 9 | Heterogeneous nuclear ribonucleoprotein M |
| **UACA_HUMAN** | 30 | 162404 | 9 | Uveal autoantigen with coiled-coil domains and ankyrin repeats |
| **RHG23_HUMAN** | 25 | 162092 | 9 | Rho GTPase-activating protein 23 |
| **PKHD1_HUMAN** | 38 | 446418 | 8 | Fibrocystin |
| **ZSWM8_HUMAN** | 32 | 197173 | 8 | Zinc finger SWIM domain-containing protein 8 |
| **FND3A_HUMAN** | 20 | 131767 | 8 | Fibronectin type-III domain-containing protein 3A |
| **ALBU_HUMAN** | 6676 | 69321 | 7 | Serum albumin |
| **HNRPC_HUMAN** | 442 | 33650 | 7 | Heterogeneous nuclear ribonucleoproteins C1/C2 |
| **ZC3HD_HUMAN** | 53 | 196519 | 7 | Zinc finger CCCH domain-containing protein 13 |
| **YLPM1_HUMAN** | 20 | 219849 | 7 | YLP motif-containing protein 1 |
| **PEX1_HUMAN** | 73 | 142778 | 6 | Peroxisome biogenesis factor 1 |
| **AT1A4_HUMAN** | 34 | 114093 | 6 | Sodium/potassium-transporting ATPase subunit alpha-4 |
| **K2C75_HUMAN** | 29 | 59524 | 6 | Keratin, type II cytoskeletal 75 |
| **RAB7A_HUMAN** | 61 | 23475 | 5 | Ras-related protein Rab-7a |
| **RHG39_HUMAN** | 41 | 121210 | 5 | Rho GTPase-activating protein 39 |
| **K2C5_HUMAN** | 31 | 62340 | 5 | Keratin, type II cytoskeletal 5 |
| **DLG1_HUMAN** | 20 | 100393 | 5 | Disks large homolog 1 |
| **PABP3_HUMAN** | 18 | 69987 | 5 | Polyadenylate-binding protein 3 |
| **IQCA1_HUMAN** | 375 | 95281 | 4 | IQ and AAA domain-containing protein 1 |
| **S14L4_HUMAN** | 45 | 46614 | 4 | SEC14-like protein 4 |
| **RAE1_HUMAN** | 45 | 73429 | 4 | Rab proteins geranylgeranyltransferase component A 1 |
| **TERA_HUMAN** | 39 | 89266 | 4 | Transitional endoplasmic reticulum ATPase |
| **MVD1_HUMAN** | 29 | 43377 | 4 | Diphosphomevalonate decarboxylase |
| **ADH4_HUMAN** | 27 | 40196 | 4 | Alcohol dehydrogenase 4 |
| **ADCY2_HUMAN** | 24 | 123523 | 4 | Adenylate cyclase type 2 |
| **ROA2_HUMAN** | 432 | 37407 | 3 | Heterogeneous nuclear ribonucleoproteins A2/B1 |
| **GRP78_HUMAN** | 128 | 72288 | 3 | 78 kDa glucose-regulated protein |
| **AFF3_HUMAN** | 101 | 133394 | 3 | AF4/FMR2 family member 3 |
| **COF1_HUMAN** | 76 | 18491 | 3 | Cofilin-1 |
| **HNRPK_HUMAN** | 66 | 50944 | 3 | Heterogeneous nuclear ribonucleoprotein K |
| **COEA1_HUMAN** | 60 | 193394 | 3 | Collagen alpha-1(XIV) chain |
| **RL12_HUMAN** | 47 | 17808 | 3 | 60S ribosomal protein L12 |
| **NMD3A_HUMAN** | 46 | 125385 | 3 | Glutamate receptor ionotropic, NMDA 3A |
| **HCD2_HUMAN** | 31 | 26906 | 3 | 3-hydroxyacyl-CoA dehydrogenase type-2 |
| **FA30A_HUMAN** | 27 | 14616 | 3 | Putative uncharacterized protein FAM30A |

Table 5.3: Membrane proteins which are sulphenated in unactivated $CD4^+$ T cells in the presence of $H_2O_2$ after 30mins

| ACCESSION | SCORE | MASS | NUM. OF SEQUENCES | DESCRIPTION |
|---|---|---|---|---|
| **ACTB_HUMAN** | 1664 | 41710 | 12 | Actin, cytoplasmic 1 |
| **UACA_HUMAN** | 45 | 162404 | 8 | Uveal autoantigen with coiled-coil domains and ankyrin repeats |
| **ALBU_HUMAN** | 2162 | 69321 | 7 | Serum albumin |
| **WDR48_HUMAN** | 17 | 76162 | 7 | WD repeat-containing protein 48 |
| **CEL_HUMAN** | 13 | 79272 | 7 | Bile salt-activated lipase |
| **HNRPC_HUMAN** | 99 | 33650 | 6 | Heterogeneous nuclear ribonucleoproteins C1/C2 |
| **NMD3A_HUMAN** | 34 | 125385 | 5 | Glutamate receptor ionotropic, NMDA 3A |
| **OPLA_HUMAN** | 32 | 137371 | 5 | 5-oxoprolinase |
| **RPC2_HUMAN** | 18 | 127702 | 5 | DNA-directed RNA polymerase III subunit RPC2 |
| **HNRH1_HUMAN** | 115 | 49198 | 4 | Heterogeneous nuclear ribonucleoprotein H |
| **AFF3_HUMAN** | 74 | 133394 | 4 | AF4/FMR2 family member 3 |
| **PLSL_HUMAN** | 65 | 70244 | 4 | Plastin-2 |
| **PDIA3_HUMAN** | 56 | 56747 | 4 | Protein disulfide-isomerase A3 |
| **FANCC_HUMAN** | 33 | 63388 | 4 | Fanconi anemia group C protein |
| **TERA_HUMAN** | 32 | 89266 | 4 | Transitional endoplasmic reticulum ATPase |
| **TMPS9_HUMAN** | 26 | 113948 | 4 | Transmembrane protease serine 9 |
| **RC3H2_HUMAN** | 21 | 131586 | 4 | Roquin-2 |
| **TANC1_HUMAN** | 55 | 202093 | 3 | Protein TANC1 |
| **IQCAL_HUMAN** | 496 | 94871 | 3 | IQ and AAA domain-containing protein 1-like |
| **GNAS1_HUMAN** | 188 | 110956 | 3 | Guanine nucleotide-binding protein G(s) subunit alpha isoforms XLas |
| **CC134_HUMAN** | 151 | 26544 | 3 | Coiled-coil domain-containing protein 134 |
| **GRP78_HUMAN** | 85 | 72288 | 3 | 78 kDa glucose-regulated protein |
| **ABCC8_HUMAN** | 24 | 176879 | 3 | ATP-binding cassette sub-family C member 8 |
| **RN111_HUMAN** | 17 | 108794 | 3 | E3 ubiquitin-protein ligase Arkadia |
| **EX3L1_HUMAN** | 17 | 81627 | 3 | Exocyst complex component 3-like protein |

Table 5.4: Membrane proteins which are sulphenated in activated $CD4^+$ T cells in the presence of $H_2O_2$ after 30min.

| ACCESSION | SCORE | MASS | NUM. OF SEQUENCES | DESCRIPTION |
|---|---|---|---|---|
| **ASXL3_HUMAN** | 29 | 241767 | 10 | Putative Polycomb group protein ASXL3 |
| **IQCAL_HUMAN** | 432 | 94871 | 7 | IQ and AAA domain-containing protein 1-like |
| **UACA_HUMAN** | 56 | 162404 | 7 | Uveal autoantigen with coiled-coil domains and ankyrin repeats |
| **ACTB_HUMAN** | 1658 | 41710 | 6 | Actin, cytoplasmic 1 |
| **HNRPC_HUMAN** | 534 | 33650 | 6 | Heterogeneous nuclear ribonucleoproteins C1/C2 |
| **C163B_HUMAN** | 41 | 159135 | 6 | Scavenger receptor cysteine-rich type 1 protein M160 |
| **ATS3_HUMAN** | 22 | 135515 | 6 | A disintegrin and metalloproteinase with thrombospondin motifs 3 |
| **ZN133_HUMAN** | 20 | 73341 | 6 | Zinc finger protein 133 |
| **ZN229_HUMAN** | 18 | 93647 | 6 | Zinc finger protein 229 |
| **ALBU_HUMAN** | 3835 | 69321 | 5 | Serum albumin |
| **PEX1_HUMAN** | 46 | 142778 | 5 | Peroxisome biogenesis factor 1 |
| **AFF3_HUMAN** | 62 | 133394 | 5 | AF4/FMR2 family member 3 |
| **ZC3HD_HUMAN** | 38 | 196519 | 5 | Zinc finger CCCH domain-containing protein 13 |
| **MVD1_HUMAN** | 24 | 43377 | 5 | Diphosphomevalonate decarboxylase |
| **HNRPK_HUMAN** | 63 | 50944 | 4 | Heterogeneous nuclear ribonucleoprotein K |

| | | | | |
|---|---|---|---|---|
| **NMD3A_HUMAN** | 45 | 125385 | 4 | Glutamate receptor ionotropic, NMDA 3A |
| **OPLA_HUMAN** | 43 | 137371 | 4 | 5-oxoprolinase |
| **CH60_HUMAN** | 42 | 61016 | 4 | 60 kDa heat shock protein, mitochondrial |
| **FANCC_HUMAN** | 39 | 63388 | 4 | Fanconi anemia group C protein |
| **SC31B_HUMAN** | 38 | 128615 | 4 | Protein transport protein Sec31B |
| **TAF5_HUMAN** | 36 | 86776 | 4 | Transcription initiation factor TFIID subunit 5 |
| **CHMP7_HUMAN** | 36 | 50879 | 4 | Charged multivesicular body protein 7 |
| **PKHD1_HUMAN** | 30 | 446418 | 4 | Fibrocystin |
| **CC134_HUMAN** | 90 | 26544 | 3 | Coiled-coil domain-containing protein 134 |
| **PTBP1_HUMAN** | 49 | 57186 | 3 | Polypyrimidine tract-binding protein 1 |
| **ROA2_HUMAN** | 37 | 37407 | 3 | Heterogeneous nuclear ribonucleoproteins A2/B1 |
| **SHOC2_HUMAN** | 35 | 64848 | 3 | Leucine-rich repeat protein SHOC-2 |
| **INT13_HUMAN** | 23 | 80174 | 3 | Integrator complex subunit 13 |
| **K2012_HUMAN** | 20 | 135223 | 3 | Uncharacterized protein KIAA2012 |
| **AT134_HUMAN** | 14 | 133900 | 3 | Probable cation-transporting ATPase 13A4 |

Table 5.5: Membrane proteins which are sulphenated after 24h in unactivated $CD4^+$ T cells.

| ACCESSION | SCORE | MASS | NUM. OF SEQUENCES | DESCRIPTION |
|---|---|---|---|---|
| **ZN229_HUMAN** | 18 | 93647 | 12 | Zinc finger protein 229 |
| **RP1L1_HUMAN** | 31 | 261049 | 11 | Retinitis pigmentosa 1-like 1 protein |
| **ZC3HD_HUMAN** | 33 | 196519 | 10 | Zinc finger CCCH domain-containing protein 13 |
| **ALBU_HUMAN** | 843 | 69321 | 9 | Serum albumin |
| **COR1A_HUMAN** | 39 | 50994 | 8 | Coronin-1A |
| **SMC6_HUMAN** | 21 | 126246 | 8 | Structural maintenance of chromosomes protein 6 |
| **HNRPU_HUMAN** | 87 | 90528 | 7 | Heterogeneous nuclear ribonucleoprotein U |
| **INT13_HUMAN** | 23 | 80174 | 7 | Integrator complex subunit 13 |
| **FLNC_HUMAN** | 21 | 290841 | 7 | Filamin-C |
| **NPAT_HUMAN** | 21 | 154195 | 7 | Protein NPAT |
| **KIF27_HUMAN** | 22 | 160184 | 6 | Kinesin-like protein KIF27 |
| **COXM1_HUMAN** | 16 | 12481 | 6 | COX assembly mitochondrial protein homolog |
| **IQCAL_HUMAN** | 105 | 94871 | 5 | IQ and AAA domain-containing protein 1-like |
| **ACTG_HUMAN** | 98 | 41766 | 5 | Actin, cytoplasmic 2 |
| **AFF3_HUMAN** | 34 | 133394 | 5 | AF4/FMR2 family member 3 |
| **POLK_HUMAN** | 19 | 98746 | 5 | DNA polymerase kappa |
| **DDX55_HUMAN** | 36 | 68503 | 4 | ATP-dependent RNA helicase DDX55 |
| **CC134_HUMAN** | 35 | 26544 | 4 | Coiled-coil domain-containing protein 134 |
| **FA30A_HUMAN** | 25 | 14616 | 4 | Putative uncharacterized protein FAM30A |
| **MEG10_HUMAN** | 25 | 122121 | 4 | Multiple epidermal growth factor-like domains protein 10 |
| **ADAM9_HUMAN** | 23 | 90497 | 4 | Disintegrin and metalloproteinase domain-containing protein 9 |
| **GRB10_HUMAN** | 21 | 67189 | 4 | Growth factor receptor-bound protein 10 |
| **MAST1_HUMAN** | 19 | 170572 | 4 | Microtubule-associated serine/threonine-protein kinase 1 |
| **SC31B_HUMAN** | 39 | 128615 | 3 | Protein transport protein Sec31B |
| **GIMA7_HUMAN** | 27 | 34487 | 3 | GTPase IMAP family member 7 |
| **SHSA7_HUMAN** | 24 | 56179 | 3 | Protein shisa-7 |
| **TRI29_HUMAN** | 22 | 65793 | 3 | Tripartite motif-containing protein 29 |
| **RL23_HUMAN** | 21 | 14856 | 3 | 60S ribosomal protein L23 |

| | | | | |
|---|---|---|---|---|
| **RUBIC_HUMAN** | 19 | 108553 | 3 | Run domain Beclin-1-interacting and cysteine-rich domain-containing protein |
| **CCD80_HUMAN** | 18 | 108106 | 3 | Coiled-coil domain-containing protein 80 |

Table 5.6: Membrane proteins which are sulphenated after 24h in activated $CD4^+$ T cells.

| ACCESSION | SCORE | MASS | NUM. OF SEQUENCES | DESCRIPTION |
|---|---|---|---|---|
| **LRP1B_HUMAN** | 15 | 515159 | 21 | Low-density lipoprotein receptor-related protein 1B |
| **RBP2_HUMAN** | 35 | 357974 | 14 | E3 SUMO-protein ligase RanBP2 |
| **UACA_HUMAN** | 38 | 162404 | 12 | Uveal autoantigen with coiled-coil domains and ankyrin repeats |
| **VWF_HUMAN** | 28 | 309058 | 11 | von Willebrand factor |
| **ACTB_HUMAN** | 3148 | 41710 | 10 | Actin, cytoplasmic 1 |
| **CH60_HUMAN** | 37 | 61016 | 10 | 60 kDa heat shock protein, mitochondrial |
| **PDS5B_HUMAN** | 35 | 164563 | 10 | Sister chromatid cohesion protein PDS5 homolog B |
| **TCF20_HUMAN** | 21 | 211641 | 10 | Transcription factor 20 |
| **ZC3HD_HUMAN** | 44 | 196519 | 9 | Zinc finger CCCH domain-containing protein 13 |
| **M3K4_HUMAN** | 29 | 181570 | 9 | Mitogen-activated protein kinase kinase kinase 4 |
| **ALBU_HUMAN** | 6454 | 69321 | 8 | Serum albumin |
| **INADL_HUMAN** | 48 | 196247 | 8 | InaD-like protein |
| **DEN4B_HUMAN** | 28 | 163743 | 8 | DENN domain-containing protein 4B |
| **DHX57_HUMAN** | 20 | 155507 | 8 | Putative ATP-dependent RNA helicase |
| **TANC1_HUMAN** | 50 | 202093 | 7 | Protein TANC1 |
| **HNRPC_HUMAN** | 52 | 33650 | 7 | Heterogeneous nuclear ribonucleoproteins C1/C2 |
| **TERA_HUMAN** | 38 | 89266 | 7 | Transitional endoplasmic reticulum ATPase |
| **SLN11_HUMAN** | 34 | 102770 | 7 | Schlafen family member 11 |
| **OPLA_HUMAN** | 34 | 137371 | 7 | 5-oxoprolinase |
| **ISK5_HUMAN** | 32 | 120637 | 7 | Serine protease inhibitor Kazal-type 5 |
| **VWA3A_HUMAN** | 28 | 133935 | 7 | von Willebrand factor A domain-containing protein 3A |
| **Z518B_HUMAN** | 23 | 119456 | 7 | Zinc finger protein 518B |
| **NPAT_HUMAN** | 16 | 154195 | 7 | Protein NPAT |
| **IQCA1_HUMAN** | 479 | 95281 | 6 | IQ and AAA domain-containing protein 1 |
| **AFF3_HUMAN** | 98 | 133394 | 6 | AF4/FMR2 family member 3 |
| **GRP78_HUMAN** | 272 | 72288 | 5 | 78 kDa glucose-regulated protein |
| **ENPL_HUMAN** | 54 | 92411 | 5 | Endoplasmin |
| **ASTN1_HUMAN** | 26 | 144820 | 5 | Astrotactin-1 |
| **FA30A_HUMAN** | 24 | 14616 | 5 | Putative uncharacterized protein FAM30A |
| **PEX1_HUMAN** | 43 | 142778 | 4 | Peroxisome biogenesis factor 1 |
| **RAE1_HUMAN** | 64 | 73429 | 4 | Rab proteins geranylgeranyltransferase component A 1 |
| **OPTN_HUMAN** | 38 | 65880 | 4 | Optineurin |
| **NMD3A_HUMAN** | 37 | 125385 | 4 | Glutamate receptor ionotropic, NMDA 3A |
| **ROA2_HUMAN** | 26 | 37407 | 4 | Heterogeneous nuclear ribonucleoproteins A2/B1 |
| **ATPB_HUMAN** | 24 | 56525 | 4 | ATP synthase subunit beta, mitochondrial |
| **LSG1_HUMAN** | 19 | 75178 | 4 | Large subunit GTPase 1 homolog |
| **ASPC1_HUMAN** | 18 | 60146 | 4 | Tether containing UBX domain for GLUT4 |
| **CP071_HUMAN** | 15 | 55647 | 4 | Uncharacterized protein C16orf71 |
| **ANT3_HUMAN** | 105 | 52569 | 3 | Antithrombin-III |

| | | | | |
|---|---|---|---|---|
| **FKBP8_HUMAN** | 99 | 44534 | 3 | Peptidyl-prolyl cis-trans isomerase FKBP8 |
| **ATPA_HUMAN** | 36 | 59714 | 3 | ATP synthase subunit alpha, mitochondrial |
| **RGPA2_HUMAN** | 27 | 210636 | 3 | Ral GTPase-activating protein subunit alpha-2 |
| **SLN14_HUMAN** | 25 | 103840 | 3 | Protein SLFN14 |
| **L1CAM_HUMAN** | 25 | 139915 | 3 | Neural cell adhesion molecule L1 |
| **AGRD2_HUMAN** | 20 | 104021 | 3 | Adhesion G-protein coupled receptor D2 |

Table 5.7: Membrane proteins which are sulphenated after 24h in unactivated $CD4^+$ T cells in the presence of $H_2O_2$ (20µM)

| ACCESSION | SCORE | MASS | NUM. OF SEQUENCES | DESCRIPTION |
|---|---|---|---|---|
| **FSIP2_HUMAN** | 31 | 780119 | 20 | Fibrous sheath-interacting protein 2 |
| **UACA_HUMAN** | 29 | 162404 | 12 | Uveal autoantigen with coiled-coil domains and ankyrin repeats |
| **APC_HUMAN** | 24 | 311455 | 12 | Adenomatous polyposis coli protein |
| **PKHD1_HUMAN** | 47 | 446418 | 10 | Fibrocystin |
| **CH60_HUMAN** | 259 | 61016 | 9 | 60 kDa heat shock protein, mitochondrial |
| **ALBU_HUMAN** | 6341 | 69321 | 8 | Serum albumin |
| **ACTB_HUMAN** | 3091 | 41710 | 7 | Actin, cytoplasmic 1 |
| **M3K4_HUMAN** | 27 | 181570 | 7 | Mitogen-activated protein kinase kinase kinase 4 |
| **ZC3HD_HUMAN** | 51 | 196519 | 6 | Zinc finger CCCH domain-containing protein 13 |
| **K2C75_HUMAN** | 26 | 59524 | 6 | Keratin, type II cytoskeletal 75 |
| **TERA_HUMAN** | 31 | 89266 | 5 | Transitional endoplasmic reticulum ATPase |
| **PEX1_HUMAN** | 141 | 142778 | 4 | Peroxisome biogenesis factor 1 |
| **HNRPC_HUMAN** | 202 | 33650 | 4 | Heterogeneous nuclear ribonucleoproteins C1/C2 |
| **FANCC_HUMAN** | 39 | 63388 | 4 | Fanconi anemia group C protein |
| **CBX4_HUMAN** | 28 | 61329 | 4 | E3 SUMO-protein ligase CBX4 |
| **DYH12_HUMAN** | 27 | 356713 | 4 | Dynein heavy chain 12, axonemal |
| **CDK12_HUMAN** | 17 | 164054 | 4 | Cyclin-dependent kinase 12 |
| **IQCAL_HUMAN** | 495 | 94871 | 3 | IQ and AAA domain-containing protein 1-like |
| **AFF3_HUMAN** | 86 | 133394 | 3 | AF4/FMR2 family member 3 |
| **RAE1_HUMAN** | 50 | 73429 | 3 | Rab proteins geranylgeranyltransferase component A 1 |
| **FA30A_HUMAN** | 34 | 14616 | 3 | Putative uncharacterized protein FAM30A |
| **AT1A4_HUMAN** | 34 | 114093 | 3 | Sodium/potassium-transporting ATPase subunit alpha-4 |
| **RHG39_HUMAN** | 32 | 121210 | 3 | Rho GTPase-activating protein 39 |
| **ACOXL_HUMAN** | 31 | 61756 | 3 | Acyl-coenzyme A oxidase-like protein |
| **SSX6_HUMAN** | 28 | 21675 | 3 | Putative protein SSX6 |
| **HERC2_HUMAN** | 20 | 526895 | 3 | E3 ubiquitin-protein ligase HERC2 |
| **CCNF_HUMAN** | 14 | 87584 | 3 | Cyclin-F |

Table 5.8: Membrane proteins which are sulphenated after 24h in activated $CD4^+$ T cells in the presence of $H_2O_2$ (20µM).

The list of proteins were further characterised based on the molecular function within cells as shown in Figure 5.9. The “number of genes” identified refers to the number of proteins identified as being sulphenated. An observable increase in the number of proteins identified

by the DCP-bio1 probe when the T cells were activated after 30min shows that activation leads to increase in sulphenic acid modification (Figure 5.9a and b). However, activation for 24h shows a decrease in the number of sulphenated proteins identified suggesting that it is reversible and occurs during early TCR signalling. $H_2O_2$ causes an increase in sulphenated proteins (Figure 5.9c and d) while activation in the presence of $H_2O_2$ shows a decrease in sulphenic acid containing proteins (Figure 5.9g and h).

Two proteins were specifically found in a sulphenated form only when cells were activated and at both time points; these were APOL2 and WNT2, and sulphenation was not detected after activation in the presence of hydrogen peroxide.

Conversely, NMDA3 was sulphenated at 30mins irrespective of treatment, but at 24 hours was sulphenated in non-activated cells but was not detected as sulphenated in activated cells.

A group of proteins was always detected as sulphenated, irrespective of treatment. These included serum albumin, actin, Heterogeneous nuclear ribonucleoproteins C1/C2, IQ and AAA domain-containing protein 1-like and Zinc finger CCCH domain-containing protein 13 shown below.

**ACTB_HUMAN**
**ALBU_HUMAN**
**HNRPC_HUMAN**
**IQCA1_HUMAN**
**ZC3HD_HUMAN**

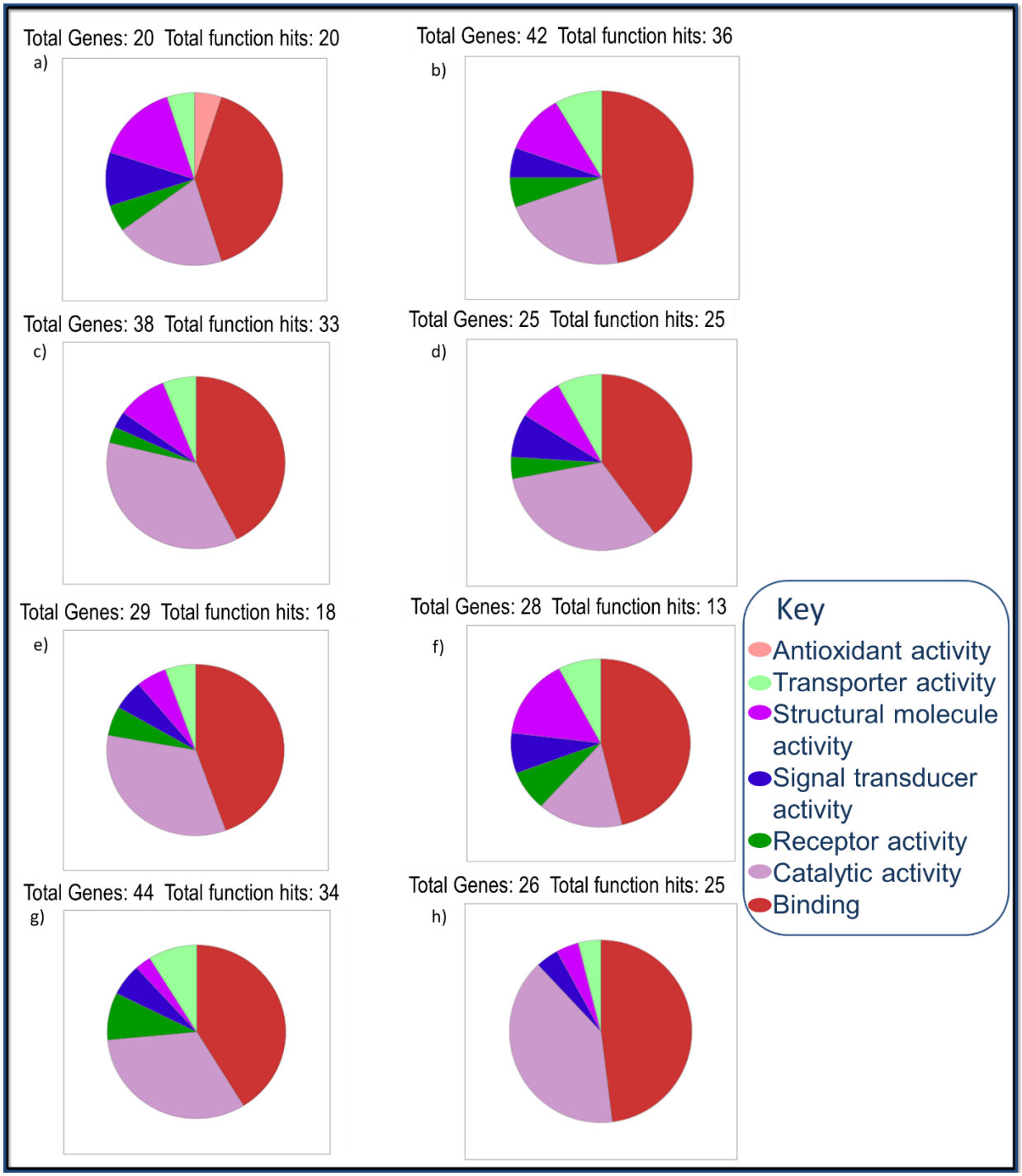



Figure 5.9: Summary of the identified proteins grouped based on their molecular function. $CD4^+$ T cells were activated in the presence or absence of $H_2O_2$. The cells were probed with DCP-Bio1 to capture sulphenated proteins during activation, and membrane were isolated and biotinylated proteins separated using SDS-PAGE. The gel lanes were cut and digested using trypsin and digested peptides were analysed by LC-MS/MS. a) Unactivated cells at 30min, b) Activated cells at 30min, c) Unactivated cells + $H_2O_2$ at 30min, d) Activated cells + $H_2O_2$ at 30min, e) Unactivated cells at 24h, f) Activated cells at 24h, g) Unactivated cells + $H_2O_2$ at 24h and h) Activated cells + $H_2O_2$ at 24h. Proteins identified by Mascot daemon database and grouped based on their molecular function by PANTHER gene ontology database (http://pantherdb.org/).

## 5.4. Discussion

This chapter has described membrane sulphenic acid modified proteins in $CD4^+$ T cells during activation for the first time and validated the effect of modulating ROS during activation as shown by others. The findings of this chapter also show the distribution of Trx1 and Prx2 on the surface during T cell activation.

Under normal physiological conditions T cells require a reducing environment for efficient T cell proliferation and activation which leads to increased cytokine production and further proliferation.

The interaction of the TCR and MHC II leads to the release of extracellular ROS including $H_2O_2$ by APC and T cells, suggesting that APC modulate T cell proliferation by regulating the availability of thiols including cysteine and thioredoxin (Hadzic et al. 2005). Data in this study shows a significant increase in surface Trx1 after activation which may play a role in reducing the thiols on the surface of T cells and APCs. As regards to the role it plays on the surface, some studies indicate that the Trx1 present on the surface of T cells may reduce certain receptors such as CD30 and CD4, which are crucial in T cell activation as T cell activation requires a reduced environment (Angelini et al. 2002; Moolla et al. 2016a; Schwertassek et al. 2007a). Furthermore, proteins and receptors on the surface are susceptible to oxidation which may lead to impairment of T cell activation, thus the –SH groups on the surface are maintained in a reduced state (Gelderman et al. 2006). The results obtained show a significant increase in surface thiols after activation explaining the alteration in redox state within the cell. The increase in surface thiols may directly enhances T cell activation and proliferation *in vitro* and *in vivo* as shown by Gelderman et al 2006. Mitogenic stimulation of T cells leads to increase in surface thiol levels (Lawrence et al. 1996).

Trx1 may modulate –SH of membrane target proteins to transduce its signal during cell-cell interaction. It is not clear yet of how Trx1 is translocated onto the membrane or its anchorage. The increase in surface Trx1 and Prx2 during activation may suggest that Trx1 may initiate

signal transduction by oxidising or reducing thiols of one or more membrane proteins or receptors and Prx2 acts as a reducing partner. In the Prx-Trx signalling pathway; $H_2O_2$ would oxidise the Prx and then transfer oxidation to Trx1 and then followed by transfer to a signalling protein (Netto & Antunes 2016). For example, a known interaction protein of Trx1 is CD4- Trx1 is responsible for regulating the activity of CD4 by dimerization. This step is known to be critical for efficient TCR-MHC II CD4 function (Cerutti et al. 2014; Moolla et al. 2016a). Additionally, migration inhibitory factor (MIF) binds to Trx1 to form a complex through disulphide bond formation and the internalisation and signalling of MIF in ATL-2 cells is enabled by Trx1 (Son et al. 2009). Other techniques have allowed detection of target molecules on the surface of lymphocytes for Trx1 such as tumour necrosis factor receptor superfamily member CD30 (TNFRSF8). The Trx1 mediated thiol disulphide exchange between Trx1 and CD30 facilitates CD30 effector functions in conditions of inflammation and infections, as CD30 is involved in the regulation of inflammation (Schwertassek et al. 2007b). The importance of membrane bound Trx1 may be the initiation of intracellular signal transduction mediated by surface Trx1. Cys35 active site can be replaced by serine which allows binding of membrane target molecules potentially entering the cells (Kondo et al. 2007a; Hara et al. 2007). This overall suggests the role of extracellular Trx1 in the interaction of membrane receptors including Trx1 which may form dimers (Nakamura et al. 2009). Hence, it would be interesting to identify the interaction partners of Trx1 on the membrane in the future.

Lymphocytes including T cells may secrete Trx1 which may act as an extracellular antioxidant or as a cytokine (Kondo et al. 2004; Rubartelli et al. 1992; Mougiakakos et al. 2010b; Sonia Salzano et al. 2014a; Riccardo Bertini et al. 1999). Once it is secreted, it can stimulate the effect of IL-2 promoting growth and survival (Gasdaska et al. 1995; Tanudji et al. 2003). In support of this, recombinant Trx1 has been shown to enhance the IL-2 cytokine effect (Léveillard & Aït-Ali 2017). Results in this chapter showed an increased trend in Trx1 secretion after T cell activation which may explain the association of Trx1 with proliferation and

activation. This also supports work done by Mougiakakos et al, whereby T cells showed increased secretion of Trx1 and blocking Trx1 decreased the –SH and resulted in $H_2O_2$ induced cell death. They suggested that the increased production of Trx1 in Treg may protect the cells from enhanced oxidative stress as seen in inflammatory conditions and hence Trx1 may exert effect in addition to antioxidative functions (Mougiakakos et al. 2010a). Moreover, Trx1 can act as a chemoattractant for monocytes and T cells (R Bertini et al. 1999).

Besides Trx1, Prx2 has been shown to be released from macrophages and modifies cell surface receptors stimulating inflammatory responses (Sonia Salzano et al. 2014a; Mullen et al. 2015b), although macrophages may act differently to stimulation compared to T cells.

$H_2O_2$ was used for further manipulating the amount of ROS during T cell activation. Physiologically, it remains unclear whether the interaction between $H_2O_2$ and T cells leads to activation or inhibition and the effect may be concentration and time dependent as low levels of $H_2O_2$ may be beneficial for the immune response. The estimated concentration of $H_2O_2$ in the microenvironment at the site of inflammation can reach up to 10-100µM (Cemerski et al. 2002). Also, a unique feature of $H_2O_2$ among other ROS is that it can freely diffuse through the membrane hence can target intracellular signalling as well as extracellular surface receptors (Holmdahl et al. 2016)

Here, $CD4^+$ T cell activation in the presence of $H_2O_2$ decreased IL-2 secretion and CD25 expression on the surface. This supports previous work showing that lower concentration of $H_2O_2$ abrogates T cell function which is not due to apoptosis (R Kiessling Aleksandra Mandic Havelka et al. 2005; Rodica Lenkei et al. 2001). This may affect further proliferation of T cells, as a decrease in IL-2 production is directly related to reduced proliferation (Hadzic et al. 2005). Also, $H_2O_2$ does not affect the distribution of Trx1 or Prx2 to the membrane. $H_2O_2$ is inhibiting T cell activation while Trx1 and Prx2 are present on the membrane. However, the presence of Trx1 and Prx2 on the membrane is prone to oxidation so it is not clear whether they are present in oxidised or reduced forms during activation or in the presence of $H_2O_2$.

Surface expression of CD25 along with IL-2 secretion was markedly decreased by $H_2O_2$. Others have shown that addition of exogenous IL-2 rescued T cell proliferation under thiol limiting conditions. This suggests that the role low molecular weight thiols is important in IL-2 signalling and is sensitive to alteration in redox state (Hadzic et al. 2005). Hence, the significant role ROS may play including $H_2O_2$ mainly depends on the concentration, the duration of any optimum concentration and superoxide production and the target of action as in which particular receptor or signalling pathway it may affect (A. V Belikov et al. 2015)

Further work here has examined the effect of NAC in $H_2O_2$ treated $CD4^+$ T cells. The presence of NAC does not reverse the effect of $H_2O_2$ in terms of CD25 expression. This suggests that increased amount of ROS in form of $H_2O_2$ may possibly inhibit T cell activation by oxidising proteins and receptors and NAC is a poor extracellular agent for mitigating the effect of $H_2O_2$, possibly because NAC does not fully reduce the thiols affected by the presence of high $H_2O_2$ concentration. Also, NAC is possibly regulating the intracellular redox signalling elevating intracellular cysteine levels suggesting that these compartments behave differently. Further work would be to observe the effects of NAC and $H_2O_2$ on surface thiols whether it is efficient in restoring surface thiols and that NAC is not metabolised. This will clarify whether the oxidation of surface thiols is leading to inefficient T cell response. Intracellular GSH was measured after 24h of activation in the presence of $H_2O_2$ and had not been restored suggesting a sustained intracellular oxidation process that was essential for signalling. This contrasts with previous work from our lab which showed that after depletion in T cells, GSH was restored within 5 hours (Grant & Griffiths 2007).

Others have shown that $CD4^+$ T cells that were activated in the presence of NAC had a significant increase in IL-2 secretion after activation with anti-CD3 and CD28 (Eylar et al. 1995) but this was not confirmed here. NAC is used as a generic antioxidant and an indicator of $H_2O_2$ involvement thus protecting proteins against oxidative damage and inactivation (Zafarullah et al. 2003). NAC may promote T cell proliferation and IL-2 secretion and possibly by

synthesising GSH which is required for DNA synthesis (Levring et al. 2015; Zhang et al. 2011). However, some effects of NAC seem not to be mediated through GSH synthesis as suggested by some experiments involving NAC or GSH inhibitors (Jiang et al. 1999; Yan et al. 1995; Laragione et al. 2003). The lack of NAC effect on thiols, GSH and T cell activation here needs further investigation.

The mechanism of how increase in ROS inhibits T cell activation is unclear but evidence suggests that higher amounts of ROS may lead to increased oxidation of certain receptors and proteins crucial for downstream intracellular signalling pathways (Phillips et al. 2010). For example, anti-CD3 stimulation in the presence of $H_2O_2$ impaired the phosphorylation of Zap-70 (Chakravarti & Abraham 2002). This further leads to the hypothesis that increased oxidative stress may lead to T cell hypo-responsiveness by further oxidation of specific intracellular proteins.

Calcium-signalling pathways may also be thiol sensitive. This hypothesis is supported by studies carried out showing that heat shock induced oxidative stress down regulated CD3 mediated $Ca^{2+}$ signalling and that NAC reversed the observed effect (Nambiar et al. 2002). Also, Bogeski et al, evaluated the effects of $H_2O_2$ and how it may affect T cell activation and proliferation. One way $H_2O_2$ in which may affect T cell differentiation is by oxidising calcium release activated channels (CRAC). The plasma membrane protein ORAI1 was found to be inhibited in the presence of $H_2O_2$ due to oxidation (Bogeski et al. 2010). Further measure of $Ca^{2+}$ in the presence of $H_2O_2$ would provide more insight on the effects of $H_2O_2$ on T cell activation with respect to $Ca^{2+}$ signalling.

This study shows an increase in thiol oxidation and sulphenic acid modification after 5min of activation. Data in this chapter has demonstrated antigen stimulation, mimicked by anti-CD3 and anti-CD28 activation, may lead to an increase in sulphenic acid formation and may cause additional proteins to be oxidised. However, what is not clear form the methodology here is whether the concentration and composition of proteins on the membrane also increases during

activation, as might be expected. Protein sulphenic acids as well known, are important and considered as a molecular switch which regulates protein function (Denu & Tanner 1998; Gupta & Carroll 2014). The mass spectrometry technique applied here has previously identified several proteins within the cytosol and cell membrane which are involved in inflammation via this molecular redox switch such as actin, integrins cell adhesion molecules and kinases (Jones & Go 2010; Go et al. 2010).

An increase T cell intracellular ROS, including $O_2^{\bullet-}$ and $H_2O_2$, has been described immediately after TCR stimulation (Devadas et al. 2002; Belikov et al. 2014; Sharon H. Jackson et al. 2004), which can react with intracellular cysteine residue to form sulphenic acid (Kettenhofen & Wood 2010). This contrasts with the extracellular reduction in surface thiols that has been reported in proliferating T cells. Hence, the effect of excess $H_2O_2$ during T cell activation on the formation of membrane sulphenic acid formation was investigated and potential proteins identified. Activated $CD4^+$ T cells show an increase in numbers of sulphenic acid modification on the membrane proteins after 5min of activation compared to unactivated T cells. It was critical to find out what proteins may be susceptible to oxidation on the membrane during T cell activation. This was achieved by prior incubation of dimedone which allows capturing of CysSOH species as they are formed following TCR stimulation. The timing of ROS induced oxidative modification is critical and hence the dimedone was incubated immediately prior to activation. Dimedone incubation traps oxidised cysteine by forming a covalent bond to CysSOH. However, it is important to note that any subsequent functional studies would be limited because this may alter the activity and or function of the protein leading to non-physiological increase in ROS (Furdui & Poole 2014; Michalek et al. 2007) . However, this issue can only be questioned when the DCP-Bio1 concentration is higher than 1mM for a longer period of time whereas this study used 0.5mM for 30min.

In the presence of dimedone during T cell activation, proteins which undergo sulphenic acid formation will form a stable bond with DCP-Bio1. Membrane proteins were isolated and

sulphenated proteins were pulled out of total membrane proteins pool using streptavidin magnetic beads. The modified proteins were separated using SDS-PAGE and each lane within the gel with different treatments were cut and digested with trypsin. Subsequent experiments by LC-MS/MS analysis and protein identification using Mascot Daemon database revealed a list of protein hits undergoing sulphenic acid modification during T cell activation under oxidative stress conditions.

The number of membrane sulphenic acid modified proteins increases when the T cells are activated after 30min. This further supports the hypothesis that activation leads to increase in sulphenic acid formation, possibly due to local ROS production. Also, unactivated cells in the presence of $H_2O_2$ (20μM) yield a similar number of sulphenated membrane proteins as activated at 30min. However, when the cells were activated in the presence of $H_2O_2$ at 30min, a decrease in sulphenic acid proteins was observed. This may suggest irreversible protein oxidation or intra or inter disulphide bond formation which is unreactive to DCP-Bio1. Additionally, the number of proteins identified to be sulphenated may be similar but the results show different proteins are oxidised under different treatment conditions. Major target of sulphenated proteins are catalytic proteins which suggest their role in intracellular signalling (Heppner et al. 2017). Also, activation may also change the total number of proteins oxidised as observed in the molecular function grouping of proteins.

Interesting sulphenated proteins were identified on the membrane of $CD4^+$ T cells at baseline i.e. untreated. Peroxiredoxin 1 was only identified to be sulphenated in unactivated $CD4^+$ T cells after 30mins. It is widely known to undergo protein modification as its main role is reduction of peroxides such as $H_2O_2$ (Hall et al. 2009). This is through the formation of sulphenic acid followed by disulphide bond formation with the resolving cysteine. Prx1 can form dimers and decamers at physiological pH (Morais et al. 2015).

PDI on the surface of cells are responsible for thiol-disulphide interchanges resulting in the formation of protein disulphide bonds (Jiang et al. 1999). Although PDI is predominantly associated in the cytosol and Golgi, it is also shown to be secreted and associated with the

plasma membrane. This chapter identified PDIA3 undergoing sulphenic acid modification on the membrane after 30minutes only under resting and activated conditions. This suggests that PDI is susceptible to early sulphenic acid at normal physiological conditions. Peroxynitrite has been shown to oxidise the redox active Cys residue to sulphenic acid which further reacts to form disulphide with adjacent Cys residues (Peixoto et al. 2018).

The importance of actin reorganisation following TCR stimulation is well defined during T cell activation and migration of T cells (Burkhardt et al. 2008). Actin was identified as a sulphenated protein under all conditions. It is closely associated with and anchored to the membrane in order to mediate cell migration and formation of the immune synapse, hence it is not unexpected to find actin within the membrane proteins that were isolated. Also, components which make up the actin cytoskeleton is shown to be sensitive to oxidation (Fratelli et al. 2002a; Klemke et al. 2008). Coronin is described as actin binding factor which is involved in protein-protein interactions, phagocytosis, cell migration and cytokinesis. Coronin in mouse T cells has been shown to be associated with the plasma membrane and play a role in TCR stimulation and T cell activation. It recruits and promotes downstream signalling pathways following TCR-CD3 stimulation (Nal et al. 2004). It is not clear whether coronin is undergoing oxidation during activation, but this chapter has identified coronin on the membrane undergoing –SOH after 30 and 24h of activation. However, coronin was also identified in unactivated cells at 30min further suggesting that it may undergo sulphenic acid modification at normal physiological conditions. Also, the treatment with $H_2O_2$ did not show coronin. Thus it is not clear whether activation leads to oxidation of coronin. On the other hand, coronin was shown to undergo posttranslational modification mediated by cyclin dependent kinase 5 (CDk5) inducing TCR mediated actin polarisation (Pareek et al. 2010)

Additionally, cofilin is a key component of actin cytoskeleton regulating the formation of new filaments (Samstag et al. 2013). The oxidation state of cofilin is important in the migration and activation of T cells (Hoffmann & Griffiths 2018). The function of cofilin is regulated by the posttranslational modification of S3. Dephosphorylation of this residue promotes actin

remodelling upon TCR stimulation via Ras and PI3K signalling. Interestingly, cofilin was shown to be sulphenated in unactivated T cells; untreated and when treated with $H_2O_2$. This is an interesting finding as cofilin has four Cys residues that can possibly be oxidised (Samstag et al. 2013) but two out of four were confirmed to be sensitive to oxidation, 147 and 139 forming sulphenic acid (Yang et al. 2014). Other studies have shown that two Cys are involved in the formation of disulphide bridge and C139 is sulfonylated under oxidative stress. (Klemke et al. 2008; Samstag et al. 2013). Furthermore, the biological function of cofilin can be affected by reversible cysteine oxidations. Inactivation of cofilin by ROS may lead to T cell hyporesponsiveness and cell death. These findings may possibly suggest the role of cofilin oxidation corresponding with the loss of S3 in T cell hypo-responsiveness.

Previous experiments show the importance of cysteine sulphenic acid for ERK1/2 phosphorylation, cell growth and calcium flux. This was shown by a study by Michalek et al, whereby T cells incubated with dimedone showed a decreased calcium influx. This was due to the binding of dimedone to sulphenic acid intermediate hence inhibiting further calcium signalling. Also, there is evidence which suggests that cysteine oxidation is required for calcium signalling (Redondo et al. 2004). Several cysteine residues which can undergo oxidation in PMCA, Orai1 and STIM1 (Hawkins et al. 2010; Bogeski et al. 2010; Galan et al. 2010; Lushington et al. 2005).

Here, NMDA receptor thiols were oxidised in all conditions after 30minutes and this protein plays a role in calcium signalling (Lombardi et al. 2001; Miglio et al. 2005; Affaticati et al. 2011). Further oxidation of this receptor may lead to decreased calcium flux in T cells as shown in chapter 3. While NMDA may not be a key receptor involved in calcium influx as it has low permeability to calcium, NMDA inhibitors have been shown to inhibit lectin induced T cell proliferation suggesting that it may be very important for T cell proliferation (Miglio et al. 2005). Interestingly, this NMDA3 was not sulphenated in activated cells at 24 hours and whether this is preferentially reduced during activation merits further investigation. Furthermore, the use of

gold compounds led to a decreased calcium flux that was associated with defective T cell response (Langston et al. 2015).

After 24h, no difference was observed in the number of proteins identified to be sulphenated between unactivated and activated and in the presence or absence of oxidative stress, however, the pattern of modified proteins was different. This highlights that sulphenic acid formation is transient and may be important in signalling.

The proteins sulphenated proteins were characterised according to their molecular function to further suggest which group of proteins on the membrane might be active during the activation process and hence sulphenated. Catalytic proteins were most affected.

Activated cells in the presence $H_2O_2$ at 24h show increased number of catalytic proteins sulphenated compared to activated cells at 24h. This may suggest a contribution of these proteins in the proliferation of T cells that maybe further modified in the presence of $H_2O_2$. For example Mitogen-activated protein kinase kinase (MAP3K4) was identified in activated cells treated with peroxide after 24 hours, in the regulation of T cell proliferation and activation (ZHANG & LIU 2002). M3K4 is shown to mediate IFNɣ in differentiating T cells (Chi et al. 2004). T cell proliferation leads to the translocation of cyclin dependent kinase 2 (Cdk2) associated with MAPK and IL-2 secretion (Blanchard et al. 2000). Also MAPK signalling inhibits Fas mediated apoptosis to allow further proliferation of activated T cells (Holmström et al. 2000). Using the same approach, Keyes et al, have shown the sulphenylation of ERK1/2 which prevents the phosphorylation of ERK. This in turn inhibits proliferative responses in a number of cells (Keyes et al. 2017).

The antioxidant protein Prx2 was identified as sulphenated. Prx1 and 2 were found to undergo irreversible oxidation at concentration of 20μM $H_2O_2$ in the cytosol of Jurkat T cells (Baty et al. 2005). Others have shown the loss of Prx thiols and oxidised products formed in the presence of $H_2O_2$ and Trx1 required for reducing the oxidised Prx, this controls the redox state within cells (Wood, Schröder, et al. 2003; Woo et al. 2003; Yang et al. 2002).

Some sulphenated proteins were identified that are mainly expected to present in the golgi, ER and mitochondria. This discovery may be due to the enrichment of membrane proteins rather than specifically exofacial membrane proteins. A smaller number of cytosolic proteins were also detected and may be contaminants form the method used to isolate membrane proteins. The kit uses mild detergents to solubilise membrane and membrane associated proteins. Intracellular sources of ROS, which is the mitochondria or exogenous $H_2O_2$ which penetrates through membrane may oxidise intracellular signalling proteins, partly an essential step (Holmdahl et al. 2016).

Although multiple mechanisms may be involved in controlling the signalling pathways, reversible cysteine modification allows the cells to modulate protein activity (Michalek et al. 2007; Charles et al. 2007). Intracellular proteins such as, PTPs including PTEN, cdc25C can be activated by oxidation (Holmdahl et al. 2016). In most cases, oxidation of the cysteine leads to decreased protein activity. In addition, SHP-2 protein oxidation in Jurkat T cells have been reported to occur within 5min of stimulation (Kwon et al. 2005). And the redox regulation of SHP2 modulates the downstream TCR signalling. Michalek et al. showed an increase in total cysteine sulphenic acid after activation of $CD8^+$ T cells suggesting that cysteine sulphenic acid modification plays a key important role in activation and proliferation of $CD8^+$ T cells (Michalek et al. 2007). However, this study specifically looked at membrane sulphenic acid formation. TCR stimulation is rapid and this thesis focused on early time points (5 and 30min). TCR stimulation leads to relocalisation of membrane bound receptors, hence moving the receptors to the surface in theory to initiate signalling. This supports the hypothessis that increased number of membrane proteins are susceptible to oxidation during TCR interaction in the presence of extracellular ROS.

Therefore, during T cell activation ROS can affect the rate of proliferation. This chapter has shown the proteins which may be susceptible to oxidation on the membrane when T cells are activated in the presence or absence of $H_2O_2$. This will provide the possibility of exploring whether these proteins are differentially affected in disease conditions, such as RA.

# Chapter 6. The effect of NOX2 activators on T cell function and CIA

## Preface

A reduced cell surface protein redox state is required for receptor mediated T cell stimulation. Increases in reactive oxygen species (ROS) are predicted to inhibit T cell receptor signalling and activation. The main objective of this study was to investigate the effects of specific NOX activating compounds on T cell activation and a model of RA. T cell activation in the presence of NOX activating compounds showed a significant decrease in IL-2 secretion and intracellular ROS after 24h. Also, ROX2 showed a decreased CIA severity in WT mice. Hence, NOX compounds have a profound effect on T cell activation.

## 6.1. Introduction

ROS play a major role in autoimmune disease progression including rheumatoid arthritis (RA). However, it was established that rodents with a lack of functional NADPH oxidase (a decreased ability to produce ROS), have increased susceptibility and severity of RA (Olsson et al. 2016; Holmdahl et al. 2016).

Collagen-induced arthritis is the most commonly used rodent model for RA. In this model, the major type of collagen found in cartilage, type II collagen is used to induce arthritis in combination with adjuvants (Courtenay et al. 1980; Trentham et al. 1977). The prominent features of RA are chronic inflammation and oxidative stress leading to bone erosion in the joints (Szabó-Taylor et al. 2012a). CIA is critically dependent on both T and B cells. It is a T cell mediated disease. Cells present in the joints are activated but respond poorly to TCR stimulation, this might be due to defective TCR signalling. Previous studies have implicated ROS and the redox state in T cell-driven arthritogenicity (Hultqvist et al. 2009b).

The cellular redox state may be influenced by the activity of NADPH oxidase complex. The phagocytic cell form of NADPH oxidase enzyme consists of several subunits which assemble during activation and are mainly responsible to generate ROS. NADPH oxidase has different forms and functions. The catalytic part is located in the membranes (NOX2 dimerising with p22 phox forming flavocytochrome b558 complex). Some regulatory components of NOX2 including Ncf1 (p47phox), Ncf2 (p67phox) and Ncf4 (p40phox) are cytosolic when the enzyme is said to be in resting state. Upon activation the Ncf1 is phosphorylated and undergoes a conformational change allowing translocation to the membrane and interaction with p22phox. The Ncf1 is necessary for the oxidative function and binding of the other subunits and hence is considered as an essential organising protein during immune activation. The Ncf1 gene was one of the first genes identified to regulate arthritis severity (Olofsson et al. 2007). The NOX2 complex is mainly expressed in phagocytes and is essential in generating ROS during inflammation. Several other cell types including T cells were shown to express phagocyte type NOX2 enzyme which acts as a major source of oxidants (Sharon H Jackson et al. 2004). Ncf1

via NOX2 was shown to be mainly responsible for continuous production of ROS by T cells. Ncf1 mutation in mice leads to increased serum levels of anti-CII antibodies which suggests the presence of activated autoreactive T cells when ROS production is inhibited.

Any direct role of thiol antioxidants in RA is not clear, however, several studies have suggested the presence of increased Trx1 in the synovial fluid of RA patients compared to healthy individuals and that plasma and Trx1 positively correlated with disease progression (Jikimoto et al. 2002; Staal et al. 1997). Also Trx1 negatively regulates apoptosis by binding to ASK1. This supports the idea that Trx1 may act as a proinflammatory molecule promoting cell proliferation and activation. Nevertheless the specific role of Prx2, a Trx1 interaction partner, has not been studied extensively in RA. A greater proportion of RA lymphocytes expressed surface Prx2 compared to healthy control (Szabó-Taylor et al. 2012a). Also, the previous chapter showed a significant increase in Trx1 and Prx2 on the surface of $CD4^+$ T cells after activation with anti-CD3 antibodies.

ROS, which can be detected upon TCR stimulation (Devadas et al. 2002), can either act as a promoting factor for T cell activation or in certain circumstances can suppress signalling effectors including ERK-pathway (Kwon et al. 2003), hence acting as negative regulators of activation. NOX activating compounds have been found promising and efficacious in the treatment of RA in rats, however the mechanism is still not understood (Hultqvist et al. 2006). Therefore, NOX2 activators were used in this chapter to observe the effect it might have on $CD4^+$ T cell activation followed by CIA. Modulation of the T cell function using has shown promising disease outcomes as opposed to immunosuppression (Cope et al. 2007).

Hence the aim of this chapter was to determine the importance of ROS in RA, by looking at CIA in mice with mutation in Ncf1 gene. The aims was to see whether a decreased ability to produce ROS affects the redox state of T cells and how efficacious NOX2 activators are in the routine treatment of mice immunised with CII.

The objectives of the chapter were as follows;

- To analyse the effects of NOX 2 activating compounds on the surface redox state during T cell activation
- To assess T cell functions in the presence of NOX 2 compounds (IL-2 and CD25; activation markers)
- To induce collagen induced arthritis (CIA) in WT and NOX2 deficient mice (Ncf1-/-)
- To analyse T cell responsiveness in CIA induced splenocytes in the presence and absence of NOX2 activity
- To validate the effect of a NOX2 activating compound on RA severity

## 6.2. Methods and Materials

### 6.2.1. Animals and disease induction.

Compounds ROX1 and ROX 2 used in this chapter are quinolinone derivatives described in US 2014/0018384 A1 patent. Ethical permit: N229-14 (Swedish research council given to Redoxis AB, Lund, SE).

For eliciting the collagen induced arthritis (CIA), 15 B6N.Q females (8 weeks old) and 17B6N.Q.Ncf1-/- (6 weeks old) females were used from Redoxis (Lund, Sweden) developed by in house breeding. The Ncf1 mutation in mice results into the impairment of the relocalisation of Ncf1 ($p47^{phox}$) protein when activated, hence inhibiting the function of NOX2 complex. Collagen type II (CII) (Chondrex, Inc, Redmond, USA) was dissolved in 0.1M acetic acid to a final concentration of 5 mg/ml and all of the mice were immunised except 3 from each group annotated as naïve. Collagen emulsion was injected (100µl subcutaneous) at the base of the tail and the mice were weighed and marked. Arthritis development was monitored using the macroscopic scoring system whereby all four limbs were monitored for swelling and redness. Each red or swollen knuckle, toe or midfoot digit scored as 1point and ankles which were swollen scored at 5points resulting in a maximum of 60points per mice. The mice were scored three times following day 19. At day 21 a boost emulsion was prepared in the lab and injected into the mice according the same procedure as used at immunisation. Peripheral blood was collected before termination for analysis and after the mice were terminated and spleens were collected at the end point.

### 6.2.2. Flow cytometric analysis of cell surface thiols, sulphenic acid and Trx1, Prx2 from whole blood

Peripheral blood was collected and RBC were lysed in a 96 well plate with 1:10 Pharmlyse (BD Biosciences) and incubated for 6mins. Following incubation the plate was centrifuged and the supernatant was discarded. The cells were incubated with antibodies specific to CD4-PE/FITC (BD Bioscience; 553048/553651 and CD8 (PB, Biolegend; 140414) (1:40) and

treated with 10μM maleimide and Alexa Fluor® 488 C5 maleimide (Life Technologies, Carlsbad, CA) for 20 min in the dark. Cells were resuspended with PBS and analysed for free surface thiol groups by Attune flow cytometry (Thermo Scientific, Sweden).

For sulphenic acid labelling, whole blood was collected and lysed in a 96 well plate with 1:10 Pharmlyse (BD Biosciences) and incubated for 6mins. Following incubation the plate was centrifuged and the supernatant was discarded. 0.1mM biotin-linked dimedone derivative (DCP-Bio1) Merck (Poole et al. 2007; Michalek et al. 2007) and 10mM iodoacetamide (IAA) (Sigma) were employed in order to block free thiols on ice for 30min. Following incubation, the cells were washed with PBS and streptavidin-PE was added (1:40, BD Bioscience) and antibodies specific to CD4-FITC (BD Bioscience; 553048/553651 and CD8-PB (Biolegend; 140414) (1:40) and incubated in the dark for 20min and analysed on flow cytometry.

For flow cytometric analysis of surface Trx1 and Prx2, peripheral blood was collected and lysed in a 96 well plate with 1:10 Pharmlyse (BD Biosciences) and incubated for 6mins. Following incubation, the plate was centrifuged and the supernatant was discarded. Cells were washed in cold wash buffer (PBS supplemented with 1% w/v BSA). Following the wash in cold wash buffer, cells were incubated with mouse monoclonal anti-Trx1 (ab16965; Abcam, 1:1000) or rabbit polyclonal anti-Prx2 (ab109367; Abcam, 1:1000) for 30 minutes at room temperature. After a further two washes, cells were incubated with goat anti-mouse polyclonal conjugated dylight-488 (ab96879; Abcam) antibody and antibodies specific to CD4 conjugated to PE/FITC (BD Bioscience; 553048/553651 and CD8-PB (Pacific blue) (Biolegend; 140414), anti-mouse Ly6G-PerCP (127653; Biolegend) (1:40) and incubated in the dark for 20min and analysed on flow cytometry (Attune NxT flow cytometer, Sweden)

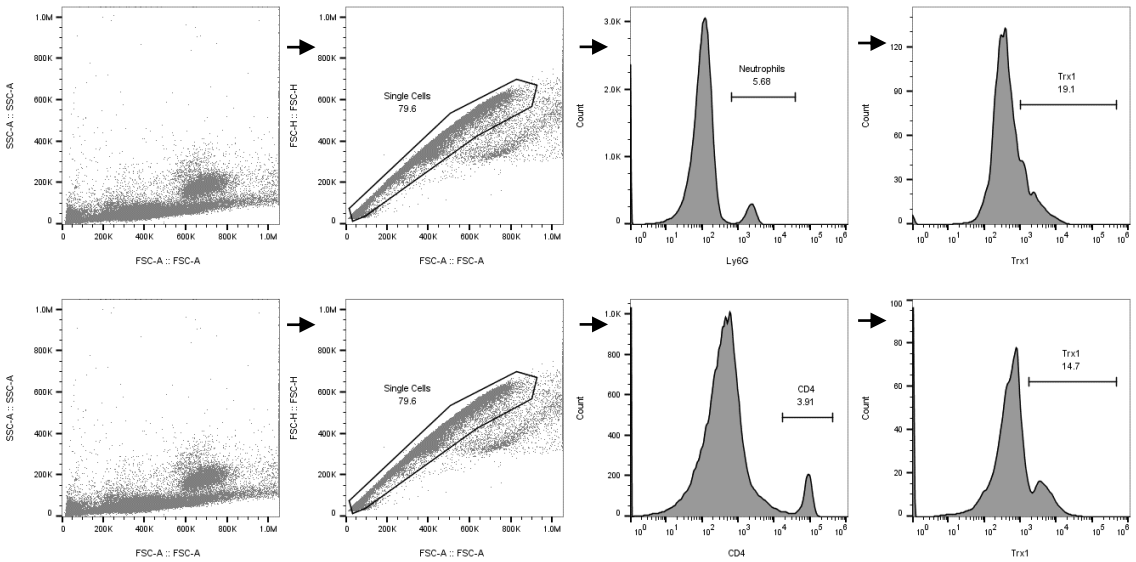



Figure 6.0: Gating strategy for T cells and neutrophils. Blood was collected and lysed with pharrmlyse. Lymphocytes were harvested, stained and analysed by flow cytometry. Gating on single, viable lymphocytes, the T cell population was identified by gating on CD4+ cells and neutrophils by gating on Ly6G+ cells. Using unstained as a negative control, the population of cells expressing Trx1/Prx2 on the surface was determined.

### 6.2.3. 2′,7′-Dichlorodihydrofluorescein diacetate (DCF-DA) oxidation by flow cytometry.

Intracellular ROS levels were measured as DCF oxidation. The non-fluorescent probe DCF-DA (Sigma Aldrich, UK) rapidly diffuses into the cytosol. In the presence of peroxide, DCF-DA oxidises to fluorescent DCF. $2x10^5$ CD4 T cells were loaded with 50µM DCF-DA 30min before the termination of the treatment. Immediately after 30min DCF-DA incubation, the cells were analysed by flow cytometry on the FL1 channel.

### 6.2.4. Extracellular ROS production measured by isoluminol

Spleens were collected from naïve mice and homogenised in 50ml of HBSS. RBC were lysed using pharmlyse and cells were further resuspended in HBSS. 25µl (12.5µM) of ROX 2 (Quinolinone derivative, patent no: US 2014/0018384 A1) was added in a 96 well plate followed by 25µl of isoluminol assay buffer (HBSS, Isoluminol (10mg/ml), HRP II (100U/ml). Luminescence was measured to eliminate the background and 50µl of cell suspension was

injected using the sample injector. ROS measurement was initiated for every min for 20minutes (Fluostar Optima, BMG Labtech).

### 6.2.5. *Ex vivo* re-stimulation assay

Spleens were collected and homogenised in 50ml of Dulbecco's Hanks balanced Salt solution (HBSS) (Gibco, Life Technologies) using 40µm cell strainers and the piston of 5ml syringe to a single cell suspension. The RBC were lysed using 1:10 Pharmlyse (BD Bioscience) for 5min and the cells were washed with HBSS. The cells were further resuspended in HBSS and cell concentration was determined by using Scepter™ cell counter (Merck, Millipore).

The cells were re-suspended in complete RPMI (10% FBS and 1% penicillin/streptomycin) at a concentration of $2x10^6$cells/ml and 100µl was plated in a 96well plated coated with or without 1µg/ml of anti-CD3ε (Biolegend; 145-2C11). Anti-CD28 (2µg/ml) (Biolegend; E18) was added to the appropriate wells along with rat CII (100µg/ml) (Chondrex) and ROX 2 (12.5µM) (Redoxis AB, Lund, Sweden) and incubated for 24h at 37°C, 5% $CO_2$ incubator.

Following stimulation, the cells were washed and supernatants were stored for IL-2 and TNF-α cytokine analysis. The cells were resuspended in PBS 1% w/v BSA for flow cytometry of surface Prx2 and Trx1 analysis.

## 6.3. Results

### 6.3.1. ROX 2 increased extracellular ROS production in WT mice splenocytes.

To investigate whether ROX 2 compound increases extracellular ROS production, splenocytes from naïve WT and Ncf1-/- mice were isolated and stimulated with ROX 2. The luminescence was recorded every minute for 20minutes. Figure 6.1 shows an increasing pattern in superoxide production in splenocytes from WT mice. In contrast, no ROS was released from splenocytes of Ncf1-/- mice after treatment with ROX 2, confirming that the compound targets functional NOX 2 to release ROS.

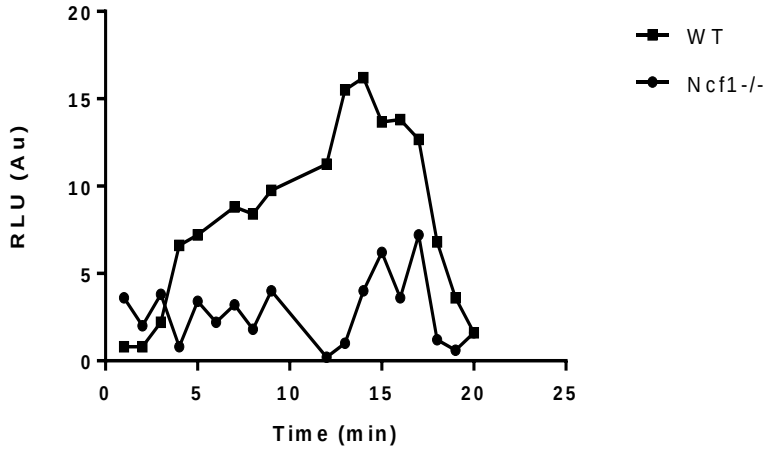



Figure 6.1: Extracellular ROS production in splenocytes from WT and Ncf1-/- mice after ROX 2 stimulation. Spleens were homogenised and single cell suspensions were prepared. RBC were lysed using BD Pharmlyse. Splenocytes were stimulated with ROX 2 in the presence of isoluminol to detect extracellular ROS production. Line plots show mean of 5 animals per group.

### 6.3.2. Activation of T cells show significant increase in surface thiols levels after 30min compared to unactivated and ROX compounds have no effect.

To understand how the surface redox state behaves after activation of $CD4^+$ T cells in the presence or absence of the NOX-2 activating compounds. T cells were activated with anti-CD3/CD28 antibodies and the cell surface reduced thiol (-SH) levels were determined by flow cytometry. Five minutes after activation T cells show no change in surface –SH levels between activated and unactivated T cells (Figure 6.2a). Additionally, ROX compounds had no effect on surface thiol levels in activate and unactivated cells. however, after 30min of activation (Figure 6.2b), a significant increase in -SH was detected on the T cell surface compared to unactivated cells and ROX compounds did not change the activation-induced surface –SH levels.

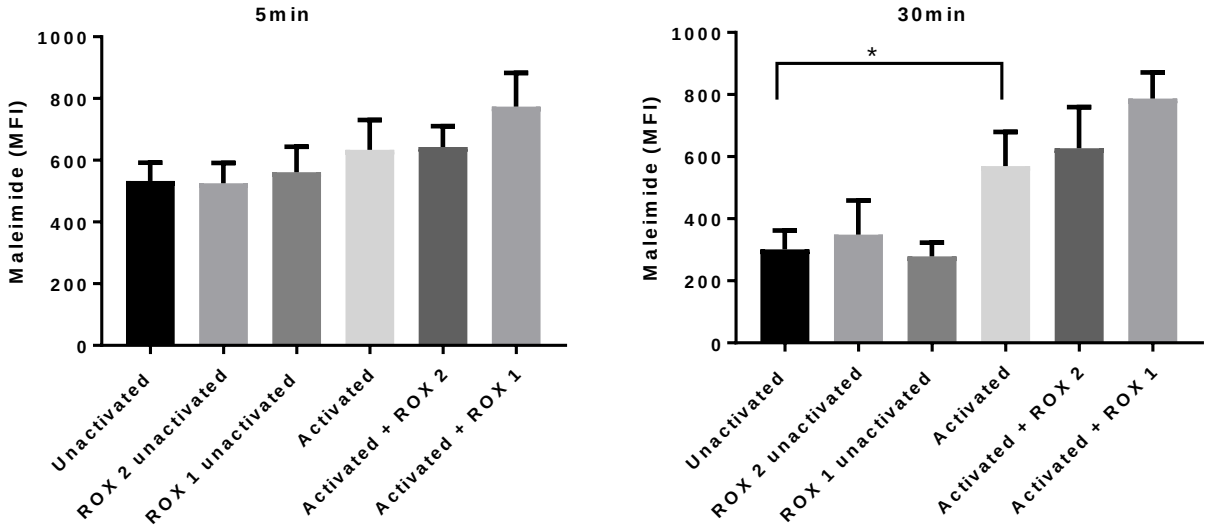



Figure 6.2: Surface thiol levels increases after T cell activation. $CD4^+$ T cells were isolated from blood and activated with 1µg/ml anti-CD3 (OKT3) and 2µg/ml anti-CD28 (CD28.2) for 24h in the presence or absence of ROX compounds; ROX 2 and ROX 1. Cells were collected after 5 and 30mins of activation and incubated with FITC-conjugated maleimide for 30min and analysed by flow cytometry. a) –SH after 5min, b) surface -SH after 30min activation. c) A schematic showing the increase in –SH after activation. Data represents three independent experiments, n=3, mean and SEM, ANOVA, Tukey's multiple comparison test *p<0.02

## 6.3.3. Activation of T cells causes an increase in sulphenic acid formation on the surface with no effect of ROX compounds.

Although ROS are a major cause of post-translational modification to macromolecules, reversible oxidation of cysteine thiols e.g. sulphenic acid (-SOH) is important for signalling proteins (Michalek et al. 2007). To understand the role of –SOH during T cell activation, this chapter looked at the surface oxidised thiols (sulphenic acid) on primary T cells after activation and in the presence of NOX-2 regulatory compounds (ROX 2 and ROX 1). Activated $CD4^+$ T cells in Figure 6.3 show an increased surface –SOH levels after 5min whereas no significant effect 30min was observed. Also, no difference in the trend was observed when the compounds were added.

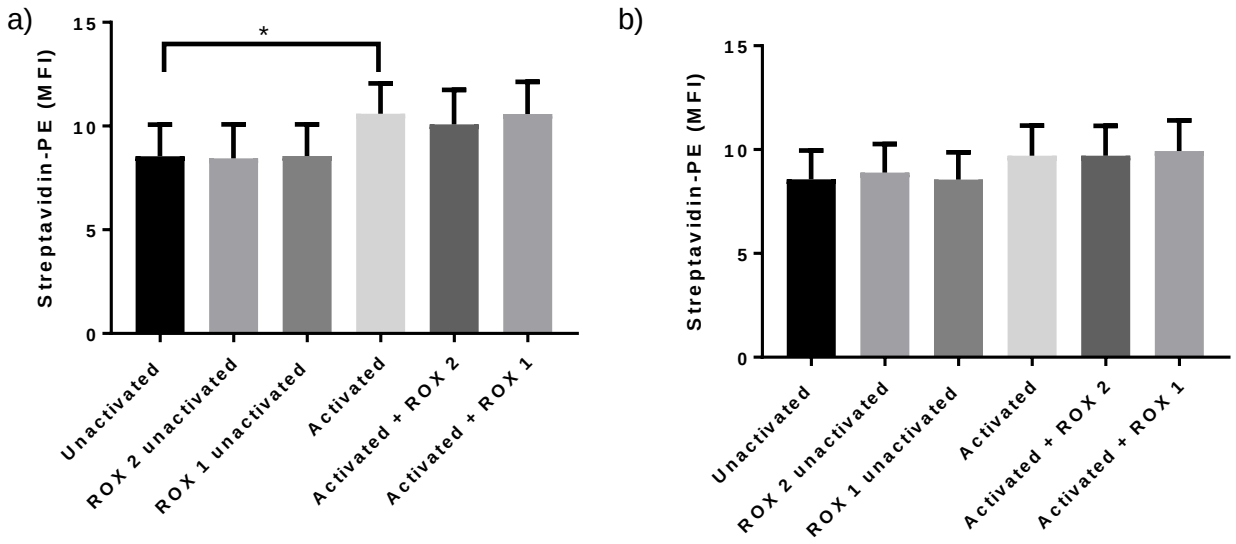



Figure 6.3: Surface -SOH increases after T cell activation. $CD4^+$ T cells were isolated from blood and activated with 1µg/ml anti-CD3 (OKT3) and 2µg/ml anti-CD28.2 for 24h in the presence or absence of compound ROX 2 and ROX 1. Cells were collected after 5 and 30mins of activation and incubated with 0.1mM DCP-Bio1 and 10mM iodoacetamide (IAA) for 30min. The cells were further washed and incubated with streptavidin-PE (1:100) for 30min on ice and analysed by flow cytometry. a) –SOH after 5min, b) surface –SOH after 30min activation. Data represents three independent experiments, n=3, mean and SEM, ANOVA, Tukey's multiple comparison test *p<0.01

### 6.3.4. Intracellular ROS is increased after 24h of activation

T cells generate ROS via at least two sources; from the mitochondrion (Laura A. Sena et al. 2013) and NOX type enzyme (Sharon H Jackson et al. 2004). In order to determine whether $CD4^+$ T cells produce peroxide during activation and whether the NOX compounds have any effect on the production of ROS in T cells, the cells were activated and intracellular ROS were measured using DCF-DA. This dye only fluoresces within the cell and upon contact with peroxides. Therefore, DCF oxidation was measured from 5 minutes after anti-CD3/CD28 activation followed by 30minutes and 24h. From figure 6.4a, an increased trend in DCF MFI (Median fluorescence intensity) was observed when T cells were treated with $H_2O_2$ after 5min. However, after 24h of activation, T cells showed a significant increase in DCF MFI fold change compared to unactivated cells suggesting increase in intracellular ROS and a similar result was observed in T cells activated in the presence of $H_2O_2$ at 20µM. ROX 2 decreased DCF MFI after 30minutes and 24h of activation. ROX 1 shows no change to DCF oxidation within early time points of activation (5 and 30minutes) but a significant decrease after 24h of activation. Moreover, $H_2O_2$ increases intracellular peroxide within 5min of incubation irrespective of activation, however, unactivated cells treated with $H_2O_2$ show decreased intracellular ROS compared to activated cells.

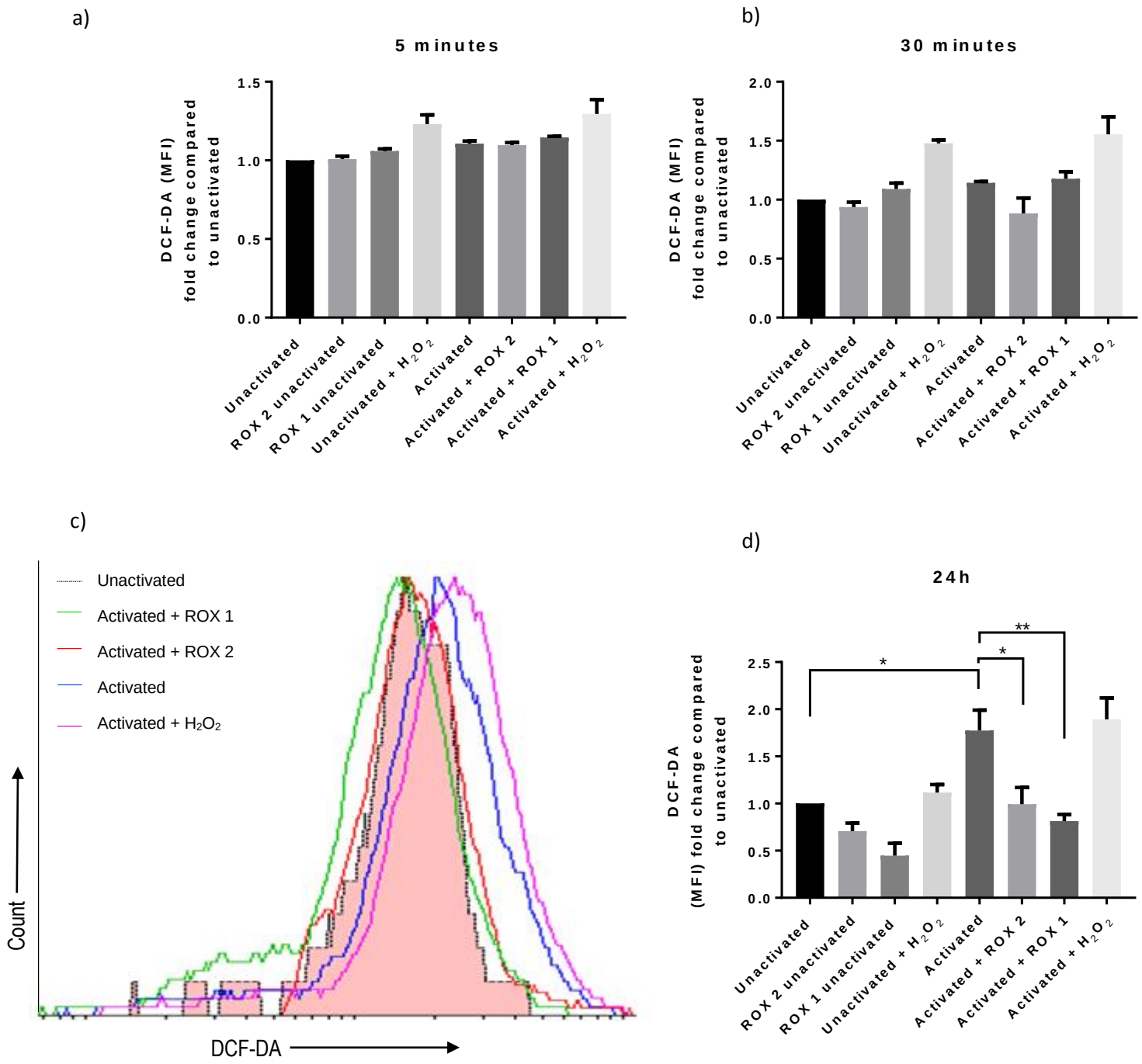



Figure 6.4. Intracellular ROS is increased 24 after T cell activation. $CD4^+$ T cells were isolated and activated with 1µl/ml anti-CD3 (OKT3) and 2µg/ml anti-CD28.2 for 24h in the presence of 12.5µM of ROX 2, ROX 1 and 20µM $H_2O_2$. . Before the end of the incubation period, cell samples were loaded with 50 µM DCFH-DA for 30min. Samples were analysed immediately for DCF fluorescence by flow cytometry and the MFI of 5000 cells was recorded. a) 5 minutes after activation, b) 30minutes after activation, c) histogram showing the Median X (pink fill-unactivated cells) & d) 24h after activation. Results are presented as the mean and SEM from three individual experiments. Ordinary one-way ANOVA, Tukey's multiple comparison test, *p<0.02, **p<0.004, n=3.

### 6.3.5. Effects of NOX-2 activating compounds on surface Trx1 and Prx2 after T cell activation.

Trx1 may have a crucial role in T cell function and proliferation (Hadzic et al. 2005). The distribution of the surface Trx1 level in activated cells was investigated in the presence or absence of ROX compounds; ROX 2 and ROX 1. Trx1 was increased on the exofacial surface during T cell activation. A significant inhibition of increased surface Trx1 levels was observed when cells were activated in the presence of ROX 1 but no difference was observed in the presence of ROX 2. To further validate that the inhibition of the increase in surface Trx1 by activation is not a result of cell death, the viability was determined after 24h. Figure 6.5 shows that the compounds (ROX 2 and ROX 1) do not affect the viability at 12.5µM after 24h, suggesting that the decrease in surface Trx1 is not a result of cell death.

Prx2 is present on the surface of lymphocytes including T and B cells (Szabó-Taylor et al. 2012b). In order to detect changes in surface Prx2 during T cell activation, surface Prx2 was analysed in the presence or absence of NOX compounds. Prx2 was increased on the exofacial surface during T cell activation. From figure 6.5a, it can be seen that surface Prx2 increase is significantly inhibited when $CD4^+$ T cells were activated in the presence of compound ROX 1. However no significant difference was observed when activated in the presence of ROX 2.

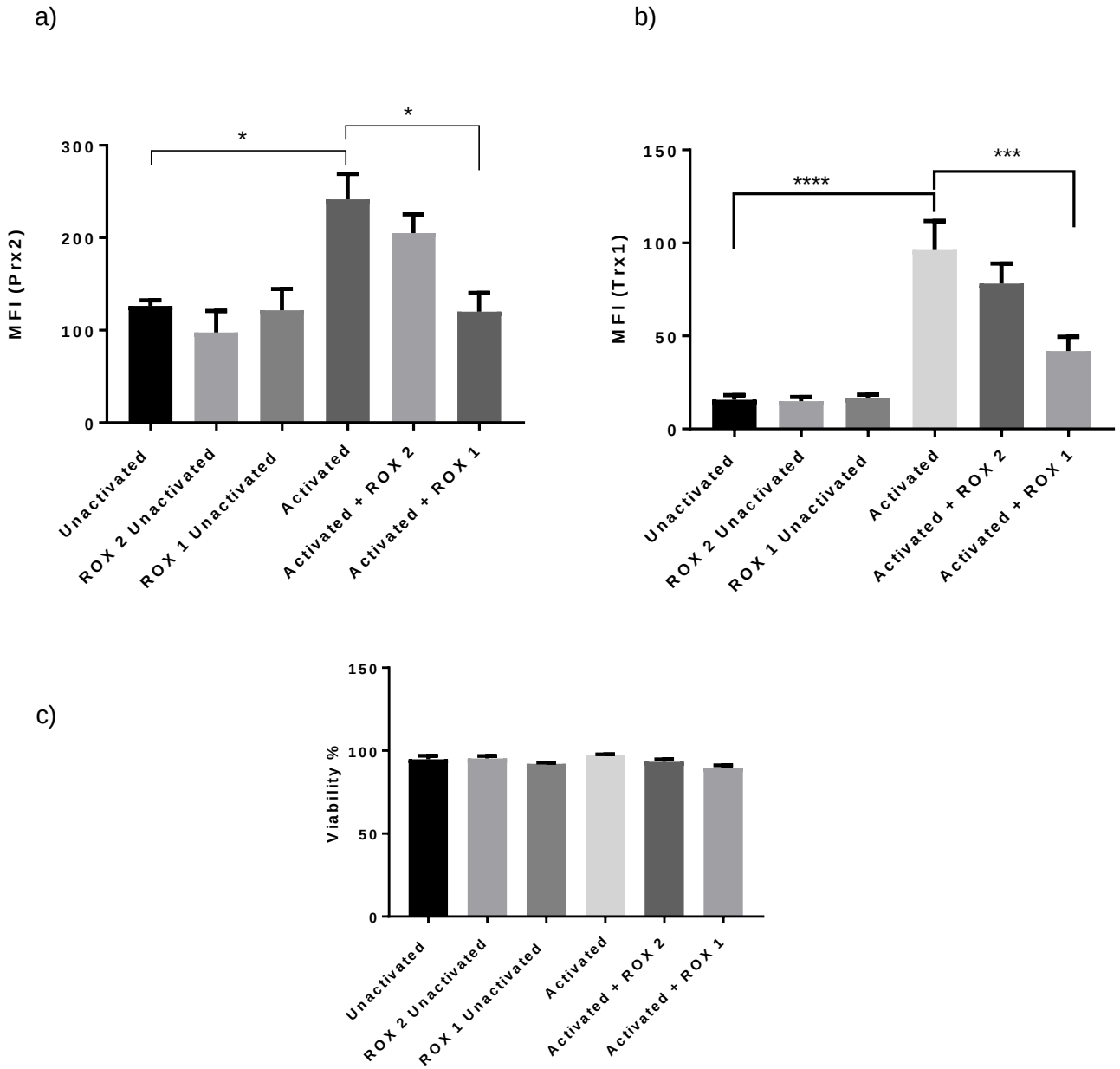



Figure 6.5: The presence of compound ROX 1 show decreased Trx1 and Prx2 expression on the surface after 24h compared to activated T cells. $1x10^6$/ml $CD4^+$ T cells were isolated and activated with 1µl/ml anti-CD3 (OKT3) and 2µg/ml anti-CD28.2 for 24h in the presence or absence of compound 12.5µM ROX 2 and ROX 1. The cells were isolated and washed with PBS/BSA 1%w/v and incubated with primary anti-Trx1 (ab16965), primary rabbit anti-Prx2 (ab109367) for 30min. The cells were washed and secondary goat anti-Mouse IgG H&L (ab96879) or goat anti-rabbit IgG Alexa-488 was added and incubated for 30min and analysed by flow cytometry. a) Surface Prx2 expression after 24h, b) Surface Trx1 expression after 24h, c) Cell viability measured by nucleocounter as described in methods after 24h. Data expressed as the difference in MFI between antigen specific and isotype control signal over 5000 events. n=3 mean and SEM, ANOVA, Tukey's multiple comparison test. ****p<0.0001, ***p<0.004, *p<0.02

## 6.3.6. Effect of compounds on $CD4^+$ T cell GSH levels during activation

GSH plays an essential role in T cell function and proliferation (Levring et al. 2015). In order to determine the effect of ROX compounds on intracellular GSH levels during activation, the cells were activated in the presence of ROX 2 and ROX 1. Activation of $CD4^+$ T cells leads to a significant increase in intracellular GSH levels after 24h as shown in Figure 6.6. However, the presence of compound ROX 1 during activation significantly prevented the increase in GSH levels after 24h while ROX 2 showed no significant difference. Also, unactivated cells treated with ROX 1 shows a decreased trend by 3 fold compared to untreated.

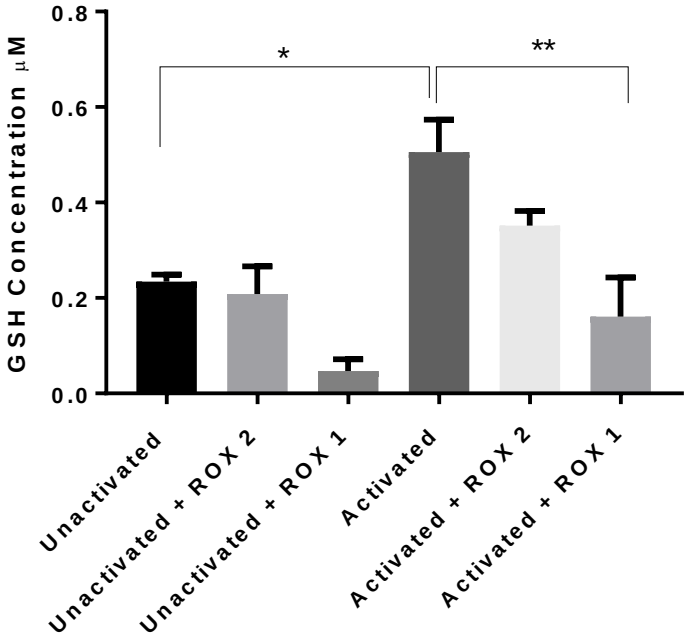



Figure 6.6: Effect of NOX compounds on intracellular GSH after 24h. $CD4^+$ T cells were isolated and activated with 1µl/ml anti-CD3 (OKT3) and 2µg/ml anti-CD28.2 for 24h in the presence or absence of compounds12.5µM (ROX 2 and ROX 1). $1x10^5$ cells were isolated and plated on a 96 well plate in duplicates, the GSH concentration was determined using GSH-Glo™ glutathione assay (Promega) using a standard curve. Data represents three independent experiments n=3, mean and SEM. Ordinary one-way ANOVA, Tukey's multiple comparison test *$p<0.03$, **$p<0.005$

### 6.3.7. NOX2 compounds inhibit T cell activation.

The secretion of IL-2 is critical and an early landmark in the activation process of $CD4^+$ T cells, which suggests further proliferation and differentiation of T cells (Sojka et al., 2004). To be able to determine the effect of NOX 2 compounds on T cell activation, $CD4^+$ T cells were activated in the presence or absence of ROX 2 and ROX 1 at 12.5µM for 24h. As expected, IL-2 secretion was increased 8-fold during the first 24 hours of activation. Figure 6.7a shows that there was a significant inhibition of IL-2 secretion when cells were activated in the presence of either of the compounds; ROX 2 (0.79±0.19ng/ml) and ROX 1 (0.59±0.11ng/ml) compared to absence of compounds (1.76±0.02ng/ml) after 24h, which suggests that these compounds inhibit the activation of T cells. The binding of the secreted IL-2 to IL-2 receptor (CD25) leads to further proliferation, we looked at the effect of these compounds on surface CD25 expression. From Figure 6.7b, CD25 expression was increased during the first 24 hours of activation. The compound ROX 2 show no significant effect on CD25 after 24h activation, however compound ROX 1 significantly inhibited the upregulation of surface CD25 after 24h suggesting that T cell activation is suppressed in the absence of ROS.

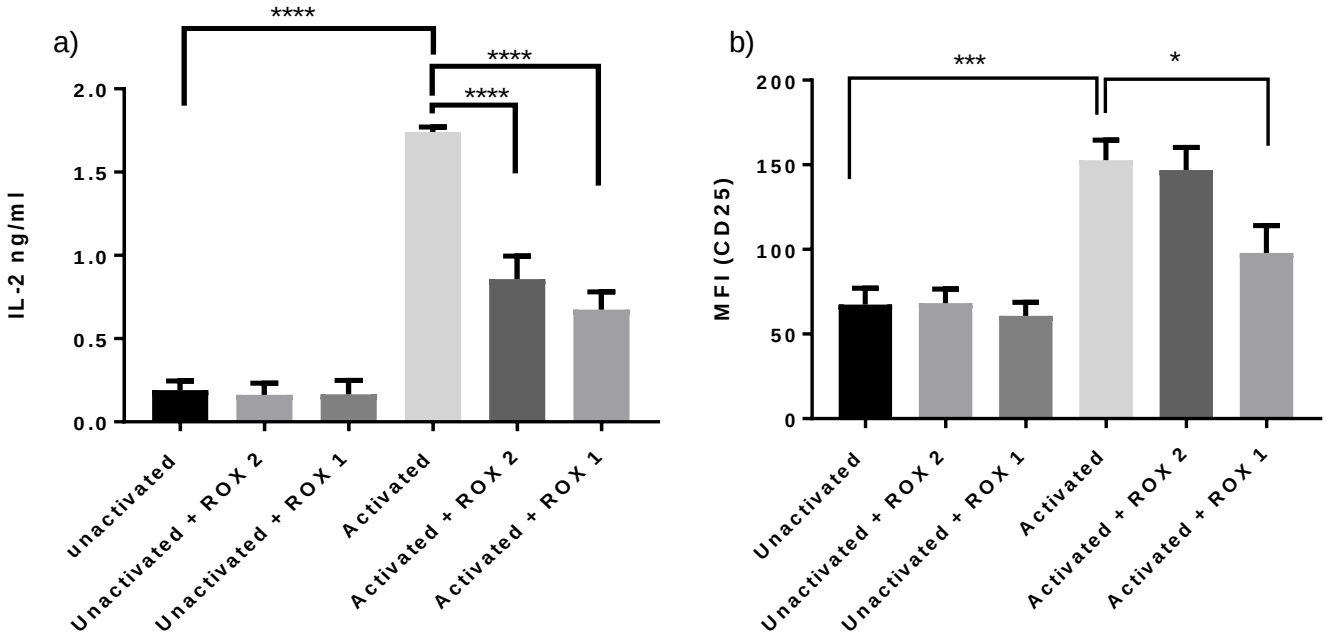



Figure 6.7: Effect of NOX-2 compounds on T cell activation. $CD4^+$ T cells were isolated and activated with 1µl/ml anti-CD3 (OKT3) and 2µg/ml anti-CD28.2 for 24. a) Supernatants from each condition were collected and IL-2 secretion was measured using a sandwich ELISA. b) Cells were washed in PBS with BSA (1%w/v) and incubated with ant-CD25 cy5 for 20mins on ice and analysed by flow cytometry. Mean values are shown with standard error of the mean. These values are the results from three independent experiments, n=3 mean and SEM, Ordinary one-way ANOVA, Tukey's multiple comparison test ****$p<0.0001$. ***$p<0.0004$, *$p<0.04$

## 6.3.8. Induction of Arthritis in B6NQ and B6NQ.Ncf1 mice

In order to investigate the effect of decreased Ncf1 and NADPH oxidase function on arthritis, collagen induced arthritis was induced in WT and Ncf1 mutated mouse using collagen type II derived in rat. From figure 6.8, Ncf1 mutated mice show a rapid disease progression after day 25 compared to WT B6 mice. This is due to the lack of functional NADPH oxidase complex, confirming that the presence of Ncf1 mutation leads to increased collagen induced arthritis (CIA).

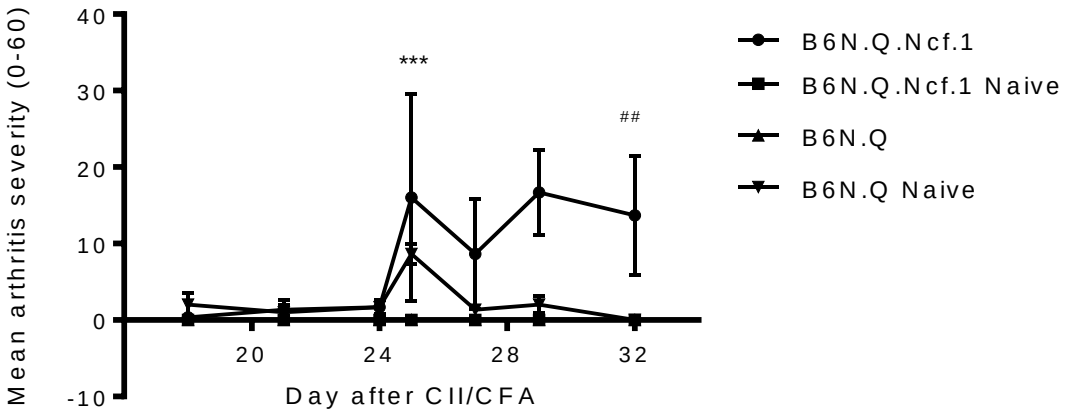
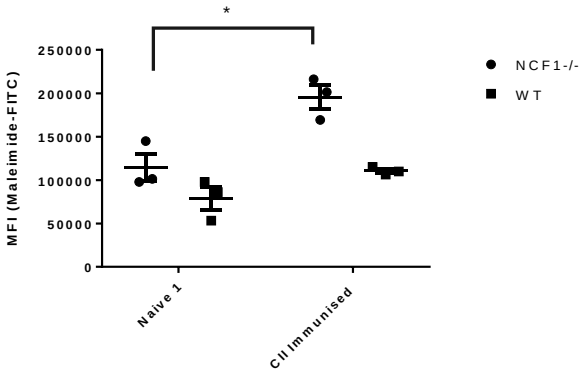


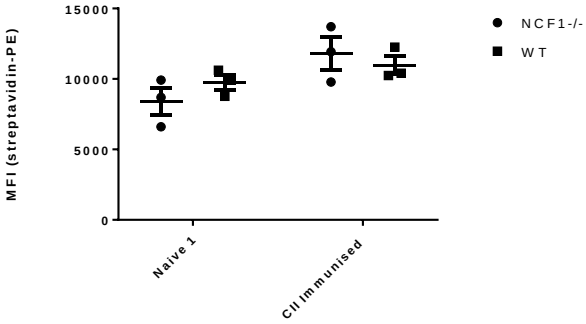

Figure 6.8: Mice with mutation in Ncf1 develop enhance arthritis compared to WT. Mice were immunised with 100μg of rat CII in complete Freud adjuvant (CFA) injected at the base of the tail. A boost at day 21 of 50μg rat CII in incomplete Freud's adjuvant was performed. Macroscoping scoring of arthritis after day 18 of immunisation. Results show 3 individual mice in each group. Two way ANOVA, multiple comparison test ***$p<0.0009$ comparing immunised NCF-/- and naïve Ncf1-/- mice. ##$p<0.05$ comparing immunised Ncf1-/- vs immunised WT mice.

### 6.3.9. CIA does not change the T cell surface redox state.

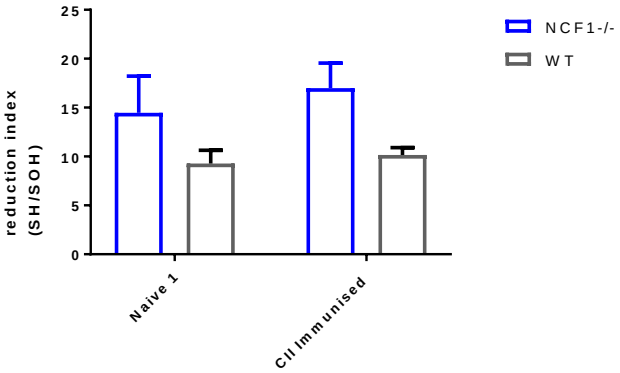
In order to investigate the surface redox state in CIA, whole blood was collected during disease period (day 28) and at termination (day 35) and surface thiols (-SH) and sulphenic acid (-SOH) were determined on the surface of $CD4^+$ T cells. Figure 6.9 below shows that Ncf1-/- mice have higher levels of $CD4^+$ T cells surface thiols in the naïve state compared to naïve WT mice at day 28. Also, the Ncf1 mutated mice show an significant increase in surface thiol levels after immunisation (Figure 6.9a) whereas no observable change in WT thiols after immunisation at day 28. At termination (day 35) wild type animals have higher levels of $CD4^+$ T cell surface thiols in the naïve state but that after immunisation there was no difference in –SH on the surface thiols between Ncf1 mutated and wild type mice (Figure 6.9c). Conversely, the–SOH was higher in two of the three naïve WT animals than in the mutants, and this difference was removed after immunisation. Arbitrary thiol oxidation and reduction indices were calculated as ratios between sulphenic acid and thiols at both time during the disease period. The overall picture at day 28 shows less proportion of reduced thiols in WT mice upon CII immunisation. Also, the analysis at day 35 highlights that the presence of NOX2 in WT animals is associated with a 50% higher oxidation of thiols and that activation of WT T cells tends towards greater surface reduction. Thiol levels on Ncf1-/- T cells were unaffected by immunisation throughout the disease period.

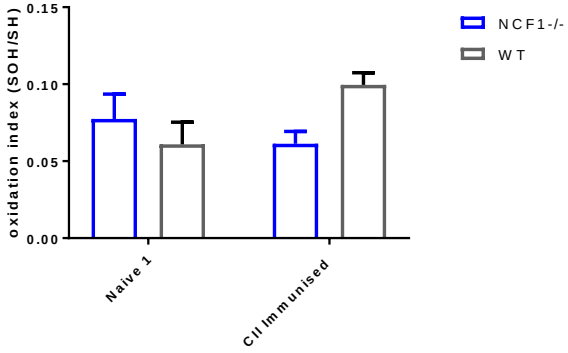
a)


*
250000
200000
150000
100000
50000
0
MFI (Maleimide-FITC)
Naive 1
CII immunised
NCF1-/-
WT

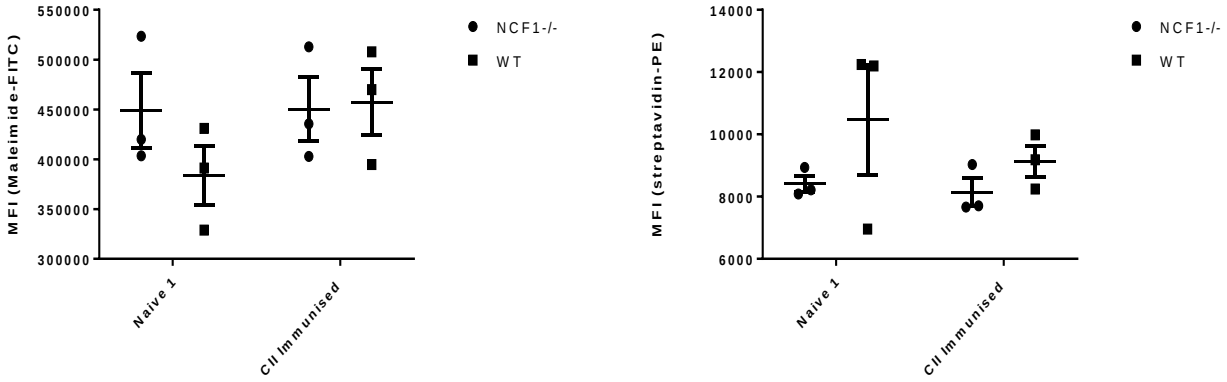

15000
10000
5000
0
MFI (streptavidin-PE)
Naive 1
CII immunised
NCF1-/-
WT

b)


25
20
15
10
5
0
reduction index (SH/SOH)
Naive 1
CII immunised
NCF1-/-
WT


0.15
0.10
0.05
0.00
oxidation index (SOH/SH)
Naive 1
CII immunised
NCF1-/-
WT

c)




d)

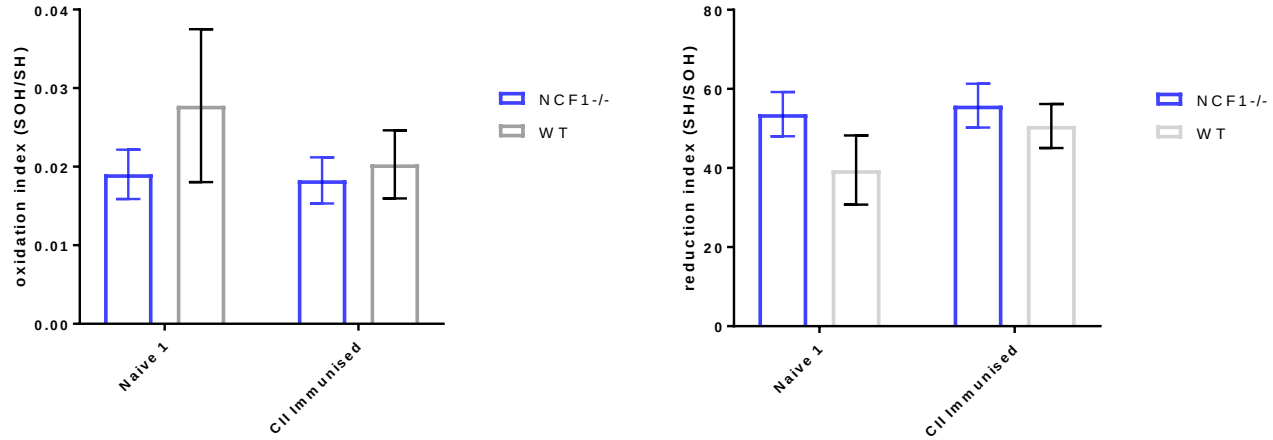
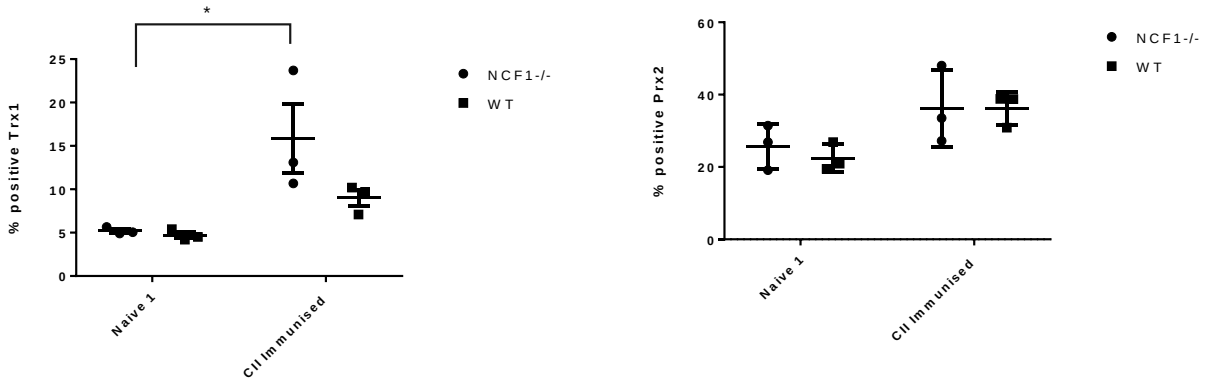



Figure 6.9: Surface thiol and sulphenic acid levels at day 28 and 35 of immunisation in WT and Ncf1-/- mice. Whole blood was collected and lysed using BD pharmlyse and washed twice with PBS. Cells incubated with FITC-conjugated maleimide for 30min and analysed by flow cytometry. Cells were incubated with 0.1mM DCP-Bio1 and 10mM iodoacetamide (IAA) for 30min. The cells were further washed and incubated with streptavidin-PE (1:100) for 30min on ice and analysed by flow cytometry. a) –SH levels and –SOH formation in CD4+ T cells at day 28, b) redox index at day 28. c) –SH levels and –SOH formation in CD4+ T cells at day 35, d) redox index at day 35. Data represents three mice per group. n=3, mean and SEM, one way ANOVA, Tukeys multiple comparison test, *p<0.049

### 6.3.10. Distribution of Trx1 and Prx2 in CD4 T cells from CIA mice

To further investigate the distribution of Trx1 and Prx2 during CIA in CD4 T cells, surface Prx2 was analysed in $CD4^+$ T cells from whole blood after day 28 and 35 of immunisation. Figure 6.10 shows no difference in % cells positive for Trx1, however, there was a significant increase in % cells positive for Trx1 in the CII immunised Ncf1-/- mice. Moreover, figure 6.10b shows no difference in % of cells positive for surface Trx1 between naïve and CII immunised Ncf1-/- mice after day35. However, a trend for increased number of Trx1 positive cells was observed in T cells from WT mice after immunisation. Furthermore, more cells tended to be positive for Prx2 in WT animals than in Ncf1-/- mice and the % of cells that were positive for surface Prx2 was increased after immunisation in Ncf1-/- and WT compared to naïve at day 35 as shown in the figure below.

a)




b)

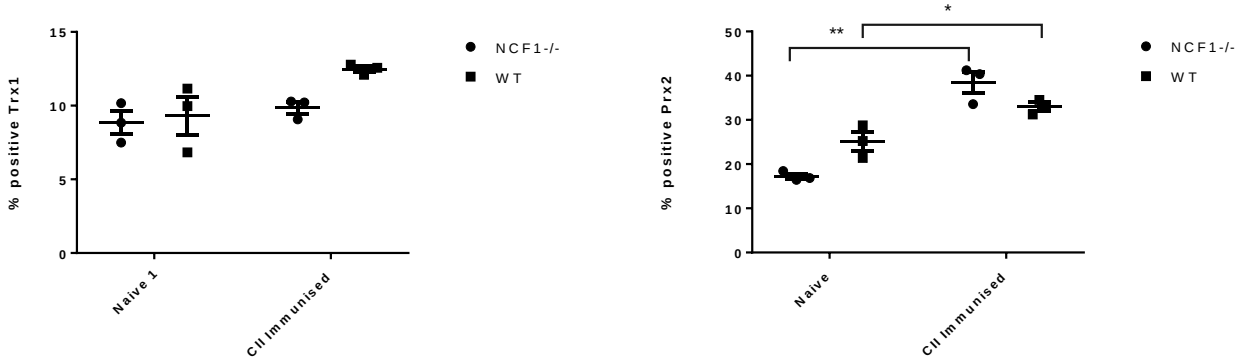
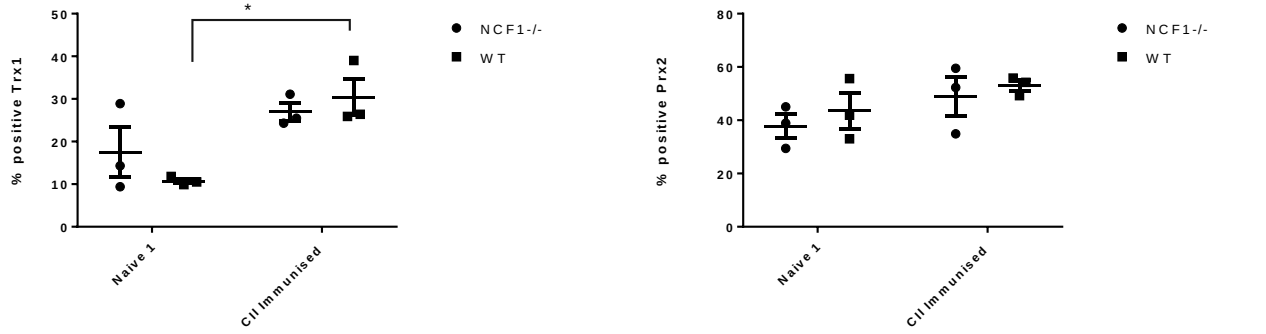



Figure 6.10. CD4$^+$ T cells from CIA mice show no difference in Trx1 but an increase in Prx2 after day 28 and 35. Whole blood was collected and lysed using BD pharmlyse and washed twice with PBS/BSA 1%w/v and incubated with primary anti-Trx1 (ab16965), primary rabbit anti-Prx2 (ab109367) for 30min. The cells were washed and secondary goat anti-Mouse IgG H&L (ab96879) or goat anti-rabbit IgG Alexa-488 was added and incubated for 30min and analysed by flow cytometry a) Surface Trx1 and Prx2 expression at day 28, b) Surface Trx1 and Prx2 expression at day35. Data expressed as % of cells expressing specific antigen on the surface. n=3 mean and SEM, ****p<0.0001, **p<0.004, *p<0.02, *p<0.01

## 6.3.11. Distribution of Trx1 and Prx2 in neutrophils from CIA mice

To further evaluate the distribution in other cells involved in inflammation, surface Trx1 and Prx2 expression was also analysed in neutrophils. More neutrophils from Ncf1-/- immunised mice were positive for surface Prx2 compared to naïve mice after day 35 but not at day 28 as shown in Figure 6.11, whereas immunisation did not change in the proportion of the WT cells expressing Prx2 on the surface as compared to naïve mice. Additionally, immunisation led to an increase in Trx1 positive neutrophils from both groups (Ncf1-/- and WT) after day 28 and 35 as shown in the figure below.

a)




b)

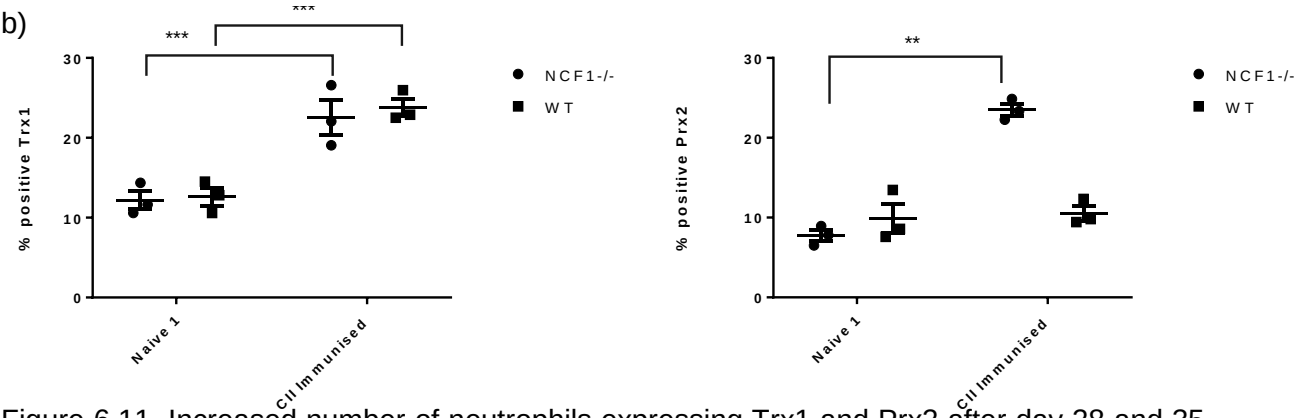



Figure 6.11. Increased number of neutrophils expressing Trx1 and Prx2 after day 28 and 35 of immunisation. Whole blood was collected and lysed using BD pharmlyse and washed twice with PBS/BSA 1%w/v and incubated with primary anti-Trx1 (ab16965), primary rabbit anti-Prx2 (ab109367) for 30min. The cells were washed and and incubated with anti-mouse Ly6G-PerCP and secondary goat anti-Mouse IgG H&L (ab96879) or goat anti-rabbit IgG Alexa-488 for 30min and analysed by flow cytometry. a) Surface Trx1 and Prx2 expression at day 28 b) Surface Trx1 and Prx2 expression at day35. Data expressed as % of cells expressing specific antigen on the surface. Data analysed from 3 mice per group (Naïve WT, immunised WT, Naïve Ncf1-/- and immunised Ncf1-/-) n=3. Two way ANOVA, Sidaks multiple comparison test. *p< 0.05, **p<0.002, ***p<0.0008

### 6.3.12. T cell responses in WT and Ncf1-/- B6 mice

To further investigate the T cell responses in naïve and immunised with CIA mice, splenocytes were isolated from WT and Ncf1-/- mice after termination. T cell responses were assessed by measuring cytokine levels, IL-2 and TNF-α secreted in the supernatants after 24h stimulation with CII and anti-CD3/CD28 antibodies. T cells from all groups of mice were activated when stimulated with anti-CD3 and CD28 as shown in Figure 6.12. In contrast, only immunised WT cells responded to CII stimulation *in vitro* whereas Ncf1-/- splenocytes responded to CII whether they had been immunised with the antigen or not.

However, when treated with ROX 2, T cells from immunised WT mice secreted significantly less IL-2 whereas naïve WT show no difference in secretion. Furthermore, strong antigen response which led to increased cytokine secretion were observed in splenocytes from Ncf1-/-, naïve Ncf1-/- and WT but no IL-2 secretion in naïve WT mice.

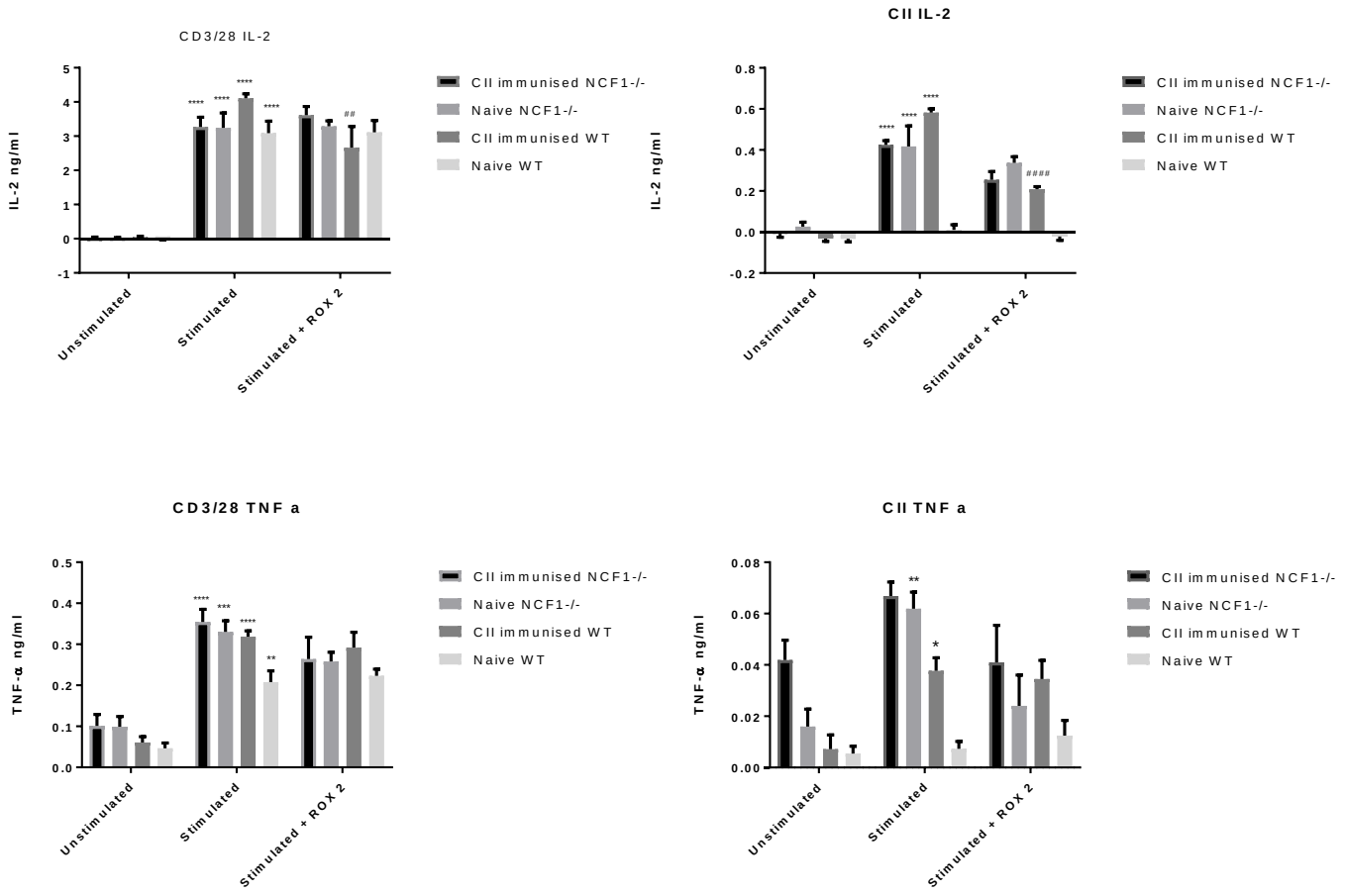



Figure 6.12: *Exvivo* stimulation of mice splenocytes with anti-CD3/CD28 and rat CII. Spleens were collected and single cell suspensions were prepared. RBC were lysed using BD pharmlyse and lymphocytes were stimulated with 1µg/ml of anti-CD3 and 2µg/ml of anti-CD28. Also splenocytes were re-stimulated with rat CII at 100µg/ml for 24h with or without ROX 2 (12.5µM). Supernatants were collected and analysed for IL-2 and TNF-α. Data analysed from 3 mice per group (Naïve WT, immunised WT, Naïve Ncf1-/- and immunised Ncf1-/-) n=3. *comparing to the unstimulated. #comparing the treatment ROX 2 to stimulated control. Two way ANOVA, Tukey's multiple comparison test. ****p<0.0001, ####p<0.0001, ##p<0.008

### 6.3.13. Surface Trx1 and Prx2 in CD4 T cells increases after activation with anti-CD3 antibodies

In order to determine the distribution of Trx1 and Prx2 in T cells re-stimulated from CIA induced mice (WT and Ncf1-/-), splenocytes were collected and re-stimulated with anti-CD3/CD28 antibodies for 24h and surface Trx1 and Prx2 was analysed. The proportion of T cells expressing Trx1 and Prx2 was significantly increased by stimulation *in vitro* as shown in Figure 6.13 but the proportion of Trx1 expressing cells was lower in Ncf1-/-. The effect of ROX 2 on surface Trx1 and Prx2 was analysed after T cell activation, the compound did not show observable effect on the distribution of Trx1 and Prx2, however, the proportion of Prx2 expressing cells tended to be lower in naïve cells that had not been previously immunised

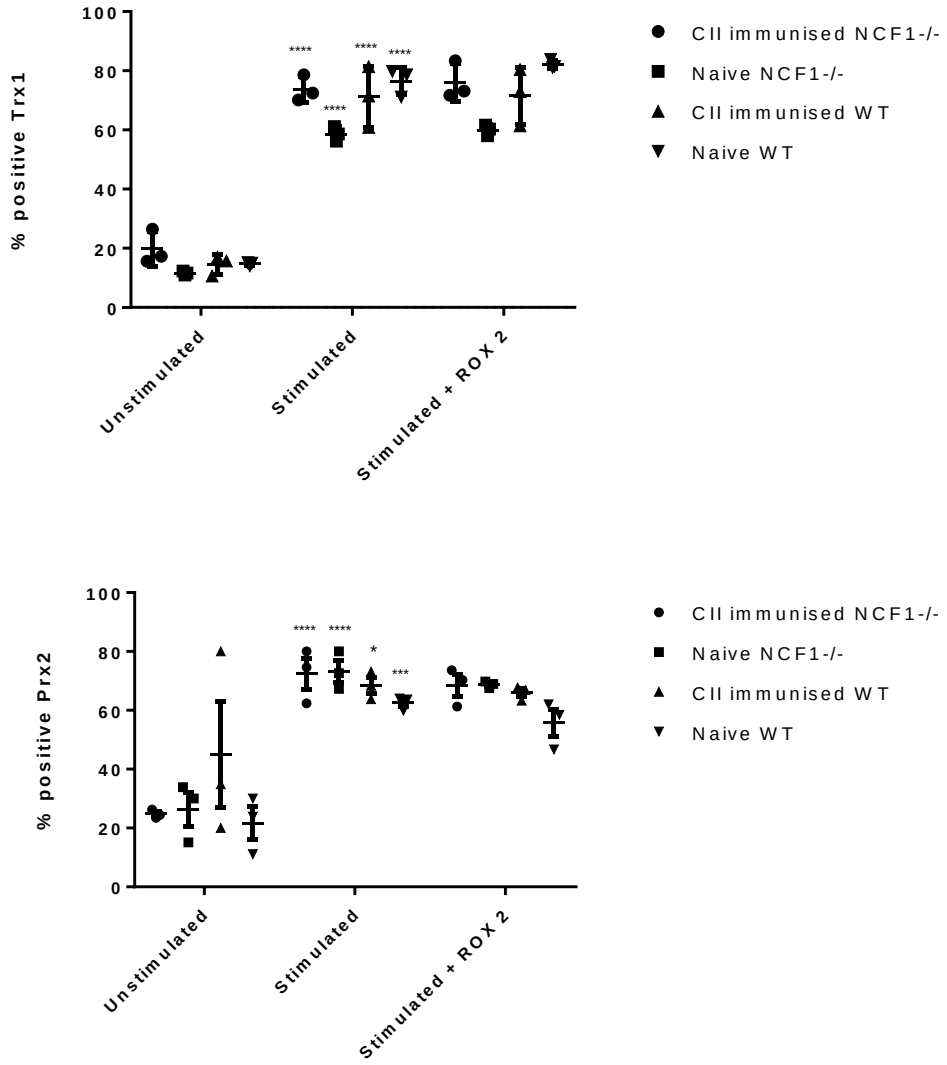



Figure 6.13: Surface Trx1 and Prx2 in CD4 T cells from immunised mice stimulated with anti-CD3/CD28. Spleens were homogenised and single cell suspensions were prepared. RBC were lysed using BD pharmlyse and lymphocytes were stimulated with 1µg/ml of anti-CD3 and 2µg/ml of anti-CD28 with or without ROX 2. Cells were collected and stained with mouse anti-Trx1 and or rabbit anti-Prx2 for 30min in PBS (1% w/v BSA). The cells were washed and further incubated with goat anti-mouse FITC and or mouse anti-rabbit in addition to anti-CD4-PE for 20mins and analysed by FC. Data analysed from 3 mice per group (naïve WT, immunised WT, Naïve Ncf1-/- and immunised Ncf1-/-) n=3. ****p<0.0001, ***p<0.0004, *p<0.02.

### 6.3.14. No change in neutrophils expressing Trx1 and Prx2 in unactivated T cells compared to activated T cells

The proportion of neutrophils expressing Trx1 or Prx2 when splenocytes were re-stimulated with anti-CD3 antibodies after immunisation was investigated. The expression of redoxins was more variable between animals after immunisation, whereas expression on non-immunised neutrophils was more consistent between animals. No difference was observed in the proportion of cells expressing Trx1/Prx2 on the surface of neutrophils. However, a trend for lower expression of Prx2 on neutrophils was observed in naïve animals that were stimulated with anti-CD3/CD28 antibodies and this was not affected by ROX. WT animal neutrophils were more sensitive to the bystander effects of splenocyte activation and showed a trend for lower proportions of cells expressing Trx1 after restimulation. This effect was lost in the presence of ROX 2.

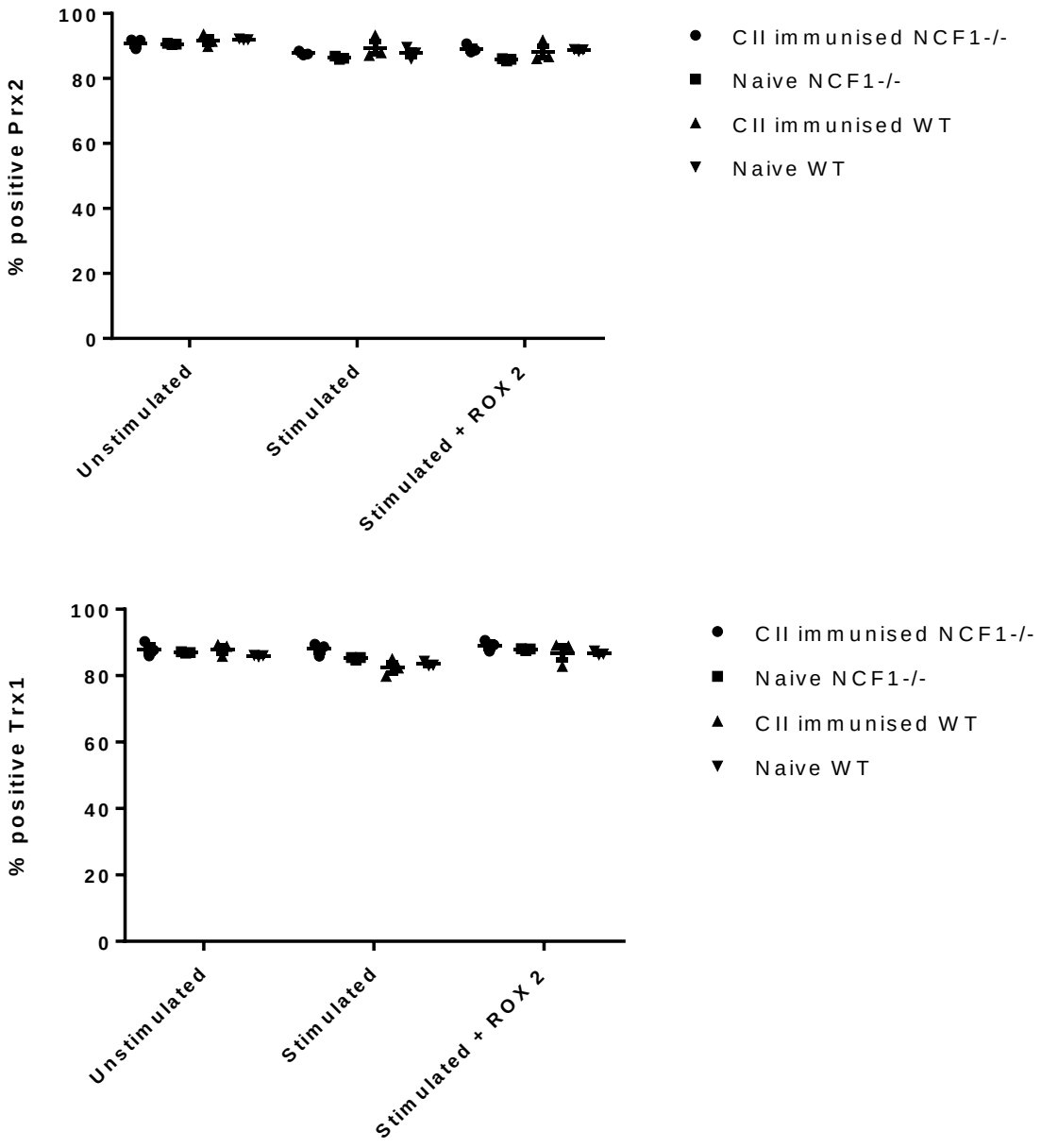



Figure 6.14: Surface Trx1 and Prx2 in neutrophils from immunised mice stimulated with anti-CD3/CD28. Spleens were homogenised and single cell suspensions were prepared. RBC were lysed using BD pharmlyse and lymphocytes were stimulated with 1ug/ml of anti-CD3 and 2ug/ml of anti-CD28 with or without ROX 2. Cells were collected and stained with mouse anti-Trx1 and or rabbit anti-Prx2 for 30min in PBS (1% w/v BSA). The cells were washed and further incubated with goat anti-mouse FITC and or mouse anti-rabbit in addition to anti-CD4-PE and anti-mouse Ly6G-PerCP for 20mins and analysed by FC. Data analysed from 3 mice per group (naïve WT, immunised WT, Naïve Ncf1-/- and immunised Ncf1-/-) n=3.

### 6.3.15. ROX 2 treated WT mice show a decreased arthritis severity while no change in disease activity was seen in Ncf1-/- mice compared to WT

The effect of NOX compounds in CIA was investigated to see if enhanced ROS production might protect against CIA in animals with functional NADPH oxidase and hence may have potential use as therapeutic targets. WT and Ncf1-/- mice were immunised with CII and treated with ROX 2 (Redoxis AB) 3 times a week after immunisation. Ncf1-/- mice showed severe arthritis with earlier onset compared to WT mice. Additionally, the compound ROX 2 has no effect on Ncf1-/- CIA immunised mice as shown in Figure 6.15. However, the mild arthritis score observed in WT mice showed a trend for decrease in the WT mice group treated with ROX 2 at a later stage of the disease.

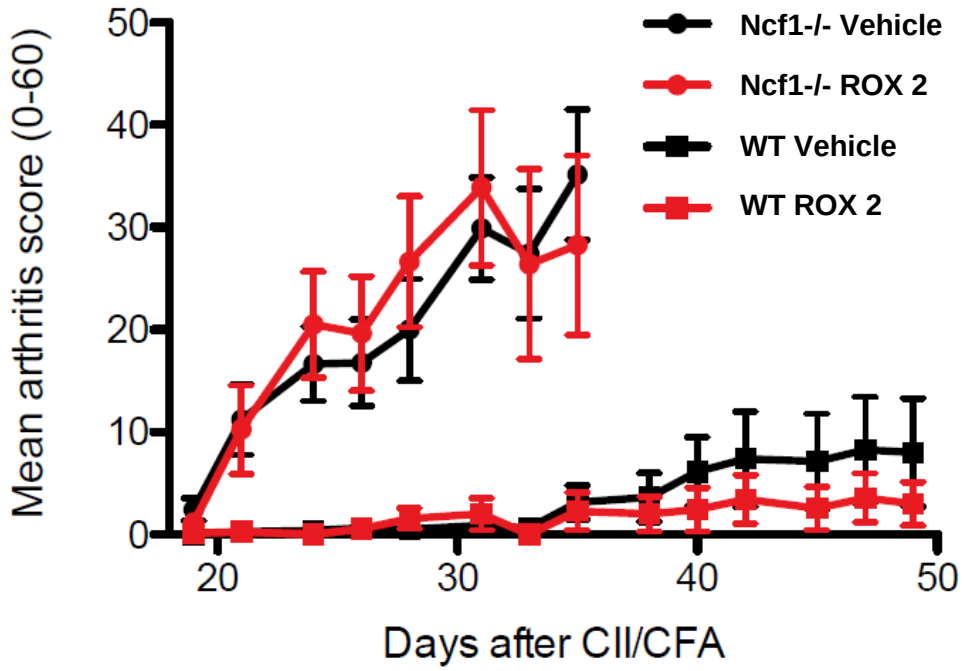



Figure 6.15: Treatment with ROX 2 show decreased severity in WT mice after day 35. WT and Ncf1-/- mice were immunised with rat CII in CFA. The mice were boosted with CII in incomplete Freund adjuvant at day 18 ad macroscoping scoring was performed from day 19 until termination. The chart represents 5mice per group.

## 6.4. Discussion

This chapter has looked at the effects of ROS inducing compounds on T cell function *in vitro*, *in vivo* and on the frequency of Trx1 and Prx2 expressing cells. The findings of this chapter have validated the effect of compounds targeting NOX2 in CIA, a model that is associated with increased T cell activation. Also, it shows increased disease severity in mice with decreased NADPH oxidase function as shown by others (Hultqvist et al. 2004).

T cell proliferation is increasingly influenced by alteration in the redox state with a majority of studies focussing on the intracellular ROS and few showed a reducing extracellular environment is beneficial for T cell proliferation (Survival et al. 2010).

Firstly this study used compounds which have been shown to increase superoxide production from NOX *in vitro* to establish a model in human primary T cells to study the effects of these compounds on T cell activation and the distribution of Trx1 and Prx2 analysing the surface redox state by looking at reduced and oxidised thiols on the surface of T cells.

NOX-2 plays an important role in regulating T cell activation by generating superoxide anion within adjacent cells during antigen presentation. ROS also play an important role intracellularly initiating a signalling cascade, possibly by oxidising proteins in the TCR pathway including PTPs (Hultqvist et al. 2009a; Salmeen et al. 2003; Sareila et al. 2011).

Results obtained from this thesis show an increase in –SH and –SOH after activation but no significant effect of ROX compounds. This suggests that the ROX compounds have no effect on overall membrane protein sulphenic acid formation. However, an increased trend of –SH was observed in the presence of ROX 1 and 2. Cell surface –SH is widely known as a critical component for T cell activation and function (Pellom et al. 2013).

The effect of ROX compounds was however unknown in terms of whether they stimulate ROS production in T cells and hence intracellular ROS was measured using DCF oxidation. $H_2O_2$ was added as a positive control. Numerous studies suggest that T cell receptor activation

leads to production of ROS (Sharon H Jackson et al. 2004). An increasing pattern was observed of DCF Median X in cells activated in the presence of $H_2O_2$ after 30minutes. Furthermore, a significant increase in intracellular ROS when T cells were activated for 24h further supports hypothesis that T cell receptor stimulation leads to increase in intracellular ROS. This may suggest $H_2O_2$ is metabolised in unactivated cells but activation of T cells require intermediate amounts of ROS. This is also consistent with the work done by Phillips et al, whereby T cells produce cytosolic ROS after mitogenic stimulation for up to 72h in culture (Phillips et al. 2010). The observation also relies on the sources of ROS. The major sources of ROS are the mitochondria and hence an increase in DCF oxidation maybe due to an increase in mitochondrial ROS and metabolic activity resulting from proliferation as compared to resting cells. Further work using dyes such as MitoSOx which detects mitochondrial ROS can provide an idea of mitochondrial superoxide generation. ROS released from the mitochondria are required for T cell activation and are induced by a calcium influx as shown by Sena et al. 2013; T cells activated in the presence of mitochondrial targeted antioxidant-Mito-Vitamin E, completely reduced IL-2 secretion (Laura A. Sena et al. 2013). Mitochondiral ROS also play an important role in T cell proliferation along with cell death (Simeoni & Bogeski 2015b; Murphy & Siegel 2013).

However, the NOX compounds show an interesting trend such that the intracellular ROS is decreased in the presence of compounds suggesting an inhibition of activation in isolated T cells. This contradicted the results from figure 6.3.1 which clearly show an increase in RLU when splenocytes were treated with ROX 2 and this increase was absent in Ncf1-/- mice. This is supported by the same compound (ROX 2) used in a study showing increasing extracellular ROS production in peritoneal cells (Kienhöfer, et al. 2017). This is interesting because it is thought that the compound may act on surface enzymes to generate ROS to target surface proteins e.g. calcium channels responsible for intracellular signalling, which may lead to increased metabolic activity including the production of ROS by mitochondria. This may be the reason it may affect T cell activation as inhibition of intracellular ROS during immune activation results in reduced $CD4^+$ T cell responses (Previte et al. 2017). Alternatively,

detecting ROS production in T cells during activation in the presence of these compounds will provide an idea of how they may affect T cell activation. ROX might inhibit signalling pathways involved during T cell activation and hence low levels of ROS produced, since temporary production of ROS is essential for receptor mediated cell signalling (Kesarwani et al. 2013b). From the data obtained a reduction in intracellular ROS may impair T cell function (Phillips et al. 2010), shown in Figure 7.2.

Although ROS plays a critical role in T cell responses, the ability to detect ROS remains a challenge. The DCF oxidation technique used in this chapter is the most widely used to detect intracellular ROS. This reagent reacts with the peroxides and generate fluorescence but they exhibit a range of limitations including the lack of specificity to a particular oxidant and highly prone to autoxidation with time resulting in increased fluorescence (Poole & Nelson 2008). T cells have been shown to produce intracellular ROS upon TCR stimulation possibly mitochondrial ROS (Belikov et al. 2014; Sharon H. Jackson et al. 2004). ROS present in the extracellular space may influence the surface redox state, but intracellular ROS from the mitochondria may play an important role in intracellular cell signalling. This suggests that the compound may vary in its action depending on the cell type, location and source of ROS.

Interestingly, when the T cells were activated in the presence or absence of ROX 2 and ROX 1, intracellular GSH levels were significantly lowered in the presence of ROX 1 in activated and unactivated cells. However unactivated cells treated with ROX 1 also showed a decrease that was not significant. This lack of significance may be due to the variability in the data obtained and the low range of detection. GSH synthesis is required for DNA synthesis which allows further T cell proliferation (Levring et al. 2015), T cell effector functions and metabolic activity (Mak et al. 2017). This is achieved by the import of oxidised cysteine through the plasma membrane $x_c^-$ transporter. Naïve T cells express very low levels of $x^-_c$ but shortly after activation, the expression is upregulated and the cells are provided with the required cystine for differentiation and proliferation (Castellani et al. 2008; Garg et al. 2011). Therefore, the

decrease in intracellular GSH might be due to the lack of cystine transporter on the membrane of T cells. Also, there is a possibility of excess GSH exported out of the cells into the extracellular environment or perhaps the compound inhibiting GSH synthesis as the measure was after 24h.

Likewise, $CD4^+$ T cells activated in the presence of NOX-2 compounds show a significant effect on surface Trx1 and Prx2. A significant decrease in T cell surface Trx1 was seen in the presence of ROX 1 after 24h suggests that compound ROX 1 (which inhibits intracellular peroxides measured as DCF oxidation) may inhibit the translocation of Trx1 to the surface. Also, another study by World et al. shows that endothelial cells treated with $H_2O_2$ for 30min leads to increased levels on Trx1 on the surface (World et al. 2011). In general redox-sensitive cysteine residues of proteins are largely dependent on Trx1, which restores their reduced state (Hanschmann et al. 2013a). Elevated serum and plasma levels of Trx1 are observed in patients suffering from RA (Jikimoto et al. 2002; Maurice et al. 1999; Lemarechal et al. 2006). Moreover, the synovial fluid of RA patients showed higher levels of Trx1 compared to healthy individuals (Yoshida et al. 1999). This finding might support the idea that high concentrations secreted into the SF is in fact due to the hypo-responsiveness of T cells in RA (Maurice et al. 1997). Studies show increase in serum Trx1 and CD30 in conditions of autoimmunity including SLE, RA. Both of these proteins play a role in the regulation of the immune response through the thiol disulphide exchange to changes in lymphocyte function (Schwertassek et al. 2007a; Horie & Watanabe 1998).

Increased secretion of IL-2 suggests that effective T cell activation upon stimulation. The interaction of IL-2 and CD25 allows T cells to differentiate and proliferate (Malek & Castro 2010). IL-2 secretion was measured by ELISA and CD25 by flow cytometry. In both cases, the data shows that IL-2 secretion is inhibited in the presence of NOX-2 compounds (ROX 2 and ROX 1) suggesting the compounds inhibit T cell activation. It is reported that certain antioxidants are likely to affect the IL-2 secretion such as mitovitamin E; this abrogates IL-2 production (Laura A. Sena et al. 2013). In contrast, others have shown that gp91phox$^{-/-}$ (NOX-

2 heme binding subunit) mice T cells have no difference in CD25 expression or IL-2 production upon anti-CD3/CD28 stimulation when compared to WT mice (Shatynski et al. 2012). These approaches have been made to further understand the functional importance of ROS in the activation of T cells. It is not clear of whether ROX 2 and ROX 1 are specific to activating NOX2. Additionally, it is not clear yet whether the decrease of IL-2 is due to the signalling pathway such as the possible oxidation of NFAT pathway involved in IL-2 gene expression (Chow et al. 1999), thus analysis of mRNA expression of IL-2 would provide more insight. It has been debated whether T cells express NOX 2 enzyme on the surface. However a study has shown that T cells express a NOX2 like enzyme on the surface responsible for generating superoxide extracellularly (Sharon H Jackson et al. 2004). Also, since NOX-2 is transmembrane protein and is made up of different subunits, identifying targets to which these compounds act will be interesting for further studies.

There is evidence suggesting that NOX derived ROS may play a role in immunosuppression in conditions such as tumours and autoimmune conditions (Kono et al. 1996; Otsuji et al. 1996; Björkman et al. 2008). Hence chapter 6 has endeavoured to look at the effect of ROS deficient cells on T cell responsiveness. Ncf1 is strongly associated with arthritis and deletion of this gene leads to arthritis progression (Olsson et al. 2016).

Here in chapter 6, mice with decreased NADPH oxidase function show increased arthritis severity compared to WT mice.

It is described previously that T cell proliferation and activity is influenced by ROS which can be endogenously or exogenously generated (Lawrence et al. 2000; Kesarwani et al. 2013a). It continues to be challenging and interesting how a mutation in Ncf1 gene leads to severe arthritis. This phenomenon was also observed in other autoimmune conditions including chronic encephalomyelitis (EAE) and SLE (Hultqvist et al. 2004; Holmdahl et al. 2016; Kienhöfer, et al. 2017). Other studies show a complete lack of ROS production due to Ncf1 deficiency leads to chronic granulomatous disease in mice (Jackson et al. 1995), and patients with this disease are also more susceptible to infections. Hence, the increased susceptibility

to infection may be due to decreased pathogen elimination. This further explains the role of ROS in not only invasion of pathogens but also immune regulation. It has been shown that co-incubation of T cells with granulocytes producing ROS inhibits T cell cytokine production, apoptosis and proliferation due to altered ROS levels (Hultqvist et al. 2009b). This evidence suggests that the treatment with NOX compounds may lead to T cell suppression by changes in the oxidation status. Secondly, a reducing environment is required for efficient T cell proliferation and activation. Increase in ROS release by NADPH oxidase can alter the redox environment hence affecting T cell mediated pathways. Thirdly, oxidation of proteins on the membrane of T cells are crucial for T cell activation including LAT which is highly sensitive to oxidation, resulting in lowered TCR signalling (Gringhuis et al. 2002). Moreover, evidence suggests that ROS is required for activation induced cell death of T cells (Hildeman et al. 1999; Goldstone et al. 1996; Sharon H Jackson et al. 2004; Hildeman et al. 2002; Bauer et al. 1998). This is required to shut down the immune response and hence protect the cells against autoimmunity (Brenner et al. 2008). A failure to eliminate T cells either by activation induced cell death does not only result in hyperactive T cells but enhances pro-inflammatory functions. The paradox is hence not clear such that; on one side ROS may lead to damage of macromolecules promoting inflammation and autoimmunity by posttranslational modifications and extracellular redox changes, but on the other hand ROS are essential in regulating the T cell response possibly by induction of apoptosis. This irony can be explained considering the extent of ROS production in subcellular compartments and the antioxidant defence in different cell types ( Phillips et al. 2010). Hence, the balance of ROS production during an immune response may indeed be important for the removal of autoreactive T cells, and in situations of a redox shift which may lead to apoptosis failure and thus survival of autoreactive T cells. A reduction in intracellular ROS levels may impair T cell function. This study has shown mice with NADPH oxidase ROS production deficiency show severe arthritis, which supports the idea that loss of ROS production and loss of intracellular ROS as shown in human and animal models (K. A. Gelderman et al. 2006), may lead to T cell mediated autoimmunity.

The surface redox state plays an essential role in the progression of the disease, hence this thesis has looked at the surface reduced and oxidised thiols and the distribution of Trx1 and Prx2 to understand the activity of T cells and surface proteins in RA. No difference in the sulphenic acid intermediate in the surface of T cells from Ncf1-/- mice was seen and this may be due to the fact that it is transient and hence no changes are detected at steady state. T cells from WT mice show a decreased reduction index suggesting that the membrane thiols are more oxidised upon CII challenge at day 28. This is supported by a study which suggests lower levels of surface thiols (-SH) in leukocytes from RA patients (Pedersen-Lane et al. 2007). Also, a decreased trend in oxidised thiols in T cells from Ncf1-/- mice may indicate the absence of ROS production hence decreased oxidation in comparison to WT mice. The increased –SH levels in naïve Ncf1-/- compared to WT may be explained by the lack of ROS. This is also supported by previous work done by Hultqvist et al, suggesting that T cell membrane proteins are less oxidised in Ncf1-/- mice and have higher levels of reduced thiols on the cell surface. -SH on the surface of T cells is regulated by the release of ROS, produced by and that the level of –SH influences activation, RA development and proliferation (K. A. Gelderman et al. 2006). Additionally, the –SH may be determined by other cells within the surrounding that are responsible for producing ROS including APC and neutrophils (Matsue et al. 2003). Hence it forms an essential step in regards to T cell function as these changes are observed during thymic selection and direct interaction of APC to T cells during antigen presentation which requires a reducing extracellular environment (Angelini et al. 2002; Oliveira-dos-Santos et al. 1998; Yan & Banerjee 2010). The present study involved 3 mice per group due to the susceptibility of CIA in B6 mice, which limits the confidence in the data shown, increasing the number of mice per group would sufficiently power the result obtained. High individual variability on the results contribute to the lack of statistical significance in the results.

The proportion of T cells expressing Trx1 on the surface show no difference between WT and Ncf1-/- mice, but an increased trend in the number of WT $CD4^+$ T cells expressing Trx1 on the surface compared to naïve $CD4^+$ T cells. The proportion of T cells expressing Prx2 on the surface was however increased in immunised mice (WT and Ncf1-/-) compared to naïve. This

chapter looked at the percentage of cells expressing Trx1/Prx2 on the surface whereas previous chapter looked at median X (surface expression). This was due to the variability in the MdX obtained from lower number of mice. Increasing the number of mice per group will provide the quantitative and powered analysis of Trx1/Prx2 expression in $CD4^{+}$ T cells in the future.

Earlier observations in the literature described that a higher number of cells expressed Prx2 on the surface of RA cells compared to normal lymphocytes but there was no difference in the secreted Prx2 levels in the plasma (Szabó-Taylor et al. 2012a). However, higher proportion of $IL17^{+ve}$ T cells showed Prx2 on the surface compared to total lymphocytes suggesting it may promote redox mediated signalling on the surface of pro-inflammatory T cell subsets. Therefore, it may be possible to suggest that Prx2 maintains a reducing environment on the surface which contributes to T cell activation seen in RA. The functional role of increased Prx2 can be studied in the future including the regulation of inflammation and apoptosis in T cell subpopulations (Mullen et al. 2015a).

Increased attention has grown over the past few years on the roles of other phagocytic cells including monocytes and neutrophils cells in the progression of the autoimmune RA. Neutrophils are predominantly present in the synovial fluid making up to 90% of the cells during the peak phase of the disease (Edwards & Hallett 1997). Studies with depletion of neutrophils *in vivo* have shown the impairment of lymphocyte and monocyte recruitment to the inflammatory sites. This possibly leads to decreased production of autoantibody in joints hence diminishing the progression of the disease (Schimmer et al. 1997; Wipke et al. 2004). Neutrophils from RA synovial fluid produce increased amount of ROS as compared to peripheral neutrophils (Capsoni et al. 2005). This chapter has looked into the distribution of Trx1 and Prx2 in neutrophils from CIA induced mice. Mice with NADPH oxidase deficiency show increased number of neutrophils expressing Prx2 on the surface suggesting the possible role of Prx2 in activated neutrophils in the periphery. Additionally, similar trend of increased neutrophils expressing exofacial Trx1 was observed in NOX deficient mice. Also, an increase

in surface Trx1 was also seen in immunised WT mice as compared to naïve. Trx1 on the surface of neutrophils may modulate clearance through phagocytic mechanisms. In mice models of RA, neutrophils have been suggested to a play a critical role in the progression of the disease by generating autoantigens (Wright et al. 2014), however they may be also powerful inducers of apoptosis in bystander cells. The T cell redox status is affected by ROS produced from macrophages/monocytes but also granulocytes, presumably neutrophils, which are believed to be strongest ROS producers (Hultqvist et al. 2007). Neutrophils and monocytes have increased NADPH oxidase activity and the redox state of these neutrophils is regulated by antioxidants (Abbas & Monireh 2000). Increased neutrophils survival in the joints associated with increased ROS production may promote oxidative damage in the joints (Phillips et al. 2010). Hence, further understanding is needed regarding the role of neutrophils in RA.

The T cell plays an important role in the development of CIA and hence the cytokine profile of T cells was assessed after re-stimulation with anti-CD3/CD28 antibodies. Restimulation of activated T cells is widely used to characterise T cell responsiveness and cytokine profile (Sharon H. Jackson et al. 2004). This chapter describes the responsiveness of T cells from CIA induced WT and Ncf1 mice upon antigen stimulation including anti-CD3/anti-CD28 and CII. Additionally, the cells were either treated with ROX 2. Upon stimulation with anti-CD3/CD28 antibodies T cells from both groups (WT and Ncf1-/-) show a significant increase in IL-2 secretion suggesting the activation of T cells in response to an antigen is not compromised by lack of NOX2. No difference in IL-2 secretion was seen in Ncf1-/- mice when stimulated in the presence ROX 2 further supporting the fact that the compound is specific to functional NADPH oxidase as a significant decrease was observed in T cells from WT mice. Furthermore, a similar result was observed when splenocytes were restimulated with CII stimulation. However the responsiveness of T cells to CII antigens was approximately 10 fold lower, the stronger cytokine response to anti-CD3 may suggest functional potential of cells from both the groups (WT and Ncf1-/-) mice and CII immunisation has no effect. CII stimulation in T cells suggests more sort of a physiological response to CII antibodies. Interestingly, cells

from naïve Ncf1-/- mice show a response to CII whereas naïve WT T cells show no secretion of IL-2 when stimulated with CII. This confirms that general responsiveness and increased T cell activation is only detectable in antigen stimulated cells which may not be affected by ROS (Gelderman et al. 2007). However, the mice weren't immunised with CII and this may be explained by the hyper-responsiveness of naïve T cells to CII in the absence of NOX2, again suggesting a regulatory role for ROS. Others have evaluated the CII specific immune response at different stages of CIA and shown that the T cell response to CII is dominated by Th1 cells that produce IL-2, which initiates pathogenesis of CIA (Rosloniec et al. 2002; Batsalova et al. 2012). Another study showed a strong proliferative responses against CII (mouse, chicken and bovine) suggesting the T cell reactivity and autoimmunity evidence (Inglis et al. 2007). A significant decrease in IL-2 from WT T cells upon CII stimulation further suggests the inhibitory effect of ROX 2 on T cell response against CII antigen. How ROX compounds inhibit T cell activation *in vitro* and *ex vivo* is unclear and hence a further experiment followed that involved treating mice immunised with CIA with ROX 2 to observe whether it affects arthritis severity *in vivo*. Similar studies have shown the prevention of T cell activation in the presence of these compounds *ex vivo* and decreased T cell transfer autoimmunity by NOX2 knockout (Hultqvist et al. 2015a). It was shown that ROS produced by NOX2 expressing cells have the ability to alter T cell reactivity and function during antigen presentation by shifting the redox state in both, intracellular and extracellular of neighbouring cells as the ROS produced was detected in intracellularly and in mitochondrial matrix (Enyedi et al. 2013). Hence a further experiment was designed to treat CIA immunised mice with ROX 2 to observe whether it affects arthritis severity in vivo.

CIA, the most common form of induced arthritis comprises of two phases, an immune response and inflammatory process (Nandakumar et al. 2003a). The development of CIA

depends on the strain with greater susceptibility in DBA1 and B10 strains (Inglis et al. 2007). B10 mice developed severe and chronic arthritis on immunisation with rat CII (Bajtner et al. 2005). This results in this chapter shows a mild arthritis in WT B6 mice. The severity of CIA depends on the breed of the mice and the CII used. Others have shown milder CIA in B6 mice compared to DBA1 suggesting B6 strain is somehow resistant to CIA (Asquith et al. 2009). This may be due to the collagen type used as chicken type 2 CII is widely capable of inducing the disease in B6 strain (Campbell et al. 2000; Campbell et al. 1998). In comparison to DBA1 mice developed arthritis in all cases of chicken, bovine and mouse CII. This in fact may explain the resistance of B6 to CIA may vary on the type of collagen used (Pan et al. 2004). Also, other factors may be the quality of CII emulsion preparation and how they have been immunised as the location at the base of the tail and a clear suspension should be carefully administered. Also, the incidence of CIA in B6 strain is variable across different sub strains of B6 (Asquith et al. 2009).

This variability may raise an interesting question on the therapeutic outline of CIA in different strains of mice. Available redox-modulatory therapies of RA include methotrexate, auranofin and penicillamine (Suarez-Almazor et al. 2000). The therapeutic action of methotrexate in B6 mice was indeed effective compared to DBA mice, as DBA mice are shown to be resistant to methotrexate treatment (Delano et al. 2005). The anti-inflammatory effect of methotrexate is to increase ROS levels which in turn reduces inflammation (Phillips et al. 2003). Auranofin, used in previous chapter is a gold compound which limits the progression of arthritis perhaps by inhibiting the activation of monocytes and other blood cells and lowered secretion of cytokines including TNF-α. additionally, several lines of evidence suggests that auranofin may act by altering the redox balance by oxidising Trx1 and activation of Nrf2 (Omata et al. 2006). however, the use of these compounds may have serious toxic side effects and proteinuria (Omata et al. 2006; Eisler 2003).

T cells in human synovial joints display markers of activation but show decreased proliferative response such as IL-2 and IFNɣ secretion, this is due to low TCR signalling which may be due

to loss of GSH (Maurice et al. 1997). However, there is little understanding on the role of Ncf1 in the determination of T cell activation. The studies so far have directed towards the intracellular ROS produced by the T cells itself as a result of metabolism (Kwon et al. 2010). Interestingly it was observed that DCs were responsible for a reduced environment during interaction with T cells and macrophages(Yan et al. 2009) which lack NOX2 function allow priming of CII reactive T cells, eventually leading to arthritis (Pizzolla et al. 2011).

This led to the hypothesis that ROS produced by activating the NOX2 expressing cells may alter T cell activation during antigen presentation (Hultqvist et al. 2009b; Holmdahl et al. 2013). ROX 2 compound was used in CIA mice as a potential therapeutic agent to investigate if enhanced ROS production by activating the NOX 2 complex reduces severity of arthritis. The results obtained were promising as the WT mice show a decreased severity in CIA at a later onset of the disease. It was also shown that the compound is targeting NOX2 complex as Ncf1-/- mice show no change in severity when treated with the compound. Similar work has been carried out using the same NOX2 agonist (ROX 2) in pristine induced lupus (PIL), where the compound led to decreased autoantibody production in WT mice compared to untreated vehicle. It also showed a strong trend towards diminishing PIL in WT mice and Ncf1-/- mice had no ROS production compared to WT (Kienhöfer, et al. 2017). This supports the idea of increased ROS by activators could serve as potential therapeutic agents by specifically targeting cells expressing NOX2. However, it is necessary to determine the consequences of targeting NOX2 in diseases as it may lead to different outcomes depending on specificity, dose and production of ROS (Hultqvist et al. 2015b). The intervention might shift the balance towards an anti-inflammatory state either by promoting apoptosis or cytokine degradation.

This chapter has described the timing of redox change in immune cells on activation in the presence and absence of NOX2 compounds and has explored the potential role of NOX2 activators during T cell activation in the context of RA. The data obtained shows that intracellular ROS, surface Trx1 and Prx2 are inhibited by ROX compounds and that this associates with lower intracellular GSH, reduced cytokine secretion and CD25 expression.

Future investigation into membrane partner proteins of Trx1 and Prx2 and their oxidation state would be of interest and to understand whether de novo DNA synthesis may be inhibited, possibly inhibiting proliferation. Together this also suggests a new strategy to modulate T cell functions by targeting NADPH oxidase complex to reduce arthritis severity. However, the relation between ROS production by granulocytes/APC and autoimmune arthritis, which is mainly mediated by T lymphocytes needs further clarification.

# 7. General Discussion

The role of ROS in T cell function is complex and its effects depend on the amount, the location and the source. Large quantities may lead to oxidative stress but controlled release of significant ROS is essential for cell signalling. Signalling ROS target free cysteines on proteins leading to reversible protein oxidation. Regardless of the source, ROS have clearly been shown to affect T cell downstream pathways and hence T cell functions. Mitochondria may be an important source of ROS (Murphy & Siegel 2013; Torrao et al. 2014), that can induce cell differentiation through metabolic programming. Considering the intracellular effects, $H_2O_2$ has been shown to directly influence the activity of protein tyrosine kinases by oxidising the Cys residues on the site other than catalytic site. (M. Lee et al. 2011; Paulsen et al. 2012; Yoo et al. 2011). A number of studies have focused on protein modifications after T cell activation, but have not selectively focused on the reversible cysteine modification, hence cysteine modification has become of increasing interest (Kesarwani et al. 2013b; Simeoni & Bogeski 2015a)

Oxidative modification of cysteine to cysteine sulphenic acid may play an essential role in protein function and signalling pathways. However further oxidation in the presence of excess ROS may lead to irreversible cysteine oxidation which may lead to protein dysfunction. There is accumulated evidence suggesting the presence of oxidatively modified proteins in disease including RA (Phillips et al. 2010). Given the essential role of cysteine oxidation as a modulator of cell signalling, cysteine sulphenic acid may act as both a product and an intermediate of signalling processes (Furdui & Poole 2014). Cysteine sulphenic acid detection has always been challenging to study, until recently when advanced techniques became available, due to its unstable nature.

This study used a biotin tagged dimedone based approach to detect surface –SOH followed by identifying specific proteins which undergo protein sulphenic acid modifications on the membrane and cytosol. The dimedone based probe conditions were optimised in chapter 3 identifying protein sulphenic acid formation in the cytosol and membrane in Jurkat T cells. This

was achieved by prior incubation of dimedone which allows capturing of CysSOH species as they are formed following TCR stimulation. The limitation of this approach may be the free probe interfering with the biotinylated proteins during affinity bead capture. Reports suggest that reactivity of dimedone towards non-CysSOH derivatives is low (Furdui & Poole 2014), but removal of the free probe would eliminate any non-specific binding. Furthermore, additional steps will be required to remove contaminants depending on downstream application. For example, sticky proteins can be removed using a pre-clearing step and stringent washing using DTT for mass spectrometry analysis.

Following the model established using Jurkat T cells to detect the sulphenated proteins and redox systems under oxidative stress, this thesis looked at the proteins susceptible to undergo sulphenic acid modification during $CD4^+$ T cell activation in the presence or absence of $H_2O_2$. T cells encounter ROS including $O2^{\bullet-}$ and $H_2O_2$ immediately after TCR stimulation in small amounts (Devadas et al. 2002; Belikov et al. 2014; Jackson et al. 2004), which can react with cysteine residue to form sulphenic acid due to increased ROS release from the phagocytes/APC (Kettenhofen & Wood 2010). Firstly, the total sulphenic acid content was analysed by flow cytometry using biotin tagged DCP-Bio1 and streptavidin-HRP which allowed fluorescent detection of –SOH proteins on the membrane shown in chapter 5.

Activated $CD4^+$ T cells show an increase in sulphenic acid modification on the membrane after 5min of activation compared to unactivated T cells. Early time points of 5 and 30min were mainly focussed upon in this study due to the transient nature of sulphenic acid intermediate and TCR signalling. It was critical to find out what proteins may be susceptible to oxidation on the membrane during T cell activation.

Protein oxidation was further studied in chapter 3 using Jurkat T cells, which show protein aggregates, possible disulphide formation and carbonylation. In the presence of $H_2O_2$, the T cell cytosolic and membrane protein show distinct bands suggesting aggregate formation, possibly due to disulphide formation as these bands were not visible under reducing

conditions. Moreover, significant bands shown in the carbonyl western blot indicate that general protein oxidation was increased in the presence of $H_2O_2$.

Using different concentrations of the $H_2O_2$, it was possible to detect the first reversible oxidation of cysteines as under concentrations of 2mM $H_2O_2$, the band intensity in streptavidin HRP western blot decreased suggesting high concentrations may lead to further protein oxidation including sulphinic or sulphonic which are not detected by DCP-Bio1, and or formation of aggregates. Therefore an optimum concentration of 200µM was used to capture the sulphenated proteins in the membrane and cytosol. Mass spectrometry analysis identified a list of proteins with suphenic acid modifications among which NMDA and Prx would be an interesting target to further explore in T cell responses.

The NMDA receptor plays a key role in T cell activation by maintaining cellular $Ca^{2+}$ influx. Mass spectrometry results indicated that NMDA receptor is susceptible to sulphenic acid modification at rested stage. In vitro analysis confirmed that increased oxidative stress induced by $H_2O_2$ decrease cellular $Ca^{2+}$ influx. While NMDA is not a key receptor for $Ca^{2+}$ signalling several other studies have shown that oxidative stress may inhibit $Ca^{2+}$ by oxidising other calcium channels such as Orai1 on the membrane of T cells (Bogeski et al. 2010).

This thesis also identified the distribution of the critical reducing enzyme/protein Prx2 and Trx1 providing insight on localisation in the membrane of T cells under different redox conditions. From the proteins identified from MS, a small number of them are involved in oxidation-reduction process as shown in the chart characterising proteins based on their molecular function, including Prxs, alpha enolase and protein disulphide isomerases. Under normal physiological conditions, these oxidised proteins are readily reduced via the presence of Trx1, GSH, Grx and Prxs (Salmeen et al. 2003). There are two prominent cellular physiological electron donors for protein reduction, GSH and Trx1 (Holmgren 2000). Oxidised Trx1 is reduced by TrxR in the presence of NADPH where as GSH reduces proteins either directly or indirectly through the enzyme Grx.

ARF depletes cellular GSH levels in the cells suggesting that Trx1 and GSH might depend on each other to respond to oxidative stress, given the relationship of Trx1 and GSH in the cytosol (Jones & Go 2010). Trx1 and GSH regulates the redox state of proteins within the cell either by scavenging peroxides via Grx and Prx/Trx1 pathways or reducing oxidised cysteine thiols (Holmgren 2000; Dickinson & Forman 2002). A broad smear in the absence of β-mercaptoethanol suggests that proteins attached to Trx1 are linked by a disulphide bridge under non-reducing conditions. Trx1 has a number of binding partners in the cytosol as stated in the literature (Carilho Torrao et al. 2013), but further work to identify Trx1 binding partners on the membrane by western blotting of the targeted protein would provide potential new significant interacting partners involved. Trx1 and Prx2 are widely associated with the membrane of lymphocytes (Carilho Torrao et al. 2013; Szabó-Taylor et al. 2012a; Mullen et al. 2015a). This thesis looked at the distribution of Trx1 and Prx2 and how it may be affected in the presence of BSO, ARF, $H_2O_2$ and T cell activation. This may explain the redistribution of Trx1 and Prx2 to shed light as Trx1 and Prx2 is exported via a leaderless secretory pathway (Mougiakakos et al. 2010b; Salzano et al. 2014a). ARF leads to an increase in surface Prx2 and Trx1. It is however not clear of how inhibiting the TrxR enzyme leads to distribution of Trx1 and Prx2 to the membrane and awaits further investigation.

The extracellular environment during an immune response is more oxidised compared to the intracellular compartment (Hanschmann et al. 2013b; Belikov et al. 2014). This may provide an explanation for the increase in the translocation of Trx1 and Prx2 to the surface during T cell activation. Potential functions have been described previously in this thesis, but primarily it could be involved in the elimination of ROS which is present in higher amounts in the milieu or due to leakage through phagosomes. It is not anticipated that the redistribution of Trx1 to the extracellular surface may influence the surface redox state but its regulatory role of catalysing chemical reduction of receptors may be promoted due to high presence of ROS on the surface. Also, the interaction between Trx1 and surface receptors such as CD30 may suggest a link to regulate further lymphocyte function in an oxidative environment (Schwertassek et al. 2007). Similarly, this study did not measure Prx2 in the culture

supernatants. A study by Salzano et al. clearly showed the release of Prx2 and Trx1 from macrophages upon LPS stimulation via a non-classical secretory pathway (Salzano et al. 2014b).

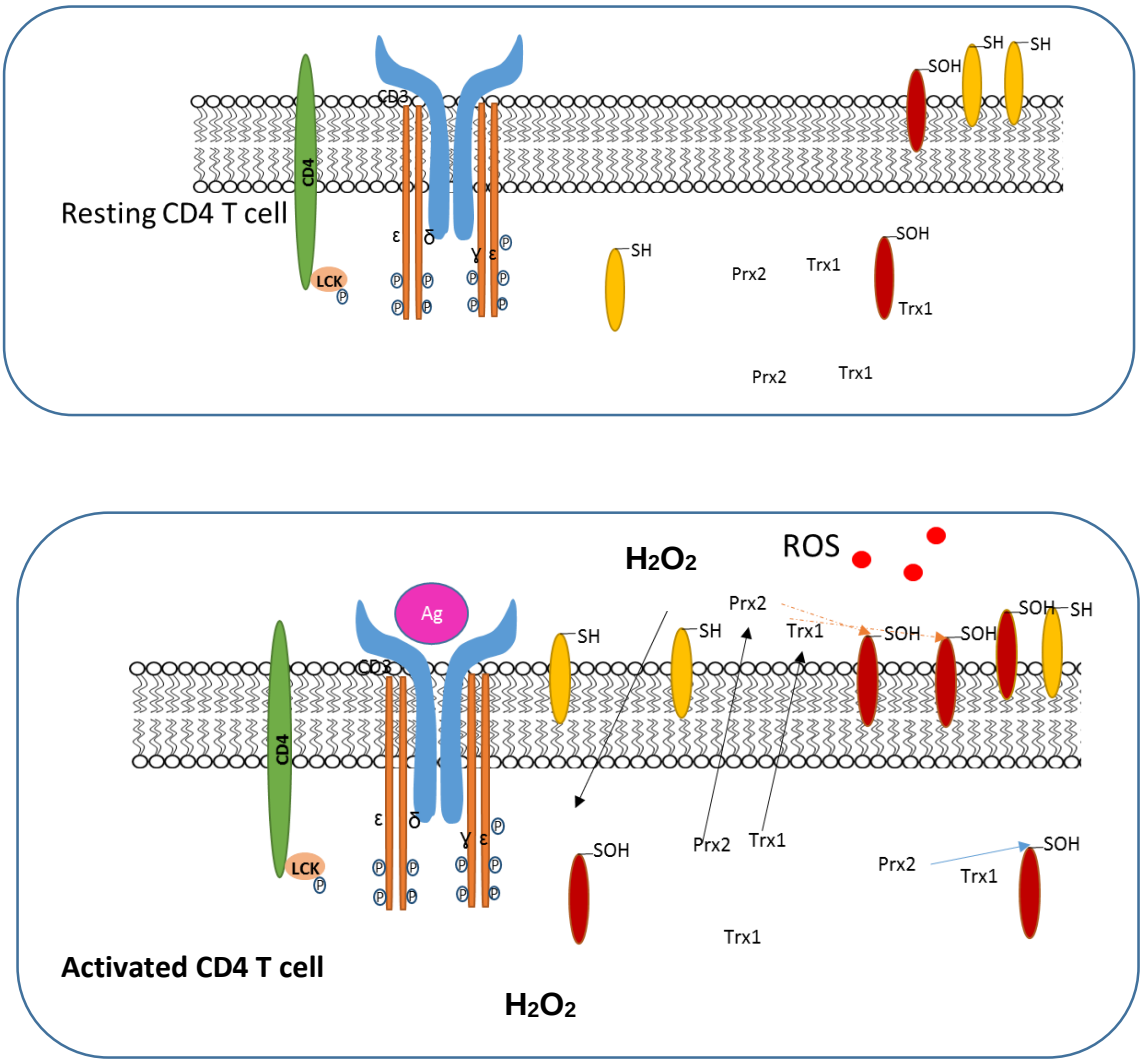



Figure 7.1: Activation of T cells. Upper panel- resting $CD4^+$ T cell, whereby no antigen interaction. Lower panel- Activated $CD4^+$ T cell showing T cell activation leads to increase in –SH and –SOH on the surface and the redistribution of Trx1/Prx2 to the membrane possibly responsible to interact with binding proteins on the surface indicated with orange dotted arrow.,

The interaction of the TCR and MHC II leads to the release of extracellular ROS including $H_2O_2$ by APC and T cells suggesting that APC modulate T cell proliferation by regulating the availability of thiols including cysteine and thioredoxin (Hadzic et al. 2005). Exogenous treatment with $H_2O_2$ shows a significant decrease in T cell activation. Surface expression of CD25 along with IL-2 secretion was markedly decreased by $H_2O_2$. Also, the decrease in GSH in the presence of $H_2O_2$ suggests T cells with depleted GSH negatively affects the adaptive immune response and T cell proliferation by inhibiting IL-2 dependent DNA synthesis and

restoration of GSH elevated Th1 cytokine response (Rose et al. 2012; Gmünder et al. 1990; Liang et al. 1989). The mechanism of how an increase in ROS affects T cell activation is unclear but evidence suggests that higher amounts of ROS in the extracellular environment may lead to increased oxidation of certain receptors and proteins and may overoxidise intracellular proteins that support signal transduction crucial for downstream TCR signalling pathways intracellularly (Phillips et al. 2010), for example protein tyrosine kinases and phosphatases including ZAP70 and calcium channels (Bogeski et al. 2010) (briefly shown in Figure 7.2 and 7.3). PTP are generally impaired by ROS where as PTK are hyper activated, both of which may modulate T cell proliferative responses, so the net effect of moderate ROS generation is for MAP kinase signalling to be active (Chiarugi & Buricchi 2007; Salmeen & Barford 2005). It is important to note the sources of ROS, that includes the NADPH oxidase from surrounding macrophages and neutrophils may have a significant impact in the intracellular ROS in T cells (Hultqvist & Holmdahl 2005). Also, to note, the mitochondria as a source of ROS at later stages of T cell activation (Belikov et al. 2014). Extracellular ROS may affect the surface proteins and surface redox state but intracellular ROS (e.g. mitochondria) may regulate the downstream signalling. Mitochondrial ROS controls T cell activation by regulating IL-2 expression (Kaminski et al. 2010).

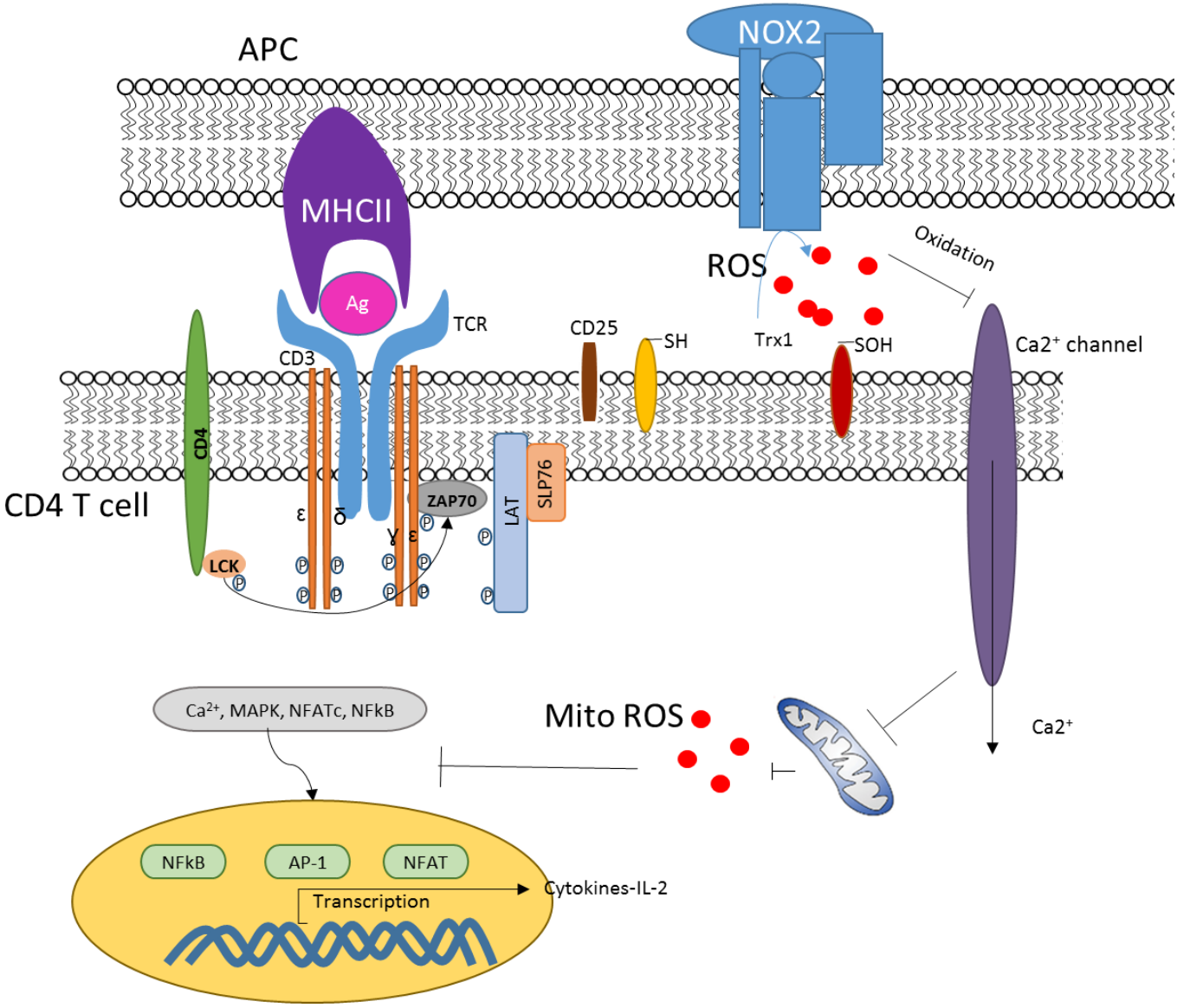



Figure 7.2: ROS signalling in T cells. Extracellular source of ROS from NADPH oxidase may lead to oxidation of proteins on the surface preventing release of ROS from the mitochondira which in turn inhibits signalling and activation.

A number of proteins identified are mainly present in the mitochondria and cytosol. This may be due to the technique used to isolate membrane proteins and hence the fraction involves enrichment rather than complete separation.

The treatment of T cell membranes with $H_2O_2$ did not yield any additionally modifiable proteins. This may be due to the transient nature of sulphenic acid modification or the limitations in the

experimental approach. It is possible that the experimental techniques and handling leads to observed baseline oxidation of susceptible thiol moieties on proteins. Therefore, care should be taken on handling, temperature and buffer conditions to minimise any additional oxidation.

This thesis then applied the knowledge gained through *in vitro* studies to investigate the role of ROS in T cell activation in a rheumatoid arthritis disease mouse model. Defective NOX is widely associated with various animal models of autoimmunity via a polymorphism in Ncf1 gene resulting in diminished production of superoxide and increased susceptibility of arthritis (Olofsson et al. 2003). ROS produced by NADPH oxidase complex affects the severity of arthritis and development as shown in this thesis and others. NOX 2 compounds were used to establish a model to analyse the effects on T cell activation and surface redox state *in vitro* using human primary $CD4^+$ T cells. Previous work showed significant decrease of RA severity by activating ROS release by NADPH oxidase and mice deficient of functional NADPH oxidase show increased severity of autoimmune disorders including RA and SLE. The compounds had a profound effect on T cell activation by inhibiting IL-2 secretion *in vitro*. Furthermore, to evaluate whether the compounds increase ROS production in T cells, intracellular ROS was measured after activation in the presence of the compounds using DCF. Interestingly, the compounds did not show an increase in intracellular ROS but a decrease after 24h of activation suggesting possible inhibition of intracellular ROS production by the mitochondria. On the other hand, splenocytes stimulated with ROX 2 showed an increase in extracellular superoxide production using isoluminol as the detector as shown in chapter 6. Subsequently, this compound has shown to release ROS by NOX2 from peritoneal cells, which includes T, B cells and macrophages, and cells from NOX deficient mice show no response to the compound (Kienhöfer et al. 2017).

In vitro studies show that Jurkat cells treated with ARF; a compound that inhibits the enzyme TrxR, show an increase in surface Trx1. This might suggest that Trx1 translocates to the membrane in the absence of reducing enzyme. The T cells from WT and Ncf1-/- mice show

no significant difference in expressing exofacial Trx1, but an increased trend in the number of immunised WT $CD4^+$ T cells expressing Trx1 on the surface compared to naïve $CD4^+$ T cells. The proportion of T cells expressing Prx2 on the surface was however increased in immunised mice (WT and Ncf1-/-) compared to naïve. This may also be supported by earlier observation in the literature that a higher number of cells expressed Prx2 on the surface of RA Th17 cells compared to normal lymphocytes (Szabó-Taylor et al. 2012a). Thus, Prx2 and Trx1 may regulate autoimmunity through their oxido-reductase activity of membrane disulphides or thiols as hypothesised previously. In general, the main limitation of this study is the low number of mice showing severity of arthritis. This is an important factor that can be overcome by the repetition of the study using a more susceptible strain to induce CIA in WT and Ncf1-/- mice. This additional work will possibly have the potential to understand the relevance of surface redox index in CIA. Despite this, this study had an innovative approach whereby the *in vitro* study was used to develop methodological optimisation. Therefore, *in vivo* study was crucial to possibly identify the effect of the compound in disease and the potential of ROS deficiency.

Promising results from the compound *in vitro* human $CD4^+$ T cells allowed follow up *ex vivo* stimulation of immunised WT and Ncf1-/- mice splenocytes with anti-CD3/CD28 and CII. A significant decrease was seen in IL-2 secretion in T cells from WT only when stimulated in the presence of ROX 2. This was an interesting finding as no effect was observed in T cells from Ncf1-/- mice in the presence of ROX 2 and hence the compound specific to functional NOX2 enzyme. Also, further experiment followed treating mice immunised with CIA with ROX 2 to observe how it affects arthritis severity *in vivo*. Routine treating the mice with this compound showed favourable outcome as the WT mice show a decreased severity in CIA at a later onset of the disease. It was also shown that the compound is targeting NOX2 complex as Ncf1-/- mice show no change in severity when treated with the compound. Similar work has been carried out using NOX2 agonist in pristine induced lupus (PIL), whereby the compound (ROX 2) led to decreased autoantibody production in WT mice compared to untreated vehicle. It also showed a strong trend towards diminishing PIL in WT mice and Ncf1-/- mice had no ROS production compared to WT (Kienhöfer et al. 2017). Moreover, one other readout to determine

the severity of arthritis either *ex vivo* stimulated cell supernatants or in plasma from these mice would be collagen II ELISA, which detect anti-type II collagen antibodies. It is understood that anti-type II collagen antibodies are significantly increased in RA NOX2 deficient mice (K. Lee et al. 2011).

From this study, the use of NOX2 agonist and the effect may vary such that; the cell type targeted to produce ROS, the location of ROS production being extracellular, cytosol or the intracellular organelles, timing of the production of ROS and the target cell or receptor. These issues needs to be clarified before further use of these agonists as therapeutic targets. According to the screening of these compounds, different compounds appeared to target various pathways and could possibly provide an insight of the molecular mechanism as to how NOX2 derived ROS may protect from autoimmunity (Hultqvist et al. 2015a).

The increased severity of arthritis in mice with Ncf1 mutation (chapter 6) was also previously shown that low ROS as a result of dysfunctional NADPH oxidase, led to higher immune response to CII in mice and rats (Holmdahl et al. 2016). NOX released ROS protects against progression of the disease by down regulating T cell activation (Gelderman et al. 2007). NADPH oxidase not only expressed in granulocytes but also expressed in APC including macrophages and not in T cells (Kobayashi et al. 1995; Mizuki et al. 1998).

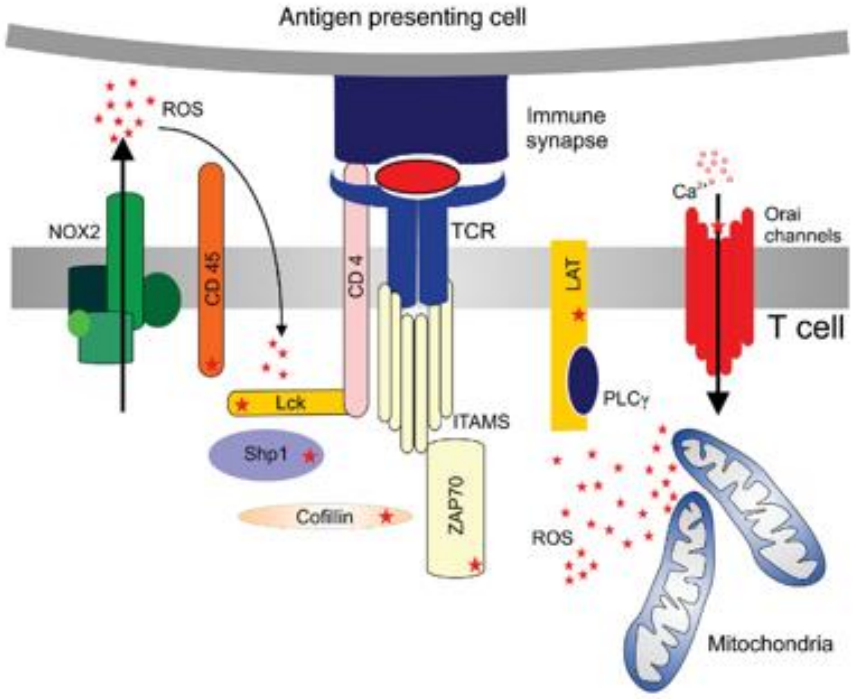



Figure 7.3: Different components of downstream TCR signalling regulated by the presence of ROS. The engagement induces a cascade leading to ROS production (red stars) extracellularly and from the mitochondria. Possible targets of oxidation indicated with a red star. Adapted from (Simeoni & Bogeski 2015a)

Also, decreased T cell receptor signalling may enhance T cell auto reactivity to antigens including CII. Sakaguchi et al. reported that a mutation in Zap70 lead to increased arthritis in mice (Sakaguchi et al. 2003). T cells from synovial joints of RA patients show increased oxidative stress which presumably affects LAT signalling suggesting possible regulatory mechanism of ROS in human RA (S. I. Gringhuis et al. 2000).

ROS play roles firstly in the interaction between the APC and T cells that occurs at several stages during T cell development in the thymus, periphery and migration, a decrease in the APC oxidative burst response may affect the interaction leading to enhanced T cell function. T cells from low ROS production mice showed efficient transfer of the disease compared to T cells from functional NOX2 (Olofsson et al. 2003). Hence, it possible that ROS produced by APC may have an impact on the interacting cell such as T cells. Restoring NOX2 functions in macrophages but not DCs by reinstating Ncf1 mutation in CD68 promoter. This showed protection from severe arthritis suggesting that surface and intracellular pathways are affected by ROS produced by APC (Gelderman et al. 2007; Hultqvist et al. 2015a). Recently, NOX2 derived ROS have also been shown to differentiate Th17 subsets into Th2 phenotype (A. V Belikov et al. 2015). Therefore, it is critical to maintain the equilibrium between the ROS produced and antioxidant levels with surface redox state possibly playing a role, and this crucial balance is believed to be disrupted in RA.

## Conclusions

This thesis has shown that protein oxidation in Jurkat T cells occurs in the presence of oxidative stress e.g. BSO, ARF and $H_2O_2$ and that proteins undergo sulphenic acid modification in the membrane and cytosol. Further manipulation of the redox state towards and oxidising potential within cells by interfering GSH or Trx1 signalling pathways increases the redistribution of Trx1 and Prx2 onto the surface. For the first time, proteins on the membrane have been identified to undergo sulphenic acid modification during T cell activation. The presence of Trx1 and Prx2 on the surface of T cells from CIA mice and any relationship with regulating other protein activity requires further understanding *in vivo*.

## Future work

Several studies can be suggested to support and complement the approach used to identify sulphenated proteins and possible disulphides formed here. The method used here *in vitro* has though identified susceptible proteins on the membrane of $CD4^+$ T cells during activation. However, to further quantify the data obtained on the oxidative sulphenic acid formation in proteins and abundance in complex samples, quantitative proteomic strategies which include label-based and label free approaches. The use of tags such as iTRAQ (isobaric tags for relative quantification), which labels primary amino acid in combination with NEM. This is useful to identify redox sensitive moieties such as sulphenic acids. Also, ICAT (isotope coded affinity tags), which is commercially available tags based on IAA reactive thiols. This can be effectively used to detect and quantify cysteine oxidation. Similarly, it is important to understand the role of sulphenic acid modification by identification and how these proteins including Trx1 and Prx2 on the surface may possibly modulate T cell activation. This can be achieved by adding exogenous recombinant Trx1 or Prx2 to determine the effects of these reducing proteins on the surface of T cells during activation. Trx1 has been shown to reduce

inflammatory response possibly by inducing the expression of genes involved in antioxidant activity. Also, presence of exogenous Trx1 will increase the chance of it binding to the surface proteins hence using advanced techniques of isolating membrane proteins and CO-IP, further studies can possibly identify the interacting partner on the membrane. There is evidence that thiol oxidation, and disulphide bond formation controls further signalling in cells and so Trx1 present will bind to target proteins. This will be an interesting study as these surface receptor can further be targeted to modulate an immune response. The first reversible oxidation is cysteine sulphenic acid which is an intermediate step before over oxidation or binding to target proteins. The physiological significance of Trx1 binding to other proteins is yet to be determined. The bands observed under non-reducing conditions can be further looked into using techniques such as mass spectrometry to identify the proteins. Analysing the expression of Trx1 at gene and protein level will aid in the better understanding of the intracellular molecular mechanisms involved. Also, it will be essential to repeat the membrane protein isolation using different techniques such as biotinylated based methods that use reagent which is cell permeable labelling the exposed primary amines on the surface of intact cells.

Since $Ca^{2+}$ signalling is one of the first early response upon antigen stimulation, the readout of calcium influx is challenging but using developed techniques such as self-injecting plate reader with real time data acquirement, it is possible to analyse calcium influx in activated T cells in the presence of $H_2O_2$ in the future to explore the effects of oxidation. This may include cells with reduced NMDA function and how they may respond to T cell activation in the presence or absence of peroxide.

In order to understand the impairment of the IL-2 T cell response in the presence of NOX2 agonists, gene expression of IL-2 would ideally be a preferable indicator. Whether the decrease in IL-2 secretion is down to the transcription level or the translation of IL-2 protein or as a result inhibited secretion. Other possible readout would be longer activation times to evaluate the effect of the compound on T cell proliferation by looking at CFSE dye dilution on

FC. These experiments would provide a better understanding on the proliferation of T cells in the presence of these compounds taking toxicity into consideration.

CIA serum redox markers can further complement the results obtained in terms of the redox status in the peripheral environment. Plasma GSH/GSSG will further provide an indication of oxidised and reduced GSH levels. Also, mouse specific ELISAs to detect plasma Trx1 in the future in WT and Ncf1-/- mice immunised with CII, detect Trx1 in plasma of these mice to be able to evaluate the presence of Trx1 in high concentration is associated with RA (Jikimoto et al. 2002). Autoantibodies against collagen type II plays an important role in the pathogenesis of RA, using CII ELISA to detect CII antibodies in the plasma of WT and Ncf1-/- mice treated with the compound would provide information of the concentration of antibodies and their role in RA incidence.

Appropriate techniques for ROS measure in the cells from the treated mice will provide an indication of what the compounds do during T cell activation, and as to whether they release or inhibiting superoxide anion radical formation thus decreasing arthritis severity

Additionally, the use of ROS inducing drugs in this study has shown promising effects on the severity of CIA. Further immunological pathways of the new compound in the treated CIA mice will complement the results obtained and provide the insight of how they may affect T cells. ROS generated form macrophages is likely to affect T cell activation during antigen presentation presumably by oxidising the TCR signalling pathway. Identification of the target mechanism would possibly allow targeting specific protein hence minimising side effects. Additional investigation of the pharmaceutical efficacy and safety may lead to the development of a strong effective ROS inducer targeting NOX2. These experiments may include looking at different T cell subsets and redox markers, specifically focussing on the proliferative response of $CD4^+$ T cells to Th17 subsets as previous work has shown increased surface Prx2 levels in human RA Th17 cells.

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