The Effect of Viral Vaccines on Natural Killer Cell Effector Function

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Declaration

I, Helen Wagstaffe, 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.

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Date   29/05/2019

Abstract

Natural killer cells are large, granular, innate lymphoid cells that lie at the interface between innate and adaptive immunity and facilitate bidirectional crosstalk between the two systems. This has led to an abundance of research into vaccination induced priming of 'adaptive' or memory-like NK cell responses. Using human peripheral blood mononuclear cells for cross-sectional and vaccination studies in two distinct virus models, cytokine-dependent NK cell activation, and the effect of vaccination on these responses was investigated.

Very low concentrations of IL-15 enhanced NK cell activation in response to inactivated influenza virus stimulation *in vitro* via augmented innate cytokine secretion from accessory cells, in particular IL-12 from myeloid dendritic cells. Similarly, after seasonal influenza vaccination, both accessory cell cytokine secretion and NK cell activation were enhanced compared to before vaccination in response to low dose IL-15 stimulation *in vitro*. This suggested that together with a vaccination-induced enhanced responsiveness to innate cytokines at the NK cell level (the generation of cytokine-induced memory like NK cells), an increased production of innate cytokines at the accessory cell level contributed to the overall enhancement in NK cell response post-vaccination.

The effect of vaccination on NK cell effector function was further investigated in PBMC from heathy volunteers vaccinated with an Ebola glycoprotein expressing, vectored, prime-boost regimen vaccine (Ad26.ZEBOV/MVA-BN-Filo). Both inactivated influenza vaccination and Ebola vaccination increased the frequency and proliferation of less differentiated, CD56^{bright} NK cells. Data described in this

thesis also demonstrate for the first time that NK cells were activated indirectly by Ebola glycoprotein induced IL-12 and IL-18, independently of Ebola vaccination, and in contrast to influenza vaccination, there was no evidence of vaccine induced boosting of these responses. NK cell responses to Ebola GP were dependent on accessory cell TLR-4 engagement but were tightly regulated by CD14$^+$ monocyte-derived IL-10. In addition to soluble antigen driven cytokine-dependent activation, 21-day post-boost vaccination serum induced robust antibody-dependent NK cell activation of more differentiated subsets that varied with vaccine arm and correlated with antibody titre.

Together, these studies demonstrate that two different types of vaccines against distinct viruses have similar impacts on the activation of less differentiated NK cells. Priming of both NK cells and myeloid cells for enhanced cytokine-dependent NK cell responses after vaccination was not replicated across different vaccination models and may be a function dependent on the unique cytokine signatures induced by the respective antigens. These studies further our understanding of NK cells in post-vaccination effector responses, and may represent an avenue for improving future design of vaccines and adjuvants.

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First and foremost, I would like to thank Martin, initially my secondary supervisor, and then primary. He has been a mentor, a lab buddy, the source of much advice and a great supervisor. At times when the words 'it's all a bit much' come to mind, he has been cool and collected. This thesis would be missing half of the results chapters if it wasn't for his persistence and patience to receive samples in time. His help in the lab has been invaluable, helping out with all sorts of odd time points, saving me from working all night and early the next morning on top! Thank you, Martin, for being ever present and supporting me throughout it all.

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Abbreviations

Ad26	Adenovirus type 26
ADCC	Antibody-dependent cellular cytotoxicity
APC	Antigen presenting cell
AS	Adjuvant-delivery system
BCG	Bacille Calmette-Guérin
BN	Bavarian Nordic
CD	Cluster of differentiation
ChAd3	Chimp Adenovirus type 3
CHMI	Controlled human malaria infection
CIML	Cytokine induced memory-like
CLP	Common lymphoid progenitor
CSP	Circumsporozoite surface protein
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DTP	Diptheria, tetanus and pertussis vaccine
EBOVAC	Ebola vaccine consortium
EVD	Ebola virus disease
Fc	Fragment crystallisable (region of an antibody)
FcR	Fc receptor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GP	Glycoprotein
GSK	GlaxoSmithKline
H3N2	Inactivated whole influenza virus (A/Victoria/361/2011)
HA	Hemagglutinin
HBs	Hepatitis B virus surface antigen
HCC	High concentration cytokines
HCMV	Human cytomegalovirus

HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HPV	Human papilloma virus
HPV	Human papilloma virus
ICAM-1	Intracellular adhesion molecule 1
IFN	Interferon
IFNAR	Interferon α/β receptor
IID	IFN-inhibiting domains
IL	Interleukin
ILC	Innate lymphoid cell
iNK	immature NK cell
IQR	Interquartile range
ITAM	Immunoreceptor tyrosine-based activating motif
J&J	Johnson and Johnson
KIR	Killer immunoglobulin-like receptors
KLR	Killer lectin-like receptors
LAMP-1	Lysosome associated membrane protein 1
LFA-1	Lymphocyte function-associated antigen 1
M	Matrix
MAb	Monoclonal antibody
MCMV	Murine cytomegalovirus
mDC	Myeloid dendritic cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mNK	Mature NK cell
mTOR	Mammalian target of rapamycin
MVA	Modified Vaccinia Ankara
NA	Neuraminidase
NCAM	Neural cell adhesion molecule

NK	Natural killer cell
NP	Nucleoprotein
NS	Non-structural
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PRR	Pattern-recognition receptor
QIV	Quadrivalent influenza vaccine
RNA	Ribonucleic acid
SEBOV	Sudan ebolavirus
SIV	Simian immunodeficiency virus
STAT	Signal transducer and activator of transcription
TB	Tuberculosis
TGF-β	Transforming growth factor beta
TIV	Trivalent influenza vaccine
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
VLP	Virus-like particle
VSV	Vesicular stomatitis virus
YF-17D	Yellow fever vaccine (17D)
YFV	Yellow fever virus
ZEBOV	Zaire ebolavirus

Chapter 1: Introduction

This chapter contains an extended version of published review article:

Vaccinating for Natural Killer Cell Effector Functions

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1.1 The Immune System

The human immune system is traditionally divided into two arms, innate and adaptive. Innate immune cells of myeloid lineage, including monocytes, macrophages and dendritic cells (DCs), and of lymphoid lineage, such as innate lymphoid cells (ILC) including natural killer (NK) cells, react within hours of infection. The innate immune system responds to conserved pathogen associated molecular patterns (PAMP) present on viral, bacterial and fungal pathogens via pattern-recognition receptor (PRR) engagement. Activation of these cells leads to non-specific, antigen-independent targeting and elimination of infected cells and extracellular pathogens. Downstream secretion of pro and anti-inflammatory cytokines and chemokines conducts recruitment of immune cells to the site of infection and enables the orchestration of subsequent immune responses. The adaptive arm, consisting of T and B lymphocytes, develops later in the course of an infection and is capable of targeting specific pathogens via rearrangement of genes encoding antigen receptors to produce a diverse array of T and B cell receptors. This response forms the basis of immunological memory and the ability to mount a more robust response upon secondary exposure.

1.2 Natural Killer Cells

NK cells are large, granular, ILCs that express a wide variety of germ line encoded receptors on their surface. Derived from the bone marrow, but along a distinct lineage from B and T cells, they make up 5-15% of human peripheral blood mononuclear cells (PBMC). NK cells are characterised phenotypically by the lack of T cell marker CD3 and/or B cell marker CD19 and the expression of CD56 (neural cell adhesion molecule; NCAM) (1). They are among the first cells to respond during primary infections and are important in the early control of viral infections including herpesviruses and influenza infections (2-5). The role of NK cells as early producers of inflammatory cytokines (such as interferon (IFN)-γ), controllers of viral load and modulators of the bidirectional cross-talk between innate and adaptive immunity has led to an abundance of research into 'adaptive' or memory-like NK cell responses and their function after vaccination.

1.2.1 Human NK cell Subsets and Differentiation

ILC1, 2 and 3 and NK cells derive from common lymphoid progenitor (CLP) cells, however, NK cells are said to derive from a distinct progenitor (NKP vs ILCP) and can be distinguished from ILC1 by the expression of transcription factor Eomes (6). NKP cells, having gained expression of the IL-15 receptor β-chain (IL-15Rβ; CD122), give rise to immature NK cells (iNK) expressing CD56, which develop into mature NK cells (mNK) and gain expression of CD94 (a component of killer lectin-like receptors; KLR) and CD16 (7, 8). The bone marrow and several secondary lymphoid tissues are thought to be the sites of NK cell development

as suggested by the identification of NKP in human tissues (9). IL-15 is critical for NK cell development; IL-15$^{-/-}$ mice (or mice deficient in any chain of the IL-15R - α, β or γ chain) lack NK cells, highlighting the definitive role for this cytokine (10). Group 2 and 3 ILCs are phenotypically and functionally different from ILC1 and NK cells; ILC2, distinguished by transcription factor GATA-3 expression, release type 2 cytokines (IL-5 and IL-13) and ILC3, dependent on transcription factor RORγt, release IL-17 (8). ILC phenotypes are summarised in **Table 1**.

	NK cells	**ILC1**	**ILC2**	**ILC3**
Lineage	-	-	-	-
CD16	+/-	-	-	-
CD56	+	+/-	-	+/-
CD94	+	-	-	-
CD117	-	-	+/-	+
CD127	-	+/-	+	+
NKp44	+/-	+/-	-	+/-
IL-1βR	-	+/-	+	+
IL-12Rβ	+	+	-	+/-
IL-17Rβ	-	-	+	-

Table 1: Phenotypes of human ILC subsets. Adapted from (11).

In human peripheral blood, mature NK cells are subtyped in to CD56^{dim} and CD56^{bright} subsets depending on the density of CD56 expression on the cell surface. The classification, first made in 1986 by Lanier *et al.*, demonstrated that low CD56 expressing NK cells (CD56^{dim}) are CD16 positive (or high) and more cytotoxic than high CD56 expressing NK cells (CD56^{bright}) that produce cytokines more efficiently (12). CD56^{bright} NK cells that make up around 8% of NK cells in human peripheral blood also become cytotoxic and CD56^{dim} cells that make up the majority of NK cells in peripheral blood (>95%) also release cytokines, blurring the distinction between the roles (1, 13). It is thought that CD56^{bright} NK cells are precursors to more differentiated CD56^{dim} NK cells, however this is still debated and under investigation (14, 15).

CD57, which is only expressed on a proportion of CD56^{dim} NK cells, is another surface marker by which human peripheral blood NK cells are categorised. First recognised as a marker of terminal T cell differentiation, CD57 is a terminally sulphated glycan carbohydrate carried on adhesion molecules (16). CD57^{+} NK cells are thought to differentiate from CD56^{dim}CD57^{-} cells (via intermediate CD57 expression) during normal ageing and activation, which is accelerated by viral infections such as human cytomegalovirus (HCMV) (discussed later) (17, 18). Less differentiated CD56^{dim}CD57^{-} NK cells are more responsive to exogenous cytokines and produce more IFN-γ than the more differentiated CD56^{dim}CD57^{+} NK cell subset (16). CD56^{dim}CD57^{+} NK cells express the highest levels of CD16 making them specialised for antibody-dependent activation, but respond less well to cytokine stimulation (17, 19). CD56^{bright} and CD56^{dim}CD57^{-} NK cells are also more proliferative, measured by Ki67 expression, and express higher levels and percentages of cytokine receptors such as IL-2Rα-chain (CD25), IL-12Rβ2 and

IL-18Rα than CD56^{dim}CD57^{+} NK cells. (18, 19). **Figure 1** shows the phenotypic and functional differences between NK cell differentiation subsets.

NK cells are widely distributed in tissues including the liver, lungs and the uterus; tissue resident NK cells, in the liver for example, can make up 50% of total lymphocytes (1). NK cells in secondary lymphoid tissues such as lymph nodes and tonsils are predominantly CD56^{bright}, whereas the majority of splenic NK cells are CD56^{dim} (1, 20). 'Tissue residency' markers of human NK cells, such as integrins involved in retaining lymphocytes in tissues, have recently been deduced, such as CD103^{+} and CD49a^{+} subsets in the liver, lung and uterus (1, 20-23).
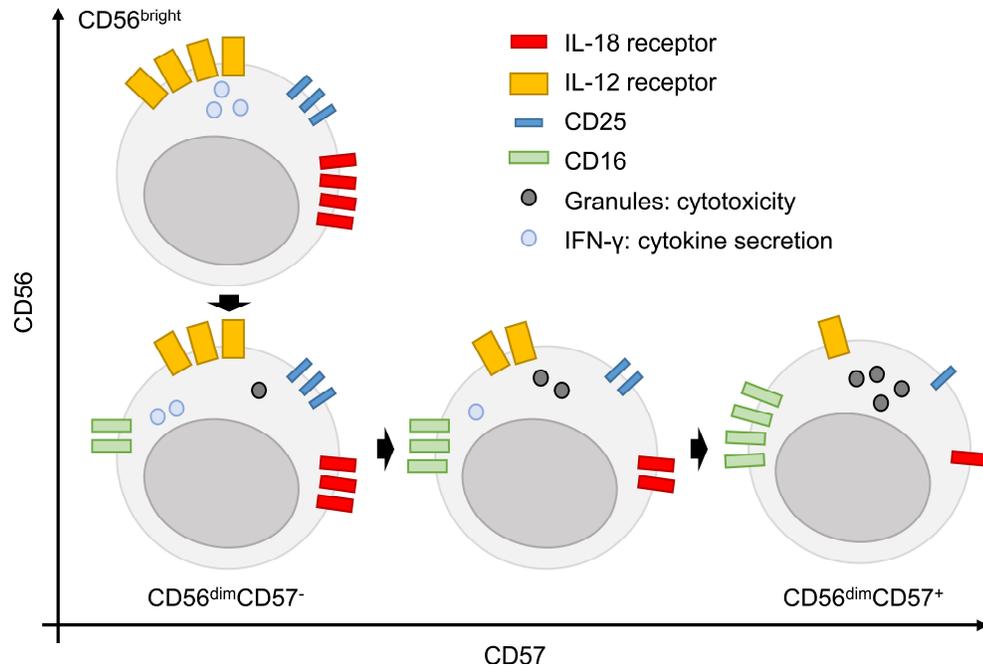
Figure 1: NK cell differentiation subsets.

The phenotype and function of less differentiated CD56^bright, and more differentiated CD56^dim NK cell subsets, which are further subtyped by CD57 expression. Expression of cytokine receptors (for IL-12, IL-18 and IL-2 (CD25)), and activating receptor CD16, IFN-γ secretion and cytotoxicity differs between subsets. Adapted from (18).

1.2.2 NK Cell Activation

NK cells are activated by several distinct but synergistic signals: direct activation by an imbalance in activating and inhibitory receptor engagement, antibody-dependent activation via CD16 crosslinking, and indirect activation by soluble cytokines and chemokines. **Figure 2** shows a schematic of how NK cells integrate innate and adaptive signals for activation. This thesis primarily focuses on antibody and cytokine-dependent NK cell activation.
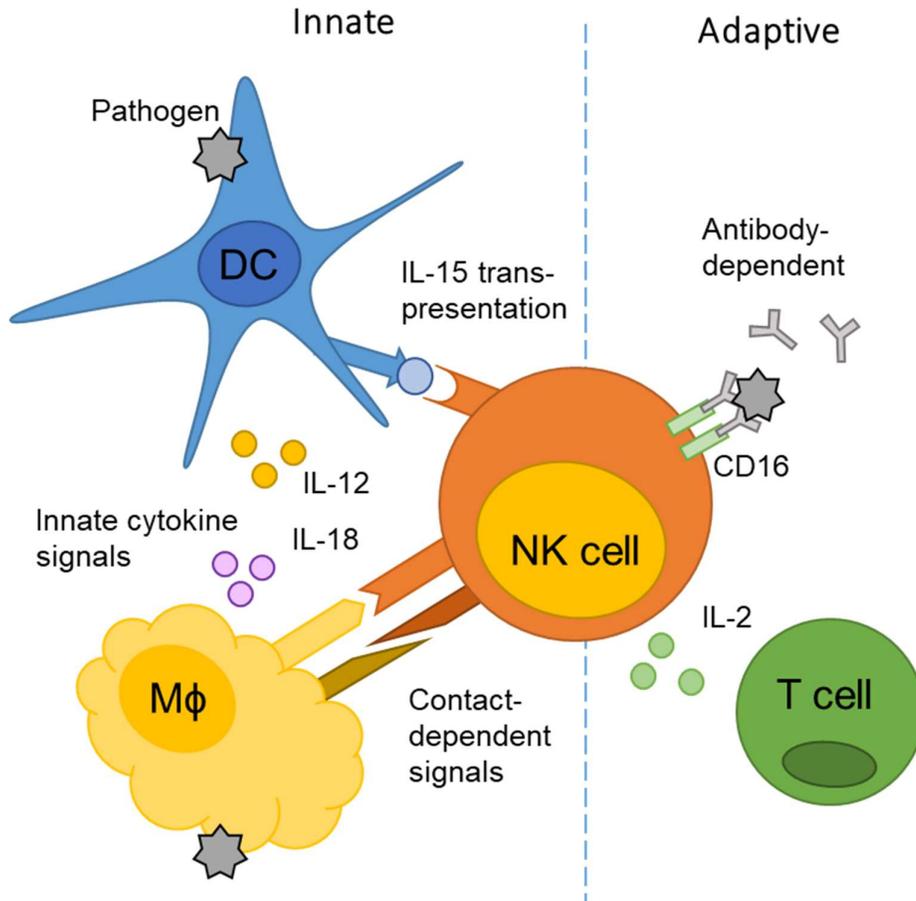
Figure 2: Integration of innate and adaptive signals for NK cell activation.

NK cells can be activated by soluble or contact-dependent signals from innate antigen presentating cells (APCs) such as macrophages (MΦ) or dendritic cells (DC), or by adaptive signals such as antibody-antigen immune complxes or T cell-derrived IL-2.

1.2.2.1 Direct Activation

NK cells form contacts with myeloid accessory cells and target cells via adhesion molecules such as lymphocyte function-associated antigen 1 (LFA-1) (24). An initial activating receptor signal, such as NKG2D-mediated recognition of upregulated stress ligands can induce a conformational change in LFA-1, allowing binding to intracellular adhesion molecule (ICAM)-1 on target cells (25). This conjugate formation is essential for cellular polarisation and is important for NK cell lytic granule delivery and cytotoxicity (26, 27). Cell-cell contact is also important for cytokine mediated NK cell responses, for example trans-presentation of IL-15 by DC at the interface of DC-NK cell interactions is important for NK cell survival, proliferation and activation (28-30). IL-12 may also be delivered in a targeted way for inducing NK cell IFN-γ secretion (31).

NK cells express germ line encoded receptors such as KLR, highly polymorphic killer immunoglobulin-like receptors (KIR) and natural cytotoxicity receptors (NCR). KLR, for example NKG2A (inhibitory receptor) and NKG2C (activating receptor), form heterodimers with CD94 and bind non-classical HLA-E molecules (32). KIR bind classical HLA-A, B and C molecules, for example KIR2DL1 (inhibitory receptor) and KIR2DS1 (activating receptor) bind HLA-C2 molecules (33). NCRs NKp30, NKp44 and NKp46 are known for inducing cytotoxicity after recognition of self-antigens upregulated in response to cellular stress and transformation (34). Contact with cells lacking ligands for inhibitory receptors or contact with cells expressing ligands for activating receptors enables direct NK cell activation. Under homeostatic conditions, the balance of these receptor interactions favours NK inhibition, stalling the killing of healthy self-cells.

However, following infection or transformation, down regulation of HLA class I and induced expression of stress ligands on the target cell tips the balance in favour of NK cell activation, a phenomenon known as missing-self (35, 36).

This cytotoxic effector mechanism is important in anti-viral immunity as many viral proteins, for example human immunodeficiency virus (HIV) Nef and Vpu proteins, mediate downregulation of HLA molecules rendering the infected cell open to NK cell killing (37-39). Alternatively, in the case of murine cytomegalovirus (MCMV), viral protein m157 can act as a ligand for NK cell inhibitory receptor Ly49C stalling NK cell activation and evading killing (40, 41). In addition to activation and inhibition of NK cell functions in the effector phase of an infection, the level of activation and requirement for additional co-stimulation is determined by a prior 'education' process whereby functional receptor-ligand interactions (KIR/HLA and NKG2A/HLA) determine NK cell sensitivity for inhibition (42). The presence or absence of KIR and their ligands has been linked to the outcome of viral infections in humans, for example KIR2DL3 with HLA-C1 ('educated' genotype) is associated with increased resolution of hepatitis C virus (HCV) infection (43, 44).

Direct interaction between human NK cell receptors and pathogen-derived molecules demonstrate modulation of NK cell activation in an antigen-specific manner. HCV and Flavivirus peptides in combination with HLA-C molecules are recognised by KIR2DS2 leading to NK cell activation and target cell lysis (45). HCMV-derived peptides stabilise HLA-E on the cell surface acting as a ligand for NKG2C, which enables activation and proliferation of NKG2C$^+$ NK cells (46). Influenza haemagglutinin (HA) was identified as a ligand for NK cell NKp46 and

NKp44 in humans, and shown to trigger cytotoxicity (47, 48). This interaction was dependent on sialyation of NKp46 and was demonstrated with HA from H1, H3 and H5 influenza viruses with different sites and degrees of binding (49, 50). The interaction in a mouse model, also dependent on sialic acid residues, was shown to be critical for preventing lethal influenza virus infection suggesting an important anti-viral mechanism (51, 52). NCRs have also been suggested to bind other viral, bacterial and parasite-derived ligands resulting in both activating and inhibitory signals (53). Recently, *Aspergillus fumigatus* hyphae was shown to be directly recognised by NK cells involving binding of CD56 inducing NK cell cytokine secretion and cytotoxicity (54, 55).

1.2.2.2 Antibody-Dependent Activation

Target cells coated with pathogen-specific antibodies or antibody-antigen immune complexes can induce NK cell antibody-dependent cellular cytotoxicity (ADCC). The constant (Fc) portion of antigen-specific antibodies (typically IgG1 and IgG3 in humans) engage activating or inhibitory Fc receptors (FcR), FcγRI and FcγRIIIa or FcγRIIa respectively. NK cells express the low affinity, activating FcR FcγRIIIa (CD16), predominantly expressed by more differentiated CD56^{dim} NK cell subsets. Crosslinking of CD16 leads to signal transduction via immunoreceptor tyrosine-based activating motifs (ITAM) and intracellular adaptor molecules CD3ζ and/or FcεR1γ (56, 57). This leads to the release of cytolytic granules containing lytic proteins such as granzyme B and perforin, a process termed degranulation (57). During degranulation, lysosome associated membrane protein-1 (LAMP-1; CD107a) within the membrane of the granule is

exposed on the surface of the cell and therefore is often used as a marker of NK cell degranulation (58). Following activation and vaccination, CD16 is cleaved from the surface of NK cells through matrix metalloprotease ADAM17 cleavage, which is thought to be a regulation mechanism limiting further antibody and cytokine-dependent activation (59-61).

Antibodies are important effector molecules that facilitate cellular immune mechanisms for killing of infected or transformed cells. Many studies are shedding light on the importance of ADCC inducing antibodies (with weak neutralising ability) in vaccine-induced protection against influenza, Ebola and HIV (62-64). NK cell function and ADCC may be utilised as a parameter for the evaluation of vaccine responses and in the design of new vaccines or post-exposure monoclonal antibody (mAb) therapies in the future.

1.2.2.3 Cytokine-Dependent Activation and Regulation

Optimal NK cell activation and regulation requires innate cytokines released from accessory cells after antigen recognition by PRRs or pathogen phagocytosis; both pro and anti-inflammatory cytokine signalling is indispensable for anti-viral immunity (65). *In vitro* studies have demonstrated unique but synergistic roles of IL-12, IL-15, IL-18 and type I IFN, as well as T cell derived IL-2 in NK cell activation. Whole PBMC or purified NK cell stimulation with cocktails of IL-12, IL-15 and IL-18 are well known to induce significant NK cell IFN-γ secretion, degranulation and CD25 upregulation (66-68).

Type I IFNs, consisting of IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω are produced in abundance by DCs after viral infection, predominantly by plasmacytoid DCs. Human IFN-α (of which there are 13 subtypes in humans) and IFN-β are critical during viral infection; both bind the heterodimeric IFN-α/β (IFNAR) receptor (two subunits, IFNAR1 and IFNAR2), expressed on all nucleated cells (69). Receptor binding results in signal transduction and phosphorylation of STAT1 and 2 and ultimately leads to transcription of IFN-stimulated genes (ISGs) (70). Type I IFN is particularly important for NK cell cytotoxicity, whether that is by binding IFNAR on NK cells directly or by indirectly promoting further pro-inflammatory cytokine release (such as IL-15 and IL-12) from DCs (71, 72). Many viruses have evolved type I IFN evasion mechanisms, highlighting the significance of type I IFN in anti-viral immune responses. Influenza virus non-structural (NS) 1 protein, for example, blocks type I IFN transcription and mRNA processing, and Ebola virus encoded VP24 and VP35 proteins use several different mechanisms to strongly antagonise host IFN responses (73, 74).

IL-12, produced by monocytes, macrophages and DCs, is a potent inducer of NK cell activation, in particular IFN-γ secretion (75, 76). Engagement with the IL-12 receptor (a dimer of IL-12Rβ1 and IL-12Rβ2) induces intracellular signalling resulting in phosphorylation of STAT4 and transcription of genes for effector function (77). Neutralisation of IL-12 has been shown to reduce NK cell IFN-γ production in many different infection models in mice and in human *in vitro* culture systems (78-80). Exogenous IL-12 therapy restored NK cell function in HIV exposed infants with impaired accessory cell cytokine secretion highlighting the importance of this cytokine in the immune response to viral infection (81).

IL-18, a member of the IL-1 family, is synthesised as a precursor protein (pro-IL-18) that is constitutively expressed in macrophages. Pro-IL-18 is cleaved in an inflammasome dependent manner by caspase-1 function forming biologically active IL-18 that is secreted (82). IL-18 binding to the IL-18R complex (IL-18Rα and IL-18Rβ) induces rapid NK cell CD25 upregulation; IL-18 also synergises with IL-12 and IL-15 for robust IFN-γ secretion (66-68, 83).

IL-2 and IL-15, produced by different arms of the immune system, have well known importance in NK cell differentiation and survival and have differential roles in NK cell activation. They have unique alpha chain receptors (IL-2Rα and IL-15Rα) but share a beta chain (IL-2Rβ/IL-15Rβ) and common gamma chain (γc) which together make up high affinity trimeric receptors on the surface of NK cells (84). IL-2 has been shown to reduce expression of CD25, γc and IL-15Rα, whereas IL-15 sustains IL-15Rα expression (85). Evidence for models where IL-2 secretion from T cells limits further IL-15 signalling after adaptive responses are mounted describe a feedback mechanism between these two cytokines (68, 85).

IL-15 trans-presentation, where IL-15 bound to IL-15Rα is presented by DC at the DC-NK cell interface, is gaining in interest as a NK cell 'priming' tool, and is being utilised for NK cell immunotherapy (86-91). The IL-15 superagonist complex ALT-803 has been shown to induce NK cell activation and was well tolerated in cancer patients in phase 1 clinical studies (90-92). Increased NK cell numbers correlated with a reduction in simian immunodeficiency virus (SIV) viral load in non-human primates treated with ALT-803, suggesting an ability to control viral replication in chronic infections (89). IL-15 complex treatment prevented the development of cerebral malaria in mice, synergy between IL-15 and IL-12/IL-21

induced immunomodulatory, IL-10-secreting NK cells perhaps reducing pathogenic T cell activity (93). Vaccines combined with IL-15 have been shown to elicit superior immune responses in mice; in these studies, enhanced DC maturation correlated with NK cell activation, although the role of NK cells in protective immunity was not evaluated (94-97).

Synergistic effects of innate cytokines and synergy between cytokines and direct or antibody-mediated activation are important for optimal NK cell responses and regulation of effector functions. IL-12, IL-15 and IL-18 independently and synergistically drive the upregulation of CD25 on NK cells, increasing responsiveness to IL-2 (68, 98). IL-12 and IL-18 independently synergise with IL-2 for increased CD25 expression and IFN-γ secretion (68). IL-12 and type I IFN each synergise to enhance antibody-dependent induction of NK cell cytotoxicity towards antibody-coated tumour cells and virally infected cells (99-101). It is suggested that co-localisation of CD16 and the IL-12 receptor on the surface of activated NK cells allows enhanced downstream signalling functions (102). IL-18 also synergises with CD16 signalling to drive NK cell degranulation (68). Cytokine stimulation has also been shown to reduce the threshold for receptor-mediated direct activation of NK cells (24).

Negative regulation of NK cell function by soluble mediators has been widely demonstrated and has an important role in optimising immune responses to infection and limiting pathology (103). TGF-β and IL-10 can inhibit NK cell function directly or indirectly via suppression of accessory cell cytokine secretion. TGF-β signalling has been shown to block the mammalian target of rapamycin (mTOR) signalling pathway, important for NK cell development and activation and leads

to downregulation of CD25 and type I IFN receptor expression (104, 105). IL-10 can also indirectly limit NK cell function by supressing cytokine secretion; DCs from *IL10^{-/-}* mice secrete more IL-12 during MCMV infection and IL-10R blockade increased TLR-induced IL-12 secretion in monocytes *in vitro* (106, 107).

1.2.2.4 NK Cell Activation in Viral Infections

PRRs such as members of the TLR family sense both the extracellular and intracellular environments for PAMPs. TLRs are expressed on many innate immune cells and recognise different pathogen-derived PAMPs. TLR-3, 7, 8 and 9 are important in recognising viral products and become exposed to viral double stranded (ds)-RNA or single stranded (ss)-RNA, for example, during endosomal internalisation of virus particles or after cell lysis (70). Cell surface expressed TLR-4, typically known for recognising bacterial LPS, has also been shown to recognise viral GPs (108, 109). TLR ligation induces signalling cascades (typically via adaptor molecules TIR domain–containing adaptor inducing IFN-β (TRIF) or myeloid differentiation primary response protein 88 (MyD88)) ultimately leading to promotion of gene transcription for pro and anti-inflammatory cytokine production, including type I IFN, IL-12, IL-15 and IL-18, with distinct kinetics relating to the viral pathogen (70, 110). These cytokines modulate the NK cell response via receptor engagement and/or cell-cell contact and signal transduction, resulting in STAT phosphorylation and transcriptional control of target genes such as ISGs (70).

NK cells shape the subsequent adaptive response by secretion of cytokines and chemokines. IFN-γ is important for T cell expansion and recruitment, memory

formation and B cell isotype class-switching (111), whereas IL-10 produced by NK cells is reported to limit T cell proliferation and effector functions (112, 113). NK cell cytotoxic functions reduce viral loads by eliminating infected cells, which modulates the amount of antigen available for T cell activation (114). Direct lysis of CD4$^+$ T cells by NK cells has also been demonstrated in viral infections, eliciting indirect effects on the CD8$^+$ T cell compartment and regulating the balance between viral clearance and immunopathology (113). The absence of NK cells in mice increases CD4$^+$ T cell activation, T follicular helper cell responses and the number of germinal centre B cells in response to lymphocytic choriomeningitis virus (LCMV) infection, demonstrating NK cells can constrain antibody generation and affinity maturation (115, 116). NK cell production of CC-chemokines (such as CCL3, CCL4 and CCL5) can inhibit viral entry and replication, for example, by competing for HIV co-receptor CCR5 (117, 118).

1.3 NK Cell Memory

Several studies have shown that NK cells can acquire some features of adaptive lymphocytes; early examples of NK cell adaptive features arose from mouse studies of hapten-induced contact hypersensitivity and MCMV. NK cells from *Rag2$^{-/-}$* mice were shown to transfer hapten-specific memory-like responses (lasting up to 4 weeks) to naive mice despite the absence of T and B cell immunity (119). The MCMV viral protein m157 on the surface of infected cells was shown to recognise the murine NK cell activating receptor Ly49H and lead to clonal expansion of effector NK cells and generation of a pool of self-renewing m157-responsive NK cells; these cells respond robustly to subsequent MCMV infection when transferred to naive mice (120). More recently, virus antigen-specific NK cell killing has been reported in rhesus macaques with characteristics of memory like NK cells. Purified splenic NK cells from rhesus macaques chronically infected with SIV demonstrated specific lysis of DCs pulsed with SIV antigens; DCs pulsed with unrelated antigens or NK cells from uninfected animals were not reactive (121).

Further to the MCMV model, as mentioned previously, a human counterpart has been proposed to involve NK cell recognition of HCMV infected host cells via the HLA-E-binding receptor NKG2C. Expanded populations of highly differentiated (NKG2C expressing) NK cells in individuals chronically infected with HCMV were first described more than a decade ago (122). Increased frequencies of NKG2C^{+} NK cells after solid organ transplant from HCMV seropositive donors were measured after HCMV reactivation in the transplant recipient (123). This expansion is thought to be mediated by stabilisation of HLA-E and increased

surface expression (46), being associated with enhanced IFN-γ production, acquisition of CD57 and KIR and a reduction in NKG2A expression (4). Further, HCMV expanded NK cells that have lost expression of several key signalling molecules, in particular FcεR1γ, SYK and EAT-2, have been described as 'adaptive' NK cells (124). The loss of these signalling molecules is associated with stable epigenetic changes of the promotor regions of key genes involved in NK cell function, including IFN-γ production (124, 125). Another phenotype of these cells is the loss of a transcription factor involved in IL-12 and IL-18 receptor expression, PLZF, leading to a reduction of these receptors on the surface (124). Adaptive NK cells show reduced responsiveness to IL-12 and IL-18 and produce less IFN-γ compared to other NK cell subsets (124). However, these NK cells do display enhanced ADCC activity towards HCMV-infected target cells, involving a shift to an FcεR1γ independent signalling pathway, suggesting they are specialised for controlling virus reinfection or reactivation (126-129). IL-12 was found to be important in both MCMV and HCMV expansions of adaptive NK cells and IL-2 was shown to be important in their maintenance (130, 131).

Cytokine induced memory-like (CIML) NK cells have been described in mouse and human *in vitro* models (132-135), and there is evidence supporting the generation of these NK cells *in vivo* after vaccination. This is introduced further in section 1.6.

1.4 Influenza Virus

In this thesis, the NK cell response to two viral vaccines will be characterised; the two viruses, influenza and Ebola, will be introduced here.

Influenza virus is a member of the Orthomyxoviridae family and consists of an enveloped, single stranded, segmented RNA genome. Eight segments encode RNA dependent RNA polymerase (PB1, PB2 and PA), nucleoprotein (NP), matrix (M), non-structural (NS), HA and neuraminidase (NA) (136) (**Figure 3**). There are 3 main types of influenza virus, A, B and C, which are classified by properties such as host preference, NP and M protein expression. More recently a new influenza genus, influenza D, has been identified (137). Influenza A viruses, responsible for a large majority of human disease, are further subtyped depending on the expression of HA and NA proteins located on the surface of the viral particle (138). These two highly expressed proteins are responsible for cell attachment and the release of new virions respectively and are primary targets for neutralising antibodies (136). Influenza A has the ability to escape any pre-existing immunity by mutating the genes for HA and NA, a process called antigenic drift, or by reassortment of gene segments between human and non-human strains, antigenic shift (138).
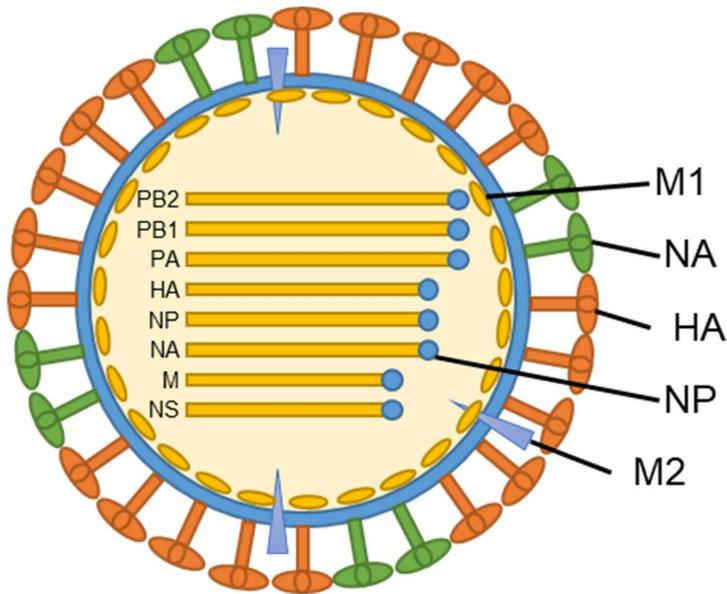
Figure 3: A schematic of Influenza A virus.

Influenza A virus has 8 segments of RNA genome expressing viral proteins RNA dependent RNA polymerase (PB1, PB2 and PA), nucleoprotein (NP), matrix (M), non-structural (NS), haemagglutinin (HA) and neuraminidase (NA). The viral particle is made up of HA and NA proteins on the surface, internal NP and matrix proteins M1 and M2. Adapted from (136).

Influenza pandemics (caused by influenza A only), such as the 1918 'Spanish flu' and 2009 'swine flu' occur when a new stain is introduced into the human population. Pandemic viruses then continue to circulate as seasonal epidemics (138). Influenza A seasonal epidemics result in 3 to 5 million cases and up to 650,000 deaths per year globally, as well as putting intolerable pressure on health systems and causing major economic losses (139). Annual variation in the predominant circulating strains of influenza viruses mitigates vaccine-induced or naturally acquired cross-protective immunity, necessitating annual revaccination of high risk groups (such as pregnant women, children of 6 months to 5 years and the elderly) (139). The effectiveness of the current seasonal influenza

vaccines depend on whether the vaccine strains, recommended by WHO biannually, are matched or mismatched to the circulating strains that season. Currently available vaccines include inactivated (delivered intramuscularly) or live attenuated (via nasal spray) tri, or quadrivalent vaccines (TIV/QIV), containing two influenza A plus one or two influenza B strains.

The influenza vaccines used for the studies in Chapter 3 were as follows: inactivated TIV from the 2015-2016 season (A/California/7/2009 (H1N1), A/Switzerland/9715293/2013 (H3N2), B/Phuket/3073/2013) or inactivated QIV from the 2017-2018 season (A/Michigan/45/2015 (H1N1), A/Hong Kong/4801/2014 (H3N2), and B/Brisbane/60/2008) (both non-adjuvant, Split Virion BP, Sanofi Pasteur). The viruses were propagated in fertilised hens' eggs from healthy chicken flocks and may contain very small amounts of ovalbumin, chicken proteins, neomycin, formaldehyde or octoxinol-9. Buffer solution contains sodium chloride, potassium chloride, disodium phosphate dihydrate, potassium dihydrogen phosphate and water for injections.

Influenza virus infection of the lung epithelia and lung resident macrophages and DCs is recognised by PRRs such as retinoic inducible gene 1 (RIG-1), TLR-3, TLR-7 and nucleotide oligomerisation domain (NOD)-like receptor (NLR) sensing of ssRNA (140). This stimulates the release of cytokines and chemokines including type I IFN which leads to antigen presentation, further cytokine/chemokine release and modulation of apoptosis and proliferation of effector cells in the lung (69). NK cells and T cells are recruited to the lung as a result of local accumulation of IL-15 and CXCL10 (IP-10) (5, 140). Murine lung NK cells become cytotoxic, secrete IFN-γ and upregulate CD25 after influenza

infection (141). DCs act as APCs to cytotoxic T lymphocytes (CTL) and T helper cells and aid recruitment to the lung where they can mediate viral clearance (142).

Cross-reactive antibodies (with neutralising and/or ADCC function) and cross-protection mediated by T cells (predominantly CD8$^+$ T cells) has been demonstrated across influenza A strains (and across influenza B strains) and forms the basis for new improved 'universal' influenza vaccine development (143-146). The leading strategy involves targeting more conserved epitopes of influenza virus proteins, for example the HA 'stalk' region (more highly conserved compared to the more accessible HA 'head' region), the M proteins or NP (147-151). Development of multivalent vaccines (combinations of HA from different strains or combinations of HA + NP/M/NA) have also shown broad protection against heterologous viruses (152). Adding adjuvants, using conserved antigenic domains of the HA gene in DNA vaccines and passive mAb immunisation are among other strategies employed. Vaccine platforms engaged include peptide and recombinant protein vaccines, virus-like particle (VLP) vaccines, DNA vaccines and viral vectored vaccines (153, 154).

1.5 Ebola Virus

Ebola virus is a member of the Filoviridae family, an enveloped, negative stranded RNA virus with a genome containing just seven genes (155). Ebola virus infection causes Ebola virus disease (EVD), a severe acute haemorrhagic fever with rapid onset of symptoms such as muscle pain and headache, leading to vomiting, damage to liver and kidney function, bleeding and death in 25% to 90% of cases depending on the outbreak (156). Since the first recoded outbreak in 1976, five species have been isolated, *Zaire ebolavirus* (ZEBOV), *Sudan ebolavirus* (SEBOV), *Tai Forest ebolavirus*, *Bundibugyo ebolavirus* (BEBOV) and *Reston ebolavirus* (155). During the West African outbreak of 2014 to 2016 (ZEBOV), over 28,000 cases were reported with more than 11,000 deaths (157). Since 2017, there has been a series of smaller outbreaks of the ZEBOV species in the Democratic Republic of the Congo that continue to be of considerable global health concern (155).

During Ebola virus infection of macrophages and DCs, dsRNA triggers intracellular RIG-I and melanoma differentiation–associated protein 5 (MDA5) receptor signalling, which leads to an initial type I IFN response. Despite high levels of IFN-α detected in the serum of infected patients and non-human primates (NHPs), viral loads continue to rise, failing to resolve infection (158). Innate immune dysregulation, including impaired DC maturation, inhibition of type I IFN signalling and excessive secretion of inflammatory cytokines such as TNF-α, IL-6 and IL-10 are hallmarks of EVD (158-160). This leads to a failure to activate effector cells, failure to control viral replication, and enables systemic dissemination and inflammation (158, 161). The most immunogenic Ebola virus

protein, the surface glycoprotein (GP) (that is shed or cleaved from the surface of infected cells) can activate non-infected monocytes and macrophages to produce inflammatory cytokines such as TNF-α, IL-1β, IL-10, IL-6, IFN-β and IL-12 *in vitro*, this perhaps exacerbates inflammation further (108, 162, 163).

Several vaccines are in development for the prevention of EVD. The most advanced of these utilise viral vectors for the expression of Ebola GP. Vectored vaccines expressing ZEBOV GP use the recombinant vesicular stomatitis virus (VSV) (rVSV-ZEBOV) and Chimp Adenovirus type 3 (ChAd3-ZEBOV) vectors. ChAd3-ZEBOV and a third viral vector expressing ZEBOV GP, adenovirus type 26 (Ad26-ZEBOV), are being tested as a prime-boost regimen combined with a multivalent Modified Vaccinia Ankara (MVA) vectored vaccine expressing ZEBOV, SEBOV, Marburg Virus GP (related Filovirus) and Tai Forest Ebola virus NP (MVA-BN-Filo). The Ad26-ZEBOV, MVA-BN-Filo vaccine, a 2-dose heterologous vaccine regimen (currently being tested by the EBOVAC consortium, Innovative Medicines Initiative) was used for the studies in chapters 4 and 5 of this thesis. Ad26-ZEBOV (Janssen Vaccines and Prevention B.V., The Netherlands) is a recombinant, replication-defective, Ad26-vectored vaccine produced in PER.C6 human cells (5×10^{10} virus particles/dose). MVA-BN-Filo (Bavarian Nordic, Denmark) is a recombinant, replication-defective, MVA-vectored vaccine manufactured in chicken embryo fibroblasts (1×10^8 $TCID_{50}$/dose), both are delivered intramuscularly. These vaccines have been shown to be safe and immunogenic in humans in phase 1, 2 and 3 trials, however, challenges remain in optimising regimen interval, determining correlates of protection and the durability of efficacy (164-166). **Table 2** details the most advanced Ebola vaccines currently in human trials.

Vaccine	Company	Design	Phase	Study Population	Reference
rVSV-ZEBOV	Merck	Zaire Ebola virus GP	Phase 1	USA	(167)
			Phase 1	Germany, Switzerland, Gabon, Kenya	(168, 169)
			Phase 1/2	Switzerland	(170)
			Phase 3	Guinea, Sierra Leone	(171)
ChAd3-ZEBOV	GSK	Zaire (and Sudan) Ebola virus GP	Phase 1	USA	(172)
			Phase 1/2	Switzerland	(173)
ChAd3-ZEBOV/MVA-BN-Filo	GSK, BN	Zaire (and Sudan) Ebola virus GP (ChAd3), Zaire/Sudan Ebola virus GP/Marburg GP/ Tai Forest Ebola virus NP (MVA)	Phase 1	Mali	(174)
			Phase 1	U.K.	(175)
			Phase 1	U.K., Senegal	(176)
Ad26-ZEBOV/MVA-BN-Filo *	J&J (Janssen), BN	Zaire Ebola virus GP (Ad26), Zaire/Sudan Ebola virus GP/Marburg GP/ Tai Forest Ebola virus NP (MVA)	Phase 1	U.K.	(166)
			Phase 1	Tanzania, Uganda	(177)
			Phase 1	Kenya	(178)

Table 2: The most advanced Ebola vaccines currently in human trials.

Information gathered and updated from (164, 165), * EBOVAC consortium vaccine used in this thesis. GSK; GlaxoSmithKline, BN; Bavarian Nordic, J&J; Johnson and Johnson.

1.6 NK Cells and Vaccination

Vaccination is a cost-effective way of reducing the burden of, eliminating, and - in exceptional cases - eradicating infectious diseases. Whilst a number of effective vaccines are currently licenced, many highly prevalent and complex diseases remain without effective prophylactic vaccines. Protective titres of neutralising antibodies and expanded populations of effector and memory B and T lymphocytes are viewed as measures of protection for many vaccines. Currently, the generation of durable antigen-specific memory forms the basis of vaccine development and evaluation of vaccine efficacy (166, 179). Developing vaccines to overcome pathogen polymorphism and complexity demands new approaches to vaccine design and evaluation; this in turn requires the identification of novel correlates of protection and determination of optimal dosing schedules. The activation of NK cells represents a potential route for further optimisation of vaccination strategies by harnessing their role as anti-pathogen effector cells which integrate innate and acquired immune responses.

An increasing number of studies in humans demonstrate activation of NK cells during recall responses to pathogens in vaccinated subjects. *In vitro* NK cell responses to components of the DTP vaccine (diphtheria toxoid, tetanus toxoid and whole cell inactivated pertussis), Bacille Calmette-Guérin (BCG) and influenza vaccine are enhanced after vaccination (18, 180-182) and heightened NK cell IFN-γ and degranulation responses have been detected after vaccination against rabies, malaria, influenza and BCG (133, 183-185). These post-vaccination responses are dependent on vaccine-specific CD4^{+} memory T cells and, in particular, their rapid secretion of IL-2.

Although the 'antigen-specificity' of these post-vaccination NK cell responses resides in the CD4^{+} T cell pool, the NK cells are also modified as a result of vaccination. Innate cytokines, which can be induced by live or killed whole pathogen vaccines or by adjuvants, are potent NK cell activators and can induce their differentiation into CIML NK cells. First described as being generated by cytokine co-culture *in vitro*, CIML NK cells have an enhanced ability to secrete IFN-γ and become cytotoxic in response to cytokine and MHC-devoid K562 cell restimulation for up to 21 days after the initial stimulation (132, 133, 135, 186). *In vitro* cytokine activation with IL-18 and IL-12 and/or IL-15 induces prolonged expression of CD25, thereby generating CIML NK cells with enhanced responsiveness (demonstrated by IFN-γ production and cytotoxicity) to picomolar concentrations of IL-2 (67). More importantly perhaps, CIML NK cells can be induced by vaccination in response to CD4^{+} T cell-derived IL-2 and myeloid cell-derived IL-12 and type I IFN, and have been implicated in the enhancement of NK cell function *ex vivo* (133, 183-185, 187-189). Vaccination-induced CIML NK cells can be detected for up to 12 weeks post-vaccination in European subjects (133) and up to 6 months in west African vaccines (189) but are not well understood. **Table 3** summarises the evidence for vaccination induced CIML NK cells.

Pathogen	Species	Vaccine	Increased Responsive-ness *In vitro*	Duration of Response	Reference
Influenza	Human	TIV	IL-12, IL-15, IL-18	3 months (UK) or 6 months (Gambia)	(133, 189)
Yellow fever virus	Human	YF-17D	IL-12	15 days	(187)
SIV	Macaque	Ad26 HIV-1, DNA-Ad26	IL-12, IL-15	38 weeks	(190)
TB	Human	BCG	IL-12, IL-18	ND	(184)

Table 3: Evidence of induction of human cytokine-induced memory-like (CIML) NK cells by vaccination.

SIV; simian immunodeficiency virus, TB; Tuberculosis, TIV; trivalent influenza vaccine, YF-17D; yellow fever 17D vaccine, BCG; Bacille Calmette-Guérin, ND, not determined.

Vaccination-induced CIML NK cells are restricted to the less-differentiated subsets of NK cells and their induction is accompanied by proliferative expansion of the least mature CD56^{bright} NK cells and CD56^{dim}CD57$^-$ subsets (133, 189). Enrichment of less differentiated NK cells in lymph nodes and effector tissues could influence the impact of CIML NK cells induced by vaccination. The highest proportion of human immature CD56^{bright} (CD16$^-$) NK cells are found in the lymph nodes (191, 192) and produce IFN-γ in response to CD4$^+$ T cell derived IL-2, thereby potentially influencing subsequent adaptive responses (192). A higher percentage of adoptively transferred pre-activated CIML NK cells were found in the lymph nodes of mice compared to control NK cells (132) and localised in the

bone marrow, spleen and blood of mice and in the bone marrow of patients with acute myeloid leukaemia (186).

The tissue localisation of NK cells amenable to cytokine mediated pre-activation may also be crucial to functional outcomes. Human liver is enriched for resident CD56^{bright} NK cells with high NCR and NKG2D expression, with strong target cell mediated degranulation but poor IFN-γ production (193). Tissue resident ILCs may also be sensitive to pre-activation by vaccine-induced cytokines. Murine liver ILC1, for example, are highly sensitive to IL-12 stimulation and produce more IFN-γ at the sites of MCMV infection than peripheral sites (194). When taken together with the emerging literature on the impact of persistent viral infections such as HCMV on NK cell function (180), it is possible that differences between or within human populations in proportions of CIML NK cells and 'adaptive' NK cells (due to differences in recent infection and vaccination history) may contribute to differences in vaccine immunogenicity and effectiveness (189, 195, 196).

Despite the likely dominance of 'HCMV-adaptive' cells (described in section 1.3) in populations with endemic HCMV infection, the generation of CIML from less differentiated NK cells persists after vaccination (189) (reviewed in reference (197)). It appears, therefore, that there is a balance of CIML and highly differentiated NK cell effectors which may be altered by vaccination. Less differentiated NK cells are shorter lived, possess higher levels of cytokine receptors and higher intrinsic proliferative capacity; vaccination may simply contribute to the homeostatic maintenance of these cells. The benefits of preferentially expanding and generating CIML NK cells from these subsets is

unknown but could be more functionally significant in young infants where highly differentiated cytotoxic effectors are lacking (18). On the other hand, loss of IL-12 responsiveness and independence of this cytokine for IFN-γ production is a well-known feature of more differentiated NK cell effectors; more focused antibody driven responses may be advantageous in restricting the potential for inflammation associated damage in older individuals.

In the remainder of the introduction, I will explore in more detail the potential role of NK cells, activated by myeloid cell-derived cytokines or by components of adaptive immunity (CD4$^+$ T cell IL-2 or pathogen-specific antibodies), as effectors of vaccination against a number of globally important infectious diseases.

1.6.1 Influenza

In vitro restimulation of PBMC from TIV-vaccinated volunteers with inactivated influenza virus induces higher frequencies of IFN-γ producing and degranulating NK cells compared to restimulation of pre-vaccination PBMC from the same people (133, 182, 198, 199). The heightened NK cell response becomes evident as early as 2 weeks post-vaccination but is normally lost by 12 weeks. Post-vaccination enhancement of NK cell IFN-γ production was dependent on IL-2 produced from CD4$^+$ T cells, whilst degranulation responses were dependent on IL-2 and on the presence of anti-influenza antibody (133, 182). A co-stimulatory role for innate myeloid cell-derived cytokines was also demonstrated by partial inhibition of TIV restimulation responses with IL-12, IL-18 and IFN-αβR2 blockade (133).

Consistent with the generation of CIML NK cells, antigen-independent *in vitro* responses to exogenous IL-12, IL-15 and IL-18 were also elevated for up to 3 months after influenza vaccination in a U.K. study (133), and for up to 6 months in African subjects (189). Enhancement of NK cell responses after influenza vaccination is therefore mediated by indirect mechanisms involving antigen-specific cellular CD4^{+} T cell and humoral responses combined with a shorter-lived CIML component. Such enhanced NK cell function after seasonal influenza vaccination may contribute to protective immunity to influenza, but, given the dependence on antigen-specific T cells and antibodies, does not in itself overcome the issue of variation in the predominant circulating strains of influenza viruses and the need for annual re-vaccination.

Development of a 'universal', cross-protective influenza vaccine is a major priority for many groups working in the influenza vaccine field, NK cell function may yet play a role. Conserved HA stalk-specific antibodies that mediate NK cell ADCC are present after natural infection and after vaccination with TIV or monovalent adjuvanted H1N1 vaccines (200). NP-specific ADCC-mediating antibodies induced by seasonal influenza vaccination demonstrate cross-reactivity with H7N9 avian influenza NP (149). As mature CD56^{dim}CD57^{+} NK cells and HCMV-induced 'adaptive' NK cells are both potent mediators of ADCC and preferentially respond to influenza antigens after vaccination (60), NK cells may be of particular importance as effectors of the next generation of universal influenza vaccines.

1.6.2 Ebola

Little is known about the role of NK cells in Ebola virus infection or vaccination*, in vitro* studies show IFN-inhibiting domains (IIDs) within Ebola viral proteins VP24 and VP35 interrupt DC maturation and type I IFN signalling impairing NK cell activation and cytotoxicity (159). Disrupting either of these IIDs restores DC maturation and NK cell activation as measured by NKp46 and CD38 expression (159). Another study showed that Ebola VLPs lacking IIDs activated NK cells and led to lysis of Filovirus infected autologous human DCs in culture and pro-inflammatory cytokine release (201). Mice can be protected against Ebola virus infection by adoptive transfer of NK cells from VP40-containing VLP treated mice (161). Increased survival of mice after post-exposure vaccination with the candidate vaccine rVSV∆G-EBOV is reversed by NK cell depletion (202); post-exposure vaccination stimulated a burst of IFN-γ release and type I IFN secretion from accessory cells, potentially kick-starting the antiviral response and overcoming the blockade caused by IIDs (202).

In humans, increased levels of NK cell activation markers were measured at day 2 and 3 after vaccination with rVSV-ZEBOV (203). Post-exposure antibody therapy has also been shown to give effective protection in animal models via ADCC activity and anti-Ebola GP mAbs induced human NK cell activation *in vitro* (204-206). These studies implicate NK cells as important effectors in protection against Ebola virus infection and in vaccine-induced immunity and raise the potential for indirect cytokine activation of NK cells to restrict virus dissemination after therapeutic vaccination.

1.6.3 Other Pathogens

1.6.3.1 Yellow Fever

The live attenuated yellow fever virus (YFV) vaccine 17D is one of the most effective vaccines developed to date; 99% of recipients are protected for more than 10 years after a single vaccination (207). For this reason, YF-17D has been used as a tool to identify highly effective early (innate) immune responses to acute viral infection in humans (187, 208). YFV infects and induces TLR-mediated signalling in hepatocytes and cells of the innate immune system such as monocytes and DCs. In mouse models of YFV infection or YF-17D vaccination, NK cells accumulate in the spleen and are major producers of IFN-γ (209, 210). Induction of innate cytokines such as IL-1α and chemokine IP-10, and up-regulation of the early activation and proliferation markers CD69 and Ki67 on NK cells is detected as early as 3 days post-vaccination in humans (187, 208, 211). NK cell activation peaks at the same time as viral load, 6 days post-vaccination, and correlates directly with a rise in plasma type I and type III IFN. Thereafter, viral load and NK cell responses decline rapidly, returning to baseline by day 10 and 15 post-vaccination respectively (187, 211).

In a study in Uganda, pre-existing IFN-γ producing NK cells in an activated immune microenvironment were associated with lower viral loads and subsequently reduced antibody titres after YF-17D vaccination (195). NK cell IFN-γ responses to YFV correlated with increased *in vitro* responsiveness of less differentiated NK cells to innate cytokines such as IL-12 after vaccination (187) suggesting that, as for influenza vaccines, YF-17D-induced accessory cell-derived cytokines may also induce development of CIML NK cells. As in influenza

vaccination, this pre-activation state is short-lived suggesting that there is no lasting imprint on the NK cell repertoire. These transient innate responses (including NK cells) may, however, synergise with antigen-specific vaccine-induced responses resulting in the formation of particularly durable and effective T and B cell-mediated immunity to YFV (187, 211). A more robust mechanistic understanding of the induction and function of CIML NK cells during infection or vaccination with YFV and other Flaviviruses will help to define their role.

1.6.3.2 HIV

HIV remains highly prevalent across the world with 1.8 million new infections estimated in 2017; lifelong treatment is required to prevent disease and death, which places a considerable burden on health systems worldwide (212). A prophylactic HIV vaccine is of utmost priority. HLA class I and KIR genotype and NK cell education influence direct killing of HIV-1 infected CD4$^+$ T cells and are associated with the rate of progression of HIV infection (213, 214). In the partially successful RV144 vaccine trial, IgG against variable regions 1 and 2 of the HIV-1 envelope GP were inversely correlated with the rate of infection (215). Indeed, RV144 induced isotypes IgG1 and IgG3 targeting the crown of the V2 loop and ADCC activity of anti-V2 antibodies has been demonstrated in human PBMC cultures (216-218).

NK cells from KIR3DL1/HLA-Bw4$^+$ or KIR2DL1/HLA-C2$^+$ donors show higher antibody-dependent cytotoxicity against HIV infected targets in the presence of anti-HIV GP120 antibody (219, 220). These studies could demonstrate an effect of NK cell education on HIV vaccine-induced effector NK cells, potentially

contributing to individual variability in vaccine outcomes. $CD57^+NKG2C^+$ memory-like NK cells are expanded in HIV-1/HCMV co-infected individuals and these cells make a potential contribution to control of viremia during primary HIV infection (221, 222). Similarly, expanded populations of peripheral blood $KLRC2^+$ NK cells (NKG2A/C) were shown in macaques infected with SIV and rhesus macaque (rh)CMV (223). Together with evidence that individuals with a degree of inherent resistance to HIV - so-called 'elite controllers' or slow progressors - mount stronger antibody-mediated NK cell activation and ADCC responses than more susceptible individuals, these studies suggest that NK cells may contribute to HIV protection and control (224).

NK cells have been implicated as antigen-specific effector cells after vaccination or infection of non-human primates with SIV. Target cells pulsed with SIV vaccine-antigen but not heterologous antigens can be lysed *in vitro* by splenic and hepatic NK cells from infected but not from uninfected animals (121). These antigen-specific responses could be detected for at least 5 years after SIV DNA/adenovirus prime-boost vaccination, suggesting that this memory-like response is long lived (121). By contrast, no significant potentiation of circulating NK cell function was observed after SIV infection or vaccination; rather, SIV infection impaired the cytotoxic response of peripheral blood NK cells (190). However, a trend towards increasing *in vitro* NK cell CD107a expression in response to IL-15 and IL-12 post-vaccination suggests that memory-like NK cells with enhanced cytokine responsiveness may have been induced in this study (190).

In HIV patients, therapeutic HIV vaccination or IL-2 treatment sustains or enhances NK cell activity (188, 225). Immunisation of chronically-infected patients with an adjuvanted HIV-1 GP120/NefTat subunit protein vaccine induces IL-2 from T helper cells and an increase in NK cell IFN-γ production *in vitro*. NK cell IFN-γ production was reduced by depletion of CD4$^+$ T cells and almost completely abrogated after blocking both IL-2 and IL-12, suggesting a role for accessory cells in full NK cell effector functions after vaccination (188). These, and other, studies highlight the potential of therapeutic vaccination to restore NK cell function during chronic HIV infection (188, 226).

1.6.3.3 Malaria

The role of NK cells in natural immunity or vaccine-induced protection against malaria infection has not been fully established (227). NK cell activation has been described to varying degrees in different experimental murine models (228, 229) and NK cells have been shown to contribute directly to the elimination of *Plasmodium (P.) falciparum* infected red blood cells (RBC) and protect against experimental cerebral malaria in mice (93, 230). *In vitro* studies of human PBMC show NK cells are readily activated by *P. falciparum* infected RBC; the resulting NK cell proliferation, IFN-γ production, CD25 and CD69 expression were further demonstrated to be dependent on IL-2, accessory cell IL-12 and IL-18 production and on cell-cell contact (83, 231-234). Long lasting NK cell activation has been reported in controlled human malaria infection (CHMI) studies, and a decrease in peripheral blood NK cell frequency early after infection suggests migration of NK cells into the tissues, possibly the liver (235-237).

RTS,S/AS01 is the most promising vaccine tested to date for human *P. falciparum* malaria. RTS,S consists of recombinant circumsporozoite surface protein (CSP) of *P. falciparum* fused to the hepatitis B virus surface antigen (HBs) and adjuvant delivery system (AS)01 formed into VLPs. PBMC collected from a RTS,S randomised controlled trial revealed post-vaccination IL-2 secretion with IFN-γ and CD69 upregulation on NK cells in response to *in vitro* restimulation with HBs or CSP. All responses were significantly higher in RTS,S vaccinees compared to control rabies vaccinated subjects (185). A weak association has been reported between IL-2 secreting CD4$^+$ T cells and time to parasitaemia, accompanied by an increase in the proportion of CD56^{bright} NK cells. Higher IFN-γ and perforin expression and protection against malaria challenge in vaccine recipients has also been reported (238). Interestingly, peripheral blood NK cell gene expression signatures were negatively correlated with RTS,S induced malaria protection, consistent with migration of activated blood NK cells to the tissues (239). *In vitro*, human NK cells also mediate ADCC against infected RBC in response to antibodies isolated from humans in malaria endemic regions, potentially indicating a role for NK cells in the presence of vaccine-induced antibody (240).

1.6.3.4 Tuberculosis

The live attenuated BCG vaccine is the only vaccine currently licensed for the prevention of tuberculosis (TB) disease caused by *Mycobacterium tuberculosis* (MTB) and is administered to over 120 million infants each year (241). NK cells are an important component of the cellular immune response to BCG, producing

more than half of the total IFN-γ after vaccination in new-borns and two-month old infants (241).

BCG, and other live vaccines such as measles vaccine, have been shown to induce non-specific effects that are beneficial to the recipient and reduce overall mortality in a community (242, 243). Potential underlying mechanisms include T cell mediated cross-reactivity and/or 'training' or 'priming' of innate immune cells, including monocytes and NK cells. Increased expression of PRR in monocytes and higher levels of IFN-γ, TNF-α and IL-1β secretion have been observed when PBMC from BCG-vaccinated individuals are restimulated with mycobacterial or unrelated antigens compared to pre-vaccination PBMCs (181). These effects persisted for up to 12 months after BCG vaccination and were partly attributed to epigenetic remodelling of key cytokine gene loci and has been termed 'trained immunity'. Similarly, increased NK cell CD69 expression in response to Pam3Cys (heterologous stimulation) *in vitro* (that correlated with higher concentrations of IL-12) and *in vivo* in humans after experimental malaria challenge has been reported post-BCG vaccination (244, 245). Interestingly, no changes in NK cell phenotype, maturation or IFN-γ production were reported in BCG trained NK cells (246), suggesting that they are not equivalent to CIML NK cells.

Enhancement of NK cell IFN-γ responses to BCG has been reported after BCG vaccination of patients with latent TB (184) and in 5-week-old infants who were BCG vaccinated at birth compared to unvaccinated controls (184); NK cell responses were completely abrogated by neutralisation of IL-12 and IL-18 (184). Consistent with studies of other whole organism vaccines, as described above,

these studies indicate that enhanced responsiveness to cytokines is a key feature of vaccine-mediated effects on NK cells.

1.6.4 The Role of Vaccine Adjuvants in Promoting NK Cell Responses

Killed whole organism or live attenuated vaccines are both highly immunogenic and particularly effective at potentiating NK cell responses; both of these traits likely reflect the presence of potent PAMPs for PRR-mediated accessory cell activation. PAMP-containing adjuvants are typically required to improve the immunogenicity of subunit or vectored vaccines, which lack these ligands. Several studies have documented enhancement of NK cell activation by adjuvants (188, 247, 248). IL-15-matured DCs exposed *in vitro* to the TLR-4 agonist AS04-adjuvanted human papilloma virus (HPV) VLP vaccine can potentiate NK cell activation and killing of HPV-infected cells compared to either IL-4 matured DCs or VLP alone; this effect was attributed to the superior cytokine-producing ability of the DCs (95). Similarly, vaccination in the presence of exogenous IL-15 enhances DC maturation and protection against lethal *Staphylococcal* enterotoxin B challenge in mice compared to vaccine alone (94).

AS03, a squalene-based adjuvant, promotes recruitment of APCs and antigen processing. A system-wide analysis of the response to AS03 adjuvanted inactivated H5N1 influenza vaccine revealed a direct correlation between IP-10, type I and II IFN production, and enhanced NK cell activation and proliferation (249, 250). Similarly, a bursin-like peptide shown to stimulate immune cells induced higher levels of IL-2 and IL-4 and increased NK cell frequencies and IFN-

γ secretion in mice vaccinated with inactivated influenza H9N2 compared to vaccine alone (251). Taken together, these studies indicate that PRR-mediated activation and maturation of accessory cells such as DCs by vaccine adjuvants increase the production of co-stimulatory cytokines leading to heightened NK cell activation. Whether these NK cells share features of CIML NK cells has not yet been formally tested.

1.7 Concluding Remarks

Although there is now considerable evidence of enhanced NK cell responses after vaccination, the functional importance of NK cells in vaccination-induced immunity is rather difficult to evaluate. The NK cell response to vaccination varies depending on the type of vaccine, the cytokine signature induced by the vaccine/adjuvant combination and subsequent accessory cell activation (**Figure 4**). The ability of NK cells to respond to signals from both innate and adaptive immune cells suggests that when one arm of the immune response is impaired, such as T cell responses in HIV infection or innate cell dysregulation in EVD, NK cells may play an important immune effector role, maximising the impact of the remaining arm of the immune system. Successful activation of APCs and induction of an early inflammatory response by a vaccine correlates with enhanced and sustained NK cell activation and function. Importantly, NK cell education by HLA-KIR or other receptor-ligand combinations may well calibrate functional capacity on induction by both adaptive and innate pathways thereby driving individual variability in vaccine induced responses. The addition of adjuvant systems to vaccines to increase accessory cell activation and therefore augmenting NK cell function including ADCC activity could play a role in the future design of new vaccines, post-exposure therapy, therapeutic cancer vaccines, regimen optimisation and evaluation of vaccine efficacy.
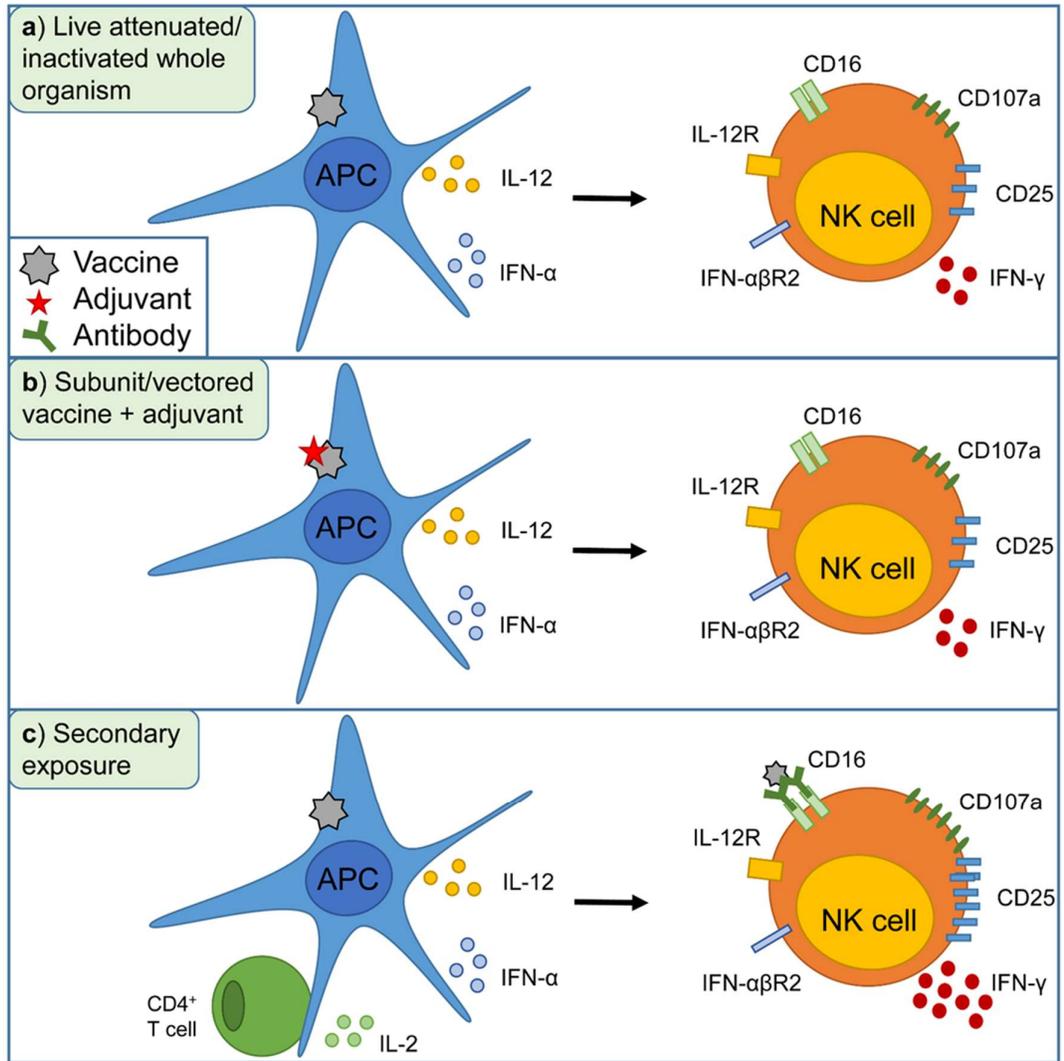
Figure 4: Accessory cell-dependent NK cell activation after vaccination.

Activation of APCs by live attenuated or inactivated whole organism vaccines induces the release of co-stimulatory cytokines which in turn leads to NK cell activation including IFN-γ release, degranulation and CD25 upregulation (a). Adjuvants promote accessory cell function for subunit or vectored vaccines in the absence of vaccine derived PAMPs (b). Upon secondary exposure, IL-2 from memory CD4+ T cells, antibody and the presence of CIML NK cells enable an enhanced response (c).

1.8 Aims and Hypothesis

The overall aim of this thesis was to investigate the effect of vaccination against viral pathogens on human NK cell phenotype and effector function upon restimulation with vaccine antigen and/or cytokines.

The central hypothesis was that vaccination primes NK cells for enhanced responsiveness to innate cytokines, leading to more robust NK cell activation upon secondary exposure to either viral innate cytokines or viral stimuli.

Objectives:

1. To determine the effect of very low doses of IL-15 on NK cell activation in response to vaccine antigens, and mechanisms involved.
2. To characterise the early effects of influenza vaccination on NK cell phenotype and activation, and cytokine-dependent enhancement of NK cell function after vaccination.
3. To analyse NK cell phenotype and function to test the hypothesis in fully naïve recipients after vaccination with a novel prime-boost Ebola vaccine regimen.
4. To examine the role of vaccine-induced antibody in the induction of NK cell responses.

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Chapter 2: IL-15 Promotes Polyfunctional NK Cell Responses to Influenza by Boosting IL-12 Production

This chapter contains the published article:

IL-15 Promotes Polyfunctional NK Cell Responses to Influenza by Boosting IL-12 Production

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Surname/Family Name	Wagstaffe		
Thesis Title	The Effect of Viral Vaccines on Natural Killer Cell Effector Function		
Primary Supervisor	Martin Goodier		

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2.1 Abstract

IL-15 is a key regulator of NK cell maintenance and proliferation and synergises with other myeloid cell-derived cytokines to enhance NK cell effector function. At low concentration, trans-presentation of IL-15 by dendritic cells can activate NK cells, whilst at higher concentrations IL-15 can act directly on NK cells, independently of accessory cells. In this study, we investigate the potential for IL-15 to boost responses to influenza virus by promoting accessory cell function. We find that co-culture of human peripheral blood mononuclear cells with inactivated influenza H3N2 virus in the presence of very low concentrations of IL-15 results in increased production of myeloid cell-derived cytokines, including IL-12, IFN-α2, GM-CSF and IL-1β, and an increased frequency of polyfunctional NK cells (defined by expression of two or more of CD107a, IFN-γ and CD25). Neutralisation experiments demonstrate that IL-15 mediated enhancement of NK cell responses is primarily dependent on IL-12 and partially dependent on IFN-αβR1 signalling. Critically, IL-15 boosted the production of IL-12 in influenza-stimulated blood myeloid dendritic cells. IL-15 co-stimulation also restored the ability of less differentiated NK cells from human cytomegalovirus seropositive individuals to respond to influenza virus. These data suggest that very low concentrations of IL-15 play an important role in boosting accessory cell function to support NK cell effector functions.

2.2 Introduction

IL-15 is essential for the survival, proliferation and functional integrity of NK cells and is being exploited to enhance NK cell-mediated immunotherapies (1, 2). IL-15 augments NK cell expression of perforin, granzyme B, natural cytotoxicity receptors NKp30 and NKp44 (3) and the activating receptor NKG2D (4). The potency of IL-15, even at very low concentrations, is partly due to its presentation at the surface of antigen presenting cells (APCs) as a complex with the α-chain of its own receptor (IL-15Rα) (5). Here, it can be presented to the same cell (cis-presentation) or to neighbouring cells (trans-presentation) such as NK cells and CD8+ T cells that express IL-15Rβ and the common γ-chain receptor (IL-15Rβγc) (6).

Dendritic cells (DCs) can be induced to present IL-15 at their surface by microbial ligands signalling through TLR and by innate cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and type I IFN (7, 8). DC-mediated NK cell activation is, in part, dependent on IL-15 that polarises to the DC-NK cell synapse during conjugate formation (9). Although IL-15 is believed to mediate the majority of its effects via trans-presentation by IL-15Rα, at high concentrations it can bind directly to IL-15Rβγc and thereby activate NK cells (10). Furthermore, at very high concentrations, free IL-15 may bind to IL-15Rα on neighbouring cells for cis- or trans-presentation (5, 11). Low concentrations of IL-15 alone induce negligible NK cell activation but IL-15 is highly synergistic with other cytokines and with recall antigens such as influenza for NK cell CD25 and IFN-γ expression (12, 13).

IL-15 trans-presentation is being explored for cancer immunotherapy; induction of constitutive expression of IL-15 and IL-15Rα by DCs or use of soluble IL-15/IL-15Rα complexes has been shown to enhance NK cell anti-tumour activity *in vitro* and in preclinical mouse studies (reviewed in (14)). Another strategy for NK cell immunotherapy includes activation of NK cells with IL-12, IL-15 and IL-18 prior to adoptive transfer where they reduce tumour growth in mice (15). Pre-activation of peripheral blood mononuclear cells (PBMC) with high concentrations of IL-15 can also restore impaired NK cell cytotoxicity of simian immunodeficiency virus (SIV)-infected macaques (16). Although these studies are consistent with direct NK cell priming by IL-15 at high concentration, or synergy with myeloid cell-derived cytokines at low concentration, the potential for IL-15 to amplify the myeloid cell response has not been thoroughly explored. However, one study has shown that TLR-induced maturation of DCs is enhanced in the presence of IL-15, leading to increased NK cell cytotoxicity towards human papilloma virus-(HPV) infected cells (17).

We hypothesised that, in addition to NK cell activation by trans-presentation and other direct effects, IL-15 could have indirect effects on the response of human NK cells to viruses by promoting NK cell activating cytokines from accessory cells. We show that very low concentrations of IL-15 (0.75ng/ml) dramatically enhance the production of IL-12, IFN-α, IL-1β and GM-CSF from myeloid accessory cells in response to influenza H3N2 and that increased production of IFN-α and, in particular IL-12, is associated with (i) heightened and sustained activation of NK cells and (ii) polyfunctionality of the responding NK cells. Furthermore, IL-15-mediated enhancement preferentially boosts IL-12

production in myeloid DCs (mDC) compared to other blood DC populations and monocytes. These studies suggest that IL-15 enhanced accessory cell function may potentiate NK cell responses, providing an additional avenue of interest for boosting NK cell effector responses in vaccination and NK cell-mediated immunotherapy.

2.3 Materials and Methods

2.3.1 Study participants

Volunteers were recruited from among staff and students at the London School of Hygiene and Tropical Medicine (LSHTM) (n=84) using an anonymised volunteer database. The study was approved by the LSHTM Research Ethics Committee (reference numbers 6237 and 6324). HCMV serostatus was determined for each donor by HCMV IgG ELISA (BioKit, Barcelona, Spain) using plasma collected from heparinised whole blood. Donors ranged in age from 20 to 77 years with a median age of 32 years. Twenty-nine (35%) of the donors were male, and 48% were HCMV seropositive.

2.3.2 PBMC isolation and *in vitro* culture assays

PBMC were isolated from heparinised whole blood using Histopaque 1077 (Sigma-Aldrich, Gillingham, U.K.) gradient centrifugation. Cells were rested for 2 hours and either used fresh (blocking experiments and IL-12 intracellular staining) or cryopreserved in liquid nitrogen (all other experiments). Before use, frozen cells were thawed and washed in RPMI 1640 supplemented with 100U/ml penicillin/streptomycin and 20mM L-glutamine (Gibco, ThermoFisher). Cells were counted using Countess II FL Automated Cell Counter (Invitrogen, ThermoFisher); average viability after thaw was 86%. 3×10^5 cells/well were cultured in RPMI 1640 supplemented as above with 5% pooled human AB serum for 6, 9 or 18 hours at 37°C in 96-well round-bottom plates with 2µg/ml inactivated whole H3N2 influenza virus (influenza A/Victoria/361/2011 (H3N2) (IVR-165)

National Institute for Biological Standards and Control, Potters Bar, U.K.), with or without recombinant human IL-15 at 0.75ng/ml (PeproTech, London, U.K.). Concentrations were determined by prior titration, H3N2 at 2μg/ml was the lowest concentration to induce significant NK cell IFN-γ upregulation without the presence of additional cytokines, 0.75ng/ml IL-15 was previously shown to be the lowest concentration to synergise with other cytokines for NK cell activation, without significant NK cell activation alone (13). Additional cultures were also stimulated with a high concentration of cytokines (HCC) consisting of IL-12 (PeproTech, London, U.K.; 5ng/ml) and IL-18 (R&D Systems, Oxford U.K.; 50ng/ml).

For blocking experiments the following antibodies were used; anti-IL-2 at 3μg/ml (Becton Dickinson (BD) Biosciences, Oxford, U.K.), rat IgG2a isotype control at 3μg/ml (eBiosciences, ThermoFisher), anti-IL-12 at 3μg/ml (BD Biosciences), anti-IFN-αβR2 at 1μg/ml (Merck Millipore, Watford, U.K.) and combined mouse IgG1 and IgG2a isotype controls at 3μg/ml final (eBiosciences). GolgiStop (Monensin; 1/1500 concentration; BD Biosciences) and GolgiPlug (Brefeldin A; 1/1000 final concentration; BD Biosciences) were added for the final 3 hours of culture. Culture supernatants were collected and stored at -80°C. For control experiments, NK cells were purified (mean purity 87%) using an NK Cell Isolation Kit (Miltenyi Biotec) and 2×10^5 NK cells were stimulated for 18 hours under the conditions described above for PBMC cultures (n=5). For IL-12 intracellular staining experiments, 2×10^6 cells/well were cultured as above for 18 hours with GolgiStop and GolgiPlug for the final 5 hours.

2.3.3 Flow cytometry and Luminex

Cells were stained in 96-well round-bottom plates for surface markers including viability marker (Fixable Viability Dye eFluor780; eBioscience) in FACS buffer (PBS containing 0.5% FCS, 0.05% sodium azide and 2mM EDTA) for 30 minutes after blocking Fc receptors for 5 minutes with Fc Receptor (FcR) Blocking Reagent (Miltenyi Biotec). Cells were then washed in FACS buffer, fixed and permeabilised using BD Cytofix/Cytoperm Kit according to the manufacturer's instructions. Cells were then stained for intracellular markers with FcR blocking for 15 minutes and washed again, finally cells were resuspended in 300µl FACS buffer and transferred to alpha tubes for acquisition on a BD LSRII flow cytometer.

Fluorophore labelled antibodies used were: anti-CD3-V500 (clone UCHT1), anti-CD56-PECy7 (clone NCAM16.2), anti-CD107a-FITC (clone H4A3), anti-HLA-DR-PE (clone TU36) (all BD Biosciences), anti-IFN-γ–APC (clone 45.B3), anti-CD86-AF488 (clone IT2.2), anti-CD11c-PerCPCy5.5 (clone 3.1), anti-CD16-PEDazzle (clone 3G8), anti-CD14-AF700 (clone 63D3) (all Biolegend, London, U.K.), anti-CD25-PerCPCy5.5 (clone BC96), anti-CD57-e450 (clone TB01), anti-CD19-PECy5 (clone HIB19), anti-CD123-e450 (clone 6H6), anti-IL-12(p40)-e660 (clone C17.8) (all eBiosciences). Cells were acquired using FACSDiva software, data were analysed using FlowJo V10 (Tree Star, Oregon, U.S.A). FACS gates were set using unstimulated cells or FMO controls, samples with less than 100 NK cell events were excluded. Concentrations of GM-CSF, IFN-α2, IFN-γ, TNF-α, IP-10, IL-1β, IL-10, IL-12p70 in cell culture supernatants were determined by Luminex technology (Merck Millipore) and Bio-Plex software (Bio-Rad, Watford, U.K.).

2.3.4 Statistics

Statistical analysis was performed using GraphPad Prism version 7.01 (GraphPad, California, U.S.A.). Functional responses were compared using Wilcoxon signed-rank test and intergroup comparisons between HCMV seropositive and HCMV seronegative individuals were performed using Mann–Whitney U test. Correlation analysis was performed using linear regression. Significance levels are assigned as *p, 0.05, **p, 0.01, ***p, 0.001, and ****p, 0.0001 for all tests. Analysis and presentation of polyfunctional NK cell data was performed using SPICE version 5.1 (downloaded from http://niaid.github.io/spice/).

2.4 Results

2.4.1 Nanogram concentrations of IL-15 boost and sustain functional NK cell responses to influenza H3N2.

To determine the effect of a low concentration of exogenous IL-15 on the frequency and kinetics of NK cell responses to influenza, IFN-γ, CD107a and CD25 were measured at 6, 9 and 18 hours after stimulation of human PBMC with inactivated H3N2 virus in the presence or absence of 0.75ng/ml recombinant human IL-15. The flow cytometry gating strategy is shown in Figure 1A-D. H3N2 alone induced high frequencies of NK cells expressing each of the three activation markers, but there was no significant response to IL-15 alone. At all time points, the percentage of expression of all markers was significantly higher when cells were cultured with H3N2 plus IL-15 compared to cells cultured with H3N2 alone (Figure 1E-G), with each marker displaying distinctly different kinetics (frequencies of cells expressing CD107a, IFN-γ and CD25 peaking at 6, 9 and 18 hours respectively). A low frequency of NK cells showed spontaneous degranulation (CD107a expression) at early time points (Figure 1E) whereas there was little or no IFN-γ production or CD25 induction in unstimulated cultures (Figure 1F, 1G). Increased responses to H3N2 in the presence of IL-15 were reflected in increased mean fluorescence intensities (MFI) for all NK cell functional markers at their optimal time points. A small, but significant, increase was observed in CD107a MFI at 6 hours (H3N2 median 747 units, interquartile range (IQR) 718–776; H3N2 + IL-15 median 768 units, IQR 735–813, p=0.0001), IFN-γ MFI increased substantially by 9 hours (H3N2 median 681 units, IQR 523–814; H3N2 + IL-15 median 1037 units, IQR 864–1179, p=0.0001), and a

significant shift in CD25 MFI was observed by 18 hours (H3N2 median 72.5 units, IQR 34.7–153.8; H3N2 + IL-15 median 371.5 units, IQR 249–508, p=0.0001). Thus, very low concentrations of IL-15 boost the NK cell response to H3N2 stimulation and lead to sustained CD107a and IFN-γ production compared with untreated cultures.

NK cells are a heterogeneous population of cells, CD56^{bright} subsets are highly responsive to cytokines whereas the more mature CD56^{dim}CD57$^+$ subset are known to be less responsive to cytokines but maintain cytotoxic function (18). We therefore analysed the response of each of these subsets to IL-15 (flow cytometry gating strategy shown in Supplementary Figure 1A). As expected, the early degranulation response was highest in the most mature (CD56^{dim}CD57$^+$) subset and IFN-γ production was highest in the less mature (CD56^{dim}CD57$^-$) subsets at the peak of each response (Supplementary Figure 1B, 1F). However, at 6 hours, IL-15 had little effect on degranulation in the CD56^{dim}CD57$^+$ NK cell population, but by 9 hours, the heightened response in the presence of IL-15 was pronounced within the CD56^{bright} and CD56^{dim} subsets (Supplementary Figure 1B, 1E). Enhancement of NK cell IFN-γ and CD25 responses to influenza by low concentrations of IL-15 was evident to a similar extent in all NK cell subsets (Supplementary Figure 1C-J).

2.4.2 IL-15 enhances NK cell polyfunctionality.

As IL-15 appeared to enhance all three NK cell functions to a similar degree, we considered the possibility that this was due to a fraction of NK cells being highly

sensitive to the effects of IL-15 and responding in a polyfunctional manner. We examined co-expression of IFN-γ with CD107a, CD25 or both at each time point (flow cytometry gating strategy shown in Figure 2A-C). No polyfunctional NK cells were detected in unstimulated cultures or in cultures containing only IL-15 (Supplementary Figure 2A-C). When cells were cultured with H3N2 alone, very few double or triple positive NK cells were detectable (Figure 2D-F). However, after 6 or 9 hours of culture with H3N2 plus IL-15, a considerable population of NK cells were double positive for IFN-γ and CD107a (Figure 2D) and by 18 hours cells co-expressing IFN-γ and CD25 (Figure 2E) together with a small but statistically significant population of triple positive NK cells (Figure 2F), were detectable. Importantly, influenza virus stimulation in the presence of as little as 0.75ng/ml of IL-15 was almost as effective at inducing polyfunctional NK cells as much higher concentrations of a combination of IL-12 and IL-18 (5ng/ml and 50ng/ml respectively) (Supplementary Figure 2A-C). Polyfunctionality of NK cells was also evident from the statistically significant correlation between expression of the different functional markers among NK cells cultured with H3N2 plus IL-15 compared to those cultured with H3N2 alone (Supplementary Figure 2D-I). Overall, therefore, IL-15 significantly increased the frequency of polyfunctional NK cells responding to influenza virus.

2.4.3 IL-15 enhances *in vitro* production of myeloid cell-derived cytokines.

Because NK cells respond to cytokines released from activated accessory cells such as DCs and monocytes, supernatants were collected from cultures at 18

hours and cytokine concentrations were measured by Luminex. H3N2 influenza virus alone induced secretion of significant concentrations of IL-12(p70), IFN-α2, IFN-γ, GM-CSF, IL-1β, TNF-α and IL-10 (Figure 3A-G). However, addition of low concentrations of IL-15 to H3N2 resulted in further significant increases in the secretion of all of these cytokines (with the exception of IL-10) (Figure 3G). IL-15-mediated enhancement of IL-12 secretion was observed in 42 of 73 donors (57.5%) with an average 2.1 fold increase in these individuals (Figure 3A). IL-15 co-stimulation increased IFN-α2 secretion in 66% of individuals compared to H3N2 alone with a median 1.2 fold increase (Figure 3B) and also enhanced IFN-γ by an average 5.6 fold (Figure 3C), consistent with IL-15 boosting of NK cell IFN-γ responses (Figure 1). In contrast, IL-15 alone only induced modest increases in IFN-γ and GM-CSF production (median increase of only 8.18pg/ml for IFN-γ and increase in GM-CSF in only 3 of 20 donors) compared with unstimulated cells (Figure 3C, 3D), and there was no effect of IL-15 alone on IL-12(p70), IFN-α2, IL-1β, TNF-α or IL-10 secretion (Figure 3A, 3B, 3E-G).

In the presence of H3N2 and IL-15, the percentage of NK cells producing IFN-γ at 18 hours was significantly correlated with secreted concentrations of both IL-12 and type I IFN (Figure 3H-M). However, there was no enhanced correlation between NK cells producing IFN-γ and GM-CSF, TNF-α or IL-1β with IL-15 (GM-CSF: r=+0.385, r=+0.391 with IL-15; TNF-α: r=+0.231, r=+0.285 with IL-15; IL-1β: r=+0.197, r=+0.331 with IL-15) suggesting that IL-12 and/or IFN-α2 might be driving the enhanced NK cell IFN-γ response. In summary, IL-15 dramatically enhances the secretion of myeloid cell-derived cytokines and the secretion of two of these cytokines (IL-12 and IFN-α2) is strongly correlated with NK cell function.

2.4.4 IL-12 is critical for IL-15-mediated enhancement of NK cell responses and generation of polyfunctional NK cells.

As IL-12 and IFN-α2 were induced by co-culturing PBMC with influenza H3N2 and IL-15, and because enhanced NK cell function was correlated with the concentrations of these two monokines, we tested the hypothesis that the effects of IL-15 on NK cells were mediated via IL-12 and/or type I IFNs. Neutralising antibodies against IL-12, IFN-αβR2 and IL-2 were added to cultures stimulated with H3N2 (with and without IL-15) for 6, 9 and 18 hours. All three NK cell functional responses were reduced in the presence of blocking antibody to IL-12 by 18 hours (Figure 4A-F) and at the earlier time point of 9 hours (not shown); in particular the frequencies of CD107a or IFN-γ producing cells induced by H3N2 plus IL-15 were reduced to those observed in the absence of IL-15 (Figure 4A-E). Degranulation was also dependent partially on type I IFNs as blockade of the IFN-αβR2 receptor led to partial reduction in CD107a expression in both IL-15 treated and untreated cultures (Figure 4A, 4D). Blocking IL-2 also reduced CD107a upregulation after H3N2 stimulation, irrespective of the presence or absence of IL-15 (Figure 4A, 4D). However, although anti-IL-2 reduced the NK cell IFN-γ response to H3N2 no such reduction was seen in response to H3N2 plus IL-15, suggesting that IL-15 may be substituting for IL-2 in this assay.

In addition to reducing the overall frequencies of CD107a$^+$, IFN-γ$^+$ and CD25$^+$ NK cells, IL-12 blockade had a marked effect on the induction of polyfunctional NK cells. IL-12 neutralisation reduced the frequencies of double and triple positive NK cells to the levels observed without IL-15 (Figure 4G-J). These data suggest that both the heightened NK cell response to H3N2 and the generation of

polyfunctional NK cells in response to IL-15 is dependent on accessory cell IL-12. Furthermore, correlations between different NK cell functional markers (as seen in Supplementary Figure 2D-I) are also seen when NK cells are stimulated with high concentrations of IL-12 and IL-18 (data not shown). Stimulation of purified NK cells with H3N2 alone or with IL-15 induced no significant NK cell activation confirming an accessory cell requirement for both the virus induced response and IL-15 mediated enhancement (IFN-γ percentage at 18 hours: H3N2 median 0.29%, IQR 0.25-0.368%, H3N2 + IL-15 median 0.465%, IQR 0.308-0.84; CD107a percentage at 18 hours: H3N2 median 0.93%, IQR 0.605-1.293%, H3N2 + IL-15 median 1.68%, IQR 0.75-2.475%; see Figure for reviewers only, appendix page 317).

2.4.5 IL-15 enhances IL-12 production by mDCs.

To determine the source of IL-15-induced IL-12, PBMC were stimulated with H3N2 in the presence or absence of IL-15 for 18 hours and stained for DC/monocyte phenotypic markers and for intracellular IL-12(p40). DC populations were gated as lineage$^-$, CD14$^-$, HLA-DR$^+$ cells, and further split in to CD123$^-$CD11c$^+$ mDC, and CD123$^+$CD11c$^-$ plasmacytoid DC (pDC) populations. The majority of IL-12-producing cells were mDCs rather than pDCs, classical (CD14$^+$CD16$^-$) or non-classical (CD14$^-$CD16$^+$) monocytes (figure 5A). Up to 3.1% of mDCs expressed IL-12 in response to H3N2 and a significantly higher proportion (4.5%) were IL-12 positive with the combination of H3N2 and IL-15 (a median 1.9 fold increase) (Figure 5A). Furthermore, IL-15-mediated enhancement of IL-12 production was observed only in DCs and was associated

with increased expression (MFI) of CD86 (Figure 5B). Little to no IL-12 was
detected in unstimulated and IL-15 alone treated mDCs (Figure 5C). Of the 14
individuals tested, 8 (57.1%) showed an increase in IL-12 production from mDCs
with H3N2 plus IL-15 compared to H3N2 alone, corresponding with the proportion
of responders determined by Luminex detection of IL-12 in supernatant (Figure
5D). Among these 8 'responders', there was also an increase in the MFI of IL-12
staining of mDCs cultured with H3N2 plus IL-15 compared to H3N2 alone (Figure
5E). These data suggest that IL-15 potentiates NK cell responses to H3N2 by
enhancing maturation (CD86 expression) and IL-12 production specifically from
mDCs.

2.4.6 Enhancement in NK cell function by IL-15 is observed in both HCMV seropositive and seronegative individuals.

We, and others, have reported altered NK cell functions in HCMV seropositive
individuals (19-22). HCMV seropositive individuals respond less well to
exogenous cytokines and vaccine antigens (including influenza H3N2), and this
is only partially explained by accelerated NK cell differentiation (21, 22). Here, we
again observed lower NK cell CD107a, IFN-γ and CD25 responses to H3N2
among HCMV seropositive donors than among HCMV seronegative donors
(Figure 6). Low concentrations of IL-15 enhanced the responses of both HCMV
seropositive and seronegative subjects but could not fully restore the NK cell
response of HCMV seropositive subjects to levels seen in HCMV seronegative
subjects (Figure 6A-C). Interestingly, however, IL-15 was able to completely
restore the IFN-γ response of CD56^{bright} and CD56^{dim}CD57$^-$ NK cells, but not

CD56^{dim}CD57^{+} cells, in HCMV seropositive individuals (Figure 6E, 6H). This suggests that IL-15 preferentially affects immature NK cells, normalising their IFN-γ response to H3N2 to levels seen in HCMV seronegative individuals. Apart from a reduced IFN-γ response, which may be accounted for by reduced production from NK cells in seropositive individuals, there was no difference in the *in vitro* production of lymphoid or myeloid cell-derived cytokines in response to H3N2 between HCMV positive and negative donors, irrespective of the presence or absence of IL-15 (Supplementary Figure 3).

2.5 Discussion

Many studies of the effect of IL-15 on NK cell activation focus on IL-15 trans-presentation or direct activation of NK cells with high concentrations of IL-15 (typically between 5 and 50ng/ml) in combination with other cytokines (5, 10). Moreover, the synergy between cytokines and pathogen-derived signals for NK cell activation has typically been studied only for high concentrations of cytokines acting on isolated NK cells, precluding consideration of potential indirect effects of the pathogen, the cytokines, or both. To characterise more deeply the potential for synergy between IL-15 and pathogen-derived signals in NK cell activation, we have conducted a comprehensive analysis of NK cell CD107a, IFN-γ and CD25 expression and myeloid cell-derived cytokine secretion in response to influenza virus H3N2 in the presence or absence of an extremely low concentration of IL-15.

Low concentrations of IL-15 enhanced the innate cytokine response to influenza virus and this increased cytokine (primarily IL-12) was associated with potentiation of NK cell function. Importantly, the concentrations of IL-12 induced by this synergistic interaction between influenza virus and IL-15 are of the same order of magnitude as the lowest concentrations of IL-12 that we have previously shown to effectively synergise with IL-15 and other common γ-chain family cytokines for NK cell activation *in vitro* (13). This concentration of IL-12 and the low concentration of IL-15 used in this study are at least ≥5-fold lower than described effective concentrations for NK cell activation with single cytokines, these may be more physiologically relevant than previously used concentrations that are unlikely to be reached *in vivo*. The low *in vitro* concentration used in this

study is within the range for the maximal serum concentration achieved therapeutically in patients with metastatic malignant melanoma or renal cancer after a low-dose transfusion of 0.3mg/kg/day recombinant human IL-15 and which resulted in increases in innate cytokines (23).

In this article, we have shown that IL-15 preferentially enhances virus-induced mDC maturation (measured by upregulation of costimulatory marker CD86) as well as cytokine secretion compared to other DC subsets and monocytes, and that, together, this heightens and sustains NK cell activation. This role for mDCs is fully in line with the known pathogen recognition repertoire and cytokine production profile of mDCs (24, 25). Similarly, the essential role of DC-derived IL-12 in NK cell activation is in line with published data, including data showing that IL-12 synergises with specific antibody for NK cell-mediated ADCC of tumours (26, 27), that NK cell activation by HPV virus-like particle (VLP)-matured DCs is reversed by IL-12 blocking antibodies (28) and that exogenous IL-12 can restore NK cell function in HIV-exposed but uninfected infants (29). Interestingly, IL-15 synergizes well *in vitro* with IL-12 and IL-18 but not with other γ-chain–dependent cytokines, including IL-2 and IL-21, suggesting some redundancy between the latter pathways (13). This is also consistent with our observation that neutralization of endogenous IL-2 did not reduce NK cell IFN-γ in response to H3N2 plus IL-15.

Enhancement of NK cell function by IL-15 is well established (30). IL-15 has also been reported to enhance DC maturation (measured by upregulation of CD40, CD86 and MHC class II expression) in mice (17, 31). In line with our observations in humans, DCs cultured with HPV-VLP matured more efficiently in the presence

of IL-15 and this correlated with enhanced NK cell activation and killing of HPV infected tumour cells (17). However, the role of IL-15 enhancement of IL-12 production by peripheral DCs has not been previously appreciated or linked to enhanced NK cell responses. Interestingly, one study demonstrated IL-15 enhancement of IL-12 secretion by a PMA-activated U937 monocytic cell line was associated with increased ability to kill intracellular *Leishmania* parasites, suggestive of a potential role of such mechanisms in protection against infection (32).

The cytotoxic and cytokine-producing roles of NK cells have traditionally been ascribed to distinct NK cell subsets (CD56^{dim} and CD56^{bright}, respectively) and this dichotomy has rather obscured the potential for NK cells to perform both functions simultaneously (i.e. to be polyfunctional). Nevertheless, polyfunctional CD56^{dim}CD62L$^+$ NK cells (with dual ability to produce IFN-γ and become cytotoxic) can be induced by stimulation with high concentrations (50ng/ml) of IL-12 and IL-18 (33) and TLR stimulation induced polyfunctional NK cells (expressing 2 or more of CD107a, IFN-γ and TNF-α) in HIV-1-exposed seronegative but not seropositive individuals (34). We defined polyfunctionality as simultaneous expression of two or more of IFN-γ, CD107a and CD25 by any NK cell subset; no polyfunctional cells were induced by IL-15 alone and very few were detected in response to influenza virus alone. However, stimulation with IL-15 and virus induced significant numbers of both double and triple-positive NK cells suggesting that synergy (likely at the level of mDCs) between IL-15 and pattern recognition receptor (PRR) signalling may be necessary for the induction of polyfunctional NK cells. This may be due, in part, to the dependence of NK

cells on IL-12 to drive IFN-γ production and on IFN-α and IL-15 to drive cytotoxicity (2, 35).

Our observation that IL-15 restored the impaired responses of HCMV seropositive individuals only among the less differentiated CD56^{bright} and CD56^{dim}CD57^{-} NK cell subsets, and that (apart from IFN-γ production) there were no differences in cytokine production by PBMCs from HCMV seropositive and seronegative donors, suggests that polyfunctionality may arise from broadening the effector function of less mature NK subsets rather than any effect on mature NK subsets. This effect is entirely consistent with IL-12 mediating IL-15 enhancement of NK cell responses, IL-12 receptor expression, and therefore responsiveness, being progressively lost during NK cell differentiation and in 'adaptive' NK cell subsets (18, 36). Moreover, these data suggest that reduced NK cell responses in HCMV seropositive individuals are not due to an accessory cell defect but may result from intrinsic changes in more differentiated NK cells (18, 22). This hypothesis, that IL-15 broadens the functional response of immature NK cells, is further supported by evidence that degranulation, IFN-γ and TNF-α production from CD56^{bright} NK cells are potently enhanced by exposure to multiple myeloma or acute myeloid leukaemia target cells after *in vivo* therapy with the IL-15R agonist ALT803 (37). The use of an IL-15/IL-15Rα superagonist may further boost NK cell responses to H3N2 virus, however, this would be expected to act on NK cells directly, as IL-15 is already complexed to its receptor, therefore potentially bypassing the accessory-dependent effects.

In summary, we have revealed an unexpected impact of very low concentrations of IL-15 on the production of cytokines, in particular IL-12 from mDCs, which, in

turn, plays a vital role in boosting NK cell responses to influenza virus (summarised in Supplementary Figure 4). In addition to increasing the overall frequencies of responding NK cells, IL-15 also promotes the generation of polyfunctional NK cells. These studies suggest that use of very low dose IL-15 may be a strategy for enhancing and broadening NK cell effector function in immunotherapy and in enhancing vaccine responses.

2.6 Acknowledgements

We thank Carolynne Stanley for recruiting and obtaining consent from study subjects and for blood sample collection.

2.7 Figures
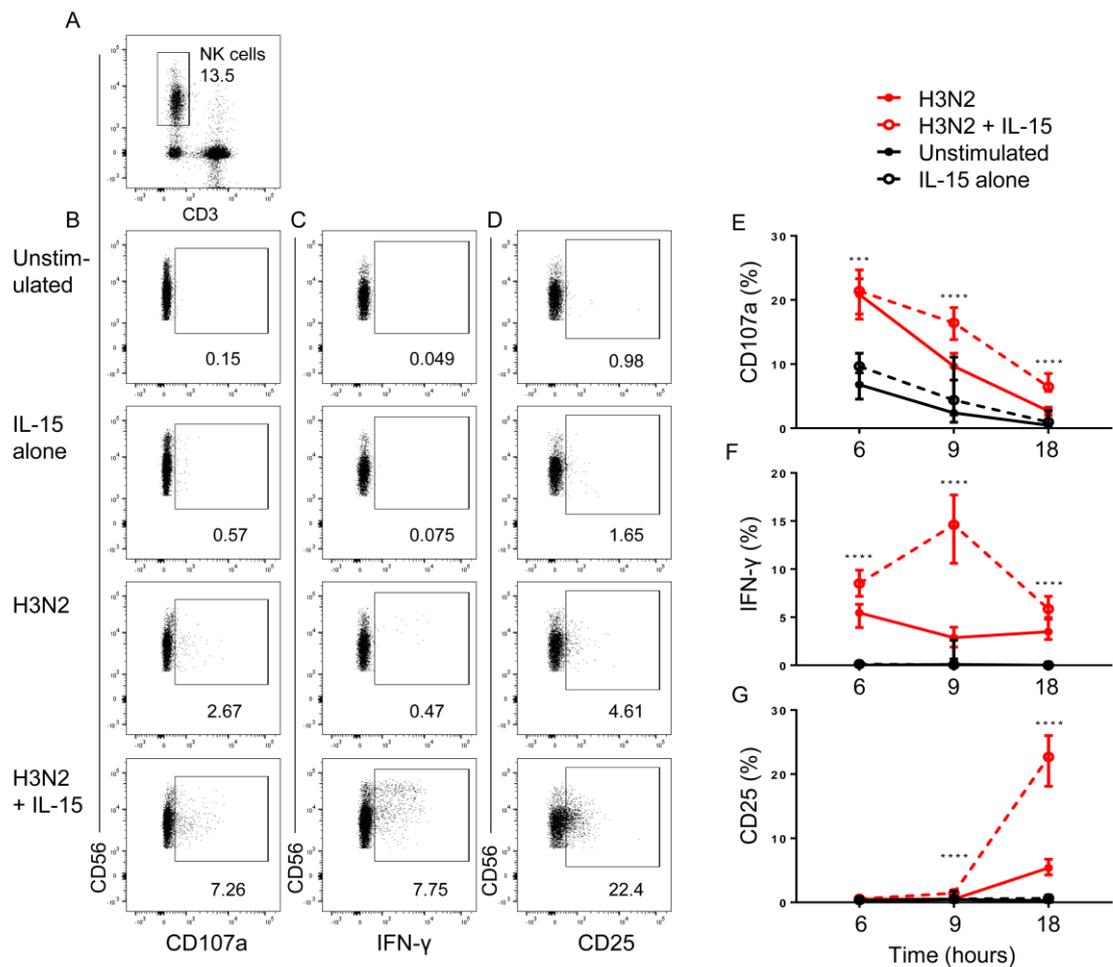
Figure 1: Nanogram concentrations of IL-15 boost and sustain functional NK cell responses to influenza H3N2.

PBMC were cultured *in vitro* for 6, 9 and 18 hours in medium alone (unstimulated; n=12) or IL-15 alone (n=12), H3N2 (n=62) or H3N2 plus IL-15 (n=62). NK cells are gated as $CD56^+CD3^-$ lymphocytes (A). Flow cytometry gating strategy for NK cell CD107a (B), IFN-γ (C), and CD25 (D) responses after 18 hours *in vitro* culture in one representative individual. Numbers shown are the percentage of total NK cells positive for each marker. Graphs show percentage of total NK cell population expressing CD107a (E), IFN-γ (F) and CD25 (G) with the median and 95% confidence interval. ***$p < 0.001$, ****$p < 0.0001$ H3N2 alone versus H3N2 plus IL-15 by the Wilcoxon signed-rank test.
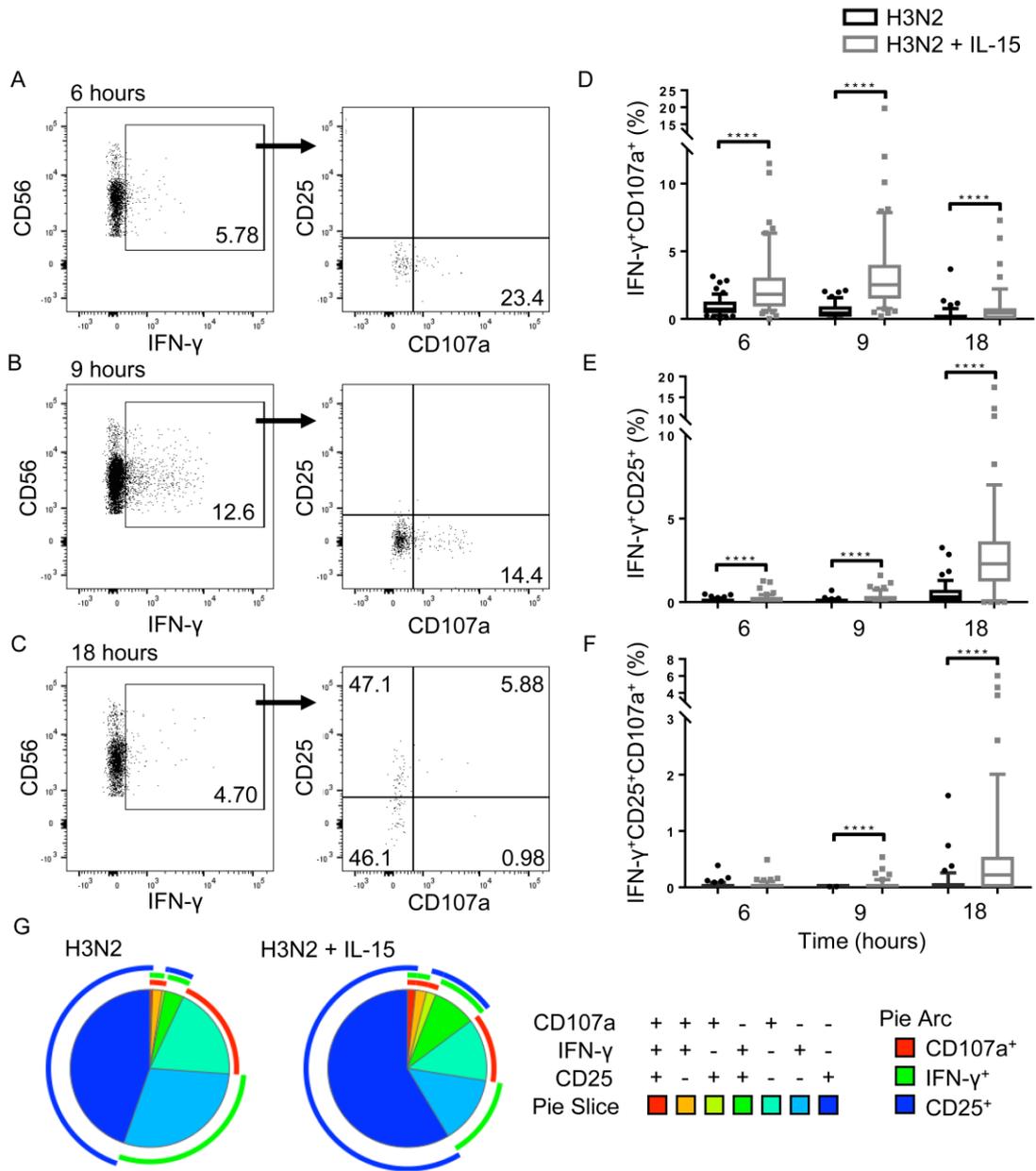
Figure 2: IL-15 enhances NK cell polyfunctionality.

CD107a and CD25 expression of IFN-γ secreting NK cells were gated at 6 (A), 9 (B) or 18 (C) hours. Plots show NK cell function in one representative donor, numbers shown are percentage of parent population after stimulation with H3N2 plus IL-15. Frequencies of IFN-γ$^+$CD107a$^+$ (D), IFN-γ$^+$CD25$^+$ (E) double positive and IFN-γ$^+$CD25$^+$CD107a$^+$ (F) triple positive NK cells after antigen stimulation with IL-15 or without IL-15 are shown for each time point (n=62). Graphs are box and whisker plots with 10-90th percentile. Median distribution of triple, double and single positive NK cells after stimulation with H3N2 alone or H3N2 with IL-15 for 18 hours are also shown in pie charts as percentage of total NK cells (pie slice) and proportion expressing each marker (pie arc) (G). ****$p < 0.0001$, Wilcoxon signed-rank test.
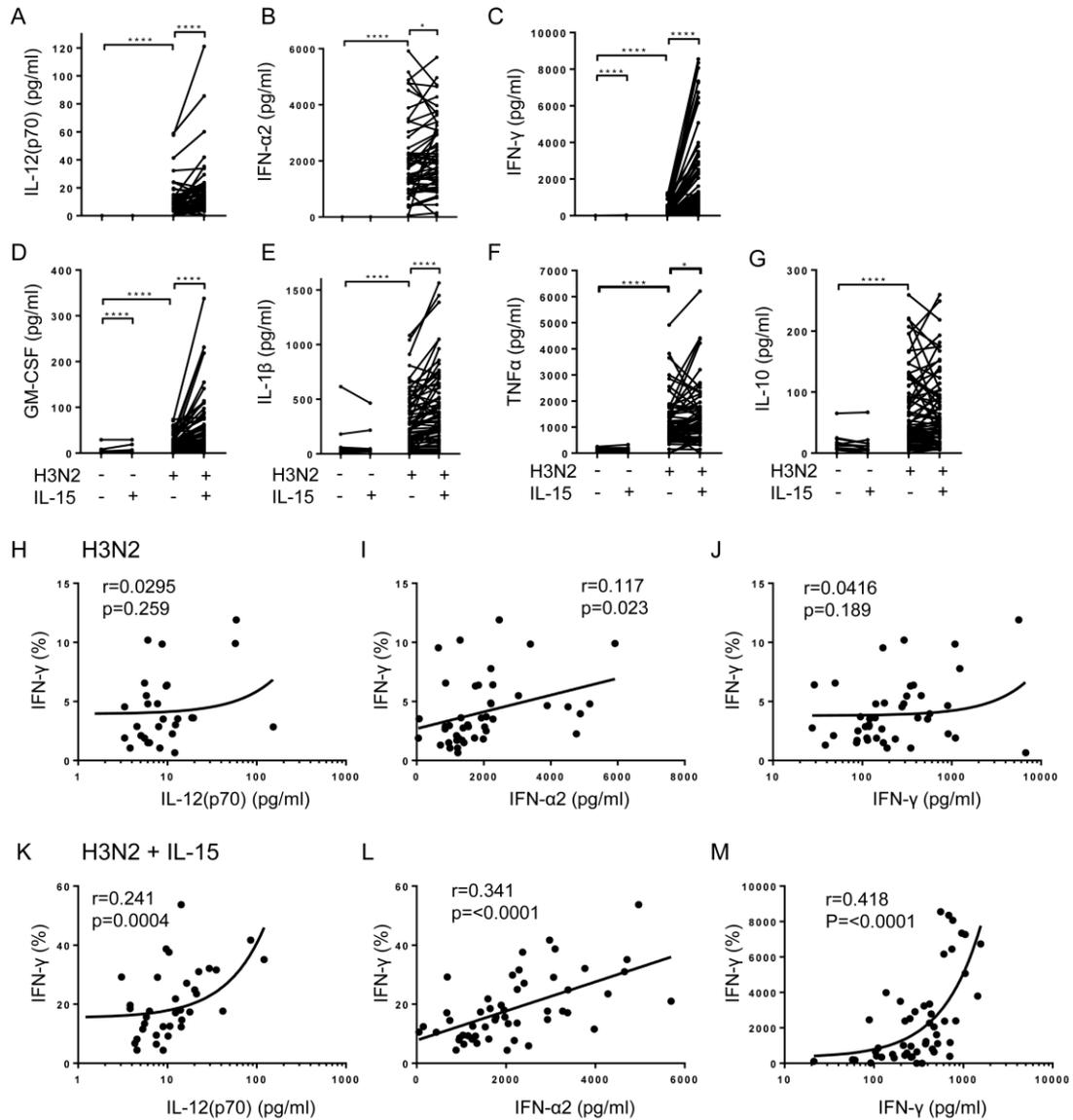
Figure 3: IL-15 enhances *in vitro* production of myeloid cell derived cytokines.

Supernatants were collected from whole PBMC cultured with medium alone (n=20) or IL-15 alone (n=20), H3N2 (n=73) or H3N2 with IL-15 (n=73) for 18 hours, and concentrations of IL-12p70 (A), IFN-α2 (B), IFN-γ (C), GM-CSF (D), IL-1β (E), TNF-α (F) and IL-10 (G) were determined by Luminex technology. The correlation between NK cell IFN-γ production determined by intracellular cytokine staining and the concentration of IL-12(p70) (H, K), IFN-α2 (I, L), IFN-γ (J, M) determined by Luminex after stimulation with H3N2 (H-J) and H3N2 with IL-15 was also determined (K-M). Graphs are before and after plots. *p < 0.05, ****p < 0.0001, Wilcoxon signed-rank test (A-G). Correlations were measured by linear regression with statistical significance determined as a p value < 0.05 (H-M).
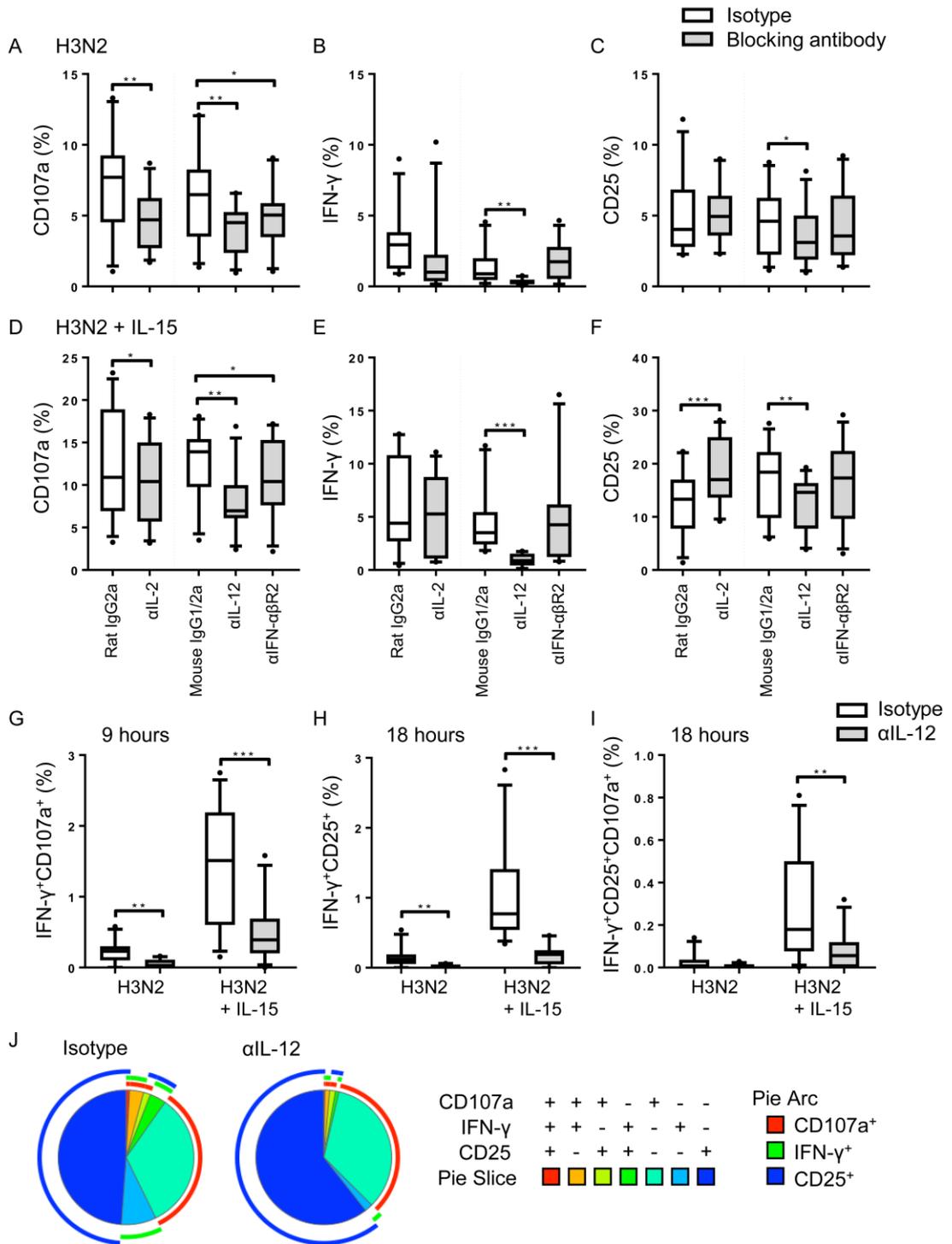
Figure 4: IL-12 is critical for IL-15-mediated enhancement of NK cell responses and generation of polyfunctional NK cells.

PBMC were cultured for 6, 9 and 18 hours with H3N2 (A-C) and H3N2 plus IL-15 (D-F) in the presence of IL-12, IFN-αβR2 and IL-2 blocking antibodies or the appropriate isotype control. Graphs show CD107a (A,D), IFN-γ (B,E) and CD25 (C,F) responses after 18 hours culture and

frequencies of IFN-γ+CD107a+ (G), IFN-γ+CD25+ (H) double positive and IFN-γ+CD25+CD107a+ (I) triple positive NK cells after 9 (peak of IFN-γ response) or 18 hours (peak of CD25 response) (n=11). Graphs are box and whisker plots with 10-90th percentile. Median distribution of triple, double and single positive NK cells after stimulation with H3N2 and IL-15 for 18 hours are also shown in pie charts as percentage of total NK cells (pie slice) and proportion expressing each marker (pie arc) (J). *p < 0.05, **p < 0.01, ***p < 0.001, Wilcoxon signed-rank test.
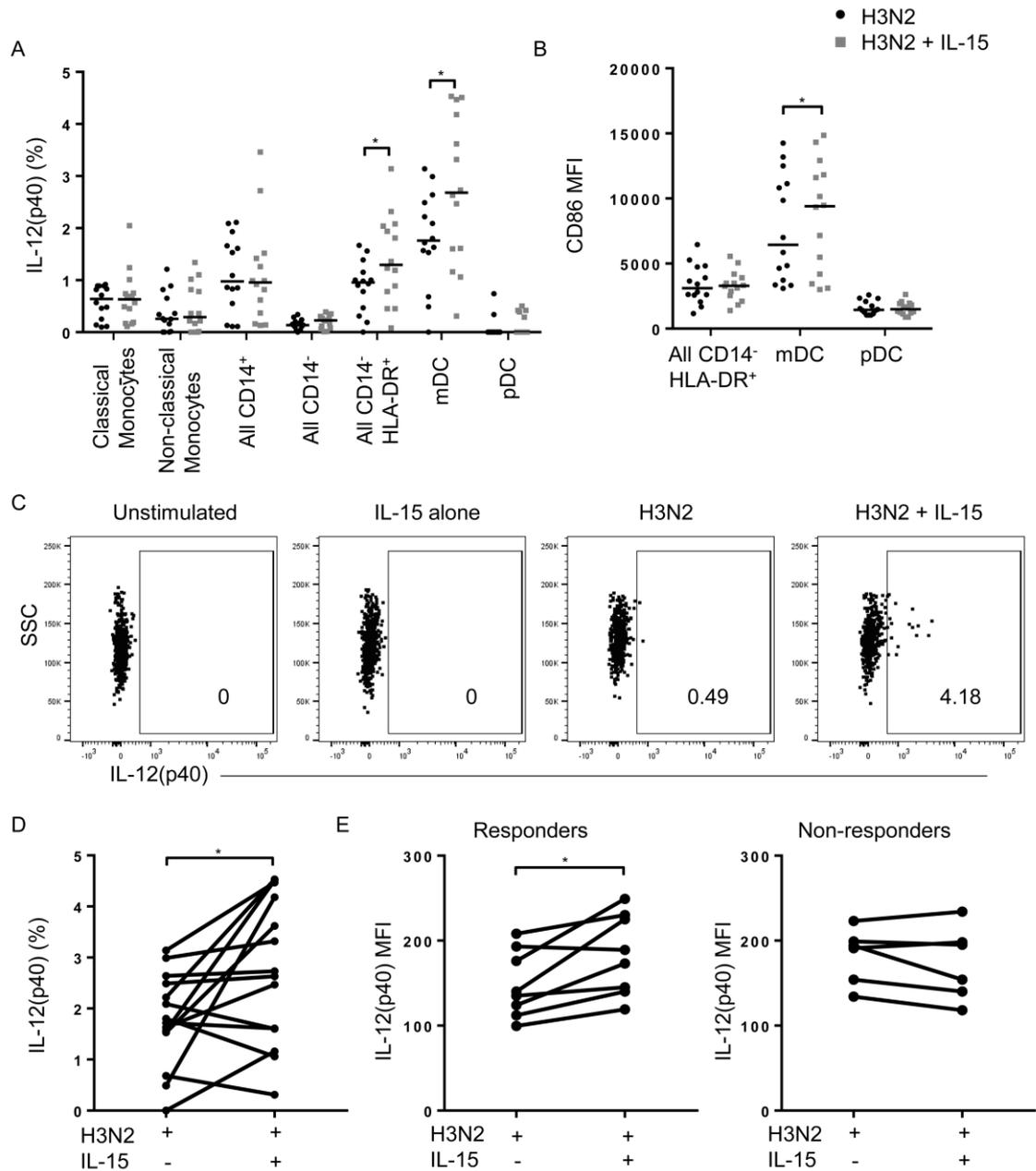
Figure 5: IL-15 enhances IL-12 production by mDCs.

PBMC were cultured with H3N2 or H3N2 with IL-15 (n=14) for 18 hours, cells were stained for DC and monocyte phenotypic markers and intracellular IL-12. Single, live cells were further gated as lineage (CD3, CD19, CD56) negative, classical (CD14$^+$CD16$^-$) and non-classical (CD14$^-$CD16$^+$) monocytes, all CD14$^+$ and CD14$^-$ cells, all CD14$^-$HLA-DR$^+$ DCs, mDCs (CD123$^-$CD11c$^+$) and pDCs (CD123$^+$CD11c$^-$). IL-12$^+$ events (A) and CD86 MFI (B) for each cell type was gated using fluorescence minus one controls and shown as one data point per donor; the horizontal line represents the median. Flow cytometry gating strategy for IL-12$^+$ mDCs is shown in one representative individual; numbers denote percentage IL-12$^+$ mDCs (C). Before and after plots

show percentage of mDCs expressing IL-12 for each individual when stimulated with H3N2 +/- IL-15 (D) and the corresponding IL-12 MFI for each responder and non-responder with IL-15 co-stimulation (E). *p < 0.05, Wilcoxon signed-rank test.
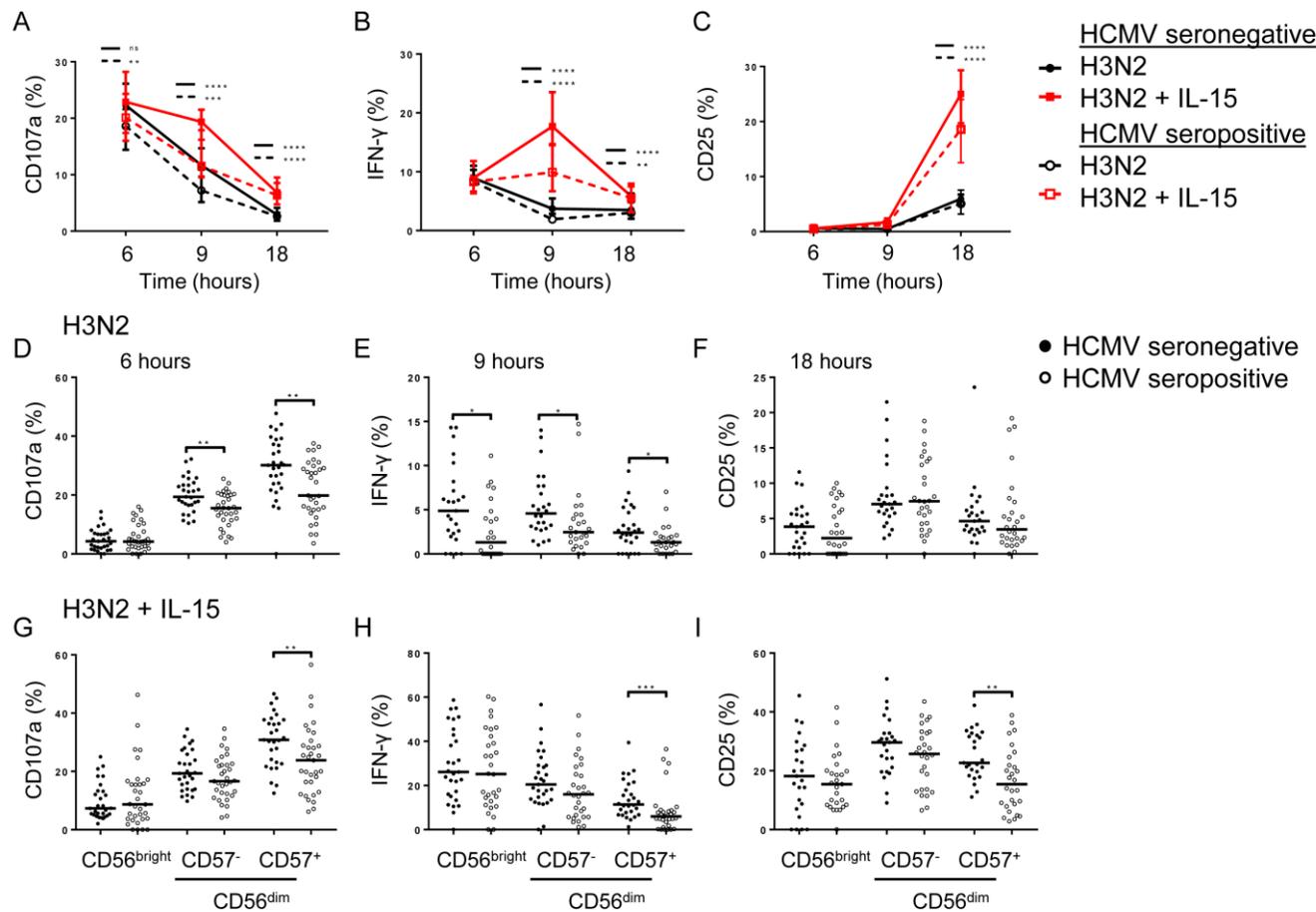
Figure 6: Enhancement in NK cell function by IL-15 is observed in both HCMV seropositive and seronegative individuals.

HCMV serostatus for each individual was determined (n=35 seropositive and n=38 seronegative). CD107a (A), IFN-γ (B) and CD25 (C) expression within each group after stimulation with H3N2 and H3N2 plus IL-15 for 6, 9 and 18 hours. NK cell responses attributed to each differentiation subset determined by the expression of CD56 and CD57, CD56^{bright}, CD56^{dim}CD57$^-$ CD56^{dim}CD57$^+$ were analysed (flow cytometry gating strategy in Supplementary Figure 1A). Graphs show median with 95% confidence interval. CD107a (D, G), IFN-γ (E, H) and CD25 (F, I) expression within each subset is shown for HCMV seropositive and seronegative individuals, only the peak time point for each response is shown. Each dot represents an individual donor; the horizontal line represents the median. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, H3N2 versus H3N2 plus IL-15, Wilcoxon signed-rank test (A-C), unpaired Mann-Whitney *U* tests (D-I). ns, not significant.

2.8 Supplementary Figures
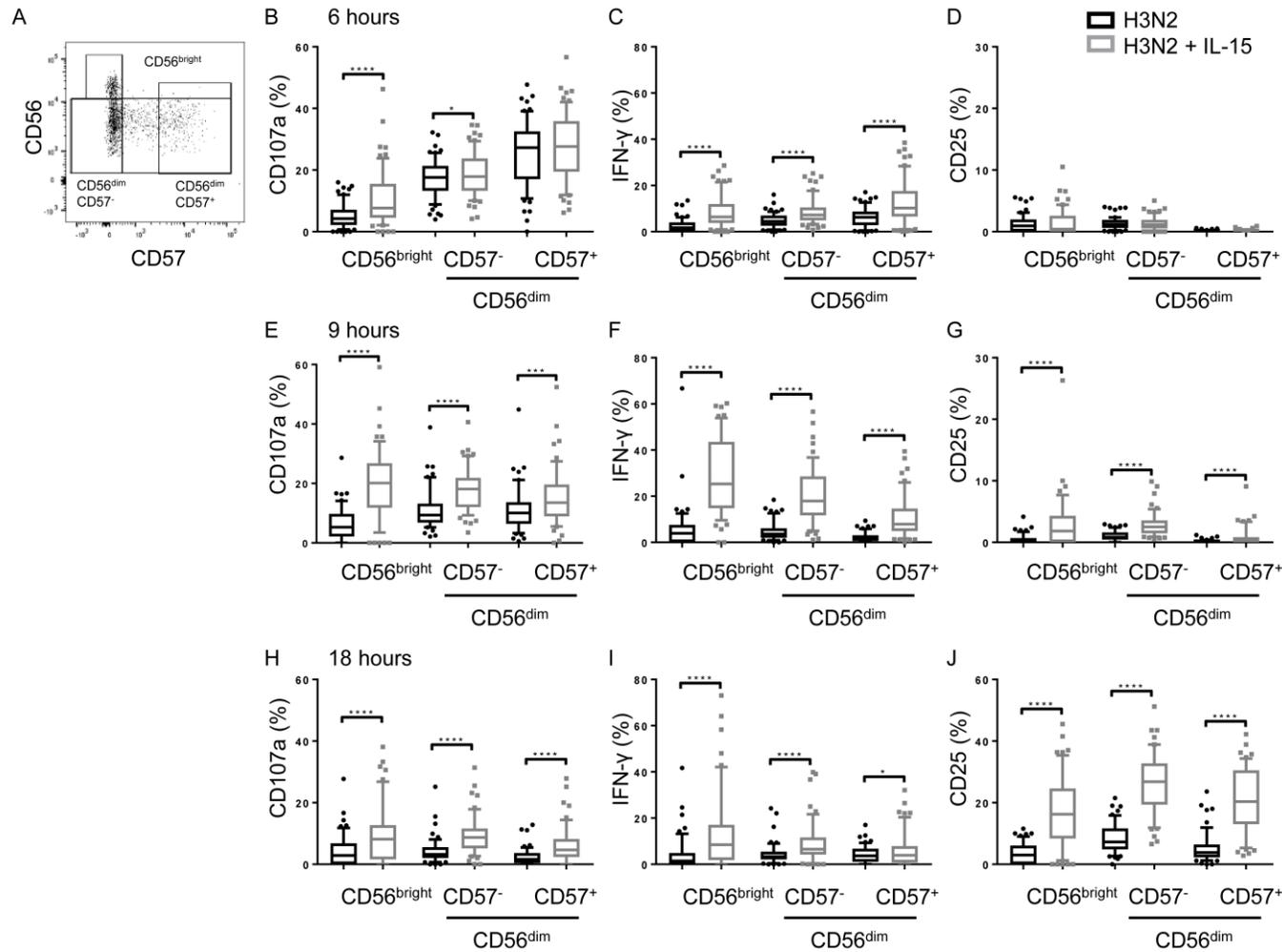
Supplementary Figure 1: IL-15 boosts NK cell functional responses to H3N2 vaccine antigen in all NK cell differentiation subsets.

Gating strategy for NK cell differentiation subsets CD56bright, CD56dimCD57- CD56dimCD57+ (A). CD107a (B, E, H), IFN-γ (C, F, I) and CD25 (D, G, J) expression within these subsets after stimulation with H3N2 and H3N2 plus IL-15 for 6 (B-D), 9 (E-G) and 18 hours (H-J), (n=62) are shown. Graphs are box and whisker plots with 10-90th percentile. *p < 0.05, ****p < 0.0001, Wilcoxon signed-rank test.

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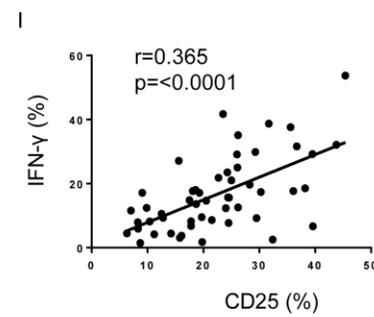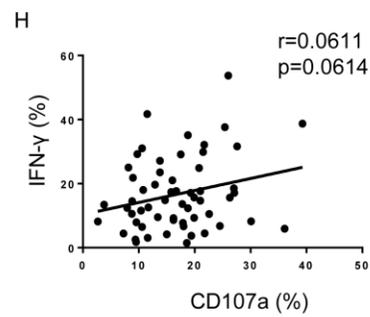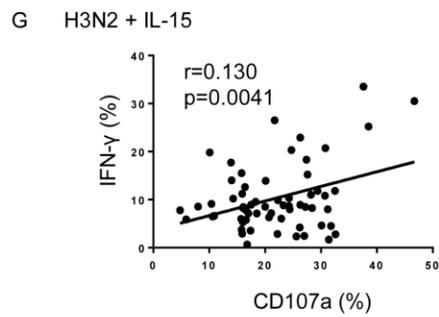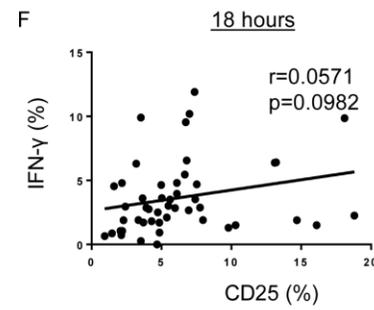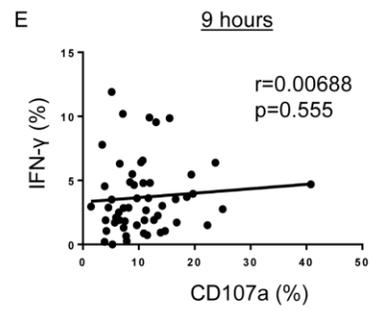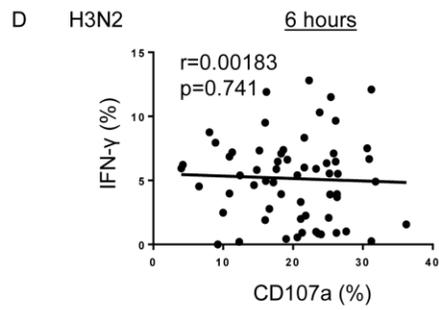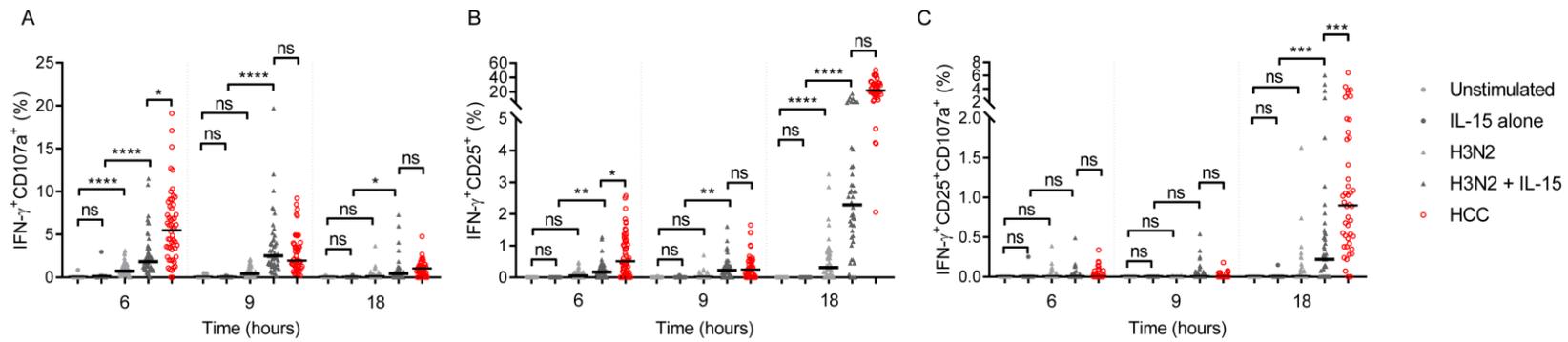
Supplementary Figure 2: No detection of polyfunctional NK cells in cultures containing IL-15 alone, the relationship between NK cell functional responses becomes more positively correlated with the presence of IL-15.

Frequencies of IFN-γ⁺CD107a⁺ (A), IFN-γ⁺CD25⁺ (B) double positive and IFN-γ⁺CD25⁺CD107a⁺ (C) triple positive NK cells after H3N2 stimulation with and without IL-15 or HCC (IL-12 5ng/ml and IL-18 50ng/ml) are shown for each time point (unstimulated and IL-15 alone; n=12, H3N2, H3N2 plus IL-15 and HCC; n=62). Each dot represents an individual donor; the horizontal line represents the median. Correlations between NK cell IFN-γ production and CD107a or CD25 expression after 6 (D, G), 9 (E, H) or 18 (F, I) hours stimulation with H3N2 (D-F) or H3N2 with IL-15 (G-I) (n=62) are shown. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA Kruskal-Wallis test with Dunns multiple comparisons. Correlations were measured by linear regression with statistical significance determined as a p value < 0.05. ns, not significant.
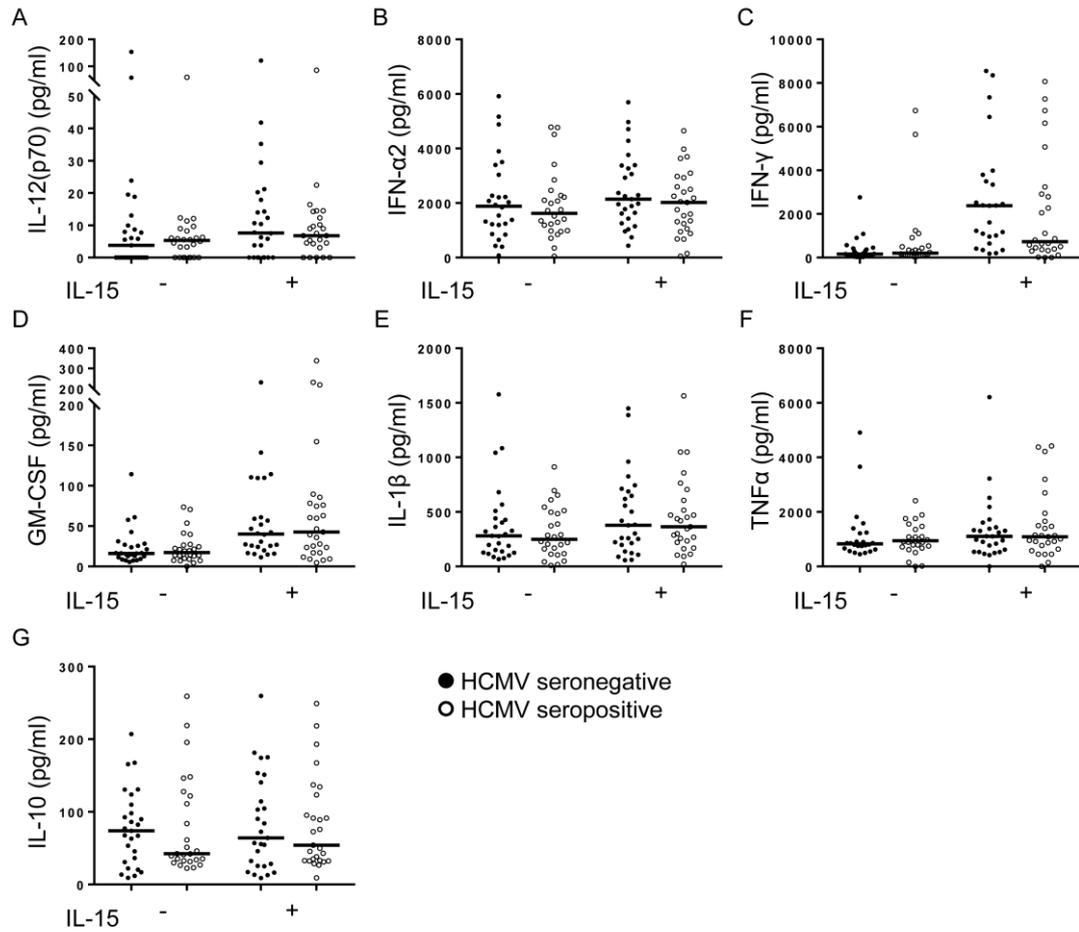
**Supplementary Figure 3: Cytokine release is not significantly different between HCMV seropositive and seronegative groups.**

Concentrations of IL-12p70 (A), IFN-α2 (B), IFN-γ (C), GM-CSF (D), IL-1β (E), TNF-α (F) and IL-10 (G) in supernatants after 18 hours stimulation were determined by Luminex technology and split in to HCMV seropositive (n=35) and seronegative (n=38) groups. Each dot represents an individual donor; the horizontal line represents the median. Not significant by unpaired Mann-Whitney U tests.
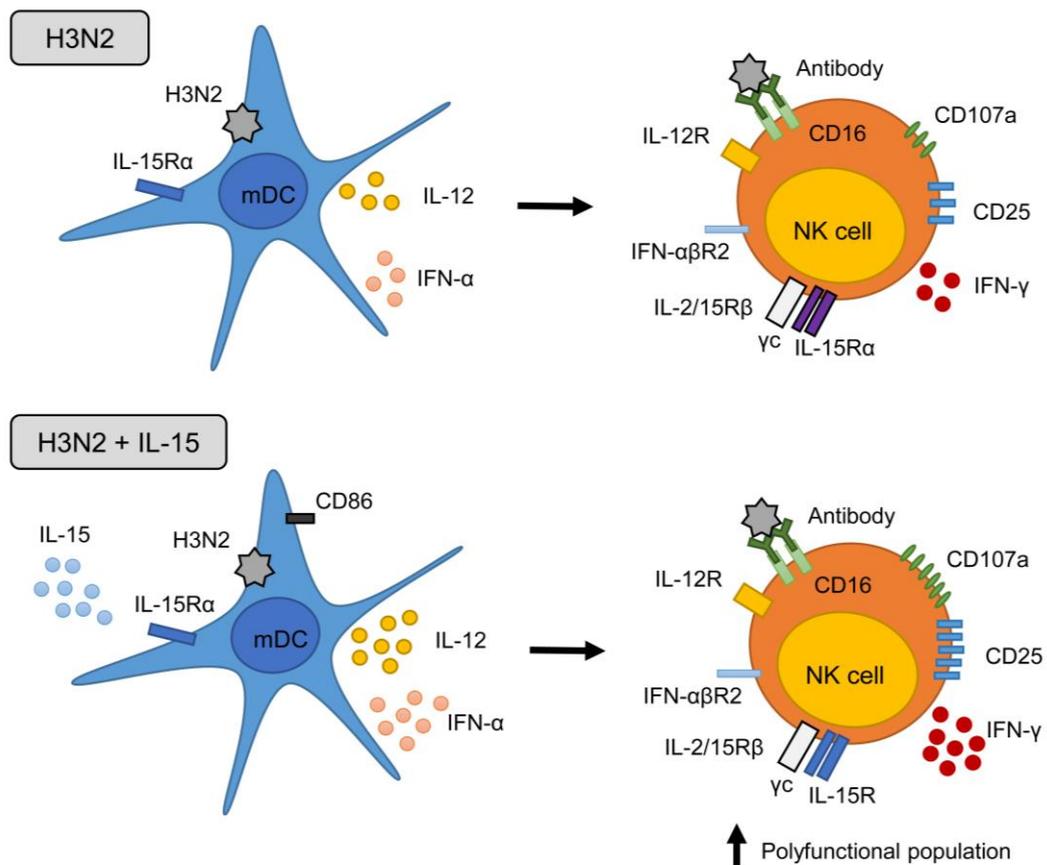
Supplementary Figure 4: A schematic representation of the effect of IL-15 on NK cell responses to H3N2.

H3N2 induced IL-12 secretion from mDCs is enhanced in the presence of very low concentrations of IL-15, leading to enhanced NK cell responses and higher frequencies of polyfunctional NK cells independently of trans-presentation or direct NK cell IL-15R binding.

2.9 References

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Chapter 3: Influenza Vaccination Primes Human Myeloid Cell Cytokine Secretion and Natural Killer Cell Function

This chapter contains the article currently under review:

Influenza Vaccination Primes Human Myeloid Cell Cytokine Secretion and Natural Killer Cell Function

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Thesis Title	The Effect of Viral Vaccines on Natural Killer Cell Effector Function		
Primary Supervisor	Martin Goodier		

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3.1 Abstract

Cytokine-induced memory like (CIML) NK cells generated in response to pro-inflammatory cytokine activation, *in vitro* and *in vivo*, can also be generated by vaccination, exhibiting heightened responses to cytokine stimulation for months after their initial induction. Our previous study demonstrated that *in vitro* NK cell responses to inactivated influenza virus were also indirectly augmented by very low doses of IL-15 which increased induction of myeloid cell-derived cytokine secretion. These findings led us to hypothesise that IL-15 stimulation could reveal a similar effect for active influenza vaccination and influence CIML NK cell effector functions. In this study, 51 healthy adults were vaccinated with seasonal influenza vaccine and PBMC were collected before and up to 30 days after vaccination. Myeloid and lymphoid cell cytokine secretion was measured after *in vitro* PBMC restimulation with low dose IL-15, alone or in combination with inactivated H3N2 virus; the associated NK cell response was assessed by flow cytometry. PBMCs collected 30 days post-vaccination showed heightened cytokine production in response to IL-15 compared to PBMCs collected at baseline, these responses were further enhanced when IL-15 was combined with H3N2. NK cell activation in response to IL-15 alone (CD25) and H3N2 plus IL-15 (CD25 and IFN-γ) was enhanced post-vaccination. We also observed proliferation of less differentiated NK cells with downregulation of cytokine receptors as early as 3 days after vaccination, suggesting cytokine stimulation *in vivo*. We conclude that vaccination-induced 'training' of accessory cells combines with the generation of CIML NK cells to enhance the overall NK cell response post-vaccination.

3.2 Introduction

NK cells are large, granular, lymphoid cells, classically categorised as cells of the innate immune system as they express activating and inhibitory receptors from germline encoded genes. The balance of activating and inhibitory signals transduced by these receptors controls the direct activation of NK cells, and this is fine-tuned by signals from innate cytokines such as IL-12, IL-15 and type I interferon (IFN) produced by myeloid accessory cells. NK cells can also augment and regulate adaptive immune responses by production of cytokines such as IFN-γ, and the adaptive response feeds back to support the NK cell response, for example via CD4$^+$ T cell IL-2 secretion (1-3). Evaluation of this bi-directional relationship has contributed to our understanding of memory-like NK cell responses (reviewed in (4)).

Cytokine pre-activated or cytokine-induced memory like (CIML) NK cells have been described in mouse and human *in vitro* cell culture models. NK cells pre-activated with combinations of IL-12, IL-15 and IL-18 displayed enhanced proliferation and IFN-γ production after cytokine restimulation; this was maintained for up to 12 weeks and through several cycles of cell division (5-7). Vaccination with whole inactivated or live attenuated viral vaccines generate CIML NK cells *in vivo*, with heightened potential for IFN-γ secretion after re-encounter of pro-inflammatory cytokines (8-10). In particular, both seasonal influenza vaccination and *in vitro* pre-activation of human PBMC with inactivated influenza virus can enhance NK cell responses to cytokine restimulation for up to 4 weeks (9). In mice, murine cytomegalovirus (MCMV) infection induces both 'antigen-specific' memory NK cells (Ly49H recognition of m157) and CIML NK

cells, suggesting that diverse subsets of memory/memory-like NK cells can be induced by a single infection (11, 12). The pro-inflammatory cytokine IL-12 was critical in MCMV induced 'antigen-specific' NK cell expansions and maintenance, the expansion of NKG2C$^+$ NK cells in human cytomegalovirus (HCMV) infection and reactivation and in the generation of CIML NK cells, suggesting this cytokine may be a common requirement for the generation of memory-like NK cells (13-15).

In human PBMC, low concentrations of exogenous IL-15 (0.75ng/ml) enhance influenza virus-induced secretion of myeloid cell derived cytokines such as GM-CSF, IFN-α, IL-12 and IL-1β and this, in turn, enhances cytokine-dependent NK cell activation (16). Moreover, NK cell IFN-γ responses to low concentrations of IL-12 and IL-18 (12.5pg/ml and 10ng/ml respectively) are enhanced after influenza vaccination (9), suggesting that vaccine induced primed or co-stimulated NK cells have a lower threshold for cytokine-induced activation.

Whilst vaccine antigen driven NK cell recall responses are known to be dependent on vaccine specific T cell derived IL-2 and antibody (9, 10, 17, 18), the mechanisms of increased NK cell responses to innate cytokines after vaccination are not well understood. Our previous studies showed that IL-15 could indirectly promote NK cell responses by boosting the *in vitro* production of accessory cell-derived cytokines in response to inactivated influenza H3N2 (16). We therefore hypothesised that IL-15 stimulation could also reveal whether a similar indirect effect could be promoted by active vaccination with seasonal trivalent or quadrivalent influenza vaccine (TIV, QIV). We examined IL-15 stimulated accessory cell derived cytokine production *in vitro* before and after

vaccination and the associated impact on NK cell function. Restimulation of human PBMC cultures with low concentrations of IL-15 alone revealed enhanced production of both myeloid and lymphoid cell-derived cytokines four weeks after vaccine administration. Co-stimulation with IL-15 and influenza H3N2 resulted in further enhancement of cytokine production and in NK cell IFN-γ and CD25 upregulation in post-vaccination compared to baseline samples. These studies contribute to the understanding of vaccine induced pre-activation of innate immune cells, and of the *in vivo* mechanisms promoting the generation of CIML NK cells in humans.

3.3 Materials and Methods

3.3.1 Study participants and sample collection

Fifty-one healthy adult volunteers (median age 39y, range 24-66y; 41% male; 41% HCMV seropositive by IgG ELISA (Biokit, Barcelona, Spain)) were recruited from amongst staff and students of the London School of Hygiene and Tropical Medicine (LSHTM). The study was approved by the LSHTM Ethical Review Committee (reference number 10336). All subjects received a single dose of 2015-2016 inactivated TIV (n=37) or 2017-2018 inactivated QIV (n=14) by the intramuscular route (Split Virion BP, Sanofi Pasteur). Heparinised blood was collected prior to vaccination (baseline; day 0), and 3 days, 7 days and 30 days post-vaccination. Whole blood was also collected from non-vaccinated volunteers for use in additional experiments. PBMC were isolated using Histopaque 1077 (Sigma-Aldrich, Gillingham, U.K.) gradient centrifugation and cryopreserved in liquid nitrogen. Plasma samples were stored at -80°C.

3.3.2 Cell cultures

Before use, cryopreserved cells were thawed, washed in RPMI 1640 supplemented with 100U/ml penicillin/streptomycin and 20mM L-glutamine (Life Technologies, Thermo Fisher), and rested for 2 hours in the absence of exogenous cytokines. Cells were counted using a Countess II FL Automated Cell Counter (Invitrogen, Thermo Fisher); the median yield was 51% and median viability by trypan blue exclusion was 89%. PBMCs (4×10^5) were either stained immediately for *ex vivo* phenotyping or cultured for 6 or 18 hours with or without

2µg/ml inactivated whole H3N2 influenza virus (influenza A/Victoria/361/2011 H3N2 (IVR-165); formalin inactivated, in PBSA buffer plus 1% (w/v) sucrose, National Institute for Biological Standards and Control, Potters Bar, U.K.), with or without 0.75ng/ml recombinant human IL-15 (PeproTech, London, U.K.) in RPMI plus 5% pooled human AB serum (Sigma-Aldrich). Additional cultures were stimulated with a high concentration of cytokines (HCC) consisting of IL-12 (5ng/ml; PeproTech) and IL-18 (50ng/ml; R&D Systems, Oxford U.K.).

For blocking experiments, the following antibodies were used; anti-IL-12 at 3µg/ml (BD Biosciences, Oxford, U.K.) and mouse IgG1 isotype control at 3µg/ml final (eBiosciences, Thermo Fisher). There was no difference in NK cell activation in the presence or absence of isotype control antibody. GolgiStop (Monensin; 1/1500 concentration; BD Biosciences) and GolgiPlug (Brefeldin A; 1/1000 final concentration; BD Biosciences) were added for the final 3 hours of all cell cultures. Culture supernatants were collected and stored at -80°C for Luminex analysis.

3.3.3 Antibody-dependent NK cell activation assay

96 well flat bottom tissue culture treated plates were coated overnight with 1µg/ml inactivated H3N2 virus at 4°C, washed and blocked with 5% FCS in RPMI 1640 supplemented as above for 30 minutes. PBMC from fresh blood of one previously tested (non-vaccinated) donor with pre or post-vaccination serum from each participant at a final concentration of 0.1% (plus 4.9% FCS) were plated on the coated plates and incubated for 5 hours at 37°C. Cells were harvested into round-

bottom plates by soaking and resuspending in cold PBS containing 0.5% FCS, 0.05% sodium azide and 2mM EDTA for staining.

3.3.4 Flow cytometry and Luminex

PBMCs were stained in 96-well round-bottomed plates as described previously (16). Briefly, cells were incubated for 5 minutes with Fc Receptor (FcR) blocking Reagent (Miltenyi Biotec) and then with a viability marker (Fixable Viability Stain 700; BD Biosciences) and antibodies to surface markers diluted in FACS buffer (PBS containing 0.5% FCS, 0.05% sodium azide and 2mM EDTA). Cells were then washed in FACS buffer, fixed and permeabilised using Cytofix/Cytoperm Kit (BD Biosciences) or Foxp3/Transcription Factor Fixation/Permeabilization Kit (eBiosciences) according to the manufacturer's instructions. Cells were then stained for intracellular markers with FcR blocking and washed again and finally, cells were resuspended for acquisition on a BD LSRII flow cytometer.

Fluorophore-labelled antibodies used were: anti-CD3-V500 (clone UCHT1), anti-CD56-PE-Cy7 (clone NCAM16.2), anti-CD107a-FITC (clone H4A3) (all BD Biosciences), anti-CD57-eFlour450 (clone TB01), anti-CD25–PerCP-Cy5.5 (clone BC96), anti-IL-18Rα-PE (clone H44) (all eBiosciences), anti-IL-12Rβ2-PerCP-Cy5.5 (clone 622509) (R&D systems), anti-CD25–FITC (clone BC96), anti-IFN-γ-APC (clone 4S.B3) and anti-Ki67-PEDazzle (clone Ki67) (all Biolegend, London, U.K). Cells were acquired using FACSDiva software, data were analysed using FlowJo V10.4 (Tree Star, Oregon, U.S.A). FACS gates were

set using unstimulated cells or FMO controls. Samples with less than 100 NK cell events were excluded from analysis.

Concentrations of GM-CSF, IFN-α2, IFN-γ, TNF-α, IP-10, IL-1β, IL-10, IL-12p70, IL-15 and IL-2 in cell culture supernatants were determined by Luminex technology, kit number HCYTOMAG-60K-09 (Merck Millipore, Watford, U.K) using Bio-Plex software (Bio-Rad, Watford, U.K.) for data acquisition.

3.3.5 Data analysis

Statistical analysis was performed using GraphPad Prism version 7.04 (GraphPad, California, U.S.A.). Differences in responses were analysed using Wilcoxon signed-rank test or one-way ANOVA Friedman test with Dunn's correction for multiple comparisons. Correlation analysis was performed using linear regression. Significance levels are assigned as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ for all tests.

3.4 Results

3.4.1 Enhanced IL-15 stimulated cytokine production after influenza vaccination.

Our previous study (16) revealed that IL-15 co-stimulation enhanced the production of myeloid cell-derived cytokines from influenza H3N2-stimulated PBMC. We therefore reasoned that influenza vaccination might also prime accessory cells for cytokine production which could be revealed on restimulation of PBMC with IL-15 *in vitro*. Baseline and day 30 post-vaccination PBMC from volunteers vaccinated with a single dose of 2015-2016 TIV were cultured *in vitro* for 18 hours with 0.75ng/ml exogenous IL-15, with or without inactivated H3N2 virus or medium alone. Cytokine concentrations in cell culture supernatants were determined by Luminex (Figure 1). In line with our previous data in unvaccinated individuals (16), baseline samples showed significant induction of IL-10 (p=0.0391) and IFN-γ (p=0.006) in response to IL-15 alone (Figure 1b, g) and influenza H3N2 stimulation increased levels of IFN-α2 (p=0.001), IL-12(p70) (p=0.002), TNF-α (p=0.008), and IFN-γ (p=0.009) compared to medium alone (Figure 1d-g). Co-stimulation of baseline, pre-vaccination samples with H3N2 and IL-15 further enhanced secretion of GM-CSF (p=0.0039), IL-1β (p=0.004), TNF-α (p=0.0039) and IFN-γ (p=0.005) compared either stimulus alone (Figure 1a, c, f, g).

We then compared post-vaccination to pre-vaccination cytokine secretion in response to each stimulation condition alone or in combination. *In vitro* restimulation of PBMC with IL-15 alone significantly increased GM-CSF (3.6 fold), IL-10 (3.6 fold), IL-1β (1.4 fold) and IFN-γ (1.5 fold) secretion at 30 days post-

vaccination compared to baseline (Figure 1a-c, g). An increase in median concentration of TNF-α (baseline, 509.4pg/ml; day 30, 1169pg/ml) was also observed in response to IL-15 alone after vaccination (Figure 1f). Vaccination did not result in increased levels of cytokines in unstimulated (medium alone) cultures, indicating that enhancement of cytokine responses after vaccination was an active process, reliant both on *in vivo* priming and *in vitro* restimulation, and not due to residual activated cells in the cultures. Although H3N2 stimulated significant levels of IFN-α2 and IL-12(p70) secretion, no enhancement post-vaccination was observed to this stimulation (Figure 1d-e). However, when cells were co-stimulated with IL-15 and H3N2, significant increases in GM-CSF, IL-10, IL-1β, IFN-α2 and IFN-γ were observed post-vaccination compared to baseline (Figure 1a-d, g). No enhancement in IL-2 was measured in cell culture supernatants in response to *in vitro* stimulation after vaccination (not shown). These data show that restimulation *in vitro* with low concentrations of exogenous IL-15 are sufficient to reveal enhanced cytokine secretion after vaccination in the absence of antigen specific stimuli. Whilst increased IFN-γ secretion is consistent with enhanced NK cell activation, GM-CSF, IL-10 and IL-1β are indicative of myeloid cell activation, suggesting that influenza vaccination primes both myeloid and lymphoid cells *in vivo* for increased cytokine production.

3.4.2 Enhanced NK cell responses to IL-15 post-vaccination.

To examine the effect of influenza vaccine-induced priming of innate immune cells on NK cell responses, baseline and 30 day post-vaccination PBMC were cultured for 6 or 18 hours *in vitro* with medium alone, IL-15 alone, H3N2 or H3N2

+ IL-15 and analysed for NK cell (CD56$^+$CD3$^-$) IFN-γ, CD25 and CD107a expression by flow cytometry; the gating strategy is shown in Figure 2a-c. There were no significant differences between responses of individuals vaccinated with 2015-2016 TIV or 2017-2018 QIV (not shown); all data were therefore pooled for analysis. NK cell IFN-γ and CD107a were induced after 6 hours stimulation with H3N2 (Figure 2a, b) and CD25 was upregulated after 18 hours (Figure 2c), NK cell activation was significantly higher after H3N2 + IL-15 co-stimulation compared to either H3N2 or IL-15 alone, consistent with our previous observations (Figure 2a-c) (16).

Importantly, when PBMCs were stimulated with H3N2 + IL-15, significantly higher percentages of NK cells expressing IFN-γ at 6 hours (median 2.6% increase; 34 of 49 donors), and CD25 at 18 hours (median 7.8% increase; 30 of 48 donors) were detected among PBMCs collected 30 days after vaccination compared to baseline (Figure 2d, f). Similarly, after IL-15 stimulation, a higher percentage of NK cells expressed CD25 in day 30 post-vaccination PBMCs than in baseline PBMCs (Figure 2f). Conversely, there were no significant differences in levels (mean fluorescence intomsity; MFI) of CD25 expression on CD25$^+$ cells after *in vitro* stimulation (IL-15 alone: baseline median MFI 165, interquartile range (IQR) 137-208; day 30 median 164, IQR 129-199; H3N2 + IL-15: baseline median 547, IQR 416-661; day 30 median 532, IQR 368-693; not shown) indicating that vaccination was increasing the frequencies of responsive NK cells rather than modulating the expression of CD25 expression *per se*. CD107a expression did not differ significantly between baseline and day 30 post-vaccination cells, irrespective of the restimulation conditions (Figure 2e). Finally, neither IFN-γ,

CD107a (Supplementary Figure 1a, b) nor CD25 (Figure 2f) expression differed significantly between baseline and post-vaccination cells after restimulation with H3N2 alone.

Whilst higher frequencies of IFN-γ^+ cells were observed among less differentiated (CD56^{bright} and CD56^{dim}CD57$^-$) NK cells, both before and after vaccination, compared to more differentiated subsets (CD56^{dim}CD57$^+$) (Figure 2g), enhanced post-vaccination CD25 and IFN-γ (but not CD107a) responses to H3N2 + IL-15 were observed across all NK cell subsets indicating that vaccination influences NK cell function independently of differentiation state (Figure 2g, h, i). In line with previous studies (8, 9, 19), we observed small but significant increases in IFN-γ^+ NK cells 30 days post-vaccination compared to baseline among cells restimulated with high concentrations of IL-12 and IL-18, consistent with an intrinsic vaccine-induced effect on NK cells (Supplementary Figure 1c-e). These data suggest that very low concentrations of IL-15 alone are sufficient to reveal vaccination mediated enhancement of total NK cell activation (CD25 expression) in post-vaccination cultures potentially through synergy with vaccine-primed cytokine secretion from accessory cells.

TIV and QIV vaccination induced significant levels of H3N2 specific antibodies (Supplementary figure 2a), therefore, we measured antibody-dependent NK cell activation (whole PBMC from one non-vaccinated control donor) to inactivated H3N2 and pre or post-vaccination plasma from each vaccinated individual. Indeed we measured significantly higher NK cell CD107a and IFN-γ expression in response to inactivated virus with post-vaccination plasma compared to baseline plasma (Supplementary Figure 2b). There was a weak positive

correlation (p=0.0006) between day 30 antibody concentration and NK cell IFN-γ secretion (Supplementary Figure 2c).

3.4.3 Vaccine induced enhancement of polyfunctional NK cell responses requires IL-15 co-stimulation.

IL-15 is a major contributor to CIML NK cell function and synergises with IL-12 and IL-18 for polyfunctionality (20), suggesting that, *in vivo*, IL-15 may play an important role in maximising anti-viral NK cell activity. Here we examined whether vaccination would promote polyfunctional NK cell responses after restimulation with IL-15 alone or in combination with H3N2 (Figure 3). Frequencies of IFN-γ$^+$CD25$^+$, CD107a$^+$IFN-γ$^+$, and CD107a$^+$CD25$^+$ polyfunctional NK cells were significantly higher in response to H3N2 + IL-15 than H3N2 alone or IL-15 alone both pre and post-vaccination (Figure 3a-c). Enhanced polyfunctional NK cell responses were observed after vaccination; significantly higher percentages of IFN-γ$^+$CD25$^+$ NK cells (median baseline, 0.38%; day 30, 0.73%) and an increase in median percentage of CD107a$^+$IFN-γ$^+$ (baseline, 0.27%; day 30, 0.40%) and CD107a$^+$CD25$^+$ (baseline, 2.4%; day 30, 2.8%) were measured in response to H3N2 + IL-15 but not to either stimulus alone (Figure 3a-c). These data suggest that, consistent with viral induction of IFN-α2 and IL-12(p70) shown in Figure 1, enhancement of polyfunctional NK cell frequencies requires viral induced signals.

3.4.4 Post-vaccination NK cell responses are dependent on IL-12.

Synergy between innate cytokines, IL-12, type I IFN and CD4$^+$ T cell derived IL-2 are required for enhanced NK cell responses after vaccination (9, 16, 17, 19).

Despite vaccination having no direct enhancement of IL-12 production *per se* compared to baseline samples (Figure 1) we assessed whether the enhancement of NK cell responses after vaccination was nonetheless dependent on IL-12. Pre and post-vaccination PBMCs were stimulated *in vitro* with H3N2 + IL-15 in the presence of anti-IL-12 blocking antibody or isotype control antibody and NK cell function was assessed. IL-12 blockade significantly reduced the percentages of IFN-γ^+ positive and CD25$^+$IFN-γ^+ double-positive NK cells at baseline and ablated the vaccination-induced enhancement of CD25$^+$IFN-γ^+ double-positive NK cells (Figure 4a). The relationship between NK cell function and IL-12(p70) secretion in 18 hour *in vitro* cultures was assessed by linear regression. Both NK cell IFN-γ expression and CD25$^+$IFN-γ^+ polyfunctionality were loosely but significantly correlated with IL-12(p70) concentration at baseline (r^2=0.19; p=0.014 and r^2=0.13; p=0.03, respectively) (Figure 4b) but were more strongly correlated at 30 days post-vaccination (r^2=0.61, p=<0.0001 and r^2=0.58, p=<0.0001, respectively) (Figure 4c). These data indicate that the enhancement of NK cell responses post-vaccination were dependent on IL-12 but that the intrinsic ability of NK cells to respond to this cytokine may also be influenced by vaccination.

3.4.5 Altered *ex vivo* NK cell phenotype and cytokine receptor expression early after TIV vaccination.

Enhanced cytokine-dependent NK cell responses observed after vaccination are restricted to less differentiated, cytokine responsive subsets (8-10, 19, 21). To determine how vaccination increases the functional potential of these less differentiated NK cells, including IL-12 responsiveness, we examined the effects of vaccination on NK cell phenotype at day 3, 7 and 30 post-vaccination by *ex vivo* analysis of expression of CD56, CD57, the proliferation marker Ki67, and cytokine receptors IL-12Rβ2, IL-18Rα and CD25 (gating strategy is shown in Supplementary Figure 3a). We observed a transient expansion of the CD56^{bright} NK cell subset, along with concomitant contraction of the CD56^{dim} subset within the total NK cell population at 7 days post-vaccination when compared to baseline or day 30 (Figure 5a). This seems to be due to proliferation of CD56^{bright} cells as demonstrated by a transient increase in the proportion of Ki67^{+}CD56^{bright} NK cells (Figure 5b) with no parallel effect on cell death (as monitored by viability dye) within both CD56^{bright} and CD56^{dim} NK cells across vaccination visits (Supplementary Figure 3b).

We observed significant downregulation of surface IL-12 receptor (IL-12Rβ2) expression on CD56^{dim}CD57^{-} cells 3 days after vaccination (Figure 5c), although we did not detect any significant vaccination-induced changes in IL-12Rβ2 expression in CD56^{bright} or CD56^{dim}CD57^{+} NK cells (Figure 5c). No changes in surface IL-18 receptor (IL-18Rα) expression in any subset was observed (Supplementary Figure 3c). There was a small yet highly significant decline in the MFI of CD25 expression on both CD56^{bright} and CD56^{dim} NK cells post-vaccination

(Figure 5d). Taken together, these data show transient proliferation of less differentiated NK cells, and a small but significant downregulation of cytokine receptors (IL-12Rβ2 and CD25) early after vaccination, consistent with vaccine induced responses to the cognate cytokines *in vivo*.

3.5 Discussion

CIML NK cells have been described in a number of different *in vitro* models and after infection and vaccination (reviewed in (22) and (23)). Similarly to *in vitro* priming with cytokines, *in vitro* priming with inactivated H1N1 virus and TIV vaccination led to enhanced NK cell responses to cytokines alone (9), suggesting that anti-viral cytokine stimulation is sufficient to generate CIML NK cells. Here, in line with previous data, we demonstrate the generation of CIML NK cells by vaccination but furthermore, for the first time, we show that vaccination also primes for heightened secretion of myeloid cell-derived cytokines that can be induced by extremely low concentrations of IL-15 *in vitro*. Overall, our data suggest that influenza vaccination primes both myeloid cell cytokine secretion and intrinsic NK cell responsiveness, which together lead to the enhanced NK cell responses observed after influenza vaccination.

These observations are in line with systems level analyses of the PBMC response to TIV influenza vaccination in which NK cell activation, type I IFN, serum IP-10 (IFN-γ-induced protein) and DC activation signatures are seen within 48 hours (24, 25) and with innate cytokine secretion from PBMCs stimulated *in vitro* with inactivated influenza virus (16). Our study represents the first demonstration of enhanced myeloid cell-derived cytokine production after viral vaccination and is reminiscent of the 'trained immunity' described in human monocytes after *Candida albicans* infection or Bacille Calmette-Guérin (BCG) vaccination, where increased TNF-α, IL-1β and IL-6 secretion occurred after secondary stimulation with the same or distinct stimuli (26, 27). Enhanced pro-inflammatory function in 'trained' monocytes is associated with activation induced epigenetic modifications

(28, 29). Jegaskanda *et al.* recently reported that accessory cell IFN-α induced in response to influenza infection primed NK cells for enhanced responsiveness to antibody-dependent signals after a 12 hour rest period (without continued exposure). Consistent with our study, the authors measured increased Ki67 expression on the pre-exposed NK cells (30).

Although measurement of cytokines in supernatant after stimulation does not identify a specific cellular source, it is likely that the high levels of GM-CSF, IL-10, IL-1β and IFN-α2 measured here were derived from myeloid accessory cells, including monocytes, macrophages and DCs. As we have previously shown that IL-12 is induced in response to H3N2 in CD123⁻CD11c⁺ myeloid DCs (mDCs) (16), we suggest this cell subset may be responsible for the low but significant production of this cytokine. In contrast, IL-10 and TNF-α are produced by CD14⁺ monocytes and IFN-α and IFN-β are produced by conventional and plasmacytoid DCs within hours of TIV vaccination (31, 32). This vaccine induced myeloid cell cytokine secretion enhancement may therefore demonstrate priming (or 'training') of monocytes, or other innate myeloid cell populations in response to viral vaccination.

Vaccine-induced enhanced cytokine secretion was revealed with low dose IL-15 stimulation, without the need for vaccine antigen-specific stimulation. This same concentration of IL-15 also enhanced secretion of myeloid cell-derived cytokines, in particular IL-12 and IFN-α in combination with H3N2 (16). This suggests that pre-activation of these cells by the inactivated H3N2 virus within the vaccine leaves them with enhanced ability to respond to IL-15 and this effect is maintained for at least 30 days after initial stimulation. This enhanced cytokine secretion

capacity has a knock-on effect on NK cell activation, with significant upregulation of CD25 in post-vaccination cultures compared to baseline in response to IL-15. In contrast to our previous study, where vaccination promoted limited enhancement of NK cells in response to H1N1 virus alone (9), enhanced responsiveness of H3N2 vaccination-primed NK cells required IL-15. This may be due to previous exposure to H3N2 by infection or vaccination leading to different levels of T cell help as suggested by comparing our previous study cohorts, lower overall induction of NK cell activation markers with H3N2 compared to other influenza viruses (such as H1N1) (9) or differences in virus specific cytokine secretion profiles. Production and trans-presentation of IL-15 is of particular significance in the lung during influenza infection, where local accumulation impacts T cell apoptosis, formation of IL-2-independent tissue resident memory T cells and recruitment of NK cells (33-36). Our previous study observed similar CIML NK cell generation with intranasal administration of live attenuated influenza vaccine (LAIV) (9), and thus the potential impacts of locally induced IL-15 in the lung after influenza vaccination or indeed infection merits further investigation.

Frequencies of IFN-γ^+ and IFN-γ^+CD25$^+$ NK cells were strongly correlated with IL-12 concentrations in cell supernatants, suggesting this cytokine is important for post-vaccination NK cell activation. Although, there was no evidence of enhanced secretion of IL-12(p70) after vaccination here (Figure 1), which may be due to immediate utilisation of secreted IL-12 *in vitro.* Similarly, we could not detect IL-2 in supernatants even though IL-2 (and IL-12) plays an essential role in NK cell responses to vaccination, supporting the suggestion that differential T

cell help is playing a role in the post-vaccination response to H3N2 (9, 17, 19). NK cells required both viral stimuli and IL-15 for enhanced polyfunctionality after vaccination, which is consistent with the need for combinations of two or more of IL-12, IL-15 and IL-18 in order to induce polyfunctionality in non-pre-activated and *in vitro* generated CIML NK cells, and IL-15 playing a major role in CIML NK cell mediated cytotoxicity (20). Vaccine induced changes in frequencies of polyfunctional NK cell cells ranged from 0.13% to 0.4%, representing up to 1/2500 of total lymphocytes. Similar frequencies are observed for typical antigen specific T cell responses to whole antigens, indicating that the NK cell response could contribute meaningfully to vaccine induced immunity.

CIML NK cells retain their characteristics through subsequent cell divisions, suggesting that their induction is not simply a short term priming effect but rather reflects an intrinsic change or differentiation state (37). Proliferation of less differentiated (CD56^{bright} and CD56^{dim}CD57^{-}) NK cell subsets after vaccination is consistent with the previously demonstrated effects of vaccination on NK cells and with other routes for generation of CIML NK cells (5, 8, 9, 19). In addition, we measured significant down regulation of IL-12 receptor at day 3 after vaccination suggesting cytokine mediated NK cell activation *in vivo*; down-regulation of IL-12Rβ2 expression after vaccination would be consistent with IL-12-mediated receptor turnover, supporting a critical role for this cytokine in CIML NK cell generation. We previously measured weak but significant trends for NK cell function, in response to cytokine stimulation, to decline with age (38), however there was no significant tend for declining NK cell function or cytokine production post-vaccination in response to IL-15 +/- H3N2 with age.

In summary, we show for the first time that influenza vaccination primes PBMCs for heightened innate cytokine production, that this can be revealed by very low concentrations of IL-15, and that this - when combined with the generation of CIML NK cells - leads to enhanced NK cell responses after vaccination. Further work is required to determine the significance of this pathway in post-vaccination immunity to influenza virus.

3.6 Acknowledgements

3.7 Figures
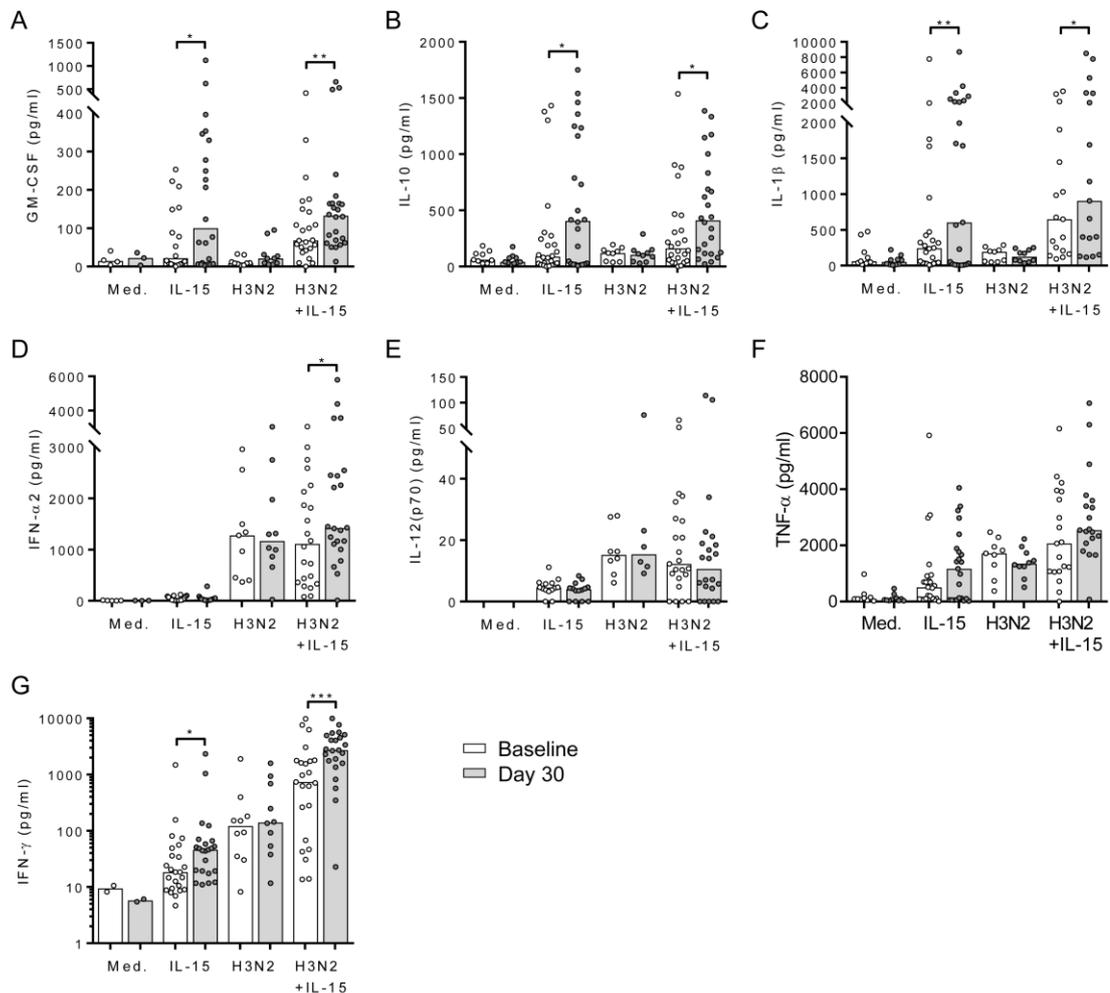
Figure 1: Influenza vaccination enhances IL-15 stimulated cytokine production.

Baseline and 30 day post-vaccination samples of PBMC were cultured with medium (n=10), 0.75ng/ml IL-15 alone (n=26), inactivated H3N2 (n=10) or H3N2 + IL-15 (n=26) for 18 hours and culture supernatants were collected. Concentrations (pg/ml) of GM-CSF (a), IL-10 (b), IL-1β (c), IFN-α2 (d), IL-12(p70) (e), TNF-α (f), and IFN-γ (g) in supernatant were determined by Luminex. Graphs show one dot per donor, with a bar representing median value. Comparisons between vaccination time points were made using Wilcoxon signed-rank test. *p < 0.05, **p < 0.01, *** p < 0.001.
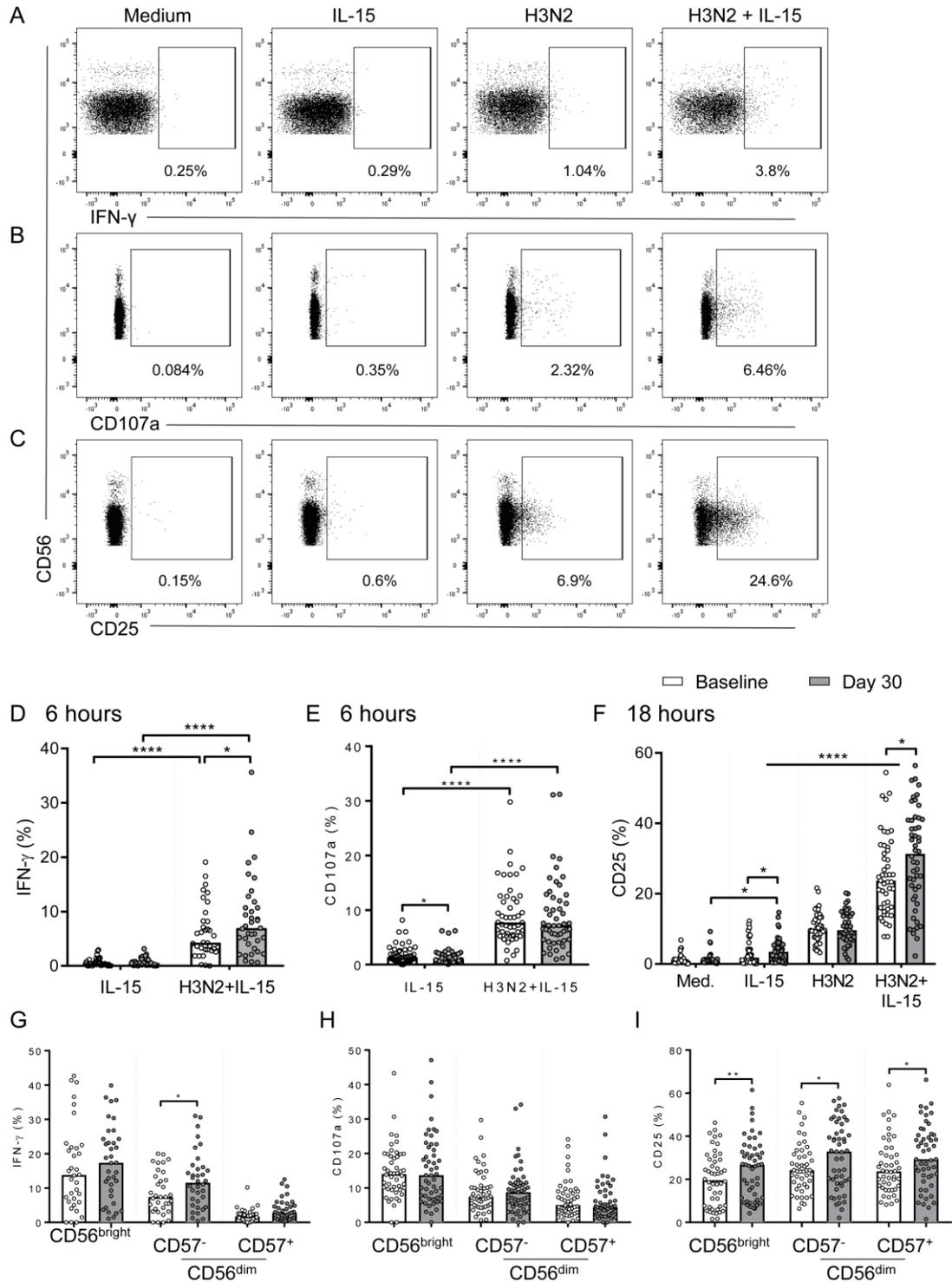
Figure 2: Enhanced NK cell responses to IL-15 post-vaccination.

Baseline and 30 day post-vaccination PBMC samples from 2015-2016 TIV (n=37) or 2017-2018 QIV (n=14) vaccinated individuals (all donors were combined for analysis), were stimulated *in vitro* with medium alone, IL-15 alone, H3N2 or H3N2 + IL-15. IFN-γ, CD107a and CD25 expression of $CD56^+CD3^-$ NK cell activation was measured after 6 (IFN-γ, CD107a) or 18 hours

(CD25) by flow cytometry. Plots show the gating strategy for IFN-γ (a), CD107a (b) and CD25 expression (c) from one representative donor at baseline. Numbers shown are the percentage of total NK cells positive for each marker. IFN-γ (d), CD107a (e) in response to IL-15 and H3N2 + IL-15 and CD25 in response to all 4 conditions (f) is shown at baseline and 30 days post-vaccination. IFN-γ (g), CD107a (h) and CD25 (i) in response to H3N2 + IL-15 attributed to NK cell differentiation subsets defined by CD56 and CD57 expression is also shown. Plots show one dot per donor with a bar representing median percentage. Comparisons between vaccination time points were made using Wilcoxon signed-rank test and between culture conditions by one-way ANOVA with Dunn's correction for multiple comparisons. *p < 0.05, **p < 0.01, ****p < 0.0001.
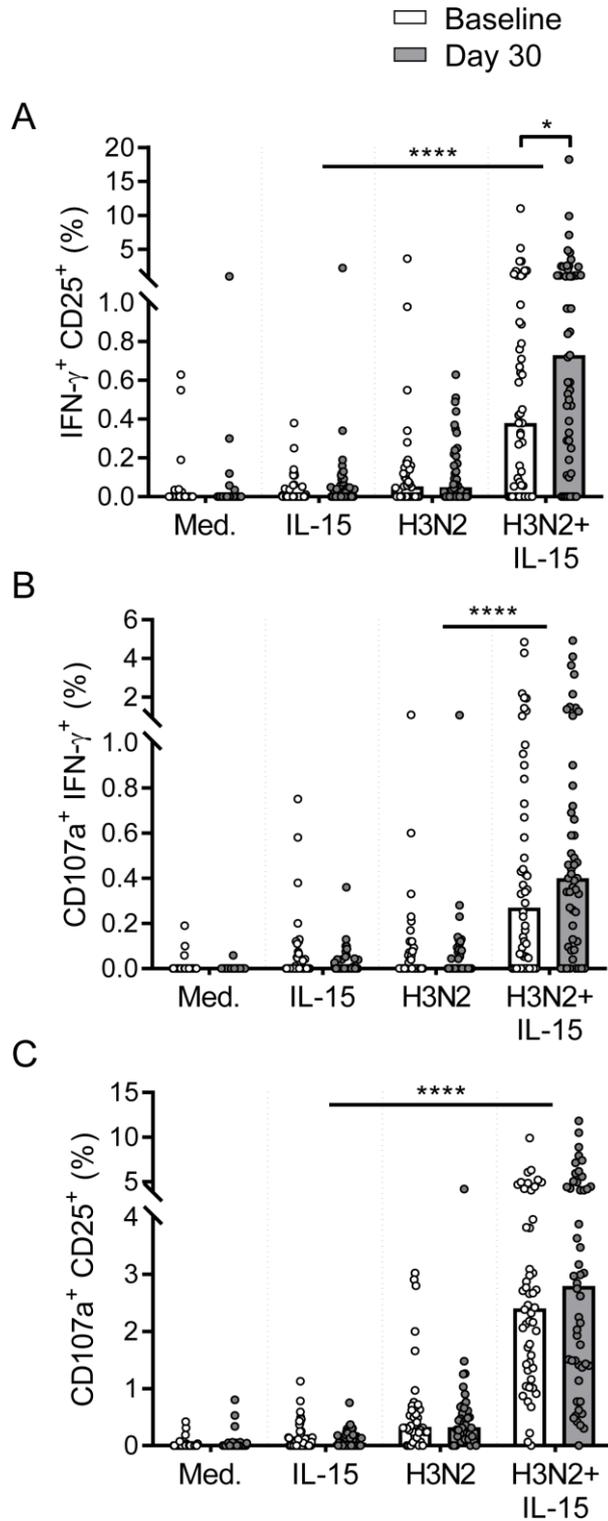
Figure 3: Vaccine induced enhancement of polyfunctional NK cell responses requires IL-15 co-stimulation.

Percentages of double positive IFN-γ⁺CD25⁺ (a), CD107a⁺IFN-γ⁺ (b) and CD107a⁺CD25⁺ (c) NK cells at baseline and 30 days post-vaccination were determined by flow cytometry after 18 hour stimulation with medium alone, IL-15 alone, H3N2 or H3N2 + IL-15 (n=51). Plots are one dot per donor with a bar representing median percentage. Comparisons between vaccination time points were made using Wilcoxon signed-rank test and between culture conditions by one-way ANOVA with Dunn's correction for multiple comparisons. *p < 0.05, ****p < 0.0001.
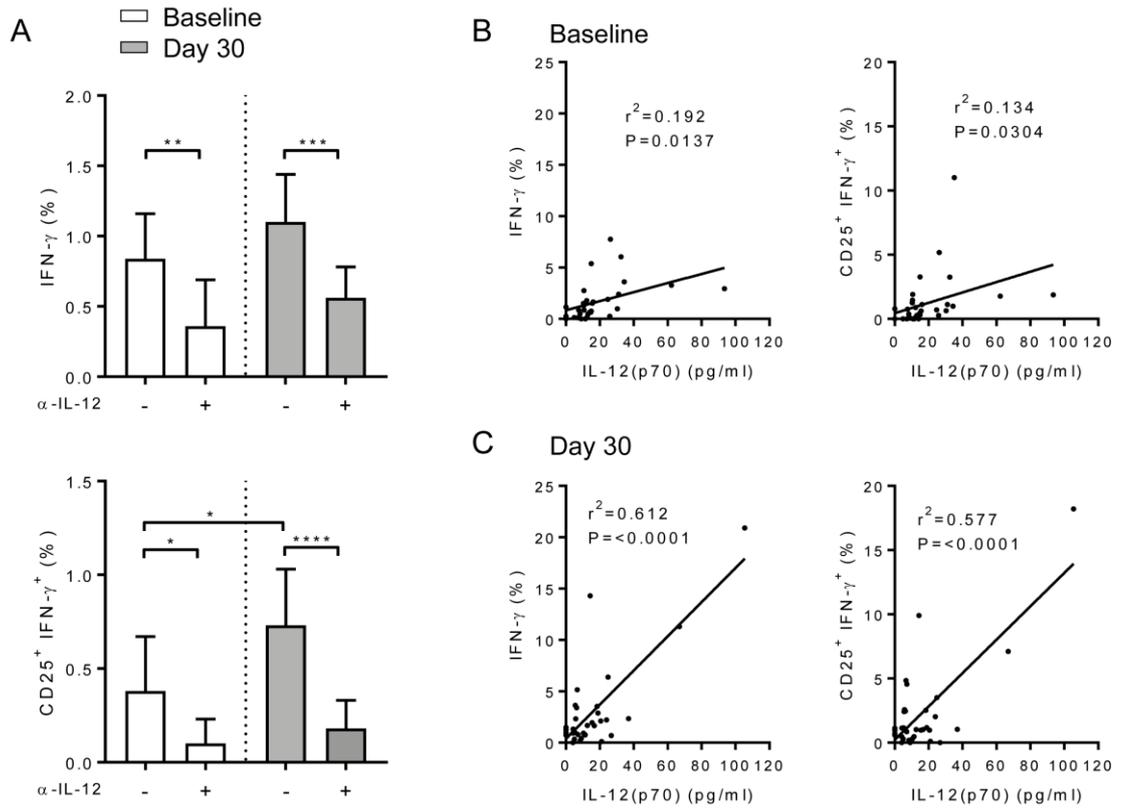
Figure 4: Post-vaccination NK cell responses are dependent on IL-12.

Baseline and 30 day post-vaccination PBMC samples were stimulated *in vitro* with H3N2 + IL-15 in the presence of an anti-IL-12 antibody for 18 hours, (a) percentage of IFN-γ⁺ and CD25⁺IFN-γ⁺ double positive NK cells were determined by flow cytometry (n=51). Graphs show median percentage with 95% confidence interval. The relationship between IFN-γ⁺ and CD25⁺IFN-γ⁺ double positive NK cells and IL-12(p70) production in response to H3N2 + IL-15 (no IL-12 blocking) at 18 hours both before (b) and after (c) vaccination was determined by linear regression, goodness of fit was determined using r^2 and significance was determined as $p < 0.05$. Comparisons between culture conditions and vaccination time points were made using Wilcoxon signed-rank test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.
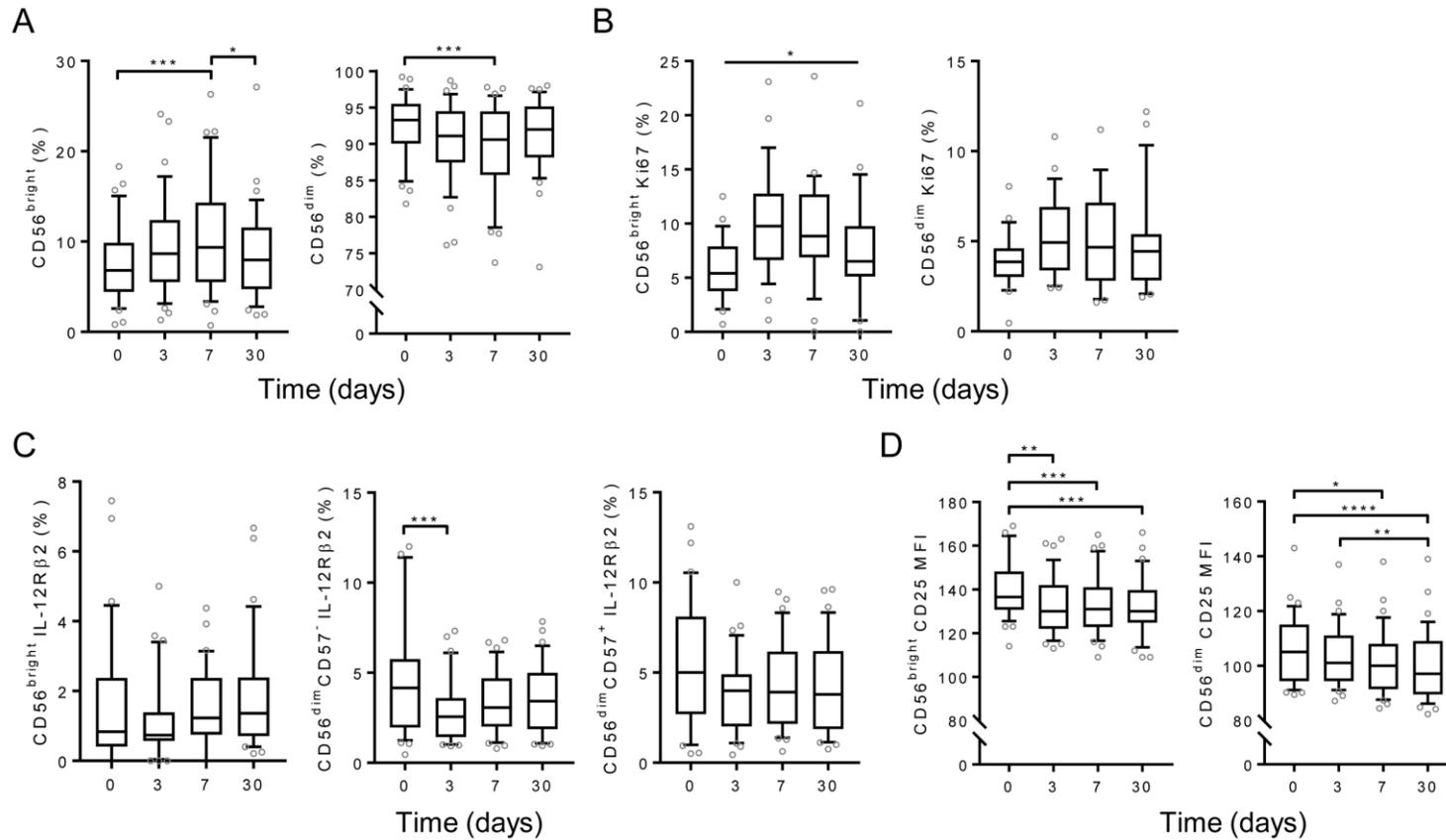
Figure 5: Altered *ex vivo* NK cell phenotype and cytokine receptor expression early after TIV vaccination.

NK cell phenotype was measured *ex vivo* after 2015-2016 TIV vaccination (n=37) at baseline, day 3, day 7 and day 30 post-vaccination. Percentage of CD56^{bright} and CD56^{dim} NK cells (a), Ki67 (b), IL-12Rβ2 (c) and CD25 MFI (d) were determined and attributed to NK cell differentiation subsets defined by CD56 and CD57 expression. Graphs show box and whisker plots with 10th-90th percentile, comparison across vaccination visits was determined by one-way ANOVA with Dunn's correction for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001, **** p < 0.0001.

3.8 Supplementary Figures
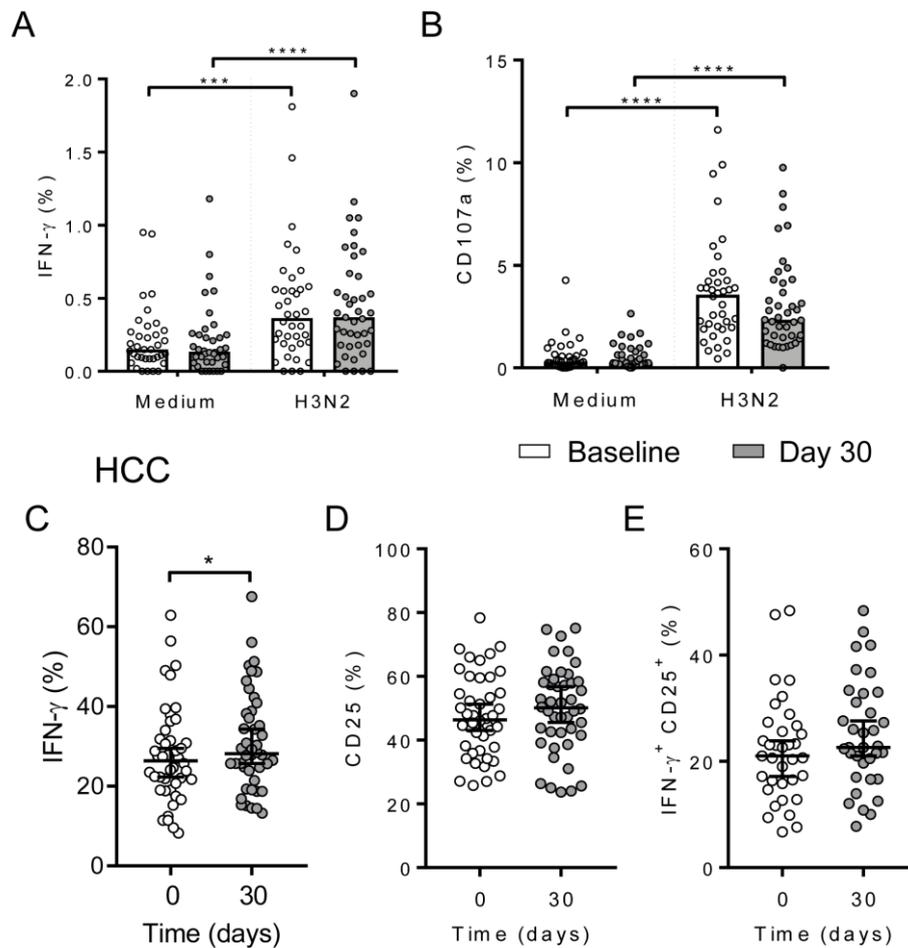
Supplementary Figure 1: Post-vaccination NK cell responses to H3N2 alone or a high concentration of cytokines.

Baseline and 30 day post-vaccination PBMC samples were stimulated *in vitro* with medium alone, H3N2 alone or a high concentration of IL-12 and IL-18 (HCC). IFN-γ, CD107a and CD25 expression of CD56+CD3- NK cells was measured after 18 hours by flow cytometry. IFN-γ (a) and CD107a expression (b) in response to medium and H3N2 alone is shown as one dot per donor with a bar at the median percentage. IFN-γ (c), CD107a (d) and IFN-γ+CD25+ expression (e) in response to HCC is also shown with a line representing the median and interquartile range. Comparisons were made using Wilcoxon signed-rank test. *p < 0.05, ***p < 0.001, ****p < 0.0001.
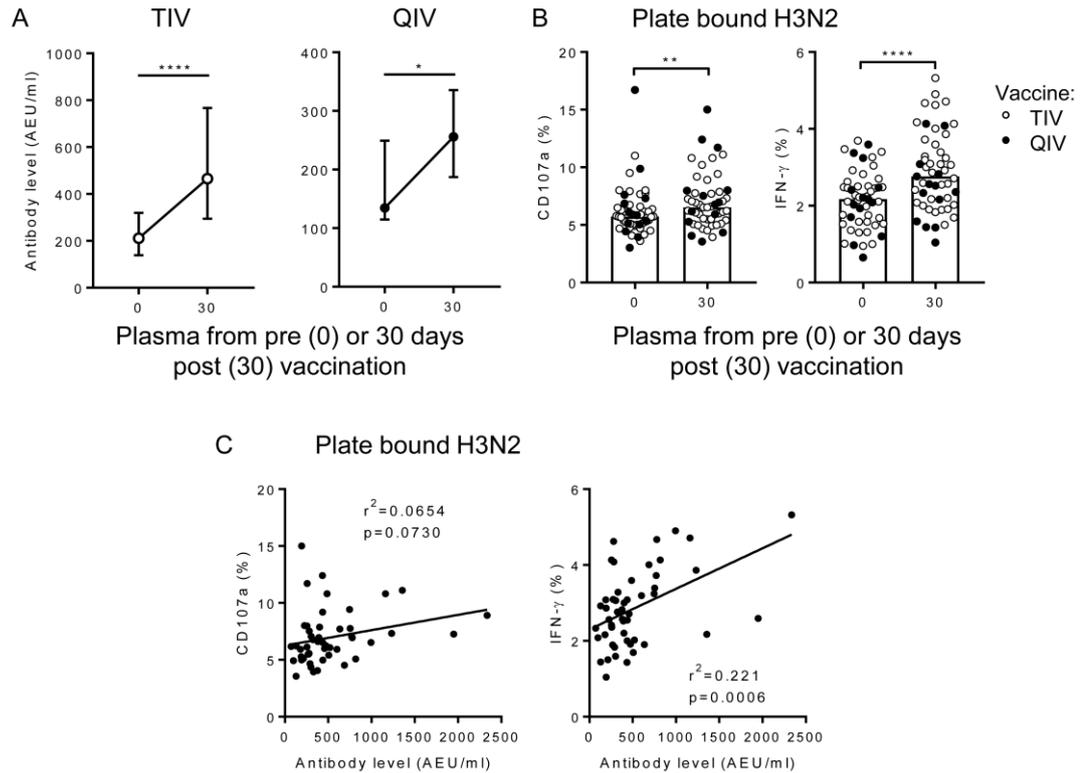
Supplementary Figure 2: Antibody-dependent NK cell responses post-vaccination.

Antibody level in baseline (0) and 30 day post-vaccination (30) plasma samples of participants vaccinated with TIV (n=37) and QIV (n=14) measured by ELISA (a). NK cell CD107a and IFN-γ expression in response to pre or post-vaccination plasma and plate bound inactivated H3N2 virus was measured in one non-vaccinated control donor (b). The correlation between antibody level and NK cell CD107a and IFN-γ expression at 30 days post-vaccination (c). Comparisons were made using one-way ANOVA with Dunn's correction for multiple comparisons. *p < 0.05, **p < 0.01, ****p < 0.0001. Correlation was determined by linear regression, goodness of fit was determined using r^2 and significance was determined as p value below 0.05. AEU; arbitrary ELISA units.
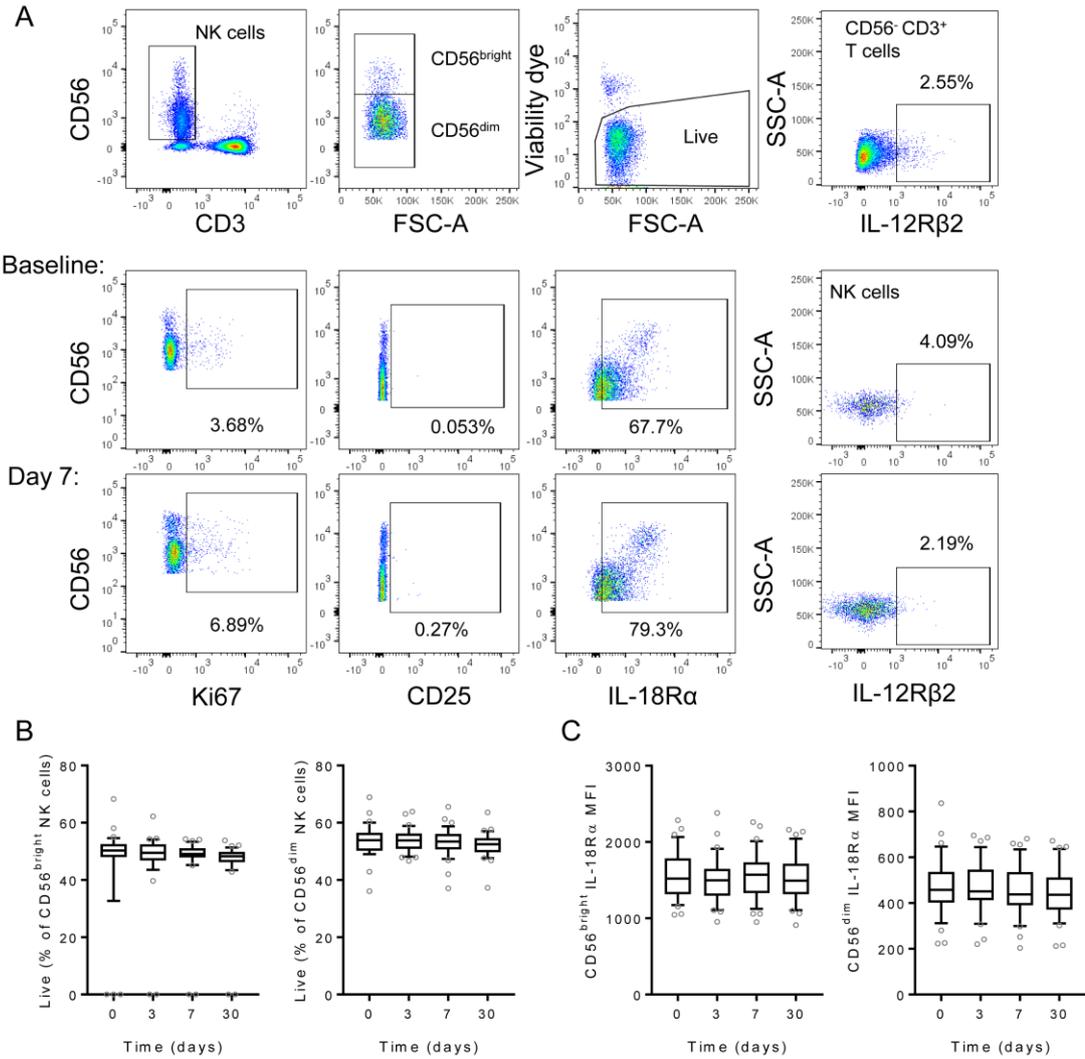
Supplementary Figure 3: *Ex vivo* flow cytometry gating strategy.

NK cell phenotype was measured *ex vivo* after 2015-2016 TIV vaccination (n=37) at baseline, day 3, day 7 and day 30 post-vaccination by flow cytometry. The flow cytometry gating strategy for CD56, live NK cells, Ki67, IL-12Rβ2, CD25 and IL-18Rα expression from one representative donor is shown (baseline and day 7 only) (a). Percentage of live CD56bright and CD56dim NK cells (viability dye negative) (b) and CD56bright and CD56dim IL-18Rα MFI (c) across vaccination visits is shown as box and whisker plots with 10th-90th percentile. Comparison were determined by one-way ANOVA with Dunn's correction for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

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Chapter 4: Ebola Virus Glycoprotein Stimulates IL-18 Dependent Natural Killer Cell Responses

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Surname/Family Name	Wagstaffe		
Thesis Title	The Effect of Viral Vaccines on Natural Killer Cell Effector Function		
Primary Supervisor	Martin Goodier		

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4.1 Abstract

NK cells are activated by innate cytokines and viral ligands to kill virus-infected cells; these functions are enhanced during secondary immune responses and after vaccination by synergy with effector T cells and virus-specific antibodies. In human Ebola virus infection, clinical outcome is strongly associated with the initial, innate cytokine response but the role of NK cells in this process has not been examined. The novel prime-boost Adenovirus type 26.ZEBOV/Modified Vaccinia Ankara-BN-Filo vaccine regimen is a safe and immunogenic Ebola glycoprotein expressing vaccine, currently undergoing phase 3 trials. In this study, we analysed the NK cell response to Ad26.ZEBOV/MVA-BN-Filo vaccination, including NK cell phenotype and function *ex vivo* and in response to recombinant Ebola glycoprotein, before and after vaccination. We show i*n vivo/ex vivo* proliferation and activation of NK cells after vaccination. Ebola glycoprotein-induced activation of NK cells *in vitro* was dependent on accessory cells and TLR-4-dependent innate cytokine secretion (predominantly from primary $CD14^+$ monocytes) and enriched within less differentiated NK cell subsets. Optimal NK cell responses were dependent on IL-18 and IL-12, whilst IFN-γ secretion was restricted by high concentrations of IL-10. This study furthers our understanding of innate immune responses to soluble Ebola glycoprotein and the impact of vaccination.

4.2 Introduction

Ebola virus infection causes a rapid onset, severe acute haemorrhagic fever (Ebola virus disease, EVD) with mortality ranging from 25% to 90% depending on the outbreak (1). The search for an effective vaccine remains a high priority as regular disease outbreaks continue on the African continent. Ebola vaccine development has focussed on the viral glycoprotein (GP) which is the only protein exposed on the surface of the mature virus particle, is essential for viral entry into host cells and is highly immunogenic (2, 3). A prime-boost vaccination approach, priming with Adenovirus type 26 (Ad26) expressing the Zaire Ebola virus GP (ZEBOV) and boosting with Modified Vaccinia Ankara (MVA) expressing ZEBOV, Sudan Ebola virus (SEBOV) and Marburg Virus GP and Tai Forest Ebola virus nucleoprotein (MVA-BN-Filo), has been shown to be safe and immunogenic in phase 1 clinical trials. Eliciting high and sustained antibody titres and antigen specific T cell responses, phase 2 and 3 clinical trials of Ad26.ZEBOV/MVA-BN-Filo are underway (4-6).

Innate immune dysregulation underlies the pathophysiology of EVD, resulting in failure to activate essential effector cell functions and consequent uncontrolled virus replication, systemic virus dissemination and inflammation (3, 7). Ebola virus infects macrophages and DCs, impairs maturation and inhibits the type I IFN response, due in part to the presence of interferon inhibiting domains (IIDs) within the viral proteins, VP24 and VP35. This, in turn, leads to impairment of NK cell function: *in vitro* studies with human peripheral blood mononuclear cells (PBMC) have shown that DC maturation, type I IFN secretion and NK cell activation are all enhanced when these Ebola virus IIDs are mutated (8, 9).

Impairment of the type I IFN response is accompanied by an excessive pro-inflammatory cytokine response (3, 10). *In vitro* studies have shown that the Ebola GP is a potent ligand for TLR-4, activating non-infected monocytic cell lines, monocyte-derived DCs and macrophages to produce cytokines such as TNF-α, IL-1β, IL-10, IL-6, IFN-β and IL-12 (11-15). Importantly, an initial type I IFN response accompanied by modest and transient IL-1β and TNF-α secretion correlates with survival among EVD patients, whereas high IL-10 associated with fatal outcome (10, 16, 17). This indicates that the very earliest interactions between the Ebola virus and the host immune system are critical for determining the outcome of infection.

There is evidence that, if they can be appropriately activated, NK cells may play a role in vaccine-induced protection from EVD. For example, murine infection with Ebola virus fails to induce an NK cell response, whereas treatment of mice with Ebola GP virus-like particles (VLPs) confers complete protection against a lethal Ebola virus infection just 3 days later; this protection was lacking after *in vivo* NK cell ablation (7). Furthermore, NK cell cytotoxicity and IFN-γ secretion have been implicated in the prolonged survival of NK cell-sufficient mice immunised with the VSV-vectored Ebola GP vaccine compared to NK cell-depleted mice (18). In humans, upregulation of the activation markers NKG2D and CD38 on NK cells was noted within 24 hours of vaccination with the rVSV-ZEBOV vaccine (19). When taken together with evidence from non-human primates of partial protection against live virus within 3 days of vaccination and full protection within 7 days, this suggests that NK cells may be able to mediate rapid and effective protection against Ebola virus (20, 21). Moreover, after vaccination, NK cells may synergise with anti-GP antibodies to clear virus-infected cells via antibody-

dependent cellular cytotoxicity (ADCC) (22, 23). The ability of an Ebola virus vaccine to ensure appropriate NK cell activation upon subsequent exposure to Ebola virus is thus likely to be an important component of vaccine efficacy.

Here, we evaluate the effect of Ad26.ZEBOV/MVA-BN-Filo prime-boost vaccination on accessory cell cytokine secretion, NK cell phenotype and NK cell effector function both *ex vivo* and in response to restimulation *in vitro* with soluble Ebola virus GP (EbovGP). We find that vaccination with Ad26.ZEBOV/MVA-BN-Filo induces *in vivo (ex vivo)* proliferation and activation of less differentiated NK cell subsets. We also find that stimulation of PBMCs (collected either before or after vaccination) with EbovGP induces TLR-4 dependent secretion of high concentrations of inflammatory cytokines (mainly from CD14$^+$ monocytes) and accessory cell-dependent NK cell activation. EbovGP induced NK cell activation was inhibited by neutralising antibodies to IL-18 (and IL-12) and was enhanced by IL-10 receptor blockade. These studies demonstrate a major role for peripheral blood monocyte cytokine secretion in control of NK cell effector function in response to EbovGP in unvaccinated and vaccinated individuals.

4.3 Results

4.3.1 Robust *in vivo/ex vivo* NK cell responses to Ad26.ZEBOV/MVA-BN-Filo vaccination.

Vaccination with several anti-viral vaccines, including influenza, has been shown to promote NK cell activation and a realignment of subsets associated with functional differentiation (24-26). We therefore analysed the effect of Ad26.ZEBOV/MVA-BN-Filo vaccination on NK cell activation and subset distribution. *Ex vivo* flow cytometric analysis of $CD56^+CD3^-$ NK cells from pre-vaccination (visit 0), post-prime (visit 1) and post-boost (visit 2) samples was performed. NK cells were divided into $CD56^{bright}$, $CD56^{dim}CD57^-$ and $CD56^{dim}CD57^+$ (or total $CD56^{dim}$) subsets ($CD56^{bright}$ representing the least differentiated and $CD56^{dim}CD57^+$ the most differentiated subset). The expression of Ki67 (a cell cycle marker of proliferation), IL-2Rα-chain (CD25) and NK cell receptors NKG2A and NKG2C was analysed for each subset (the flow cytometry gating strategies are shown in Supplementary Figure 1a). Initially, samples from all five vaccination groups were pooled for analysis.

When data for all vaccination groups were combined, there was a significant increase in the representation of $CD56^{bright}$ NK cells within total NK cells and a corresponding decrease in the frequency of $CD56^{dim}$ NK cells across vaccination visits (Figure 1a). $CD56^{bright}$ NK cells had the highest intrinsic capacity to proliferate, reflected in the higher percentage expression of Ki67 in this subset (Figure 1b), followed by $CD56^{dim}CD57^-$ cells. There was a significant increase in the frequency of $CD56^{bright}$ Ki67$^+$ and $CD56^{dim}CD57^-$ Ki67$^+$ NK cells between visit 1 and visit 2, suggesting that proliferation of less differentiated NK cells may

explain their increasing frequency (as in Figure 1a). There was no significant change in the proportion of more highly differentiated (CD56^{dim}CD57$^+$) NK cells expressing Ki67 (Figure 1b).

Consistent with the expression of the inhibitory receptor NKG2A on less differentiated NK cell subsets, a significant increase in frequency of NK cells expressing NKG2A was observed at visit 2, with no significant change in expression of the corresponding activating receptor, NKG2C (Figure 1c). There was a small but significant increase post-boost in the percentage of CD56^{dim} (but not CD56^{bright}) NK cells expressing CD25 (median 0.74% at visit 1; 0.88% at visit 2) (Figure 1d). The proportion of CD25$^+$ NK cells was positively correlated with the frequency of proliferating (Ki67$^+$) cells at 21 days post-boost, further suggesting an association between NK cell activation and proliferation in response to vaccination (Figure 1e). No effect of vaccination was observed on the percentage or mean fluorescence intensity (MFI) of NK cells expressing CD16 (the low affinity IgG receptor III, FcγRIII) (Supplementary Figure 1b). These data indicate proliferation of less differentiated NK cells in response to Ad26.ZEBOV/MVA-BN-Filo vaccination.

Overall, no significant changes in *ex vivo* NK cell phenotype and function were observed after the primary vaccination but significant NK cell proliferation and CD25 expression were observed after the secondary (boost) vaccination but with a diversity of responses among individuals. To investigate any effects of the order and/or timing of the priming and boosting vaccinations, NK cell responses were reanalysed by vaccination group. Increasing CD56^{bright} and decreasing CD56^{dim} NK cell frequencies after vaccination was indicated by a trend in all groups except

group 4 and reached significance by one-way ANOVA between baseline and post-boost in groups 3 and 5 only (Supplementary Figure 2a, b). Furthermore, there was a significant increase in CD56^{bright} Ki67$^+$ and CD56^{dim} CD25$^+$ NK cells between baseline and post-boost in group 4 only (Supplementary Figure 2c, d). These data suggest that the Ad26 prime/MVA boost vaccine regimen induced a more robust NK cell response than MVA prime/Ad26 boost. However, these effects were small and may lack statistical significance due to a lack of power in this subgroup analysis.

4.3.2 NK cell CD107a and CD25, but not IFN-γ upregulation in response to EbovGP stimulation *in vitro.*

To determine the effect of Ad26.ZEBOV/MVA-BN-Filo vaccination on NK cell responses to soluble EbovGP, baseline, visit 1 and visit 2 PBMCs were cultured for 8 and 18 hours with 10µg/ml EbovGP. Frequencies of NK cells expressing CD107a and IFN-γ (at 8 hours) or CD25 and CD16 (at 18 hours) were analysed by flow cytometry (gating strategies are shown in Figure 2a). There were no significant differences in response to EbovGP between vaccination groups (not shown); therefore, all five vaccination groups were combined for analysis. *In vitro* stimulation with EbovGP induced a significant increase in the proportion of NK cells expressing CD107a (Figure 2b) and CD25 (Figure 2c) at the cell surface compared to unstimulated cultures (medium alone). EbovGP stimulation had no effect on NK cell IFN-γ (at 8 or 18 hours) or CD16 expression (Figure 2d, e). The effect of EbovGP on markers of NK cell function did not differ across vaccination

visits (Figure 2b-e) suggesting the effect of EbovGP on NK cells is entirely innate and unaffected by vaccination.

Given that there was no effect of vaccination on the NK response to EbovGP, the analysis of NK cell function by differentiation subset was restricted to the baseline data set (Figure 3). This analysis revealed that IFN-γ secretion was restricted to the less differentiated CD56^{bright} and CD56^{dim}CD57^{-} subsets and that significant induction of IFN-γ by EbovGP was detected only within the CD56^{dim}CD57^{-} subset (Figure 3a). By contrast, CD107a and CD25 upregulation in response to EbovGP was seen in all NK cell subsets (Figure 3b, c), with a significantly higher CD25 expression in the CD56^{bright} subset compared to CD56^{dim} subsets (Figure 3c). The majority of CD25^{+} NK cell events were CD56^{dim}CD57^{-} (60.5%) (Figure 3d). Overall, these data demonstrate that EbovGP induces markers associated with NK cell cytotoxicity (CD107a) and activation (CD25), with a much lesser impact on IFN-γ secretion, and that these responses seem to be innate and not enhanced by vaccination.

4.3.3 High concentrations of inflammatory cytokines induced by EbovGP *in vitro.*

NK cells are able to respond to cytokines secreted from activated accessory cells in response to viral stimuli. To quantify cytokine production in response to EbovGP stimulation, baseline and 21-day post-boost vaccination PBMC samples were stimulated with EbovGP *in vitro* for 18 hours and cytokine concentrations in cell supernatants were measured by Luminex. EbovGP induced secretion of high concentrations of IL-10, IL-1β, IFN-α2, GM-CSF, TNF-α and IFN-γ from PBMCs

at baseline and post-boost samples compared to medium alone, where minimal concentrations were observed (Figure 4). Particularly high concentrations of IL-10 (median 3151pg/ml at baseline), IL-1β (median 3995pg/ml at baseline), GM-CSF (median 461pg/ml at baseline) and TNF-α (median 5542pg/ml at baseline) were measured in response to EbovGP (Figure 4a, b, d, e). IFN-α2 secretion was also significantly enhanced by EbovGP however the absolute concentrations of this cytokine were low (median 6.2pg/ml at baseline) compared to the other myeloid cell-derived cytokines (Figure 5c). Similarly, a low concentration of IL-12(p70) (maximum 6.6pg/ml) was detectable by Luminex in only a small number of individuals (13 of 71 at baseline and 9 of 71 at post-boost; not shown). Conversely, there was no increase in IP-10 secretion over medium alone and IL-15 was not detected (not shown).

With the exception of a small but significant reduction in EbovGP-induced TNF-α concentration in cultures of post-boost PBMCs (4647pg/ml post-boost compared to 5542pg/ml at baseline) (Figure 4e), there was no effect of vaccination on cytokine concentrations. When vaccination groups were analysed separately, concentrations of GM-CSF in group 3, IFN-α2 in group 4 and IL-10 and TNF-α in group 5 were significantly reduced at visit 2 compared to baseline (supplementary figure 3a, c, d, e) suggesting that reductions in cytokine responses were limited to the Ad26 prime/MVA boost regimen. In summary, EbovGP stimulated the release of high concentrations of IL-10, IL-1β, GM-CSF and TNF-α from PBMCs, indicative of myeloid cell activation, with lower concentrations of IFN-α2, IL-12 and IFN-γ detected.

4.3.4 Myeloid accessory cell cytokine dependent NK cell activation.

Vaccination independent activation of less differentiated, cytokine-responsive NK cell subsets accompanied by high levels of myeloid cell-derived cytokine secretion, led us to hypothesise that the NK cell response to EbovGP is a function of indirect NK cell activation. To test this hypothesis, we compared IFN-γ, CD107a and CD25 expression among PBMCs and purified NK cells from healthy (non-vaccinated) control subjects (n=4) stimulated *in vitro* with EbovGP (Figure 5a-c). Expression of CD107a, IFN-γ and CD25 in the CD56^{bright} NK cell population (in which significant induction was measured) were determined by flow cytometry as before. IFN-γ (in 4 of 4 donors), CD107a (in 4 of 4 donors) and CD25 (in 3 of 4 donors) expression was markedly reduced in purified NK cells compared to PBMCs (Figure 5a-c) indicating that accessory cell-derived stimuli are required for optimal NK cell responses to EbovGP.

To determine the precise nature of the accessory cell-dependent stimuli that drive NK cell responses to EbovGP, we stimulated whole PBMCs from (non-vaccinated) control subjects with EbovGP in the presence of neutralising antibodies to IL-2, IL-12, IL-15, IL-18 and IFN-αβR2. The blockade of IL-18 significantly reduced the frequency and MFI of NK cell CD25 expression (Figure 5d, e, Supplementary Figure 4a), with blockade of IL-12 also significantly reducing CD25 expression within the CD56^{bright} NK cell subset (Figure 5f, g). CD107a expression was also impaired by IL-18 blockade, reflected in the CD56^{bright} and CD56^{dim}CD57$^-$ subsets (Figure 5h, Supplementary Figure 4a). There was no effect of IL-12 or IL-18 blockade on NK cell IFN-γ expression (Figure 5i, Supplementary Figure 4a). Conversely, neutralisation of IL-2 or IL-15,

or IFN-αβR2 blockade, had no significant effect on NK cell activation in any NK cell subset (not shown). In summary, these data suggest optimal NK cell CD25 and CD107a expression in response to EbovGP stimulation is dependent on myeloid cell-derived IL-18 and IL-12.

As both IL-12 and IL-18 were not amenable to detection by Luminex assay of cell culture supernatants, we next sought to measure these responses to EbovGP using high sensitivity ELISA for secreted IL-18 and flow cytometry for intracellular IL-12 (gating strategy shown in Supplementary Figure 5a). There was a significant increase in IL-18 measured in supernatant after 18 hours stimulation with EbovGP (median 47.6pg/ml, range 16.8-183.5pg/ml) (Figure 5j), which correlated significantly with increasing NK cell CD25 expression (Figure 5k). We were able to detect IL-12(p40)$^+$ cells by flow cytometry with significantly higher frequencies of IL-12(p40)$^+$ cells in CD14$^-$CD11c$^+$ myeloid DCs (mDC), total CD14$^-$ cells and CD14$^+$ monocytes compared to medium alone, and the highest frequencies being observed in mDC (median 0.19%) and monocyte (median 0.22%) populations (Figure 5l).

4.3.5 Regulation of NK cell IFN-γ production by EbovGP induced IL-10.

IL-10 is an essential immunoregulatory cytokine that is typically upregulated in response to inflammation (27). Having detected very high concentrations of IL-10 in supernatants of EbovGP-stimulated PBMCs (Figure 4a) we explored the relationship between IL-10 production and NK cell function. NK cell IFN-γ expression significantly negatively correlated with IL-10 secretion in 18 hour cultures in both baseline (r=-0.331, p=0.0218) (Figure 6a) and 21 days post-boost

PBMC (r=-0.324, p=0.0157; not shown) suggesting that IL-10 induced by EbovGP might restrict the NK cell IFN-γ response. Therefore, PBMC from (non-vaccinated) control subjects were cultured for 18 hours with EbovGP in the presence of a blocking monoclonal antibody to the IL-10 receptor (IL-10R) or the appropriate isotype control antibody. IL-10R blockade resulted in significantly higher frequencies of IFN-γ$^+$ (Figure 6b) and CD25$^+$ (Figure 6c) NK cells (and a significant increase in CD25 MFI; median 349.5 with IL-10R blockade; 110.5 with isotype control; p=0.0002; not shown) compared to isotype control treated cultures. CD107a was significantly enhanced by IL-10R blockade in the CD56^{dim}CD57$^+$ NK cell subset only (Supplementary Figure 4b), and IL-10R blockade particularly enhanced IFN-γ responses in CD56^{bright} and CD56^{dim}CD57$^-$ NK cell populations (Supplementary Figure 4b).

To determine the cellular source of the cytokines induced by EbovGP, PBMC were cultured with EbovGP for 18 hours, stained for intracellular IL-10, GM-CSF and TNF-α and analysed by flow cytometry (gating strategy shown in Supplementary Figure 5a). IL-10 was expressed predominantly in CD14$^+$ monocytes (median 6.0%) with little or no evidence of expression in B cells, mDCs, CD14$^-$, NK cells or T cells (Figure 6e). Back-gating confirmed that the majority of IL-10$^+$ cells were CD19$^-$CD14$^+$ monocytes (Figure 6f). GM-CSF expression was also essentially restricted to monocytes whereas mDCs and monocytes produced similar amounts of TNF-α (Supplementary Figure 5b, c). In summary, monocytes are the predominant source of inflammatory cytokines in response to EbovGP in primary peripheral blood and monocyte-derived IL-10 negatively regulates NK cell IFN-γ secretion and CD25 expression. This

immediate, robust IL-10 response helps to explain the lack of IFN-γ expression by NK cells in response to EbovGP both before and after vaccination (Figure 2).

4.3.6 EbovGP-induced NK cell activation is TLR-4 dependent.

EbovGP stimulates cytokine secretion in human monocytic cell lines and *in vitro* generated monocyte-derived DCs and macrophages in a TLR-4-dependent fashion (11, 13-15). TLR-4 is expressed at high levels on human peripheral blood monocytes, as well as other myeloid lineage cells including macrophages and granulocytes (28). We therefore assessed the effect of blocking TLR-4 on cytokine secretion (measured by Luminex) and NK cell activation (by flow cytometry) in response to EbovGP within PBMC from (non-vaccinated) control subjects. TLR-4 blockade significantly reduced secretion of IL-10 (0.3 fold-reduction; 7 of 7 donors) (Figure 7a), IL-1β, GM-CSF and IFN-γ but had no overall effect on IFN-α2 or TNF-α secretion (Figure 7b). Parallel effects were observed among NK cells where there was a partial, but significant, decrease in frequencies of IFN-γ⁺ (median 49.6% decrease frequency) and CD25⁺ (median 14.6% decrease frequency) CD56^bright NK cells in the presence of TLR-4 blocking antibodies (Figure 7c, d). Overall, these data indicate that NK cell activation by EbovGP is mediated, at least in part, via ligation of TLR-4 on primary human monocytes and the induction of accessory cytokines.

4.4 Discussion

In the 2014-2015 Ebola virus outbreak in West Africa, almost 30,000 cases of EVD were reported with more than 11,000 deaths (29). In 2019, Ebola virus continues to be a considerable global health concern, with outbreaks currently in the Democratic Republic of the Congo. Detailed understanding of the immune response to Ebola virus infection, efficacy and mechanisms of protection induced by Ebola virus vaccines would greatly assist in efforts of prevention and containment of future outbreaks. We analysed the effect of the prime-boost regimen Ad26.ZEBOV/MVA-BN-Filo vaccine on human NK cell phenotype *ex vivo* and primary human innate cell function in response to soluble Ebola GP *in vitro*. We demonstrate NK cell activation, proliferation and expansion of less differentiated NK cells up to 21 days after boost vaccination. We have found that CD14^{+} monocytes are key responders to Ebola virus GP, rapidly producing a range of inflammatory cytokines in a TLR-4-dependent manner. This led to NK cell activation (CD25 expression) and function (degranulation); dependent on myeloid cell-derived IL-12 and IL-18. However, this potentially crucial, rapid NK cell effector response is almost completely abrogated by the very high levels of IL-10 that are secreted as part of the acute myeloid cell response to EbovGP.

Activation and proliferation of NK cells after vaccination has been demonstrated with both inactivated and live attenuated vaccines. Jost *et al.* demonstrated upregulation of CD69 and CD25 and increased numbers of CD56^{bright} NK cells at day 4 post-influenza vaccination (25) and Marquardt *et al.* observed heightened NK cell Ki67 expression (peaking at day 10) after yellow fever vaccination (26). We have previously reported increased percentages and proliferation of

CD56^{bright} NK cells at day 3 and up to 4 weeks after influenza vaccination ((24) and unpublished data – Chapter 3 of this thesis). Here, we detected heightened CD56^{bright} NK cell proliferation up to 78 days after primary vaccination (21 days post-boost) and an increase in the proportion of CD56^{bright} NK cells from as early as day 15 post-primary vaccination until at least 21-days post booster vaccination. Further, increased expression of CD25 by NK cells post-vaccination may indicate the potential for T cell derived IL-2 to contribute to NK cell proliferation and activation (24, 30, 31). Of potential relevance for future development of this vaccine, these responses were more robust after Ad26 prime/MVA boost than after MVA prime/Ad26 boost. This work demonstrates - for the first time - activation of cytokine-responsive NK cells by a vectored, Ebola GP-expressing vaccine without the need for additional pathogen receptor-mediated stimulation by adjuvant or the whole organism, although these experiments cannot exclude effects of the viral vectors.

The pathogenesis of EVD is closely linked to the very high levels of pro-inflammatory cytokines induced by the infection (10, 16, 17). We show for the first time within primary human PBMC cultures, that Ebola GP stimulated the secretion of high levels of IL-1β, GM-CSF and TNF-α, both before and after vaccination. This inflammatory response was accompanied by an equally rapid and potent IL-10 response and somewhat lower levels of IL-12, IL-18 and IFN-α2. These data - in a highly relevant *ex vivo* system – corroborate previous observations from human cell lines and *in vitro* generated monocyte-derived DCs and macrophages (8, 11-13). The relatively low levels of important NK cell and T cell-activating cytokines together with the abundance of IL-10 suggest the generation of a tightly regulated cytokine environment within hours of exposure

to soluble EbovGP *in vitro*. Rapid production of IL-10 in response to a potent pro-inflammatory stimulus is a well-described feature of the human homeostatic response; in preventing a life-threatening cytokine storm, this can also influence the emerging adaptive response (27). Indeed, pro and anti-inflammatory cytokines both indirectly correlate with survival after EVD indicating that IL-10 itself, although associated with anti-inflammatory properties does not predict protection from disease (10).

CD14$^+$ monocytes were the main source of both inflammatory (GM-CSF, TNF-α and IL-12) and anti-inflammatory (IL-10) cytokines within hours of EbovGP stimulation. Both types of monocyte response and the downstream NK cell response were TLR-4-dependent confirming prior studies that the Ebola virus GP is recognised by TLR-4 on the surface of monocytes, inducing inflammatory cytokine secretion (11, 13, 15, 32). Interestingly, cytokine responses to EbovGP were lower in cells collected after Ad26 prime/MVA boost vaccination regimen than in baseline (pre-vaccination) cells. We wondered whether this might be due to neutralisation of EbovGP by vaccine-induced anti-GP antibodies, but cytokine responses did not differ significantly in control cultures in the presence of pooled pre or post-boost vaccination serum (data not shown) suggesting that vaccine induced antibody was not responsible for this difference. Moreover, the Ad26 prime/MVA boost regimen induced lower anti-GP antibody titres 21 days post-boost than did MVA prime/Ad26 boost, suggesting that monocyte cytokine responses are not related to antibody titre (4). This observation thus merits further investigation, perhaps indicating vaccine induced intrinsic control of Ebola GP-induced inflammatory mediators.

EbovGP induces cytokine-dependent activation of peripheral blood NK cells *in vitro*, independent of prior Ad26.ZEBOV/MVA-BN-Filo vaccination. This innate response, which is particularly enriched in less differentiated NK cell subsets, is consistent with the proliferation and activation of the least differentiated, CD56^{bright} NK cells after vaccination itself (measured *ex vivo*). This suggests that, as seen *in vitro*, Ebola GP within the vaccine potentially stimulates innate, cytokine-dependent NK cell activation *in vivo*. Finally, *in vitro*, IL-18 and to a lesser extent, IL-12 from myeloid accessory cells were required for optimal NK cell degranulation and CD25 upregulation. This is the first demonstration of indirect, IL-18 and IL-12 dependent NK cell effector function in response to Ebola virus GP in human PBMC.

It is well established that enhanced NK cell responses after vaccination, are mediated in part by IL-2 from antigen-specific T cells and vaccine induced antibody (24, 26, 30, 33-35). Moreover, incubation of NK cells with antigen-antibody complexes cross-links and downregulates CD16 on the surface of NK cells (34, 36). However, in this study, despite evidence of moderate induction of IL-2^{+}IFN-γ^{+}TNF-α^{+} triple positive T cells and anti-GP antibodies by Ad26.ZEBOV/MVA-BN-Filo vaccination (4), there was no enhancement of the NK cell response, or downregulation of CD16 in response to EbovGP, post-vaccination compared to baseline (that is in the presence of 1% immune sera).

This innate NK cell activation by EbovGP, combined with an apparent lack of enhancement of NK cell responses by the adaptive immune response, is in complete contrast to previous observations with yellow fever, BCG and influenza vaccination (24, 26, 35) and might plausibly be linked to the monocyte IL-10

response. In support of this hypothesis, a system-wide analysis of the immune response to the rVSV-ZEBOV Ebola vaccine suggested that vaccination-induced inflammatory monocytes negatively regulated the immune response (19). Additionally, IL-10 blockade can restore antigen-specific T cell-derived IL-2-dependent activation of NK cells in other viral infection models (37, 38). NK cell responses to Ebola GP, including IFN-γ secretion, were augmented by low dose IL-15 co-stimulation (data not shown), previously shown to boost accessory cell IL-12 and IFN-α secretion in response to influenza virus *in vitro* (39). This could suggest that boosting certain NK cell activating pro-inflammatory cytokines could override the effects of high concentrations of IL-10.

In summary, for the first time, we have characterised the NK cell response to a novel Ebola virus GP-encoding, viral-vectored vaccine. We find that the robust TLR-4-dependent, monocyte-derived, innate cytokine response to Ebola GP both stimulates and moderates the NK cell effector response. This study contributes to the knowledge of Ebola vaccine induced immunity and the effect of Ebola GP on the innate immune system, aiding the continued development of novel Ebola vaccines.

4.5 Materials and Methods

4.5.1 Study participants and samples

Cryopreserved PBMCs (with corresponding serum samples) were obtained from participants enrolled in the EBL1001 single-centre, randomised, placebo-controlled, observer blind trial conducted in Oxford, U.K. as described (ClinicalTrials.gov Identifier: NCT02313077) (4). Eligible, healthy adults (n=72), aged 18 to 50 years, were randomised into four groups, with a fifth group subsequently added by a protocol amendment, to receive the Ad26.ZEBOV/MVA-BN-Filo vaccine according to one of five vaccination schedules. The vaccine comprises monovalent Ad26.ZEBOV expressing the GP of the Ebola Zaire virus (Mayinga variant) (Crucell Holland N.V. (now Janssen Vaccines & Prevention B.V.), The Netherlands) and multivalent MVA-BN-Filo expressing the GP of the Sudan and Zaire Ebola viruses and Marburg virus together with Tai Forest virus nucleoprotein (Bavarian Nordic, Denmark). Groups 1 and 2 received MVA-BN-Filo as prime on day 1 and Ad26.ZEBOV as boost on either day 29 or 57 respectively; groups 3, 4 and 5 received Ad26.ZEBOV as prime and MVA-BN-Filo as boost on days 29, 57 or 15 respectively.

Samples were collected immediately prior to primary vaccination (baseline, visit 0), post-prime (day 29, 57 or 15 depending on group; visit 1) and 21 days post-boost (day 50, 78 or 36 depending on group; visit 2) (Table 1). HCMV serology was conducted on the baseline serum sample of each donor by HCMV IgG ELISA (BioKit, Barcelona, Spain): 26 of 72 volunteers (36%) were HCMV seropositive, 44 were HCMV seronegative and two were indeterminate. Additional non-vaccinated, healthy, adult volunteers (n=16) were recruited for subsequent *in vitro*

experiments from among staff and students at the London School of Hygiene and Tropical Medicine (LSHTM) using an anonymised volunteer database.

	Vaccine schedule	PBMC samples received for this study		
		Baseline (Visit 0)	Post-prime (Visit 1)	21d post-boost (Visit 2)
Group 1 (n=15)	MVA-Ad26	Day 1	Day 29	Day 50
Group 2 (n=15)	MVA-Ad26	Day 1	Day 57	Day 78
Group 3 (n=14)	Ad26-MVA	Day 1	Day 29	Day 50
Group 4 (n=14)	Ad26-MVA	Day 1	Day 57	Day 78
Group 5 (n=14)	Ad26-MVA	Day 1	Day 15	Day 36

Table 1: Vaccination regimen and PBMC samples used in this study.

MVA, MVA-BN-Filo; Ad26, Ad26.ZEBOV.

4.5.2 *In vitro* cellular assays

Cryopreserved PBMCs were thawed, washed in RPMI 1640 supplemented with 100U/ml penicillin/streptomycin and 20mM L-glutamine (Gibco, ThermoFisher) and rested for 2 hours. Fresh PBMC were isolated from heparinised whole blood using Histopaque 1077 (Sigma-Aldrich, Gillingham, U.K.) gradient centrifugation. All cells were counted using Fastread counting slides (Immune Systems); the average cell yield after thaw was 5.8×10^6 per vial. Trial PBMC were stained immediately *ex vivo* or cultured in 96-well round-bottom plates in RPMI 1640 supplemented as above and with 1% autologous (visit 0, 1 or 2) serum and 10µg/ml purified recombinant Ebola virus GP (EbovGP), Mayinga variant, prepared in Hek293F cells (Crucell Holland B.V.) for 8 and 18 hours at 37°C.

For additional 18 hour experiments, fresh PBMC from non-trial donors were stimulated with 10µg/ml EbovGP in RPMI supplemented as above and with 5% FCS. The following blocking antibodies or isotype control antibodies were used, all at 3µg/ml; anti-IL-2 (Becton Dickinson (BD) Biosciences, Oxford, U.K.), anti-IL-10R (Biolegend), rat IgG2a isotype control (eBiosciences, ThermoFisher), anti-IL-12 (BD Biosciences), anti-IL-15 (eBiosciences), anti-IL-18 (MBL, U.S.A), mouse IgG1 isotype control (eBiosciences). Anti-IFN-αβR2 (Merck Millipore, Watford, U.K.) and mouse IgG2a isotype control (eBiosciences) were used at a final concentration of 1µg/ml. *In vitro* blockade of TLR-4 was performed in the presence of 5µg/ml anti-TLR-4 rabbit polyclonal anti-sera or isotype matched control reagent with irrelevant specificity (Invivogen, UK).

To determine accessory cell dependency, NK cells were purified using NK Cell Isolation Kits (Miltenyi Biotec, Germany) and magnetic bead separation (MACS). Purified NK cells were on average 92.8% pure and were cultured as above for 18 hours in 5% FCS. GolgiPlug (Brefeldin A; 1/1000 final concentration; BD Biosciences) and GolgiStop (Monensin; 1/1500 concentration; BD Biosciences) were added to all *in vitro* cultures for the final 3 hours of culture. Cells were then stained with fluorophore labelled antibodies for flow cytometry and culture supernatants were collected and stored at -80°C for cytokine analysis by Luminex/ELISA.

4.5.3 Flow cytometry

Cells were stained for surface markers including a viability marker (Fixable Viability Stain 700; BD Biosciences) in FACS buffer (PBS, 0.5% FCS, 0.05% sodium azide and 2mM EDTA) for 30 minutes in 96-well round bottom plates after blocking Fc receptors for 5 minutes with Fc Receptor (FcR) Blocking Reagent (Miltenyi Biotec). Cells were then washed in FACS buffer, fixed and permeabilised using Cytofix/Cytoperm Kit (BD Biosciences) or Foxp3/Transcription Factor Fixation/Permeabilisation Kit (eBiosciences) according to the manufacturer's instructions. Cells were then stained for intracellular markers with FcR blocking for 20 minutes and washed again. Finally cells were resuspended in FACS buffer and analysed using a BD LSRII flow cytometer. Cells were acquired using FACSDiva software and data were analysed using FlowJo V10 (Tree Star, Oregon, U.S.A). FACS gates were set using unstimulated cells or FMO controls. Samples with less than 100 NK cell events were excluded from the analysis (<4% of samples evenly distributed across all groups).

The following fluorophore labelled antibodies were used: anti-CD3-V500 (clone UCHT1) (BD Biosciences), anti-CD56-BV605 (clone HCD56), anti-IFN-γ-BV785 (clone 4S.B3), anti-IFN-γ-APC (clone 4S.B3), anti-CD25-BV785 (clone BC96), anti-CD11c-BV785 (clone 3.9), anti-CD14-AF700 (clone 63D3), anti-GM-CSF-PE-Dazzle (clone BVD2-21C11), anti-TNF-α-FITC (clone MAb11), anti-IL-10-PE (clone JES3-9D7) (all Biolegend, London, U.K.). Anti-CD16-APC (clone CB16), anti-CD25-PerCPCy5.5 (clone BC96), anti-CD57-e450 (clone TB01), Ki67-PerCP-eFluor710 (clone 20Raj1), anti-CD19-PECy7 (clone HIB19), anti-IL-12-

eFlour660 (clone C8.6) (all eBiosciences), anti-NKG2A-PE-Vio770 (clone REA110) (Miltenyi Biotec), anti-NKG2C-PE (clone 134591) (R&D systems). Anti-CD107a-FITC (clone H4A3) (BD Biosciences) was added to the culture at 2μl per 100μl for the whole culture period.

4.5.4 Luminex and IL-18 ELISA

Concentrations of GM-CSF, IFN-α2, IFN-γ, TNF-α, IP-10, IL-1β, IL-10, IL-12p70, IL-15 in cell culture supernatants were determined by Luminex technology (Merck Millipore) using the xPONENT 4.1 software for data acquisition. The concentration of IL-18 was determined by ELISA (R&D Systems).

4.5.5 Statistics

Statistical analysis was performed using GraphPad Prism version 7.04 (GraphPad, California, U.S.A.). Functional responses were compared using Wilcoxon signed-rank test or one-way ANOVA Friedman test with Dunn's correction for multiple comparisons and correlations were made using linear regression or spearman's correlation analysis. Significance levels are assigned as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$ for all tests.

4.5.6 Study approval

Written informed consent was received from all participants prior to inclusion in the study. The trial protocol and study documents were approved by the National Research Ethics Service (reference number 14/SC/1408) and the LSHTM Research Ethics Committee (reference number 14383).

4.6 Acknowledgements

4.7 Figures

Figure 1: Robust NK cell responses to Ad26.ZEBOV/MVA-BN-Filo vaccination *ex vivo/in vivo*.

NK cell phenotype at baseline (visit 0), visit 1 (day 29, 57 or 15 post-prime) and visit 2 (21 days post-boost) was analysed *ex vivo* by flow cytometry (gating strategy is shown in Supplementary Figure 1), n=72. Frequencies of CD56bright and CD56dim (a), CD56bright Ki67+, CD56dimCD57- Ki67+ and CD56dimCD57+ Ki67+ (b), NKG2A+ and NKG2C+ (c), CD56bright CD25+ and CD56dim CD25+ NK cells (d) were determined. The relationship between total NK cell CD25 and Ki67 expression at 21 days post-boost (e) was also determined by Spearman's coefficient. Graphs show box and whisker plots with median, interquartile range (IQR) (box) and 10th-90th percentile (whiskers). Comparisons across vaccination visits were performed using one-way ANOVA with Dunn's correction for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001.
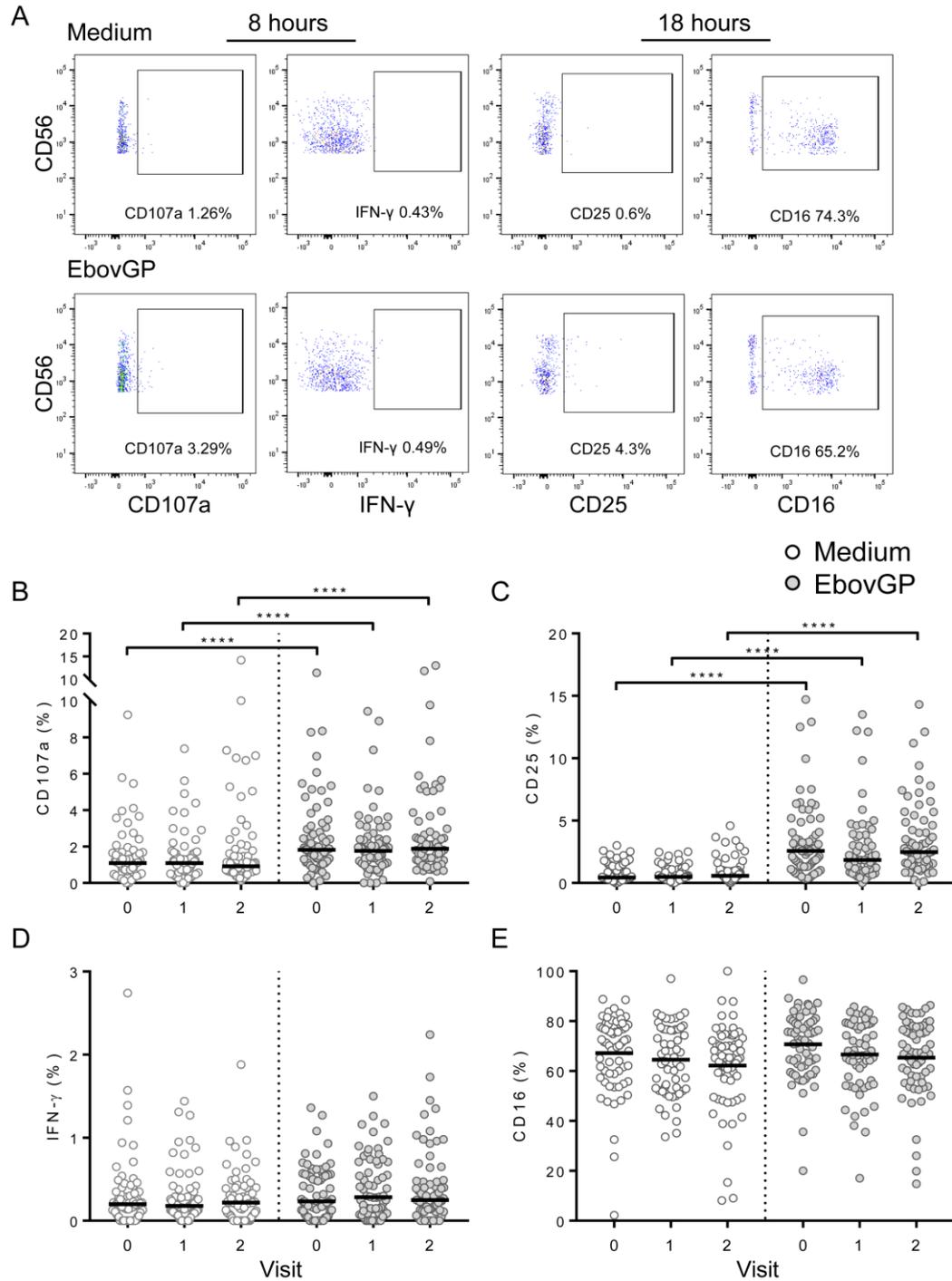
Figure 2: NK cell CD107a and CD25, but not IFN-γ expression upregulation in response to EbovGP stimulation *in vitro*.

Whole PBMC from baseline (visit 0), visit 1 (day 29, 57 or 15 post-prime) and visit 2 (21 days post-boost) were stimulated with EbovGP or left unstimulated (medium) for 8 and 18 hours, n=72. Cells were stained for NK cell activation markers and analysed by flow cytometry. Frequencies of CD107a and IFN-γ, measured at 8 hours or CD25 and CD16 measured at 18 hours were gated using medium alone controls, plots shown from one representative donor (a). Graphs show NK

cell CD107a (b), IFN-γ (c), CD25 (d) and CD16 (d) expression as one point per donor with a line representing the median. Comparisons across vaccination visits were performed using one-way ANOVA with Dunn's correction for multiple comparisons and between conditions by Wilcoxon signed-rank test. ****$p < 0.0001$.
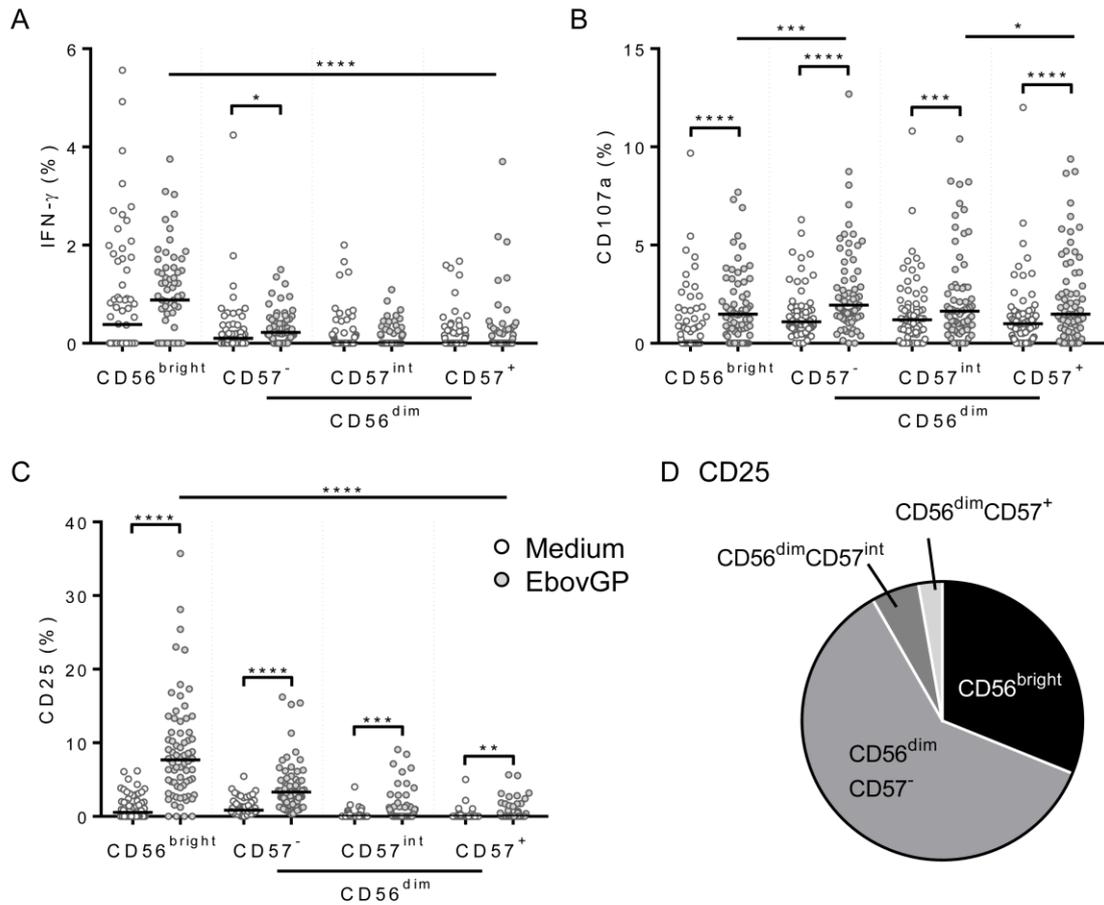
Figure 3: Less differentiated NK cells respond strongly to EbovGP stimulation *in vitro*.

NK cell IFN-γ (a) and CD107a (b), measured at 8 hours and CD25 (c), measured at 18 hours in response to medium alone and EbovGP in baseline (visit 0) samples only was analysed according to NK cell differentiation subset determined by CD56 and CD57 expression (CD56^{bright}, CD56^{dim}CD57^{-}, CD56dimCD57$^{intermediate\ (int)}$ and CD56^{dim}CD57^{+}), n=72. The proportion of CD25^{+} NK cell events per subset determined by back-gating is also shown as a pie chart (d). Graphs show one point per donor with a line representing the median. Comparisons across NK cell subsets were performed using one-way ANOVA with Dunn's correction for multiple comparisons and between conditions by Wilcoxon signed-rank test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
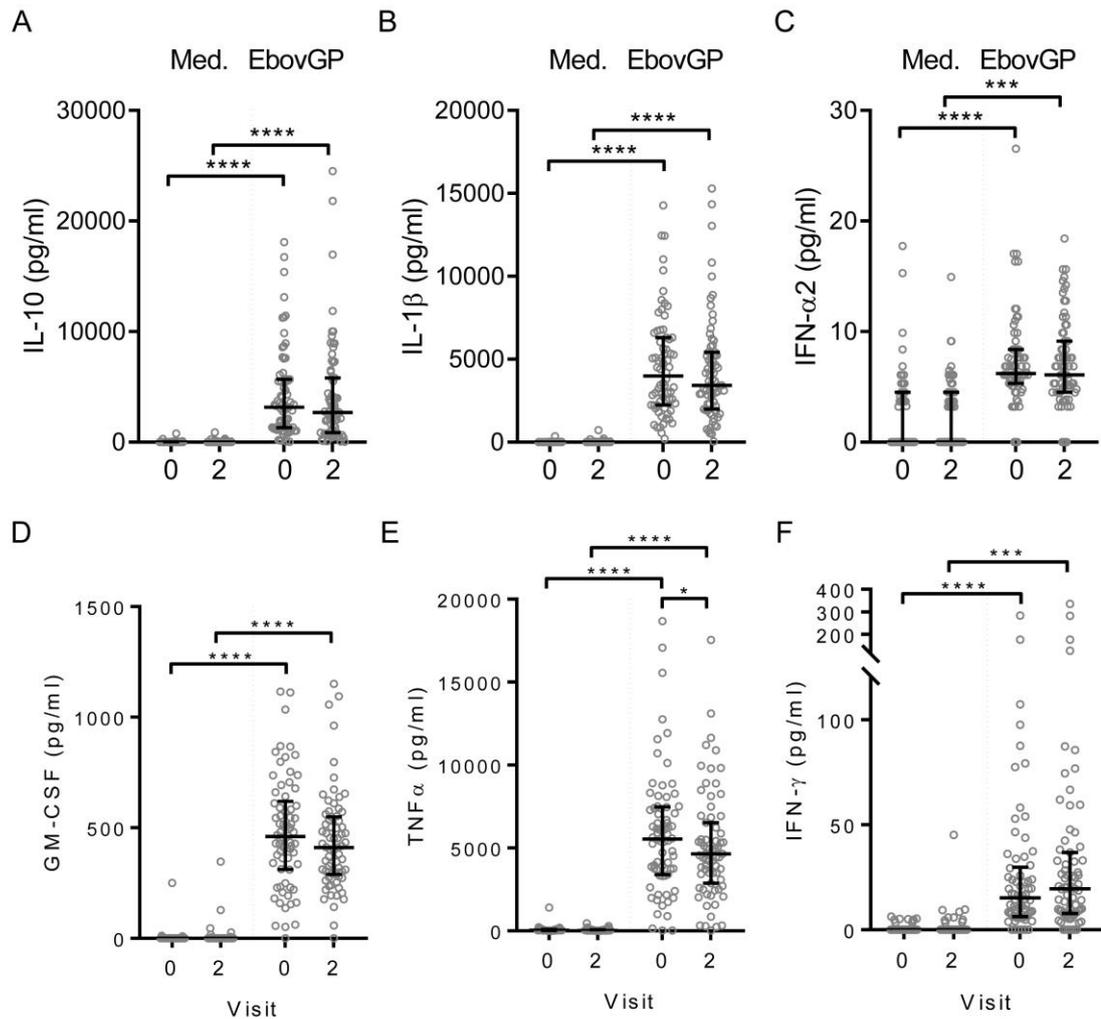
Figure 4: High concentrations of inflammatory cytokines induced by EbovGP stimulation *in vitro*.

Supernatants were collected from baseline (visit 0) and post-boost (visit 2) PBMC after 18 hours stimulation with EbovGP and concentrations of IL-10 (a), IL-1β (b), IFN-α2 (c), GM-CSF (d), TNF-α (e) and IFN-γ (f) were determined by Luminex, n=72. Graphs show one point per donor with the median and IQR. Comparisons were performed using one-way ANOVA with Dunn's correction for multiple comparisons. *p < 0.05, ***p < 0.001, ****p < 0.0001.
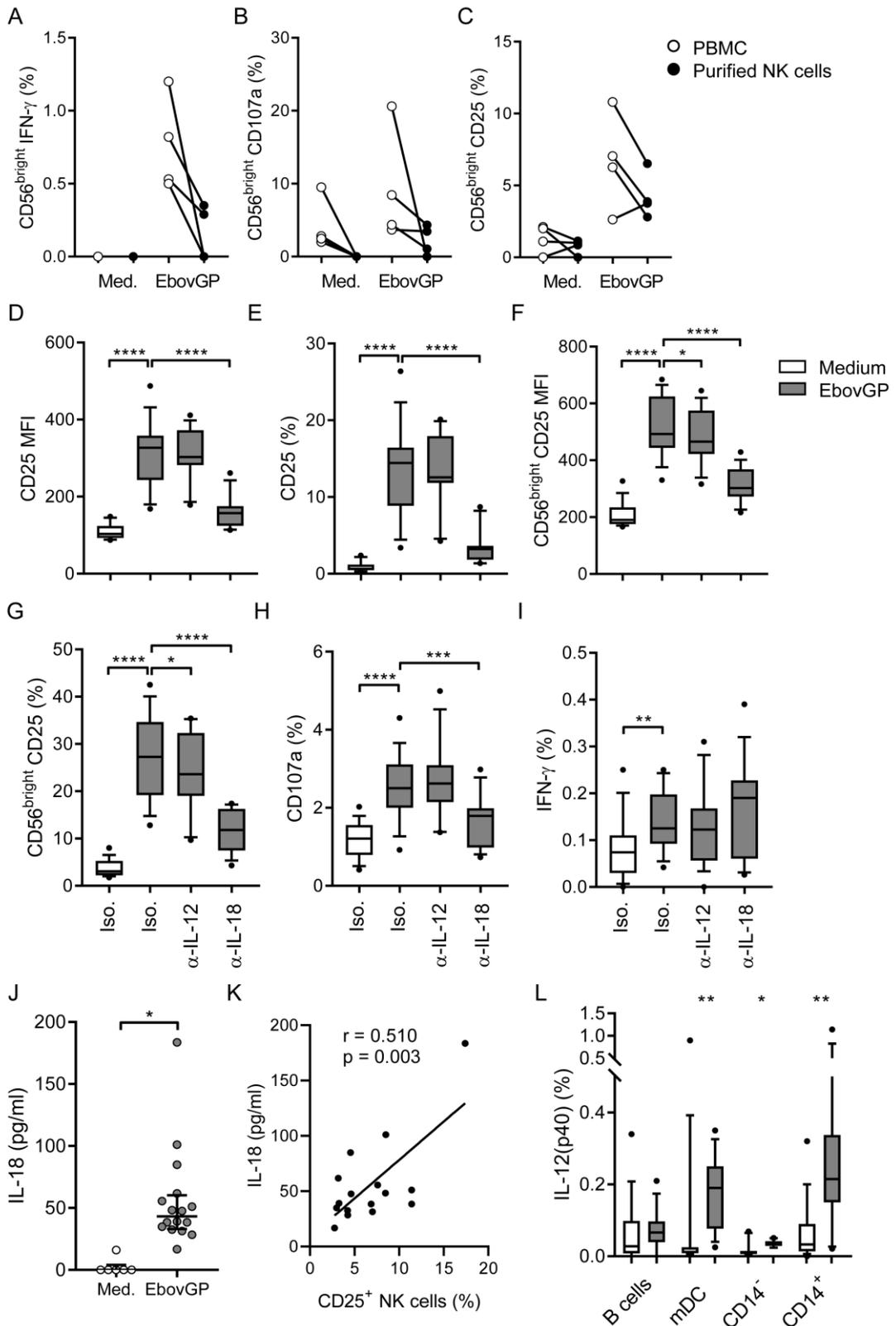
Figure 5: Myeloid accessory cell cytokine-dependent NK cell activation.

Non-vaccinated control PBMC or purified NK cells were stimulated with EbovGP or left unstimulated (medium). PBMC were also left unstimulated or stimulated with EbovGP in the

presence of blocking antibodies against IL-12 and IL-18 or appropriate isotype control (Iso.), NK cell function was analysed by flow cytometry, n=16. Graphs show CD56^{bright} IFN-γ, CD107a and CD25 expression (a-c), total NK cell CD25 percentage or MFI (d, e) or CD56^{bright} CD25 percentage or MFI (f, g) and total NK cell CD107a and IFN-γ expression (h, i). IL-18 in culture supernatant and intracellular IL-12 of unstimulated and EbovGP stimulated PBMC were determined by ELISA and flow cytometry respectively, the relationship between IL-18 and total NK cell CD25 expression was determined by Spearman's coefficient (j-l). IL-12(p40)$^+$ B cells (CD19$^+$), myeloid DC (mDC; CD19$^-$CD14$^-$CD11c$^+$), total CD14$^-$ and total CD14$^+$ cells were gated as per gating strategy in Supplementary Figure 5a. Graphs show box and whisker plots with median, IQR (box) and 10th-90th percentile (whiskers) or one point per donor. Comparisons were performed using Wilcoxon signed-rank test and correlations were determined using Spearman's correlation. *p <0 .05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
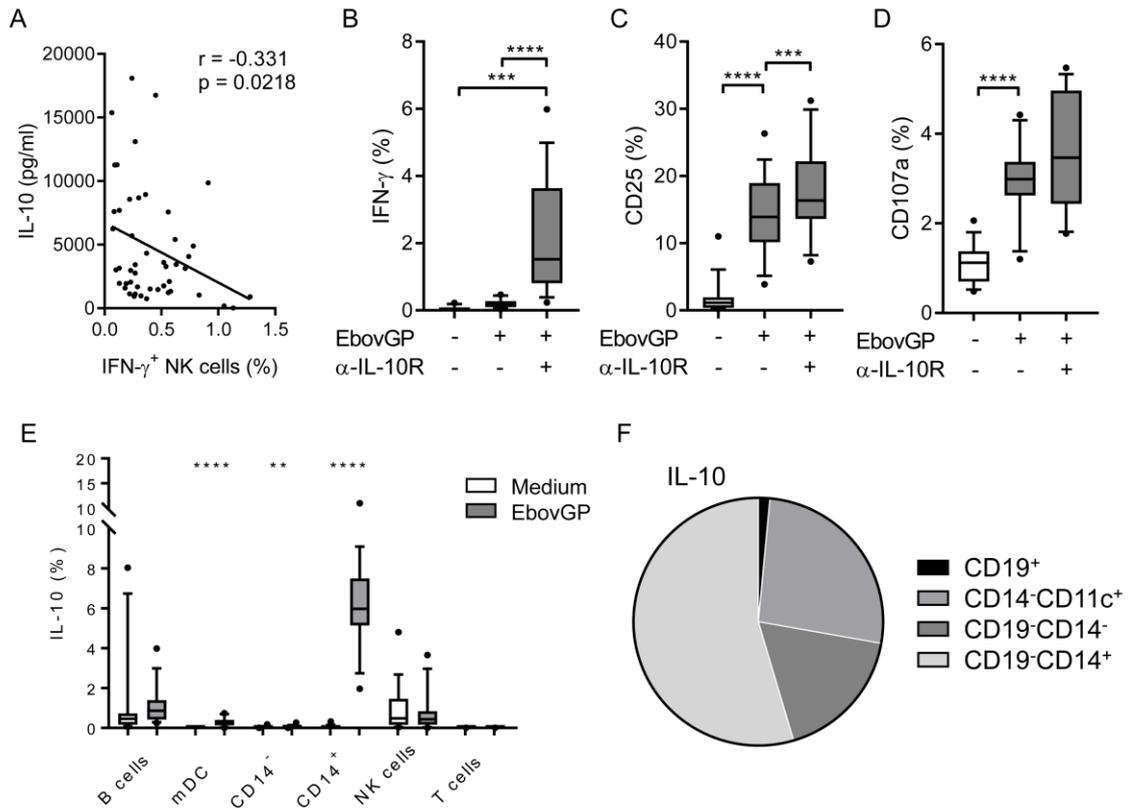
Figure 6: Regulation of NK cell IFN-γ production by EbovGP induced IL-10.

The correlation between NK cell IFN-γ secretion determined by intracellular staining and IL-10 secretion measured by Luminex in response to EbovGP (in baseline trial samples) was determined by Spearman's coefficient, n=72 (a). Non-vaccinated control PBMC were stimulated in the presence of blocking antibodies against IL-10R or isotype control, n=16 (b-f). Total NK cell IFN-γ (b), CD107a (c) and CD25 (d) expression was determined. Intracellular IL-10 was also measured by flow cytometry (gating strategy as per Supplementary Figure 5a) in B cells (CD19$^+$), myeloid DC (mDC; CD19$^-$CD14$^-$CD11c$^+$), total CD14$^-$ and total CD14$^+$ cells, NK cells (CD3$^-$CD56$^+$) and T cells (CD3$^+$) (e). The proportion of IL-10$^+$ events per cell type determined by back-gating is also shown as a pie chart (f). Graphs show box and whisker plots with median, IQR (box) and 10th-90th percentile (whiskers). Comparisons were performed using Wilcoxon signed-rank test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
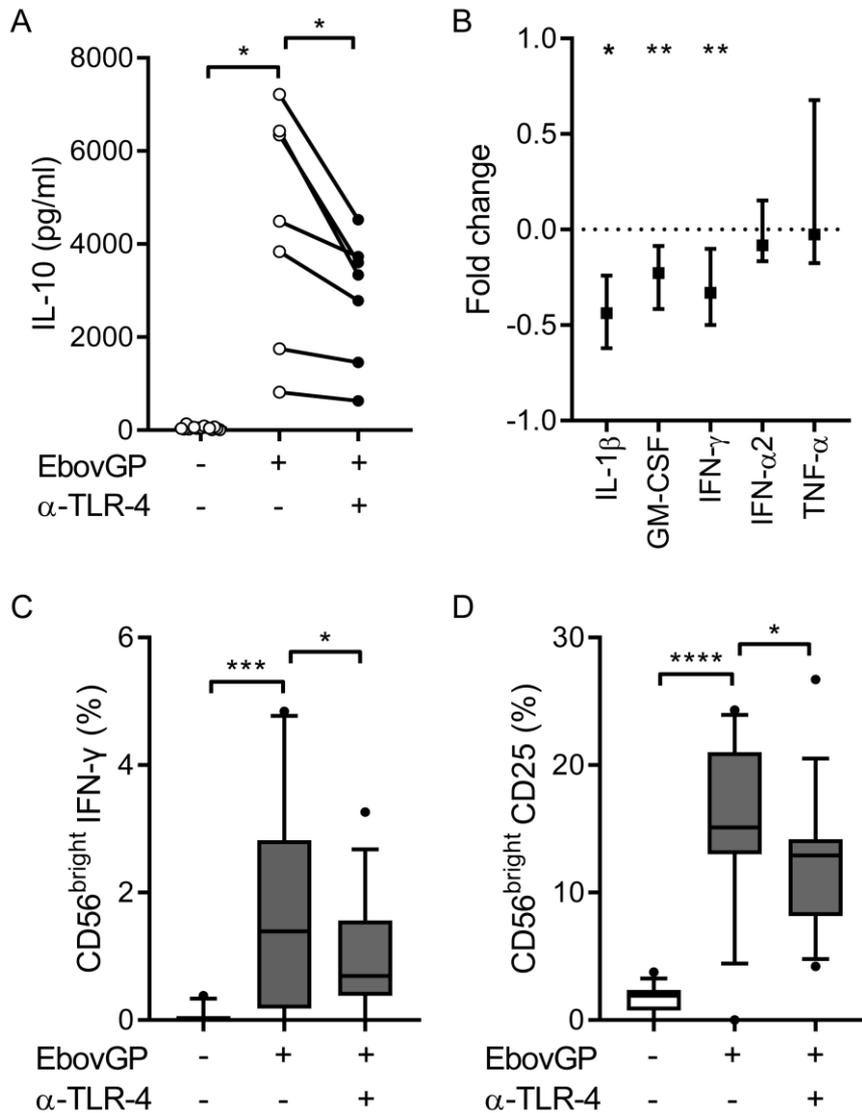
Figure 7: EbovGP induced NK cell activation is dependent on interaction with TLR-4.

Non-vaccinated control PBMC were stimulated in the presence of blocking antibodies against TLR-4 or isotype control, n=16. Supernatants were collected and concentrations of IL-10, IL-1β, GM-CSF, IFN-γ, IFN-α2 and TNF-α were measured by Luminex. Graphs show IL-10 concentration as one dot per donor (showing 7 of 16 with values below ELISA cut-off value of 10,000pg/ml) (a) and IL-1β, GM-CSF, IFN-γ, IFN-α2 and TNF-α as fold change between isotype control and TLR-4 blockade (b). Expression of CD56^{bright} NK cell IFN-γ (c) CD25 (d) were determined after 18 hours by flow cytometry. Graphs show median with IQR or box and whisker plots with median, IQR (box) and 10th-90th percentile (whiskers). Comparisons between conditions were performed using Wilcoxon signed-rank test. *p < 0.05, ***p < 0.001, ****p < 0.0001.

4.8 Supplementary Figures
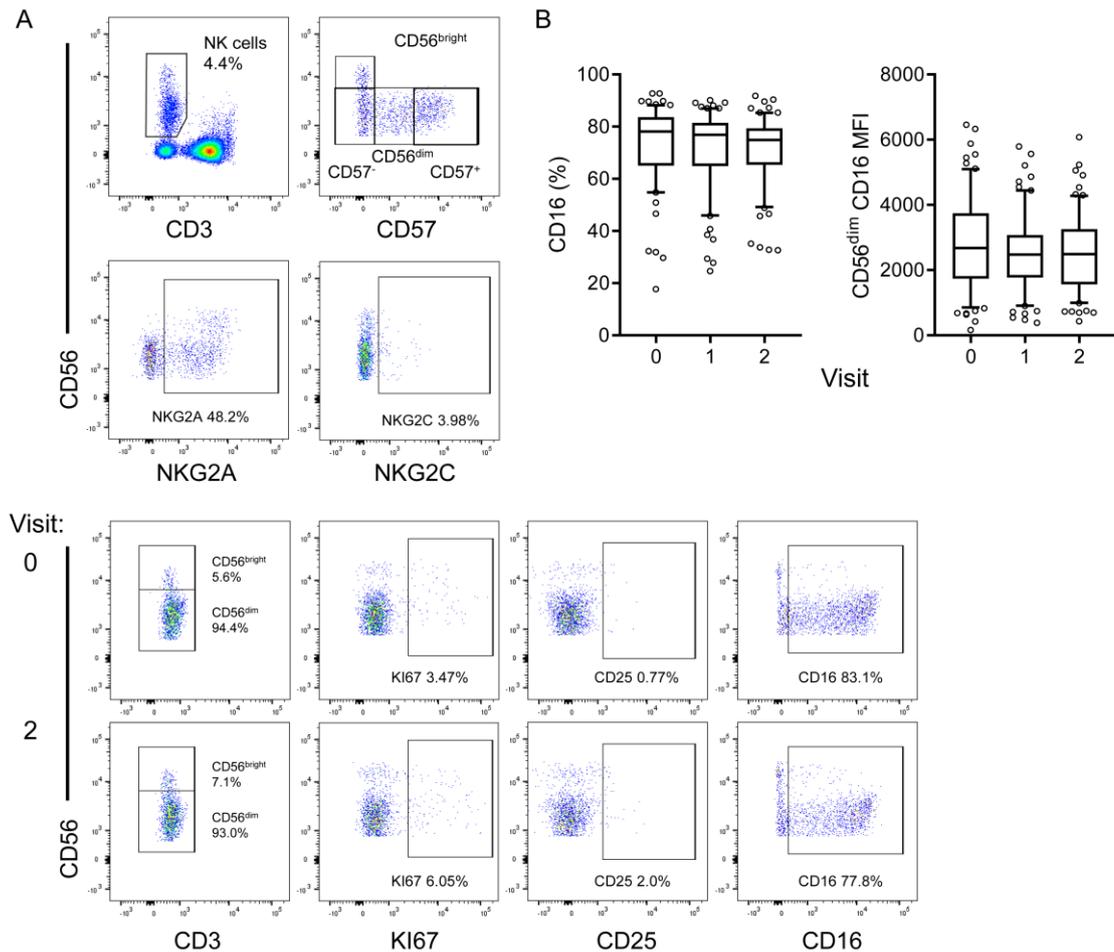
Supplementary Figure 1: Flow cytometric gating strategy for *ex vivo* NK cell phenotype analysis.

Plots demonstrate strategy for NK cell subset, NKG2A, NKG2C, Ki67, CD25 and CD16 gating in one representative donor (a). NK cell CD16 expression (percentage and MFI) at baseline (visit 0), visit 1 (day 29, 57 or 15 post-prime) and visit 2 (21 days post-boost) after vaccination with Ad26.ZEBOV/MVA-BN-Filo was analysed (b). Graphs show box and whisker plots with median, interquartile range (box) and 10th-90th percentile (whiskers). Comparisons across vaccination visits were performed using one-way ANOVA with Dunn's correction for multiple comparisons.
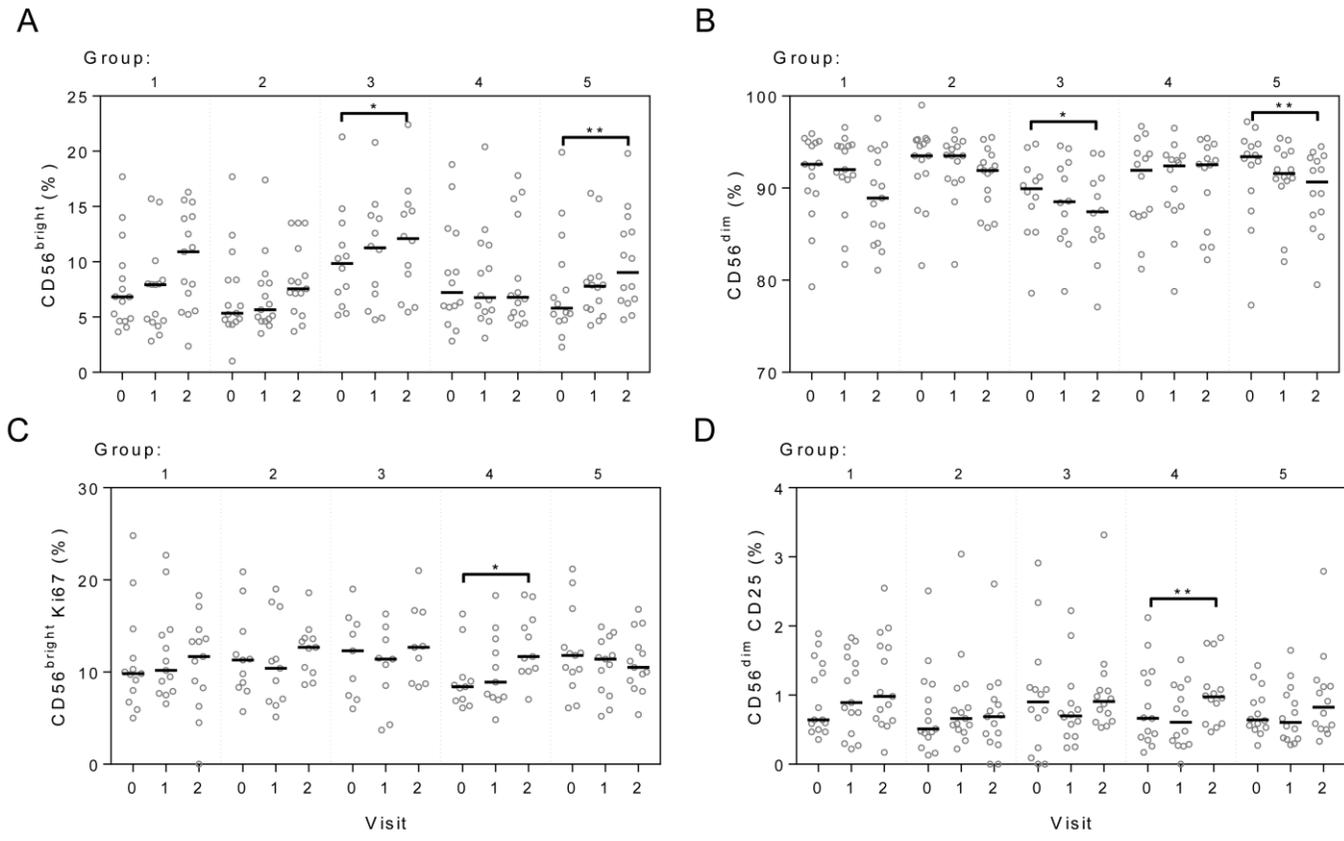
Group 1: MVA-Ad; boost d29
Group 2: MVA-Ad; boost d57
Group 3: Ad-MVA; boost d29
Group 4: Ad-MVA; boost d57
Group 5: Ad-MVA; boost d15

Supplementary Figure 2: NK cell phenotype *ex vivo* according to vaccination regimen group.

NK cell phenotype at baseline (visit 0), visit 1 (day 29, 57 or 15 post-prime) and visit 2 (21 days post-boost) after vaccination with Ad26.ZEBOV/MVA-BN-Filo was analysed *ex vivo* by flow cytometry. Data was analysed according to vaccine regimen group (1-5) as indicated, n=15 (groups 1-2), n=14 (groups 3-5). Frequencies of CD56bright (a), CD56dim (b), CD56bright Ki67+ (c) and CD56dim CD25+ (d) NK cells were determined. Graphs show one point per donor with a line representing the median. Comparisons across vaccination visits were performed using one-way ANOVA with Dunn's correction for multiple comparisons. *p < 0.05, **p < 0.01.

Supplementary Figure 3: Cytokine concentrations according to vaccination regimen group.

Group 1: MVA-Ad; boost d29
Group 2: MVA-Ad; boost d57
Group 3: Ad-MVA; boost d29
Group 4: Ad-MVA; boost d57
Group 5: Ad-MVA; boost d15

Supernatants were collected from baseline (visit 0) and post-boost (visit 2) PBMC after 18 hours stimulation with EbovGP *in vitro* and concentrations of IL-10 (a), IL-1β (b), IFN-α2 (c), GM-CSF (d), TNF-α (e) and IFN-γ (f) were determined by Luminex. Data was analysed according to vaccine regimen group (1-5) as indicated, n=15 (groups 1-2), n=14 (groups 3-5). Graphs show one point per donor with a line representing the median. Comparisons were performed using Wilcoxon signed-rank test. *p < 0.05.
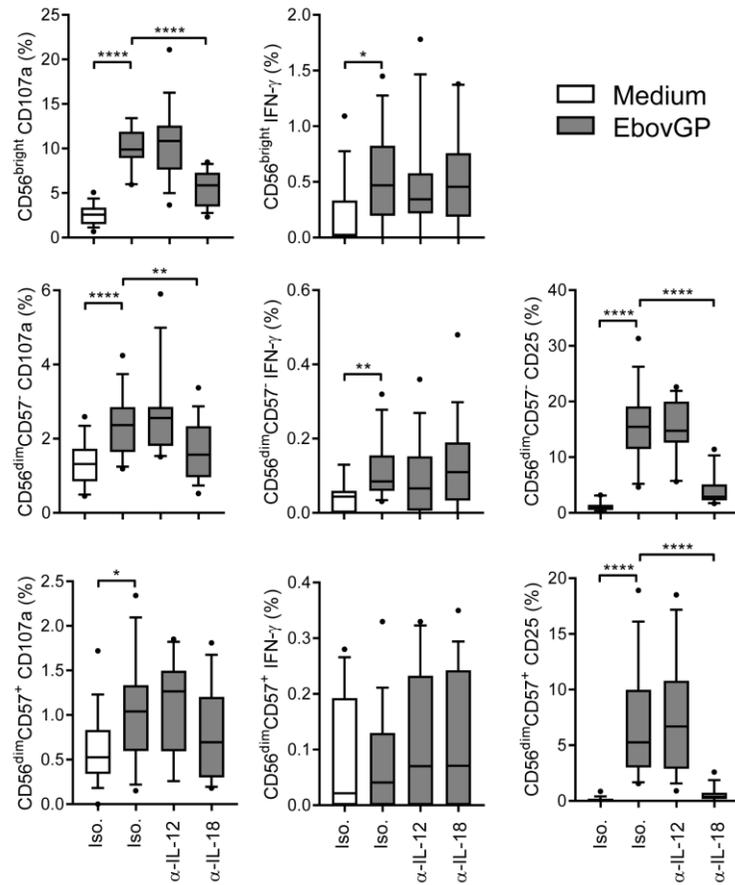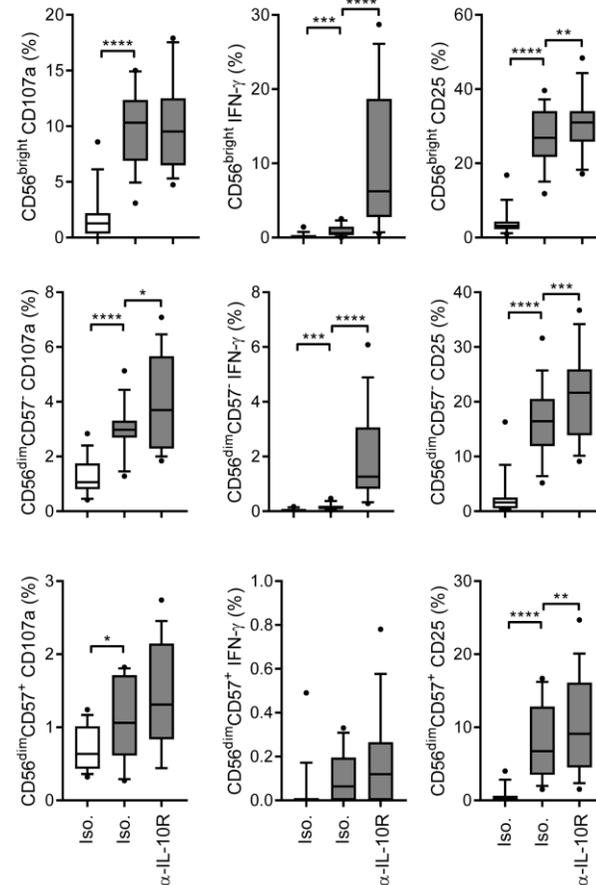
Supplementary Figure 4: NK cell function in response to EbovGP and blocking antibodies according to NK cell subset.

Non-vaccinated control PBMC were stimulated with EbovGP in the presence of blocking antibodies against IL-12, IL-18 and IL-10R or appropriate isotype control, n=16. CD56^{bright}, CD56^{dim}CD57$^-$ and CD56^{dim}CD57$^+$ NK cell CD107a, IFN-γ and CD25 expression was determined by flow cytometry. Graphs show box and whisker plots with median, interquartile range (box) and 10th-90th percentile (whiskers). Comparisons between conditions were performed using Wilcoxon signed-rank test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

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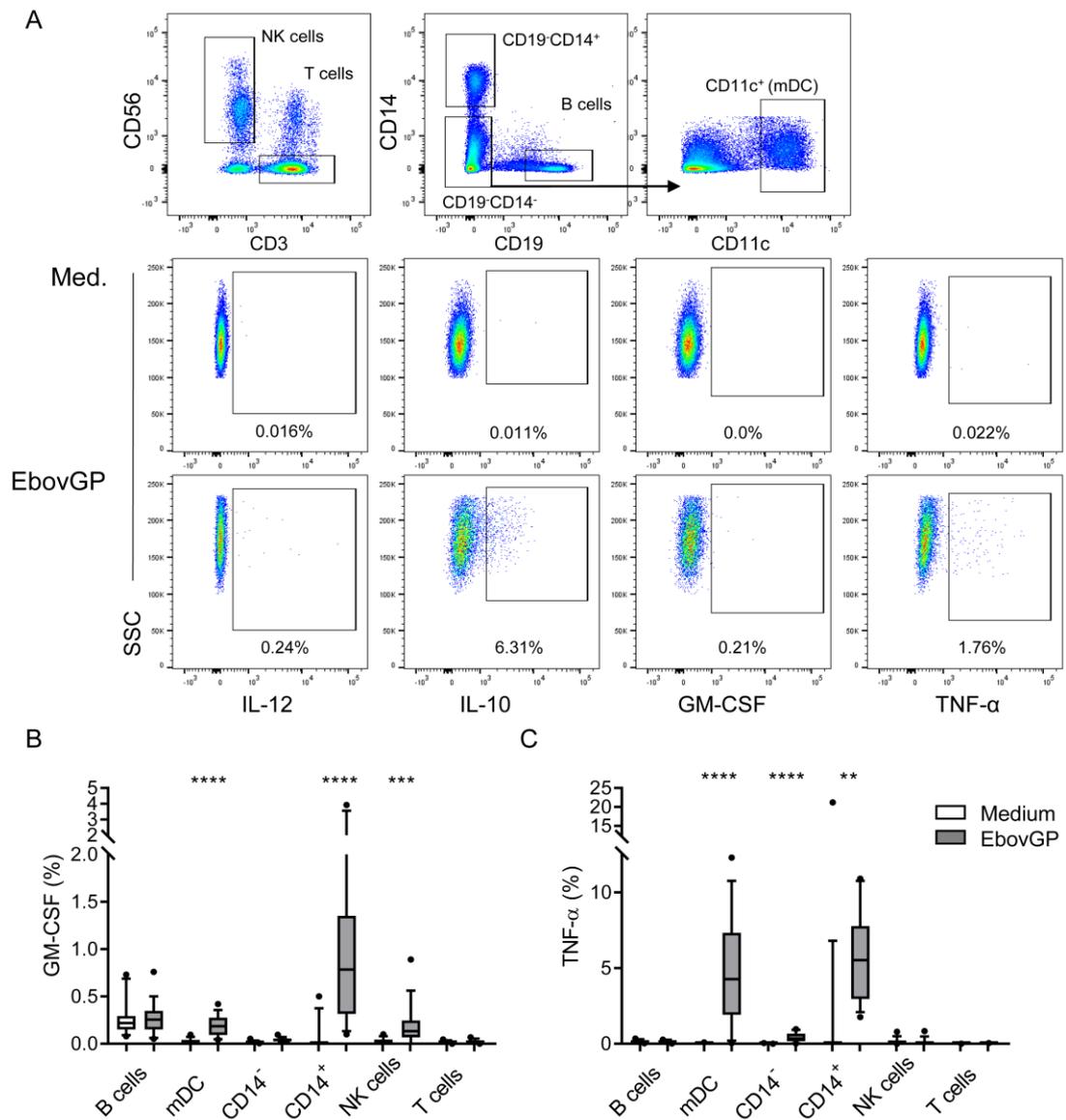
Supplementary Figure 5: Intracellular cytokine staining gating strategy and GM-CSF and TNF-α expression.

Intracellular IL-12, IL-10, GM-CSF and TNF-α expression in response to EbovGP stimulation of non-vaccinated control PBMC was measured in B cells (CD19+), myeloid DC (mDC; CD19-CD14-CD11c+), total CD14- and total CD14+ cells, NK cells (CD3-CD56+) and T cells (CD3+). Plots show gating strategy from one representative donor, and gates for intracellular cytokines in CD14+ monocytes. Graphs show box and whisker plots with median, interquartile range (box) and 10th-90th percentile (whiskers). Comparisons were made between unstimulated and stimulated, using Wilcoxon signed-rank test. *p < 0.05, **p < 0.01, ****p < 0.0001.

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5.1 Abstract

Antibody Fc-mediated functions such as antibody-dependent cellular cytotoxicity contribute to vaccine-induced protection against viral infections. Weak neutralisation but strong Fc-mediated function of anti-Ebola glycoprotein antibodies suggest that Fc-dependent activation of effector cells, including NK cells, may be important for vaccine induced protection against Ebola virus infection. We analysed the impact of anti-Ebola glycoprotein antibody in the serum of U.K. based volunteers vaccinated with the novel prime-boost Adenovirus type 26.ZEBOV/Modified Vaccinia Ankara-BN-Filo vaccine regimen on primary human NK cell activation. We demonstrate induction of CD107a and IFN-γ expression, with reciprocal downregulation of CD16, in more differentiated NK cell subsets in response to *in vitro* stimulation with Ebola glycoprotein and post-prime/post-boost vaccination serum. These responses varied significantly with vaccine regimen and, using a uniform responder cell preparation, NK cell activation was found to be strongly correlated with anti-glycoprotein antibody titre, suggesting at least partial dependence on antibody titre for optimal NK cell activation. Stimulation of NK cells from multiple donors with a standardised antigen/immune serum cocktail, revealed the impact of NK cell differentiation phenotype on antibody-dependent NK cell activation, with highly differentiated NKG2C$^+$CD57$^+$ NK cells being the most responsive. This study thus highlights the dual importance of vaccine-induced antibody titre and NK cell differentiation status in promoting Fc-mediated activation of NK cells after vaccination. Antibody mediated NK cell activation may be a useful readout for evaluating vaccine efficacy in different populations.

5.2 Introduction

Determining correlates of protection for Ebola vaccines has proved difficult and ambiguous (1). Some anti-Ebola antibodies possess strong neutralising capacity (2, 3), however, antibodies with limited neutralising activity were protective in animal models and human *in vitro* culture systems suggesting that neutralisation alone presents an incomplete mechanistic picture of *in vivo* protection (3-5). In humans, Ebola specific antibodies induce antibody-dependent cellular cytotoxicity (ADCC) in peripheral blood NK cells and NK cell lines *in vitro*; in light of this, Fc-mediated function in anti-Ebola monoclonal antibody (mAb) therapy and vaccine induced protection is gaining in interest (5-7). Analysis of the primary response to the candidate Ebola vaccine, rVSV-ZEBOV, has revealed a correlation between early NK cell activation and anti-Ebola antibody titre in humans (8). Also, protection of non-human primates against Ebola virus challenge is associated with a low IgG2:IgG1 antibody isotype ratio, compatible with ADCC as a major mechanism of protection (9). In mice, experimental Ebola (or related filovirus) vaccines demonstrate induction of anti-glycoprotein (GP) antibodies with weak neutralising activity and robust ADCC function that was critical for protection (10-12). Taken together, these studies suggest Fc functions of anti-Ebola antibodies contribute to protection and may be exploited for improving vaccine and therapeutic mAb efficacy and as markers of vaccine induced immunity.

NK cells, like other innate immune effector cells, express one or more Fc receptors (FcR) on their surface allowing activation of cell-mediated antibody-dependent anti-viral functions (13). Antibody-dependent phagocytosis (ADP) of

virus or virally-infected cells by monocytes, macrophages and neutrophils, and ADCC mediated by NK cells, promote the clearance of infected cells, reducing viral load and viral dissemination. NK cell ADCC is principally mediated by crosslinking of FcγRIIIa (CD16) by the Fc region of immunoglobulins – typically subclasses IgG1 and IgG3 in humans – which leads to activation of immunoreceptor tyrosine-based activation motifs (ITAM) and downstream pathway activation. Ultimately, killing proceeds by the release of lytic granules (containing granzyme B and perforin) from activated NK cells inducing apoptosis of virally infected cells. Cross-linking of CD16 by antibody induces its cleavage from the NK cell surface (14-16), however, despite this NK cells can move on to kill multiple targets providing effective and rapid clearance of infected cells (17). Fc functions of broadly neutralising antibodies have been shown to be indispensable for protection against influenza virus infection (18, 19). However, the role of Ebola vaccine induced antibody-dependent NK cell functions is unknown.

The novel Ad26.ZEBOV/MVA-BN-Filo prime-boost vaccine regimen has shown promising results in phase 1 trials; high levels of anti-Ebola GP specific antibody are sustained for at least 360 days (20-22). However, different vaccine regimens (differing in the order and timing of prime/boost) induce substantially different serum antibody titres in U.K. based volunteers at both post-prime and post-boost time points (21). Therefore, the purpose of this study was to assess the ability of post-Ad26.ZEBOV/MVA-BN-Filo vaccination sera to mediate antibody-dependent NK cell function using a 6-hour *in vitro* ADCC assay with immobilised Ebola GP (EbovGP) as antigen. We observe robust, antibody-dependent

activation of NK cells in whole human peripheral blood mononuclear cell (PBMC) preparations cultured with EbovGP in the presence of serum from Ad26.ZEBOV/MVA-BN-Filo-vaccinated individuals. NK cell activation varied depending on vaccine regimen; NK cell degranulation and IFN-γ secretion correlated positively with mean antibody titres. Antibody-dependent NK cell activity also varied between NK cell donor consistent with NK cell differentiation phenotype influencing the potency of these responses.

5.3 Materials and Methods

5.3.1 Study participants and samples

Eligible, healthy volunteers (n=72, age range 18-50 years, 36% HCMV seropositive by IgG ELISA; Biokit, Barcelona, Spain) were recruited to take part in the EBL1001 (EBOVAC consortium) single-centre, randomised, placebo-controlled, observer blind Ebola vaccine trial in Oxford, U.K.; see Milligan *et al.* for additional methodology (21) (ClinicalTrials.gov Identifier: NCT02313077). Active vaccination comprises monovalent Ad26.ZEBOV expressing the GP of the Ebola Zaire virus (Mayinga variant) and multivalent MVA-BN-Filo expressing the GP of the Sudan and Zaire Ebola viruses and Marburg virus together with Tai Forest virus nucleoprotein (NP) (Crucell Holland N.V. - now Janssen Vaccines & Prevention B.V., The Netherlands and Bavarian Nordic, Denmark). Participants were randomised to receive vaccinations as follows; groups 1 and 2 received MVA-BN-Filo as prime on day 1 and Ad26.ZEBOV as boost on either day 29 or 57 respectively, groups 3, 4 and 5 received Ad26.ZEBOV as prime and MVA-BN-Filo as boost on days 29, 57 or 15 respectively.

Serum samples were obtained as shown in Table 1; anti-Ebola GP antibody concentrations were determined by ELISA as previously reported (21). Additional whole blood samples were obtained from non-vaccinated volunteers from among staff and students of LSHTM. PBMC were isolated using Histopaque 1077 gradient centrifugation and cryopreserved in liquid nitrogen or used immediately. Written informed consent was received from all participants prior to inclusion in the study. The trial protocol and study documents were approved by the National

Research Ethics Service (reference number 14/SC/1408) and the LSHTM Research Ethics Committee (reference number 14383).

	Serum samples used in this study			
	Vaccine schedule	Baseline (Visit 0)	Post-prime (Visit 1)	21d post-boost (Visit 2)
Group 1 (n=15)	MVA-Ad26	Day 1	Day 29	Day 50
Group 2 (n=15)	MVA-Ad26	Day 1	Day 57	Day 78
Group 3 (n=14)	Ad26-MVA	Day 1	Day 29	Day 50
Group 4 (n=14)	Ad26-MVA	Day 1	Day 57	Day 78
Group 5 (n=14)	Ad26-MVA	Day 1	Day 15	Day 36

Table 1: Vaccination regimen and samples used in this study.

MVA, MVA-BN-Filo; Ad26, Ad26.ZEBOV.

5.3.2 *In vitro* culture assays

For antibody-dependent NK cell activation assays, 10µg/ml purified Ebola GP (EbovGP), Mayinga variant (Crucell Holland B.V.) was immobilised on 96 well, flat-bottom tissue culture plates overnight at 4°C, washed, blocked with 5% FCS in RPMI 1640 supplemented with 100U/ml penicillin/streptomycin and 20mM L-glutamine (Gibco, ThermoFisher) for 30 minutes and washed again. Fresh PBMC from one individual (non-vaccinated) donor were washed in RPMI 1640 supplemented as above and counted using a Countess II FL automated cell counter (Invitrogen, ThermoFisher). PBMC were seeded (3×10^5/well) onto the antigen-coated plates together with pre or post-vaccination serum at various concentrations (with total serum concentration made up to 5% with FCS), and

incubated for 6 hours at 37°C. Alternatively, cryopreserved PBMC from multiple (non-vaccinated) donors were thawed, washed and seeded onto the antigen-coated plates with pooled pre or post-vaccination serum from vaccination group 2 (MVA-BN-Filo prime and Ad26.ZEBOV boost at day 57).

Anti-CD107a-FITC (clone H4A3; BD Biosciences) was added to the cultures (at 2μl per well) for the entire culture period and GolgiStop (Monensin; 1/1500 concentration; BD Biosciences) and GolgiPlug (Brefeldin A; 1/1000 final concentration; BD Biosciences) were added for the final 3 hours of culture. Positive control cultures comprised the CD20 expressing human Burkitt′s Lymphoma cell line (RAJI; ECACC, Salisbury, U.K.) in conjunction with monoclonal anti-CD20 (Rituximab; Ritxan®; Genentech, San Francisco, U.S.A.) at varying concentrations. In all cases, cells were harvested into round-bottom plates by soaking and resuspension in cold PBS (containing 0.5% FCS, 0.05% sodium azide and 2mM EDTA) for staining.

5.3.3 Flow cytometry

Cells were stained in 96-well round-bottom plates as described previously (23). Briefly, cells were blocked with Fc Receptor (FcR) Blocking Reagent (Miltenyi Biotech, Germany) according to manufacturer's instructions and stained with fluorophore labelled antibodies for surface markers including viability marker (Fixable Viability Stain 700; BD Biosciences) in FACS buffer (PBS, 0.5% FCS, 0.05% sodium azide and 2mM EDTA). Cells were washed in FACS buffer, fixed and permeabilised using Cytofix/Cytoperm Kit (BD Biosciences) according to the manufacturer's instructions. Cells were then stained for intracellular markers with

further FcR blocking for 20 minutes and washed again. Finally cells were resuspended in FACS buffer and acquired using a BD LSRII flow cytometer. Cells were acquired using FACSDiva software, data were analysed using FlowJo V10 (Tree Star, Oregon, U.S.A). FACS gates were set using unstimulated cells or FMO controls, samples with less than 100 NK cell events were excluded from the analysis.

Fluorophore labelled antibodies used were: anti-CD3-V500 (clone UCHT1) (BD Biosciences), anti-CD56-BV605 (clone HCD56) and anti-IFN-γ-BV785 (clone 45.B3) (Biolegend, London, U.K.), anti-CD16-APC (clone CB16), anti-CD57-e450 (clone TB01) (eBiosciences) and anti-NKG2C-PE (clone 134591) (R&D systems). Cells were acquired using FACSDiva software, data were analysed using FlowJo V10 (Tree Star, Oregon, U.S.A).

5.3.4 Statistics

Statistical analysis was performed using GraphPad Prism version 7.04 (GraphPad, California, U.S.A.). Functional responses were compared using Wilcoxon signed-rank test or one-way ANOVA Friedman test with Dunn's correction for multiple comparisons. Correlation analysis was performed using linear regression. Significance levels are assigned as *p, 0.05, **p, 0.01, ***p, 0.001, and ****p, 0.0001 for all tests.

5.4 Results

5.4.1 Ad26.ZEBOV/MVA-BN-Filo Ebola vaccine induced antibody-dependent NK cell activation in vitro.

To assess the effect of Ad26.ZEBOV/MVA-BN-Filo induced anti-GP antibody on antibody-dependent NK cell activation, whole PBMC from one non-vaccinated donor were cultured with plate bound EbovGP plus pre or post-vaccination serum for 6 hours. Initially, serial dilutions of pooled pre-vaccination (baseline) and post-boost serum were performed and $CD3^{-}CD56^{+}$ NK cell CD107a surface expression was assayed by flow cytometry (gating strategy shown in Figure 1a and Supplementary Figure 1a). Significant NK cell expression of CD107a was induced with the lowest concentration (0.63%) of post-boost serum (1.32% $CD107a^{+}$ NK cells compared to 0.055% with pre-immune serum) and the prevalence of $CD107a^{+}$ NK cells increased with increasing concentrations of post-boost serum, consistent with an antibody-dependent response (Supplementary Figure 1b). A concentration of 5% post-boost serum was selected for use in for subsequent assays as it induced a strong CD107a response but a low background response to pre-vaccination serum or FCS control (11.1% $CD107a^{+}$ NK cells with 5% post-boost serum; 0.54% $CD107a^{+}$ with 5% pre-vaccination serum and 0.11% $CD107a^{+}$ with 5% FCS) (Supplementary Figure 1b).

NK cell CD107a, CD16 and IFN-γ expression was then measured in response to 5% serum collected pre (visit 0), post-prime (visit 1) or post-boost (visit 2) vaccination with Ad26.ZEBOV/MVA-BN-Filo from each individual study participant (n=72) (gating strategy shown in Figure 1a-c). Initially, data from all

five vaccination arms were combined for analysis. Significantly higher frequencies of CD107a$^+$ NK cells were observed with post-prime serum compared to pre-vaccination serum and was further enhanced with post-boost serum (median 2.39% post-prime, 8.24% post-boost) (Figure 2a). CD56^{dim} NK cell CD16 expression measured by mean fluorescence intensity (MFI) decreased significantly in cells cultured with post-prime serum and there was a further, more pronounced, decrease in CD16 expression in cells cultured with post-boost serum (median MFI 8990 post-prime, 4020 post-boost) (Figure 2b). Frequencies of NK cells producing IFN-γ in response to post-prime serum were low but significantly higher than in response to pre-vaccination serum, and again, this was dramatically increased with post-boost serum (median 0.28% post-prime, 1.17% post-boost) (Figure 2c).

The effect of Ad26.ZEBOV/MVA-BN-Filo-induced anti-GP antibody on antibody-dependent NK cell activation was analysed according to NK cell differentiation subset (gating strategy is shown in Supplementary Figure 1c). The NK cell subset distribution of the single donor used in for this assay was 10.2% CD56^{bright}, 28.3% CD56^{dim}CD57$^-$, 45.2% CD56^{dim}CD57$^+$ and 16.3% CD56^{dim}CD57$^+$NKG2C$^+$ NK cells (Supplementary Figure 1d). NK cell CD107a expression was induced equally in less differentiated CD56^{bright} and more differentiated CD56^{dim} NK cell subsets and in subsets further subdivided into moderately and highly differentiated CD56^{dim}CD57$^-$ and CD56^{dim}CD57$^+$(NKG2C$^+$) cells, respectively (Figure 2d). This was consistent with significant CD16 downregulation observed in all NK cell subsets (Figure 2e). Basal CD16 expression increased with increasing differentiation status (CD56^{dim}CD57$^-$ < CD56^{dim}CD57$^+$ <

CD56^{dim}CD57^{+}NKG2C^{+}) and CD16 expression was maintained at higher MFI post-boost in the most differentiated subsets (Figure 2e). CD107a was induced within the CD56^{bright} NK cell population in response to post-vaccination serum, however the overall contribution of this subset to total NK cell CD107a expression was significantly less than CD56^{dim} subsets (less than 5%, p=<0.0001) (Figure 2g). IFN-γ expression in response to post-prime and post-boost serum was attributed to CD56^{dim} NK cells and no increase in expression was measured in the least differentiated CD56^{bright} NK cell subset (Figure 2f).

The most highly differentiated CD56^{dim}CD57^{+} and CD56^{dim}CD57^{+}NKG2C^{+} NK cell subsets showed the most extensive CD16 downregulation and contained the highest frequencies of IFN-γ producing cells (Figure 2e, f). Over half (58%) of all the NK cells producing IFN-γ in response to post-boost serum were CD56^{dim}CD57^{+} NK cells, with 25% of IFN-γ^{+} cells being CD56^{dim}CD57^{+}NKG2C^{+} (Figure 2h). Consistent with antibody-dependent activation of more differentiated NK cell subsets, anti-CD20 (Rituximab) and CD20-expressing RAJI cells also preferentially induced NK cell degranulation and IFN-γ expression in CD56^{dim}CD57^{+} and CD56^{dim}CD57^{+}NKG2C^{+} cells (Supplementary Figure 2). These data suggest that anti-Ebola GP antibody induces antibody-dependent NK cell activation, including IFN-γ secretion, in more differentiated NK cell subsets.

5.4.2 Variation in antibody-dependent NK cell activation by vaccine regimen.

The anti-GP antibody titres in the serum samples of Ad26.ZEBOV/MVA-BN-Filo vaccinated individuals varied depending on the vaccination regimen (21). We

therefore analysed the impact of vaccine regimen on antibody-dependent NK cell activation. There was significant upregulation of CD107a, IFN-γ and downregulation of CD16 with post-boost serum in all groups compared to pre-vaccination serum responses, however responses differed significantly between study arms (Figure 3a-c).

Groups 1 and 2 (MVA prime/Ad26 boost) resulted in the strongest induction of CD107a and IFN-γ expression and the lowest CD16 MFI of all 5 groups (Figure 3a-c). Serum collected after MVA prime alone did not induce NK cell activation, but significant induction of CD107a, IFN-γ and downregulation of CD16 was seen with post-Ad26 boost serum (when compared to both pre-vaccination and post-prime vaccination serum) (Figure 3a-c, Supplementary Table 1). By contrast, in groups 3, 4 and 5 (Ad26 prime/MVA boost) there was induction of NK cell responses to post-Ad26 prime serum and IFN-γ (groups 3 and 4), CD107a and CD16 downregulation (group 4 only) were further enhanced by post-MVA boost serum (Figure 3a-c, Supplementary Table 1). Earlier MVA boost in group 3 and group 5 did not result in further significant NK activation over that with post-Ad26 prime serum (except weak boosting of IFN-γ in group 3) and group 5 resulted in the weakest overall response (Figure 3a-c, Supplementary Table 1). This suggests that Ad26 prime induces sufficient titres of antibody for a robust NK cell response that is further increased by MVA boost, whilst MVA alone does not induce sufficient antibody (or antibody of the correct isotype or subclass) to mediate ADCC. This is in line with data on the effect of vaccine regimen on anti-GP antibody concentration (21).

We next analysed the relationship between geometric mean anti-GP antibody titres (determined by Milligan *et al.* (21)) and median expression of NK cell activation markers. There was a significant positive correlation between post-boost antibody concentration and median NK cell CD107a$^+$ and IFN-γ$^+$ frequencies and a negative correlation with CD16 downregulation (Figure 3d-f). Both geometric mean antibody concentrations and NK cell ADCC responses were highest in groups 1 and 2 and lowest in group 5. Despite a large difference in antibody concentration between group 1 and 2 (MVA prime/Ad26 boost at day 29 and day 57 respectively), NK cell CD107a expression and CD16 downregulation were comparable (Figure 3d, e). Similarly, differences in antibody titres between group 3 and 4 (Ad26 prime/MVA boost at d29 and d57 respectively) did not manifest as big differences in NK cell activation (Figure 3d, e). This suggests that sufficient antibody titres can be achieved with early (d29) boost to obtain optimal NK cell degranulation responses. By contrast, IFN-γ production, was very strongly correlated with antibody titre with no saturating effect of increasing mean antibody titre, suggesting that increasing levels of anti-GP antibodies will be associated with stronger NK cell cytokine secretion (Figure 3f). There was no significant correlation between antibody titre and NK cell function per group at post-prime time points (Supplementary Figure 3a-c), consistent with requirement of boost-vaccination for strong antibody-dependent NK cell responses.

5.4.3 Antibody-dependent activation varies by NK cell donor.

The studies described above (Figures 1-3) employed a consistent source of PBMC from a single donor whilst varying the source of serum, such that variation in NK cell antibody-dependent activation between serum donor and vaccination group could be related to differences in antibody titre. In order to analyse the effect of donor variation, whole PBMC from non-vaccinated donors were cultured with plate bound EbovGP and pooled pre or post-boost vaccination serum samples from group 2 (MVA prime/Ad26 boost). NK cell CD107a (14 of 16 responding), CD16 downregulation (16 of 16 donors responding) and IFN-γ (13 of 16 donors responding) was induced with pooled post-boost serum compared to pooled pre-vaccination serum (Figure 4a-c). This suggests that cells from the majority of donors respond to Ad26.ZEBOV/MVA-BN-Filo-induced antibodies for NK cell activation and also indicates that, in addition to antibody titre, factors intrinsic to the responding NK cells influence individual ADCC responses.

The extent of NK cell differentiation is influenced by age and infections such as human cytomegalovirus (HCMV) (24, 25). We therefore analysed NK cell activation in response to pooled post-boost vaccination serum according to NK cell differentiation subset. Amongst the individuals tested, frequencies of the most highly differentiated CD56^{dim}CD57^{+}NKG2C^{+} NK cells varied widely (with 5 of 16 donors showing frequencies above 10%) and overall there was a wide range of frequencies of the different subsets (CD56^{bright} range 1.8-18.6%; CD56^{dim}CD57^{-} 17.6-68.1%; CD56^{dim}CD57^{+} 7.9-66.1%; CD56^{dim}CD57^{+}NKG2C^{+} 0-32.5%) (Figure 4d). NK cell CD107a^{+} expression after co-culture with antigen and post-boost sera was apparent in all NK cell subsets (Figure 4e), as was CD16

downregulation (Figure 4f). IFN-γ was significantly upregulated in post-boost serum in CD56^{dim}CD57$^-$ and CD56^{dim}CD57$^+$ subsets with the highest frequency of IFN-γ expression being observed within the CD56^{dim} NK cell subsets. The individuals with a frequency of CD56^{dim}CD57$^+$NKG2C$^+$ NK cells above 10% contributed the higher NK cell IFN-γ production of this subset (Figure 4g), and almost half (46.9%) of total NK cell IFN-γ production was attributed to this subset (Figure 4h). These data demonstrate that differences in NK cell differentiation status introduce additional inter-donor variation for in NK cell ADCC responses.

5.5 Discussion

We have shown that antibodies to Ebola virus GP induced by the Ad26.ZEBOV/MVA-BN-Filo vaccine activate robust NK cell degranulation and IFN-γ secretion within 6 hours of co-culture with Ebola GP antigen. These NK cell responses are highly variable depending on vaccine regimen and are directly correlated with post-boost antibody titres. Furthermore, these antibody-dependent NK cell responses are markedly enriched in (although not limited to) the more differentiated (CD56^{dim}) NK cell subsets and differences between donors in the differentiation status of their NK cells therefore adds to the heterogeneity of ADCC responses seen post-vaccination. Thus, these data demonstrate that assessment of antibody-dependent NK cell effector function has potential as an informative correlate of vaccine efficacy.

The strong positive correlation between post-boost antibody titre and NK cell activation suggests this is a robust read-out of antibody-dependent effector function that, to some extent, depends on antibody titre. Interestingly, NK cell CD107a expression did not further increase with higher antibody titres induced by delayed boost vaccination (d57) compared to earlier boost (d29) suggesting a threshold for maximum NK cell degranulation was reached. However, NK cell IFN-γ expression was higher with later boost (d57) compared to the earlier boost (d29). The lack of correlation between antibody titre and NK cell responses after a single vaccine dose (i.e. post-prime) highlights a requirement for a two dose vaccine regimen to induce robust NK cell responses and may reflect effects of antibody affinity maturation and isotype/subclass switching in addition to any effects of overall antibody concentration.

Of interest, NK cell activation post-Ad26.ZEBOV/MVA-BN-Filo vaccination required relatively high serum concentrations. We have observed similar levels of NK cell degranulation with, for example, up to 40-fold lower concentrations of post-seasonal influenza vaccination serum (16); likely prior exposure to influenza (and possibly prior vaccination) may result in production of higher anti-influenza antibody titres and induction of high avidity antibodies with a broad range of specificities. In addition, in the assays used here, only antibodies able to bind to the plate bound Ebola GP were able to contribute to the ADCC response; the contribution of antibodies to the Ebola virus nucleoprotein (contained in the MVA vaccine) was not assessed.

Our data highlight a high level of variation in frequencies of activated NK cells subsets both within a single donor and between donors in response to post-vaccination antibody. Many variables effect NK cell ADCC function including FcR polymorphisms (26), antibody glycosylation (27) and cytokine mediated regulation (28). Antibody binding epitopes can also affect the ADCC functional capacity of vaccine induced antibodies (3). Studies suggest that neutralising antibodies bind epitopes on the core of Ebola GP potentially inhibiting virion cell entry whereas antibodies with Fc function bind epitopes on the upper domains that are exposed on GP presented on the surface of infected cells and remain on the shed form of GP (6, 29). Ebola GP returning to the surface of the infected cell and the liberation of soluble GP for immune complex formation could present opportunities for NK cell ADCC. However, GP shed from infected cells has been shown to bind anti-GP antibodies and block, rather than facilitate, their activity (30).

More differentiated CD57^{hi} (NKG2C^{hi}) NK cells express high levels of CD16 on their surface, exhibit high antibody-dependent responses to viral antigens and display reduced responsiveness to cytokine stimulation, due in part to reduced cytokine receptor expression (16, 31-36). Our data is thus consistent with antibody-dependent NK cell activation. HCMV infection is highly prevalent, with seropositivity rates of up to 60% in adults in developed countries, near universal in developing countries, and strongly influences NK cell function in response to viral antigens (25, 37) (and reviewed in (38)). HCMV infection leads to the accumulation of highly differentiated NK cells expressing NKG2C with a mature (CD56^{dim}CD57^{+}) and 'adaptive' (FcεRγ^{-}) phenotype. HCMV expanded populations of NK cells show enhanced IFN-γ secretion in response to antibody coated targets, owing to epigenetic modification of the IFN-γ gene promotor (36, 39). HCMV serostatus therefore may impact antibody-dependent NK cell activation, and perhaps vaccine-induced protection after Ad26.ZEBOV/MVA-BN-Filo vaccination.

In separate studies, we demonstrated soluble EbovGP induced inflammatory cytokine secretion and cytokine-dependent activation of less differentiated NK cells, with high levels of IL-10 controlling NK cell IFN-γ secretion (unpublished data, Chapter 4 of this thesis). This present study, utilising plate bound antigen, suggests that anti-GP antibody in post-vaccination serum strongly activates NK cell CD107a and IFN-γ secretion in more differentiated NK cell subsets via Fc-dependent mechanisms. Frequencies of IFN-γ^{+} NK cells measured in this study (median 1.17% at post-boost) may represent substantially higher percentages than Ebola GP-specific T cell IFN-γ measured by ELISPOT previously (maximum

940 spot forming units/million PBMC (0.094%)) (21). This leads us to hypothesise that NK cell IFN-γ induced by ADCC in response to vaccine antibody, may be of increased importance during post-vaccination exposure to Ebola virus by limiting reliance on non-specific inflammatory mediators and associated downregulation by IL-10.

In summary we have shown that vaccination with Ad26.ZEBOV/MVA-BN-Filo induces antibodies capable of inducing strong antibody-dependent NK cell activation that strongly correlated with antibody titre. This response varied widely between donors, was robust in the majority tested and was further enriched in, but not limited to, highly differentiated $CD57^+NKG2C^+$ NK cells. This study highlights NK cells as potential mediators of immunity to Ebola infection where responses are determined by both the level of vaccine induced antibody and by the differentiation status of effector NK cells. Antibody-dependent NK cell function may help better define the protective capacity of vaccine induced antibodies when combined with standard antibody level or neutralisation assays.

5.6 Acknowledgements

5.7 Figures
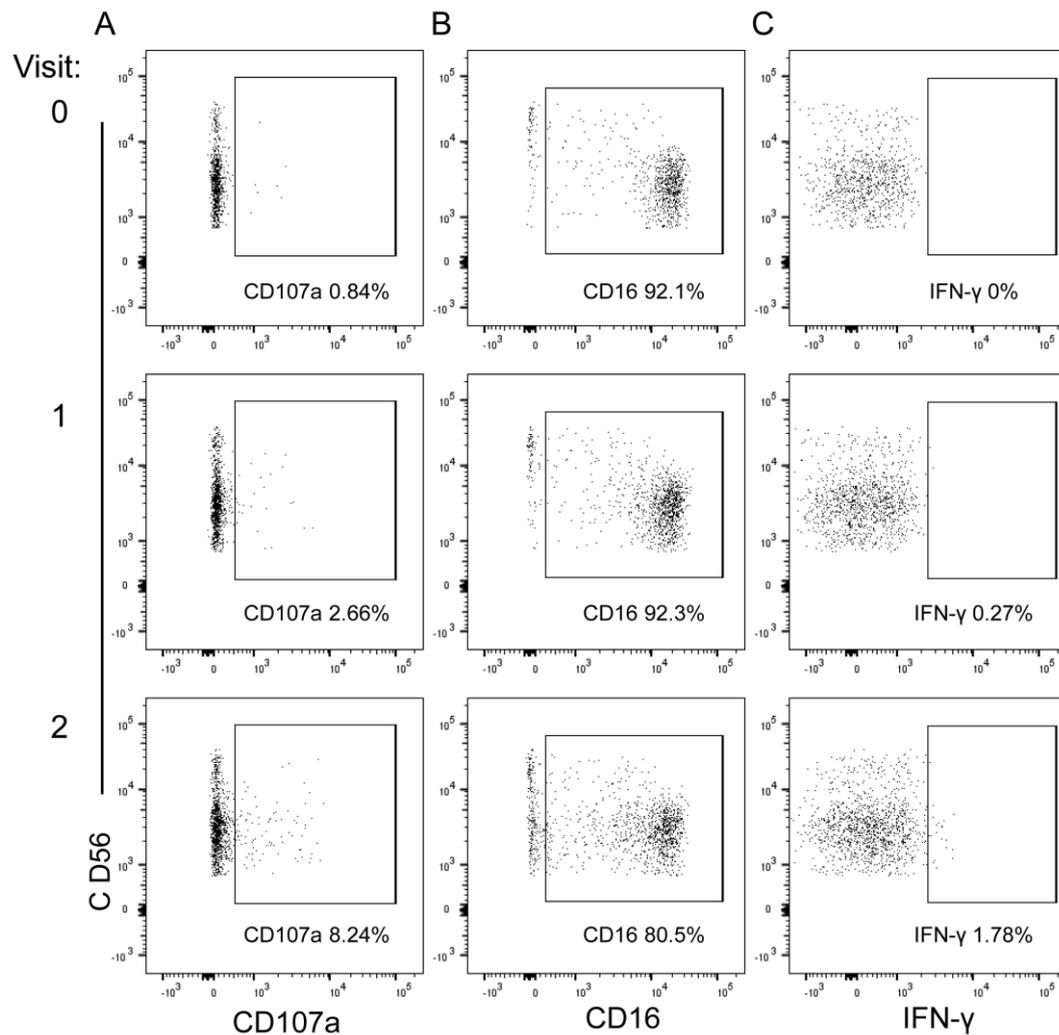
Figure 1: Gating strategy for NK cell CD107a, CD16 and IFN-γ expression.

Flow cytometry plots show CD3⁻CD56⁺ NK cell (gating strategy shown in Supplementary Figure 1a) CD107a (a), CD16 (b) and IFN-γ (c) expression in response to 5% pre (visit 0), post-prime (visit 1) and post-boost (visit 2) vaccination serum and plate bound EbovGP antigen. Whole PBMC from one non-vaccinated single donor were used for NK cell activation assays in figures 1-3, the NK cell differentiation phenotype of the donor is shown in Supplementary Figure 1c and d.
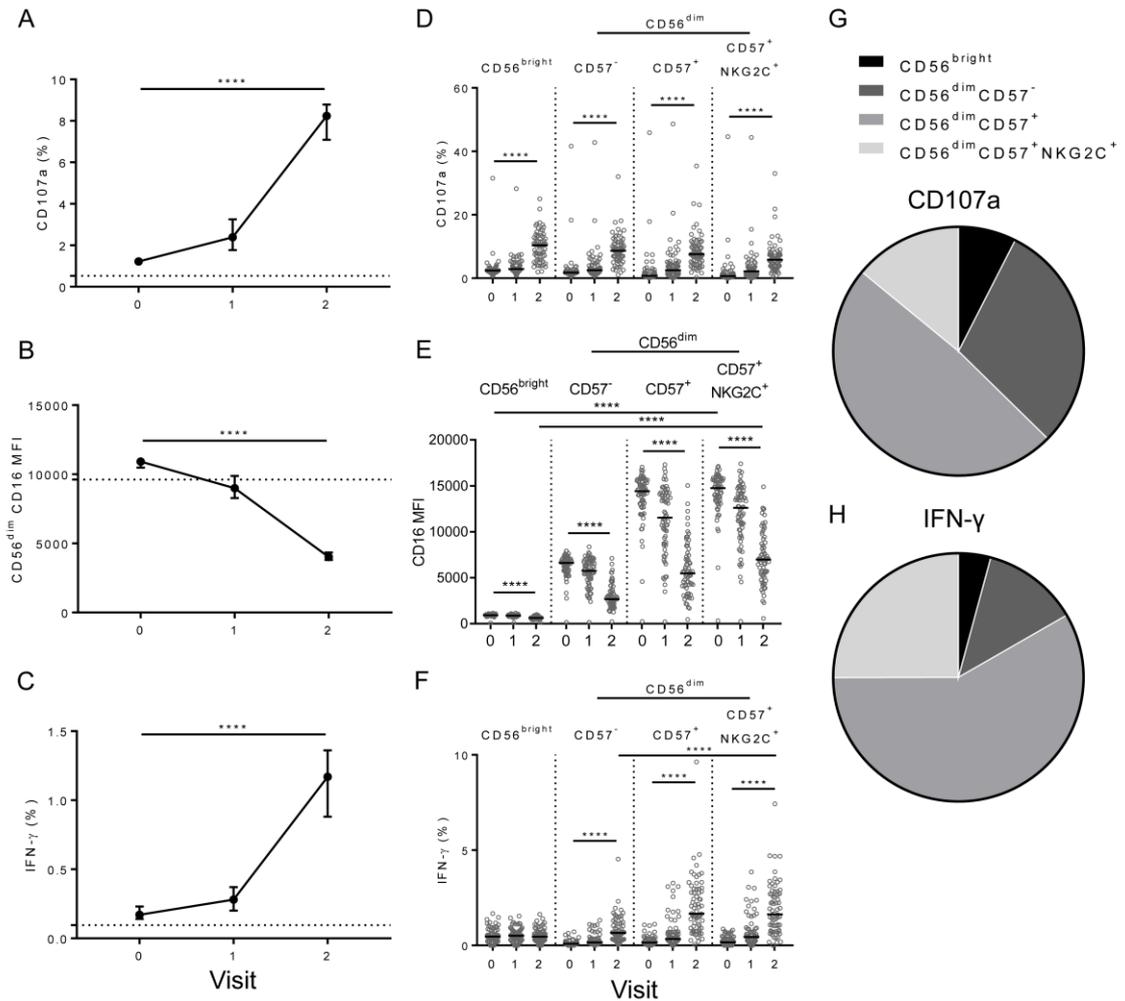
Figure 2: Antibody-dependent NK cell responses to plate bound EbovGP after Ad26.ZEBOV/MVA-BN-Filo vaccination.

The median and 95% confidence interval of NK cell CD107a (a), $CD56^{dim}CD16$ MFI (b) and IFN-γ (c) responses to each pre (visit 0), post-prime (visit 1) and post-boost (visit 2) vaccination serum sample are shown – all vaccine arms combined (n=72). NK cell CD107a (d), CD16 (e) and IFN-γ (f) responses were analysed according to NK cell differentiation subset defined by CD56, CD57 and NKG2C expression (gating strategy shown in Supplementary Figure 1c). Each individual donor serum sample is represented by a dot with a line at the median. The proportion of total NK cell CD107a (g) and IFN-γ (h) expression (post-boost) attributed to each subset is shown as a pie graph with each slice representing the median. Comparisons across vaccination visits and between subsets was performed using one-way ANOVA with Dunn's correction for multiple comparisons. ****$p < 0.0001$.
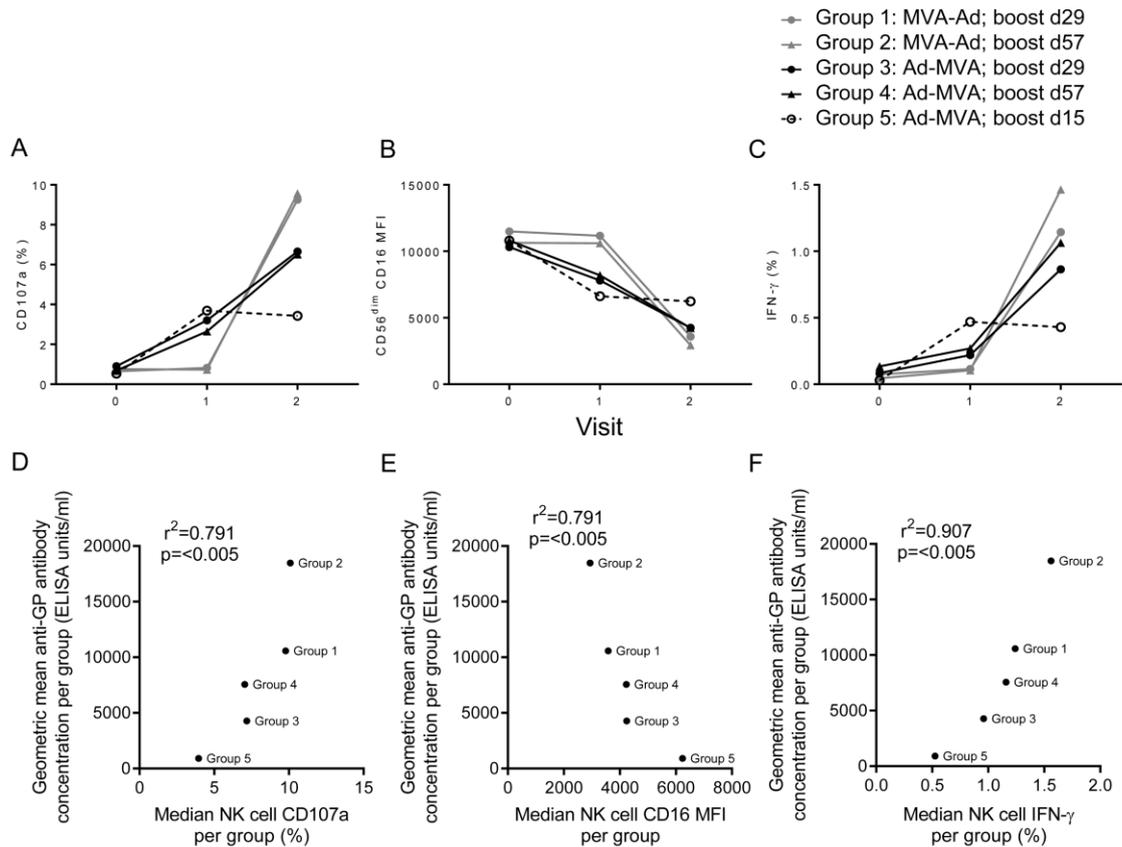
Figure 3: NK cell activation varies with vaccine regimen.

NK cell CD107a (a), CD56^{dim}CD16 MFI (b) and IFN-γ (c) expression was plotted according to vaccine regimen for pre (visit 0), post-prime (visit 1) and post-boost (visit 2) vaccination time points, graphs show median only. Comparisons between groups using one-way ANOVA with Dunn's correction for multiple comparisons was performed and summarised in Supplemntary Table 1. Geometric mean anti-GP antibody concentration per study arm was correlated with median NK cell CD107a (d), CD16 (e) and IFN-γ (f) expression, plots show one dot per group. Correlation was determined by linear regression and significance was defined as p < 0.05.
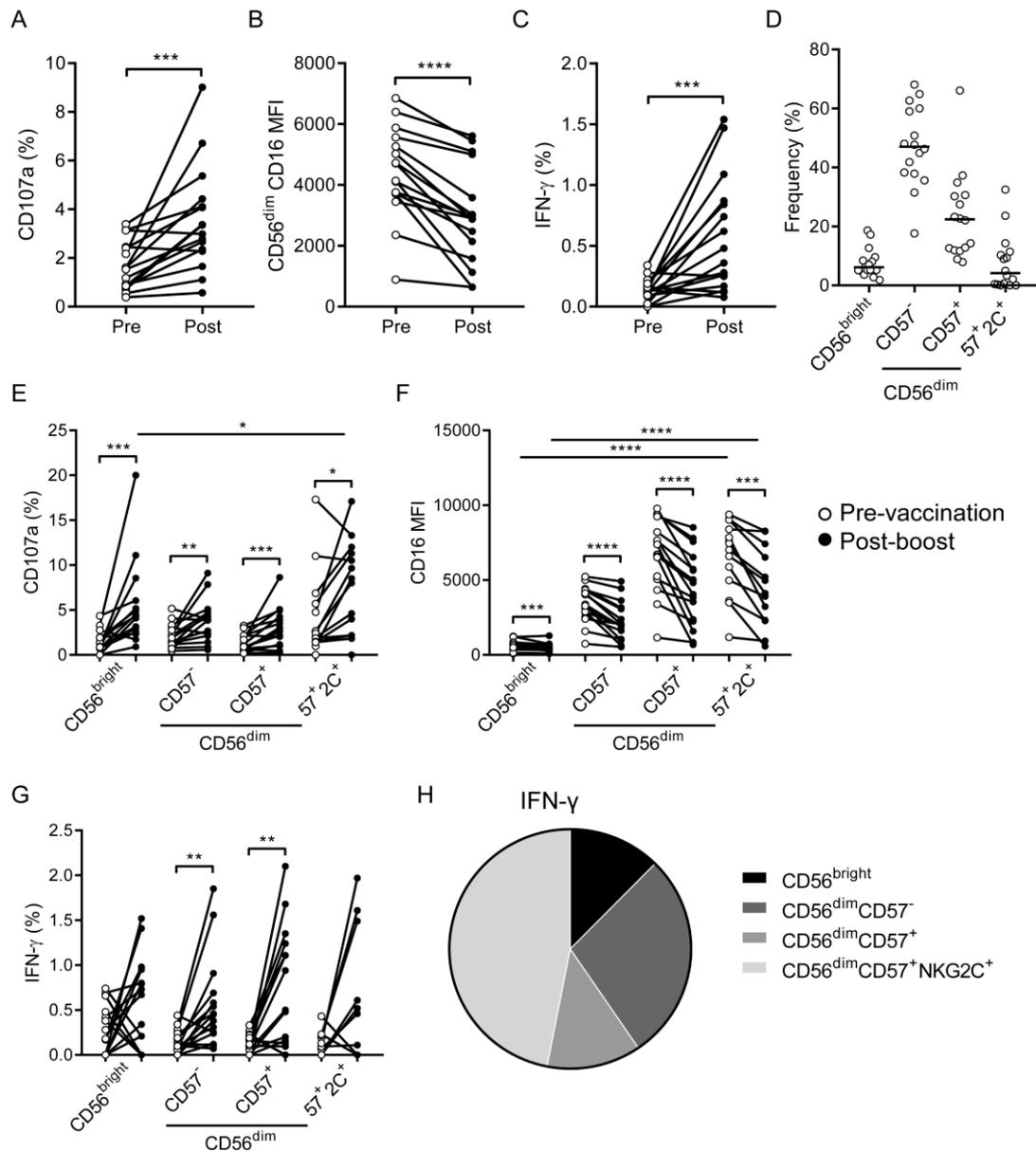
Figure 4: NK cell activation varies with NK cell donor.

NK cell CD107a (a), CD56^{dim}CD16 MFI (b) and IFN-γ (c) expression (multiple non-vaccinated donors, n=16) in response to 5% pooled (group 2) pre and post-boost vaccination serum and plate bound EbovGP. The NK cell subset frequency distribution is shown for each donor (d). The NK cell CD107a (e), CD16 MFI (f) and IFN-γ (g) responses were also analysed according to NK cell differentiation subset and the proportion of total NK cell IFN-γ attributed to each subset is shown as a pie graph with each slice representing the median (h). Graphs show before and after plots with a line connecting each donor or one dot per donor with a line representing the median. Comparisons between pre and post serum responses was performed using Wilcoxon signed-rank test and between subsets using one-way ANOVA with Dunn's correction for multiple comparisons. *p < 0.05, **p < 0.01, ****p < 0.0001.

5.8 Supplementary Material

Group	Visit	P value		
		CD107a (%)	CD16 (MFI)	IFN-γ (%)
1	0 vs. 1	ns	ns	ns
	0 vs. 2	<0.0001	0.0002	0.0004
	1 vs. 2	0.001	0.0002	0.0004
2	0 vs. 1	ns	ns	ns
	0 vs. 2	0.003	<0.0001	0.0002
	1 vs. 2	0.0004	0.0008	0.0008
3	0 vs. 1	0.0421	0.0138	ns
	0 vs. 2	<0.0001	<0.0001	<0.0001
	1 vs. 2	ns	ns	0.0322
4	0 vs. 1	ns	0.0245	ns
	0 vs. 2	<0.0001	<0.0001	<0.0001
	1 vs. 2	0.0138	0.0245	0.0075
5	0 vs. 1	0.0052	0.0024	0.011
	0 vs. 2	0.011	ns	0.0417
	1 vs. 2	ns	ns	ns

Supplementary Table 1: Group wise statistical analysis.

Statistical analysis of NK cell CD107a, CD16 and IFN-γ expression in response to plate bound EbovGP and pre (visit 0), post-prime (visit 1) and post-boost (visit 2) vaccination serum between visits within each group. One-way ANOVA with Dunn's correction for multiple comparisons, significance defined as $p < 0.05$. ns, non-significant.
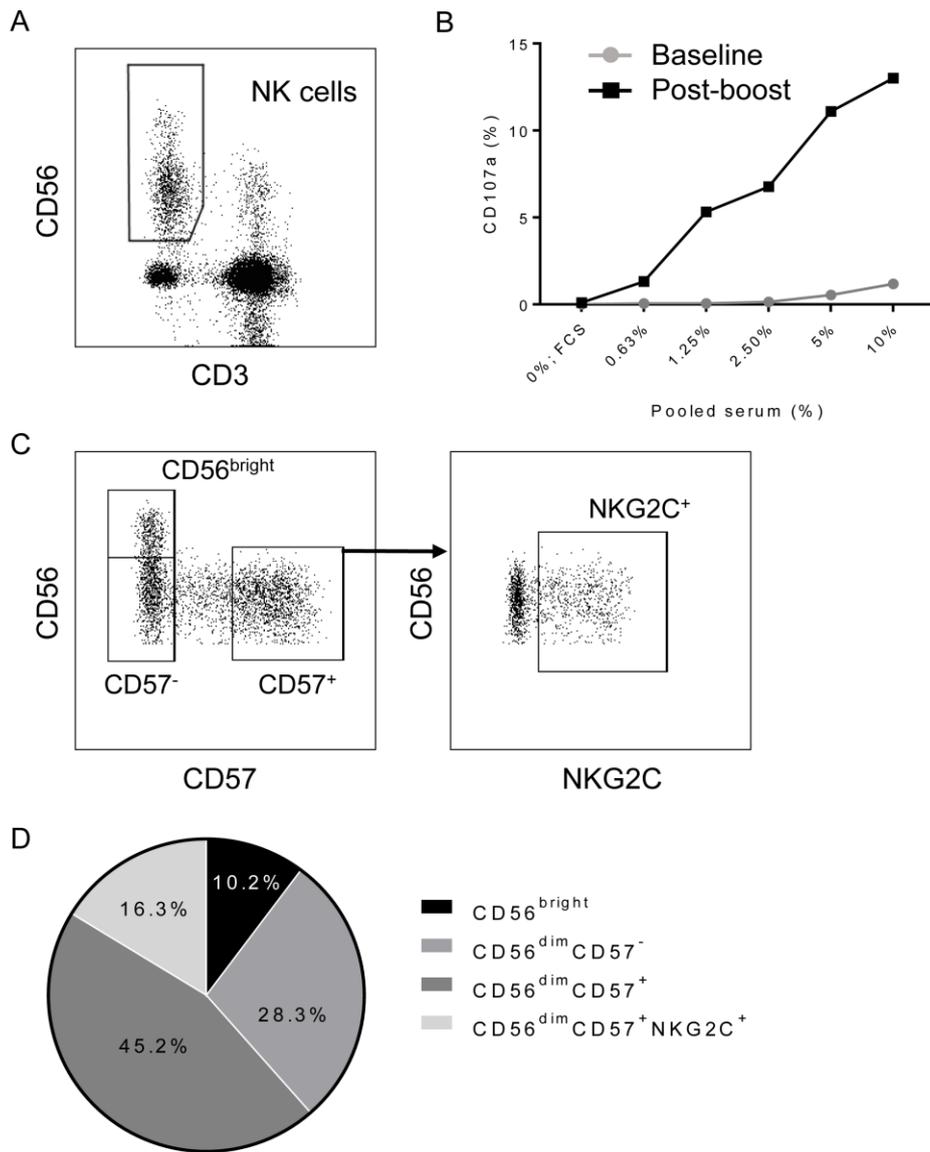
Supplementary Figure 1: NK cell gating strategy.

CD3$^-$CD56$^+$ NK cells were gated for activation marker analysis (a). The effect of increasing concentrations of pooled baseline and post-boost serum on frequencies of CD107a$^+$ NK cells in response to EbovGP (b). The gating strategy (c) and proportions (d) of NK cell differentiation subsets defined by CD56 and CD57 expression in the single PBMC donor used in Figures 1-3.
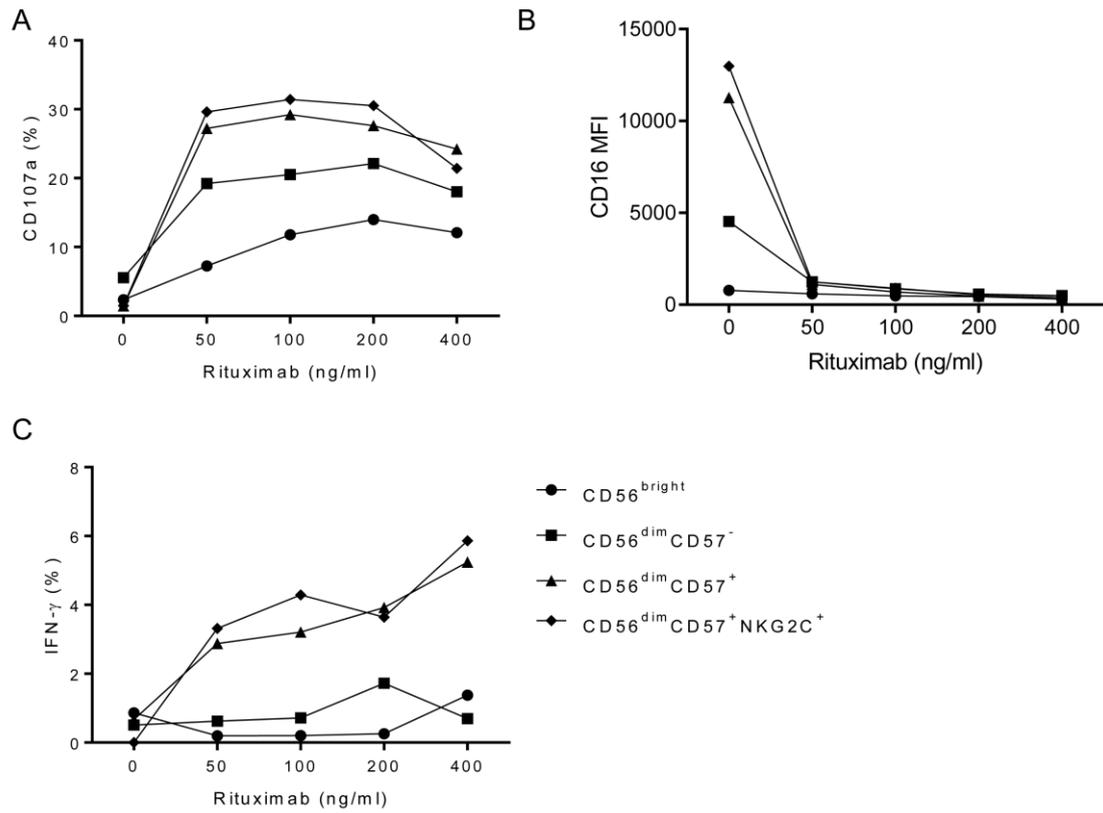
Supplementary Figure 2: The effect of increasing concentrations of Rituximab on NK cell activation.

NK cell CD107a (a), CD16 MFI (b) and IFN-γ (c) expression in response to CD20-expressing cell line (RAJI) and anti-CD20 antibody (Rituximab) was analysed by NK cell differentiation subset.
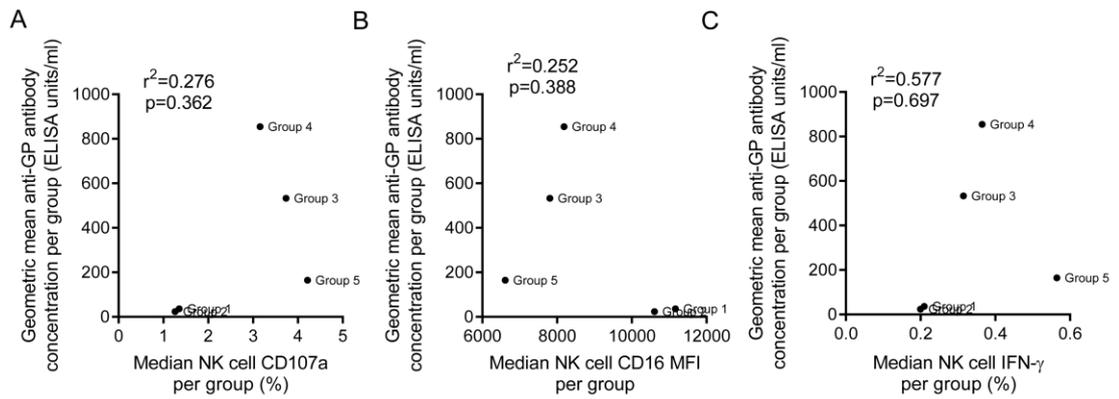
Supplementary Figure 3: Correlation between NK cell activation and antibody titre at post-prime time point.

Geometric mean anti-GP antibody concentration per group was correlated with the median NK cell CD107a (a), CD56^{dim} CD16 MFI (b) and IFN-γ (c) expression at the post-prime time point. Plots show one dot per group. Correlation was determined by linear regression and significance was defined as p < 0.05.

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Chapter 6: Discussion

6.1 Summary of Findings

The aim of this thesis was to investigate the effect of viral vaccination and restimulation with vaccine antigen and/or cytokines on human NK cell phenotype and function. I hypothesised that vaccination primes NK cells for enhanced responsiveness to innate cytokine stimulation leading to heightened effector responses post-vaccination. I have described a previously unappreciated mechanism of indirect NK cell functional enhancement by a very low concentration of IL-15. The effects of vaccination with whole inactivated influenza and vectored prime-boost Ebola vaccines on NK cell cytokine and antibody-dependent activation have also been characterised. The specific cytokine signature secreted in response to vaccination and *in vitro* restimulation proved important in determining the magnitude and functional characteristics of the NK cell response after vaccination, therefore combined analysis of cytokine secretion and associated NK cell activation was performed throughout this project. This lead to key findings that vaccine induced priming within the myeloid accessory cell pool contributes to post-vaccination NK cell responses and that vaccine-antigen induced IL-10 can suppress NK cell function. The findings presented in this thesis have furthered our understanding of NK cells as effectors of vaccine induced immunity. Here, I will summarise these and suggest directions for future work.

6.1.1 IL-15 Mediated Enhancement of NK Cell Function

IL-15 is important for NK cell development, survival and activation, the potency of which has previously been attributed to both trans-presentation on neighbouring DCs and synergy with other innate cytokines. IL-15 is also known to recruit NK cells to the lung after influenza infection, enhance DC maturation and recruit and modulate activation of T cells (1-3). However the role of IL-15 in enhancing DC cytokine secretion has not been previously appreciated and linked to enhanced NK cell responses against viral challenge.

I have shown that a very low dose of IL-15 (0.75ng/ml), which was unable to stimulate NK cells directly, enhanced NK cell responses to inactivated H3N2 influenza virus by boosting accessory cell cytokine secretion. **Table 1** summarises the concentrations of cytokines measured by Luminex after H3N2 vs H3N2 + IL-15 simulation in this study. IL-15 not only increased the frequency of responding NK cells but also increased the frequency of NK cells performing two or more functions (polyfunctional). This suggests a broadening of functional capacity against influenza virus, perhaps reflecting the increased concentrations of not only IL-12 (for enhanced IFN-γ secretion) but also IFN-α for the promotion of cytotoxicity. Interestingly, just under half (42-43%) of donors did not display enhanced IL-12 production in response to H3N2 + IL-15 compared with H3N2 alone. This observation of 'responders' and 'non-responders' may reflect differences in the frequency of accessory cells amenable to IL-15 boosting, or differential responsiveness to IL-15 within the myeloid cell pool between donors. Recent influenza virus exposure or vaccination leading to differential T cell help

or indeed primed or trained innate immunity could introduce donor-donor variation of this kind.

Although increases in functional NK cell percentages were small, this may represent a substantial proportion of the total lymphocyte response in peripheral blood and may be relevant in localised tissue environments such as the lung. The synergising concentrations of IL-15 employed here may be more physiologically relevant than the 5-fold higher concentrations typically used in NK cell *in vitro* culture (4). Increased concentrations of IL-15 (as well as IP-10, TNF, IL-10 and IFN-γ) have been measured in nasal lining fluid of live attenuated influenza vaccine (LAIV) vaccinated individuals demonstrating potential for this mechanism of NK cell enhancement in the lung after LAIV vaccination and indeed influenza infection (1, 5). It would be interesting to further investigate the effect of low doses of IL-15 on DC cytokine secretion, perhaps by measuring expression of IL-15 receptor components, IL-15 trans-presentation or the role of DC/NK cell contact in peripheral blood, but also in the lung using LAIV vaccination as a model for infection. Luminex analysis and immunophenotyping of nasal lining fluid and nasal cells applied in recent studies (5), may reveal whether these priming effects also have relevance in tissues after vaccination or infection.

Cytokine	Median cytokine concentration (pg/ml)		
	H3N2	**H3N2 + IL-15**	**P value**
GM-CSF	13.9	27.9	<0.0001
IFN-α2	1041	1271	0.0107
IL-10	25.7	21.4	NS
IL-12(p70)	5.1	8.9	<0.0001
IL-1β	224	314.9	<0.0001
TNF-α	902.2	1106	0.0341
IFN-γ	139.2	806.3	<0.0001

Table 1: Summary of cytokine concentrations secreted *in vitro* in response to H3N2 vs H3N2 + IL-15 stimulation of whole human PBMC.

Data taken from chapter 2. Median concentration in pg/ml measured by Luminex (n=73). Red; increase over H3N2 alone. P value determined by Wilcoxon signed-rank test, significant values determined as p < 0.05. NS; not significant.

It would be interesting to examine whether the impacts of IL-15 are unique to the signals induced by H3N2 (or other influenza viruses) or whether these are found in response to other viruses. In this context, I have found that co-stimulation of whole PBMC with EbovGP and 0.75ng/ml rhIL-15 also induced a heightened NK cell functional response compared to both IL-15 alone and EbovGP alone (**Appendix Supplementary Figure 1**). The frequency of IFN-γ$^+$ NK cells was significantly higher in the presence of IL-15, which in the absence of IL-15 was not induced over medium alone. This suggested that consistent with whole inactivated H3N2 virus stimulation, IL-15 enhanced the *in vitro* NK cell response to soluble recombinant EbovGP, most likely indirectly via boosting innate cytokine secretion for synergistic responses. Measurement of the cytokine concentrations secreted in response to EbovGP + IL-15 compared with that induced by EbovGP alone would certainly help evaluate this observation further.

6.1.2 Influenza Vaccine Enhanced Innate Cytokine Secretion and NK Cell Function

Previously, enhanced NK cell responses after influenza vaccination were demonstrated upon stimulation with vaccine strain influenza virus *in vitro*, this was dependent on antigen-specific T cell derived IL-2 and antibody (6, 7). Both *in vitro* and *in vivo* in mice, NK cells were primed in response to exogenous cytokine exposure (IL-12, IL-15 and IL-18) to mount more robust responses upon secondary exposure to innate cytokine stimulation (8, 9). NK cells were also primed by inactivated influenza vaccination *in vivo* enabling a heightened response to exogenous IL-12 and IL-18 stimulation *in vitro* in the absence of antigen stimulation; these cytokine primed or pre-activated NK cells are termed CIML NK cells (6, 10). In this thesis, I have shown that influenza vaccination primes both myeloid accessory cells and NK cells for enhanced cytokine secretion and activation in response to H3N2 and IL-15 stimulation, up to 30 days post-vaccination. These findings demonstrate that the function of vaccination primed NK cells, with increased responsiveness to innate cytokines (CIML NK cells), is further promoted in an environment with synergistic signals from enhanced accessory cell cytokine secretion and adaptive signals such as IL-2.

The most interesting finding of this study was the response to low dose IL-15 alone; I observed secretion of myeloid cell-derived cytokines such as GM-CSF, IL-10 and IL-1β in post-vaccination samples where little was induced at baseline. This was reflected in the NK cell response where there was significantly higher frequencies of CD25$^+$ NK cells post-vaccination compared to baseline in response to IL-15 alone. Co-stimulation with H3N2 + IL-15 also revealed vaccine

enhanced cytokine secretion and NK cell function that was magnified over that of IL-15 alone. **Table 2** summarises the concentrations of cytokines measured by Luminex in response to H3N2 + IL-15 simulation before and after vaccination in this study.

It is likely that pro-inflammatory cytokines released after vaccination in response to PRR stimulation led to the generation of CIML NK cells that may be sensitive to even the smallest increases in cytokine concentrations (such as IL-12 and IFN-α) induced by low dose IL-15 *in vitro*. This, in combination with the first finding (chapter 2), highlights an unappreciated role for very low doses of IL-15 in post-vaccination anti-viral immunity in the context of pre-activated/primed or co-stimulated innate immune cells. With this study, I have made some progress on assessing the functional differences in innate immune cells before and after vaccination. However, the mechanisms involved in vaccine-induced enhancement of innate immunity and their functional significance or contribution to vaccine-induced protection are not comprehensively understood. Further research could lead to improvements in the design of vaccine adjuvants, new vaccines or therapeutic strategies to boost (non-antigen specific) innate immunity through vaccination.

Cytokine	Median cytokine concentration (pg/ml)		
	Baseline	Day 30	P value
GM-CSF	68.1	132.7	0.0035
IFN-α2	1113	1419	0.0263
IL-10	160.7	411.6	0.0196
IL-12(p70)	12.3	10.6	NS
IL-1β	648.9	906.5	0.0386
TNF-α	2067	2545	NS
IFN-γ	737.9	2729	0.0006

Table 2: Summary of cytokine concentrations secreted *in vitro* in response to H3N2 + IL-15 stimulation before (baseline) and after (day 30) influenza vaccination.

Data taken from chapter 3. Median concentration in pg/ml measured by Luminex (n=26). Red; increase over baseline. P value determined by Wilcoxon signed-rank test, significant values determined as $p < 0.05$. NS; not significant.

Trained immunity, described in monocytes as induction of a non-specific memory, is underpinned by epigenetic modifications; histone trimethylation, keeping promotor regions open for rapid cytokine gene transcription, was increased up to 3 months after BCG vaccination (with possible effects lasting up to 1 year) (11-13). A study in which lower viremia upon yellow fever virus challenge was measured 1 month after BCG vaccination compared with unvaccinated controls suggested a role of non-specific trained immunity in protection against non-related viral pathogens. In this study, monocyte-derived IL-1β was a crucial component of trained immunity (14). We measured enhanced IL-1β secretion in response to both IL-15 alone and H3N2 + IL-15 restimulation after influenza vaccination, perhaps suggesting the presence of trained monocytes in our system. Intracellular staining experiments suggested mDCs are important producers of IL-12 in response to influenza virus, and blocking experiments demonstrated IL-12 was critical for CIML NK cell function after influenza

vaccination. Yet trained immunity in DC subsets or indeed in response to viral stimuli has not been reported before. Whether it is possible for DC subsets to be trained in response to virus exposure during vaccination and undergo epigenetic modification resulting in lasting modulation would be an interesting avenue to explore.

It is conceivable that epigenetic modifications are also involved in mediating heightened NK cell responsiveness to cytokines after vaccination. Promotor hypermethylation, was associated with signalling molecule SYK and FcεR1γ deficiency in HCMV driven expansions of 'adaptive' NK cells (15, 16). Demethylation, increasing gene accessibility, of the conserved non-coding sequences (CNS-1) of the *IFNG* locus in cytokine primed NK cells has also been demonstrated (17). CIML NK cells have comparable amounts of IFN-γ mRNA transcripts to naïve NK cells and the properties of CIML NK cells (enhanced IFN-γ secretion) were maintained in daughter cells after proliferation suggesting post-transcriptional/post-translational, heritable mechanisms (8, 9). However, epigenetic modifications have not yet been linked to enhanced cytokine responsiveness in NK cells after vaccination. Previous studies document the loss of influenza vaccine priming effects on NK cells by 12 weeks in U.K. based volunteers suggesting CIML NK cells, although perhaps retained after cellular proliferation, represent a transient state, with a currently unknown benefit (6).

To further our understanding, RNA transcriptional analysis of unstimulated and IL-15 stimulated pre and post-influenza vaccinated PBMC from the study described in Chapter 3 is currently underway. This could identify changes in steady state gene expression which persist after vaccination, perhaps identifying

cell populations primed for rapid responsiveness to TLR signalling (myeloid) and inflammatory mediators (NK cells). Analysis of IL-15 stimulated pre and post-vaccination PBMC could characterise the pathways upregulated by IL-15 in vaccine primed vs baseline samples. Cellular metabolism is also increasing in interest in regards to trained or pre-activated innate immune cells; studies have indicated a shift towards aerobic glycolysis in NK cells after cytokine stimulation and enhanced glycolysis in trained compared to naïve monocytes (18, 19). Assessing epigenetic modifications and glucose metabolism pathways in NK cells and accessory cells after viral vaccination would be of particular interest for future work.

6.1.3 Altered NK Cell Phenotype after Vaccination

Previous data on the effect of vaccination on human peripheral blood NK cells showed activation (CD69 and CD25 expression) and proliferation (Ki67 expression) after live attenuated and whole inactivated vaccinations (6, 20, 21). I have demonstrated a transient change in NK cell phenotype after both inactivated influenza vaccination and vectored Ad26.ZEBOV/MVA-BN-Filo Ebola vaccination; the proliferative increase in frequency of CD56^{bright} NK cells was a comparable effect of the two vaccines. As less differentiated NK cell subsets express a wider compliment of cytokine receptors including CD25, IL-12Rβ2 and IL-18Rα, a cytokine-dependent activation of the less differentiated subsets *in vivo* in response to vaccination could be responsible. This is remarkable in the context of a viral vectored vaccine vs a whole organism vaccine, in the absence of whole organism PAMPs or adjuvants for additional PRR signalling. In light of the

cytokine response to EbovGP alone, perhaps this is not so unexpected, however, we cannot rule out effects of the viral vectors (Ad26 and MVA), utilised in this Ebola vaccine. Pre-existing immunity (neutralising antibodies) to Ad26 is lower than other viral vectors such as Ad5, however internalisation and genome delivery may expose PAMPs such as viral DNA, eliciting an immune response (22). Similarly, the conformation and structure of the virally vectored Ebola GP expressed after vaccination is unknown.

6.1.4 Characterisation of NK Cell Responses to Ebola GP Stimulation and Vaccination

I have thoroughly investigated innate cytokine and antibody-dependent activation of NK cells in response to Ebola GP before and after Ad26.ZEBOV/MVA-BN-Filo Ebola vaccination. Soluble Ebola GP induces high levels of innate cytokines (TNF-α, IL-1β, IL-10, IL-6, IFN-β and IL-12) in monocytic cell lines, monocyte-derived DCs and macrophages (23-26). However, the effect of this cytokine induction on cytokine-dependent NK cell function in primary whole human PBMC cultures has not been thoroughly evaluated. In this thesis, I measured whole human PBMC cytokine secretion in response to Ebola GP stimulation by Luminex, the cytokine signature and quantity was striking and merited further investigation. In comparison to H3N2 stimulation, EbovGP stimulated very high concentrations of GM-CSF, IL-10, IL-1β and TNF-α, but lower concentrations of IFN-α2 and IL-12(p70) (**Table 3**). This high level cytokine secretion (described as a cytokine storm) has been shown in the plasma of infected humans, and has

been likened to septic shock syndrome and pandemic 1918 influenza virus infection (27-29).

Cytokine	Median cytokine concentration (pg/ml)	
	EbovGP	H3N2
GM-CSF	460.9	13.9
IFN-α2	6.2	1041
IL-10	3151	25.7
IL-12(p70)	ND	5.1
IL-1β	3995	224
TNF-α	5542	902.2
IFN-γ	15.2	139.2

Table 3: Comparison of cytokine concentrations secreted *in vitro* in response to H3N2 and EbovGP stimulation.

Median concentration in pg/ml measured by Luminex (n=72 EbovGP, n=73 H3N2). Red; increase vs other antigen stimulation. ND; none detected.

The findings of chapter 4 are consistent with others showing TLR-4 dependent recognition of Ebola GP by human innate immune cells of myeloid lineage, leading to the secretion of inflammatory cytokines (24, 26). I have shown human CD14$^+$ monocytes in primary whole mononuclear cell preparations are key responders to soluble Ebola GP, where previous studies have been confined to human monocyte cell lines or *in vitro* differentiated DC and macrophages. Our study may be more relevant to vaccination (i.e. soluble GP in the absence of replicating virus) and subsequent environmental challenge, and also implicates CD14$^+$ monocytes for future *in vitro* study of innate immune responses to Ebola GP. Moreover, I have shown for the first time that human NK cell upregulation of

CD25 and degranulation after Ebola GP stimulation *in vitro* is dependent on IL-12 and IL-18 induced by the GP.

TLR-4 activates two signalling pathways, the MyD88 and TRIF pathways, leading to activation of NF-κB (for pro-inflammatory cytokine production) and IRF3 (for type I IFN production) respectively, both have been shown to be activated in response to Ebola VLP stimulation in mice (30). IL-18 production requires inflammasome activation and caspase-1 dependent cleavage into the mature, bio-active form (31). In THP-1 cells (human monocytic cell line), IL-1β and IL-18 were induced in response to Ebola GP stimulation via caspase-1 dependent NLRP3 inflammasome activation (25). NLRP3 inflammasome activity is thought to be primed by microbial ligand-dependent activation of NF-κB (via TLR engagement), and then activated by a secondary signal such as Ca^{2+} signalling, K^+ efflux, reactive oxygen species (ROS) production or ATP (31). Although, in human monocytes, LPS induced TLR-4 signalling alone was shown to activate NLRP3 inflammasome activity without the need for a second signal (alternative inflammasome activation) (32). LPS induced IL-18 production in mouse bone marrow-derived macrophages was dependent on type I IFN signalling suggesting an indirect mechanism and requirement for additional cytokine stimulation (33), however, IFNαβR2 blockade did not reduce NK cell function in response to EbovGP in our *in vitro* system. IL-12 and the observed high levels of IL-10 are likely a result of direct TLR-4 signalling pathways. RNA transcriptional analysis of unstimulated and EbovGP stimulated whole PBMC and purified CD14+ monocytes to identify such pathways is underway.

Ad26.ZEBOV/MVA-BN-Filo vaccination, in contrast to influenza vaccination, did not lead to an enhancement in NK cell function or innate cytokine secretion in response to restimulation post-vaccination. Instead, NK cell activation *in vitro*, in particular IFN-γ secretion, was limited and highly regulated by monocyte derived IL-10. It is conceivable that EbovGP expression *in vivo* after vaccination causes a similarly tightly regulated immune environment, a hypothesis supported by monocyte-mediated negative regulation of the immune response to the rVSV-ZEBOV Ebola vaccine (34). IL-10 supresses macrophage and DC cytokine secretion and T cell derived IL-2, factors important for CIML NK cell generation and enhanced NK cell function post-vaccination (35-37). This perhaps goes some way to explain the lack of CIML NK cell generation and NK cell recall responses to EbovGP stimulation in post-Ad26.ZEBOV/MVA-BN-Filo PBMC samples. It would therefore be interesting to analyse post-Ad26.ZEBOV/MVA-BN-Filo vaccination NK cell responses to EbovGP in the presence of IL-10 blockade.

Vaccination did lead to lower median concentrations of some inflammatory cytokines (significantly so for TNF-α) in response to EbovGP compared to baseline (**Table 4**). Interestingly, this observation was limited to Ad26 prime/MVA boost groups that exhibited more robust T cell cytokine responses (IL-2$^+$IFN-γ$^+$TNF-α$^+$ triple positive) (38-40). As inflammatory cytokines are associated with increased likelihood of death in EVD (29), it may be preferable that there is no detectable post-vaccination enhancement of these responses. Furthermore, many of these responses (IL-1β, TNF-α and GM-CSF) are already of a log magnitude higher than those induced by H3N2 so may not be amenable to boosting. It is hoped that vaccine induced virus-specific immunity could limit

inflammatory responses upon challenge via the induction of neutralising or ADCC antibody function or via cytotoxic T cells restricting Ebola viral loads or by antibody blockade of Ebola GP stimulatory activity.

Cytokine	Median cytokine concentration (pg/ml)		
	Baseline	**Post-boost**	**P value**
GM-CSF	460.9	410.5	0.211
IFN-α2	6.2	6.1	0.655
IL-10	3151	2700	0.449
IL-12(p70)	ND	ND	-
IL-1β	3995	3428	0.341
TNF-α	5542	4647	0.0232
IFN-γ	15.2	19.5	0.111

Table 4: Summary of cytokine concentrations secreted *in vitro* in response to EbovGP stimulation before (baseline) and after (post-boost) Ad26.ZEBOV/MVA-BN-Filo vaccination.

Data taken from chapter 4. Median concentration in pg/ml measured by Luminex (n=26). Green; decrease over baseline. P value determined by Wilcoxon signed-rank test, significant values determined as $p < 0.05$. ND; none detected.

In additional experiments, 5% pooled post-vaccination serum was unable to mimic the post-vaccination reduction in EbovGP induced inflammatory cytokine secretion in non-vaccinated, control PBMC, suggesting that vaccine induced cell intrinsic changes rather than antibody-mediated blockade may be playing a role. Treatment of mice with an Ebola GP VLP vaccine that increased survival after Ebola challenge, attenuated viral replication and the Ebola-induced inflammatory cytokine response (41). This reduced inflammatory response was linked to robust type I IFN responses regulating cytokine secretion by inducing IL-10, supressing inflammasome activity and the formation of mature IL-1β and IL-18 (42). I did

observe a small but significant decrease in NK cell CD25 expression in control PBMC in the presence of post-boost serum compared to baseline serum suggesting possible antibody-mediated blockade of EbovGP interaction with TLR-4 (**Appendix Supplementary Figure 2**). Further studies on the effect of vaccine-induced antibody on cytokine secretion and NK cell function and the effect of IL-10 blockade on the inflammatory response would contribute to the understanding of these observations.

6.1.5 Antibody-Dependent NK Cell Activation after Vaccination

I have also shown that anti-GP antibody in the serum of Ad26.ZEBOV/MVA-BN-Filo vaccinated individuals induced robust antibody-dependent NK cell activation against immobilised EbovGP. This response strongly correlated with antibody titre suggesting this is a robust read out for Fc-dependent antibody induced by vaccination. Individual antibody level data has been requested from the vaccine consortium in order to perform more detailed correlations between antibody titre and NK cell function at an individual level. This study implicates antibody-activated, more differentiated NK cells as important effectors of post-vaccination immunity against Ebola virus infection, not only for cytotoxicity but also for IFN-γ secretion when cytokine-dependent activation may be limited. I also found anti-GP antibody-dependent NK cell responses varied widely between donors in the presence of a constant antibody source (pooled post-boost sera), which was partly due to differences in NK cell differentiation subset frequency. It would be interesting to analyse antibody-dependent NK cell function in multiple Ad26.ZEBOV/MVA-BN-Filo vaccinated individuals to determine the extent to which the combined impact of antibody titre and NK cell differentiation determines NK cell function. In this context, the impact of HCMV serostatus, which is associated with a polarisation towards antibody-dependent responses, can also be assessed.

Both the production of innate inflammatory mediators and the NK cell functional differentiation profile are altered by age (43). The frequency of CD56^{dim}CD57^{+} NK cells increases over a lifetime, with a reciprocal decrease in CD56^{bright} NK cells; overall, NK cell IFN-γ secretion and responses to innate cytokine stimulation

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reduces while cytotoxicity is maintained (44, 45). In African populations, where HCMV seropositivity is near universal by the age of three, this differentiation process proceeds rapidly in childhood (46). Therefore, further work is needed to evaluate the effect of infant vaccination in the context of higher levels of inflammatory cytokines and reduced NK cell differentiation and in African populations where both age an exposure to HCMV may influence the outcome of the effector NK cell response. The more refined antibody-dependent mechanisms may be preferential over the non-specific inflammatory cytokine-dependent mechanisms for NK cell activation to reduce viral loads and contribute IFN-γ. **Figure 1** shows a schematic summary of EbovGP induced NK cell function.
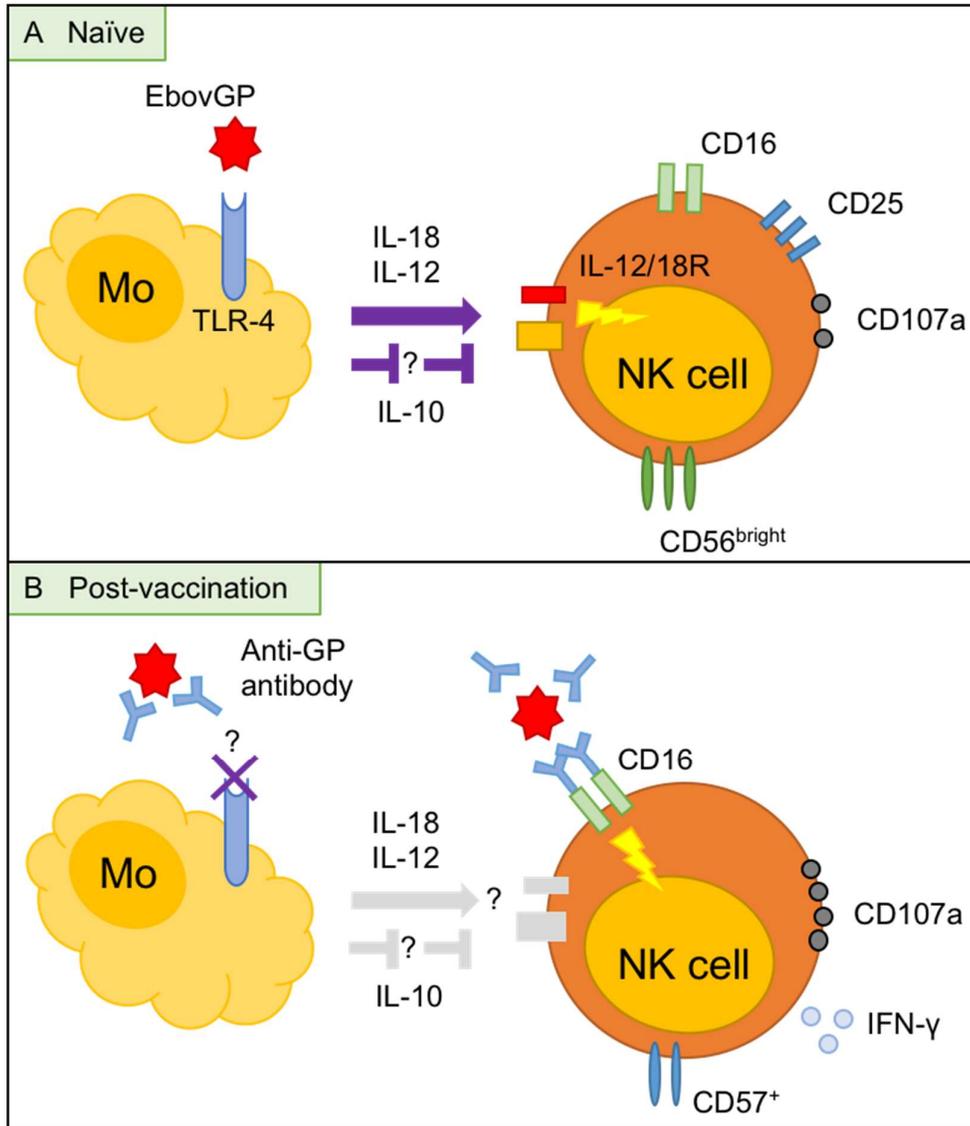
Figure 1: Mechanisms for NK cell activation in response to Ebola GP challenge in a naïve and vaccinated individuals.

Naïve donor; less differentiated NK cell (CD56bright) activation in response to monocyte (Mo) derived IL-12 and IL-18 released after to EbovGP TLR-4 stimulation. Monocyte derived IL-10 mediated inhibition of NK cell IFN-γ secretion, most likely indirectly via suppression of further innate cytokine secretion (a). Post-vaccination; more differentiated NK cell (CD57+) activation in response to anti-GP antibody via CD16 crosslinking, perhaps independent of monocyte derived IL-12 and IL-18. Anti-GP antibody blockade of GP stimulatory effects via TLR-4 and/or IL-10 mediated blockade of antigen-specific T cell activation may also play a role in regulating the post-vaccination NK cell response (b).

6.2 Conclusions

In conclusion, the generation of CIML NK cells by vaccination and subsequent memory-like NK cell recall responses upon restimulation is a process uniquely dependent on the cytokine signature secreted by the particular vaccine antigen involved and/or the type of vaccine administered. Although both vaccines studied during this thesis induced proliferation and expansion of less differentiated NK cells, this did not universally lead to vaccine-enhanced NK cell function. The utilisation of IL-15 to boost accessory cell cytokine secretion and therefore NK cell stimulation during vaccination could lead to better NK cell effector functions upon challenge, and perhaps cross-strain or universal protection. However, such boosting of innate immune function may only be beneficial in the context of certain viral infections and could be detrimental for others, including Ebola, which cause overt inflammatory responses. Finally, NK cell antibody-dependent activation in response to post-vaccination serum gathered as part of a vaccine trial, could be an informative and correlative readout of vaccine-induced antibody function and protection.

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Appendices

Supplementary Data
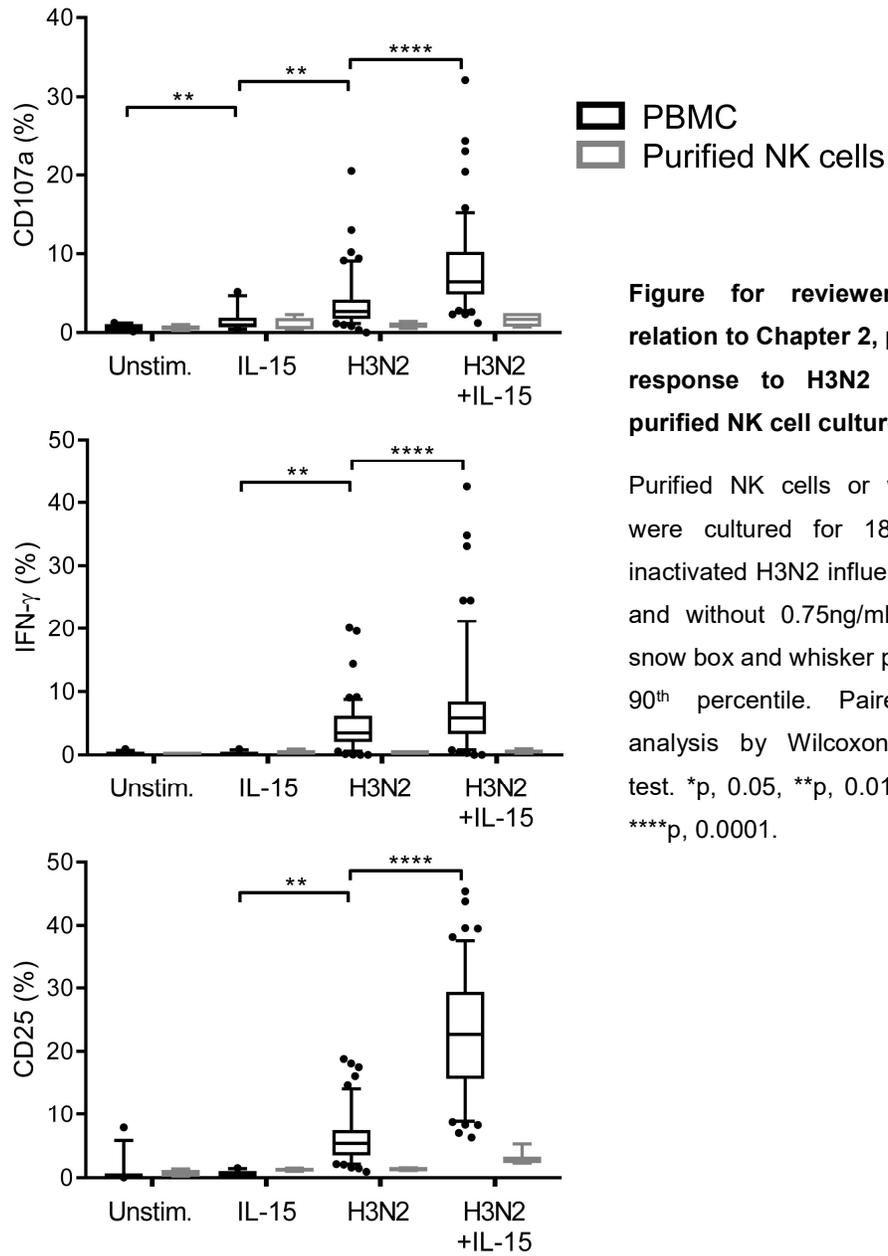
Figure for reviewers only; in relation to Chapter 2, page 126: No response to H3N2 or IL-15 in purified NK cell cultures.

Purified NK cells or whole PBMC were cultured for 18 hours with inactivated H3N2 influenza virus with and without 0.75ng/ml IL-15. Plots snow box and whisker plots with 10th-90th percentile. Paired statistical analysis by Wilcoxon signed-rank test. *p, 0.05, **p, 0.01, ***p, 0.001, ****p, 0.0001.
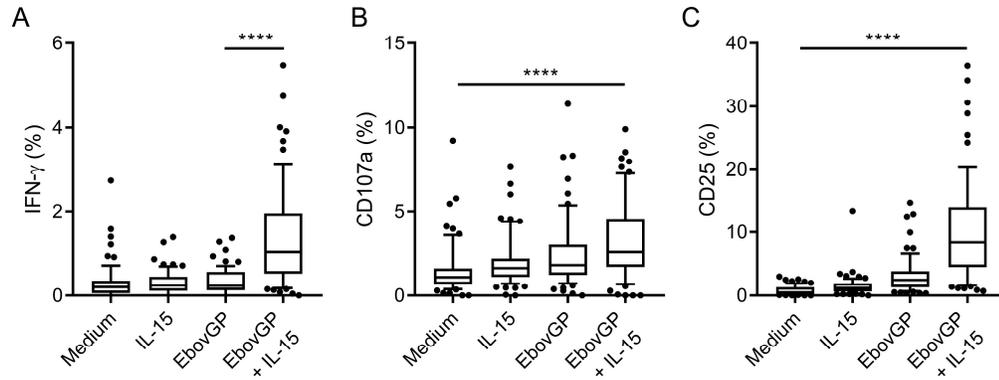
Appendix Supplementary Figure 1: Nanogram concentrations of soluble IL-15 boosts the NK cell response to EbovGP.

Whole PBMC from baseline (visit 0) were stimulated with 10µg/ml EbovGP alone, 0.75ng/ml IL-15 alone, EbovGP plus IL-15 or left unstimulated (medium) for 18 hours, n=72. Cells were stained for NK cell activation markers and analysed by flow cytometry. Frequencies of IFN-γ+, CD107a+ and CD25+ NK cells were gated using medium alone controls; gating strategies are shown in Chapter 4, Figure 2a. Graphs show NK cell IFN-γ (a), CD107a (b) and CD25 (c) expression as box and whisker plots with median, interquartile range (IQR) (box) and 10th-90th percentile (whiskers). Comparisons across conditions were performed using one-way ANOVA with Dunn's correction for multiple comparisons. ****p < 0.0001.

Appendix Supplementary Figure 2: Ad26.ZEBOV/MVA-BN-Filo vaccine-induced antibody mediated reduction in NK cell CD25 expression.

Non-vaccinated control PBMC were stimulated with EbovGP or left unstimulated in the presence of pooled pre (baseline; visit 0) or post (post-boost; visit 2) vaccination serum for 18 hours, n=16. NK cell CD25 expression was determined by flow cytometry; gating strategies are shown in Chapter 4, Figure 2a. Graphs show CD56^{bright} NK cell CD25 percentage (a) and MFI (b), CD56^{dim}CD57$^-$ (c) and CD56^{dim}CD57$^+$ (d) CD25 percentage. Graphs show one point per donor with median and IQR. Comparisons between conditions were performed using Wilcoxon signed-rank test. *p < 0.05, **p < 0.01, ****p < 0.0001.

Publications (PDF published versions)

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REVIEW

Vaccinating for natural killer cell effector functions

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Abstract

Vaccination has proved to be highly effective in reducing global mortality and eliminating infectious diseases. Building on this success will depend on the development of new and improved vaccines, new methods to determine efficacy and optimum dosing and new or refined adjuvant systems. NK cells are innate lymphoid cells that respond rapidly during primary infection but also have adaptive characteristics enabling them to integrate innate and acquired immune responses. NK cells are activated after vaccination against pathogens including influenza, yellow fever and tuberculosis, and their subsequent maturation, proliferation and effector function is dependent on myeloid accessory cell-derived cytokines such as IL-12, IL-18 and type I interferons. Activation of antigen-presenting cells by live attenuated or whole inactivated vaccines, or by the use of adjuvants, leads to enhanced and sustained NK cell activity, which in turn contributes to T cell recruitment and memory cell formation. This review explores the role of cytokine-activated NK cells as vaccine-induced effector cells and in recall responses and their potential contribution to vaccine and adjuvant development.

Keywords: accessory cell, cytokines, NK cell, vaccination.

INTRODUCTION

Vaccination is a cost-effective way of reducing the burden of, eliminating and – in exceptional cases – eradicating infectious diseases. Whilst a number of effective vaccines are currently licenced, many highly prevalent and complex diseases remain without effective prophylactic vaccines. Protective titres of neutralising antibodies and expanded populations of effector and memory B and T lymphocytes are viewed as measures of protection for many vaccines. Currently, the generation of durable antigen-specific memory forms the basis of vaccine development and evaluation of vaccine efficacy.[1,2]

Developing vaccines to overcome pathogen polymorphism and complexity demands new approaches to vaccine design and evaluation; this in turn requires the identification of novel correlates of protection and determination of optimal dosing schedules. The activation of Natural Killer (NK) cells represents a potential route for further optimisation of vaccination strategies by harnessing their role as antipathogen effector cells which integrate innate and acquired immune responses.

NK cells are large, granular, type 1 lymphoid cells that express a wide variety of germline-encoded receptors on their surface. Direct NK cell activation is controlled by the balance between

signals transduced by inhibitory and activating receptors; NK cells are also activated indirectly by innate cytokines such as type I interferons (IFN), IL-12, IL-15 and IL-18, released from accessory cells.[3] Because NK cells do not rearrange receptors to permit antigen-specific recognition, they are normally classified as cells of the innate immune system. NK cells are among the first cells to respond during primary infection and contribute to the early control of viral infections including herpesviruses and influenza infections.[4–8] However, NK cells can also augment and shape the subsequent adaptive response by secretion of cytokines (including IFN-γ) and chemokines (such as IP-10, MIP-1α and MIP-1β), reducing viral loads by killing infected cells, inhibiting viral entry and replication by production of chemokines which compete for human immunodeficiency virus (HIV) coreceptor CCR5[9] and by controlling expansion of the CD4^{+} T cell compartment.[10,11] In turn, the secondary (adaptive) immune response can activate NK cells via secretion of cytokines such as IL-2 from CD4^{+} T cells and via FcγRIIIa (CD16)-dependent recognition of antigen–antibody complexes.[12–14]

Several studies have shown that NK cells can acquire some features of adaptive lymphocytes, raising the possibility that the memory-like properties of these cells could be induced or enhanced by vaccination. Early examples of NK cell adaptive features arose from mouse studies of murine cytomegalovirus (MCMV) and hapten-induced contact hypersensitivity. The MCMV viral protein m157 on the surface of infected cells was shown to recognise NK cell Ly49H receptor and leads to clonal expansion of effector NK cells and generation of a pool of self-renewing m157-responsive NK cells; these cells respond robustly to subsequent MCMV infection when transferred to naive mice.[15] NK cells from Rag2^{−/−} mice were shown to transfer hapten-specific memory-like responses (lasting up to 4 weeks) to naive mice despite the absence of T and B cell immunity.[16] More recently, virus antigen-specific NK cell killing has been reported in rhesus macaques,[17] and there are suggestions of pathogen-specific responses among human NK cells. These include influenza hemagglutinin (HA) antigen recognition by NK cell NKp46 and HLA-E stabilisation by HCMV peptides for recognition by NK cell CD94/NKG2C heterodimers.[18–20]

An increasing number of studies in humans demonstrate activation of NK cells during recall responses to pathogens in vaccinated subjects. *In vitro* NK cell responses to components of the DTP vaccine (diphtheria toxoid, tetanus toxoid and whole cell inactivated pertussis), Bacille Calmette–Guérin (BCG) and influenza vaccine are enhanced after vaccination[14,21–23] and heightened NK cell IFN-γ and degranulation responses have been detected after vaccination against rabies.[24] In contrast to the memory responses described above, these postvaccination responses are dependent on vaccine-specific CD4^{+} memory T cells and, in particular, their rapid secretion of IL-2.[23,24]

Although the 'antigen-specificity' of these postvaccination NK cell responses resides in the CD4^{+} T cell pool, the NK cells are also modified as a result of vaccination. Innate cytokines, which can be induced by live or killed whole pathogen vaccines or by adjuvants, are potent NK cell activators and can induce their differentiation into cytokine-induced memory-like (CIML) NK cells. First described as being generated by cytokine coculture *in vitro,* CIML NK cells have an enhanced ability to secrete IFN-γ and become cytotoxic in response to cytokine and MHC-devoid K562 cell restimulation for up to 21 days after the initial stimulation.[13,25–27] *In vitro* cytokine activation with IL-18 and IL-12 and/or IL-15 induces expression of CD25, thereby generating CIML NK cells with enhanced responsiveness (demonstrated by IFN-γ production and cytotoxicity) to picomolar concentrations of IL-2.[28] More importantly perhaps, CIML NK cells can be induced by vaccination in response to CD4^{+} T cell-derived IL-2 and myeloid cell-derived IL-12 and type I interferons, and have been implicated in the enhancement of NK cell function *ex vivo.*[13,24,29–33] Vaccination-induced CIML NK cells can be detected for up to 12 weeks postvaccination in European subjects[13] and up to 6 months in west African vaccines.[33] Table 1 summarises the evidence for vaccination-induced CIML NK cells.

Immature CD56^{bright} and CD56^{dim}CD57^{−} NK cells are more responsive to exogenous cytokines and produce more IFN-γ than do the more mature, predominantly cytotoxic, CD56^{dim}CD57^{+} NK cell subset.[34] Accordingly, vaccination-induced CIML NK cells are restricted to the less differentiated subsets of NK cells and their induction is accompanied by proliferative expansion of the least mature CD56^{bright} NK cells and CD56^{dim}CD57^{−}NKG2C^{−/+} subsets.[13,33] Enrichment of less differentiated NK cells in lymph nodes and effector tissues could influence the impact of CIML NK cells induced by vaccination. The highest proportion of human

Table 1. Evidence of induction of human cytokine-induced memory-like (CIML) NK cells by vaccination

Pathogen	Host species	Vaccination	Increased responsiveness *in vitro*	Duration of response	References
Influenza	Human	TIV	IL-12, IL-15, IL-18	3 months (UK) or 6 months (Gambia)	Goodier et al.[60] and Darboe et al.[33]
YFV	Human	YF-17D	IL-12	15 days	Marquardt et al.[30]
SIV	Macaque	Ad26 HIV-1, DNA-Ad26	IL-12, IL-15	38 weeks	Vargas-Inchaustegui et al.[77]
TB	Human	BCG	IL-12, IL-18	ND	Suliman et al.[29]

ND, not determined.

immature CD56^{bright} (CD16^{−}) NK cells are found in the lymph nodes[12,35] and produce IFN-γ in response to CD4^{+} T cell-derived IL-2, thereby potentially influencing subsequent adaptive responses.[12] A higher percentage of adoptively transferred pre-activated CIML NK cells were found in the lymph nodes of mice compared to control NK cells[25] and localised in the bone marrow, spleen and blood of mice and in the bone marrow of patients with acute myeloid leukaemia.[27] The tissue localisation of NK cells amenable to cytokine-mediated pre-activation may also be crucial to functional outcomes. Human liver, in contrast to secondary lymphoid tissues, is enriched for resident CD56^{bright} NK cells with high natural cytotoxicity receptor and NKG2D expression, strong target cell-mediated degranulation but poor IFN-γ production.[36] Tissue-resident innate lymphoid cells (ILC) which are phenotypically distinct from NK cells may, however, also be sensitive to pre-activation by vaccine-induced cytokines. Murine liver ILC-1, for example, is highly sensitive to IL-12 stimulation and produces more IFN-γ at the sites of MCMV infection.[37] When taken together with the emerging literature on the impact of persistent viral infections (such as human cytomegalovirus infection (HCMV); see below) on NK cell function,[14] it is possible that differences between or within human populations in proportions of CIML NK cells (due to differences in recent infection and vaccination history) may contribute to differences in vaccine immunogenicity and effectiveness.[33,38,39]

Expanded populations of highly differentiated NK cells in individuals chronically infected with HCMV were first described more than a decade ago.[40] Many of these highly differentiated cells were subsequently shown to have undergone key intrinsic changes such as the loss of the signalling molecules FcεRγ, SYK and EAT-2, associated with stable epigenetic changes in the promotor regions of genes involved in NK cell function, including IFN-γ.[41–43] These 'adaptive' NK cells display enhanced antibody-dependent cellular cytotoxicity (ADCC) activity towards HCMV-infected target cells suggesting they are specialised for controlling virus reinfection or reactivation and antigen-specific.[44,45] However, despite the likely dominance of such adaptive cells in populations with endemic HCMV infection, the generation of CIML from less differentiated NK cells persists after vaccination[33] (reviewed in ref. 46). It appears, therefore, that there is a balance of CIML and highly differentiated NK cell effectors which may be altered by vaccination. Less differentiated NK cells are shorter-lived, possess higher levels of cytokine receptors and higher intrinsic proliferative capacity; vaccination may simply contribute to the homoeostatic maintenance of these cells. The benefits of preferentially expanding and generating CIML NK cells from these subsets are unknown but could be more functionally significant in young infants where highly differentiated cytotoxic effectors are lacking.[47] On the other hand, loss of IL-12 responsiveness and independence of this cytokine for IFN-γ production is a well-known feature of more differentiated NK cell effectors; more focused antibody-driven responses may be advantageous in restricting the potential for inflammation associated damage in older individuals.

In the remainder of this review, we explore in more detail the potential role of NK cells, activated by myeloid cell-derived cytokines or by components of adaptive immunity (CD4^{+} T cell IL-2 or pathogen-specific antibodies), as effectors

of vaccination against a number of globally important infectious diseases.

INFLUENZA

Seasonal influenza epidemics result in 3–5 million cases of influenza globally and up to half a million deaths every year as well as putting intolerable pressure on health systems and causing major economic losses.[48] Annual variation in the predominant circulating strains of influenza viruses mitigates vaccine-induced or naturally acquired cross-protective immunity, necessitating annual revaccination of high-risk groups (pregnant women, children of 6 months to 5 years and the elderly).[48] A cross-protective 'universal' influenza vaccine is a major priority for influenza vaccine development.

Influenza virus induces secretion of innate cytokines (including IFN-α, IL-12 and IL-18) from accessory cells such as macrophages and dendritic cells (DCs); in turn, these cytokines support the very rapid activation of NK cells (within hours of infection).[49,50] These activated NK cells are cytotoxic, secrete IFN-γ and upregulate cytokine receptors such as CD25 (IL-2Rα)[51] and can reciprocally activate DCs, thereby promoting T cell recruitment to sites of infection and to lymph nodes.[52] *In vitro* restimulation of peripheral blood mononuclear cells (PBMC) from trivalent influenza vaccine (TIV)-vaccinated volunteers with inactivated influenza virus induces much higher frequencies of IFN-γ producing and degranulating NK cells compared to restimulation of prevaccination PBMC from the same people.[13,18,23,53] The heightened NK cell response becomes evident as early as 2 weeks postvaccination but is normally lost by 12 weeks. Postvaccination enhancement of NK cell IFN-γ production was dependent on IL-2 produced from CD4+ T cells, whilst degranulation responses were dependent on IL-2 and on the presence of anti-influenza antibody.[13,23] A costimulatory role for innate myeloid cell-derived cytokines was also demonstrated by partial inhibition of TIV restimulation responses with IL-12, IL-18 and IFN-αβR2 blockade.[13]

Indeed, consistent with the generation of CIML NK cells, antigen-independent *in vitro* responses to exogenous IL-12 and IL-18 were also elevated for up to 3 months after influenza vaccination in a UK study,[13] but this response was detected for up to 6 months in African subjects.[33]

Enhancement of NK cell responses after influenza vaccination is therefore mediated by indirect mechanisms involving antigen-specific cellular CD4+ and humoral responses combined with a shorter-lived CIML component. Such enhanced NK cell function after seasonal influenza vaccination may contribute to protective immunity to influenza, but, given the dependence on antigen-specific T cells and antibodies, does not in itself overcome the need for regular revaccination. However, the search for a 'universal influenza vaccine' has identified the conserved 'stalk' of the polymorphic HA molecule[54] and other nonvaccine antigens[55] as possible targets of broadly neutralising antibodies which mediate ADCC.[56,57] Stalk-specific antibodies that mediate NK cell ADCC are present after natural infection and after vaccination with TIV or monovalent adjuvanted H1N1[58] and nucleoprotein (NP)-specific ADCC-mediating antibodies induced by seasonal influenza vaccination demonstrate cross-reactivity with H7N9 avian influenza NP.[59] As mature CD56dimCD57+ NK cells and HCMV-induced 'adaptive' NK cells are both potent mediators of ADCC and preferentially respond to influenza antigens after vaccination,[60] NK cells may be of particular importance as effectors of the next generation of universal influenza vaccines.

YELLOW FEVER

The live attenuated yellow fever virus (YFV) vaccine 17D is one of the most effective vaccines developed to date; 99% of recipients are protected for more than 10 years after a single vaccination.[61] For this reason, YF-17D has been used as a tool to identify highly effective early (innate) immune responses to acute viral infection in humans.[30,62] YFV infects and induces TLR-mediated signalling in hepatocytes and cells of the innate immune system such as monocytes and DCs. In mouse models of YFV infection or YF-17D vaccination, NK cells accumulate in the spleen and are major producers of IFN-γ.[63,64] Induction of innate cytokines such as IL-1α and chemokine IP-10 (CXCL10), and upregulation of the early activation and proliferation markers CD69 and Ki-67 on NK cells are detected as early as 3 days postvaccination in humans.[30,62,65] NK cell activation peaks at the same time as viral load, 6 days postvaccination and correlates directly with a rise in plasma type I and type III interferons. Thereafter, viral load and NK cell responses

decline rapidly returning to baseline by day 10 and 15 postvaccination, respectively.[30,65]

In a study in Uganda, pre-existing IFN-γ producing NK cells in an activated immune microenvironment were associated with lower viral loads and subsequently reduced antibody titres after YF-17D vaccination.[38] NK cell IFN-γ responses to YFV correlated with increased in vitro responsiveness of less differentiated NK cells to innate cytokines such as IL-12 after vaccination[30] suggesting that, as for influenza vaccines, YF-17D-induced accessory cell-derived cytokines may also induce development of CIML NK cells. As in influenza vaccination, this pre-activation state is short-lived suggesting that there is no lasting imprint on the NK cell repertoire. These transient innate responses (including NK cells) may, however, synergise with antigen-specific vaccine-induced responses resulting in the formation of particularly durable and effective T cell- and B cell-mediated immunity to YFV.[30,65] A more robust mechanistic understanding of the induction and function of CIML NK cells during infection or vaccination with YFV and other flaviviruses will help to define their role.

HUMAN IMMUNODEFICIENCY VIRUS

HIV remains highly prevalent across the world with 2.1 million new infections estimated in 2015; lifelong treatment is required to prevent disease and death, which places a considerable burden on health systems worldwide.[66] A prophylactic HIV vaccine is of utmost priority. HLA-I and killer cell immunoglobulin-like receptor (KIR) genotype and NK cell education influence killing of HIV-1-infected CD4$^+$ T cells and are associated with the rate of progression of HIV infection.[67,68] In the partially successful RV144 vaccine trial, IgG against variable regions 1 and 2 of the HIV-1 envelope glycoprotein was inversely correlated with the rate of infection.[69] Indeed, RV144 induced isotypes IgG1 and IgG3 targeting the crown of the V2 loop demonstrating the potential for NK cell ADCC induction.[70,71] NK cells from KIR3DL1/HLA-Bw4$^+$ or KIR2DL1/HLA-C2$^+$ donors show higher cytotoxicity against HIV-infected targets in the presence of anti-HIV gp120 antibody, highlighting the influence of NK cell education to HIV vaccine-induced effector NK cells and potentially contributing to individual variability in vaccine outcomes.[72,73] CD57$^+$NKG2C$^+$ memory-like NK cells are expanded in HIV-1/HCMV co-infected individuals, and these cells make a potential contribution to control of viremia during primary HIV infection.[74,75] Together with evidence that individuals with a degree of inherent resistance to HIV – so-called elite controllers or slow progressors – mount stronger antibody-mediated NK cell activation and ADCC responses than more susceptible individuals, these studies suggest that NK cells may contribute to HIV protection and control.[76]

NK cells have been implicated as antigen-specific effector cells after vaccination or infection of nonhuman primates with simian immunodeficiency virus (SIV); target cells pulsed with SIV vaccine antigen but not heterologous antigens can be lysed in vitro by splenic and hepatic NK cells from infected but not from uninfected animals.[17] These antigen-specific responses could be detected for at least 5 years after SIV DNA/adenovirus prime-boost vaccination, suggesting that this memory-like response is long-lived.[17] By contrast, no significant potentiation of circulating NK cell function was observed after SIV infection or vaccination; rather, SIV infection impaired the cytotoxic response of peripheral blood NK cells.[77] However, a trend towards increasing in vitro NK cell CD107a expression in response to IL-15 and IL-12 postvaccination suggests that memory-like NK cells with enhanced cytokine responsiveness may have been induced in this study.[77]

In HIV patients, therapeutic HIV vaccination or IL-2 treatment sustains or enhances NK cell activity.[32,78] Immunisation of chronically infected patients with an adjuvanted HIV-1 Gp120/NefTat subunit protein vaccine induces IL-2 from T helper cells and an increase in NK cell IFN-γ production in vitro; NK cell IFN-γ production was reduced by depletion of CD4$^+$ T cells and almost completely abrogated after blocking both IL-2 and IL-12, suggesting a role for accessory cells in full NK cell effector functions after vaccination.[32] These, and other, studies highlight the potential of therapeutic vaccination to restore NK cell function during chronic HIV infection.[32,79]

EBOLA

Several vaccines are in development for the prevention of Ebola virus disease (EVD). Two vectored vaccines that express the glycoprotein (GP) from the Zaire strain of Ebola (ZEBOV) and use the recombinant vesicular stomatitis virus and Chimp Adenovirus type 3 (rVSV-ZEBOV and

ChAd3-ZEBOV, respectively) are the most advanced of these.[80] Ebola virus has a wide range of host cell targets including macrophages and DCs, infection of which aids viral dissemination and crucially leads to immune dysregulation.[81] Little is known about the role of NK cells in Ebola virus infection but *in vitro* studies show IFN-inhibiting domains (IIDs) within Ebola viral proteins VP24 and VP35 interrupt DC maturation and type I IFN signalling leading to somewhat impaired NK cell activation and cytotoxicity.[82] Disrupting either of these IIDs restores DC maturation and NK cell activation as measured by NKp46 and CD38 expression.[82] Another study showed that Ebola virus-like particles (VLPs) lacking IIDs activated NK cells and led to lysis of filovirus-infected autologous human DCs in culture and pro-inflammatory cytokine release.[83]

Activation of the early inflammatory response and release of cytokines such as IP-10, IL-1β, IL-6 and TNFα, correlated with survival from EVD in humans[81,84] and mice can be protected against Ebola by adoptive transfer of NK cells from VP40 containing VLP-treated mice.[85] Increased survival of mice after postexposure vaccination with the candidate vaccine rVSVΔG-EBOV is reversed by NK cell depletion;[86] postexposure vaccination stimulated a burst of IFN-γ release and type I IFN secretion from accessory cells, potentially kick-starting the antiviral response and overcoming the blockade caused by IIDs.[86] Postexposure antibody therapy has also been shown to give effective protection in animal models via ADCC activity.[87,88] These studies implicate NK cells as important effectors in protection against Ebola virus infection and in vaccine-induced immunity and raise the potential of indirect cytokine activation of NK cells to restrict virus dissemination after therapeutic vaccination.

MALARIA

The role of NK cells in natural immunity or vaccine-induced protection against malaria infection remains to be established.[89] NK cell activation has been described to varying degrees in different experimental murine models[90,91] and NK cells have been shown to contribute directly to the elimination of *Plasmodium falciparum*-infected red blood cells (RBC) in a humanised mouse model.[92] *In vitro* studies of human PBMC show NK cells are readily activated by *P. falciparum*-infected RBC; the resulting NK cell

proliferation, IFN-γ production, CD25 and CD69 expression were further demonstrated to be dependent on IL-2 and accessory cell IL-12 and IL-18 production and on cell–cell contact.[93–96] In humans, long-lasting NK cell activation has been reported in controlled human malaria infection (CHMI) studies; a decrease in peripheral blood NK cell frequency early after infection suggests migration of NK cells into the tissues, possibly the liver.[97–99]

RTS,S/AS01 is the most promising vaccine tested to date for human *P. falciparum* malaria. RTS,S consists of recombinant circumsporozoite surface protein (CSP) of *P. falciparum* fused to the hepatitis B virus surface antigen (HBs) and adjuvant delivery system (AS)01 formed into VLPs. PBMC collected from a RTS,S randomised controlled trial revealed postvaccination IL-2 secretion with IFN-γ and CD69 upregulation on NK cells in response to *in vitro* restimulation with HBs or CSP. All responses were significantly higher in RTS,S vaccines compared to control rabies vaccinated subjects.[31] A weak association has been reported between IL-2 secreting CD4+ T cells and time to parasitaemia, accompanied by an increase in the proportion of CD56bright NK cells, higher IFN-γ and perforin expression, and protection against malaria challenge in vaccine recipients has also been reported.[100] Interestingly, peripheral blood NK cell gene expression signatures were negatively correlated with RTS,S-induced malaria protection, consistent with migration of activated blood NK cells to the tissues,[101] which implies that peripheral NK cell responses to malaria play a minimal role in vaccine responses.

TUBERCULOSIS

The live attenuated BCG vaccine is the only vaccine currently licenced for the prevention of tuberculosis disease (TB) caused by *Mycobacterium tuberculosis* (M.tb) and is administered to over 120 million infants each year.[102] NK cells are an important component of the cellular immune response to BCG, producing more than half of the total IFN-γ after vaccination in newborns and 2-month-old infants.[102]

BCG, and other live vaccines such as measles vaccine, have been shown to induce nonspecific effects that are beneficial to the recipient and reduce overall mortality in a community.[103,104] Potential underlying mechanisms include T cell-mediated cross-reactivity and/or 'training' or

'priming' of innate immune cells, including monocytes and NK cells. Increased expression of pattern recognition receptors (PRR) in monocytes, and higher levels of IFN-γ, TNFα and IL-1β secretion have been observed when PBMC from BCG-vaccinated individuals are restimulated with mycobacterial or unrelated antigens, compared to prevaccination PBMCs.[22] These effects persisted for up to 12 months after BCG vaccination and were partly attributed to epigenetic remodelling of key cytokine gene loci and have been termed 'trained immunity'. Similarly, increased NK cell CD69 expression in response to Pam3Cys has been reported in post-BCG vaccination samples from infants and correlated with higher concentrations of IL-12 secretion.[105] Interestingly, no changes in NK cell phenotype, maturation or IFN-γ production were reported in BCG-trained NK cells,[106] suggesting that they are not equivalent to CIML NK cells.

Enhancement of NK cell IFN-γ responses to BCG has been reported after BCG vaccination of patients with latent TB[29] and in 5-week-old infants who were BCG-vaccinated at birth compared to unvaccinated controls;[29] NK cell responses were completely abrogated by neutralisation of IL-12 and IL-18.[29] Consistent with studies of other whole organism vaccines, as described above, these studies indicate that enhanced responsiveness to cytokines is a key feature of vaccine-mediated effects on NK cells.

THE ROLE OF VACCINE ADJUVANTS IN PROMOTING NK CELL RESPONSES

Killed whole organism or live attenuated vaccines are both highly immunogenic and particularly effective at potentiating NK cell responses; both of these traits likely reflect the presence of potent pathogen-associated molecular patterns (PAMPs)
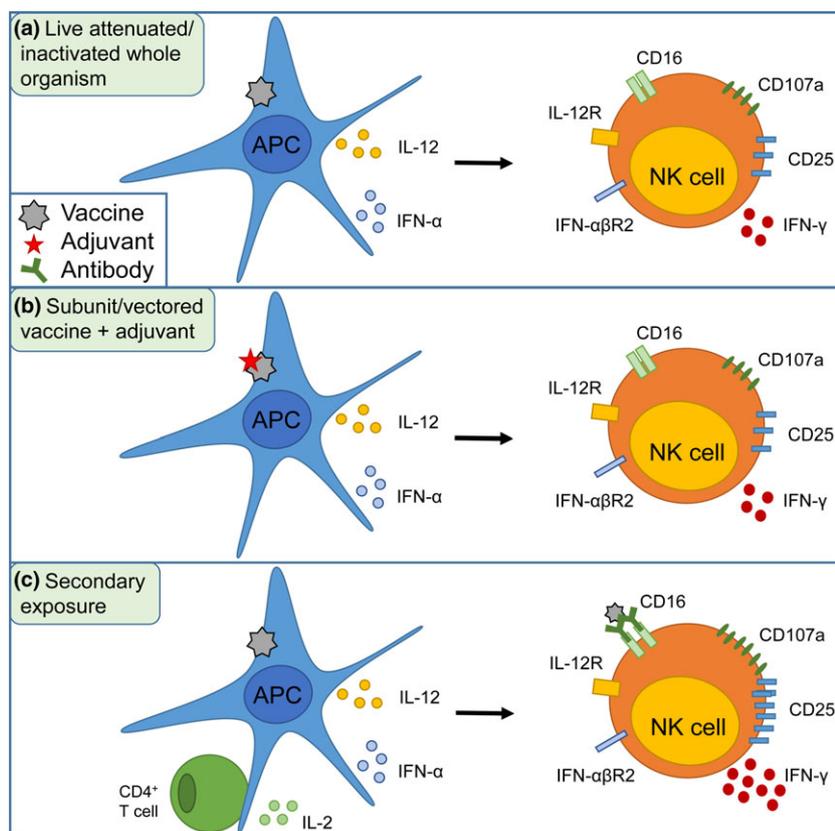
Figure 1. Accessory cell-dependent NK cell activation after vaccination. **(a)** Activation of APCs by live attenuated or inactivated whole organism vaccines induces the release of costimulatory cytokines which in turn leads to NK cell activation including IFN-γ release, degranulation and CD25 upregulation. **(b)** Adjuvants promote accessory cell function for subunit or vectored vaccines in the absence of vaccine-derived PAMPs. **(c)** Upon secondary exposure, IL-2 from memory CD4+ T cells, antibody and the presence of CIML NK cells enable an enhanced response.

for PRR-mediated accessory cell activation. PAMP-containing adjuvants are typically required to improve the immunogenicity of subunit or vectored vaccines, which lack these ligands. Several studies have documented enhancement of NK cell activation by adjuvants.[32,107,108] IL-15-matured DCs exposed *in vitro* to the TLR-4 agonist AS04-adjuvanted human papilloma virus (HPV) VLP vaccine can potentiate NK cell activation and killing of HPV-infected cells compared to either IL-4-matured DCs or VLP alone; this effect was attributed to the superior cytokine-producing ability of the DCs.[109] Similarly, vaccination in the presence of exogenous IL-15 enhances DC maturation and protection against lethal staphylococcal enterotoxin B challenge in mice compared to vaccine alone.[110]

AS03, a squalene-based adjuvant, promotes recruitment of antigen-presenting cells (APCs) and antigen processing. A system-wide analysis of the response to AS03-adjuvanted inactivated H5N1 influenza vaccine revealed a direct correlation between IP-10, type I and II interferon production, and enhanced NK cell activation and proliferation.[111,112] Similarly, a bursin-like peptide shown to stimulate immune cells induced higher levels of IL-2 and IL-4 and increased NK cell frequencies and IFN-γ secretion in mice vaccinated with inactivated influenza H9N2 compared to vaccine alone.[113] Taken together, these studies indicate that PRR-mediated activation and maturation of accessory cells such as DCs by vaccine adjuvants increase the production of costimulatory cytokines leading to heightened NK cell activation. Whether these NK cells share features of CIML NK cells has not yet been formally tested.

CONCLUDING REMARKS

Although there is now considerable evidence of enhanced NK cell responses after vaccination, the functional importance of NK cells in vaccination-induced immunity is rather difficult to evaluate. The NK cell response to vaccination varies depending on the type of vaccine, the cytokine signature induced by the vaccine/adjuvant combination and subsequent accessory cell activation (Figure 1). The ability of NK cells to respond to signals from both innate and adaptive immune cells suggests that when one arm of the immune response is impaired, such as T cell responses in HIV infection or innate cell dysregulation in EVD, NK cells may play an important immune effector role, maximising the impact of the remaining arm of the immune system. Successful activation of APCs and induction of an early inflammatory response by a vaccine correlate with enhanced and sustained NK cell activation and function. Importantly, NK cell education by HLA-KIR or other receptor-ligand combinations may well calibrate functional capacity on induction by both adaptive and innate pathways thereby driving individual variability in vaccine-induced responses. The addition of adjuvant systems to vaccines to increase accessory cell activation and therefore augmenting NK cell function including ADCC activity could play a role in the future design of new vaccines, postexposure therapy, therapeutic cancer vaccines, regimen optimisation and evaluation of vaccine efficacy.

CONFLICT OF INTEREST

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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IL-15 Promotes Polyfunctional NK Cell Responses to Influenza by Boosting IL-12 Production

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IL-15 is a key regulator of NK cell maintenance and proliferation and synergizes with other myeloid cell–derived cytokines to enhance NK cell effector function. At low concentrations, *trans*-presentation of IL-15 by dendritic cells can activate NK cells, whereas at higher concentrations it can act directly on NK cells, independently of accessory cells. In this study, we investigate the potential for IL-15 to boost responses to influenza virus by promoting accessory cell function. We find that coculture of human PBMCs with inactivated whole influenza virus (A/Victoria/361/2011) in the presence of very low concentrations of IL-15 results in increased production of myeloid cell–derived cytokines, including IL-12, IFN-α2, GM-CSF, and IL-1β, and an increased frequency of polyfunctional NK cells (defined by the expression of two or more of CD107a, IFN-γ, and CD25). Neutralization experiments demonstrate that IL-15–mediated enhancement of NK cell responses is primarily dependent on IL-12 and partially dependent on IFN-αβR1 signaling. Critically, IL-15 boosted the production of IL-12 in influenza-stimulated blood myeloid dendritic cells. IL-15 costimulation also restored the ability of less-differentiated NK cells from human CMV-seropositive individuals to respond to influenza virus. These data suggest that very low concentrations of IL-15 play an important role in boosting accessory cell function to support NK cell effector functions. *The Journal of Immunology*, 2018, 200: 2738–2747.

I nterleukin-15 is essential for the survival, proliferation, and functional integrity of NK cells and is being exploited to enhance NK cell–mediated immunotherapies (1, 2). IL-15 augments NK cell expression of perforin, granzyme B, natural cytotoxicity receptors NKp30 and NKp44 (3), and the activating receptor NKG2D (4). The potency of IL-15, even at very low concentrations, is due, in part, to its presence as a complex with the α-chain of its own receptor (IL-15Rα) at the surface of APCs (5), where it can be presented to the same cell (*cis*-presentation) or to neighboring cells (*trans*-presentation), such as NK cells and CD8$^+$ T cells that express IL-15Rβ and the common γ-chain receptor (IL-15Rβγc) (6).

Dendritic cells (DCs) can be induced to present IL-15 at their surface by microbial ligands signaling through TLR and by innate cytokines, such as GM-CSF and type I IFN (7, 8). DC-mediated NK cell activation is dependent, in part, on IL-15 that polarizes to the DC–NK cell synapse during conjugate formation (9). Although IL-15 is believed to mediate the majority of its effects via *trans*-presentation by IL-15Rα, at high concentrations it can bind directly to IL-15Rβγc and, thereby, activate NK cells (10). Furthermore, at very high concentrations, free IL-15 may bind to IL-15Rα on neighboring cells for *cis*- or *trans*-presentation (5, 11). Low concentrations of IL-15 alone induce negligible NK cell activation, but IL-15 is highly synergistic with other cytokines and with recall Ags, such as influenza, for NK cell CD25 and IFN-γ expression (12, 13).

IL-15 *trans*-presentation is being explored for cancer immunotherapy; induction of constitutive expression of IL-15 and IL-15Rα by DCs or the use of soluble IL-15/IL-15Rα complexes has been shown to enhance NK cell antitumor activity in vitro and in preclinical mouse studies (reviewed in Ref. 14). Another strategy for NK cell immunotherapy includes activation of NK cells with IL-12, IL-15, and IL-18 prior to adoptive transfer, which reduces tumor growth in mice (15). Preactivation of PBMCs with high concentrations of IL-15 can also restore impaired NK cell cytotoxicity of SIV-infected macaques (16). Although these studies are consistent with direct NK cell priming by IL-15 at high concentration, or synergy with myeloid cell–derived cytokines at low concentration, the potential for IL-15 to amplify the myeloid cell response has not been thoroughly explored. However, one study has shown that TLR-induced maturation of DCs is enhanced in the presence of IL-15, leading to increased NK cell cytotoxicity toward human papillomavirus (HPV)-infected cells (17).

We hypothesized that, in addition to NK cell activation by *trans*-presentation and other direct effects, IL-15 could have indirect effects on the response of human NK cells to viruses by promoting NK cell–activating cytokines from accessory cells. We show that very low concentrations of IL-15 (0.75 ng/ml) dramatically enhance the production of IL-12, IFN-α, IL-1β, and GM-CSF from myeloid accessory cells in response to inactivated whole influenza

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Abbreviations used in this article: DC, dendritic cell; HCMV, human CMV; H3N2, inactivated whole influenza virus (A/Victoria/361/2011); HPV, human papillomavirus; IQR, interquartile range; mDC, myeloid DC; MFI, mean fluorescence intensity; pDC, plasmacytoid DC.

virus (A/Victoria/361/2011) (H3N2) and that increased production of IFN-α and, in particular, IL-12 is associated with heightened and sustained activation of NK cells and polyfunctionality of the responding NK cells. Furthermore, IL-15–mediated enhancement preferentially boosts IL-12 production in myeloid DCs (mDCs) compared with other blood DC populations and monocytes. These studies suggest that IL-15–enhanced accessory cell function may potentiate NK cell responses, providing an additional avenue of interest for boosting NK cell effector responses in vaccination and NK cell–mediated immunotherapy.

Materials and Methods

Study participants

Volunteers were recruited from the staff and students at the London School of Hygiene and Tropical Medicine ($n = 84$) using an anonymized volunteer database. The study was approved by the London School of Hygiene and Tropical Medicine Research Ethics Committee (reference numbers 6237 and 6324). Human CMV (HCMV) serostatus was determined for each donor with HCMV IgG ELISA (Biokit, Barcelona, Spain) using plasma collected from heparinized whole blood. Donors ranged in age from 20 to 77 y, with a median age of 32 y. Twenty-nine (35%) of the donors were male, and 48% were HCMV seropositive.

PBMC isolation and in vitro culture assays

PBMCs were isolated from heparinized whole blood using Histopaque 1077 (Sigma-Aldrich, Gillingham, U.K.) gradient centrifugation. Cells were rested for 2 h and were used fresh (blocking experiments and IL-12 intracellular staining) or were cryopreserved in liquid nitrogen (all other experiments). Before use, frozen cells were thawed and washed in RPMI 1640 supplemented with 100 U/ml penicillin/streptomycin and 20 mM L-glutamine (Life Technologies, Thermo Fisher). Cells were counted using a Countess II FL Automated Cell Counter (Invitrogen, Thermo Fisher); average viability after thaw was 86%. A total of 3×10^5 cells per well was cultured in RPMI 1640 supplemented as above with 5% pooled human AB serum for 6, 9, or 18 h at 37°C in 96-well round-bottom plates with 2 μg/ml H3N2 (IVR-165; National Institute for Biological Standards and Control, Potters Bar, U.K.), with or without 0.75 ng/ml recombinant human IL-15 (PeproTech, London, U.K.). Concentrations were determined by prior titration; 2 μg/ml H3N2 was the lowest concentration to induce significant NK cell IFN-γ upregulation without the presence of additional cytokines, and 0.75 ng/ml IL-15 was previously shown to be the lowest concentration to synergize with other cytokines for NK cell activation, without significant NK cell activation alone (13). Additional cultures were stimulated with a high concentration of cytokines consisting of IL-12 (5 ng/ml; PeproTech) and IL-18 (50 ng/ml; R&D Systems, Oxford U.K.). The following Abs were used for blocking experiments: anti–IL-2 (3 μg/ml; BD Biosciences, Oxford, U.K.), rat IgG2a isotype control (3 μg/ml; eBioscience, Thermo Fisher), anti–IL-12 (3 μg/ml; BD Biosciences), anti–IFN-αβR2 (1 μg/ml; Merck Millipore, Watford, U.K.), and combined mouse IgG1 and IgG2a isotype controls (3 μg/ml final; eBioscience). GolgiStop (monensin; 1/1500 concentration) and GolgiPlug (brefeldin A; 1/1000 final concentration; both from BD Biosciences) were added for the final 3 h of culture. Culture supernatants were collected and stored at −80°C. For control experiments, NK cells were purified (mean purity 87%) using an NK Cell Isolation Kit (Miltenyi Biotec), and 2×10^5 cells were stimulated for 18 h under the conditions described above for PBMC cultures. Cells were stained with NK cell activation markers as before. For IL-12 intracellular staining experiments, 2×10^6 cells per well were cultured as above for 18 h, with GolgiStop and GolgiPlug for the final 5 h.

Flow cytometry and Luminex

Cells were stained in 96-well round-bottom plates for surface markers, including viability marker (Fixable Viability Dye eFluor 780; eBioscience) in FACS buffer (PBS containing 0.5% FCS, 0.05% sodium azide, and 2 mM EDTA) for 30 min after blocking Fc receptors for 5 min with Fc Receptor Blocking Reagent (Miltenyi Biotec). Cells were then washed in FACS buffer and fixed and permeabilized using a BD Cytofix/Cytoperm Kit, according to the manufacturer's instructions. Cells were then stained for intracellular markers with FcR blocking for 15 min and washed again; finally, cells were resuspended in 300 μl of FACS buffer and transferred to alpha tubes for acquisition on a BD LSR II flow cytometer. The following fluorophore-labeled Abs were used: anti-CD3–V500 (clone UCHT1), anti-CD56–PE–Cy7 (clone NCAM16.2), anti-CD107a–FITC (clone H4A3),

anti-HLA-DR–PE (clone TU36) (all from BD Biosciences), anti-IFN-γ–allophycocyanin (clone 45.B3), anti-CD86–Alexa Fluor 488 (clone IT2.2), anti-CD11c–PerCP-Cy5.5 (clone 3.1), anti-CD16–PE/Dazzle (clone 3G8), anti-CD14–Alexa Fluor 700 (clone 63D3) (all from BioLegend, London, U.K.), anti-CD25–PerCP-Cy5.5 (clone BC96), anti-CD57–eFluor 450 (clone TB01), anti-CD19–PE–Cy5 (clone HIB19), anti-CD123–eFluor 450 (clone 6H6), and anti–IL-12(p40)–eFluor 660 (clone C17.8) (all from eBioscience). Cells were acquired using FACSDiva software, and data were analyzed using FlowJo v10 (TreeStar, Ashland, OR). FACS gates were set using unstimulated cells or fluorescence minus one controls, and samples with <100 NK cell events were excluded. Concentrations of GM-CSF, IFN-α2, IFN-γ, TNF-α, IP-10, IL-1β, IL-10, and IL-12p70 in cell culture supernatants were determined using Luminex technology (Merck Millipore) and Bio-Plex software (Bio-Rad, Watford, U.K.).

Statistics

Statistical analysis was performed using GraphPad Prism version 7.01 (GraphPad, La Jolla, CA). Functional responses were compared using the Wilcoxon signed-rank test, and intergroup comparisons between HCMV-seropositive and HCMV-seronegative individuals were performed using the Mann–Whitney U test. Correlation analysis was performed using linear regression. Significance levels are assigned as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$ for all tests. Analysis and presentation of polyfunctional NK cell data were performed using SPICE version 5.1 (downloaded from https://niaid.github.io/spice/).

Results

Nanogram concentrations of IL-15 boost and sustain functional NK cell responses to H3N2

To determine the effect of a low concentration of exogenous IL-15 on the frequency and kinetics of NK cell responses to influenza, IFN-γ, CD107a, and CD25 were measured at 6, 9, and 18 h after stimulation of human PBMCs with H3N2 in the presence or absence of 0.75 ng/ml recombinant human IL-15. The flow cytometry gating strategy is shown in Fig. 1A–D. H3N2 alone induced high frequencies of NK cells expressing each of the three activation markers, but there was no significant response to IL-15 alone. At all time points, the percentage of expression of all markers was significantly higher when cells were cultured with H3N2 plus IL-15 compared with cells cultured with H3N2 alone (Fig. 1E–G), with each marker displaying distinctly different kinetics (frequencies of cells expressing CD107a, IFN-γ, and CD25 peaking at 6, 9, and 18 h, respectively). A low frequency of NK cells showed spontaneous degranulation (CD107a expression) at early time points (Fig. 1E), whereas there was little or no IFN-γ production or CD25 induction in unstimulated cultures (Fig. 1F, 1G). Increased responses to H3N2 in the presence of IL-15 were reflected in increased mean fluorescence intensities (MFIs) for all NK cell functional markers at their optimal time points. A small, but significant, increase was observed in CD107a MFI at 6 h (H3N2 median 747 units, interquartile range [IQR] 718–776; H3N2+IL-15 median 768 units, IQR 735–813, $p \leq 0.0001$), IFN-γ MFI increased substantially by 9 h (H3N2 median 681 units, IQR 523–814; H3N2+IL-15 median 1037 units, IQR 864–1179, $p \leq 0.0001$), and a significant shift in CD25 MFI was observed by 18 h (H3N2 median 72.5 units, IQR 34.7–153.8; H3N2+IL-15 median 371.5 units, IQR 249–508, $p \leq 0.0001$). Thus, very low concentrations of IL-15 boost the NK cell response to H3N2 stimulation and lead to sustained CD107a and IFN-γ production compared with untreated cultures.

NK cells are a heterogeneous population of cells; CD56^{bright} subsets are highly responsive to cytokines, whereas the more mature CD56^{dim}CD57^{+} subset is known to be less responsive to cytokines but maintains cytotoxic function (18). Therefore, we analyzed the response of each of these subsets to IL-15 (flow cytometry gating strategy shown in Supplemental Fig. 1A). As expected, the early degranulation response was highest in the
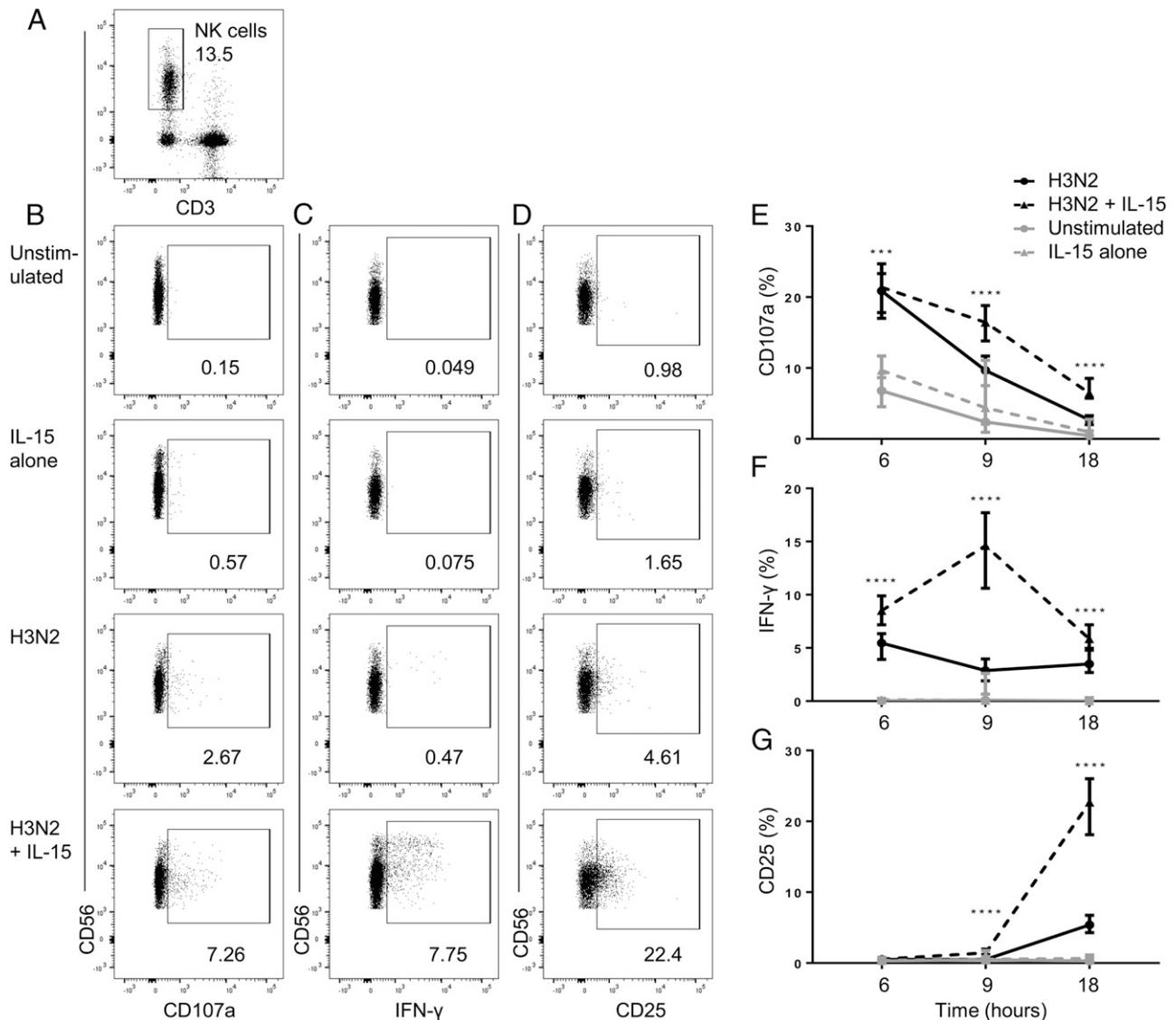
FIGURE 1. Nanogram concentrations of IL-15 boost and sustain functional NK cell responses to H3N2. PBMCs were cultured in vitro for 6, 9, and 18 h in medium alone (unstimulated; $n = 12$), IL-15 alone ($n = 12$), H3N2 ($n = 62$), or H3N2 plus IL-15 ($n = 62$). (**A**) NK cells are gated as CD56$^+$CD3$^-$ lymphocytes. Flow cytometry gating strategy for NK cell CD107a (**B**), IFN-γ (**C**), and CD25 (**D**) responses after an 18-h in vitro culture in one representative individual. Numbers shown are the percentage of total NK cells positive for each marker. Graphs show the percentage of the total NK cell population expressing CD107a (**E**), IFN-γ (**F**), or CD25 (**G**). Data are median and 95% confidence interval. ***$p < 0.001$, ****$p < 0.0001$ H3N2 alone versus H3N2 plus IL-15 by the Wilcoxon signed-rank test.

most mature (CD56^{dim}CD57$^+$) subset and IFN-γ production was highest in the less mature (CD56^{dim}CD57$^-$) subsets at the peak of each response (Supplemental Fig. 1B, 1F). However, at 6 h, IL-15 had little effect on degranulation in the CD56^{dim}CD57$^+$ NK cell population, whereas, by 9 h, the heightened response in the presence of IL-15 was pronounced within the CD56^{bright} and CD56^{dim} subsets (Supplemental Fig. 1B, 1E). Enhancement of NK cell IFN-γ and CD25 responses to influenza by low concentrations of IL-15 was evident to a similar extent in all NK cell subsets (Supplemental Fig. 1C–J).

IL-15 enhances NK cell polyfunctionality

Because IL-15 appeared to enhance all three NK cell functions to a similar degree, we considered the possibility that this was due to a fraction of NK cells being highly sensitive to the effects of IL-15 and responding in a polyfunctional manner. We examined coexpression of IFN-γ with CD107a, CD25, or both at each time point (flow cytometry gating strategy shown in Fig. 2A–C). No polyfunctional NK cells were detected in unstimulated cultures or in

cultures containing only IL-15 (Supplemental Fig. 2A–C). When cells were cultured with H3N2 alone, very few double- or triple-positive NK cells were detectable (Fig. 2D–F); however, after 6 or 9 h of culture with H3N2 plus IL-15, a considerable population of NK cells was double positive for IFN-γ and CD107a (Fig. 2D); by 18 h, cells coexpressing IFN-γ and CD25 (Fig. 2E), together with a small, but statistically significant, population of triple-positive NK cells (Fig. 2F), were detectable. Importantly, influenza virus stimulation in the presence of as little as 0.75 ng/ml IL-15 was almost as effective at inducing polyfunctional NK cells as much higher concentrations of a combination of IL-12 and IL-18 (5 and 50 ng/ml, respectively, Supplemental Fig. 2A–C). Polyfunctionality of NK cells was also evident from the statistically significant correlation between expression of the different functional markers by NK cells cultured with H3N2 plus IL-15 compared with those cultured with H3N2 alone (Supplemental Fig. 2D–I). Overall, therefore, IL-15 significantly increased the frequency of polyfunctional NK cells responding to influenza virus.
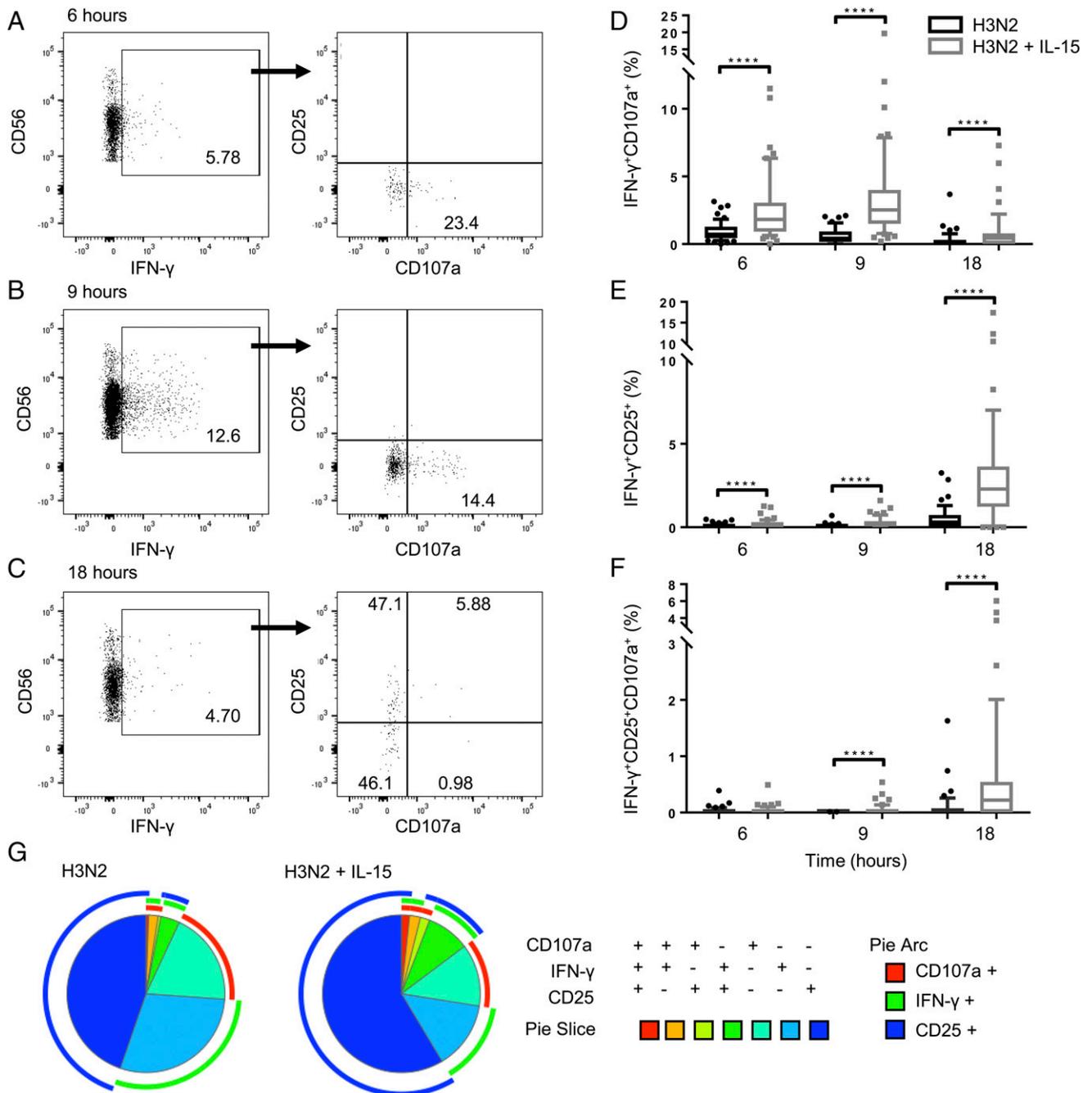
FIGURE 2. IL-15 enhances NK cell polyfunctionality. CD107a and CD25 expression of IFN-γ–secreting NK cells was gated at 6 h (**A**), 9 h (**B**), or 18 h (**C**). Plots show NK cell function in one representative donor; number is the percentage of the parent population after stimulation with H3N2 plus IL-15. Frequencies of IFN-γ⁺CD107a⁺ double-positive (**D**), IFN-γ⁺CD25⁺ double-positive (**E**), and IFN-γ⁺CD25⁺CD107a⁺ triple-positive (**F**) NK cells, after Ag stimulation with IL-15 (gray) or without IL-15 (black), at each time point ($n = 62$). Graphs are box-and-whisker plots with 10–90th percentiles. (**G**) Median distribution of triple-, double-, and single-positive NK cells after stimulation with H3N2 alone or H3N2 with IL-15 for 18 h are shown as pie charts as the percentage of total NK cells (pie slice) and the proportion expressing each marker (pie arc). ****$p < 0.0001$, Wilcoxon signed-rank test.

IL-15 enhances in vitro production of myeloid cell–derived cytokines

Because NK cells respond to cytokines released from activated accessory cells, such as DCs and monocytes, supernatants were collected from cultures at 18 h, and cytokine concentrations were measured by Luminex. H3N2 alone induced secretion of significant concentrations of IL-12 (p70), IFN-α2, IFN-γ, GM-CSF, IL-1β, TNF-α, and IL-10 (Fig. 3A–G). However, addition of low concentrations of IL-15 to H3N2 resulted in further significant increases in the secretion of all of these cytokines, with the

exception of IL-10 (Fig. 3G). IL-15–mediated enhancement of IL-12 secretion was observed in 42 of 73 donors (57.5%), with an average 2.1-fold increase in these individuals (Fig. 3A). IL-15 costimulation increased IFN-α2 secretion in 66% of individuals compared with H3N2 alone, with a median 1.2-fold increase (Fig. 3B), as well as enhanced IFN-γ by an average of 5.6-fold (Fig. 3C), consistent with IL-15 boosting of NK cell IFN-γ responses (Fig. 1). In contrast, IL-15 alone induced only modest increases in IFN-γ and GM-CSF production (median increase of only 8.18 pg/ml for IFN-γ and an increase in GM-CSF in only 3 of
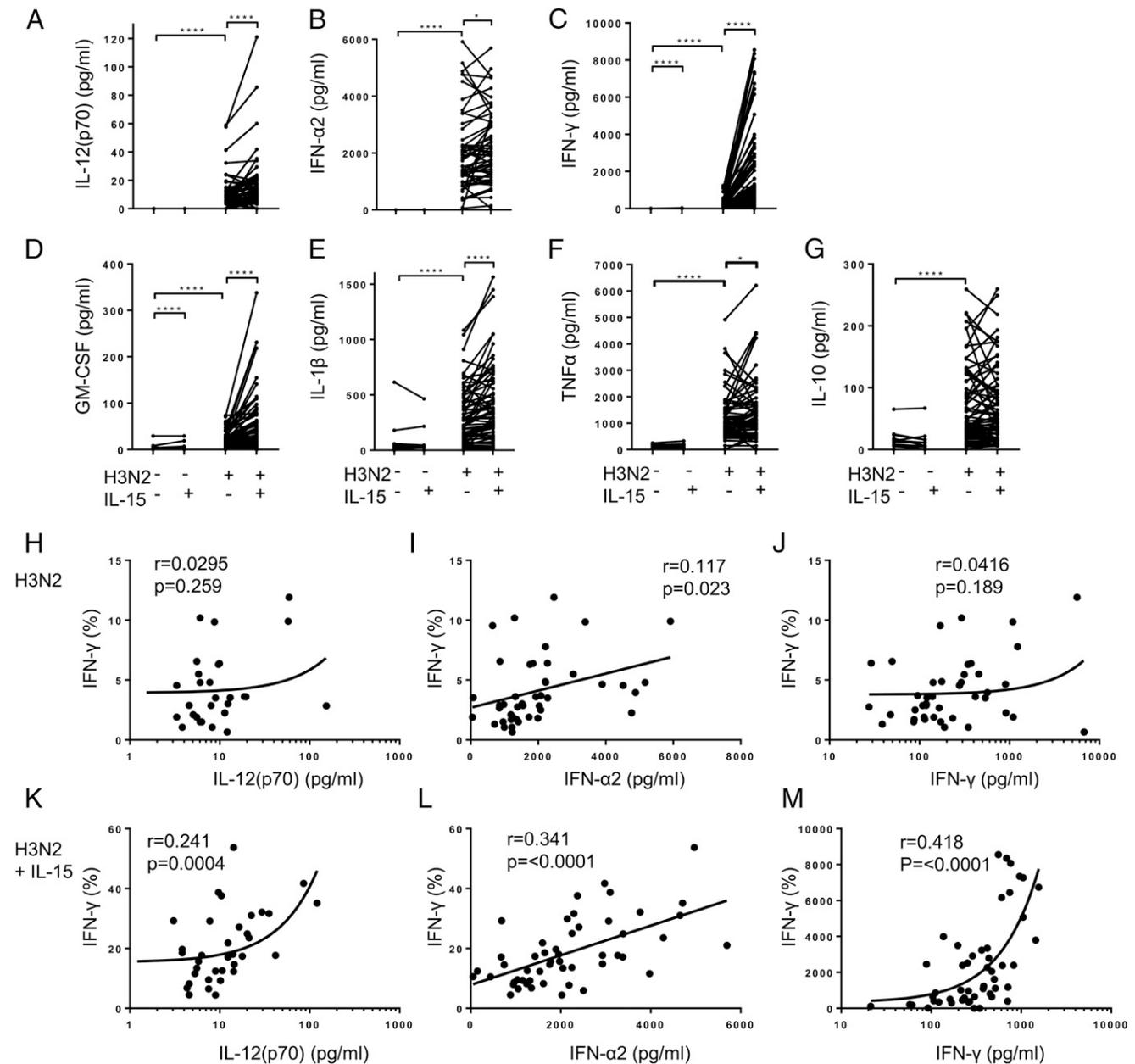
FIGURE 3. IL-15 enhances in vitro production of myeloid cell–derived cytokines. Supernatants were collected from whole PBMCs cultured with medium ($n = 20$), IL-15 ($n = 20$), H3N2 ($n = 73$), or H3N2 with IL-15 ($n = 73$) for 18 h, and concentrations of IL-12p70 (**A**), IFN-α2 (**B**), IFN-γ (**C**), GM-CSF (**D**), IL-1β (**E**), TNF-α (**F**), and IL-10 (**G**) were determined by Luminex technology. The correlation between NK cell IFN-γ production was determined by intracellular cytokine staining, and the concentration of IL-12 (p70) (**H** and **K**), IFN-α2 (**I** and **L**), and IFN-γ (**J** and **M**) was determined by Luminex after stimulation with H3N2 (**H**–**J**) or H3N2 with IL-15 (**K**–**M**). (**A**–**G**) Graphs are before-and-after plots. *$p < 0.05$, ****$p < 0.0001$, Wilcoxon signed-rank test. (**H**–**M**) Correlations were measured by linear regression, with statistical significance determined as a p value < 0.05.

20 donors) compared with unstimulated cells (Fig. 3C, 3D), and IL-15 alone had no effect on IL-12 (p70), IFN-α2, IL-1β, TNF-α, or IL-10 secretion (Fig. 3A, 3B, 3E–G).

In the presence of H3N2 and IL-15, the percentage of NK cells producing IFN-γ at 18 h was significantly correlated with the secreted concentrations of IL-12 and type I IFN (Fig. 3H–M). However, there was no enhanced correlation between NK cells producing IFN-γ and GM-CSF, TNF-α, or IL-1β with IL-15 (GM-CSF: $r = +0.385$, $r = +0.391$ with IL-15; TNF-α: $r = +0.231$, $r = +0.285$ with IL-15; IL-1β: $r = +0.197$, $r = +0.331$ with IL-15), suggesting that IL-12 and/or IFN-α2 might be driving the enhanced NK cell IFN-γ response. In summary, IL-15 dramatically enhances the secretion of myeloid cell–derived cytokines, and the

secretion of two of these cytokines (IL-12 and IFN-α2) is strongly correlated with NK cell function.

IL-12 is critical for IL-15–mediated enhancement of NK cell responses and generation of polyfunctional NK cells

Because IL-12 and IFN-α2 were induced by coculturing PBMCs with H3N2 and IL-15, and because enhanced NK cell function was correlated with the concentrations of these two monokines, we tested the hypothesis that the effects of IL-15 on NK cells were mediated via IL-12 and/or type I IFNs. Neutralizing Abs against IL-12, IFN-αβR2, and IL-2 were added to cultures stimulated with H3N2 (with and without IL-15) for 6, 9, and 18 h. All three NK cell functional responses were reduced in the presence of

blocking Ab to IL-12 by 18 h (Fig. 4A–F), as well as at the earlier time point of 9 h (data not shown); in particular, the frequency of CD107a- or IFN-γ–producing cells induced by H3N2 plus IL-15 was reduced to that observed in the absence of IL-15 (Fig. 4A–E). Degranulation was also dependent, in part, on type I IFNs, because blockade of the IFN-αβR2 receptor led to a partial reduction in CD107a expression in cultures treated or not with IL-15 (Fig. 4A, 4D). Blocking IL-2 also reduced CD107a upregulation after H3N2 stimulation, irrespective of the presence or absence of IL-15 (Fig. 4A, 4D). However, although anti–IL-2 reduced the NK cell IFN-γ response to H3N2, no such reduction was seen in the response to H3N2 plus IL-15, suggesting that IL-15 may be substituting for IL-2 in this assay.

In addition to reducing the overall frequencies of CD107a⁺, IFN-γ⁺, and CD25⁺ NK cells, IL-12 blockade had a marked effect on the induction of polyfunctional NK cells. IL-12 neutralization reduced the frequencies of double- and triple-positive NK cells to the levels observed without IL-15 (Fig. 4G–J). These data suggest that the heightened NK cell response to H3N2 and the generation of polyfunctional NK cells in response to IL-15 are dependent on accessory cell IL-12. Furthermore, correlations between different NK cell functional markers (as seen in Supplemental Fig. 2D–I) are also seen when NK cells are stimulated with high concentrations of exogenous IL-12 and IL-18 (data not shown). Stimulation of purified NK cells with H3N2 alone or with IL-15 induced no significant activation, confirming an accessory cell requirement for the virus-induced response and IL-15–mediated enhancement (IFN-γ percentage at 18 h: H3N2 median 0.29%, IQR 0.25–0.368%, H3N2+IL-15 median 0.465%, IQR 0.308–0.84; CD107a percentage at 18 h: H3N2 median 0.93%, IQR 0.605–1.293%, H3N2+IL-15 median 1.68%, IQR 0.75–2.475%).

IL-15 enhances IL-12 production by mDCs

To determine the source of IL-15–induced IL-12 within PBMCs, stimulations were performed with H3N2 in the presence or absence of IL-15 for 18 h, and cells were stained for DC/monocyte phenotypic markers and for intracellular IL-12(p40). DC populations were gated as lineage⁻CD14⁻HLA-DR⁺ cells and further split into CD123⁻CD11c⁺ mDC and CD123⁺CD11c⁻ plasmacytoid DC (pDC) populations. The majority of IL-12–producing cells were mDCs rather than pDCs, classical (CD14⁺CD16⁻) monocytes, or nonclassical (CD14⁻CD16⁺) monocytes (Fig. 5A). Up to 3.1% of mDCs expressed IL-12 in response to H3N2, and a significantly higher proportion (4.5%) were IL-12⁺ with the combination of H3N2 and IL-15 (a median 1.9-fold increase) (Fig. 5A). Furthermore, IL-15–mediated enhancement of IL-12 production was observed only in DCs and was associated with increased expression (MFI) of CD86 (Fig. 5B). Little to no IL-12 was detected in unstimulated mDCs or in those treated with IL-15 alone (Fig. 5C). Of the 14 individuals tested, 8 (57.1%) showed an increase in IL-12 production by mDCs with H3N2 plus IL-15 compared with H3N2 alone, corresponding with the proportion of responders determined by Luminex detection of IL-12 in supernatant (Fig. 5D). Among these eight responders, there was also an increase in the MFI of IL-12 staining of mDCs cultured with H3N2 plus IL-15 compared with H3N2 alone (Fig. 5E). These data suggest that IL-15 potentiates NK cell responses to H3N2 by enhancing maturation (CD86 expression) and IL-12 production specifically from mDCs.

Enhancement in NK cell function by IL-15 is observed in HCMV-seropositive and -seronegative individuals

We (19, 20), and other investigators (21, 22) have reported altered NK cell functions in HCMV-seropositive individuals.

HCMV-seropositive individuals also respond less well to exogenous cytokines and vaccine Ags (including H3N2), and this is only partially explained by accelerated NK cell differentiation (19, 20). We again observed lower NK cell CD107a, IFN-γ, and CD25 responses to H3N2 among HCMV-seropositive donors than among HCMV-seronegative donors (Fig. 6). Low concentrations of IL-15 enhanced the responses of HCMV-seropositive and -seronegative subjects but could not fully restore the NK cell response of HCMV-seropositive subjects to the level seen in HCMV-seronegative subjects (Fig. 6A–C). Interestingly, however, IL-15 completely restored the IFN-γ response of CD56^bright and CD56^dimCD57⁻ NK cells, but not CD56^dimCD57⁺ cells, in HCMV-seropositive individuals (Fig. 6E, 6H). This suggests that IL-15 preferentially affects immature NK cells, normalizing their IFN-γ response to H3N2 to the level seen in HCMV-seronegative individuals. With the exception of a reduced IFN-γ response, which may be accounted for by reduced production from NK cells in seropositive individuals, there was no difference in the in vitro production of lymphoid or myeloid cell–derived cytokines in response to H3N2 between HCMV-seropositive and -seronegative donors, irrespective of the presence or absence of IL-15 (Supplemental Fig. 3).

Discussion

Many studies of the effect of IL-15 on NK cell activation focus on IL-15 *trans*-presentation or direct activation of NK cells with high concentrations of IL-15 (typically between 5 and 50 ng/ml) in combination with other cytokines (5, 10). Moreover, the synergy between cytokines and pathogen-derived signals for NK cell activation has typically been studied only for high concentrations of cytokines acting on isolated NK cells, precluding consideration of the potential indirect effects of the pathogen, the cytokines, or both. To characterize more deeply the potential for synergy between IL-15 and pathogen-derived signals in NK cell activation, we have conducted a comprehensive analysis of NK cell CD107a, IFN-γ, and CD25 expression and myeloid cell–derived cytokine secretion in response to H3N2 in the presence or absence of an extremely low concentration of IL-15.

Low concentrations of IL-15 enhanced the innate cytokine response to influenza virus, and this increased cytokine (primarily IL-12) was associated with potentiation of NK cell function. Importantly, the concentrations of endogenous IL-12 induced by this synergistic interaction between influenza virus and IL-15 are of the same order of magnitude as the lowest concentrations of IL-12 that we have previously shown to effectively synergize with IL-15 and other common γ-chain family cytokines for NK cell activation in vitro (13). The measured concentration of IL-12 and the concentration of IL-15 used in this study are ≥5-fold lower than the described effective concentrations for NK cell activation with single cytokines and may be more physiologically relevant than the higher concentrations previously studied in vitro. The low in vitro concentration used in this study is within the range for the maximal serum concentration achieved therapeutically in patients with metastatic malignant melanoma or renal cancer after a low-dose transfusion of 0.3 μg/kg/day recombinant human IL-15 and which resulted in increases in innate cytokines (23).

In this article, we have shown that IL-15 preferentially enhances virus-induced mDC maturation (measured by upregulation of costimulatory marker CD86), as well as cytokine secretion, compared with other DC subsets and monocytes and that, together, this heightens and sustains NK cell activation. This role for mDCs is fully in line with the known pathogen-recognition repertoire and cytokine-production profile of mDCs (24, 25). Similarly, the
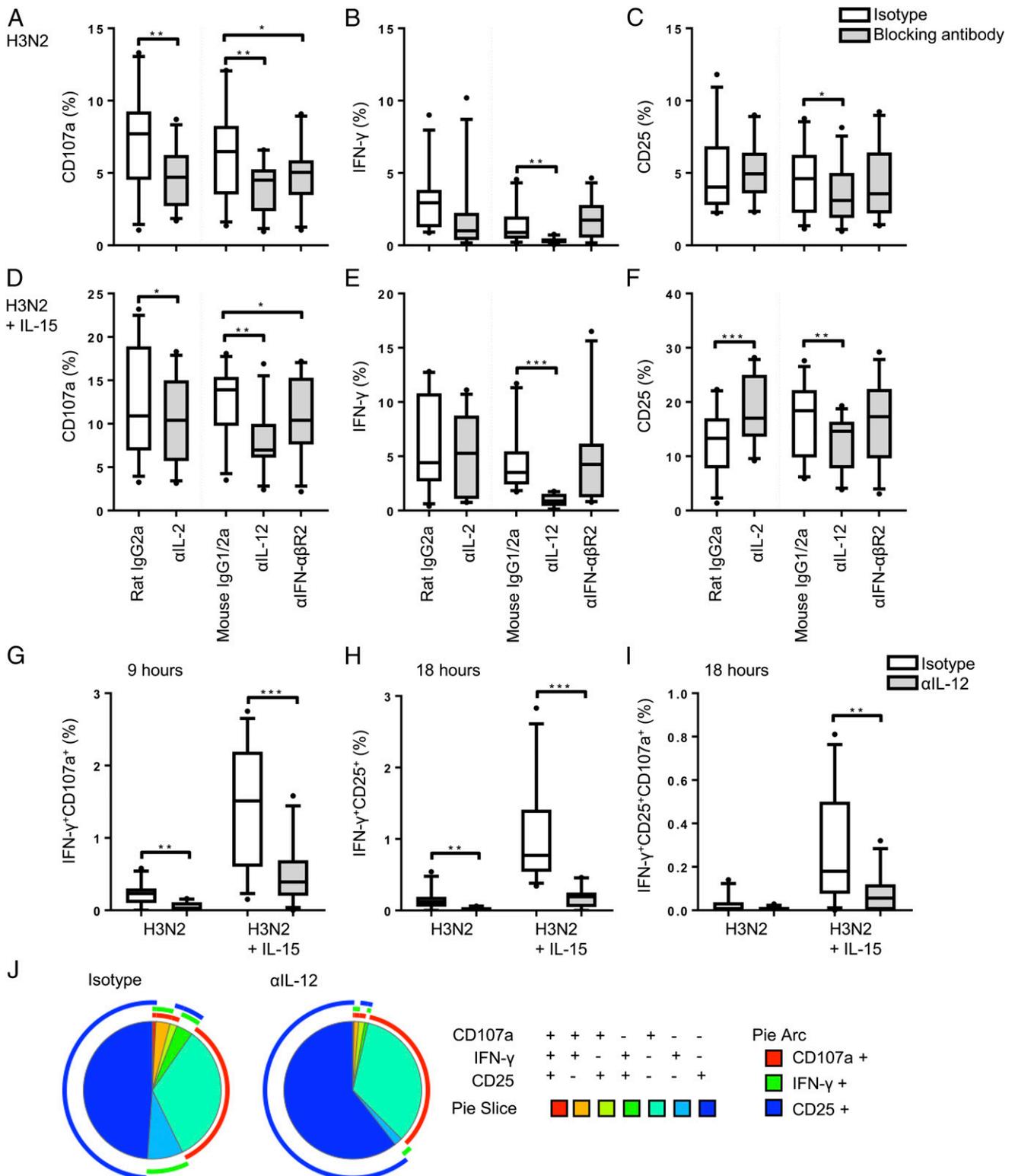
FIGURE 4. IL-12 is critical for IL-15–mediated enhancement of NK cell responses and generation of polyfunctional NK cells. PBMCs were cultured for 6, 9, and 18 h with H3N2 (**A–C**) and H3N2 plus IL-15 (**D–F**) in the presence of IL-12–, IFN-αβR2–, or IL-2–blocking Ab or the appropriate isotype control. Graphs show CD107a (A and D), IFN-γ (B and E), and CD25 (C and F) responses after an 18-h culture, and frequencies of IFN-γ⁺CD107a⁺ (**G**), IFN-γ⁺ CD25⁺ (**H**) double-positive and IFN-γ⁺CD25⁺CD107a⁺ triple-positive (**I**) NK cells after 9 h (peak of IFN-γ response) or 18 h (peak of CD25 response) (*n* = 11). Graphs are box-and-whisker plots with 10–90th percentile. (**J**) Median distributions of triple-, double-, and single-positive NK cells after stimulation with H3N2 and IL-15 for 18 h are shown as the percentage of total NK cells (pie slice) and the proportion expressing each marker (pie arc). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Wilcoxon signed-rank test.

essential role for DC-derived IL-12 in NK cell activation is in line with published data, including data showing that IL-12 synergizes with specific Ab for NK cell–mediated Ab-dependent cell–medi-

ated cytotoxicity of tumors (26, 27), that NK cell activation by HPV-like particle–matured DCs is reversed by IL-12–blocking Abs (28), and that exogenous IL-12 can restore NK cell function
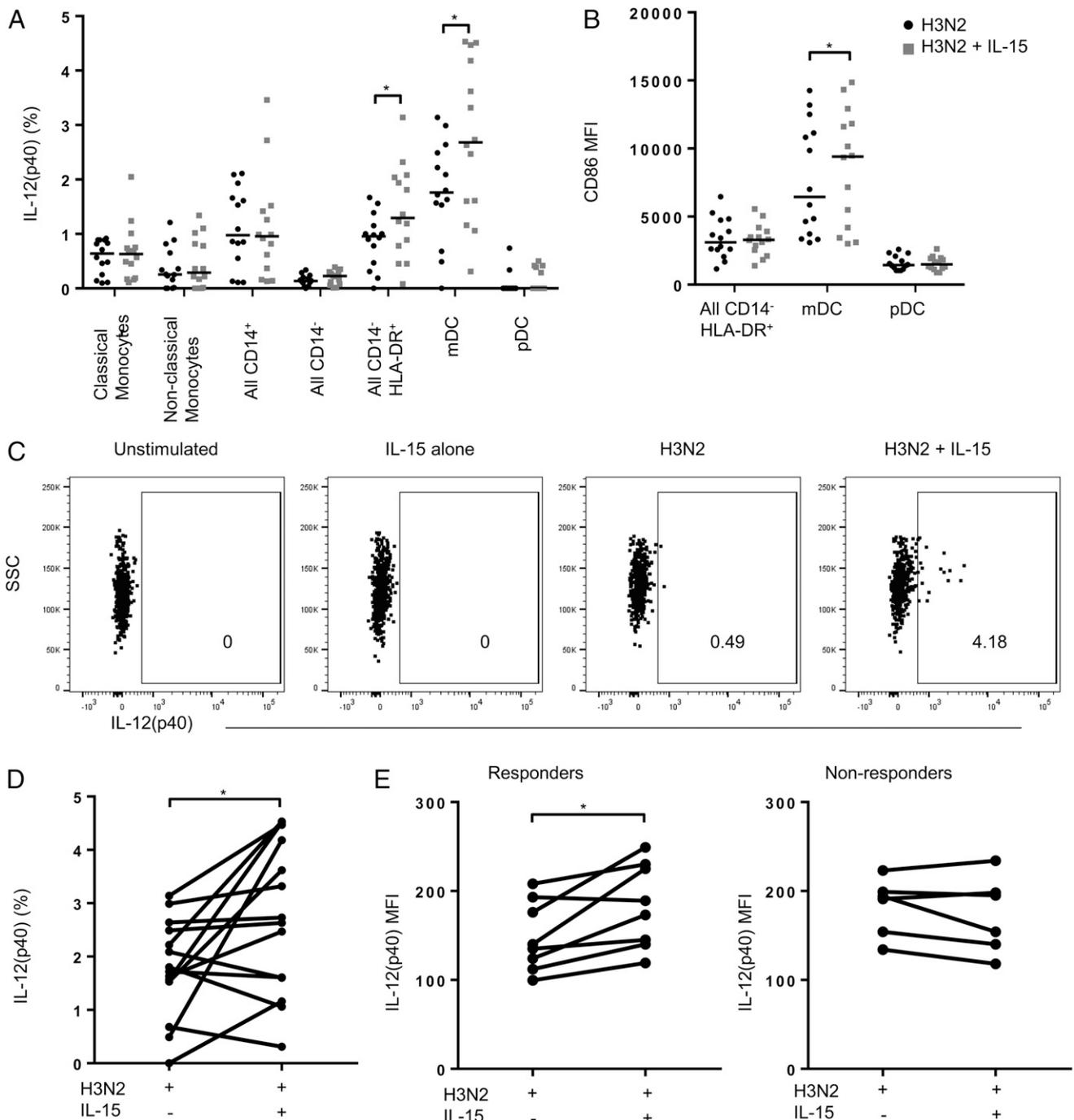
FIGURE 5. IL-15 enhances IL-12 production by mDCs. PBMCs were cultured with H3N2 or H3N2 with IL-15 ($n = 14$) for 18 h, and cells were stained for DC and monocyte phenotypic markers and intracellular IL-12. Single live cells were further gated as lineage (CD3, CD19, CD56) negative, classical (CD14$^+$CD16$^-$), and nonclassical (CD14$^-$CD16$^+$) monocytes, all CD14$^+$ and CD14$^-$ cells, all CD14$^-$HLA-DR$^+$ DCs, mDCs (CD123$^-$CD11c$^+$), and pDCs (CD123$^+$CD11c$^-$). IL-12$^+$ events (**A**) and CD86 MFI (**B**) for each cell type was gated using fluorescence minus one controls and shown as one data point per donor; the horizontal line represents the median. (**C**) Flow cytometry gating strategy for IL-12$^+$ mDCs is shown in one representative individual; numbers denote the percentage of IL-12$^+$ mDCs. Before-and-after plots show the percentage of mDCs expressing IL-12 for each individual when stimulated with H3N2 $^{+/-}$ IL-15 (**D**) and the corresponding IL-12 MFI for each responder and nonresponder with IL-15 costimulation (**E**). *$p < 0.05$, Wilcoxon signed-rank test.

in HIV-exposed, but uninfected, infants (29). Interestingly, IL-15 synergizes well in vitro with IL-12 and IL-18 but not with other IL-2Rγ-chain–dependent cytokines, including IL-2 and IL-21, suggesting some redundancy between the latter pathways (13). This is also consistent with our observation that neutralization of endogenous IL-2 did not reduce NK cell IFN-γ in response to H3N2 and IL-15.

Enhancement of NK cell function by IL-15 is well established (30). IL-15 has also been reported to enhance DC maturation

(measured by upregulation of CD40, CD86, and MHC class II expression) in mice (17, 31). In line with our observations in humans, DCs cultured with HPV-like particle matured more efficiently in the presence of IL-15, and this correlated with enhanced NK cell activation and killing of HPV-infected tumor cells (17). However, the role of IL-15 enhancement of IL-12 production by peripheral DCs has not been previously appreciated or linked to enhanced NK cell responses. Interestingly, one study demonstrated that IL-15 enhancement of IL-12 secretion by a PMA-activated U937 monocytic cell line was
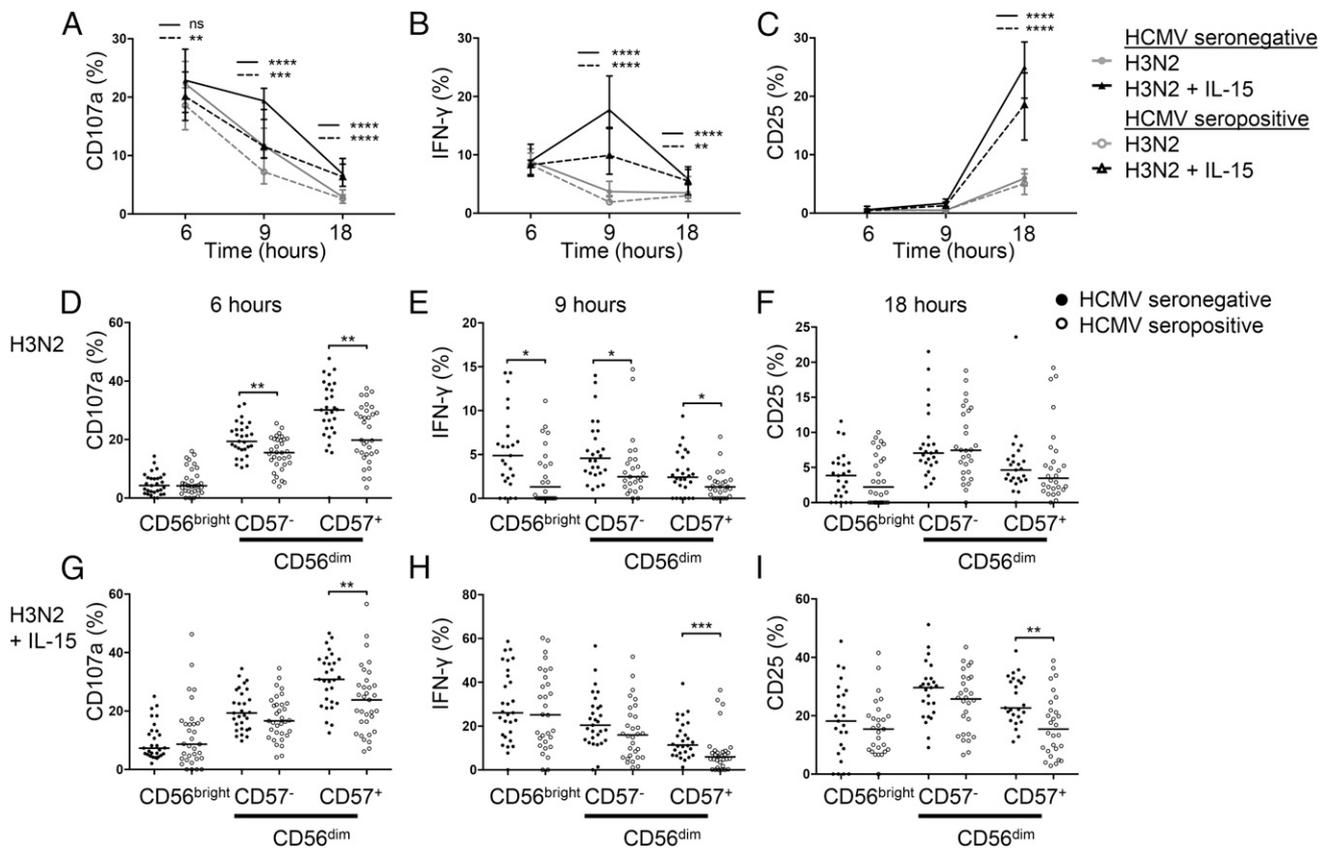
FIGURE 6. Enhancement in NK cell function by IL-15 is observed in HCMV-seropositive and -seronegative individuals. HCMV serostatus for each individual was determined ($n = 35$ seropositive and $n = 38$ seronegative). CD107a (**A**), IFN-γ (**B**), and CD25 (**C**) expression within each group after stimulation with H3N2 or H3N2 plus IL-15 for 6, 9, or 18 h. NK cell responses attributed to each differentiation subset determined by the expression of CD56 and CD57, CD56^{bright}, CD56^{dim}CD57$^-$, and CD56^{dim}CD57$^+$ were analyzed (flow cytometry gating strategy in Supplemental Fig. 1A). Graphs show median with 95% confidence interval. CD107a (**D** and **G**), IFN-γ (**E** and **H**), and CD25 (**F** and **I**) expression within each subset is shown for HCMV-seropositive and -seronegative individuals; only the peak time point for each response is shown. Each dot represents an individual donor; the horizontal line represents the median. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, H3N2 versus H3N2 plus IL-15, Wilcoxon signed-rank test (A–C), unpaired Mann–Whitney U test (D–I). ns, not significant.

associated with increased ability to kill intracellular *Leishmania* parasites, suggestive of a potential role for such mechanisms in protection against infection (32).

The cytotoxic and cytokine-producing roles of NK cells have traditionally been ascribed to distinct NK cell subsets (CD56^{dim} and CD56^{bright}, respectively), and this dichotomy has rather obscured the potential for NK cells to perform both functions simultaneously (i.e., to be polyfunctional). Nevertheless, polyfunctional CD56^{dim} CD62L$^+$ NK cells (with dual ability to produce IFN-γ and become cytotoxic) can be induced by stimulation with high concentrations (50 ng/ml) of IL-12 and IL-18 (33), and TLR stimulation induced polyfunctional NK cells (expressing two or more of CD107a, IFN-γ, and TNF-α) in HIV-1–exposed seronegative, but not seropositive, individuals (34). We defined polyfunctionality as simultaneous expression of two or more of IFN-γ, CD107a, and CD25 by any NK cell subset; no polyfunctional cells were induced by IL-15 alone, and very few were detected in response to influenza virus alone. However, stimulation with IL-15 and virus induced significant numbers of double- and triple-positive NK cells, suggesting that synergy (likely at the level of mDCs) between IL-15 and pattern recognition receptor signaling may be necessary for the induction of polyfunctional NK cells. This may be due, in part, to the dependence of NK cells on IL-12 to drive IFN-γ production and on IFN-α and IL-15 to drive cytotoxicity (2, 35).

Our observation that IL-15 restored the impaired responses of HCMV-seropositive individuals only among the less differentiated CD56^{bright} and CD56^{dim}CD57$^-$ NK cell subsets, as well as that (with the exception of IFN-γ production) there were no differences in cytokine production by PBMCs from HCMV-seropositive and -seronegative donors, suggests that polyfunctionality may arise from broadening the effector function of less mature NK subsets rather than any effect on mature NK subsets. This effect is entirely consistent with IL-12–mediated IL-15 enhancement of NK cell responses, IL-12R expression, and, therefore, responsiveness being progressively lost during NK cell differentiation and in adaptive NK cell subsets (18, 36). Moreover, these data suggest that reduced NK cell responses in HCMV-seropositive individuals are not due to an accessory cell defect but may result from intrinsic changes in more-differentiated NK cells (18, 22). This hypothesis, that IL-15 broadens the functional response of immature NK cells, is further supported by evidence that degranulation and IFN-γ and TNF-α production from CD56^{bright} NK cells are potently enhanced by exposure to multiple myeloma or acute myeloid leukemia target cells after in vivo therapy with the IL-15R agonist ALT803 (37). The use of an IL-15/IL-15Rα superagonist may further boost NK cell responses to H3N2; however, this would be expected to act on NK cells directly, because IL-15 is already complexed to its receptor, therefore potentially bypassing accessory cell–dependent effects.

In summary, we have revealed an unexpected impact of very low concentrations of IL-15 on the production of cytokines, in

particular IL-12 from mDCs, which, in turn, plays a vital role in boosting NK cell responses to influenza virus (summarized in Supplemental Fig. 4). In addition to increasing the overall frequencies of responding NK cells, IL-15 promotes the generation of polyfunctional NK cells. These studies suggest that the use of very low dose IL-15 may be a strategy for enhancing and broadening NK cell effector function in immunotherapy and in enhancing vaccine responses.

Acknowledgments

Disclosures

The authors have no financial conflicts of interest.

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Ethics Approvals

i) IL-15 study (6324 and 6237)

ii) Influenza vaccination study (10336)

iii) Influenza vaccination study amendment (10336-1)

iv) EBOVAC vaccination study (14383)

Observational / Interventions Research Ethics Committee

Eleanor Riley
Professor of Immunology
IID/ITD
LSHTM

14 January 2013

Dear Professor Riley,

Study Title:	**The role of human cytomegalovirus (HCMV) in driving phenotypic and functional differentiation of Natural Killer cells**
LSHTM ethics ref:	**6324**

Thank you for your letter of 11 January 2013, responding to the Observational Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
LSHTM ethics application	n/a	
Protocol	V2	11/01/2013
Information Sheet NK CMV	V2.A1	13/11/2012
Consent form	V1	09/10/2012
HCMV study-Data capture form	V1	26/10/2012
Sample recruitment email		

After ethical review

Any subsequent changes to the application must be submitted to the Committee via an E2 amendment form. All studies are also required to notify the ethics committee of any serious adverse events which occur during the project via form E4. At the end of the study, please notify the committee via form E5.

Yours sincerely,

Professor Andrew J Hall
Chair
ethics@lshtm.ac.uk
http://intra.lshtm.ac.uk/management/committees/ethics/

Observational / Interventions Research Ethics Committee

Eleanor Riley
Professor of Immunology
IID/ITD
LSHTM

7 September 2012

Dear Professor Riley,

Study Title: The role of Natural Killer cells in Immunity induced by influenza vaccines
LSHTM ethics ref: 6237

Thank you for your letter of 15 August 2012, responding to the Interventions Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered by the Committee.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
LSHTM ethics application	n/a	10/07/2012
Protocol	V3	03/07/2012
Information Sheet	V4	14/08/2012
Consent form	V2	17/05/2012
Sample recruitment email	V2	14/08/2012

After ethical review

Any subsequent changes to the application must be submitted to the Committee via an E2 amendment form.   All studies are also required to notify the ethics committee of any serious adverse events which occur during the project via form E4. An annual report form (form E3) is required on the anniversary of the approval of the study and should be submitted during the lifetime of the study. At the end of the study, please notify the committee via form E5.

Yours sincerely,

<Not signed to avoid delay>

Dr Wenzel Geissler
Anthropologist
Member of Committee and Acting Chair for Application
ethics@lshtm.ac.uk
http://intra.lshtm.ac.uk/management/committees/ethics/

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Observational / Interventions Research Ethics Committee

Dr Martin Goodier
Lecturer in Immunology
Department of Immunology and Infection (IID)
Infectious and Tropical Diseases (ITD)
LSHTM

6 October 2015

Dear Martin

Study Title: The role of Natural Killer cells in killing influenza virus-infected cells after vaccination

LSHTM Ethics Ref: 10336

Thank you for responding to the Interventions Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Protocol / Proposal	Sponsor_letter_QA747	04/08/2015	V1.1
Advertisements	MG SAMPLE RECRUITMENT EMAIL V1	13/08/2015	V1
Investigator CV	CV_Goodier_LSHTM ethicsV2	13/08/2015	V2
Investigator CV	CV_EMR_LSHTM ethics	13/08/2015	V1
Investigator CV	CV_RB_LSHTM ethics	14/08/2015	V08/15
Information Sheet	MG Influenza Vacc Participant Information sheet V1.2	05/10/2015	V1.2
Protocol / Proposal	MG Influenza Vacc Data capture form V1.2	05/10/2015	V1.2
Covering Letter	Goodier 10336 response	06/10/2015	1
Information Sheet	MG Influenza Vacc Informed Consent_Participant with impartial witness V1.2	15/10/2015	V1.2
Protocol / Proposal	MG New Protocol Influenza V1.2_LSHTM	15/10/2015	V1.2

After ethical review

The Chief Investigator (CI) or delegate is responsible for informing the ethics committee of any subsequent changes to the application. These must be submitted to the Committee for review using an Amendment form. Amendments must not be initiated before receipt of written favourable opinion from the committee.

The CI or delegate is also required to notify the ethics committee of any protocol violations and/or Suspected Unexpected Serious Adverse Reactions (SUSARs) which occur during the project by submitting a Serious Adverse Event form.

An annual report should be submitted to the committee using an Annual Report form on the anniversary of the approval of the study during the lifetime of the study.

At the end of the study, the CI or delegate must notify the committee using an End of Study form.

All aforementioned forms are available on the ethics online applications website and can only be submitted to the committee via the website at: http://leo.lshtm.ac.uk

Additional information is available at: www.lshtm.ac.uk/ethics

Yours sincerely,

Professor John DH Porter
Chair

Improving health worldwide

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Dr Martin Goodier

LSHTM
Keppel Street
London
WC1E 7HT

12 October 2017

Dear Martin,

Study Title: The role of Natural Killer cells in killing influenza virus-infected cells after vaccination

LSHTM MSc Ethics ref: 10336 - 1

Thank you for submitting your amendment for the above research project.

Your amendment has been assessed by the Research Governance & Integrity Office and has been approved as a non-substantial change. The amendment does not require further ethical approval from the observational ethics committee.

List of documents reviewed:

Document Type	File Name	Date	Version
Other	Influenza Vacc Informed Consent_Participant with impartial witness_V2Amend01	11/10/2017	2
Other	Influenza Vacc Participant Information sheet_V2Amend01	11/10/2017	2
Other	Participant Recruitment Email_V2Amend01	11/10/2017	2
Other	Protocol Influenza_V2Amend01	11/10/2017	2

Any subsequent changes to the application must be submitted to the Committee via an Amendment form on the ethics online applications website: http://leo.lshtm.ac.uk .

Best of luck with your project.

Yours sincerely,

Rebecca Carter

Research Governance Coordinator

Ethics@lshtm.ac.uk
http://www.lshtm.ac.uk/ethics/

Improving health worldwide

Observational / Interventions Research Ethics Committee

Dr Martin Goodier
Assistant Professor
Department of Immunology and Infection (IID)
LSHTM

17 October 2017

Dear Dr Martin Goodier ,

Study Title: EBOVAC WP3 Exploratory Natural Killer Cell assays

LSHTM ethics ref: 14383

Thank you for your application for the above research, which has now been considered by the Observational Committee.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Local Approval	14_SC_1408 Favourable_opinion_on_further_information-11_12_2014	11/12/2014	1
Information Sheet	Ebola Participant Information and ICF (V4 21-Jan-2015) clean	21/01/2015	V4
Protocol / Proposal	VAC52150EBL1001_Protocol_Amend_5_Clean	03/03/2015	Amendment 5
Protocol / Proposal	VAC52150EBL2001_Protocol_Amend_4_1Sep2016 (004)	01/09/2016	Amend_4
Investigator CV	CV. M. Goodier July 2017	06/07/2017	1
Investigator CV	Helen Wagstaffe CV 2017	09/07/2017	1

After ethical review

The Chief Investigator (CI) or delegate is responsible for informing the ethics committee of any subsequent changes to the application. These must be submitted to the Committee for review using an Amendment form. Amendments must not be initiated before receipt of written favourable opinion from the committee.

The CI or delegate is also required to notify the ethics committee of any protocol violations and/or Suspected Unexpected Serious Adverse Reactions (SUSARs) which occur during the project by submitting a Serious Adverse Event form.

An annual report should be submitted to the committee using an Annual Report form on the anniversary of the approval of the study during the lifetime of the study.

At the end of the study, the CI or delegate must notify the committee using an End of Study form.

All aforementioned forms are available on the ethics online applications website and can only be submitted to the committee via the website at: http://leo.lshtm.ac.uk

Additional information is available at: www.lshtm.ac.uk/ethics

Yours sincerely,

Professor John DH Porter
Chair

ethics@lshtm.ac.uk

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