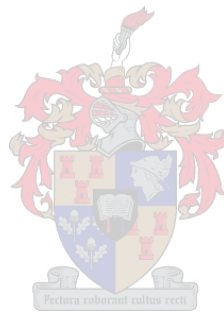


# Investigation of Regulatory B-cell Responses during *Mycobacterium tuberculosis* Exposure

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## Abstract

Tuberculosis (TB) remains a global health challenge due to limited understanding of the complex host immune responses required for the successful control and eradication of invading *Mycobacterium tuberculosis* (*M.tb*) bacilli. To date, T-cells and macrophages have been regarded as the principle immune cells responsibly for protective anti-TB immunity. However, emerging evidence has revealed a fundamental role of B-lymphocytes (B-cells) during *M.tb* infection; although the function of B-cells in TB disease incidence and progression remains ill-defined. We hypothesize that B-cells influence anti-TB immune responses through modulation of T-cell activation, and that the microenvironment in which these interactions occur greatly impacts immune cell function, with an emphasis on B-cells.

Healthy participants that were either pre-exposed or unexposed to *M.tb* were recruited for the study. Within the pilot study investigating the effect of B-cells on T-cell function: peripheral blood was collected, and B- or T-cell enriched using magnetic-activated cell sorting (MACS) bead technology for downstream analysis. Following sample collection, autologous T-cells were co-cultured with H37Rv/BCG-stimulated B-cells, pulsed with or without cluster of differentiation 40 Ligand (CD40L) and interleukin-5 (IL5). The resulting B- and T-cell phenotypic frequencies and cytokine profiles were subsequently evaluated. To determine the effect of the microenvironment complexity on cell function, peripheral blood was collected, and B-cell frequency and function (immunoglobulin isotype profile) determined in whole blood, peripheral blood mononuclear cells (PBMCs) and untouched, isolated B-cells following 24-hour stimulation with toll-like receptor 9 agonist (TLR9a) or H37Rv.

Our results demonstrate that B-cells are able to modulate T-cell activation and function. We found that Bacillus Calmette-Guérin (BCG) and H37RV alike are able to induce killer/regulatory B-cell phenotypes in QuantiFERON positive and negative participants. Furthermore, the microenvironment was found to have a substantial effect on B-cell function with noticeable changes in B-cell development, shown by a decrease in mature B-cell frequencies following cellular isolation. Likewise, impairment of humoral responses, indicated by hampered ability to produce certain immunoglobulin isotypes in response to antigenic stimulation, were observed for isolated B-cells.

Collectively, these findings indicate the potential influence B-cells have in anti-TB immune responses through modulation of T-cell behaviour, underscoring the role B-cells may play in initiating and guiding the immune response against *M.tb*. Our study highlights the need to further study B-cells as therapeutic targets in novel TB prevention strategies. Additionally, our study identifies significant limitations associated with the use of cell isolation studies for inference of cell function during health and disease. Findings from these studies, while informative, should be interpreted carefully, as the *in vitro* microenvironment may have considerably influenced the observed results.

## Opsomming

Tuberkulose (TB) bly 'n wêreldwye gesondheidsuitdaging as gevolg van die beperkte begrip van die komplekse gasheerimmunitets reaksies wat benodig word vir die suksesvolle beheer en uitwissing van indringer *Mycobacterium tuberculosis* (*M.tb*) bakteriee. Tot op datum is T-selle en makrofages as die primêre immuunselle verantwoordelik vir beskermende anti-TB immunitet beskou. Opkomende bewyse het egter 'n fundamentele rol van B-limfositete (B-selle) tydens *M.tb* infeksie geopenbaar; alhoewel die funksie van B-selle in TB-siekte voorkoms en progressie steeds ondefinieerbaar is. Ons vermoed dat B-selle anti-TB immuunresponse beïnvloed deur modulasie van T-sel aktivering, en dat die mikro-omgewing waarin hierdie interaksies voorkom grootliks die immuunsel funksie beïnvloed, met die klem op B-selle.

Gesonde deelnemers wie blootgestel was aan *M.tb* is vir die studie gewerf. Binne die loodsstudie wat die effek van B-selle op T-selffunksie ondersoek het, is perifere bloed ingesamel, en B- of T-sel verryk met behulp van magnetiese-geaktiveerde sel sortering (MACS) kraal tegnologie vir stroomaf analise. Na volgende monsterversameling, is autoloë T-selle gekweek met H37Rv/BCG-gestimuleerde B-selle, gepulseer met of sonder groepering van differensiasie 40 Ligand (CD40L) en Interleukine-5 (IL5). Die gevolglike B- en T-sel fenotipiese frekwensies en sitokienprofile is vervolgens geëvalueer. Om die effek van die mikro-omgewingskompleksiteit op selffunksie te bepaal, is perifere bloed ingesamel, en B-sel frekwensie en funksie (immunoglobulien isotipe profiel) is bepaal in volbloed, perifere bloedmononukleêre selle (PBMCs) en onaangeraakte geïsoleerde B-selle na 24 -uur stimulasie met tollike receptor 9 agonist (TLR9a) of H37Rv.

Ons resultate toon dat B-selle T-sel aktivering en funksie kan moduleer. Ons het bevind dat Bacillus Calmette- Guérin (BCG) en H37Rv gelyk is aan regulatoriese B-sel fenotipes in QuantiFERON positiewe en negatiewe deelnemers. Verder is gevind dat die mikro-omgewing 'n wesentlike effek op B-selffunksie het met merkbare veranderinge in B-selontwikkeling, getoon deur 'n afname in volwasse B-sel frekwensies na sellulêre isolasie. Net so is waargeneem vir die geïsoleerde B-selle waardedaling van humorale response, aangedui deur die belemmerde vermoë om sekere immunoglobulien isotipes te produseer in reaksie op antigeen stimulasie.

Gesamentlik dui hierdie bevindinge op die moontlike invloed wat B-selle in anti-TB immuunresponse het deur modulasie van T-selgedrag, wat die rol wat B-selle kan speel in die inisiatief en leiding van die immuunrespons teen *M.tb* beklemtoon. Ons studie beklemtoon die noodsaaklikheid om B-selle verder as terapeutiese teikens in nuwe TB-voorkomingstrategieë te bestudeer. Daarbenewens identifiseer ons studie beduidende beperkings wat verband hou met die gebruik van sel isolasie studies vir inferensie van selffunksie tydens gesondheid en siekte. Bevindings uit hierdie studies, terwyl insiggewend, moet versigtig geïnterpreteer word, aangesien die *in vitro* mikro-omgewing die waargenome resultate aansienlik beïnvloed het.



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In loving memory of  
Stanley John Dechristie Kenny

*“When great trees fall,  
rocks on distant hills shudder,  
lions hunker down in tall grasses,  
and even elephants lumber after safety.*

*When great trees fall in forests,  
small things recoil into silence,  
their senses eroded beyond fear.*

*When great souls die,  
the air around us becomes light, rare, sterile.  
We breathe, briefly.  
Our eyes, briefly, see with a hurtful clarity.  
Our memory, suddenly sharpened,  
examines, gnaws on kind words unsaid,  
promised walks never taken.*

*Great souls die and our reality,  
bound to them, takes leave of us.  
Our souls, dependent upon their nurture,  
now shrink, wizened.  
Our minds, formed and informed  
by their radiance, fall away.  
We are not so much maddened  
as reduced to the unutterable ignorance  
of dark, cold caves.*

*And when great souls die,  
after a period peace blooms,  
slowly and always irregularly.  
Spaces fill with a kind of  
soothing electric vibration.  
Our senses, restored, never  
to be the same, whisper to us.  
They existed. They existed.  
We can be. Be and be  
better. For they existed.*

- Maya Angelou

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## List of Abbreviations

$\mu\text{g}$	: microgram
$\mu\text{L}$	: microliter
$\mu\text{m}$	: micrometers
%	: percentage
max	: maximum
min	: minutes
mL	: milileter
Ng	: nanogram
$^{\circ}\text{C}$	: degrees Celsius
xg	: relative centrifugal force

### A

AICD	: Activation-induced cell death
AIDS	: Aquired immune deficiency syndrome
ANOVA	: Analysis of variance
APCs	: Antigen presenting cells
APRIL	: A proliferation-inducing ligand

### B

Be-1	: B-effector 1
B-cells	: B-lymphocytes
BAFF	: B-cell activating factor
BCG	: Bacillus Calmette-Guérin
BCR	: B-cell receptor
$B_{\text{reg}}$	: Regulatory B-lymphocytes
BTK	: Bruton tyrosine kinase

### C

CD	: Cluster of differentiation (e.g. CD95)
CD40L	: CD40 ligand
CFP-10	: 10kDa culture filtrate antigen
CFU	: Colony forming units
$\text{CO}_2$	: Carbon dioxide

## **D**

DMSO : Dimethyl sulfoxide

## **E**

EDTA : Ethylenediaminetetraacetic acid

## **F**

FACS : Fluorescence-activated cell sorting

FasL : Fas ligand

FBS : Fetal bovine serum

FCS : Fetal calf serum

FDR : False discovery rate

FO B-cells : Follicular B-lymphocytes

FoxP3 : Forkhead box P3

FSC : Forward-scatter area

## **H**

HIV : Human immunodeficiency virus

## **I**

ICAM : Intercellular adhesion molecule 1

IFN- $\beta$  : Interferon-beta

IFN- $\gamma$  : Interferon-gamma

Ig : Immunoglobulin (e.g. IgG1)

IL : Interleukin (e.g. IL-10)

IL5R $\alpha$  : interleukin-5 receptor-alpha

## **L**

LPS : Lippopolysaccharide

LSD : Least significant difference

LTBI : Latent tuberculosis infection

## **M**

<i>M.tb</i>	: <i>Mycobacterium tuberculosis</i>
mAB	: Monoclonal antibody
MACS	: Magnetic-activated cell sorting
MDSC	: Myeloid-derived suppressor cell
MHC	: Major histocompatibility complex
MOI	: Multiplicity of infection
MZ B-cells	: Marginal zone B-lymphocytes

## **N**

NaHep	: Sodium heparin
NF- $\kappa$ B	: Nuclear factor kappa-light-chain-enhancer of activated B-cells
NK cells	: Natural killer cells

## **P**

PBMC	: Peripheral blood mononuclear cell
PBS	: Phosphate buffered saline
PI3K	: Phosphoinositide 3-kinase
PMA	: Phorbol 12-myristate 13-acetate

## **Q**

QFN	: QuantiFERON
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## **R**

RPMI	: Roswell Park Memorial Institute medium
RSV	: Resveratrol

## **S**

SEA	: Schistosomal egg antigens
sFasL	: Soluble Fas ligand
SSC	: Side-scatter area
Stat3	: Signal transducer and activator of transcription 3

## **T**

T-cells	: T-lymphocytes
T1 B-cells	: Transitional 1 B-lymphocytes
T2 B-cells	: Transitional 2 B-lymphocytes
TB	: Tuberculosis
tB <sub>res</sub>	: Tumor-evoked regulatory B-lymphocytes
TE	: Effector T-lymphocytes
TGF- $\beta$	: Transforming growth factor-beta
Th	: T-helper lymphocytes
TLR	: Toll-like receptor
TLR9a	: Toll-like receptor 9 agonist
TNF- $\alpha$	: Tumor necrosis factor-alpha
T <sub>reg</sub>	: Regulatory T-lymphocytes

## **W**

WHO	: World Health Organization
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## Chapter 1: Introduction

In 2017, tuberculosis (TB) was ranked the ninth leading causes of death worldwide, causing more deaths than human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS). The World Health Organization (WHO) estimated 10.4 million new TB cases globally in 2016, and the existence of latent infection in nearly 2 billion individuals (World Health Organization, 2017). The etiological agent of TB, is *Mycobacterium tuberculosis* (*M.tb*), a slow growing organism with a doubling time of 12-24 hours under optimal conditions (Delogu et al., 2013). *M.tb* typically infects the lungs, resulting in a disease formally known as pulmonary TB. However, *M.tb* can also affect other sites such as the brain, eye or lymph nodes, in which case the infection is known as extra pulmonary TB. *M.tb* transmission occurs when an individual with active pulmonary TB coughs or sneezes, expelling tubercle bacilli in droplets into the air, which are inhaled by the host. In the alveoli of the lungs, alveolar macrophages phagocytose the bacteria, of which most are eliminated by the host's innate immune response. In some cases, bacilli survive this initial defense and actively replicate within the macrophages, or disperse to adjacent cells and eventually organs, causing active disease and eliciting an adaptive immune response.

An estimated 1.3 million TB deaths occurred among HIV-negative individuals and an additional 374 000 deaths among HIV-positive individuals in 2016 (World Health Organization, 2017). The incidence of TB on the WHO African region continent ranks amongst the highest globally, and accounts for 25% of the global total of TB cases (World Health Organization, 2017). Importantly, the WHO African region together with the WHO South-East region accounted for 85% of the total TB deaths in both HIV-positive and HIV-negative individuals. The WHO proposed a strategy in 2015 called the End TB Strategy, in which it sets specific targets to be met in order to achieve a 90% reduction in TB deaths and an 80% reduction in TB incidence by 2030. Most TB deaths that occur could be prevented with early diagnosis and appropriate treatment. However, most of the high TB burden areas include low to middle income countries who lack the resources needed to adequately achieve this.

Studies have projected an increase in the prevalence of TB worldwide, particularly in developing countries, due to the introduction of agriculture, civilization, increased human population density, inadequate and delayed TB diagnosis and unsuccessful TB treatment (Albuquerque et al., 2007; Amante and Ahemed, 2015; Dooley et al., 2011; Tachfouti et al., 2011). The probability of contracting TB disease is much higher in individuals with a compromised immune system, such as infection with HIV/AIDS, as well as in individuals exposed to high risk factors for TB infection which include malnutrition, alcohol abuse, diabetes, smoking and indoor air-pollution (Ismail and Bulgiba, 2013; Tachfouti et al., 2011; Thuy et al., 2007). These risk factors and co-infections are very typical in countries, where poverty, insufficient infrastructure and inadequate access to health facilities are common. Currently, the African health care system is insufficient, and has led to selection of enhanced virulent strains, which persist in low density populations, causing TB disease reactivation following long periods of latent infection



(Comas et al., 2013; Gandhi et al., 2006; Pillay and Sturm, 2007; Van Soolingen et al., 1997). As such TB poses a major health, social and economic burden on low and middle-income countries, such as South Africa, due to the high costs of medical care and poor living conditions.

The ultimate goal for the WHO End TB Strategy and researchers in health and medicine is to improve the overall health and well-being of the country to ensure a bright future (World Health Organization, 2017). In order for these targets to be achieved, provisions for TB treatment and prevention need to be made, and investment into TB research and translation of such research into clinically relevant outputs is paramount. Improvements to the current systems used to diagnose and treat TB disease form part of this much needed effective change, and strategies that aim at increasing TB preventative treatment in high risk population groups and developing effective, less harmful and timely treatments will help facilitate this.

Research into the mechanisms by which the host adaptive immune system responds to infection with *M.tb* have indicated T-cells (Kaufmann, 2002; Lalvani et al., 1998; Orme et al., 1993; Ribeiro-Rodrigues et al., 2006) and macrophages (Kamath et al., 1999; Kaufmann, 2002; Sharma et al., 2007) as the main line of defense against invading pathogens. Further studies revealed the ability of B-cells to modulate T-cell activation, as well as macrophage and T-cell responses and function (Bénard et al., 2018; Du Plessis et al., 2016). As such, B-cell function may directly affect the immune response to T-cell driven protection and macrophage polarization against *M.tb*, and ultimately play a key role in disease development and progression. Importantly, only approximately 10% of individuals exposed to *M.tb* develop active disease, while the remaining 90% are able to effectively contain the bacteria (World Health Organization, 2017). This is believed to be due to a sufficient balance between effector and regulatory immune responses that are able to contain and eliminated *M.tb* before infection is established (Du Plessis, *et al.* 2016). Studies investigating the presence of B-cells during TB disease have indicated dysfunction and decreased frequencies of B-cells following *M.tb* infection (Joosten et al., 2016). Furthermore, the occurrence of regulatory killer B-cells during TB illustrated the presence of this cell type in moderate frequencies in healthy individuals; however following infection with *M.tb* a significant decrease in this population was observed (van Rensburg, *et al.* 2016). In accordance with successful TB treatment, these regulatory killer B-cell frequencies were observed to increase to levels similar to that of healthy controls - suggesting a protective role of this cell type in TB disease (van Rensburg, *et al.* 2016). It is thus hypothesized that the absence of regulatory killer B-cells may be vital in TB disease onset and progression, as alterations in this cell population frequency and function has been associated with TB disease on numerous occasions. The exact mechanisms by which regulatory B-cells (CD19<sup>+</sup> CD24<sup>++</sup> CD38<sup>++</sup>) and more specifically the regulatory killer B-cells subtype (CD19<sup>+</sup> CD38<sup>+</sup> CD178<sup>+</sup>) may modulate the immune response to TB is undefined. It is further suggested that B-cell derived cytokines and cell surface factors play a key role in eliciting the postulated protective immune responses.

Interestingly, regulatory killer B-cells have been shown to express a cell surface receptor known as FasL (CD178) (van Rensburg, *et al.* 2016; Lundy, *et al.* 2015), a surface protein belonging to death ligand family. These death ligands mediate cell death through cell-cell contact with immune cells expressing the co-receptor Fas (CD95), causing in the activation of an intracellular cascade that results in cell apoptosis; hence the name “killer” B-cells (Lundy, 2009). It is speculated that these regulatory killer B-cells provide protection against *M.tb* infection by: 1) enhancing *M.tb* killing capacity of macrophages that have internalized the bacteria into a phagosome and 2) control the spread and survival of the bacilli by inducing apoptosis of infected cells, thought to express the Fas co-receptor as a result of cell activation and stress due to *M.tb* infection (Lundy, *et al.* 2002). This induction of programmed cell death facilitates the elimination of cellular reservoirs, responsible for harboring the bacteria and providing a niche for their survival.

To date, there is limited knowledge as to the exact role of immune cell types previously thought to have little importance during *M.tb* infection. An example of such a cell type is B-cells. The primary function of B-cells is antibody production and secretion; due to the intracellular nature of the pathogen, many researchers believed that these effector cells would have minimal impact on the survival of *M.tb* and thus focused their efforts elsewhere. Studies have subsequently identified several functions of B-cells other than antibody secretion, including antigen presentation, complement activation and regulation of cellular activation and function of additional cells types via cytokine secretion and cell-cell interactions (Du Plessis, *et al.* 2016; Bénard, *et al.* 2017). As such, the purpose of this project was to investigate the modulatory effects of B-cells, whose role within TB disease incidence and progression remains incompletely defined. Reports have suggested a clinically relevant role of regulatory B-cells, at the early stages of *M.tb* infection and have implied active interaction with T-cells and macrophages - in turn influencing disease outcome (Rao, *et al.* 2015). We hypothesize that B-cells have the ability to regulate T-cell function, and that B-cell phenotype plays a key role in this interaction. Additionally, we hypothesize that microenvironment complexity may be a modulator of B-cell population distribution and could contribute to B-cell phenotype and function, thereby impacting which functional markers or cell surface factors are expressed.

Accordingly, this research project aimed to study the interaction between B- and T-cells and the resulting effects of this intercommunication on T-cell function in order to understand the important role B-cells may play in the immune response to *M.tb*. Apart from establishing the interaction of isolated B- and T-cells in the context of TB, concerns with the translation of the observed isolated B-cell response to events in whole blood and *in vivo* remains. As such, the response of B-cells to a particular stimulus in various compartments of isolation (e.g. whole blood, PBMC fraction and pure B-cells) was investigated to determine whether *in vitro* study observations, made from cellular responses within an artificial microenvironment, represent viable events within the body. Should the observed responses of isolated B-cells prove homogenous to those within whole blood *ex vivo*, identification of the relevant abundance

of different functional immune cell subsets during various stages of *M.tb* infection/exposure may prove useful for diagnosis, disease progression and treatment monitoring.

To achieve our aims, we evaluated the effect of *M.tb* exposed B-cells on the T-cell population distribution and cytokine secretion by assessing the cell surface receptor expression and cytokine production of T-cells using flow cytometry following stimulation. Different populations of cells can be readily distinguished by the expression of a unique combination of intracellular molecules and cell surface membrane receptors. As previously described, T-cells are regarded as the primary immune cells responsible for TB disease management, as such modulation of the function of these cells may have direct implications on TB susceptibility. Many T-cell populations have been implicated in anti-TB responses, each having varied effects on the overall outcome of infection. Investigation as to which T-cell population *M.tb* exposed B-cells induce will provide insight into how these cells contribute to the host defence in combating *M.tb* infection. Additionally, we determined the effects of microenvironment complexity on B-cell function by stimulating various sample fractions obtained throughout isolation of B-cells from whole blood. The cell surface receptor expression of B-cells in each fraction was then determined using flow cytometry and the type and magnitude of immunoglobulin induced and secreted under each condition evaluated using multiplex cytokine analysis.

Targeting of B-cells to modulate anti-TB T-cell responses could be a promising host-based treatment strategy to combat *M.tb* infection and TB disease, should B-cells prove efficient in directing the immune response to *M.tb*. This potential prevention therapy or TB treatment strategy may confer additional protective immunity, resulting in better prognosis and successful treatment outcome; thereby contributing to the attainment of the WHO End TB Strategic plan (World Health Organization, 2017).

## **Chapter 2:**

The work presented in this chapter has been formatted in the style of Immunotherapy journal to which it was submitted for publication.

## **Modulation of the host Immune Responses by B-cells**

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**Keywords:** Regulatory B-cells, Regulatory T-cells, immune tolerance, autoimmunity, immune modulation

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## Abstract

The role of B-cells in immunogenic responses has become increasingly important over the past decade, focusing on a new B-cell subtype: regulatory B ( $B_{reg}$ ) cells. These  $B_{reg}$  cells have been shown to possess potent immunosuppressive activities and have identified as key players in disease control and immune tolerance. In this review the occurrence of this cell type in various conditions, along with evidence supporting discovered functions and proposed purposes will be explored. An example of such regulatory functions includes the induction or suppression of various T-cell phenotypes in response to a particular stimulus. Should  $B_{reg}$  cells prove effective in mediating immune responses, and correlate with favourable disease outcome, they may serve as a novel therapeutic to combat disease and prevent infection. However, the induction, functioning and stability of these cells remains unclear and further investigation is needed to better understand their role and therapeutic efficacy.

### 1. Background

Immunity refers to the ability of an organism to withstand disease and is conferred by cells, tissues and molecules of the immune system (Paul, 2013). These elements mediate illness by coordinating an immune response to prevent disease and eradicate established infections. The importance of immunity is illustrated by individuals with compromised immune systems, in which defective immune response results in diminished health and increased susceptibility to life threatening conditions (Lundy, 2009). An immune response occurs when infiltrating foreign microorganisms are encountered by immune cells within the body. Recognition of these foreign bodies by circulating immune cells results in their subsequent activation and initiation of a cascade of events to control infection and confer protection against disease.

The immune system can be divided up into two parts, namely innate and adaptive immunity. Innate immunity, also known as naïve immunity due to the fact that it is encoded within the genome and is always present, provides the first line of defense against invading pathogens. It functions to prevent entry of microbes and facilitate rapid elimination of microbes that have successfully enter the body (Zabriskie, 2009). Adaptive immunity, also known as acquired or specific immunity, is the long-lasting second line of defense that requires stimulation and differentiation to activate and combat disease. Adaptive immunity is less rapid in initial encounter of foreign substances, however once adaptive immune cells have encountered a particular organism, they 'remember' this organism through development of memory cells which remain in circulation to enable faster response in the event of re-infection (Abbas et al., 2014).

Adaptive immunity can be further divided into two subsections, namely cell-mediated immunity and humoral immunity, based on the cell types and molecules they produce in respond to invading microbes. Cell-mediated immunity is facilitated by a collection of cells known as T lymphocytes (T-cells). The main

functions of these cells include elimination of intracellular microbes and stimulation of additional immune cells to combat infection (Abbas et al., 2014; Zabriskie, 2009). Humoral immunity is mediated by proteins known as antibodies, derived from B-lymphocytes (B-cells). These antibodies act to block infection, neutralise foreign substances within the body, activate innate immune responses (such as the complement system) and eliminate microbes (Capra et al., 1999; Slifka et al., 1998).

Conventionally, B-cells have been regarded as positive regulators of the immune response and key contributors to disease pathology, based on their ability to produce antibodies (Abbas et al., 2014; Mauri and Bosma, 2012). This review aims to consolidate current knowledge of effector B-cell functioning, with newly discovered and prospective functional roles of B-cells, in order to elucidate their role in autoimmune disease, cancer and infection. Understanding how and why these effector B-cells mediate the immune response by influencing the functioning of other immune cells, will better enable researchers to harness their therapeutic potential.

## **2. B-cell origin and development**

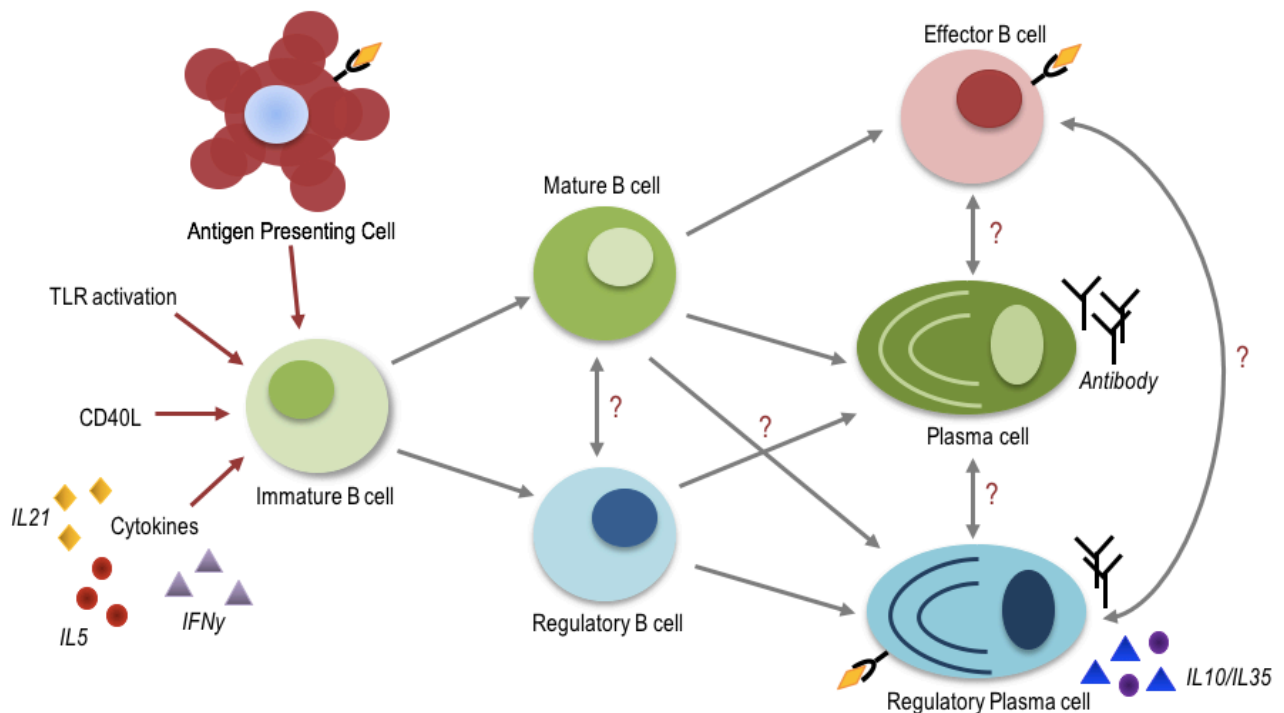
B-lymphocytes arise from a stem cell, the common lymphoid precursor, within the bone marrow (Paul, 2013). Immature B-cells go through a process of selection in which self-reactive cells are destroyed. B-cells that pass the selection criteria enter into circulation and peripheral lymphoid organs, where they encounter and respond to foreign antigens (Abbas et al., 2014; Pier et al., 2004). When naïve B-cells encounter and recognize foreign microbes, antigen-specific lymphocytes are activated, proliferate and differentiate into effector and memory cells (Rosser and Mauri, 2015). These effector cells perform key immune functions that prevent infection and eliminate microbes. The precise phenotype, and in turn function, of these effector cells depends on the stimulus encountered and the surrounding environment (Baba et al., 2015).

Over the past decade, a variety of newly discovered B subsets have arisen, collectively known as regulatory B-cells ( $B_{\text{regs}}$ ) (Daïen et al., 2014; Rosser and Mauri, 2015; Shao et al., 2014; Siewe et al., 2013).  $B_{\text{regs}}$  are immunosuppressive cells responsible for immune tolerance and modulation through the expression of various cytokines, such as interleukin-10 (IL-10) and IL-35, as well as the expression of various cell surface markers (Carter et al., 2012; Liu et al., 2016; Matsumoto et al., 2014; Nova-Lamperti et al., 2016; Shen et al., 2014; Wang et al., 2014). This  $B_{\text{reg}}$  population is further divided, depending on the phenotype of the cell, the molecules secreted and the predicted function. The exact origin of these  $B_{\text{regs}}$  are still unclear, although many researchers have identified pathways, such as the phosphoinositide 3-kinase (PI3K) (Cantley, 2002; Jellusova and Rickert, 2016) and Bruton tyrosine kinase (BTK) or B-cell receptor (BCR) pathway (Petro et al., 2000; Takata and Kurosaki, 1996), essential for the development and functioning of these cells. As such, further investigation is needed to determine the exact molecular changes that occur, in order to fully characterise these cells.

It was initially postulated that B<sub>regs</sub> originated following the expression of a B<sub>reg</sub>-specific lineage factor (Rosser and Mauri, 2015). However, this theory gives little explanation as to observed heterogeneity of phenotypes within this population. Additionally, no study to date has been successful in identifying unique transcription factors needed for B<sub>reg</sub> development (van de Veen et al., 2013). An alternative, and widely accepted theory states that under certain environmental conditions, this regulatory phenotype can be induced in circulating B-cell of any type (memory, effector, plasma) (Matsumoto et al., 2014; Shen et al., 2014). This alternative hypothesis supports the large phenotypic variance observed when comparing B<sub>reg</sub> cell subsets from various immunological disorders. The observed plasticity of B<sub>reg</sub> phenotypes is believed to arise due to varying environmental conditions, as well as differential time of stimulation (Mauri and Bosma, 2012; van Rensburg et al., 2017). According to numerous studies, inflammatory signals are essential for B<sub>reg</sub> differentiation, and different inflammatory environments induce distinct B<sub>reg</sub> populations (Carter et al., 2011, 2012; Evans et al., 2007; Mizoguchi et al., 2002). Additionally, Matsumoto and colleagues (2014) implicated the lymph nodes, in addition to the spleen, as locations for B<sub>reg</sub> maturation, supporting the second theory of B<sub>reg</sub> development in which any immature B-cell can be induced into a regulatory phenotype (Matsumoto et al., 2014).

Activation of naïve B-cells occurs through binding of microbial antigens in circulation, or presented by CD4<sup>+</sup> helper T-cells/antigen presenting cells (APCs), to the B-cell receptor (Petro et al., 2000; Rao et al., 2015; Takata and Kurosaki, 1996). Importantly, in order for activation of B-cells to occur, a costimulatory signal is required. This takes place by a variety of surface receptors, including Toll-like receptors (TLRs), CD40 and CD138 (Mauri and Bosma, 2012; Rosser and Mauri, 2015). Numerous publications have indicated environmental stimuli required to introduce B<sub>reg</sub> development, namely CD40L, IL5, TLR9 agonist CpG and LPS (Lundy et al., 2015; van Rensburg et al., 2017; Rosser and Mauri, 2015; Van Rensburg et al., 2017) (see Figure 2.1). A study by Lundy and Boros (2002) illustrated the induction of a B<sub>reg</sub> subset in response to schistosomal infection (Lundy and Boros, 2002). Importantly, induction of this B<sub>reg</sub> population, in response to schistosomal egg antigens (SEA), was dependant on the presence of IL-10 and IL-4 (Lundy and Boros, 2002). Furthermore, a study by Shao and colleagues (2014) illustrated the importance of CD40 in the differentiation of B<sub>regs</sub> (Shao et al., 2014). This highlights the necessity of co-stimulation in the activation and differentiation of B<sub>regs</sub> (Abbas et al., 2014; Paul, 2013). Additionally, killer B<sub>reg</sub> functioning was found to be dependent on CD4<sup>+</sup> T-cell-derived IL-10, creating a negative feedback loop to reduce apoptosis following down modulation once decreased inflammation is achieved (Lundy and Boros, 2002). Inhibitors also play a key role in modulating the functioning of B-cells, in which substances such as BTK inhibitors (Advani et al., 2013), NF-κB inhibitors (Wu et al., 1996) and Stat3 inhibitors (Zhang et al., 2016) have been found to impact B-cell activation, differentiation and functioning. Investigation into the effect these inhibitors have on immune modulation by B-cells has provided great insight into molecular pathways essential to their functioning.





**Figure 2.1. Proposed Developmental Pathways for regulatory B ( $B_{reg}$ ) Cell Differentiation.**

During development, immature B-cells can be activated by a variety of stimuli, depending on the microenvironment to which they are exposed. After activation, immature B-cells can differentiate into either regulatory B-cells ( $B_{regs}$ ) or mature B-cells. It is probable that these  $B_{regs}$  can mature into multifunctional immune cells, in which they are able to secrete antibodies, as well as produce regulatory cytokines and perform B-cell effector functions such as antigen presentation. In accordance with the large heterogeneity found in the  $B_{reg}$  population, it is postulated that  $B_{regs}$  can also differentiate from mature B-cells with during or after terminal effector function differentiation. **Adapted from** (Rosser and Mauri, 2015)

### 3. Functional role of B-cell subsets

The commonly known effector cells in the B-lymphocyte lineage are antibody-producing cells, known as plasma cells (Abbas et al., 2014; Capra et al., 1999; Paul, 2013; Pier et al., 2004). These plasma cells secrete antibodies during and long after infection to confer protection against infiltrating microbes (Slifka et al., 1998). Antibodies are able to bind a wide variety of targets with a complementary epitope, depending on their class, and mediate their effector functions (Abbas et al., 2014; Slifka et al., 1998; van de Veen et al., 2013). These include opsonization of bacterial cells, neutralization of secreted bacterial proteins and antibody-dependant cellular toxicity (Achkar et al., 2015; Rao et al., 2015). Naïve B-cells express either membrane bound IgM or IgD. Upon activation these effector cells are able to undergo a process known as isotype switching in which they are able to produce antibodies with other heavy-chain classes (Abbas et al., 2014; Paul, 2013; Pier et al., 2004).

Over the past two decades, researchers have attributed several additional functions of effector B-cells apart from antibody production. These include opsonization, complement activation, antigen presentation, cytokine production and most recently suppressive capacity (Abbas et al., 2014; Du Plessis et al., 2016; Mauri and Bosma, 2012; Rao et al., 2015; Rosser and Mauri, 2015; Zabriskie, 2009). Antigen presentation by B-cells occurs via the MHC class II pathway which is recognised and bound by antigen-specific CD4<sup>+</sup> T-cells, resulting in the induction of effective T-cell immune responses during disease, contributing to early protection (Abbas et al., 2014; Capra et al., 1999; Paul, 2013; Pier et al., 2004; Rao et al., 2015). The suppressive activity of B-cells has been attributed to a variety of molecules, found to modulate immune responses and immune cell frequencies (Blair et al., 2010; Carter et al., 2012; Liu et al., 2016; Lundy, 2009; Lundy et al., 2015; Matsumoto et al., 2014; Nova-Lamperti et al., 2016; van Rensburg et al., 2017; Shen et al., 2014; Wang et al., 2014). The relevance of this recently attributed function will be discussed in more detail below. Depicted in the table 2.1 is a list of B<sub>regs</sub> markers that have been identified in literature, as well as their proposed and/or documented function(s).

### 4. Evidence for the modulation of immune responses by B-cell during disease

The importance of B-cells have been shown in numerous diseases, such as rheumatoid arthritis (Edwards and Cambridge, 2006; Hirano et al., 1988), cancer (Berntsson et al., 2016; Lee-Chang et al., 2013; Lundgren et al., 2016; Mor et al., 2000; Ou et al., 2015; Shao et al., 2014; Tadmor et al., 2011; Zhou et al., 2014) , systemic lupus erythematosus (Blair et al., 2010), inflammatory bowel syndrome (Defendenti et al., 2011) and others, in which absence or impaired functioning of these immune cells has been associated with immune complications and poor prognosis. Studies investigating the dysfunction and decreased frequencies of IL-10 producing B-cells have implicated their significant role in immune balance within the body (Carter et al., 2012; Liu et al., 2016; Matsumoto et al., 2014; Nova-Lamperti et al., 2016). Numerous murine models involving IL-10 deficient B-cells illustrated a skewed proinflammatory response, by an increase in Th17 and Th1 responses, and a decrease in T<sub>reg</sub>

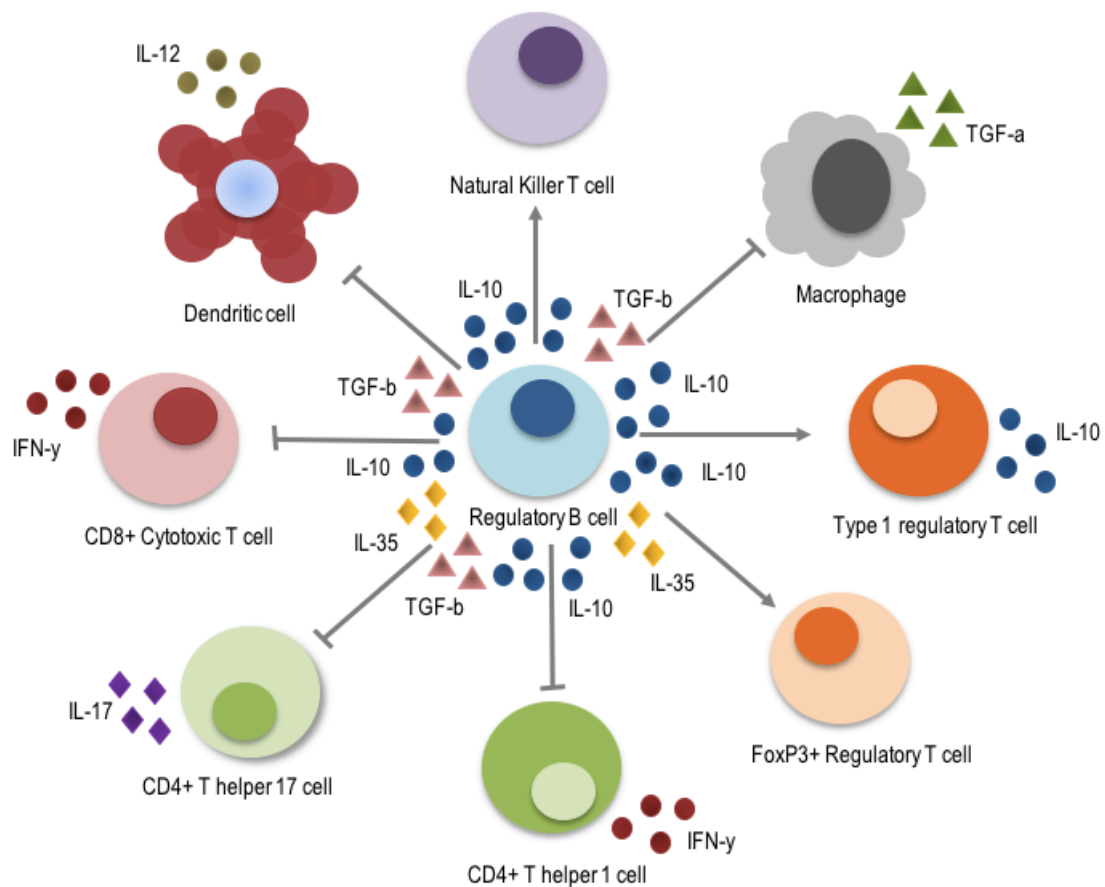
<i>B<sub>reg</sub></i> Marker	Phenotype	Proposed Function	Reference
<i>CD138, IgM</i>	CD19 <sup>+</sup> CD24 <sup>hi</sup> CD38 <sup>hi</sup> CD138 <sup>+</sup> IgM <sup>+</sup>	Plasma B <sub>reg</sub>	(Lundy and Boros, 2002; Mauri and Bosma, 2012; Shen et al., 2014; Van Rensburg et al., 2017)
<i>CD27</i>	CD19 <sup>+</sup> CD24 <sup>hi</sup> CD27 <sup>+</sup>	Memory B <sub>reg</sub>	(Mauri and Bosma, 2012)
<i>IL-10</i>	CD19 <sup>+</sup> CD24 <sup>hi</sup> CD38 <sup>hi</sup> IL10 <sup>+</sup>	Decrease Th1/Th17; increase T <sub>reg</sub>	(Carter et al., 2012; Flores-Borja et al., 2013; Lundy, 2009; Nova-Lamperti et al., 2016; van Rensburg et al., 2017)
<i>FasL (CD178)</i>	CD19 <sup>+</sup> CD24 <sup>hi</sup> CD38 <sup>hi</sup> CD178 <sup>+</sup> CD5 <sup>+</sup>	Induction of T-cell apoptosis via binding to Fas	(Lundy, 2009; Lundy and Boros, 2002; Lundy and Fox, 2009; Lundy et al., 2015; Mor et al., 2000; van Rensburg and Loxton, 2018a; van Rensburg et al., 2017)
<i>IL-35</i>	CD19 <sup>+</sup> CD24 <sup>hi</sup> CD38 <sup>hi</sup> IL35 <sup>+</sup>	Decrease Th1/Th17; increase T <sub>reg</sub> immune cells	(Mauri and Bosma, 2012; Shen et al., 2014; Wang et al., 2014)
<i>TGF-B</i>	CD19 <sup>+</sup> CD24 <sup>hi</sup> CD38 <sup>hi</sup> TGF-b <sup>+</sup>	Induction of T <sub>regs</sub>	4, 12, 13, 17(Carter et al., 2012; Liu et al., 2016; Lundy et al., 2015; Mauri and Bosma, 2012; Rosser and Mauri, 2015; Tadmor et al., 2011)
<i>Stat3</i>	CD19 <sup>+</sup> CD5 <sup>+</sup> IL10 <sup>+</sup>	Induction of T <sub>regs</sub>	(Evans et al., 2007; Zhang et al., 2016)
<i>TNFa</i>		Induction of IL-10 producing Bregs	(Carter et al., 2012; Lundy and Boros, 2002; Sedger et al., 2002; Van Rensburg et al., 2017)

**Table 2.1. Regulatory B-cell (B<sub>reg</sub>) subsets and their proposed functions.** Depicted is a comprehensive list of the phenotypic descriptions used within current literature to describe regulatory B-cell subsets, along with the suggested immune function of these cell populations.

development (Carter et al., 2012; Lee-Chang et al., 2013; Liu et al., 2016; Nova-Lamperti et al., 2016). The requirement of B<sub>regs</sub> in immunosuppressive activities and immune modulation is further illustrated in work by Wang and colleagues (2014), who stated that B<sub>regs</sub> negatively regulate monocyte cytokine production through IL-10 dependant mechanisms (Wang et al., 2014). These findings illustrate the capacity of B-cells in controlling regulatory hierarchy, there by controlling the immune responses to maintain homeostasis (see Figure 2.2). This balance between immune suppression and activation is key in preventing autoimmunity and maintaining immune tolerance (Abbas et al., 2014; Carter et al., 2012; Evans et al., 2007; Mauri and Bosma, 2012; Rao et al., 2015). Additional studies have identified a B<sub>reg</sub> phenotype commonly referred to as killer B<sub>regs</sub> (Lundy, 2009; Lundy and Boros, 2002; Lundy et al., 2015; Mor et al., 2000; van Rensburg et al., 2017; Van Rensburg et al., 2017). These killer B<sub>regs</sub> express death-inducing ligand Fas ligand (FasL), known to induce apoptosis of activated CD4<sup>+</sup> T-cells (Lundy et al., 2015; van Rensburg et al., 2017). It is believed that these cells mediate inflammation and immune responsiveness through Fas-mediated mechanisms (Lundy, 2009; Lundy and Boros, 2002; Lundy et al., 2015).

#### 4.1 B-cells in cancer

An example of the importance of immune balance has been illustrated in multiple tumor studies (Berntsson et al., 2016; Lee-Chang et al., 2013; Lundgren et al., 2016; Mor et al., 2000; Shao et al., 2014; Tadmor et al., 2011; Zhou et al., 2014), in which an increase in B<sub>reg</sub> cells frequency corresponds to poor disease outcome. During these investigations, B<sub>regs</sub> were found to suppress anti-tumor activity of pathogenic immune cells, resulting in tumor growth and metastasis (Berntsson et al., 2016; Lee-Chang et al., 2013; Lundgren et al., 2016; Ou et al., 2015; Schwartz et al., 2016; Tadmor et al., 2011). In such cases, suppression of the immune system is disadvantageous and ultimately contributes to disease pathology. Studies have found a significant positive correlation between T<sub>reg</sub> (CD4<sup>+</sup> CD25<sup>+</sup> CD127<sup>low</sup>) and MDSC (CD14<sup>+</sup> HLA-DR<sup>-</sup>) frequencies with B<sub>reg</sub> (CD19<sup>+</sup> CD24<sup>hi</sup> CD38<sup>hi</sup> IL10<sup>+</sup>) frequencies in cancer patients (Carter et al., 2012; Flores-Borja et al., 2013; Liu et al., 2016; Nova-Lamperti et al., 2016; Schwartz et al., 2016). Various investigations proposed that these tumor-evoked B<sub>regs</sub> (tB<sub>res</sub>) induce a TGF- $\beta$ -dependant conversion of FoxP3<sup>+</sup> regulatory T-cells (T<sub>regs</sub>) from non-T<sub>reg</sub> CD4<sup>+</sup> cells (Flores-Borja et al., 2013; Kessel et al., 2012; Liu et al., 2016), which suppressing anti-tumor activity of CD8<sup>+</sup> T-cells and NK cells, in turn protect cancer cells (Kessel et al., 2012; Lee-Chang et al., 2013; Rao et al., 2015). Studies have also identified the occurrence of killer B<sub>regs</sub> in various cancer models (Lundy et al., 2015; Mor et al., 2000). In these models, killer B<sub>regs</sub> promote tumor survival and suppress anti-tumor activities through Fas-dependant mechanisms, to induce apoptosis of activated CD4<sup>+</sup> T-cells, preventing activation and functioning of cytotoxic T-cells. In certain cancer cases, anti-CD20 antibody treatment results in depletion of CD20<sup>+</sup> B-cells, impeding primary tumour metastasize, facilitating favourable prognosis (Lee-Chang et al., 2013; Schwartz et al., 2016b). However, in other instances anti-CD20 antibody treatment has been found to enrich CD20<sup>-</sup> tB<sub>regs</sub> which are potent anti-tumor effector cells,



**Figure 2.2. Functional Properties of regulatory B-cells ( $B_{reg}$ s).** Through the expression of various cell surface markers and soluble factors,  $B_{reg}$  cells are capable of modulating the differentiation and function of several immune cells, including monocytes, dendritic cells and T-cells. The influence of  $B_{reg}$  cells on other immune cells, and in turn the effect of the elicited response, depends on the environment in which they are found. As such,  $B_{reg}$  occurrence could be beneficial or detrimental to the overall outcome of a condition. **Adapted from** (Rosser and Mauri, 2015)

leading to enhanced tumor growth and metastasis (Lee-Chang et al., 2013). To date, no simple or specific method exists to inactivate  $B_{\text{regs}}$ . Although, some promise has been shown with various compounds including resveratrol (RSV) (Lee-Chang et al., 2013; Wang et al., 2017), ibrutinib (Advani et al., 2013; Castillo et al., 2016) and Idelalisib (Brown et al., 2014; Lannutti et al., 2011). Low, noncytotoxic doses of RSV have been shown to inactivate Stat3 phosphorylation and acetylation, a constitutively expressed pathway involved in TGF- $\beta$  generation, and thus  $tB_{\text{reg}}$  functioning (Lee-Chang et al., 2013). Idelalisib is a potent inhibitor of the PI3K pathway, which has been shown to be vital for proper B-cell activation and functioning (Lannutti et al., 2011). Zhang and colleagues (2016) also showed a strong positive correlation between  $CD5^+ CD19^+$  cells with Stat3 expression levels in patients with lung and prostate cancer (Zhang et al., 2016). In a study by Chang, C.L. and colleagues (2013), RSV-treated mice were found to have significantly lower  $tB_{\text{reg}}$  and  $T_{\text{regs}}$ , and that their  $tB_{\text{regs}}$  had lost all suppressive activity, when compared to untreated mice (Lee-Chang et al., 2013). RSV relieved the immunosuppressive effects of  $tB_{\text{regs}}$  by blocking the generation of  $T_{\text{regs}}$ , providing evidence of the relevance of  $B_{\text{regs}}$  in immune regulation and disease outcome (Wang et al., 2017).

#### *4.2 B-cells in autoimmunity*

$B_{\text{reg}}$  cells have also been shown to play key roles in autoimmunity (Edwards and Cambridge, 2006; Firl et al., 2017; Lundy et al., 2015; Matsumoto et al., 2014; Wang et al., 2014), in which deficiency of IL-10 producing  $B_{\text{regs}}$  have been associated with autoimmunity (Liu et al., 2016; Mauri and Bosma, 2012; Mauri and Menon, 2015; Ray and Dittel, 2017). It has been suggested that these cells mediate their effector function via passive or active immune suppressive mechanisms (Lundy, 2009). During passive immune suppression, deactivation or decreased functioning of effector cells is observed, including cytokine withdrawal, antigen clearance and T-cell anergy (Lundy, 2009). It is speculated that  $B_{\text{regs}}$  suppress autoimmune pathogenic T-cell activity through antigen-specific MHCII mechanisms (Lundy et al., 2015). In contrast, active immune suppression involves cytokine secretion or activation-induced cell death (AICD) (Alderson and Lynch, 1998; Anel et al., 2007; Sharpe et al., 2007). Increasing amounts of evidence support the hypothesis of AICD as a mechanism by which  $B_{\text{regs}}$  inflict their suppressive activity (Sedger et al., 2002; Takahashi et al., 1994). The occurrence of killer  $B_{\text{regs}}$  has been shown in an autoimmune model, following stimulation with LPS or PMA/ionomycin (Lundy and Boros, 2002; Lundy et al., 2015; Mauri and Bosma, 2012; Van Rensburg et al., 2017). Importantly, studies investigating the role of B-cells in autoimmunity have focused mainly on IL-10 production (Carter et al., 2011; Lundy et al., 2015; Matsumoto et al., 2014; Mauri and Bosma, 2012), and not FasL expression. Adoptive transfer of killer  $B_{\text{regs}}$  from FasL-deficient mice have been shown to induce immune tolerance, providing probable evidence of key FasL mechanisms needed (Lundy et al., 2015). Mixed results have been shown regarding B-cell-deficiency models, in which the presence of  $B_{\text{regs}}$  during disease have conflicting outcomes. In some cases,  $B_{\text{reg}}$  frequencies were associated with decreased inflammatory conditions and favourable disease outcome (Carter et al., 2012; Lundy and Fox, 2009; Lundy et al., 2005); while other cases

indicated an exacerbated disease condition, correlating with  $B_{reg}$  frequencies (Daien et al., 2014; Johansson et al., 2001; Matsushita et al., 2008; Svensson et al., 1998). In some instances, self-reactive B-cells are believed to revert to a regulatory phenotype in an attempt to eliminate or inactivate other autoreactive cells, conferring protection against autoimmunity (Lundy et al., 2015). Alternatively, self-reactive B-cells could contribute to inflammation and autoimmunity by chronic humoral responses, thereby contributing to immune pathology (Carter et al., 2011; Johansson et al., 2001). This potentially harmful self-reactive B-cell responses could account for instances where B-cell depletion results in better disease prognosis.

#### *4.3 B-cells in transplantation*

Following transplantation, innate immune cells distinguish between self and donor antigens, resulting in immune stimulation (Abbas et al., 2014; Capra et al., 1999; Zabriskie, 2009). As expected, T-cells are mainly responsible for allograft rejection (Firl et al., 2017; Ingulli, 2010). However, in order for these T-cells to function, they require activation from other immune cells (Benichou and Thomson, 2009; Felix and Allen, 2007; Ng et al., 2010). B-cells are responsible for allopeptide presentation to T-cells, providing co-stimulation essential for activation and differentiation of memory T-cells (Bharat and Mohanakumar, 2007; Leddon and Sant, 2010). As such alloresponsiveness is dependent on this B--T-cell interaction (Benichou and Thomson, 2009; Ng et al., 2010; Vongwiwatana et al., 2003; Whitmire et al., 2009). A study by Firl and colleagues (2017) showed that B-cell depletion correlated with increased allograft rejection due to increased alloreactivity (Firl et al., 2017). During chronic inflammation,  $B_{regs}$  skew T-cells development to a regulatory phenotype ( $T_{regs}$ ) and maintain this population to impose immune tolerance (Adams and Newell, 2012; Bharat and Mohanakumar, 2007; Matsumoto et al., 2014; Rosser and Mauri, 2015). Therefore,  $B_{regs}$  could promote tolerance of mismatched MHC transplants by inducing  $T_{regs}$  suppression and inflammatory alloreactivity (Adams and Newell, 2012; Chesneau et al., 2013; Firl et al., 2017; Flores-Borja et al., 2013; Vongwiwatana et al., 2003). Another theory is the induction of apoptosis of self-reactive T-cells by killer  $B_{regs}$  (Alderson and Lynch, 1998; Sharpe et al., 2007). Importantly, B-cells do not always foster tolerance; In acute and chronic allograft rejection, plasma cells secrete donor-specific antibodies responsible for complement fixation and antibody-dependant T-cell-mediated cytotoxicity, resulting in allograft rejection (Adams and Newell, 2012; Chesneau et al., 2013; Matsumoto et al., 2014, 2014; Ray and Dittel, 2017). Henceforth, understanding the complex mechanisms involved in alloresponses, and conditions needed to induce regulatory B-cell phenotypes to increase immune tolerance, are key to achieving beneficial outcomes in transplantation procedures.

#### *4.4 B-cells during infectious disease*

Inflammation is the primary immune response to infection, resulting in effective pathogenic clearance and activation of the wound healing cascade (Abbas et al., 2014; Medzhitov, 2008). Innate effector B-cells have been shown to promote Th1 and Th17 development through B-effector 1 (Be-1) cytokine



production (e.g. IFN- $\gamma$ ), important for antimicrobial immune responses (Baba et al., 2015; Du Plessis et al., 2016; Lundy and Boros, 2002; Mauri and Bosma, 2012). However if unresolved could result in injury to the host (Rao et al., 2015; Rosser and Mauri, 2015). As such, anti-inflammatory regulatory mediators, such as B<sub>regs</sub>, are needed to suppress the immune response, via production of Be-2 cytokines (e.g. IL-4) and prevent tissue damage by subverting the Th1 and Th17 response (Achkar et al., 2015; Carter et al., 2012; Kessel et al., 2012; Shen et al., 2014). A study by van Rensburg and colleagues (2017) investigated the role of B-cells in the context of *Mycobacterium tuberculosis* infection (van Rensburg et al., 2017). Stimulation of isolated B-cells with BCG, and co-stimulatory molecules (CD40L), resulted in the induction of B<sub>regs</sub> expressing FasL (unpublished results). Additionally, van Rensburg and colleagues (2017) detected the induction of B<sub>regs</sub>, expressing FasL, following successful anti-tuberculosis treatment (van Rensburg et al., 2017; Van Rensburg et al., 2017). In these studies, higher phenotypic frequencies of FasL<sup>+</sup> IL5RA<sup>+</sup> CD40<sup>+</sup> PD-1<sup>+</sup> CD38<sup>+</sup> B<sub>regs</sub> cells, although not significant, were found in healthy controls compared to TB patients (van Rensburg et al., 2017; Van Rensburg et al., 2017). Following anti-TB treatment, phenotypic expression levels of these markers increased.

Gene expression analysis revealed significant increases in FasL, CD19, APRIL and IL5RA<sup>31</sup> (Van Rensburg et al., 2017), while cytokine expression analysis revealed significant increases in sFasL levels (Du Plessis et al., 2016; van Rensburg et al., 2017), following anti-TB treatment. This implies that an increase in B-cell activation (killer B<sub>reg</sub> activity) during TB disease corresponds to reversion towards a healthy state. A study by Lundy and colleagues (2015) illustrated that T-cell surface expression of FasL represents just a fraction of the total FasL protein produced by activated killer B<sub>regs</sub> (Lundy et al., 2015). These findings explain the variations of significance in van Rensburg and colleagues' investigation. Collectively, these results suggest that the killer B<sub>reg</sub> cell population are crucial in the immune response and confer a protective role during infection. According to Rao and colleagues (2015), this suggested protection is as a result of B-cell-induced apoptosis of *Mtb* reservoirs, decreasing the bacterial load, associated cytokine profiles and interaction with T-cells during disease (Rao et al., 2015). These results are in agreement with others involving parasitic infection (e.g. schistosomal infection) in which an increase in the killer B<sub>reg</sub> subset, response to SEA exposure, was found to correlate with CD4<sup>+</sup> T-cell apoptosis, decreased granuloma size and better treatment outcome (Lundy and Boros, 2002; Lundy et al., 2015). Interestingly, B-cells from uninfected mice, exposed to SEA, showed potent killing functioning, while B-cells from infected mice showed enhanced killing capacity, indicating that primed B-cells have heightened FasL expression and advanced function (Lundy and Boros, 2002). Comprehensively, results for each of the studies suggest that killer B<sub>regs</sub> may possess a constitutive effector function in non-diseased conditions, linking with van Rensburg and colleagues' findings that increased B<sub>reg</sub> frequencies in infected patients corresponds to a healthy state. Nevertheless, B<sub>reg</sub> presence during disease is not always favourable. To date, a limited number of studies have focused on the immunomodulatory role of B<sub>regs</sub> in intracellular infection. Investigation of B<sub>reg</sub> function during HIV infection has associated B<sub>reg</sub>



functioning with immune dysfunction and poor disease outcome (Siewe et al., 2013). Accordingly, further research is required in order to characterize the role of these killer B<sub>regs</sub> in disease.

## 5. Future Advances

Current literature indicates vast functional roles of B-cells in immune modulation, in which evidence of the regulatory role of B-cells has been shown in numerous diseases. However, in order to fully elucidate the role of these B<sub>regs</sub>, additional functional and transcriptional studies are required to uncover the basic operation of these cells and how they elicit their control. Remarkably, Lundy and colleagues (2015) investigated the potential uses of killer B<sub>regs</sub> in mediating peripheral immune tolerance through the induction and use of killer B<sub>reg</sub>-derived FasL expressing exosomes (Lundy et al., 2015). Self-reacting B-cells have been shown to express FasL (Firl et al., 2017), thus it is hypothesized that these cells can synthesize and secrete FasL expressing exosomes, with incorporated MHC II self-Ag presentation, to induce apoptosis of other self-reactive cells throughout the body, implementing immune tolerance (Alderson and Lynch, 1998; Firl et al., 2017; Sharpe et al., 2007). Currently, cellular immunity mediated by adoptive transfer of regulatory lymphocytes is used to treat autoimmunity and allergic responses, however this procedure has many drawbacks (Chesneau et al., 2013; Firl et al., 2017; Leddon and Sant, 2010; Lundy and Boros, 2002; Shen et al., 2014).

A newly developed, robust method for experimentally producing human B-cell derived FasL<sup>+</sup> MHCII<sup>+</sup> exosomes provides therapeutic potential for patient-customized treatment, with many benefits and far less shortcomings (Lundy et al., 2015). However, investigation into the safety and efficacy of this method remains unknown. Studies involving various stimuli and inhibitors have begun uncovering essential pathways needed for B<sub>reg</sub> generation and functioning (Advani et al., 2013; Brown et al., 2014; Lannutti et al., 2011; Lee-Chang et al., 2013; Takata and Kurosaki, 1996), however these only touch the surface with regards to the mechanisms B<sub>regs</sub> employ. The relative importance of each molecule expressed or secreted by B<sub>regs</sub> in immune modulation would enable better understanding of potential interactions with other immune cells. Determination of the contribution of each cell subset, in this case B<sub>regs</sub>, and their impact on disease occurrence and progression could have vast implications for future vaccine development and treatment regimens (Du Plessis et al., 2016; Lundy, 2009; Van Rensburg et al., 2017). Moreover, identification of a unique B<sub>reg</sub> cell-surface marker could permit its potential use as a biomarker, to diagnose disease occurrence and monitor treatment response to treatment. The potential of B<sub>regs</sub> remains infinite, while our knowledge is limiting.

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## **Chapter 3:**

The work presented in this chapter has been formatted in the style of PLoS ONE journal to which it was submitted for publication and is currently under review.



## **Isolation of B-cells using Miltenyi MACS bead Isolation Kits**

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## Abstract

This article describes the procedures used to isolate pure B-cell populations from whole blood using various Miltenyi magnetic-activated cell sorting (MACS) bead Isolation kits. Such populations are vital for studies investigating the functional capacity of B-cells, as the presence of other cell types may have indirect effects on B-cell function through cell-cell interactions or by secretion of several soluble molecules. B-cells can be isolated by two main approaches: 1) Negative selection - in which B-cells remain “untouched” in their native state; this is advantageous as it is likely that B-cells remain functionally unaltered by this process. 2) Positive selection – in which B-cells are labelled and actively removed from the sample. We used three Negative B-cell isolation kits as well as the Positive B-cell isolation kit from Miltenyi and compared the purity of each of the resulting B-cells fractions. Contamination of isolated B-cell fractions with platelets was the conclusive finding for all of the isolation techniques tested. These results illustrate the inefficiency of current available MACS B-cell isolation kits to produce pure B-cell populations, from which concrete findings can be made. As such we suggest cell sorting as the preferred method for isolating pure B-cells to be used for downstream functional assays.

### 1. Background

The immune system consists of a collection of cell types responsible for maintaining our health by fighting off infection, eradicating foreign materials and battling disease (Abbas et al., 2014; Capra et al., 1999; Lundy, 2009; Slifka et al., 1998; Zabriskie, 2009). B-lymphocytes (B-cells), an immune cell type that forms part of the adaptive immune response, contribute fundamentally to the balance between health and disease. B-cells perform a multitude of effector functions, including antigen presentation, antibody production, cytokine secretion, opsonization, complement activation and immune modulation (Abbas et al., 2014; Capra et al., 1999; Du Plessis et al., 2016b; Mauri and Bosma, 2012; Paul, 2013; Pier et al., 2004; Rao et al., 2015; Rosser and Mauri, 2015; Zabriskie, 2009). The activation state of B-cells influences the effect they have on the immune response and ultimately determines whether or not their presence is beneficial or harmful to the host.

B-cells interact directly with other immune cells, such as macrophages, T-cells and dendritic cells, through receptor-mediated mechanisms as well as indirectly through the secretion of various molecules. For instance, B-cells present a captured antigen via major histocompatibility complex (MHC) to a T-cell clone within a secondary lymphoid organ resulting in cellular activation, clonal expansion and elicitation of an immune response. This is an example of cell-contact immune modulation. Moreover, B-cells may enhance the function of already activated immune cells through indirect means. For example, antibody secretion by plasma cells (differentiated effector B-cells) enables microbe opsonization which targets foreign material for phagocytosis by circulating macrophages by increasing binding affinity and uptake by endocytosis.

Similarly, B-cell function is influenced by the presence and interaction with other cell types. Several studies have illustrated the necessity of co-stimulation by other cell types via MHC presentation, co-receptor engagement and cytokine encounter for B-cell activation and differentiation (Breloer et al., 2007; Cazac and Roes, 2000; Chambers and Allison, 1997; Chen and Flies, 2013; Elgueta et al., 2009; Jang et al., 2015; Kretschmer et al., 2007; McKenzie et al., 1993; Müller et al., 1991; Takatsu, 1997). An example of receptor-mediated mechanisms that influence B-cell function is the CD40-CD40L interaction that occurs between B-cells and T-cells, required for cellular maturation and survival (Chambers and Allison, 1997; Chen and Flies, 2013; Elgueta et al., 2009). Additionally, cytokines such as interleukin-2, 4, 6, 21, transforming-growth factor beta (TGF- $\beta$ ) and interferons (IFNs) (Cazac and Roes, 2000; Chen and Flies, 2013; McKenzie et al., 1993; Sowa et al., 2009; Takatsu, 1997) produced by activated immune cells bind to various receptors on the B-cell surface, such as the B-cell receptor, CD21, membrane-bound immunoglobulin and toll-like receptors (TLRs), initiating intracellular signalling pathways that regulate B-cell differentiation and activation. Dysregulation or impaired function of B-cells can result in detrimental consequences for the host; therefore, studies investigating the behavior of B-cells and their contribution to observed immune response are of great importance.

The study of human B-cell populations for functional and/or mechanistic purposes are best performed in the absence of other cell types. The presence of these other cell types may alter B-cell function, either through direct contact or indirectly by the production and secretion of soluble factors like cytokines (Igaz et al., 2001; McKenzie et al., 1993; Wrenshall et al., 1999). B-cells constitute roughly 10-20% of the total lymphocytes population within whole blood (Autissier et al., 2010; Corkum et al., 2015; Kleiveland, 2015), as such studies on whole blood, or isolated peripheral blood mononuclear cells (PBMCs) are not suitable for investigating in-depth B-cell specific function. It is thus important to validate isolation procedures that are used to obtain these desirable pure B-cell populations, as they ultimately determine the reliability and accuracy of such studies.

Currently, a range of B-cell isolation kits from various companies are available. Miltenyi B-cell isolation kits are among the most popular to be used for obtaining pure B-cell populations from human (Du Plessis et al., 2016; Joosten et al., 2016; Lundy and Boros, 2002; Qiu et al., 2016; van Rensburg and Loxton, 2018; Xu et al., 2008; Yanaba et al., 2008; Yoshizaki et al., 2012) and animal samples (Jang et al., 2015; Martinez-Martin et al., 2017; Tsui et al., 2018; Yanaba et al., 2008). As such, a large proportion of the research currently inferring conclusions regarding B-cell function rely heavily on the efficacy of these kits to isolate B-cells from whole blood. This paper reviews the ability of various B-cell isolation kits available from Miltenyi to isolate pure B-cell populations from human blood, based on the purity of the obtained sample fractions.

## 2. Methods

### 2.1 Participant Recruitment and Sample collection/preparation

Ethical approval was obtained from the ethics committee of Stellenbosch University (N16/05/070) and the City of Cape Town City Health. The study was conducted according to the Helsinki Declaration and International Conference of Harmonisation guidelines. For this study, we recruited 27 healthy individuals. On several occasions, varying amounts of peripheral blood was collected in Sodium Heparin tubes and processed as described below. Each blood draw, and subsequent cell isolation procedure, was recorded as a separate event (amounting to a total of 68 cell isolation procedures). Written informed consent was obtained from all study participants.

### 2.2 Isolation of peripheral blood mononuclear cells (PBMC) from whole blood

In this section, the various protocols amendments used to isolate B-cells from whole blood are described. It should be noted that Miltenyi isolation kits used to obtain enriched pure B populations required a pre-isolation of PBMCs from whole blood. As such the protocol for PBMC isolation refers to the initial processing of blood samples, before the use of either of the Miltenyi isolation kits.

#### *2.2.1 Isolation of mononuclear cells from peripheral blood using the Ficoll-density Gradient method*

##### *Materials*

9mL Sodium Heparin (NaHep) tubes (Lasec)	Disposable Pipettes
Phosphate-buffered saline (PBS; Lonza)	Trypan Blue (Sigma)
50mL Falcon centrifugation tubes	Heamocytometer
Ficoll-Histopaque Plus Media (GE Healthcare)	Microscope

##### Centrifuge

Note: Unless otherwise notes, performed all steps in biosafety cabinet under sterile conditions.

##### *Methods*

1. Collect peripheral blood from donor in NaHep tubes – This step is done by a professional healthcare worker in a medical examination room. Blood was processed within two hours of blood draw.
2. In 50mL Falcon tube, add 15mL of Ficoll-Histopaque Plus media
3. In separate 50mL Flacon tube, dilute peripheral blood in 1:1 ratio with PBS (to a max volume of 35mL)
4. Gently layer the diluted blood from step 3 onto the Ficoll from step 2
5. Centrifuge at 400xg for 25 min at room temperature with the accelerator and brake off.
6. Use a sterile Pasteur pipette to carefully remove the upper plasma layer. In a circular motion, collect the opaque PBMC band at the Ficoll interface and transfer into a new 50mL Falcon tube
7. Wash the PBMCs twice in 50mL of PBS, centrifuge at 400xg for 10 min at room temperature with the brake and accelerator set to max.
8. Count the cells to determine cell number and viability.

### 2.2.2 Additions and Alterations to PBMC isolation protocol

#### A. Addition of Platelet wash step to PBMC isolation procedure

The alteration steps listed replace step 7 in the PBMC isolation method described in section 1 above. This was done to decrease the debris/platelet population found in the PBMC sample. This was done by altering the speed at which the isolated PBMC's were washed, in an attempt to prevent pelleting and retention of the platelets during the washing steps. It is assumed that the degree of platelet contamination within the PBMC fraction is likely to affect the purity of the isolated B-cell fraction, and thus should be limited. Three different approaches were used, each tested in a separate set of experiments multiple times by various lab technicians:

1. Wash the PBMCs twice in 50mL of PBS, centrifuge at 300xg for 10 min at room temperature
2. Wash the PBMCs twice in 50mL of PBS, centrifuge at 200xg for 10 min at room temperature
3. Wash the PBMCs twice in 50mL of PBS, centrifuge at 120xg for 15 min at room temperature

### 2.3 Isolation of B-cells from mononuclear cells using the Miltenyi B-cell Isolation kits

Miltenyi isolation kits are based on a simple process, in which biotin conjugated antibody-labelled mononuclear cells are separated from unlabeled cells via a column in the presence of a magnetic field. During negative selection, the cell type of interest (in this case B-cells) remains unlabeled/"untouched" and in its native state, while the remaining unwanted mononuclear cells (in this case T-cells, NK cells and monocytes) are targeted via biotin labelled-antibodies specific for cell surface receptor(s) of those cell types. During positive selection, the cell type of interest (in this case B-cells) are specifically targeted, labelled and actively magnetically removed from the sample by retention in the column in the presence of a magnetic field. These cells are then retrieved by plunging the column in the absence of a magnetic field.

#### 2.3.1 Isolation of B-cells from mononuclear cells by negative selection using the Miltenyi B-cell Isolation kit II

##### Materials

Miltenyi B-cell Isolation Kit II (For detailed description of kit components see supplementary 3.1)

MACS buffer (PBS) containing 0.5% (v/v) fetal bovine serum (FBS, heat-inactivated, Hyclone) and 2mM EDTA

Standard MACS Isolation materials:   MACS Magnet and magnet stand  
  LS Columns  
  15mL Falcon centrifugation tubes  
  Centrifuge

Note: Unless otherwise noted, all steps were performed in biosafety cabinet under sterile conditions, at 4°C on ice in the dark. LS columns and MACS buffer were stored at 4°C prior to procedure while MACS magnet and magnet stand were stored at -20°C.

*Methods*

The kit was operated according to the manufacturer's instructions; For detailed information on this procedure see supplementary 3.1.

*2.3.2 Additions and Alterations to original protocol***A. Addition of Miltenyi Dead cell removal kit to isolation protocol**

The addition of this isolation kit was done in attempt to decrease the "cell debris/platelet" population found within isolated B-cell fractions. This kit was used according to the manufacturer's recommendations (For detailed description of kit components see supplementary 3.2) and integrated into the isolation procedure described in section 2.3.1 was done using three different approached:

1. Perform dead cell removal procedure before B-cells isolation procedure
2. Perform dead cell removal procedure simultaneously to B-cell isolation (Addition of dead cell staining buffer together with negative isolation kit buffers – MACS buffer volumes altered to maintain stipulated staining volume of B-cell isolation kit II)
3. Perform dead cell removal procedure after B-cells isolation procedure

**B. Addition of Miltenyi CD61 platelet removal kit to isolation protocol**

The addition of this isolation kit was done in attempt to decrease the "cell debris/platelet" population found within isolated B-cell fractions. This kit was used according to the manufacturer's recommendations (For detailed description of kit components see supplementary 3.3) and integrated into the isolation procedure described in section 2.3.1 was done using three different approached:

1. Perform CD61 platelet removal procedure before B-cells isolation procedure
2. Perform CD61 platelet removal procedure simultaneously to B-cell isolation (Addition of CD61 staining buffers together with negative isolation kit buffers - MACS buffer volumes altered to maintain stipulated staining volume of B-cell isolation kit II)
3. Perform CD61 platelet removal procedure after B-cells isolation procedure

*2.3.3. Isolation of B-cells from mononuclear cells by negative selection using the Miltenyi Naïve B-cell Isolation kit**Materials*

Miltenyi Naïve B-cell Isolation Kit (For detailed description of kit components see supplementary 3.4)

Standard MACS Isolation materials (listed in methods section 2.3.1)

*Methods*

The kit was operated according to the manufacturer's instructions; For detailed information on this procedure see supplementary 3.4.

#### *2.3.4. Isolation of B-cells from mononuclear cells by negative selection using the Miltenyi CD43 Microbeads kit*

##### *Materials*

Miltenyi CD43 Microbeads Kit (For detailed description of kit components see supplementary 3.5)

MACS buffer (PBS containing 5% fetal bovine serum)

Standard MACS Isolation materials (listed in methods section 2.3.1)

##### *Methods*

The kit was operated according to the manufacturer's instructions; For detailed information on this procedure see supplementary 3.5.

#### *2.3.5. Isolation of B-cells from mononuclear cells by positive selection using the Miltenyi CD19 positive Isolation kit*

##### *Materials*

Miltenyi CD19 positive Isolation Kit (For detailed description of kit components see supplementary 3.6)

Standard MACS Isolation materials (listed in methods section 2.3.1)

##### *Methods*

The kit was operated according to the manufacturer's instructions; For detailed information on this procedure see supplementary 3.6)

#### *2.4. Isolation of T-cells from mononuclear cells by negative selection using the Miltenyi Pan T-cell Isolation kit*

##### *Materials*

Miltenyi Pan T-cell Isolation Kit (For detailed description of kit components see supplementary 3.7)

Standard MACS Isolation materials (listed in methods section 2.3.1)

##### *Methods*

The kit was operated according to the manufacturer's instructions; For detailed information on this procedure see supplementary 3.7)

#### *2.5 Fluorescent-activated cell sorting (FACS) of isolated B-cell from samples pre-processed using the Miltenyi B-cell isolation kit II*

Lymphocytes were FACS sorted from biological samples, that had already undergone MACS isolation sample using the B-cell isolation kit II, based on their forward-scatter area (FSC) and side-scatter area (SSC) properties. In theory, B-cells should account for the majority (above 90%) of the cells within the lymphocyte population. Therefore, this additional separation process should remove particles within the sample that do not form part of the lymphocyte population, essentially purifying the sample. Sorting based on size and cellular complexity rather than fluorescence was done to limit the level of manipulation and processing that the cells were exposed to, as this may have negative effects on

downstream processes as previously mentioned. Prior to sorting the cell suspensions were filtered through 0.35µm filter to remove any cell aggregates that could block the fluidics lines within the FACS instrument. Samples underwent two sorting steps: 1) an enrichment sort - to remove excessive debris without compromising the cell numbers by sorting all cells of interest, and 2) a pure sort - to completely eliminate cell debris while sacrificing cell yield by only sorting the cells of interest that were not flanked by contaminating cells.

## 2.6 Immunofluorescence staining and flow cytometric analysis of various cell fractions to confirm determine the purity of isolated samples

Sample purity is of vital importance; this purity check validates the isolation technique as sufficient in isolating the cell type of interest and that conclusions drawn from downstream experiments are conclusive based on the measured cellular responses of the isolated cells and not by the presence/influence of other cell types.

The isolated fraction was stained with anti-human mAb specific for CD19 (to determine the proportion of isolated B-cells in the lymphocyte population i.e. “B-cell purity”) and anti-human mAb specific for CD36 (to determine the proportion of platelets making up the cell debris population i.e. “Platelet Contamination”). The differentiation of cell debris from lymphocytes was determined using FSC and SSC, a due to the fact that cell debris, dead cells and platelets are of a smaller size and less cellular complexity compared to lymphocytes. The gating strategy used to evaluate sample purity of isolated B-cells (see Suppl Figure 3.1 and 3.2.) and T-cells (see Supp Figure 3.3) is displayed in Supplementary data. The resulting data was analyzed using FlowJo v10 software (Oregon, USA).

## 2.7 Statistical Analysis

Data analysis of the flow cytometry plots was done using FlowJo V10 (Treestar, USA) and the resulting stats analyzed using Prism 7 Software (San Diego, CA). Statistical differences between groups was calculated using a non-parametric Kruskal-Wallis test with a Dunn’s multiple comparisons test. Alternatively, when applicable a multiple non-parametric unpaired student t-test was used to calculate statistical differences between groups for a list of independent variables. Linear regression analysis was performed using Prism 7 Software. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with an FDR of 1%, was used to correct for multiple testing. Statistical significance is indicated by an asterisk, in which the  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*) and  $p < 0.0001$  (\*\*\*\*) or by letters in which data points with different letters indicated statistical differences.



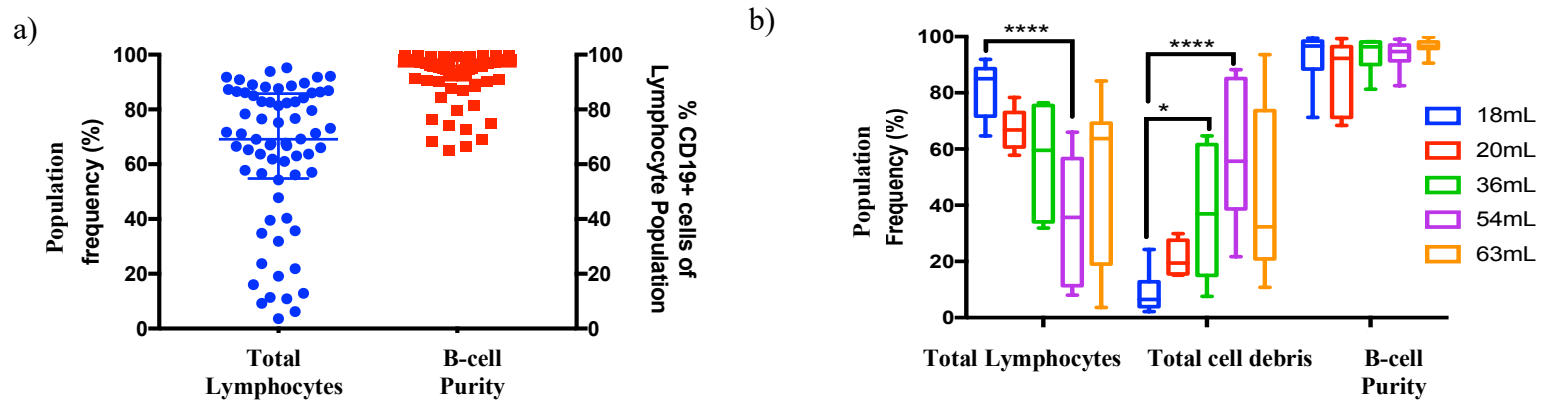
### 3. Results and Discussion

The purpose of cell isolation methods is to obtain a pure cell population of interest, that can be used to investigate cell structure and function without the influence of other cell types. As such, the presence of contaminants in an isolated sample are undesirable and defeat the objective of isolation procedures. The methods described in this paper using various Miltenyi B-cell isolation kits result in such a phenomenon. The desired purity of a cell-type within an isolated sample equates to 90% or more of the total cell population. Key factors used to assess the purity of isolated samples within this study included: 1) lymphocyte population frequency - the proportion (%) of lymphocytes within the sample, 2) debris population frequency - the proportion (%) of cell debris within the sample, 3) Platelet frequency – the proportion (%) of CD36<sup>+</sup> within the debris population and 4) B-cell purity –the proportion (%) of CD19<sup>+</sup> within the lymphocyte population. It should be highlighted that the acceptable > 90% isolated cell purity stated in the data sheet of the tested isolation kits is only achieved when examining the cellular content of the lymphocyte population, while excluding all cell debris from analysis (Figure 3.1a). This type of analysis is termed “gated purity” and overlooks the composition of the isolated sample as a whole. This isolation method is sufficient for studies only focusing on cell surface receptor expression analysis but not functional downstream experiments, due to the presence of cellular contaminants within the final sample fraction. Thus “gated purity” should not be the only purity of concern when assessing the efficiency of isolation procedures. In almost all instances, with the exception of the MACS CD19 positive isolation kit, platelets were found to be a large contaminant within the isolated samples, making up a large portion of the total cell content within the isolated fraction (Table 3.1). Platelets were identified using flow cytometry based on their size, cellular complexity and expression of the cell surface marker CD36 (Oquendo et al., 1989; Silverstein, 2009). This large variation of platelet contamination may be as a result of individual sample variation in the number of platelets per liter of human peripheral blood – the reference range has been reported as 150-450 x 10<sup>9</sup> per liter (Brecher and Cronkite, 1950; Bull et al., 1965; Lozano et al., 1998).

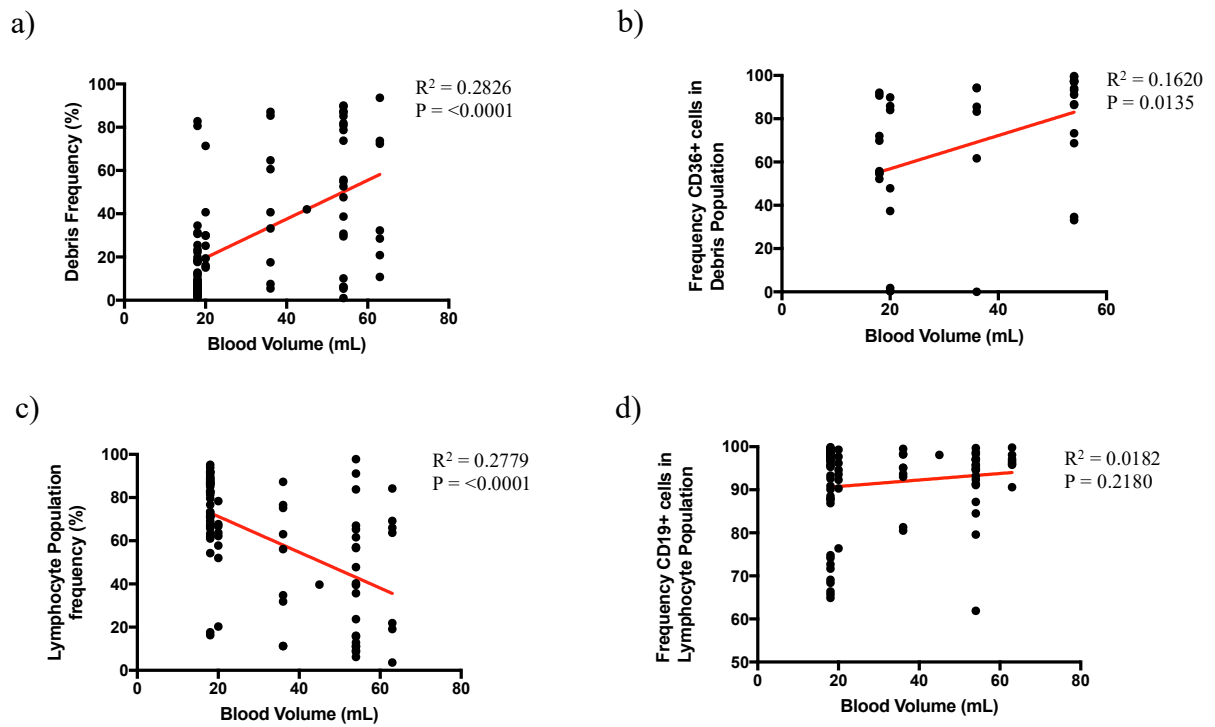
Consequently, the volume of peripheral blood used for the isolation procedure, and in turn the number of platelets comprised within the whole blood sample, had a significant effect on the obtained sample purity (Figure 3.1b). A significant difference was observed between the investigated blood volumes when investigating it's the effect on each of the factors used to assess sample purity following the isolation process. Upon further analysis it was found that a positive relationship exists between whole blood volume and the frequency of the debris population (Figure 3.2a), as well as platelet contamination (Figure 3.2b). Conversely, a negative relationship exists between whole blood volume and the frequency of the lymphocyte population (Figure 3.2c). Notably, the frequency of CD19<sup>+</sup> cells within the lymphocyte population remained unaffected by whole blood volume (Figure 3.2d).

	Total Lymphocytes (% of sample cellular content)		Total cell debris (% of sample cellular content)		Platelet population (% of Debris population)		B-cell Purity (%CD19+ cells of lymphocyte population)		Blood Volume (mL)	N
	Median	Range	Median	Range	Median	Range	Median	Range	Range	
<i>Naive B kit</i>	11,20	-	87,10	-	94,40	-	96,90	-	36	1
<i>CD43+ kit</i>	11,30	-	85,40	-	83,30	-	61,90	-	36	1
<i>CD19+ kit</i>	52,00	20,30-62,20	40,70	30,00-71,40	0,39	0,26-1,82	92,40	91,10-96,20	20	3
<i>B-cell Isolation Kit II + CD61 removal kit</i>	70,90	65,80-73,40	9,38	7,61-18,60	54,90	52,20-69,90	99,00	65,70-99,30	18	3
<i>B-cell Isolation Kit II + Dead cell removal kit</i>	17,60	16,30-63,30	80,60	34,50-82,80	90,70	72,00-92,00	98,70	98,30-99,50	18	3
<i>B-cell Isolation Kit II</i>	69,15	3,63-95,20	17,85	1,48-93,60	86,55	0,014-99,70	95,75	64,90-99,90	18-63	68

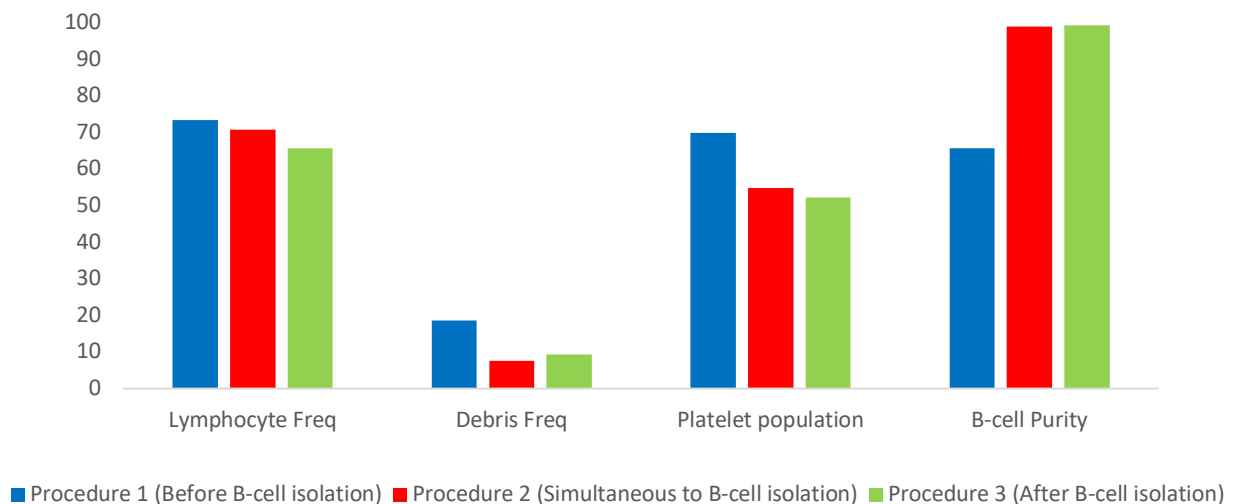
**Table 3.1. Summary of B-cell isolation kits tested.** This table includes the various parameters used to evaluate the efficiency of each of the Miltenyi B-cell isolation kits tested. The statistical comparison between the various parameters listed above is graphically illustrated in Figure 3.1.



**Figure 3.1. Analysis of isolated B-cell sample purity obtained using commercially available MACS B-cell isolation kit II (n =68).** (a) Sample purity following negative MACS bead isolation using B-cell Isolation Kit II only. The left axis illustrates total lymphocytes as a percentage of all cellular content within the isolated sample, while the right axis illustrates B-cell purity as a percentage of the lymphocyte population (b) Effect of blood volume on effectiveness of MACS B-cell Isolation Kit II. Statistical differences between blood volumes was calculated using a non-parametric Kruskal-Wallis test with a Dunn’s multiple comparisons test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistical significance is indicated by an asterisk, in which the  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*) and  $p < 0.0001$  (\*\*\*\*).



**Figure 3.2. Relationship between B-cell isolation quality and sample volume for all B-cell isolation kits tested (n=79).** (a) Linear regression between blood volume and frequency of debris population (b) Linear regression between blood volume and total lymphocyte population (c) Linear regression between blood volume and frequency of CD36+ platelets within debris population (d) Linear regression between blood volume and frequency of CD19+ cells in lymphocyte population.



**Figure 3.3. Analysis of isolated B-cell sample purity obtained using the MACS B-cell isolation kit II with the addition of a CD61 platelet removal kit.** The implementation of the CD63 platelet removal kit was done in three different ways, namely before isolation with MACS B-cell isolation kit II (Procedure 1, n=1), simultaneously with MACS B-cell isolation kit II (Procedure 2, n=1) and after isolation with MACS B-cell isolation kit II (Procedure 3, n=1).

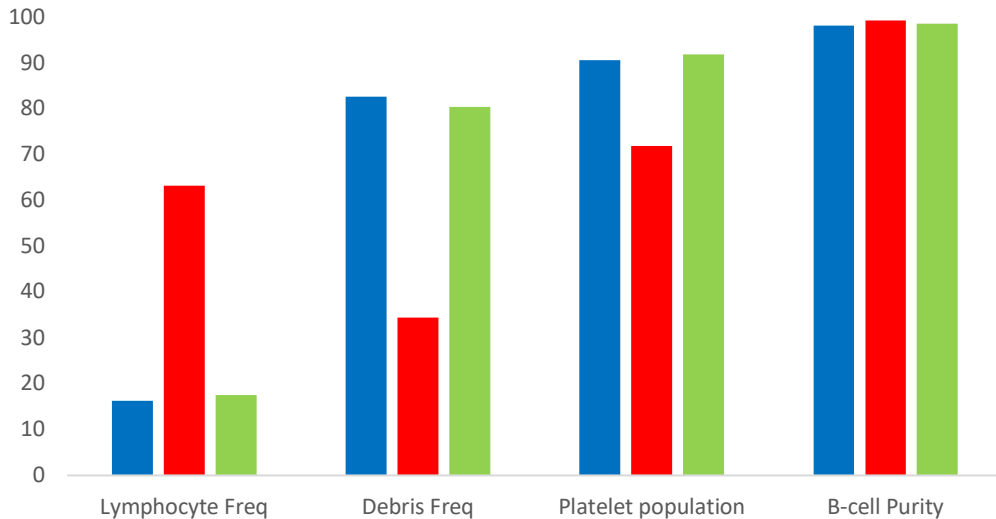
As previously stated, significantly less platelet contamination was observed in samples isolated with the MACS CD19 positive isolation kit, when compared to the negative B-cell isolation kit II (Table 3.1). However, the obtained isolated sample comprised of a large proportion of 'cell debris' (range 30-70%). Characteristically, dead cells are identified in flow cytometry by changes in their light scatter; generally they exhibit decreased FSC and increased in SSC properties (Muirhead et al., 1985; Penttila et al., 1975; Schmid et al., 2001, 1992), hence are suspected to make up this debris population. During positive selection surface receptors on the cell type of interest are targeted for labeling. Theoretically, positive selection should result in highly pure cell populations to be obtained, as all non-labelled cells (including cell debris) should simply elute from the column leaving only the labeled cell type of interest. However, non-specific binding may occur resulting in unwanted cell types contaminating the final sample. Dead cells have been known to bind non-specifically to antibodies (Schmid et al., 2001, 1992) and could thus be the major source of the observed contamination. Nevertheless, further investigation is required in order to determine the source of this 'contamination'. Ideally, negative isolation would be the method of choice as the cell type of interest surface receptors remain unbound and thus functionally unaltered by this process, whereas positive selection introduces the risk of possible cell activation and ultimately functional alteration of the cell, which may complicate downstream processes. Possible downstream processes include but are not limited to investigating cellular activation following exposure to a particular molecule/drug. This *ex vivo* manipulation could result in altered B-cell function to either enhance or diminish the measured B-cell response resulting in artefactual observations.

Of the three negative B-cell isolation kits investigated, one of the kits, namely the CD43 microbead isolation kit, did not contain any anti-biotin CD36<sup>+</sup> monoclonal antibody (mAb), which would explain the large degree of platelet contamination within these samples (Table 3.1, Suppl Figure 3.1a). However, a large population of CD36<sup>+</sup> platelets were identified within isolated samples that underwent processing using either of the other two negative B-cells isolation kits that contain an anti-biotin CD36 mAb (Table 3.1), namely the B-cell isolation kit II (Suppl Figure 3.2a and b) or the Naïve B-cell isolation kit (Suppl Figure 3.1b), for effective removal of CD36 positive cells (including platelets). A possible reason for the observed phenomenon is inadequate concentration of this cell surface receptor mAb within the antibody cocktail solution, thereby influencing the obtained sample purity. This supports the observed effects of whole blood volume on the overall effectiveness of the isolation process, in which an increase in the volume of starting sample, hence platelet numbers, resulting in decreased efficiency in removal of CD36<sup>+</sup> platelets. Due to the large individual variability of platelet counts per participant, it is difficult to determine how much anti-biotin CD36 mAb is adequate.

Various procedures were implemented into the isolation procedure utilizing the MACS negative B-cell isolation kit II in an attempt to decrease the platelet contamination and 'cell debris' population found within isolated samples. These modifications including the addition of a MACS CD61 platelet removal kit to determine whether or not this would improve the resulting sample purity (Suppl Figure 3.2c).

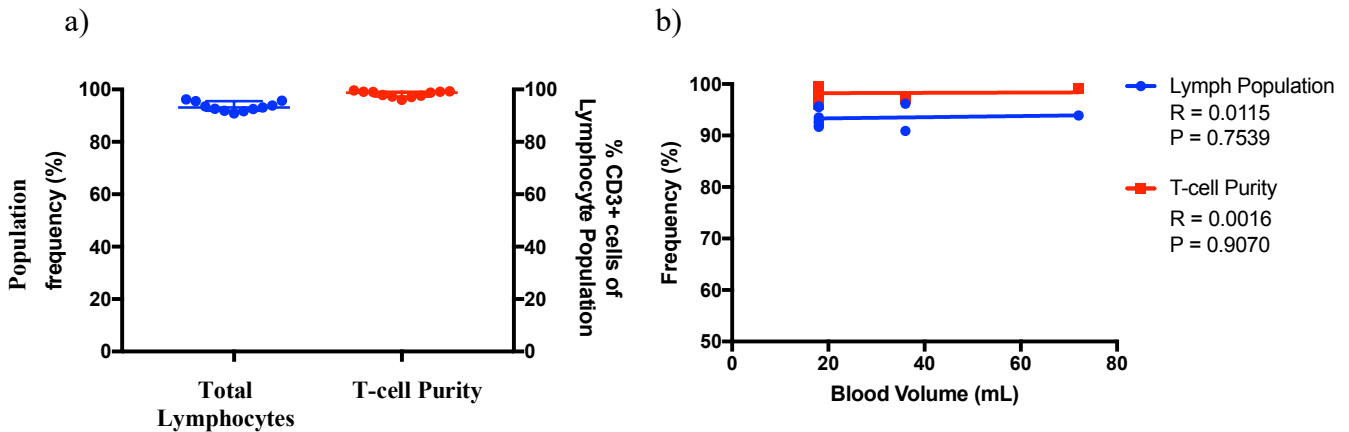
While a general improvement in the obtained sample quality was observed as seen by a reduction in the frequency of the debris population within the isolated sample, these results were not statistically significant from either of the other isolation methods (Table 3.1). It should be noted that when comparing the effectiveness of the addition of the MACS CD61 microbead kit to other isolation methods, the results from procedure I, II and III were pooled. When subjectively relating each of the implementation methods separately, no methods was perceived to be superior (Figure 3.3). Furthermore, the addition of a MACS dead cell removal kit was implemented to determine whether this would improve sample quality in cases where platelet contamination within the “cell debris” population was infrequent (Suppl Figure 3.2d). No improvement in the resulting sample quality was observed (Table 3.1). It should be noted that when comparing the effectiveness of the addition of the MACS dead cell removal kit to other isolation methods, the results from procedure I, II and III were pooled. Notable, when subjectively relating each of the implementation methods separately, procedure II was perceived to yield sample purity of a superior quality (Figure 3.4). As such, further investigation utilizing method II should be performed in order to better evaluate the usefulness of this kit in improving B-cell isolation. Additionally, the effectiveness of this MACS dead cell removal kit was not investigated in combination with the MACS CD19 positive isolation kit, in which dead cells are the suspected cause of contamination within isolated samples (Suppl Figure 3.1c). Accordingly, additional investigation is required before a conclusive decision can be made with regards to the efficiency of the modification in refining the isolation process.

Interestingly, when performing T cell isolations using the MACS Pan T cell negative isolation kit available from Miltenyi, limited debris/platelet contamination within the resulting isolated fraction was observed (Figure 3.5a). Additionally, sample volume was observed to have no confounding effect on the resulting sample purity (Figure 3.5b). These results thereby illustrate that our PBMC isolation technique and MACS isolation methods are up to standard and are not the cause of this recurring contamination issue. Instead, these results emphasize the inefficiency of current B-cell isolation kits available from Miltenyi to produce pure B-cell populations from which concrete findings can be made. Given that it is standard practice for immunology research to be conducted on immune cells in isolation *ex vivo* (Du Plessis et al., 2016; Jang et al., 2015; Joosten et al., 2016; Lundy and Boros, 2002; Martinez-Martin et al., 2017; Qiu et al., 2016; Tsui et al., 2018; van Rensburg and Loxton, 2018; Xu et al., 2008; Yanaba et al., 2008, 2008; Yoshizaki et al., 2012), it is crucial that any external influences, such as the presence of contaminating cells within an isolated sample, be acknowledged as these factors may contribute to the measured physiological responses, resulting in artefactual observations. It is thus proposed that the Miltenyi B-cell isolation kits are suboptimal and need further optimization in order to achieve the desired cell isolation results for which these kits are intended. The presence of platelets and cell debris within isolate cell samples to be used for downstream functional assays is a significant drawback - as these “contaminants” may influence the function of cells under investigation. Platelets and cell debris



■ Procedure 1 (Before B-cell isolation) ■ Procedure 2 (Simultaneous to B-cell isolation) ■ Procedure 3 (After B-cell isolation)

**Figure 3.4. Analysis of isolated B-cell sample purity obtained using the MACS B-cell isolation kit II with the addition of the dead cell removal kit.** The implementation of the dead cell removal kit was done in three different ways, namely before isolation with MACS B-cell isolation kit II (Procedure 1, n=1), simultaneously with MACS B-cell isolation kit II (Procedure 2, n=1) and after isolation with MACS B-cell isolation kit II (Procedure 3, n=1).



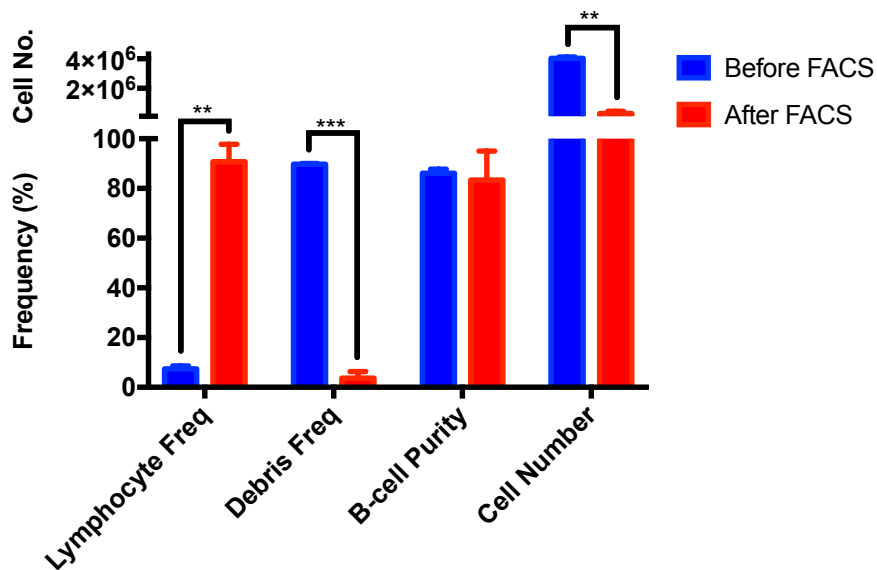
**Figure 3.5. Analysis of isolated T cell sample purity obtained using the MACS Pan T cell isolation kit (n=11).** (a) Sample purity following negative MACS bead isolation. The left axis illustrates total lymphocytes as a percentage of all cellular content within the isolated sample, while the right axis illustrates T-cell purity as a percentage of the lymphocyte population. As illustrated, platelet/cell debris was successfully removed, and a pure T cell population obtained, as shown by CD3+ cells (b) Linear regression between blood volume and efficiency of MACS Pan T-cell isolation kit with reference to frequency of lymphocyte population and frequency of CD3+ cells within lymphocyte population (T-cell Purity).

contribute to the microenvironment, that is the *in vitro* setting, in which these cells are investigated as they secrete various substances such as cytokines (in the case of platelets) or cellular content (in the case of dead cells) known to have an effect on the activation and function of surrounding immune cells (Elzey et al., 2005; Kral et al., 2016). Thus, the observed immune response of investigated cells may not be identical to those where pure sample isolates were used as the cellular function may have been altered by the substances released from these contaminating cells. Evidence has illustrated that platelets have the capacity to modulate B and T cell function - and potentially drive their operation (Sowa et al., 2009). One such way in which platelets may achieve this is through the expression of mRNA found within their cytoplasm (Denis et al., 2005; Power et al., 1995; Soslau et al., 1997). The resulting proteins include a variety of molecules known to influence cell function, including cell surface receptors involved in cellular activation and various cytokines (Elzey et al., 2005; Kral et al., 2016; Sowa et al., 2009). These findings are of huge concern, as several studies inferring B-cell function downstream utilizing these kits to obtain “pure” B-cell populations, have not acknowledged the presence of these platelets within their isolated samples. Therefore, any conclusions made from isolated cell studies that utilized these B-cell isolation kits, need to consider the presence of these platelets within the sample, and note the limitations when reporting their findings contributing to B-cell function.

Lastly, the addition of a fluorescent-activated cell sorting (FACS) step, following B-cell isolation with the Miltenyi B-cell isolation kit II, was investigated to determine whether this could improve sample purity. The resulting sample fraction was found to have significantly improved sample purity, however cell number was compromised using this method (Figure 3.6). The gating strategy used to evaluate sample purity of FACS B-cells is displayed in Suppl Figure 4. Based on the findings of this paper, the addition of FACS sorting to this isolation procedure for obtaining pure B-cell populations is recommended, as this technique was found to significantly reduce the platelet contamination within the isolated sample fractions. Consequently, the tested B-cell isolation kits would better serve as a pre-enrichment step prior to cell sorting rather than an isolation technique alone. This is highly beneficial in comparison to traditional cell sorting as it allows for the isolation of naïve B-cells, whereas B-cells sorted directly from whole blood or PBMC samples requires labeling of the cells of interest with a fluorescent tag for removal from cell suspension. This is disadvantageous as it may result in cell activation through engagement of the B-cell receptor (CD19) resulting in altered immune function, as well as the increased amount of time needed to sort the B-cells out of these dense cell populations, resulting in excessive costs. As such, the use of the commercially available MACS isolation kits is not discouraged but rather recommended as an additional pre-step to cell sorting procedures.

Various FACS sorting platforms exist (eg. BD, SONY, Beckman Coulter) including the MACSQuant-Tyto (Miltenyi Germany) and use of the services offered should be to achieve the desired sample purity. However, in terms of the research cost, processing time and the volume of blood required to obtain sufficient B-cell numbers for functional assays downstream, this method may not be feasible for most laboratories. A possible improvement to reduce the loss in cell number could be to label the sample with a CD36 mAb and actively sort the platelets within the debris population, using the pure sort method, based on their FSC and SSC properties as well as fluorescence; followed by a pure sort of the lymphocyte population based on FSC and SSC, with less abort rates as the sample will comprise of significantly less cell debris. Importantly, B-cell isolation kits from additional companies was not investigated within this study. Therefore, the results depicted within this paper do not dissuade the efficacy of other B-cell isolation kits. However, it would be advisable to do a comparative study to determine the efficacy of the kits not tested.





**Figure 3.6. Analysis of isolated B-cell sample purity obtained using the MACS B-cell isolation kit II, followed by cell sorting based on FSC and SSC (n=2).** Statistical differences between sorting conditions was calculated using multiple non-parametric unpaired student t-tests. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistical significance is indicated by an asterisk, in which the  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*)

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## Supplementary Information

### Supplementary 3.1



## B Cell Isolation Kit II human

Order no. 130-091-151

<b>Components</b>	<p><b>1 mL B Cell Biotin-Antibody Cocktail, human:</b> Cocktail of biotin-conjugated monoclonal antibodies against CD2, CD14, CD16, CD36, CD43, and CD235a (Glycophorin A).</p> <p><b>2 mL Anti-Biotin MicroBeads:</b> MicroBeads conjugated to monoclonal anti-biotin antibodies (isotype: mouse IgG1).</p>
<b>Capacity</b>	For 10 <sup>9</sup> total cells.
<b>Product format</b>	All components are supplied in buffer containing stabilizer and 0.05% sodium azide.
<b>Storage</b>	Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial labels.

#### Safety information

**For research use only. Not intended for any animal or human therapeutic or diagnostic use.**

**Before use, please consult the Safety Data Sheet for information regarding hazards and safe handling practices.**

#### Cell separation protocols

**Fully automated cell labeling and separation using the autoMACS<sup>®</sup> Pro Separator**

**Alternatively:**

**Manual magnetic labeling**

↓

or

**Subsequent manual separation**

↓

**Subsequent automated cell separation using the autoMACS<sup>®</sup> Pro Separator**

#### General notes

▲ For tips concerning sample preparation, magnetic labeling and separation, visit [www.miltenyibiotec.com/faq](http://www.miltenyibiotec.com/faq) and [www.miltenyibiotec.com/protocols](http://www.miltenyibiotec.com/protocols).

▲ For product-specific background information and applications of this product, refer to the respective product page at [www.miltenyibiotec.com/130-091-151](http://www.miltenyibiotec.com/130-091-151).

#### Reagent and instrument requirements

- Buffer: Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS<sup>®</sup> BSA Stock Solution (# 130-091-376) 1:20 with autoMACS<sup>®</sup> Rinsing Solution (# 130-091-222). Degas buffer before use, as air bubbles could block the column.
- (Optional) Pre-Separation Filters (30 µm) (# 130-041-407) to remove cell clumps.
- Choose the appropriate MACS Separator and MACS Columns:

Column	Max. number of labeled cells	Max. number of total cells	Separator
M5	10 <sup>7</sup>	2×10 <sup>9</sup>	MiniMACS, OctoMACS
LS	10 <sup>8</sup>	2×10 <sup>9</sup>	MidiMACS, QuadroMACS
autoMACS	2×10 <sup>8</sup>	4×10 <sup>9</sup>	autoMACS Pro

▲ **Note:** When using this kit the unwanted cell fraction is labeled and the target cells remain unlabeled. Depending on the target cell frequency, the labeled fraction can therefore represent the majority of the total cells.

To avoid blocking of the column, do not exceed the max. number of labeled cells per column. Estimate the number of labeled cells in the sample, split the sample if necessary and use the appropriate number of separation columns.

#### Fully automated cell labeling and separation using the autoMACS<sup>®</sup> Pro Separator

▲ Refer to the user manual for instructions on how to use the autoMACS<sup>®</sup> Pro Separator.

▲ All buffer temperatures should be ≥10 °C.

▲ Place tubes in the following Chill Rack positions:

**position A** = sample, **position B** = negative fraction, **position C** = positive fraction.

1. For appropriate resuspension volumes and cell concentrations, please visit [www.automacspro.com/autolabeling](http://www.automacspro.com/autolabeling).
2. Switch on the instrument for automatic initialization.
3. Go to the **Reagent** menu and select **Read Reagent**. Scan the 2D barcode of each reagent vial with the barcode scanner on the autoMACS Pro Separator. Place the reagent into the appropriate position on the reagent rack.
4. Place sample and collection tubes into the Chill Rack.
5. Go to the **Separation** menu and select the reagent name for each sample from the **Labeling** submenu (the correct labeling, separation, and wash protocols will be selected automatically).
6. Enter sample volume into the **Volume** submenu. Press **Enter**.
7. Select **Run**.
8. Collect enriched B cell fraction at position B = negative fraction.

**Manual magnetic labeling**

- ▲ Work fast, keep cells cold, and use pre-cooled solutions (2–8 °C).
- ▲ Volumes for magnetic labeling given below are for up to 10<sup>7</sup> total cells. When working with fewer cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly.
- ▲ For optimal performance it is important to obtain a single-cell suspension before magnetic labeling.

1. Prepare cells and determine cell number.
2. Resuspend cell pellet in 40 µL of buffer per 10<sup>7</sup> total cells.
3. Add 10 µL of Biotin-Antibody Cocktail per 10<sup>7</sup> total cells.
4. Mix well and incubate for 5 minutes in the refrigerator (2–8 °C).
5. Add 30 µL of buffer per 10<sup>7</sup> total cells.
6. Add 20 µL of Anti-Biotin MicroBeads per 10<sup>7</sup> total cells.
7. Mix well and incubate for 10 minutes in the refrigerator (2–8 °C).
8. Proceed to subsequent magnetic cell separation.

▲ **Note:** A minimum of 500 µL is required for magnetic separation. If necessary, add buffer to the cell suspension.

**Subsequent manual cell separation**

- ▲ Always wait until the column reservoir is empty before proceeding to the next step.

9. Place column in the magnetic field of a suitable MACS Separator. For details refer to the respective MACS Column data sheet.
10. Prepare column by rinsing with the appropriate amount of buffer:
  - MS: 500 µL      LS: 3 mL
11. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells, representing the enriched B cells.
12. Wash column with the appropriate amount of buffer. Collect unlabeled cells that pass through and combine with the effluent from step 11.
  - MS: 3×500 µL      LS: 3×3 mL
13. (Optional) Remove column from the separator and place it on a suitable collection tube. Pipette the appropriate amount of buffer onto the column. Immediately flush out the magnetically labeled non-B cells by firmly pushing the plunger into the column.
  - MS: 1 mL      LS: 5 mL

**Subsequent automated cell separation using the autoMACS® Pro Separator**

- ▲ Refer to the user manual for instructions on how to use the autoMACS® Pro Separator.

- ▲ All buffer temperatures should be ≥10 °C.

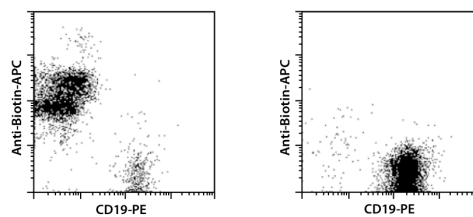
- ▲ Place tubes in the following Chill Rack positions:

**position A** = sample, **position B** = negative fraction, **position C** = positive fraction.

9. Prepare and prime the instrument.
10. Follow the instructions that are given in the user manual.
11. The program “Depletes” is recommended. Collect enriched B cells at position B = negative fraction.

**Example of a separation using the B Cell Isolation Kit II**

Isolation of untouched B cells from human PBMCs using the B Cell Isolation Kit II, an LS Column, and a MidiMACS™ Separator. Cells are fluorescently stained with CD19-PE and Anti-Biotin-APC. Cell debris and dead cells were excluded from the analysis based on scatter signals and PI fluorescence.



For more information or assistance refer to our technical support.

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## Supplementary 3.2



## Cell Isolation Kits

### Components of MACS® Dead Cell Removal Kit

- MACS® colloidal super-paramagnetic Dead Cell Removal MicroBeads. The product is supplied as a suspension containing stabilizer.
- MACS 20× Binding Buffer stock solution. The buffer has to be diluted 20-fold with sterile, double distilled water prior to use (see Preparation of 1× Binding Buffer).

These MACS products are susceptible to bacterial contamination. **Handle under sterile conditions!**

### Storage Conditions

Store protected from light at 4 °C. Do not freeze.

### Instruments Required

Magnetic cell separators MiniMACS™, MidiMACS™, VarioMACS™ or SuperMACS™.

MACS Column(s) MS or LS (plus MS or LS adapter for use in combination with VarioMACS or SuperMACS).

### Protocol for Dead Cell Removal

#### Preparation of 1× Binding Buffer

Per  $10^7$  total cells, dilute 0.25 mL of 20× Binding Buffer Stock Solution with 4.75 mL of sterile, double distilled water. Alternatively, the total amount of 25 mL of 20× Binding Buffer Stock Solution can be diluted with 475 mL of sterile, double distilled water. Store at 4 °C. **Important: Handle under sterile conditions!**

#### Magnetic labeling

Collect cells e.g. from cell culture.

Centrifuge cells at 300×g. Remove supernatant completely and resuspend cell pellet in 100 µL of Dead Cell Removal MicroBeads per approximately  $10^7$  total cells (e.g. petri dish Ø 9 cm: about  $1 \times 10^7$  cells, one mouse spleen: about  $1 \times 10^8$  cells). For fewer cells, use same volume. Mix well and incubate for 15 minutes at room temperature (20–25 °C).

#### Preparation of MACS® Column

Choose a positive selection column type MS (for up to  $10^7$  dead cells and up to  $2 \times 10^8$  total cells) or LS (for up to  $10^8$  dead cells and up to  $2 \times 10^9$  total cells) and place the column in the magnetic field of a suitable MACS® Separator (see “Column Data Sheets”).

Prepare column by rinsing with 1× Binding Buffer (MS: 500 µL; LS: 3 mL; for details, see “Column Data Sheets”).

#### Magnetic separation

Apply cell suspension in suitable amount of 1× Binding Buffer onto the column (MS: 500-1000 µL; LS: 1–10 mL; see “Important Notes”). Let the negative cells pass through. Rinse with appropriate amount of 1× Binding Buffer (MS:  $4 \times 500$  µL; LS:  $4 \times 3$  mL). Collect effluent as live cell fraction.

## Dead Cell Removal Kit

10 mL Dead Cell Removal MicroBeads  
25 mL 20× Binding Buffer Stock Solution

Order No. 130-090-101

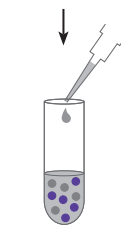


### How to eliminate dead cells using Dead Cell Removal Kit

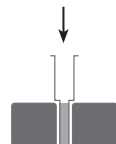
1. Prepare 1× Binding Buffer by dilution of 20× Binding Buffer Stock Solution.



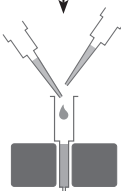
2. Collect cells, centrifuge at 300×g and remove supernatant completely.



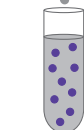
3. Resuspend cells in Dead Cell Removal MicroBeads. Mix well and incubate for 15 min at room temperature.



4. Place column in MACS Separator, prepare by rinsing with 1× Binding Buffer.



5. Apply cell suspension onto the column. Rinse with 1× Binding Buffer.



6. Collect effluent as live cell fraction.

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### Important Notes

- ▲ To increase the efficiency of the magnetic removal of dead cells, the live cell fraction can be passed over a second, freshly prepared column. Alternatively, the whole procedure (Magnetic Labeling and Magnetic Separation) can be repeated.
- ▲ Dead Cell Removal MicroBeads are susceptible to bacterial contamination. **Handle under sterile conditions.**
- ▲ Use 1× Binding Buffer prepared from 20× Binding Buffer Stock Solution supplied with the Dead Cell Removal Kit for all magnetic labeling, washing and selection steps. Binding of Dead Cell Removal MicroBeads requires Ca<sup>2+</sup>. The presence of the ion chelator EDTA will abolish binding. 1× Binding Buffer is optimized for best Dead Cell Removal MicroBeads binding. The use of a different buffer may lead to poor dead cell removal efficiency.
- ▲ **Attention:** Working on ice or incubating in the refrigerator requires increased incubation times for MACS MicroBeads. Incubate at **room temperature** (20–25 °C).
- ▲ Dead cells without any remnants of the plasma membranes ("stripped" nuclei) cannot be removed using Dead Cell Removal MicroBeads due to lack of accessible antigen.
- ▲ When working with cell samples containing platelets (e.g. blood samples), wash samples carefully at low centrifugation speed (200×g) in order to remove platelets. Use buffer containing the ion chelator EDTA for these washing steps. Dead Cell Removal MicroBeads bind to activated platelets. Activated platelets also bind to leukocytes (e.g. monocytes). In this case, viable cells bound to activated platelets would be retained in the magnetic field and reduce the recovery of living cells.

### Background Information

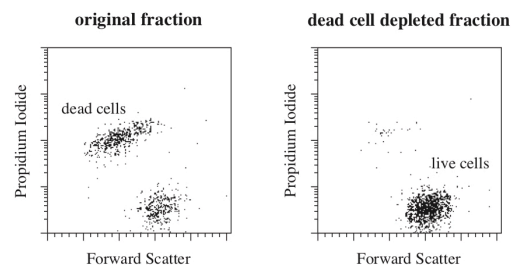
Dead Cell Removal MicroBeads recognize an antigen in the plasma membrane of apoptotic as well as dead cells. For the MACS dead cell depletion, cells are magnetically labeled with Dead Cell Removal MicroBeads and passed through a separation column. The magnetically labeled dead cells are retained in the column while the unlabeled living cells are collected in the flow-through. Using MACS Dead Cell Removal Kit, even early apoptotic cells with an intact cellular membrane are removed.

### Options for the Analysis of Dead Cell Removal

Removal of dead cells can be analyzed by microscopy with membrane exclusion dyes like Trypan Blue or by flow cytometric analysis using Propidium Iodide.<sup>1</sup>

### Example for elimination of dead cells from tissue culture using Dead Cell Removal Kit

Dead cells were eliminated from cultured Jurkat cells by labeling of cells with Dead Cell Removal MicroBeads and separation over an LS Column in the magnetic field of a MidiMACS Separator. Dead Cells can be fluorescently stained with Propidium Iodide.



Forward Scatter versus staining with Propidium Iodide.

### References

1. Coder, DM (1997) Assessment of Cell Viability. In: Robinson, JP *et al.* (eds.) Current Protocols in Cytometry, p. 9.2.1-9.2.14, John Wiley & Sons Inc., New York

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## Supplementary 3.3



## CD61 MicroBeads

### human

Order no. 130-051-101

#### Index

1. Description
  - 1.1 Principle of MACS® Separation
  - 1.2 Background and product applications
  - 1.3 Reagent and instrument requirements
2. Protocol
  - 2.1 Sample preparation
  - 2.2 Magnetic labeling
  - 2.3 Magnetic separation
3. Example of a separation using CD61 MicroBeads
4. References
5. Special cell separation protocol: Megakaryocyte isolation

#### 1. Description

<b>Components</b>	2 mL CD61 MicroBeads, human: MicroBeads conjugated to monoclonal mouse anti-human CD61 antibodies (isotype: mouse IgG1).
<b>Size</b>	For 10 <sup>9</sup> total cells, up to 100 separations.
<b>Product format</b>	CD61 MicroBeads are supplied as a suspension containing stabilizer and 0.05% sodium azide.
<b>Storage</b>	Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label.

##### 1.1 Principle of MACS® Separation

First, the CD61<sup>+</sup> cells are magnetically labeled with CD61 MicroBeads. Then, the cell suspension is loaded onto a MACS® Column which is placed in the magnetic field of a MACS Separator. The magnetically labeled CD61<sup>+</sup> cells are retained on the column. The unlabeled cells run through and this cell fraction is depleted of CD61<sup>+</sup> cells. After removal of the column from the magnetic field, the magnetically retained CD61<sup>+</sup> cells can be eluted as the positively selected cell fraction.

##### 1.2 Background and product applications

CD61 MicroBeads can be used for the isolation or depletion of megakaryocytes and their precursors from bone marrow<sup>1,3</sup> and for the removal of platelets from peripheral blood cell preparations. The CD61 antigen is also known as the integrin β3-subunit. CD61 combines with CD41 to form the heterodimeric gpIIb/gpIIIa complex, which is present on human megakaryocytes and platelets, mediating cell adhesion processes. Together with CD51, CD61 forms the vitronectin receptor, which is present on platelets as well as on a variety of other cell types like osteoclasts and endothelial vessel cells. CD61 MicroBeads were reported to cross-react with canine platelets.

#### Examples of applications

- CD61<sup>+</sup> cells from bone marrow cells are isolated for studies on megakaryocytopoiesis.
- Positive selection or depletion of cells expressing human CD61 antigen.
- Isolation of megakaryocytes from human bone marrow (see: 5. Special cell separation protocol: Megakaryocytes isolation).<sup>1</sup>
- Depletion or isolation of platelets from human peripheral blood.
- Isolation or depletion of leukocyte-platelet conjugates from human peripheral blood.

#### 1.3 Reagent and instrument requirements

- Buffer: Prepare a solution containing phosphate-buffered saline (PBS) pH 7.2, 0.5% BSA and 2 mM EDTA by diluting MACS BSA Stock Solution (# 130-091-376) 1:20 in autoMACS™ Rinsing Solution (# 130-091-222). Keep buffer cold (4–8 °C). Degas buffer before use, as air bubbles could block the column.
  - ▲ Note: EDTA can be replaced by other supplements such as anticoagulant citrate dextrose formula-A (ACD-A) or citrate phosphate dextrose (CPD). BSA can be replaced by other proteins such as human serum albumin, human serum or fetal calf serum. Buffers or media containing Ca<sup>2+</sup> or Mg<sup>2+</sup> are not recommended for use.
- MACS Columns and MACS Separators: CD61<sup>+</sup> cells can be enriched from bone marrow, peripheral blood or cultivated cells by using MS, LS or XS Columns (positive selection). CD61 MicroBeads can be used for depletion of CD61<sup>+</sup> cells on LD, CS or D Columns. Cells which strongly express the CD61 antigen can also be depleted using MS, LS, or XS Columns. Positive selection or depletion can also be performed by using the autoMACS Separator.

Column	Max. number of labeled cells	Max. number of total cells	Separator
<b>Positive selection</b>			
MS	10 <sup>7</sup>	2×10 <sup>8</sup>	MiniMACS, OctoMACS, VarioMACS, SuperMACS
LS	10 <sup>8</sup>	2×10 <sup>9</sup>	MidiMACS, QuadroMACS, VarioMACS, SuperMACS
XS	10 <sup>9</sup>	2×10 <sup>10</sup>	SuperMACS
<b>Depletion</b>			
LD	10 <sup>8</sup>	5×10 <sup>8</sup>	MidiMACS, QuadroMACS, VarioMACS, SuperMACS
CS	2×10 <sup>8</sup>		VarioMACS, SuperMACS
D	10 <sup>9</sup>		SuperMACS
<b>Positive selection or depletion</b>			
autoMACS	2×10 <sup>8</sup>	4×10 <sup>9</sup>	autoMACS

▲ Note: Column adapters are required to insert certain columns into the VarioMACS™ or SuperMACS™ Separators. For details see the respective MACS Separator data sheet.

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- (Optional) Fluorochrome-conjugated CD61 antibody for flow-cytometric analysis, e.g., CD61-PE (# 130-081-501), CD45-FITC (# 130-080-202), CD45-PE (# 130-080-201), CD45-APC (# 130-091-230).
- (Optional) Propidium iodide (PI) or 7-AAD for flow-cytometric exclusion of dead cells.
- (Optional) Pre-Separation Filters (# 130-041-407) to remove cell clumps.

## 2. Protocol

### 2.1 Sample preparation

When working with anticoagulated peripheral blood or buffy coat, peripheral blood mononuclear cells (PBMCs) should be isolated by density gradient centrifugation, e.g. using Ficoll-Paque™. For details see section General Protocols in the User Manuals or visit [www.miltenyibiotec.com/protocols](http://www.miltenyibiotec.com/protocols).

When working with tissues, prepare a single-cell suspension by a standard preparation method.

For details see section General Protocols in the User Manuals or visit [www.miltenyibiotec.com/protocols](http://www.miltenyibiotec.com/protocols).

▲ Note: Dead cells may bind non-specifically to MACS MicroBeads. To remove dead cells, we recommend using density gradient centrifugation or the Dead Cell Removal Kit (# 130-090-101).

### 2.2 Magnetic labeling

▲ Work fast, keep cells cold, and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and non-specific cell labeling.

▲ Volumes for magnetic labeling given below are for up to  $10^7$  total cells. When working with fewer than  $10^7$  cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for  $2 \times 10^7$  total cells, use twice the volume of all indicated reagent volumes and total volumes).

▲ For optimal performance it is important to obtain a single-cell suspension before magnetic separation. Pass cells through 30 µm nylon mesh (Pre-Separation Filters, # 130-041-407) to remove cell clumps which may clog the column.

1. Determine cell number.
2. Centrifuge cell suspension at  $300 \times g$  for 10 minutes. Aspirate supernatant completely.
3. Resuspend cell pellet in 80 µL of buffer per  $10^7$  total cells.
4. Add 20 µL of CD61 MicroBeads per  $10^7$  total cells.
5. Mix well and incubate for 15 minutes at 4–8 °C.  
▲ Note: Working on ice may require increased incubation times. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling.
6. (Optional) Add staining antibodies, e.g., add 10 µL of CD61-PE (# 130-081-501), and incubate for 5 minutes at 4–8 °C.
7. Wash cells by adding 1–2 mL of buffer per  $10^7$  cells and centrifuge at  $300 \times g$  for 10 minutes. Aspirate supernatant completely.
8. Resuspend up to  $10^8$  cells in 500 µL of buffer.

▲ Note: For higher cell numbers, scale up buffer volume accordingly.

▲ Note: For depletion with LD Columns, resuspend up to  $1.25 \times 10^8$  cells in 500 µL of buffer.

9. Proceed to magnetic separation (2.3).



### 2.3 Magnetic separation

▲ Choose an appropriate MACS Column and MACS Separator according to the number of total cells and the number of CD61<sup>+</sup> cells. For details see table in section 1.3.

#### Magnetic separation with MS or LS Columns

1. Place column in the magnetic field of a suitable MACS Separator. For details see respective MACS Column data sheet.
2. Prepare column by rinsing with appropriate amount of buffer:  
MS: 500 µL      LS: 3 mL
3. Apply cell suspension onto the column.
4. Collect unlabeled cells that pass through and wash column with appropriate amount of buffer. Perform washing steps by adding buffer three times. Only add new buffer when the column reservoir is empty.  
MS:  $3 \times 500$  µL      LS:  $3 \times 3$  mL  
Collect total effluent; this is the unlabeled cell fraction.
5. Remove column from the separator and place it on a suitable collection tube.
6. Pipette an appropriate amount of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.  
MS: 1 mL      LS: 5 mL

▲ Note: To increase the purity of the magnetically labeled fraction, it can be passed over a new, freshly prepared column.

#### Magnetic separation with XS Columns

For instructions on the column assembly and the separation, refer to the XS Column data sheet.

#### Depletion with LD Columns

1. Place LD Column in the magnetic field of a suitable MACS Separator. For details see LD Column data sheet.
2. Prepare column by rinsing with 2 mL of buffer.
3. Apply cell suspension onto the column.
4. Collect unlabeled cells which pass through and wash column with  $2 \times 1$  mL of buffer. Collect total effluent. This is the unlabeled cell fraction.

#### Depletion with CS Columns

1. Assemble CS Column and place it in the magnetic field of a suitable MACS Separator. For details see CS Column data sheet.
2. Prepare column by filling and rinsing with 60 mL of buffer. Attach a 22G flow resistor to the 3-way-stopcock of the assembled column. For details see CS Column data sheet
3. Apply cell suspension onto the column.
4. Collect unlabeled cells which pass through and wash column with 30 mL buffer from the top. Collect total effluent. This is the unlabeled cell fraction.

### Depletion with D Columns

For instructions on column assembly and separation, refer to the D Column data sheet.

### Magnetic separation with the autoMACS™ Separator

▲ Refer to the autoMACS™ User Manual for instructions on how to use the autoMACS Separator.

1. Prepare and prime autoMACS Separator.
2. Place tube containing the magnetically labeled cells in the autoMACS Separator. For a standard separation, choose one of the following separation programs:

Positive selection: "Possel"

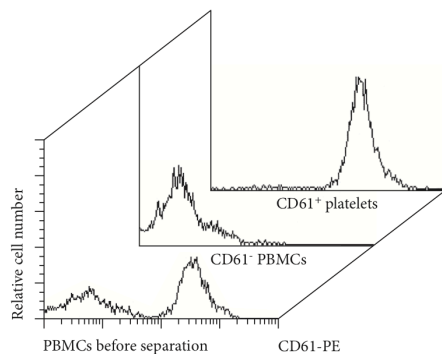
Depletion: "Depletes"

▲ Note: Program choice depends on the isolation strategy, the strength of magnetic labeling and the frequency of magnetically labeled cells. For details see autoMACS User Manual: "autoMACS Cell Separation Programs".

3. When using the program "Possel", collect positive fraction outlet port pos1. This is the purified CD61<sup>+</sup> cell fraction.  
When using the program "Depletes", collect unlabeled fraction from outlet port "neg1". This is the CD61<sup>-</sup> cell fraction.

### 3. Example of a separation using CD61 MicroBeads

Separation of PBMCs using CD61 MicroBeads and a MiniMACS™ Separator with an MS Column. The cells are fluorescently stained with CD61-PE (# 130-081-501). Cell debris and dead cells were excluded from the analysis based on scatter signals and PI fluorescence.



### 4. References

1. Schmitz, B. *et al.* (1994) *Eur. J. Hematol.* 52: 267-275. [104]
2. Komor, M. *et al.* (2005) *Stem cells* 23: 1154-1169.
3. Appel, S. *et al.* (2006) *Blood* 107: 3265-3270.

### Warnings

Reagents contain sodium azide. Under acidic conditions sodium azide yields hydrazoic acid, which is extremely toxic. Azide compounds should be diluted with running water before discarding. These precautions are recommended to avoid deposits in plumbing where explosive conditions may develop.

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## 5. Special cell separation protocol: Megakaryocyte isolation

Megakaryocytes can be isolated from bone marrow preparations using CD61 MicroBeads, which are developed for separation of human cells based on the expression of the CD61 antigen. CD61 is expressed on thrombocytes, megakaryocytes, osteoclasts and endothelial vessel cells. For MACS separation, megakaryocytes are magnetically labeled using CD61 MicroBeads. The magnetically labeled cells are retained on a MACS<sup>®</sup> Column in the magnetic field of a MACS Separator.

### Instrument and reagent requirements

- Buffer: Prepare a solution containing phosphate-buffered saline (PBS) pH 7.2, 0.5% BSA and 2 mM EDTA by diluting MACS BSA Stock Solution (# 130-091-376) 1:20 with autoMACS<sup>™</sup> Rinsing Solution (# 130-091-222). Keep buffer cold (4–8 °C). Degas buffer before use, as air bubbles could block the column.
  - ▲ Note: EDTA can be replaced by other supplements such as anticoagulant citrate dextrose formula-A (ACD-A) or citrate phosphate dextrose (CPD). BSA can be replaced by other proteins such as human serum albumin, human serum or fetal calf serum. Buffers or media containing Ca<sup>2+</sup> or Mg<sup>2+</sup> are not recommended for use.
- CD61 MicroBeads (# 130-051-101).
- Large Cell Separation Columns (# 130-042-202).
- RPMI 1640 (# 130-091-440).
- RPMI/FCS/EDTA: RPMI 1640 containing 25% FCS and 2 mM EDTA.
- Percoll<sup>™</sup> (1.05 g/mL).
- (Optional) Fluorochrome-conjugated CD61 antibody for flow cytometric analysis, e.g., CD61-PE (# 130-081-501).

### Isolation of megakaryocytes from bone marrow

- ▲ If cells cannot be separated on the day of harvest, store cells at 4 °C.
  - ▲ Remove all cell clumps during the cell preparation.
1. Collect human spongiosa material (e.g. from ribs).
  2. Take up spongiosa material in 5 mL of RPMI 1640 per approximately 0.5 cm<sup>3</sup> of spongiosa material.
  3. Incubate cells in 6 well plate at 37 °C, 5% CO<sub>2</sub> for 4 hours under agitation to detach the bone marrow cells from the bone.
  4. Subsequently, incubate cells overnight in 25 cm<sup>2</sup> tissue flask at 37 °C, 5% CO<sub>2</sub> to eliminate stroma cells and fibroblasts. Collect the non-adherent cells.
  5. Wash cells by adding RPMI/FCS/EDTA, centrifuge at 200×g for 10 minutes, remove supernatant completely. Repeat washing procedure once and resuspend cells in 7 mL RPMI/FCS/EDTA.
  6. Carefully layer 7 mL of diluted cell suspension over 7 mL of Percoll (1.05 g/mL).
  7. Centrifuge for 30 minutes at 400×g at 20 °C in a swinging-bucket rotor without break.
  8. Aspirate the upper layer leaving the mononuclear cell layer undisturbed at the interphase.
  9. Carefully collect interphase cells and wash them in PBS/BSA/EDTA. Centrifuge for 10 minutes at 200×g at 20 °C. Repeat this wash step.



### Magnetic labeling of 1×10<sup>7</sup> total cells in suspension

1. Resuspend 1×10<sup>7</sup> cells with PBS/BSA/EDTA in a final volume of 80 µL.
2. Add 20 µL CD61 MicroBeads.
3. Mix well and incubate for 15 minutes at 4–8 °C.
3. (Optional) Add fluorochrome-conjugated CD61 antibody, e.g., CD61-PE (# 130-081-501), at the titer recommended by the manufacturer and incubate for an additional 5–10 minutes at 4–8 °C.
4. Wash cells by adding 1–2 mL of buffer per 10<sup>7</sup> cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
5. Resuspend up to 10<sup>8</sup> cells in 500 µL of buffer.



### Magnetic separation

1. Place Large Cell Separation Column (without flow resistor) in the MACS Separator.
2. Prepare column by rinsing with 500 µL of PBS/BSA/EDTA.
3. Apply cell suspension onto the column.
4. Collect unlabeled cells which pass through and wash column with 3×500 µL of PBS/BSA/EDTA. Perform washing steps by adding buffer three times, each time once the column reservoir is empty. Collect total effluent. This is the unlabeled cell fraction.
5. Remove column from the separator and place it on a suitable collection tube.
6. Pipette appropriate 1 mL of PBS/BSA/EDTA onto the column. Immediately flush out fraction with the magnetically labeled cells by firmly applying the plunger supplied with the column.
  - ▲ Note: To increase the purity of the magnetically labeled fraction, it can be passed over a new, freshly prepared column.
7. Elute positive fraction as described above and proceed to analysis by flow cytometry or fluorescence microscopy.



## Supplementary 3.4



## Naive B Cell Isolation Kit II human

Order no. 130-091-150

<b>Components</b>	<p><b>1 mL Naive B Cell Biotin-Antibody Cocktail, human:</b> Cocktail of biotin-conjugated monoclonal antibodies against CD2, CD14, CD16, CD27, CD36, CD43 and CD235a (Glycophorin A).</p> <p><b>2 mL Anti-Biotin MicroBeads:</b> MicroBeads conjugated to monoclonal anti-biotin antibodies (isotype: mouse IgG1).</p>
<b>Capacity</b>	For 10 <sup>9</sup> total cells.
<b>Product format</b>	All components are supplied in buffer containing stabilizer and 0.05% sodium azide.
<b>Storage</b>	Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial labels.

### Safety information

**For research use only. Not intended for any animal or human therapeutic or diagnostic use.**

**Before use, please consult the Safety Data Sheet for information regarding hazards and safe handling practices.**

### Cell separation protocols

**Fully automated cell labeling and separation using the autoMACS® Pro Separator**

Alternatively:

**Manual magnetic labeling**

↓

Subsequent manual separation

or

↓

Subsequent automated cell separation using the autoMACS® Pro Separator

### General notes

▲ For tips concerning sample preparation, magnetic labeling and separation, visit [www.miltenyibiotec.com/faq](http://www.miltenyibiotec.com/faq) and [www.miltenyibiotec.com/protocols](http://www.miltenyibiotec.com/protocols).

▲ For product-specific background information and applications of this product, refer to the respective product page at [www.miltenyibiotec.com/130-091-150](http://www.miltenyibiotec.com/130-091-150).

### Reagent and instrument requirements

- **Buffer:** Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS® BSA Stock Solution (# 130-091-376) 1:20 with autoMACS® Rinsing Solution (# 130-091-222). Degas buffer before use, as air bubbles could block the column.
- (Optional) Pre-Separation Filters (30 µm) (# 130-041-407) to remove cell clumps.
- Choose the appropriate MACS Separator and MACS Columns:

Column	Max. number of labeled cells	Max. number of total cells	Separator
M5	10 <sup>7</sup>	2×10 <sup>8</sup>	MiniMACS, OctoMACS
LS	10 <sup>8</sup>	2×10 <sup>9</sup>	MidiMACS, QuadroMACS
autoMACS	2×10 <sup>8</sup>	4×10 <sup>9</sup>	autoMACS Pro

▲ **Note:** When using this kit the unwanted cell fraction is labeled and the target cells remain unlabeled. Depending on the target cell frequency, the labeled fraction can therefore represent the majority of the total cells.

To avoid blocking of the column, do not exceed the max. number of labeled cells per column. Estimate the number of labeled cells in the sample, split the sample if necessary and use the appropriate number of separation columns.

### Fully automated cell labeling and separation using the autoMACS® Pro Separator

▲ Refer to the user manual for instructions on how to use the autoMACS® Pro Separator.

▲ All buffer temperatures should be ≥10 °C.

▲ Place tubes in the following Chill Rack positions:

**position A** = sample, **position B** = negative fraction, **position C** = positive fraction.

1. For appropriate resuspension volumes and cell concentrations, please visit [www.automacspro.com/autolabeling](http://www.automacspro.com/autolabeling).
2. Switch on the instrument for automatic initialization.
3. Go to the **Reagent** menu and select **Read Reagent**. Scan the 2D barcode of each reagent vial with the barcode scanner on the autoMACS Pro Separator. Place the reagent into the appropriate position on the reagent rack.
4. Place sample and collection tubes into the Chill Rack.
5. Go to the **Separation** menu and select the reagent name for each sample from the **Labeling** submenu (the correct labeling, separation, and wash protocols will be selected automatically).
6. Enter sample volume into the **Volume** submenu. Press **Enter**.
7. Select **Run**.
8. Collect enriched naive B cell fraction at position B = negative fraction.

140-000-877.07  
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**Manual magnetic labeling**

- ▲ Work fast, keep cells cold, and use pre-cooled solutions (2–8 °C).
- ▲ Volumes for magnetic labeling given below are for up to 10<sup>7</sup> total cells. When working with fewer cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly.
- ▲ For optimal performance it is important to obtain a single-cell suspension before magnetic labeling.

1. Prepare cells and determine cell number.
2. Resuspend cell pellet in 40 µL of buffer per 10<sup>7</sup> total cells.
3. Add 10 µL of Biotin-Antibody Cocktail per 10<sup>7</sup> total cells.
4. Mix well and incubate for 5 minutes in the refrigerator (2–8 °C).
5. Add 30 µL of buffer per 10<sup>7</sup> total cells.
6. Add 20 µL of Anti-Biotin MicroBeads per 10<sup>7</sup> total cells.
7. Mix well and incubate for 10 minutes in the refrigerator (2–8 °C).
8. Proceed to subsequent magnetic cell separation.

▲ Note: A minimum of 500 µL is required for magnetic separation. If necessary, add buffer to the cell suspension.

**Subsequent manual cell separation**

- ▲ Always wait until the column reservoir is empty before proceeding to the next step.

9. Place column in the magnetic field of a suitable MACS Separator. For details refer to the respective MACS Column data sheet.
10. Prepare column by rinsing with the appropriate amount of buffer:
 

MS: 500 µL	LS: 3 mL
------------	----------
11. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells, representing the enriched naive B cells.
12. Wash column with the appropriate amount of buffer. Collect unlabeled cells that pass through and combine with the effluent from step 11.
 

MS: 3×500 µL	LS: 3×3 mL
--------------	------------
13. (Optional) Remove column from the separator and place it on a suitable collection tube. Pipette the appropriate amount of buffer onto the column. Immediately flush out the magnetically labeled non-B and non-naive B cells by firmly pushing the plunger into the column.
 

MS: 1 mL	LS: 5 mL
----------	----------

**Subsequent automated cell separation using the autoMACS® Pro Separator**

- ▲ Refer to the user manual for instructions on how to use the autoMACS® Pro Separator.

- ▲ All buffer temperatures should be ≥10 °C.

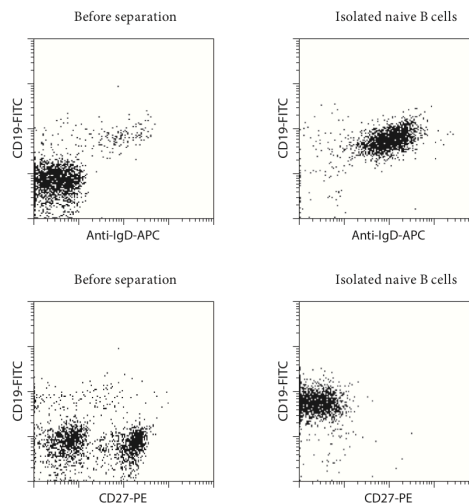
- ▲ Place tubes in the following Chill Rack positions:  
**position A** = sample, **position B** = negative fraction,  
**position C** = positive fraction.

9. Prepare and prime the instrument.

10. Follow the instructions that are given in the user manual.
11. The program “Depletes” is recommended. Collect enriched naive B cells at position B = negative fraction.

**Example of a separation using the Naive B Cell Isolation Kit II**

Isolation of untouched naive B cells from human PBMCs using the Naive B Cell Isolation Kit II and an LS Column. The cells are fluorescently stained with CD19-FITC, CD27-PE, and Anti-IgD-APC. Cell debris and dead cells were excluded from the analysis based on scatter signals and propidium iodide fluorescence.



For more information or assistance refer to our technical support.

Check out Miltenyi Biotec's flow cytometry solutions at [www.miltenyibiotec.com/MACSQuant](http://www.miltenyibiotec.com/MACSQuant) and explore the extensive antibody portfolio at [www.miltenyibiotec.com/antibodies](http://www.miltenyibiotec.com/antibodies).

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Supplementary 3.5



**Magnetic cell sorting**

**Index**

1. Description
  - 1.1 Principle of MACS® separation
  - 1.2 Background and product applications
  - 1.3 Reagent and instrument requirements
2. Protocol
  - 2.1 Sample preparation
  - 2.2 Magnetic labeling
  - 2.3 Magnetic separation
3. Example of a separation using CD43 MicroBeads
4. References

**1. Description**

**Components** 2 mL CD43 MicroBeads, human: MicroBeads conjugated to monoclonal anti-human CD43 antibodies (isotype: mouse IgG1).

**Size** For 10<sup>9</sup> total cells, up to 100 separations.

**Product format** CD43 MicroBeads are supplied as a suspension containing stabilizer and 0.05% sodium azide.

**Storage** Store protected from light at 4–8 °C. Do not freeze. The expiration date is indicated on the vial label.

**1.1 Principle of MACS® separation**

First the CD43<sup>+</sup> cells are magnetically labeled with CD43 MicroBeads. Then, the cell suspension is loaded onto a column which is placed in the magnetic field of a MACS® Separator. The magnetically labeled CD43<sup>+</sup> cells are retained on the column. The unlabeled cells run through and this cell fraction is depleted of CD43<sup>+</sup> cells. After removal of the column from the magnetic field, the magnetically retained CD43<sup>+</sup> cells can be eluted as the positively selected cell fraction.

**1.2 Background and product applications**

CD43 MicroBeads are developed for the isolation of untouched human B cells based on the magnetic depletion of cells that express the CD43 antigen. CD43 (leukosialin, sialophorin) is a cell surface glycoprotein that is suggested to be involved in adhesion, anti-adhesion, and signal transduction processes.<sup>1</sup> The CD43 antigen is expressed on most leukocytes, i.e. T cells, NK cells, granulocytes, monocyte and macrophages, hematopoietic stem cells and platelets but not on erythrocytes.<sup>2–4</sup> Among B cells, CD43 is expressed on activated B cells and plasma cells but not on resting B cells, e.g. naive B cells.<sup>2</sup> In bone marrow, CD43 is found on pro-B cells but is down regulated during transition to the pre-B cell stage.

**CD43 MicroBeads**  
human

Order No. 130-091-333

**Examples of applications**

- Positive selection or depletion of cells expressing the human CD43 antigen.
- Isolation of untouched resting B cells from peripheral blood mononuclear cells (PBMC), body fluids (e.g. bronchial lavage) or single-cell suspensions from tissue (e.g. lymphoid tissue).

**1.3 Reagent and instrument requirements**

- **Buffer (degassed):** Prepare a solution containing PBS (phosphate buffered saline) pH 7.2, 0.5% BSA and 2 mM EDTA by diluting MACS BSA Stock Solution (# 130-091-376) 1:20 with autoMACS® Rinsing Solution (# 130-091-222). Keep buffer cold (4–8 °C).
  - ▲ Note: EDTA can be replaced by other supplements such as anticoagulant citrate dextrose formula-A (ACD-A) or citrate phosphate dextrose (CPD). BSA can be replaced by other proteins such as human serum albumin, human serum or fetal calf serum. Buffers or media containing Ca<sup>2+</sup> or Mg<sup>2+</sup> are not recommended for use.
- **MACS Columns and MACS Separators:** Choose the appropriate MACS Separator and MACS Columns according to the number of labeled cells and to the number of total cells.

Column	max. number of labeled cells	max. number of total cells	Separator
MS	10 <sup>7</sup>	2×10 <sup>8</sup>	MiniMACS, OctoMACS, VarioMACS, SuperMACS
LS	10 <sup>8</sup>	2×10 <sup>9</sup>	MidiMACS, QuadroMACS, VarioMACS, SuperMACS
XS	10 <sup>9</sup>	2×10 <sup>10</sup>	SuperMACS
autoMACS	2×10 <sup>8</sup>	4×10 <sup>9</sup>	autoMACS

- ▲ Note: Column adapters are required to insert certain columns into VarioMACS or SuperMACS. For details, see MACS Separator data sheets.
- (Optional) Fluorochrome-conjugated antibodies against a B cell lineage marker (e.g. CD19-APC # 130-091-248, CD19-PE # 130-091-247, CD20-FITC # 130-091-108, or CD20-PE # 130-091-109), CD43 and CD235a (Glycophorin A).
- (Optional) PI (propidium iodide) or 7-AAD for flow cytometric exclusion of dead cells.
- (Optional) Pre-Separation Filter (# 130-041-407).

**2. Protocol**

**2.1 Sample preparation**

When working with anticoagulated peripheral blood or buffy coat, PBMC should be isolated by density gradient centrifugation (see "General Protocols" in the User Manuals or visit [www.miltenyibiotec.com](http://www.miltenyibiotec.com)).

- ▲ Note: Remove platelets after density gradient separation: resuspend cell pellet in buffer and centrifuge at 200×g for 10–15 minutes at 20 °C. Carefully remove supernatant. Repeat washing step and carefully remove supernatant.

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When working with tissues, prepare a single-cell suspension by a standard preparation method (see "General Protocols" in the User Manuals or visit [www.miltenyibiotec.com](http://www.miltenyibiotec.com)).

▲ **Note:** Dead cells may bind non-specifically to MACS MicroBeads. To remove dead cells, we recommend using density gradient centrifugation (e.g. Ficoll-Paque™) or the Dead Cell Removal Kit (# 130-090-101).



## 2.2 Magnetic labeling

▲ **Work fast, keep cells cold, and use pre-cooled solutions.** This will prevent capping of antibodies on the cell surface and non-specific cell labeling.

▲ **Volumes for magnetic labeling given below are for up to  $10^7$  total cells.** When working with fewer than  $10^7$  cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for  $2 \times 10^7$  total cells, use twice the volume of all indicated reagent volumes and total volumes).

▲ **For optimal performance it is important to obtain a single-cell suspension before magnetic separation.** Pass cells through 30  $\mu$ m nylon mesh (Pre-Separation Filter # 130-041-407) to remove cell clumps which may clog the column.

1. Determine cell number.
2. Centrifuge cell suspension at  $300 \times g$  for 10 minutes. Pipette off supernatant completely.
3. Resuspend cell pellet in 80  $\mu$ L of buffer per  $10^7$  total cells.
4. Add 20  $\mu$ L of CD43 MicroBeads per  $10^7$  total cells.
5. Mix well and incubate for 15 minutes at 4–8 °C.  
▲ **Note:** Working on ice may require increased incubation times. Higher temperatures and/or longer incubation times lead to non-specific cell labeling.
6. (Optional) Add staining antibodies, e.g. add 10  $\mu$ L of CD19-PE (# 130-091-247), and incubate for 5 minutes at 4–8 °C.
7. Wash cells by adding 1–2 mL of buffer per  $10^7$  cells and centrifuge at  $300 \times g$  for 10 minutes. Pipette off supernatant completely.
8. Resuspend up to  $10^8$  cells in 500  $\mu$ L of buffer.  
▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.
9. Proceed to magnetic separation (2.3).



## 2.3 Magnetic separation

▲ **Choose an appropriate MACS Column and MACS Separator** according to the number of total cells and the number of CD43<sup>+</sup> cells (see table in section 1.3).

### Magnetic separation with MS or LS Columns

1. Place column in the magnetic field of a suitable MACS Separator (see "Column data sheets").
2. Prepare column by rinsing with appropriate amount of buffer:  
MS: 500  $\mu$ L      LS: 3 mL.

3. Apply cell suspension onto the column.

4. Collect unlabeled cells which pass through and wash column with appropriate amount of buffer. Perform washing steps by adding buffer three times, each time once the column reservoir is empty.

MS: 3×500  $\mu$ L      LS: 3×3 mL.

Collect total effluent. This is the unlabeled cell fraction.

5. Remove column from the separator and place it on a suitable collection tube.
6. Pipette appropriate amount of buffer onto the column. Immediately flush out fraction with the magnetically labeled cells by firmly applying the plunger supplied with the column.

MS: 1 mL      LS: 5 mL.

▲ **Note:** To increase the purity of the magnetically labeled fraction, it can be passed over a second, freshly prepared column.

### Magnetic separation with XS Columns

For instructions on the column assembly and the separation, refer to the "XS Column data sheet".

### Depletion with LD Columns

1. Place LD Column in the magnetic field of a suitable MACS Separator (see "LD Column data sheet").
2. Prepare column by rinsing with 2 mL of buffer.
3. Apply cell suspension onto the column.
4. Collect unlabeled cells which pass through and wash column with  $2 \times 1$  mL of buffer. Collect total effluent. This is the unlabeled cell fraction.

### Depletion with CS Columns

1. Assemble CS Column and place it in the magnetic field of a suitable MACS Separator (see "CS Column data sheet").
2. Prepare column by filling and rinsing with 60 mL of buffer. Attach a 22G flow resistor to the 3-way-stopcock of the assembled column (see "CS Column data sheet").
3. Apply cell suspension onto the column.
4. Collect unlabeled cells which pass through and wash column with 30 mL buffer from the top. Collect total effluent. This is the unlabeled cell fraction.

### Depletion with D Columns

For instructions on column assembly and separation, refer to the "D Column data sheet".

### Magnetic separation with the autoMACS™ Separator

▲ **Refer to the "autoMACS™ User Manual" for instructions on how to use the autoMACS™ Separator.**

1. Prepare and prime autoMACS Separator.

- Place tube containing the magnetically labeled cells in the autoMACS Separator. For a standard separation, choose following separation programs:

Positive selection: "Possel"

Depletion: "Deplete"

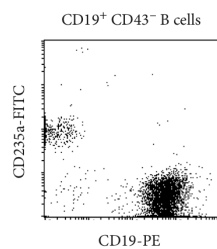
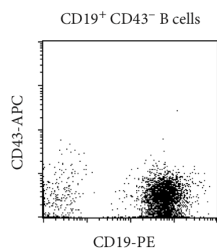
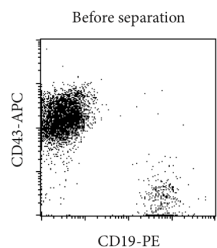
▲ Note: Program choice depends on the isolation strategy, the strength of magnetic labeling and the frequency of magnetically labeled cells. For details see autoMACS User Manual: "autoMACS Cell Separation Programs".

- When using the program "Possel", collect positive fraction (outlet port "pos1"). This is the purified CD43<sup>+</sup> cell fraction.

When using the program "Deplete", collect unlabeled fraction (outlet port "neg1"). This is the CD43<sup>-</sup> cell fraction containing the untouched resting B cells.

### 3. Example of a separation using CD43 MicroBeads

Separation of PBMC using CD43 MicroBeads and a MidiMACS<sup>™</sup> Separator with an LS Column. The cells are fluorescently stained with CD19-PE (# 130-091-247), CD43-APC and CD235a (Glycophorin A)-FITC. Cell debris and dead cells were excluded from the analysis based on scatter signals and PI fluorescence.



### 4. References

- Woodman *et al.* (1998) *J Exp Med.* 188: 2181-2186
- Barclay *et al.* eds. 1997. *The Leucocyte Antigen FactsBook.* Academic press. London
- Moore *et al.* (1994) *J. Immunol* 153: 4978-4987
- Remold-O'Donnell (1987) *Biochemistry.* 26: 3908-39134.

### Warnings

Reagents contain sodium azide. Under acidic conditions sodium azide yields hydrazoic acid, which is extremely toxic. Azide compounds should be diluted with running water before discarding. These precautions are recommended to avoid deposits in plumbing where explosive conditions may develop.

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This MACS<sup>™</sup> product is for *in vitro* research use only and not for diagnostic or therapeutic procedures.

## Supplementary 3.6



## CD19 MicroBeads

### human

Order no. 130-050-301

#### Contents

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  - 1.1 Principle of the MACS<sup>®</sup> Separation
  - 1.2 Background information
  - 1.3 Applications
  - 1.4 Reagent and instrument requirements
2. Protocol
  - 2.1 Sample preparation
  - 2.2 Magnetic labeling
  - 2.3 Magnetic separation
3. Example of a separation using the CD19 MicroBeads
4. References

#### 1. Description

<b>Components</b>	2 mL CD19 MicroBeads, human: MicroBeads conjugated to monoclonal anti-human CD19 antibodies (isotype: mouse IgG1).
<b>Capacity</b>	For 10 <sup>9</sup> total cells, up to 100 separations.
<b>Product format</b>	CD19 MicroBeads are supplied in buffer containing stabilizer and 0.05% sodium azide.
<b>Storage</b>	Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label.

##### 1.1 Principle of the MACS<sup>®</sup> Separation

First, the CD19<sup>+</sup> cells are magnetically labeled with CD19 MicroBeads. Then, the cell suspension is loaded onto a MACS<sup>®</sup> Column which is placed in the magnetic field of a MACS Separator. The magnetically labeled CD19<sup>+</sup> cells are retained within the column. The unlabeled cells run through; this cell fraction is thus depleted of CD19<sup>+</sup> cells. After removing the column from the magnetic field, the magnetically retained CD19<sup>+</sup> cells can be eluted as the positively selected cell fraction.

##### 1.2 Background information

CD19 MicroBeads have been developed for the separation of human B cells based on the expression of the CD19 antigen. CD19 is a 95–120 kDa glycosylated transmembrane protein that is critically involved in signal transduction processes that regulate development, activation, and differentiation of B lymphocytes. CD19 is expressed on lineage B cells from the early lineage-committed pro-B cell stage to the B cell blast stage including most malignant B cells. Expression is down-regulated during the differentiation into plasma cells. Furthermore, CD19 is expressed on follicular dendritic cells.

#### 1.3 Applications

- Positive selection or depletion of CD19<sup>+</sup> cells.
- Isolation or depletion of B cells from peripheral blood mononuclear cells (PBMCs), body fluids (e.g. bronchial lavage), or single-cell suspensions from tissues (e.g. lymphoid or tumor tissue).

#### 1.4 Reagent and instrument requirements

- Buffer: Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS BSA Stock Solution (# 130-091-376) 1:20 with autoMACS<sup>™</sup> Rinsing Solution (# 130-091-222). Keep buffer cold (2–8 °C). Degas buffer before use, as air bubbles could block the column.
  - ▲ Note: EDTA can be replaced by other supplements such as anticoagulant citrate dextrose formula-A (ACD-A) or citrate phosphate dextrose (CPD). BSA can be replaced by other proteins such as human serum albumin, human serum, or fetal bovine serum. Buffers or media containing Ca<sup>2+</sup> or Mg<sup>2+</sup> are not recommended for use.
- MACS Columns and MACS Separators: CD19<sup>+</sup> cells can be enriched by using MS, LS, or XS Columns or depleted with the use of LD, CS, or D Columns. Cells which strongly express the CD19 antigen can also be depleted using MS, LS, or XS Columns. Positive selection or depletion can also be performed by using the autoMACS or the autoMACS Pro Separator.

Column	Max. number of labeled cells	Max. number of total cells	Separator
<b>Positive selection</b>			
MS	10 <sup>7</sup>	2×10 <sup>8</sup>	MiniMACS, OctoMACS, VarioMACS, SuperMACS
LS	10 <sup>8</sup>	2×10 <sup>9</sup>	MidiMACS, QuadroMACS, VarioMACS, SuperMACS
XS	10 <sup>9</sup>	2×10 <sup>10</sup>	SuperMACS
<b>Depletion</b>			
LD	10 <sup>8</sup>	5×10 <sup>8</sup>	MidiMACS, QuadroMACS, VarioMACS, SuperMACS
CS	2×10 <sup>8</sup>		VarioMACS, SuperMACS
D	10 <sup>9</sup>		SuperMACS
<b>Positive selection or depletion</b>			
autoMACS	2×10 <sup>8</sup>	4×10 <sup>9</sup>	autoMACS, autoMACS Pro

▲ Note: Column adapters are required to insert certain columns into the VarioMACS<sup>™</sup> or SuperMACS<sup>™</sup> Separators. For details see the respective MACS Separator data sheet.

- (Optional) Fluorochrome-conjugated CD19 or CD20 antibodies for flow cytometric analysis, e.g., CD19-PE (# 130-091-247), CD19-APC (# 130-091-248), CD20-PE (# 130-091-109), or CD20-FITC (# 130-091-108). For more information about other fluorochrome-conjugates see [www.miltenyibiotec.com](http://www.miltenyibiotec.com).

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- (Optional) Propidium iodide (PI) or 7-AAD for flow cytometric exclusion of dead cells.
- (Optional) Dead Cell Removal Kit (# 130-090-101) for the depletion of dead cells.
- (Optional) Pre-Separation Filters (# 130-041-407) to remove cell clumps.

## 2. Protocol

### 2.1 Sample preparation

When working with anticoagulated peripheral blood or buffy coat, peripheral blood mononuclear cells (PBMCs) should be isolated by density gradient centrifugation, for example, using Ficoll-Paque™. For details see the General Protocols section of the respective separator user manual. The General Protocols are also available at [www.miltenyibiotec.com/protocols](http://www.miltenyibiotec.com/protocols).

▲ **Note:** To remove platelets after density gradient separation, resuspend cell pellet in buffer and centrifuge at 200×g for 10–15 minutes at 20 °C. Carefully aspirate supernatant. Repeat washing step.

When working with tissues or lysed blood, prepare a single-cell suspension using standard methods. For details see the General Protocols section of the respective separator user manual. The General Protocols are also available at [www.miltenyibiotec.com/protocols](http://www.miltenyibiotec.com/protocols).

▲ Dead cells may bind non-specifically to MACS MicroBeads. To remove dead cells, we recommend using density gradient centrifugation or the Dead Cell Removal Kit (# 130-090-101).



### 2.2 Magnetic labeling

▲ Work fast, keep cells cold, and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and non-specific cell labeling.

▲ Volumes for magnetic labeling given below are for up to 10<sup>7</sup> total cells. When working with fewer than 10<sup>7</sup> cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for 2×10<sup>7</sup> total cells, use twice the volume of all indicated reagent volumes and total volumes).

▲ For optimal performance it is important to obtain a single-cell suspension before magnetic separation. Pass cells through 30 μm nylon mesh (Pre-Separation Filters, # 130-041-407) to remove cell clumps which may clog the column. Wet filter with buffer before use.

▲ Working on ice may require increased incubation times. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling.

1. Determine cell number.
2. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend cell pellet in 80 μL of buffer per 10<sup>7</sup> total cells.
4. Add 20 μL of CD19 MicroBeads per 10<sup>7</sup> total cells.
5. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).

6. (Optional) Add staining antibodies, e.g., 10 μL of CD19-PE (# 130-091-247), and incubate for 5 minutes in the dark in the refrigerator (2–8 °C).
7. Wash cells by adding 1–2 mL of buffer per 10<sup>7</sup> cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
8. Resuspend up to 10<sup>8</sup> cells in 500 μL of buffer.
  - ▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.
  - ▲ **Note:** For depletion with LD Columns, resuspend up to 1.25×10<sup>8</sup> cells in 500 μL of buffer.
9. Proceed to magnetic separation (2.3).



### 2.3 Magnetic separation

▲ Choose an appropriate MACS Column and MACS Separator according to the number of total cells and the number of CD19<sup>+</sup> cells. For details see table in section 1.4.

#### Magnetic separation with MS or LS Columns

1. Place column in the magnetic field of a suitable MACS Separator. For details see the respective MACS Column data sheet.
2. Prepare column by rinsing with the appropriate amount of buffer:
 

MS: 500 μL	LS: 3 mL
------------	----------
3. Apply cell suspension onto the column.
4. Collect unlabeled cells that pass through and wash column with the appropriate amount of buffer. Collect total effluent; this is the unlabeled cell fraction. Perform washing steps by adding buffer three times. Only add new buffer when the column reservoir is empty.
 

MS: 3×500 μL	LS: 3×3 mL
--------------	------------
5. Remove column from the separator and place it on a suitable collection tube.
6. Pipette the appropriate amount of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
 

MS: 1 mL	LS: 5 mL
----------	----------
7. (Optional) To increase the purity of CD19<sup>+</sup> cells, the eluted fraction can be enriched over a second MS or LS Column. Repeat the magnetic separation procedure as described in steps 1 to 6 by using a new column.

#### Magnetic separation with XS Columns

For instructions on the column assembly and the separation refer to the XS Column data sheet.

#### Depletion with LD Columns

1. Place LD Column in the magnetic field of a suitable MACS Separator. For details see LD Column data sheet.
2. Prepare column by rinsing with 2 mL of buffer.
3. Apply cell suspension onto the column.

4. Collect unlabeled cells that pass through and wash column with 2x1 mL of buffer. Collect total effluent; this is the unlabeled cell fraction. Perform washing steps by adding buffer two times. Only add new buffer when the column reservoir is empty.

**Depletion with CS Columns**

1. Assemble CS Column and place it in the magnetic field of a suitable MACS Separator. For details see CS Column data sheet.
2. Prepare column by filling and rinsing with 60 mL of buffer. Attach a 22G flow resistor to the 3-way stopcock of the assembled column. For details see CS Column data sheet.
3. Apply cell suspension onto the column.
4. Collect unlabeled cells that pass through and wash column with 30 mL buffer from the top. Collect total effluent; this is the unlabeled cell fraction.

**Depletion with D Columns**

For instructions on column assembly and separation refer to the D Column data sheet.

**Magnetic separation with the autoMACS™ Separator or the autoMACS™ Pro Separator**

- ▲ Refer to the respective user manual for instructions on how to use the autoMACS™ Separator or the autoMACS Pro Separator.
- ▲ Buffers used for operating the autoMACS Separator or the autoMACS Pro Separator should have a temperature of ≥ 10 °C.
- ▲ Program choice depends on the isolation strategy, the strength of magnetic labeling, and the frequency of magnetically labeled cells. For details refer to the section describing the cell separation programs in the respective user manual. Program recommendations below refer to separation of human PBMCs.

**Magnetic separation with the autoMACS™ Separator**

1. Prepare and prime the instrument.
2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube at the uptake port and the fraction collection tubes at port neg1 and port pos1.
3. For a standard separation choose one of the following programs:
  - Positive selection: "Possel"  
Collect positive fraction from outlet port pos1.
  - Depletion: "Depletes"  
Collect negative fraction from outlet port neg1.

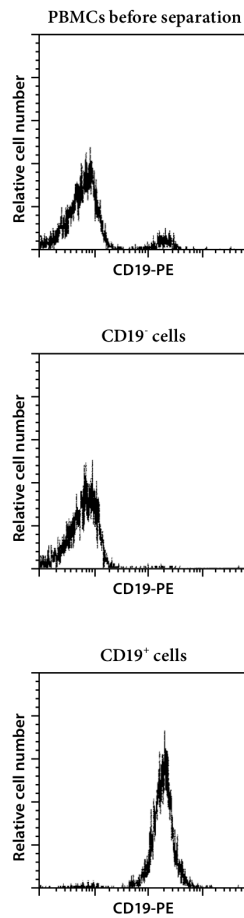
**Magnetic separation with the autoMACS™ Pro Separator**

1. Prepare and prime the instrument.
2. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube in row A of the tube rack and fraction collection tubes in rows B and C.

3. For a standard separation choose one of the following programs:
  - Positive selection: "Possel"  
Collect positive fraction in row C of the tube rack.
  - Depletion: "Depletes"  
Collect negative fraction in row B of the tube rack.

**3. Example of a separation using the CD19 MicroBeads**

CD19<sup>+</sup> cells were isolated from human PBMCs using CD19 MicroBeads, an MS Column, and a MiniMACS™ Separator. Cells are fluorescently stained with CD19-PE (# 130-091-247). Cell debris and dead cells are excluded from the analysis based on scatter signals and PI fluorescence.



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## Supplementary 3.7



## Pan T Cell Isolation Kit human

Order no. 130-096-535

<b>Components</b>	<p><b>1 mL Pan T Cell Biotin-Antibody Cocktail, human:</b> Cocktail of biotin-conjugated monoclonal antibodies against CD14, CD15, CD16, CD19, CD34, CD36, CD56, CD123, and CD235a (GlycophorinA).</p> <p><b>2 mL Pan T Cell MicroBead Cocktail, human:</b> MicroBeads conjugated to monoclonal anti-biotin antibody (isotype: mouse IgG1) and monoclonal anti-CD61 antibody (isotype: mouse IgG1).</p>
<b>Capacity</b>	For 10 <sup>9</sup> total cells.
<b>Product format</b>	All components are supplied in buffer containing stabilizer and 0.05% sodium azide.
<b>Storage</b>	Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial labels.

**Safety information**

For research use only. Not intended for any animal or human therapeutic or diagnostic use.

Before use, please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

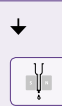
**Cell separation protocols**

Fully automated cell labeling and separation using the autoMACS<sup>®</sup> Pro Separator

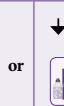
Alternatively:



Manual magnetic labeling



Subsequent manual separation

Subsequent automated cell separation using the autoMACS<sup>®</sup> Pro Separator**General notes**

▲ For tips concerning sample preparation, magnetic labeling and separation, visit [www.miltenyibiotec.com/faq](http://www.miltenyibiotec.com/faq) and [www.miltenyibiotec.com/protocols](http://www.miltenyibiotec.com/protocols).

▲ For product-specific background information and applications of this product, refer to the respective product page at [www.miltenyibiotec.com/130-096-535](http://www.miltenyibiotec.com/130-096-535).

**Reagent and instrument requirements**

- Buffer: Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS<sup>®</sup> BSA Stock Solution (# 130-091-376) 1:20 with autoMACS<sup>®</sup> Rinsing Solution (# 130-091-222). Degas buffer before use, as air bubbles could block the column.
- (Optional) Pre-Separation Filters (30 µm) (# 130-041-407) to remove cell clumps.
- Choose the appropriate MACS Separator and MACS Columns:

Column	Max. number of labeled cells	Max. number of total cells	Separator
LS	10 <sup>8</sup>	2×10 <sup>9</sup>	MidiMACS, QuadroMACS
autoMACS	2×10 <sup>8</sup>	4×10 <sup>9</sup>	autoMACS Pro

▲ Note: When using this kit the unwanted cell fraction is labeled and the target cells remain unlabeled. Depending on the target cell frequency, the labeled fraction can therefore represent the majority of the total cells. To avoid blocking of the column, do not exceed the max. number of labeled cells per column. Estimate the number of labeled cells in the sample, split the sample if necessary and use the appropriate number of separation columns.



Fully automated cell labeling and separation using the autoMACS<sup>®</sup> Pro Separator

▲ Refer to the user manual for instructions on how to use the autoMACS<sup>®</sup> Pro Separator.

▲ All buffer temperatures should be ≥10 °C.

▲ Place tubes in the following Chill Rack positions:  
**position A** = sample, **position B** = negative fraction,  
**position C** = positive fraction.

1. For appropriate resuspension volumes and cell concentrations, please visit [www.automacspro.com/autolabeling](http://www.automacspro.com/autolabeling).
2. Switch on the instrument for automatic initialization.
3. Go to the **Reagent** menu and select **Read Reagent**. Scan the 2D barcode of each reagent vial with the barcode scanner on the autoMACS Pro Separator. Place the reagent into the appropriate position on the reagent rack.
4. Place sample and collection tubes into the Chill Rack.
5. Go to the **Separation** menu and select the reagent name for each sample from the **Labeling** submenu (the correct labeling, separation, and wash protocols will be selected automatically).
6. Enter sample volume into the **Volume** submenu. Press **Enter**.
7. Select **Run**.
8. Collect enriched T cell fraction at position B = negative fraction.

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**Manual magnetic labeling**

- ▲ Work fast, keep cells cold, and use pre-cooled solutions (2–8 °C).
- ▲ Volumes for magnetic labeling given below are for up to 10<sup>7</sup> total cells. When working with fewer cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly.
- ▲ For optimal performance it is important to obtain a single-cell suspension before magnetic labeling.

1. Prepare cells and determine cell number.
2. Resuspend cell pellet in 40 µL of buffer per 10<sup>7</sup> total cells.
3. Add 10 µL of Pan T Cell Biotin-Antibody Cocktail per 10<sup>7</sup> total cells.
4. Mix well and incubate for 5 minutes in the refrigerator (2–8 °C).
5. Add 30 µL of buffer per 10<sup>7</sup> total cells.
6. Add 20 µL of Pan T Cell MicroBead Cocktail per 10<sup>7</sup> total cells.
7. Mix well and incubate for 10 minutes in the refrigerator (2–8 °C).
8. Proceed to subsequent magnetic cell separation.

▲ **Note:** A minimum of 500 µL is required for magnetic separation. If necessary, add buffer to the cell suspension.

**Subsequent manual cell separation**

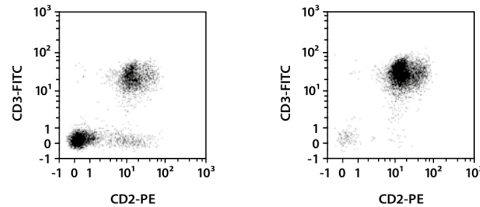
- ▲ Always wait until the column reservoir is empty before proceeding to the next step.
9. Place LS Column in the magnetic field of a suitable MACS Separator. For details refer to the respective MACS Column data sheet.
  10. Prepare column by rinsing with 3 mL of buffer.
  11. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells, representing the enriched T cells.
  12. Wash column with 3 mL of buffer. Collect unlabeled cells that pass through, representing the enriched T cells, and combine with the effluent from step 11.
  13. (Optional) Remove column from the separator and place it on a suitable collection tube. Pipette 5 mL of buffer onto the column. Immediately flush out the magnetically labeled non-T cells by firmly pushing the plunger into the column.

**Subsequent automated cell separation using the autoMACS® Pro Separator**

- ▲ Refer to the user manual for instructions on how to use the autoMACS® Pro Separator.
  - ▲ All buffer temperatures should be ≥10 °C.
  - ▲ Place tubes in the following Chill Rack positions:  
**position A** = sample, **position B** = negative fraction,  
**position C** = positive fraction.
9. Prepare and prime the instrument.
  10. Follow the instructions that are given in the user manual.
  11. The program “Depletes” is recommended. Collect enriched T cells at position B = negative fraction.

**Example of a separation using the Pan T Cell Isolation Kit**

Untouched T cells were isolated from human PBMCs by using the Pan T Cell Isolation Kit, an LS Column, and a MidiMACS™ Separator. The cells were fluorescently stained with CD3-FITC and CD2-PE and analyzed by flow cytometry using the MACSQuant™ Analyzer. Cell debris and dead cells were excluded from the analysis based on scatter signals and propidium iodide fluorescence.



For more information or assistance refer to our technical support.

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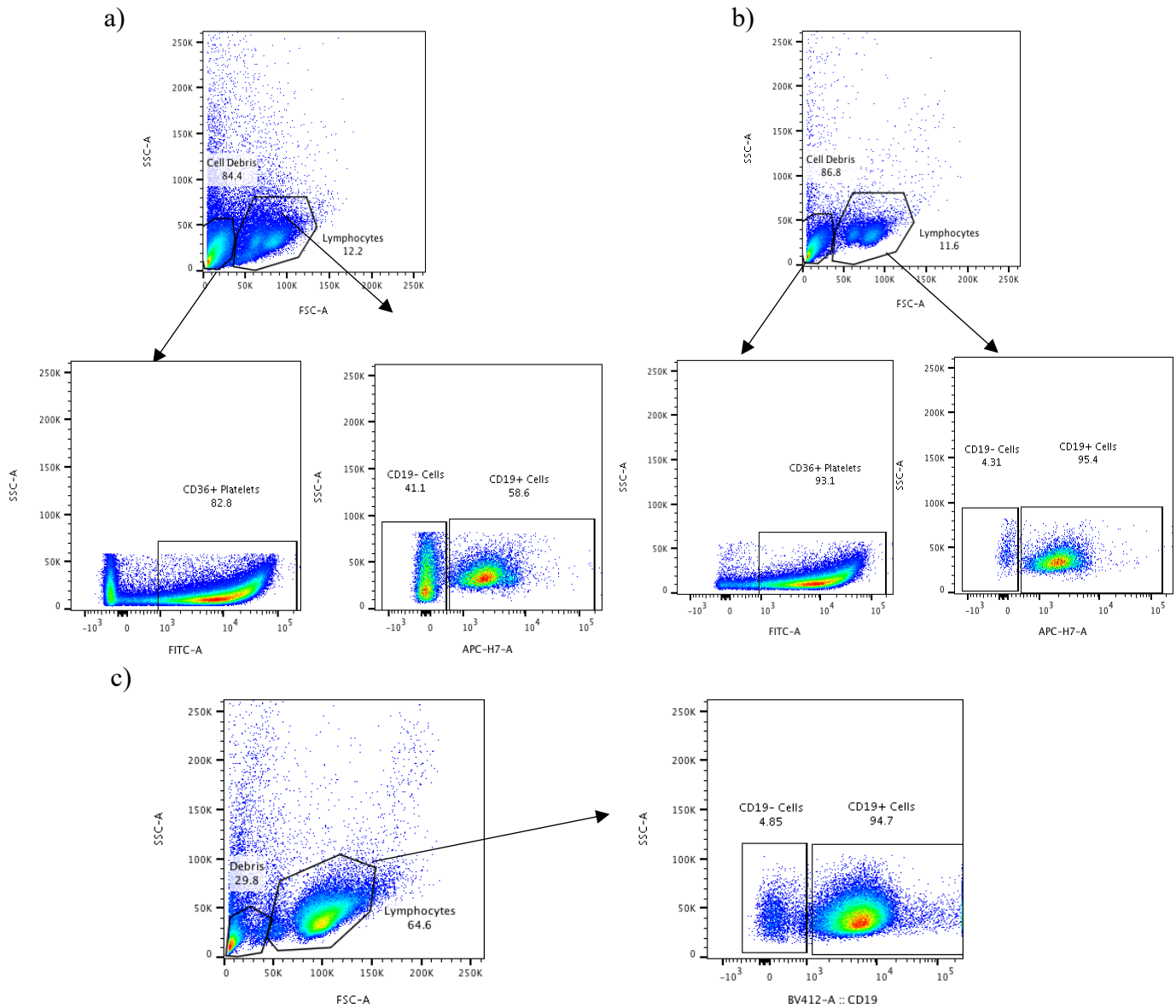
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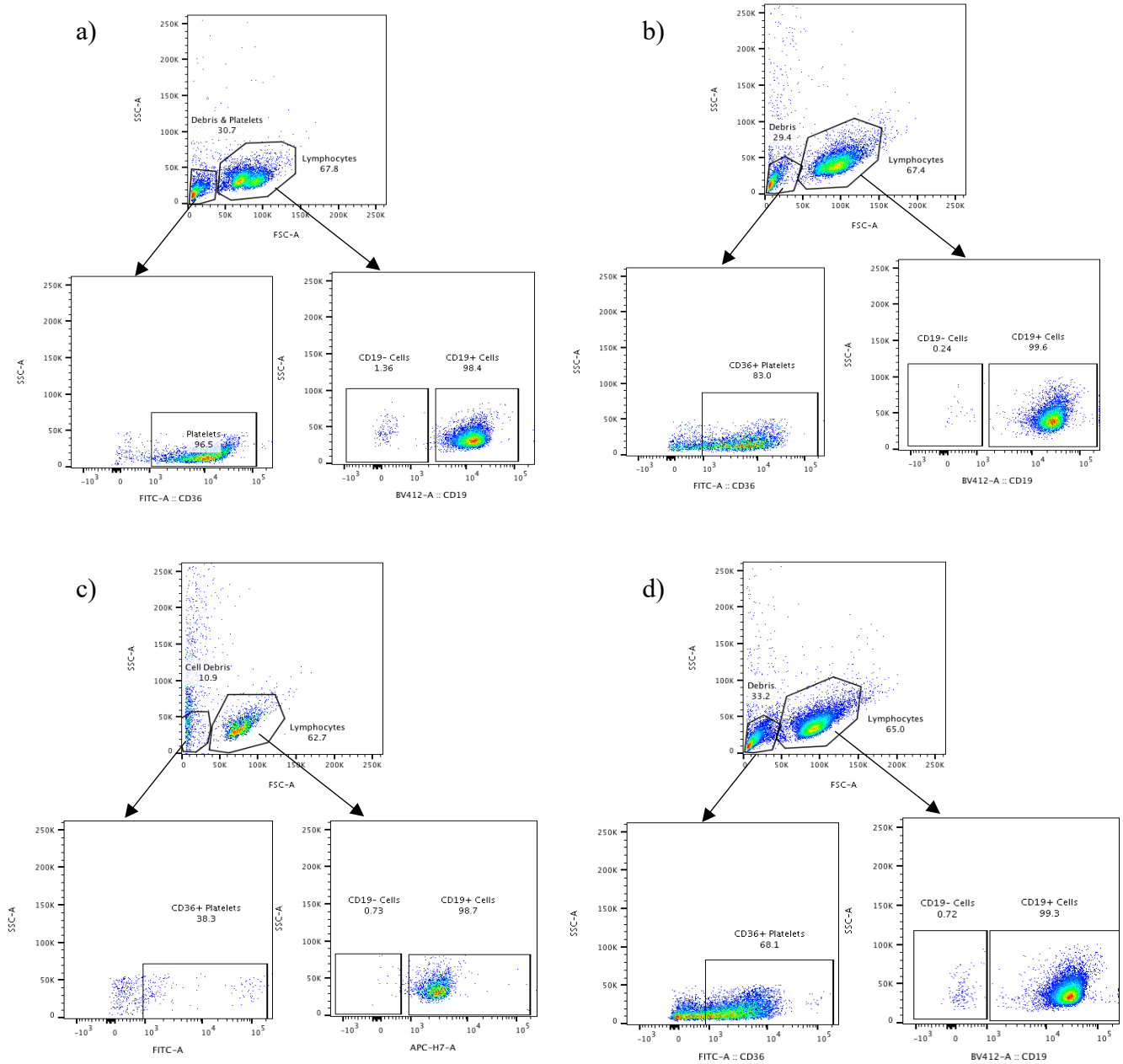
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## Supplementary Data

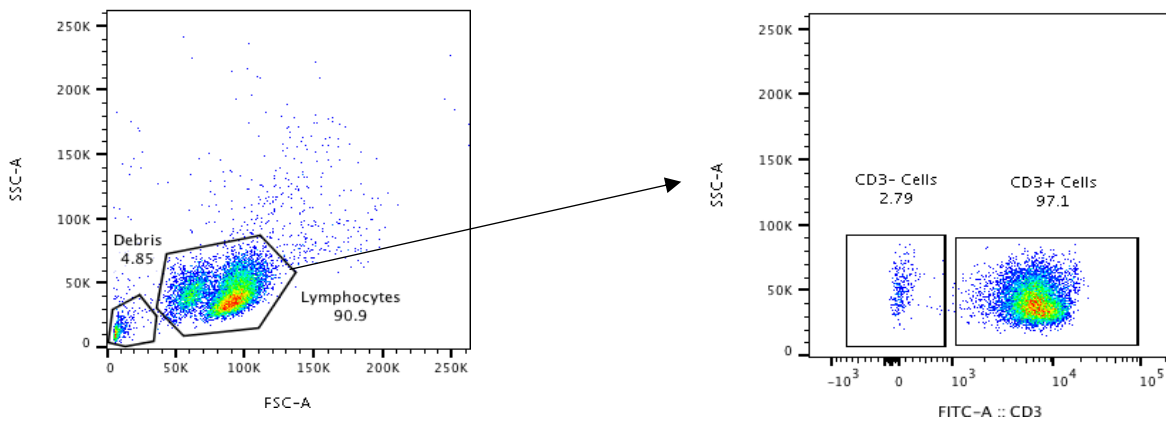


**Supl Figure 3.1. Flow cytometric analysis of isolated B-cell sample purity obtained using the naive MACS isolation kits. (a) CD43 microbeads kit (n = 1) (b) Naïve B-cell Isolation kit (n = 1) (c) CD19 positive Isolation kit (n = 3). These sample were not stained with CD36 mAb and thus the proportion of platelets that make up the 'Debris' population cannot be confirmed.**

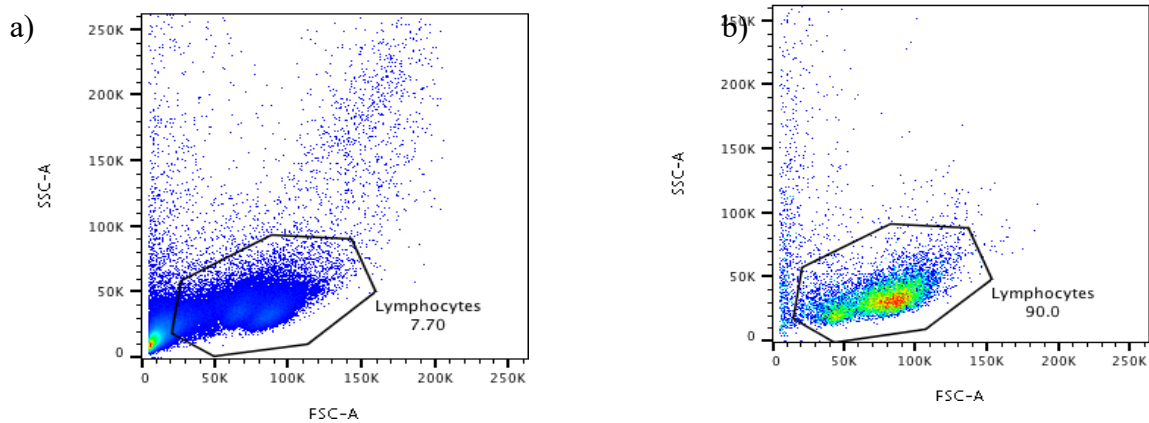




**Suppl Figure 3.2. Flow cytometric analysis of isolated B-cell sample purity obtained using the MACS B-cell isolation kit II.** (a) Using the normal PBMC method (n = 16) (b) Using the altered platelet wash method (n= 4) (c) Addition of CD61 platelet removal kit (n = 3) (d) Addition of dead cell removal kit (n = 3).



**Suppl Figure 3.3. Flow cytometric analysis of isolated T cell sample purity obtained using the MACS Pan T cell isolation kit.** Sample purity following negative MACS bead isolation in which platelet/cell debris was successfully removed, as shown in SSC-A vs FSC-A, and a pure T cell population obtained, as shown by CD3+ cells (n = 11).



**Suppl Figure 3.4. Flow cytometric analysis of isolated B-cell sample purity obtained using the MACS B-cell isolation kit II, followed by cell sorting based on FSC and SSC.** (a) Sample purity of MACS bead isolated B-cell sample (b) Sample purity of MACS bead isolated B-cell sample followed by two cell sorting steps, resulting in successfully removal of undesirable platelet contamination (n=2).

## **Chapter 4:**

The work presented in this chapter has been formatted in the style of a manuscript for submission to an immunology journal.

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Participant recruitment, sample collection, processing, acquisition and data analysis of results within BCG pilot study was performed by Ilana van Rensburg during her MSc (2015-2016).

**Modulation of T-cell populations and cytokine profiles by antigen experienced B-cells during latent *Mycobacterium tuberculosis* infection**

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**Keywords:** Regulatory B-cells, tuberculosis, immune modulation, co-culture, polarisation

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**Abstract**

**Rationale:** Emerging evidence implicate B-cells as important players in the defense against *Mycobacterium tuberculosis* (*M.tb*). This study aimed to identify potential mechanisms by which B-cells modulate T-cell function, the cells regarded as the principle immune cells involved in eradicating TB disease.

**Method:** Autologous T-cells were co-cultured with H37Rv/BCG-stimulated or naïve B-cells that had been pulsed with or without CD40L and IL5, to induce a regulatory killer B-cell subtype. The resulting B- and T-cell phenotypic frequencies and cytokine profiles were evaluated by multiparameter analysis.

**Results:** Killer (FasL<sup>+</sup>) B-cell frequencies were elevated in *M.tb* healthy, QuantiFERON (QFN) negative individuals when compared to healthy QFN positive individuals. Elevated levels of Fas-expressing T-cells was observed in QFN positive individuals compared to QFN negative individuals following co-culture of autologous T-cells with H37Rv-stimulated B-cells. Additionally, B-cells, naïve or pre-stimulated, were observed to induce alterations in effector T-cell (TE) and T<sub>reg</sub> frequencies. Wherein, a decrease in TE and T<sub>reg</sub> frequencies for QFN positive and negative participants was observed following co-culture of T-cells with pre-stimulated B-cells. Interestingly, an increase in TE frequencies of QFN negative individuals was observed following co-culture with H37Rv-stimulated B-cells compared to naïve B-cells. Similarly, enhanced T<sub>reg</sub> frequencies were observed, for QFN positive and negative, following co-culture with H37Rv-stimulated B-cells compared to naïve B-cells. Finally, alterations in cytokine production by CD4<sup>+</sup> and CD8<sup>+</sup> T-cells was observed, such as the induction of bi-functional and multifunctional T-cells, of both QFN positive and negative individuals following co-culture with unstimulated or H37Rv/BCG stimulated B-cells.

**Conclusion:** Naïve B-cells, as well as killer B-cells, are able to influence T-cell behavior by modulating phenotype development and cytokine secretion, emphasizing the potential important involvement of B-cells in initiating and guiding the immune response against *M.tb*.

## 1. Introduction

Tuberculosis (TB) remains the leading cause of death from a single infectious agent worldwide, with approximately 10.4 million active TB cases, resulting in an estimated 1.67 million TB deaths in 2016 (World Health Organization, 2017). The etiological agent of TB disease is *Mycobacterium tuberculosis* (*M.tb*) (Delogu et al., 2013), an air-borne pathogen easily spread in over-populated areas amid poor living conditions - where lack of hygiene and poor-ventilation contribute to the unsuccessful eradication of this bacillus (Schwander and Dheda, 2011; World Health Organization, 2017). Currently, the only vaccine available for TB prevention is the Bacillus Calmette-Guérin (BCG) vaccine, which has been shown to have variable efficacy (Colditz et al., 1994; Romano and Huygen, 2012; Sepulveda et al., 1992), offering reasonable protection against disseminated TB in infants, while providing minimal protection against the primary form of the disease, pulmonary TB, which predominantly affects adolescents and adults (Kaufmann and Gengenbacher, 2012; Li et al., 2017). Partial understanding of the complex interplay between immune cells and the cellular responses required for successful control and eradication of *M.tb* infection, has resulted in poor vaccine design (Andersen, 2001). Consequently, novel vaccine candidates fail to elicit an immune response superior or equivalent to that of BCG, and are unsuccessful in improving TB prevention therapies (Andersen and Kaufmann, 2014; Baldwin et al., 2009; Gupta et al., 2007; Kaufmann, 2006, 2000; Williams et al., 2005). This has resulted in poor development and progression of innovative therapeutics against TB disease (Cliff et al., 2015; Ottenhoff and Kaufmann, 2012). As such, a re-evaluation of the importance of other cell types previously thought to have minor influence on the effectiveness of anti-TB response has followed (Achkar and Casadevall, 2013; Balu et al., 2011).

Increasing evidence suggests that B-cells and immunoglobulins play a significant role in modulating the immune response to *M.tb* (Chan et al., 2014; Li et al., 2017). For decades, it was assumed that B-cells had minimal influence on the survival of *M.tb* due to the fact that antibody production was considered to be the primary function of B-cells. Multiple studies involving several other intracellular pathogens have shown great promise with antibody-mediated immunity (Achkar and Casadevall, 2013; Balu et al., 2011; Buccheri et al., 2009; Chan et al., 2014; Hamasur et al., 2004; Teitelbaum et al., 1998), underscoring the possibility that protective humoral immune responses may exist for TB. In addition, recent findings have revealed non-humoral functions of B-cells, including immune modulation and cytokine production, which contribute to the successful suppression of *M.tb* infection (Bao and Cao, 2014; Bénard et al., 2018; Blair et al., 2010; Carter et al., 2012, 2011; Chesneau et al., 2013; Du Plessis et al., 2016b; Elgueta et al., 2009; Kurt-Jones et al., 1988a; León et al., 2014; Matsumoto et al., 2014; Nova-Lamperti et al., 2016; Schwartz et al., 2016; Whitmire et al., 2009; Yoshizaki et al., 2012). Thus far, it is unclear whether or not B-cell derived antibodies directly target mycobacterial antigens or if they stimulate a general immunomodulatory effect to target the inflammatory response to TB disease, resulting in protective immune function (Li et al., 2017). Furthermore, mouse models have shown that mice with dysfunctional

B-cells, or depleted B-cells, tend to have increased bacterial burdens (Maglione et al., 2007), illustrating a key role of B-cells in immunity. As such, deeper insight into the function of B-cells and their role in inducing protective immune responses against TB disease is required.

T-cells, together with macrophages, are regarded as the central immune cells responsible for TB disease management (Cliff et al., 2015; Kaufmann, 2002; Lalvani et al., 1998; McKenzie et al., 1993; Orme et al., 1993; Ribeiro-Rodrigues et al., 2006; Schwander and Dheda, 2011; Scriba et al., 2016; Sharma et al., 2007; Stevens et al., 1988). The modulation of T cell function is largely associated with TB disease outcome. Thus, the objective of this study was to elucidate the influence B-cells have on T-cell polarisation, phenotype development and function - in the context of *M.tb* infection. This was achieved by co-culture of isolated T-cells with autologous *M.tb*-stimulated B-cells and comparing the resulting T-cell population to determine whether any variations occurred. A subpopulation of B-cells, known as killer B-cells was recently discovered (Lundy, 2009; Lundy et al., 2015; van Rensburg et al., 2017; Van Rensburg et al., 2017; van Rensburg and Loxton, 2018a). The frequency and function of these killer B-cells during TB disease has been investigated, in which decreased levels of killer B-cells were observed in TB-infected individuals compared to healthy controls and successfully treated TB patients (Van Rensburg et al., 2017), suggesting an essential role of killer B-cells in effective anti-TB immunity. In light of the above, changes in T-cell activation and function following killer B-cell induction were investigated, to afford deeper insight into possible mechanisms by which these cells exert their therapeutic effects.

## 2. Methods

### 2.1 Ethics Statement

*The current study consists of two parts: BCG Pilot study and H37Rv Pilot study*

Ethical approval was obtained from the health research ethics committee of Stellenbosch University for the BCG pilot (N10/01/013) and H37Rv pilot (N16/05/070) and from the City of Cape Town City Health. The study was conducted according to the Helsinki Declaration and International Conference of Harmonisation guidelines. Written informed consent was obtained from all study participants.

### 2.2 Study Participants

For both studies, 10 healthy participants were recruited (5 individuals with a positive QuantiFERON (QFN) status and 5 individuals with a QFN negative status) – BCG pilot in the year 2016 and H37Rv pilot during 2017. The QFN status was suggestive of exposure to *M.tb*, and participants with a positive status were considered latently infected. Recruited participants did not present any clinical symptoms of TB, with no previous record of active disease. All participants for this study were HIV negative. QFN positive and negative participants were matched according to socio-economic background. The studies were performed in Cape Town, South Africa.

### 2.3 B-cell Isolation

Heparinized peripheral blood (80ml) was collected and peripheral blood mononuclear cells (PBMCs) isolated using the ficoll-histopaque (GE Healthcare Life Sciences, USA) density gradient method. Subsequently, B-cells were negatively isolated from PBMCs using MACS bead technology, according to the manufacturer's instructions, with the B-cell isolation kit II (Miltenyi Biotec, South Africa). Enriched B-cells were then cryopreserved (90% Fetal Calf Serum (FCS), Lonza, South Africa and 10% Dimethyl sulfoxide (DMSO), Sigma-Aldrich, St, Louis, MO) and stored in liquid nitrogen for future use. Purity of the enriched B-cell samples was confirmed by flow cytometry using anti-human CD19 mAb. All samples had a gated B-cell purity (% CD19<sup>+</sup> cells within lymphocyte population – exclude debris population portion of sample) above 90%.

### 2.4 T-cell Isolation

On a separate occasion, heparinized peripheral blood (20ml) was collected to obtain autologous T-cells for co-culture. PBMCs were isolated as described above, and T-cells subsequently isolated from PBMCs using MACS bead technology with the Pan T-cell isolation kit (Miltenyi Biotec, South Africa). Enriched T-cells were cryopreserved (90% FCS and 10% DMSO) and stored in liquid nitrogen for future use. Purity of the enriched T-cell samples was confirmed by flow cytometry using anti-human CD3 mAb. All samples had a T-cell purity (% CD3<sup>+</sup> cells within sample – including lymphocyte and debris population) above 90%.



## 2.5 Co-culture Stimulation

Isolated B-cells (250 000 cells/well) were cultured in 96-well round bottom plates, in a total of 200uL complete media (RPMI plus L/Glutamine (Sigma, USA) supplemented with 10% FCS) at 37°C and 5% CO<sub>2</sub>. B-cells were stimulated with BCG Danish 1331 (1.25 x 10<sup>6</sup> CFU/mL), or H37Rv at a MOI of 10:1, for 3 hours. Where applicable, IL-5 (Sigma, USA) was added at 50 ng/ml and CD40L (Sigma, USA) at 2ug/ml (for BCG pilot study) or 1ug/ml (for H37Rv pilot study). A detailed description of the stimulatory conditions can be found in Suppl Table 4.1.

### *BCG Pilot study*

Following the above mentioned 3-hour incubation, culture supernatants were removed, and isolated T cells added (500 000 cells/well) to the respective wells containing activated B-cells (2:1 ratio). These cells were co-cultured in the presence of brefeldin A (Sigma, USA) at 5ug/mL, immediately, for 20 hours. Following stimulation, culture supernatant was removed, and the cells cryopreserved (90% FCS and 10% DMSO) for phenotypic receptor analysis by flow cytometry at a later time.

### *H37Rv Pilot study*

Following the 3-hour incubation, culture supernatants were harvested, filtered and stored at -80°C for “early” B-cell cytokine immune response evaluation (pre-co-culture = time point 1). Isolated T cells were added (500 000 cells/well) to the respective wells containing activated B-cells (2:1 ratio). These cells were co-cultured for 3 hours prior to the addition of brefeldin A (Sigma, USA) at 5ug/mL followed by incubation for an additional 16 hours’ stimulation. Following this 19-hour incubation, culture supernatants were harvested, passed through a filter of 0.22µm (to remove any bacilli that may be contained within the sample) and stored at -80°C for analysis of ‘late’ B-cell cytokine immune response (post-co-culture = time point 2). Cells were fixed in fixation buffer (Biolegend, California) for 30 min at 37°C and immediately stained for phenotypic receptor analysis by flow cytometry.

## 2.6 Cytokine Analysis by Luminex

### *H37Rv Pilot study*

Quantification of soluble factors within cell culture supernatant, was determined using the MAGPix and Bioplex platforms (Bio-rad Laboratories, California, USA). The analytes of interest included APRIL, BAFF, FasL, ICAM-1, IFN-β, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A, IL-21, TNF-α, TGF-β1, TGF-β2 and TGF-β3, assayed according to the manufacturer’s recommendations.

## 2.7 Phenotype Analysis by Flow Cytometry

For culture conditions to which no T cells were added, a B-cell specific antibody panel was used, whereas culture conditions in which T cells were present, a T cell specific antibody panel was used. For the purpose of intracellular staining, all cells were cultured with Brefeldin A as previously described. The gating strategies used for each of the panels can be found in Suppl Figures 4.1 to 4.5.

*BCG Pilot study*

The T-cell antibody panel consisted of CD3-FITC, CD4-HV500, CD8-APC-Cy7, CD25-PE, IFN- $\gamma$ - PE/Cy7, TNF- $\alpha$ - APC and IL-2-PerCP-Cy5.5 (All from Beckton Dickenson (BD), Germany). Cells were stained in 1x permeabilization buffer (Biolegend, California) for 30min at room temperature in the dark after, which the cells were washed and acquired on a BD LSR II (BD Biosciences). The resulting data was analyzed using FlowJo v10 software (Treestar, USA).

*H37Rv Pilot study*

The B-cell antibody panel consisted of IgM-FITC, CD24-BV510, CD38-PE/Cy7, CD5-PerCP/Cy5.5, CD125 (IL5RA)-PE (All from BD, Germany), CD19-BV605, IL-10-BV421 (All from Biolegend, California) and CD178 (FASL)-APC (Miltenyi Biotec, South Africa). The T-cell panel consisted of CD3-PerCP, CD95-APC/Fire750, IL-2-BV421 (All from Biolegend, California), CD4-BV510, CD8-FITC, CD25-PE, IFN- $\gamma$ -PE/Cy7 and TNF- $\alpha$ - APC and (All from BD, Germany). Cells were stained in 1x permeabilization buffer (Biolegend, California) for 1 hour at room temperature in the dark with the respective antibody panels, after which the cells were washed, individual participant samples pooled (according to QFN status and stimulation condition) and acquired on a BD LSR II (BD Biosciences). The resulting data was analyzed using FlowJo v10 software (Treestar, USA).

**2.8 Statistical Analysis***BCG Pilot study*

The exported frequencies of the flow cytometry plots analyzed using FlowJo V10 (Treestar, USA), were analyzed using Prism 7 Software (San Diego, CA). Comparisons between groups was calculated using the Mann Whitney U-test, and differences within groups was calculated using the Kruskal-Wallis with Dunns multiple comparisons post-hoc test. Statistical significance is indicated by an asterisk, in which the  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*).

*H37Rv Pilot study*

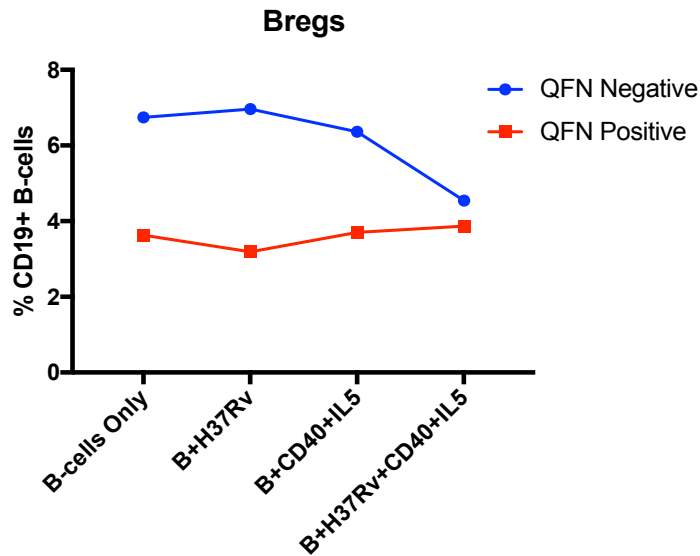
Data analysis for all Luminex results was performed using Statistica 12 software (Statsoft, Ohio, USA) and Prism 7 Software (San Diego, CA). Raw data was checked for normality using normality plots. Statistical differences between culture conditions was calculated using a three-way or four-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistical significance is indicated by an asterisk, in which the  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*) or by letters, in which groups denoted with different letters indicate statistical differences. Data analysis of the flow cytometry plots was done using FlowJo V10 (Treestar, USA) and the resulting frequencies of pooled cells plotted using Prism 7 Software (San Diego, CA). Note: isolated B-cell samples were pooled prior to flow cytometry analysis, due to inadequate cell

numbers compromising acquisition event rate, thereby prohibiting the performance of statistical analysis.

### 3. Results

#### 3.1 Regulatory B-cell ( $B_{reg}$ ) frequencies

The frequency of  $B_{reg}$  in response to immune stimulation and *M.tb* challenge was investigated.  $B_{regs}$  were defined by the phenotype  $CD19^+CD24^{hi}CD38^{hi}$ . For each of the investigated stimulation conditions, a general pattern of elevated mean  $B_{regs}$  frequencies was observed for the QFN negative group compared to the QFN positive group (Figure 4.1). However, the significance of these differences could not be investigated due to the samples being pooled. Interestingly, CD40L/IL5 stimulation was observed to have varying effects on  $B_{reg}$  frequencies depending on QFN status of the participants. A minor increase in measured mean  $B_{reg}$  frequencies was observed for the QFN positive group following antigenic stimulation of B-cells (CD40L+IL5 3.70%; H37Rv+CD40L+IL5 3.87% vs Unstimulated 3.63%), whereas a decrease in mean  $B_{reg}$  frequencies was observed for the QFN negative group in response to antigenic stimulation (CD40L+IL5 6.36%; H37Rv+CD40L+IL5 4.54% vs Unstimulated 6.74%). Notably, the implication of these differences could not be determined, due to lack of insight into individual sample variation as previously stated.



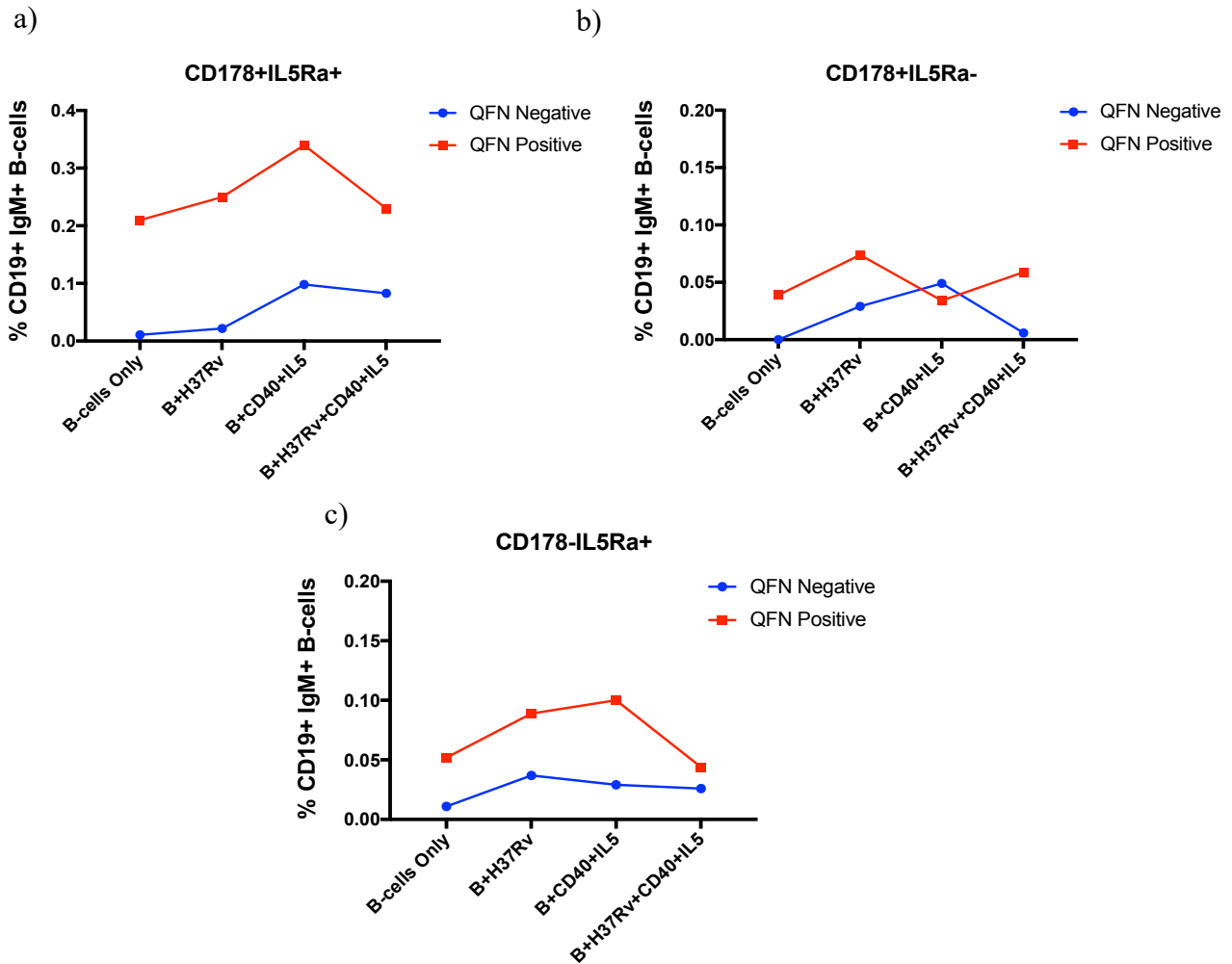
**Figure 4.1. Evaluation of regulatory B-cell ( $B_{reg}$ ) frequencies following antigenic stimulation for the H37Rv pilot study.** The frequency of  $B_{regs}$  was expressed as a percentage of CD19<sup>+</sup> B-cells, in which  $B_{regs}$  were defined by the phenotype CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup>. Differences in the mean  $B_{regs}$  frequencies between the QFN positive and negative group were investigated for the relevant stimulatory conditions. Samples were pooled according to QFN status and stimulation condition prior to flow cytometry analysis, prohibiting the performance of statistical analysis.

### 3.2 FasL-expressing/Killer B-cell frequencies

Isolated B-cells were pulsed with CD40L and IL5, in combination with H37Rv infection, to evaluate the relative expression of FasL by B-cells following *M.tb* infection. Following stimulation, the frequency of killer B-cells in response to the various culture conditions was investigated. The expression of CD178 (FasL) was evaluated in association with CD125 (IL5R $\alpha$ ), as signalling via this cell surface receptor has been implicated in killer B-cell development. Killer B-cells were defined by the phenotype CD19<sup>+</sup>CD5<sup>+</sup>IgM<sup>+</sup>CD38<sup>+</sup>CD178<sup>+</sup>. A pattern of increased mean killer B-cells (CD178<sup>+</sup>) frequencies, regardless of IL5R $\alpha$  expression, was observed for the QFN positive group (Figure 4.2a, H37Rv 0.25%; CD40L+IL5 0.34%; H37Rv+CD40L+IL5 0.23% vs Unstimulated 0.21%; and Figure 4.2b, H37Rv 0.074%; CH37Rv+D40+IL5 0.059 vs Unstimulated 0.039%) in response to antigenic stimulation compared to unstimulated B-cells. Similarly, a pattern of increased mean killer B-cells (CD178<sup>+</sup>) frequencies, regardless of IL5R $\alpha$  expression, was observed for the QFN negative group (Figure 4.2a, H37Rv 0.022%; CD40L+IL5 0.098%; H37Rv+CD40L+IL5 0.083% vs Unstimulated 0.011%; and Figure 4.2b, H37Rv 0.029%; CD40L+IL5 0.049%; H37Rv+CD40L+IL5 0.006% vs Unstimulated 0.000%) in response to stimulation with either H37Rv or CD40L and IL5R $\alpha$  compared to unstimulated B-cells.

Furthermore, a pattern of increased mean IL5R $\alpha$  expression by B-cells, regardless of FasL expression, was observed for the QFN positive group (Figure 4.2a; Figure 4.2c, H37Rv 0.089%; CD40L+IL5 0.100%; H37Rv+CD40L+IL5 0.044% vs Unstimulated 0.052%) following stimulation with either H37Rv or CD40L and IL5R $\alpha$  compared to unstimulated B-cells. Likewise, a pattern of increased mean IL5R $\alpha$  expression by B-cells, regardless of FasL expression, was observed for both the QFN negative group (Figure 4.2a; Figure 4.2c, H37Rv 0.037%; CD40L+IL5 0.029%; H37Rv+CD40L+IL5 0.026% vs Unstimulated 0.011%) following stimulation with either H37Rv or CD40L and IL5R $\alpha$  compared to unstimulated B-cells.

In all instances, a higher incidence of killer-B frequencies was observed for the QFN positive group compared to the QFN negative group (Figure 4.2), with the exception of FasL<sup>+</sup>IL5R $\alpha$ <sup>-</sup> killer B-cells in response to CD40L and IL5 stimulation (Figure 4.2b). Notably, the significance of these differences could not be investigated due to the pooling of samples as previously described. As such, the implication of these differences could not be determined, due to lack of insight into individual sample variation.

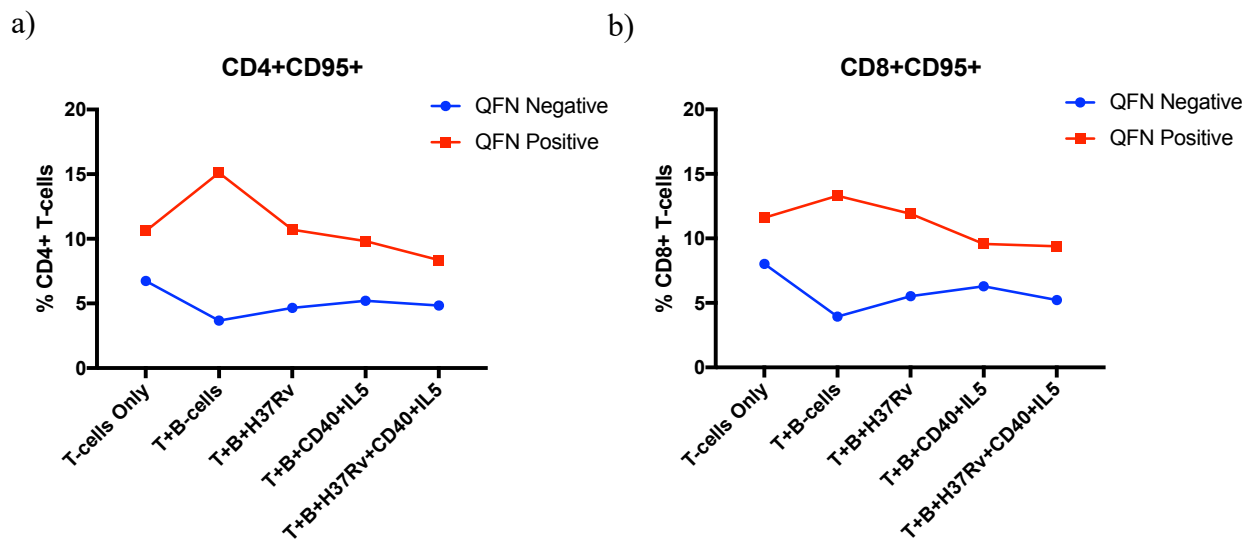


**Figure 4.2. Evaluation of Killer (FasL-expressing) B-cell frequencies following antigenic stimulation for the H37Rv Pilot study.** The frequency of killer B-cells was expressed as a percentage of CD19+IgM+ B-cells, in which killer B-cells were defined by the phenotype CD19+CD5+IgM+CD38+CD178+. Additionally, expression of interleukin-5 receptor alpha (IL5R $\alpha$ ) was investigated as engagement of this receptor with IL5 is associated with killer B-cell development. Differences in the mean frequencies between the QFN positive and negative group were investigated for the relevant stimulatory conditions. (a) IL5R $\alpha$ -expressing killer B-cell frequencies (CD125+CD178+) for the various stimulatory conditions. (b) Killer B-cell frequencies (CD125-CD178+) for the various stimulatory conditions. (c) IL5R $\alpha$ -expressing B-cell frequencies (CD125+CD178-) for the various stimulatory conditions. Samples were pooled according to QFN status and stimulation condition prior to flow cytometry analysis, prohibiting the performance of statistical analysis.

### 3.3 Fas-expressing T-cell frequencies

A subtle increase in mean Fas expression levels by CD4<sup>+</sup> (Figure 4.3a, 15.1% vs 10.6%) and CD8<sup>+</sup> (Figure 4.3b, 13.3% vs 11.6%) T-cells was observed for the QFN positive group following co-culture with unstimulated B-cells. In contrast, co-culture of T-cells with unstimulated B-cells for the QFN negative group resulted in a decrease in Fas expression by CD4<sup>+</sup> (Figure 4.3a, 3.67% vs 6.74%) and CD8<sup>+</sup> (Figure 4.3b, 3.94% vs 8.03%) T-cells. However, the inference of these differences could not be determined, due to pooling of samples prior to flow cytometry analysis, prohibiting insight into individual sample variation.

Remarkably, co-culture of T-cells with autologous pre-stimulated B-cells resulted in decreased mean frequencies of CD4<sup>+</sup>Fas<sup>+</sup> (8.36-10.7% vs 15.1%) and CD8<sup>+</sup>Fas<sup>+</sup> T-cells (9.39-11.9% vs 13.3%) for the QFN positive group, whilst increased the mean frequencies of CD4<sup>+</sup>Fas<sup>+</sup> (4.66-5.21% vs 3.67%) and CD8<sup>+</sup>Fas<sup>+</sup> T-cells (5.23-6.29% vs 3.94) was observed for the QFN negative group under the same stimulatory conditions (Figure 4.3). As previously alluded to, the statistical significance of these differences could not be determined due to pooling of samples.



**Figure 4.3. Evaluation of Fas-expressing T-cell frequencies following co-culture with autologous pre-stimulated B-cells for the H37Rv Pilot study.** The frequency of Fas<sup>+</sup> (CD95<sup>+</sup>) for each of the investigated T-cells populations was expressed as a percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, in (a) and (b) respectively. A control condition, in which T-cells were cultured in the absence of pre-stimulated B-cells, was used as the reference of basal T-cell phenotypic frequencies. Differences in the mean Fas-expression between the QFN positive and negative group were investigated for the relevant stimulatory conditions. (a) CD4<sup>+</sup>Fas<sup>+</sup>(CD95<sup>+</sup>) T-cell frequencies for the various stimulatory conditions (b) CD8<sup>+</sup>Fas<sup>+</sup>(CD95<sup>+</sup>) T-cell frequencies for the various stimulatory conditions. Samples were pooled according to QFN status and stimulation condition prior to flow cytometry analysis, prohibiting the performance of statistical analysis

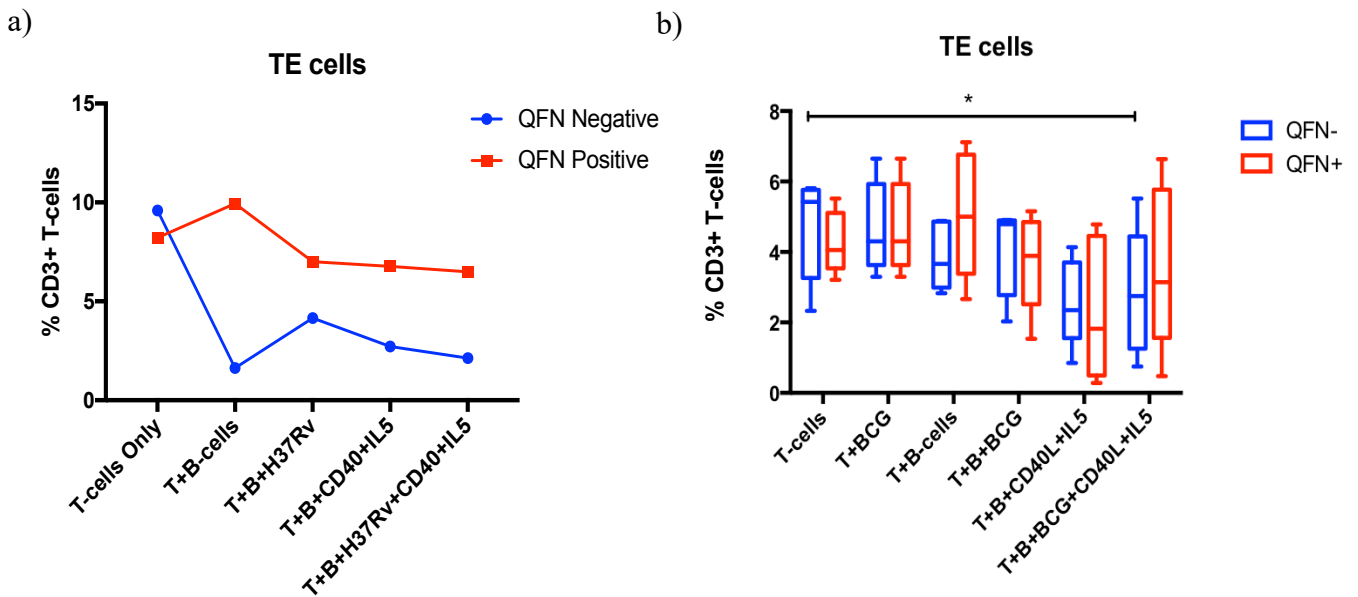


### 3.4 Effector T-cell and Regulatory T-cell frequencies

The ability of B-cells to alter T-cell function was evaluated, by assessing alterations in effector T-cell (TE) phenotypic frequencies following co-culture with autologous, pre-stimulated B-cells. Effector T-cells were defined by the phenotype CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>. Within the H37Rv Pilot study, a pattern of decreased mean TE frequencies was observed for both the QFN positive (6.49-7.00% vs 8.22%) and negative group (1.63-4.16% vs 9.60%) for all culture conditions in which autologous T-cells were co-cultured with pre-stimulated B-cells, compared to the control condition consisting of T-cells only (Figure 4.4a). Paradoxically, an increase in mean TE frequencies were observed following co-culture of T-cells with unstimulated B-cells for the QFN positive group (Figure 4a, 9.94% vs 8.22%). Markedly, the implication of these differences could not be determined, due to pooling of samples prior to flow cytometry analysis, prohibiting insight into individual sample variation.

Within the BCG pilot study, a similar pattern of decreased TE frequency was observed, although not significant in all cases, for both QFN positive and negative individuals, for all culture conditions where pre-stimulated B-cells were present compared to T-cells only (Figure 4.4b). Notably, a significant decrease in TE frequencies was observed for QFN negative individuals following coculture with BCG+CD40L+IL5 stimulated B-cells compared to T-cells only ( $p=0.01$ , Figure 4.4b). Following culture of T-cells with unstimulated B-cells, a non-significant increase in TE frequencies was observed for QFN positive individuals.

Interestingly, elevated mean frequencies of TE were observed for the QFN positive group in all instances where T-cells were cultured in combination with autologous B-cells, compared to the QFN negative group. However, in the case of the BCG pilot study, no significant differences were observed.



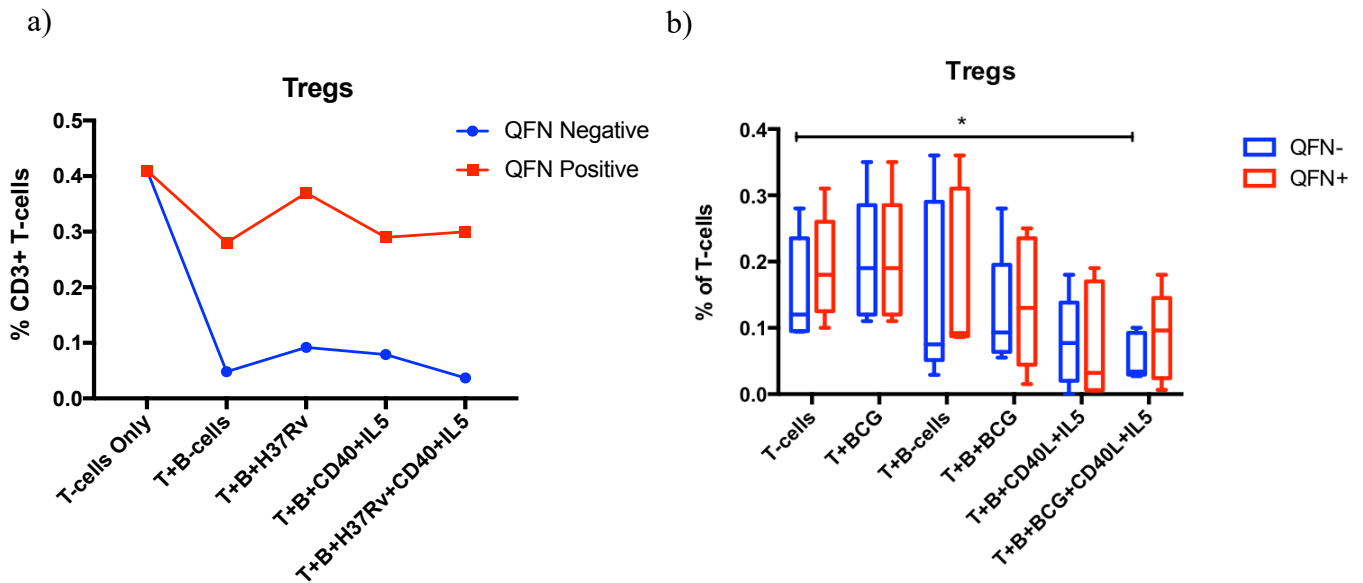
**Figure 4.4. Evaluation of effector T-cell (TE) frequencies following co-culture with autologous pre-stimulated B-cells.** The frequency of TE was expressed as a percentage of CD3<sup>+</sup> T-cells, in which TEs were defined by the phenotype CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>. A control condition, in which T-cells were cultured in the absence of pre-stimulated B-cells, was used as the reference for basal T-cell phenotypic frequencies. Differences in mean TE frequencies between the QFN positive and negative groups were investigated for the relevant stimulatory conditions. (a) TE frequencies for the various stimulatory conditions observed for H37Rv Pilot study. Samples were pooled according to QFN status and stimulation condition prior to flow cytometry analysis, prohibiting the performance of statistical analysis (b) TE frequencies for the various stimulatory conditions observed for BCG pilot study. Whisker denote min and max. Comparisons between groups was calculated using the Mann Whitney U-test, and differences within groups was calculated using the Kruskal-Wallis with Dunns multiple comparisons post-hoc test. Statistical significance is indicated by an asterisk (\*), where the  $p < 0.05$ .

Additionally, the ability of B-cells to modulate regulatory T-cell ( $T_{reg}$ ) phenotypic frequencies following co-culture was investigated. Regulatory T-cells were defined by the phenotype  $CD3^+CD4^+CD25^{hi}$ . Additional  $T_{reg}$  markers such as CD127 and FoxP3 were not included in this analysis, limiting the validity of the defined cell population as true  $T_{regs}$ . Within the H37Tv pilot study, a decrease in mean  $T_{reg}$  frequencies was observed for both the QFN positive (0.28% vs 0.41%) and negative group (0.048% vs 0.41%) following co-culture of autologous T-cells with unstimulated B-cells (Figure 4.5a). Likewise, a pattern of decreased mean  $T_{reg}$  frequencies were observed, for the QFN positive (Figure 4.5a, 0.29-0.37% vs 0.41%) and negative group (Figure 4.5a, 0.037-0.092% vs 0.41%), for all culture conditions where pre-stimulated B-cells were present compared to T-cells only. Cell viability was not assessed post-co-culture, hence the observed decrease in  $T_{reg}$  numbers could be a result of cell death rather than B-cell modulation.

A comparison of mean  $T_{reg}$  frequencies between the various stimulatory conditions, for both the QFN positive and negative group, revealed that H37Rv stimulation resulted in slightly higher  $T_{reg}$  levels compared to CD40 and IL5 stimulation (QFN positive: 0.37% vs 0.29%; QFN negative: 0.092% vs 0.079%), as well as H37Rv in combination with CD40 and IL5 (QFN positive: 0.37% vs 0.3%; QFN negative: 0.092% vs 0.037%). Once more, the implication of these differences could not be determined, due to pooling of samples prior to flow cytometry analysis, prohibiting insight into individual sample variation.

Oppositely, an increase in  $T_{reg}$  frequencies was observed for both QFN positive and negative individuals within the BCG pilot study, following co-culture of T-cells with unstimulated B-cells (Figure 4.5b). On the contrary, a pattern of decreased  $T_{reg}$  frequency, similar to that of the H37Rv pilot study, was observed for both QFN positive and negative individuals, for all culture conditions where pre-stimulated B-cells were present compared to T-cells only (Figure 4.5b).

Interestingly, elevated mean frequencies of  $T_{regs}$  were observed for the QFN positive group in all instances where T-cells were cultured in combination with autologous B-cells, compared to the QFN negative group. Again, in the case of the BCG pilot study, no significant differences were observed.



**Figure 4.5. Evaluation of regulatory T-cell ( $T_{reg}$ ) frequencies following co-culture with autologous pre-stimulated B-cells.** The frequency of  $T_{regs}$  was expressed as a percentage of  $CD3^+$  T-cells, in which  $T_{regs}$  were defined by the phenotype  $CD3^+CD4^+CD25^{hi}$ . A control condition, in which T-cells were cultured in the absence of pre-stimulated B-cells, was used as the reference for basal T-cell phenotypic frequencies. Differences in mean  $T_{reg}$  frequencies between the QFN positive and negative groups were investigated for the relevant stimulatory conditions. (a)  $T_{reg}$  frequencies for the various stimulatory conditions observed for H37Rv Pilot study. Samples were pooled according to QFN status and stimulation condition prior to flow cytometry analysis, prohibiting the performance of statistical analysis (b)  $T_{reg}$  frequencies for the various stimulatory conditions observed for BCG pilot study. Whisker denote min and max. Comparisons between groups was calculated using the Mann Whitney U-test, and differences within groups was calculated using the Kruskal-Wallis with Dunns multiple comparisons post-hoc test. Statistical significance is indicated by an asterisk (\*), where the  $p < 0.05$ .

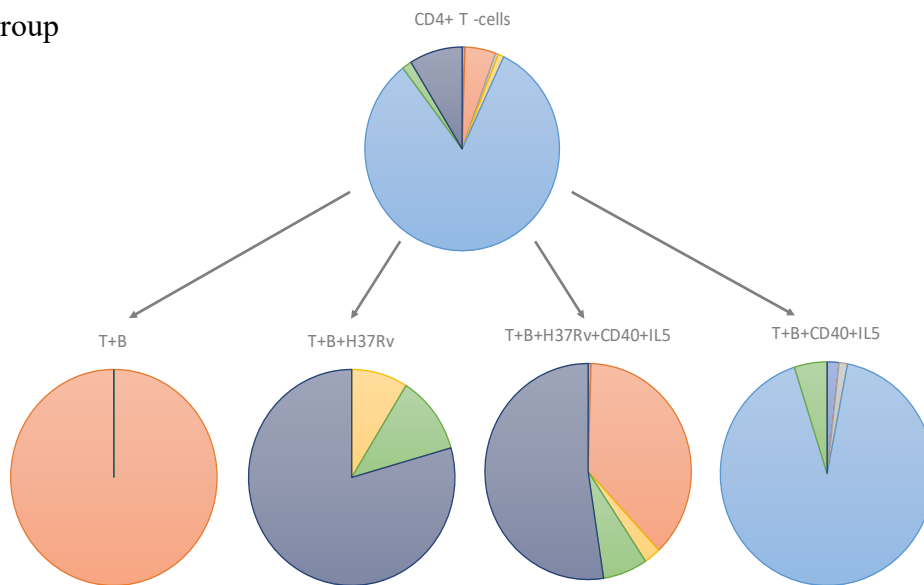
### 3.5 T-cell cytokine production profiles

The modulatory effect of B-cells on T-cell cytokine production was evaluated, to determine whether or not B-cells had the ability to manipulate T-cell function. The results obtained from the H37Rv pilot study for each of the stimulation conditions with respect to QFN status are initially described, followed by the results observed within the BCG pilot study for clear comparison.

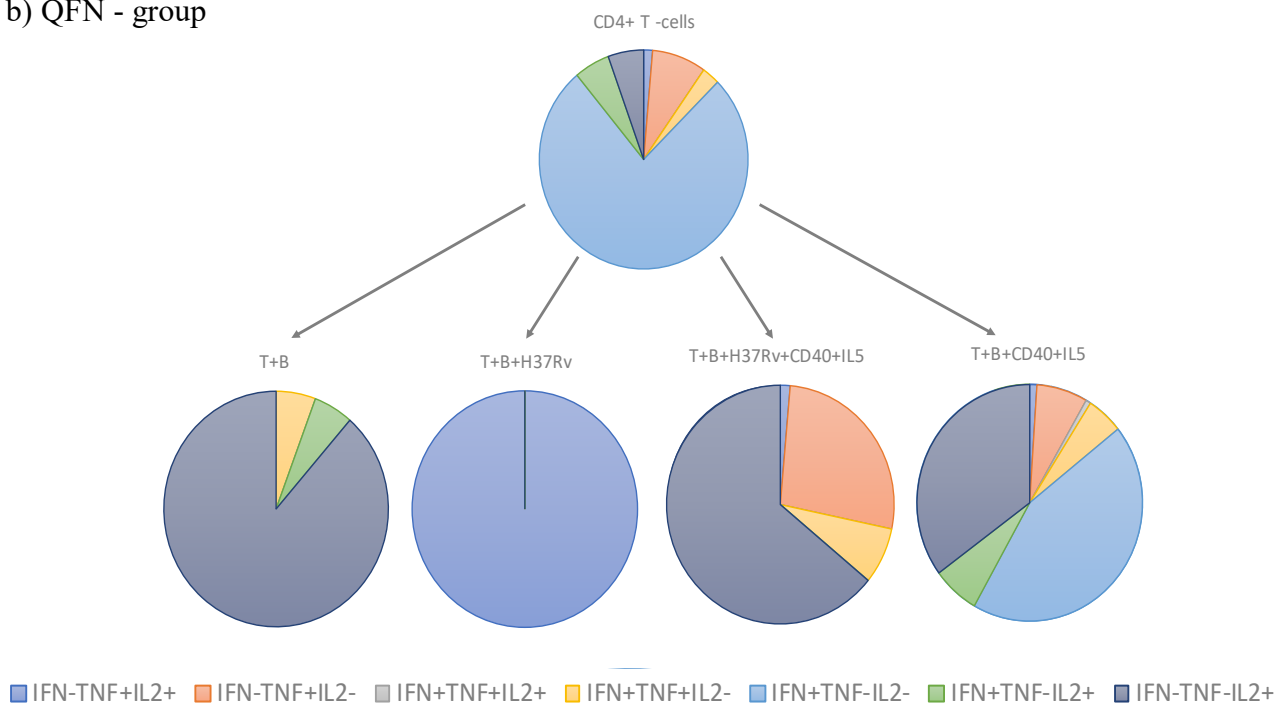
The following CD4<sup>+</sup> T-cells cytokine profiles were observed for the QFN positive group: Unstimulated B-cells within the H37Rv pilot study (Figure 4.6a) induced CD4<sup>+</sup>TNF $\alpha$ <sup>+</sup> T-cells. In contrast, unstimulated B-cells within the BCG pilot study (Suppl Figure 4.6a) induced CD4<sup>+</sup>IL2<sup>+</sup>T-cells, as well as CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>IL2<sup>+</sup> T-cells (ns,  $p>0.05$ ). B-cells pre-stimulated with H37Rv or BCG induced a similar cytokine milieu to that of unstimulated B-cells within the BCG pilot study, with the addition of CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> T-cells (ns,  $p>0.05$ ). B-cells stimulated with CD40L and IL5 (i.e. induction of FasL expression), within the H37Rv pilot study, resulted in increased CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>IL2<sup>+</sup>, CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup> and CD4<sup>+</sup>IL2<sup>+</sup>T-cells. Similarly, B-cells stimulated with CD40L and IL5, within the BCG pilot study, induced CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>IL2<sup>+</sup> T-cells (ns,  $p>0.05$ ). Lastly, B-cells stimulated with H37Rv in combination with CD40L and IL5 increased CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup>, CD4<sup>+</sup>IL2<sup>+</sup>, CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>IL2<sup>+</sup> and CD4<sup>+</sup>TNF $\alpha$ <sup>+</sup> T-cells, whereas B-cells stimulated with BCG in combination CD40L and IL5 induced CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> and CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> T-cells (ns,  $p>0.05$ ).

The CD4<sup>+</sup> T-cells cytokine profiles observed for the QFN negative group included: Unstimulated B-cells within the H37Rv pilot study induced CD4<sup>+</sup>IL2<sup>+</sup>, CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>IL2<sup>+</sup> and CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> T-cells (Figure 4.6b); whereas, unstimulated B-cells within the BCG pilot study (Suppl Figure 4.6b) induced an increase in CD4<sup>+</sup>TNF $\alpha$ <sup>+</sup>, CD4<sup>+</sup>TNF $\alpha$ <sup>+</sup>IL2<sup>+</sup>, CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>IL2<sup>+</sup> and CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> T-cells (ns,  $p>0.05$ ). B-cells pre-stimulated with H37Rv induced CD4<sup>+</sup>TNF $\alpha$ <sup>+</sup>IL2<sup>+</sup> T-cells (ns,  $p>0.05$ ), while B-cells stimulated with BCG induced upregulation of CD4<sup>+</sup>TNF $\alpha$ <sup>+</sup> T-cells. B-cells stimulated with CD40L and IL5 within the H37Rv pilot study resulted in a distinct alteration in the cytokine milieu, in which CD4<sup>+</sup>IL2<sup>+</sup>, CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>, CD4<sup>+</sup>TNF $\alpha$ <sup>+</sup>, CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> and CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>IL2<sup>+</sup> T-cells were induced. In comparison, B-cells stimulated with CD40L and IL5 within the BCG pilot study resulted in the induction of CD4<sup>+</sup>IL2<sup>+</sup>, CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup> and CD4<sup>+</sup>TNF $\alpha$ <sup>+</sup> T-cells. Finally, B-cells stimulated with H37Rv in combination with CD40L and IL5 increased CD4<sup>+</sup>IL2<sup>+</sup>, CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> and CD4<sup>+</sup>TNF $\alpha$ <sup>+</sup> T-cells. Comparably, B-cells stimulated with BCG in combination with CD40L and IL5 increased CD4<sup>+</sup>IL2<sup>+</sup>, CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> and CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>IL2<sup>+</sup> T-cells (ns,  $p>0.05$ ).

a) QFN + group



b) QFN - group



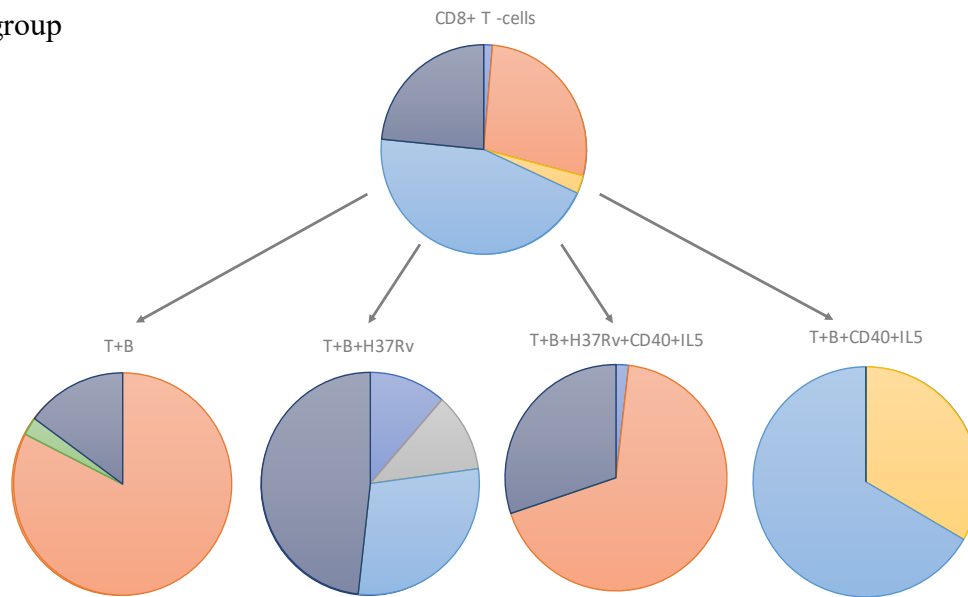
**Figure 4.6. Cytokine secretion profile of CD4<sup>+</sup> T-cells following co-culture with pre-stimulated B-cells for the H37Rv Pilot study.** The proportion of cells expressing various combinations of cytokines is calculated as a function of the total frequency of cytokine producing cells. Alterations in cytokine production for each of the culture conditions was compared to the control condition consisting of T-cells only. The control condition served as a representation of basal cytokine production levels; these background levels were subtracted from the various stimulatory conditions and the resulting profiles reported (a) Cytokine profiles of CD4<sup>+</sup> T-cells for each of the stimulatory conditions observed for the QFN positive group. (b) Cytokine profiles of CD4<sup>+</sup> T-cells for each of the stimulatory conditions observed for the QFN negative group.

The CD8<sup>+</sup> T-cells cytokine profiles observed for the QFN positive group demonstrated: Unstimulated B-cells within the H37Rv pilot study induced CD8<sup>+</sup>TNF $\alpha$ <sup>+</sup>, CD8<sup>+</sup>IL2<sup>+</sup> and CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup>IL2<sup>+</sup> T-cells (Figure 4.7a), whereas, unstimulated B-cells within the BCG pilot study (Suppl Figure 4.7a) did not induce any change within the cytokine milieu. B-cells stimulated with H37Rv increased the number of CD8<sup>+</sup>IL2<sup>+</sup>, CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup> and CD8<sup>+</sup>TNF $\alpha$ <sup>+</sup>IL2<sup>+</sup> T-cells, as well as multifunctional (CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup>IL2<sup>+</sup>) T-cells. While B-cells pre-stimulated with BCG induced CD8<sup>+</sup>TNF $\alpha$ <sup>+</sup>, CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> and CD8<sup>+</sup>TNF $\alpha$ <sup>+</sup>IL2<sup>+</sup> T-cells (ns, p>0.05). B-cells stimulated with CD40L and IL5 within the H37Rv pilot study induced CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup> and CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> T-cells, and B-cells stimulated with H37Rv in combination with CD40L and IL5 increased CD8<sup>+</sup>IL2<sup>+</sup> and CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup> T-cell frequencies. In contrast, B-cells stimulated with CD40L and IL5 within the BCG pilot study resulted in an increase in CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup>, CD8<sup>+</sup>IL2<sup>+</sup>, CD8<sup>+</sup> TNF $\alpha$ <sup>+</sup> IL2<sup>+</sup> and multifunctional T-cells (ns, p>0.05). B-cells stimulated with BCG in combination with CD40L and IL5 induced as similar cytokine milieu, with the addition of CD8<sup>+</sup>TNF $\alpha$ <sup>+</sup> T-cells (ns, p>0.05).

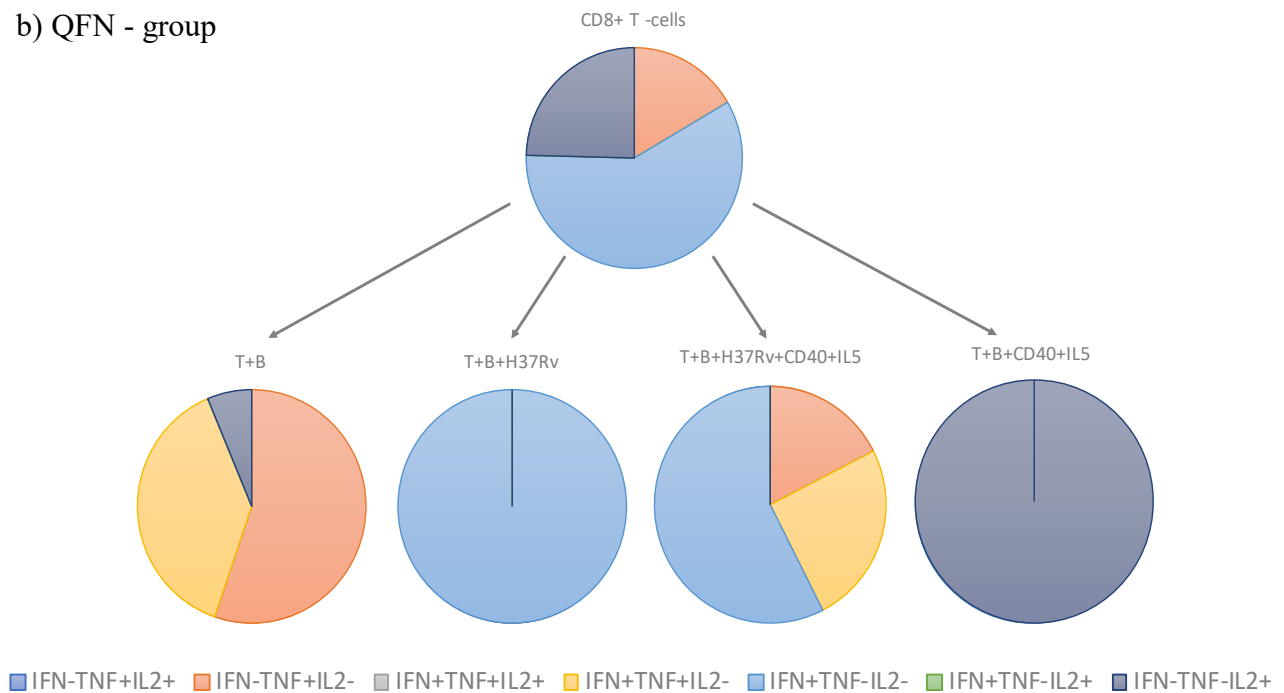
Finally, the CD8<sup>+</sup> T-cells cytokine profiles observed for the QFN negative group showed: Unstimulated B-cells, within the H37Rv pilot study, induced CD8<sup>+</sup>IL2<sup>+</sup>, CD8<sup>+</sup>TNF $\alpha$ <sup>+</sup> and CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> T-cells (Figure 4.7b). In contrast, unstimulated B-cells within the BCG pilot study (Suppl Figure 4.7b) induced an increase in CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup>, CD8<sup>+</sup>IL2<sup>+</sup> and CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup>IL2<sup>+</sup> T-cells. B-cells stimulated with H37Rv resulting in increased CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup> T-cell frequencies, whilst B-cells pre-stimulated with BCG induced CD8<sup>+</sup> TNF $\alpha$ <sup>+</sup> and CD8<sup>+</sup> TNF $\alpha$ <sup>+</sup>IL2<sup>+</sup> T-cells (ns, p>0.05). B-cells stimulated with CD40L and IL5, within the H37Rv pilot study, induced CD8<sup>+</sup>IL2<sup>+</sup> T-cells. Similarly, B-cells stimulated with CD40L and IL5, within the BCG pilot study, resulted in an increase in CD8<sup>+</sup>IL2<sup>+</sup> T-cells, as well as CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup> T-cells (ns, p>0.05). Lastly, B-cells stimulated with H37Rv in combination with CD40L and IL5 induced CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup>, CD8<sup>+</sup>TNF $\alpha$ <sup>+</sup> and CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> T-cells, whereas B-cells stimulated with BCG in combination with CD40L and IL5 increased CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup> and CD8<sup>+</sup>IL2<sup>+</sup> and CD8<sup>+</sup>TNF $\alpha$ <sup>+</sup> T-cells (ns, p>0.05).

Importantly, no significant differences between the QFN positive and negative groups were observed for either CD4<sup>+</sup> or CD8<sup>+</sup> T-cell cytokine responses within the BCG pilot study, whilst significance within the H37Rv pilot study could not be evaluated due to pooling of samples according to QFN status and stimulation condition prior to flow cytometry analysis.

a) QFN + group



b) QFN - group



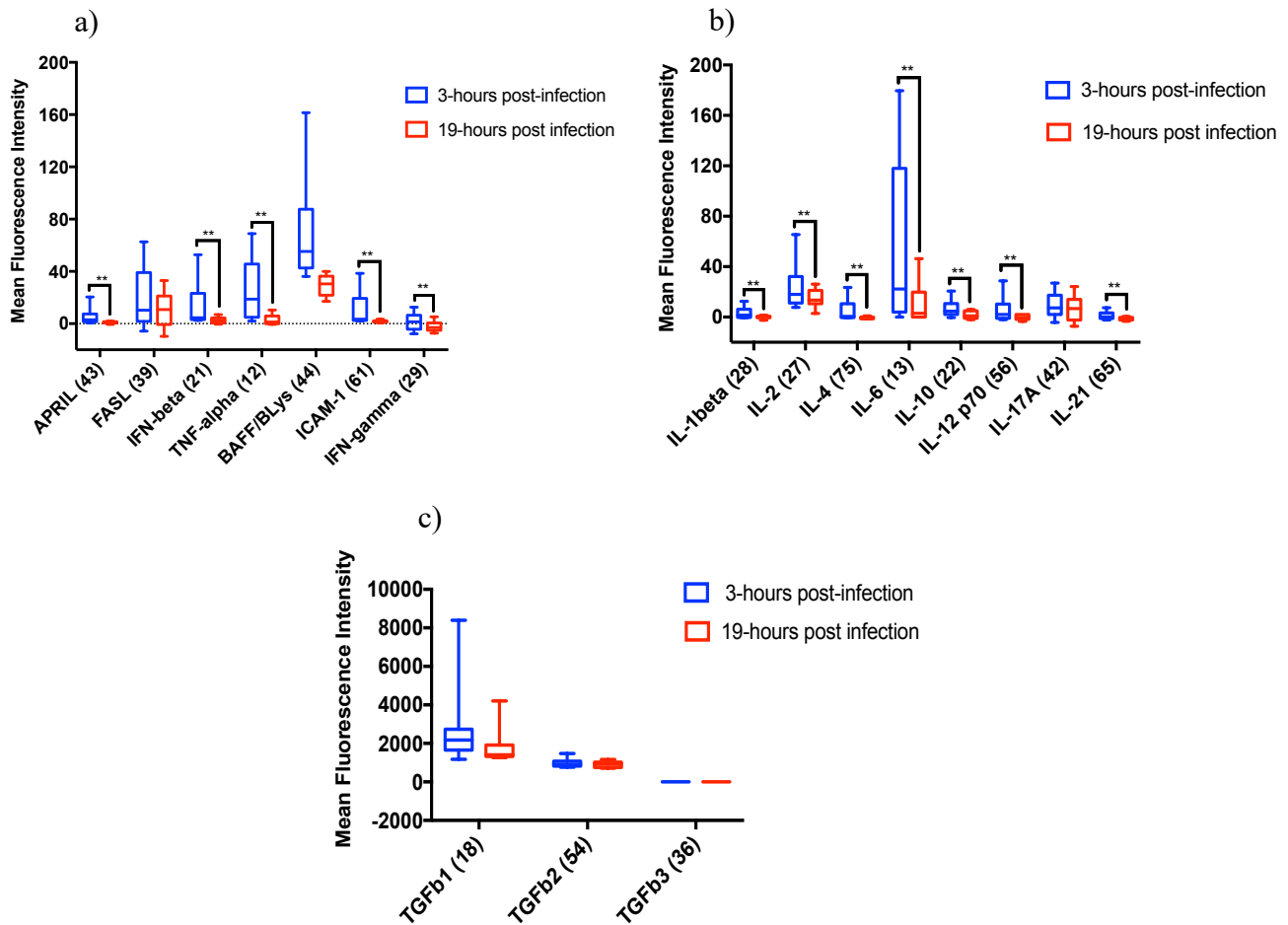
**Figure 4.7. Cytokine secretion profile of CD8<sup>+</sup> T-cells following co-culture with pre-stimulated B-cells for the H37Rv Pilot study.** The proportion of cells expressing various combinations of cytokines is calculated as a function of the total frequency of cytokine producing cells. Alterations in cytokine production for each of the culture conditions was compared to the control condition consisting of T-cells only. The control condition served as a representation of basal cytokine production levels; these background levels were subtracted from the various stimulatory conditions and the resulting profiles reported (a) Cytokine profiles of CD8<sup>+</sup> T-cells for each of the stimulatory conditions observed for the QFN positive group. (b) Cytokine profiles of CD8<sup>+</sup> T-cells for each of the stimulatory conditions observed for the QFN negative group.



### 3.6 B-cell cytokine responses

The cytokine responses of B-cells following antigenic stimulation was investigated. Cell culture supernatants were collected at different time points, namely 3-hours post stimulation (denoted as time point 1) and 19-hours post co-culture with autologous T-cells (denoted as time point 2).

Differences between cell culture supernatants of stimulated B-cells (in the absence of autologous T-cells), collected at time point 1 and time point 2, were investigated to determine the effects of culture time on B-cell cytokine secretion. A general pattern of decreased cytokine expression was observed for all investigated analytes at time point 2 compared to time point 1 (Figure 4.8). Significant differences were observed for the majority of the measured cytokines, namely APRIL ( $p=0.0040$ ), ICAM-1 ( $p=0.0044$ ), IFN- $\beta$  ( $p=0.0040$ ), IFN- $\gamma$  ( $p=0.0057$ ), IL-1 $\beta$  ( $p=0.0040$ ), IL-2 ( $p=0.0089$ ), IL-4 ( $p=0.0040$ ), IL-6 ( $p=0.0090$ ), IL-10 ( $p=0.0040$ ), IL-12p70 ( $p=0.0041$ ), IL-21 ( $p=0.0040$ ) and TNF- $\alpha$  ( $p=0.0090$ ); with the exception of soluble FasL (sFasL) ( $p=0.0597$ ), BAFF ( $p=0.0230$ ), IL17A ( $p=0.2376$ ) and all three isotypes of TGF $\beta$  (TGF $\beta$ 1,  $p=0.0827$ ; TGF $\beta$ 2,  $p=0.2725$ ; TGF $\beta$ 3,  $p=0.4004$ ). Interestingly, QFN status (Suppl Figure 4.8) and stimulation conditions (Suppl Figure 4.9) had no significant effect on the overall cytokine production by B-cells. However, a significant increase in relative cytokine expression of APRIL ( $p=0.0134$ ), IFN- $\beta$  ( $p=0.0105$ ), IL-1 $\beta$  ( $p=0.0061$ ), IL-2 ( $p=0.0030$ ), IL-4 ( $p=0.0118$ ), IL-10 ( $p=0.0097$ ), IL-12 p70 ( $p=0.0238$ ) and IL21( $p=0.0360$ ), was observed following infection with H37Rv compared to unstimulated controls. Similarly, a significant increase in the expression of APRIL ( $p=0.0413$ ), IFN- $\beta$  ( $p=0.0233$ ), IL-1 $\beta$  ( $p=0.0210$ ), IL-2 ( $p=0.0268$ ), IL-4 ( $p=0.0258$ ), IL-10 ( $p=0.0166$ ), IL-12 p70 ( $p=0.0386$ ) and IL21( $p=0.0123$ ), was observed following infection with H37Rv compared to CD40L and IL5 stimulate samples. Moreover, a considerable increase in ICAM-1 ( $p=0.0093$ ) and IFN- $\gamma$  ( $p=0.0346$ ) was observed following H37Rv infection compared to the unstimulated control, while negligible differences were observed compared to CD40L and IL5 stimulation, as well as CD40L and IL5 in combination with H37Rv. A considerable increase in TNF- $\alpha$  was observed for all stimulatory conditions, namely H37Rv ( $p=0.0414$ ), CD40L and IL5 ( $p=0.0208$ ) as well as CD40 and IL5 in combination with H37Rv ( $p=0.0049$ ), compared to the unstimulated control. Likewise, a considerable increase in IL-6 was observed for the stimulatory conditions CD40L and IL5 ( $p=0.0145$ ) as well as CD40 and IL5 in combination with H37Rv ( $p=0.0019$ ), compared to the unstimulated control. Lastly, the combined effects of QFN status and stimulation condition on B-cell cytokine expression was investigated (Suppl Table 4.2). For all cases, no significant difference between QFN positive and negative individuals for each of the stimulatory conditions was observed (Suppl Figure 4.10), but rather a trend of heightened cytokine responses in response to H37Rv infection observed for all individuals, regardless of QFN status.



**Figure 4.8. Effect of culture time on cytokine secretion by B-cells following antigenic stimulation for the H37Rv Pilot study.** Cell culture supernatants were collected at different time points, namely 3-hours post stimulation and 19-hours post co-culture with autologous T-cells. Whisker denote 10-90 percentile. Statistical differences between culture conditions was calculated using a four-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR), with a FDR of 1%, was used to correct for multiple testing. Statistical significance is indicated by an asterisk, in which  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*). (a) Effect of culture time on B-cell secretion factors following antigenic stimulation of B-cells. (b) Effect of culture time on interleukin (IL) production following antigenic stimulation of B-cells. (c) Effect of culture time on TGFβ isotype secretion following antigenic stimulation of B-cells.

In addition, differences between cell culture supernatants collected 19-hours post co-culture, in which B-cells were culture with/without autologous T-cells, was investigated to determine whether the presence of T-cells had any effect/contribution to the microenvironment. Once more, the effect of QFN status on cytokine expression was investigated, in which no significant difference between QFN positive and negative individuals was observed (Suppl Figure 4.11). Moreover, the effects of stimulation condition on measured cytokine levels was investigated (Suppl Figure 4.12). A significant decrease in BAFF was observed for the culture condition in which T-cells were cultured alone compared to conditions in which B-cells were present, namely B-cells only ( $p=0.0000$ ), B+H37Rv ( $p=0.0000$ ), B+CD40L+IL-5 ( $p=0.0000$ ), B+CD40L+IL-5+H37Rv ( $p=0.0000$ ), T+B ( $p=0.0000$ ), T+B+H37Rv ( $p=0.0000$ ), T+B+CD40L+IL-5 ( $p=0.0000$ ) and T+B+CD40L+IL-5+H37Rv ( $p=0.0000$ ) (Suppl Figure 4.12e). Similarly, a significant decrease in TGF- $\beta$ 1 was observed for the culture condition in which T-cells were cultured alone compared to the conditions B-cells only ( $p=0.0024$ ), B+H37Rv ( $p=0.0015$ ), B+CD40L+IL-5 ( $p=0.0005$ ), B+CD40L+IL-5+H37Rv ( $p=0.0012$ ), T+B ( $p=0.0016$ ), T+B+H37Rv ( $p=0.0044$ ), T+B+CD40L+IL-5 ( $p=0.0016$ ) and T+B+CD40L+IL-5+H37Rv ( $p=0.0134$ ) (Suppl Figure 4.12p). Furthermore, a significant increase in sFasL expression was observed following culture of unstimulated B-cells with autologous T-cells compared to B-cells alone ( $p=0.0375$ ), B+H37Rv ( $p=0.0439$ ), B+CD40L+IL-5 ( $p=0.0479$ ), B+CD40L+IL-5+H37Rv ( $p=0.0113$ ), T+B+H37Rv ( $p=0.0452$ ) and T+B+CD40L+IL-5 ( $p=0.0310$ ).

Once more, the combined effects of QFN status and stimulation condition on observed cytokine expression was investigated. For majority of cases, no difference was observed between QFN positive and negative individuals for each of the stimulatory conditions (Suppl Table 4.3). However, a significant increase in sFasL expression ( $p=0.0006$ ) was observed for QFN negative compared to QFN positive individuals following culture of T-cells with unstimulated B-cells (Suppl Figure 4.13b). Similarly, a significant increase in the expression of IFN- $\beta$  ( $p=0.0349$ ) and ICAM-1 ( $p=0.0168$ ) was observed for QFN negative individuals following co-culture of T-cells with H37Rv stimulated B-cells compare to QFN positive individuals (Suppl Figure 4.13c and f, respectively). Additionally, a significant increase in the expression of IL-2 ( $p=0.0475$ ) was observed for QFN negative individuals following co-culture of T-cells with H37Rv stimulated B-cells compare to QFN positive individuals (Suppl Figure 4.13i). Finally, a significant increase in the expression of IL-17A ( $p=0.0007$ ) was observed for QFN negative individuals following co-culture of T-cells with CD40L and IL5 stimulated B-cells compare to QFN positive individuals (Suppl Figure 4.13n).

#### 4. Discussion

Immune cells capable of modulating macrophage and T-cell function would remarkably impact anti-TB immunity and TB disease outcome. Recent advances in TB research have highlighted the importance of additional cells types, such as B-cells and NK cells, in combating *M.tb* infection (Achkar et al., 2015; Balu et al., 2011; Chan et al., 2014; Du Plessis et al., 2016b; Kaufmann, 2002; Li et al., 2017; Maglione et al., 2007; Rao et al., 2015). The importance of B-cells in T-cell activation and function in terms of traditional immunological roles has been extensively researched (Abbas et al., 2014; Capra et al., 1999; Paul, 2013; Pier et al., 2004). For instance, antigen presentation by B-cells greatly impacts T-cell behavior, influencing T-cell differentiation and proliferation (Crawford et al., 2006; Heesters et al., 2016; Kurt-Jones et al., 1988a; Lanzavecchia, 1985). Furthermore, it has been revealed that T-cells acquire additional signaling (apart from antigen presentation) in order for complete activation to occur. This includes direct engagement of co-receptors as well as indirect signaling via soluble factors such as cytokines, illustrating the role microenvironment complexity has on influencing cell function (Abbas et al., 2014; Chambers and Allison, 1997; Kurt-Jones et al., 1988b; Lanzavecchia, 1985). These complex cellular interactions and the physiological relevance of each cell type during immune activation translates to considerable changes in resulting host immunity. Emerging evidence suggests that the relationship between B- and T-cells extends far beyond this simple interaction, and that B-cell may play a more valuable role in priming T-cell immune responses by regulating effector and regulatory T-cell frequencies (Carter et al., 2012, 2011; Flores-Borja et al., 2013; Kessel et al., 2012; León et al., 2014; Linton et al., 2003).

Thus, the aim of this study was to evaluate the ability of B-cells to modulate T-cell function – in the context of *M.tb* exposure - by investigating changes in T-cell phenotypic frequencies and cytokine profiles following co-culture with autologous pre-stimulated B-cells. For the purpose of this investigation, QFN positive and QFN negative individuals were recruited and analysed as separate cohorts. The rationale behind comparing the observed immune responses in relation to QFN status is the fact that individuals with a QFN positive status represented healthy individuals presumed to have possible latent TB infection (LTBI); while the latter represented healthy, uninfected individuals. LTBI may be defined as a state of persistence immune response to stimulation by *M.tb* antigens, while presenting no clinical symptoms of active disease (WHO, 2018). In theory, the immune response of QFN positive individuals to *M.tb* infection should differ in magnitude and response time compared to QFN negative individuals - due to altered basal immune function and acquisition of adaptive memory. It is assumed that QFN negative individuals will have heightened immune reaction in response to immunogenic stimulation compared to QFN positive individuals, whose immune cells may experience cellular exhaustion due to constant antigenic stimulation. However, more investigation into this occurrence is required to validate this perception. Importantly, QFN positive individuals do not have active TB disease, indicating that their immune system is able to successfully contain or eliminate *M.tb*

infection and halt progression to clinical disease. As such, understanding the complex functioning of immune cells within this cohort may reveal mechanisms that these individuals employ for the successfully combat of *M.tb* infection. Evidently, the absence of an active TB cohort within the study design is a confounding limitation as all observations made within this study simply inferred the proposed significance during TB disease, rather than confirming the events.

Changes in B-cell phenotypic frequencies in response to *M.tb* infection were assessed to evaluate the potential modulatory effects *M.tb* stimulated B-cells may have on anti-TB immune responses. Currently, no distinct phenotypic signature exists for the classification of B<sub>regs</sub> or killer B-cells, due to the large heterogeneity observed within these populations. Therefore, identification of the various B-cell populations of interest was done using a combination of markers listed within literature and those previously investigated by members within our research group (Blair et al., 2010; Buffa et al., 2013; Flores-Borja et al., 2013; van Rensburg et al., 2017; van Rensburg and Loxton, 2018b). B<sub>regs</sub> were identified by co-expression of the cell surface markers CD19 (the B-cell receptor), CD38 (a marker of activation) and CD24 (a marker of cell maturation). Similarly, killer B-cells were identified by co-expression of CD19 and CD38, with the addition of CD178 (Fas Ligand) and interleukin-5 receptor alpha (IL5R $\alpha$ ). It is important to note that due to limited cell numbers, samples were pooled prior to flow cytometry analysis, prohibiting assessment of individual sample distribution and statistically significant differences between cohorts or stimulation conditions. Consequently, observations made from the resulting data depict the general trends for each of the experimental conditions under comparison and is not based on statistical analysis. Markedly, a limitation of the obtained results may be skewing of the data by the inclusion of an outlier within the pooled sample fraction, resulting in artefactual observations that are not indicative of genuine immune responses within the general population. As such, any assumptions made from the resulting pilot data should be interpreted with caution. Additional experiments that address these restrictions are required before any convincing conclusions can be made.

According to literature, B<sub>regs</sub> function within the immune system entails suppression of effector immune responses, such as those elicited by CD4<sup>+</sup> and CD8<sup>+</sup> T-cells (Carter et al., 2012, 2011; Flores-Borja et al., 2013; Kessel et al., 2012; Liu et al., 2016; Lundy and Boros, 2002; Nova-Lamperti et al., 2016; Siewe et al., 2013). In many cases, B<sub>reg</sub> frequencies have been found to correlate with poor disease prognosis - due to suppression of protective immune responses (Advani et al., 2013; Berntsson et al., 2016; Brown et al., 2014; Johansson et al., 2001; Lannutti et al., 2011; Liu et al., 2016; Lundgren et al., 2016; Ou et al., 2015; Siewe et al., 2013; Svensson et al., 1998; Tadmor et al., 2011). Whilst in other cases B<sub>reg</sub> frequencies have been associated with improved health outcome - due to effective control of pro-inflammatory immune responses that if unregulated result in pathological immune conditions (Abbas et al., 2014; Carter et al., 2012, 2011; Chesneau et al., 2013; Daien et al., 2014; Evans et al., 2007; Matsumoto et al.,

2014; Matsushita et al., 2008; Ray and Dittel, 2017; Wang et al., 2014). Therefore, the frequency of these cells during health and disease contributes significantly to the well-being of the host. Elevated  $B_{reg}$  levels were observed for the QFN negative group for all stimulatory conditions, compared to the QFN positive group. These results are in agreement with the BCG pilot study conducted by van Rensburg and colleagues (van Rensburg and Loxton, 2018), in which elevated levels of  $B_{regs}$  were observed for QFN negative individuals compared to QFN positive individuals, regardless of simulation condition. As previously mentioned, studies have demonstrated dysregulation and decreased frequencies of B-cells during TB disease (Du Plessis et al., 2016a; Joosten et al., 2016b; Van Rensburg et al., 2017), illustrating that *M.tb* infection considerably affects B-cell function. This evidence supports the patterns observed within the study, in which B-cell activation and differentiation of the QFN positive group was less proficient compared to the QFN negative group, linking to the aforementioned assumption that QFN negative individuals exhibit heightened immune responses compared to QFN positive individuals. Intriguingly, a marginal increase in mean  $B_{reg}$  frequencies was observed for the QFN positive group following antigenic stimulation, while a reduction in mean  $B_{reg}$  frequencies was discovered for the QFN negative group. These results call attention to possible differences in immune cell function between QFN positive and negative individuals.

Studies investigating CD38 expression by B-cells associated increased expression with protection against apoptosis (Hamblin et al., 2002), and suggested possible involvement of CD38 in augmenting B-cell receptor signaling (Deaglio et al., 2001). Thus, increased levels of CD19<sup>+</sup>CD38<sup>+</sup> B-cells may be associated with heightened immune response and improved host immunity. As aforementioned, QFN positive individuals are presumed to be latently infected and are thus exposed to constant antigenic stimulation (Getahun et al., 2015; WHO, 2018). This persistent stimulation could incite cellular exhaustion, resulting in compromised immune function and inapt immune responses – attributing to the obtained results of lower mean  $B_{reg}$  frequencies within the QFN positive group (Henao-Tamayo et al., 2011; Torrado et al., 2011; Wherry and Kurachi, 2015). Conversely, the observed heightened suppressive immune responses of the QFN positive group following antigenic stimulation may permit effective bacterial containment preventing progression to active disease. Further investigation into the effects of QFN status on immune cells function is necessary before any presumptions can be made regarding the potential significance of these differences.

Moreover, the frequency of killer B-cells following antigenic stimulation was investigated. An increase in mean killer-cell frequencies was observed, for both the QFN positive and negative group, following antigenic stimulation with H37Rv, CD40L and IL5, as well as CD40 and IL5 in combination with H37Rv. Similarly, increased frequencies of IL5 $\alpha$ -expression on B-cell were observed for both the QFN positive and negative group following antigenic stimulation with H37Rv, CD40L and IL5, as well as CD40 and IL5 in combination with H37Rv. Again, these results are in agreement with the BCG pilot study conducted



by van Rensburg and colleagues (van Rensburg and Loxton, 2018), in which elevated levels of killer B-cells were observed for QFN positive and negative individuals following antigenic stimulation with BCG and CD40L in combination with IL-5. Decreased killer B-cell frequencies have been associated with active TB disease; though, upon successful TB treatment these frequencies were observed to increase to levels similar to that of healthy controls (Du Plessis et al., 2016a; Joosten et al., 2016b; Van Rensburg et al., 2017; van Rensburg et al., 2017). This implies a fundamental role of killer B-cells in the successful elimination of *M.tb* infection. Unexpectedly, a shared pattern of enhanced FasL and IL5R $\alpha$  expression was observed for the QFN positive group compared to the QFN negative group, suggesting that *M.tb* exposure did not have an effect on killer B-cell development. A possible cause for the minor increase in mean killer B-cell frequencies observed for the QFN positive group may be a result of persistent *M.tb* antigenic stimulation during LTBI, resulting in mounted effector immune responses that if unrestricted, may cause severe immunopathology. Therefore, the observed responses may be a mechanism employed by LTBI participants to prevent progression to active TB. However, further investigation into this phenomenon is required before any assumptions can be made.

Fas (CD95) is a cell surface antigen expressed by T-cells following cellular activation (Arase et al., 1995; Singer and Abbas, 1994; Stalder et al., 1994a; Süss and Shortman, 1996). Fas plays an essential role in immune modulation through activation-induced cell death, in which encounter of Fas with FasL, triggers signalling cascade initiating receptor-mediated apoptosis of Fas-expressing cells (Arase et al., 1995; Lundy et al., 2015; Lundy and Boros, 2002; Stalder et al., 1994a; Süss and Shortman, 1996; van Rensburg et al., 2017; Van Rensburg et al., 2017). Studies investigating the occurrence of activation-induced cell death in CD4<sup>+</sup> T-cells has identified B-cells and NK cell as key players in mediating effector T-cell frequencies via this mechanism (Alderson and Lynch, 1998; Lundy and Boros, 2002; Sedger et al., 2002; Stalder et al., 1994b; Süss and Shortman, 1996; Tinhofer et al., 1998). Dysregulation of activation-induced elimination of effector T-cells has been associated with an imbalance of effector versus suppressor cell frequencies, impacting the ability of the immune system to adequately combat disease. Hence, the degree of Fas and FasL expression, as well as the efficiency of this regulatory process, greatly influenced disease progression and observed outcome. Considering the hypothesized role of killer B-cells during *M.tb* infection, the expression of Fas (CD95) by CD4<sup>+</sup> and CD8<sup>+</sup> T-cells was investigated following co-culture with autologous pre-stimulated B-cells. A marginal increase in mean Fas expression by CD4<sup>+</sup> and CD8<sup>+</sup> T-cells was observed for the QFN positive group following co-culture with unstimulated B-cells, as well as for the QFN negative group following co-culture of T-cells with pre-stimulated B-cells. Oppositely, a decrease in mean Fas expression by CD4<sup>+</sup> and CD8<sup>+</sup> T-cells was observed for the QFN negative group following co-culture of T-cells with unstimulated B-cells, as well as for the QFN positive group following co-culture of T-cells with pre-stimulated B-cells. Notably, a common trend of enhanced Fas expression by CD4<sup>+</sup> and CD8<sup>+</sup> T-cells for the QFN positive group was observed compared to the QFN negative group. These results, together with the observed killer B-cell

frequencies, illuminate the relative importance and degree to which killer B-cells may influence protective anti-TB immune response. These FasL-mediated mechanisms may be beneficial through regulation of effector T-cell phenotypic frequencies and eradication of cellular reservoirs that aid invading bacilli in immune evasion during *M.tb* infection.

As previously alluded to, T-cells have been regarded as key players in anti-TB immunity, with extensive supporting evidence to substantiate this statement (Cliff et al., 2015; Kaufmann, 2002; Lalvani et al., 1998; McKenzie et al., 1993; Orme et al., 1993; Ribeiro-Rodrigues et al., 2006; Schwander and Dheda, 2011; Scriba et al., 2016; Sharma et al., 2007; Stevens et al., 1988). This study does not intend to refute this claim, but rather emphasize an important fact that additional immune cells with the ability to modulate T-cell behaviour play as crucial a role in anti-TB immunity, as they primarily shape protective T-cell immune responses against *M.tb* infection. Decreased mean frequencies of TE were observed for the QFN positive and negative group following co-culture of T-cells with pre-stimulated B-cells. This is in accord with previous studies in which  $B_{reg}$  were shown to suppress Th1 and Th17 responses while promoting  $T_{reg}$  development (Carter et al., 2012, 2011; Flores-Borja et al., 2013; Kessel et al., 2012; León et al., 2014; Linton et al., 2003). Similarly, decreased mean  $T_{reg}$  frequencies were observed, for both the QFN positive and negative group, for all culture conditions in which T-cells were co-cultured with unstimulated as well as pre-stimulated B-cells. Collectively, these results support the notion that B-cells are capable of regulating T-cell function by influencing T-cell population frequencies.

Interestingly, a general pattern of enhanced TE and  $T_{reg}$  frequencies was observed for the QFN positive group compared to the QFN negative group. The induction of suppressive  $T_{regs}$  during TB disease could result in impaired ability of the host to effectively contest infection, resulting in immune evasion and progression to active disease. This notion is supported by literature, in which studies investigating the effect of  $T_{reg}$  development during TB disease revealed that depletion of these cells correlated with effective containment and improved bacterial clearance (Chen et al., 2007; Mahnke et al., 2007). This shared pattern of increased cellular frequencies of the QFN positive group for majority of the investigated cell phenotypes (with the exception of  $B_{regs}$ ) suggests dissimilarities in immune cell activation and function between *M.tb*-infected and uninfected individuals, and may allude to possible explanations as to why some individuals are able to successfully prevent *M.tb* infection, while individuals with active TB disease or LTBI, have increased susceptibility.

Recent discoveries have suggested an essential role of multifunctional  $CD4^+$  T-cells, that is T-cells that produce interferon-gamma ( $IFN\gamma$ ), tumor necrosis factor alpha ( $TNF\alpha$ ) and interleukin-2 (IL-2) simultaneously, in the fight against *M.tb* infection. Several studies have demonstrated that the induction of multifunctional  $CD4^+$  T-cells is associated with protection and better disease prognosis (Derrick et al., 2011; Lindenstrøm et al., 2009); owing to enhanced anti-TB immune responses and increased killing



capacity. Conversely, several reports have identified a correlation between elevated multifunctional CD4<sup>+</sup> T-cells frequencies and disease incidence (Caccamo et al., 2010; Forbes et al., 2008); likely due to excessive pro-inflammatory immunity resulting in immune pathology and exacerbated disease. These conflicting findings are as a result of an incomplete understanding of the complex biological processes required for effective elimination of *M.tb* infection and the spectrum of disease states within TB disease. As such, the physiological relevance of these multifunctional CD4<sup>+</sup> T-cells during *M.tb* infection, in combination with additional effector and regulatory cells, is still unclear. In some instances, in which equilibrium between pro-inflammatory and anti-inflammatory immune responses exists, multifunctional CD4<sup>+</sup> T-cells may promote bacterial killing and successful infection control. Dissimilar, in the event of impaired immune cell function and dysregulated effector cell frequencies, multifunctional CD4<sup>+</sup> T-cells may provoke pathological outcomes. Thus, the exact role of these multifunctional CD4<sup>+</sup> T-cell in driving the immune response against *M.tb* infection requires further investigation.

Current research investigating T-cell cytokine production during TB disease focuses of CD4<sup>+</sup> T-cell rather than CD8<sup>+</sup> T-cell responses, due to the nature in which *M.tb* is internalized and processed for presentation by antigen presenting cells. Essentially, bacilli are phagocytosed and processed for presentation via major histocompatibility complex (MHC) Class II, of which CD4<sup>+</sup> T-cells are the cellular targets. Whereas, CD8<sup>+</sup> T-cell activation occurs through presentation via MHC Class I, in which foreign proteins within the cytosol are tagged for processing and for presentation. A possible explanation enabling *M.tb* antigen presentation to CD8<sup>+</sup> T-cell by MHC Class I is translocation of the internalized bacilli for the phagosome to the cytosol via several resistance mechanisms. Henceforth, we aimed to investigate the cytokine profiles of both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells to gain deeper insight into the contribution of each of these cell type in anti-TB immunity. Observation within the H37Rv pilot study demonstrated the ability of pre-stimulated or naive B-cells, for both the QFN positive and negative group, to modulate CD4<sup>+</sup> and CD8<sup>+</sup> T-cell cytokine milieu, via the suppression or induction of monofunctional, bi-functional and multifunctional T-cells. Similar observations were made within the BCG pilot study, in which unstimulated as well as pre-stimulated B-cells, while insignificant, had the capacity to alter cytokine secretion profiles of both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells.

Interestingly, CD8<sup>+</sup> T-cells were observed to be the main source of enhanced multifunctional T-cell activity, drawing attention to the prominence of these cells in manipulation of resulting anti-TB immune responses. Slight discrepancies were observed when comparing the observed CD4<sup>+</sup> and CD8<sup>+</sup> T-cell cytokine profiles for the QFN positive and negative groups within the H37Rv and BCG pilot study. However, the physiological relevance of these differences was trivial, in which small alterations within cell number were observed, which would have had no noticeable effect on immune function. A study by Soares et al., (2008) showed the induction of complex T-cell cytokine profiles, including the generation of multifunctional T-cells, following stimulation of whole blood with BCG. These findings support the

observations made in this study, in which *M.tb* infection resulted in the development of intricate effector T-cell responses shown by the induction of multifaceted cytokine producing T-cells. Therefore, this study revealed the importance of B-cells in driving the anti-TB immune responses through regulation of T-cell population development. One way in which B-cells may elicit this control is via the secretion of immune modulatory cytokines that have been shown to influence T-cell function. Alternatively, cell-cell contact, enabling cell surface receptor engagement, may facilitate manipulation of T-cell behavior by B-cells. Studies investigating the relative physiological importance of each of the aforementioned mechanisms are required before any assumptions regarding this complex immune interaction can be made.

Research has demonstrated the ability of B-cell to interact with additional cell types by receptor-mediated mechanisms and via the secretion of several soluble molecules (Carter et al., 2012, 2011; DeFuria et al., 2013; Elgueta et al., 2009; Flores-Borja et al., 2013; Huard et al., 2004; Kessel et al., 2012; Kurt-Jones et al., 1988a; León et al., 2014; Linton et al., 2003; Liu et al., 2016; Lund and Randall, 2010; Lundy and Boros, 2002; Moulin et al., 2000; Ng et al., 2010; Nova-Lamperti et al., 2016; Opata et al., 2015; Siewe et al., 2013; Tadmor et al., 2011; Yoshizaki et al., 2012). As with most immune cells, the cytokine profile of B-cells is altered in response to stimulation. Hence, the cytokine profile of B-cells following stimulation and cellular activation would impact the general microenvironment in which these cells are situated, and ultimately influence the function of surrounding immune cells. Importantly, each of these molecules have varying effects on T-cell activation; several cytokines may induce a pro-inflammatory immune response via the induction of Th1 and Th17 responses (Lund and Randall, 2010; Mangan et al., 2006), while others stimulate an anti-inflammatory Th2 response or the development of a regulatory T-cell phenotype (Linton et al., 2003). As one may recall, a function of B-cells includes antigen presentation (Crawford et al., 2006; Heesters et al., 2016; Kurt-Jones et al., 1988a; Lanzavecchia, 1985); therefore B-cells are among the first immune cell types within adaptive immunity to respond to antigenic challenge. In this way, B-cells activation may greatly impact resultant T-cell function, through co-stimulation via peptide presentation, co-receptor engagement and cytokine expression.

A significant increase in the expression of several cytokines (APRIL, ICAM-1, IFN $\gamma$ , IFN- $\beta$ , , IL-1 $\beta$ , IL-2, IL-4, IL-10, IL-12 p70 and IL21) was observed following co-culture of B-cells stimulation with H37Rv compared to unstimulated controls and B-cells pulsed with CD40 and IL5, suggests the induction of a heightened immune response following infection with *M.tb*. Within the host, a dynamic relationship exists between cytokine production and resultant immune responses. In the event of a physiological stimulus, transient cytokine production and consequential immune responses arises to counteract shifts within the host biological system and return the cellular environment to homeostasis to maintain a state of health (Bocci, 1988). As such, the secretion of these cytokines is dependent on the stimulus

encountered. IFN production is onset following viral or bacterial infection and is responsible for containment of pathogens for effective elimination. IFN $\gamma$  in particular, has been profoundly associated to *M.tb* infection, in which its relative production by T-cells has been linked to effective TB disease control; whilst copious secretion has been linked to increased disease severity (Bocci, 1988; Dlugovitzky et al., 1999). IL-1 $\beta$  has been revealed as an important mediator of pro-inflammatory immune responses and is involved in a variety of functions including cellular proliferation, differentiation, activation and cellular communication (Awomoyi et al., 2005). Similarly, IL12 and 21 secretion are observed to promote the production of IFN $\gamma$  and tumor necrosis factor alpha (TNF $\alpha$ ), thereby induces differentiation of T-cells into Th1 cells (Cooper et al., 1997; Flynn et al., 1995; Korn et al., 2007).

Together, secretion of the abovementioned cytokines by B-cells would induce pro-inflammatory effector T-cells in response to the investigated stimulatory conditions. These responses have been shown to be essential during the initial stages of *M.tb* infection, stressing the prominent role B-cells may play during TB disease. On the other hand, IL-2, 4 and 10 have been associated with immune tolerance and the induction suppressive anti-inflammatory responses (Li et al., 2001; North, 1998; Redford et al., 2011). Although, IL-2 has been found to promote effector T-cell differentiation during initial stages of infection - aiding in fighting off infection contributing to the induction of Th1 cells (Millington et al., 2007). Similarly, APRIL, IL-10, IL-21 and IL-4 have been shown to play a crucial role in B-cell proliferation, differentiation and isotype switching – all processes that are crucial for protective anti-TB immunity during the early stages of infection (Aversa et al., 1993; Bryant et al., 2007; Rousset et al., 1992; Vincent et al., 2013).

Differences between cytokine profiles of B-cells cultured with/without autologous T-cells was investigated to determine the effect of co-culture on the resulting immune environment. An insignificant increase in the expression of BAFF and TGF- $\beta$ 1 by QFN positive individuals was observed compared to QFN negative individuals, wherein BAFF and TGF- $\beta$ 1 production was observed to be dependent on the presence of B-cells within the sample, as shown by the significant decrease in these cytokine levels in supernatants where T-cells were cultured alone compared to all other conditions where autologous B-cells were present. B-cell activating factor (BAFF) is a member of the TNF ligand family and plays an important role in B-cell function by promoting of B-cell proliferation and differentiation (Chen et al., 2009; Van Rensburg et al., 2017; Vincent et al., 2013). Similarly, TGF- $\beta$ 1 is responsible for regulation of immune responses by controlling cellular proliferation and differentiation of several immune cell types (Biancheri et al., 2014). Thus, the relative expression of these cytokines by B-cells may contribute substantially to the proposed beneficial role of B-cells during TB disease. Furthermore, a significant increase in sFasL, ICAM-1, IFN- $\beta$ , IL-2 and IL-17 was observed for QFN negative individuals in response

to antigenic stimulation compared to QFN positive individuals, in which sFasL expression was enhanced following culture of B-cells with autologous T-cells.

The secretion of sFasL by B-cells has been widely investigated by Lundy and colleagues (Lundy, 2009; Lundy et al., 2015), in which they describe its secretion as a mechanism of immune regulation by B-cells. It is insinuated that FasL-expressing B-cells would have a beneficial impact on TB disease prognosis through the regulation of pro-inflammatory immune responses that if left unrestrained, result in immune pathology and exacerbated disease conditions (Lundy, 2009; van Rensburg et al., 2017; Van Rensburg et al., 2017; van Rensburg and Loxton, 2018a). A likely mechanisms by which these B-cells may mediate this protection is through FasL-mediated apoptosis of activated CD4<sup>+</sup> T-cells (Alderson and Lynch, 1998; Lundy and Boros, 2002; Sedger et al., 2002; Stalder et al., 1994b; Süß and Shortman, 1996; Tinhofer et al., 1998) that develop as a result of antigenic stimulation. This theory of immune regulation by B-cells is in agreement with studies investigating the role of FasL-expressing B-cells during autoimmunity and parasitic infection (Chervonsky et al., 1997; De Maria and Testi, 1998; Hu et al., 1999; Zhang et al., 2008). Additionally, ICAM-1 expression has been revealed to play an important role in mycobacterial uptake and antigen processing. Studies have found increased expression of ICAM-1 following infection of macrophages with *M.tb* (Bohnet et al., 1994; Ghosh and Saxena, 2004). It has been suggested that this upregulation facilitates protective anti-TB immune responses by macrophages during *M.tb* infection. Thus, this significant decline in sFasL and ICAM-1 expression by QFN positive individuals indicates possible dysregulation of crucial anti-TB mechanisms attributing to increased susceptibility to *M.tb* infection (Ghosh and Saxena, 2004). Moreover, IFN- $\beta$  production has been associated with enhanced IFN- $\gamma$  secretion during *M.tb* infection, and is suspected to assist in effective anti-TB immunity (Zhang et al., 2018). A clinical trial investigating the use of IL-2 immunotherapy in TB disease indicated enhanced bacterial clearance, suggesting potential role in anti-TB immunity (Johnson et al., 1997, 1995). In summary, these findings illustrate the dysregulation of cytokine expression by QFN positive individuals and underlines possible protective cellular functions irregularly during TB disease that facilitate the establishment of infection and progression to active disease.

It is important to note the presence of contaminating platelets (median proportion of total sample content 58.15%; range 29.1 - 87.5%) within isolated B-cells samples prior to co-culture with autologous T-cells (Suppl Figure 4.13). Evidence has shown that platelets contribute to the *in vitro* cellular microenvironment and have the capacity to modulate the function of B- and T-cells (Sowa et al., 2009). One way in which platelets achieve this is via the expression of cytoplasmic mRNA encoding (Denis et al., 2005; Power et al., 1995; Soslaw et al., 1997) for various proteins, such as cytokines, known to effect the activation and function of surrounding immune cells (Elzey et al., 2005; Kral et al., 2016). As such, these observed findings may have been influenced by the incidence of contaminating platelets and should be interpreted with this in mind. Additionally, the effects of *ex vivo* manipulation on the observed

results must be taken into consideration as artefactual observations may have incurred that do not reflect physiologically relevant events within whole organism.

## **5. Concluding Remarks**

In our studies, B-cells had the ability to modulate T-cell function, and thus play an important role in directing the immune response against *M.tb* infection. Collectively, the results demonstrate the ability of B-cells to modulate T-cell activation and function through control of T-cell population dynamics and cytokine profiles. This emphasizes the importance of investigating the role of additional cell types within TB disease incidence and progression, previously thought to have minimal contribution to the general disease outcome. The physiological implications of the results includes the possibility for improved vaccine design through targeting of B-cell function to induce protective CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses that facilitate superior protection from *M.tb* infection compared to the current commercially available BCG vaccine. Despite this, the results described in this paper serve as preliminary data and further investigation into the mechanisms of this multifaceted cellular interaction is required, in which a larger sample size and the addition of an active TB cohort are included, before any definitive assumptions can be made.

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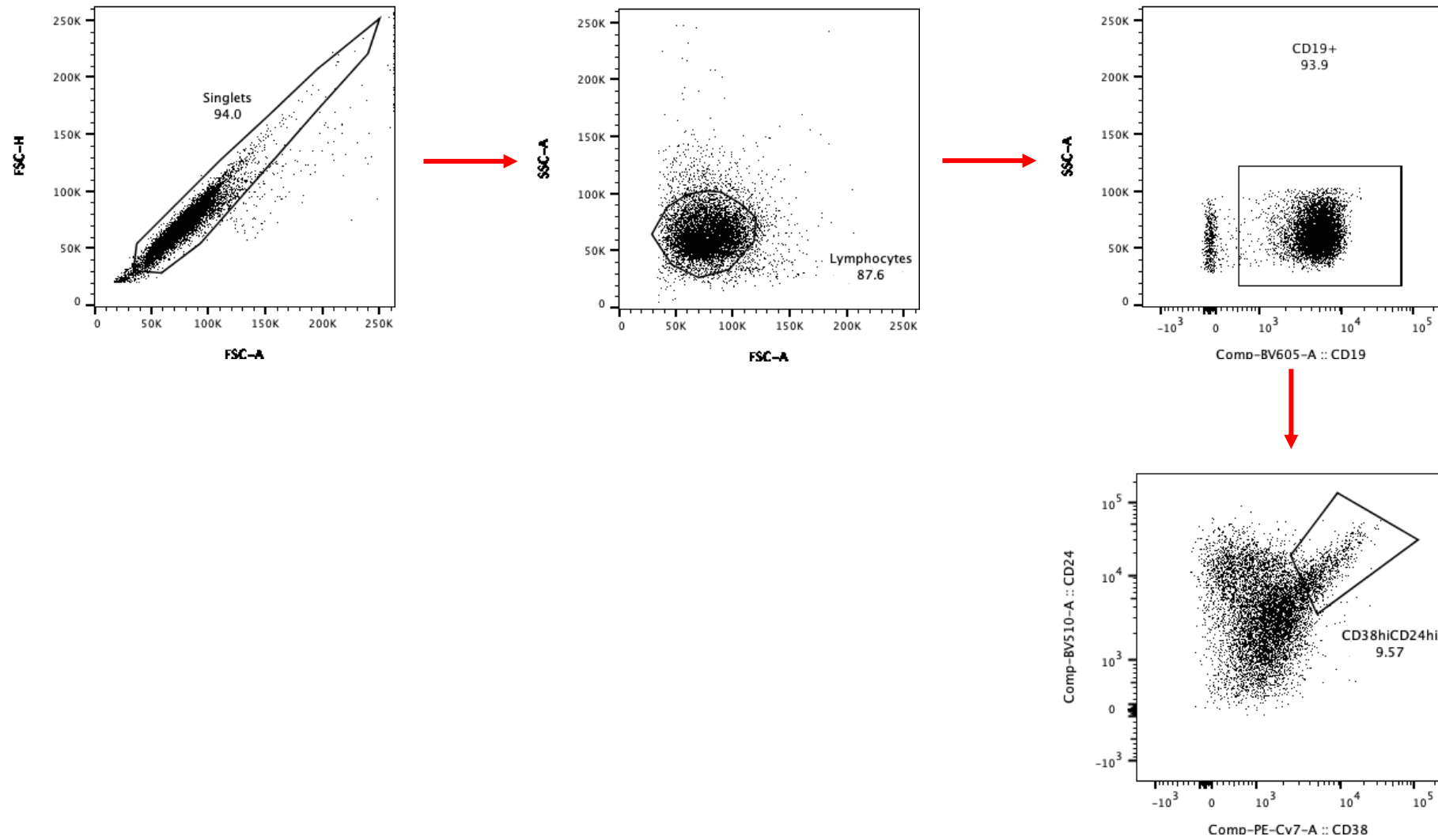
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**Supplementary Information**

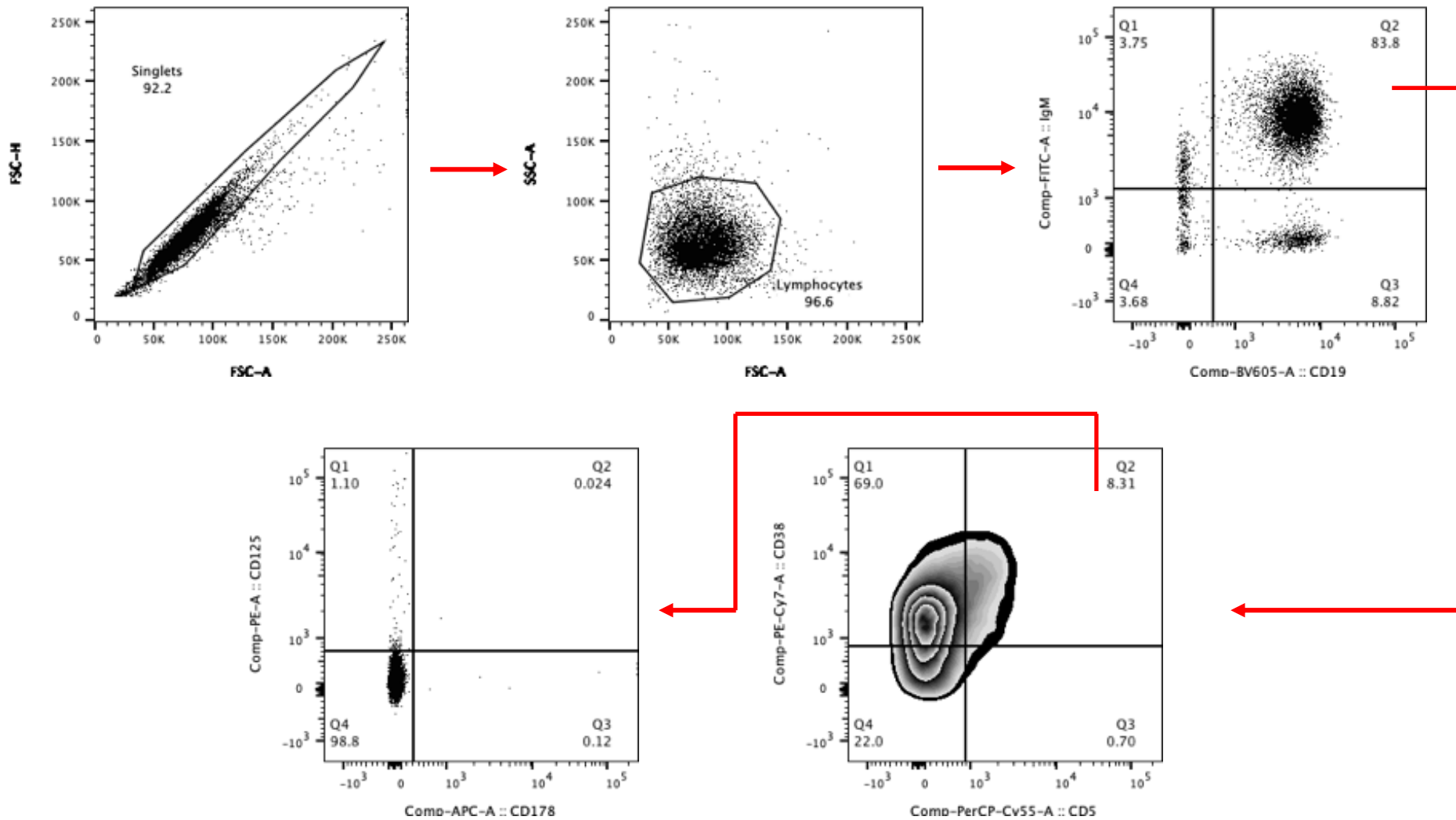
<i>Condition</i>	<i>B-cells</i>	<i>BCG or H37Rv</i>	<i>CD40L &amp; IL5</i>	<i>T-cells</i>
<b>1</b>	X			
<b>2</b>	X	X		
<b>3</b>	X		X	
<b>4</b>	X	X	X	
<b>5</b>				X
<b>6</b>	X			X
<b>7</b>	X	X		X
<b>8</b>	X		X	X
<b>9</b>	X	X	X	X
<b>10</b>		X		X

**Suppl Table 4.1. Description of stimulatory and co-culture conditions.** B-cells were seeded at a concentration of  $2.5 \times 10^5$ /well, while T-cells were seeded at a concentration of  $5 \times 10^5$ /well in a 96-well round bottom plate in a total volume of 200uL. The BCG pilot study utilized BCG ( $1.25 \times 10^6$  CFU/mL) as their *M.tb* antigen, while the H37Rv pilot study stimulated cells with the virulent *M.tb* laboratory strain H37Rv (MOI 10:1). Where applicable, IL-5 (Sigma, USA) was added at 50 ng/ml and CD40L (Sigma, USA) at 2ug/ml. Stimulatory condition 10, indicated in red, was incorporated into the BCG pilot study only. As such comparison between the studies for this condition cannot be made.



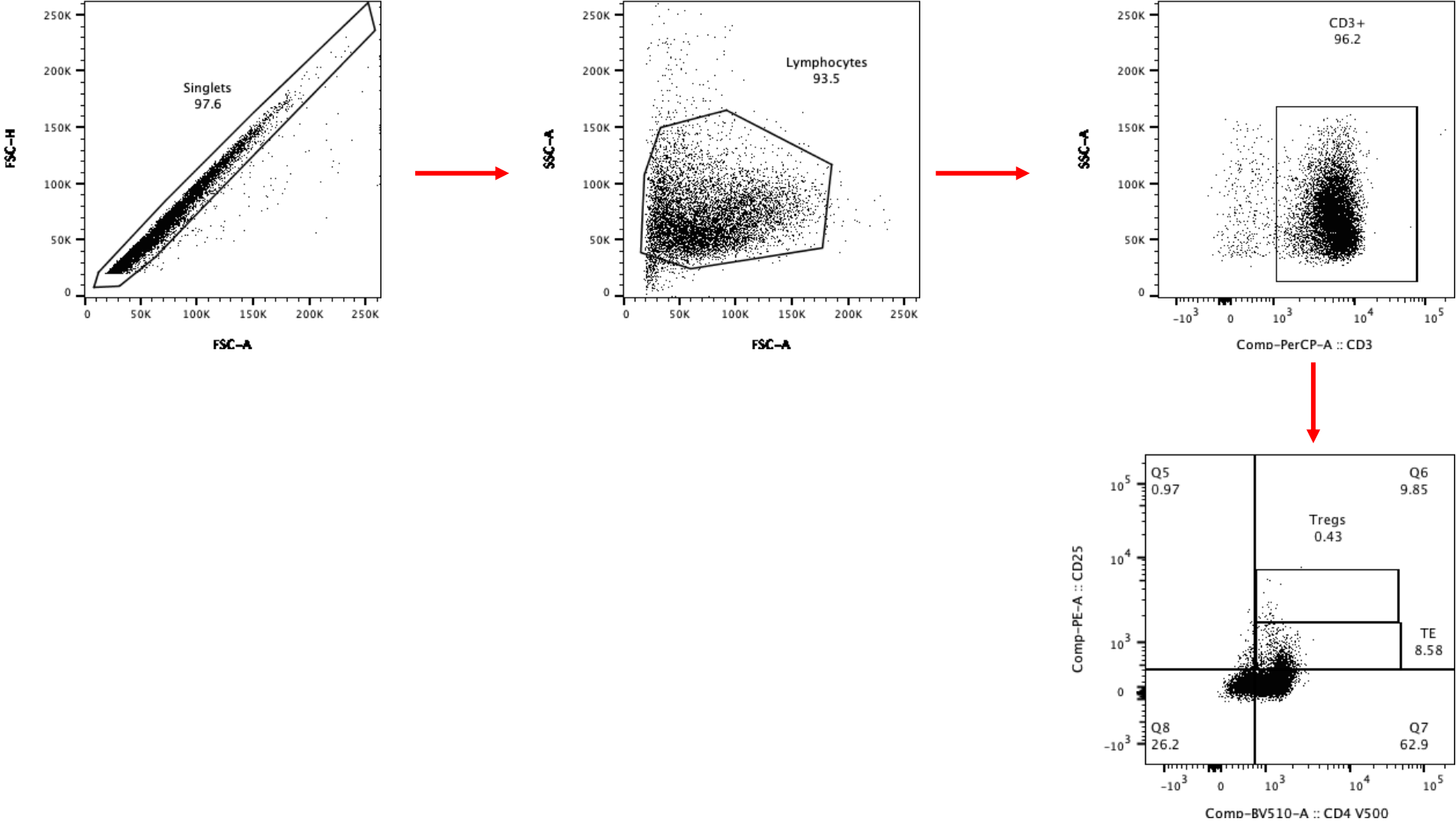
Suppl Figure 4.1. Gating strategy for Regulatory B-cells for the H37Rv pilot study

\*Note: Fluorescence-minus-one (FMO) control samples were used to determine appropriate gating cut-off to increase accuracy of distinguishing between populations



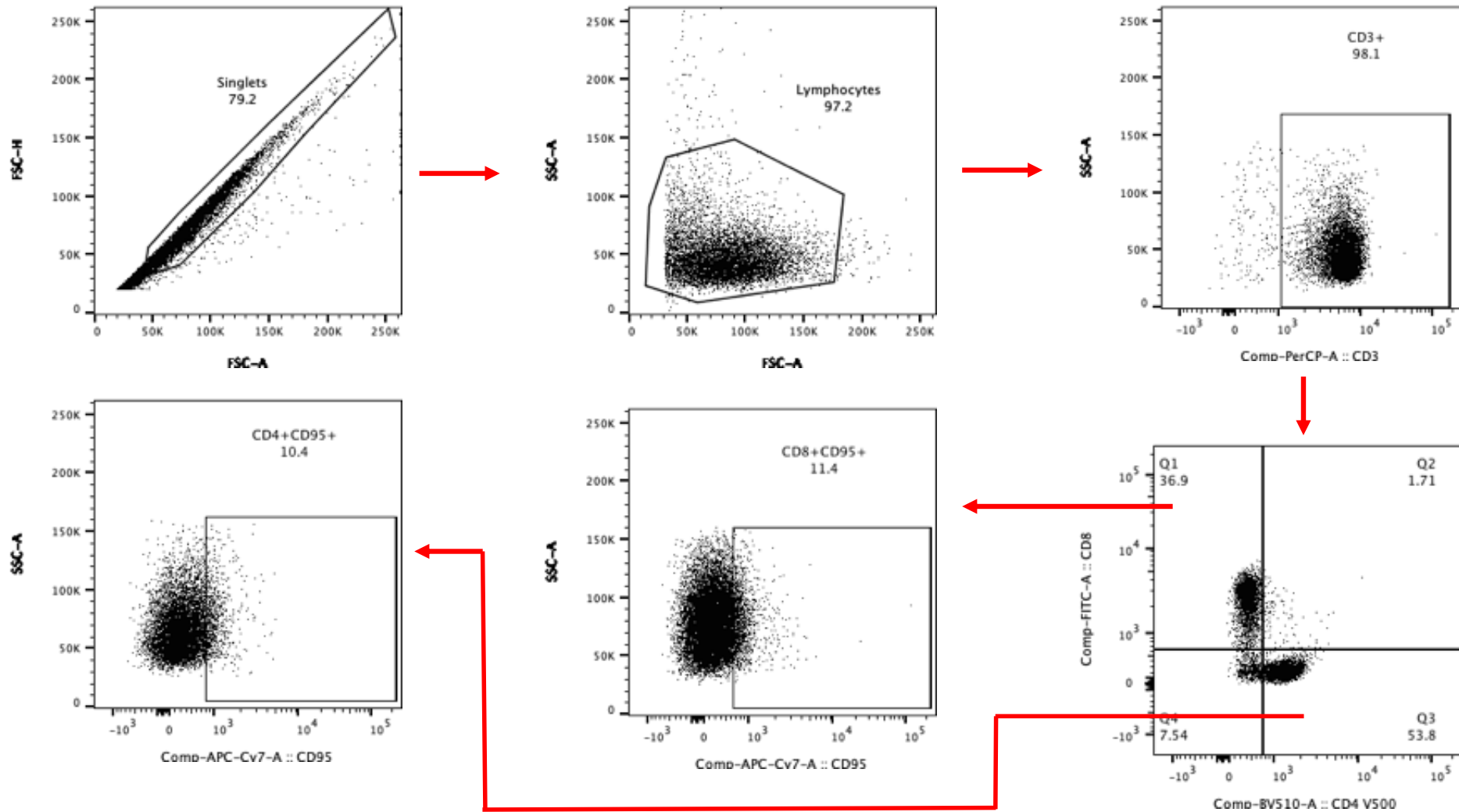
Suppl Figure 4.2. Gating strategy for Killer B-cells for H37Rv pilot study

\*Note: Fluorescence-minus-one (FMO) control samples were used to determine appropriate gating cut-off to increase accuracy of distinguishing between populations



Suppl Figure 4.3. Gating strategy for Effector and Regulatory T-cells for the H37Rv and BCG pilot study

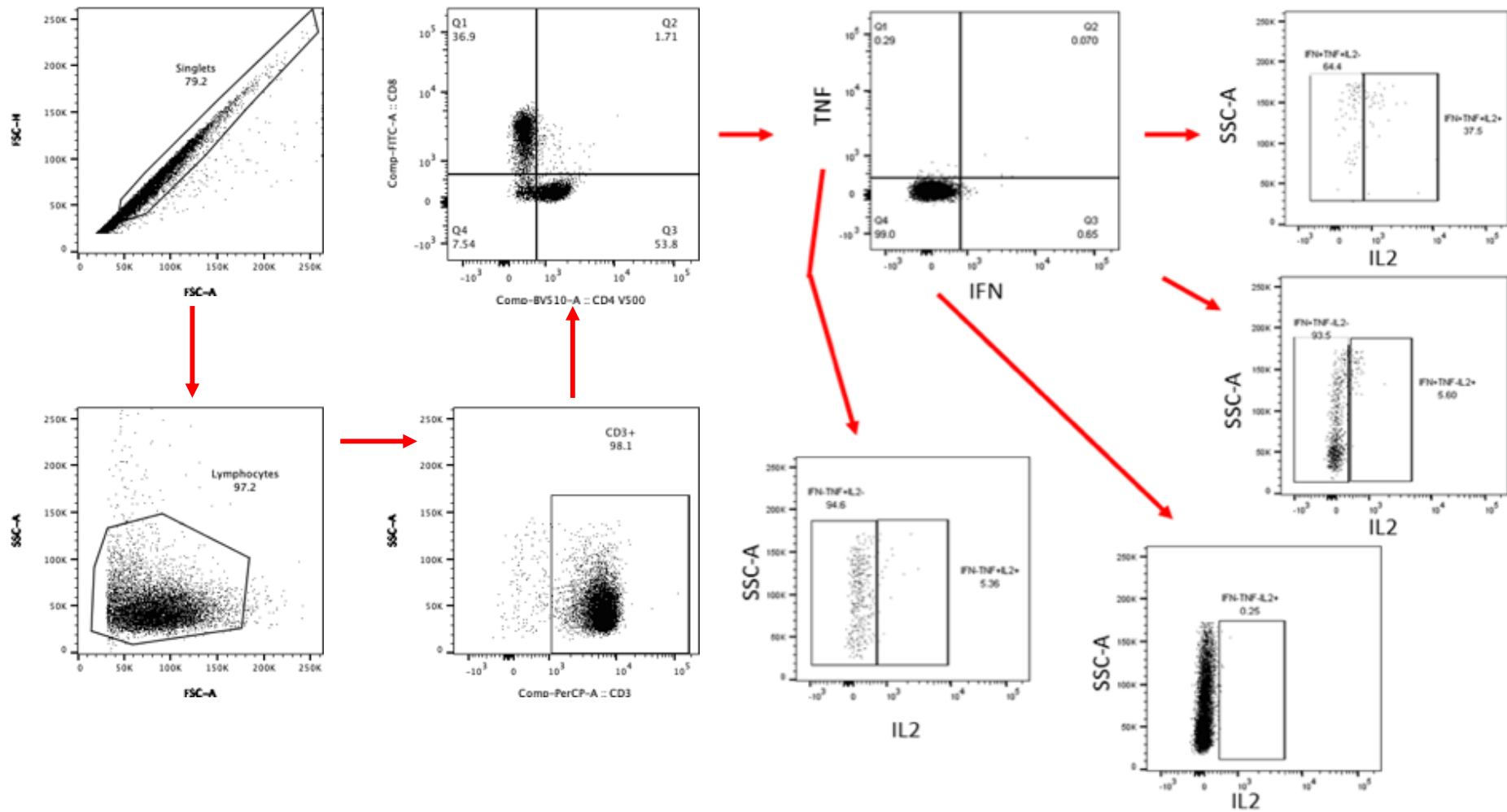
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Suppl Figure 4.4. Gating Strategy for Fas-expressing T-cells for the H37Rv pilot study

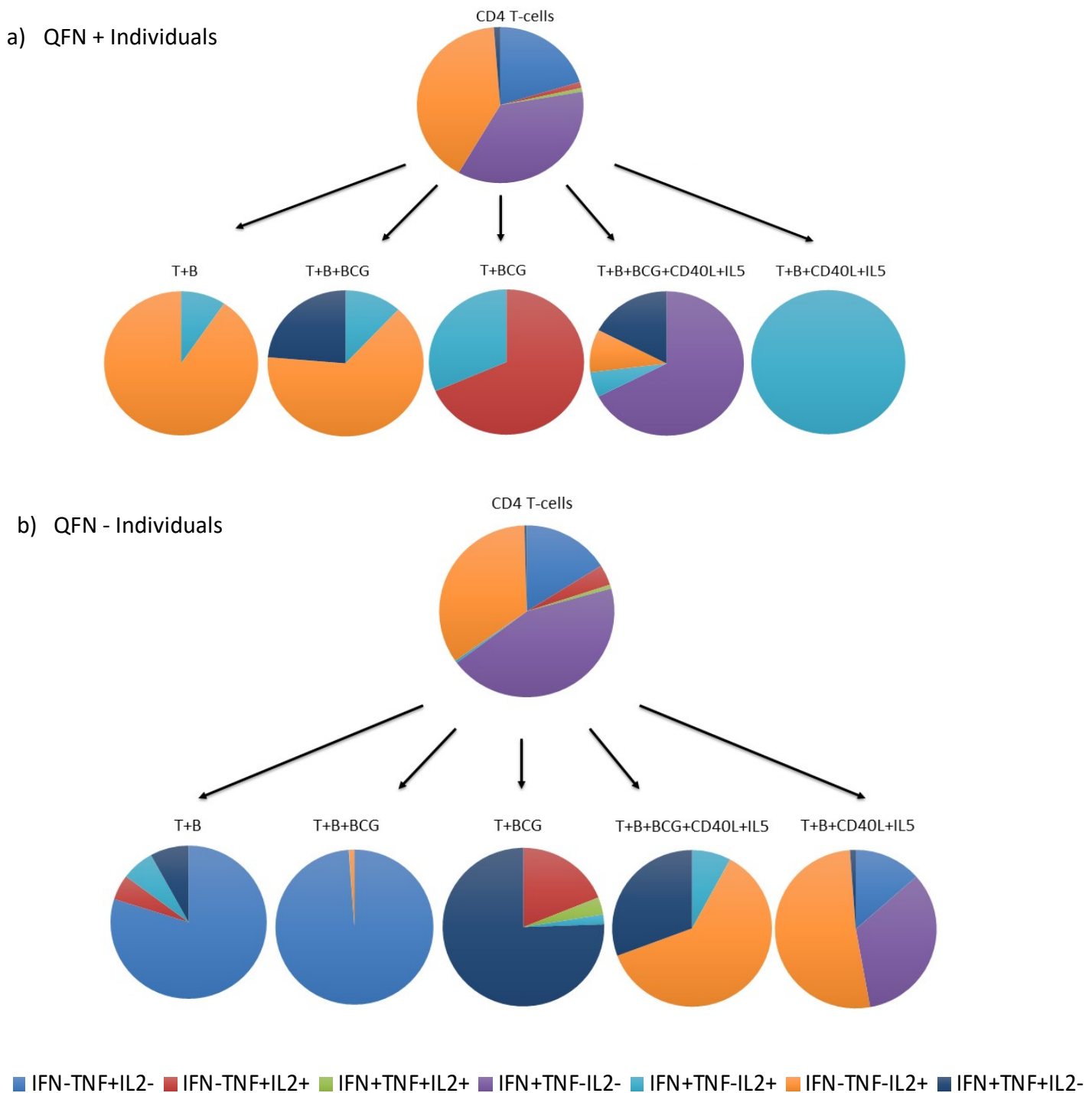
\*Note: Fluorescence-minus-one (FMO) control samples were used to determine appropriate gating cut-off to increase accuracy of distinguishing between populations



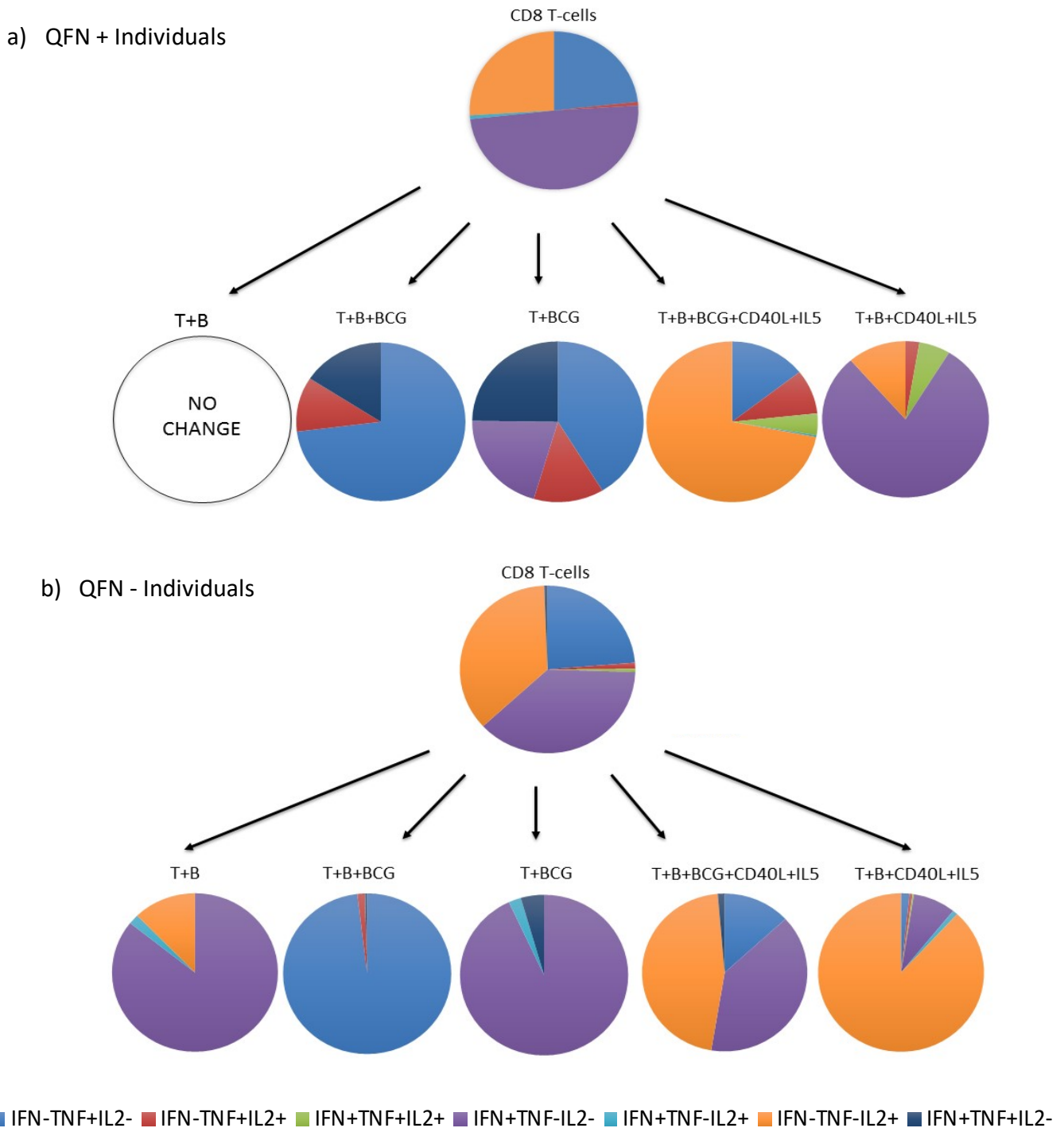


Suppl Figure 4.5. Gating strategy for cytokine expression evaluation of T-cells for the H37Rv and BCG pilot study

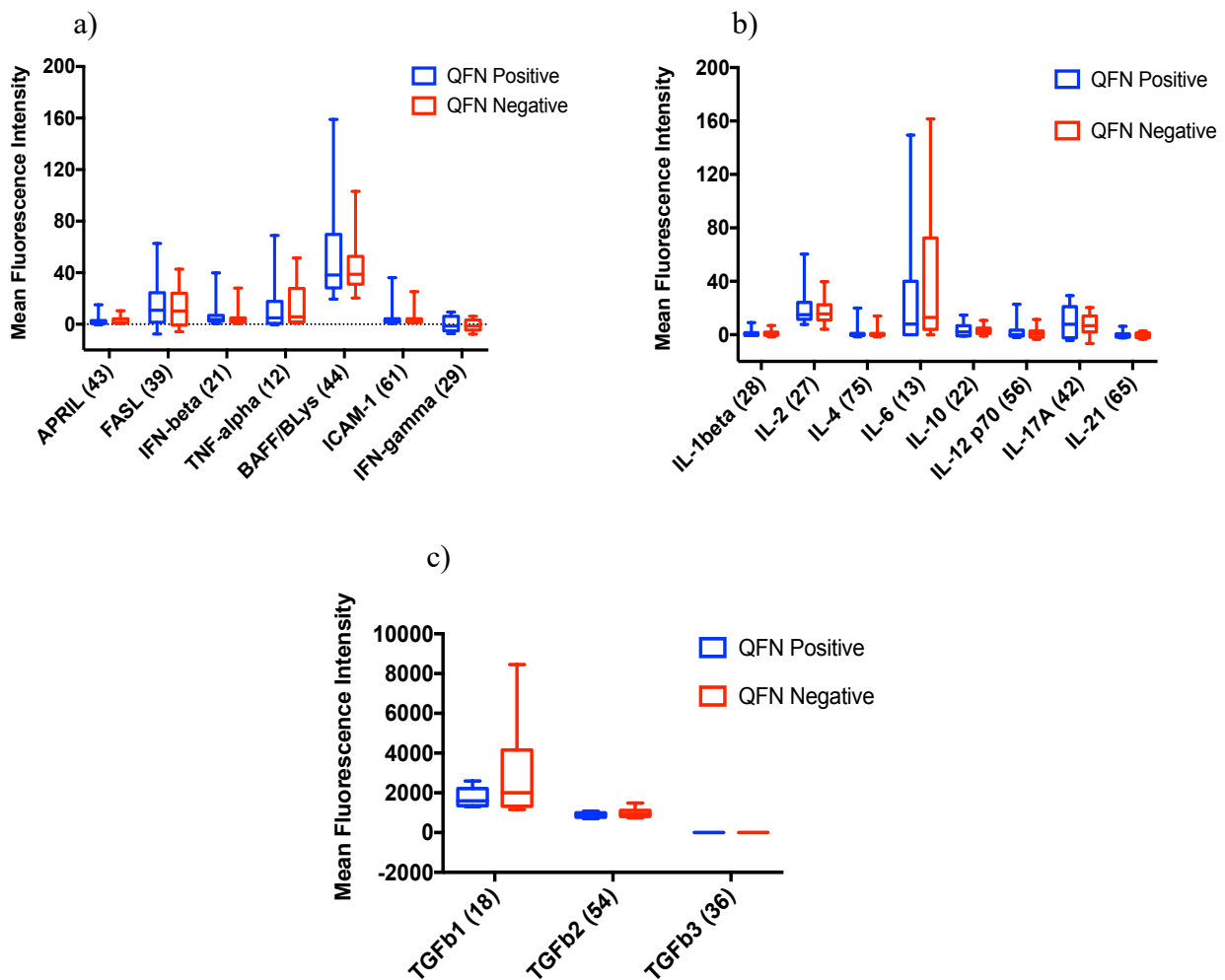
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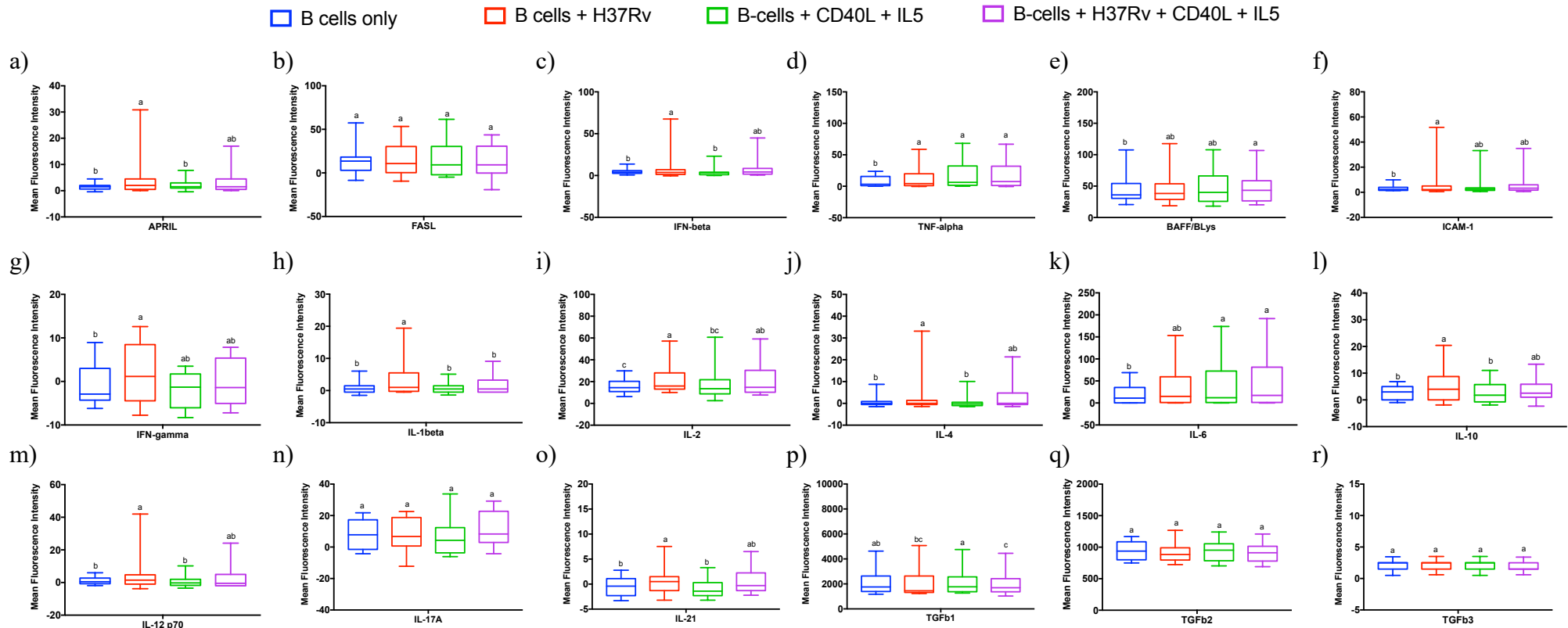
**Suppl Figure 4.6. Cytokine secretion profile of CD4<sup>+</sup> T-cells following co-culture with pre-stimulated B-cells for the BCG pilot study.** The proportion of cells expressing various combinations of cytokines is calculated as a function of the total frequency of cytokine producing cells. Alterations in cytokine production for each of the culture conditions was compared to the control condition consisting of T-cells only. The control condition served as a representation of basal cytokine production levels; these background levels were subtracted from the various stimulatory conditions and the resulting profiles reported (a) Cytokine profiles of CD4<sup>+</sup> T-cells for each of the stimulatory conditions observed for QFN positive individuals. (b) Cytokine profiles of CD4<sup>+</sup> T-cells for each of the stimulatory conditions observed for QFN negative individuals.



**Suppl Figure 4.7. Cytokine secretion profile of CD8<sup>+</sup> T-cells following co-culture with pre-stimulated B-cells for the BCG pilot study.** The proportion of cells expressing various combinations of cytokines is calculated as a function of the total frequency of cytokine producing cells. Alterations in cytokine production for each of the culture conditions was compared to the control condition consisting of T-cells only. The control condition served as a representation of basal cytokine production levels; these background levels were subtracted from the various stimulatory conditions and the resulting profiles reported (a) Cytokine profiles of CD8<sup>+</sup> T-cells for each of the stimulatory conditions observed for QFN positive individuals. (b) Cytokine profiles of CD8<sup>+</sup> T-cells for each of the stimulatory conditions observed for QFN negative individuals.



**Suppl Figure 4.8. Effect of QuantiFERON status on cytokine secretion by B-cells following antigenic stimulation for the H37Rv Pilot study.** Cell culture supernatants were collected at different time points, namely 3-hours pre-co-culture (Time point 1) and 19-hours post-co-culture with autologous T-cells (Time point 2). Whisker denote 10-90 percentile. Statistical differences between culture conditions was calculated using a four-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR), with a FDR of 1%, was used to correct for multiple testing. Statistical significance is indicated by an asterisk, in which  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*). (a) Effect of culture time on B-cell secretion factors following antigenic stimulation of B-cells. (b) Effect of culture time on interleukin (IL) production following antigenic stimulation of B-cells. (c) Effect of culture time on TGF $\beta$  isotype secretion following antigenic stimulation of B-cells.

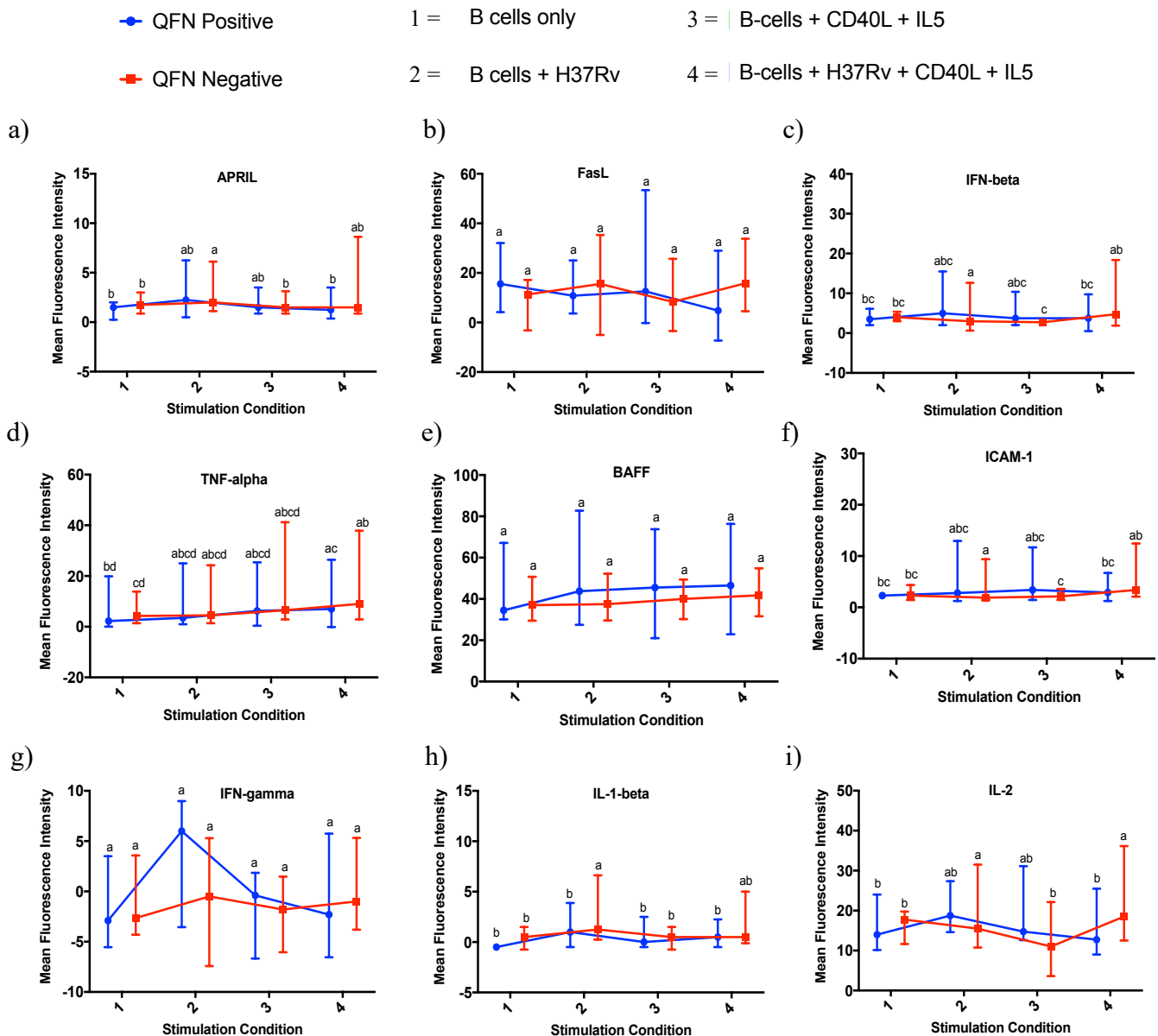


**Suppl Figure 4.9. Effect of stimulation condition on cytokine secretion by B-cells following antigenic stimulation for the H37Rv Pilot study.**

Cell culture supernatants were collected at different time points, namely 3-hours pre-co-culture (Time point 1) and 19-hours post co-culture with autologous T-cells (Time point2). Whisker denote 10-90 percentile. Statistical differences between culture conditions was calculated using a four-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR), with a FDR of 1%, was used to correct for multiple testing. Statistical differences are indicated by letters, in which groups denoted with different letters indicate statistical differences. Effect of stimulation condition on secretion of (a) APRIL (b) soluble FasL (c) IFN- $\beta$  (d) TNF- $\alpha$  (e) BAFF (f) ICAM-1 (g) IFN- $\gamma$  (h) IL-1 $\beta$  (i) IL-2 (j) IL-4 (k) IL-6 (l) IL-10 (m) IL-12 p70 (n) IL-17A (o) IL-21 (p) TGF $\beta$ -1 (q) TGF $\beta$ -2 (r) TGF $\beta$ -3

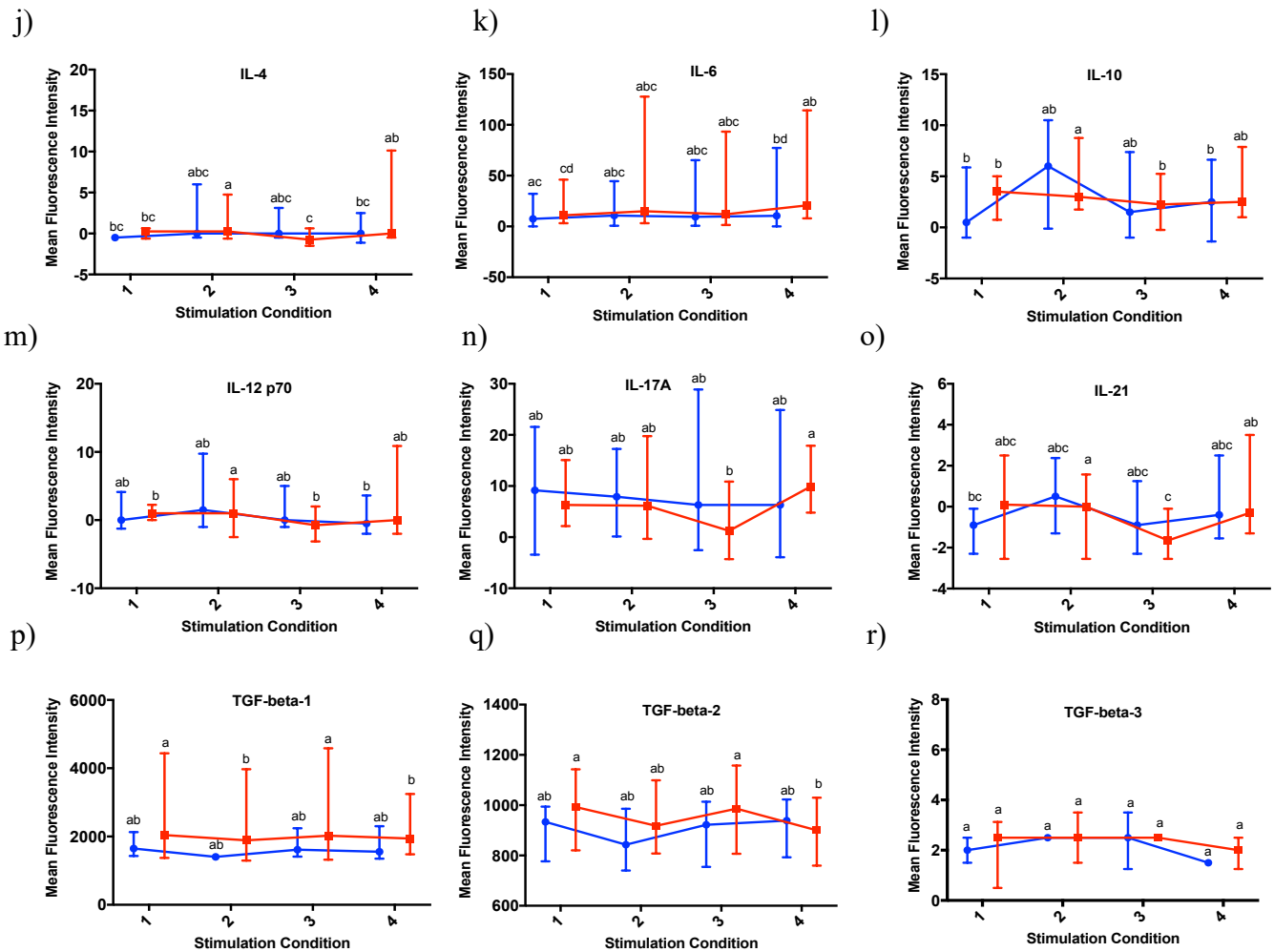
<i>Analyte</i>	<i>Time Point</i>		<i>Stimulation</i>		<i>QFN Status</i>		<i>Stimulation*</i>	
	<i>P-value</i>	<i>q-value</i>	<i>P-value</i>	<i>q-value</i>	<i>P-value</i>	<i>q-value</i>	<i>P-value</i>	<i>q-value</i>
<i>APRIL</i>	0.00212	0.00396	0.6774	0.76970	0.55541	0.99141	0.12716	0.19822
<i>sFasL</i>	0.08274	0.05969	0.86329	0.87192	0.97053	0.99141	0.13118	0.19822
<i>IFN-β</i>	0.00143	0.00396	0.04686	0.10635	0.68867	0.99141	0.03755	0.16292
<i>TNF-α</i>	0.01008	0.00898	0.02728	0.10635	0.84289	0.99141	0.87777	0.89657
<i>BAFF</i>	0.02955	0.02296	0.18945	0.24601	0.51968	0.99141	0.78763	0.89657
<i>ICAM-1</i>	0.00347	0.00438	0.05850	0.10635	0.92505	0.99141	0.02657	0.16101
<i>IFN-γ</i>	0.00506	0.00568	0.13859	0.20188	0.67763	0.99141	0.49327	0.64055
<i>IL-1β</i>	0.00205	0.00396	0.03131	0.10635	0.49703	0.99141	0.08817	0.19822
<i>IL-2</i>	0.00885	0.00894	0.00991	0.10635	0.65602	0.99141	0.00280	0.05090
<i>IL-4</i>	0.00235	0.00396	0.05243	0.10635	0.71816	0.99141	0.05377	0.16292
<i>IL-6</i>	0.01067	0.00898	0.01173	0.10635	0.57861	0.99141	0.82375	0.89657
<i>IL10</i>	0.00202	0.00396	0.03882	0.10635	0.60442	0.99141	0.14055	0.19822
<i>IL-12 p70</i>	0.00283	0.00408	0.09407	0.15547	0.82944	0.99141	0.10820	0.19822
<i>IL-17A</i>	0.37638	0.23759	0.49002	0.59390	0.96870	0.99141	0.14174	0.19822
<i>IL-21</i>	0.00073	0.00396	0.05489	0.10635	0.51599	0.99141	0.08296	0.19822
<i>TGF-β1</i>	0.12276	0.08266	0.02339	0.10635	0.40323	0.99141	0.01036	0.09417
<i>TGF-β2</i>	0.45872	0.27253	0.14436	0.20188	0.43993	0.99141	0.04925	0.16292
<i>TGF-β3</i>	0.71364	0.40043	0.82922	0.87192	0.98159	0.99141	0.88769	0.89657

**Suppl Table 4.2. Evaluation of cytokine secretion by B-cells at different time points following antigenic stimulation for the H37Rv Pilot study.** Cell culture supernatants were collected at different time points, namely 3-hours pre-co-culture (Time point 1) and 19-hours post-co-culture with autologous T-cells (Time point 2). Statistical differences between culture conditions was calculated using a four-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistically significant p-values are highlighted in red. The adjusted p-value is denoted 'q-value'.



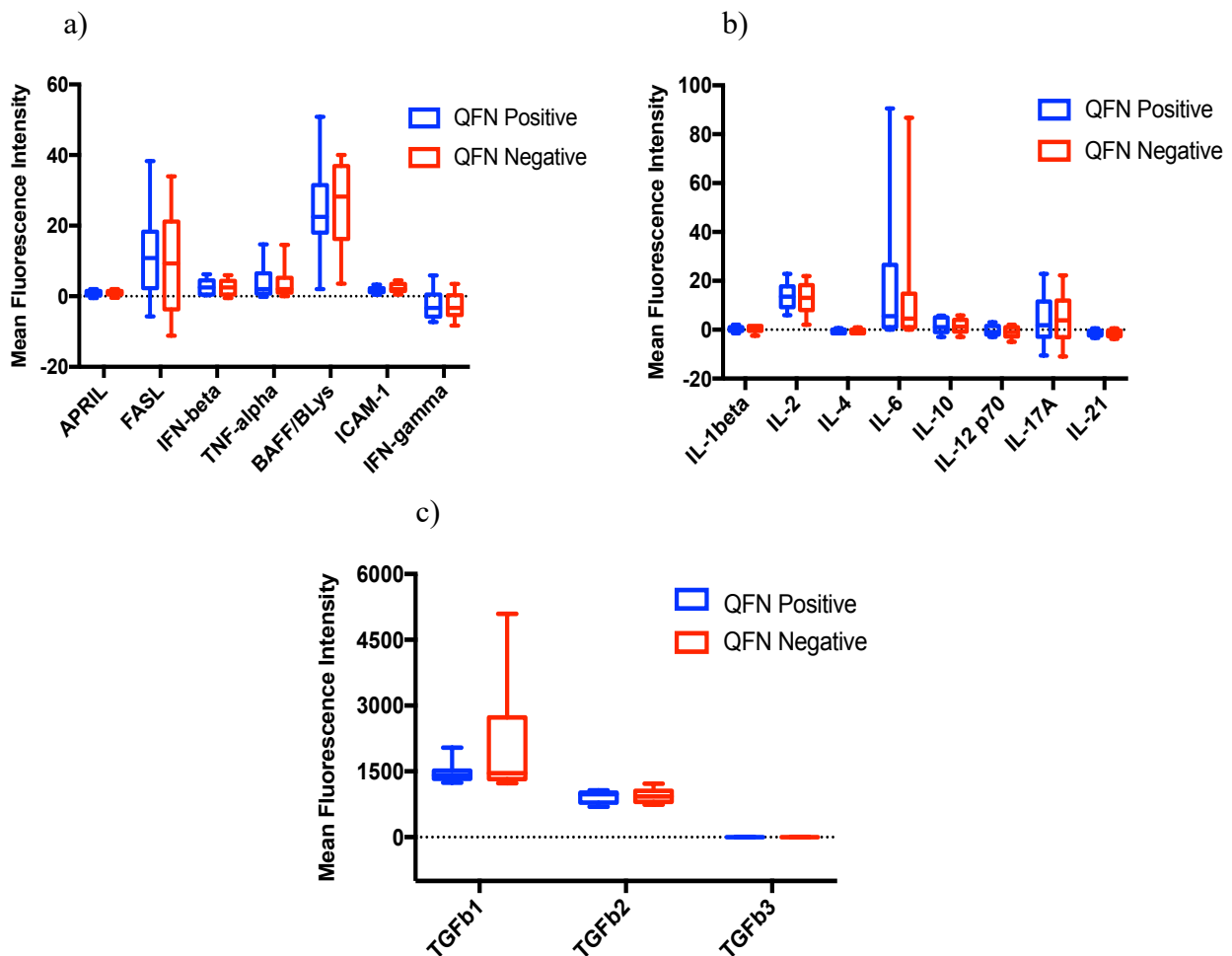
**Suppl Figure 4.10. Effect of stimulation condition and QuantIFERON status in combination on cytokine secretion by B-cells following antigenic stimulation for the H37Rv Pilot study.** Cell culture supernatants were collected at different time points, namely 3-hours pre-co-culture (Time point 1) and 19-hours post co-culture with autologous T-cells (Time point 2). Median with interquartile range plotted. Statistical differences between culture conditions was calculated using a four-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistical differences are indicated by letters, in which groups denoted with different letters indicate statistical differences. Effect of stimulation condition and QFN status in combination on secretion of (a) APRIL (b) soluble FasL (c) IFN- $\beta$  (d) TNF- $\alpha$  (e) BAFF (f) ICAM-1 (g) IFN- $\gamma$  (h) IL-1 $\beta$  (i) IL-2

● QFN Positive                      1 = B cells only                      3 = B-cells + CD40L + IL5  
■ QFN Negative                      2 = B cells + H37Rv                      4 = B-cells + H37Rv + CD40L + IL5

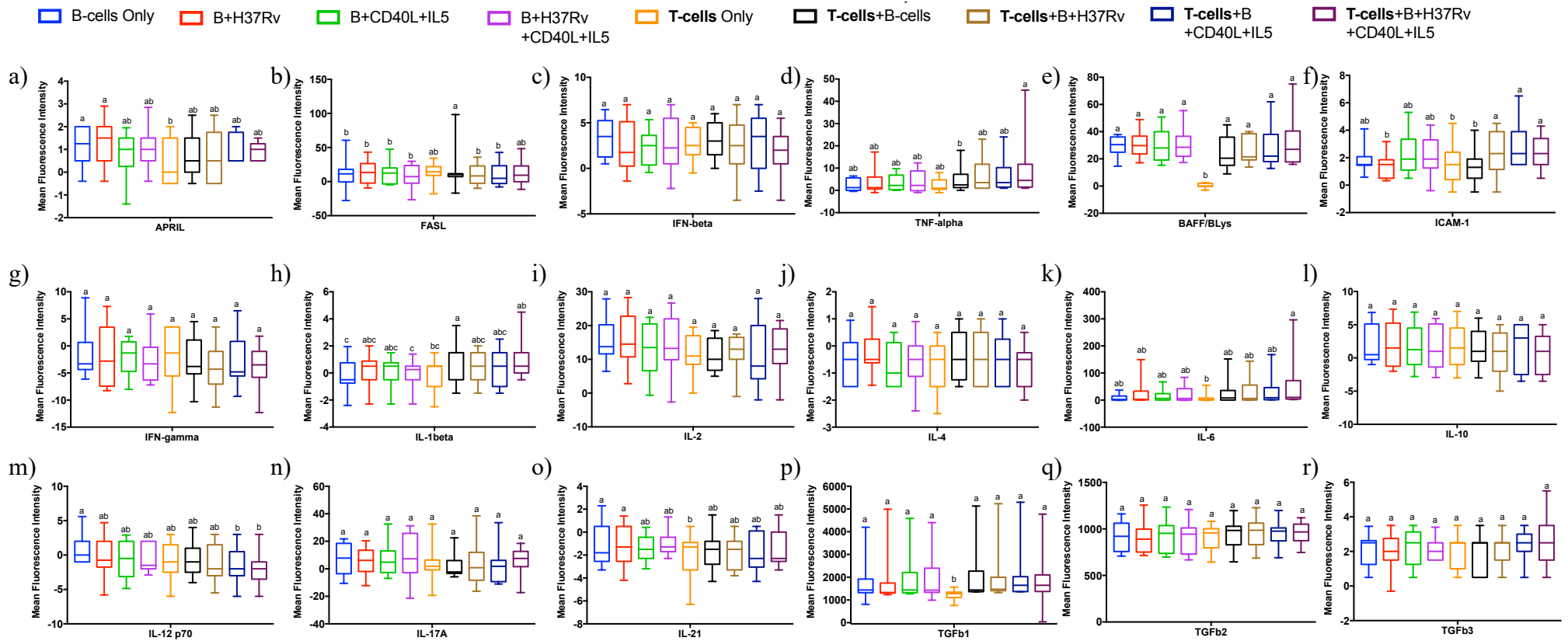


**Suppl Figure 4.10 continued. Effect of stimulation condition and QFN status in combination on cytokine secretion by B-cells following antigenic stimulation for H37Rv Pilot study.** Cell culture supernatants were collected at different time points, namely 3-hours pre-co-culture (Time point 1) and 19-hours post-co-culture with autologous T-cells (Time point 2). Median with interquartile range plotted. Statistical differences between culture conditions was calculated using a four-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistical differences are indicated by letters, in which groups denoted with different letters indicate statistical differences. Effect of stimulation condition and QFN status in combination on secretion of (j) IL-4 (k) IL-6 (l) IL-10 (m) IL-12 p70 (n) IL-17A (o) IL-21 (p) TGFβ-1 (q) TGFβ-2 (r) TGFβ-3.





**Suppl Figure 4.11. Effect of QuantiFERON status on cytokine secretion profiles of B- and T-cells following co-culture for the H37Rv Pilot study.** Cell culture supernatants were collected 19-hours post-co-culture with/without autologous T-cells (Time point 2). Whisker denote 10-90 percentile. Statistical differences between culture conditions was calculated using a three-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistical significance is indicated by an asterisk, in which  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*). (a) Effect of QFN status on B-cell secretion factors following antigenic stimulation of B-cells. (b) Effect of QFN status on interleukin (IL) production following antigenic stimulation of B-cells. (c) Effect of QFN status on TGF- $\beta$  isotype secretion following antigenic stimulation of B-cells.

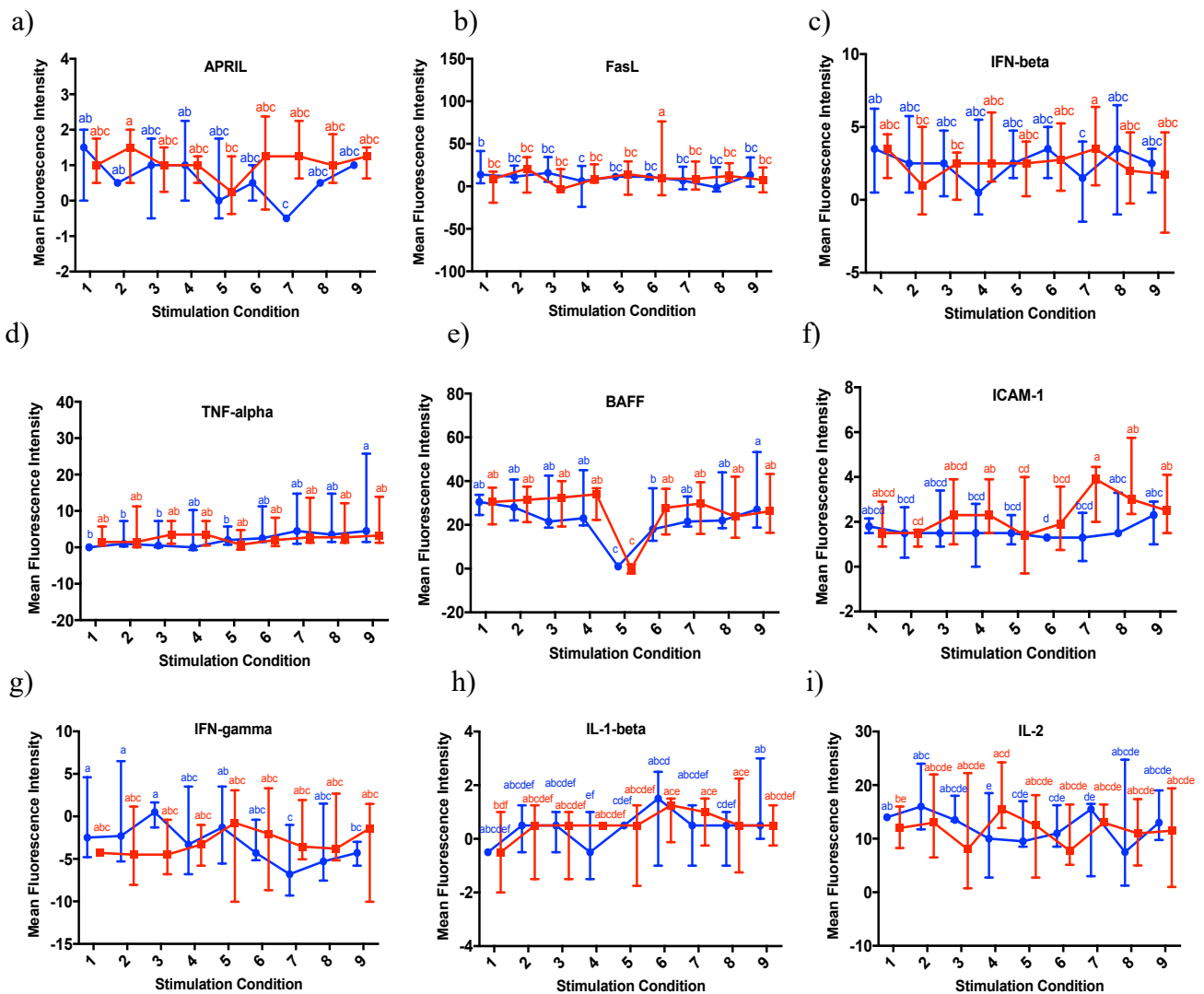


**Suppl Figure 4.12. Effect of stimulation condition on cytokine secretion profiles of B- and T-cells following co-culture for the H37Rv Pilot study.** Cell culture supernatants were collected 19-hours post-co-culture with/without autologous T-cells (Time point 2). Whisker denote 10-90 percentile. Statistical differences between culture conditions was calculated using a three-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistical differences are indicated by letters, in which groups denoted with different letters indicate statistical differences. Effect of stimulation condition on secretion of (a) APRIL (b) soluble FasL (c) IFN- $\beta$  (d) TNF- $\alpha$  (e) BAFF (f) ICAM-1 (g) IFN- $\gamma$  (h) IL-1 $\beta$  (i) IL-2 (j) IL-4 (k) IL-6 (l) IL-10 (m) IL-12 p70 (n) IL-17A (o) IL-21 (p) TGF $\beta$ -1 (q) TGF $\beta$ -2 (r) TGF $\beta$ -3

<i>Analyte</i>	<i>Stimulation</i>		<i>QFN Status</i>		<i>Stimulation*</i>	
	<i>P-value</i>	<i>q-value</i>	<i>P-value</i>	<i>q-value</i>	<i>P-value</i>	<i>q-value</i>
<i>APRIL</i>	0.24858	0.75096	0.48594	0.92337	0.71412	0.86551
<i>sFasL</i>	0.38573	0.81350	0.35355	0.92337	<b>0.00779</b>	0.07081
<i>IFN-<math>\beta</math></i>	0.68833	0.86298	0.64539	0.92337	0.16233	0.36449
<i>TNF-<math>\alpha</math></i>	0.48589	0.83427	0.81625	0.92337	0.94585	0.95996
<i>BAFF</i>	<b>0.00000</b>	<b>0.00000</b>	0.86344	0.92337	0.95046	0.95996
<i>ICAM-1</i>	<b>0.03325</b>	0.19030	0.27593	0.92337	0.18044	0.36449
<i>IFN-<math>\gamma</math></i>	0.88702	0.89977	0.95727	0.96684	0.06191	0.18627
<i>IL-1<math>\beta</math></i>	0.14214	0.61014	0.80587	0.92337	0.41646	0.58240
<i>IL-2</i>	0.94327	0.89977	0.81281	0.92337	0.07172	0.18627
<i>IL-4</i>	0.59065	0.86298	0.56306	0.92337	0.27587	0.44199
<i>IL-6</i>	0.70365	0.86298	0.80432	0.92337	0.92567	0.95996
<i>IL10</i>	0.79769	0.89977	0.45388	0.92337	0.29174	0.44199
<i>IL-12 p70</i>	0.26242	0.75096	0.67093	0.92337	0.05617	0.18627
<i>IL-17A</i>	0.68532	0.86298	0.26530	0.92337	<b>0.00282</b>	0.05127
<i>IL-21</i>	0.33997	0.81350	0.69598	0.92337	<b>0.04040</b>	0.18362
<i>TGF-<math>\beta</math>1</i>	<b>0.02174</b>	0.18664	0.43682	0.92337	0.22916	0.41661
<i>TGF-<math>\beta</math>2</i>	0.92098	0.89977	0.83167	0.92337	<b>0.02552</b>	0.15465
<i>TGF-<math>\beta</math>3</i>	0.42641	0.81350	0.55124	0.92337	0.46140	0.59916

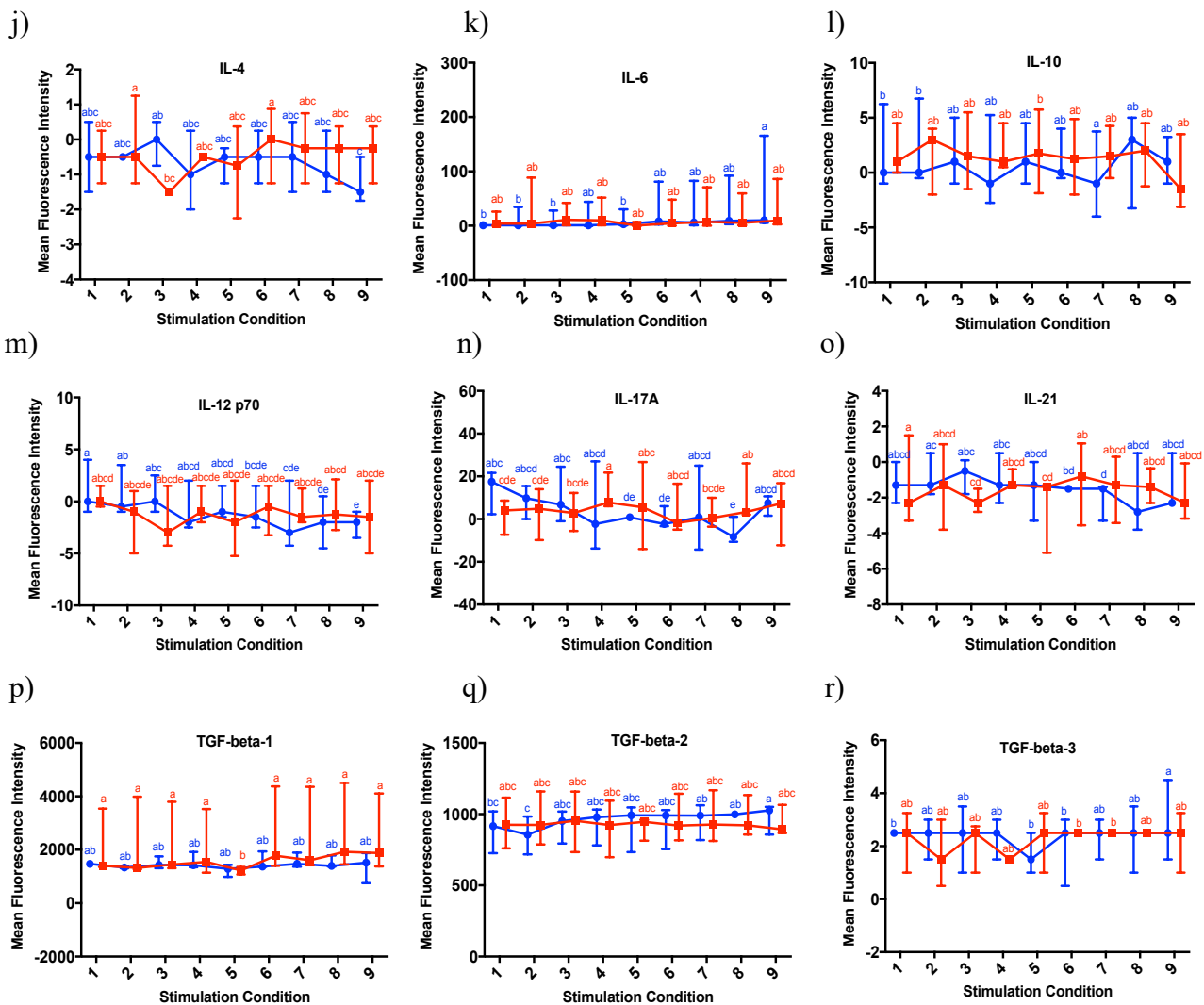
**Suppl Table 4.3. Evaluation of cytokine secretion profiles of B- and T-cells following co-culture for the H37Rv Pilot study.** Cell culture supernatants were collected 19-hours post-co-culture with/without autologous T-cells (Time point 2). Statistical differences between culture conditions was calculated using a three-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistically significant p-values are highlighted in red. The adjusted p-value is denoted 'q-value'.

■ QFN Negative    1 = B-cells Only    3 = B+CD40L+IL5    5 = T-cells Only    7 = T-cells+B+H37Rv    9 = T-cells+B+H37Rv +CD40L+IL5  
■ QFN Positive    2 = B+H37Rv    4 = B+H37Rv +CD40L+IL5    6 = T-cells+B-cells    8 = T-cells+B +CD40L+IL5

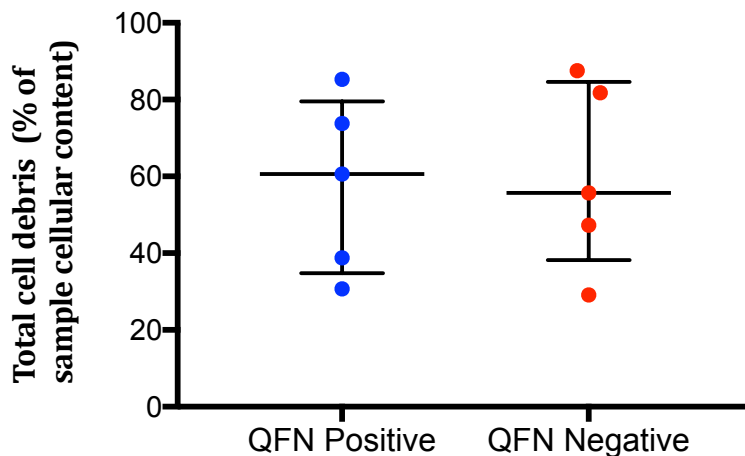


**Suppl Figure 4.13. Effect of stimulation condition and QuantIFERON status in combination on cytokine secretion profiles of B- and T-cells following co-culture for the H37Rv Pilot study.** Cell culture supernatants were collected 19-hours post-co-culture with/without autologous T-cells (Time point 2). Median with interquartile range plotted. Statistical differences between culture conditions was calculated using a three-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistical differences are indicated by letters, in which groups denoted with different letters indicate statistical differences. Effect of stimulation condition and QFN status in combination on secretion of (a) APRIL (b) soluble FasL (c) IFN- $\beta$  (d) TNF- $\alpha$  (e) BAFF (f) ICAM-1 (g) IFN- $\gamma$  (h) IL-1 $\beta$  (i) IL-2

■ QFN Negative    1 = B-cells Only    3 = B+CD40L+IL5    5 = T-cells Only    7 = T-cells+B+H37Rv    9 = T-cells+B+H37Rv+CD40L+IL5  
■ QFN Positive    2 = B+H37Rv    4 = B+H37Rv+CD40L+IL5    6 = T-cells+B-cells    8 = T-cells+B+CD40L+IL5



**Suppl Figure 4.13 continued. Effect of stimulation condition and QuantiFERON status in combination on cytokine secretion profiles of B- and T-cells following co-culture for the H37Rv Pilot study.** Cell culture supernatants were collected 19-hours post-co-culture with/without autologous T-cells (Time point 2). Median with interquartile range plotted. Statistical differences between culture conditions was calculated using a three-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistical differences are indicated by letters, in which groups denoted with different letters indicate statistical differences. Effect of stimulation condition and QFN status in combination on secretion of (j) IL-4 (k) IL-6 (l) IL-10 (m) IL-12 p70 (n) IL-17A (o) IL-21 (p) TGFβ-1 (q) TGFβ-2 (r) TGFβ-3



**Suppl Figure 4.14. Degree of cell debris/platelet contamination in isolated B-cell samples from QuantiFERON positive and negative individuals used in B- and T-cells co-culture experiment for the H37Rv Pilot study.** B-cell isolation was achieved using the Miltenyi B-cell Isolation kit II, from a starting volume of 54mL whole blood. Median with interquartile range plotted. Sample purity was assessed by flow cytometry, based on forward and side scatter properties, using a CD19 monoclonal antibody (mAb) to identify B-cells within the lymphocyte population and a CD36 mAb to identify platelets within the cell debris population.

## **Chapter 5:**

The work presented in this chapter has been formatted in the style of a manuscript for submission to an immunology journal.

**The Effect of Microenvironment Complexity on B-cell activation and function during latent *Mycobacterium tuberculosis* infection**

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**Keywords:** Regulatory B-cells, tuberculosis, immune modulation, microenvironment

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## Abstract

**Rationale:** Studies on isolated cells, while important for investigating the function of a particular immune cell type, may not reflect the responses that occur *in vivo* under the same circumstances – due to the presence of additional cell types. This study aimed to investigate the effect of microenvironment complexity (i.e. degree of cell isolation) on cell function, with an emphasis on B-lymphocytes.

**Method:** B-cells were isolated from whole blood, during which a sample fraction was retained following each isolation procedure (whole blood, PBMC's, isolated B-cells). The various sample fractions were then stimulated with TLR9a or H37Rv and the resulting B-cell phenotypic frequencies and immunoglobulin profiles of each stimulation condition for all sample types determined.

**Results:** Alterations in the immunoglobulin profile and killer B-cell phenotypic frequencies were observed for each of the investigated sample types, regardless of stimulation condition. Under the same stimulation conditions, while in an isolated cellular environment, secretion of immunoglobulin isotypes IgA, IgG2 and IgG3 by B-cells was hampered. Additionally, a progressive decrease in the quantity of all immunoglobulin isotypes within each sample type was observed following successive isolation. The observed differences in the immunoglobulin profile highlight the importance of cell-cell communication between B-cells and additional cell types for B-cell activation. Furthermore, a decrease in marginal zone B-cell frequencies and an increase in T1 B-cells was observed following cell isolation, indicating impaired B-cell development in response to antigenic stimulation in isolation. In contrast, increased frequencies of killer B-cells were observed following cellular isolation, suggesting a biased shift in augmented immune response *in vitro*. Further discovery showed a relationship between sample type and stimulation condition in which the elicited immune response to a particular stimulus differed significantly between sample types.

**Conclusion:** Microenvironment complexity plays a substantial role in immune cell polarisation and function. Humoral B-cell function, as well as development of mature B-cell populations, was impaired following isolation, likely due to a lack of co-stimulatory signals from additional cell types, thereby compromising their ability to elicit an efficient immune response. Thus, observations inferring B-cell function from isolated cell studies, while useful and informative, should be interpreted carefully, as the *in vitro* microenvironment may have considerably influenced the observed results.

## 1. Introduction

Over the past two decades, researchers have increased the use of cell culture and isolated tissue samples for biological research as an alternative to *in vivo* animal studies, due to the large cost and strict regulatory conditions involved (Adams and Larson, 2007; Brown Jr, 1997; Murphy, 1991). For this reason, cell culture studies have formed the fundamental basis of a variety of research topics (Anderson and Wilbur, 1952; Arase et al., 1995; Chess et al., 1974; Dounce, 1943; Jonuleit et al., 2001; O'doherty et al., 1993; Tsuda et al., 1974). While the information gained from these isolated cell studies provides valuable insight into biological mechanisms under investigation, they do not account for the many factors that control these physiological responses *in vivo*. Numerous studies have illustrated the ability of various cell types to modulate host immune response under different conditions (Bénard et al., 2018; Blair et al., 2010; Carter et al., 2012; Maglione and Chan, 2009; Mauri and Bosma, 2012; McKenzie et al., 1993; Shen et al., 2014; Sowa et al., 2009; Xu et al., 2008). The presence and activation of these cell types may contribute significantly to the function of a cell type of interest, through directing the mounting immune response. As such, absence of these cells during isolated cell studies may result in artificial observation and inaccurate assumptions regarding the role of a cell population during health and disease.

The benefits of isolated cell culture techniques include, the ability to investigate the direct effect of a substance or compound on a cell type of interest, and the short time needed to conduct multiple experiments (Murphy, 1991). However, *in vivo*, endogenous enzymes and various biological processes may interact with said drug or compound under investigation prior to it reaching its cellular target, thereby contributing to any physiological responses observed (Igaz et al., 2001; Wrenshall et al., 1999). The interaction of these biological processes and mechanisms present within whole organism are still ill-defined. Consequently, there is no way to simulate all of the possible interactions that may occur to account for this phenomenon *in vitro*. For this reason, variations in observations between *in vitro* isolated cell studies and *in vivo* investigations exist. The effects of isolation on investigated cellular responses is evident in many studies (Kondo and Magee, 1977; Murphy, 1991; Sanders et al., 1983), in which a particular substance/condition produced a measured immune response in whole organism, while having minimal/no effect on isolated target cells or tissue; or vice versa. This is due to the many mechanisms that act simultaneously within the immune system to regulate all biological processes, some inhibitory while others stimulatory (Carter et al., 2012; Chen, 2004; Kessel et al., 2012; Schlessinger, 2000). The absence of these processes during isolated cell culture attributes to the altered responses observed.

Importantly, each of the investigative techniques (whole organism, tissue culture or isolated cell studies) is not more beneficial than the next – individually they provided vital information needed to fill the knowledge gaps currently within the scientific field. Isolated cell studies enable investigation into cellular functional capacity and complex immune mechanisms employed by cells that may be masked by the presence of other cell types through direct cell-cell interactions or indirectly by the secretion of several soluble molecules (Igaz et al., 2001; McKenzie et al., 1993; Wrenshall et al., 1999). Moreover, such studies allow for investigation into cellular cytotoxicity, minimum effective dose and immune modulatory screening prior to whole animal studies (Combes et al., 2003; Kirkland et al., 2007; Knight and Breheny, 2002), saving both time and money in the process. This information must simply be interpreted cautiously, keeping in mind the effects microenvironment complexity may have on the experimental outcome. Ultimately, all research needs to be translated into clinically relevant events in whole, living organism; intrinsically there will always be a need for live animal testing following isolation studies for proof of concept and to ensure compound safety (Hajar, 2011; Murphy, 1991; Wall and Shani, 2008).

Emerging evidence has acknowledged B-cells as fundamental in the defense against *Mycobacterium tuberculosis* (*M.tb*). Prior to the participation of B-cells in host immune responses, development and migration of precursors cells known as transitional B-cells from the bone marrow to the spleen is required; where they give rise to mature B-cells in response to antigenic stimulation (Abbas et al., 2014; Paul, 2013). Immature transitional 1 (T1) B-cells (CD19<sup>+</sup>CD21<sup>-</sup>CD23<sup>-</sup>) form the foundation from which transitional 2 (T2) B-cells (CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup>) and mature B-cells sequentially derive (Petro et al., 2002). Notably, studies have demonstrated the expansion of mature B-cells from both T1 and T2 B-cells (Loder et al., 1999). However, T2 B-cells have been found to be more receptive to cellular activation and proliferation, in comparison to T1 B-cells (Chung et al., 2003; Mackay and Browning, 2002). Importantly, T2 B-cells are the principal source from which mature B-cells such as marginal zone (MZ) B-cells (CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>-</sup>) and follicular (FO) B-cells (CD19<sup>+</sup>CD21<sup>-</sup>CD23<sup>+</sup>) develop (Cariappa et al., 2001; Kuroda et al., 2003; Martin and Kearney, 2002). As such, the presence and regulation of these T2 B-cells dramatically affects the course of mounted immune responses. This complex developmental process of B-cells has been shown to be partially affected during TB disease, in which dysregulation expression of genes involved in B-cell development have been found.

Recent studies investigating the role of B-cells during tuberculosis (TB) disease revealed impaired B-cell function and decreased B-cell frequencies during active disease (Joosten et al., 2016a; Van Rensburg et al., 2017). A regulatory B-cell ( $B_{reg}$ ) subtype, killer B-cells, was recently discovered and has been implicated in a variety of immune conditions, including *M.tb* infection (Lundy, 2009; Lundy et al., 2015; van Rensburg and Loxton, 2018a). Functional analysis of these cells in numerous health settings identified the role of these cells as potential immune modulators responsible for controlling the immune response during disease and infection. (Chesneau et al., 2013; Lundy, 2009; Lundy and Boros, 2002; Lundy et al., 2015; Matsushita et al., 2008). A study by van Rensburg and colleagues (van Rensburg et al., 2017) investigated the frequency of these killer  $B_{regs}$  during active TB disease; in which a decrease in the frequency of killer B-cells was observed for TB diseased individuals when compared to healthy individuals. Upon successful TB treatment, these killer B-cell frequencies returned to levels comparable to that of healthy controls, suggesting a pivotal role of these  $B_{regs}$  in protective anti-TB immunity (Joosten et al., 2016a; van Rensburg et al., 2017; Van Rensburg et al., 2017). The objective of this study was to elucidate the influence of microenvironment complexity on B-cell polarisation and function - in the context of TB disease. Current research findings inferring the role of B-cells during *M.tb* infection utilized isolated cell culture techniques to investigate their functional capacity. As such, the observed physiological response may simply be a result of the controlled laboratory environment in which the cells were studied and may not reflect events that occur within whole organism, in the presence of additional immune cells and endogenous factors. In turn, biased conclusions as to the relative importance of this cell type during TB disease may have occurred - resulting in exaggerated or understated findings concerning their physiological relevance.

## 2. Methods

### 2.1 Ethics Statement

Ethical approval was obtained from the health research ethics committee of Stellenbosch University (N16/05/070) and the City of Cape Town City Health. The study was conducted according to the Helsinki Declaration and International Conference of Harmonisation guidelines. Written informed consent was obtained from all study participants.

### 2.2 Study Participants

For this pilot study, we recruited 23 healthy individuals (15 individuals with a negative QuantiFERON (QFN) status) in the year 2018. The QFN status was suggestive of exposure to *M.tb*, and participants with a positive status were considered latently infected. Recruited participants did not present with any clinical symptoms of TB and had no previous record of active disease. All participants for this study were HIV negative. QFN positive and negative participants were matched according to socio-economic background. The study was performed in Cape Town, South Africa.

### 2.3 B-cell Isolation

Heparinized peripheral blood (18ml) was collected, of which 3mL was set aside for stimulation. From the remaining whole blood, peripheral blood mononuclear cells (PBMCs) were isolated using the ficoll-histopaque (GE Healthcare Life Sciences, USA) density gradient method. A fraction of the PBMC's ( $3 \times 10^6$  cells) was set aside for stimulation. Subsequently, B-cells were negatively isolated from the remaining PBMCs using MACS bead technology, according to the manufacturer's instructions, with the B-cell isolation kit II (Miltenyi Biotec, South Africa). Once all the sample fractions had been collected, the cells were stimulated as described below. Purity of the enriched B-cell samples was confirmed by flow cytometry using anti-human CD19 mAb. All samples with resulting gated purity of above 90% were included in analysis.

### 2.4 Stimulation assays

Whole blood (1mL blood/well), PBMC's ( $1 \times 10^6$  cells/well) and isolated B-cells (100 000 cells/well) were stimulated under 3 conditions: unstimulated, H37Rv ( $1 \times 10^6$  CFU) or TLR9a (Sigma, USA) at 50 ng/ml. PBMCs and isolated B-cells were cultured in 96-well round-bottom plates in a total of 200uL complete media (RPMI plus L/Glutamine (Sigma, USA)) supplemented with 10% Fetal Calf Serum (FCS, Lonza, South Africa). Whole blood samples were incubated in a 24-well flat bottom plate in a total of 1.1mL (in which stimulants were diluted in complete media and added to 1mL blood). All sample fractions were incubated at 37°C and 5% CO<sub>2</sub> for 24 hours. Following incubation, the plasma (in case of whole blood) and culture supernatants (in the case of PBMCs and isolated B-cells) were harvested, passed through a filter of 0.22µm (to remove any bacilli that may be contained within the sample) and stored at -80°C for measurement of immunoglobulin secretion. The cells were then fixed with 4%

paraformaldehyde (eBioscience, USA) for 30min at 37°C, washed with phosphate buffered saline (PBS (Lonza, South Africa) and cryopreserved (90% FCS and 10% Dimethyl sulfoxide (DMSO), Sigma-Aldrich, St, Louis, MO) in liquid nitrogen for future analysis by flow cytometry.

## **2.5 Immunoglobulin isotype analysis by Luminex technology**

Quantification of immunoglobulins within the plasma and culture supernatant, following the 24-hour stimulation, was determined using the MAGPix and Bioplex platforms (Bio-rad Laboratories, California, USA). The immunoglobulins included IgA, IgE, IgG1, IgG2, IgG3, IgG4 and IgM. The experiments were performed according to the kit manufacturer's recommendations.

## **2.6 Phenotype Analysis by Flow Cytometry**

The antibody panel for cell surface receptor analysis consisted of: CD19-BV605, CD21-PE/Dazzle, CD23-BV421, CD5-PerCP/Vio770, CD125 (IL5RA)-PE (All from Biolegend, California), CD3-FITC (BD, Germany) and CD178 (FASL)-APC (Miltenyi Biotec, South Africa). Cells were stained for 1 hour at room temperature in the dark, washed and acquired on a BD LSR II (BD Biosciences). The resulting data was analyzed using FlowJo v10 software (Treestar, USA). The gating strategies used can be found in Suppl Figures 5.8 to 5.10.

## **2.7 Statistical Analysis**

Quantification of the immunoglobulin isotypes by Luminex was expressed as a percentage of the total immunoglobulin and statistical analysis performed on the relative percentage contribution of each of the isotypes within a sample. The rationale of standardizing the data was that whole blood, comprising of plasma, contained circulating antibody that were present prior to stimulation, while PBMCs and B-cells were cultured in fresh media. This would result in large significant differences between whole blood and other sample types being observed based on the quantity of immunoglobulin present rather than the immunoglobulin isotype expression patterns, and in turn functional capacity of the B-cells. Data analysis for all Luminex results was performed using Statistica 12 software (Statsoft, Ohio, USA) and Prism 7 Software (San Diego, CA). Raw data was checked for normality using normality plots. Statistical differences between sample fractions (Whole Blood, PBMC, B-cells), QFN status and culture conditions (unstimulated, TLR9a, H37v) was calculated using a four-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing.

Data analysis of the flow cytometric plots was done using FlowJo V10 (Treestar, USA) and the resulting cell frequencies analyzed using Statistica and Prism 7 Software. Note: isolated B-cell samples were pooled prior to flow cytometry analysis, due to inadequate cell numbers compromising acquisition event rate, thereby prohibiting the performance of statistical analysis. Statistical differences between

sample fractions (Whole Blood and PBMC), QFN status and culture conditions (unstimulated, TLR9a, H37v) was calculated as described for the Luminex data. Raw data was checked for normality using normality plots and winsorised using the Huber mean and MAD as necessary to reduce the residual distribution into close agreement with a normal distribution. Statistical significance is indicated by an asterisk, in which the  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*) or by letters, in which groups denoted with different letters indicate statistical differences.

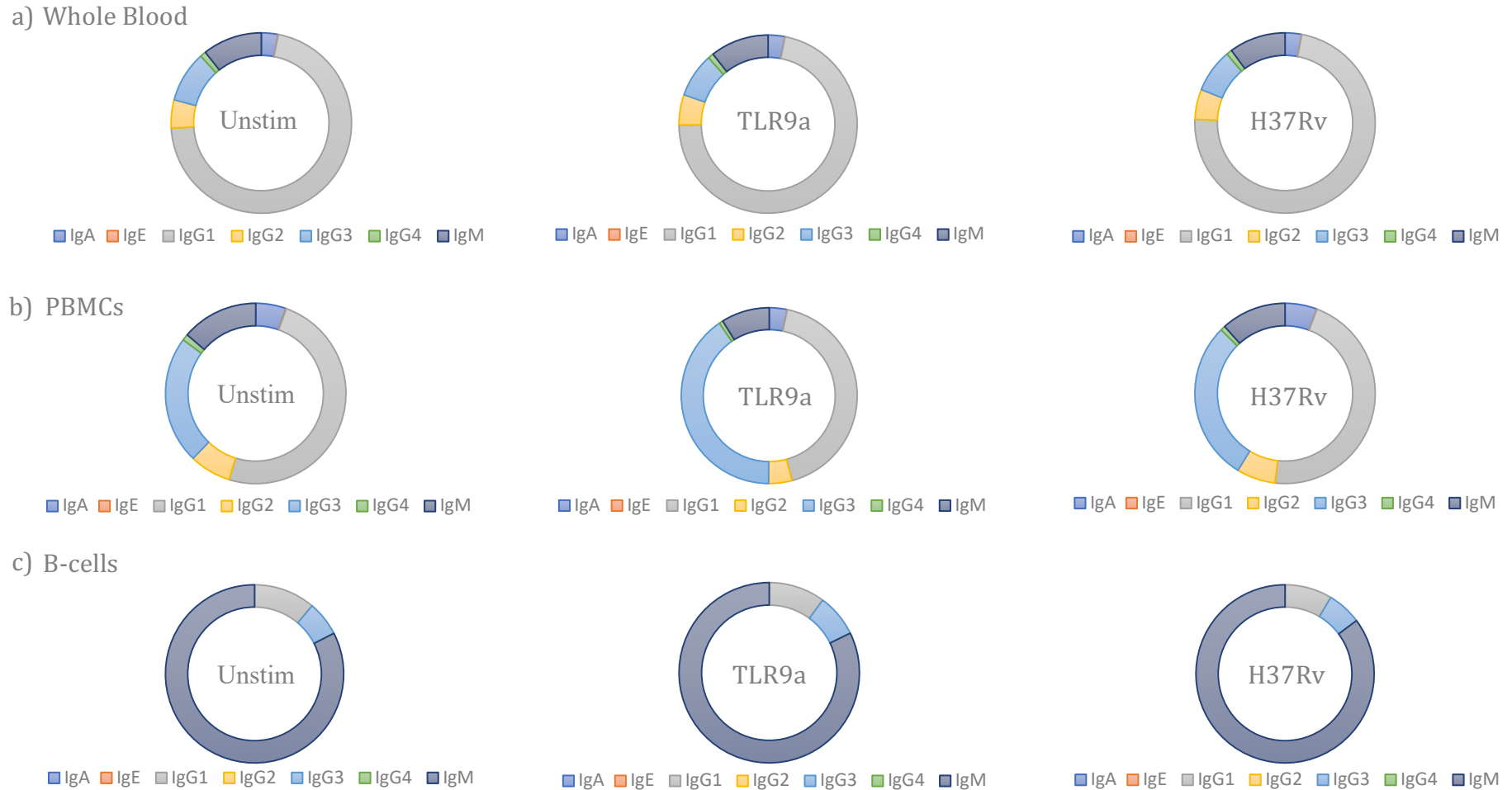
### 3. Results

#### 3.1 First order effects on Immunoglobulin expression profile

The mechanisms employed by B-cells toward facilitating enhanced anti-TB immunity remains unresolved. Subsequently, the effects of microenvironment complexity i.e. the degree of cellular isolation on B-cell function was investigated. This was achieved by examining the immunoglobulin expression patterns within culture supernatants following stimulation of B-cells in various sample fractions (whole blood, PBMCs and isolated B-cells).

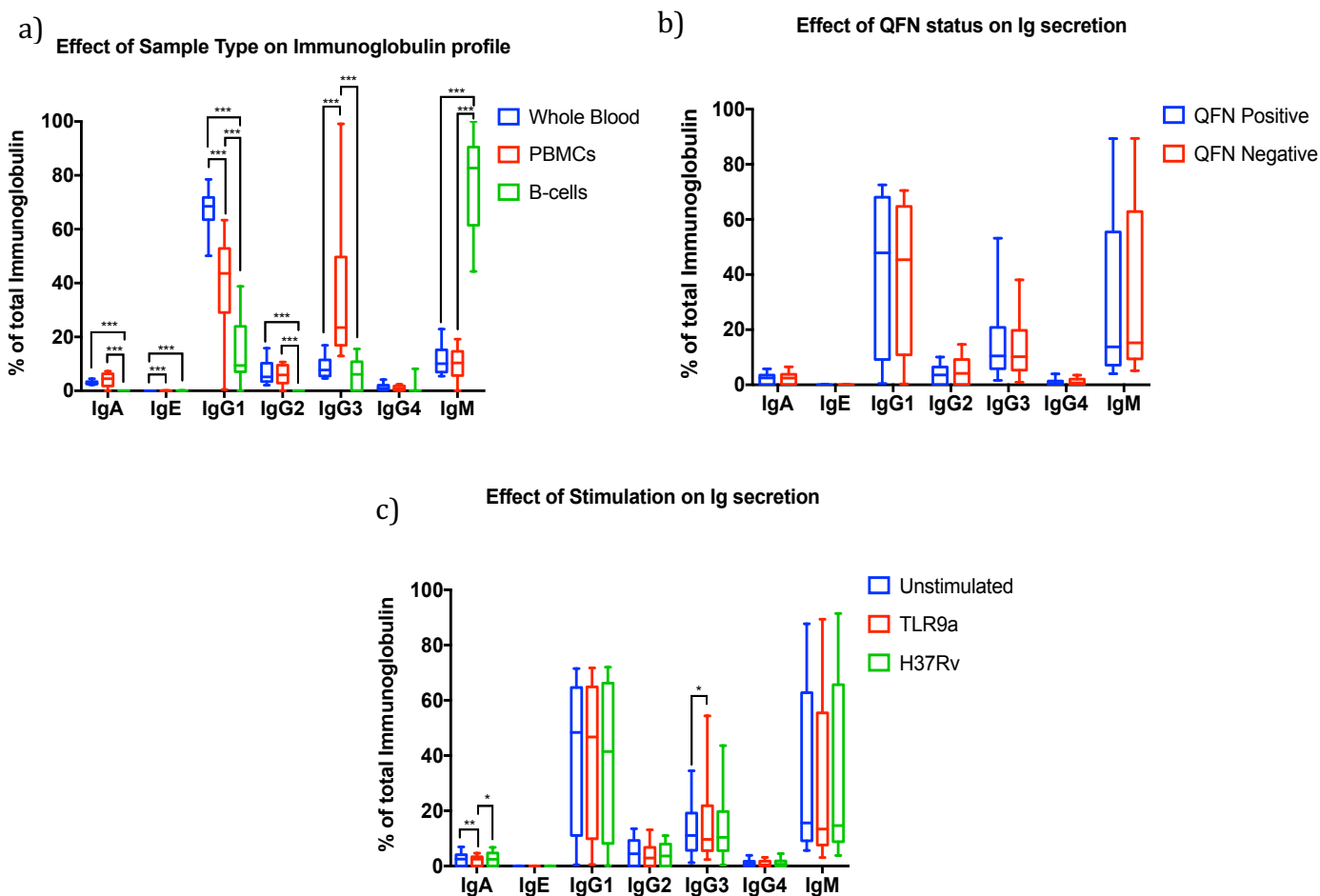
Sample type was observed to considerably influence B-cell performance (Figure 5.1). Altered immunoglobulin profiles were observed across the various sample fractions, regardless of stimulation condition. A significant difference ( $p < 0.05$ ) in the immunoglobulin profile was observed for all isotypes, except IgG4, following isolation of B-cells from whole blood (Figure 5.2a). In some instances, the relative abundance of an isotype within a given sample set, such as IgE ( $p = 0.0001$ ), IgG3 ( $p = 0.0000$ ) and IgA (ns,  $p > 0.05$ ), was observed to increase following PBMC isolation. However, in majority of cases a decrease in the relative abundance of an immunoglobulin isotype, specifically IgG1 ( $p = 0.0000$ ), IgG2 (ns,  $p > 0.05$ ), IgG4 (ns,  $p > 0.05$ ) and IgM (ns,  $p > 0.05$ ), within a given sample set was observed following PBMC isolation. A significant decrease in the relative abundance of most isotype, including IgA ( $p = 0.0000$ ), IgG1 ( $p = 0.0000$ ) and IgG2 ( $p = 0.0000$ ), was observed following isolation of B-cells compared to whole blood, whilst an increase in the relative abundance of IgE ( $p = 0.0004$ ) and IgM ( $p = 0.0000$ ) was found. Likewise, a substantial decrease in the relative abundance of most isotypes, IgA ( $p = 0.0000$ ), IgG1 ( $p = 0.0001$ ), IgG2 ( $p = 0.0000$ ) and IgG3 ( $p = 0.0000$ ), was observed following isolation of B-cells compared to the PBMC sample fraction, whereas an increase in the relative abundance of IgM ( $p = 0.0000$ ) was found. Incidentally, the effect of sample type on the abundance of the various immunoglobulin isotypes was investigated to discern the effect of cellular isolation on the magnitude of subsequent B-cell responses. A significant difference in quantified immunoglobulin levels was observed for all isotypes following each successive isolation procedure (Figure 5.3). To account for the obviously limitations involved in comparing plasma supernatants to culture supernatants, only differences between PBMCs and isolated B-cells were considered. A significant decrease in the observed concentration of all immunoglobulin isotypes, IgA ( $p = 0.00$ ), IgE ( $p = 0.0000$ ), IgG1 ( $p = 0.0000$ ), IgG2 ( $p = 0.00$ ), IgG3 ( $p = 0.0000$ ), IgG4 ( $p = 0.00$ ) and IgM ( $p = 0.0000$ ), was observed for isolated B-cell culture supernatants when comparing to those of PBMC samples.



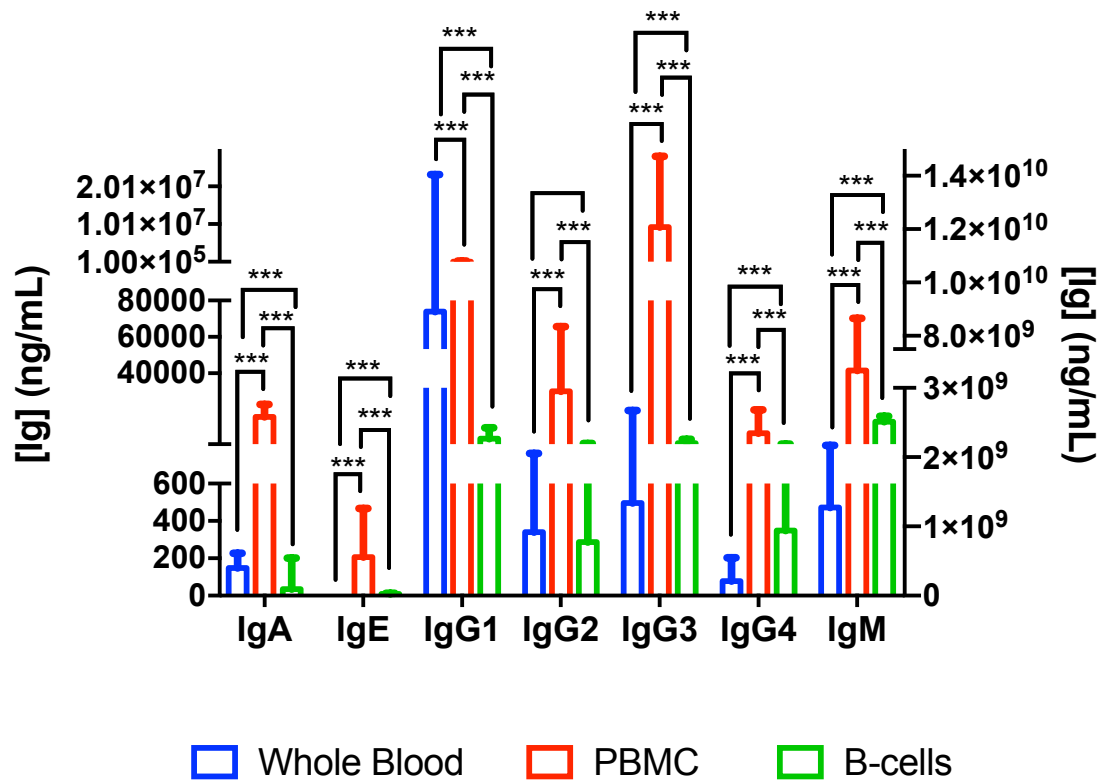


**Figure 5.1. Immunoglobulin profile of supernatants obtained from each of the stimulatory conditions of the various cellular fractions (n=23).** Following a 24-hour stimulatory period, plasma/culture supernatants of each of the stimulatory conditions for all sample types was collected and the immunoglobulin secretion profile determined using Luminex. (a) Representation of the average secretion of each isotype within whole blood for each stimulatory condition (b) Representation of the average secretion of each isotype within the PBMC fraction for each stimulatory condition (c) Representation of the average secretion of each isotype within the isolated B-cell fraction for each stimulatory condition.





**Figure 5.2. Evaluation of the effect of various experimental factors on B-cell immunoglobulin profile (n=23).** Following a 24-hour stimulatory period, plasma/culture supernatants of each of the stimulatory conditions for all sample types was collected and the immunoglobulin profile determined using Luminex. The Immunoglobulin isotype levels depicted are reported as a percentage of the total immunoglobulin within a given sample. Median with interquartile range plotted. Statistical differences between culture conditions was calculated using a four-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistical significance is indicated by an asterisk, in which the  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*). (a) Effects of sample type i.e. microenvironment complexity on immunoglobulin isotype abundance following antigenic stimulation (b) Effects of QuantiFERON (QFN) status on immunoglobulin isotype abundance following antigenic stimulation (c) Effects of stimulatory conditions on immunoglobulin isotype abundance following antigenic stimulation.



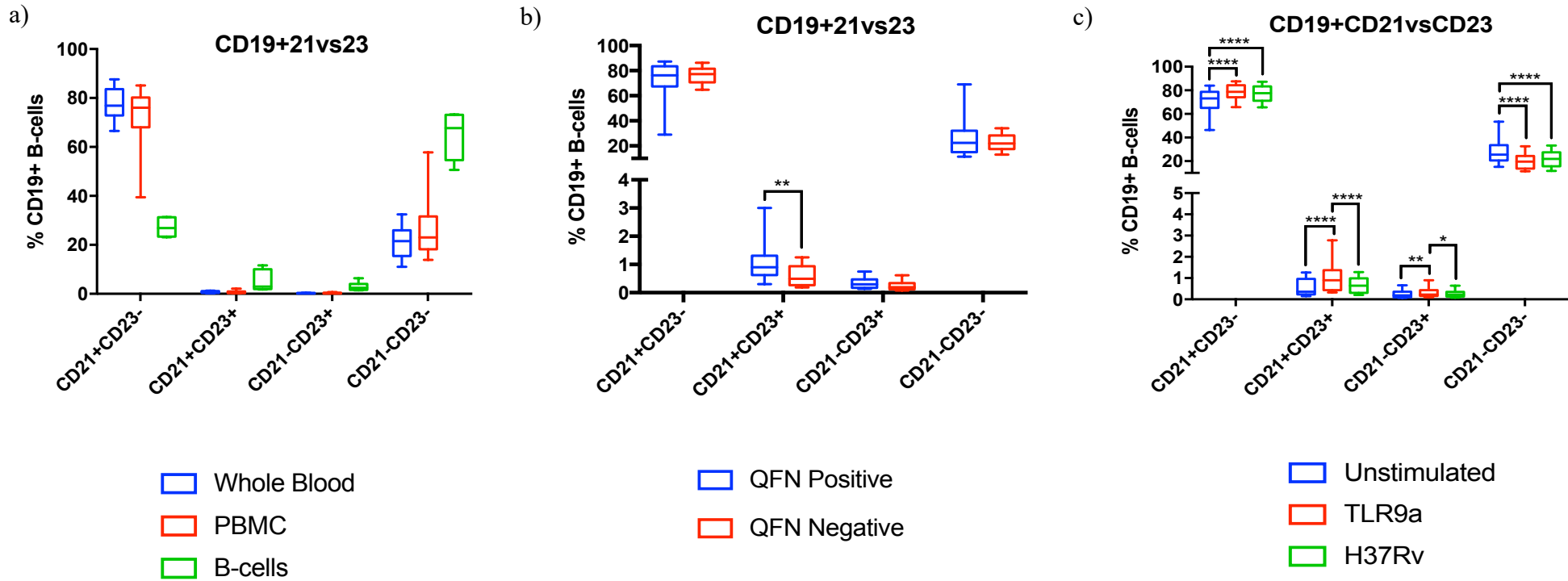
**Figure 5.3. Evaluation of the effect of sample type on observed immunoglobulin isotype concentration (n=23).** Following a 24-hour stimulatory period, plasma/culture supernatants of each of the stimulatory conditions for all sample types was collected and the immunoglobulin secretion profile determined using Luminex. The observed concentration for each immunoglobulin isotypes within a given sample is reported. Median with interquartile range plotted. Statistical differences between culture conditions was calculated using a four-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistical significance is indicated by an asterisk, in which the  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*). The scale on the left represent the measured immunoglobulin concentration (ng/mL) within the PBMC and B-cell sample supernatants, while the right scale left represent the measured immunoglobulin concentration (ng/mL) within whole blood sample supernatants. Two scales, indicating the same variable, were utilized for ease of display due to the large difference in [Ig] between sample types.

Additionally, the effects of QFN status and stimulation condition on immunoglobulin profiles were investigated. We investigated whether or not *M.tb*-exposed (QFN positive) individuals with immune memory would respond differently to *M.tb* challenge compared to QFN negative individuals. In all instances, QFN status was found to have no effect on the relative abundance of the various immunoglobulin isotypes (Figure 5.2b). To conclude, the impact of stimulation condition on immunoglobulin profiles were investigated to determine the effect of *M.tb* infection on B-cell performance. For majority of immunoglobulin isotypes, stimulation condition was found to have no effect on the measured immunoglobulin abundance (Figure 5.2c). However, a significant decrease in IgA levels were observed following TLR9 stimulation for all sample types compared to unstimulated controls ( $p=0.0038$ ) and H37Rv stimulated ( $p=0.0292$ ) samples. Conversely, an increase in IgG3 levels were observed following TLR9 stimulation for all sample types compared to unstimulated controls ( $p=0.0482$ ).

### 3.2 B-cell Development

In addition to investigating alterations within the immunoglobulin profile of B-cells, variations in the phenotypic frequencies of various B-cell subsets was investigated to evaluate the effect of microenvironment complexity on cell function. The expression of the cell surface receptors CD21 and CD23 by B-cells was examined to determine the proportion of B-cells within various developmental stages following antigenic stimulation. Taking into account the magnitude B-cell development has on elicited host immunity, the effects of microenvironment complexity on B-cell maturity and expansion was investigated. Following a 24-hour stimulatory period, cells from all stimulatory conditions for each of the cellular fractions was collected and phenotypic frequencies of the various B-cell population determined using flow cytometry. It is important to note that due to limited cell numbers, isolated B-cell samples (B-cells only) were pooled according to QFN status and stimulatory conditions prior to flow cytometry analysis, prohibiting the assessment of individual sample distribution and statistically significant differences between B-cells only and other microenvironment conditions. Consequently, observations made from the resulting data focus principally on difference between whole blood and PBMC, while inferring the physiological implications of the trends observed for isolated B-cells.

The effect of sample type on B-cell development was investigated, in which no significant difference in T1, T2, MZ and FO B-cell frequencies was observed between whole blood and PBMCs. However, a shared pattern of decreased CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>-</sup> (MZ) B-cells, whilst an increase in CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup> (T2) B-cells, CD19<sup>+</sup>CD21<sup>-</sup>CD23<sup>+</sup> (FO) B-cells and CD19<sup>+</sup>CD21<sup>-</sup>CD23<sup>-</sup> (T1) B-cells was observed for all sample types following each successive isolation procedure (Figure 5.4a). Regrettably, the significance of alterations in these B-cell frequencies for isolated B-cell samples cannot be analyzed, however a sizeable difference in the investigated frequencies is apparent. These results indicate the potential impact cell isolation has on B-cell development in response to stimulation *in vitro*.



**Figure 5.4. Analysis of developmental B-cell phenotypic frequencies.** Following a 24-hour stimulatory period, cells from each of the stimulatory conditions, for all sample types, was collected and the B-cell phenotypic frequencies determined using flow cytometry. Notable, statistical significance was only investigated for whole blood and PBMC sample type as isolated B-cell samples for the respective QFN groups were pooled (according to stimulation condition) prior to flow analysis, due to inadequate cell numbers. Whiskers denote 10-90 percentile. Statistical differences between culture conditions was calculated using a four-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistical significance is indicated by an asterisk, in which the  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*). (a) Effects of sample type on the developmental state of B-cells (b) Effects of QuantiFERON (QFN) status on the developmental state of B-cells (c) Effects of stimulation condition on the developmental state of B-cells.

Additionally, the effects of QFN status and stimulation conditions on B-cell development was investigated. For majority of the investigated populations, QFN status was found to have no significant effect on the observed B-cell frequencies (Figure 5.4b). A general trend of decreased frequencies for all populations was observed for QFN negative individuals compared to QFN positive individuals. In accordance, a significant decrease in the observed frequency of CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup> (T2) B-cells ( $p=0.0012$ ) was observed for QFN negative individuals. Furthermore, the effects of stimulation condition were examined (Figure 5.4c). A common shift of increased B-cell frequencies was observed for most of the population subsets investigated following TLR9a stimulation compare to unstimulated controls. A significant increase in the frequency of CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>-</sup> (MZ) B-cells, was observed following TLR9a stimulation ( $p=0.0000$ ) and *M.tb* infection ( $p=0.0000$ ), compared to unstimulated cells. Similarly, a significant increase in CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup> (T2) B-cells and CD19<sup>+</sup>CD21<sup>-</sup>CD23<sup>+</sup> (FO) B-cells was observed in response to TLR9a stimulated compare to unstimulated ( $p=0.0000$ ;  $p=0.0061$ ) and H37Rv stimulated ( $p=0.0000$ ;  $p=0.0198$ ) samples, respectively. Contrariwise, a significant decreases in CD19<sup>+</sup>CD21<sup>-</sup>CD23<sup>-</sup> (T1) B-cells was observed for samples stimulated with either TLR9a ( $p=0.0000$ ) or H37Rv ( $p=0.0000$ ) compared to unstimulated controls.

### 3.3 Killer/FasL-expressing B-cell frequencies

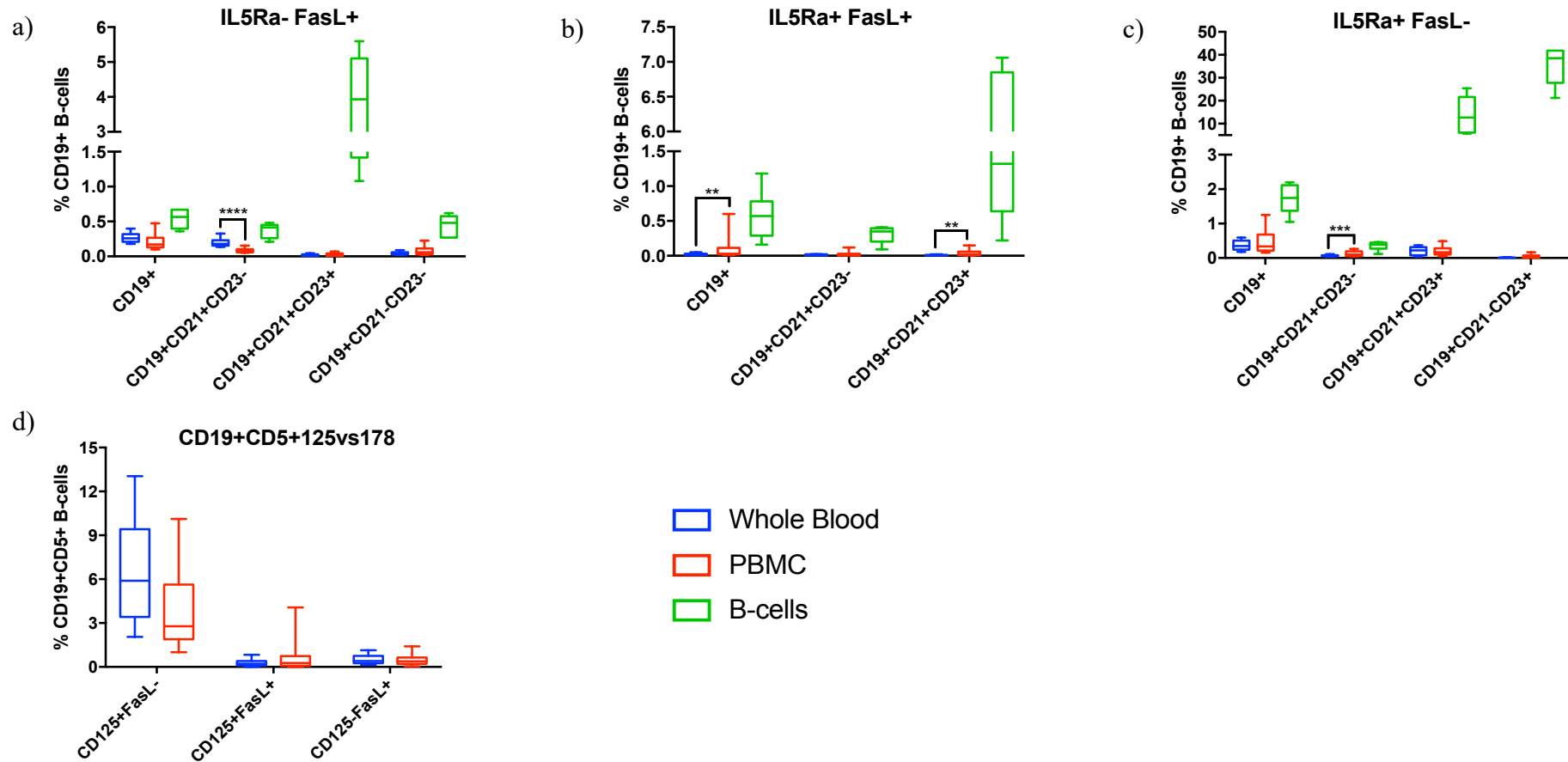
As previously stated, recent finding within the TB research field have revealed a particular B-cell subset, killer B-cells, and instrumental players in the fight against TB disease (Lundy, 2009; van Rensburg and Loxton, 2018b; van Rensburg et al., 2017; Van Rensburg et al., 2017). The proposed function of these cells in health and disease is regulation of effector and suppressive immune responses through activated-cell death mechanisms involving the FasL-Fas interaction and the induction of regulatory T-cells (T<sub>regs</sub>) and myeloid-derived suppressor cell (MDSCs), respectively (Kessel et al., 2012; Liu et al., 2016; Lundy et al., 2015). Taking into consideration the relative importance of killer B-cells in health and disease, the effects of microenvironment complexity of the development of this cell population was investigated.

Currently, no defined phenotype exists for the identification of killer B-cells within biological samples, due to the large heterogeneity observed within these cell populations. Thus, the phenotypic signatures used within this study was based on a combination of cell surface markers previously identified by members within our research group and those listed in literature (Lundy, 2009; Mauri and Menon, 2015; van Rensburg and Loxton, 2018b; van Rensburg et al., 2017). Killer B-cells were defined by the co-expression of CD19 and FasL (CD178), with the addition of various supplementary cell surface markers to distinguish diverse killer B-cell subsets within the population. The separation of killer B-cell subclasses was achieved by evaluating the co-expression of CD5 with FasL, as well as CD21 and CD23 individually and in combination. The expression of CD178 was evaluated in association with CD125/interleukin-5 receptor alpha (IL5R $\alpha$ ) as expression and engagement of this receptor is involved

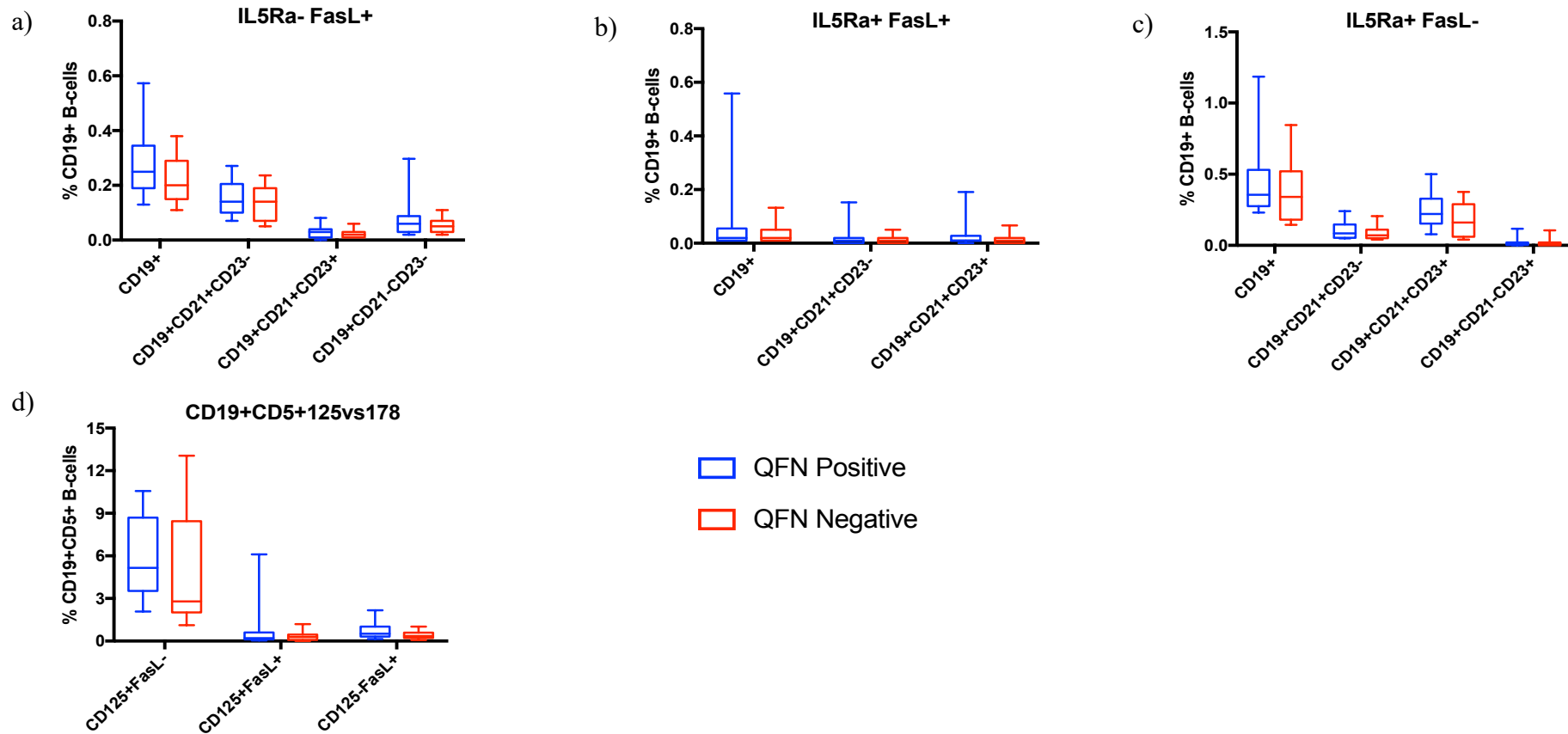
in immune signalling responsible for killer B-cell development. As previously noted, isolated B-cell samples for each of the stimulatory conditions were pooled prior to flow cytometry analysis. Consequently, observations made from the resulting data focus primarily on difference between whole blood and PBMC, while inferring the physiological implications of these trends for isolated B-cells.

The effect of sample type of killer B-cell phenotypic frequencies is apparent upon initial inspection, in which alterations in the frequency of FasL<sup>-</sup>-expressing CD19<sup>+</sup> cells is observed for all population subsets. A general pattern of increase killer B-cell levels was observed for all population subsets following cellular isolation (Figure 5.5a-d). In accordance, a significant increase in CD19<sup>+</sup>IL5Ra<sup>+</sup>FasL<sup>+</sup> B-cells ( $p=0.0017$ ), CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup>IL5Ra<sup>+</sup>FasL<sup>+</sup> B-cells ( $p=0.0029$ ) and CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>-</sup>IL5Ra<sup>+</sup>FasL<sup>-</sup> B-cells ( $p=0.0002$ ) was observed following PBMC isolation. Conversely, a significant decrease in CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>-</sup>IL5Ra<sup>-</sup>FasL<sup>+</sup> B-cells ( $p=0.0000$ ) and was observed following PBMC isolation. Regrettably, the significance of alterations in killer B-cell frequencies for isolated B-cell samples cannot be analyzed, however an indication of the vast differences in killer B-cell frequencies is evident. It can be assumed that large physiological implications may result from this difference, thereby influencing the observed immunological responses under investigation. Additionally, the effects of QFN status and stimulation conditions on killer B-cell frequencies was investigated. In all instances, QFN status was found to have no significant effect on killer B-cell frequencies (Figure 5.6a-d). However a general trend of decreased killer B-cell frequencies was observed for QFN negative individuals was observed.

The effects of stimulation condition on killer B-cell frequencies was well-defined (Figure 5.7a-d). In which a general pattern of increased killer B-cell frequencies was observed for all population subsets following TLR9a stimulation compare to unstimulated or H37Rv stimulated samples. Significant increases for the population subsets, CD19<sup>+</sup>IL5Ra<sup>+</sup>FasL<sup>+</sup> B-cells ( $p=0.0058$ ), CD19<sup>+</sup>CD21<sup>-</sup>CD23<sup>-</sup>IL5Ra<sup>-</sup>FasL<sup>+</sup> B-cells ( $p=0.0044$ ), CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup>IL5Ra<sup>+</sup>FasL<sup>+</sup> B-cells ( $p=0.0075$ ), CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup>IL5Ra<sup>+</sup>FasL<sup>-</sup> B-cells ( $p=0.0081$ ) and CD19<sup>+</sup>CD21<sup>-</sup>CD23<sup>+</sup>IL5Ra<sup>+</sup>FasL<sup>-</sup> B-cells ( $p=0.0153$ ), was observed following TLR9a stimulation compared to unstimulated cells. Similarly, a significant increase in CD19<sup>+</sup>IL5Ra<sup>+</sup>FasL<sup>+</sup> B-cells ( $p=0.0003$ ), CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>-</sup>IL5Ra<sup>+</sup>FasL<sup>+</sup> B-cells ( $p=0.0025$ ), CD19<sup>+</sup>CD21<sup>-</sup>CD23<sup>-</sup>IL5Ra<sup>+</sup>FasL<sup>+</sup> B-cells ( $p=0.0013$ ) was observed for TLR9a stimulated samples compare to H37Rv stimulated samples. Likewise, a pattern of marginally increased levels of IL5Ra<sup>+</sup>FasL<sup>+</sup> and IL5Ra<sup>+</sup>FasL<sup>-</sup> B-cells was observed for all population subtypes following H37Rv stimulation compared to unstimulated controls (Figure 5.7b-d). A significant increase for the population subsets CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup>IL5Ra<sup>+</sup>FasL<sup>+</sup> B-cells ( $p=0.0276$ ) and CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup>IL5Ra<sup>+</sup>FasL<sup>-</sup> B-cells ( $p=0.0103$ ) was observed when comparing unstimulated verses *M.tb* stimulated samples. On the contrary, a tendency of minor decreases in IL5Ra<sup>+</sup>FasL<sup>+</sup> B-cells was observed for all population subtypes following H37Rv infection compared to unstimulated controls, although insignificant in all cases ( $p>0.05$ ) (Figure 5.7a and d).

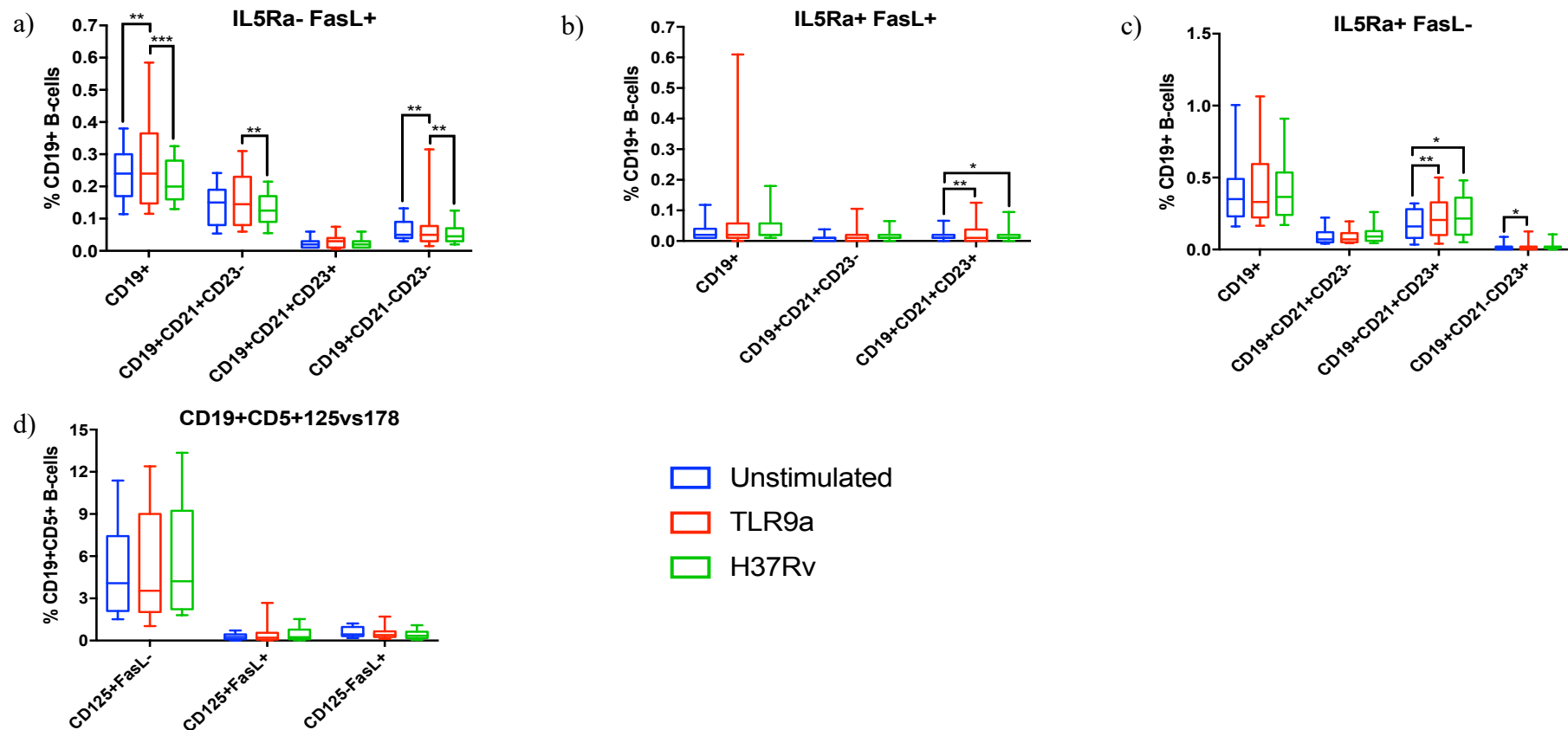


**Figure 5.5. Evaluation of the effect of sample type on killer B-cell phenotypic frequencies.** Following a 24-hour stimulatory period, cells from each of the stimulatory conditions, for all sample types, was collected and the B-cell phenotypic frequencies determined using flow cytometry. Notable, statistical significance was only investigated for whole blood and PBMC sample type as isolated B-cell samples for the respective QFN groups were pooled (according to stimulation condition) prior to flow analysis, due to inadequate cell numbers. Whiskers denote 10-90 percentile. Statistical differences between culture conditions was calculated using a four-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistical significance is indicated by an asterisk, in which the  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*). Effects of sample type on (a) IL5Ra-FasL<sup>+</sup> B-cells (b) IL5Ra+FasL<sup>+</sup> B-cells (c) IL5Ra+FasL<sup>-</sup> B-cells (d) CD19+CD5<sup>+</sup> FasL<sup>-</sup> expressing B-cells



**Figure 5.6. Evaluation of the effect of QuantiFERON status on killer B-cell phenotypic frequencies.** Following a 24-hour stimulatory period, cells from each of the stimulatory conditions, for all sample types, was collected and the B-cell phenotypic frequencies determined using flow cytometry. Notable, statistical significance was only investigated for whole blood and PBMC sample type as isolated B-cell samples for the respective QFN groups were pooled (according to stimulation condition) prior to flow analysis, due to inadequate cell numbers. Whiskers denote 10-90 percentile. Statistical differences between culture conditions was calculated using a four-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistical significance is indicated by an asterisk, in which the  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*). Effects of QFN status on (a) IL5Ra-FasL<sup>+</sup> B-cells (b) IL5Ra<sup>+</sup>FasL<sup>+</sup> B-cells (c) IL5Ra<sup>+</sup>FasL<sup>-</sup> B-cells (d) CD19<sup>+</sup>CD5<sup>+</sup> FasL-expressing B-cells.





**Figure 5.7. Evaluation of the effect of stimulation condition on killer B-cell phenotypic frequencies.** Following a 24-hour stimulatory period, cells from each of the stimulatory conditions, for all sample types, was collected and the B-cell phenotypic frequencies determined using flow cytometry. Notable, statistical significance was only investigated for whole blood and PBMC sample type as isolated B-cell samples for the respective QFN groups were pooled (according to stimulation condition) prior to flow analysis, due to inadequate cell numbers. Whiskers denote 10-90 percentile. Statistical differences between culture conditions was calculated using a four-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistical significance is indicated by an asterisk, in which the  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*) and  $p < 0.0001$  (\*\*\*\*). Effects of stimulation condition on (a) IL5Ra-FasL<sup>+</sup> B-cells (b) IL5Ra<sup>+</sup>FasL<sup>+</sup> B-cells (c) IL5Ra<sup>+</sup>FasL<sup>-</sup> B-cells (d) CD19<sup>+</sup>CD5<sup>+</sup> FasL-expressing B-cells.

## Discussion

Over the past century, the standard quality of scientific research conducted has progressed tremendously in terms of the development of state of the art technologies, improved scientific techniques, advanced data analysis and the implementation of rigorous validation processes. Examples of the aforementioned superior scientific advances includes the ability to analyse biological samples at a single cell level (Wang and Bodovitz, 2010; Wheeler et al., 2003), multiplexing of gene expression and phenotypic analysis using the 10x genomics platform (Coombe et al., 2016; Jiao and Schneeberger, 2017) and the evolution of powerful imaging tools enabling in-depth investigation into cell structure, composition and intercellular communication (Neu et al., 2010; Tezera et al., 2017). All of these progressions have fundamentally changed the way we conduct immunological research and have facilitated the discovery of many phenomena, providing crucial knowledge needed to improve health management, disease control, drug development and more. Alongside these advances come new challenges for research, such as those encountered following the standard application of isolated cell studies for the investigation of cell function. This technique enables investigation into the development and mechanisms employed by a specific cell population in response to a drug or stimulus, devoid of external influences that may otherwise mask the resulting immune response. However, these studies do not account for the multidimensional immune intervention by additional cells types that occurs *in vivo*. As previously mentioned, an abundance of ill-defined intricate cellular interactions occurs amongst immune cells within the host. This cellular communication results in physiological events that shape the immune response to a particular stimulus. During cell isolation studies these events are amiss, and may result in altered immune cell function. In such cases, erroneous discernment as to the physiological role of the investigated cell population during health and disease may result; markedly impeding the discovery of physiological phenomenon vital for effective disease management and enhanced immune protection.

In accordance, the purpose of this pilot study was to determine the effect of microenvironment complexity on B-cell function. Additionally, the effect of cell isolation on the functional capacity of B-cells during *M.tb* infection was investigated, to determine the significance of the use of isolated cell culture techniques in studies inferring the role of this cell type during TB disease. Current research findings have signified the importance of B-cells during *M.tb* infection, in which absence or impaired function of this immune cell type has been associated with poor disease prognosis (Achkar et al., 2015; Bénard et al., 2018; Du Plessis et al., 2016a, 2016b; Joosten et al., 2016b; Rao et al., 2015). For decades, the primary function of B-cells was considered to be antibody secretion, forming part of the adaptive humoral response (Abbas et al., 2014; Capra et al., 1999; Zabriskie, 2009). These humoral immune response were considered to be effective in controlling the growth and survival of extracellular invading pathogens exclusively. However, recent investigations analysing the efficiency of antibody-mediated immunity against several intracellular pathogens, including *M.tb*, have since disproven this notion

(Achkar and Casadevall, 2013; Balu et al., 2011; Buccheri et al., 2009; Chan et al., 2014; Hamasur et al., 2004; Teitelbaum et al., 1998). In addition, studies have indicated non-humoral B-cell function, such as immune modulation through receptor engagement and cytokine expression, as key mechanisms by which these cells contribute to the successful control of *M.tb* infection (Bénard et al., 2018; Chen et al., 2007; Cooper et al., 1997; Du Plessis et al., 2016b; van Rensburg and Loxton, 2018b; Van Rensburg et al., 2017). As such, the influence of *in vitro* isolated cell culture studies on B-cell development and function is of great importance, as currently observational findings inferring the physiological role of these cells during TB disease utilize this techniques (Du Plessis et al., 2016b; van Rensburg and Loxton, 2018b). Intrinsically, the results from such studies form the foundation upon which new TB drugs, host-directed therapies and TB vaccines are based.

In light of the above, the impact of microenvironment complexity (i.e. the degree of cellular isolation) on immunoglobulin expression patterns and B-cell development was investigated. Antibody profiles were assumed to be a direct indication of the relative functional capacity of B-cells within the investigated samples. Notable, the presence of circulating antibody within the plasma samples compromises the inference of B-cell activity within whole blood samples and is a limitation of the study. Considerable changes in the immunoglobulin profile were observed across the different sample types, in which the relative percentage contribution of each of the measured isotypes, with the exception of IgG4, differed significantly. Sample type, rather than stimulation condition, had a significant effect on the observed immunoglobulin profile. More specifically, a significant decrease in the relative abundance of IgG1 was observed following PBMC isolation compared to whole blood samples, while a significant increase in the relative abundance of IgG3 was found. Similarly, a significant decrease IgG1, as well as IgA and IgG2, was observed following B-cell isolation compare to whole blood, whilst a substantial increase in the relative abundance of IgE and IgM was observed. The same pattern in the immunoglobulin expression was observed when comparing isolated B-cell samples with PBMCs, in which a decrease in the abundance of IgA, IgG1 and IgG2, as well as IgG3 was observed; whereas an increase in IgM levels was found. Importantly, the observed 'increase/decrease' in antibody levels is not equivalent to the concentration of these isotypes within a given sample but rather indicates the relative immunoglobulin diversity within the cellular microenvironment. Bearing this in mind, the effect of sample type on the observed concentration for each of the immunoglobulin isotypes was investigated; to discern the effect of cellular isolation on the magnitude of elicited B-cell responses. The physiological implications of altered immunoglobulin production have been extensively reviewed in several disease states, where deficiency has been associated with increased susceptibility to bacterial infection (Franz et al., 1997; Hermans et al., 1976; Twomey et al., 1969).

The quantity of immunoglobulin [Ig] secreted for each isotype was greatly impacted by sample type, in which a systematic decrease in antibody levels was observed following successive cellular isolation. It

is important to note that immunoglobulins been shown to have a half-life of between 5-21 days (Anderson et al., 2006; Kim et al., 2007; Waldmann and Strober, 1969). Thus, circulating levels of antibodies were present within the plasma of whole blood samples prior to stimulation, whereas cells within the PBMC and isolated B-cell fraction were incubated in fresh media. This may have resulted in possible artefactual observations regarding the relative reduction in immunoglobulin levels for whole blood samples compare to PBMC and isolated B-cell samples. As such, significance of the observed [Ig] decrease was only considered between PBMCs and isolated B-cells. For all isotypes, an immense decrease in antibody quantification was observed following isolation. Collectively, these results illustrated that isolation procedures profoundly hindered the ability of B-cells to secrete several immunoglobulin isotypes; underscoring the fact that the presence of additional cell types is required for augmented B-cell activation and function.

Research has implicated IgA and IgG as leaders in protective anti-TB humoral immunity (Abebe et al., 2018; Achkar et al., 2015; Balu et al., 2011). A murine study investigating the role of humoral immunity in TB disease associated IgA deficiency with increased susceptibility to *M.tb* infection (Achkar et al., 2015). Additionally, IgA has been found to be differentially expressed during *M.tb* infection; in which differences in the relative expression of IgA can be used to discriminate between clinically diseased, *M.tb*-infected and uninfected individuals (Abebe et al., 2018). The exact mechanisms by which these immunoglobulins achieve this protective effect is still unknown, and further investigation into their cellular targets is needed to better understand the role they play during TB disease (Li et al., 2017). As cited afore, an isolated cellular environment was observed to significantly impair the ability of B-cells to produce various antibody isotypes, including IgA, IgG1 and IgG2. Hence, studies evaluating the effectiveness of B-cells to control *M.tb* infection, within an isolated environment, may incur imprecise conclusions due to diminished competence of B-cells to suitably response to antigenic stimulation. It has been well documented that B-cells require co-stimulation from T-cell and dendritic cells in order to undergo isotype/class switching (Coffman et al., 1993; Fayette et al., 1997; Le Bon et al., 2001a; McAdam et al., 2001; Stevens et al., 1988). Notable, IgA secretion is associated with mucosal immunity, and is a key defence mechanism employed at the site of disease (Williams, et al. 2004; Woof & Kerr, 2006) . As such, investigation as to the effect of isolated cell studies on B-cells at the site of infection may provide valuable insight into anti-TB immunity. Mechanisms by which T-cells and dendritic cell induce this process are, directly through cell-cell interactions with adjacent B-cells and indirectly through the secretion of various soluble molecules (Coffman et al., 1993; Fayette et al., 1997; Le Bon et al., 2001b; Stevens et al., 1988). Therefore, experiments investigating the humoral response of B-cells during TB disease in isolation could lead investigators to believe that B-cells are ineffective in protecting the host against *M.tb* infection and that the observed humoral responses are insufficient in neutralizing invading bacilli. Whereas, studies utilising animal models to study anti-TB humoral immunity *in vivo* and *ex vivo* analysis of human samples from healthy and active TB participants has proved that humoral responses

do in fact aid in the defence against *M.tb* infection (Achkar and Casadevall, 2013; Achkar et al., 2015; Balu et al., 2011; Casadevall and Pirofski, 2006; Chan et al., 2014; Li et al., 2017; Maglione and Chan, 2009; Teitelbaum et al., 1998). This emphasizes the need to validate experimental observations in several independent experiments, utilizing difference techniques, before making any definitive conclusions regarding the relative importance of a cell type in health and disease.

Furthermore, the effect of sample type on B-cell phenotypic frequencies was investigated. As previously stated, due to limited cell numbers, isolated B-cells were pooled according to QFN status and stimulation condition prior to flow cytometry analysis. Consequently, observations made from the resulting phenotypic data focus primarily on difference between whole blood and PBMCs, while inferring the physiological implications of the mean B-cell frequencies observed for isolated B-cell samples. The effect of sample type on B-cell development was investigated via evaluation of the relative frequencies on T1, T2, MZ and FO B-cells within each sample type following antigenic stimulation. Interestingly, no significant difference in the frequency of all B-cell subsets was observed between whole blood and PBMCs. However, a shared pattern of decreased MZ B-cells was observed for all sample types following each successive isolation procedure. In contrast an increase in T1, T2 and FO B-cells was observed for all sample types following cellular isolation. Regrettably, the significance of alterations within isolated B-cell samples could not be determined, however a parallel trend with sizeable difference in the investigated frequencies was evident.

The involvement of MZ B-cells in T-independent early adaptive immune responses is well established, in which amplified inclination for plasma cell differentiation and superior induction of Th1 expansion has been shown by MZ B-cells in comparison to FO B-cells (Attanavanich and Kearney, 2004; Crawford et al., 2006; Lopes-Carvalho et al., 2005). As such, MZ B-cells are regarded as primarily responsible for protective humoral and effector T-cell immune response. In contrast, activation of FO B-cells occurs via T-cell dependant mechanisms, and is thus involved in late immune responses (Balázs et al., 2002; Martin et al., 2001). FO B-cells have been shown to primarily be involved in memory B-cell development; while some FO B-cells may differentiate into plasma cells for the expansion of long-lived humoral immune cells (Calame, 2001; Kerfoot et al., 2011). Hence, the regulation of MZ B-cell development during immunogenic challenge substantially influences host immune responses. These results indicate the potential impact of cell isolation on B-cell derived immune responses; in which decreased frequencies of MZ B-cells was found. Thus, impaired B-cell development, as a result of diminished microenvironment complex due to cellular isolation, may result in the manifestation of inappropriate cellular responses to antigenic stimulation *in vitro*. The subsequent implication of this for immunology research inferring cell function utilizing isolated cell culture techniques is paramount.

Lastly, the effect of sample type on killer B-cell phenotypic frequencies was investigated. A significant increase in B-cell frequencies for all population subsets, with the exception of IL5Ra-FasL<sup>+</sup> MZ B-cells, was observed following PBMC isolation. Inversely, a significant decrease in the aforementioned FasL-expressing MZ B-cell subset was observed following PBMC isolation. Again, the significance of alterations observed for isolated B-cell samples could not be determined, however similar trends illustrating vast differences in B-cell frequencies was observed for isolated B-cell samples. It is well to remember that the induction and maintenance of killer B-cells during immunogenic challenge greatly impacts the resulting immune response through suppression of effector T-cell function via receptor-mediated apoptosis (Alderson and Lynch, 1998; Arase et al., 1995; Lundy, 2009; Lundy and Boros, 2002; Lundy et al., 2015; van Rensburg et al., 2017; Stalder et al., 1994; Süss and Shortman, 1996). The expression of Fas, the cellular target of FasL, by activated T-cells has been widely reviewed, in which an increase in Fas expression by effector CD4<sup>+</sup> T-cells following immunogenic stimulation has been shown (Arase et al., 1995; Singer and Abbas, 1994; Stalder et al., 1994a; Süss and Shortman, 1996). Upon engagement of Fas with FasL, activation of an intracellular signalling cascade follow, resulting in commencement of receptor-mediated apoptosis (Alderson and Lynch, 1998; Arase et al., 1995; Lundy, 2009; Lundy and Boros, 2002; Lundy et al., 2015; van Rensburg et al., 2017; Stalder et al., 1994; Süss and Shortman, 1996). This delicate balance between pro-inflammatory, anti-inflammatory and immune suppressive function ultimately determines the incident of successful disease management or immunopathological effects. As such, it is suspected that physiological alterations in the frequencies of killer B-cells, such as the considerable increase observed within the pilot study following successive B-cell isolation, could deleteriously influencing immunological responses under investigation and result in improper diagnosis as to the relative position of a cell type for a particular immune condition. For instance, adequate levels of killer B-cells during acute inflammation may assistance be beneficial, by preventing exacerbated immunopathology while having modest effect effector function (van Rensburg and Loxton, 2018a; van Rensburg et al., 2017; Van Rensburg et al., 2017). Whereas, infrequent/abundant killer B-cell frequencies may have detrimental effects on the observed clinical outcome. Examples of such instances include autoimmune disease (De Maria and Testi, 1998; Sharpe et al., 2007) and cancer, respectively (Lee-Chang et al., 2013; Schwartz et al., 2016; Tadmor et al., 2011).

In addition to the effects of sample type on B-cell function, the effects of stimulation condition were investigated. Currently, a large gap in TB research with respect to the role of previously underappreciated cell types in TB disease exists. Majority of studies investigating and implying the role of these neglected cell types utilize isolated cell culture techniques. As such, determination as to the relative impact of *M.tb* infection on B-cell function was investigated. The effect of stimulation condition on immunoglobulin isotype abundance was investigated, in which upon first glance no obvious alterations in the immunoglobulin profile were observed. However upon further analysis, statistically significant differences between TLR9a stimulation and unstimulated samples, as well as TLR9a



stimulation and *M.tb* infection samples, for the secretion of IgA were observed. In both cases, TLR9a stimulation significantly decreased the relative abundance of IgA within the microenvironment. Moreover, a statistically significant difference in the relative abundance of IgG3 was observed between TLR9a stimulation and unstimulated samples. In which an increase in the relative abundance of IgG3 was observed in response to TLR9a stimulation. No significant difference between H37Rv stimulated and unstimulated samples was observed indicating that *M.tb* exposure did not have an immediate effect of humoral immunity.

Furthermore, the effects of stimulation condition on B-cell phenotypic frequencies was examined. A general pattern of enhanced B-cell development was observed following TLR9a stimulation compare to unstimulated controls. Wherein, a significant increase in T2 B-cell and FO B-cell frequencies was observed following TLR9a stimulated. Similarly, a significant increase in the frequency of MZ B-cells, was observed in response to TLR9a stimulation as well as *M.tb* infection compared to unstimulated control. In constant, a significant decreases in T1 B-cells was observed in response to TLR9a stimulation as well as *M.tb* infection compared to unstimulated control. The observed results are in agreement with findings in literature, in which TLR9a stimulation has been shown to induced MZ B-cell development (Lenert et al., 2005). The induction of MZ B-cells following H37Rv stimulation indicates that *M.tb* challenge evokes early non-humoral B-cell responses that may play an importance role in the host defence against *M.tb* infection. These responsibilities may include the stimulation of T-cell activation and proliferation to facilitate augmentation of protective effector CD4<sup>+</sup> T-cell responses (Attanavanich and Kearney, 2004; Crawford et al., 2006; Lopes-Carvalho et al., 2005), shown to be highly correlated with enhanced bacterial containment and clearance (Forbes et al., 2008; Maglione et al., 2007).

Moreover, the effects of stimulation condition on killer B-cell frequencies was investigated, in which a general pattern of increased killer B-cell frequencies was observed for all population subsets following TLR9a stimulation. In accordance, a significant increases in CD19<sup>+</sup>IL5Ra<sup>+</sup>FasL<sup>+</sup> B-cells, IL5Ra<sup>+</sup>FasL<sup>+</sup> T1 B-cells, IL5Ra<sup>+</sup>FasL<sup>+</sup> T2 B-cells, IL5Ra<sup>+</sup>FasL<sup>-</sup> T2 B-cells and IL5Ra<sup>+</sup> FasL<sup>-</sup> MZ B-cells was observed following TLR9a stimulation, compared to unstimulated samples. Likewise, a significant increase in the cellular frequencies of CD19<sup>+</sup>IL5Ra<sup>+</sup>FasL<sup>+</sup> B-cells, IL5Ra<sup>+</sup>FasL<sup>+</sup> T1 B-cells and IL5Ra<sup>+</sup>FasL<sup>+</sup> MZ B-cells was observed following TLR9a stimulation, compare to H37Rv stimulated samples. Additionally, a pattern of increased levels of IL5Ra-expressing B-cells, although not significant in all cases, was observed for all population subtypes following H37Rv infection. Specifically, a significant increase in IL5Ra<sup>+</sup>FasL<sup>+</sup> T2 B-cells and IL5Ra<sup>+</sup>FasL<sup>-</sup> T2 B-cells was observed following H37Rv infection, compared to unstimulated samples. Opposingly, following H37Rv infection, a minor decreases, although not statistically relevant, in IL5Ra<sup>+</sup>FasL<sup>+</sup> B-cells was observed for all stimulation conditions compared to unstimulated controls. These results underscore the ability of B-cell to actively response to *M.tb* challenge and insinuate the

possibility that B-cells may be involved in directing and shaping the immune response during TB disease.

According to the World Health Organization (WHO), one third of world's population is estimated to be latently infected with *M.tb* (Corbett et al., 2003; Dodd et al., 2014; Houben and Dodd, 2016; World Health Organization, 2017). Latent tuberculosis infection (LTBI) can be defined as a state of constant immune response to stimulation by *M.tb* antigens, while exhibiting no clinical manifestation of active disease (Getahun et al., 2015; World Health Organization, 2017). The occurrence of LTBI can be determined by a simple blood test, known as the IFN $\gamma$  release assay; wherein heightened levels of IFN $\gamma$  production in response to stimulation with *M.tb* specific antigens, such as ESAT-6 and CFP-10, are indicative of immunogenic memory and thus pre-exposure to *M.tb*. Individuals observed to have this amplified response are regarded as latently infected and are denoted QFN positive. It is assumed that the immune response elicited in QFN positive individuals in response to a particular stimulus may differ significantly from QFN negative individuals in their course, magnitude and response time.

Consequently, the effect of QFN status on immunoglobulin expression patterns was investigated, in which no significant difference in the relative abundance of each of the immunoglobulin isotypes was observed between QFN positive and negative individuals. Likewise, the effects of QFN status on B-cell development was investigated. A general pattern of decreased B-cell frequencies was observed for all investigated populations for QFN negative individuals compared to QFN positive individuals (Figure 4b). For majority of the investigated populations, namely T1 B-cells, MZ B-cells and FO B-cells, QFN status was found to have no significant effect on the observed B-cell frequencies, whilst, a significant decrease in the observed frequency of T2 B-cells was observed for QFN negative individuals. These results are intriguing as T2 B-cells are regarded as the principal precursors for both MZ and FO B-cells and have been shown to be heavily involved in effector T-cell development (Cariappa et al., 2001; Kuroda et al., 2003; Martin and Kearney, 2002). Therefore, alterations in the frequency of this cell population could largely impact successive immunological responses. Last but not least, the effects of QFN status on killer B-cell frequencies was investigated, in which a general pattern of decreased killer B-cell frequencies was observed for QFN negative individuals was observed. In all instances, QFN status was found to have no significant effect on all investigated killer B-cell populations. Notably, a limitation of this study included the relatively small sample size, which may account for the insignificant differences between QFN positive and negative individuals observe.

Apart from investigating the effects of each of the experimental conditions on the relative expression of the various immunoglobulin isotypes and B-cell development, the effect of these factor in combination was investigated. In doing so, the effects of a single variable on another experimental condition could be examined to identify whether or not a relationship between the two existed. Firstly, the effect of QFN



status in combination with sample type was investigated. In all cases, factors in combination with QFN status were observed to have modest effects, while in some instances statistical significance was found. With regards to the effect on QFN status and sample type in combination on the relative abundance of each immunoglobulin isotype, no significance was observed. Though, a discrepancy in the responsiveness of B-cells between QFN positive and negative individuals was observed, in which immunoglobulin responses of QFN positive individuals were found to be less intense compared to QFN negative individuals. Likewise, an inconsequential effect of QFN status in relation to sample type was observed for the induction of killer B-cells. In contrast, a substantial difference in the frequency of T2 B-cells was observed in the PBMC fraction of QFN positive individuals compared to QFN negative individuals, while no difference was observed in whole blood. Additionally, no significance difference in the frequency of T1 B-cells, MZ B-cells or FO B-cells between QFN positive and negative individuals was observed for whole blood or PBMC samples. Collectively, these results indicated that although not significant in all instances, QFN status had a moderate effect on the immune response elicited following antigenic challenge. Wherein, alterations within the humoral response of QFN positive individuals was observed to be less receptive to stimulation. Oppositely, QFN positive individuals were found to have heightened immune responses to antigenic challenge with regards to the induction of various B-cell populations, particularly FasL-expressing B-cells. The physiological implications of this phenomenon include predisposition of QFN positive individuals to develop suppressive immune responses following immune challenge. As previously disclosed abundance of these killer B-cells during a particular physiological condition may impose effector function and exacerbate disease conditions.

In conclusion, the effect of stimulation condition and sample type in combination was investigated (refer to supplementary). For all investigated immune responses, a general theme was observed in which PBMCs were found to be more susceptible to antigenic stimulation compared to whole blood or isolated B-cells. A significant decrease in the relative abundance of IgA was observed for PBMCs following TLR9a stimulation compared to unstimulated controls, while no difference in the immunoglobulin profile of all other isotypes was observed for whole blood, PBMCs or isolated B-cells in response to antigenic stimulation. Correspondingly, a significant increase in T2 B-cell frequencies was observed for PBMCs following TLR9a stimulation compared to unstimulated controls, while no difference was observed for whole blood. Equally, a significant increase in T1 B-cell frequencies was observed for PBMC following H37Rv infection compared to unstimulated controls, while no difference was observed for whole blood. Dissimilar, a significant increase in MZ B-cell frequencies was observed for all stimulatory conditions in whole blood compared to PBMCs. Further investigation revealed QFN positive individuals to be the cause of this observed response. Finally, the effect of stimulation condition in combination with sample types on killer B-cell frequencies was examined. A significant increase of all IL5Rα<sup>+</sup>FasL<sup>+</sup> B-cell subpopulations was observed for PBMCs following TLR9a stimulation or H37Rv infection compared to the unstimulated control, while a decrease was observed in whole blood samples under the same conditions.

These results demonstrating the impact microenvironment composition has in guiding immune cell activation and function, give prominence to the fact that cellular isolation profoundly affect cell function. Additionally, the stupefaction of these complex interaction underscores the basis for the use isolated cell studies to investigate cellular function, in an attempt to limit the degree of external factors influencing the observed results. This allows for the assumption, with complete certainty, that the measured output for a cell population is in response to a particular drug or stimulus. However, it is important to remember that this isolated interaction is not indicative of real-life scenarios. Composite cellular interactions exist *in vivo* that may drastically influence the function of the cell type of interest resulting in a different reaction of these cells to the same drug or stimulus in whole organism.

### **Concluding Remarks**

Our study illustrates that microenvironment complexity i.e. sample type had a profound impact of the activation and function of B-cells. Wherein, a burdened ability of B-cells to secrete various antibody classes, as well as dysregulated induction of B-cell subtypes such as killer B-cells and altered B-cell development, was observed following cell isolation. This emphasizes the impact that isolated cell studies may have on the measured immune responses, and that while isolated cell culture techniques allow for in-depth functional analysis, the resulting findings may be artefactual observations and if not interoperated cautiously can lead to inappropriate conclusions regarding authentic cellular function. As a final confirmation of scientific discovery all *in vitro* results should be verified in several independent experiments, utilizing various experimental techniques (including *in vivo* animal model) to ensure the integrity of the obtained results.

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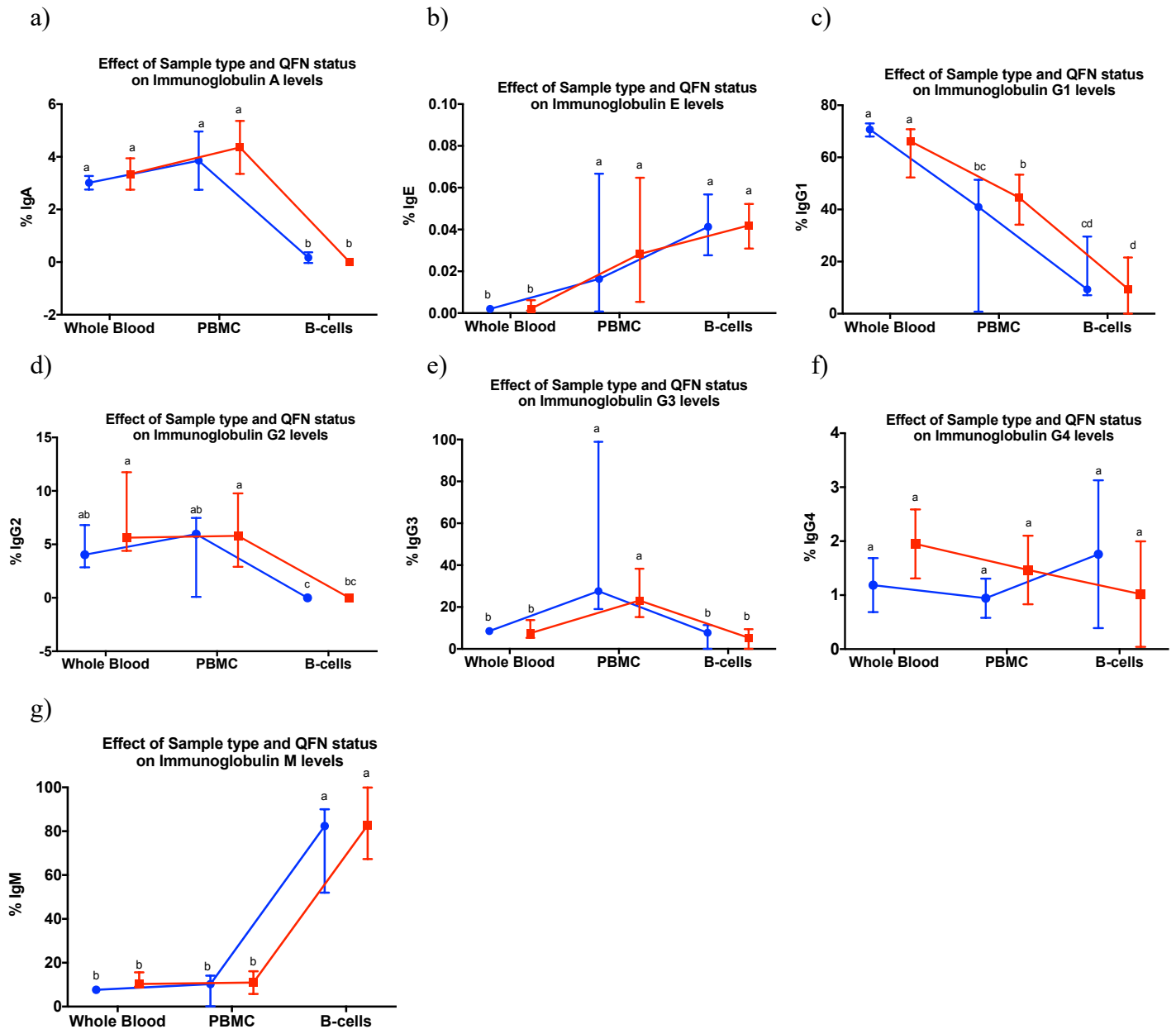
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## Supplementary Information

### 1. *Second and third order effects on Immunoglobulin expression profile*

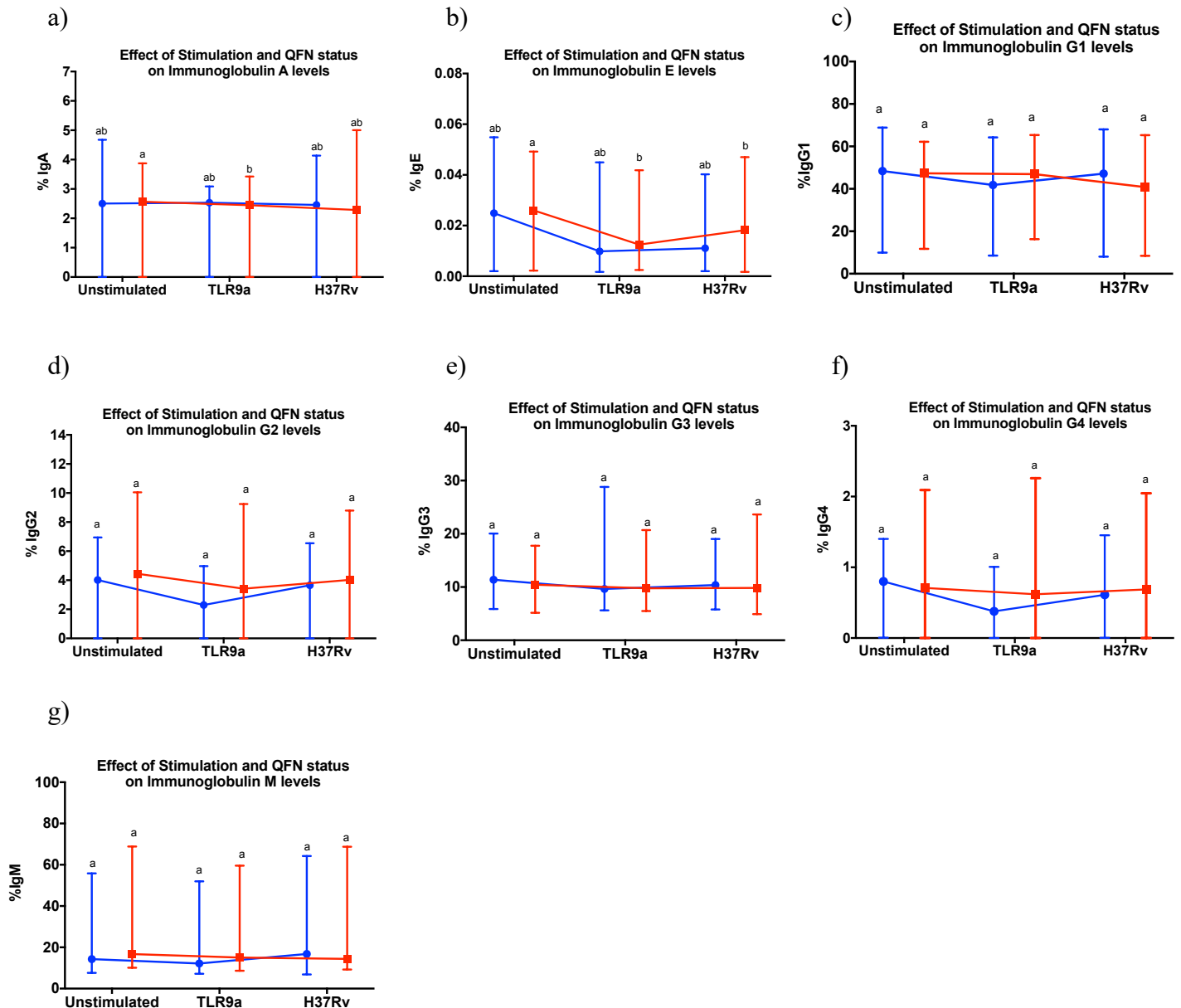
In addition to investigating the effects of each of the experimental conditions on the relative abundance of the various immunoglobulin isotypes, the effect of these factor in combination was investigated. The reason for this was to observe if a relationship between either (denoted second-order interactions) or all (denoted the third-order interaction) of the experimental conditions existed. Consequently, the effects of QFN status and sample type in combination on immunoglobulin expression patterns was investigated (Suppl Figure 5.1). No significant difference ( $p>0.05$ ) was found when investigating the general correlation between QFN status and sample type. These results illustrate that the consequential effect of QFN status and sample type on immunoglobulin expression patterns are independent of one another. Likewise, the effects of QFN status and stimulation condition in combination was investigated (Suppl Figure 2). In all cases, no significant difference ( $p>0.05$ ) between QFN positive and negative individuals was observed for each of the stimulation conditions; whilst significant differences between stimulation conditions within a particular QFN group were detected. A significant decrease in IgA was observed following TLR9a stimulation for QFN negative individual ( $p=0.0177$ ). A similar trend was seen for QFN positive individuals however these differences were not significant (Suppl Figure 5.2a). Similarly, a significant decrease in IgE was observed for QFN negative individuals following TLR9a stimulation ( $p=0.019$ ) or H37Rv infection ( $p=0.0287$ ) compared to the unstimulated control; whilst a trivial decrease was observed for QFN positive individuals (Suppl Figure 5.2b). A marginal decrease in IgG1 was observed for QFN positive individuals in response to TLR9a stimulation (ns,  $p>0.05$ ). Comparably, a decrease in IgG1 was observed in response to TLR9a stimulation (ns,  $p>0.05$ ), as well as H37Rv infection ( $p>0.05$ ), compared to the unstimulated control for QFN negative individuals (Suppl Figure 5.2c). A general trend of decreased IgG2 was observed for QFN negative individuals following antigenic stimulation (ns,  $p>0.05$ ), whereas a decrease in IgG2 for QFN positive individuals was only observed in response to TLR9a stimulation (ns,  $p>0.05$ ) (Suppl Figure 5.2d). The same immunogenic response was observed for both QFN positive and negative individuals with regards to IgG3 abundance, in which a minor decrease was observed following antigenic stimulation (ns,  $p>0.05$ ) (Suppl Figure 2e). No difference in IgG4 abundance was observed for QFN negative individuals following antigenic stimulation, whilst a minor decrease was observed for QFN positive individuals following TLR9a stimulation (ns,  $p>0.05$ ) (Suppl Figure 5.2f). To finish, no difference in IgM abundance was observed for both QFN positive and negative individuals following antigenic stimulation (ns,  $p>0.05$ ) (Suppl Figure 5.2g). These results indicate a slight discrepancy in the activation state of B-cells between QFN positive and negative individuals, in which immunoglobulin responses of QFN positive individuals were found to be less responsive to antigenic stimulation compared to QFN negative individuals. However, these differences were not significant.

● QFN Positive      ■ QFN Negative



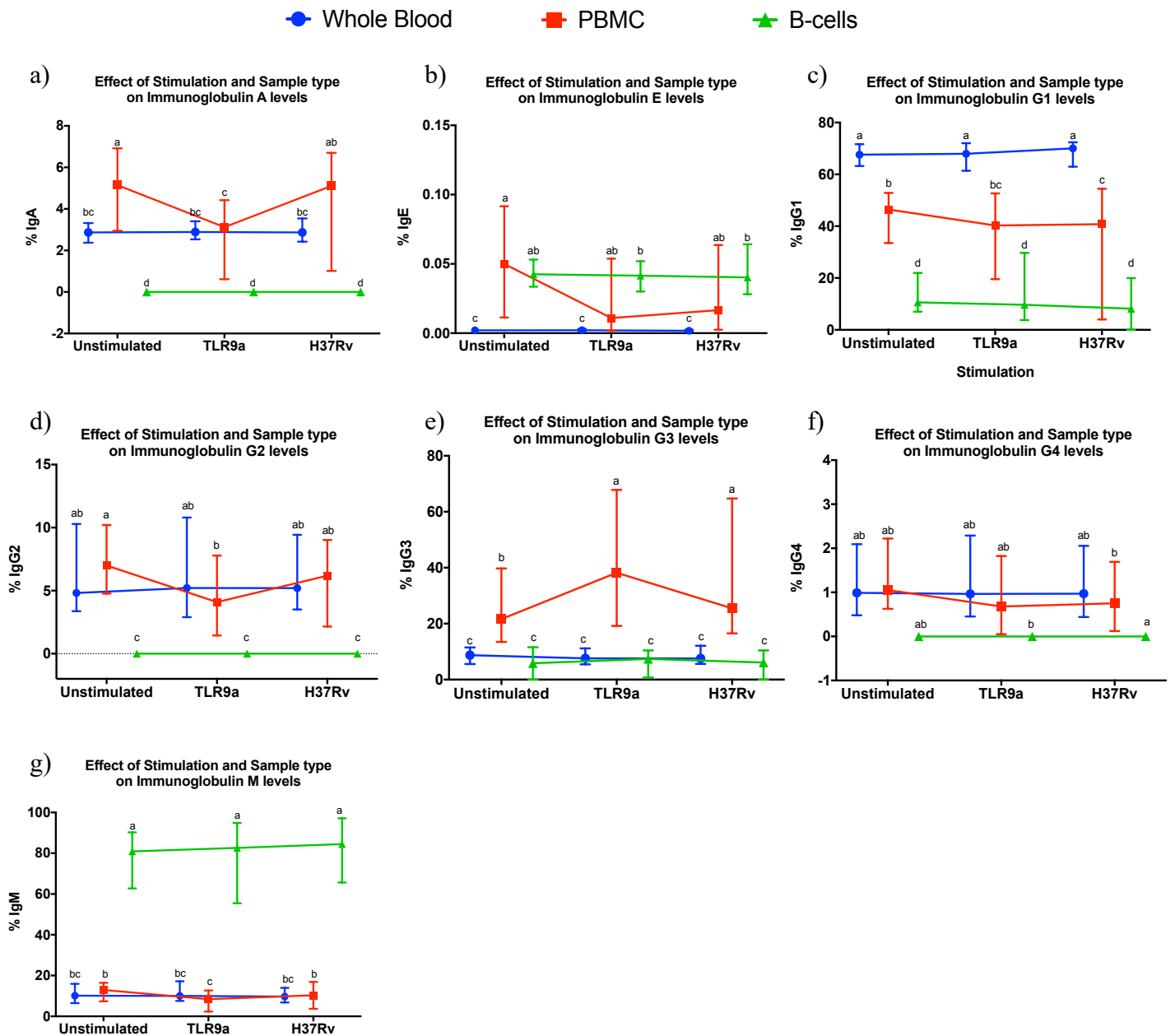
**Suppl Figure 5.1. Effect of QuantiFERON status and sample type in combination on immunoglobulin expression pattern of B-cells following antigenic stimulation.** Following a 24-hour stimulatory period, plasma/culture supernatants of each of the stimulatory conditions for all sample types was collected and the immunoglobulin isotype profile determined using Luminex. Median with interquartile range plotted. Statistical differences between culture conditions was calculated using a four-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistical differences are indicated by letters, in which groups denoted with different letters indicate statistical differences. Effect of QuantiFERON (QFN) status and sample type in combination on secretion of (a) IgA (b) IgE (c) IgG1 (d) IgG2 (e) IgG3 (f) IgG4 (g) IgM

◆ QFN Positive      ■ QFN Negative

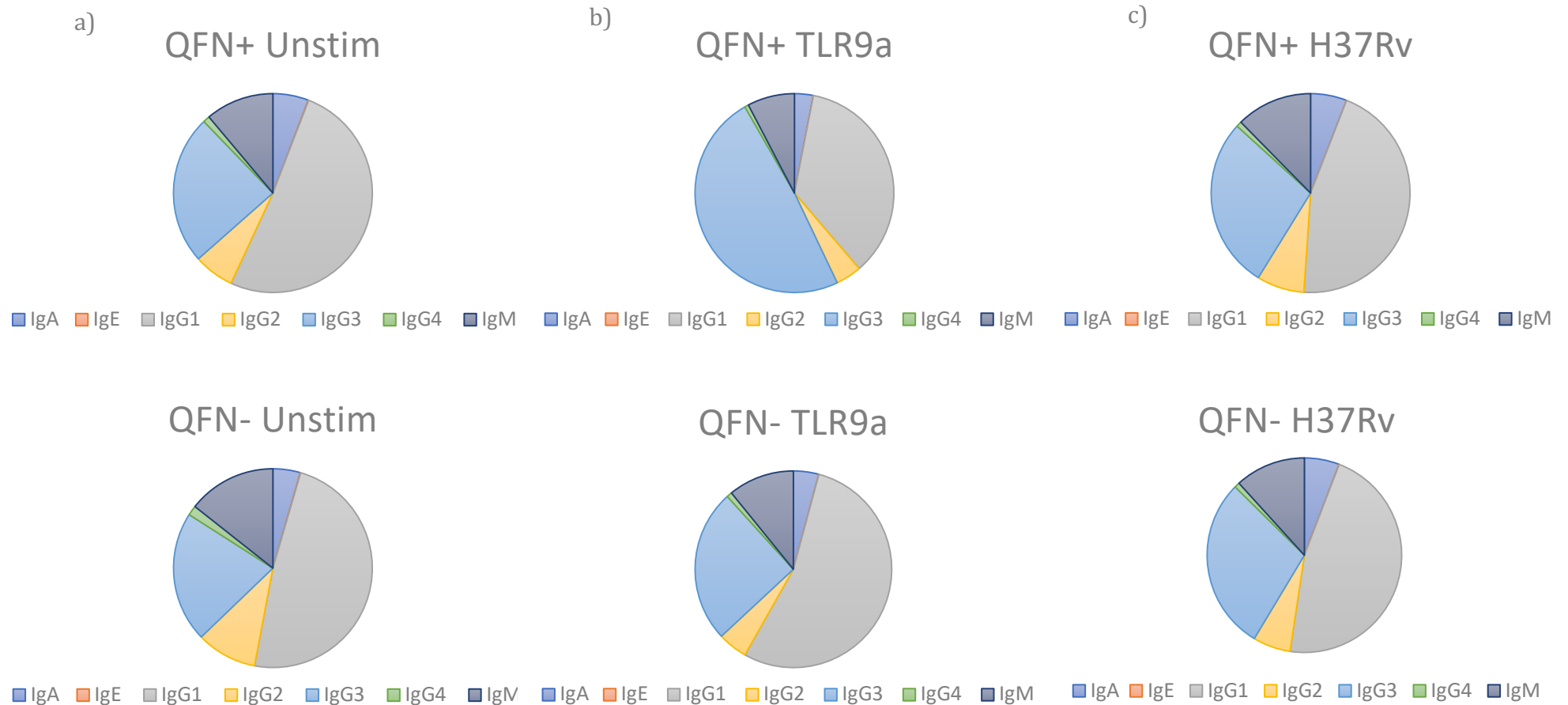


**Suppl Figure 5.2. Effect of QuantiFERON status and stimulation condition in combination on immunoglobulin expression pattern of B-cells following antigenic stimulation.** Following a 24-hour stimulatory period, plasma/culture supernatants of each of the stimulatory conditions for all sample types was collected and the immunoglobulin isotype profile determined using Luminex. Median with interquartile range plotted. Statistical differences between culture conditions was calculated using a four-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistical differences are indicated by letters, in which groups denoted with different letters indicate statistical differences. Effect of QuantiFERON (QFN) status and stimulation condition in combination on secretion of (a) IgA (b) IgE (c) IgG1 (d) IgG2 (e) IgG3 (f) IgG4 (g) IgM

Furthermore, the effect of stimulation condition and sample type in combination on immunoglobulin expression patterns was investigated (Suppl Figure 5.3). Upon inspection, a general theme was observed in which PBMCs were found to be more susceptible to antigenic stimulation compared to whole blood or isolated B-cells. For example, a significant decrease in IgA levels was detected for PBMCs following TLR9a stimulation compared to unstimulated ( $p=0.0000$ ) or H37Rv stimulated ( $p=0.0000$ ) samples; while no significant difference for was observed for whole blood or isolated B-cell samples (Suppl Figure 5.3a). Additionally, a significant difference in IgA between whole blood and PBMC ( $p=0.0124$ ), whole blood and B-cells ( $p=0.0000$ ) and PBMC and B-cells ( $0.0000$ ) for unstimulated samples was observed; as well as between whole blood and B-cells ( $p=0.0000$ ) and PBMCs and B-cells ( $p=0.0000$ ) for TLR9a stimulation and H37Rv infection, respectively. A comparable pattern was observed for all immunoglobulin isotypes with regards to the variable immunoglobulin profile of PBMCs in comparison to whole blood or isolated B-cells. Wherein, significant variations were observed for IgG1 (Suppl Figure 5.3c), IgG2 (Suppl Figure 5.3d), IgG3 (Suppl Figure 5.3e) and IgM (Suppl Figure 5.3g), while similar but non-significant differences were observed for IgE (Suppl Figure 5.3b) and IgG4 (Suppl Figure 5.3f). Lastly, the effect of QFN status, stimulation condition and sample type in combination on immunoglobulin expression patterns was investigated. In all instances, the overall effect of these factors in combination resulted in insignificant alterations to the observed immunoglobulin isotype levels. In spite of this, a slight discrepancy was observed when comparing the immunoglobulin profile of PBMCs following TLR9a stimulation (Figure 5.3b). Wherein an increase in the relative abundance of IgG3 ( $p=0.0262$ ) was observed for QFN positive compared to QFN negative individuals (Suppl Figure 5.4). However, this was not the case for all other stimulatory conditions for whole blood (Suppl Figure 5.5), PBMCs or isolated B-cells (Suppl Figure 5.6). Depicted in Suppl Figure 5.7 is an example of the multifaceted relationship between all investigated experimental conditions for a single immunoglobulin isotype. The resulting perplexity of this complex interaction highlights the rationale behind the use isolated cell studies to investigate cellular function, in order to limit the degree of external factors influencing the observed results. A summary of the significance of each of the various first, second and third order interactions investigated is described in Suppl Table 5.1.

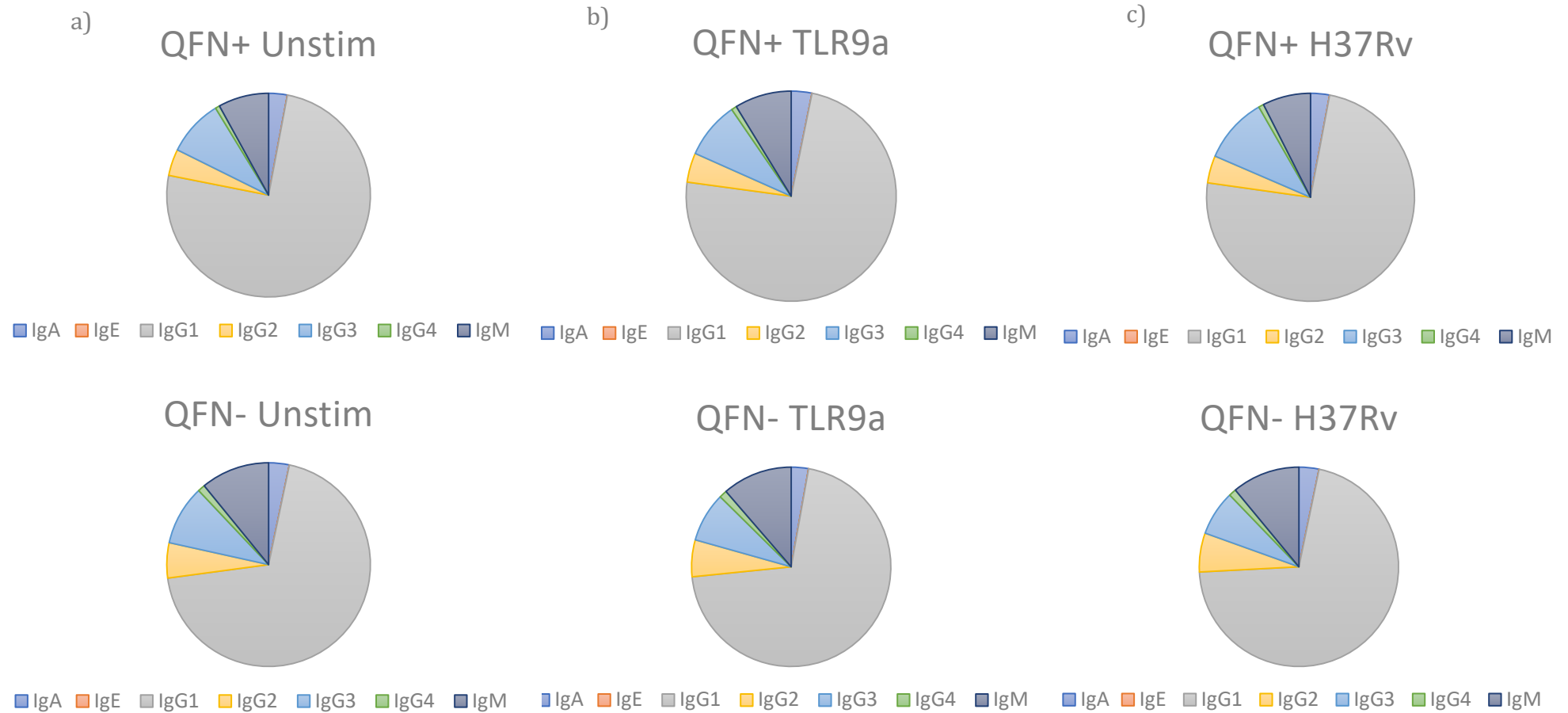


**Suppl Figure 5.3. Effect of stimulation condition and sample type in combination on immunoglobulin expression patterns of B-cells following antigenic stimulation.** Following a 24-hour stimulatory period, plasma/culture supernatants of each of the stimulatory conditions for all sample types was collected and the immunoglobulin isotype profile determined using Luminex. Median with interquartile range plotted. Statistical differences between culture conditions was calculated using a four-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistical differences are indicated by letters, in which groups denoted with different letters indicate statistical differences. Effect of stimulation condition and sample type in combination on secretion of (a) IgA (b) IgE (c) IgG1 (d) IgG2 (e) IgG3 (f) IgG4 (g) IgM

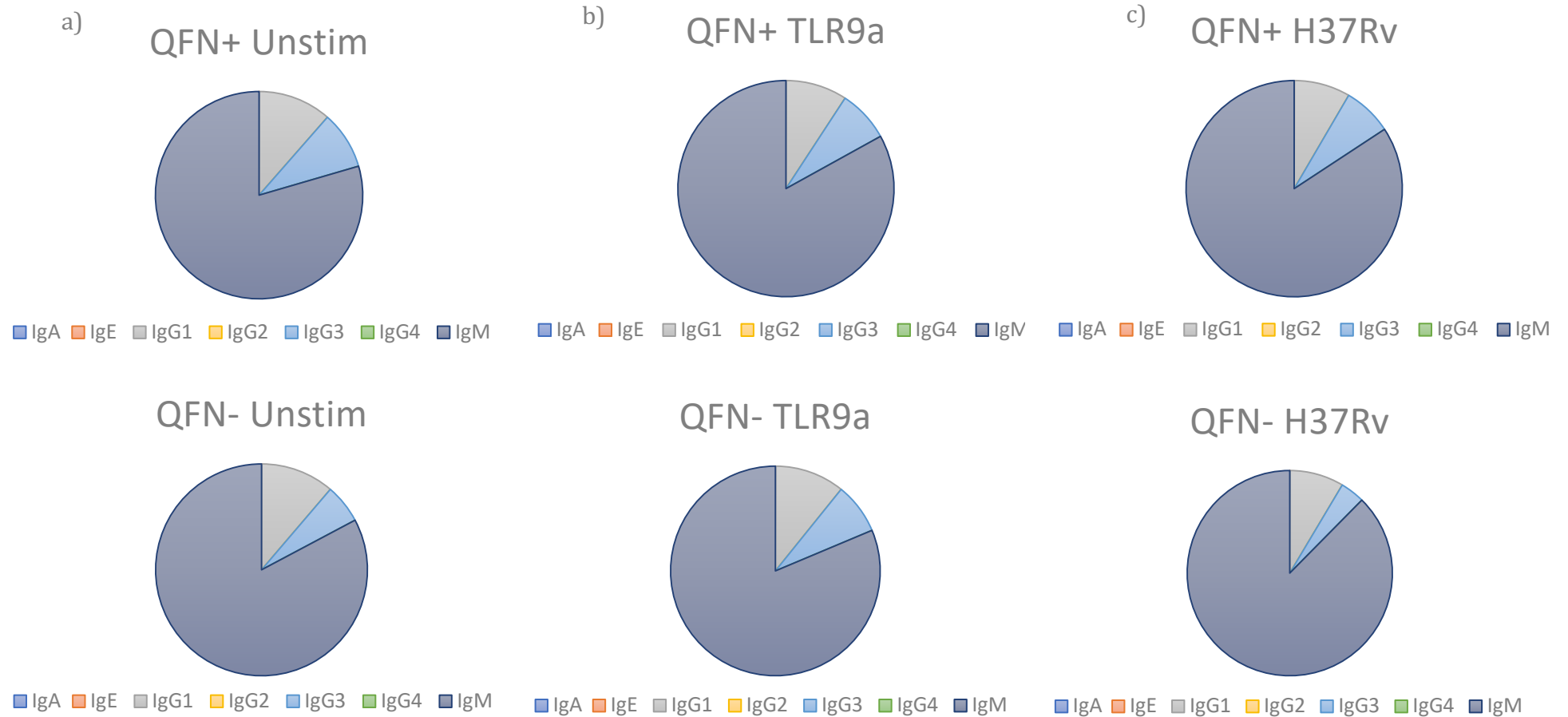


**Suppl Figure 5.4. Comparison of Immunoglobulin expression patterns of QuantiferON positive and negative participants for each of the stimulatory conditions in PBMCs.** Following a 24-hour stimulatory period, plasma/culture supernatants of each of the stimulatory conditions for all sample types was collected and the immunoglobulin isotype profile determined using Luminex. (a) Representation of the average secretion of each isotype by QuantiferON (QFN) positive and negative individuals within unstimulated PBMCs (b) Representation of the average secretion of each isotype by QFN positive and negative individuals within TLR9a stimulated PBMCs (c) Representation of the average secretion of each isotype by QFN positive and negative individuals within *M.tb*-stimulated PBMCs.



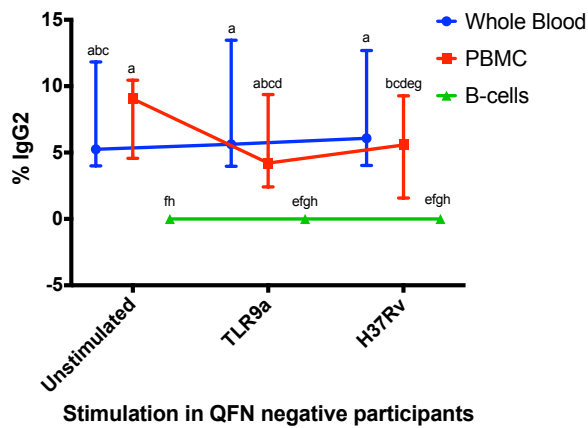


**Suppl Figure 5.5. Comparison of Immunoglobulin expression patterns of QuantiFERON positive and negative participants for each of the stimulatory conditions in whole blood.** Following a 24-hour stimulatory period, plasma/culture supernatants of each of the stimulatory conditions for all sample types was collected and the immunoglobulin isotype profile determined using Luminex. (a) Representation of the average secretion of each isotype by QuantiFERON (QFN) positive and negative individuals within unstimulated whole blood (b) Representation of the average secretion of each isotype by QFN positive and negative individuals within TLR9a stimulated whole blood (c) Representation of the average secretion of each isotype by QFN positive and negative individuals within *M.tb*-stimulated whole blood.

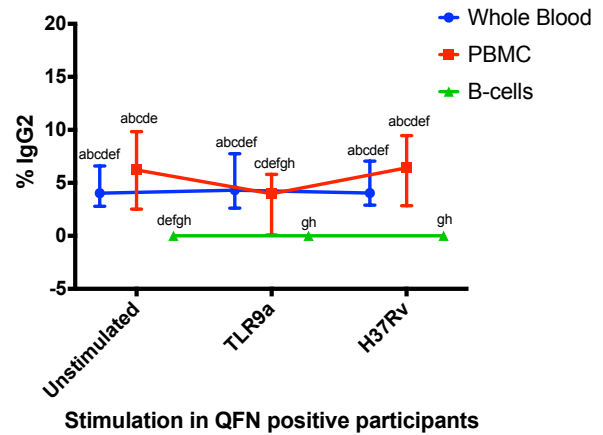


**Suppl Figure 5.6. Comparison of Immunoglobulin expression patterns of QuantiFERON positive and negative participants for each of the stimulatory conditions in Isolated B-cells.** Following a 24-hour stimulatory period, plasma/culture supernatants of each of the stimulatory conditions for all sample types was collected and the immunoglobulin isotype profile determined using Luminex. (a) Representation of the average secretion of each isotype by QuantiFERON (QFN) positive and negative individuals within unstimulated B-cells (b) Representation of the average secretion of each isotype by QFN positive and negative individuals within TLR9a stimulated B-cells (c) Representation of the average secretion of each isotype by QFN positive and negative individuals within *M.tb*-stimulated B-cells.

a) Effect of Stimulation, Sample type and QFN status on Immunoglobulin G2 levels



b) Effect of Stimulation, Sample type and QFN status on Immunoglobulin G2 levels



**Suppl Figure 5.7. Evaluation of the effects of third order interactions with respect to the investigated factors on immunoglobulin expression patterns.** Following a 24-hour stimulatory period, plasma/culture supernatants of each of the stimulatory conditions for all sample types was collected and the immunoglobulin isotype profile determined using Luminex. An example of the various second and third order interactions within the data set is depicted. The Immunoglobulin levels depicted are reported as a percentage of the total immunoglobulin within a given sample. Whiskers denote 10-90 percentile. Statistical differences between culture conditions was calculated using a four-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistical differences are indicated by letters, in which groups denoted with different letters indicate statistical differences. (a) Collective effect of negative status, stimulation condition and sample type on immunoglobulin G2 secretion (b) Collective effect of positive status, stimulation condition and sample type on immunoglobulin G2 secretion

<i>Effect</i>	<b>IgA</b>		<b>IgE</b>		<b>IgG1</b>		<b>IgG2</b>		<b>IgG3</b>		<b>IgG4</b>		<b>IgE</b>	
	P-value	q-value	P-value	q-value	P-value	q-value	P-value	q-value	P-value	q-value	P-value	q-value	P-value	q-value
<i>QFN_status</i>	0.73	0.91	0.90	0.91	0.65	0.91	0.50	0.91	0.48	0.91	0.78	0.91	0.22	0.91
<i>Stimulation</i>	<b>0.01</b>	0.07	0.15	0.34	0.20	0.34	0.33	0.39	0.14	0.34	0.53	0.54	0.24	0.34
<i>Sample type</i>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	<b>0.00</b>	0.59	0.09	<b>0.00</b>	<b>0.00</b>
<i>QFN_status*</i>	0.72	0.79	0.33	0.69	0.62	0.79	0.14	0.69	0.39	0.69	0.78	0.79	0.29	0.69
<i>Stimulation</i>														
<i>QFN_status*</i>	0.77	0.91	0.91	0.92	0.18	0.82	0.53	0.82	0.40	0.82	0.28	0.82	0.58	0.82
<i>Sample type</i>														
<i>Stimulation*</i>	<b>0.00</b>	<b>0.00</b>	<b>0.02</b>	0.06	0.35	0.33	0.35	0.33	0.05	0.10	0.20	0.30	0.38	0.33
<i>Sample type</i>														
<i>QFN_status*</i>	0.20	0.35	0.61	0.85	0.99	1.00	<b>0.04</b>	0.28	0.15	0.35	0.72	0.85	0.11	0.35
<i>Stimulation*</i>														
<i>Sample type</i>														

**Suppl Table 5.1. Fixed effects observed for immunoglobulin expression patterns of B-cells.** Statistical differences between culture conditions was calculated using a four-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini, Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistically significant p-values are highlighted in red. The adjusted p-value is denoted 'q-value'.

## 2. Second and third order effects on B-cell Development

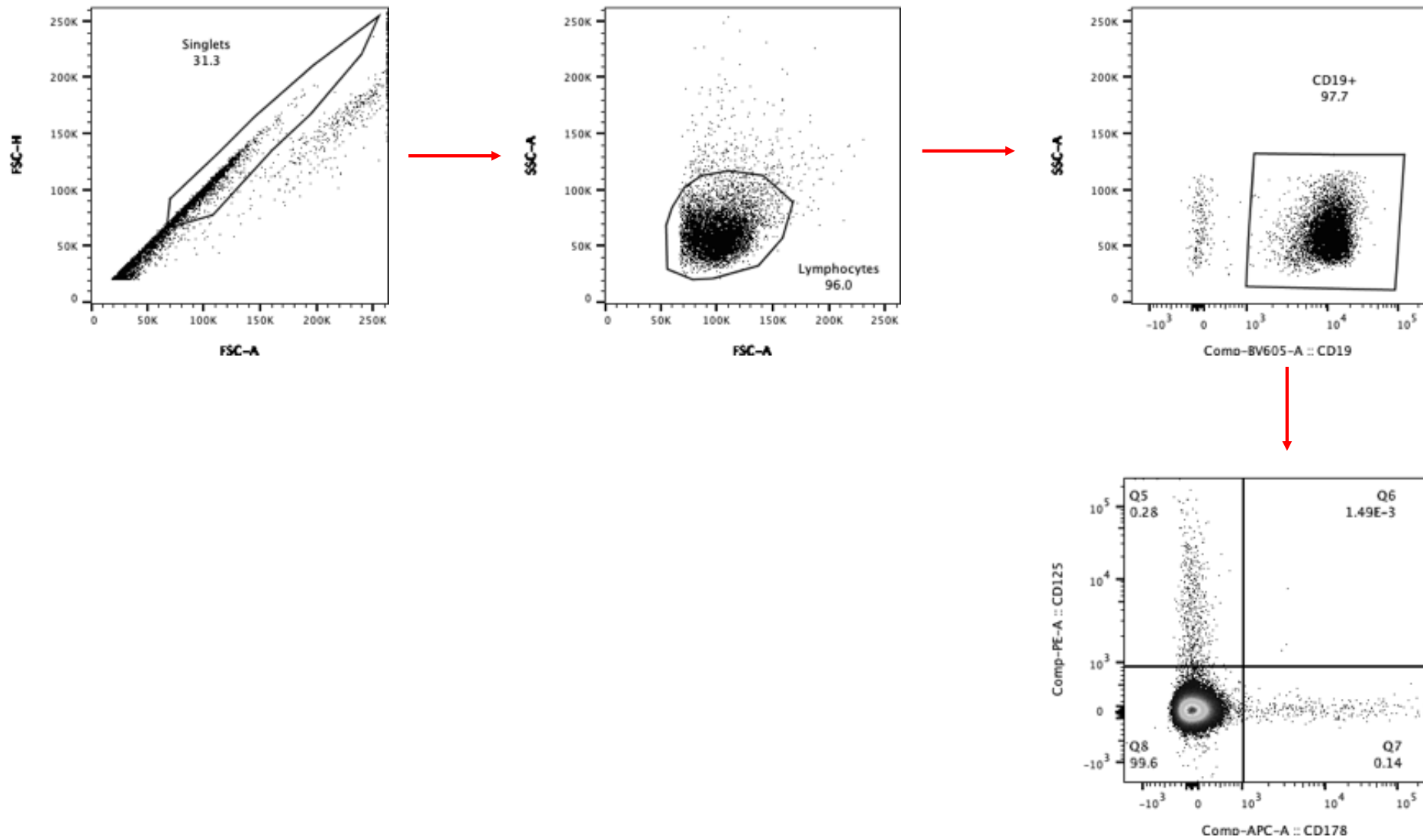
The effect of each experimental conditions in combination on the B-cell development was investigated (Suppl Table 5.2). The effects of QFN status and sample type in combination on T1, T2, MZ and FO B-cell frequencies was examined, in which no significance difference ( $p > 0.05$ ) was observed between QFN positive and negative individuals for all but one population. A substantial difference was observed for CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup> (T2) B-cells ( $p = 0.0033$ ), in which increased frequencies were observed in the PBMC fraction for QFN positive individuals compared to QFN negative individuals while no difference was observed in whole blood. Moreover, the effects of QFN status and stimulation condition in combination was investigated. Generally, no effect on the investigated B-cell frequencies was observed ( $p < 0.05$ ); however, a sizeable impact was observed for a single population, namely CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup> (T2) B-cells ( $p = 0.0002$ ). In which QFN positive individuals were observed to have increased cellular frequencies following TLR9a stimulation, compared to all other stimulatory conditions in both QFN positive and negative individuals. Lastly, the effect of stimulation condition and sample types in combination was investigated. Remarkably, a significant relationship was observed for all but one of the investigated cell phenotypes, specifically CD19<sup>+</sup>CD21<sup>-</sup>CD23<sup>+</sup> (FO) B-cells ( $p = 0.1443$ ), demonstrating the prominent role microenvironment composition plays in manipulating immune cell activation and function. A substantial increase in CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>-</sup> (MZ) B-cells ( $p = 0.0000$ ) was observed for all stimulatory conditions in whole blood compared to PBMC. Conversely, a considerable increase in CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup> (T2) B-cells ( $p = 0.0003$ ), with enhanced immune response to TLR9a stimulation, was observed for PBMCs compared to whole blood. Upon further investigation, it was discovered that QFN positive individuals were solely responsible for the former observed response ( $p = 0.0034$ ). Equally, increased levels of CD19<sup>+</sup>CD21<sup>-</sup>CD23<sup>-</sup> (T1) B-cells ( $p = 0.0000$ ), with enhanced immune response to *M.tb* infection and higher basal levels within unstimulated samples, was observed for PBMCs compared to whole blood samples.

<i>Effect</i>	<b>QFN Status</b>		<b>Stimulation</b>		<b>Sample type</b>		<b>QFN*Stim</b>		<b>QFN*Type</b>		<b>Stim*Type</b>		<b>QFN*Stim*Type</b>	
	<b>(QFN)</b>	<b>(QFN)</b>	<b>(Stim)</b>	<b>(Stim)</b>	<b>(Type)</b>	<b>(Type)</b>	<b>(Type)</b>	<b>(Type)</b>	<b>(Type)</b>	<b>(Type)</b>	<b>(Type)</b>	<b>(Type)</b>	<b>(Type)</b>	<b>(Type)</b>
	P-value	q-value	P-value	q-value	P-value	q-value	P-value	q-value	P-value	q-value	P-value	q-value	P-value	q-value
<i>CD19+ Cell Population</i>														
CD21+CD23-	0.24	0.37	0.00	0.00	0.03	0.05	0.13	0.19	0.20	0.34	0.00	0.00	0.12	0.52
CD21+CD23+	0.00	0.00	0.00	0.00	0.28	0.26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CD21-CD23+	0.15	0.37	0.01	0.02	0.05	0.06	0.10	0.19	0.10	0.34	0.14	0.13	0.57	0.65
CD21-CD23-	0.32	0.42	0.00	0.00	0.04	0.06	0.31	0.34	0.25	0.34	0.00	0.00	0.25	0.54
CD125+FasL-	0.31	0.42	0.25	0.23	0.07	0.08	0.05	0.13	0.23	0.34	0.70	0.46	0.21	0.54
CD125+FasL+	0.72	0.74	0.35	0.29	0.00	0.00	0.43	0.41	0.26	0.34	0.00	0.00	0.46	0.62
CD125-FasL+	0.11	0.37	0.00	0.00	0.25	0.25	0.00	0.00	0.04	0.29	0.18	0.14	0.90	0.88
CD21+CD23-CD125+FasL-	0.24	0.37	0.63	0.46	0.00	0.00	0.28	0.33	0.68	0.73	0.38	0.28	0.61	0.65
CD21+CD23-CD125+FasL+	0.21	0.37	0.14	0.14	0.02	0.04	0.14	0.19	0.14	0.34	0.00	0.00	0.11	0.52
CD21+CD23-CD125-FasL+	0.73	0.74	0.01	0.02	0.00	0.00	0.12	0.19	0.14	0.34	0.05	0.06	0.29	0.55
CD21+CD23+CD125+FasL-	0.10	0.37	0.01	0.02	0.53	0.44	0.05	0.13	0.05	0.29	0.04	0.05	0.60	0.65
CD21+CD23+CD125+FasL+	0.18	0.37	0.02	0.03	0.00	0.00	0.04	0.13	0.09	0.34	0.00	0.00	0.06	0.52
CD21+CD23+CD125-FasL+	0.22	0.37	0.11	0.13	0.03	0.05	0.14	0.19	0.35	0.43	0.49	0.34	0.22	0.54
CD21-CD23+CD125+FasL-	0.50	0.61	0.04	0.05	0.01	0.03	0.39	0.39	0.53	0.61	0.02	0.03	0.37	0.62
CD21-CD23-CD125-FasL+	0.04	0.23	0.00	0.00	0.02	0.04	0.00	0.00	0.21	0.34	0.12	0.12	0.47	0.62
CD5+CD125+FasL-	0.72	0.74	0.39	0.30	0.34	0.30	0.28	0.33	0.75	0.76	0.18	0.14	0.92	0.88
CD5+CD125+FasL+	0.84	0.80	0.35	0.29	0.09	0.10	0.53	0.45	0.25	0.34	0.00	0.00	0.42	0.62
CD5+CD125-FasL+	0.02	0.17	0.14	0.14	0.99	0.78	0.54	0.45	0.80	0.76	0.96	0.59	0.23	0.54

**Suppl Table 5.2. Fixed effects observed for of B-cell population distribution.** Statistical differences between culture conditions was calculated using a four-way mixed model ANOVA. Comparisons within groups was calculated using the Fishers LSD post-hoc test. A two-way step-up Benjamini , Krieger and Yekutieli False Discovery rate (FDR) approach, with a FDR of 1%, was used to correct for multiple testing. Statistically significant p-values are highlighted in red. The adjusted p-value is denoted 'q-value'.

### 3. *Second and third order effects on Killer/FasL-expressing B-cell frequencies*

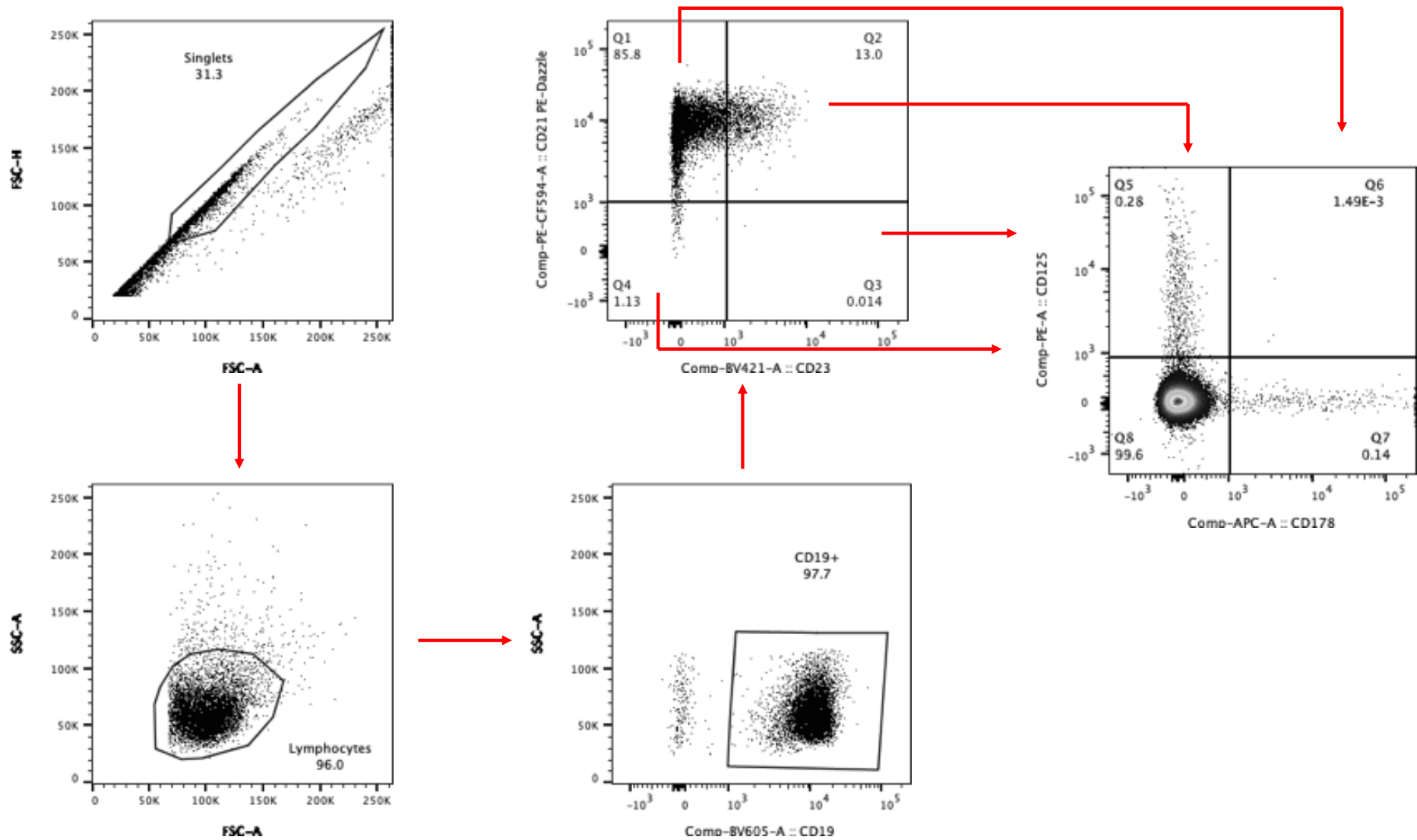
The effect of each experimental conditions in combination on the killer B-cell levels was investigated (Suppl Table 5.2). The effects of QFN status and sample type in combination on killer B-cell frequencies was explored, in which no significance difference ( $p > 0.05$ ) was observed between QFN positive and negative individuals for each sample type (Suppl Table 5.2). These results illustrate the inconsequential effect of QFN status and sample type in relation to one another on the induction of killer B-cells. Likewise, the effects of QFN status and stimulation condition in combination was investigated. Interestingly, for majority of the killer B-cell subsets no effect was observed, while a considerable effect was observed for two IL5Ra-FasL<sup>+</sup> populations, namely CD19<sup>+</sup>IL5Ra-FasL<sup>+</sup> ( $p = 0.0041$ ) and CD19<sup>+</sup>CD21<sup>-</sup>CD23<sup>-</sup>IL5Ra-FasL<sup>+</sup> B-cells ( $p = 0.0047$ ). For each of these cases, QFN positive individuals were observed to have increased cellular frequencies following TLR9a stimulation compared to all other stimulatory conditions in both QFN positive and negative individuals. Finally, the effect of stimulation condition and sample types in combination on killer B-cell frequencies was examined, to which rather imperative findings arose. For all killer B-cell subsets, the frequencies of cells co-expressing IL5Ra<sup>+</sup>FasL<sup>+</sup> were observed to be significantly affected. In all cases, following TLR9a stimulation or H37Rv infection, an increase in killer B-cell frequencies, specifically CD19<sup>+</sup>IL5Ra<sup>+</sup>FasL<sup>+</sup> B-cells ( $p = 0.0005$ ), CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>-</sup>IL5Ra<sup>+</sup>FasL<sup>+</sup> B-cells ( $p = 0.0003$ ), CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup>IL5Ra<sup>+</sup>FasL<sup>+</sup> B-cells ( $p = 0.0040$ ) and CD19<sup>+</sup>CD5<sup>+</sup>IL5Ra<sup>+</sup>FasL<sup>+</sup> ( $p = 0.0009$ ), was observed compared to the unstimulated control of PBMC samples, while a decrease was observed in whole blood samples. This discovery accentuates the point that isolation can dramatically affect cell function, resulting in incongruent observations between studies utilizing different sample types.



Suppl Figure 5.8. Gating strategy for FasL-expressing CD19<sup>+</sup> B-cells

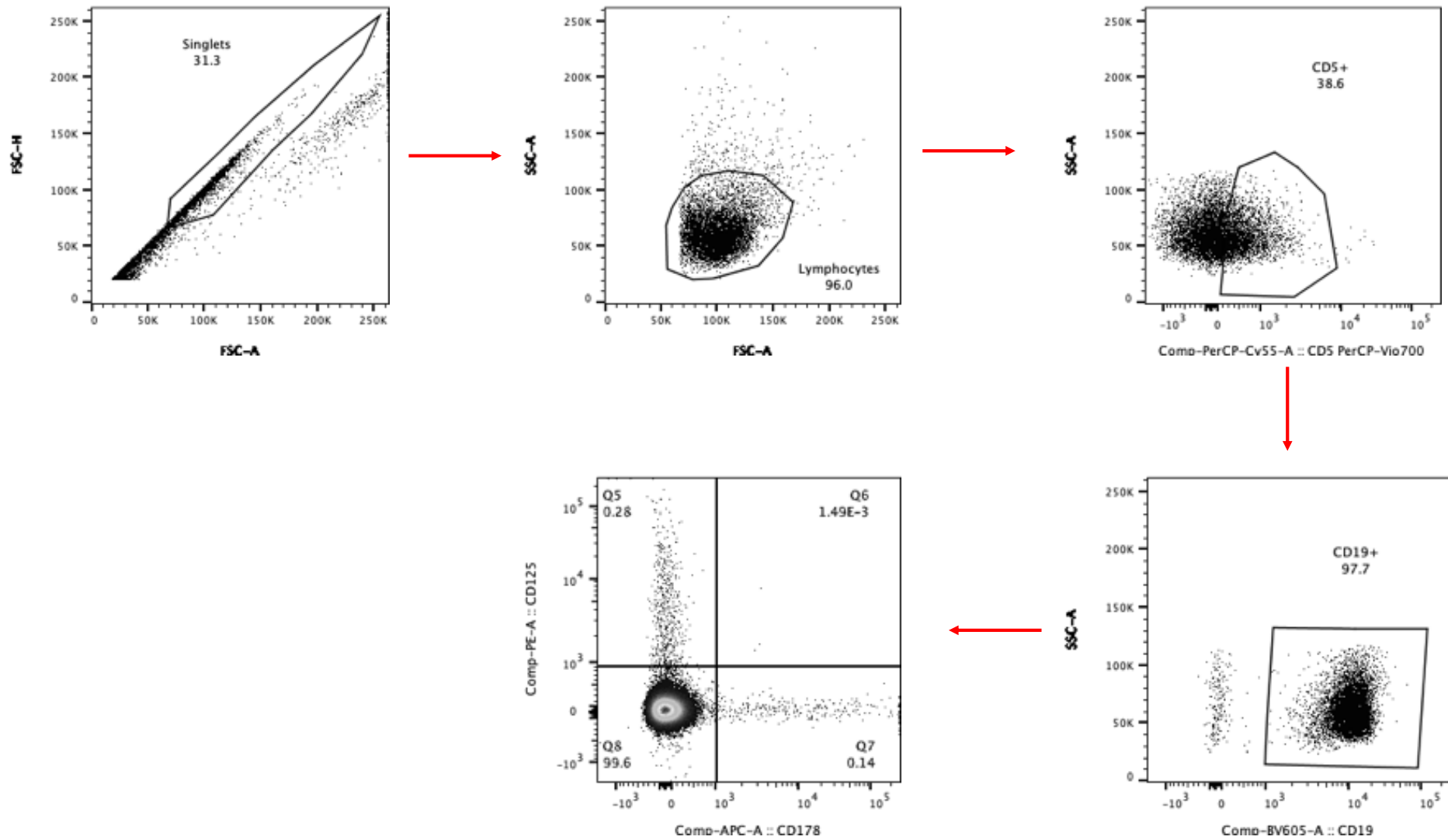
\*Note: Fluorescence-minus-one (FMO) control samples were used to determine appropriate gating cut-off to increase accuracy of distinguishing between populations





Suppl Figure 5.9. Gating strategy for T1, T2, MZ and FO B-cells

\*Note: Fluorescence-minus-one (FMO) control samples were used to determine appropriate gating cut-off to increase accuracy of distinguishing between populations



Suppl Figure 5.10. Gating strategy for CD5+CD19+ B-cells

\*Note: Fluorescence-minus-one (FMO) control samples were used to determine appropriate gating cut-off to increase accuracy of distinguishing between populations

## Chapter 6: Summary and Concluding Remarks

To gain insight into the multitude of processes required for effective control and eradication of *M.tb*, a comprehensive overview of the complex immune interactions that occur within the host is necessary. This includes understanding the relative significance of each cell type in determining the fate of TB incidence and progression. Thus far, an incomplete understanding of the role of each cell type within TB disease exists, attributing to the slow progress made towards improved TB prevention strategies (Andersen, 2001). Initial studies investigating anti-TB humoral responses of B-cells during TB disease showed enhanced protection to *M.tb* infection; however, these responses were shown to be inessential for successful *M.tb* eradication (De Valliere et al., 2005; Manivannan et al., 2012). Conversely, recent findings have suggested a clinically relevant role of B-cells during the early stages of *M.tb* infection (Achkar et al., 2015; Bénard et al., 2018; Du Plessis et al., 2016; Joosten et al., 2016; Rao et al., 2015). Researchers have since discovered several non-humoral functions of B-cells whose incidence and influence during TB disease remain ill defined. Characterization of various B-cell subtypes, such as B<sub>regs</sub> or killer B-cells, presumed to be responsible for these non-humoral responses have been well examined within autoimmunity (Bao and Cao, 2014; De Maria and Testi, 1998; Sharpe et al., 2007; Vincent et al., 2013), cancer (Lee-Chang et al., 2013; Mor et al., 2000; Schwartz et al., 2016; Shao et al., 2014) and transplantation (Adams and Newell, 2012; Chesneau et al., 2013; Firl et al., 2017). However, no conclusive discoveries as to the physiological relevance of these B-cell subtypes during TB disease have been made. This emerging evidence led us to think that these B-cell subsets, particularly killer B-cells, may have a vital role during *M.tb* infection. Hence, the purpose of this research project was to investigate the potential role of B-cells in the defense against *M.tb*. As previously alluded to, cell types capable of modulating the function of T-cells and macrophages may contribute substantially to the disease incidence and progression.

We hypothesize that B-cells possess the ability to regulate T-cell function either through direct cell-cell interaction or indirectly through the secretion of various soluble molecules. In accordance, this project aimed to study the interaction between B- and T-cells in the context of *M.tb* infection to determine the potential immunomodulatory role B-cells may play during TB disease. Studies have inferred the importance of several T-cell populations during TB disease, in which some populations have been shown to correlate with protection (Derrick et al., 2011; Lindenstrøm et al., 2009) while others have been found to correlate with poor disease prognosis (Caccamo et al., 2010; Forbes et al., 2008). Therefore, the induction of specific T-cell populations by B-cells following *M.tb* challenge could ultimately determine the fate of infection. To achieve our aims, we evaluated the effect of pre-stimulated B-cells on T-cell population distribution and cytokine secretion, and provided insight into the relative contribution of these cells to the host defense in combating *M.tb* infection. In line with our hypothesis, B-cells were observed to influence T-cell behavior through modulation of T<sub>reg</sub> and T<sub>E</sub> phenotypic frequencies, as well

as via alterations within the cytokine milieu of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells. These results are in agreement with findings within literature, in which B<sub>regs</sub> have been shown to modulate MDSC and T<sub>reg</sub> phenotypic frequencies (Carter et al., 2012; Flores-Borja et al., 2013; Kessel et al., 2012), illustrating the potential immune modulatory capability of B-cells during health and disease. This emphasizes the need for further investigation as to the potential role of cell types previously thought to have minimal involvement in protective anti-TB immunity. Furthermore, these findings underscore the prospect that B-cells play a fundamental role in initiating and guiding the immune response against *M.tb*. The physiological implications of this includes the possibility for host directed therapy targeting B-cell function to induce protective CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses, as well as the development of improved prevention therapies that target protective humoral and non-humoral B-cells responses to afford superior protection from *M.tb* infection. Nevertheless, before any definite inference regarding the efficiency of B-cell to modulate T-cell function can be made, further studies aimed at elucidating the intricate interaction between B- and T-cells is required. Deeper insight into the relationship amongst B-cell activation and T-cell priming regarding the mechanisms involved in intercellular signaling and the extent of these responses are crucial to understanding how to target B-cells for immunotherapy and why this would benefit the host against *M.tb*-exposure.

Apart from understanding of this intercommunication between B- and T-cells in the context of TB, concerns with the translation of this interaction and response in whole organism ensued. Isolated cell studies provide valuable insight into the various biological mechanisms implemented by cells to elicit effector functions in response to a particular stimulus devoid of external factors. However, partial understanding of the complex intercellular interactions that occur within *in vivo*, due to the presence of additional cell types, may result in artefactual observations and imprecise analyses regarding the function of a cell population during a particular immune condition. We hypothesize that microenvironment complexity (i.e. the degree of cellular isolation) affects B-cell function, thereby impacting the measured immune responses. Consequently, this project aimed to determine the effects of microenvironment complexity on cell function, with an emphasis on B-cells, and the influence this may have on the prospect of isolated *in vitro* cell studies to reflect accurate events within whole organism. Additionally, the effect of cell isolation on the functional capacity of B-cells during *M.tb* infection was investigated, to determine the significance of employment of isolated cell culture techniques in studies inferring the role of this cell type during TB disease. Presently, observational findings inferring the physiological role of these cells during TB disease utilize isolated cell culture techniques (Du Plessis et al., 2016; van Rensburg and Loxton, 2018). Inherently, the results from such studies form the foundation upon which all assumptions regarding the importance of this cell type during disease are based. These deductions in turn affect the decisions relating to the development of new TB drugs, host-directed therapies and TB vaccines in terms of the possible cell types for targeting.

Therefore, we evaluated the immune response of B-cells within various compartments of isolation (e.g. whole blood, PBMC fraction and pure B-cells) following antigenic stimulation, to determine whether the sample type had an effect on the resulting cellular responses. Evaluation of the resulting immunoglobulin profiles of B-cells following antigenic stimulation (including *M.tb* infection) was performed. To our knowledge, B-cells are the only immune cell type capable of immunoglobulin secretion, thus immunoglobulin secretion was regarded as a direct measure of the B-cell function. Additionally, B-cell phenotypic frequencies were investigated to conclude the effect of sample type on B-cell development. In line with our hypothesis, microenvironment complexity was found to substantially influence immune cell function, in which impaired humoral B-cell function and altered B-cell development was observed following successive isolation procedures. This was illustrated by a hampered ability of B-cells to secrete various immunoglobulin isotypes, namely IgA, IgG1 and IgG3, in isolation - likely due to a lack of co-stimulatory signals from additional cell types. Furthermore, concurred mature B-cell development following cellular isolation was observed, illustrated by a decline in MZ B-cell frequencies and an increase in T2 B-cell levels. Similarly, dysregulation of killer B-cell induction, shown, in which significant increases in observed cellular levels, following isolation was found; emphasizing the impact isolation had on the capability of B-cells to elicit an efficient immune response. In light of the above, caution should be taken when interoperating observations acquired from cell isolation studies, as the *in vitro* microenvironment may have considerably influenced the observed findings. Furthermore, these results accentuate the possibility that B-cells play a more prominent role in anti-TB immunity than currently assumed, and further enquiry as to the function of these cells during TB disease is suggested.

## Project limitations and future research prospective

A short coming of this project included the small sample sizes used within each of the studies. Moreover, all participants were recruited within the same general demographic area. These factors could have contributed to artefactual observations regarding the significance of a particular experimental condition. As such, assumptions made from this pilot data should be interpreted with this in mind. Moreover, the absence of an active TB cohort as well as other lung disease (OLD) controls within the co-culture pilot study represents a drawback, as our observations were based solely on data from healthy QFN positive and negative individuals. Participants within our study are recruited from highly endemic TB areas (World Health Organization, 2017), where it is suggested that more than 50% of the population are exposed to *M.tb* challenged by the time they reach adolescence. However, only 10% of exposed individuals go on to develop active disease, underscoring the ability of their immune system to overcome infection. In the current context, understanding the role of B-cells during healthy conditions (this includes *M.tb*-exposed individuals) are more suited to our research questions as it provides the opportunity to study cellular responses essential for the successful combat of *M.tb* infection. As such, the applicability of these investigated physiological events is assumed during TB disease, rather than authenticated. Consequently, these results should be regarded as purely preliminary, highlighting the need for more in-depth analysis of the function of B-cells during *M.tb* challenge during different disease states. Prospective studies of active TB disease cohorts should increase the sample size before any conclusions regarding the relative significance of this intercellular interaction as a potential target for innovative anti-TB host therapies can be made. Additionally, investigation as to the function and inter-communication of the investigated cell types at the site of disease, rather than in the periphery, may provide valuable pertaining to the primary anti-TB immune mechanisms responsible for successful *M.tb* control and/or eradication.

Recently, the incidence of killer B-cell during TB disease had been accentuated (Lundy, 2009; Lundy et al., 2015; van Rensburg and Loxton, 2018; van Rensburg et al., 2017; Van Rensburg et al., 2017). However, the function of these cells along with possible mechanisms they employ to elicit the suggested protective immune responses during *M.tb* infection remain undefined. Thus, a practical future endeavor would involve full characterization of these naturally occurring killer B-cells using a variety of advance techniques, including single-cell FACS sorting, whole genome sequencing, receptor expression analysis, protein expression analysis and metabolic pathway mapping. This would improve the basic understanding we have of killer B-cell function and the physiological impact these cells may have on anti-TB immune responses.

Moreover, limited research investigating the role of killer B-cells *in vivo* has occurred. The results obtained within this study highlighted the significant effect microenvironment complexity has on cellular function. Thus, investigating the protective capacity of regulatory killer B *in vivo* may provide vital information as to the physiological relevance of this cell type in anti-TB immunity that would otherwise be masked/absent during *in vitro* culture. This can be achieved by induction of killer B-cells prior and preceding *M.tb* infection in murine models, as well as pre- and post- treatment. Such enquiry will provide insight into efficacy of this cell type to prevent, control and eliminate *M.tb* through modulation of adaptive immune responses, enhancing host-derived protection against TB infection. Importantly, evaluation of several sample types, including the blood, lung tissue and lymphatic system will provide a “full” picture of the augmented immune response that occur prior to establishment of disease and following successful infection control. This “complete” study approach will provide valuable insight into the complex communication between various systems within the body during TB disease, as well as uncover alterations in the immune profile within a specific sample type that would have otherwise gone unnoticed using an isolated experimental approach.

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