Mechanisms of natural killer cell activation by *Plasmodium falciparum*-infected erythrocytes and influenza vaccine

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I, Samuel Roy Sherratt, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Natural Killer (NK) cells are immune effector cells capable of responding to infected cells through cytotoxic activity and production of pro-inflammatory cytokines. In recent years there has been increasing interest in studying how vaccine- or pathogen-induced antibodies promote NK cell responses to pathogens through the immunoglobulin G receptor CD16.

The initial work described in this thesis uses imaging flow cytometry to examine the ability of human NK cells to interact with erythrocytes parasitised with the human malaria parasite *Plasmodium falciparum* (PRBCs). Although several limitations of this technology became apparent, I was able to establish that conjugates between NK cells and PRBCs form within 30 minutes of co-incubation and persist for up to 3 hours. PRBCs were observed forming conjugates with both $CD56^{bright}$ and $CD56^{dim}CD57^{+}$ NK cells.

The role of antibody in promoting NK cell responses via CD16 was then assessed in two models. Firstly, an *in vitro* model using NK cells and plasma from individuals vaccinated with trivalent influenza vaccine was used to establish assay conditions and to investigate the mechanisms by which CD16 is downregulated after antibody-dependent activation. In this model, I have shown that influenza antigen-antibody complexes are able to induce downregulation of CD16 on NK cells, resulting in degranulation. These antibody dependent functions are more potent in highly differentiated $CD56^{dim}CD57^{+}$ NK cells. Using imaging flow cytometry, I have also shown that downregulation of CD16 after ligation is due to shedding of the molecule from the surface of the NK cell, rather than through a process of internalisation.

Secondly, I established an *in vitro* system to investigate the impact of antibody on NK cell responses to PRBCs in Gambian and UK resident individuals. This study also assessed the contributions of different NK cell differentiation subsets to these responses. I have shown that NK cells are able to mount a robust antibody-dependent response to PRBCs, involving production of IFN-γ and degranulation. I have also shown that NK cells vary in their responsiveness to antibody stimulation, with 'adaptive' $FcεRIγ^{-}$ and 'differentiated' $CD57^{+}$ NK cells demonstrating enhanced responses to anti-malarial antibodies. Finally, I have shown that the cytokines interleukin 12 and interleukin 18 contribute to the anti-malarial antibody-dependent response in certain NK cell subsets, as does ligation of CD2 on the NK cell.

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CHAPTER 1

Introduction

1.1 Basic biology of the natural killer cell

Natural killer (or NK) cells are large granular lymphocytes traditionally considered to be a bridge between the innate and adaptive immune systems. Although a product of the lymphocytic progenitor pathway [1], unlike other lymphocytic cells such as T-cells, NK cells possess an innate capacity to respond to cancerous or infected host-cells. This activity is mediated through a large number of germline-encoded receptors capable of recognising pro-inflammatory molecules and changes in the expression of ligands associated with infection or cellular dysfunction (reviewed in [2]).

In humans, NK cells typically constitute 5-15% of peripheral blood mononuclear cells (PBMCs).[3]. They are typically characterised as cells expressing the surface marker CD56 (albeit to varying degrees depending on the NK cell subset) whilst lacking the T-cell co-receptor CD3. Generic human NK cells are thus phenotypically classified as CD56+ CD3- cells. Within this basic subset there is considerable diversity in both the receptors NK cells express and their consequent immunoreactivity and function, as will be discussed in more detail over the following sections (reviewed in [4]).

1.2 Natural killer cell functions

NK cells are responsible for responding to a wide range of dysfunctional and infectious cellular targets, including cancer cells [5], cells infected with intracellular bacteria such as *Salmonella* [6], and cells infected with viruses such as Human Cytomegalovirus (HCMV) [7]. There is also increasing evidence that NK cells can respond through a range of mechanisms to host cells infected with intracellular parasites such as malaria, as will be discussed later in greater detail (reviewed in [4]).

The function of NK cells in instances of infection or cancer can be broadly broken down into two categories; production of pro-inflammatory cytokines to upregulate immune responses indirectly, and direct killing of target cells through cytotoxic activity. These

functions can be triggered by a range of direct and indirect mechanisms, explored in detail in the following sections.

1.2.1 Cytotoxic activity and killing of cellular targets

The titular function of NK cells is to kill infected or cancerous cellular targets through cytotoxic activity. This requires physical contact between the NK cell and its target, leading to a multi-stage killing process.

Initial contacts between NK cells and potential targets are mediated by NK-expressed integrins such as lymphocyte function-associated antigen 1 (LFA1) and macrophage-1 antigen (MAC1), which cluster at sites of contact [8]. These receptors have a large number of known ligands, though the most relevant for adhesive purposes appear to be the intercellular adhesion molecule family, or ICAMs (reviewed in [9]). After making initial adhesive contacts, a balance of activating and inhibitory signals will determine whether the NK cell choose to attack the bound target cell. The receptors that mediate this will be discussed in detail in sections 1.2.1.1 and 1.2.1.4.

If the NK cell recognises a target cell as something it should attack, preliminary cellular contacts are reinforced as NK cells begin to form a tight ‘immunological synapse’ with the target. The immunological synapse consists of two distinct zones of receptors that polarise towards the site of contact; an outer ring of adhesion molecules (including LFA-1 and MAC1) known as the peripheral supramolecular activation cluster (pSMAC), and a central zone of activating receptors known as the central supramolecular activation cluster (cSMAC) [10]. This process is mediated by formation of F-actin filaments within the cell from a pool of monomeric G-actin which reorganise the NK membrane, increasing the surface area of the cellular contact [11]. Once this synapse is fully formed, the intracellular microtubule-organising center (MTOC) gravitates towards the site of contact, forming a microtubule network throughout the cell which serves to guides cytotoxic granules towards the contact site [12]. These granules contain cytotoxic mediators such as perforin and granzymes A and B. Once released, perforin proteins insert themselves into the membrane of the target cell, forming a larger pore structure consisting of multiple perforin subunits [13]. Granzyme A and B then enter the target cell through this pore, inducing cell death through lysis of cytoskeletal and intracellular proteins leading to rapid cell death [14].

NK cells are also capable of expressing so called ‘death’ ligands, such as Fas ligand (FasL) and TNF-related apoptotic inducing ligand (TRAIL) [15]. These ligands bind Fas and TRAIL receptors commonly expressed on target cell surfaces, inducing cell death

through activation of the caspase cascade. Expression of these ligands is upregulated in response to signals sent by NK activating receptors such as CD16 and LFA-1, leading to enhanced cytotoxicity [16].

NK cytotoxic functions can be mediated through three primary mechanisms; antibody-independent cellular cytotoxicity mediated through a wide range of germline-encoded activating and inhibitory receptors, antibody-dependent-cellular cytotoxicity (ADCC) utilising B-cell produced immunoglobulin G which binds to the NK immunoglobulin receptor CD16, and exposure to various pro-inflammatory cytokines which upregulate NK cell cytotoxic functions by binding to cytokine receptors (Fig. 1.3). These mechanisms of activation will be explored in more detail in the following sections.

1.2.1.1 'Missing self' and cytotoxic activity in response to innate receptors

During antibody-independent cytoxicity, NK cells initially recognise suitable tumour cells or infected host cells through innate germline-encoded receptors that 'read' the surface of the intended target (reviewed in [2]). These receptors can be either activating or inhibitory, and bind a vast range of both host and pathogen-derived ligands (Fig. 1.2).

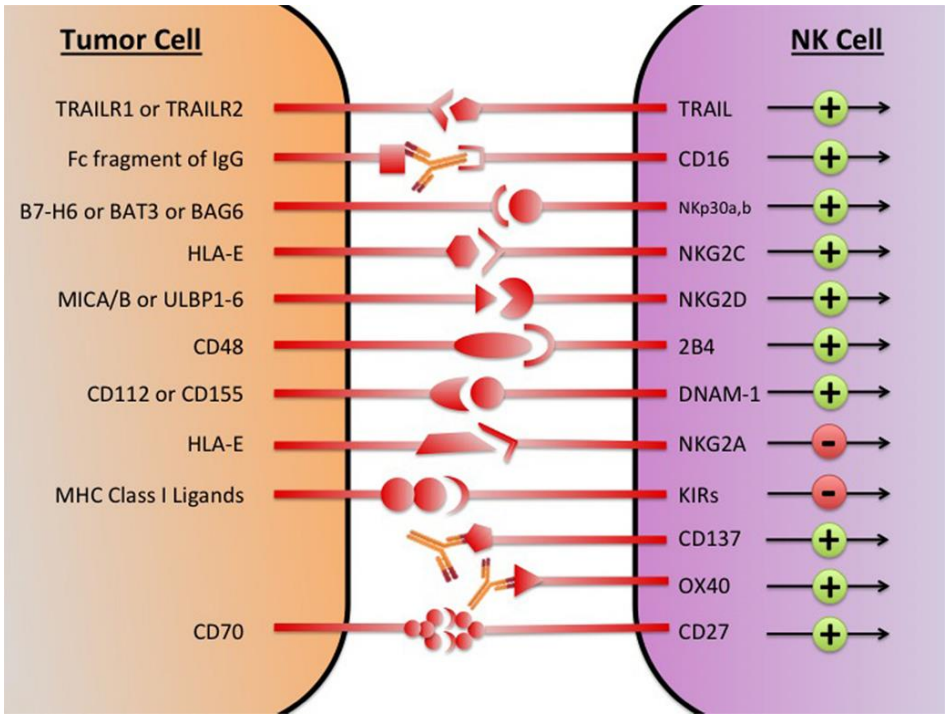


Figure 1.2 Examples of m**ajor NK cell receptors and their ligands**
NK cells are capable of degranulating in response to infected or cancerous cells, releasing cytotoxic mediators such as perforin and granzymes. The transition of the NK cell to an activated state is mediated by a wide range of activating and inhibitory receptors. The balance of activating versus inhibitory signals determines if the NK cell becomes cytotoxic. Figure republished in accordance with the Creative Commons attribution licence from Chester et al (2015) *Frontiers in Immunology* [245]

Many NK receptors interact with 'classical' Major Histocompatibility Class 1 molecules (MHC-I) that are constitutively expressed on almost all human cell types [17]. Classical MHC-I molecules are composed of three alpha chain subunits in association with β2-microglobulin. Under homeostatic conditions their function is to bind host-derived or 'self' peptides produced through proteolytic processes in the cell and present them to CD8+ cytotoxic T-cells via the T-cell receptor (TCR) [18]. If the proteins are recognised as being 'self', T-cells are not activated. In the event a cell is infected or cancerous, pathogen-derived or cancerous mutant proteins will be presented by classical MHC-I molecules instead, leading to activation of CD8+ T-cells with complementary TCRs and subsequent killing of the afflicted cell (reviewed in [19]).

The expression of classical MHC-I molecules is often downregulated by host cells during infection or cancer leading to evasion from killing by cytotoxic T cells [20,21]. NK cells express a range of receptors that bind classical MHC-I, many of which provide inhibitory signals to the NK cell. In the event classical MHC-I expression is lost during infection or cancer, NK cells become activated, acting as a secondary safeguard. This concept is generally known as the 'Missing Self' hypothesis. Host cells which are not recognised as being immunologically healthy or 'self' due to the downregulation of MHC-I are considered dangerous and a valid target for NK cell cytotoxic attack [22].

Killer Immunoglobulin-Like Receptor family (or KIRs) represent the principal family of NK receptors which detect classical MHC-I on target cells. The KIR receptors have either two or three extracellular immunoglobulin domains, called 2D or 3D, and either a long (L) or a short (S) intracellular domain [23]. The KIR family is large and polymorphic, currently consisting of 15 expressed receptors with both inhibitory and activating members. These receptors utilise intracellular immunoreceptor tyrosine-based inhibitory or activation motifs (ITIM or ITAM) to send either inhibitory or activating signals on recognition of their respective MHC-I ligands, though most are inhibitory (reviewed in [24]). KIR family members with established HLA ligands bind classical MHC-I molecules such as HLA-A, HLA-B or HLA-C, although several recognise non-classical MHC-I molecules, including KIR 2DL4 and KIR3DS1, which bind HLA-G and HLA-F respectively. Additionally, the ligands for several other KIR are currently unknown (reviewed in [25]). In the case of most KIRs which bind classical HLA, the loss of this molecule on targets due to cellular stress leads to downregulation of inhibitory signals, skewing the NK cell towards activation (reviewed in [26]).

In recent years KIRs have also been found to play a role in so-called NK cell 'education', or 'licensing'. Developing NK cells expressing inhibitory KIRs for self HLA demonstrate

enhanced responsiveness (both degranulation and cytokine production) to other activating signals, whereas NK cells lacking inhibitory KIR signals or expressing activating KIR signals for self-HLA become hyporesponsive. This process is believed to allow NK cells to develop a rudimentary form of self-tolerance, preventing excessive cytotoxic activities from being directed towards other self-cells [27].

KIR proteins exhibit considerable diversity between individuals due to a predilection for individual variation in the content and copy number of genes carried, as well as frequent allelic polymorphisms. They consequently assemble into a diverse range of haplotypes that are broadly designated into classes A and B. Both haplotypes consists of a basic genetic framework containing the genes KIR2DL4, KIR3DL2, KIR3DL3 and the pseudogene KIR3DP1, but vary in the other genes carried [28]. Group B haplotypes are characterised by the presence of one or more of the KIR genes KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5 and KIR3DS1 [23]. Group A haplotypes lack these genes. Many group B haplotype genes are activating, and as a result B haplotypes generally have more genes encoding activating KIR than A haplotypes (reviewed in [29]).

In addition to expressing receptors that recognise classical HLA molecules, NK cells also express receptors targeting several ‘non-classical’ HLA molecules. Non-classical MHC-I molecules share a certain level of structural homology with the classical molecules but differ both in their mechanism of action and in the receptors they interact with. Examples include the molecule HLA-E, which binds signal peptides derived from classical HLA molecules and acts as a ligand for members of the NKG2 family of C-type lectins [30]. These include NKG2C; an activating NK receptor that dimerises with the transmembrane glycoprotein CD94, and NKG2A; an inhibitory receptor that also dimerises with CD94 and provides overriding signals to prevent activation through NKG2C [31]. A related receptor,NKG2D, forms an activating homodimer that binds a diverse range of HLA homologues such as MICA and MICB that are overexpressed on stressed cells (reviewed in [32]).

However, loss of inhibition associated with the downregulation of HLA is itself not sufficient to drive NK cell activation, NK cells also express a range of receptors which provide the necessary activating signals through direct targeting of pathogenic ligands or host stress-induced molecules. These include the Natural Cytotoxicity Receptor family (NCRs); consisting of NKp30, NKp44 and NKp46, which bind a broad spectrum of ligands, including viral and bacterial molecules, in addition to host cellular ligands expressed in response to cellular stress (reviewed in [4]). Specific examples include binding of viral haemagglutinins expressed by viruses such as influenza and vaccinia

to NKp30 and NKp46 [33,34]; binding of mycobacterium cell wall components to NKp44 [35]; and binding of host molecules such as heparin sulphate and HLA-B-associated transcript 3 (BAT3) by NKp30 and NKp46 [36,37]. Additional research has also suggested that the NCRs may be involved in killing of intracellular parasites such as *Plasmodium falciparum*, with at least one study suggesting that NKp30 and NKp46 may also bind Duffy binding-like (DBL) domain of *P. falciparum* erythrocyte membrane protein-1 (PfEMP–1), expressed on parasitized erythrocytes [38].

Additionally, activating adhesion molecules such as LFA-1 and the costimulatory-receptor DNAM-1 (CD226) have been shown to play a role in mediating NK cell interactions with both cellular models and pathogenic targets such as *Plasmodium falciparum*-infected erythrocytes, as discussed later in more detail [39], [40].

While most activating germline activating receptors such as the NCRs and NKG2D are expressed on the majority of NK cells to some degree (though expression may vary between NK differentiation subsets within a single individual), others including NKG2C and the activating KIR exhibit considerable heterogeneity in their expression due to genetic differences between individuals. NKG2C expression is known to be reduced or even absent in some individuals due to a relatively common ~16kb deletion mutation present in approximately 20% of the global population. Consequently around 4% of individuals are homozygous negative for the *NKG2C* gene with a corresponding absence of surface expression. [41].

1.2.1.2 Cytotoxic activity in response to cytokine stimulation

Cytotoxic activity by NK cells can also be upregulated by exposure to pro-inflammatory cytokines produced or presented by a range of accessory peripheral blood mononuclear cells (PBMCs).

In viral infections plasmacytoid and myeloid dendritic cells can be activated by binding of their Pattern-Recognition Receptors (PRRs) to viral molecules such as nucleic acids, causing them to produce Type I interferons including interferons α and β (IFN-α and IFN-β) (reviewed in [42]). These interferons act to further activate plasmacytoid dendritic cells in a positive feedback loop, but have also been demonstrated to regulate NK cell expansion and differentiation in response to virally-infected cells by binding to the interferon-α/β receptor [43]. Type I interferons have also been demonstrated to play a vital role in inducing NK cell cytotoxic responses against tumour cells [44], as well as intracellular parasites such as malaria in our own groups' published work[45].

Studies investigating the cytotoxic response of NK cells in both cancer and infection models have shown that pro-inflammatory interleukins such as IL-12 and IL-18 produced by accessory monocytes or myeloid DC can also significantly upregulate the degranulation of NK cells in response to tumours and intracellular pathogens. In models of metastatic melanoma IL-12 and IL-18 have been shown to significantly upregulate NK cell cytotoxic activity as well as expression of the IL-2 receptor CD25, suggesting a synergistic effect of these cytokines [46]. In studies investigating the interaction between NK cells and haematological tumour lines IL-12 has been demonstrated to upregulate expression of cytotoxic effector molecules such as TRAIL and perforin, as well as inducing phosphorylation of signalling molecules such as STAT1, STAT4, and ERK1/2, which regulate cytotoxic activity by NK cells [47]. In mice models of cytomegalovirus infection, binding of viral DNA to the dendritic PRR Toll-like Receptor 9 has been demonstrated to lead to production of IL-12 by dendritic cells, leading to enhanced NK cell responses [48]. Similarly, in in mouse models of herpes infection a deficiency in IL-18 has been associated with reduced NK cell cytolytic capacity and increased viral load [49]. In influenza models a deficiency in either IL-12 or IL-18 has also been associated with significantly reduced production of granzyme B, an essential cytotoxic mediator [50].

Production of interleukin IL-15 by both plasmacytoid and myeloid dendritic cells can also enhance NK cell cytotoxic responses. In parasitic models of infection, IL-15 has also been shown to upregulate NK cell meditated lysis of *Cryptosporidium*-infected epithelial cells due to upregulation of the activating NK receptor NKG2D, leading to enhanced parasite clearance [51]. NK cell cytotoxic activity has been shown to be upregulated against K562 tumour cell lines when cultured in contact with IL-15 producing dendritic cells, leading to significantly higher expression of co-stimulatory receptors such as CD25, NKG2D and NKp30 [52].

T-cell derived IL-2 has also been shown to upregulate NK cell cytotoxic activity in both cancer and infection models. In *in vivo* models of lymphoma, supplementation with IL-2 has been shown to restore NK cell cytotoxic activity in NK cells suffering from loss-of-function mutations [53]. In models of cytomegalovirus infection T-cell derived IL-2 has also been shown to significantly upregulate NK cell responses to virally-infected macrophages [54]. In *in vitro* models of *Plasmodium falciparum* infection exogenous IL-2 has also been shown to upregulate cytotoxic responses against parasitized erythrocytes [55]. As with IL-12 and IL-18 the effects of IL-2 on NK cells appear to synergise with other cytokines, as studies have shown that IL-2 enhances the response to IL-12 through upregulation of the IL-12 receptor and associated intracellular signalling molecules such as STAT4 [56].

1.2.1.3 Cytotoxic activity in response to physical contact with accessory cells

Physical contact between NK cells and accessory cells such as macrophages and dendritic cells is required to present monocyte-derived cytokines to NK cells and induce optimal IFN-γ production. Optimal IL-12 release by peripheral blood myeloid dendritic cells appears to require the formation of an immunological synapse between activated dendritic cells and NK cells, while optimal 'priming' of NK cells by IL-15 requires trans-presentation of the cytokine to the NK cell via the IL-15 receptor alpha chain expressed on the surface of dendritic cells [57,58], [59]. These cytokines have also been demonstrated to play a vital role in upregulating NK cell cytotoxic responses, indicating that physical contact between NK cells and myeloid accessory cells also leads to enhanced cytotoxicity.

Studies have shown NK cell cytotoxic activity against tumour cell lines is enhanced when cultured in contact with IL-15 producing dendritic cells, suggesting upregulation of NK cytotoxic responses by IL-15 may be dependent on physical trans-presentation of the cytokine via the IL-15 alpha chain [52]. Additional tumour studies have shown that presentation of IL-12 by accessory monocytes significantly upregulates expression of cytotoxic mediators such as perforin, and that this process appears to be partially dependent on physical interactions between NK cells and monocytes [47]. In mouse models Il-15, IL-18 and IFN-β produced by macrophages has also been shown to upregulate NK cell cytotoxicity directed against target cells in a process that is partially contact-dependent, though soluble versions of these cytokines also appear to have some effect [60].

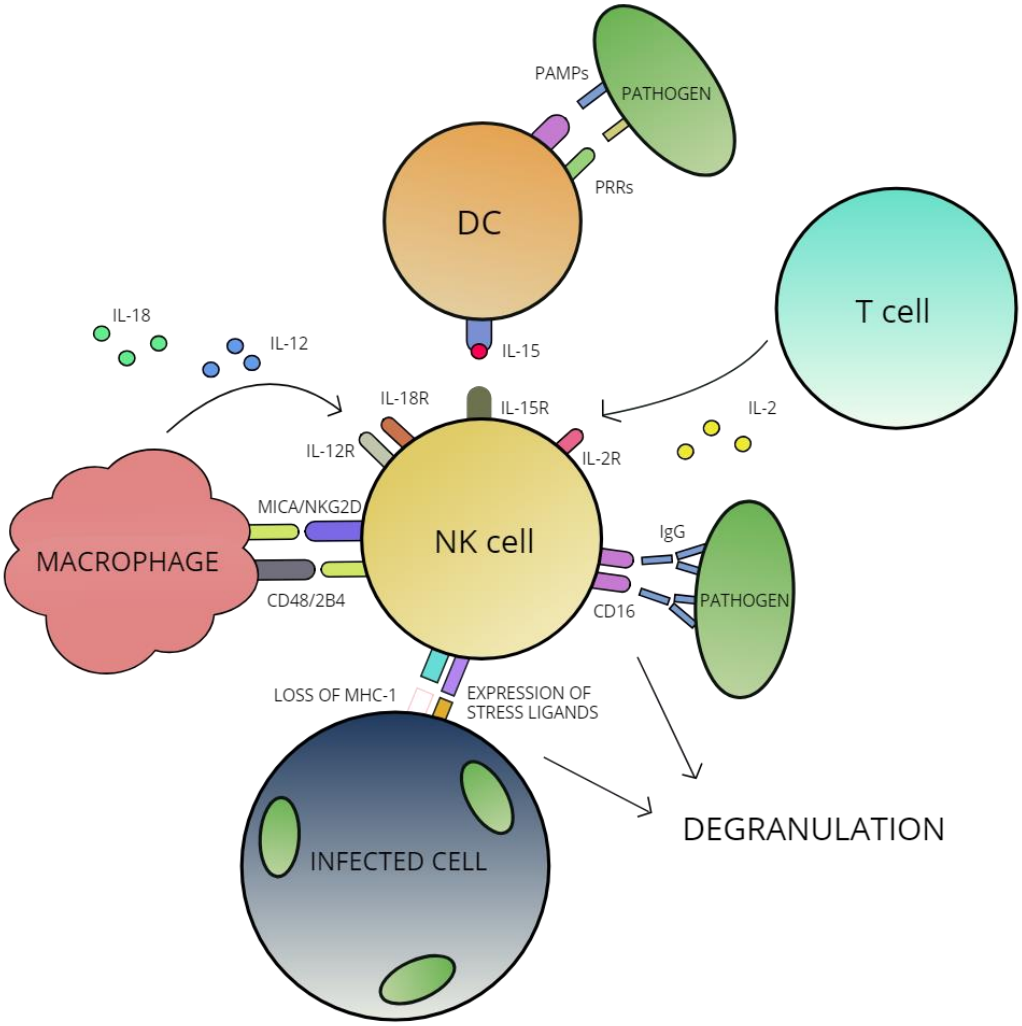
In addition to optimising the presentation of pro-inflammatory cytokines, physical contact between NK cells and accessory monocytes can lead to direct interactions between NK receptors and activating ligands, leading to enhanced cytotoxicity. In mouse models, macrophages can upregulate cytotoxic killing of tumour cell lines by NK cells by upregulating their own expression of HLA-like ligands for the NK activating receptor NKG2D [60]. Similar results have been found in human *in vitro* models, where increased expression of the NKG2D ligand MICA enhances the cytotoxicity of both NK and CD8+ T-cells [61]. Coincubation of NK cells with macrophages activated by low-dose LPS increased has also been shown to induce expression of NK cell activating receptors such as NKp46 as well as increase expression of cytotoxic molecules such as perforin via physical interactions between macrophage ligands and the NK cell receptor 2B4. This leads to enhanced killing of both tumour cell lines and virally-transformed target cells by NK cells [62].

Studies have also demonstrated the ability of dendritic cells to activate NK cell cytotoxic functions through physical contacts. In cancer models, binding between dendritic-expressed ligands and the NK receptor NKp30 has been shown to upregulate NK cell proliferation and killing of lymphoid tumour cell lines through cytotoxic activity [63]. Increased expression of MICA and MICB on dendritic cells in response to IFN-α also appears to upregulate NK cell killing of tumour cells lines by binding the NK activating receptor NKG2D [64]. Additional studies have shown that the adhesion molecule and activating receptor LFA-1 expressed on NK cells can also mediate interactions with dendritic cells, triggering the release of cytotoxic perforin and granzymes [65].

1.2.1.4 Cytotoxic activity in response to antibody-antigen complexes

In instances of antibody-dependent cellular cytotoxicity (ADCC), B-cell produced immunoglobulin G binds to pathogen- and cancer-specific epitopes, forming antibody-antigen complexes. The Fc region of bound IgG1 and IgG3 antibodies bind to the principal NK cell immunoglobulin receptor CD16 (also known as FcyRIIIa). When multiple antibodies bind CD16 receptors in close proximity on the NK cell surface, the CD16 receptors become 'cross-linked', sending activating signals through immunoreceptor tyrosine-based activation motifs (ITAMs) to either the CD3ζ and/or FcεRIγ adaptor proteins [66]. These adaptor proteins then transduce activating signals to the downstream cytosolic signalling pathway consisting of the classical SYK-family of tyrosine kinases [67], which ultimately trigger the release of lytic granules from the NK cell towards the opsonised cell target [68]. As will be discussed in more detail in section 1.2.2.3, this process can also induce the production of IFN-γ by the NK cell [69].

Antibody-dependent cellular cytotoxicity by NK cells has been shown to play a vital role in the immune response to both virally-infected cells and cancers [70,71]. Studies have demonstrated that these antibody-dependent cytotoxic responses can be upregulated by co-stimulation with pro-inflammatory cytokines such as IL-12 and also by CD4+ T cell-derived interleukin-2 (IL-2) [72]. Further information regarding antibody-dependent responses will be discussed in the section dealing with adaptive NK cell subsets.



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KEY

DC = dendritic cell

IgG = immunoglobulin G

PAMPs = pathogen-associated molecular patterns

PRRs = pattern recognition receptors

MHC-I = major histocompatibility complex class 1 molecules

CD48 = cluster of differentiation 48

2B4 = cluster of differentiation 244

CD16 = immunoglobulin G Fc domain receptor

MICA = MHC class I polypeptide-related sequence A

Figure 1.3 Natural killer cells degranulate in response to several forms of stimulation

NK cells are capable of degranulating in response to infection or cancer, subsequently releasing cytotoxic mediators such as perforin and granzymes which destroy dysfunctional or infected host cells. This process is regulated by a range of signals; including exposure to pro-inflammatory cytokines such as IL-2, IL-12, IL-15 and IL-18; direct contact with accessory PBMCs such as macrophages and monocytes through stress ligands such as MICA and MICB; binding of antibody-antigen complexes to the NK cell, leading to crosslinking of the IgG receptor CD16; and direct activation of the NK cell by infected or cancerous cells due to expression of stress ligands or downregulated expression of 'self' MHC-I (HLA) molecules.

1.2.2 Pro-inflammatory cytokine production and the importance of IFN-γ

A central role of NK cells in mounting an innate immune response to tumours and pathogens is the production of cytokines designed to upregulate the activity of other immune cells. Depending on the activating stimulus, these can include TNF-α, TNF-β, GM-CSF, IL-10 and IL-13 [97].

Also amongst the primary pro-inflammatory cytokines produced by activated NK cells is Interferon gamma (IFN-γ), currently the only known member of the type II class of interferons (reviewed in [73]). IFN-γ has been demonstrated to upregulate the activity of a range of immune cells in response to a variety of pathogens and cancers, and thus plays an important role in both the innate and adaptive immune responses.

The receptor for IFN-γ (IFN-γR) is widely expressed on a range of both immune and non-immune cells, though it is most prominently expressed on cells of myeloid lineage such as monocytes [74]. Once bound to its receptor, IFN-γ upregulates cellular activities such as phagocytosis, proliferation and pro-inflammatory functions through activation of the JAK-STAT pathway (reviewed in [75]). Specific examples include upregulating the production of bactericidal reactive oxygen species inside human mast cells in response to bacteria such as *Staphylococcus aureus* [76], activation of cytotoxic CD8+ T-cell responses to cancers such as intraocular tumours [77], and upregulation of cytokine production and antigen presentation by accessory cells such as macrophages [78].

NK cells are thought to be an important early source of IFN-γ in many infection models prior to the activation of an adaptive T-cell response [79], [80], [81]. In *in vitro* models of malaria infection for example, our own research group has demonstrated NK cells to be the primary source of IFN-γ during short-term incubation with malaria-infected cells when the T-cell response is minimal [82].

Production of IFN-γ by NK cells in instances of infection or cancer is regulated by a range of signals, including exposure to pro-inflammatory cytokines, direct receptor-mediated contact with activated accessory PBMCs such as macrophages and monocytes, and binding of antibody-antigen complexes to the NK cell (Fig. 1.1). These sources of stimulation will be explored in more detail in the following sections.

1.2.2.1 IFN-γ production in response to pro-inflammatory cytokines

Production of IFN-γ by NK cells in instances of infection or cancer is regulated by a range of signals, including exposure to a range of pro-inflammatory and regulatory cytokines (Table 1.1).

CYTOKINE	NK CYTOKINE RECEPTOR (SUBUNITS)	PRODUCED BY
Interleukin 1 (IL-1) [83]	IL-1R (IL-R1 and IL-1RAcP)	Multiple cell types
Interleukin 2 (IL-2) [84]	IL-2R (IL-2Rα, IL-2/IL-15Rβ and common γ_c chain)	T-cells, dendritic cells
Interleukin 12 (IL-12) [85]	IL-12R (IL-12Rβ1 and IL-12Rβ2)	Dendritic cells, macrophages
Interleukin 15 (IL-15) [86]	IL-15R (IL-15Rα, IL-2/IL-15Rβ and common γ_c chain)	Dendritic cells, macrophages
Interleukin 18 (IL-18) [87]	IL-18R (IL-18Rα and IL-18Rβ)	Dendritic cells, macrophages
Interleukin 21 (IL-21) [88]	IL-21R (IL-21Rα and common γ_c chain)	Regulatory T-cells
Type I Interferons e.g. IFNα, IFNβ [89]	IFNAR (IFNAR1 and IFNAR2)	Multiple cell types

Table 1.1 List of cytokines capable of activating NK cell activity and their primary cellular sources

The interleukins IL-12, IL-15 and IL-18 in particular have been shown to be an important activator of NK IFN-γ production in several in vitro models of innate immune activity [90], [91], [92]. These cytokines are produced primarily by monocytes, macrophages and dendritic cells (DC) that have been activated through direct recognition of pathogenic targets. Monocytes express innate pattern recognition receptors (PRRs) designed to detect extracellular and intracellular pathogen-associated molecular patterns (PAMPs). These PRRs include protein families such as the Toll-like receptors (TLRs) and C-type lectins (CLRs), which when bound to their cognate ligands induce activation of the monocyte and production of pro-inflammatory cytokines (reviewed in [93]). Specific examples include induction of IL-18 production in circulatory and liver resident macrophages when exposed to hepatitis C [94], and induced production of IL-12 in macrophages exposed *in vitro* to *Acanthamoeba castellanii,* an opportunistic amoeba [95]. Production of the interleukin IL-15 by dendritic cells can also be important in the 'priming' of NK cells in instances of infection, upregulating the expression of other cytokine receptors and increasing their responsiveness to these other pro-inflammatory cytokines [57,58].

Later, during the adaptive immune response, T-cell derived IL-2 has also been demonstrated to be an important for activation and clonal expansion of NK cells. The $CD56^{bright}$ subset of NK cells constitutively express the high affinity CD25/CD122 $\alpha\beta$ heterodimer and respond to picomolar concentrations of IL-2 [96]. CD25 is also upregulated on the larger $CD56^{dim}$ NK cell subset after NK cell activation by accessory

cytokines [97]. Therefore, by combining with accessory cell-derived IL-12, IL-15 and IL-18, IL-2 provides a synergistic signal leading to both enhanced IFN-γ production and increased NK cell proliferation. For example, our own research group has demonstrated that in models of both tetanus and rabies vaccination IL-2 production by CD4+ memory T-cells significantly upregulates production of IFN-γ by NK cells [98], [99]. Additionally, *ex vivo* studies have demonstrated that a numerical and functional decline in T-cells due to active HIV infection leads to reduced IFN-γ production by NK cells in response to secondary bacterial infections [100].

1.2.2.2 IFN-γ production in response to physical contact with accessory cells

In addition to exposure to soluble cytokines, direct contact with peripheral blood mononuclear cells (PBMCs) such as dendritic cells and macrophages can induce production of IFN-γ by NK cells. Physical contact between NK cells and PBMCs can both optimise *trans* presentation of cytokines to NK cells by monocytes and allow for other direct activating interactions between NK activating receptors and PBMC expressed ligands.

As previously discussed in section 1.2.2.1, several studies have also suggested that physical contact between dendritic cells and NK cells is required for optimal activation of NK cells by dendritic-derived cytokines. Optimal IL-12 release by dendritic cells appears to require contact between dendritic cells and NK cells, while optimal 'priming' of NK cells by IL-15 requires trans-presentation of the cytokine to the NK cell via the IL-15 receptor alpha chain [59], [101].

In addition to presenting cytokine through physical contact however, dendritic cells can also express stress-induced ligands that are capable of directly activating NK cells by binding to activating receptors. These include Major Histocompatability Class I Chain-related proteins A and B (MICA and MICB) which form activating contacts with the NK cell surface receptor NKG2D. Expression of MICA and MICA on virally-infected dendritic cells has been shown to inducing production of IFN-γ by NK cells in several viral infection models [64] [102].

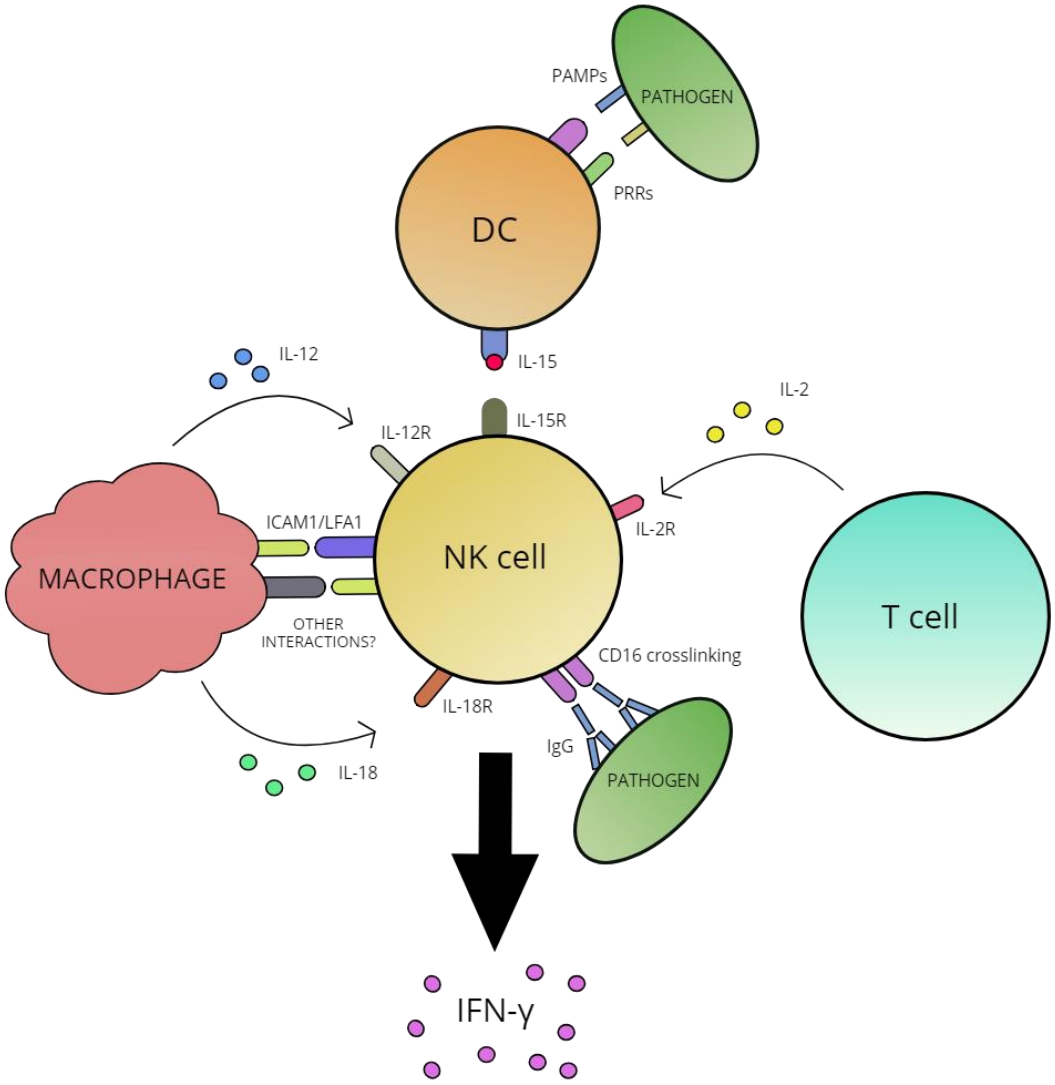
Contacts between macrophages and monocytes and NK cells are also stabilised directly through interactions between adhesion molecules such as LFA-1 expressed on NK cells and ICAM-1 expressed on these accessory cells [103], [104]. Blocking of these interactions has been demonstrated to result in significantly decreased IFN-γ production by NK cells in models of malarial parasitic infection [103], [105]. The pro-

inflammatory cytokine IL-18 has been demonstrated to increase expression of ICAM-1 on monocytes, suggesting a positive feedback loop whereby activated monocytes produce this cytokine and enhance their own ability to activate NK cells through adhesive contacts [106].

Several studies have indicated that such physical interactions between NK cells and accessory PBMCs may be vital for inducing an optimal response to pathogens. In models of *Salmonella* infection for example, NK cells have been shown to produce significant quantities of IFN-γ in response to *Salmonella*-infected macrophages through a contact-dependent mechanism [6]. In studies investigating chronic hepatitis C infection, virally induced downregulation of MICA and MICB expression on dendritic cells leads to reciprocal downregulation in IFN-γ production by NK cells [64]. Similar observations have been made in studies investigating autoimmune disorders such as rheumatoid arthritis. Physical contact between NK cells and monocytes in these disorders induces reciprocal activation, leading to production of IFN-γ by NK cells and TNF-α by monocytes, suggesting these kinds of cell-cell contacts can also play a maladaptive role by mediating autoinflammation [107]

1.2.2.3 IFN-γ production in response to antibody-antigen complexes and co-stimulation

Binding of antibody-antigen complexes to the NK cell has been shown to induce IFN-γ production in models of cancer and viral infections such as HIV [108], [109], [110]. This occurs via binding of antibody to the high-affinity Fc receptor for immunoglobulin G (FcγRIIIA/ CD16), which is expressed on the surface of the majority of NK cells. When multiple antibodies bind CD16 receptors in close proximity on the NK cell surface, the CD16 receptors become 'cross-linked', sending activating signals through the CD3ζ and FcεRIγ adaptor proteins [66], which transduce activating signals to downstream signalling pathways and trigger transcription of the IFN-γ gene [111],[112]. Several studies have suggested that this response can be upregulated by co-stimulation with pro-inflammatory cytokines such as IL-12 and IL-2, particularly in models of breast cancer [113,114]. More recent studies have also suggested that IL-18 is involved in the upregulation of IFN-γ production during the antibody-dependent CD16 response [109]. In leukaemia models, physical contact with accessory cells such as monocytes has also been shown to upregulate IFN-γ production by NK cells in response to antibody, suggesting there may be additional regulatory pathways requiring physical contact with monocyte ligands or physical presentation of cytokines [115].



KEY

DC = dendritic cell

IgG = immunoglobulin G

PAMPs = pathogen-associated molecular patterns

PRRs = pattern recognition receptors

ICAM1 = intercellular adhesion molecule 1

LFA1 = lymphocyte function-associated antigen 1

CD16 = immunoglobulin G Fc domain receptor

Figure 1.1 Natural killer cells produce IFN-γ in response to several forms of stimulation

Production of IFN-γ by NK cells in infection or cancer is regulated by a range of signals; including exposure to pro-inflammatory cytokines such as IL-2, IL-12, IL-15 and IL-18; direct contact with accessory PBMCs such as macrophages and monocytes through receptors such as ICAM1, and binding of antibody-antigen complexes to the NK cell, leading to crosslinking of the IgG receptor CD16.

1.3 NK cell subsets: 'differentiation' and 'adaptation'

Though all NK cell are capable of producing pro-inflammatory IFN-γ and killing cells through cytotoxic activity, the extent to which any one individual NK cell will perform these activities is dependent on its state of functional differentiation. This will determine the expressed receptor repertoire, and which epigenetic modifications regulate its activity. Research conducted over the last few years has increasingly suggested that prior exposure to infected or cancerous cells can induce different forms of cellular differentiation in NK cells, leading to altered phenotype and subsequent altered function.

1.3.1 Differentiation: CD56 downregulation and CD57 expression

In human peripheral blood, there are two major subsets of NK cells as defined by the degree of CD56 expression. '$CD56^{bright}$' cells (hereafter referred to as 'Brights'), which typically represent around 10% of peripheral NK cells, are the least differentiated subset of NK cell, as evidenced by expression of the stem cell factor receptor c-kit, their long telomeres, high prevalence in blood relative to other subsets after haemopoietic stem cell transfer, and ability to differentiate into $CD56^{dim}$ NK cells (hereafter referred to as 'Dims') [116–118]. These cells are highly responsive to pro-inflammatory cytokines such as IL-12, IL-18, IL-15 and IL-2, and constitutively express high levels of cytokine receptors, including IL-12R and the high affinity IL2-R heterodimer CD25/CD122 (IL-2Rα and IL-2Rβ). Although a minor subset of blood NK cells, individual Brights consequently produce exceptionally high levels of IFN-γ in response to cytokine stimulation [90]. Conversely, these cells express low levels of activating NK cell receptors such as NKG2C and activating KIR, little or none of the immunoglobulin G receptor CD16, and high levels of the inhibitory C-type lectin-like receptor NKG2A (reviewed in [119]), thereby demonstrating limited responsiveness to direct contact with target cells or antibody-antigen complexes [120].

Brights also produce minimal amounts of cytotoxic mediators such as perforin and granzymes, and express low levels of death ligands such as TRAIL [121]. However, studies have demonstrated that 'priming' of Brights with cytokines such as IL-15 can increase their production of perforin and granzyme B in addition to increasing expression of TRAIL, leading to enhanced cytotoxic capacity [122]. As such, although they are still capable of killing cells through cytotoxic activity, they do not appear to be particularly predisposed to this function.

As NK cells encounter sources of activation they begin to differentiate, with reduced levels of CD56 [123] (Fig. 1.4). These 'Dims' express higher levels of receptors such as CD16 and KIRs and lower levels of NKG2A, becoming more efficient at both antibody-dependent and antibody-independent cytotoxicity (reviewed in [119]). Conversely they express lower levels of cytokine receptor subunits such as CD25, becoming less responsive to pro-inflammatory cytokines such as IL-2 and subsequently demonstrate reduced IFN-γ production in response to pro-inflammatory cytokines during long-term immune responses [124]. However, as these cells make up the bulk of the NK cell population, they are generally still responsible for the majority of IFN-γ produced in models of infection or cancer, and recent studies have indicated that they are capable of potent short-term IFN-γ production when activated through receptors such as NKp30 and NKp46 [125].

Between 30-60% of Dims in healthy adults also express a secondary surface maker, CD57, which is associated with late differentiation, telomere shortening and reduced proliferative capacity. CD57+ Dims (hereafter referred to as 'Mature Dims') display even further reduction in their capacity to respond to pro-inflammatory cytokines, including IL-12 and IL-18, and to produce IFN-γ in this context [126]. Conversely, Mature Dims contain higher frequencies of cells expressing KIR, CD16, and NKG2C, often associated with increased surface expression of these molecules (Fig. 1.4). As such, Mature Dims are highly efficient at both antibody-independent and antibody-dependent cytotoxic killing of cell targets (reviewed in [127]).

Brights, Dims and Mature Dims are therefore thought to exist on a functional spectrum, differentiating in response to exposure to pro-inflammatory cytokines [126] and cellular targets such as virally-infected cells [128]. Several studies have identified HCMV as a potent effector of NK cell maturation to Mature Dims [128], [129]. Greater frequencies of Dim and Mature NK cells versus Bright NK cells have also been associated with increased age [130], though currently it is unclear whether age itself is a causal factor or simply an indirect measure of the extent of prior exposure to infection.

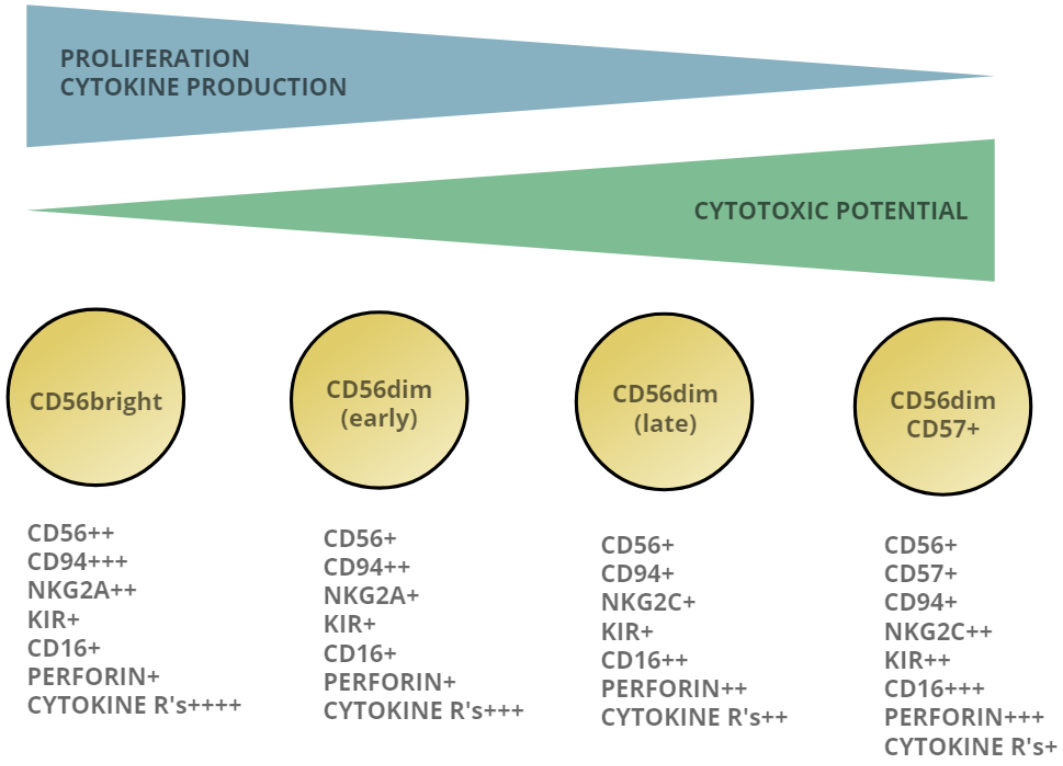


Figure 1.4 Models for the CD56/CD57 differentiation pathway of natural killer cells in response to activation and age

$CD56^{bright}$, $CD56^{dim}$ and $CD56^{dim}$ CD57+ NK cells are thought to exist on a functional spectrum of differentiation. Undifferentiated $CD56^{bright}$ cells exhibit greater proliferative capacity and responsiveness to cytokines, whilst differentiated $CD56^{dim}$ and CD57+ cells exhibit greater cytotoxic potential. Differences in effector functions between subsets are tied to differential expression of surface receptors and internal cytotoxic mediators. NK cells progress down the differentiation pathway in response to exposure to pro-inflammatory cytokines and cellular targets such as virally-infected cells.

1.3.2 'Adaptation': downregulation of FcεRIγ and functional changes in response to HCMV

Recent research has revealed that, in addition to classical functional differentiation, human NK cells from HCMV infected individuals also modulate their intracellular signalling pathways to achieve functional adaptation. Early studies suggested that a NK cells expressing the activating NK receptor NKG2C in combination with its lectin co-receptor CD94 were expanded in response to HCMV-infected host cells [131,132]. Although these cells had a late differentiation phenotype, they demonstrated increased IFN-γ production, particular in the presence of HCMV antigens, leading to the suggestion that they had 'adapted' to HCMV infection [133]. HCMV responsive NK cells were characterised by increased expression of receptors including NKG2C, CD2, CD57 and KIR, but reduced expression of the activating receptors NKp46 and NKp30. (reviewed in [134]). Though originally considered to have adapted to HCMV specifically, subsequent studies have confirmed that this is not the case. Though particularly reactive to HCMV antigens, 'adaptive' NK cells have also demonstrated increased IFN-γ production in response to other targets such as K562 tumour cells, suggesting that while HCMV may drive the development and expansion of these cells the mechanisms by which they become activated afterwards may be more generalised [135].

Studies by Schlums et al and Lee et al in 2015 demonstrated that these changes in adaptive NK receptor expression and subsequent function are due to underlying epigenetic modifications that downregulate the expression of various intracellular adaptor molecules. Hypermethlyation of promoter regions within the genome leads to silencing of certain genes, and subsequent altered function of the NK cell [136] [137].

The resulting modifications include downregulated expression of the transmembrane adaptor molecule FcεRIγ, which is involved in regulating the activity of various NK receptors. Conversely, adaptive NK cells maintain normal expression levels of the alternative adaptor molecule CD3ζ which is involved in regulating the activity of many of the same receptors. FcεRIγ and CD3ζ have been shown to associate with the NK activating receptors NKp46 and NKp30 as either homodimers or a heterodimer including both proteins [138], [139], [5]. Additional studies have shown that FcεRγ1- NK cells express lower levels of NKp46 and NKp30, and demonstrate consequent reduced activation in the presence of certain tumour lines [140].

The NK cell Fc receptor CD16 also associates with FcεRIγ and CD3ζ, and demonstrates an increased activating capacity on FcεRIγ- cells. Several studies have demonstrated that FcεRIγ- NK cells have increased degranulation and IFN-γ production

when stimulated with antibodies directed against cells infected with viruses such as HCMV, HSV-1 and influenza [141,142]. Preliminary published abstracts have also suggested potentially greater antibody-dependent responses against intracellular parasites [143,144].

The downregulation of FcεRIγ in adaptive NK cells is driven by reduced expression of the transcription factor PLZF, which initiates changes in DNA methlylation [145]. PLZF has been shown to bind to the promotor region of FcεRIγ, in addition to other NK adaptor molecules such as SYK and EAT2 that are also frequently downregulated through hypermethylation in adaptive NK cells [137].

In addition to reduced expression of natural cytotoxicity receptors such as NKp30, NKp44 and NKp46, 'adaptive' NK cells exhibit reduced responsiveness to pro-inflammatory cytokines such as IL-2, IL-12 and IL-18 when provided without other forms of activation. DNA analysis has suggested that this may be driven by downregulated transcription of the genes encoding the receptors for these cytokines [137]. However, recent evidence has shown that IL-18 can upregulate IFN-γ and TNFα production of adaptive NK cells in the presence of cancer cells, indicating that the cytokine responsiveness of 'adaptive' NK cells may be rescued in the presence of alternate forms of activation [146].

Though previously thought to be a purely NKG2C+ population, studies have identified the presence of FcεRγ1- NK cells in individuals with inactivating mutations in the NKG2C gene locus [147]. Additional studies have also suggested that in NKG2C- donors specifically, these responses are mediated partly through the engagement of the NK cell surface receptor CD2. CD2 is known to bind a range of ligands expressed on both target cells and accessory PBMCs, such as the lymphocyte differentiation marker CD48, the adhesion molecule CD58 and the glycoprotein CD59 which protects against activation of the complement pathway [148]. Binding of CD2 to CD58 in particular has been shown to synergise with antibody crosslinking of CD16, leading to enhanced cytotoxic reactions through increased phosphorylation of intracellular signalling molecules [149].

It is worth noting that whilst these particular NK cells are referred to as 'adaptive', they have globally enhanced ADCC responses to a range of antigenic targets and as such they cannot be said to have clonal antigen specificity in the sense of an adaptive T-cell or B-cell. This does however raise the interesting possibility that the development of adaptive NK cells in response to HCMV may have the potential to shape the NK cell response not only to this specific pathogen, but to any future infectious or cancerous

target antigens NK cells encounter. It is also possible that the antibody-dependent activity of these cells against different pathogens may vary due to synergistic effects of cytokines such as IL-18 or engagement of modulatory receptors such as CD2 or NKG2C, the costimulatory effects of which may be more prominent in certain infection models than others.

Whilst most studies have identified HCMV as the primary driver of adaptation in NK cells, more recent research has suggested that this adaptive state can also be induced by exposure to cells infected with HIV, suggesting that there may be other sources of adaptive differentiation signals beyond HCMV [150].

1.4 Malaria; biology, symptoms and transmission

1.4.1 Developmental cycle of the malaria parasite

Malaria is a severe insect-borne infectious disease most commonly found in developing African and Asian countries [151]. The causative agents of malaria are members of the *Plasmodium* genus of protozoan parasites, capable of infecting a diverse range of mammalian and non-mammalian hosts, from humans to lizards [152], [153]**.** Female members of the Anopheles genus of mosquitoes transmit the parasites while they feed on the blood of host animals. During a single bite, anywhere between a dozen and several hundred motile and infectious malaria sporozoites can be transmitted into the skin of the host from the salivary glands of the mosquito [154]. The parasites then migrate to the blood stream to initiate development within the non-*Anopholean* host [155].

The developmental pathway of the *Plasmodium* parasite within vertebrate hosts consists of several life stages. After sporozoites enter the blood, they travel to the liver and enter the hepatocytic cells where they then begin to develop into large multinucleated schizonts. These eventually divide into dozens of smaller merozoites, which then lyse the hepatic cells and enter the bloodstream to infect erythrocytes. Once inside erythrocytes the parasites undergo asexual replication. Merozoites develop over ~48 hours from adolescent trophozoites into more schizonts, which then divide and repeat the merozoite cycle, constantly lysing and invading new erythrocytes (Fig. 1.5). Some blood stage parasites differentiate instead into male and female gametocytes that, when ingested in the mosquito blood meal, are activated to produce gametes and initiate a sexual replication cycle inside the *Anopheles* host (reviewed in [156]). These stages exhibit both conserved and stage-specific proteomic expression [157].

1.4.2 Global malarial epidemiology and the importance of research

At present an estimated 3 billion people are at risk of developing malaria, the majority of whom live in Sub-Saharan African and South Asia. In 2016 alone there were an estimated 216 million cases of malaria worldwide leading to just under half a million deaths, with children under 5 years old accounting for the vast majority [151]. Africa overwhelming experiences the most malarial deaths worldwide, accounting for 91% of all malaria deaths in 2016, followed by South-East Asia with 6% of all deaths [151].

The most common methods used to manage malarial infection include long-lasting insecticides used to treat bed nets and spray housing, chemoprevention in at-risk individuals and the use of combination drug cocktails to treat individuals already infected [151]. Efforts to develop a viable malarial vaccine are also ongoing, though these have experienced recent setbacks with the publication of disappointing clinical trial results for the RTS-S vaccine previously considered to be the most promising candidate for a commercial vaccine [158]

Regardless, it is generally recognised by researchers in this area that development of a viable vaccine is desirable for use in addition to other pre-existing methods of malarial control, and will require continued research into the interaction between the human immune system and *Plasmodium* parasites. Of particular interest are the species *Plasmodium falciparum* and *Plasmodium vivax*, as together these species are responsible for the majority of lethal malaria cases worldwide. *Plasmodium falciparum* is the most prevalent species in sub-Saharan Africa (and consequently the most prevalent worldwide given the global burden of the disease primarily falls in this region), accounting for 99% of estimated malaria cases in Africa in 2016. Outside of Africa, *Plasmoidum vivax* is the most common species in the Americas, representing 64% of malaria cases, and represents above 30% of cases in South-East Asia and the Eastern Mediterranean [151]. In this thesis I will focus on investigating human natural killer cell interactions with *P. falciparum.*

1.4.3 Malarial pathology and immunology

1.4.3.1 Innate immunity and the pro-inflammatory balance

The complex nature of the *Plasmodium* life cycle dictates an equally complex anti-malarial immune response from the human host. The initial stages of infection in the skin and liver provoke a relatively muted immune response in humans, with minimal innate involvement and limited production of antibody even in individuals with prior exposure [159]. Though the reasons for this are not definitively understood, it is suspected that the relatively high proportion of regulatory T-cells present in the skin under normal homeostatic conditions may inhibit early immune responses to the sporozoite stage of *Plasmodium* [160]. Meta-analyses of malarial vaccine studies have also suggested that *Plasmodium*-specific regulatory T-cells are induced during initial infection, potentially inhibiting early responses to sporozoites as well as later stages of infection [161].

In human hosts the most pathogenic stage of the malaria developmental cycle is the intra-erythrocyte asexual cycle resulting in the repetitive infection and lysis of red blood cells (Fig. 1.5)**.** Structural changes in the surface membrane of infected erythrocytes can cause the cells to become sequestered in the vascular endothelium, blocking off small blood vessels. Rupturing of erythrocytes during merozoite release can also significantly reduce the number of circulating erythrocytes in the blood stream, causing anaemia. Reduced oxygen supply to the tissue due to blockage of vessels and anaemia can in turn lead to systemic lactic acidosis in the body, and in the brain can lead to severe symptoms such as seizures and coma (reviewed in [162]). A cyclical fever is also indicative of the condition, roughly correlating with the parasite's own ~48 hour asexual developmental cycle [163].

In addition to being the most clinically problematic, asexual blood-stage malaria is by far the most immunogenic. Studies conducted in both human and animal models have shown a role for various innate immune cells in the response to early blood-stage infection, inhibiting the growth of parasites and promoting inflammation. *Plasmodium* pathogen associated molecular patterns such as GPI glycolipid anchors [164]**)** and malarial DNA [165] can bind to host toll-like receptors such as TLR-2 and TLR-9 respectively, while parasite-derived hemozoin binds to NOD-like receptors present in the myeloid inflammasome [166]. Interactions between parasite-derived ligands such as these and their cognate host receptors induce pro-inflammatory responses in innate immune cells, including phagocytosis of infected erythrocytes and production of reactive oxygen species by macrophages, production of pro-inflammatory IL-12 and IL-

18 by dendritic cells, and production of pro-inflammatory IFN-γ by γδ T-cells [167]. NK cells have also been shown to play an important role in shaping the early immune response to blood-stage malaria, as will be discussed in more detail in a later section.

Many studies conducted over the last 20 years have indicted a vital role for pro-inflammation in controlling parasitaemia. An early study by Luty et al on discovered an association in African children between higher IFN-γ levels and increased resistance to reinfection after a mild episode of malaria [168]. Later studies published by Roestenberg et al have also found associations between production of IFN-γ and IL-2 and protection against infectious challenge in experimental human models of infection involving vaccination with whole sporozoites [169,170]. Additional studies have also indicated that excessive regulatory immune responses lead to negative clinical outcomes. In 2005 Walther et al discovered that increased frequency of regulatory T-cells producing anti-inflammatory cytokines such as IL-10 was associated with higher parasitaemia in humans [171], whilst in 2006 Prakash et al suggested that anti-inflammatory cytokines were associated with increased susceptibility to cerebral malaria in Indian patients [172].

While it has been demonstrated that pro-inflammatory immunity is vital for promoting clearance of parasites, excessive inflammatory immune responses to infected cells have also been suggested to exacerbate the symptoms of infection. High levels of pro-inflammatory cytokines such as IL-1, IL-6, IL-12, IFN-γ, and TNFα have been specifically implicated in this respect [172]. An early study by Riley et al. in 1991 demonstrated an association between IFN-γ production after in vitro stimulation with malaria antigens and an increased likelihood of developing fever and malaise during *in vivo* infection in a sample of African children [173]. A later study by Walther et al. also found a similar association between IFN-γ levels in blood plasma and fever in an experimental model of malarial infection [171]. Additional studies conducted by our own lab group on children from Ghana have also suggested that TNFα and IL-12 production by various peripheral blood mononuclear cell types are associated with increased incidence of fever [174], [175].

These studies suggest that an optimal response to malarial infection requires a delicate balance of pro-inflammatory and anti-inflammatory activity designed to prevent parasite replication without provoking a pathological autoimmune response.

1.4.3.2 Acquired immunity and the importance of antibody

In instances of chronic or secondary infection with malarial the adaptive immune system becomes active, leading to gradually increasing protection against re-infection. This is often reflected in the demographics of malarial infection, as typically most infections occur in the young, who also generally suffer the most severe symptoms and consequently make up the majority of malarial deaths worldwide [151]. In the case of *Plasmodium falciparum* specifically most infections in holoendemic areas occur in childhood, characterised by high parasitaemia and clinical symptoms such as fever and severe anaemia. With age, semi-protective immunity develops with repeated exposures, resulting in a reduced likelihood of infection if bitten and asymptomatic infections if exposed [176].

It has been known for many decades that production of anti-malarial antibodies is a vital component of blood-stage immunity, as several early studies have demonstrated that the passive transfer of anti-malarial immunoglobulin G from malaria-immune adults to partially immune children can rapidly reduce parasitaemia and symptoms of fever [177]. Anti-malarial antibodies can confer protection through several mechanisms which can involve either direct complement-mediated destruction of parasitised cells or the neutralisation of pathogenic effects. In passive transfer studies anti-malarial antibodies acquired from immune donors have been shown to induce antibody-dependent cellular cytotoxicity of infected cells by monocytes in partially immune recipients [178]. In cellular studies opsonisation of infected erythrocytes with anti-malarial IgG1 and IgG3 have been shown to induce phagocytosis of infected cells [179]. Antibodies targeting the parasite-derived protein PfEMP1 have also been shown to be able to block binding of infected erythrocytes to host-express adhesion molecules such as CD36, thrombosponsin and ICAM-1, and therefore may play a role in inhibiting the sequestration of infected erythrocytes in the vascular endothelium which is typically considered a major contributor to pathology [180]. Additionally, antibodies targeting parasite-derived molecules such as GPI glycolipid anchors have been shown to neutralise the pro-inflammatory activity of *Plasmodium falciparum* in cellular models, whilst also minimising pathogenic effects of infection such as malarial acidosis, pulmonary oedema and cerebral malaria in murine models infected with *Plasmodium berghei* [181].

Over time and with repeated infectious exposures, individuals can achieve an increasing natural immunity to malaria with increasing breadth and depth of the anti-

malarial immunoglobulin repertoire. Consequently, the symptoms of clinical malaria are most common in children, as adults who have survived multiple exposures display increased resistance to the development of severe symptoms. True sterilising immunity is largely considered unlikely however, as parasites can continue to be detected in the blood up until at least 60 years old, even in asymptomatic adults [162].

Many studies have indicated that antibody-independent acquired immune mechanisms also contribute protection against malaria. In *ex vivo* studies conducted on human volunteers inoculated with low doses of live *falciparum*-infected erythrocytes, secondary challenge with malarial antigens can result in a Th1-biased CD4+ and $CD8^+$ T cell response involving proliferation of T cells, production of pro-inflammatory IFN-γ and increased nitric oxide synthesis in peripheral blood mononuclear cells [182]. Similar results have also been observed in murine models infected with *Plasmodium yoelii* [183]. Additional studies have also suggested that the acquired T cell response to malaria can help shape the innate NK cell response through production of IL-2, as will be discussed later in more detail in section 1.5 [184] [185].

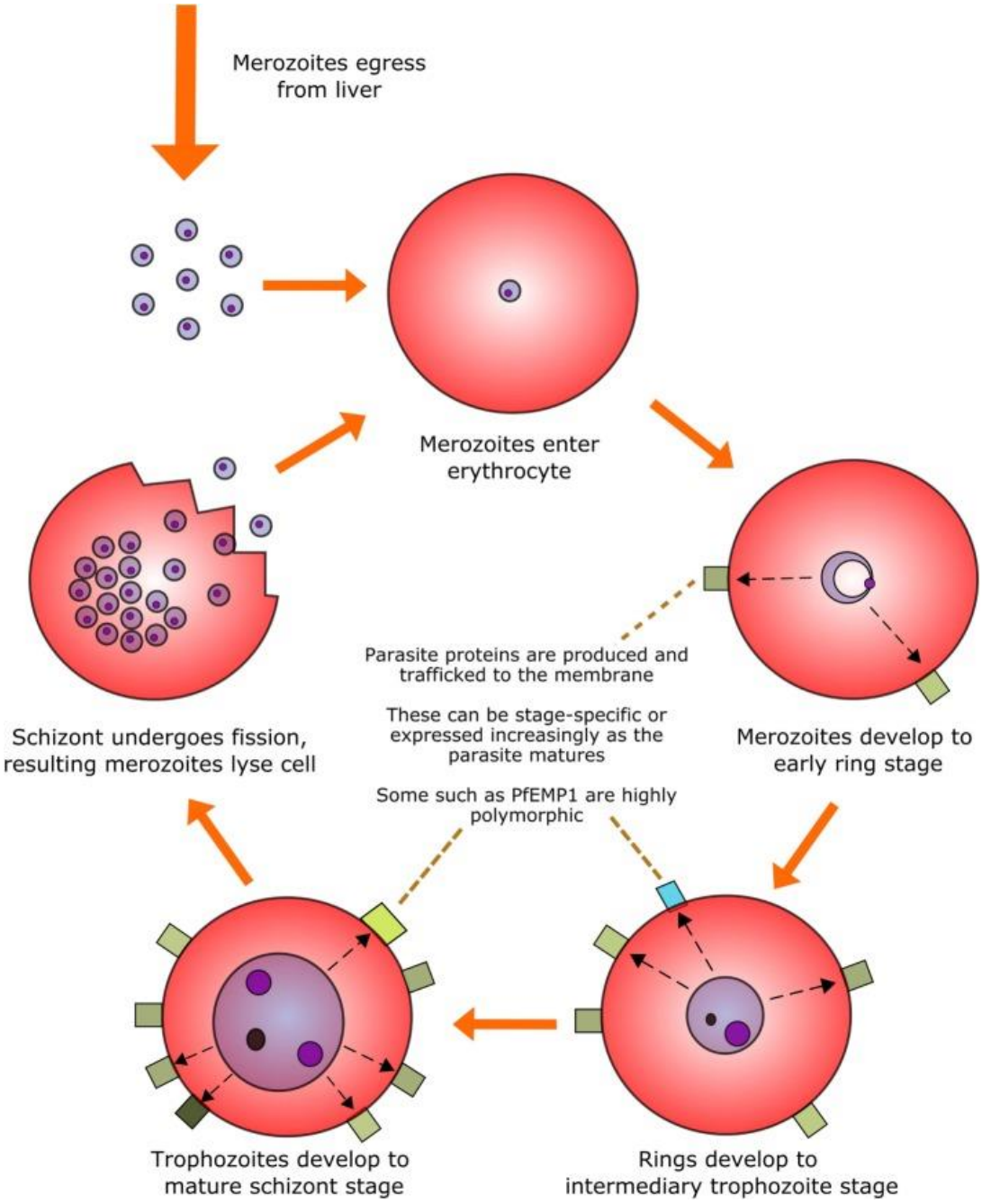


Figure 1.5 The intra-erythrocytic asexual replication cycle of malaria parasites

After initial infection of the host and replication in the liver, merozoites are released into the blood stream where they penetrate healthy erythrocytes. The merozoite will then metabolize host haemoglobin to fuel its own development into the mature schizont stage while restructuring the erythrocytic membrane to aid in nutrient transfer, rosetting and sequestration. Parasite proteins such as PfEMP1 are produced, some of which are exported to the erythrocyte surface. The mature schizont will then undergo replicative fission, forming 8–32 merozoites which lyse the cell membrane and re-enter the bloodstream. In in the case of *P. falciparum*, this entire process will take approximately 48 hours, corresponding with the cyclical fever indicative of malarial infection.

Figure republished with permission from Wolf, Sherratt and Riley (2017) *Frontiers in Immunology* [246]

1.5 Natural killer cells and malaria

1.5.1 Production of anti-malarial pro-inflammatory cytokines by NK cells

Multiple studies conducted over the last few decades have suggested there may be a vital role for the production of IFN-γ in mounting an early successful immune response against malaria. In children, IFN-γ production has been found to associate with protection against severe malarial symptoms as well as delayed incidence of re-infection within one year of initial contraction of the disease [168]. In experimental models of vaccination with *Plasmodium falciparum* sporozoites, production of IFN-γ by PBMCs has also been found to associate with long-term protection against malaria [169,170]. In cellular studies, production of IFN-γ in addition to other pro-inflammatory cytokines such as TNFα has also been associated with lower parasite burden and high haemoglobin concentration in the blood, suggesting specific mechanisms for these protective effects [174,175].

Evidence for a beneficial effect of IFN-γ production is mixed however, as research has also suggested that excessive production of pro-inflammatory cytokines may contribute to the autoimmune pathology believed to contribute to clinical symptoms. Several studies have suggested an association between production of IFN-γ during either natural or experimental infection with malaria and increased likelihood of developing fever and fatigue [173], [171]. Additional studies have suggested a link between higher levels of several pro-inflammatory cytokines, including IFN-γ, and increased frequency of febrile episodes in young patients [175], [174]. Taken together, these studies suggest that while IFN-γ is necessary for a robust anti-malarial response excessive production may contribute to autoimmune pathology.

Given that one of the primary functions of NK cells is the production of IFN-γ, there has naturally been significant interest in studying the activities of this particular cell type in instances of malarial infection. Early studies typically assumed that classical CD4+ helper and CD8+ cytotoxic T-cells were the predominant source of malaria-induced IFN-γ as a consequence of researchers utilizing techniques that could not differentiate between T-cells and NK cells [186,187].

More recent studies by both our own lab group and others have confirmed that NK cells produce significant quantities of IFN-γ in response to erythrocytes infected with *Plasmodium falciparum* in both *in vitro* cellular models of infection and controlled human infection models [188], [189]. These models have suggested that NK cells may be particularly important in providing an early source of IFN-γ during infection, with cellular studies

previously published by our own research group indicating that NK cells begin to produce IFN-γ within only 6 hours of co-culture with infected erythrocytes [82], though this response varies significantly between individuals [190]. Some studies have suggested that the optimal production of IFN-γ by NK cells in response to malaria parasites is dependent on physical contact with infected target cells [188]. The heterogeneity in IFN-γ responses may therefore be a result of variation in the NK germline receptors expressed by different individuals, though this is somewhat speculative as the specific receptor-ligand pairings that mediate interactions between NK cells and malaria-infected targets are largely unknown.

Additional signals such as pro-inflammatory cytokines and physical contacts with accessory peripheral blood mononuclear cells have also been demonstrated to upregulate NK production of IFN-γ in response to malaria. Our own research group has demonstrated that in cellular models soluble signals such as IL-12 and IL-18 produced by myeloid accessory cells during initial infection [45] [188] and IL-2 produced by memory CD4+ T-cells later on during the adaptive immune response have both been shown to upregulate cytokine production by NK cells (Fig. 1.6) [184]. In controlled human infection models the presence of T-cells appears particularly crucial to induce IFN-γ production by NK cells in response to malaria, with depletion of the CD3 subset leading to significant downregulation of the NK response through a partially IL-2 dependent mechanism [185].

The specific physical contacts that occur between NK cells and accessory cells in instances of malarial infection are largely uncharacterised, but include binding of LFA-1 expressed on the NK cell surface to ICAM-1 expressed on PBMCs [103]. Transpresentation of IL-15 to NK cells by dendritic cells is also speculated to occur as soluble IL-15 has been demonstrated to upregulate NK IFN-γ cells to *falciparum*-infected erythrocytes [191].

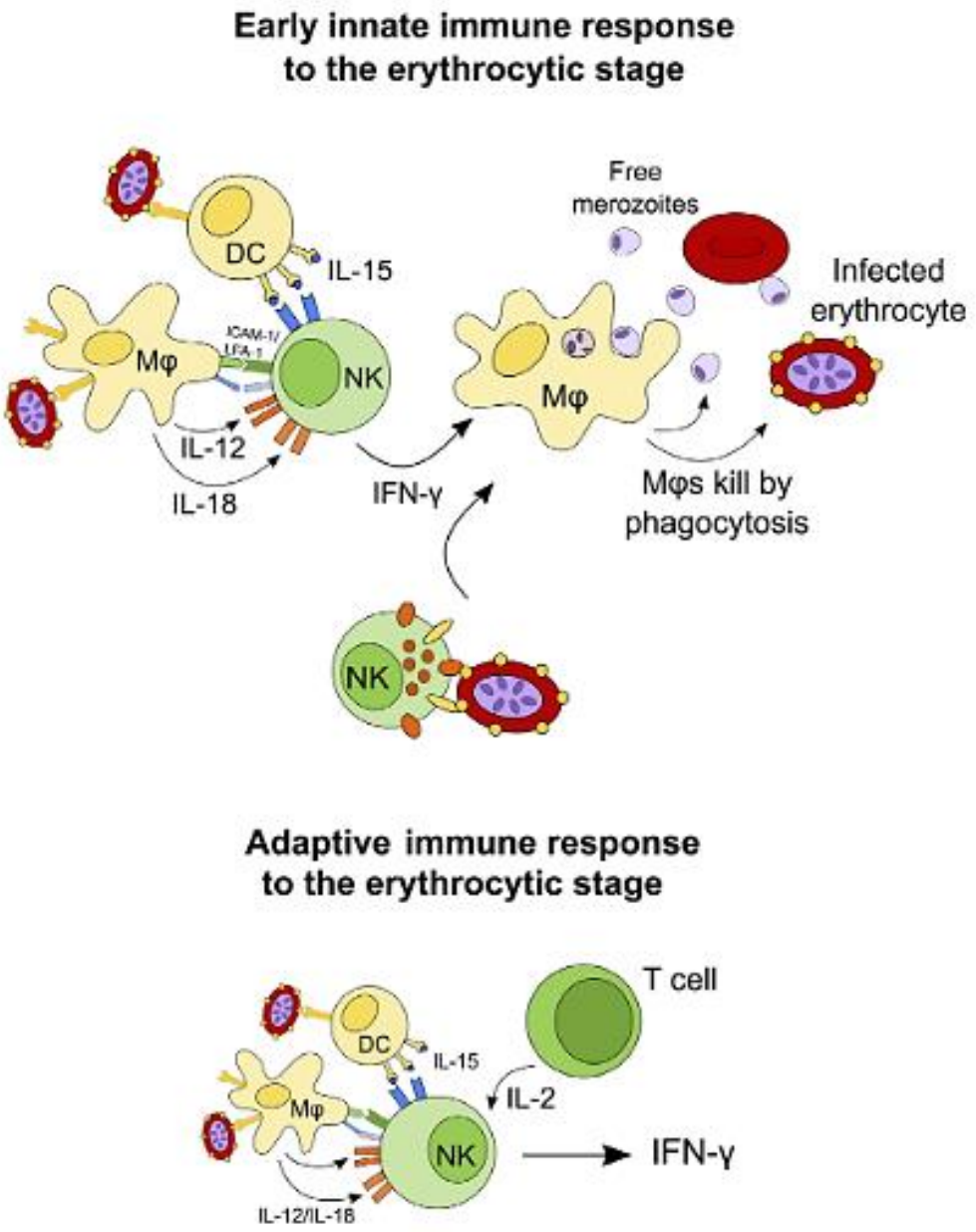


Figure 1.6 Established model for the production of IFN-γ by natural killer cells during malarial infection

During the asexual erythrocytic stage of infection, NK cells are activated by cytokines such as IL-12, IL-15 and IL-18 produced by macrophages and dendritic cells. Some evidence also suggests NK cells are activated by direct contact with infected erythrocytes. NK cells in turn release IFN-γ to stimulate the pro-inflammatory activity of other immune cells. Examples include activation of macrophages that subsequently phagocytose infected erythrocytes. Once the adaptive immune response has developed, T-cells contribute IL-2 to enhance the ongoing NK cell response.
Figure adapted with permission from Wolf, Sherratt and Riley (2017) *Frontiers in Immunology* [246]

1.5.2 Natural killing of malaria-infected erythrocytes through cytotoxic activity

A growing body of research has also suggested that NK cells can form direct functional contacts with erythrocytes infected with *Plasmodium falciparum*, facilitating their activation and subsequent cytotoxic activity and cytokine production. Early flow cytometry studies conducted by our lab have suggested that direct conjugates may form between NK cells stained for the CD56 marker and infected erythrocytes stained for glycophorin A after as little as 30 minutes of co-culture [188]. Immortalised NK92 cell lines have also been imaged forming so called 'rosettes' consisting of one NK cell bound to many infected erythrocytes [103], while additional studies have also observed purified NK cells forming what appear to be functional cytotoxic contacts with erythrocytes infected with *Plasmodium falciparum* as determined by the apparent reorganisation of cytoskeletal F-actin within the conjugated NK cells [190]. More recently, studies utilising video microscopy have shown NK cells forming conjugates and apparently killing malaria-infected erythrocytes in a humanised mouse model of malarial infection [40]. Additional studies conducted using controlled human infection models have also demonstrated a significant decrease in the proportion of NK cells found in the blood after early malarial infection, potentially due to migration to the afflicted tissues [192].

Whilst these studies have presented convincing evidence that NK cells can form functional conjugates with malaria-infected erythrocytes, there are significant gaps in our current knowledge both regarding the receptors that facilitate these interactions and whether there is any significant variance in the ability of different NK cell subsets to form these conjugates.

Erythrocytes typically do not express classical or non-classical HLA class I molecules that act as ligands for inhibitory NK cell receptors such as the KIRs, or do so at very low levels [193]. Despite this, healthy erythrocytes remain unchallenged by NK cells due to a simultaneous lack of ligands for activating NK cell receptors such as the NCRs or activating KIRs, allowing them remain immunological 'silent'. This raises questions as to which altered-host or parasite-derived ligands bind to which NK receptors in order for NK cells to recognise and respond to erythrocytes after they are infected with malaria.

Several research groups have attempted to block various NK receptors and surface molecules during malarial cytotoxicity assays to assess their contribution to the anti-malarial response; but few of the receptors tested have demonstrated any significant role in conjugate formation or subsequent cytotoxicity. One study has suggested that the NK receptor NKp30 can bind the Duffy binding-like domain of *Plasmodium*

falciparum erythrocyte membrane protein-1 (PfEMP-1), a protein widely expressed on parasitized erythrocytes [38], though subsequent studies have thoroughly disputed this result [40]. PfEMP-1 has also been found to bind to chondroitin sulfate A (CSA), a glycosaminoglycan modification of many cell surface proteins, including those found on NK cells. However, whilst it has been shown that binding of PfEMP1 to CSA appears to mediates adhesion of infected erythrocytes to NK92 cell lines, it does not seem to be required to induce NK cell activation, refuting its importance [103].

Currently the only NK receptor that has been repeatedly suggested to play an important role in the formation of conjugates with infected erythrocytes is the activating receptor and adhesion molecule LFA-1, and to a lesser extent the costimulatory receptor DNAM-1 [40,103]. However, studies disagree on whether LFA-1 binds directly to an unknown ligand on infected erythrocytes [40] or simply facilitates the activation of NK cells by binding ICAM-1 present on the surface of accessory cells within PBMC [103].

Adding to these complexities, most if not all of the studies that have thus far been performed in this area have failed to take into account any variability in receptor repertoire that may occur between different subsets of primary NK cells. NK cell receptor expression is known to vary considerably as NK cells differentiate from Brights to Mature Dims, and several studies have subsequently noted a significant difference in the ability of these subsets to respond to physical contact with infected targets [128,194] (reviewed in [127]). In the case of studies utilising NK92 cell lines any diversity amongst NK receptor repertoires is simply not represented, as these cells are clonal and typically present as undifferentiated Brights. Some results acquired using this cell line have also failed to hold true when experiments are repeated using primary NK cells [103], and in other cases have proven difficult to replicate even using the same line [195]. In other studies which use primary NK cells for imaging, whilst there is presumably considerable diversity present amongst the NK cells in terms of differentiation and receptor expression, this is generally not noted or analysed [40,196].

Furthermore, several of these studies utilise models of infection that are unrepresentative of malarial infection *in vivo*. In particular, most studies use NK92 tumour cell lines or purified NK cells where accessory cells are absent. These cannot be assumed to provide a realistic representation of conjugate formation due to the lack of potential interactions with accessory PBMCs that have been demonstrated by both our own group and others to provide essential costimulatory signals for NK cells in instances of malarial infection, such as pro-inflammatory cytokines and physical contacts [45,103,184,188,191]. Additional studies also utilise NK cells grown under extremely

unusual conditions, such as humanised mouse models that require contact supplementation with high dose cytokine in order to keep the NK cells viable [40].

Given these issues, it would be helpful for future research in this area to develop an experimental *in vitro* imaging system, which allows us to assess whether NK cells preferentially form conjugates with malaria-infected erythrocytes *whilst* using NK cells obtained from human donors with representative subset diversity and allowing for potential co-stimulatory signals from autologous accessory PBMCs. Such a system would allow us to determine whether different NK subsets preferentially form conjugates under representative conditions. If so, this may help identify additional receptor-ligand pairings involved in these interactions.

Developing such a system is therefore one of the aims of this thesis, and the subject will therefore be discussed in greater detail in the relevant chapter.

1.5.3 Antibody-dependent responses to malaria in NK cells

Whilst there are an increasing number of studies investigating cytokine and innate receptor induced IFN-γ production and cytotoxic conjugate formation by NK cells in response to malaria-infected erythrocytes, with the exception of a few published abstracts investigating antibody-dependent responses in cellular models of naïve subjects, there are currently no full published studies investigating antibody-dependent NK cell responses to malaria in naturally-exposed individuals [143,144]. Given the continued focus of the global research community on development of an effective anti-malarial vaccine [158] the importance of NK cell IFN-γ production and cytotoxic activities in controlling malarial infection [40,173], and recent studies indicating the existence of adaptive NK cells with enhanced antibody-dependent activity [137,141,142], this seems an important omission. There is a need both to evaluate the generic NK cell response to malaria through antibody-dependent mechanisms in a representative model of exposure, as well as to evaluate the specific role of adaptive NK cells in mounting these responses.

A primary aim of this thesis will therefore be to investigate antibody-dependent IFN-γ and cytotoxic activity of NK cells in response to malaria-infected erythrocytes, with specific focus on the activity of adaptive NK cells. This subject will therefore be discussed in greater detail in the relevant chapter.

CHAPTER 2

Materials and Methods

In this chapter, I will give generic descriptions of the methods used in this thesis. Experiment-specific methods will be described in the relevant chapters with more detailed information.

2.1 Cell and plasma donors

2.1.1 European donors for malaria and influenza vaccine studies

50 healthy volunteers were recruited from within the London School of Hygiene and Tropical Medicine (LSHTM) through an anonymised blood donation system operated by Carolynne Stanley, a trained phlebotomist. Ethical approval for this system was acquired from the LSHTM Ethics Committee under the ethics reference numbers 5520 and 6237, and informed consent was obtained from all donors as stipulated by LSHTM and the appropriate regulatory bodies. 50ml of venous blood was collected in sterile Falcon tubes with 2 I.U. per ml of sodium heparin added to prevent coagulation. Plasma and peripheral blood mononuclear cells (PBMCs) from these donors were isolated from venous blood by myself, Dr Martin Goodier and Dr Carolyn Nielsen and either used immediately or cryopreserved for cold storage and later use. 40 of these donors had been previously tested for HCMV exposure prior to this study by Dr Carolyn Nielsen using an ELISA assay testing for the presence of anti-HCMV antibodies.

2.1.2 Gambian donors for malaria studies

641 donors were recruited from sites of known high-malarial transmission in the Gambia. Ethical approvals to collect samples were acquired from the Gambian Government and Medical Research Council Gambian joint ethics committee (reference numbers SCC 1269 and SCC 1449) as well as the LSHTM ethics committee (ethics reference number 6034). Informed consent was also obtained from all donors as required by local regulations. Plasma and PBMCs from these donors were isolated from venous blood and cryopreserved in Gambia before shipment to LSHTM for later use. The previous malarial exposure of these donors was determined by ELISA assay

assessing the presence of antibodies in blood plasma directed against apical merozoite antigen (AMA-1) and 66 positive donors selected for inclusion in experiments. Due to the high prevalence of HCMV in the Gambia, all of these individuals tested positive for anti-HCMV IgG.

2.1.3 Anti-AMA1 ELISA of plasma samples

Gambian plasma samples were tested for the presence of antibodies targeting AMA1, a malarial antigen expressed by infectious merozoites. The presence of anti-AMA1 antibodies is a commonly used criterion to confirm previous exposure to malaria.

Selected plasma samples were diluted 1 in 200 with reconstitution buffer (PBS, 0.05% Tween 20, 1g/L sodium azide) in 96-well flat-bottom deep well plates.

AMA1 stock antigen consisting of 0.5 µg AMA1 protein per ml was diluted 1/5836 in coating buffer (distilled water, 1.59g/L Na_2CO_3, 2.93g/L $NaHCO_3$) and 50ul of the resulting solution added to wells of 96-well flat-bottom Immulon 4 HBX plates (Thermofisher). These plates were covered to prevent evaporation and left to incubate overnight at 4^{o}C.

ELISA plates were then washed three times in washing solution (PBS, 0.05% Tween 20) and allowed to dry. 150ul of blocking solution (PBS, 0.05% Tween 20, 1% skimmed milk powder) was added to each well and allowed to incubate at room temperature for 3 hours. Plates were then washed three more times in washing solution and allowed to dry.

45ul of blocking solution was then added to each well, followed by 5ul of the previously diluted 1 in 200 plasma samples to form a final dilution of 1 in 2000. Positive and negative control plasmas were added to each ELISA plate to generate a standard reference curve. Plates were then covered and incubated overnight at 4^{o}C to allow binding of antibody to AMA1-coated wells.

Plates were then washed 5 more times in washing buffer and allowed to dry. Horseradish peroxidase-conjugates rabbit anti-human immunoglobulin G stock was then diluted in washing solution 1 in 15000. 50ul of this solution was then added to each well of the ELISA plates and incubated at room temperature for 3 hours. Plates were then washed again 5 times in washing solution and allowed to dry. 100ul of Tetramethylbenzidine (TMB) Liquid Substrate (Sigma) was then added to each well and

incubated at room temperature in the dark for 15 minutes. The ELISA reaction was then halted by adding 50ul of 0.2M sulphuric acid to each well.

Plates were read immediately using a Dynex Technologies MRX II Microplate Absorbance Reader run at 450nm. Standard curves and positive/negative cutoffs were determined by Dr Joe Biggs, with the positive cutoff based on the mean optical density plus two standard deviations.

2.2 PBMC purification, freezing, and thawing

2.2.1 PBMC purification

Peripheral blood mononuclear cells (PBMCs) were purified from whole blood samples by slowly layering 25ml of blood onto 15ml of Histopaque 1077 (Sigma-Aldrich) in a 50ml sterile tube before centrifugation at 500g for 25 minutes with the centrifuge brake turned off. Once these cells formed a visibly distinct layer between Histopaque 1077 and blood plasma, they were transferred to a fresh tube using a 25ml stripette and washed twice with RPMI 1640 (Thermofisher), centrifuging at 500g for 10 minutes with maximum braking. Viable cells were then mixed at a 1:1 dilution with Trypan blue dye and then counted using a FastRead 102 haemocytometer (Immune Systems). Cells were then resuspended in a culture medium consisting of RPMI 1640 (Thermofisher) supplemented with 2mM L-glutamine at the desired cell concentration.

2.2.2 PBMC freezing for nitrogen storage

PBMCs were suspended at a concentration of 2×10^7 cells/ml in RPMI 1640 (Thermofisher) with 10% FCS and 2mM L-glutamine. Cells were diluted 1:1 with freezing medium consisting of 80% FCS with 20% DMSO (Sigma) and aliquoted into 1.5ml cryotubes which were then placed in pre-chilled cryogenic freezing containers filled with isopropyl alcohol (Nalgene). Cells were frozen and allowed to reach a temperature of -80^{o}C before being transferred to long-term liquid nitrogen storage. (N.B. Research has indicated that cryopreservation in DMSO and sera has no significant effects on NK phenotype when performed correctly [197])

2.2.3 Recovery of frozen PBMCs

Cryopreserved PBMCs were thawed for use by pipetting warm RPMI 1640 onto the frozen cells and transferring the thawed cells into 20ml warm RPMI quickly to avoid cell death by prolonged exposure to unfrozen DMSO. Cells were centrifuged at 500g for 10 minutes and washed again with RPMI 1640 before being resuspended in RPMI 1640 supplemented with 2mM l-glutamine at the required cell concentration.

2.2.4 NK cell enrichment

When necessary, NK cells were enriched from PBMC mixtures using a MACS NK Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. PBMCs were suspended in PBS supplemented with 1% bovine serum albumin (BSA) and 1% EDTA at a concentration of 2.5 x 10^8 cells/ml. Cells were incubated with 20ul of NK Cell Biotin-Antibody Cocktail per 40ul of cell suspension (per 10^7 cells) for 5 minutes at 4^{o}C. 60ul of PBS buffer was then added per 60ul of cell suspension (per 10^7 cells). 40ul of NK Cell MicroBead Cocktail was then added per 120ul of cell suspension of cells (per 10^7 cells) and incubated for 10 minutes at 4^{o}C. The resulting cell suspension was then topped up with PBS buffer to a final volume of 2ml, and run through an MS MACs Separator column placed within a magnetic field. The enriched runoff was collected and washed twice with RPMI 1640 before being resuspended in the appropriate cell medium.

2.3 Culture of asexual-stage *Plasmodium falciparum*

2.3.1 Thawing frozen erythrocytes infected with ring-stage *Plasmodium falciparum*

Cryopreserved erythrocytes infected with ~3% ring-stage *Plasmodium falciparum* (either 3D7-strain or transgenic 3D7-strain tagged with GFP) were thawed for use by briefly warming the cells in a 37^{o}C water bath for approximately 30 seconds until the frozen cell mass liquefied. Filtered deionised water supplemented with 3.5% NaCl was then added drop-wise to the thawed cell suspension until reaching a 1:1 ratio. The cells were then spun at 800g for 2 minutes with a slow brake and the supernatant removed and discarded. This process was repeated once more before resuspending the remaining infected erythrocytes in 10mls of parasite culture medium consisting of RPMI 1640 (Life Technologies) with the following modifications: 2.3 g/L sodium bicarbonate, 4 g/L dextrose, 5.957 g/L Hepes, 0.05 g/L hypoxanthine, 5 g/L AlbuMAX II (Thermo

Fisher Scientific) and 0.292 g/L L-glutamine. Parasitised erythrocytes were then cultured at 37ºC in an incubator supplied with a 5% CO_2, 5% O_2 and 90% N_2 gas mixture.

2.3.2 Routine culturing of *Plasmodium falciparum*

Erythrocytes infected with *Plasmodium falciparum* were cultured in 23.25ml of parasite culture medium (as above) supplemented with 1.25ml of packed fresh red blood cells to form a final culture of 25mls at 5% haematocrit kept in a 75ml filtered top flask (Nunc). Red blood cells were obtained from the National Blood Service, London on a weekly basis and kept at 4ºC for storage. Parasitaemia was kept at between 0.5-2.5% for routine culture to prevent excessive depletion of culture nutrients. For cultures at the correct parasitaemia, culture medium was changed every day by spinning entire cultures at 800g for 2 minutes with a slow brake before removing the supernatant and resuspending cells in fresh medium. For cultures with parasitaemia >2.5%, 5mls of infected culture were removed and added to 19mls of fresh culture medium supplemented with 1ml fresh packed red blood cells to form a 1:5 dilution with 5% haematocrit. For experiments requiring high parasitaemia cultures were allowed to continue replicating to the required point, taking care to change culture medium regularly. Cultures were tested regularly for Mycoplasma contamination with a LookOut Mycoplasma QPCR Detection Kit (Sigma-Aldrich). Parasitaemia was monitored by blood films fixed with methanol and stained using 0.2µm filtered 10% giemsa solution (VWR Chemicals) for 10 minutes at room temperature.

2.3.3 Percoll purification of late-stage *Plasmodium falciparum* schizonts for synchronisation and experiments

In order to obtain purified cultures of late-stage schizonts for creation of synchronised cultures and experimentation, 25ml *Plasmodium falciparum* cultures were allowed to reach a parasitaemia of ~10-12%. 60% Percoll solution was made using 30mls Percoll plus 17 mls RPMI 1640 supplemented with 3ml 10X PBS. This solution was filter sterilised using 0.25 micron syringe filters before use. Parasite cultures were then spun at 800g for 2 minutes with slow brake and resuspended in culture medium at a haematocrit of ~40%. 2mls of 40% parasite culture were then slowly layered onto 4mls of prewarmed 60% Percoll solution before being spun at 800g for 10 minutes with no brake. Erythrocytes infected with late-stage schizonts formed a distinct layer above

uninfected erythrocytes and erythrocytes infected with early ring and trophozoite stage parasites, allowing them to be pipetted off. The late-stage schizonts were then washed twice in culture medium to remove any traces of Percoll, and are either recultured to form a synchronised culture or used directly in experiments requiring purified schizonts.

2.3.4. Culturing uninfected erythrocytes for experiments

Uninfected erythrocytes from pooled donors were obtained from the National Blood Service, London on a weekly basis and kept at 4°C for storage. Uninfected erythrocytes were cultured under the same conditions using the same culture media as described for infected erythrocytes so as to control for environmental variables that could affect erythrocyte phenotype.

2.4 PBMC/NK cell assays

2.4.1 Assessing NK cell functional markers through flow cytometry

For functional assays PBMCs were either thawed or purified using the protocols described in sections 2.2.1 and 2.2.3. PBMCs were either used directly or used to collected enriched NK cells using the protocol described in section 2.2.4. Either PBMCs or enriched NK cells were incubated at 37°C with 5% CO_2 in wells of 96-well U-bottom plates (ThermoFisher) under the required conditions for each experiment, specified in the relevant chapters. FCS was used as a control for Ig-free human plasma in order to maintain consistency with our previous publications, where we have shown that FCS is a viable substitute for Ig-free human plasma in NK cell culture [198]. GolgiStop containing Monensin (BD Biosciences) was added to cultures to form a final 1/1500 concentration and GolgiPlug containing Brefeldin A (BD Biosciences) added to form a 1/1000 final concentration after either 15 hours (for 18 hour assays) or 1 hour (6 hour assays) to prevent protein trafficking from the endoplasmic reticulum and allow a measurable build-up of intracellular IFN-γ. Anti-CD107a antibody (ef660-conjugated, clone 1D4B, BD Biosciences) was added during culturing at 1µl per 100ul of culture suspension to allow sufficient staining of recycled CD107a.

After the required period of incubation, cells were centrifuged at 500g for 5 minutes at maximum brake and the resulting supernatant removed by flicking the plate over a waste container. The cells were then washed twice with 200ul cold FACS buffer (PBS supplemented with 1% FCS, 1% EDTA and 0.01% sodium azide). Cells were then

incubated for 30 minutes at 4°C in the dark with 10µl total of the required surface antibody cocktail made up with FACS buffer. Cells were then rewashed with 200ul FACS buffer, taking care to avoid excessive exposure to light. Cells were then incubated in 75µl Foxp3/Transcription Factor Staining Buffer (eBioscience) for 25 minutes at room temperature in the dark. Cells were then washed with 200ul 1X Permwash (BD Biosciences) twice at 500g with maximum brake and the supernatant again removed by flicking. Cells were then stained with 10µl total of the required intracellular antibody cocktail made up with Permwash for 30 minutes at 4°C in the dark. Cells were then washed again with 200ul Permwash, then resuspended in 300µl FACS buffer, transferred to alpha tubes (Alpha labs) and stored at 4°C until run on a BD LSRII Flow Cytometer (BD Biosciences) using the appropriate laser set. Data was collected using the complementary FACSDiva software (BD Biosciences) and analysed using FlowJo, v.10 (FlowJo LLC). Fluorescence compensation was carried out using OneComp eBeads Compensation Beads (eBioscience) stained with single antibodies Cell samples were run until a minimum of 1000 CD56+ CD3- events were acquired. FMO controls were run for all staining panels in order to determine background fluorescence and allow accurate discrimination of positive versus negative signals.

2.4.2 Assessing NK cell conjugate formation with PRBCs through imaging flow cytometry

For conjugation assays PBMCs were purified from whole blood using the protocol described in section 2.2.1. PBMCs were then incubated at 37°C with 5% CO_2, 5% O_2 and 90% N_2 in 100ul wells of 96-well U-bottom plates (ThermoFisher) with either healthy erythrocytes or erythrocytes infected with transgenic GFP-expressing *Plasmodium falciparum* schizonts purified using the Percoll method described in section 2.3.5 (GFP parasites originally obtained from Professor BM Cooke, University of Monash). PBMC and erythrocytes were cultured under the required conditions for each experiment, specified in the relevant chapter.

After the required period of incubation, cells were centrifuged at 500g for 5 minutes at maximum brake and the resulting supernatant removed by gentle aspiration. The cells were then washed once with 200ul cold FACS buffer (PBS supplemented with 1% FCS, 1% EDTA and 0.01% sodium azide). Cells were then incubated in 75ul of 4% paraformaldehyde for only 15 minutes at room temperature to allowing fixation of conjugates whilst preventing erythrocyte lysis. Cells were then washed again with 200ul FACS buffer once at 500g with maximum brake and the supernatant removed by gentle

aspiration. Cells were then incubated for 30 minutes at 4ºC in the dark with 12µl total of the required surface antibody cocktail. Cells were then rewashed with 200ul FACS buffer, taking care to avoid excessive exposure to light. Cells were then resuspended in 50µl FACS buffer at a final concentration of 8 x 10^7/ml PBMCs and erythrocytes combined, transferred to 1.5ml Eppendorf tubes (Sigma) and run immediately on an ImageStreamX Mark II Imaging Flow Cytometer (Amnis) using the appropriate lasers to induce fluorescence. Data was collected using INSPIRE acquisition software (Amnis) and analysed using IDEAS analytical software (Amnis). CD56+ CD3- events were gated using IDEAS, sorted based on level of GFP or glycophorin A expression and inspected visually to confirm whether the gated events were in fact NK-RBC conjugates based on the proximity of CD56+ CD3- NK cells to glycophorin A+ erythrocytes. Fluorescence compensation was carried out using cells stained with single antibodies to avoid conflict with the tracking beads used in the ImageStream sheath fluid. Cell samples were run until a minimum of 1000 CD56+ CD3- events were acquired. FMO controls were run for all staining panels in order to determine background fluorescence and allow accurate discrimination of positive versus negative signals.

2.4.3 Assessing mechanisms of CD16 downregulation on NK cells exposed to flu antigen-antibody complexes through imaging flow cytometry

For CD16 imaging assays PBMCs were purified from whole blood using the protocol described in section 2.2.1. PBMCs were then incubated for 5 hours at 37ºC with 5% CO_2 in 100ul wells of 96-well U-bottom plates (ThermoFisher) with trivalent flu vaccine supplemented with either 5% FCS or 1% pooled immune plasma collected by Dr Martin Goodier and Helen Wagstaffe as described in section 2.1.1.

After the required period of incubation, cells were centrifuged at 500g for 5 minutes at maximum brake and the resulting supernatant removed by flicking plates. The cells were then washed once with 200ul cold FACS buffer (PBS supplemented with 1% FCS, 1% EDTA and 0.01% sodium azide). Cells were then incubated for 30 minutes at 4ºC in the dark with 12µl total of the required surface antibody cocktail. Cells were then rewashed with 200ul FACS buffer, taking care to avoid excessive exposure to light. Cells were then incubated in 75µl Foxp3/Transcription Factor Staining Buffer (eBioscience) for 25 minutes at room temperature in the dark. Cells were then washed

with 200ul 1X Permwash (BD Biosciences) twice at 500g with maximum brake and the supernatant again removed by flicking. Cells were then stained with 10µl total of the required intracellular antibody cocktail made up with Permwash for 30 minutes at 4^{o}C in the dark. Cells were then washed again with 200ul Permwash, then resuspended in 50µl FACS buffer and,run immediately on an ImageStreamX Mark II Imaging Flow Cytometer (Amnis) using the appropriate lasers to induce fluorescence. Data was collected using INSPIRE acquisition software (Amnis) and analysed using IDEAS analytical software (Amnis). CD56+ CD3- events were gated using IDEAS and inspected visually to assess CD16 staining. Fluorescence compensation was carried out using cells stained with single antibodies to avoid conflict with the tracking beads used in the ImageStream sheath fluid. Cell samples were run until a minimum of 1000 CD56+ CD3- events were acquired. FMO controls were run for all staining panels in order to determine background fluorescence and allow accurate discrimination of positive versus negative signals.

CHAPTER 3

Developing an in vitro system to image conjugate formation between natural killer cells and erythrocytes infected with *Plasmodium falciparum*

3.1 Introduction

3.1.1 NK cell conjugate formation with malaria-infected erythrocytes

Several studies have provided evidence that NK cells are able to discern the difference between uninfected erythrocytes and erythrocytes infected with malaria parasites, forming stable conjugates with the latter.

In early flow cytometry studies conducted by Artavanis-Tsakonas et al. in 2003, fluorescent events positive for both NK markers and erythrocyte markers were found to occur more frequently when the erythrocytes were infected with *Plasmodium falciparum*, suggesting the preferential formation of conjugates with infected cells. Physical contact between NK cells and infected erythrocytes was also found to be necessary for production of IFN-γ by NK cells [188].

In 2005 Korbel et al. directly observed physical conjugates forming between purified human NK cells and *P.falciparum*-infected erythrocytes after as little as 15 minutes co-culture using confocal microscopy [190]. In 2007, Baratin et al. similarly observed the formation of 'rosettes' between immortalised NK92 clonal NK cells and *P. falciparum*-infected erythrocytes using light microscopy, each rosette consisting of multiple erythrocytes bound to a single NK92 cell. However, these rosettes could not be observed forming between infected erythrocytes and NK cells derived from peripheral blood [103].

More recently in 2014, Chen et al. directly observed purified NK cells interacting with and killing *P. falciparum*-infected erythrocytes using live video microscopy. On average, NK cell conjugates with infected erythrocytes lasted 11-fold longer with than with uninfected erythrocytes, indicating an ability for NK cells to discern between the two [40].

3.1.2 Evidence for cytotoxic killing of infected erythrocytes as a result of conjugation

In additional to observing NK cells preferentially forming conjugates with *Plasmodium falciparum*-infected erythrocytes versus uninfected, several studies have suggested that these conjugates lead to killing of infected erythrocytes through NK cell cytotoxic activity.

An early *in vitro* study by Orago and Facer in 1991 demonstrated that erythrocytes infected with the blood-stage parasite can induce production of cytotoxic mediators from purified peripheral NK cells, leading to apparent killing of infected erythrocytes as determined by chromium release assays [55]. A later study by Hermsen et al. in 2003 showed that there are significantly higher levels of cytotoxic granzymes A and B in the plasma of individuals infected with *Plasmodium falciparum* in both natural and experimental models of infection. This latter study suggests parasitised cells also provoke cytotoxic functions in immune cells *in vivo*, though NK cells were not specifically implicated in this response [199].

In 2005 Korbel et al. observed the reorganisation of cytoskeletal F-actin in purified NK cells bound to *P. falciparum*-infected erythrocytes, indicating the formation of an active immune synapse between NK cells and parasitised cells. This response varied considerable between donors however, with some individuals displaying no reorganisation of F-actin in response to infected erythrocytes [190].

More recently, Chen et al. demonstrated that co-incubation of purified NK cells with *Plasmodium falciparum*-infected erythrocytes leads to significantly reduced parasitaemia, suggesting NK cells have an inhibitory effect on parasite replication. Video microscopy assays conducted in the same study also suggested that direct conjugate formation between purified NK cells and infected erythrocytes led to ‘flattening’ of the erythrocytes, suggested they had been killed through cytotoxic activity [40].

3.1.3 Receptors involved in mediating conjugates

While there is a growing body of evidence suggesting that NK cells can conjugate with and cytotoxically kill malaria-infected erythrocytes, the receptor-ligand interactions that mediate this process are not well understood. Erythrocytes do not express MHC-I (HLA)

molecules, and as such it is generally assumed that any interactions between NK cells and *falciparum*-infected erythrocytes must involve NK cell receptors that do not bind HLA.

One early study has suggested that the NK receptors NKp30 and NKp46 may bind Duffy binding-like (DBL) domain of *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1), expressed on the surface of parasitized erythrocytes [38]. However, this result has since been disputed by a study by Baratin et al. suggesting that PfEMP-1 is not required to induce NK cell activation in the presence of parasites [103].

More recently in 2014 Chen et al. performed a systematic assay utilising antibodies to block the activity of various NK receptors on purified NK cells co-incubated with *falciparum*-infected erythrocytes; including NKG2D, NKp30, NKp44, NKp46, 2B4, CD2, DNAM-1, and LFA-1. Of these, only the blocking of DNAM-1 and LFA-1 led to any significant increase in subsequent parasitaemia, suggesting these co-receptors may be involved in mediating NK cell conjugation with infected erythrocytes [40]. These results concur with earlier work by Baratin et al. which suggested that blocking of LFA-1 significantly reduced NK cell activity in the presence of PRBCs, though Baratin et al. suggested that this was due to its role in binding ICAM-1 expressed on accessory PBMCs such as macrophages [103]. Additional work by Baratin et al. has suggested macrophages provide co-stimulatory signals to NK cells in *in vitro* models of *falciparum* infection [105], and it may be possible that LFA-1 is involved in both direct conjugation to parasitised cells and indirect activation of NK cells through binding of activated accessory PBMCs. However, the ligand(s) for LFA-1/DNAM-1 on PRBCs are currently unknown.

Additional work by Baratin et al. has suggested than the proteoglycan molecule chondroitin sulphate A (CSA) expressed on the surface of NK92 cells, an NK cell tumour line, mediates conjugation of these cells to PRBCs, potentially by binding to the parasite-expressed molecule PfEMP-1, although this appeared not to be required to induce NK cell activation. These results could not be replicated with purified NK cells obtained from peripheral blood however, suggesting this result may not be representative of *in vivo* infections [103].

3.1.4 Limitations of current methodologies

While there is a growing body of research indicating that NK cells may be able to form functional cytotoxic conjugates with erythrocytes infected with *Plasmodium falciparum*, there are several limitations of the models currently in use by researchers.

Firstly, several studies utilise models that exclude the possibility of co-stimulatory interactions between NK cells and accessory PBMCs such as macrophages. Many of the conjugation studies conducted thus far utilise NK cell lines such as NK92 or purified peripheral NK cells incubated alone with parasitised cells [103], [40], [55]. Several studies have suggested that production of IFN-γ by NK cells in the presence of PRBCs is induced in part by direct contact with accessory PBMCs [45], [105], and it is possible that cytotoxic responses may be similarly regulated.

Secondly, many of these studies fail to take into account the diversity in receptor expression and consequent function of different NK cell subsets. NK cells in peripheral blood occupy a spectrum of maturity, from undifferentiated $CD56^{bright}$ NK cells to the differentiated $CD56^{dim}$ CD57+ cells. These cells exhibit varied expression of natural cytotoxicity receptors such as the NCR family, as well as CD16 and KIR receptors [127]. Several studies have also suggested that $CD56^{dim}$ NK cells express higher levels of LFA-1, a receptor suggested to be involved in mediating cytotoxic conjugate formation between NK cells and PRBCs [200,201]. Studies utilising NK92 cell lines fail to account for this diversity as these cells are clonal and typically considered to be $CD56^{bright}$ [195]. Other studies utilising purified peripheral NK cells can be assumed to have a better representation of NK diversity, but this is generally not accounted for during imaging or cytometric assays, and is not considered as a variable in any analyses of conjugate formation with parasitised cells [40].

It would be of value for future research in this area to develop an *in vitro* imaging system that would take these issues into consideration.

3.2 Aims, Hypotheses and Objectives

The aim of the study in this chapter was to develop an *in vitro* imaging system that would facilitate imaging of conjugates between erythrocytes and primary peripheral NK cells. This system would then be used to assess whether NK cells preferentially form conjugates with *Plasmodium falciparum*-infected erythrocytes versus healthy, whilst allowing for a) possible co-stimulatory interactions with accessory PBMCs, and b) categorisation of conjugates based on NK cell CD56 and CD57 expression, or 'maturity'.

My hypotheses were that CD56+ human NK cells preferentially form conjugates with infected erythrocytes versus uninfected, and that the frequency of conjugation would vary depending on the differentiation status of NK cells.

My objectives were to purify peripheral blood mononuclear cell mixtures containing NK cells from the whole blood of healthy donors, co-incubate them for varying time periods with either infected or uninfected erythrocytes, fix the cells to stabilise any conjugates that have formed, and then stain and image the cells using an ImageStreamX Mark II Imaging Flow Cytometer (Amnis).

3.3 Methods

3.3.1 PBMC donors

Five healthy volunteers were recruited from within the London School of Hygiene and Tropical Medicine (LSHTM) through an anonymised blood donation system operated by Carolynne Stanley, a trained phlebotomist. Donors are summarised in Table 3.1 by age, sex, nationality and malarial status. Ethical approval for this system was acquired from the LSHTM Ethics Committee under the ethics reference number 5520, and informed consent was obtained from all donors as stipulated by LSHTM and the appropriate regulatory bodies. 50ml of venous blood from each donor was collected in sterile Falcon tubes with 2 I.U. per ml of sodium heparin added to prevent coagulation**.**

Age/Years		Nationality		Sex		Malarial Status	
Mean	28	White British	5	Male	2	Naïve	5
Median	27	Other	0	Female	3	Exposed	0
Range	18-42						

Table 3.1 Age, nationality, sex and malarial status of donors for this study

3.3.2 Incubating PBMCs with uninfected or infected erythrocytes

PBMCs were isolated from whole blood using the Histopaque 1077 method described in Section 2.2.1. PBMCs were washed and resuspended in parasite culture medium consisting of RPMI 1640 with 5% Albumax II and 1% Hypoxanthine (Gibco) supplemented with 2mM L-glutamine. Erythrocytes infected with a schizont-stage transgenic GFP-expressing 3D7 strain of *Plasmoidum falciparum* were purified to a parasitaemia of >95% using the Percoll method described in Section 2.2.5 and resuspended in parasite culture medium.

PBMCs and either *Plasmoidum*-infected (PRBC) or uninfected (URBC) erythrocytes were then incubated at either a 1:1, 1:3 or 1:9 ratio for either 60, 120 or 180 minutes in 100ul total medium in wells of a 96-well U-bottom plate as described in Section 2.4.2. Cell mixtures were then fixed and stained with 12ul of the antibody cocktail described in Table 3.2 using the method described in Section 2.4.2;

Antibody	**Clone**	**Fluorophore**	**Supplier**	**Volume per well**
Mouse Anti-Human CD56	NCAM16.2	PE-Cy7	BD Biosciences	3µl
Mouse Anti-Human CD3	SK7	APC- eFluor® 780	eBioscience	3µl
Mouse Anti-Human CD57	TB01	eFluor® 450	eBioscience	3µl
Recombinant Human Anti-Human Glycophorin A	REA175	PE	Miltenyi Biotec	3µl

Table 3.2 List of fluorescent antibodies used to stain the surface of NK cells for Imagestream

3.3.3 ImageStreamX Mark II data acquisition

After fixation and staining PBMC and erythrocyte cell mixtures were resuspended in 50µl FACS buffer at a final concentration of 8 x 10^7/ml cells, transferred to 1.5ml Eppendorf tubes (Sigma) and run immediately on an ImageStreamX Mark II Imaging Flow Cytometer (Amnis) using the appropriate lasers to induce fluorescence. Single-stained cell preparations were used to compensate for spectral overlap. Fluorescence positive events were collected using INSPIRE acquisition software (Amnis) and analysed using IDEAS analytical software (Amnis).

Events were first gated on above average Gradient Root Mean Square (RMS) value. High Gradient RMS indicates that an image has a high number of different pixel shades, indicating that it is in focus (Fig. 3.1A). CD56+ CD3- events were then gated to identify NK cells, and then subsequently gated again on levels of CD56 and CD57 expression to categorise NK cells by into three 'maturity' subsets; $CD56^{bright}$ CD57-, $CD56^{dim}$ CD57-, and $CD56^{dim}$ CD57+ (Fig. 3.1A). CD56+ CD3- events were then sorted and ranked based on levels of GFP-parasite expression (Fig. 3.1B) or glycophorin A-erythrocyte membrane expression (Fig. 3.1C) to assess the presence of infected or healthy erythrocytes. 'Double positive' events with at least one NK cell and one erythrocyte were then inspected visually to confirm whether the cells were directly conjugated.

3.4 Results

3.4.1 CD56+ NK cells preferentially form conjugates with erythrocytes infected with *Plasmodium falciparum* schizonts versus healthy erythrocytes

PBMCs were incubated with either medium alone, healthy erythrocytes or parasitized erythrocytes at a 1:3 ratio in parasite culture medium for 60 minutes before being fixed, stained and imaged using an ImageStreamX Mark II. The percentage of CD56+ NK cells forming conjugates with erythrocytes was assessed via the method described in section 3.3.3. The gating strategy used to categorise NK subsets is shown in Figure 3.1A, while the ranking strategy used to determine the presence or absence of conjugates is shown in Figures 3.1B and 3.1C. Representative examples of a non-conjugated NK cell and conjugated NK cell are shown in Figures 3.2A and 3.2B respectively.

No conjugates were found in medium controls as expected, confirming that the Histopaque 1077 PBMC purification had adequately removed all erythrocyte contamination (Fig. 3.2C).

CD56+ CD3- NK cells cell were observed forming conjugates with uninfected erythrocytes in all but one of the individuals tested, with a mean of 1.05% of NK cells forming conjugates after 60 minutes incubation (Fig. 3.2C). However in all individuals tested there was a distinct increase in the frequency of CD56+ CD3- NK cells forming conjugates when incubated with erythrocytes parasitised with schizont-stage *Plasmodium falciparum*, with a mean of 2.54% of NK cells forming conjugates (Fig. 3.2C). This suggested that the NK cells of all donors were able to distinguish between infected and uninfected cell targets. The frequency of conjugation remained unexpectedly low however, so it was decided to further optimise this assay.

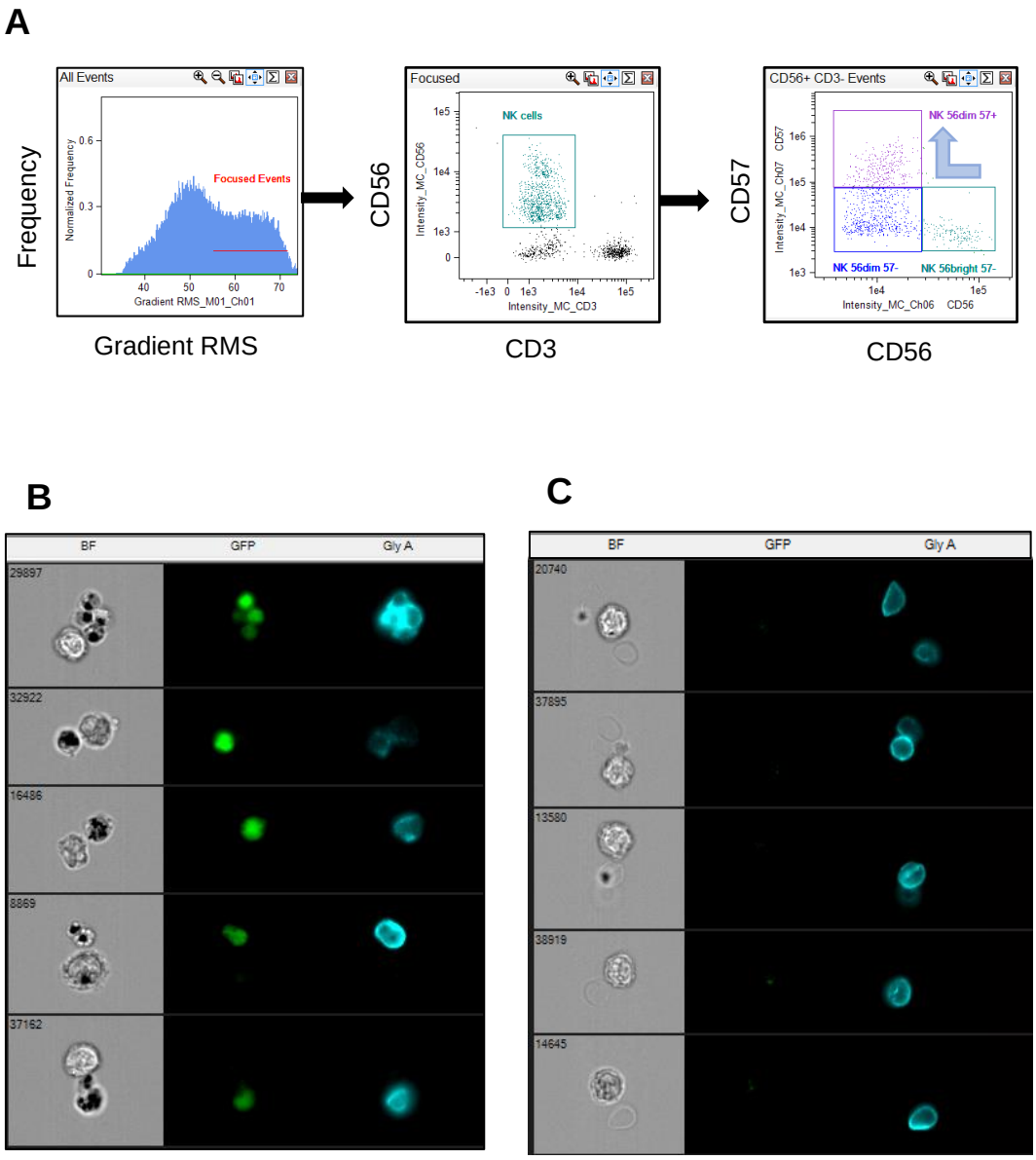


Figure 3.1. Representative IDEAS analysis of NK cells and erythrocytes imaged using confocal flow cytometry

PBMCs were purified from whole blood and incubated with either healthy or parasitized erythrocytes at a 1:3 ratio in parasite culture medium for 60 minutes before being fixed, stained and imaged using an ImageStreamX Mark II. (n = 5)

A) Flow diagram demonstrating method of identifying in-focus $CD56^{dim}$ CD3- NK cells belonging to 3 CD56/CD57 subsets in ascending order of differentiation, or 'maturity'; $CD56^{bright}$ CD57-, $CD56^{dim}$ CD57-, and $CD56^{dim}$ CD57+. The blue arrow on the third diagram shows the pathway of differentiation from least differentiated $CD56^{bright}$ cells to most differentiated $CD56^{dim}$ CD57+.

B-C) Representative example of CD56+ CD3- events from one donor being ranked by B) parasite GFP expression or C) erythrocyte membrane glycophorin A expression to assess frequency of conjugates

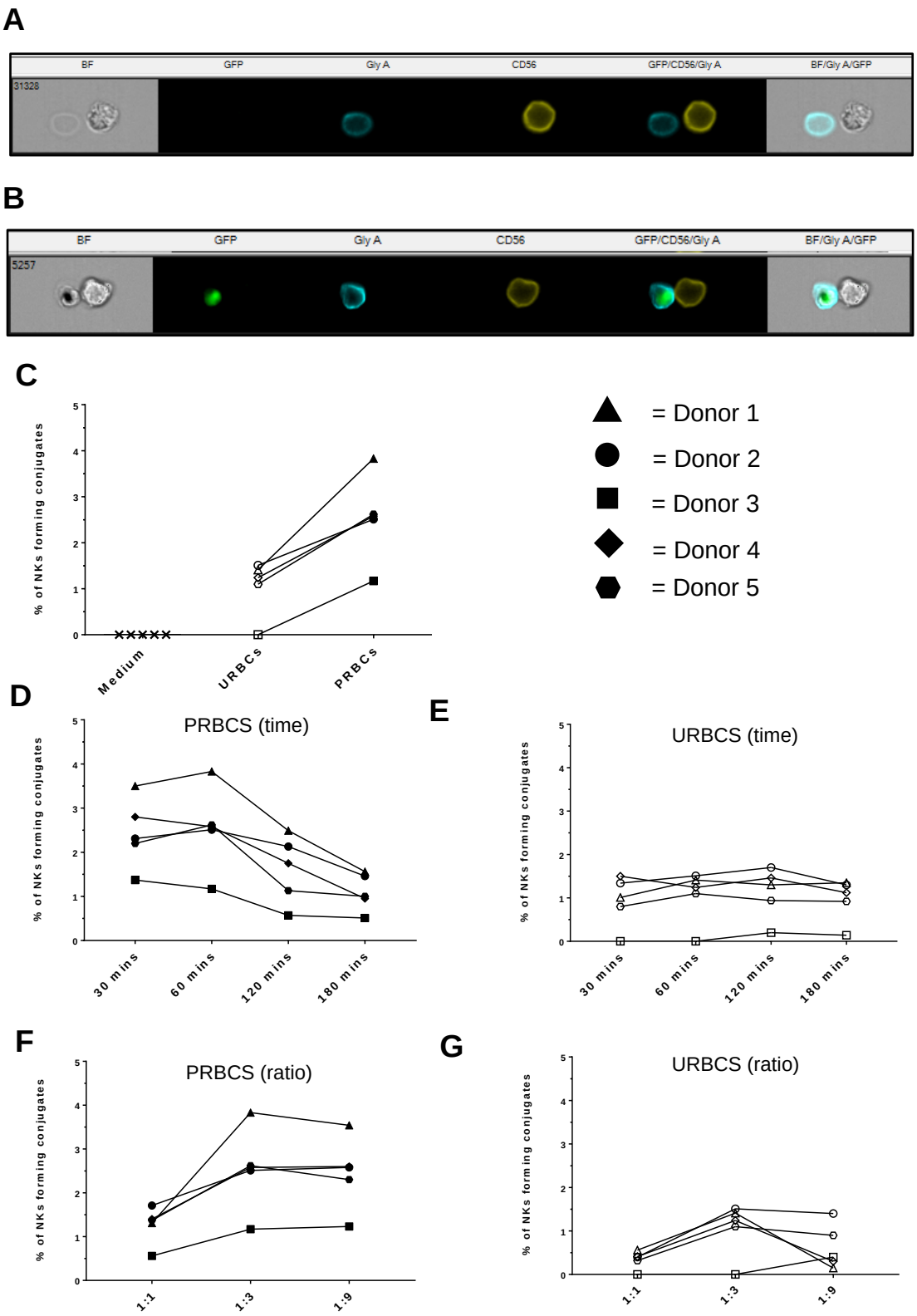


Figure 3.2. Results of conjugation assays between CD56+ NK cells and either uninfected or infected erythrocytes

A-B) Representative images of CD56+ NK cells incubated with A) unconjugated healthy erythrocytes or B) conjugated erythrocytes infected with *Plasmodium falciparum* schizonts. (BF = ^{bright} field; GFP = parasite expressing green fluorescent protein; Gly A = erythrocyte membrane stained with anti-glycophorin A antibody; CD56 = NK cell membrane stained with anti-CD56 antibody)

C) PBMCs were incubated with either medium alone, healthy erythrocytes or parasitized erythrocytes at a 1:3 ratio for 60 minutes before imaging to assess conjugate frequency. Each line represents one donor without replicates.

D-E) PBMCs were purified from whole blood and incubated with either D) PRBCs or E) healthy erythrocytes at a 1:3 ratio for 60, 120 or 180 minutes before imaging to assess conjugate frequency. Each line represents one donor without replicates.

F-G) PBMCs were incubated with either F) PRBCs or G) healthy erythrocytes at a 1:1, 1:3 or 1:9 ratio for 60 minutes before imaging to assess conjugate frequency. Each line represents one donor without replicates.

3.4.2 The percentage of CD56+ NK cells forming conjugates with erythrocytes infected with *Plasmodium falciparum* schizonts declines over time

In a time course assay conducted over 180 minutes, PBMCs from all donors were incubated with either healthy or parasitized erythrocytes at a 1:3 ratio. Samples were taken after 30, 60, 120 and 180 minutes before being fixed, stained and imaged as previously described.

Conjugates were observed forming between CD56+ CD3- NK cells and infected erythrocytes at all time points, but were most frequent at the second earliest time point of 60 minutes incubation with a mean conjugate frequency of 2.54%, though conjugate frequency was almost as high after only 30 minutes with a mean of 2.44% (Fig. 3.2D). After 120 minutes the mean conjugate frequency declined to 1.61%, and finally to 1.1% after 180 minutes (Fig. 3.2D). No such effect was observed with uninfected erythrocytes, where the frequency of conjugation remained low throughout the assay with a mean frequency of 0.93% after 30 minutes incubation, 1.05% after 60 minutes, 1.12% after 120 minutes and 0.97% after 180 minutes (Fig. 3.2E). This suggested that the NK cells of all donors were able to distinguish between infected and uninfected cell targets until at least 180 minutes after incubation, at which point the frequency of conjugates was similar for both infected and uninfected erythrocytes.

3.4.3 The percentage of CD56+ NK cells forming conjugates with erythrocytes infected with *Plasmodium falciparum* schizonts increases with increased ratio of erythrocytes

In a titration assay conducted over 60 minutes, PBMCs from all donors were incubated with parasitized erythrocytes at either a 1:1, 1:3 or 1:9 ratio before being fixed, stained and imaged as previously described.

Conjugates were observed forming between CD56+ CD3- NK cells and infected erythrocytes at all ratios, but were infrequent at a PBMC to PRBC ratio of 1:1 with only a mean of 1.27% of CD56+ CD3- NK cells forming conjugates with parasitised targets (Fig. 3.2E). This increased to a mean of 2.54% at a ratio of 1:3, indicating a titre dependent effect. However, at a ratio of 1:9 the mean frequency of NK cells forming conjugates with parasitised targets remained relatively steady at 2.47%, indicating this effect is saturated beyond a certain ratio (Fig. 3.2E).

A similar effect was observed with uninfected erythrocytes, with the mean frequency of conjugation increasing from 0.34% at a 1:1 ratio to 1.05% at a 1:3 ratio before declining slightly to 0.64% at 1:9 (Fig. 3.2F). However, the mean frequency of conjugation with uninfected erythrocytes was lower than with PRBCs for all ratios, with a difference of 0.93-1.9% in mean conjugate frequency between uninfected and infected erythrocytes (Fig. 3.2F). This provided further evidence that the NK cells of all donors were able to distinguish between infected and uninfected cell targets.

3.4.4 Conjugates form between *Plasmodium falciparum*-infected erythrocytes and all NK cell differentiation stages

PBMCs were purified from whole blood and incubated with parasitized erythrocytes at a 1:3 ratio in parasite culture medium for 60 minutes to optimise conjugate formation according to previous results (Fig. 3.2D, Fig. 3.2F). Cells were then fixed, stained subgated into three differentiation subsets ascending from least to most differentiated; $CD56^{bright}$ CD57-, $CD56^{dim}$ CD57- and $CD56^{dim}$ CD57+ (gating strategy shown in Fig. 3.1A).

Conjugates could be seen forming between PRBCs and $CD56^{bright}$ CD57- NK cells (Fig. 3.3A), $CD56^{dim}$ CD57- NK cells (Fig. 3.3B) and $CD56^{dim}$ CD57+ NK cells (Fig. 3.3C), indicating the potential of NK cells at all differentiation stages to interact with PRBCs.

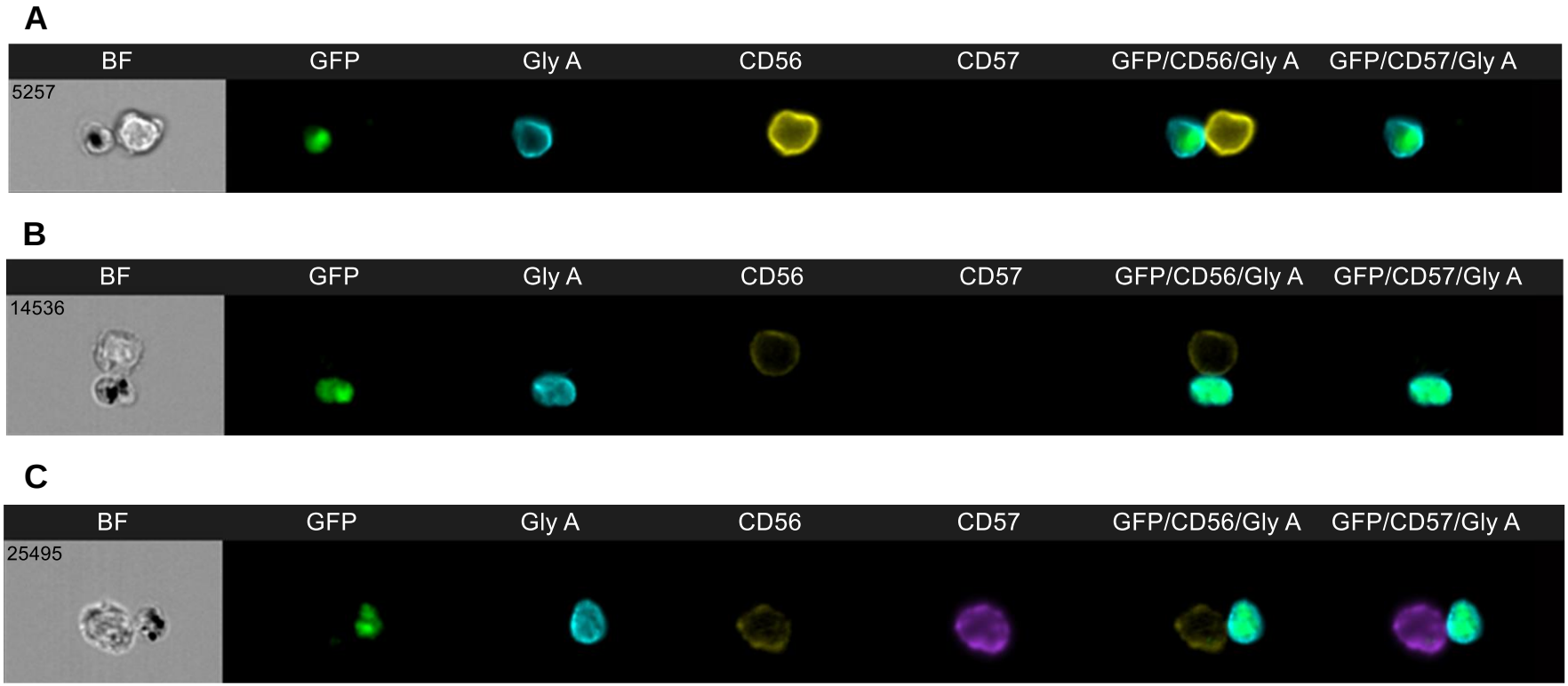


Figure 3.3. Representative images of CD56+ CD3- NK cell subsets forming conjugates with *Plasmodium falciparum*-infected erythrocytes

A-C) Representative images of erythrocytes infected with schizont-stage *Plasmodium falciparum* forming conjugates with; A) $CD56^{bright}$ CD57- NK cells; B) $CD56^{dim}$ CD57- NK cells; C) $CD56^{dim}$ CD57+ NK cells (BF = ^{bright} field; GFP = parasite expressing green fluorescent protein; Gly A = erythrocyte membrane stained with anti-glycophorin A antibody; CD56 = NK cell membrane stained with anti-CD56 antibody; CD57 = NK cell membrane stained with anti-CD57 antibody) (n = 5)

3.4.5 ImageStream has limited application for enumeration of NK cell subset specific conjugates

The initial aim of this assay was to determine whether any of the aforementioned NK differentiation subsets are more or less likely to form conjugates with PRBCs in comparison to the other subsets. If so, this would potentially indicate selective expression of receptors on certain subsets that mediate conjugate formation. Unfortunately, the ImageStreamX Mark II imaging flow cytometer assay established in this study proved an inviable method to determine this. The reasoning for this will be discussed in greater detail in the relevant discussion section.

3.5 Discussion

A number of studies conducted by various research groups over the years have suggested that NK cells are able to preferentially form conjugates with erythrocytes infected with malaria parasites in comparison to uninfected erythrocytes cultured under the same conditions [40,103,188,190]. However, many of these studies utilise purified NK cells [61] or NK92 cell lines [103] when conducting conjugation assays, ignoring the potential role of cytokine exposure or physical contact with accessory cells in upregulating conjugate formation. Numerous studies conducted in viral and cancer models have indicated that NK cell cytotoxic activity against targets is enhanced when cultured in contact with accessory cells within PBMCs such as macrophages and dendritic cells, involving both enhanced presentation of cytokines such as IL-15, IL-12, IL-18 and IFN-β [52], [47], [60], as well as direct activation of NK cell receptors by PBMC-expressed ligands such as MICA and MICB [60], [62].

Additionally, though the role of accessory cell contact in mediating cytotoxic responses against malaria-infected cells has not been fully explored, several studies have indicated that IFN-γ production by NK cells in response to malaria is upregulated by physical contact between PBMCs [103], [105]. Given that many of the signals required for IFN-γ production by NK cells are also necessary for cytotoxic activity, it is reasonable to assume that optimal cytotoxic responses against malaria may also require such contact-dependent signals.

Most if not all of the aforementioned conjugation studies also do not distinguish between different NK cell subsets. In peripheral blood NK cells fall along a spectrum of cytotoxic 'maturity'; ranging from undifferentiated, or 'naïve', $CD56^{bright}$ CD57- NK cells expressing low levels of activating NK cell receptors such as NKG2C, KIRS and CD16 (reviewed in [119]), and differentiated $CD56^{dim}$ CD57+ NK cells expressing higher levels of CD16 and KIRs and lower levels of inhibitory NKG2A (reviewed in in [119]). Consequently the latter are typically considered to be the most efficient at antibody-independent cytotoxic killing of cancerous and virally-infected cellular targets. It therefore seems plausible that these cells could form cytotoxic conjugates more frequently with targets infected with an intracellular parasite such as *Plasmodium*.

In this study I therefore set out to develop an *in vitro* imaging assay that would permit imaging of primary NK cells obtained from human peripheral blood forming conjugates with *Plasmodium falciparum*-infected erythrocytes whilst allowing for accessory cell contact during incubation. I also set out to stain and image these conjugates using

markers of NK cell maturity to assess whether different NK subsets varied in their ability to form conjugates.

3.5.1 Primary human CD56+ NK cells preferentially form conjugates with erythrocytes infected with *Plasmodium falciparum* schizonts, but this occurs less frequently than previously reported

Numerous studies conducted over the previous few decades by various research groups have suggested that NK cells are able to discern the difference between uninfected erythrocytes and erythrocytes infected with malaria parasites. In flow cytometry studies using peripheral PBMCs incubated with either healthy or infected cells, fluorescent events positive for both NK markers and erythrocyte markers are much more frequent when erythrocytes are infected with *P. falciparum* schizonts [188]. In confocal imaging studies using purified NK cells conjugates can also be seen forming between purified human NK cells and infected erythrocytes after a short time in co-culture [190]. Additional imaging studies using NK92 cell lines have observed 'rosettes' consisting of multiple erythrocytes bound to a single NK92 cell forming with infected erythrocytes but not uninfected, though these rosettes could not be observed forming between infected erythrocytes and NK cells derived from peripheral blood [103].

In this study PBMCs were purified from whole blood and incubated with either healthy erythrocytes or parasitized erythrocytes under optimal conditions in parasite culture medium. Cell mixtures were then imaged using an ImageStreamX Mark II to determine the percentage of CD56+ NK cells forming conjugates with erythrocytes. In keeping with expectations, in all individuals tested there was a noticeable increase in the frequency of NK cells forming conjugates when incubated with erythrocytes parasitised with schizont-stage *Plasmodium falciparum* (Fig. 3.2C). This suggested that the NK cells of all donors were able to distinguish between infected and uninfected cell targets in keeping with prior published results obtained by other researchers [40,103,188,190].

However, the frequency of conjugation between infected erythrocytes and NK cells remained surprisingly low even under optimised conditions, as previous researchers have repeatedly reported much higher values. In 2003 Artavanis-Tsakonas et al. suggested that NK cells can form what appear to be conjugates with infected erythrocytes at a frequency of near 60% when co-cultured for 30 minutes at a 1:3 ratio (PBMCs:PRBCs) [188]. These conjugates were defined as any event testing 'double-positive' using flow cytometry with CD56 as an marker for NK cells and glycophorin A

as a marker for erythrocytes [188]. However, as Artavanis-Tsakonas et al noted themselves in their study, these events could not be confirmed definitively as conjugates as the events were not imaged directly. Prior research by other imaging groups has indicated that there is transfer of cellular membrane material from target cells to NK cells during the formation of an immune synapse [202]. It is therefore possible that the high frequency of CD56+ glycophorin A+ events found by Artavanis-Tsakonas et al using cytometry may have been due in part to transfer of glycophorin A+ erythrocyte membrane material to NK cells after conjugation. This would still suggest a high degree of conjugation had taken place between NK cells and infected erythrocytes, though it creates difficulty in using cytometry alone to distinguish between active conjugates consisting of NK and target cell bound together and NK cells that have retained prior membrane material from previous encounters.

In 2005 Korbel et al also noted a high frequency of conjugate formation between NK cells and infected erythrocytes when imaged using confocal microscopy, with conjugate frequencies of between 40-50% after only 15 minutes incubation at a 1:3 ratio (NK cells :PRBCs) [190]. Though confocal imaging can be considered a more reliable technique than flow cytometry, it is worth noting that this study utilised purified primary NK cells for assays rather than mixed PBMCs. The absence of physical interference from accessory cells and relatively high ratio of NK cells to infected targets may have resulted in greater physical contact between NK cells and targets in comparison to the assay described in this thesis, leading to artificially higher frequencies.

In 2007 Baratin et al reported a similar result as Korbel et al, observing NK cells forming rosettes with infected erythrocytes at the relatively high frequency of 26.7% after one hour of co-culture at a 1:10 ratio (NK92s:PRBCs) [103]. However, these results suffer from similar issues to those reported by Korbel et al, as Baratin et al utilised a NK92 cell line rather than primary PBMCs which may have overinflated the frequency of conjugation. Furthermore, NK92 cells are a tumour line derived from early stage NK cells, and do not truly reflect the functional differentiation of primary NK cells found in blood.

Regardless of variation between the absolute frequency of conjugation found in this study and the results of other researchers, this study confirms prior observations that CD56+ CD3- NK cells preferentially form conjugates with erythrocytes infected with *Plasmodium falciparum* compared to uninfected erythrocytes.

3.5.2 Conjugation between primary human CD56+ NK cells and erythrocytes infected with *Plasmodium falciparum* schizonts occurs rapidly but declines over time

Previous studies investigating conjugate formation between NK cells and infected erythrocytes have suggested that this process occurs fairly rapidly in co-culture, with some studies suggesting that NK cells are able to begin forming conjugates in less than an hour of co-culture [190]. The results of the assays described in this thesis support these conclusions, as conjugates between primary NK cells and infected erythrocytes could be observed forming between primary NK cells and infected erythrocytes within only 30 minutes co-culture and at a higher frequency than with uninfected control erythrocytes (Fig. 3.2D).

Prior research has suggested that the frequency of conjugates forming between NK cells and infected erythrocytes declines rapidly in co-culture. In their 2003 flow cytometry study, Artavanis-Tsakonas et al found that the frequency of conjugation between NK cells and infected erythrocytes declined by over 50% after only two hours of co-culture, eventually reaching frequencies similar to with uninfected control cells by 150-180 minutes [188]. During the study described in this thesis it was decided to evaluate whether this result could be replicated using high-throughput imaging as a more reliable technique of assessing conjugate formation over time.

A time course assay was carried out where PBMCs were purified from whole blood and incubated with parasitised erythrocytes for either 30, 60, 120 or 180 minutes. At the earlier time points of 30 and 60 minutes the mean frequency of conjugation was roughly equivalent but began to decline after 120 minutes. After 180 minutes co-culture conjugate frequency between NK cells and infected erythrocytes declined to a level on par with uninfected erythrocytes, similar to the effect seen by Artavanis-Tsakonas et al [188].

There are several potential explanations for these results. Perhaps the simplest explanation may be that as time passes, NK cells lyse infected erythrocytes through cytotoxic activity and consequently there are fewer and fewer erythrocytes are available for conjugation at later time points. In prior studies cytotoxic mediators have been detected in cellular supernatants after co-culture of NK cells with *Plasmodium*-infected erythrocytes [55], and at least one research group has observed NK cells directly lysing

infected erythrocytes after conjugation [40] which would seem to support this interpretation.

Several studies have also demonstrated that NK cells participate in 'serial killing', conjugating to and killing up to 5 separate target cells sequentially before being depleted of cytotoxic capacity unless restimulated with cytokine or antibody [203] [204]. This can involve both depletion of cytotoxic mediators such as perforin and granzymes [204], as well as downregulation of receptors used to form activating contacts such as CD16, 2B4 and DNAM-1 [205]. Additional studies have suggested that once NK cells have exhausted their killing capacity they may become apoptotic [206]. The decline in conjugate frequency between NK cells and infected erythrocytes seen in this study may therefore be a result of serial lysis of infected erythrocytes by NK cells, leading to both fewer viable erythrocyte targets and fewer effectors as NK cells become anergic or die through apoptosis.

3.5.3 Conjugate formation by distinct CD56/CD57 NK cell subsets

One of the initial aims of this study was to determine whether different CD56/CD57-defined NK cell subsets are more likely to form conjugates with parasitised erythrocytes. If so, this would potentially indicate selective expression of receptors on certain subsets that mediate conjugate formation. This could then be investigated with conjugation assays utilising blocking antibodies against receptors that are known to vary between subsets to determine their role in conjugate formation [126]. Unfortunately, the ImageStreamX Mark II imaging flow cytometer assay study proved to be an impractical method to determine this.

Acquisition of fluorescent events is considerably slower using ImageStream relative to conventional flow cytometry due to the need to image each event as it passes through the flow cell using a set of confocal cameras. Renting the ImageStream equipment is also a far more expensive technique than in-house flow cytometry, with per-hour costs in excess of seven times higher than conventional techniques. The combination of these factors prohibited a thorough investigation into the relative ability of different NK cell maturity subsets to form conjugates with parasitised cells, as acquiring a sufficiently high number of CD56+ events for each maturity subset to perform a valid comparison could not be achieved within an acceptable budget.

Distinct CD56/CD57 maturity subsets are clearly present in these data (Fig. 7A), and conjugates could be observed forming between NK cells of each of these subsets and

infected erythrocytes (Fig. 9A-9C). However, comparing the absolute frequency of conjugates between subsets becomes unreliable when subsets comprise of only a hundred or few hundred total acquired events, particularly when conjugates are infrequent. Under these conditions, a difference of only a few events can translate into large percentage differences. As with all techniques utilising fluorescence, ImageStream is vulnerable to the effects of cellular autofluorescence or non-specific antibody staining resulting in a certain level of fluorescent noise [207]. Whilst with high event numbers these issues are generally considered negligible, their capacity to skew the interpretation of data is much higher with low event numbers. Given these issues, it was decided that ImageStream is an unreliable technique to evaluate this hypothesis under our original parameters.

Previous researchers have reported observing much higher frequencies of conjugate formation when using purified NK cells or NK cell lines rather than mixed peripherical blood mononuclear cells, with frequencies often in the double-digits [188], [190], [103]. Consequently, one possible method of compensating for the issue of low conjugate frequency in future work with ImageStream may be to abandon the concept of allowing for accessory PBMC contact and cytokine production and simply use purified peripheral NK cells or an NK92 cell line. Whilst this would diminish the extent to which the results could be extrapolated to real *in vivo* infections, it would perhaps allow for more efficient data acquisition and subsequent analysis of the relative conjugation frequencies of CD56/CD57 subsets. Additionally, whilst physical accessory cell contact with PBMCs would be difficult to mimic under these conditions, the effect of cytokines such as IL-12 and IL-18 produced by accessory cells could perhaps be simulated by supplementation of culture medium with soluble cytokines.

CHAPTER 4

Investigating mechanisms of CD16 downregulation during antibody-dependent natural killer cell responses

4.1 Introduction

4.1.1 CD16: function and expression on NK cells

CD16 (FcγRIII) is an activating receptor belonging to the immunoglobulin superfamily of proteins. It is ubiquitously expressed on $CD56^{dim}$ NK cells, and its expression is upregulated alongside increased expression of CD57 as NK cells differentiate into a more differentiated state, resulting in significantly enhanced antibody-dependent responses in this subset [126]. CD16 binds the Fc region of immunoglobulin G molecules produced by B-lymphocytes. When multiple CD16 molecules bind immunoglobulin in proximity the receptors become cross-linked, activating NK cells to direct cytotoxic activities against opsonised cellular targets, and in some circumstances, to produce pro-inflammatory cytokines such as IFN-γ and TNFα [208,209].

Signalling through CD16 occurs by association with either CD3ζ or FcεRIγ, transmembrane protein chains containing immunoreceptor tyrosine-associated activation motifs (ITAMs) used to initiate signalling cascades [66], [210]. Cross-linking of CD16 and subsequent phosphorylation of ITAM residues results in increased intracellular calcium levels, inducing the activity of nuclear factors NFATp and NFATc which initiate DNA transcription [211].

4.1.2 Downregulation of CD16 after ligation of immunoglobulin G

Once CD16 has been cross-linked by antibody and has successfully transduced an activating signal to the NK cell, it is rapidly downregulated on the surface of the cell [212]. ADAM17, a metalloproteinase expressed by the majority of leukocytes, mediates this process. ADAM17 plays a broad role in shedding of leukocyte receptors such as TNFRI and adhesion molecules such as CD62L in addition to CD16 [213]. After CD16 ligation by IgG, ADAM17 cleaves CD16 at a membrane proximal cleavage region, leading to reduced sensitivity to antibody-dependent activation [214]

4.1.3 CD16 downregulation: a role for internalisation

Several studies have validated the role of ADAM17 in CD16 cleavage from the NK surface resulting in downregulated antibody-sensitivity [213,214]. However, there are sporadic reports indicating that a second mechanism of CD16 downregulation involving internalisation into the cell may also be involved.

In 2007 Cecchetti et al performed a series of experiments utilising confocal microscopy to image NK cells after stimulation with antibody, and reported seeing CD16 enter the cytoplasm from the cell surface [215]. This process was dependent on a functional actin cytoskeleton, and Cecchetti et al therefore theorised that CD16 may also be internalised into the cell for recycling or degradation via actin-dependent endocytosis as is the case with many other NK receptors [215], [216].

Subsequent attempts to replicate these results have seemingly contradicted these findings, though a limited number of studies have investigated the issue [212] [213]. There remains a valid question therefore whether CD16 downregulation on antibody-stimulated NK cells is purely a result of shedding by ADAM17, or whether a secondary mechanism exists by which CD16 is internalised for possible recycling or degradation.

4.2 Aims, Hypotheses and Objectives

The aims of the study in this chapter were to establish a reliable assay that induces downregulation of CD16 and degranulation in NK cells in response to influenza antigen and immune plasma. This assay will then be used to investigate whether the downregulation of CD16 seen on NK cells after this stimulation is purely a result of metalloprotease-mediated shedding from the cell surface, or whether a second mechanism exists by which CD16 is internalised into the cell.

My hypotheses were that influenza antigen-antibody complexes would induce significant downregulation of CD16 on NK cells leading to degranulation, and that this downregulation would be partly a result of internalisation of CD16 into NK cells.

My objectives were to purify NK cells from the whole blood of healthy donors, incubate them in the presence of influenza-immune serum acquired from vaccinated donors and trivalent influenza vaccine antigen, stain and fix the cells, and then run the resultant cell mixtures through either an LSRII flow cytometer (BD Biosciences) to assess CD16 downregulation and functional activity, or through an ImageStreamX Mark II Imaging Flow Cytometer (Amnis) to image the spatial distribution of CD16.

4.3 Methods

4.3.1 European PBMC donors for Imagestream

5 healthy volunteers were recruited from within the London School of Hygiene and Tropical Medicine (LSHTM) through an anonymised blood donation system operated by Carolynne Stanley, a trained phlebotomist. Donors are summarised in Table 4.1 by age, nationality and sex. Ethical approval for this system was acquired from the LSHTM Ethics Committee under the ethics reference number 6237, and informed consent was obtained from all donors as stipulated by LSHTM and the appropriate regulatory bodies. 50ml of venous blood from each donor was collected in sterile Falcon tubes with 2 I.U. per ml of sodium heparin added to prevent coagulation**.** Peripheral blood mononuclear cells were purified for use in imaging experiments.

Age/Years		**Nationality**		**Sex**	
Median	34	White British	5	Male	1
Range	27-53	Other	0	Female	4

Table 4.1 Age, nationality and sex of donors for this assay

4.3.2 Influenza-immune European PBMC and plasma donors for flow cytometry

Prior to the current study, 17 healthy volunteers had been recruited from within the London School of Hygiene and Tropical Medicine (LSHTM) through an anonymised blood donation system operated by Carolynne Stanley, a trained phlebotomist. Donors are summarised in Table 4.2 by age and vaccination status. All donors received a single dose of 2012–2013 seasonal trivalent influenza vaccine (TIV) by the intramuscular route (Split Virion BP, Sanofi Pasteur MSD), performed by the study clinician Dr Ron Behrens who provided medical supervision for the original visit as well as follow-up care in the event of side effects. Ethical approval for this system was acquired from the LSHTM Ethics Committee under the ethics reference number 6237, and informed consent was obtained from all donors as stipulated by LSHTM and the appropriate regulatory bodies. 50ml of venous blood from each donor was collected in sterile Falcon tubes with 2 I.U. per ml of sodium heparin added to prevent coagulation. Blood samples were collected immediately after vaccination and at various time points after vaccination. Purification and storage of frozen PBMC and plasma for use in future

experiments and quantitation of anti-TIV IgG was coordinated Dr Martin Goodier at LSHTM.

Age/Years		Influenza Vaccines Received Prior to Study		Vaccines Received During Study	
Median	37.5	None	17	Intramuscular TIV	17
Range	21-63	Intramuscular TIV	0	Other	0
		Intranasal TIV	0		

Table 4.2 Donor **ag**e, prior vaccine status and vaccines received for this assay

4.3.3 Incubating PBMCs with trivalent flu vaccine for imaging flow cytometry

PBMCs were isolated from whole blood using the Histopaque 1077 method described in Section 2.2.1. PBMCs were washed and resuspended in RPMI 1640 supplemented with 2mM L-glutamine. 2×10^6 freshly isolated PBMC were then cultured for a total of 5 hours with inactivated TIV (Split Virion BP, Sanofi Pasteur MSD) in 100ul total medium supplemented with either 5% fetal calf serum or 1% pooled immune plasma acquired from influenza-exposed donors (PIP) diluted with FCS to give a total concentration of 5% serum in wells of a 96-well U-bottom plate as described in Section 2.4.3. PBMCs were then stained with 12ul of the antibody cocktail described in Table 4.3 using the method described in section 2.4.3.

Antibody	Clone	Fluorophore	Supplier	Volume per well
Mouse Anti-Human CD56	B159	PE-Cy7	BD Biosciences	3µl
Mouse Anti-Human CD3	UCHT1	PE	BD Biosciences	3µl
Mouse Anti-Human CD16	3G8	APC	Biolegend	3µl
Mouse Anti-Human CD57	TB01	E450	eBioscience	3µl

Table 4.3. List of fluorescent antibodies used to stain the surface of NK cells for ImageStream

After staining cells were washed twice in FACS buffer (PBS, 1% EDTA, 1% FCS, 0.01% sodium azide) a blocking step was performed for an additional 15 minutes with 3ul of unconjugated Mouse Anti-Human CD16 (Clone:3G8, BD Biosciences) diluted in 12ul total volume of FACS buffer to ensure all remaining CD16 surface epitopes were bound by antibody. Cells were then fixed, permeabilised and stained for a further 30 minutes with 3ul of Mouse Anti-Human CD16 PE-Dazzle 594 (Clone:3G8, Biolegend) diluted in

12ul total volume of FACS buffer to stain intracellular CD16 before being washed as described in Section 2.3.4.

4.3.4 ImageStreamX Mark II data acquisition

After staining and fixation PBMC were resuspended in 50µl FACS buffer at a final concentration of 8 x 10^7 cells/ml, transferred to 1.5ml Eppendorf tubes (Sigma) and run immediately on an ImageStreamX Mark II Imaging Flow Cytometer (Amnis) using the appropriate lasers to induce fluorescence. Single-stained cell preparations were used to compensate for spectral overlap. Fluorescence positive events were collected using INSPIRE acquisition software (Amnis) and analysed using IDEAS analytical software (Amnis). Cell samples were run until a minimum of 1000 CD56+ CD3- events were acquired.

Events were first gated on above average Gradient Root Mean Square (RMS) value. High Gradient RMS indicates that an image has a high number of different pixel shades, indicating that it is in focus (Fig. 4.1A). CD56+ CD3- events were then gated to identify NK cells, and then subsequently gated again on levels of surface CD16 expression to categorise NK cells by into three functional subsets; CD16negative, CD16reduced, and CD16strong (Fig. 4.1A). Events were inspected visually to determine the presence or absence of internalized CD16.

4.3.5 Incubating PBMCs with trivalent flu vaccine for functional flow cytometry

Frozen PBMC samples from European donors were thawed using the protocol described in section 2.2.3. PBMCs were thawed from unimmunised donors and the same donors 2, 4, 6 or 12 weeks after immunisation with the 2012–2013 seasonal trivalent influenza vaccine (TIV) by the intramuscular route (Split Virion BP, Sanofi Pasteur MSD). 2 x 10^5 PBMCs from each donor and timepoint were incubated for 6 hours at 37ºC with 5% CO_2 in wells of 96-well U-bottom plates (ThermoFisher) with autologous plasma alone or autologous plasma with TIV. GolgiStop containing Monensin (BD Biosciences) was added to cultures to form a final 1/1500 concentration and GolgiPlug containing Brefeldin A (BD Biosciences) added to form a 1/1000 final concentration after 1 hour of incubation. Anti-CD107a antibody (FITC-conjugated, clone H4A3, BD Biosciences) was added during culturing at 1µl per 100ul of culture suspension.

After the required period of incubation, cells were washed and stained for 30 minutes at 4^{o}C in the dark with 10µl total of the surface antibody cocktail described in Table 4.4 using the protocol as described in section 2.4.1. Dead and dying cells were excluded using APC-efluor780-conjugated fixable viability dye (eBioscience).

Antibody	Clone	Fluorophore	Supplier	Volume per well
Mouse Anti-Human CD56	B159	PE-Cy7	BD Biosciences	1ul
Mouse Anti-Human CD3	UCHT1	V500	BD Biosciences	1ul
Mouse Anti-Human CD57	TB01	eFluor® 450	eBioscience	1ul
Mouse Anti-Human CD16	CB16	APC	eBioscience	1ul
Mouse Anti-Human CD69	FN50	PE	eBioscience	1ul

Table 4.4. List of fluorescent antibodies used to stain the surface of NK cells for flow cytometry

Cells were then washed, fixed, rewashed and resuspended in 300µl FACS buffer. Cells were transferred to alpha tubes (Alpha labs) and stored at 4^{o}C until run on a BD LSRII Flow Cytometer (BD Biosciences) using the appropriate laser set. Data was collected using the complementary FACSDiva software (BD Biosciences) and analysed using FlowJo, v.10 (FlowJo LLC). Fluorescence compensation was carried out using OneComp eBeads Compensation Beads (eBioscience) stained with single antibodies. This assay was performed in collaboration with Dr Martin Goodier and Chiara Lusa, an MSc student.

4.3.6 Statistical analyses

Statistical analyses were performed with Prism 7.03 (Graphpad), as specified for each experiment. Data were excluded when the gated cell subset contained fewer than the required minimum number of cells. All statistical tests are two-sided, with p values stated as follows; **** <p 0.0001; *** p< 0.001; ** p< 0.01; * p< 0.05. Specific statistical tests and sample sizes are stated in the relevant figure legends.

4.4 Results

4.4.1 CD56+ CD3- NK cells downregulate CD16 after stimulation with influenza trivalent vaccine antigen and immune plasma but not with either alone

Before conducting CD16 internalisation experiments, we first attempted to establish a reliable assay that induces downregulation of CD16 and subsequent degranulation in NK cells in response to influenza antigen and immune plasma. We used a combination of imaging and conventional flow cytometry to establish this assay.

For imaging experiments, PBMCs were purified from whole blood and incubated with trivalent flu vaccine containing influenza antigen supplemented with either 5% fetal calf serum or 1% pooled immune plasma (PIP) for 6 hours. Cells were then stained, fixed and imaged using an ImageStreamX Mark II. CD56+ CD3- NK cells were gated based on CD16 surface expression level as described in section 4.3.4. (gating shown in Fig. 4.1A and 4.1B)

All donors tested demonstrated a distinct decrease in the proportion of CD56+ CD3- NK cells expressing ‘strong’ levels of surface CD16 after treatment with TIV and PIP in comparison to TIV with control FCS (Fig.4.1C). The percentage of cells expressing strong levels of CD16 decreased from a mean of 47.8% in response to FCS to mean of 20.2% in response to PIP. All donors demonstrated a decrease in NK cells expressing strong levels of CD16, though there was some degree of heterogeneity in this response with a decrease of between 7.6-47.7% for all donors.

At the same time CD56+ CD3- NK cells were observed in all donors exhibiting a complementary reduction in the levels of surface CD16 after treatment with TIV in the presence of PIP, but not with FCS (Fig. 4.1D). The percentage of cells expressing ‘reduced’ levels of CD16 increased from a mean of 19% in response to FCS to mean of 44.2% in response to PIP. Interestingly, substantial heterogeneity was observed in the extent of CD16 down-regulation responses despite the same pooled immune plasma being used in the different individuals tested. This indicated that cell intrinsic factors may influence the extent of the antibody-dependent response in different individuals.

A representative example of an NK cell stained and imaged for CD16 is shown in Figure 4E.

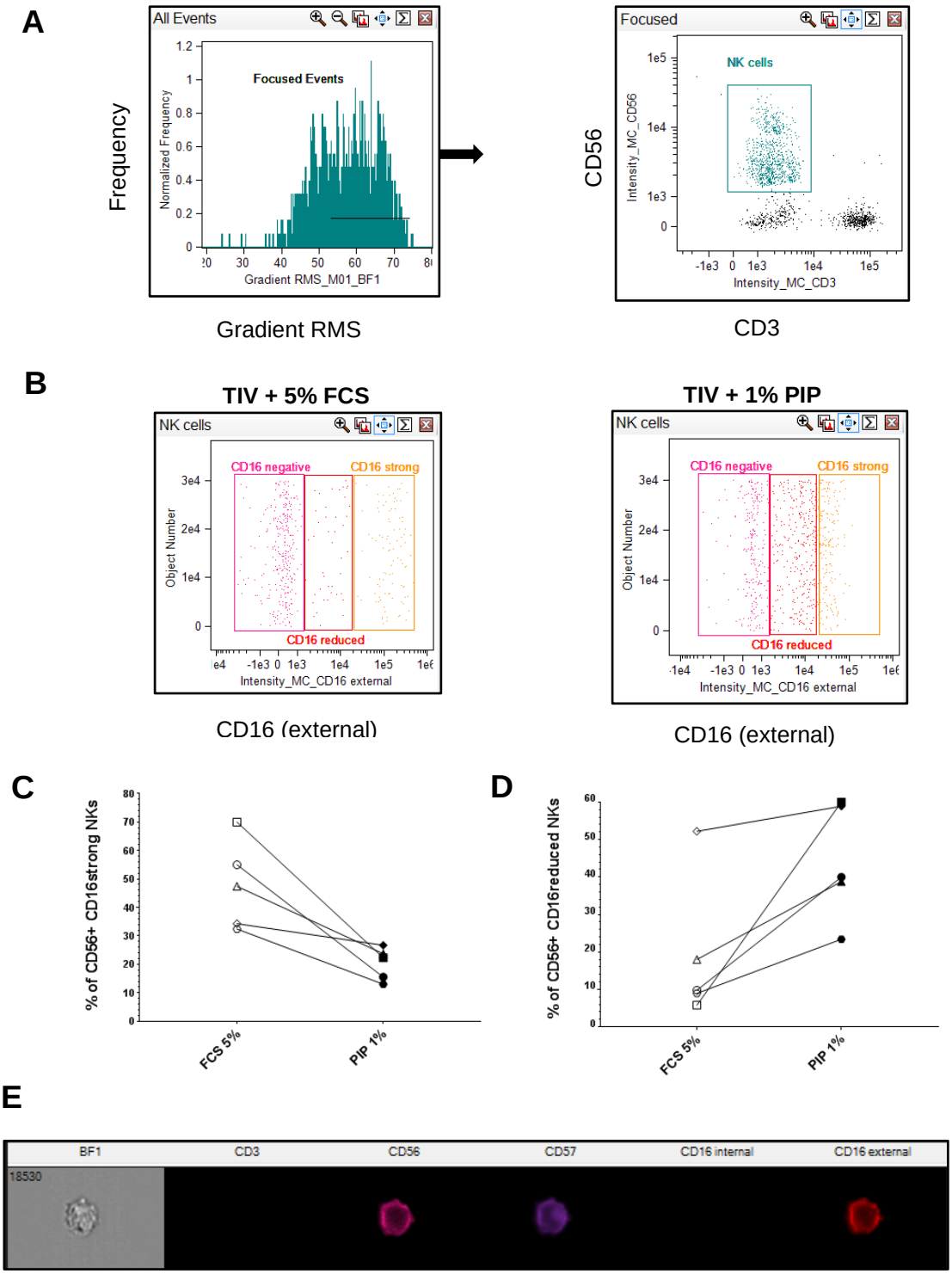


Figure 4.1 Representative ImageStream gating and imaging of TIV-exposed NK cells

PBMCs incubated with trivalent influenza vaccine (TIV) supplemented with either 5% fetal calf serum (FCS) or 1% pooled immune plasma (PIP) in RPMI 1640 with 2mM L-glutamine for 5 hours before imaged using an ImageStreamX Mark II.

A) Flow diagram demonstrating method of identifying in-focus $CD56^{dim}$ CD3- NK cells

B) Examples of CD56+ CD3- events being gated by surface CD16 expression into 3 CD16 subsets; negative, reduced and strong, after incubation with TIV and either FCS (left) or PIP (right)

C-D) PBMCs were incubated with TIV supplemented with either 5% FCS or 1% PIP for 5 hours before flow imaging to assess the percentage of CD56+ CD3- NK cells in either the A) CD16strong or B) CD16reduced subsets

E) Representative image of unstimulated CD56+ CD3- CD57+ CD16strong NK cell (BF = ^{bright} field; CD3 = marker for T cells [absent]; CD56 = anti-CD56 antibody; CD57 = anti-CD57 antibody; CD16 internal = internal anti-CD16 antibody [absent]; CD16 external = surface anti-CD16 antibody

For conventional flow cytometry experiments plasma from 17 donors at 7 time points (immediately after vaccination; or 2, 4, 6, 12. 24 or 36 weeks after intramuscular influenza vaccination) were thawed from frozen stocks. Median anti-influenza antibody titres in the plasma were assessed via ELISA by Dr Martin Goodier and Chiara Lusa, a MSc student, and were found to significantly increase shortly after vaccination, before declining slowly over the following weeks (Fig. 4.2A). Median baseline antibody titres in the study group were 429.9 (IQR = 299.4-767.2) Arbitrary Elisa Units (AEU), while the highest median anti-influenza antibody titres were found 2 weeks after vaccination (1027.0 AEU, IQR:742-1089). It was decided to use plasma from this time point in future experiments assessing functional responses to antibody.

PBMCs from the 17 donors were incubated with 1% autologous plasma collected immediately after vaccination or 2 weeks after vaccination; either alone or with TIV. PBMCs were incubated under these conditions for 6 hours before being stained, fixed and imaged using an LSRII flow cytometer as described in section 4.3.5.

Total $CD56^{dim}$ as well as CD56dim CD57- and CD56dim CD57+ NK cells significantly downregulated CD16 in response to TIV and immunised plasma collected immediately after vaccination in comparison to cells incubated with plasma alone, though this effect was relatively small ($p< 0.05$ for both subsets, Fig. 4.2D).

In contrast, downregulation of CD16 was much more pronounced in all subsets in response to TIV and immunised plasma collected 2 weeks after vaccination in comparison to plasma alone ($p<0.001$ for all subsets, Fig. 4.2D). CD16 MFI was significantly lower in all subsets when incubated with plasma collected 2 weeks post vaccination than plasma collected immediately ($p<0.001$ for all subsets, Fig. 4.2D).

Downregulation of CD16 in the presence of immune plasma and trivalent influenza vaccine antigen was dependent on the presence of antibody, as pooled immune plasma depleted of IgG (7.6 AEU anti-TIV IgG) was unable to induce significant downregulation of CD16 compared to IgG replete pooled immune plasma (413.4 AEU). Trivalent influenza vaccine antigen alone was also unable to induce CD16 downregulation in the absence of immune plasma, indicating the need for antigen-antibody complexes to induce this effect (Fig. 4.2E).

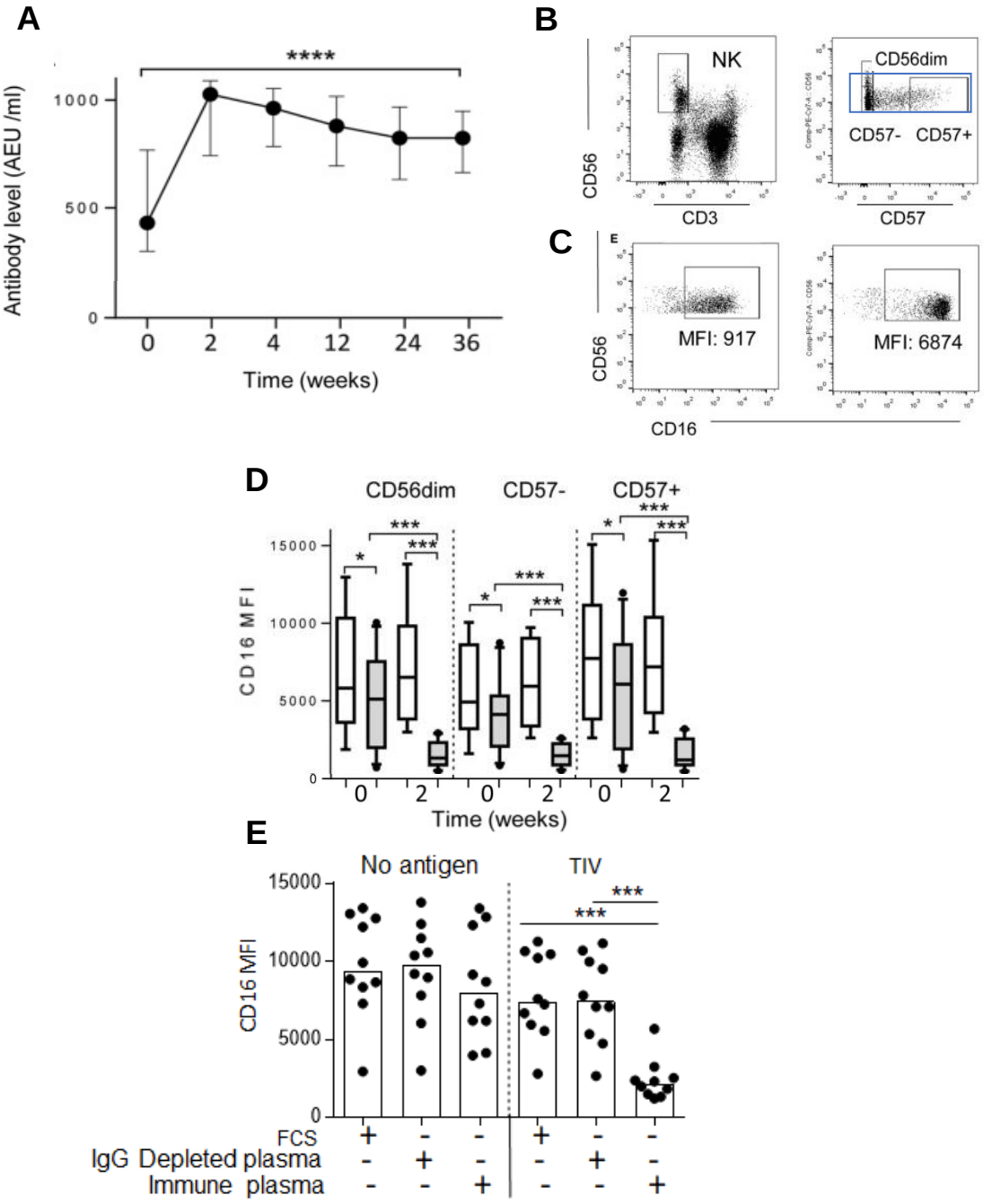


Figure 4.2 Representative flow cytometry gating of TIV-exposed NK cells and graph of anti-influenza ELISA titres over time

A) Graph showing median anti-influenza antibody titres with interquartile ranges at various timepoints after vaccination with intramuscular influenza vaccine (n = 17, one-way repeated measures ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

B) Diagram of gating strategy, including NK cell gate (left), and NK maturity subset gate (right).

C) Diagram of gating strategy for NK CD16 expression. PBMCs incubated with 1% pooled immune plasma either with (right) or without (left) trivalent influenza vaccine (TIV) for 6 hours.

D) PBMCs incubated with 1% pooled plasma collected either prior to vaccination (x-axis = 0) or two weeks post vaccination (x-axis = 2) with (shaded bars) or without (white bars) trivalent influenza vaccine for 6 hours (n = 17, Mann–Whitney U test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Data represent median values with interquartile ranges.

E) PBMCs incubated with either FCS, IgG depleted plasma or immune plasma in either the presence or absence of trivalent influenza vaccine antigen (TIV) for 5 hours (n = 10, Mann–Whitney U test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

These assays were performed in collaboration with Dr Martin Goodier and Chiara Lusa, an MSc student. Figures adapted from Goodier, Lusa and Sherratt et al (2016) *Frontiers in Immunology*

4.4.2 CD56+ CD3- NK cells degranulate after stimulation with TIV antigen and immune plasma and this effect is most prominent in highly differentiated cells

In conventional flow cytometry experiments, PBMCs from 17 donors were incubated with 1% pooled plasma collected immediately after intramuscular vaccination or 2 weeks after vaccination; either alone or with TIV. PBMCs were incubated under the conditions as described in section 4.3.5.

$CD56^{dim}$ NK cells and $CD56^{dim}$ CD57- and $CD56^{dim}$ CD57+ subsets significantly upregulated presentation of CD107a in response to TIV antigen combined with either baseline (week 0) or post- vaccination plasma (week 2) collected immediately after vaccination in comparison to parallel cultures with no antigen. However, this effect was relatively small ($p< 0.05$ for both subsets, Fig. 4.3A).

However, while $CD56^{dim}$ NK cells and differentiation subsets responded to TIV and plasma collected at either time point, the degranulation response was significantly higher in both subsets in response to TIV and immunised plasma collected 2 weeks after vaccination in comparison to plasma collected at baseline (week 0) ($p<0.05$ for all subsets, Fig. 4.3A).

The degranulation response was also noticeably stronger in the CD57+ subset, both when incubated with TIV and plasma collected immediately or TIV and plasma collected after two weeks. After incubation for 6 hours with TIV and plasma collected immediately after vaccination 18% of CD57+ cells begun degranulating in comparison to 10% of CD57-, while after incubation with TIV and plasma collected after 2 weeks 22% of CD57+ cells begun degranulating in comparison to 12% of CD57- (Fig. 4.3A).

Downregulation of CD16 MFI and percentage of NK cells degranulating were significantly inversely correlated in both the $CD56^{dim}$ CD57- ($p< 0.01$, Fig. 4.3B) and $CD56^{dim}$ CD57+ ($p<0.001$, Fig. 4.3B) subsets. Interestingly, this correlation was stronger for the more differentiated CD57+ subset. Increasing immune plasma concentration was also associated with both increased downregulation of CD16 and increased degranulation, most prominently in CD57+ cells which constitutively have higher expression of CD16 (Fig. 4.3C-D). This correlation could indicate that downregulation of CD16

was due to binding of anti-influenza antigen-antibody complexes to NK cells and subsequent degranulation of those cells. This would be consistent with the increased capacity of highly differentiated $CD56^{dim}$CD57+ NK cells to respond to antigen-antibody complexes.

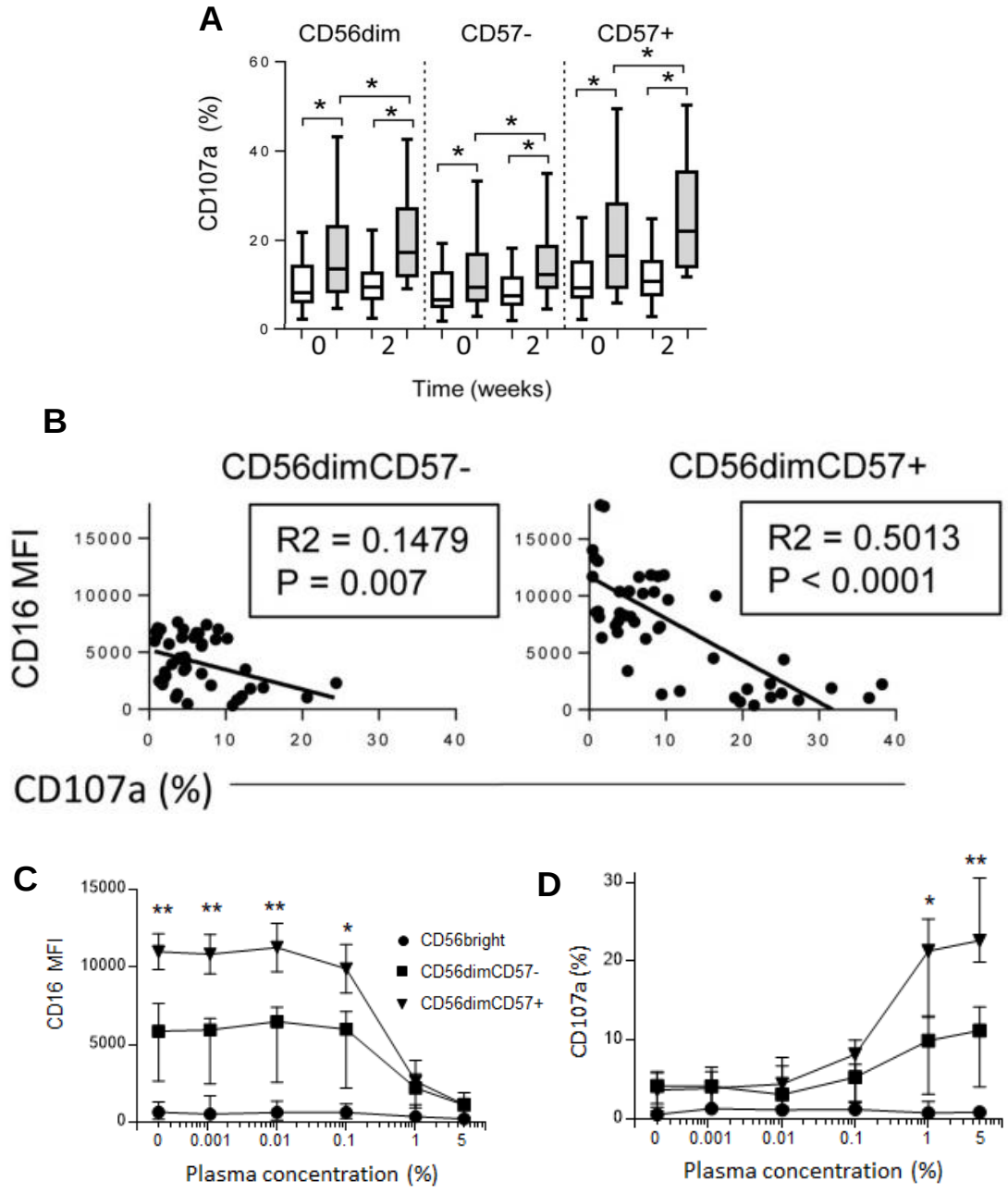


Figure 4.3 Reciprocal loss of CD16 and activation of degranulation in response to immune plasma and TIV

A) PBMCs were thawed from frozen stocks and incubated with 1% pooled plasma collected either prior to vaccination (x-axis = 0) or two weeks post vaccination (x-axis = 2) in RPMI 1640 with 2mM L-glutamine either with (shaded bars) or without (white bars) trivalent influenza vaccine (TIV) for 6 hours before being stained for CD107a. Cells were then fixed and run through an LSRII flow cytometer (n = 17, Mann–Whitney U test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Data represent median values with interquartile ranges.

B) Correlation plots of average NK cells CD16 MFI (y-axis) versus percentage of NK cells presenting CD107a (x-axis) after stimulation with TIV and pooled immune plasma in either $CD56^{dim}$ CD57- NK cells (left plot) or $CD56^{dim}$ CD57+ NK cells (right plot) (n = 17, linear regression)

C-D) Plots of median NK cell CD16 MFI (C) or mean percentage of NK cells expressing CD107a (D) for 3 NK cell differentiation subsets after stimulation with TIV and plasma, vs plasma concentration (x-axis) (n = 17, p-values refer to comparisons between $CD56^{dim}$CD57− and $CD56^{dim}$CD57 + NK cell subsets using Mann–Whitney U test. *p < 0.05, **p < 0.01, ***p < 0.001)

These assays were performed in collaboration with Dr Martin Goodier and Chiara Lusa, an MSc student. Figures adapted from Goodier, Lusa and Sherratt et al (2016) *Frontiers in Immunology*

4.4.3 Downregulation of CD16 on CD56+ CD3- NK cells stimulated with antibody-influenza antigen complexes does not involve internalisation of CD16

For imaging experiments, PBMCs were purified from whole blood and incubated with trivalent flu vaccine containing influenza antigen supplemented with either 5% fetal calf serum or 1% pooled immune plasma as described in section 4.3.4. Cells were run through an ImageStreamX Mark II imaging flow cytometer and gated on CD16 surface expression level, then examined visually for CD16 internal fluorescence.

No visible internalisation of CD16 was seen in the CD16reduced subset after treatment with TIV and PIP despite visible downregulation of CD16 on the surface of the cells (Fig. 4.4B) in comparison to the CD16strong subset (Fig. 4.4A). Additionally, no internalisation of CD16 was seen in either the CD16negative (Fig. 4.4C) or CD16strong subsets (Fig. 4.4A). These results were consistent in the control FCS treatment, with no internalisation of CD16 seen in any subset after treatment with TIV and FCS. These results suggested no apparent mechanism for CD16 internalisation after stimulation with influenza antibody-antigen complexes.

A

CD16strong CD56+ CD57- CD3- NK cell after TIV + 1% PIP

B

CD16reduced CD56+ CD57- CD3- NK cell after TIV + 1% PIP

C

CD16negative CD56+CD57- CD3- NK cell after TIV + 1% PIP

Figure 4.4 Representative IDEAS analysis of TIV-exposed NK cells

PBMCs were incubated with trivalent influenza vaccine (TIV) supplemented with either 5% fetal calf serum (FCS) ot 1% pooled immune plasma (PIP) in RPMI 1640 with 2mM L-glutamine for 5 hours before being stained, fixed and imaged using an ImageStreamX Mark II (n = 5 donors). A minimum of 5000 NK cells were analysed per donor.

A-C) Representative images of CD56+ NK cells incubated with TIV supplemented with 1% PIP in either the C) CD16strong D) CD16reduced or E) CD16negative subsets (BF = ^{bright} field; CD3 = marker for T cells [absent]; CD56 = NK cell membrane stained with anti-CD56 antibody; CD57 = NK cell membrane stained with anti-CD56 antibody [absent in these examples]; CD16 internal = internal anti-CD16 marker [absent]; CD16 external = NK cell membrane stained with anti-CD16 antibody

4.5 Discussion

It has been observed repeatedly by several research groups that CD56+ CD3- NK cells downregulate surface expression of the Fc receptor CD16 after it binds antibody-antigen complexes [212]. It has also been firmly established that this process of downregulation involves the metalloprotease ADAM17, which cleaves CD16 at a membrane proximal cleavage region, leading to reduced sensitivity to antibody-dependent activation [214]. However, it has also been suggested that a second mechanism for downregulation of CD16 may exist, involving internalisation into the cell for recycling or degradation via actin-dependent endocytosis [216]. This hypothesis is based on the results of imaging studies purporting to demonstrate CD16 being internalised into NK cells after stimulation [215], though subsequent efforts to replicate these results have been conflicting [212].

Consequently, it was decided to investigate this hypothesis using high-throughput imaging flow cytometry to assess definitively whether such a secondary mechanism of downregulating CD16 exists. An influenza vaccine model of antibody-dependent activation was used to test the mechanisms of CD16 downregulation, as our group has previously established that a combination of trivalent influenza vaccine and immune plasma produce significant antibody-dependent responses in NK cells [217]. However, as the CD16 cellular machinery is largely invariant and does not require antigen-specificity, it is assumed that these results can be generalised to the generic antibody-dependent response [66,210].

4.5.1 CD56+ CD3- NK cells downregulate surface-expressed CD16 after stimulation with influenza antigen-antibody complexes

In this study, two separate but complementary methods were used to assess whether CD16 is downregulated on the surface of NK cells after stimulation with influenza antigen-antibody complexes; conventional flow cytometry and imaging cytometry.

In imaging experiments, donor PBMCs stimulated with trivalent influenza vaccine antigen (TIV) and pooled immune plasma (PIP) had higher proportions of NK cells expressing reduced levels of CD16 and lower proportions of NK cells expressing high levels of CD16 in comparison to controls (Fig. 4.1C-D).

In keeping with previously published results, this suggested that NK cells in all donors tested has downregulated CD16 on their NK cells in response to antibody-antigen

complexes formed by mixing immune plasma with influenza vaccine antigen [217]. This suggested that CD16 had either been shed from the surface of NK cells by the action of ADAM17 metalloprotease [213] or possibly internalised after binding antibody-antigen complexes and transducing an activation signal to the cells.

In conventional flow cytometry experiments stimulation with TIV and plasma caused both $CD56^{dim}$ CD57- and $CD56^{dim}$ CD57+ NK cells to significantly reduce in median CD16 MFI in comparison to cells incubated with plasma alone (Fig. 4.2C). This suggested that NK cells of both subsets downregulate CD16 on their cell surface in response to antibody-antigen complexes formed in the presence of both flu antigen and immune plasma, as our research group has previously seen in our published work [217]. This response was also significantly greater when using plasma collected 2 weeks after intramuscular vaccination rather than plasma collected immediately (Fig. 4.2C), likely a result of the significantly higher anti-influenza antibody titres detected at the 2 week timepoint leading to a greater concentration of antibody-antigen complexes (Fig. 4.2A).

It must be noted however that the apparent downregulation in CD16 seen in these experiments may also be due to experimental errors, such as antigen-antibody complexes blocking access to CD16 by anti-CD16 labelling antibodies, or fixation and permeabilization of cells prior to staining leading to deformation of the CD16 surface receptor and potentially preventing binding of anti-CD16 labelling antibody. In future experiments these possibilities could be accounted for by using multiple staining panels utilising different clones of anti-CD16 designed to bind to different sites on the receptor. If CD16 downregulation is consistent regardless of the site targeted for labelling, this would indicate that downregulation is due to genuine downregulation of CD16 rather than methodological issues arising from blocking or deformation of any one specific binding site. Alternatively, the amount of CD16 receptor present in the surrounding surface medium could be quantified using proteomic techniques. A greater quantity of soluble CD16 present in surrounding medium after stimulation would also indicate CD16 had been downregulated through shedding rather than simply being unlabelled.

4.5.2 Downregulation of CD16 after stimulation with flu antigen-antibody complexes leads to degranulation, and this is more prominent in CD57+ cells

In conventional flow cytometry experiments, both $CD56^{dim}$ CD57- and $CD56^{dim}$ CD57+ cells demonstrated significantly higher presentation of CD107a on their cell surface after stimulation with TIV and plasma, indicating they had begun degranulating (Fig.

4.3A). Linear regression analysis revealed a significant inverse correlation between average CD16 MFI and the percentage of NK cells presenting CD107a, suggesting that binding of flu antigen-antibody complexes to NK cells is associated with degranulation by NK cells (Fig. 4.3B).

This result is in keeping with the established literature on the subject. Several research groups have published data in cellular, animal and human *ex vivo* models indicating that NK cells exhibit degranulation and subsequent cytotoxic activity in response to influenza antigen-antibody complexes binding and crosslinking CD16, leading to transduction of activation signals into the cell [218–220]. Our own results further validate this model.

Whilst both $CD56^{dim}$ CD57- and $CD56^{dim}$ CD57+ cells demonstrated significantly higher presentation of CD107a on their cell surface after stimulation with TIV and immunised plasma, this response was noticeably higher in CD57+ cells (Fig. 4.3A). This was also in keeping with our expectations based on the established literature on NK maturity subsets. CD57+ NK cells are known to express significantly higher levels of CD16 than their CD57- counterparts, leading to enhanced antibody-dependent cytotoxic responses and cytokine production [126,127]. Our own results confirm this, as CD57+ cells expressed noticeable higher levels CD16 at baseline in response to negative control stimuli than CD57- whilst also demonstrating higher levels of degranulation and CD16 downregulation as a consequence of antibody-dependent activation (Fig. 4.3A-C)

4.5.3 Downregulation of CD16 on CD56+ CD3- NK cells stimulated with antibody-influenza antigen complexes does not involve internalisation of CD16

Various studies conducted over the years have confirmed that the downregulation in CD16 seen on NK cells stimulated by antibody-antigen complexes is due at least in part to the activity of metalloprotease molecules such as ADAM17. These molecules cleave CD16 from from the NK surface, resulting in downregulated responsiveness to antibody stimulation [213,214].

However, there have been occasional reports suggesting that a second mechanism of CD16 downregulation may exist, by which CD16 is internalised into the cell for possible degradation or recycling. Most prominently, in 2007 Cecchetti et al performed a series of confocal microscopy experiments imaging NK cells after stimulation with anti-CD16 antibody, and reported CD16 entering the cytoplasm from the cell surface [215]. This process appeared to be dependent on a functional actin cytoskeleton, leading to the

theory that in adding to being shed from the surface via metalloprotease activity CD16 may also be internalised into the cell via actin-dependent endocytosis [215]. Subsequent attempts to replicate these results have seemingly contradicted Cecchetti et al's findings, though the number of studies investigating the issue is minimal [212] [213].

Sadly our own results also contradict these findings. After stimulating NK cells with influenza antigen-antibody complexes we were able to visualise a reduction in surface CD16 as expected, but no CD16 was detected inside cells after intracellular staining (Fig. 4.4A-C).

A possible explanation for this discrepancy may the difference in methods used by researchers investigating this issue. In Cecchetti et al's study NK cells were stimulated directly with a fluorescent anti-CD16 antibody designed to induce activation and internalisation of CD16 without the need for antigen. The presence of the fluorescent anti-CD16 antibody inside the cell after incubation was considered to be evidence that the receptor had been internalised through actin dependent endocytosis [215]. In our own study NK cells were stimulated with influenza antigen-antibody complexes, perhaps representing a more realistic approximation of CD16 activation *in vivo*, and did not internalise CD16 (Fig. 4.4A-C). It may be that stimulating CD16 directly with the Fab region of an antibody induces internalisation of CD16 or the antibody itself through a mechanism not involved in the usual process of CD16 activation, whereby it binds the Fc region of antibody-antigen complexes.

It should noted however that subsequent attempts to replicate Cecchetti et al's results using a variety of methods have also failed to result in detectable internalisation of CD16. In 2013 Romee et al published a study where anti-CD16 antibodies were used to directly activate CD16, similar to the technique used by Cecchetti et al. Subsequent immunoblotting revealed little to no evidence of CD16 internalisation [213]. Also in 2013, Peruzzi et al published a study utilising phorbol 12-myristate 13-acetate (PMA) as an inducer of CD16 downregulation, and reported seeing no internalisation of CD16 through confocal microscopy [212]. Peruzzi et al have also reported that downregulation of CD16 also appears to be unaffected by exposure to endocytic pathway inhibitors such as phenylarsine oxide (PAO), suggesting internalisation of CD16 does not occur through actin-dependent endocytosis [212]. Additional work by our own group has also demonstrated that inhibition of the metalloprotease ADAM17 in the presence of the ADAM17 inhibitor TAPI-1 results in significant reduction of CD16 shedding after stimulation with flu antibody-antigen complexes, indicating that shedding of CD16 on

NK cells in instances of viral infection is largely mediated by metalloprotease cleavage [198].

Recently published evidence by Srpan et al has also indicated that shedding of CD16 from the surface of NK cells after engagement with opsonised targets results in increased motility, promoting serial engagement of NK cells with multiple targets. Inhibition of this process through the use of pharmacological inhibitors and NK cells transfected to express a noncleavable form of CD16 resulted in decreased motility of NK cells with subsequent negative consequences on the strength of observed immune responses. This perhaps offers an explanation as to why CD16 is preferentially shed from the surface of NK cells post-engagement rather than internalised, as the former may promote faster disengagement of NKs from target cells, allowing for more efficient serial killing [221].

Our data lend support to the mechanism of downregulation of CD16 on NK cells post-activation is largely the result of cleavage rather than internalisation. Regardless, we were able to establish a robust assay to detect CD16 downregulation and degranulation in response to antigen and immune plasma. These studies also indicate a stronger and more rapid downregulation of CD16 is achieved in the presence of antigen-antibody complexes than in response to previously tested forms of activation, such as high concentrations of cytokines. The influence of NK cell differentiation on these responses has also been demonstrated. In the next chapter these assays will be applied to assess NK cell antibody-dependent responses to malaria with further investigation of the impact of NK cell differentiation status.

CHAPTER 5

Investigating antibody-dependent adaptive natural killer cell responses to erythrocytes infected with *Plasmodium falciparum*

5.1 Introduction

5.1.1 Development of a malarial vaccine and the importance of antibody

An estimated 3 billion people are currently at risk of developing malaria, the majority of whom live in Sub-Saharan African and South Asia. Each year the disease results in hundreds of thousands of deaths, the majority of which are in children [151].

At present the most widely used methods to manage malarial infection include long-lasting insecticides along with combination drug therapies used for chemoprevention and treatment of infected patients [151]. Whilst these methods have met with considerable success the past few decades, it is widely recognised that future effects to curb and eventually eradicate malaria as a public health concern would be greatly aided by development of an effective vaccine. Efforts to develop such a vaccine have been ongoing for many years now, with variable success (reviewed in [222]). The most promising candidate for a viable vaccine in recent years is RTS,S/AS01, a recombinant protein vaccine consisting of *Plasmodium falciparum* circumsporozoite protein (CSP) fused to hepatitis B surface antigen, combined with the AS01 adjuvant [223]. Implementation of this vaccine has experienced recent setbacks however, particularly with the publication of Phase 3 results for RTS,S/AS01 where protective efficacy amongst children was shown to wane relatively rapidly [224] [158].

Nonetheless, development of an effective and safe malaria vaccine remains a primary goal of the public health community [158]. As with all vaccines, the goal of an efficient anti-malarial vaccine is to illicit strong antibody-dependent responses targeted against malarial epitopes [225] [226] [227]. Consequently, it is of considerable interest to the research community to continue studying the adaptive antibody-dependent response of the immune system to the most problematic species of malaria.

5.1.2 Antibody-dependent responses to malaria in NK cells; a dearth of research

As of 2018 there is a considerable body of research on the antibody-independent response of human natural killer cells to malaria. Studies have confirmed that NK cells produce significant quantities of IFN-γ in response to erythrocytes infected with *Plasmodium falciparum* in *in vitro* cellular models of infection in the absence of antibody [188], with previously studies published by our own lab indicating that NK cells begin to produce IFN-γ within only 6 hours of co-culture with infected erythrocytes [82]. Production of IFN-γ in stances of malarial infection has also been found to associate with protection against severe symptoms as well as delayed incidence of re-infection [168].

Additional studies have confirmed that NK cells are able to mount cytotoxic responses against malaria-infected erythrocytes in the absence of antibody. Imaging studies performed by several groups have seen the formation of an active immune synapse between NK cells and parasitised cells [190] [40] whilst some have claimed to see direct killing of parasitized erythrocytes by NK cells as indicated by 'flattening' of infected cells after incubation with NK cells [40]. Others have demonstrated that erythrocytes infected with the blood-stage parasite can induce production of cytotoxic mediators from NK cells both *in vitro* [55], and *in vivo* [199].

In contrast, with the exception of a few published abstracts, there is little to no published research investigating antibody-dependent NK cell responses to malaria [143,144]. Given the continuing focus of the global research community on development of an effective malaria vaccine designed to trigger potent anti-malarial antibody production, this would seem to be an important lapse.

5.1.3 Adaptive NK cells and the coincidence of HCMV and malaria

Recent research performed in the last few years has indicated that NK cells are capable of differentiating into a functionally active 'adaptive' phenotype in response to cells infected with certain viruses, predominantly human cytomegalovirus (HCMV) [131,132]. Though originally these cells were considered to have adapted to HCMV specifically, subsequent studies have confirmed that these cells have both increased antibody-independent response to certain tumour lines [135] as well as increased antibody-dependent responses to cells infected with viruses such as HSV-1 and influenza [141,142]. Preliminary published abstracts have also suggested potentially greater antibody-dependent responses against intracellular parasites, though as of yet there are no full published studies on the subject [143,144].

This altered 'adaptive' functionality appears to be driven by epigenetic modifications resulting in altered function. Several studies have confimed that 'adaptive' NK cells have reduced expression of the transcription factor PLZF, leading to hypermethylation of certain promotor DNA regions [145]. This leads to downregulated expression of adaptor molecules such as FcεRγ1, SYK and EAT2 [137], leading to enhanced antibody-dependent responses through as-of-yet unclear mechanisms. Additional studies have also suggested that demethylation of cytokine promoter regions also occurs as a result of differentiation down the adaptive pathway, leading to enhanced production of IFN-γ in response to ligation of receptors such as NKG2C and 2B4 [228].

Many of the areas of the world where malaria is still present also have high rates of HCMV infection, most prominently in Africa, South-East Asia and South America [151]. In Africa in particular HCMV is endemic in many countries, with 85% of infants infected by one year of age in countries such as Gambia [229] and Zimbabwe [230]. Some studies estimate that by the time of adolescence, HCMV infection is virtually universal in Sub-Saharan Africa [231].

If the assumption that HCMV drives expansion of adaptive NK cells is correct, and that these cells exhibit increased ability to respond to pathogens via antibody-dependent mechanisms, it would be a reasonable hypothesis that individuals from these areas could demonstrate increased antibody-dependent responses to pathogens such as malaria. Given this prospect, research into the NK cell antibody-dependent response to malaria becomes even more imperative. Consequently, in this study I have set out to investigate the antibody-dependent response to malaria in NK cells, with a specific focus on the adaptive NK cell activity of HCMV-exposed Africans.

5.2 Aims, Hypotheses and Objectives

The primary aims of the study in this chapter were fourfold;

1) To investigate the magnitude of the antibody-dependent NK cell response to malaria in comparison to the antibody-independent response, with a specific focus on IFN-γ production and degranulation given the importance of these activities in mounting an effective anti-malarial immune defence

2) To investigate the effect of co-stimulatory factors such as cytokines and accessory cell contact on antibody-dependent NK cell response to malaria

3) To evaluate the contribution of 'adaptive' FcεRIγ- NK cells and differentiated CD57+ NK cells to the antibody-dependent anti-malarial response

4) To investigate the specific effects of co-stimulatory factors on the antibody-dependent response of FcεRIγ- and CD57+ NK cells

My hypotheses were that NK $CD56^{dim}$ NK cells would be significantly activated into producing IFN-γ and degranulating by the presence of malaria-infected erythrocytes and malaria exposed plasma; that these responses would be mediated primarily through anti-malarial antibody-dependent mechanisms but may involve additional co-factors; that adaptive and/or differentiated NK cells subsets would respond significantly differently to this stimuli than canonical and/or undifferentiated subsets, and that the precise combination of stimuli that activate these subsets may vary due to phenotypic differences.

My objectives were to purify peripheral blood mononuclear cell mixtures containing NK cells from the whole blood of either malaria-exposed Gambians or malaria-naïve Europeans, as well as autologous plasma from both groups. I would then incubate these cells either with or without matched autologous plasma in the presence of either uninfected erythrocytes or erythrocytes infected with *Plasmodium falciparum*. I would then stain and fix the cells using markers that would allow me to parse $CD56^{dim}$ NK cells by adaptive and differential subsets, and use flow cytometry to assess antibody-dependent functional activity with an emphasis on degranulation and IFN-γ production.

5.3 Methods

5.3.1 European PBMC and plasma donors

Prior to the current study, several hundred healthy malaria-naive volunteers were recruited from within the London School of Hygiene and Tropical Medicine (LSHTM) through an anonymised blood donation system operated by Carolynne Stanley, a trained phlebotomist. Donors are summarised in Table 5.1 by age, nationality, sex, malarial status and HCMV status. Ethical approval for this system was acquired from the LSHTM Ethics Committee under the ethics reference numbers 5520 and 6237, and informed consent was obtained from all donors as stipulated by LSHTM and the appropriate regulatory bodies. 50ml of venous blood from each donor was collected in sterile Falcon tubes with 2 I.U. per ml of sodium heparin added to prevent coagulation**.** Peripheral blood mononuclear cells and plasma were purified and frozen for use in experiments by Dr Martin Goodier, Dr Asia Wolf and Dr Carolyn Nielsen. Prior to the current study, plasma samples were tested via ELISA for the presence of antibodies against human cytomegalovirus by Dr Carolyn Nielsen. 20 HCMV+ donors and 20 HCMV- donors were selected for use in this study, with a total of 40 European donors in total.

Age/Years		Ethnicity		Sex		Malarial Status		HCMV Status	
Mean	38.5	White British	40	Male		Naïve	40	Negative	20
Median	48	Other	0	12		Exposed	0	Positive	20
Range	21-63			Female					
				28					

Table 5.1 Age, nationality, sex, malarial status and HCMV status of donors for this assay

5.3.2 Gambian PBMC and plasma donors

Prior to the current study, 641 volunteers were recruited from malaria-exposed sites in Gambia. Ethical approval for this system was acquired from the Gambian Government/ MRC Gambia joint ethics committee (ethics reference numbers SCC 1269 and SCC 1449) and the LSHTM ethics committee (ethics reference number 6034) and informed consent was obtained from all donors as stipulated by LSHTM and the appropriate regulatory bodies. 50ml of venous blood from each donor was collected in sterile Falcon tubes with 2 I.U. per ml of sodium heparin added to prevent coagulation**.** Peripheral blood mononuclear cells and plasma were purified frozen and shipped to LSHTM by Dr

Martin Goodier, Dr Carolyn Nielsen and Dr Asia Wolf for use in experiments. Plasma samples were then tested via ELISA for the presence of antibodies against apical membrane antigen 1 (AMA1), a malaria-derived protein. 66 donors who tested positive for prior malarial exposure were selected for use in this study. Donors are summarised in Table 5.2 by age, nationality, malarial status and HCMV status.

Age/Years		Nationality		Malarial Status		HCMV Status	
Mean	18	Gambian	66	Naïve	0	Negative	0
Median	16	Other	0	Exposed	66	Positive	66
Range	1-49						

Table 5.2 Age, nationality, sex, malarial status and HCMV status of donors for this assay

5.3.3 Anti-AMA1 ELISA of Gambian plasma

Plasma samples from 641 Gambian donors were diluted 1 in 200 with reconstitution buffer (PBS, 0.05% Tween 20, 1g/L sodium azide) in 96-well flat-bottom deep well plates.

AMA1 antigen-coated Immulon ELISA plates were prepared as described in section 2.1.3. 45ul of blocking solution (PBS, 0.05% Tween 20, 1% skimmed milk powder) was then added to each well, followed by 5ul of the previously diluted 1 in 200 plasma samples to form a final dilution of 1 in 2000. Positive and negative control plasmas were added to each ELISA plate to generate a standard reference curve. ELISA plates were then incubated, washed and activated using the HRP-TMB system described in section 2.1.3.

Plates were read immediately using a Dynex Technologies MRX II Microplate Absorbance Reader run at 450nm. Standard curves and positive/negative cut-offs were determined by Dr Joe Biggs, with the positive cut-off based on the mean optical density plus two standard deviations.

5.3.4 Assessing NK cell antibody-independent responses to *Plasmodium falciparum*-infected erythrocytes through flow cytometry

Frozen PBMC samples from Gambian and European donors were thawed using the protocol described in section 2.2.3. 2 x 10^5 PBMCs were incubated for 18 hours at 37ºC with 5% CO_2 in wells of 96-well U-bottom plates (ThermoFisher) under the conditions

described in Table 5.3. GolgiStop containing Monensin (BD Biosciences) was added to cultures to form a final 1/1500 concentration and GolgiPlug containing Brefeldin A (BD Biosciences) added to form a 1/1000 final concentration after 15 housr of incubation. Anti-CD107a antibody (ef660-conjugated, clone 1D4B, BD Biosciences) was added during culturing at 1µl per 100ul of culture suspension.

PBMCs per well	Incubated with;	Total volume per well
2 x 10^5	Culture medium alone	100µl
2 x 10^5	High Dose Cytokines (Il-12 and IL-18)	100µl
2 x 10^5	6 x 10^5 uninfected erythrocytes (URBCs)	100µl
2 x 10^5	6 x 10^5 *P. falciparum*-infected erythrocytes (PRBCs)	100µl

Table 5.3. List of PBMC culture conditions used for functional flow cytometry

After the required period of incubation, cells were washed and stained for 30 minutes at 4^{o}C in the dark with 10µl total of the surface antibody cocktail described in Table 5.4 using the protocol as described in section 2.4.1.

Antibody	Clone	Fluorophore	Supplier	Volume per well
Mouse Anti-Human CD56	B159	BV605	Biolegend	0.5µl
Mouse Anti-Human CD3	UCHT1	V500	BD Biosciences	0.5µl
Mouse Anti-Human CD57	TB01	ef450	eBioscience	0.5µl

Table 5.4. List of fluorescent antibodies used to stain the surface of NK cells for flow cytometry

Dead and dying cells were also stained using APC-conjugated fixable viability dye (eBioscience). Cells were then washed thoroughly with buffer, fixed and stained intracellularly with 10µl of the intracellular antibody cocktail described in Table 5.5 using the protocol described in section 2.4.1.

Antibody	Clone	Fluorophore	Supplier	Volume per well
Mouse Anti-Human IFN-γ	4S.B3	BV785	Biolegend	0.25µl

Table 5.5. List of fluorescent antibodies used to internally stain NK cells for flow cytometry

Cells were then washed, resuspended in 300µl FACS buffer, transferred to alpha tubes (Alpha labs) and stored at 4^{o}C until run on a BD LSRII Flow Cytometer (BD Biosciences) using the appropriate laser set. Data was collected using the complementary FACSDiva software (BD Biosciences) and analysed using FlowJo, v.10 (FlowJo LLC).

Fluorescence compensation was carried out using OneComp eBeads Compensation Beads (eBioscience) stained with single antibodies.

5.3.5 Assessing NK cell antibody-dependent responses to *Plasmodium falciparum*-infected erythrocytes through flow cytometry

Frozen PBMC samples from Gambian and European donors were thawed using the protocol described in section 2.2.3. 2 x 10^5 PBMCs were incubated for 6 hours at 37ºC with 5% CO_2 in wells of 96-well U-bottom plates (ThermoFisher) under the conditions described in Table 5.6. GolgiStop containing Monensin (BD Biosciences) was added to cultures to form a final 1/1500 concentration and GolgiPlug containing Brefeldin A (BD Biosciences) added to form a 1/1000 final concentration after 1 hour of incubation. Anti-CD107a antibody (ef660-conjugated, clone 1D4B, BD Biosciences) was added during culturing at 1µl per 100ul of culture suspension.

PBMCs per well	Incubated with;	Total volume per well
2 x 10^5	Culture medium alone	100µl
2 x 10^5	4 x 10^4 K562 cells	100µl
2 x 10^5	4 x 10^4 Raji cells with 1µg/ml IgG1 isotype	100µl
2 x 10^5	4 x 10^4 Raji cells with 1µg/ml Rituximab	100µl
2 x 10^5	6 x 10^5 uninfected erythrocytes (URBCs)	100µl
2 x 10^5	6 x 10^5 (URBCs) with 1% autologous plasma	100µl
2 x 10^5	6 x 10^5 *P. falciparum*-infected erythrocytes (PRBCs)	100µl
2 x 10^5	6 x 10^5 (PRBCs) with 1% autologous plasma	100µl

Table 5.6. List of PBMC culture conditions used for functional flow cytometry

After the required period of incubation, cells were washed and stained for 30 minutes at 4ºC in the dark with 10µl total of the surface antibody cocktail described in Table 5.7 using the protocol as described in section 2.4.1.

Antibody	Clone	Fluorophore	Supplier	Volume per well
Mouse Anti-Human CD56	B159	BV605	Biolegend	0.5µl
Mouse Anti-Human CD3	UCHT1	V500	BD Biosciences	0.5µl
Mouse Anti-Human CD57	TB01	ef450	eBioscience	0.5µl
Mouse Anti-Human NKG2C	FAB138P	PE	R&D Systems	0.5µl
Mouse Anti-Human CD16	3G8	PE/Dazzle	Biolegend	0.5ul
Mouse Anti-Human CD94	REA113	PE/Vio770	Miltenyi Biotec	1ul
Mouse Anti-Human CD2	RPA-2.10	PerCP/Cy5.5	Biolegend	0.5ul

Table 5.7. List of fluorescent antibodies used to stain the surface of NK cells for flow cytometry

Dead and dying cells were also stained using APC-conjugated fixable viability dye (eBioscience). Cells were then stained with 10µl of the intracellular antibody cocktail described in Table 5.8 using the protocol described in section 2.4.1.

Antibody	Clone	Fluorophore	Supplier	Volume per well
Mouse Anti-Human IFN-γ	4S.B3	BV785	Biolegend	0.25µl
Anti-Human FcεRIγ	Polyclonal	FITC	MIllipore	0.15ul

Table 5.8. List of fluorescent antibodies used to internally stain NK cells for flow cytometry

Cells were then washed and run on a BD LSRII Flow Cytometer (BD Biosciences) using the appropriate laser set. Data was collected using FACSDiva software (BD Biosciences) and analysed using FlowJo, v.10 (FlowJo LLC). Compensation was carried out using OneComp eBeads Compensation Beads (eBioscience) stained with single antibodies. A representative gating strategy is outlined in Figure 5.1.

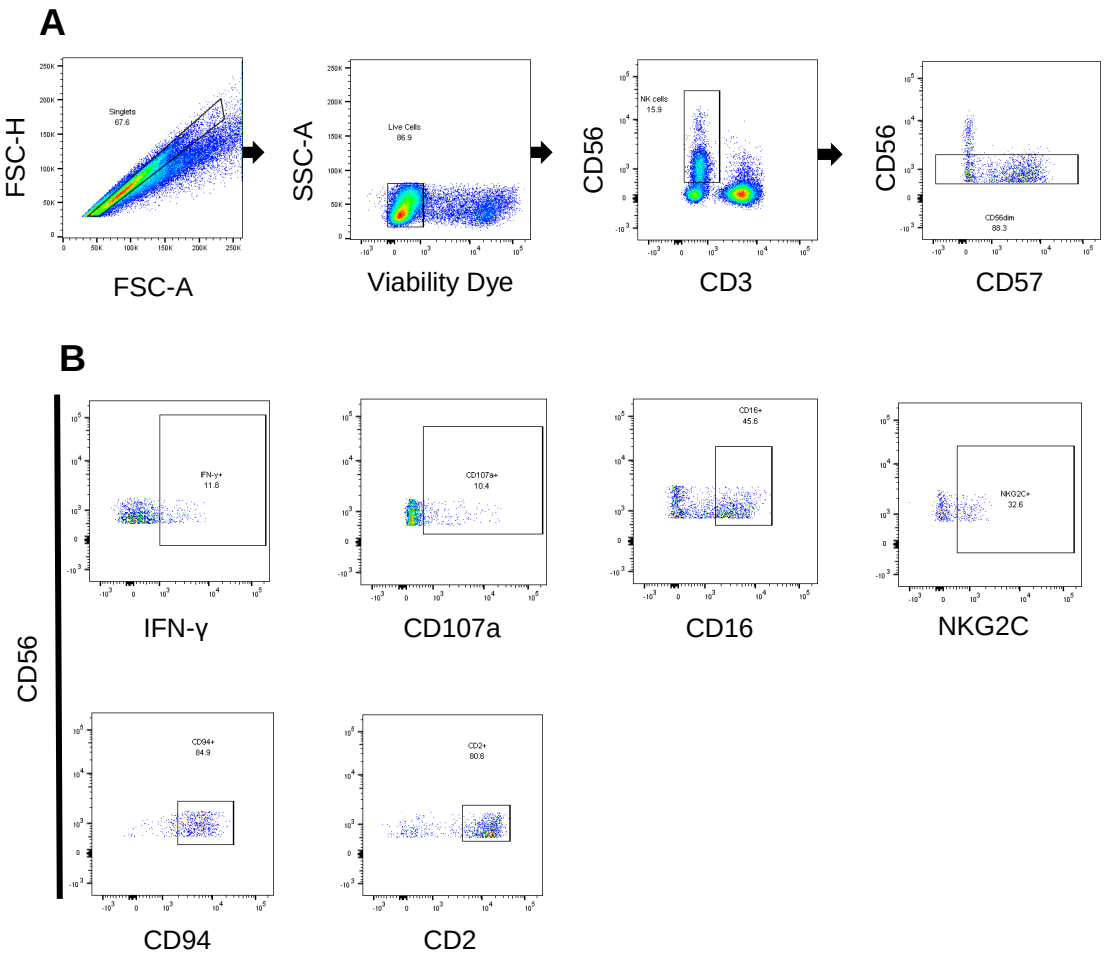


Figure 5.1 **Representative FACS plots of NK cells stimulated by PRBCs and 1% autologous plasma.** PBMCs were stained and fixed as described in section 3.3.4

A) Flow diagram demonstrating method of identifying viable $CD56^{dim}$ CD3- NK cells

B) FACS plots demonstrating method of assessing expression of functional markers on $CD56^{dim}$ CD3- NK cells; (top row, left to right) IFN-γ, CD107a, CD16, NKG2C, (bottom row, left to right) CD94, CD2

5.3.6 Statistical analyses

Statistical analyses were performed with Prism 7.03 (Graphpad), as specified for each experiment. Data were excluded when the gated cell subset contained fewer than the required minimum number of cells. All statistical tests are two-sided, with p values stated as follows; **** <p 0.0001; *** p< 0.001; ** p< 0.01; * p< 0.05. Specific statistical tests and sample sizes are stated in the relevant figure legends. Two-way paired or unpaired t-tests were used to compare control and experimental data when sample sizes were sufficiently large, or two-way paired or unpaired Wilcoxon tests when not. Pearson's correlations were used to determine trends between data. For data sets where n =<10 no formal analyses were performed as the data lacked sufficient statistical power.

5.3 Results

5.3.1 Both Gambian and European CD56^{dim} NK cells produce IFN-γ and degranulate after long-term incubation with erythrocytes infected with *Plasmodium falciparum*

Previous research by both our own lab group and others has indicated that European NK cells are induced to produce IFN-γ and degranulate in response to long-term incubation with *Plasmodium falciparum*-infected erythrocytes (PRBCs) through cytokine and potentially germline-receptor mediated mechanisms [82,184,188,190]. Before assessing antibody-dependent responses over a shorter period of incubation, we decided to validate these prior observations in both Gambian and European control individuals by incubating them either with medium alone, with a high dose concentration of cytokines IL-12 and IL-18 (HDC) as a positive control, with uninfected erythrocytes (URBCs) or with parasitised erythrocytes (PRBCs) as described in section 5.3.4.

When incubated with HDC there was a large significant increase in the frequency of both European and Gambian CD56^{dim} NK cells producing IFN-γ than when incubated with medium alone (Fig. 5.2A-B, $p<0.0001$ for both).

When incubated with PRBCs there was a small but highly significant increase in the frequency of both European and Gambian CD56^{dim} NK cells producing IFN-γ than when incubated with URBCs (Fig. 5.2A-B, $p<0.0001$ for both).

Similar responses were observed with degranulation. When incubated with HDC there was a large significant increase in the frequency of both European and Gambian CD56^{dim} NK cells degranulating than when incubated with medium alone (Fig. 5.2C-D, $p<0.0001$ for both).

When incubated with PRBCs there was a small significant increase in the frequency of both European and Gambian CD56^{dim} NK cells degranulating than when incubated with URBCs (Fig. 5.2C-D, $p<0.0001$ for both).

Having confirmed our prior observations that CD56^{dim} NK cells are capable of producing pro-inflammatory cytokine and degranulating in response to parasitised cells over prolonged incubation, we then moved on to evaluating antibody-dependent responses to this stimulation after short-term incubation with or without the presence of immune plasma.

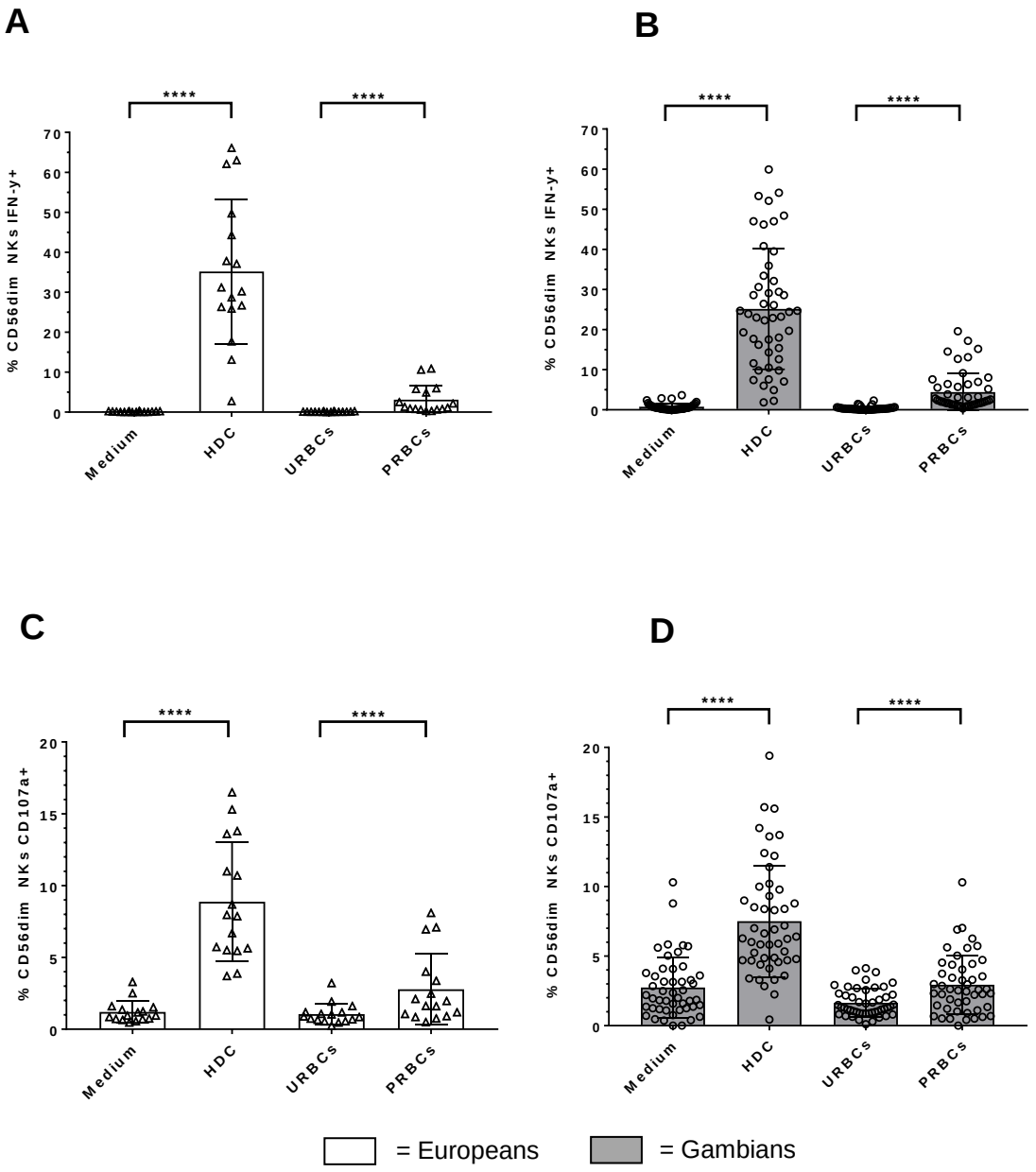


Figure 5.2 **Gambian and European antibody-independent CD56^{dim} NK responses to control treatments and PRBCs A-B)** PBMCs were thawed and incubated with; (bars, left to right) culture medium alone, High Dose Cytokines (HDC), URBCs or, PRBCs for 18 hours. Cells were stained and fixed for IFN-γ; A) Europeans B) Gambians; (Gambian n value=48, paired t-tests, European n value=16, paired Wilcoxon tests, **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05).
C-D) PBMCs were thawed and incubated with; (bars, left to right) culture medium alone, High Dose Cytokines (HDC), URBCs or, PRBCs for 18 hours. Cells were stained and fixed for CD107a as a marker of degranulation; C) Europeans D) Gambians value=48, paired t-tests, European n value=16, paired Wilcoxon tests, **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05).

5.3.2 Gambian CD56^{dim} NK cells produce IFN-γ in response to short-term incubation with erythrocytes infected with *Plasmodium falciparum,* but European CD56^{dim} NK cells do not

PBMCs isolated from European individuals (n=40) were incubated at a 1:3 ratio for 6 hours with either URBCs or PRBCs. Cells were then stained and fixed for cellular and functional markers, then compared to Gambian cells treated under the same conditions (n=66).

When incubated with PRBCs alone, there was no significant increase in the frequency of European CD56^{dim} NK cells producing IFN-γ than when incubated with URBCs (Fig. 5.3A). In contrast, a mean of 1.48% (SD±2.52) of Gambian CD56^{dim} NK cells produced IFN-γ in response to PRBCs versus a mean of 0.41% (SD±0.38) in response to URBCs ($p=0.001$, Fig. 5.3B).

5.3.3 The short-term IFN-γ response of CD56^{dim} NK cells from malaria exposed Gambian individuals produce IFN-γ response to short-term incubation with PRBCs is upregulated in the presence of autologous plasma

When PBMCs from Gambians were incubated with PRBCs in the presence of autologous plasma, the percentage of CD56^{dim} NK cells producing IFN-γ was also significantly higher in contrast to incubation with URBCs with autologous plasma. A mean of 2.77% (SD±4.06) of CD56^{dim} NK cells produced IFN-γ in response to PRBCs with plasma versus a mean of 0.47% (SD±0.46) in response to URBCs with plasma ($p<0.0001$, Fig. 5.3B). The response to PRBCs and autologous plasma was heterogenous, with some donors expressing no IFN-γ at all in response to this combination of stimuli, while in at least one individual up to 28.3% of CD56^{dim} NK cells expressed IFN-γ (Fig. 5.3B).

Overall, the frequency of CD56^{dim} NK cells producing IFN-γ in Gambian individuals was significantly higher in response to PRBCs in the presence of autologous plasma than with PRBC alone ($p<0.05$, Fig 5.3B). This suggested that the production of IFN-γ was upregulated by the presence of autologous plasma beyond what was seen in response to parasitised cells alone.

In contrast there was no significant difference in the percentage of $CD56^{dim}$ NK cells expressing IFN-γ when incubated with URBC alone or URBC with autologous plasma, suggesting that autologous plasma in the absence of malarial antigens was not sufficient to provoke increased production of IFN-γ (Fig. 5.3B).

5.3.3 Autologous plasma promotes higher frequencies of $CD56^{dim}$ NK cells to produce IFN-γ in response to PRBCs in Gambians than in European individuals

Europeans donors demonstrated no significant IFN-γ in response to incubation with PRBCs alone in comparison to URBCs. However, when incubated with PRBCs and autologous plasma, there was a significant increase in the frequency of European $CD56^{dim}$ NK cells producing IFN-γ than when incubated with URBCs and autologous plasma. A mean of 1.26% (SD±2.62) of European $CD56^{dim}$ NK cells produced IFN-γ in response to PRBCs in the presence of autologous plasma versus a mean of 0.35% (SD±0.34) in response to URBCs in the presence of plasma ($p=0.05$, Fig. 5.3A).

While this response was relatively minimal it was nonetheless unexpected given the malaria-naïve status of our European donors, perhaps indicating that expression of IFN-γ in these donors in response to parasitised cells and plasma may be due to unknown antibody-independent mechanisms. These will be discussed in the relevant section.

The frequency of $CD56^{dim}$ NK cells from European individuals producing IFN-γ was still significantly lower than Gambian individuals however, where 2.77% of $CD56^{dim}$ NK cells produced IFN-γ in response to PRBCs in the presence of plasma in comparison to 1.26% in Europeans ($p<0.05$, Fig. 5.3C). This difference in responsiveness was also obiovus when observed as fold-change in interferon production (Fig. 5.4D). There was no significant difference between Gambians and Europeans in the percentage of $CD56^{dim}$ NK cells producing IFN-γ in response to URBCs and plasma (Fig. 5.3C).

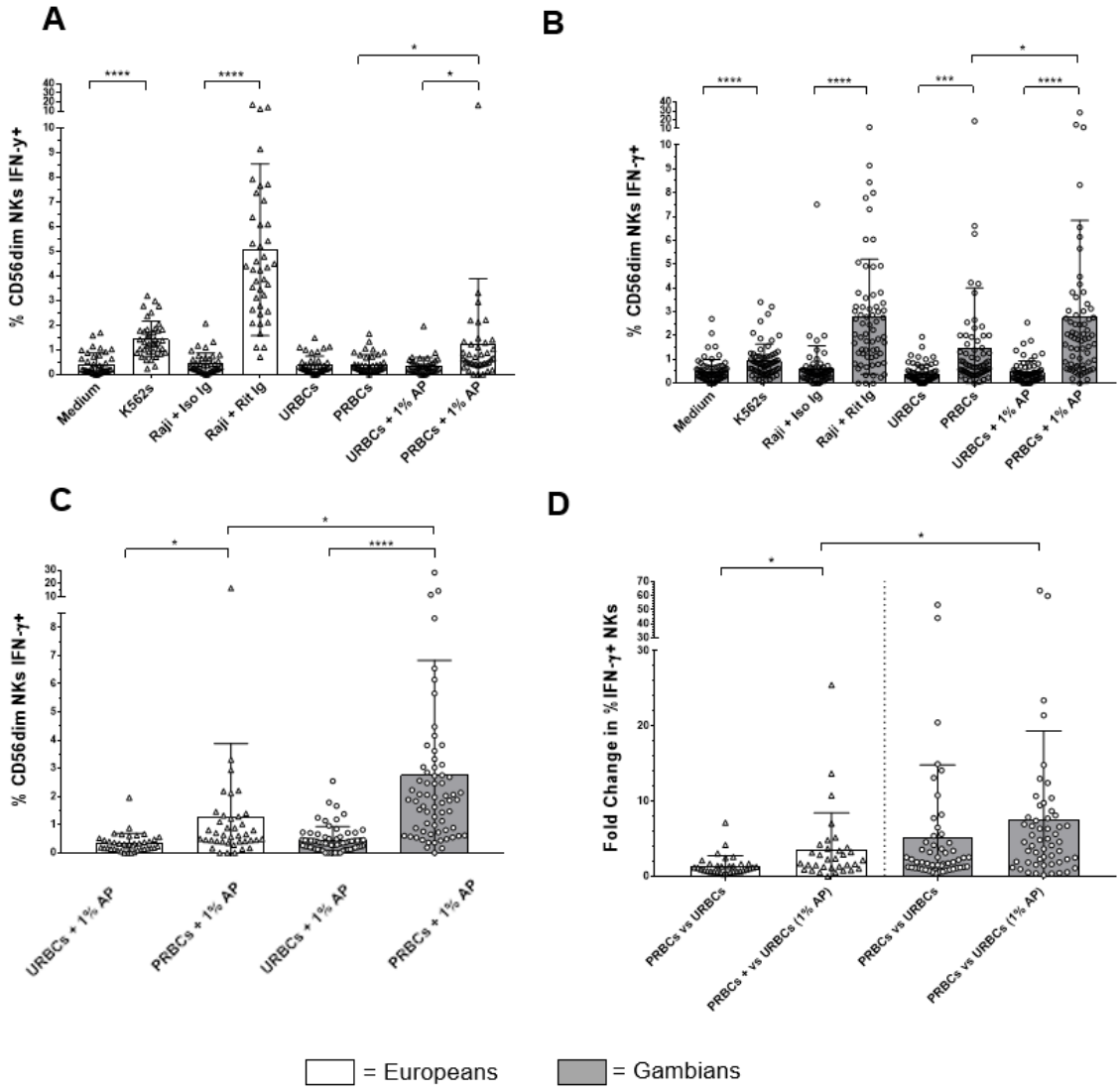


Figure 5.3 **European and Gambian CD56^{dim} NK** IFN-γ **responses to control treatments and PRBCs with autologous plasma.**

A-B) European (A) or Gambian (B) PBMCs were thawed and incubated with; (bars, left to right) culture medium alone, K562 cells, Raji cells with IgG1 isotype control antibody, Raji cells with Rituximab antibody, URBC, URBC with 1% autologous plasma, PRBC, or PRBC with 1% autologous plasma. Cells were stained and fixed for IFN-γ. (European n value = 40; Gambian n value = 66, paired t-tests, **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05).

C) European (left bars) and Gambian (right bars) PBMCs were thawed and incubated with URBCs, URBCs 1% autologous plasma, PRBCs, or PRBCs with 1% autologous plasma. Cells were stained and fixed for IFN-γ; (European n value = 40; Gambian n value = 66, within-group paired t-tests and between-group unpaired t-tests, **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05).

D) Fold change in the percentage of Gambian and European CD56dim NK cells expressing IFN-y in response to either PRBCs vs URBCs (left bars) or PRBCs + 1% autologous plasma vs URBCs + 1% autologous plasma (right bars) (Gambian n value= 66, European n value=40, within-group paired and between-group unpaired t-tests, **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05).

5.3.4 CD56^{dim} NK cells mount weak antibody-independent degranulation responses to PRBCs

PBMCs isolated from European individuals (n=40) were incubated at a 1:3 ratio for 6 hours with either healthy erythrocytes or erythrocytes infected with schizont-stage *Plasmodium falciparum*. Cells were then stained and fixed for cellular and functional markers, then compared to Gambian cells treated under the same conditions (n=66).

When incubated with PRBCs, the frequency of European CD56^{dim} NK cells degranulating was significantly higher than when incubated with URBCs, with a mean of 9.01% (SD±7.23) CD56^{dim} NK cells presenting CD107a in response to infected cells versus a mean of 8.01% (SD±5.64) in response to URBCs ($p>0.05$, Fig. 5.4A). A similarly upregulated degranulation response was seen in Gambian CD56^{dim} NK cells, where a mean of 4.62% (SD±3.51) of CD56^{dim} NK cells degranulated in response to parasitised cells in comparison to a mean of 3.52% (SD±2) in response to URBCs ($p=0.001$, Fig. 5.4B).

The mean degranulation response was relatively small in both Europeans and Gambians, though considerably heterogenous between individuals, with between 0.88% and 9.73% of CD56^{dim} NK cells degranulating for most Gambian donors. In at least one individual the percentage of CD56^{dim} NK cells degranulating reached 25%, though this was considerably higher than all other donors and may be anomalous (Fig. 5.4B).

5.3.5 Upregulation of PRBC-induced NK cell degranulation in the presence of autologous plasma

When PBMC from Gambians donors were incubated with PRBC in the presence of autologous plasma, the frequency of CD56^{dim} NK cells expressing CD107a was significantly higher in contrast to cells incubated with URBC under the same conditions, with a mean of 19% (SD±11.8) of CD56^{dim} NK cells degranulating versus a mean of 4.36% (SD±3.38) respectively ($p<0.0001$, Fig. 5.3B). The range of degranulation responses to infected erythrocytes with autologous plasma was extremely heterogenous within the group, ranging from 3.35% of CD56^{dim} NK cells in the weakest responders to 51.1% in the strongest responders. (Fig. 5.4B).

The frequency of CD56^{dim} NK cells degranulating in Gambian donors was also significantly higher in response in the presence of autologous plasma than with PRBCs

alone, equating to an increase in mean frequency of 13.38% ($p<0.0001$, Fig. 5.4B). This suggested that degranulation of $CD56^{dim}$ NK cells was mediated by the presence of autologous plasma and not simply due to parasitised cells alone.

In contrast there was no significant difference in the percentage of CD107a+ $CD56^{dim}$ when comparing URBCs alone or with autologous plasma, suggesting that autologous plasma alone without the presence of malarial antigens was not sufficient to provoke a degranulation response in Gambian individuals (Fig.5.4B).

5.3.6 Stronger plasma-dependent degranulation responses in Gambian individuals compared to European control subjects

In response to incubation with PRBCs and autologous plasma, there was a significant increase in the frequency of European $CD56^{dim}$ NK cells degranulating than when incubated with URBCs and autologous plasma. A mean of 14.08% (SD±10.56) of European $CD56^{dim}$ NK cells degranulated in response to parasitised cells with plasma versus a mean of 10.03% (SD±10.23) in response to URBCs with plasma ($p=0.05$, Fig. 5.4A).

While this increase was relatively small, it was nonetheless unexpected given the malaria-naïve status of our European donors. This could perhaps indicate that expression of IFN-γ in these donors in response to parasitised cells and plasma may be due to unknown antibody-independent mechanisms, which will be discussed in the relevant section of the discussion.

The European degranulation response to parasitised cells and plasma was also significantly lower than in Gambian donors, where 19.04% (SD±11.8) of CD56dim NK cells produced plasma in response to parasitised cells and plasma in comparison to 14.08% (SD±10.56) in Europeans ($p<0.05$, Fig. 5.4C).

Unexpectedly, there was a significant difference in the frequency of CD56dim NK cells producing IFN-γ in response to URBCs and plasma between Gambians and Europeans, with Europeans far more likely to degranulate in response to this control. 10.03% (SD±10.23) of European CD56dim NK cells degranulated in response to URBCs and plasma in comparison to only 4.36% (SD±3.38) in Gambians (Fig. 5.4C).

Given the relatively high background degranulation of European CD56 NK cells in response to uninfected controls, we decided to perform an additional analysis of these results as a function of fold-change in the percentage of cells degranulating in response

to experimental stimuli versus controls (Fig. 5.4D). This allowed us to compensate for high background degranulation in Europeans by expressing results as a measure of change from background values rather than absolute values. When analysed in this format, it became clear that $CD56^{dim}$ NK cells from Gambian individuals are far more responsive to cytotoxically PRBCs and plasma than those from Europeans donors. $CD56^{dim}$ NK cells from Gambians demonstrated a mean x6.22 fold-change in the percentage of cells degranulating in response to PRBCs with plasma versus URBCs with plasma, in comparison to a mean of only x1.78 fold-change in Europeans ($p<0.0001$, Fig. 5.4D).

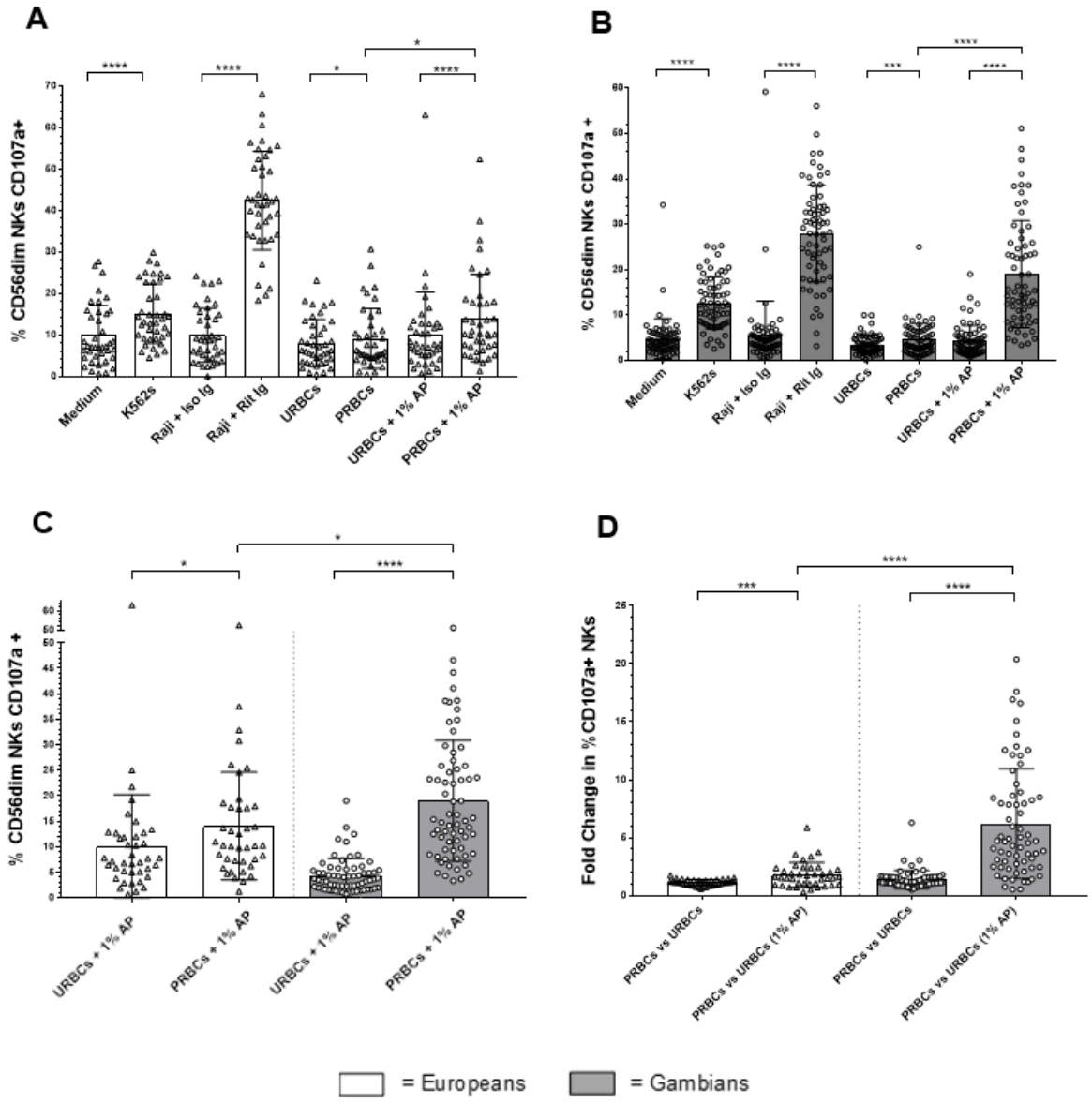


Figure 5.4 **European and Gambian CD56^{dim} NK** degranulation **responses to control treatments and PRBCs with autologous plasma.**

A-B) European (A) or Gambian (B) PBMCs were thawed and incubated with; (bars, left to right) culture medium alone, K562 cells, Raji cells with IgG1 isotype control antibody, Raji cells with Rituximab antibody, URBC, URBC with 1% autologous plasma, PRBC, or PRBC with 1% autologous plasma. Cells were stained and fixed for CD107a as a marker of degranulation. (European n value = 40; Gambian n value = 66, paired t-tests, **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05).

C) European (left bars) and Gambian (right bars) PBMCs were thawed and incubated with URBCs, URBCs 1% autologous plasma, PRBCs, or PRBCs with 1% autologous plasma. Cells were stained and fixed for CD107a; (European n value = 40; Gambian n value = 66, within-group paired t-tests and between-group unpaired t-tests, **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05).

D) Fold change in the percentage of Gambian and European CD56dim NK cells expressing CD107a in response to either PRBCs vs URBCs (left bars) or PRBCs + 1% autologous plasma vs URBCs + 1% autologous plasma (right bars) (Gambian n value= 66, European n value=40, within-group paired and between-group unpaired t-tests, **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05).

5.3.7 Shedding of CD16 from CD56^{dim} NK cells from malaria-exposed Gambian donors is strongly upregulated by autologous plasma

A significantly reduced frequency of CD56^{dim} NK cells from Gambian individuals expressed CD16 after 6 hours of incubation with PRBCs in comparison to cells incubated with URBCs, indicating activation-induced background shedding of CD16 in the absence of autologous plasma. A mean of 76.46% (SD±13.3) of CD56^{dim} NK cells expressed CD16 when incubated with URBCs versus 71.45% (SD±17.1) when incubated with parasitized cells (p=0.001, Fig. 5.5A).

Whilst this decrease was statistically significant, in absolute terms it was relatively small, with the percentage of CD56^{dim}s expressing CD16 only decreasing by a mean of 5.01% when exposed to infected erythrocytes versus uninfected.

However, when PBMCs from Gambian individuals were incubated with infected erythrocytes in the presence of autologous plasma, the frequency of CD56^{dim} NK cells shedding CD16 was far greater. A mean of 74.89% (SD±14.9) of CD56^{dim} NK cells expressed CD16 when incubated with URBCs versus 41.6% (SD±29.66) when incubated with PRBCs, representing a mean decrease of 33.29% in the percentage of CD56^{dim} NK cells expressing when exposed to PRBCs vs URBCs in the presence of autologous plasma (p<0.0001, Fig. 5.5A). This strong response could indicate that Gambian CD56^{dim} NK cells could be responding to PRBCs through an antibody-dependent mechanism in the presence of autologous plasma, perhaps due to anti-malarial immunoglobulins present in malaria-exposed Gambian plasma.

5.3.8 The frequency of CD56^{dim} NK cells downregulating CD16 in response to PRBCs and autologous plasma is significantly higher in Gambian individuals

In response to incubation with PRBCs and autologous plasma, CD56^{dim} NK cells from European individuals were significantly more likely to downregulate CD16 than when incubated with URBCs and autologous plasma. A mean of 71.41% (SD±19.2) of CD56^{dim} NK cells expressed CD16 in response to parasitised cells with plasma versus a mean of 81.49% (SD±10.7) in response to URBCs with plasma (p=0.05, Fig. 5.5B).

This response was unexpected given the malaria-naïve status of our European donors. This could perhaps indicate that expression of IFN-γ in these donors in response to

parasitised cells and plasma may be due to unknown plasma-dependent mechanisms, to be discussed in the relevant section.

The mean downregulation of CD16 by NK cells from European donors was also significantly lower than that seen in Gambian donors, where only a mean of 41.6% of $CD56^{dim}$ NK cells expressed CD16 after incubation with parasitised cells and plasma in comparison to 71.41% in Europeans ($p<0.05$, Fig. 5.5C). This difference was far more apparent when observed as fold-change in CD16 expression (Fig. 5.5D).

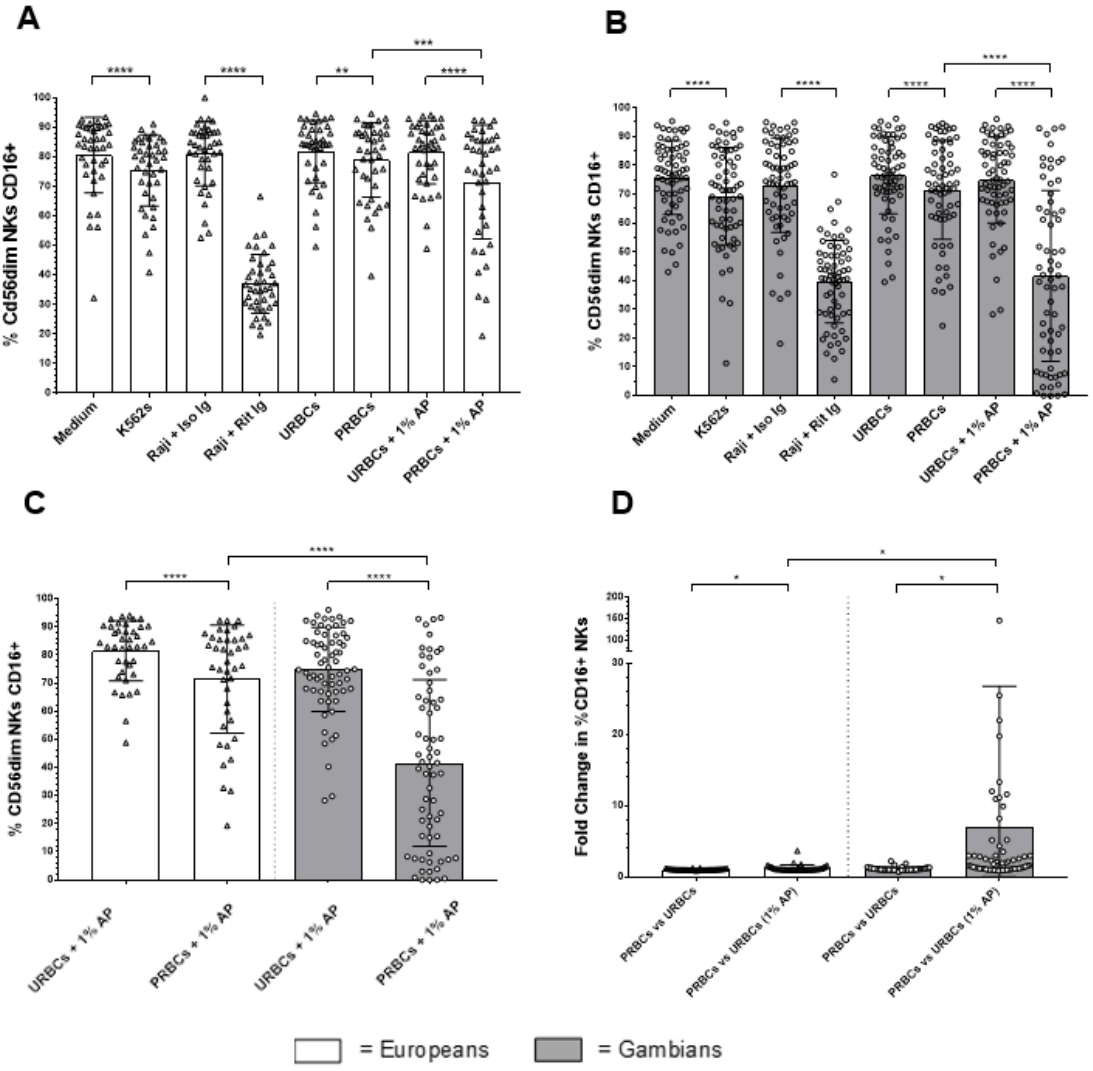


Figure 5.5 **European and Gambian CD56^{dim} NK** CD16 **responses to control treatments and PRBCs with autologous plasma.**

A-B) European (A) or Gambian (B) PBMCs were thawed and incubated with; (bars, left to right) culture medium alone, K562 cells, Raji cells with IgG1 isotype control antibody, Raji cells with Rituximab antibody, URBC, URBC with 1% autologous plasma, PRBC, or PRBC with 1% autologous plasma. Cells were stained and fixed for CD16. (European n value = 40; Gambian n value = 66, paired t-tests, **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05).

C) European (left bars) and Gambian (right bars) PBMCs were thawed and incubated with URBCs, URBCs 1% autologous plasma, PRBCs, or PRBCs with 1% autologous plasma. Cells were stained and fixed for CD16; (European n value = 40; Gambian n value = 66, within-group paired t-tests and between-group unpaired t-tests, **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05).

D) Fold change in the percentage of Gambian and European CD56dim NK cells expressing CD16 in response to either PRBCs vs URBCs (left bars) or PRBCs + 1% autologous plasma vs URBCs + 1% autologous plasma (right bars) (Gambian n value= 66, European n value=40, within-group paired and between-group unpaired t-tests, **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05).

5.3.9 Downregulation of CD16 on Gambian CD56^{dim} NK cells in response to incubation with PRBCs and malaria-exposed plasma correlates with degranulation

The percentage of Gambian CD56^{dim} NK cells downregulating CD16 when incubated with PRBCs and autologous plasma was plotted against the percentage of cells producing IFN-γ and presenting CD107a under the same culture conditions in order to determine whether antibody-dependent activation and shedding of CD16 was associated with CD56^{dim} functional activity.

A significant positive correlation was found between the downregulation in CD16 and CD107a presentation, suggesting the degranulation of Gambian CD56^{dim} NK cells in response to parasitised cells and plasma was perhaps mediated by binding of anti-malarial antibody-antigen complexes to the NK cell ($p<0.0001$, Fig. 5.6A).

No significant correlation was found between decrease in CD16 expression and IFN-γ production, indicating that while the presence of autologous plasma does significantly increase IFN-γ production by CD56^{dim} NK cells from Gambian individuals, this may involve multiple co-stimulatory factors (Fig. 5.6B).

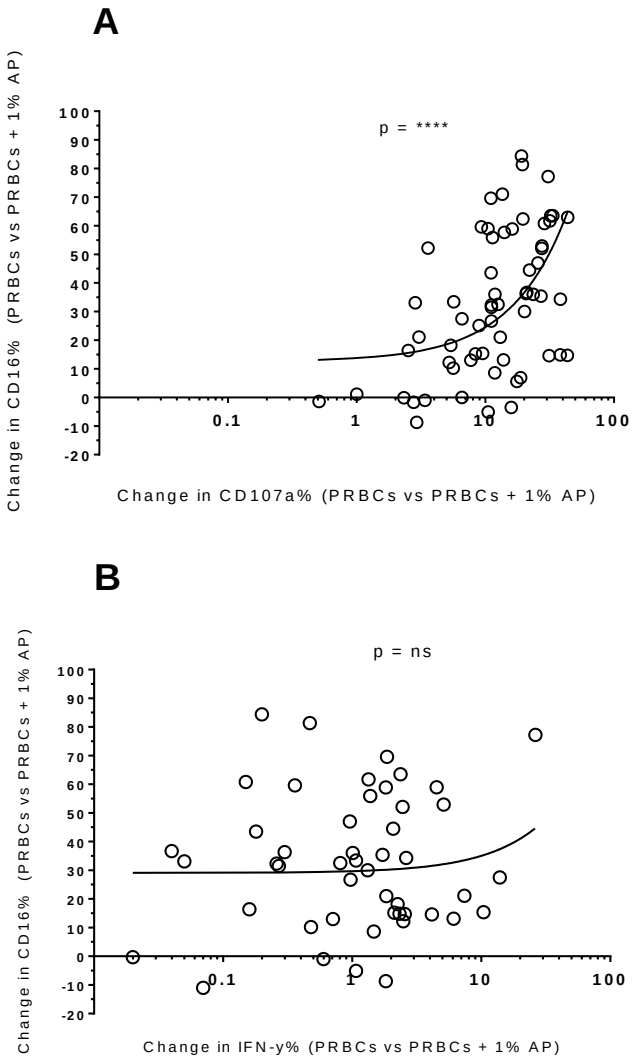


Figure 5.6 **Correlations of Gambian CD56^{dim} NK responses to PRBCs with autologous plasma.**
A-B) Correlations of change in the percentage of CD56^{dim} NK cells expressing CD16 (PRBCs vs PRBCs + 1% autologous plasma) against; A) change in percentage of CD56^{dim} NK cells producing CD107a; B) change in percentage of CD56^{dim} NK cells presenting IFN-γ (Gambian n value=66, Pearson correlation coefficients, **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05)

5.3.10 IFN-γ production by Gambian CD56^{dim} NK cells in response to PRBCs and malaria-exposed plasma is upregulated by the cytokines IL-12 and IL-18, as well as ligation of the CD2 receptor

No significant correlation was found between the percentage of Gambian CD56^{dim} NK cells producing IFN-γ and the percentage of cells shedding CD16 after stimulation with PRBCs and autologous plasma (Fig. 5.6B). However, the percentage of CD56^{dim} NK cells producing IFN-γ was still significantly upregulated when cultured with parasitised cells and plasma versus parasitised cells alone (Fig. 5.3B).

There were two potential explanations for this result. IFN-γ production by CD56^{dim} NK cells could be completely unrelated to anti-malarial antibody-antigen complexes binding to CD16 present on NK cells, and instead be the result of an alternative pathway of activation induced by autologous plasma. Alternatively, IFN-γ production under these conditions could still involve binding of antibody-antigen complexes to NK cells, but also involve additional sources of co-stimulation that confound an absolute direct correlation between downregulation of CD16 and IFN-γ production.

We hypothesised that autologous plasma may synergise with the early production of pro-inflammatory cytokines by accessory cells present in the PBMC cultures, leading to indirect activation of CD56^{dim} NK cells. We therefore elected to test the effect of neutralising or blocking antibodies to inhibit potential sources of NK cell co-stimulation on the immune plasma-dependent response.

Inhibition assays utilised neutralising antibodies against cytokines IL-2, IL-12 and IL-18 which are known to co-stimulate NK cells and have previously been shown to be induced in response to PRBC alone. , Blocking antibodies against the pro-inflammatory cytokine receptor IFNαβR on NK cell and the adhesion receptor CD2 which is known to mediate interactions between NK cells and accessory cells were also tested. PBMC isolated from Gambian individuals (n=28) were incubated at a 1:3 ratio for 6 hours with erythrocytes infected with schizont-stage *Plasmodium falciparum* and autologous immune plasma and the relevant blocking antibody or isotype control.

Blocking of soluble IL-12 significantly decreased the frequency of CD56^{dim} NK cells producing IFN-γ in response to PRBC and autologous immune plasma compared to isotype-matched control antibody. The mean percentage of CD56dim NK cells producing IFN-γ when incubated with IL-12 blocking antibody was 2% (SD±1.5) in comparison to 3% (SD±3.51) with isotype control, representing a modest but significant

decrease in frequency of 1% ($p<0.05$, Fig. 5.7A). Blocking of soluble IL-18 also significantly decreased the frequency of $CD56^{dim}$ NK cells producing IFN-γ in comparison to incubation with isotype control. The mean percentage of $CD56^{dim}$ NK cells producing IFN-γ when incubated with IL-18 blocking antibody was 2.18% (SD±2.04) in comparison to 3% (SD±3.51) with isotype control, representing a modest mean decrease of 0.82% ($p<0.05$, Fig. 5.7A). Blocking of the CD2 receptor also significantly decreased the frequency of $CD56^{dim}$ NK cells producing IFN-γ in comparison to incubation with isotype control antibody. The mean percentage of $CD56^{dim}$ NK cells producing IFN-γ when incubated with CD2 blocking antibody was 2.07% (SD±1.74) in comparison to 3% (SD±3.51) with isotype control, representing a mean decrease of 0.93% ($p<0.05$, Fig. 5.7A). Blocking of IL-2 and the IFNαβ receptor had no significant effect on IFN-γ production in the presence of parasite erythrocytes and autologous plasma.

We next determined whether IL-12, IL-18 and CD2 were *required* for IFN-γ production by $CD56^{dim}$ NK cells in the presence of accessory cells and immune autologous plasma, or whether plasma alone was capable of inducing this response. To do this we repeated the previous 6 hour incubation using enriched NK cells isolated through negative selection to remove accessory PBMCs that could serve as a source of co-stimulatory cytokines or receptor-mediated signals. CD2 blocking antibody was also added to some samples to prevent the possibility of NK cells providing each other with activating CD2 ligands.

NK cells were enriched using a MACS NK Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions, as described in section 2.2.4. NK cells were enriched to >80% purity. When the original functional assays were repeated using enriched NK cells a significantly higher frequency of $CD56^{dim}$ NK cells produced IFN-γ after incubation with PRBCs and autologous plasma than PRBCs alone despite the absence of accessory PBMCs to act as a source of CD2 ligands, IL-12 or IL-18. A mean of 0.14% CD56 NK cells expressed IFN-γ after incubation with PRBCs alone in comparison to a mean of 1.05% after incubation with PRBCs in the presence of plasma (Fig. 5.7B). This suggested that while IL-12, IL-18 and CD2 may upregulate production of IFN-γ by $CD56^{dim}$ NK cells in the presence of PRBCs and autologous plasma, NK cells are still able to produce IFN-γ in the absence of these signals. The co-stimulatory effects of IL-12, IL-18 and CD2 were noticeable however, as only a mean of 1.05% of isolated $CD56^{dim}$ NK cells expressed IFN-γ in response to parasitized cells and plasma in comparison to 2.77% when using mixed PBMCs ($p<0.0001$, Fig. 5.7B).

While this may suggest that NK cells were able to mount plasma-dependent responses to PRBCs in the absence of accessory cell factors, it must be noted that the enrichment process was not 100% efficient, with potential implications for these results. This will be discussed in more detail in the relevant discussion section.

5.3.11 Degranulation by Gambian CD56^{dim} NK cells in response to PRBCs and malaria-exposed autologous plasma does not require IL-2, IL-12, IL-18, or the CD2 or IFN$\alpha\beta$ receptors

A significant correlation was found between the percentage of CD56^{dim} NK cells producing presenting CD107a and the percentage of cells shedding CD16 after stimulation with PRBCs and autologous plasma suggesting degranulation under these conditions is significantly associated with anti-malarial antibody-antigen complexes binding to CD16 ($p<0.0001$, Fig. 5.6A). However, it remained possible that additional co-factors such as pro-inflammatory cytokines provided by PBMCs could also contribute to the degranulation response. Therefore we also assessed degranulation responses as measured by presentation of CD107a in the same blocking and enriched NK assays previously mentioned.

Blocking of IL-2, IL-12, IL-18 and the IFN$\alpha\beta$ and CD2 receptors had no significant effect on degranulation, suggesting that degranulation was not dependent on these factors (Fig. 5.7C).

In keeping with these results, when the original functional assays were repeated using enriched Gambian NK cells rather than mixed PBMCs a noticeably higher percentage of CD56^{dim} NK cells still demonstrated degranulation after 6 hours incubation with parasitised cells and autologous plasma in comparison to incubation with parasitised cells alone. A mean of 17.54% CD56 NK cells expressed IFN-γ after incubation with parasitised cells alone in comparison to a mean of 23.23% after incubation with parasitised cells and plasma (Fig. 5.7D).

5.3.12 Shedding of CD16 by CD56^{dim} NK cells in response to PRBCs and malaria-exposed plasma does not require accessory factors

In order to assess that shedding of CD16 by Gambian CD56^{dim} NK cells was primarily dependent on anti-malarial antibodies present in autologous plasma rather than other

factors, the percentage of cells shedding CD16 was also assessed in blocking assays. Blocking of IL-2, IL-12, IL-18, CD2 and the IFN$\alpha\beta$ receptor had no significant effect on the percentage of cells shedding CD16, suggesting that this effect was perhaps driven primarily by binding of anti-malarial antibody-antigen complexes to CD16 (Fig. 5.7E).

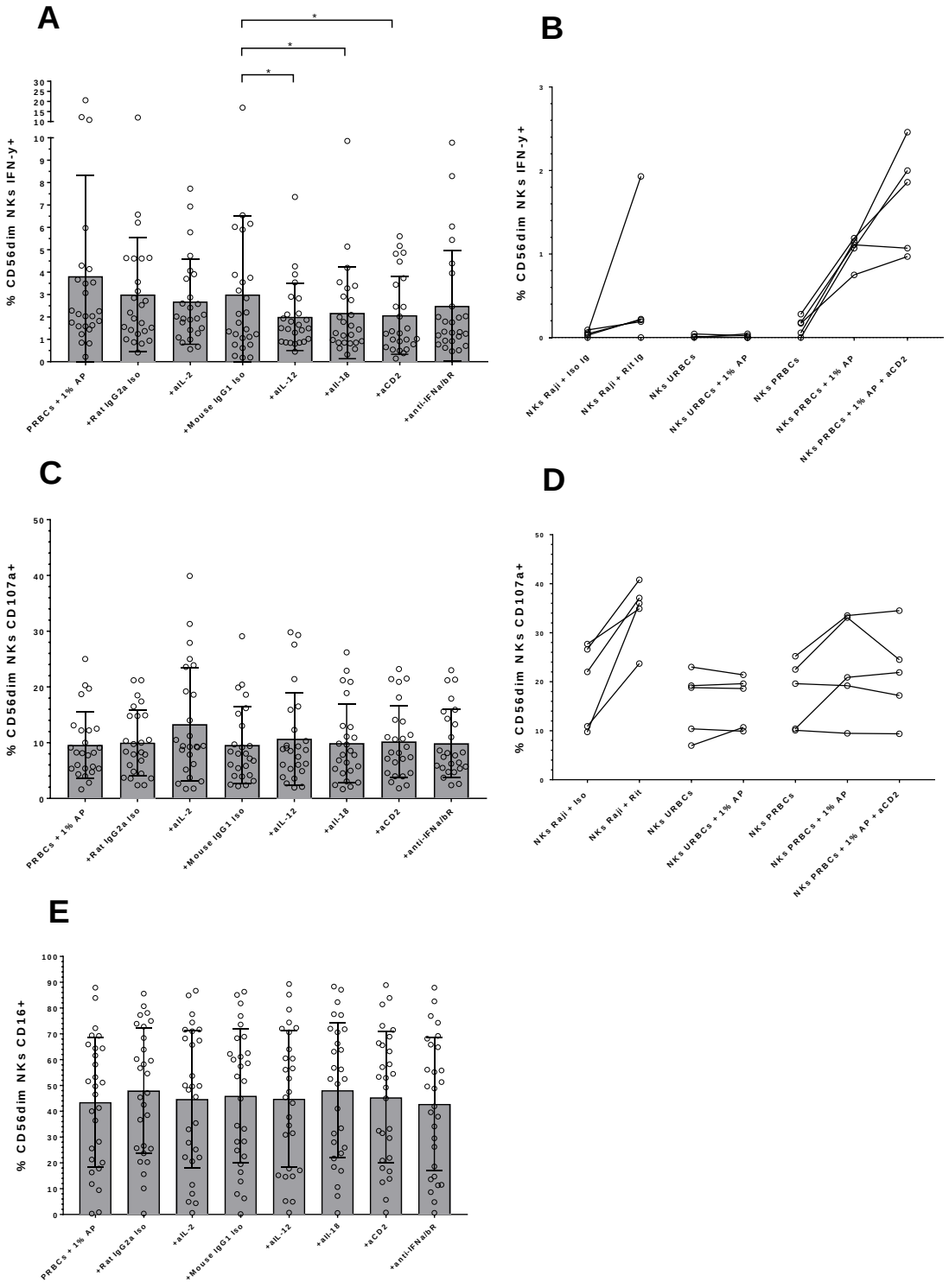


Figure 5.6 **Gambian CD56^{dim} NK responses to PRBCs with autologous plasma in the presence of blocking antibodies or after enrichment**

A, C, E) PBMCs were thawed and incubated with either (bars, left to right) PRBCs, PRBCs with 1% autologous plasma, or PRBCs with 1% autologous plasma and additional antibodies; rat IgG2a isotype control; anti-IL-2 Ig; mouse IgG1 isotype control; anti-IL-12 Ig; anti-IL-18 Ig; anti-CD2 Ig; or anti-IFNα/βR Ig. Cells were fixed and stained for functional markers; A) IFN-γ; C) CD107a; D) CD16 (n value=28, paired Wilcoxon test, **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05).

B, D) NK cells were enriched and incubated with either (bars, left to right) Raji cells with mouse IgG1 isotype control, Raji cells with Rituximab, URBCs, URBCs with 1% autologous plasma, PRBCs, PRBCs with 1% autologous plasma, or PRBCs with 1% autologous plasma and anti-CD2 Ig. Cells were fixed and stained for functional markers; B) IFN-γ; D) CD107a (n value=5)

5.3.13 Anti-AMA-1 plasma titres do not correlate with IFN-γ production or degranulation by CD56^{dim} NK cells in response to PRBCs and plasma

Prior to the current study, the Gambian PBMC and plasma donors used in this study (n= 66) were tested for prior malarial exposure via an ELISA assay testing for the presence of antibodies against apical membrane antigen 1 (AMA-1), a common malarial antigen expressed on merozoites. The plasma of all donors tested positive for antibodies against AMA-1 but varied considerably in their relative concentration of this antibody.

We hypothesised that the heterogeneity seen in Gambian donors in their CD56^{dim} IFN-γ production, degranulation and CD16 downregulatory responses to parasitised cells and plasma could be due to individual differences in the quality or quantity of anti-malarial antibodies produced by donors. We therefore elected to analyse whether anti-AMA-1 titres correlated with these responses in our Gambian cohort.

No significant correlation was found between IFN-γ production and anti-AMA-1 plasma titres. This indicated that while the presence of autologous plasma does significantly increase IFN-γ production by Gambian CD56^{dim} NK cells through partially antibody-driven mechanisms, antibodies directed against AMA-1 specifically are not a primary contributor to this response (Fig. 5.8A).

Similarly, no significant correlation was found between degranulation and anti-AMA-1 plasma titres. This suggested that while the presence of autologous plasma significantly upregulates degranulation by Gambian CD56^{dim} NK cells against parasitised cells through a primarily antibody-driven mechanism, antibodies directed against AMA-1 specifically are not a primary contributor to this response either (Fig. 5.8B).

Surprisingly, a significant positive correlation was found between downregulation in CD16 and anti-AMA-1 titres ($p<0.05$, Fig. 5.8C). When this trend was inspected visually however, it became apparent that this was largely due to a correlation between CD16 downregulation and anti-AMA-1 titres at only the very highest relative concentrations of anti-AMA-1, as indicated by a the steepness of the curve at the distal end of the x-axis (Fig. 5.8C). Potential explanations for this trend will be discussed in more detail in the relevant discussion section.

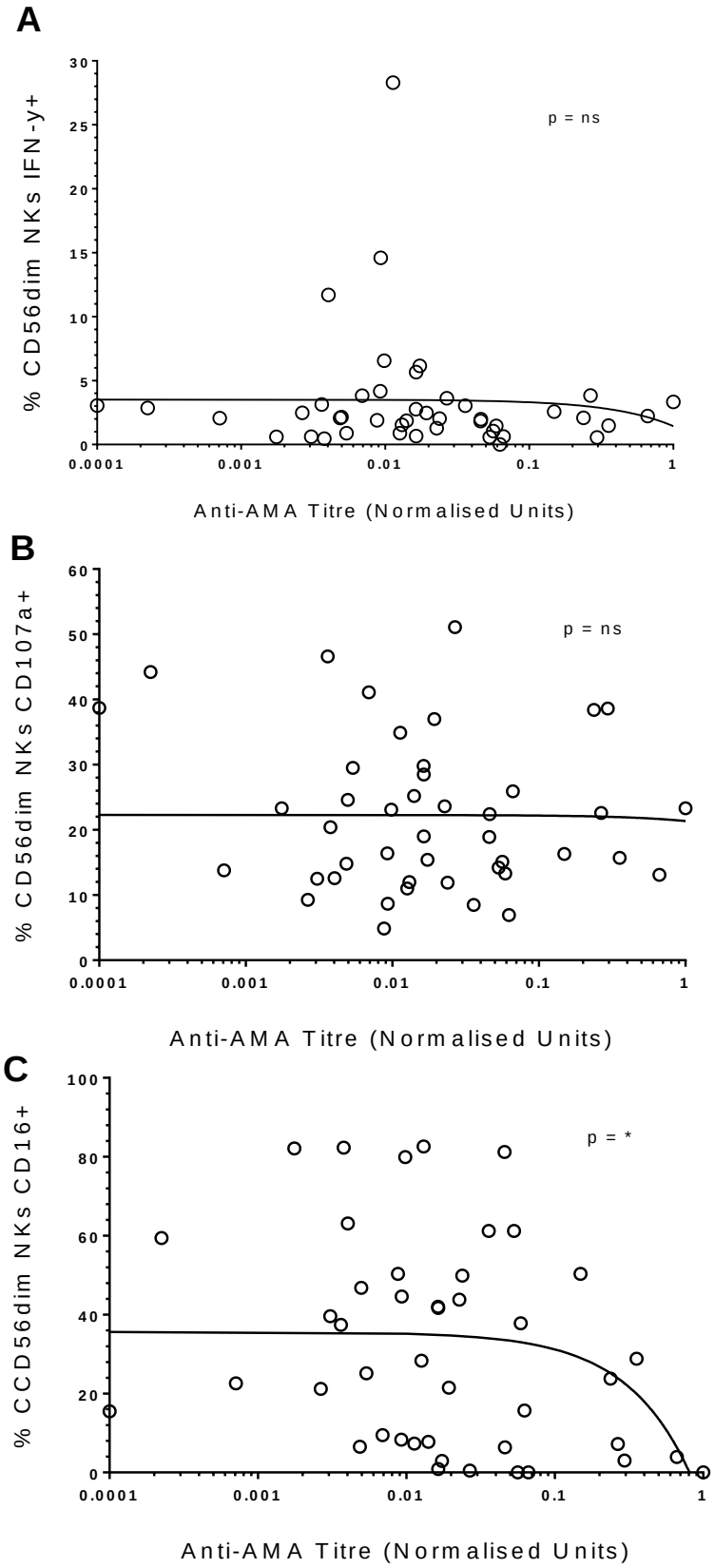


Figure 5.8 **Correlations of Gambian NK responses to parasitized erythrocytes and autologous plasma versus normalised anti-AMA titres**

Gambian plasma was tested via ELISA for titres of antibody targeting apical membrane antigen 1 to assess prior malarial exposure. PBMCs from anti-AMA positive individuals were thawed and incubated with PRBCs at a 1:3 ratio with 1% autologous plasma. Cells were incubated for 6 hours at 37ºC before being stained and fixed for functional markers. Correlation plots show anti-AMA titres versus percentage of $CD56^{dim}$ NK cells expressing; A) IFN-γ; B) CD107a; C) CD16 (n value=47, Pearson correlation coefficient, **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05).

5.3.14 High frequencies of CD56^{dim} FcεRIγ- CD57- and CD56^{dim} FcεRIγ- CD57+ NK cells in Gambian individuals compared to both HCMV+ and HCMV- Europeans

Published studies by several research groups have indicated that prior infection with human cytomegalovirus (HCMV) drives the expansion of differentiated CD57+ CD56^{dim} NK cells, as well a recently identified CD56^{dim} subset tentatively referred to as 'adaptive' NK cells characterised by reduced expression of the adaptor molecule FcεRIγ. Both differentiated and 'adaptive' CD56^{dim} NK cells have exhibited enhanced capacity to respond to antibody in models of viral infection and cancer. Given our interest in antibody-dependent responses to malaria and the endemicity of HCMV in the Gambia, we chose to investigate the relative contribution of these cells to the IFN-γ and degranulation responses we previously observed.

As the Gambia is endemic for HCMV with an estimated ~85% of individuals infected by 12 months of age [229], we expect relatively high frequencies of both differentiated and adaptive NK cells amongst this population. In contrast, epidemiological studies of Europe indicate an average HCMV infection frequency of below 80% in Caucasian adults of childbearing age, with infection rates as low as 30-45% in certain demographics [232]. To assess this we compared the total proportion of CD56^{dim} NK cells that were either FcεRIγ+ CD57- (hereafter referred to as 'Canonical Immature'), FcεRIγ- CD57- (hereafter 'Adaptive Immature'), FcεRIγ+ CD57+ ('Canonical Mature') or FcεRIγ- CD57+ ('Adaptive Mature') in 66 Gambians, 20 HCMV+ European controls and 20 HCMV- European controls.

Gambian individuals had significantly fewer Canonical Immature NK cells than both HCMV+ and HCMV- European donors, with a mean of 40.06% (SD±19.48) in Gambians in comparison to a mean of 48.91% (SD±17.71) in HCMV+ Europeans ($p<0.01$%, Fig. 5.9B) and 63.14% (SD±14.93) in HCMV- European donors ($p<0.0001$%, Fig. 5.9B).

Conversely, Gambian individuals had significantly more Adaptive Immature NK cells than both HCMV+ and HCMV- European individuals, with a mean of 13.47% (SD±10.47) in Gambians in comparison to 8.9% (SD±8.18) in HCMV+ Europeans ($p<0.05$, Fig. 5.9B) and 6.9% (SD±4.43) in HCMV- Europeans ($p<0.05$, Fig. 5.9B).

Gambian donors also had significantly fewer Canonical Mature NK cells than both HCMV+ and HCMV- European donors, with a mean of 18.09% (SD±11.33) in

Gambians in comparison to a mean of 28% (SD±16.45) in HCMV+ Europeans ($p<0.01\%$, Fig. 5.9B) and 27.34% (SD±11.4) in HCMV- European individuals ($p<0.01\%$, Fig. 5.9B).

Finally, Gambians also had significantly more Adaptive Mature NK cells than both HCMV+ Europeans and HCMV- Europeans, with a mean of 28.28% (SD±21.55) in Gambians versus 14.18% (SD±14.33) in HCMV+ Europeans ($p<0.01\%$, Fig. 5.9B) and 2.59% (SD±5.05) in HCMV- Europeans ($p<0.0001$, Fig. 5.9B**)**.

On average a mean of 41.75% of total $CD56^{dim}$ NK cells in Gambians were Adaptive (either Immature or Mature) in comparison to 23.08% in HCMV+ Europeans and 9.49% in HCMV- Europeans (Fig. 5.9B).

A mean of 46.46% of total $CD56^{dim}$ NK cells in Gambians were Mature (either Canonical or Adaptive) in comparison to 42.18% in HCMV+ Europeans and 29.92% in HCMV- Europeans (Fig. 5.9B).

5.3.15 Downregulation of FcεRIγ is significantly associated with higher expression of CD2 and NKG2C in Gambian individuals

Previously published studies by other research groups have indicated that downregulated expression of FcεRIγ is associated with increased expression of the activating receptor NKG2C as well as increased expression of the activating receptor CD2 in European individuals. We chose to evaluate this in our sampled Gambian individuals to confirm that $CD56^{dim}$ NK cells from Gambian donors downregulating FcεRIγ exhibited the same adaptive phenotype as seen in European counterparts.

Ex vivo staining of Gambian $CD56^{dim}$ NK cells revealed that a significantly greater proportion of Adaptive Immature NK cells expressed CD2 in comparison to Canonical Immature, though the absolute difference was relatively small (mean difference 5.42%, $p<0.05$, Fig. 5.9C). Adaptive Mature NK cells were also significantly more likely to express CD2 than their Adaptive Immature counterparts, and this difference was far greater (mean difference 21.3%, $p<0.0001$, Fig. 5.9C). Adaptive Mature cells were the most likely to express CD2 of all subsets, with a mean of 90.98% of these cells expressing the receptor.

Expression of CD57 without loss of FcεRIγ did not significantly increase CD2 expression. From this we conclude that loss of FcεRIγ is associated with increased

expression of CD2, and this effect seems to be most prominent on differentiated CD57+ NK cells; though expression of CD57+ alone seems to have no significant effect on CD2 expression.

Staining of Gambian CD56^{dim} NK cells also revealed that a significantly greater proportion of Adaptive Immature NK cells expressed NKG2C in comparison to Canonical Immature (mean difference 10.84%, $p<0.001$, Fig. 5.9D). Expression of CD57 without loss of FcεRIγ also significantly increased NKG2C expression (mean difference 13.64%, $p<0.0001$, Fig. 5.9D), though Adaptive Mature cells were still significantly more likely to express NKG2C than cells expressing CD57 without concurrent loss of FcεRIγ (mean difference 8%, $p<0.0001$, Fig. 5.9D). From this we conclude that both loss of FcεRIγ and expression of CD57 are associated with increased expression of NKG2C, and this effect seems to be synergistic, with Adaptive Mature NK cells the most likely to express NKG2C overall with a mean of 26% of these cells being NKG2C+ (Fig. 5.9D).

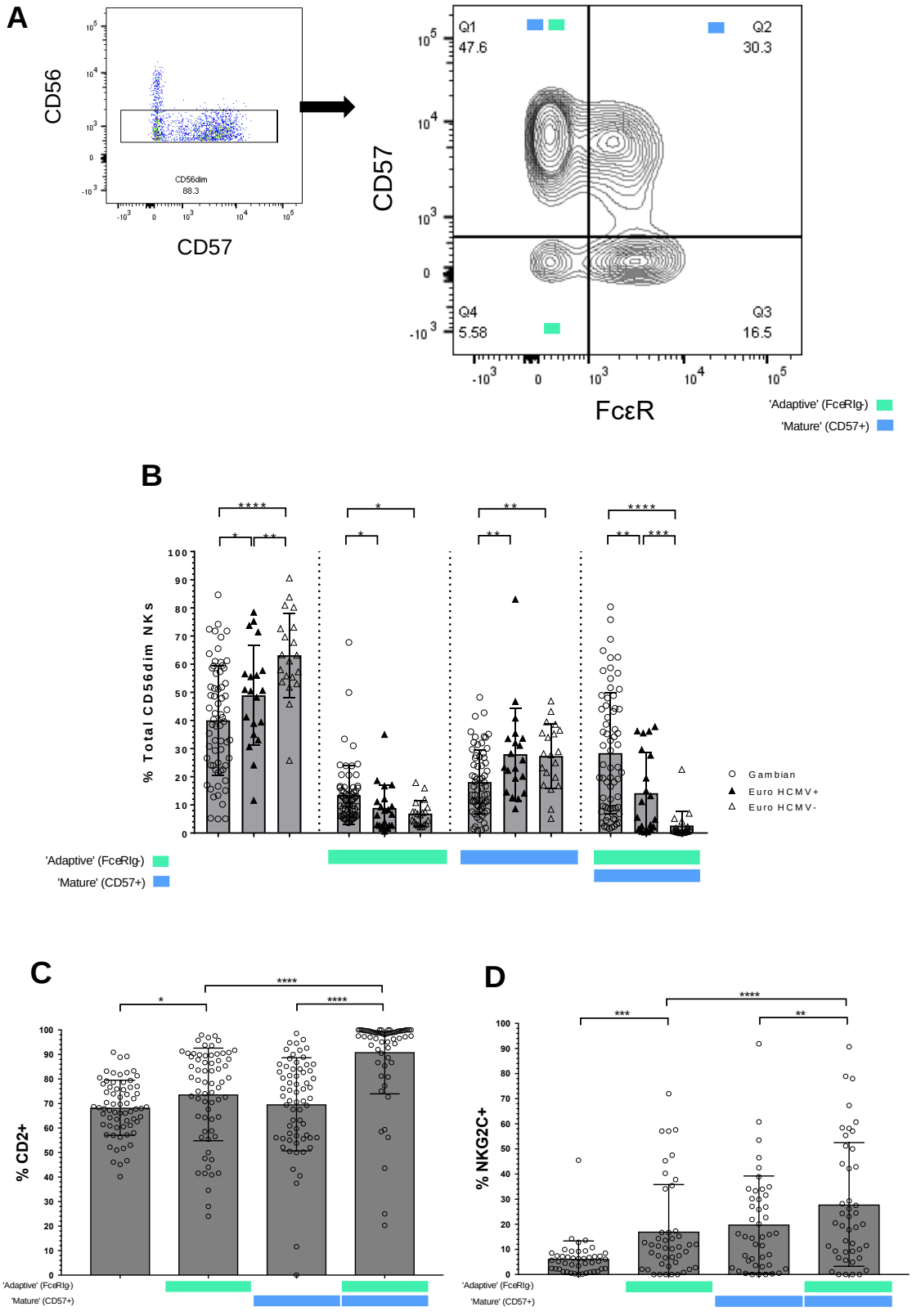


Figure 5.9 **Gating strategy, phenotype and frequency of FcεRIγ/CD57-defined subsets for European and Gambian donors**

A) Flow diagram demonstrating method of identifying CD56^{dim} FcεRIγ/CD57-defined NK cell subsets

B) Frequencies **of** CD56^{dim} FcεRIγ/CD57-defined subsets in Gambians, HCMV+ European and HCMV- European donors, expressed as a percentage of total CD56^{dim} NK cells (Gambian n value = 66, HCMV+ European n value = 20, HCMV- European n value = 20, unpaired t-tests, **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05).

C-D) Percentage of CD56^{dim} FcεRIγ/CD57-defined subsets expressing C) CD2 and D) NKG2C in Gambian donors (n value=66, paired t-tests, **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05).

5.3.16 Gambian CD56^{dim} Adaptive and Mature NK cells are more likely to produce IFN-γ in response to antibody-dependent control stimuli than less differentiated subsets

Published studies by several research groups have indicated that both differentiated and 'adaptive' CD56^{dim} NK cells from European donors have enhanced capacity to produce IFN-γ in response to antibody-antigen complexes in models of viral infection and cancer. We wished to confirm this in our Gambian donors using Raji cells and Rituximab antibody as an antibody-dependent control stimulus.

Both Adaptive Immature and Canonical Mature NK cells were significantly more likely to produce IFN-γ in response to control stimuli than Canonical Immature cells, suggesting that both maturity and adaptation led to increased sensitivity to activation via antibody. A mean of 4.17% (SD±3.13) of Canonical Immature NK cells produced IFN-γ in response to Raji cells and Rituximab in comparison to a mean of 7.42% (SD±6) for Adaptive Immature ($p<0.01$, Fig. 5.10A) and a mean of 5.71% (SD±4.01) for Canonical Mature cells ($p<0.01$, Fig. 5.10A).

Whilst FcεRIγ- adaptation alone led to higher production of IFN-γ than CD57+ alone, this effect appeared to be synergistic, as cells that were both Adaptive and Mature were the most likely to produce IFN-γ of all subsets. A mean of 9.63% (SD±7.46) of Adaptive Mature NK cells produced IFN-γ, significantly more than Adaptive Immature ($p<0.01$, Fig. 5.9A) or Canonical Immature ($p<0.01$, Fig.5.10A).

5.3.17 Gambian CD56^{dim} Adaptive Immature NK cells are significantly more likely to produce IFN-γ- than other subsets in response to PRBCs and autologous plasma

We next chose to investigate whether Adaptive or Mature CD56^{dim} NK cells also exhibited similar enhanced antibody-dependent IFN-γ production in response to infected erythrocytes in our Gambian donors.

All FcεRIγ/CD57-defined subsets were more likely to produce IFN-γ in response to infected erythrocytes and autologous plasma than URBCs with plasma, indicating an antibody-dependent response to PRBCs but not URBCs (Fig. 5.10A).

There was no significant difference in the percentage of cells producing IFN-γ between FcεRIγ/CD57-defined subsets except for Adaptive Immature NK cells. These cells were by far the most likely to produce IFN-γ, with a mean of 12.95% (SD±12.33) producing IFN-γ in comparison to a mean of 7.18% (SD±5.69) in Canonical Immature cells ($p<0.05$, Fig. 5.9A). Adaptive Immature cells were more likely to produce IFN-γ even in comparison with Adaptive Mature cells (mean = 7.29%, SD±4.67, $p<0.05$, Fig. 5.10A) though the latter had previously demonstrated the highest responses to antibody-dependent control stimuli (Fig. 5.10A).

These results did not conform to expectations based on our antibody-dependent Raji controls (Fig. 5.10A), suggesting that antibody alone may not determine the IFN-γ response of these cells. Previous experiments in this chapter on the total $CD56^{dim}$ NK cells population suggested that, in addition to anti-malarial antibodies, PBMC-derived IL-12 and IL-18 as well as the adhesion receptor CD2 play a role in the NK IFN-γ response to parasitised cells and plasma. We hypothesised that these co-factors may affect the IFN-γ production of different FcεRIγ/CD57- defined subsets to varying degrees. This was further investigated by analysing the effects of blocking antibodies on the responses of these subsets in section 5.3.20.

5.3.18 Both Adaptive and Mature Gambian $CD56^{dim}$ NK cells are more likely to degranulate in response to antibody-dependent control stimuli than less differentiated subsets

Published studies by several research groups have indicated that both 'mature' and 'adaptive' $CD56^{dim}$ NK cells from European donors have enhanced capacity to degranulate in response to antibody in models of viral infection and cancer. We wished to confirm this in our Gambian donors, again using Raji cells and Rituximab antibody as an antibody-dependent control stimulus.

Both Adaptive Immature and Canonical Mature NK cells were significantly more likely to degranulate in response to control stimuli than Canonical Immature cells, though this effect was minimal with Canonical Mature cells and far more prominent with Adaptive Immature. A mean of 31.58% (SD±11.39) of Canonical Immature cells degranulated in response to Raji cells and Rituximab in comparison to a mean of 43.6% (SD±11.82) for Adaptive Immature cells ($p<0.0001$, Fig. 5.10B) and a mean of 33.93% (SD±11.31) for Canonical Mature cells ($p<0.05$, Fig. 5.10B).

A combination of adaptation and maturity did not appear to significantly upregulate the degranulation response beyond adaptation alone. A mean of 45.71% (SD±13.07) of Adaptive Mature cells degranulated, which while significantly more than Canonical Mature cells ($p<0.0001$, Fig. 5.10B), was not significantly higher than the mean number of Adaptive Immature cells seen degranulating under the same conditions.

5.3.19 Higher frequency of degranulation within CD56^{dim} Adaptive Mature NK cell populations in response to PRBCs and autologous plasma

We next chose to investigate whether adaptive or differentiated CD56^{dim} NK cells also exhibited similar enhanced antibody-dependent degranulation in response to infected erythrocytes in our Gambian donors.

All FcεRIγ/CD57-defined subsets were more likely to degranulate in response to infected erythrocytes and autologous plasma than URBCs with plasma, indicating an antibody-dependent response to PRBCs but not URBCs (Fig. 5.10B).

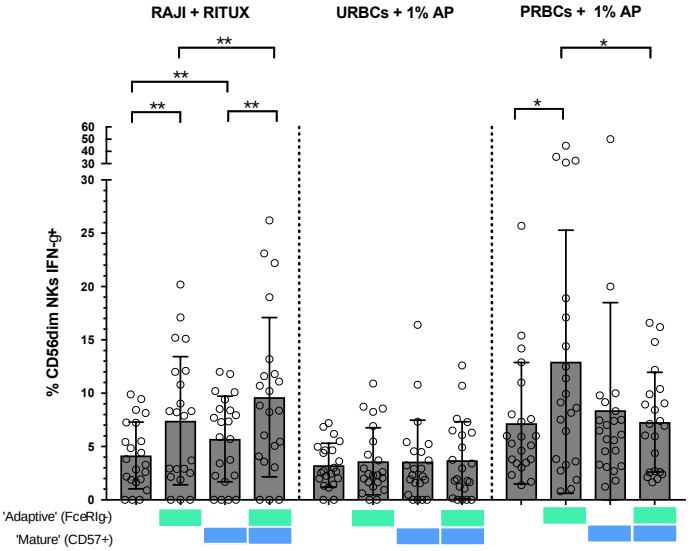
Both Adaptive Immature and Canonical Mature NK cells were significantly more likely to degranulate in response to control stimuli than Canonical Immature cells, suggesting that both maturity and adaptation led to increased sensitivity to activation via antibody. A mean of 27.57% (SD±15.16) of Canonical Immature cells degranulated in response to parasitized cells and plasma in comparison to a mean of 34.79% (SD±15.18) for Adaptive Immature cells ($p<0.01$, Fig. 5.10B) and a mean of 35.29% (SD±21.69) for Canonical Mature cells ($p<0.05$, Fig. 5.10B).

There was no significant difference between the percentage of cells degranulating between Adaptive Immature and Canonical Mature NK cells. Adaptation and maturity did not appear to be synergistic, as although cells that were Adaptive Mature were more likely to degranulate on average this difference was not significant from cells that were Adaptive or Mature alone (mean 37.59%, SD±21.72, Fig. 5.10B).

These results did not conform to expectations based on our antibody-dependent Raji controls where FcεRIγ- adaptation appeared to be the primary driver of antibody-dependent granulation whilst CD57+ maturity had little to no effect (Fig. 5.10B). This suggested that antibody alone may not determine the degranulation response of CD57+ cells in response to parasitized cells. However, previous experiments on the total CD56^{dim} NK cells population did not indicate any role for PBMC-derived IL-2, IL-12 or

IL-18 or the receptors IFNαβR or CD2 in the NK degranulation response to parasitized cells and plasma, suggesting this may be due to an untested co-factor (Fig. 5.10B).

A



B

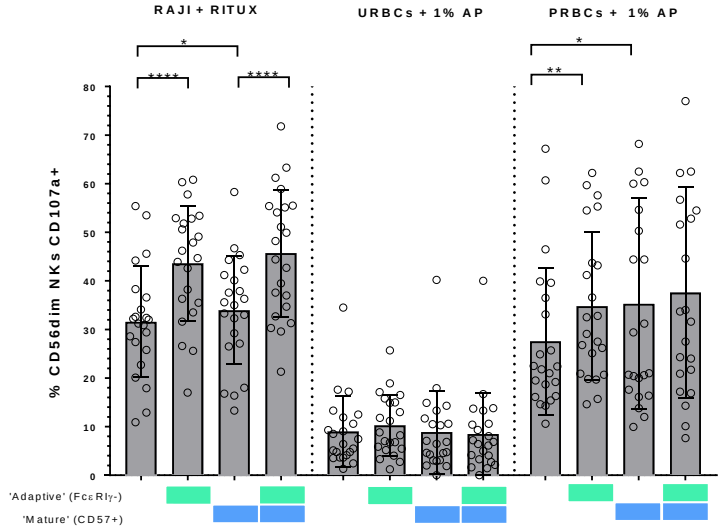


Figure 5.10 **IFN-γ and degranulation responses of Gambian CD56^{dim} FcεRIγ/CD57-defined subsets**

A-B) Percentage of CD56^{dim} FcεRIγ/CD57-defined subsets expressing A) IFN-γ or B) CD107a in response to (trisections, left to right) Raji cells and Rituximab antibody, uninfected erytrhocytes and 1% autologous plasma (AP), or parasitized erythrocytes and 1% autologous plasma

(n value = 22, paired t-tests, **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05

5.3.20 Production of IFN-γ by Gambian CD56^{dim} Canonical Immature NK cells is partially dependent by IL-12, IL-18 and CD2

Blocking assays utilised antibodies against the IL-2, IL-12 and IL-18 as well as antibodies against the adhesion receptor CD2. PBMC isolated from Gambian individuals (n=28) were incubated at a 1:3 ratio for 6 hours with PRBC, autologous plasma and the relevant blocking antibody or isotype control.

Blocking of soluble IL-12 significantly decreased the percentage of Canonical Immature FcεRIγ+ CD57- NK cells producing IFN-γ in comparison to the IgG1 isotype control antibody. The mean percentage of Canonical Immature cells producing IFN-γ when incubated with IL-12 blocking antibody was 1.12% (SD±0.8) in comparison to 1.83% (SD±1.31) with isotype control, representing a mean decrease of 0.71% ($p<0.01$, Fig. 5.11A).

Blocking of IL-18 also significantly decreased the frequency of Canonical Immature cells producing IFN-γ in comparison to IgG1 isotype control antibody. The mean percentage of Canonical Immature cells producing IFN-γ when incubated with IL-18 blocking antibody was 0.91% (SD±0.85) in comparison to 1.83% (SD±1.31) with isotype control, representing a mean decrease of 0.92% ($p<0.001$, Fig. 5.11A).

Additionally, blockade of the NK CD2 receptor significantly decreased the frequency of Canonical Immature cells producing IFN-γ. The mean percentage of Canonical Immature cells producing IFN-γ when incubated with CD2 blocking antibody was 1.02% (SD±0.88) in comparison to 1.83% (SD±1.31) with isotype control, representing a mean decrease of 0.81%. ($p<0.001$, Fig. 5.11A).

These data indicate that the antibody dependent responses of less differentiated, canonical NK cells are more dependent on accessory cytokines and co-stimulatory molecules than other subsets.

5.3.21 Enhanced production of IFN-γ by Gambian CD56^{dim} Adaptive Immature NK cells is mediated by IL-12

Blocking of soluble IL-12 significantly decreased the percentage of Adaptive Immature NK cells producing IFN-γ in comparison to the IgG1 isotype control antibody. The mean percentage of Adaptive Immature cells producing IFN-γ when incubated with IL-12

blocking antibody was 8.95% (SD±8.01) in comparison to 13.76% (SD±12.66) with isotype control, representing a mean decrease of 4.81% ($p<0.05$, Fig. 5.11B). In contrast, blocking of IL-18 and the CD2 receptor had no significant effect on IFN-γ production in the presence of parasite erythrocytes and autologous plasma (Fig. 5.11B).

These results indicated that the production of IFN-γ by Adaptive Immature NK cells in response to parasitized cells and plasma was mediated by both antibody and IL-12, potentially explaining the significantly high levels of IFN-γ production found in this subset. This issue will be discussed in more detail in the relevant discussion section.

In both Canonical Mature and Adaptive Mature NK cells blocking of IL-12, Il-18 and CD2 appeared to have no significant effect on the percentage of cells producing IFN-γ (Fig. 5.11C-D).

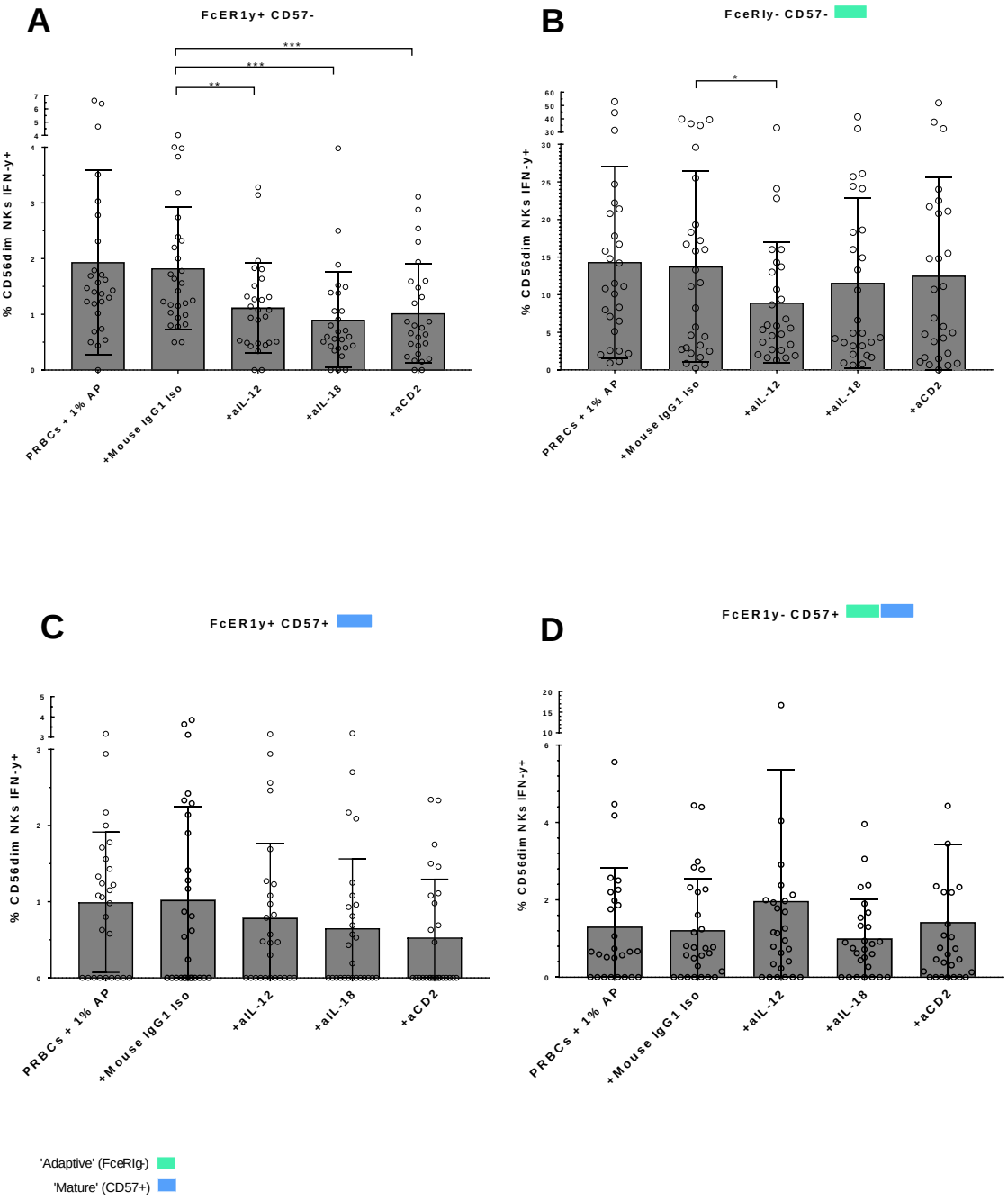


Figure 5.11 **Gambian CD56^{dim} NK IFN-γ responses to PRBCs with autologous plasma in the presence of blocking antibodies**

A-D) PBMCs were thawed and incubated with either (bars, left to right) PRBCs with 1% autologous plasma alone, or PRBCs with 1% autologous plasma and either; mouse IgG1 isotype control; anti-IL-12 antibody; anti-IL-18 antibody; or anti-CD2 antibody. Cells were stained and fixed for functional markers; A) IFN-γ; B) CD107a; C) CD16 (n value=28, paired Wilcoxon test, **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05).

5.4 Discussion

In this study I have attempted to characterise the NK antibody-dependent response to *Plasmodium falciparum* malaria in a representative *in vitro* model of infection, using mixed peripheral blood mononuclear cells and autologous plasma from confirmed malaria-exposed Gambians and malaria-naïve Europeans.

Given the recent interest in so-called 'adaptive' NK cells and their seemingly enhanced capacity to respond to antibody, I have also specifically characterised the role of this particular subset in the antibody-dependent response to malaria in this Gambian cohort. I have also examined the role of pro-inflammatory co-factors on the NK cell antibody-dependent response to infected erythrocytes, utilising blocking assays and other techniques to investigate the influence of pro-inflammatory cytokines and accessory leukocytes on this response.

5.4.1 Plasma-independent IFN-γ production by CD56^{dim} NK cells in response to blood-stage malaria is significantly greater in Gambians than Europeans

In this study, cryopreserved PBMCs from 66 Gambian donors with prior malarial exposure and 40 European donors without prior exposure were incubated with *Plasmodium*-infected erythrocytes (PRBCs) or uninfected (URBCs); either alone, or in the presence of immune or non-immune autologous plasma. Cells were then stained, fixed and imaged using an LSRII flow cytometer to determine the expression of various functional markers. Production of pro-inflammatory IFN-γ and presentation of CD107a as a measure of cytotoxic activity were used to assess the relative responses of CD56^{dim} NK cells cultured under these conditions, while downregulation of CD16 was used to determine the contribution of antibody-antigen complexes to these responses.

CD56^{dim} NK cells from Gambian donors were significantly more likely to produce IFN-γ and degranulate when cultured for a short period of 6 hours in the presence of PRBCs than when cultured with URBCs (Fig. 5.3A-B). This result concurs with previous work our group has published on the subject, where we have seen NK cells producing IFN-γ after a short period incubated with PRBCs [82,188] as well as demonstrating signs of cytotoxic killing [190]. IFN-γ production in response to infected erythrocytes was also considerably heterogenous, with some donors expressing no IFN-γ at all in response to parasitised cells, while in others nearly a fifth of CD56^{dim} NK cells produced the

cytokine. This is also in keeping with our previously published observations on the subject where the antibody-independent NK response to parasitised cells varied largely between donors [190].

Curiously however, $CD56^{dim}$ NK cells from European donors did not produce significant levels of IFN-γ when incubated for 6 hours with PRBCs in comparison to controls. This conflicts with previous observations published by our group, where NK cells obtained from European donors produced noticeable levels of IFN-γ in response to incubation with *falciparum*-infected erythrocytes after only 6 hours of co-culture [82]. The number of donors tested in our previous studies were considerably smaller than in the study described in this thesis however, and given the heterogenicity we have also observed in these responses it may be that a small sample size does not give reliable results. Additionally, while noticeable levels of IFN-γ were observed in some donors after 6 hours in previous studies, this response did not reach optimal in all donors until much later at around 24 hours. This is more in keeping with the results of our longer-term assays where both Gambian and Europeans were able to produce IFN-γ after being cultured in the presence of PRBCs for 18 hours.

Regardless, it appears that the acute antibody-independent response of Gambian $CD56^{dim}$ NK cells to infected erythrocytes is more potent than that of Europeans, particularly in regard to IFN-γ production. Given the heterogenicity of the observed in the antibody-independent IFN-y responses to malaria, it is clear that donors within a single population differ in the magnitude by which they respond to this parasite, possibly as a result of differential expression of receptors that mediate activation. It is possible that these differences manifest both at an inter-group as well as inter-individual level as a result of either genetic differences or immunological adaptation to the local microflora, causing Gambian donors to respond more potently in comparison to Europeans. It is worth noting that all the sampled European donors were of White British ethnicity, and as such it is very likely that genetic differences exist between these donors and native Gambians.

Previous work conducted by Dr Asia Wolf in our lab demonstrated higher baseline responsiveness to pro-inflammatory cytokines in African populations in Uganda, partly as a result of higher baseline expression of activating cytokine receptors such as CD25 [191]. It is possible that NK cells from Gambian individuals may demonstrate similarly high expression of cytokine receptors which may mediate activation in instances of malarial infection. Previous studies have identified IL-12 and IL-18 produced by myeloid accessory cells [188] and (speculatively) trans-presentation of IL-15 by dendritic cells [191]

as important antibody-independent activating signals for NK cells in instances of malarial infection. Consequently, future work on the subject should involve investigating the expression of the receptors for these cytokines on Gambian NK cells versus the cells of Europeans.

In addition, while the specific physical contacts that occur between NK cells and accessory cells in instances of malarial infection remain largely uncharacterised, binding of LFA-1 expressed on the NK cell surface to ICAM-1 expressed on PBMCs has been suggested to mediate activation [103]. It may also be worth investigating whether expression of this receptor varies between European and Gambian populations in future work.

The receptors which mediate direct interactions between NK cells and infected erythrocytes are similarly unknown or disputed, but should any be definitively identified in the coming years it would also be beneficial to investigate whether their expression varies between Gambian and European populations.

5.4.2 Significant enhancement of IFN-γ production to PRBCs in the presence of autologous plasma is likely due in part to anti-malarial antibodies, but further work is required to confirm this

$CD56^{dim}$ NK cells from Gambian donors were significantly more likely to produce IFN-γ and degranulate when cultured in the presence of erythrocytes infected with late-stage *Plasmodium falciparum* than when cultured with URBCs. These responses were relatively small, but were greater enhanced in the presence of autologous plasma.

This indicated that NK cells were perhaps responding to anti-malarial antibody-antigen complexes through CD16, resulting in considerable greater production of pro-inflammatory cytokines and enhanced cytotoxic responses in comparison to the antibody-independent response.

In support of this explanation, plasma from malaria-naïve Europeans elicited a much smaller IFN-γ response and weaker CD16 downregulation in autologous $CD56^{dim}$ NK cells. This would be in keeping with the interpretation that NK responses to PRBCs in the presence of plasma were perhaps driven primarily by anti-malarial antibodies, as European donors were malaria-naïve and thus would have no such antibodies.

However, the European response to parasitised erythrocytes in the presence of plasma was still significantly greater compared to the response to parasitised erythrocytes alone, suggesting that non-immune plasma can still upregulate NK cell responses to parasitised erythrocytes to a certain extent through alternative (albeit weaker) antibody-independent mechanisms. These could include residual pro-inflammatory cytokines possibly present in European plasma, inflammatory factors produced by accessory cells in response to PRBCs capable of inducing moderate downregulation of CD16, or soluble blood components such as haem which are known to be immunogenic [213]. Variance in the composition and concentration of such plasma-derived proteins between European and Gambian donors could also perhaps explain the differences in their responses to uninfected erythrocytes in the presence of plasma.

Future work in this area should take into account the possibility that plasma may contain immune mediators other than antibody, and perhaps test for their presence using techniques such as multiplex immunoassays. Alternatively, future work in this area may involve the use of purified anti-malarial antibodies rather than mixed immune plasma, allowing for the assessment of antibody-dependent responses without concern for other potentially confounding immunological mediators. This would also allow us to control for any differences between the composition of Gambian and European plasma.

At the time of writing there are currently no full published studies on the autologous antibody-dependent NK response to malaria in exposed subjects. As such these results are relatively novel, though they appear to be in concert with a recent study published by Arora et al in 2018 where non-autologous pooled malaria-immune plasma was shown to illicit significant antibody-dependent cytotoxic responses against parasitised cells in malaria-naïve NK cells obtained from US donors [233]**.** While this is encouraging, the use of pooled plasma and relatively low sample numbers in the aforementioned study prohibits too great a comparison to our own results where we have taken care to match self-plasma to self-cells from naturally-exposed donors in order to better replicate the variation in anti-malarial responses that are likely to exist *in vivo*.

5.4.3 Plasma-dependent production of IFN-γ by CD56^{dim} NK cells in response to blood-stage malaria is upregulated by IL-12 and IL-18 produced by accessory peripheral blood mononuclear cells

Blocking experiments utilising antibodies directed against various different factors known to activate NK cells revealed that the cytokines IL-12 and IL-18 play a role in

upregulating the plasma-dependent production of IFN-γ in response to PRBCs. Several studies have suggested that antibody-dependent production of IFN-γ by NK cells in response to other targets such as cancer cells can also be upregulated by co-stimulation with pro-inflammatory cytokines such as IL-12, IL-2 and IL-18, offering some support for these data [113,114], [109].

IL-12 and IL-18 do not appear to be strictly necessary to induce plasma-dependent IFN-γ production in response to malaria however, as subsequent experiments carried out using enriched NK cells rather than mixed PBMCs suggested that immune plasma alone may be sufficient to provoke a response (Fig. 5.6D). Assuming that the primary source of IL-12 and IL-18 during the anti-malarial response are myeloid dendritic cells and CD14+ monocytes as our research group has previously confirmed [45,188], IFN-γ production by NK cells in the absence of these cells would suggest that the cytokines they produce are not obligatory to elicit a response. However, it must be noted that the enriched NK cells used in these assays were not 100% pure due to limitations of the enrichment process, allowing for the possibility that a small number of contaminatory dendritic cells and/or monocytes may have been present in the final solution. Consequently these results cannot be said to be entirely definitive. In future experiments it may be worth repeating these experiments with a pure NK cell line in order to control completely for the possibility of confounding cellular contamination.

When the Gambian $CD56^{dim}$ response was broken down by FcεRIγ/CD57-defined subset it was revealed that the different subsets had very different reactions to IL-12 and IL-18. Canonical Immature NK cells proved to be the most responsive to both cytokines, exhibiting a significant decrease in IFN-γ production when either was blocked. Previously published research by others in the field has indicated that both Immature CD57- cells and Canonical FcεRIγ+ cell have increased sensitivity to IL-12 and IL-18 in comparison to their Mature or Adaptive counterparts. In both Canonical FcεRIγ+ and Immature CD57- cells higher affinity for IL-12 has been linked to higher levels of transcription of the IL12Rβ2 gene which encodes the beta 2 subunit of the IL-12 receptor [126,137]. In Canonical cells specifically it has also been discovered that transcription of the IL18RAP gene which encodes an accessory subunit of the IL-18 receptor is as much as four-fold higher than in adaptive NK cells [137]. Consequently, the fact that we find Canonical Immature $CD56^{dim}$ NK cells to be the most responsive to IL-12 and IL-18 in our own study is well in keeping with the established view of these cells as highly cytokine-responsive.

IFN-γ responses to PRBCs within Adaptive Immature NK cells in contrast were unresponsive to IL-18, though surprisingly they retained the ability to respond to IL-12, which may explain why IFN-γ production in this subset was so significant. This will be discussed in more detail in section 5.4.5.

In contrast, neither Canonical Mature nor Adaptive Mature NK cells responded to either IL-12 or IL-18, suggesting IFN-γ production by these cells was primarily induced by antibody-antigen complexes with little requirement for accessory cytokines. Whilst recent results published by Hammer et al in 2018 have suggested that Adaptive Mature NK cells can respond to IL-18 when also stimulated by antibody [146], we found no evidence for this in our own study. We note however that in Hammer et al's study NK cells were artificially supplemented with IL-18, whereas in our own study the source of IL-18 is assumed to be monocytes present within the PBMCs as we have observed in our previously published work [45,188]. Consequently, it may be that the concentrations of IL-18 present in culture media in our own assays were not high enough to observe this effect. Future work should perhaps include repeat antibody-stimulation assays directed against malaria with or without the presence of supplemental cytokine to assess this issue more directly.

Blocking of these factors did not appear to have any significant effect on the general degranulation response of $CD56^{dim}$ NK cells to blood-stage malaria, and also did not appear to affect CD16 expression. This suggested that cytotoxic responses to blood-stage malaria are largely driven directly by immunoglobulin-opsonised parasitised cells with minimal input from other soluble accessory factors (Fig. 5.6B-C).

5.4.4 Plasma-dependent production of IFN- $CD56^{dim}$ NK cell response to blood-stage malaria is upregulated by binding of the NK CD2 receptor to accessory blood mononuclear cells

Blocking of the NK receptor CD2 significantly downregulated plasma-dependent production of IFN-γ in response to PRBCs (Fig. 5.6A). CD2 has several known ligands, including the lymphocyte differentiation marker CD48, the adhesion molecule CD58, and the glycoprotein CD59 [234]. These ligands can be expressed on a range of cells, including NK cells themselves, as well as both erythrocytes and accessory peripheral blood mononuclear cells such as monocytes [234,235]. Binding of CD2 to CD58 expressed on target cells has been shown to synergise with antibody crosslinking of CD16, leading

to enhanced antibody-dependent NK cell responses through increased phosphorylation of intracellular signalling molecules [149]. Binding of CD2 to CD58 expressed on accessory monocytes has also been shown to mediate presentation of cytokines to CD2-expressing cells, also leading to enhanced NK cell responses [228]. Consequently, the downregulation in IFN-γ production seen in $CD56^{dim}$ NK cells during the antibody-dependent response to malaria after blocking of CD2 may be due to multiple mechanisms. CD2 may mediate interactions between $CD56^{dim}$ NK cells and each other, parasitised erythrocyte targets, accessory PBMCs, or all of the above.

However, when the CD2 blocking assay was repeated using enriched NK cells incubated alone with PRBCs and immune plasma, there was no noticeable decrease in IFN-γ production in comparison to unblocked controls (Fig. 5.6D). This indicated that CD2 was not involved in mediating interactions between either NK cells and erythrocyte targets or NK cells and each other, but rather interactions between NK cells and non-NK accessory PBMCs.

When the $CD56^{dim}$ IFN-γ response was broken down by FcεRIγ/CD57-defined subset, only Canonical Immature FcεRIγ+ CD57- NK cells demonstrated any significant downregulation of IFN-γ production as a result of blocking CD2. These cells also demonstrated the greatest sensitivity to cytokine, responding to both IL-12 and IL-18. Previous work by other researchers has suggested that binding of CD2 expressed on T-cells to CD58-expressing monocytes can mediate presentation of cytokines such as IL-12, leading to enhanced T-cell activation [228]. Here we suggest that a similar mechanism exists for NK cells during the antibody-dependent response, by which CD2-expressing NK cells bind accessory PBMCs, leading to increased exposure to cytokines such as IL-12 and IL-18, which upregulate the antibody-dependent response.

Consequently, future work in this area should include blocking these cytokines and CD2 simultaneously. If blocking of CD2 does not significantly reduce activation beyond what is activated by blocking cytokines alone, it would confirm the role of CD2 in mediating cytokine presentation by accessory cells.

Blocking of CD2 did not appear to have any significant effect on the general degranulation response of $CD56^{dim}$ NK cells to blood-stage malaria, and also did not appear to affect CD16 expression. This again suggested that cytotoxic responses to blood-stage malaria during the antibody-dependent response are largely driven by immunoglobulin.

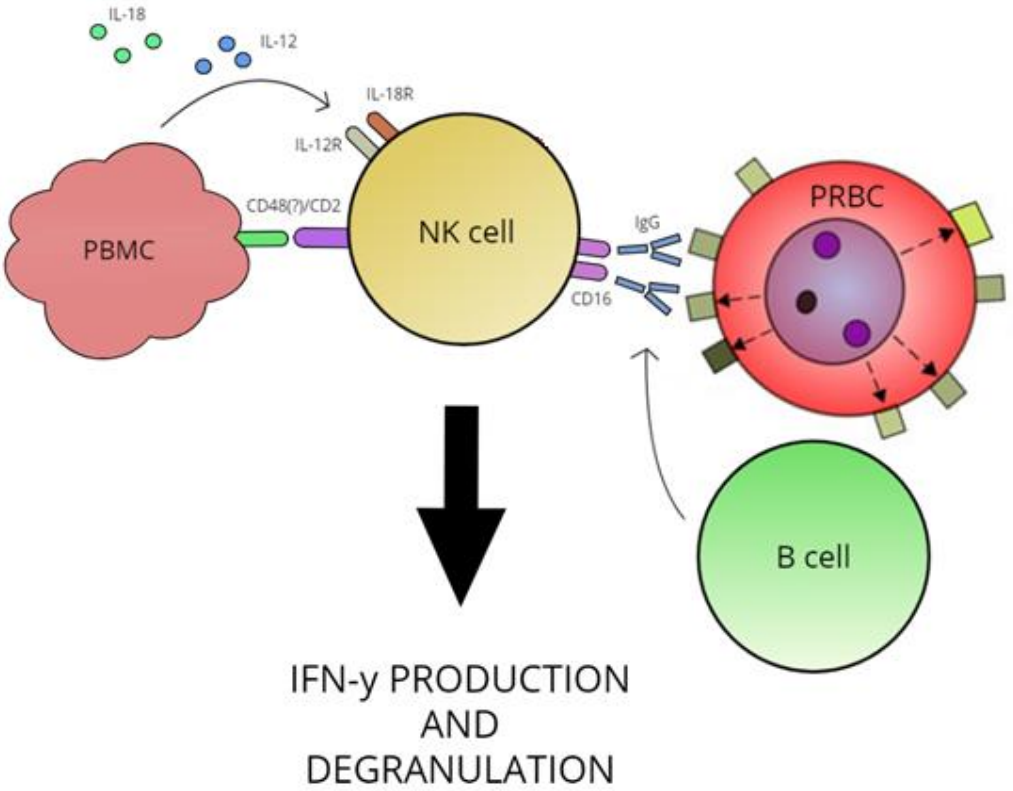


Figure 5.12. Proposed m**odel for activation of natural killer cells during the antibody-dependent response to malarial infection**

Previous research has established that NK cells are activated to produce IFN-γ during the adaptive immune response to malaria by IL-2 produced by activated T-cells. Based on the results of the current study, we also propose that NK cells can also be activated by binding of anti-malarial antibody-antigen complexes to the CD16 receptor, resulting in both production of IFN-γ and degranulation. We also propose that IFN-γ production in response to anti-malarial antibody is upregulated by IL-12 and IL-18 produced by peripheral blood mononuclear cells, as well as binding of the CD2 receptor to the same.

5.4.5 Adaptive Immature CD56^{dim} FcεRIγ- CD57- NK cells exhibit the strongest plasma-dependent IFN-γ response to malaria, but this is lost upon maturation to CD57+ cells

Adaptive NK cells are known to be more responsive to antibody-dependent stimulation, leading to enhanced production of IFN-γ in response to virally-infected cells and tumours [141] [144]. This altered functionality is thought to be driven by reduced expression of the transcription factor PLZF, leading to hypermethylation of certain promotor DNA regions [145] and subsequent downregulated expression of certain adaptor molecules [137]. Our own data confirmed this, as both Adaptive Immature and Adaptive Mature NK cells demonstrated significantly enhanced IFN-γ production in response to Raji tumour cells and Rituximab antibody.

This would explain why Adaptive Immature cells exhibit greater IFN-γ production during the plasma-dependent response to malaria than their Canonical Immature counterparts, as the latter lack the epigenetic modifications that make adaptive cells more responsive to activation through the CD16 Fc receptor. This would also lend weight to our interpretation that the upregulation of immune responses seen in NK cells in response to infected cells and malaria-exposed plasma was due to the presence of anti-malarial antibodies in the latter.

It is curious however that IFN-γ production within the Adaptive Immature subset was greater than in the Adaptive Mature subset. The latter are considered to be even more responsive to antibody-dependent stimulation as a result of increased expression of the CD16 receptor associated with CD57+ maturity [126]. Our own assays also confirmed that Adaptive Mature cells were the most likely to produce IFN-γ in response to Raji tumour targets and Rituximab antibody.

These results indicated that the antibody-dependent response of Adaptive Immature cells may be dependent on additional activating co-factors not applicable to their Mature counterparts. Subsequent blocking assays revealed that IFN-γ production by Adaptive Immature cells is partially mediated by IL-12, whereas Adaptive Mature CD57+ cells appear to be largely unresponsive to this cytokine. This offers an explanation for the higher responsiveness of Adaptive Immature cells relative to Adaptive Mature as the former likely possess the increased sensitivity to antibody indicative of Adaptive NK cells [141] [144] but also retain some ability to integrate cytokine into these responses.

Adaptive Mature cells in contrast are also sensitive to antibody but do not appear to be able to respond as well to cytokine.

These results are somewhat contradicted by the established literature on the subject. Early studies conducted on Adaptive NK cells by Schlums et al in 2015 indicated that they express lower levels of transcription of the genes encoding subunits of the IL-12 and IL-18 receptors, and consequently demonstrate reduced capacity to respond to these cytokines when provided without other forms of stimulation [137]. A more recent study by Hammer et al published in 2018 has suggested that Adaptive NK cells are able to respond to IL-18 when supplemented with it during both germline receptor mediated and antibody-dependent responses to target cells, whilst remaining largely unresponsive to IL-12 [146]. It is worth noting however that in the latter study 'adaptive' NK cells were defined as being purely FcεRIγ- CD57+ (what we would term 'Adaptive Mature'), whilst in our own study it is the FcεRIγ- CD57- 'Adaptive Immature' NK cells that demonstrated responsiveness to IL-12 during the antibody-dependent response. Adaptive Immature cells have been previously observed in the work of Schums et al [137] but were not investigated phenotypically in terms of differences in transcription factor, adaptor protein or cytokine receptor expression. It could therefore be that this subset represents an intermediate population with important functional characteristics against certain infectious agents.

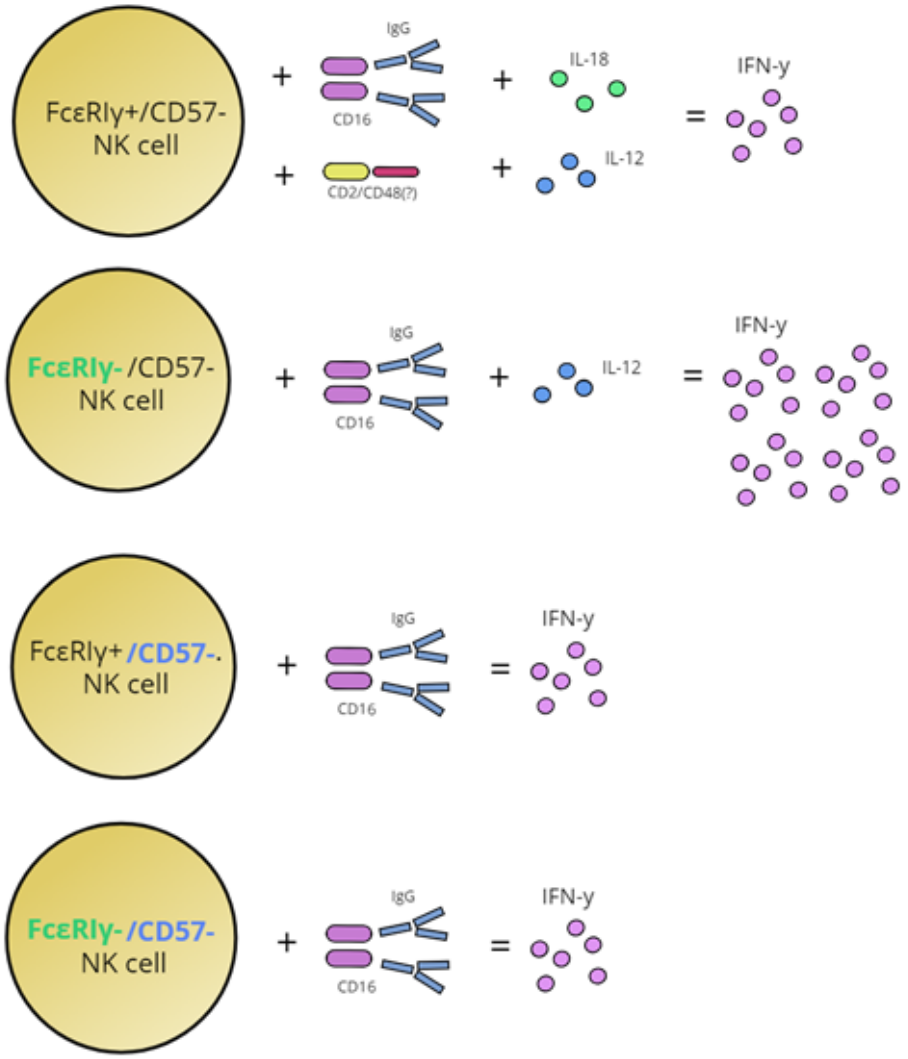


Figure 5.13. Proposed m**odel for differential induction of IFN-γ production by NK cell FcεRIγ/CD57-defined subsets during the antibody-dependent response to erythrocytes infected with *Plasmodium falciparum***

Based on the results of the current study, we propose that the activating co-factors that influence the production of IFN-γ by NK cells during the antibody-dependent response to malaria vary depending on the FcεRIγ/CD57-defined status of the cell. FcεRIγ+ CD57- cells are able to integrate IL-12, IL-18 and CD2 as co-factors, and FcεRIγ- CD57- are able to integrate IL-12, whilst FcεRIγ+ CD57+ and FcεRIγ- CD57+ cells seem to produce IFN-γ primarily in response to antibody alone.

5.4.6 The nature of the malarial antigen driving antibody-dependent anti-malarial responses

To select Gambian donors with prior malarial exposure for use in these assays, plasma samples from 641 Gambian donors were tested with ELISA for the presence of antibodies directed against apical membrane antigen 1 (AMA1). AMA1 is a protein is expressed on the surface of blood-stage merozoites, and is involved in the invasion of healthy erythrocytes [236]. Naturally-acquired antibodies directed against AMA1 are often found in the plasma of patients infected with *Plasmodium falciparum*, and due to its limited antigenic diversity it is considered to be both a potential vaccine candidate and a useful marker for use in ELISA assays testing for prior malarial exposure [237].

AMA1 is only expressed on the free merozoite stage of *Plasmodium falciparum*, and is generally not expressed on the surface of infected erythrocytes containing late-stage schizonts as utilised in the incubation assays in this study. Consequently, we did not expect to find any significant relationship between plasma titres of anti-AMA1 antibodies and $CD56^{dim}$ NK cell activation against this stage.

To our surprise we found that high titres of anti-AMA1 antibodies in plasma appear to associate significantly with subsequent downregulation of CD16 during the antibody-dependent NK cell response to schizont-stage malaria ($p<0.05$, Fig. 5.7C). This effect was only apparent at the very highest titres of anti-AMA1 however, and we found no such correlation between anti-AMA1 titres and direct functional markers of NK cell activation such as IFN-γ production (Fig. 5.7A) or degranulation (Fig. 5.7B). Consequently, it seems unlikely that anti-AMA1 antibodies themselves are capable of inducing downregulation of CD16 during the NK cell response to schizont-stage malaria. Rather we suggest that high titres of anti-AMA1 may be indicative of high anti-malarial antibodies in general, and as such the association between high titres of anti-AMA1 antibodies and downregulation of CD16 is largely coincidental rather than functional.

Though AMA1 is not expressed on the surface of schizont-infected erythrocytes, there are several other malarial antigens that are, such as *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1), which mediates parasite sequestration through binding to vascular endothelial cells. Antibodies against PfEMP1 constitute most of the antibodies generated during the antibody-dependent response to malaria, and consequently it is considered to be an immunodominant peptide [238]. Recent research conducted by Arora et al in 2018 has confirmed that antibodies against

PfEMP1 present in pooled malaria-immune plasma can elicit cytotoxic responses against parasitised erythrocytes by non-autologous NK cells, as do custom antibodies directed against parasite-derived RIFIN molecules which are also expressed on the erythrocyte surface [239]**.** The use of pooled non-autologous plasma and custom antibodies in the aforementioned study prohibits us from generalising these results too extensively however, as the quality and quantity of antibodies directed against PfEMP1 and RIVIN proteins may vary between donors *in vivo*. Nonetheless, future work in this area will likely involve conducting assays designed to neutralise these antigens through antibody blocking or knockout mutation to observe the resulting effect on NK cell antibody-dependent responses in a more representative model of infection.

5.3.7 HCMV exposure is associated with increased frequencies of Adaptive and Mature NK cells

The differentiation of $CD56^{dim}$ NK cells into a functionally distinct 'Adaptive' phenotype appears to be mediated primarily by exposure to cells infected with certain viruses, the most widely reported of which is human cytomegalovirus (HCMV) [131,132]. In areas of the globe where malaria is still present HCMV infection is also generally widespread, particularly in Africa, South-East Asia and South America [151]. In Gambia HCMV is endemic, with 85% of infants infected by one year of age in countries such as Gambia [229]. In contrast infection rates in Europe are far lower on average, particularly amongst Caucasians whom make up 100% of our European donors [232]. Consequently we hypothesised that our Gambian donors would possess higher frequencies of both Adaptive Immature and Adaptive Mature NK cells than HCMV- European controls.

Happily, our results appear to confirm this hypothesis. After gating $CD56^{dim}$ NK cells by FcεRIγ/CD57-defined subset, Gambian donors demonstrating significantly higher frequencies of both Adaptive subsets than European HCMV- donors, and consequently also demonstrate lower frequencies of both Canonical subsets. In addition, Gambian donors demonstrated significantly higher frequencies of Mature CD57+ cells in comparison to HCMV- Europeans with 46.46% Gambian cells expressing CD57 in comparison to only 29.92% in HCMV- Europeans. Prior work by both our own research group and others has suggested that exposure to HCMV-infection also drives expansion of CD57+ cells [240,241], a theory that is further substantiated by these results.

Unexpectedly, Gambian donors also demonstrated significantly higher frequencies of Adaptive CD56^{dim} NK cells and significantly lower frequencies of Canonical cells than European donors who were HCMV+ (Fig. 5.8D). This can perhaps be explained by differences in the average period of time the average HCMV+ Gambian is infected with the virus versus the average HCMV+ European of the same age. In Gambia 85% of infants are infected by one year of age and maintain chronic infections throughout life, whereas in most European countries less than 80% of individuals are infected by reproductive age [229], [242]. Consequently it is possible for a Gambian adult and a European adult of the same age to both test seropositive for HCMV whilst having been infected for very different periods of time. As a result HCMV+ Gambians would logically possess more adaptive NK cells than HCMV+ Europeans of the same age due to longer periods of HCMV exposure.

CHAPTER 6

Overview of Results and Plans for Future Work

In this thesis, I have made six main findings with the potential for future investigation.

6.1 NK cells preferentially form conjugates with malaria-infected erythrocytes

I have confirmed that human natural killer cells are able to distinguish between uninfected erythrocytes and erythrocytes infected with schizont-stage *Plasmodium falciparum*, and preferentially form conjugates with the latter, as well as demonstrating for the first time that both undifferentiated and differentiated NK cells can form these conjugates. Conjugate formation is transient, the majority of these being lost within 3 hours of co-culture.

I was unable to determine the relative frequencies of conjugation between infected erythrocytes and either undifferentiated or differentiated NK cells, as was one of my original aims. This was due to the low frequency of conjugation events and innate limitations of the imaging methodology used. In future work this issue could be addressed by making alterations to the experimental protocol designed to increase the frequency of conjugation events.

Though an original aim of the study was to assess the impact of the presence of accessory cells on conjugate formation, the presence of additional cell types appears to have limited the extent to which NK cells could interact with infected erythrocytes directly. In contrast, other researchers have previously been able to achieve much higher conjugate frequencies using purified peripheral NK cells or NK92 tumour lines [190], [103]. Whilst NK92 cells are inappropriate for my own aims given their lack of phenotypic diversity [195], using purified peripheral NK cells would allow for more frequent contacts to occur between NK cells and infected targets whilst still maintaining the diversity in NK cell differentiation I wish to study.

It may also be possible to simulate the presence of accessory PBMCs to a certain extent using supplementary cytokines and soluble ligands, though this may be a somewhat limited approach given that several studies have suggested that full physical contact is required between NK cells and activating accessory cells to optimise presentation of

cytokines such as IL-12 and IL-15, as well as activating ligands such as MICA and MICB [22], [26], [60].

In addition, although the original aim of this study was to evaluate the frequency of conjugation in an antibody-independent context, I observed potent degranulation responses in NK cells as a result of stimulation with anti-malarial antigen-antibody complexes in the assays described in Chapter 5. Therefore, it may also be informative to perform the conjugation assays described in Chapter 3 with the addition of malaria-immune plasma to ascertain whether antibody ligation increases the frequency of conjugation between NK cells and infected erythrocytes, and whether this varies based on the differentiation and adaptive status of NK cell subsets. Furthermore, it would be informative to analyse the stability of antibody induced conjugates to test whether these can be detected beyond the 2-3 hour time frame observed in the absence of antibody or whether antibody mediated conjugated formation would also be rapidly lost, for example through the cleavage of CD16.

6.2 CD16 shedding after antibody stimulation is not due to internalisation

I have demonstrated through a reliable imaging-based methodology that the downregulation of the NK cell Fc receptor CD16 seen after stimulation with antibody-antigen complexes is due to shedding of CD16 alone, and does not involve internalisation of CD16 as has been previously reported.

The results of this study are relatively definitive, and in keeping with observations made both by our own lab group and other researchers using alternative techniques [213], [212], [198]. The clinical consequences of CD16 shedding on the NK anti-malarial response however remains a relevant avenue of research. As the antibody-dependent response of NK cells to malaria is dependent on this receptor, it would be interesting to evaluate how quickly levels of CD16 are able to regenerate to homeostatic levels after downregulation in response to anti-malarial antibodies. Given that CD16 is shed rather than internalised and recycled, we can safely assume that the reconstitution of homeostatic levels of CD16 expression require *de novo* synthesis and export of CD16. Any delays in this process would limit the capacity of NK cells to respond to future antibody-dependent stimuli, perhaps with functional consequences on the ability of NK cells to shape the immune response through IFN-γ production and clearance of parasitised cells through cytotoxicity. Research by our own lab in an influenza model of antibody-dependent stimulation has suggested that expression levels of CD16 can take

several weeks to normalise in-vitro after stimulation with antibody-antigen complexes [198], and recently published research by other groups has found similar results in a lymphoma model where serial downregulation of CD16 via antibody stimulation was also associated with reduced capacity to produce perforin [221]. Future work in this area may therefore involve assessing the impact of CD16 shedding on long-term antibody-dependent NK responses to malaria. Work by our lab has also indicated that the use of metalloprotease inhibitors designed to prevent cleavage of CD16 by the metalloprotease ADAM-17 results in sustained degranulation responses by NK in response to influenza [198]. It would be interesting to run a similar assay in a malaria model to assess the impact of inhibiting CD16 shedding on the anti-malarial immune response and parasite clearance.

Conversely, recent research published by Srpan et al in 2018 has suggested that shedding of CD16 on NK cells in response to antibody-stimulation can actually result in a significant upregulation of antibody-*independent* serial killing of target cells, mostly notably by increasing expression of the activating NKG2D receptor and easing cellular motility [221]. Uninfected erythrocytes are not known to express ligands for NKG2D, and a prior study by Chen et al in 2014 confirmed that antibody-independent cytotoxic killing of *falciparum*-infected erythrocytes is not dependent on this receptor [40].

However, Srpan et al did not test receptors other than NKG2D, therefore it is possible that the upregulation seen in antibody-independent killing capacity after antibody-induced shedding of CD16 may also involve increased expression of other molecules. Several studies have indicated that the antibody-independent response to malaria in NK cells is mediated by the activating co-receptors LFA-1 and DNAM-1 [103], [40]. Additional future work may involve testing the expression levels of these receptors after antibody-dependent stimulation.

6.3 CD56^{dim} NK cells can respond robustly to malaria through antibody-dependent mechanisms

I have shown for the first time in a full study that human natural killer cells have robust antibody-dependent responses to erythrocytes infected with *Plasmodium falciparum* in the presence of autologous anti-malarial antibodies, and that this response involves both production of pro-inflammatory interferon gamma and cytotoxic degranulation.

Whilst stimulation of NK cells with anti-malarial antigen-antibody complexes provoked significant levels of degranulation, in the current study I was not able to ascertain the

effects of degranulation on parasite growth or viability due to time constraints. In future work it may be valuable to assess the effects of NK cell cytotoxicity on parasites directly through growth inhibition assays designed to measure the effects of cytotoxic activity on parasite population growth [40], or Chromium-51 release assays designed to detect the lysis of infected erythrocytes [243].

Additionally, whilst the study described in this thesis strongly suggested a role for anti-malarial antibodies in stimulating the NK cell response to malaria, I was not able to determine which malarial antigens expressed on infected erythrocytes were the most immunogenic. Research published in this area during the preparation of this thesis has confirmed that antibodies directed against *Plasmodium*-derived PfEMP1 and RIFIN proteins can elicit cytotoxic responses against parasitised erythrocytes by NK cells in models using knockout strains of malaria lacking expression of these proteins and pooled immune-plasma [239]. While we generally avoid the use of pooled plasma in our own work as it does not allow us to assess the role of diversity in donor responses, using similar knockout parasite strains in our own assays would allow us to determine the contribution of various malarial antigens to the antibody-dependent NK cell response whilst taking into account potential variation in antibody repertoire between individuals.

6.4 The antibody-dependent response of CD56^{dim} NK cells to malaria involves additional co-factors

I have shown for the first time that the antibody-dependent response of human natural killer cells to erythrocytes infected with *Plasmodium falciparum* is partially dependent on interleukins 12 and 18 produced by accessory cells, as well as binding of the NK cell expressed CD2 to ligands within PBMCs.

Whilst ligation of the CD2 receptor led to upregulation of the antibody-dependent response to malaria in NK cells, in the described study I was not able to determine which specific ligand(s) on which specific accessory peripheral blood mononuclear cells bound NK-expressed CD2 due to time constraints.

Previous work by other researchers has confirmed that binding of CD2 expressed on lymphocytes to CD58-expressing monocytes can mediate presentation of cytokines such as IL-12, which my own work has also identified as an important source of NK cell activation during the anti-malarial response [228]. Alternatively, it is possible that ligation of CD2 itself leads to an upregulated response, as in addition to serving as an

adhesion receptor CD2 is also capable of transducing co-activating signals through the CD3 zeta subunit of the CD16 complex [244].

CD2 has several described ligands, including CD48, CD58, and CD59 [130]. These ligands can be expressed on a range of cells, including various accessory peripheral blood mononuclear cells. Consequently, in order to fully investigate the role of this receptor in the antibody-dependent NK cell response to malaria, future assays will likely involve a combination of blocking antibodies directed against these ligands as well as targeted depletion of specific accessory cell subsets in order to determine which cells provide which signals. Additional assays could include the simultaneous use of blocking antibodies against CD2 and blocking antibodies against accessory cell-derived cytokines to determine whether CD2 ligation has any effect independent of cytokine presentation.

6.5 The antibody-dependent response of CD56^{dim} NK cells to malaria is enhanced in 'Adaptive' FcεRIγ- and 'Mature' CD57+ NK cells

I have shown for the first time in full study that the antibody-dependent response of human natural killer cells to erythrocytes infected with *Plasmodium falciparum* varies significantly depending on whether NK cells are FcεRIγ positive or negative (here referred to as 'Canonical' or 'Adaptive' respectively), and/or CD57 positive or negative (here referred to as 'Mature' or 'Immature'). Adaptive Immature NK cells appear to be the most likely to produce interferon gamma during the antibody-dependent response to malaria due to their enhanced responsiveness to antibody and ability to integrate IL-12, whilst both Adaptive and Mature NK cells have enhanced antibody-dependent cytotoxic responses that appear to be primarily driven by anti-malarial antibodies. I have also demonstrated that HCMV exposure is associated with the differentiation of these subsets, and that Gambians possess significantly higher frequencies of these cells than Europeans individuals, likely due to endemic exposure to HCMV from childhood.

In future work it would be interesting to examine the extent to which a larger Adaptive and/or Mature NK cell compartment contributes to the clearance of parasites. Future work is planned where this will be investigated via NK cell activation assays and parasite growth inhibition assays incubating NK cells from either HCMV negative or positive donors with parasitised erythrocytes in the presence of pooled malaria-immune plasma. This approach would allow us to evaluate to what extent a larger adaptive or

differentiated NK cell compartment effects the anti-malarial response whilst controlling for anti-malarial antibody titres.

6.6 CD56^{dim} Adaptive Immature NK cells form an intermediary subset with the capacity to response to both anti-malarial antibody and IL-12

Lastly, I have shown for the first time that the different FcεRIγ/CD57-defined human natural killer cell subsets respond differently to the co-factors IL-12, Il-18 and CD2 during the antibody-dependent response to malaria. Canonical Immature NK cells (FcεR1γ+CD57-) are able to integrate all of these signals during the antibody dependent response to malaria, while Adaptive Immature NK cells (FceR1γ-CD57-) can integrate only IL-12 and make superior antibody dependent IFN-γ responses.

In future it would be of value to investigate the underlying phenotypic changes that distinguish canonical undifferentiated NK cells from other subsets, particularly in terms of transcription factor, adaptor protein or cytokine receptor expression. Early studies conducted on Adaptive mature FcεRIγ- CD57+ NK cells by Schlums et al indicated that they express lower levels of transcription of the genes encoding subunits of the IL-12 and IL-18 receptors, and as a result demonstrate reduced capacity to respond to these cytokines [137]. Adaptive Immature cells were also found to be present, but were not investigated by gene expression profiling. It could therefore be that this subset represents an intermediate population which retains some ability to respond to IL-12, either innately, or after induction through exposure to antibody-antigen complexes.

6.7 Summary

In summation, I believe that I have demonstrated that anti-malarial immunity in humans may depend not only on the breadth and depth of the antibody repertoire but also on the differentiation and adaptation state of antibody dependent effector cells, including NK cells. Further in-vitro experiments and field studies tracking the association these factors with malarial infection and clinical symptoms could address this hypothesis further, and provide useful insights into the interplay between the innate and adaptive anti-malarial responses.

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