Analysis of cytokine induced phosphorylation of STAT3 in peripheral blood mononuclear cells by flow cytometric and western blot assays

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Declaration of Originality

The work described in this dissertation was carried out in the Department of Pharmacology of the University of Pretoria under the supervision of Dr AD Cromarty. I declare that this dissertation is my own original work submitted for the degree Master of Science. Where other people's work has been used (either from a printed source, Internet or any other source), this has been properly acknowledged and referenced in accordance with departmental requirements.

I have not used work previously produced by another student or any other person to hand in as my own.

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Abstract

Signal transducer and activator of transcription (STAT) is a family of intracellular proteins that are responsible for carrying the signal from the cell surface to the nucleus in response to specific ligands. Once in the nucleus, STATs activate the transcription of specific genes. To date, seven human STATs have been identified. Among these STATs, STAT3 is considered as oncogenic. It activates genes that block apoptosis and inhibits antitumor immune responses (1). STAT3 is also essential in early embryogenesis and plays a role in cell growth and survival, differentiation and apoptosis depending on the target tissue.

Analysing STAT3 signalling provides insights into pathology and can be used as a tool for diagnosis, prognosis and therapy development. Traditionally, western blot has been used to analyse cell signalling but it is impractical in analysing rare cell populations or providing information at the single cell level. Moreover, it is a demanding and time consuming technique that offers qualitative and less sensitive analysis. The rapid evolution in the multi-parametric flow cytometry and the availability of both epitope specific antibodies and sophisticated software facilitate the wide application of this technology in cell signalling studies. Flow cytometry has the ability to resolve different subcellular sets in a heterogeneous population, collects data at a single cell level and correlates multiple markers simultaneously. However, it requires highly standardized protocols for maximal sensitivity.

The aim of this study was to assess the dose and the time response of both total STAT3 and pSTAT3 to *in vitro* stimulation with either IL-6 or IL-10 in peripheral blood mononuclear cells (PBMC). This assessment was done using both the flow cytometry and the western blot techniques.

The results of this study showed that lower doses of IL-6 (1 & 10 ng/ml) were not sufficient to induce phosphorylation of STAT3. However, following stimulation with 100 ng/ml of IL-6, no significant change in the level of total STAT3 could be detected in either lymphocytes or monocytes from 3 different donors using either the FC500 or the Accuri cytometer. Using the FC500 cytometer, a small but insignificant increase in the pSTAT3 was seen in the lymphocytes and monocytes. A significant increase in STAT3 phosphorylation was only observed for monocytes after 15 minutes stimulation with 100 ng/ml of IL-6 using the Accuri flow cytometer.

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When the fluorescent labelled antibodies used in the flow cytometric assays were used for western blot probing, western blot analysis of stimulated cell lysates with 100 ng/ml IL-6 detects proteins of a low molecular weight than STAT3 or pSTAT3 which may explain the flow cytometric results of IL-6 stimulation.

In IL-10 stimulation experiments, lower doses (1 and 10 ng/ml) tested by flow cytometric and western blot techniques demonstrated insignificant STAT3 phosphorylation induction. Following stimulation with either 50 or 100 ng/ml IL-10, no significant change in the total STAT3 was seen in either lymphocytes or monocytes when using the Accuri flow cytometer. However, stimulation with 100 ng/ml IL-10 induces STAT3 phosphorylation from 10 minutes through 30 minutes in both lymphocytes and monocytes. Longer times were required and high inter-individual variability was noticed for the activation of STAT3 after stimulation with 50 ng/ml IL-10.

By using different antibodies from those used in the flow cytometric assay; the western blot results were comparable with the flow cytometric findings following stimulation with 100 ng/ml IL-10.

The addition of phosphatase inhibitors during the flow cytometric protocol didn't show any increase in the STAT3 phosphorylation. However, using paraformaldehyde for fixation and methanol for permeabilisation significantly decreased the mean fluorescence intensity of the PE conjugated antibodies comparing to the BD commercial fixation and permeabilisation buffers.

The onset and the signal intensity of “in house” chemiluminescence mixture for western blot detection of STAT3 were comparable to the commercial ECL reagent used. However, the background of the “in house” mixture increased with time and was higher than with the commercial product. Upon longer exposure, the background increased enough to cause signal loss.

In spite of the number of advantages of the flow cytometric assay compared to the western blot assay, these results are highly dependent on the specificity and the selectivity of the used antibodies. Furthermore, flow cytometry requires a highly standardized protocol to be able to assess the normal level of signalling proteins which could be later applied to detect abnormalities. It is suggested that the antibodies used in the flow cytometric assay be tested by western blot to confirm their selective detection of the target protein before their use in the flow cytometric analysis.

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Abbreviations

ACN	Acetonitrile
ADC	Analogue digital converter
%	Percent
°C	Degree Celsius
µl	Microlitre
µm	Micrometre
BCA	Bicinchoninic acid
BD	Beckton Dickinson
BSA	Bovine serum albumin
Ca	Calcium
CCD	Charged-couple device
CD	Cluster of differentiation
CIS	Cytokine inducible SH_2 protein
Cu	Cupper
DBD	DNA binding domain
DDT	Dithiothreitol
DMSO	Dimethyl sulfoxide
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ESI	Electrospray ionization
F/M	Paraformaldehyde/methanol

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FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FL	Filter
Fraction V	Fraction five
FSC	Forward scatter
g	Centrifugal force
GAS	Gamma activated site
GH	Growth hormone
g	Gram
gp	Glycoprotein
HCL	Hydrochloric acid
HRP	Horse radish peroxidase
IAA	Iodoacetamide
IgG	Immunoglobulin G
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-6Rα	Interleukin 6 receptor subunit alpha.
IL-10R	Interleukin 10 receptor
INF	Interferon
JAK	Janus kinase
JH	Janus homology
KCl	Potassium chloride
KDa	Kilodalton
LPS	Lipopolysaccharides

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M	Molar
MDa	Mega Dalton
MeOH	Methanol
MFI	Mean fluorescence intensity
mg	Milligram
MHC	Major histocompatibility complex.
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
MMP	Matrix metalloproteinase
MW	Molecular weight
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
ng	Nano gram
NH_4Cl	Ammonium chloride
NH_4HCO_3	Ammonium bicarbonate
NK	Natural killer
p CA	Para coumaric acid
PerCP Cy5.5	Peridinin chlorophyll protein combines with a cyanine dye
pSTAT3	Phosphorylated signal transducer and activator of transcription
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline.

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PE	Phycoerythrin
PFA	Paraformaldehyde
PI	Phosphatase inhibitor
PIAS	Protein inhibitor of activated STAT
p-Tyr	Phosphorylated tyrosine
PVDF	Polyvinylidene difluoride
RT	Room temperature
S	Serine
SDS	Sodium dodecyl sulphate
SH2	Src homology 2
SOCS	Suppressor of cytokine signalling
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TAD	Transcriptional activation domain
TBS	Tris buffer saline
TBST	Tris buffer saline plus Tween 20
TNF	Tumour necrosis factor
TYK2	Tyrosine kinase 2
V	Volt
Y	Tyrosine
α	Alpha
β	Beta
γ	Gamma
γc	Gamma chain

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Chapter 1: Introduction

1.1 Intracellular signalling

In order to respond to changes in their immediate environment, cells must be able to receive and process signals that originate outside their borders (2). The individual cell not only has the ability to receive signals but it can also send messages to other cells. Each cell can receive many signals simultaneously and these signals can be integrated to a selected action in the cell through a certain signalling pathway.

There are a large number of intracellular signalling pathways responsible for transmitting information within the cell (3). These pathways are categorized into two groups depending on the type of the stimuli they respond to. Some of the signalling pathways respond to the information generated from internal metabolic messengers. However, the majority of the signalling pathways are activated via external stimuli which could be chemical such as growth factors or cytokines or mechanical such as pressure or sound. When a signalling molecule binds to a specific cell membrane receptor, conformational changes in this receptor will be initiated leading to activation of an internal signalling pathway. Receptors are made up of successive sequences with the recognition motif for a ligand (exposed to the outside of the cell), a trans membrane segment that provides the binding of the receptor to the plasma membrane, and finally an intracellular sequence with enzyme activity (4). Generally, a cascade of biochemical reactions will be triggered in these intracellular signalling pathways to carry the signal from the cell surface to internal effectors in a process known as intracellular signal transduction. The targets of such signalling pathways frequently include transcription factors that function to regulate gene expression (3). These signalling pathways play an important role in cell survival, proliferation, differentiation and apoptosis. Furthermore, they can play a role in the tumour formation process in which signal dysregulation can lead to uncontrolled proliferation and apoptosis unresponsiveness.

Some of the intracellular signalling pathways that are activated in response to external stimuli are illustrated in the following diagram (Figure 1).

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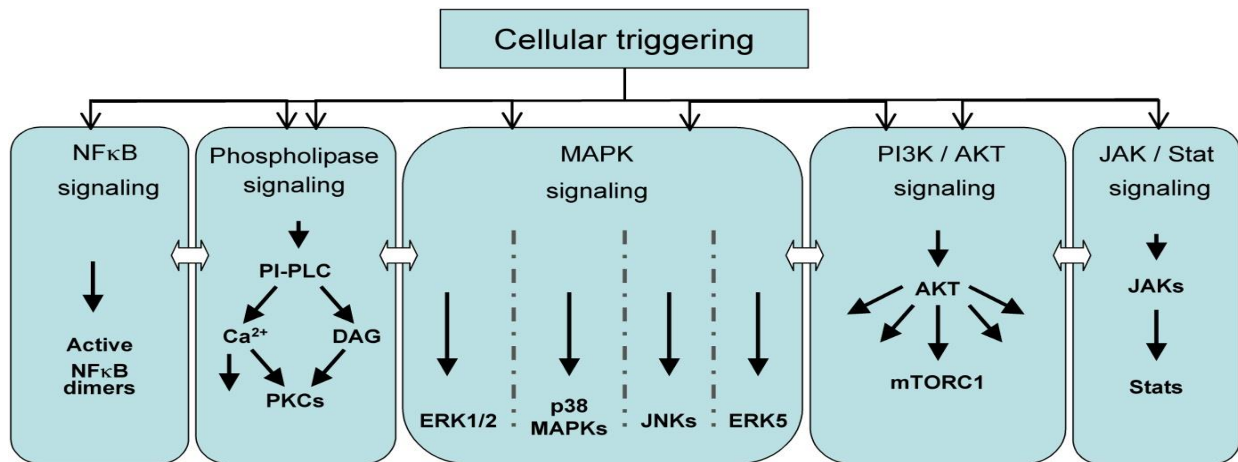

Figure 1: Intracellular signalling pathways that respond to external stimuli. Adapted with permission from (Nunès JA,et al. 2012) (4).

1.2 JAK/STAT pathway

The Janus kinase - signal transducer and activator of transcription (JAK/STAT) pathway is one of the intensely studied and best understood intracellular signalling cascades but still some gaps in the activation and regulation mechanisms have not been explained. This pathway is a handful of pleiotropic cascades used to transduce a multitude of signals for cell development and homeostasis (5).Therefore, dysregulation in this pathway is commonly associated with malignancies and immune disorders. It is regulated by many intrinsic and environmental stimuli, which add plasticity to the response of a cell or tissue (6).

Various cytokines and growth factors activate this pathway upon binding to specific receptors. In contrast to growth factor receptors which have intrinsic tyrosine kinase activity, most of the STAT-activating cytokine receptors are associated with a cytoplasmic protein called Janus kinase (JAKs) (7). JAK is relatively large protein of more than 1100 amino acids and has a molecular mass of 120 - 140 KDa. Alignment of the JAK sequences reveals seven regions of homology, named JH1 to JH7, from the carboxyl to the amino terminus (8) which is illustrated in Figure 2.

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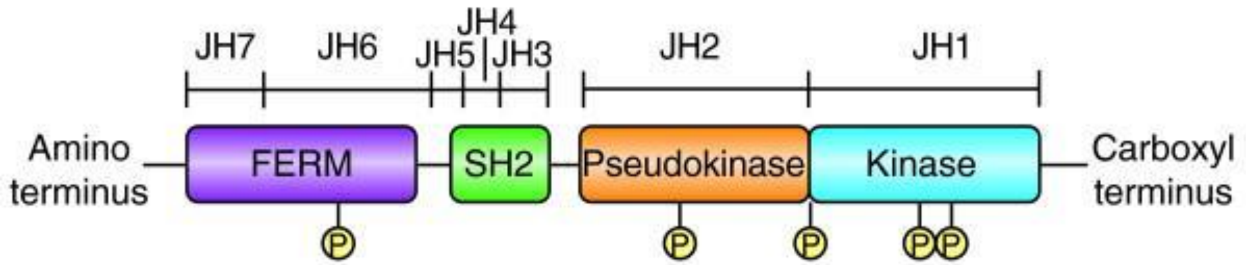

Figure 2: A schematic representation of the primary structure of Janus kinases (JAKs). Taken with permission from (Yamaoka K,et al. 2004) (9).

While the JH1 has the kinase domain responsible for the enzymatic activity; the JH2 has a pseudo kinase domain which is important to regulate the normal kinase activity. The function of SH2 domain which is shared between JH3 and JH4 is still unclear. JH6-JH7 is also called the FERM domain, mediates the adhesion of the JAK to trans membrane receptors.

The JAK family consists of four members: Janus kinase 1 (JAK1), Janus kinase 2 (JAK2), Janus kinase 3 (JAK3) and Tyrosine kinase 2 (TYK2). Initially, studies of interferon’s signalling showed that JAK1 or TYK2-deficient cells were unresponsive to IFNα/β, whereas JAK1 or JAK2-deficient cells were unresponsive to IFNγ. Subsequent studies indicate that the JAKs can be activated by not only interferon but also by other cytokines. Although specific JAKs are activated through each cytokine receptor and may partially contribute to specificity, the JAK kinases by themselves are clearly not an absolute determinant of the specificity in cytokine signalling because many different cytokines activate the same JAKs (10). In contrast to the JAK3 which selectively associate with γc receptors, the JAK1 is widely associated with different types of receptors such as INF receptors, gp130 cytokine receptors and γc receptors. Like JAK1, JAK2 is widely expressed and is involved in signalling by single chain hormone receptors, the common β_chain family, and certain members of the class II receptor cytokine family (11). TYK2, the founding member of the JAK family, appears to be most important in mediating the biological response to IL-12 and LPS (12).

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Table 1: The different cytokines that involved in the activation of each JAK member

JAK	**Cytokine**
JAK1	γc cytokines, gp130 cytokines, IL9, IL10, IFNα, IFNβ, IFNγ, IL13, IL15, IL4.
JAK2	Erythropoietin, gp130 cytokines, IL13, IL12, prolactin, IFNγ, GH, IL5.
JAK3	IL9, IL4, IL7, IL15, γc cytokines.
TYK2	IL13, IL2, IFNα, IFNβ, IL10.

Binding of a cytokine to its corresponding receptors initiates conformational changes in the cytoplasmic portion of the receptor, initiating activation of receptor associated members of the JAK family of kinases (12). Activated JAKs induce the phosphorylation of the receptor tyrosine kinase residues, creating a docking site for cytoplasmic phospho-tyrosine binding SH2 domain proteins such as STAT proteins. After being recruited to the receptors, STATs are also phosphorylated by the JAKs, bind to the SH2 domain of a second STAT leading to either homo or hetero dimerisation and rapidly translocate to the nucleus. Once in the nucleus, the activated STAT dimers bind to consensus DNA recognition motifs, called gamma activated sites (GAS), in the promoter regions of cytokine inducible genes, resulting in transcriptional activation (13). A remarkable feature of this system is that newly induced STAT-DNA binding activity can be detected in the nucleus within minutes of cytokine binding (14).

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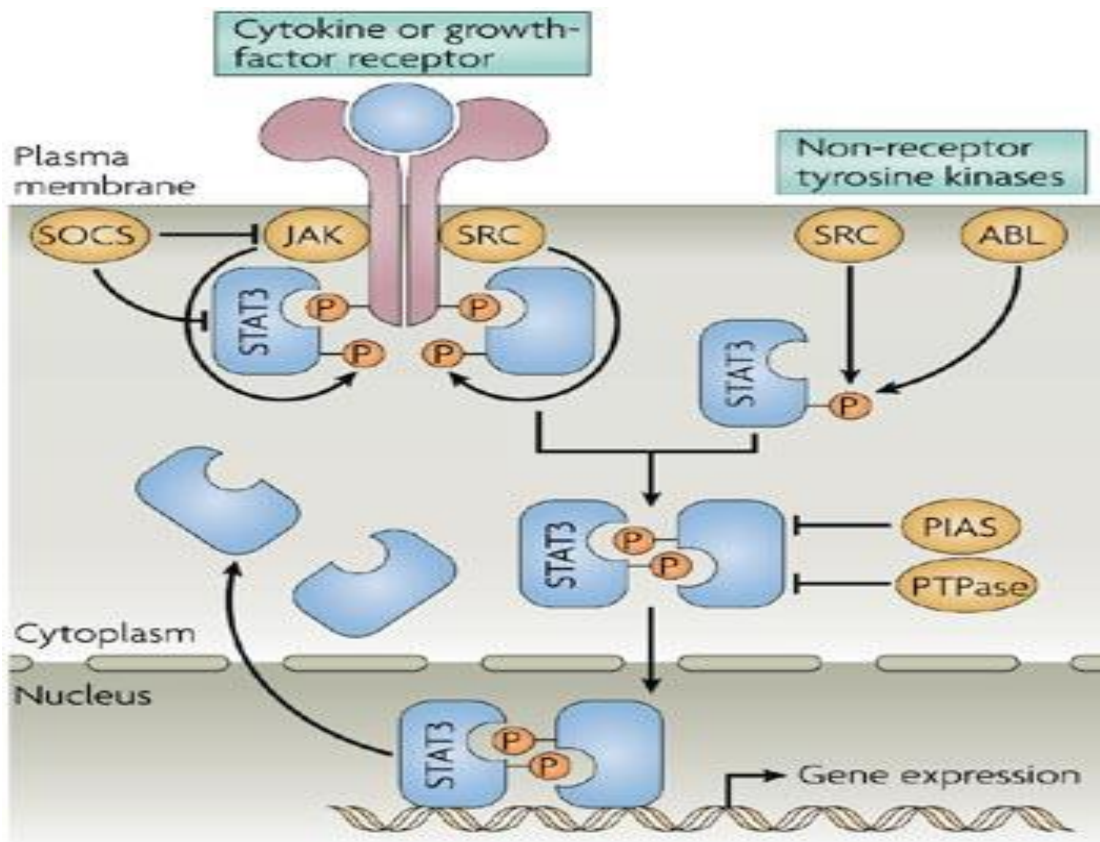

Figure 3: STAT3 signalling pathway. Upon binding of a cytokine to its receptor, activation of the receptor associated JAK will be initiated. Activated JAKs subsequently phosphorylate the receptor's tyrosine residues creating a site for STAT3 phosphorylation. Post phosphorylation, pSTAT3 molecules dimerise and translocate to the nucleus to enhance certain gene transcription. Modified with permission from (Yu et al, 2007) (15).

1.2.1 Negative regulation of the JAK/STAT pathway

Negative regulation of the JAK/STAT pathway can be achieved at several different levels, including down regulation of the receptor/ligand complex, degradation of signalling intermediates, inactivation of positive regulators by dephosphorylation, and activation of specific suppressors (16). Three families of proteins: the phosphotyrosine phosphatases, the SOCS proteins (Suppressors of Cytokine Signalling) and the PIAS (Protein Inhibitor of Activated STAT) are involved in this process (17). As tyrosine phosphorylation is an important step to enhance the signalling cascade, tyrosine phosphatases can down regulate the cytokine signalling by dephosphorylating the tyrosine residues in signalling intermediates such as the membrane receptors, JAK, and the activated STATs either in the cytoplasm or in the nucleus. The SOCS proteins are synthesized following cytokine stimulation and

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inhibit cytokine signalling by inhibiting JAK kinase activity or by binding to cytokine receptors (18). An interesting property of SOCS proteins is that they specifically affect the kinetics of JAK–STAT-signalling termination, but have no effect on the initial activation process or signalling strength (19). The eight identified members of the SOCS family: SOCS1 – SOCS7 and cytokine inducible SH2 (CIS) containing proteins, can also mediate proteosomal degradation of ubiquitin ligases at the receptor complex by a negative feedback of the signal. The transcriptional activity of STATs can be inhibited by a family of proteins named protein inhibitor of activated STAT (PIAS) (20). This family has four members: PIAS1, PIAS3, PIASx and PIASy. PIAS proteins bind only to the dimeric form of STATs and this indicates the importance of cytokine stimulation for mediating the PIAS-STAT association. Apparently, these members follow different mechanisms for inhibiting STAT mediating gene activation. In contrast to PIAS1 and PIAS3 which prevent DNA recognition by blocking the STAT- DNA binding activity, PIASx and PIASy inhibit this transcriptional activation by enhancing other co repressors. A nuclear form of STATs phosphatase called (TC45) inactivates STATs proteins by dephosphorylating the STATs dimers in the nucleus to be recycled back to the cytoplasm.

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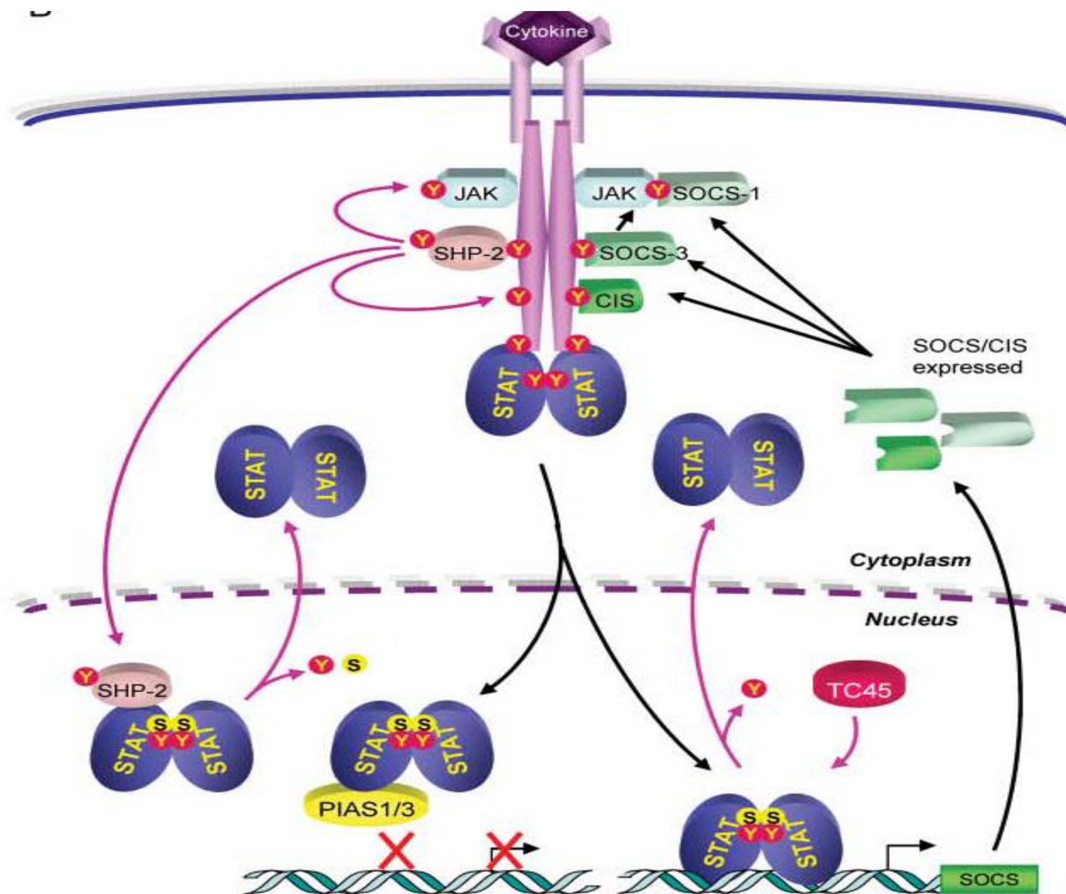

Figure 4: Negative regulation of STAT signalling at different levels. Following cytokine binding to its receptor and the activation of the receptor bounded JAKs, activated JAKs induce the phosphorylation of the receptor creating a docking site for the recruitment and phosphorylation of tyrosine residues (Y) of SHP-2, SOCS/CIS proteins and either tyrosine or serine (S) residues of STAT protein. Activation of SHP-2 dephosphorylates the receptor, JAKs and STATs while the expression of SOCS/CIS proteins leads to negative feedback inhibition of STATs activity. PIAS 1/3 block the binding of STAT's dimers to the DNA. TC45 is a nuclear phosphatase that dephosphorylates STATs in the nucleus to be shuttled back to the cytoplasm. Taken from (Lim CP,et al. 2006) (21) and reproduced by permission of The Royal Society of Chemistry.

1.3 Cytokine activated STAT

Subsequent to the characterization of different cytokines, numerous studies have been conducted to investigate cytokine induced gene expression through the JAK-STAT paradigm. Cytokines are small signalling proteins, peptides or glycoproteins transiently produced in low concentrations by specific immune cells and affect other target cells. On the target cells, the cytokine binding to the extracellular moiety of the receptor induces a coordinated series of events within the cell initiated by the

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receptors intracellular moiety that leads to changed gene expression patterns, altered cytoskeleton organization, or release of secretory vesicles (22). Due to their pleiotropic activities, different cell types can respond differently to a single cytokine. Cytokines play pivotal roles in a variety of responses including the immune response, haematopoiesis, embryogenesis and oncogenesis (23).

Regardless of the when and where cytokine receptors and JAKs associate, their close apposition at the membrane is required to stimulate the kinase activity of the JAK following cytokine binding (24) which, in turn, enhances the activation of intracellular STATs. Each of the seven human STATs can be activated by different cytokines or growth factors. Although, both IL-10 and IL-6 act via the same signalling pathway (JAK/STAT) and activate STAT3, the intracellular protein that is the focus of this study, they elicit different biological responses.

1.3.1 Interleukin 10

Interleukin 10 (IL-10) is 18.5 KDa, homodimeric protein that is encoded by *IL-10 gene* which is located on q32 on chromosome 1. It is a pleiotropic cytokine with diverse immunoregulatory activities. In response to different stresses, IL-10 is produced by monocytes, macrophages and both T and B lymphocytes. By acting on different cell types, it regulates growth and/or differentiation of B cells, NK cells, cytotoxic and helper T cells, mast cells, granulocytes, dendritic cells, keratinocytes, and endothelial cells (25). However, it not only suppresses macrophages activation but also inhibits their cytokine secretion. Additionally, it is considered a potent anti-inflammatory cytokine as it has the ability to repress the expression of the pro-inflammatory cytokines such as TNFα, IL-1, IL-6 and IL-8. Moreover, it was reported that IL-10 produced by monocytes have a strong auto regulatory feedback activity on T cell activation due to its ability to down regulate Class II MHC antigen on these cells (26). Taking these properties together, it seems that IL-10 can regulate both the inflammatory and the immune response. Therefore, dysregulation of IL-10 cytokine results in acute and chronic inflammation as well as autoimmune diseases (27). Recently, studies have shown a contradictory effect of IL-10 in different cancer types. Not only its immunosuppressive and anti-inflammatory functions but also its ability to modulate apoptosis and suppress angiogenesis may explain its anti-tumour activity. However, these functions may also explain the opposing effect of IL-10 as its immunosuppressive effect may favour tumour escape from an immune attack. In

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addition, it can activate some anti- apoptotic genes through activation of STAT3. These dramatically opposing effects of IL-10 might depend on interactions with either cytokines or factors found in the tumour microenvironment, as it is unlikely that IL-10 functions in isolation (28).

IL-10 receptor is a heterotetramer complex consisting of two identical (IL-10R1) subunits and two accessory (IL-10R2) subunits. The IL-10R1 chain plays a dominant role in mediating high affinity ligand binding and signal transduction, whereas the IL-10R2 subunit is thought to be required for signalling only (29). Binding of IL-10 to its receptor induces the activation of STAT3 which, in turn, decreases the expression of inflammatory cytokines. Moreover, STAT3 promotes the expression of SOCS3 which negatively regulates IL-10/STAT3 signalling. Although IL-10 transduces signalling through the JAK/STAT pathway, this does not exclude the potential for parallel activation pathways (30) which can explain the diverse function of a single cytokine on a certain type of cells.

1.3.2 Interleukin 6

Interleukin 6 (IL-6) is a single chain glycoprotein that is encoded by *IL-6* gene which is located on chromosome 7. It is produced by different cell types such as monocytes, macrophages and T and B-lymphocytes in response to various stimuli including IL-1, INF, TNF, bacterial endotoxins and viral infection. Stimulated monocytes, the main source of IL-6, produce five different forms with molecular masses ranging between 21 - 29 KDa. IL-6 exerts its biological activities through a unique receptor system that is mainly expressed in haemopoietic cells. The receptors are a heterodimer complex that is composed of two different subunits: alpha subunit and the glycoprotein 130 subunit. While the alpha subunit (IL-6Rα) is responsible for ligand binding, the glycoprotein subunit (gp) is important for the signal transduction. Gp130 constitutively associates with JAK1, JAK2, or TYK2 and contains five tyrosine motifs for the phosphorylation dependent recruitment of signalling molecules such as STAT3 (31). By binding to its receptor, IL-6 induces homo or hetero dimerisation of the gp130 subunits, leading to activation of the receptor associated JAKs and subsequently phosphorylation of the cytoplasmic STATs.

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IL-6 is also a pleiotropic cytokine with a wide range of biological activities in immune regulation, haematopoiesis, inflammation and oncogenesis (32). Indeed, it promotes inflammatory events through the expansion and activation of T cells, differentiation of B cells, and the induction of acute-phase reactants by hepatocytes (33). Overproduction of IL-6 has been detected in acute and chronic inflammatory diseases. Recently, it has been found that high levels of IL-6 are associated with certain types of cancer. This may be due to the regulatory effect of IL-6 in chronic inflammation which can create a cellular microenvironment beneficial to cancer growth (34). In addition, it activates STAT molecules which are essential in transcribing genes responsible for cell proliferation and apoptosis. Taken together, these findings indicate that blockade of IL-6 signalling may be an effective therapy for many chronic inflammatory and autoimmune diseases and might be used for the prevention of inflammation-associated cancer (35).

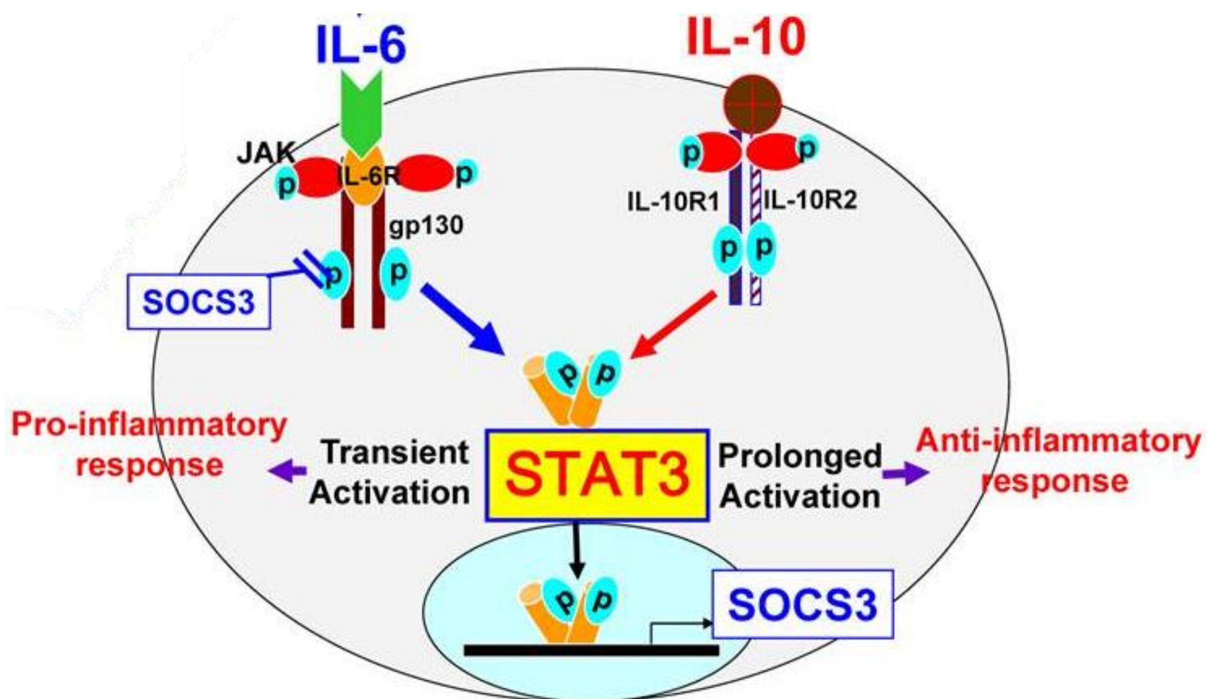

Figure 5: STAT3 signalling following IL-6 and IL-10 binding to their receptors. Modified from (Wang H,et al. 2011) (36). Article distributed under the terms of the Creative Commons License (http://creativecommons.org/licenses/by-nc-nd/3.0/).

Upon binding to their corresponding receptors, both IL-6 and IL-10 can activate STAT3 and subsequently up regulate the expression of SOCS3. However, these cytokines have different sensitivities towards the feedback inhibition of SOCS3.

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While SOCS3 can rapidly inhibit the IL-6 signalling activation by binding to gp130 subunit, it does not affect IL-10 activation of STAT3. This explains the rapid and transient activation of STAT3 by IL-6 compared with the prolonged IL-10 induced STAT3 activation.

1.4 Signal transducer and activator of transcription (STAT)

Originally, STAT proteins were discovered during Interferon (IFN) signalling studies. The initial finding of a family of related proteins, each activated by a different cytokine receptor, suggested that these proteins would fulfil the requirements predicted for carriers of intracellular signalling information capable of retaining the specificity inherent in cytokine receptor interactions (37). As their name implies, this family of proteins has the dual function of transducing signals from the cell surface to the nucleus and activation of gene transcription (38). To date, seven STAT members have been identified: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 (39). Their diversity in response to extracellular stimuli is contributed to their difference in amino acid sequence, tissue distributions, and their ability to form both homo and hetero dimers. They are rapidly activated in response to ligand receptor coupling and are recruited to the intracellular domain of the receptor through specific binding between STAT Src-homology 2 (SH2) and the receptor phosphotyrosine residues (6). Although this activation is essential to induce gene transcription, it has been found that STAT 1 and 3 (and probably other STATs) play important roles in mediating gene expression without tyrosine phosphorylation (40). Extended stimulation of the pathway to form phosphorylated STAT3 has been reported to lead to a remarkable increase in unphosphorylated STAT3 level by activating its gene expression and this increased level of unphosphorylated STAT3 persists for several days, long after the initial amount of pSTAT3 has returned to baseline levels (41). By involvement in other signalling pathways, unphosphorylated STAT can transcriptionally activate other genes distant from those activated by the phosphorylated STAT (42). Furthermore, accumulating observations suggest that dimerisation of STAT proteins is independent on tyrosine phosphorylation and can be taken place prior to activation. The dimers of the unphosphorylated STAT can exist in two crystal structures. The dimer is either formed by antiparallel interactions between the coiled-coil and DNA binding domain or the STATs can joint in a parallel manner keeping their N-termini lodged in between.

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Structurally, STATs have different functional motifs such as an amino terminus that plays a role in dimerisation, a coiled coil domain that can be involved in interactions with other proteins, a central DNA binding domain (DBD), a Src homology 2 (SH2) domain, a conserved tyrosine residue that is phosphorylated in response to stimuli, and a carboxyl transcriptional activation domain (TAD) (43). STATs can be phosphorylated at either a tyrosine site close to the carboxyl domain or at serine site within the transcriptional domain. Although the carboxyl segment is the one responsible for transcriptional activation in all STATs, the other domains still play a role in this activation.

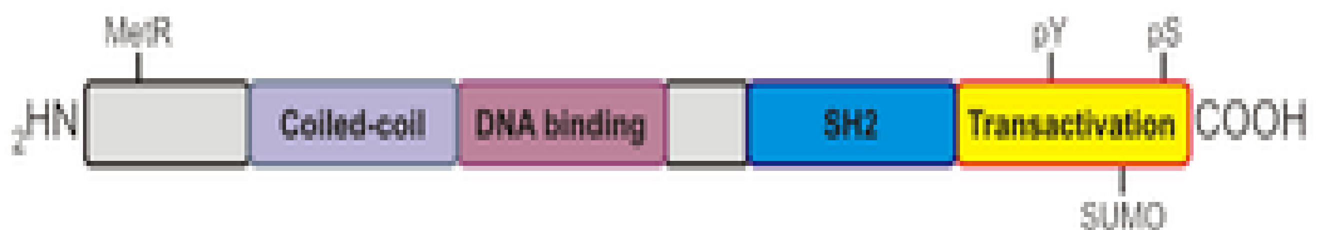

Figure 6: STAT functional domains (Modified from Wikipedia with a public permission from the author). Available at en.wikipedia.org*/wiki/STAT_protein.*

1.4.1 STAT3

STAT3 is a cytoplasmic protein encoded by *STAT3* gene which is located at position 21.31 on the long q arm of chromosome 17. It is activated in response to several cytokines and growth factors, including IL-6, IL-10, epidermal growth factor (EGF), and interferon (IFNα). STAT3 is also weakly activated in response to other cytokines, including IFNγ (44).

STAT3 is structurally similar to other STATs members, having the six domains that previously mentioned. Upon binding to the phosphotyrosine site on the receptor, STAT3 is activated by phosphorylation of the tyrosine residue (Y705) or the serine residue (S727). The tyrosine phosphorylation, in turn, promotes dimerisation and translocation of STAT3 to the nucleus where the transcription of specific genes is activated. However, the serine phosphorylation has been reported to enhance the recruitment of cofactors potentiating tyrosine phosphorylation (45) and maximal transcriptional activation (46) of responsive genes.

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Conversely, other studies (47, 48) have shown that serine phosphorylation either inhibits or has no effect on tyrosine phosphorylation or DNA binding. Thus, the kinases that phosphorylate STATs on serine-727 and the functional significance of serine phosphorylation will likely vary according to extracellular stimulus, cell type, and activation status of the cell (49).

Some of the identified STAT3 targeted genes are alpha-2-macroglobulin, fibrinogen, alpha1 acidic glycoprotein and matrix metalloproteinase (MMP)-1, MMP-3, MMP-9 (50).

Functionally, STAT3 is essential for early embryogenesis and it is the only STAT member where its ablation leads to embryogenic lethality. It is also plays a role in cell growth, survival, differentiation and apoptosis. This contradictory involvement of STAT3 in different opposing processes appears to be a target tissue dependent. In addition, STAT3 participates in a variety of biological processes such as the induction of acute phase protein synthesis in hepatocytes, the regulation of haematopoiesis and the immune response (51). Studies of tissue-STAT3 gene deletions have shown that STAT3 is critical for epithelial cell apoptosis and involution in the post-lactating mammary gland, skin remodelling and keratinocyte migration, macrophage inactivation and down regulation of inflammatory cytokines in T-helper cell responses (52). Activated STAT3 limits the responsiveness of activated macrophages and other haematopoietic cells to inflammatory signals and tumour antigens (53).

Of great current interest is the persistently active STAT3, which is known to occur in a wide variety of human tumours (54) such as prostate, pancreas, breast and blood cancers. Being oncogenic, STAT3 activates genes that block apoptosis, favour cell proliferation and survival, promote angiogenesis and metastasis, and inhibit antitumor immune responses (1). Studies are being performed to assess the potential value and the therapeutic effect of STAT3 in cancer treatment. Although the inhibition of tyrosine kinases could potentially activate alternative pathways leading to increased metastasis and tumour growth, *STAT3* gene ablation in diverse tumour models results in the inhibition of tumour growth (55). Nevertheless, STAT3 has emerged as an important target for cancer immunotherapy, either alone or in conjunction with other promising immunotherapeutic approaches (56), and

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discovering specific, effective and safe drugs is still a big challenge. Researchers are working hard to identify selective inhibitors of STAT3 without affecting the activity of STAT1 which in contrast to STAT3 has a tumour suppressor activity despite having a similar amino acids sequence.

Analysing STAT3 signalling in primary cells is a key to understanding its physiological relevant function (13). The ability to measure disrupted signalling across multiple pathways within multiple cell types provides a framework to assess the network state of the disease for an individual patient at one moment in time (14). This can apply in many diverse areas including the characterization of signalling pathways in normal immune response to antigenic stimulation and microbial challenge, alteration of signalling networks that occur in cancer and autoimmune diseases and high throughput, high content drug discovery (16). These measurements provide insights into pathology and can be used as a tool for diagnosis, prognosis and therapy development.

Traditionally, cell signalling analysis has been performed using western blots on large populations of homogenous cells (17). Recently, the development of multi-parametric flow cytometry, the availability of phosphoprotein-specific antibodies and sophisticated software has improved not only the analysis outcome but the convenience and time as well.

1.5 Flow cytometry

Flow cytometry has been long known simply as a research tool of immunologists used to identify various cellular characteristics or phenotype of primary cell samples (57). Recently, it has been used for a range of different applications such as immuno-subphenotyping, intracellular function assessment, cell cycle analysis, cell viability and apoptosis and kinetic studies. Major advances have been made in both flow cytometry instrumentation and applications, expanding the number of possible simultaneous analysis parameters to 13 or more (58). Concomitant with this development, our understanding of immunology and stem cell biology has matured tremendously (59).

As its name implies, it is the measurement of cells in a flowing system. However, it is newly begun to be used for the analysis of particles that are not considered as cells such as viruses, chromosomes and DNA fragments.

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1.5.1 General principle

The basic principle of flow cytometry depends on passing thousands of cells per second through a light beam (mainly laser light) and measuring the light that is scattered or emitted from each cell. To achieve these measurements, a flow cytometer employs lasers, fluidics, optics, detectors, measuring circuits, and computer electronics (60).

In brief, the sample is injected into the central channel of the fluidics system that is surrounded by a fast flowing sheath fluid in a narrow channel that produces a drag effect on the central channel creating a single ordering of the particles in the sample called ”hydrodynamic focusing”.

After hydrodynamic focusing, each particle passes individually through a beam of laser light (61). Lasers have the advantage of producing an intense beam of monochromatic light which in some systems may be tuned to several different wavelengths (62). As the cell pass through, the laser light is scattered in different directions giving information about the physical properties of the particles analysed. Forward scatter tends to be more sensitive to the size and surface properties and can be used to distinguish live from dead cells while side scatter tends to be more sensitive to inclusions within cells and can be used to distinguish granulated from non-granulated cells (61). Depending on both forward and side scatter, different cell types in a heterogeneous population can be identified. For example, in the simple discrimination between peripheral blood cells, monocytes and granulocytes show higher forward and side scatter than Lymphocytes and circulating dendritic cells (intermediate forward and side scatter) while platelets and erythrocytes show low forward and side scatter (63).

Additional photo-detectors in a flow cytometer are used to provide information about the biochemical characteristics of a cell; they collect light of different colours, emitted by fluorescent molecules within or on the cell surface when the molecules absorb laser light and emit light of a longer wavelength (64). These fluorescent molecules could be natural molecules such as flavins and NADPH or fluorochromes that are conjugated to antibodies directed against a molecule of interest. Emitted light is given off in all directions and is collected via optics that direct the light to a series of filters and dichroic mirrors that isolate particular wavelength bands (65) by blocking

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certain and transmitting others. The light scattering and colour discriminated fluorescence of the cells is picked up by detectors that convert the intensity of light into a digital signal which can be statistically analysed. Two types of detectors are mainly used: the silicon photodiodes detector which is sensitive to the forward scatter signalling and the photomultiplier tube detector which is commonly used for both scatter and florescence detection. Once the light signals, or photons, strike one side of the photomultiplier tubes or the photodiode, they are converted into a proportional number of electrons that are multiplied, creating a greater electrical current (66). The voltage of this current is then subjected to either a linear or logarithmic amplification. The logarithmic amplification is widely used especially for fluorescence signal measurement. This amplification has the advantage of compressing the scale of strong signals and expanding the scale of weak signals. Finally, the voltage signal converts to digital signal by analogue-digital converter (ADC). A computer connected to the flow cytometer captures the digital signals and analyses the data by specific software. The data can be presented in different ways such as density plots, counter diagram and single parameter histograms.

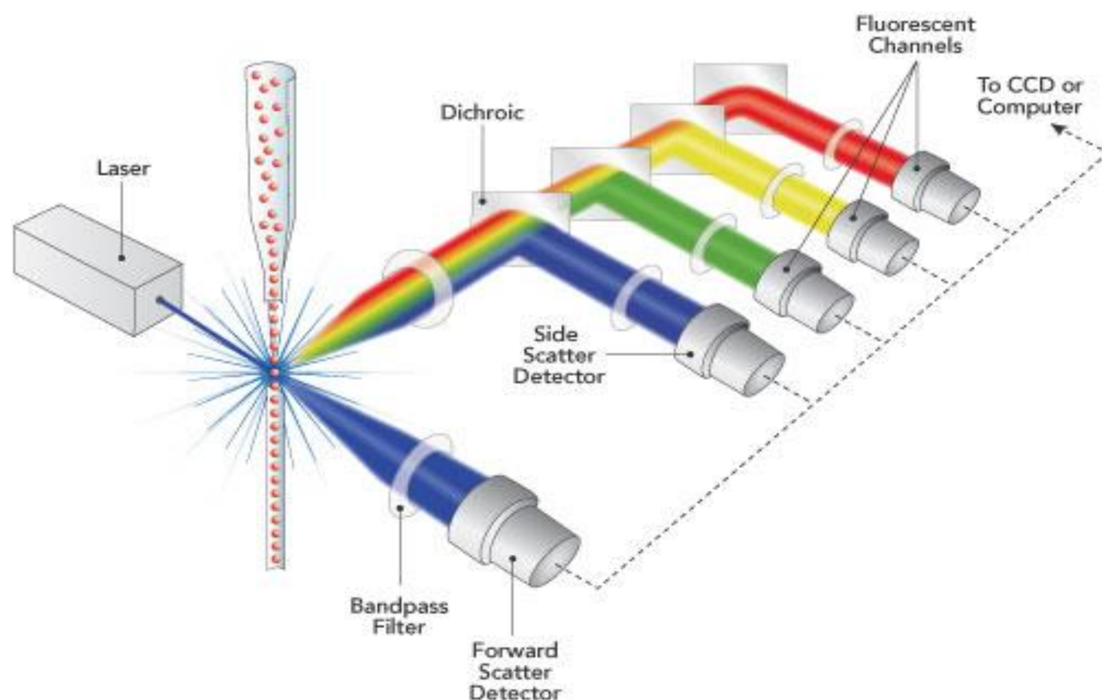

Figure 7: The major components of flow cytometer (modified with permission from Semrock), *www.semrock.com/flow-cytometry.aspx*.

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1.5.2 Clinical application of flow cytometry

Flow cytometry is one of the rare disciplines that historically entered into the clinical area before its capabilities were fully translated to the basic sciences (57). Its applications have been grown in the past decade and recently it is widely applied in various clinical fields such as molecular biology, immunology, pathology and medicine.

Owing to the simultaneous multi-parametric measuring capability of flow cytometry, many different phosphoproteins can be analysed instantaneously, allowing the interrogation of multiple upstream and downstream signalling components within individual cells (67). By comparing the differences in the signalling networks between healthy and diseased cells, signalling dysregulation associated with a particular disease can be identified. However, as signalling abnormalities can also happened in some genetic disorders, combining both the immunophenotyping of the cell population with the signalling network may be a better predictive tool for diagnosis and therapy development. Sequential measurements of the network state over time may provide important insights into the pathophysiological characterisation (68).

The smaller, less expensive instruments and an increasing number of clinically useful antibodies are creating more opportunities for routine clinical laboratories to use flow cytometry in the diagnosis and management of disease (65). Additionally, it is recently involved in clinical trials of therapy development to assess the specificity and the efficacy of the drugs.

1.6 Flow cytometric analysis of intracellular phosphorylated proteins

Recent advances in the instrumentation and commercially available reagents for multiparametric flow cytometry have encouraged the development of intracellular staining techniques to assess the status of signalling proteins that, when phosphorylated, translocate to the nucleus, such as STATs (69). However, measuring intracellular antigens by flow cytometry is not a simple process as the phosphorylation states of individual signalling elements change rapidly in response to stimuli and therefore may be subjected to changes during sample collection, storage, preparation and staining that may also obscure the signalling state of the condition under study (70). Therefore, optimization of the analysis protocol is important to get relevant results. Furthermore, some points should be taken into

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consideration for successful application such as rapid termination of the transient signalling, efficient permeabilisation of the cells and identification of cells of interest in heterogeneous population.

In general, flow cytometric detection of intracellular phospho-proteins involves cell stimulation, fixation, permeabilisation and probing with the appropriate fluorophore conjugated antibody.

1.6.1 Cell stimulation

Live cells of interest are activated using a specifically chosen modulator (e.g. growth factors, cytokines, hormones, and may include drugs) at predetermined concentrations and exposure times; subsequent to the modulation event (71). There are several points that should be taken into account for optimal stimulation. Importantly, Ca^{++} should be included in the stimulation media as it is an essential element in the cell signalling pathway. Secondly, the optimal temperature for cell stimulation is 37°C which should be kept constant during the stimulation periods as any slight change in temperature can dramatically affect the protein phosphorylation. Thirdly, the time of activation should be as brief as possible to be sure that direct signalling events following stimulation are being analysed (69).

1.6.2 Fixation

Because of the transient nature of intracellular signalling events, fixation techniques used for phospho-specific analysis must be rapid and efficient at freezing signalling to prevent dephosphorylation or further phosphorylation (58). Choosing the appropriate fixative is important step as some fixatives such as glutaraldhyde may increase the autofluoresence of single cells. Other fixatives may affect the scatter properties of the cells leading to difficulty in gating a specific subpopulation in heterogeneous cell mixture. Recently, it was found that formaldehyde fixation followed by methanol permeabilisation was the method of choice for flow cytometric analysis of many key intracellular signalling molecules of the immune system especially those of the STAT protein family (72). Formaldehyde performs two critical roles. Firstly, it freezes cellular processes by cross-linking proteins to one another and to themselves, likely creating a lattice of static proteins within the cell and preventing further signalling (65). Secondly, it stabilizes cell structure, a process which could be considered critical for experiments involving human peripheral blood

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cells (65). However, crosslinking fixatives can also cause artefacts either by linking low molecular-weight antigens to larger structural proteins or by causing steric blockage of antibody access to the antigenic epitopes (73).

1.6.3 Permeabilisation

In order to help the probed antibodies to get access to their intracellular phosphorylated target proteins, the cells must first be treated with a permeabilising agent that produces pores in the outer cell membrane. Two general types of permeabilising agents are commonly used: organic solvents, such as methanol and acetone, and detergents such as saponin, Triton X-100 and Tween-20 (74). Generally, detergents are mainly used to permeabilise cholesterol containing membranes, allowing for efficient cytoplasmic proteins detection while alcohol permeabilisation provides efficient nuclear proteins detection. Detergents can reversibly permeabilise the cell membrane by solvating the sterol molecules without affecting the mitochondria or nuclei membranes. This permeabilisation maintains sufficient cell integrity enabling for cell types differentiation. Furthermore, it does not alter membrane expression of many antigens (75).

Methanol has a rapid and effective permeabilising effect over other permeabilising agents. It is thought that alcohols fix and permeabilise cells by dehydrating them and solubilizing molecules out of the plasma membrane (76). This dehydrating effect allows for efficient access of antibodies even to nuclear antigens. Additionally, methanol permeabilisation has the ability to denature proteins. Protein denaturation is critical in staining intracellular phosphorylated proteins which primarily dimerise following activation. Methanol also has the benefit of allowing samples to be stored over time, a consideration for clinical samples or samples in which the analysis is not immediately possible (77).

Conversely, methanol permeabilisation compromises the labelling of some surface antigens and dramatically changes the cells light scattering profile leading to difficulty in subpopulation gating due to changing scatter characteristics. Furthermore, treating the cells with methanol can lead to cell clumping which can be eliminated by prior fixation with paraformaldehyde and constant gentle vortex mixing of the cells during methanol addition.

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Careful consideration should therefore be given not only to the choice of permeabilisation buffers, but also to the cell surface antibody clones to be used during the experiment (78). Moreover, limitations such as insufficient access to target antigen, cell losses, formation of small cellular aggregates, alteration of cell morphology, and light scatter patterns are commonly encountered during membrane permeabilisation and can hamper the consistency and reproducibility of the results (79).

1.6.4 Monoclonal antibody probing

In recent years, specific and high-affinity monoclonal antibodies have been developed that recognize a wide range of phosphorylation sites on signalling proteins (80). These antibodies are combined with flow cytometry to perform quantitative multi-parameter analysis of single cells (81). Either direct or indirect staining methods can be applied and each has its advantages and limitations. The direct method is a single step in which the cells are incubated with antibody directly conjugated with specific fluorochromes. Although this technique is rapid and simple it is more expensive and less sensitive.

On the other hand, the indirect method depends on incubating the cells with unlabelled primary antibody, wash and then incubates with secondary antibody labelled with specific fluorochromes. However, despite of its sensitivity, inexpensiveness and its ability to amplify the signal, it increases the staining time and the potential for cross reaction. Additionally, the primary antibody should be generated from species differ from the secondary antibody generated species.

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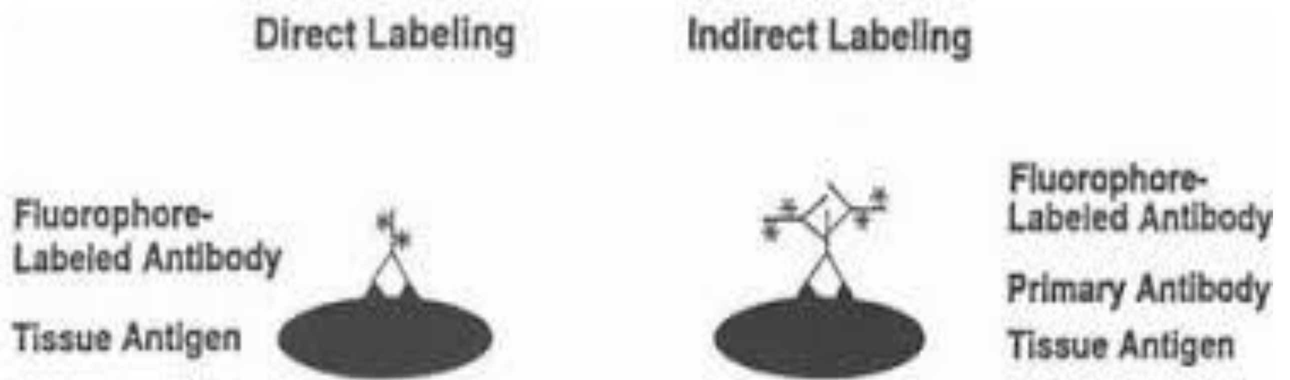

Figure 8: Schematic diagram of both direct and indirect staining. Modified from (Javois LC, 1999) (82) with permission from Springer.

Two characteristics should be considered upon choosing the antibody conjugated fluorophore. Firstly, it should be excited by the wavelength of the laser light present in the flow cytometer used for the analysis and its emission wavelength should be detected by the available filters sets on the instrument. Secondly, the fluorophore should not affect the antibody binding ability.

Accurate measurement of antigen-positive cells by flow cytometry can be hampered by background fluorescence of antigen-negative cells and other particles (83). Three causes can lead to background fluorescence and increased noise: auto fluorescence, spectral overlap and nonspecific antibody binding.

Auto fluorescence is a result of the excitation of the naturally present molecules in the cells and this can be minimized by comparing the stained cells with fully processed, unstained sample.

When more than one fluorochrome is used in the same experiment, there is a great chance for their emission spectra to overlap leading to a difficulty in measuring the fluorescence emitted from each fluorochrome. To overcome this problem, it is better to use fluorochromes with very different emission spectra. If this is not possible, then fluorescent colour compensation should be applied in which the percent of the emission bleed of one of the fluorochromes in a specific filter should be subtracted from the total fluorescence that is detected by this filter. Fortunately, modern software can automatically apply this step.

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Generally, nonspecific antibody binding is defined as binding of the antibody to other epitopes rather than the one it is designed against. Not only using a blocking agent but also the optimization of the antibody concentration should be considered to minimize the undesirable non-selective binding of the antibody. Figure 9 summarizes the main steps in the protocol for intracellular antigen detection by flow cytometry (78).

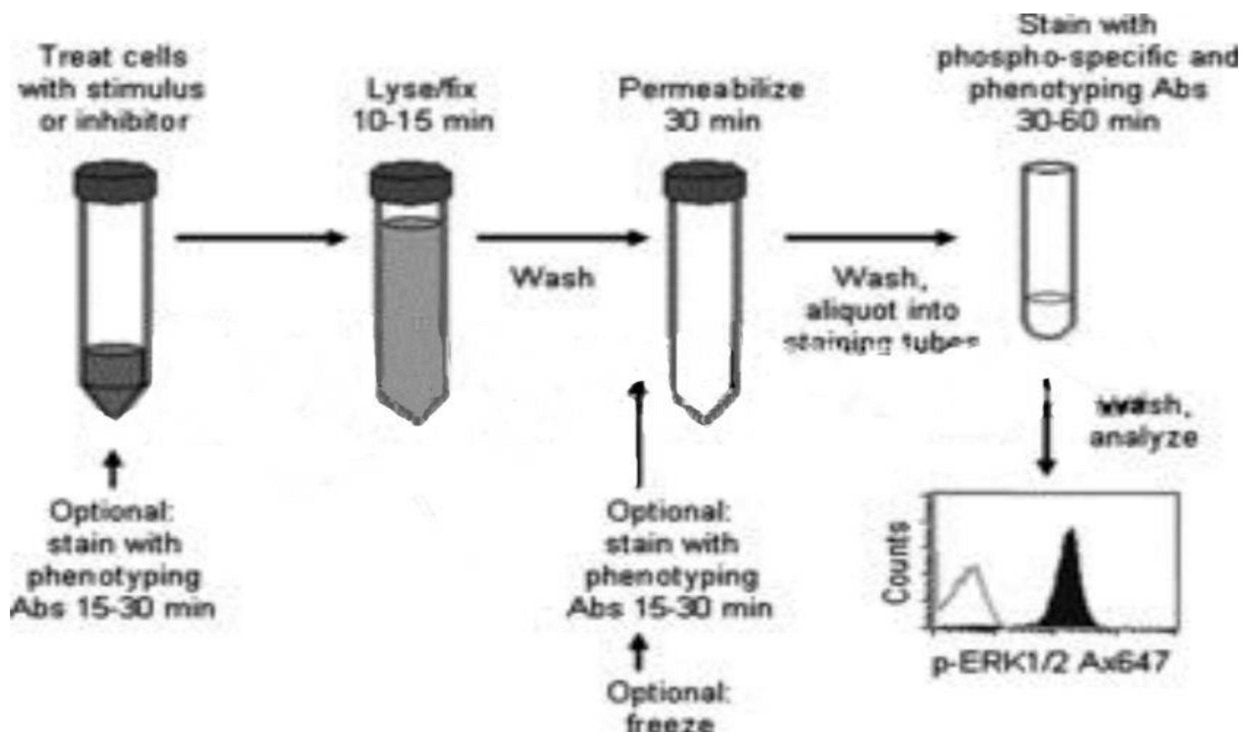

Figure 9: The main steps in the protocol for intracellular antigen detection by flow cytometry. Adapted from (Suni MA ,et al. 2011) (78)) with a permission agreement from Springer.

1.7 Advantages and limitations of flow cytometry

Flow cytometry has been verified as a rapid and quantitative technique in the clinical research. It has the ability to distinguish heterogeneous cell populations, collects data from single cells and correlates multiple markers simultaneously. It allows the analysis of rare subpopulations due to the small sample size requirement for this technique. When compared with other strategies, flow cytometry offers a time saving technique with less manipulation and separation steps. Furthermore, it offers greater sensitivity and allows one to identify individual cells with differences in responsiveness, rather than a mean value of the total cell population (84). On the

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other hand, flow cytometry still has some limitations. Firstly, it cannot give information about the localization of the antigen. Secondly, it lacks the sensitivity in detecting low abundant signalling proteins leading to a low signal to noise ratio. Thirdly, different subpopulations with similar markers are required the employment of more fluorophores leading to more signal spillover. Fourthly, as the cells pass through a fluid stream, the flow cytometric analysis is restricted to cells in suspension which can be difficult to get in some cells types. Moreover, there is loss of architectural relationships between different cell structures (85). Finally, In many cases, the fluorescent intensities of the phosphorylated proteins of interest are still quite weak in most phosphoflow applications which may limit the sensitivity of detecting and measuring more subtle changes in signalling pathways and signalling intensity in cells (86). Due to all these limitations, it is advisable to compare the results of flow cytometry technique with other strategies results such as western blot for validation.

1.8 Western blot assay

The need for a technique that could allow the determination of specific antigens specificity of antisera led to the development of a method that allowed the production of a replica of proteins, which had been separated electrophoretically on polyacrylamide gels, on to a membrane that is probed using specific antibodies (87). This method is referred as Western blotting or Immunoblotting which was developed in the late 1970s by Harry Towbin for ribosomal protein detection (88). However, it is now used in many applications in science.

Generally, this assay includes a sequence of several steps such as sample preparation, gel electrophoresis, electro blotting onto a membrane, blocking the membrane, probing and detection of the protein.

During the sample preparation of intact cells, a cell lysis buffer should be used to enable cell lysis and protein extraction. This should be done at cold temperature and in the presence of both protease and phosphatase inhibitors to avoid protein degradation and dephosphorylation of modified proteins. It is prudent to consider that the lysis buffer should allow for optimal protein extraction without affecting the gel electrophoresis, immunoblotting or the protein antigenic sites.

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Differences in the protein levels between the tested samples can be roughly quantified by normalizing the level of expression of the target protein to a loading control protein with constant levels of expression, such as the cytoskeletal β-actin.

Gel electrophoresis is mainly used to separate the proteins depending on their molecular weight, electric charge, isoelectric point or combination of all these factors. The most common type of electrophoresis uses polyacrylamide gels and buffers loaded with sodium dodecyl sulphate (SDS) (88). In short, the sample should be diluted with a loading buffer which mainly contains SDS and a reducing agent. Treatment with this combination facilitates protein denaturation and removes the secondary and tertiary structure by breaking the disulfide bonds. Furthermore, it binds in a fixed mass ratio to create negatively charged proteins in the lysate, allowing for separation according to their molecular weight. Glycerol should be added to the loading buffer to increase the density of the samples, helping them to sink and layer in the gel wells. Bromophenol blue is an indicator that is also added to the buffer to indicate the sample during loading and as a migration marker. When the voltage is applied to the polyacrylamide gel, the proteins migrate through the gel at different speeds according to their molecular weight resulting in different bands in each sample lane. Molecular weight markers, a mixture of proteins with known molecular weights, can be run in parallel with the sample to monitor the progress of running and orient the immunoblotting.

After electrophoresis, an electro-blotting method is applied to transfer the separated proteins from the polyacrylamide gel onto a nitrocellulose or polyvinylidene difluoride (PVDF) membrane to make them accessible to a probe antibody. Nitrocellulose membrane was used for a long time as it is cheaper and has excellent protein binding affinity. However, it is brittle and can’t be used in repeated probing. On the other hand, PVDF membrane possesses more mechanical strength for re-probing, but can exhibit higher background staining which should be considered.

The electrophoresis gel and blotting membrane are assembled into a sandwich along with several sheets of filter paper which protect the gel and blotting membrane and help to ensure close contact between their surfaces (89).

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Due to the high protein binding affinity of the membrane, a nonspecific protein binding blocking step should be performed prior to the probing with the selected primary antibodies. This step will decrease the "noise", helping the antibody to bind to its specific target protein. Often, non-fat dried milk is used for this purpose, however, bovine serum albumin (BSA) is the blocking agent of choice for phospho-protein detection when using pan-phospho-tyrosine, -serine, or –threonine antibodies, as milk contains many phosphorylated proteins (60).

Finally, the membrane is incubated with primary antibody to probe the protein of interest. For better sensitivity, optimal antibody concentration should be determined. In order to reveal the location of the probed antibody, direct or indirect detection can be used. In contrast to the direct detection in which the primary antibody is covalently linked to a label molecule (enzyme or fluorescent dye), the indirect detection relies on exposing the primary antibody-probed membrane to a secondary antibody directed against the primary antibody and conjugated with the label molecule.

Protein visualization can be accomplished by colorimetric, fluorescent, chemiluminescent, or radioactive detection (90). By using the appropriate instrument, fluorescent tags can easily be observed and the fluorescence signal can be measured. The most commonly used secondary-linked enzyme is a horse radish peroxidase (HRP) which can be detected by either colorimetric or chemiluminescent substrates.

Colorimetric detection depends on the reaction between the enzyme and a soluble dye resulting in a precipitation of an insoluble highly coloured form of the dye, with different colour next to the enzyme, which can be evaluated by spectrophotometry. On the other hand, the efficiency of the western blot was improved with the discovery of the chemiluminescence technique that allows the use of non-radioactive reagents and an increased sensitivity and higher stability of the detection signal (91). Enhanced Chemiluminescence (ECL) detection depends on exposing the enzyme to a chemiluminescent substrate producing luminescence related to the amount of the protein. However, it should be taken into consideration that the enzymatic reaction is dynamic and change over time making it necessary to optimize the reaction time and imaging (92). This luminescence can be detected by either a photographic film or charged-couple device camera (CCD camera). Although the X-ray film is still widely

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used and more sensitive, CCD imaging systems eliminate the hassles of film handling, have a large dynamic range and exposure control and accompanying analytical software that enables densitometry analysis (93).

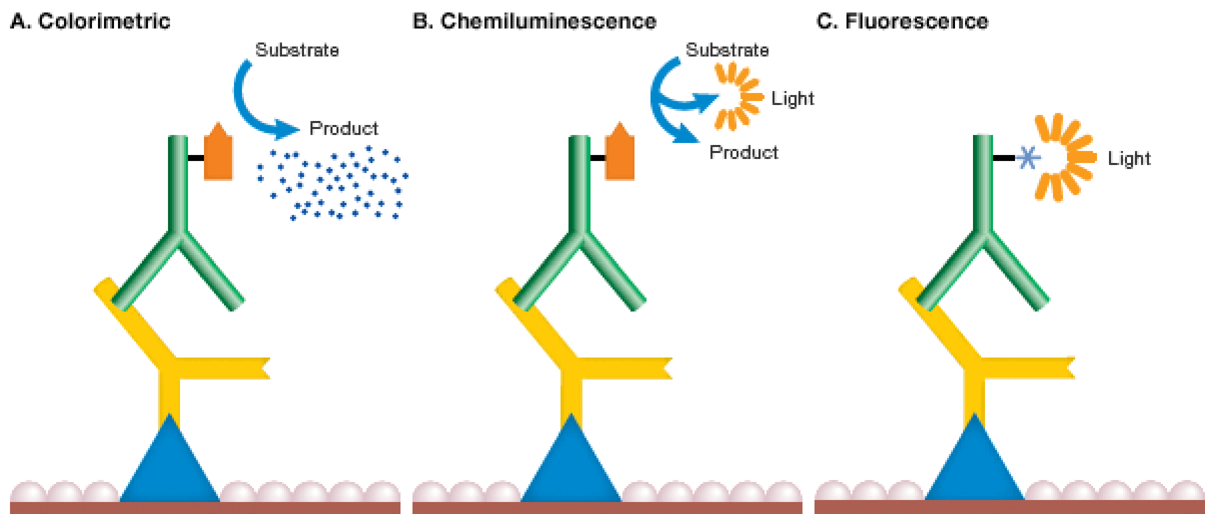

Figure 10: A diagram illustrating the three different western blot detection techniques, A: Colorimetric where a highly coloured precipitate forms due to the enzymatic activity conjugated to the secondary antibody, B: Chemiluminescent where light is generated in place of a coloured product and C: Fluorescent where the conjugated detection dye is fluorescent when exposed to the correct light (modified from Bio Rad with permission).

Once exposures have been captured, blots can be washed in a buffer and then “stripped,” which involves removing bound antisera to enable reuse of the blot (88). Although the blots can be stripped and re-probed several times, recurrent stripping and re-probing may affect the signal intensity.

Overall, Western blotting is a powerful tool for protein detection which takes advantage of the high efficiency of protein separation from electrophoresis, and high sensitivity and specificity of immunodetection (94). Its higher sensitivity is owed to its ability to detect as minute amount of protein as 0.1ng. Moreover, the indirect staining can contribute to the sensitivity of the assay by intensifying weak signals. Both separation of different proteins by electrophoresis and the use of highly specific antibodies are major contributors to the specificity of the assay.

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On the other hand, western blot is a multi-step technique and therefore is a time and effort consuming assay. Furthermore, optimization for each step and component are required to avoid false results.

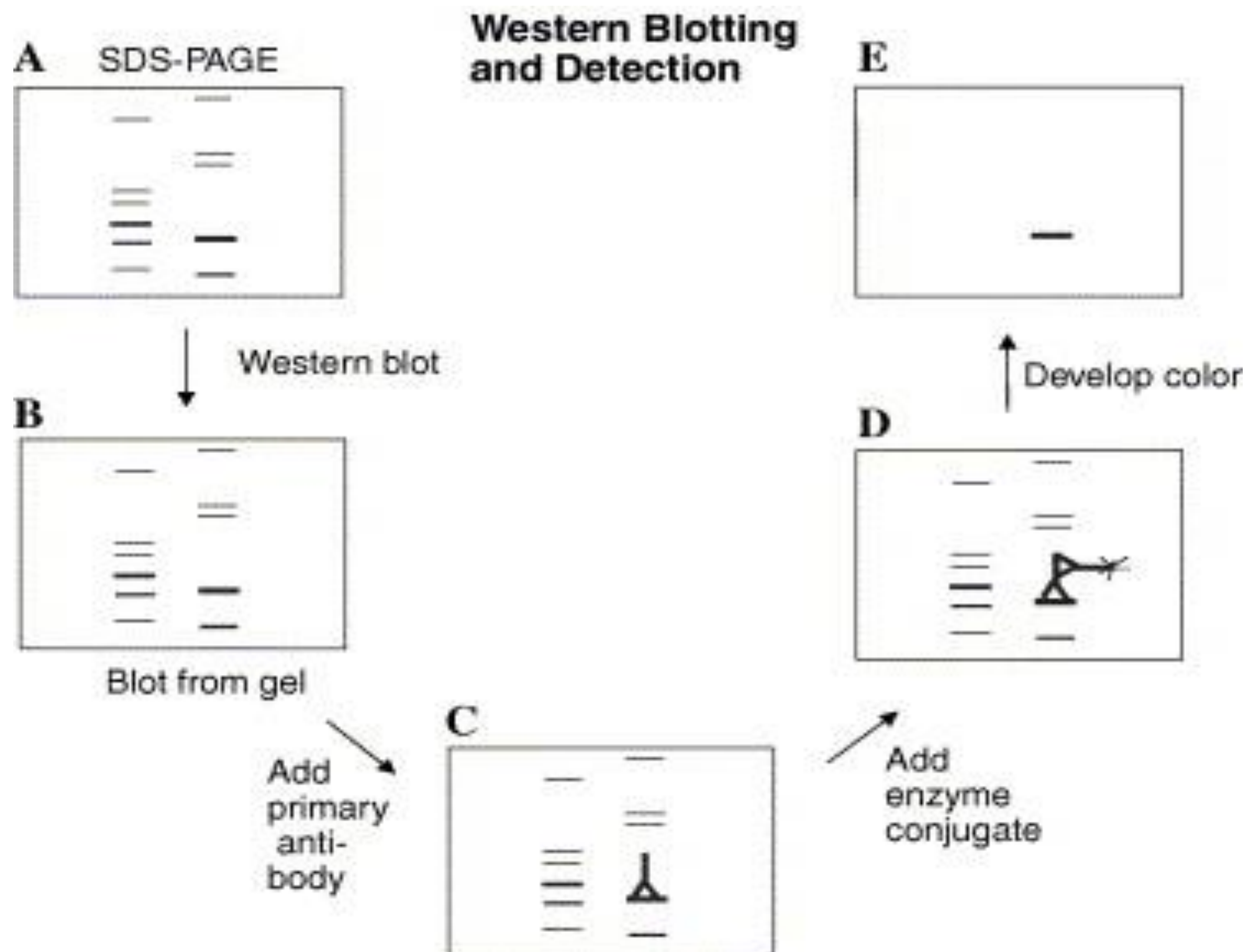

Figure 11: Schematic presentation of the sequence of steps in a western blotting assay including the initial Polyacrylamide gel electrophoresis (PAGE), trans-blotting, blocking, probing with primary and secondary antibodies and final detection. Taken from (Kurien BT,et al. 2006) (95) with agreement license from Elsevier.

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Table 2: Comparison between western blot and flow cytometry in intracellular phosphoprotein analysis

Western blot	**Flow cytometry**
Requires homogeneous population	Heterogeneous cell population can be used
Gives an average value of the cells in the sample	Gives information at a single cell level
One parameter analysis	Multi-parametric analysis
Not applicable to study rare cell subpopulations	Applicable to study rare cell subpopulations
Needs large sample size	Smaller sample size can be used
Localization of intracellular antigen can be determined in fractionated cells	Can't determine the localization of the intracellular antigen.
Qualitative or semi-quantitative results are mainly obtained	Both quantitative and qualitative results can be obtained.

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1.9 Aim

- The primary aim of this study was to assess the dose and time responses of STAT3 and pSTAT3 in PBMC *in vitro* after IL-6 or IL-10 stimulation using flow cytometry and the traditional western blot assays.

- The secondary aim was to optimize the experimental conditions and protocol for the maximal detection of STAT3 and pSTAT3 proteins following cytokine stimulation.

1.10 Objectives

- To isolate active and viable PBMC from fresh whole blood of healthy volunteers.
- To resolve and analyse the monocytes and the lymphocytes separately depending on the difference in their physical properties by flow cytometry.
- To confirm the identities of cell populations for gating based on combined fluorescence and light scatter properties.
- To determine the dose response of cytokine stimulation on STAT3 and pSTAT3 in normal PBMC.
- To investigate the kinetics of STAT3 and pSTAT3 at different time intervals after stimulation in normal PBMC.
- To compare Flow cytometry results with Western blot results for the same samples.

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Chapter 2: Materials and methods

2.1 Flow cytometric assay reagents

PBMC were isolated from heparin uncoagulated whole blood collected from healthy donors after signing a written consent. The blood was collected from three donors on different days. However, the same experiment was done on each blood sample on the same day of collecting blood. No samples were frozen for future analysis.

Heparin was purchased from Sigma Aldrich (St Louis, USA). It was dissolved in water to prepare 30 mg/ml solution and kept at 4°C. This stock solution was added to the collected whole blood in a ratio 1:1000.

Histopaque-1077 and RPMI media were purchased from Sigma Aldrich (St Louis, USA) and were stored at 4°C. $RPMI^+$ was made up by adding 10% of FCS to RPMI media.

Ammonium chloride was used to lyse the contaminating red blood cells and was prepared by dissolving 8.3 g NH_4Cl (Merck), 1 g Na_2HCO_3 and 74 mg EDTA in 1L distilled water.

Foetal calf serum was purchased from PAA (Pasching, Austria). Prior to its use, the serum was inactivated by heating at 56°C for 45 minutes. The serum was added to the RPMI media to get a final concentration of 10%.

Bovine serum albumin BSA (# sc2323) was purchased from Santa Cruz Biotechnology and stored at 4°C. BSA was used as a blocking agent.

Phosphate buffer saline (PBS) was prepared by dissolving 4.62 g of premixed FTA-Haemagglutination buffer powder, purchased from (BD Bioscience, South Africa), which contains 7.65 g sodium chloride, 1.27 g disodium phosphate, 0.10 g monosodium phosphate and 0.21 g mono potassium phosphate in 500 ml deionised water and stored at 4°C.

Human recombinant Interleukin 6, purchased from (BD Bioscience, South Africa), was reconstituted under aseptic conditions with sterile PBS buffer containing not less than 0.5 mg/ml human plasma (according to the manufacture instructions). This stock solution was diluted to a final concentration of 5.1µg/ml, divided into 20 µl aliquots and stored at -80°C.

Human recombinant Interleukin 10 (Bio-Vision, USA) was used as another stimulant. It was reconstituted with distilled water to a concentration of 0.1µg/ml

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and stored at -20°C in 10 µl aliquots. Before its use as a stimulant, the stock solution in the aliquot was diluted to a final concentration of 5.1 µg/ml using PBS buffer.

Paraformaldehyde 4% (Merck) or BD Cytofix fixation buffer (# 554655 purchased from BD Bioscience, South Africa) was used for cells fixation.

Either ice cold methanol 99.5% obtained from Romil, or BD phosflow Perm buffer III (# 558050, purchased from BD Bioscience, South Africa), was used for permeabilisation.

The staining buffer was prepared by dissolving 1% BSA in PBS, pH 7.4, filtered with 0.2µm filter and prepared freshly to avoid sodium azide addition.

The following antibodies were purchased from BD Biosciences:

- Mouse anti-STAT3-PE (catalogue # 560391) was used to label the intracellular total STAT3 protein.
- Mouse anti-pSTAT3-PerCP Cy5.5 (pY705) (catalogue # 560114) was used to label the phosphorylated STAT3 protein.
- Anti-CD14-FITC was used as monocyte surface marker antibody for gating conformation.

Two different cytometers were used to analyse the cells:

- Beckman Coulter FC500 flow cytometer situated in the Department of Pharmacology, University of Pretoria.
- Accuri C6 flow cytometer which was kindly loaned to the department by BD biosciences.

2.2 Flow cytometric assay method

2.2.1 Isolation of cells

Thirty millilitres of heparinized blood of a healthy donor were carefully loaded on top of 15 ml of Histopaque-1077 to set up a step density gradient. The blood was centrifuged at 500 x *g* for 25 minutes at ambient temperature. The supernatant plasma layer was removed and the next cloudy band that contained the peripheral blood mononuclear cells was pipetted into a new sterile 50 ml tube. RPMI media was added to the tube and the cells were centrifuged for 15 minutes at 300 x *g.* Following removal of the supernatant, the pellet was resuspended in the residual volume (approximately 80 -130 µl) and 15 ml of cold ammonium chloride was added to the cells and allowed to stand on ice for 10 minutes to lyse the remaining red blood cells.

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Then, the cells were centrifuged for 10 minutes at 300 x *g.* Finally, the cells were washed with RPMI and the cell pellet was resuspended in one millilitre of $RPMI^+$. The cells were counted using a haemocytometer and the cells' suspension was diluted to a concentration of 2 x 10^6cells/ml in $RPMI^+$ medium.

2.2.2 The effect of the sequence of fixation and permeabilisation on CD14 staining efficiency

Some antibodies do not bind to their epitopes when probing fixed/permeabilised cells and can result in high background signal or decrease in the positive staining (96). Moreover, methanol permeabilisation does compromise detection of some surface antigens and makes population sub gating more difficult (77). Taking these facts into consideration, it is absolutely critical to evaluate the effect of paraformaldehyde fixation and methanol permeabilisation on the surface marker staining.

Following the isolation of the PBMC as described above, the cells were treated in different sequences to assess the effect of each preparation step on the outcome of probing with anti-CD14-FITC. Initially 1 x 10^5 cells in 50 µl were pipetted in 3 different test tubes. 10 µl of the surface marker antibody (anti-CD14-FITC) was added to the first test tube and left in the dark for 30 min at RT. At the end of the incubation period, the cells were washed with PBS and fixed with a final concentration of 2% paraformaldyde then permeabilised with a final concentration of 90% ice chilled methanol as described below. These cells were analysed directly to create a gate of control cells. The second set of cells was fixed first with a final concentration of 2% paraformaldehyde then washed with PBS and probed with 10 µl of anti-CD14-FITC and finally permeabilised with a final concentration of 90% ice chilled methanol. The third set of cells was fixed with a final concentration of 2% paraformaldehyde and permeabilised with a final concentration of 90% ice chilled methanol before they were surface probed with 10 µl of anti-CD14-FITC. Monocytes were gated according to both their high side scatter characteristics and high CD14-FITC fluorescence. The analysis was done using the Beckman Coulter FC500 cytometer.

2.2.3 Time response of STAT3 and pSTAT3 to IL-6 or IL-10 stimulation

The cells were freshly prepared for each experiment and used within the same day of isolation. No cells were frozen or kept for the following days.

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2.2.3.1 Cell stimulation

One millilitre of the previous cell suspension was pipetted into test tubes and acclimatised at 37°C in an incubator for one hour. One hour resting time is ample time to warm the cells for optimal stimulation without affecting the basal phosphorylation level that often increased with longer resting periods (96). The cells were then stimulated with either different concentrations (1, 10 and 100 ng/ml) of either IL-6 or IL-10 at 37°C or for different time intervals (5, 10, 15 and 30 minutes) leaving one untreated sample as a control.

2.2.3.2 Cell fixation and permeabilisation

At the end of the treatment period, the fixative agent was quickly added to the cells and left for 10 minutes at 37°C. Two different fixative agents were tested: 4% paraformaldehyde to achieve a 2% final concentration or equal volume of pre-warmed BD Cytofix fixation buffer (according to the manufacture instructions). The fixed cells were washed with 1 ml PBS at RT and centrifuged for 6 minutes at 600 x *g* to remove the fixative agent. The supernatant was discarded and the pellet was resuspended in the residual medium (approximately 80 -130 µl) by vortex mixing. To permeabilise the cells, either 1 ml of 99.5% ice chilled methanol was added to the cell suspension to achieve a 90% final concentration or 1 ml of chilled Perm Buffer III was used. The permeabilising agent was added slowly while gently vortex mixing the cells to avoid cell clumping. To complete the permeabilisation, the cells were then allowed to stand on wet ice for 30 minutes.

2.2.3.3 Cell washing and staining

Prior to flow cytometric staining, a thorough washing was done to completely remove the permeabilising agent. Any residual permeabilising agent can negatively affect the fluorescence and the staining of protein fluorochromes such as PE and PerCp-Cy5.5. Therefore, following permeabilisation the cells were initially washed with 2ml PBS alone then centrifuged for 6 minutes at 600 x *g* to collect the cell pellet. Washing the permeabilised cells with staining media should be avoided at this stage because any traces of residual methanol could precipitate the BSA protein in the staining buffer. The washing step was then repeated twice with 2 ml staining buffer. Washing and incubating the cells with staining buffer can reduce the nonspecific binding of the antibodies. Following resuspension of the cell pellet in the residual

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volume of the staining buffer, 100 µl of staining media was added to the cell suspension. Then, 50 µl of the cell suspension was transferred into appropriate flow cytometer tubes and incubated with 10 µl of each anti-STAT3-PE and anti-pSTAT3-PerCp-Cy5.5 simultaneously for 1 hour at RT in the dark (according to the supplier instructions). Finally, the cells were washed with 2 ml staining buffer, centrifuged for 6 minutes at 600 x *g,* the supernatant discarded and the pellet resuspended in 200 µl of staining buffer for flow cytometric analysis.

2.2.3.4 Flow cytometric analysis

Both Beckman Coulter FC 500 flow cytometer and the Accuri flow cytometer were used to analyse the samples. CXP software was used for acquisition of events on Beckman Coulter while C Flow plus software was used for acquisition on Accuri. Gating of monocyte and lymphocyte populations was created according to forward and side scatters properties. This gating was then confirmed using anti-CD14-FITC that binds to the CD14 present as a selective monocytes surface antigen protein. The mean fluorescent intensity of each antibody was calculated and used as an indication of the relative quantity of the protein probed with each antibody. Table 3 below summarizes the setup of both instruments.

Table 3: The setup of the Beckman Coulter and the Accuri cytometer

	Beckman Coulter cytometer	Accuri cytometer
Laser	Blue 448 nm	Blue 448 nm and Red 640 nm
Software	CXP	C Flow
Event rate	10,000	100,000
FITC fluorescence detection channel	FL1	FL1
PE fluorescence detection channel	FL2	FL2
PerCP Cy5.5 fluorescence detection channel	FL3	FL3
Colour compensation between PE and PerCP Cy5.5	No colour compensation was needed	No colour compensation was needed

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2.2.4 The effect of fixation and permeabilisation with paraformaldehyde and methanol (F/M) or commercial buffers on the fluorescence intensity of the antibodies

Cells were stimulated with 100 ng/ml of IL-10 for 15 minutes. At the end of the stimulation period, the cells were either fixed with 2% paraformaldehyde or BD Cytofix buffer for 10 minutes. Paraformaldehyde fixed cells were permeabilised with 90% methanol on ice while the cells fixed with the commercial buffer were then permeabilised with BD Perm Buffer III (as described above). Following several repeats of washing, initially with PBS then with staining buffer, both groups of cells were probed with anti-STAT3-PE and anti-pSTAT3-PerCP Cy5.5 for one hour. The fluorescence intensity for each antibody was measured in each group by the Accuri cytometer.

2.2.5 The effect of adding phosphatase inhibitors during fixation in flow cytometric analysis

It has been reported that fixation and permeabilisation techniques did not completely abolish all phosphatase activity (77). However, addition of phosphatase inhibitors is not necessary when using methanol permeabilisation due to its ability to abrogate the majority of phosphatase activity (77). Taking into consideration that fixation is done using warm buffer; this may increase the phosphatase activity. Therefore, the effect of adding phosphatase inhibitors during the fixation step was tested to determine if this addition increased the measured fluorescence intensity.

PBMC were either left untreated or treated with 100 ng/ml of IL-10 for 15 minutes. At the end of the stimulation period, the cells were either fixed with equal volume of warmed-Cytofix buffer alone or with warmed Cytofix buffer mixed with 10 µl of phosphatase inhibitor II. Following fixation, the cells were permeabilised with chilled Perm Buffer III for 30 minutes on ice. After washing three times, the cells were incubated with anti-STAT3-PE and anti-pSTAT3-PerCp Cy5.5 for one hour in the dark. Then, the cells were washed, resuspended in staining buffer and analysed using the Accuri cytometer.

2.3 Western blot assay's reagents

The following consumables were purchased from Bio-Rad, (Johannesburg, SA):

- Criterion™ TGX stain-free midi precast gradient gels (4 - 20%).

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- Trans-Blot Turbo, midi format, 0.2 µm PVDF membranes with blot filters for semi-dry blotting.
- Precision plus protein standards.

Goat anti-rabbit IgG conjugated to horse radish peroxidase (HRP) was purchased from Abcam, and was used as a secondary antibody to visualise the blotted protein of interest.

The following antibodies were purchased from Sigma Aldrich (St Louis, USA):

- Rabbit anti-STAT3 (# 056K1250) to probe the native STAT3 proteins on the blot membrane.
- Rabbit anti phospho-STAT3 (p-Tyr 705) (#511711045) to probe the phosphorylated STAT3 proteins on the blot membrane.

Phosphatase inhibitor cocktail II which is a mixture of inhibitors for acid and alkaline phosphatases as well as tyrosine phosphatases was purchased from Sigma Aldrich (St Louis, USA).

Protease inhibitor cocktail was also purchased from Sigma Aldrich (St Louis, USA).

Commercial chemiluminescence mixture was purchased from (Vacutec, SA) and was used according to the manufacture instructions.

An “in house” chemiluminescence mixture was prepared, according to the recipe described below, and its performance was compared with the commercial mixture.

All required reagents were purchased from Sigma Aldrich (St Louis, USA).

The membrane was visualized using a Bio-Rad ChemiDoc XRS Molecular Imager at the CSIR, Pretoria.

The following buffers were prepared and used during the technique:

<u>Tris buffer saline (TBS)</u>

Tris buffered saline solution was prepared by dissolving 8 g NaCl, 3 g Tris base and 0.2 g KCl in 800 ml deionised water and the pH of the solution was then adjusted to 8.0 with 1 M HCL. Finally, the total volume was completed to one litre with deionised water.

<u>Lysis buffer</u>

A solution of 1 ml of lysis buffer containing 100 mM NaCl, 10 mM Tris and 1% Triton X100 was made in deionised water. Just before use, 10 µl phosphatase inhibitor

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cocktail II and 5 µl protease inhibitor solution were added to the buffer solution and mixed well.

Laemmli 2x sample buffer

A solution consisting of 4% SDS, 20% glycerol, 125 mM Tris, pH 6.8, 0.02% Bromophenol blue and 10% β mercaptoethanol was made up in deionised water. The buffer was divided into 1 ml aliquots and stored at -20°C.

Gel electrophoresis running buffer 10X

Running buffer was prepared by dissolving 15 g Tris base, 72 g glycine and 5 g SDS in 500 ml deionised water. The solution was stirred well but gently to avoid foaming of the buffer concentrate. This is diluted 1/10 in deionised water when making up the running buffer for the reservoir.

Blocking buffer

The blocking buffer consists of 3 g of BSA that was dissolved in 100 ml TBS buffer prepared as described above. Then, 0.05% Tween-20 was added to the BSA solution and mixed gently.

Stripping buffer

To make up this buffer, 15 g glycine, 1 g SDS and 10 ml Tween-20 were dissolved in one litre of deionised water and the pH of the solution was adjusted to 2.2 with HCL.

2.4 Bicinchoninic acid (BCA) protein assay reagents

The following reagents were prepared, as described below, to be used in this assay:

Reagent A was prepared by dissolving 1 g bicinchoninic acid (BCA), 2 g sodium carbonate, 0.16 g sodium tartrate, 0.4 g sodium hydroxide and 0.95 g sodium bicarbonate in 90 ml deionised water and the pH was adjusted to 11.25 with 10 M NaOH. Finally, the solution was made up to 100 ml with deionised water.

To prepare Reagent B, 0.4 g cupric sulphate (5 x hydrated) was dissolved in 10 ml deionised water.

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Different concentrations of powdered fraction V BSA in TBS buffer solution (0.1, 0.2, 0.25, 0.4, 0.5, 1 and 2 mg/ml) were prepared from a stock solution of 5 mg/ml by serial dilution.

2.5 Preparation of "in house" chemiluminescence mixture

This mixture was prepared according to Haan *et al* (97), and all the reagents were obtained from Sigma Aldrich (St Louis, USA).

Initially, two stock solutions were made in DMSO and were used for the preparation of Detection Solution (A). These were: Stock 1 which was consisting of 250 mM luminol and Stock 2 which was consisting of 100 mM *para*-coumaric acid and both were stored at ambient temperature in the dark.

Additionally, a dilution solution of 100 mM Tris in deionised water was prepared and the pH was adjusted to 8.5 (Detection Solution B).

An activation solution of 30% hydrogen peroxide was required for the detection.

Using the above stock solutions, Detection Solution A was prepared by mixing 100 µl Stock 1 (luminol) and 40 µl Stock 2 (*p*-coumaric acid) with 10 ml of the 100 mM Tris, pH 8.5.

To prepare the active detection solution: 5 ml of Detection Solution A was mixed with 5 ml of Detection Solution B and 3.1 µl of 30% hydrogen peroxide solution.

2.6 Western blot assay's methods

2.6.1 Cell preparation

Following the isolation of the PBMC as mentioned above, the cells were either left untreated in complete media or treated with different concentrations (1, 10 and 100 ng/ml) of either IL-6 or IL-10 for different time intervals (5, 10, 15 or 30 minutes) at 37°C. At the end of the incubation time, the cells were lysed by incubating them in the lysis buffer for 15 min. The total protein concentration in each of the lysates was analysed using BCA assay as described below. Then, the samples were mixed 1:1 by volume with 2X Laemmli sample buffer, boiled at 100°C for 5 minutes and the appropriate volume of solubilised protein loaded onto the electrophoresis gels.

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2.6.2 BCA assay

BCA assay depends on a colorimetric reaction for the quantification of the total amount of protein in a sample. Generally, the reaction has two steps:

Initially, the protein reduces the Cu^{++} in the cupric sulphate solution to Cu^{+} ions. The amount of Cu^{+} ions formed is proportional to the amount of protein in the sample (91).

Secondly, each ion of Cu^{+} chelates two molecules of bicinchoninic acid, forming a purple colour.

Initially, a working solution was prepared by mixing Reagent A (green colour) with Reagent B in a ratio A: B is 50: 1.

A volume of 5 µl of each of the different BSA standards and the samples were added to individual wells in a 96 well microplate. Then, 250 µl of the working solution was added to each well and incubated for 30 minutes in the dark. Finally, the absorbance was measured at 570 nm on a plate reader. The mean of the blank replicates was subtracted from each of the standards and the unknown samples absorbance values. A standard curve was generated by plotting the concentration of each standard solution versus its blank corrected absorbance. The concentration of the unknown sample can be determined from this curve and reported in µg/ml protein by the spectrometer software.

2.6.3 Gel electrophoresis

The samples were loaded into wells of a gradient polyacrylamide gel (4 – 20%) at 30 µg protein in each lane. The two outer lanes were loaded with Precision Plus molecular mass marker solution. The running buffer was diluted 1/10 and used to run the gel electrophoresis. The proteins were separated at 200 V for approximately 45 minutes at which time the tracking dye reached the bottom of the gel.

The gel was removed from the cassette, briefly rinsed and scanned in a Bio-Rad Gel Doc EZ Imager system using the stain free tray which requires no prior protein staining. If better sensitivity was required the gels were stained in Aqua colloidal Coomassie stain for 3 hours and destained in deionised water for 60 minutes and again scanned using the white tray in the Bio-Rad Gel Doc EZ Imager system.

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2.6.4 The semi Immunoblotting

Semi-dry electro-blotting transfer of the proteins was done using a Trans-blot Turbo Transfer System (Bio-Rad). The proteins were transferred from the gel to the PVDF membrane (Bio-Rad) purchased as a kit that included a pre-cut membrane sandwiched between two buffer containing filter pads. The standard transfer protocol was used that transfers the proteins in 30 minutes. To confirm that all the proteins had been transferred from the gel to the membrane, the gel was scanned again with the Bio-Rad Gel Doc EZ Imager system.

After protein transfer was confirmed, the membrane was blocked with the blocking buffer at 4°C overnight with gently shaking. Following blocking, the membrane was washed with TBS + 0.2% Tween-20 (TBST) 5 times, 10 minutes each, with gentle agitation and sufficient volume to cover the whole membrane by at least 3 mm.

2.6.5 Membrane staining

According to the manufacture recommendation, the membrane was incubated with 1:1000 working dilution of 1 mg/ml rabbit anti-STAT3 in TBST under constant agitation for 2 hours at RT. Then, the antibody solution was discarded and the membrane washed 5 times using TBST buffer for 10 minutes per wash. After that, the membrane was probed with 1/2000 dilution (the supplier suggested working dilution) of 1 mg/ml secondary antibody in TBST for one hour RT. The membrane was then washed for further five times, ten minutes each, with TBST under constant agitation.

2.6.6 Detection

For protein detection, either a commercial or a self-made chemiluminescence mixture was used (as prescribed above) and incubated with the membrane for 5 minutes. The membrane was then briefly blotted to remove excess reagent, centred on the imaging plate and imaged by Bio-Rad ChemiDoc XRS Imager for different time intervals with the settings allowing all light to reach the detection camera.

2.6.7 Membrane stripping

Following the detection of the total STAT3, the membrane was stripped twice with a stripping buffer for 5 - 10 minutes at RT. Then, the blot was washed twice with PBS for 10 minutes under constant agitation and twice with TBST for 5 minutes. The blot

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was probed then with 1:1000 working dilution of 1 mg/ml rabbit anti-pSTAT3 in TBST for 2 hours at RT (according to the supplier recommendations). Following repeated washing steps as described above, the membrane was probed with the same dilution of the secondary antibody used before for one hour at RT. Finally, the membrane was washed 5 times, 10 minutes each, using TBST with gentle agitation then incubated with the chemiluminescence mixture and visualized on the ChemiDoc XRS system as described above.

2.7 In gel digestion's reagents

Colloidal Coomassie blue dye was purchased from Vacutec, SA.

Ammonium bicarbonate (NH_4HCO_3) was bought from (BDH laboratory reagents, England). Three different solutions were prepared with NH_4HCO_3:

I. 50 mM NH_4HCO_3 in deionised water, pH=8.
II. 50 mM NH_4HCO_3 / 50% MeOH, stored at 4°C.
III. 25 mM NH_4HCO_3 in 50% ACN, stored at 4°C.

Gradient HPLC grade acetonitrile (ACN) was purchased from (Merck, Germany), and diluted with deionised water to prepare 75% solution.

Dithiothreitol (DTT) was bought from Sigma Aldrich, SA. A 1 M DTT stock solution was prepared by dissolving 0.154 g of DTT in 1 ml deionised water. Then, a working solution containing 10 µl of the 1 M DTT solution, 495 µl of 50 mM NH_4HCO_3 and 495 µl of deionised water was prepared.

Iodoacetamide (IAA) was also purchased from Sigma Aldrich, SA. 55 mM fresh IAA solution was prepared in 25 mM NH_4HCO_3 and stored in the dark.

Sequence Grading Trypsin was bought from Promega, USA. 200 µl of the supplied buffer in the kit was added to a vial containing 20 µg of lyophilised trypsin. Then, 20 µl from this trypsin stock was added to 90 µl of 50 mM NH_4HCO_3 and 90 µl of deionised water to prepare 10 ng/µl trypsin solution.

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2.8 In gel digestion's method

Several sources of extraneous protein can contaminate the gel and affect the final results of the analysis. Therefore, to minimize contamination some precaution steps should be done prior and during the procedure such as double gloved hands, wearing a lab coat, tying the hair back and avoid wearing woollen clothes. Additionally, the procedure should be carried out in a laminar flow cabinet without flow and all the surfaces should be wiped with 70% ethanol prior to use. Importantly, powder-free gloves should be worn all the time and should be changed when touching anything that has not been previously washed with ethanol.

Following electrophoresis, the gel was stained with Coomassie blue dye for one hour to visualize the proteins bands. Bands of interest were then carefully cut from the gel using a cleaned sharp blade, and the bands separately cut into small pieces on a new microscope slide that had been cleaned with water and ethanol. Each bands gel piece were placed into a 1.5 ml micro reaction vial and treated separately. For destaining, 200 µl of 50 mM NH_4HCO_3/ 50% MeOH was added to the gel pieces and shaken for 20 min. After discarding the supernatant, this destaining step was repeated. Then 100 µl of 75% ACN was added to the gel pieces and shaken for 20 min. The supernatant was discarded and the gel pieces were completely dried using the Speed Vac. For irreversible breakage of the protein disulfide bonds and tertiary structure, 25 µl of 10 mM DTT in 25 mM NH_4HCO_3 was added to the dried gel pieces and left for one hour at 60°C. The gel pieces were then incubated with 500 µL ACN for 10 minutes. After discarding the supernatant, 25 µl of 55 mM iodoacetamide in 25 mM NH_4HCO_3 was added to the gel pieces and left for 20 minutes in the dark at room temperature to irreversibly alkylate and exposed SH groups in the protein. The gel was then washed with 100 µl NH_4HCO_3 in deionised water for 10 minutes, dehydrated with 100 µl of 25 mM NH_4HCO_3 in 50% ACN for 5 minutes and finally, completely dried with Speed Vac.

To digest the proteins into peptides, 50 µl of freshly prepared trypsin solution was added to the reduced and alkylated gel pieces and incubated at 37°C for 20 hours. Following the enzymatic cleavage, the tube was briefly centrifuged at high *g* force and the digest solution was transferred into a clean micro reaction vial. To the gel pieces, 50 µL of a solution containing 50% ACN and 5% formic acid in deionised

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water was added and incubated for 30 minutes with shaking to extract the peptides with different physical and chemical properties. This extract was added to the previous extract and this step was repeated 3 times to ensure complete extraction of the peptides from the gel. The samples were then completely dried with the Speed Vac. Then, these samples were handed out to the CSIR where they subjected to LC-MSMS to analyse and identify proteins present in the gel bands. The peptides were reconstituted in 50 µl acetonitrile and chromatographically separated on C18 column (100 x 2.1 mm) using an acetonitrile gradient from 5 to 50% over 32 min. The eluted peptides were directly introduced into an ESI source of a 4000 QTrap triple quadruple mass spectrometer and sequenced by low energy fragmentation and comparing the resulting y and b ions to a mammalian protein database. The protein identification analysis was done using Protein Pilot 4 (AB Sciex). .

2.9 Statistical analysis

The flow cytometric experiments were done in duplicates and the mean fluorescence intensity of the unstimulated and stimulated cells were compared. A ranked Krushkal Wallis test was used for statistical analysis of the data using Graph Pad Prism 5 software. P value < 0.05 was considered statistically significant.

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Chapter 3: Results

3.1 Gating for lymphocytes and monocyte populations

Gating individual population in a heterogeneous PBMC mixture can be created according to the difference in the physical properties (cell size and granularity) of each cell population and thereafter the difference in its forward and side scatter properties. Gating of lymphocytes and monocytes based on their forward and side scatter intensities were shown in Figure 12 below. Lymphocytes have low forward and side scatter properties compared to monocytes.

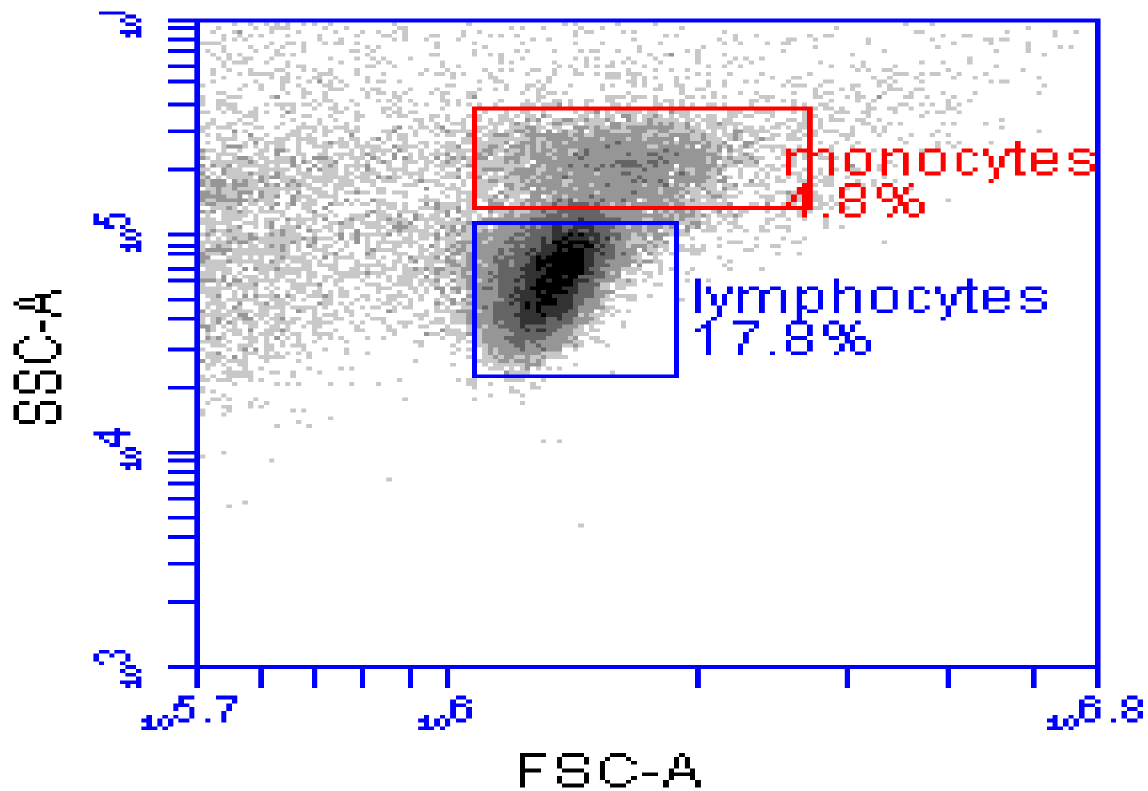

Figure 12: An example of gating of the combined lymphocyte and monocyte populations depending on forward and side scatter properties. Compared to monocytes, lymphocytes are smaller and non-granular cells; therefore they have both lower FSC and SSC. Data was collected using the Accuri flow cytometer.

3.2 Gating for monocytes using scatter and fluorescence characteristics

To enable accurate and consistent gating of the treated cells, a combination of fluorescence and scatter parameters were applied. Anti-CD14-FITC was used to label the monocyte population. Thus, the excluded population in the PBMC sample

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will be the lymphocytes. The dot plot in Figure 13 shows the fluorescence of anti-CD14-FITC on the x-axis versus the SSC on the y-axis. In comparison to lymphocytes, the monocyte population has higher SSC and CD14-FITC fluorescence intensity in Filter 1 (FL1).

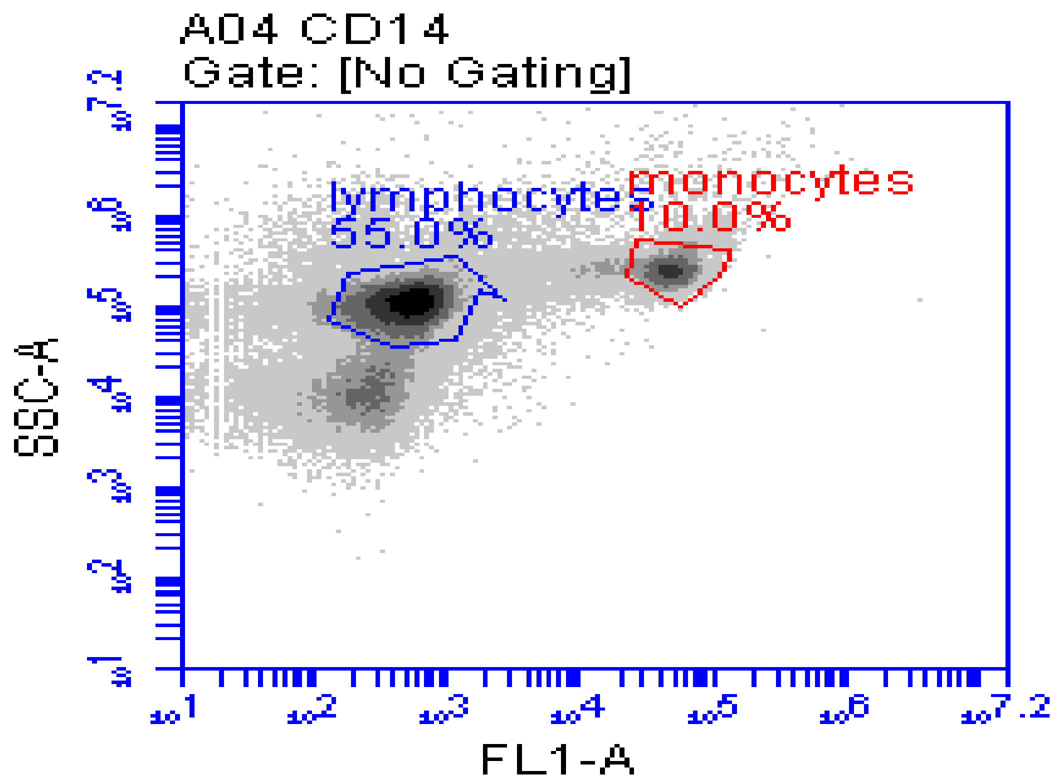

Figure 13: An example of gating of the CD14 positive monocytes based on the MFI of anti-CD14-FITC using the Accuri cytometer. Monocytes have the highest side scatter and show much higher fluorescence intensity in Filter 1 (FL1). This was performed to confirm that the correct gating of the monocytes was applied. The other major population in the PBMC sample will be the T and B lymphocytes.

3.3 The effect of the sequence of fixation and permeabilisation on CD14 staining efficiency

The effect of paraformaldehyde fixation and methanol permeabilisation on CD14 probing was presented in Figure 14. Apparently, fixation didn't affect the monocyte probing and subsequently their gating (Gate B) did not need to be adjusted. The monocytes can be identified in the same gated region that was created using probed cells with no pre-treatment. Neither the fluorescence of FITC nor the SSC seemed to be affected. However, probing the cells post permeabilisation dramatically decreased

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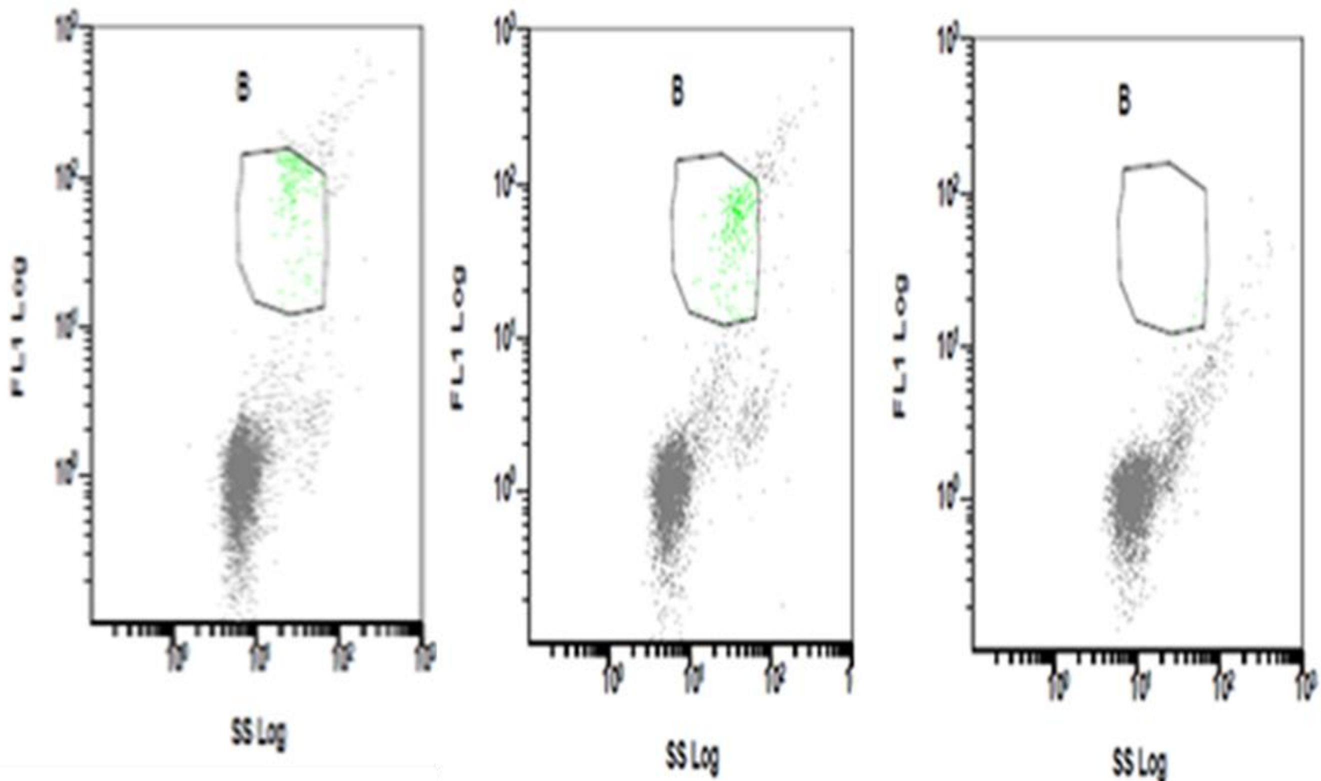
the MFI of the FITC fluorophore and thereafter the cells show no fluorescence within the gated region.



Probed, fixed and permeabilised **Fixed, probed and permeabilised** **Fixed, permeabilised and probed**

Figure 14: The effect of cell fixation and permeabilisation on CD14 staining and monocyte gating. PBMC were stained with anti-CD14-FITC before any treatment (left panel) or stained either after fixation with paraformaldehyde (middle panel) or after fixation and permeabilisation with 90% methanol (right panel). Monocyte gating was created according to fluorescence intensity before any treatment. Following fixation, monocytes still show high fluorescence in approximately the same region of interest created using untreated cells demonstrating that the fluorescence had not been affected by fixation. However, the next step of permeabilisation using methanol leads to a significant decrease of the fluorescence due to loss of surface antigen.

Due to the effect of the sample treatment on the antibody probing and final fluorescent signal, the sequential probing was followed during the protocol in which cells were probed for the surface proteins either before or after fixation but not after permeabilisation.

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3.4 Time response of STAT3 and pSTAT3 to 100 ng/ml IL-6 in three donors using Beckman Coulter cytometer

The response of isolated PBMC to 100 ng/ml IL-6 stimulation was investigated in both lymphocyte and monocyte cell populations. Lower concentrations of 1&10 ng/ml of IL-6 were initially tested in the preliminary studies without showing any detectable response on the STAT3 or pSTAT3 (data not shown). In this experiment, 2% paraformaldehyde was used to fix the cells and 90% methanol was used for permeabilisation as described in the method section. The results were expressed as a fold change of the MFI of the untreated cells which was calculated according to the following formula

$$Fold\ change\ =\ MFI\ of\ stimulated\ cells/MFI\ of\ unstimulated\ cells$$

Figure 15 represents the response of STAT3 to 100 ng/ml IL-6 stimulation. No specific pattern was seen in the level of STAT3 between the three donors. Compared to the baseline level of STAT3 in unstimulated cells, slight variations in the level of STAT3 were observed in both stimulated lymphocytes (panel A) and monocytes (panel B). However, these changes were not significant and it appears that the total level of STAT3 remains essentially constant even after stimulation.

The kinetics of the phosphorylated STAT3 in response to stimulation with 100 ng/ml of IL-6 was illustrated in Figure 16. A slight increase in the phosphorylation of STAT3 was detected after 10 minutes of stimulation in both lymphocytes (panel C) and monocytes (panel D). However, this increase in STAT3 phosphorylation was statistically insignificant in comparison to the unstimulated cells.

Inter-individual variability in the response of both STAT3 and pSTAT3 to IL-6 stimulation can be noticed between the three healthy donors although these differences were very small and were not statistically significant.

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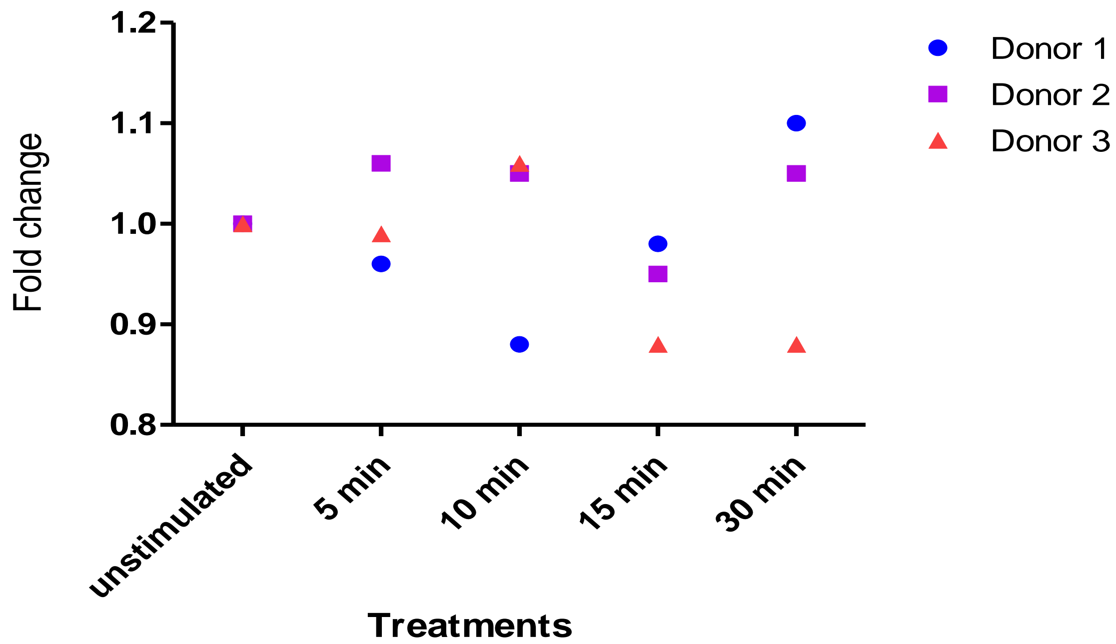
A)



B)

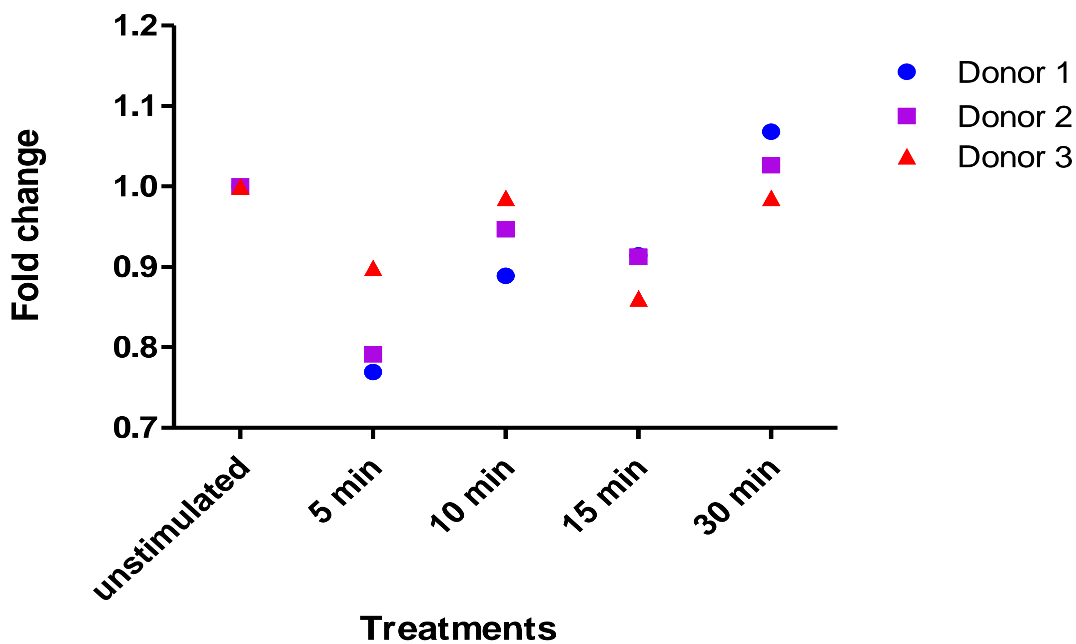


Figure 15: Time response of total STAT3 to 100 ng/ml of IL-6 in PBMC of 3 different donors by FC500 Beckman Coulter cytometer. PBMC were stimulated for different time intervals, fixed with 2% paraformaldyde and permeabilised with 90% methanol. The response of STAT3 was expressed as a fold change in the MFI in A) lymphocytes and B) monocytes population.

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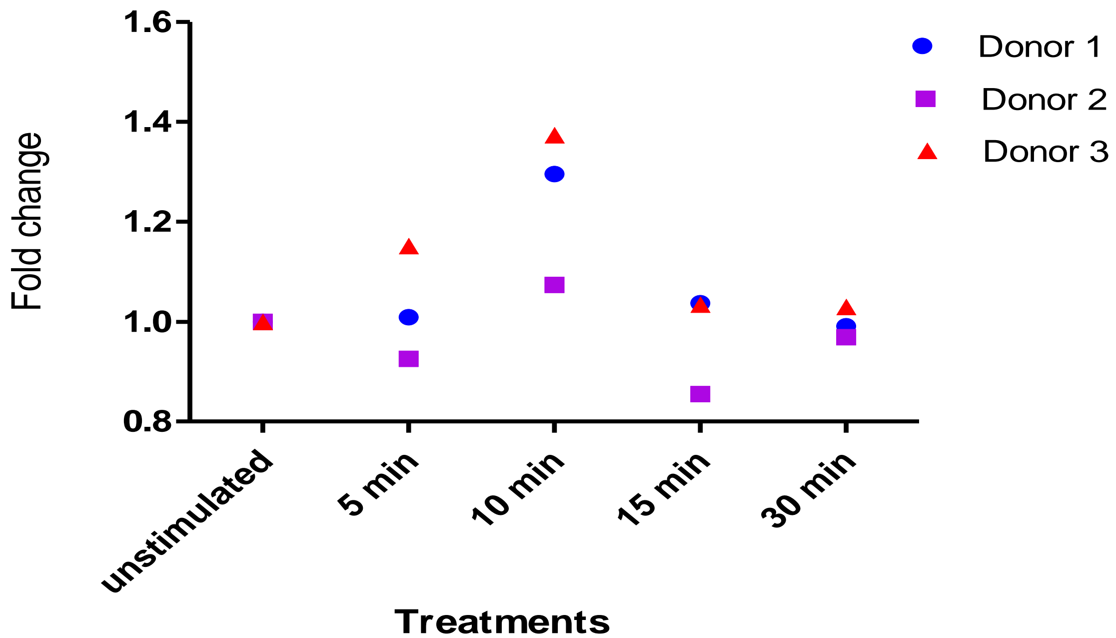
C)



D)

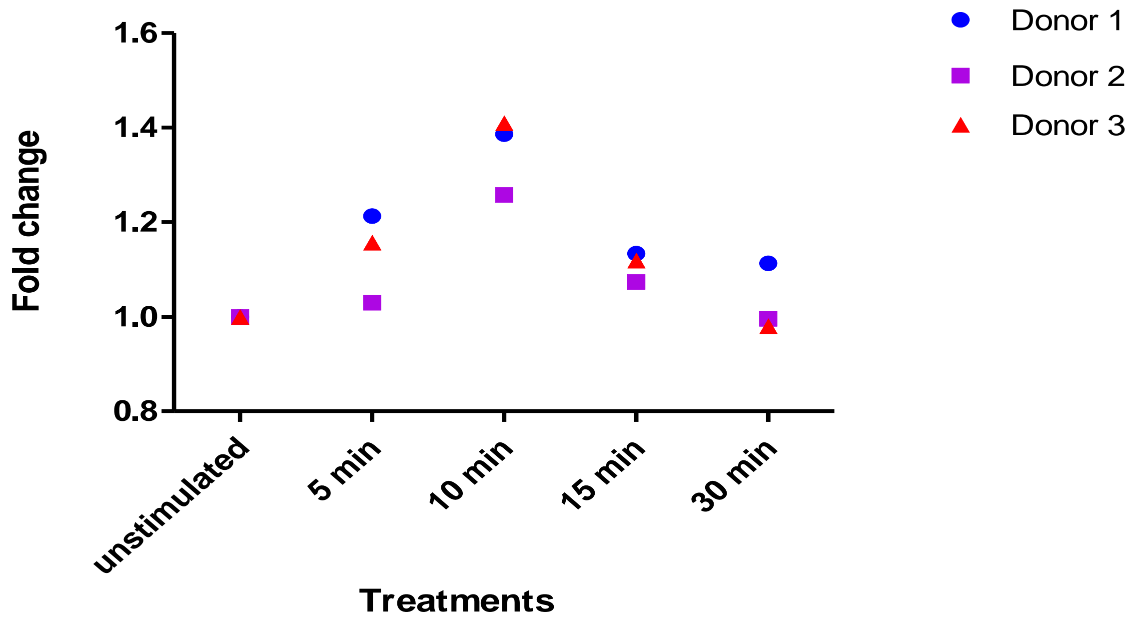


Figure 16: Time response of pSTAT3 to 100 ng/ml of IL-6 in PBMC of 3 different donors by FC500 Beckman Coulter cytometer. PBMC were stimulated for different time intervals, fixed with 2% paraformaldyde and permeabilised with 90% methanol. The response of both pSTAT3 was expressed as a fold change in the MFI in C) lymphocytes and D) monocytes population.

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3.5 Time response of STAT3 and pSTAT3 to 100 ng/ml IL-6 by Accuri cytometer

The response of STAT3 and pSTAT3 to 100 ng/ml IL-6 were investigated using the Accuri flow cytometer that is reported to have a wider dynamic range and easier to adjust for intrinsic fluorescence of cells.

The duplicate fold changes in MFI over the untreated cells were plotted for one donor (Donor 1). The results of the time response of STAT3 and pSTAT3 in both lymphocytes and monocytes are represented in Figures 17 and 18 respectively.

Compared to the baseline level of untreated cells, a slight, consistent but non-significant decrease in the level of STAT3 was detected (Figure 17) following stimulation of either lymphocytes (panel A) or monocytes (panel B) with 100 ng/ml IL-6. These results confirmed the thought that the cells seem to keep a constant level of total STAT3 even after stimulation.

On the other hand, a slight increase in the pSTAT3 was detected following stimulation with 100 ng/ml of IL-6 (Figure 18) in both lymphocytes (panel C) and monocytes (panel D). The maximal level of phosphorylation was reached at 15 minutes post treatment. However, this increase was considered statistically insignificant in the lymphocytes with p value > 0.05. In the monocytes, the increase of phosphorylation after 15 minutes was the only time point showing a statistically significant increase ($p < 0.05$). The higher variance in the MFI after 30 minutes stimulation contributes to a statistically insignificant increase despite the level of pSTAT3 was still as high as the level after 15 minutes stimulation.

Although it is difficult to compare these results with the previous ones because the analysis was done on a different cytometer, however, the pattern of the response of both STAT3 and pSTAT3 was very similar.

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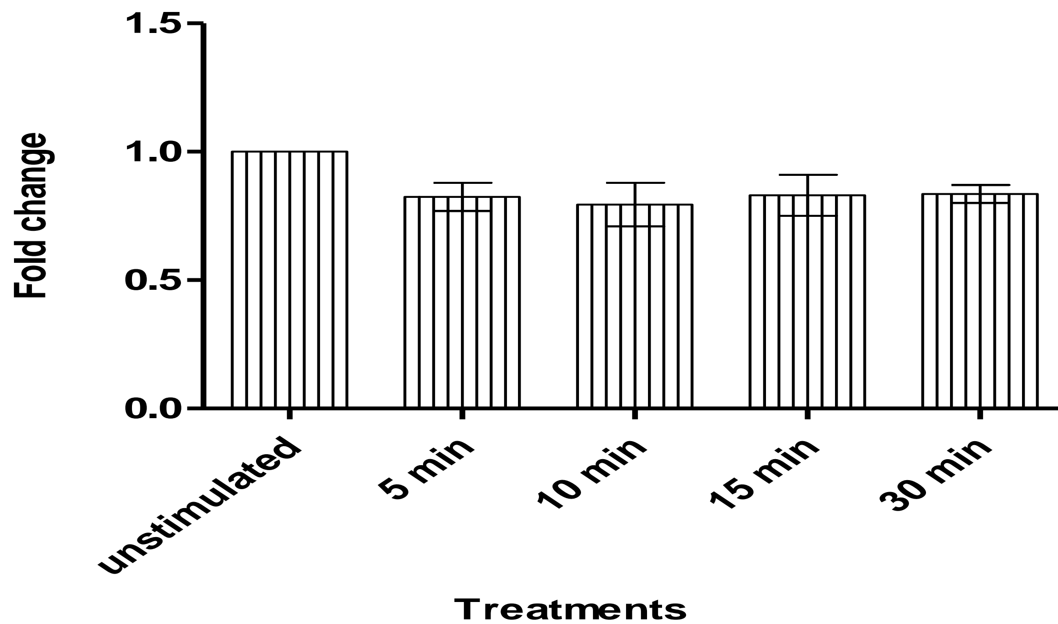
A)



B)

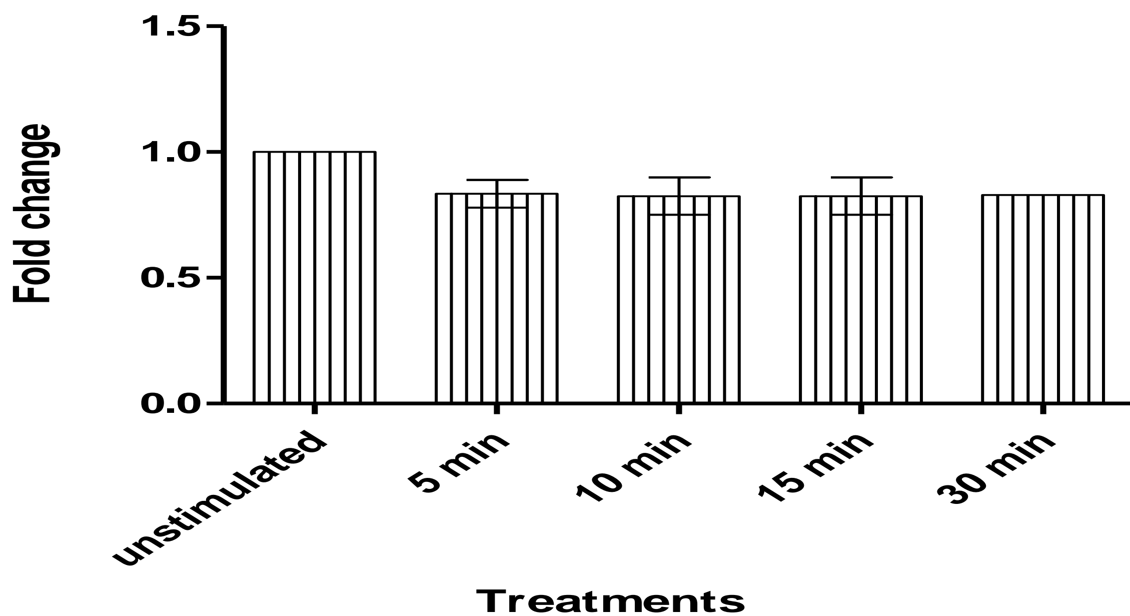


Figure 17: Time response of STAT3 to 100 ng/ml IL-6 stimulation in A) lymphocytes and B) monocytes. PBMC were either left untreated or treated with 100 ng/ml IL-6 for different time intervals (5, 10, 15 and 30 minutes). The cells were fixed with 2% paraformaldyde and permeabilised with 90% methanol. Analysis was done using the Accuri flow cytometer. The response of the STAT3 was expressed as a fold change of MFI of the treated cells to the untreated cells. This experiment was repeated twice on the same day using blood from the same donor.

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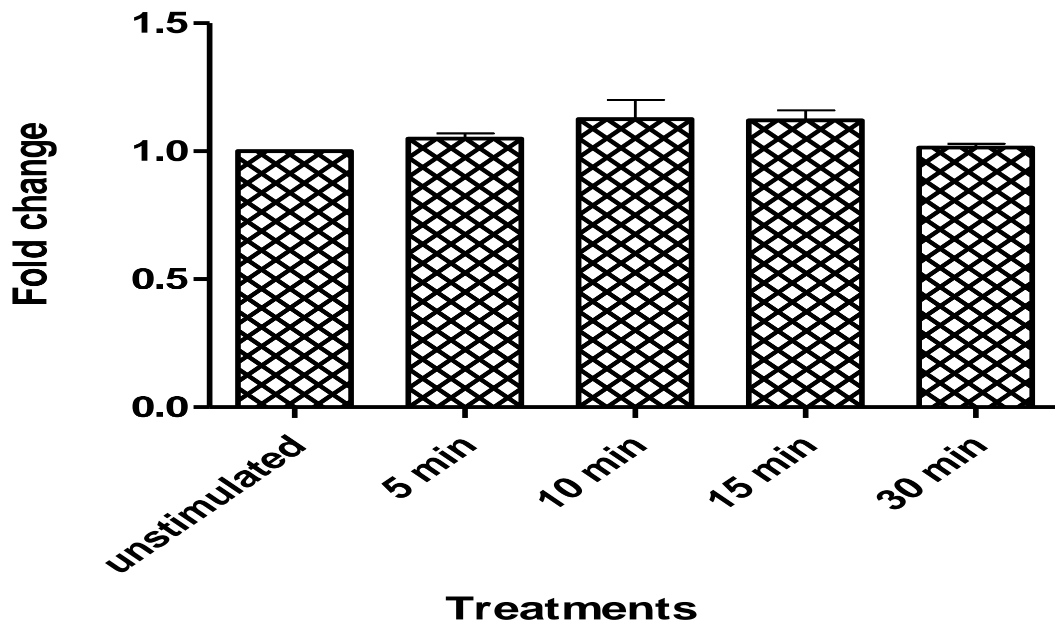
C)



D)

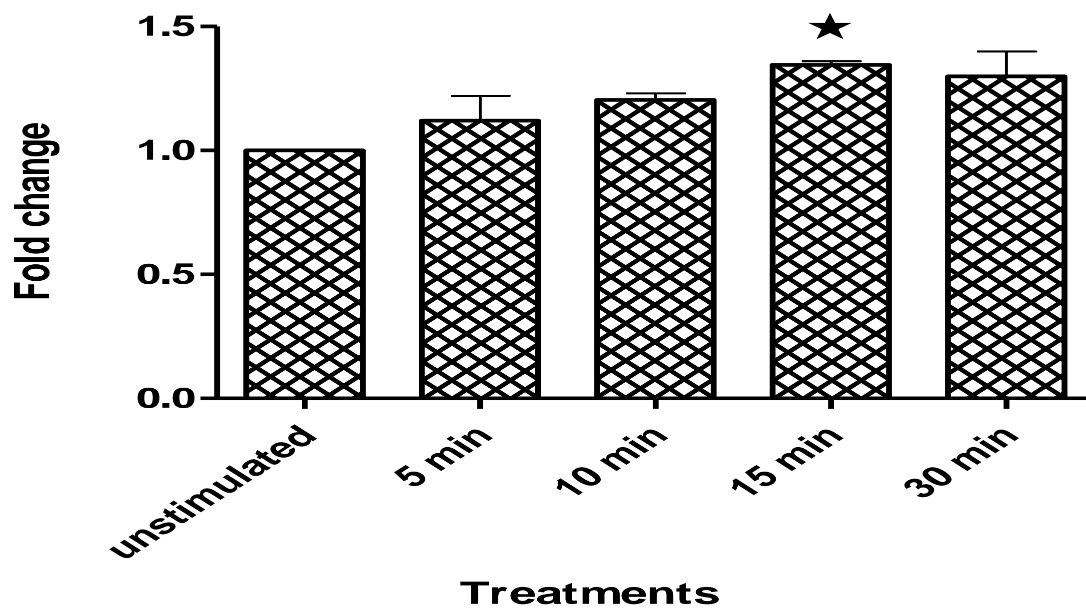


Figure 18: Time response of pSTAT3 to 100 ng/ml IL-6 stimulation in C) lymphocytes and D) monocytes. PBMC were either left untreated or treated with 100 ng/ml IL-6 for different time intervals (5, 10, 15 and 30 minutes). The cells were fixed with 2% paraformaldyde and permeabilised with 90% methanol. Analysis was done using the Accuri flow cytometer. This experiment was repeated twice on the same day using blood from the same donor. (*) $p < 0.05$.

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3.6 The effect of fixation and permeabilisation with paraformaldehyde and methanol (F/M) or commercial buffers on the fluorescence intensity of the antibodies

Prior to study the kinetic of STAT3 following IL-10 stimulation, the effect of F/M fixation and permeabilisation on the PE and PerCP Cy5.5 fluorescence intensities was compared with BD commercial fixation and permeabilisation buffers.

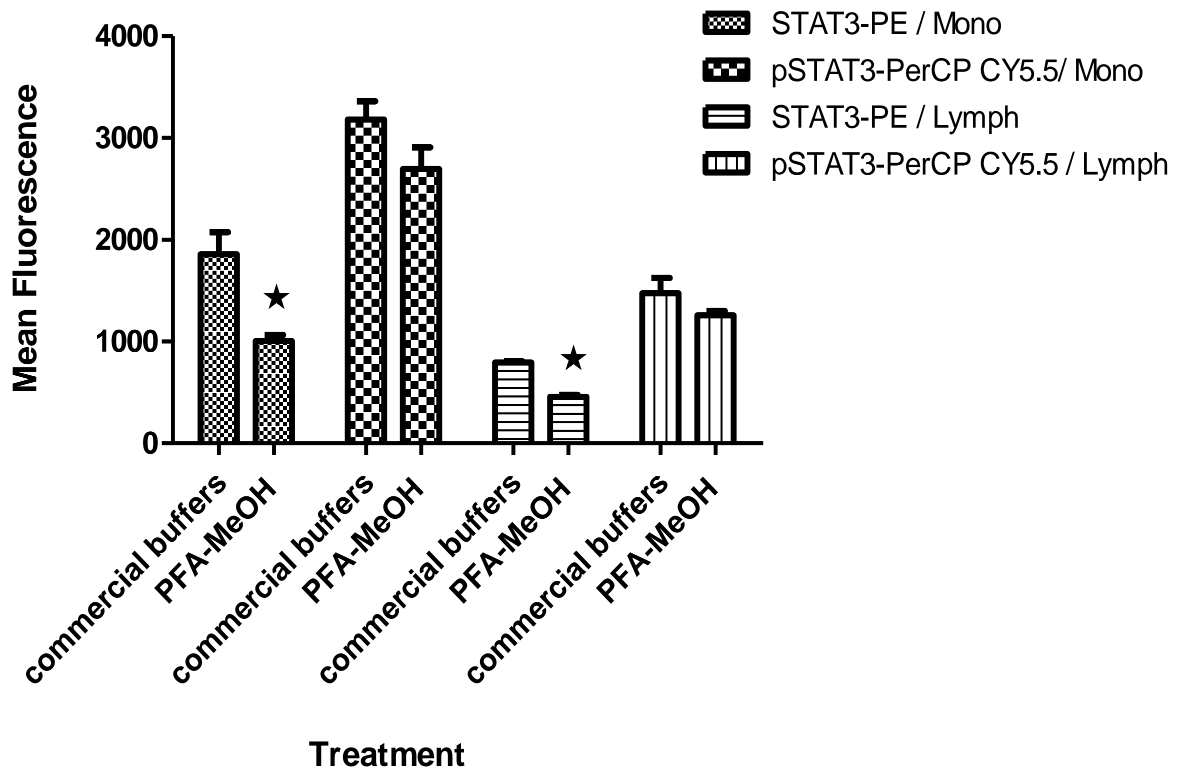

Figure 19: The mean fluorescence intensities of anti-STAT3-PE and anti-pSTAT3-PerCp Cy5.5 in Cells that were fixed and permeabilised with either paraformaldehyde/methanol or using BD commercial buffers in both monocytes and lymphocytes. The cells were analysed using Accuri cytometer. (*) $p < 0.05$.

Compared to the BD fixation and permeabilisation buffers, paraformaldehyde fixation and methanol permeabilisation resulted in a significant decrease in the fluorescence intensity of PE conjugated antibody ($p < 0.05$) while, they didn't significantly affect

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the MFI of PerCP Cy5.5 conjugated antibody. This decrease in fluorescence intensity was seen in both lymphocyte and monocyte cell populations.

Based on these observations, the BD commercial buffers were used in the IL-10 experiments.

3.7 The effect of adding phosphatase inhibitors during fixation in flow cytometric analysis

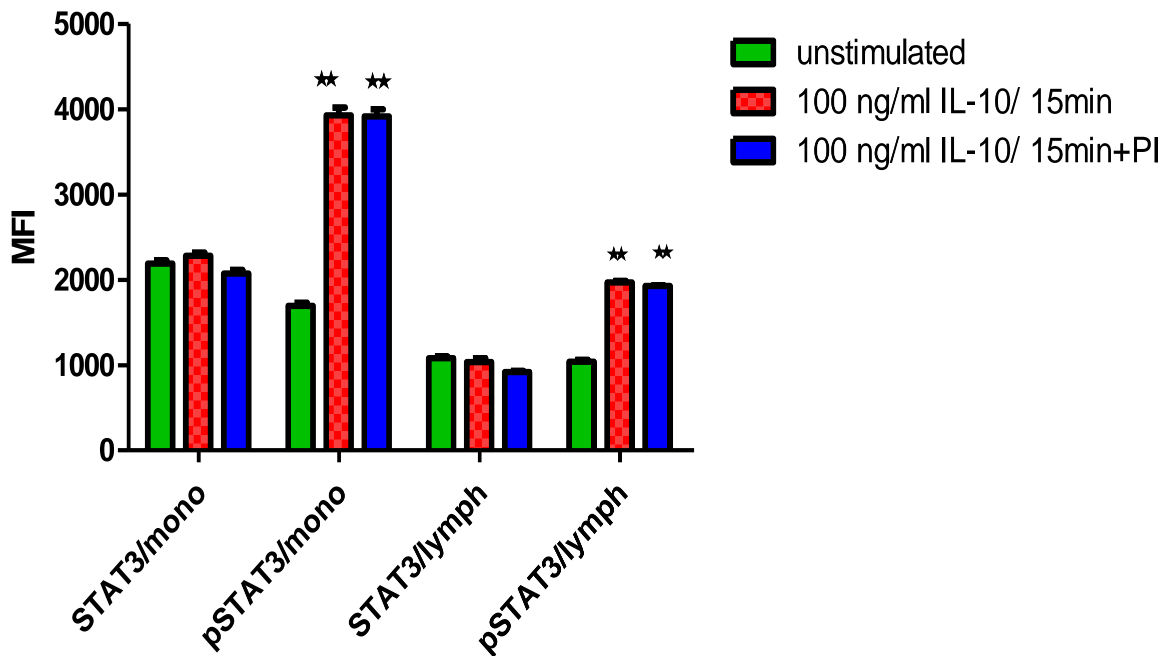

Figure 20: The effect of adding Phosphatase inhibitors during fixation in flow cytometric analysis. Following stimulation with 100 ng/ml of IL-10 for 15 minutes, the cells were fixed either with Cytofix buffer alone or combined with phosphatase inhibitor II. The cells were then permeabilised with Perm buffer III and incubated with anti-STAT3-PE and anti-pSTAT3 PerCP Cy5.5 for one hour. Analysis was done using an Accuri cytometer and the MFI was reported. (**) $p < 0.01$.

It is notable from Figure 20 that the level of STAT3 seems to be unresponsiveness to stimulation with IL-10. Agreeing with the response of STAT3 to IL-6 stimulation, very minor changes can be seen in both populations. However, an increase in the STAT3 phosphorylation level can be detected after 15 minutes stimulation in both monocyte and lymphocyte populations ($p < 0.01$). Despite the activation of both populations by

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IL-10, the phosphorylation in the monocytes was almost double that in the lymphocytes.

Surprisingly, no effect of the addition of phosphatase inhibitor was seen on the phosphorylation and consequently on the MFI of either antibodies in the entire populations. Accordingly, it appears that there is no need for the addition of phosphatase inhibitor during the fixation as it seems that the buffers used can arrest the activity of phosphatase enzymes.

3.8 Time response of STAT3 and pSTAT3 to 50 ng/ml or 100 ng/ml IL-10 in 3 donors

To study the kinetics of STAT3 following stimulation with IL-10, the cells from the same three healthy donors were fixed and permeabilised using BD buffers as described in the method section. Lower concentrations of IL-10 (1 or 10 ng/ml) that were tested in the preliminary studies were not sufficient to induce phosphorylation of STAT3. Therefore, higher concentrations (50 ng/ml or 100 ng/ml) of IL-10 were used in this experiment.

From Figure 21, it is noticed that in response to stimulation with either 50 ng/ml or 100 ng/ml IL-10, no significant change in the total STAT3 was detected in either lymphocytes (panel A) or monocytes (panel B). Once again, it seems that STAT3 keeps a constant level even after stimulation.

However, following stimulation with 100 ng/ml of IL-10, an increase in the phosphorylation of STAT3 was detected after 10 minutes stimulation and kept high at later time point (Figure 22). This activation was noticed in both lymphocytes (panel A) and monocytes (panel B) of the three donors. In response to 50 ng/ml of IL-10, longer time was needed to induce a significant increase in the phosphorylation of STAT3 compared to the unstimulated cells. Moreover, inter-individual variability in responding to 50 ng/ml of IL-10 was noticed between the three donors.

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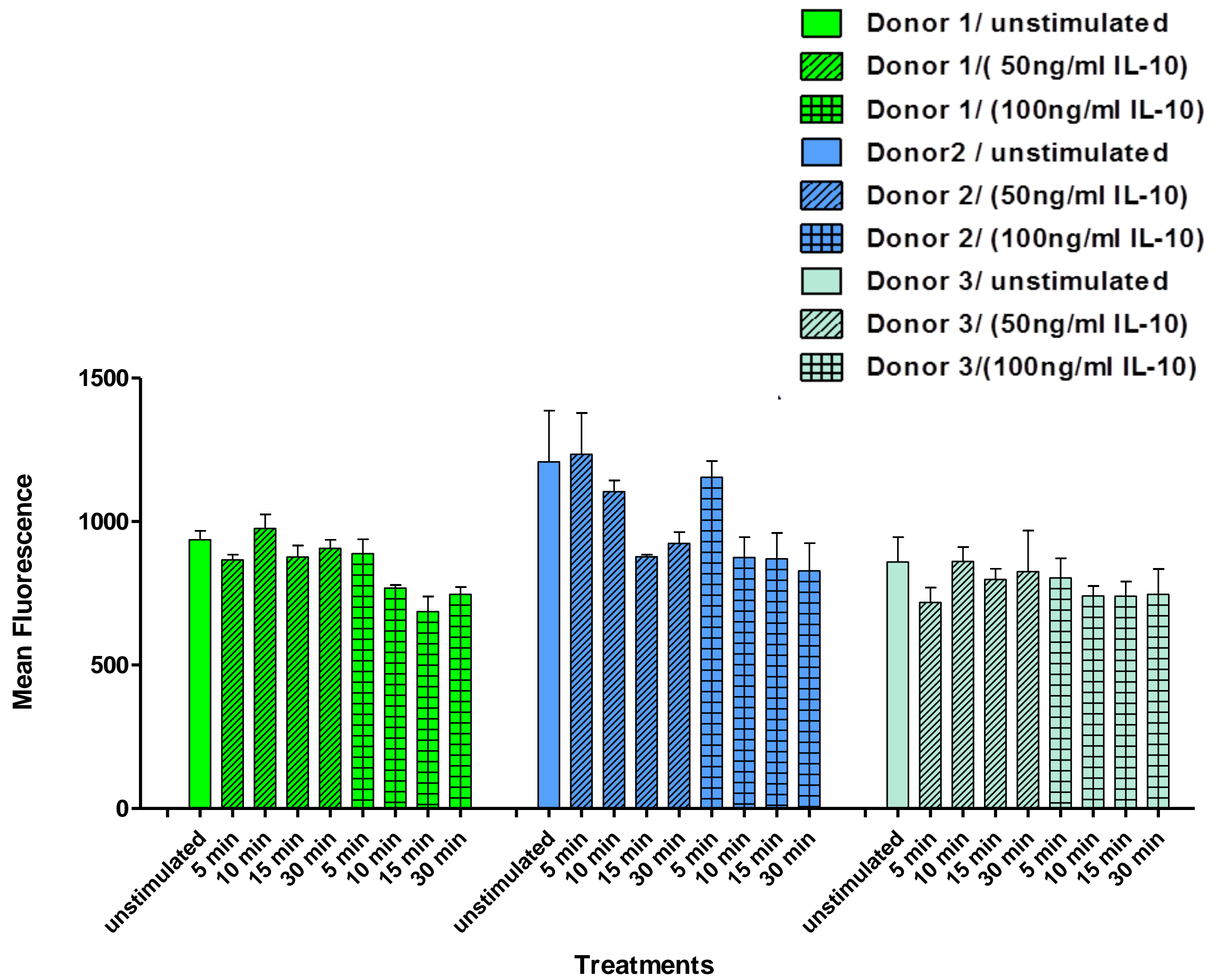
STAT3 changes in 3 donors

A)



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B)

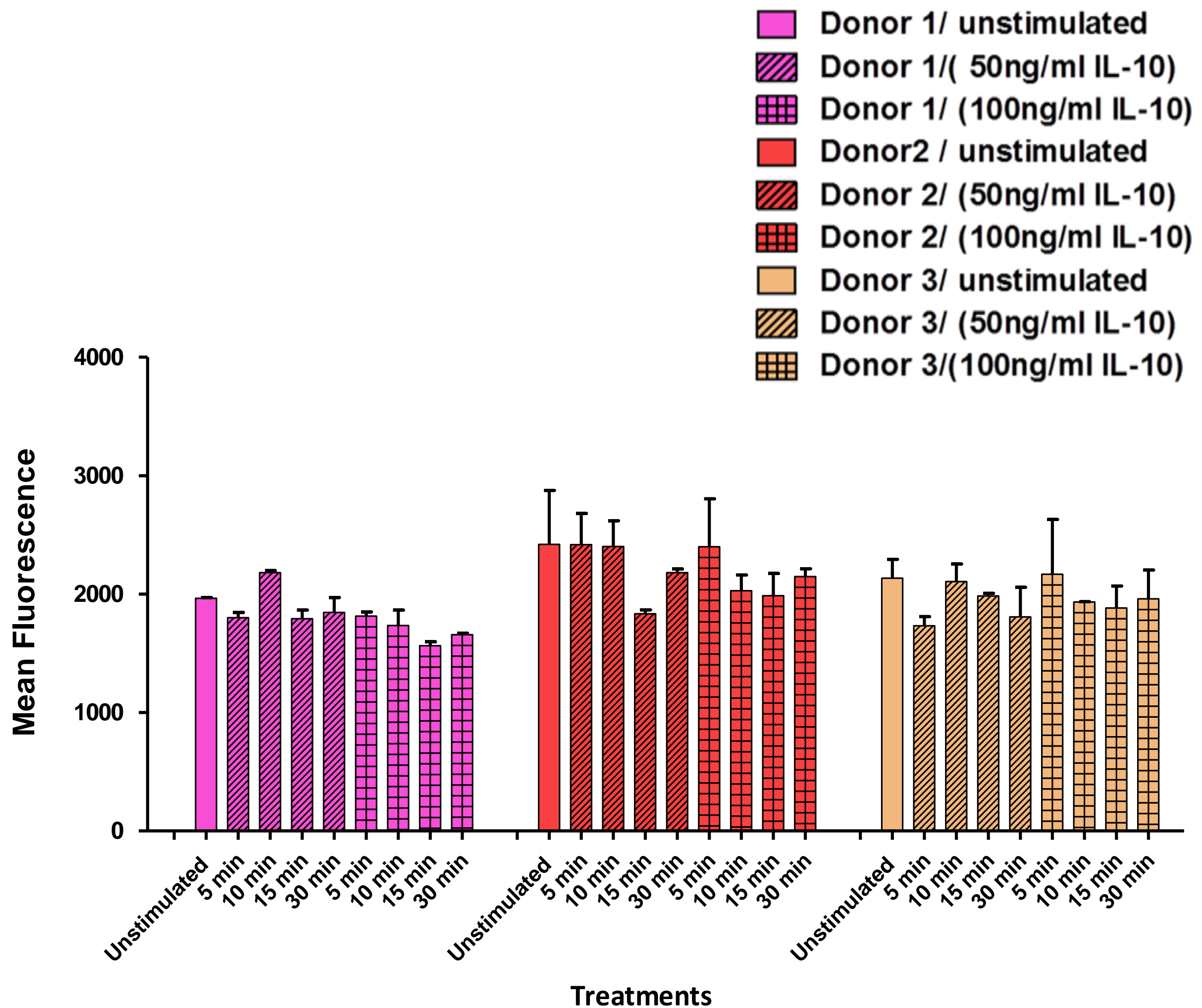

Figure 21: Three donors' kinetics of STAT3 following stimulation with 50ng/ml or 100ng/ml of IL-10 in A) Lymphocytes and B) monocytes. PBMC were fixed with BD fixation buffer and permeabilised by BD Perm buffer III. The cells were incubated with both anti-STAT3-PE and anti-pSTAT3-PerCp Cy5.5 for one hour. This experiment was repeated twice on the same day and the samples were analysed using Accuri cytometer. No significant change in either lymphocytes or monocytes total STAT3 was detected in comparison to the unstimulated cells in the 3 healthy donors.

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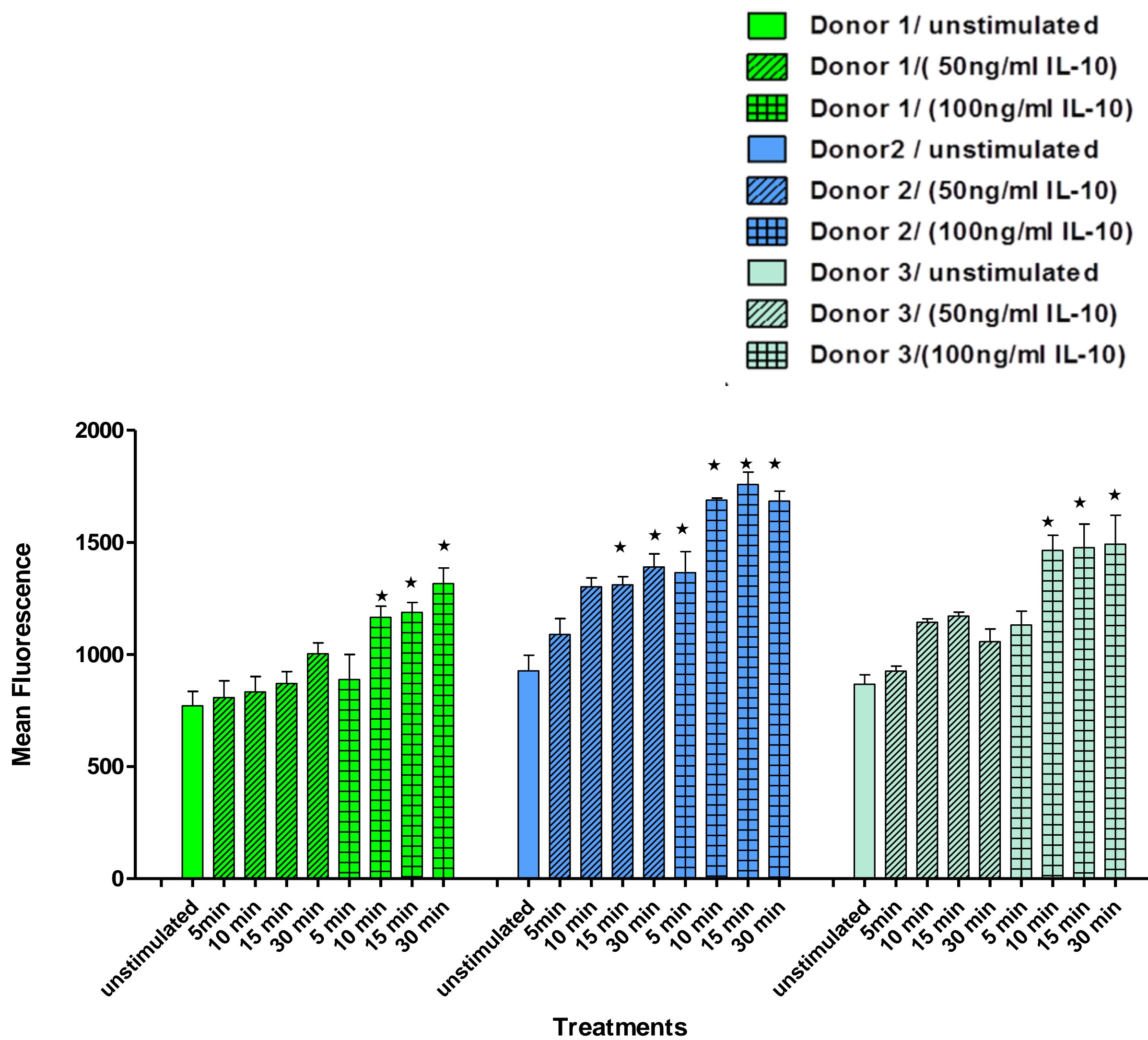
pSTAT3 changes in 3 donors

A)



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B)

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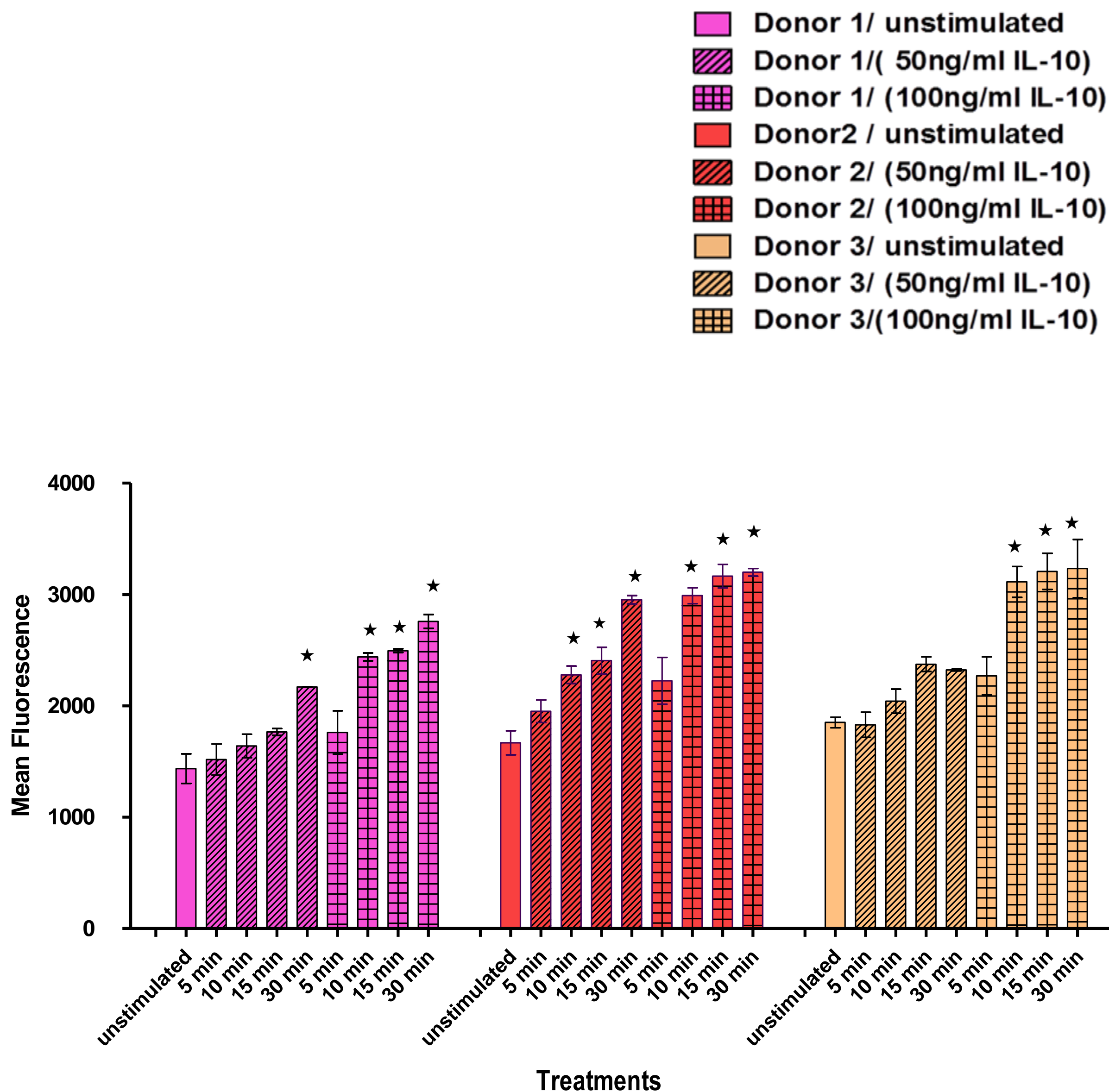

Figure 22: Three donors' kinetics of pSTAT3 following stimulation with 50ng/ml or 100ng/ml of IL-10 in A) Lymphocytes and B) monocytes. PBMC were fixed with BD fixation buffer and permeabilised by BD Perm buffer III. The cells were incubated with both anti-STAT3-PE and anti-pSTAT3-PerCp Cy5.5 for one hour. This experiment was repeated twice on the same day and the samples were analysed using Accuri cytometer. An increase in the phosphorylation level of STAT3 was noticed following 10 minutes of stimulation with 100 ng/ml of IL-10 in both lymphocytes and monocytes. Inter individual variability in responding to 50 ng/ml of IL-10 was noticed between the 3 donors. (*) $p < 0.05$.

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3.9 Western blot analysis of STAT3 and pSTAT3 following IL-6 stimulation

Western blot analysis of IL-6 stimulated cell lysates were done as described in the method section and were probed using the same primary fluorescent antibodies that were used in the flow cytometric analysis to test for their specificity. The experiment was repeated three times. In each repeat, the fluorescently conjugated antibodies detected lower molecular weight protein bands (MW = 46 - 68 KDa) than the STAT3 protein. Moreover, no bands were detected in the expected molecular mass region of STAT3 protein (MW = 89 - 92 KDa). There were however two very distinct bands visualised at molecular weights of 65 KDa and at approximately 46 KDa, as shown in Figure 23 below, where the stimulation time verses band intensity was assessed. To complicate the results further, no differences were observed between the bands when probed with either BD anti-STAT3-PE (Figure 23/ right panel) or with BD anti-pSTAT3-PerCP CY5.5 (Figure 23/ left panel).

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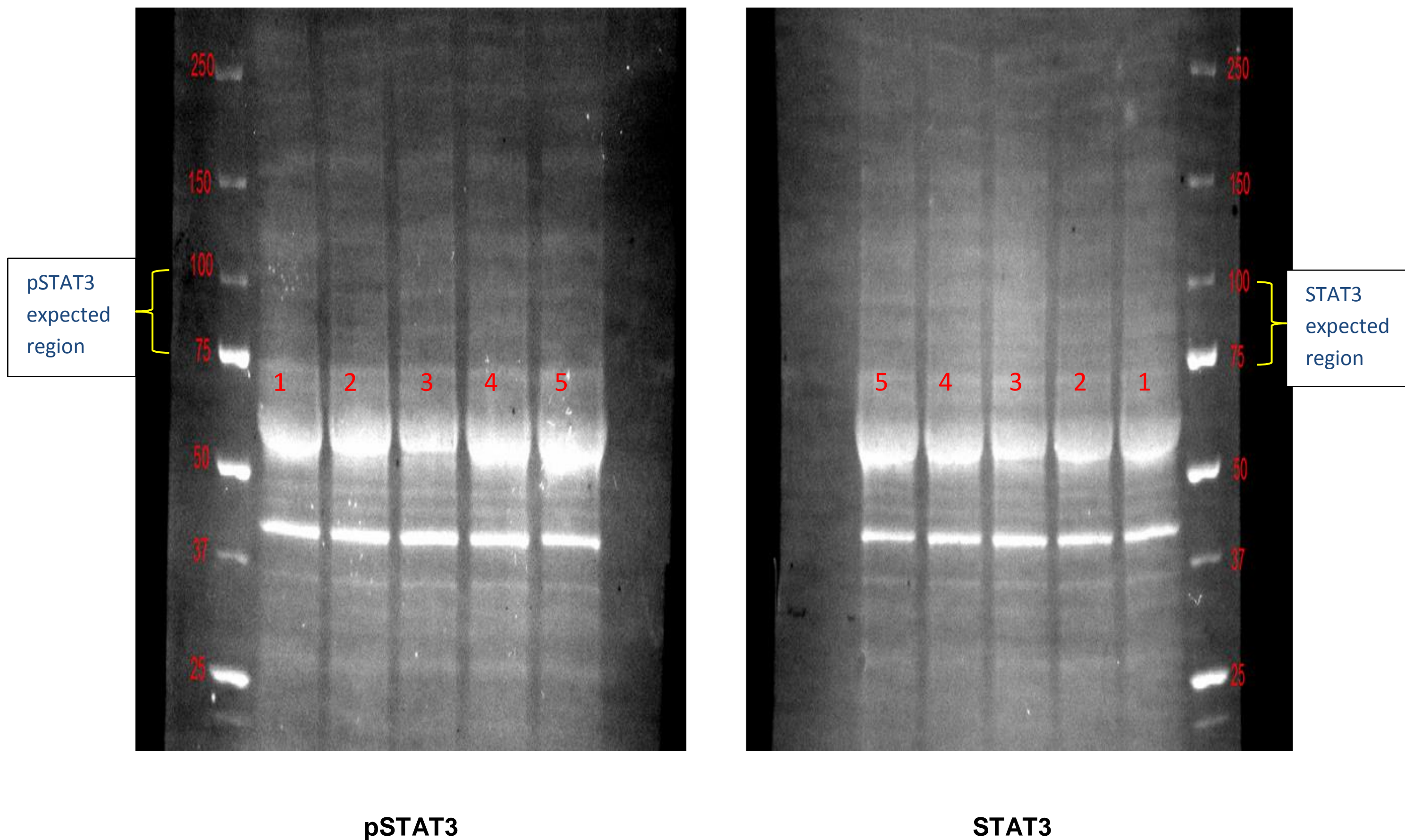

Figure 23: Western blot analysis of time response of 100 ng/ml of IL-6 stimulation. 1) Unstimulated cells. 2) 5 minutes of stimulation. 3) 10 minutes of stimulation. 4) 15 minutes of stimulation. 5) 30 minutes of stimulation. The samples were run on the same gel and blotted on the same membrane. The membrane was then cut into two pieces and each piece was incubated with either anti-STAT3 or anti-pSTAT3 antibodies. Fluorescence detection using the Bio-Rad ChemiDoc XRS was used for visualization. STAT3 and pSTAT3 bands are expected to be detected in the MW region of 100 - 75 KDa.

3.10 Mass spectrometer analysis of the in gel digestion of the upper dense bands in Figure 23

The results of nano LC mass spectrometer analysis of the peptides of the upper dense bands that excised from the gel represented in Figure 23 are presented in Table 4. It is noted that the most abundant protein detected in the 65 KDa protein bands was serum albumin from *Bos Taurus* (N1). However, no fragments or peptides of STAT3 can be detected in these bands.

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Table 4: ESI mass spectrometer analysis of in-gel digestion of the proteins in the dense band of IL-6 stimulated cells lysate gel electrophoresis

Proteins Detected

N	Unused	Total	% Cov	Accessio...	Name	Species	Peptides(95%)
1	75.96	75.96	68.5	AAN17824	AF542068 NID: - Bos taurus	Bos taurus	90
2	27.90	27.90	39.4	S21097	alpha-1-antitrypsin precursor - bovine	bovine	19
3	12.00	12.00	32.0	A35714	fetuin precursor - bovine	bovine	11
4	8.02	8.02	12.9	Q2KIQ9_...	SERPINA3 protein.- Bos taurus (Bovine).	Bos taurus	4
5	7.16	7.16	15.5	Q9NTI9_...	Lymphocyte cytosolic protein 1 (L-plastin).- Hom...	Homo sapiens	5
6	6.12	73.78	68.2	AAI02743	BC102742 NID: - Bos taurus	Bos taurus	83
7	4.00	4.00	8.0	Q4FZU2...	Hypothetical protein.- Rattus norvegicus (Rat).	Rattus norvegicus	2
8	4.00	4.00	9.1	S13560	translation elongation factor EF-Tu.A - Salmonel...	Salmonella typhimurium	2
9	4.00	4.00	13.1	CAC88237	Sequence 1 from Patent WO0164857.- Rhodoc...	Rhodococcus rhodochrous	2
10	3.07	3.07	8.1	S64635	pyruvate kinase (EC 2.7.1.40), muscle splice for...	human	2
11	2.00	2.00	2.3	AAI03070	BC103069 NID: - Bos taurus	Bos taurus	1
12	1.64	1.64	7.8	TRPGTR	trypsin (EC 3.4.21.4) precursor - pig (tentatives...	pig	2

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3.11 Mass spectrometer analysis of the in gel digestion of the thin lower bands in Figure 23

Table 5: ESI mass spectrometer analysis of in-gel digestion of the proteins in the prominently probe sensitive thin lower band of of IL-6 stimulated cells lysate gel electrophoresis

N	Unused	Total	% Cov	Accessio...	Name	Species	Peptides(95%)
1	78.55	78.55	74.5	Q6QAQ1...	Cytoskeletal beta actin (Fragment).- Sus scrofa (...	Sus scrofa	101
2	26.86	26.86	57.8	Q5R496_...	Hypothetical protein DKFZp459A052.- Pongo py...	Pongo pygmaeus	19
3	24.57	24.57	27.1	K2C1_H...	Keratin, type II cytoskeletal 1 (Cytokeratin-1) (C...	Homo sapiens	12
4	22.85	22.85	28.5	AAN17824	AF542068 NID: - Bos taurus	Bos taurus	14
5	18.00	18.00	22.4	VIME_RAT	Vimentin.- Rattus norvegicus (Rat).	Rattus norvegicus	9
6	11.87	11.87	34.1	CAG46678	CR541880 NID: - Homo sapiens	Homo sapiens	7
7	9.02	9.02	20.1	CAA82315	HSKERAT9 NID: - Homo sapiens	Homo sapiens	4
8	7.67	10.48	16.6	Q4VAQ2...	Keratin 2A (Epidermal ichthyosis bullosa of Sie...	Homo sapiens	4
9	6.86	6.86	17.6	A35714	fetuin precursor - bovine	bovine	3
10	6.81	6.81	9.2	SEPT9_...	Septin-9 (MLL septin-like fusion protein) (MLL s...	Homo sapiens	3
11	6.00	6.00	25.1	TRPGTR	trypsin (EC 3.4.21.4) precursor - pig (tentatives...	pig	4
12	5.83	5.83	22.2	S27383	elastase inhibitor - human	human	4
13	5.74	5.74	15.2	AAX43878	AY891951 NID: - synthetic construct	synthetic constr...	3
14	5.32	5.32	15.4	Q53FG3...	Interleukin enhancer binding factor 2 variant (Fr...	Homo sapiens	3
15	4.74	4.74	9.4	S00755	pleckstrin - human	human	2
16	4.37	4.37	18.5	Q8N175_...	Keratin 10.- Homo sapiens (Human).	Homo sapiens	2
17	4.01	22.33	54.1	ABF99754	DP000009 NID: - Oryza sativa (japonica cultivar...	Oryza sativa	20
18	4.01	4.01	15.4	Q9BQ80...	Ribonuclease/angiogenin inhibitor.- Homo sapie...	Homo sapiens	2
19	4.00	4.00	16.3	S33438	laminin receptor, 34/67K - Chinese hamster	Chinese hamster	2
20	4.00	4.00	8.6	Q5ZIH1_...	Hypothetical protein.- Gallus gallus (Chicken).	Gallus gallus	2
21	3.80	3.80	11.5	Q6F3E7_...	Elongation factor-1 alpha.- Lampetra japonica (J...	Lampetra japoni...	2
22	3.28	3.28	9.1	S21097	alpha-1-antitrypsin precursor - bovine	bovine	2
23	2.81	2.81	7.0	AAI03070	BC103069 NID: - Bos taurus	Bos taurus	1
24	2.70	2.70	17.1	S43440	3-oxoacyl-CoA thiolase - human	human	1
25	2.48	30.10	45.7	AAC80574	ECCACTNII NID: - Echinococcus granulosus	Echinococcus g...	26
26	2.30	2.30	17.9	Q96FG8...	ACAT1 protein.- Homo sapiens (Human).	Homo sapiens	1
27	2.18	2.18	4.9	AAL40393	AF085235 NID: - Homo sapiens	Homo sapiens	1
28	2.03	2.03	17.3	S30377	cytochrome c - American alligator	American alligator	1
29	2.01	25.64	43.4	Q27S91_...	Beta-actin.- Culex pipiens pipiens (Northern hou...	Culex pipiens pi...	29
30	2.00	78.55	80.3	ATHUG	actin gamma 1 - human	human	95
31	2.00	69.00	80.0	Q3TIJ9_...	TIB-55 BB88 cDNA, RIKEN full-length enriched l...	Mus musculus	87
32	2.00	37.39	61.0	AAA82601	DDU27834 NID: - Diphyllobothrium dendriticum	Diphyllobothriu...	36
33	2.00	21.43	45.4	Q6SQL8...	Actin (Fragment).- Lepidochitona cinerea.	Lepidochitona c...	23
34	2.00	19.35	40.2	Q9NC31...	Actin (Fragment).- Chiroteuthis veranyi.	Chiroteuthis ver...	22
35	2.00	2.00	19.4	THIO_EC...	Thioredoxin 1 (TRX1) (TRX).- Escherichia coli O...	Escherichia coli O	1
36	2.00	2.00	6.6	S78515	single-stranded nucleic acid-binding protein CB...	mouse	1
37	2.00	2.00	12.6	DECHG3	glyceraldehyde-3-phosphate dehydrogenase (ph...	chicken	1
38	2.00	2.00	3.7	S58529	alpha-complex protein 1 - human	human	1
39	2.00	2.00	6.4	Q5GN72...	Alpha-1-acid glycoprotein precursor (Fragment)....	Bos taurus	1
40	1.92	2.01	8.7	KRHUE	keratin 14, type I, cytoskeletal - human	human	1

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Table 5 above summarised the different proteins that have been detected in the nano LC analysis of the lower excised thin bands from the gel in Figure 23. Of note, cytoskeletal beta actin (N1) was the major human protein detected in these bands. Actin is well known as an intracellular house-keeping protein which expected to be found in all human cells including PBMC. However, No STAT3 protein or STAT3 peptide fragments were also detected in these bands.

Keratin is the common contaminant in the in gel digestion procedure. Therefore, it is expected to be seen in the results of the bands analysis. However, due to the precautions that were taken during the samples preparation (mentioned in the method section), contaminant keratin was detected in very small amounts (N7, N8 and N16). Note that N3 and N40 are cytoskeletal keratins found in most human cells and would be derived from the monocyte cytoskeleton and not contaminants.

3.12 Non-specific anti-STAT3-PE detection of western blot of denatured BSA sample

To investigate if the fluorescently conjugated antibodies can also non-specifically bind to BSA fraction V, a western blot analysis of a prepared sample that just contained BSA fraction V was done.

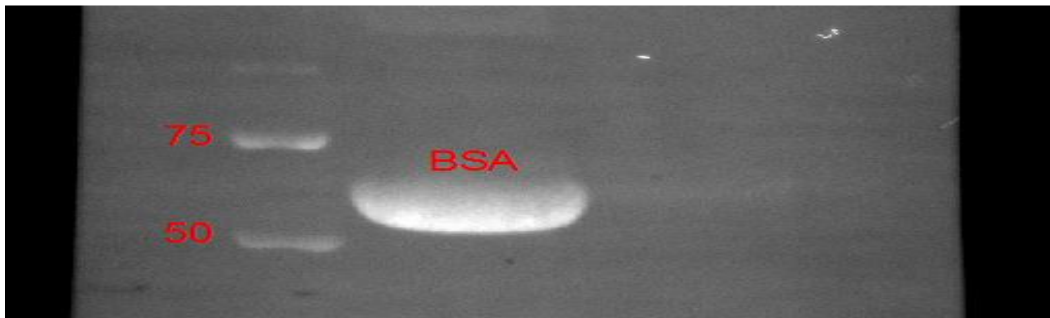

Figure 24: Western blot of denatured BSA that was detected by anti-STAT3-PE. The blot was imaged using Bio-Rad ChemiDoc XRS using the fluorescent protocol.

Surprisingly, Figure 24 showed that the fluorescently conjugated antibodies that were used in the flow cytometric assay, in concentrations suggested by the supplier, bound non-specifically not only the FCS but also to BSA Fraction V. However, the same antibodies didn’t detect the BSA that was used for blocking non-specific protein binding of the membrane. Due to all the previous troubleshootings with the

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fluorescently conjugated antibodies, these antibodies were excluded in the western blot analysis of the IL-10 stimulation experiments and replaced with primary and secondary antibodies suitable for chemiluminescent western blotting.

3.13 Time response of STAT3 and pSTAT3 following stimulation with IL-10 by western blot

For the dose response experiment, different concentrations (1, 10 and 100 ng/ml) of IL-10 were used to stimulate PBMC for 15 minutes while to evaluate the kinetic response, cells were stimulated with 100 ng/ml IL-10 for different time intervals as described in the method section. However, antibodies that were purchased from Sigma Aldrich (described in the reagents section) were used for probing. The commercial chemiluminescence mixture was used for detection and the Bio-Rad ChemiDoc XRS was used for visualization. STAT3 was detected within the expected molecular weight region. It is detected in unstimulated cells as well as within cells that were stimulated with different doses of IL-10. Following stimulation with 100 ng/ml IL-10, Figure (25/A) showed that the level of STAT3 didn't significantly change following stimulation at different time intervals in comparison to the unstimulated cells' STAT3 level. This finding confirms the thought that the cells have a constant level of the total STAT3 and this matched with what was seen with the flow cytometric results.

Figure (25/B) showed that lower doses of IL-10 were insufficient to induce phosphorylation of STAT3. However, 100 ng/ml of IL-10 can induce the phosphorylation of STAT3. The increase in the pSTAT3 was detected after 5 min of stimulation and reached the maximum level after 15 min of stimulation. Those observations was also matched with what have been seen using the flow cytometric assay.

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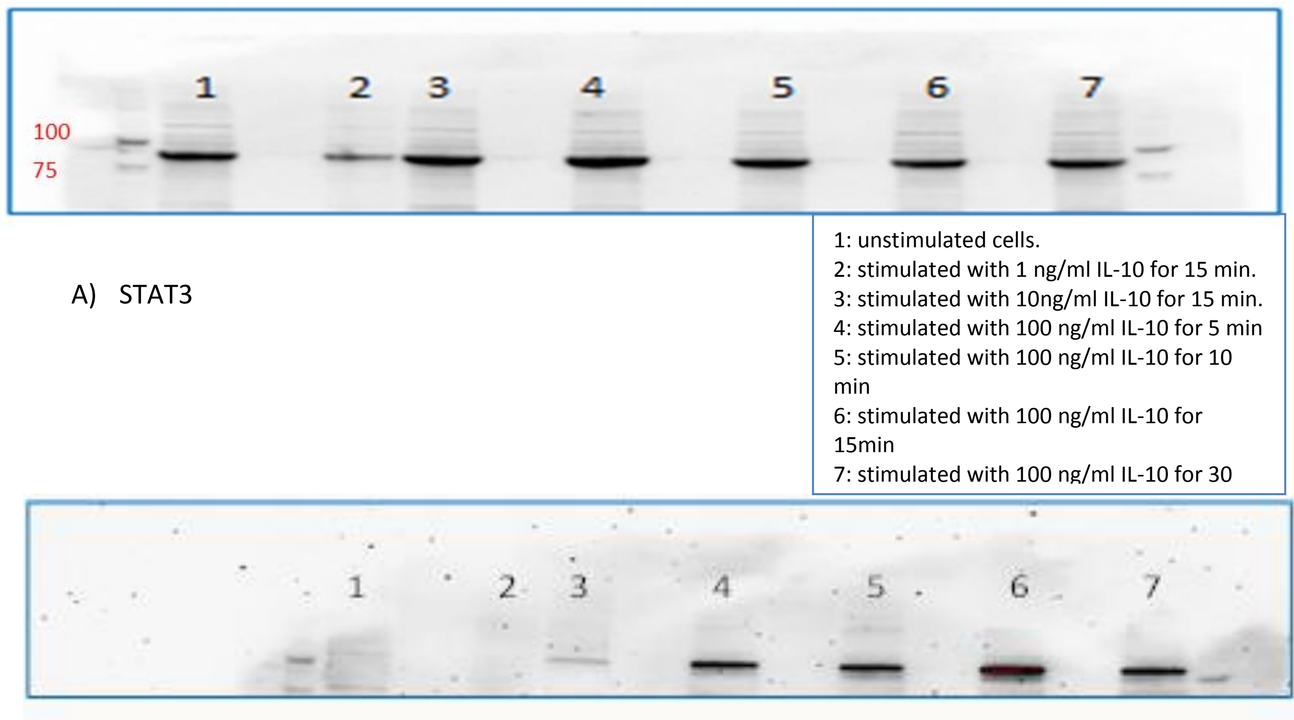

B) pSTAT3

Figure 25: Western blots of lysates from PBMC which were stimulated with different doses of IL-10 for 15min or with 100 ng/ml of IL-10 for different time intervals. STAT3 (panel A) was still detected following stimulation with different doses of IL-10 and at different stimulation times. The phosphorylation of STAT3 (panel B) was increased with higher doses of IL-10 and it reached the maximum level at 15 minutes. Commercial ECL solution was used for detection and the blots were visualized by Chemi-Doc XRS system.

3.14 Comparison between the performance of a commercial and “in house” chemiluminescence mixtures

The performance of “in house” and a commercial chemiluminescence mixture was compared in the detection of the western blots. 10 ml mixture of either the “in house” or the commercial detection solution was prepared as described in the method section and incubated with the blot for 5 minutes. Then, the blot was centred on the imaging plate and imaged by Bio-Rad ChemiDoc XRS Imager for different time intervals. The performance of the two detection solutions was represented in Figure 26 below.

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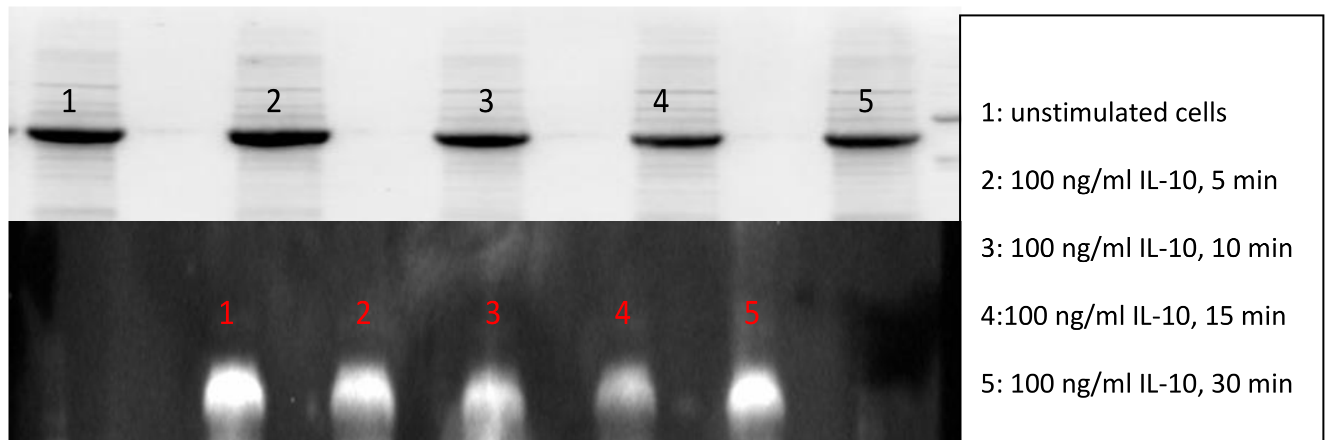

Figure 26: Kinetic of STAT3 protein in response to 100 ng/ml IL-10 using either commercial ECL (the top row) or "in house" chemiluminescence mixture (the bottom row). Lysates of stimulated PBMC were separated by gel electrophoresis and blotted to PVDF membranes. The blots were incubated with rabbit anti-STAT3, probed with goat anti-rabbit IgG conjugated to HRP and visualized by Chemi-Doc XRS system.

Both the onset and the signal intensity of the "in house" ECL solution (the bottom row) were comparable to the commercial ECL solution (the top panel) in detecting the STAT3 protein. However, the background from the "in house" chemiluminescence mixture was much higher than the commercial one and the background was increased dramatically with time leading to signal loss with longer exposure time as presented in Figure 27.

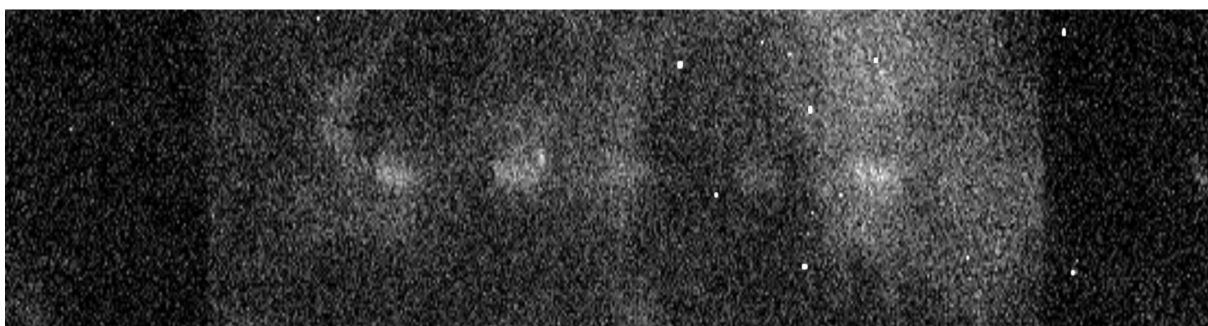

Figure 27: Detection of western blot with in house ECL mixture after 4 minutes of exposure time. Note the high background relative to the signal. The ChemiDoc XRS system automatically adjusts the signal intensity.

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Chapter 4: Discussion and Conclusion

Two different relative quantitation techniques for assessing expressed protein, flow cytometry and the traditional western blot have been applied and compared in the analysis of cytokine induced phosphorylation of intracellular STAT3 protein. Compared to the western blot, flow cytometric methods have much fewer requirements on the number of input cells; it requires less time for processing and last but not least, is also much more cost effective (76). It allows the visualization of the cell population of interest without further separation steps. Moreover, it allows simultaneous multi-parametric analysis (98) of not only the intracellular STAT3 proteins but also the surface marker proteins that can be used to phenotype selected cell populations. In this study; both the flow cytometry and the western blot technique showed a comparable qualitative trend in the detected changes of pSTAT3 following IL-10 stimulation only.

In the flow cytometric approach, the cytometric analysis was applied using two different cytometers: a FC500 from Beckman Coulter and an Accuri from Becton Dickinson. The mean fluorescence intensity of the samples differed greatly depending on the instrument used despite the same antibodies being used. An added confounder was that the experimental cells were collected from different donors on different days. Moreover, different batches of reagents were used during the sample preparation steps. For these reasons, the comparison between the experimental results was extremely difficult. However, the trends and patterns of activation can be compared and was calculated as a fold change in the MFI of stimulated cells in comparison to the unstimulated cells.

As opposed to any other tissue in the body, cells being part of the immune system display a huge diversity and hundreds of subsets can be identified even within the same lineage (99). One of the important features of flow cytometric analysis is the ability to identify the cell population of interest in a heterogeneous cell mixture. Selecting this identified group of cells and performing further analysis on only those identified cells is referred to as gating, which can be done based on either the physical properties of the cells of interest, such as forward and side scatter, or/and

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the fluorescence intensity of fluorochrome conjugated antibody against specific surface protein of these cells.

PBMC is a mixture of several distinct cell populations: monocytes and T and B lymphocytes. Based on the differences in the physical properties between these two populations which results in characteristic laser light scatter properties, the cells of interest were gated. As previously described, forward scatter is more sensitive to the size of the cell while the side scatter correlates with the cell granularity. Monocytes are large but not so granular while lymphocytes are small, non-granulated cells. Therefore, monocytes produce a higher forward and side scatter in comparison to lymphocytes. However, it should be taken into consideration that the physical properties of the cells can be severely affected by the sample preparation steps required to make the cell interior accessible to the probe antibodies and thus, in turn, can lead to difficulty in establishing a reliable gating. Additionally, the change in the physical properties means that the gating that established using unprocessed cells can't be applied to fixed/permeabilised cells. To overcome this problem, the combination of the fluorescence intensity of the fluorochrome conjugated antibody against a unique surface marker expressed selectively by only one of these populations along with the scatter property of this population were used to identify selected cell populations. Anti-CD14-FITC was used to stain the CD14 antigen which is selectively and intensely expressed on the surface of monocytes but not on lymphocytes. Depending on both the side scatter properties and the fluorescent intensity of the CD14-FITC antibody, the CD14 positive monocytes can be identified. Combining the fluorescence of a population specific marker antibody with the scatter characteristics greatly improved the accuracy of correct gating of a specific cell population.

It is well-known that any fixation or permeabilisation procedure may negatively impact the intensity of cell surface expression (100). Therefore, when developing the protocol for intracellular phosphoprotein analysis, the effect of fixation and permeabilisation buffers on the surface markers probing should be tested. In this study, probing for the CD14 surface marker was still detectable after paraformaldehyde fixation while it was completely supressed following permeabilisation with alcohol. Although alcohol permeabilisation allows for efficient access of the antibody to an intracellular antigen, such as STAT3, it can harshly

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compromise some surface marker antigens such as CD14 and affects the fluorescence of some fluorophores such as PE. Therefore, to ensure proper gating within the heterogeneous sample, a sequential staining was followed in this study in which surface marker probing was done before permeabilisation. Importantly, sequential staining is limited to small fluorophore conjugated antibodies which are not affected by methanol treatment, such as FITC.

Although it is possible to make in house flow cytometric fixation and permeabilisation buffers, these buffers would need to vary in composition depending on the antibodies and the fluorochromes used as well as the phosphorylated protein being studied (101). Traditionally, most protocols for flow cytometric analysis of phosphorylated STAT used paraformaldehyde for fixation and methanol for permeabilisation (102, 103). However, a number of commercial kits using proprietary reagents are now available. The effect of Beckton Dickinson "fix/perm" buffers were tested on the fluorescent intensity of the intracellular antibodies compared with the effect of the F/M buffers. Surprisingly, in comparison with the BD commercial buffers, the F/M buffers significantly decreased the fluorescence intensity of the PE fluorochrome without significantly affected the fluorescence intensity of the PerCP Cy5.5 fluorochrome. In line with these observations, the BD commercial fix/perm buffer kit was used in IL-10 stimulation experiments.

In order to draw a conclusion about flow cytometric assay's results, it should be kept in mind that this procedure is highly dependent on the affinity and selectivity of the antibody used against a specific epitope on the protein of interest. Lack of specificity may occur as a result of several factors such as: the antibody may bind to a common epitope on several different proteins or bind to many cell types via their non-specific (Fc) ends (104). Monocytes are well known to bind non-specifically to different antibodies due to their Fc region binding capability. Using a low concentration of a protein based blocking reagent, such as BSA in the assay solution can minimize the nonspecific binding. Some publications reported the cross reactivity of the antibodies used with serum proteins or assay immunoglobulin and suggested the pre-screening of the antibodies for both low background and high sensitivity (105),(106).

Directly conjugated antibodies are becoming widely available which are more practical when probing with multiple antibodies. In addition, their use reduces the

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probing steps. Importantly, attention should be taken in the choice of the fluorochrome that is conjugated to the antibody. This strategy depends on the lasers available on the instrument used, the amount and location of the antigen, the compatibility of the fluorochrome with the sample preparation reagents used and the "spill over" into adjoining fluorescence channels in a situation where more than one fluorochrome conjugated antibody was used. PE and PerCP CY5.5 conjugated antibodies were used to stain intracellular STAT3 and pSTAT3 respectively as their emission wavelength are wide enough apart to avoid the need for colour compensation.

The kinetics of STAT3 in response to different cytokine stimulation were studied with respect to the response to the stimulus, as well as whether the duration of the elicited response played a critical role in determining the magnitude of downstream signalling (107). When comparing the two different cytometers that were used, their results were not completely similar. In response to 100 ng/ml IL-6 stimulation, Beckman Coulter analysis showed a variation in the response of the total STAT3 between the three donors while the maximal phosphorylation of STAT3 was detected within 10 minutes in both lymphocytes and monocytes. On the other hand, when using the Accuri cytometer, a slight non-significant decrease in the total STAT3 was detected following IL-6 stimulation and the maximal phosphorylation was seen within 15 minutes in both cell populations tested. However, this increase in phosphorylation was only significant in the monocytes.

The differences in the results between the two instruments could be due to several different reasons. Initially, the voltage and the gain had to be adjusted each time before use in the FC500 cytometer while no adjustment was required for the Accuri cytometer due to the very wide dynamic range of the detectors. Therefore, any improper adjustment of parameters when using the FC500 could significantly affect the results. Secondly, gating the population of interest on Beckman Coulter was quite difficult especially post fixation and permeabilisation while the Accuri software has some features that enable easier and more consistent gating. Thirdly, only 10,000 events were collected in each sample in the FC500 which was later established that this number of events contained too few monocytes to obtain reliable results. Therefore, the events rate was increased to 100,000 when the Accuri was used. Finally, a major advantage of the Accuri is that the re-analysis of the

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results can be performed after the completion of sample analysis even if optimal data collection parameters were not used.

Due to the reasons highlighted above, the Accuri was used for the analysis of all the IL-10 induced STAT3 phosphorylation experiments. Different concentrations (1, 10, 50 and 100 ng/ml) of IL-10 were tested. Preliminary investigations showed that lower concentrations of IL-10 (1 and 10 ng/ml) were unable to induce detectable phosphorylation while 50 or 100 ng/ml of IL-10 can induce a dose dependent activation of STAT3. Following stimulation with 100 ng/ml of IL-10, the level of total STAT3 appeared not to be changed in either of the tested cell populations. However, this stimulation induced a rapid and significant increase in the level of pSTAT3 that peaks between 10 - 30 minutes. Notable inter-individual variability in STAT3 phosphorylation was observed when using 50 ng/ml of IL-10 for stimulation.

Many articles have reported the ability of IL-6 to strongly and rapidly activate not only STAT3 (58, 60, 84, 96, 108) but also the expression of SOCS3 protein (109). The maximal level of tyrosine phosphorylation, a measure of STAT3 activation, could be reached by 15 minutes (84, 109) or within 20 - 30 minutes (31) following stimulation. This activation of STAT3 by phosphorylation was not observed when using the FC500 cytometer and only a small effect was observed using the Accuri cytometer. This may have been due to the loss of stimulant activity with time or related to storage instability of the cytokine. These parameters could however not be tested as the IL-6 had been aliquoted on purchase and the possibility of stability issues was only proposed when the last aliquots were being used.

Some studies have reported a STAT3 phosphorylation induction in response to IL-10 stimulation as early as 5 minutes post exposure (84, 96, 110, 111) whereas translocation to the nucleus is not maximal until at least 30 minutes (111) which matches what was observed at the highest concentration of IL-10 used in this study.

Activation of STAT3 is reported to be rapid, with a maximum accumulation of phosphorylated STAT in the nucleus by 30 minutes after initiation, encompassing 20 - 25% of total STATs (21). These activated STATs are rapidly inactivated by dephosphorylation with a half-life of phosphorylated STAT being estimated to be ≤ 15 min (21). In spite of the majority of the STATs in the cell being activated, cycling through the nucleus and reappearance in the cytoplasm in dephosphorylated state, it

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was found that the total amount of 35S-labeled STAT1 was the same at the beginning (112) and at the end of IFN stimulation cycle.

Following IL-10 stimulation, both Alas *et al* (110) and Chaudhry *et al* (113) reported that no change in the amount of STAT3 expression was observed. Similarly, Liu *et al* (114) observed that IL-6 induced STAT3 phosphorylation in a dose-dependent manner, but did not alter the expression of total STAT3. Those observations matched with what was seen in this study regarding the level of total STAT3 following stimulation with both cytokines used.

Recently, different models have been proposed for the STAT3 signalling paradigm. The standard model assumed that all inactive STATs are in free monomeric state in the cytoplasm which dimerise subsequent to phosphorylation and are rapidly imported to the nucleus for transcriptional activation (115), (116).

The recent statosome model emerged following the observation of a small pool of monomeric STAT3 in the cytoplasm and the existence of inactive homo- or hetero-dimers prior to stimulation in the absence of phosphorylation (117). This model predicts that STAT3 associates with other proteins and the sizes of these molecular complexes are in the range of 200 – 400 KDa (statosome I) and 1 – 2 MDa (statosome II) (115, 118). The status of STAT3 within multiple equilibria of monomeric versus dimeric, free versus multi-protein complex-associated, and cytoplasmic versus nuclear forms which more likely have a substantial impact on the kinetics of STAT3 signalling (119).

Recently, nucleo-cytoplasmic shuttling of STAT3 was shown to be a dynamic process that involves constitutive shuttling of unstimulated STAT3 in the absence of cytokine stimulation (120).

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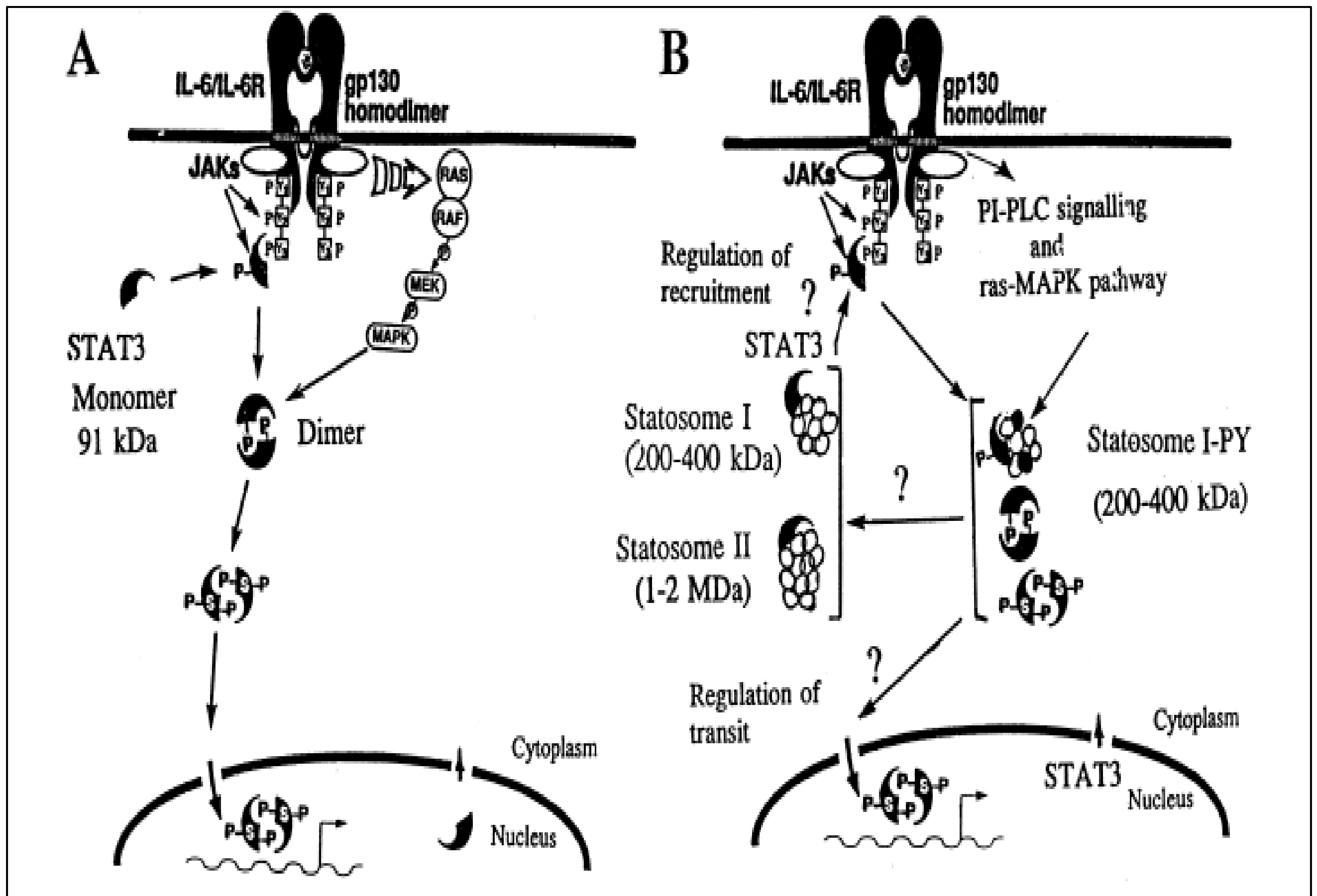

Figure 28: Models for STAT3 signalling. A) The standard model B) The statosome model. Modified from (Sehgal PB,2000) (121), with agreement licence from Elsiever.

Technically, flow cytometry is a sensitive and less demanding approach that is valuable in studying any activation pathway for which antibody reagents exist that discriminate between a native and an activation modified protein (122). Once the protocol has been optimized and the normal response profile to certain cytokines has been identified, the method can be used to detect signalling dysregulation associated with different immunological disorders. Furthermore, this can be used to identify patients with mutations that result in defective receptor proteins, JAKs, or STATs that retain the antigenic epitopes but are nevertheless dysfunctional (123). These results provide a comprehensive and physiologically relevant framework for comparison of signalling networks that have become altered in pathological disease states (124) and monitor the development of pharmacological intervention for these diseases. Despite the advantages of this technique, probing for intracellular pSTAT is a difficult multistep procedure due to the low and transient STAT expression level as well as the variable extent of phosphorylation that appears to be donor blood

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dependent. In addition to the inter-individual variation, inter-instrument variations were also observed in this study. Thus the urgent need for the consensus on the protocols is becoming essential to avoid discrepancies in the results reported when different protocols were used.

Although the western blot analysis is a reliable technique, it has some drawbacks such as the need for high cell numbers (>2 × 10^6 cells), difficulties in precise quantification of the signal, the impossibility of distinguishing the status of signalling molecules in cell subsets without sorting or separating beforehand, and its unsuitability for high-throughput analysis (125). In this study, western blot assay demonstrated comparable trends to those observed using the flow cytometric technique for the assessment of STAT3 response to IL-10. However, it gave a measure of the average phosphoprotein changes in the total samples without taking into account the level of expression in each cell population. This could present a skewed result when only a small subpopulation of cells responds to the stimulant.

Unlike lysate-based approaches, which pool protein from many cells, the sensitivity of flow cytometric methods is limited by the amount of phospho-protein present within a single cell (126). Thus, the specificity and the selectivity of the antibodies remains a limiting issue which should be validated carefully for the target in question prior to their application (127). Namiki *et al* as recently as 2008 stated “To date, the antibody generation is something of a gamble, with no guarantee for the sensitivity or selectivity of an antibody raised against an epitope from a protein of interest, and whether the antibody will be effective across all desired techniques” (128). When the fluorescently conjugated antibodies were used in the flow cytometric analysis of IL-6 stimulation experiments, insignificant detection of the phosphorylation of STAT3 was observed. Therefore, these antibodies were tested using the western blot assay on the same experiments.

Despite the several drawbacks of the western blot assay, one of its advantages that it provides information about the molecular weight of the detected proteins while the flow cytometry lacks this property. By loading a molecular weight marker during the electrophoresis step, the molecular weight of the antibody detected protein can be estimated. Hence, western blot yields additional information about the mass of the phosphoproteins detected and thereby an indication of the specificity of the phospho-

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specific antibodies applied in the setup (129). However, in this study, when the fluorescently conjugated antibodies were used in the western blotting of STAT3 response to IL-6 experiments, they detected two bands with lower molecular weight than the STAT3 molecular weight (MW 89 - 92 KDa). As the possibility of cross-reactivity or a truncated form of the STAT3 could have been present in these bands, these possibilities were investigated by performing a mass spectrometer based proteomic analysis of the bands identified by the fluorescent antibodies. The antibody identified protein bands were carefully cut from the gel and subjected to the in-gel digestion procedure using trypsin to digest the protein into peptide fragments. These peptides were then run on a nano LC system to separate and identify the peptides from which the possible proteins that exist in the detected bands could be inferred and whether STAT3 peptides were present in any of the excised detected bands.

Surprisingly, the major protein that was detected in one of these bands was serum albumin from *Bos Taurus* which was used as blocking buffer in both the flow cytometric and the western blot analysis. This was an unexpected result as the PVDF membrane had been blocked with a Fraction V BSA solution and if there was cross-reactivity, the entire membrane should have given a high background and not been limited to a single band per gel lane. Careful assessment of the experimental procedure revealed that the stimulation media for the PBMC contained 10% FCS to maintain cells survival and enhance stimulation. This FCS is not processed beyond inactivation of the complement proteins by a heating process that does not alter the serum albumin which is a major component of FCS whereas Fraction V is prepared using both heat and ethanol extraction which could remove the cross-reactive epitope from the Fraction V BSA used for membrane blocking.

It is well known that western blot is done on denatured sample of cell lysate. Therefore, when western blot of denatured sample of BSA was probed with one of the fluorescently conjugated antibodies, the antibody bound non- specifically to the denatured BSA while it didn’t detect the blocking BSA. One of the explanations to this finding is that sample preparation and denaturation might expose certain antibody binding epitope. Additionally, nonspecific binding may be attributed to either too much protein in the sample which can create adsorptive surface to certain

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IgG or to a highly sensitive detection system which can uncover the nonspecific binding.

The finding that implies cross-reactivity with FCS which was used in all the experiments as an additive to the culture media used during cell incubations may explain the apparent unresponsive results that were seen in the flow cytometric assays.

Several points should be highlighted regarding the western blot assay. Initially, protease and phosphatase enzymes play a critical and rapid role in terminating the phosphorylation and breaking down the protein. Therefore, the addition of protease and kinase inhibitors is crucial during western blotting sample preparation but not in the flow cytometric analysis, as shown in Figure 20, due to rapid fixation in the flow cytometric sample preparation which would destroy the proteolytic and phosphatase activities. Secondly, optimization of the technique should be done in a preliminary study for proper electrophoretic separation and optimisation of detection parameters including membrane blocking conditions.

Thirdly, although the antibodies that were used in the western blot differ from those used in the flow cytometry, it is possible that the antibodies would bind differently to proteins in paraformaldehyde or alcohol-fixed cells than in unfixed cell extracts, since the protein structure might be modified during these preparation steps (130). For these reasons, the results of the two techniques cannot be directly compared quantitatively. However, they show a good agreement with respect to the relative change in the kinetics of the probed proteins.

For western blot detection, many commercial chemiluminescence mixtures are available to improve the sensitivity of the detection and these differ in the price, sensitivity, ease of use and compatibility. Due to the high cost and the limited amount of the sensitive commercial ECL, in house chemiluminescent mixture was prepared and its performance was compared with a commercial enhanced chemiluminescent product. Compared to the commercial kit, the in house ECL offers an initial good performance with a rapid onset and intense signals at lower cost. The pCA-ECL is a robust product that is easy to handle and works well with most antibodies (131). However, the background observed with the pCA-ECL solution was greater than that observed with the commercial product and longer exposure times

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must be avoided. As the background of the in house product increases continuously, reading the membrane within the first 5 minutes of exposure is important to avoid signal swamping especially for light bands.

In conclusion, flow cytometry is a novel methodology that can detect phosphorylation of signalling effectors in multiple, rare cellular populations within peripheral blood (132). In contrast to the older techniques such as western blot, it offers a rapid, sensitive and more quantitative analysis of cell signalling pathways. However, several technical considerations should be taken into account during the protocol set up. Moreover, the specificity of antibodies needs to be tested in control experiments to avoid false-positive results due to non-specific binding to tissue components or recognition of epitopes shared by several molecules (133). Once the protocol has been optimized, it can be applied for the identification of normal and disrupted signalling cascades such as the JAK/STAT3 signalling pathway. Dysregulation of STAT3 has been associated with several diseases including cancer (55, 134, 135) and inflammatory conditions such as rheumatoid arthritis (136), systemic lupus erythematous (137-140) and inflammatory bowel diseases (140, 141).

Due to its ability to measure impaired signals, studies are being performed to validate the insertion of flow cytometry in the clinical laboratories for diagnosis and in the field of drug development. However, this transition requires strictly standardized protocols and the use of specific antibodies as well as a well-defined control samples.

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Chapter 5: Limitations and future work

This study has been faced with several limitations that affected the quality and the outcomes of the research. These limitations are highlighted below and should be taken into consideration for future work.

- The major limitation in this project was the high cost of the antibodies and related reagents which limits the number of replicates that was done in each experiment and subsequently the quality of the statistical data analysis.
- Some difficulties in obtaining significant results were due to reagents that were not received at the same time but at separate time intervals. This waiting time can affect the activity of some antibodies. Furthermore, some antibodies were shipped and stored in inappropriate conditions.
- The PBMC were freshly isolated each time before starting with the flow cytometry experiment which was a time consuming procedure to remove the contaminating red blood cells and to isolate the populations of cells to be tested.
- Some of the experiments have been done using blood collected from the same three healthy donors while other experiments were carried out on a blood sample collected from one of these donors. Due to biological variations, inter-individual variations in the response of STAT3 to different cytokines have been observed which made the direct comparison between the results of different donors quite difficult.
- The Accuri cytometer was provided by the BD for a limited period only. Therefore, further analysis on this instrument has not been possible after the termination of the trial period.
- Lack of experience and limited knowledge in instrument setup, cytometer controls and sample analysis parameters affected the results collected using the FC500 flow cytometer as any error in initial voltage settings resulted in data loss and inappropriate gating lead to misleading results.
- It was also noticed that the population of the debris and the unwanted cells was too large in comparison to the two cell populations of interest. This made gating on the FC500 cytometer very challenging.

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- Finally, technical variations in pipetting, incubation time and washing steps may interfere with the final outcome, due to the short incubation times and the sensitivity of the cells to stimulant concentrations in the ng/ml range.

However, despite the shortcomings, many valuable lessons and pointers for future research have been gained throughout this work. Moreover, some helpful guidelines in setting up similar comparative research have been established.

Despite the ease and the simplicity of flow cytometric experiments compared to western blotting, some suggestions should be highlighted for future work:

Firstly, concerning the inter-individual variability that have been noticed in this study, future work should be done to assess the effect of different factors such as gender, race and age on the signalling pathways. Alternatively, to avoid the influence of these confounding factors, it is preferable to take the sample from one individual. However, if this is done, the results can't be generalized as being applicable to the whole population.

Secondly, prior to starting with similar work, the effect of all variable parameters should be studied and optimized in the preliminary work to obtain consistent and reproducible results.

Thirdly, this work had been done on PBMC cells that were isolated from the whole blood. Being freshly isolated each time, this eliminates the effect of freeze/ thaw cycle on the phosphorylation level. However, one should consider that the separation steps can potentially affect the loss of some important cells. Additionally, a given stimulus can trigger a variety of different responses dependent on cell type that can lead to the production of an array of cytokines and other intercellular communication molecules (142). Moreover, many of the phospho-epitopes of interest are part of signalling networks that respond to the environment and have extremely rapid and high turnover, the interval and manipulations used to eliminate erythrocytes from samples have the potential to introduce artifacts (70). Therefore, to get measurements in a physiologically relevant environment, whole blood sample should be used without any purifying steps. In addition, whole blood analysis is essential in diagnostic and drug pharmacodynamic monitoring studies.

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Lastly, although in a non-diseased state, mediators such as cytokines may have consistent network activation and end results, the same mediators may have drastically altered cellular response in a diseased state because of extensive signalling pathway crosstalk (143). Notably, many diseases are associated with disrupted signalling in different pathways as well as in different cell types. Therefore, to get a general insight to the disease disrupted network and further on to the treatment development, the involved signalling pathways should be assessed simultaneously.

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Appendix I: Ethical approval to collect blood from healthy donors

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 13/04/2011 and Expires 13/04/2014.

UNIVERSITEIT VAN PRETORIA
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YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

11/09/2013

Approval Certificate
New Application

Ethics Reference No.: 301/2013

Title: Analysis of cytokine induced phosphorylation of STAT3 in peripheral blood monomuclear cells by flow cytometric and western blot assays.

Dear Mrs. R Elhussiny

The **New Application** as supported by documents specified in your cover letter for your research received on the 1/07/2013, was approved by the Faculty of Health Sciences Research Ethics Committee on the 11/09/2013.

Please note the following about your ethics approval:
- Ethics Approval is valid for 2 years.
- Please remember to use your protocol number (301/2013) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

DR R SOMMERS; MBChB; MMed(Int); MPharmMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee
University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

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Appendix II: Informed Consent

INFORMATION LEAFLET AND INFORMED CONSENT FOR NON-CLINICAL RESEARCH (e.g. educational, health systems or nonclinical operational research)
Consent and assent

If there are children younger than 7 years in your study, the parents give consent on their behalf and you will need to adapt the information leaflet by substituting "you" with "your child".
For children between 7 and 18 years, parents give consent for their child to participate in the study and the child gives assent. Adapt the form below for that purpose too. Both information leaflets and the consent /assent form have to be included with your application.

TITLE OF STUDY:
Analysis of cytokine induced phosphorylation of STAT3 in peripheral blood mononuclear cells by flow cytometric and western blot assays.

Dear Participant,

1) INTRODUCTION

We invite you to participate in a research study. This information leaflet will help you to decide if you want to participate. Before you agree to take part you should fully understand what is involved. If you have any questions that this leaflet does not fully explain, please do not hesitate to ask the investigator Mrs Roba Elhussiny.

2) THE NATURE AND PURPOSE OF THIS STUDY

The aim of this study is to optimize a flow cytometric technique to measure STAT3 in normal blood mononuclear cells. STAT3 is one of the signalling proteins in the cell that is responsible for carrying the signal from the surface to the nucleus in response to specific stimulants. STAT3 is over activated in certain types of cancer. Analysing STAT3 signalling provides insights into pathology and can be used as a tool for diagnosis, prognosis and therapy development. Traditionally western blot has been used to analyse cell signalling but this technique is time consuming and has several drawbacks. Recently, flow cytometry have been involved in clinical research for rapid and more quantitative analysis. The results of the flow cytometry technique that will be obtained from this study will be compared with the traditional western blot assay, also obtained from this study, in order to provide a more convenient and time saving technique. Once the protocol has been optimized in healthy donors, it can be applied to detect signalling dysregulation in cancer and immunological diseases.
You as normal healthy donors are a very important source of information by donating blood to carry out this study.

3) EXPLANATION OF PROCEDURES TO BE FOLLOWED

This study involves the quantification of the normal level of STAT3 in normal peripheral blood mononuclear cells by two different assays in different donors. Therefore, we will ask you to donate 60 ml of blood to carry out these assays.

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The blood will be drawn for laboratory research only. The blood from each donor will be collected early in the morning in heparinized tubes by a nurse in the clinical research unit and then taken to the lab to isolate the peripheral mononuclear cells and run the assays. In the department of pharmacology's lab, the blood will be centrifuged to obtain the PBMC and the experiment will be done on those cells. The unused component of the blood will be discarded according to the lab protocols for biological samples.

4) RISK AND DISCOMFORT INVOLVED

There are no risks in participating in the study.

You may have some pain during blood drawing process or swelling after blood drawing.

You will be asked to be early in the morning in the clinic to donate the blood which may cause some discomfort.

The blood drawing process will take about 15 minutes of your time.

5) POSSIBLE BENEFITS OF THIS STUDY

Apart from getting the results from your tests, there will be no other direct benefit for you. However, the results of the study will help in the optimization of rapid and sensitive technique to measure STAT3 in healthy donors and then compare its level in abnormal patients. These measurements provide insight into the pathology and can be used as a tool for diagnosis, prognosis and therapy development in the future.

6) WHAT ARE YOUR RIGHTS AS A PARTICIPANT?

Your participation in this study is entirely voluntary. You can refuse to participate or stop at any time during the study without giving any reason. Your withdrawal will not affect you in any way.

7) HAS THE STUDY RECEIVED ETHICAL APPROVAL?

This study has been screened by and received written approval from the Research Ethics Committee of the Faculty of Health Sciences at the University of Pretoria and has been approved from the MSc Committee of the Faculty of Health Sciences at the University of Pretoria. This study conforms to the Declaration of Helsinki as amended and is to be performed under the ICH guidelines for good clinical practice.

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8) INFORMATION AND CONTACT PERSON

The contact persons for the study are:

Mrs Roba Elhussiny
Cellphone: xxxxxxxxx
E- mail: xxxxxxxxxxxx

Dr .Duncan Cromarty
Telephone: xxxxxxxxxx
E-mail: xxxxxxxxxxxxxxxxxx

If you have any questions about the study please contact us at the previous numbers.

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9) COMPENSATION
Your participation is voluntary. No compensation will be given for your participation.

10) CONFIDENTIALITY
All information that you give will be kept strictly confidential. Once we have analysed the information, no one will be able to identify you. Research reports and articles in scientific journals will not include any information that may identify you.

CONSENT TO PARTICIPATE IN THIS STUDY
I confirm that the person asking my consent to take part in this study has told me about nature, process, risks, discomforts and benefits of the study. I have also received, read and understood the above written information (Information Leaflet and Informed Consent) regarding the study. I am aware that the results of the study, including personal details, will be anonymously processed into research reports. I am participating willingly. I have had time to ask questions and have no objection to participate in the study. I understand that there is no penalty should I wish to discontinue with the study and my withdrawal will not affect the study in any way.
I have received a signed copy of this informed consent agreement.
Participant's name ……..(Please print)
Participant's signature: ....………………… Date............................
Investigator’s name: Mrs Roba Elhussiny
Investigator’s signature ....…………………. Date: 10-06-2013
Witness's Name ....…………….................(Please print)
Witness's signature ....………………….... Date…........................

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