

Natural Killer Cells: A Coherent Model for Their Functional Role in *Mycobacterium tuberculosis* Infection

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Key Words

Natural killer cells · Tuberculosis · Mycobacteria · Natural cytotoxicity receptors · Toll-like receptor

Abstract

Tuberculosis is still a leading cause of bacterial infection worldwide, with an estimate of over two billion people latently infected with *Mycobacterium tuberculosis* (MTB). A delicate interplay between MTB and the host's innate and acquired immune system can influence the outcome of the infection, which ranges from pathogen elimination to the establishment of a latent infection or a progressive disease. Although the host cell-mediated adaptive immune response is of vital importance in the control of MTB infection, growing evidence indicates that innate immune cells may greatly influence the outcome of the interaction between the bacterium and the host. Among the cell populations likely to play a role in the host immune response to MTB, natural killer (NK) cells have recently attracted considerable interest. This review is dedicated to dissecting the role of NK cells in immunity to tuberculosis, reporting the most relevant findings and providing a working model of the possible contribution of NK cells in early and late events associated with MTB infection.

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Introduction

Tuberculosis (TB) is still the leading cause of death due to bacterial infection worldwide. According to recent estimates, *Mycobacterium tuberculosis* (MTB), the causative agent of human TB, caused 8.6 million cases of active disease and 1.3 million deaths globally in 2012 [1]. Nevertheless, thanks to the concerted action of host innate and adaptive immunity, most infected individuals do not develop clinical signs of TB, but rather establish a long-lasting relationship with the bacterium that persists in a dormant state in the lung, at least for as long as immunity remains competent [2].

Although it is generally agreed that the IL-12-Th1-IFN- γ (interleukin 12-T helper 1 cell-interferon gamma) axis plays a crucial role in immune protection against TB [3], it has become progressively evident that the host immune response to MTB is multifaceted and that numerous cell subsets, apart from Th1 lymphocytes, are required for an optimal response [2]. Among the cell populations likely to play a role in host immune response to MTB, natural killer (NK) cells have recently attracted considerable interest. Initially identified for their marked cytotoxic activity against tumours and virally infected cells without need of a prior sensitization, NK cells have

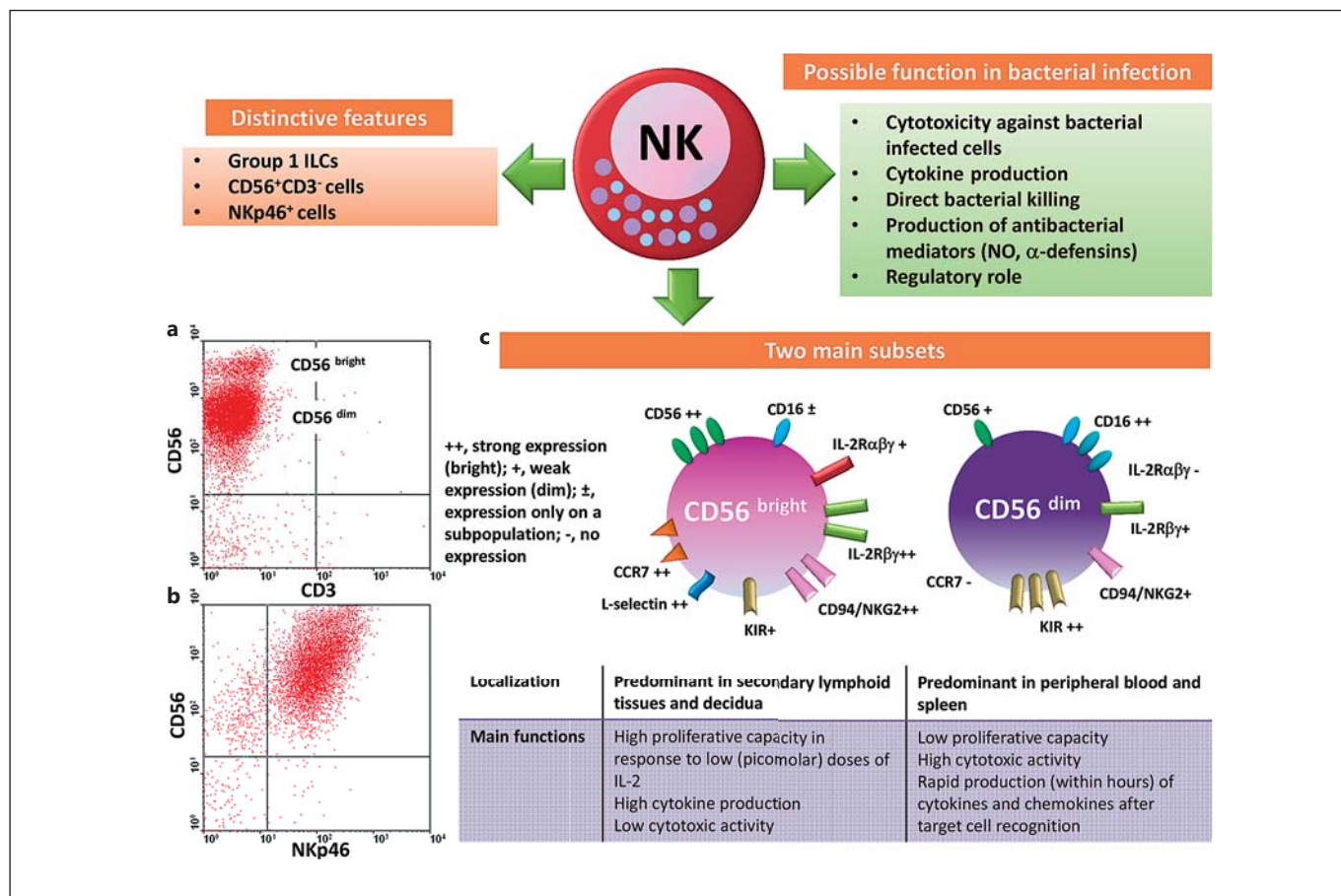


Fig. 1. Overview of NK cell biology and function in bacterial infections. NK cells can be phenotypically defined by the surface expression of the CD56 marker and the lack of expression of CD3. The activating receptor NKp46 (NCR1) is also expressed on virtually all human NK cells. NKp46 and CD56 are also expressed by some group 3 ILCs, very rare cells in the peripheral blood. **a, b** Flow cy-

tometric analysis of human peripheral-blood NK cells stained with fluorescent-labelled anti-CD56 and anti-CD3 (**a**) or anti-CD56 and anti-NKp46 (**b**) monoclonal antibodies. **c** The 2 main NK cell subsets CD56^{bright} and CD56^{dim} differ in terms of expression of phenotypic markers, tissue distribution and biological functions.

long been considered as the innate immune cells involved mainly in anti-viral and anti-tumour immunity. It is now clear that they can perform a range of immunological functions including the production of great amounts of immunoregulatory cytokines, the lysis of cells infected with intracellular bacteria, the production of anti-bacterial mediators (e.g. nitric oxide, α -defensins and granulysin), the regulation of functions of other cell types and, in certain circumstances, direct bactericidal activity, which underlines their contribution to anti-bacterial immunity [4].

The aim of this article is to dissect the role of NK cells in TB by reviewing the most relevant findings obtained from in vitro and in vivo studies and the clinical setting, and to evaluate the growing amount of data in this re-

search field, providing a working model on the possible contribution of NK cells in early and late events associated with MTB infection.

Biology of Human NK Cells

NK cells represent 10–15% of the lymphocytes in the peripheral blood, and are identified phenotypically by the expression of the CD56 marker and the lack of expression of CD3. It is now evident that NK cells are far from being a homogenous cell population; instead, they comprise different cell subsets with distinct phenotypes, functional activities and tissue localization [5, 6] (fig. 1). The surface density of the CD56 marker enables identification of 2 distinct

main subpopulations of NK cells. The majority of peripheral NK cells (approx. 90%) exhibit low-density expression of CD56 (CD56^{dim}) and express high levels of the FcγIII receptor (CD16). In contrast, about 10% of peripheral-blood NK cells express CD56 at high levels (CD56^{bright}) but lack or express low levels of the CD16 marker [5]. Of note, CD56^{bright} cells are the most represented NK cells in the lymph nodes; they constitutively express the high- and intermediate-affinity IL-2 receptor and promptly expand in vitro and in vivo in response to low doses of IL-2 [7].

Recently, NK cells were classified as the prototypical members of the group 1 innate lymphoid cells (ILCs) [8]. ILCs are a family of developmentally related cells that are emerging as important effectors of innate immunity and have a central role in tissue remodelling. They are divided into 3 groups based on their ability to produce IFN-γ (group 1: ILC1s and NK cells), IL-5 and IL-13 (group 2: ILC2s) and IL-17 and/or IL-22 (group 3: ILC3s and lymphoid-tissue inducer cells) [8]. Although there are no studies reporting ILCs in mycobacterial infection, recent evidence suggests that they have important effector functions during the early stages of immune responses against various microorganisms including several bacterial species [8].

The best-characterized effector function of NK cells is cytotoxicity; target cells include tumour cells, cells infected with viruses or intracellular bacterial pathogens and, as more recently reported, immature dendritic cells (iDC) [9]. Following monokine stimulation, NK cells also produce a wide range of cytokines including IFN-γ, tumour necrosis factor alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF) and other soluble factors that positively or negatively regulate the functions of other innate or adaptive immune cells [4]. NK cell effector functions can be preferentially assigned to the 2 major NK cell subsets, with the CD56^{dim} subset being intrinsically highly cytotoxic and the CD56^{bright} subset being the primary source of immune-regulatory cytokines [5], although recent studies revealed that CD56^{dim} are also capable of a rapid secretion of cytokines [10, 11].

NK cell activity is under the control of a complex network of signals triggered by ligands binding to different, germ-line-encoded, cell-surface-expressed inhibitory and activating receptors [11]. The balance between activating and inhibitory signals determines if and how NK cells will undergo activation. Inhibitory receptors include killer immunoglobulin-like receptors (KIR), which inhibit NK cytotoxic functions following the recognition of major histocompatibility complex class I (MHC I) molecules on target cells [12]. Some of the best-characterized

activating NK cell receptors are the natural cytotoxicity receptors (NCRs), comprising three molecules NKp30 (CD337), NKp44 (CD336) and NKp46 (CD335), the NKG2D, 2B4 or the DNAX accessory molecule 1 [11–14].

The strategies used by NK cells to recognize their targets are diverse. The first includes the recognition of host-derived or pathogen-encoded ligands, the expression of which is up-regulated in transformed or infected cells ('induced self-recognition'). The second relies on the inhibitory recognition of self-proteins that are expressed by normal cells but down-regulated by infected or transformed cells ('missing self-recognition') [15]. One of the most interesting advances in the biology of NK cells over the past few years came from demonstrating that they can express 'pathogen recognition receptors', suggesting that a third strategy by which NK cells may undergo the activation and expression of effector functions is the direct recognition of 'pathogen-associated molecular patterns' (PAMPs) [16, 17]. In this context, the pioneer work of Chalifour et al. [18] is particularly significant. They demonstrated that two bacterial PAMPs, i.e. the outer membrane protein A from *Klebsiella pneumoniae* (KpOmpA) and flagellin from *Escherichia coli* may directly stimulate human NK cells through Toll-like receptor (TLR)2 and TLR5, respectively. These proteins induced IFN-γ production by NK cells and up-regulated the expression of α-defensins, which are anti-microbial peptides endowed with a rapid and direct bactericidal activity and immune-regulating properties [19]. The KpOmpA and flagellin stimulatory capacity was highly enhanced in the presence of exogenous IL-2 or proinflammatory cytokines such as IL-1β, IL-12, IL-15 or IFN-α, but not IL-10, suggesting that although able to directly respond to PAMPs, NK cell functions are tightly regulated by soluble factors released by bystander cells. It is now increasingly evident that direct sensing of microbial pathogens may represent a previously unappreciated mechanism of NK cell participation to the first line of defence against infectious agents. NK cell receptors potentially involved in the direct recognition of microbial ligands are numerous and include several members of the TLR family [18, 20–25], intracellular receptors like NOD2 [26] or members of the NCR family [21, 27–37] (table 1).

Although the past decade has seen a net advance in the knowledge of NK cell biology, much has still to be elucidated. The recent evidence that NK cells may express properties that have been previously attributed only to cells of adaptive immunity (e.g. immunological memory) [38–40] render the study of these cells extremely interesting both at a basic and a clinical level because of the po-

Table 1. NK cell receptors potentially involved in the direct recognition of microbial ligands

NK cell receptor	Ligand	Source	Effect on NK cell function	Reference
<i>TLR</i>				
TLR2	OmpA	<i>Klebsiella pneumoniae</i>	α -defensin and IFN- γ production	[18]
	lipophosphoglycans	<i>Leishmania major</i>	IFN- γ and TNF- α production	[20]
	peptidoglycan	<i>M. tuberculosis</i>	CD69 expression, stimulation of NKp44 expression	[21]
	unknown	<i>M. bovis</i> BCG	CD69 and CD25 expression, IFN- γ and TNF- α production	[22]
	HpaA lipoprotein	<i>Helicobacter pylori</i>	IFN- γ production	[23]
TLR3	dsRNA	virus	CD69 and CD25 expression, IFN- γ and TNF- α production, cytotoxicity	[24]
TLR4	FimH	<i>E. coli</i>	CD69 expression, cytotoxicity and IFN- γ production	[25]
TLR5	flagellin	<i>E. coli</i>	IFN- γ production and cytotoxicity	[18]
TLR9	CpG	bacterial DNA	CD69 and CD25 expression, IFN- γ and TNF- α production, cytotoxicity	[24]
<i>NOD</i>				
NOD2	muramyl-dipeptide	bacterial peptidoglycan	CD69 expression, IFN- γ production	[26]
<i>NCR</i>				
NKp30	pp65	Human cytomegalovirus	inhibition of cytotoxicity	[27]
	viral HA	poxvirus, Vaccinia virus	inhibition of cytotoxicity (blocks NKp30-triggered activation; stimulates activation through NKp46)	[28]
	PfEMP-1	<i>Plasmodium falciparum</i>	CD69 and CD25 expression, granzyme B and perforin production, cytotoxicity	[29]
NKp44	ENVgp	West Nile virus, dengue	cytotoxicity, IFN- γ production	[30]
	viral HA and HN	Sendai virus, influenza virus, Newcastle disease virus	cytotoxicity	[28, 31–33]
	arabinogalactan, mycolic acid	<i>M. tuberculosis</i> (<i>M. bovis</i> BCG)	possible secondary role in maintaining activation	[21]
	unknown	<i>Pseudomonas aeruginosa</i> , <i>Nocardia farcinica</i> , <i>M. avium</i> , <i>M. smegmatis</i>	unknown	[34]
NKp46	viral HA and HN	Sendai virus, poxvirus, influenza virus, Newcastle disease virus	cytotoxicity	[32, 35, 36]
	unknown	<i>Fusobacterium nucleatum</i>	TNF- α production	[37]
	PfEMP-1	<i>P. falciparum</i>	CD69 and CD25 expression, granzyme B and perforin production, cytotoxicity	[29]

tential for exploiting them for immune-prophylactic and/or therapeutic strategies, not only in the field of oncology but also for infectious diseases [4].

NK Cells and TB: in vitro Studies

Several in vitro studies support the role of NK cells in anti-mycobacterial immunity. It is well established that NK cells can lyse monocytes infected with MTB [41], *My-*

cobacterium avium complex [42] or *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) [43] and that the killing activity is highly enhanced by IL-2 or IL-12 [41]. The mechanisms underlining NK cell lysis of MTB-infected cells were recently elucidated. At least two NK cell-activating receptors, NKG2D and NKp46, are involved in the NK cell-mediated lysis of human MTB-infected monocytes [44, 45]. The stress-induced UL-16-binding protein (ULBP1), one of the cellular ligands of NKG2D, is up-regulated in a TLR2-dependent manner in MTB-infected

monocytes, and anti-ULBP1 inhibits NK cell lysis of infected monocytes, suggesting that the lysis is mediated through interactions between NKG2D and ULBP1 [45]. The same results were also obtained with alveolar macrophages, the most physiologically relevant mononuclear phagocytes involved in the defence against MTB. Immunoprecipitation with an anti-NKp46 antibody followed by mass spectrometry identified vimentin, a type-III cytoskeletal protein, as a putative NKp46 cellular ligand on MTB-infected monocytes [46]. Although enhanced on NK cells exposed to MTB-infected monocytes, NKp30 was not found to be involved in the lysis of mononuclear phagocytes by NK cells [45], but is shown to play a major role in the NK lysis of iDC [9]. Furthermore, although NK cells co-cultured with MTB-infected monocytes produced IFN- γ , cytotoxicity against infected cells was unaffected by the addition of anti-IFN- γ antibodies, suggesting that IFN- γ does not play a role in this process [44].

NK cells can also restrict MTB intracellular growth [47, 48]. It has been reported that the co-culture of infected mononuclear phagocytes with purified NK cells results in killing of intracellular MTB within 24 h [47]. NK cell-mediated killing of intracellular MTB is not associated with either IFN- γ production or the release of cytotoxic granules by NK cells or by Fas-Fas ligand interaction, but requires direct contact between NK cells and MTB-infected monocytes [47]. Supernatants from IL-12-stimulated human NK cells could activate human macrophages to inhibit the intracellular growth of *M. avium* and this ability was decreased by treatment of the supernatant with anti-TNF- α or anti-GM-CSF antibodies, but not by anti-IFN- γ antibodies [49]. Partially in agreement with this, Silver et al. [50] reported that non-adherent cells, isolated from the peripheral blood of purified protein derivative-negative subjects, depleted of both CD4⁺ and CD8⁺ T cells, displayed an inhibitory capacity towards MTB growth in human monocytes, but that this limitation of MTB intracellular growth was not significantly reduced by neutralizing antibodies to IFN- γ , TNF- α or IL-12. These findings suggest that phagocyte killing of intracellular mycobacteria is only partially mediated by soluble factors secreted by NK cells and also involves contact-mediated mechanisms. Interestingly, it has been reported that NK cells can contribute to immune defence against MTB through the production of IL-22, which inhibits intracellular mycobacterial growth by enhancing phagolysosomal fusion [51]. A role for glutathione, alone and in conjunction with IL-2 and IL-12, in activating the ability of NK cells to control the growth of MTB inside human monocytes has also been demonstrat-

ed [52], highlighting that multiple mechanisms might be involved in restricting MTB intracellular growth by human NK cells.

Direct Mycobacterial Recognition and the Role of NK Cell Receptors in the Direct Sensing of MTB

In 1996, we formulated the hypothesis that human NK cells may directly interact with and respond to mycobacteria [53]. By using an in vitro system that enabled the evaluation of the proliferative response of distinct cell subsets within heterogeneous cell populations, we demonstrated that the incubation of monocytes infected with live or killed MTB or *M. avium* with populations enriched in T cells and NK cells resulted in a prevalent expansion of CD3⁺ T cells (both CD4⁺ and CD8⁺). In contrast, when the same cell populations (containing <1% CD14⁺ cells) were directly stimulated with extracellular mycobacteria, CD16⁺/CD3⁻ NK cells were the major cell subset contributing to the overall proliferative response [53]. Subsequent studies by our group demonstrated that BCG directly stimulates highly purified human NK cells from the peripheral blood of healthy subjects, in the absence of monocyte/macrophages or IL-12, and induces CD69 and CD25 expression and proliferation, IFN- γ production and cytotoxic activity of NK cells [54, 55]. These effector functions were also induced upon stimulation with killed BCG or mycobacterial cell-wall preparations, and were totally abrogated when the NK cells and the bacteria were separated by a membrane with 0.2- μ m pores, which inhibits cell-bacteria contact but not the passage of soluble factors [54]. Together, such results suggested a direct interaction between BCG surface components and human NK cells that promotes the activation of their effector functions. Interestingly, distinct NK cell subsets differentially contributed to the overall in vitro NK cell response to BCG. The CD56^{bright} subset was mainly responsible for IFN- γ production and proliferative responses, whereas the CD56^{dim} subset was preferentially involved in the expression of perforin and granzyme A, and was in conjugate formation with target cells (K562), suggesting a major role of this latter subset in the cytotoxic effector functions of NK cells in response to BCG [55].

It is generally agreed that the activation of NK cell functions requires signals derived from soluble factors produced by accessory cells (e.g. IL-12 and IL-2). Our finding that intact mycobacteria may represent a sufficient stimulus to promote NK cell responses, even in the absence of accessory cell-derived cytokines, has been con-

firmed in some studies [22] but not in others [56, 57]. Interestingly Marcenaro et al. [22] reported that, while in some donors (high responders), elevated levels of IFN- γ were detected in NK cells cultured with BCG alone or with BCG and an anti-IL12 antibody, in others (low responders), the presence of IL-12 was required for IFN- γ production to be evident. Of note, unlike IFN- γ , the production of TNF- α by NK cells stimulated with BCG displayed only a minor variation in cells cultured in the presence or absence of exogenous IL-12 [22]. A certain degree of variability in the NK cell response to intact mycobacteria among healthy donors has also been noticed by us [unpubl. obs.] and others [58], and it may explain, at least in part, the discrepancies observed in different studies. The recent discovery that NK cells may express immunological memory [38–40] suggests the attractive hypothesis that the variability in NK cell responses could be related to differences among donors in the degree of sensitization to mycobacterial antigens, due to previous exposure to BCG vaccination or environmental mycobacteria. Another explanation for the inter-individual variability in the NK cell responses could be related to the expression of specific human leukocyte antigen/KIR haplotypes. It has recently been proposed that KIR B haplotypes are correlated to a higher responsiveness of NK cells to extracellular mycobacteria [58].

Recent studies [21, 22, 34] have allowed for the identification of the putative NK cell receptors and corresponding mycobacterial ligands involved in the direct recognition of mycobacteria by NK cells, further supporting evidence that the direct recognition of microbial ligands is a previously unappreciated way by which NK cells participate in anti-mycobacterial immunity. It has been suggested that at least two NK cell receptors are involved in the direct sensing of mycobacteria by NK cells. The first one is NKp44, a member of the NCR family, the expression of which is highly up-regulated on NK cells (mainly CD56^{bright} cells) following direct stimulation with BCG [34], but not in response to MTB-infected mononuclear phagocytes [45]. A soluble form of NKp44, chimera for the Fc fragment of the human IgG (NKp44-Fc), but not analogous forms of the other NCRs (NKp30-Fc and NKp46-Fc), was shown to bind BCG as well as other mycobacterial species, including the highly virulent laboratory strain MTB H37Rv [34]. In contrast, NKp44-Fc was not able to bind several common Gram-positive or Gram-negative bacteria, with the exception of *Pseudomonas aeruginosa* and *Nocardia farcinica*, the latter known to have structural similarities with the members of the *Mycobacterium* genus in that it contains mycolic acid (MA) in the bacterial cell wall [34].

Another NK cell receptor involved in the direct sensing of mycobacteria by NK cells is TLR2 [21, 22], a receptor also implicated in MTB recognition by antigen-presenting cells. Employing several purified MTB-derived cell wall components, we showed in an ELISA assay that NKp44-Fc can bind the mycobacterial ‘cell-wall core’ mycolyl-arabinogalactan-peptidoglycan (mAGP) as well as MA and arabinogalactan (AG) from MTB, but not many other cell wall components from mycobacteria or Gram-positive and Gram-negative bacteria [21]. Furthermore, pre-incubation of NKp44-Fc with mAGP, MA and AG inhibited the binding of NKp44-Fc to whole BCG in a dose-dependent manner. Conversely, a soluble form of TLR2-histidine chimera was able to bind mAGP and peptidoglycan (PG) from MTB, but not AG or MA [21].

Moreover, 1- μ m diameter polystyrene beads coated with mAGP (to mimic the bacterial surface) and incubated with human NK cells in vitro were able to induce the expression of CD25, CD69 and NKp44 on NK cells and the production of IFN- γ at levels comparable to those obtained with BCG-stimulated cells, but AG and MA used alone were not. Mycobacterial PG was the only component of mAGP able to activate NK cells and to induce expression of NKp44 when used alone [21]. The addition of a TLR2-blocking antibody in the cultures, but not of an NKp44-blocking antibody, significantly reduced CD69 expression on the NK cells stimulated with the mAGP-coated beads and abolished IFN- γ production. Overall, these results provide evidence that components abundant in the mycobacterial cell wall are possible ligands of NKp44 (AG and MA) and TLR-2 (PG), respectively, and that the interaction of TLR2 with its ligand(s) was able to promote activation and IFN- γ production by NK cells [21].

Unlike NKp30 and NKp46, NKp44 is not expressed or is only poorly expressed on resting NK cells but is induced promptly after cell activation by, for instance, IL-2 treatment [59]. Interestingly, different from what was observed with resting cells, when purified NK cells were pre-incubated with IL-2 to induce NKp44 expression, and then re-stimulated with mAGP-coated beads or with live BCG in the presence of the NKp44 blocking antibody, there was a statistically significant inhibition of CD69 expression. This suggests that NKp44 may play a secondary role in NK cell activation by mycobacterial ligands [21]. Interestingly, expression of NKp44, together with NKp30 and NKp46, was demonstrated in a subset of human group 3 ILCs (NCR⁺ ILC3s) [8], suggesting that these cells might be involved in the direct recognition of mycobacteria.

Taken together, these results suggest a two-step hypothesis on the possible role of TLR2 and NKp44 in the response to MTB [21]. TLR2 may represent the primary receptor involved in NK cell activation by mycobacterial cell-wall components. Direct recognition of mycobacterial PG (or PG-associated components) by TLR2 or IL-2 released by surrounding T cells may promote NK cell activation and NKp44 induction. Once the cells are activated, NKp44 may interact with its putative mycobacterial ligands. This secondary interaction could signal the persistency of bacteria in the extracellular space and, therefore, the need of maintaining/prolonging NK cell activation, contributing to the protective response (e.g. IFN- γ production). Alternatively, MTB binding to NKp44 could induce tissue damage and immunopathology as a consequence of an exaggerated proinflammatory response, favouring bacterial dissemination. Finally, the second signal could cause NK cells to undergo apoptosis because of an excessive activation, and it thus represents a mechanism exploited by MTB to evade immune response. This possibility is supported by the recent finding that BCG seems to modulate the functions of NKp44⁺CD56^{bright} NK cells driving this subset to produce IFN- γ before subsequent programmed cell death [57].

NK Cell Interaction in vitro with Other Cells of the Immune System and Regulation of the Response to MTB

NK-DC Interaction

DC are important mediators of the cellular immune response to MTB. They are the major professional antigen-presenting cells and represent an important link between the early innate and late adaptive immune responses to a number of pathogenic microorganisms. DC are highly represented in the lung mucosa at the site of MTB infection, where they display an immature phenotype (the iDC) specialized for antigen uptake and processing. After interacting with pathogens or pathogen-derived products, DC undergo a maturation process during which their capacity to internalize antigens decreases, while their ability to present antigens and migrate to draining lymph nodes is highly up-regulated. Once in the secondary lymphoid tissues, DC interact with T cells, playing a pivotal role in T cell priming through the cell surface expression of MHC and co-stimulatory molecules and the secretion of immunoregulatory cytokines such as IL-12 [60].

The existence of an intense crosstalk between DC and NK cells in vitro is well documented [61, 62]. In the con-

text of mycobacterial infection, it has been demonstrated that in the presence of killed MTB as a maturation stimulus, monocyte-derived iDC activate human peripheral-blood NK cells, resulting in an enhanced expression of the CD69 early activation marker and an induction of cytolytic activity against the NK-resistant Daudi cell line [56]. This effect was mainly dependent on a cell-to-cell contact, as neutralizing anti-IL-12 or anti-IL-2 monoclonal antibody did not abolish CD69 expression and the induction of NK cell cytotoxicity. Reciprocally, NK cells cultured with iDC in the presence of MTB strongly enhanced DC maturation evaluated as CD86 expression [56]. Both cell-to-cell interaction and cytokines were responsible for DC maturation by NK cells [56]. In another study, Ferlazzo et al. [9] reported that human NK cells activated by DC infected with live BCG were able to lyse autologous uninfected iDC, while infected DC were much more resistant to NK cell-mediated cytotoxicity due to the up-regulation of human leucocyte antigen class I molecules. Killing of iDC by NK cells is mediated by the activating receptor NKp30, while the NKp46 or NKp44 receptors play a minor role [63]. Thus, NK cells seem to discriminate between infected and uninfected or not properly activated DC, mediating what has been defined a 'quality control' system that regulates the quality and the intensity of antigen presentation during an infection [64].

The possibility that the reciprocal DC-NK interactions demonstrated in vitro may occur also in vivo during TB infection is, at the moment, only an assumption. Interestingly, in vivo evidence for DC activation by NK cells has been provided in viral infections [65]. Recently, by using an experimental model of anti-cancer vaccination, evidence of iDC lysis in vivo by NK cells was obtained; it resulted in an enhanced, protective, cancer-specific immune response [66]. These findings suggest that similar NK-based vaccination strategies aimed to improve DC activity could also have potential in immune interventions against TB and may represent an important area for future investigation.

It has been proposed that there are at least two different anatomical sites where DC-NK interactions occur in vivo [61]. The first is the site of inflammation. It can be argued that following MTB infection, NK cells are recruited in the lung from peripheral blood (mainly highly cytotoxic CD56^{dim} NK cells that predominate in the circulation) and could participate in the early immune response to the pathogen by selecting the most suitable DC for subsequent migration to lymph nodes, thereby contributing to optimizing the phase of 'antigen loading'. The second possible site of DC-NK interaction is

in the secondary lymphoid tissues, where mature DC (mDC) migrate to present antigens to T cells. At this site, NK cells (mainly cytokine-producing CD56^{bright} cells that predominate in the lymph nodes) may become activated by T cell-derived IL-2 and/or by directly responding to mycobacterial ligands that interact with their receptors (TLR/NCR) [21]. As a consequence of these multiple activating signals, NK cells may produce IFN- γ and other cytokines and undergo a bidirectional activation process with DC that contributes to the phase of 'T cell priming'. Interestingly, in this regard, we have previously demonstrated that, in the absence of IFN- γ , DC infected with MTB or stimulated with combinations of pathogen recognition receptor ligands that simulate MTB, produce high levels of IL-23 and IL-10, but only low amounts of IL-12p70 [67]. In contrast, the priming of DC with IFN- γ restores their capacity to produce high levels of IL-12p70, the main cytokine involved in Th1 polarization, with only a minor effect on IL-23 production, and reduces DC production of IL-10. Thus, the release of IFN- γ by NK cells at the site of T cell polarization could be crucial to render DC able to generate the proper cytokine milieu for the development of a protective Th1 cell phenotype.

NK-T Cell Interaction

Growing evidence obtained in the mouse model and in humans suggests that NK cells can exert immunoregulatory roles by positively or negatively regulating the activity of different T cell subsets [68]. In this respect, it has been demonstrated in vitro that, after depletion of NK cells by PBMCs from healthy sensitized donors, the proliferation of $\gamma\delta$ T cells in response to heat-stable, low-molecular-weight antigens from MTB is highly reduced [69–71]. The regulatory activity of NK cells on $\gamma\delta$ T cell proliferation seemed to be dependent on the production of cytokines other than IFN- γ by NK cells as well as by a direct NK- $\gamma\delta$ T cell contact via the adhesion molecule CD54 [71]. Similarly, the depletion of NK cells in PBMCs from healthy tuberculin reactors impaired the ability of CD8⁺ T cells to lyse MTB-infected monocytes and reduced the frequency of MTB-specific, IFN- γ producing CD8⁺ T cells [72]. In this case, the NK cell regulatory activity on CD8⁺ T cell functions was dependent on the production of IFN- γ by NK cells that, in turn, elicited the monocyte release of IL-15 and IL-18, two monokines implicated in MTB-specific CD8⁺ T cell expansion [72].

The possible immunoregulatory effect of NK cells on T cells extends to T regulatory cells (Tregs), a subset of CD4⁺ T lymphocytes that expresses high levels of CD25

and FoxP3, and is involved in the down-regulation of immune responses. Through the secretion of inhibitory cytokines such as IL-10 and transforming growth factor beta (TGF- β) or by cell-cell contact-dependent mechanisms, Tregs play an important physiological role in preventing excessive inflammation and subsequent tissue damage [73]. Nevertheless, an unbalanced response of Tregs induced by certain bacterial pathogens, including MTB, has been correlated with a detrimental host response [73, 74]. Of note, the ability of human NK cells to lyse Tregs that expand in vitro in response to MTB, but not freshly isolated Tregs, has been demonstrated [75]. This NK cell-mediated inhibition of Treg expansion was not mediated by IFN- γ , but rather by the recognition of the self-induced stress protein ULBP1 by NKG2D and NKp46 NK cell-activating receptors [75].

Tregs are present in the blood and at disease sites in patients with TB [76], and their frequency in the pleural fluid correlates inversely with local MTB-specific immunity [77]. Thus, it is tempting to speculate that NK cell control of MTB-specific Treg expansion could contribute to mitigate Treg suppression of effector immune responses and/or to ameliorate the efficacy of anti-TB vaccines [78].

Collectively, these studies point towards a possible contribution of NK cells in regulating multiple aspects of the innate as well as the adaptive immune response against MTB. Compared to other bacterial pathogens, MTB seems to display the unique ability to delay the initiation of the adaptive immune response, and this seems, at least partially, to be due to a failure of infected DC to migrate from the lung to the draining lymph nodes [79]. Thus, the ability of NK cells to promote DC activation and migration to secondary lymphoid tissue could be important in the early stages of the infection to accelerate the process of T cell priming and antigen presentation, a point that undoubtedly deserves more investigation. On the other hand, the ability of NK cells to kill iDC while sparing mDC may contribute to the development of an optimal T cell polarization. Finally, the ability of NK cells to positively regulate CD8⁺ and $\gamma\delta$ ⁺ T cell functions while suppressing Treg expansion may suggest that they are somehow involved in the maintenance of the delicate balance between the effector and regulatory arm of the antigen-specific immune response. Although experimental evidence supports the view that NK cells may contribute to the immune response to MTB at several levels, the possibility that they might also be detrimental as a consequence of excessive activation should not be disregarded [16].

NK Cells and Mycobacterial Infection: Studies in the Mouse Model

There are inherent difficulties to comparing studies of NK cells between humans and mice because of inter-species differences in the expression of various cell surface markers [80]. For example, CD56 is not expressed by mouse NK cells, which have been identified by their expression of CD49b/NKPRPC1 in the absence of CD3. Mice NK lack KIRs but express lectin-like Ly49 receptors that are functionally equivalent to human KIRs. Both human and mouse NK cells express the CD94/NKG2 heterodimer and the important activating receptor NKG2D, although its ligands differ between the two species. Among other activating receptors, mouse NK cells express NKp46 but lack both NKp30 and NKp44. Identification of NK cell subsets in mice that are comparable with the 2 human subsets, CD56^{bright} and CD56^{dim}, has been hampered by the lack of CD56 expression by mouse NK cells. However, it was recently discovered in mice that NK cells are differentially distributed in tissues according to the expression of CD27 [80]. While CD27⁺ NK cells constitute the major NK cell population in the lymph nodes, similar to the human CD56^{bright} subset, the CD27⁻ NK subset dominates the blood and spleen as CD56^{dim} does in humans. However, as the CD27⁺ subset appears to be more reactive than CD27⁻ NK cells with respect to cytotoxicity, cytokine production and proliferation, functionally, these 2 NK cell subsets in mice do not appear to be distinct as observed in humans. In mice, another small NK cell population resident in the thymus has been suggested to represent an equivalent to the human CD56^{bright} subset. These thymic NK cells express CD127 and are potent cytokine producers but display low cytotoxicity like the human CD56^{bright} subset [80].

Taken together, these observations underline that, although in the mouse there are NK subsets with functional resemblances to human NK cells, the phenotypes are not always directly comparable. As a consequence, it might simply not be possible to extrapolate results obtained in mouse TB infection models to humans.

Early studies in the mouse model demonstrated that antibody (anti-asialo-GM1)-mediated depletion of NK cells renders mice extremely susceptible to infection by *M. avium* complex [81]. This finding was not confirmed in later reports, which suggested that NK cells are not critical for resistance to this infection in immunocompetent mice [82, 83]. SCID (severe combined immunodeficiency) mice are deficient of T cells but capable of exhibiting detectable levels of resistance to mycobacterial in-

fections. Studies on SCID mice highlighted the possibility that murine NK cells are heterogeneous and that the production of IFN- γ by NK cells, rather than their cytolytic activity, is involved in the restriction of *M. avium* growth [83].

Infection of C57BL/6 mice by aerosol exposure to MTB resulted in an evident increase of NK1.1⁺CD3⁻ cells (NK cells) in the lung at 7 days from the start of infection and peaking at day 14 when the NK cells represented approximately 10% of the total lung cells [84]. Over the course of the infection, lung NK cells also displayed a significant increase in the expression of the early activation marker CD69 and produced IFN- γ and perforin, but not IL-4. Nevertheless, depletion of NK cells by anti-asialo-GM1 antibody had no evident influence on the bacterial load in the lung, questioning the primary role of NK cells in the context of MTB infection of immunocompetent mice. A detailed analysis of the possible role of NK cells in the murine innate response to MTB was provided by Feng et al. [85], who used different sets of targeted mutant mice and/or antibody-mediated, cytokine-depleted mice. They demonstrated that in T cell-deficient RAG^{-/-} animals, NK cells are the main cellular source of IFN- γ . This NK cell-derived IFN- γ was shown to be implicated in the induction of the microbicidal mediator nitric oxide synthase 2 in the lungs of MTB-infected RAG^{-/-} animals and in promoting a classic, rather than the alternative, macrophage activation program [86]. Interestingly, the same study also demonstrated that NK cell-dependent IFN- γ production negatively regulates neutrophil response in infected lungs and protects animals from severe lung pathology.

Altogether, data from mouse models suggest that in immunocompetent hosts, NK cells may provide a dispensable source of activities that partially overlap with those of other immune cells, but that in animals with compromised T cell function, they could represent an essential barrier to bacterial growth.

Evidence of a Role of NK Cells in Human TB: Clinical Studies

In humans, several clinical studies have explored the potential association between NK cell counts in the peripheral blood (CD3⁻ CD56⁺) and TB [87–91]. In some reports, a reduction in the frequency of peripheral-blood NK cells has been observed in TB patients when compared to healthy donors [87, 88]. However, other studies have not found a difference [90, 91] or have reported an

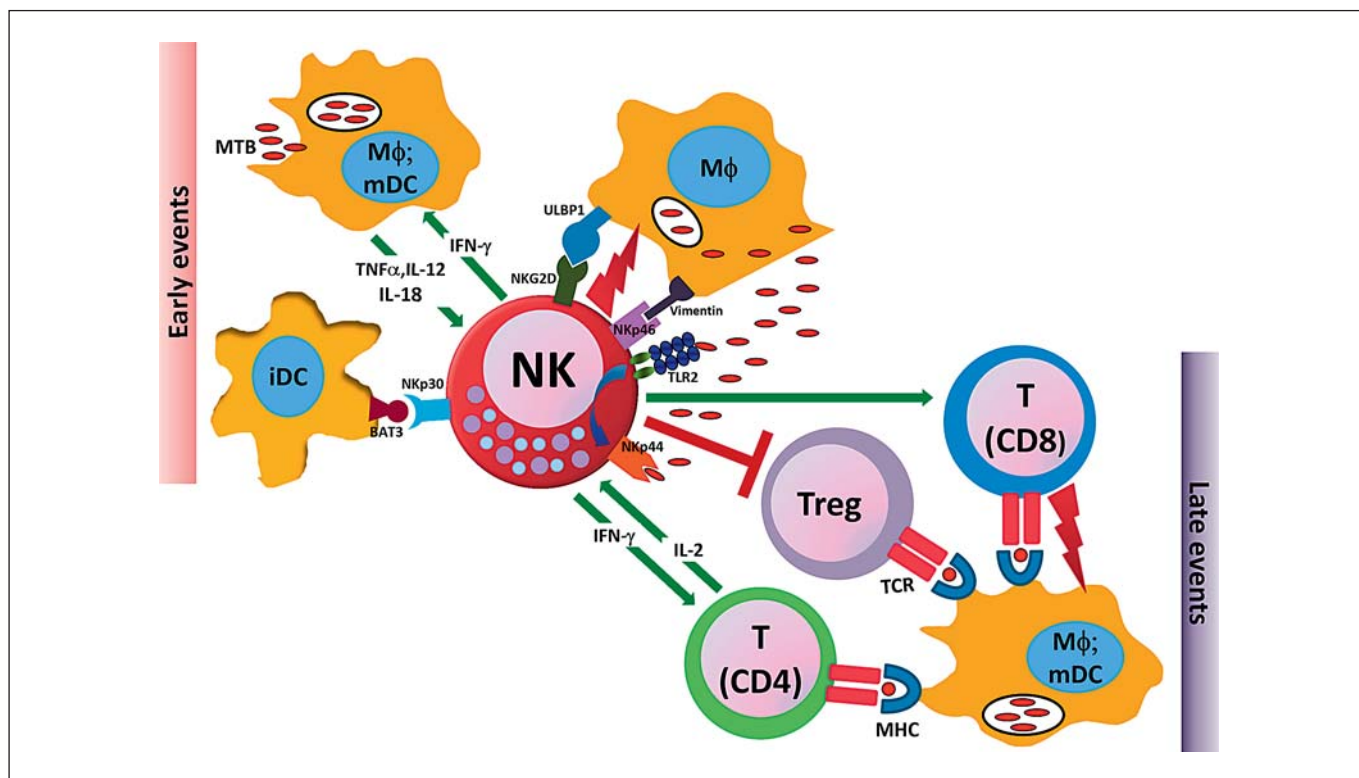


Fig. 2. Possible role of NK cells in the early and late events of MTB infection. During the early stages of MTB infection in the lung, NK cells are activated by proinflammatory cytokines released by infected macrophages or DC and provide an early source of IFN- γ and other macrophage-activating soluble factors. NK cells also lyse iDC or alveolar macrophages (M Φ) through the recognition of stress-induced cellular ligands (BAT3, ULBP1 and vimentin) by NK cell-activating receptors (NKp30, NKG2D and NKp46). Bacteria released by infected cells may directly interact with NK cell receptors such as TLR2 and NKp44, undergoing a two-step pro-

cess that eventually maintains and prolongs cell activation. Later on, IFN- γ and other cytokines released by activated NK cells may contribute to the phase of T cell priming by shaping the subsequent T cell response towards a Th1 phenotype. Once T cells have been recruited at the infection site and become activated following antigen recognition, NK cells may undergo a bidirectional interaction with them, by being activated by IL-2 released from activated T cells and playing an immunoregulatory role in other T cell activities.

increase in the peripheral-blood NK cell count in TB patients compared to healthy individuals [89]. The discrepancy encountered in the number of NK cells in the peripheral blood of TB patients compared to healthy donors in different studies could be due to: (1) differences in the stage and gravity of the TB infection in the patients included in the studies [92], (2) the MTB strain involved or (3) the presence/absence and duration of anti-TB antibiotic therapy [87, 91]. Moreover, it is essential to compare the number of NK cells in the peripheral blood and at sites of local infection (e.g. the pleural fluid, bronchioles and alveoli and lung tissue), as the migration of NK cells from the peripheral blood to sites of local infection, with a prevalence of CD56^{bright} NK cell subset in the latter, has been demonstrated [58, 93].

In addition, in some studies, the effector functions (i.e. cytotoxicity, IFN- γ production and activation) of NK cells obtained from TB patients were assessed following re-stimulation *in vitro* [44, 87, 91, 94–96]. The cytotoxic activity of NK cells against natural tumoral target cell-lines or autologous mononuclear phagocytes was the most studied effector function, and the vast majority of studies reported an impaired cytotoxic activity of NK cells in TB patients [44, 86, 90, 94–96]. In a few reports in which the NK cell activity was monitored before and after anti-TB treatment, the NK cell cytotoxic activity was restored upon successful antibiotic treatment [90, 94]. The results demonstrating a correlation between the NK cell activity and the progress of the disease may suggest a role for NK cells in TB infection.

Only a few studies have investigated the presence of human NK cells at local TB infection sites. One demonstrated that NK cells are present in the pleural fluid from TB patients but not in the pleural fluid with other pathologies [93]. These NK cells at the site of TB infection were enriched in the CD56^{bright} subset due to selective apoptosis of CD56^{dim} NK cells induced by soluble factors present in the TB effusions. Furthermore, MTB induced IFN- γ production by CD56^{bright} NK cells in vivo which was not dependent on the presence of CD3⁺, CD19⁺ or CD14⁺ cells, suggesting a direct interaction between MTB and NK cells at the infection site [93]. Another recent study provides evidence that NK cells may infiltrate both early and late human lung granulomatous lesions during active TB, suggesting that NK cells play a role at different stages of MTB infection [59]. The same study reported an association between a KIR haplotype (KIR B) and ex vivo NK cell responses to mycobacteria, indicating that NK cell genetics may represent one of the susceptibility traits of the human population to TB [59]. Overall, these studies demonstrate the presence of functional NK cells able to interact directly with MTB in the infected tissues of TB patients, suggesting that these cells are taking part in the immune response during pulmonary TB. However, the exact role of NK cells in the course of TB infection requires further investigation in order to be fully clarified.

NK Cells: A New Player in the Protective Response against MTB Infection? Model of the Possible Role of NK Cells in TB Pathogenesis

In conclusion, evidence from in vitro studies, animal models and clinical data suggest that NK cells may represent a new player in the host immune response to MTB infection (fig. 2). During the early phase of MTB infection, following activation by cytokines released by accessory cells or by cell-to-cell contact-mediated signals, NK cells may provide an early source of IFN- γ before the onset of the adaptive T cell response. Although this production of IFN- γ does not seem to be involved in the lysis of infected cells [44] or the restriction of MTB intracellular growth [47], both mediated by the NK cells, it might be crucial for establishing the optimal proinflammatory cytokine milieu by enhancing the production of IL-12 and lowering the production of IL-10 [67]. NK cells can also exert cytotoxic activity against iDC and monocytes/macrophages that are not well-equipped to control the intracellular growth of MTB, with the consequent release of the bacteria into

the extracellular space. This may allow NK cells to interact directly with whole bacteria or bacteria-derived products through the recognition of mycobacterial ligands by activating NK cell receptors [21, 34]. Later in the process, the production of IFN- γ by activated NK cells (mainly CD56^{bright}) may contribute to shaping the T cell response towards a Th1-protective phenotype. Once T cells are activated by recognition of mycobacterial antigens, they may produce IL-2, and a two-directional relationship may be established between different T cell subsets and NK cells at the site of antigen processing and presentation (e.g. the secondary lymphoid tissues) which regulates the activation and function of MTB-specific T cells.

NK cells have long been categorized as components of innate immunity. However, several lines of developmental, phenotypic and functional evidence suggest that they are closely related to T and B cells and may display hallmarks of adaptive immunity cells including the ability to develop immunological memory [38–40]. A full understanding of the role of NK cells in anti-mycobacterial immunity may open new possibilities for NK cell-based prophylactic and/or therapeutic strategies against TB.

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