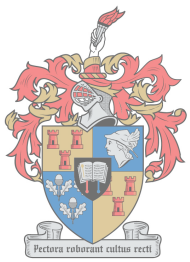
Correlation of the Mycobacterial killing capacity of Natural killer cells with the number of activating Killer immunoglobulin-like receptors expressed on their surfaces.

By

Talani van Schalkwyk



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Declaration

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Glossary

µL	Microliter
ANOVA	Analysis of variance
aKIR	activating killer immunoglobulin-like receptor
APC	Allophycocyanin
APC	Antigen presenting cell
APC_Cy7	Allophycocyanin cyanine dye 7
bp	Basepair
BCG	Bacillus Calmette-Guérin
BSL3	Biosafety level 3
CCL	Chemokine (c-c motif) ligand
CD	Cluster of differentiation
CFU	Colony Forming unit
CNV	Copy number variation
CXCL	Chemokine (c-x-c motif) ligand
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRB1	DR beta 1
EDTA	Ethylendiaminetetraacetic acid
FACS	Fluorescent activating cell sorting
FBS	Fetal bovine serum
FSC-A	Forward scatter-area
FSC-H	Forward scatter-height
GM-CSF	Granulocyte macrophage colony stimulating factor
HCl	Hydrochloric acid
hIL-2	Human interleukin-2
HIV	Human immune deficiency virus
HLA	Human leucocyte antigen
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
iKIR	Inhibiting killer immunoglobulin-like receptor
IL-15	Interleukin-15
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-23	Interleukin-23
IL-12	Interleukin-12
iNK	Immature natural killer
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
kb	Kilo-base
KIR	Killer immunoglobulin-like receptor
KZN	Kwazulu-Natal
LDH	Lactate dehydrogenase

LILR	Leucocyte immunoglobulin-like receptor
LN	Liquid nitrogen
LS	Least square
LRC	Leucocyte receptor complex
LSD	Least significant difference
LTBI	Latent tuberculosis infection
M.africanum	*Mycobacterium africanum*
M.bovis	*Mycobacterium bovis*
mM	Millimolar
M.tb	*Mycobacterium tuberculosis*
MHC	Major histocompatibility complex
MIC	MHC class 1 polypeptide-related sequence
Min	Minute
MIP-1α	Macrophage inflammatory protein 1- alpha
mL	Millilitre
mNK	Mature natural killer
MOI	Multiplicity of infection
MTC	*Mycobacterium tuberculosis complex*
NaOH	Sodium hydroxide
NCAM	Neural cell adhesion molecule
ng	Nanogram
NK	Natural Killer
nm	Nanometer
OD	Optical density
OR	Odds ratio
PAMP	Pathogen associated molecular pattern
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
Pe-Cy7	Phycoerythrin cyanine dye 7
PerCP-Cy5.5	Peridinin Chlorophyll Protein Complex Cyanine dye 5.5
PMT	Photomultiplier tubes
pNK	Precursor natural killer
PRR	Pattern recognition receptor
PTB	Pulmonary tuberculosis
pH	Potential of hydrogen
QC	Quality control
Rcf	Relative centrifugal force
RANTES	regulated on activation, normal T cell expressed and secreted
RPMI	Roswell Park Memorial Institute
ROS	Reactive oxygen specie
RT	Room temperature
S	Seconds
SAC	South African Coloured

SSC-A	Side scatter-area
SSP	Sequence specific primer
TB	Tuberculosis
TE	Tris-EDTA
Th	T helper
TNF-α	Tumor necrosis factor-alpha
Treg	Regulatory T
U	Units
UTR	Untranslated region
UV	Ultra violet
v/v	Volume per volume
vs	Versus
WC	Western Cape
WHO	World Health Organisation
w/v	Weight per volume
ZN	Ziehl-Neelsen
γδT	Gamma-delta T

Abstract

Tuberculosis (TB) is a curable disease, but continues to kill and remains a major health problem because of its high mortality rate throughout the world. While infection with the causative agent, *Mycobacterium tuberculosis (M.tb)* is necessary for the development of TB, it is not sufficient to cause disease in most infected individuals.

Only approximately 5-15% of infected, immunocompetent individuals progress to active clinical disease whereas the remainder will never develop the disease. The precise factors associated with progression to clinical disease are still largely uncharacterized. However the involvement of host genetics in TB has been proven unequivocally and, in concert with environmental factors and the causative bacterium, can influence the outcome of disease. Natural killer (NK) cells are important in the innate and adaptive system and are regulated by two receptor superfamilies with one of those being the immunoglobulin-like superfamily (killer immunoglobulin-like receptors).

An investigation of the role of killer immunoglobulin-like receptors (KIRs) in TB susceptibility found that an overexpression (≥ 5) of the activating killer immunoglobulin-like receptors (aKIRs) offers protection against developing active TB disease. In the present study the aim was to compare the efficiency of mycobacterial killing of NK cells of individuals with five or more aKIRs versus individuals with less than five aKIRs.

The *KIR* genotypes of 30 individuals were determined using a PCR sequence-specific primers method. Five individuals with less than five aKIRs (group 1) and five individuals with five or more aKIRs (group 2) were identified. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using density gradient centrifugation. NK cell populations were isolated from the PBMCs through negative selection. The NK cells were stimulated with *M.tb H37Rv* at two time points (1 hour & 24 hours), either with or without interleukin-2. CFU counts were used to determine the mycobacterium's viability. No statistical significant difference was observed between the two groups ($p = 0.60$).

The levels of cytokine production from NK cells following the stimulations were determined by Luminex immunoassays. A higher expression of interferon-gamma (IFN-γ), perforin, interleukin-17A (IL-17A) and regulated on activation, normal T cell expressed and secreted (RANTES) was observed for group 2. However only RANTES expression was statistically significant ($p = 0.04$). This pilot study was the first to contribute towards a protocol to determine the killing efficiency of

NK cells against fighting TB infection while incorporating the *KIR* genotype. Furthermore this study is also one of few that investigated the direct and extracellular killing of *M.tb H37Rv*.

Future work includes using different techniques to determine mycobacteria viability as well as incorporating the *HLA* genotypes. Ligand recognition assays can also be used to determine the involved ligand specificity. In the long run this can be used to manipulate NK cells to generate an increased immune protection against developing active TB.

Opsomming

Tuberkulose (TB) is 'n siekte wat genees kan word; tog eis dit steeds menselewens en bly dit een van die grootste gesondheidsprobleme wêreldwyd as gevolg van die hoë sterftesyfer. Hoewel infeksie met die patogeen *Mycobacterium tuberculosis (M.tb)* nodig is vir die ontwikkeling van TB, is dit alleen nie genoegsaam om die siekte by die meeste geïnfekteerde individue te veroorsaak nie.

Slegs ongeveer 5-15% geïnfekteerde, immuunbevoegde individue ontwikkel die kliniese aktiewe toestand van die siekte, terwyl die res nooit die aktiewe siekte kry nie. Die presiese faktore wat die progressie van die siekte veroorsaak, is nog nie vasgestel nie. Die betrokkenheid van gasheergenetika in TB speel egter onteenseglik 'n rol en, saam met omgewingsfaktore en die oorsaaklike bakterie, beïnvloed dit die uitkoms van die siekte. Natuurlike doderselle (NK) speel ‘n belangrike rol in die immuunsisteem en word deur twee reseptor superfamilies gereguleer, waarvan een die immunoglobulienagtige superfamilie is (witbloed immunoglobulienagtige reseptore).

'n Ondersoek na die rol van witbloed immunoglobulienagtige reseptore (KIRs) in TB vatbaarheid het aangedui dat individue wat vyf of meer aktiverende immunoglobulienagtige reseptore (aKIRs) gehad het, minder geneig was om aktiewe TB te ontwikkel. Hierdie studie se doel was om die effektiwiteit van mikobakteriële doding deur NK-selle van individue met vyf of meer aKIRs te vergelyk met individue met minder as vyf aKIRs.

Die *KIR* genotipe van 30 individue is bepaal deur die gebruik van ‘n PCR sekwensspesifieke metode. Vyf individue met minder as vyf aKIRs (groep 1) en vyf met vyf of meer aKIRs (groep 2) is geïdentifiseer vir verdere studies. Periferale bloed mononukleêre selle (PBMCs) is geïsoleer vanuit bloed, deur die digtheidsgradient sentrifugasie metode.

NK-selle is deur negatiewe seleksie geïsoleer vanuit die PBMCs. Die NK-selle is met *M.tb H37Rv* gestimuleer (1 uur & 24 uur), met of sonder interleukin-2. CFU-tellings is gebruik om die mikobakterieë se lewensvatbaarheid vas te stel. Geen beduidende verskil tussen die twee groepe is waargeneem nie ($p = 0.60$).

Vlakke van sitokienproduksie deur NK-selle na stimulasie, is deur Luminex immunoanalise vasgestel. Daar was 'n hoër uitdrukking van interferon-gamma (IFN-γ), perforien, interleukin-17A (IL-17A) en RANTES deur groep 2. Slegs die verskil in RANTES uitdrukking was statisties beduidend ($p = 0.04$). Hierdie navorsing was die eerste om by te dra tot ‘n protokol om vas te stel

wat die doodmaaksukses van NK-selle is as manier om TB-infeksie te beveg terwyl die *KIR* genotipe geïnkorporeer word. Hierdie studie is ook een van net 'n paar wat die direkte en ekstrasellulêre doodmaak van *M.tb H37Rv* ondersoek.

Toekomstige werk sluit die gebruik van ander tegnieke in om vas te stel wat die lewensvatbaarheid van *M.tb* is. Die *HLA* genotipes van die individue moet ook geïnkorporeer word. Ligand spesifisiteit kan ook bepaal word en dit kan gebruik word om NK-selle te manipuleer om die immuunsisteem te verbeter teen *M.tb* infeksie.

I dedicate this thesis to my parents,
Leon van Schalkwyk and Magda van Schalkwyk.

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Chapter 1: Introduction

1.1 Tuberculosis

Tuberculosis (TB) is a curable disease, but continues to kill and remains a major health problem because of its high mortality rate throughout the world[1]. In one of the earliest descriptions of TB, Greek physician Clarissimus Galen describes TB as an ulceration of the lungs, chest or throat[2]. It has now been established that TB is an infectious disease that is caused by the *Mycobacterium tuberculosis (M.tb)* complex (MTC)[2]. This complex consists of closely related bacterial species, namely, *M.tb*, *M. bovis*, *M. africanum*, *M. bovis* Bacillus Calmette-Guérin (BCG), *M. microti*, *M. canetti* and *M. pinnipedii*[3–6]. *M.tb* is a rod shaped, non-spore forming, obligatory aerobic pathogen that has a predilection for the lung tissue which is rich in oxygen supply and thus *M.tb* largely causes pulmonary TB (PTB) in humans[7–9]. Necrotising granulomatous inflammation, usually observed in the lung (~85% of cases), is therefore a pathological characterisation of TB[7].

During the infection period (between onset cough & initiation of treatment) transmission is known to occur, thus it would be ideal if people experiencing TB symptom would be promptly identified and diagnosed[10,11]. Transmission occurs through the expulsion of an airborne droplet nuclei from infected individuals that contains viable bacilli through coughing, sneezing or talking in close proximity[12]. The bacillary load, duration and proximity of exposure will have an influence on the transmission[13]. The symptoms of TB are mainly, night sweats, fever, weight loss and persistent, non-remitting cough[14].

1.2 Global epidemic

TB is one of the main causes of death due to a single infectious agent worldwide[15,16] . The World Health Organization (WHO) already declared TB a global health emergency in 1993. Even though there was a 22% decrease in TB deaths between 2000 and 2015, the disease was still one of the top 10 causes of death worldwide in 2015[1]. TB is a significant health problem globally with developing countries being the most affected as indicated in figure 1.1[1]. In 2016 more than 10 million people fell ill with TB and 1.7 million died with 95% of these deaths occurring in low and middle income countries[17]. Developed countries such as the United States and Australia have a TB incidence of less than 10 per 100 000 whereas the incidence of TB in developing countries like South Africa and Swaziland exceeds 1000 per 100 000[1]. The six countries that accounted for 60% of the new TB cases in 2015 were India, China, Nigeria, Indonesia, Pakistan and South Africa[15].

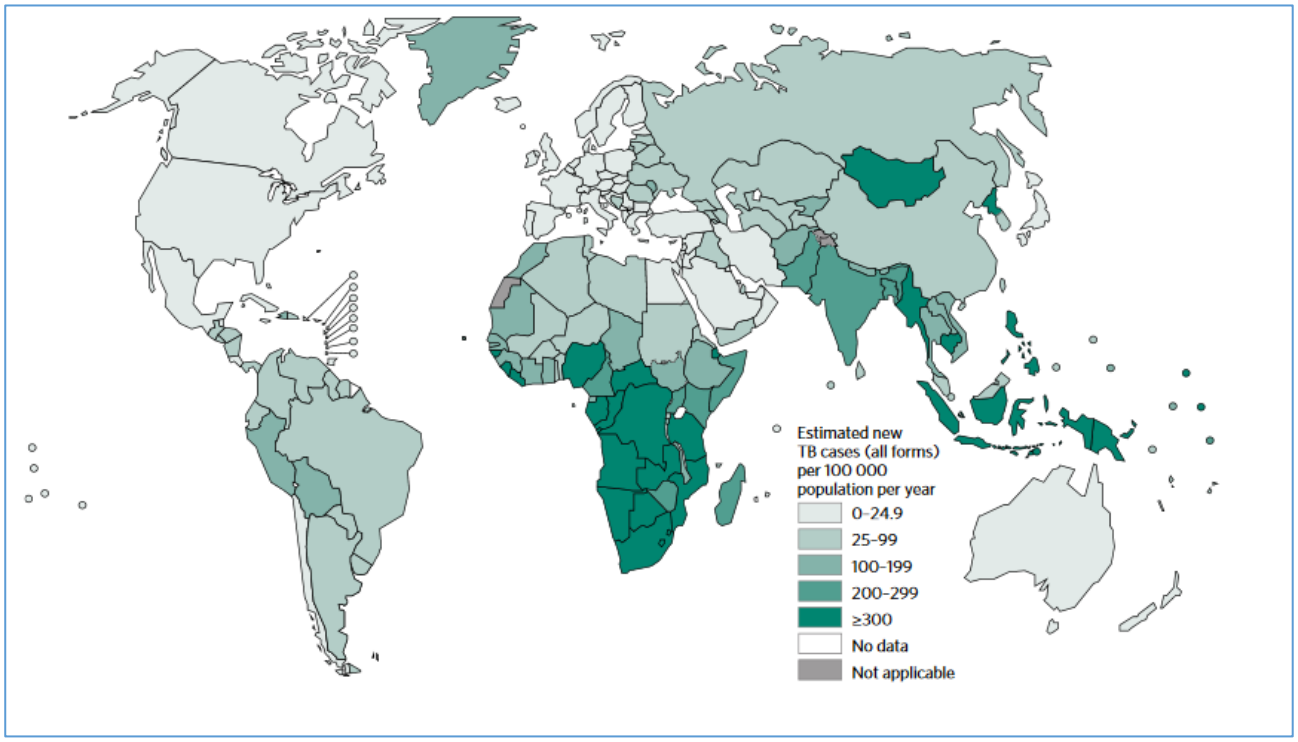


Figure 1.1: The estimated TB incidence rates in 2015. Developing countries have a higher incidence of new cases per 100 000 than developed countries. Adapted from World Health Organization Global Tuberculosis Report, 2016[1].

1.3 Influence of TB in South Africa

One of the countries with the highest TB incidence and subsequent mortality rates in the world is South Africa, with the human immunodeficiency virus (HIV) pandemic having a detrimental effect[18,14]. In 2015, the three districts with the highest TB burden were KwaZulu-Natal (KZN), Western Cape (WC) and Eastern Cape (EC) (Figure 1.2)[19]. In the WHO African region the proportion of TB cases living with HIV were 31% and exceeded 50% in some parts of Southern Africa[1]. HIV is an encumbrance to lowering the national TB incidence rate since it increases the lifetime-risk of converting from sub-clinical infection to active disease from 1 in 10 to 1 in 3[20]. In 2013, it was estimated that 10.4 million people had TB-HIV co-infection which was about 13% of the global incident caseload, with 80% of these causes occurring in Africa[7] and in 2015 it was also found that 35% of HIV deaths were due to TB[17] .

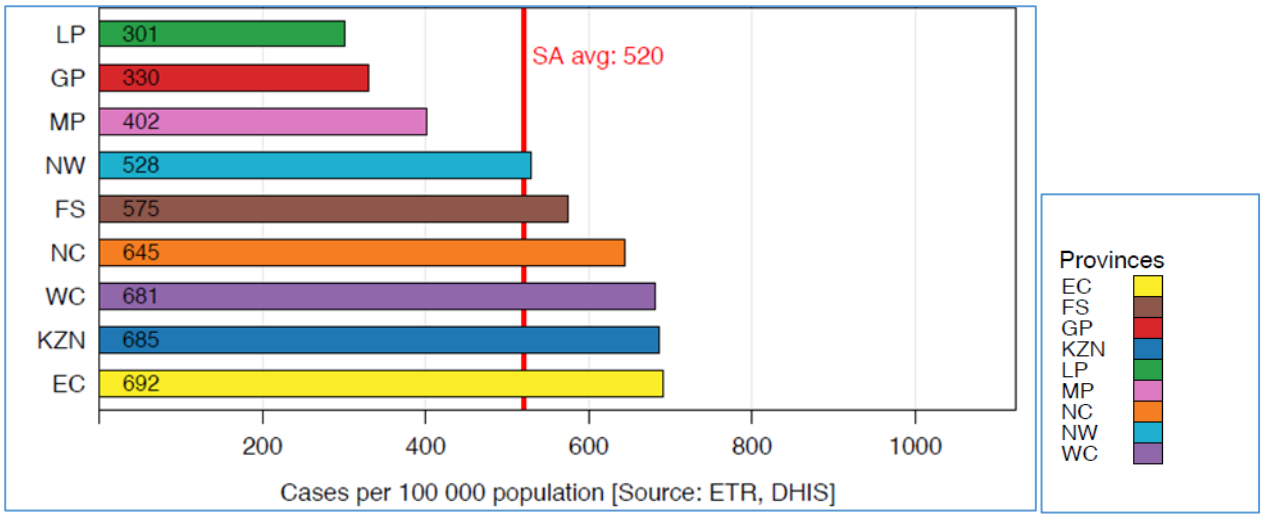


Figure 1.2: Incidences of TB in nine South African provinces. Eastern Cape (EC), Kwazulu-Natal (KZN) and Western-Cape (WC) had the highest incidence. Adapted from District Health Barometer, 2016[19]. (FS, Free state; GP, Johannesburg ; LP, Limpopo province; MP, Mpumalanga; NC, Northern Cape; NW, North West).

1.4 Susceptibility to TB

Only 5-15% of infected, immunocompetent individuals progress to active clinical disease whereas the remainder will never develop the disease[21]. The precise factors associated with the progression to clinical disease is still largely uncharacterized[8,22]. A key question that remains unanswered is why the majority of infected individuals stay healthy[23]. Overall, one-third of the world's population is infected with *M.tb* without displaying any symptoms, this group of individuals are described as latently infected[24]. Latent TB infection (LTBI) refers to the condition in which *M.tb* remains viable in the macrophage without a large amount of metabolic activity, infected individuals remain free of symptoms and tissue damage[18,25]. Once infected the individual is at highest risk for developing the disease within two years[26].

Other possible outcomes after *M.tb* infections are also known, namely (1) fail to register an infection, (2) become infected but then clear the infection, or (3) immediately progress to active disease. These outcomes were illustrated in 1926, in Lübeck, Germany when the same live dose of virulent *M.tb* was accidentally given to 251 infants within the first 10 days of their life. Forty seven of these children showed no evidence of active TB disease, 77 died and 127 had radiological signs of disease[27–29]. The wide range of responses after *M.tb* infection is one of the aspects that contributes to the distinctiveness of this infectious disease[30].

There are vast differences between diverse populations with regards to TB susceptibility which adds to the complexity of the disease. These differences can be due to various reasons[31]. Factors that contribute to the variation among populations and the magnitude of risk includes lifestyle, economic status, HIV infection, diabetes, occupational exposure and immune-suppressive therapy[7,23,28,32,33]. In addition to these, the virulence of the causative organism and the genetic makeup of the infected host influences the progression from infected status to active disease[23].

1.4.1 TB and host genetics

Before Koch discovered the causative bacterium, the initial belief was that TB was an inherited disorder. Subsequent inquests into TB disease aetiology revealed a very complex and multifactorial disease since disease susceptibility is influenced by environmental, host and pathogen factors[34] (Figure 1.3). It has also been established that human genetics contributes greatly to TB susceptibility and the estimated heritability range from 36% to 80%[35–38]. Various twin studies indicated that monozygotic twins (essentially identical in their genetic makeup) showed higher concordance for the development of TB than dizygotic twins[35,39,40] as well as a higher concordance in terms of their cellular immune response[37]. Additional compelling evidence from several case-control association studies, animal models of disease, whole-genome linkage scans and genome-wide association studies (GWAS), substantiate the involvement of human genetics in TB susceptibility[41].

Genetic susceptibility studies of TB are complex, since TB disease does not follow a Mendelian inheritance pattern and two genomes, the bacterium and the host, as well as the environment contribute to the outcome[41]. Family-based linkage studies and population-based case-control studies indicate that TB susceptibility in the general population is the result of a large number of genes that are inherited in a complex manner which makes genetic studies more convoluted[42,43]. The genes that are associated with active disease is not necessarily the same as those that contribute to infection risk, but play a role in course of disease and risk of disease development[44].

Numerous gene polymorphisms that influence host TB susceptibility in the general population have been identified, indicating the polygenic nature of the disease[45–48]. An improved understanding of how the host-genetic make-up differs between individuals who remain healthy and those who develop active TB disease can possibly lead to the identification of genes and variants that can help explain these varying outcomes.

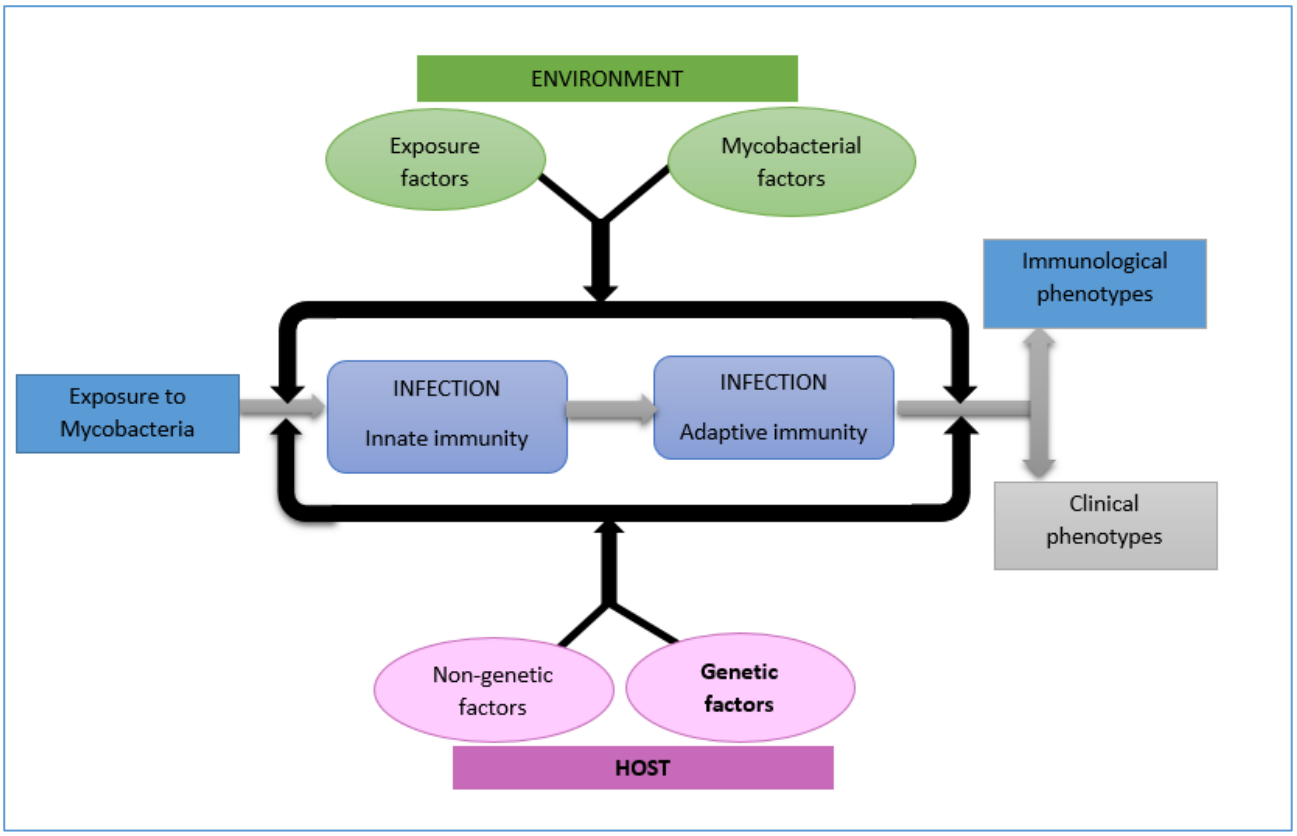


Figure 1.3: The various factors that have an effect on the outcome of infection with *M.tb*. Adapted from Casanova et al. 2002[34].

1.5 Host immune response

The human body is constantly invaded by a wide range of microbes, including bacteria, fungi, viruses and parasites. Healthy individuals protect themselves against the replication and spread of these various microbes by means of their immune system. The host immune response plays an important role in the outcome of TB infection and its components are encoded for by host genes[22]. It is thought that the human factors governing whether an individual will progress to active disease or not are assumed to be those regulating the immunological state of the host[41]. Currently it is not known which immune system genes regulate an individual's disease outcome following exposure[49]. The immune system can be divided into two parts known as innate and adaptive immunity and host protection depends on a wide range of innate and adaptive responses[50].

1.5.1 Innate immune response

The skin acts as the first line of defence, which is impermeable to microbes if intact. Additional non-specific defence mechanisms responsible for repelling most infectious agents include, lysozymes, stomach acids or entrapment in the sticky mucous of the throat or nose[51]. The innate immune system is the next line of defence if infectious organisms manage to penetrate the first line defences. This system does not require memory in order to prevent spreading of microbes and is important in establishing host-pathogen interactions[18].

Innate immunity is non-specific and pathogens are identified when antigen presenting cells (APCs) recognize pathogen associated molecular patterns (PAMPs) via membrane associated pattern recognition receptors (PRRs), such as toll-like receptors, mannose receptors and cluster of differentiation (CD) 14 receptors (Figure 1.4)[52,53]. After recognition these cells take up the bacilli and this results in activation of signalling pathways which leads to production of predominantly pro-inflammatory cytokines[52,53]. These cytokines subsequently activate lymphocytes to mount immune and inflammatory responses (Figure 1.4). The innate system is thus responsible for engulfing and destroying the bacilli during the process of phagocytosis[54]. This system requires a variety of independent cellular responses from many different host immune cell types including macrophages, neutrophils, dendritic cells (DCs) and natural killer (NK) cells[55]. Persistent microbes that are not destroyed by the innate immune system will activate the adaptive immune system.

1.5.2 Adaptive immune response

Adaptive responses differ from the innate immune system since they are specific and develop slowly on first exposure to a new pathogen[51]. An essential feature of adaptive immunity is its ability to obtain a long lasting protection, which leads to a quicker response during subsequent exposure. The adaptive immune system is classified into two broad classes known as the humoral response and cell-mediated immune response[54]. Both of these involve T helper (Th) lymphocytes that are produced by Th1 and Th2 maturation pathways[56] (Figure 1.4). During the humoral responses, B lymphocytes gets activated to secrete antibodies which will circulate in the bloodstream and bind to foreign antigens (substance that is capable of eliciting an adaptive immune response) which then stimulates the production of antibodies[51].

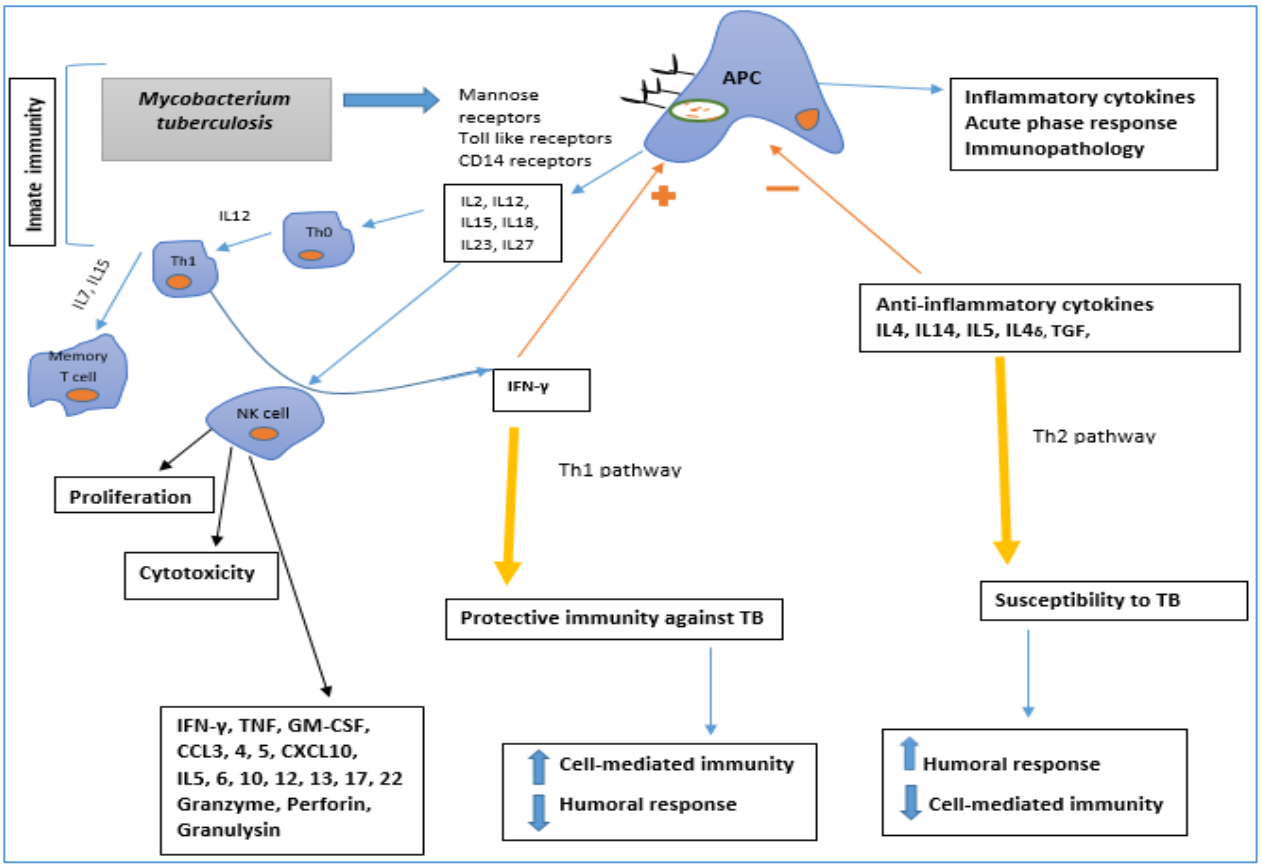


Figure 1.4: An overview of the immune response to *M.tb*. Antigen-presenting cells (APC) such as DCs and macrophages takes up the bacilli and produce cytokines. This activates lymphocytes that mount immune and inflammatory responses resulting in *M.tb* infection control. Adapted from Meya & McAdam, 2007[48].

The primary adaptive immune response to *M.tb* infections is cell-mediated immunity and T lymphocytes plays an important role in this response[57]. Protective cell-mediated immunity against *M.tb* is associated with the development of Th1 responses together with the production of Th1 cytokines such as interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and interleukin-2 (IL-2). The release of other cytokines like IL-4 leads to the development of Th2 responses which supports B lymphocyte growth and differentiation[58]. While the Th1 cells direct the cell-mediated immunity, the Th2 cells drive the humoral immunity through up-regulation of antibody production as depicted in figure 1.4. Although T lymphocytes mainly target intracellular microbes, the up-regulation of antibodies during the humoral response is important to recognise extracellular microbes and to prevent invasive pathogens. The cells of the innate system are still very important in the adaptive system, specifically acting as APCs and setting the stage for the adaptive response[54].

NK cells are important in both the innate and adaptive immunity and forms a bridge between the two systems. They are employed when cells experience various forms of stress and are known as effector cells of innate immunity[59,60]. Even though NK cells are seen as part of the innate immune system, they

display features of adaptive immune cells[59,61]. There are four phases in the adaptive immune response against infections and the first one is known as the expansion phase. During this phase the naïve T lymphocytes proliferate to mediate effector functions[62–64]. The three other phases are known as contraction -, memory maintenance- and the recall response phase[61,65–67]. It has been shown that NK cells undergo all four of these phases. NK cells also functions in synchronicity with other cells of the immune system and are important in setting the stage for an appropriate adaptive immune response, for example through their interactions with DCs[68,69].

1.5.3. NK cells and their interactions with other immune cells

1.5.3.1 NK and DCs

Dendritic cells link the innate immune sensing of the environment to the initiation of adaptive immune responses. The cross talk between this cell type and NK cells have been well documented and in the context of mycobacterial infections, NK cells enhance DC maturation[69,70,71]. Maturation can be further enhanced by the production of IFN-γ and TNF. IL-15 recognition on DCs triggers a molecular program which leads to NK cells lytic activity[72]. DCs act as the main APC to prime naïve T cells to mount a suitable adaptive response to pathogens[59]. It also appears that NK cells discriminate between infected, uninfected or improperly activated DCs[73] which then act as a mechanism to control quality and intensity of antigen presentation during an infection[74]. DC-NK interactions occur *in vivo* at the site of inflammation and it is thought that following *M.tb* infection, NK cells are recruited to the lung from peripheral blood (PB). Therefore these cells participate in the early immune response to the pathogen by selecting the most suitable DC for migrating to the lymph nodes[74], which contributes to optimizing the phase of antigen loading.

1.5.3.2 NK and T cells

The activity of different T cell subsets can be positively or negatively regulated by NK cells[75]. For example, the proliferation of the gamma-delta (γδ) T cell subset seems to be dependent on the production of cytokines as well as direct contact between these cells and NK cells[76]. Additionally, the depletion of NK cells in peripheral blood monocytes (PBMCs) from healthy tuberculin responders impaired the ability of $CD8^+$ T cells to lyse *M.tb* infected monocytes and reduced the frequency of $CD8^+$ T cells producing *M.tb* specific IFN-γ[77]. The immunoregulatory effect of NK cells also extends to another subset of T cells, known as the T regulatory cells (Treg). These cells are present in the blood and at the site of disease in patients with TB[78]. The frequency of these cells in the pleural fluid correlates inversely with local *M.tb*-specific immunity[79].

It is therefore possible that NK cell control of *M.tb* specific Treg expression could contribute to reduce Treg suppression of affected immune responses. These studies points toward a potential role of NK cells in regulating multiple aspects of the immune response against *M.tb* infections. It is also possible that NK cells are involved in the maintenance of the balance between the effector and regulator arms of the antigen specific immune response because of the fact that it positively regulates $CD8^+$ and $\gamma\delta^+$ T cell functions, while supressing Treg cells. Dissecting the immune system of the host is complex and goes beyond the scope of this thesis, which will mainly focus on NK cells.

1.6 Natural Killer (NK) Cells

1.6.1 Characteristics

NK cells are bone-marrow derived lymphocytes and comprise 5-10% of PB circulating lymphocytes, however this percentage can vary with age[80,81]. These cells are a heterogeneous population since they consist of different subsets with distinct phenotypes, tissue localization (NK cells are also found in the spleen, liver and lungs[82]) and functional activities[83]. NK cells are larger than resting lymphocytes and phenotypically defined as $CD56^+$ $CD3^-$ in human peripheral blood[84]. CD16, which is a receptor for Immunoglobulin G (IgG), also known as FcγIII, is commonly used to identify NK cells[85,86]. Neural cell adhesion molecule (NCAM or CD56) is also expressed on NK cells, but is not as widely expressed[87].

Together CD16 and CD56 forms the characteristic markers of NK cells and these cells can be subdivided into different populations based on the expression of the two surface markers[83]. Two major subsets are $CD56^{bright}CD16^{dim}$ and $CD56^{dim}CD16^{bright}$. The major circulating subset is $CD56^{dim}CD16^{bright}$ since this subset represents at least 90% of all peripheral blood NK cells[83]. The $CD56^{dim}CD16^{bright}$ subset is mostly known as "mature" NK cells, however it still remains controversial whether these subsets represent different maturational stages of NK cells or are terminally differentiated populations[88,89]. NK cell maturation and receptor expression profile is depicted in figure 1.5. The "immature" NK cells are more enriched in lymphoid tissues and at sites of inflammation[73].

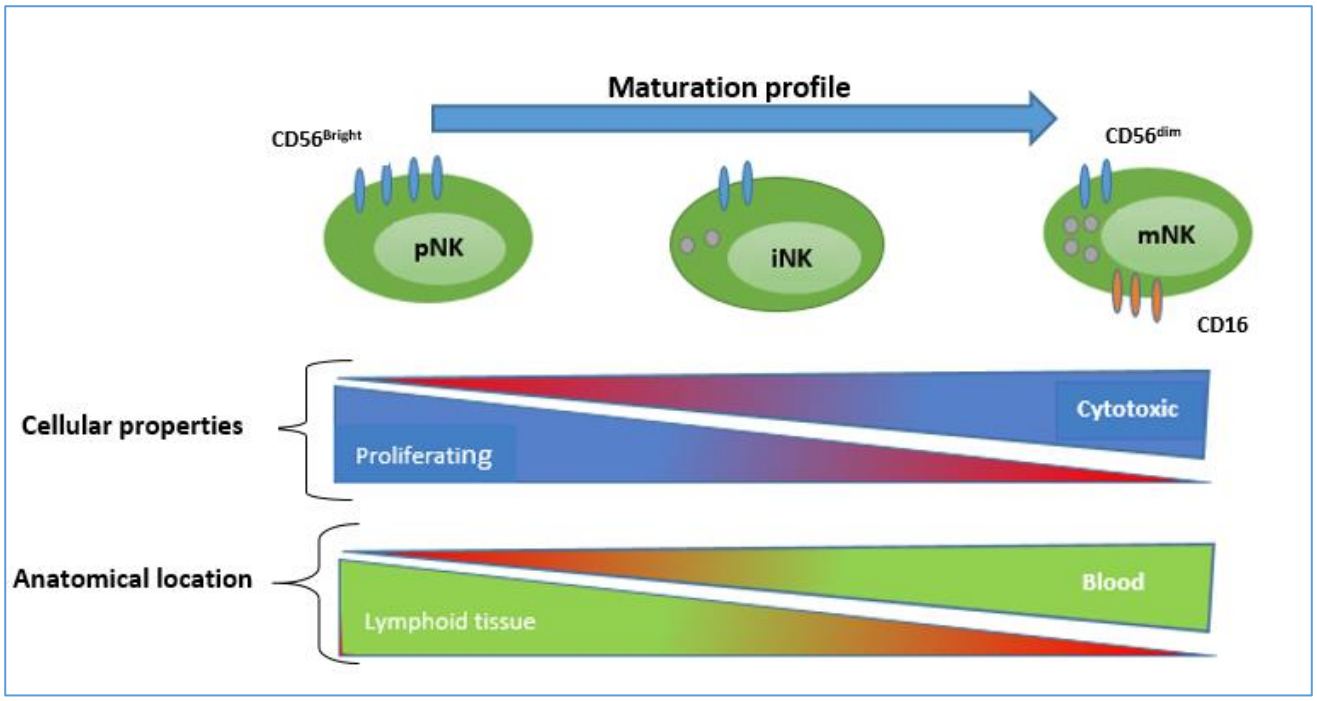


Figure 1.5: Maturation profile of human NK cells, representing the major NK cell markers. Adapted from Chanvillard et al. 2013[90]. pNk, precursor NK cells; iNK, immature NK cells; mNK, mature NK cells.

1.6.2 Functions

In 1975, Hans Wigzell and co-worker's established that a leukaemia cell line could be lysed by cells that had the morphology of small lymphocytes but lacked T- and B-cell characteristics[91]. This was the first known report on NK cells[92]. The capacity of NK cells to kill target cells and their expression of FcγIII receptors was demonstrated there after by Herberman and colleagues[93]. It was known that NK cells are also active against virus-infected cells[94], but the confirmation of NK cell activity against bacterial infections was only confirmed much later[92]. The first report demonstrating this NK cell function showed that these immune cells could lyse *Shigella flexneri*–infected HeLa cells[95], *Legionella pneumophila*–infected monocytes[96] or *M.tb*–infected monocytes[97]. NK cells do not show germ-line receptor rearrangement and the effector functions of these cells are controlled through an extensive repertoire of activating and inhibitory receptors.

The effector functions of the cells vary depending on the cellular microenvironment[98]. Although NK cells were first identified for their ability to kill tumour cells without activation, it is now known that these cells have various other functions[99]. A primary role of NK cells is to provide early protection against infectious agents while the adaptive immune system is activating[100]. This elimination of infectious agents is beneficial to the host since it prevents exaggerated adaptive immune responses that could lead to immunopathology[101].

The mechanisms through which NK cells protect against bacterial infections are not extensively characterized and only a few studies have investigated the roles of NK cells in bacterial infections of the lung[102]. The role of NK cells against infections can be both deleterious and protective[92,103]. Previous studies found that freshly purified NK cells are able to lyse human monocytes infected with BCG[104], *M. avium* complex[105] or *M.tb*[106]. NK cells can mediate the early killing of intracellular *M.tb*[97] and can promote the killing of *M.tb* either directly or indirectly[107]. When NK cells bind to the microorganism, direct cytotoxicity can occur via cytotoxic molecules, and indirect killing occurs through secreted cytokines[108]. These cytokines activates macrophages and other members of the immune system which results in the killing of microorganisms[108]. The hypothesis that human NK cells directly interacts and responds to mycobacteria was made as early as 1996[109]. A direct interaction between NK cells and mycobacteria can promote the activation, proliferation, cytokine production and lytic activity in the absence of an antigen-specific T cell response during early infection. This mechanism can then help destroy intracellular bacteria by ensuring sufficient levels of macrophage-activating cytokines or a cytotoxic activity against infected cells, when the majority of macrophages may be inadequately equipped to destroy intracellular bacteria[109].

It is therefore possible that NK cell proliferation are dependent on direct bacteria-cell contact[109]. Specifically, it was shown that the proliferation of NK cells after exposure to extracellular BCG was not caused by soluble factors released by the bacteria, but was due to a direct interaction between NK cells and mycobacteria[110]. The same observation was made when NK cells were stimulated with heat killed BCG/ mycobacterial cell-wall preparations, but this was not observed when the cells and bacteria were separated by a 0.22µm pore membrane. The membrane inhibited cell-bacteria contact, but not the passage of soluble factors. Intact mycobacteria may thus be a sufficient stimulus to promote NK cell responses, even without accessory cell derived cytokines[109,111,112]. This was seen in most studies, but not in all[69,113]. *In vitro* studies support the role of NK cells in anti-mycobacterial immunity. Multiple mechanisms contribute to the restriction of intracellular *M.tb* growth by NK cells. Induction mechanisms of NK cell functions depend on the type of antigen[109]. T cell responses are modulated by NK cells which limits the availability of APCs[114]. NK cells are activated by various cytokines, including IL-2, IL-18, IL-12 and IL-15, and through an imbalance between activating and inhibitory receptors[72,115,116]. Direct recognition of PAMPs can also further activate these cells.

Several clinical studies have investigated the potential association between NK cell counts and TB[80,117,118]. Some studies reported a reduction in the frequency of NK cells in TB patients when compared to healthy controls[118], whereas other studies show an increase in NK cell count in TB

patients[119]. Migration of NK cells from peripheral blood to the site of infection has also been demonstrated[30,120]. Furthermore the effector functions of NK cells obtained from TB patients have also been investigated and the majority of these studies reported impaired cytotoxic activity of NK cells in TB patients[121–123]. NK effector functions clearly play a role during TB infections with the two main effector responses being target cell killing (cytotoxicity) and cytokine production[99].

1.6.2.1 Cytotoxicity

NK cells and $CD8^+$ T cells are known as the most prominent cytolytic effector cells within the immune system[124]. $CD56^{dim}$ NK cells produce low levels of cytokines but are potent cytotoxic effector cells[83]. Target cells includes cells infected with viruses or intracellular bacterial pathogens, tumour cells and immature DCs[125]. This response of NK cells is mediated by cytotoxic molecules which is stored in specialized exocytic organelles called secretory lysosomes[108]. Secretory lysosomes are dual function organelles[124,126] and target cell recognition induces exocytosis of cytotoxic contents[108]. The major cytotoxic proteins contained within the NK cells are perforin, granzymes and granulysins[127–129]. Perforin belongs to the membrane-attack-complex protein family[130]. This protein facilitates the entry of granzymes and granulysins into the target cell cytoplasm[108,131], but the mechanism is still unclear[127]. Perforin can also complete additional cytolytic functions in the target cell after the transport of the cytotoxic molecules[132]. Then lastly, granulysins are proteins that can interact with lipids and two isoforms of granylysin exist in NK cells[133]. Granulysin can destroy extracellular *M.tb* on its own but requires the presence of perforin to kill intracellular *M.tb*[133].

The major constituent in NK cells is granzyme B which is part of the serine protease family[134]. When an immune synapse is formed between the target cell and the NK cells, the cytotoxic granules move into a synaptic cleft and the granzymes are delivered into the target cell[127]. Granzyme B causes target cell apoptosis and this occurs through direct activation of caspases 3 and 7[134]. Apoptosis can also occur through proteolysis of the protein Bid as illustrated in figure 1.6. This enzyme can have an effect in the absence of perforin, but the full effect will not be observed[134]. NK cells rapidly respond to the activation of signals and can be directly cytolytic without the need of proliferation or transcription[49]. On average NK cells can kill four target cells each, but subsequently perforin and granzyme B levels decrease and the cell is worn out[135]. IL-2 treatment of NK cells restores their cytotoxicity, presumably due to increased expression of granzymes and perforin[108]. The activity of NK cells must be regulated to prevent auto reactivity against the host[136]. NK cell cytotoxicity is regulated by two receptor families[137].

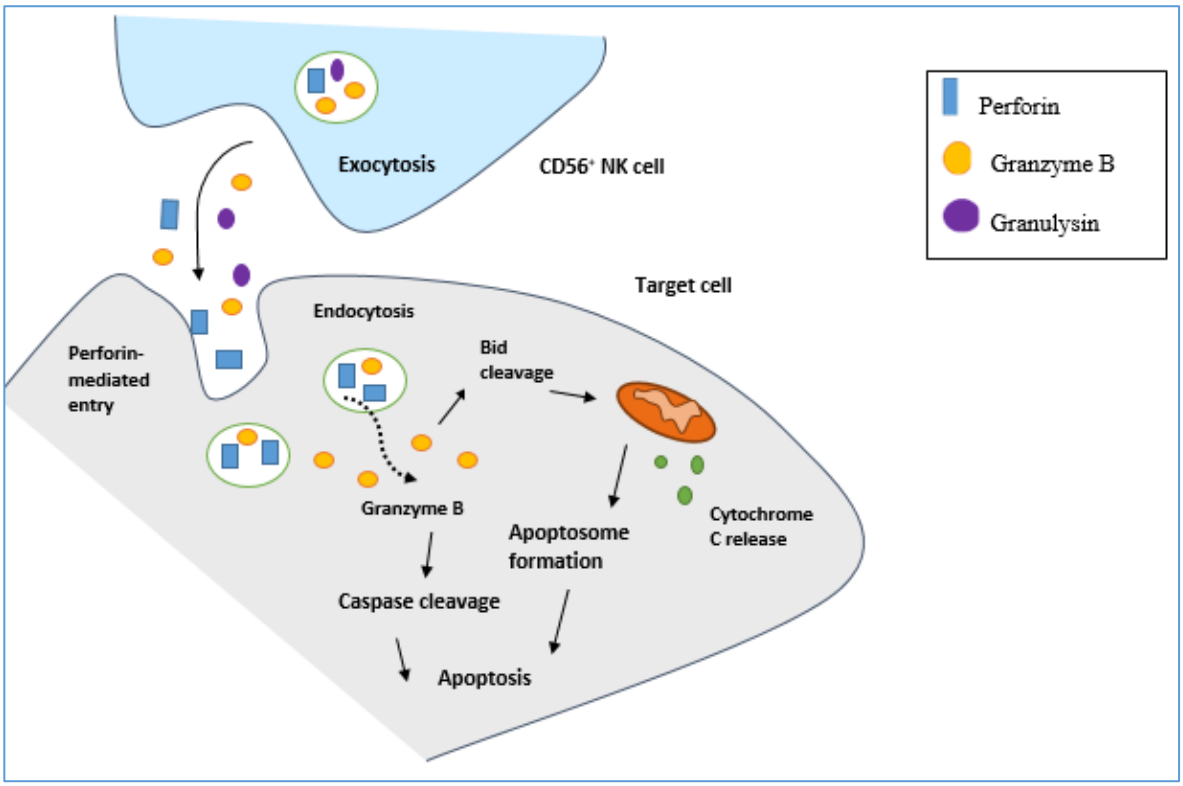


Figure 1.6: NK cell cytotoxicity. Granzyme B, granulysin and perforin are released from NK cell granules. Granzyme B enters the target cell cytoplasm with assistance from perforin and initiates apoptosis. Adapted from Hiebert et al. 2012[138].

1.6.2.2 Cytokines

Although the lytic ability of NK cells against a variety of cell targets is crucial, cytokine production by these immune cells is also of significant importance and has an immense influence on host-immunity[109]. Cytokines are required for protection against disease, but also contribute to the progression or development of pathophysiology[139]. Cytokines have a short half-life and induce biological effects by binding to the extracellular moiety of specific transmembrane receptor proteins in the outer membrane of cells. This binding induces a coordinated series of intracellular events leading to functional changes in these cells[140]. Previous studies established that cytokines also play a major role in determining the outcome of *M.tb* infection[141].

Cytokines control initial infection and can promote or maintain the adaptive T cell responses that mediates host resistance[142]. Therefore, cytokines plays a major role in the innate and adaptive immune response. The $CD56^{bright}$ NK cells have the ability to produce high levels of immunoregulatory cytokines and can also produce chemokines like Macrophage inflammatory protein 1- alpha (MIP-1α) and Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES)[115].

Cytokine regulation in human NK cells is understudied[143]. Some of the cytokines produced by NK cells and their role in the immune system will be discussed here.

Interferon-gamma (IFN-γ):

Interferons are a group of proteins and glycoproteins that are produced in response to infections. IFN-γ has pleiotropic adjuvant effects on host defences and is one of the key cytokines in the control of *M.tb* infections. It plays a critical role in the regulation of inflammatory responses and is important in host protection from a number of infectious agents[144–147].

It is known that IFN-γ gene defects in humans makes them prone to serious mycobacterial infections[148]. In certain infectious diseases, an inadequate IFN-γ response or inability to respond to IFN-γ is associated with persistent infection or even increased host mortality[145,146,149]. The primary source of this cytokine was considered to be conventional $CD4^+$ and $CD8^+$ T cells, but it is now known that innate lymphocytes, NKT cells, NK cells and γδT cells produce IFN-γ in response to *M.tb* stimulation[141]. The production of IFN-γ by T cells can be induced after recognition of specific antigens by the T cells whereas NK cell production of IFN-γ is not antigen specific and can be driven by DCs or macrophage derived IL-12[150,151]. The IFN-γ produced stimulates macrophage activation which subsequently increase cytokine synthesis leading to increased NK cell IFN-γ production[152]. Processed bacterial products can also increase the cytokine stimulation of IFN-γ production. The release of both inhibitory and stimulatory cytokines balances the regulation of IFN-γ production by NK and Th1 cells[152]. Chace et al. showed that NK cells do not secrete IFN-γ after challenge with bacterial DNA, however in the presence of macrophages, IFN-γ is produced, and that the requirement for the presence of macrophages can be replaced with exogenous IL-12[149].

Interleukin-12 (IL-12):

This heterodimeric cytokine is mostly produced by phagocytic cells in response to bacterial products and intracellular parasites[147]. IL-12 is produced during early infection and stimulates the production of IFN-γ from NK and T cells[60]. This pathway links the innate with the Th1 adaptive response to *M.tb*. It also acts as growth factor for activated T and NK cells and enhances the cytotoxic activity of NK cells[150]. IL-12 (*in vivo*) acts in three states during innate resistance and adaptive immune response to infection[150]. In the first stage, IL-12 production induce NK and T cells to produce IFN-γ. This contributes to phagocytic cell activation and inflammation. IL-12 and IL-12 induced IFN-γ favour Th1 cell differentiation by priming $CD4^+$ T cells for high IFN-γ production during the second stage.

In the third stage, IL-12 contributes to optimal IFN-γ and contributes to the proliferation of differentiated Th1 cells in response to antigens[150]. Hence IL-12 represents a functional bridge between early non-specific innate resistance and antigen-specific adaptive immunity. IL-12 can be strongly inhibited by IL-10 and therefore contributions of IL-10 and IL-12 may determine whether IFN-γ based inflammation response is sufficient to combat the infection[149].

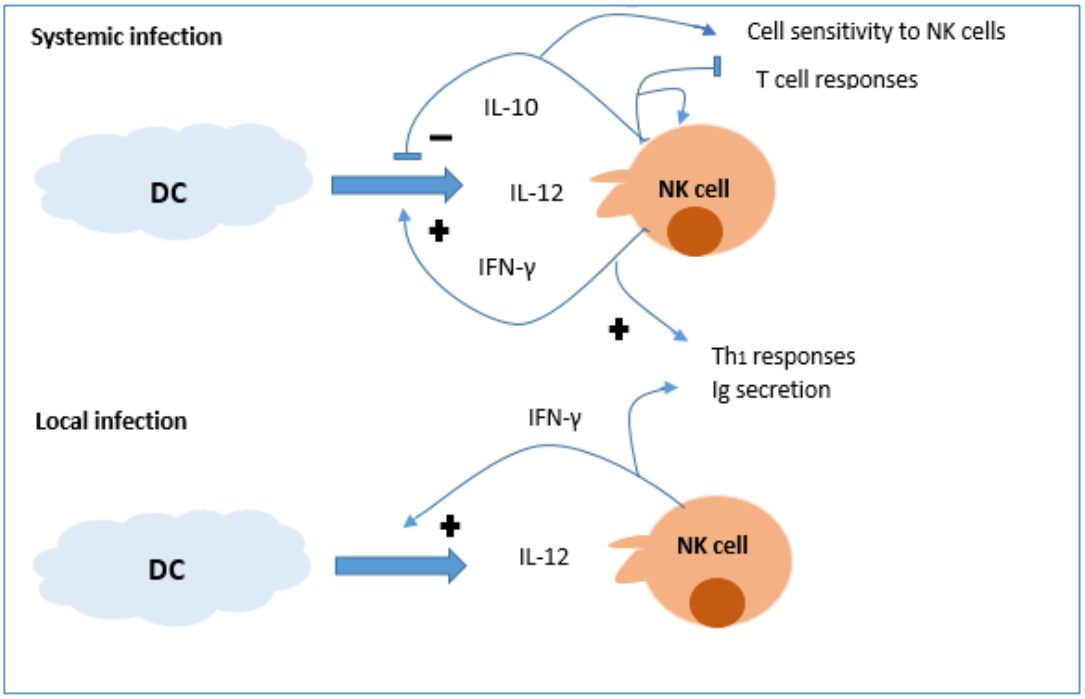


Figure 1.7: IL-12 gets produced by APC. This is a potent inducer of NK IFN-γ. During a systemic infection: Increased IL-12 production leads to IFN-γ + IL-10 production by NK cells with an outcome that will be in favour of the immunosuppressive function of IL-10 on T cell and APC responses. Local infections: this leads to a low IL-12 production which then only triggers IFN-γ production by NK cells and results in a positive feedback loop between NK cells and APC. Adapted from Vivier & Ugolini, 2009[153].

Interleukin-10 (IL-10):

IL-10 is an anti-inflammatory cytokine. Earlier studies have struggled to demonstrate IL-10 production by NK cells, but it was subsequently shown that NK cells produce IL-10, in smaller amounts compared to the amount produced by T cells or monocytes[154–157]. This cytokine can directly regulate innate and adaptive Th1 and Th2 responses as well as supress pro-inflammatory responses in tissue[157].

It also directly inhibits T cell, macrophages and NK cell responses and APC functions of cells that are infected with mycobacteria[158] (Figure 1.7). The macrophage-deactivating properties of this cytokine lead to a decrease in the production of IFN-γ as previously discussed. This cytokine is also a major regulator of IL-12 dependent immune response and directs the Th1 pathway (Figure 1.7). When IL-10 gets produced by NK cells it can lead to decreased inflammatory processes and increased NK cell

effector responses[153]. This contributes to the evidence that NK cells exert regulatory functions[153]. Additionally, when isolated NK cells are stimulated with IL-2, they produce low levels of IL-10.

Tumor necrosis factor (TNF):

TNF is a potent inflammatory cytokine and modulates early inflammatory responses to a variety of environmental, infectious and immunological stimuli[159]. This cytokine is also part of the group of cytokines that are involved in stimulating the acute phase response[160] and plays multiple roles in the immune and pathologic responses to TB. It is involved in the regulation of cell survival, affects cell migration within tissues in *M.tb* infections, has pro-inflammatory properties and regulates IFN-γ positively[60,161,162]. This cytokine also plays a major role in the formation and maintenance of granulomas[162]. Individuals undergoing TNF modulation treatment for chronic inflammatory diseases provided the first evidence that TNF is necessary for preventing reactivation of TB[112]. Anti-TNF therapy interferes with innate immune responses such as phagolysomal maturation as well as cell-mediated responses which includes IFN-γ secretion by memory T cells and increased T cell activity[112,163]. Mice deficient in TNF do not form proper granulomas in their lungs and these mice are unable to control *M.tb* infections[164,165]. Clinical studies also found associations between TNF blockers and progression from LTBI to TB[112,163]. TNF is known to support anti-TB immunity through secretion of chemokines[166], induction of macrophage apoptosis[167] and the upregulation of adhesion molecules[168]. It acts in synergy with IFN-γ to induce antimicrobial activity via induction of reactive oxygen and nitrogen intermediates[160].

Granulocyte macrophage colony stimulating factor (GM-CSF):

This prominent haematopoietic cytokine immune regulator can be released by a multitude of cell types[169]. GM-CSF can expand DC numbers, enhance their immunostimulatory capacity[170] and in the context of TB, GM-CSF may also contribute to the chemokine/cytokine milieu responsible for granuloma formation in the lung[171]. Careful regulation of pulmonary GM-CSF levels may be critical in sustaining protection against chronic TB disease since over expression of GM-CSF in lungs impairs protective immunity against *M.tb*[172]. This is one of the cytokines that NK cells produce that are capable of enhancing blood phagocytic activity and increases its microbicidal capacity[173]. GM-CSF is a heavily glycosylated cytokine and can recruit circulating monocytes and lymphocytes to enhance their functions in the host response. This cytokine is an important mediator in lung inflammatory models, controls macrophage numbers and TLR-4 expression[169]. A study done by Nambiar et al. found that the predominant effect of GM-CSF on T cell immune responses is exerted through the increase presence of IL-12 secreting DCs[172].

Interleukin-17A (IL-17A):

IL-17A is an inflammatory cytokine which initiates inflammation[174,175]. It can induce production of many neutrophilic granulocyte attracting chemokines (chemokine (c-x-c motif) ligand (CXCL)-1, 2, 5, 8) and T cell attracting chemokines (chemokine (c-c motif) motif (CCL)-2,7,20) in epithelial and endothelial cells[176,177]. Both polymorphic and mononuclear cells can be regulated by IL-17 during mycobacterial infection and IL-17 responses are largely dependent on IL-23[178]. IL-17 is mainly produced by subsets of T cells known as Th17 and Th22 but it is also produced by NK cells[140,178].

Interleukin-22 (IL-22):

IL-22 is a novel type of immune mediator that increases innate immunity of tissue cells, enhances tissue regeneration and protects from tissue damage[140]. The primary source of IL-22 are Th17 and or Th22 cells, however NK cells can also produce IL-22 at mucosal sites[179–181]. The production of this cytokine by NK cells can inhibit intracellular growth of *M.tb* through enhancing phagolysosomal fusion[182]. The type of cells that actually contributes to IL-22/IL-17A production *in vivo* depends on the kind and extent of inflammation, the type of pathogen and the site of pathogen entry[140]. The role of this cytokine against extracellular bacteria in the lung has been characterized in a mouse model of *Klebsiella pneumonia* induced *pneumoniae*[180,183,184]. IL-22 contributes to optimal host defence and does not require T cells for its production. In addition to this, NK cells is also able to produce IL-22 in the absence of T cells[102].

The IL-22 receptor is expressed on several epithelial tissues, including human bronchial epithelial cells[102] and a beneficial role of IL-22 in host defence against extracellular bacteria includes maintaining barrier integrity and the up-regulation of bactericidal proteins[179,180,185]. NK cells can also contribute to the production of IL-22 through indirect effects which is mediated by IL-6 and TNF-α NK cell products which can then induce IL-22 in T cells[186,187]. The rs2227473 polymorphism, in the IL-22 promoter was shown to be associated with TB susceptibility in the Chinese population[188].

The secretion of cytokines mediates important regulatory capabilities and is central to immunity and immunopathologies. It is clear that the immune response to mycobacterial infections involves a plethora of cytokines which exhibit a multitude of cellular effects and this complex interplay has ultimately a big influence on the outcome of infection[160]. The immediate response of NK cells must be tightly regulated since it can hold severe consequences in the event of inappropriate NK cell activation.

1.6.3 Receptors

NK cells differ from T and B cells in that T and B cells possess a single antigen receptor that dominates activation and development; whereas NK cells rely on a vast combinative array of receptors[189]. There are a multitude of activating and inhibitory receptors and the net balance of signals from these receptors makes it possible for the NK cells to effectively kill their targets, while maintaining self tolerance[136]. The initial hypothesis regarding NK cell activation is known as the 'missing self' or induced self-hypothesis[136,190]. This hypothesis postulates that the incomplete/absence of major histocompatibility complex (MHC) class I molecules in a normal cell would be sufficient to make it susceptible to NK cells without other changes being required[191]. The loss of expression or down regulation of MHC class I molecules releases the inhibitory signal to NK cells and permits their activation[136].

The two main receptor systems of the NK cells includes members from the C-type lectin-like domain superfamily and immunoglobulin-like (Ig) superfamily[136,190]. The receptors of these two families are preformed and non-rearranging and variability is a direct result of the genetically defined subset of genes present for each family. This process differs from T and B cells which undergo rearrangement of gene clusters. Complex combinatorial expression patterns forms with the development of NK cells.

NK cells are not MHC restricted and eliminate target cells which have downregulated expression of MHC class I. NK cells kills cells that are characterized by absent/down-regulated, modified MHC or human leucocyte antigen (HLA) class I molecules[192]. These molecules are expressed ubiquitously on healthy cells and therefore provide NK cells with a means of identifying 'self'[136]. The two main receptor superfamilies have complementing MHC specificities: Ig superfamily (KIR molecules) are specific for HLA-A, -B, -C allotype subsets and HLA-G ligands[193,194] and C-type lectin-like domain superfamily (CD94/NKG2 lectin like molecules) are specific for HLA-E and MHC class I polypeptide-related sequence A (MICA).

The expression of the receptors differs within the two subsets ($CD56^{bright}CD16^{dim}$ and $CD56^{dim}CD16^{bright}$) of NK cells and this allows for differential cytotoxic activities, trafficking properties and proliferative responses[81]. The regulation of NK cell responses is not only due to a simple balance but can also be controlled by soluble factors including cytokines, chemokines and other secreted ligands of NK cell receptors[195]. Both superfamilies include inhibitory and activating receptor variants. Inhibitory and activating receptors are a complex group of receptors that uses opposing signalling motifs to stimulate or inhibit the cells effector responses[196].

1.6.3.1 Activating receptors

NK cells express more activating receptors than inhibitory receptors[136]. The cells will mount an effector response when a critical threshold of activating signalling exceeds the counterbalance of the inhibitory receptors[189]. Down-regulation of MHC class I or loss of its expression leads to activation of NK cells[136]. Activating receptors contain the immunoreceptor tyrosine-based activation motifs (ITAM)[189], however ITAM is not detected in the cytoplasmic tails of NKp46 and NKp44[197]. ITAM-bearing proteins all contains an aspartate residue within their transmembrane region. This charged residue is needed for stable associations within the associated receptors[189]. There are a number of different receptors that transduce activating signals through adaptor molecules and NK cells use several different activating pathways[136]. Dominant activating receptors on NK cells are NKG2D, DNAX accessory molecule (DNAM-1) and natural cytotoxicity receptors (NCRs).

NCRs are a family of receptors which includes NKp46, NKp44, NKp30 and NKp80[197]. The expression of NKp44 is confined to activated NK cells, whereas NKp30 and NKp46 is expressed on all NK cells[199]. It is thought that this receptor initiates a pathway of NK cell triggering during the process of non-MHC restricted cytotoxicity by activated NK cells. NKp30 co-operate with NKp46 and NKp44 in the induction of cytotoxicity against a variety of target cells[200]. NKp80 is expressed on all NK cells and mAB-mediated cross-linking of NKp80 resulted in induction of cytolytic activity.

NKG2D is also expressed by NK cells as well as subsets of T cells[198]. This is a homodimeric activating receptor and member of the C-type lectin family. DNAM-1, CD2, CD16 and CD9 are surface molecules expressed by NK cells that have been shown to trigger NK cell mediated cytotoxicity in redirected killing assays[197]. However none of these molecules appear to be directly involved in the triggering of natural cytotoxicity[196]. Different receptor/ligand interactions may be responsible for the activation of NK cells upon interaction with target cells.

1.6.3.2 Inhibitory receptors

Inhibitory receptors that are specific for autologous MHC class I molecules are expressed by almost all mature NK cells[201,202]. It was found that inhibition regulates NK cell function[136]. Some inhibitory NK receptors are specific for MHC class I, whereas other inhibitory receptors binds non-MHC ligands[189].

Two receptor families that play the most prominent role in NK regulation by MHC class I are the KIR family and the lectin-like CD94/NKG2A receptor family[136]. Both these receptors synergize to generate NK cells that are responsive to changes in MHC class I expression[136]. Other inhibitory receptors

include leucocyte immunoglobulin-like receptors (LILRs) and the Ly49 receptors[189]. The inhibitory receptors are diverse but share a common signalling motif in their cytoplasmic regions[189].

This motif is called the immunoreceptor tyrosine-based inhibitory motif (ITIM). When ITIM-bearing receptors engage their ligands, a tyrosine residue is phosphorylated and this results in the recruitment of the lipid phosphatase SHIP-1. This degrades phosphatidylinositol-3,4,5-trisphosphate to phosphatidylinositol-3,4-bisphosphate[203]. These phosphates are recruited to the interface between the NK cells and its target cells and this suppresses NK cell responses[203].

1.7 Killer immunoglobulin-like receptors (KIRs)

Killer immunoglobulin-like receptors (KIRs) belong to the immunoglobulin superfamily and are type 1 transmembrane glycoproteins. The genes are highly polymorphic and are encoded on chromosome 19q13.4 within the leucocyte receptor complex (LRC). All of the *KIR* genes are encoded within a range of 160kb genomic sequence, and they cluster together with a genetic distance shorter than 3kb[193]. This receptor family has unique structures which enables them to recognize MHC class I molecules with locus and allele-specificity[204]. There are extensive functional, genetic and expression diversity within the KIRs that can impact NK cells at several levels. The *KIR* genes are diverse and vary at both allelic polymorphism and gene content[194,205]. Moreover, KIRs are clonally expressed in a stochastic manner to generate a repertoire of NK cells expressing different combinations of KIRs in the same individual[194,201]. Every person has an unique repertoire of NK cells due to their different set of inherited genes and alleles coding for a different NK cell receptor and secondly, due to stochastic expression of these genes among the cells from the same individual[206]. This may affect cytokine production in response to *M.tb* which leads to innate viability and thus different levels of susceptibility to *M.tb* infection[30]. This could be one of the reasons why there is such a vast difference between individuals outcome after infection with *M.tb*.

1.7.1 Nomenclature

KIR gene nomenclature is defined by the WHO committee and is subcategorized by considering the number of extracellular domains and the length of their cytoplasmic tails. The number of extracellular domains (Ig-domains) can either be two or three (2D or 3D)[136]. There are thus two structural groups into which KIRs can be identified: KIR2D or KIR3D. KIRs are also classified as activating or inhibitory. Activating KIRs (aKIRs) (with ITAM) contain short cytoplasmic tails and are designated as 2DS and 3DS. The long cytoplasmic tails are in general associated with the ITIM, and inhibitory KIRs (iKIRs) are classified as 2DL and 3DL[136]. To date, 15 distinct *KIR* gene loci, including two pseudogenes, have been identified. Each *KIR* gene encodes either an activating or inhibitory KIR with the exception of *KIR2DL4*, which shares structural features with both activating and inhibitory KIR[136]. The expression of *KIR* genes are highly complex.

1.7.2 Haplotypes

There are numerous haplotypes with different gene content segregation in human populations. *KIR* genes are highly polymorphic and KIR haplotypes differ in terms of variation in the number and types of genes present among individuals[207]. There are four genes (*2DL4*, *3DP1*, *3DL2* and *3DL3*) that are present in all haplotypes and they are known as framework genes[208]. These framework genes have been found in all individuals tested to date, with very few exceptions[209]. Highly homologous *KIR* genes are separated by short and equally homologous intergenic sequences.

Haplotypes can be classified as A or B based on the number of aKIRs present[210]. A haplotype contains only one aKIR gene (*2DS4*) whereas B haplotypes can contain one or more of the genes encoding aKIRs (*2DS1*, *2DS2*, *2DS3*, *2DS5*, *3DS1*)[204,209]. Another difference between the two haplotypes is that haplotype B is variable; whereas haplotype A has a set number of *KIR* genes (9 genes) (Figure 1.8). Deletions and duplications of genes have led to many different haplotypes[209]. KIRs that are expressed on NK cells becomes fixed through methylation of unexpressed *KIR* genes and patterns passed on to daughter cells during cell division[211]. It appears that activating isoforms of the KIR family have evolved more rapidly than the iKIRs. This can maybe be due to selection imposed by pathogens.

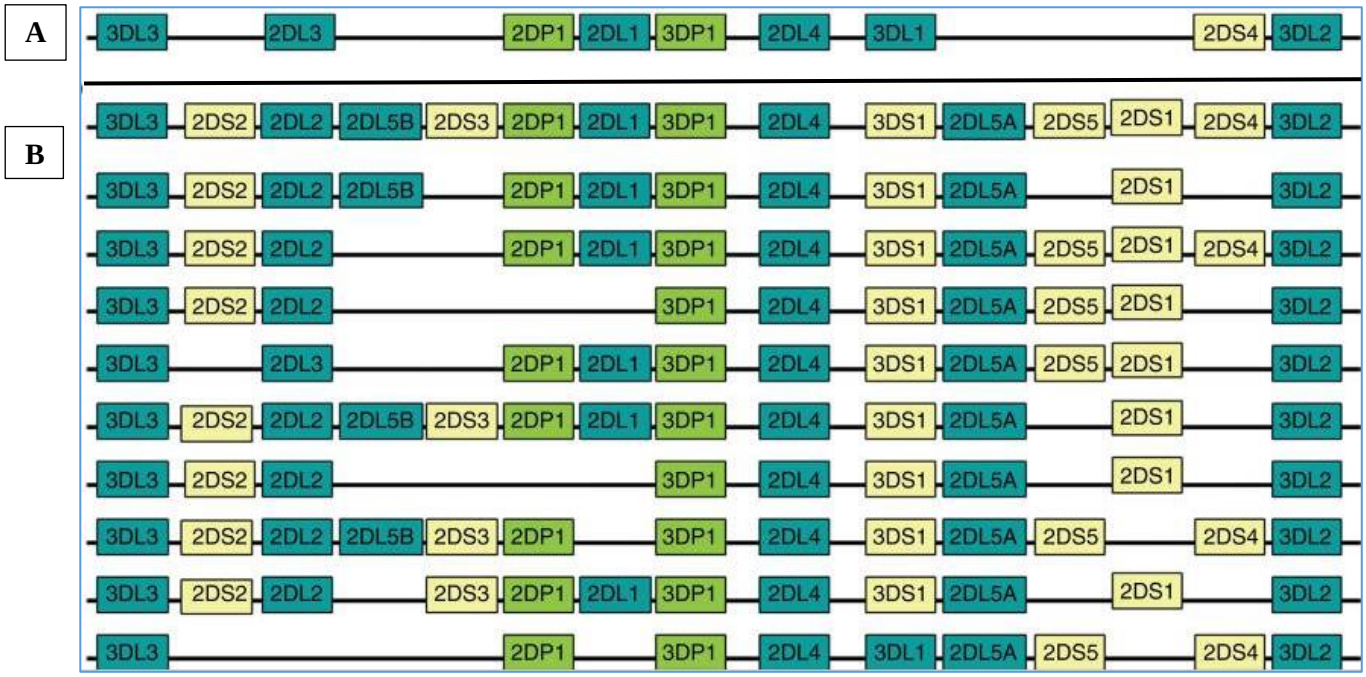


Figure 1.8: The two haplotype groups of killer immunoglobulin-like receptors (KIR). (A) Indicates haplotype A and (B) indicates haplotype B. Adapted from Middleton & Gonzelez, 2010[209].

1.7.3 KIRs and TB

It is known that variations in the repertoire of *KIR* alleles expressed by NK cells between individuals are associated with known differences in susceptibility and resistance to diseases[212]. Previous studies revealed that the expression of HLA/KIR haplotypes was a crucial determinant of NK cell responsiveness to tumour cell lines and pathogen-associated signals[213,214]. Consequently, a number of studies have investigated the role of KIRs in TB susceptibility.

Portevin et al. showed that *KIR* B haplotypes correlated with a higher responsiveness to extracellular mycobacteria and that this association was driven by *KIR2DS3* and *KIR2DS5*[30]. This could suggest a possible link between *KIR* genotype and the ability of the respective NK cell repertoire to react to mycobacteria[30]. Other studies have also addressed the influence of *KIR* genotypes on TB in different ethnic populations. In a Lebanese population, the *KIR* A haplotype was found to be more prevalent in TB patients when compared to healthy controls[215]. No associations were found in the Iranian population[18]. A cross-sectional study compared *KIR* gene frequencies and haplotypes of individuals with active TB and latent TB, to TB and HIV negative controls. *KIR2DL2*, *KIR2DL5*, *KIR2DL5B*, *KIR2DS2* and *KIR2DS3* differed significantly in this study conducted in Manitoba[49]. Another study with participants from Xalapa, Veracruz and Mexico found that patients with *KIR2DL3* differed significantly with the controls[216].

There is extensive variation between *KIR* genotypes in populations according to different geographical regions and different ethnic groups. This can be due to the presence or absence of different KIRs in individual haplotypes and allelic polymorphism of *KIR* genes[217,218]. A study that was conducted in the South African Coloured (SAC) population showed that the number and type of aKIRs were associated with TB disease. In this study several *KIR* associations with TB were identified[22]. The 15 *KIR* genes were genotyped and the frequencies of all the genes were similar, except for *KIR3DS1*[22]. This gene was found at a higher frequency in the controls when compared to the TB cases. The presence of this gene lowered the odds (OR = 0.65, p = 0.014) of developing TB in the SAC population. This study also analysed the *KIR* genes by the number of activating or inhibitory genes present and it was observed that individuals with more than five aKIRs had a lower risk of developing TB (OR = 0.67, p = 0.046). One of the reasons that individuals with more aKIRs had a greater NK cell response to *M.tb* infections might be due to an increased IFN-γ response; whereas a lack of aKIRs might lead to insufficient production of *M.tb* killing cytokines[30].

Scope of the thesis

TB is a complex disease and only approximately 5-15% of infected, immunocompetent individual's progress to active clinical disease. *M.tb* infection is required for the development of TB, however it is not necessarily sufficient for causing active disease. The precise host genetic factors that contributes to individuals being more susceptibility to TB remains a long-standing research challenge. The identification of genetic factors that contribute to an individual's ability to be protected against disease can translate into strategies to be used for therapeutic purposes and personalised medicine. A previous study in our group determined that certain *KIR* genes may alter susceptibility to TB and an overexpression (≥ 5) of aKIRs offers protection against developing TB disease. The research presented in this thesis aimed to compare the efficiency of mycobacterial killing by NK cells isolated from individuals with five or more aKIRs compared to individuals with less than five aKIRs. We hypothesised that NK cells from healthy individuals with five or more aKIRs would be able to kill invading mycobacteria more efficiently than those with less than five aKIRs. Individual's *KIR* genotypes will be determined using a PCR-SSP genotyping protocol, after which PBMCs will be isolated from whole blood using density gradient centrifugation. Pure NK cell populations will then be isolated from the PBMCs with the EasySep™ Human NK Cell Isolation Kit (Stemcell technologies, Canada). The isolated NK cells will be stimulated with *M.tb H37Rv* and viability will be determined using CFUs. Cytokine expression will also be determined using a Luminex immunoassay platform.

Objectives:

1) To identify five individuals with five or more aKIRs and five individuals with less than five aKIRs using PCR-based genotyping.
2) To determine whether NK cells expressing five or more aKIRs are more efficient at killing mycobacteria compared to NK cells expressing less than five aKIRs.
3) To determine the levels of cytokine production following mycobacterial stimulation in individuals with five or more aKIRs compared to those with less than five aKIRs.

Chapter 2: Materials and Methods

2.1 Study participants and sample collection

Study participants were recruited from the Department of Biomedical Sciences, Stellenbosch University, Cape Town, South Africa. The participants were healthy without any symptoms of TB and were recruited based on verbal reporting of never having had active TB. Given that the estimated frequency of five or more aKIRs in the South African population is 20%[22], we recruited 30 control individuals for genotyping in order to identify individuals expressing five or more aKIRs and individuals with less than five aKIRs. From each individual, 4mL of venous blood was collected by a trained phlebotomist in an EDTA tube (Kendon Medical supplies, South Africa) for DNA extraction (Figure 2.1). Following genotyping of all 30 control individuals, a further 40mL of venous blood was drawn from 10 selected individuals in EDTA tubes for PBMC isolations over a three week period. The 10 selected individuals consisted of five with less than five aKIRs and the other five individuals had five or more aKIR genes. In this thesis "group 1" will refer to individuals with less than five aKIRs and "group 2" will refer to individuals with five or more than five aKIRs. All participants gave written informed consent to participate in the study. The study was conducted in accordance with the best ethical practices as stated in the Declaration of Helsinki (2013) and was approved by the Health Research Ethics committee of Stellenbosch University, South Africa (project number S16/03/040).

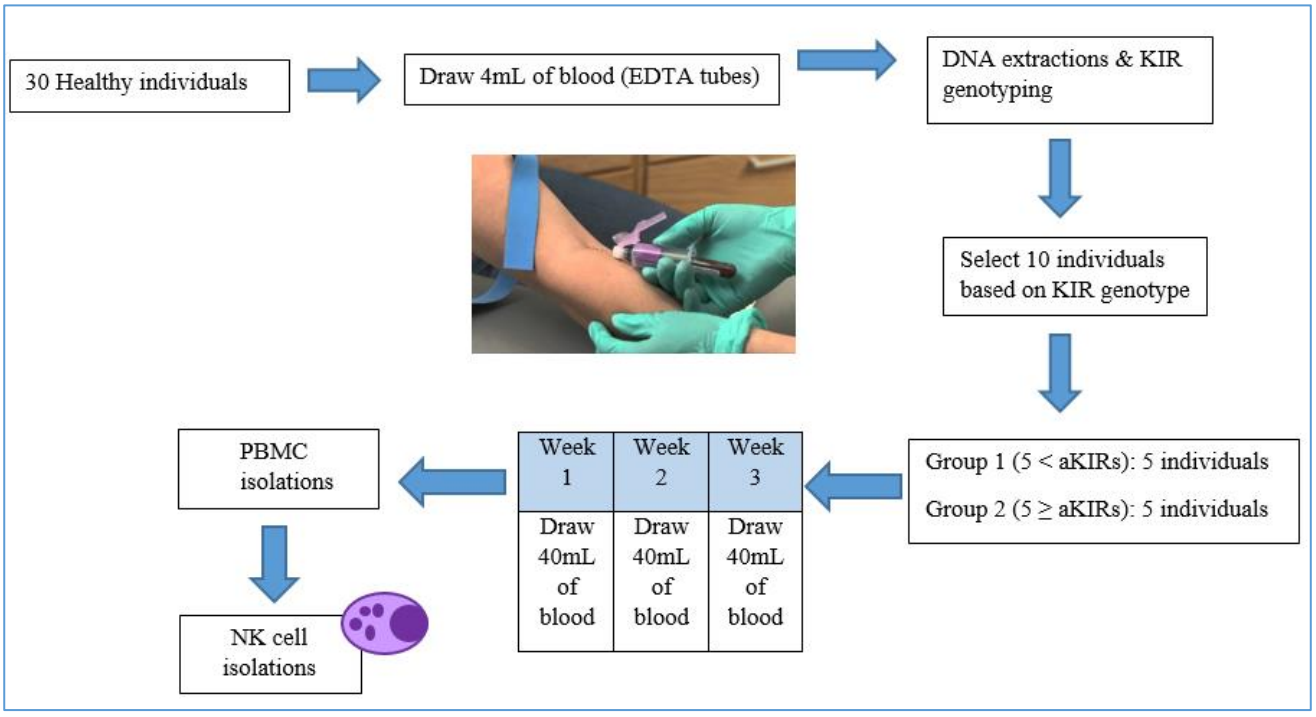


Figure 2.1: Diagram indicating the sample collection process.

2.2 DNA extractions

DNA was extracted with the Illustra Nucleon BACC3™ Genomic DNA extraction kit (GE healthcare, UK) following the instructions of the manufacturer. This protocol can be briefly subdivided into five steps as indicated in figure 2.2. The genomic DNA was extracted within 24 hours from venous blood collected in tubes containing EDTA anti-coagulant.

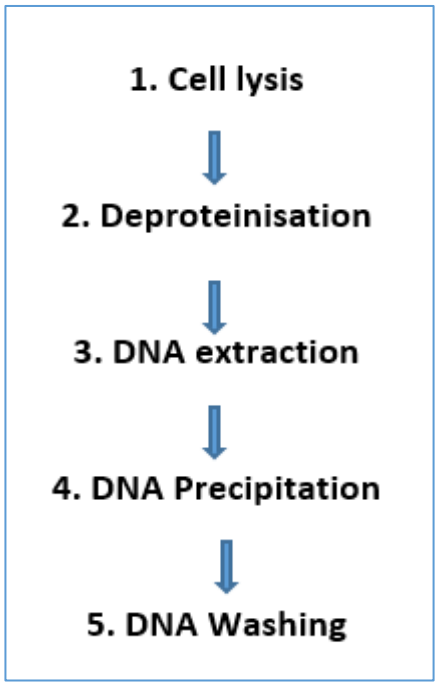


Figure 2.2: Steps during the DNA extraction protocol.

To initiate cell lysis, 4mL blood from each participant was transferred to a 50mL tube and filled with reagent A (*Appendix I*) to approximately 5x the blood volume and shaken for 2 minutes. The tubes were covered with parafilm (Sigma-Aldrich, South-Africa) and centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 3220 rcf, 4°C for 20 minutes. The supernatant was decanted without losing the pellet and centrifuged (Centrifuge 5810R, Eppendorf, Germany) again at 3220 rcf, 4°C for 20 minutes. After the second centrifugation, the supernatant was decanted and the pellet was air dried. Two mL of reagent B and 5µL proteinase K was added to the pellet. The pellet was dissolved at 37°C (37°C Incubator, Merret®, Germany) while shaking for ± 2 hours. For deproteinisation, 500µL sodium perchlorate was added to the dissolved pellet in 15mL tubes and mixed (± 20 inversions). DNA was extracted from the solution by adding 2mL chloroform and inverting until mixed. Thereafter 300µL resin was very slowly added close to the surface so that it formed a middle layer as indicated in figure 2.3, which was then centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 800 rcf, 4°C for 8 minutes.

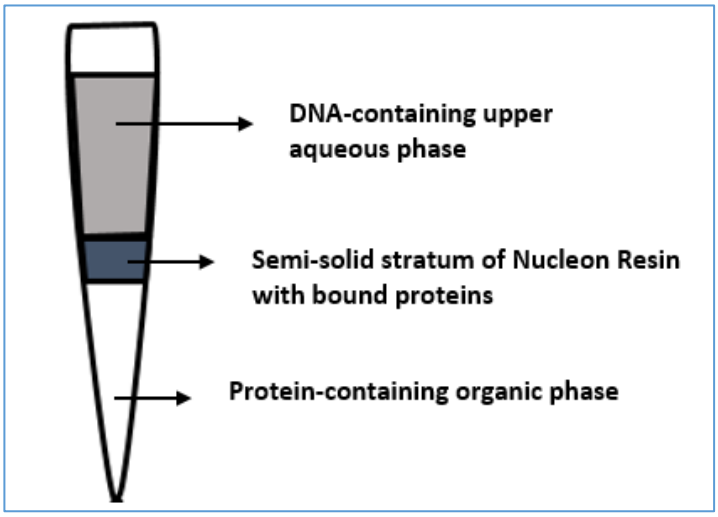


Figure 2.3: Extraction with chloroform treatment nucleon resin. Adapted from Illustra Nucleon BACC3™ Genomic DNA extraction kits product booklet.

After the centrifugation step, the top layer was transferred into a 15mL tube without disturbing the resin. Cold absolute ethanol was added (2x the volume of the pipetted top layer) and left overnight at 4°C. DNA was fished and added to a 2mL Eppendorf tube which was centrifuged (Eppendorf, Germany) at 14 000 rcf for 7 minutes. The supernatant was decanted and the DNA was washed with 70% ethanol and then centrifuged (Eppendorf, Germany) at 14 000 rcf for 7 minutes. The ethanol was completely decanted after which the Eppendorf tube was placed upside down to air dry. TE buffer (*Appendix I*) was added according to the size of the pellet and the DNA concentrations were determined using the Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, South Africa) and the Nanodrop v3.7.1 Software. Samples were diluted to a final working stock solution of 100ng/µL and stored at -20°C.

2.3 *KIR* genotyping

The absence or presence of *KIR* genes was determined using the gene-specific PCR-sequence specific primers (SSP) method[219]. This method is based on the 3'-mismatched principle where primers are designed which will and will not allow amplification.

2.3.1 Gene-Specific PCR-SSP protocol

Each *KIR* gene has two sets of primers that aligned to two different regions of the gene (except for *KIR2DS1*). The primer sequences that were used for this method are listed in table 2.2.

The PCR Mastermix was prepared for each sample in a total volume of 215µL (Table 2.1). One µL (100µg/µL) of both the forward and reverse primers per gene were added to an Eppendorf tube and 4µL of the PCR cocktail was added to each tube for a total PCR volume of 6µL. For the negative control 4µL of PCR cocktail was added and 2µL of the dH_20 and DR beta 1 (*DRB1*) was used as the positive control.

Table 2.1: Preparation of the PCR Mastermix.

PCR Mastermix	**Volume (µL)**
Kapa Taq readymix (Kapa Biosystems, Cape Town)	125
Distilled water (dH_20)	60
DNA	30

PCR reactions were done on the GeneAmp PCR system 9700 (Applied Biosystems, Germany) with the following cycling programme: 3 min at 94°C; 5 cycles of 15 s at 94°C ; 15 s at 65°C ; 30 s at 72°C ; 21 cycles of 15 s at 94°C; 15 s at 60°C; 30 s at 72°C ; 4 cycles of 15 s at 94°C; 1 min at 55°C; 2 min at 72°C, with a final 7 min extension step at 72°C.

PCR amplicons were loaded together with 5µL Cresol Loading Dye (Sigma-Aldrich, South Africa). Gels were stained with ethidium bromide (Sigma-Aldrich, South Africa), and 100bp Universal DNA ladder (Kapa Biosystems, South Africa) was loaded to confirm the size of the amplified products. All PCR reactions were electrophoresed on a 1% agarose gel (*Appendix I*) (Sigma-Aldrich, South Africa) at 200V for 15 minutes. The amplicons were visualized with UV light. Gel visualization and image capturing was completed using the G-Box (Syngene, UK) and the Gene Snap program was used. Both primers should be visible if the gene is present and if the gene is absent, no product should be visible. Samples that showed faint bands were repeated to confirm if the gene was present or not.

Table 2.2: List of primer sequences used for *KIR* typing by the PCR-SSP method.

Name	Sequence (5' – 3')	Exon	Size (bp)
2DLIF1	GTTGGTCAGATGTCATGTTTGAA	4	146
2DL1R1	GGTCCCTGCCAGGTCTTGCG	4	
2DL1F2	TGGACCAAGAGTCTGCAGGA	8	330
2DL1R2	TGTTGTCTCCCTAGAAGACG	3'UTR	
2DL2F1	CTGGCCCACCCAGGTCG	4	173
2DL2R1	GGACCGATGGAGAAGTTGGCT	4	
2DL2F2	GAGGGGGAGGCCCATGAAT	5	151
2DL2R2	TCGAGTTTGACCACTCGTAT	5	
2DL3F1	CTTCATCGCTGGTGCTG	7	550
2DL3R1	AGGCTCTTGGTCCATTACAA	8	
2DL3F2	TCCTTCATCGCTGGTGCTG	7	800
2DL3R2	GGCAGGAGACAACTTTGGATCA	9	
2DL4F1	CAGGACAAGCCCTTCTGC	3	254
2DL4R1	CTGGGTGCCGACCACT	3	
2DL4F2	ACCTTCGCTTACAGCCCG	5	288
2DL4R2	CCTCACCTGTGACAGAAACAG	5	
2DL5F1	GCGCTGTGGTGCCTCG	3	214
2DL5R1	GACCACTCAATGGGGGAGC	3	
2DL5F2	TGCAGCTCCAGGAGCTCA	5	191
2DL5R2	GGGTCTGACCACTCATAGGGT	5	
2DS1F1	CTTCTCCATCAGTCGCATGAA	4	
2DS1F2	CTTCTCCATCAGTCGCATGAG	4	102
2DS1R1	AGAGGGTCACTGGGAGCTGAC	4	
2DS2F1	TTCTGCACAGAGAGGGGAAGTA	4	175
2DS2R1	GGGTCACTGGGAGCTGACAA	4	

2DS2F2	CGGGCCCCACGGTTT	5	240
2DS2R2	GGTCACTCGAGTTTGACCACTCA	5	
2DS3F1	TGGCCCACCCAGGTCG	4	242
2DS3R1	TGAAAACTGATAGGGGGAGTGAGG	4	
2DS3F2	CTATGACATGTACCATCTATCCAC	5	190
2DS3R2	AAGCAGTGGGTCACTTGAC	5	
2DS4F1	CTGGCCCTCCCAGGTCA	4	204
2DS4R1	TCTGTAGGTTCCTGCAAGGACAG	4	
2DS4F2	GTTCAGGCAGGAGAGAAT	5	197/219
2DS4R2	GTTTGACCACTCGTAGGGAGC	5	
2DS5F1	TGATGGGGTCTCCAAGGG	4	126
2DS5R1	TCCAGAGGGTCACTGGGC	4	
2DS5F2	ACAGAGAGGGGACGTTTAACC	4	178
2DS5R2	ATGTCCAGAGGGTCACTGGG	4	
3DL1F1	CGCTGTGGTGCCTCGA	3	191
3DL1R1	GGTGTGAACCCCGACATG	3	
3DL1F2	CCCTGGTGAAATCAGGAGAGAG	4	186
3DL1R2	TGTAGGTCCCTGCAAGGGCAA	4	
3DL2F1	CAAACCCTTCCTGTCTGCCC	3	211
3DL2R1	GTGCCGACCACCCAGTGA	3	
3DL2F2	CCCATGAACGTAGGCTCCG	5	130
3DL2R2	CACACGCAGGGCAGGG	5	
3DS1F1	AGCCTGCAGGGAACAGAAG	8	300
3DS1R1	GCCTGACTGTGGTGCTCG	3’UTR	
3DS1F2	CCTGGTGAAATCAGGAGAGAG	4	180
3DS1R2	GTCCCTGCAAGGGCAC	4	

3DL3F1	GTCAGGACAAGCCCTTCCTC	3	232
3DL3R1	GAGTGTGGGTGTGAACTGCA	3	
3DL3F2	TTCTGCACAGAGAGGGGATCA	4	165
3DL3R2	GAGCCGACAACTCATAGGGTA	4	
2DP1F1	GTCTGCCTGGCCCAGCT	3	205
2DP1R1	GTGTGAACCCCGACATCTGTAC	3	
2DP1F2	CCATCGGTCCCATGATGG	4	89
2DP1R2	CACTGGGAGCTGACAACTGATG	4	
DRB1F1	TGCCAAGTGGAGCACCCAA	Intron 3	796
DRB1R1	GCATCTTGCTCTGTGCAGAT	Intron 3	

*bp, base pair; UTR, untranslated region
*numbering based on KIR alignment from http://www.ebi.ac.uk/ipd/kir/[220]

2.4 Peripheral blood mononuclear cell (PBMC) isolation

The PBMCs were isolated from peripheral blood within four hours of drawing, using density gradient centrifugation protocol (SUN immunology research group, Stellenbosch). Briefly, 15mL density medium (ficoll-paque plus) (Sigma-Aldrich, South Africa) was added to 50mL tubes and the diluted blood (1:1 with 1x Phosphate buffer saline (PBS)) (*Appendix I*) was slowly pipetted over the ficoll-paque layer (Figure 2.4). The 50mL tubes were centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 400 rcf for 25 minutes at room temperature, with no brakes and zero acceleration. The plasma layer was removed (top layer as indicated in figure 2.4) and the PBMC interface was removed using a Pasteur pipette and washed with PBS by centrifugation (Centrifuge 5810R, Eppendorf, Germany) at 400 rcf, 10 minutes at RT. Thereafter the pellet was resuspended in residual PBS and washed again with PBS (centrifuge at 400 rcf, 10 minutes at RT). The viability of the PBMCs were estimated by trypan blue dye (Thermo Fisher Scientific, South Africa) exclusion using a Countess™ II FL Automated cell counter (Thermo Fisher Scientific, South Africa). The total number of viable cells were calculated as indicated in *appendix II*.

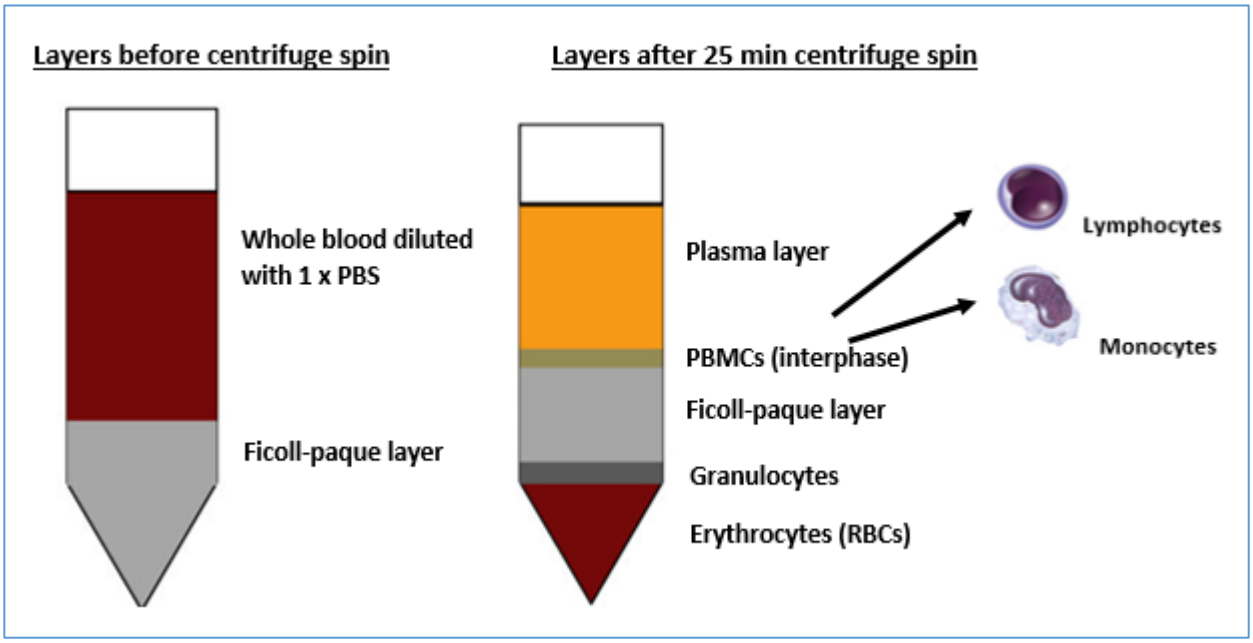


Figure 2.4: Overview of the layers that form before and after the centrifuge step during the isolation of PBMCs. The PBMCs interphase can be subdivided into lymphocytes and monocytes. RBCs, red blood cells; PBS, phosphate buffer saline.

2.5 Cryopreservation

The cryomedia which consists of 10% Dimethyl sulfoxide (DMSO): heat inactivated fetal bovine serum (FBS) (Sigma-Aldrich, South-Africa) was prepared (*Appendix I*). Two mL of cryomedia was prepared per cryovial. The PBMCs were resuspended in residual PBS (Sigma-Aldrich, South Africa) by gentle flicking and the cryomedia was added to the cells drop by drop while on ice. The cells were transferred to pre-cooled 2mL cryovials (Merck, South Africa) and frozen in a Nalgene Cryo freezing container (Sigma-Aldrich, South Africa) at -80°C overnight. This method lowers specimen temperature by approximately 1°C per hour. The vials were removed within 24 hours and transferred to liquid nitrogen (LN).

2.6 Thawing PBMCs

Cryovials were removed from LN and transferred into a 37°C water bath (Imperial III water bath, Imperial Scientific works, India) for 10 minutes with an occasional gentle "flick" during thawing. After adequate thawing the vials were removed from the water bath and immediately sterilized by spraying the outside with 70% ethanol.

2.6.1 Removing DMSO from PBMCs

DMSO (Sigma-Aldrich, South Africa) is used as a cryoprotectant and added to cell media to reduce ice forming, thereby preventing cell death during the freezing process. It is used in a slow-freeze method after which cells can be stored in LN. In order to remove this toxic substance from the frozen stocks and to ensure viability of the cells, the following method was used: 1mL of pre-warmed (37°C) thawing media (*Appendix I*) was added to each cryovial in a drop wise manner and the cells were gently resuspended 3-5 times. Each sample consisted of 30 cryovials and thus 6 x 50mL tubes were prepared per sample. Five of the cryovials were transferred to each 50mL tube. An additional 45mL of thawing media (*Appendix I*) was added very slowly. All tubes were centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 400 rcf for 10 minutes at RT. After centrifugation the waste was decanted and the pellet was resuspended in residual media. A final volume of 45mL thawing media was added to each 50mL tube and centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 400 rcf for 10 minutes at RT.

2.7 Cell counting

A 1:10 dilution of cells: trypan blue was prepared. Ten µL of cells (resuspended in cryomedia) were pipetted into a sterile Eppendorf tube and 80µL of sterile 1xPBS was added to the cells. Thereafter 10µL of trypan blue (Thermo Fisher Scientific, South Africa) was added to the diluted cells. After thorough mixing, 10µL of the sample mixture was added into the filling notch in the counting chamber of the prepared Neubauer haemocytometer (Celeromics technologies, Spain). The chamber was then placed on the platform of a microscope (Nikon TMS, Nikon instruments Inc., USA) and the cells were visualised under the lowest magnification.

The Countess™ II FL Automated cell counter (Thermo Fisher scientific, South Africa) was used as an additional method to count the cells. For each cell count using the Countess™ II FL Automated cell counter (Thermo Fisher scientific, South Africa), 10µL cells, 10µL trypan blue and 80µL sterile 1xPBS were mixed together. Ten µL of this mixture was gently pipetted into the half-moon shaped sample loading area of the loading Countess chamber ™ slide (Thermo Fisher scientific, South Africa). The sample is loaded by capillary action. The entire counting area was covered with the cell suspension (no bubbles must be present) to ensure accurate results of the cell viability. The sample mixture was left to settle for 30 seconds and then the slide was inserted into the slide port. The total number of cells was calculated as indicated in *appendix II*.

2.8 NK cell isolation

The EasySep™ Human NK Cell Isolation Kit (Stemcell technologies, Canada) was used for the NK cell isolations. This kit uses antibody recognizing specific cell surface markers to target non-NK cells. The unwanted cells are labelled with antibodies and magnetic particles, and separated with the use of a magnet. The desired cells (NK cells) are then poured into a new 15mL tube. This is a form of negative selection. For the NK cell isolation, PBMCs were thawed and the pellet was resuspended in 900µL thawing media and 100µL DNase I solution (Thermo Fisher Scientific, South Africa) followed by 20 minutes incubation at 4°C. Thereafter, the NK cells were isolated according to the protocol as suggested by the manufacturer (Stemcell technologies, Canada) with a few minor adaptations. The incubation times were increased from 5 minutes to 10 minutes and isolations were done at 4°C. Furthermore, after adding the isolation cocktail to the sample it was placed on a spinning wheel (Eppendorf, Germany). An overview of the adapted protocol is given in figure 2.5.

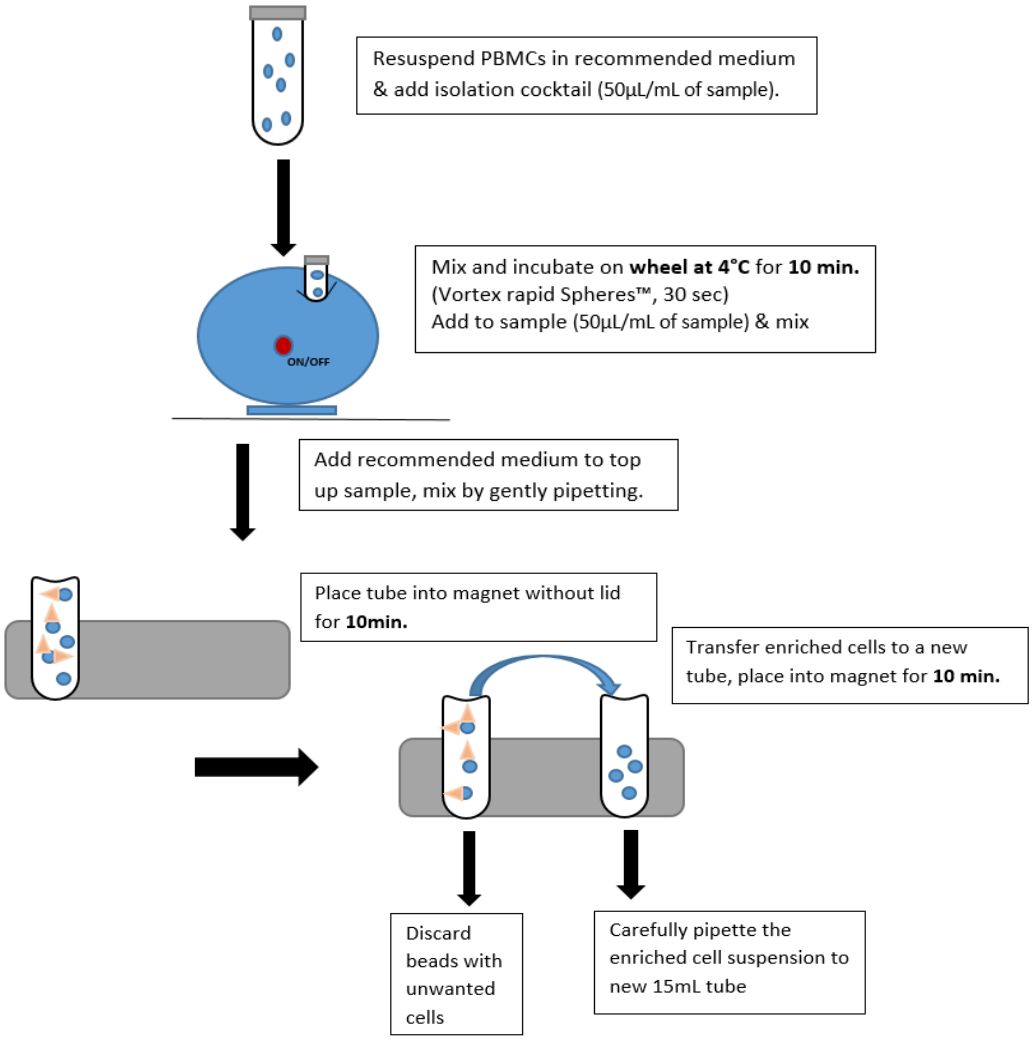


Figure 2.5: Overview of the adapted EasySep™ Human NK cell isolation protocol (Stemcell Technologies, Canada). This is a negative selection technique and the kit targets non-NK cells for removal with antibodies recognizing specific surface markers. The unwanted cells are labelled with antibodies & magnetic particles.

After pipetting the enriched cell suspension into a new 15mL tube, it is centrifuged at 400 rcf for 10 minutes, RT. The supernatant is poured off and the pellet is resuspended in 1mL RPMI-1640 (LONZA Group, Switzerland) and 2% FBS (Sigma Aldrich, South Africa). Thereafter the cell count and the viability of the cells was determined by using a Countess™ II FL Automated cell counter (Thermo Fisher scientific, South Africa). Cell counting calculations that were used can be seen in *appendix II*.

2.9 Fluorescent activating cell sorting (FACS) analysis

Flow cytometry is a method that characterizes single cells using different parameters such as size, granularity, extracellular surface and intracellular surface. Fluorescent activating cell sorting (FACS) analysis is a specialized type of flow cytometry which can be used to sort a heterogeneous mixture of biological cells into sub-populations based on fluorescent labelling. Cells stained using fluorophore-conjugated antibodies can be separated from one another depending on which fluorophore they have been stained with. To assess the purity of the isolated NK cell populations using the EasySep™ Human NK cell isolation kit (Stemcell technologies, Canada), FACS analysis was used. A fraction of the thawed PBMCs from each sample was used as a control as well as the EasySep™ D magnetic particles (Stemcell technology, Canada) that were used for isolating the NK cells. The cells were acquired on a FACSCANTO II instrument (BD Biosciences, USA) and analysed with the Flowjo V10 program (*https://www.flowjo.com*).

2.9.1 Antibodies and fluorescent reagents

Fluorophore-conjugated monoclonal antibodies (Biolegend, USA) used for flow cytometry are listed in table 2.3. The emission and absorption spectrum of each fluorophore is indicated in *appendix III*.

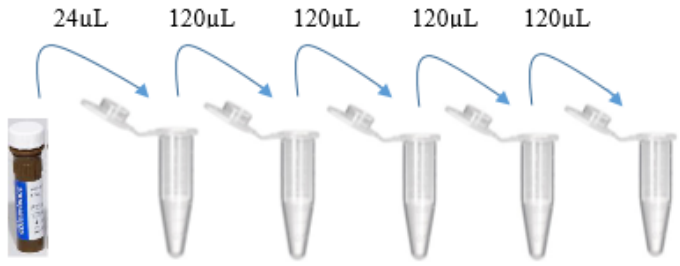
Table 2.3: The fluorophore-conjugated monoclonal antibodies.

Fluorophore	Antibody	Dilutions	Volume added [µL]
APC_Cy7	CD3	1:160	0.25
PerCP_Cy5.5	CD4	1:80	1.25
PE_Cy7	CD8	1:40	2.50
APC	CD56	1:80	1.25

2.9.2 Antibody titration

The first step in a multicolour flow panel is to do antibody titrations to determine the most efficient quantity of each antibody for optimal staining, with minimal wastage of unbound antibody[221]. In this process a known amount of cells were stained with decreasing concentrations of antibodies.

Previously isolated PBMCs were used for this optimization step. Cells were added to 50mL tubes and 22mL FACS buffer was added. For each antibody, a dilution series was made (Figure 2.6). One mL of cells in buffer was added to each FACS tube (round-bottom polystyrene tubes) (The Scientific group, South Africa). FACS tubes were centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 250 rcf for 10 minutes. The supernatant was decanted and the FACS tubes were tapped on a paper towel to dry. One hundred µL of prepared antibodies were pipetted into each FACS tube and 100µL staining buffer was added to an unstained FACS tube. FACS tubes were vortexed (Vortex whirl mixer, Stuart® equipment, UK) and incubated in the dark at 4°C for 30 minutes. Thereafter 1mL staining buffer was added to each tube and the FACS tubes were centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 250 rcf for 10 minutes. Two percent formaldehyde (*Appendix I*) (Sigma-Aldrich, South Africa) was used to fix the cells and 100µL was added to each tube. This was resuspended and vortexed (Vortex whirl mixer, Stuart® equipment, UK). Thereafter 1mL of staining buffer was added and centrifuged at 400 rcf for 5 minutes. The FACS tubes were left in 200µL staining buffer at 4°C in the dark until analysed. The results were analysed using FlowJo V10 program (*https://www.flowjo.com*) to determine the best signal-to-noise ratio (*Appendix III*). Data were analysed by determining the mean of positive (signal) and negative (noise) populations for each antibody.



Staining buffer	216µL	120µL	120µL	120µL	120µL
Dilution	1:10	1:20	1:40	1:80	1:160

Figure 2.6: Antibody titration dilution series. The staining buffer consisted of 2% FBS and PBS. Each Eppendorf tube was vortexed before addition to the next Eppendorf tube.

2.9.3 Instrument quality control

Various steps were taken to ensure that the instrument operates optimally on a daily basis. These checks were performed through the Cytometer Setup & Tracing application (CS&T). After an appropriate instrument warm-up period, the photomultiplier tubes (PMT) and amplifier gains were set to a standard configuration. Thereafter a standard particle was analysed and monitored for mean of fluorescence level and coefficient of variation.

2.9.4 Compensation

The goal of colour compensation is to correctly quantify each dye with which a cell is labelled. This is done by subtracting a portion of one detector's signal from another, leaving only the desired signal. Five FACS tubes (The Scientific group, South Africa) were prepared as indicated in table 2.4. All the compensation beads were anti-mouse (BD Biosciences, USA) except for CD4, which was anti-rat (BD Biosciences, USA) since its isotype is rat IgG2b. The negative control tube was analysed on the BD FASCANTO II (BD Bioscience, USA) followed by the tubes containing the various compensation beads. A cut off value of 100 000 events was chosen. Data analysis was performed using FlowJo V10 program (*https://www.flowjo.com*).

Table 2.4: Overview of tubes during the compensation step.

Compensation (beads)	**Tube 1 Comp-Unstained**	**Tube 2 Comp-CD3**	**Tube 3 Comp- CD4**	**Tube 4 Comp-CD8**	**Tube 5 Comp-CD56**
CD3 APC-Cy7 [µL]		6.25			
CD4 PerCP-Cy5.5 [µL]			1.25		
CD8 PE-CY7 [µL]				2.5	
CD56 APC [µL]					1.25
Comp beads negative (drop)	1	1	1	1	1
Comp beads positive (drop)	1	1	1	1	1
FACS Buffer [µL]	100	100	100	100	100

2.9.5 Fluorescent minus one (FMO)

FMO control is used to ensure that the spread of fluorophores into the channel of interest is correctly identified. The control contained all the fluorophores in the panel, except the fluorophore being tested (Table 2.5). Cells were resuspended in 1mL washing buffer (*Appendix I*) and added to each FACS tube (round-bottom polystyrene tubes) (The Scientific group, South Africa). FACS tubes were centrifuged (Eppendorf, Germany) at 400 rcf for 5 minutes and 100µL of washing buffer was added to each tube. Thereafter antibodies (BioLegend, USA) were vortexed and added as indicated in table 2.5. Cells were washed twice and resuspended in 200µL washing buffer. The FACS tubes were left in the dark at 4°C until they were analysed.

Table 2.5: Overview of which tubes contained which antibodies.

Antibodies	**Tube 1 Unstained**	**Tube 2 Stained**	**Tube 3 FMO-CD3**	**Tube 4 FMO-CD4**	**Tube 5 FMO-CD8**	**Tube 6 FMO-CD56**
CD3 APC-Cy7 [µL]		6.25		6.25	6.25	6.25
CD4 PerCP-Cy5.5 [µL]		1.25	1.25		1.25	1.25
CD8 PE-CY7 [µL]		2.5	2.5	2.5		2.5
CD56 APC [µL]		1.25	1.25	1.25	1.25	
FACS Buffer [µL]	100	88.75	95.0	90.0	91.25	90.0
Total Volume [µL]	**100**	**100**	**100**	**100**	**100**	**100**

*Blue blocks indicate that the antibody was not added. FMO, fluorescent minus one.

2.9.6 Sample preparation for FACS

Cells were resuspended in 1mL FACS buffer (*Appendix I*), centrifuged (RT, 5 minutes at 400 rcf) (Eppendorf, Germany) and the supernatant was discarded. Antibodies were added to each tube (Table 2.3). The FACS tubes were vortexed (vortex whirl mixer, Stuart® equipment, UK) and incubated at RT in the dark for 30 minutes. After the 30 minute incubation, 1mL FACS buffer was added, centrifuged (400 rcf, 5 minutes, RT) and the supernatant was discarded. This step was repeated twice. Five hundred µL FACS buffer was added to each FACS tube and stored in the dark at 4°C until samples were analysed using the BD FACSCANTO II (BD Biosciences, USA) with a cut off value of 50 000 events. Sample preparation steps are indicated in figure 2.7.

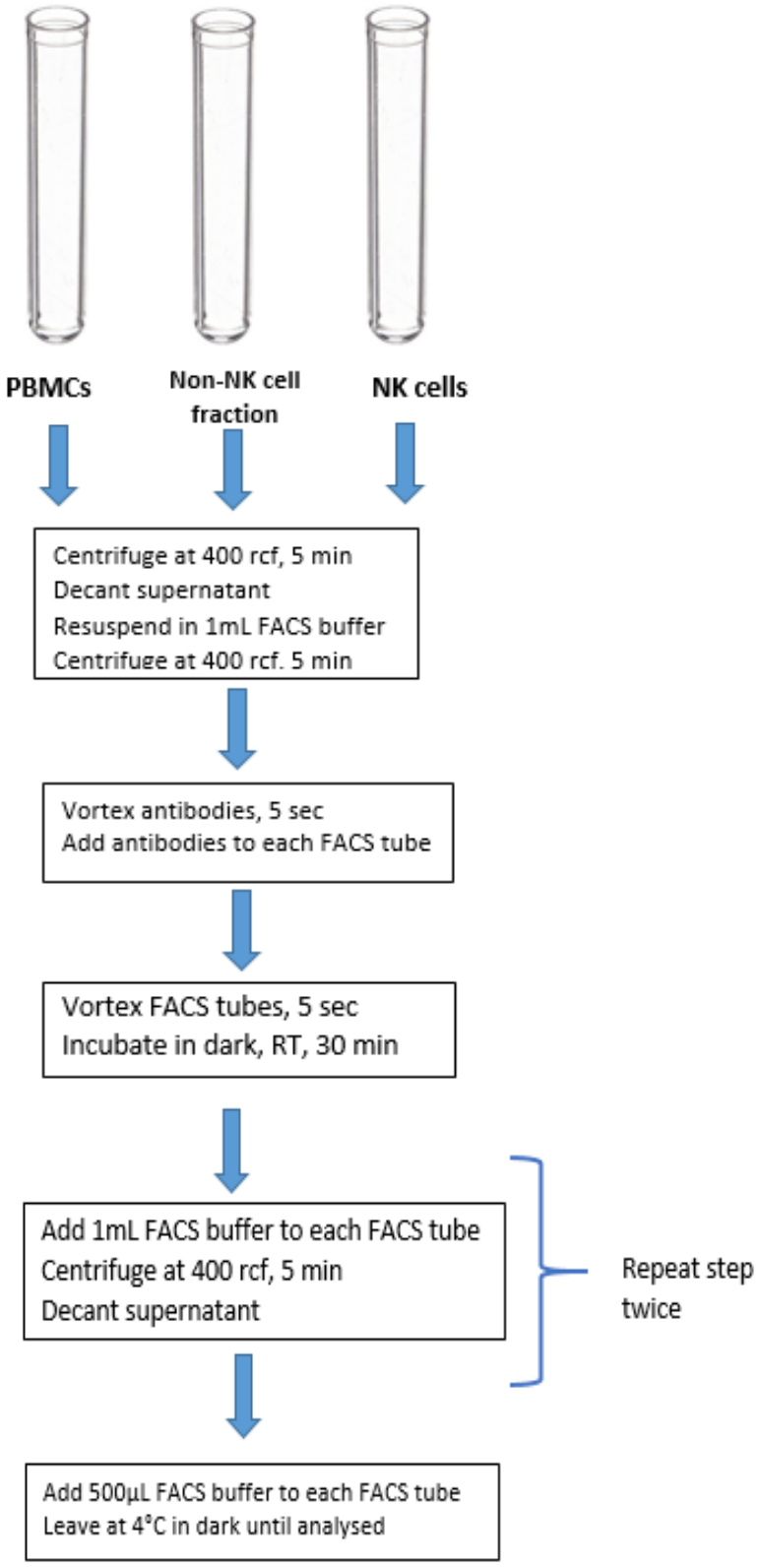


Figure 2.7: Sample preparation for FACS. A fraction of the PBMCs were stored before isolating the NK cells. The PBMCs and a non-NK cell fraction were used as controls during the analysis.

2.10 Bacterial strains and culture conditions

M.tb H37Rv was grown in a biosafety level-3 (BSL3) facility in T25 flasks (Thermo Fischer scientific, South Africa) in Middelbrook 7H9 medium (BD Biosciences, USA) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) (BD Bioscience, South Africa) and 0.2% (v/v) glycerol (Sigma-Aldrich, South Africa) (*Appendix I*). One mL of *M.tb H37Rv* stock was thawed and added to 5mL of the Middlebrook 7H9 medium. The flask was placed in the Merret® incubator (Merret, Germany) at 37°C for ± 5 days. The optical density (OD) at 600nm was taken every 2nd day until an OD_{600} of 1 was reached (OD of 1 ~ 1 x 10^8 CFU/mL)[222]. Thereafter the culture was kept at RT to prevent further growth of the bacteria. The culture medium did not contain Tween 80 because this detergent possibly alters the mycobacterial cell wall and solubilizes various proteins and lipids[223] .

2.11 Ziehl-Neelsen stain

This bacteriological stain is used to identify acid-fast organisms, mainly mycobacteria. Initially, Carbol fuchsin stains every cell. After destaining with acid-alcohol, only non-acid-fast bacteria are destained since they do not have a thick, waxy lipid layer like acid-fast bacteria. When counter stain is applied, non-acid-fast bacteria captures the stain and become blue when viewed under the microscope. Acid-fast bacteria retain Carbol Fuchsin so they appear red. *M.tb H37Rv* was cultured until an OD_{600} of 0.8 was reached and then the stocks were frozen at -80°C. ZN stain was completed for the *M.tb H37Rv* stocks to confirm that no contamination was present.

Five µL fixative (10% formalin) (Merck, South Africa) was added to a microscope slide. Thereafter 20µL *M.tb H37Rv* culture was added, smeared onto the slide and heat fixed for 2 hours at 95 °C (Dry block heater, Thermo Fischer Scientific, South Africa). The slide was flooded with Carbol Fuchsin and heated with a flame until steaming point, left for 5 minutes and rinsed with clean water. The slide was flooded with acid-alcohol, left for 2 minutes, rinsed with clean water and flooded with Methylene Blue for 2 minutes. After rinsing with water the slide was air dried for 20 minutes and examined using a four objective light microscope at 60X (oil immersion) magnification (LW Scientific, South Africa).

2.12 Stimulations of NK cells with mycobacteria

After NK cell isolations, the NK cells were seeded in 96 well plates (Merck, South Africa) (amount of cells per well depended on the total amount of cells isolated). The layout for the plate is shown in figure 2.8 (Each block represents a well).

NK cells were either stimulated with 50U recombinant interleukin 2, human (hIL-2) (Sigma-Aldrich, South Africa) or seeded without hIL-2. Each sample was completed on a separate 96 well plate. All samples were analysed in triplicate. *M.tb H37Rv* was passaged 15 times through a 1 mL/cc insulin syringe (Avacare®, South Africa) without creating any bubbles. The bacteria was filtered through 5µm filters (Merck, South Africa) and passaged with a 1mL/cc insulin syringe (Avacare®, South Africa) before adding the *M.tb H37Rv* to each well containing the NK cells. The amount of *M.tb H37Rv* that was added to each well depended on the amount of NK cells seeded per well. A multiplicity of infection (MOI) of five was used. This indicates the ratio of agents to infection targets. Thus there were five times more bacteria than cells in each well. Calculations used to calculate the amount of cells and the amount of bacteria that must be added per well is indicated in *appendix II*.

For the 1 hour stimulation the NK cells were incubated in the Shell lab incubator (Sheldon manufacturing, USA) at 37°C in air containing 5% CO_2 for 1 hour and the 24 hour stimulation was left in the incubator for 24 hours before the supernatants was plated for CFUs. *M.tb H37Rv* in RPMI-1640 media (LONZA group, Switzerland) without NK cells was used as control.

***H37Rv*_1h** RPMI media	***H37Rv*_1h** RPMI media	***H37Rv*_1h** RPMI media	***H37Rv*_1h** NK cells RPMI media MOI; 5:1	***H37Rv*_1h** NK cells RPMI media MOI; 5:1	***H37Rv*_1h** NK cells RPMI media MOI; 5:1
***H37Rv*_1h** NK cells RPMI + hIL2 media MOI; 5:1	***H37Rv*_1h** NK cells RPMI + hIL2 media MOI; 5:1	***H37Rv*_1h** NK cells RPMI + hIL2 media MOI; 5:1			
***H37Rv*_24h** RPMI media	***H37Rv*_24h** RPMI media	***H37Rv*_24h** RPMI media	***H37Rv*_24h** NK cells RPMI media MOI; 5:1	***H37Rv*_24h** NK cells RPMI media MOI; 5:1	***H37Rv*_24h** NK cells RPMI media MOI; 5:1
***H37Rv*_24h** NK cells RPMI + hIL2 media MOI; 5:1	***H37Rv*_24h** NK cells RPMI + hIL2 media MOI; 5:1	***H37Rv*_24h** NK cells RPMI + hIL2 media MOI; 5:1			

Figure 2.8: The 96 well-plate layout used when stimulating the isolated NK cells with *M.tb H37Rv*. Stimulations were done for a 1 hour and 24 hour period.

2.13 Colony forming units (CFUs)

After the NK cells were stimulated with *M.tb H37Rv*, the supernatant was used to make a dilution series ranging from 10^{-1} to 10^{-7} as indicated in figure 2.9. Serial dilutions of the supernatants were plated on Middelbrook 7H11 agar plates supplemented with 10% OADC (BD Bioscience, USA) and 5mL glycerol (Sigma-Aldrich, South Africa) (*Appendix I*). The Middlebrook 7H11 agar plates were incubated at 37°C for ±3 weeks inverted in the Merret® incubator (Merret, Germany). Following this, the single bacterial colonies on each plate were counted.

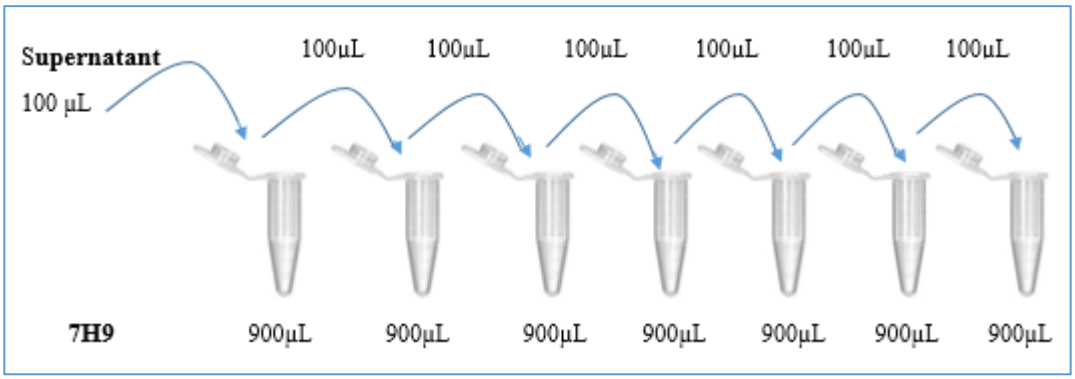


Figure 2.9: The dilution series that was used to determine the colony forming units (CFUs) of *M.tb H37Rv* after stimulation.

2.14 Cytokine expression

2.14.1 Human CD8 + T- cell Magnetic bead panel (Milliplex® map Kit, EMD)

Milliplex® map is based on the Luminex xMAP® technology and in the present study, a 17-plex panel was used (EMD millipore's Milliplex® map Human CD8 + T-cell magnetic bead panel). This panel was used for the simultaneous quantification of the following in the culture supernatant samples: Perforin and Granzyme B.

2.14.1.1. Preparation of tissue culture supernatant and reagents for immunoassay

Two hundred µL of each sample was stored in polypropylene cryovials (Merck, South Africa) at -20°C in the BSL3 facility after each stimulation. Before running the assay all the samples were filtered using 0.2µm Millex® syringe filters (EMD Millipore, USA). The samples were thawed at RT and then vortexed (Vortex whirl mixer, Stuart® equipment, UK) for 5 seconds. Thereafter the samples were centrifuged (Eppendorf, Germany) for 2 minutes at 2000 rpm.

2.14.1.2 Preparation of reagents for immunoassay

Antibody-immobilized beads

Each antibody-bead vial was sonicated (Bransonic Ultrasonic bath sonicator) (Sigma-Aldrich, South Africa) for 30 seconds and then vortexed (Stuart® equipment, UK) for 1 minute. Thereafter 80µL of each antibody-bead vial was added to a mixing bottle and bead diluent was added to make up the final volume to 5.6mL (since two plates will be run). The mixed beads were vortexed (Stuart® equipment, UK) well before use.

Quality controls (QC)

Quality control 1 and 2 was reconstituted with 250µL deionized water. The vial was inverted several times and vortexed (Stuart® equipment, UK). The vials were left for 5-10 minutes before use.

Wash buffer

The 10x wash buffer was brought to RT and mixed to bring all the salts into solution and then 60mL of the wash buffer was diluted with 540mL deionized water.

Preparation of standards

Six polypropylene tubes were labelled, Standard 1 to Standard 6. One hundred and fifty µL of Assay buffer was added to all of the tubes. Serial dilutions were then prepared by adding 50µL of the reconstituted Standard 7 to Standard 6 (mix well) and then 50µL of Standard 6 to Standard 5 etc. as indicated in figure 2.10.

Table 2.6: Preparation of CD8 + T cell standard (standard 7) and working standards.

Standard tube	**Volume of Deionized Water to add**	**Volume of Standard to add**
Standard 7	250µL	0
Standard tube	**Volume of Assay Buffer to add**	**Volume of Standard to add**
Standard 6	150µL	50µL of Standard 7
Standard 5	150µL	50µL of Standard 6
Standard 4	150µL	50µL of Standard 5
Standard 3	150µL	50µL of Standard 4
Standard 2	150µL	50µL of Standard 3
Standard 1	150µL	50µL of Standard 2

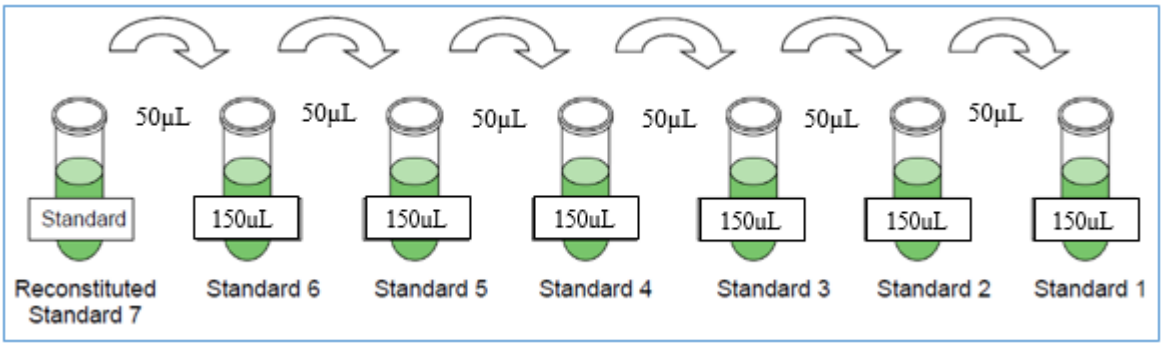


Figure 2.10: The workflow for the preparation of the working standards. Adapted from booklet Human CD8+ T-cell magnetic Bead panel kit booklet (EMD Millipore, USA).

2.14.1.3 Assay procedure for Human CD8 + T- Cell Magnetic Bead panel

All reagents were allowed to reach RT (20-25°C) before use. The assay was performed as indicated in figure 2.11. After the 16-18 hour incubation step, the plates were washed twice using Bio-plex Pro™ wash station (Bio-rad, USA).

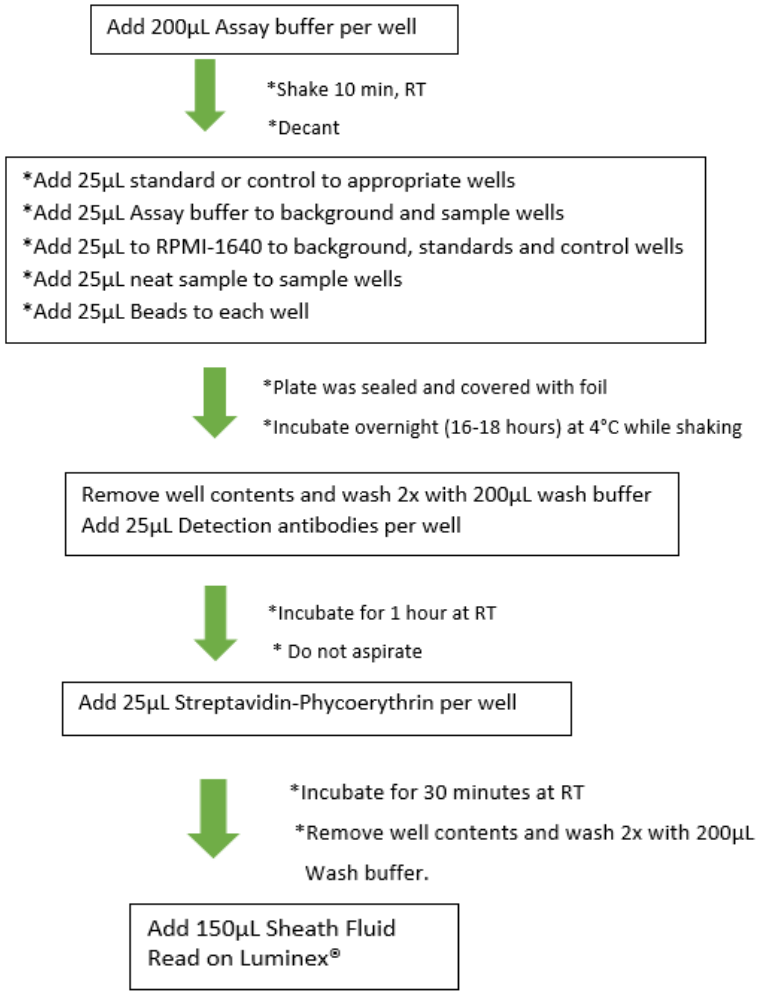
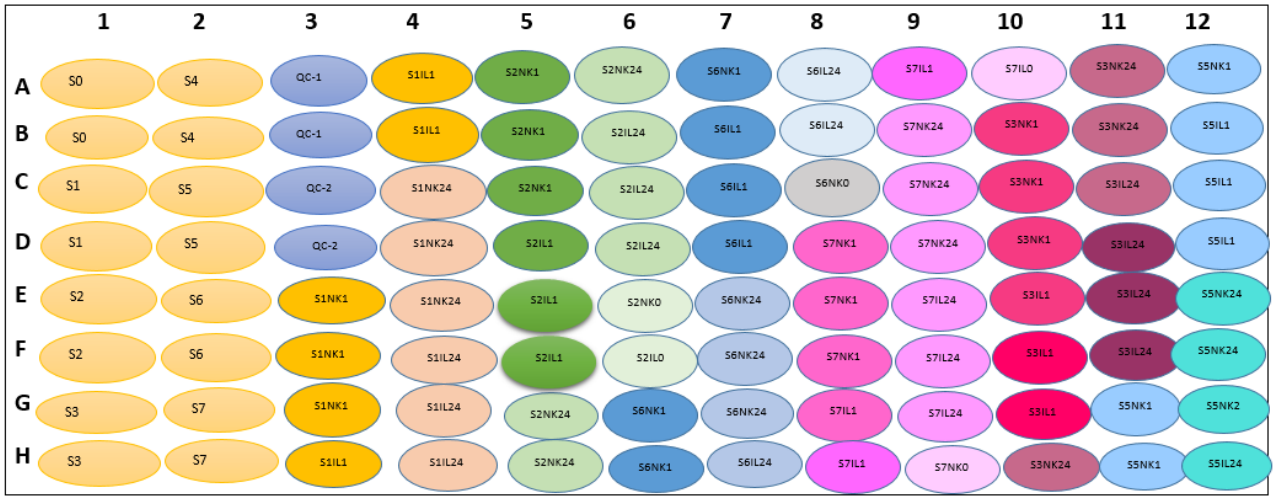


Figure 2.11: Assay procedure of the Human CD8 + T-cell magnetic Bead panel (EMD Millipore, USA).

The plates were analysed on the Luminex® Bioplex 200 system (Bio-rad, USA) with the program, Bioplex manager. Calibration and verification was done as part of the daily setup. The plate layout is indicated in figures 2.12 and 2.13.

A



B

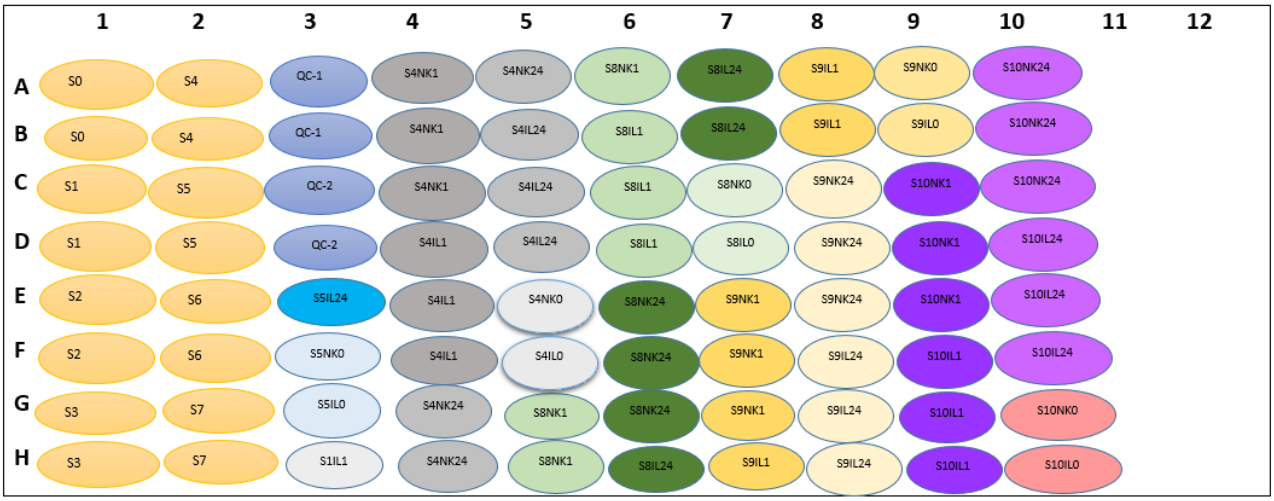


Figure 2.12: The 96 well -plate layout of plate 1 (A) and plate 2 (B) for Human CD8 + T cell magnetic bead assay. Standard 0 = blank, standard 1 = low concentration standard, standard 6 = Highest concentration std, QC1 = Assay Quality control 1, QC2 = Assay Quality Control 2. Evaluate QC1 and QC2 as recommended by kit manufacturer and the interpolate control just like any other sample. Each circle represents a well. Each sample was analysed in triplicate for each time point as well as for stimulations with and without hIL-2.

2.14.2 Human Cytokine/Chemokine magnetic bead panel (Milliplex® map kit, EMD)

This panel was used for the simultaneous quantification of the following cytokines in the culture supernatant samples: GM-CSF, IFN-γ, IL-10, IL-17A, RANTES and TNF-α. The same steps were followed for the preparation of the samples and reagents. The standards that were used are indicated in table 2.7.

2.14.2.1 Preparation of reagents for immunoassay

Preparation of Antibody-immobilized Beads

Individual bead vials were used. Each antibody-bead vial was sonicated (Bransonic Ultrasonic bath sonicator) (Sigma-Aldrich, South Africa) for 30 seconds and then vortexed (Stuart® equipment, UK) for 1 minute. Eighty µL of each antibody-bead vial was added to the mixing bottle and then to bring to a final volume of 5.6mL, bead diluent was added. All the other reagents were prepared as described above.

Table 2.7: Preparation of Human cytokine standard and working standards

Standard concentration (pg/mL)	**Volume of Deionized Water to add**	**Volume of Standard to add**
10,000	250µL	0
Standard concentration (pg/mL)	**Volume of Assay Buffer to add**	**Volume of Standard to add**
2000	200µL	50µL of 10 000pg/mL
400	200µL	50µL of 2000pg/mL
80	200µL	50µL of 400pg/mL
16	200µL	50µL of 80pg/mL
3.2	200µL	50µL of 16pg/mL

The assay procedure that was used for Human Cytokine/Chemokine magnetic bead kit (EMD Millipore, USA) were also used for this kit. The overview of the assay is given in figure 2.12. These plates were run on Luminex MAGPIX® (Luminex Xmap technology, Bio-rad, USA) with the program, Bioplex manager. Calibration and verification was done using the Calibration and performance verification kit (MAGpix, Bio-rad, USA).

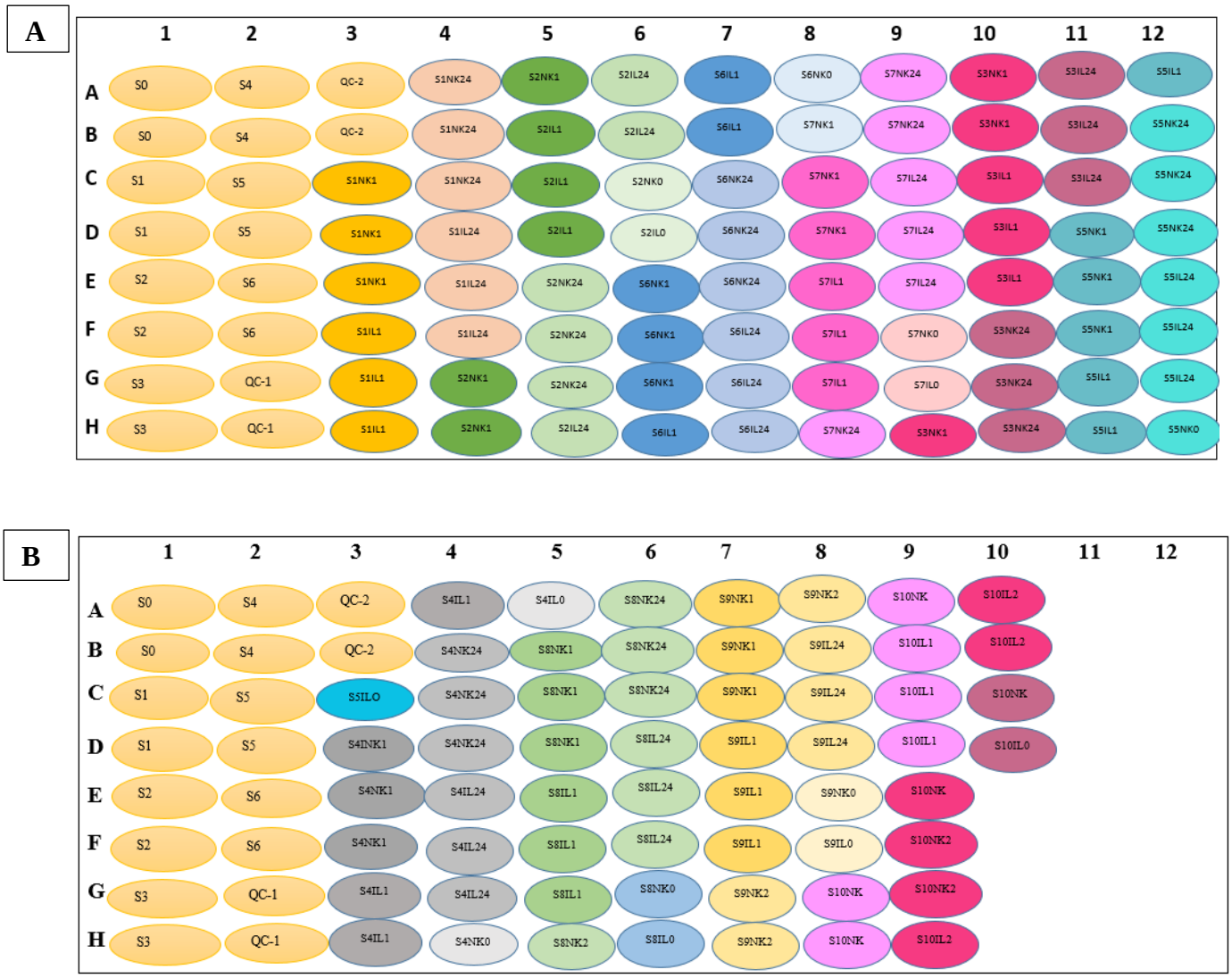


Figure 2.13: The 96-well plate layout of plate 1 (A) and plate 2 (B) for the Human Cytokine/Chemokine magnetic bead assay. Standard 0 = blank, standard 3.2 = low concentration standard, standard 10 000= Highest concentration std, QC1 = Assay Quality control 1, QC2 = Assay Quality Control 2. Evaluate QC1 and QC2 as recommended by kit manufacturer and the interpolate control just like any other sample. Each circle represents a well. The samples was done in triplicates for each time point as well as for stimulations with and without hIL-2.

2.15 Statistical analysis

2.15.1 CFU counts analysis

For the CFU counts analysis the R stats package v.3.2.4, (https://cran.r-project.org/bin/windows/base/) and nmle package v3.1-131 (https://cran.r-project.org/package=nlme) was used to perform the statistical analysis. A p-value of 0.05 or less was seen as statistically significant.

Quantile-Quantile (Q-Q) plot

A quantile-quantile (Q-Q) plot is a plot of the quantiles of the first data set against the quantiles of the second data set. Therefore a Q-Q plot was used to determine if the CFU counts were normally distributed (Figure 2.14).

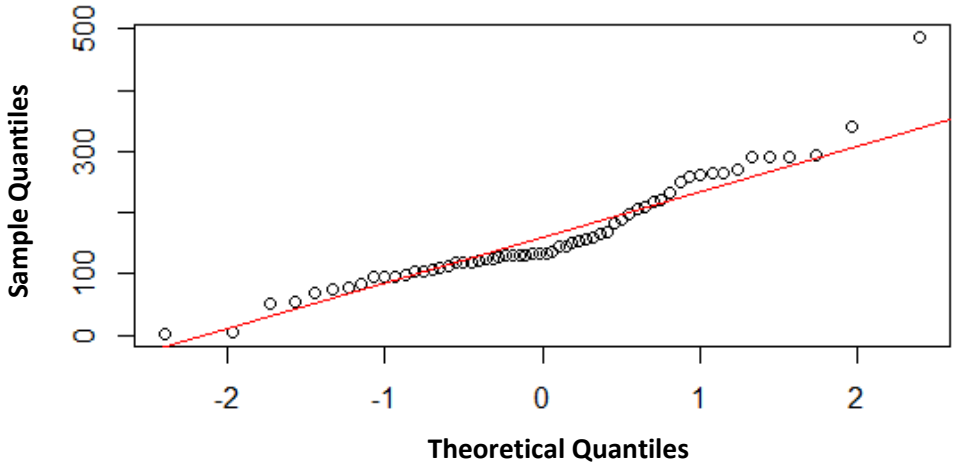


Figure 2.14: Quantile-Quantile plot. Data are normal.

Mixed effect model

A mixed effect model was used to determine if there was a significant association between CFU counts and the amount of *KIR*s present, while correcting for NK cell purity, NK cell subsets and time. A mixed effect model contains both random and fixed effects. In this model the *KIR* genotype was set as the categorical factor (the two groups) and the NK cell purity, NK cell subsets ($CD56^{dim}$ /$CD56^{bright}$) and time was set as fixed effects. The random effect is the variance between samples in order to account for natural variation between individuals. A few models were tested with Analysis of variance (ANOVA) to determine the model with the best fit (based on the model with the lowest odds ratio), which was the random effects mixed model. In this model the CFU counts were modelled as a function of *KIR* genotype and time, while taking into account the interaction between the *KIR* genotype, NK cell purity and NK cell subsets.

Fixed effect only model

A fixed effect model is a statistical model where the model parameters are fixed (non-random quantities). This model was used to determine whether the random effect (Variation between samples) is significantly affecting the model.

2.15.2 Luminex multiplex immunoassay data analysis

The cytokines, perforin, IL-17A, IFN-γ and granzyme B, and the chemokine, RANTES were analysed. IL-10, GM-CSF and TNF-α was left out of the analysis because of missing data (OOR<). The program Statistica 64 (http://statistica.io/) was used to perform the statistical analysis.

Normal probability plot

A normal probability plot is a graphical technique used to assess whether data are normally distributed. Raw data are plotted against a theoretical normal distribution so that the points form a straight line and any deviation from this line indicates departures from normality. A normal probability plot for all 5 (Perforin, IL-17A, IFN-γ, granzyme B and RANTES) cytokines was computed (*Appendix V*). The residuals (difference between the observed value and the estimated value of the quantity of interest) were plotted against the expected normal value.

Mixed effect model

For the mixed effect model, the fixed effects was set as the KIR group, stimulation and time.

Three way repeated measures ANOVA (Analysis of variance)

A three way ANOVA determines how a response or outcomes is affected by three factors. This is used to determine if there is an interaction between three independent variables, namely the *KIR* genotype (the two groups), stimulation, time and a continues dependent variable (samples). A p-value of 0.05 or less was seen as statistically significant.

Fisher's least significant difference (LSD) test

Following the three way ANOVA analysis, a Fisher's LSD test was done. This test can be performed after an analysis of variance, were the F-ratio lead to rejection of the null hypothesis (H_0) to determine which means lead to the rejection of the null hypothesis. The test compares the means of the *KIR* genotype, stimulation and time. The result of IFN-γ were transformed using the logarithm base-10 to reduce variance in the distribution.

Chapter 3: Results

3.1 Demographics of study participants

All participants (n = 30) were recruited from the Division of Molecular Biology and Human Genetics (Stellenbosch University, South Africa). None of these subjects had a history of active TB or had clinical signs or symptoms of active TB at the time of the study. All the participants were over the age of 21. The self-identified ethnicity of both groups were 80% Caucasian and 20% South African Coloured. Table 3.1 shows the age and sex proportions of the groups.

Table 3.1: Sample characteristics of individuals.

Samples		Total number	Age (Mean ± SD)	No. of Males (Prop)
KIR genotyping samples		30	28.08 ± 4.08	7 (0.23)
Selected cohort	**Group 1**	5	29.8 ± 7.79	1 (0.20)
	Group 2	5	30 ± 4.30	3 (0.6)

*SD, Standard deviation
*Prop, Proportion

3.2 *KIR* genotyping

Thirty individuals were genotyped for the six aKIRs to identify individuals with five or more aKIRs. The six aKIRs are *KIR2DS1*, *2DS2*, *2DS3*, *2DS4*, 2DS5, and *3DS1*[22,224]. *KIR2DL4* is a framework gene and can be either activating or inhibitory in function. However this gene was included as an aKIR in our study, since it carries only a single ITIM and displays very weak inhibitory potential[225]. Figure 3.1 is a representation of the genotyping results from one individual. The *KIR* genotyping results of all 30 individuals are indicated in *appendix VII* as well as the frequencies observed for each gene. The most predominant haplotype observed within this study was the B haplotype with a frequency of 80% within the starting cohort (n = 24) (*Appendix VII*). However within group 1, the A haplotype was predominant (60%). This was classified on the basis that haplotype B has one or more of the following genes, *2DS1-2DS5* and *3DS1* whereas these genes were absent in haplotype A. If only one aKIR gene, *2DS4*, was present, it is classified as haplotype A[220,226].

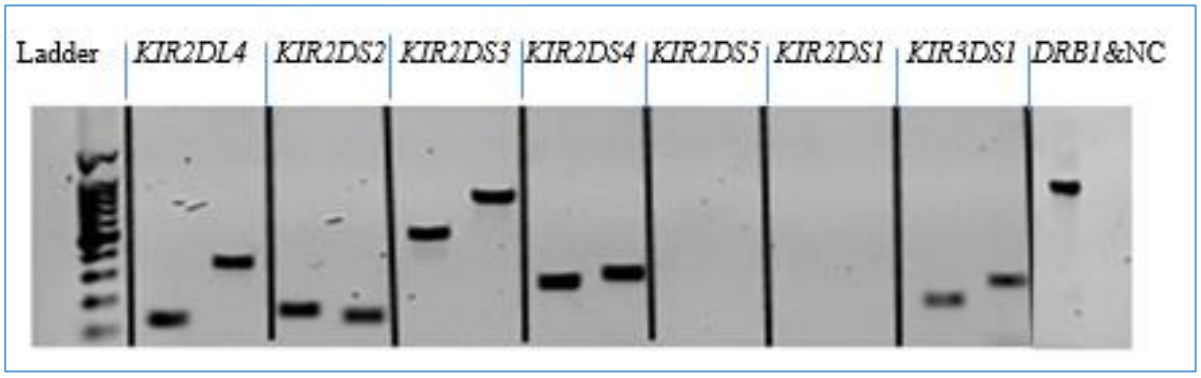


Figure 3.1: Agarose gel image of *KIR* genotyping. The following KIRs were identified as present due to successful amplification: *KIR2DL4*, *KIR2DS2*, *KIR2DS3*, *KIR2DS4* and *KIR3DS1*. *KIR2DS5* and *KIR2DS1* were not present (no amplification). *DRB1* was used as positive control. NC, Negative control.

Ten individuals were selected for functional analysis, depending on their *KIR* genotype (Table 3.2). Five individuals with less than five aKIRs (group 1) and five with five or more aKIRs (group 2) were selected. The genotyping success rate for the *KIR2DL4* gene was 87% and 80% for *KIR2DS4* (*Appendix VII*). However; *KIR2DS4* was only observed at a frequency of 50% within the 10 selected individuals.

Table 3.2: The *KIR* genotypes of the 10 participants. Samples 1-5 had less than 5 aKIRs and samples 6-10 had 5 or more aKIRs. *KIR2DL4* is known to be a framework gene and as expected, this gene was observed in all the samples. The amount of total aKIRs is indicated in the last column.

Group	Sample	*KIR2DL4*	*KIR2DS1*	*KIR2DS2*	*KIR2DS3*	*KIR2DS4*	*KIR2DS5*	*KIR3DS1*	Total *aKIRs*
1	**Sample 1**	yes	no	no	no	no	yes	no	**2**
1	**Sample 2**	yes	no	no	no	no	no	no	**1**
1	**Sample 3**	yes	no	no	no	no	no	no	**1**
1	**Sample 4**	yes	no	no	no	no	no	no	**1**
1	**Sample 5**	yes	no	no	no	yes	no	no	**2**
2	**Sample 6**	yes	no	yes	yes	yes	no	yes	**5**
2	**Sample 7**	yes	yes	no	yes	yes	yes	yes	**6**
2	**Sample 8**	yes	no	yes	yes	no	yes	yes	**5**
2	**Sample 9**	yes	no	yes	no	yes	yes	yes	**5**
2	**Sample 10**	yes	no	yes	no	yes	yes	yes	**5**

3.3 ZN staining of *M.tb H37Rv*

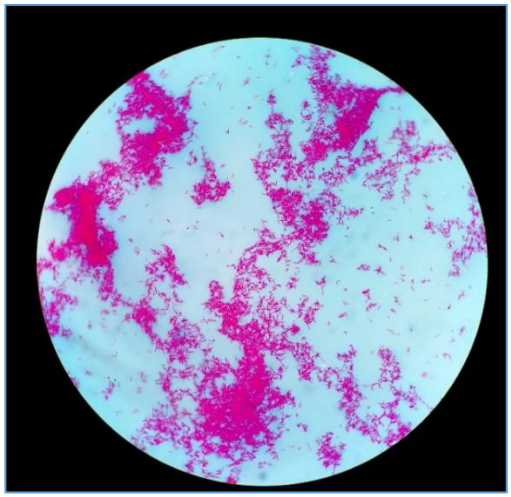


Figure 3.2: Light microscope image of *M.tb H37Rv* after ZN staining.

Figure 3.2, shows the ZN staining of *M.tb H37Rv* to determine the purity of the culture. As seen in the figure, only bright red bacilli were present, indicating a pure culture, free of other bacterial contaminants.

3.4 Assessing the purity of the isolated NK cell populations using FACS

The signal-to-noise-ratio for each antibody was calculated and antibody titration curves were plotted to determine the saturating concentration. The saturation points are indicated in figure 3.3. At this point there is an optimal separation of the negative and positive populations. These points represents the concentrations that will generate specific staining with the least amount of background noise.

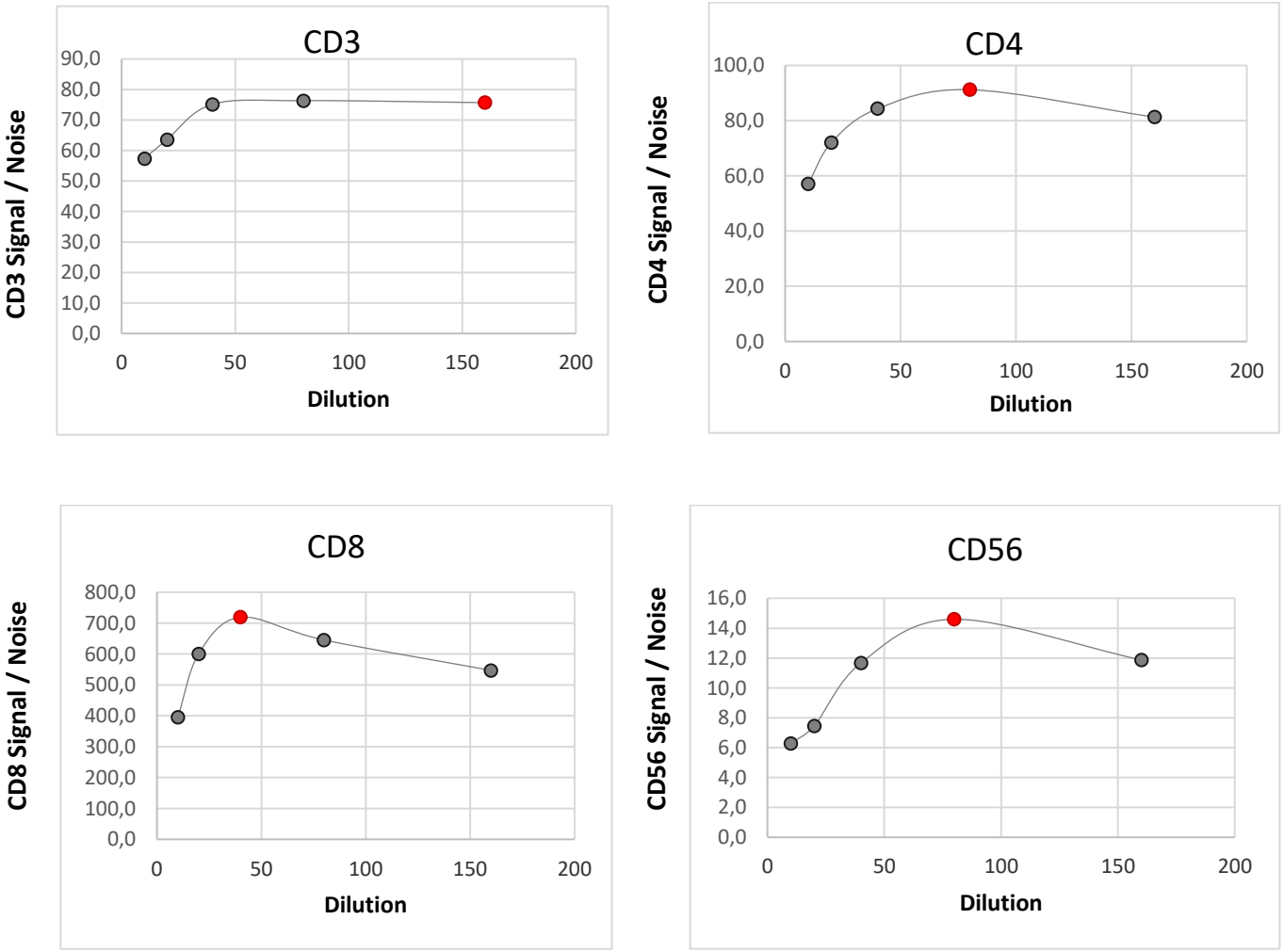


Figure 3.3: Antibody titration plots. The evaluation of different antibodies volumes by staining PBMCs with each antibody. The dots in red show the optimal concentration, which was used for further FACS analysis. The y-axis indicates the signal-to-noise ratio (cell count).

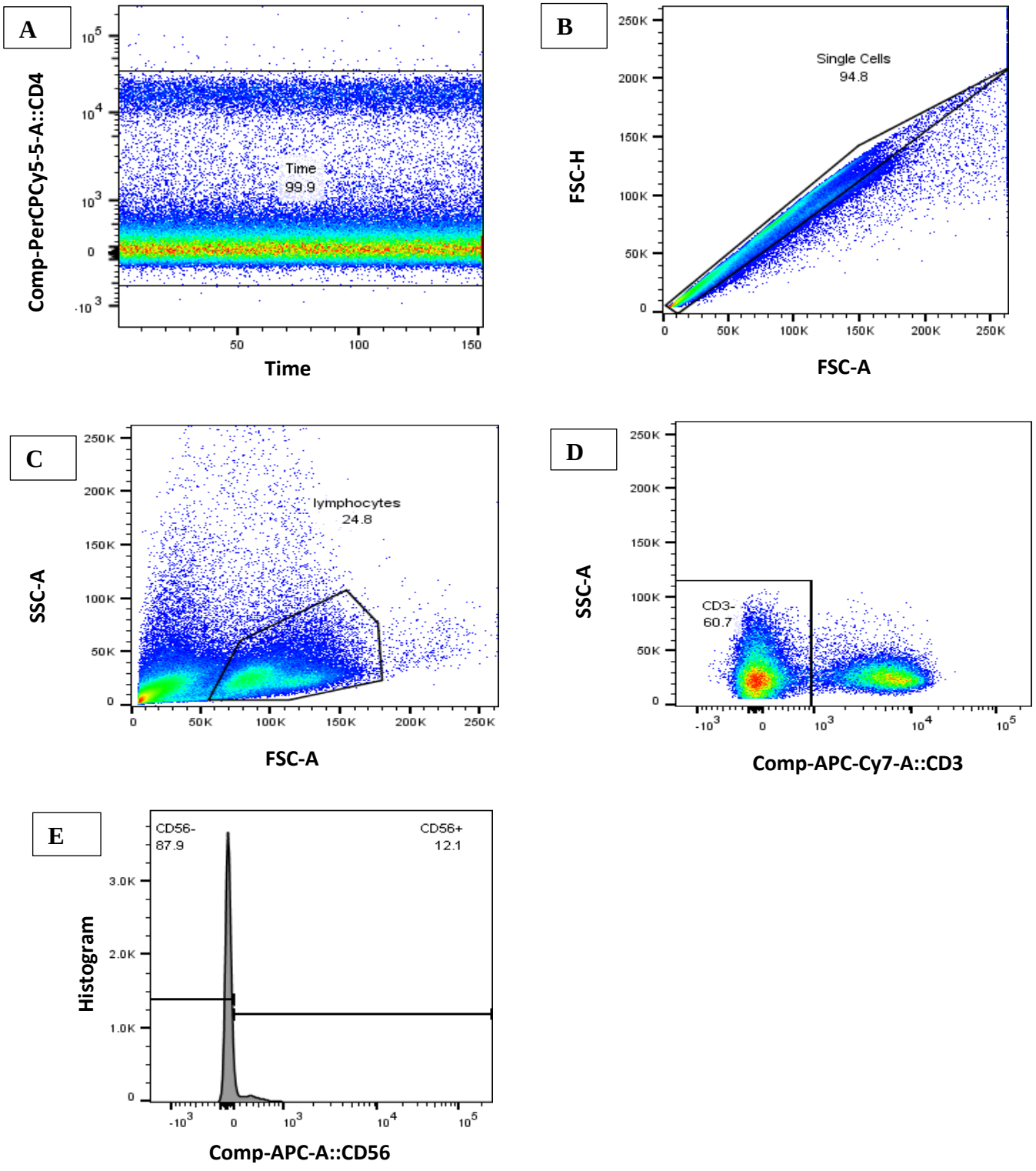


Figure 3.4: Gating strategy. (A) Time gating was included to check for any instrument interruption. (B) Single cells were included using forward-scattered height (FSC-H) vs forward-scattered area (FSC-A). (C) The lymphocyte population was selected using FSC-A and side scatter area (SSC-A). (D) The CD3 negative population was selected. (E) The CD3 negative population was further subdivided into $CD3^-$ cells expressing CD56. This was then subdivided into $CD56^+$ and $CD56^-$ populations.

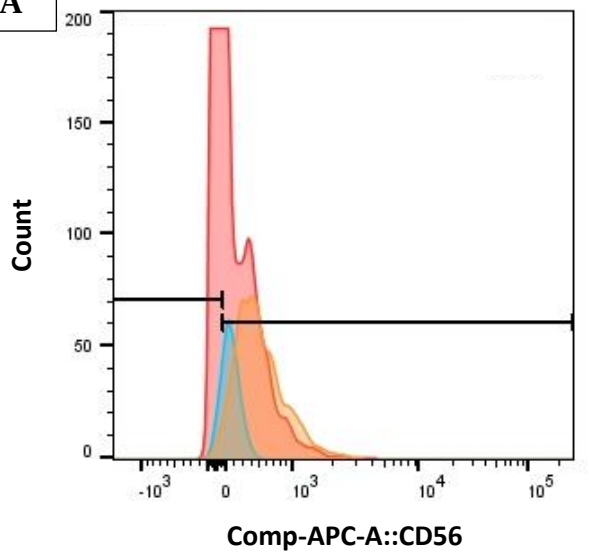
The percentage of $CD3^-$ cells was determined using the gating strategy indicated in figure 3.4. Data were plotted versus time to visualize the cell flow. This was used to control for any unstable flow of cells and to ensure that there were no interruptions. Thereafter single cells were identified by gating on forward scatter-height (FSC-H) versus forward scatter-area (FSC-A). The lymphocyte population was then selected and divided into $CD3^-$ cells. This was further divided into $CD56^+$ and $CD56^-$ populations. The isolated NK cell population purities varied between 40% and 88% (mean = 69.95 ± SD = 14.74). The frequencies of $CD56^+$ and $CD56^-$ within the isolated NK cell populations are indicated in table 3.3.

Table 3.3: The purity of the isolated NK cell populations. The frequencies of the $CD56^+$ and $CD56^-$ cells within the NK cell populations is indicated in the last two columns. Frequencies were calculated based on FACS results obtained with the FlowJo® V10 program.

Sample	Purity of NK cell population (%)	$CD56^+$ (%)	$CD56^-$ (%)
Sample 1	74.6%	94.9%	5.14%
Sample 2	86.3%	97.0%	2.99%
Sample 3	70.8%	93.7%	6.29%
Sample 4	39.6%	96.3%	3.66%
Sample 5	73.0%	86.5%	13.5%
Sample 6	85.6%	96.0%	4.01%
Sample 7	52.4%	93.8%	6.25%
Sample 8	68.9%	94.3%	5.70%
Sample 9	87.6%	98.9%	1.10%
Sample 10	60.7%	64.9%	35.1%

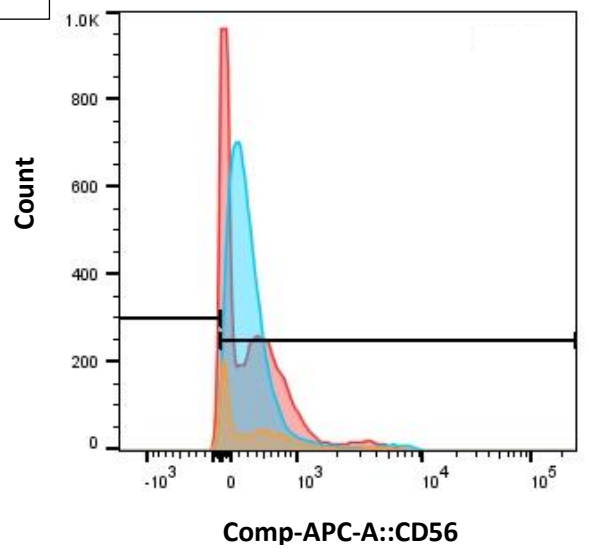
The fraction of NK cells in the PBMC populations as well as the amount of NK cells that were isolated for each sample differed. Figure 3.5 is a representation of two samples. The graphs of the remaining samples are shown in *appendix IV*. Some of the isolated NK cells were still attached to the magnetic particles after the NK cell isolation.

A



	Sample Name	Subset Name	Count
	Sample 3-NK cells	$CD3^-$	2290
	Sample 3-non-NK fraction	$CD3^-$	819
	Sample 3-PBMC	$CD3^-$	26080

B



	Sample Name	Subset Name	Count
	Sample 5-NK cells	$CD3^-$	3305
	Sample 5-non-NK fraction	$CD3^-$	16139
	Sample 5-PBMC	$CD3^-$	17742

Figure 3.5: Panel A shows the NK cell counts of Sample 3 in the two different cell populations and the Non-NK cell fraction. The natural killer (NK) cell counts within the NK cell population are indicated in light orange, the NK cell counts within the non-NK cell fraction are indicated in blue and the NK cell counts within the PBMC population are indicated in red. Panel B shows the NK cell counts for Sample 5. Graphs were made using the Layout Editor of the FlowJo® V10 program.

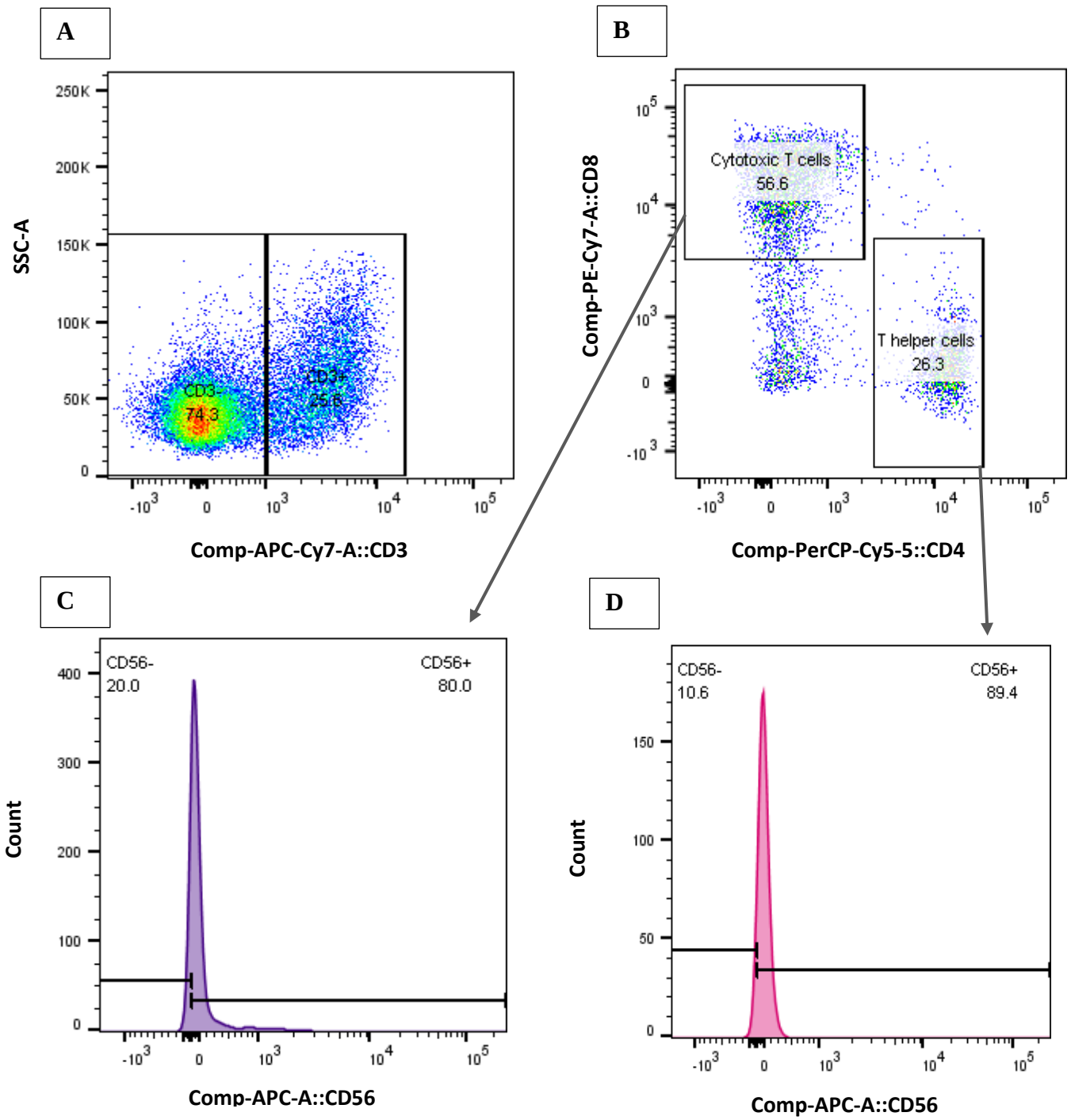


Figure 3.6: Gating strategy to identify the presence of other cell populations. (A) The CD3 population was selected by gating for $CD3^+$. (B) The cytotoxic T cells and Th cells were subsequently selected. (C) Number of $CD56^+$ cells within the cytotoxic T cell populations. (D) Number of $CD56^+$ cells within the T helper cell population.

After assessing the purity of the isolated NK cell populations, FACS analysis was done to determine whether other cell types were present. The gating strategy is indicated in figure 3.6. Cytotoxic T cells, Th cells and NKT cells were identified (Table 3.4). The frequencies varied between the samples, however, cytotoxic T cells were constantly found at a higher frequency when compared with the Th cells (14.32% vs 5.7%).

Table 3.4: The frequencies of other cell types observed within the isolated NK population. Frequencies were calculated based on FACS results obtained with the FlowJo® V10 program.

Sample	**T cells ($CD3^+$)**	**Cytotoxic T cells ($CD3^+$ $CD8^+$)**	**T helper cells ($CD3^+$ $CD4^+$)**	**NKT cells**	
				($CD8^+CD56^+$)	**($CD4^+CD56^+$)**
Sample 1	25.2%	56.6%	26.3%	80%	89.4%
Sample 2	12.7%	6.83%	3.41%	100%	100%
Sample 3	27.8%	12.2%	1.78%	100%	100%
Sample 4	59,9%	4.05%	0.95%	95.6%	95.3%
Sample 5	25.5%	3.03%	1.38%	100%	82.9%
Sample 6	12.2%	21.3%	3.56%	92.7%	95.6%
Sample 7	47.1%	6.35%	2.04%	100%	100%
Sample 8	26.2%	7.13%	0.30%	94.8%	100%
Sample 9	10.8%	22.6%	7.80%	90.1%	94.7%
Sample 10	40.20%	3.08%	3.16%	80%	90%

The $CD56^{dim}$ subset was increased compared to the $CD56^{bright}$ subset within each isolated NK cell population (Table 3.5). The gating strategy for the identification of the CD56 subsets is indicated in figure 3.7.

Table 3.5: The total counts of the $CD56^{-}$, $CD56^{dim}$ and $CD56^{bright}$ subsets.

Sample	$CD56^{-}$	$CD56^{dim}$	$CD56^{bright}$
Sample 1	33.8	56.4	5.75
Sample 2	23.2	66.7	5.00
Sample 3	13.1	79.1	3.59
Sample 4	34.6	50.8	3.20
Sample 5	35.8	41.8	4.11
Sample 6	20.8	72.7	2.20
Sample 7	18.0	75.2	3.87
Sample 8	7.54	89.3	1.94
Sample 9	40.7	33.0	2.75
Sample 10	32.9	48.6	0.38

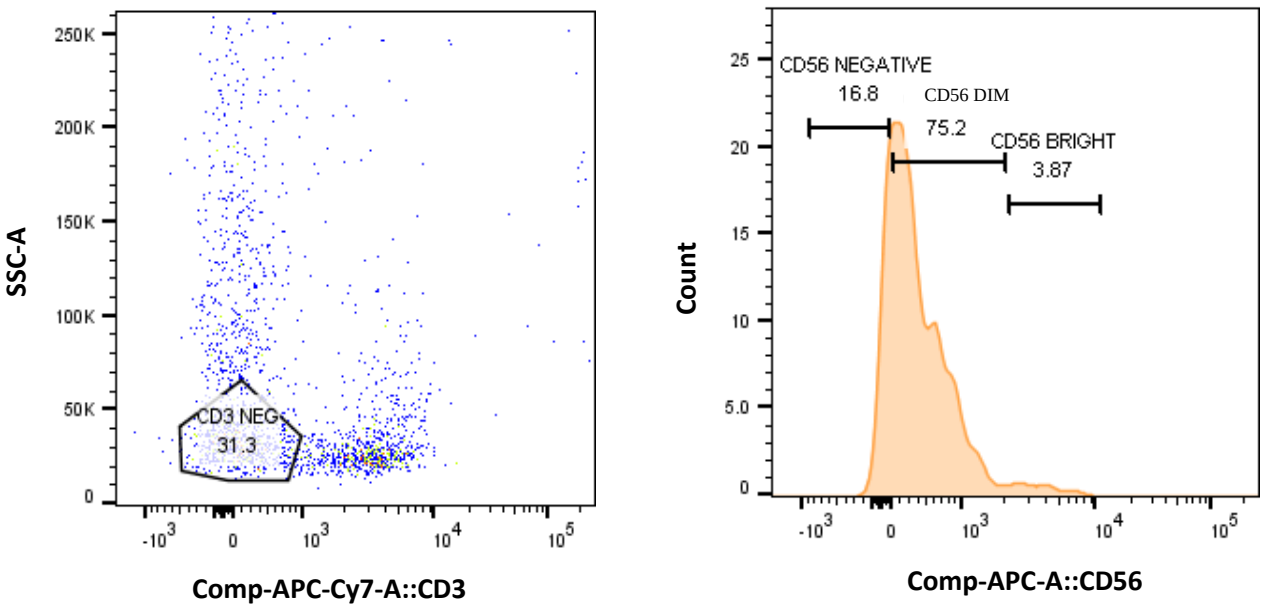


Figure 3.7: Gating strategy for identifying NK cell subsets. The CD3 negative population was selected and followed by $CD56^{negative}$, $CD56^{dim}$ and $CD56^{bright}$ cells.

3.5 Assessing the mycobacterial killing capacity of the NK cells.

No significant difference in mycobacterial killing efficiency was found between the two groups (p = 0.57, Figure 3.8). In addition there was no significant association found between the CFU counts and the NK cell purity (p = 0.17), NK cell subsets (p = 0.12) or stimulation time (p = 0.14). Even though no statistical significant difference was found, it was visually observed that the growth of *M.tb H37Rv* was less when NK cells and hIL-2 was added compared to the control (*M.tb H37Rv*, without NK cells/hIL-2). This difference was not observed when comparing the NK cells stimulated with *M.tb H37Rv* only versus cells that were stimulated with hIL-2 and *M.tb H37Rv* (data not shown).

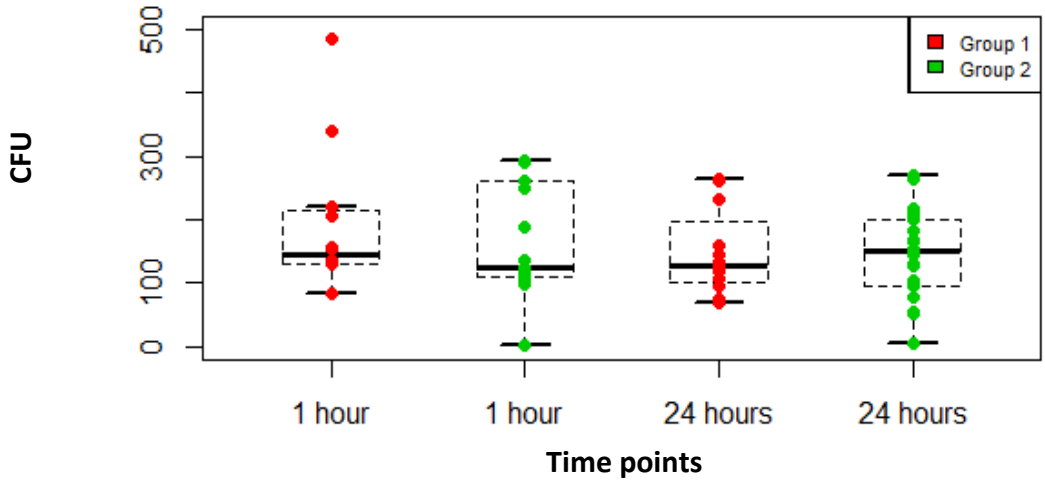


Figure 3.8: Mycobacterial killing efficiency of NK cells isolated from group 1 (aKIR < 5) and group 2 (aKIR ≥ 5) individuals. Stimulations were done at 1 hour and 24 hours. Group 1 is indicated in red and group 2 is indicated in green. Graph shows means and SD readings from 10 individual experiments performed in triplicates. The y-axis indicates the CFU count and the x-axis the time points. CFU, colony forming units.

3.6 Cytokine quantification

The expression of RANTES, perforin, IL-17A, IFN-γ and granzyme B after *M.tb H37Rv* stimulation was determined in the culture supernatant using a Luminex immunoassay platform.

3.6.1 The effect of the number of aKIRs on cytokines expression

A higher expression was observed for RANTES, perforin, IL-17A and IFN-γ in group 2 (Figure 3.9). However only the difference in RANTES expression between the two groups was significant (p = 0.04) (Figure 3.9A). Granzyme B expression, however was higher in group 1, but this was not statistically

significant (p = 0.12). The observations indicated in figure 3.9 were only when no other factors (stimulation types/time) were taken into account.

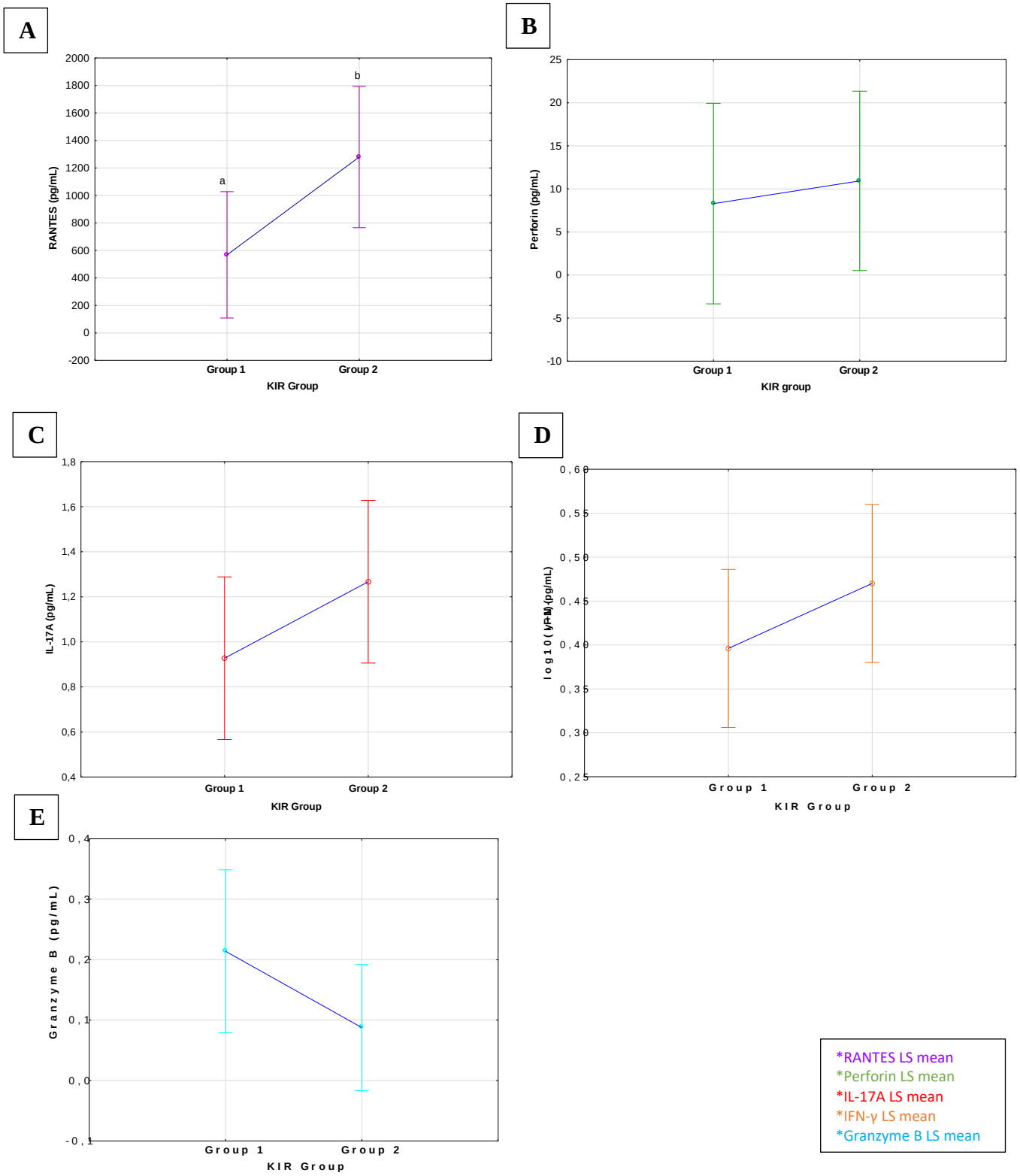


Figure 3.9: The Least Square (LS) means of cytokine expression between the KIR groups. Vertical bars denote 0.95 confidence intervals. (Different letters between two vertical lines indicate statistical significance) (A) RANTES, (B) Perforin, (C) IL-17A, (D) IFN-γ , (E) Granzyme B.

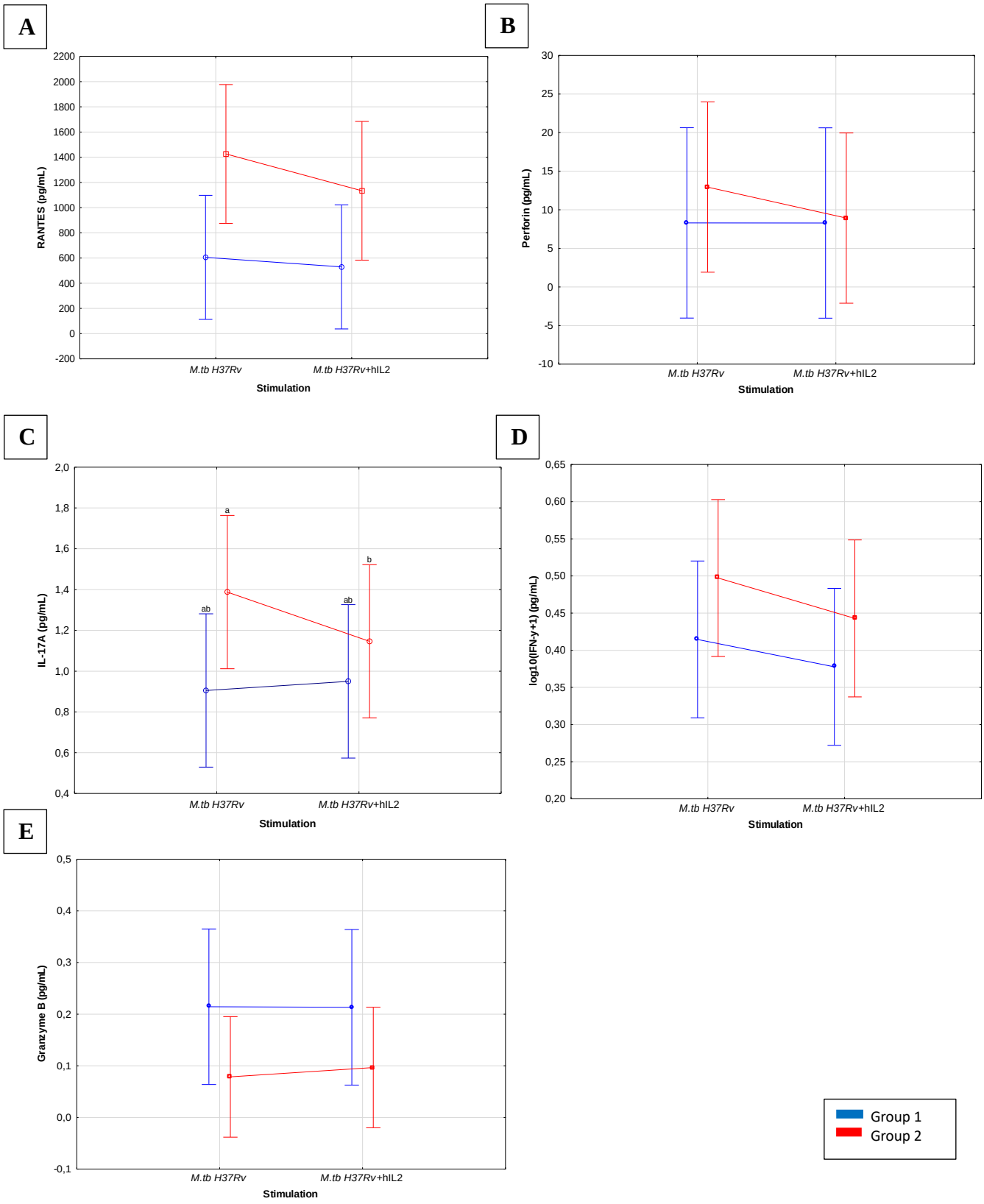


Figure 3.10: The LS mean of cytokine expression, taking KIR group and stimulation into account. Vertical bars denote 0.95 confidence intervals. (Different letters between two vertical lines indicate statistical significance). (A) RANTES, (B) Perforin, (C) IL-17A, (D) IFN-γ , (E) Granzyme B.

Figure 3.10 shows the comparison of cytokine expression between group 1 and group 2 following stimulation with either *M.tb H37Rv* or *M.tb H37Rv*+ hIL-2. All of the investigated cytokines, with the exception of granzyme B showed a higher expression when stimulated with *M.tb H37Rv* only. RANTES expression remained increased in group 2, compared to group 1, after the type of stimulation was taken into account; however this was not statistically significant (p = 0.37). A significant difference between the two different stimulations was observed for IL-17A in group 2 (p = 0.05) (Figure 3.10C). Increased IL-17A expression was observed after *M.tb H37Rv* only stimulation within group 2 (mean = 1.39; SE = 0.16).

Furthermore when taking into account the time points and stimulation types, perforin expression was significantly higher at 1 hour (mean = 14.04; SE = 3.77) compared to the 24 hour time point (mean = 7.21; SE = 3.77), when stimulated with *M.tb H37Rv* only (p = 0.02). When stimulated with *M.tb H37Rv*+ hIL-2, no difference was observed between the two time points as indicated in figure 3.11A. Granzyme B expression was also higher at 1 hour when stimulated with *M.tb H37Rv* only, however this was not statistically significant.

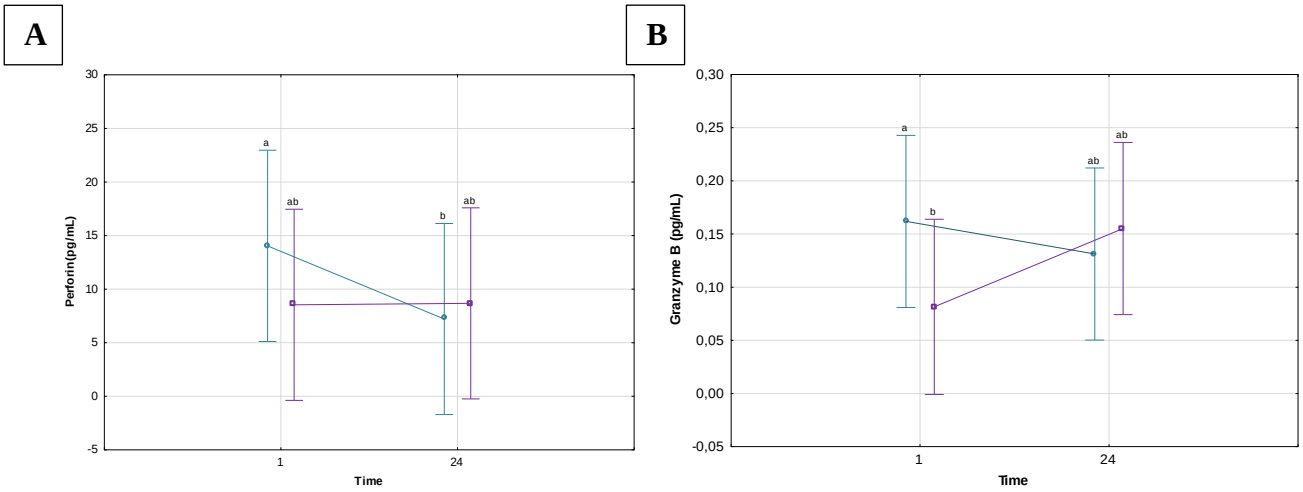


Figure 3.11: The LS means of the cytokine expression and the influence of stimulation and time on the expression. Vertical bars denote 0.95 confidence intervals. (Different letters between two vertical lines indicates statistical significance). (A) Perforin, (B) Granzyme B. Stimulations with *M.tb H37Rv* is indicated in blue; *M.tb H37Rv*+hIL-2 is indicated in purple.

Figure 3.12 indicates how cytokine expression are affected when taking into account, KIR genotype, stimulation type and time. Group 2 had an increased RANTES expression compared to group 1, with the highest expression observed 24 hours after stimulation with *M.tb H37Rv* only (Figure 3.12A). This was also the case for IFN-γ expression 24 hours after stimulation with *M.tb H37Rv*; however for group 1, expression was higher at the 1 hour time point when compared to the 24 hour time point when

stimulated with *M.tb H37Rv* only. None of these observations were statistically significant. Higher granzyme B expression was observed for group 1 when stimulated with *M.tb H37Rv* at both time points when compared to group 2. However, overall, no third order interaction was observed significant for any of the cytokines.

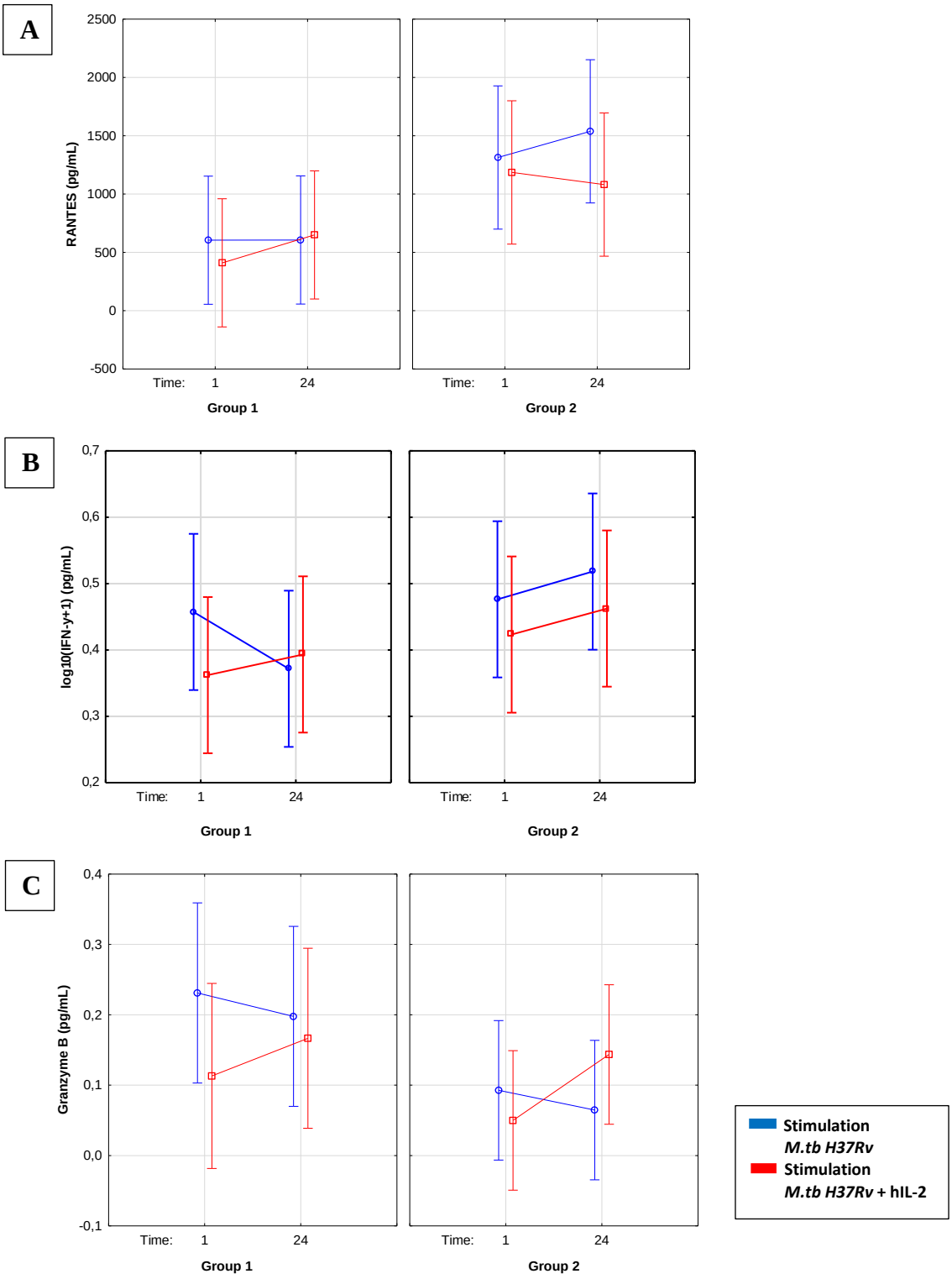


Figure 3.12: LS means of cytokine expression when taking KIR group, stimulation and time into account. **(**A) RANTES, (B) IFN-γ, (C) Granzyme B.

Chapter 4: Discussion

Susceptibility to *M.tb* infection and disease progression depends on the complex interaction between host genetic factors, the causative pathogen and the environment. It is known that a great majority of individuals never develop active TB disease after infection with the causative pathogen[21]. Despite intensive scientific investigations, the exact factors contributing to this favourable outcome still remain unknown. Genetic epidemiology studies have provided robust evidence that genetic variants contribute to TB susceptibility, but functional verification of these statistical associations have not received the required attention. It was previously found that individuals with five or more aKIRs are protected from developing active TB[22]. In this study we therefore aimed to compare the mycobacterial killing efficiency of NK cells, isolated from individuals with less than five aKIRs (group 1), to that of individuals with five or more aKIRs (group 2), and to characterise the cytokine expression profiles of the NK cells after stimulation with *M.tb H37Rv* or *M.tb H37Rv*+hIL-2.

4.1 *KIR* genotypes

It is well known that *KIR*s are highly polymorphic, that unrelated individuals rarely have identical *KIR* genotypes and that ethnic groups differ remarkably in the distribution of *KIR* genotype frequencies[227,228]. KIRs are one of the main receptor superfamilies expressed on NK cells. During development, NK cells are programmed to express unique combinations and numbers of KIRs resulting in the clonal diversity of receptors on NK cells in peripheral blood[201]. Five hundred and ninety four different *KIR* genotypes have been reported in populations worldwide and it is believed that this diversity may alter the susceptibility/resistance to a number of pathogens through ligand-receptor interactions as well as through cytokine release[229–231]. The first objective of this study was to identify individuals with five or more aKIRs and individuals with less than five aKIRs. Thirty individuals were genotyped, of which 17% had five or more aKIRs. This frequency of aKIRs correlates with a previous South African study[22]. South Africa is known to be culturally diverse, with four predominant ethnic groups, namely the Black South African, Caucasians, South African Coloured and South African Indian[232]. Each of these population groups have their own distinct demographic history. The ethnicity of the participants selected within the two groups were the same since it is known that *KIR* genes vary between ethnically and geographically distinct populations and would therefore influence the outcomes[233–236].

The *KIR2DL4* gene is unusual since it contains a cytoplasmic ITIM, which is a feature typical of inhibitory KIR as well as a positively charged amino acid in the transmembrane region, suggesting an activating function. This gene has inhibitory as well as activating functions. A previous study has shown that the cytoplasmic domain of *KIR2DL4* can exhibit a strong Src homology 2 containing protein tyrosine phosphatase (SHP) 1 independent inhibitory function in isolation when the 2DL4 transmembrane arginine residue is mutated or when fused to the extracellular transmembrane domains of *KIR3DL1*[237,238]. On the contrary it has been shown that the engagement of *KIR2DL4* on resting cells results in IFN-γ production and IL-2 activated NK cells respond to *KIR2DL4* by increasing both IFN-γ and cytotoxicity[239]. It was also found that this gene does not have a strong inhibitory signal when compared to other iKIRs, like *KIR3DL1* and that some activation potential was observed for 2DL4 isoforms that lacked the cytoplasmic ITIM, indicating that ITIM doesn't influence the activating function[240]. Possible reasons for this are the presence of only a single ITIM and the context of the unique *KIR2DL4* cytoplasmic tail, which may dampen the inhibitory function of the ITIM[238]. There for *KIR2DL4* gene was defined as an aKIR in the current study. Furthermore this study is based on work done by Salie et al. which included this gene as an aKIR[22]. However, since the gene was present in all the individuals in group 2 it could have been possible to exclude it and adjust the cut off four or more aKIRs (*2DS1*, *2DS2*, *2DS3*, *2DS4*, *2DS5* and *3DS1*) for group 2.

Salie et al. identified a protective effect for the number of aKIR genes in the SAC population. In addition to this, these researchers showed that individuals with a copy of the *KIR3DS1* gene were less likely to develop active TB[22]. In contrast to this finding, the presence of the *KIR3DS1* gene was associated with TB susceptibility in the Lur population (China)[241] and in the Chinese Han population[242]. These findings suggest that this gene has diverse effects across ethnicities or could be in linkage disequilibrium with the true disease-contributing gene or variant. In our cohort, none of the individuals in group 1 had a copy of the *KIR3DS1*, while participants in group 2 all carried the gene. This study did not investigate haplotypes but it is known that haplotype A consists of one aKIR (*2DS4*) whereas haplotype B has five or more aKIRs (*2DS1*-*2DS5* & *3DS1*)[226]. This correlates with the absence of *KIR3DS1* gene in our study participants. The predominant haplotype observed within this study was haplotype B. This correlates with the study conducted by Salie et al.[22] . No statistically significant difference was observed for the killing of mycobacteria between the two groups and therefore it can be said that no protective effect was observed for the *KIR3DS1* gene, however this should be further investigated in a larger sample set.

KIR2DS4 is the only aKIR gene present on haplotype A and two different amplicon sizes can be obtained with this gene. The variant of *KIR2DS4* gene has a 22bp deletion in exon 5[243]. Therefor when 219bp product gets detected when the primer pair in exon 5 is amplified it means that the full length gene is present. However when a 197bp is detected it means that the deletion variant is present. The two types of allele are distributed unequally in different populations[244]. The deletion allele is frequently (80%) observed within Caucasians. This would suggest that A-haplotype homozygotes of this ethnicity often have no aKIR, besides *KIR2DL4*[245]. The majority of individuals would therefore be expected to have at least one copy of this gene. In this study we only determined whether *KIR2DS4* was present and did not investigate the size of the fragment. The gene was observed with a frequency of 80% in the overall cohort. However; only 50% of the 10 selected individuals carried this gene which was not expected. Possible reasons for the low observation of *KIR2DS4* within the 10 selected individuals can be because of possible failed assays. It would have been of interest to determine the size of the gene fragments by using QIAxcel automated gel electrophoresis system (Qiagen, USA)[246,247]. Previous studies also used a nested PCR to isolate *KIR2DS4* alleles with and without the known exon 5 deletion[248]. This method was not used in the current study and therefore it is possible that some alleles of *KIR2DS4* were not detected. The PCR-SSP method used in this study is simple and robust but have the drawback of relying on the amplification of DNA fragments spanning from 0.5-2k bps, which tends to fail in low-quality DNAs[244]. However; a positive control was used, which showed that the DNA quality of the samples were not low in quality.

The *KIR* genotypes were determined based on the absence/presence of *KIR* genes. Thus the copy number of the genes were not taken into consideration. The expression of *KIR* genes on NK cells are random and is determined by polymorphisms, gene content and stochastic epigenetic regulation at promoter level[249]. The underlying impact of KIR copy number variation (CNV) on human NK cell repertoires and functionality remains unknown[249]. CNV leads to considerable diversity for B haplotypes, but not for A haplotypes[250]. There for individuals carrying a duplication/deletion of genes on A haplotypes would have no selective advantage whereas extreme B haplotypes containing between four and 15 genes are consistent with a survival advantage of variability[250]. In this study we did not consider CNV, but it would be of interest since CNV can lead to expression differences which may be important to susceptibility to some diseases. For example CNV of *3DLS1* influences HIV control and it has been found that expression differences of 2DL3 interacting with HLA-C also may have an effect on resolution of hepatitis C virus infection[251,252].

A previous study also found that individuals with multiple *KIR* gene copies had higher frequencies of responding cells[249]. Copy number can be measured using a quantitative PCR comparative Ct method[210,253]. Furthermore variations between individuals in the repertoire of KIR alleles expressed can also be associated with differences in response to pathogen associated signals.

4.2 NK cell isolations

NK cells were isolated from PBMCs using a negative selection kit. Using this method for the isolation of cells results in "untouched" cells that are suitable for functional assays. However, obtaining pure isolated cells is challenging because it requires the design of the perfect depletion cocktail that is specific only to the cells of interest and will result in high purity. It has been shown that the purity of isolated NK cells varies (51.50% - 81.97%) depending on the kit used, despite the kits employing the same underlying method[254]. Therefore the negative selection method that was used in our study might have affected NK cell purity.

The purity of the isolated NK populations for the samples in this study varied from 40% to 88%. This variation between the samples (even though the same technique and kit was used) can be due to various reasons. The NK cell content within the PBMCs of each sample varies and purities might be lower when starting with samples that contained less than 10% NK cells[255]. Additionally, PBMCs were cryopreserved prior to NK cell isolation. Cryopreservation has been shown to influence the percentage of NK cells[256] and the cytotoxic function of these cells in some studies[257,258], while in others no effect was detected[259,260]. Therefore, the exact consequences of using cryopreserved PBMCs remains unclear. However, the cryopreservation of the PBMCs in this study was required for its successful execution.

To determine whether other cell types were present within the isolated NK cell population, further gating was done (Section 3.4). The presence of T cells were investigated by selecting for $CD3^+$ expression markers. Both cytotoxic T cells ($CD8^+$) and T helper cells ($CD4^+$) were identified within the $CD3^+$ T cell gate. The presence of these cell types following negative selection may be due to the ability of NK cells to express CD8 and CD4 markers[261,262]. Although it is unknown exactly which antibodies were used in the EasySep™ Human NK isolation cocktail due to proprietary reasons, it is possible that since NK cells can also express CD4 and CD8 that anti-CD4 and anti-CD8 antibodies are not present.

Subsequently, these cell types could not have been labelled and therefore not effectively removed. Alternatively, lymphocyte receptors for antigens are derived by a stochastic process and therefore recognition of epitopes by receptors will never be absolute[263,264]. NK cells can be subdivided into two major subsets, namely $CD56^{bright}CD16^{dim}$ and $CD56^{dim}CD16^{bright}$, based on the relative expression of their surface markers. We determined the frequencies of the two subsets in PBMCs from each participant and found that the $CD56^{bright}$ subset was consistently lower than the $CD56^{dim}$ subset. The mean was 3.28% for the $CD56^{bright}$ subset and 61.36% for the $CD56^{dim}$ subset. This was expected since it is known that the major circulating NK cell subset in blood of healthy individuals is $CD56^{dim}CD16^{bright}$ with a frequency of 90% and a maximum of 10% for the $CD56^{bright}$ [82,265]. It is also known that the proportion, numbers and subset frequencies of NK cells can differ between individuals due to age, ethnicity and gender[266–268]. It has been shown that the $CD56^{bright}$ subset decreases with age whereas the $CD56^{dim}$ subset increases, however, this was not observed in this study[266,269]. The participant ages in this study did not differ significantly between the groups and no participant was older than 50 years or younger than 21.

Peripheral blood is complex since it contains a variety of cell types and the relative proportions of these cell types can also differ tremendously between individuals[270]. It is known that PBMCs only contain a small fraction (up to 15%) of NK cells which add to the complexity of isolating these cells[81]. Even though there was a variation in purity this did not significantly influence the outcomes of the killing capacity between the two groups (Section 3.5)

4.3 Mycobacterial killing capacity of NK cells

The second objective of this study was to determine whether NK cells expressing five or more aKIRs are more efficient at killing mycobacteria compared to NK cells with less than five aKIRs. NK cells were initially only thought to be vital against viral infections and cancers, now however, it is widely accepted that these cells play a crucial role in protection and control of bacterial infections[65,85,91]. The two main effector responses of these cells are their ability to be cytotoxic and produce cytokines[99].

4.3.1 Cytotoxicity

Stimulation of NK cells was done using *M.tb H37Rv* (in the presence or absence of hIL-2) and CFUs were counted to determine the killing efficiency of the NK cells between groups 1 and 2. No statistical significant difference was found when comparing the killing efficiency of NK cells from individuals in group 1 with that of the individuals in group 2. It was observed that the growth of *M.tb H37Rv* decreased in the presence of NK cells and hIL-2. This study possibly had too few samples for statistical purposes since it is known that there is a lot of variance when looking at CFU counts. Therefore more samples will lead to less variation and as a result, higher confidence.

It is known that human NK cells mediate the killing of intracellular mycobacteria within the first 24 hours of co-culture with infected monocytes[97], while other studies also showed that NK cells inhibited intracellular growth of avirulent strains, *H37Rva* and *M.avium*[131,271]. In the present study, we did not make use of co-cultured infected monocytes and stimulations were done using extracellular *M.tb* instead. NK cells can interact directly (in the absence of antigen specific T cell response, APC, IL-12) with various pathogens, including *M.tb*, which promotes NK cell activation[109,192,272,273].

Previous studies that investigated direct interactions between NK cells and mycobacteria observed that receptors, such as the NKp44 receptor is involved in the direct binding[274]. It is uncertain how KIRs interact directly with mycobacteria. Therefore it would have been of interest to determine the activation states of the cells by looking at differentially expressed surface markers. The NK cells for this study mostly had a mature phenotype since the dominant subset within both groups in the isolated NK cell populations was the $CD56^{dim}$ subset. This subset is known to be more involved in cytotoxic killing compared to the $CD56^{bright}$ subset[90]. In this study the NK cell subsets did not significantly contribute to the outcomes of the NK cells killing efficiency between the two groups. The functions of the different NK cell subsets can be affected by diverse cellular microenvironmental signals[98]. It is known that the functional response of NK cells depends on the recognition of soluble factors (pro-inflammatory cytokines) as well as the ligands expressed on the cell surface of target cells[275,276]. Therefore, signals from the microenvironment are integrated by NK cells. The cellular microenvironment in our study did not reflect the "normal" cellular microenvironment of NK cells within peripheral blood. This may be a reason why no significant differences were detected between the killing capacity of the cells since it is known that in peripheral blood the abundance of stimulatory cytokines may be needed to maintain NK cells in the cytotoxic state to combat infections[98]. The purity of the isolated NK cell populations varied between 40–88% and therefore other cell types were present.

However, the cell types other than NK cells, that were present, are possibly not the cell types needed for NK cells to effectively function. Even though it has been shown that *M.tb* is sufficient stimulus to promote NK cell responses without other immune cells or accessory derived cytokines it has been shown that activated DCs and T cells are needed to secrete abundant IL-2,-12,-18 and -21 which will drive NK cell activation as well as their ability to kill[98].

It is also possible that the varying outcomes of studies investigating the killing efficiency of NK cells are due to differences in virulence between the strains. Most of the studies that found direct associations used attenuated strains rather than *M.tb H37Rv*, therefore it is possible that stimulating with different strains might lead to different NK cell effector outcomes[109,111]. The time of the stimulations may also have an effect. In this study stimulations were only done for 1 hour and 24 hours. This correlates with some studies[97,111], whereas other studies had longer stimulation time points' varying from 48 hours up to three days[110,113]. A previous study found that NK cells stimulated with IL-2 and *M.bovis* BCG resulted in progressive release of IFN-γ up to 72 hours co-culture. However; the addition of *M.bovis* BCG after the first 48 hours culture in the presence of IL-2 did not affect the ability of the NK cells to lyse their target cells and a decrease in cytotoxicity was observed after 72 hours mycobacterial exposure[113]. Furthermore, another study found that when monocyte depleted cell populations were directly cultured with live bacteria, a marked proportion of NK cells were detected among the responding cells. The starting from 3-4 days *in vitro* stimulations[110].

NK cells interact with various other immune cells. Human NK cells lyses *M.tb* infected macrophages *in vitro* resulting in the production of IFN-γ and IL-22 from $CD8^+$ T cells and the enhancement of phagolysomal fusion which inhibits intracellular growth of *M.tb*[182,277,278]. Therefore, the efficient killing of mycobacteria by NK cells can be strongly influenced by the presence or absence of other immune cells. The immune system is very complex and interlinked. While it is clear that other factors also have a vast effect on the killing ability of these cells this goes beyond the scope of the study.

The killing ability of NK cells is known to be stimulated by IL-12[106,271,275]. However in the current study NK cells were stimulated with a low dose of hIL-2. This cytokine was first identified as a T cell growth factor[279,280]. IL-2 is derived from activated T cells and can enhance NK cell response toward infection *in vivo* and can activate NK cells *in vitro*[281,282]. Another study showed that NK cells are incapacitated in IL-2-$\beta^{-/-}$ mice, suggesting that this cytokine may have an important role in maintaining NK cells activity[283].

Comprehensive activation of NK cells is needed for NK cells to fulfil their roles as sentinels. IL-2, -15, -12, -18 and -21 all play a role in activating NK cells[195]. The stimulation of the NK cells with hIL-2 was therefore expected to increase the cytotoxic function. Nevertheless this was not observed when comparing the NK cells that were stimulated with *M.tb H37Rv* only versus cells that were stimulated with hIL-2 and *M.tb H37Rv*. A relatively low concentration was used to stimulate the cells so even though this cytokine has the capacity to stimulate and quantify a response, the low dose (50U/mL) might not have been sufficient to activate NK cells. Previous studies have used much higher doses ranging from 100 – 500U/mL[284–286]. Furthermore it is possible that the cells acquire additional signals since it has been shown that the expansion of NK cells are much higher in the presence of other cell types, including monocytes[284,287]. Even though no significant difference was found between the killing efficiency of NK cells expressing five or more aKIRs versus those expressing less, this is the only functional study thus far that attempted to link the number of aKIRs with the outcomes of the mycobacterial killing capacity of the cells.

Lastly, genetic association studies may produce varying results in different populations. Evidence shows that differences in disease outcomes and inflammation between human populations can be attributed to genetic diversity in populations[288–290]. The genes that are known to be the most diverse within the human genome are involved with immune responses[291]. Significant genetic influences on both humoral and cell-mediated responses have been observed[36]. The highly polymorphic and or clustered characteristics of immune response genes can be an indication of evolutionary pressure and selection[292].

One of the characteristic features of the MHC region is the fact that it is polymorphic which contributes to a wide range of variation. This feature is essential for the host to respond to several pathogens since the peptide grooves are coded for by these polymorphic regions[293,294]. Furthermore most human KIRs, which are also highly polymorphic, are specific for polymorphic determinants of HLA class I molecules. This adds to the complexity of understanding and studying *KIR* genes because the role that these genes play in disease susceptibility will be greatly influenced by the HLA class I molecules.

4.3.2 Cytokine expression

Another reason why NK cells are important in innate immunity is due to their ability to produce a variety of cytokines and chemokines. The $CD56^{bright}$ subset is commonly known as the cytokine producing subset of the NK cells.

Cytokines that are produced affect the functions of other cell types as well as the survival of the cells itself and mediate in part their cytotoxic function. The outcome of *M.tb* infections are also largely determined by the cytokines that are present and cytokine production influences both innate and adaptive immunity[295]. Therefore, the third objective of this study was to quantify the cytokines produced after mycobacterial stimulations and to determine if the expression levels of the cytokines are influenced by the number of aKIRs present. Identifying a possible link between innate and adaptive immunity through cytokines that are produced by NK cells may lead to possible new designs of adjuvants and vaccines against TB.

It has been proposed that a decrease of aKIRs expression on NK cells can lead to insufficient production of *M.tb* killing cytokines[296]. Varying outcomes of immune-regulated diseases between different populations have been observed which can also be due to differential expression of cytokines[297]. This adds to the variation of the effect of cytokines as well as the complexity of the network through which NK cells help to control *M.tb* infection. Even though we found no statistical significant difference in expression due to the amount of aKIRs, a higher expression was still observed for all the cytokines, except for granzyme B. This may be because the sample size was too small to observe any significant difference between the groups. It would thus be necessary to repeat this pilot project with additional samples. Furthermore, baseline controls can also be used to compare cytokine expression before and after the stimulations. It is also possible that the minimal requirement for cytokine secretion was not met. Cytokine expression *in vivo* is influenced by various factors including, immune complexes, cell-cell contact, microbial species and other types of cytokines. Therefore to determine cytokine expression *in vitro* is complex and difficult. The longest infection duration in this study was 24 hours and it is possible that this was not long enough for significant cytokine production. It would therefore be of interest to include longer infection time points. Moreover, comparing cytokine expression within active TB cases to cytokine expression within healthy controls will give insight into which cytokines play important role in the immune response to *M.tb*. Hence investigating the expression of cytokines derived from active TB cases would also be of importance.

The immune system's cellular components cross-talk through cytokines, thus cytokines play an essential role in modulating the immune cell function[298]. Chemokines are also important for an effective inflammatory response due to their ability to modulate and promote leukocyte migration[298]. RANTES, also known as (C-C motif) ligand 5 (CCL5), is a chemokine produced by NK cells.

We found that RANTES expression was significantly higher in group 2 when compared to group 1. Chemokine production is an early feature of NK cell responses and can be triggered with relatively weak activating signals and requires less stimulation than what is needed for cytokine production[115]. Therefore this might contribute to why only a statistical significant increase was observed for RANTES expression and not for any of the cytokines investigated. Furthermore, CCL4, CCL5 and CCL22 are produced by NK cells *in vitro* without any stimulation[299–301]. CC chemokine receptors, like CCR1 until 9, are not significantly expressed on the surface of resting NK cells. However NK cells express CCR2, -4, -5 and CCR8 after activation with IL-2 or IL-15 *in vitro*[302]. Therefore, we would have expected to see an increase in RANTES expression after stimulation with hIL-2; however this was not observed, possibly due to low dose stimulation.

The fact that RANTES expression was significantly higher in the group with more than five aKIRs (group 2) can possibly contribute to the mechanism that individuals with five or more aKIRs are more protected against developing active TB disease, since this chemokine has been found to stimulate greater levels of killing[298]. However this was not consistently observed throughout previous studies[303,304]. Furthermore CCL2 - 8 have been shown to promote cytotoxic granule release by resting polyclonal NK cells[305,306]. The concentrations of these chemokines that causes the stimulation of granule exocytosis were similar to those that enhance NK cell cytolytic activity. We therefore speculate that chemokines increase NK cell lysis of target cells by facilitating the discharge of NK cell cytotoxic granules[306].

Both granzyme B and perforin are known to be the major cytotoxic proteins that are contained within the NK cells. These cytotoxic granules in NK cells are preformed before cell activation, therefore a balance of inhibitory and activating receptors are important for immunosurveilance[307]. NK cell lytic synapse occurs before molecular patterning is completed and molecular reorganization at the synapse seems to be dependent on regulatory signals, with signals from KIRs playing an important role as well. This regulation ensures that NK cells carry out their surveillance function as effectively as possible[307].

Perforin facilitates the entry of granzymes into the target cell cytoplasm; however, this mechanism by which this occurs remains unclear. When an immune synapse is formed between a target cell and NK cells, the cytotoxic granules moves into a synaptic cleft and the granzymes are then delivered into the target cell[307].

Granzyme B expression was relatively higher in group 1 compared to group 2. Perforin is required for granzyme delivery into target cells, however less perforin expression was observed within the group 1, suggesting it was utilised elsewhere. This could result in higher granzyme B expression. The pathway for perforin and granzyme B secretion is distinct from cytokine expression since perforin is secreted from cytotoxic granules. This mechanism allows NK cells to simultaneously recruit immune cells and kill target cells. The low levels of granzyme B (especially in group 2) and perforin expression may also be due to NK cells exhaustion after carrying out their killing functions[308,309]. Cytotoxicity is mainly achieved through the release of granzyme B and perforin into the immunological synapse and therefore a loss of cytotoxicity can be because of inhibition of these two compounds[129,135].

A reduction of granzyme B and perforin content was observed in a previous study after the cells were incubated with the target cells[135]. In the present study a slight decrease in perforin expression was observed at 24 hours when stimulated with *M.tb H37Rv* only, whereas for granzyme B expression, a higher expression was observed when stimulated with *M.tb H37Rv* + hIL-2 at 24 hours. Previous studies also found an increase in perforin and granzyme B content when cultured with IL-2[135]. The expression and activation of these two proteins varies between different clonal populations[310]. Perforin expression differed significantly depending on the time and the type of stimulation. $CD56^{dim}$ NK cells' perforin expression is 10-fold higher when compared with $CD56^{bright}$ NK cells[81]. The current study did not differentiate between $CD56^{dim}$ and $CD56^{bright}$ cells when measuring expression.

Another influence on cytokine expression is the multiple cytokine receptors. Cytokines plus activating NK cell receptors provide combined signals which results in robust NK cell effector responses. Therefore it is thought that NK cells can change the extent of response and sense differing extents of inflammation and pathogenicity[67,311]. NK cells were stimulated with a combination of cytokines (IL-2, IL-15 and IL-12) and a higher IFN-ɣ secretion was observed when compared to when the cells were only stimulated with IL-2[301]. In our study IFN-γ expression was lower when stimulated with *M.tb H37Rv* and hIL-2 compared to when stimulated with *M.tb H37Rv* in the absence of hIL-2. This might be due to the fact that IL-12 was not present, since previous studies found that IL-2 alone cannot stimulate the release of IFN-γ. IL-2 acts synergistically with IL-12 to secret IFN-γ and another study found that IFN-γ half-life is increased when both IL-12 and IL-2 are used in combination[312].

However in other studies, IL-2 directly stimulated IFN-γ production from activated NK cells in the absence of other stimuli[313,314]. Secretion of IFN-γ in a previous study was tightly controlled and needed engagement of multiple different receptors[115]. It is therefore possible that resting NK cells need supplementary signals to produce detectable levels of IFN-γ. These additional signals can possibly be provided upon cell-cell interaction with accessory cells or additional cytokines[315]. Cytokine receptors which are expressed on NK cells also play an important role in NK cell effector outcomes when ligated through a number of intracellular signalling pathways[311]. However this study only focused on the aKIR receptors. Previously it was found that individuals with a greater number of aKIRs had a greater response to *M.tb* infection due to an increased IFN-γ release. IFN-γ is one of the main cytokines involved in protection against *M.tb* since it is known to activate macrophages[141].

In this study a slight increase in IFN-γ expression was observed in the group with five or more aKIRs. This can explain why individuals with more than five aKIRs were shown to be more protected against developing active TB disease even though the higher expression was not statistically significant in this study. It has also been found that regulation of IFN-γ production is dependent on the balance of both inhibitory and stimulatory cytokines released from Th1 cells and NK cells[152]. In this study no other cell types were present, therefore optimal IFN-γ production was not fully observed. Macrophages were shown to be required for IFN-γ production[316] and since no macrophages were co-cultured with the NK cells in this study, this could explain the low cytokine expression. It has also been found that the production of immunoregulatory cytokines from $CD56^{bright}$ NK cells is dependent on monokine stimulations and qualitative differences are seen, depending on the specific monokines that are present[81]. The effect of exogenous cytokines on NK cells have been well studied. It is known that NK cells can proliferate in response to IL-2, -4, -7, -12 and IL-15[317–320] and that IL-2 and IL-15 cause a greater NK cell proliferation compared to other cytokines[301]. IL-2 and IL-15 represents the best studied cytokine activators of NK cells. IL-2 is central for NK cell development, homeostasis, proliferation induction and enhancement of cytotoxic effector mechanisms. $CD56^{dim}$ cells express βγ common chains of IL-2 and IL-15 receptors. These cells therefore demonstrate an increase in cytotoxicity, proliferation and cytokine secretion after stimulation with IL-2 or IL-15. Whereas $CD56^{bright}$ cells express high affinity αβγ heterodimers and proliferate with IL-2 alone and produce an abundance of IFN-γ [321].

Resting $CD56^{dim}$ cells are more cytotoxic against NK sensitive targets than $CD56^{bright}$ cells[67,322]. However when $CD56^{bright}$ cells were activated with IL-2, an enhanced cytotoxicity against NK targets compared to $CD56^{dim}$ was exhibited[322]. In this study we did not investigate the variation of cytokines between NK cell subsets; however this would be of interest in follow up studies, because it is clear that the different NK cell subsets are one of many factors that influence NK cells cytokine expression. IL-2 and IL-15 also induce IL-17 production in PBMCs[323], however in this study IL-17A expression was significantly higher in the group with five or more aKIRs when stimulated with *M.tb H37Rv* in the absence of hIL-2. IL-17A contributes to protection against *M.tb* infections through early neutrophil recruitment, enhancing reactive oxygen species (ROS) production and migration of inflammatory cells at early stages of infection[324–326]. IL-17A increased NK cell number and their cytotoxic function and *M.tb H37Rv* induced IL-17 production in neutrophils which was dependent on IL-6 and IL-23 in previous studies[112,327]. Clearly optimal cytokine production is influenced by the presence of other cytokines. Even though IL-17A contributes to protection against *M.tb* infection, this cytokine is also known to add to the pathogenesis of autoimmune and inflammatory disease. *M.tb* infection and autoimmune diseases have been found to be related since *M.tb* is a strong immunogen[328]. Therefore type-I immunity is essential for protection against microbial pathogens but should be tightly controlled since uncontrolled type-1 cellular immune responses can cause immunopathology, tissue damage and disease[327]. The expression as well as the control of cytokines are important for immunopathology and immunity.

4.4 Limitations of the study

Limited sample size

The first limitation of the study was the small sample size. Genes encoding iKIRs are nearly always present in populations at frequencies greater than 90%; however the frequencies of aKIRs are much lower. Therefore to increase sample size, a larger cohort will be needed to increase the amount of individuals in group 2. Furthermore, this was a pilot study and therefore the protocol established, during our study to determine the killing efficiency of NK cells against fighting TB infection while incorporating the *KIR* genotype, can be used in a larger scale study.

KIR genotyping

Some individuals could have more copies of a certain gene when compared to other individuals, since the CNV were not determined. Furthermore, there were no internal positive control for the specific primers used during the genotyping method. Bx genotype with all the genes present would have been

beneficial to use as a positive control. The presence/absence of only seven *KIR* genes were determined but it would have been of interest to genotype all 15 *KIR* genes.

PBMCs cryopreservation

The NK cells could only be isolated once all the PBMCs were isolated from the whole blood for each sample and since blood was drawn over a period of three weeks, cryopreservation was needed. The fact that the PBMCs had to be cryopreserved before the NK cells could be isolated also made it more challenging. Cryopreservation of PBMCs is a standard procedure even though it can potentially induce significant changes in phenotype and/or function. It has been shown that cryopreservation does not have an effect on cells[259,260]. However there are studies that found that cryopreservation influence PBMCs and the true effects of cryopreservation on NK cells remain unknown[256]. A study showed that after standard freezing and thawing of PBMCs, the percentage of $CD56^{+}CD3^{-}$ NK cells did not change[329]. The percentage of viable active NK cells also stayed consistent, however it seemed that the cytotoxic function of the these cells were reduced[257,258]. After the thawing process, the initial amount of viable PBMCs decreased, which resulted in a decrease in the number of NK cells that could be isolated.

NK cell isolations

Isolation of NK cells from PBMCs is complex because peripheral blood only contains a small fraction of NK cells. This study required the isolation of "untouched" NK cells and therefore a negative isolation kit was used instead of a positive isolation method. Negative isolation leaves the NK cells "untouched", however it is more difficult to isolate a pure population of cells. Additionally the FACS analyses showed for some samples the NK cells were still attached to the magnetic that also contributed to decreased numbers of isolated NK cells. The culturing of NK cells is challenging and it was not feasible to obtain viable NK cells for longer than 24 hours without stimulation. We therefore stimulated the cells with hIL-2[254]. It is known that hIL-2 causes a change in the phenotype of NK cells and can also affect the function of the cells[85]. Downstream experiments in this study were therefore done with and without hIL-2.

Determining mycobacterial survival.

CFU counts were used to determine the mycobacterium's viability. Two advantages of using this method, only viable bacteria are counted and this method has the capacity for counts of any number of bacteria; however disadvantages of using this method, takes ± 3 weeks to obtain results, not very reproducible and count can be affected by minor bacterial clumps[330].

Therefore, the LIVE/DEAD® Baclight Bacterial viability kit (https://www.thermofisher.com/order/catalog/product/) or cytotoxic assays can be used to determine NK cells killing efficiency. These techniques might better represent the mycobacterium's viability.

HLA class I genotyping

Another limitation of this study was that the *HLA* genes of the participants were not taken into consideration since it is known that most human KIRs are specific for *HLA* class I molecules[331]. Therefore, the *HLA* class I molecules will greatly affect the role of *KIR* genes in disease susceptibility. It is known that the *HLA* region is the most polymorphic in the human genome[332]. Since this was a pilot study, including another factor into the analysis would have decreased the study power.

Determining cytokine expression

TNF-α, GM-CSF and IL-10 were excluded from the analysis due to missing and out of range (OOR) data. Higher sample volume would be needed for the samples that were OOR due to low signal for the standard curve. However when OOR data were observed due to samples containing analyte concentrations higher than highest standard point, the sample can be diluted and reanalysed for that analyte. Furthermore, we could not compare how cytokine expression differed between baseline and after infections because of the limited amount of NK cells isolated from PBMCs.

4.5 Future studies

If proven that individuals with more aKIRs are protected against developing active TB, follow up studies can be done to find the mechanism behind this effect. The effector functions involved and the exact role that more aKIRs play in the immune response against *M.tb* can be further investigated. Furthermore cotransfection and coimmunoprecepitation can be used to determine the association of aKIRs and adaptor molecules. Ligand recognition assays can also be used to determine the involved ligand specificity. In the long run this can be used to manipulate NK cells to generate an increased immune protection against developing active TB.

NK cells can be stimulated with *M.tb* in the presence of an APC, for example, stimulating NK cells in the presence of macrophages. This will facilitate the investigation of the NK killing capacity through an indirect pathway. The Pierce™ Lactate dehydratase cytotoxicity kit (Thermo Fisher scientific, South Africa) can be used to measure LDH released into the media from damaged cells as a biomarker for cellular cytotoxicity and cytolysis.

The point of contact between the NK cells and mycobacteria can also be investigated by using high-resolution imaging techniques such as super-resolution confocal microscopy and to identify the receptors that are involved in the activation of the immune response against *M.tb* infection. Flow cytometry can be used to identify the effect of *M.tb* stimulation on the expression markers of the NK cells and subsets before and after stimulations. Identifying the effect of *M.tb* stimulation on NK cells can lead to a possible identification of the effect of the infection on the phenotype of the cells. It is known that NK cells exhibit different phenotypes with diverse properties. If the mature phenotypes of NK cells can be increased it is possible that sufficient effector functions against *M.tb* can be secured.

It would be of interest to compare the effector functions of NK cells between healthy controls and active TB cases. Finally, *HLA* class I genotypes should be incorporated and more samples should be included for each group. Cytokine expression between the different NK cell subsets can also be further investigated.

4.6 Concluding remarks

A case-control association study found that an overexpression of aKIR genes may protect against TB disease development. To functionally validate this, the mycobacterial killing efficiency of NK cells from individuals with five or more aKIRs were compared to individuals with less than five aKIRs. Even though the exact protective role of NK cells against *M.tb* due to the number of aKIRs expressed on their surfaces is still unclear, the protocols generated in this thesis can be used by follow up studies. Furthermore this pilot study is the first to incorporate the number of aKIRs while investigating the effector functions of NK cells against mycobacteria. Additionally, this is one of the few studies that investigated the antimicrobial defences of NK cells in direct response to mycobacteria. Identifying genes that confer susceptibility to TB disease can be used in developing personalised medicine. Furthermore knowledge of the effector functions of NK cells during *M.tb* infection can be used to modify NK cells and may contribute to developing novel strategies to improve immunosurveillance. Future work will include repeating this study with bigger sample size and determining the effect of indirect mycobacterial stimulations on NK cell phenotype.

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Appendices

Appendix I

Reagents

1. DNA extractions

TE buffer

1.21g Tris-HCl

0.372g EDTA
Add 800mL dH20
Adjust the pH to 8.0 with concentrated HCl
Make up 1L with dH20
Autoclave for 20 min at 121°C and store at RT

Reagent A
10mM Tris-HCl
320mM Sucrose
5mM MgCl2
1% (v/v) Triton x-100, pH 8.0
Combine reagents in 80% of the volume required, mix to dissolve
Adjust pH to 8.0 using 40% (w/v) NaOH
Autoclave aliquots at 121°C for 15 min

2. *KIR* genotyping

1% Agarose gel

1g agarose
100mL (1x) SB Buffer
Microwave for 2-3 min
add 5µL ethidium bromide (10mg/mL) when temperature reaches ±55°C

Loading dye
5 µL Cresol loading dye

3. PBMC isolations

Phosphate buffered saline (PBS)
1 tablet PBS
dH20 up to final volume of 200mL
Autoclave for 20 min at 121°C and store at 4°C

4. Cryopreservation

Cryomedia

10% DMSO
45mL FBS
Filter
*Do not freeze & thaw FBS more than once

Thawing media

445mL RPMI-1640
50mL FBS
5mL Penicillin Streptomycin
Filter FBS and Penicillin Streptomycin

5. NK cell isolations

0.5M EDTA

93.06g EDTA
400mL dH20
Adjust pH to 8.0 using ± 10g NaOH pellets
Make up to 500mL with dH20
Autoclave at 121°C for 20min and store at RT

Recommended media

293.4mL PBS
6mL FBSS
0.6mL (1mM EDTA)
Autoclave PBS at 121°C for 20min, let cool down and add FBS + EDTA

NK cell culture media

450mLRPMI-1640
50mL FBS
50U/mL hIL-2
Store at 4°C

6. Bacterial culture

Middelbrook 7H9 medium

Suspend 4.7g of 7H9 powder in 900mL dH20 (supplemented with 2mL glycerol)
Autoclave at 121°C for 10 min
Aseptically add 100mL of Middelbrook OADC Enrichment to medium when cooled (45°C)

Middelbrook 7H11 Agar plates
Suspend 19g 7H11 powder in 900mL dH20 (supplemented with 5mL glycerol)
Autoclave at 121°C for 10 min
Aseptically add 100mL of Middelbrook OADC to medium when cooled (45°C)
Aseptically pour in petri dishes and let air dry in hood

7. FACS
FACS/Staining buffer

2% FBS
PBS

Washing buffer

1x PBS

Fixative buffer

2% formaldehyde
PBS

Appendix II
Calculations

Cell counting

Haemocytometer

In order to determine the number of cells per mL, the number of cells in each of the five larger square blocks was counted. The amount of the cells in these five squares was added together and divided by five to get an average number of cells for each of the 25 large squares of the central quadrant of the haemocytometer. The average number of cells was subsequently multiplied by 25 to get an estimated average number of cells within the large central quadrant. The formula used to determine the number of cells/ml is as follows:

Number of cells/mL = number of cells x dilution factor x 10^4 (because the depth of the counting chamber is 0.1mm)

Cell counting

Countess™ II FL automated cell counter

A) $C_A = N_A \times (\frac{D}{2})$

B) $T = C_A \, x \, V$

Cell counted calculation by Countess™ II FL Automated cell counter. (A) Determining the concentration of cells counted, (N_A = Live cell counts as displayed by automated reader, D = Dilution of cells with trypan Blue). (B) Calculating the total number of cells counted, (V= Resuspension volume of cells in cryomedia in mL)

Calculation for the stimulations with *M.tb H37Rv*

Concentration [ ] = $\frac{Average\ CFU}{Volume\ (ml)\ that\ you\ plated\ X\ dilution\ factor}$

Number of *M.tb H37Rv* per NK cells

MOI = $\frac{Volume\ X\ Concentration}{Number\ of\ cells\ per\ well}$ * MOI = 5

Appendix III

Antibody titration values

Table 1A: Fluorophores and their absorption and emission spectra.

Fluorophore	Excitation max (nm)	Excitation Laser Lines (nm)	Emission max (nm)	Instrument
APC_Cy7	650	595,633,635,647	774	BD FACSCANTO II
PerCP_Cy5.5	482	488	690	BD FACSCANTO II
PE_Cy7	496,565	488	774	BD FACSCANTO II
APC	645	595,633,635,647	660	BD FACSCANTO II

*nm, nanometer

Table 2A: CD3 antibody titration dilution values.

Antibody + fluorophore	Median CD3 positive	Median CD3 negative	Dilution	Ratio pos/neg
CD3 APC-Cy7	7800	136	10	57.4
CD3 APC-Cy7	7629	120	20	63.6
CD3 APC-Cy7	7049	93.8	40	75.1
CD3 APC-Cy7	6474	84.8	80	76.3
CD3 APC-Cy7	5737	75.8	160	75.7

Table 3A: CD4 antibody titration dilution values.

Antibody + fluorophore	Median CD4 positive	Median CD4 negative	Dilution	Ratio pos/neg
CD4 PerCP-Cy55	13701	240	10	57.1
CD4 PerCP-Cy55	14119	196	20	72.0
CD4 PerCP-Cy55	14250	169	40	84.3
CD4 PerCP-Cy55	13956	153	80	91.2
CD4 PerCP-Cy55	11377	140	160	81.3

Table 4A: CD8 antibody titration dilution values.

Antibody + fluorophore	Median CD8 positive	Median CD8 negative	Dilution	Ratio pos/neg
CD8 PE-Cy7	32486	82.2	10	395.2
CD8 PE-Cy7	44764	74.5	20	600.9
CD8 PE-Cy7	48021	66.8	40	718.9
CD8 PE-Cy7	39765	61.6	80	645.5
CD8 PE-Cy7	27410	50.1	160	547.1

Table 5A: CD56 antibody titration dilution values.

Antibody + fluorophore	Median CD56 positive	Median CD56 negative	Dilution	Ratio pos/neg
CD56 APC	590	93.8	10	6.3
CD56 APC	642	86.1	20	7.5
CD56 APC	839	71.9	40	11.7
CD56 APC	918	62.9	80	14.6
CD56 APC	793	66.8	160	11.9

Appendix IV

FACS data

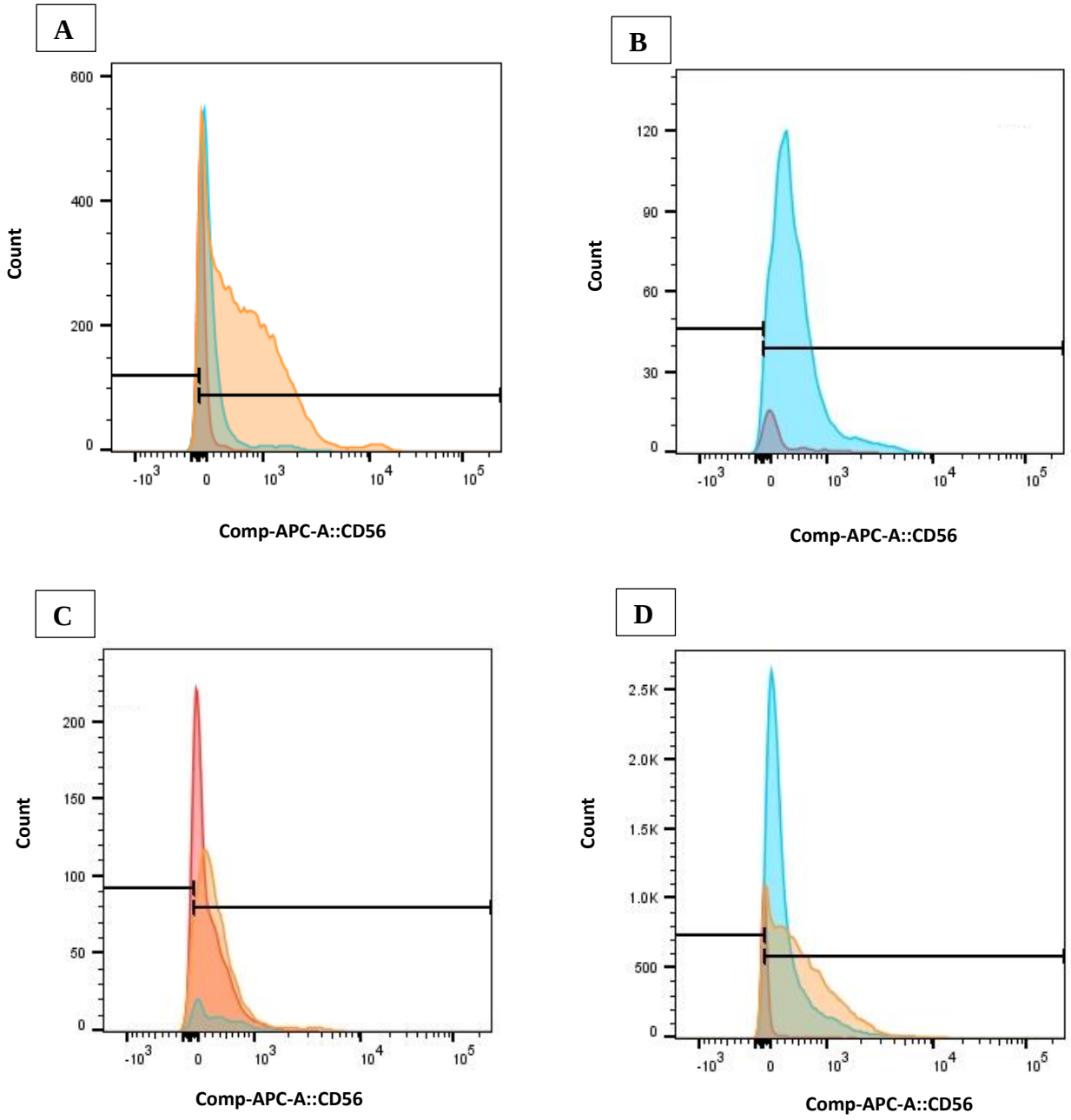

Figure 1A: Graphs showing the overlay of the NK cell counts within each population. Orange, NK cells; Blue, non-NK fraction; Red, PBMCs. (A) sample 1, (B) sample 2 , (C) sample 4 , (D) sample 6.

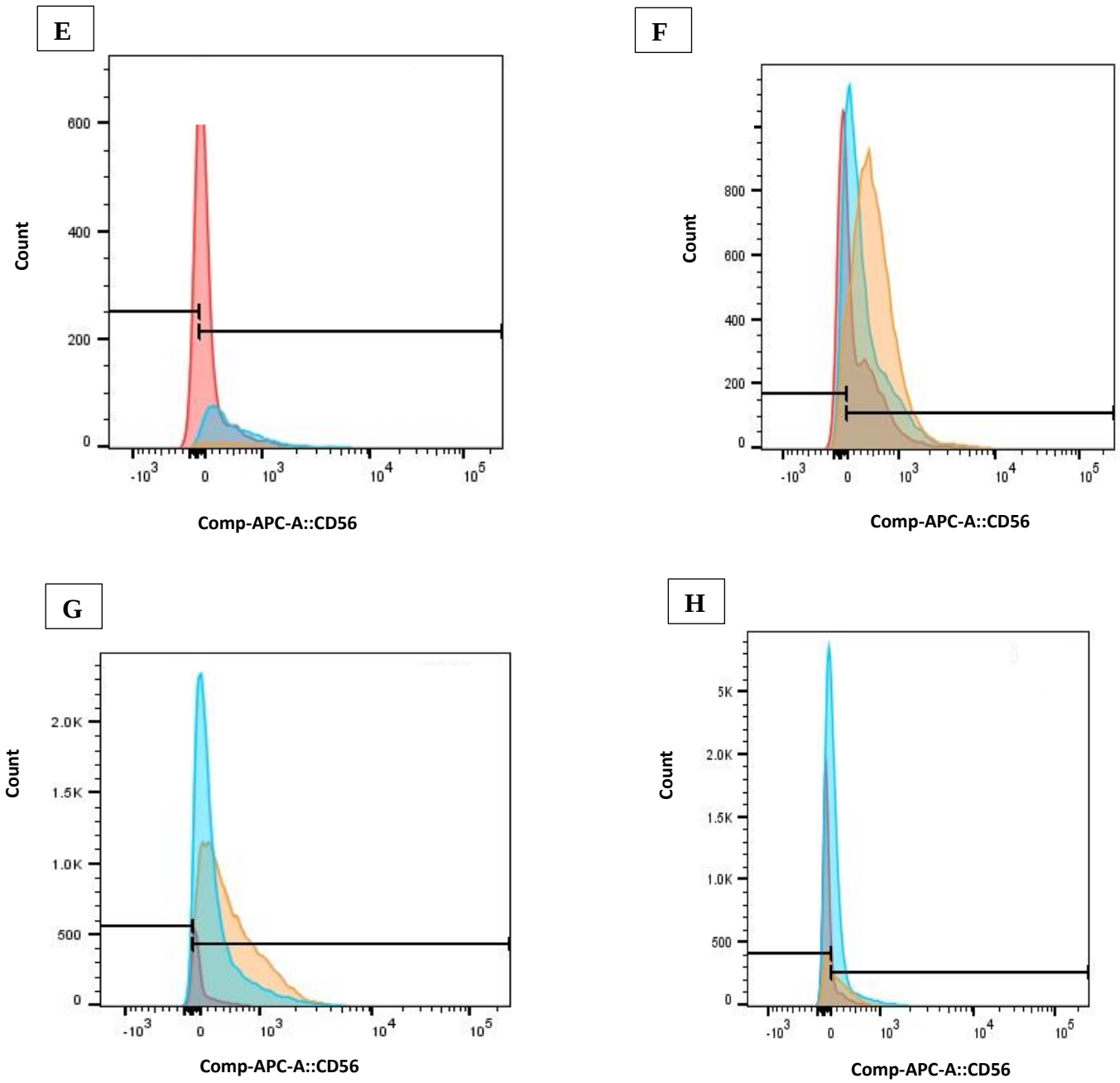

Figure 1A continued: Graphs showing the overlay of the NK cell counts within each population. Orange, NK cells; Blue, non-NK fraction, Red, PBMCs. (E) sample 7, (F) sample 8, (G) sample 9, (H) sample 10.

Table 6A: The frequency of the $CD3^-$ subset for each sample are indicated in the last column. X indicates that data are missing (cells not present).

Sample name	Subset Name	Count	Frequency (%)
Sample 1_NK cells	$CD3^-$	18219	74.6%
Sample 1_Non-NK fraction	$CD3^-$	6681	25.3%
Sample 1_PBMCs	$CD3^-$	3309	14.6%
Sample 2_NK cells	$CD3^-$	3383	86.3%
Sample 2_Non-NK fraction	$CD3^-$	303	87.1%
Sample 2_PBMCs	$CD3^-$	x	x
Sample 3_NK cells	$CD3^-$	2290	70.8%
Sample 3_Non-NK fraction	$CD3^-$	819	98.6%
Sample 3_PBMCs	$CD3^-$	26080	27.2%
Sample 4_NK cells	$CD3^-$	3119	39.6%
Sample 4_Non-NK fraction	$CD3^-$	527	82.1%
Sample 4_PBMCs	$CD3^-$	3310	14.3%
Sample 5_NK cells	$CD3^-$	3305	73%
Sample 5_Non-NK fraction	$CD3^-$	16139	67.4%
Sample 5_PBMCs	$CD3^-$	17742	43.1%
Sample 6_NK cells	$CD3^-$	40219	85.6%
Sample 6_Non-NK fraction	$CD3^-$	44491	33.6%
Sample 6_PBMCs	$CD3^-$	6636	33.1%
Sample 7_NK cells	$CD3^-$	544	50.6%
Sample 7_Non-NK fraction	$CD3^-$	2639	62.5%
Sample 7_PBMCs	$CD3^-$	9155	14.3%
Sample 8_NK cells	$CD3^-$	32073	68.9%
Sample 8_Non-NK fraction	$CD3^-$	26748	67.1%
Sample 8_PBMCs	$CD3^-$	18800	46.4%
Sample 9_NK cells	$CD3^-$	41136	87.6%
Sample 9_Non-NK fraction	$CD3^-$	40226	91.5%

Sample 9_PBMCs	$CD3^-$	4714	21.8%
Sample 10_NK cells	$CD3^-$	7171	60.7%
Sample 10_Non-NK fraction	$CD3^-$	32645	69.3%
Sample 10_PBMCs	$CD3^-$	13066	36.3%

Appendix V

Normal probability plots

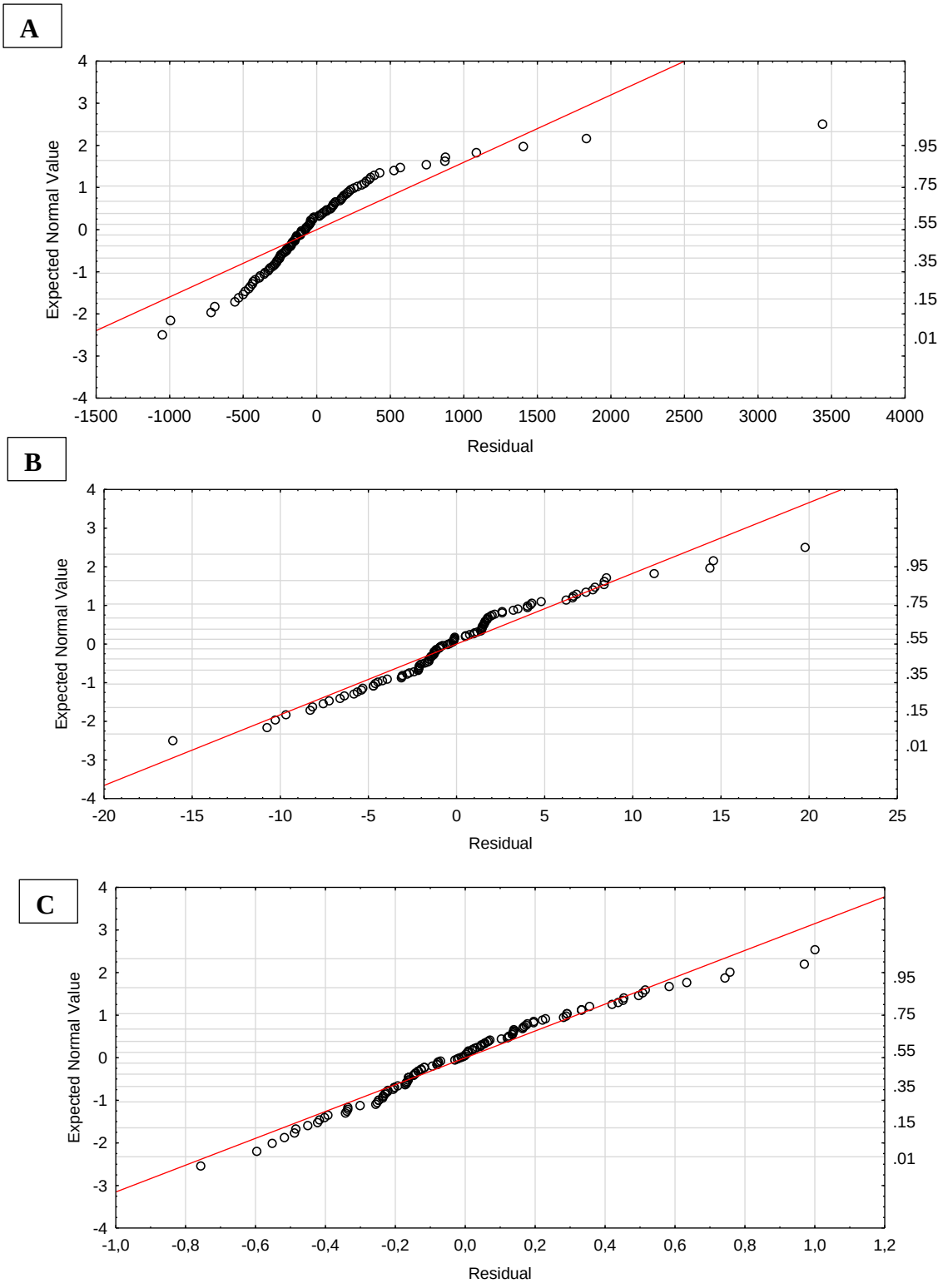

Figure 2A: Normal probability plot. (A) RANTES (B) Perforin (C) IL-17A.

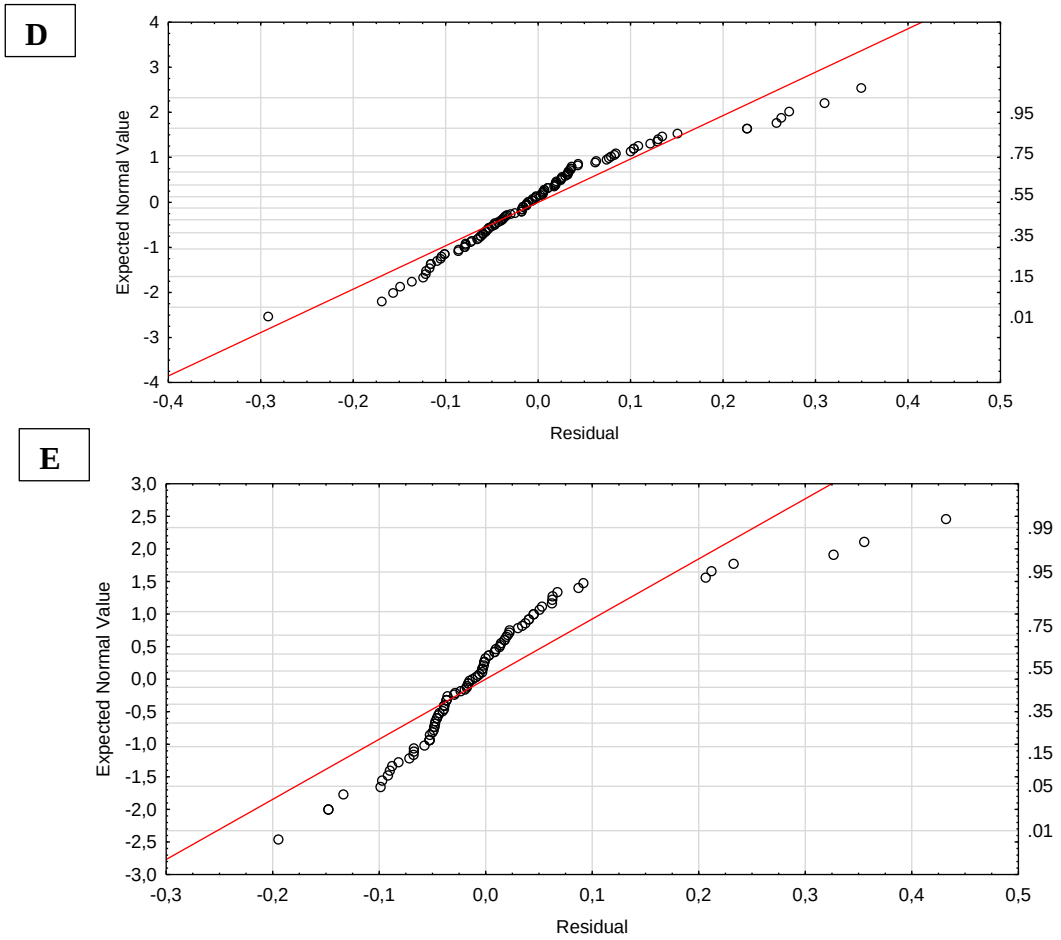

Figure 2A continued: Normal probability plot. (D) Log- 10 of IFN-γ, (E) Granzyme B, outlier is removed.

Appendix VI
List of suppliers

Reagents	
EDTA	Thermo Fisher Scientific
HCl	Sigma-Aldrich
Agarose	Sigma-Aldrich
Cresol loading dye	Sigma-Aldrich
Cryovials	Merck
PBS	Sigma-Aldrich
DMSO	Sigma-Aldrich
FBS	Sigma-Aldrich
Formaldehyde	Sigma-Aldrich
RPMI-1640	LONZA Group
PEN-STREP	Thermo Fisher Scientific
hIL-2	Merck
Middelbrook 7H9	Becton Dickson
Middelbrook 7H11	Becton Dickson
Glycerol	Sigma-Aldrich
OADC	BD Bioscience
Ficoll-plaque plus	Sigma-Aldrich
Trypan Blue	Thermo Fisher Scientific
Dnase	Thermo Fisher Scientific
Universal DNA ladder	Kapa Biosystems
Kappa Taq readymix	Kapa Biosystems
Ethidium bromide	Sigma-Aldrich

Kits	
EasySep™Human NK Cell Isolation kit	Stemcell technologies
Nucleon BACC3™ Genomi DNA extraction kit	GE Healthcare
Human CD8+ T-cell Magnetic bead Panel	EMD Millipore
Human Cytokine/chemokine bead Panel	EMD Millipore

Antibodies	
CD3 APC-Cy7	BioLegend
CD4 PerCP-Cy5.5	BioLegend
CD8 PE-Cy7	BioLegend
CD66 APC	BioLegend

Equipment

0.2µm filters	Thermo fisher scientific
1mL/cc insulin syringe	Supra
24 well plates	Whitehead Scientific
37°C Incubator	Merret
4 Objective Light Microscope	LW Scientific
5% CO2, 37°C shell lab Incubator	Sheldon manufacturing
5µm filters	Milex
96 well plates	Greiner Bio-one
Automatic plate washer	Bio-Rad
Centrifuge 5810R	Eppendorf
Consort electrophoresis power supply ps500xt	Hoffer scientific instruments
Countess automated cell counter	Thermo Fisher scientific
Countess loading plates	Thermo Fisher scientific
Dry block heater	Thermo Fischer Scientific
EDTA tubes	Kendon Medical supplies
Eppendorf tubes (1mL, 2mL)	Sigma-Aldrich
FACS tubes	The Scientific group
FACSCANTO II	BD Biosciences
G-box	Syngene
Gel casting tray	Bio-Rad
Gloves	Avacare
Half 90mm petri dishes	Thermo Fisher scientific
Hypodermic Needles 21GX1	Avacare
Imperial III water bath	Imperial Scientific works
Magnet	Stemcell technology
Magnetic stirrer	Velp Scientific
Micropore tape	Clicks pharmacy
Microscope	Nikon instruments Inc
Mr frosty	Thermo fisher scientific
Nanodrop ND-1000	Inqaba Biotechnology
Neubour haemocytometer	Celeromics
OD reader	WPA Biowave
Orbital Shaker incubator LM-530	Yih Der
Parafilm	Sigma-Aldrich
Pipette tips filtered (10µL,20µL 200µL, 1000µL)	Trefflab
Pipettes (10µL, 20µL 200µL, 1000µL)	Trefflab
Sonicator	Branson
Spinning wheel	Eppendorf
Syringes	Avacare
Titer Plate Shaker	Eppendorf
Vortex whirl Mixer	Stuart equipment

Programs

FlowJo®	*https://www.flowjo.com/*
R version 3.2.4	*https://cran.r-project.org/bin/windows/base/*
nmle version 3.1-131	*https://cran.r-project.org/package=nlme*
Statistica	*http://statistica.io/*

Appendix VII

KIR genotypes

Table 7A: The a*KIR* genes that were absent/present. The last column indicates the frequencies of observing the amount of aKIRs.

Sample	*2DL4*	*2DS1*	*2DS2*	*2DS3*	*2DS4*	*2DS5*	*3DS1*	Total	Frequency (%)
Sample 2	yes	no	no	no	no	no	no	**1**	**20**
Sample 3	yes	no	no	no	no	no	no	**1**	
Sample 4	yes	no	no	no	no	no	no	**1**	
Sample 13	no	no	no	no	yes	no	no	**1**	
Sample 14	no	no	no	no	yes	no	no	**1**	
Sample 15	no	no	no	no	yes	no	no	**1**	
Sample 1	yes	no	no	no	no	yes	no	**2**	**27**
Sample 5	yes	no	no	no	yes	no	no	**2**	
Sample 11	yes	no	no	no	yes	no	no	**2**	
Sample 16	yes	no	no	no	yes	no	no	**2**	
Sample 17	yes	no	no	no	yes	no	no	**2**	
Sample 19	yes	no	no	no	yes	no	no	**2**	
Sample 26	yes	no	no	no	yes	no	no	**2**	
Sample 28	yes	no	no	no	yes	no	no	**2**	
Sample 12	yes	no	yes	yes	no	no	no	**3**	**27**
Sample 20	yes	no	yes	no	yes	no	no	**3**	
Sample 21	no	no	no	yes	yes	no	yes	**3**	
Sample 22	yes	no	yes	no	yes	no	no	**3**	
Sample 23	yes	no	no	no	yes	no	no	**3**	
Sample 24	yes	no	yes	no	yes	no	no	**3**	
Sample 27	yes	no	yes	no	yes	no	no	**3**	
Sample 29	yes	no	no	no	yes	yes	no	**3**	
Sample 18	yes	no	yes	yes	yes	no	no	**4**	**10**
Sample 25	yes	no	no	no	yes	yes	yes	**4**	
Sample 30	yes	no	no	no	yes	yes	yes	**4**	
Sample 6	yes	no	yes	yes	yes	no	yes	**5**	**17**
Sample 8	yes	no	yes	yes	no	yes	yes	**5**	
Sample 9	yes	no	yes	no	yes	yes	yes	**5**	
Sample 10	yes	no	yes	no	yes	yes	yes	**5**	
Sample 7	yes	yes	no	yes	yes	yes	yes	**6**	

Table 8A: The total count and frequencies of each gene observed within the cohort (n = 30).

Gene	Absent	Present	Frequency (%)
KIR2DL4	4	26	87
KIR2DS1	29	1	3
KIR2DS2	20	10	33
KIR2DS3	24	6	20
KIR2DS4	6	24	80
KIR2DS5	22	8	27
KIR3DS1	22	8	27

Table 9A: *KIR* gene profiles of the 30 individuals.

Sample	Haplotype	*2DL4*	*2DS1*	*2DS2*	*2DS3*	*2DS4*	*2DS5*	*3DS1*	Total *aKIR*s	Frequency (%)
Sample 2	AA	■							**1**	
Sample 3	AA	■							**1**	**10**
Sample 4	AA	■							**1**	
Sample 13	AA					■			**1**	
Sample 14	AA					■			**1**	**10**
Sample 15	AA					■			**1**	
Sample 1	Bx	■					■		**2**	**3**
Sample 5	Bx	■				■			**2**	
Sample 11	Bx	■				■			**2**	
Sample 16	Bx	■				■			**2**	**23**
Sample 17	Bx	■				■			**2**	
Sample 19	Bx	■				■			**2**	
Sample 26	Bx	■				■			**2**	
Sample 28	Bx	■				■			**2**	
Sample 12	Bx	■		■	■				**3**	**3**
Sample 21	Bx				■	■		■	**3**	**3**
Sample 20	Bx	■		■		■			**3**	
Sample 22	Bx	■		■		■			**3**	**13**
Sample 24	Bx	■		■		■			**3**	
Sample 27	Bx	■		■		■			**3**	
Sample 23	Bx	■				■			**3**	**3**
Sample 29	Bx	■				■	■		**3**	**3**
Sample 18	Bx	■		■	■	■			**4**	**3**
Sample 25	Bx	■				■	■	■	**4**	**7**
Sample 30	Bx	■				■	■	■	**4**	
Sample 6	Bx	■		■	■	■		■	**5**	**3**
Sample 8	Bx	■		■	■		■	■	**5**	**3**
Sample 9	Bx	■		■		■	■	■	**5**	**7**
Sample 10	Bx	■		■		■	■	■	**5**	
Sample 7	Bx	■	■		■	■	■	■	**6**	**3**

*black blocks indicate the gene is present, white blocks indicate gene is absent. Our *KIR* genotype designations are in concordance with that of the Allele Frequency Net Database (AFND; http://www.allellefrequencies.net)[229]