Immunological basis of early clearance of *Mycobacterium tuberculosis* infection: the role of natural killer cells

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Summary

Tuberculosis kills more people than any other single infectious disease globally. Despite decades of research, there is no vaccine to prevent TB transmission. Bacille Calmette-Guerin (BCG) vaccine developed a century ago has little effect on pulmonary TB and does not control transmission. Lack of an effective vaccine emanates from lack of knowledge on correlates of protective immunity on which to base vaccine design and development. However, some household contacts who are extensively exposed to *Mtb* infection remain persistently negative to tuberculin skin test and interferon-gamma assay. These individuals called "resisters" clear *Mtb* infection early before the development of acquired immunity. The immunological basis of early *Mtb* clearance is yet to be established, however, innate lymphocytes such as monocytes/macrophages, dendritic cells, neutrophils and natural killer cells, and innate like T cells such as mucosal associated invariant T cells, invariant natural killer T cells and gamma-delta (γδ) T cells have been implicated in this early protection. One of the cells that has attracted increasing attention in recent years, in protection against *Mtb* is the natural killer cell. Emerging data from animal and epidemiological studies indicate that NK cells may play a significant role in the fight against *Mtb*. NK cells express various surface markers to recognize and kill both *Mtb* and *Mtb*-infected cells. In this review, recent advances in our understanding of NK cells in the fight against *Mtb* early during infection, with emphasis on cohort studies, will be presented.

Key words: tuberculosis; NK cells; innate immune cells; early clearance; Immunity; cytokines

Introduction

Tuberculosis (TB), mainly caused *Mycobacterium tuberculosis (Mtb*), is the leading cause of death among infectious diseases. In 2018, an estimated 10 million people developed clinical TB and an estimated 2.2 people are *Mtb* infected globally [1]. Current control strategy in endemic countries depends on passive case finding and treatment of active cases, based on directly observed treatment short course recommended by WHO [2] The United Nations sustainable Development Goal (Target 3.3) aims at ending the TB epidemic by reducing TB related deaths by 90% by 2030 [1]. However, such an ambitious goal may not be achieved without an efficacious vaccine. BCG vaccine developed a century ago does not prevent TB transmission and efforts to develop of a new vaccine to replace BCG achieved little success because of lack of knowledge on correlates of protective immunity. On the other hand, over 90% of *Mtb* infected individuals do not develop clinical TB, implying that they are immune

protected [3]. Several studies have shown that some household contacts do not acquire infection despite extensive exposure to *Mtb* [4-6]. These individuals clear infection early and are referred to as “resisters”. The “resister” phenotype is defined based on negativity of test results of interferon-gamma-release assay (IGRA) and tuberculin skin test (TST). Since the two tests depend on recall response (immunological memory), this early clearance is attributed to innate and innate-like immune cells [4, 5]. These innate cells include monocytes/macrophages; dendritic cells (DCs), neutrophils, and NK cells. There are also reports of innate-like T cells such as mucosal-associated invariant T cells, gamma-delta T cells [7,8] and invariant natural killer T cells [9] playing a role in early *Mtb* clearance. In recent years, NK cells have received increasing attention in controlling *Mtb* infection. NK cells express various surface markers that recognize *Mtb* cell components and *Mtb* infected cells. These cells employ direct and indirect mechanisms to kill *Mtb* and infected cells. In this review, recent data on the role of NK cells on early *Mtb* clearance, with emphasis on longitudinal studies will be presented.

Evidence of Early *Mtb* clearance

Early clearance of *Mtb* infection is defined as the eradication of infective *Mtb* before the development of acquired immunity [5]. In TB endemic setting, exposure to *Mtb* has, at least, three possible outcomes. There are individuals (5-10%) who develop clinical disease after primary infection. Another group of individuals (90%) acquires *Mtb* infection but do not develop clinical TB. These individuals are believed to have latent *Mtb* infection. LTB is defined clinically by a reactive TST, indicating a delayed-type hypersensitivity (DTH) response to intradermal injection of *Mtb*-derived purified proteins or a T cell response to *Mtb*-specific antigens in the absence of clinical and radiological findings [10]. Both innate and adaptive immune cells and their products are involved in controlling clinical TB in LTB infection [11-13]. However, some household contacts who are extensively exposed to *Mtb* remain negative to TST or IGRA. These individuals are named “resisters” and clear *Mtb* infection early before the development of acquired immunity [4, 5].

Observation of persistent TST negativity of some individuals despite heavy exposure to *Mtb* was reported in nursing students as early as 1940s [14]. Case control studies in the 1960s showed the existence of resistant individuals to *Mtb* infection following extended exposure. This evidence comes from the US personnel aboard USS destroyer who shared the same confined environment with index cases for 6 months. In this case, 7 out of 308 (10%) developed active TB, while 7 (10%) of the crew members remained negative for TST after 6 months in the same ship with index cases. Another example is an evaluation of nursing students in the pre-antibiotic era, which showed TST negative individuals despite extended exposure to *Mtb* [15]. Differences in susceptibility to *Mtb* among close contacts of TB index cases was suggested from a systematic review report [6], where close to 50% of close contacts remained uninfected. Results of some early studies suggest that rates of resistance can be as high as 70% of heavily *Mtb* exposed close contacts [16-19]. Although the proportion of resistant individuals reported above could be due to the inherent shortcomings of TST, the presence of *Mtb* resistant household contacts in an endemic setting is unquestionable.

In recent years, several studies in different endemic communities [reviewed in 20] have established persistently TST or IGRA negative individuals in longitudinal studies over a period of 2 years. In these studies, the proportion of individuals who cleared infection early ("resisters") ranged from 3.4% in South Africa [21] to 26.8% in Uganda [22]. However, the true proportion of resisters in a given endemic community is yet to be established as most of these studies used either TST or IGRA. These two tests depend on recall response/immunological memory and do not discriminate between clinical TB and *Mtb* infection or exposure to *Mtb* and non-environmental mycobacteria. Moreover, these two tests have inherent shortcomings in terms of sensitivity and specificity and test results are influenced by other factors such as duration and intensity of contact, and quality of aerosol [4, 23].

However, there are recent studies [24, 25] that used both TST and IGRA to determine the true proportion of resisters longitudinally. For instance, a study in India [24] examined 799 household contacts in culture confirmed TB index cases in 355 households at baseline, 4-6 months and 12 months. They found 52 (6.5%) in 39 households to be resisters. The authors found no epidemiological factors associated with the resister phenotype based on random effects Poisson regression.

Another study in Uganda [25], followed-up 407 HIV negative household contacts for more than 2 years using both TST and IGRA. The study concluded that resistance to latent infection in adults, who have had close contact with pulmonary TB patients living in TB-endemic areas, is a stable outcome of *Mtb* exposure. Repeated longitudinal measurements with two different immune assays and extended follow-up provide enhanced discriminatory power to identify this resister phenotype and avoid misclassification [25].

Immunological basis of early *Mtb* clearance

Our knowledge of immune protection against *Mtb* infection is incomplete, especially about the resister phenotype. Sine early *Mtb* clearance occurs before the development of acquired immunity, innate immune cells such as monocytes/macrophages, dendritic cells, neutrophils and natural killer cells are believed to be responsible for this early response [4, 5, 26, 27]. In the lungs, alveolar macrophages (AMs) are the first innate cells to encounter *Mtb*. However, the number of other mononuclear cell subsets recruited to the infected lungs increases by 20-30-fold following *Mtb* infection [28, 29]. Examination of the distribution of *Mtb* in these cell subsets on day 14 post-infection shows that the pathogen was equally distributed in AMs, myeloid DCs, and neutrophils [28]. Moreover, recent findings indicate that monocytes, DCs, neutrophils, epithelial cells, endothelial cells and fibroblasts are recruited to the lungs following cytokine signaling by AMs [reviewed in 31-32].

While these innate cells are critical for early anti-mycobacterial responses, they are also targets of infection by the pathogen and serve as niches for bacterial replication, disease progression, and dissemination of the pathogen to different organs and tissues [32, 33]. Moreover, recruitment of neutrophils serves as an early line of defense against *Mtb* infection via secretion of antimicrobial molecules and inflammatory mediators. After recruitment, neutrophils recognize bacteria either directly or through Fc-R and complement

receptors, and phagocytose the bacteria [34, 35]. However, neutrophils also serve as niches for bacterial replication, can impede immunity against *Mtb* [32]), and mediate immunopathology during *Mtb* infection [32, 35].

An innate immune cell that has attracted increasing attention in recent years in host defense against *Mtb* is the NK cell. NK cells belong to the same group of innate immune cells such as monocytes/macrophages, dendritic cells, and neutrophils. Unlike the above innate phagocytic cells, NK cells do not serve as a niche for *Mtb* and do not disseminate the pathogen. This feature combined with the strategic distribution of in lymphoid non-lymphoid tissues and organs make NK cells critically important in the fight against *Mtb*.

The biology of NK cells

Natural killer cells constitute a large granular lymphocytes and belong to group 1 of innate lymphoid cells (ILC1) [36] and express CD56 (neural cell adhesion molecule, NCAM) and lack CD3 and CD19 [37]. NK cells are distributed in the blood, lymphoid organs (including the thymus, and spleen), and non-lymphoid organs (including the lungs, liver, the uterus, as well as tissues such as skin) [38-40]. In humans, NK cells subsets exhibit major functional differences in their cytotoxicity, cytokine production and homing capabilities [41]. Based on CD56 density on the cell surface, NK cells are two types, namely, CD56 ^{bright} and CD56 ^{dim}, which have different phenotypic properties. CD56 ^{bright} NK cells have the capacity to produce large quantities of cytokines, while CD56 ^{dim} NK cells are more cytotoxic and express more killer immunoglobulin (Ig)-like receptors (KIR) as well as Fc-Ɣ receptor III (Fc-GR III, CD16) [41-43]. NK cells express activating and inhibitory receptors on their surface, which define their functional properties [39, 40, 44, 45]. NK cells lyse target cells that express insufficient or lack MHC-1 molecules, whereas cells that express MHC-I molecule are not affected.

Inhibitory receptors

Inhibitory receptors specific for MHC-1 antigens tightly regulate NK cell-mediated cytotoxicity and cytokine production. The inhibitory signal from the MHC-1 specific receptor is essential for hematopoietic target cells to avoid destruction by NK cells. This concept is termed “missing self” [46-48]. Such MHC-1 recognizing inhibitory receptors form three families of NK cell surface receptors, namely, KIRs, LIRs (leucocyte Ig-like receptors), and NKG2A (natural killer group 2A) [39, 40]. KIRs, which are members of the Ig super family, are type-1 transmembrane molecules that recognize classical human leukocyte antigens A, B, and C (HLA class IA [49-51]. LIRs, also known as ILTs (Ig-like transcripts), form the second set of receptors and mainly recognize non-classical HLA-G (class IB) HLA class IA molecules. LIRs belong to the same Ig superfamily as KIRs. NKG2A, a member of the NKG2 group of seven receptors, namely A,B, C, D, E, F and H, dimerize with CD94 to form the NKG2A/CD94 receptor. It belongs to the c-type lectin family of receptors that recognizes non-classical HLA-E class I molecule as its ligand [52].

Activating receptors

Natural cytotoxicity receptors (NCRs) represent the group of NK cell surface activating receptors that include NKp46, NKp30, and NKp44. Nkp46 also known as natural cytotoxicity

receptor 1 (NCR1), is a 46 kDa transmembrane protein belonging to the Ig superfamily. In humans, NKp46 is expressed by all CD56 ^{dim} CD16^{+}, and CD56 ^{bright} CD16^{-} human NK cells, irrespective of their activation status [53]. NKp30, also called natural cytotoxicity receptor 3 (NCR3), is a 30-kDa protein expressed on all mature resting and activated NK cells [54]. These receptors, as well as NKG2D (natural killer group 2D) recognize ligands expressed on target cells [55-57]. CD16 (Fc-YR III, also an activating receptor, is expressed mainly by CD56 ^{dim} NK cell subset and is essential for ADCC against IgG-coated target cells [42, 58].

Mtb recognition by NK cells

NK cells use non-antigenic specific mechanisms to exert effector functions and pattern recognition receptors recognize pathogen associated molecular patterns and are essential components of the NK cell-mediated innate immune response against *Mtb* [39, 45]. Various components of *Mtb* cell wall can bind directly to NKp44 of NK cells [59] and NK cells can recognize stress molecules upregulated on the surface of *Mtb* infected cells [60]. For example, Nkp44 directly binds to *Mtb* cell wall components, such as arabinogalactan-peptidoglycan as well as mycolic acids and arabinogalactan derivatives [59, 61]. On the other hand, NKp46 was reported to play a dominant role in the lysis of mononuclear phagocytes infected with *Mtb* via recognition of vimentin expressed on the surface of *Mtb*-infected cells [60, 62].

Human NK cells directly recognize *Mtb* by the binding of TLR2 and NKp44 to peptidoglycan and other components of the cell walls, respectively, and then become activated [59, 61, 63]. In one study, in T cell deficient mice, it was demonstrated that NK cell mediated early defense against *Mtb* infection via IFN-γ [64, 65]. In humans, NK cells in the peripheral blood stimulated with *Mtb* or BCG upregulated IFN-γ expression [66, 67].

Evidence of NK cell response to _Mtb_ from epidemiological studies

One earlier study has shown that the pleural fluid of TB patients was enriched with IFN-γ producing CD56 ^{bright} NK cells due to selective apoptosis of cytotoxic CD56 ^{dim} NK cells induced by soluble factors present in TB effusions [68]. A longitudinal study on a cohort of South African adolescents found that the frequency of NK cells in the peripheral blood could inform disease progression, therapeutic response and lung inflammation of patients with active TB. This group has shown that NK cells from individuals with LTB display elevated levels of cytotoxicity and increased frequency [69]. In a study carried to assess the contribution of NK cells against *Mtb* infection a cross-sectional assessment of NK cell phenotype and function in four distinct group of individuals, pre-treatment TB patients, post-treatment TB patients, household contacts, and TST negative individuals was made. The results showed significant decrease in IFN-γ expression and degranulation in NK cells of TB patients, with no variation in NK cell frequencies. On the other hand, CD57 expression a marker for advanced NK cell differentiation was significantly lower in cases post-treatment compared to pretreatment. Finally, NKG2C, an activation marker and imprinted-memory marker, has significantly increased in TST+ (latently infected) compared to TB cases and TST^{-} resistant individuals [70].

Moreover, to determine NK cell phenotype and functional responses to *Mtb* using flow cytometry, Harris *et al*. [71], compared three groups: QuantiFERON (QFT-positive and QFT-negative adults in TB endemic setting in Kisumu, Kenya, and compared NK cell responses to those of *Mtb*-naïve healthy adult controls in the US. The results showed distinct CD56 ^{dim} NK cell phenotype that differentiated the Kenyan and US groups. In addition, among Kenyan participants, NK cells from QFT-positive individuals with latent *Mtb* infection were characterized by significant down-regulation of NKp44 and the inhibitory receptor TIGIT, compared with QFT-negative individuals. Moreover, the distinct CD56 ^{dim} phenotypic profiles in Kenyan individuals correlated with dampened NK cell responses to tumor cells and diminished activation, degranulation, and cytokine production following stimulation with *Mtb* antigens, compared with *Mtb*-naïve US healthy adult controls. Put together, these data provide evidence that phenotypic and functional profiles of NK cells are modified in TB endemic setting [71].

Possible Mechanisms of *Mtb* killing by NK cells

NK cells use two suggested mechanisms in controlling *Mtb* infection, direct and indirect mechanisms. Frist, NK cells are cytotoxic lymphocytes that lyse cells infected with intracellular pathogens [72]. The cytolytic function of NK cells can initiate primarily through degranulation and death receptor ligation, and is critical for the clearance of diseased and dysfunctional cells [73, 74]. Second, NK cells can produce a variety inflammatory cytokines in response to activation receptor stimulation as well as inflammatory cytokine-induced activation signaling [75, 76].

Direct mechanisms

Direct mechanism of NK cell-mediated control of infection follow three steps: 1) target cell recognition, 2) target cell contact and immunological synapse (IS) formation, and 3) NK cell-induced target cell death [39]. The main direct mechanism of NK cell cytotoxicity is through cytoplasmic granules containing perforin, granulysin, and granzymes, as well as several death receptors that can initiate apoptosis (Fig.1.). Perforin belongs to the membrane attack complex protein family, and inserts into target cellular membrane to function as a pore similar to the C5-9 membrane attack complex of the complement system [77]. Perforin pores are used to facilitate transport of granulysin and granzyme into the target cell cytoplasm. Granzymes are a family of serine proteases with many members, the major constituent in NK cells being granzyme B. This enzyme can initiate apoptosis of the target cell through direct activation of caspases 3 and 7 or through proteolysis of the protein Bid. Cleavage of Bid allows the active fragment to move to the mitochondrial membrane and form a pore complex with Bax and Bak, promoting the exit of cytochrome c into the cytosol, thereby initiating the formation of a caspase-activating complex [78]. Granzyme B can also mediate nuclear destruction in the presence of perforin, and granzymes may be able to mediate caspase-independent cell death pathways [74].

Second, direct mechanisms of cell NK cell cytolysis is through apoptosis. Fas (CD95) is a TNF receptor family transmembrane death receptor responsible for cell lysis, whose ligand (Fas L) is expressed in NK cells [79, 80]. The Fas receptor can be found on most cell types in the

body, and is of particular interest in the context of macrophage expression. Upon Fas-FasL ligation, a death-inducing signaling complex (DISC) forms, composed of multiple proteins, including Fas, Fas-associated death domain (FADD), and caspase-8. Activation of caspase-8 by DISC initiates the extra mitochondrial apoptotic pathway [80]. Macrophages infected with *Mtb* undergo NK-mediated apoptosis through this Fas pathway to limit viability of *Mtb* [81].

Third, NK cells express CD40L, the ligand of CD40 that expressed on antigen presenting cells and macrophages [82, 83]. After ligation, CD40 leads to an upregulation of co-stimulatory molecules CD80 and CD86 on the cell surface of macrophages as well as generation of nitric oxide (NO) when accompanied by IFN-γ [84].

NK cell-mediated pro-inflammatory cytokine production

Upon stimulation by *Mtb* antigens, NK cells produce pro-inflammatory cytokines (Fig.1) such as IFN-γ, TNF-α, IL-22, granulocyte-monocyte colony-stimulating factor (GM-CSF) and these cytokines exert their effects on infected cells. For instance, IFN-γ released by NK cells can triggers numerous intracellular effector mechanisms within macrophages such as activation of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase type 1 and 2 (NOX1.2) as well as NO synthase type 2, leading to formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), respectively [85, 86]. Superoxide can spontaneously generate hydrogen peroxide (H2O2) and hydroxyl (HO-) radicals [87, 88]. ROS and RNS can react further with each other to generate NO2 and peroxynitrate (OONO-) [87, 89, 90]. These various reactive species contribute antimicrobial oxidative destruction to membrane lipids and proteins, DNA, and enzymes [87]. IFN-γ also upregulates the expression of IgG Fc-γ-RI in monocytes to increase opsonization-dependent phagocytosis [85]. NOX2 is recruited to phagosomes by IFN-γ stimulation where it catalyzes the production of superoxide (O2-) from O2 and NADPH [88, 91]. NOS2 catalyzes the conversion of L-arginine and O2 into NO and citrulline [91].

Second, TNF-α released by NK cells contributes to macrophage mitochondrial ROS formation through TNF-α receptor (TNFR1) complex association with NOX1, induction of NOX2, and through a receptor interacting serine-threonine kinase-1 and 3-dependent pathway, which can lead to programmed necrosis [92-94].

Third, some studies have shown that human NK cells produce IL-22 in addition to IFN-γ and TNF-α. IL-22 is a member of the IL-10 cytokine family that is produced by special immune cell populations including CD4+ and CD8+ T cells, which display either a protective or a pathogenic role in chronic inflammatory diseases [95]. IL-22 plays an important role in host defense and homeostasis through production of antimicrobial peptides [96] and has been shown *in vitro* to inhibit *Mtb* intracellular growth by enhancing phagolysosomal fusion [97].

Antibody-dependent NK cell cytotoxicity

It is generally believed that early *Mtb* clearance is conferred by innate cells before the development of adaptive immunity. However, early *Mtb* clearance and the resister phenotype are based on TST and IGRA negativity and these two tests depend on T cell memory (recall response), implying that information on antibody (Ab) responses during early

Mtb clearance is lacking. However, Abs responsive innate immune cells bearing Fc-R have been reported in TB granulomas, suggesting that they may play a role in the anti-microbial response. For instance, it has been shown that Abs against *Mtb* lipoarabinomannan enhance bacterial opsonization and restrict intracellular growth [98]. Beyond opsonization, Abs direct innate immune antimicrobial activity via their constant Fc domains following engagement of Fc-R found on all innate immune cells [98]. Abs enhance cytolysis of infected target cells by NK cells and complement. Using an unbiased Ab profiling approach Lu *et al* [98] have shown that individuals with LTB and active TB have distinct *Mtb*-specific Ab responses, such that LTB infection is associated with unique Ab Fc functional profiles, selective binding to Fc-γ-RIII, and distinct Ab glycosylation patterns. A recent cohort study in Uganda, Lu LL *et al* [99], reported that resisters possess IgM, class-switching IgG antibody responses and non-IFN-γ T cell responses to *Mtb* specific proteins early secreted antigen target-6 and culture filtrate protein 10. Compared to subjects with classic LTB, resisters displayed enhanced antibody avidity and distinct *Mtb*-specific IgG Fc profiles.

Conclusions

Tuberculosis remains the most important infectious disease that kills over 1.5 million people annually. In addition, there are an estimated 2.2 billion people believed to be *Mtb* infected globally. However, there are household contacts of index cases, who despite extensive exposure to *Mtb* persistently remain TST and/or IGRA negative. These individuals, referred to as "resisters", are believed to clear *Mtb* infection early before the development of adaptive immunity. Although several innate immune cells and innate-like T cells are involved in response to *Mtb* infection, NK cells are increasingly recognized as playing a vital role in defense against *Mtb* infection. NK cells are strategically distributed in lymphoid and non-lymphoid tissues and organs, including the lungs, which makes them increasingly relevant to early *Mtb* protection. NK cells non-specifically bind to *Mtb* cell wall components through various receptors (TLR2, NKp46, NKp30, NKp44, NKG2D, and CD16). NK cells can also recognize *Mtb* infected cells through up-regulated IFN-γ expression, and KIR, and NKG2D receptors. Moreover, NK cells kill the pathogen and infected cells using different mechanisms, including destroying infected cells via cytolysis, apoptosis, and production of cytokines (IFN-γ, TNF-α, and IL-22). Moreover, there are also NK cell mechanisms that target the pathogen, including antibody-dependent NK cell cytotoxicity and generation of reactive nitrogen and oxygen species. There is convincing evidence from cohort studies in endemic communities that NK cells are involved in early *Mtb* clearance. Thus, the above attributes of NK cells and their capacity to develop innate memory ("trained immunity") will make them ideal for future research in vaccine design and development against *Mtb* infection.

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