Natural Killer cell function

after vaccination

in an African (Gambian) population

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DECLARATION

I, Alansana Darboe, 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.

Signature: .....

Alansana Darboe, July 2016.

I commence in the name of Allah

The Most Beneficent The Most Merciful

ABSTRACT

Emerging evidence suggests that NK cells could be important in the early effector response induced by vaccination, supported by vaccine antigen-specific CD4 IL-2 production and antigen-antibody immune complexes. 'Memory-like' NK cells, with heightened responsiveness can be also generated by pre-activation with cytokines.

I found that NK cell differentiation is accelerated in Africans in The Gambia compared to age-matched UK residents and that this is linked to reduced functional NK cell responses to cytokines. This effect may also relate to a high burden of human cytomegalovirus (HCMV) infection in this population, with all Gambian study subjects infected by 3 years of age. There is also significant variation in lymphoid and myeloid cell populations with increasing age. Additionally, I found that a deletion of the *NKG2C* gene, a receptor important for recognition of HCMV infected cells, results in delayed NK cell differentiation. Furthermore, the allele frequency of the *NKG2C* gene deletion is higher in The Gambia compared to other countries studied to date. The frequency of the deletion allele did not change with age.

I went on to investigate the impact of this advanced differentiation phenotype on NK cell responses in two vaccination studies: Gambian subjects of all ages made negligible NK cell CD107a, CD25, and IFN-γ responses to influenza or DTPiP vaccine antigens. No enhancement of these responses was observed after vaccination. However, vaccination resulted in intrinsic changes to NK cells with enhancement of NK cell IFN-γ responsiveness to exogenous cytokines. The main source of IFN-γ was derived from a population of CD56dimNKG2C+CD57- NK cells. These less differentiated cells may retain some capacity to control HCMV infection, and at the same time represent a possible target for generation of 'memory-like' NK cells in vivo in vaccine induced NK cell responses.

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LIST OF ABBREVIATIONS

BCG: *Mycobacterium bovis* Bacillus Calmette-Guerin

Bcl-xl: B-cell lymphoma-extra large

Bcl-2: B-cell lymphoma 2

Bp: Base pairs

CD: Cluster of Differentiation

cGAS: cyclic GMP-AMP Synthase

CI: Confidence Interval

CLR: C-type lectin receptors

CpG: 5'—C—phosphate—G—3'

CTL: Cytotoxic Lymphocyte

DC: Dendritic Cell

dH_2O: Distilled water

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic acid

DNAM-1: DNAX Accessory Molecule-1

dNTPS: Deoxynucleotide Triphosphate Solution

DTPiP: Diphtheria, Tetanus, Pertussis and inactivated Poliomyelitis 1 & 2 3 virus

EBNA-1: Epstein Barr Virus nuclear antigen 1

EBV: Epstein Barr Virus

EC: Ethics Committee

ELISA: Enzyme-Linked Immunosorbent assay

Eomes: Eomesodermin

ETs: E26 transformation-specific

E4bp4: Nuclear factor, interleukin 3 regulated

FACS Buffer: Fluorescence Activated Cell Sorting Buffer

FCS: Heat Inactivated Foetal Calf Serum

FLT3L: FMS-Like Tyrosine kinase 3 Ligand

Gab2: Growth-factor-receptor-bound protein 2-associated binder-2 GG: The Gambia Government

HBsAg: Hepatitis B virus surface antigen

HCC: High Concentration of cytokines

HCMV: Human cytomegalovirus

HLA: Human Leukocyte Antigen

HRP: Horseradish peroxidase

H1N1: Haemagglutinin 1 Neuraminidase 1 influenza A virus antigen

H3N2: Haemagglutinin 3 Neuraminidase 2 influenza A virus antigen

Id2: DNA-binding protein inhibitor ID-2

IE: Immediate Early

IFN: Interferon

IFN-γ: Interferon-γ (gamma)

Ig: Immunoglobulin

IL: Interleukin

rIL-12: Recombinant human Interleukin-12

rIL-18: Recombinant human Interleukin-18

ITAM: Immunoreceptor Tyrosine-based Activation Motif

ITIM: Immunoreceptor Tyrosine-based Inhibition Motif

K562: Human erythroleukaemic cell line K562

KIR: Killer Immunoglobulin-like Receptor

LCC: Low Concentration of Cytokines

LFA-1: Lymphocyte Function-Associated antigen 1

LPS: Lipopolysaccharide

LSHTM: London School of Hygiene and Tropical Medicine

MAPK: Mitogen-activated protein kinases

MCMV: Murine cytomegalovirus

mDC: Myeloid Dendritic Cells

MFI: Mean Fluorescence Intensity

MHC: Major Histocompatibility complex

MIP-1α: Macrophage Inflammatory Protein 1-alpha

MIP-1β: Macrophage Inflammatory Protein 1-beta

MRC: Medical Research Council

MRC ING: Medical Research Council International Nutrition Group

NCR: Natural Cytotoxicity Receptors

NCAM: Neural Cell Adhesion Molecule

NK: Natural Killer

NKG2D: Natural-Killer Group 2, member D

OAS: Oligoadenylate synthase

OPD: Ortho-Phenylenediamine

PAMP: Pathogen Associated Molecular Patterns

PBMC: Peripheral Blood Mononuclear Cells

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

pDC: Plasmacytoid Dendritic Cells

Ph.D.: Doctor of Philosophy

PI3K: Phosphoinositol-3-kinase

PKB: Protein Kinase B

PSG: Penicillin, Streptomycin, and L-glutamine

PT: Pertussis Toxin

PRR: Pattern Recognition Receptors

RANTES: Regulated And Normal T cell Expressed and Secreted

Ras: Rat Sarcoma

RNA: Ribonucleic Acid

RPM: Revolutions per minute

RPMI: RPMI-1640

RIG-I like Retinoic acid-inducible gene 1-like

SCC: Scientific Coordinating Committee

SLT: Secondary Lymphoid Tissues

STAT: Signal Transducer and Activator of Transcription

T-bet: Homologous transcription factor Tbx21

TCR: T Cell Receptor

TIV: Trivalent Influenza Vaccine

TLR: Toll-Like Receptors

TMB: 3,3′,5,5′-Tetramethylbenzidine

TNF-α: Tumour Necrosis Factor

TOX: Thymocyte selection-associated high-mobility group box protein

TT: Tetanus Toxoid

1 CHAPTER 1:

INTRODUCTION

1.1 INTRODUCTION

The human immune system has evolved to recognize and eliminate or control harmful pathogens from our body whilst at the same time having other mechanisms that tolerate self-antigens. Traditionally, the immune system can be divided into two parts, which are the innate immune system and the adaptive immune system.

The innate immune system is the first line of immune responses that recognise antigens by using germ-line encoded pattern-recognition receptors (PRR) to identify conserved pathogen associated molecular patterns (PAMP) present on viral, bacterial and fungal pathogens (Iwasaki and Medzhitov, 2015). When the PRR bind these pathogens, this stimulates an inflammatory response and other innate defence mechanisms essential in pathogen clearance. PRR include cell membrane surface sensors, such as C-type lectin receptors (CLR), toll-like receptors (TLR), leucine-rich repeat containing receptors, as well as intracellular sensors of nucleic acid like OAS proteins and cGAS and RIG-I like receptors (Iwasaki and Medzhitov, 2015). These PRR are very important in the induction of the adaptive immune responses, as they define the type and origin of the infection and instruct adaptive lymphocytes to clear the infection. Generally, the innate immune system uses two modes of recognition: cell-intrinsic recognition mediated by cytosolic sensors and cell-extrinsic sensor recognition expressed by specialised cells like myeloid cells. The detection of extracellular pathogens is mainly induced by plasma membrane receptors on innate cells such as dendritic cells and macrophages expressing surface TLR1, 2, 4, 5 & 6 and CLR (dectin 1 and 2). These receptors specialise in recognising conserved pathogen structures such as LPS and flagellin. Intracellular sensors in the endosome including TLR3, 7 8 & 9 identify components of intracellular pathogens, in particular, bacterial CpG DNA and viral RNA and DNA (Iwasaki and Medzhitov, 2015).

Innate leukocytes include cells of both myeloid and lymphoid origin. These include granulocytes (neutrophils, eosinophils, basophils), classical antigen presenting cells (monocytes, macrophages and myeloid and plasmacytoid dendritic cells (DCs)) and natural killer cells. The activation of these innate immune cells induces the secretion of a series of chemokines and pro- or anti-inflammatory cytokines important in pathogen clearance, tissue repair, and recruitment of other immune cells. It is the combination of these secretory factors at a given site of infection that can influence the outcome of an immune response.

Another mechanism of innate pattern recognition is via identification of ‘missing self’ on infected cells. This approach is employed by natural killer cells to detect stressed and pathogen-infected host cells (Iwasaki and Medzhitov, 2015, Kiessling et al., 1975a). Briefly, inhibitory receptors on natural killer cells identify MHC class I molecules thereby blocking cytotoxic killing of normal cells. However, virally-infected cell and transformed cells decrease their expression of MHC class I molecules; in this manner the inhibitory signals are not activated and then natural killer cells are licensed to mediate target cell cytotoxicity.

The second line of immune defence, the adaptive immune system, is mainly made up of two types of immune cells, namely, T lymphocytes and B lymphocytes and their secreted molecules. There are two phases of adaptive immune responses; that is primary and secondary adaptive responses. The primary adaptive response is activated the first time the host encounters the pathogen and triggers antigen processing and presentation by accessory cells to antigen-specific T cells and B cells, along with co-stimulation signals, which stimulates an immune response. This also leads to differentiation of memory cells. The secondary adaptive response is the immune responses induced by a second infection with a previously encountered antigen or pathogen. A similar pattern of activation is initiated but this time because of the presence of clonally expanded memory cells induced during the primary response, the secondary adaptive responses is faster and more targeted to control the antigen or pathogen.

The T and B lymphocyte lineages possess the important property of immunological memory. Immunological memory is described as the capacity of immune cells to generate qualitatively and quantitatively enhanced immune responses following reinfection with a similar pathogen or antigen. T lymphocytes express T cell receptors (TCR) which recognise antigenic peptides from pathogens when presented in the context of major histocompatibility complex (MHC) class I and class II molecules. B lymphocytes express surface immunoglobulins which act as B cell receptors (BCR) for recognition of protein (and carbohydrate) antigens in tertiary conformation. The unique specificity of mature T and B cell receptors for individual peptides and proteins, combined with the ability of these cells to clonally expand upon reactivation against a similar antigen or pathogen, allows the adaptive immune system to develop memory.

The initial antigenic priming of adaptive immune responses normally requires several days and the generation of sufficient numbers of mature memory T and B cells can take weeks to fully develop and can often involve multiple exposures to a particular infection. Innate immune mechanisms, therefore, provide critical control within the first few days of an infection before the development of effective adaptive control mechanisms.

Thus, the interplay between these innate and adaptive immune responses is crucial in determining the outcome of a given infection or responses to vaccination. Any imbalance in any one of these two main responses could lead to immunodeficiency or autoimmunity diseases or chronic infections.

Natural Killer cells are typically activated early in infection as a consequence of the production of cytokines by PAMP-activated cells of granulocyte and myeloid lineages. In addition, NK cells can express receptors for T cell-derived cytokines and antibodies, making them uniquely placed to bridge immediate early innate responses with adaptive T and B cell responses. At the same time, NK cells possess several effector functions, including cytolytic activity and cytokine production, which can contribute to the control of infections during primary and secondary immune responses.

Natural killer cell have a natural cytotoxic capacity to eliminate transformed tumours or virally infected cells without the need for prior priming or exposure, thus, the name ‘natural killer’. Apart from been cytotoxic, NK cells are can also secret cytokines that can modulate neutrophil, dendritic cell, and macrophages (Moretta et al., 2005). They provide an essential early innate source of pro-/ anti-inflammatory cytokines including interferon-γ, tumour necrosis factor-α, interleukin-10 in diverse physiological and pathological situations (Vivier et al., 2011). NK cells also produce growth factors including interleukin-3, granulocyte colony-stimulating factor, and granulocyte macrophage colony-stimulating factor, although their role in growth is not fully elucidated. Additionally, NK cells also secret different types of chemokines such as C-C motif chemokine Ligand- 2 (CCL)-2, CCL3, and C-X-C motif chemokine Ligand 8 important in immune cell co-localization and trafficking during immune responses (Walzer et al., 2005, Vivier et al., 2011). Specifically, T cell responses in secondary lymphoid tissues can directly be modulated by IFN-γ secreted by NK cells following migration from peripheral tissue to the lymph nodes, and the cytokines produced by NK cells can also indirectly modulate dendritic cells function by killing immature DC

(Martin-Fontecha et al., 2004, Vivier et al., 2011). NK cells through the induction of viral target cell debris can enhance CD4, CD8 cytotoxic lymphocyte function and IgG immune responses (Robbins et al., 2007, Krebs et al., 2009). In contrast, NK cells can negatively influence T lymphocyte immune responses (Andrews et al., 2010).

NK cells also express receptors to adhere to endothelial cells. Some of these receptors include CD62L, which binds to addressins, α4β1 integrin which binds to vascular cell adhesion protein 1 and CX3C chemokine receptor 1 which binds to membrane bound C-X3-C motif ligand 1 (Vivier et al., 2008). C-X3-C motif ligand 1 can induce NK cells to kill endothelial cells leading to its contribution to vascular pathogenesis during injury, in transplantation and certain viral infections (Yoneda et al., 2000, Bolovan-Fritts and Spector, 2008, Rieben and Seebach, 2005). In contrast, a subset of NK cells promotes angiogenesis during pregnancy. Specifically, decidual NK cells can produce proangiogenic factors like placental growth factor and vascular endothelial growth factors to allow placental development during pregnancy (Hanna et al., 2006).

This thesis explores how NK cells mature in an African population in response to infection and how vaccination influences the functional responses of these cells.

1.1.1 Natural Killer cells

Natural Killer (NK) cells are immune effector cells that are derived from haematopoietic stem cells and specifically originate from common lymphoid progenitor cells. NK cells belong to a group of related lymphocytes called innate lymphoid cells (ILC). They are classified as Group 1 ILC, developing from Id2 progenitor cells through interleukin-15 (IL-15) and thymocyte selection-associated high-mobility group box (TOX) protein expression and, in common with cytotoxic T cell subsets, express NFIL3, Eomes and T-bet transcription factors (Walker et al., 2013, Kiessling et al., 1975b, Kiessling et al., 1975a). In humans, NK cells make up 5-15% of the peripheral blood lymphocyte population. They are large granular lymphocytes that express germ-line encoded inhibitory and activating receptors which regulate the recognition of transformed and intracellular pathogen-infected cells (Kiessling et al., 1975b, Freud and Caligiuri, 2006). In contrast to T and B lymphocytes, which undergo antigen-specific T cell receptor and B cell immunoglobulin gene assortment, NK cells do not rearrange their germ-line receptor genes. NK cell receptors nonetheless exhibit considerable diversity. Through these receptors, NK cells survey target cells by binding to classical MHC class I, MHC class

I related molecules, co-stimulatory ligands and cytokines in order to achieve an equilibrium between becoming cytotoxic or cytokine-producing effector cells against transformed or infected somatic cells and being tolerant to healthy somatic cells (Caligiuri, 2008, Colucci et al., 2003). Furthermore, in contrast to T and B lymphocytes, NK cells do not require priming to be activated in order to produce cytokines and chemokines to modulate other immune cells (Colucci et al., 2003).

1.1.2 Natural Killer cell development

1.1.2.1 Developmental subsets in humans

NK cells are derived from cluster of differentiation 34+ (CD34+) bone marrow haematopoietic stem cells and their development and maturation comprises progressive alterations of cellular phenotype and function. Using cell surface receptors, human NK cell development has been categorized into five stages, namely, pro-NK, pre-NK, immature NK (iNK), CD56bright NK and CD56dim NK cells. However, these stages are not necessarily sequential; it only means that at some stage of NK cell development the cells would have expressed characteristics of the above mentioned stages. The process of NK cell development involves different organs as they migrate from the bone marrow to secondary lymphoid tissues (SLT) where maturation occurs before they emerge as functional effector cells. For these cells to fully mature, they require essential signals such as FMS-like tyrosine kinase 3 ligand (FLT3L), interleukin 15 (IL-15) and c-kit ligand (Briercheck et al., 2010, Grzywacz et al., 2010).

Until now, a unique biomarker for the identification of all human NK cells has not been identified and therefore, a combination of biomarkers are used to define NK cell phenotype. Using flow cytometry, human NK cells are defined as lymphocytes that express CD56 (the 140-kDa isoform of neural cell adhesion molecule 1) but which lack expression of the universal T cell receptor component CD3 epsilon (Caligiuri, 2008). CD56 is acquired between the pre-NK and the iNK stage of NK cell development. Following the latter stage, NK cells acquire high levels of CD56 (CD56bright). However, these cells later lose some expression of CD56 and gain or lose the C-type lectin-like receptor component CD94, becoming CD56dim NK cells. Although, they acquire additional receptors such as CD16 (FcRγIII receptor, low

affinity receptor for the Fc portion of Immunoglobulin G) and killer immunoglobulin-like receptors (KIR) (Briercheck et al., 2010).

Due to the expression of high levels of CCR7 and CD62L on CD56bright cells, these cells home to and are enriched in SLT, where they encounter DCs and macrophages. These myeloid accessory cells produce cytokines that activate NK cells and promote the secretion of inflammatory cytokines, including interferon-γ, (IFN-γ), which in turn further potentiate antigen-presenting cell function. However, CD56dim NK cells are mainly involved in immune-surveillance for signs of atypical expression of MHC class I molecules on the surface of transformed or infected cells, in a process termed 'missing-self' recognition. Apart from CD56dim NK cells, which are abundant in blood, spleen and bone marrow, all the other NK cell intermediate stages are predominantly present in SLT (Briercheck et al., 2010, Caligiuri, 2008).

It has not yet been formally proven that NK cell maturation takes place in SLT, although existing evidence suggests that SLT might be important in NK cell development as there is increased expression of CCR7 and CD62L on the intermediate pre, pro and iNK cells, and also because of the enriched presence of membrane bound IL-15 important in NK development on resident DCs. However, the presence of a sizeable compartment of CD56bright cells in blood suggests that their maturation may not be exclusively confined to SLT (Briercheck et al., 2010).

Two main NK cell populations have been described in peripheral human blood: CD56dim CD16bright which represents about 90% of peripheral NK cell and CD56brightCD16-/dim, accounting for the remaining 10%. The CD56bright cells principally have cytokine-producing and immunoregulatory functions whilst CD56dim cells are enriched for cells exhibiting both cytotoxicity and cytokine secretion. Some researchers divide NK cells into five subsets on the basis of their CD16 expression, namely: CD56bright CD16-, CD56bright CD16dim, CD56dim CD16-, CD56dim CD16+ and CD56- CD16+ (Poli et al., 2009, Cooper et al., 2001a, Domaica et al., 2012).

1.1.2.2 Mechanisms of NK cell development

Expression of interleukin 15 receptor common β (CD122) and γ chains (CD132) is required for the development of NK cells through the activation of STAT5a and

STAT5b. Human and mouse data show that NK cells lacking these STAT molecules were unable to differentiate from progenitors to immature NK cells (Eckelhart et al., 2011, Kofoed et al., 2003, Cichocki et al., 2013). STAT5 signalling molecules are thought to exert their effects on NK cells by activation of phosphoinositol-3-kinase (PI3K), PKB and Ras and MAPK signalling pathways via growth-factor-receptor-bound protein 2-associated binder-2 (Gab2) and additionally by promotion of expression of anti-apoptotic genes like Mcl-1, Bcl-xl and Bcl-2 (Nyga et al., 2005, Debierre-Grockiego, 2004). IL-15 receptor is particularly known to induce the activation of the E4bp4 transcription factor which is essential in NK cell development. It has been shown that mice deficient in E4bp4 lack mature NK cells and that E4bp4 transcription factor induces the expression of GATA3 and Id2 critical in the development of NK cells from NK progenitor cells to immature NK cells (Gascoyne et al., 2009). However, the development of immature to mature NK cells also requires the transcription factor thymocyte selection-associated high-mobility group box protein (TOX). The expression of Id2 did not salvage NK cell maturation of these cells and it should be noted that TOX-deficient NK progenitor cells express normal levels of IL-15 receptors on the cell surface (Aliahmad et al., 2010). Eomes and the homologous transcription factor Tbx21 (T-bet) constitute another set of critical transcription factors important in NK cell maturation. T-bet mainly influences maturation from NK progenitor cells to immature NK cells, whilst Eomes affects later stages of maturation (Gordon et al., 2012). Other transcription factors that are still essential for normal NK cell development include the ETs-family transcription factors PU.1, Spil and GATA2 (Cichocki et al., 2013).

1.1.3 Natural Killer cell receptors

NK cell function is regulated in order to achieve a balance between tolerance to healthy cells and cytotoxicity against malignant or infected cells. Such regulation involves a sophisticated repertoire of surface receptors expressed by NK cells. These receptors comprise three main groups, namely: natural killer cytotoxicity receptors (NCR: including NKp46, NKp30 and NKp44), which may directly recognise viral ligands (Mandelboim et al., 2001, Mandelboim and Porgador, 2001); immunoglobulin-like receptors, including inhibitory or activating killer immunoglobulin-like receptors (KIR) (such as KIR2DL1 or KIR2DS1), and Leucocyte immunoglobulin-like receptors (LIR), which recognise classical and non-classical MHC class I; and C-type lectin-like receptors like CD94/NKG2A and NKG2C/E, which recognise HLA-E and HL-A G (see Table 1.1)(Farag et al., 2002, Almeida-Oliveira et al., 2011, Dorak, 2012).

Additionally, NK cells also express CD16, an antibody binding receptor and co-stimulatory receptors such as 2B4, DNAM-1 and CD2 essential in effective NK cell immune responses (Vivier et al 2011).

Table 1.1: Natural killer receptor family.

NK Receptor Family	Known Ligands
NCR (NKp30, NKp44, NKp46)	Influenza haemagglutinin
CD94/NKG2 Receptors	MHC Class Ib (HLA-E)
KIR	HLA-A, -Bw, -Cw, -G
(KIR and LIR)	LIR binds MHC Class Ia -G

Adapted from (Dorak, 2012)

1.1.4 Direct receptor mediated NK cell activation

Ligands for NK cell surface molecules CD16, NKG2D, LFA-1, DNAM-1, and CD244 receptors expressed on *Drosophila* cells have been incubated with human NK cells to investigate the production of chemokines and cytokines through target cell-mediated NK activation. Fauriat et al showed that CD56dim NK cells were more potent cytokine and chemokine producers than CD56bright NK cells upon target cell mediated activation. RANTES, MIP-1α, and MIP-1β chemokines were produced within an hour of activation, whilst, IFN-γ and TNF-α were released later (3 and 5 hours, respectively). Activation of NKG2D, CD16 and 2B4 were enough to induce chemokine release. However, IFN-γ and TNF-α release necessitated additional receptor activation (Fauriat et al., 2010).

NCRs belong to the immunoglobulin superfamily of receptors. NKp46, NKp80, and NKp30 are expressed on resting NK cells whilst NKp44 is induced after IL-2 stimulation. Inhibition of these receptors blocks NK cell activation (Pegram et al., 2011). Tumour cells can release nuclear factor HLA-B associated transcripts which can bind NKp30 (Pogge von Strandmann et al., 2007). NKp30 is also an important receptor mediating NK cell interactions with dendritic cells (Ferlazzo et al., 2002,

Moretta, 2002). NKp30 also binds B7-H6 on K562 tumour cells (Pegram et al., 2011, Brandt et al., 2009). NKp80 interacts with activation-induced C-type lectin to enhance lysis of tumour cells. NKp46 and NKp44 are known to bind to influenza virus haemagglutinin (Mandelboim and Porgador, 2001).

KIR receptors play an essential role in maintaining tolerance to self-tissue antigens. KIR receptors bind to class I polymorphic HLA-A, B and C molecules. Their binding to HLA results in signal transduction of inhibitory or activating receptors. Activating KIR (for which there are 6 different genes) have DAP-12 (with immunoreceptor tyrosine-based activating motif) while inhibitory KIR (for which there are 9 genes) have immunoreceptor tyrosine-based inhibitory motif in their cytoplasmic domain (Pegram et al., 2011, De Re et al., 2015).

In relation to adaptive immune responses, antibodies can directly activate NK cells via CD16. CD16 is a low affinity FcγRIIIa receptor mainly expressed on CD56dim NK cells (Oboshi et al., 2016, Leibson, 1997). This receptor cross-link immune complexes that are formed when IgG binds an antigen by specifically recognising the constant (Fc) region of the IgG antibody. Binding of the immune complex activates CD16 immunoreceptor tyrosine-based activating motif (ITAM) in the cytoplasmic domain and signals via CD3ζ and MAP kinase pathways to activate NK cells in what is called antibody-dependent cellular cytotoxicity (ADCC). CD16 activation results in the release of cytolytic granules that contain lytic proteins such as perforin and granzymes. Cytolytic granule membranes contain lysosome associated membrane protein-1 (LAMP-I or CD107a); when these granules fuse with the NK cell membrane during degranulation, the CD107a is exposed on the surface of the cell. This allows surface expression of CD107a to be used as a surrogate marker of NK cell degranulation (Burkhardt et al., 1989, Tschopp and Nabholz, 1990, Cooper et al., 2001a). The interaction of CD16 with immune complexes in the presence of IL-18 can induce both surface CD107a expression and IFN-γ secretion (Nielsen et al., 2016, Srivastava et al., 2013).

It is also possible that NK cells may also directly be triggered via TLRs. This has been shown in human NK cells where early endosomes contain TLR9. KIR3DL2+ NK cells were directly activated through co-internalisation of KIR3DL2-CpG oligodeoxynucleotide complex in these cells (Sivori et al., 2010).

1.1.5 Cytokine mediated regulation of Natural Killer cell activation

During an infection, innate immune cells are activated either through direct recognition of pathogenic molecules or indirectly by immune signalling molecules produced by accessory cells, which leads to initiation, amplification and modulation of immune responses to protect against the infection. In addition to the functional consequences of direct activating and inhibitory receptor ligation, signals produced by accessory cells as a result of the recognition of pathogen-associated molecular patterns (PAMP) during an infection influence the activation of NK cells (Horowitz et al., 2012b, Newman and Riley, 2007, Karre, 2008).

Negative regulation of NK cells can, for instance, be mediated through transforming growth factor-β (TGF-β) or interleukin 10 (IL-10) released by accessory cells (Newman and Riley, 2007). Cytokines produced by monocytes and DCs in response to TLR ligation promote NK cell activation in a cell-contact dependent manner; these signals override the inhibitory signal produced by ligation of MHC class I molecules (Evans et al., 2011, Horowitz et al., 2010, Newman and Riley, 2007, Long et al., 2008). The accessory cells also produce cytokines and chemokines for the recruitment of NK cells, including type I interferons (IFN-α & β) important in sustaining NK cell cytotoxic function. Interleukin-12 (IL-12) is essential in driving NK cell type II interferon (IFN-γ) secretion (Horowitz et al., 2012b) (Figure 1.2).

Accessory cells activate NK cells using both contact and cytokine dependent mechanisms. This has been demonstrated with different bacterial and viral infections (Newman and Riley, 2007). Consequently, some viruses have evolved to inactivate NK cells, an immune evasion mechanism that highlights the importance of NK cells in viral clearance. For example, vaccinia virus produces IL-18 binding proteins that inhibit accessory cell-derived IL-18 in order to deactivate and evade NK cell cytotoxicity (Reading and Smith, 2003). Other accessory cells, like plasmacytoid DCs (pDCs) play an important role in viral clearance. In later stages of murine cytomegalovirus (MCMV) infection, NK cytotoxic function is sustained by pDC-derived IFN-α in a TLR9 dependent manner; at the same time, IFN-γ secretion by NK cells is reduced due to decreased availability of IL-12 (Delale et al., 2005). However, during early stages of infection, myeloid differentiation primary response 88 MyD88/TLR9

NK cell induction can be maintained by IFN-α and IL-12 secretion (Delale et al., 2005, Tabeta et al., 2004, Newman and Riley, 2007).

Additionally, myeloid DCs (mDCs) induce NK cell activation through both cytokine and contact-dependent processes. In mice, MCMV infected CD11b+ mDC can activate NK cells via IFN-α and NKG2D contact-dependent pathways, whilst IFN-γ secretion is maintained by IL-18 produced by DCs. Myeloid DC TLR9 signalling was shown not to be essential in activating NK cells through this pathway (Andoniou et al., 2005). However, these cells required TLR2/3 signalling to stimulate NK cells (Barbalat et al., 2009, Szomolanyi-Tsuda et al., 2006). Thus, different cytokines can induce NK cell responses. Henceforth, I will focus on the cytokines relevent to our vaccine model.

Interferon–γ (IFN-γ) is a type II interferon required for both innate and adaptive immunity. The main source of IFN-γ is CD8+ T cells and T helper 1 CD4 cells and natural killer (NK) cells (Schroder et al., 2004, Yu et al., 2006). It should be noted that either an excess or a deficiency in IFN-γ production can seriously influence both autoimmunity as well as pathogen and malignant tumour clearance in humans and in mice, highlighting the importance of regulating IFN-γ in protective immunity (Jouanguy et al., 1999, Street et al., 2002, Bouma and Strober, 2003). Functionally, IFN-γ potentiates anti-microbial and anti-tumour activation pathways through different routes, such as enhancing the activity of antigen presenting cells like macrophages via increased antigen processing and presenting capacity; or by attracting and facilitating the maturation and effector functions of different types of cells (Schroder et al., 2004).

Antigen presenting cells, such as macrophages and dendritic cells produce pro-inflammatory cytokines like interleukin (IL)-12, IL-18, and IL-15, which can induce IFN-γ release (Cooper et al., 2001b, Fehniger et al., 1999). IL-12, IL-18 and IL-15 can induce the transcription factor T-bet, which can control the production of IFN-γ in NK cells and T cells (Yu et al., 2006). NK cells are known in both human and mice to constitutively express IFN-γ messenger RNA available for rapid immune responses (Hammarlund et al., 2016).

On the other hand, IL-12 is a key cytokine in mediating IFN-γ secretion. IL-12 signals through IL-12 receptor β1 and β2 heterodimer subunits (Chua et al., 1994, Presky et al., 1996, Yu et al., 2006). The binding of IL-12 to its receptors on NK cells triggers

the activation of the Janus kinases Tyk2 and Jak2. These kinases relay the signal by phosphorylating tyrosine and serine amino acids to activate signal transducer and activator of transcription factor (STAT)-4 and p38 mitogen-activated protein kinase leading to the production of IFN-γ by NK cells (Watford et al., 2004, Zhang and Kaplan, 2000, Visconti et al., 2000).

In parallel, IL-18 on NK cells signals through the binding of its receptor subunit interleukin 18 receptor-α (IL-18Rα) (or IL-1 receptor related protein) and interleukin 18 receptor accessory protein (IL-18RAP) (or accessory protein-like receptors) (Akira, 2000, Born et al., 1998). The activation of these receptors can trigger STAT-3, MAPK and MyD88-NFκB signalling pathways (Nakanishi et al., 2001). However, IL-18 alone does not significantly stimulate IFN-γ release, due to the low affinity of the IL-18R binding receptor. Nonetheless, type I interferon, interferon-α (IFN-α) through the induction of MyD88 and IL-12 in combination can substantially upregulate IL-18 receptors (IL-18Rα and IL-18RAP) (Sareneva et al., 2000, Yu et al., 2006, Nakanishi et al., 2001, Akira, 2000). The synergy of IL-12 and IL-18 signalling induces a range of transcription factors such as AP-1, NFκB, STAT-4. The binding sites for these transcription factors are situated around the *IFNG* locus (Akira, 2000, Nakanishi et al., 2001).

Thus, taken together, in appreciation of NK cell function it is important to consider how accessory cells influence NK cells. These cells could prove to be very important sources of chemokines and cytokines vital in the recruitment and activation of NK cells. However, immune cell phenotype is known to be influenced by herpes viruses, especially, human cytomegalovirus (HCMV) infection. HCMV is recognized to influence NK cell differentiation. Therefore, it is relevant that we understand how these viruses affect NK cell function.

1.1.6 Human cytomegalovirus

HCMV belongs to a family of viruses called *Herpesviridae*, which is divided into three subfamilies: alpha, beta and gamma. HCMV belongs to the betaherpesviruses sub family. It is made up of four main components which are: the core, the capsid, the tegument and the envelope. HCMV has about 230 genes of which 54 are membrane proteins present on the envelope of the virus. As with all herpesviruses, HCMV expresses gB and gH/gL glycoproteins important for viral entry into the host cell (Britt,

2008, Vanarsdall and Johnson, 2012, Hanley and Bollard, 2014). HCMV infection is one of the leading causes of congenital mental retardation and deafness. These viruses can be secreted in saliva and breastmilk, which can lead to early postnatal infection in children. In premature infants, HCMV infection can cause pneumonia, neutropenia and thrombocytopenia. HCMV infection is also common in immunodeficient, and immunosuppressed patients (Muntasell et al., 2013).

HCMV viruses reside in myeloid lineage and CD34+ haematopoietic cells (Hanley and Bollard, 2014). As HCMV infects a new cell it translates the immediate early (IE) proteins within 2 hours, without the requirement of new viral protein synthesis (Hanley and Bollard, 2014). These IE proteins are essential in viral replication; suppression of these IE proteins is associated with HCMV latency and expression is correlated with reactivation (Paulus and Nevels, 2009). It is thought that granulocyte-macrophage colony stimulating factor and tumour necrosis factor-α induce monocyte differentiation into dendritic cells or macrophages. This process activates the viral IE-1 promoter and induces HCMV reactivation (Bunde et al., 2005, Hanley and Bollard, 2014). How these cytokines induce activation is still under investigation, but it is clear that CD8 T cells specific for IE protein are essential for viral control (Bunde et al., 2005).

The immune system uses different mechanisms to control HCMV infection, ranging from innate immune cytokine secretion through cellular immunity to humoral immunity. This process involves T cells, B cells and antibodies as well as NK cell effector functions (Muntasell et al., 2013). Recent data suggest that HCMV induced NKG2C+ NK cell populations may help in tumour control during malignant disease (acute myeloid leukaemia) after stem cell transplantation (Green et al., 2013, Ito et al., 2013, Foley et al., 2012). It is also known that NK cells are important in herpes viral control in humans (Biron et al., 1989). This highlights the importance of NK cells in providing protective immune responses against herpesviruses in humans. However, NK cells are not the only immune cell important for viral control; it has been shown that HCMV gB and gH glycoproteins can induce Toll Like Receptor-2 on fibroblasts via NFκB to promote pro-inflammatory cytokine release for viral control (Boehme et al., 2006).

Antibodies against HCMV are an important mechanism of viral control. It has been revealed in animal that HCMV specific antibodies protect the animal from death but do not prevent HCMV infection (Hanley and Bollard, 2014, Bratcher et al., 1995). In

humans, the immune system generates a range of antibodies against viral proteins like pp65 on the viral tegument, gB, gH and IE-1 glycoproteins (Britt et al., 1990).

Cellular immunity is important for viral control and it has been demonstrated that the percentage of CD8 specific T cells generated in response to HCMV antigens in seropositive individuals can reach around 10% of circulating CD8 T cell population (Olsson et al., 2000, Hanley and Bollard, 2014), and mainly targets IE proteins and pp65 receptors (Wills et al., 1996, Sylwester et al., 2005). Similar to CD8 T cells, 9% of CD4 T cell respond to HCMV antigens in infected people (Hanley and Bollard, 2014). These CD4 and CD8 T cells recognize 59% of all viral open reading frames (Sylwester et al., 2005). However, both CD8 and CD4 T cells are essential in viral control of HCMV infection and reactivation (Hanley and Bollard, 2014).

Despite all of these protective mechanisms that our immune system uses to control HCMV, this virus has developed diverse mechanisms of immune evasion for both innate and adaptive immune cells. HCMV has Unique Short (US) and Unique Long (UL) gene regions in its genome encoding gene products. These gene products help in multiple ways to avoid either immune recognition or immune cell effector function. Table 1.2 summarises the different mechanisms by which HCMV can inhibit NK cell function (Hanley and Bollard, 2014). HCMV gene products US2, US3, US6, US10 and US11 inhibit HLA class I molecule surface expression, resulting in reduced detection by CD8 T cells (Wilkinson et al., 2008).

UL40 is a HCMV conserved viral peptide presented on HLA-E class Ib molecules. The UL40 sequence of which includes a peptide that mimics the signal sequence peptide of HLA-Ia molecules which binds with high affinity to HLA-E. Its function is not fully understood, but it is known to stabilise HLA-E expression on the surface of infected cells, thereby triggering the inhibitory CD94/NKG2A+ receptor and thus evading NK cell killing (Braud et al., 1998).

Table 1.2: Mechanisms of HCMV immune evasion.

HCMV Gene	Mechanism of evasion	Effect on immune system
UL18	MHC Class I homologue	Inhibition of NK cell via KIR
UL40	HLA-E	Inhibition of NK cell via CD94/NKG2A
UL142, UL16	Down regulation of ligands	Retention of MICA (by UL142), MICB, ULBP1 and ULBP2 (by UL-16)
US2, US3, US6, US10, US11	MHC Class I down-regulation	Decreased presentation of HCMV antigens to CD8+ T cells
UL83 (pp65)	HCMV-IE-1 sequestration	T cells cannot target first genes expressed upon reactivation
UL36, UL37	Inhibitors of apoptosis	Decrease in phagocytosis of infected cell by antigen presenting cells
UL111a	IL-10 homolog	Immune suppression

Adapted from (Hanley and Bollard, 2014).

1.1.7 CD94/NKG2C acts as an activating receptor for HCMV

Natural killer group 2A (NKG2A) and natural killer group 2C (NKG2C) are type II cell surface membrane receptors that originate from the CD94/NKG2 C-type lectin family of receptors and are covalently attached to the CD94 glycoprotein (Lazetic et al., 1996, Heidenreich et al., 2012) to form heterodimeric receptors. They are encoded by the NK gene complex on human chromosome 12 (Guma et al., 2004). These two receptors recognise nonamer peptides derived from leader sequences of classical MHC class I molecules, Human Leucocyte Antigen-A HLA-A, -B, -C and -G, presented on the non-classical MHC class Ib molecule Human Leucocyte Antigen-E (HLA-E) on target cells. HLA-E molecules are expressed at low frequency on nucleated cells and function as ligands for CD94/NKG2 receptors on CD8 T cells and NK cells (Braud et al., 1998, Gong et al., 2012). HLA-E class Ib can be presented as HLA-E^{R107} having Arginine at position 107 or as HLA-E^{G107} with Glycine instead (Sullivan et al., 2008). This class Ib molecule has also been shown to bind Hsp60 and present some pathogen peptides but their specific role in this is still unknown (Ulbrecht et al., 1998, Nattermann et al., 2005, Michaelsson et al., 2002). Importantly, a peptide from HCMV, UL40 binds with high affinity to HLA-E and can stabilise the expression of HLA-E on HCMV infected cells (Tomasec et al., 2000); polymorphism within the viral peptide has been presented to modulate the affinity of the NKG2C receptor binding (Heatley et al., 2013) (Table 1.2).

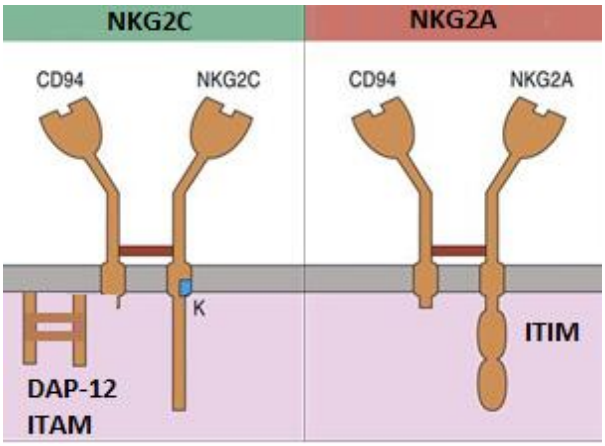

Figure 1.1: CD94/NKG2 Receptors (NKG2C and NKG2A) receptors.

Activating receptor NKG2C binds DAP12 which contains an intracellular immunoreceptor tyrosine-based activating motif (ITAM). NKG2A receptors have an intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM) inducing an inhibitory signal. Adapted from (Roitt I et al., 2006).

NKG2A is an inhibitory receptor containing an intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM). This motif recruits protein tyrosine phosphatase containing SH2 domain-1 (SHP-1) to inhibit NK cell function (Guma et al., 2004). NKG2C, in contrast, binds DAP12, which contains an intracellular immunoreceptor tyrosine-based activating motif (ITAM) and induces NK cell activation (Figure 1.1) (Gong et al., 2012). The binding affinity of the inhibitory CD94/NKG2A for HLA-E is higher than the affinity of the activating CD94/NKG2C receptors; this is potentially important in providing tolerance against NK cell targeted cytotoxicity. CD56bright NK cells express more CD94/NKG2A than do CD56dim cells (Beziat et al., 2010).

Exposure to human cytomegalovirus appears to have a unique relationship with NK cell activation and receptor expression. NK cell activation and preferential expansion of NKG2C+ NK cells can be induced with human cytomegalovirus (HCMV)-infected fibroblasts in culture and NKG2C expansion is seen ex vivo in HCMV healthy seropositive individuals (Beziat et al., 2012, Guma et al., 2006). It has been suggested that HCMV might trigger NKG2C+ NK cell expansion and differentiation thereby changing the NK cell compartment. CD56dim NK cells expressing high levels of NKG2C+ have been shown to be terminally differentiated and polyfunctional. Terminally differentiated CD56dim NKG2C+ NK cells have been characterised as

cells with low frequencies of NKG2A, NKp46, NKP30, CD161 and Siglec-9 whilst comprising a high frequency of LIR-1, KIR and CD57 expressing cells. Following detailed analysis of NKG2C receptors, two subsets have been identified, that is NKG2Cdim NK cells, which express NKG2A and NKG2Chi NK cells, which do not have NKG2A receptors. However, both of these cells equally express the activating receptors NKG2D and CD16 (Muntasell et al., 2013). NKG2Chi NK cells are found in approximately half of HCMV seropositive individuals and NKG2Cdim NK cells are seen in some HCMV+ in cord blood and all HCMV- negative people (Muntasell et al., 2013).

Furthermore, these NKG2C+ NK cells have limited IFN-γ secretion capacity following interleukin-12/-18 (IL-12/-18) stimulation whilst retaining high cytotoxic capacity (Beziat et al., 2012, Beziat et al., 2010). However, IL-12 driven activation of NKG2C+ NK cells can induce expression of NKG2A, possibly providing a negative-feedback mechanism to control the activated signalling pathway (Saez-Borderias et al., 2009). NKG2A+ KIR- NK cells are nonetheless cytotoxic towards immature DC, which is controlled by HLA-E expression on the DC (Della Chiesa et al., 2003). It should be noted that NKG2C receptors can also be expressed by T lymphocytes such as TRCαβ, CD4, CD8, and TCRγδ T cells (Muntasell et al., 2013).

1.1.8 HCMV promotes NK cell differentiation (CD57 expression)

HCMV infection induces NKG2C+CD57+ NK cells (Hendricks et al., 2014, Lopez-Verges et al., 2011). It is though that in acute HCMV infection NKG2C+ NK cells expand and acquire CD57 molecules on their surface as they differentiate to become mature cells (Lopez-Verges et al., 2011). Consequently, NK cells have been observed to acquire CD57 expression with maturation and age. CD57 (HNK-1) is a terminally sulfated trisaccharide N-glycan neural cell adhesion molecule expressed on CD56dim NK cells and CD28-CD8+ T cells. However, it is predominantly expressed in different cell types of the nervous system (Focosi et al., 2010). CD57 has been shown to bind to CD62L, P-selectin, laminin and amphoterin and CD57-dependent adhesion has been associated with cell-to-cell and cell-to-matrix interactions during cell migration processes. Expression of CD57 on T cells is associated with replicative immunosenescence. These cells have impaired ability to undergo cell division cycles and they are vulnerable to activation induced cell death.

Nonetheless, they still have the potential to secrete cytokines (Focosi et al., 2010, Nielsen et al., 2013).

CD57 expression on CD56dim NK cells may occur due to remodelling of NK cell subsets towards terminal differentiation (Gayoso et al., 2011). CD57+ NK cells show increased activation after contact with MHC-deficient target cells but reduced responsiveness to cytokine-dependent activation (Bjorkstrom et al., 2010, Lopez-Verges et al., 2010, Nielsen et al., 2013). These cells have a highly mature phenotype with increased cytotoxicity abilities, including antibody dependent cytotoxicity using CD16 receptors whilst having shortened telomeres and reduced proliferative capacity (see Table 1.3) (Lopez-Verges et al., 2010, Solana et al., 2012). It is proposed that NK cell differentiation progresses from CD56bright to CD56dim CD57- to CD56dim CD57+ NK cells (Gayoso et al., 2011).

It has been observed that the frequency of NK cells expressing activating receptors NKp46/30, NKG2D and inhibitory CD94-NKG2A significantly decreases with age whilst the population expressing inhibitory KIR and LIR-1 receptors increase with age, suggesting a change in the balance of receptors regulating NK cell function (Lutz et al., 2005, Sundström et al., 2007). Le Garff-Tavernier and colleagues (2010) compared NK cell function and phenotype in healthy new-born, middle-age, old and in the very old individuals. This study had similar findings to the proposed shift in receptor expression, demonstrating that cord blood had low frequencies of KIR and LIR-1 expressing cells (Le Garff-Tavernier et al., 2010, Almeida-Oliveira et al., 2011).

Table 1.3: NK cell maturation stages.

Maturation from CD56bright to CD56dimCD57- to CD56dim CD57+

CD56bright	**CD56dimCD57-**	**CD56dimCD57+**
Cytokine+++	Natural cytotoxicity	Natural cytotoxicity
CD16-/^{dim}	CD16+	CD16++
CD94/NKG2A^{high}	CD94+	CD94+/-
KIR-	KIR+	KIR++
Long telomeres		Shorter telomeres
High proliferation		Limited proliferation

Adapted from (Solana et al., 2012).

1.1.9 'Memory' Natural Killer cells

1.1.9.1 MCMV- and hapten-associated NK memory in mice

Contemporary advances in the understanding of NK cell biology suggest that NK cells may have immunological 'memory' or recall potential during some secondary viral infections. This has been shown through the enhancement of NK cell activation observed in secondary antigen re-exposure. This NK cell 'memory-like' characteristic was first observed during murine cytomegalovirus virus (MCMV) infection. It has been demonstrated that Ly49H+ NK cells expand in primary MCMV infection after binding to MCMV m157 protein. Following re-exposure of these 'memory' NK cells to MCMV, these cells were protective against MCMV infection as they had increased potential to secret cytokines, degranulate and replicate compared to 'naïve' NK cells (Sun et al., 2009, Horowitz et al., 2012b). Another form of NK cell 'memory' has been shown in murine adoptive transfer of primed, hepatic Ly49C-I (+) NK cells. These cells induced contact hypersensitivity reactions following adoptive transmission of NK cells from donors who had previously been sensitized with 2, 4-dinitrofluorobenzene and oxazolone hapten molecules (O'Leary et al., 2006).

In humans, equivalent populations of HCMV-driven 'memory' NK cell can be generated by contact-dependent interaction of NKG2C receptors with infected HLA-E expressing target cells, and CD14+ monocytes IL-12 production (Cerwenka and Lanier, 2016, Rolle et al., 2014). However, whether 'memory' NK cells can differentiate in the absence of NKG2C (e.g. in homozygous *NKG2C* gene deletion donors) is still unknown, although in this case expansion may be driven by activating KIR (KIR2DS2, KIR2DS4 and KIR3DS1) suggesting a possible role for these activating KIR in 'memory' NK cell expansion (Beziat et al., 2013, Muntasell and Pupuleku, 2016, Della Chiesa et al., 2014).

1.1.9.2 'Adaptive' NK cells

HCMV has more recently been shown to be associated with NK cell differentiation resulting in the generation of 'adaptive' NK cells which tend to express high levels of NKG2C, CD16, LIR-1 and CD57 whilst they lack NKG2A, CD161, NKp46, and NKp30 although there are exceptions to this surface phenotype (Vieira Braga et al., 2015). Early NK cells express cell signalling proteins common to T, B and myeloid lineages essential to its effector functions. However, late mature, highly differentiated 'adaptive' NK cells lose the expression of certain B and myeloid cell related signalling proteins, which correlates with hyper-methylation in the promoter regions of these genes, the methylation pattern of these NK cells being very similar to those of cytotoxic effector CD8 T cells (Schlums et al., 2015, Tesi et al., 2016). HCMV seropositive individuals have 'adaptive' NK cells that are FcεRγ- and have reduced expression of SYK and EAT-2 signalling molecules (Zhang et al., 2013, Schlums et al., 2015). These cells had similar properties to cytotoxic T cells and have enhanced expression of DAP12-coupled activating receptors (Schlums et al., 2015). 'Adaptive' NK cells can be defined as cells that have lost expression of the transcription promyelocytic leukemia zinc finger molecule (PLZF) and often also lack FcεRγ-, SYK and EAT-2 signalling molecules. These transcription factors can regulate chromatin remodelling, thereby influencing gene expression (Schlums et al., 2015).

'Adaptive' NK cells have reduced cytokine responsiveness, which is in particular strongly associated with diminished expression of PLZF. Interestingly, these cells do not respond to autologous T cell-mediated activation (Schlums et al., 2015, Lee and Maeda, 2012) which could have implications for NK cell responses to vaccines. In addition, PLZF is known to regulate the target genes of IL-12 and IL18 receptors resulting in reduced responsiveness to cytokines (Gleimer et al., 2012, Schlums et

al., 2015). PLZF has been shown to be essential in inducing IFN-stimulated genes important in IFN-γ secretion in NK cells and patients with PLZF mutations have an altered NK cell phenotype where these cells produce increased amount of IFN-γ (Xu et al., 2009, Eidson et al., 2011).

1.1.9.3 Cytokine-induced 'memory-like' NK cells

Cytokines also play an important role in the generation of 'memory-like' NK cells, as it has been observed that mice that lack IL-12, IFN-γ and IFN-α receptors fail to develop memory NK cells. However, it should be noted that using human PBMC and HCMV infected fibroblasts, only IL-12 (and not IL-15, IL-18 or the IFN-α receptor) was shown to be important in generating 'adaptive' NKG2C NK cells (Rolle et al., 2014). However, in humans, no single cytokine was found to be uniquely required to generate 'memory-like' NK cells (Cooper et al., 2009). Conversely, IL-12 is known to be redundant in HCMV infected individuals, as IL-12 functional mutations did not influence HCMV infection control (Bustamante et al., 2008). In mice, IL-12 and IL-18 secreted in response to MCMV infection, induce the up-regulation of the high affinity chain IL-2 receptor, CD25 (IL-2Rα) (Lee et al., 2012, Ni et al., 2012). Also in humans, up-regulation of CD25 after cytokine pre-activation enhances NK cell responsiveness to low concentration of IL-2 (Leong et al., 2014).

Cytokine-induced memory NK cells can be generated, through in vitro incubation of mouse NK cells with IL-12, IL-15, and IL-18 and these expanded NK cells have an increased ability to secret IFN-γ up to 4 months post adoptive transfer (Keppel et al., 2013, Cooper et al., 2009). The same IFN-γ potentiation is seen in human NK cells that are treated with IL-12, IL-15, and IL-18 then re-stimulated with cytokine or target cell line (Romee et al., 2012, Ni et al., 2012). These cytokine-induced 'memory-like' NK cells are similar to HCMV driven NKG2C+ NK cells, as they both have stable demethylation of the *IFNG l*ocus conserved non-coding sequence 1 (CNS1). This evidence suggests that 'memory-like' NK cells can be generated in the absence of antigens (Cerwenka and Lanier, 2016).

In summary, in humans, 'adaptive' NK cells have been defined as NKG2ChiCD57+ cells lacking NKG2A receptors. These cells are also known to lack FcεRIγ, SYK and EAT-2 signalling molecules with low expression of PLZF transcription factor. In individuals homozygous for the NKG2C gene deletion, these cells express inhibitory KIR2DL for self HLA class I ligands and activating KIR2DS2, KIR2DS4 and KIR3DS1

in some instances. Conversely, cytokine-induced 'memory-like' NK cells, which may be distinct from adaptively expanded NK cell populations, express NKG2C, CD94, NKG2A and CD69 receptors and tend to lack CD57 (Cerwenka and Lanier, 2016, Romee et al., 2012).

1.1.10 Natural Killer cells and vaccination

Evidence thus far suggests that there is a mechanism for induction of 'memory-like' NK cells after vaccination which resembles the pre-activation of NK cells by cytokines such as IL-12, IL-15 and IL-18. These 'memory-like' NK cells may have enhanced responsiveness to CD4+ T cell derived IL-2; moreover, IL-2 production is also enhanced post-vaccination (Figure 1.2) (Horowitz et al., 2012b, Long et al., 2008, He et al., 2004, O'Leary et al., 2006, Cooper et al., 2009). This recall antigen-induced IL-2-mediated activation may prove to be valuable in enhancing NK cell anti-pathogen effector function after vaccination. Horowitz et al. (2010) have shown that CD69, an early activation marker, CD107a a degranulation marker, IFN-γ production and perforin release by NK cells were all enhanced following vaccination with inactivated rabies vaccine in an antigen-specific T cell and IL-2-dependent manner (Figure 1.2). These NK cells responded faster than CD8+ T cells following re-exposure to rabies antigens and secreted most of the IFN-γ within the first 24 hours. Similar NK cell recall potentiation has been observed in response to several different vaccines, including the RTS,S/AS01 malaria vaccine, Bacillus Calmette-Guérin (BCG), Hepatitis B (HB) and influenza vaccines (He et al., 2004, Long et al., 2008, Evans et al., 2011, Horowitz et al., 2012a). However, this recall response is heavily influenced by the HCMV infection status of the vaccine recipient (Nielsen et al., 2015, Goodier et al., 2016). This raises the possibility that the NK response to vaccination may be very different in the UK (where HCMV prevalence is relatively low) compared to countries (such as The Gambia) where HCMV infection is almost universal and occurs very early in life (Goodier et al., 2014). Thus, in this thesis I present an investigation of NK cell responses to trivalent influenza vaccine and diphtheria, tetanus, pertussis and polio virus vaccination in The Gambia.

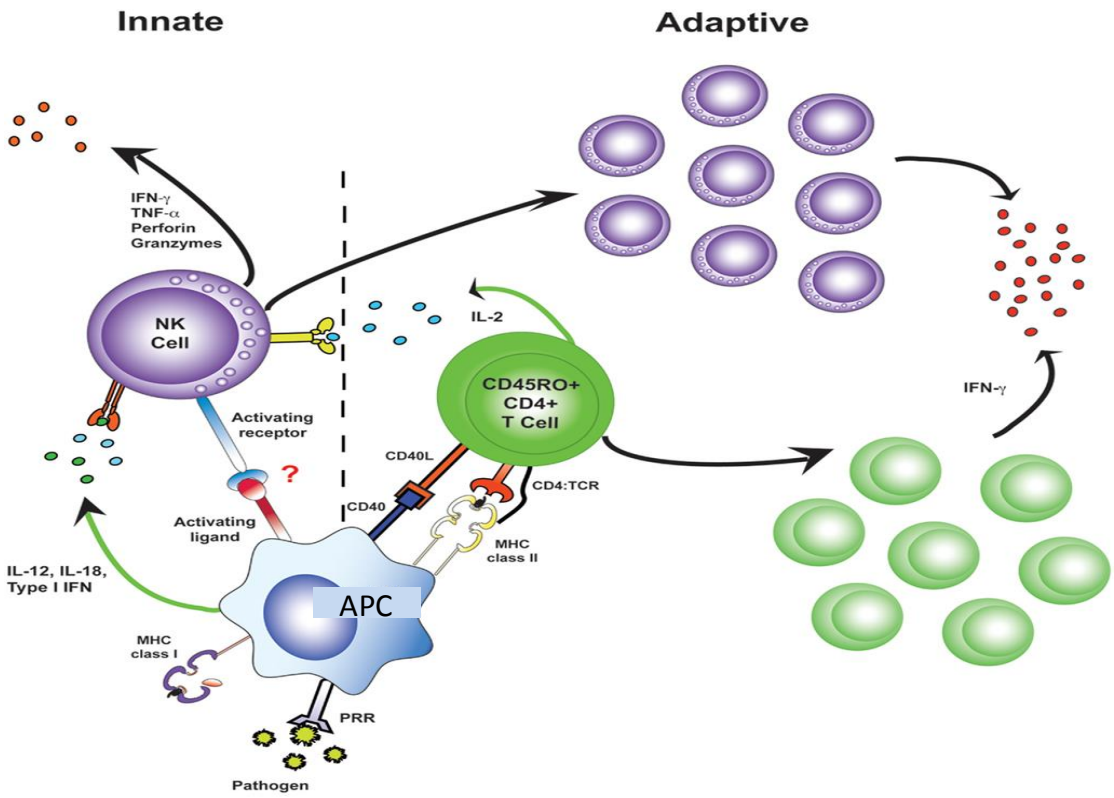

Figure 1.2: Recall NK cell activation in adaptive immune responses.

Pathogen invasion during primary infection stimulates PRR of antigen presenting cells (APC) to prompt innate signals (cytokines & receptors activation) to induce a limited NK cell activation (left of dotted line). In an adaptive immune response, re-exposure to a similar pathogen simultaneously induces antigen presenting cell signals and a potent antigen-specific CD4+ T cells IL-2 secretion, leading to a rapid and robust 'adaptive' NK cell secretion of effector molecules, killing of transformed target cells and proliferation. Image from (Horowitz et al., 2012b)

1.1.11 A Knowledge gap in vaccine responses

Non-heritable environmental influences on the human immune system are emerging as a key factor driving immune response heterogeneity including differential vaccine responses (Brodin et al., 2015). In particular, chronic infections like HCMV infection are known to drive not only maturation and differentiation of NK cells but also of other immune cells. Variation in vaccine efficacy and effectiveness in developed and developing countries is emerging as a new and important area of research (Lamberti et al., 2016), as it is becoming apparent that these variations in efficacy can partially be attributed to the heterogeneity of the immune system of these different populations. Differences in immune phenotype and function could in turn be attributed to different environmental exposure to acute and chronic infections.

Although vaccination has saved more lives than any other medical intervention, millions of children still die of vaccine preventable diseases, particularly in sub-Saharan Africa (Feikin et al., 2016, Lamberti et al., 2016). This highlights the gap of knowledge that still exists on understanding how vaccines work and how to improve the efficacy of the current expanded programme of immunization. Also, with the emerging of contagious diseases like Zika and Ebola viruses, as well as the persistence of endemic infectious diseases like malaria and tuberculosis, we need to develop better mechanisms of activating and regulating T cells, B cells and other innate immune cells like natural killer cells, to provide effective initial and late immunological responses that can protect different populations.

We still do not fully understand how natural killer cells coordinate with other immune cells in protective responses generated by vaccination, and how this varies between populations. Understanding the genotypic and phenotypic differences within the natural killer cell population and also between different human populations will enhance our abilities to develop better vaccines in order to save lives and improve health. This is particularly important in developing countries like The Gambia, where many children still die of vaccine preventable diseases and there is a high burden of chronic infections, including HCMV.

1.1.12 Aims of this research project

The aims of this Ph.D. project were to characterise natural killer cell genotype, phenotype and function in an African (Gambian) population, where there is a high burden of human cytomegalovirus infection and to investigate how this influences natural killer cell function after vaccination.

Specifically, the following objectives were studied:

1. I investigated how natural killer cells differentiated and matured with increasing age in a Gambian cohort (Chapter 3). I hypothesised that natural killer cell phenotype and function would change with increasing age.

2. I evaluated natural killer cell function following a single vaccination with trivalent influenza vaccine immunization, with responses followed up to 24 weeks post vaccination (Chapter 4). I hypothesised that natural killer cell function would be potentiated after primary vaccination in a CD4+ IL-2 dependent and B cell antibody dependent manner.

3. Finally, I examined the role of natural killer cells after secondary booster vaccination with diphtheria, tetanus, pertussis and poliovirus vaccine with NK cells responses followed up to 4 weeks after boosting (Chapter 5). I hypothesised that natural killer cell function would be enhanced post booster vaccination through T and B cell responses.

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2 CHAPTER 2:

MATERIALS & METHODS

2.1 INTRODUCTION

The aim of my Ph.D. was to characterise Natural Killer (NK) cell genotype, phenotype and effector function in Gambians, particularly focusing on natural killer cell effector function after vaccination. For this purpose, four different studies were conducted during the period January 2012 to March 2016.

Initially, as described in Chapter 3, I investigated in detail the phenotypic differentiation of natural killer cells and related this to their functionality, to the presence of an NK cell receptor gene deletion and to infection with human cytomegalovirus. The data presented in this study were derived from 191 individuals, between the ages of 1 to 49 years, resident in the villages of Keneba, Manduar, and Kantong Kunda, in The Gambia. Based on the observation of a high frequency of a deletion allele for the activating NK cell receptor, NKG2C in this study, we extended these observations to further genotype 1485 individuals from the Keneba Biobank, between the ages of 1 to 88 years.

In Chapter 4, the role of natural killer cells in primary vaccine-induced responses using 2012-2013 seasonal trivalent influenza vaccine (TIV) was investigated in 68 subjects from Keneba, Manduar, and Kantong Kunda, The Gambia. Because of the strong impact of age and HCMV infection on NK cell function observed in Chapter 3, this vaccination study was stratified into three age-defined groups. Additionally, the impact of giving a booster (secondary) vaccination in enhancing NK cell responses was investigated in children.

Subsequently in Chapter 5, I investigated the role of NK cells in booster vaccination using the ‘Repevax’ booster vaccine containing Diphtheria, Tetanus, Pertussis and inactivated Poliomyelitis virus (DTPiP).

Thus, all of the samples used in these studies were collected in The Gambia, specifically in Kiang West, Lower River Region of The Gambia. However, the samples for the ‘Repevax’ booster study were collected from Sukuta, Kombo North District, West Coast Region, The Gambia.

2.2 THE STUDY POPULATION

The Republic of The Gambia is a sub-Saharan African country in West Africa. It is bordered by Senegal on three sides and on the western side by the northern Atlantic Ocean. The population of The Gambia was 1,967,709 as of 2015, with a rural population of 40.4% and an urban population of 59.6% (The United States Central Intelligence Agency, 2016).

Most of the data presented in Chapter 3 and Chapter 4 were collected from the Kiang West Longitudinal Population Study Cohort (Hennig et al., 2015). This cohort consists of 36 villages with a population size of about 14 000 people as shown in Figure 2.1. This population comprises 79.9% Mandinka, 16.2% Fula, and 2.4% Jola ethnicities. The Kiang West Demographic Surveillance System captures all inhabitants of this region every three months. All residents of this region are given a specific West Kiang number that is used to identify the person and also track their movement within this region or outside the specified demographic zone (Hennig et al., 2015). It is through this system that the potential list of study participants of any study in the area is generated using the specific study criteria (based on specified inclusion and exclusion characteristics).

With regards to the ‘Repevax’ booster vaccination study, a demographic surveillance system was not available in Sukuta, therefore, potential study participants were recruited through a local youth organization called ‘Service for Peace’ and through door-to-door compound visits within the area. Sukuta is a peri-urban town of Kombo North District and forms part of the 9 districts of Brikama Area Council (Figure 2.2). This district is populated by 344,756 people. Sukuta is predominantly of Mandinka ethnicity (The Gambia Bureau of Statistics, 2013).

Figure 2.1: Map of West Kiang, Lower River Region, The Gambia.

District of West Kiang showing the locations of Keneba, Manduar, and Kantong Kunda villages. (Picture taken from Google map image).

Figure 2.2: Map of Sukuta, Kombo North District, West Coast Region, The Gambia.

This map indicates the location of Sukuta and MRC Unit The Gambia, Fajara. (Picture taken from Google map image).

2.3 ETHICAL APPROVALS

The London School of Hygiene & Tropical Medicine Ethics Committee, The MRC Unit The Gambia Scientific Coordinating Committee (SCC) and The Gambia Government/ MRC Joint Ethics Committee all gave approval for each of the studies presented in this thesis. Each study is identified through the SCC number below:

I. SCC number: 1269, Immune correlates of breakthrough infections of HBV after vaccination, approved on the 10th of May 2012 (Chapter 3).

II. SCC number: L2015.17, Control of CMV infections and NK cell receptor genotypes in West Kiang residents (adjunct to L2012.44 Exome variation in West Kiang residents; SCC1185, L2010.97 and L2012.31 (Keneba Biobank); SCC1269: Immune correlates of breakthrough infections of HBV after vaccination, approved on the 8th of June 2015 (Chapter 3).

III. SCC number: 1309, Age-related alterations in natural killer cell function after influenza vaccination, approved on the 31st of December in 2012 (Chapter 4).

IV. SCC number: 1372, Does booster vaccination enhance IL-2 driven NK cell responses, approved on the 16th of May 2014 (Chapter 5).

The Gambia Medicines Board also gave approval for the importation of the two vaccines used in the vaccination studies, see Appendix XVII and XX for the approval letters. The approval letters for all studies are attached on Appendix XII to XL at the end of the thesis.

Additionally, before each study started, a village meeting was held with the Alkalos (local name for the head of the village) and village elders to inform them of the study aims and intentions. The samples collected and the different laboratory techniques performed to investigate our hypotheses are set out below.

2.4 PERIPHERAL BLOOD MONONUCLEAR CELL SEPARATION

In the two vaccination studies presented in Chapters 4 and 5, 30 ml of blood from adults and 5 ml from children, collected in sodium heparinised (BD vacutainer, Oxford, UK) tubes, were layered onto 15 ml and 6 ml of Lymphoprep ™ (Axis-Shield, Stockport, UK), respectively, to separate peripheral blood mononuclear cells (PBMC), using density gradient centrifugation. The tubes were spun for 30 minutes at 1800 revolutions per minute (rpm) at 22°C, with zero brake. Two to four ml of plasma were aliquoted and stored at -80°C for future antibody or cytokine analysis by ELISA or multiplex immunoassay or for autologous plasma cell culture. PBMC were transferred into fresh 50 ml Falcon tubes and washed with RPMI PSG (RPMI 1640 containing 2 mM L-glutamine, 100 IU/ml of Penicillin and Streptomycin (Gibco® Life Technology, ThermoFisher Scientific, USA)), centrifuging at 1600 rpm for 7 minutes. Supernatants were then discarded and the cells re-suspended. Twenty ml of medium was added for adult samples or 5 ml of RPMI for samples from children and cells were filtered through a 70 µm nylon cell strainer (BD Falcon cell strainer, California, USA), 5 ml of RPMI was used to wash the filter. Cell counting was then performed after diluting the suspension 1:1 in Trypan Blue (0.4% Sigma-Aldrich, USA). Following the removal of PBMC for ex vivo staining, the remaining PBMC were cryo-preserved in freezing medium (5% DMSO in Fetal Calf Serum, FCS) at -80^{o}C (1 X 10^{7} PBMC per vial) overnight in Nalgene cryo-boxes containing isopropanol (Mr. Frosty™ Freezing Container, Thermo-Fisher Scientific Inc, Waltham, USA) before they were transferred into liquid nitrogen. These cells were at the end of the week transported from the Keneba field station to the main MRC Laboratories at Fajara using a Liquid Nitrogen (LN_2) dry shipper and were stored in the Natural Killer cell project liquid nitrogen dewar.

Ex vivo staining is defined as PBMC that have been isolated and stained without in vitro stimulation. And in vitro staining is defined as PBMC that have been cultured in vitro with various exogenous stimuli for 5 or 18 hours before staining, as appropriate.

2.5 RECOVERY OF CRYOPRESERVED PBMC

PBMC vials were transported from the LN_2 room to the lab using a 'Mr Frosty™' container cooled to -80ºC or on dry ice, where available. Following wiping of vials with 70% ethanol to decontaminate exposed surfaces, 20 ml RPMI (supplemented with 1%v/v Penicillin, Streptomycin and L-Glutamine solution (PSG), (Gibco, Paisley, UK), pre-warmed in a 37ºC water-bath was used to quickly thaw the PBMC using a 1 ml pipette, the remaining vials being kept on dry ice or in the Mr Frosty until required. Cells were washed by centrifugation at 1700 rpm for 10 minutes. The supernatant was discarded to remove the freezing medium containing dimethyl sulfoxide (DMSO). Then another 20 ml of RPMI (PSG) with 200 µl of FCS was added and re-spun at 1700 rpm for 7 minutes. Following this spinning, the supernatant was discarded and the cells re-suspended in 3 ml of 0.5% FCS RPMI in a 4 ml sterile capped tube (Falcon polypropylene, USA). A 1:1 dilution of samples with Trypan Blue was then used to count the cells. After counting the samples were spun at 1500 rpm for 10 minutes and the cells re-suspended at a concentration of 2 $X10^7$ PBMC/ml in the required final volume. The PBMC were allowed to rest for 30 minutes before the in vitro T cell assay, while for the in vitro NK cell assays, the cells were rested for at least 3 hours prior to stimulation.

2.6 IN VITRO PBMC CULTURE CONDITIONS

All NK cell assays were conducted in a final volume of 100 µl per well with 2.5 $X10^5$ PBMC per well, whereas, the T cell assays were conducted in a final volume of 200 µl with 5 $X10^5$ PBMC per well. All assays were carried out in RPMI containing 10% pooled AB serum (Sigma-Aldrich, USA) or 10% autologous plasma. The precise PBMC culture conditions are described in each specific chapter but were generally as follows: Medium alone: No antigen 0.1% FCS RPMI; Target antigen: vaccine/target antigen in 0.1% FCS RPMI; Low Concentration of Cytokines (LCC) 12.5pg/ml recombinant rIL-12 (Peprotech, London, UK) + 10ng/ml rIL-18 (R&D systems, Abingdon, UK) in RPMI (+PSG) 1% FCS; High Concentration of Cytokine (HCC) 5ng/ml rIL-12 + 50ng/ml rIL-18 in RPMI (+PSG) 1% FCS.

In order to assess vaccine responses, I added a low concentration of cytokine (LCC) of rIL-12 and rIL-18 to the vaccine antigens. This low concentration cannot by itself activate NK cells but compensates for the lack of PAMPs in the purified subunit vaccine antigens; PAMPs are required to induce production of accessory cytokines (such as IL-12 and IL-18) by antigen presenting cells and these cytokines are essential for NK activation.

In order to assess the functional capacity of the NK cells, we cultured the PBMC overnight for 18 hours at 37ºC, 5% CO_2. Generally, the functional characteristics of the NK cells were assessed by using CD107a as a degranulation marker, CD25 as a proliferation marker and IFN-γ as cytokine production potential.

In assessing NK cell cytoxicity, we used CD107a, which is an endosomal marker that fuses with cell membrane during degranulation to release granules containing perforin and granzymes essential in cytotoxicity (Al-Hubeshy et al., 2011, Aktas et al., 2009). As CD107a is expressed on the surface it is used as a measure for degranulation. However, because it is known that CD107a can be recycled on the cell surface of NK cells, we added anti-CD107a antibodies at the beginning of the culture to allow for the cumulative quantification of CD107a degranulation (Betts et al., 2003).

Resting NK cell express low levels of CD25 (IL-2Rα), this is a high affinity receptor for IL-2 and functions in combination with CD132 (common γ chain) and CD122 (common β chain) which are constitutively expressed on resting NK cells. Therefore, increased expression of CD25 signifies NK cell activation and also increased sensitivity to IL-2.

IFN-γ is an important cytokine essential in NK cell responses to infection and tumours. It helps in inducing the activation of macrophages (phagocytes) and also Th1 immune responses. As it is secreted from the NK cell, I used Brefeldin A and Monensin to block secretion and allow the cytokine to accumulate; I then stained the cells for intracellular IFN-γ. Brefeldin A reallocates intracellular proteins from cis/medial Golgi to the endosomal reticulum, while Monensin prevents protein secretion by blocking Golgi transmembrane protein trafficking from the endoplasmic reticulum to the Golgi (Schuerwegh et al., 2001). This mechanism of blocking allows the accumulation and detection of IFN-γ in the NK cells.

2.7 EX VIVO STAINING OF PBMC FOR FLOW CYTOMETRY

After counting, the PBMC were washed by spinning at 1600 rpm for 10 minutes at 22°C and re-suspended at 2 X 10^7 cells/ ml. Fifty µl of the cell suspension containing 1 X 10^6 cells were taken from each individual PBMC sample for staining for each antibody panel tested in flow cytometry. The specific conditions are given in the respective chapters of the thesis.

The general, the ex vivo staining procedure is as follows: 50 µl of cell suspension per sample was split into replicate wells on a 96 well U-bottomed tissue culture plate and then washed in FACS buffer (0.1% Foetal Calf Serum (FCS), 5mM EDTA, pH8 and 0.05% Sodium Azide). Specific combinations of antibodies were added to each well according to the panel specifications and cells were stained for 30 minutes.

Following staining, the cells were washed to remove excess unbound antibodies by spinning at 1800 rpm for 5 minutes and fixed with 75 µl of Cytofix/Cytoperm (BD Biosciences, California, USA) for 15 minutes in the dark at room temperature. The Cytofix buffer was washed off using 175 µl of 1X Perm wash (BD Biosciences, California, USA) by centrifugation at 2000 rpm for 5 minutes. The supernatant was discarded and cells re-suspended. Cell surface panels were re-suspended in 300 µl of FACS buffer and reserved for acquisition. Intracellular staining panels were further stained, for 15 minutes in the dark at room temperature, to detect intracellular cytokines. Following incubation, the plate was again washed in 1X Perm wash and cells re-suspended in 300 µl FACS buffer and transferred from the microtitre plate to 1.2 ml alpha micro-tubes (Alpha Laboratories Ltd, Hampshire, UK). The samples were acquired within 2-3 days post staining.

For staining after in vitro culture, the precise antibody panels used are detailed in the individual results chapters.

2.8 COMPENSATION OF FLOW CYTOMETERS

To control and monitor the flow cytometer analyser settings, daily alignment beads were run before acquiring the stained samples. Compensation beads were prepared

weekly, by adding 2 drops of the positive and negative BD comp beads (BD Biosciences, California) into 400 µl of FACS Buffer. Then, 50 µl of the bead suspension was added to each of the 8 single fluorochrome tubes. One µl of each individual fluorochrome-conjugated anti-human monoclonal antibody was added to a separate tube and incubated at 4ºC for 15 minutes. Subsequently, the beads were washed by centrifugation at room temperature for 5 minutes at 1800 rpm. These beads were re-suspended in 300µl FACS Buffer and were used to compensate the analysers. PBMC were acquired in The Gambia by means of either a Cyan™ ADP (Dako) using Summit™ Software or LSRIII® Fortessa (BD Biosciences, Oxford, UK) flow cytometer using FacsDiva® software, and in London we acquired using LSRII® (BD Biosciences, Oxford, UK), flow cytometer using FacsDiva® software.

Analysis was performed using FlowJo® (TreeStar, USA). FlowJo analyses were done by using fcs files. For each file a singlet gate was drawn to select only single cell data using forward scatter height (FSC-H) against forward scatter area (FSC-A) (Figure 2.3A). This step allows the exclusion of clumps or doublets or triplets or large number of cells that might mislead the interpretation of the result. Then the singlets are used to create the lymphocyte gate, using FSC-A on the x-axis and side scatter area (SSC-A) on the y-axis. The FSC-A indicates cell size while SSC-A indicates the relative cellular granularity (cell complexity). This lymphocyte gating strategy also allows the differentiation of live and dead cells as it excludes dead cells with lower FSC and higher SSC. Because of the limited number of channels per panel, this gating strategy allowed us to gate out dead cells without having to use a dead cell marker (Figure 2.3).

Using the lymphocyte gate, NK cells were defined as CD3-CD56+ lymphocytes (Figure 2.3C). In the literature, NK cell CD56bright and CD56dim subsets are defined as CD56+ and CD16 receptors, however, it has been observed that this strategy of gating (using CD16) is not reliable because in vitro NK cell stimulation can lead to downregulation of CD16 making it difficult to track the cell population of interest. So the strategy I used in this thesis, to identify NK cell CD56bright and CD56dim subsets was using any of the following strategy where it was appropriate: (1) CD56 vs CD3 using LSRII (Chapter 3); (2) CD56 vs SSC-log using the Cyan (Chapter 4); (3) CD56 vs FSC-H using the LSRIII Fortessa (Chapter 5) to distinguish the CD56bright and CD56dim NK cell subsets. Where less than 100 events were obtained in the NK cell gate, that sample was excluded from the analysis. Also where less than 50

CD56bright events were obtained, this population was excluded from the analysis of the functional data.

The CD56bright and CD56dim NK cells can be separated based on their CD56 expression, CD56bright express more CD56 than CD56dim NK cells. For further maturation analyses, CD56bright NK cells do not express CD57 (a late differentiation marker), whilst CD56dim NK cells can be identified into three subsets based on CD57 expression. Creating CD56dimCD57-, CD56dimCD57intermediate (or int) and CD56dimCD57+ NK cells (Figure 2.3D).

NK cell functional activation was measured by gating CD56 against CD107a for degranulation (Figure 2.3I), CD56 against CD25 (Figure 2.3G), and CD56 vs IFN-γ (Figure 2.3H).

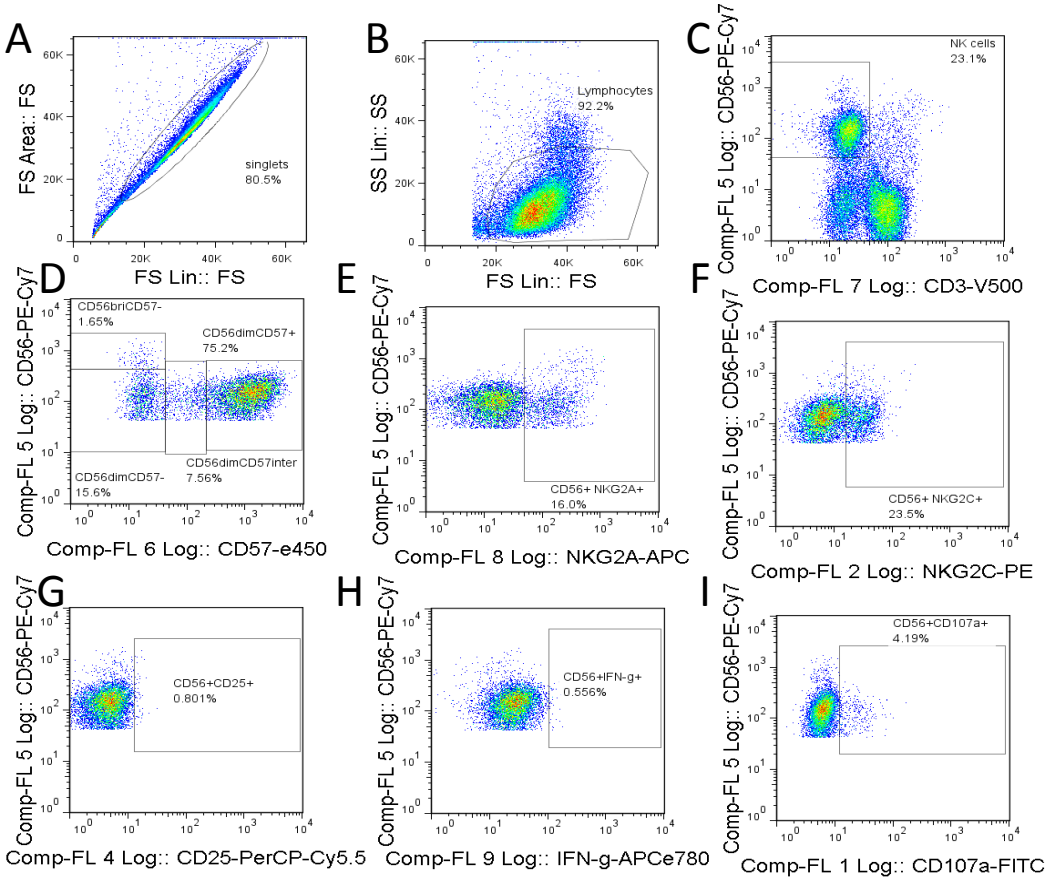

Figure 2.3: General gating strategy used in this thesis.

(A) Singlet gate, (B) lymphocyte gate, (C) NK cell gate, (D) CD57 NK subsets, (E) NKG2A expression, (F) NKG2C expression and (G) CD25 (H) IFN-γ and (I) CD107a expression. Representative dot plots from participant S1 visit 1 (29 years) recruited from the influenza vaccination study.

2.9 ENZYME-LINKED IMMUNOSORBENT (ELISA) ASSAYS

2.9.1 BioELISA cytomegalovirus kit

A commercially available kit was used to measure HCMV-pp65 antigen specific-IgG in the plasma samples from the individuals studied in Chapter 3 and 4 (Biokit, Barcelona, Spain) (BIOKIT, 2012). In brief, human IgG antibodies against CMV were detected in both plasma and serum. A 96 well plate was pre-coated with CMV pp65 antigens and 100 µl of diluted samples and controls were incubated for 1 hour at 37ºC to allow the binding of CMV specific antibodies. Plasma samples were initially diluted 1:500 and repeated at higher (1:1000) or lower (1:100) dilution where samples were out of range on the standard curve. After sample incubation the plate was washed 3X with 300 µl of washing buffer (Phosphate buffer containing 1% Tween 20 and 0.01% Thimerosal).

One hundred µl of rabbit anti-human IgG antibody conjugated to peroxidase was added in all wells except the blank well and incubated for 30 minutes at 37ºC to detect bound anti-CMV IgG. Excess, unbound, conjugated antibodies were removed by washing 3X with 300 µl of wash buffer. One hundred µl of the substrate TMB (3,3′,5,5′-Tetramethylbenzidine) solution was then added in each well including the blank well and was allowed to develop for 10 minutes, the reaction being stopped with 100 µl of 1N sulphuric acid. The Optical Density (OD) values were read at 450nm using the Dynex Technology; MRX TC II Dynex Technologies (USA) ELISA plate reader. Microsoft Excel (Microsoft 2007) was used to plot a linear graph using the OD calibrators standard curve on each plate and to calculate the unknown concentration of HCMV IgG plasma antibodies.

2.9.2 Epstein-Barr Virus ELISA kit

Levels of Epstein-Barr Virus (EBV) antibodies were monitored in our study cohorts using a commercially available kit to measure IgG specific for Epstein-Barr Virus nuclear antigen 1 (EBNA-1) (Euroimmun, Lubeck, Germany). These IgG antibodies

are only detected late in infection and remain at stable levels in serum long after infection (Euroimmun, 2014). The procedure is briefly described below:

The plates were commercially pre-coated with EBNA-1 antigen and 100 µl of the calibrators, positive and negative controls and the diluted samples were added into their respective wells according to the plate layout. The plates were then incubated for 30 minutes at room temperature (+18ºC to +25ºC). Following the incubation, the plates were washed three times using 300 µl of the kit wash buffer (1X dilution). One hundred µl of enzyme conjugate (peroxidase-labelled anti-human IgG) was then added to each well and the plate was incubated for 30 minutes at room temperature. After washing 3 times with 300 µl of wash buffer as above, 100 µl of chromogen/substrate (TMB/H_2O_2) solution was added to each well and the colour was allowed to develop for 15 minutes at room temperature. The reaction was stopped by adding 100 µl of stop solution (0.5M sulphuric acid). The photometric measurement was done at 450nm wavelength using the MRX TC II Dynex Technologies (USA) ELISA plate reader, in London. Microsoft Excel (Microsoft 2007) was used to plot a linear graph using the known OD calibrators on each plate and to calculate the unknown concentration of EBV IgG antibodies.

2.10 NKG2C RECEPTOR GENOTYPING

In both the natural killer maturation (Chapter 3) and influenza vaccination (Chapter 4) studies, genotyping was performed on each subject to detect the presence or absence of the gene encoding NKG2C in extracted DNA samples according to the method of Miyashita et al (Miyashita et al., 2004).

DNA extraction was done using Promega DNA extraction kit (Promega Corporation, Madison, USA) (Promega Corporation, 2016). Two hundred µl of whole blood was stored at -80ºC until DNA extraction. Concisely, the whole blood was thawed for 15 minutes, then transferred into pre-labeled sterile 1.7ml PCR microtubes. Six hundred µl of cell lysis solution was added and mixed. The blood was incubated for 10 minutes at room temperature. The tube was spun at 16 000xg (gravitational force) for 40 seconds at room temperature. The supernatant was discarded without disturbing the pellet. Subsequently, 500 µl of cell lysis solution was added and incubated for 10 minutes then spun at 16 000xg for 40 seconds and the supernatant discarded as above. Additionally, 200µl of nuclear lysis solution added to the microtubes, the

pipette was used to dissolve the pellets by pipetting down and up about 5-6 times. If clumps were still visible the tubes were left at room temperature for about 1 hour and 30 minutes to allow the clumps to dissolve. Then, 100 µl of nuclear lysis solution was added if the clumps persisted. One hundred µl of protein precipitation solution was added to the nuclear lysate and mixed by vortexing for 10-20 seconds. After this, the tube was spun for 3 minutes at 16000xg at room temperature.

Subsequently, the supernatant was transferred after spinning in fresh tubes containing 200ul of 70% isopropanol, then mixed until the thread-like strands were visible. The tube was spun again at 16000xg for 2 minutes. The supernatant was discarded and 200ul of 70% ethanol was added to the tubes and mixed gently by inverting the tube several times to wash the DNA pellet and the side of the microtube. Finally, the tube was spun at 16000xg for 2 minutes, the supernatant was discarded, the tube was inverted for 10-15 minutes to remove excess ethanol and the DNA was suspended by addition of 20µl of DNA rehydration solution to the tubes. The DNA was stored at 2-8ºC until NKG2C assay was done.

A Phusion® High-Fidelity Polymerase Chain Reaction (PCR) kit was used to amplify the wild type *NKG2C* gene or the deletion mutant using sequences internal to the gene or in 5' and 3' flanking regions, respectively. A master mix of PCR reagents were prepared (Table 2.1). For each sample <100ng/ml of DNA was used for the PCR reaction. The primers for detection of wild type *NKG2C* genotypes were NKG2C200_F (5'-AGTGTGGATCTTCAATGATA-3') and NKG2C200_R (5'-TTTAGTAATTGTGTGCATCCT-3'), yielding a 200bp band product. The primers for detecting the *NKG2C* deletion were BREAK411_F (5'ACTCGGATTTCTATTTGATGC3') and BREAK411_R (5'ACAAGTGATGTATAAGAAAAAG3'), yielding a 411bp band product.

Table 2.1: NKG2C receptor genotyping PCR master mix:

The reagent content of the Phusion® kit master mix reaction for NKG2C genotyping.

PHUSION (HF)	1x (µL) per sample
Buffer 5X (Red cap)	4.0
dNTPS 10mM	0.4
PF201 (10uM)	0.5
PR201 (10uM)	0.5
PF411 (10uM)	1.0
PR411 (10uM)	1.0
TAQ DNA polymerase	0.1
Total	7.5

The reactions were amplified on an AB Applied Biosystems Veriti^{TM} Thermal Cycler using the following temperature and time specifications: Step 1 cycle X10: 95ºC for 3 seconds, 94ºC, 65ºC & 72ºC each 30 seconds for annealing. Step 2 cycle X26: 94ºC, 55ºC & 72ºC each for 30 seconds and 72ºC for 10 seconds and 10ºC maintained at infinity. Subsequently, the samples were run on a 1.5% agarose gel for 50 minutes at 120 Volts. *NKG2C* homozygous gene deletion had 411 bp (base pairs) band only and homozygous *NKG2C* gene had 200 bp band only, whilst heterozygous *NKG2C* donors had both bands of 200 bp and 411 bp. The image was acquired using GeneSnap software.

2.11 STATISTICAL ANALYSIS

Non-parametric Wilcoxon matched paired tests were performed to analyse paired sample data within the study groups and the Kruskal-Wallis test was used for unpaired comparisons between groups. Linear trend analyses were done using repeated measure ANOVA. GraphPad Prism (GraphPad Software 6) was used to prepare the diagrams and statistical analysis. Significant differences between groups were defined as having a p value of *$p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

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3 CHAPTER 3:

RAPID NATURAL KILLER CELL MATURATION WITH AGE IN A GAMBIAN POPULATION WITH WIDESPREAD HCMV INFECTION

3.1 DECLARATION

Some of the work presented in this chapter has been published as:

Martin R. Goodier, Matthew J. White, **Alansana Darboe**, Carolyn M. Nielsen, Adriana Goncalves, Christian Bottomley, Sophie E. Moore and Eleanor M. Riley. Rapid natural killer cell differentiation in a population with near universal human cytomegalovirus infection is attenuated by *NKG2C* deletions. *Blood,* 2014; 124(14):2213-22; DOI 10.1182/blood-2014-05-576124.

The full manuscript is attached in the Appendix on page I to XI. Specifically, for these data, I coordinated the logistics of study design and participant recruitment, collected blood samples, organised participant and sample transport between the villages and from Keneba to Fajara, and sample shipment to London. I contributed to PBMC separation and staining, flow cytometry data acquisition and data analysis. In addition, I ran the HCMV IgG antibody assays, carried out the NKG2C genotyping and analysed these data.

Paper authorship

Contribution: M.R.G. designed research, performed experiments, analysed and interpreted data and wrote the manuscript; M.J.W. designed and performed research and analysed and interpreted data. **A.D. designed and performed research**; C.M.N. performed experiments and analysed data. A.G. performed research; C.B. analysed data; S.M. designed research and coordinated recruitment of study subjects and E.M.R. designed research, supervised data collection, directed data analysis and wrote the manuscript.

3.2 INTRODUCTION

Natural Killer (NK) cells are effector lymphocytes that are essential in the control of viral infections and tumour cells (Lanier, 2005, Kiessling et al., 1975). The phenotype of these cells is known to change with age. NK cells differentiate from less mature CD56bright NK cells to more mature CD56dim NK cells. CD56bright cells express c-kit and high levels of the C-type lectin-like receptor CD94/NKG2A, CD62L and natural cytotoxicity receptors (NCR) NKp30 and NKp46, and lack the expression of KIR, CD16 and CD57 receptors/ molecules (Bjorkstrom et al., 2010, Cooper et al., 2001, Juelke et al., 2010, Lopez-Verges et al., 2010). These cells constitutively express high levels of cytokine receptors that make them highly responsive to exogenous cytokines. On the contrary, the more mature CD56dim NK cells express CD16 and variable levels of NKG2A, KIR, and NCRs, despite this variability they still preserve their ability to release IFN-γ and have greater cytotoxic potential (Cooper et al., 2001).

The variability of expression of these receptors on CD56dim NK cells correlates with the acquisition of CD57 on their surface (Bjorkstrom et al., 2010, Lopez-Verges et al., 2010, Juelke et al., 2010). CD56dim NK cells lacking expression of CD57, that is CD56dimCD57- NK cells, resemble CD56bright NK cells both in phenotype and function (White et al., 2014). In contrast, CD56dimCD57+ NK cells have lower proliferative capability with shorter telomeres and reduced competence to secrete IFN–γ. Nonetheless, these cells are highly proficient at degranulation via their CD16/FcγRIII antibody receptors (Bjorkstrom et al., 2010, Lopez-Verges et al., 2010, White et al., 2014). CD57 expression is highly correlated with the acquisition of NKG2C activating receptors on NK cells, but their developmental and functional association is still poorly comprehended (Saghafian-Hedengren et al., 2013, Wu et al., 2013).

Inflammation is believed to make an important contribution to the process of NK cell differentiation, specifically, during infections and in conditions which result in loss of immune homeostasis. Nonetheless, the mechanism whereby inflammatory pathways influence NK cell differentiation in the long-term, is still not fully elucidated (Hazeldine and Lord, 2013). This interpretation is based on evidence showing that CD57 (a marker of late differentiation) could be induced on NK cells in culture with high concentrations of IL-2 (Lopez-Verges et al., 2010).

More significantly, however, NKG2C+ NK cell populations are expanded in human cytomegalovirus (HCMV) seropositive individuals and when NK cells are co-cultured with HCMV infected fibroblasts (Guma et al., 2004, Guma et al., 2006b, Mela and Goodier, 2007, Tanaka et al., 2009). This process is mediated, at least in part by the stabilisation of the CD94-NKG2C ligand HLA-E on the cell surface by HCMV encoded peptides (Borrego et al., 1998, Djaoud et al., 2016, Rolle et al., 2014). Furthermore, other studies have shown that during acute HCMV infection, CD57+NKG2Chi NK cells are highly expanded (Lopez-Verges et al., 2011) and this has also been seen in people co-infected with HCMV and other viruses including Epstein Barr virus (EBV) (Saghafian-Hedengren et al., 2013), hantavirus (Bjorkstrom et al., 2011), hepatitis viruses (Beziat et al., 2012a) and chikungunya virus (Petitdemange et al., 2011). Importantly, however, cytokines including IL-15 can contribute to NK cell expansion in response to HLA-E expressing or HCMV-infected target cells (Guma et al., 2006a, Beziat et al., 2012b).

In Caucasians, NK cell differentiation increases gradually with increasing age. It has also been observed that there is significant variability in NK cell phenotype and function with increasing age in these populations, comparing cord blood, young children, adults and the elderly (Almeida-Oliveira et al., 2011, Hayhoe et al., 2010, Le Garff-Tavernier et al., 2010, Lutz et al., 2005, Sundstrom et al., 2007). Young children have greater proportions of CD56brightCD16- and NKG2A+NKG2C- NK cells compared to adults, and younger adults have increased proportions of these cells compared to the elderly (Almeida-Oliveira et al., 2011, Hayhoe et al., 2010, Le Garff-Tavernier et al., 2010, Lutz et al., 2005, Sundstrom et al., 2007). Likewise, expression of NCR+ and NKG2D+ NK cell receptors is reported to decline with increasing age, associated with diminished expression of CD62L and gain of CD57 (Almeida-Oliveira et al., 2011, Bjorkstrom et al., 2010, Juelke et al., 2010, Sundstrom et al., 2007). NK cell cytokine secretion also declines with increasing age, whereas cytotoxic capacity is maintained (Hazeldine and Lord, 2013, Le Garff-Tavernier et al., 2010, Nogusa et al., 2012). On the other hand, little is known about the phenotypic and functional capacity of NK cells in older children and teenagers.

The pathways through which NK cell differentiation is influenced by age are still unknown. In HCMV seronegative individuals receiving allogeneic haematopoietic stem cell transplant, the initial NK cell subsets that repopulate in circulation are the CD56bright or CD56dimCD94+ NK cells. CD57+ and KIR+ NK cells re-appear after around 12 months (Bjorkstrom et al., 2010, Della Chiesa et al., 2012). In contrast,

among HCMV seropositive donors who reactivate HCMV after transplantation, NKG2C+CD57+ NK cells can be identified within 3 months and the patient's pre-transplantation repertoire is restored within 6 months (Della Chiesa et al., 2013b). This indicates that HCMV infection is a substantial driver of NK cell differentiation (Della Chiesa et al., 2012, Foley et al., 2012b).

The commonest cause of congenital infection is human cytomegalovirus, leading to sensorineural hearing loss and intellectual impairment (Dollard et al., 2007, Griffiths and Lumley, 2014). Worldwide about 0.7% of neonates are born with congenital HCMV infection. From these 13.5% and 12.7% have symptoms at birth and after follow-up, respectively. The awareness of HCMV infection worldwide is generally low both in developed and developing countries (Cannon et al., 2012). Additionally, HCMV infection is a common complication of transplantation, mostly, in haematopoietic stem cell transplantation. This can occur through the reactivation in the transplant recipient or through primary infection (Azevedo et al., 2015).

The prevalence of HCMV infection may have severe consequences, especially, increasing the risk of acquiring additional infections, development of cancers or autoimmune diseases. Evidence indicates that NK cell phenotype and function could be altered by certain infections in people and that the extent of this may be determined by the occurrence of HCMV and other viruses. To better understand the ability of NK cells to adapt to HCMV and other infections across the lifespan and the consequences for NK cell functional differentiation, we performed detailed characterisation of NK cell phenotype and function in Gambian children and adults known to have a high burden of HCMV and other infections.

3.3 METHODS & MATERIALS

3.3.1 Study subjects

Study approvals were obtained from the ethics committees of The Gambia Government/Joint MRC Unit The Gambia and London School of Hygiene and Tropical Medicine. The first part of this chapter details a study of NK and T cell functional phenotype in relation to herpes infection and NKG2C genotype in 191 study participants aged 1-49 years, recruited from the villages of Keneba, Manduar, and Kantong Kunda. Subsequently, NKG2C genotyping studies were extended to incorporate 1485 individuals extending across the age range (aged 1-88 years) recruited from the entire West Kiang District, in Lower River Region, The Gambia, including the above mentioned villages.

Informed consent was obtained from all study participants, including parental/guardian consent from those under the age of 17 years. Anyone with pregnancy, or having an acute or chronic infection/ disease, or known to be HIV seropositive, was excluded from the study. Study participants' plasma was assayed for IgG against HCMV (BioKit, Spain), tetanus toxoid (HOLZEL Diagnostika, Cologne, Germany), Hepatitis B surface antigen (DiaSorin, Vercelli, Italy) and Epstein Barr Virus nuclear antigen (Euroimmun, Germany). Subject characteristics are shown in Table 3.1.

3.3.2 Hepatitis B surface Antigen ELISA

Briefly, direct competitive sandwich assay technique was used to determine the amount of HBsAg specific antibodies in this cohort (DiaSorin S.p.A., Vercelli, Italy), the plate wells were pre-coated with human Hepatitis B surface Antigens (HBsAg), then the diluted samples and calibrators having anti-HBs antibodies were added and incubated for 2 hours at 37ºC then washed using a wash buffer. The enzyme tracer made of anti-human HBsAg conjugated to horseradish peroxidase (HRP) was added and incubated for 1 hour at 37ºC. This enzyme allowed binding of HBs specific antibodies still present in the well after the wash. Subsequently, the wells are washed and the chromogen/substrate was added for 30 minutes at room temperature, then a blocking reagent was added to stop the reaction and the plate read using 450nm wavelength on the ELISA plate reader. The calibrator OD values were used to prepare a standard curve which was plotted against known IU/L concentration (DiaSorin S.p.A., Vercelli, Italy).

3.3.3 Tetanus toxoid ELISA

The HOLZEL Tetanus toxoid IgG ELISA kit was used to determine the amount of Tetanus-specific IgG in our samples (HOLZEL diagnostika, Cologne, Germany). Tetanus antigens were pre-coated on the plate surface of the wells. Then, the standards and diluted plasma samples were added into the wells to allow binding between the Tetanus-specific IgG antibodies in the plasma/ standards and Tetanus toxoid antigens on the plate. The plates were incubated for an hour at room temperature, then washed with 1X wash solution, in order to remove unbound antibodies. Subsequently, anti-human IgG peroxidase conjugate was added and incubated for 30 minutes. After washing as above, the TMB (3,3′,5,5′-Tetramethylbenzidine) substrate solution was added and incubated for 20 minutes, allowing colour development in the wells. The reaction was stopped by adding a stop solution. The resulting OD values were measured at 450 nm wavelength. The known standard concentrations in IU/ml were plotted against the standard OD values to determine the unknown sample concentrations.

3.3.4 Peripheral blood mononuclear cells (PBMC) preparation and culture

PBMC were isolated by density gradient centrifugation (Histopaque, Sigma, UK), as described in Chapter 2. We analysed cells both ex vivo and after 18 hours cell culture with cytokines [5ng/ml rhIL-12 (Peprotec, London, UK) plus 50ng/ml rhIL-18 (R&D systems, Oxford, UK)] or K562 cells (an E:T ratio of 2:1) in 10% pooled AB serum (Sigma-Aldrich, USA). Anti-CD107a FITC-conjugated (BD Biosciences) was added throughout the PBMC culture. Brefeldin A and Monensin (BD Biosciences) were added after 15 hours.

3.3.5 Flow cytometry

PBMC were incubated with combinations of the following monoclonal antibodies: Panel 1: anti-CD3-V500, anti-CD94-FITC, anti-NKG2A-APC, anti-CD8-PeCy7, anti-CD57-e450, and anti-CD16-APC-e780 (All Ebiosciences), anti-NKG2C-PE (R&D Systems), and anti-CD56-PeCy7 (BD Biosciences). Panel 2: anti-CD3-V500, anti-CD4-PE, anti-CD45RA-APC-H7, anti-CD8-PeCy7, anti-CD27-FITC, anti-CD28-PeCy7, and anti-CCR7-APC (All Ebiosciences), and anti-CD56-PeCy7 (BD

Biosciences). Panel 3: anti-CD45-FITC, anti-CD11c-PE, anti-CD19-PeCy5, anti-CD123-efluor450, and anti-CD14-APCe780 (All Ebiosciences). Panel 4: anti-CD3-V500, anti-CD57-e450, anti-CD25PE, and anti-IFN-γ-APC-efluor780 (All Ebiosciences), anti-CD56-PeCy7, and anti-CD107a-FITC (both BD Biosciences). PBMC were acquired on an LSRII® flow cytometer using FacsDiva® software. And analysis was performed using FlowJo® (TreeStar).

3.3.6 NKG2C genotyping

Whole blood (200ul) was used for DNA extraction (Wizard genomic DNA extraction kit, Promega, UK) and NKG2C genotype was assayed by PCR using Phusion® High Fidelity PCR kits as described in Chapter 2 (New England Biolabs, UK) (Miyashita et al., 2004).

3.3.7 Statistical analysis

Statview and Stata version 13.1 and GraphPad Prism version 6 were used for statistical analysis. Cubic splines in linear regression models; p-values (F-test) and R-squared values were obtained from these models, using a non-linear effects of age. ANOVA was used to assess responses of individuals of different genotype. Wilcoxon signed rank test was used to compare differences between NK cell subsets.

3.4 RESULTS

3.4.1 High rates of HCMV and EBV infection in the study population

HCMV infection rates are high in Africa and therefore, as expected, only 4 of the 191 individuals were HCMV seronegative; seronegative individuals were aged between 1 and 3 years, suggesting HCMV infection occurs within the first 3 years of life within this entire population, with a prevalence of 97.9% (Table 3.1). Interestingly, anti-HCMV antibody titres were significantly higher in children under 10 years old than in older individuals suggesting that optimal control of HCMV infection takes several years to develop (Table 3.1). Epstein Barr virus (EBV) infection was also common, with 75% of the entire cohort being sero-positive for EBV nuclear antigen (EBNA). EBNA sero-positivity rates were lowest in children ≤ 2 years old and anti-EBNA titres

tended to be higher in older children and adolescents aged under 15 years than in older individuals (Table 3.1).

Table 3.1: Cohort characteristics.

Age group, years	n (male/female)	HCMV IgG1, n (%)	HCMV IgG titer, IU/mL, median (range)	EBV nuclear antigen IgG1, n (%)	EBV nuclear antigen IgG titer, IU/mL, median (range)	NKG2C genotype, n (%)*		
						+/+	+/-	-/-
1-2	23 (9/14)	20 (86.9)	487.5 (81.8-845.2)†	12 (52.2)	107.0 (48.5-178.6)	11 (47.8)	10 (43.4)	2 (8.7)
3-5	19 (6/13)	18 (94.7)	288.4 (80.9-1681.8)	13 (68.4)	134.0‡ (32.5-328.7)	7 (37.8)	10 (52.6)	2 (10.5)
6-9	18 (11/7)	18 (100)	361.1 (89.2-2200.2){	16 (88.9)	103.6 (33.1-219.7)	8 (47.0)	7 (41.2)	2 (11.8)
10-12	20 (10/10)	20 (100)	215.4 (43.4-1693.6)	18 (90.0)	119.3§ (37.2-359.5)	8 (44.4)	8 (44.4)	2 (11.1)
13-15	23 (10/13)	23 (100)	252.6 (51.5 21057.9)	16 (70.0)	114.6 (29.7-193.4)	11 (47.8)	10 (43.4)	2 (8.7)
16-19	23 (11/12)	23 (100)	177.6 (61.2-678.1)	18 (78.2)	99.9 (23.9-195.2)	10 (47.6)	8 (38.1)	3 (14.3)
20-25	22 (11/11)	22 (100)	252.5 (81.5-828.4)	19 (86.4)	93.9 (27.6-171.7)	11 (52.4)	8 (38.1)	2 (9.5)
26-39	22 (13/9)	22 (100)	165.9 (39.0-968.4)	19 (86.4)	88.8 (24.9-272.7)	14 (73.7)	3 (15.7)	2 (10.5)
40-49	21 (10/11)	21 (100)	191.2 (53.5-735.2)	13 (61.9)	73.4 (24.0-183.2)	14 (70.0)	4 (20.0)	2 (10.0)
Total	191 (91/100)	187 (97.9)	252.6 (39-2200.1)	145 (75.9)	101.8 (23.9-359.5)	94 (51.9)	68 (37.6)	19 (10.5)

***NKG2C genotypes were obtained from a total of 181 individuals.**

†Significantly higher anti-HCMV IgG titers compared with 16- to 19-year-olds and all groups older than 26 years; p<0.05, analysis of variance.

‡Significantly elevated anti-EBV nuclear antigen IgG titers compared with all groups older than 16 years.

{Significantly higher anti-HCMV IgG titers compared with all groups older than 16 years; p<0.01, analysis of variance.

§Significantly elevated anti-EBV nuclear antigen IgG titers compared with all groups older than 20 years.

3.4.2 Redistribution of NK cell numbers and frequencies with age

CD56bright NK cells are the least differentiated subset of NK cell in human peripheral blood. They are the only NK cells to express the stem cell factor c-kit and constitutively express a number of cytokine receptors. In contrast CD56dim cells are phenotypically and functionally more differentiated. NK cell numbers and frequencies, and the distribution of CD56bright and CD56dim subsets, were therefore analysed by age group (Figure 3.1). The gating strategy for NK cells and CD56bright and dim subsets is shown in Figure 3.1A. Consistent with previous reports which cover American and European subjects across the life-span (Almeida-Oliveira et al., 2011, Hayhoe et al., 2010, Le Garff-Tavernier et al., 2010, Lutz et al., 2005, Sundstrom et al., 2007), the proportion of NK cells among peripheral blood lymphocytes increased significantly with age and was maximal around 15 years of age (Figure 3.1B). Within the total NK cell population, the proportion of CD56bright NK cells reduced rapidly and significantly with increased age (Figure 3.1C) and the frequency of CD56dim cells increased (Figure 3.1D), the overall NK cell subset distribution being stable from about 10 years of age and above (Figure 3.2).The absolute number of peripheral blood CD56bright and CD56dim NK cells decreased with age, and therefore the increased frequency of CD56dim cells in older individuals did not compensate for the overall reduction in total NK cell numbers (Figure 3.2, Table 3.2). Two distinct phases of phenotypic NK cell differentiation occur with a transition at around 10 years of age (Figure 3.2).

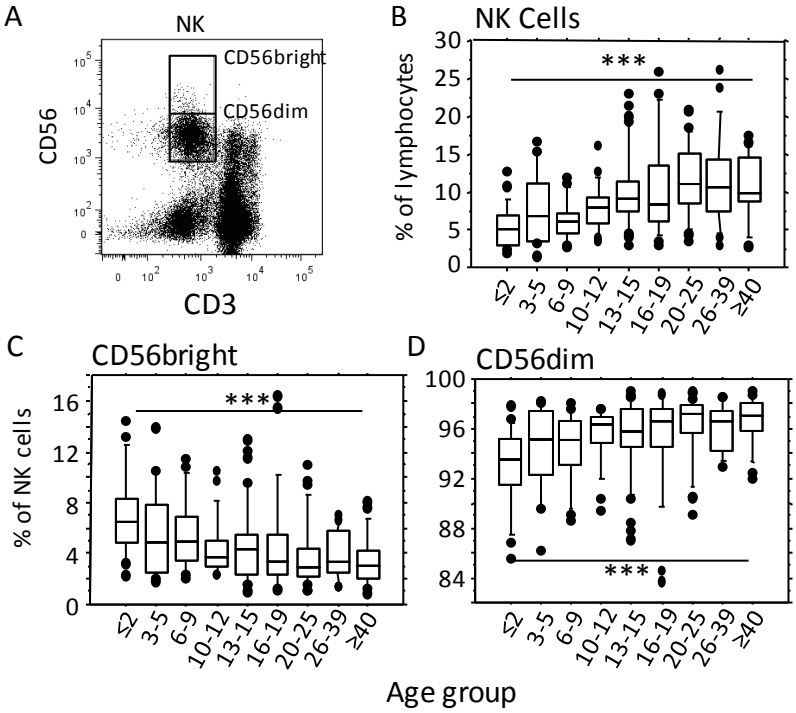

Figure 3.1: Age-related changes in NK cell frequencies.

(A) Natural killer cells were identified within PBMC after gating on singlets and viable lymphocytes. CD56+CD3- NK cells were then subsequently gated into CD56bright and CD56dim subsets. Frequencies of (B) all NK cells, (C) CD56bright, and (D) CD56dim NK cells, are shown for each age group. Representative dot plot from a 20 year old male donor. Horizontal bars represent median values, boxes extend from the 25th to the 75th percentile and whiskers represent the 95th percentiles. Asterisks represent significant trends across the entire cohort (*** $p<0.001$, F-test).

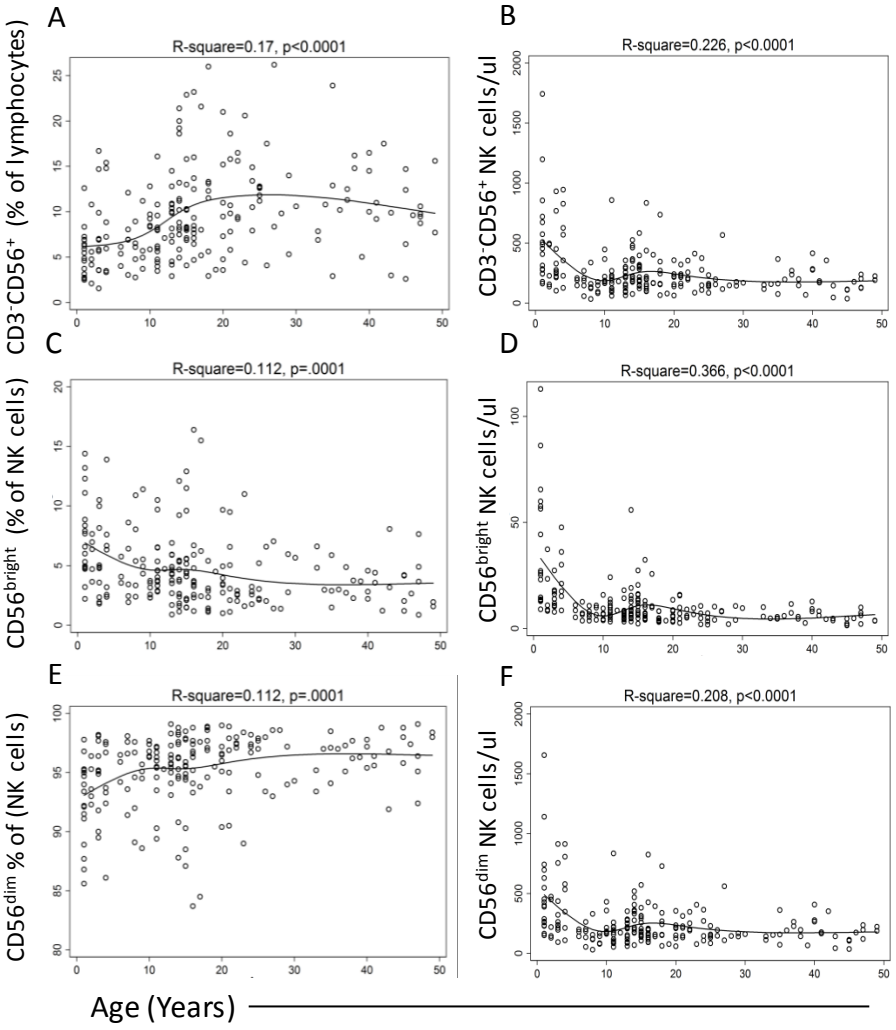

Figure 3.2: Cubic spline models showing changes in NK cell and NK cell subset distribution with age across the entire cohort.

Linear regression models were fitted as a natural cubic spline to the data in which age was included. Figure 3.2 A, C and E, percentages of NK cells and CD56bright and CD56dim subsets corresponding to Figure 3.1 (and as gated in Figure 3.1A). Figure 3.2 B, D and F, absolute numbers of CD3-CD56+ and CD56bright and CD56dim subsets. R2 and p values (F-test) are shown.

Table 3.2: Changes in absolute numbers of NK cell subsets in different age groups.

[1] Cells/µl blood, median (range)

Age group	$CD3^-CD56^+$	$CD56^{br}$ $CD16^-$	$CD56^{dim}CD16^+$	$CD94^+$	$NKG2A^+$	$NKG2C^+$	$CD57^+$
≤2	359 (54-1587)[1]	17 (5-89)	343 (117-1614)	291 (46-1463)	217 (44-777)	40 (0-905)	97 (4-1226)
3-5	188 (80-839)	14 (4-41)	253 (84-924)	158 (41-752)	122 (51-487)	40 (0-351)	69 (19-588)
6-9	96 (24-669)	8 (3-15)	150 (32-327)	81 (14-245)	44 (13-286)	41 (0-239)	36 (5-495)
10-12	154 (40-656)	6.5 (1.7-24)	163 (51-818)	110 (24-571)	59 (22-235)	27 (0-271)	64 (14-393)
13-15	227 (65-584)	9 (3-45)	280 (90-759)	129 (54-403)	59 (20-274)	35 (0-344)	80 (20-349)
16-19	150 (28-774)	6 (3-22)	193 (50-924)	121 (17-671)	43 (20-327)	39 (0-343)	68 (8-529)
20-25	179 (53-364)	6 (2-18)	201 (28-374)	143 (23-323	52 (26-185)	35 (0-259)	89 (9-288)
26-39	110 (20-161)	6 (1-17)	177 (65-544)	89 (10-213)	44 (17-132)	12 (0-82)	60 (16-126)
40-49	115 (24-249)	5 (1-12)	168 (36-408)	99 (20-217)	43 (13-134)	36 (0-128)	73 (13-189)

Early and very marked changes in NK cell phenotype contrasted with more gradual changes in the differentiation phenotype of T cells (Figure 3.3). Consistent with previous studies (Czesnikiewicz-Guzik et al., 2008, Koch et al., 2008), there was a steady decline in the frequencies of naïve $CD4^+$and $CD8^+$T cell, with accompanying increases in the proportions of effector memory (TEM) and central memory T cells (TCM). However, in contrast to previous studies (Czesnikiewicz-Guzik et al., 2008, Koch et al., 2008), the proportion of terminally differentiated T effector memory cells expressing CD45RA (TEMRA) was already high in young children, particularly observed within CD8+ T cells and this did not vary significantly with increasing age. High frequencies of highly differentiated T cells are likely due to frequent exposure to pathogens in infancy in our study population (Table 3.3 and 3.4).

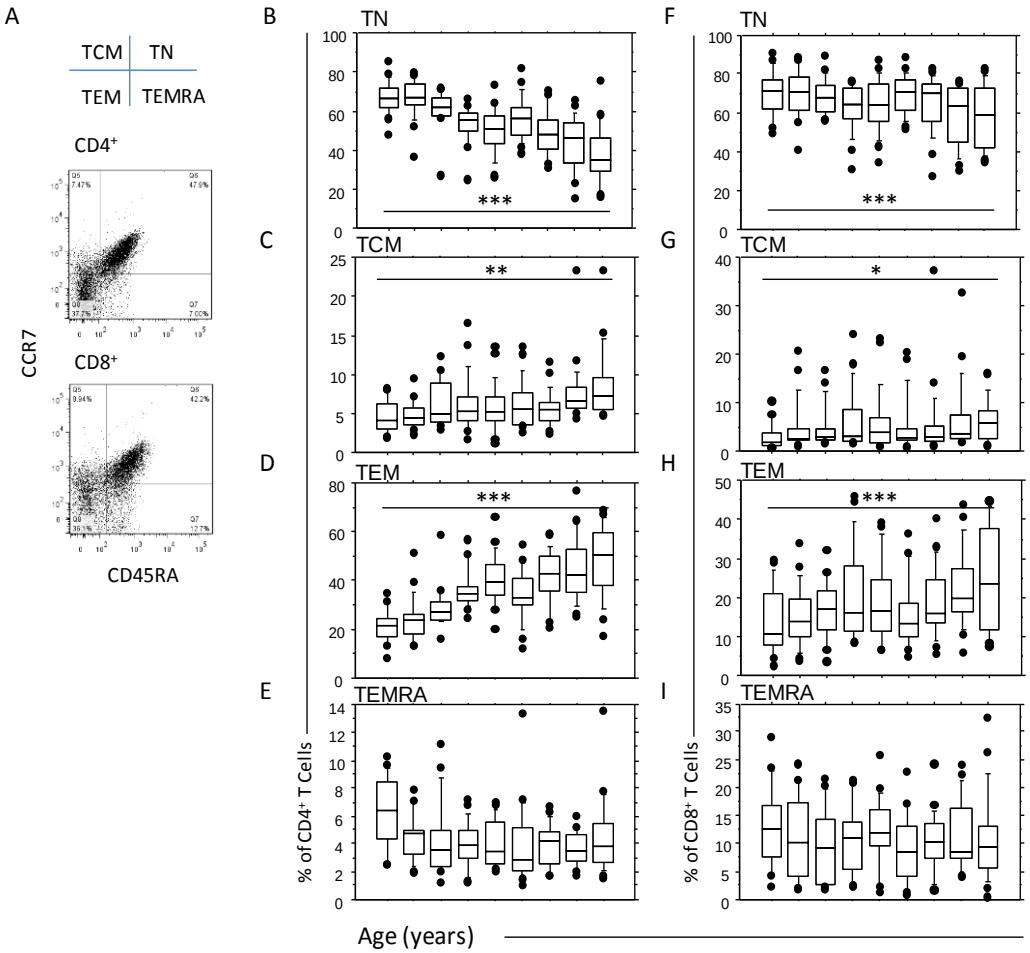

Figure 3.3: Changes in T cell maturation with age.

(A) T cell differentiation status was assessed after gating on CD4 or CD8 and subsequently on naïve (TN), central memory (TCM), effector memory (TEM) and terminally differentiated effector memory (TEMRA) using a combination of CD45RA and CCR7. Frequencies of naïve and memory subsets are shown in age stratified groups for $CD4^+$ (B-E) and $CD8^+$ T cells (F-I). Age group stratification is defined on Figure 3.1. Representative dot plot from a 20 year old male donor. Horizontal bars represent median values, boxes extend from the 25th to the 75th percentile and whiskers represent the 95th percentiles. Asterisks represent significant trends across the entire cohort (* $p<0.05$, ** $p< 0.01$, *** $p<0.001$, F-test).

Table 3.3: Changes in $CD4^+$T cell memory and senescence with age.

[1] Cells/μl blood, median (range).

Age group	$CD3^+CD4^+$ T cells	$CD4^+$ Naive	$CD4^+$ TCM	$CD4^+$ TEM	$CD4^+$ TEMRA	$CD4^+$ $CD28^-$ $CD57^-$
≤2	3381 (1437-9315)[1]	1923 (963-7201)	197 (37-369)	682 (206-1673)	177 (78-535)	921 (343-3270)
3-5	2461 (1187-4508)	1646 (708-2692)	104 (50-426)	493 (296-2317)	88 (56-188)	497 (195-1871)
6-9	1056 (469-1727)	620 (283-1150)	58 (18-152)	263 (98-719)	32 (11-162)	285 (89-527)
10-12	1012 (583-2144)	564 (291-1181)	60 (17-194)	344 (162-1000)	43 (8-106)	230 (26-925)
13-15	1140 (748-2010)	622 (255-931)	58 (14-240)	441 (216-955)	45 (17-82)	356 (69-1154)
16-19	1064 (608-1638)	557 (235-1172)	52 (16-168)	379 (109-523)	29 (9-155)	290 (31-415)
20-25	946 (394-1695)	417 (186-827)	46 (20-173)	360 (90-693)	39 (8-73)	287 (109-591)
26-39	732 (425-1760)	322 (76-931)	49 (28-160)	302 (160-752)	25 (10-86)	241 (98-954)
40-49	702 (333-1478)	250 (55-881)	52 (17-196)	335 (113-718)	25 (9-81)	198 (83-528)

Table 3.4: Changes in $CD8^+$ T-cell memory and senescence with age. [1] Cells/μl blood, median (range).

Age group	$CD3^+CD8^+$ T cells	$CD8^+$ Naive	$CD8^+$ TCM	$CD8^+$ TEM	$CD8^+$ TEMRA	$CD8^+$ $CD28^-$ $CD57^-$
≤2	1730 (494-3917) [1]	1245 (270-2890)	30 (6-394)	389 (89-1135)	167 (31-938)	408 (146-1747)
3-5	977 (582-3659)	746 (353-2023)	24 (13-203)	217 (150-1881)	84 (25-498)	245 (86-1518)
6-9	474 (242-1085)	306 (176-640)	14 (7-183)	134 (41-338)	43 (5-127)	115 (25-449)
10-12	466 (230-1467)	311 (144-880)	18 (5-249)	169 (72-698)	61 (6-186)	124 (8-646)
13-15	553 (210-1969)	349 (132-1234)	23 (3-142)	231 (42-776)	74 (6-324)	172 (16-1164)
16-19	448 (176-806)	314 (110-594)	19 (3-118)	171 (29-260)	46 (4-123)	101 (8-230)
20-25	399 (102-1132)	259 (77-706)	16 (2-132)	170 (23-413)	44 (5-275)	112 (28-648)
26-39	313 (109-1192)	215 (41-705)	12 (4-66)	131 (76-519)	30 (5-211)	102 (34-664)
40-49	338 (118-575)	183 (52-365)	15 (5-60)	143 (47-373)	24 (1-137)	82 (29-326)

We have defined three distinct populations within CD56dim NK cells according to expression of the differentiation marker CD57: CD57-, CD57+ and those with intermediate CD57 expression (CD57int) (White et al., 2014) (Figure 3.4A). The frequency of CD57-CD56dim within NK cells declined significantly with age with parallel increases observed in the proportions of CD57+ NK cells (Figure 3.4B). The proportion of CD57int cells changed little across the age range, consistent with cells transitioning at a constant rate through this stage of differentiation (Figure 3.4B). Strikingly, and in contrast to the linear differentiation towards memory phenotype observed for T cells, this was a biphasic process, with the most marked changes in CD57-defined subsets evident in children up to 5 years of age, and change occurring by 10 years of age (Figure 3.5 A-C).

The frequency of NK cells expressing CD94, which normally combines as a heterodimer with either NKG2A and NKG2C at the cell surface, remained stable throughout life, suggesting that there may also be limited variation in proportions of NK cells expressing either NKG2A or NKG2C (Figure 3.4C-F, 3.5D). However, within

CD94+ NK cells, the frequency of NKG2A+ cells declined with increasing age (Figure 3.4F, p=0.03, ANOVA) with parallel increases in the proportions of NKG2C+ cells (Figure 3.4F), p=0.02, ANOVA). Increasing proportions of NKG2C+ NK cells did not result in an overall increase in NKG2C+ NK cell numbers due to the overall decline in absolute NK cell numbers Table 3.2.

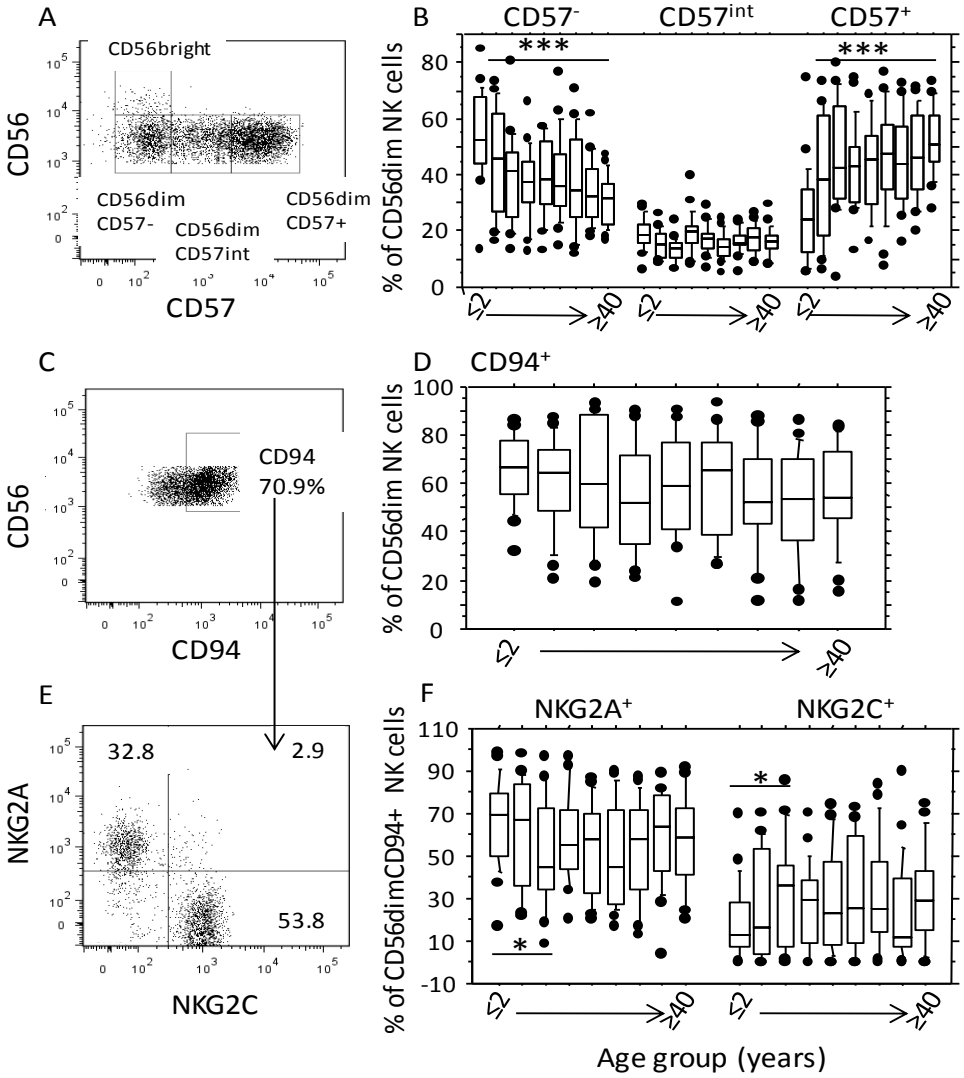

Figure 3.4: Age related changes in frequencies of CD57 and C-type lectin-like receptor expressing NK cell subsets.

(A) CD56dim cells were gated into CD57-, CD57intermediate (int) and CD57+ subsets. The CD57- population was gated using an isotype matched control reagent and the CD57+ gate was set at an MFI of 3000. (B) Frequency distribution by age group of $CD57^-$, CD57int and CD57+ subsets within the CD56dim NK cell population. Asterisks denote statistically significant trends for changes in NK cell subset frequency by age (*** $p<0.001$, F-test). (C, E) Gating strategy for CD94+NKG2A+ and CD94+NKG2C+ cells within the CD56dim NK cell subset. Frequencies of CD94+ (D), NKG2A+ and NKG2C+ (F) NK cells by age group. Asterisks denote statistically significant differences in frequencies of NKG2A+ and NKG2C+ cells by age group (* $p<0.05$, ANOVA). (A, C, E) representative dot plot from a 29 year old male donor. Horizontal bars represent median values, boxes extend from the 25th to the 75th percentile and whiskers represent the 95th percentiles. Age groups are as shown in Figure 3.1.

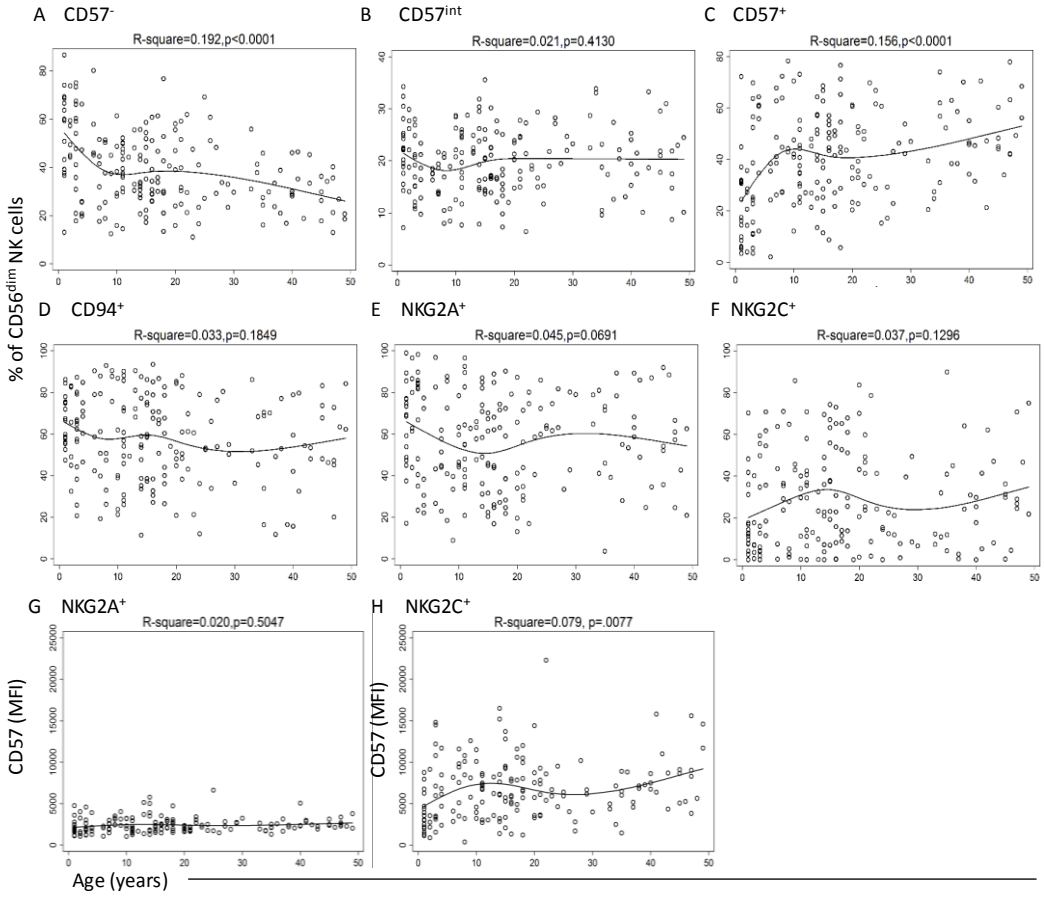

Figure 3.5: Cubic spline models showing changes in NK cell maturation and C-type lectin-like receptor defined NK cell subsets with age across the entire cohort.

(A-C) Models of changes in frequencies of CD57-defined subsets corresponding to data shown in Figure 3.4. (D-F) Changes in the frequencies of CD94, NKG2A and NKG2C expressing NK cells, (as gated in Figure 3.4C and E). G, H. Changes in MFI of CD57 on NKG2A$^+$ and NKG2C$^+$ NK cells (corresponding to data shown in Figure 3.4F). R^2 and p values (F-test) are shown.

3.4.3 NKG2C+ cells contribute to increases in highly differentiated CD57+ NK cells

The contribution of changes in the frequencies of NKG2A+ and NKG2C+ NK cells to increased proportions of CD57+ cells with age were then investigated (Figure 3.6).

Frequencies of CD57int and CD57+ NK cells within NKG2A+ cells increased significantly with increasing age, with a reciprocal decline in the frequencies of CD56dim CD57- cells (Figure 3.6A). However, the majority of NKG2A+ NK cells remained relatively undifferentiated (CD57-) even in the oldest study subjects (Figure 3.6A).

Conversely, NKG2C+NK cells contain a high frequency of CD57+ cells even in infants under 2 years old; the majority of NKG2C+ NK cells co-express CD57+ by 5 years of age (Figure 3.6B). The geometric mean fluorescence intensity (MFI) for CD57 expression was very low on NKG2A+ NK cells over the entire age span but the MFI of NKG2C expression increased significantly in NKG2C+ cells with increasing age (Figure 3.6C, 3.5G, 3.5H) consistent with pronounced and rapid differentiation of NKG2C+ NK cells in this cohort. Rapid expansion and differentiation within the NKG2C+ NK cell subset is most likely linked to acquisition of HCMV infection in utero or perinatally in this Gambian study population. Moreover, anti-HCMV IgG titre was weakly negatively associated with the proportion of CD57+ NK cells (Figure 3.7), consistent with an association between advanced NK cell differentiation and control of HCMV infection.

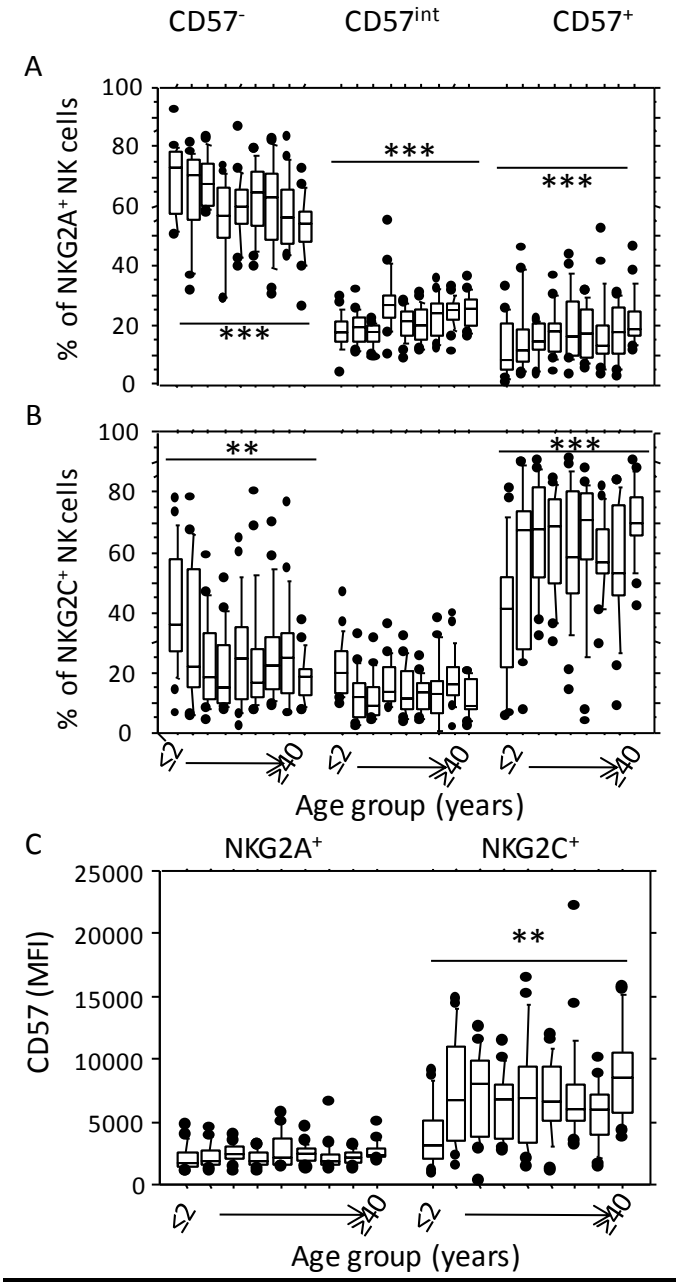

Figure 3.6: CD57 is preferentially expressed on NKG2C+ NK cells.

CD56dim NK cells were gated as shown in Figure 3.1A and the frequency of CD57-, CD57int and CD57+ cells was determined within (A) CD94/NKG2A+ or (B) CD94/NKG2C+NK cells, by age group. (C) Mean fluorescence intensity (MFI) for CD57 expression on NKG2A$^+$ and NKG2C$^+$ NK cells by age group. Horizontal bars represent median values, boxes extend from the 25th to the 75th percentile and whiskers represent the 95th percentiles. Asterisks denote statistically significant trends by age within each subset (**p<0.01, *** p<0.001, F-test). Age groups are as shown in Figure 3.1.

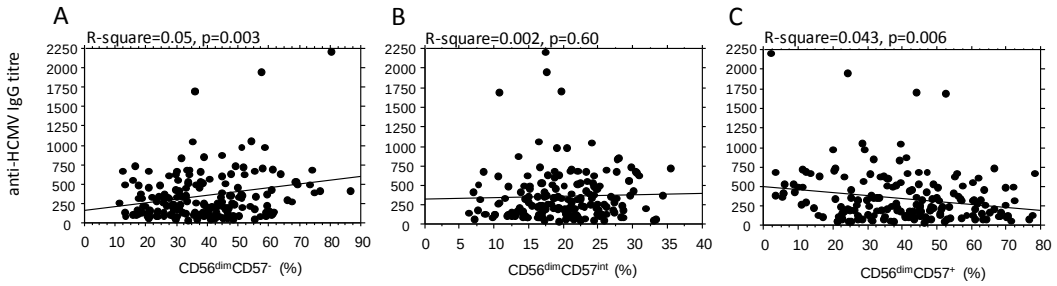

Figure 3.7: The frequency of mature CD57+ cells is inversely correlated with HCMV IgG titre.

A positive correlation is observed between HCMV IgG titre and the percentage of CD57- NK cells (A) and a negative correlation with the percentage of CD57+ NK cells (C) whereas no significant effect is observed for the CD57intermediate (int) subset (B). R^2 and p values (F-test) are shown.

Infection with Epstein Barr Virus (EBV) has been associated with altered NK cell phenotype in HCMV exposed Europeans (Saghafian-Hedengren et al., 2013). A minor increase in CD56dim cell frequencies was observed in EBNA IgG seropositive subjects (Figure 3.8C) whereas no impact of EBV status was observed on the overall distribution or MFI of other NK cell differentiation markers or subsets (Figure 3.8A, B, D-G). This is consistent with other studies showing no significant impact on NKG2C+CD57+ NK cells during acute EBV co-infection in HCMV infected subjects (Hendricks et al., 2014).

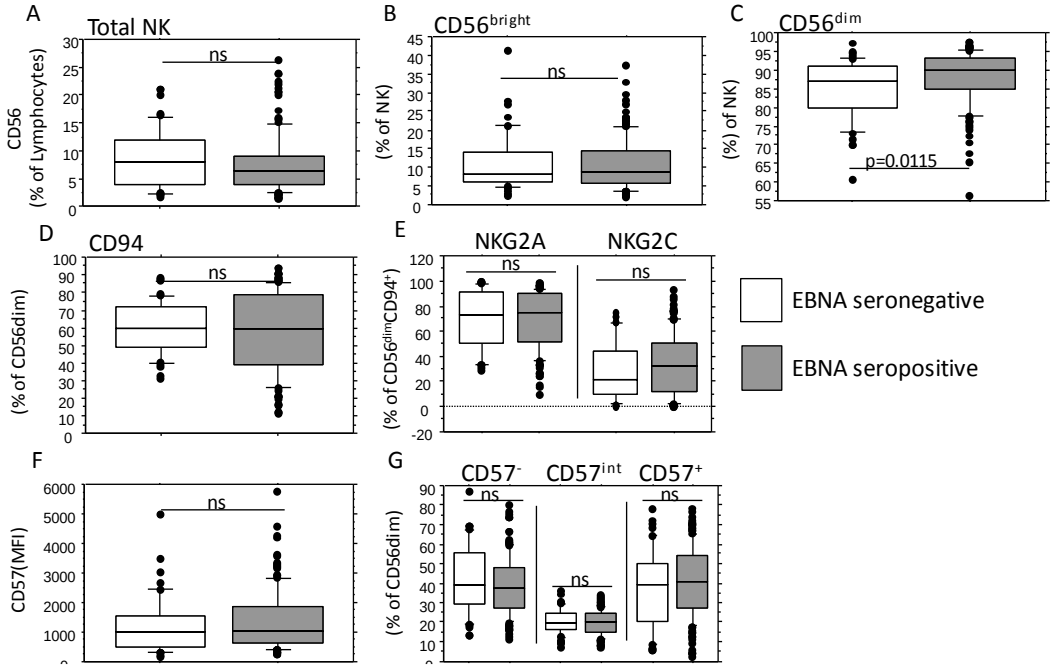

Figure 3.8: Limited impact of EBV serostatus on NK cell subset distribution.

The frequencies of total NK cells (A) CD56bright (B) CD56dim (C) CD94 (D) NKG2A (E) NKG2C (F) and the Mean Fluorescence Intensity (MFI) for CD57 (G) and frequencies of CD57 defined subsets (H) were compared in EBNA sero-negative and sero-positive individuals. *p<0.01.

3.4.4 Rapid functional maturation of NK cells during childhood in The Gambia

The consequences of these phenotypic changes were then investigated to determine whether the differentiation towards CD57+ NK cells influenced functional capacity. NK cell degranulation (CD107a), CD25 and IFN-γ expression were assessed by flow cytometry within PBMC cultured in vitro with K562 target cells, with high concentrations of cytokines (rIL-12 and rIL-18; HCC) or left unstimulated in tissue culture medium only (Figure 3.9). Spontaneous low level degranulation and IFN-γ production was observed amongst unstimulated cells from children below the age of 10 years, perhaps indicating ongoing NK cell activation in vivo in infants in our study population (Figure 3.9C, I). K562 cells consistently stimulated increased NK cell degranulation but this did not vary across the age range (Figure 3.9D). In contrast, degranulation and upregulation of CD25 and IFN-γ production in response to HCC (Figure 3.9E,H,K) were all strongly age-related, with significantly higher frequencies of responding cells being observed in children under the age of 10 years compared to older subjects, using cubic spline model (Figure 3.10).

We next investigated to what extent the redistribution of CD57-defined NK cell subsets with increasing age contributed to overall changes in NK cell function. Degranulation and CD25 and IFN-γ expression were therefore analysed across the age range within gated CD56bright, CD56dimCD57-, CD57int and CD57+ subsets. The spontaneous (medium alone) NK cell degranulation (CD107a expression) observed in children was mainly associated with activity within CD56dimCD57- cells (Figure 3.11A) whilst spontaneous CD25 and IFN-γ production was mainly observed within CD56bright cells (Figure 3.11B,C). CD107a and CD25 expression were observed in all NK cell subsets after incubation with K562 cells. Although these responses did not vary significantly with age, they were significantly higher in CD57- cells compared to CD57int and CD57+ cells (Figure 3.11D,E). Less mature NK cells express higher levels of the NKp30 activating receptor (a receptor for B7-H6 on K562 cells) which could explain the preferential responsiveness of CD56dimCD57- NK cells (Brandt et al., 2009, Fiegler et al., 2013). Unsurprisingly, little IFN-γ secretion from any NK cell subset was observed in response to K562 cells after 18 hours of stimulation (Figure 3.11F).

CD57- NK cells contained the highest frequencies of cells degranulating in response to cytokine stimulation (Figure 3.11G) and both cytokine-induced CD25 expression

and IFN-γ production declined with progressive NK cell differentiation, being highest in the CD56bright subset and lowest in the CD56dimCD57+ subset (Figure 3.11H, I). A tendency towards increasing CD107a and CD25 expression was observed with increasing age in CD57int and CD57+ NK cells after cytokine stimulation (Figure 3.11 G, H), which however only reached significance when infants less than 2 years old were compared with the oldest adults ($p<0.01$; ANOVA with correction for multiple comparisons).

Although small age-associated changes in NK cell function may occur within CD57-defined subsets, overall changes in NK cell function with age is most strongly affected by the redistribution of these subsets within NK cells.

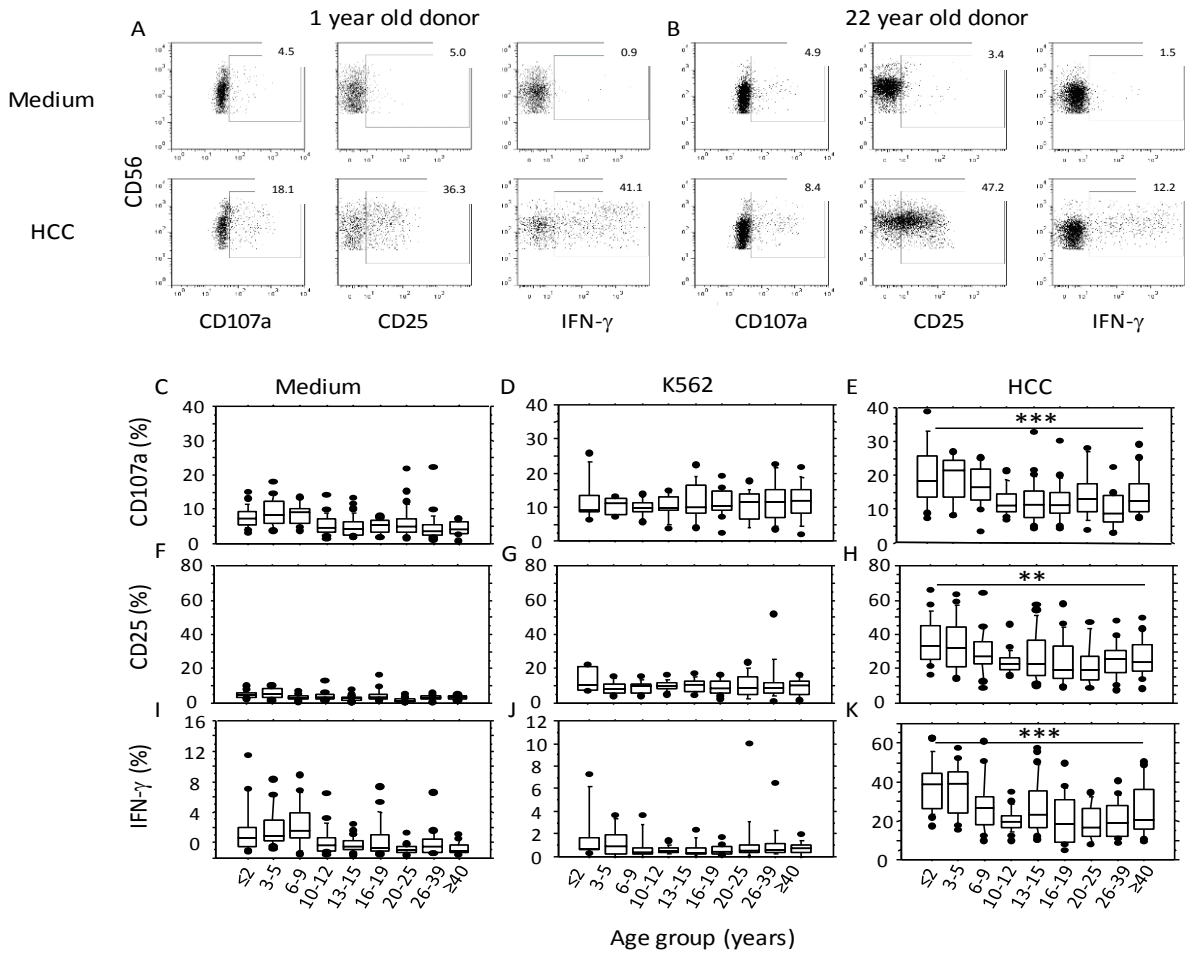

Figure 3.9: Age-associated changes in NK cell function.

Example flow cytometry plots are shown for CD3-CD56+ lymphocytes from a 1 year old (A) and a 22 year old (B), cultured in medium alone (upper panels) or stimulated with high concentrations of IL-12+ IL-18 (HCC, lower panels) and assayed for degranulation (CD107a), CD25 and IFN-γ expression. C-K: NK cells were assayed for degranulation (C-E), CD25 (F-H) or IFN-γ (I-K) expression after in vitro culture in medium alone (C, F, I) or with K562 target cells (D, G, J) or IL-12 + IL-18 (HCC; E, H, K). Horizontal bars represent median values, boxes extend from the 25th to the 75th percentile and whiskers represent the 95th percentiles. Asterisks denote significant age related trends for frequencies of NK cells expressing CD107a, CD25 or IFN-γ (p<0.01;*****

p<0.001, F-test).

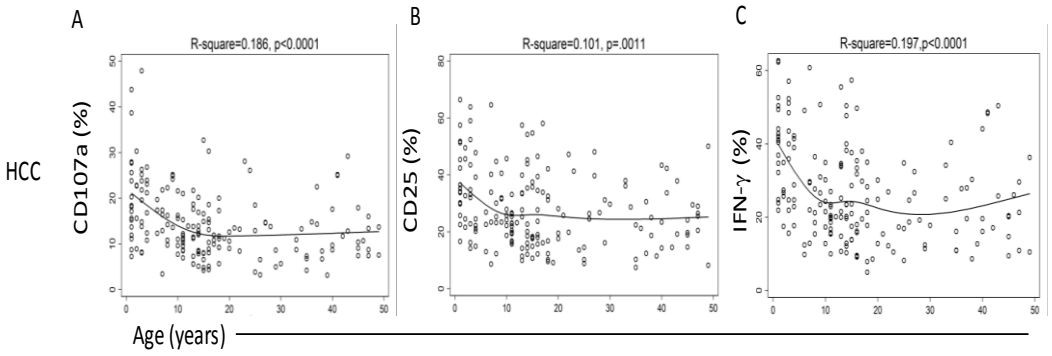

Figure 3.10: Cubic spline models showing changes in rIL-12 + rIL-18 induced NK cell function with age across the entire cohort.

A. CD107a B. CD25 and C. IFN-γ expression in gated $CD3^{-}CD56^{+}$ NK cells. (Models correspond to data shown in Figure 3.9E, H, K) R^2 and p values (F-test) are shown.

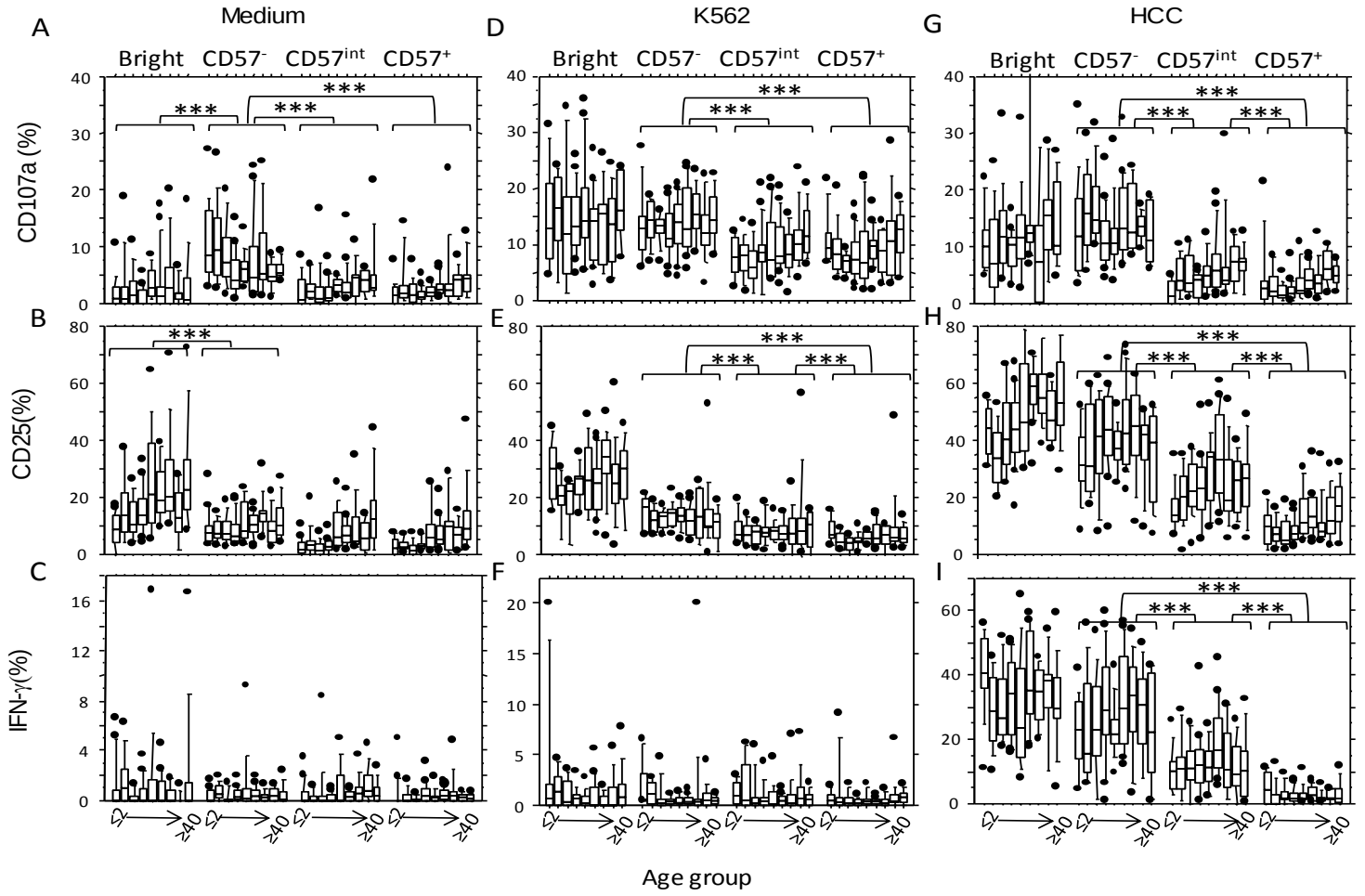

Figure 3.11: NK cell function reflects CD57 expression, irrespective of age.
Bright (CD56brightCD57$^-$), CD57- (CD56dimCD57$^-$),CD57int (CD56dimCD57int) and CD57+ (CD56dimCD57+) NK cell subsets were analysed for CD107a (A, D, G),CD25 (B, E, H) or IFN-γ (C, F, I) after in-vitro culture in medium alone (A-C), with K562 target cells (D-F) or with IL-12 + IL-18 (HCC; G-I).Horizontal bars represent median values, boxes extend from the 25th to the 75th percentile and whiskers represent the 95th percentiles. There were no significant age-related trends in response within any of the subsets. Asterisks denote statistically significant differences between CD57$^-$, CD57int and CD57+subsets. (p<0.001 for all comparisons,

Wilcoxon-signed rank).

3.4.5 Impact of NKG2C genotype on NK cell numbers and phenotype

Several studies in different populations have reported a gene deletion at the *NKG2C* locus which results in a loss of surface expression of the activating receptor NKG2C in NK cells from homozygous *NKG2C-/-* individuals (Miyashita et al., 2004, Muntasell et al., 2013, Rangel-Ramirez et al., 2014, Noyola et al., 2012). NKG2C genotype was assessed by a single tube PCR assay (Miyashita 2004). We genotyped 181 of the subjects in this study, as described in Chapter 2; a representative agarose gel is shown in Figure 3.12A. Nineteen of 181 individuals tested from this cohort (10.5%) were *NKG2C-/-* (and lacked surface expression of *NKG2C* gene) whereas 68 individuals were heterozygous (37.6%), resulting in a *NKG2C* allele frequency of 29.3%. *NKG2C-/-* individuals were distributed evenly across age groups and between the sexes (Figure 3.12B, Table 3.1).

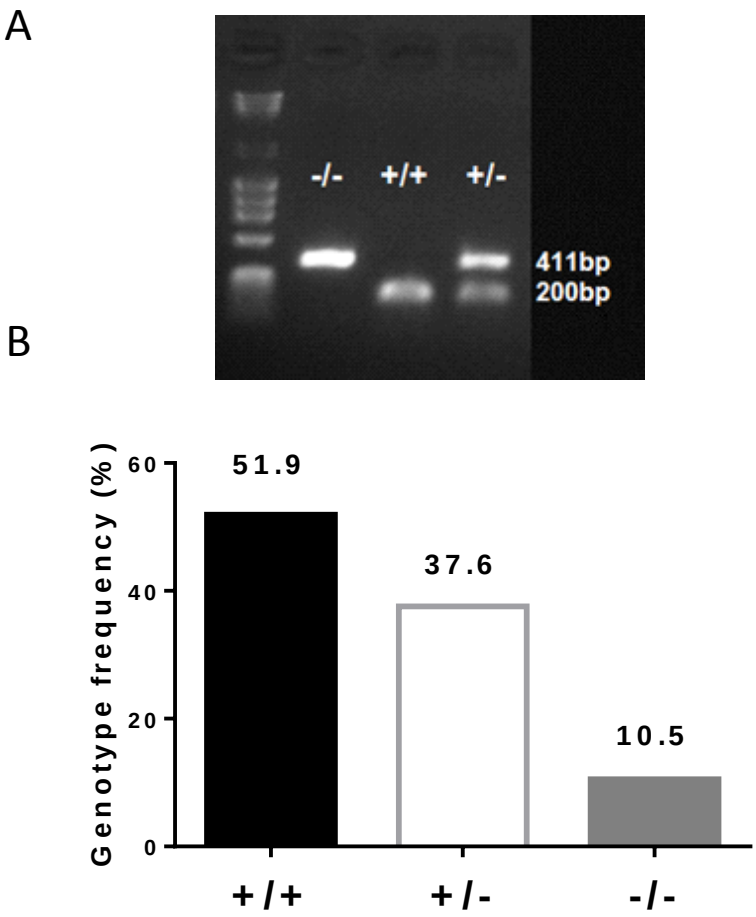

Figure 3.12: NKG2C genotype distribution in the cohort and representative example of an NKG2C genotyping gel.

(A) Representative example of NKG2C genotyping for 3 study subjects. Lane 1 = base pair ladder; Lane 2 = homozygous deletion for NKG2C; Lane 3 = homozygous wild type; Lane 4 = heterozygote. The *NKG2C* gene deletion gives a band at 411 bp (base pairs) and the wild type genotype gives a band at 200bp. Heterozygotes have both the 200bp and 411bp bands. (B) Percentage NKG2C genotype frequency. The dark bar represents homozygous NKG2C, open bar depicts heterozygous individuals, whilst the grey bar represents *NKG2C* gene deletion. Data presented is derived from 181 individuals.

Consistent with published data, *NKG2C* genotype did not affect the overall proportions of total NK cells and CD56bright or CD56dim subsets (Noyola et al., 2012), although *NKG2C-/-* children below 10 years of age had lower absolute numbers of NK cells compared to children heterozygous for the deletion (Figure 3.13D). Strikingly, *NKG2C-/-* individuals had significantly lower frequencies of CD56dimCD94+ NK cells compared to *NKG2C+/-* and *NKG2C+/+* individuals (Figure 3.14A). Absolute numbers of NKG2A+ cells were unaffected by genotype (Figure 3.15B), whereas consistent with the observed effects on the frequencies of these cells, the absolute numbers of CD94+ cells were significantly reduced in *NKG2C-/-* individuals (Figure 3.15A).

The ratios of CD94+NKG2A+ to CD94+NKG2C+ cells are therefore likely to be determined by the expansion of CD94+NKG2C+ cells rather than a switch from NKG2A+ cells to NKG2C+ NK cells. A significant gene dosage effect was observed on the frequencies of NK cells with NKG2C surface expression with individuals heterozygous for *NKG2C* having display both intermediate frequencies (Figure 3.14B) and absolute numbers (Figure 3.15A,D) of CD94+NKG2A+ and CD94+NKG2C+ cells. The MFI for NKG2C expression was partially reduced in *NKG2C+/-* compared to *NKG2C+/+* individuals within the entire cohort, (Figure 3.16A) and after splitting the cohort by two age groups (Figure 3.16B) although this did not reach statistical significance in either case. Significantly lower frequencies of CD57+ NK cells were observed in *NKG2C-/-* children (under 10 years of age) compared to both heterozygous and homozygous *NKG2C+* children, a reciprocal increase being observed in the proportions of CD57- and CD57int NK cells (Figure 3.14C). No impact of NKG2C genotype was observed on the frequencies of NK cells expressing CD57 in individuals over 10 years old, constituent with maximal differentiation being achieved by this age.

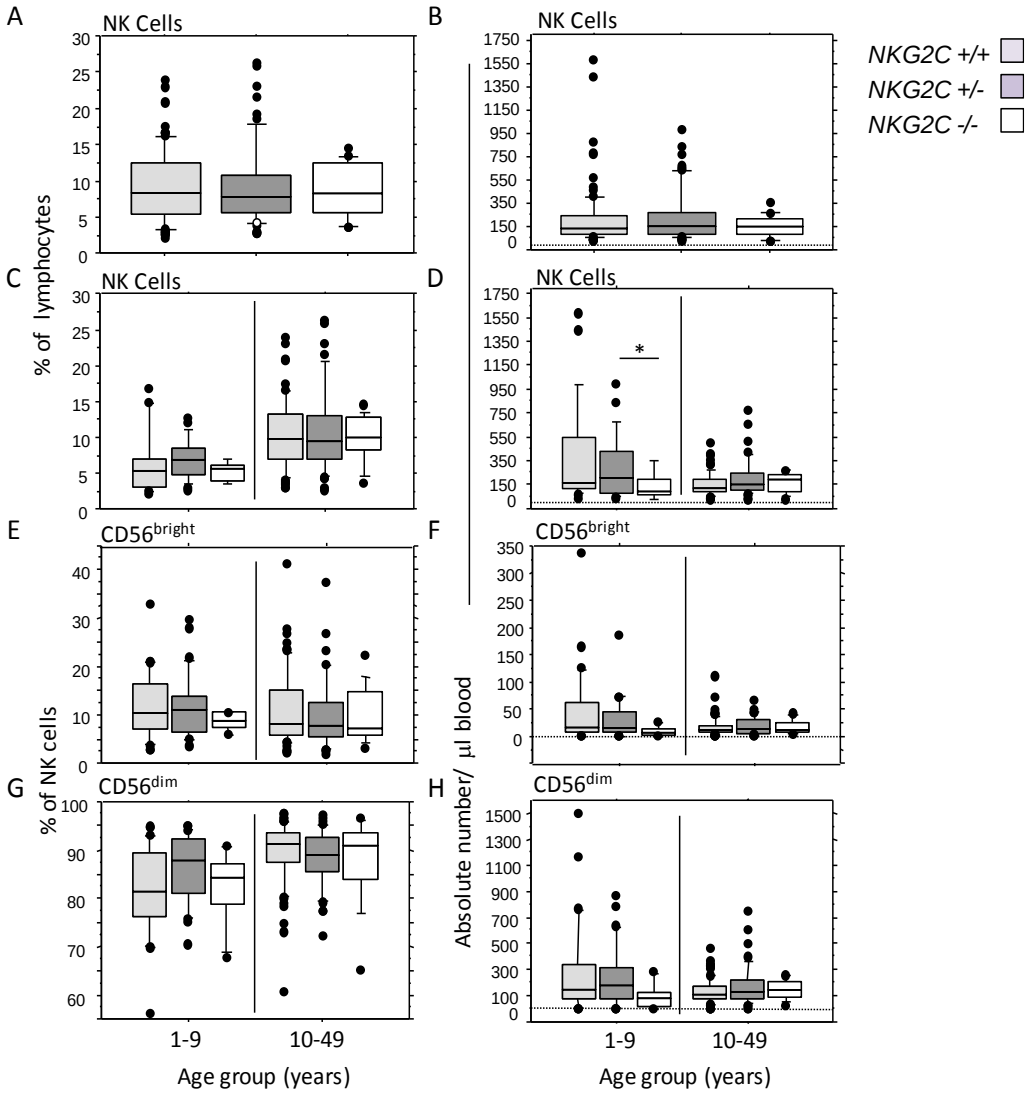

Figure 3.13: NKG2C genotype has limited effects on frequencies and absolute numbers of NK, CD56bright and CD56dim cells in children.

Frequencies and absolute numbers of NK cells across the entire cohort (A, B) and (C, D) in groups stratified according to age (<10 and 10-49 years old). Frequencies (E,G) and (F,H) absolute numbers of CD56bright and CD56dim NK cells.

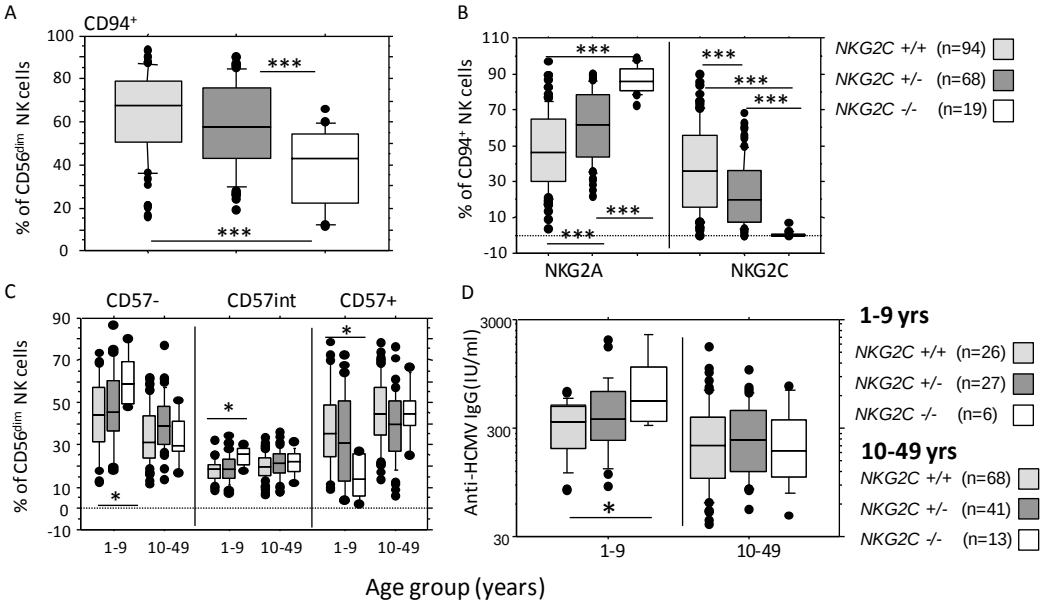

Figure 3.14**: Effect of NKG2C genotype on NK cell maturation phenotype and HCMV antibody titre.**

(A) Frequency of CD94+ cells within the CD56dim NK cell population in individuals with zero (*NKG2C-/-*), one (*NKG2C+/-*) or two (*NKG2C+/+*) copies of the NKG2C gene. (B) Impact of NKG2C genotype on the frequencies of CD94+ NK cells expressing either NKG2A+(NKG2C-) or NKG2C+(NKG2A-) cells. (C) Impact of NKG2C genotype on the frequency of CD57-, CD57int and CD57+ NK cells in subjects<10 and ≥ 10 years of age. (D) Anti-HCMV antibody titres by age (years) and NKG2C genotype. Horizontal bars represent median values, boxes extend from the 25th to the 75th percentile and whiskers represent the 95th percentiles. Asterisks denote statistically significant differences between genotypes for all comparisons shown ($p<0.05$, *** $p< 0.001$, ANOVA).

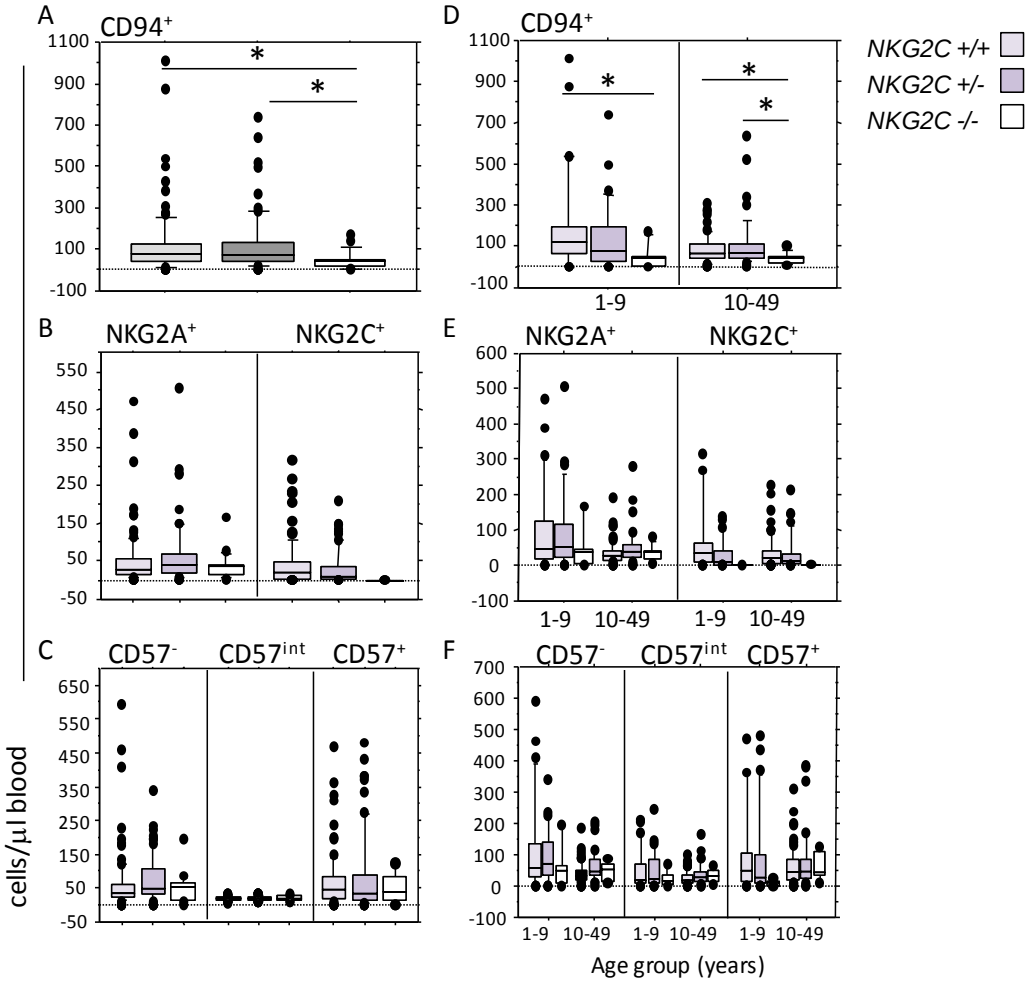

Figure 3.15: Impact of NKG2C genotype on absolute numbers of NK cell subsets.

(A) CD94+ NK cells, (B) NKG2A+ or NKG2C+ NK cells and (C) CD57 defined NK cell subsets are shown for all study subjects. (D-F) Effect of NKG2C genotype on study subjects grouped according to ages <10 or 10-49 years old; (D) CD94+ NK cells, (E) NKG2A+ or NKG2C+ NK cells or (F) CD57-defined subsets. * $p<0.05$, ANOVA.

We then hypothesised that because NKG2C is directly involved in responses to HCMV, individuals lacking expression of NKG2C may have less effective control of HCMV infection. Lack of recognition of HCMV-infected cells would also be consistent with the NK cells of NKG2C-/- children being overall less differentiated than cells of wild type or heterozygous children (as shown above in Figure 3.14C). To investigate whether *NKG2C* genotype affects control of HCMV, we examined the relationship between age, genotype and anti-HCMV antibody titres (Figure 3.14D). In this case we assumed that high anti-HCMV IgG titres reflected poorer control of HCMV infection.

Anti-HCMV antibody titres were significantly higher in NKG2C-/- compared to NKG2C+/+ children (under 10 years of age) (Figure 3.14D) consistent with more limited control of HCMV infections leading to more frequent virus reactivations which result in boosting of antibody responses in these children. No relationship was observed between NKG2C genotype and anti-HCMV IgG titre in older individuals. Furthermore, the observed effect appeared to be HCMV specific as no effect of *NKG2C* genotype was observed in children on the titres of antibodies to infant tetanus toxoid, hepatitis surface antigen and EBV vaccine antigens (Figure 3.17A, B C).

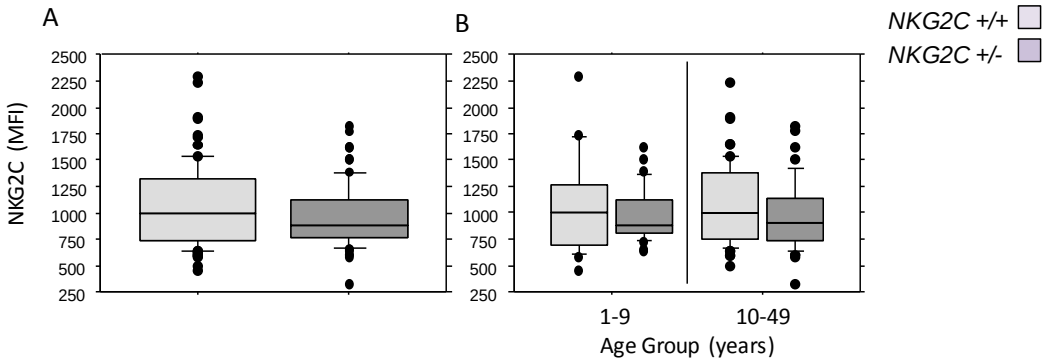

Figure 3.16**: Expression levels of NKG2C in *NKG2C*+/- and +/+ individuals.**

The mean fluorescence intensity (MFI) is shown for NKG2C in (A) all +/+ and +/- study subjects and (B) in individuals grouped into age strata (<10 and 10-49 years old).

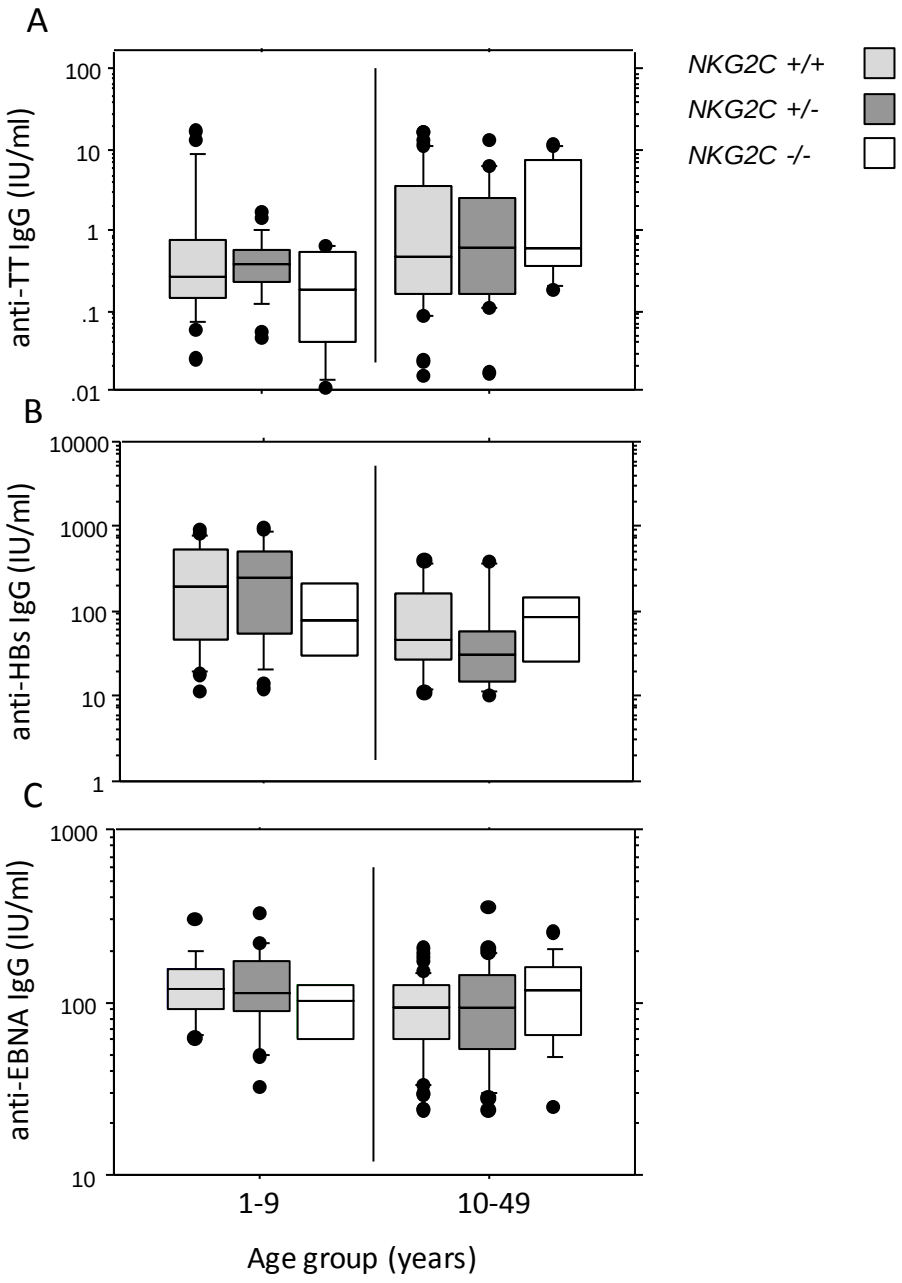

Figure 3.17: NKG2C genotype does not affect antibody titres to vaccine antigens.

No significant impact of genotype is observed on titres of antibody against Tetanus Toxoid (anti-TT IgG, IU/ml) (A) or Hepatitis B surface antigen (anti-HBsAg, IU/ml) (B) and Epstein-Barr Virus nuclear antigen (anti-EBNA IgG, IU/ml) (C) in children (<10 years old) or in adults (10-49 years old).

3.4.6 Comparison of NK cell phenotype and functionality between matched Gambian and UK donors

The burden of HCMV in our Gambian cohort as shown in Table 3.1 was 97.9% while in our UK cohort it was 36% (Goodier et al., 2016). As we have observed this variation in HCMV exposure, we wanted to compare NK cell phenotype and function between these two populations with very different HCMV exposure. The data was reanalysed and compared with age-matched UK donors between the ages of 20-49 years.

Although HCMV+ UK subjects had lower proportions of NKG2A+ NK cells than HCMV- UK subjects, the Gambian subjects had a significantly higher proportions of NK cells expressing both NKG2A and NKG2C receptors when compared to either group of UK subjects (Figure 3.18A, B). The HCMV- UK donors had the lowest proportions of NKG2C+ NK cells (Figure 3.18B). Similarly, Gambian subjects had higher proportions of NK cells expressing the late differentiation marker CD57+ NK cells compared to both HCMV+ and HCMV- UK donors (Figure 3.18C). Functionally, the NK cells of Gambian subjects were less likely to express CD25 or IFN-γ after stimulation with cytokines than either UK set of subjects (Figure 3.18 E, F). However, there was no significant difference in NK cell degranulation (CD107a) between the three groups (Figure 3.19D).

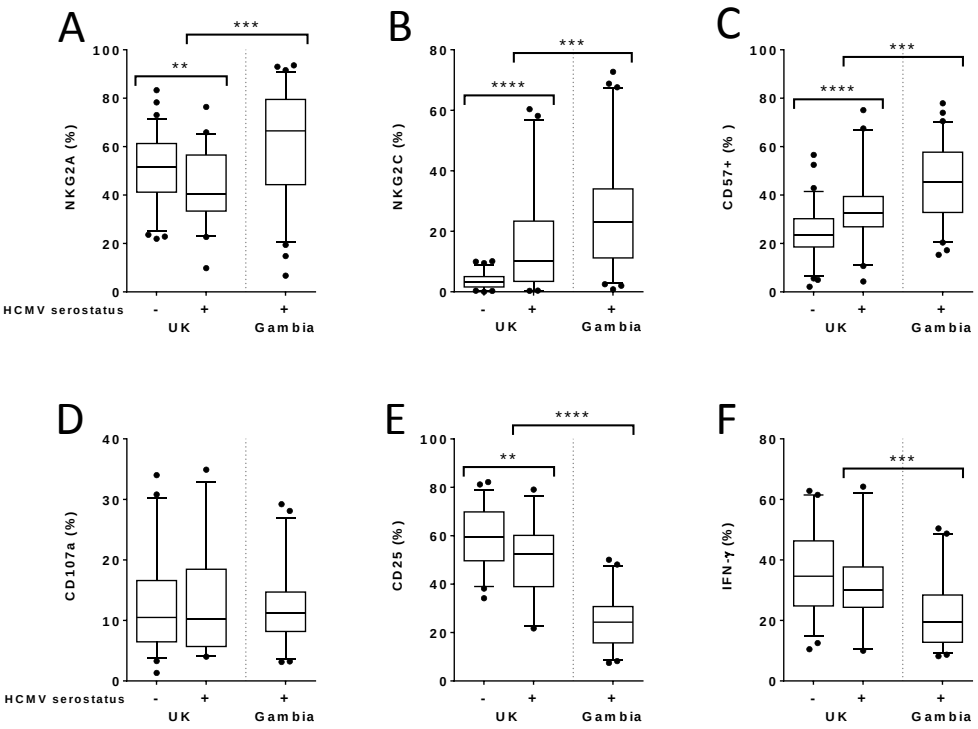

Figure 3.18: Elevated frequencies of C-type lectin-like receptor positive NK cell subsets and reduced NK cell functional capacity in Gambian compared to UK adults.

The frequencies of CD57+ (A), NKG2A+ (B) and NKG2C+ (C) within CD56dim NK cells are compared between Gambian adults (n=65) and HCMV seronegative (n=78) and HCMV seropositive UK adults (n=43) aged between 20 and 49 years. Frequencies of cells expressing CD107a (D), CD25 (E) and IFN-γ (F) were determined within total NK cells. PBMC (D, E, F) were cultured in high concentrations of cytokines alone (rIL-12 5ng/ml + rIL-18 50ng/ml).Boxes represent median values and boxes extend from the 25th to the 75th percentile and whiskers represent the 95th percentiles. Asterisks denote statistically significant ** p<0.01, *** p<0.001, p<0.0001, Mann-Whitney U test.

3.4.7 Extension of NKG2C genotyping study to a larger cohort from the West Kiang region of The Gambia

The genotyping studies described above in the context of our initial studies on NK differentiation and HCMV infection were extended to a larger sample size in order to obtain a more precise estimate of the allele frequencies in The Gambia and to extend the analysis to a wider age range, incorporating samples from adults aged 50-88 years old. In addition, ongoing studies, which have not yet been completed and are therefore not presented in this thesis, aim to further establish the relationship between anti-HCMV IgG titre and NKG2C genotype in this extended cohort. To further investigate the frequency of NKG2C within our study population, 1825 DNA samples were processed for NKG2C genotyping using the Phusion® kit as described in Chapter 2. We excluded 340 of these samples from the analysis for inconclusive results and/or duplication. After filtering out these, 1485 samples had appropriate NKG2C genotype data used for analysis. Of these, sex was known for only 1479 subjects. There were 870 females and 609 males within this cohort with the age range of 1 to 88 years (Table 3.5).

Table 3.5: Characteristics of the extended cohort.

This table shows the distribution of sex and age within our cohort.

Age Group	Male	Female	Total
1-10	415	345	760
11-20	140	165	305
21-30	5	93	98
31-40	2	126	128
41-50	9	66	75
51-60	11	38	49
61-70	18	25	43
71-80	8	10	18
81-90	1	2	3
Total	**609**	**870**	**1479***

*Sex data available for only 1479 individuals, instead of the overall 1485 samples processed.

3.4.7.1 NKG2C genotype distribution within the West Kiang region

The *NKG2C* allele frequency in our initial cohort was 29.3%, substantially higher than in other reported countries with average frequency of 20% (Table 3.6) (Miyashita et al., 2004, Li et al., 2015). We therefore increased our sample size to confirm the validity of this observation. Consequently, we analysed DNA samples from 1485 individuals from West Kiang across different age groups. Surprisingly, an allele frequency of 36.9% was observed, higher than in our initial studies. We identified 225 individuals who were homozygous for the deletion allele (15.2%) while 645 individuals (43.4%) were heterozygotes and 615 (41.4%) were homozygous for the wild type *NKG2C* allele (Figure 3.19).

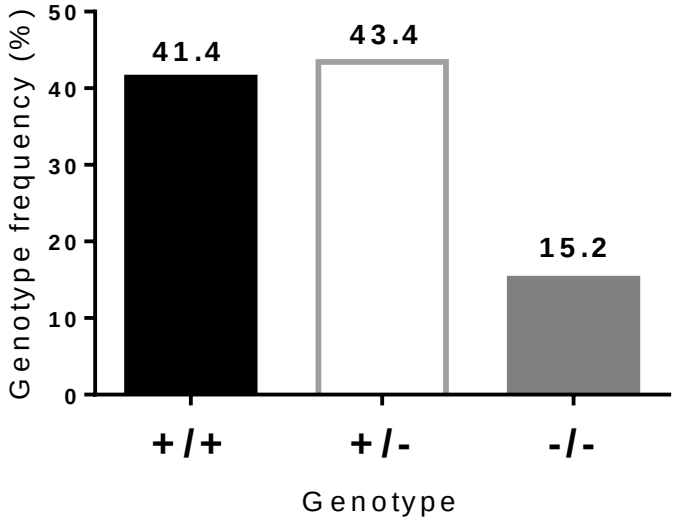

Figure 3.19: Frequency of NKG2C genotype distribution in West Kiang, The Gambia.

Distribution of NKG2C genotypes within West Kiang District, The Gambia. The dark bar represents *NKG2C*+/+ homozygous gene, the open bar depicts *NKG2C*+/- heterozygous gene and the grey bar represents *NKG2C*-/- homozygous gene deletion. This data was obtained from the DNA of 1485 individuals within West Kiang.

The genotype frequencies observed within our extended cohort do not correspond with Hardy-Weinberg (H-W) equilibrium (Chi Squared, p=0.009). The expected frequency for the homozygous *NKG2C* gene deletion if in H-W equilibrium should be 13.6% instead of 15.2% (see calculation below). A recent publication of adults sampled in different regions of The Gambia however estimates a homozygous *NKG2C* deletion frequency of 13.5% and an overall frequency for the deletion of 36.2%, (Goncalves et al., 2016) similar to our observation of an allele of frequency of 36.9%. It should be noted in our extended cohort that almost half of the samples were collected from children below 10 years of age. Lack of conformity to H-W equilibrium may result from sampling bias as some of these children may be closely related and there is an increased representation of heterozygous individuals (644) increasing the probability of detecting the deletion and reducing the homozygous NKG2C+ genotype frequency.

Calculation of Hardy-Weinberg equilibrium

Observed numbers

+/+= 615; -/+= 645; -/-= 225

Allele frequency of NKG2C gene and NKG2C gene deletion=

((2X homozygous NKG2C gene number) + heterozygous NKG2C number)/ (2* total number)

Allele frequency NKG2C gene= ((2*615) +645)/ (2*1485) = 0.631 and

Allele frequency NKG2C deletion= ((2*225) +645)/ (2*1485) =0.369

Expected numbers

Formula= Homozygous NKG2C gene² X 2 (heterozygous) X homozygous NKG2C deletion²

Homozygous NKG2C gene: (0.631) X (0.631)1485 = 591, 39.9%

Heterozygous NKG2C gene: 2(0.631) X (0.369)1485 = 692, 46.6%

homozygous NKG2C deletion: (0.369) X (0.369)1485 = 202, 13.6%

3.4.7.2 NKG2C genotype in different regions of the world

Table 3.6: Distribution of NKG2C genotype around the world.

Characteristic	Mexico	Germany	Holland	Japan	Spain	China	Tanzania	Gambia∞	Gambia West Kiang
N=	300	280	105	245	137	1123	509	787	1485
Genotype n (%)									
+/+	240 (80)	196 (70)	67 (63.8)	156 (63.7)	83 (60.6)	n/a	329 (64.6)	316 (40.2)	615 (41.4)
+/-	58 (19.3)	70 (25)	34 (32.4)	79 (32.2)	48 (35)	n/a	156 (30.7)	363 (46.1)	645 (43.4)
-/-	2 (0.66)	14 (5)	4 (3.8)	10 (4.1)	6 (4.4)	n/a	24 (4.7)	108 (13.7)	225 (15.2)
% allele deletion	**10.33**	**17.5**	**20**	**20.2**	**21.9**	**20.3-26.8**	**20**	**36.2**	**36.9**

Reference: Mexico (Rangel-Ramirez et al., 2014); Germany (Noyola et al., 2012); Holland (Miyashita et al., 2004); Japan (Miyashita et al., 2004); Spain (Moraru et al., 2012); China (Li et al., 2015); Tanzania (Goncalves et al., 2016); The Gambia∞ (Goncalves et al., 2016).

3.4.7.3 NKG2C genotype distribution with age

A selective advantage of NKG2C deletion allele carriage over the life course, or a sampling bias within our cohort leading to H-W disequilibrium might be expected to result in differences in genotype across the lifespan. We therefore assessed genotype frequencies within individuals stratified by age to assess if *NKG2C* allele frequency changed with age, in particular whether there was any bias towards carriage of the deletion allele within the youngest age group. No significant variation of NKG2C genotype was observed with age (Figure 3.20A). Furthermore, despite over half of the individuals tested being under 10 years of age within this sample (760/1485 individuals; Figure 3.20B, Table 3.5) no difference in the distribution of NKG2C genotypes was observed compared to the other age-defined strata. Table 3.6 shows the distribution of *NKG2C* allele frequencies around the world showing that it is higher in The Gambia than in other known countries.

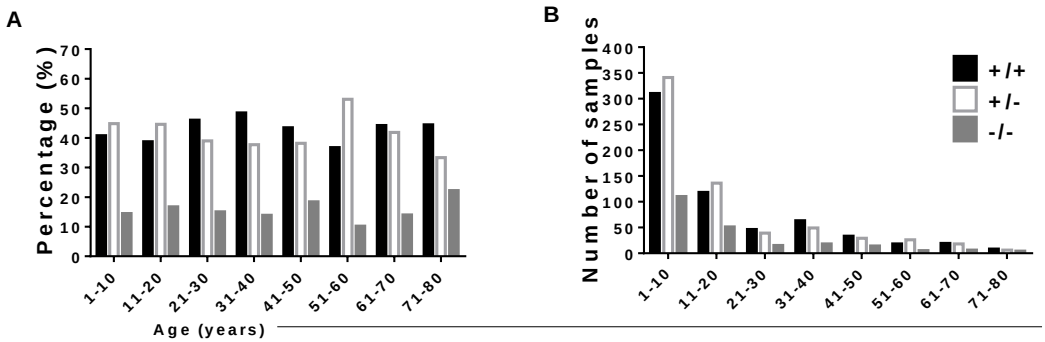

Figure 3.20: NKG2C genotype distribution with age.

Percentage (A) and absolute numbers (B) of individuals with each NKG2C genotype. The dark bar represents *NKG2C*+/+ homozygous gene, the open bar depicts *NKG2C*+/- heterozygous gene and the grey bar represents *NKG2C*-/- homozygous gene deletion. This data was obtained from the DNA of 1485 individuals between the ages of 1-80 years within West Kiang. Chi-square test.

3.5 DISCUSSION

NK cells differentiate to mature status as people age. At the same time, it is now being recognized that genetic and environment factors also influence this differentiation process. These factors create heterogenic populations of NK cells that are genetically, phenotypically and functionally diverse at the epidemiological level (Parham and Moffett, 2013) as well as within the host (Horowitz et al., 2013). NK cell function can considerably be altered by these factors (Le Garff-Tavernier et al., 2010, Luetke-Eversloh et al., 2013, Yu et al., 2013) and emerging evidence suggests that this could be linked to different health outcomes (Hazeldine and Lord, 2013).

NK cell differentiation can significantly be influenced by age, by affecting mainly their phenotype and function. However, it is still unclear if this is an intrinsic ageing process or a marker of prolonged, cumulative environmental exposures (Almeida-Oliveira et al., 2011, Hayhoe et al., 2010, Le Garff-Tavernier et al., 2010, Lutz et al., 2005, Sundstrom et al., 2007). This confounding effect can clearly be demonstrated in HCMV infection, making it difficult to untangle the effects of infection from intrinsic age and NK cell function (Foley et al., 2012a, Foley et al., 2012b, Guma et al., 2004). Most NK cell publications do not account for HCMV infection status thereby impeding data interpretation. Recent studies from our group, however, demonstrate that changes in NK cell phenotypic and functional characteristics do indeed vary with age in HCMV negative individuals but that these changes are exacerbated at all ages in HCMV positive subjects (Nielsen et al, 2015). To address this issue further, comprehensive phenotypic and functional studies are needed from diverse genetic populations in different settings and across the lifespan.

The results presented in this chapter represent a comprehensive study of NK cell phenotype and function from infancy to mature adulthood, the first such study in The Gambia where the confounding effects of HCMV infection status are moderated due to high prevalence of this infection since infancy.

Generally, we observed that the proportions of less mature CD56bright and CD56dimCD57- NK cells decreased and proportions of CD56dimCD57+ NK cells increased with age. On the other hand, transitional CD57int NK cells were present at similar frequencies in all age groups, signifying that maturation of CD57- to CD57+ NK cells may occur at a comparable rate irrespective of age. Age associated changes in CD57- and CD57+ NK cell proportions may therefore reflect differential rates of proliferation or apoptosis of these two phenotypes rather than altering rates of cell

conversion. It should be noted that the rate of proliferation and apoptosis in human NK cells is reportedly very high but how this varies between CD57 subsets is unclear. Also, less mature CD56bright NK cells have longer telomeres than differentiated CD56dim NK cells (Lutz et al., 2011, Ouyang et al., 2007, Chan et al., 2007).

A prominent observation in this cohort is the high proportion of fully differentiated, CD56dimNKG2C+CD57+ NK cells in young children and infants. These subsets of NK cell represent up to 50% of all NK cells in infants (1-2 years) and up 80% of NK cells in children (6-9 years), with mature adult proportion (~30-70%) reached by the age of 10 years. In Caucasians, the frequency of CD57+ NK cells increases from zero at birth (cord blood) to a median frequency of ~50% in adults (Le Garff-Tavernier et al., 2010) with higher proportions in HCMV+ (30-70%) than in HCMV- individuals (25-50%) (Campos et al., 2014). The proportion of CD57+ NK cells in HCMV-infected Gambians adults is notably higher than in age-matched HCMV-infected UK adults, signifying more extensive NK cell maturation in Gambians. HCMV infection may occur earlier in life in Gambians than in UK donors and this may account for this observation or it might be due to higher exposure in The Gambia to other acute/chronic infections which drive NKG2C+CD57+ NK cell polarization in HCMV+ individuals. Further studies comparing HCMV+ and HCMV- children from regions with lower HCMV acquisition will help to answer this.

In this cohort, co-infection of HCMV and EBV did not significantly alter NK differentiation cell phenotype or function, with the notable exception of a modest enrichment of CD56dim cells in co-infected individuals. To some extent this is in agreement with other studies showing that EBV+ HCMV co-infection, but not EBV alone was correlated with mature NK cell differentiation compared to HCMV infection only (Saghafian-Hedengren et al., 2013) and is consistent with slight enhancement of differentiation from CD56bright to CD56dim NK cells observed elsewhere (Hendricks et al., 2014). In addition, it is thought that HCMV infection influences NK and T cell differentiation by distinct mechanisms, because the biphasic kinetic of NK cell differentiation is not reflected by a comparable differentiation pattern for memory T cell populations (Bengner et al., 2014).

Age associated differences in NK cell functionality were mainly due to variations in the frequencies of CD57- and CD57+ NK cells. Cytokine-induced CD107a, CD25 expression and IFN-γ secretion all decline with acquisition of CD57 expression, in

European adults (Bjorkstrom et al., 2010, Juelke et al., 2010, Lopez-Verges et al., 2010, White et al., 2014). This also corresponds with diminished IL-12 and IL-18 surface receptors (White et al., 2014). This association between CD57 expression and NK cell function is also observed in the Gambians studied here. Therefore, as CD57 expression particularly on NKG2C+ NK cells is to a great degree associated with HCMV infection, it seems that early in life HCMV infection rapidly skews the entire NK cell repertoire to receptor-mediated or antibody dependent cytotoxicity at the expense of cytokine-driven responses (Costa-Garcia et al., 2015, Muntasell and Pupuleku, 2016, Noyola et al., 2012, Lopez-Botet et al., 2014). This phenomenon of skewed NK cell function is much more manifest among HCMV sero-positive Gambian adults than HCMV sero-positive UK adults, again probably reflecting an earlier age of HCMV infection or increased occurrence of co-infections in The Gambia. Deficiencies in NK cell functionality early in life in some infants could contribute to links between perinatal HCMV infection, stunted growth and increased rates of hospitalisation, as observed in Zambian children (Gompels et al., 2012).

Consistent with the high prevalence of HCMV infection early in life in our cohort, and the recognition of CD57+NKG2C+ NK cell expansion in HCMV+ individuals (Guma et al., 2004), frequencies of NKG2C+ cells were high in all age groups in our cohort. Adult frequencies of NKG2C+ cells were achieved by the age of 6-9 years, however, the very young children had a lower frequency than older age groups. This observation suggests that expansion of the NKG2C+ NK cell subset commences early after HCMV infection and may continue for some years. This is consistent with data from transplant recipients with acute HCMV infection or reactivation where the proportion of NKG2C+ NK cells surges within 4 weeks of infection/reactivation and continues to rise for at least 12 months (Foley et al., 2012a, Foley et al., 2012b, Lopez-Verges et al., 2011). Also, it has previously been shown that higher frequencies of NKG2C+ NK cells were observed in HCMV+ compared to HCMV- children under the age of 2 years (Monsivais-Urenda et al., 2010).

Both in African and Caucasian adults, NKG2C+ NK cells induced by HCMV infection tend to co-express CD57 (Lopez-Verges et al., 2011) although both the frequency of NKG2C+ cells expressing CD57 and the median MFI of CD57 expression were lower in children under the age of 2 years than in older individuals. By contrast, CD57 is expressed only at low intensity on NKG2A+ NK cells within all age groups. We observed, as previously described, that NKG2A+ NK cells express low levels of CD57. This is consistent with a model in which HCMV UL40 peptides bind to HLA-E

leading to its stabilisation on the surface of HCMV infected cells where this drives NK cell expansion, differentiation and expression of NKG2C+ NK cell (the activating receptor on NK cells for HLA-E) and CD57. At the same time such stabilisation would be expected to inhibit differentiation and expansion of cells expressing NKG2A+ which is the inhibitory receptor for HLA-E (Della Chiesa et al., 2013a, Guma et al., 2006b, Prod'homme et al., 2012, Ulbrecht et al., 2000).

It should be noted that CD94/NKG2C and CD94/NKG2A may not be the only NK cell receptors for HCMV recognition (Prod'homme et al., 2007, Arnon et al., 2005, Della Chiesa et al., 2014a). *NKG2C-/-* gene deletion was associated with delayed – but not complete lack of - NK cell differentiation and maturation. Importantly, *NKG2C+/+* individuals had higher proportions of NKG2C+ NK cells than *NKG2C+/-* subjects (Muntasell et al., 2013) and the proportion and absolute numbers of CD94+ cells also correlated with *NKG2C* copy number, consistent with the hypothesis that NKG2C+ NK cell numbers expand by proliferation rather than by transformation from NKG2A+ NK cells. Interestingly, normal frequencies of CD56dim NK cells in *NKG2C-/-* individuals with reduced overall expression of CD94, raises queries about which supplementary NK cell receptors might be expressed on these cells in order to maintain NK cell homeostasis and HCMV latency. HCMV reactivation in recipients of *NKG2C-/-* stem cells drives differentiation of functional KIR+NKG2A- NK cells (Della Chiesa et al., 2014b) suggesting that activating KIR may compensate for CD94/NKG2C gene deletion. Importantly persistent expansions of KIR+NKG2A-NKG2C-NK cells have been reported in HCMV sero-positive adults, in particular, cells expressing activating KIR2DS1 and KIR2DS4 (Beziat et al., 2013).

NKG2C$^{-/-}$ donors had lower frequency of CD57+ NK cells than in those with one or more copies of *NKG2C,* particularly children aged under 10 years, consistent with activation and expansion of NKG2C+ cells prior to their acquisition of CD57. Recent studies by Muntasell et al are also relevant in this context where activating KIR+ expansions are enriched in NKG2C+/- and -/- individuals (Muntasell and Pupuleku, 2016). It would be interesting to identify whether delayed NK cell differentiation in HCMV-infected *NKG2C-/-* subjects is seen in other populations, whether this confers any survival benefit or whether this is offset by impaired control of HCMV (as implied by the significantly higher anti-HCMV antibody titres). These studies will require large sample size to achieve statistical power, which will depend on both the prevalence of both the *NKG2C* gene deletion allele and HCMV infection. The 36.9% allele frequency of the *NKG2C* deletion in The Gambia cohort is higher than that recorded

elsewhere. Our observation is consistent with that of a recent study done by Gonclaves et al, where they show a similar frequency in The Gambia (36.2% allele frequency, Table 3.6) (Goncalves et al., 2016). Although in our study the prevalence of homozygous *NKG2C* gene deletion did not conform to Hardy-Weinberg equilibrium, we think this might have been because of the bias of the sampling of our cohort, including sampling of people from the same families.

The *NKG2C* deletion frequency ranges from 10% in Mexico, 20% in Japan, Spain, Holland, Tanzania and China to 36% in The Gambia. It would be interesting to further investigate the reason for the observed disparity between Tanzania and The Gambia as these two populations might have the same level of HCMV infection exposure (Manicklal et al., 2013). The reason for these disparity is still unknown (Muntasell et al., 2013, Rangel-Ramirez et al., 2014, Zeng et al., 2013) but it is possible that the deletion offers some selective advantage e.g. via mechanisms operating in utero. Mother to child transmission of HCMV in utero is likely to be common in our study population. For example, it may be that reduction of overt immune responses to HCMV in the placenta reduces the likelihood of placental damage and placental insufficiency. If so, then balancing selection may be operating with heterozygous individuals able to control HCMV infection without incurring damage in utero.

Even though there is almost universal HCMV infection in our cohort, we have observed a diverse array in NK cell phenotype and function within each age defined strata. This heterogeneity could be partially explained by host genetic variability (Horowitz et al., 2013), although, there is also a potential for variability in key viral proteins which interact with the innate immune system or which regulate viral fitness to play a role. Co-infection of HCMV with other pathogens could also shape NK cell maturation (Bjorkstrom et al., 2011, Petitdemange et al., 2011), (Saghafian-Hedengren et al., 2013, Saghafian-Hedengren et al., 2009). Additional studies will be required to understand whether inflammatory cytokines induced in response to other pathogens co-stimulate and promote NK cell differentiation or whether certain pathogens mediate costimulatory effects via specific ligands for receptors on CD57+NKG2C+ NK cells.

In conclusion, this study demonstrates rapid phenotypic and functional NK cell differentiation in a population with almost universal prevalence of HCMV infection. The expression of CD57 and/or NKG2C receptors appears to significantly influence NK cell phenotype. These findings highlight the need to further investigate the impact

of early life HCMV infection on the efficacy of NK cell protective immune responses against different infections in ageing.

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4 CHAPTER 4:

THE IMPACT OF INFLUENZA VACCINATION ON NK CELL RESPONSES IN HCMV+ GAMBIANS

4.1 INTRODUCTION

Annually, about 3-5 million cases of influenza are reported worldwide and 250-500,000 of these cases are fatal (UK Department of Health, 2012). Influenza viruses are RNA viruses belonging to the *Orthomyxoviridae* family. They cause infection of the respiratory tract and are transmitted from person-to-person via sneeze or cough droplets. There are three types of influenza viruses, namely, influenza virus A, B and C. Influenza A and B occur more frequently than C, which is why it is not included in seasonal influenza vaccines. Healthy individuals recover from influenza infection after about a week. However, influenza infection can cause severe morbidity and mortality in the very young and in adults over 65 years and those at high risk of chronic heart, lung and kidney diseases (UK Department of Health, 2012, World Health Organization, 2012). The importance of influenza as a global health problem has been confirmed during the 2009 influenza A (H1N1) pandemic, causing 59 million cases, about 200,000 hospitalizations and over 12,000 deaths in the United States alone. This pandemic mainly affected those over 50 years (Bautista et al., 2010). These facts warrant resources to be focused on influenza virus research and its treatment.

The best manner to protect people against influenza is by giving them seasonal influenza vaccination. The seasonal influenza vaccines provide protection against three circulating influenza virulent strains. These strains usually include two type A influenza virus strains and one type B strain. These vaccines are expected to induce the production of memory B and T cells within 2 weeks of vaccination (UK Department of Health, 2012). The UK Department of Health (2012) recommends that all pregnant women, those with underlying chronic diseases and all those over 65 years of age should receive a single dose of influenza vaccine. Children aged 6 months to 9 years are recommended to receive a booster shot at least 4 weeks after the primary vaccination if they have not previously received an influenza vaccine (UK Department of Health, 2012).

Both adaptive and innate immunity are altered with ageing. Such immune maturation has important consequences for the ability of individuals to mount protective immune responses, including those elicited by vaccines. It is important to study the innate immune response in young individuals where the immune system is not fully developed and in the elderly where the immune system is fully matured as this may

have consequences on the quality and magnitude of the vaccine-induced responses (Almeida-Oliveira et al., 2011).

In healthy ageing, the CD56bright NK cell subset decreases in frequency whilst the CD56dim subset increases with age resulting in an overall augmentation in NK cell numbers in human peripheral blood. Studies in healthy elderly subjects have furthermore demonstrated that whilst the overall proportion of NK cells increased, their cytotoxic capacity is unaltered. Preservation of NK cell cytotoxicity in the elderly is associated with better health status (Gayoso et al., 2011).

However, seasonal influenza vaccines do not always provide full protection against influenza infection in the young and the very old. Meta-analyses have shown that the efficacy of influenza vaccines has been inconsistent and, in some cases, very low. This raises the need to develop improved vaccines that would offer better protection (Grubeck-Loebenstein et al., 2009, Gross et al., 1995, Osterholm et al., 2012, Jefferson et al., 2012). Currently, new vaccines are tested using correlates of memory CD4 T helper 1 for macrophage mediated responses, CD4+ T helper 2 for B cells, antibody production and CD8+ recall immune responses to judge how useful and effective vaccines would be. For vaccines with limited efficacy there is a need to develop novel mechanisms to enhance protection. Optimising CD4 T cell derived IL-2 mediated NK activation or NK cell-mediated ADCC may be a useful strategy to render vaccines more effective.

The aim of this research project was to examine alteration of natural killer cell phenotype and effector function up to 24 weeks post seasonal trivalent influenza vaccination and to study the impact of age, in a Gambian population with very high prevalence of HCMV infection in people of all ages. And our secondary aim was to assess the potential of NK cell activation after booster vaccination.

4.2 STUDY OBJECTIVES

The following primary objectives were investigated:

1. To describe the ex vivo phenotype of CD45+ white blood cells, mainly peripheral myeloid and lymphoid subsets, including detailed NK cell subset phenotypic analysis, before and up to 6 months after influenza vaccination.
2. To describe in vitro peripheral blood NK cell responses (CD107a, CD25, and IFN-γ) to influenza antigens among vaccinated participants and the stability of these responses up to 6 months after vaccination using the trivalent influenza vaccine (TIV) antigen and also single strain antigens of the vaccine.
3. To describe the effect of booster vaccination with influenza vaccine on NK cell function 9 months after primary vaccination in children.
4. To compare the functional phenotype and magnitude of NK responses among individuals of different ages: that is 2-6 years, 20-30 years and 60-75 years of age.
5. To assess the role of influenza-specific CD4+ helper T cell IL-2 production and vaccine-induced antibodies in supporting NK cell responses.

4.3 METHODS & MATERIALS

4.3.1 Study subjects

This study was approved by the Medical Research Council (MRC) Scientific Coordinating Committee (SCC), The Gambia Government and MRC Joint Ethics Committee (SCC reference number 1309), London School of Hygiene and Tropical Medicines Observational / Interventions Research Ethics Committee (LSHTM Ethics reference 6331) and The Republic of The Gambia Medicines Board.

The timeline was planned such that sample collection time points fall outside the influenza season, to reduce the impact of natural exposure, which in this region occurs annually between October and December (World Health Organization, 2012).

In February 2013, following written informed consent from study participants or their legal guardians, 68 healthy participants were recruited from three West Kiang villages, namely, Keneba, Manduar, and Kantong Kunda in West Kiang in the Lower

River Region of The Gambia. These study participants were selected to represent one of the three age-stratified groups: 2-6, 20-30 and 60-75 years.

Study subjects were enrolled by a trained nurse excluding anyone with chronic disease, infections or influenza-like signs and symptoms during the previous 3 months and anyone with an axillary temperature of ≥ 38°C. Pregnant women and individuals potentially allergic to vaccine products and anyone with previous history of influenza vaccination were also excluded.

Axillary temperature was taken and whole blood samples collected at baseline (pre-vaccination). Participants were then vaccinated with the 2012-2013 seasonal Trivalent Sanofi Pasteur inactivated influenza split virion vaccine (Batch number J8322; expiry July 2013). Vaccine safety monitoring was done through a home visit by a nurse within a week of vaccination to monitor possible adverse reactions to the vaccine. Subsequent blood samples were taken at 1, 3 and 6 month(s) post-vaccination.

For children aged between 2 and 6 years, it was required that a second dose be given to boost the vaccine response. Ethical permission was obtained to delay this booster vaccination until after the completion of the 6 months follow-up visit (to allow comparison of responses to a single vaccination across all age groups) however, this meant that the booster vaccination would be due at a time when the 2012-2013 seasonal influenza virus vaccine would have expired. Therefore, the equivalent 2013-2014 seasonal influenza virus vaccine (Batch number K7231-4; June 2014) was given instead.

In the primary vaccination study, we administered intramuscularly the WHO recommended vaccine strains for the 2012-2013 influenza vaccine for northern hemisphere winter: the trivalent Influenza A/California/7/2009 (H1N1) pdm09-like virus; Influenza A/Victoria/361/2011 (H3N2)-like virus; and Influenza B/Wisconsin/1/2010-like virus, trade mark Sanofi Pasteur MSD. In the children, the secondary booster vaccination was the 2013-2014 influenza virus season vaccine for northern hemisphere winter: the quadrivalent Influenza A/California/7/2009 (H1N1) pdm09-like virus; Influenza A/Victoria/361/2011 (H3N2)-like virus derived from influenza A/Texas/50/2012; and Influenza B/Massachusetts/02/2012 virus, trademark Sanofi Pasteur MSD.

4.3.2 Ex vivo staining of freshly isolated PBMC

The ex vivo staining was done as described in Chapter 2. Briefly, after counting, the cells were washed by spinning at 1600 rpm for 10 minutes at 22°C and re-suspended at 2 X 10^7 cells/ ml. Fifty µl of the cell suspension containing 1 X 10^6 cells were taken for staining with 4 ex vivo panels per sample. Namely:

Panel 1 NK cell functional panel: CD107a-FITC; NKG2C-PE, CD25-PerCP-Cy5.5, CD56-PE-Cy7, CD57-e450, CD3-V500, NKG2A-APC, IFN-γ-APC-e780;

Panel 2 NK cell phenotype panel: CD27-FITC, NKG2C-PE, CD8-PerCP-Cy5.5, CD56-PE-Cy7, CD57-e450, CD3-V500, LIR-1-APC, CD16-APC-e780; (data not presented)

Panel 3 Dendritic cell, monocyte/ macrophage, T and B cell phenotype: CD45-FITC, CD11c-PE, CD19-PerCP-Cy5.5, CD56-PE-Cy7, CD123-e450, CD3-V500, CD40-APC, CD14-APC-e780;

Panel 4: T cell phenotype panel: CD27-FITC, CD4-PE, CD8-PerCP-Cy5.5, CD28-PE-Cy7, CD57-e450, CD3-V500, CCR7-APC, CD45RA -APC-H7.

The samples were analysed by flow cytometry within 2-3 days of collection and staining. PBMC were acquired using Cyan ADP flow cytometer and LSRII flow cytometer on FacsDiva® software. All FACS data analyses were performed using FlowJo® (TreeStar) as described in Chapter 2.

4.3.3 HCMV & EBV ELISA

The HCMV and EBV assays were performed as described in Chapter 2. Sixty eight samples were assayed for plasma human cytomegalovirus IgG (BioELISA CMV IgG 3000-1216, Barcelona, Spain) and anti-Epstein-Barr virus nuclear antigen (anti-EBNA-1) IgG (EI 2793-9601 G, Euroimmun Medizinische Labordiadnostika, Lubeck, Germany).

4.3.4 Influenza virus antigen ELISA

Briefly, 0.5 µg/ml of vaccine (Trivalent Influenza Vaccine (TIV), H1N1, H3N2 & influenza B) antigens were coated on 96-well plates overnight at 4^0C. The following

day the plates were washed 4 times with washing buffer (containing 250mL of 20X PBS + 2.5mL of Tween 20 + 4.7475L dH_2O) then blocked with blocking buffer (containing 1% skimmed milk powder in washing buffer) for an hour at room temperature, and subsequently washed 4 times again. Fifty µl of standard, negative control, AB plasma, blank or serum samples were added per well, sealed and incubated for 2 hours at 37^0C. Post sample incubation, the plates were washed 7 times and 50 µl of 1:15 000 dilution of horseradish peroxidase HRP-conjugate (Promega Cooperation, Madison, USA) was added and incubated at room temperature for 1 hour 30 minutes. Plates were subsequently washed 7 times. One hundred µl of ortho-phenylenediamine (OPD) (Sigma, P9187, Saint Louis, USA) was added per well and plates were incubated at room temperature for 12 minutes in the dark. The stop solution of 25 µl 2M H_2SO_4 sulphuric acid was used to stop the reaction in all wells and the plates read at 492 nm using Dynex technologies MRX TC II reader. A standard curve of known Optical density (OD) was plotted against known Arbitrary ELISA Units/ml (AEU/ml) concentration for each ELISA plate, and the unknown values were derived using the standard curve. Further dilutions (1:100 and 1:2000) were performed for samples with initial readings below or above the standard curve.

4.3.5 In vitro cell culture assay

The specific in vitro procedures are described in Chapter 2. In order to assess the functional capacity of the NK cells, we cultured the PBMC overnight for 18 hours at 37ºC, 5% CO_2. Most of the NK cells were cultured using 10% human male AB serum (Sigma-Aldrich®, Saint Louis, USA) conditions apart from the autologous plasma experiments at the end of this chapter. Generally, the functional characteristics of the NK cells were assessed by using CD107a as a degranulation marker, CD25 as an activation marker and IFN-γ for cytokine production potential. The rationale of the markers is discussed in Chapter 2.

4.3.6 T cell in vitro culture

PBMC were cultured at 37ºC, 5% CO_2, in 10% human AB serum (Sigma-Aldrich®, Saint Louis, USA). The PBMC were cultured with or without TIV antigens (2.5µg/ml) for 5 hours and Brefeldin A and Monensin added after 2 hours of in vitro culture.

4.3.7 Statistical analysis

Non-parametric Wilcoxon matched paired tests were employed to analyse paired sample data within the study groups and Kruskal-Wallis tests were used for unpaired comparisons between different age groups and subsets, where applicable. GraphPad Prism (GraphPad Software 6) was used to prepare the diagrams and for statistical analysis. Significant difference between subsets was defined as having a p value of *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

4.4 RESULTS

The aim of this study was to evaluate the induction of seasonal TIV antigen-specific CD4+ T helper cell-dependent and antibody-dependent NK cell activation, after primary and secondary influenza vaccinations. The secondary aims were to observe how long these responses would last post vaccination and how the NK cell responses are affected by age.

Overall, I collected matched samples for 68 individuals, of which 22 were children in the youngest (2-6 years) age groups, 21 were young adult (20-30 years) and 25 were in the oldest (60-75) age group. The median age in each group was 3.9, 21.7, and 65 years, respectively. Similar numbers of male and female donors were recruited within each age group (Table 4.1).

It is well known that NK cell maturation can be influenced by human cytomegalovirus (HCMV) infection (Guma et al., 2004, Guma et al., 2006, Lopez-Verges et al., 2011). It has previously been shown in Chapter 3, that our study population has a high prevalence of HCMV infection, therefore, we went on to examine the exposure to HCMV and Epstein-Barr virus (EBV) in our vaccination cohort using ELISA to determine the amount of IgG antibodies specific to these two herpes viruses. Table 4.1 shows that, as expected, HCMV is highly prevalent in our cohort with only two individuals having a negative IgG antibody ELISA result, one in the youngest age group and one in the oldest age group. Consistent with the findings in our previous study, children had a higher median anti-HCMV IgG (398 IU/ml) titre than young adults (238 IU/ml), although not significant in this case. However, a significant increase in the median antibody titre was observed in 60-75 (580 IU/ml)year olds compared to children, Kruskal-Wallis test ∞ (Table 4.1). This extends the data shown

in Chapter 3, where the oldest subjects were under 50 years and suggest HCMV reactivation may be occurring in the elderly in our cohort (Table 4.1).

There was no significant variation in anti-EBNA IgG levels between the age groups, although EBV seroprevalence increased with age (Table 4.1). In this cohort, the overall frequencies of individuals homozygous for the NKG2C wild type gene was 44.1%, heterozygous individuals were 42.6% and 13.2% were homozygous for the *NKG2C* gene deletion (therefore allele frequency = 34.6%).

Table 4.1: Cohort characteristics: Baseline NKG2C genotype, HCMV and EBV IgG antibody levels.

∞HCMV IgG antibody levels significantly different between the youngest and oldest age groups (*p<0.05).

Age Group			HCMV IgG+	HCMV IgG titer, IU/ml	EBV nuclear antigen	EBV nuclear antigen IgG titer	NKG2C genotype n (%)		
(Years)	N (Male/Female)	Median Age	n (%)	Median (range)	IgG+, n (%)	RU/ml, median (range)	+/+	+/-	-/-
2-6	22 (11/11)	3.9	21 (95.5)	398 (35-2942)∞	19 (86.4)	76 (17-185)	12 (54.5)	7 (31.8)	3 (13.6)
20-30	21 (13/8)	21.7	21 (100)	238 (54-798)	19 (90.5)	103 (8-178)	7 (33.3)	10 (47.6)	4 (19.0)
60-75	25 (13/12)	65.0	24 (96.0)	580 (72-8618)∞	24 (96.0)	76 (3-192)	11(44.0)	12 (48.0)	2 (8.0)
Total	**68 (37/31)**		**66 (97.1)**	**398 (35-8618)**	**62 (91.2)**	**85 (3-185)**	**30 (44.1)**	**29 (42.6)**	**9 (13.2)**

In order to understand the role of NK cells in vaccination, we need to identify the specific subsets of NK cells that are circulating in peripheral blood. It has previously been shown that mature NK cells have limited ability to respond to exogenous cytokine stimulation but remain able to mediate ADCC (White et al., 2014, Nielsen et al., 2015). The different types of cells that might influence our vaccine responses were examined, namely, T cells, B cells, antigen presenting cells and NK cells.

4.4.1 Significant variation of ex vivo phenotype of white blood cells between the different age groups

We examined the proportions of PBMC expressing the following lymphoid and myeloid markers: CD19+CD14-: B cells, CD11c+CD14-: myeloid DC, CD123+CD11c-: plasmacytoid DC, CD14+CD11c-: Monocytes, CD56+CD3-: NK cells, and CD3+CD56-: T cells. CD45 was used to identify myeloid as well as lymphoid cells. The gating strategy is shown in Figure 4.1.

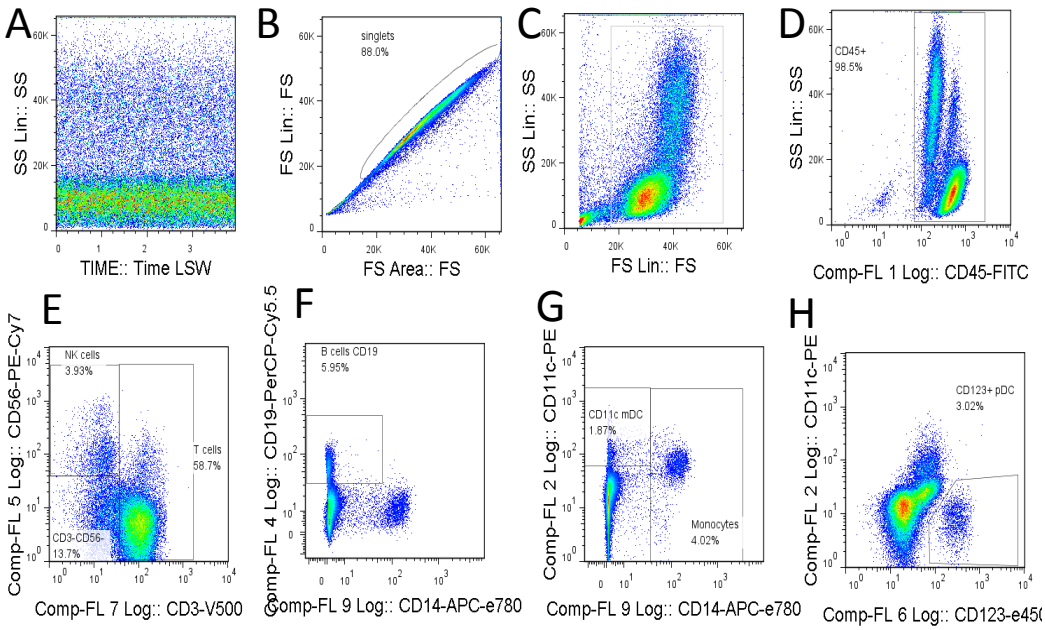

Figure 4.1: Lymphoid and myeloid cell gating strategy

Figure 4.1A illustrates the time gate, (B) singlet gate, (C) lymphocyte and myeloid cells gate, (D) CD45 white blood cells gate, (E) CD56+CD3- NK cells and CD3+CD56- T cells, (F) CD19+CD14- B cells, (G) CD14+CD11c+ monocytes and CD11c+CD14- myeloid DC, and (H) plasmacytoid DC. Representative dot plots from participant S11 visit 1 (20 years-young adult) recruited from the influenza vaccination study.

The proportion of NK cells increases with age (Figure 4.2A), and a similar observation was also seen in the absolute number of NK cells (data not shown). The median proportion of NK cells increased from 5% in the children to about 8% in the young adult group. The older adult group had median NK cell frequency of 10%. There was no observable difference between the age groups in the overall proportions of T cells (Figure 4.2B) but the frequency of B cells (Figures 4.2C) and absolute number of B cells (data not shown) significantly decreased with increasing age. Monocyte frequency (Figure 4.2D) was not different between the children and the older adults. However, there was a significant increase in the proportion of monocytes between children and the younger adults. Myeloid dendritic cell (mDC) frequency increased with age (Figure 4.2E) between the children and older adults, but not when comparing children and younger adults, however, the frequency of plasmacytoid dendritic cells (pDC) did not vary with age (Figure 4.2F). Generally, there was no significant difference observed in these lineage marker defined cells pre- and post-vaccination (data not shown).

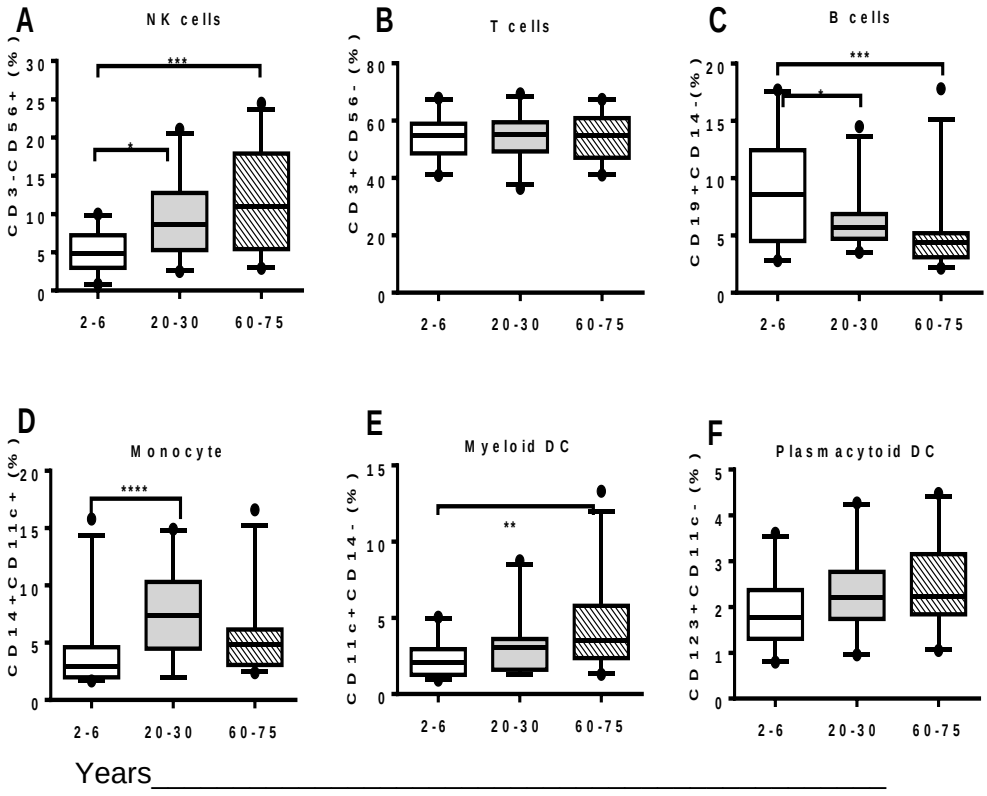

Figure 4.2: Age-related variation in baseline proportions of lymphoid and myeloid cell populations.

Lymphoid and myeloid cell subset distribution at baseline between the three age groups (2-6, 20-30, 60-75 years). These cells were stained ex vivo without stimulation. Data are shown for 68 subjects. (A) CD56+CD3- NK cells, (B) CD3+CD56- T cells, (C) CD19+CD14- B cells, (D) CD14+CD11c+ monocytes, (E) CD11c+CD14- myeloid DC, and (F) plasmacytoid DC. In box and whisker plots, the horizontal bar indicates median frequency, the boxes extend 25th-75th percentile range and the whiskers indicate 95th percentiles. Statistical analysis was performed on samples using Kruskal-Wallis test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.4.2 T cell subset ex vivo phenotype varies with age

4.4.2.1 Changes in proportions of naive and effector CD4 T cells with age

CD4+ T helper cell production of IL-2 is an essential component in the potentiation of NK cells by immunization (Horowitz et al., 2010). This implies that any variation with age in the size of the CD4+ T helper cell population may influence NK cell activation. There was no significant difference in CD3 and CD8 expression amongst the age-stratified groups, however, the CD4 population seems to increase with age (data not shown). The T cell phenotype may affect proliferative and phenotypic characteristics pre- and post-vaccination and the proportions of different memory subsets may vary with age. T cell subsets were assessed by removing doublets and identifying CD3+CD4+ cells and CD3+CD8+ cells and using CD45RA and CCR7 markers for the characterization of naïve, effector memory (EM), central memory (CM) and terminally differentiated effector memory (TEMRA) cells. Mature and potentially senescent CD4+ and CD8+ T cells were identified using CD28 and CD57 expression.

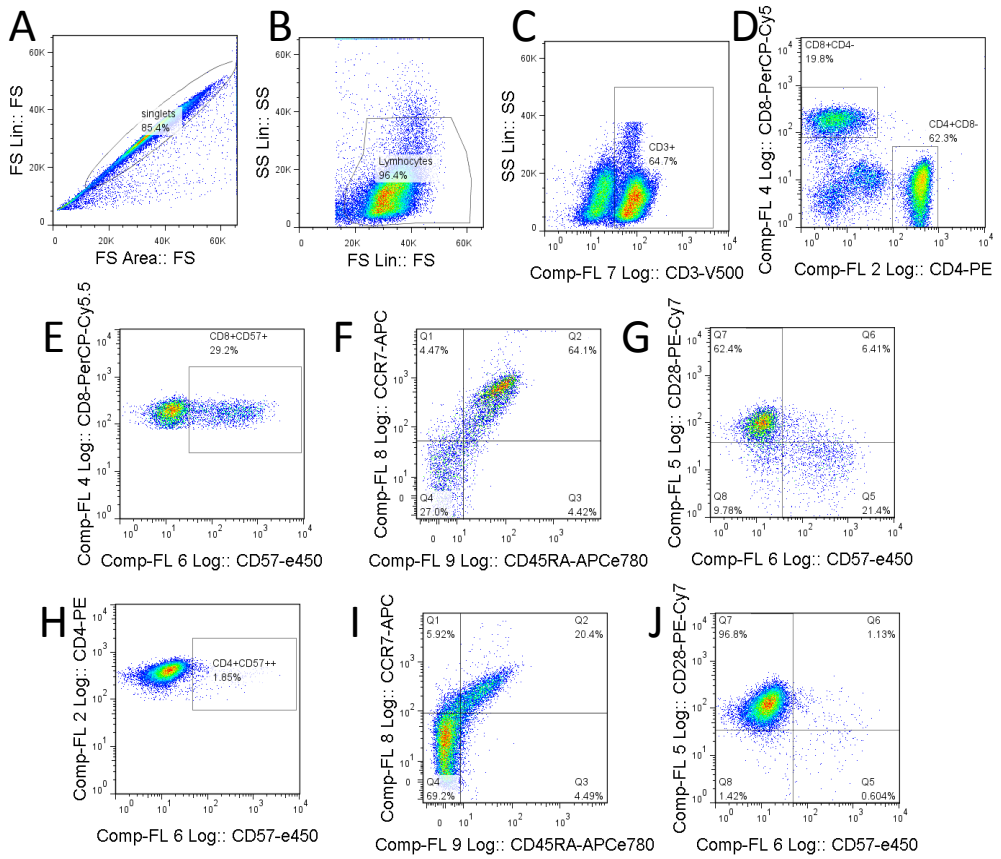

Figure 4.3: Gating strategy for T lymphocytes subsets.

(A) singlet gate, (B) lymphocyte gate, (C) CD3+ T cells, (D) CD3+CD8+ T cells and CD3+CD4+ T cells, (E) CD57 expression on CD3+CD8+ T cells and (H) CD57 expression on CD3+CD4+ T cells. T cell CCR7 and CD45RA-defined CD8+ (F) and CD4+ (I) subsets and terminally differentiated CD28-CD57+ CD8+ (G) and CD4+ (J) T cells. Representative dot plots from participant S9 visit 4 (74 years) recruited from the influenza vaccination study.

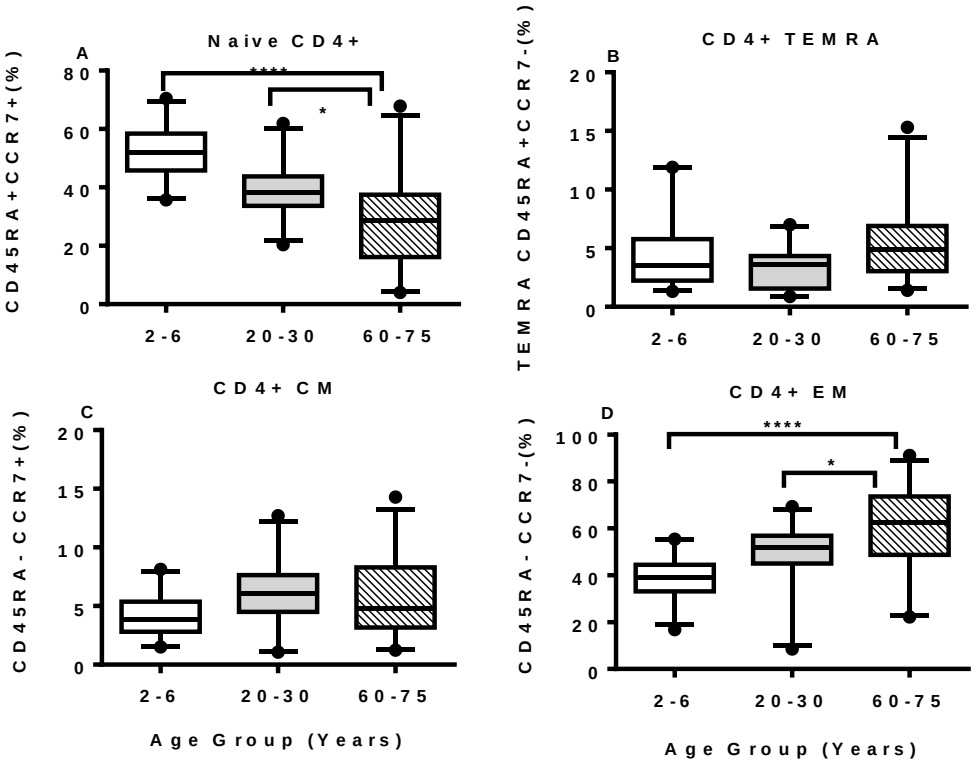

Figure 4.4: Baseline frequencies of CD4+ T helper cell subsets vary with age.

CD4 T cell subset distribution at baseline is shown for the three age groups (2-6, 20-30, 60-75 years), gated from CD3+CD4+ T cells. Data are shown for 68 subjects, (A) CD45RA+CCR7+ naïve T cells, (B) CD45RA+CCR7- TEMRA cells, (C) CD45RA-CCR7+ central memory cells, and (D) CD45RA+CCR7+ effector memory T cells. In box and whisker plots, the horizontal bar indicates median frequency, the boxes extend 25^{th}-75^{th} percentile range and the whiskers indicate 95^{th} percentiles. Statistical analysis was performed on samples using Kruskal-Wallis test, *p<0.05, ****p<0.0001.

Consistent with expectations, a decrease in naive CD4+ and CD8+ T cell subsets, and a parallel increase in effector memory subsets were observed with age. As expected, the naïve CD4+ helper T cell (CD3+CD4+CD45RA+CCR7+) population was considerably higher in children with a median proportion of 50% compared to the other two groups (Figure 4.4A). These data demonstrate a general decrease in the naïve CD4 T helper population with age. By contrast, the EM (CD3+CD4+CD45RA-CCR7-) population increased with age (Figure 4.4D). The older adults had the highest frequency of EM (about 60%), which was significantly higher than among the children. The children had the lowest median proportion of CD4+ effector memory (EM) cells. Neither the CD4+ central memory (CM) (CD3+CD4+CD45RA-CCR7+) nor

the terminally differentiated effector memory (TEMRA) cells (CD3+CD4+CD45RA+CCR7-) differed in frequency between the groups (Figure 4.4 B, C). Also, there was no significant different between proportions of CD4+ T helper cell subsets pre- and post-vaccination (data not shown).

4.4.2.2 Proportions of naive and effector CD8 T cells vary with age

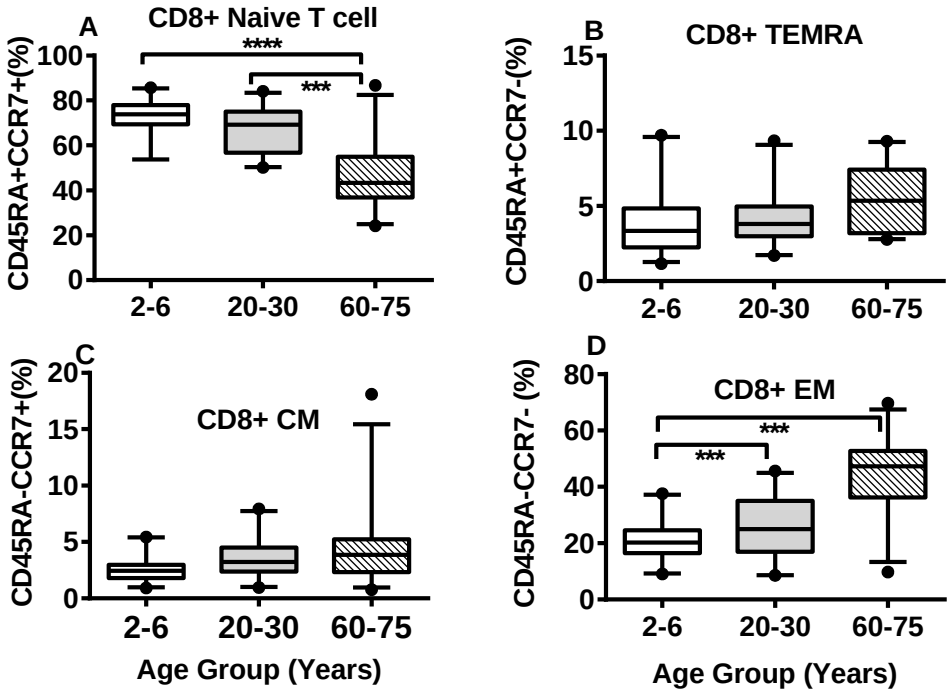

Figure 4.5: Baseline frequencies of CD8 T helper cell subsets vary with age.

CD8 T subset distribution at baseline between the three age groups (2-6, 20-30, 60-75 years), gated from CD3+CD8+ T cells. These cells were stained ex vivo without stimulation. Data are shown for 68 subjects. (A) CD45RA+CCR7+ naïve cells, (B) CD45RA+CCR7- TEMRA cells, (C) CD45RA-CCR7+ central memory cells, and (D) CD45RA+CCR7+ effector memory CD8+ T cells. In box and whisker plots, the horizontal bar indicates median frequency, the boxes extend 25^{th}-75^{th} percentile range and the whiskers indicate 95^{th} percentiles. Statistical analysis was performed on samples using Kruskal-Wallis test, ***p<0.001, ****p<0.0001.

Cytotoxic CD8+ T cells kill target cells in a MHC class I restricted manner. This mechanism of targeted cell death is important in complementing recognition of target cells missed by NK cells. The proportion of CD8+ naïve T cells (CD3+CD8+CD45RA+CCR7+) significantly decreases with age (Figure 4.5A). More than 70% of CD8+ T cells were naïve in children aged 2-6 years, whilst this

proportion was approximately 65% among younger adults and as low as 40% in older adults. The frequency of CD8+ effector memory (EM) increases (CD3+CD8+CD45RA-CCR7-) cells with age (Figure 4.5D). Children had EM median percentage of about 20%, whilst older adult adults had a frequency of over 40%. Proportions of both CD8+ TEMRA (CD3+CD8+CD45RA+CCR7-) and CD8+ CM (CD3+CD8+CD45RA-CCR7+) were not significantly different between the age groups (Figure 4.5 B,C).

4.4.2.3 Changes in T cell senescence markers with age

Highly differentiated CD4+ and CD8+ T cells acquire CD57 and lose the expression of the co-stimulatory molecule CD28 and several groups have proposed that these phenotypes are associated with functional senescence (Fulop et al., 2013). I therefore, investigated whether there were age-related changes in T cell phenotype evident in this cohort which could impact on vaccine responsiveness. The oldest adults displayed a higher frequency of CD4+ CD28-CD57+ cells compared to children and young adults (Figure 4.6A). Interestingly and similarly to the observations described in Chapter 3, a high frequency of CD28-CD57+ CD8+ T cells was observed in children and older adults. The young adults had a significantly lower proportion of CD28-CD57+ CD8 T cells than the other age groups (Figure 4.6B).

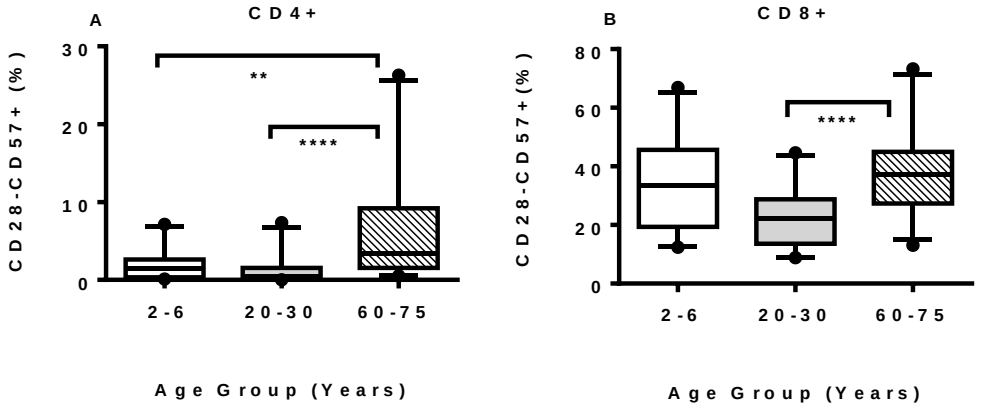

Figure 4.6: Baseline expression of CD28 and CD57 on T cell subsets changes with age.

CD28-CD57 expression on CD4 and CD8 T cell subsets at baseline among the three age groups (2-6, 20-30, 60-75 years), gated from CD3+ T cells. These cells were stained ex vivo without stimulation. Data are shown for 68 subjects. Frequency of CD4+CD28-CD57+ (A) and CD8+CD28-CD57+ (B) T cells, the horizontal bar indicates median frequency, the boxes extend 25th-75th percentile range and the whiskers indicate 95th percentiles. Statistical analysis was performed on data using Kruskal-Wallis test, **p<0.01, ***p<0.001.

4.4.3 Baseline, ex vivo NK cell subset proportions change with age

In view of the increasing proportion of NK cells observed with age, I wanted to further characterise the NK cell phenotype in this study population to assess variations that may exist between the young and old and which could impact on NK cell function. At the same time, we also looked for ex vivo differences in functional markers before and 1 month after vaccination. The gating strategy is shown below (Figure 4.7).

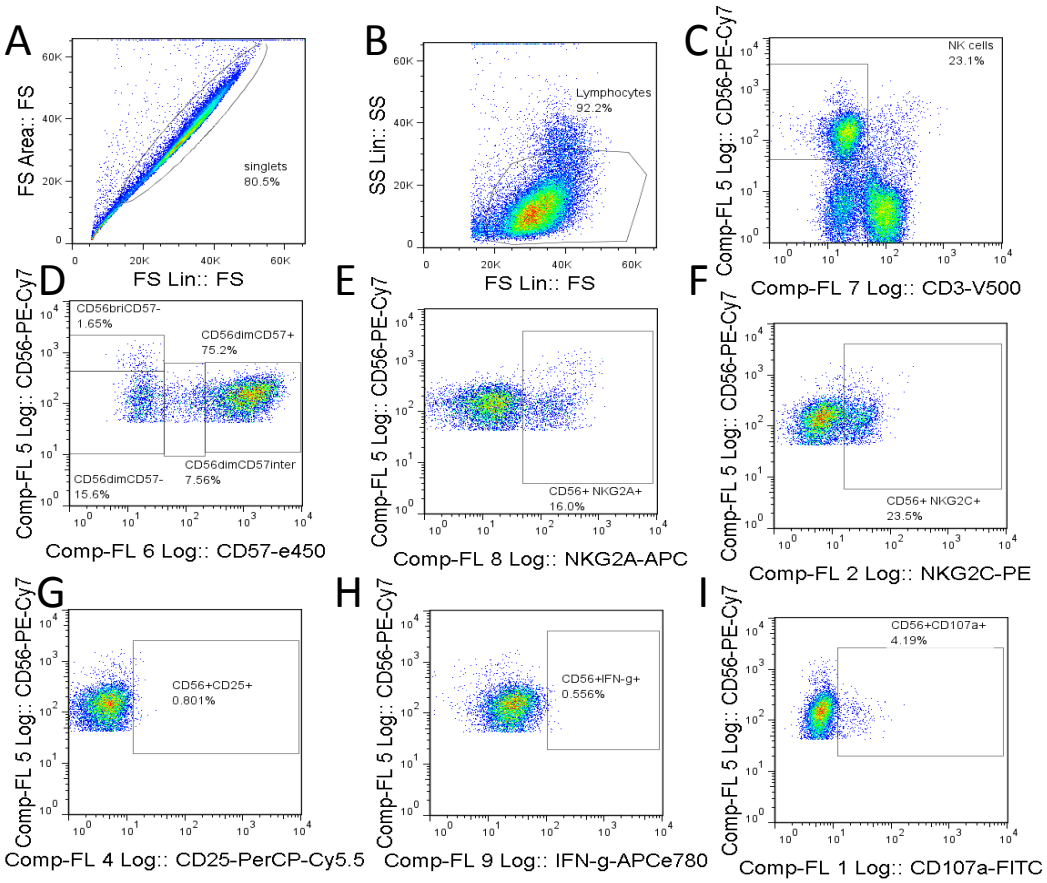

Figure 4.7: Gating strategy for NK cell subsets.

(A) Singlet gate, (B) lymphocyte gate, (C) NK cell gate, (D) CD57 NK subsets, (E) NKG2A expression, (F) NKG2C expression and (G) CD25 (H) IFN-γ and (I) CD107a expression. Representative dot plots from participant S1 visit 1 (29 years) recruited from the influenza vaccination study.

4.4.3.1 Increase in proportions of NK cells is mainly due to enrichment for CD56dim cells

In this ex vivo analysis, NK cells have been defined as CD56+CD3- lymphocytes and subsequently, subpopulations of CD56bright and CD56dim populations were also identified (Figure 4.8).

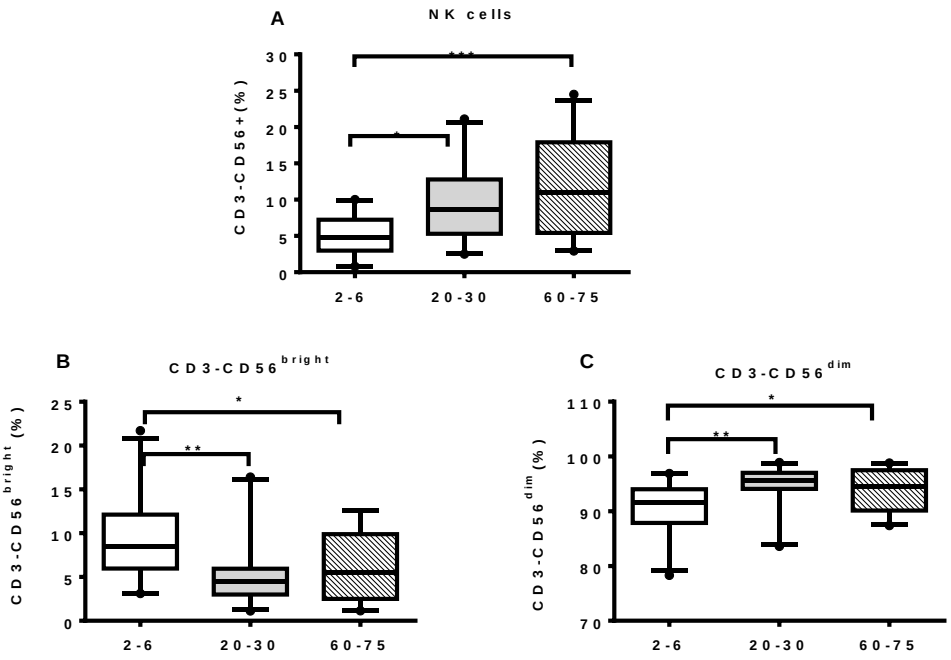

Figure 4.8: Baseline frequencies of total (CD3-CD56+), CD56bright and CD56dim NK cell subsets with age.

NK cell subsets at baseline between the three age groups (2-6, 20-30, 60-75 years), gated from CD56+CD3- NK cells. These cells were stained ex vivo without stimulation. (A) Proportion of all lymphocytes that are CD56+CD3- NK cells and (B,C) proportion of NK cells that are CD56bright (B) or CD56dim NK cells (C). In box and whisker plots, the horizontal bar indicates median frequency, the boxes extend 25th-75th percentile range and the whiskers indicate 95th percentiles. Statistical analysis was performed on samples using Kruskal-Wallis test, *p<0.05, **p<0.01, ***p<0.001.

At baseline, and consistent with the data shown in Chapter 3, the 2-6 years age group had a significantly higher frequency of CD56bright cells than the other two age groups (Figure 4.8B). The median proportion of CD56bright cells was about 9%. This was higher than the other two groups, which were around 5% for both age groups. As expected, the increased frequency of CD56bright cells resulted in children having a significantly lower frequency of CD56dim cells than the older age groups (Figure 4.8C). This was about 91% for the children and 95% and 94% for the 20-30 and 60-75 years age groups, respectively. Thus, this result indicates that children had a greater number of CD56bright cells than adults; however, they had a lower proportion of CD56dim.

4.4.3.2 The proportion of CD57+ NK cells increases with age

CD57+ expression was not different between the age groups in the CD56bright NK cells, (Figure 4.9A) but CD56dim NK cells expressing CD57+ increased with age (Figure 4.9B). Young children expressed a median of about 50% CD57+ cells. The young adults had a median frequency of 60% whilst in the older adults the frequency was about 65%.

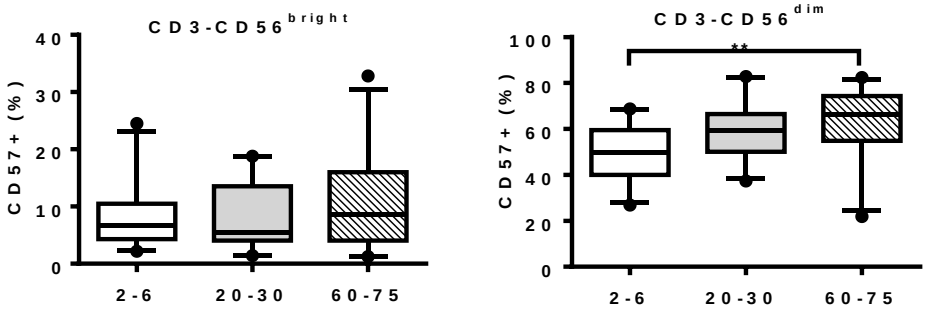

Figure 4.9: Baseline ex vivo CD57+ expression on CD56dim NK cells increases with age.

CD57 expression on NK cells subsets at baseline between the three age groups (2-6, 20-30, 60-75 years), gated from CD56+CD3- cells. These cells were stained ex vivo without stimulation. Data are shown for 68 subjects. Frequency of CD57 on (A) CD56bright and (B) CD56dim NK cells. In box and whisker plots, the horizontal bar indicates median frequency, the boxes extend 25^{th}-75^{th} percentile range and the whiskers indicate 95^{th} percentiles. Statistical analysis was performed on samples using Kruskal-Wallis test, **p<0.01.

HCMV is known to be highly prevalent in our study cohort as shown in Table 4.1. NKG2C is an activation receptor on NK cells that recognises HCMV infected cells and the expansion of the NKG2C+ subset has been associated with HMCV infection. The expression of NKG2A, an inhibitory receptor, and NKG2C on NK cells is mutually exclusive, meaning that most of the cells have either NKG2A or NKG2C, but not both (Figure 4.10B). Figure 4.10A demonstrates the negative correlation that exists between NK cells expressing NKG2C and NKG2A in the 2-6 years old age group within our study population. Interestingly, considerable heterogeneity is observed for the expression of these markers in the children, which may reflect age at HCMV infection, viral load or host genotype.

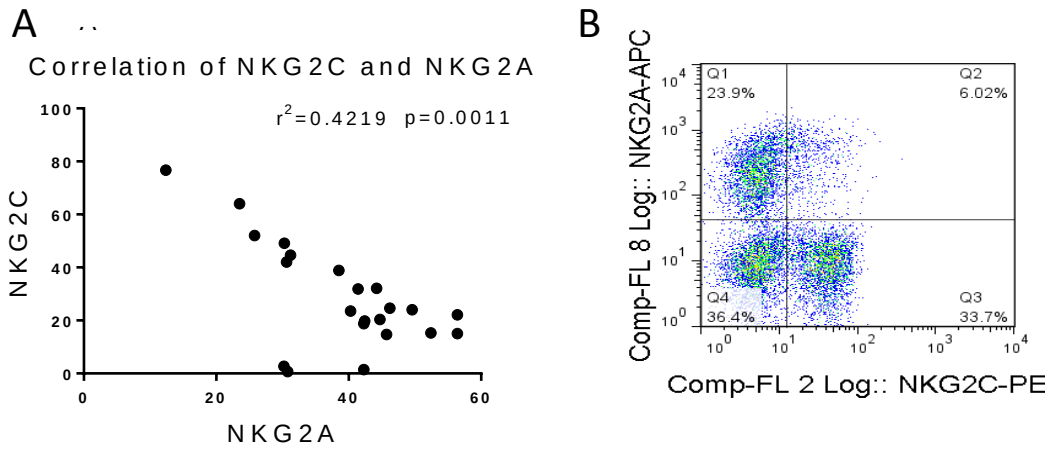

Figure 4.10: Mutually exclusive expression of NKG2A and NKG2C on NK cells of children (2-6 years).

(A) There is a negative correlation between proportions of NK cells expressing NKG2C and NKG2A receptors. (B) Shows gating of NKG2A+ and NKG2C+ NK cells, gated from CD56+CD3- NK cells. These cells were stained ex vivo without stimulation. Data are shown for 22 children; each data point represents an individual donor.

The proportion of NKG2A+ NK cells was significantly higher in children aged 2-6 years compared to young adults (Figure 4.11A). However, consistent with an early maturation of NK cells in The Gambia, this observed difference was no longer significant comparing young and old adults. The children had a median CD56+NKG2A+ percentage of about 50%, whilst both younger and older adults had median percentages of less than 40%. This was also seen after influenza vaccination.

NKG2C expression on NK cells was not different between children and adults (Figure 4.11B). Children had a median percentage NKG2C+ of about 30%, whilst the young

and old adults had median NKG2C+ frequency of 21% and 30%, respectively. A similar pattern was also observed post influenza vaccination, and is consistent with known high rates of HCMV infection early in life in the study population. Therefore, high frequencies of NKG2C+ NK cells are already apparent by 6 years of age in this cohort.

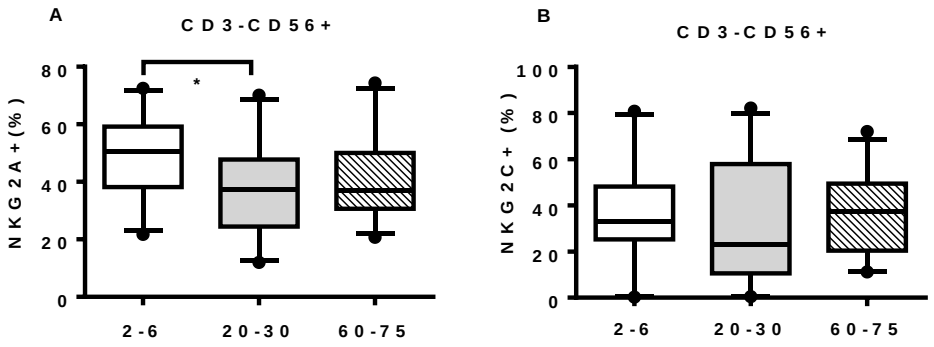

Figure 4.11: Ex vivo, baseline proportions of NK cells expressing NKG2A and NKG2C varies with age.

Proportion of NKG2A and NKG2C on NK cells subsets at baseline between the three age groups (2-6, 20-30, 60-75 years), gated from CD56+CD3- NK cells. These cells were stained ex vivo without stimulation. Data are shown for 68 subjects. Frequency of NKG2A+ (A) and NKG2C+ (B) NK cells. In box and whisker plots, the horizontal bar indicates median frequency, the boxes extend 25th-75th percentile range and the whiskers indicate 95th percentiles. Statistical analysis was performed on samples Kruskal-Wallis test, *p<0.05.

4.4.3.3 NKG2A is highly expressed on CD56brightCD57- NK cells, whereas CD56dimCD57+ (CD57+) NK cells are enriched for NKG2C expression

In order to assess whether differences in the frequencies of NKG2A+ and NKG2C+ NK cells between adults and children was related to the differentiation phenotype, the proportions of these cells were examined within CD57-defined subsets. Higher frequencies of CD56brightCD57- NK cells expressed NKG2A than the other CD57 subsets (Figure 4.12A) and NKG2A expression decreased as NK cells differentiate from CD56brightCD57-(CD56bri) to CD56dimCD57- (CD57-) through CD57int (CD57int) to CD56dimCD57+ (CD57+) (Figure 4.12A). This observation is not affected by age, as there was no difference between the age groups. Similar patterns

were seen after influenza vaccination (data not shown). Conversely, higher proportions of CD56dimCD57+ (CD57+) cells expressed NKG2C compared to the CD57- subsets (Figure 4.12B). In summary, no difference was observed in the subset distribution of NKG2C+ NK cells between different age groups. However, as a significant increase in the overall frequencies of CD56dimCD57+ NK cells was observed between adults and children this is likely to result in higher numbers of highly differentiated CD57+NKG2C+ cells with increasing age.

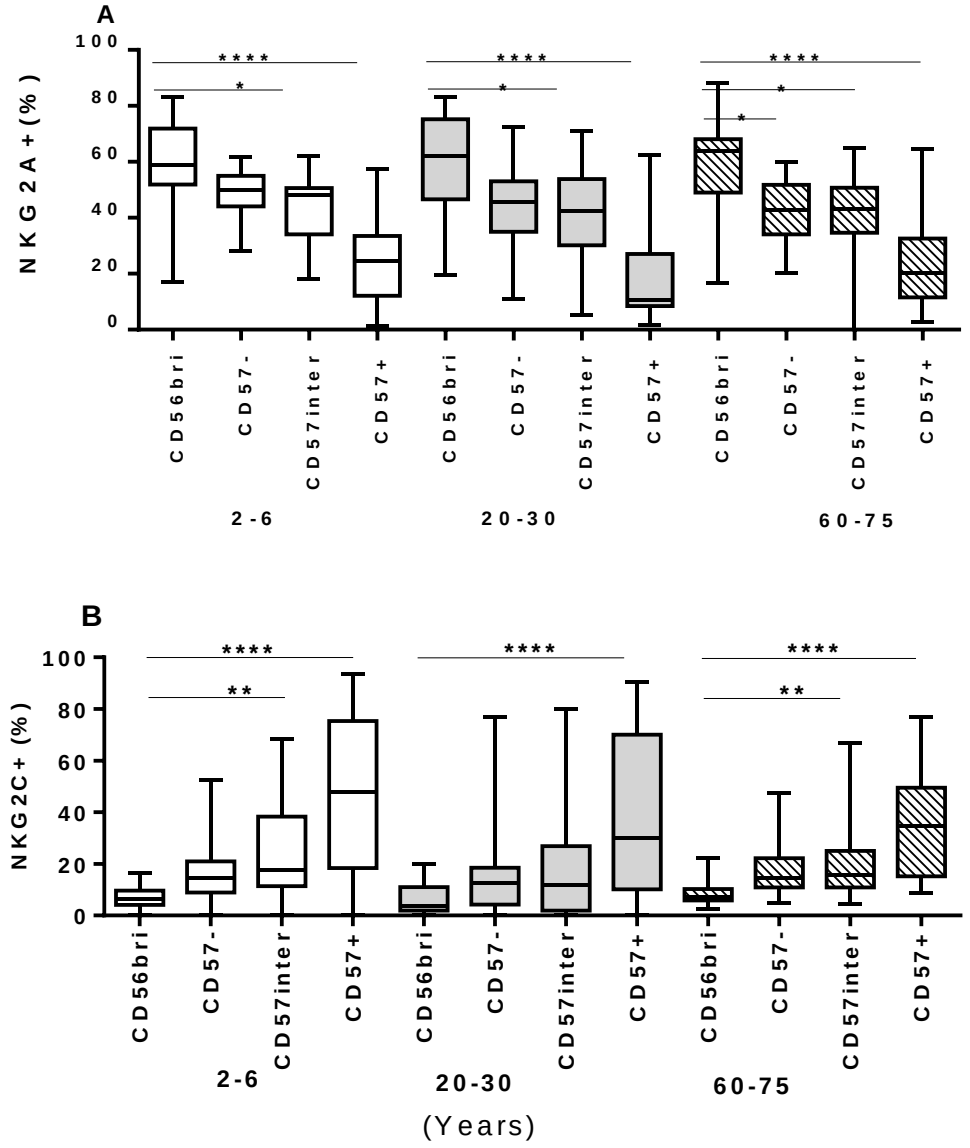

Figure 4.12: Baseline expression of NKG2A decreases and NKG2C increases, with the acquisition of CD57 receptors.

Expression of NKG2A and NKG2C in CD57-defined NK cell subsets (CD56bright, CD56dimCD57-, CD56dimCD57inter, and CD56dimCD57+) within each of the three age groups (2-6, 20-30, 60-75 years), gated from CD56+CD3- NK cells. These cells were stained ex vivo without stimulation. Data are shown for 68 subjects. Frequency of NKG2A (A) and NKG2C (B) NK cells among all NK cells. In box and whisker plots, the horizontal bar indicates median frequency, the boxes extend 25th-75th percentile range and the whiskers indicate 95th percentiles. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, *p<0.05, **p<0.01, ****p<0.0001.

4.4.3.4 The effect of vaccination on NK cell functional markers ex vivo

The expression of NK cell functional markers was investigated ex vivo to test whether any residual vaccine induced effects persisted 1 month after vaccination. The only significant effect of vaccination was that CD107a expression was slightly but significantly decreased in children post vaccination compared to pre-vaccination, consistent with a higher frequency of CD56bright cells in the children (Figure 4.13A). Ex vivo staining reveals no significant expression of CD25 and IFN-γ before vaccination and no significant up-regulation up to 4 weeks post-vaccination (Figure 4.13 B,C).

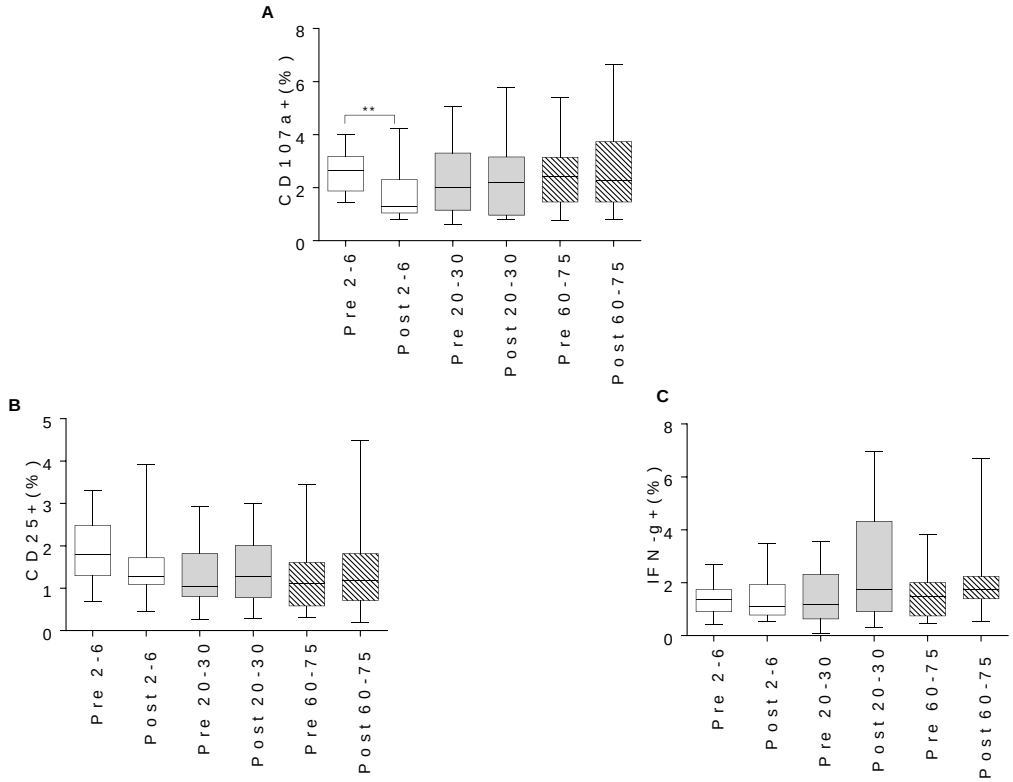

Figure 4.13: Ex vivo expression of CD25, CD107a, and IFN-γ by NK cells before and after influenza vaccination.

Ex vivo NK cell (A) CD107a, (B) CD25, and (C) IFN-γ frequencies at pre- and post-vaccination within the three age groups (2-6, 20-30, 60-75 years), gated from CD56+CD3- NK cells. These cells were stained ex vivo without stimulation. Data are shown for 68 subjects. In box and whisker plots, the horizontal bar indicates median frequency, the boxes extend 25th-75th percentile range and the whiskers indicate 95th percentiles. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, *p<0.05.

4.4.3.5 Pre- and post-vaccination NK cell responses to in vitro restimulation with influenza antigen

I hypothesized that trivalent influenza virus (TIV) immunization will induce influenza antigen-specific CD4+ helper T cells and influenza-specific antibodies which enhance NK cell activation and effector function on re-exposure to similar antigens. Because different people will have different levels of pre-existing influenza IgG antibodies, I initially standardised our experiment using pooled AB+ serum in all culture conditions.

No change in the frequencies of CD107a, CD25 or IFN-γ expressing NK cells were observed after vaccination following restimulation of PBMC with TIV antigens alone or TIV in combination with low concentrations of accessory cell cytokines (LCC: rIL-12 12.5pg/ml + rIL-18 10ng/ml) (Figures 4.14, 4.16, and 4.17). Baseline NK cell responses to antigens were not significantly different from those observed at 4, 12 and 24 weeks after vaccination. However, the addition of TIV antigens induced a significant CD107a production (****$p<0.0001$) at baseline, 4, 12 and 24 weeks after vaccination (Figures 4.14).

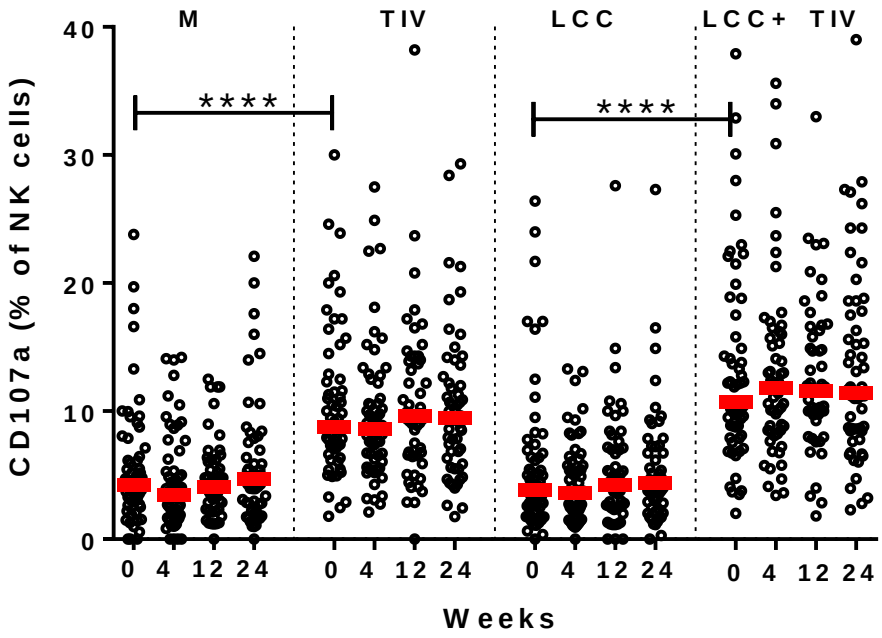

Figure 4.14: CD107a+ NK cells were not induced by in vitro restimulation post influenza vaccination.

Frequencies of CD107a+ NK cells at baseline (Week 0) compared to 4, 12 and 24 weeks post TIV vaccination, gated from CD56+CD3- cells. The cells were cultured in (M) Medium alone, TIV alone, (LCC) low concentration of cytokines [rIL-12: 12.5 pg/ml & rIL18: 10 ng/ml] alone and LCC plus TIV vaccine antigen. Data are shown for 61 subjects; each dot representing the frequency of CD107a+ NK cells from a single individual, the red bar represents the median frequency. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, ****p<0.0001.

In addition, we hypothesised that influenza antigen-driven NK cell responses to influenza antigens may decline with increasing age of vaccinated individuals as a result of either impaired T or B lymphocyte responses or of age-associated changes in NK cell phenotype and function. However, there was no significant difference in CD107a expression between the three different age groups (Figure 4.15). This analysis is based on LCC/TIV as this gave the optimal responses in our experiment.

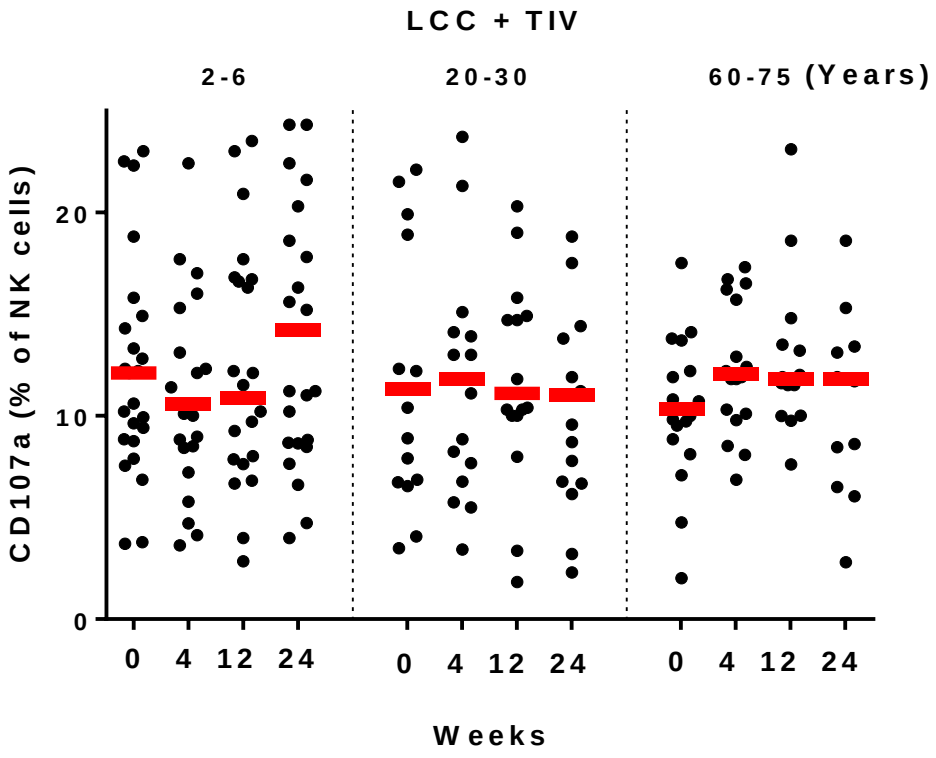

Figure 4.15: No significant difference in TIV-induced NK cell CD107a+ expression between age groups after vaccination.

NK cell CD107a expression in response to LCC or LCC/TIV at baseline (Week 0) compared to 4, 12 and 24 weeks post TIV vaccination, stratified by age group (2-6, 20-30, 60-75 years), gated from CD56+CD3- cells. The cells were cultured in (LCC) low concentrations of cytokines [rIL-12: 12.5 pg/ml & rIL18: 10 ng/ml] plus TIV antigen (LCC+ TIV). Data are shown for 61 subjects; each dot representing the frequency of CD107a+ NK cells from a single individual, the red bar represents median frequency. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test.

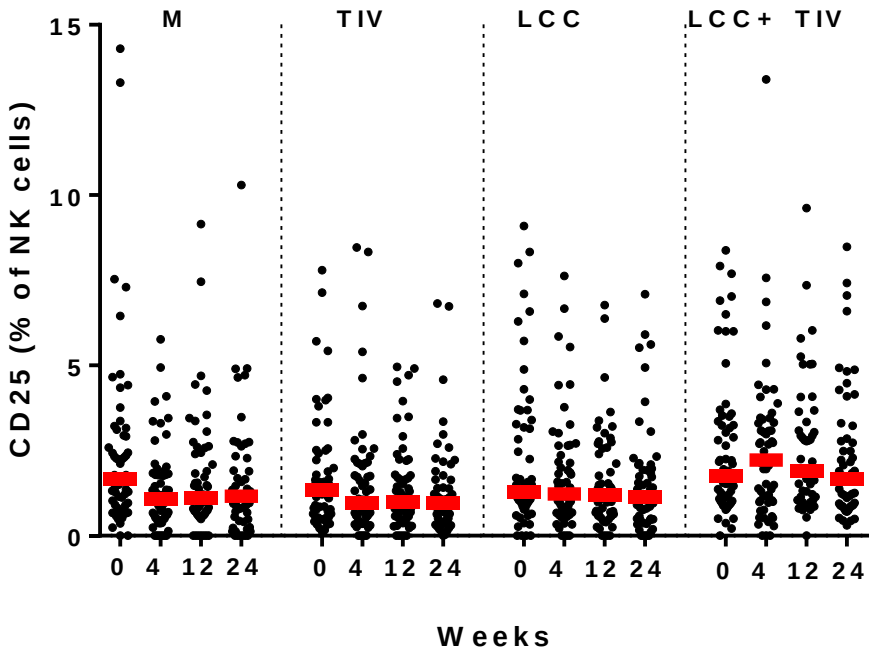

Figure 4.16: CD25+ expression by NK cells after in vitro restimulation with TIV antigens was not potentiated post influenza vaccination.

Frequency of NK cells expressing CD25 after in vitro restimulation at baseline (Week 0) compared to 4, 12 and 24 weeks post TIV vaccination, gated from CD56+CD3- NK cells. The cells were cultured in (M) Medium alone, TIV alone, (LCC) low concentrations of cytokines [rIL-12: 12.5 pg/ml & rIL18: 10 ng/ml] alone and plus TIV vaccine antigen (LCC+ TIV). Data are shown for 61 subjects; each dot representing the frequency of CD25+ NK cells from a single individual, the red bar represents median frequency. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test.

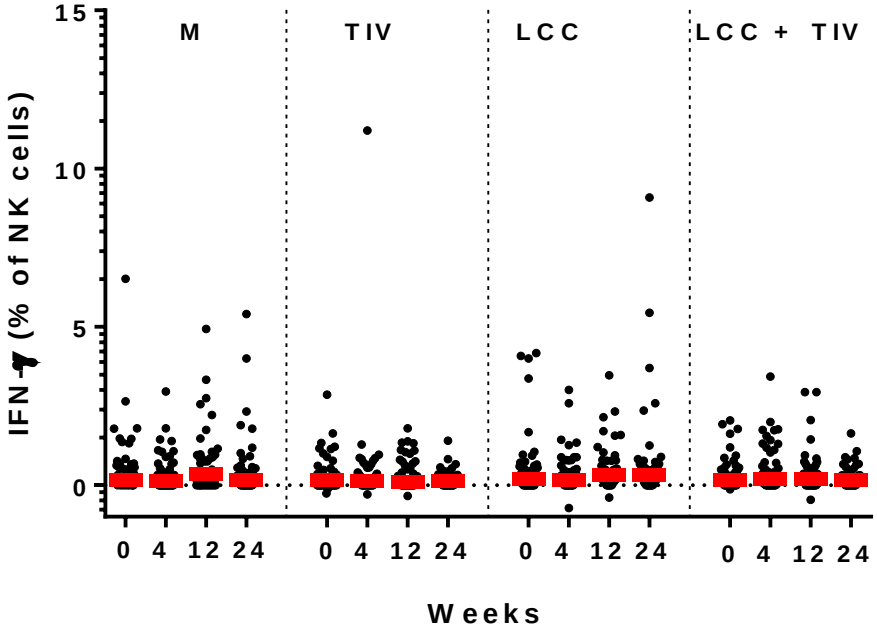

Figure 4.17: No significant potentiation of NK cell IFN-γ production in response to TIV post influenza vaccination.

Proportion of IFN-γ by NK cells after in vitro restimulation at baseline (Week 0) compared to 4, 12 and 24 weeks post TIV vaccination, gated from CD56+CD3- cells. The cells were cultured in (M) Medium alone, TIV alone, (LCC) low concentrations of cytokines [rIL-12: 12.5 pg/ml & rIL18: 10 ng/ml] alone and plus TIV vaccine antigen (LCC+ TIV). Data are shown for 61 subjects; each dot representing the proportion of IFN-γ+ NK cells from a single individual, the red bar represents median frequency. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test.

4.4.3.6 Vaccination enhances NK cell IFN-γ responses to high concentrations of accessory cell cytokines

The effector function of NK cells post-vaccination was also assessed after stimulation in vitro with high concentrations of rIL-12 and rIL-18 in combination. I did not observe a change in CD107a and CD25 expression in response to rIL-12 and rIL-18 after vaccination (Figure 4.18A, B). However, I saw a significant enhancement of NK cell IFN-γ responsiveness to rIL-12 and rIL-18 up to 24 weeks post-vaccination (Figure 4.19C).

Changes in cytokine-induced IFN-γ production were then assessed within the three age-defined strata. Considerably higher frequencies of IFN-γ producing NK cells were

observed within the youngest age group compared to older people at baseline (0) and 24 weeks only (Figure 4.20). Although there is a trend towards the enhancement of IFN-γ production post influenza vaccination in those aged between 2-6 and 20-30 years, it was only statistically significant in the oldest age group (60-75 years, **p<0.01, (Figure 4.20).

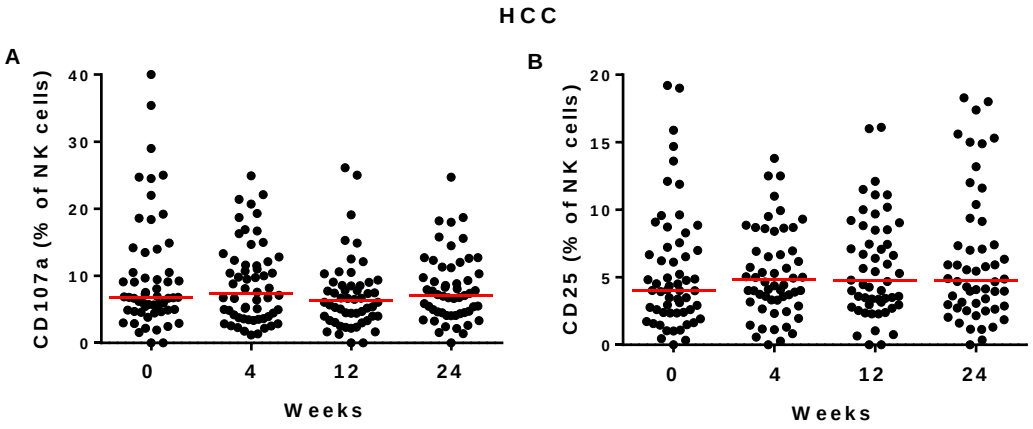

Figure 4.18: High concentrations of cytokines did not potentiate NK cell CD107a and CD25 responses post-vaccination.

NK cell CD107a and CD25 expression after in vitro stimulation with HCC at baseline (Week 0) and 4, 12, and 24 weeks post TIV vaccination, gated from NK cells. PBMC were cultured in high concentrations of cytokines alone (rIL-12 5ng/ml + rIL-18 50ng/ml). Data are shown from 65 subjects, each individual dot representing the frequency of CD107a+ or CD25+ NK cells before and after vaccination, the red bar represents median frequency. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test.

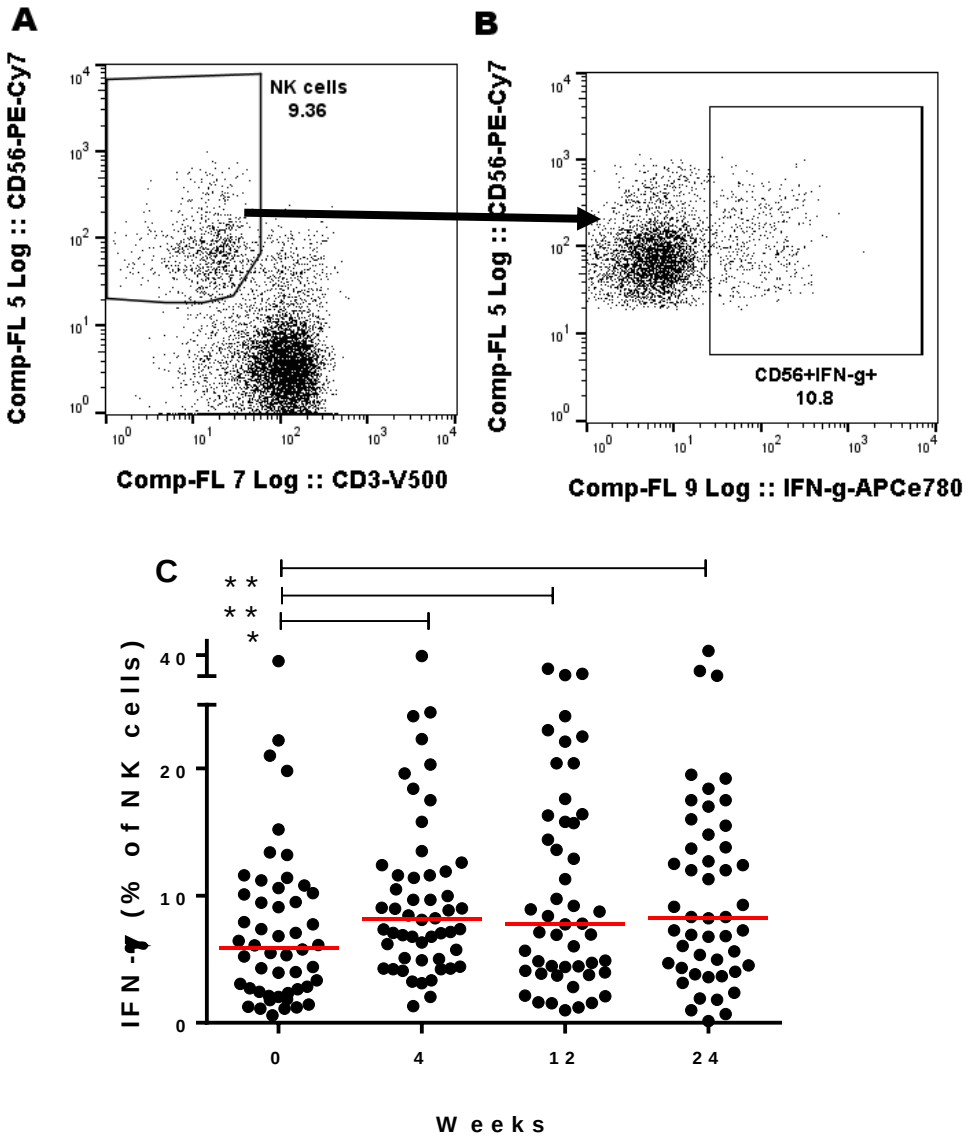

Figure 4.19: NK cell IFN-γ responses to high concentrations of cytokines are significantly enhanced up to 24 weeks post vaccination.

NK cell IFN-γ (C) expression after restimulation with HCC at baseline (Week 0) NK cells and 4, 12 and 24 weeks post TIV vaccination, (A,B) gated from NK cells. PBMC were cultured in high concentrations of cytokines alone (rIL-12 5ng/ml + rIL-18 50ng/ml). Data are shown from 65 subjects; each dot represents the frequency of IFN-γ+ NK cells in a single individual before and after vaccination, red bar represents median frequency. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, *p<0.05, **p<0.01. Representative dot plots from participant S11 visit 1 (21.6 years) recruited from the influenza vaccination study.

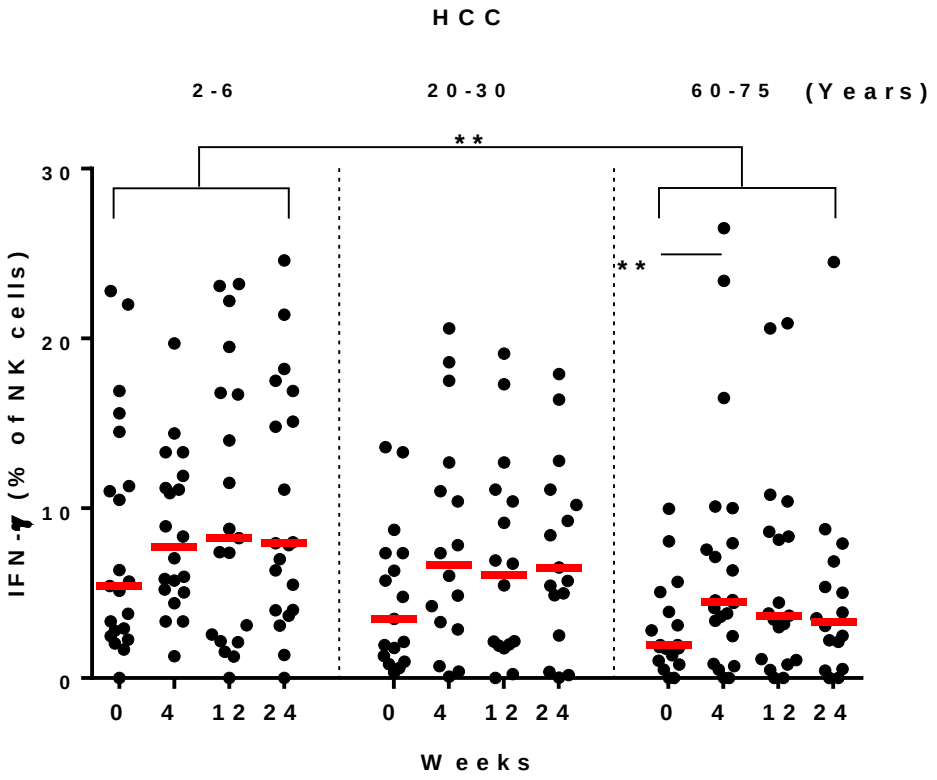

Figure 4.20. Vaccination-induced potentiation of NK cell IFN-γ response to cytokines in old adults only (60-75 years).

Age-stratified (2-6, 20-30, 60-75 years) NK cell IFN-γ expression after in vitro restimulation with HCC at baseline (Week 0) NK cells and 4, 12 and 24 weeks post TIV vaccination, gated from CD56+CD3- cells. PBMC were cultured in high concentrations of cytokines alone (rIL-12 5ng/ml + rIL-18 50ng/ml). Data are shown from 61 subjects; each dot represents the frequency of IFN-γ+ NK cells in a single individual before and after vaccination, the red bar represents median frequency. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, **p<0.01.

4.4.3.6.1 Vaccination-induced enhancement of IFN-γ production occurs mainly within CD56bright and CD56dimCD57- NK cells

As there was a significant potentiation of IFN-γ production in response to high cytokine concentrations post-vaccination, and as less well differentiated NK cells are known to respond better to cytokines, we wanted to investigate the source of the IFN-γ within the CD56- and CD57-defined NK cell subsets. No overall change in the proportion of the CD57 subsets was observed post-vaccination (Figure 4.21B). However, IFN-γ was primarily produced by CD56bright and CD56dimCD57- (CD57-) NK cells (Figure 4.22). Furthermore, it is these subsets which show a significant enhancement in the frequencies of IFN-γ production in post-vaccination samples in response to high concentrations of cytokines, with no enhancement being observed within CD56dimCD57+ (CD57+) NK cells (Figure 4.22).

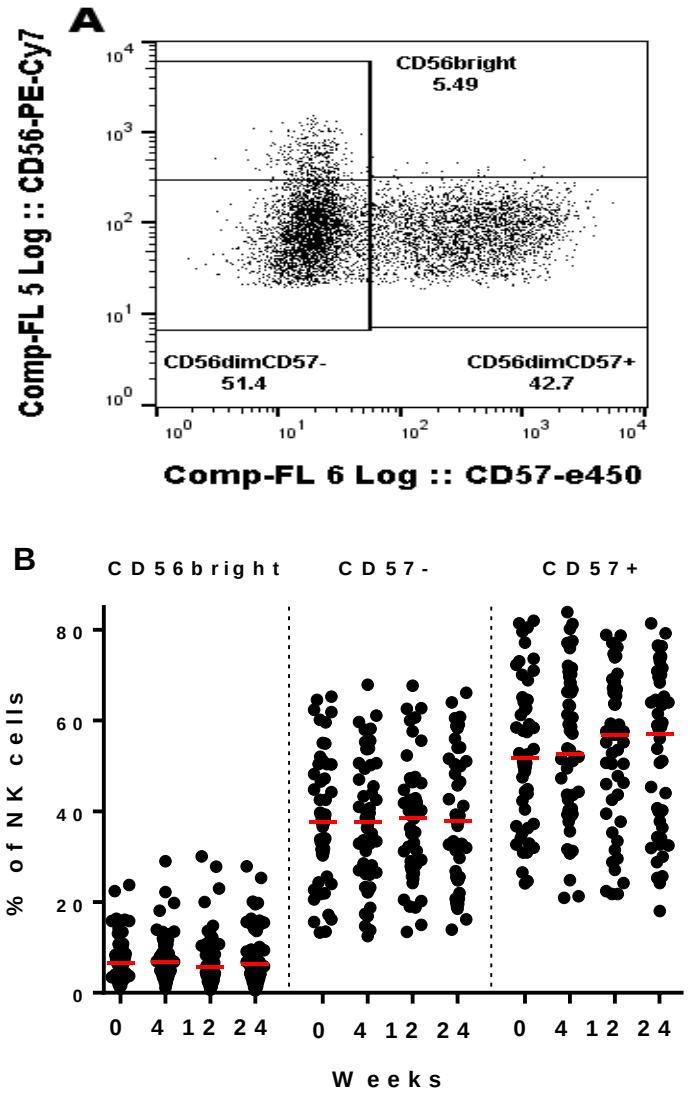

Figure 4.21: No change in the frequencies of CD56 and CD57-defined NK cell subsets after vaccination.

Frequencies of CD56 and CD57-defined (CD56bright, CD56dimCD57- and CD56dimCD57+) NK cell subsets (B) at baseline (Week 0) NK cells compared to 4, 12 and 24 weeks post TIV vaccination, (A) gated from CD56+CD3- cells. PBMC were cultured in high concentrations of cytokines alone (rIL-12 5ng/ml + rIL-18 50ng/ml). Data are shown from 65 subjects; each dot represents the frequency of NK cells in a single individual before and after vaccination, the red bar represents median frequency. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test. Representative dot plots from participant S47 visit 1 (2.2 years) recruited from the influenza vaccination study.

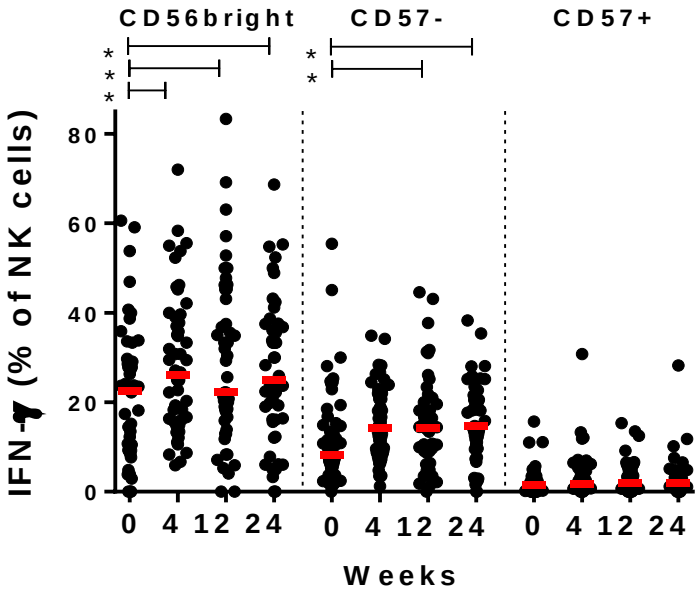

Figure 4.22: Post vaccination enhancement of IFN-γ production by high concentration of cytokines was due to increased IFN-γ production by CD56bright and CD56dimCD57- NK cells.

HCC induced IFN-γ production among CD56bright, CD56dimCD57- and CD56dimCD57+ NK cell subsets at baseline (Week 0) NK cells compared to 4, 12 and 24 weeks post TIV vaccination, gated from CD56+CD3- cells. PBMC were cultured in high concentrations of cytokines alone (rIL-12 5ng/ml + rIL-18 50ng/ml). Data are shown from 65 subjects; each dot represents the frequency of IFN-γ+ NK cells in a single individual before and after vaccination, the red bar represents median frequency. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, *p<0.05.

4.4.3.6.2 Enhanced frequencies of IFN-γ producing CD56dim NK cells within both NKG2C-CD57- and NKG2C+CD57- subsets after influenza vaccination

It has been shown in Chapter 3 that there is a high exposure of HCMV infection within Gambian population, with nearly all individuals being sero-positive by 3 years of age (Bello, 1992, Goodier et al., 2014, Bello and Whittle, 1991).

As expansions of NKG2C+/CD57+ NK cells have been directly linked to HCMV infection and these cells come to dominate the NK cell repertoire in our Gambian study subjects from an early age (Chapter 3), to specifically delineate which NK subset was producing the IFN-γ, we categorised the CD56dim NK cells according to the expression of these late markers of differentiation, that is, NKG2C and CD57. There was no change in the frequency of NKG2C and CD57-defined subsets before and after vaccination (Figure 4.23C), except for a small but apparently significant decrease, in the proportion of NKG2C-CD57+ NK cells at 12 weeks post vaccination compared to baseline.

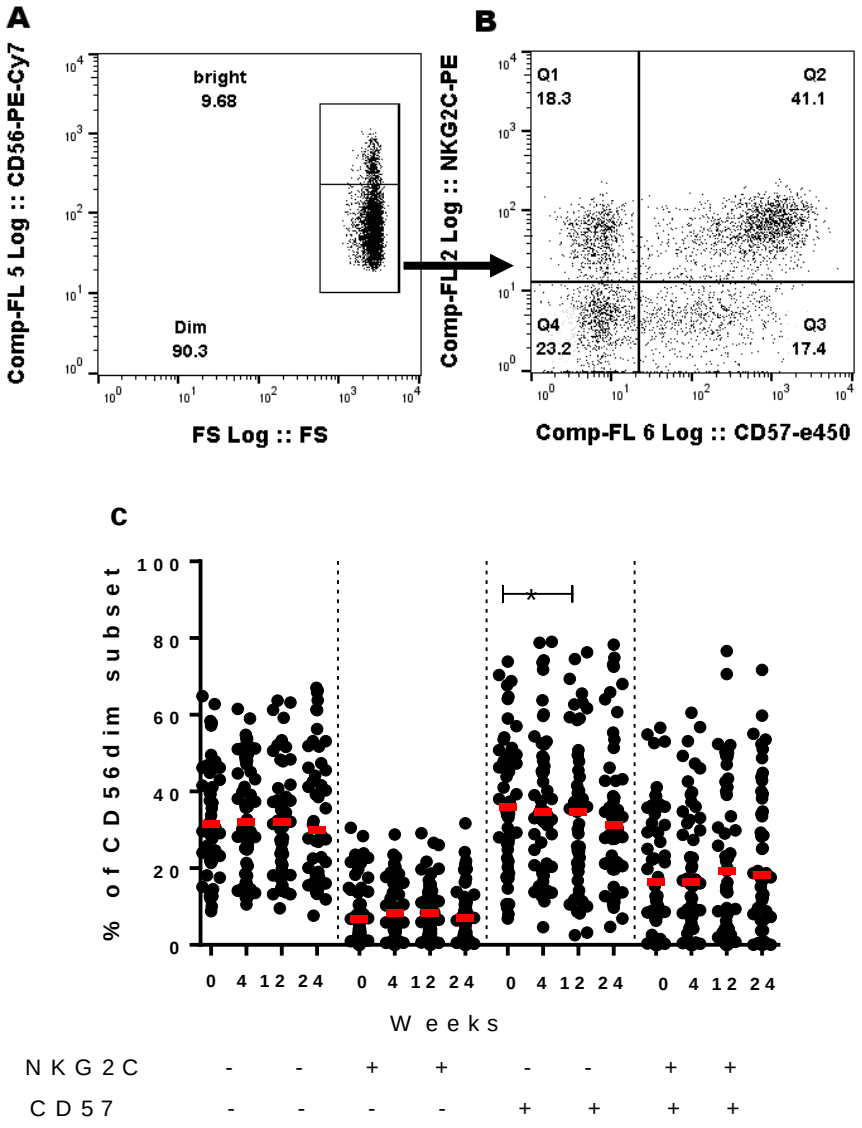

Figure 4.23: The frequency of NKG2C and CD57-defined subsets does not change post vaccination.

Frequency of NKG2C and CD57-defined NK cells subsets, (NKG2C-CD57-, NKG2C+CD57-, NKG2C-CD57+, and NKG2C+CD57+) at baseline (Week 0) compared to 4, 12 and 24 weeks post TIV vaccination, gated from CD56dim cells. PBMC were cultured in high concentrations of cytokines alone (rIL-12 5ng/ml + rIL-18 50ng/ml). Data are shown from 65 subjects; each dot represents the frequency of NK cells in a single individual before and after vaccination. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test. Representative dot plots from participant S57 visit 1 (63.6 years) recruited from the influenza vaccination study.

Higher frequencies of NK cells producing IFN-γ in response to HCC were observed within both NKG2C-CD57- and NKG2C+CD57- subsets but not within CD57+ cells (Figure 4.24). These data are consistent with CD56dimCD57- cells making superior responses to cytokines, whether or not they express NKG2C. Enhancement of IFN-γ production in CD57-NKG2C+ cells also support a model whereby HCMV-driven expansions of these cells, which maintain strong cytokine responsiveness, occurs prior to the acquisition of CD57.

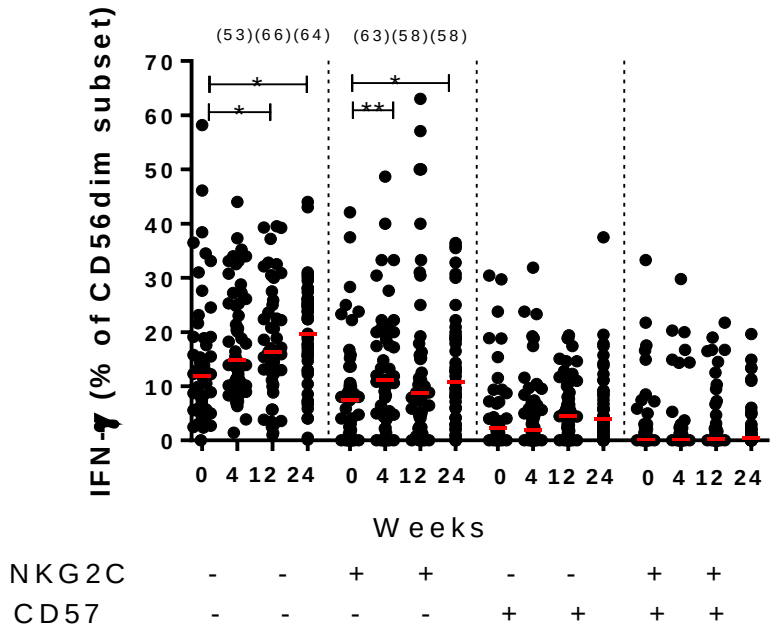

Figure 4.24: Increased HCC-induced IFN-γ among NKG2C-CD57- and NKG2C+CD57- CD56dim NK cells after vaccination.

HCC-induced IFN-γ production by distinct NKG2C/CD57-defined NK cells subsets, (NKG2C-CD57-, NKG2C+CD57-, NKG2C-CD57+, and NKG2C+CD57+) at baseline (Week 0) compared to 4, 12 and 24 weeks post TIV vaccination, gated from CD56dim NK cells. Values in brackets () indicate the percentage of responders post vaccination. PBMC were cultured in high concentrations of cytokines alone (rIL-12 5ng/ml + rIL-18 50ng/ml). Data are shown from 65 subjects; each dot represents the frequency of NK cells in a single individual before and

after vaccination. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, *p<0.05, **p<0.01.

4.4.3.7 The impact of vaccine-induced antibodies on NK cell responses

In order to investigate the role of vaccine-induced influenza-specific antibodies in NK cell responses, the amount of IgG specific for the trivalent vaccine and its three component strains were estimated in plasma. Overall, we observed a significant enhancement of trivalent influenza virus (TIV)-specific antibodies post-vaccination (Figure 4.25A). However differences were observed in the patterns of antibody responses between age groups with 60-75 year old subjects having limited enhancement of antibody responses to TIV and H3N2 virus whilst showing induction of anti-influenza B and anti-H1N1 IgG antibodies (Figure 4.25 D,H,L,P). In contrast, antibody responses to TIV and influenza B were significantly enhanced in children and young adults after vaccination (Figures 4.25 B,C,F,J,G). However, H1N1 IgG antibodies were only weakly induced in the children, while anti-H3N2 antibodies were not induced in the young adults (Figure 4.25 N,K, respectively).

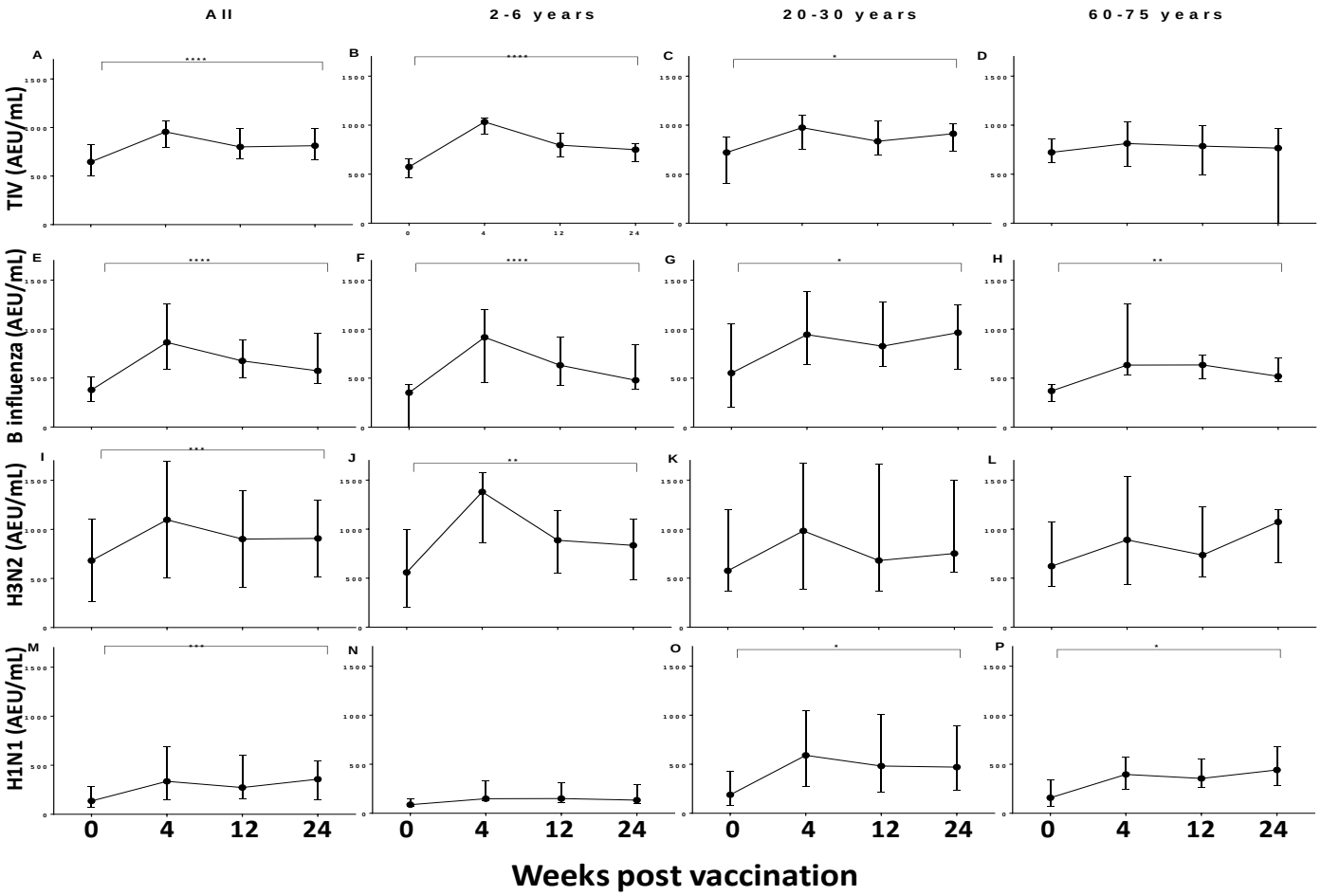

Figure 4.25: IgG antibody levels (expressed as Arbitrary ELISA Units (AEU)/mL) before and up to 24 weeks post TIV vaccination. Plasma IgG antibodies were measured by ELISA to whole TIV antigen (A-D), or to individual component antigens (E-P) in the entire cohort (A,E,I,M) or separately in each age group (B.C.D.F.G.H.J.K.L.N.O.P). Dots indicate median values, with 25th and 75th percentile range. (*p<0.05, ***p<0.001; ****p<0.0001, linear trend analysis using repeated measure ANOVA).

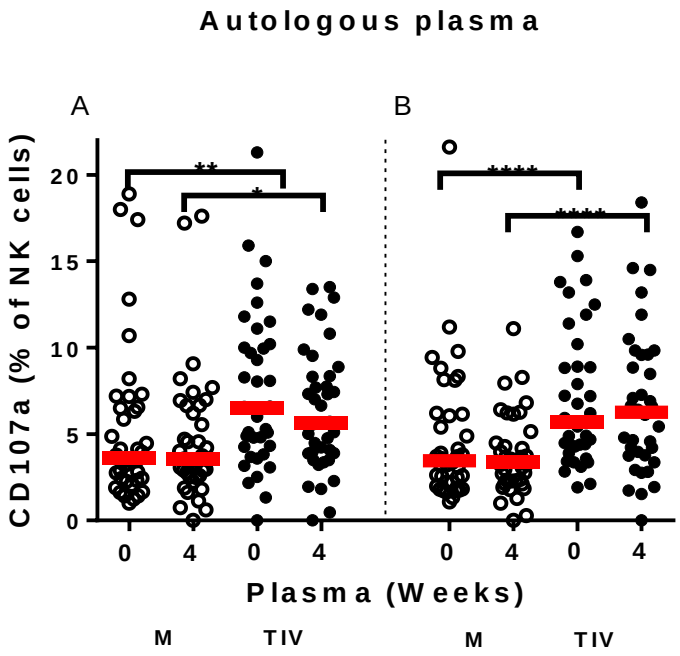

Figure 4.26: NK cell CD107a responses to TIV antigens when cultured in autologous plasma collected at baseline or at 4 weeks post vaccination.

NK cells collected at baseline (A) or 4 weeks after vaccination (B) were cultured in vitro with medium alone or with TIV antigen in the presence of autologous plasma collected at baseline or at 4 weeks after vaccination and analysed for CD107a expression. Population gated from CD56+ cells. Each dot represents the proportion of all NK cells expressing CD107a for one individual. The red bar indicates the median. Wilcoxon signed-rank test, *p<0.05, **p<0.01, ****p<0.0001.

Compared to medium alone, TIV antigen induced significant NK cell CD107a expression whether the cells were cultured in baseline plasma or post vaccination plasma (Figure 4.26); responses were similar for NK cells collected at baseline or at 4 weeks post vaccination. These data suggest that even baseline plasma samples contained sufficient antibody to TIV to allow ADCC-like activation of NK cells to take place. Because the availability of cells from the young children was limited, these experiments were performed mainly on samples from young adults and the oldest age group and it is important to bear in mind that the antibodies to TIV and H3N2 were not significantly enhanced by vaccination in the oldest age group (Figure 4.25).

As we had a variable range of antibody responses post vaccination, we decided to split the analysis of NK cell CD107a frequencies based on the baseline antibody titre

to compare people with low IgG antibody titre to those who already had high IgG antibody at baseline.

Looking at the responses of NK cells taken at baseline and 4 weeks after vaccination, we observed a trend towards individuals with low baseline titres to have an increased CD107a response to antigen in plasma taken after vaccination, whereas those with higher titres tended to have reduced responses, although none of these effects were significant (Figure 4.27). These effects are however consistent with previous observations in European subjects where high levels of anti-influenza antibody are induced by vaccination which tend to suppress NK cell degranulation responses (Goodier et al, 2016). Similarly, there were no significant difference in CD25 and IFN-γ responses between people with low IgG and high IgG at baseline both for pre-and post-vaccination NK cells (Figure 4.28, 4.29).

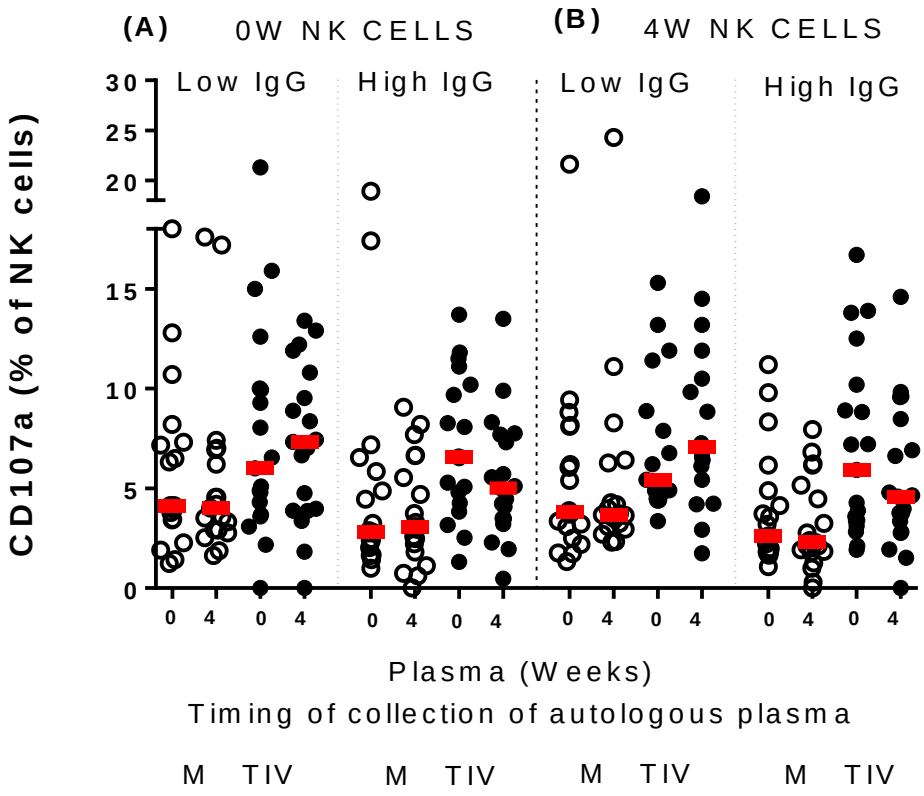

Figure 4.27: CD107a responses to TIV and autologous plasma stratified by baseline anti-TIV.

The frequency of NK cells collected either at baseline (A) or 4 weeks post vaccination (B) expressing CD107a after in vitro culture with medium alone (M) or with TIV antigens (TIV) in the presence of autologous plasma collected at baseline (0) or 4 weeks after vaccination (4). Data are stratified by the titre of anti-TIV IgG at baseline (Low = below and High = above the median value for the cohort, respectively). Data are shown for 38 subjects; each dot representing the frequency of CD107a+ NK cells from a single individual, the red bar represents median frequency. Statistical analysis was performed on paired samples using Mann-Whitney U test.

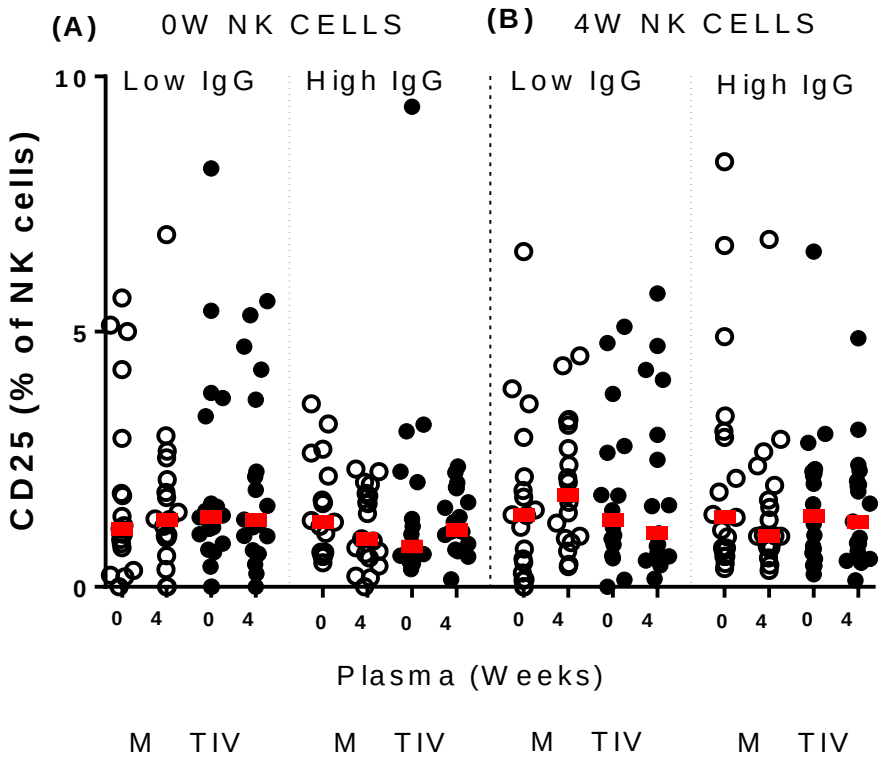

Figure 4.28: CD25 responses to TIV and autologous plasma stratified by baseline anti-TIV.

The frequency of NK cells collected either at baseline (A) or 4 weeks post vaccination (B) expressing CD25 after in vitro culture with medium alone (M) or with TIV antigens (TIV) in the presence of autologous plasma collected at baseline (0) or 4 weeks after vaccination (4). Data are stratified by the titre of anti-TIV IgG at baseline (Low = below and High = above the median value for the cohort, respectively). Data are shown for 38 subjects; each dot representing the frequency of CD25+ NK cells from a single individual, the red bar represents median frequency. Statistical analysis was performed on paired samples using Mann-Whitney U test.

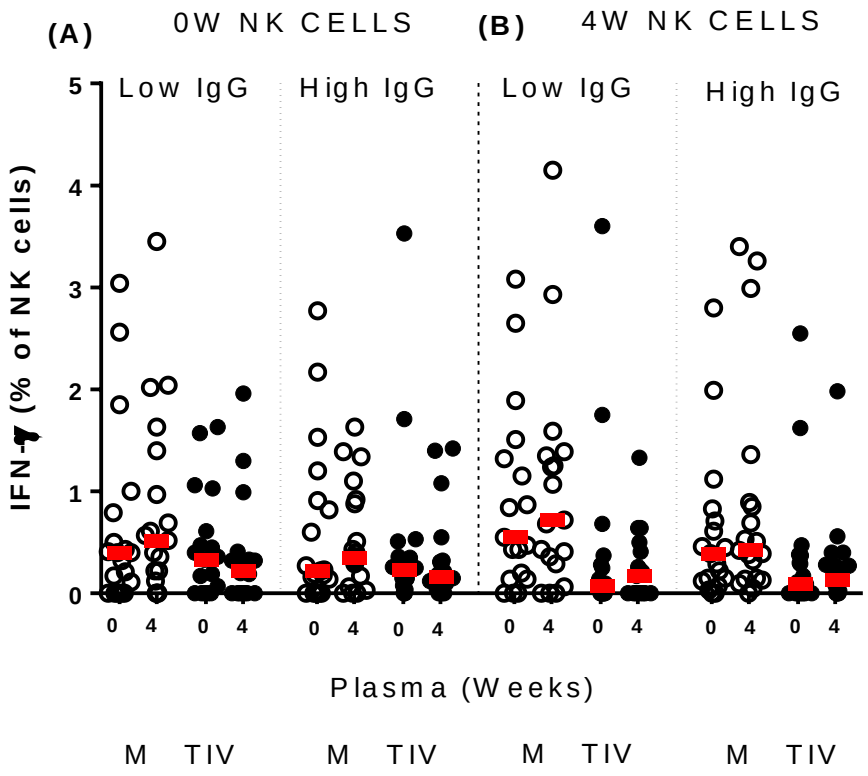

Figure 4.29: IFN-γ responses to TIV and autologous plasma stratified by baseline anti-TIV titre.

The frequency of NK cells collected either at baseline (A) or 4 weeks post vaccination (B) expressing IFN-γ after in vitro culture with medium alone (M) or with TIV antigens (TIV) in the presence of autologous plasma collected at baseline (0) or 4 weeks after vaccination (4). Data are stratified by the titre of anti-TIV IgG at baseline (Low = below and High = above the median value for the cohort, respectively). Data are shown for 38 subjects; each dot representing the frequency of IFN-γ + NK cells from a single individual, the red bar represents median frequency. Statistical analysis was performed on paired samples using Mann-Whitney U test.

4.4.3.8 Antibody dependence of NK cell response to influenza virus antigen

In order to confirm the role of IgG antibodies in antigen-driven NK cell CD107a responses at 4 weeks, we used H3N2 antigen to compare responses in complete serum and IgG depleted serum (Figure 4.30). To minimise variations due to differences in antibody titre, a single pool of AB serum was used for all assays. Although we did not have sufficient cells remaining from enough individuals to achieve statistical significance in these experiments, overall CD107a NK cells

responses decreased when the cells are cultured in IgG depleted AB serum compared to complete serum.

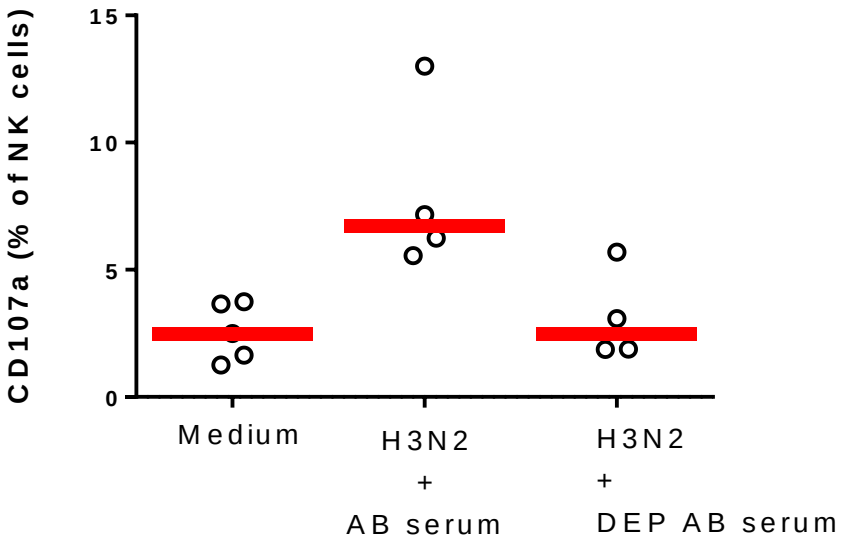

Figure 4.30: Effect of IgG depletion on NK cell CD107a responses to H3N2 antigen.

NK cells collected at 4 weeks after vaccination were PBMC cultured with or without H3N2 antigen in complete AB serum (10%) or IgG-depleted AB (DEP AB) serum (10%) and analysed for CD107a expression. Each dot represents data for one donor; the red bar represents median frequency, data from 4 subjects.

4.4.3.9 Influenza booster vaccination did not enhance NK cell responses in children

As none of the subjects studied here had been vaccinated previously and our antibody data indicated variable exposure to natural infection we took advantage of the required booster vaccination administered to the children in our cohort to test whether this would promote stronger NK cell responses. The booster vaccine was administered 36 weeks (9 months) after the primary vaccination and the samples were collected two weeks after boosting (i.e. 38 weeks after the primary vaccination). There was no significant enhancement of CD107a, CD25 or IFN-γ responses among the NK cells (Figure 4.31 A,B,C) two weeks post booster vaccination. Consistent with previous data that a single vaccination enhanced NK cell IFN-γ responsiveness to HCC cytokines (Figure 4.19C), enhancement of the IFN-γ response to HCC was maintained for at least 36 weeks after vaccination and was further enhanced by booster vaccination in some, but not in all children (Figure 4.32).

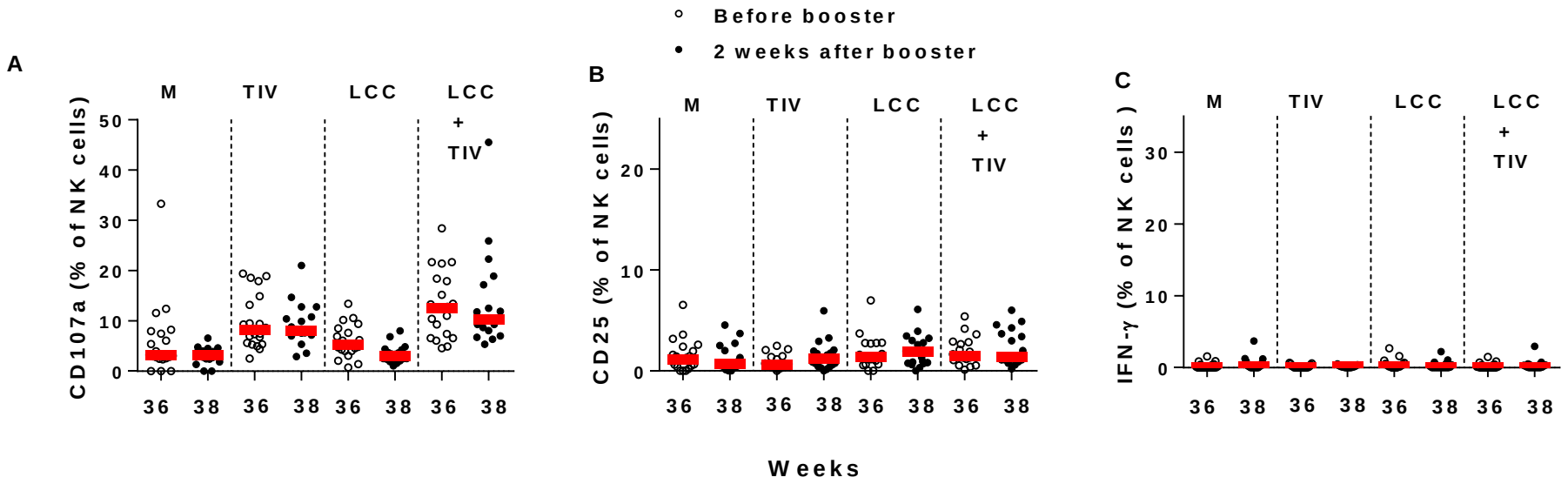

Figure 4.31: No significant enhancement of NK cell response after booster vaccination in children.

NK cell CD107a, CD25 and IFN-γ responses to TIV with or without LCC pre- and post booster vaccination in children (2-6 years), gated from CD56+. The cells were cultured in (M) Medium alone, TIV alone, (LCC) low concentration of cytokine [rIL-12: 12.5 pg/ml & rIL18: 10 ng/ml] alone or with TIV vaccine antigen (LCC+ TIV). Data are shown for 21 subjects; each dot representing the frequency of CD107a+, CD25+ or IFN-γ+ NK cells from a single individual, the red bar indicates the median frequency. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, *p<0.05.

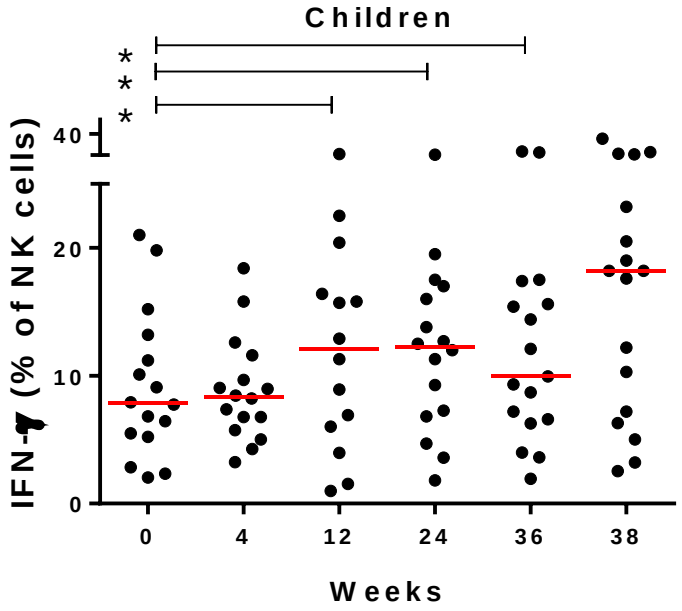

Figure 4.32: Booster vaccination enhances NK cell responsiveness to accessory cell cytokines in some children.

NK cell IFN-γ responses to HCC in children at baseline (Week 0) compared to 4, 12, 24, 36 and 38 weeks post TIV vaccination. Booster vaccination was given at 36 weeks, cell populations were gated from CD56+ NK cells. PBMC were cultured in high concentrations of cytokines alone (rIL-12 5ng/ml + rIL-18 50ng/ml). Data are shown from 21 subjects, each dot represents the frequency of IFN-γ NK cells in a single individual before and after vaccination, the red bar indicates the median frequency. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, *p<0.05.

4.4.4 No significant induction of CD4 IL-2 post vaccination

CD4+ T helper cell production of IL-2 is an essential component in the potentiation of NK cells in vaccination (Horowitz et al., 2010, White et al., 2014). We hypothesised that CD4 T IL-2 will enhance NK cell responses in our cohort after vaccination. Therefore, we examined the production of IL-2 from CD4 T cell pre and post influenza vaccination. To our surprise we did not observe IL-2 production both in the presence of TIV antigens compared to cells cultured in medium alone, except 12 weeks after vaccination in TIV antigen restimulated cultures (Figure 4.33D). This observation

could partly justify why we did not see potentiation of NK cell responses after vaccination in our cohort.

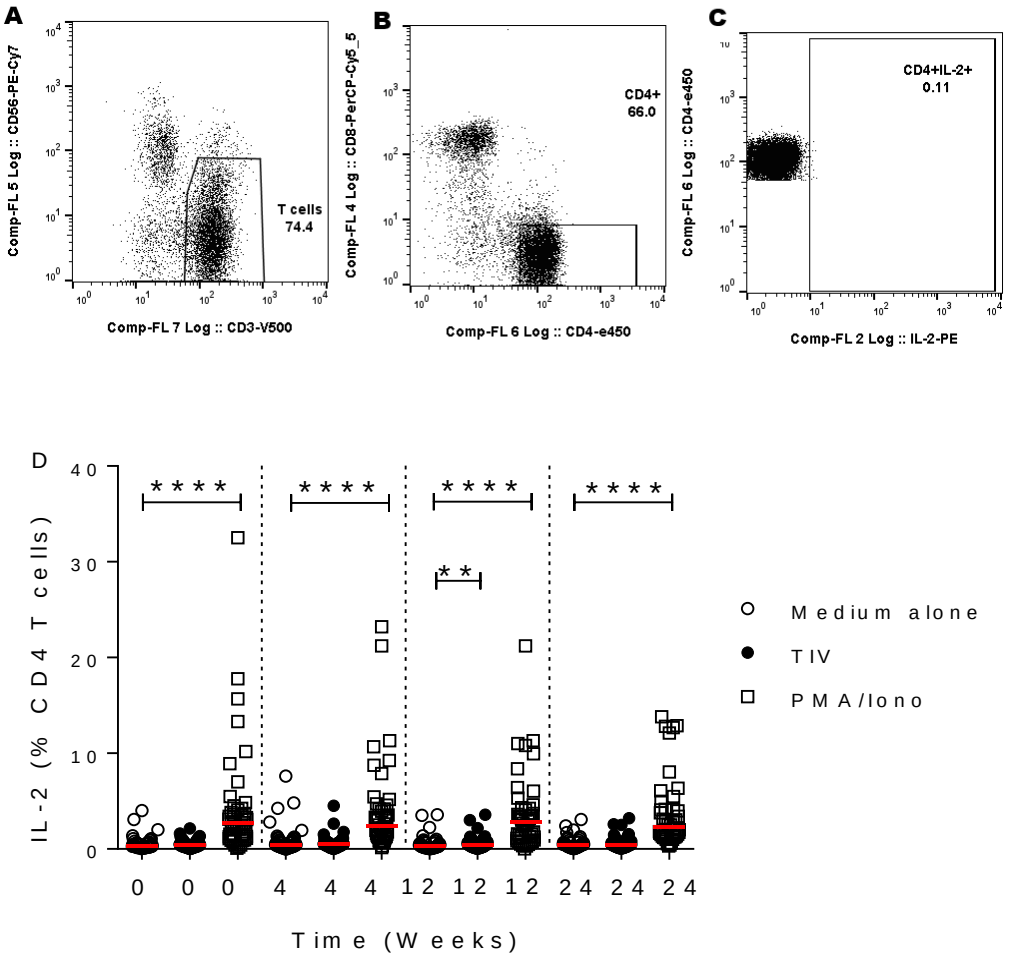

Figure 4.33: No significant change in CD4 T cell IL-2 secretion post vaccination.

CD4 IL-2 responses (D) at baseline (Week 0) compared to 4, 12, and 24, post TIV vaccination. (A-C) Gating strategy for the analysis of CD4 IL-2 function. PBMC were cultured in medium (M) alone and the presence of TIV antigens (2.5 µg/ml) and PMA/iono for 5 hours. Data are shown from 64 subjects, each dot represents the frequency of IL-2+ CD4+ T cell in a single individual before and after vaccination, the red bar indicates the median frequency. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, *p<0.05.

4.4 DISCUSSION

Vaccination is aimed at priming naïve antigen specific T and B cells to expand and differentiate into memory effector cells essential for the clearance of microbial pathogens. Vaccine-induced memory cells should direct innate effector cell activation on subsequent exposure to infectious agents to provide protective immunological responses. NK cells have the potential to be activated by pathogen-specific antibody cross-linking of FcγRIII (CD16) and by IL-2 derived from CD4+ T cells after vaccination. The latter mechanism has been demonstrated using rabies, malaria, hepatitis B virus, and BCG vaccines (Evans et al., 2011, Horowitz et al., 2010, Horowitz et al., 2012). Memory CD4+ T-cell derived IL-2 is important in influenza vaccination, as it has previously been shown that NK cell IFN-γ production was elevated in a T cell-dependent manner following influenza infection (He et al., 2004, Long et al., 2008).

In this study, I observed negligible enhancement of NK cell responses up to 24 weeks post influenza vaccination. This impaired responsiveness could partially be attributed to the high prevalence of HCMV infection in this population. In a parallel study done in the UK using the same vaccines and protocol, NK cell IFN-γ IL-2 dependent responses and antibody-dependent responses to influenza virus were significantly potentiated up to 4 weeks post-vaccination. However, consistent with the data presented here, this response was significantly higher in HCMV sero-negative subjects compared to HCMV sero-positive subjects, highlighting the importance of HCMV infection in NK cell effector function (Goodier et al., 2016). It is likely that the HCMV sero-positive status of our study cohort could partially explain why we see this low level of responsiveness to influenza vaccination. It is well known that HCMV drives NK cells to become CD57+NKG2C+ mature cells; this phenotype is known to have limited ability to respond to cytokine-mediated activation, which would reduce their ability to respond to T cell IL-2 (White et al., 2014). Another reason why we observed negligible NK cell responses post vaccine could be that there was no significant induction of CD4 T cell IL-2 secretion post-vaccination. Meaning that, there is no antigen-driven IL-2 available to enhance NK cell responses in vitro.

Using exogenous high concentrations of cytokines, however, we saw a potentiation of NK cell IFN-γ production post vaccination. This effect is consistent with the data from a UK cohort, where a similar enhancement of cytokine responses was observed after vaccination, importantly only in HCMV sero-positive individuals (Goodier et al, 2016).

Furthermore, enhancement of cytokine-induced IFN-γ production occurred predominantly within the CD56bright and CD56dimCD57- NK cells consistent with a dominant effect on less differentiated NK cells.

Nielsen et al (2015) have previously shown that HCMV sero-positive individuals have impaired NK cell responses to pertussis and H1N1 influenza vaccine antigens. They also showed that CD56dimCD57+ NK cells did not respond to IL-2 induction and released lower amounts of IFN-γ (Nielsen et al., 2015, White et al., 2014). This is consistent with what we have observed in our study, as CD56dimCD57+ NK cells produced negligible amount of IFN-γ before and post vaccination when stimulated with exogenous cytokines. However, it should be noted that the acquisition of CD57 and NKG2C and low levels of IL-18Rα expression on HCMV positive NK cells does not fully explain the impaired responses seen in HCMV infected people (White et al., 2014, Nielsen et al., 2015). Enhancement of CD56dimNKG2C+CD57- NK cell after vaccination to exogenous cytokines, suggests that these cells are similar to the less differentiated NK cells which have been generated in vitro by cytokines pre-activation and have been described as cytokine-induced 'memory-like' NK cells (Cooper et al., 2009, Berrien-Elliott et al., 2015). These cells might also be comparable to those described by Goodier et al., showing that CD56dimCD57- NK cells expressed more Ki67+ and CD71+ receptors and were more sensitive to cytokine responses (Goodier et al., 2016). However, that study was performed in European donors with limited expansions of NKG2C+ NK cells and was therefore not unable to detect immature CD57-NKG2C+ NK cells (Goodier et al 2016). In addition, the booster vaccine that was given to the children showed that a secondary influenza booster vaccine was still not sufficient to induce NK cell responses in vitro, although there was potentiation of NK responsiveness to exogenous cytokines in most donors.

The secondary aim of the study was to examine age-related differences between the children and the older adults. Our in vitro and ex vivo results show that there is a significant variation in the number of NK cells, naïve CD4+ and CD8+ T cells and B cells between the different study age groups. Although NK cell proportions were higher in the older adult group, the proportions of naïve CD4+ and CD8+ T cells and B cells were considerably lower than in the children. This has implications in terms of developing an effective vaccination response to prevent influenza infections across different age groups. The availability of these cells is important in order to develop an

effective adaptive immune response. The diminished number of naïve CD4+ T cells in the older adults might have had a great impact on the ability to generate IL-2 producing influenza-specific CD4+ T cells and therefore on the potency of NK cell activation through vaccination. It has been shown that pre-existing memory CD4+ T cells were more important in providing protection in influenza vaccinated individuals than CD8+ T cells (Wilkinson et al., 2012).

Specifically, we have shown that the proportion and absolute numbers of total CD56+ and CD56dim NK cells increased, whilst the number of CD56bright NK cells diminished with increasing age. Furthermore, CD57 has previously been shown to be a marker of terminal T cell and NK cell differentiation; CD56dimCD57+ NK cells exhibit a more mature status than CD56dimCD57- NK cells and are enriched for cells expressing high levels of the C-type lectin-like receptor NKG2C on their surface (Poli et al., 2009, De Colvenaer et al., 2011, Lopez-Verges et al., 2011). CD56dimCD57+ cells also have a greater potential to be activated via direct receptor activation than indirect cytokine-mediated activation and these cells were shown to have reduced amount of IL-12Rβ2 mRNA than CD57- NK cells and have poor proliferative capacity (Lopez-Verges et al., 2010). In addition to changes in memory T cell subsets, these differences in functional NK cell subsets could have significant impact on the ability of different age-defined groups to respond to vaccine antigens.

In this study, no significant NK cell activation was observed ex vivo at 4 weeks post vaccination relative to baseline samples. This indicates that any systemic NK cell activation resulting from vaccination may be transient. Previous studies done by Long and colleagues (Long et al., 2008) have demonstrated that there was no significant change in NK cell and T cell proportions post vaccination (up to 8 weeks), nonetheless, there was an elevated production of IFN-γ by NK cells and T cells in 7 out of 8 vaccinated subjects in vitro. NK cells were the main source of IFN-γ in that study, 9 out of 10 subjects previously receiving an influenza vaccine prior to re-vaccination (Long et al., 2008). Another study in subjects immune to influenza A, showed that CD56bright NK cells were the main source of IFN-γ production compared to CD56dim NK cells. Blocking of IL-2 or depletion of T cells inhibited NK responses and addition of recombinant IL-2 reinstated the response (He et al., 2004).

The mechanism of expansion of NKG2C+ NK cells is not yet fully understood. This type of cell can be observed in our youngest age group (2-6 years), implying that there are likely modulatory effects of HCMV in the NK cell repertoire in children

(Noyola et al., 2012). Serological testing for HCMV in our study population indicates 97.1% seroprevalence. HCMV is known to drive NK cell maturation acquiring NKG2C+ and CD57+ receptors, cells bearing this receptor combination being defined as terminally differentiated NK cells in HCMV sero-positive individuals. A high frequency of NKG2C+ NK cells is also observed in HIV, Hantavirus, Chikungunya, HBV, and HCV infections, however in all cases, these were associated with concomitant HCMV infection (Beziat et al., 2012, Petitdemange et al., 2011, Bjorkstrom et al., 2011).

In conclusion, there is significant variation in NK cell, T cell and B cell numbers between old and young subjects. Furthermore, there is a significant alteration of phenotypic characteristics of NK cell subsets with increasing age, partially associated with HCMV infection, which might have impacted the efficiency of vaccine-driven NK cell effector function post primary and secondary influenza vaccination. CD4 T cell IL-2 secretion was not normally induced or enhanced after vaccination, however, significant induction of influenza-specific antibodies was observed. Nevertheless, increased NK cell responsiveness to cytokines was observed post vaccination, which was mainly driven by CD56bright and NKG2C+CD57- NK cells.

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5 CHAPTER 5:

DIPHTHERIA, TETANUS, PERTUSSIS,

& INACTIVATED POLIOVIRUS

BOOSTER VACCINATION STUDY

5.1 DECLARATION

Bakary Sanneh of the Infant Immunology Platform did the measurements of Diphtheria, Tetanus Toxoid and Pertussis antibody immunoassays. In addition, the Poliomyelitis 1 & 3 viruses neutralizing assays were done by Elishia Roberts, Scientific Officer at the Infant Immunology Platform, MRC Unit The Gambia, Vaccine & Immunity Theme, Fajara.

5.2 INTRODUCTION

Vaccination can potentiate NK cell responses through antigen-specific memory CD4 T cells, via immune-complexes and by intrinsic changes to NK cells induced by innate cytokines (Horowitz et al., 2010, Horowitz et al., 2012, Goodier et al., 2016). Chapter 4 demonstrated that seasonal influenza virus vaccination resulted in enhancement of the NK cell response to cytokines, despite having no significant impact on the in vitro response to vaccine or virus antigens. In that project none of the individuals studied were previously vaccinated against influenza and children, despite receiving a booster vaccination after 9 months, did not demonstrate vaccine induced enhancement of in vitro NK cell responses to antigen. With this observation in mind, I wanted to investigate the effect of administration of a more potent secondary vaccination. Therefore, I used booster vaccination with diphtheria toxin, tetanus toxoid, acellular pertussis and inactivated poliomyelitis conjugated vaccine (DTPiP), diseases against which all study subjects had been vaccinated in infancy, to see if this booster vaccination will potentiate NK cell responses.

One possibility for the absence of significant antigen-driven NK cell responses is that absence of previous vaccination and limited natural exposure to influenza could result in insufficient antigen-specific CD4+ T cells to support these responses, as shown in Chapter 4. The individual components of the DTPiP vaccine are known to induce protective antibodies and several studies have demonstrated that these components, or vaccines related to DTP, enhance the frequencies of antigen-specific CD4+ T cells producing IL-2 in vaccinated subjects (Sharma and Pichichero, 2012).

Severe diphtheria is caused only when the aerobic gram-positive bacillus *Corynebacterium diphtheria* are infected by a bacteriophage leading to the generation of toxins which inhibit cellular protein synthesis and lead to tissue damage (Centers for Disease Control and Prevention, 2016). Diphtheria/acellular pertussis/ tetanus toxoid (DPT) vaccination can induce IL-2 producing CD4+ T cells in adults (Sharma and Pichichero, 2012).

Tetanus is caused by an exotoxin called tetanospasmin, which is produced by *Clostridium tetani* bacterium. Despite the existence of very effective vaccines, tetanus still kills about 50 000 newborns and infants every year, mainly due to low coverage of immunization and poor medical health care facilities, specifically, in developing countries (Stock, 2015). Li Causi et al showed that tetanus booster vaccination induced memory CD4 T helper cell function and had bystander effects on central

memory T cells (Li Causi et al., 2015). IL-2 production by CD4+ T cells induced by tetanus vaccine can help NK cell responses promoting Th1 protective immunity (Garcia-Knight et al., 2015). Tetanus toxoid (TT)-specific CD4+ T cell IL-2 production was also induced after vaccination with the Meningococcal, C- and Y-Tetanus Toxoid (TT) conjugate vaccination. These data indicate that tetanus vaccination can promote potent CD4+ T cell production to help strengthen Th1 mediated immunity (Fuery et al., 2015).

The bacterium *Bordetella pertussis* is generally known to be an extracellular pathogen but it is now emerging that it can avoid immune recognition by infecting respiratory epithelial cells. It evades destruction by remaining in non-acidic lysosome-associated membrane-protein-1-negative vesicles (Lamberti et al., 2013). Th1 immunity is considered to be essential in the clearance of *B. pertussis* (Warfel and Merkel, 2013, Ryan et al., 1997). This protective mechanism is proven by the fact that the whole cell pertussis vaccine induces Th1 responses while acellular pertussis vaccine induces less-protective Th2 and Th17 responses, partially explaining the recent pertussis outbreaks (Higgs et al., 2012). Children who received whole pertussis vaccines were better protected than those who received the acellular pertussis vaccine (Klein et al., 2013). It has previously been shown that NK cells help *B. pertussis* clearance via IL-12 mediated production of IFN-γ, which potentiates macrophages and stimulates Th1 immune responses (Byrne et al., 2004). Protective immunity after pertussis vaccination is not lifelong (suggested to last between 4-12 years in children) highlighting the need for booster vaccination after this period (Wendelboe et al., 2005).

Poliovirus is an enterovirus belonging to the *Picornaviridae* family and causes paralytic diseases. Although there has been a significant reduction of polio globally, there are still a small number of countries where this disease is still endemic, with occasional outbreaks in other countries that are considered ‘polio free’. There are three types of poliomyelitis virus: type 1, 2 and 3. Type 2 is considered to be eradicated, type 3 is on the verge of eradication and type 1 is the one mainly in circulation (Bandyopadhyay et al., 2015, Racaniello, 2006). It has long been shown in mice that polio-specific CD4+ Th1 clones can confer protection of immunized mice and that the protection mechanism was dependent on both T and B cell contributions

(Mahon et al., 1995). It is also known that oral polio vaccine in combination with adjuvant can induce IL-2 and IFN-γ production (Dietrich et al., 2014).

Thus, there is sufficient evidence to indicate that these DTPiP vaccine antigens have the potential to induce CD4+ T helper cell IL-2 production and other supporting factors (e.g. antibodies), which could promote NK cell effector function after vaccination. We, therefore, investigated the impact of booster vaccination with Diptheria, Tetanus and Pertussis and Polio (Repevax) in adults with a record of prior vaccination. Thirty males between the ages of 20-35 years were recruited from Sukuta, The Gambia. Baseline blood samples were collected before giving a single dose of Repevax (which contains diphtheria toxoid, tetanus toxoid, acellular pertussis and inactivated poliomyelitis virus types 1, 2 & 3). Subsequently, a follow-up sample was collected 4 weeks post booster vaccination.

This DTPiP vaccine was used to investigate the effect of a single booster vaccination of the four different antigens on lymphocyte-dependent effector NK cell responses. Specifically, we examined the following objectives:

1. To investigate the levels of antibodies against Diphtheria toxin (DT), Tetanus (TT), Pertussis, and Poliomyelitis at baseline and 4 weeks post vaccination.

2. To assess, the peripheral blood NK cell responses to DT, TT, Pertussis and Polio in-vitro before and after booster vaccination in individuals who had initially been vaccinated during infancy.

5.3 METHODS & MATERIALS

Approval of this study was obtained from The London School of Hygiene & Tropical Medicine Ethics Committee, The MRC Unit The Gambia Scientific Coordinating Committee and The Gambia Government/ MRC Joint Ethics Committee and The Gambia Medicines Board (SCC application number: 1372v2, title: 'Does booster vaccination enhance IL-2 driven NK cell responses). This study was conducted in Sukuta, Kombo North district, West Coast region. We recruited 30 adult males between the age of 20-35 years, who had previously been vaccinated with DTP and oral polio vaccine when they were children and had not received a booster since childhood.

A single dose of Diphtheria toxoid, Tetanus toxoid, acellular component Pertussis and inactivated Poliomyelitis Virus Type 1, 2 & 3 (DTPiP) – trademark Repevax (Sanofi Pasteur MSD)- was given intramuscularly to all 30 subjects after collecting 30ml of baseline peripheral blood. Then, four weeks later another 30ml of peripheral blood was collected. All cells were separated and frozen as described in Chapter 2. PBMC were collected from 30 subjects at baseline, however, 4 weeks post booster vaccination we had 28 subjects. Data for 18 of these subjects will be presented in this chapter, as the remaining samples were saved for the in vitro autologous plasma assay because of limited number of available paired cells.

5.3.1 PBMC separation

PBMC were recovered from cryopreservation as described in Chapter 2 and were allowed to rest for 3-4 hours. NK cells were cultured in vitro, overnight for 18 hours at 37ºC, 5% CO_2, with 10% Human AB serum (Sigma-Aldrich®, Saint Louis, USA) with or without vaccine antigens: DTPiP vaccine at 0.5 IU/ml; single diphtheria toxin (1µg/ml) [NIBSC 69/017]; single tetanus toxoid (7.5 µg/ml) [NIBSC 02/232]; single pertussis toxin (1:400 000 dilution, *B. pertussis* [NIBSC vaccine strain 88/522] and single inactivated poliomyelitis (5 IU/ml) [NIBSC 12/104] vaccine antigens with or without low concentrations of rIL-12/18 (rIL-12: 12.5 pg/ml & rIL18: 10 ng/ml) or high concentrations of rIL-12/18 (rIL-12: 5 ng/ml & rIL18: 50ng/ml).

The functional characteristics of the NK cells were assessed using CD107a as a degranulation marker, CD25 as an activation marker and IFN-γ as a marker of cytokine production potential. The rationale and gating strategy for these markers are discussed in Chapter 2. The following fluorochromes were used to investigate the NK

cells: CD107a-FITC; NKG2C-PE, CD25-PerCP-Cy5.5, CD56-PE-Cy7, CD57-e450, CD3-V500, IFN-γ-APC-e780. Anti-CD107a was added to the cultures at the start of the culture period. Brefeldin A (GolgiPlug, BD Biosciences, Oxford, U.K.) 1/1000 final concentration and Monensin (GolgiStop, BD Biosciences, Oxford, U.K.) 1/500 final concentration were added at 15 hours to allow accumulation of intracellular IFN-γ. PBMC were acquired using LSRIII® Fortessa flow cytometer on FacsDiva® software. All FACS data analyses were performed using FlowJo® (TreeStar), the gating strategy is described in Chapter 2.

5.3.2 DTaP4 Multiplex Immunoassay antibody assay

The DTaP4 multiplex immunoassay (Bio-Rad laboratories, Inc, USA) was used to examine the antibody levels pre- and post-vaccination. This technique simultaneously detects plasma specific IgG antibody levels to diphtheria toxin, tetanus toxoid, and *Bordetella pertussis*.

Briefly, 100 µl of 1X PBS (Phosphate Buffered Saline 0,01 M; pH 7.2) was added to pre-wet the wells of the filter plate. Serial dilution of the pertussis standard and the diphtheria-tetanus standard and QC sera were prepared in serum dilution buffer (1X PBS Phosphate Buffered Saline 0,01 M; pH 7.2). Then, the subject samples were diluted at 1/200 and 1/4,000 dilution in serum dilution buffer. Additionally, the bead solution was set up at 4,000 beads/region/25 µl in serum dilution buffer. A vacuum was applied to the filter plate at 5 mmHg for 2-5 seconds using a vacuum manifold. The plate was blotted to remove the leftover solution. The multiplex bead solution containing diphtheria toxin, tetanus toxoid, and *Bordetella pertussis* conjugated to carboxylated microspheres (Bio-Rad laboratories, Inc, USA) was diluted in bead activation buffer (1xPBS, 2.5 mg/ml 1-ethul-3-(-3dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and 2.5 mg/ml N-hydroxy-sulphosuccinimide (Sulfo-NHS)) mixed and then 25 µl was added to each well. Subsequently, 25 µl of blank (serum dilution buffer), standard dilution series, QC sera and samples were added to the plate according to the plate layout. The plate was covered with aluminium foil, shaken for 30 seconds at 1100 rpm (revolutions per minute) on a shaker and was incubated for 30 minutes at room temperature, shaking at 600 rpm. At that time, the plate was washed three times with 100 µl of wash buffer (Phosphate Buffered Saline 0,01 M; pH 7.2).

R-phycoerythrin conjugated goat anti-human IgG (γ chain specific) (Jackson ImmunoResearch laboratories, Inc, USA) detection antibody was added to the

respective wells, covered with aluminium foil, vortexed for 30 seconds at 1100 rpm and incubated for 30 minutes at room temperature, shaken at 600 rpm. Then, the plates were washed three times as mentioned above, and subsequently, read using Bio-Plex 200 system and the Bio-Plex Manager Software 4.1 was used for data analysis.

5.3.3 Polio 1 & 3 virus neutralization assay

The following polio virus strains were used: Poliomyelitis 1 virus: X2245 diluted to 1:1,000,000 and Poliomyelitis 3 virus: X2246 diluted to 1:150,000, in 2% Fetal Calf Serum (FCS; Invitrogen, USA) in Dulbecco's Modified Eagle's Medium (DMEM).

Briefly, 25 µl of the subjects' plasma or the reference serum 498 (HW) were diluted at 1:4 in sterile 1X PBS medium and complement was inactivated by incubation at 56^{o}C for 30 minutes. Then, 50 µl of medium was added to each well of a flat-bottom plate. At that point, standard reference serum, positive control (containing 50 µl of medium and 50 µl of virus), and negative control (containing 100 µl of medium only), and diluted subject samples were further serially diluted 8 times to a final dilution of 1:1024, and added to the plate; all samples were run in duplicate. Then 50 µl of diluted virus was added to each well except the negative control wells. Subsequently, the plate was incubated at 37ºC, 5% CO_2 for an hour.

During this incubation period, the Human Epithelial type 2 (HEp2) Cincinatti cells were stripped and washed and re-suspended in 2% FCS in Dulbecco's Modified Eagle's Medium (DMEM) at a concentration of 2 x 10^5/ml. Following the hour incubation, 100 µl of cell suspension was added to each well and then incubated at 37^{o}C, 5% CO_2. After 4 days, the cytopathic effect (CPE) was read as positive [+] or negative [-] in each well. Negative was defined as well having less than 50% cell death by using a microscope. The neutralizing antibody titre was defined as the highest sample dilution where there was no cytopathic effect.

5.3.4 Statistical analysis

Non-parametric Wilcoxon matched paired tests were performed to analyse paired sample data within the study groups and Kruskal-Wallis tests were used for unpaired comparisons between different subsets. GraphPad Prism (GraphPad Software 6) was used to prepare the diagrams and for statistical analysis. Significant difference between subsets was defined as having a p value of *$p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

5.4 RESULTS

In order to examine how NK cell effector function is influenced by secondary vaccination, it was important to understand the potential role of immune complexes. Thus, levels of IgG vaccine-specific antibodies were determined pre (Week 0) and post (4 weeks) booster vaccination. In contrast to the influenza vaccination study (Chapter 4) where, typically, annual exposure to influenza viruses will boost antibody titres, diphtheria, pertussis, polio, and tetanus are only rarely encountered in the community nowadays and individuals who had received a booster vaccination after childhood were excluded. We expected, therefore, that subjects would have very low levels of antibodies at baseline but that these might be boosted by revaccination, enabling us to investigate the role of IgG in vaccine-induced NK cell responses.

5.4.1 DTPiP vaccine-specific antibody assays

5.4.1.1 Potent induction of IgG antibodies to diphtheria, tetanus, and pertussis toxins post vaccination

Protective antibody levels for anti-diphtheria and anti-tetanus toxoid are defined as >0.01IU/ml and for anti-pertussis toxin as >16 EU/ml (Hammarlund et al., 2016, Kwon et al., 2012).

Prior to booster vaccination (week 0), the median IgG antibody level against diphtheria toxin was 0.030 IU/ml but this increased to 1.323 IU/ml 4 weeks post vaccination (Figure 5.1). Considering a protective level of greater than 0.01 IU/ml, 7/28 (25%) people had non-protective levels of diphtheria antibody at baseline, whilst all had protective levels post vaccination. Additionally, 26/28 (93%) subjects had protective anti-tetanus antibody levels prior to booster vaccination and all had protective levels after boosting with median titres increasing from 1.8 IU/ml at baseline to 7.5 IU/ml after vaccination (Figure 5.2). Prior to booster vaccination, 19/28 (68%) subjects had below protective levels of anti-pertussis toxin antibody with one individual failing to reach protective levels after boosting (Figure 5.3); at baseline, the median titre was 7.9 EU/ml and this increased to 112.2 EU/ml post vaccination.

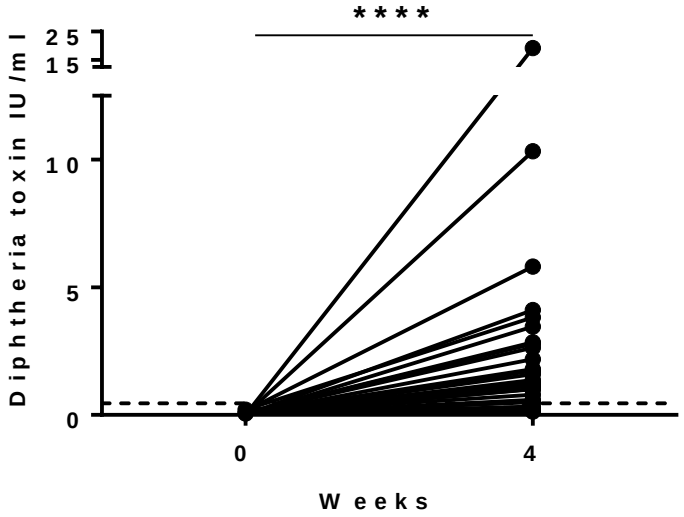

Figure 5.1: Concentration of serum IgG antibodies to diphtheria toxin before and after booster vaccination.

DTaP4 multiplex immunoassay was used to determine the level of plasma diphtheria toxin IgG antibodies before (Week 0) and 4 weeks post vaccination. Samples were tested from 28 subjects. Each dot represents data from one individual before and after booster. The dotted line indicates the protective level of diphtheria toxin antibodies. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, ****p<0.0001.

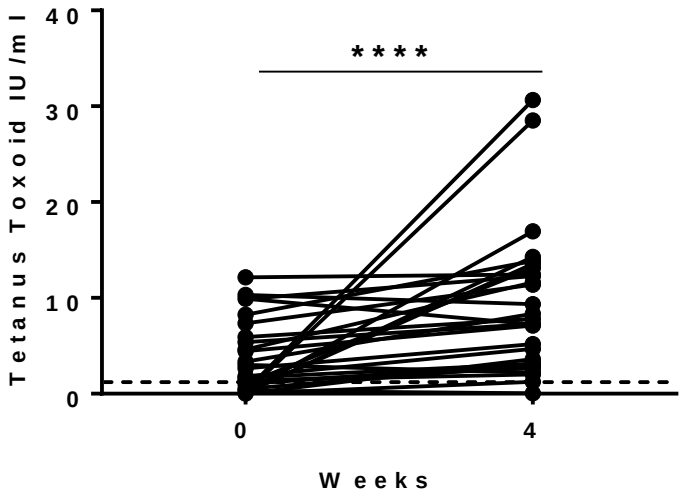

Figure 5.2: Concentration of serum IgG antibodies to tetanus toxoid before and after booster vaccination.

DTaP4 multiplex immunoassay was used to determine the level of plasma tetanus toxoid IgG antibodies before (Week 0) and 4 weeks post vaccination. Samples were tested from 28 subjects. Each dot represents data from one individual before and after booster. The dotted line indicates the protective level of tetanus toxoid antibodies. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, ****p<0.0001.

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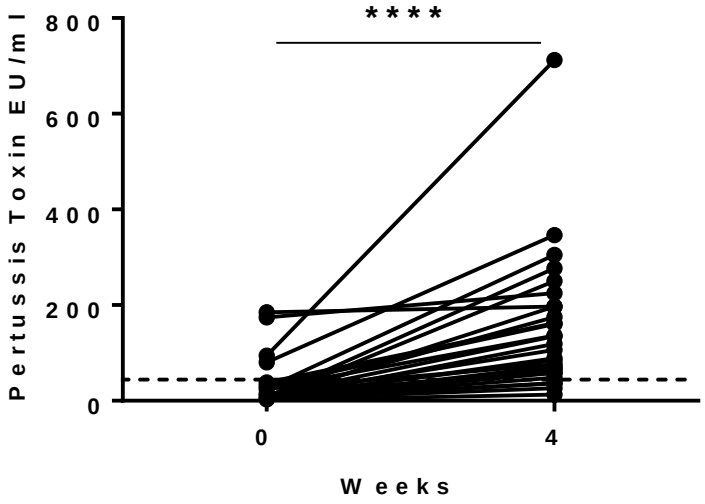

Figure 5.3: Concentration of serum IgG antibodies to pertussis toxin before and after booster vaccination.

DTaP4 multiplex immunoassay was used to determine the level of plasma pertussis toxin IgG antibodies before (Week 0) and 4 weeks post vaccination. Samples were tested from 28 subjects. Each dot represents data from one individual before and after booster. The dotted line indicates the protective level of pertussis toxin antibodies. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, ****p<0.0001.

5.4.1.2 Poliomyelitis 1 & 3 virus neutralization assay

Viral neutralization is a key antibody defence mechanism against viral infection. Neutralizing antibodies can function in different pathways such as causing aggregation of viral particles preventing them from infecting cells, or by blocking viral attachment through steric interference, capsid stabilization and structural changes. They can also block endocytosis or block viral un-coating via capsid stabilization and viral fusion interference (Klasse, 2014, Greenspan, 2001, Dimmock, 1993, Klasse and Sattentau, 2002).

The assay for poliomyelitis virus specific IgG used in this study relies on inhibition of virus induced cytopathic effects (prevention of lysis of HEp 2 cells). The cut-off value for protective polio antibody is greater than 1:8 dilution (Diedrich et al., 2002, Arya and Agarwal, 2007). As shown in Figure 5.4, both Polio 1 & 3 antibodies significantly induced antibodies post booster, however, one person in the Polio 1 assay had a lower titre after the booster, this could have been a technical error.

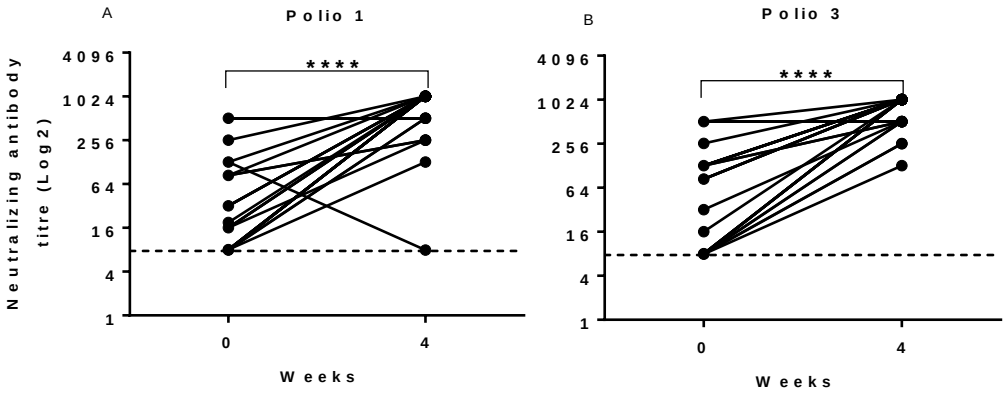

Figure 5.4: Titres of neutralizing antibodies to plasma poliomyelitis 1 and 3 virus before and after booster vaccination.

Plasma samples were cultured for 4 days with Human Epithelial type 2 (HEp2) Cincinatti cells in 2% FCS in Dulbecco's Modified Eagle's Medium (DMEM) at a concentration of 2x 10^5/ml in the presence of live Polio 1 or Polio 3 viruses. Cytopathic effects (CPE) was determined as negative [-] result, where there was less than 50% cell death per well, as read using a microscope. The level of neutralizing antibody titre was defined as the highest sample dilution where there was no cytopathic effect. Data are shown from 28 subjects; each dot represents the neutralising titre for a single individual before and after vaccination. The dotted line indicates protective polio neutralizing antibody titre. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, ****p<0.0001.

5.4.2 In vitro responses of NK cells before and after vaccination

As the aim of this study was to investigate the effect of booster vaccination, on NK cells, in vitro cultured PBMC responses at baseline were compared with those at 4 weeks after vaccination. PBMC were cultured for 18 hours under standard incubation conditions using 10% pooled human AB serum. However, this setup can only examine IL-2+ CD4 T cell-dependent responses or intrinsic changes to NK cells: DTPiP-specific antibodies present in the pooled human AB serum could contribute to vaccine driven NK cell ADCC responses in this system, but would not be expected to vary between baseline and post-vaccination samples. The impact of changes in antibody titre post-vaccination requires experiments using an autologous serum, which is either intact or depleted of IgG antibodies. This will be studied in future experiments using cryopreserved PBMC and stored pre and post vaccination plasma.

5.4.3 Booster vaccination does not enhance NK cell responses to individual vaccine component antigens

NK cell CD107a and CD25 expression and IFN-γ production in response to vaccine components - Diptheria Toxin (DT), Tetanus Toxoid (TT), whole cell *B. pertussis* (PT) and inactivated poliomyelitis virus (P) - were determined before and after vaccination. Stimulations were performed with antigen alone or in the presence of low concentrations of cytokines (rIL-12 12.5ng/ml + rIL-18 10ng/ml) to compensate for any loss of antigen presenting cell populations among cryopreserved PBMC and the lack of PAMPs in subunit antigens.

All of the single antigens alone, except poliomyelitis antigen, induced NK cell CD107a+ expression both at baseline and 4 weeks post booster vaccination (Figure 5.5). Degranulation (CD107a) in the presence of low concentrations of cytokine alone (LCC) was not significantly different from that in medium alone. However, in combination with the vaccines antigens, the frequency of CD107a+ NK cells was significantly increased in both baseline and 4 weeks NK cells. These data indicate that NK cells can respond to these four pathogens, at least in the presence of human serum that likely contains specific IgG antibodies. In the presence of a standard human AB serum, booster vaccination did not, however, result in enhancement of CD107a responses except in poliomyelitis antigen plus cytokine stimulated cultures ($p < 0.05$) (Figure 5.5).

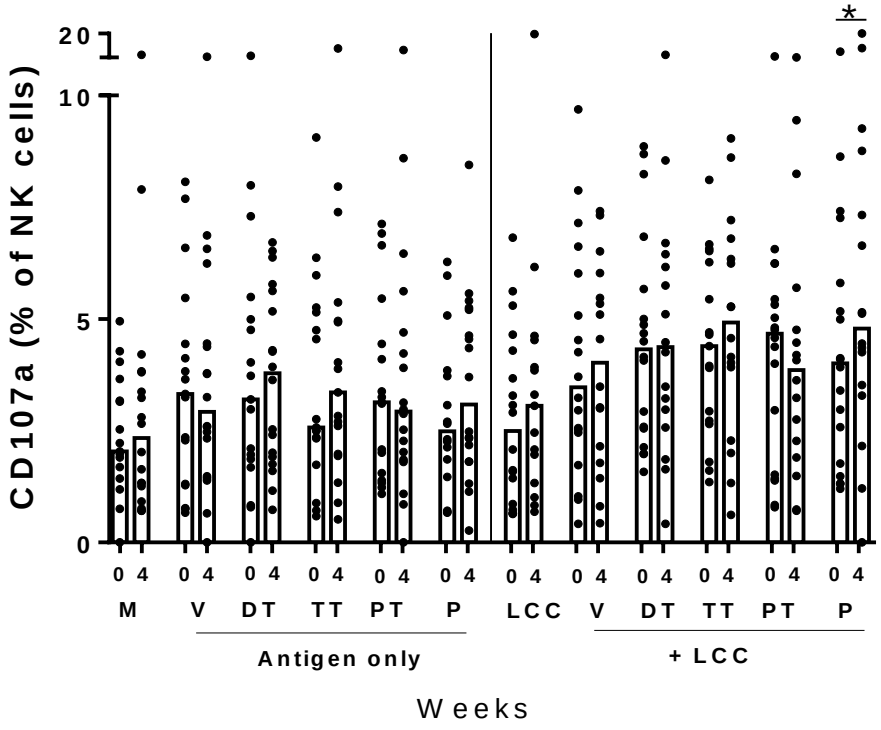

Figure 5.5: No significant difference in CD56+CD107a expression pre and post vaccination.

Frequencies of CD107a+ NK cells expression baseline (Week 0) compared to 4 weeks NK cells post vaccination, gated from CD56+ cells. These cells were cultured in (M) Medium alone, (LCC) low cytokine concentration [rIL-12: 12.5 pg/ml & rIL18: 10 ng/ml], (V) DTPiP vaccine antigen, (DT) diphtheria toxin, (TT) tetanus toxoid, (PT) pertussis toxin, (P) inactivated poliomyelitis vaccine antigens. Data are shown for 18 subjects, each dot representing the frequency of CD107a+ cells from a single individual. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, *p<0.05. All comparisons between 0 and 4 weeks were non-significant, except where indicated (*). Responses to antigen were significantly higher than responses to medium alone, and responses to LCC plus antigen were significantly higher than responses to LCC alone: Week 0 and Week 4, antigen only: medium vs: DTPiP, p<0.02; DT, p<0.01; TT, p<0.02; PT, p<0.006; Polio, not significant]. Week 0 and Week 4, antigen plus LCC: LCC vs LCC plus DTPiP p<0.02; plus DT p<0.001; plus TT p<0.0002; plus PT p<0.002; plus polio p<0.0003.

The expression of CD25 was also assessed within total NK cells before and after vaccination (Figure 5.6). No significant induction of CD25 was observed on NK cells after stimulation with vaccine antigen alone. LCC alone significantly enhanced CD25 expression (**p<0.002) on NK cells both in the absence or presence of antigens.

Nevertheless, the frequencies of CD25+ NK cells did not differ between cultures treated with LCC alone compared to antigen plus LCC, except for LCC plus PT (*p<0.02) at baseline only. Moreover, CD25 was not activated post vaccination as there were no significant differences between baseline and 4 weeks NK cell CD25 expression.

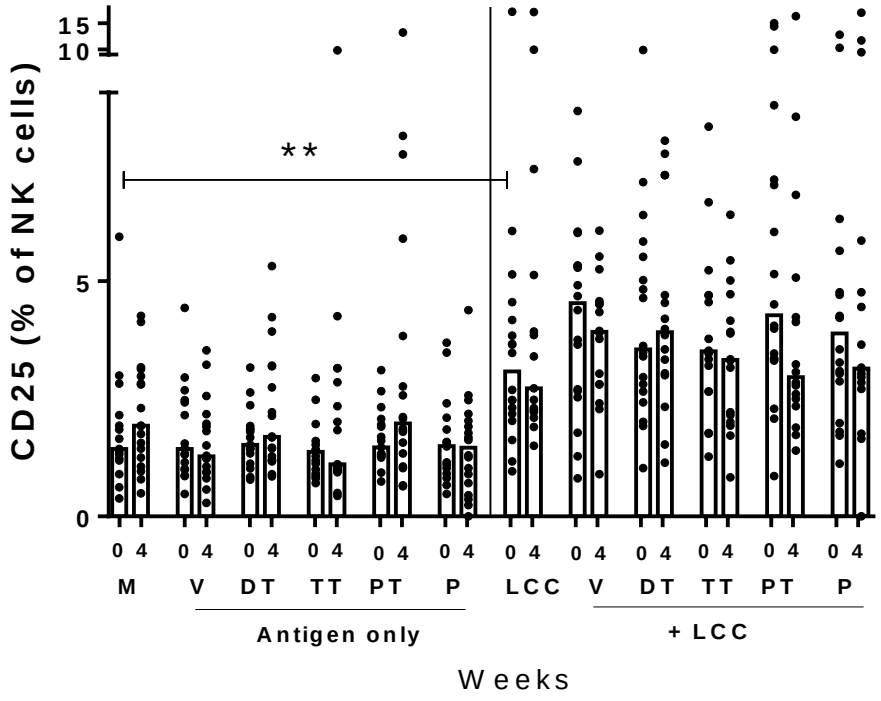

Figure 5.6: CD25 expression on NK cells after in vitro restimulation with vaccine antigens, pre- and post- booster vaccination.

CD25 expression in NK cells taken at baseline compared to 4 weeks post vaccination, gated from CD56+ cells. PBMC were cultured in (M) Medium alone, (LCC) low cytokine concentration [rIL-12: 12.5 pg/ml & rIL18: 10 ng/ml], (V) DTPiP vaccine antigen, (DT) diphtheria toxin, (TT) tetanus toxoid, (PT) pertussis toxin, (P) inactivated poliomyelitis vaccine antigens. Data are shown for 18 subjects, each dot representing CD25 expression on NK cells from a single individual before and after vaccination. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, **p<0.01.

Negligible IFN–γ production was detected by intracellular staining of NK cells both before and after vaccination (Figure 5.7). Vaccine antigens alone and in combination with LCC could not induce IFN-γ production and no significant increment was observed in these responses after vaccination. A single exception was observed

when LCC was used in combination with DT antigens in samples taken after booster vaccination where a higher frequency of IFN-γ+ NK cells was observed compared to LCC alone (*p<0.04).

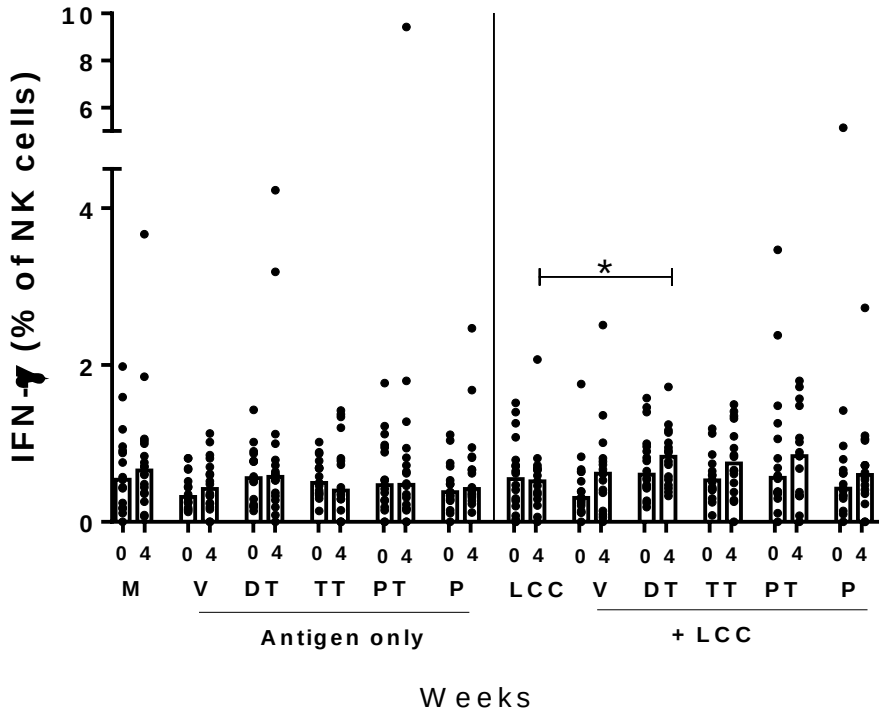

Figure 5.7: IFN-γ expression by NK cells after in vitro restimulation with vaccine antigens, pre- and post- booster vaccination.

IFN-γ expression in total NK cells measured at baseline (Week 0) compared to 4 weeks NK cell post vaccination, gated from CD56+ cells. Cells were cultured in (M) Medium alone, (LCC) low cytokine concentration [rIL-12: 12.5 pg/ml & rIL18: 10 ng/ml], (V) DTPiP vaccine antigen, (DT) diphtheria toxin, (TT) tetanus toxin, (PT) pertussis toxin, (P) inactivated poliomyelitis vaccine antigens. PBMC were cultured in 10% human AB serum for 18 hours under standard culture conditions. Data are shown from 18 subjects, each dot representing the frequency of IFN-γ+ NK cells for one donor before and after vaccination. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, *p<0.05.

5.4.3.1 Enhancement of NK cell responses to high concentrations of cytokines

NK cell responsiveness to high concentrations of rIL-12 (5ng/ml) and rIL-18 (50ng/ml) (in the absence of vaccine antigen) has been shown to be enhanced after influenza vaccination both in Gambians (Chapter 4 of this thesis) and in a UK vaccination cohort (Goodier et al, 2016). High concentrations of cytokines (HCC) were therefore used to test if this effect was specific to influenza vaccination or whether similar effects could also be observed after booster vaccination with DTPiP.

There was a significant increase in the frequency of CD56bright NK cells post-vaccination among unstimulated PBMC and a corresponding, reciprocal decrease in the proportion of CD56dim NK cells (Figure 5.8C).

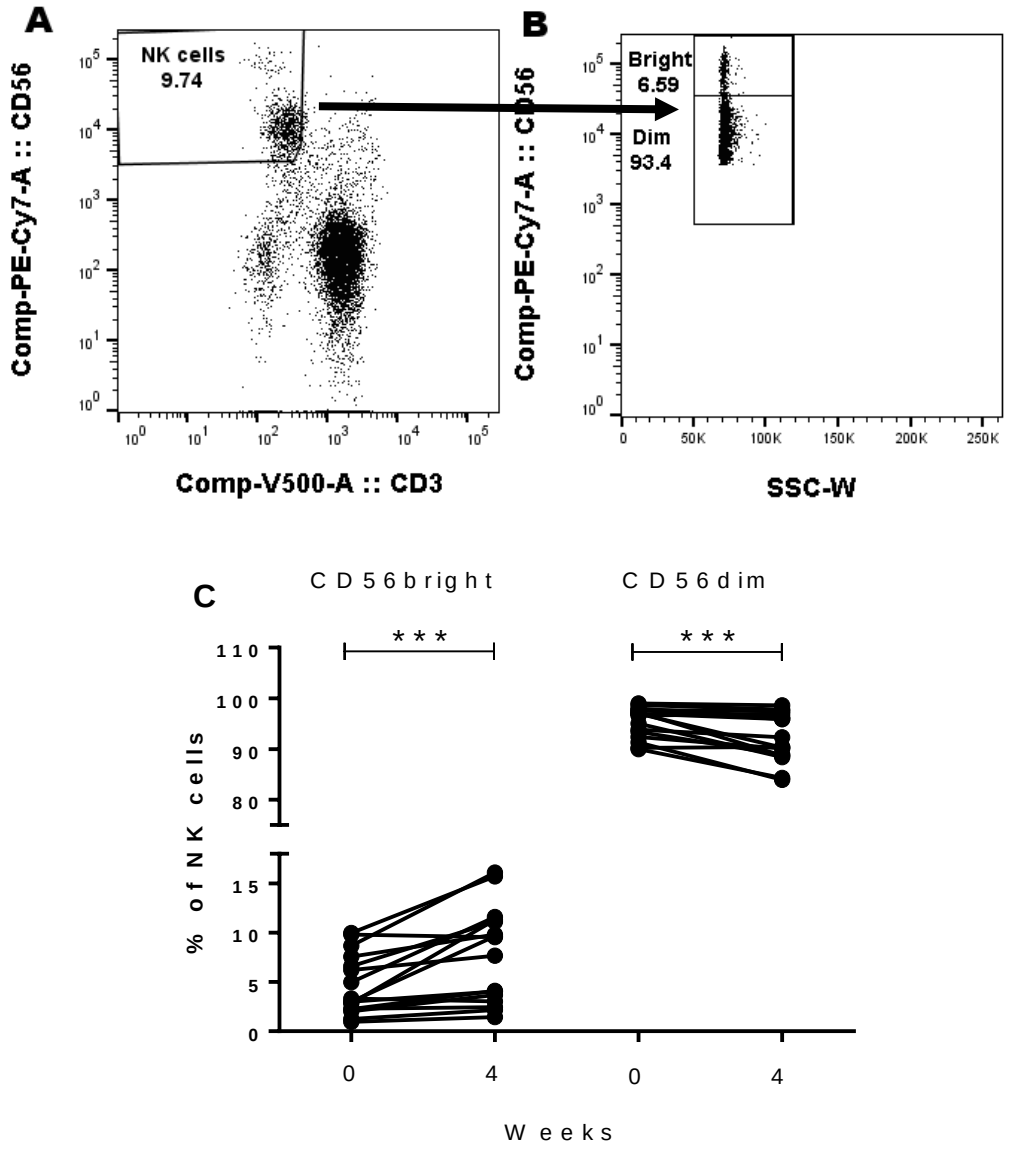

Figure 5.8: Frequencies of CD56bright and CD56dim NK cells pre- and post – booster vaccination.

(C) CD56bright and CD56dim NK cell frequency at baseline (Week 0) and 4 weeks post vaccination, gated from CD56+ cells. PBMC were cultured in medium alone, without stimulation. Figure (A, B) shows representative dot plot of cells cultured in medium alone from subject number 23 visit 1 cells, age 22 years. Data are shown from 18 subjects, each individual dot representing the frequency of CD56bright and CD56dim NK cells subsets before and after vaccination for one subject. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, ***p<0.001.

No change in the frequencies of CD107a+ or CD25+ NK cells in response to HCC was observed between baseline and 4 weeks post-vaccination (Figure 5.9 A,B). Strikingly, and similar to the effect observed after influenza vaccination, a significant potentiation of NK cell IFN-γ production in response to HCC occurred 4 weeks post booster vaccination (**p<0.0021) compared to baseline NK cells (Figure 5.10C). These data support a model where different vaccines could potentiate NK cell responses to exogenous cytokines through common mechanisms.

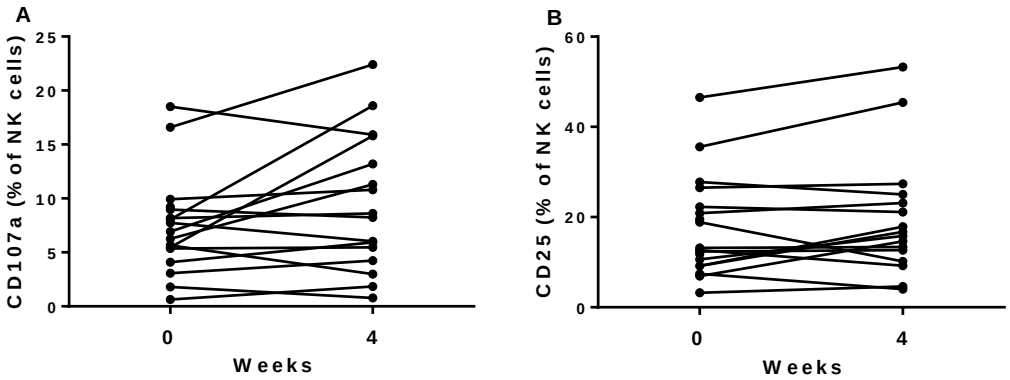

Figure 5.9: NK cell CD107a and CD25 expression in response to high concentrations of cytokine, before and after vaccination.

NK cell CD107a and CD25 expression at baseline (Week 0) and 4 weeks post vaccination, gated from CD56+ cells. PBMC were cultured in high concentrations of cytokines alone (IL-12 5ng/ml + IL-18 50ng/ml). Data are shown from 18 subjects, each individual dot representing the frequency of CD107a+ or CD25+ NK cells from one subject before and after vaccination. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test.

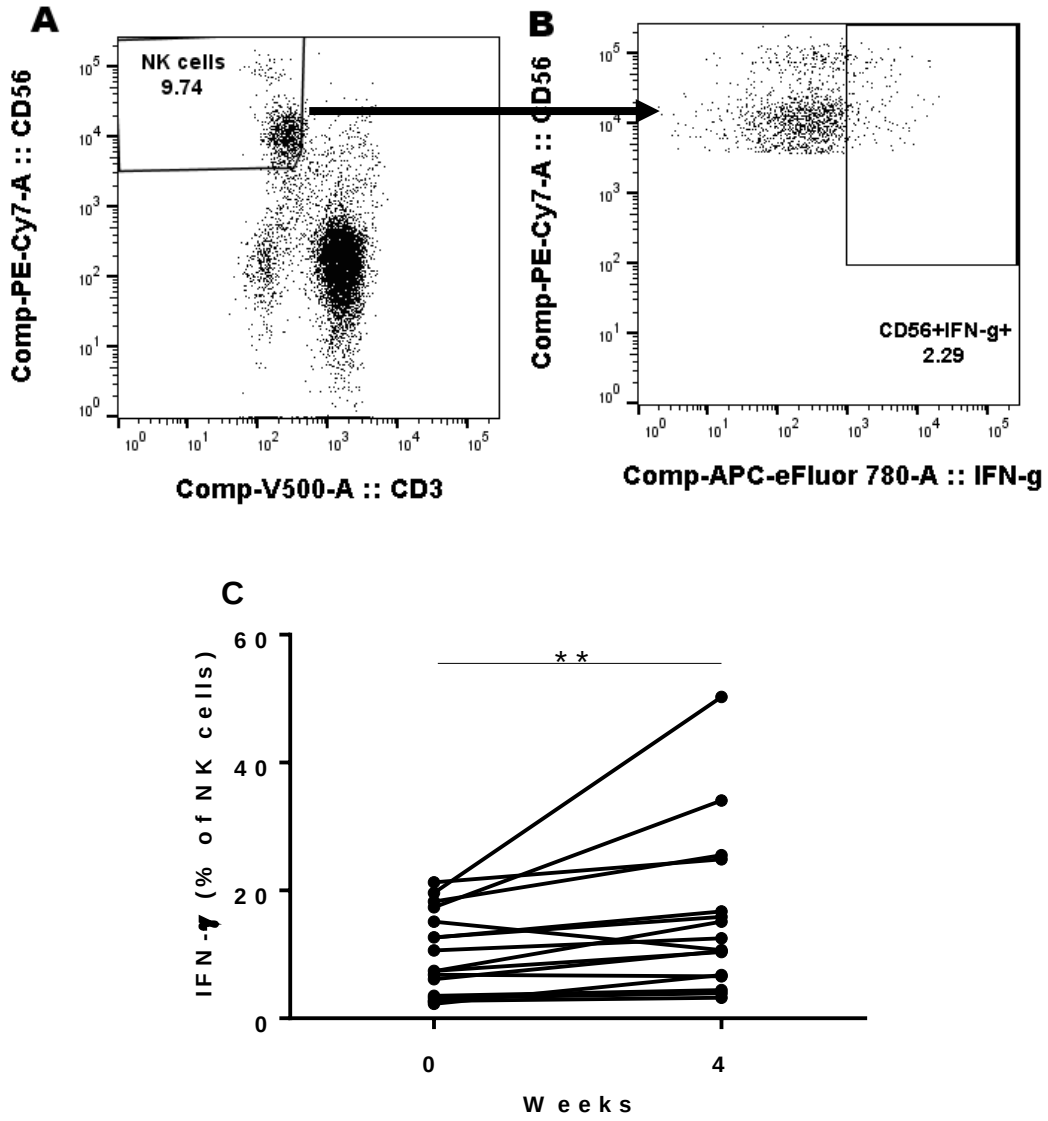

Figure 5.10: NK cell IFN-γ production in response to high concentrations of cytokine, before and after vaccination.

(C) NK cell IFN-γ expression from baseline (Week 0) NK cells and 4 weeks post vaccination. PBMC were cultured in high concentrations of cytokines alone (IL-12 5ng/ml + IL-18 50ng/ml). Figure (A, B) shows a representative dot plot of cells cultured in high concentrations of cytokines from subject number 23 visit 1 cells, age 22 years. Data are shown from 18 subjects, each dot represents the frequency of IFN-γ+ NK cells in a single individual before and after vaccination. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, **p<0.01.

5.4.3.2 Proportions of CD56- and CD57-defined NK cell subsets after vaccination

Our studies indicate that NK cells from Gambian subjects are highly differentiated with greater skewing towards a CD56dimCD57+ phenotype compared to UK cohorts (Chapter 3). Furthermore, influenza vaccination in UK subjects was recently demonstrated by our group to promote changes in the proportions of CD56 and CD57-defined NK cell subsets (Chapter 3 and Goodier et al, 2016). Influenza vaccination resulted in enrichment of CD56dimCD57- NK cells, detected ex vivo and these changes were restricted to HCMV+ individuals (Goodier et al, 2016). As all the individuals in our study cohort are likely to have been exposed to HCMV, we analysed whether DTPiP vaccination had an impact on the distribution of NK cell subsets according to differentiation status.

As already shown in Figure 5.8, it can clearly be seen in Figure 5.11B that the frequency of CD56bright (CD56bri) NK cells within unstimulated PBMC significantly increases post booster vaccination. However, changes in the proportion of either CD57dimCD57-(CD57-) NK cells or CD56dimCD57+ NK cells were not statistically significant indicating that the increase in the proportion of CD56bright cells is not at the expense of any particular subset of CD56dim cells. These data suggest that DTPiP vaccination may promote the expansion of immature CD56bright NK cells, which could influence overall NK cell function especially in response to exogenous cytokines.

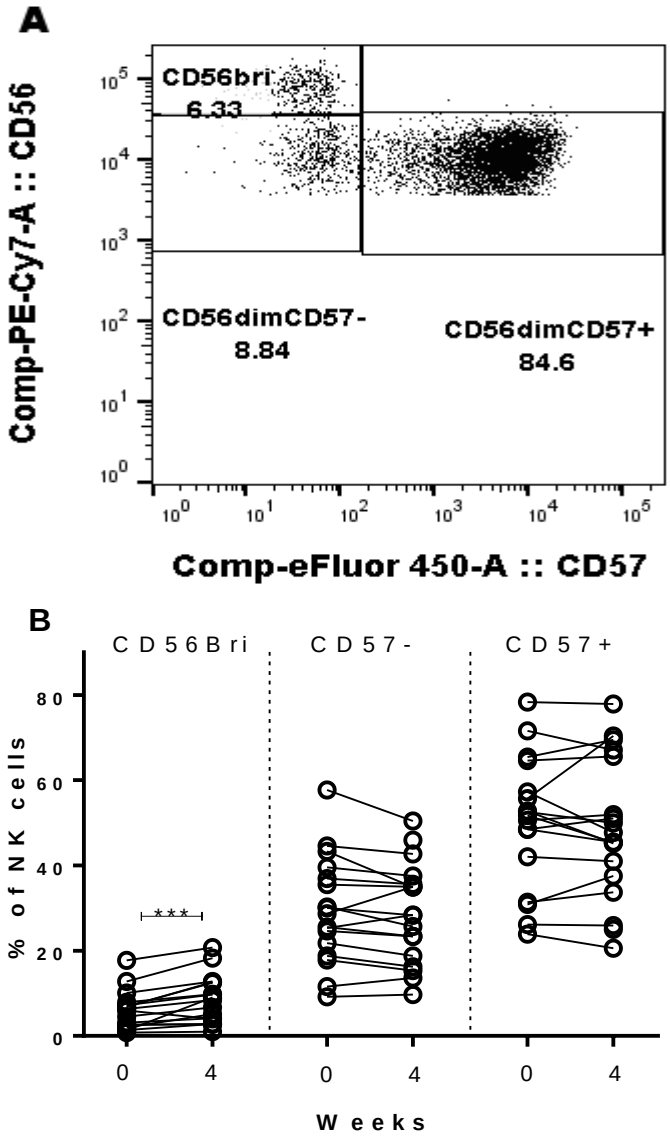

Figure 5.11: Proportions of CD56 and CD57-defined NK cells pre- and post-booster vaccination.

PBMC were gated as NK cells (CD56+CD3-) (A) and analysed as CD56bright, CD56dimCD57- and CD56dimCD57+ NK cells (B). Dots represent data derived from PBMC cultured with medium alone without any stimulation. Figure (A) shows a representative dot plot of cells cultured in medium alone from subject number 23 visit 1 cells, age 22 years. Data are shown for 18 subjects, each dot representing frequencies of NK cell subsets in a single individual before and after vaccination. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, ***p<0.001.

Previous studies in this thesis and elsewhere have shown that vaccination causes a shift in the subset distribution of NK cells, as shown in this chapter for CD56bright NK cells (Figure 5.8C and 5.11B). There may be, however, additional intrinsic effects on the function of individual NK cell subsets (Chapter 4 and Goodier et al, 2016). IFN-γ responses to HCC were therefore analysed to test whether booster vaccination enhanced the ability of particular CD56/CD57-defined NK cell subsets to respond to cytokines. As previously described (Chapter 4 and elsewhere) CD56bright NK cells, being the least mature cell subset contained the highest frequencies of IFN-γ producing cells in response to HCC, whilst CD56dimCD57+ NK cells had the lowest (Figure 5.12). However, analysis of IFN-γ production within CD56bright and CD57-defined CD56dim NK cell subsets revealed a significant enhancement of IFN-γ production within both CD56dimCD57- and CD56dimCD57+ NK cells (*$p<0.027$ and *$p<0.049$, respectively) post-vaccination (Figure 5.12). A strong trend was also observed towards enhancement of IFN-γ production within CD56bright NK cells after vaccination but this did not reach statistical significance (Figure 5.12). These data suggest that DTPiP booster vaccination not only expands the cytokine-producing CD56bright population but also enhances cytokine responsiveness in CD56dim NK cells which are more differentiated and which have intrinsically less IFN-γ production capacity than CD56bright NK cells.

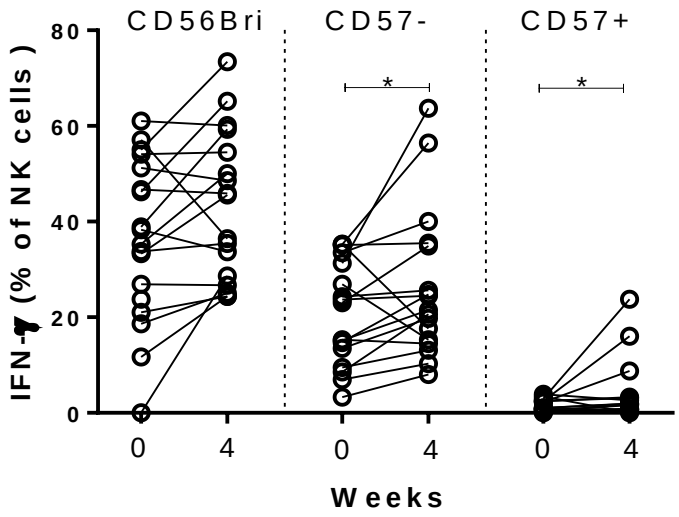

Figure 5.12: IFN-γ production in response to HCC pre- and post-booster vaccination in CD56 and CD57-defined NK cell subsets.

Frequencies of IFN-γ producing cells within CD56 and CD57-defined NK cell subsets before and after vaccination, gated from CD56+ cells. NK cells were cultured in high concentration of cytokines alone (rIL-12 5ng/ml + rIL-18 50ng/ml). Data are shown from 18 subjects, each individual dot representing the frequency of IFN-γ+ cells within NK cell subset for each individual before and after vaccination. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, *p<0.05.

5.5 ENHANCEMENT OF IFN-γ PRODUCTION IN NKG2C+CD57- NK CELLS AFTER VACCINATION

Potential changes in the frequencies and/or role of NKG2C+CD57+ NK cells, known to be expanded in HCMV+ individuals (Lopez-Verges et al., 2011, Schlums et al., 2015), and CD57-NKG2C+ NK cells were then investigated before and after booster vaccination. Based on NKG2C and CD57 expression, CD56dim NK cells were separately categorised into NKG2C-CD57-, NKG2C+CD57-, NKG2C-CD57+, and NKG2C+CD57+ NK cells subsets (Figure 5.13B). There was no significant change in the frequencies of any of these subsets after vaccination (Figure 5.13C).

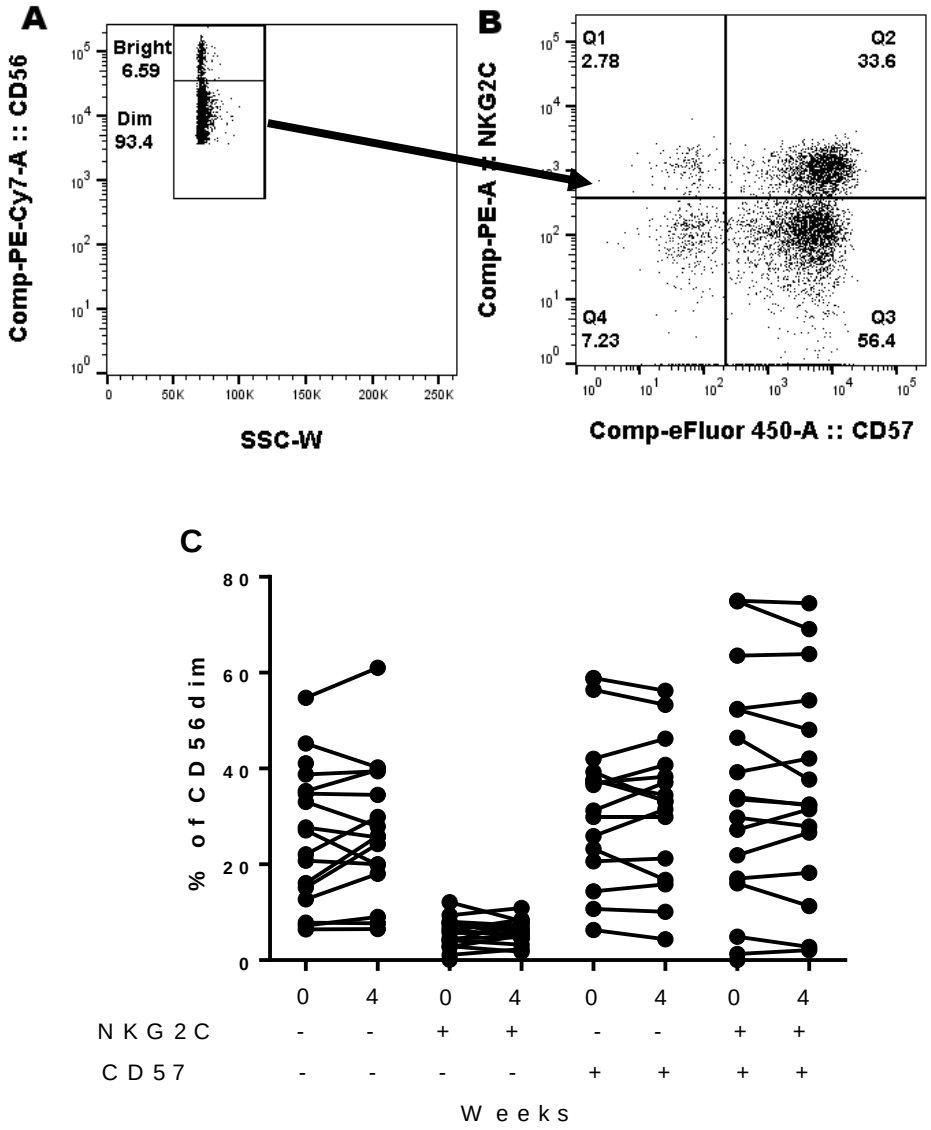

Figure 5.13: Frequencies of NKG2C- and CD57-defined CD56dim NK cell subsets do not change post vaccination.

Proportion of NKG2C and CD57-defined NK subsets pre and post vaccination. NK cells were gated within PBMC after culture in high concentration of cytokine alone (rIL-12 5ng/ml + rIL-18 50ng/ml). Figure (A) shows a representative dot plot of cell cultured in high concentrations of cytokines from subject number 23 visit 1 cells, age 22 years. Data are shown from 18 subjects, each dot represents a single data point of frequency of CD56dim before and after vaccination. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test.

Finally, in order to establish whether differentiation status impacted on the ability of CD56dim NK cells to produce IFN-γ in response to HCC before and after vaccination, the frequencies of IFN-γ producing cells were estimated within NKG2C and CD57-defined subsets of CD56dim cells. Interestingly although NKG2C-CD57- cells contained the highest frequencies of IFN-γ producing NK cells in response to HCC before vaccination with NKG2C+CD57+ cells the lowest, less mature NKG2C+CD57- cells contained high frequencies of IFN-γ producing cells post vaccination (Figure 5.14). Furthermore, although a trend towards increased frequencies post-vaccination of IFN-γ producing NK cells after HCC stimulation was observed within all subsets, this was only significant within the NKG2C+CD57- NK subset (Figure 5.14, **p<0.002). Indicating that these might be the ones driving the potentiation of NK cell responsiveness to cytokines post vaccination.

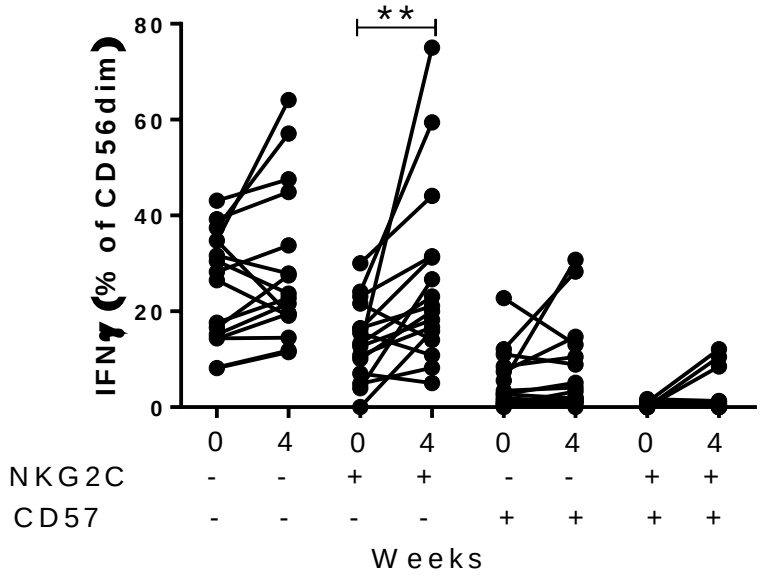

Figure 5.14: HCC-induced IFN-γ production by different subsets of CD56dim NK cells pre- and post-booster vaccination.

Frequencies of NKG2C and CD57-defined NK cell subsets expressing IFN-γ before and after vaccination. NK cells were gated after culture of PBMC in high concentrations of cytokines alone (rIL-12 5ng/ml + rIL-18 50ng/ml). Data are shown from 18 subjects, each dot representing single individual proportion of CD56dim IFN-γ before and after vaccination. Statistical analysis was performed on paired samples using Wilcoxon signed-rank test, **p<0.01.

5.6 DISCUSSION

In this study, we provide evidence that booster vaccination of adults against diphtheria toxin, tetanus toxin, pertussis and polio (DTPiP) after primary vaccination in childhood did not potentiate NK cell responses against vaccine antigens in Gambians donors. However, the booster vaccination significantly enhanced NK cell IFN-γ responsiveness to exogenous cytokines. These results confirm the pattern we saw in Chapter 4 using an influenza vaccine model, where enhancement of IFN-γ producing NK cell frequencies was also only seen after culture with high concentrations of cytokines (Chapter 4). This evidence thus supports a model where different vaccines could potentiate NK cell cytokine responses through common mechanisms, although further work will be required to identify the factors involved.

Nielsen et al have shown that HCMV-infected individuals display significantly reduced vaccine antigen-induced NK cell responses (IFN-γ, CD25 and CD107a) regardless of their differentiation status, sex and anti-HCMV IgG titre, when compared to HCMV- individuals (Nielsen et al., 2015). It is hypothesised that this decreased capacity could be attributed to the redistribution of the functional NK cell repertoire and differentiation status driven partially by HCMV infection. HCMV infection induces NK cell maturation towards NKG2C+CD57+ NK cell phenotype, which is known to have reduced responsiveness to cytokines (and reduced IL-12Rβ2 and IL-18Rα receptors) (White et al., 2014), resembling the NK cell phenotype of the elderly (Nielsen et al., 2015).

IFN-γ production in response to exogenous cytokines diminishes with the acquisition of CD57 (White et al., 2014). It has previously been shown that HCMV-infected individuals have higher frequencies of CD56dimCD57+ and CD56dimNKG2C+CD57+ NK cells than HCMV-negative individuals and that CD57+ NK cells produced less IFN-γ in response to stimuli (Nielsen et al., 2015, Goodier et al., 2016, Guma et al., 2006a, Guma et al., 2006b, Lopez-Verges et al., 2010). As there was a significant disparity in IFN-γ production between HCMV-positive and negative donors across all CD57 and NKG2C defined subsets, the overall variation in the frequencies of different subsets could not fully account for the defect in vaccine responsiveness (Nielsen et al., 2015, White et al., 2014). Thus, HCMV infection seems to affect NK cell responsiveness in multiple ways, of which distorting NK cell subset distribution is just one.

Comparing the two Gambians studies (Chapters 4 and 5), we consistently failed to see vaccine antigen driven NK cell responses post vaccination, although both studies revealed induction of IFN-γ responses to exogenous cytokines. This is in stark contrast to studies in European (UK) donors (Goodier et al., 2016), wherein influenza vaccination induced a potent vaccine antigen-driven NK cell responses up to 4 weeks post-vaccination. However, all of these studies – in UK donors and in the Gambia - show potent induction of IFN-γ within CD56bright and CD56dimCD57- NK cells in response to HCC (Goodier et al., 2016). The IFN-γ was mainly produced by CD56dimNKG2C+CD57- NK cells. Also, both my studies and those in UK revealed an increase in the proportions of CD56bright NK cells, suggesting that vaccination may induce NK cell proliferation or repopulation of the blood from tissues or bone marrow by less differentiated NK cells.

Goodier et al found that in HCMV-infected European donors, the ratio of CD57- to CD57+ NK cells was augmented 2 weeks post vaccination. These authors proposed that, in HCMV-positive donors, inferior IFN-α release and lower proportions of CD25 expressing NK cells could be a limiting factor for NK cell responsiveness to CD4 T cell IL-2 (Goodier et al., 2016). I have also observed limited IFN-α secretion by PBMC from Gambian donors after stimulation with influenza H3N2 antigen (unpublished, Darboe, A.).

Comparing CD56 and NKG2C receptors only, the NKG2C- NK cell subset produced more IFN-γ than NKG2C+ NK cells. When the association between NKG2C and CD57 receptors was assessed in combination, although NKG2C- and CD57-defined NK cell proportions did not change, IFN-γ production potential was mainly enhanced among NKG2C+CD57- NK cells post vaccination, however, CD107a, CD25 activation did not change post immunization. This data contrasts to some extent with previous studies which reported that NKG2C+CD57+ NK cells acquired epigenetic modifications within the IFN-γ locus, through demethylation of the activation-induced proximal upstream conserved non-coding sequence-1 (CNS1) (Luetke-Eversloh et al., 2014b). Interestingly, these cells also had similar demethylation pattern to memory-like NK cells stimulated with IL-12, IL-18 and IL-15. It may be that NK cell receptor mediated events, which are not necessarily provided by vaccination, play a stronger role modifying the function of highly mature NK cells. CNS1 demethylation potentiated IFN-γ transcriptional activity was observed following NKG2C and 2B4 receptor cross-linking (Luetke-Eversloh et al., 2014b). Additionally, it was also

demonstrated that mature NK cells (CD56dim) express superior IFN-γ competence to CD56bright NK cells after stimulation through activating receptors (NKp30, NKp46, CD2, NKG2D, CD16 and by K562). CD56dim NK cells express higher IFNG mRNA, T-bet (TBX21) mRNA, than CD56bright NK cells and within the CD56dim subsets, CD62L+CD57-KIR- cells express less mRNA than CD62L-CD57+KIR+ NK cells (Luetke-Eversloh et al., 2014a). In summary, my data show intrinsic potentiation of IFN-γ production potential of NK cells post booster immunization. This observation is not limited to one type of vaccination and is not antigen dependent.

Cytokine- or receptor-mediated signals induced by vaccines could affect different NK cell subsets. To test this hypothesis, future experiments could include pre-activation of NK cells of HCMV infected individuals with vaccine or cytokines in vitro followed by examination for enhancement of cytokine or receptor-mediated IFN-γ production after long-term in vitro culture. The effects of cytokines and of cross-linking NKG2C or other receptors could also be compared before and after vaccination in HCMV infected subjects. The hypothesis is that CD56dimNKG2C+CD57- NK cells could be more potently activated in culture after exposure to vaccine-induced cytokines, as these demethylate the IFNG locus, giving access to activating transcription factors required for IFN-γ secretion. In contrast, after vaccine-induced pre-activation, more mature NKG2C+CD57+ cells may be better restimulated by receptor cross-linking.

Although HCMV antibody levels were not tested in this study, we already know through our different studies that we have a very high prevalence of HCMV infection in The Gambia, as seen in Chapters 3 and 4. HCMV infection is known to influence NK cell repertoires with accumulation of mature NKG2C+ NK cells which have been reported in several studies to have superior antibody dependent function (Schlums et al., 2015, Tesi et al., 2016, Lee et al., 2015). This phenotype of NK cells is highly prevalent in our study population indicating that the majority of subjects in this study are indeed HCMV sero-positive.

There was potent induction of diphtheria, tetanus, pertussis toxins and poliomyelitis specific IgG antibodies post vaccination. One of the limitations of the current study is that I did not have time to run autologous plasma assays to look at the effect of antibody responses post vaccination, specifically, looking at the role of CD16 in the NK cell assay. This will be done in the coming months when the samples are received from The Gambia. The clear difference between the baseline and the 4 weeks post vaccination IgG antibody titres for the four individual DTPiP vaccine

components will enable clear analysis of antibody-dependent NK cell activation in this system.

A further limitation of this DTPiP study is that we did not perform NKG2C genotyping in these subjects, which may have permitted comparison of responses between NKG2C+/+ and NKG2C+/- individuals in this small cohort. However, few subjects completely lacked NKG2C expression by flow cytometry.

In conclusion, no enhancement of vaccine antigen-driven NK cell responses was observed after secondary vaccination with DTPiP vaccine (Repevax). However, and consistent with studies on influenza vaccination in both Gambian and UK cohorts, we clearly saw a significant increase in NK cell IFN-γ production post vaccination in response to exogenous cytokines. This enhancement was mainly observed within NKG2C+CD57- NK cells but the effect was not exclusive to this subset. Future studies could potentially inform us of possible pathways to induce NK cell effector function in vaccination, especially, in HCMV-infected individuals. The role of CD56dimNKG2C+CD57- NK cells could be investigated by assessing demethylation patterns at the IFN-γ locus pre- and post-vaccination. This NK cell subset, with intermediate differentiation status, might hold the key to potentiating NK cells effector functions in responses to vaccination in HCMV-infected people.

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6 CHAPTER 6:

GENERAL DISCUSSION

6.1 HCMV INFECTION

A high prevalence of HCMV infection was observed in all of our Gambian study cohorts. HCMV infection and reactivation is largely asymptomatic in healthy people and can remain latent for life in an individual. However, HCMV infection is known to be associated with poor growth, development, and morbidity in children (Gompels et al., 2012). HCMV seroprevalence in Western countries varies from 36-77% and in sub-Saharan Africa it ranges from 98-100%. The rate of HCMV congenital infection in The Gambia is about 5.4% (van der Sande et al., 2007), 2.9% in South Africa and 3.8% in Zambia (Adland et al., 2015). Almost universal sero-positivity is reached by the age of 3 years in The Gambia, as we have seen in Chapter 3. This is probably due to high rates of breastfeeding (Dop, 2002), over-crowded houses (Alao et al., 2009), and co-infection with other herpes viruses (Schaftenaar et al., 2014).

6.2 NKG2C GENOTYPE

A high frequency of the *NKG2C* gene deletion was observed in our Gambian cohorts compared to other countries, which has since been confirmed by studies performed in distinct regions of The Gambia and neighbouring West African countries (Goodier et al, 2014, Goncalves et al, 2016, Chapter 3). The selective pressure that drives the high prevalence of this gene deletion is still unknown. However, I observed no alteration of the distribution of the gene deletion across the life course (Chapter 3) supporting the idea that the NKG2C receptor is a redundant receptor whose function can be substituted by other NK cell activating receptors and is therefore not critical for survival. Specifically, the significant difference in allele frequency of *NKG2C* gene deletion between West Africa (33.2%) and East Africa (20.9%) (Goncalves et al., 2016), raises further questions, because, the level of exposure to HCMV infection between these two regions, is similar. This suggests that there may be other HCMV independent drivers of this gene deletion. The origin and mechanism of selection of this gene deletion in different populations are also unknown. It is hypothesised that being heterozygous might provide selective advantage in a particular environment and thus the deletion might be under balancing selection (Goncalves et al., 2016). Many immune receptors are maintained through balancing selection, this includes receptors like MHC molecules and KIR haplotypes (Parham and Moffett, 2013, Karlsson et al., 2014).

Of note, in our initial study (Chapter 3), young children with this gene deletion had high anti-HCMV antibody titres, possibly signifying inferior control of HCMV infection or frequent HCMV reactivation in these children. However, this effect is not seen in those over 10 years of age, suggesting that the NKG2C receptor is not crucial in lifelong control of HCMV infection and that other receptors like self HLA-specific KIR might compensate for the lack of NKG2C (Beziat et al., 2013, Beziat et al., 2012, Goncalves et al., 2016). It would be interesting in our future studies to investigate the role of these other NK cell receptors in the control of HCMV infection in *NKG2C* negative individuals as well as how this affects NK cell differentiation and function. For example, CD2 co-stimulation, in people with the *NKG2C* gene deletion, was shown to be essential for the provision of 'signal 2' in ADCC-mediated NK activation (Liu et al., 2016).

Consistent with another study showing reduced NK cell differentiation (Muntasell et al., 2013a, Muntasell et al., 2013b), *NKG2C* gene deletion in our study was correlated to the acquisition of CD57. Importantly *NKG2C* heterozygous subjects had lower frequencies of NKG2C+ NK cells compared with homozygous *NKG2C* gene subjects suggesting that a single copy of *NKG2C* helps to balance the control of HCMV infection with the rate of NK cell differentiation. The presence of CD94+ NK cells was associated with *NKG2C* copy numbers suggesting that NKG2C+ cells proliferate additionally to the normal process and clonally expand instead of transforming from NKG2A+ NK cells (Chapter 3).

6.3 NK CELL DIFFERENTIATION

In Chapter 3, I showed that the proportion of CD57- NK cell subsets decreased and CD57+ NK cells increased with age. Gambians have a highly differentiated NK cell repertoire compared to Europeans. HCMV sero-negative European adults express about 25%-50% NKG2C+CD57+ NK cells whilst HCMV sero-positive Europeans have about 30-70% of these cells. In The Gambia, the frequency of these cells is significantly higher even in the very young reaching about 80% by the age of 9 years. Age-matched Gambian and UK donors show marked variability in NK cell phenotype and function, even after comparing HCMV sero-positive Gambian and UK donors. This variation can possibly be explained by the early acquisition of HCMV infection in Gambians and these individuals might have higher prevalence of other chronic infections, which may contribute to this effect (Bjorkstrom et al., 2011, Lopez-Verges et al., 2011, Petitdemange et al., 2011).

6.4 NATURAL KILLER CELL RESPONSES AFTER VACCINATION

In contrast to Europeans receiving primary trivalent influenza vaccination, we did not observe enhancement of CD107a, CD25, or IFN-γ in vitro to vaccine antigen after vaccination in Gambians (Chapter 4). In UK donors, IFN-γ secretion was enhanced up to 4 weeks post vaccination (Goodier et al., 2016). Other studies in North Americans have shown enhancement of CD56brightCD25+NK cells post influenza vaccination and alteration of plasma cytokine concentrations in acute influenza infection (Jost et al., 2011). Additionally, it has been previously demonstrated that influenza vaccination can activate NK cells via NKp46 (Dou et al., 2015). These results indicate that influenza vaccination typically enhances NK cells responses to vaccine antigens in European and North American populations, whereas this effect was not observed in our African study population.

Indeed, the lack of enhancement of antigen-stimulated NK cell responses after influenza vaccination does not appear to be specific to this vaccine as secondary DTPiP booster vaccination also did not enhance NK cell responses in Gambians in vitro (Chapter 5). A possible explanation for these negligible responses is that HCMV infected donors have highly differentiated NK cells, expressing NKG2C+ and CD57+ receptors, which have reduced capacity to up-regulate both IL-12Rβ2 and IL-18Rα receptors, essential in the induction of IFN-γ, CD25, and CD69 (White et al., 2014). In turn, reduced induction of CD25 could impair T cell dependent NK cell responses. Although, these mature NK cells highly express CD16, the principal antibody Fc receptor on NK cell surface, we still did not see potent NK cell responses in the presence of post vaccination plasma (Chapter 4). It is possible our experimental setup preferentially promotes cytokine-mediated NK activation instead of receptor-mediated activation. Nevertheless, this is unlikely because it has previously been shown by Goodier et al (Goodier et al., 2016) and Nielsen et al (Nielsen et al., 2015) that depletion of antibodies from post-vaccination plasma significantly reduced influenza antigen-driven NK cell responses. This dependence was also confirmed here in a limited number of Gambian individuals. Direct stimulation of NK cell activating receptors can, however, lead to potent IFN-γ secretion in highly differentiated NK cells, due to the demethylation of IFN-γ locus in these cells (Luetke-Eversloh et al., 2014b, Luetke-Eversloh et al., 2014a). The main difference between my observations in this study and other studies that described differentiated NK cells,

is that the activation conditions in those experiments were mainly receptor-mediated activation, whereas vaccine antigen-driven pathways of NK cell activation appear to be mainly cytokine dependent (Goodier et al., 2016, White et al., 2014, Nielsen et al., 2015). Another point is that the highly differentiated NK cells in those studies were identified as cells that not only highly express CD57 and NKG2C but also have increased demethylation of the *IFNG* locus (Luetke-Eversloh et al., 2014b). We cannot therefore assume that vaccination caused demethylation at this locus in the cells in our study. Future analysis would try to delineate my observations in terms of 'adaptive' NK cell characteristics in addition to NKG2C and CD57, including, for example, reduced expression of specific signalling molecules including FcεRγ and transcription factor PLZF (Schlums et al., 2015, Tesi et al., 2016).

Human adaptive NK cells have been defined as NKG2ChiCD57+, lacking NKG2A receptors. These cells are known to lack FcεRIγ, SYK and EAT-2 signalling molecules, and have low expression of PLZF transcription factor. PLZF has been shown to be vital in the control of NK cell cytokine receptor expression, including IL-12 and IL-18 receptors (Cerwenka and Lanier, 2016, Schlums et al., 2015). In relation to function, it is clear that cytokines play a significant role in the generation of 'adaptive' NK cells, especially IL-12 but not IL-15, IL-18 or IFN-α (Rolle et al., 2014). It is thought that these mature 'adaptive' NK cells differentiate from NKG2Chi FcRγ+ to NKG2Chi FcRγ- cells, these NKG2Chi cells produce TNF-α independent of FcRγ expression (Muntasell and Pupuleku, 2016).

In contrast, cytokine-induced 'memory-like' NK cells can be generated through brief pre-activation in vitro with combinations of cytokines in the absence of receptor cross-linking, including IL-12+IL-15, IL-12+IL18 and CD16+IL-12 (Romee et al., 2012). 'Memory-like' NK cells do not express KIR receptors and are CD57-, however, they express CD94, NKG2C, NKG2A, NKp46 and CD69 markers and these cells secrete a lot of IFN-γ. Currently, the mechanism of this activation is unknown but the levels of IFN-γ mRNA did not change in pre-activated cells compared to control cells. This suggests that the control of IFN-γ in these cells is not at the transcript level, and this might be at post transcription or translation levels (Romee et al., 2012).

Cytokine-induced 'memory-like' NK cells make large amounts of IFN–γ after re-stimulation with cytokines or a target cell line (Romee et al., 2012, Ni et al., 2012). At the transcription level, these cytokine-induced 'memory-like' NK cells are similar to HCMV-driven NKG2C+ NK cells, as they both have stable demethylation of the *IFNG*

locus conserved non-coding sequence 1 (CNS1). These lines of evidence show, however, that 'memory' NK cells can be generated in the absence of antigens (Cerwenka and Lanier, 2016, Luetke-Eversloh et al., 2014b). However, maintenance of both adaptive type and cytokine-driven 'memory-like' NK cells has been shown in different systems to be dependent on T cell derived IL-2, for which antigen–dependence is necessary.

6.5 ENHANCED NK CELL RESPONSIVENESS TO CYTOKINES IN HCMV INFECTED INDIVIDUALS AFTER VACCINATION

In Gambians, vaccination enhanced NK cell IFN-γ responsiveness to exogenous cytokines. This was observed using both the trivalent influenza vaccine and the DTPiP vaccine. Vaccination augmented the frequency of CD56bright cells and post-vaccination enhancement of IFN-γ was driven by CD56dimNKG2C+CD57- NK cells and to a lesser extent, CD56dimNKG2C-CD57- cells. NKG2C+CD57- NK cells are likely to be less differentiated than CD57+ cells and respond better to cytokine-driven responses, with higher expression of IL-12 and IL-18 receptors than differentiated NKG2C+CD57+ NK cells. These cells probably also express more Ki67 and CD71 post vaccination as shown by Goodier et al. (Goodier et al., 2016). This CD56dimNKG2C+CD57- NK cell subset was present at a similar frequency to CD56bright cells in our study subset and could therefore make a significant contribution to overall NK cell IFN-γ production. Importantly, as NKG2C+ NK cells are found in a lower proportion of European donors and are present at lower frequencies in these individuals (Chapter 3), the vaccine studies described in our Gambian cohorts with high frequencies of these cells are the first where it has been possible to analyse NK cell vaccine responses in less well differentiated CD56dimNKG2C+CD57- cells. These CD56dimNKG2C+CD57- cells may be functionally related to the cytokine-induced 'memory-like' NK cells described above and represent a potential target for understanding of the development of NK cell memory in vivo. Because, they are less mature CD56dim NK cells, which lack CD57, it would be interesting to investigate the patterns of activating and inhibitory receptors expressed on these NKG2C+ NK cells. For example, does this population coincide with transitory populations of NKG2A+NKG2C+ NK cells which are normally rare in European and American subjects? And are NKG2C+CD57- cells transitory cells between cytokine-induced 'memory-like' NK cells and fully differentiated 'adaptive' NK

cells which express high levels of NKG2C and CD57 but which have alternative signalling requirements?

Maintenance of both 'adaptive' type and cytokine-driven 'memory-like' NK cells has been shown in different systems to be dependent on T cell derived IL-2, for which antigen–dependence is necessary (Kamimura and Lanier, 2015). Importantly, in contrast to our observations, human cytokine-induced 'memory-like' NK cells have enhanced CD25 expression, which made them more sensitive to low concentrations of IL-2 (Leong et al., 2014); we did not observe CD25 upregulation post vaccination in our donors. However, degranulation function was neither enhanced nor accelerated in cytokine-induced 'memory-like' NK cells (Cooper et al., 2009), consistent with our results for CD107a expression after cytokine stimulation of post-vaccination NK cells.

We observed in Chapter 3 that the mean fluorescence intensity (MFI) of NKG2C receptor in individuals homozygous for the *NKG2C* gene was higher than the MFI of heterozygous subjects. It is possible that NK cells from heterozygous donors might respond better to cytokines than homozygous donors as their NK cells may be less well-differentiated and may be enriched for NKG2C+CD57- cells.

Taken together, these data show that NK cells in Gambians are highly differentiated and have impaired cytokine-mediated responses to vaccine antigens. However, vaccination did induce enhanced intrinsic NK cell responsiveness to exogenous cytokines and this was mainly driven by NKG2C+CD57- NK cells. These less well-differentiated cells may retain some capacity to control HCMV infection, but at the same time represent a possible target for generation of 'memory-like' NK cells in vivo post vaccination.

6.6 T CELL HELP FOR NK CELL RESPONSES

In the age cross-sectional studies shown in Chapters 3 and 4, proportions of naive T cells decreased while central memory and effector T cells increased with age, as expected and consistent with other studies (Koch et al., 2008, Czesnikiewicz-Guzik et al., 2008). However, terminally differentiated effector memory T cell frequencies were already high in children under 2 years of age and did not vary significantly after this age. This might imply very intense exposure of infants to microbial antigens early in life. There was also a significant variation of lymphoid and myeloid subsets between the different age groups, raising the possibility that the different mechanisms of activation of NK cells, particularly by myeloid cell subsets, could vary across different

age groups. In addition to the advanced differentiation status of the NK cells in the cohorts tested, a lack of significant induction of TIV specific T cell responses at baseline and after vaccination (with the exception of week 12) could account for negligible NK cell IFN-γ responses to influenza antigens. This weak T cell response likely reflects less frequent seasonal exposure to influenza in The Gambia as supported by your own antibody data (in the case of H1N1) and also by surveillance data from other West African regions (World Health Organization, 2012).

In Gambian children, HCMV infection has been shown to drive CD8+ T cell differentiation without affecting immune responses to other antigens. These children had a higher proportion of differentiated CD8 T cells, mainly driven by the loss of CCR7, CD27, and CD28 receptors with the acquisition of CD57. This subset of CD8 T cells was maintained up to at least 2 years after HCMV infection. However, there was no significant increase in CD4 T cell differentiation within this time frame and the cellular responses to measles vaccine were not affected (Miles et al., 2008b, Miles et al., 2008c).

Miles et al also showed that children between the ages of 4-5 years did not have different magnitude of CD4+ T cell responses compared to adults, though CD4+ T cell co-expression of IFN-γ and CD40L was higher than co-expression of IFN-γ and IL-2, implying a potential switch to costimulatory interactions with CD40+ cells, including B cells and myeloid cells. Most responding CD4+ T cells were CD27+CD28+ to HCMV antigens; however, IFN-γ was mainly produced by the CD27-CD4+ T cells. Generally, it was found that CD4+ T cells of Gambian children produced a weak response to HCMV antigens which was mainly driven by bifunctional responses of IFN-γ and CD40L then followed by IFN-γ and IL-2 (Miles et al., 2008a).

6.7 ANTIBODY RESPONSE TO VACCINATION AND ANTIBODY-MEDIATED NK CELL RESPONSES

Annual influenza season in Gambia and Senegal is from October to December (World Health Organization, 2012). In my study, vaccination induced increased antibody production to most of the influenza strains present in the vaccine. However, the antibody data indicated that children (2-6 years) had not been exposed to H1N1 strain but also made low responses after a single dose of TIV (Chapter 4). Conversely, the adults also had low level of H1N1 antibodies at baseline most of

these were significantly enhanced post vaccination. This observation suggests a low level of natural exposure to H1N1 influenza in this population but older individuals may have had memory B or T cells that lead to a more potent antibody response after a single dose of vaccine. The oldest age group (60-75 years) could not induce potent TIV and H3N2 influenza antibodies post vaccination.

Highly differentiated NK cells in HCMV-infected subjects have increased potential for activation through their high levels of CD16 receptors. This would facilitate ADCC, especially when the antibody can recognise different strains of influenza, although, there was no induction of NK cell potentiation using 4 weeks autologous plasma (Chapter 4). NKG2ChiCD57+ NK cells express high levels of CD16, these cells have the ability to be activated through immune-complexes (Luetke-Eversloh et al., 2014b, Luetke-Eversloh et al., 2014a). This is relevant to our Gambian studies where highly differentiated NKG2C+CD57+ NK cells are abundant and likely to include adaptively expanded NK cells, even in children. It may be that these adaptively expanded cells show optimal levels of antibody-mediated degranulation even in baseline samples due to the availability of sufficient IgG antibodies in baseline plasma. The DTPiP study, however, provides an opportunity to test this further (discussed below), as these individuals are mainly negative for antibodies against vaccine antigens in baseline samples.

In addition to CD16 cross-linking, adaptive expansions of NK cells have improved responses mediated by other receptors. Because in our system we are using soluble antigens to stimulate NK cells, it could be that other signals which are needed to co-stimulate optimal antibody-CD16 dependent responses of highly differentiated NK cells are missing, (for example CD2 (Liu et al., 2016) or ligands for NCR (Draghi et al., 2007)). These signals would normally be provided in infected cells. To fully investigate this possibility, future studies will need to compare the responses of ‘adaptive’ NK cells to antigen-antibody complexes using infected target cells or by providing co-stimulation of other receptors by cross-linking them with antibodies.

6.8 IMPLICATIONS OF THIS RESEARCH

In order to properly stimulate NK cells after vaccination, we need to better understand how these cells can be activated to induce effector responses and at the same time control it to avoid immune pathology. NK cell activation by vaccines requires balancing the signals of the innate and adaptive immune responses that are triggered

in primary infections, innate cytokines such as IL-12, IL15, IL-18, IFN-α, IFN-β, contact-dependent signals and, in secondary infection, via T cell mediated cytokines like IL-2, IL-21 or IFN-γ. The basic mechanism of NK cell activation involves early inflammatory IL-15 and IL-18 production by antigen presenting cells (macrophages, monocytes and dendritic cells) acting on the resting NK cell receptors IL-15R and IL-18Rα, which induces CD25. Up-regulation of CD25 increases the sensitivity of NK cells to IL-2 (via high affinity IL2Rαβγ), which can be important in the induction of IL-12Rβ2 receptors thereby increasing sensitivity to both IL-12 and IL-2 leading to secretion of IFN-γ (Nielsen et al., 2013, Nielsen et al., 2016, Goodier et al., 2016). Cytokine-induced 'memory-like' NK cells have been shown to be less differentiated without expression of CD57 molecule and KIR receptor. As observed in this thesis, these CD56dimNKG2C+CD57- NK cells may prove to be potential targets for vaccine-induced responses, because they might share some similarities with these cytokine-induced 'memory-like' NK cells.

Furthermore, in the understanding of NK cell activation, IL-2 and IL-15 receptors share the common signalling receptor chains CD132 (common γ chain) and CD122 (β subunit) that induce STAT5 signalling. Resting NK cells are more responsive to IL-15 than IL-2 in initial activation, however, up-regulation of CD25 on activated NK cells increases the responsiveness of IL-2 rather than IL-15 (Nielsen et al., 2016). This homeostatic regulation of NK cell activation ensures responsiveness to IL15 in an innate immune response and IL-2 in an adaptive immune response. IL-2 can decrease IL-15 receptor transcripts (Pillet et al., 2011). Thus, it would be important to understand the mechanism of NK activation both in HCMV infected and uninfected individuals in relation to the cells and cytokines mentioned above.

6.9 FUTURE WORK

6.9.1 HCMV antibody and NKG2C genotype

Chapter 3 of this thesis, demonstrated that children below 10 years with homozygous *NKG2C* gene deletion had a higher titres of HCMV specific-antibodies compared to *NKG2C* heterozygous and *NKG2C+* homozygous children. This observation possibly indicates an impaired ability to properly control HCMV infection early in life in children

completely lacking the *NKG2C* gene or could suggest more frequent reactivation of the infection in these people.

In the coming months, I would like to investigate the following objectives within the larger cohort of individuals who have been genotyped for NKG2C and are described in Chapter 3.

1. To investigate the correlation of HCMV antibody titre with NKG2C genotype and how this is influenced by age in these 1485 individuals.
2. To pilot the possibility of detecting viral HCMV in plasma or urine of children in West Kiang, so that this can be related to NKG2C genotype and anti-HCMV IgG plasma antibody titre and directly test whether children lacking NKG2C excrete virus more often than those with NKG2C.

6.9.2 NK cell DTPiP assay

In the near future, cryo-preserved PBMC and plasma samples from the NK cell DTPiP vaccination study will be sent to London where the role of CD16 and immune complex- mediated NK cell activation will be tested by comparing antigen responses of cells cultured in autologous plasma taken before and after vaccination. It is known that highly differentiated NK cells express higher levels of CD16, and therefore understanding how cross-linking of this receptor affects the responses of NKG2C+CD57- and NKG2C+CD57+ NK cells will be important for our understanding of vaccine responses.

I will therefore investigate the following:

1. Determine CD4+ T cell IL-2 secretion to vaccine antigens after booster DTPiP vaccination.
2. Examine the role of vaccine-induced antibodies in NK cell activation and how this relates to NK cell differentiation.
3. Where feasible, investigate the demethylation patterns of NKG2C+ and CD57+ NK cell subsets pre and post vaccination.

6.9.3 Influence of HCMV infection on immune repertoire

As discussed in Chapter 3, 4, and 5, I have partially shown that HCMV infection can significantly influence lasting phenotypic and functional immune responses, especially affecting responses of natural killer cells important in early innate immunity and of T cells essential for adaptive immunity. These immunological outcomes have serious implications concerning human health and disease, particularly in the developing countries where HCMV infection is very common. As innate and adaptive immune cells are biased towards a specific cellular phenotype and functional capacity, this could considerably influence the efficacy and effectiveness of the immune response to a specific pathway. Consequently, virulent viral and bacterial pathogens and other microbes can exploit this HCMV-induced immune defect to evade immune recognition and killing. Thus, understanding in detail how HCMV infection can shape the immune system will instruct us on means of considering the extent of this infection in different groups of individuals so that we can prevent and develop safer and better vaccines. In addition, such knowledge could possibly lead to the use of anti-viral drugs or HCMV vaccines to manipulate HCMV infection and in turn influence immune differentiation if this is found to be important.

With this in mind, we plan in future to investigate in detail the natural history of human cytomegalovirus infection and its impact on immune phenotype and function in children. The overall aim of this research project would be to understand how HCMV modulates the human immune repertoire, immune function and how this influences human health outcomes. In spite of universal HCMV infection in The Gambia, considerable heterogeneity in T cell and NK cells exists in different age groups. This observation might imply that other unidentified factors apart from HCMV infection might be influencing immune cell phenotype differentiation and maturation, thereby, affecting health outcome measures. We plan to specifically look at understanding what determines the time of HCMV infection, latency and reactivation, and viral load. We will also investigate the factors that drive HCMV driven immune differentiation. Finally, we will examine how the timing and nature of HCMV infection influence immune phenotype and function and health outcomes. The overall goal would be to define the time, level, reactivation, viral genotype, genetic complexity of HCMV infections in relation to host age, NKG2C genotype, NK cell and T cell immune differentiation and effector function, nutritional status and clinical history.

Thus, knowing the different strains of HCMV that circulate within our study cohort will inform us about the spread of the virus and its transmission. This information will help answer the long awaited question of how HCMV infection is transmitted in children and thus what interventions might be required to reduce early infection. At the same time, we will also understand its impact on the immune system, especially, on T cell and NK cells.

In the future, because of the high prevalence of this infection, it would be informative to perform a clinical survey to assess whether congenital HCMV disease represents a significant burden on the Gambian population. If so, it would be ideal to consider performing a HCMV vaccination study if a safe, immunogenic and reliable candidate vaccine can be available in the coming years. Live attenuated CMV vaccines have, however, only given a modest protection to date (Schleiss and Heineman, 2005).

A phase II vaccine trial of recombinant gB subunit of HCMV with MF59 adjuvant had only 50% efficacy in vaccinated women. However, in this study, there was a reported case of congenital HCMV (cHCMV), which might raise concerns about the effectiveness of using this type of vaccine, although the placebo group had 3 cases of cHCMV (Pass et al., 2009). In another phase II trial the same recombinant gB subunit vaccine was shown to decrease viraemia after kidney transplantation and enhance antibody levels (Dasari et al., 2013). Furthermore, there is currently a phase I study investigating the effects of giving a HCMV DNA vaccination to both sero-positive and sero-negative healthy donors. The intention is to give the vaccine to determine if the donors would mount an effective immune response to both HCMV gB and pp65 antigens (Astellas Pharma Global Development Inc., 2016). In addition, two other studies are under way both looking at vaccination in stem cell transplantation patients using CMVpp65-A*0201 peptide vaccine and multi-peptide cytomegalovirus (CMV)-modified vaccinia Ankara (MVA) vaccine (National Cancer Institute, 2016, National Cancer Institute & Diavax Biosciences, 2016).

Before an effective vaccine can be developed, it will be important to understand the immune correlates that provide protection of HCMV infection in both the infected and uninfected individuals. This can be done through vaccination of either uninfected mothers or newborns, who are at high risk of infection. Congenital HCMV occurs in 1-2 % of sero-positive women, which is about one third of all congenital infections. This increases to 40% incidence of congenital infection in sero-negative women who experience primary HCMV infection during pregnancy, suggesting that prior exposure

to HCMV may provide some form of protection against congenital HCMV infection. Also congenitally infected infants born to mothers with primary HCMV infection during pregnancy are more likely to experience clinical sequelae at birth and to develop more severe neurological defects than those born to mothers infected prior to pregnancy. Furthermore, CMV-specific CD4+ T cells and antibodies against HCMV gH/gL/UL123, UL130, and UL131 glycoproteins essential for viral entry have been correlated with maternal protection in pregnancy (Schleiss and Heineman, 2005, Bialas and Permar, 2016). However, because of ethical and safety concerns of these clinical vaccine trials, animal models (e.g. rhesus monkeys) would provide the most appropriate tools to comprehend the mechanism of CMV infection.

6.10 IMPACT OF RESEARCH ON THE GAMBIA POPULATION

Although HCMV infection is asymptomatic in immune competent individuals, it is a significant contributor of pathology in immunosuppressed and immunocompromised people. This raises the question of how HCMV infection impacts the Gambian population and how this influences immune responses and co-infection with other bacterial and viral pathogens. This might mean that children in The Gambia born at high risk of acquiring HCMV either at birth or as they grow, could stand at a disadvantage compared to children that are not infected with this virus. It also means that the high prevalence of this infection might be affecting people in different ways that have not as yet been measured or studied. For example, looking at how early HCMV infection affects children's cognition, hearing, growth and development would be an interesting and relevant area to investigate. If HCMV is found to have a significant impact on human health in The Gambia it would highlight the need to inform Gambian women of the existence of this viral transmission, how this could affect their pregnancies, and how they could protect their children from this viral transmission after birth and throughout life.

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In the name of Allah the Most Beneficent the Most Merciful