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SPECIAL FEATURE REVIEW

Regulation of the human NK cell compartment by pathogens and vaccines

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Abstract

Natural killer cells constitute a phenotypically diverse population of innate lymphoid cells with a broad functional spectrum. Classically defined as cytotoxic lymphocytes with the capacity to eliminate cells lacking self-MHC or expressing markers of stress or neoplastic transformation, critical roles for NK cells in immunity to infection in the regulation of immune responses and as vaccine-induced effector cells have also emerged. A crucial feature of NK cell biology is their capacity to integrate signals from pathogen-, tumor- or stress-induced innate pathways and from antigen-specific immune responses. The extent to which innate and acquired immune mediators influence NK cell effector function is influenced by the maturation and differentiation state of the NK cell compartment; moreover, NK cell differentiation is driven in part by exposure to infection. Pathogens can thus mould the NK cell response to maximise their own success and/or minimise the damage they cause. Here, we review recent evidence that pathogen- and vaccine-derived signals influence the differentiation, adaptation and subsequent effector function of human NK cells.

Keywords: differentiation, malaria, NK cells, vaccines, viruses

HUMAN NK CELL DIFFERENTIATION – STRAIGHT FROM INNATE TO ADAPTIVE?

NK cells differentiate from haematopoietic bone marrow precursors under the influence of innate cytokines and stromal cell-derived factors and, in humans, are defined by the expression of CD56, whilst lacking CD3epsilon. It has been estimated that there are more than 100 000 distinct NK cell phenotypes in human peripheral blood alone1; lymphoid and tissue-resident and migratory NK cells add to this diversity.2 The distribution of these different NK cell subsets in the circulation, their tissue residence or their capacity to home to different tissues undoubtedly influences their capacity to respond to damaged, cancerous or infected cells.

Initial observations pointed to a potential differentiation pathway for peripheral blood NK cells in which cells expressing high levels of CD56 were considered as less differentiated precursors of cells with lower CD56 expression.3 Several important pieces of evidence support this broad phenotypic characterisation including the higher intrinsic proliferative capacity and greater telomere length of CD56bright NK cells compared to CD56dim NK cells.4–6 However, any relationship between
CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells is unlikely to be direct and the exact relationship remains uncertain. A recent single-cell transcriptomic analysis indicated the presence of eight major differentiation phenotypes of peripheral blood of NK cells; some of these have the potential to interconvert in response to cytokine signals whilst others may represent more terminally differentiated lineages.\textsuperscript{7} More recently, cord blood ILC-1-like precursors were shown to differentiate into CD56\textsuperscript{dim}KIR\textsuperscript{-}NKG2A Peforin\textsuperscript{GzB} NK cells on OP9 stromal cells in the presence of interleukin (IL)-2, IL-7 and IL-15.\textsuperscript{8} However, NK cells derived under these conditions contained relatively low frequencies of CD16\textsuperscript{+} NK cells.\textsuperscript{8}

CD56\textsuperscript{bright} NK cells express high levels of IL-12R, IL-15R and IL-18R enabling them to respond very efficiently to these cytokines. CD56\textsuperscript{dim} NK cells express lower levels of these receptors, instead expressing markers associated with more advanced differentiation (CD57), functional education in the context of HLA-ligands [Killer cell immunoglobulin-like receptors (KIR)], antibody-dependent activation (CD16 and CD32) and cytotoxicity (granzyme B and perforin).\textsuperscript{9} However, these different differentiation states do not necessarily correlate with a cell’s ability to integrate innate or adaptive signals \textit{per se}. A subset of CD56\textsuperscript{bright} NK cells, for example, expresses the high-affinity IL-2 receptor heterodimer (CD25/CD122), responds to picogram concentrations of IL-2 and interacts with CD4\textsuperscript{+} T cells in secondary lymphoid tissues, suggesting that these cells amplify the responses of antigen-specific memory T cells.\textsuperscript{10} However, CD56\textsuperscript{dim} NK cells that express high levels of Fc\gammaRIII (CD16) could be viewed as adapted for integration of antibody-dependent signals but also acquire the high-affinity IL-2R on activation.\textsuperscript{11} Furthermore, detailed examination of transcription factor and transmembrane signalling adaptor expression of human blood NK cells reveals a polarisation of CD56\textsuperscript{dim} NK cell functional phenotypes from ‘canonical’ cells (expressing the proteomyelocytic zinc finger (PLZF) molecule) to ‘adaptive’ NK cells (lacking the expression of this transcription factor).\textsuperscript{12,13} Reduced expression of PLZF is associated with loss of Fc\gammaRI\gamma and associated signalling components including Syk and EAT-2 and alternative signalling via the CD3\zeta chain.\textsuperscript{12,13}

Paradoxically, \textit{in vitro} cross-linking of NKG2C combined with long-term IL-15 stimulation not only induces expansion of highly differentiated ‘adaptive’ CD56\textsuperscript{dim}CD57\textsuperscript{-}NKG2C\textsuperscript{-} NK cells but also promotes their co-expression of NKG2A and the checkpoint inhibitors PD1 and LAG3 and induces trans-differentiation from CD45RA to CD45RO isoform expression.\textsuperscript{14} Hence, chronic stimulation may promote further differentiation of NK cells. Interestingly, CD56\textsuperscript{bright} NK cells and a subset of CD56\textsuperscript{dim} NK cells also lack PLZF and Fc\gammaRI\gamma suggesting that there is significant plasticity in signalling pathways across distinct NK cell differentiation stages.\textsuperscript{13} In many ways, this plasticity of NK cell differentiation and adaptation should not be surprising considering the role of epigenetic changes in the diversification process. ‘Adaptive’ NK cells undergo gene silencing via methylation of loci associated with expression and responsiveness to innate cytokines whereas demethylation promotes a lower threshold for activation via alternative pathways including at the IFN-\gamma locus.\textsuperscript{13}

**NK CELL DIFFERENTIATION IS DRIVEN, IN PART, BY HCMV**

The spectrum of NK cell differentiation varies between individuals reflecting, in part, their prior infection history.\textsuperscript{7} Pathogens express immune-activating molecules (pathogen-associated molecular patterns) or induce tissue damage-associated ‘danger’ signals that contribute to NK cell activation either directly or via cytokines produced by intermediary ‘accessory’ cells such as monocytes and macrophages. The phenotypic and functional repertoire of NK cells responding to a distinct pathogen, and the magnitude of that response, will therefore vary according to the strength and duration of these pathogen-derived signals.

A role for infection in driving the differentiation and functional adaptation of human NK cells is particularly well documented for human cytomegalovirus (HCMV). Expansions of NK cells expressing CD57 and NKG2C (a receptor recognising cognate HLA-E stabilised on HCMV-infected cells by peptides from the UL40 viral protein) and lacking PLZF and Fc\gammaRI\gamma are frequently found in bone marrow transplant patients experiencing HCMV infection or reactivation and are elevated in frequency in HCMV seropositive compared to seronegative individuals.\textsuperscript{12} However, expansions of NK cells lacking Fc\gammaRI\gamma, EAT-2 and Syk1 but with intermediate levels of CD57 expression are also observed in HCMV seronegative individuals,
highlighting the potential for peripheral NK cell differentiation to be driven by other mechanisms.\textsuperscript{15} The potential for a non-linear, HCMV-independent pathway for functional NK cell diversification is further supported by the observation of dynamic, intra-individual fluctuations in the frequencies of these cells over a protracted period of time in HCMV seronegative individuals.\textsuperscript{15}

HCMV-infected myeloid cells and fibroblasts are in many ways the archetypic example of pathogen-induced NK cell activation and differentiation, both directly via binding of HCMV-induced ligands on infected host cells to NK cell surface receptors including NKG2C, LILRB1 and activating KIR and indirectly through the action of pro-inflammatory cytokines, interleukin-2 and antibodies emanating from other immune cells.\textsuperscript{16,17}

Human ‘adaptive’ NK cells (defined by the expression of NKG2C or loss of PLZF/Fc\textsuperscript{eR1g} associated pathways and induced by cognate interaction between NKG2C and HLA-E on HCMV-infected cells) share many features of murine ‘memory’ NK cells induced by binding of the murine cytomegalovirus (MCMV) m157 protein to NK Ly49h receptors.\textsuperscript{18} In both cases, NK cell expansion and differentiation are supported by accessory cell secretion of IL-12 and leads to generation of effector cells with superior killing activity and control of CMV infection.\textsuperscript{18,19}

Additionally, ‘cytokine-induced-memory-like’ (CIML) NK cells can be generated in vitro in both humans and mice with combinations of accessory cytokines (IL-12, IL-18 and IL-15); these cells respond to subsequent in vitro stimulation by enhanced cytokine secretion, and this enhanced activity is retained after adoptive transfer.\textsuperscript{20,21} CIML NK cells are distinguishable from human adaptive NK cells and murine memory NK cells in their lack of specificity for individual pathogens and their independence from cognate CMV-NKG2C/Ly49h interactions (reviewed in Pahl et al.\textsuperscript{22}).

In the ‘real world’ of complex individual infection histories, these categorisations may be less clear with adaptive/memory cells acquiring additional CIML-like properties after in vivo exposure to acute infection, inflammation or vaccination. CIML NK cells are likely to be further expanded and differentiated by subsequent CMV infection. Nevertheless, the dominant role of HCMV in promoting the differentiation and functional adaptation of human NK cells (especially after infection in early life) has the potential to directly influence responses to third party infections. HCMV-associated ‘adaptive’ NK cells in humans have enhanced capacity for antibody-mediated activation and killing of infected target cells (antibody-dependent cellular cytotoxicity, ADCC)\textsuperscript{12,13,18} although their long-term fate during HCMV infection or reactivation may ultimately depend on the presence or absence of co-stimulatory signals.

Endemicity of HCMV infection is greatly influenced by environment, for which geographical location and ethnicity have been used as proxies. Generally speaking, HCMV seroprevalence increases with equatorial proximity, with the highest prevalence being reported in sub-Saharan Africa.\textsuperscript{23} Rates of HCMV infection in infants and children are also highest in these settings with between 83\% and 98\% of individuals becoming infected by the age of 14 years.\textsuperscript{23} In a UK population, higher seroprevalence of HCMV (and, incidentally, herpes simplex virus, HSV) was observed in children of Asian heritage than amongst children of white British heritage raising the possibility that immune differentiation, and thus disease susceptibility or presentation, may vary amongst communities of differing ethnicity within the same geographical location.\textsuperscript{24} NK cell differentiation occurs noticeably more rapidly (i.e. in younger age groups) in settings of high HCMV endemicity with, for example, maximal expansion of CD57\textsuperscript{NKG2C\textsuperscript{c}} and CD57\textsuperscript{NKG2A\textsuperscript{c}} NK cells being reached by 10 years of age in an African population with near universal HCMV infection in the first year of life.\textsuperscript{25} This is accompanied by loss of NK cell responsiveness to innate cytokines but maintenance of robust antibody-dependent activation\textsuperscript{25} and emergence of high frequencies of ‘adaptive’ Fc\textsuperscript{eR1g}\textsuperscript{CD56\textsuperscript{dim}} NK cells in childhood and early adulthood.\textsuperscript{26,27} Similar effects are observed in temperate areas of the northern hemisphere, but over a more protracted period, with advanced NK cell differentiation mostly occurring later in life in these lower HCMV seroprevalence settings.\textsuperscript{28–30}

**THE ROLE OF OTHER PATHOGENS IN NK CELL DIFFERENTIATION**

Whilst HCMV infection is associated with robust NK cell differentiation and adaptation, perhaps
setting the stage for subsequent responses, other pathogens also influence the expansion and differentiation of particular NK cell subsets. In experimental settings, several examples have emerged of direct activation of NK cells by binding of pathogen-encoded ligands to either invariant or polymorphic NK cell receptors. One recent, well-characterised example is that of a conserved peptide epitope from bacterial recombinase A (rec A) that is presented by HLA-C*0501 and recognised by the human NK cell receptor KIR2DS4 enabling NK cell activation by diverse bacteria including Brucella, Campylobacter, Chlamydia and Helicobacter species. Similarly, a highly conserved epitope in the helicase of flaviviruses is presented by HLA-C*0102 and binds to KIR2DS2, inducing NK cell degranulation and cytotoxicity and several HIV-derived peptides have been identified that either provoke or inhibit NK cell responses in the context of KIR-HLA interactions. By contrast, the fungal pathogen Aspergillus fumigatus is reported to bind directly to NK cell CD56, resulting in NK cell activation, beta chemokine production and a reduction in CD56 expression. Whilst A. fumigatus appears to interact with both CD56bright and CD56dim NK cell subsets, fungal activation reportedly affects expression of other NK receptors which may be unevenly distributed across the NK cell differentiation spectrum, including NKG2D and NKp46. Furthermore, A. fumigatus-derived signals synergise with indirect, dendritic cell-derived signals to activate, and prevent exhaustion of, less differentiated NK cell subsets. Direct interaction of influenza A virus haemagglutinin with NKp46, NTB-A and 2B4 phenotypes. Varicella-Zoster virus (VZV), for example, targets CD56dim NK cells, resulting in acquisition of CD57 but, paradoxically, reducing surface CD16 expression despite the absence of VZV-specific antibodies in the culture system. Less differentiated NK cells can be infected during chronic active Epstein–Barr virus infection: EBV-infected CD56dim NK cells (with CD2−CCR7−CD11a−CD11b−NKG2A+/NKG2C−NKG2D−/CD57− phenotype) exhibit increased STAT1 phosphorylation and Akt activation with consequences for viral latency. Evidence is also accumulating of indirect changes in NK cell phenotype and function in people actively infected with a variety of, mostly viral, infections (summarised in Table 1); potential points of interaction between distinct pathogens and different NK cell differentiation subsets are summarised in Figure 1. In many cases, these infections seem to augment the effects of underlying HCMV infection, although data on HCMV infection are lacking in some studies (Table 1). For example, many of the effects of chronic HIV-1 infection on NK cells are indistinguishable from those associated with recurrent infection/ reactivation of HCMV including loss of CD56bright cells and emergence of CD57−NKG2C−FcyR1y− cells. Indeed, reversal of NKG2A/NKG2C frequencies over a 24-month period of antiretroviral therapy likely reflects enhanced immune control of HCMV infection. Accelerated acquisition of highly differentiated, ‘adaptive’ NK cell phenotypes is observed in HIV-1-infected individuals in Europe but these effects are less marked in HIV-1+ Africans amongst whom NK cell differentiation is typically already well advanced due to HCMV infection early in life. However, persistent untreated HIV-1 infection further extends NK cell differentiation with accumulation of CD56−NKG2C+ cells. Proteomic analysis suggests that these cells emerge from a CD56dim precursor population but it as yet unclear whether their differentiation is driven by HIV-1 infection itself (with or without HCMV co-infection), whether opportunistic infections (including with fungal pathogens such as A. fumigatus) also drive this NK cell differentiation, or whether the activating signals are direct (pathogen ligands binding to NK cell receptors) or indirect (e.g. mediated by inflammatory cytokines).

Influenza infection is associated with reduced frequencies of CD56bright NK cells, coincident with NK cell production of antiviral and inflammatory cytokines, as well as upregulation of activation/functional markers (CD69, CD38 and granzyme B) and a tendency for increased proliferation of both CD56bright and CD56dimCD16− NK cells. Crucially, a small population of CD49a+/CD16−CXCR3+ NK cells with potential lung homing capacity has been identified during acute influenza infection and equivalent cells isolated from lung tissue have been shown to have antiviral activity in vitro. Importantly, many of these influenza-induced phenotypic changes are

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Table 1. Impacts of active infections on human NK cell differentiation

| Pathogen           | Impact on NK cell differentiation                                                                                                                                                                                                                                                                                                                                 | References |
|--------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Dengue virus       | Robust proliferation across the differentiation spectrum although CD56<sup>bright</sup> and less differentiated CD56 NK cells dominate, emergence of skin homing CLA<sup>+</sup> NK cell phenotype                                                                                                                                                                                                                   | 54         |
| Ebola virus        | Early acute reduction in CD56<sup>bright</sup> NK cells precedes proliferation of both CD56<sup>bright</sup> and CD56<sup>dim</sup> subsets. Emergence of CD56<sup>+</sup>CD16<sup>+</sup> NK cells persisting after EVD recovery                                                                                                                                                                                     | 69,71      |
| Hantavirus         | IL-15 and HLA-E dependent expansions of CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells. High proportion of HCMV co-infected individuals                                                                                                                                                                                                                                                | 52         |
| Hepatitis C virus  | Redefinition of CD56<sup>bright</sup> and CD56<sup>dim</sup> cell subsets in both acute infection and self-resolving infections. Reduced CD56<sup>dim</sup> NK cell frequencies and expansion of CD56<sup>+</sup>CD16<sup>+</sup> NK cells. HCMV co-infection not reported                                                                                                                   | 55,56,58   |
| HIV-1              | Reduced frequencies of CD56<sup>bright</sup> and expansion of CD56<sup>dim</sup>CD57<sup>+</sup>NKG2C<sup>+</sup> NK cells and adaptive NK cells in chronic infection. Increased frequencies of CD56<sup>+</sup>CD16<sup>+</sup> NK cells. Partial resolution after treatment. Likely role of HCMV co-infection                                                                                                                                 | 41–46      |
| Influenza virus    | Acute infection is associated with reduced CD56<sup>bright</sup> NK cell frequencies. Activation and proliferation across the NK cell differentiation spectrum. Emergence of CD49α<sup>+</sup>CD16<sup>+</sup>CXCR3<sup>+</sup> with lung homing capacity                                                                                                                                   | 48,49      |
| Malaria            | Increased frequencies of activated Nkp30<sup>+</sup> cells across differentiation spectrum in CHM with CD56<sup>dim</sup> subsets dominating the responses. Emergence of CD38<sup>dim</sup>HLA-DR<sup>+</sup>CD45RO<sup>+</sup> NK cells in sickle cell trait with low parasitaemia. Role of HCMV co-infection not reported                                                                                                                  | 60,61      |
| Mycobacterium      | Enrichment of CD45RO<sup>+</sup> NK cells in pleural effusions of pulmonary tuberculosis patients                                                                                                                                                                                                                                                                         | 64,65      |
| SARS-CoV-2         | Activation of CD56<sup>bright</sup> NK cells early in infection. High frequency and increased proliferation of CD56<sup>dim</sup>NKG2C<sup>+</sup>Ksp37<sup>+</sup> associated with disease severity. Adaptive expansions present in a higher proportion of HCMV<sup>+</sup> patients with severe COVID-19 compared to control or mild disease. Enrichment of CD56<sup>dim</sup>CD57<sup>+</sup> NK cells in ARDS | 74,78      |

Figure 1. Schematic summary of the natural killer cell differentiation pathway with potential points of interaction with different pathogens. Many viral pathogens induce innate cytokines and type 1 interferons which activate and expand less differentiated CD56<sup>bright</sup> and CD56<sup>dim</sup>CD57<sup>+</sup> NK cells during early/acute infection. Viral pathogens also utilise direct mechanisms including via activating KIR-HLA interactions, in some cases synergising with IL-15. HCMV promotes expansion of adaptive NK cells, forming a potential template for the role of persistent viral infections and chronic or recurrent infections (e.g. by malaria parasites) to promote further expansion of adaptive NK cells and/or their terminal differentiation, as defined by the loss of CD56 (CD56<sup>-</sup>) and/or the expression of CD45RO.
short-lived, with reversal to pre-infection characteristics during convalescence, compatible with redistribution and regulation of the NK cell response.\textsuperscript{49}

The importance of infection in promoting activation of relatively undifferentiated, tissue-resident NK cells and migration to distinct anatomical sites is also evident in recall responses to VZV and hepatitis B viruses.\textsuperscript{50,51} NK cells with characteristics of liver-resident NK cells (CD56\textsuperscript{high}CXCR6\textsuperscript{+}CD16\textsuperscript{−}NKGD2\textsuperscript{−}CD69\textsuperscript{−}CD62L\textsuperscript{−}) were enriched in skin blisters after challenge with VZV skin test antigen,\textsuperscript{50} and CD49\textsubscript{α}CD16\textsuperscript{−} human liver NK cells acquire epigenetic modifications and migrate to the skin after hepatitis B vaccination.\textsuperscript{51}

Hantavirus infection leads to rapid and persistent in vivo expansion of NK cells across the differentiation spectrum including CD57\textsuperscript{−}NKGD2\textsuperscript{−} NK cells.\textsuperscript{52} Such expansions are associated with elevated levels of IL-15 and with the Hantavirus-driven upregulation of HLA-E on infected cells. Furthermore, over 80% of infected individuals were HCMV seropositive in the study, consistent with the possibility that Hantavirus further expands cells initially expanded by HCMV infection.\textsuperscript{52} In this context, the activation of more differentiated NK cells is supported by hantavirus-induced upregulation of IL-15-IL-15R on epithelial cells.\textsuperscript{53}

Acute dengue virus infection is associated with robust proliferation of all NK cell subsets, irrespective of their differentiation status, although CD56\textsuperscript{bright} and CD56\textsuperscript{dim}CD57\textsuperscript{−} NK cells dominate the response; this is associated with increased IL-18 concentrations in plasma (and in experimentally induced skin blister fluid) and activation of IL-18-induced signalling pathways.\textsuperscript{54} In this case, the cells preferentially homing to the skin were CD56\textsuperscript{bright} and expressed a unique combination of chemokine receptors, including CLA-1 which is typically associated with skin homing.\textsuperscript{54}

Acute hepatitis C virus (HCV) infection leads to increased blood frequencies of CD56\textsuperscript{bright} cells, and correspondingly decreased frequencies of CD56\textsuperscript{dim} NK cells, although both subsets of NK cells appear activated.\textsuperscript{55} However, NK cell frequencies subsequently normalised in patients who went on to clear their infections and in those who developed chronic infection and no long-term impacts on NK cells were reported.\textsuperscript{55} Similarly, another study reported increased frequencies of CD56\textsuperscript{−} NK cells and concomitantly reduced frequencies of CD56\textsuperscript{dim}, NKGD2\textsuperscript{+}, NKp30\textsuperscript{+} and NKp46\textsuperscript{−} NK cells during HCV infections.\textsuperscript{56} Importantly, frequencies of NKGD2\textsuperscript{−} and CD94\textsuperscript{−} NK cells were increased in acute and chronic infection but not in those whose infections resolved, whereas frequencies of NKp30\textsuperscript{+}, NKp46\textsuperscript{+} and NKGD2\textsuperscript{−} cells were lower in those who resolved their infections than in those who became chronically infected.\textsuperscript{56} Consistent with these observations, a recent study of HCMV seropositive patients reported that high frequencies of adaptive CD57\textsuperscript{−}Fc\textsubscript{c}:R1\textsuperscript{γ} NK cells and elevated expression of PD-1 were associated with high viral load in chronic HCV infection and were reduced after direct-acting antiviral therapy (DAA).\textsuperscript{57} In a separate study, chronic HCV infection was similarly associated with higher frequencies of CD57\textsuperscript{−} and PD-1\textsuperscript{+} NK cells compared to uninfected control individuals.\textsuperscript{58} However, although PD1 expression frequency reduced after DAA, proportions of CD57\textsuperscript{−} and KLRG1\textsuperscript{−} cells increased after treatment, suggesting ongoing expansion of highly differentiated NK cells.\textsuperscript{58} The slightly different NK cell outcomes in these two studies may be explained by differences in HCMV status, HCV viral genotype, viral load and treatment regimen.\textsuperscript{57,58}

**IMPACTS OF CHRONIC, PERSISTENT OR RECURRENT INFECTION ON NK CELL DIFFERENTIATION – EVIDENCE FROM MALARIA AND TUBERCULOSIS**

Human malaria infections provide insights into the impacts of chronic and repeated infection on the NK cell compartment. Blood stage malaria parasites can induce high concentrations of inflammatory and NK cell-activating cytokines with associated NK cell activation seen in animal models and human co-culture systems \textit{in vitro}.\textsuperscript{59} Experimental, controlled human malaria infections (CHMI) have given valuable insights into the activation of NK cells during primary infection in malaria-naïve individuals. Surprisingly, despite the known role for inflammatory cytokines (IL-12, IL-18) in activating less differentiated NK cells, the primary acute response during CHMI is characterised by activation (CD69 expression) of NKp30\textsuperscript{+} NK cells distributed across the differentiation spectrum but with CD56\textsuperscript{dim} cells dominating over CD56\textsuperscript{bright} cells\textsuperscript{60}; interestingly, these expansions were closely linked to the course of parasitaemia and resolved after treatment.\textsuperscript{60} It is likely that both expansion and homing of
activated CD56\textsuperscript{bright} NK cells to – and their retention in – secondary lymphoid tissues contribute to redistribution of NK cell subsets during active malaria infection.

The potential for chronic or repeated malaria infections to influence NK cell phenotype and function is illustrated by the identification of a novel, activated CD38\textsuperscript{HLA-DR\textsuperscript{CD45RO\textsuperscript{+}} NK cell population in individuals with sickle cell trait who have persistent low-density parasitaemia.\textsuperscript{61} A possible explanation for this is that chronic in vitro stimulation of NK cells via NKG2C in combination with IL-15 induces CD45 isoform switching on CD57\textsuperscript{/}NKG2C\textsuperscript{+} cells, resulting in a CD45RO\textsuperscript{+} population with high proliferative potential, characteristic of central memory T cells.\textsuperscript{14} Increased expression of checkpoint inhibitors including TIM3 and PD-1 has also been observed after repeated malaria exposure with increasing age in endemic populations,\textsuperscript{62} again with parallels to the emergence of LAG3 and PD1\textsuperscript{+} adaptive NK cells after activating receptor (NKP30, NKG2D, NKG2C) or HCMV-mediated activation in vitro.\textsuperscript{14} Interestingly, a CD25\textsuperscript{+}CD45RO\textsuperscript{+} NK cell population has been identified in patients with metastatic melanoma treated with anti-PD-1\textsuperscript{62,63} further supporting a potential for progressive differentiation of NK cells in a manner analogous to that seen in T cells. There is also evidence that persistent Mycobacterium tuberculosis infections, and the associated chronic inflammatory response, lead to the emergence of CD45RO\textsuperscript{+} NK cells and enrichment of these cells in the pleural effusion fluid of pulmonary tuberculosis patients.\textsuperscript{64} These cells are potent producers of both IL-22 and IFN-\gamma that are implicated, respectively, in tissue repair and inflammation.\textsuperscript{65} Whether these NK cell expansions rely on prior infection or co-infection with other pathogens is unknown although there is some evidence that the course of tuberculosis can be modulated by HCMV co-infection.\textsuperscript{66,67}

EMERGING PATHOGENS AND NK CELLS

As described above, an abundance of experimental and observational studies suggests a role for infections, especially repeated or persistent parasite or viral infections, in gradual and extensive NK cell differentiation. Furthermore, studies of emerging pathogens are providing additional insights into the relative impacts of innate and adaptive immune pathways in inducing NK cell differentiation. Notably, severe systemic inflammation is a hallmark of severe Ebola and SARS-CoV-2 (COVID-19) disease; this inflammation may in turn induce expansion and differentiation of NK cells with potential long-term consequences.

Ebola virus is an example of a novel pathogen with only very limited adaptation to a human host. Ebola virus-induced overproduction of the NK cell-activating inflammatory cytokines IFN-\alpha2 and IL-18, insufficiently counterbalanced by anti-inflammatory cytokines (IL-10), is associated with poor outcomes in Ebola virus disease (EVD).\textsuperscript{68} During EVD, there is a rapid reduction in the frequency of CD56\textsuperscript{bright} NK cells, within days of infection, and a subsequent increase in the frequencies of proliferating CD56\textsuperscript{bright} and CD56\textsuperscript{dim} cells.\textsuperscript{69} In vitro, Ebola virus glycoprotein directly induces the secretion of both pro-inflammatory cytokines (including IL-18 which contributes significantly to NK cell activation, degranulation and IFN-\gamma production) and anti-inflammatory IL-10 (which restricts these responses).\textsuperscript{70} In this scenario, NK cell responses are dominated by CD56\textsuperscript{bright} and CD56\textsuperscript{dim}CD57\textsuperscript{+} cells, raising the possibility that these less differentiated, cytokine-producing cells may act to further amplify the inflammatory cascade (and thus disease severity) in vivo. Interestingly, increases in the frequency of CD56 CD16\textsuperscript{+} NK cells are observed up to 2 years after recovery from severe EVD, suggestive of persistent activation and/or terminal differentiation from CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cells.\textsuperscript{47,71} However, in experimental studies in mice, adoptive transfer of dendritic cells exposed to virus-like particles expressing the Ebola virus glycoprotein can prime NK cells to protect against subsequent lethal Ebola virus infection, raising the possibility that NK cell activation by direct binding of a viral ligand together with cytokine-mediated co-stimulation could induce protective NK cell effector mechanisms.\textsuperscript{72} Indeed, human NK cells expressing NKP30 have been implicated in the cytotoxic response to Ebola virus after interaction with infected dendritic cells.\textsuperscript{73}

More recently, NK cells have been implicated in both protection against and pathogenesis of SARS-CoV-2 infection (COVID-19). As in other viral infections, and irrespective of outcome in terms of disease, innate inflammatory pathways are triggered early in SARS-CoV-2 infection. CD56\textsuperscript{bright} NK cells are activated, and their increased
expression of perforin and granzyme B is associated with inflammatory markers of disease severity, in particular with high concentrations of IL-6.\textsuperscript{74} In addition, reduced expression of CD16 on CD56\textsuperscript{dim} NK cells in people with COVID-19\textsuperscript{75} is consistent with ongoing activation of this subset by SARS-CoV-2 antigen/antibody immune complexes.\textsuperscript{76} Importantly, HCMV infection and the associated expansion of the subset of adaptive CD56\textsuperscript{dim}NKG2C\textsuperscript{+} NK cells were associated with severe COVID-19. Over 90% of severe COVID-19 cases in this cohort were HCMV\textsuperscript{+} (compared with \~60% of controls and 80% of mild COVID-19 cases).\textsuperscript{74} More importantly, \~65% of people with severe COVID-19 had expansion of adaptive NK cells compared with only 10–20% of controls and those with moderate disease.\textsuperscript{74} Whether these expansions of adaptive NK cells in HCMV\textsuperscript{+} people were driven by SARS-CoV-2, or whether they predated SARS-CoV-2 infection, is not known but these data do raise the possibility that adaptive NK cells may contribute to tissue damage in COVID-19. In support of this hypothesis, in COVID-19 patients these adaptive cells also express Ksp37, a marker of cytotoxic lymphocytes associated with lung disease,\textsuperscript{77} and, despite their lower intrinsic proliferative capacity, also express markers of recent proliferation.\textsuperscript{74} This is reminiscent of the increased proliferative capacity of adaptive CD45RO\textsuperscript{+} NK cells in chronic malaria infection as discussed above\textsuperscript{61,74} and is also consistent with reports of enrichment of highly differentiated CD56\textsuperscript{dim}CD57\textsuperscript{+} NK cells with high proliferative capacity in the peripheral blood in a patient with SARS-CoV-2 infection and acute respiratory distress syndrome (ARDS).\textsuperscript{78}

A cluster of genes enriched in terminally differentiated CD8\textsuperscript{+} T cells and CD56\textsuperscript{dim}CD57\textsuperscript{+} NK cells was downregulated in the blood of children with multisystem inflammatory syndrome after SARS-CoV-2 infection\textsuperscript{79} suggesting that there is no straightforward relationship between NK cell subset activation and disease severity. However, a causal relationship between activation of highly differentiated NK cells and severe COVID-19 is, as yet, unproven and homing of less differentiated CD56\textsuperscript{dim} NK cells to the tissues during severe disease may also contribute to alterations in cell frequencies in peripheral blood.

The expansion of CD56\textsuperscript{dim}NKG2C\textsuperscript{+} NK cells in COVID-19 patients also raises the possibility that increased expression of HLA-E in conjunction with inflammatory mediators may mediate this adaptive NK cell expansion.\textsuperscript{74} Indeed, a recent study suggested that a peptide derived from the SARS-CoV-2 spike 1 protein can stabilise HLA-E on lung epithelial cells leading to activation of NKG2C\textsuperscript{+} NK cells.\textsuperscript{80} Interestingly, an in silico study also identified SARS-CoV-2 peptides predicted to bind HLA-C alleles recognising activating KIR.\textsuperscript{81}

**VACCINATION AND NK CELLS**

An indirect role for NK cells in regulating virus- or vaccine-induced responses has been highlighted in several experimental systems. For example, NK cells have been shown to regulate CD4\textsuperscript{+} T-cell responses and follicular T helper cell (Tfh) activity, thereby influencing the breadth and depth of the antibody response and effector CD8\textsuperscript{+} T-cell response to vaccinia virus and LCMV infections in murine models.\textsuperscript{82,84} Moreover, adaptive/highly differentiated NK cells, which express higher levels of the endosomal effector protein RAB111FIP5, impact on the generation of broadly neutralising antibodies in HIV-1-infected individuals.\textsuperscript{85}

However, mounting evidence also suggests that NK cell differentiation is also affected by vaccination (summarised in Table 2). Less differentiated CD56\textsuperscript{bright} and CD56\textsuperscript{dim}CD57\textsuperscript{−} NK cells are activated after influenza virus vaccination\textsuperscript{48,86,87} with evidence suggesting that common \gamma chain cytokines (IL-2, IL-12 and IL-15) prime myeloid dendritic cells to secrete IL-12 to support NK cell responses.\textsuperscript{88,89} Post-vaccination increases in the frequencies of these less differentiated CD56\textsuperscript{dim}CD57\textsuperscript{−} NK cells in HCMV\textsuperscript{+} individuals are consistent with IL-2 family cytokines also supporting maintenance and expansion of less differentiated, cytokine-responsive subsets. However, more differentiated CD56\textsuperscript{dim}CD57\textsuperscript{+} cells are also enhanced post-vaccination in the presence of influenza-specific antibody rather than relying on cytokines for their activation.\textsuperscript{76} In line with this, increased frequencies of CD56\textsuperscript{dim}CD16\textsuperscript{−}NKG2C\textsuperscript{−} (CD57\textsuperscript{−} and CD57\textsuperscript{+}) NK cells are observed up to 14 days after seasonal influenza vaccination in individuals with high titres of haemagglutination inhibiting antibodies.\textsuperscript{90}

A recent study demonstrated increased cytotoxic and proliferative responses to antigen-pulsed monocyte-derived dendritic cells by highly differentiated CD56\textsuperscript{dim}CD57\textsuperscript{−}KLRG1\textsuperscript{−} NK cells after hepatitis B subunit vaccination.\textsuperscript{91} Interestingly,
these responses did not require the presence of immune serum. Yellow fever vaccine 17D induces proliferation and expansion of less differentiated CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells; these cells also demonstrate enhanced responsiveness to \textit{in vitro} restimulation with innate cytokines.\textsuperscript{92} Similarly, increased absolute numbers of CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells are observed within 3 days of immunisation with the rVSV-ZEBOV Ebola virus vaccine; increased NK cell expression of CXCR6 is an independent correlate of rVSV-ZEBOV vaccine responsiveness whilst reduced frequencies of NKG2D\textsuperscript{+} and increased frequencies of NKP30 and KIR\textsuperscript{+} NK cells are inversely correlated with plasma cytokine and chemokine concentrations, indicating likely recirculation of NK cell subsets within 72 h of vaccination.\textsuperscript{93} The Ad26.ZEBOV Ebola virus vaccine also activates CD56\textsuperscript{bright} NK cells as assessed by the induction of CD25 and Ki67 expression up to 14 days after the second dose.\textsuperscript{70} The extent to which different vaccine vectors, delivery platforms and adjuvating systems impact on NK cell differentiation will likely depend on their primary cellular tropism, interactions with pattern recognition receptors and downstream production of NK cell-activating cytokines; this aspect of vaccination has as yet received very little attention.

There are also many examples of potent, Fc receptor-mediated degranulation and cytokine production by more highly differentiated NK cells in response to vaccination-induced antibody.\textsuperscript{76,94–96} Moreover, subunit, virally vectored and whole viral vaccines, with or without chemical adjuvants, will provide distinct cytokine signatures for co-stimulation of NK cell responses with type 1 interferons, IL-15 and IL-18, all described to support antibody-dependent responses of adaptive NK cells.\textsuperscript{27,95,97,98} However, it is not known whether these cytokine- and antibody-mediated signals contribute to further differentiation of these particular NK cells.

**CONCLUSIONS**

Exposure to an infection, be it in early life, with a novel pathogen or after vaccination, is accompanied by bystander, accessory-cell-dependent proliferation and expansion of the ‘less differentiated’ pool of NK cells. Cumulative exposure to diverse pathogens, exacerbated by chronic or recurrent infection, can lead to progressive activation and expansion of ‘more differentiated’ NK cells working in tandem with the adaptive immune response. Cytokines from antigen-specific T cells and pathogen-specific antibodies amplify these NK cell responses, thereby co-opting NK cells into the adaptive immune response. Important questions remain, however, as to how pathogens or vaccines influence NK cell differentiation and functional diversification and what this means for the ability of NK cells to then contribute to protection from, or susceptibility to, both infectious and non-communicable diseases. Amongst these are the potential for maternal antibodies to protect the infant from infection by non-neutralising mechanisms and to induce NK cell activation and differentiation in early life. Differentiation of CD3\textsuperscript{+} CD16\textsuperscript{+} cells from cord blood ILC1-like precursors provides an opportunity for antibody-dependent effector cells to contribute to immune responses, perhaps earlier in life than hitherto appreciated. The extent to which NK cell subsets re-equilibrate after a pathogen is controlled or eliminated, and the mechanisms whereby uncontrolled infections (e.g. Hantavirus, severe SARS-CoV-2) or chronic or repeated infections (e.g. hepatitis C and malaria) induce lasting effects on the NK cell repertoire, are other key issues that need further investigation (Figure 1). The role, and the longer-term functional

| Vaccine     | Impact on NK cell differentiation subset                                                                 | References |
|-------------|----------------------------------------------------------------------------------------------------------|------------|
| Ebola       | Increased absolute numbers of CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK subsets. Increased Ki67 and CD25 expression in CD56\textsuperscript{bright} NK cell | 70,93      |
| HBSAg       | Priming of CD56\textsuperscript{dim} CD57\textsuperscript{+} KLRG1\textsuperscript{+} NK cell subset revealed on \textit{in vitro} restimulation | 91         |
| Influenza   | Activation, proliferation or expansion of CD56\textsuperscript{bright} and CD56\textsuperscript{dim} CD57\textsuperscript{+} NK cells. Priming of CD56\textsuperscript{bright} and CD56\textsuperscript{dim} CD57\textsuperscript{+} NK cells for enhanced response to cytokines. Increased frequencies of CD56\textsuperscript{dim} CD16\textsuperscript{+} NKG2C\textsuperscript{+} (both CD57\textsuperscript{+} and CD57\textsuperscript{+}) NK cells in individuals with high HAI titres after vaccination | 48,86,90   |
| Yellow Fever| Proliferation and expansion of CD56\textsuperscript{bright} and CD56\textsuperscript{dim} CD57\textsuperscript{+} subsets | 92         |

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consequences, of IL-15 in the expansion and terminal differentiation of adaptive NK cells also merits further investigation in the context of different pathogens. In summary, individual experiences of infection and vaccination over the life course will shape not only the adaptive immune cell population but also the NK cell population, with consequences for susceptibility to subsequent infections and, potentially, non-communicable disease such as cancers as inflammatory conditions.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Martin R Goodier: Conceptualization; Writing-original draft; Writing-review & editing. Eleanor M Riley: Conceptualization; Writing-review & editing.

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