RED BLOOD CELLS: THE IMMUNE SYSTEM'S HIDDEN REGULATOR

INVESTIGATION INTO THE ROLE OF RED BLOOD CELLS IN INFLAMMATORY CYTOKINE SIGNALLING

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The University of Sydney

Faculty of Medicine

DECLARATION

I certify that the content of this thesis is my own work. This thesis has not previously been submitted for a degree, nor has it been submitted as part of requirements for a degree to any other university of institution other than the University of Sydney.

I certify that all the information sources and literature used in the preparation of this thesis are indicated. I also certify that the thesis is an original piece of research and that it has been written by me. Any help and assistance that I have received in my research work and the preparation of the thesis itself have been appropriately acknowledged and is summarised as follows:

A/Prof. Benjamin Herbert: Project design, data interpretation, thesis editing
Dr. Edmond Breen: Statistical analysis of multiple cytokine data in Chapter 4 and Chapter 5

The research presented in this thesis was initiated at Macquarie University and was approved by the Macquarie University Human Research Ethics Committee (reference number: 5201100827). I transferred this degree to the University of Sydney in July 2015, and this study was then approved under the Northern Sydney Coast Human. Research Ethics Committee of NSLHD and CCLHD (reference number: 1201-046M). The work outlined in this thesis was completed within the Northern Clinical School of the University of Sydney under the supervision of A/Professor Benjamin Herbert.

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PUBLICATIONS

The data in this thesis are included in three patent applications, the first of which is due to publish in April 2017. As such, the results of this thesis are confidential and have not yet been published in peer-reviewed journals. Further detail on these patents are outlined below and are included in Appendix I.

Title of patent	Date of filing	Status
Blood preparation and profiling	October 2015	PCT
Therapeutic methods using erythrocytes	December 2015	PCT
Blood profiling with protease inhibitors	December 2016	Provisional

ABSTRACT

Red blood cells are the most abundant cell type in mammals, although, they are mostly described as inert carriers of haemoglobin that function only in gas exchange and transport. Evidence is now mounting that these enucleate cells are more complex than previously understood. Studies have reported that red blood cells from healthy individuals regulate immune cell activity and maturation, but red blood cells from inflammatory disease cohorts are dysfunctional. Red blood cells are known to bind a small number of chemokines and have been described as a sink for these molecules, and the loss of this activity is correlated with disease progression.

This results of this thesis support a broader role for red blood cells in regulating inflammation by acting as a cytokine buffer and modulating cell activity. The aims and hypotheses of this thesis were founded on the discovery that red blood cells are a major reservoir for the pro-inflammatory cytokine, macrophage migration inhibitory factor (MIF); in fact, they contribute 1000-fold more per millilitre than plasma. Red blood cells were also identified to be a major reservoir of 30 additional cytokines, chemokines, and growth factors. Further investigation showed that red blood cells bind and release significant quantities of these proteins, a function that can be modulated by other cells and by enzyme inhibitors. Incubating red blood cells with a cancer cell line (A549 cells) resulted in the significant increase of eight pro-tumorigenic cytokines in the red blood cell lysates. These primed red blood cells altered the activity of lymphocytes by stimulating the proliferation of T cells compared to controls, and promoted the expression of cell activation markers.

This study supports the hypothesis that red blood cells act as a buffer for cytokines through binding and release, and that alterations in red blood cells from cell-to-cell interactions affects the activity of T cells. This thesis proposes that red blood cells have multiple functions and the results have implications for the study of inflammation, the role of red blood cells in diagnostics, and on the development of red blood cell derived therapeutics.

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ABBREVIATIONS

ABCA1	ATP binding cassette transporter subfamily 1
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
BP	Bio-Plex standard spike
BSA	Bovine serum albumin
CD markers	Cluster of differentiation markers
CFSE	5(6)-carboxyfluorescein diacetate N-succinimidyl ester
CI	Confidence interval
CM	Conditioned media
CO_2	Carbon dioxide
CTACK	Cutaneous T-cell attracting chemokine
CXCR2	C-X-C chemokine receptor type 2
DARC	Duffy antigen receptor for chemokines
DDT	D-dopachrome tautomerase
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
G-CSF	Granulocyte stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
HALI	Hyperoxia-induced injury
HGF	Hepatocyte growth factor
IFN-α2	Interferon alpha 2
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-1ra	Interleukin-1 receptor antagonist
IL-2	Interleukin-2

IL-2ra	Interleukin-2 receptor subunit alpha
IL-3	Interleukin-3
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-7	Interleukin-7
IL-8	Interleukin-8
IL-9	Interleukin-9
IL-10	Interleukin-10
IL-12(p40)	Interleukin-12 subunit p40
IL-12(p70)	Interleukin-12 subunit p70
IL-13	Interleukin-13
IL-15	Interleukin-15
IL-16	Interleukin-16
IL-17	Interleukin-17
IL-18	Interleukin-18
IP-10	Interferon gamma-induced protein 10
ISO-1	(S,R)-3-(4-Hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid
KCl	Potassium chloride
kDa	Kilodalton
LIF	Leukaemia inhibitory factor
LPS	Lipopolysaccharide
MCHC	Mean corpuscular haemoglobin concentration
MCP-1	Monocyte chemotactic protein 1
MCP-3	Monocyte chemotactic protein 3
M-CSF	Macrophage colony-stimulating factor
MCV	Mean corpuscular volume
MFI	Mean fluorescence intensity
MIF	Macrophage migration inhibitory factor
MIG	Monokine induced by gamma interferon
MIP-1α	Macrophage inflammatory protein 1 alpha
MIP-1β	Macrophage inflammatory protein 1 beta
MQ-H_2O	Milli-Q water
mRNA	Messenger RNA

MSC	Mesenchymal stem cell
NaCl	Sodium chloride
NAP-2	Neutrophil-activating peptide-2
β-NGF	Beta nerve growth factor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PDGF-BB	Platelet derived growth factor
PHA-P	Phytohemagglutinin-P
PI	Propidium iodide
PLT	Platelet
pRBC	Primed red blood cells
pRBC-H	Red blood cells primed with A549 cells at a ratio of 1000:1
pRBC-L	Red blood cells primed with A549 cells at a ratio of 100:1
PrI	Protease inhibitor
RANTES	Regulated on activation, normal T cell expressed and secreted
RBC	Red blood cell
RBC-CM	Red blood cell conditioned media
RDW	Red blood cell distribution width
rMIF	Recombinant MIF
RNA	Ribonucleic acid
ROS	Reactive oxygen species
ROSC	Return of spontaneous circulation
RPMI-1640	Roswell Park Memorial Institute-1640 medium
SCF	Stem cell factor
SCGF-β	Stem cell growth factor-beta
SD	Standard deviation
SDF-1α	Stromal cell-derived factor 1-alpha
TNF-α	Tumour necrosis factor alpha
TNF-β	Tumour necrosis factor beta
TRAIL	TNF-related apoptosis-inducing ligand
upRBC	Unprimed red blood cells
VEGF	Vascular endothelial growth factor
WBC	White blood cell

CYTOKINES: ALTERNATE NAMES

Cytokine name used in thesis	Alternate cytokine name
bFGF	FGF-β
CTACK	CCL27
DDT	MIF-2
Eotaxin-1	CCL11
G-CSF	CSF-3
GM-CSF	CSF-2
GRO-α	CXCL1
GRO-α	MGSA
GRO-3	CXCL3
IL-8	CXCL8
IP-10	CXCL10
MCP-1	CCL2
MCP-3	CCL7
M-CSF	CSF-1
MIG	CXCL9
MIP-1α	CCL3
MIP-1β	CCL4
NAP-2	CXCL7
RANTES	CCL5
SDF-1α	CXCL12

PREAMBLE

The research outlined in this thesis was a result of a discovery whilst investigating macrophage migration inhibitory factor (MIF) as a potential biomarker for osteoarthritis. In a double-blind placebo controlled trial of stem cell therapy for osteoarthritis, our industry partner (Regeneus Pty Ltd) identified that MIF serum levels were significantly reduced over time following stem cell treatment. This lead to the hypothesis that MIF may be an optimal biomarker to track treatment outcome following stem cell therapy.

This thesis was initiated to optimise the protocol of blood collection, handling, and processing for MIF analysis. The following data was produced by the thesis candidate. In the preliminary stages of research for this thesis, initial results found that the method of sample processing could result in substantially varied MIF concentrations (Figure X.1.). Blood collected from a single individual at one time point could be processed using different methods to produce serum or plasma samples that, in turn, produced varied results (Figure X.1.).
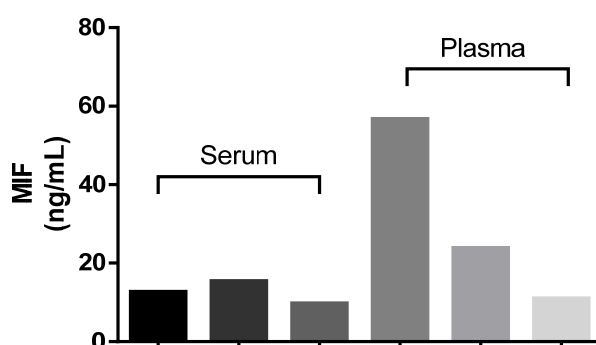
Figure X.1.   Concentration of macrophage migration inhibitory factor (MIF) in serum and plasma samples collected from a single individual produced with a variety of different commercially available collection tubes, as measured by enzyme-linked immunosorbent assay ($n = 1$).

In an effort to correct for these variations by reducing the time between venepuncture and storage, the MIF concentration in whole lysed blood was analysed. For this analysis, whole blood was collected and immediately snap frozen to achieve complete cellular lysis. The intention of this was to determine the total MIF concentration in whole blood that had not been

subjected to a variety of sample preparation methods or the time required for this processing. This whole blood lysate was then analysed using a MIF ELISA (enzyme-linked immunosorbent assay). The results of this were unexpected and demonstrated that the concentration of MIF in whole blood lysate was approximately 1000-fold higher than plasma or serum levels (Figure X.2).
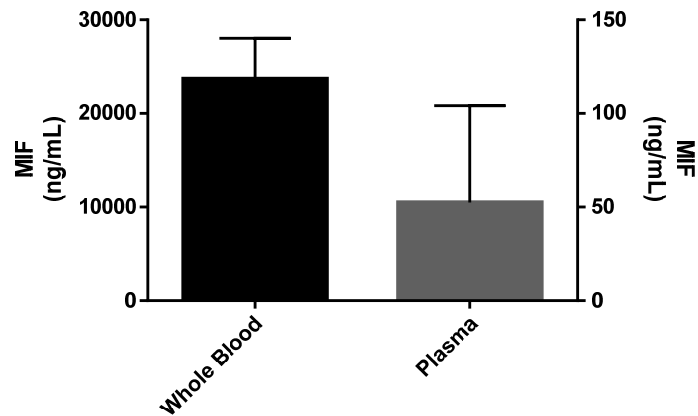
Figure X.2. Concentration of macrophage migration inhibitory factor (MIF) in whole blood lysate (according to one millilitre of whole blood) or in plasma as measured by ELISA. Data are presented as mean ± standard deviation ($n = 5$).

CHAPTER

1

INTRODUCTION

This introduction provides an overview of mature red blood cells and the role they play in the immune system. The historical view of red blood cells is that, although they are the most abundant cell type in humans, they are inert bystanders in inflammatory processes and function only in the transport of gas down an oxygen gradient. This chapter introduces the complexity of the development, structure, and function of mature red blood cells. The role that red blood cells may play in inflammation is also investigated with particular emphasis on the interaction between red blood cells and inflammatory molecules (cytokines). Finally, as addressed in the aims of this thesis, an outline of the potentially detrimental implications on the quantification of these cytokines in samples of blood will be outlined.

1.1   BIOLOGY OF RED BLOOD CELLS

Red blood cells are a unique cell type in mammals and they are a crucial component of human medical treatment, with over 100 million units of blood collected worldwide every year. When mature, these cells lack a nucleus and organelles, and are the most abundant cell type in humans. As a result of their seemingly simple structure they were previously believed to have a single role, which was the transport of gas. Evidence is now mounting that red blood cells are more complicated than once thought and are clearly performing important secondary roles. Although these cells lack organelles, they have been shown to contain an antioxidant complex which responds quickly to the local environment, are regulated by a rich tapestry of enzymes, and contain more than 2000 unique proteins of which a number are still listed as having 'unknown functions'. The intricacies of these cells are still being unravelled, even today.

1.1.1   Red blood cell development and death

Mammalian mature red blood cells are enucleate and are the most abundant cell type in the human body with a total of approximately 25 trillion cells, which equates to almost two thirds of the total number of cells in the body[1,2]. Mature red blood cells arise from nucleated erythroid progenitor cells in the bone marrow and they survive for approximately 120 days in the circulation before being degraded.

1.1.1.1   Red blood cell development

The majority of the maturation of red blood cells from progenitor cells occurs in the bone marrow, including red blood cell enucleation[3]. Approximately 2.5 million immature red blood cells are released from the bone marrow every second to maintain total red cell number in the circulation[4]. These immature red blood cells are known as reticulocytes and make up approximately 1 % of the circulating red cells[5]. Although reticulocytes lack a nucleus, when they are released from the bone marrow they have not yet lost their organelles and thus have the capacity to translate protein. The process of reticulocyte maturation to a mature red blood cell takes approximately 2 days and includes the elimination of all organelles (with the exception of the proteasome) and the restructuring of the cell membrane into the classic bi-concave shape[6–8]. The mitochondria from these cells are removed by a combination of enzymatic degradation and cell excretion in vacuoles[9,10]. Less is understood about the

elimination of the other organelles, but it has been proposed to include secretion in exosomes or autophagy, also known as self-digestion[11]. Whilst the cells are still nucleated, proteins and enzymes integral for red blood cell function such as haemoglobin are produced and remain in the cells even after loss of the nucleus and other organelles.

1.1.1.2 *Red blood cell death*

Mature red blood cells survive for approximately 120 days in the circulation before being phagocytosed by macrophages in the liver and the spleen[12,13]. These cells present a 'self-maker', CD47, during their lifespan which prevents engulfment, but as they age they become senescent and the level and/or distribution of CD47 on the cell surface can change[14,15]. This alteration in the level of CD47 stimulates the macrophages in the liver and the spleen to remove these old cells from the system[15,16].

1.1.2 Structure of red blood cells

In the absence of organelles, the cytoplasm is largely composed of haemoglobin, a cytoskeleton, and a 20S proteasome. The haemoglobin is required for the primary role of gas exchange, the cytoskeleton maintains the classic bi-concave shape of the cell, and the proteasome is tasked with controlled degradation of unwanted or oxidatively damaged proteins[6,17]. Red blood cells, and in particular their membranes, are required to survive repeated mechanical stress in their movements through the circulation and the microvasculature[18]. The biconcave shape of these cells and the cytoskeleton enables the required flexibility under these sheer forces (Figure 1.1). Unlike the cytoskeletons of most cells, the skeleton of the red blood cells sits directly underneath the cell membrane and does not traverse across the cytoplasm[19]. This structure is tethered to the membrane and as such, enables the cells to squeeze through the microvasculature at high speeds without permanent damage.
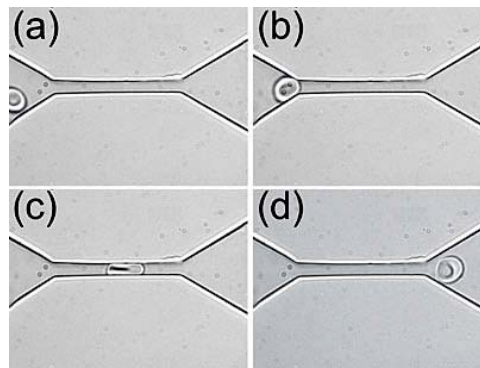
Figure 1.1. Illustration of the deformability of a human red blood cell as it moves through a microfluidic channel as presented in Li *et al.*[18].

The distinctiveness of red blood cells can be largely attributed to its cellular membrane, the most complicated component of red blood cells. The red blood cell membranes have a high proportion of membrane-associated enzymes[20,21], and they display specific blood group antigens. In addition to these unique features, these membranes also contain many of the regular components on membranes of nucleated cells, including ligand receptors[22] and transmembrane transport channels[23]. Curiously, red blood cell membranes can be isolated by centrifugation at approximately 16,000 g[24]. This number is six times lower than what is needed to isolate other cellular membranes which require ultracentrifugation at approximately 100,000 g[25]. The reason for this disparity may be a result of the abnormal cytoskeleton tethered to the membrane, but this feature is minimally discussed in the literature. Red blood cells are also particularly susceptible to osmotic changes in the environment and can be readily dehydrated or lysed. Following hypotonic lysis, the red blood cell membranes are capable of resealing if the tonicity is restored[26]. These reformed membranes are referred to as red blood cell ghosts. In light of this, lysis has been used as a method to deplete the cells of haemoglobin and other intracellular soluble components, and load these empty, theoretically inert, ghosts with target molecules such as drugs or cytokines[27,28]. Slavin *et al.* demonstrated that bFGF-loaded red blood cell ghosts promoted wound healing and extended the duration of therapeutic benefit compared with application of the recombinant protein alone[28]. This supposition that red blood cell ghosts are inert carriers may not be entirely accurate. Almost all of the enzymatic machinery of these cells is located on the red blood cell membrane, so exclusion of the cytoplasmic component of the cell is unlikely to render the membrane non-functional. In fact, it has been demonstrated that the enzymatic activity of red blood cell membranes is retained as

well as the function of the calcium and sodium pumps[29,30]. Whilst the biological implications of this are not understood, it is worthy of further investigation.

1.1.3 Function and activity of red blood cells

1.1.3.1 Gas exchange and oxidative stress

The primary documented function of red blood cells is gas exchange wherein they transport oxygen from the lungs to oxygen-poor tissues. Intracellular haemoglobin in these cells accounts for more than 95 % of the total cellular proteins and the iron core of these molecules enable the binding and release of oxygen or carbon dioxide as needed. The limitation of transporting oxygen on a regular basis is the resulting exposure to high levels of oxidative stress. Over time, haemoglobin is slowly oxidised by the endogenous reactive oxygen species (ROS) into methemoglobin at a rate of approximately 4 % per day[31]. This oxidation damages the protein to the point that it can no longer bind oxygen for transport[32]. Any additional release of ROS during this process is readily neutralised by the available intracellular antioxidant factors[33]. In addition to the intracellular oxidative stress pressure, exogenous ROS produced by cells such as T cells are also removed from the circulation by red blood cells[34]. As they can bind and readily neutralise these potentially damaging ions, red blood cells have been proposed as an oxidative sink for ROS[34,35]. In order to attenuate oxidative stress-induced damage, the red blood cells have a highly complex antioxidant system that encompasses both enzymatic (catalase and glutathione peroxidase)[33,36] and non-enzymatic factors (glutathione and ascorbic acid)[37,38]. Whilst these processes are highly effective, cell damage occurs over the lifespan of red blood cells.

The antioxidant system in red blood cells is mostly cytosolic, which makes the management of oxidative stress on the membrane less efficient. A build-up of ROS at the cell membrane typically leads to cell deformability and lipid peroxidation of the membrane, subsequently causing haemolysis[39]. If not managed quickly, oxidative stress-induced haemolysis can cause local tissue damage due to the unavoidable release of ROS[40]. In a healthy system, this is typically managed by younger red blood cells which are more readily able to mop up exogeneous ROS. However, this system does not work perfectly in inflammatory environments. As identified by Castillo *et al*., open heart surgery can cause further tissue damage as a result of ROS release[40] and the free ROS observed in the local environment is likely a direct result of haemolysis[41]. Thus, red blood cells play a key role in maintaining

oxidative homeostasis, and any dysfunction of that activity is likely to cause detrimental downstream effects.

1.1.3.2 *Enzymes in red blood cells*

Enzymes in red blood cells and their membranes are ubiquitous. In the cytosol, enzymes such as catalase and glutathione peroxidase minimise the effects of oxidative stress[33,36], and a protease complex (the 20S proteasome) degrades damaged proteins[6]. In addition, the red blood cell membrane contains a glycolytic enzyme complex that is necessary for cellular metabolism[20], a number of ATPases to mediate ion transfer across the membrane[21], and proteolytic enzymes[42,43]. This diverse group of enzymes is responsible for much of the observed activity of red blood cells. Surprisingly, a functional nitric oxide synthase has also been identified in both the membrane and the cytosol of red blood cells[44]. This report is a key example of the growing understanding around the complexity of red blood cell enzymes. Whilst many of the enzymes mentioned above were identified decades ago, this nitric oxide synthase was identified much more recently. Red blood cells have been considered for many years as a sink for nitric oxide and due to the high concentration of haemoglobin, have been described as metabolisers of this molecule. Contrary to conventional opinion, Kleinbongard *et al.* demonstrated that the red blood cells are not only a sink, but are also actively producing functional nitric oxide though a specific synthase[44]. Thus, the network of enzymes in red blood cells is complex and dictates much of the cellular activity *in vivo*; this points to functions beyond conventional red blood cell biology.

1.1.3.3 *Metabolism (glycolysis)*

Although mature red blood cells do not contain any organelles, they still need to metabolise glucose to manage their energy requirements and so, in the absence of mitochondria, anaerobic glycolysis by the Embden-Meyerhof pathway occurs. A glycolytic enzyme complex located on the red blood cell membrane is responsible for this activity[20]. Pathologies associated with these enzymes typically cause non-spherocytic haemolytic anaemia (premature degradation of red blood cells), a condition that can be lethal if left untreated[45].

1.1.3.4 *Red blood cell proteome and gene expression*

There has been growing interest over the recent years in determining the full proteome of red blood cells. Optimising sample preparation techniques and analysis parameters has been challenging due to the overwhelming amount of haemoglobin in these cells compared to the concentration of all other proteins[46]. In light of those challenges, the most recent reviews report

that 2289 unique proteins have now been identified in red blood cells[47]. This represents a collection of studies that have identified smaller numbers of unique proteins. Pasini *et al.* assessed the structural and functional distribution of the proteins they identified in the red blood cell membrane[48]. As illustrated by Figure 1.2, more than half of the identified proteins were directly associated with the membrane or the cytoskeleton. Surprisingly, Pasini *et al*. identified that 6 % of the identified proteins were organellar proteins, including specific transport proteins for the Golgi network, the endoplasmic reticulum, and the mitochondria[48].
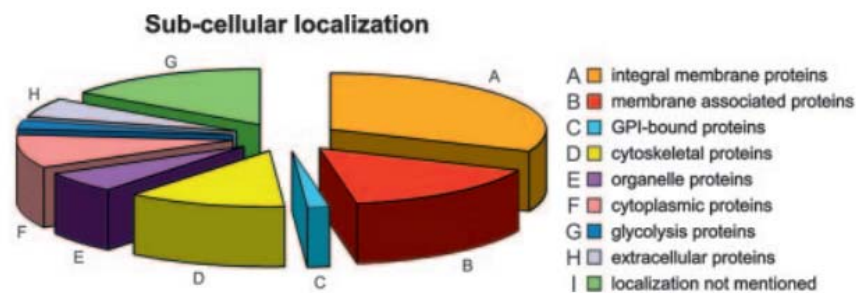
Figure 1.2.   Subcellular localisation of the identified membrane proteins as illustrated in Pasini *et al.*[48].

Similarly, another study identified the presence of translation initiation factors and proteins for cellular defence, as well as the expected protein categories[49]. Of note, this study also reported that approximately 24 % of the total observed proteins had unknown functions and could not be categorised[49]. The complexity does not end here, with total RNA transcripts of 1019 genes now reported to be present in red blood cells[50]. These genes were identified as being relevant for many normal cellular processes including metabolism (52 %), programmed cell death (5 %), and even some genes for translation/transcription of protein (up to 5 %)[50]. Red blood cells contain considerably more active cellular components than their primary role would suggest and may be more complex than conventionally described. The complexity of these cells and the presence of unknown molecules warrants significant further investigation.

1.2   RED BLOOD CELLS AND INFLAMMATION

Red blood cells are the most abundant cell type in the body and are directly exposed to a wide variety of tissues on their journey through the circulatory system. For many years, these cells have been described to be inert bystanders rather than participants in inflammatory processes. However, it is now clear that red blood cells are involved in many pathologies, although the specific nature of their involvement for the most part has not yet been elucidated, such as in the case of anaemia of chronic inflammation. Red blood cells themselves have also been shown to be immuno-stimulatory to specific cellular populations, and can cause cytokine storm events following their transfusion. It appears that we are only just scratching the surface of red blood cells and their role in inflammation, disease, and repair.

1.2.1   Red blood cells in diagnostics

Although red blood cells are largely believed to be inert, in regular clinical practice, red blood cell characteristics are collected and are used in diagnostics. These diagnostic processes are entirely biased towards evaluating the oxygen binding capacity of these cells with a focus on diagnosing different types of anaemia. A low red blood cell count, variations in the average size of the cells (mean corpuscular volume, MCV), or variations in the average amount of haemoglobin in each cell (mean corpuscular haemoglobin concentration, MCHC) can all be indicative of anaemia and are all collected as part of the standard full blood count panel. Outside of this, there is only one test used regularly that assesses red blood cells and is expressive of non-specific inflammation. This simple test has been used since the 19th century and it assesses the rate of erythrocyte sedimentation. A very high rate of sedimentation (compared to a normal range) indicates broad spectrum inflammation such as an infection or an auto-immune condition[51,52]. However, this is not an optimal biomarker of inflammation, as other plasma-derived markers such as C-reactive protein have shown to be more reliable and are more responsive to changes in the inflammatory microenvironment[53].

Although not yet used clinically for this purpose, Lippi *et al.* investigated the analysis of another red blood cell characteristic as a diagnostic marker for inflammation[54]. In this extensive study of almost 4000 participants, they identified that red blood cell distribution width (RDW) was closely correlated with inflammation as indicated by erythrocyte sedimentation rate and level of C-reactive protein. This correlation of RDW with inflammation has received a bit of

attention over the last five years. High RDW values have been reported as a risk factor for increased mortality in cardiovascular patients[55], diabetics[56], and patients with chronic lymphocytic leukemia[57]. Curiously, this increased risk of mortality is not isolated to specific diseases. Patel *et al.* demonstrated that high RDW values was a strong predictor of mortality in people over the age of 45 and was independent of anaemia and the presence of other diseases[58]. They hypothesised that this observation was a result of increased inflammation and oxidative stress[58]. In light of these results, analysis of red blood cells as an indicator of inflammation or overall health is likely a valuable addition to the standard blood analysis panels in clinic.

1.2.2   Anaemia of chronic inflammation

Anaemia of chronic inflammation is defined as normocytic, normochromatic anaemia, where the size and the haemoglobin load of the red blood cells is normal, but the anaemia is caused by there being fewer red blood cells in the circulation. The reduced number of red blood cells is a result of both increased red blood cell destruction and impaired differentiation of these cells[59]. Anaemia of chronic inflammation presents as a secondary condition to other chronic inflammatory diseases such as infection, cancer, or auto-immune conditions[60,61]. Whilst there is still a lot left to understand about this condition, a key molecule in its pathogenesis has now been identified[62]. High levels of this molecule, hepcidin, slows iron export into local tissue, thus resulting in lower levels of available iron[63]. This activity is in turn regulated by the presence of pro-inflammatory cytokines such as IL-6[64]. Simultaneously in the pathogenesis of anaemia of chronic inflammation, the production of new red blood cells from the bone marrow is restricted by low levels of the red blood cell differentiation hormone, erythropoietin[65], and by inhibiting the maturation of red blood cell progenitors in the bone marrow due to inflammatory cytokines[59].

Adequate treatment for the anaemia of chronic inflammation does not yet exist, and the recommendation is to focus on treating the underlying condition, as the anaemia typically improves once the primary condition is alleviated[66]. It is not yet understood if this anaemia is a result of the chronic inflammation and is a biological response to mediate the inflammation, or if the chronic inflammation is in part a result of the anaemia itself, and if the red blood cells are playing a role in the modulation of the levels of inflammatory molecules during disease progression.

1.2.3 Transfusion medicine

Red blood cell transfusions are the most common blood derived therapeutic and have been used widely over the last century. As a transplant, they are generally well tolerated as donor matching only needs to be met at the blood type level. Due to its extensive history, the area of transfusion medicine has now moved away from optimising the treatment for the patient and into optimising the storage parameters for the blood products to extend their use. At the moment, red blood cells are stored at 4 °C for up to 42 days before use[67]. Although there is a lot known about the cause of most of the adverse reactions that can occur with red blood cell transfusion, there remains the controversial issue of the storage lesion. The storage lesion refers to the red blood cell deformability that can occur during long term storage. The debate regarding the implications of using these altered cells as a treatment is ongoing[68]. Whilst there are many studies that report a correlation between the transfusion of older (deformed) red blood cells and the occurrence of adverse events[69,70], there are also studies that report no effect of storage duration at all[71]. This debate has been going on now for decades, and it doesn't appear that an answer will be reached any time soon.

A lot of analyses have been done in an attempt to determine exactly what occurs in red blood cells during storage. The morphology of these cells is known to change substantially over time, wherein they become more rigid and are more susceptible to haemolysis[72]. There are also a number of reports that suggest that these cells undergo oxidative damage during storage and begin to take on the qualities of senescent red blood cells[73]. Whilst others have focused more on the issue of cytokine release from stored blood[74,75]. Some adverse events following red blood cell transfusion were hypothesised to be a result of contaminating white blood cells which secrete a range of inflammatory proteins during storage[76]. Thus, transfusion of a blood pack containing both the contaminating white blood cells and the resulting cytokines could elicit a detrimental immune reaction. However, the evidence now suggests that leukodepletion does not entirely abolish the cytokine release[74] and that in a number of situations it is no different to non-depleted red blood cells[77,78]. Weisbach *et al.* demonstrated that stored leukodepleted red blood cells actually produced significantly more IL-8 than the non-leukodepleted red blood cells[74].

Regardless of these differences in red blood cell storage, there are longer term effects that have been noted as a result of red blood cell transfusions. For example, following hip replacement surgery (arthroplasty), 31 % of people who received red blood cell transfusions experienced wound healing delays and disruptions; this was significantly higher than the non-transfused

group[79]. Further, people receiving multiple transfusions typically have higher levels of circulating CD8+ cytotoxic T cells which has been suggested to be the cause of the immunosuppression observed following transfusions[80,81]. In fact, in a systematic review of the literature on the use of red blood cell transfusions, the authors concluded that these transfusions were strongly correlated with increased morbidity and mortality, and that on average, the risks of transfusion outweighed any potential benefit[82]. Infection was identified as a primary, independent, risk factor for red blood cell transfusion. Of note, this risk of infection was not correlated with transfusion-transmitted infections, but instead with transfusion-dependent immunomodulation resulting in increased susceptibility to infection[82,83]. These results and other similar studies demonstrate that the red blood cells that are transfused have long term and potentially lethal immunological effects, although the mechanisms of such are not well understood.

1.2.4　　　　Red blood cells and their interactions with other cells

Most of the investigation into the activity of immune cells was, and still is for the most part, done in single cell cultures containing the target immune cell. The dogma of immunology teaches that the other cells of the blood (such as red blood cells or platelets) are inert and would not contribute to the overall activity of the immune system. However, all of the recent research into red blood cells suggests that this is unlikely to be the case. In response to the changing climate around red blood cells and their involvement in inflammatory conditions, a few laboratories across the world have started to investigate the specific interaction between red blood cells and other cell types. The majority of this investigation has focused on the role red blood cells play in altering the function of immune cells.

1.2.4.1　　　　T lymphocytes

One of the first reports in this area documented the effect of red blood cells on freshly isolated T cells *in vivo*[34]. T cells are a subset of lymphocytes, and any increase in proliferation of these cells *in vitro* following stimulation is used as a model of immune activation. This initial paper reported that by stimulating T cells and treating them with fresh, autologous red blood cells their activity was significantly altered[34]. Red blood cells stimulated these T cells to proliferate more than the untreated control, and were also found to be protective for these cells against apoptosis[34]. These results have since been replicated under similar conditions and does not appear to be dependent on the ratio of red blood cells to T cells, but is dependent on the red blood cell being intact[84–86]. These studies have led to the question of whether or not this state

of T cell activation is actually its natural state *in vivo*, as they are always in contact with red blood cells.

In particular disease or inflammatory states, red blood cells have been shown to be dysregulated in this activity in that they do not have the same effect on T cells that healthy red blood cells do[85,87,88]. As an example, red blood cells isolated from patients with carotid atherosclerosis were found to be no longer protective against T cell apoptosis[85], and red blood cells isolated after hip arthroplasty were no longer immunogenic to the T cells unlike the cells isolated prior to the surgery[87]. It has been hypothesised that these results are representative of a dysregulation of T cell homeostasis that occurs *in vivo*, and may be involved in potentiating the conditions[85].

1.2.4.2   Dendritic cells

Red blood cells from healthy donors have been shown to restrict the maturation of dendritic cells, and in the process, the secretion of IL-12(p40)[89,90]. This is proposed to be a mechanism for controlling the overstimulation of dendritic cells and the hyper release of pro-inflammatory cytokines *in vivo*[90]. However, in carotid atherosclerosis this system is dysfunctional similar to the observed effects on T cell survival. Red blood cells collected from patients with carotid atherosclerosis were less able to prevent the maturation of dendritic cells, and thus the secretion of pro-inflammatory cytokines[89].

1.2.4.3   Other immune cells

There is minimal other literature on the interaction between red blood cells and immune cells other than T cells or dendritic cells. A 30 year old study reported that natural killer cells, when in the presence of red blood cells, were more cytotoxic towards tumour cells[91]. Virella *et al.* investigated the effect of autologous red blood cells on B cell activity and they reported a significant increase in the secretion of immunoglobulins and of the pro-inflammatory cytokine, IFN-γ[92]. Finally, a recent study reported that red blood cells promoted eosinophil migration through endothelial cells by scavenging the chemokine RANTES, thus suggesting a role for red blood cells in allergic inflammation[93].

1.2.4.4   Fibroblasts

The interaction between red blood cells and fibroblasts, a non-immune cell type, has been evaluated extensively by one laboratory. This group demonstrated that the presence of red blood cells in a culture of fibroblasts alter the function and secretome of these cells. Unlike the effect of red blood cells on T cell proliferation and survival, red blood cells suppressed the proliferation of fibroblasts and stimulated apoptosis[94]. Red blood cells also promoted fibroblast

mediated contraction of collagen[95], the secretion of the chemokine IL-8[96], and also the secretion of matrix metalloproteinases[97]. This modulation of fibroblast activity illustrates a role for red blood cells in the remodelling process of wound healing.

1.2.4.5 Mechanism of action

The mechanism of action for this modulation of cellular function by red blood cells has not yet been investigated in detail and thus there are multiple theories. Profumo *et al.* investigated the cause of the dysfunction in the red blood cells from carotid atherosclerosis patients that had altered T cell activity[85]. They reported that the result could be replicated in healthy red blood cells by subjecting them to oxidative stress, as such they concluded that it was a result of oxidative imbalance in the red blood cells[85]. Similarly, it was reported that treatment with healthy red blood cells reduced the level of oxidative stress in the target T cell population[34]. The report by Antunes *et al.* has been the only study thus far to perform an investigation into the factors responsible for the activity of healthy red blood cells on T cells[86]. In a series of experiments, they identified that the causative agent(s) were present in the red blood cell conditioned media, and were soluble protein factors that were sensitive to heat[86]. However, they were not able to identify the specific molecule(s) responsible for the activity. In the promotion of eosinophil migration, Kanda *et al.* attributed their results to red blood cells being able to bind the chemokine RANTES out of the media and thus play a role in chemotaxis[98]. There is growing evidence that these signalling molecules (cytokines and chemokines) at the centre of inflammatory processes may also be interacting with red blood cells, thus giving them a role in inflammation.

1.3 CYTOKINES IN RED BLOOD CELLS

Cytokines are key mediators and signalling molecules in inflammation. Of the few reports on cytokines and red blood cells, the majority are restricted to discussion of the Duffy antigen receptor for chemokines (DARC) which is present on red blood cells, and the Gardos channel on red blood cells which is regulated by DARC (Figure 1.3). Outside of these areas, there is minimal other literature. Although there are some reports on cytokines in red blood cells, no study has attempted to quantify these proteins in a unit of these cells. This information is crucial for understanding the role of cytokine interactions with red blood cells in blood and the implications of haemolysis.
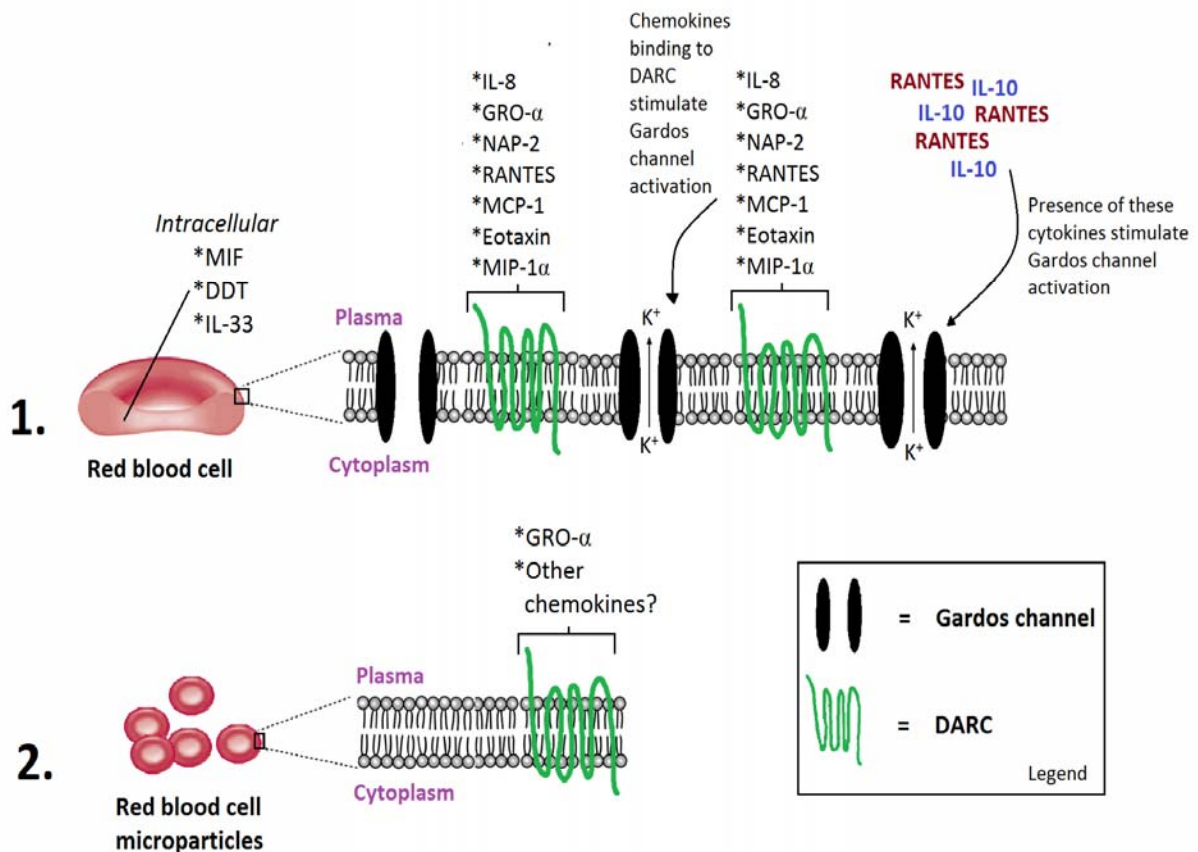
Figure 1.3. Schematic of the current understanding of the interaction between cytokines and red blood cells.

1.3.1   Duffy antigen receptor for chemokines (DARC)

DARC was first identified on red blood cells in 1950 and was described as a new blood group[99]. Since, it has been reported that DARC is not only present on red blood cells but is also expressed on some endothelial cells[100]. Since its identification, the majority of research on this receptor on red blood cells has focused on its role in malarial pathogenesis. *Plasmodium species*, the parasites that causes malaria, have been shown to use DARC on red blood cells to invade the cells and thus progress the infection[101]. More than 95 % of West Africans[102], and 68 % of African Americas[103] are negative for the Duffy antigen receptor on their red blood cells, but are still positive for the receptor on their endothelial cells[104]. This has likely developed as an evolutionary advantage against malaria.

1.3.1.1   Chemokine binding to red blood cells

In addition to its role in malaria pathogenesis, another potential role of DARC was identified in 1991. Darbonne *et al.* reported the presence of a receptor on the surface of red blood cells that could readily bind IL-8 and implicated red blood cells as a biological sink for this chemokine[24]. Following this report, the same receptor was then described to be promiscuous as it was able to bind not only IL-8 but also some C-X-C (IL-8, GRO-α, NAP-2) or C-C (RANTES, MCP-1) chemokines[105]. Further analysis of this receptor identified that it was in fact DARC[22,106]. In the 20 years following these reports, only Eotaxin and MIP-1α have been identified as additional ligands for DARC[22,107,108]. Of the few cytokines that have been identified, the concentration of these in the red blood cells from healthy or diseased cohorts have never been reported. Due to the broad binding capacity of DARC on red blood cells, they have been implicated as sinks for circulating inflammatory markers[24,109,110]. Although this receptor is structurally similar to G-protein-coupled chemokine receptors with its seven transmembrane domains, unlike these receptors, ligation of DARC does not appear to activate intracellular chemokine signal transduction pathways[22]. In 1993, Neote *et al.* reported that RBC-bound IL-8 had no effect on mobilising Ca^{2+} ion stores, which is indicative of signal transduction with G-protein-coupled receptors[105]. Since that report, no other evidence of signal transduction has been identified. Similarly, no reference to phosphorylation of the receptor in the membrane or its ligand(s) have been reported in the literature. These results have been used to support the theory that the primary function of chemokine ligation of DARC is not in chemokine signalling, but instead as an inflammatory sink for these markers[22,111]. Investigation into the concentration of these cytokines in the red blood cells of healthy individuals or

individuals with inflammatory conditions may provide valuable information regarding disease state in the identification of biomarkers.

Evidence is gathering that DARC on red blood cells, and cytokine ligation of this receptor plays a role in inflammatory disease. Whilst DARC negative individuals are less susceptible to malarial infection, they have been reported to have a higher risk of developing inflammatory conditions such as graft rejection[112] or prostate cancer[113]. This is theorised to be a result of the reduced availability of a DARC mediated inflammatory sink[114]. Although the cytokine binding capacity of red blood cells in DARC negative individuals is lost, some capacity to bind excess inflammatory markers is retained by the DARC positive endothelial cells. This has been evidenced in animal knockout models. In mice lacking DARC from the red blood cells only, the overall level of IL-8 and MCP-1 being bound was reduced compared to the DARC positive control[109]. However, binding was still higher than that observed in the complete DARC knockout model (no DARC on the red blood cells or the endothelial cells)[109]. In addition to altered binding capacity of the cells, the inflammatory response to stimuli was exaggerated when DARC was not present in the animals[109].

Fukuma *et al*. sought to further investigate the role of DARC on red blood cells as an inflammatory sink for cytokines[107]. In this study, they treated DARC positive and negative mice with recombinant protein and monitored the protein half-life. They reported that in DARC negative mice, these chemokines were rapidly cleared upon administration. However, the half-life of the proteins in the peripheral circulation was significantly higher in the DARC positive animals due to red blood cell sequestration. With these results, they hypothesised two roles for cytokine binding to DARC on red blood cells in managing inflammation. First, that DARC on red blood cells acts as an inflammatory sink to remove excess chemokines, and second, that it acts as a reservoir of these cytokines to release when they are needed[107]. The mechanisms behind chemokine release from DARC have since been investigated. Release of MCP-1 (a C-C chemokine) from DARC is activated by two identified mechanisms, (1) clotting or (2) treatment with unfractionated heparin[115]. This discovery supports the hypothesis of Fukuma *et al.*, that DARC is involved with both binding and releasing cytokines as needed[107]. Release of chemokines in response to clotting identifies a key role of DARC in inflammation. *In vivo*, clotting occurs at the site of injury or in the case of thrombi, and red blood cells are key to both of these processes. Thus, a bolus of chemokines released from red blood cells at the site of injury or thrombi may play a role in the resulting inflammatory processes. Humphries *et al*. identified that in a thrombus, MCP-1 levels increase and remain high until resolution and that

treatment with MCP-1 leads to accelerated thrombus clearance[116]. Red blood cells in the thrombus are likely to be a major source of this chemokine. Similarly, lung inflammation in response to lipopolysaccharide stimulation has been shown to be modulated by DARC. Neutrophil migration into the lungs in response to inflammatory stimuli is attenuated and the migration of mononuclear inflammatory cells is increased in DARC knockout models[109,117,118]. Local levels of DARC ligand chemokines are also reported to be higher in the lungs of these stimulated mice[117,118]. The results of these studies propose a notable role for cytokines binding to red blood cells in modulating immune responses.

1.3.1.2 *Red blood cell microparticles*

Red blood cell derived microparticles have also been demonstrated to be positive for the DARC receptor[119]. This chemokine binding receptor is not only present on the membranes of these microparticles, but it also retains its capacity to bind GRO-α out of solution[119]. Although ligand binding to DARC is possible on microparticles, its binding affinity is reduced compared to intact red blood cells[119]. The number of microparticles released from red blood cells increases over time with storage and the presence of these microparticles have been implicated as a potential risk for red blood cell transfusion[120]. Interaction with platelets has been demonstrated to stimulate the release of GRO-α from allogenic microparticles[119]. This models a biological interaction that may occur *in vivo* following red blood cell transfusion, which may play a role in the observed adverse reactions.

1.3.1.3 *Gardos channel*

The Gardos channel, present on red blood cells, regulates cell volume by transporting potassium ions across the cell membrane[121] and plays an important role in promoting red blood cell dehydration in sickle cell anaemia[23]. Haemoglobin concentration in red blood cells increases as a result of cell dehydration which subsequently enhances the rate of haemoglobin polymerisation, a key characteristic of sickled red blood cells[122,123]. High levels of cytokines have been observed in the plasma of sickle cell anaemia patients, and these cytokines have been demonstrated to play a role in its pathogenesis[124]. Rivera *et al.* reported that the presence of select cytokines (including IL-10 and RANTES) at high levels increased the Gardos channel activity by up to 80 %[125]. Further investigation into this interaction identified that this activity was mediated, at least in part, by cytokine binding to DARC on red blood cells[125,126]. DARC positive red blood cells were found to be 17x more dense (and thus more dehydrated) than DARC negative red blood cells and the density of the DARC positive cells could be modulated

with the addition of RANTES or IL-8[126]. Whilst there are no reported downstream effects of cytokine binding to DARC on red blood cells, these studies suggest a functional role of cytokines on red blood cells that is additional to its role as a chemokine sink.

1.3.2 Macrophage migration inhibitory factor (MIF)

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine and an oxido-reductase enzyme that was first identified in 1966[127]. Since its identification, MIF has been shown to play a critical role in a wide range of inflammatory conditions. These conditions include sepsis, rheumatoid arthritis, and preeclampsia, and patients with these conditions typically have higher levels of the protein in their serum or plasma[128–130]. As such, considerable research has focused on MIF as a potential biomarker. However, MIF as a biomarker is complicated by the fact that many cells have been shown to produce and secrete MIF. This protein was originally identified in lymphocytes[127], and has since been identified in macrophages and other inflammatory cells[98,131], adipocytes[132], liver cells[133], and many other cell types. It is even secreted from the pituitary gland in response to infection[134]. There are reports, although few, that suggest that another source of MIF is mature red blood cells.

Shortly after its identification, Fox *et al*. reported in 1974 that the activity of MIF from fetal calf serum was attenuated when red blood cells were present[135]. They hypothesised that this loss of activity was indicative of MIF being bound to the red blood cells and becoming inactivated. Since that report, red blood cells have been postulated to not only bind, but also harbour MIF. A strong correlation between haemoglobin concentration and the concentration of MIF in plasma or serum has been observed[136,137]. This rise in MIF concentration has been attributed to red blood cell derived MIF. However, this has been the extent of the investigation. Up until this point no study has sought to quantify the concentration of MIF in a unit of red blood cells nor to investigate its activity. In fact, it has been recommended to just avoid analysis of MIF where red blood cells may be present in order to minimise inference[137], but this view is particularly short-sighted. Determining the concentration of MIF in red blood cells and its chemokine activity is likely to be crucial in understanding the inflammatory processes following haemolysis *in vivo*. MIF has two major documented functions that appear to be dependent on its cellular location. Secreted MIF has a primary role as a pro-inflammatory cytokine which modulates the migration of inflammatory cells[138], and is dependent on its tautomerase active site[139]. Whilst the secondary role of MIF is largely intracellular and is a specific oxidoreductase activity which has been demonstrated to regulate cellular oxidative

stress[140,141], this is likely the primary role that it is playing in red blood cells.

The value in this analysis is made clearer by looking at a condition where MIF plays a key role, such as sepsis. MIF has been reported to be significantly elevated in the plasma of septic patients, and high levels of this protein have been correlated with early death in patients with severe sepsis[142]. Similarly, by inhibiting MIF, the survival rate of patients with severe sepsis improves signficantly[143,144]. Haemolysis is a common complication of sepsis, and haemoglobin has even been proposed as a potential biomarker for the condition[145]. In fact, Adamzik *et al.* reported that free haemoglobin levels were twice as high in the non-survivors of severe sepsis compared to the survivors[145]. It is likely that at least some of the MIF detected in the plasma of severe septic patients is a result of haemolysis and red blood cell release. Thus, assessment of where the MIF has come from and determining what concentration of MIF can be attributed to red blood cells could lead to improved therapeutic targets for treating sepsis, a condition with very high mortality (approximately 30 %). At the very least, this warrants further investigation.

1.3.3 Other cytokines in red blood cells

1.3.3.1 *D-dopachrome tautomerase (DDT)*

D-dopachrome tautomerase (DDT), also known as MIF-2, has been described as a functional homologue of MIF[146]. Although DDT cannot replicate the same oxido-reductase activity of MIF, it shares its tautomerase and cytokine activity[147,148]. MIF and DDT bind to the same cell surface receptor complex formed between CD44-CD74, and ligation of this receptor leads to the same downstream effects[146]. These proteins tend to be localised to the same tissues and areas of the body, in fact, DDT has even been identified in red blood cells. Bjork *et al.* reported that red blood cells were responsible for approximately 99 % of the D-dopachrome tautomerase activity in whole blood and identified the presence of DDT in these cells[149]. The reason for its localisation in red blood cells is unknown, particularly since it does not share the same oxido-reductase activities of MIF.

1.3.3.2 *Interleukin 33 (IL-33)*

IL-33 has also been identified in the red blood cell lysates of healthy individuals[150]. Notably, IL-33 is a nuclear protein that is released from dying or dead cells. As such, its presence in red blood cells, an enucleate cell, is unexpected. Wei *et al.* identified that IL-33 was expressed in erythroid progenitor cells, thus hypothesising that the protein had been present in the cells since they were nucleated[150]. IL-33 can be released from apoptotic or necrotic cells[151] and its release

has been shown to stimulate the secretion of T$_H$2 cytokines and play a key role in promoting allergic inflammation[152]. Haemolysis of red blood cells would thus result in a release of this cytokine into the soluble fraction of blood. In sickle cell anaemia patients, higher levels of IL-33 in plasma were observed with increased levels of haemolysis[150]. This may have implications in the pathogenesis of the condition.

1.4 CURRENT ANALYTICAL STANDARDS

1.4.1 Analysis and processing of blood

Plasma and serum are the two most common components of blood analysed in research and clinical testing, and soluble disease markers in these fluids are regularly used as indicators of immune or disease status[153]. In the collection of these samples, the integrity of the other blood cells needs to be maintained for accurate, reproducible results. However, blood cells are particularly susceptible to stress induced by mechanical processing techniques. If blood processing takes longer than a few minutes, white blood cells are known to secrete a range of factors into the plasma or serum component that can interfere with results[154]. Similarly, excessive centrifugation or sample manipulation can lead to the activation and degranulation of platelets also resulting in higher detectable levels of cytokines[155]. With the knowledge that red blood cells may harbour a range of cytokines, careful processing of blood components is of paramount importance. As identified by Sobierajski *et al.*, haemolysis in the process of isolating serum or plasma can result in high concentrations of detectable MIF[137]. Although panels of cytokines are not monitored on regular basis in clinical diagnostics, their analysis in research and clinical trials is becoming common. Thus, any source of interference in the quantification of proteins in serum or plasma, such as haemolysis, could confound results. The full implications of this however will not be understood until the concentration of these cytokines in red blood cells is determined and reported.

1.4.1.1 Cell washing

Although red blood cells account for approximately 50 % of the total volume of whole blood, in diagnostics they are discarded more often than they are analysed. Outside of the diagnostic arena, the protein content of red blood cells does occasionally get evaluated. There is a small number of studies that have investigated the proteome of red blood cells and their membranes[48,49]. However, the protocol for red blood cell isolation in these analyses encourages excessive washing of the target cells in an isotonic buffer[48,49]. Extensive washing such as this is strongly discouraged in the isolation of other blood cells such as neutrophils to avoid cell activation[156], yet it is part of regular practice for red blood cell isolation, probably because the cells are considered to be transcriptionally and translationally inert and incapable of secretory

activity. Optimised methods for gentle isolation of red blood cells is likely to produce cells more representative of their native state.

1.4.2   Case study: interleukin-8

Interleukin-8 (IL-8) is regularly quantified in serum or plasma in clinical research. This protein is a chemokine and it is present in plasma at reported concentrations of approximately 7 pg/mL in healthy individuals and is associated with a number of conditions such as prostate cancer, kidney injury, and coronary syndrome[157–159]. In 1991, Darbonne *et al*. identified the presence of IL-8 in red blood cells and investigated their capacity to readily bind this protein[24]. They demonstrated that with the addition of IL-8 to the plasma fraction of whole blood, red blood cells bound the majority of this protein out of solution[24]. Whilst this was an interesting finding, it highlights an issue in quantifying IL-8 in only plasma or serum. If serum IL-8 levels were low, it would not be known if this was a true result or whether the red blood cells had bound the excess IL-8 during sample processing, thus creating an artefact. Without knowledge of the level of IL-8 bound to the red blood cells, it is difficult to elucidate the true abundance of the protein. As an example, people lacking DARC on their red blood cells tend to have higher circulating levels of IL-8 than people positive for DARC, which suggests that a fraction of the missing IL-8 is likely still there, it is just bound to red blood cells[160]. This adds variance in IL-8 quantification even within the healthy population. These confounding factors in IL-8 quantification in blood is likely to be similar for any other cytokines that are associated with red blood cells. Quantification these cytokines in a unit of red blood cells *in addition to* plasma or serum is necessary to elucidate the severity of the issue, and following that, analysis of both components may prove to be useful in identifying disease specific biomarkers.

1.5 PROJECT DETAILS

1.5.1 Project outline

There is growing evidence that red blood cells are more complex than was once thought, nevertheless, investigation into red blood cells and their response to the inflammatory micro-environment has not received a lot of attention. There are relatively few publications that have investigated the relationship between cytokines and red blood cells, and out of those, none have followed up the analysis by investigating the effect of this on immune cell activity. In fact, none have sought to quantify these analytes in a unit of red blood cells. Most of the literature on cytokines associated with red blood cells is isolated to discussion on cytokine binding to DARC, with a small selection of literature that has identified the presence of other cytokines in red blood cells not affiliated with DARC. Preliminary data to this thesis identified the presence of MIF in red blood cells at 1000-fold higher than the plasma concentration. This discovery prompted further analysis into red blood cell derived MIF (Chapter 3), the presence of other red blood cell derived cytokines (Chapter 4), and the modulation of the cytokine profile of red blood cells and the effect on lymphocyte activity (Chapter 5).

1.5.2 Study hypothesis

The hypothesis of this study is that red blood cells are a key player in inflammation and that this activity is reliant upon their interaction with cytokines.

1.5.3 Study aims

The overall aim of this thesis was to quantify red blood cell-derived cytokines and to determine the role of these cells in the immune response. The specific aims of the study were:

1. To elucidate the presence and the activity of macrophage migration inhibitory factor in red blood cells.
2. To identify and quantify other cytokines associated with red blood cells.
3. To modulate the cytokine profile of red blood cells using a variety of techniques including incubation with recombinant protein or through cell-to-cell interaction.
4. To modulate the activity of lymphocytes by using red blood cells with altered cytokine profiles.

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CHAPTER

2

MATERIALS AND METHODS

This chapter includes a summary of the materials, equipment, reagents, chemicals, and solutions used or referred to in this thesis. This chapter also details the methods that are common across multiple chapters. References to the relevant sections of the thesis that detail the methods used is also included.

2.1 MATERIALS

The materials and equipment that were used to complete the experimental work presented in this thesis are outlined in Table 2.1. The reagents, chemicals, and cell lines used to complete the experimental work presented in this thesis are outlined in Table 2.2 and Table 2.3. Detail on the formulation and storage parameters for the solutions prepared and used for the completion of this thesis are outlined in Table 2.4.

Table 2.1. Materials and equipment.

Materials/Equipment	Company
35 μm nylon cell strainer	Becton Dickinson, USA
Annexin V detection kit	BioLegend, USA
BD Calibrite™ beads	Becton Dickinson, USA
Bio-Plex Pro Human Cytokine 21-plex assay	Bio-Rad, USA
Bio-Plex Pro Human Cytokine 27-plex assay	Bio-Rad, USA
Bio-Plex Pro II Wash Station	Bio-Rad, USA
FACSAria III Cell Sorter	Becton Dickinson, USA
FACScalibur Flow Cytometer	Becton Dickinson, USA
Hematology analyzer, Coulter AcT Diff	Beckman Coulter, USA
IncuCyte™ FLR	Essen BioScience, USA
K_2EDTA vacutainers, 3 mL or 9 mL	Becton Dickinson, USA
Luminex® 200 system, Bio-Plex	Bio-Rad, USA
MIF ELISA	R&D Systems, USA
Nuclear Factor Fixation and Permeabilisation Buffer Set	BioLegend, USA
Pierce BCA (bicinchoninic acid) Protein Assay Kit	ThermoFisher Scientific, Australia
Synergy 2 Multi-Mode plate reader	BioTek, USA
Ultra-0.5 Centrifugal Filter Unit, 3K membrane	Merck Group, Germany

Table 2.2.   Reagents and chemicals.

Reagent/Chemical	Supplier
(S,R)-3-(4-Hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid (ISO-1)	Merck Millipore, Australia
5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE)	Sigma Aldrich, USA
Annexin V-FITC	BioLegend, USA
Antibiotic-antimycotic (100x)	Life Technologies, USA
Bis-Tris	Sigma Aldrich, USA
Bovine serum albumin (BSA)	Sigma Aldrich, USA
Crystal violet	Merck Millipore, Australia
Dextran, molecular weight 450-650 kDa	Sigma Aldrich, USA
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, USA
Dulbecco's Modified Eagle Medium (DMEM) with sodium pyruvate	Life Technologies, USA
Ethylenediaminetetraacetic acid, disodium salt (EDTA)	Sigma Aldrich, USA
Fetal bovine serum (FBS)	AusGeneX, Australia
Ficoll-Paque	GE Healthcare, Australia
Haemoglobin, human	Sigma Aldrich, USA
Glacial acetic acid	ThermoFisher Scientific, USA
L-3,4 dihydroxyphenylalanine methyl ester	ThermoFisher Scientific, USA
L-glutamine (200 mM)	Sigma Aldrich, USA
Methanol	Chem-Supply, Australia
Mo anti-human CD3-APC antibody, clone: SK7	BioLegend, USA
Mo anti-human CD3-FITC antibody, clone: UCHT1	BioLegend, USA
Mo anti-human CD4-APC antibody, clone: RPA-T4	BioLegend, USA
Mo anti-human CD44-PE antibody, clone: 515	Becton Dickinson, USA
Mo anti-human CD45-FITC antibody, clone: H130	Affymetrix eBioscience, USA
Mo anti-human CD74-FITC antibody, clone: MB741	Becton Dickinson, USA
Mo anti-human CD8a-APC antibody, clone: HIT8a	BioLegend, USA
Mo anti-human GATA-3-PE antibody, clone: 16E10A23	BioLegend, USA
Mo anti-human T-bet-PE antibody, clone: 4B10	BioLegend, USA

Reagent/Chemical	Supplier
Mo IgG1,κ Iso Control-APC, clone: MOPC-21	BioLegend, USA
Mo IgG1,κ Iso Control-FITC, clone: MOPC-21	BioLegend, USA
Mo IgG1,κ Iso Control-PE, clone: MOPC-21	BioLegend, USA
Mo IgG1,κ Iso Control-PE, clone: MOPC-21	Becton Dickinson, USA
Mo IgG1κ Iso Control-FITC, clone: P3.6.2.8.1	Affymetrix eBioscience, USA
Mo IgG$_{2a}$, κ Iso Control-FITC, clone: G155-178	Becton Dickinson, USA
Mo IgG2b,κ Iso Control-PE, clone: MPC-11	BioLegend, USA
Penicillin-streptomycin (100x)	Sigma Aldrich, USA
Phosphate buffered saline tablets (PBS)	Astral Scientific, Australia
Phytohemaggultinin-P	Sigma Aldrich, USA
Potassium chloride	Sigma Aldrich, Australia
Protease inhibitor cocktail, mini cOmplete (PrI)	Roche, Switzerland
Recombinant MIF	Jomar Bioscience, Australia
Red blood cell lysis solution	Sigma Aldrich, USA
RPMI-1640 with sodium bicarbonate	Sigma Aldrich, USA
Sodium chloride (NaCl)	Sigma Aldrich, USA
Sodium periodate (NaIO$_3$)	VWR Chemicals, Australia
Trypan blue solution (0.4%)	Sigma Aldrich, USA
TrypLE	Life Technologies, USA

Table 2.3.   Cell lines.

Cell line	Supplier
A549 cells	Bill Walsh research laboratory, Kolling Institute, Australia
Jurkat cells	Perinatal research laboratory, Kolling Institute, Australia

Table 2.4.   Solution formulations.

Solution (concentration)	Formulation
Bis-Tris (200 mM)	A 200 mM Bis-Tris solution was prepared by dissolving Bis-Tris in Milli-Q water
CFSE (1 mM and 5 μM)	A stock solution of 1 mM CFSE was prepared by dissolving CFSE in DMSO; stock was stored in aliquots at minus 20 °C; as needed, aliquots were diluted further to working concentration of 5 μM in PBS
Dextran solution (6 %)	For a 6 % (w/v) dextran solution, dextran powder was dissolved in NaCl (0.15 M) and stored at room temperature
EDTA (5 mM)	A 5 mM solution of EDTA was prepared by dissolving EDTA in Milli-Q water
Haemoglobin (25 mg/mL)	Solutions of haemoglobin were prepared fresh for calibration curves by dissolving haemoglobin in PBS, starting at 25 mg/mL
ISO-1 (20 mM and 5 mM)	ISO-1 powder was dissolved in DMSO (100 %) to make a 20 mM stock; stock was stored in aliquots at minus 20 °C; as needed, aliquots were diluted further to working concentration 5 mM in methanol
L-3,4 dihydroxyphenylalanine methyl ester (400 mM and 12 mM)	L-3,4 dihdyroxphenylalanine methyl ester powder was dissolved in Milli-Q water to make a 400 mM stock; stock was stored in aliquots at minus 20 °C; as needed, aliquots were further diluted to working concentration 12 mM in Milli-Q water
Recombinant MIF (1 mM)	Recombinant MIF was dissolved in PBS to 1 mM stock; stock was stored in aliquots at minus 80 °C

Solution (concentration)	Formulation
PHA-P (5 M)	A 5 M stock solution was prepared by dissolving PHA-P in PBS; stock was stored in aliquots at minus 20 °C
Potassium chloride, KCl (0.6 M)	KCl was dissolved in Milli-Q water to prepare a 0.6 M solution which was stored at room temperature
Sodium chloride, NaCl (0.15 M)	0.15 M solution prepared by dissolving NaCl in Milli-Q water
Sodium periodate (400 mM and 24 mM)	Sodium periodate was dissolved in Milli-Q water to make a 400 mM stock; stock was stored in aliquots at minus 20 °C; as needed, aliquots were diluted further in Milli-Q water to make a 24 mM working solution
Flow cytometry staining buffer	For a flow cytometry staining buffer, a 0.5 % BSA solution was prepared by dissolved BSA in PBS; solution was stored in aliquots at 4 °C
Turks solution	Turks solution was prepared by adding glacial acetic acid to a final concentration of 1 % (v/v) and crystal violet to a final concentration of 0.05% into Milli-Q water; solution was stored at room temperature

2.2 METHODS

2.2.1 Plasma and blood cell isolation

Plasma and blood cells were isolated from EDTA anti-coagulated whole blood (k_2 vacutainers, BD Biosciences) within four hours of collection. After isolation, the blood components were either used fresh or were frozen at -80 °C and were subjected to three freeze-thaw cycles at -80 °C prior to analysis.

2.2.1.1 Red blood cells (protocol 1)

Red blood cells were isolated by dextran sedimentation (protocol 1) as in Chapter 3 and Chapter 4. Whole blood was added to a dextran solution (6 % w/v in 0.15 M sodium chloride) at a ratio of 2:1 in a 15 mL falcon tube. This tube was left upright at room temperature for 1 hour to enable the red blood cells to sediment to the bottom of the tube (Figure 2.1). After the incubation, the upper white blood cell and platelet rich layer was discarded and the top of the red blood cell dense lower layer was also discarded to minimise contamination. The remaining red blood cell fraction was collected and was washed once in phosphate buffered saline (PBS, 500 *g*, 5 minutes).
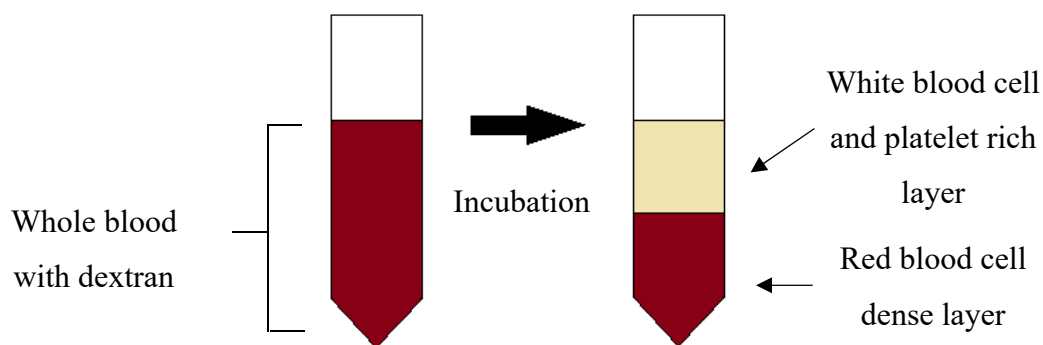
Figure 2.1. Schematic of dextran sedimentation of red blood cells from whole blood.

2.2.1.2 Red blood cells (protocol 2)

Red blood cells were isolated by an optimised dextran sedimentation method (protocol 2) as in Chapter 5. Whole blood was centrifuged (1500 *g*, 10 minutes) and the plasma layer was discarded. The red blood cell dense pellet was then resuspended in an equal volume of sodium

chloride (0.15 M) in a 15 mL falcon tube. A 6 % solution of dextran (w/v in 0.15 sodium chloride) was added to the cell suspension at a ratio of 1:4 (dextran:cells). This cellular suspension was then left at room temperature for 30 minutes to enable the red blood cells to sediment to the bottom of the tube (Figure 2.1). The upper white blood cell and platelet rich layer was discarded along with the top of the lower red blood cell layer to minimise contamination. The remaining red blood cell fraction was then collected and washed once in phosphate buffered saline (PBS, 500 g, 5 minutes).

2.2.1.3   *White blood cells (total population)*

White blood cells were isolated following dextran sedimentation (protocol 1). A 3 mL aliquot of whole blood was added to 1.5 mL of 6 % dextran solution (w/v in 0.15 sodium chloride) in a 15 mL falcon tube. This tube was then left to incubate for 60 minutes, after which the white blood cell rich fraction was collected (Figure 2.1). The white blood cell suspension was diluted in 10 mL of PBS and the cells were then isolated by centrifugation (1000 g, 10 minutes). Following isolation, any contaminating red blood cells were lysed by resuspending the cell pellet in 3 mL of Milli-Q water for 0.5 minutes. Addition of 1 mL of potassium chloride (0.6 M) restored isotonicity to the cell suspension. The remaining white blood cells were then pelleted and were washed once in 10 mL of PBS (1000 g, 5 minutes).

2.2.1.4   *Peripheral blood mononuclear cells (PBMCs)*

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using density gradient cell separation. Whole blood was diluted in an equal volume of PBS and was then carefully layered over Ficoll-Paque (2:1 ratio respectively, GE Healthcare). This was then centrifuged for 40 minutes (400 g, no brake). After centrifugation, the PBMC layer which had formed at the interface between the plasma and the Ficoll-Paque was isolated (

Figure 2.2) and was washed once with PBS (500 g, 5 minutes). Any contaminating red blood cells in the PBMC fraction were lysed with red blood cell lysis solution (Sigma Aldrich) for 10 minutes at 37 °C, and the remaining white blood cells were then pelleted and were washed once in PBS (1000 g, 5 minutes).
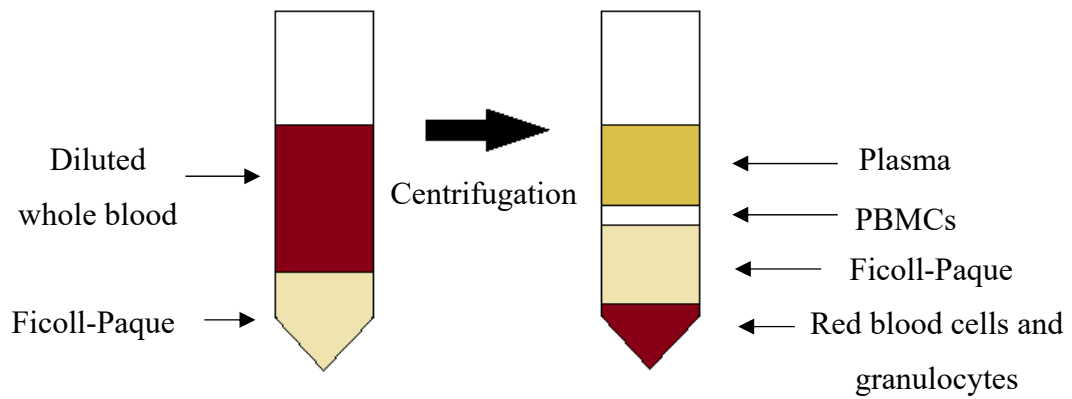
Figure 2.2. Schematic of Ficoll-Paque isolation of peripheral blood mononuclear cells (PBMCs) from whole blood.

2.2.1.5 Platelets

Platelet-rich plasma was prepared by slow centrifugation of whole blood (100 g, 20 minutes). After this, the upper, platelet rich layer was collected (Figure 2.3) and contaminating cells were progressively removed by subsequent cycles of centrifugation of the upper isolated platelet rich fraction (100 g, 5 minutes). Platelets were then pelleted by centrifugation and were washed twice in PBS (1000 g, 5 minutes).
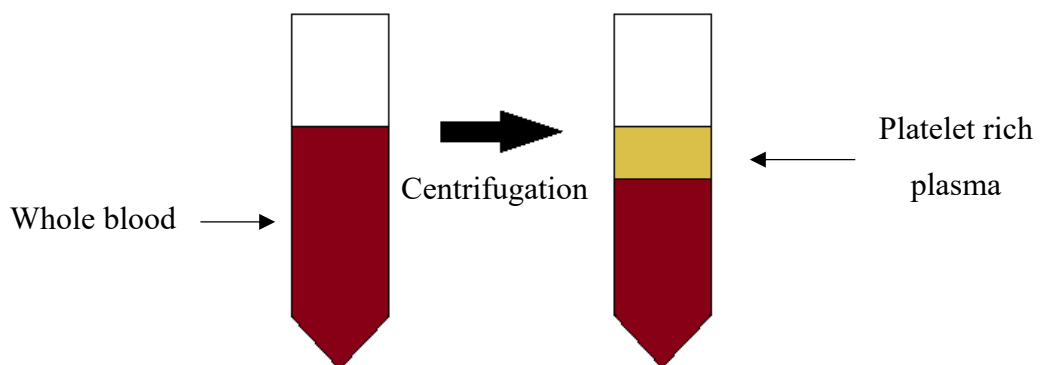
Figure 2.3. Schematic of isolation of platelet rich plasma from whole blood.

2.2.1.6 Plasma

Plasma was isolated from anticoagulated whole blood by centrifugation at room temperature (1500 g, 10 minutes). Samples were stored at -80 °C prior to analysis.

2.2.2 Cell counting

Blood cells and immortalised cell lines used in analyses were counted by variety of methods. White blood cell and platelet depletion of enriched red blood cell populations were also quantified.

2.2.2.1 Haematology analyser

The cells in whole blood, red blood cell, white blood cell, and platelet populations were quantified using a haematology analyser (Coulter Act Diff, Beckman Coulter). Depletion of white blood cells and platelets from the enriched red blood cell populations was calculated according to the following equation using the values collected from the haematology analyser:

Equation 2.1. Percentage depletion of contaminating cells from purified red blood cells.

$$100 - \left(\frac{\frac{a}{b} \times d}{c} \times 100 \right) = Percentage\ depletion$$

Where,

a = Number of red blood cells in 1 µL of whole blood

b = Number of red blood cells in 1 µL of the enriched red blood cell sample

c = Number of white blood cells (or platelets) in 1 µL of whole blood

d = Number of white blood cells (or platelets) in 1 µL of the enriched red blood cell sample

2.2.2.2 Haemocytometer with trypan blue

An aliquot of cell lines in suspension (A549 cells or Jurkat cells) was diluted in an equal volume of trypan blue solution (0.4 %). Of the diluted cell suspension, 10 µL was applied to the haemocytometer and the number of live (unstained) and dead (trypan blue stained) cells were counted to determine the concentration of cells per mL.

2.2.2.3 Haemocytometer with Turks solution

PBMCs were quantified using Turks solution. An aliquot of PBMCs was diluted in Turks solution at a ratio of 1:20 (PBMCs : Turks solution). The Turks solution lysed any contaminating red blood cells in the sample. Of the diluted cell suspension, 10 µL was applied

to the haemocytometer and the number of PBMCs were counted to determine the concentration of cells per mL.

2.2.3 Protein quantification

For the quantification of cytokines, a direct sandwich ELISA was initially utilised to identify the concentration of macrophage migration inhibitory factor (MIF) in blood samples. The ELISA is one of the most common techniques used to quantify the concentration of a single analyte, such as is outlined in Chapter 3. Further experimentation in this thesis introduced a need to assess the concentration of a wide variety of cytokines, which was achieved by using a multiplex Luminex® assay. This assay uses the same principle as the direct sandwich ELISA wherein a capture antibody is used to bind the analyte out of solution, and a subsequent detection antibody is added to sandwich the analyte and to enable quantification. The primary difference between the two is that the capture antibody in the ELISA is directly bound to the well of the plate, whilst the Luminex® capture antibody is bound to a specific colour-coded bead. In this assay, the bead colour is specific for an analyte, thus other bead colours can be used to differentiate between multiple analytes. Per analyte, these Luminex® kits are substantially cheaper, more time efficient, and require lower volumes of sample than ELISAs. For this reason, the Luminex® kits were chosen as a preferential technique. For both the ELISA and the Luminex® kits, the capture antibodies were human anti-mouse and the detection antibodies were human anti-goat. The antibody clones for these kits are unknown as they are proprietary.

2.2.3.1 *Multiplex assay – Luminex®*

For cytokine quantification of cell lysates and conditioned media samples, two Luminex® multi-plex assays was utilised. The first was the 27-plex human cytokine panel that assays for FGF basic, Eotaxin-1, G-CSF, GM-CSF, IFN-γ, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF, and the second was the 21-plex human cytokine panel that assays for IL-1α, IL-2Ra, IL-3, IL-12(p40), IL-16, IL-18, CTACK, GRO-α, HGF, IFN-α2, LIF, MCP-3, M-CSF, MIF, MIG, β-NGF, SCF, SCGF-β, SDF-1α, TNF-β, TRAIL (Bio-Plex Pro 27-plex and 21-plex, Bio-Rad). The assays were performed according to manufacturer's instructions as follows. The protocol is summarised in Figure 2.4.
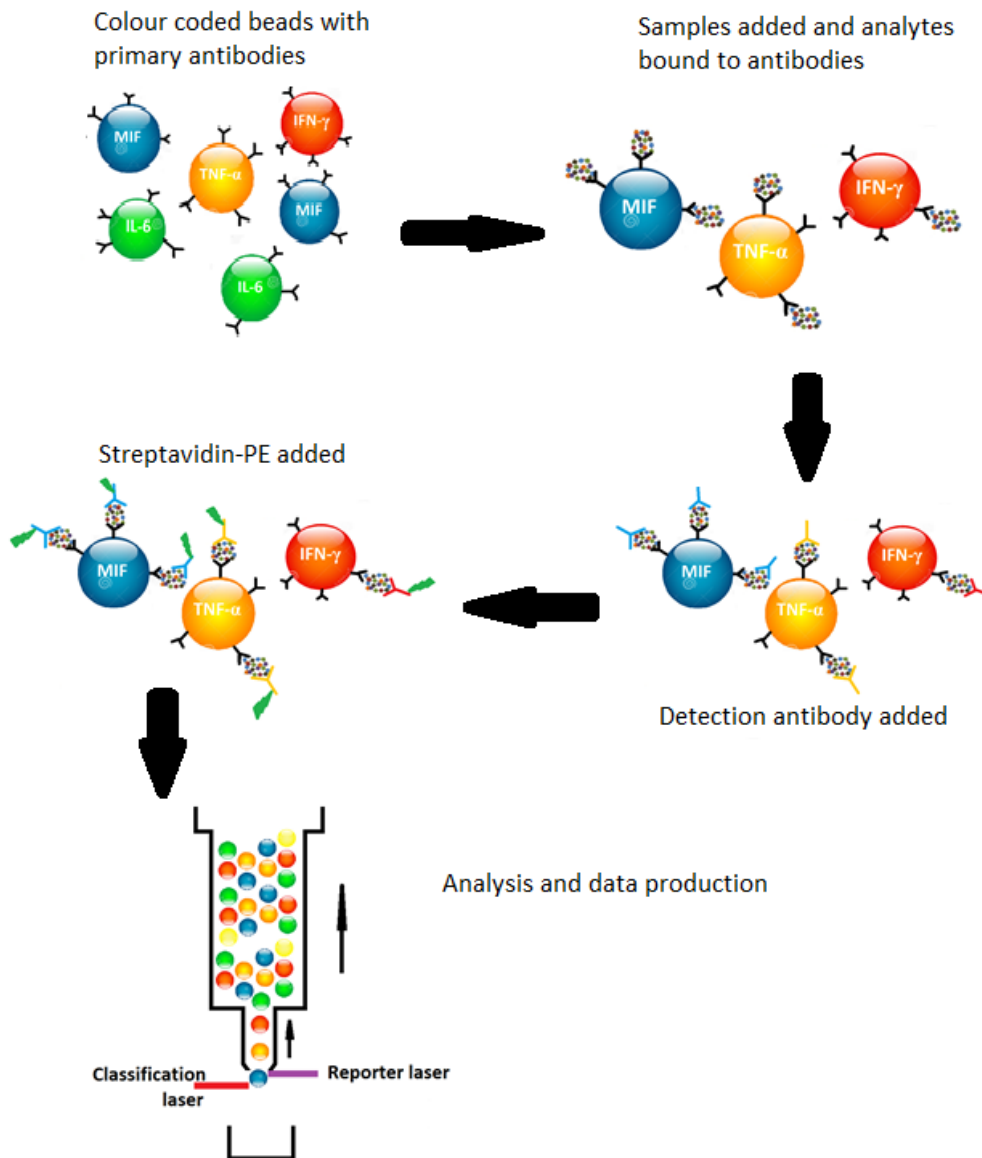
Figure 2.4.   Schematic of multi-plex assay workflow.

To start, all reagents were brought to room temperature. The Bio-Plex standards were reconstituted with 500 µL of the provided standard diluent. This solution was mixed well then incubated on ice for 30 minutes. During this time, the 10x magnetic beads were resuspended in the provided assay buffer to a 1x concentration. These beads were left to incubate at room temperature for 20 minutes. Following incubation of both solutions, the reconstituted standards were diluted in the provided standard diluent where 128 µL of the standard was added to 72 µL to produce the most concentrated standard. Serial dilution of this standard was then performed as a 1:4 dilution series to produce a total of 8 standards for each kit. To each well of the provided

96 well plate, 50 µL of the diluted magnetic beads was added and washed twice with the provided wash buffer and magnetic wash station (Bio-Plex Pro II wash station). After washing, 50 µL of the standards, blanks, and samples were added to the relevant wells. This plate was then sealed and protected from light during incubation at room temperature on a microplate orbital shaker (850 rpm, 30 minutes). Following incubation, the plate was washed three times with the provided wash buffer (Bio-Plex Pro II wash station). The 10x detection antibodies were diluted 1:10 in the provided detection antibody diluent. Of this solution, 25 µL was added to each well. The plate was then sealed and protected from light during incubation at room temperature on a microplate orbital shaker (850 rpm, 30 minutes). Following incubation, the plate was washed three times with the provided wash buffer (Bio-Plex Pro II wash station). The 100x Streptavidin-PE solution was diluted 1:100 in the provided assay buffer. Of the diluted Streptavidin-PE solution, 50 µL was added to each well. The plate was the sealed and protected from light during incubation at room temperature on a microplate orbital shaker (850 rpm, 10 minutes). Following incubation, the plate was washed three times with the provided wash buffer (Bio-Plex Pro II wash station). The beads in each well were then resuspended in 125 µL of the provided assay buffer and the plate was sealed and protected from light during incubation at room temperature on a microplate orbital shaker (1200 rpm, 1 minute). The plate was then read using the Luminex® 200 system (Bio-Plex, Bio-Rad) according to the settings outlined in Table 2.5.

Table 2.5.   Bio-Plex manager settings for plate analysis.

Parameter	Analysis setting
Beads per region	50
Bead map	100 region
Sample size	50 µL
DD gates (low)	5,000
DD gates (high)	25,000
PMT	Low RP1

The calibration curve for each cytokine was analysed with 5 parametric logistic curve regression suing Bio-Plex manager software (ver. 5.0, Bio-Rad). Standard values were considered acceptable if the points fell within 80 - 120 % of the expected values.

2.2.3.2   Haemoglobin quantification

Concentration of haemoglobin in red blood cell conditioned media samples was quantified by assessing absorbance at 414 nm with comparison to a calibration curve (Synergy 2 Multi-Mode plate reader). Peaks at this wavelength are indicative of free haemoglobin in the solution. To minimise interference, red blood cell membranes were removed by centrifugation of the conditioned media samples (16,000 g, 15 minutes, 4 °C). Following centrifugation, 100 µL of the membrane-free samples were added to individual wells of a 96-well plate. A calibration curve was also prepared by a serial dilution of human-derived haemoglobin (Sigma Aldrich). For this calibration curve, 100 µL was also added to wells of the 96-well plate, and the absorbance of the plate was read at 414 nm (Synergy 2 Multi-Mode plate reader).

2.3 METHODOLOGY INDEX

In this thesis, each results chapter includes a detailed description of all the methods used for the outlined experimental work. The location of the detailed methods in the thesis is summarised in Table 2.6.

Table 2.6. Reference to methods in thesis

Method	Thesis section reference
Blood collection	3.2.1, 4.2.1, 5.2.1, B.1.1
Bicinchoninic acid assay	4.2.5
Cytokine multiplex assay	4.2.7, 5.2.7
Enzyme linked immunosorbent assay	3.2.7, B.1.3
Flow cytometry	3.2.8, 5.2.5
Haemoglobin quantification	3.2.6, 4.2.6, 5.2.6
Isolation of red blood cell cytosol and membranes	3.2.3, 5.2.4.3
Plasma and blood cell isolation	3.2.2, 4.2.2, 5.2.2, B.1.2
Proliferation assay	5.2.4
Protein binding to red blood cells	3.2.5, 4.2.4
Protein recovery efficiency	3.2.4, 4.2.8
Red blood cell conditioned media preparation	3.2.5, 4.2.3
Red blood cell isolation (protocol 1)	3.2.2, 4.2.2, B.1.2
Red blood cell isolation (protocol 2)	5.2.2
Red blood cell priming	5.2.3
Tautomerase activity assay	3.2.9
Statistical analysis	3.2.10, 4.2.9, 5.2.8, B.1.4, E.1.1

CHAPTER

3

IDENTIFICATION OF RED BLOOD CELLS AS THE PRIMARY RESERVOIR OF MACROPHAGE MIGRATION INHIBITORY FACTOR IN WHOLE BLOOD

This chapter investigates the presence of macrophage migration inhibitory factor (MIF) in red blood cells. Red blood cells are widely accepted to be inert carriers of oxygen and haemoglobin, but there is growing evidence that they play a much more critical role in immune function. MIF is a key cytokine in disease and aids in managing oxidative stress. In a previous study in our laboratory, MIF was identified as a potential biomarker for osteoarthritis. MIF levels in plasma decreased significantly after mesenchymal stem cell therapy for this condition in a clinical trial. As the number of samples and the number of people processing the samples increased, more variability than expected in MIF levels was observed, making statistical analysis difficult. In an attempt to determine whether blood processing effects were responsible for the differences, a series of experiments with snap-frozen whole blood was completed. The discovery that MIF was present in whole lysed blood at concentrations 1000-fold higher than plasma led to a series of additional experiments that ultimately produced the work detailed in this chapter. As described here, freshly isolated red blood cells from healthy individuals were collected and the concentration of MIF was quantified using an enzyme linked immunosorbent assay (ELISA). The capacity of cells to bind and release MIF, and investigation into the enzymatic activity of MIF was investigated.

3.1   INTRODUCTION

Macrophage migration inhibitory factor (MIF) plays a central role in promoting inflammation. This 12.5 kDa cytokine is a key component of the early stage inflammatory response that is associated with innate immunity. To enable this response, MIF is produced at high concentrations and is retained intracellularly for rapid secretion in the active form, a homotrimer[1]. It is secreted by both immune cells (such as macrophages and T cells) and organ specific cells (such as hepatocytes and osteoblasts), and unlike other cytokines, it is also secreted by the pituitary gland in response to infection[2]. MIF binds to cells via a cell surface receptor complex formed between CD44 and CD74, which initiates a series of signalling cascades[3]. MIF functions, as its name suggests, in preventing the random migration of macrophages from the site of inflammation and as a chemokine that recruits other immune cells[4]. In addition, MIF functions to protect cells from apoptosis and oxidative stress[5,6]. Interestingly, the pro-inflammatory function of MIF is dependent on an enzymatic active site within its trimeric structure[7]. As such, its enzymatic and pro-inflammatory activity are closely related.

Interest in MIF has expanded since its discovery in 1966[8] and it has been implicated in a range of acute and chronic inflammatory conditions[9–12]. For this reason, MIF has been frequently proposed as a potential disease biomarker[10–14]. In a cohort of osteoarthritis patients, Liu *et al.* identified significantly increased levels of MIF in serum and synovial fluid samples when compared to healthy controls and also reported a correlation between serum levels of MIF and increasing grade severity of osteoarthritis[10]. Similarly, Todros *et al.* identified that serum levels of MIF were significantly higher in women who had severe preeclampsia when compared to women with uncomplicated pregnancies[11]. However, the involvement of this protein in so many biological processes and the wide range of plasma MIF concentrations in healthy participants (24.7 ± 13.2 ng/mL)[15] makes its use as an independent biomarker challenging[16].

One contributing factor to this variability may be the source of circulating MIF. Levels of MIF are most often monitored in serum or plasma collected from peripheral blood and the source is typically attributed to white blood cell secretion[17,18]. More recently, platelets have been identified as a previously unrecognised source of MIF which supports the growing literature around the involvement of platelets in inflammation[19,20]. In contrast to white blood cells and platelets, there is minimal literature on the cytokines associated with the most common cell type in blood, the red blood cell. There are studies indicating that red blood cells bind

inflammatory chemokines of the C-X-C or C-C family in addition to MIF[30-32]. However, no information on the concentrations of cytokines associated with red blood cells could be found. The preliminary observation that led to the study in this chapter was the quantification of MIF at approximately 30 µg/mL in one millilitre of whole blood. The literature on inflammatory signalling in disease and injury does not consider red blood cells as a source of cytokines, which may need to be re-evaluated in the light of these findings.

An example of elevated MIF after injury that may be in part a result of red blood cell-sourced MIF is post-cardiac arrest syndrome. In healthy volunteers the plasma concentration of MIF is typically between 1 and 50 ng/mL[21], which is 1000 times higher than most other cytokines. Although the plasma level of healthy controls is high compared to other cytokines, after injury the MIF level can be significantly elevated. Pohl *et al.* investigated the role of MIF in post-cardiac arrest syndrome where patients exhibited return of spontaneous circulation (ROSC)[22]. In patients after ROSC, MIF plasma levels at admission to the intensive care unit were significantly higher compared to those in healthy volunteers (173 ± 45 ng/mL vs 18 ± 2 ng/mL). Red blood cells are susceptible to free radical-induced lipid peroxidation, which occurs during ischemia-reperfusion injury as a result of cardiac arrest[23]. Increased haemolysis is observed as a result of ischemic-reperfusion injury and one hypothesis discussed is that oxidative stress causes haemolysis[24,25]. Acute haemolysis would cause a spike in MIF levels, which may be interpreted as an inflammatory response when the underlying cause may be cellular damage. Pohl *et al.* found a close relationship between circulating MIF levels and cellular damage but not with an inflammatory syndrome[22]. This result supports the hypothesis that the increased MIF observed post-cardiac arrest is a result of haemolysis.

Red blood cells are the most abundant cell type in the body and are present at upwards of 5 billion cells per millilitre of whole blood[26]. Although the conventional view is that red blood cells only function in gas exchange, it now appears that the role of this enucleate cell may be more complex. Proteomic and transcriptomic analyses have identified 2289 unique proteins in red blood cells[27] and transcripts of 1019 different genes[28]. A series of studies on the red blood cell soluble proteome over the last decade have classified the majority of identified proteins as being involved in either cellular metabolism or regulation of transport[29]. Within that, the main functional areas are catabolism, proteolysis, and energy production (glucose metabolism). These functional groups are consistent with the reality that mature red blood cells are actually a pool of red blood cells of different ages, on a predominantly destructive pathway. Whilst these categories account for much of the red blood cell proteome, approximately one quarter of the identified proteins are associated with organelles that are not present in mature red blood

cells or are not present in their active form. The presence of such large numbers of transcripts and mature proteins in red blood cells raises the question of functionality versus chance. Are these proteins present in red blood cells in a functional capacity or as the result of an imperfect process during maturation and ejection of the nucleus and other organelles? Red blood cells have also been shown to interact with some inflammatory cytokines including IL-8, RANTES, and MCP-1 through binding to the Duffy antigen receptor for chemokines (DARC) on the surface of red blood cells[30–32], as such they may also actually be involved in regulating inflammation.

D-dopachrome tautomerase (DDT), or MIF-2, is an active enzyme that has been identified within red blood cells[33]. The structural similarities between DDT and MIF have been researched extensively, and it has been reported that these proteins share 34 % amino acid sequence identity in humans[34–36]. DDT is encoded by a gene adjacent to MIF and has an overlapping functional spectrum with MIF. The DDT protein and MIF share the same cell surface receptor complex, CD74-CD44, and induce similar cell signalling and effector functions. Furthermore, an analysis of the signalling properties showed that they work cooperatively, and that neutralisation of DDT *in vivo* significantly decreases inflammation[34]. In fact, these proteins appear to be localised to the same areas throughout the body, with the testis being the only organ studied thus far that has a significant difference in expression levels[34]. It is likely that DDT and MIF are both present in red blood cells, however, their intracellular function remains unknown. Although there is minimal literature on this point, Mizue *et al.* suggested that MIF is indeed present in red blood cells after seeing an increase in MIF levels which correlated to the concentration of haemoglobin[21]. In order to explore this further, the aims and hypothesis of the study presented in this chapter were as follows.

3.1.1   Chapter hypothesis

It is hypothesised that red blood cells are the primary reservoir for MIF in whole blood and that this MIF is enzymatically active.

3.1.2   Chapter aims

1. To quantify MIF in human red blood cells.
2. To investigate the role of MIF in red blood cells, particularly its enzymatic activity.

3.2 METHODS

3.2.1 Blood collection

This study was approved by the Macquarie University Human Research Ethics Committee (5201100827) and by the Northern Sydney Coast Human Research Ethics Committee of NSLHD and CCLHD (1201-046M). Written informed consent was obtained from all participants before enrolment.

Whole blood was collected from healthy volunteers by venepuncture ($n = 10$; female: 3, male: 7). The age range of these participants was 21 - 47 years. Blood was combined with EDTA (ethylenediaminetetraacetic acid) for anticoagulation (K_2EDTA vacutainers, Becton Dickinson). Blood and blood components were isolated at room temperature within four hours of collection. All samples were subjected to three freeze-thaw cycles at -80 °C prior to analysis to ensure complete cellular lysis. Samples were stored at -80 °C until analysis.

3.2.2 Plasma and blood cell isolation

The components of whole blood were isolated using a range of techniques. Collected cell populations (whole blood, red blood cells, white blood cells, and platelets) were isolated and frozen immediately for analysis of cell lysates or were used fresh for storage experiments (Section 3.2.5). Concentration and purity of whole blood, red blood cell, white blood cell, and platelet populations were determined using a haematology analyser (Coulter AcT Diff, Beckman Coulter).

3.2.2.1 Whole blood and plasma

Whole blood was separated into aliquots and frozen at specific concentrations directly after anticoagulation. Plasma was isolated from anticoagulated whole blood by centrifugation (1500 *g*, 10 minutes, 23 °C) and was frozen at -80 °C.

3.2.2.2 Red blood cells and white blood cells

For isolation of the blood cells, whole blood was added to dextran solution (6 % w/v dextran in 0.15 M sodium chloride) at a 2:1 ratio (blood:dextran). This solution was gently mixed and left upright for 60 minutes at 23 °C for red blood cell sedimentation. After sedimentation, two layers formed and were collected and transferred into individual tubes. The lower, red blood cell dense layer, was washed once in phosphate buffered saline (PBS, 500 *g*, 5 minutes) and

the red blood cell pellet was isolated. The upper layer, rich in white blood cells, was washed once in PBS at 1000 g for 10 minutes. Any contaminating red blood cells were lysed by the addition of excess Milli-Q water (MQ-H$_2$O, 6:1 original whole blood volume) for 0.5 minutes. Isotonicity was restored by the addition of potassium chloride (0.6 M, 1:3 potassium chloride:MQ-H$_2$O). Remaining cells were pelleted and washed in PBS at 1000 g for 5 minutes.

3.2.2.3 *Platelets*

For isolation of platelets, platelet rich plasma was prepared by centrifugation of whole blood (100 g, 20 minutes, 23 °C). The upper, platelet dense layer was collected and contaminating cells were removed by two subsequent cycles of centrifugation of the platelet rich plasma and collection of the upper soluble fraction (100 g, 20 minutes, 23 °C). Platelets were then pelleted and washed twice in PBS (1000 g, 5 minutes, 23 °C).

3.2.3 Isolation of red blood cell cytosol

Red blood cell membranes were removed from cell lysates of purified red blood cells to collect the cytosolic fraction (after three freeze-thaw cycles). The cell membranes were isolated by centrifugation (16,000 g, 15 minutes, 4 °C) after which the upper, cytosolic fraction was collected and was stored at -80 °C until analysis.

3.2.4 Recovery efficiency

Recombinant MIF (rMIF, 50 ng/mL, R&D Systems) was spiked into red blood cell lysates immediately prior to analysis of samples on the MIF ELISA. A control sample was also analysed to factor in any dilution effect. For the controls, the sample diluent (MIF ELISA sample diluent, R&D systems) was added into the corresponding red blood cell lysates at the same volume as the recombinant MIF spike. The recovery efficiency of recombinant MIF was calculated using the following equation:

Equation 3.1. Recombinant protein recovery efficiency.

$$\frac{A + B}{C} \times 100 = Percentage\ recovery$$

Where,

       A = Concentration of un-spiked control

       B = Concentration of recombinant protein (spiked)

       C = Final assayed concentration of sample

3.2.5 Red blood cell conditioned medium and binding of MIF

To evaluate red blood cell release of MIF and protease activity during incubation, purified red blood cells were suspended in PBS (200 x 10^6 cells/mL) with or without protease inhibitors (PrI, mini cOmplete protease inhibitor cocktail tablets, Roche) and were incubated for 24 hours (5 % CO_2, 37 °C). After incubation, the red blood cells were removed by centrifugation (500 g, 5 minutes). The red blood cell pellet and the conditioned PBS were collected and frozen separately at -80 °C. Purified red blood cells were also incubated in the presence of recombinant MIF (2 ng/mL, Jomar Bioscience) as above in PBS and with or without protease inhibitors (24 hours, 5 % CO_2, 37 °C). After incubation, the red blood cell pellet and the conditioned PBS was collected and frozen separately at -80 °C. The '*in silico*' spike was calculated according to the following equation:

Equation 3.2. Calculation of concentration of *in silico* spike.

$$A + B = In\ silico\ spike$$

Where,

A = Concentration of MIF secreted in un-spiked control

B = Concentration of recombinant protein (spiked)

3.2.6 Haemoglobin quantification

Levels of free haemoglobin in plasma and red blood cell conditioned media were measured by spectrophotometric analysis as previously described[37] on a Synergy 2 plate reader (BioTek). Absorbance peaks at 414 nm are indicative of free haemoglobin. A standard curve of haemoglobin (Sigma Aldrich) at known concentrations was analysed at 414 nm.

3.2.7 Enzyme-linked Immunosorbent Assay (ELISA)

Concentrations of MIF were measured using a MIF ELISA (R&D Systems) which was run according to manufacturer's instructions. The absorbance data for the MIF ELISA were collected with a Synergy 2 plate reader (BioTek) at 450 nm with absorbance correction at 570 nm. The calibration curve was analysed using a log/log curve fit using GraphPad Prism software (ver. 6, USA).

3.2.8   Immunophenotyping

Immunophenotyping of the purified red blood cell population was performed by flow cytometry on a FACSCalibur flow cytometer with a 488 nm argon laser (Becton Dickinson) with data collection using CELLQuest Pro software (ver. 5.2.1). Sample preparation proceeded as follows. Fresh purified red blood cells were diluted in PBS and this solution was passed through a 35 µm cell strainer (Becton Dickinson) to produce a single cellular suspension. This cell suspension was then equally divided up into six flow cytometry tubes such that each contained at least 100,000 cells. Following this, the cells were washed twice in PBS with 2 % FBS (fetal bovine serum, 5 minutes, 1000 g, 23 °C). The resulting cell pellet was resuspended in 50 µL of PBS with 2 % FBS (v/v) and 5 µL of the isotype controls, or the monoclonal antibodies for CD45, CD44, or CD74 (Table 3.1). This solution was incubated at room temperature for 10 minutes. After incubation, the cells were washed twice in PBS (5 minutes, 1000 g, 23 °C). Cells were resuspended in 100 µL of PBS and were immediately analysed by flow cytometry. A minimum of 10,000 events were counted per sample.

Table 3.1.   Monoclonal antibodies for immunophenotyping of red blood cells.

Antibody	Fluorochrome	Clone
Mouse anti-human CD44	PE	515
Mouse anti-human CD45	FITC	HI30
Mouse anti-human CD74	FITC	M-B741
Mouse IgG1,κ Iso Control	PE	MOPC-21
Mouse IgG1κ Iso Control	FITC	P3.6.2.8.1
Mouse IgG$_{2a}$, κ Iso Control	FITC	G155-178

3.2.9   MIF tautomerase activity

Red blood cell derived MIF was tested for dopachrome tautomerase activity as previously described[38]. Briefly, *L*-dopachrome methyl ester was prepared shortly before use by adding 100 µL of *L*-3,4 dihydroxyphenylalanine methyl ester (12 mM; Thermo Fisher Scientific) and 100 µL of sodium periodate (24 mM, AnalaR) to 3800 µL of reaction buffer (50 mM Bis-Tris, 1 mM EDTA, pH 6.2). This solution was used immediately. Immediately prior to analysis, 200 µL of the reaction buffer was added to 20 µL of 40 nM MIF in a 96-well plate. In this assay, a decrease in absorbance is indicative of enzymatic activity and was monitored at 475 nm over 10 minutes. A negative control (no MIF) was included in this assessment to

monitor any non-enzymatic decrease in absorbance over the 10-minute period. For analysis of enzymatic activity, the following solutions were tested: 1) recombinant MIF, 2) whole red blood cell lysate (including membranes), and 3) the red blood cell cytosolic fraction. For MIF inhibition, 10 µL of the MIF solutions (80 nM) were incubated with 10 µL of ISO-1 ((S,R)-3-(4-Hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid, 5 mM in methanol, Merck Millipore), or 10 µL of the vehicle (methanol 75 % v/v in DMSO, dimethyl sulfoxide) for 15 minutes at room temperature before analysis. ISO-1 was reconstituted in DMSO to 20 mM, it was then diluted to 5 mM in 100 % methanol (v/v). This was optimised for MIF inhibition of red blood cell lysates as DMSO negatively interfered with the samples.

3.2.10   Statistical analysis

Data were evaluated using two-tailed, paired t-tests to assess statistical significance between groups with significance levels of $p < 0.05$. For comparison of three or more groups, a one-way ANOVA was used with Geisser-Greenhouse correction for sphericity and correction for multiple comparisons was performed (Sidak correction) with significance levels of $p < 0.05$. Statistical analysis and graphing of results was performed using GraphPad Prism software (ver. 6, USA). Flow cytometry results were analysed and illustrated using Flowing Software 2 (ver. 2.5.1, Finland). Where relevant, fold change after treatment was determined and presented as mean ± confidence interval (CI), and concentration values were presented as mean ± standard deviation (SD). Data analysed in this study were assessed as normally distributed according to the Kolmogorov-Smirnov test. The concentration of cytokines in blood components was presented according to the relative contribution in one millilitre of whole blood. This calculation was performed for each cell population according to the following equation:

Equation 3.3. Concentration of protein in one millilitre of whole blood.

$$\frac{A}{B} \times C = Contribution\ to\ whole\ blood$$

Where,

   A = Original cell number of population in 1 mL of whole blood

   B = Number of cells assayed per mL

   C = Concentration of analyte per mL

For the remainder of this chapter, the term 'significant' will be used to indicate a statistically significant result of $p < 0.05$ unless otherwise specified.

3.3 RESULTS

3.3.1 Red blood cell purity

The purity of enriched red blood cell fractions was calculated by comparing the ratio of white blood cells or platelets to red blood cells in the whole blood sample and to the enriched sample. Following dextran sedimentation, white blood cells were depleted by approximately 80 % and platelets were depleted by approximately 97 % (Table 3.2). Thus, for every one million red blood cells there were 210 white blood cells and 4,050 platelets.

Table 3.2.   Purity assessment of enriched red blood cell fractions.

RBC purity (%)*	WBC depletion (%)*	PLT depletion (%)*
99.97 ± 0.01	79.0 ± 5.1	97.3 ± 2.9

*Data are presented as mean ± standard deviation ($n = 10$)

3.3.2 Distribution of MIF between blood components

Red blood cells contribute the highest concentration of MIF to one millilitre of whole blood when compared to the other major components (Table 3.3). Red blood cells contribute 127 ± 19 % of the total MIF in whole blood, whilst white blood cells, platelets, and plasma contribute approximately 1.7 % combined. Although white blood cells contain the highest concentration of MIF per cell (34.1 ± 9.8 fg/cell), the prevalence of red blood cells is 1000 times higher than white blood cells. Thus, they are the primary reservoir for MIF in peripheral blood.

Table 3.3.   Distribution of MIF across major components of blood and corresponding average concentration per cell.

Blood Fraction	Percentage of MIF contribution (%) *	Total MIF contribution to 1 mL whole blood (ng/mL) *	Measured MIF/cell (fg/cell) *	Previously reported MIF/cell (fg/cell)
Whole blood	100	23,678 ± 4,365	n/a	n/a
Red blood cells	127 ± 19	31,241 ± 9,129	5.6 ± 0.9	n/a
White blood cells	1.1 ± 0.8	251 ± 173**	34.1 ± 9.8	3.9-1000[#]
Platelets	0.4 ± 0.2	92 ± 14**	0.3 ± 0.2	0.3[##]
Plasma	0.1 ± 0.1	26 ± 26**	n/a	n/a

*Data are presented as mean ± standard deviation ($n = 5$)

**Data are significantly different from whole blood ($p < 0.05$)

[#]Intracellular concentration of macrophages[17,19]

[##]Intracellular concentration of platelets[19]

3.3.3   Recovery efficiency

To investigate the extent of sample interference by factors such as haemoglobin, red blood cell lysates were spiked with recombinant MIF (final concentration of 2 ng/mL) immediately prior to analysis by ELISA to determine recovery efficiency. Recombinant MIF recovery was between 90 - 105 % (97 ± 7 %) for the red blood cell lysates. These results are similar to the reported recovery of recombinant MIF from EDTA plasma (80 - 108 %) and serum (94 - 104 %) (R&D systems, MIF ELISA product validation).

3.3.4   Location of MIF on red blood cells

The MIF concentration of the cytosolic fraction of red blood cells was compared to the whole red blood cell lysate. To isolate the cytosolic fraction, the red blood cell membrane was removed by centrifugation from the lysate. There was a significant decrease in the concentration and the fold change of the MIF measured in the cytosolic fraction compared to the whole lysate (Figure 3.1). There was an average fold change of 0.89 in the concentration of MIF between the lysate and the cytosol samples. This indicates that approximately 10 % of the MIF was bound to or was associated with the red blood cell membrane.
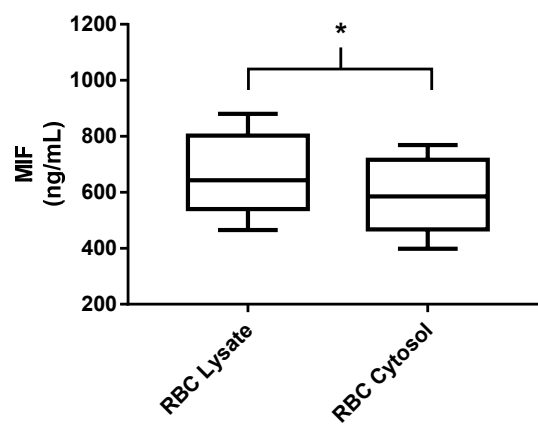
Figure 3.1.   MIF concentration in whole red blood cell lysate (per 10 million cells) and the concentration of MIF in the cytosolic fraction of the lysate (per 10 million cells) as measured by ELISA. Data are presented as mean fold change $\pm$ 95% CI, $n = 5$, statistically significant (*) if $p < 0.05$.

3.3.5   MIF receptors

Immunophenotyping of red blood cells was performed to investigate the presence of CD44 and CD74 receptors on red blood cells. These results demonstrate that whilst the red blood cells were positive for half of the MIF receptor complex, CD44, and that they were negative for the other half of the complex, CD74 (Figure 3.2). As anticipated, the analysed red blood cells were also negative for CD45, a leukocyte specific marker (Figure 3.2).
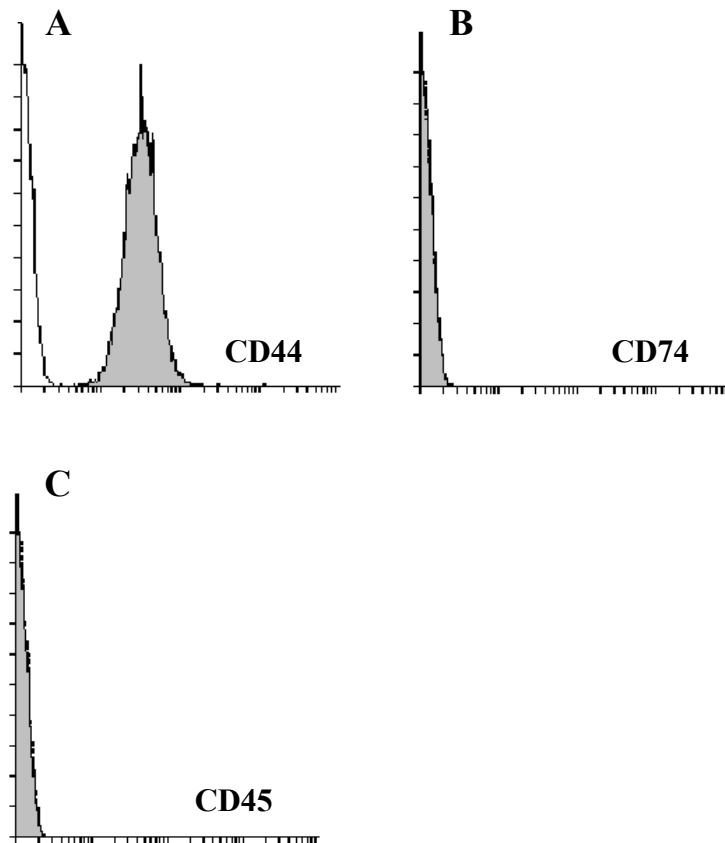
Figure 3.2.   Fresh red blood cells collected from healthy volunteers were stained for the presence of CD markers and the immunophenotype was determined by flow cytometry. A comparison of antibody labelled red blood cells (filled histogram) and the respective IgG negative controls (empty histogram) demonstrates that the red blood cells were positive for the presence of (A) CD44 and were negative for the presence of (B) CD74 and (C) CD45. Data are presented as overlay histograms.

3.3.6   Red blood cell incubation and MIF release (conditioned media)

MIF was released from red blood cells after overnight incubation at 37 °C in PBS (6.5 ± 1.4 ng/mL). The addition of protease inhibitors during incubation did not have a significant effect on the release of MIF or on the red blood cell lysates. After incubation, approximately 1 % (control: 1.1 ± 0.3 %; PrI: 1.3 ± 0.4 %) of the original intracellular concentration of MIF (control, T0) was released into the PBS (Figure 3.3).
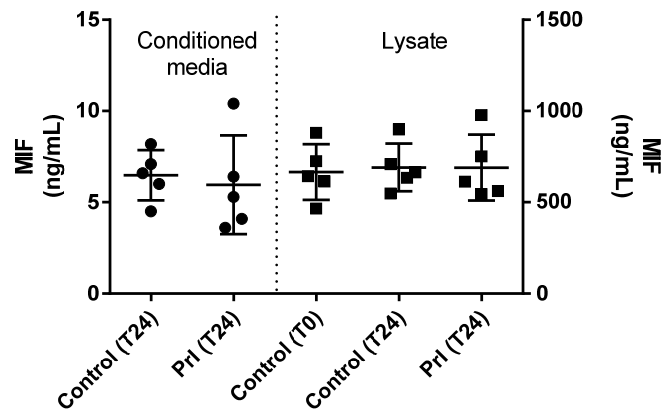
Figure 3.3. Red blood cells were incubated for 24 hours at 37 °C in 5 % CO_2 (200 x 10^6 cells per mL) in the presence of PBS alone (control) or PBS with protease inhibitors (PrI). After incubation the conditioned PBS (conditioned media, ●) and remaining whole cells (lysate, ■) were separated and frozen to lyse all intact cells. Samples were analysed by ELISA. Conditioned media samples (●) are presented against the left y-axis, and lysate samples (■) are presented against the right y-axis. Data are presented mean ± SD, $n = 5$, data are statistically significant (*) if $p < 0.05$.

3.3.7 Red blood cell binding of MIF

Recombinant MIF was added to suspensions of red blood cells which were then incubated at 37 °C for 24 hours. The '*in silico*' spike refers to the expected MIF concentration in the supernatant with the addition of rMIF. There was no significant difference between the concentration of MIF in the spiked sample and the predicted concentration (in silico spike) in the conditioned media or lysates of the control (Figure 3.4a and Figure 3.4c). However, with the addition of protease inhibitors during incubation there was significantly less ($p = 0.036$) MIF measured in the conditioned media fraction of the spiked sample when compared to the '*in silico*' spike (Figure 3.4b). Assay controls were run which demonstrated that the protease inhibitors themselves did not bind MIF out of solution (2.4 ± 0.7 ng/mL vs 2.2 ± 0.3 ng/mL, for rMIF vs rMIF + PrI respectively). As such, the results indicate that the cells were directly involved in the reduced levels of MIF. This difference was not mirrored in the corresponding protease inhibitor treated lysates (Figure 3.4d).
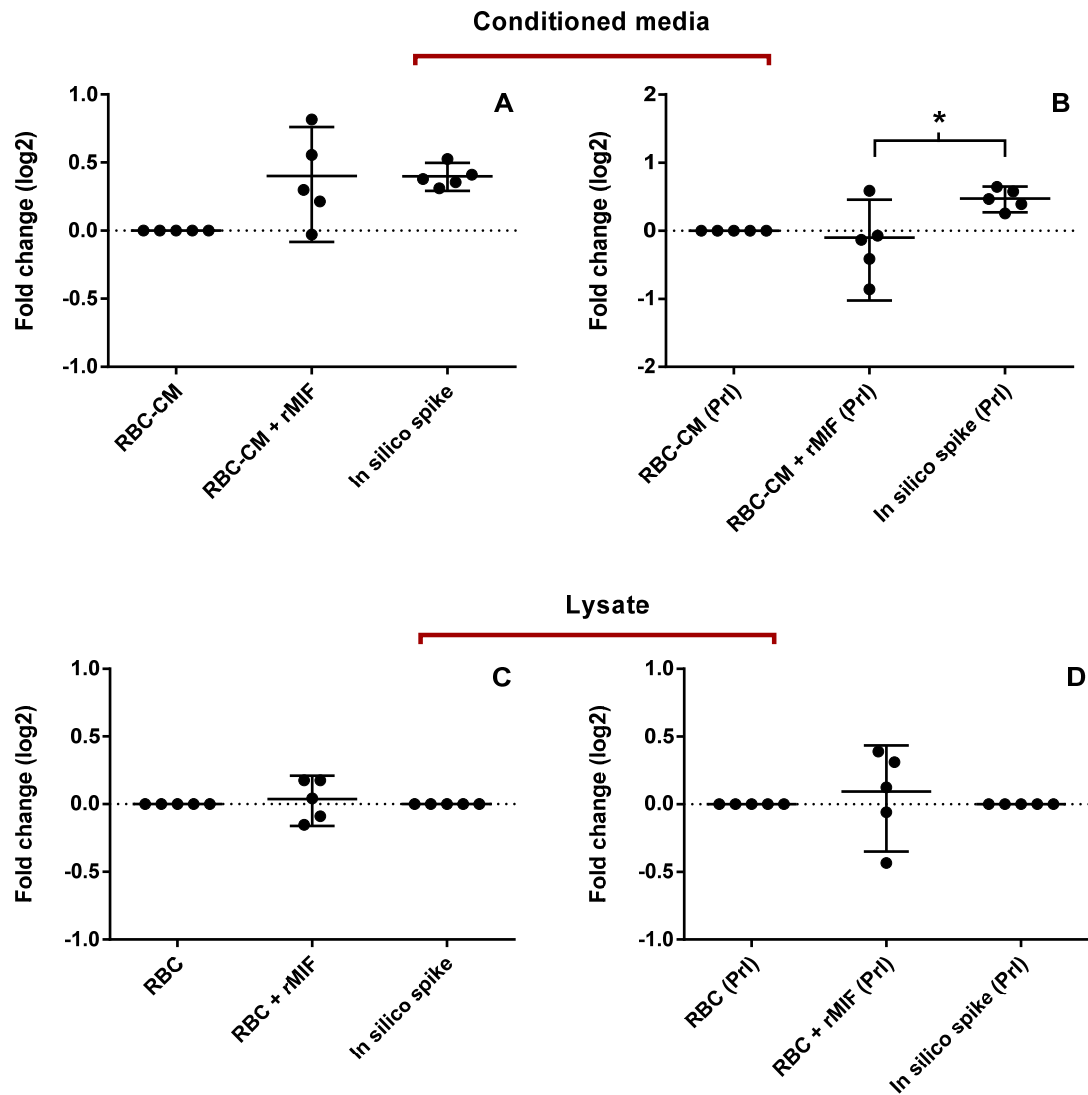
Figure 3.4. Red blood cells were incubated for 24 hours at 37 °C in 5 % CO_2 (200 x 10^6 cells per mL) in the presence of PBS alone (control), PBS with protease inhibitors (PrI), or PBS with recombinant MIF (rMIF) spiked in at a final concentration of 2 ng/mL to solutions with or without protease inhibitors (RBC + rMIF (PrI) or RBC + rMIF respectively). The expected concentration after spike is represented as '*in silico* spike'. After incubation the conditioned PBS (conditioned media, A and B) and remaining whole cells (lysate, C and D) were separated and frozen to lyse all intact cells. Samples were analysed by ELISA. Data are presented as the fold change from the PBS control (A and C) or the PBS + PrI control (B and D) with mean fold change ± 95% CI, $n = 5$. Data are statistically significant (*) if $p < 0.05$.

3.3.8   Concentration of haemoglobin in red blood cell conditioned media

The concentration of haemoglobin in plasma samples and in red blood cell conditioned media was quantified by monitoring absorbance at 414 nm. The level of haemoglobin in red blood cell conditioned media samples was significantly less than that measured in plasma with means of 0.2 mg/mL and 0.6 mg/mL respectively (Figure 3.5). There was no significant difference between the concentration of haemoglobin in red blood cell conditioned media with or without protease inhibitors (Figure 3.5).
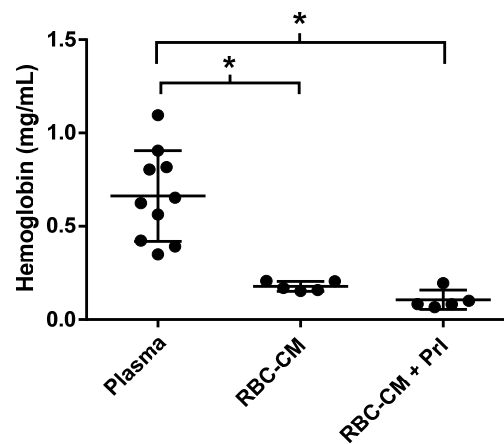
Figure 3.5.   Concentration of haemoglobin in plasma and the red blood cell conditioned media (200×10^6 cells per mL) incubated in PBS with and without protease inhibitors (RBC-CM + PrI and RBC-CM respectively) over 24 hours at 37 °C. Haemoglobin concentration was measured by absorbance at 414 nm. Data are presented as mean $\pm$ SD, $n \geq 5$, statistically significant (*) if $p < 0.05$.

3.3.9 MIF tautomerase activity

Consumption of the substrate *L*-dopachrome methyl ester in the presence of MIF is indicative of the tautomerase activity of the protein and is represented by a decrease in measured absorbance. The addition of red blood cell lysates and cytosol (containing 10 ng of MIF) to the substrate steadily decreased the measured absorbance over the 10-minute period indicating enzymatic activity (Figure 3.6). This reaction progressed at a comparable rate to the recombinant protein. There was no significant difference between the rate of substrate breakdown between the red blood cell cytosol and the whole lysate.
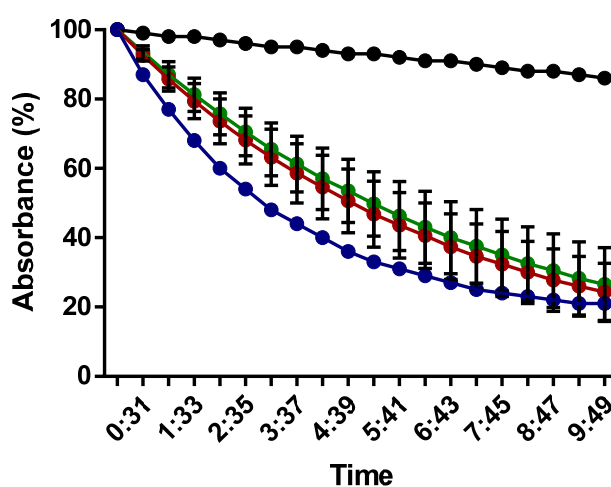
Figure 3.6. Dopachrome tautomerase activity of MIF was monitored in solutions of 10 ng of MIF and (black) an assay blank containing no MIF. A decrease in absorbance over 10 minutes was indicative of enzymatic consumption of the substrate *L*-dopachrome methyl ester as seen with (blue) recombinant MIF, (red) red blood cell lysate, and (green) red blood cell cytosol. Data are presented as mean ± SD, $n = 5$, statistically significant (*) if $p < 0.05$.

The addition of the MIF inhibitor, ISO-1, to recombinant MIF resulted in an inhibition of the tautomerase activity (Figure 3.7a). For recombinant MIF, the vehicle caused little interference with the assay, however this was not the case for the red blood cell samples. Although the chosen vehicle was optimised for the red blood cell samples, the addition of the vehicle without the inhibitor caused notable interference (Figure 3.7b and Figure 3.7c). After monitoring the absorbance for 10 minutes in the lysates, the PBS control had decreased to 24 % ± 8.3 compared to 44 % ± 12.4 for the vehicle control (Figure 3.7b). Despite the interference, there was a significant difference between the vehicle control and the inhibitor samples at the

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conclusion of the assay for both the lysate and the cytosol (lysate: $p = 0.036$; cytosol: $p = 0.005$). This difference was more prominent in the cytosol samples. This indicates that there had been inhibition of MIF in red blood cell samples although it was not complete under the conditions tested.
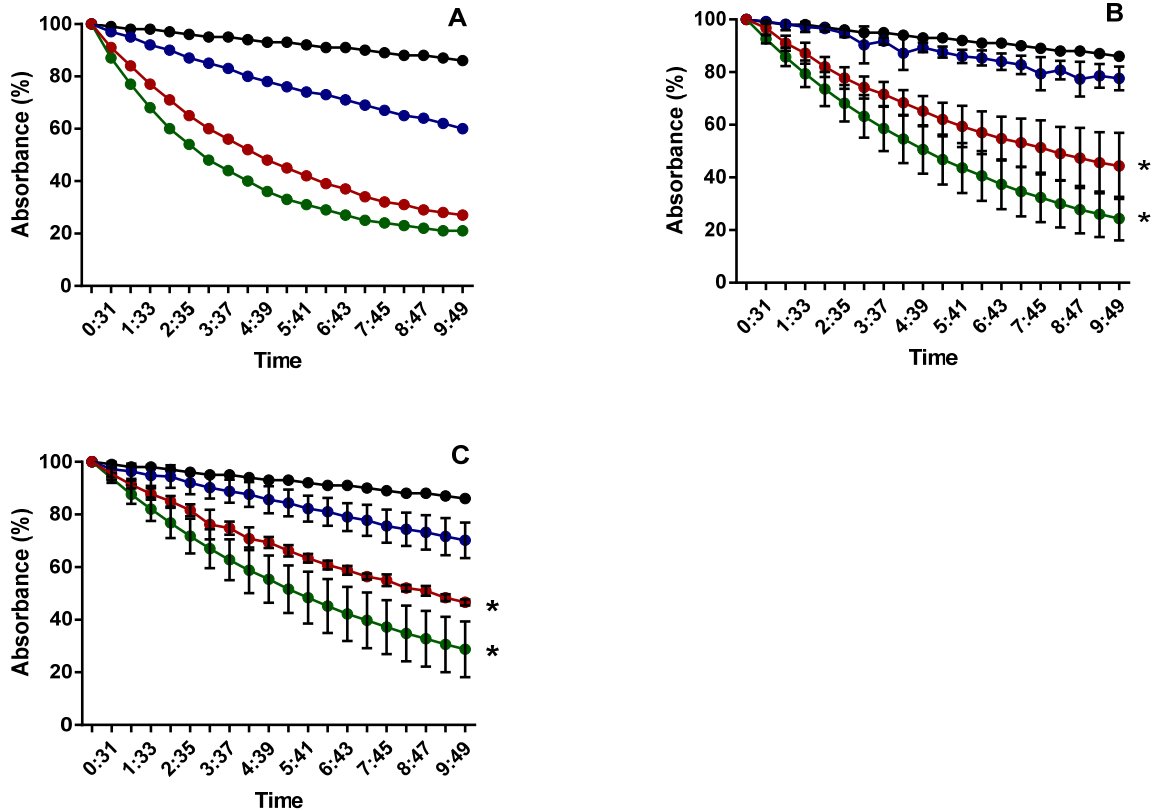
Figure 3.7. Dopachrome tautomerase activity of MIF and its inhibition using ISO-1 was monitored for (A) recombinant MIF, (B) red blood cell lysate, and (C) red blood cell cytosol. MIF samples were incubated with PBS (green), 5mM ISO-1 in vehicle (blue), or vehicle (red) prior to analysis. A decrease in absorbance was monitored at 450 nm and was indicative of enzymatic consumption of substrate. An assay blank (black) which contained no MIF was also monitored to determine level of non-enzymatic loss of absorbance. Data are presented as mean $\pm$ SD, $n = 5$, statistically significant compared to the ISO-1 sample (*) if $p < 0.05$.

3.4   DISCUSSION

The purpose of this study was to assess the distribution of MIF across the components of peripheral blood to identify potential reservoirs that may alter serum levels during blood processing. In investigating this, the red blood cell component of blood was identified as a major source of MIF. Although others had previously identified that MIF was associated with red blood cells, a crucial aspect of this study was the quantification of this protein, which revealed the dominant role of red blood cells as a MIF reservoir. In conventional biomarker research, MIF is monitored in the plasma fraction of blood, and white blood cells have been attributed as the primary reservoir for this protein[17,18]. More recently, platelets were also reported to contain MIF and MIF mRNA[19]. The research in this chapter demonstrates that the other major component of blood, red blood cells, not only contains a store of MIF but that they account for the majority of MIF that is present in whole blood (Table 3.3). By using blood collected from healthy volunteers, red blood cells were isolated and were identified to contain 90 % of red blood cell derived MIF in the cytosol and that the remaining 10 % was associated with the red blood cell membrane (Figure 3.1). An activity assay was performed to assess if the MIF inside red blood cells was in its active state or if it required modification upon release and it was demonstrated to be enzymatically active within the cell (Figure 3.6). It was also identified that red blood cells release approximately 1 % of their total MIF into the surrounding solution when incubated at 37 °C for 24 hours (Figure 3.3).

The results of this study indicate that red blood cells are likely to have functions beyond gas exchange, particularly in the inflammatory process. This study showed that red blood cells can release MIF into solution, however, the factors that may influence this were not explored in detail. In the subsequent chapters, some of the stimuli that can affect release from red blood cells were tested.

3.4.1   MIF in red blood cells

In a report aiming to optimise blood collection techniques for MIF analysis, Sobierajski *et al.* demonstrated that as haemolysis occurred over time, levels of MIF also increased at a linear rate[15]. They concluded that red blood cell associated MIF may be the cause of this artefact. Confirming this observation in our study, high levels of MIF associated with red blood cells were identified (Table 3.3). It was also demonstrated that the recovery efficiency of

recombinant MIF from red blood cell lysates was as good, if not slightly better than the reported recovery efficiencies in serum and plasma (R&D systems, MIF ELISA product validation). This result indicates that there were no additional interfering factors (such as haemoglobin) in red blood cell lysates which may compromise MIF detection.

3.4.1.1 *MIF on the red blood cell membrane*

The concentration of MIF, on a per cell basis, for white blood cells and platelets was consistent with reports from the literature[17,19]. Although red blood cells contain at least 8-fold less MIF per cell than white blood cells, the abundance of red blood cells in whole blood means that their contribution of MIF outweighed any other component by more than 100-fold (Table 3.3). Analysis of the MIF concentration in the cytosolic fraction compared to the total cell lysate (which included red blood cell membranes) indicated that approximately 10 % of the detectable MIF may actually reside on the red blood cell membrane (Figure 3.1). In order to further investigate this, immunocytochemistry would be a beneficial technique in identifying the localisation and distribution of MIF in and/or on red blood cells. If MIF does reside on the red blood cell membrane, its binding receptor(s) at this stage are unknown. Using immunophenotyping, it was observed that the most well-known MIF receptor complex (CD44-CD74) does not exist on the membrane (Figure 3.2). MIF has been reported to bind to other receptors including CXCR2[4], however there is no literature on the presence of CXCR2 on red blood cells. Interestingly the Duffy antigen receptor for chemokines (DARC), which is abundant on the red blood cell surface, shares a number of binding partners with the MIF receptor CXCR2[39]. These shared binding partners include a number of cytokines such as IL-8, GRO-1, and GRO-3[39]. This study has shown that the primary receptor for MIF is not present on red blood cells, thus it is hypothesised that MIF, like other cytokines, is also a shared binding partner of CXCR2 and DARC. This hypothesis was not in the scope of this current study, but future research into the MIF content of red blood cells in DARC knockout models may be useful to test this hypothesis.

3.4.2 **MIF release and binding: a passive or active process**

3.4.2.1 *MIF release from red blood cells*

In this study, it was observed that approximately 1 % of red blood cell associated MIF was released when incubated at 37 °C (Figure 3.3). In these conditioned media samples, the amount of haemoglobin detected accounts for approximately 750,000 red blood cells per millilitre,

which is approximately 0.4 % of the starting cell number (Figure 3.5). Therefore, over half of the MIF detected in the conditioned media was released from intact red blood cells.

In other cells, MIF is secreted by non-classical pathways, which are not well understood in the protein secretion literature. One proposed pathway utilises the ATP binding cassette transporter subfamily 1 (ABCA1)[40]. When the transporter was inhibited on monocytes, the secretion of MIF after lipopolysaccharide stimulation was significantly attenuated[40]. This transporter primarily regulates cellular cholesterol and phospholipid homeostasis, and is present on red blood cells[41,42]. In red blood cells, it is unclear whether active protein release occurs, or whether MIF is released from intact red blood cells via a passive system. However, if active release of MIF occurs from red blood cells, the ABCA1 mechanism may enable it.

3.4.2.2 Red blood cell binding of MIF

Red blood cells have been described as an inflammatory sink for chemokines to regulate circulating levels[32,43]. In this thesis, it was hypothesised that red blood cells perform a similar role for MIF; acting as a buffer to regulate free cytokine in plasma. An earlier report proposed that red blood cells could bind exogenous MIF, thereby removing the inhibition of macrophage migration[44]. They demonstrated that after red blood cells were incubated with FBS, MIF activity in the serum, as measured by migration inhibition, decreased. They postulated that this decrease was a result of MIF being bound to the red blood cells. To further explore the potential binding of MIF, the study in this chapter incubated red blood cells with recombinant MIF for 24 hours and then assayed the solution for extracellular MIF. Red blood cell binding of exogenous MIF should have decreased the media concentration, however it was not significantly different to the expected levels in the control samples (*in silico* spike). In a variation of the experiment, the addition of protease inhibitors during incubation did have an effect (Figure 3.4). There was significantly less MIF in the conditioned media in comparison to the '*in silico*' spike after incubation in the presence of protease inhibitors, indicating either an attenuation in MIF release or an increase in MIF binding to the red blood cells. Discussion on the possible causes of this will follow in the next section (Section 3.4.2.3).

3.4.2.3 Proteases and red blood cells

Proteases play a key role in the binding and secretion of cytokines in other cell types through processing of pro-proteins to active cytokines and in modulating the activity of receptors[45]. Red blood cells do not have protease activated receptors, which are a sub-family of G-protein coupled receptors[46]. However, receptors such as DARC have the 7-transmembrane structure

associated with G-protein coupled receptors, without the intracellular signal transduction domain[47]. Proteases and enzymes play a crucial role in red blood cells in the absence of organelles. Red blood cells utilise enzymes for a variety of functions including the production of prostaglandins as well as their inactivation, and the production of nitric oxide using endogenous nitric oxide synthases[48–50]. By incubating red blood cells with protease inhibitors, some enzyme activity may have been suppressed causing the observed reduction in exogenous MIF (Figure 3.4). The protease inhibitors used in this study are a cocktail of broad spectrum inhibitors, that act on serine-, cysteine-, and metalloproteases. Future work in this area would benefit from studying the effect of individual inhibitors on the binding and release activity of red blood cells. In order to investigate this further, use of stable isotope labelled or xeno-protein would provide valuable information. These techniques could be used to track the movement of the protein and to determine whether or not it was internalised or modified by protease activity.

3.4.2.4   *Red blood cell depletion*

Although it was not investigated in this study, one area of interest for further research may be the effect of depleting the cells of MIF. One hypothesis for the lack of binding observed in this study (Figure 3.4) was that the cells may already be saturated with MIF, which may also induce feedback inhibition. Depleting the cells of this protein may enable MIF to bind more readily to the red blood cells. Specific methods of depletion have not been investigated in detail in this project, but it has been observed that methods involving extensive washing of the cells may result in this type of depletion (Appendix B). If depleting the cells did result in a higher binding capacity for MIF, there is a possibility that the cells could be depleted *in vitro* and then used as a therapeutic for diseases associated with excess levels of MIF such as sepsis or rheumatoid arthritis[7,9]. This type of therapy may have utility as an alternative to anti-MIF treatments.

3.4.3   **Evidence for a dual role**

MIF is a unique cytokine that functions both as an inflammatory chemokine and as an enzyme. The inflammatory activity of MIF is dependent on the enzymatic active site, thus these two functions are linked[51]. Swope *et al.* showed that by blocking the enzymatic active site, MIF had a reduced capacity to activate neutrophils[51]. Most studies that investigate the activity of MIF use protein that has been secreted from white blood cells. The study in this chapter investigated the activity of intracellular MIF. A key finding, that has relevance to the capacity of red blood cells to affect the immune response, was that intracellular MIF is enzymatically active and thus likely to be functional as a chemokine (Figure 3.6).

3.4.3.1 Intracellular: Oxidoreductase activity

The less frequently discussed enzymatic function of MIF is its thiol-oxidoreductase activity and its ability to scavenge reactive oxygen species[52,53]. This study demonstrated that there is a high concentration of MIF in red blood cells, which may be explained by assuming that the primary function is intra-cellular oxidoreductase activity. The chemokine activity of MIF from red blood cells is likely to be observed by either release from intact cells or in much higher concentrations after red blood cell lysis. Nguyen *et al.* identified that the oxidoreductase activity is retained in a specific peptide fragment of MIF which is independent of its tautomerase activity[52]. This activity has since been demonstrated in a number of cell types. MIF deficient fibroblasts contained 2.3-fold more intracellular reactive oxygen species (ROS) than the MIF-positive controls[53] and in neurons the increase in ROS after stimulation was decreased by intracellular administration of rMIF[54]. The effect of MIF on oxidative stress has also been demonstrated on whole organisms. Recently, MIF has been shown to play a crucial role in healthy lung development in neonates[55]. Hyperoxia-induced lung injury (HALI) is lung damage that can result from exposure to high levels of oxygen such as in a neonatal intensive care unit. In a recent article, Sun *et al.* identified that MIF plays an important regulatory role in dealing with high levels of oxygen[56]. When MIF was knocked down, there was a higher level of mortality among the mice. Similarly, altered expression of MIF in pre-term infants was associated with the incidence of bronchopulmonary dysplasia[57]. For premature mouse pups, only 8 % of MIF deficient mice survived compared to 75 % of the wild type mice[55]. The low mortality rate of MIF deficient mice was closely correlated with less mature lungs than the control group.

Red blood cells are burdened with substantial oxidative stress due to their primary oxygen-carrying function. To combat this, they contain a number of known mechanisms, such as the 20S proteasome, to deal with this stress and catabolise damaged proteins. Intracellular MIF is likely to be another part of the red blood cell stress mechanism. Investigation in the activity of MIF as an oxidoreductase in red blood cell lysates was complicated by the fact that red blood cells contain a variety of different oxidoreductase enzymes. Thus, investigation into this activity would require protein purification to isolate the red blood cell derived MIF in its native form. Quantifying the oxidoreductase activity of red blood cell derived MIF would be interesting, but was not in the scope of this study.

3.4.3.2   *Extracellular: Cytokine activity*

MIF has also been described to have a number of roles in inflammation. It was originally identified as the factor that inhibited macrophages from migrating away from the site of inflammation[8]. Since then, it has been identified as a chemotactic attractant for monocytes, a modulator of glucocorticoid activity, and involved in neutrophil activation[17,19,51]. Since MIF has multiple enzymatic and chemotactic functions, studying the cytokine activity in isolation is not straightforward. Glucocorticoid regulation by MIF is associated with its oxidoreductase activity and the leukocyte activation is associated with its tautomerase activity[51,52]. The migration assay used to measure MIF activity involves the quantification of cell migration in the presence of MIF compared to controls. Numerous problems have been reported with the assay due to cell aggregation, surface adhesion, and passive migration, which lead to high standard deviations and only semi-quantitative results[58]. In addition, there have been reports of difficulty in purifying active MIF, which have led to suggestions that tertiary structural changes or an unknown co-factor affects the activity[58].

The data presented in this chapter indicate that MIF is present at a concentration of approximately 30 µg/mL in whole blood, which is one million times higher than the pg/mL levels usually reported for cytokines in plasma. Given that acute injury events, such as those reported by Pohl *et al.*[22] can cause the release of high quantifies of MIF locally and systemically, it seems likely that a mechanism for the attenuation of MIF chemokine activity would exist.

3.4.3.3   *Other: Tautomerase activity*

Substantial research has been done on investigating the tautomerase activity of MIF with a particular emphasis on the development of MIF inhibitors[7,59]. In spite of this, its biological substrate is yet to be identified and the reason for MIF having this activity remains unknown. A number of chemical substrates for MIF tautomerase activity have been identified (such as D-dopachrome, L/D-dopachrome methyl ester, or *p*-hydroxyphenyl-pyruvate) and are used to quantify the level of activity *in vitro*. D-dopachrome tautomerase (DDT), or MIF-2, catalyses the same substrates *in vitro*, but its activity is not inhibited by the same chemical, ISO-1[35].

This study identified that red blood cell lysates and cytosols are enzymatically active and are active at a rate that is comparable to recombinant MIF. Considering that the majority of MIF is present in the cytosolic fraction of red blood cells (Figure 3.1) it is not surprising that there was not a significant difference in the rate of substrate degradation between the whole lysate

and the cytosol (Figure 3.6). Inhibition of recombinant MIF, lysate derived MIF, and cytosolic MIF using ISO-1 resulted in a significant attenuation of the breakdown of the substrate (Figure 3.7). However, it was difficult to quantify how effective this inhibition was in red blood cells samples. For ISO-1 to act effectively as an inhibitor of MIF activity, it required a high concentration (~50 %) of organic solvent to be present. The most widely reported solvent for ISO-1 is DMSO. Although the presence of DMSO did not affect recombinant MIF, it interfered with the red blood cell samples and the addition of the DMSO vehicle alone turned the solutions opaque. This interference has been documented in the literature[60], so to circumvent this, the effects of a range of other organic solvents were tested and methanol was chosen as the optimal solvent. Methanol did not impede the inhibitory effect of ISO-1 and it had the lowest background interference for red blood cell samples. The background effect of methanol as a vehicle on the samples was less for the cytosolic fraction (Figure 3.7c) than for the lysates (Figure 3.7b) as some precipitation was observed in the lysate samples at the conclusion of the incubation with the sample.

Although MIF inhibition did occur, it was not possible to quantify the level of inhibition with the current assay and the complex nature of the red blood cell lysate. It is not clear if the tautomerase activity was a result of MIF enzymatic activity alone, or if it was a combination of MIF and D-dopachrome tautomerase enzymatic activity. Future studies should target the identification of MIF inhibitors that are compatible with complex protein solutions such as cell lysates.

3.4.4   Implications in disease

High plasma levels of MIF are often correlated with increased inflammation. A key aspect to consider, regarding the microgram quantities of MIF residing in red blood cells, is the implications of cell lysis in disease or injury. Red blood cell lysis can occur following injury, which would release high concentrations of enzymatically and chemotactically active MIF. This immediate release of high levels of MIF may actually play a role in the early stages of the immune response to injury. There is considerable literature on the protective effects of MIF and a recent paper on an animal model of ischemia reperfusion injury after myocardial infarction demonstrated that MIF exerts both pro-inflammatory effects and protective effects in cardiomyocytes[61].

It is important to note that the data enclosed in this chapter was collected using blood from healthy volunteers, and as such, analysis of blood isolated from specific disease cohorts in the

future would be of value. MIF is frequently cited as being present at increased concentrations in the plasma of diseased cohorts when compared to the healthy controls[10-12]. It is not unreasonable, therefore, to hypothesise that the concentration of MIF in, or being released by, red blood cells may also differ in these groups. Further research in this area should seek to determine if there is any correlation between red blood cell associated MIF and the initiation, progression, or the protection against inflammatory disease.

MIF in red blood cells is likely present for a number of reasons. It is part of the mechanism by which the red blood cell manages oxidative stress, and it is able to exert its pro-inflammatory and protective functions upon lysis. Like MIF, red blood cells too are playing multiple roles. Whilst their primary role is in a gas transport and exchange, they are also likely to be involved in the immune response. Further investigation into this alternative role for red blood cells will be outlined in Chapter 4.

3.5 CONCLUSION

The results of this chapter demonstrate that *in vivo* haemolysis would result in the release of high concentrations of active MIF. Unless this excess MIF is modulated or deactivated in other ways, this released MIF would exert its pro-inflammatory signalling activity on surrounding cells. MIF has been described as an important factor in a wide variety of diseases and a review of the literature presents a rich tapestry of its interactions in many cell types, including both pro-inflammatory and protective effects in the same injury model. The identification and quantification of this cytokine in its active form in the most abundant cell type in the body (red blood cells) is likely to contribute to the understanding of a number of these MIF associated conditions. MIF is present in whole blood in microgram quantities per millilitre, however, during normal function, over 90 % is sequestered in the red blood cell cytosol. The release of MIF in microgram per millilitre concentrations after injury suggests that highly effective mechanisms are present to suppress its chemokine activity. Given the literature on the protective functions of MIF, an alternative hypothesis is that extracellular MIF has a cell and tissue protective function that may be its primary role after acute injury[62].

This discovery could have implications on conditions such as haemolytic anaemia, wound healing and clot formation, sepsis, and adverse events following red blood cell transfusions. More research is required to elucidate the involvement of red blood cell derived MIF in a variety of processes, but this discovery may change the way that disease is researched, including its progression and the involvement of the immune system. Just as 'junk DNA' was discovered to be anything but 'junk', so too are red blood more active than was once thought. Subsequent chapters in this thesis investigate the presence of other cytokines and growth factors in red blood cells that may be working in concert with MIF to communicate with the immune system.

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CHAPTER

4

INVESTIGATION AND QUANTIFICATION OF A PANEL OF CYTOKINES IN RED BLOOD CELLS

This chapter investigates the presence of a panel of 48 cytokines in red blood cell lysates isolated from healthy volunteers. The previous chapter of this thesis demonstrated that red blood cells are the primary reservoir for macrophage migration inhibitory factor in whole blood and that this protein was enzymatically active. Upon the discovery that this pro-inflammatory protein was present in red blood cells at 1000-fold the concentration that is detected in plasma, it was desirable to know if this was to be observed for any other inflammatory cytokines. This chapter will expand on that investigation by quantifying the levels of 47 additional cytokines in red blood cells and in plasma using multi-plex technology. In addition, the binding and release of these cytokines from red blood cells will be assessed with consideration of the role of proteases in these activities. This investigation is based on the premise that red blood cells are able to act as a buffer in the blood for inflammatory molecules by binding and releasing these proteins as needed.

4.1 INTRODUCTION

Analysis of clinical blood samples is typically one of the first steps in diagnosing disease and is an important part of the research process of seeking new diagnostic markers. Monitoring the level of markers such as liver enzymes or C-reactive protein in the plasma component of blood can be indicative of inflammatory changes that occur in a wide range of conditions. Testing only plasma or serum simplifies the analysis by removing the cellular components of the blood. This is valuable, but it represents an incomplete profile of the relevant markers. The plasma is only one part of a much more complex, living tissue that rapidly responds to stress by altering the levels of signalling proteins that are also commonly studied markers[1]. After blood is collected for analysis, the sample must be stabilised to avoid changes in the plasma levels of disease markers. A number of factors, such as choice of anticoagulant, temperature, time, cell lysis, or platelet activation can result in release of cytokines and other proteins into the plasma thus invalidating the resulting analysis[2]. In the previous chapter, macrophage migration inhibitory factor (MIF) was shown to be present in red blood cells at levels 1000-fold higher than the typical plasma concentration (Chapter 3). After blood collection, the plasma level of MIF could be significantly altered by relatively low levels of haemolysis or by release of MIF by intact red blood cells. In this chapter, the analysis of potential biomarkers associated with red blood cells was widened to include 47 additional cytokines, chemokines, and growth factors that are commonly studies as biomarkers of disease. Importantly, the analysis included the total concentration in red blood cell lysates and the concentration released by intact red blood cells. Blood is composed of three main cell types (white blood cells, red blood cells, and platelets) suspended in plasma. An understanding and analysis of the other components of blood can provide some critical information in disease populations. Differential white blood cell counts and morphological changes in leukocytes can provide valuable diagnostic information that supplement other tests and clinical assessments[3]. These cells are known to produce and respond to a number of cytokines to either promote or suppress inflammatory processes[4,5] and more recent research into white blood cells has revealed that they have significant roles in some diseases and would potentially have value in diagnostics[6,7]. Platelets are specialised, enucleate, cellular components of blood that function in the coagulation cascade to stop bleeding by aggregating and clotting blood vessel injuries. The role of platelets in signalling, has been the subject of recent research and they have been demonstrated to contain mRNA and a range of

inflammatory signalling proteins, which may play a role in inflammatory diseases such as atherosclerosis[8–10]. Platelets are an easily harvested source of cytokines and growth factors and have been harnessed as a therapeutic for inflammatory conditions, such as the use of platelet rich plasma for osteoarthritis[11]. Red blood cells on the other hand are understudied and are generally understood to be primarily involved in respiratory gas exchange. As a result of this and their extremely high haemoglobin content, which is considered a contaminant in biomarker studies, red blood cells are discarded more often than they are analysed. The use of red blood cells for diagnostics is typically limited to monitoring cell number, concentration of haemoglobin, and erythrocyte sedimentation rate[12]. Although red blood cell research has been relatively slow, some studies have shown that red blood cells are multifunctional and may be more complex than previously understood.

Red blood cells can enhance cytotoxicity mediated by natural killer cells against tumour cells *in vitro* in a dose-dependent manner[13]. Red blood cells, or soluble factors released by these cells, can also stimulate the secretion of pro-inflammatory markers from lung fibroblasts[14]. These studies demonstrate that red blood cells may be an important component of the immune system and are capable of signalling or receiving signal from other cell types. In 1974, Fox *et al.* reported that red blood cells could 'mop up' endogenous macrophage migration inhibitory factor (MIF)[15], which they postulated was due to red blood cell binding and others have reported similar activity with the Duffy antigen receptor for chemokines (DARC). DARC is a receptor on the surface of most red blood cells and has been studied as the primary binding site for the pathogen *Plasmodium vivax*[16]. However, as Neote *et al.* identified, this receptor is "promiscuous" and has a number of binding targets[17]. These targets include some chemokines from the C-X-C or C-C cytokine families including IL-8, RANTES, and MCP-1. They proposed that the role of this receptor may be to act as a chemokine sink for modulating inflammation. Since this report in 1993, few additional cytokines and inflammatory proteins have been identified on or in red blood cells[18–20].

The previous chapter of this thesis demonstrated that red blood cells are the primary reservoir of the pro-inflammatory cytokine MIF in whole blood (Chapter 3). This cytokine is present in red blood cells in its active pro-inflammatory state within the cell cytosol, suggesting that it is primed for activity upon red blood cell lysis. This further supports the theory that the red blood cells may in fact play an important role in inflammation. Red blood cells are devoid of organelles, but they have developed alternative processes to continue functioning throughout their lifetime. MIF is just one of the many active enzymes within red blood cells. Red blood cells contain an enzymatic structure known as the 20S proteasome[21]. This proteasome is crucial

for managing oxidative stress and protein damage within the cell in concert with other enzymes such as catalase and glutathione peroxidase[22,23]. Membrane bound enzyme complexes regulate glycolysis[24], and intracellular enzymes function in protein and molecule modification within red blood cells[25]. Oonishi *et al.* reported that mechanical stress stimulated the red blood cells to produce more cytosolic PGE_1 and PGE_2[25]. They postulated, with support from previous literature[26,27], that enzymes were responsible for modifying the molecules into their active, detectable state when stressed.

Previous studies have identified a small number of chemokines associated with red blood cells, however, the role of red blood cells in signalling remains poorly understood. In this chapter, the analysis of purified red blood cells was widened to include 47 additional cytokines, chemokines, and growth factors that are commonly studied as biomarkers of disease. In addition, the analysis explored factors that may affect the binding and release of cytokines from red blood cells, including enzymatic control. It is of interest to understand how red blood cells may be involved in the homeostasis of whole blood by releasing and binding of signalling proteins.

4.1.1 Chapter hypothesis

It is hypothesised that red blood cells are associated with more cytokines than MIF alone, and that red blood cells can bind and release these cytokines in response to their environment.

4.1.2 Chapter aims

1. To quantify the levels of a panel of 48 inflammatory cytokines, chemokines, and growth factors in human red blood cells.
2. To investigate the capacity of red blood cells to bind and release these cytokines.
3. To determine the role of proteases in cytokine binding and release.

4.2 Methods

4.2.1 Blood collection

Whole blood was collected from healthy volunteers by venepuncture ($n = 10$; female: 6, male: 4) directly into EDTA vacutainers (K_2EDTA vacutainers, BD Biosciences). The age range of these participants was 23 - 47 years. All fractions of blood were collected and processed at room temperature within four hours of collection. All samples were stored at -80 °C and were subjected to three freeze-thaw cycles at -80 °C to ensure complete cellular lysis prior to analysis.

This study was approved by the Macquarie University Human Research Ethics Committee (5201100827) and by the Northern Sydney Coast Human Research Ethics Committee of NSLHD and CCLHD (1201-046M). Written consent was collected from all participants before participation in this study.

4.2.2 Plasma and blood cell isolation

Plasma and blood cells were isolated from EDTA anti-coagulated whole blood. Plasma and white blood cells at known concentrations were collected and frozen immediately for future analysis. Red blood cells were collected and were either frozen immediately or were used fresh for storage and protein binding experiments (Section 4.2.3 and 4.2.4). Cell number and purity was determined using a haematology analyser (Coulter AcT Diff, Beckman Coulter).

4.2.2.1 Plasma

Plasma was isolated by centrifuging whole, anticoagulated blood at 1500 *g* for 10 minutes at room temperature. The plasma fraction was collected and stored at -80 °C.

4.2.2.2 Red blood cells and white blood cells

Red blood cells and white blood cells were isolated using dextran sedimentation as follows. Whole blood was added to a 6 % solution of dextran (w/v in 0.15 M sodium chloride.) at a 2:1 ratio. This solution was left at room temperature for 1 hour for the red blood cells to sediment to the bottom of the tube. After this time the upper white blood cell rich layer and the lower red blood cell fraction were separated and added into individual tubes. The lower red blood cell fraction was washed once in phosphate buffered saline (PBS, 500 *g*, 5 minutes) and the remaining red blood cell pellet was either frozen (-80 °C) or used fresh. The upper, white blood

cell rich layer was washed once in PBS (1000 g, 10 minutes). The supernatant was discarded, and any contaminating red blood cells were lysed by hypotonic shock by resuspending the cell pellet in 3 mL Milli-Q water for 30 seconds. After this time, isotonicity was restored by adding 1 mL potassium chloride (0.65 M) and the solution was diluted up to 15 mL with PBS. The remaining cells were pelleted and washed twice in PBS (1000 g, 5 minutes) and the remaining cell pellet was resuspended in PBS and frozen immediately at -80 °C.

4.2.3   Red blood cell conditioned medium

After isolation, red blood cells were either frozen immediately in PBS (400 million cells per mL) to produce red blood cell lysates (T0) or they were incubated for 24 hours in PBS (T24, 200 million cells per mL, 37 °C, 5 % CO_2). Red blood cells were incubated with or without protease inhibitors (1x, mini cOmplete protease inhibitor cocktail tablets, Roche). After incubation, the conditioned media was collected by centrifugation (500 g, 5 minutes), and the remaining red blood cell pellet was resuspended to a concentration of 400 million cells per mL. All samples were stored at -80 °C, and were subjected to three freeze/thaw cycles before analysis.

4.2.4   Red blood cell protein binding

To assess whether or not red blood cells could bind exogenous protein out of solution, they were incubated for 24 hours (T24, 37 °C, 5 % CO_2) in the presence of Bio-Plex recombinant protein standards reconstituted and diluted in PBS (27-plex and 21-plex human cytokine panels, Bio-Rad). Recombinant protein standards (25 µL, Bio-Plex standard-3) were diluted in 175 µL of PBS with or without protease inhibitors (1x, mini cOmplete protease inhibitor cocktail tablets, Roche). Red blood cells were incubated at 200 million cells per mL according to the conditions outlined in Table 4.1. An aliquot of untreated red blood cells was also frozen prior to incubation (T0) in PBS (400 million cells per mL).

Table 4.1. Incubation conditions for red blood cell (RBC) protein binding analysis at 37 °C, 5 % CO_2.

RBCs (200 million cells per mL)	PBS	Recombinant Protein	Protease Inhibitors
Condition 1 (Control)	✓	✗	✗
Condition 2 (BP)	✓	✓	✗
Condition 3 (PrI)	✓	✗	✓
Condition 4 (BP-PrI)	✓	✓	✓

After incubation, the resulting conditioned media was isolated by centrifugation (500 *g*, 5 minutes), and the remaining red blood cell pellet was resuspended to a concentration of 400 million cells per mL. All samples were stored at -80 °C, and underwent three freeze/thaw cycles before analysis.

4.2.5 Bicinchoninic acid assay

To evaluate the amount of protein that was released by the red blood cells, the red blood cell conditioned media was analysed using the bicinchoninic acid (BCA) assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific) according to the manufacturers instructions. Standards were prepared for the calibration curve by serial dilution of bovine serum albumin. To run the assay, 25 µL of the sample, standard, or blank controls were incubated with 200 µL of the assay kit reagent solution (50:1, Reagent A:B) in a clear flat-bottom 96 well plate. This solution was briefly mixed using an orbital shaker (500 rpm, 0.5 minutes), after which the plate was covered and incubated at 37 °C for 30 minutes. Following incubation, the absorbance was measured at 562 nm on a plate reader (Synergy 2 plate reader, BioTek). The calibration curve was analysed using four-parameter curve fit on GraphPad Prism software (ver. 6, USA).

4.2.6 Haemoglobin quantification

Levels of free haemoglobin in plasma samples and red blood cell conditioned media samples were monitored by assessing absorbance at 414 nm (Synergy 2 plate reader, BioTek) as previously described[28]. Peaks at this wavelength are indicative of free haemoglobin. Red blood cell membranes were removed from the conditioned media samples prior to analysis by centrifugation at 16,000 *g* for 15 minutes. A haemoglobin calibration curve was prepared using

purified haemoglobin (Sigma Aldrich) and the resulting data were analysed on GraphPad Prism software (ver. 6, USA).

4.2.7 Cytokine multiplex assay

Two multiplex assays were used in this study, the first was the 27-plex human cytokine panel that assays for FGF basic, Eotaxin-1, G-CSF, GM-CSF, IFN-γ, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF, and the second was the 21-plex human cytokine panel that assays for IL-1α, IL-2Ra, IL-3, IL-12(p40), IL-16, IL-18, CTACK, GRO-α, HGF, IFN-α2, LIF, MCP-3, M-CSF, MIF, MIG, β-NGF, SCF, SCGF-β, SDF-1α, TNF-β, TRAIL (Bio-Plex Pro 27-plex and 21-plex, Bio-Rad). The assays were performed according to manufacturer's instructions using an automated magnetic wash station (Bio-Plex Pro II, Bio-Rad) for the washing steps. The assays were run on the Luminex® 200™ system (Bio-Rad) and fluorescence values were collected. The calibration curve for each cytokine was analysed with 5 parametric logistic curve regression using Bio-Plex manager software (ver. 5.0, Bio-Rad, USA). Standard values were considered acceptable if the points fell within 80 - 120 % of the expected values.

4.2.8 Recovery efficiency

To assess any signal interference in the red blood cell lysates, the Bio-Plex recombinant protein standards were spiked into the lysates (25 µL of Bio-Plex standard-3 into 75 µL of lysate). A control was also run, wherein the sample diluent was added at the spiking volume to the red blood cell lysates to account for any dilution effect. These samples were analysed using the 27-plex and 21-plex human cytokine panels (Bio-Plex Pro, Bio-Rad). The recovery efficiency for each cytokine was determined using the following equation:

Equation 4.1. Recombinant protein recovery efficiency.

$$\frac{A + B}{C} \times 100 = Percentage\ recovery$$

Where,

    A = Concentration of un-spiked control

    B = Concentration of recombinant protein (spiked)

    C = Final assayed concentration of sample

4.2.9　　　Statistical analysis

The multiplex assay data are presented as either concentration or fold change of fluorescence. Protein concentration (BCA assay) and haemoglobin concentration of plasma and conditioned media samples were statistically evaluated using two-tailed, paired t-test to assess statistical significance ($p < 0.05$) between groups. Graphing of results was performed using GraphPad Prism software (ver. 6, USA). The concentration of cytokines in blood components was presented according to the relative contribution in one millilitre of whole blood. This calculation was performed for each cell population according to the following equation:

Equation 4.2. Concentration of protein in one millilitre of whole blood.

$$\frac{A}{B} \times C = Contribution\ to\ whole\ blood$$

Where,

  A = Original cell number of population in one millilitre of whole blood

  B = Number of cells assayed per millilitre

  C = Concentration of analyte per millilitre

Statistical analysis of raw fluorescence responses from cytokine data were performed using 'R' version 3.2.3 (2015-12-10, R: A Language and Environment for Statistical Computing). Mixed-effects modelling was done using lmer[29]. The significance of interaction terms and interaction means and their associated standard errors were obtained using the Phia package[30], for post-hoc analysis. Multiple test correction was done according to Holm's method[31]. To analyse the fluorescence response, the following mixed-effects model, in R notation, was used:

Equation 4.3. Mixed-effects model for statistical analysis of cytokine data.

$$log2(FI) \sim Fluid * Cytokine + Treatment * Cytokine + Treatment: Fluid$$
$$+ (1|ID) + (1|Treatment: Fluid: Plate)$$

Where the log2 of fluorescence responses (Fl) was modelled using 3 fixed effects, (1) Fluid (2 levels, lysate and secretion), (2) Cytokine (either 27 or 21 levels), and (3) Treatment (4 levels, Control, PrI, BP, BP-PrI) plus their 1st degree interactions, together with two random effects defined as (1:ID), where ID represented subject identifier, and (1|Treatment:Fluid:plate) and

where plate was a factor containing 2 levels. The random effects account for patient-to-patient variability and for differences with respect to Treatment and Fluid groupings across the 2 cytokine plates.

For the remainder of this chapter, the term 'significant' will be used to indicate a statistically significant result of $p < 0.05$ unless otherwise specified.

4.3 RESULTS

4.3.1 Red blood cell purity

The purity of the enriched red blood cell populations was calculated according to the efficiency of white blood cell and platelet depletion from whole blood samples. Dextran sedimentation of whole blood resulted in depletion of approximately 82 % of white blood cells and 98 % of platelets from the enriched red blood cell fractions (Table 4.2). Thus, for every one million red blood cells there were approximately 175 contaminating white blood cells and 3,450 contaminating platelets.

Table 4.2. Purity assessment of enriched red blood cell (RBC) fractions ($n = 10$).

RBC purity (%)	White blood cell depletion (%)	Platelet depletion (%)
99.97 ± 0.01	82.5 ± 6.8	97.7 ± 3.0

*Data are presented as mean ± SD

4.3.2 Cytokine profile of plasma and red blood cells

The cytokine profile of red blood cells, white blood cells, and plasma was analysed by using an antibody-mediated multiplex bead array. White blood cell correction for red blood cell cytokine concentration was achieved by determining the cytokine load of a known number of white blood cells and then calculating the load of the contaminating white blood cells. Of the 48 cytokines that were assayed for, 44 were present in the plasma fraction and 35 were present at detectable levels in the red blood cells of one or more biological replicate after correction for white blood cell contamination (Table 4.3). When calculated back to the relative contribution of the plasma and red blood cells in one millilitre of whole blood, the red blood cells represented the largest reservoir for 69 % of the cytokines (33 in 48) compared to the plasma. Across the 48 cytokines analysed, there were 31 where the level of the cytokine in red blood cells substantially exceeded the plasma level (> 2-fold increase). In these proteins, the range of fold increase in red blood cells versus plasma was 3.6 to 3970 (i.e. the red blood cell:plasma ratio). In these 31 cytokines, the median fold change was 11.3. Two cytokines, MIG and SCGF-β, were higher in plasma than in the red blood cell population (Table 4.3).

There were three cytokines that were present at high levels in white blood cells but returned anomalous results in the purified red blood cells (IL-1ra, PDGF-bb, SCGF-β). For these cytokines, the expected concentration for white blood cell contamination in red blood cell populations was more than what was detected. The concentration of IL-1ra in white blood cells (when corrected to the number of white blood cells contaminating the red blood cell populations) was predicted to be present at a mean of 1572.8 pg/mL across all six biological replicates. However, in the purified red blood cells, IL-1ra was detected in only one biological replicate at 299.6 pg/mL, which is 5-fold lower than predicted. Similar discrepancies were observed with SCGF-β and IL-2 (Table 4.3). The mean depletion of white blood cells and platelets were 83 % and 98 % respectively. The small number of contaminating cells may not be representative of the whole heterogeneous population, which may account for the anomalous results.

Table 4.3. Concentration of cytokines in EDTA plasma and red blood cells (RBCs) in one millilitre of whole blood with correction for white blood cell contamination ($n = 6$).

Cytokine	No. of subjects	Plasma* pg/mL of whole blood		No. of subjects	RBCs* pg/mL of whole blood		Corrected for WBC contamination (pg/mL)		White blood cell (WBC) contribution pg/mL of total		No. of subjects
		Concentration	SD		Concentration	SD	Concentration	SD	Concentration	SD	
Pro-inflammatory											
IFN-α2	6	**8.9**	2	6	256.2	130	**256.2**	130.0	-	-	0
IFN-γ	6	**17.7**	9.5	5	106.4	56.5	**105.3**	56.2	1.2	0.2	6
IL-1α	2	2.5	0.3	0	-	-	-	-	3.8	0.6	2
IL-1β	5	0.4	0.1	0	-	-	-	-	1.2	0.2	6
IL-5	5	2.5	1.8	3	28.2	12.6	28.2	12.6	-	-	0
IL-9	2	2	1.5	0	-	-	-	-	1.1	1.2	3
IL-12(p70)	5	**2.5**	1	3	19.8	15.4	**15.0**	16.3	3.9	1.4	6
IL-15	0	-	-	0	-	-	-	-	3.0	1.4	2
IL-17	3	**7.5**	1.8	3	87.9	50.2	**70.1**	46.6	23.0	6.5	6
IL-18	6	**6.2**	3.2	6	505.4	251.5	**488.2**	247.9	17.2	7.9	6
MIF	6	**28.1**	3.8	6	111,633	56,912	**111,545**	56,954	87.9	64.7	6
TNF-α	6	**7.1**	3.3	2	79.7	62.5	**54.3**	76.5	21.4	8.4	6
TNF-β	3	**3.1**	0.9	2	31.7	11.4	**31.7**	11.4	-	-	0
TRAIL	6	**25.2**	11.7	4	262.6	63.2	**247.8**	66.0	20.9	8.0	4

Cytokine	Plasma*			RBCs*					White blood cell (WBC) contribution		
	No. of subjects	pg/mL of whole blood		No. of subjects	pg/mL of whole blood		Corrected for WBC contamination (pg/mL)		pg/mL of total		No. of subjects
		Concentration	SD		Concentration	SD	Concentration	SD	Concentration	SD	
Anti-inflammatory											
IL-1ra	5	20.8	6.6	1	299.6	-	-	-	1572.8	252.7	6
IL-2ra	6	**29.3**	5.3	3	282.6	31.7	**280.9**	28.8	6.2	5.3	3
IL-4	4	**0.4**	0.2	2	4.6	0.8	**3.3**	0.9	1.4	0.2	6
IL-10	1	1.4	-	0	-	-	-	-	-	-	0
IL-13	5	2.0	1.0	3	2.3	2.3	1.4	2.3	0.9	0.1	6
Chemokines											
CTACK	6	**36.3**	9.3	5	222.2	85.7	**131.4**	100.5	93.28	33.6	6
Eotaxin-1	5	**6.4**	2.2	6	79.3	38.4	**74.6**	38.4	4.8	0.9	6
GRO-α	3	**6.0**	1.7	4	187.3	47.8	**184.0**	54.3	4.8	0.9	6
IL-8	5	1.5	0.3	1	25.4	-	-	-	24.6	15.7	6
IL-16	6	**46.6**	11.2	5	2904.9	3231.7	**2904.9**	3231.7	-	-	0
MCP-1	0	-	-	2	55.0	6.8	36.1	7.7	15.5	4.9	6
MCP-3	4	23.4	5.5	0	-	-	-	-	-	-	0
MIG	6	70.2	34.5	0	-	-	-	-	3.7	0.7	3
MIP-1α	6	**0.5**	0.3	4	5.2	2.3	**3.9**	2.1	1.5	0.4	6
MIP-1β	6	**6.3**	3.5	2	42.7	56.3	**25.7**	48.9	9.4	7.3	6
RANTES	6	**510.7**	268.5	6	8744	11,785	**5618**	10,489	3125.8	1488.3	6
SDF-1α	6	**121.9**	11.3	6	872.4	206.2	**811.8**	193.4	60.5	34.0	6

Cytokine	Plasma*			RBCs*					White blood cell (WBC) contribution		
	No. of subjects	pg/mL of whole blood		No. of subjects	pg/mL of whole blood		Corrected for WBC contamination (pg/mL)		pg/mL of total		No. of subjects
		Concentration	SD		Concentration	SD	Concentration	SD	Concentration	SD	
Growth Factors											
bFGF	6	**4.8**	2.0	6	197.1	72.4	**147.2**	87.3	49.9	18.2	6
G-CSF	6	**5.3**	3.6	3	148.8	79.0	**131.1**	79.1	16.0	3.1	6
GM-CSF	0	-	-	5	939.7	460.2	772.3	538.5	185.3	85.1	6
HGF	6	**39.8**	7.8	6	800.3	490.5	**691.2**	500.3	109.1	31.5	6
IL-3	6	**18.1**	2.0	6	345.8	209.7	**334.2**	217.6	17.4	6.8	4
IL-7	5	**1.7**	0.9	2	34.6	5.2	**34.0**	6.2	1.2	0.2	2
IP-10	6	132.5	142.7	4	147.8	87.1	134.0	83.3	15.0	3.5	6
M-CSF	6	**4.3**	3.2	3	115.6	7.9	**99.5**	13.2	17.0	4.3	5
β-NGF	5	1.2	0.2	0	-	-	-	-	-	-	0
PDGF-bb	6	**20.3**	17.4	5	1073.5	2004.9	**115.4**	2095.6	1018.9	353.1	6
SCF	6	**6.5**	1.5	1	54.1	-	**51.9**	-	1.9	0.6	4
SCGF-β	3	944.3	563.9	0	-	-	-	-	118.7	69.2	4
VEGF	5	**2.1**	0.8	6	113.9	50.3	**82.8**	53.7	31.1	7.5	6
Cytokines with multiple functions											
IL-2	0	-	-	0	-	-	-	-	15.9	6.6	6
IL-6	2	0.5	0.1	0	-	-	-	-	1.6	0.7	4
Il-12(p40)	1	**44.5**	-	3	1914.7	357.9	**1693.7**	254.7	301.1	130.9	6
LIF	6	**5.7**	3.2	3	119.5	27.5	**119.5**	27.5	-	-	0

*Data are presented as mean ± SD

Cytokines in **bold are ≥ 2 fold higher in the red blood cell lysates (corrected for WBC contamination) compared to plasma

4.3.3   Recovery efficiency

To assess whether there was any interfering factors in red blood cell lysates that may confound quantitative results, the recovery efficiency of recombinant protein in red blood cell lysates was determined. A recovery efficiency of < 100 % indicated that the assay was not detecting all of the available cytokines, and a recovery efficiency of > 100 % indicated that the assay was reporting more cytokine than was actually present. For this study, the mean recovery efficiency in red blood cell lysates for 42 of 48 of the cytokines fell between 70 and 120 % (Table 4.4). For approximately 75 % of the cytokines, the available protein was below 100 % mean recovery. There were four cytokines (mostly chemokines) that had mean recovery efficiency of over 120 %, including IFN-α2 (166.6 ± 34.2 %), GRO-α (225.1 ± 177.3 %), SDF-1α (364.9 ± 401.9 %), and MCP-3 (369.8 ± 326.3 %). Quantitative data collected for these cytokines will need to be used with caution. The recovery efficiency of MIF, as determined by Bio-Plex, was 91.0 ± 10.4 % (Table 4.4). This recovery efficiency was consistent with the results obtained using the MIF ELISA in Chapter 3, which reported a recovery of 97 ± 7 % (Section 3.3.3).

Table 4.4. Percentage of protein recovery after recombinant protein spike into red blood cell lysates as measured by fluorescence ($n = 5$, mean $\pm$ SD).

Cytokine	Protein recovery after spike[*]	
	%	**Standard deviation**
Pro-inflammatory		
IFN-α2	166.6	34.2
IFN-γ	94.7	28.7
IL-1α	77.4	21.2
IL-1β	77.5	2.6
IL-5	75.5	3.1
IL-9	75.2	3.6
IL-12(p70)	76.3	33.8
IL-15	67.1	3.9
IL-17	76.1	3.4
IL-18	89.7	29.4
MIF	91.0	10.4
TNF-α	81.9	6.1
TNF-β	93.6	34.7
TRAIL	101.8	19.3
Anti-inflammatory		
IL-1ra	82.7	16.7
IL-2ra	110.3	14.9
IL-4	86.5	10.4
IL-10	75.2	14.1
IL-13	83.3	8.1
Cytokines with multiple functions		
IL-2	78.2	4.7
IL-6	70.2	16.9
IL-12(p40)	111.6	77.9
LIF	80.5	6.2

Cytokine	Protein recovery after spike*	
	%	Standard deviation
Chemokines		
CTACK	119.0	15.9
Eotaxin-1	86.3	23.9
GRO-a	225.1	177.3
IL-8	72.3	17.5
IL-16	117.0	30.0
MCP-1	68.6	28.1
MCP-3	369.8	326.3
MIG	105.1	94.1
MIP-1α	88.7	15.4
MIP-1β	93.2	61.0
RANTES	86.4	15.9
SDF-1α	364.9	401.9
Growth factors		
bFGF	94.7	48.2
G-CSF	75.2	14.5
GM-CSF	81.0	9.4
HGF	103.7	14.7
IL-3	114.4	26.2
IL-7	81.7	3.1
IP-10	75.8	7.4
M-CSF	98.9	6.5
B-NGF	75.3	29.4
PDGF-bb	71.3	18.4
SCF	95.5	36
SCGF-β	92.1	26.2
VEGF	79.5	6.6

4.3.4   Cytokines in red blood cell conditioned media

Red blood cell conditioned media was prepared by incubating red blood cells in a protein free media (PBS) for 24 hours at 37 °C and the resulting conditioned media was assayed for levels of inflammatory markers. The concentration of red blood cells used to produce the conditioned media was approximately 25x lower than the concentration of cells in whole blood (whole blood contains approximately 5 billion red blood cells/mL). Even so, detectable levels of 40 of the 48 cytokines were present in the conditioned media for one of more biological replicates (Table 4.5). There were 18 cytokines that were released at concentrations higher than what was detected in plasma. For example, TNF-α was present at 61.5 ± 50.6 pg/mL in conditioned media (Table 4.5) and 7.1 ± 3.3 pg/mL in plasma (Table 4.3). IL-8 was also detected at high concentrations in the conditioned media compared to circulating plasma levels (979.6 ± 873.6 vs 1.5 ± 0.3 respectively). The detectable concentration of IL-8 varied substantially between biological replicates; the range of IL-8 in the conditioned media was 291 - 2641 pg/mL (Table 4.5). This variability was also observed for a number of other cytokines including RANTES (86 - 502 pg/mL), TNF-α (8 - 127 pg/mL), and MIF (688 - 2597 pg/mL) (Table 4.5). Although the absolute concentration differed depending on the assay used, similar variability between biological replicates was observed for the release of MIF from red blood cells as measured by ELISA in Chapter 3 (4519 - 8177 pg/mL, Figure 3.3). For eight of the cytokines, including IL-2 and IL-6, levels were undetectable in red blood cell lysates (Table 4.3), but were detected in the red blood cell conditioned media (6.9 ± 2.6 pg/mL for IL-2 and 5.5 ± 3.4 pg/mL for IL-6).

Table 4.5. Cytokines in red blood cell (RBC) conditioned media after 24 hours at 37 °C (200 million cells/mL) ($n = 6$, mean ± SD).

Cytokine	RBC secretion		
	Concentration (pg/mL)	Standard deviation	No. of subjects
Pro-inflammatory			
IFN-α2	18.8	3.5	5
IFN-γ	3.7	2.2	4
IL-1α	8.6	3.5	6
IL-1β	25.1	21.2	6
IL-5	0.2	0.2	2
IL-9	0.9	1.0	2
IL-12(p70)	1.0	-	1
IL-15	9.9	4.6	6
IL-17	11.0	3.1	2
IL-18	3.4	1.4	5
MIF	1496.2	753.2	6
TNF-α	61.5	50.6	6
TNF-β	-	-	0
TRAIL	21.2	7.1	4
Anti-inflammatory			
IL-1ra	-	-	0
IL-2ra	12.2	5.7	6
IL-4	0.5	0.2	5
IL-10	0.3	-	1
IL-13	0.4	0.4	2
Cytokines with multiple functions			
IL-2	6.9	2.6	5
IL-6	5.5	3.4	5
IL-12(p40)	80.5	40.9	5
LIF	-	-	0

Cytokine	RBC secretion		
	Concentration (pg/mL)	Standard deviation	No. of subjects
Chemokines			
CTACK	2.7	1.7	2
Eotaxin-1	7.3	-	1
GRO-a	-	-	0
IL-8	979.6	873.6	6
IL-16	17.5	7.4	5
MCP-1	7.9	2.3	5
MCP-3	-	-	0
MIG	5.5	4.5	6
MIP-1α	2.2	2.7	6
MIP-1β	30.7	35.2	6
RANTES	303.5	173.0	6
SDF-1α	67.2	26.7	6
Growth factors			
bFGF	35.5	9.6	6
G-CSF	10.2	2.9	5
GM-CSF	78.0	30.7	6
HGF	38.3	17.3	6
IL-3	24.6	11.8	6
IL-7	0.2	-	1
IP-10	11.0	12.3	2
M-CSF	4.9	3.5	5
B-NGF	-	-	0
PDGF-bb	32.6	57.9	6
SCF	-	-	0
SCGF-β	-	-	0
VEGF	17.1	6.2	6

4.3.5 Concentration of haemoglobin in red blood cell conditioned media

The concentration of haemoglobin in red blood cell conditioned media was quantified by monitoring the absorbance of plasma and red blood cell conditioned media samples at 414 nm. The level of haemoglobin in the conditioned media samples were significantly lower than the haemoglobin concentration of the plasma samples (Figure 4.1). There was no significant difference in the concentration of haemoglobin for any of the conditioned media samples. The mean concentration of haemoglobin in the conditioned media samples corresponds to approximately 0.5 - 1.5 % of the original red blood cell number.
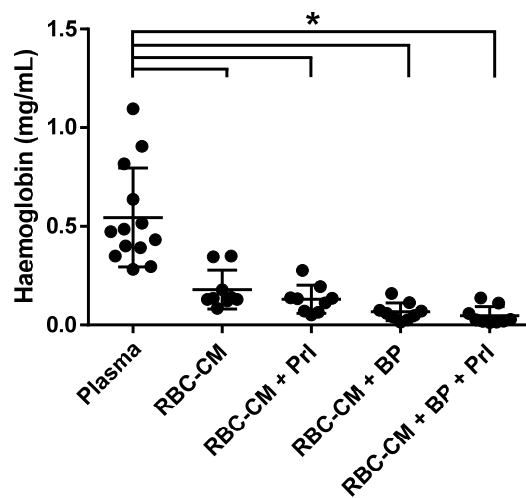
Figure 4.1. Concentration of haemoglobin in plasma and the conditioned media of red blood cells (2×10^8 cells per mL) incubated in PBS with and without protease inhibitors (RBC-CM + PrI and RBC-CM respectively) or with Bio-Plex standard recombinant protein with or without protease inhibitors (RBC-CM + BP + PrI and RBC-CM + BP) over 24 hours at 37 °C. Haemoglobin concentration was measured by absorbance at 414 nm. Data are presented as mean ± SD, $n \geq 5$, statistically significant (*) if $p < 0.05$.

4.3.6 Modulation of red blood cell cytokine profile and conditioned media

To assess the effect of protease inhibitors on the cytokine profile of red blood cells, purified red blood cells were incubated with and without protease inhibitors (PrI) for 24 hours at 37 °C. The cytokine profile of each fraction was assessed and the fold change between the control and

the PrI treatment was determined. The fold change of a selection of cytokines is presented in Figure 4.2; the data for the remaining cytokines can be found in Appendix C.

A downward trend in the concentration of cytokines was observed in both the conditioned media and the red blood cell lysate following PrI treatment (Appendix C). When corrected for the conditioned media and the lysate, the adjusted means and standard errors from the fitted fixed-effect statistical model showed that the level of IL-1b, IL-8, and MIF were significantly reduced with PrI treatment. Treatment of red blood cells with PrI resulted in less IL-8 in both the conditioned media and in the red blood cell lysate after incubation (fold change of 0.1 ± 0.07 and 0.2 ± 0.1 for each respectively). A decrease in TNF-α was observed in both the conditioned media and the lysate (fold change of 0.5 ± 0.3 and 0.7 ± 0.2 respectively) and the same was observed for IL-6 (fold change of 0.7 ± 0.3 and 0.8 ± 0.1 respectively). An increase in the concentration of detectable cytokines after PrI treatment was not common, but was observed for IL-16 in the lysate of red blood cells (fold change of 2.2 ± 0.6). The effect of PrI treatment on the red blood cells was variable between biological replicates for each cytokine. For GM-CSF, there was minimal variability between replicates with a fold change and standard deviation of 1.02 ± 0.05 for PrI conditioned media. Whereas, the effect of PrI on RANTES concentration in conditioned media varied substantially between each biological replicate with a corresponding fold change and standard deviation of 1.51 ± 1.25.
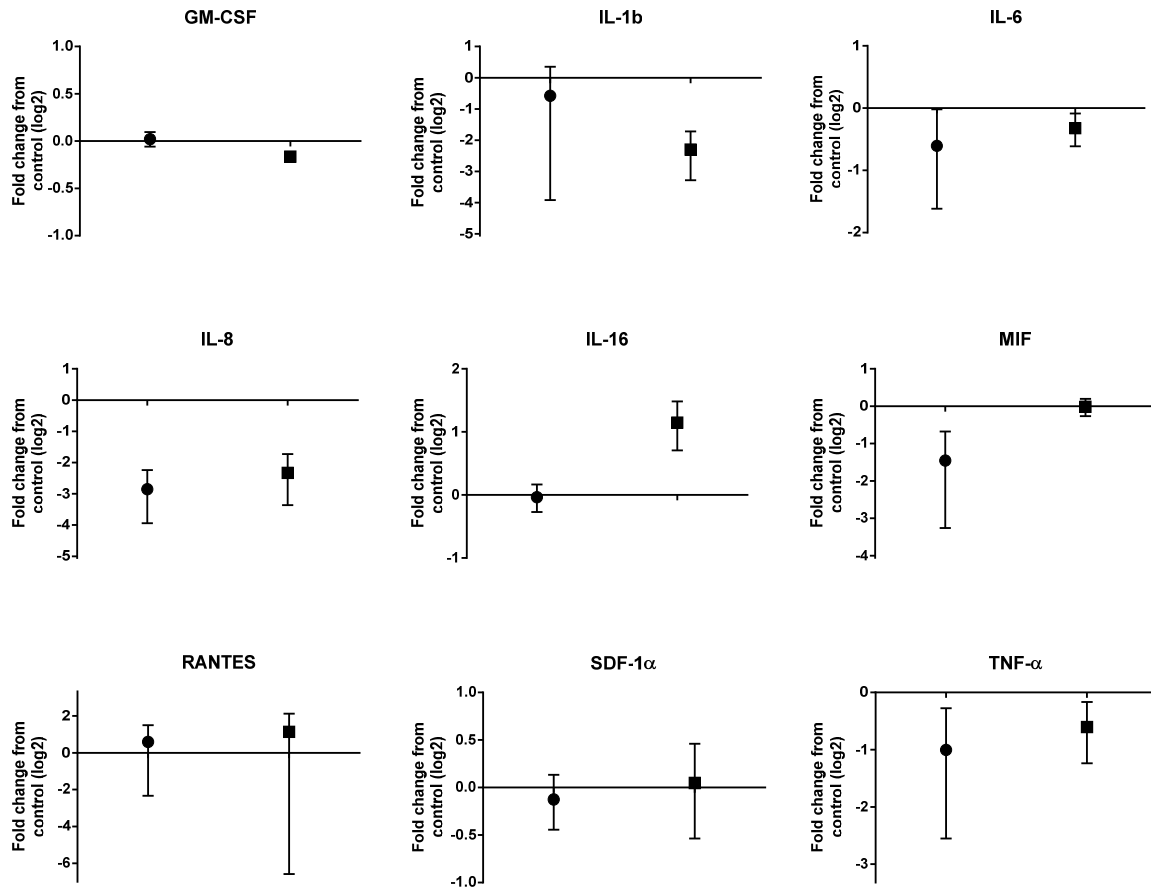
Figure 4.2. Summary of cytokines released from red blood cells into PBS over 24 hours and in the corresponding red blood cell lysate at 37 °C with or without protease inhibitors (PrI) as measured by Bio-Plex and reported as fluorescence (100 million cells in 1 mL PBS). Data are presented as mean fold change after addition of PrI ± 95% CI, where ● represents fold change in conditioned media of PrI treatment compared to the no PrI control, and ■ represents fold change in fluorescence in lysate of red blood cells treated with PrI compared to the no PrI control ($n = 6$).

4.3.7 Total protein in red blood cell conditioned media

The amount of protein present in red blood cell conditioned media with and without protease inhibitors was quantified using the BCA assay. The mean concentration of protein in the conditioned media was slightly higher in the protease inhibitor treated samples when compared to the untreated controls ($114.1 \pm 38.7\,\mu g/mL$ and $106.9 \pm 49.4\,\mu g/mL$ respectively). However, there was no significant difference between the amount of protein in conditioned media with and without protease inhibitor treatment.

4.3.8 Red blood cell protein binding and modulation

Red blood cells were incubated with recombinant protein (Bio-Plex kit standards) in PBS for 24 hours at $37\,°C$. To assess if any recombinant protein bound to the red blood cells, the concentration of cytokines in the lysate was assessed after incubation. The fold change of a selection of cytokines is presented in Figure 4.3, the data for the remaining cytokines can be found in Appendix D. The level of a number of cytokines in the red blood cell lysates increased after incubation with recombinant protein (Figure 4.3). The red blood cell lysate had 3-fold more IL-1ra (fold change of 2.9 ± 1.6) in the recombinant protein treated samples when compared back to the control, and 12-fold more β-NGF (fold change of 12.1 ± 4.2). The concentration of IL-12(p70) also increased with the addition of recombinant protein, it had a fold change of 2.8 ± 0.8 for the control and 3.7 ± 0.5 for the protease inhibitor treated samples. Treatment with protease inhibitors had variable effects depending on the cytokine. There was a significant difference between the control sample and the protease inhibitor treated samples for IL-8, GRO-α, and MIG. Treatment with protease inhibitors resulted in more IL-8 detected in the lysate, and less GRO-α and MIG compared to the untreated control (Figure 4.3). For six of the cytokines, including MIF and SDF-1α, there was no change in the lysate concentration following the addition of recombinant protein with or without protease inhibitors (Figure 4.3).
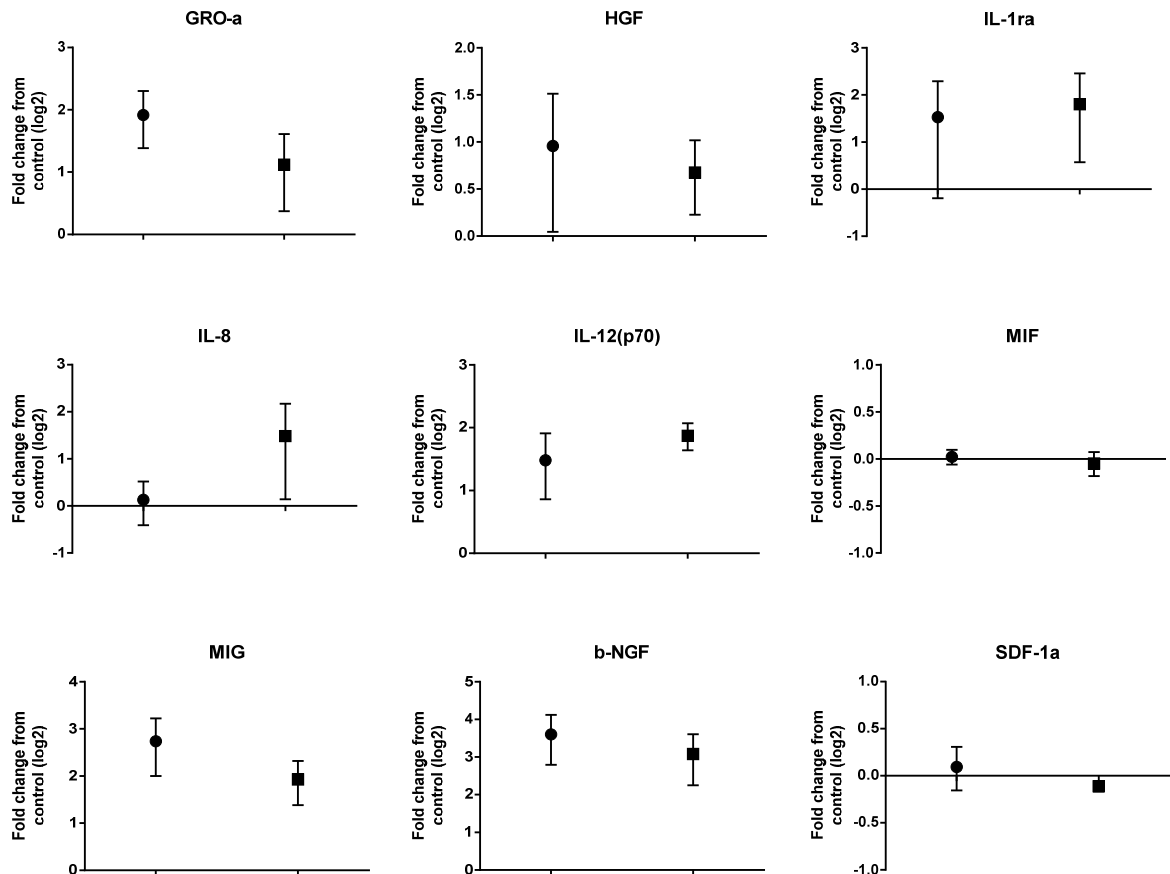
Figure 4.3. Summary of cytokines in red blood cell lysate after incubation into PBS over 24 hours at 37 °C with or without recombinant protein and protease inhibitors (PrI) as measured by Bio-Plex and reported as fluorescence (100 million red blood cells in 1 mL PBS). Data are presented as fold change after addition of recombinant protein ± 95% CI, where ● represents fold change in cytokine profile of lysate with exogenous recombinant protein compared to control, and ■ represents fold change in cytokine profile of lysate with PrI, with addition of exogenous recombinant protein compared to control ($n = 5$).

4.3.9 Red blood cell cytokine binding

Red blood cells were incubated at 37 °C for 24 hours in the presence of protease inhibitors and/or recombinant protein (Bio-Plex standards in PBS). Individual cytokine data for the resulting red blood cell lysates are outlined in Section 4.3.8. For analysis of treatment effect on red blood cell lysates, the cytokine data were analysed using fixed-effects statistical models

across pairwise comparisons between treatments (Section 4.2.9). There was no significant difference in cytokine concentration between the untreated control and protease inhibitor treated control on either the 27-plex or 21-plex panel. However, a downward trend in the cytokine levels in red blood cell lysates was observed with the addition of protease inhibitors (Figure 4.4). Red blood cells that were incubated with recombinant protein had significantly higher cytokine concentration across both panels when compared to the controls (Table 4.6). The increase in lysate cytokine concentration was consistent in both untreated and protease inhibitor treated red blood cells for the 27-plex panel and the 21-plex panel (Table 4.6).
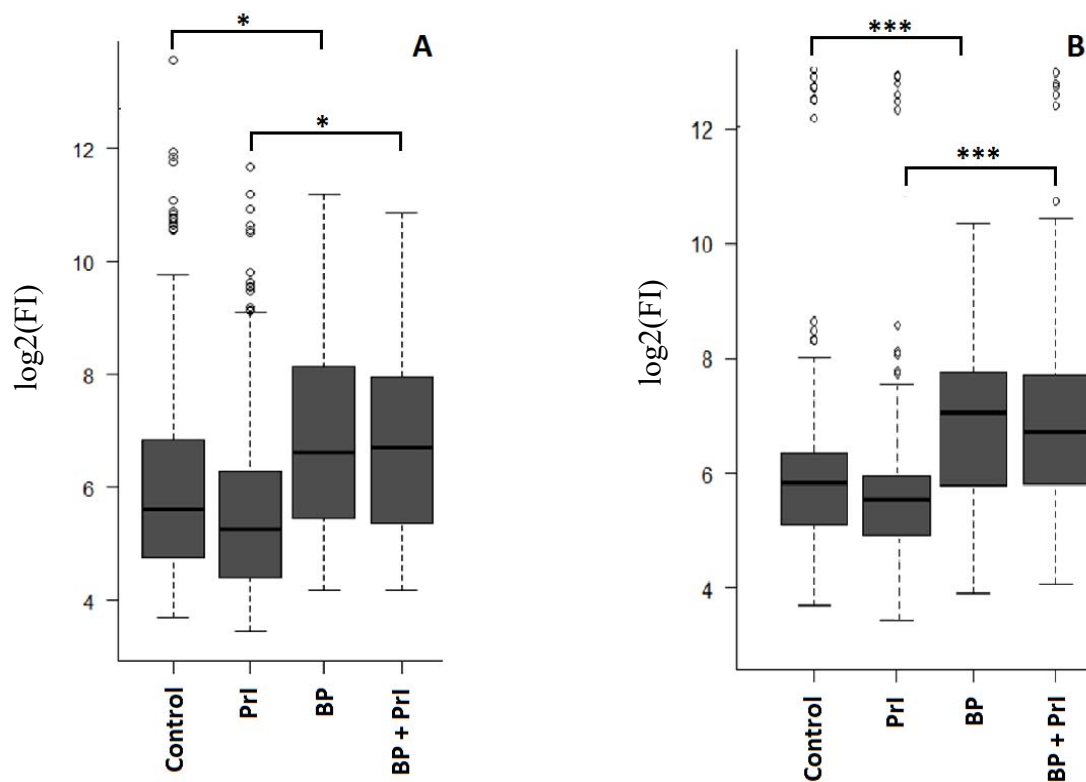
Figure 4.4. Boxplot summaries of cytokine log2 transformed raw fluorescence response data for red blood cell lysates collected using (A) the Bio-Plex human 27-plex panel and (B) the Bio-Plex human 21-plex panel ($n = 5$). Cells were incubated for 24 hours (37 °C, 5 % CO_2) with or without protease inhibitors (PrI) and with or without recombinant protein (BP). The conditions are presented from left to right as control, PrI, BP, and BP-PrI. Data are statistically significantly different (*) if $p < 0.05$, (**) if $p < 0.01$, or (***) if $p < 0.001$.

Table 4.6. Statistical analysis of the effect of treatment according to sample type (red blood cell lysate) with pairwise treatment comparison for red blood cells incubated for 24 hours (37 °C, 5 % CO_2) with or without protease inhibitors (PrI) and with or without recombinant protein (BP)[#] ($n = 5$).

Comparison	Sample type	Pr(>Chisq)	Significance[##]
27-plex			
Control vs PrI	Red blood cell lysate	0.642	
Control vs BP	Red blood cell lysate	0.038	*
PrI vs BP-PrI	Red blood cell lysate	0.003	**
BP vs BP-PrI	Red blood cell lysate	1.000	
21-plex			
Control vs PrI	Red blood cell lysate	0.265	
Control vs BP	Red blood cell lysate	8.58×10^{-9}	***
PrI vs BP-PrI	Red blood cell lysate	1.66×10^{-12}	***
BP vs BP-PrI	Red blood cell lysate	0.719	

Analysis performed on cytokine log2 transformed raw fluorescence data

For Pr(>Chisq): * $\leq$ 0.05; ** $\leq$ 0.01; *** $\leq$ 0.001

4.4 DISCUSSION

In the previous chapter of this thesis, MIF was detected in red blood cells at high concentrations and in its active form. This discovery prompted the aim of this current study, which was to investigate whether more cytokines than MIF alone were associated with red blood cells. The results of this study revealed that 35 of the 48 cytokines assayed were present in red blood cell lysates of healthy volunteers (Table 4.3) and for the 31 of these cytokines that were higher in the red blood cell lysates than in the plasma, there was a median fold increase of 11.3 (range 3.6 to 3970-fold). Unlike any other study to date, the concentrations of these cytokines in red blood cell lysates were quantified and were reported according to their relative abundance in whole blood (Table 4.3). The panel of cytokines identified in red blood cells cover a range of pro- and anti-inflammatory factors, chemokines, and growth factors. A number of these cytokines are regularly monitored in plasma or serum for biomarker studies[1] and the expression and secretion of these cytokines are thought to be limited to specific subsets of white blood cells[32]. The identification of these cytokines associated with red blood cells indicates that an additional, previously unknown, level of cytokine interactions may exist in blood. This study also showed that red blood cells are not only reservoirs for cytokines, but that they may also release and bind them in a process that is independent of haemolysis, however further investigation is required to confirm this (Table 4.5 and Figure 4.1). After storage in a protein free solution, red blood cell conditioned media contained a broad range of pro- and anti-inflammatory factors, chemokines, and growth factors. When exogenous cytokines were spiked into red blood cell storage media, the resulting cell lysates had significantly increased cytokine concentrations indicating cell binding (Figure 4.3). Finally, treatment with protease inhibitors, resulted in modulation of the concentration of these cytokines in the conditioned media and in the red blood cell lysates (Figure 4.2 and Figure 4.3). The results of this study further demonstrate that, alongside its gas exchange function, red blood cells may play a role in cytokine signalling. Red blood cells are associated with a variety of cytokines and are able to secrete or bind these cytokines when exposed to different stimuli.

4.4.1 Cytokines in red blood cells

4.4.1.1 Classes of cytokines identified in red blood cells

Red blood cells have been identified in the literature as a reservoir for a small number of cytokines. In 1991, red blood cells were reported to be a chemotactic sink for IL-8, and in 1993 the C-X-C class chemokines MGSA (aka GRO-α), NAP-2, and IL-8 were reported to bind to red blood cells[17,33]. In the study outlined in this chapter, of the 17 C-X-C chemokines in the family, three (18 %) were identified in the lysates of red blood cells after correcting for white blood cell contamination, specifically GRO-α, IP-10, and SDF-1α (Table 4.3 and Table 4.7). Neote *et al*. also reported the presence of RANTES and MCP-1 of the C-C chemokine family[17], and Fukuma *et al*. reported the presence of Eotaxin[18]. These findings were confirmed by the current study, which also identified RANTES, Eotaxin, and MCP-1 in red blood cell lysates. In addition, five more C-C chemokines were also identified out of a total of 27 chemokines in the family which adds up to 30 % C-C chemokines being associated with red blood cells (Table 4.3 and Table 4.7). The study in this chapter has revealed other classes of cytokines that are also associated with red blood cells as outlined in Table 4.7. In a recent study, red blood cells were identified as a major source of the nuclear protein IL-33, which is part of the IL-1 cytokine superfamily[34]. They found that levels of IL-33 increased in the plasma of sickle cell patients who had experienced varying levels of haemolysis. The study in this chapter identified IL-18 in red blood cells, which represents 6 % of the IL-1 superfamily (Table 4.3 and Table 4.7). In addition to those already mentioned, a range of growth factors (including bFGF, GM-CSF, and VEGF), chemokines (IL-16), and pro- and anti-inflammatory cytokines (including IFN-γ, TNF-α, and IL-2ra) were also identified.

Table 4.7.   Families of cytokines that were identified in red blood cell lysates ($n =$ the number of proteins in each family and $b =$ the number of analytes available on the assayed Bio-Plex kits).

Family	Cytokine
C-C chemokines ($n = 27$; $\boldsymbol{b} = 7$)	CTACK
	Eotaxin
	MCP-1
	MCP-3
	MIP-1α
	MIP-1β
	RANTES
CSF family ($n = 3$; $b = 3$)	G-CSF
	GM-CSF
	M-CSF
C-X-C chemokines ($n = 17$; $b = 5$)	GRO-α
	IP-10
	SDF-1α
FGF growth factors ($n = 22$; $b = 1$)	bFGF
GM-CSF/IL-5/IL-3 family ($n = 3$; $b = 3$)	GM-CSF
	IL-3
	IL-5
IFN family (Type I) ($n = 17$; $b = 1$)	IFN-α2
IFN family (Type II) ($n = 1$; $b = 1$)	IFN-γ
IL-1 superfamily ($n = 11$; $b = 4$)	IL-18

Family	Cytokine
IL-6 family ($n = 11$; $b = 3$)	G-CSF
	LIF
IL-12 family ($n = 5$; $b = 2$)	IL-12(p40)
	IL-12(p70)
IL-17 family ($n = 6$; $b = 1$)	IL-17
MIF family ($n = 2$; $b = 1$)	MIF
PDGF growth factors ($n = 10$; $b = 2$)	PDGF-bb
	VEGF
TNF superfamily ($n = 19$; $b = 3$)	TNF-α
	TNF-β
	TRAIL
Unclassified	HGF
	IL-2ra
	IL-4
	IL-7
	IL-13
	IL-16

4.4.1.2   Red blood cell contribution to whole blood cytokine level

In clinical studies, optimal sample preparation is crucial for reproducible results[35]. These techniques generally focus on minimising white blood cell and platelet activation during plasma or serum isolation. The results of this study highlight that haemolysis can too cause interference. Even minimal haemolysis that may not be macroscopically detectable (haemolysis of $\geq$ 0.125 % of red blood cells in whole blood)[28] could interfere with the results of the clinical study. For example, IL-16 was present at 62 times the concentration in red blood cells when compared to plasma. Haemolysis of 0.125 % of red blood cells (releasing approximately 0.19 mg/mL of haemoglobin) could release approximately 5 pg/mL of IL-16, which is 10 % of the mean plasma concentration (46.6 pg/mL). This undetectable amount of haemolysis could quickly confound results.

Although the cytokine concentration in red blood cells identified in this study indicates that they are a major reservoir in blood, the full range of cytokines associated with red blood cells remains to be determined. In addition, the non-denaturing red blood cell lysis method (freeze-thaw cycles) used to ensure compatibility with the antibody assay, may not have produced accurate results for every cytokine. An example of this phenomenon is IL-8 which has been reported in multiple studies as being associated with red blood cells. It was observed in this study that with correction for white blood cell contamination, the levels of IL-8 in red blood cell lysates were not detectable (Table 4.3). However, after red blood cell incubation in protein free media, IL-8 levels increased significantly in the red blood cell lysates and red blood cell conditioned media (Table 4.5). Discussion on why this may have occurred will be continued in Section 4.4.2.1.

4.4.1.3   Assay and sample validity

The recovery efficiencies reported here are similar to reported values for plasma assayed on the Luminex platform[36,37], with the exception of the cytokines with very high recovery efficiencies including IFN-α2, GRO-α, SDF-1α, and MCP-3 (Table 4.4). Belabani *et al.* reported on the accuracy of Luminex based multiplex assays and their acceptance criteria for recovery efficiencies was determined to be between 75 -125 %[36]. Using that criteria, 39 of the 48 cytokines analysed in this study fell within the acceptable range for red blood cell lysates.

To correct for any white blood cell contamination in the enriched red blood cell samples, white blood cells were isolated and the concentration of cytokines per cell was determined. Using these data, the theoretical white blood cell contribution to the total concentration for each cytokine was calculated according to the number of remaining white blood cells (Table 4.3).

This enabled an estimate of the red blood cell cytokine contribution to whole blood. For some cytokines, the white blood cell contamination was negligible. For example, the total concentration of MIF in the red blood cell lysate was 111,633 ± 56,912 pg/mL, whilst the white blood cell contribution was 87.9 ± 64.7 pg/mL. This correction calculation had a more substantial effect on other cytokines. For example, the white blood cells contributed a third of the total CTACK measured in the red blood cell lysates (total concentration: 222.2 pg/mL; corrected concentration: 131.4 pg/mL). The validity of this correction mechanism was brought into question with the results of cytokines such as IL-1ra. The theoretical white blood cell contribution of IL-1ra was calculated to be 1572.8 ± 252.7 pg/mL and IL-1ra was detectable in the white blood cell lysates of all six biological replicates. However, the actual measured concentration of IL-1ra in the red blood cell lysates (with white blood cell contamination) was far below this. In fact, IL-1ra was only detectable in one biological replicate of the red blood cell lysates, and was present at 5-fold less than the white blood cell mean (Table 4.3). So, where did the IL-1ra go and why was it not detected? One possible reason for this involves the chosen cell isolation method in this study. The process of isolating the white blood cells was approximately 30 minutes longer than the isolation of the red blood cells and involved additional steps. During this process, additional cell activation and/or communication between the components may have occurred to stimulate the expression of IL-1ra and possibly other cytokines. The white blood cell population also contained higher numbers of platelets than that present in the red blood cell isolates. Platelets are a source of some cytokines and this may have resulted in artificially high concentrations detected in the white blood cell lysates, such as that observed for PDGF-bb (Table 4.3). The percentage of contaminating white blood cells after dextran fractionation is very low and it may be that these cells are a specific cell type and not representative of the whole heterogenous white blood cell population. If this is the case it may also be that the cytokine concentration in the contaminating cells is different from the whole white blood cell population, which could account for the discrepancy in analytes such as IL-1ra. The low concentration of white blood cells in the purified red blood cells was insufficient for flow cytometry analysis and this type of analysis could only be achieved on much larger blood samples than were available for this study.

At this point, it is clear that signalling between the components of blood is more complex that previously understood. In addition, fractionation of blood into its elements is not as straightforward as once thought. Collecting, anticoagulating, and isolating individual components will inevitably introduce artefacts especially if major reservoirs of analytes are perturbed during the processing. An optimal cell isolation methodology, which produces

biologically relevant plasma and blood cells, will require rapid sample processing and minimal manipulation.

4.4.1.4   *Assay dependent discrepancy in MIF concentration*

The concentration of MIF in red blood cells was the subject of Chapter 3. Levels were measured using an R&D Systems MIF ELISA and were reported as 31,241 ng in one millilitre of whole blood (Chapter 3, Table 3.3). In this chapter, MIF was one of a number of cytokines measured using a multiplex bead-based array (Bio-Plex, Bio-Rad) and levels were reported as 112 ng in one millilitre of whole blood (Table 4.3). This is a discrepancy of approximately 280x between the two assay types. An even larger discrepancy of 929x was observed for the MIF plasma levels according to each assay (26 ng/mL vs 0.028 ng/mL for the ELISA and Bio-Plex respectively). According to the manufacturers of each kit, the antibody pair for each assay (capture antibody and detection antibody) is sourced from the same supplier (R&D Systems) and despite experimentation, the reason for this difference remains unknown. Although the antibodies are from the same species and are sourced from the same supplier, the specific antibody clones in each assay are proprietary and thus it is impossible to know at this stage if they are the same. It is well accepted in the literature that using different antibodies will result in different concentrations of the analyte being detected[38], however differences of up to 1000-fold are not typical even with different antibodies.

The discrepancies observed for MIF is supported by the literature in plasma or serum collected from healthy volunteers, but is never discussed in detail. In the published literature, MIF concentration measured using MIF ELISAs are reported in ng/mL[39–41], and pg/mL for Luminex bead-based arrays[42–44]. The potential reasons for this are numerous. For instance, the chemistry required to bind the antibodies to the plate for the ELISA is different than what is required to bind to the magnetic beads (hydrophobic interactions vs covalent bonding respectively). This may have resulted in altered binding capacity and strength between the two kits, and thus differing results. Additionally, the physical availability of the antigen binding site of the antibody is likely to be different in each assay. Attaching antibodies to a spherical bead may have resulted in reduced availability of the binding site to the protein in comparison to the ELISA, particularly for the larger forms of MIF such as the homotrimer. At this stage, the cause of the discrepancy remains unknown, however it was not in the scope of this study to resolve these technical issues. Although there is a discrepancy in MIF levels between assays, the trends between groups are consistent. For both assays the difference in MIF concentration between plasma and red blood cell lysates (according to contribution to one millilitre of whole blood)

is between 1000x - 5000x (Table 3.3 and Table 4.3). As such, any in depth analysis of MIF should still be valid if comparisons are done using the same assay type. From a review of the literature, MIF appears to be the only cytokine on the Bio-Plex panel that is reported at such vastly different concentrations between assay types.

4.4.2　　　Red blood cell conditioned media

Analysis of red blood cell conditioned media has not been widely reported in the scientific literature. Out of six publications, the effect of red blood cell conditioned media on a number of cell types has been investigated. One study reported that T cell proliferation was stimulated in the presence of red blood cell conditioned media which was comparable with intact red blood cell-mediated proliferation[44]. Whilst Fredriksson *et al.* observed that culturing fibroblasts in the presence of intact red blood cells or red blood cell conditioned media stimulated the secretion of IL-8 and the expression of the corresponding mRNA[14]. They were able to replicate this effect by culturing fibroblasts in the presence of TNF-α and IL-1β, both of which were cytokines detected in the red blood cell conditioned media in this chapter (Table 4.5). Interestingly, the cytokine profile of the conditioned media was not investigated in any of these studies. In contrast, an evaluation of the cytokine profile of red blood cell conditioned media was the focus of this chapter.

After incubation in PBS without serum, a wide range of cytokines were detected in the conditioned media of red blood cells (Table 4.5). The concentration of haemoglobin in the red blood cell conditioned media indicated lysis of 0.5 - 1.5 % of the original number of red blood cells. For 40 cytokines, the concentration in the conditioned media was higher than what could be attributed to the red blood cell lysis. For IL-16, the concentration in the conditioned media was 10 % of the cell lysate concentration prior to culture and for IFN-γ, 88 % of the initial lysate concentration was measured in the conditioned media. This suggests that the proteins were being released or shed in a mechanism that is independent of haemolysis.

4.4.2.1　　　Concentration of cytokines in conditioned media

When normalised for cell number, the concentration of 32 of the cytokines in the conditioned media were higher than that detected in the red blood cell lysate prior to incubation (Table 4.5). IFN-α2 was detected at approximately 180 % of the initial lysate concentration, and VEGF was detected at 500 % of the initial lysate concentration. The cytokine with the largest difference between pre-culture lysate concentrations and the concentration in the conditioned media was IL-8 (Table 4.3 and Table 4.5). With white blood cell correction, IL-8 was below the limits of

detection for red blood cells lysates prior to incubation. After incubation at 37 °C for 24 hours, 979.6 ± 873.6 pg/mL of IL-8 was released in the red blood cell conditioned media. The lack of detectable IL-8 in the red blood cell lysates was unexpected as it is one of the few cytokines that has been identified by multiple reports as being present in and released from red blood cells[33,45,46]. The cytokines that exhibit this increase in concentration may have been bound to chaperones or to a complex prior to incubation. The limitations of immunoassays in detecting complex bound cytokines have been previously described[47–49]. The goal of this chapter was to determine which cytokines were present in red blood cells and additionally which cytokines were bound or released by red blood cells. The methods were chosen to maintain biological activity rather than an attempt to obtain absolute quantification. In the next chapter, the effect of red blood cells on other cell types is explored and so, the purpose of this chapter was to provide some insight into which cytokines may be involved in signalling. An extensive survey of sample preparation methods aimed at dissociating cytokines to enable absolute quantification was beyond the scope of this study.

An increase in secreted cytokines from stored red blood cells is generally attributed to white blood cell contamination, but a 1999 study suggested otherwise[45]. That study compared the cytokine release after storing whole blood for 42 days to red blood cells and leukodepleted red blood cells[45]. They reported that the storage of whole blood resulted in the lowest concentration of secreted IL-8 (1.7 ± 7.0 pg/mL) and that the leukodepleted red blood cells secreted the highest concentration of IL-8 (26.8 ± 31.0 pg/mL). Further to that, warming the leukodepleted red blood cells to room temperature for 24 hours during the 42-day period resulted in even higher IL-8 release (125.8 ± 158.6 pg/mL). These results suggest that perturbation of these cells stimulated the release of IL-8. In light of this study, it is likely that the reason for the increased cytokine concentration in the stored red blood cell conditioned media is not simply white blood cell contamination, but possibly also the presence of chaperones or else a result of cell activation. Further research in this area is required to elucidate whether some red blood cell-associated cytokines are bound and therefore inaccessible to the immuno-assay. Further to this, a study that explores potential binding partners and the conditions that promote binding and release of cytokines would be valuable in situations where stored red blood cells are used clinically.

4.4.3 Modulation of the red blood cell cytokine profile

4.4.3.1 Binding of recombinant protein

Red blood cells have been described as potential sinks for inflammatory cytokines[33,50,51]. In support of this hypothesis, there was a significant increase in the concentration of cytokines in the red blood cells lysates after incubation with recombinant protein (Figure 4.4), with lysate levels increasing in 41 of the 48 cytokines (Appendix D). These results suggest that the red blood cells bound the cytokines out of solution. Red blood cells have been observed to inactivate and sequester IL-8 out of whole blood until cellular saturation through ligation of the Duffy antigen receptor for chemokines (DARC)[33]. Similar reports have been made for MCP-1 and MIP-1α[52,53]. Although there is some literature around cytokine binding to DARC, there is surprisingly little reference to the implications of this for red blood cells. DARC is present in abundance on red blood cells, endothelial cells, and on platelets and yet, most reports focus on the implications of this binding partnership on endothelial cells. It is not clear what receptors or molecules the other red blood cell-associated cytokines identified in this chapter may be binding to or if they are being internalised as a result. Future, more targeted studies are required to identify these binding sites.

4.4.3.2 Protease inhibitor treatment

Proteases and protease inhibitors play an important role in blood. For example, one of the most abundant proteins in plasma, alpha-2-macroglobulin, is a pan-inhibitor that acts to bind and inhibit proteases. Red blood cells are a rich source of a range of proteases and enzymes and this study has indicated that enzymes may play a role in cytokine binding and release[21,54]. The addition of a cocktail of protease inhibitors (Roche cOmplete cocktail) to red blood cells during incubation altered the cytokine profile of the resulting conditioned media and lysate (Figure 4.2). The Roche cOmplete cocktail inhibits serine-, cysteine-, and metallo-proteases all of which have been reported in red blood cells[55–57]. This treatment resulted in an overall reduction in the level of detectable cytokines in both the conditioned media and the lysates (Figure 4.4). This modulation was most notable with IL-8, wherein the concentration of detectable cytokine in both the conditioned media and lysate was 5-fold lower than the untreated control (Figure 4.2). In 1991 it was observed that IL-8 bound reversibly to red blood cells via the DARC[33]. It was reported that brief incubation (15 minutes) with a cocktail of proteases prevented cellular binding by 100 %[33]. Treatment with protease inhibitors in this chapter resulted in the inverse, where cellular binding increased by 3-fold (Figure 4.3). An increase in binding with protease

inhibitors was also observed for IL-1β and IL-5 (Appendix D), and a decrease in protein release into the conditioned media was observed for MIF and TNF-α (Appendix D).

Red blood cells are devoid of the classic secretion pathways that are well documented in other cells types due to the lack of membrane bound organelles. As a suggestion for alternative mechanisms, Schnabel *et al.* observed that coagulation and treatment with heparin drove MCP-1 release from DARC on red blood cells[52]. At this point, the exact mechanism of binding or release of these cytokines is not clear, but the results of this study suggest that proteases and protease inhibitors amongst other factors may be involved. The Roche cOmplete cocktail contains serine-, cysteine-, and metallo-protease inhibitors, however, it is not clear whether they were internalised or not. For this reason, future investigation in this area will require analysis of the effect of individual enzyme inhibitors.

4.4.4　　　　Clinical significance

The results of this study may have implications on understanding the pathology of haemolytic conditions such as sickle-cell disease and paroxysmal nocturnal haemoglobinuria. In these disorders, the increase in free haemoglobin in the plasma has been attributed to causing complications such as platelet activation and inflammation[58,59]. In the light of these results, haemolysis would also lead to the release of a range of cytokines into the plasma that may have down-stream effects on neighbouring cells. These results also suggest that red blood cells may play a crucial role in blood in regulating circulating cytokine levels by binding and releasing signalling proteins and the cells may be contributing to blood homeostasis. If red blood cells are actively binding circulating proteins out of plasma, then they may too be representative of the inflammatory state of the participant which has potential implications in diagnostics.

4.5   CONCLUSION

The results of this study may be from *in vitro* experiments on isolated blood components, but they make one thing quite clear. The locations and total quantities of signalling molecules such as cytokines are different to those described in the literature and as such, the signalling processes in blood are probably more complex. In one aspect, the conclusion from these experiments is that a reductionist biomarker discovery approach of analysing plasma or serum in isolation may be limiting, and a large piece of the puzzle may be lost in the process. Techniques such as monitoring the relative levels of panels of cytokines *between* the cell types and plasma in blood may prove to be more beneficial in disease research as opposed to determining the absolute concentrations in plasma or serum alone. In addition, analysis of overall trends and patterns in the cytokine data in this study is a valuable way to examine this type of analysis (Figure 4.4). Blood and its signalling network is highly complex, and analysis of individual cytokines may not be representative of the whole picture.

This study demonstrated that red blood cells have an extensive cytokine profile and that said profile can be modulated by adjusting their local environment, such as with the introduction of extracellular recombinant protein or with the inhibition of proteases. This raises the question of what may be modulating the cytokine profile of red blood cells *in vivo*. This list is potentially endless, but the effect of different cell types in the local vicinity is particularly interesting. More investigation into this will follow in Chapter 5.

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CHAPTER

5

MODULATION OF RED BLOOD CELL CYTOKINE PROFILE AND THE EFFECT ON IMMUNE CELL ACTIVITY

This chapter investigates the effect of cell-to-cell interactions on the red blood cell cytokine profile and activity. The previous chapter of this thesis demonstrated that the cytokine profile of red blood cells could be modulated under certain conditions, with the addition of exogenous protein and/or protease inhibitors. This chapter will expand on that investigation by assessing another mechanism by which the cytokine profile of red blood cells can be modulated. Red blood cells were incubated with a proliferating intact cancer cell line (red blood cell priming with a cancer cell line). Any changes in the red blood cell or the red blood cell conditioned media as a result of priming were evaluated by monitoring how these components altered the activity of immune cells. Although the effect of protease inhibitors on cytokine binding and release from red blood cells was discussed in Chapter 3 and Chapter 4, they were not included in this study. The aim of this chapter was to model biological interactions between cells that may occur in vivo. As such, the inclusion of protease inhibitors in this model would not have been representative of in vivo conditions.

5.1 INTRODUCTION

Red blood cells are emerging as an important regulator of cell function and survival. A small, but important body of literature has demonstrated that the inclusion of red blood cells in culture with other cell types can initiate changes in the secretion profile and in the activity of those cells. Red blood cells have been shown to interact *in vitro* with fibroblasts to stimulate the secretion of IL-8[1] and matrix metalloproteinases[2], and to modulate the growth and apoptosis of those cells[3]. Red blood cells can also bind RANTES secreted by endothelial cells, and by doing so, regulate the migration of eosinophils[4]. This interaction between red blood cells and inflammatory cells has been studied, however, binding and release of cytokines by red blood cells are not usually discussed as potential mechanisms of action. In 1988, it was observed that the presence of red blood cells was required to enhance natural killer cell cytotoxicity against tumour cells[5]. Similarly, red blood cells have been shown to suppress dendritic cell maturation and excess secretion of pro-inflammatory cytokines[6], and to play a role in B cell activation[7]. Particular emphasis has been placed on the role of red blood cells in T cell proliferation and survival[8–10]. Incubation of T cells or mixed lymphocyte populations with red blood cells can result in a reduction of apoptosis and a promotion of lymphocyte proliferation, in particular proliferation of cytotoxic T cells (CD8+ cells)[10–12]. Red blood cells also stimulate the release of inflammatory cytokines including TNF-α and IFN-γ in a dose dependent manner[13]. It has been suggested that this activity is dependent on red blood cell derived-protein factors that may be haemoglobin or peroxiredoxin II[9]. Whilst other groups hypothesise that this activity is a result of red blood cells reducing oxidative stress and damage in T cells by protecting cell membrane integrity[14]. This protection is dependent on the red blood cells being intact and in close proximity to the T cells.

In recent years, the dysregulation of red blood cells in inflammatory conditions has been investigated and it has been reported that red blood cells isolated from participants with carotid atherosclerosis have an attenuated capacity to protect T cells from apoptosis[15]. This result was also replicated by subjecting healthy red blood cells to excessive oxidative stress. Red blood cells isolated from the same disease group were also unable to regulate dendritic cell maturation[16] and the result of this has been directly correlated with atherosclerotic plaque progression[17]. In other study, following hip arthroplasty, red bloods were less able to stimulate proliferation of T cells when compared to the samples collected before the surgery[18]. Similarly,

storage of red blood cells for 2-3 weeks produced this same, suppressive effect on both autologous and allogeneic T cells[19]. A reduction in IL-2 was observed with red blood cell treatment, however the proliferation could not be rescued by addition of recombinant IL-2. The implications of these results are not currently understood, but it was hypothesised that the dysregulation of the interaction between red blood cells and T cells may interfere with T cell homeostasis *in vivo*[15]. Dysregulated immune activity and chronic inflammation can promote tumour development and protection from apoptosis[20]. A549 cells are a human non-small cell lung carcinoma cell line that has been investigated extensively due to its capacity to suppress an immune response. The literature has reported that this activity is modulated at least in part by tumour-derived cytokine release. It was demonstrated that this cell line, amongst others, expressed the toll-like receptor TLR4[21]. Ligation of this receptor induced the release of a range of immunosuppressive and proangiogenic cytokines (TGF-β, VEGF, and IL-8) which promoted cell growth and resistance to apoptosis[21].

As outlined in the previous chapter (Chapter 4), red blood cells are capable of both binding and releasing cytokines in response to their local environment. This result was induced by incubation with a known cocktail of recombinant proteins. The study outlined in this chapter aimed to investigate a biological interaction of inducing cytokine binding to red blood cells. *In vivo*, red blood cells come into contact with a wide array of cell types and the proteins that they secrete. In capillaries, red blood cells are forced to deform in order to pass through the microvasculature, and direct cell-to-cell contact occurs with endothelial cells and other tissue cells in the process[22]. In the lungs, gas exchange occurs across the capillary and alveolar walls, however, gases are not the only molecules transported and exchanged by red blood cells. Red blood cells are carriers of drugs, such as steroids and anticonvulsants, that partition between plasma protein binding and red blood cells[23]. Exchange of protein-binding molecules occurs between red blood cells and other cells in the restricted confines of capillaries[23]. Molecules, including proteins, bound to the surface of red blood cells appear to play a key role in cell function and are likely to modulate exchanges with other cells. Washed red blood cells have altered functions[24,25] and may have diminished levels of cytokine (Appendix B). On this basis, the hypothesis of the chapter was that interaction with other cell types would modify the red blood cell cytokine profile and subsequently those cell-contact primed red blood cells would have an altered effect on immune cells.

In this study, the effect of priming red blood cells with A549 cancer cells on lymphocyte proliferation, survival, and function was investigated. The experimental ratios of A549 cells to

red blood cells were based on literature describing the structure and cellular makeup of lung capillaries[26,27]. Red blood cell speed, flux, and density in capillaries have been published for humans, as well as rodents and amphibians and these datasets were used to approximate the number of cells for the priming experiments[26]. The total capillary surface area has been estimated, using morphometric assessment, to range from 0.01 m^2 in a mouse lung[28] to 126 m^2 in a human lung[29]. The surface area of endothelial cells is approximately 1100 μm^2 per cell, which is consistent across species[26]; this demonstrates that the increased capillary surface area is a direct result of increased numbers of endothelial cells. This is relevant to the priming experiments conducted in this chapter because a growing tumour is made of proliferating cells and neovascularisation. For the purposes of the experiments in this chapter, it was assumed that the A549 cells were structurally similar to endothelial cells, which they can mimic[30]. Endothelial cells that are seeded at 1.8 million cells per T75 culture flask (as in this study), provided stable growth, would be the equivalent of an endothelial surface area of 20 cm^2, which represents 0.0015 % of the total lung capillary surface area.

In the analysis of peripheral blood mononuclear cells (PBMCs), the response of the T cell population (CD3+ cells) was of particular interest. Subsets of this population was also analysed with focus on the response of CD4+ cells and CD8+ cells. CD4+ cells in a lymphocyte group are described as T helper cells, whilst CD8+ cells in a lymphocyte group are referred to as cytotoxic T cells.

5.1.1 Chapter hypothesis

It is hypothesised that priming red blood cells with a cancer cell line will change the cytokine profile of these red blood cells and that these cells will alter immune cell activity.

5.1.2 Chapter aims

1. To investigate if the cytokine profile of red blood cells would change following incubation with a cancer cell line.
2. To evaluate the activity of peripheral blood mononuclear cells in response to treatment with naïve red blood cells.
3. To determine if priming red blood cells with a cancer cell line would induce altered activity in the peripheral blood mononucleated cells.

5.2 METHODS
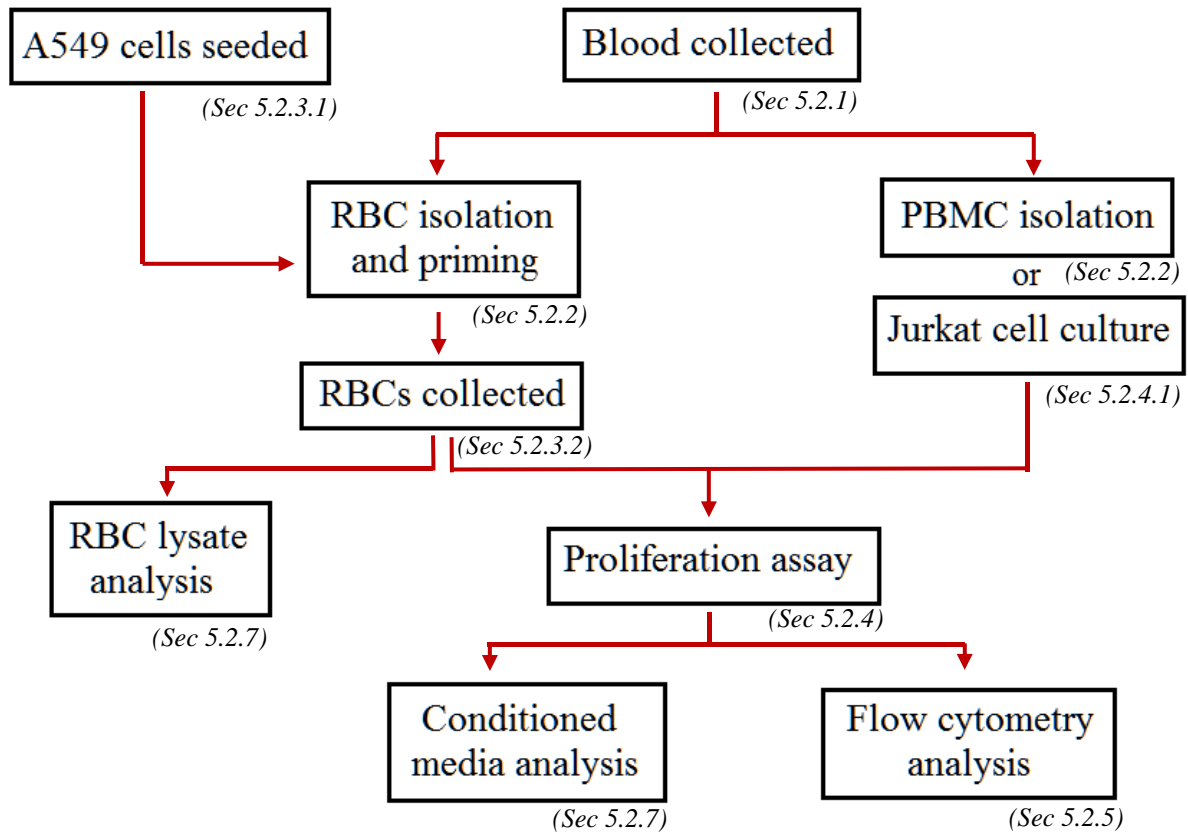
Figure 5.1. Overview of experimental design.

5.2.1 Blood collection

Whole blood was collected from healthy volunteers by venepuncture ($n = 12$; female: 7, male: 5) directly into EDTA vacutainers (K$_2$EDTA vacutainers, BD Biosciences). The age range of these participants was 25 - 48 years. All fractions of blood were collected and processed at room temperature within four hours of collection. For multiplex analyses all samples were stored at -80 °C and were subjected to three freeze-thaw cycles at -80 °C prior to analysis to ensure complete cellular lysis.

This study was approved by the Northern Sydney Coast Human Research Ethics Committee of NSLHD and CCLHD (1201-046M). Written consent was collected from all participants before participation in this study.

5.2.2 Blood cell isolation

Red blood cells and peripheral blood mononucleated cells (PBMCs) were isolated from EDTA anti-coagulated whole blood. Red blood cells were collected and were either frozen immediately or were used fresh for priming experiments (Section 5.2.3.2). PBMCs were used fresh for proliferation experiments (Section 5.2.4). A haematology analyser (Coulter Act Diff, Beckman Coulter) was used to determine cell number and purity of populations.

5.2.2.1 Red blood cell isolation

Red blood cells were isolated using dextran sedimentation as follows. Whole blood was centrifuged (1500 g, 10 minutes) and the upper plasma layer was discarded. The remaining cell pellet was resuspended in an equal volume of sodium chloride (0.15 M). Dextran (6 % w/v in 0.15 M sodium chloride) was then added to this cellular suspension at a 1:4 ratio (dextran:cell suspension). This solution was left at room temperature for 30 minutes for red blood cells to sediment to the bottom of the tube. After this time the upper white blood cell rich layer was discarded and the lower red blood cell fraction was isolated. The red blood cell fraction was washed once in phosphate buffered saline (PBS, 500 g, 5 minutes) and the remaining red blood cell pellet was either frozen (-80 °C) or used fresh (Section 5.2.3.2).

5.2.2.2 Peripheral blood mononucleated cells (PBMCs)

PBMCs were isolated from whole blood using density gradient separation. Whole blood diluted in an equal volume of PBS was carefully layered on top of Ficoll-Paque (2:1 ratio respectively, GE Healthcare) and was centrifuged without a brake (400 g, 40 minutes). After centrifugation, the PBMC layer at the interface between the plasma and the Ficoll-Paque was isolated and washed once with PBS (500 g, 5 minutes). Any contaminating red blood cells were lysed with red blood cell lysis solution (Sigma Aldrich) for 10 minutes at 37 °C.

5.2.3 Priming red blood cells with A549 cells

5.2.3.1 A549 cell culture

A549 cells were expanded in A549 culture media (DMEM with 10 % FBS and 1 % antibiotic-antimycotic, v/v) at 37 °C and 5 % CO_2. Cells were passaged twice a week with TrypLE when the cells reached confluence. Cells were counted using a haemocytometer and viability was determined by trypan blue staining.

5.2.3.2   *Priming conditions*

To prime the red blood cells, A549 cells were seeded into T25 flasks and T75 flasks at a concentration of 0.1 x 10^6 cells per mL of ADSC culture media (total volume: 6.25 mL and 18 mL for T25 and T75 flasks respectively) and were incubated for 24 hours to ensure adherence to the plate (37 °C, 5 % CO_2). After incubation, the following conditions were prepared using freshly isolated red blood cells (Table 5.1). For red blood cell priming, the total volume of culture media in the T75 flasks was 18 mL and in the T25 flasks was 6.25 mL.

Table 5.1.   Red blood cell (RBC) priming conditions with red blood cells at ratios of 1:100 and 1:1000 at 37 °C, 5 % CO_2 for 72 hours.

Condition	Label	Flask size	A549 cells number	Red blood cell number
A549 cells	-	T75	1.8 x 10^6	-
A549 cells	-	T25	0.625 x 10^6	-
A549 cells:RBCs (1:100)	Primed (pRBC-L)	T75	1.8 x 10^6	180 x 10^6
A549 cells:RBCs (1:1000)	Primed (pRBC-H)	T25	0.625 x 10^6	625 x 10^6
RBCs	Unprimed (upRBC-L)	T75	-	180 x 10^6
RBCs	Unprimed (upRBC-H)	T25	-	625 x 10^6

Cells were then incubated for 72 hours at 37 °C with 5 % CO_2. Following incubation, the conditioned media with the red blood cells in suspension was collected and the red blood cells were isolated by centrifugation out of the conditioned media (500 *g*, 10 minutes). Any remaining particulates in the conditioned media were removed by centrifugation (2000 *g*, 10 minutes) after which it was stored at -80 °C. The red blood cells were washed once with PBS and counted using a haematology analyser (Coulter Act Diff, Beckman Coulter). The red blood cells were then either used intact for proliferation assays (Section 5.2.4), or were frozen at -80 °C to produce red blood cell lysates.

5.2.3.3   *A549 cell imaging*

A549 cells were seeded into 12-well plates at 0.025 x 10^6 cells per well. These cells were incubated for 24 hours (37 °C, CO_2), after that point A549 cells were either treated with red blood cells at a ratio of 1:100 or 1:1000, or left untreated (A549 culture media, 1.5 mL per

well). After incubation for a further 72 hours (37 °C, CO$_2$) the red blood cells and conditioned media were discarded, and the adherent A549 cells were washed well with PBS. Images of the A549 cells were collected using the IncuCyte FLR, and the level of confluence for each well was calculated using IncuCyte image analysis software (ver. 2011A Rev2) using the mean of the well (9 images collected per well).

5.2.4 Proliferation assay

5.2.4.1 *Jurkat cell culture*

Jurkat cell culture media consisted of RPMI-1640 with FBS (10 % or 2.5 %, v/v), penicillin and streptomycin (1 % v/v), and L-glutamine (200 mM solution, 1 % v/v). Cells were maintained at a concentration between 0.2 x 10^6 and 1 x 10^6 cells/mL in culture. Jurkat cells were expanded with 10 % FBS (v/v) and were sub-cultured into 2.5 % FBS six days prior to initiation of the proliferation assay. Dead cells were removed using Ficoll-Paque once a week by layering 5 mL of the cell suspension over 5 mL of Ficoll-Paque. This was then centrifuged with no brake (400 *g*, 40 minutes), and the live cells at the interface of the PBS layer and the Ficoll-Paque layer were collected, washed twice with PBS and returned to culture. Cells were counted using a haemocytometer and viability was determined with trypan blue staining.

5.2.4.2 *PBMC cell culture*

After isolation, PBMCs were put into culture for 3 days until initiation of the proliferation assay (37 °C, 5 % CO2). PBMCs were cultured in RPMI-1640 with FBS (10 % v/v), penicillin and streptomycin (1% v/v), and L-glutamine (200 mM solution, 1 % v/v). After culture, only the cells still in suspension were collected, thus depleting the PBMC population of monocytes (which are plate adherent). An aliquot of the cells was diluted in Turks solution (1:20) and counted using a haemocytometer.

5.2.4.3 *Preparation of red blood cell lysates, cytosol, and membranes*

Frozen primed (pRBC) and unprimed (upRBC) red blood cells (Section 5.2.3) were subjected to three freeze-thaw cycles to ensure complete cellular lysis, thus producing red blood cell lysates. The lysates were clarified by centrifugation to remove particulates (2000 *g*, 5 minutes). The red blood cell cytosolic fraction was isolated by removing the cellular membranes from the red blood cell lysates by centrifugation (16,000 *g*, 20 minutes). The resulting membranes were washed twice with Milli-Q water (16,000 *g*, 20 minutes) before being resuspended in RPMI-1640 media.

5.2.4.4 CFSE labelling

Jurkat cells or PBMCs (1 million cells per mL) were stained with CFSE (5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester) at a final concentration of 5 µM in PBS for 10 minutes at 37 °C. After staining, the cells were washed once with FBS to quench the excess CFSE. Immediately after labelling, the cells were analysed by flow cytometry and staining efficiency was determined to be greater than 99 %.

5.2.4.5 Proliferation assay conditions

Proliferation assays were performed with CFSE stained Jurkat cells or PBMCs for up to 6 days with low serum media (RPMI-1640, 1 % FBS, 1 % penicillin/streptomycin, 1 % L-glutamine, v/v). Jurkat cells (0.25×10^6) were cultured in 12-well plates, with a total culture media volume of 2.5 mL for 6 days (37 °C, 5 % CO_2). PBMCs (1.5×10^6) were cultured in 6-well plates, with a total culture media volume of 5 mL for 5 days. For analysis of apoptosis and expression of transcription factors, unstained PBMCs were cultured under the same conditions. PBMCs were stimulated with PHA-P (Phytohemagglutinin-P, 5 µg/mL), and Jurkat cells were left unstimulated. Jurkat cells and PBMCs were cultured with or without autologous red blood cells at a ratio of 1:10 (Jurkat/PBMCs to red blood cells). As outlined in Section 5.2.3.2, the red blood cells had been either primed (incubated with A549 for 72 hours) or unprimed (incubated alone for 72 hours). In variations of the proliferation assay, Jurkat cells were treated with red blood cell lysates, cytosols, or membranes (from primed or unprimed cells) at concentrations equivalent to 2.5×10^6 cells per well. Additionally, Jurkat cells were treated with conditioned media (undiluted) from red blood cell priming conditions (Section 5.2.3.2).

At the conclusion of the culture period, the conditioned media from each sample was collected and stored at -80 °C for future analysis. The PBMCs and Jurkat cells were isolated and processed for flow cytometry analysis as outlined below (Section 5.2.5).

5.2.5 Cell staining and flow cytometry

Following cell culture, the PBMCs and Jurkat cells were isolated by centrifugation (1000 *g*, 10 minutes). The resulting conditioned media was collected and stored (-80 °C) and the remaining cells were prepared for flow cytometry analysis. For each sample, 1×10^4 events of the stained target population were acquired and analysed by flow cytometry using Cell Quest Pro software (ver. 5.2.1) and Flowing Software (ver. 2.5.1).

5.2.5.1 Jurkat cell analysis

Following culture, Jurkat cells were isolated by centrifugation (1000 g, 10 minutes). In red blood cell treated conditions, the red blood cells were lysed with red blood cell lysis buffer (10 minutes, 37 °C). The Jurkat cells were then washed in PBS before being analysed by flow cytometry (FACScalibur) to monitor level of CFSE fluorescence.

5.2.5.2 PBMC analysis

Following culture, PBMCs were isolated by centrifugation (1500 g, 10 minutes). In red blood cell treated conditions, the red blood cells were lysed with red blood cell lysis buffer (10 minutes, room temperature). The PBMCs were then washed once in PBS before commencing staining with the following antibodies (Table 5.2). Cells stained with CFSE were only co-stained with APC conjugated antibodies.

Table 5.2. Antibodies and proteins used for immunofluorescence staining of PBMCs.

Antibody/Protein	Fluorochrome	Clone
Anti-human CD3 (Mo IgG1, κ)	APC	SK7
Anti-human CD3 (Mo IgG1, κ)	FITC	UCHT1
Anti-human CD4 (Mo IgG1, κ)	APC	RPA-T4
Anti-human CD8a (Mo IgG1, κ)	APC	HIT8a
Anti-human GATA-3 (Mo IgG2b, κ)	PE	16E10A23
Anti-human T-bet (Mo IgG1, κ)	PE	4B10
Annexin V	FITC	-
Isotype control (Mo IgG1, κ)	FITC	MOPC-21
Isotype control (Mo IgG1, κ)	APC	MOPC-21
Isotype control (Mo IgG1, κ)	PE	MOPC-21
Isotype control (Mo IgG2b, κ)	PE	MPC-11

Cells were stained in two steps, for extracellular markers in the first step (CD3, CD4, CD8, or corresponding isotype controls) and intracellular markers in the second step (T-bet, and GATA-3, or corresponding isotype controls). For staining of CD3, CD4, and/or CD8, 1 x 10^6 cells were resuspended in 100 µL staining buffer (PBS, 0.5 % bovine serum albumin (BSA) w/v) with the relevant antibodies or corresponding isotype controls and were incubated at 4 °C for

30 minutes in the dark. Cells were then washed once in ice cold staining buffer (1500 g, 5 minutes). In the second step of the staining protocol for GATA-3 and T-bet, the cells were fixed and permeabilised (Nuclear Factor Fixation and Permeabilisation Buffer Set, BioLegend) according to the manufacturers instructions. The cells were then resuspended in 100 µL of the permabilisation buffer with the relevant antibodies or corresponding isotype controls and were incubated at 4 °C for 30 minutes in the dark. After the incubation, the cells were washed twice in PBS/0.1 % BSA (1500 g, 5 minutes) and were finally resuspended in 300 µL PBS for flow cytometry analysis. Multi-colour flow cytometry (FACScalibur) was used to monitor the level of CFSE fluorescence in specific cellular populations and to monitor the expression of transcription factors in CD8+ populations. For the purposes of compensation, BD Calibrite[TM] beads and single colour controls for CFSE, FITC, PE, and APC were used to optimise detection. The proliferation index of the CFSE labelled PBMCs was determined by monitoring the percentage of cells in each generation as previously described[31].

5.2.5.3 *Apoptosis analysis*

Following cell surface staining, PBMCs were washed once in Annexin V binding buffer (BioLegend) and 0.1×10^6 cells were resuspended in 100 µL of Annexin V binding buffer (BioLegend) with 5 µL of 1 mg/mL propidium iodide (PI) and the corresponding Annexin V-FITC antibody. These cells were incubated for 15 minutes in the dark at room temperature after which the cell suspension was diluted in 400 µL of Annexin V binding buffer. The cells were then analysed immediately by flow cytometry.

5.2.6 **Haemoglobin quantification**

Levels of free haemoglobin in plasma samples and conditioned media samples were monitored by assessing absorbance at 414 nm (Synergy 2 Multi-Mode plate reader) as previously described[32]. Peaks at this wavelength are indicative of free haemoglobin. Red blood cell membranes were removed from the conditioned media samples prior to analysis by centrifugation at 16,000 g for 15 minutes. A haemoglobin calibration curve was prepared using haemoglobin (Sigma Aldrich, USA) at known concentrations and was analysed on GraphPad Prism software (ver. 6, USA).

5.2.7 Cytokine multiplex assay

In this study, two multiplex assays were utilised. The first was the 27-plex human cytokine panel that assays for FGF basic, Eotaxin-1, G-CSF, GM-CSF, IFN-γ, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF, and the second was the 21-plex human cytokine panel that assays for IL-1α, IL-2Ra, IL-3, IL-12(p40), IL-16, IL-18, CTACK, GRO-α, HGF, IFN-α2, LIF, MCP-3, M-CSF, MIF, MIG, β-NGF, SCF, SCGF-β, SDF-1α, TNF-β, TRAIL (Bio-Plex Pro 27-plex and 21-plex, Bio-Rad). The assays were performed according to manufacturer's instructions using an automated magnetic wash station (Bio-Plex Pro II, Bio-Rad) for the washing steps. The assays were run on the Luminex® 200^{TM} system (Bio-Rad) and fluorescence values were collected. The calibration curve for each cytokine was analysed with 5 parametric logistic curve regression using Bio-Plex manager software (ver. 5.0, Bio-Rad, USA). Standard values were considered acceptable if the points fell within 80 - 120 % of the expected values. Conditioned media samples from A549-red blood cell culture and PBMC cultures were left undiluted for analysis. The conditioned media samples from the Jurkat cultures were concentrated 5x with centrifugal concentrators (3kD minimum cut off) for multiplex analysis.

5.2.8 Statistical analysis

Comparison of multiple treatment groups for haemoglobin concentration or flow cytometry data were statistically evaluated using a one-way ANOVA with correction for multiple comparisons (Tukey correction) to assess statistical significance. Data were statistically significant if $p < 0.05$. Graphing of results was performed using GraphPad Prism software (ver. 6, USA). Flowing Software (ver. 2.5.1) was used to create data histograms and dot plots for flow cytometry data and for data analysis. Multi-plex cytokine data were presented as fold change of fluorescence and are presented as mean ± confidence interval (CI). Concentration values are presented as mean ± standard deviation (SD).

Statistical analysis of raw fluorescence responses was performed using 'R' version 3.2.3 (2015-12-10, R: A Language and Environment for Statistical Computing). Mixed-effects modelling was done using lmer[33]. The significance of interactions terms and interaction means and their associated standard errors were obtained using the Phia package[34], for post-hoc analysis. Multiple test correction was done according to Holm's method[35]. To analyse the fluorescence response, the following mixed-effects model, in R notation, was used:

Equation 5.1. Mixed-effects model for statistical analysis of cytokine data.

$$\log2(FI) \sim Cytokine * Treatment + (1|kit\!:\!ID)$$

The analysis was performed separately for red blood cells lysates and conditioned media samples. Where the log2 of fluorescence responses (Fl) was modelled using 2-way mixed effects ANOVA, (1) Cytokine (48 levels), and (2) Treatment (2 levels for red blood cell lysates: unprimed red blood cells, or primed red blood; 4 levels for conditioned media: none, PHA, unprimed red blood cells, or primed red blood cells) plus their interactions, together with one random term defined as (1|kit:ID), where ID represented subject identifier and where kit represented the 27-plex and 21-plex cytokine panels (2 levels). The random effects account for patient-to-patient variability and for differences with respect to kit groupings across the 2 cytokine plates; it also accounted for the non-independence in the data due to multiple samples per subject. Results of the mixed-effects model validation is included in Appendix E.

For the remainder of this chapter, the term 'significant' will be used to indicate a statistically significant result of $p < 0.05$ unless otherwise specified.

5.3 RESULTS

5.3.1 Red blood cell purity

Red blood cell purity was determined by calculating the depletion of white blood cells and platelets from whole blood to an enriched red blood cell population. This enriched red blood cell population was produced through dextran sedimentation of red blood cells. The dextran sedimentation protocol utilised in this chapter was slightly different to the protocol outlined in Chapter 3 and Chapter 4. Here, dextran sedimentation occurred after the plasma was removed as opposed to sedimentation from complete whole blood. This small method change resulted in improved white blood cell depletion than previously seen in Chapter 4, Table 4.2 (mean: 97 %, vs 83 %). The platelet depletion remained unchanged with a mean of 98% depletion. Thus, for every one million red blood cells there were approximately 28 white blood cells and 2,700 platelets.

Table 5.3. Purity assessment of enriched red blood cell (RBC) fractions ($n = 12$).

RBC purity (%)	White blood cell depletion (%)	Platelet depletion (%)
99.995 ± 0.004	97.2 ± 3.4	98.2 ± 3.8

*Data are presented as mean $\pm$ SD

5.3.2 Priming red blood cells with A549 cells

For priming experiments, fresh red blood cells were collected and were incubated with or without a lung cancer cell line (A549 cells) for 3 days at 37 °C and 5 % CO_2 to produce 'primed' (pRBC) or 'unprimed' (upRBC) red blood cells. Proliferation of A549 cells in the presence or absence of red blood cells was determined and any changes in the cytokine profile of these primed or unprimed red blood cells were also investigated.

5.3.2.1 A549 cell proliferation

A549 cells were cultured under three conditions and following incubation the confluence of the A549 cells in each well was determined. There was no significant difference between the confluence of the A549 cells that were cultured alone or in the presence of red blood cells.

5.3.2.2 Conditioned media from A549 cells

There was no significant difference in the concentration of cytokines in the conditioned media from A549 cells or A549 cells with red blood cells. In addition, there was no significant difference in the concentration of haemoglobin in the red blood cell conditioned media with or without A549 cells. The levels of haemolysis in the conditioned media for each sample corresponds to approximately 1 % of the original inoculation.

5.3.2.3 Cytokine profile of red blood cell lysates

Following incubation, the resulting upRBCs and pRBCs were collected and their lysates were analysed to establish the cytokine profile. The fold change of a representative selection of cytokines from the low prime group (red blood cells primed with A549 cells at 100:1 respectively, pRBC-L) is presented in Figure 5.2; the data for the remaining cytokines can be found in Appendix F. For the upRBC group, the level of 17 cytokines in red blood cells changed following incubation, whilst 26 cytokines changed after incubation for the pRBC group (Appendix F). A significant increase in the level of eight cytokines in the pRBCs compared to upRBCs was observed. The significant changes between the upRBC and the pRBC group were observed for chemokines (IL-8, MCP-1 and GRO-α), growth factors (bFGF, M-CSF, and VEGF), and pro-inflammatory cytokines (IL-9, IL-12p70) (Figure 5.2). The largest observed increase in concentration in the red blood cells was bFGF, where, the concentration changed from a mean of 22 ± 18 pg/mL in the upRBCs, to 983 ± 414 pg/mL in the pRBCs. Similarly, the concentration of GRO-α increased from a mean of 6 ± 6 pg/mL in the unRBC group to 259 ± 214 pg/mL in the pRBC group.

The cytokine profile of the red blood cell from the high ratio prime group (red blood cells primed with A549 cells at 1000:1 respectively, pRBC-H) was very similar to the pRBC-L, however the differences between the primed and unprimed cells was less pronounced. As an example, GRO-α was 30 ± 28 fold higher with priming in the pRBC-L group, whilst it was only 20 ± 10 fold higher with priming in the pRBC-H group. This trend was also observed for IL-8, and MCS-F.
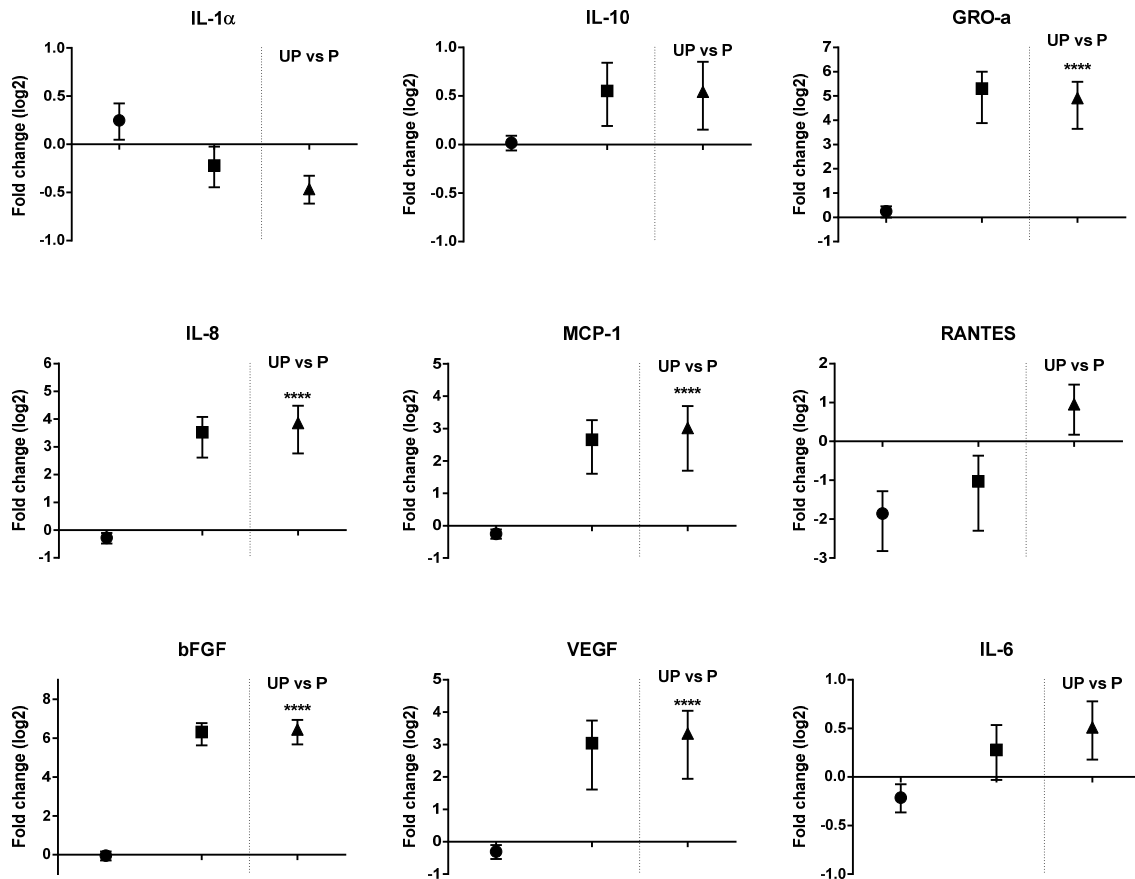
Figure 5.2. Summary of cytokines in the red blood cell lysates prior to incubation (T0) and of primed (pRBC) and unprimed (upRBC) red blood cells after incubation (T3, primed at 1:100 for A549:RBCs) as measured by Bio-Plex and reported as fluorescence (100 million cells in 1 mL PBS). Data are presented as mean fold change of fluorescence $\pm$ 95 % CI ($n = 12$), where ● represents the fold change for unprimed red blood cells before and after incubation and ■ for primed cells before and after incubation, and ▲ represents the fold change in fluorescence between unprimed and primed red blood cells (T3). Data are statistically significantly different (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$, (****) if $p < 0.0001$.

5.3.3 **Red blood cell treatment of Jurkat cells**

In this study, proliferation of a leukemic T cell line (Jurkat cells) was monitored over 6 days with and without red blood cell treatment and was quantified by monitoring the dilution of cellular CFSE staining. Cytokine release from Jurkat cells during incubation was also monitored.

5.3.3.1 *T cell proliferation*

A reduction in CFSE staining was observed for the Jurkat cells that had been treated with upRBCs, and a further reduction was observed in the Jurkat cells that had been treated with pRBCs (Figure 5.3). This is representative of Jurkat cell proliferation that was stimulated in the presence of red blood cells.
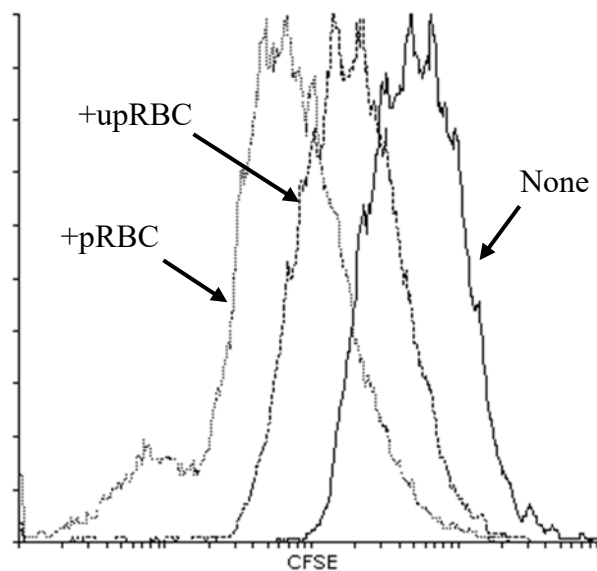
Figure 5.3. Histogram shows CFSE fluorescence loss in untreated Jurkat cells (none), Jurkat cells treated with unprimed red blood cells (+upRBC), or Jurkat cells treated with primed red blood cells (+pRBC) as measured by flow cytometry. A shift in the histogram peak to the left indicates Jurkat cell proliferation over 6 days at 37 °C and 5 % CO_2.

When treated with upRBCs, Jurkat cells proliferated significantly more than the untreated cells, with a mean fold increase of 2.4 (Figure 5.4a). In addition, Jurkat cells treated with pRBCs proliferated significantly more than the corresponding upRBCs ($p < 0.0001$) regardless of the RBC-A549 priming ratio (Figure 5.4a). Although the trends were the same, there were some differences between the groups. The red blood cells primed at a ratio of 1:100 (pRBC-L) produced a larger proliferative response in the Jurkat cells than the red blood cells primed at a ratio of 1:1000 (pRBC-H), with a mean fold change from the untreated control of 8.3 and 5.3 respectively. This difference was highly statistically significant with a p-value of 0.0008. For this reason, subsequent proliferation experiments were conducted on the pRBC-L (1:100 priming ratio of A549:RBC) group only.

No increase in proliferation was observed for Jurkat cells treated with the culture media blank or with red blood cell conditioned media (Figure 5.4b). However, treatment with conditioned media from A549 cells and A549-red blood cell conditioned media resulted in significantly more proliferation than the untreated cells ($p < 0.0001$) with mean fold changes of 2.9 and 3.5 respectively (Figure 5.4b). Of note, the addition of red blood cells to the A549 culture produced conditioned media that was significantly more stimulatory to the Jurkat cells than the conditioned media from A549 cells alone.

An overall increase in Jurkat cell proliferation was observed with treatment of red blood cell fractions. The lysate or cytosol from red blood cells significantly increased proliferation by approximately 2-fold (Figure 5.4c). This was consistent for the lysates and cytosols isolated from both upRBCs and pRBCs and there was no difference between either of these groups. In addition, the increase observed for these conditions was consistent with the proliferation associated with intact, upRBCs, where each group resulted in a 2 to 2.5-fold increase in proliferation of the Jurkat cells (Figure 5.4a). Although lower proliferation was observed with red blood cell membrane treatment in comparison to red blood cell lysate and cytosol treatment (mean of 1.4-1.9 vs 2.2-2.6), the membranes stimulated the Jurkat cells to proliferate significantly more than the untreated controls (Figure 5.4c). In fact, the only difference between the upRBC and pRBC groups in the analysis of red blood cell fractions was observed with membrane treated Jurkat cells. Membranes isolated from pRBCs significantly promoted Jurkat cell proliferation when compared to the upRBC membranes ($p = 0.0031$).
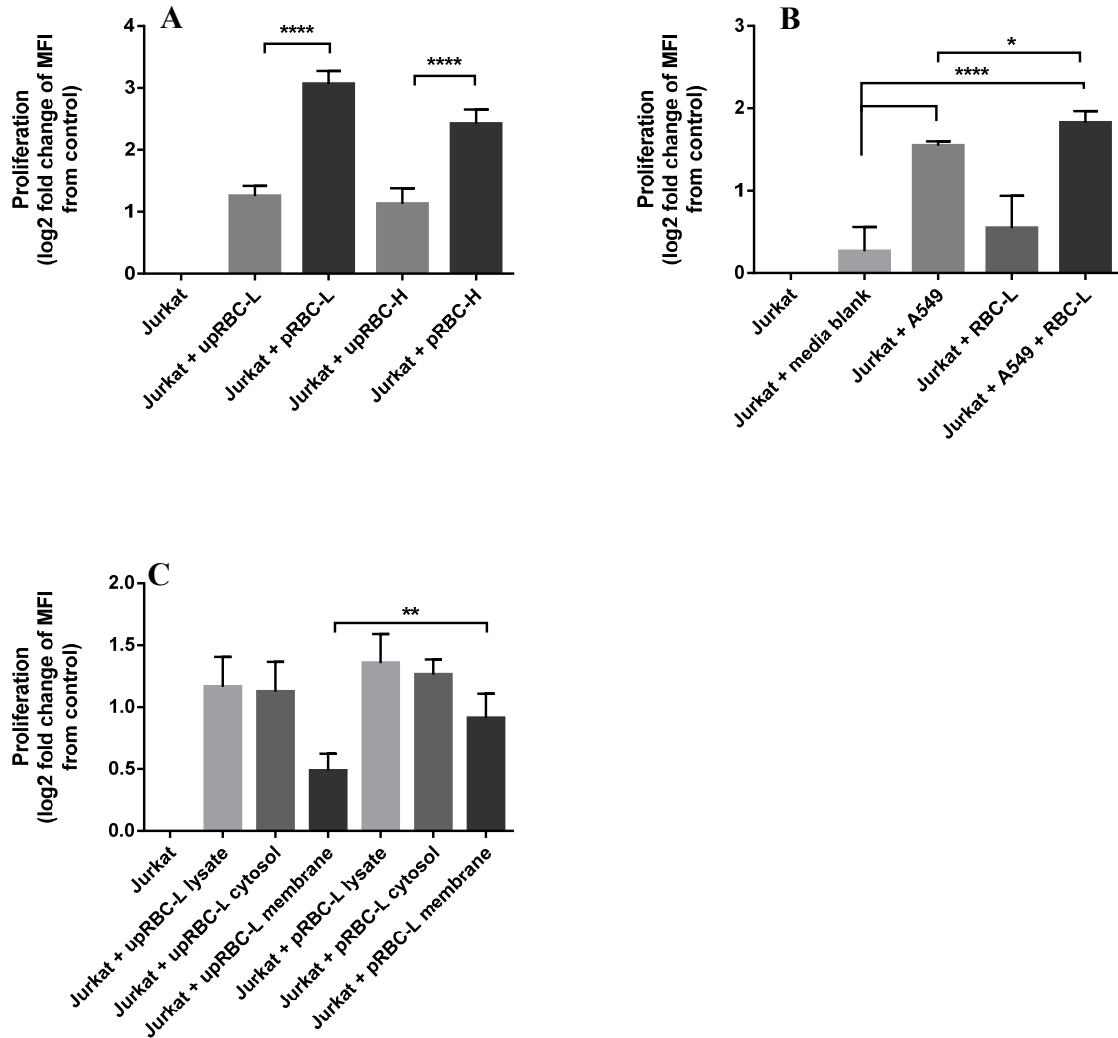
Figure 5.4. Graphical representation of Jurkat cell proliferation following treatment with (A) intact primed (pRBCs) or unprimed (upRBCs) cells which were primed at 1:100 (pRBC-L) or 1:1000 (pRBC-H) for A549 cells:RBCs, (B) conditioned media from A549 cells, RBCs, or A549/RBC culture (cells primed at 1:100 for A549:RBCs), or (C) red blood cell lysate, cytosol, or membranes isolated from upRBCs and pRBCs (cells primed at 1:100 for A549:RBCs). Data are presented as fold change of loss of CFSE fluorescence from untreated Jurkat cells after 6 days in culture at 37 °C with 5 % CO_2 (mean ± SD, $n = 5$). Data are statistically significantly different (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$, (****) if $p < 0.0001$.

5.3.3.2 *Cytokine prolife of Jurkat cell conditioned media*

The level of cytokines in the conditioned media from Jurkat cells in all culture conditions was evaluated using multiplex cytokine assays. The fold change of a representative selection of cytokines is presented in Figure 5.5; the data for the remaining cytokines can be found in Appendix G. In the pRBC group, the level of 22 cytokines increased, and none decreased (Appendix G) in comparison to the upRBC group. In the upRBC groups, secretion of only IL-16 increased in comparison to the untreated group, and only IL-7 decreased. Of the 14 cytokines that were significantly different between upRBC and pRBC treatment, all were higher in the pRBC group. Of those cytokines, there was an even distribution across the functional categories. Notably, of the five anti-inflammatory cytokines that are on the multiplex panels, the levels of four of them were significantly higher in the pRBC group when compared to the upRBC group (Appendix G).

For the conditioned media from Jurkat treated with primed red blood cells, there were five cytokines with a mean fold increase from the untreated control of greater than 10 (IL-8, GRO-α, MCP-1, RANTES, and VEGF). The concentration of each of these cytokines changed substantially (Figure 5.5). For example, the concentration of GRO-α was below the limit of the standard curve (< 7.3 pg/mL) for the upRBC group, but was present at 2443 ± 748 pg/mL (mean ± SD) in the conditioned media of the pRBC group. Similarly, IL-8 increased from a mean of 7 ± 3 pg/mL in the upRBC groups to 2317 ± 2420 in the pRBC group. Although the lysate concentration of these cytokines was also higher in the pRBC group compared to the upRBC group, these increased levels cannot only be attributed to red blood cell release or lysis. Primed red blood cells incubated alone in the same conditions released 3.5-fold less GRO-α than the Jurkat cells treated with the same primed cells. Likewise, primed red blood cells released 4-fold less IL-8 than primed red blood cell treatment group.
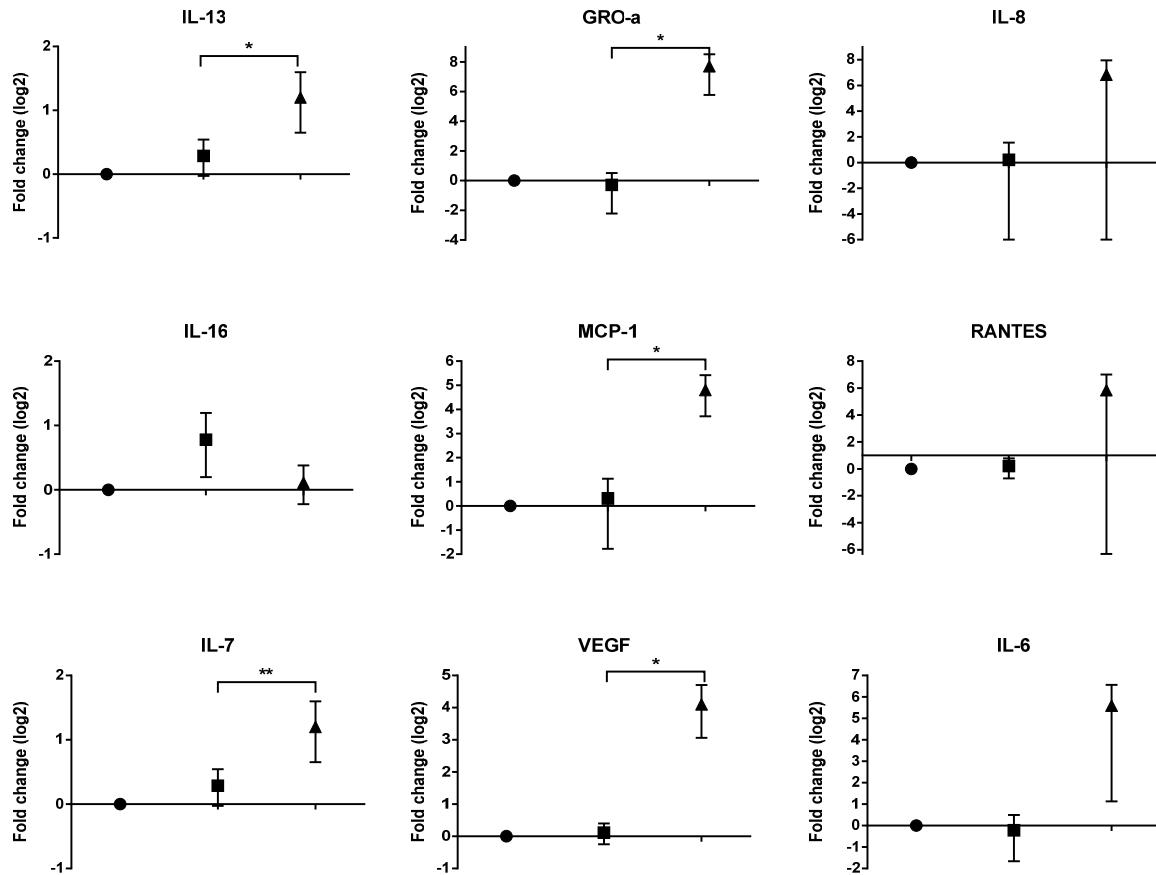
Figure 5.5. Summary of cytokines in Jurkat conditioned media as measured by Bio-Plex and reported as fluorescence. Data are presented as mean fold change from the untreated Jurkat control in fluorescence $\pm$ 95 % CI ($n = 5$), where ● represents the fold change for untreated Jurkat, ■ for Jurkat cells treated with unprimed red blood cells (upRBC), and ▲ for Jurkat cells treated with primed red blood cells (pRBC). Data are statistically significantly different from the untreated Jurkat control (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$, (****) if $p < 0.0001$.

5.3.3.3 *Concentration of haemoglobin in red blood cell conditioned media*

The concentration of haemoglobin in conditioned media from red blood cells and red blood cells incubated with Jurkat cells was quantified by monitoring the absorbance of the solutions at 414 nm. The concentration of haemoglobin in the conditioned media samples corresponds to the lysis of 68,000 to 375,000 red blood cells (Figure 5.6). With a starting inoculation of 2.5×10^6, this corresponds to lysis of a minimum of 3 % and a maximum of 15 % of the total red blood cell number. In a comparison between upRBC and pRBC conditioned media samples, there is a trend towards more lysis in the upRBC samples. However, the only statistically significant difference was between upRBC-L and pRBC-L (primed at 1:100 with A549 cells) incubated with Jurkat cells for 6 days (Figure 5.6).
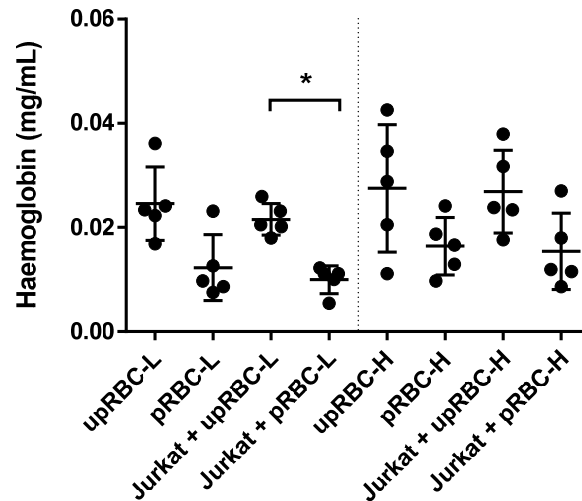
Figure 5.6. Concentration of haemoglobin in the conditioned media of unprimed or primed red blood cells (2.5×10^6 red blood cells per condition) incubated alone or with Jurkat cells for 6 days at 37 °C with 5 % CO_2. Red blood cells were primed at 1:100 (pRBC-L) or 1:1000 (pRBC-H) with A549 cells prior to incubation. Haemoglobin concentration was measured by absorbance at 414 nm. Data are presented as mean $\pm$ SD, $n = 5$, statistically significantly different (*) if $p < 0.05$.

5.3.4 Red blood cell treatment of peripheral blood mononuclear cells

The effect of pRBCs and upRBCs on the activity and expression of peripheral blood mononuclear cells (PBMCs) was investigated. PBMCs were treated with red blood cells, and the level of proliferation, apoptosis, cytokine secretion and expression of transcription factors in T cells was quantified.

5.3.4.1 T cell proliferation

Proliferation of CD3+, CD4+, and CD8+ T cells was monitored by tracking CFSE dilution across 5 days in culture. Multiple peaks in the CFSE histograms indicate new cellular generations, and a shift in the CFSE peak to the left indicates dilution of CFSE and thus proliferation. Treatment with PHA-P and upRBCs stimulated increased proliferation compared to PBMCs treated with PHA-P alone. This result was consistent for CD3+, CD4+, and CD8+ cells (Figure 5.7). Treatment with pRBCs stimulated the generation of a number of additional peaks to the left of the original peak in each of the gated populations. Specifically, when treated with pRBCs, CD8+ T cells underwent more cell division cycles than the CD4+ cells (Figure 5.7).
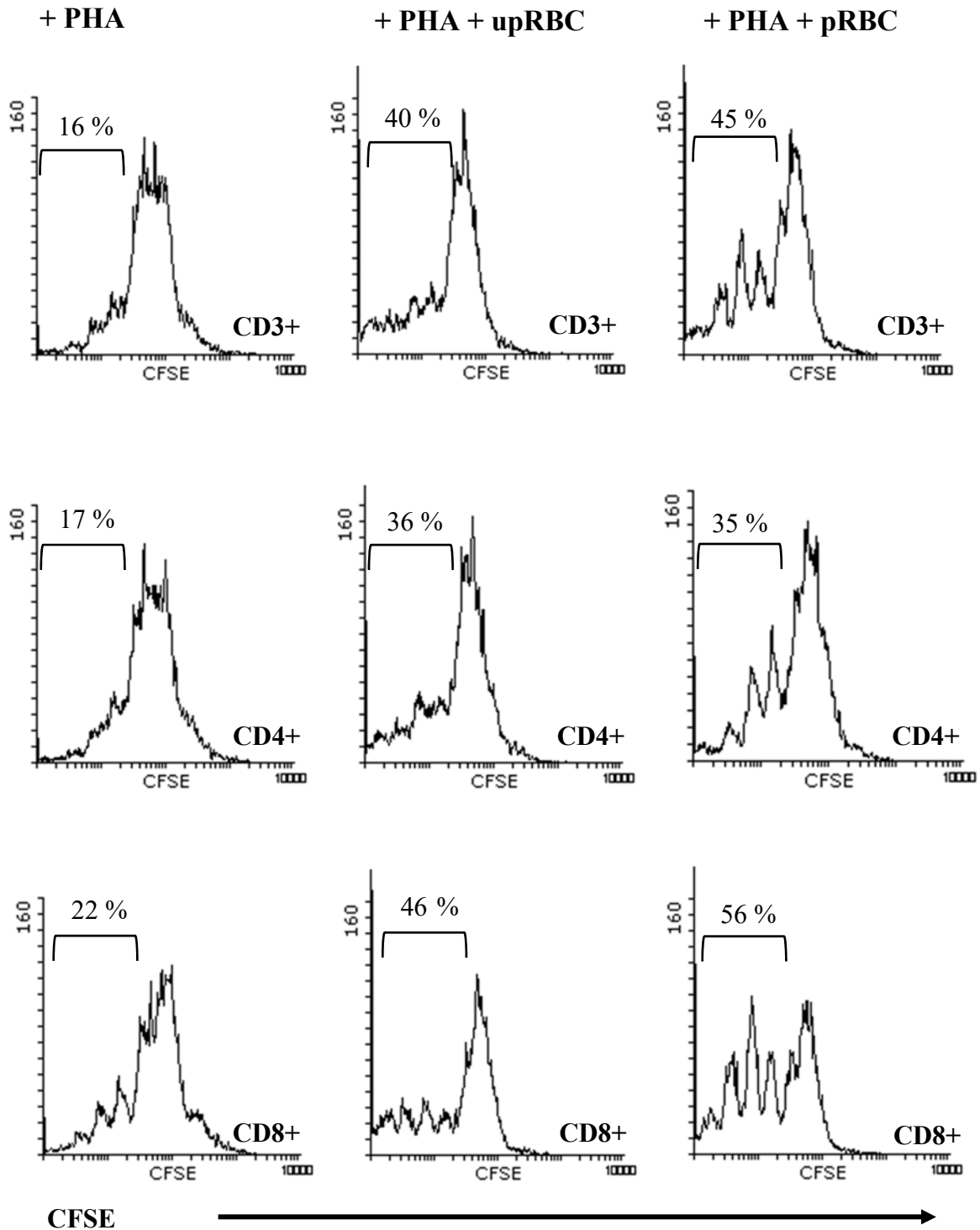
Figure 5.7. Histograms show CFSE fluorescence loss in PBMCs (peripheral blood mononuclear cells) treated with PHA-P (+ PHA), PHA-P and unprimed RBCs (+PHA + upRBC), or PHA-P and primed RBCs (+PHA + pRBC). The histograms outline proliferation for CD3+ cells, CD4+ cells, and CD8+ cells as measured by flow cytometry. A shift in the histogram peaks to the left indicates cell proliferation over 5 days at 37 °C and 5 % CO_2.

T cell proliferation was quantified by recording the percentage of cells with a particular level of CFSE staining (gating parameters outlined in Figure 5.7). Treatment with PHA-P and pRBCs resulted in a significant increase in proliferation for CD3+ cells, CD4+ and CD8+ cells when compared to PBMCs treated with PHA-P alone (Figure 5.8). Between pRBC and upRBC treatment, there was a significant increase in CD8+ cell proliferation with pRBC treatment and there was an upward trend in cellular proliferation for CD3+ cells. This was not observed for CD4+ cells (Figure 5.8).
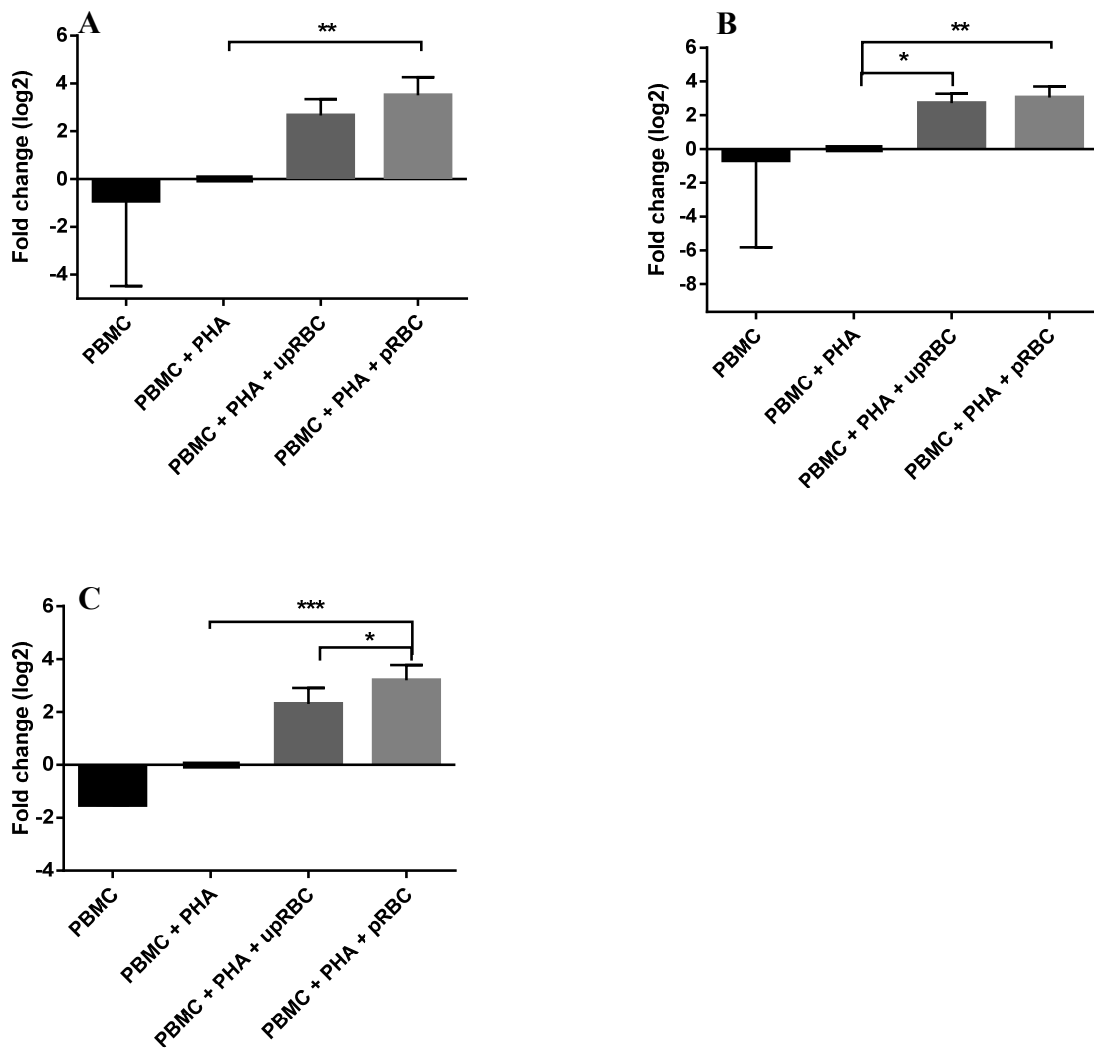
Figure 5.8. Graphical representation of proliferation of PBMCs (peripheral blood mononuclear cells) following treatment with nothing (PBMC), with PHA-P (PBMC + PHA), with PHA-P and unprimed red blood cells (PBMC + PHA + upRBC), or with PHA-P and primed red blood cells (PBMC + PHA + pRBC) after 5 days in culture at 37 °C with 5 % CO_2

(mean $\pm$ SD, $n = 6$). Figures represent proliferation of (A) CD3+ cells, (B) CD4+ cells, and (C) CD8+ cells from a PBMC population as fold change of proliferation index from loss of CFSE fluorescence (MFI). Data are statistically significantly different (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$.

5.3.4.2 *Proportion of CD4+ and CD8+ cells*

Monitoring the proportion of CD4+ cells and CD8+ cells in a CD3+ T cell population revealed that treatment only significantly affected the proportion of CD8+ cells, whilst the proportion of CD4+ cells remained largely unchanged. Inclusion of red blood cells and PHA-P in the PBMC culture (upRBCs or pRBCs) resulted in significantly more CD8+ cells than the untreated comparison (Figure 5.9).
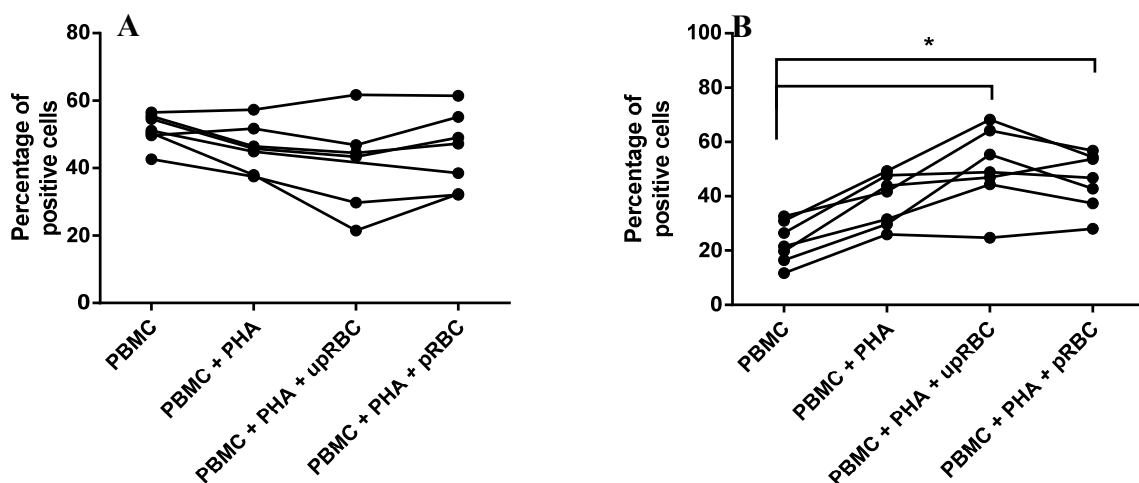
Figure 5.9. Percentage of (A) CD4+ cells or (B) CD8+ cells, in a CD3+ population of PBMCs (peripheral blood mononuclear cells) following treatment with nothing (PBMC), with PHA-P (PBMC + PHA), with PHA-P and unprimed red blood cells (PBMC + PHA + upRBC), or with PHA-P and primed red blood cells (PBMC + PHA + pRBC) for 5 days in culture at 37 °C and 5 % CO_2. Data are statistically significantly (*) if $p < 0.05$, $n = 7$.

5.3.4.3 *Apoptosis*

Apoptosis of PBMCs was measured by Annexin V/PI co-staining and flow cytometry analysis. It was demonstrated that red blood cells were protective against PHA-P induced apoptosis of PBMCs. This protection was evident for both CD4+ and CD8+ cells (Figure 5.10). The proportion of live cells (Annexin V-/PI-) was significantly lower in PBMCs treated with PHA-P when compared to untreated PBMCs (Figure 5.10). Both pRBCs and upRBCs appeared to have a protective capacity on PHA-P treated PBMCs, and the proportion of live cells in these groups was consistent with that of untreated PBMCs (Figure 5.10).
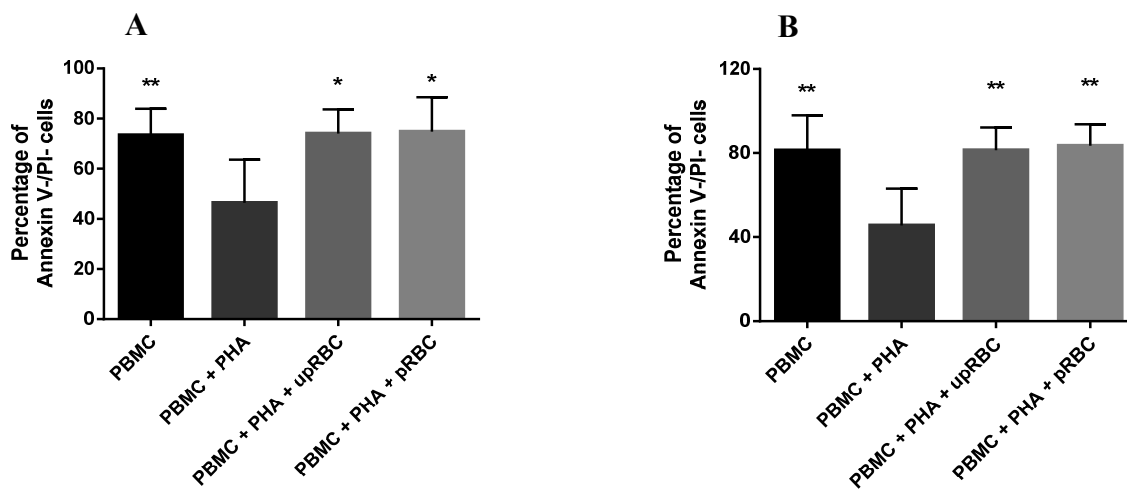
Figure 5.10. Percentage of Annexin V-/PI- cells in (A) CD4+ populations or (B) CD8+ populations from PBMCs (peripheral blood mononuclear cells) treated with nothing (PBMC), PHA-P (PBMC + PHA), PHA-P and unprimed red blood cells (PBMC + PHA + upRBC), and PHA-P and primed red blood cells (PBMC + PHA + pRBC) for 5 days at 37 °C and 5 % CO_2 (mean ± SD, $n = 5$). Data are statistically significantly different from PBMCs treated with PHA-P (*) if $p < 0.05$, (**) if $p < 0.01$.

The proportion of early and late apoptotic cells (Annexin V+/PI-, and Annexin V+/PI+) increased with PHA-P treatment for both CD4+ and CD8+ cells (Figure 5.11). The numbers of early and late apoptotic cells were reduced in the presence of red blood cells and were at similar levels to the unstimulated PBMCs.
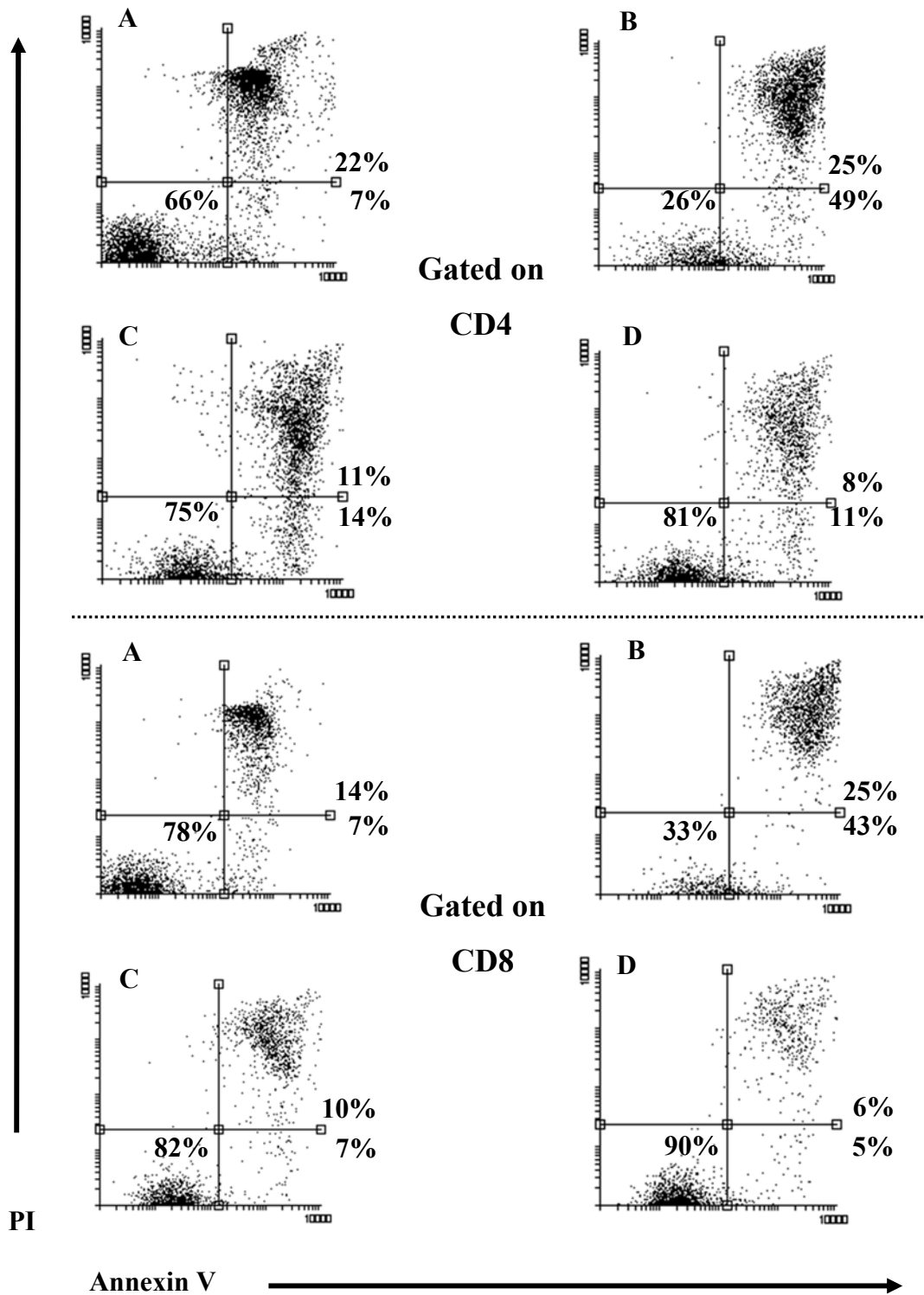
Figure 5.11. Dot plots of Annexin V and PI staining of CD4+ and CD8+ cells from PBMCs treated with (A) nothing, (B) PHA-P, (C) PHA-P and unprimed red blood cells (upRBCs), and (D) PHA-P and primed red blood cells (pRBCs) for 5 days at 37 °C with 5 % CO_2.

5.3.4.4   *Expression of transcription factors*

The expression of two transcription factors (GATA-3 and T-bet) in a CD8+ population were quantified using flow cytometry. PHA-P treatment of PBMCs stimulated a significantly higher expression of both transcription factors (Figure 5.12). Treatment with both PHA-P and upRBCs suppressed the expression of each transcription factor and there was no significant difference between those treated with the upRBC group and the untreated PBMCs (Figure 5.12). This suppression was not observed for the PBMCs treated with PHA-P and pRBCs. Instead, the level of expression of GATA-3 and T-bet were closer to the levels reported for the PHA-P treated samples (Figure 5.12). When compared to the PHA-P sample, the mean fold change in GATA-3 expression was 0.47 for the upRBC treatment and 0.84 for the pRBC treatment. Similarly, the mean fold change in T-bet expression was 0.37 for the upRBC treatment and 0.71 for the pRBC treatment. Of note, although there was significantly less expression of T-bet in the pRBC group, there was a clear increase in expression when compared to the upRBC group (Figure 5.12). The same trend was observed for GATA-3 expression.
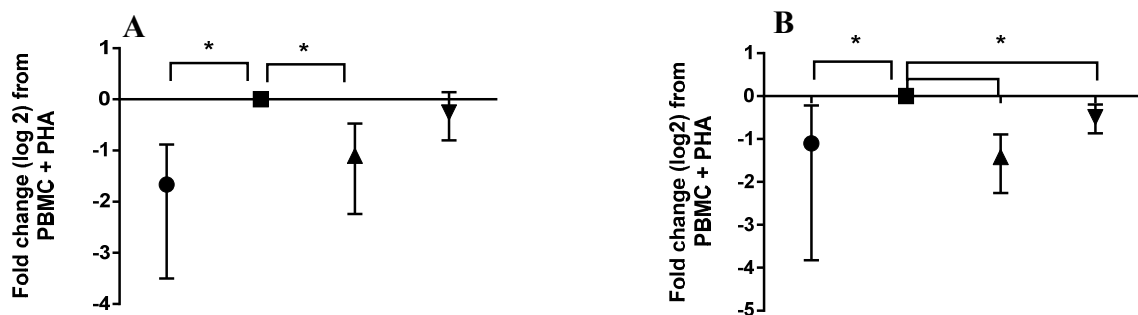
Figure 5.12. Expression of transcription factors in CD8+ population of PBMCs (peripheral blood mononuclear cells) treated with ● nothing, ■ PHA-P, ▲ PHA-P and unprimed red blood cells (upRBCs), or ▼ PHA-P and primed red blood cells (pRBCs), for 5 days at 37 °C and 5 % CO_2 (mean ± min/max, $n = 7$). Data are presented as fold change from PBMCs treated with PHA-P in expression of (A) GATA-3 and (B) T-bet. Data are statistically significantly different from ■ PBMCs treated with PHA-P (*) if $p < 0.05$.

5.3.4.5 Cytokine profile of PBMC conditioned media

The cytokines in the conditioned media of PBMCs from all cultures conditions were quantified using Bio-Plex assays. The fold change of a representative selection of cytokines is presented in Figure 5.13; the data for the remaining cytokines can be found in Appendix H. Treatment with PHA-P significantly stimulated increased secretion of 33 cytokines. Treatment with upRBCs resulted in five significantly altered cytokines when compared to the PHA-P treated control, whilst eight cytokines changed significantly in the pRBC group.

Treating PBMCs with pRBCs altered the secretion profile in comparison to treatment with upRBCs. Between the two conditions, significant differences were observed for three cytokines. Of those cytokines, one was IL-5, a pro-inflammatory cytokine, one was MCP-3, a chemokine, and one was IL-2, a cytokine with multiple functions (Appendix H). IL-5 was significantly higher ($p < 0.0001$) in the conditioned media of the pRBC group when compared the upRBC group (Figure 5.13). A number of cytokines followed this same trend but were not statistically significant including IL-4, IL13, IL-17, and G-CSF. Of the cytokines with a significant difference between red blood cell treatment and PHA-P stimulation alone, all were higher in the pRBC group (Figure 5.13).

The increase in concentration observed following treatment with red blood cells (upRBCs or pRBCs) could not be wholly attributed to red blood cell release or lysis, regardless of the red blood cell lysate concentration following priming. As an example, PBMCs treated with PHA-P alone released 153 ± 67 pg/mL of IL-17 into the conditioned media. This was significantly lower than the amount released from PBMCs treated with red blood cells (Figure 5.13). For example, upRBCs incubated alone under the same conditions released 7.5 ± 1.1 pg/mL of IL-17, and the PBMCs treated with the upRBCs released 645 ± 268 pg/mL of IL-17. Similarly, the pRBC equivalent released 7.0 ± 5.1 pg/mL of IL-17 when incubated alone, or 891 ± 282 pg/mL of IL-17 when PBMCs were treated with the primed red blood cells. The amount released by red blood cells alone does not explain the observed increase.
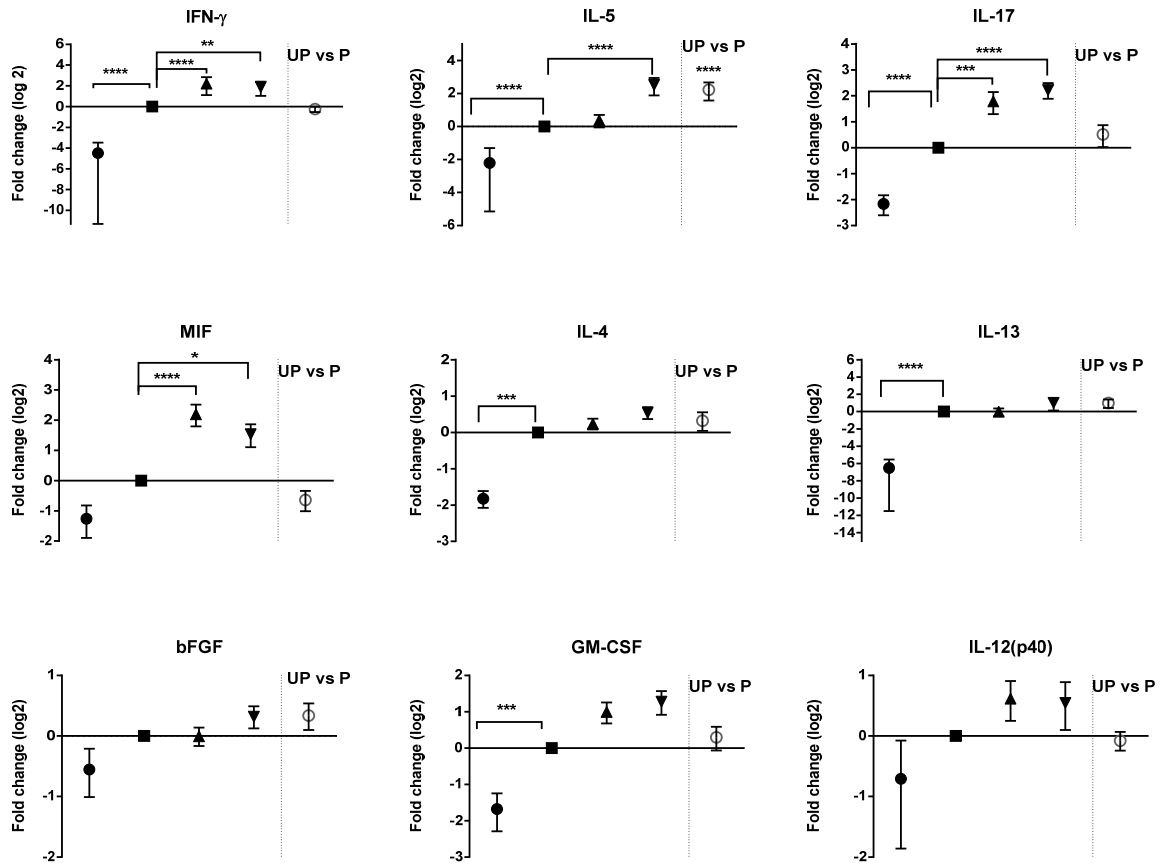
Figure 5.13. Summary of cytokines in PBMC (peripheral blood mononuclear cells) conditioned media as measured by Bio-Plex and reported as fluorescence ± 95 % CI ($n = 7$). Data are presented as mean fold change from ■ PBMC treated with PHA-P, to ● untreated PBMCs, ▲ for PBMCs treated with PHA-P and unprimed red blood cells (upRBC), and ▼ for PBMCs treated with PHA-P and primed red blood cells (pRBC). Fold change from PBMCs treated with PHA-P and upRBCs to PBMCs treated with PHA-P and pRBCs represented by ○ (open circle). Data are statistically significantly different from PBMCs treated with PHA-P (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$, (****) if $p < 0.0001$.

5.3.4.6 *Concentration of haemoglobin in red blood cell conditioned media*

Haemoglobin levels in the conditioned media from red blood cells and red blood cells incubated with PBMCs was quantified by monitoring the absorbance at 414 nm. The concentration of haemoglobin in the pRBC conditioned media samples was significantly lower than upRBC samples (Figure 5.14). This was true for both red blood cells incubated alone or red blood cells incubated in the presence of PBMCs (Figure 5.14). The concentration of measured haemoglobin corresponded to the lysis of 1,375,000 cells for the upRBC group and 153,000 cells for the pRBC group. This represents 9 % and 1 % of the starting inoculation for the upRBC and pRBC groups respectively.
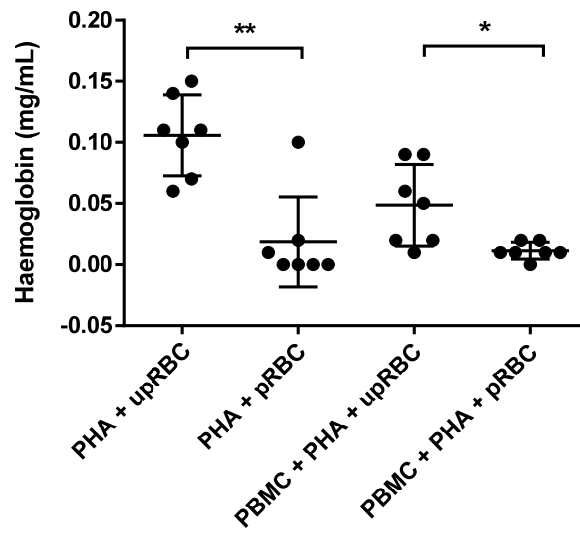
Figure 5.14. Concentration of haemoglobin in the conditioned media of unprimed (upRBC) or primed (pRBC) red blood cells (15 x 10^6 red blood cells per condition) incubated alone or with PBMCs (peripheral blood mononuclear cells) and PHA-P for 5 days at 37 °C with 5 % CO_2. Red blood cells were primed at 1:100 with A549 cells prior to incubation. Haemoglobin concentration was measured by absorbance at 414 nm. Data are presented as mean ± SD, $n = 7$, statistically significantly different (*) if $p < 0.05$, (**) if $p < 0.01$.

5.4   DISCUSSION

The previous chapters of this thesis identified that a range of inflammatory cytokines, growth factors, and chemokines are associated with red blood cells isolated from healthy individuals. These red blood cells were also observed to bind and release these cytokines, and this activity was modulated under certain conditions. The aim of this study was to investigate a cellular interaction mechanism of modulating this response. Specifically, the effect of interaction with a cancer cell line to modulate the binding and release of cytokines from red blood cells was investigated. The role of red blood cells in the activity of immune cells has been reported in the literature[4,9,12] and a small number of papers have outlined how red blood cells can become dysregulated in an inflammatory condition or following long term storage[16,18,19]. This dysregulation was illustrated through the altered effect of red blood cells on corresponding immune cell populations. In the light of these reports, this study was designed to model the communication between proliferating tumour cells and red blood cells *in vivo* and any resulting dysregulation in red blood cells. As observed in this chapter, priming red blood cells with a cancer cell line had an altered cytokine profile (Figure 5.2). These primed cells (pRBCs) also had an increased capacity to stimulate proliferation of a leukemic T cell line (Jurkat cells) and freshly isolated peripheral blood mononuclear cells (PBMCs) (Figure 5.4 and Figure 5.8) and this proliferation was biased towards the stimulation of CD8+ cytotoxic T cells (Figure 5.9). Unprimed red blood cells (upRBCs) attenuated the PHA-P mediated expression of transcription factors in the CD8+ population, however pRBCs did not produce the same attenuation (Figure 5.12). In addition, pRBCs stimulated the secretion of higher levels of IL-4, IL-5, and IL-13 from PBMCs (Figure 5.13). The results of this study support the hypothesis that red blood cells play an important role in immune cell regulation, and that this role can be dysregulated in response to interactions with biological stimuli.

5.4.1   **Red blood cell priming with A549 cells**

Angiogenesis and modification of the immune response are well-known factors in the progression of non-small cell lung carcinomas (NSCLC)[21,36]. With angiogenesis and vessel formation in tumours, red blood cells can contact and directly interact with cancer cells through vasculogenic mimicry[30]. It was hypothesised that this cell-to-cell contact would alter the cytokine profile of the corresponding red blood cells. To test this hypothesis, red blood cells

were incubated for three days with or without an adherent NSCLC line (A549 cells) and the resulting cytokine profile of the red blood cells was evaluated.

In the determination of the ratio of red blood cells to endothelial cells, the rate of red blood cell flow through capillaries was investigated. The literature determines that a 1 cm long capillary would have approximately 350 red blood cells moving at 0.9 mm/sec, therefore, approximately 2300 red blood cells would pass through this vessel in 1 minute[27]. This equates to just under 10 million red blood cells in 72 hours. If this number was used *in vitro* with 1.8 million A549 cells, the ratio would be approximately 1:6. It is difficult to obtain data on how many times a given red blood cell will pass over the same point over their 120-day lifespan. A key consideration in the experimental setup was to use sufficient red blood cells to enable the subsequent detection of cytokines. The final experimental design used two ratios, 1:100 and 1:1000, which ensured that there were sufficient red blood cells and assumed repeat transit of red blood cells between 20 and 2000 times.

5.4.1.1   *Cytokine profile*

This study identified that red blood cells incubated with a NSCLC cell line (A549 cells) were loaded with inflammatory cytokines as a result of the incubation. The levels of eight cytokines were significantly higher in these primed cells including IL-8, bFGF, and VEGF (Figure 5.2). These cytokines have been implicated as important in the progression of NSCLC. Zhao *et al.* reported that NSCLC cells express various cytokines that promote tumour growth and angiogenesis, including VEGF and bFGF[37] and both of these cytokines have been correlated with poor patient prognosis[38]. Intracellular bFGF promotes NSCLC tumour growth and stimulates secretion of VEGF[39]. The red blood cells primed with cancer cells in this study also contained significantly higher levels of three chemokines, IL-8, GRO-α and MCP-1 (Figure 5.2). Similarly, these have also been implicated in tumour growth and angiogenesis[40–42]. Although IL-10 was not significantly increased in this study, there was an upward trend in the lysate concentration of pRBC when compared to upRBC (Figure 5.2). IL-10 is discussed as a source of immunosuppressive activity from A549 cells[43] and is a T_H2 cytokine that supports an immunosuppressive environment and improves the metastatic potential of lung cancer cells[44]. These results demonstrate that following cell-to-cell contact, red blood cells could be primed with a group of pro-tumorigenic proteins. As reported in Chapter 4, red blood cells have a capacity to bind and release a wide variety of cytokines depending on availability. Shen *et al.* investigated the role of DARC on red blood cells on cytokine binding in a model of prostate cancer[45]. Similar to the study outlined in this chapter, they identified increased binding of IL-

8 and GRO-α in the presence of red blood cells, but unlike this study, the concentration of VEGF was unchanged. Although in each study the red blood cells were being primed by a secondary cell line, the final cytokine profile was slightly different. Following these results, it is not unreasonable to hypothesise that other cell lines with different secretion profiles would result in altered red blood cell cytokine profiles. However, further investigation is required to confirm this.

It is likely that the increase of cytokines observed in the red blood cell lysates was a result of decreased feedback inhibition. In the cancer cell culture where no red blood cells were present, the secretion of cytokines would be inhibited once the optimal extracellular concentration had been reached. However, with red blood cells present and continuously binding cytokines out the conditioned media, it is likely that the cancer cells would continue to produce and secrete cytokines to maintain optimal cytokine levels in the conditioned media, and thus the red blood cell levels would also continue to increase. In support of this, there were no significant differences in cytokine levels in the conditioned media of the A549 cells alone, or A549 cells incubated with red blood cells.

In situ, red blood cells are continuously moving throughout blood vessels and interact directly with a variety of cell types along the way. Blood is pumped through the whole body every minute, on average, and red blood cells remain in the circulation for 120 days[46]. The speed of blood flow varies inversely with the total cross-sectional area of the blood vessels, therefore as the cross-sectional area of the vessels increases, such as in capillaries, the velocity of flow decreases[47]. Blood flow is slowest in the capillaries, which enables time for exchange of gases and nutrients. With each cycle, the red blood cells are being repeatedly exposed to inflammatory signals whilst in motion. Although the results of this experiment were interesting and may indicate that red blood cells are primed *in vivo*, it cannot be said to represent a perfect model of biological interactions with red blood cells. In this set of experiments, the cancer cells were adherent to the bottom of the flask and the red blood cells were added in suspension to the media in the flask in static culture conditions. Over the three-day incubation period, the red blood cells settled to the bottom of the flask and did not move during this time. Agitation of the culture flasks to achieve a homogenous suspension of cells was not performed in this study as red blood cells are typically quite fragile following collection, thus there was a risk that this agitation over an extended period of time may result in excessive haemolysis. Haemolysis would raise two key issues, the first being that haemolysis would reduce the yield of red blood cells and would alter the ratio of red blood cells to the adherent cell line. Secondly, as indicated

by the previous chapters of this thesis, haemolysis would result in a release of a variety of cytokines into the conditioned media. This release of cytokines would likely have an effect on the release of cytokines from the adherent cell line (due to feedback inhibition) and on the resulting cytokine profile of the remaining red blood cells. The study outlined in this chapter was a preliminary experiment to identify if red blood cells could be primed by biological stimuli, however further studies in this area may benefit from investigation into shaking or movement during the process of red blood cell priming.

Oonishi *et al.* reported that prostaglandin E_1 and E_2 production was stimulated in red blood cells in a model of mechanical stress in microcirculation[47]. There is no literature at this point as to what effect the shearing forces of the red blood cells moving through blood vessels would have had on their capacity to bind and release cytokines, but it is likely that the incorporation of movement and pressure changes would influence the cytokine profile of the red blood cells. The aim of this study was not designed to perfectly assess the role of red blood cells in cancer. Instead, the cancer cell line was used as a model to test whether red blood cells could be changed and dysregulated following interactions with biological stimuli. Comprehensive analysis of the role of red blood cells in cancer progression would require analysis of clinical samples or animal models.

5.4.2 Effect of primed cells on T cell proliferation and survival

T cell proliferation is typically used as a model of immune cell activation. Upon presentation with an antigen or a stimulatory signal, T cells undergo clonal expansion and cell differentiation[48]. Apoptosis of these cells is a crucial process to maintain homeostasis following immune stimulation[49]. In this study, the effect of red blood cells on the proliferation and survival of T cells was evaluated. Most literature in this area has been focused on T cells, although a handful of papers have also looked at B cells and dendritic cells. The experiments in this chapter were performed on a leukemic T cell line (Jurkat cells) and on a mixed cell population of freshly isolated PBMCs. Analysis of this complex cell population was done to more closely model what may be occurring *in vivo*. However, in such models, it is difficult to elucidate the role and effect of the different cell types engaged in crosstalk. As such, where cytokines are the primary analytes, there would be value in the analysis of isolated lymphocyte populations in contact with red blood cells. Transcriptomics would be an alternative method, which may prove useful in the analysis of mixed populations such as PBMCs after stimulation

with red blood cells. Prior to RNA extraction the mixed population could be rapidly and gently separated using magnetic-activated cell sorting, which is available from a number of suppliers.

5.4.2.1 *Jurkat cell proliferation: treatment with intact red blood cells*

Jurkat cells are a leukemic T cell line that are frequently used to model T cell activity. Increased proliferation of these cells is used as an indication of stimulation with an immunogenic agent. This study demonstrated that upRBCs stimulated the proliferation of Jurkat cells by approximately 2-fold (Figure 5.4). Of the few publications investigating the effect of healthy red blood cells on T cell proliferation, one looked at the effect on Jurkat cells[9]. This study observed an approximately 5-fold increase in the proliferation of Jurkat cells in the presence of naïve red blood cells. The differences between the two studies here may be an artefact of red blood cell storage prior to analysis. In the study described in this chapter, the red blood cells were stored at 37 °C for three days before analysis, whereas in the published study, the red blood cells were stored at 4 °C for a non-specified period of time. Long term storage at 4 °C has been demonstrated to affect the capacity of red blood cells to stimulate proliferation of T cells[19,50], as such, short term storage at 4 °C or otherwise may have also had an effect on the level of T cell proliferation following treatment with those red blood cells.

Jurkat cells treated with red blood cells primed with cancer cells (pRBC) proliferated significantly more than the upRBC group (Figure 5.4). A549 cells were incubated with two different concentrations of red blood cells (low and high) to produce two groups of primed cells (pRBC-L, or pRBC-H respectively). Both groups of primed cells stimulated increased proliferation of the Jurkat cells, however, this effect was most pronounced in the pRBC-L group. This group stimulated 8.3-fold more proliferation than the untreated controls, whilst the pRBC-H groups stimulated only 5.3-fold more proliferation than the untreated controls (Figure 5.4). It is unclear as to why the pRBC-L group was more immunogenic than the pRBC-H group, but it may be a result of the concentration of available soluble factors secreted from the A549 cells being diluted across more red blood cells or simply an artefact of the static culture conditions. Fonseca *et al*. demonstrated that the activity of red blood cells was reliant upon them being intact and in direct contact with the immune cells[14]. Whilst the red blood cells were being primed by the cancer cells, they were left to settle to the bottom of the flask, and thus were in constant contact with the A549 cells. In the pRBC-H group, the layer of red blood cells was much more substantial than in the pRBC-L group and so the amount of direct contact between the cells may be have reduced. In turn, this may have reduced the immunogenic potential of these red blood cells. Following these data, the pRBC-L group was chosen as the

optimal group for stimulating T cell proliferation through cancer cell priming. All of the following T cell experiments were performed on the pRBC-L primed group only.

5.4.2.2 *Jurkat cell proliferation: treatment with red blood cell lysate, cytosols, or membranes*

Jurkat cells treated with red blood cell components including lysates, cytosols, or membranes did stimulate increased proliferation (Figure 5.4), with no significant difference observed between the upRBC and pRBC groups for the lysate and cytosol samples. However, this was not the case for the red blood cell membranes. The membranes isolated from pRBCs stimulated the Jurkat cells to proliferate significantly more than the membranes of the upRBCs with a mean fold change of 1.9 and 1.4 respectively (Figure 5.4). These results demonstrate that priming red blood cells with cancer cells resulted in a modification of the red blood cell membrane that made the membranes more immunogenic. This result is not surprising, as some inflammatory cytokines are known to bind to specific receptors on the red blood cell membrane[51,52] and upon lysis both sides of the membrane would be available for binding, which may have resulted in an increase in non-specific binding.

5.4.2.3 *Jurkat cell proliferation: treatment with red blood cell conditioned media*

The conditioned media from A549 cells alone stimulated the Jurkat cells to proliferate significantly more than the media blank control with a fold change of 3.5 compared to 1.2-fold for the media control (Figure 5.4). Of note, the conditioned media from the red blood cells and the A549 cells incubated together stimulated the Jurkat cells to proliferate significantly more (3.5-fold) than the conditioned media of either component alone (2.9-fold for A549 cells, 1.5-fold for red blood cells) (Figure 5.4). It has been previously demonstrated that the immunogenic activity of naïve red blood cells was mediated by soluble protein factors and that these factors were present in the non-vesicle fraction of the red blood cell conditioned media[9]. The increase in proliferation observed with the conditioned media in this study was a result of a change in one or more soluble factors and this change was dependent upon the inclusion of red blood cells in the culture. Together, the results of these experiments support the observation that a soluble factor may be mediating the stimulatory activity of red blood cells.

Antunes *et al.* reported that conditioned media from red blood cells alone stimulated the Jurkat cells to proliferate as much as the intact red blood cells[9], however, this result was not replicated in the study outlined in this chapter. No significant difference was observed between treatment with red blood cell conditioned media and the media control (Figure 5.4). There are a few

differences between the studies that may explain why no further proliferation was observed. In the study outlined by Antunes *et al*., they used 6x more red blood cells to produce the conditioned media and the red blood cells were incubated in a serum-free media. In contrast, in this study the red blood cell conditioned media was produced in a culture media that contained 10 % FBS. This high level of FBS and the low level of red blood cells is likely that reason that no difference was observed between the red blood cell conditioned media and the corresponding media blank.

5.4.2.4 *Jurkat cell cytokine release: treatment with intact red blood cells*

The level of cytokines in Jurkat conditioned media was increased following treatment with pRBCs compared to upRBCs, with 14 cytokines present at significantly higher levels in the pRBC group (Section 5.3.3.2). Unstimulated Jurkat cells secrete minimal inflammatory cytokines (Figure 5.5). Treatment with red blood cells was sufficient to stimulate the cells to secrete a number of cytokines. As observed with Jurkat cell proliferation, treatment with pRBCs produced a different profile than upRBCs. This demonstrates that the red blood cells were functionally altered as a result of priming. Whilst the results of the Jurkat cells are interesting, they are limited in their ability to model normal immune activity. Inactivated, these cells typically do not secrete many cytokines, and when they are activated, the signalling pathways become dysregulated[53]. For this reason, more biologically relevant analyses were performed using fresh peripheral blood mononuclear cells.

5.4.2.5 *PBMC proliferation: treatment with intact red blood cells*

In a mixed cell model, proliferation of T cells (CD3+) and subsets of T cells (CD4+ and CD8+ cells) were evaluated using CFSE staining. A small number of papers have investigated the effect of red blood cell treatment on the proliferation of cells in a PBMC population. In these papers, a consistent increase in proliferation of PBMCs following treatment with naïve red blood cells has been reported[9,10,12–15]. However, as there are so few papers reporting the proliferation of fresh T cells treated with red blood cells, there is no uniform set of methods that are used. As such, comparison of results between the papers was challenging.

In the study outlined in this chapter, cell proliferation was significantly increased with pRBC treatment for CD3+, CD4+, and CD8+ cells (Figure 5.8). Treatment with pRBCs was demonstrated to stimulate an increase in the proliferation of CD8+ cells but not CD4+ cells (Figure 5.8). In a CD3+ population, the number of CD4+ cells, unlike CD8+ cells, did not change with phytohemagglutinin (PHA-P) stimulation or with PHA-P stimulation and red

blood cell treatment (Figure 5.9). The number of CD8+ cells increased significantly with red blood cell treatment (Figure 5.9). This increase in the number of cytotoxic T cells (CD8+ cells) following red blood cell treatment has been reported previously in both PBMC cultures and purified CD3+ cultures[11,12]. It is not clear if the reason for this selective expansion of CD8+ cells *in vitro* is simply a result of a faster doubling time than CD4+ cells or if it was true preferential expansion. These results were obtained in an *in vitro* model, which comes with its own limitations. In this study, the proportion of red blood cells to lymphocytes was 1:10, which is well out of the range of the normal ratio (1:3000). However, this was not viable for an *in vitro* study. In addition, the experiments were performed over a restricted time frame (5 days), which may have limited the overall CD4+ cell proliferation. Patients receiving multiple red blood cell transfusions, a similar preferential expansion of CD8+ cells has been observed[54,55]. In one study, participants receiving multiple transfusions for sickle cell anaemia or haemophilia had significantly higher levels of CD8+ T cells than the non-transfused controls[54]. It has been suggested that this preferential CD8+ cell proliferation may be one of the reasons for the immunosuppression that is observed following red blood cell transfusions[8], however more work is required to investigate this hypothesis. In addition, the cause and effect of the increased proliferation of the CD8+ cells with primed red blood cell treatment of PBMCs observed in this study warrants further investigation.

5.4.2.6   *PBMC survival and apoptosis: treatment with intact red blood cells*

PHA-P is a stimulant for PBMC proliferation, however treatment with PHA-P also stimulates apoptosis in those same cells. In this study, the number of apoptotic and live cells was determined by assessing Annexin V staining of T cell subsets. The percentage of live cells dropped significantly from a mean of 73 % or 81 % for CD4+ and CD8+ cells respectively in the untreated group, to 46 % in the PHA-P stimulated group for both CD4+ and CD8+ cells (Figure 5.10). Treatment with red blood cells (either upRBCs or pRBCs) protected the CD4+ and CD8+ cells from PHA-P induced apoptosis (Figure 5.10). This protective activity of red blood cells on PBMCs has been well documented in the literature[9,12,15]. It has been shown that this promotion of cell survival in response to red blood cells is reliant on direct cell to cell contact and is specific to T cells and is independent of monocyte activity[14].

5.4.3 T cell activation

In order to further elucidate if there was any shift towards either a T_C1 (immuno-potentiating) or T_C2 (immunosuppressive) profile following upRBC or pRBC treatment, the expression of two transcription factors and the cytokine secretion prolife of PBMCs was assessed.

5.4.3.1 Expression of transcription factors

The expression of the transcription factors GATA-3 and T-bet are typically analysed in conjunction with cytokine expression to determine the differentiation of CD8+ T cells into a T_C1 or T_C2 subtype. Upregulation of T-bet is indicative of a T_C1 profile, and upregulation of GATA-3 is indicative of a T_C2 profile. This is the first report on the expression of GATA-3 and T-bet in a CD8+ T cell population following treatment with red blood cells. Contrary to expectation, these results did not illustrate differentiation of CD8+ cells into one subtype or another, but instead documented the overall activation of these cells. Stimulation with PHA-P alone promoted the expression of both GATA-3 and T-bet in CD8+ T cells in comparison to the unstimulated control (Figure 5.12). Treatment with upRBCs in conjunction with PHA-P supressed this expression of both transcription factors such that it resembled the expression of the unstimulated PBMCs. However, this suppression was not observed with pRBC treatment. These cells were not able to suppress this expression to the same extent as the upRBCs, and as such it resulted in a very similar profile to stimulation with PHA-P alone (Figure 5.12).

Upregulation of GATA-3 expression in CD8+ cells has been put forward as a potential biomarker for immune dysfunction[56,57]. T-bet expression in CD8+ cells drives differentiation into effector cells[58] and is associated with an immune response to viral infection[59]. The attenuation in the expression of these transcription factors following treatment with upRBCs suggests a role for red blood cells in regulating immune activation and differentiation. This role, however, was dysregulated following red blood cell priming with cancer cells. Buttari *et al*. demonstrated that red blood cells from carotid atherosclerosis patients were also dysregulated and were no longer able to promote maturation of dendritic cells[16]. The results of the study outlined in this chapter provide further evidence that that red blood cells can be functionally altered in their ability to interact with immune cells.

5.4.3.2 Cytokine release

Cytokine release from CD8+ T cells in combination with other markers can be indicative of a shift towards a specific differentiation subtype. Secretion of INF-γ or IL-12 is correlated to a T_C1 subtype, and secretion of IL-4, IL-5, and/or IL-13 is correlated to a T_C2 subtype of CD8+

T cells. In this study, the secretion of T_C1 cytokines was low and the levels did not change significantly between the upRBC and pRBC groups. T_C2 cytokines on the other hand, did change with treatment (Figure 5.13). The level of IL-4, IL-5, and IL-13 was released at higher concentrations from the PBMCs treated with pRBCs, than those treated with upRBCs (Figure 5.13). Of those three, only IL-5 was significantly different in the pRBC group compared to the upRBC group. However, there is a clear overall trend towards a T_C2 cytokine profile. Of the publications reporting the effect of red blood cells on T cells, most did not investigate cytokine release, but one study that did, reported a significant increase in the concentration of IFN-γ released from freshly isolated lymphocytes treated with red blood cells when compared to the PHA activated lymphocytes[15]. This result was replicated in this current study with an increase of IFN-γ following red blood cell treatment and was observed with both upRBC and pRBC with a mean fold change of approximately 4-fold (Figure 5.13).

The concentration of haemoglobin in the PBMC conditioned media from the pRBC treated group was significantly lower than the upRBC group (Figure 5.14). This indicates that by priming the red blood cells, they were more resistant to haemolysis. The Gardos channel on red blood cells is responsible for maintaining cell volume and is regulated by cytokine binding to the Duffy antigen receptor for chemokines (DARC)[60]. A number of the cytokines that bound in high concentrations to the red blood cells after priming are also responsible for regulating this channel (including IL-8, RANTES)[60]. High levels of these cytokines or high expression of DARC is correlated with increased dehydration in red blood cells[61]. This may be the mechanism behind the lower level of haemolysis observed in the primed red blood cell group. By binding high levels of the relevant cytokines, the Gardos channel would have been activated and the cells would have been dehydrated. Gardos channel activation in red blood cells has been shown to be protective against haemolysis[62]. In saying that, the focus of this study was on the relationship between cytokines and red blood cells, as such, only the 48 cytokines on the multi-plex panel was analysed. The cytokines on these kits are unlikely to cover all of the changes that may have occurred to the red blood cells as a result of priming. Although this hypothesis fits the observed results, it does not rule out interactions with other molecules or factors that were not investigated. In this study, the secretion profile from a mixed cell population (PBMCs) was evaluated. As such, the exact cellular source of these cytokines could not be determined. Future experiments are required to determine the source of the cytokines and thus the differentiation status of the relevant cells. This can be accomplished by more comprehensive flow cytometry analysis or analysis of gene expression.

5.4.4   Biological significance and therapeutic potential

The results of this chapter demonstrate that red blood cells can be manipulated to alter their cytokine profile and their influence on T cells. Similar dysregulation of red blood cell activity has been demonstrated in disease (carotid atherosclerosis)[15], following surgery[18], or following haemodialysis[63]. Further investigation around what causes this dysregulation *in vivo* and what role it may play in disease may be valuable in disease research. The experiments in this chapter were not designed to perfectly model the role of red blood cells in NCSLC. However, the results of these experiments do suggest that if there is interaction between the red blood cells and the cancer cells there may be some downstream immunological effects *in vivo*. In order to validate this, red blood cells isolated from NSCLC clinical samples will need to be collected and analysed to elucidate if their interaction with immune cells are altered in comparison to healthy controls. NSCLC currently has a poor prognosis and a low median survival time[64]. Thus, further understanding about the biology of the condition may be valuable in identifying therapeutic targets or developing earlier diagnostics. This study also identified that the cytokine profile of red blood cells changed significantly with exposure to NSCLC cells. Analysis of the cytokine profile of red blood cells isolated from patients may by useful for the identification of disease specific biomarkers.

Since red blood cells are considered to be inert, they have been advocated as optimal biological carriers of antigens or drugs for cancer immunotherapy[65,66]. Loading red blood cells with tumour specific antigens has been demonstrated to stimulate an extended T cell response in mice[65]. The results of this chapter demonstrated that, without the addition of antigens or drugs, red blood cells can be modified to become more immunogenic or to selectively activate populations of immune cells. These results were achieved by priming red blood cells with a cancer cell line, however priming red blood cells with other cell types such as mesenchymal stem cells may create different immunogenic profiles. As such, primed red blood cells could be valuable in the future as for personalised immunotherapies.

5.5 CONCLUSION

The results of this chapter can be summarised as follows:

- Red blood cells were 'primed' following co-incubation with a NSCLC cell line, which was illustrated by a change in the red blood cell cytokine profile.

- 'Unprimed' red blood cells played a role in stimulating the proliferation of leukemic and freshly isolated T cells.

- Red blood cells primed with a NSCLC cell line resulted in enhanced proliferation of leukemic T cells and fresh CD8+ T cells, decreased protection against PHA driven activation of T cells, and altered the secretion profile of peripheral blood mononuclear cells.

Red blood cells isolated from healthy individuals are able to modulate immune cells by promoting T cell proliferation, protecting these same cells from apoptosis, and suppressing stimulant-driven activation of CD8+ T cells. These results support the hypothesis that red blood cells play a key role in modulating the immune system *in vivo*. In addition to that, this study highlights that red blood cells and their interactions with the immune system are malleable. A dysregulation in red blood cell activity has been suggested in disease, and has now been observed through intentional alteration of the cells and their cytokine profile by exposing them to a NSCLC cell line. In the presence of these altered red blood cells, T cells were stimulated to proliferate to a greater degree, were no longer protected from stimulant-driven activation, and were driven to release a variety of cancer-related cytokines. This study identifies the potential for red blood cell involvement in disease or in their use as disease biomarkers. A further step would be the investigation of primed red blood cells as potential vehicles for immunotherapies.

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CHAPTER

6

DISCUSSION AND CONCLUSIONS

This chapter discusses the results of this thesis in the wider context of red blood cell biology, diagnostics, and the potential for cytokine priming to enable new red blood cell based therapeutics. This chapter also outlines the conclusions and future work required to further explore these areas.

6.1   Discussion

The overall hypothesis of this thesis was that through interaction with cytokines, red blood cells play a role in inflammation and act as a buffer for inflammatory mediators. In support of this hypothesis, the results of this thesis demonstrated that red blood cells are a major reservoir in whole blood for at least 31 cytokines, chemokines, and growth factors (Figure 6.1). This included the identification of enzymatically active macrophage migration inhibitory factor (MIF) in red blood cell lysates at concentrations 1000-fold higher than the typical plasma level. In addition, red blood cells were found to bind both recombinant cytokines and cytokines released from cancer cells, and the resulting primed red blood cells were able to modulate the activity of T cells (Figure 6.1). These results support the primary hypothesis and demonstrate a need for further investigation into this activity. At this point in 2016, literature in this field of research is starkly lacking, but the implications are likely to be crucial in understanding disease processes such as the occurrence and progression of anaemia, or in optimising red blood cell therapeutics such as red blood cell storage and transfusion. Without further, more in depth research, it is difficult to make any definitive conclusions about what these results will mean in disease. But, in light of this thesis, what *is* clear is that the discussion around red blood cells is in dire need of change. The therapeutic use of blood transfusion has been routine for one hundred years and the literature is replete with examples of adverse events, inflammatory responses, and elevated plasma cytokines. Despite the wealth of literature documenting altered plasma cytokine levels after transfusion, this thesis appears to be the first study to report the quantification of cytokines in red blood cells.
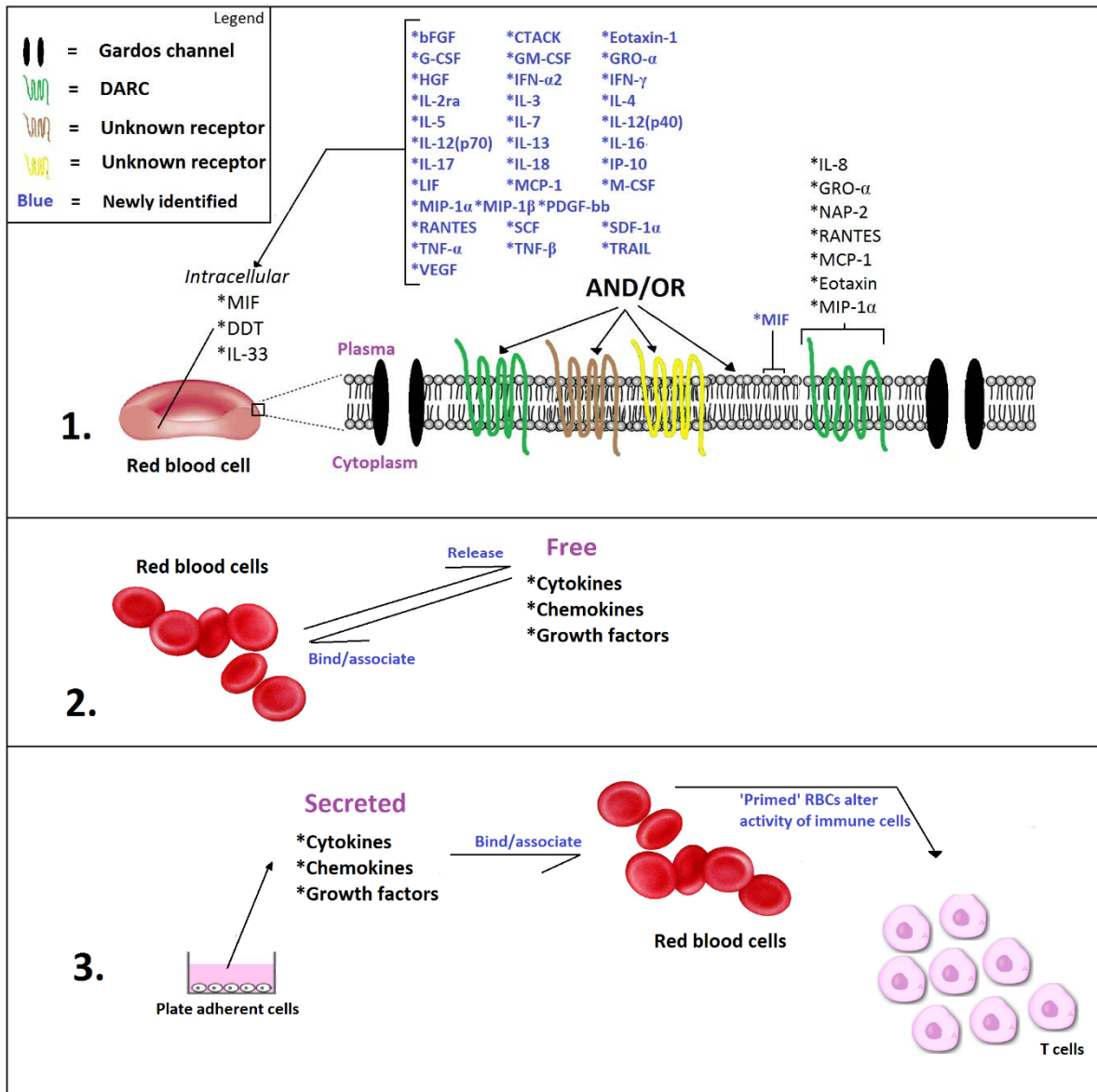
Legend

‖	=	**Gardos channel**
〰	=	**DARC**
〰	=	**Unknown receptor**
〰	=	**Unknown receptor**
Blue	=	**Newly identified**

*bFGF   *CTACK   *Eotaxin-1
*G-CSF   *GM-CSF   *GRO-α
*HGF   *IFN-α2   *IFN-γ
*IL-2ra   *IL-3   *IL-4
*IL-5   *IL-7   *IL-12(p40)
*IL-12(p70)   *IL-13   *IL-16
*IL-17   *IL-18   *IP-10
*LIF   *MCP-1   *M-CSF
*MIP-1α *MIP-1β *PDGF-bb
*RANTES   *SCF   *SDF-1α
*TNF-α   *TNF-β   *TRAIL
*VEGF

*IL-8
*GRO-α
*NAP-2
*RANTES
*MCP-1
*Eotaxin
*MIP-1α

AND/OR

*MIF

Intracellular
*MIF
*DDT
*IL-33

Plasma

1. Red blood cell

Cytoplasm

2. Red blood cells — **Release** → **Free** *Cytokines *Chemokines *Growth factors ← **Bind/associate**

3. **Secreted** *Cytokines *Chemokines *Growth factors — **Bind/associate** → Red blood cells — 'Primed' RBCs alter activity of immune cells → T cells

Plate adherent cells

Figure 6.1. Schematic of the interaction between cytokines and red blood cells as identified in this thesis.

6.1.1   Red blood cell processing

There is substantial variation in the methods used to isolate and process red blood cells. The majority of reports isolate these cells by centrifugation and removal of the plasma and the buffy coat[1,2], or by centrifugation of whole blood over a density gradient such as Ficoll-Paque or Lymphoprep[3,4]. Although these methods are rapid and straightforward, they do not remove granulocytes which make up approximately 50 % of the total white blood cells in blood nor are

platelets completely removed from these cell pellets[5,6]. Contamination of red blood cell populations with white blood cells and platelets however, is rarely discussed as a limitation in red blood cell research as depletion of these populations is infrequently quantified and reported, if at all. In addition to this, there is considerable variability in the reported length of time between blood collection and sample processing. As an example, red blood cells can be classified as 'fresh' even up to 14 days of storage[7], whilst other studies classify 'fresh' as cells processed immediately following collection[8]. These variations in processing times can have significant effects on the integrity of the sample[9]. The chosen method in this study, dextran sedimentation, is primarily used in the literature to isolate neutrophils from red blood cells as most other isolation methods do not sufficiently separate these two cellular components[5]. This method was initially chosen as it depletes most white blood cells (with greater than 80 % depletion) and most platelets (with greater than 95 % depletion) from the red blood cell pellet, and that it is also a very simple method that can be used to isolate cells from large volumes of blood with minimal manipulation[10–12]. It now appears that this method may have been more beneficial than initially recognised. Conventional red blood cell isolation methods such as centrifugation typically include extensive washing of the cells in an isotonic buffer[13,14] and the justification for this is to ensure the removal of plasma proteins and to improve the purity of the red blood cells[15]. In proteomic analyses of red blood cells, this processing can be particularly rigorous. For example, in a report on the analysis of the proteome of red blood cell membranes, the isolated cells were centrifuged, filtered, washed four times in an isotonic buffer, and finally the cells were lysed and the resulting membranes were washed another four or five times[14]. This processing method may be optimal for the identification of transmembrane or structural proteins, but in the identification of proteins bound to the surface of the cell membrane this is unlikely to be effective.

Amongst all of the proteomic analyses of red blood cells, no studies have yet been able to identify the presence of cytokines as reported in this thesis. There are likely to be a number of reasons for this such as issues with the dynamic range of the proteins; the abundance of these cytokines is approximately 100 million-fold lower than the abundance of haemoglobin in red blood cells. However, this issue is not likely helped by the vigorous washing procedures typically done during sample preparation. As identified in the literature, at least some of the cytokines associated with red blood cells are bound to receptors on the surface of the cells[16,17]. It is likely that excessive processing of the cells would result in the removal of some, if not all, of the bound cytokines, consequently further reducing their relative abundance. This is

observed in platelets, wherein excessive washing or vigorous processing techniques have been shown to promote their degranulation and subsequently an increase in the secreted detectable cytokine levels[18]. Although the platelet literature is clear on the effects of processing, the only other enucleate cells in humans, red blood cells, are not described as signalling cells and are regarded as transcriptionally and translationally inert and incapable of secretory activity. In the proteomics literature on red blood cells, they are treated as bags of protein that can be washed and purified without altering the proteome. The focus of red blood cell proteomics studies appears to have been on the technical challenge of identifying proteins against the overwhelming background of haemoglobin rather than elucidating a biological role of red blood cells beyond gas exchange. In contrast to the typical proteomic methods, dextran sedimentation is a very gentle process and does not require extensive washing. In this procedure, the red blood cells were left to slowly sediment over 30 minutes to 1 hour in the presence of high molecular weight dextran after which the purified red blood cells were collected and were washed once in phosphate buffered saline. Thus, the integrity of the binding partners on the surface of the red blood cells (such as cytokines) are likely to be protected more than other techniques would enable. In future studies, optimised blood fractionation methods, such as dextran sedimentation, will be crucial to achieve consistent and reliable analysis of cytokines in red blood cells.

6.1.2 Transfusion of red blood cells

Washing red blood cells appears to have some therapeutic benefit in transfusion medicine. The Australian Red Cross Service offers washed red blood cells for specific indications including patients with allergic reactions or in response to severe reactions with unwashed red blood cell packs. In fact, washing red blood cells following storage appears to be correlated with reduced incidence of adverse events and in some cases, improved outcomes such as in mice with haemorrhagic shock[19]. One of the benefits of this procedure is the depletion of inflammatory proteins from the blood pack[19]. This depletion is achieved by both removal of the storage media which contains inflammatory proteins[19], and also likely by the removal of loosely bound cytokines from the surface of red blood cells. Cholette *et al.* reported that children undergoing cardiac surgery had reduced post-operative inflammation with transfusion of washed red blood cells compared to transfusion of unwashed cells[20]. This effect was represented by resulting lower serum levels of inflammatory cytokines and a change in trend towards reduced mortality[20]. However, there appears to be a delicate balance required in the amount of washing

needed to achieve this therapeutic benefit. Excessive washing increases red blood cell osmotic fragility and can lead to increased haemolysis following transfusion which can cause downstream adverse effects[21–23]. What is not well understood, in light of this thesis, is the mechanism behind these results. The reduction in resulting serum levels of inflammatory cytokines observed with washed red blood cell transfusion may be a result of depleting the blood pack of these same cytokines and so attenuating any inflammatory response to the transfusion. But it may also be that the process of washing the red blood cells actually deplete the cells of cytokines. This may then enable them to bind more cytokines upon transfusion, thus resulting in an overall reduction in the levels of circulating inflammatory molecules. Analysis of the cytokine profile and the activity of red blood cells is required to determine the mechanisms behind the effect of storing, washing, and transfusing red blood cells.

Overall, the literature on red blood cell transfusions, the storage lesion, and optimising storage parameters has very little agreement[24]. One reason for this may be that the entire area of red blood cell interactions with cytokines has been underappreciated until this point, particularly in the field of transfusion medicine. Whilst there is some literature that has evaluated the cytokine load of the red blood cell storage medium over time[25–27], there is no literature that thoroughly investigates the cytokine load of red blood cells in these conditions. The results of this study demonstrate that red blood cells are capable of both binding and releasing at least 31 different cytokines, chemokines, and growth factors in high concentrations (Chapter 4). Thus, even if no significant increase in cytokine concentration in the storage media is observed, the red blood cells may still be involved in a continual process of binding and releasing cytokines to maintain a particular extracellular cytokine load, the process of which may result in potentially detrimental cellular alterations. This activity was observed during the priming of red blood cells with the lung cancer cell line in Chapter 5 of this thesis. Although there were no significant differences in the concentration of cytokines in the conditioned media from the lung cancer cells with or without red blood cells, the red blood cells themselves bound high quantities of bFGF, IL-8, and VEGF amongst others (Figure 5.2). Thus, if the cytokine analysis was isolated to the conditioned media alone, the conclusions would indicate that there was no significant effect of red blood cells. When in fact, the cytokine profile of these cells was significantly altered and their effect on T cell activity was also significantly modulated. Any cytokine changes that are likely occurring in the red blood cells during storage may be responsible, at least in part, for the progression of this 'storage lesion'. By ignoring the red blood cells in the analysis of cytokines in the storage lesion, the issues presented by stored red

blood cells may never be totally resolved. The study in this thesis revealed significant changes in the red blood cell cytokine profile within 72 hours, but red blood cells are currently being stored for weeks before transfusion. An ideal study of stored red blood cells would, at the very least, quantify cytokines in red blood cells over a time course to establish whether significant changes occur and when.

6.1.3 Red blood cells in disease

The results of this thesis have significant implications in the field of diagnostics. This study identified that red blood cells are a major reservoir of a range of cytokines, chemokines, and growth factors. For the 31 cytokines with a higher red blood cell concentration than plasma, the range was 3.6 to 3970-fold higher in red blood cells than plasma with a median fold increase of 11.3 (Table 4.3). With the quantification of these inflammatory molecules in serum or plasma becoming progressively more common in clinical research[28–30], the implications of their identification in red blood cells is two-fold. The first is that accurate and reproducible quantification of these cytokines can be complicated by red blood cells during processing. As indicated in Chapter 4, even undetectable levels of haemolysis (0.125 %) can be sufficient to release interfering concentrations of analytes such as IL-16 into the plasma or serum. In addition, Schnabel *et al.* reported that the coagulation that occurs in the production of serum can stimulate the release of the chemokine MCP-1 from red blood cells[31]. Thus, the presence of these cytokines needs to be considered when developing optimised methods for the isolation of plasma and serum. The second implication of this discovery is in the field of biomarker identification. As demonstrated in this thesis, the cytokine profile of red blood cells can change in response to their environment through binding and releasing of these molecules. As such, red blood cells have been described as a buffer for inflammatory molecules in blood. If this is true *in vivo*, then these cells are likely to have a different cytokine profile in patients with chronic inflammatory conditions. As identified by Profumo *et al.* and Yu *et al.*, the red blood cells isolated from patients with atherosclerosis[32] or following hip arthroplasty[33] were dysfunctional in how they modulated T cell activity. Similar dysfunction was observed in the study outlined in Chapter 5, where the dysfunction was also coupled with a significantly altered red blood cell cytokine profile. The hypothesis is that these two factors are linked, and that altered cytokine profiles would also be observed in patients who have chronic inflammatory conditions. If this hypothesis is correct, then the analysis of red blood cells may be useful in diagnostics and in monitoring these conditions.

Anaemia of chronic inflammation is a well-known condition that occurs as a complication in many chronic inflammatory diseases including auto-immune diseases and infection[34,35]. The pathogenesis of this condition is poorly understood, and thus far there are no adequate treatments for it[36]. At this point, the recommended therapy is the management of the underlying inflammatory condition[36]. The reduced number of red blood cells observed in this anaemic condition may well contribute to a dysregulated red blood cell buffering system, and as such, the chronic inflammation may be closely related to the eventuation or progression of the anaemia. Chronic inflammation can be damaging to the normal function of cells. A recent study identified that chronic exposure of the pro-inflammatory cytokine, IL-1, on haematopoietic stem cells severely restricted the cellular capacity to self-renew, but the self-renewal was restored upon cessation of IL-1 exposure[37]. In Chapter 4 of this thesis, IL-1α and IL-1β were both demonstrated to be bound by red blood cells. Thus, red blood cells may be a mechanism for managing this over-exposure of IL-1 molecules *in vivo*, and the dysregulation of which could have detrimental down-stream effects.

An example of attenuation of this cytokine binding has been observed with the knockout of one specific receptor on red blood cells, the Duffy antigen receptor for chemokines (DARC). Interference with this chemokine sink system has been shown to be involved in the pathogenesis of some inflammatory conditions including prostate cancer[38] and graft rejection[39]. Shen *et al.* demonstrated that mice lacking DARC had increased prostate cancer tumour growth and tumour vessel density when compared to DARC positive mice[38]. Notably the concentration of pro-angiogenic chemokines - that are known ligands for DARC - were significantly higher in the tumour of DARC negative mice[38]. With these results, the authors concluded that the chemokine binding activity of red blood cells was directly related to tumour management by clearing the tumour of pro-angiogenic chemokines and thus restricting angiogenesis. The binding and release of a much wider population of cytokines, chemokines, and growth factors were identified as part of this thesis, as such, interference with the red blood cell binding activity is likely to have even more widespread effects.

6.1.4   Red blood cells: the new immunotherapy?

Immunotherapies are designed to stimulate the host immune system to attack a tumour or disease. The use of these therapies in cancer treatment have been under investigation for many years, and more recently, red blood cells have been advocated as an optimal vehicle for the

delivery of these drugs or antigens[40,41]. These cells have been proposed due to the relatively extensive understanding about their biocompatibility, the mechanisms of cell clearance following transfusion, and the observed extended therapeutic effects[40]. Banz *et al*. reported that red blood cell ghosts, loaded with a T-cell specific antigen, substantially prevented tumour growth and they demonstrated that the engulfment of antigens in red blood cells promoted an extended and more efficacious response than treatment with the free antigen[40].

In the investigation into priming red blood cells with a lung cancer cell line in Chapter 5, it was demonstrated that these primed red blood cells were altered in such a way that they stimulated increased T cell proliferation compared to naïve red blood cells. Whilst the results of this thesis established that the red blood cells primed with a lung cancer cell line (A549 cells) may be more immunosuppressive than immunogenic, the potential for using this priming platform in immunotherapy development is still valid. If these red blood cells can be induced to promote an immunosuppressive T cell response, then reason would suggest that, with appropriate priming, the opposite would also be possible wherein an immunogenic T cell response is promoted. In order to determine the benefits or limitations of using primed red blood cells as a therapeutic, substantial further research is still required. In support of this continued investigation, these primed red blood cells likely retain the same beneficial features of red blood cells ghosts loaded with antigens. For example, like ghosts, these cells are likely to be biocompatible, will be cleared regularly from the system, and are capable of binding and releasing the relevant proteins. The difference, however, lies in that these red blood cells are still biologically functional, wherein the intact primed red blood cells stimulated a stronger response in the T cells than the membranes alone (Figure 5.4) and have undergone less manipulation during sample processing. The cancer cells used in the priming experiments in Chapter 5 were grown in serum containing media. Further studies on red blood cell priming with cancer cells grown under altered conditions would be beneficial to determine whether conditions such as serum starvation, hypoxia, hyperoxia, or pressure produce immunogenic red blood cells.

There are numerous reports on the use of cytokines as antigens in immunotherapies for cancer. These studies have highlighted the use of cytokines such as IL-2, IL-15, and IL-21 in stimulating T cell responses with promising results in cancer models and in clinical trials[42,43]. The focus of the research in this thesis was on primarily on the relationship between red blood cells and a group of 48 commonly studied cytokines, including key players in inflammation, immune cell recruitment, and regeneration. As such, the cytokine profile of these primed red

blood cells was deduced and conclusions regarding T cell activity was drawn solely on the analysis of these cytokines. However, the results of the interaction between red blood cells and the cancer cell line was likely more complex than an exchange of cytokines. The level of oxidative stress in the red blood cells may have changed and the transfer of cancer specific antigens to the red blood cells may have also occurred. Thus, it is reasonable to hypothesise that additional factors could have been partly responsible for the observed changes in T cell proliferation and activation. This transfer of immunogenic material, whether they be cytokines or antigens, is key to the development of an immunotherapy. Priming red blood cells with other cell types may produce a different cytokine profile and immunogenic response to what was observed in this study which may be prove to be more or less beneficial as a therapeutic.

The therapeutic benefit of primed red blood cells is unlikely to be isolated to stimulating the immune response in cancer. These cells may also be beneficial in extending the therapeutic benefit of anti-inflammatory cells such as mesenchymal stem cells (MSCs). In recent years, much of the therapeutic benefit of MSCs has been attributed to their secretome[44,45]. These cells have been shown to secrete an array of anti-inflammatory molecules[46,47] and have been demonstrated to be efficacious a range of conditions[44,45,48]. However, the issue with treating an inflammatory condition with a proteinaceous solution such as conditioned media is the potentially low half-life upon treatment[40,45]. Priming red blood cells with mesenchymal stem cells or with their conditioned media, may produce a similar, but slow-release, therapeutic benefit compared to treating with the conditioned media alone. Investigation into what changes, if any, are observed in red blood cells primed with other cell types or with cell-free solutions (such as conditioned media) is likely to provide some insight into the use of these cells as therapies designed to modulate the immune response.

6.2 CONCLUSIONS AND FUTURE DIRECTIONS

The results and investigations outlined in this thesis are novel and highlight a burgeoning area of interest. The quantification of 48 cytokines, chemokines, and growth factors in red blood cells using multi-plex cytokine technology has never before been reported. These results illustrate that red blood cells are more complex than generally accepted, and that there is value from a research and clinical perspective in their analysis. It also demonstrated that they are an important, signalling component of whole blood and are involved in the function of the immune system as summarised in Figure 6.1.

The investigation into the interactions between red blood cells and cytokines is very much in its infancy, and as a result, there are many pathways that future investigations could take. The majority of these investigations could be categorised under three primary topics.

1. Improved understanding of the molecular interactions between red blood cells and cytokines

2. Investigation into the profile and function of these cells in disease cohorts for use in diagnostics

3. Development and optimisation of red blood cell based therapeutics

The results of this thesis have outlined the starting ground for investigation into these areas of red blood cell research and as such have led to more questions than originally anticipated. The aim of the study was to investigate and quantify the concentration of a large group of cytokines with a focus on the modulation of these proteins and the implications of this modulation on immune cells. With this focus, elucidating the molecular processes behind the binding and release of these cytokines from red blood cells was not within the scope of this study. Thus, it would be of interest in future studies to more closely assess these molecular interactions. This could include the identification of where these cytokines localise after binding (intracellularly or on the cell membrane), and, if they bind to the cell membrane, then investigation into whether this involves binding to a specific receptor or if it is non-specific binding to membrane components. DARC is known to function as a promiscuous receptor for multiple chemokines such as IL-8 and RANTES. These chemokines have different specific receptors on other cell types, which lend weight to the hypothesis that red blood cells function as an independent sink for cytokines. Based on this hypothesis, the other cytokines identified in red blood cells in this

thesis probably bind via different receptors to those on non-red blood cells. As identified in this study, preliminary investigations demonstrated a role of proteases in the binding and release of these cytokines from red blood cells by using a cocktail of protease inhibitors to modulate this activity. Investigation into the activity of individual enzyme inhibitors could be useful in delineating which enzymes play a part in cytokine binding and release and may provide further understanding to this process. This thesis covered the analysis of 48 cytokines, chemokines, and growth factors, which are the most commonly analysed inflammatory proteins in blood. However, outside of these groups there are many more cytokines that may also be associated with red blood cells. In addition, the process of binding and release of cytokines from red blood cells is likely to involve other known proteins acting in newly described roles as chaperones. Future research into red blood cell signalling will benefit from investigation into which other proteins are present and may play a role in the inflammatory process. To achieve this, non-specific proteomics methods such as mass spectrometry will be required. This approach will need to be coupled with gentle red blood cell isolation methods to avoid washing away the target molecules. Collection of blood from a much larger cohort of healthy individuals would enable the investigation into whether there are normal ranges for these cytokines in red blood cells and to determine any trends correlated with specific demographics such as age, gender, race, or blood type. In addition to this, expanding the analysis of blood in disease groups from plasma or serum to the inclusion of red blood cells, may provide further understanding to the disease processes and inflammatory state of the patient. Value in this analysis is likely to come from time course sampling with quantification of red blood cell cytokine levels and also from investigation into the interactions between these cells and other cells types such as T cells or dendritic cells as has already been demonstrated in atherosclerosis[32]. This investigation into red blood cells in disease cohorts may also be valuable in the area of biomarker discovery. In order to further understand the effect and impact of the storage lesion of stored red blood cells, investigation into the cytokine profile of these cells at different times during storage may prove to be important. Finally, the investigations of this thesis identified that red blood cells could be primed with a cell line to alter its cytokine profile and its interaction with CD8+ T cells. These results could lead to the development of red blood cell-based therapeutics, however substantial research in this area is still required. This could include evaluating the effect of priming red blood cells with other cell types to see if a different T cell, or other immune cell response could be promoted. Identification of promising *in vitro* results would also need to be supported by investigation into the safety and efficacy of these primed cells in animal models of inflammation.

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CHAPTER

7

APPENDICES

This chapter includes information on human ethics approval for the experiments of this thesis and supplementary data for Chapter 3, Chapter 4, and Chapter 5.

APPENDIX

A

A.1 ETHICS APPROVAL

A.1.1 Macquarie University

Ethics committee: Macquarie University Human Ethics Review Committee (Human Research)

Ethics approval number: 5201100827

Date of ethics approval: 01 May 2014 - 30 May 2016

A.1.2 Northern Sydney Local Health District

Ethics committee: Northern Sydney Coast Human Research Ethics Committee of Northern Sydney Local Health District and Central Coast Local Health District

Ethics approval number: 1201-046M

Date of ethics approval: 02 December 2016 - present

APPENDIX

B

B.1   METHODS

B.1.1   Blood collection

This study was approved by the Macquarie University Human Research Ethics Committee (5201100827) and by the Northern Sydney Coast Human Research Ethics Committee of NSLHD and CCLHD (1201-046M). Written informed consent was obtained from all participants before enrolment.

Whole blood was collected from healthy volunteers by venepuncture ($n \geq 4$). Blood was combined with EDTA (ethylenediaminetetraacetic acid) for anticoagulation (K_2EDTA vacutainers, Becton Dickinson). Blood and blood components were isolated and aliquoted at room temperature within four hours of collection. All samples were subjected to three freeze-thaw cycles at -80 °C to ensure complete cellular lysis before protein analysis. Samples were stored at -80 °C until analysis.

B.1.2   Red blood cell isolation

Red blood cells were isolated and frozen immediately for analysis. Concentration and purity of red blood cells were determined using a haematology analyser (Coulter AcT Diff, Beckman Coulter) or by flow cytometry (FACSAria III, Becton Dickinson).

B.1.2.1   *Dextran sedimentation*

Whole blood was added to dextran solution (6 % w/v dextran in 0.15 M sodium chloride) at a 2:1 ratio (blood:dextran). This solution was gently mixed and left upright for 60 minutes at 23 °C for red blood cell sedimentation. After sedimentation, two layers formed and were isolated and transferred to individual tubes. The lower, red blood cell dense layer, was washed twice in phosphate buffered saline (PBS, 500 g, 5 minutes) and the remaining red blood cell pellet was isolated. Relative purity of cells was determined using a haematology analyser (Coulter AcT Diff, Beckman Coulter) and the cells were then frozen at -80 °C.

B.1.2.2 Fluorescence activated cell sorting (FACS)

Whole blood was diluted ten times in a wash buffer (PBS + 2 % FBS (fetal bovine serum)). A 50 µL aliquot of the cell suspension was then washed twice in FACS wash buffer by centrifugation (1000 g, 5 minutes). The resulting cell pellet was resuspended in 50 µL FACS wash buffer with 5 µL of CD45-FITC (Table B.1). This solution was incubated in the dark at room temperature for 15 minutes. After incubation, the cells were washed twice in the wash buffer (1000 g, 5 minutes). Cells were resuspended up to 500 µL the wash buffer for sorting. Red blood cells were sorted using a FACSAria III flow cytometer with four lasers and cell separation by negative staining. Red blood cells and white blood cells were gated from platelets according to size using forward scatter and side scatter. Red blood cells were then gated from white blood cells according to lack of CD45 fluorescence. This final gated population (red blood cells) was then sorted into an individual collection tube. Purity of the population was determined by analysing the collected population for CD45 fluorescence on the flow cytometer. Cells were then pelleted and frozen at -80 °C.

Table B.1.   Monoclonal antibody used for FACS sorting of red blood cells.

Antibody	Fluorochrome	Clone
Mouse anti-human CD45	FITC	HI30

B.1.3   Enzyme-linked Immunosorbent Assay (ELISA)

Concentrations of MIF were measured using a MIF ELISA (R&D Systems, USA), which was run according to manufacturer's instructions. The absorbance data for the MIF ELISA were collected with a Synergy 2 plate reader (BioTek) at 450 nm with absorbance correction at 570 nm. The calibration curve was analysed using a log/log curve fit using GraphPad Prism software (ver. 6, USA).

B.1.4   Statistical analysis

Data were evaluated using two-tailed, paired t-tests to assess statistical significance between groups with significance levels of $p < 0.05$. Statistical analysis and graphing of results was performed using GraphPad Prism software (ver. 6, USA). Concentration values are presented as mean ± standard deviation (SD).

B.2   RESULTS

B.2.1   Red blood cell purity

Purity of the red blood cell populations was determined by counting the levels of remaining white blood cells using a haematology analyser for dextran sorted cells or using flow cytometry for the FACS sorted cells. The purity of the red blood cell populations was slightly higher in the red blood cells sorted with FACS (Table B.2).

Table B.2.   Purity of red blood cell populations according to isolation technique.

Isolation technique	RBC purity (%)*	WBC depletion (%)*
Dextran	99.97 ± 0.01	79.0 ± 5.1
FACS	≥ 99.99	≥ 90

*Data are presented as mean $\pm$ standard deviation ($n \geq 4$)

B.2.2   MIF concentration according to isolation technique

The average concentration of MIF in red blood cells was significantly lower in the cells that were sorted by FACS (Figure B.1) compared to when the cells were separated by dextran sedimentation. Dextran sedimentation yielded a total concentration that was approximately 5 times higher than that observed in FACS sorted cells. Although there is a substantial discrepancy in concentrations, that appears to be dependent on the isolation method, these levels are still much higher than what is seen in other blood components including plasma, white blood cells, and platelets (Chapter 3, Table 3.3).
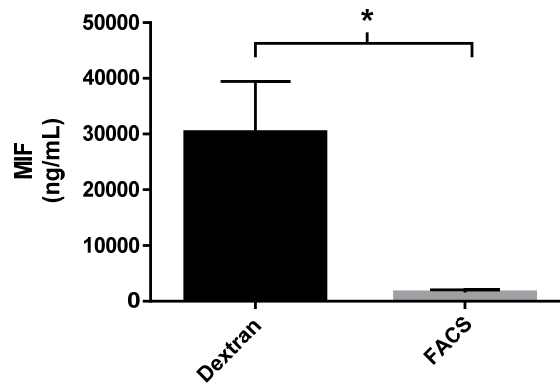
Figure B.1. Concentration of MIF in red blood cells purified using dextran sedimentation and fluorescence activated cell sorting (FACS). Concentration is normalised to their relative contribution to one millilitre of whole blood. Data are presented as mean $\pm$ SD, $n \geq 4$, statistically significant (*) if $p < 0.05$.

B.3 DISCUSSION

These results demonstrate that the amount of MIF that is measured in red blood cell populations is dependent on the method of cell recovery. Although each method tested here resulted in similar purities (Table B.2), the MIF levels differed extensively (Figure B.1). Differences in white blood cell contamination of the fractions would account for less than 1 % of the observed concentration (Chapter 3, Table 3.3), indicating that the impurities were not the contributing factor. Likewise, the time from collection to sample storage was less than 3 hours for both methods which would enable the same duration of time for cell-to-cell interactions to occur, indicating that this is also not a factor. Prior experimentation (data not shown here) demonstrated that the presence of dextran does not alter to the level of MIF measured.

The chief difference between the two populations is how extensively they were processed. The dextran sedimentation method is relatively gentle and involves minimal washing steps. With the FACS method, the cells undergo a number of washing steps and are sent at high speed through the flow cytometer for cell sorting. It is the hypothesis that this extensive washing and manipulation of the red blood cells in the FACS method depleted the cells of MIF.

Cell activation is a common complication in blood processing, and care needs to be taken to avoid activation of white blood cells and platelets[1]. The results of this study suggest that this

may also be the case for red blood cells. For the purposes of continued red blood cell analysis in this thesis, dextran sedimentation was chosen as the preferred cell isolation method. This is because the method was reproducible, large volumes of blood could be processed at once, and it was relatively gentle on the cells.

Aside from highlighting the complications in isolating and analysing pure red blood cell populations, the results of this study may be used to further understand the mechanics of red blood cells. As outlined in Chapter 3, red blood cells may be able to bind extracellular MIF (Figure 3.4). If the cells were depleted of MIF (for example by FACS or extensive washing) their secretion and binding activities may be altered. If the cells that were collected from healthy individuals were in fact already saturated with MIF, it follows that MIF depletion from red blood cells may enable the cells to bind more of this protein. If this is the case, then red blood cells could be used as a therapeutic to remove excess circulating levels of MIF in diseases that are associated with high levels of the protein such as sepsis[2].

B.4   REFERENCES

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APPENDIX

C

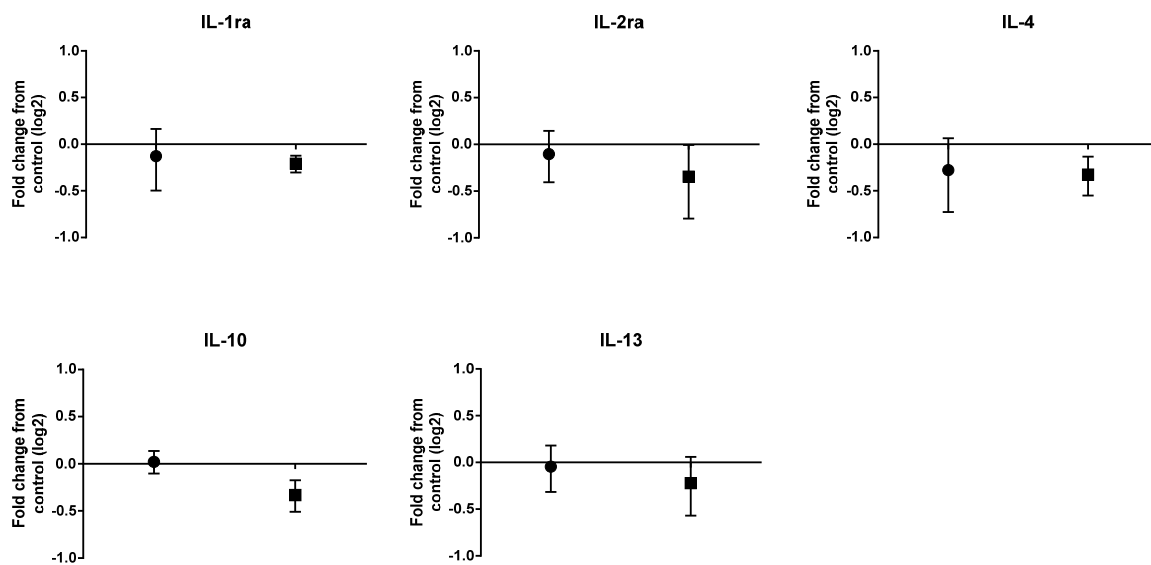--

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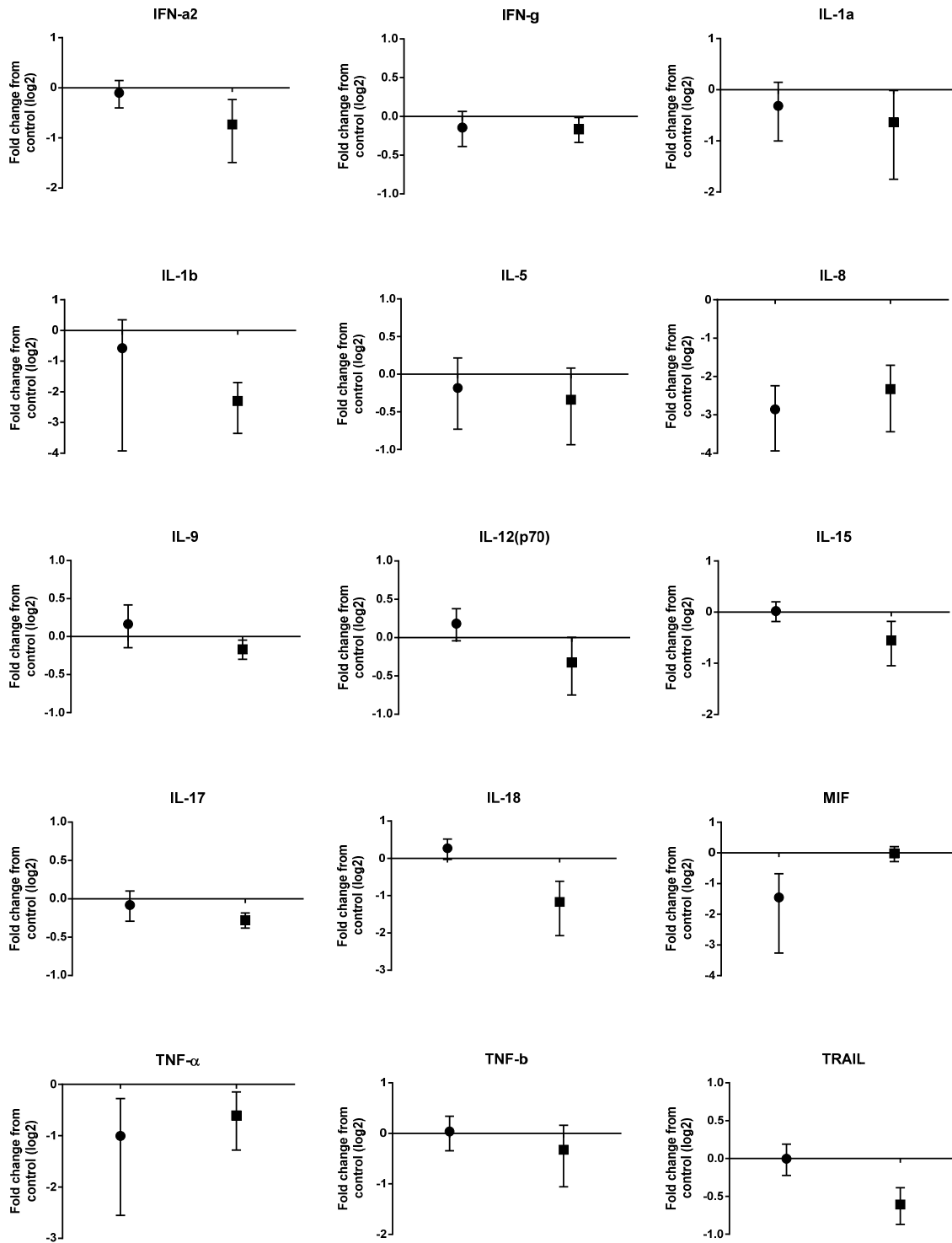
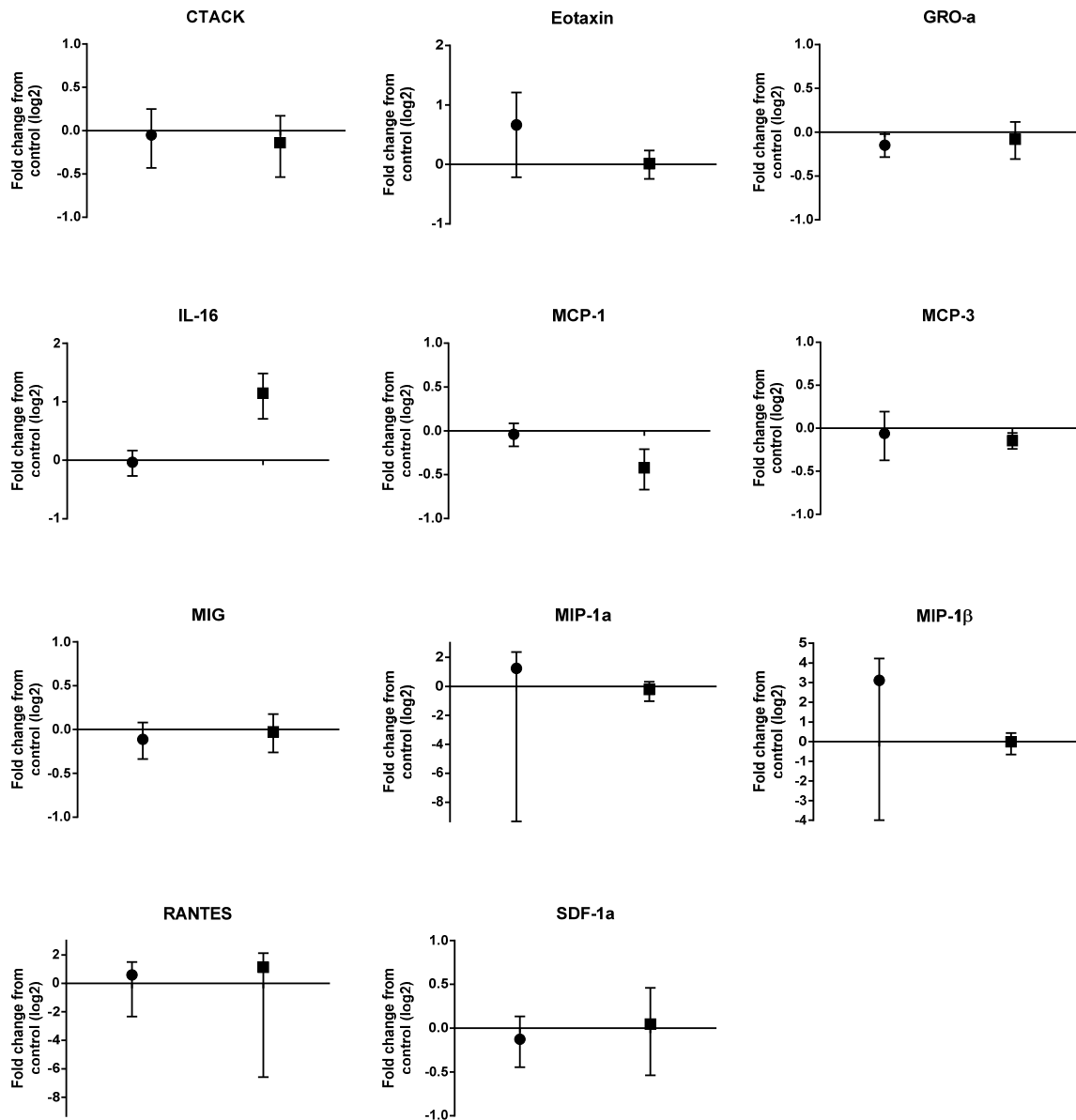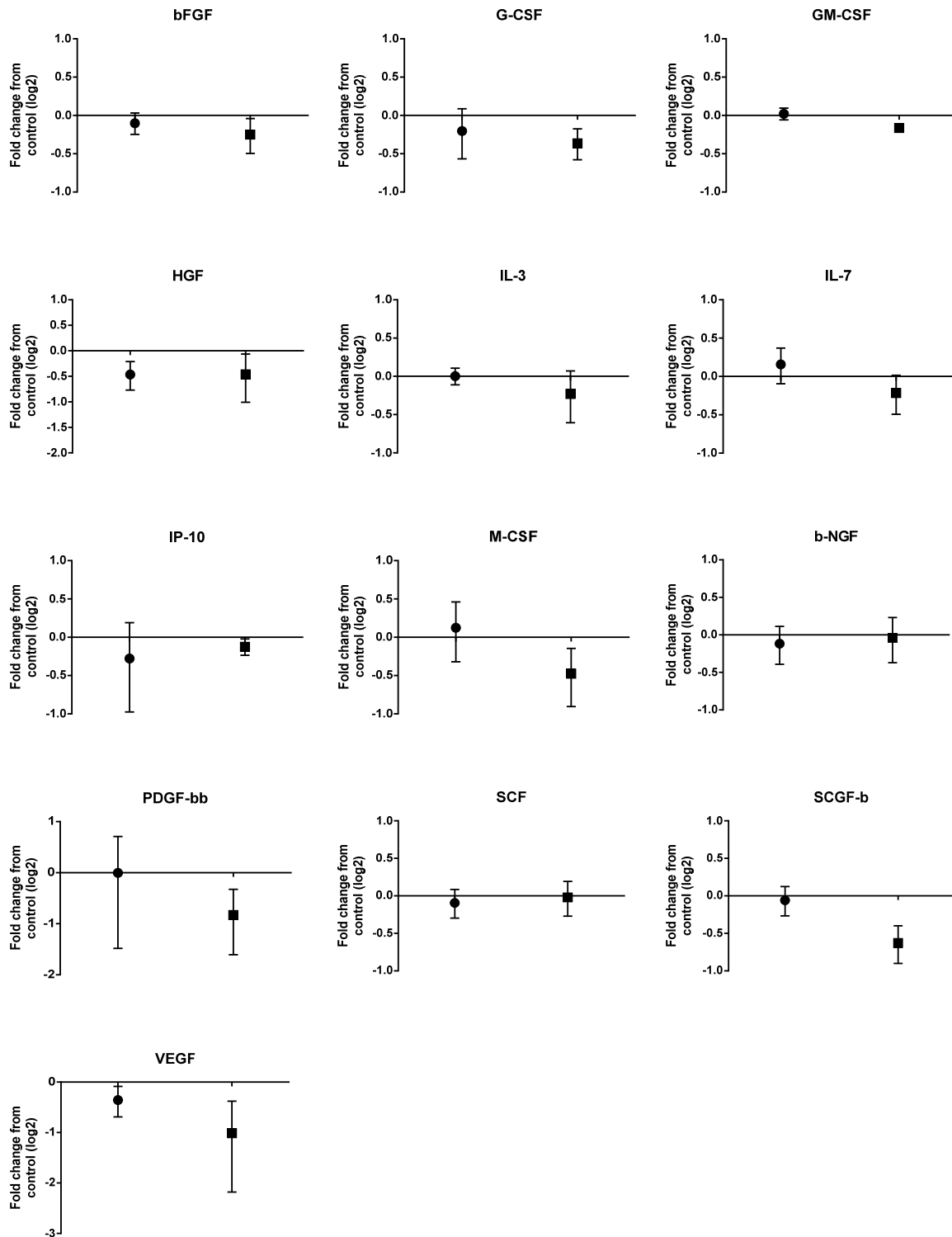**Figure C.1.** Summary of **pro-inflammatory cytokines** released from red blood cells into PBS over 24 hours and in the corresponding red blood cell lysate at 37 °C with or without protease inhibitors (PI) as measured by Bio-Plex and reported as fluorescence (100 million cells in 1 mL PBS). Data are presented as mean fold change after addition of PI ± 95% CI, where ● represents fold change in conditioned media of PI treatment compared to the no PI control, and ■ represents fold change in fluorescence in lysate of red blood cells treated with PI compared to the no PI control ($n = 6$).
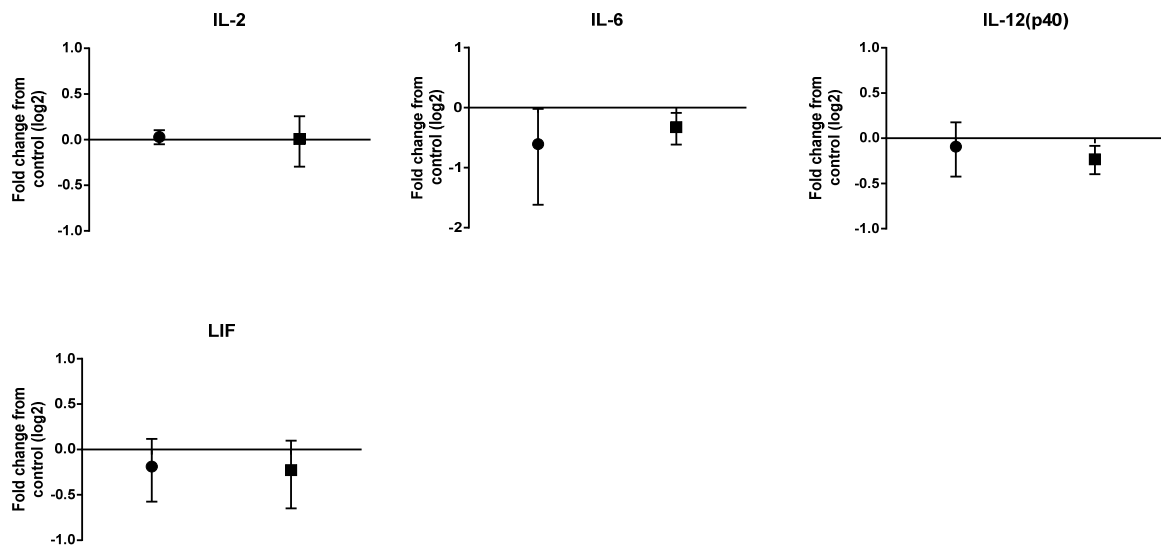

Figure C.2. Summary of **anti-inflammatory cytokines** released from red blood cells into PBS over 24 hours and in the corresponding red blood cell lysate at 37 °C with or without protease inhibitors (PI) as measured by Bio-Plex and reported as fluorescence (100 million cells in 1 mL PBS). Data are presented as mean fold change after addition of PI ± 95% CI, where ● represents fold change in conditioned media of PI treatment compared to the no PI control, and ■ represents fold change in fluorescence in lysate of red blood cells treated with PI compared to the no PI control ($n = 6$).
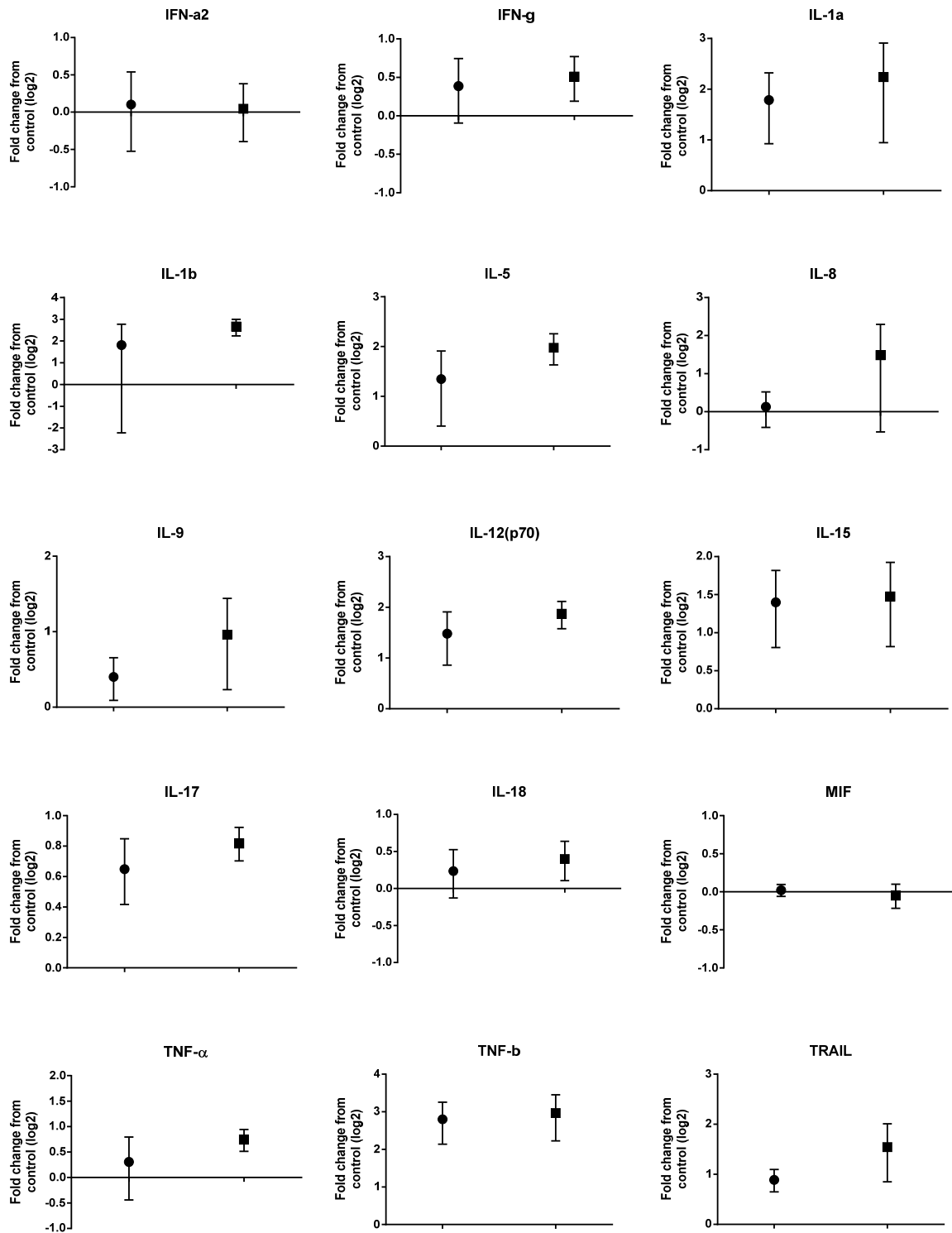
Figure C.3. Summary of **chemokines** released from red blood cells into PBS over 24 hours and in the corresponding red blood cell lysate at 37 °C with or without protease inhibitors (PI) as measured by Bio-Plex and reported as fluorescence (100 million cells in 1 mL PBS). Data are presented as mean fold change after addition of PI ± 95% CI, where ● represents fold change in conditioned media of PI treatment compared to the no PI control, and ■ represents fold change in fluorescence in lysate of red blood cells treated with PI compared to the no PI control (*n* = 6).

Figure C.4. Summary of **growth factors** released from red blood cells into PBS over 24 hours and in the corresponding red blood cell lysate at 37 °C with or without protease inhibitors (PI) as measured by Bio-Plex and reported as fluorescence (100 million cells in 1 mL PBS). Data are presented as mean fold change after addition of PI ± 95% CI, where ● represents fold

change in conditioned media of PI treatment compared to the no PI control, and ■ represents fold change in fluorescence in lysate of red blood cells treated with PI compared to the no PI control ($n = 6$).

Figure C.5. Summary of **cytokines with multiple functions** released from red blood cells into PBS over 24 hours and in the corresponding red blood cell lysate at 37 °C with or without protease inhibitors (PI) as measured by Bio-Plex and reported as fluorescence (100 million cells in 1 mL PBS). Data are presented as mean fold change after addition of PI ± 95% CI, where ● represents fold change in conditioned media of PI treatment compared to the no PI control, and ■ represents fold change in fluorescence in lysate of red blood cells treated with PI compared to the no PI control ($n = 6$).

APPENDIX

D

IFN-a2   **IFN-g**   **IL-1a**

IL-1b   **IL-5**   **IL-8**

IL-9   **IL-12(p70)**   **IL-15**

IL-17   **IL-18**   **MIF**

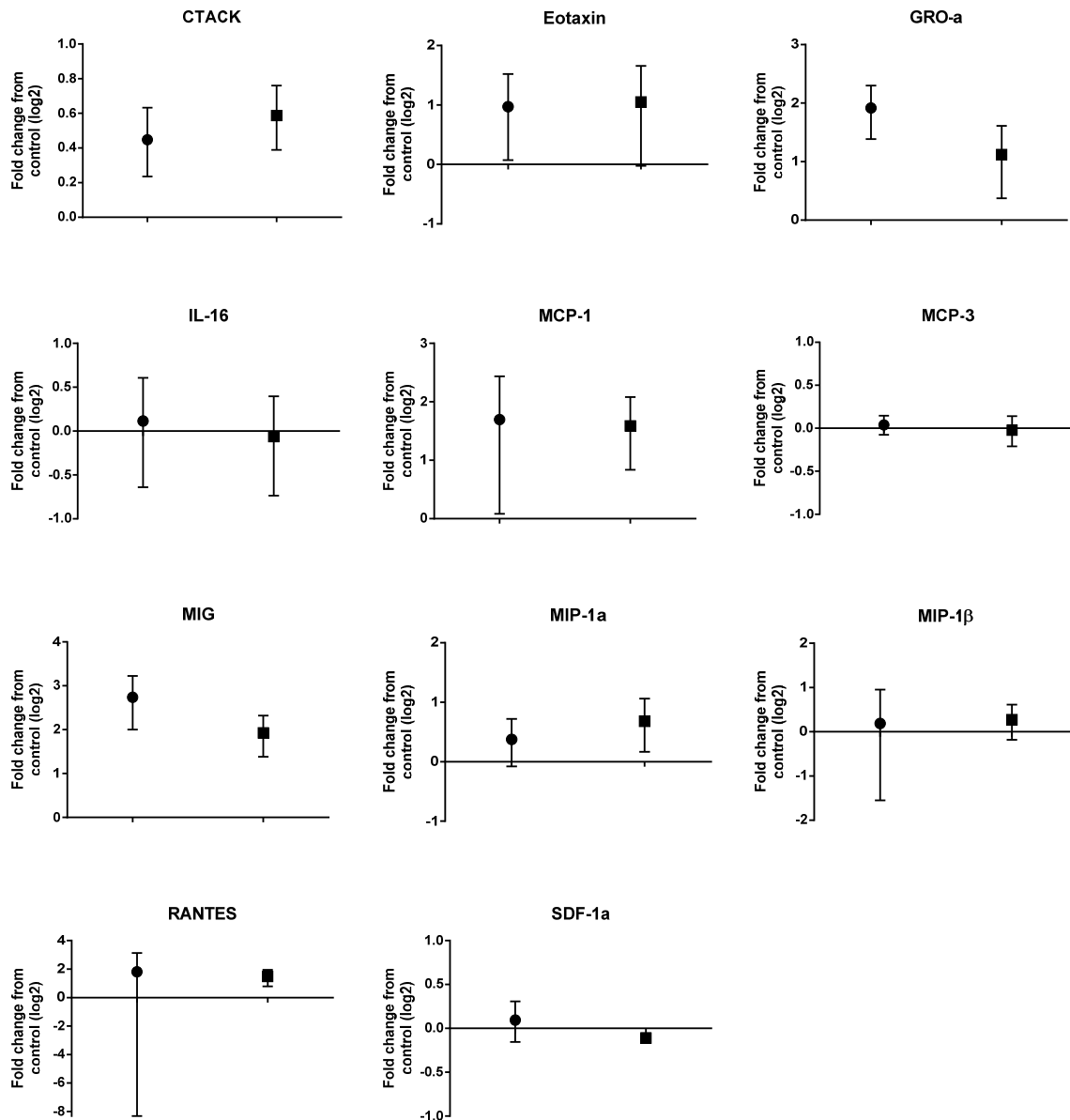**TNF-α**   **TNF-b**   **TRAIL**

Figure D.1. Summary of **pro-inflammatory cytokines** in red blood cell lysate after incubation into PBS over 24 hours at 37 °C with or without recombinant protein and protease inhibitors (PI) as measured by Bio-Plex and reported as fluorescence (100 million red blood cells in 1 mL PBS). Data are presented as fold change after addition of recombinant protein ± 95% CI, where ● represents fold change in cytokine profile of lysate with exogenous recombinant protein compared to control, and ■ represents fold change in cytokine profile of lysate with PI, with addition of exogenous recombinant protein compared to control ($n = 5$).
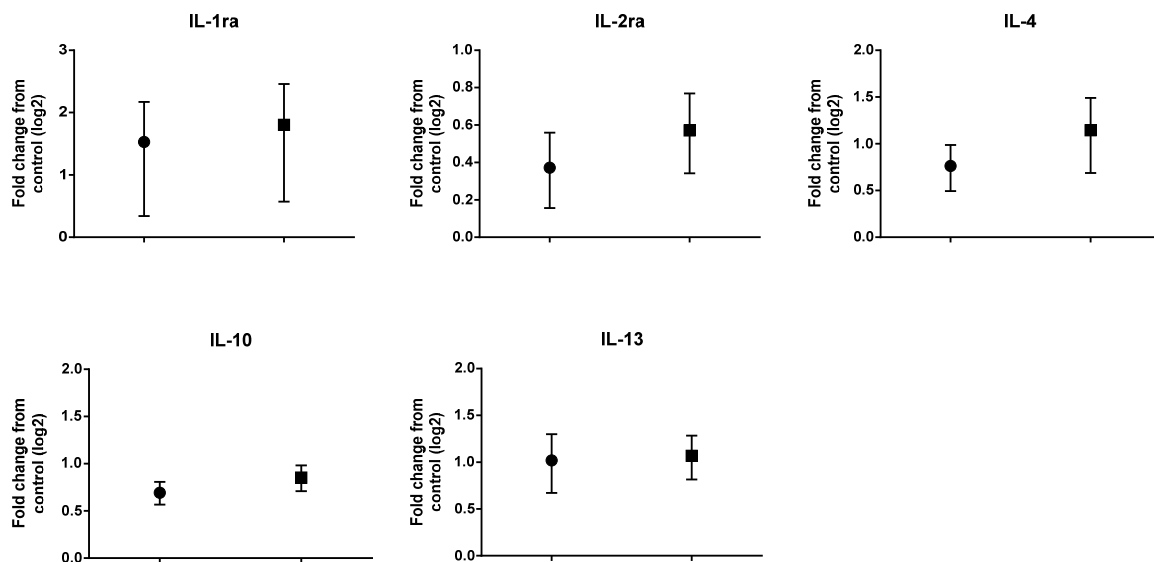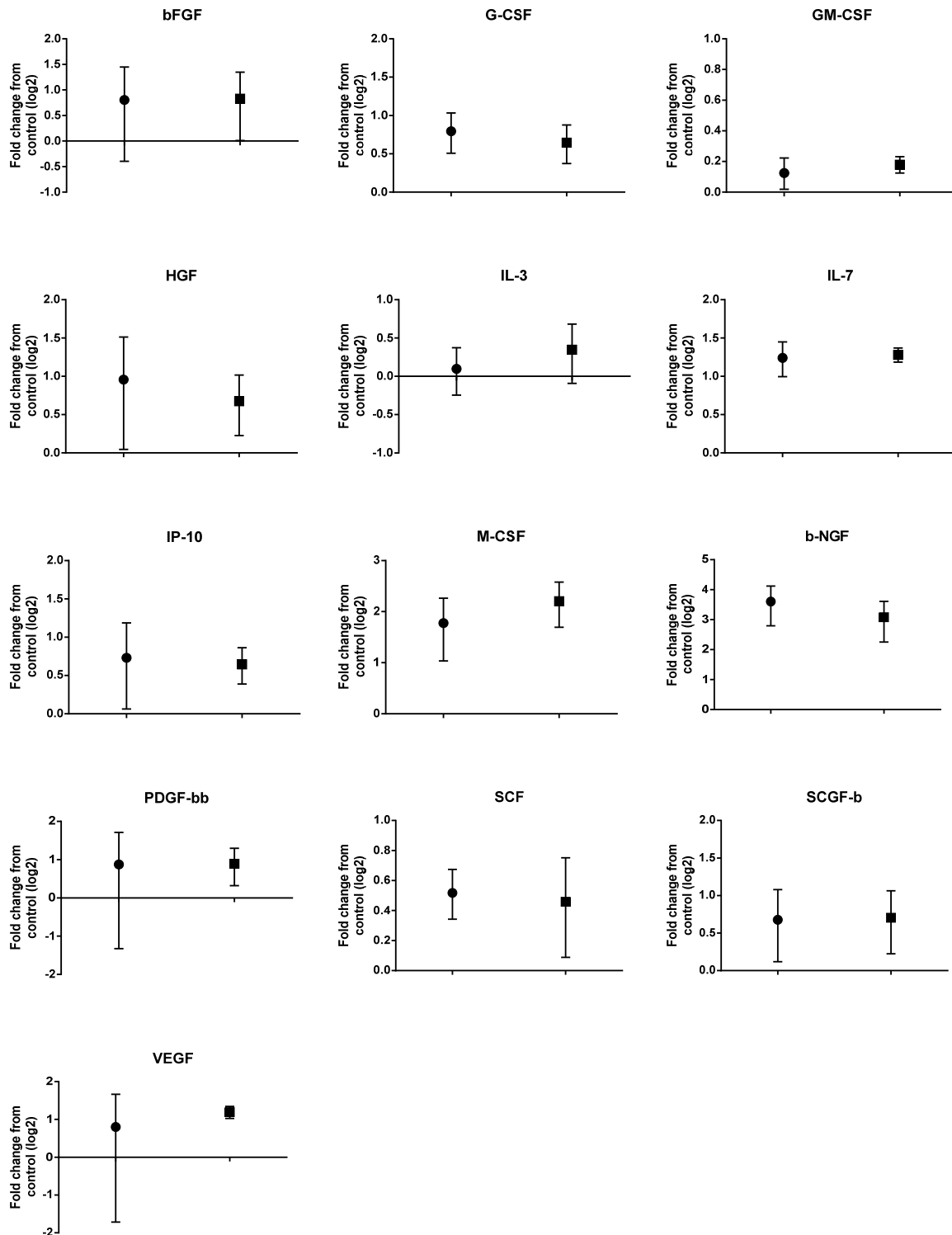

Figure D.2. Summary of **anti-inflammatory cytokines** in red blood cell lysate after incubation into PBS over 24 hours at 37 °C with or without recombinant protein and protease inhibitors (PI) as measured by Bio-Plex and reported as fluorescence (100 million red blood cells in 1 mL PBS). Data are presented as fold change after addition of recombinant protein ± 95% CI, where ● represents fold change in cytokine profile of lysate with exogenous recombinant protein compared to control, and ■ represents fold change in cytokine profile of lysate with PI, with addition of exogenous recombinant protein compared to control ($n = 5$).
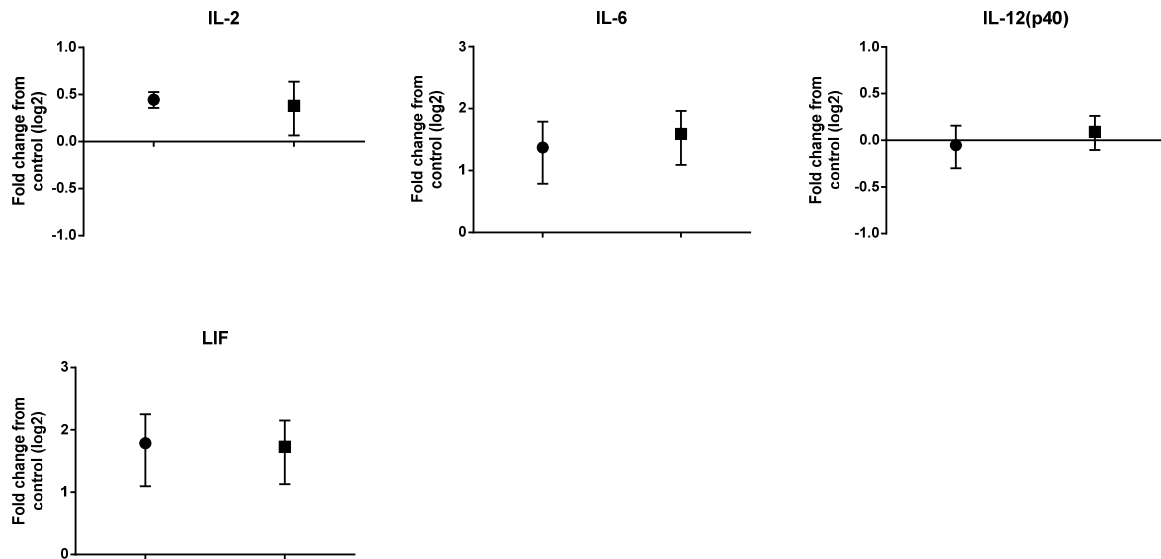
Figure D.3. Summary of **chemokines** in red blood cell lysate after incubation into PBS over 24 hours at 37 °C with or without recombinant protein and protease inhibitors (PI) as measured by Bio-Plex and reported as fluorescence (100 million red blood cells in 1 mL PBS). Data are presented as fold change after addition of recombinant protein ± 95% CI, where ● represents fold change in cytokine profile of lysate with exogenous recombinant protein compared to control, and ■ represents fold change in cytokine profile of lysate with PI, with addition of exogenous recombinant protein compared to control (*n* = 5).

Figure D.4. Summary of **growth factors** in red blood cell lysate after incubation into PBS over 24 hours at 37 °C with or without recombinant protein and protease inhibitors (PI) as measured by Bio-Plex and reported as fluorescence (100 million red blood cells in 1 mL PBS). Data are presented as fold change after addition of recombinant protein ± 95% CI, where ● represents fold change in cytokine profile of lysate with exogenous recombinant protein

compared to control, and ■ represents fold change in cytokine profile of lysate with PI, with addition of exogenous recombinant protein compared to control ($n = 5$).

Figure D.5. Summary of **cytokines with multiple factors** in red blood cell lysate after incubation into PBS over 24 hours at 37 °C with or without recombinant protein and protease inhibitors (PI) as measured by Bio-Plex and reported as fluorescence (100 million red blood cells in 1 mL PBS). Data are presented as fold change after addition of recombinant protein ± 95% CI, where ● represents fold change in cytokine profile of lysate with exogenous recombinant protein compared to control, and ■ represents fold change in cytokine profile of lysate with PI, with addition of exogenous recombinant protein compared to control ($n = 5$).

APPENDIX

E

E.1   METHODS

E.1.1   Statistics analysis of multiplex Luminex® data

Statistical analysis of raw fluorescence responses was performed using 'R' version 3.2.3 (2015-12-10, R: A Language and Environment for Statistical Computing). Mixed-effects modelling was done using lmer[1]. The significance of interactions terms and interaction means and their associated standard errors were obtained using the Phia package[2], for post-hoc analysis. Multiple test correction was done according to Holm's method[3]. To analyse the fluorescence response, the following mixed-effects model, in R notation, was used:

Equation E.1 Mixed-effects model for statistical analysis of cytokine data.

$$\log2(FI) \sim Cytokine * Treatment + (1|kit:ID)$$

The analysis was performed separately for red blood cells lysates and conditioned media samples. Where the log2 of fluorescence responses (Fl) was modelled using 2-way mixed effects ANOVA, (1) Cytokine (48 levels), and (2) Treatment (2 levels for red blood cell lysates: unprimed red blood cells, or primed red blood; 4 levels for conditioned media: none, PHA, unprimed red blood cells, or primed red blood cells) plus their interactions, together with one random term defined as (1|kit:ID), where ID represented subject identifier and where kit represented the 27-plex and 21-plex cytokine panels (2 levels). The random effects account for patient-to-patient variability and for differences with respect to kit groupings across the 2 cytokine plates, it also accounted for the non-independence in the data due to multiple samples per subject. Goodness-of-fit of the model for the data was determined by visual analysis of the residuals on a Q-Q plot and deviation of the mean from zero on a box plot.

E.2 RESULTS AND DISCUSSION

To validate the model for use with this dataset, a visual inspection of the residuals was completed. The following plots demonstrate that the data were normally distributed and there was minimal deviation from zero (Figure E.1). Similarly, the Q-Q plots demonstrate that the data is linear and the model is a good fit with small residuals (Figure E.2). This data demonstrates that the model used in this analysis was appropriate for the dataset. The goodness-of-fit for the model used in the red blood cell lysate analysis had a similar profile to the plots observed here. Thus, the models in this study were appropriate for the datasets.

Figure E.1. Box plots of the residuals and distribution of cytokine data from the PBMC conditioned media as measured by Bio-Plex and reported as fluorescence ± 95 % CI (*n* = 7).
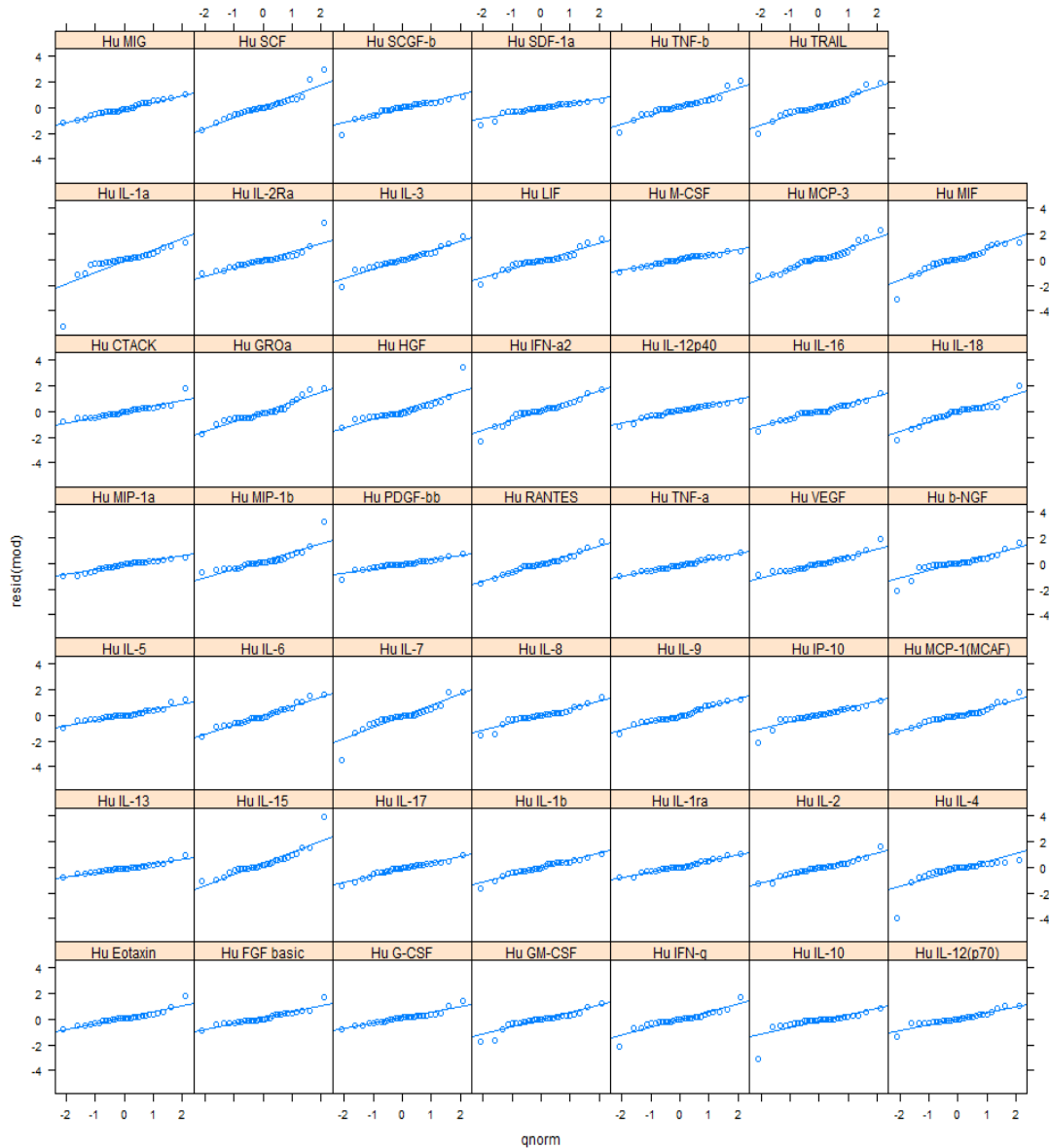
Figure E.2. Q-q-plots testing for normality of residuals from the cytokine data in the PBMC conditioned media as measured by Bio-Plex and reported as fluorescence (*n* = 7).

E.3 REFERENCES

1. Bates, D., Mächler, M., Bolker, B. M. & Walker, S. C. Fitting linear mixed-effects models using lme4. *J Stat Softw* **67,** 1–51 (2014).

2. Rosario-Martinez, H. De. Phia: post-hoc interaction analysis. *R Package version 01-3* (2015).

3. Holm, S. A simple sequentially rejective multiple test procedure. *Scand J Stat* **6,** 65–70 (1979).

APPENDIX

F

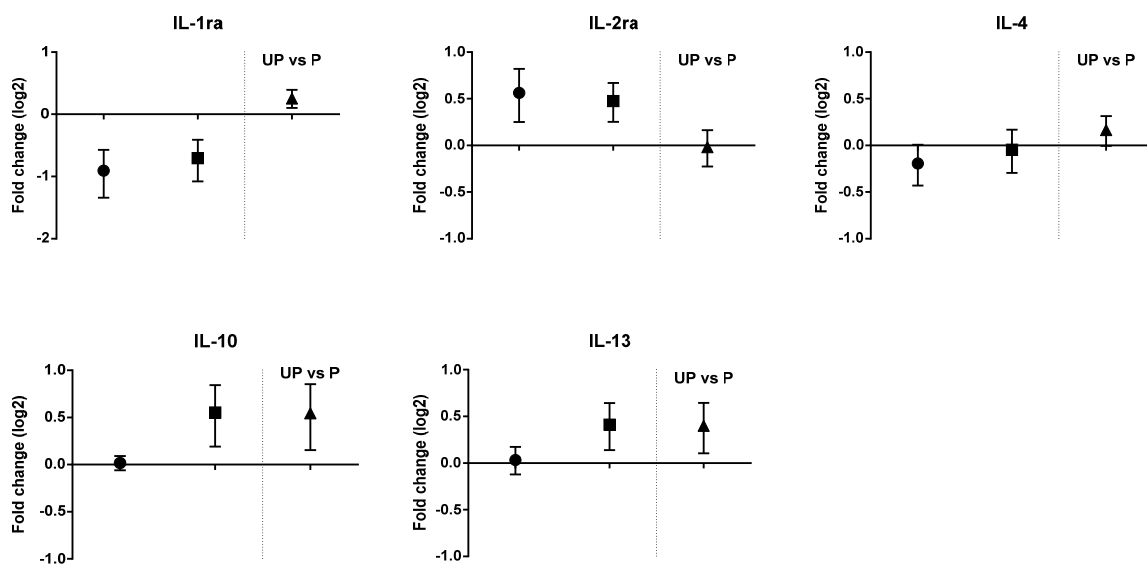--
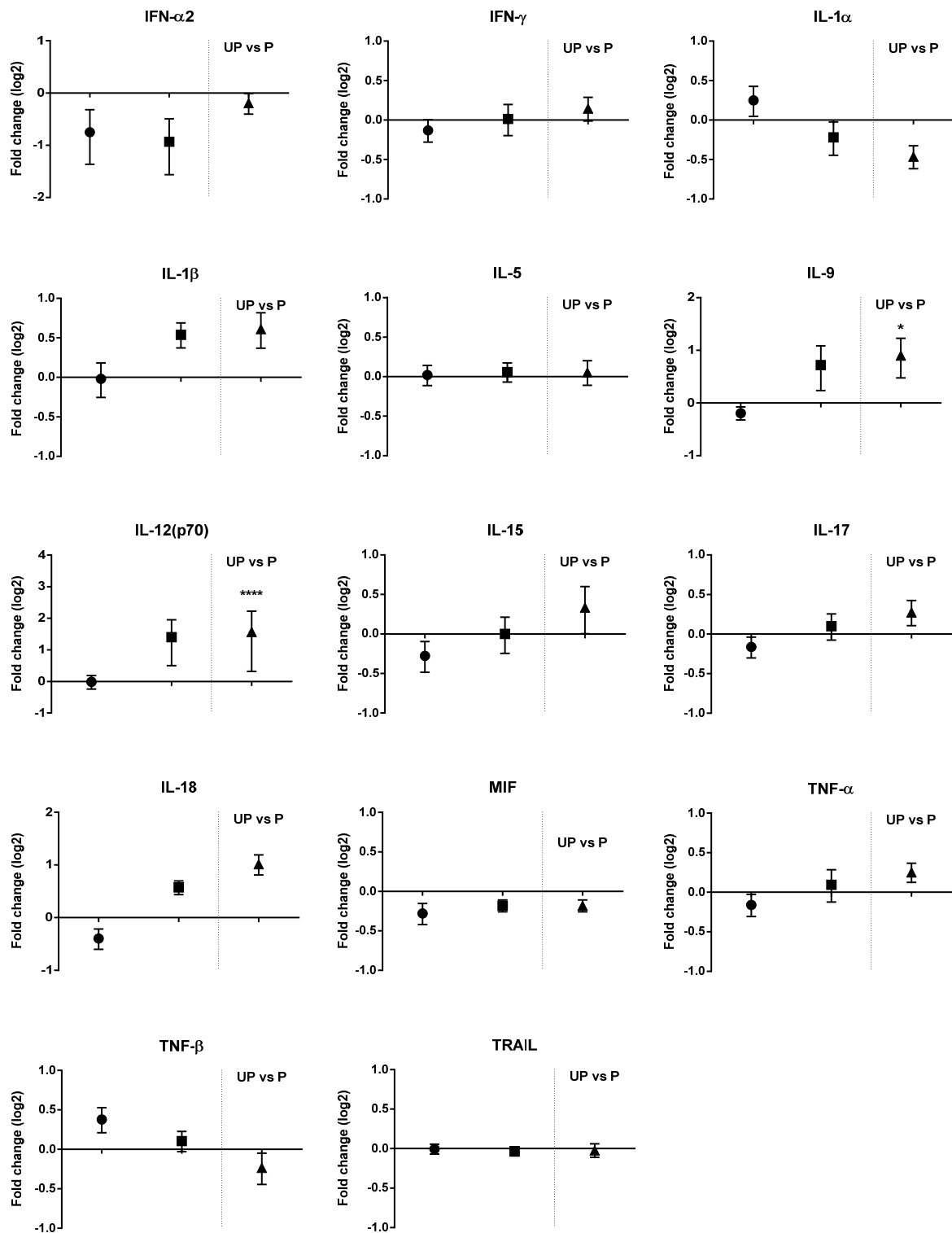
Figure F.1. Summary of **pro-inflammatory cytokines** in the red blood cell lysates prior to incubation (T0) and of primed (pRBC) and unprimed (upRBC) red blood cells after incubation (T3) as measured by Bio-Plex and reported as fluorescence (100 million cells in 1 mL PBS). Data are presented as mean fold change of fluorescence ± 95 % CI (*n* = 12), where ● represents the fold change for unprimed red blood cells before and after incubation and ■ for primed cells before and after incubation, and ▲ represents the fold change in fluorescence between unprimed and primed red blood cells (T3). Data are statistically significant different (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$, (****) if $p < 0.0001$.
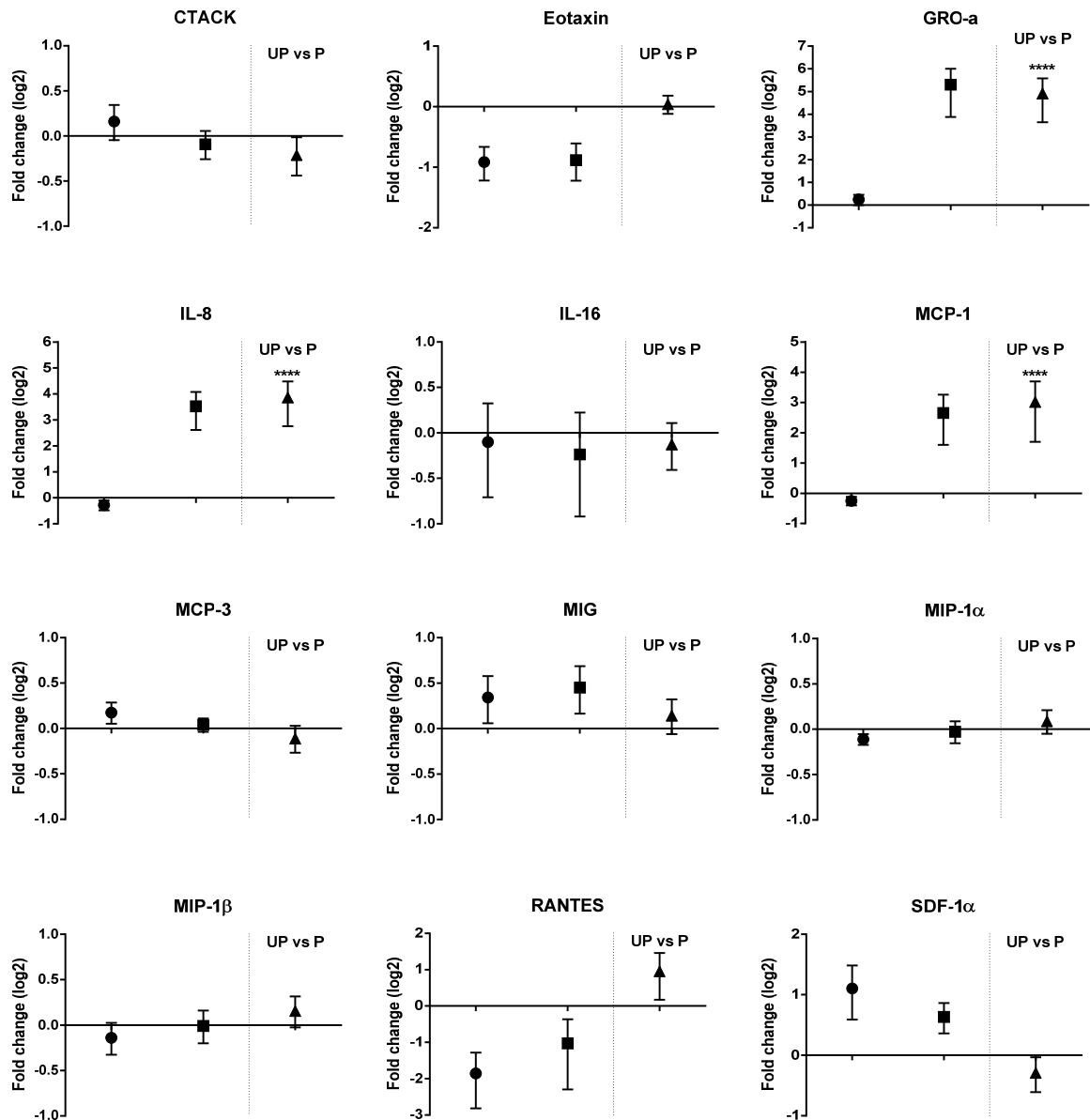

Figure F.2. Summary of **anti-inflammatory cytokines** in the red blood cell lysates prior to incubation (T0) and of primed (pRBC) and unprimed (upRBC) red blood cells after incubation (T3) as measured by Bio-Plex and reported as fluorescence (100 million cells in 1 mL PBS). Data are presented as mean fold change of fluorescence ± 95 % CI (*n* = 12), where ● represents the fold change for unprimed red blood cells before and after incubation and ■ for primed cells before and after incubation, and ▲ represents the fold change in fluorescence between unprimed and primed red blood cells (T3). Data are statistically significant different (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$, (****) if $p < 0.0001$.
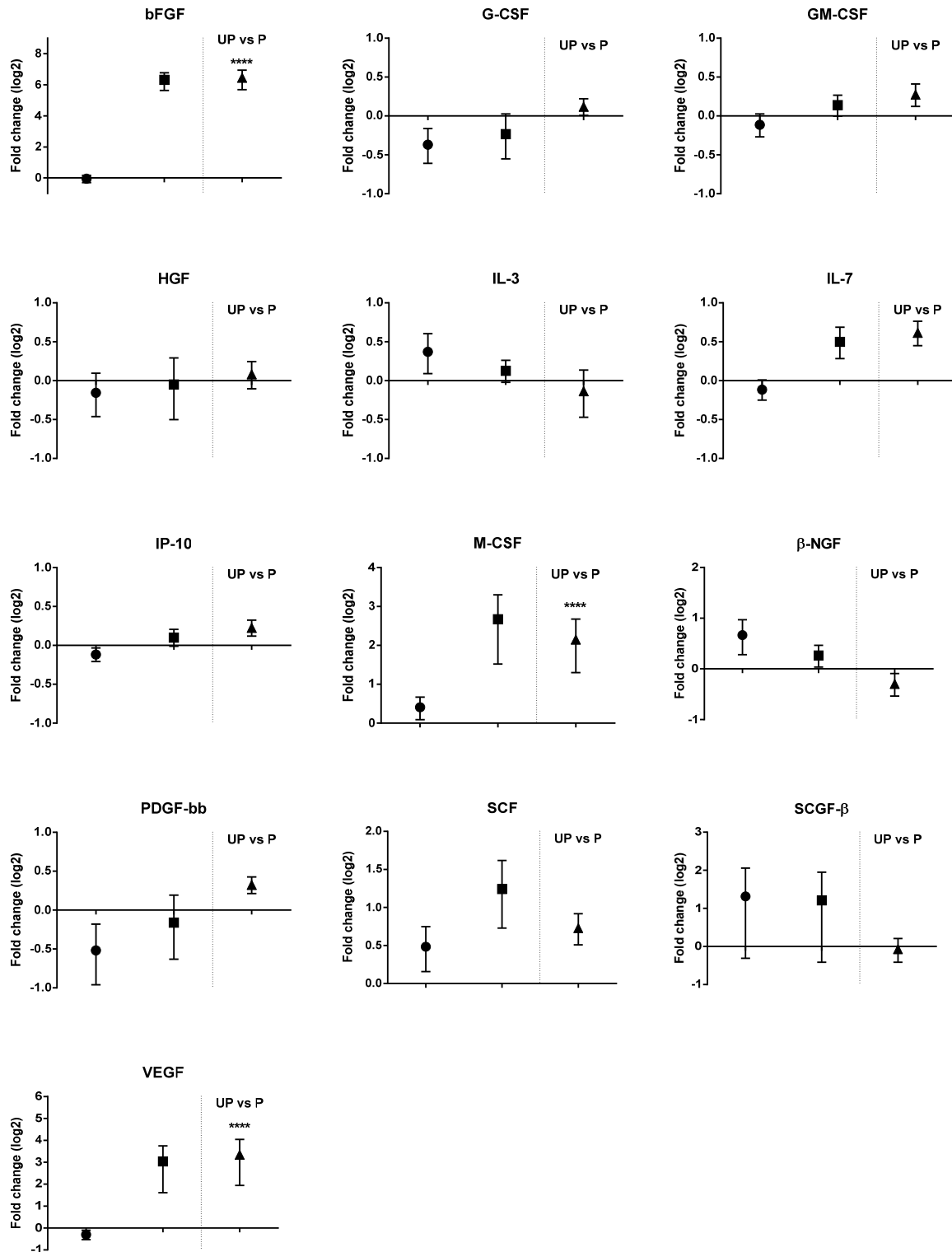
Figure F.3. Summary of **chemokines** in the red blood cell lysates prior to incubation (T0) and of primed (pRBC) and unprimed (upRBC) red blood cells after incubation (T3) as measured by Bio-Plex and reported as fluorescence (100 million cells in 1 mL PBS). Data are presented as mean fold change of fluorescence ± 95 % CI ($n = 12$), where ● represents the fold change for unprimed red blood cells before and after incubation and ■ for primed cells before and after incubation, and ▲ represents the fold change in fluorescence between unprimed and primed red blood cells (T3). Data are statistically significant different (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$, (****) if $p < 0.0001$.
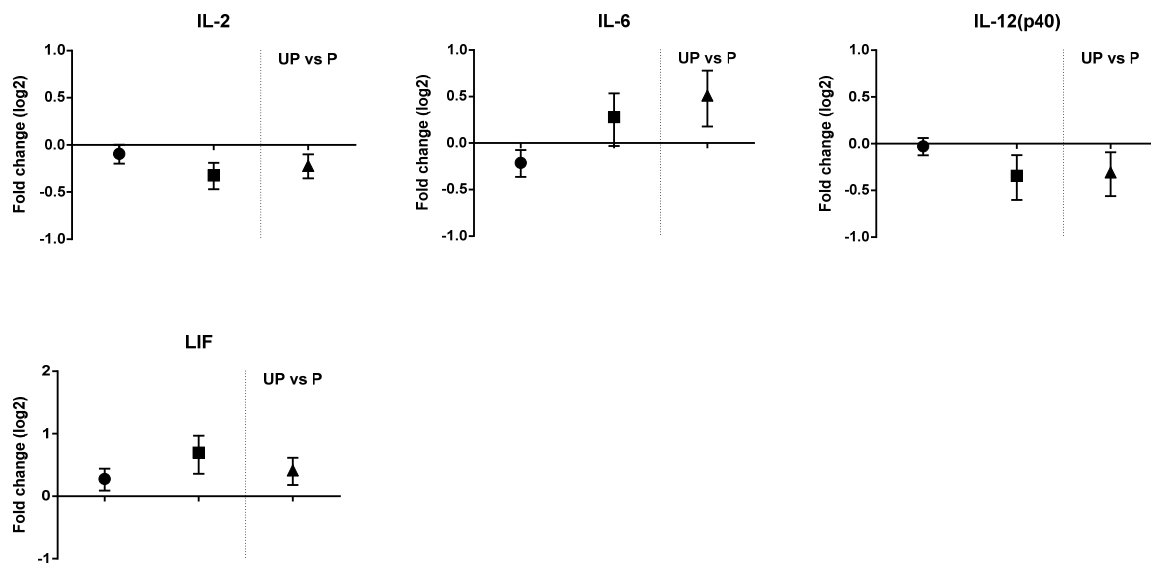
Figure F.4. Summary of **growth factors** in the red blood cell lysates prior to incubation (T0) and of primed (pRBC) and unprimed (upRBC) red blood cells after incubation (T3) as measured by Bio-Plex and reported as fluorescence (100 million cells in 1 mL PBS). Data are presented as mean fold change of fluorescence ± 95 % CI (n = 12), where ● represents the fold change for unprimed red blood cells before and after incubation and ■ for primed cells before

and after incubation, and ▲ represents the fold change in fluorescence between unprimed and primed red blood cells (T3). Data are statistically significant different (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$, (****) if $p < 0.0001$.

Figure F.5. Summary of **cytokines with multiple functions** in the red blood cell lysates prior to incubation (T0) and of primed (pRBC) and unprimed (upRBC) red blood cells after incubation (T3) as measured by Bio-Plex and reported as fluorescence (100 million cells in 1 mL PBS). Data are presented as mean fold change of fluorescence ± 95 % CI ($n = 12$), where ● represents the fold change for unprimed red blood cells before and after incubation and ■ for primed cells before and after incubation, and ▲ represents the fold change in fluorescence between unprimed and primed red blood cells (T3). Data are statistically significant different (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$, (****) if $p < 0.0001$.

APPENDIX

G

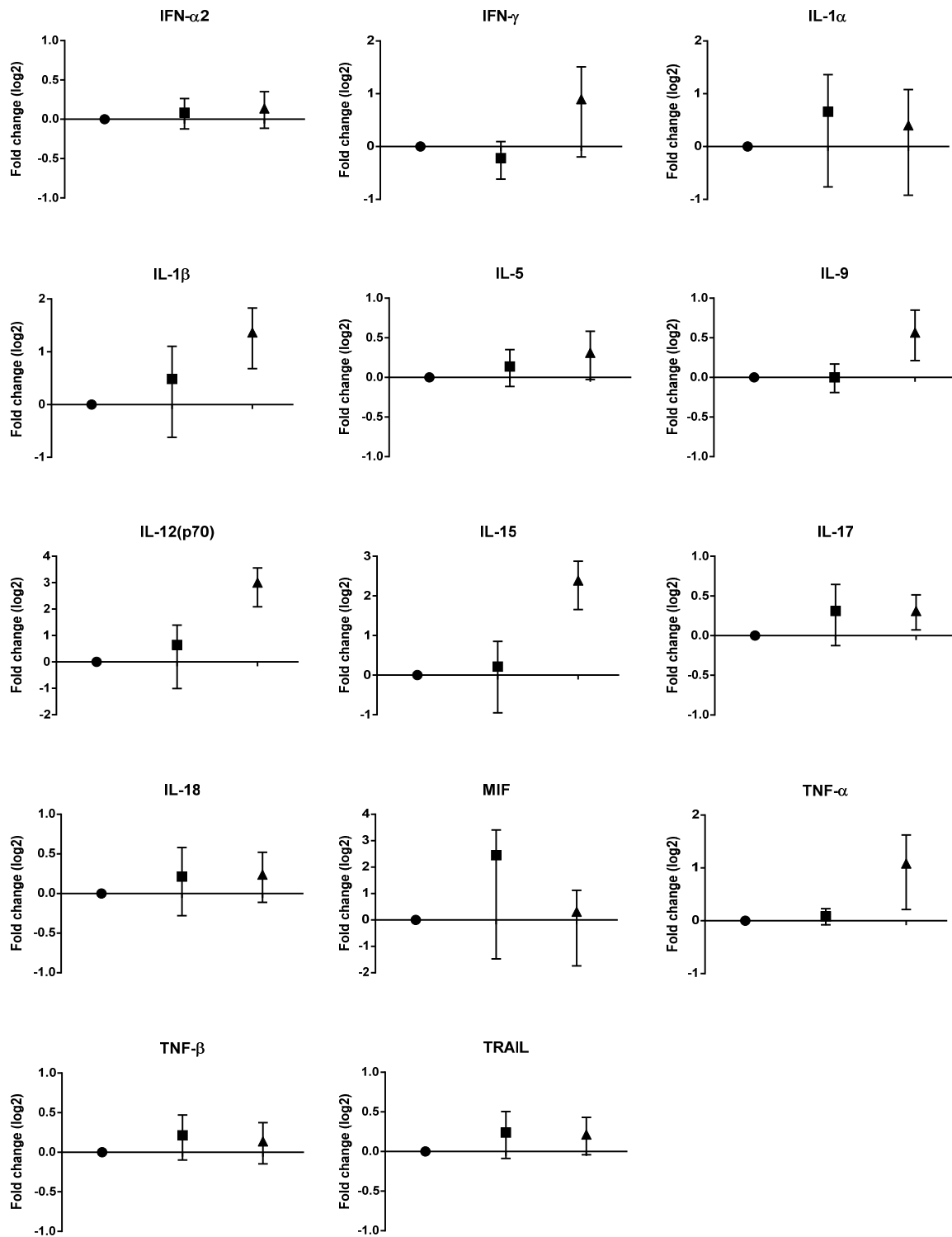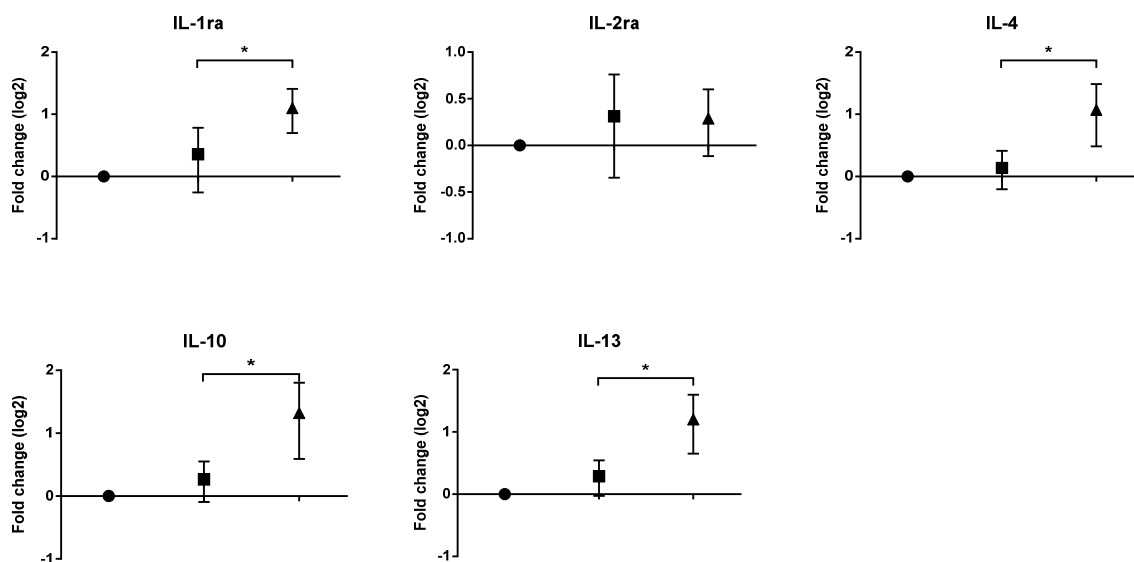--

Figure G.1. Summary of **pro-inflammatory cytokines** in Jurkat conditioned media as measured by Bio-Plex and reported as fluorescence. Data are presented as mean fold change from the untreated Jurkat control in fluorescence $\pm$ 95 % CI ($n = 5$), where $\bullet$ represents the fold change for untreated Jurkat, $\blacksquare$ for Jurkat cells treated with unprimed red blood cells (upRBC), and $\blacktriangle$ for Jurkat cells treated with primed red blood cells (pRBC). Data are statistically significant different from the untreated Jurkat control (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$, (****) if $p < 0.0001$.
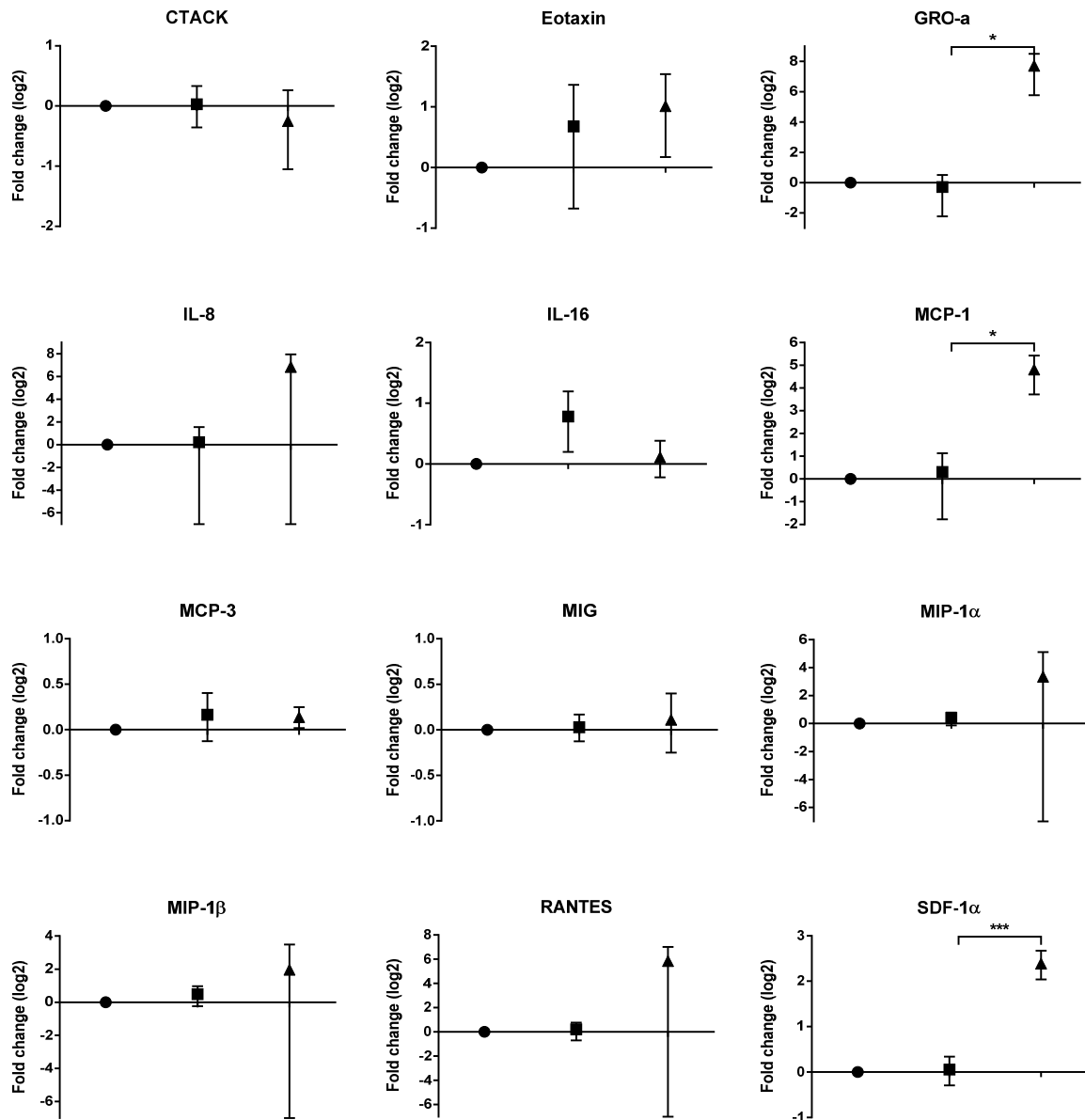

Figure G.2. Summary of **anti-inflammatory cytokines** in Jurkat conditioned media as measured by Bio-Plex and reported as fluorescence. Data are presented as mean fold change from the untreated Jurkat control in fluorescence $\pm$ 95 % CI ($n = 5$), where $\bullet$ represents the fold change for untreated Jurkat, $\blacksquare$ for Jurkat cells treated with unprimed red blood cells (upRBC), and $\blacktriangle$ for Jurkat cells treated with primed red blood cells (pRBC). Data are statistically significant different from the untreated Jurkat control (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$, (****) if $p < 0.0001$.
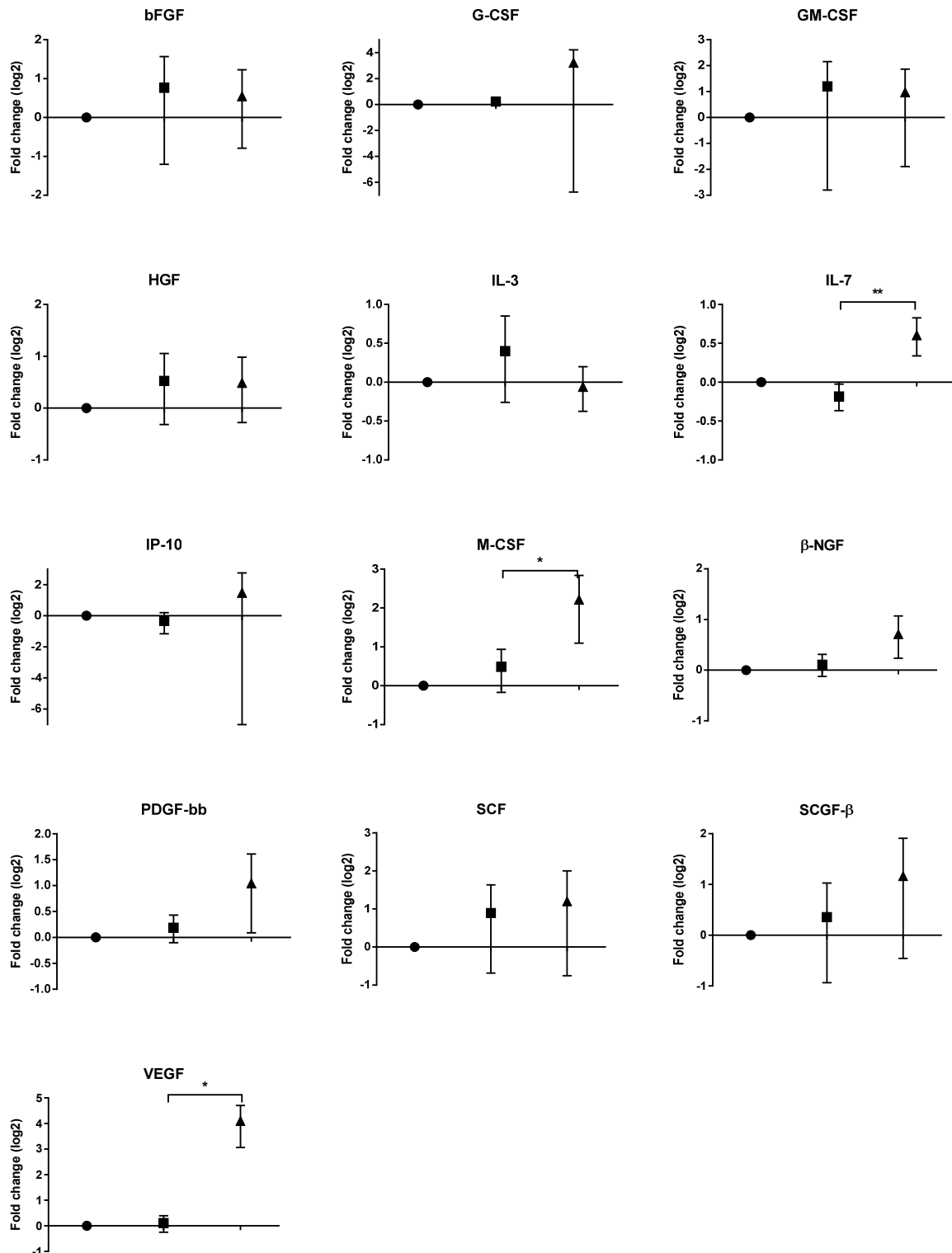
Figure G.3. Summary of **chemokines** in Jurkat conditioned media as measured by Bio-Plex and reported as fluorescence. Data are presented as mean fold change from the untreated Jurkat control in fluorescence $\pm$ 95 % CI ($n = 5$), where ● represents the fold change for untreated Jurkat, ■ for Jurkat cells treated with unprimed red blood cells (upRBC), and ▲ for Jurkat cells treated with primed red blood cells (pRBC). Data are statistically significant different from the untreated Jurkat control (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$, (****) if $p < 0.0001$.
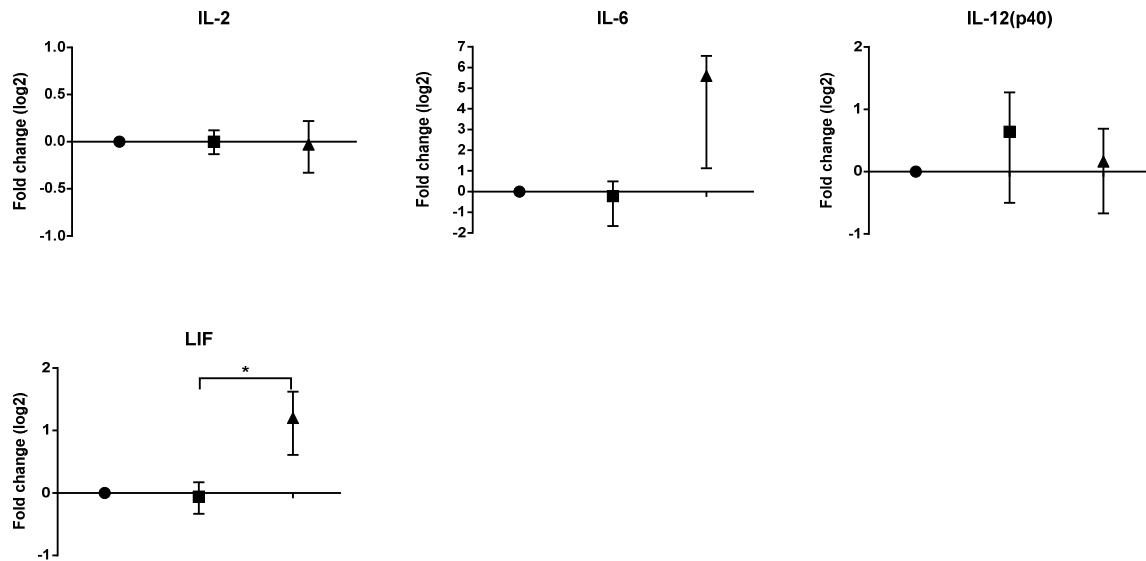
Figure G.4. Summary of **growth factors** in Jurkat conditioned media as measured by Bio-Plex and reported as fluorescence. Data are presented as mean fold change from the untreated Jurkat control in fluorescence $\pm$ 95 % CI ($n = 5$), where ● represents the fold change for untreated Jurkat, ■ for Jurkat cells treated with unprimed red blood cells (upRBC), and ▲ for Jurkat cells treated with primed red blood cells (pRBC). Data are statistically significant

different from the untreated Jurkat control (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$, (****) if $p < 0.0001$.

Figure G.5. Summary of **cytokines with multiple functions** in Jurkat conditioned media as measured by Bio-Plex and reported as fluorescence. Data are presented as mean fold change from the untreated Jurkat control in fluorescence $\pm$ 95 % CI ($n = 5$), where ● represents the fold change for untreated Jurkat, ■ for Jurkat cells treated with unprimed red blood cells (upRBC), and ▲ for Jurkat cells treated with primed red blood cells (pRBC). Data are statistically significant different from the untreated Jurkat control (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$, (****) if $p < 0.0001$.

APPENDIX

H

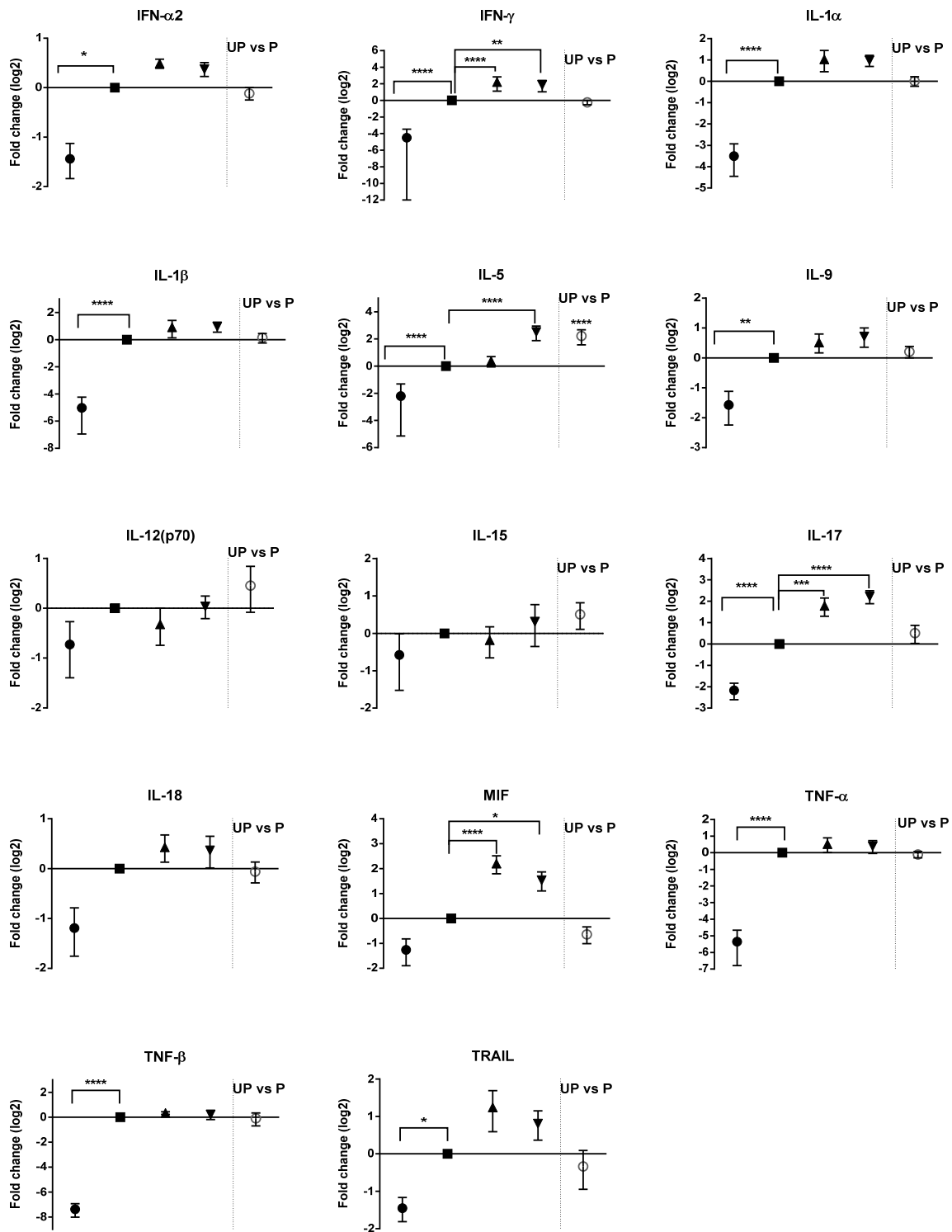--

Figure H.1. Summary of **pro-inflammatory cytokines** in PBMC (peripheral blood mononuclear cells) conditioned media as measured by Bio-Plex and reported as fluorescence ± 95 % CI (*n* = 7). Data are presented as mean fold change from ■ PBMC treated with PHA-P, to ● untreated PBMCs, ▲ for PBMCs treated with PHA-P and unprimed red blood cells (upRBC), and ▼ for PBMCs treated with PHA-P and primed red blood cells (pRBC). Fold change from PBMCs treated with PHA-P and upRBCs to PBMCs treated with PHA-P and pRBCs represented by ○ (open circle). Data are statistically significant different from PBMCs treated with PHA-P (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$, (****) if $p < 0.0001$.
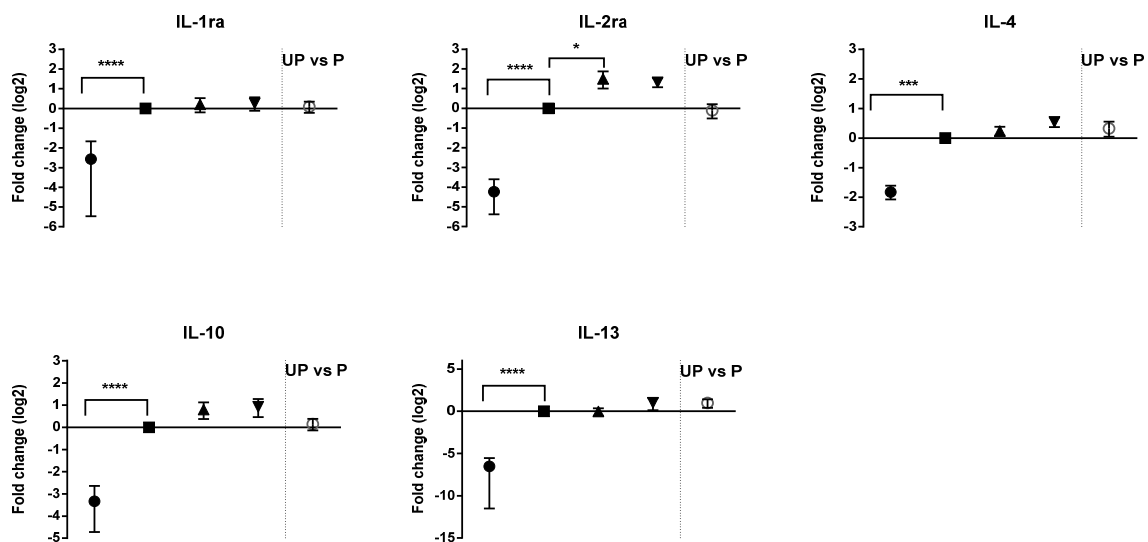

Figure H.2. Summary of **anti-inflammatory cytokines** in PBMC (peripheral blood mononuclear cells) conditioned media as measured by Bio-Plex and reported as fluorescence ± 95 % CI (*n* = 7). Data are presented as mean fold change from ■ PBMC treated with PHA-P, to ● untreated PBMCs, ▲ for PBMCs treated with PHA-P and unprimed red blood cells (upRBC), and ▼ for PBMCs treated with PHA-P and primed red blood cells (pRBC). Fold change from PBMCs treated with PHA-P and upRBCs to PBMCs treated with PHA-P and pRBCs represented by ○ (open circle). Data are statistically significant different from PBMCs treated with PHA-P (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$, (****) if $p < 0.0001$.
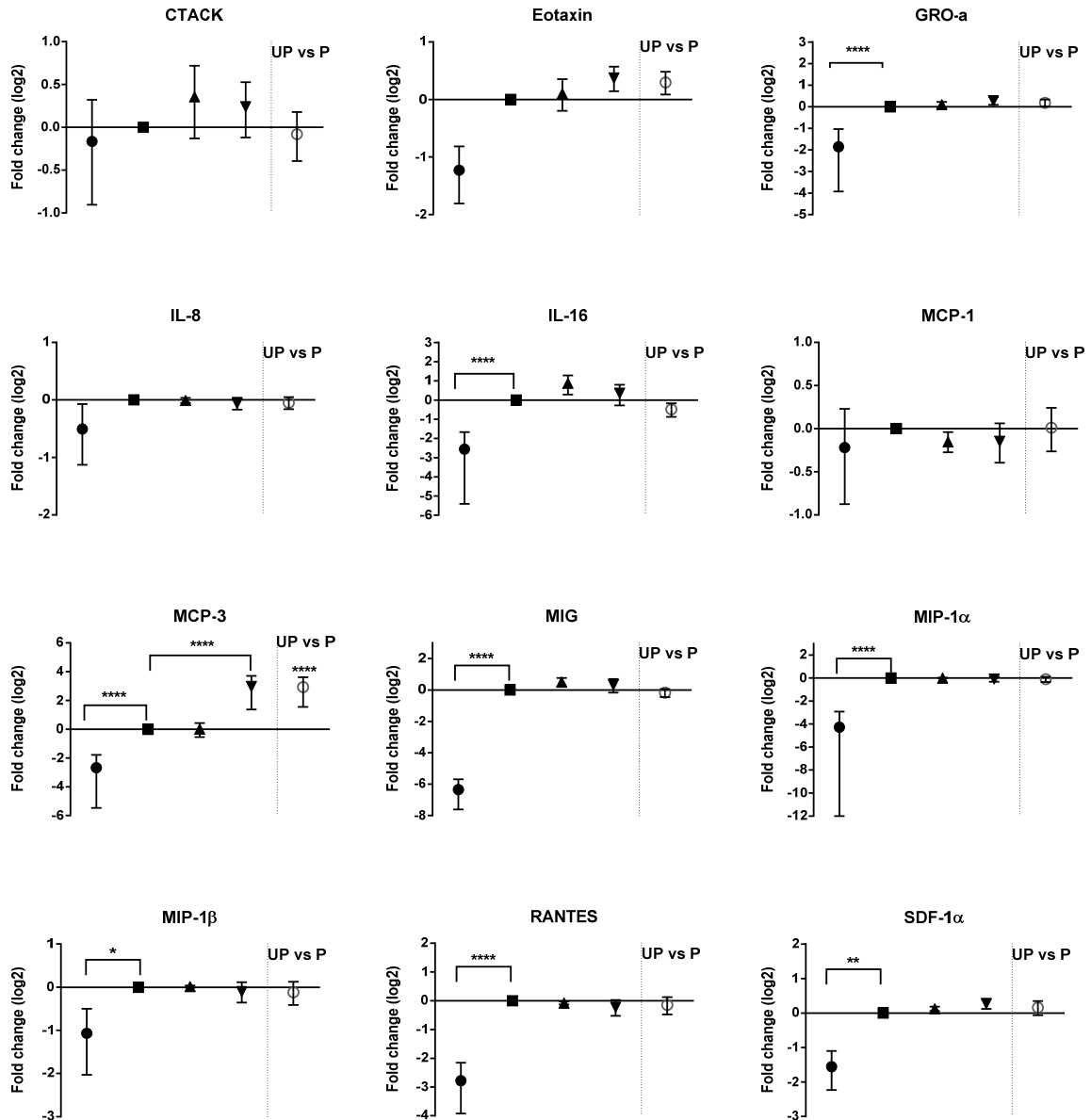
Figure H.3. Summary of **chemokines** in PBMC (peripheral blood mononuclear cells) conditioned media as measured by Bio-Plex and reported as fluorescence $\pm$ 95 % CI ($n = 7$). Data are presented as mean fold change from ■ PBMC treated with PHA-P, to ● untreated PBMCs, ▲ for PBMCs treated with PHA-P and unprimed red blood cells (upRBC), and ▼ for PBMCs treated with PHA-P and primed red blood cells (pRBC). Fold change from PBMCs treated with PHA-P and upRBCs to PBMCs treated with PHA-P and pRBCs represented by ○ (open circle). Data are statistically significant different from PBMCs treated with PHA-P (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$, (****) if $p < 0.0001$.
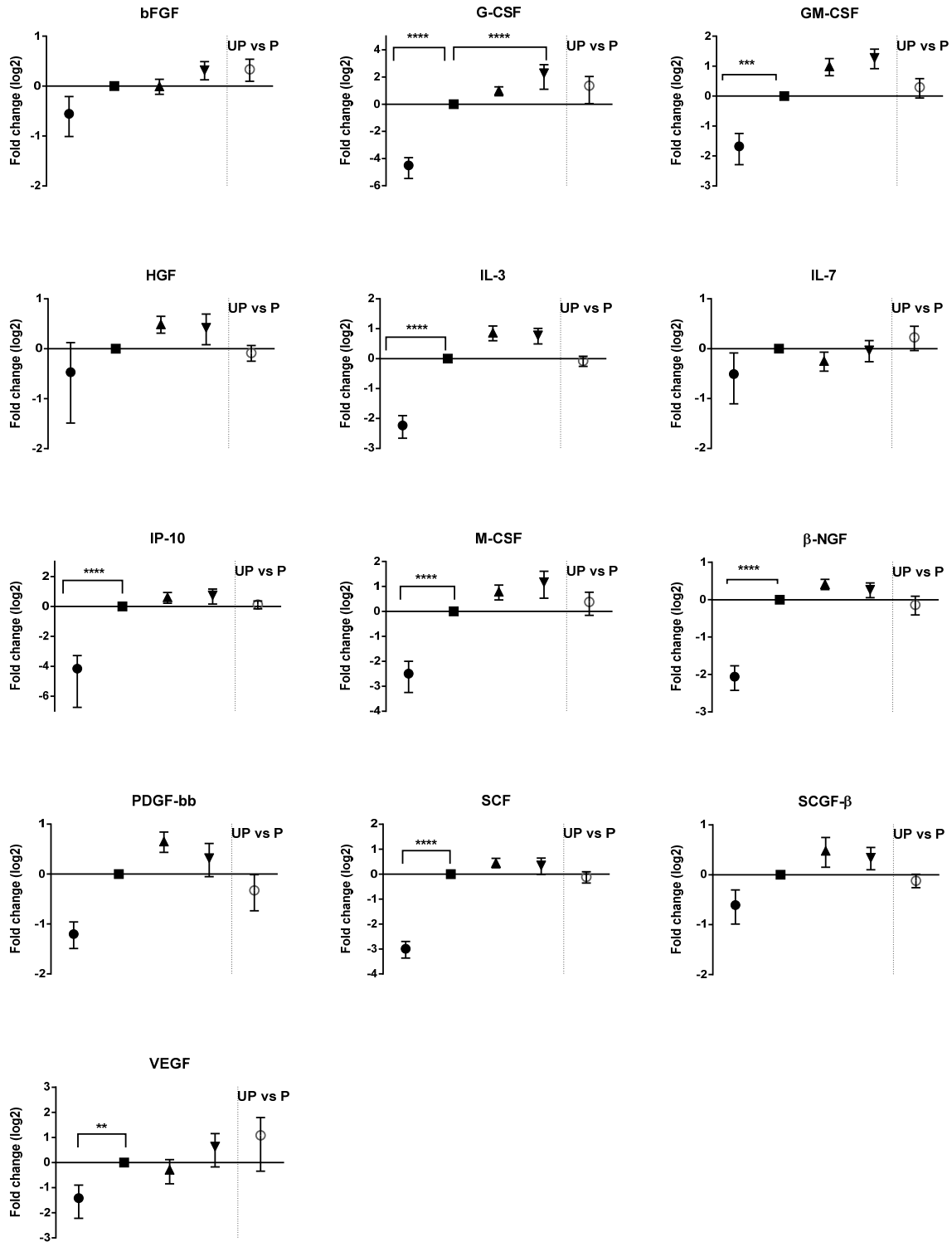
Figure H.4. Summary of **growth factors** in PBMC (peripheral blood mononuclear cells) conditioned media as measured by Bio-Plex and reported as fluorescence ± 95 % CI (*n* = 7). Data are presented as mean fold change from ■ PBMC treated with PHA-P, to ● untreated PBMCs, ▲ for PBMCs treated with PHA-P and unprimed red blood cells (upRBC), and ▼ for PBMCs treated with PHA-P and primed red blood cells (pRBC). Fold change from PBMCs

treated with PHA-P and upRBCs to PBMCs treated with PHA-P and pRBCs represented by ○ (open circle). Data are statistically significant different from PBMCs treated with PHA-P (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$, (****) if $p < 0.0001$.
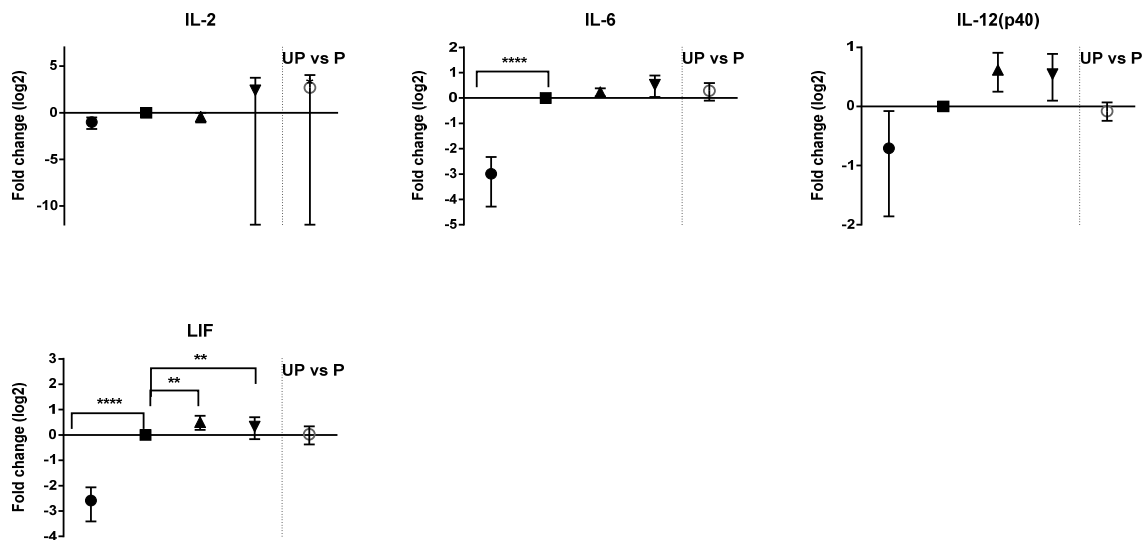

Figure H.5. Summary of **cytokines with multiple functions** in PBMC (peripheral blood mononuclear cells) conditioned media as measured by Bio-Plex and reported as fluorescence ± 95 % CI ($n = 7$). Data are presented as mean fold change from ■ PBMC treated with PHA-P, to ● untreated PBMCs, ▲ for PBMCs treated with PHA-P and unprimed red blood cells **(upRBC)**, and ▼ for PBMCs treated with PHA-P and primed red blood cells **(pRBC)**. Fold change from PBMCs treated with PHA-P and **upRBCs** to PBMCs treated with PHA-P and **pRBCs** represented by ○ (open circle). Data are statistically significant different from PBMCs treated with PHA-P (*) if $p < 0.05$, (**) if $p < 0.01$, (***) if $p < 0.001$, (****) if $p < 0.0001$

APPENDIX

I

The data in this thesis have been included in three patent applications as outlined below.

Title of patent	Date of filing	Status
Blood preparation and profiling	October 2015	PCT
Therapeutic methods using erythrocytes	December 2015	PCT
Blood profiling with protease inhibitors	December 2016	Provisional