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CD56 negative NK cells: origin, function, and role in chronic viral disease

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Natural killer (NK) cells serve as a first line of defense against acute viral infections. Immunogenetic association data suggest that NK cells also influence the course of chronic viral infections, such as infections with HIV-1 and hepatitis C virus (HCV). Chronic stages of these infections have a negative impact on NK cell function and promote the appearance of phenotypically and functionally abnormal NK cells. In this paper, we summarize available data on CD56^{neg} NK cells, an aberrant NK cell subset found in small numbers in healthy individuals and at elevated levels in individuals chronically infected with HIV-1 and HCV. We discuss current knowledge of CD56^{neg} NK cells, with a particular emphasis on their accumulation during chronic infection and the possible consequences of this for the host.

Natural killer (NK) cells in viral infection

NK cells are important in the control of viral infections [1]. They can, without previous sensitization, lyse virus-infected cells. NK cells also influence the transition between innate and adaptive immune responses via the production of cytokines and chemokines. They also affect the expression of co-stimulatory ligands for T and B cells [2]. In mice, NK cells contribute to the control of several viruses, including murine cytomegalovirus, influenza virus, ectromelia poxvirus and herpes simplex virus 1 [3]. Humans deficient in NK cell function have increased susceptibility to certain viral infections [4]. NK cells do not express antigen-specific receptors on their surface. However, certain NK cell receptors have evolved to recognize specific viral proteins [5–7]. Viruses, in turn, have developed strategies to avoid NK cell recognition [8,9]. This occurs, for example, through modulation of NK cell receptor ligands on the surface of infected cells or by direct interaction with NK cells.

Chronic viral infections have a significant impact on NK cells. This is particularly evident in HIV-1- or hepatitis C virus (HCV)-infected humans, whose NK cells undergo numerous phenotypic and functional changes [10–13]. In this review, we focus on one feature of NK cells in chronic viral infection that has attracted recent attention: the appearance of large numbers of functionally altered CD56^{-}CD16^{+} NK cells (hereafter termed 'CD56^{neg} NK cells) (Figure 1). We summarize the literature on this NK cell subset, discuss the underlying mechanisms that drive their accumulation in chronically infected patients, and discuss the consequences this might have for the host.

Identification of CD56^{neg} NK cells in chronic viral infections

NK cells can be divided into subsets based on their expression of CD56 and CD16. In peripheral blood, CD56^{dim}CD16^{+} NK cells dominate, whereas CD56^{bright}CD16^{-} NK cells make up a smaller population [14]. Although CD56^{neg} NK cells exist in healthy individuals, they are rare, and represent at most a few percent of total NK cells in the blood (Box 1). In an initial study published 15 years ago, increased numbers of CD56^{neg} NK cells in patients with chronic HIV-1 infection were reported [15]. Since then, several investigators have confirmed this finding [16–24]. Expansion of CD56^{neg} NK cells have more recently also been described in patients with chronic HCV infection (Figure 2) [25,26]. This expansion appears to develop during the chronic state of infection, because individuals with primary HIV-1 infection have normal or modestly increased numbers of CD56^{neg} NK cells in peripheral blood (Figure 2) [18,23,27].

Analysis of NK cells in patients with chronic HIV-1 infection initially led to reports of reduced NK cell counts in peripheral blood [28,29]. However, when CD56^{neg} NK cells were included in the analysis, chronic HIV-1 infection did not seem to cause a drop in total NK cell numbers [20]. Instead, the infection gave rise to an increase in CD56^{neg} NK cells that represented 20–40% of all NK cells, and 3–6% of all lymphocytes. This increase occurred primarily at the expense of CD56^{dim} NK cells, whereas numbers of CD56^{bright} NK cells remained stable. An analogous redistribution between NK cell subsets is seen in HCV infection, although the shift towards higher numbers of CD56^{neg} NK cells is not as dramatic as in patients with HIV-1 [26].

Numbers of CD56^{neg} NK cells in patients with HIV-1 infection have differed between studies, despite rather similar cohorts of patients. This might, at least in part, have been due to the different strategies utilized to identify the cells. NK cells are conventionally defined as CD56^{+}CD3^{-} lymphocytes. CD16 is expressed on many leukocytes, including monocytes, neutrophils, 6-sulfo LacNAc^{+} dendritic cells (slanDCs) [30], and subsets of CD8 and γδ T cells [30–32]. Thus, when stepping away from the standard CD56^{+}CD3^{-} definition of NK cells, safety measures are likely to be needed for correct identification of CD56^{neg}

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  doi:10.1016/j.it.2010.08.003

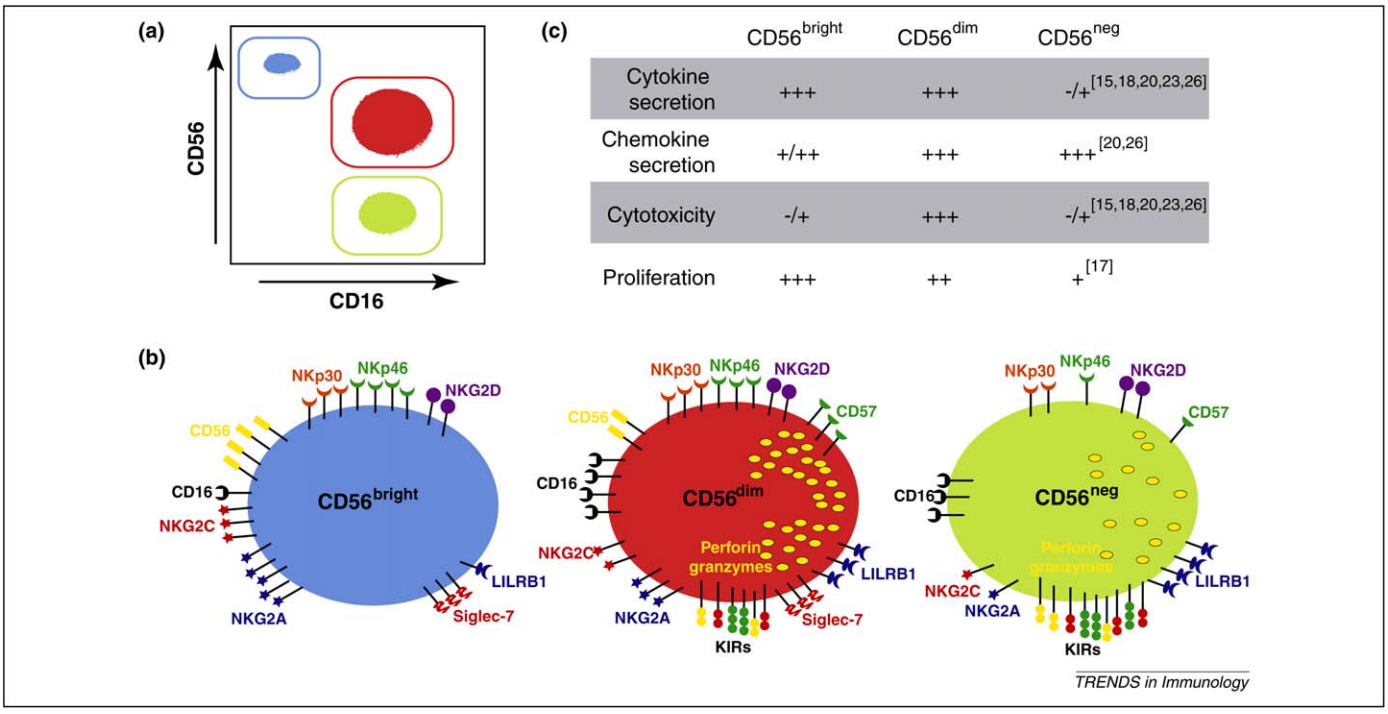

Figure 1. Phenotypical and functional properties of CD56^{bright}, CD56^{dim}, and CD56^{neg} NK cells. **(a)** Schematic illustration of CD56 and CD16 expression on CD3$^-$CD4$^-$CD14$^-$CD19$^-$ lymphocytes with gates on CD56^{bright} (blue), CD56^{dim} (red), and CD56^{neg} (green) NK cells. **(b)** Relative expression levels of activation and inhibitory receptors on CD56^{bright} (blue), CD56^{dim} (red), and CD56^{neg} (green) NK cells from peripheral blood schematically illustrated with 1–4 molecules based on a compilation of results in the literature [15,17,19,20,23–26]. **(c)** Functions of CD56^{bright}, CD56^{dim}, and CD56^{neg} NK cells from peripheral blood schematically graded as −/+, +, ++, or +++, based on a summary of results in the literature.

NK cells (Box 1) [33,34]. Early studies have excluded only CD3$^+$ cells [15,18,19,21,22], whereas later studies have relied on multicolor flow cytometry for a more precise identification of CD56^{neg} NK cells, by exclusion of cells that express CD3, CD4, CD14 and CD19, and in some cases, CD11c [20,25–27]. Other investigators have also employed purification techniques to isolate NK cells before determining CD56^{neg} NK cell numbers [16,17]. However, the latter could result in co-purification of contaminating CD56^{neg}CD16$^+$ slanDCs [30,35]. Therefore, we propose that cells that express CD3, CD4, CD14, slan or CD19 should be excluded to ensure accurate identification of CD56^{neg} NK cells.

Box 1. What is an NK cell?

To define an NK cell is not as straightforward as defining a T or B cell, for which rearranged T or B cell receptors give these cells unique features. The original characterization of NK cells as ''naturally occurring killer lymphocytes with specificity for tumor cells'' was based purely on a functional basis [51,52]. Since then, different combinations of cell surface molecules have been used to identify NK cells. In humans, NK cells are classically defined as CD56$^+$CD3$^-$ lymphocytes. However, CD56 is not present in the mouse, is expressed by subsets of T cells, and appears late on human NK cells during development [46,51]. An alternative cross species phenotypical definition has been proposed whereby NK cells are identified as NKp46$^+$CD3$^-$ cells [51]. However, this can be disputed after the discovery of NKp46$^+$ lymphoid tissue inducer-like cells in the gut mucosa [53]. Altogether, this brings a degree of uncertainty when classifying new subsets of cells as NK cells, and calls for use of multiple approaches to reach the NK cell definition. CD56^{neg} NK cells, found at low frequencies in healthy individuals and in increasing numbers during chronic viral infection, lack the expression of CD56. However, the cells express NKp46, albeit at low levels under certain conditions, and display a variegated expression of numerous other NK cell-associated molecules. Furthermore, CD56^{neg} NK cells stain negatively for lineage markers for other subsets of immune cells, and importantly, contain intracellular effector molecules. This phenotypic profile, together with functional features of these cells that are similar to those of classical NK cells, provide a platform for classification of this subset of cells as *bona fide* NK cells. However, many questions on the biology of CD56^{neg} NK cells remain unanswered.

Characteristics of CD56^{neg} NK cells

NK cell function depends on the expression pattern of activation and inhibitory receptors, and on the ability of these cells to respond to cytokines and chemokines (Box 2) [36]. Furthermore, for an NK cell to respond functionally upon recognition of target cells, it must express at least one inhibitory receptor for a self-ligand, such as MHC class I molecules, which are present in the host; a process referred to as 'NK cell education' [37,38]. Thus, the phenotype of CD56^{neg} NK cells can give clues to their functional properties (Figure 1).

When compared to CD56^{dim} NK cells, CD56^{neg} NK cells from patients with HIV-1 infection express lower levels of some activation receptors such as the natural cytotoxicity receptors (NCRs) NKp30 and NKp46, whereas the expression of NKG2D is unchanged [17,20]. This pattern is less clear in healthy individuals [20]. By contrast, patients infected with HCV alone have CD56^{neg} NK cells that express NKp30, NKp46 and NKG2D at levels similar to those of CD56^{dim} NK cells [26]. A common feature of CD56^{neg} NK cells is the low perforin expression reported for patients infected with HIV-1 or HCV, as well as for healthy individuals [18,25,26].

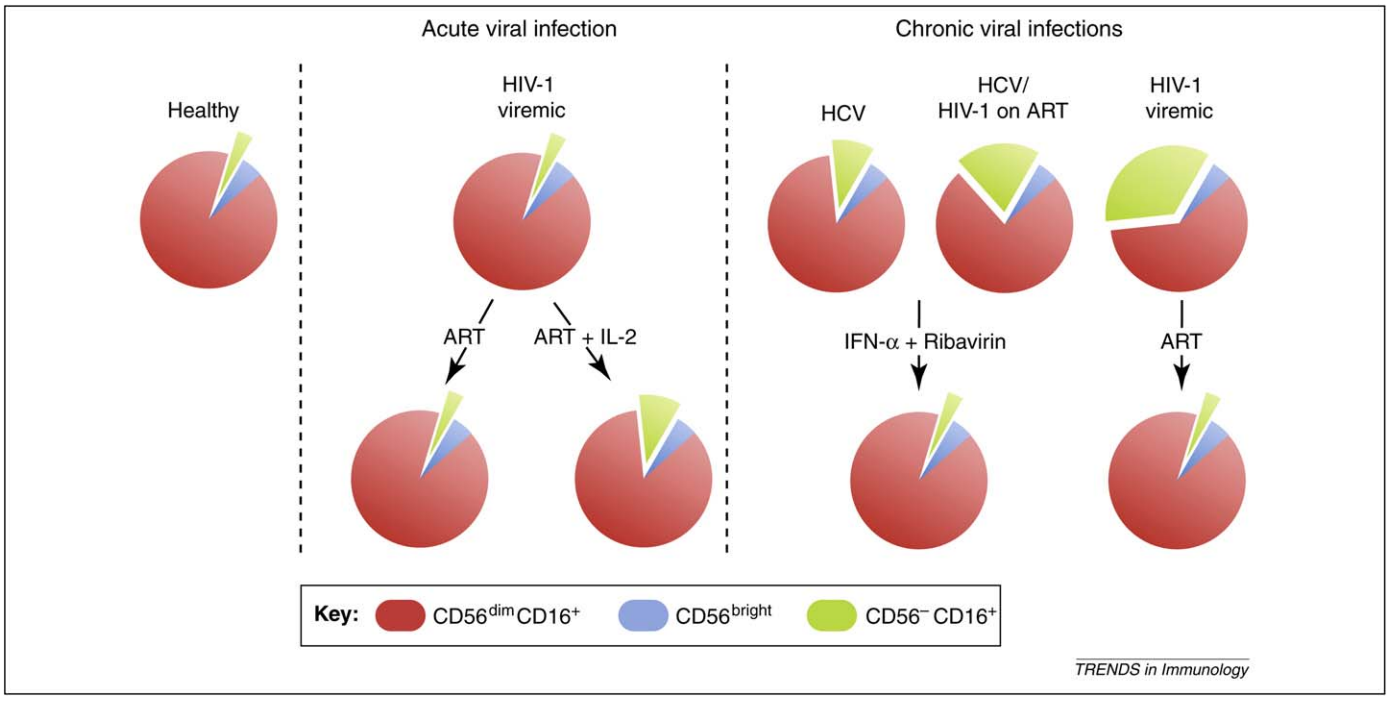

Figure 2. Modulation of CD56^{neg} NK cell numbers *in vivo* after antiviral treatment and/or immunotherapy. Representative pie charts compiled from available literature show the numbers of peripheral blood CD56^{neg} NK cells in acute [18,23,27] and chronic [15–18,20–26] viral infections (top row). CD56^{neg} NK cells are increased in numbers during chronic HCV or HIV infection. Modulation of the relative size of the CD56^{neg} NK cell subset after therapy is depicted by arrows [23,25,27] (bottom row). Treatment with IFN-α and ribavirin, for chronic HCV, or ART for chronic HIV, returns the numbers of CD56^{neg} NK cells to levels found in healthy individuals.

Investigations of inhibitory receptors, such as killer cell immunoglobulin-like receptors (KIRs), have yielded discrepant results. An initial study has documented increased expression of KIR2DL2, KIR2DL3 and LILRB1 on CD56^{neg} NK cells, as compared to CD56^{+} NK cells in patients with HIV-1 infection [17]. In another study, expression of KIR2DL1 was lower on CD56^{neg} NK cells [20]. A common aspect is the low expression of NKG2A on CD56^{neg} NK cells from HIV-1 [17,20], HCV [26], and HIV-1/HCV co-infected individuals [25]. Furthermore, CD56^{neg} NK cells do not express inhibitory Siglec-7 [23]. Despite the progress in research on CD56^{neg} NK cells, many issues concerning their detailed phenotype remain unexplored, including expression patterns of chemokine and cytokine receptors, capacity to express tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and FasL, as well as the presence of adhesion molecules (Boxes 1 and 3).

Box 2. Surface receptors that regulate NK cell function

NK cell specificity for target cells and the ensuing effector functions are dependent on signaling from a wide array of receptors that are expressed on the surface of the NK cell. This process of activation involves contact, adhesion, polarization, and degranulation towards the target cell, and different groups of receptors have distinct functions during the process [36].

- **Activation receptors**
  A multitude of activation receptors exist and these are important for making contact with target cells, to polarize granules and for subsequent release of perforin and other cytotoxic molecules [36]. NKG2D is one activation receptor that is expressed by all NK cells and it recognizes certain stress-induced molecules on target cells [54].
- **Inhibitory receptors**
  According to the 'missing-self' hypothesis [55], inhibitory receptors preserve tolerance to self and determine NK cell specificity. Inhibitory receptors, such as KIRs and NKG2A, are stochastically expressed on subsets of NK cells and many of these have classical, or non-classical, MHC class I molecules as their ligands [36,56].
- **Adhesion molecules**
  NK cells express β1 and β2 integrins, such as lymphocyte function-associated antigen-1, which recognize, for example, different intercellular adhesion molecules on target cells. These interactions are important for NK cell adhesion to target cells, immune synapse formation and granule polarization [36].
- **Apoptosis-inducing molecules**
  In addition to production and release of cytokines and effector molecules, NK cells can also mediate killing via apoptosis-inducing molecules such as TRAIL and FasL, which are released upon activation and bind to death receptors on target cells [57].

Functional skewing of CD56^{neg} NK cells

NK cells perform a multitude of antiviral functions, including production of cytokines, such as interferon (IFN)-γ, and killing of infected cells through directed release of perforin and granzymes, as well as via TRAIL. The function of CD56^{neg} NK cells has been examined in patients with chronic viral infections, and to a lesser extent, in healthy individuals (Figure 1). When compared to CD56^{dim} NK cells, CD56^{neg} NK cells from patients with HIV-1 exhibit a low capacity to degranulate and to produce IFN-γ in response to the human HLA-class-I-negative erythroleukemia cell line K562 [15,18,20,23]. CD56^{neg} NK cells are also less able than CD56^{dim} NK cells to respond with antibody-dependent cellular cytotoxicity (ADCC), or to phorbol 12-myristate 13-acetate (PMA)/ionomycin stimulation [15,18,23]. CD56^{neg} NK cells from patients with HCV infection also produce lower amounts of cytokines and degranulate to a lesser extent compared to CD56^{dim} NK cells [26]. Long-term culture with interleukin (IL)-2 (6–28 days) does not restore the functional capacity of CD56^{neg} NK cells [17]. However, CD56^{neg} NK cells from patients

Box 3. Outstanding questions in the study of CD56^{neg} NK cells

Clearly, from the present review, a number of questions remain unanswered. Important topics that merit further investigation are listed below.

- Tissue distribution/chemokine receptor expression. Are CD56^{neg} NK cells found only in peripheral blood or also in tissues? Furthermore, are these cells found in other conditions besides the ones discussed here?
- What is the maturation status of CD56^{neg} NK cells? Comprehensive studies of phenotype, function, proliferative capacity, and level of senescence in this subset of cells are mandated.
- With high expression of inhibitory KIRs, the prediction would be that a significant proportion of CD56^{neg} NK cells are 'educated', and consequently, functional. What then is the mechanism behind the paradoxically reduced functionality of CD56^{neg} NK cells?
- Are CD56^{neg} NK cells exhausted or merely functionally altered? Is the molecular signature of CD56^{neg} NK cells altered in relation to CD56^{dim} and CD56^{bright} NK cells?
- What is the relationship between CD56^{neg} NK cells found in healthy individuals and CD56^{neg} NK cells that expand numerically in patients with chronic viral infections? Are these the same cells or two distinct subsets?

with HIV-1 infection proliferate to a similar degree as CD56^{dim} NK cells after stimulation with IL-2 [17]. Together, these results suggest that CD56^{neg} NK cells from patients with chronic viral infections are defective in their capacity to perform natural cytotoxicity and ADCC, and to produce IFN-γ. However, more studies that compare *ex vivo* CD56^{neg} NK cells from patients with HIV-1 and/or HCV infection with those from healthy individuals should help determine the impact of viral disease on the function of CD56^{neg} NK cells (Box 3).

CD56^{neg} NK cells are a source of chemokines

In addition to cytotoxic activity and production of antiviral cytokines, NK cells also produce macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4), and RANTES (CCL5) [39]. These three chemokines bind to CC-chemokine receptor 5 (CCR5) and inhibit HIV-1 entry into CD4-expressing target cells [40]. This is probably one of the main ways by which NK cells suppress HIV-1 replication [41,42]. CD56^{neg} NK cells from patients with HIV-1 infection still release substantial amounts of MIP-1β (Figure 1) [20]. A similar profile of MIP-1β release has also been observed for CD56^{neg} NK cells from patients with HCV infection [26]. To what extent CD56^{neg} NK cells also produce MIP-1α and RANTES remains to be investigated. However, because the minimal requirements of receptor engagement for NK cell chemokine secretion appear to be similar for MIP-1α, MIP-1β and RANTES, one can speculate that CD56^{neg} NK cells also secrete these two chemokines [43]. The underlying explanation for the ability of CD56^{neg} NK cells to produce chemokines, in the absence of cytokine production or degranulation activity, might be that the threshold for activation is lower for induction of chemokines [43].

The data reviewed above support a model in which CD56^{neg} NK cells have largely lost, or do not gain, the capacity to produce antiviral cytokines and kill infected cells. Yet, these cells retain the ability to release pro-inflammatory chemokines. A similar sequential loss of function occurs during T cell exhaustion in settings of chronic immune activation [44,45]. Whether CD56^{neg} NK cells are an important source for CCR5-binding chemokines *in vivo,* and if they possess the capacity to inhibit HIV-1 replication, remain unanswered (Box 3).

Modulating CD56^{neg} NK cells by cytokines and antiviral treatment

Several strategies have been employed to understand the mechanism of CD56^{neg} NK cell accumulation. This includes efforts to modulate the function and phenotype of the cells *in vitro*, longitudinal monitoring of patients with chronic viral infections during immunotherapy and antiviral therapy, and careful assessment in clinical cohorts with distinct characteristics (Figure 2). Stimulation with IL-2 *in vitro* converts the CD56^{neg} phenotype to a CD56^{+} phenotype in NK cells isolated from patients with chronic HIV-1 infection [17]. However, whether IL-2 can also influence the altered expression of activation receptors in these cells remains unclear. Similar studies of CD56^{neg} NK cells from healthy individuals have shown that stimulation with IL-2, but not IFN-α or the combination of IL-2 and IFN-α, results in upregulation of CD56 [25].

HIV-1 long-term non-progressors and patients who successfully suppress their viral load after antiretroviral therapy (ART) have CD56^{neg} NK cell numbers that resemble those found in healthy subjects, which suggests that HIV-1 viral load has a direct impact on CD56^{neg} NK cell numbers [18,21,23]. By contrast, patients with chronic HIV-1 who fail to suppress their viral load, or only partially suppress it, have CD56^{neg} NK cell numbers similar to those found in patients with persistent viremia [18,21]. However, normalization of CD56^{neg} NK cell counts upon effective ART takes more than 12 months; long after the viremia is suppressed to undetectable levels. Thus, CD56^{neg} NK cell expansion might not be a direct consequence of high viral load.

In contrast to the findings in patients who are undergoing ART alone, anti-HCV therapy using IFN-α and ribavirin in HCV and HIV-1 co-infected patients who are also receiving ART results in reversion of CD56^{neg} NK cell numbers to levels found in healthy individuals after only 4 weeks of treatment [25]. This is probably not a direct consequence of IFN-α, because IFN-α has no effect on CD56 expression levels in CD56^{neg} NK cells when tested *in vitro* [25]. The increase in CD56^{neg} NK cells in HCV-infected patients might indicate a more fundamental disturbance in innate cellular immunity, because pretreatment levels of CD56^{neg} NK cells correlate with HCV treatment outcome [26].

Treatment of patients with acute HIV-1 infection reveals yet another scenario [27]. Here, CD56^{neg} NK cell numbers correlate with viral load in the untreated patients and, as in patients with chronic HIV-1, levels decreases after the initiation of ART. The effect of treatment is more rapid than in patients with chronic disease, because numbers of CD56^{neg} NK cells are normalized within 6 months. On the contrary, the combined administration of ART and IL-2 in these patients causes an increase in CD56^{neg} NK cell numbers. This is unlike the outcome of studies performed *in vitro* in which IL-2 converts the CD56^{neg} NK cell

phenotype into $CD56^{+}CD16^{+}$ [17,25]. Overall, the studies reviewed above provide some indications of the underlying mechanisms for expansion of $CD56^{neg}$ NK cells. However, discrepancies exist, especially between results obtained *in vitro* and *in vivo* (Box 3).

On the origin of $CD56^{neg}$ NK cells

Human NK cells develop through different intermediary and more committed precursors into $CD56^{bright}$ NK cells [46], and the $CD56^{bright}$ NK cell subset contains predecessors of $CD56^{dim}$ NK cells [47]. Furthermore, $CD56^{dim}$ NK cells undergo a continuous differentiation process with coordinated functional and phenotypic changes [48,49]. It will be important to determine if $CD56^{neg}$ NK cells represent a terminal subset of NK cells; whether they arise from a mixed population of mature NK cells with altered characteristics; or if they represent an expansion from an immature precursor stage.

So-called 'stage 3' immature NK (iNK) cells are the first committed NK cell precursors and are the subset that precedes $CD56^{bright}$ NK cells in development [46]. iNK cells express low levels of CD56 and NCRs, upregulate CD56 after stimulation with IL-15, and are restricted to granulocyte–macrophage colony-stimulating factor production after stimulation. Thus, this subset of cells shares characteristics with $CD56^{neg}$ NK cells. However, $CD56^{neg}$ NK cells express many NK cell-specific receptors not found on the iNK cells, including KIRs, CD94/NKG2A, NKG2D and CD16 [17,26]. Furthermore, $CD56^{neg}$ cells still possess, albeit to a reduced extent, the capacity to kill target cells and produce cytokines, which are not features of iNK cells [15,26]. It, thus, appears unlikely that $CD56^{neg}$ NK cells are descendents of iNK cells. Effector molecule expression by $CD56^{neg}$ NK cells further supports the presumption that these cells are more closely related to $CD56^{dim}$ NK cells. However, comprehensive studies of these subsets, similar to those employed to visualize mouse NK cell development and differentiation involving gene-expression profiling [50], are still needed.

Dissection of mature $CD56^{dim}$ NK cell differentiation is under way [48]. When placed into this process, $CD56^{neg}$ NK cells share characteristics with early as well as more highly differentiated $CD56^{dim}$ NK cells. Low expression of CD94/NKG2A, but higher expression of KIRs within the $CD56^{neg}$ NK cell subset, is characteristic of more differentiated $CD56^{dim}$ NK cells [17,26]. By contrast, $CD56^{neg}$ NK cells express low levels of CD57 and proliferate upon stimulation; both of which are features of less-differentiated $CD56^{dim}$ NK cells [15,17,26]. In summary, the known phenotypic and functional properties of $CD56^{neg}$ NK cells indicate that they might originate from the early stage of $CD56^{dim}$ NK cell differentiation. Whether their aberrant differentiation into $CD56^{neg}$ NK cells is reversible or not remains an important unanswered question (Box 3).

Concluding remarks

Although $CD56^{neg}$ NK cells might appear featureless at first glance, the evidence discussed here highlights these cells as a sizeable subset of lymphocytes, which represent up to 40% of all NK cells present in peripheral blood of patients chronically infected with HIV-1 or HCV. A detailed functional assessment reveals a unique profile skewed towards chemokine production, which suggests that these cells possess direct antiviral properties and the potential to direct adaptive immune responses. However, key questions remain to be answered regarding tissue distribution, development, and functional regulation (Box 3).

Acknowledgements

The authors are supported by grants from the Swedish Research Council, the Swedish Cancer Society, the Stockholm County Council, the Karolinska Institutet, and the Karolinska University Hospital. N.K.B. is a recipient of a Karolinska Institutet MD/PhD award.

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