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NK cell immunity in *Mycobacterium tuberculosis* and HIV infection

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Abstract

NK cell immunity in *Mycobacterium tuberculosis* and HIV infection

Mycobacterium tuberculosis (Mtb) is the causative agent of tuberculosis (TB) disease and is one of the most lethal infectious diseases known throughout human history. Most individuals that become infected establish latent infection and have a low risk of reactivation. Currently, it is estimated that one fourth of the global population has latent Mtb infection (LTBI). Each year, 10 million people develop active TB disease, resulting in 1.5 million deaths annually. Although the correlates of protective immunity to Mtb remain undefined, co-infection with human immunodeficiency virus (HIV) is one of the greatest risk factors for reactivation of LTBI and progression to TB disease. The increased risk of TB disease in people living with HIV has highlighted an important role for CD4 T cells in controlling Mtb infection, although many other immune cell populations also contribute to a successful immune response to Mtb. Natural killer (NK) cells have the capacity to rapidly respond to pathogens and kill infected cells, and increasing evidence indicates that NK cells also mediate a wide range of immunomodulatory effects that contribute to shaping the immune response generated after infection or vaccination.

NK cells are classically associated with cancer immunity but more recently have been shown to be involved in immune response to many infectious diseases. In the context of Mtb, NK cells secrete cytokines and cytotoxic molecules that have antimicrobial functions and have also been shown to suppress Mtb growth in macrophages. NK cell activity is regulated through expression of various activating and inhibitory receptors, the expression of which changes in the setting of inflammation, infection, and cancer, and can give rise to differentiation of NK cell populations with specialized functions. We hypothesized that phenotypic and functional profiles of NK cells are modified in the setting of persistent Mtb infection and that co-infection with HIV may further dysregulate NK cell profiles, thus potentially contributing to the increased risk of active TB disease in people living with HIV.

To address these hypotheses, we analyzed NK cell responses in peripheral blood samples collected from cohorts of healthy, TB-unexposed adults in the United States and from cohorts of adults in western Kenya across a spectrum of Mtb and HIV infection states. We used multi-parameter flow cytometry to conduct detailed NK cell phenotyping studies and functional responses to both tumor cell lines and to Mtb antigens. We found that the expression of certain receptors by NK cells in Kenyan adults is markedly different from US adults and that expression of these receptors correlates with dampened effector functions to Mtb antigens in Kenyan adults. By analysis of HIV-infected and HIV-uninfected Kenyan adults with LTBI, we found that HIV infection is associated with perturbations in NK cell phenotype and reduced NK cell reactivity to antibody-coated target cells; however, NK cell cytokine production and degranulation following stimulation with Mtb antigens is similar between HIV-infected and HIV-uninfected Kenyan adults. Overall, our data provide novel insights into the effect of Mtb and HIV infection on the phenotypic and functional profiles of NK cells. These findings will help inform future studies designed to define NK cell correlates of protection against acquisition of Mtb infection and progression to TB disease.

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Chapter 1. Introduction

1.1 History and Discovery of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis (Mtb) is one of the oldest and most deadly pathogens that has existed during human history; indeed, it is hypothesized that Mtb, the causative agent of tuberculosis (TB), is the deadliest bacterial pathogen of all time (1). Mummies from ancient Egypt have been shown to have skeletal deformities characteristic of severe forms of Mtb infection (2). Furthermore, these characteristic lesions are depicted in Egyptian art and most recently DNA sequencing has positively identified Mtb in the lesions of mummified lungs (3, 4). In Greece Hippocrates wrote about TB disease, at the time referred to as "aphorisms", describing the diseases prevalence in young people and the deadly outcomes of these diseased individuals (1). In Western Europe, TB epidemics of the 18th and 19th centuries were notorious and believed to have a mortality rate as high as 900 per 100,000 (1). Similar to Hippocrates observation, many young individuals succumbed to the disease. Obviously, TB had been a plague on human kind for thousands of years with no relief in sight. It was not until the 1800s when crucial discoveries would be made leading to the modern age of Mtb research.

Mtb is an acid-fast bacterium that primarily leads to infection of the lung. The existence of TB disease has been well documented for centuries but the causative agent for this disease was not known. It was not until 1865 when the French physician Jean-Antoine Villemin demonstrated that TB disease was caused by a transmissible agent after performing a series of critical experiments showing that rabbits develop disease after being inoculated with fluid from lung lesions of deceased TB patients (5). Villemin was also the first person to theorize a link between transmission and crowded living conditions, one of the highest risk factors for Mtb exposure.

In 1882, 17 years after Villemin's initiall findings, Robert Koch identified Mtb as the causative agent of TB disease. This advancement was aided by the development of a new staining technique (acid fast staining) and the creation of solid media, both methods pioneered by Koch (6). Using these techniques, Koch observed bacilli in the lesions of Mtb infected individuals. After observing the organism associated with disease, Koch became interested in providing definitive evidence that the bacterium was the causative agent of disease. This led to the creation of Koch's Postulates which state that for a suspected infectious agent to be the cause of disease it must be demonstrated that: 1) the bacteria must be present in every case of the disease, 2) the bacteria must be isolated from the diseased host and be grown in pure culture, 3) specific disease reproduced in hosts inoculated with the pure culture, 4) the bacteria must be recovered from the experimentally infected host (7). Indeed, with the use of acid-fast staining and solid cultures, Koch successfully isolated Mtb, and through additional inoculation experiments with rabbits, Koch fulfilled the criteria for his proposed postulates. At the time of Koch's discovery, TB disease was responsible for one in every 8 deaths in Europe (7, 8). To this day, TB remains the leading cause of death due to a single infectious agent(9), thus, there is still a great need to further study Mtb in order to develop vaccines and therapeutic options to fully eradicate this deadly pathogen.

1.2 Global burden of tuberculosis

It is estimated that in 2018 there were approximately 10 million new cases of TB and 1.5 million deaths due to TB. Approximately 90% of new cases were spread throughout high burden countries in South-East Asia, Africa and the Western Pacific (9). An estimated 8.6% of these 10 million TB cases worldwide occur in people living with HIV (9).

One of the major obstacles in eradicating TB is the development of drug-resistant TB. There has been a spike in drug resistance due to decreased drug adherence leading to increased bacterial mutation and development of drug resistant strains. In 2018 there were approximately half a million new cases of rifampicin-resistant TB; of these cases, close to 80% were resistant to multiple drugs (9). A majority of drug resistant cases are found in India, China and Russia. Currently, screening for and treating drug resistant Mtb is a major goal in eradication of disease.

One of the majors steps in eradicating TB is the development of an effective vaccine. To date, there is no vaccine that prevents pulmonary TB disease in adults. The only liscensed vaccine that has proven to have some form of protection is Bacille Calmette-Guerin (BCG) vaccine (9). The vaccine, initially developed over 90 years ago, is composed of live attenuated *Mycobacterium bovis*, and is currently one of the most widely used vaccines in the world (9). Currently, universal BCG vaccination is recommended in 157 countries (9). BCG has demonstrated efficacy in preventing severe forms of TB disease in children suchs as miliary disease and TB meningitis (10). However, protection against these severe forms is lost in adulthood as the efficacy of the vaccine wanes. A major goal of Mtb research is the development of a vaccine that is long lasting and protects against all forms of TB disease. The generation of such a vaccine would greatly help in the overarching goal of erradicting TB.

HIV patients with TB disease have a significant increase of succumbing to other opportunistic infections compared to individuals infected with HIV alone (11, 12). Both of these infections potentiate one another by employing mechanisms that compromise the immune system. CD4 T cells produce IFNy which activates Mtb infected macrophages. CD4 T cell depletion is a hallmark of HIV infection. With the loss of CD4 T cells there is decreased IFNy and thus

macrophages are less efficient to contain Mtb, leading to higher rates of reactivation. TNFα is important in maintaining the structural integrity of the granuloma and is decreased in HIV infection. The effect of this decreased cytokine production can be seen in the granulomas of TB patients with HIV. These individuals have malformed granulomas with atypical cellular infiltrate, highlighting the effects of HIV on granuloma maintenance (13).

1.3 Symptoms of Mtb Infection

After pulmonary exposure and infection with Mtb, a majority of individuals will develop a primary lung granuloma which will wall off the bacterium and stop systemic spread of the infection. These asymptomatic individuals who are able to generate an immune response to keep the bacterium at bay and stop its spread to extra pulmonary sites are thought to have latent Mtb infection (LTBI). For all individuals that become infected, it is estimated that 90% will develop latent infection and 10% will develop active TB disease (14). Patients with active disease experience constitutional symptoms (cough, fever, night sweats, and weight loss) and may demonstrate evidence of disease by chest x-ray (15). It is important to note that individuals with active disease can generate cough-induced aerosol contaminated with bacterium and are capable of exposing healthy individuals to Mtb. LTBI individuals are not capable of infecting other individuals since their sputum does not contain Mtb. Latently infected immunocompetent individuals carry a 5-10% life-time risk of developing active disease (16). This occurs when the granuloma is not sufficient to contain the bacteria to the lung. Typically this re-activation event is associated with immunodeficiency or insults to the immune system, such as HIV infection resulting in immune suppression.

1.4 Diagnostic Tests in Mtb

Properly identifying individuals that are latently infected with Mtb is central to identifying those at high risk of developing active TB disease and also designing vaccine and therapeutic strategies to prevent the spread of infection. The two main tests used to identify individuals exposed to Mtb are the Tuberculin Skin Test (TST) and Interferon Gamma Release Assay (IGRA). Both assays rely on immune memory-dependent reactivity to Mtb antigens. In the TST, purified protein derivative (PPD) from Mtb is used to elicit a delayed type hypersensitivity response in individuals exposed to Mtb (17). The test is administered at the forearm by using a needle and syringe to deliver 0.1 mL of 5 tuberculin units into the dermis of the individual being tested. Individuals with a memory response from previous Mtb exposure will develop a raised induration on their skin, indicative of an inflammatory response (17). The size of the induration is measured to determine if the test is negative or positive. TST is limited in the fact that it is not specific to Mtb and can produce false-positive results due to cross-reactivity with non-tuberculosis mycobacteria and with prior BCG vaccination.

The IGRA assay is more specific for Mtb and is composed of overlapping peptides representing the immunodominant Mtb antigens ESAT-6, CFP-10, and TB-7.7. In one type of IGRA known as the QuantiFERON-TB Gold assay (QFT), blood is collected and stimulated with Mtb antigen for 24hrs. Similar to TST, this test measures responses from memory T cells generated from previous Mtb exposure. Supernatant from the blood is used to perform an ELISA for quantification of IFNγ, produced by T cells specific for ESAT-6, CFP-10, and/or TB-7.7. Results are measured and compared to a standard cutoff. The qualitative results of IGRAs are reported as

either negative, positive or indeterminate. The T-SPOT.TB test (T-spot) is another commonly used assay to identify exposed individuals. This assay are similar to the QFT, although the T-Spot assay utilizes an ELISPOT platform for measuring the number of IFNγ-producing T cells in peripheral blood monuclear cells (PBMCs). Limitations to all of these assays for Mtb exposure and infection include variable rates of detection based on age and immune status. Older individuals and the immunocompromised have lower responses in these assays. Differing responses based on demographic information of the patient is important for interpreting results. These assays are also limited in their ability to distinguish between individuals that have cleared the bacterium versus individuals that harbor persistent Mtb infection.

Another limitation of the diagnostic assays for Mtb exposure and infection are that it is not possible to distinguish LTBI from active TB disease. Patients with active TB disease are generally symptomatic and may have unexplained weight loss, fever and night sweats. To further diagnose active TB, staining can be performed to identify acid fast bacterium. Because other species of mycobacteria are also acid fast, culturing must be performed from sputum samples to verify which mycobacterial species are present. Along with culturing Mtb, drug susceptibility testing is also performed to gauge drug resistance of the bacterium. Culture and susceptibility testing take weeks to perform. The Xpert MTB/RIF assay is a rapid nucleic acid amplification test that significantly cuts down on the time needed to identify both Mtb in the sputum and the presence of rifampin resitance mutations (18). Active disease patients are highly contagious and must be placed on antibiotic therapy to treat the infection.

1.5 TB Treatment

Before the start of the antibiotic era there were limited options to treat TB. Sanatoriums were commonly used between the 18th and 20th centuries in an attempt to stop the spread of disease by isolating those already infected (19). At the time, it was believed that isolation, rest and fresh air would help to alleviate the symptoms of disease. With the discovery of penicillin in 1928 by Alexander Fleming the focus of Mtb treatment transitioned from isolation to drug based approaches. Subsequent experimentations into microbial susceptibility led to the first antibiotic treatment for Mtb, streptomycin, discovered by Shatz and Waksman in 1944 (20).

Newly diagnosed TB diseased individuals are primarily prescribed four drugs including rifampin, isoniazid, pyrazinamide and ethambutol (21). There are two phases of treatment that are based on the lifecycle of the bacillus. In the initiation phase treatment focuses on killing replicating bacteria. Symptoms and infectiousness decrease during this phase as the bacterial burden decreases. Ethambutol, isoniazid, pyrazinamide and rifampicin are used in combination for 2 months during initiation (22). The second phase of treatment targets semi-dormant bacilli that are harder to kill due to the initial drug treatment being dependent on actively replicating cells. This phase lasts 4 months with rifampicin and isoniazid being prescribed. The use of antibiotic treatment has played a large role in helping to decrease the incidence and mortality of TB disease. However, primarily through nonadherence with TB treatment, drug resistant strains of Mtb have developed and have significantly complicated eradication of TB.

In individuals with LTBI living in low TB incidence countries, TB preventive therapy options include three months of weekly isoniazid plus rifapentine, 4 months of daily rifampin, or 6-9 months of daily isoniazid (23). Guidelines for LTBI treatment vary by geographic location and in many cases, treatment for LTBI is not routinely recommended in high TB burden settings due to

high risk of re-exposure and re-infection. However, exceptions include WHO recommendations for treatment of LTBI in people living with HIV and infants and children under the age of 5 years who are household contacts of an active TB case, even in high TB burden countries (24).

1.6 Transmission of Mtb

Transmission of Mtb when individuals inhale bacterium containing droplets produced from the coughing of active TB patients. Once these droplets are inhaled they can travel through the large airways, ultimately reaching the alveoli located deep within the lungs. Along the way to the alveoli innate host mechanisms including mucous, ciliated epithelia, lysosymes and IgA and IgG antibodies attempt to neutralize and clear Mtb from the lung (25). In the alveoli the bacilli are taken up by local macrophages that kill or limit the bacterium in an attempt to halt the spread of infection. Through several immune evasion mechanisms, the bacterium is not always eliminated by the macrophage and instead begins to replicate and spread to epithelial and endothelial cells in the local environment (26). At the same time, the bacterium is recognized by several receptors expressed by macrophages leading to activation and production of pro-inflammatory cytokines and chemokines. These inflammatory mediators recruit innate cells to the site of infection including neutrophils, dendritic cells (DCs), natural killer (NK) cells and macrophages. If the innate immune system is not capable of clearing the initial infection then cells from the adaptive immune system (T cells and B cells), primed by DCs that have migrated to the draining lymph node, will be recruited to the site of infection in order to contain the bacterium and halt the spread of infection from the lungs.

In many cases, macrophages are not able to completely clear the infection and the bacterium persists. Over time, these bacteria-laden macrophages transform into epithelial like cells and form tight junctions with adjacent cells to wall off the bacterium and form the granuloma (27). The structure of the granuloma is composed centrally of predominately macrophages while lymphocytes make up the peripheral portions of this immune structure. CD4 T cells in particular are important in helping to maintain the granuloma by producing IFNγ, a potent activator for macrophages. The granuloma is thought to be a protective structure but it is not entirely clear why the immune system is incapable of eradicating the contained bacterium (28). If the granuloma fails to form or the integrity of the granuloma becomes compromised, infected individuals are at high risk of developing active disease. Immature granulomas have been seen in immunocompromised individuals that are unable to produce IL-12, IFNγ or TNFα (29, 30).

1.7 Cytokines Associated with Containment of Mtb

There is evidence that certain mediators are important for a robust and protective response to Mtb. In the absence of these immune mediators, protection is lost in latently infected individuals and risk of reactivation increases dramatically. Similarly, uninfected individuals that are not capable of producing these products have increased risk of becoming infected after being exposed with Mtb. IFNγ is important in maintaining the granuloma and is a key contributor to protection in individuals with latent infection. Previous studies in IFNγ-deficient mice challenged with Mtb resulted in normal granuloma formation but a decrease in the ability to produce nitrogen intermediates leading to uncontrolled bacterial growth and increased rates of mortality compared to wild type mice (31). In humans, individuals with inborn IFNγ receptor mutations are

especially susceptible to several mycobacterial species. These individuals do not respond when treated with antibiotics but have a positive response when IFNγ is administered (32). These data suggest that mycobacteria immunity is dependent on immune cell activation.

Th1 polarized CD4 T cells are the primary producers of IFNγ during the response to Mtb and in the absence of CD4 T cells there is a coordinate loss of production. CD4 T cell depletion is commonly seen in certain immunodeficiencies and in uncontrolled HIV infection (33, 34). Tumor necrosis factor alpha (TNFα) is another important mediator produced by T cells, DCs, and macrophages in response to Mtb. Unlike IFNγ, TNFα is important in granuloma formation, in addition to maintenance of the granuloma structure (35, 36). In mouse studies, the inability to produce TNFα results in increased susceptibility to Mtb infection (36). After Mtb infection, animals that receive anti-TNFα antibodies have decreased granuloma integrity noted by the formation of necrotic granulomas (37). Both of these studies highlight the importance of TNFα in the earliest stages of immunity and for the duration of disease. In humans, evidence for the protective effects of TNFα can be seen in reactivation of active TB in latently infected individuals treated with anti-TNFα medications for rheumatoid arthritis and Crohn's disease (38).

1.8 Recognition of Mtb by the Innate Immune Response

Cells of the innate immune system, including macrophages, neutrophils and DCs, express numerous pattern recognition receptors (PRRs) that recognize pathogens such as Mtb. These receptors are capable of recognizing pathogen-associated molecular patterns (PAMPs) produced by the bacterium. Recognition by PRR leads to activation of the immune cell and uptake of the pathogen. These initial activation events tailor both the overall innate and adaptive immune

response and formation of the granuloma. The three major family of receptors that recognize Mtb include the Toll-like receptors (TLR), C-type lectin receptors (CLR) and Nod-like receptors (NLR).

TLRs are a family of receptors that expressed intracellularly and on the surface of innate immune cells. This family of receptors have similar motifs but are diverse in the PAMPS that they recognize. MyD88 is an important adaptor molecule in TLR signaling. In MyD88-/- mice, challenge with Mtb led to macrophages and DCs incapable of producing TNF, IL-12, and nitrous oxide (NO) (39). Interestingly, based on IFNγ production, these animals had a normal Th1 response but these mice still had remarkable pathology scores compared to wild type controls. TLR2, TLR4 and TLR9 are the TLR receptors that recognize Mtb ligands (40, 41). TLR2 is expressed on the surface of innate immune cells and recognizes several Mtb antigens including the lipoproteins LprA and LprG, lipomannan and phosphatidyl-myo-inositol mannoside (PIM) (42-45). TLR2-/- mice challenged with Mtb have a reduced ability to form granulomas and are unable to control infection (41). Importantly, TLR2 stimulation is required for macrophage TNFα production (46). In addition to MyD88 dependence, TLR4 signals through a TIR-domain containing adapter-inducing interferon B (TRIF) to increase genes important in host protection (47). Both TLR2 and TLR4 are capable of inducing IL-6, an important inflammatory cytokine, in monocytes during Mtb infection (48). TLR9 recognizes unmethylated DNA and in DCs stimulation through this receptor is important for IL-12 production. Previous studies in TLR9-/- mice have shown that upon challenge, DCs from these animals have reduced IL-12p40 expression and decreased Th1 polarization resulting in decreased control of Mtb infection (49, 50).

C-type lectin receptors contain a domain that recognizes carbohydrate molecules through a Ca2+ dependent mechanism. These receptors are abundant on alveolar macrophages, monocytes and DC cells. Mtb uses these receptors to enter alveolar macrophages and stimulate the anti-inflammatory cytokines IL-4 and IL-13 (51). Many cell wall ligands can be recognized by mannose receptor but the primary antigen most heavily studied is Mannose-Capped Lipoarabinomannan (Man-LAM) (52).

Dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) is an important receptor that is important for adhesion/trafficking and as a PRR (53). DC-SIGN binds Man-LAM, similar to the Mannose Receptor. Once engaged, DC-SIGN allows for phagocytosis of the bacterium and initiation of anti-inflammatory immune response by increasing the production of IL-10. The induction of inhibitory signaling helps to delay DC migration to lymph nodes and thus postponing initiation of the adaptive immune response.

1.8.1 Formation of the Granuloma

The alveolar macrophages located in the airway are the first innate cells to come into contact with Mtb after the bacilli is inhaled into the lung (54). Alveolar macrophages phagocytose the bacterium through binding to PRRs. Through a mechanism mediated by the Mtb ESX-1 secretion system, the infected alveolar macrophages transverse the airway epithelium to the lung parenchyma (54). IL-1 produced by these alveolar macrophages localizes DCs and neutrophils to the site of infection, providing more targets for the bacterium and effectively leading to dissemination of the pathogen. The innate cells attempt to clear the pathogen but through immune escape mechanisms Mtb is able to evade the immune system. In mouse models

and human macrophages, Mtb uses phthiocerol dimycoceroserate (PDIM) to mask surface PAMPS being detected by activated macrophages (55). At the same time, phenolic glycolipids (PGLs) are expressed and help recruit permissive macrophages to site of infection (55). These escape mechanisms allow Mtb to establish a persistent infection in the lung. During the course of infection, assuming that the bacterium is not cleared, several components of the immune system will attempt to contain the infection, ultimately leading to the formation of the granuloma. Below is a description of the role that innate immune cells play in response to Mtb infection.

1.8.2 Macrophages

After phagocytosis the macrophage will attempt to kill the bacterium through phagosome-lysosome fusion. The bacterium evades this immune mechanism by inhibiting lysosomal maturation. Phosphatidylinositol 3-phosphate (PI3P) is an important in trafficking the phagosome to the lysosome (56). Mtb inhibits this process by inhibiting the enzymes responsible for maturation and phagosomal targeting of PI3P (57).

These mechanisms allow for bacillus to survive and replicate in the infected macrophage. Autophagy is an intrinsic mechanism used to eliminate aggregated protein, damaged organelles and microbes in cell cytoplasm and this process is initiated in the presence of stress such as starvation and infection. Phagocytosis of Mtb is linked to autophagy via TLR4 and consequent upregulation of autophagosome markers including LC3, and ATG5/7 (58). The protective importance of autophagy is observed in mice with deficiency of the autophagosome-dependent proteins Atg5 and LysM (59). These animals are highly susceptible to Mtb infection and also have

abnormally high expression of IL-1α (59), suggesting that autophagy not only limits bacterial replication but is also important for limiting inflammation.

Once activated, macrophages and monocytes produce a large number of important cytokines and chemokines that help shape the innate and adaptive immune response, thus tailoring the course of infection. These cytokines attract and activate other immune cells to the site of infection and in some instances also dampen the immune response. The primary cytokines produced by macrophages upon activation are TNFα, IL-12, IL-1β and IL-10.

TNFα is proinflammatory cytokine and is important in the formation and maintenance of the granuloma (35). In the macrophage, TNFα is important in increasing the cellular production of nitric oxide synthase, increasing the mediators necessary to kill intracellular Mtb (60). Mice deficient in TNFα expression are more susceptible to disease upon infection with Mtb (61). The importance of TNFα in Mtb protection in humans has been observed in individuals undergoing anti-TNFα treatment: Crohn's disease patients treated with anti-TNFα antibody Infliximab have a significant increase in reactivation and development of active TB disease associated with drug therapy (62). Further evidence for the importance of TNFα in human immunity towards Mtb comes from genetic linkage studies showing that specific polymorphisms of TNFα are associated with TB disease (63, 64).

IL-12 is a proinflammatory heterodimer cytokine made of two subunits, p40 and p35. This cytokine is primarily responsible for polarization of CD4 T cells to a T helper type 1 (Th1) fate and thus increasing the production of IFNγ by T cells. After Mtb infection of IL-12p40-/- mice, these animals were less capable of limiting bacterial growth, had decreased IFNγ produced by both innate and adaptive immune cells and less T cell recruitment at the site of infection, compared

with wild type mice (65). The importance of IL-12 in the immune response to Mtb in humans has been shown in individuals with inborn errors of IL-12. The most common mutation involves IL-12p40 (66). Overall, IL-12 plays a central role in the production of IFNγ by both the innate and adaptive immune system and thus is important in protection in Mtb infection.

IL-1β is primarily produced by macrophages, monocytes and neutrophils. It is one of the most important inflammatory cytokines in the immune response to Mtb. IL-1-deficient mice infected with Mtb developed larger granulomas and had macrophages that were not able to produce high levels of NO, compared with wild type mice (67). Interestingly, IL-1 supplementation in knock out animals did not alter pathological findings, although macrophage NO production was increased (67). These data suggest that IL-1 is important in the initial events of the immune response to Mtb.

IL-10 suppresses inflammation during the Mtb infection by inhibiting the activation of macrophages leading to a decrease in NO production, failure to form the phagolysosome and decreased polarization of Th1 cells, and decreasing the production of IFNγ (68). The antagonism of IFNγ and decreased activation of macrophages is a major mechanism used by Mtb to evade the immune system. In mice, the overexpression of IL-10 has been shown to increase the susceptibility to reactivation during chronic stage of infection. In this same study, IL-10 overexpression was also associated with decreased TNFα, IL-12p40 and antigen-specific IFNγ secretion (69). In humans with active TB disease, high levels of IL-10 can be found in the serum and may be a biomarker for reactivation (70).

1.8.3 Neutrophils

The inflammatory environment created by the activated macrophage leads to the recruitment and activation of neutrophils to the site of infection. These short-lived cells are numerous during the acute phase of infection. Once neutrophils migrate to the site of infection they recognize the bacterium through PAMPs and these cells are further activated by proinflammatory cytokines such as TNFa and IL-1β. Engulfed bacteria are encased in the phagosome and are killed using mechanisms similar to the macrophage including phagolysosomal fusion and reactive oxygen species. In addition to these mechanisms, neutrophils also contains several bactericidal molecules to kill the bacterium such as cathepsin G, defensins, proteinases, elastase and lysozyme (71, 72). These effectors are also released into the extracellular environment primarily through the release of neutrophil extracellular traps (NETs) leading to restriction and extracellular killing of Mtb (72). Neutrophils are capable of phagocytosing Mtb but previous studies in mice suggest that these cells are not efficient at killing the bacteria (73). In humans, the presence of neutrophils has been associated with the ability to control Mtb in whole blood (74). Although the exact role of neutrophils in protection during Mtb infection is not fully understood, these cells represent another arm of the innate immune response that help to contain the bacterium and contribute to the structure of the granuloma.

1.8.4 Natural Killer Cells

Similar to macrophages and neutrophils, natural killer (NK) cells express PRR that can be activated by binding to Mtb ligands (75). IFNγ production by NK cells activates antimicrobial effector functions of macrophages. Mtb can bind also directly to the natural cytotoxicity receptor (NCR) NKp44 on NK cells, and human NK cells can lyse Mtb-infected macrophages *in vitro* via

interactions with c-type lectins and NCRs expressed on NK cells (76), and suppress growth of Mtb in infected monocytes (77, 78). Moreover, NK cells are capable of directly killing Mtb through a granulysin and perforin dependent mechanism (79). NK cells are found in the granuloma of infected individuals (80). Active TB is associated with phenotypic changes of NK cells, including downregulation of the activating receptors NKp30 and NKp46, compared to healthy controls (81). NK cells play a role in Mtb infection but the exact mechanism are not fully understood. A better understanding of NK cell responses in Mtb is an important avenue to explore for potential new vaccine strategies and biomarkers to track disease states.

1.8.5 Dendritic Cells

Dendritic cells (DCs) are one of the first cells to respond to Mtb and their central role is to bridge the innate and adaptive arms of the immune system. DCs can directly recognize Mtb through the binding of DC-SIGN and mannose receptor. Previous studies investigating DCs in TB diseased patients suggest that DC-SIGN is the major pathway for the uptake of Mtb by this subset in the lungs (82).

After activation and uptake of the bacterium, DCs migrate to the lymph nodes to activate T cells and begin the adaptive immune response that is critical to protection in Mtb infection. Infected DCs are inhibited from migrating to the LNs and thus the initiation of the adaptive immune system is delayed, allowing Mtb time to establish infection in the lung. In these infected cells lipoarabinomannan (LAM) binds to DC-SIGN leading to the production of IL-10 and decreasing migration, activation and expression of costimulatory molecules (83). Another

mechanism used by LAM to inhibit DCs is through the inhibition of IL-12 (51), thus leading to decreased polarization of Th1 cells.

1.9 The Adaptive Immune Response to Mtb

After delayed migration, infected DCs make their way to the mediastinal lymph nodes in order to activate T cells and initiate adaptive immunity (84). In mice, this delayed response has been shown to be dependent on antigen load in the lymph node and independent of higher bacterial loads in the lung (85). These data provide evidence for the requirement of infected DCs migrating to the lymph node in order to establish adaptive immunity. In addition to delayed migration, infected DCs entering the lymph node also have decreased MHC class II expression and thus are limited in presenting antigen to activate CD4 T cells (85).

Mtb-specific CD4 T cells are crucial in the development and maintenance of the granuloma and protection against reactivation in latently infected individuals. CD4 T cells are responsible for producing a majority of the IFNγ needed to activate infected macrophages, limiting the spread of the bacterium and preventing development of active disease. Mice deficient in CD4 T cells or IFNγ production quickly succumb to Mtb infection and individuals with inborn errors in the IL12/IFNγ axis are highly susceptible to mycobacterial diseases (31, 86). Although IFNγ is certainly important in the protective response toward Mtb, mice deficient in CD4 T cells have only slight changes to global IFNγ expression upon challenge with Mtb but still have high mortality (87). These data suggest that CD4 T cells contribute in other ways to disease outside of the production of IFNγ.

The quality of the memory CD4 T cell response can be quantified based on the expression of the number of cytokines that are expressed by the memory CD4 T cell compartment. In Mtb infection, Th1 cytokines commonly measured include IFNγ, TNFα and IL-2. Polyfunctional memory CD4 T cells are considered to be more robust and provide better immunity (88). LTBI is associated with memory CD4 T cells producing all three cytokines while TB disease is associated with a loss of IL-2 production capacity by Mtb-specific CD4 T cells (89-91). Further studies are needed to better understand the association between the phenotype and function of Mtb-specific CD4 T cell responses with Mtb outcomes.

Th17 CD4 memory T cells are a subset of CD4 T cells distinguished by their expression of the cytokines IL-17, IL-21 and IL-22. Similar to the Th1 polarized memory CD4 T cells, these cells are also associated with protection against TB disease in mouse models of infection (92). Th17 CD4 T cells are important in mucosal immunity and their role includes neutrophil recruitment and regulation of tissue damage (93, 94). Mtb infection in mouse models have shown that a lack of IL-17 production is associated with increased bacterial load and decreased co-localization between CD4 T cells and macrophages (95). IL-17 has also been shown to support Th1 development in vaccine studies by decreasing IL-10 expression (96).

1.10 The Role of NK Cells in the Host Immune Response

NK cells are a subset of large granular lymphocytes and are a member of the innate immune system. They were originally discovered as an unknown set of cells capable of spontaneously lysing tumor cell lines without the need of previous exposure or priming. Later, experiments involving transplant models in mice revealed that NK cells kill cells that do not have

MHC molecules matching the host expressed MHC (97, 98). The exact receptor responsible for this interaction was not known at the time. It is now appreciated that NK cells have several activating and inhibitory receptors that recognize a wide range of MHC and non-MHC ligands (99). These receptors can be divided into four major families based on their structure and the type of ligand recognized: C-type lectins recognize MHC-like molecules; killer cell immunoglobulin-like receptors (KIR) are mostly inhibitory and recognize MHC class I molecules; natural cytotoxicity receptors (NCRs) bind ligands that are yet to be identified; and Fc-receptors that bind antibodies (100). Summation of all of the inhibitory and activating signals determines if the NK cell will become activated or remain inactivated (101).

CD56 and CD16 are the main lineage markers that identify NK cells. CD56 expression can also be used to stratify NK cells into three distinct populations including $CD56^{bright}$, $CD56^{dim}$, and $CD56^{negative}$ subsets (102). $CD56^{bright}$ cells are believed to be the most naïve NK cell type and when these cells become activated CD56 is downregulated leading to the generation of the other two CD56 subsets. $CD56^{dim}$ cells are the major subset found in the peripheral blood while $CD56^{bright}$ cells are least abundant in the blood and primarily produce cytokines (103). By contrast, $CD56^{bright}$ cells predominante in the tissues.

1.10.1 NK Cell Function

The role of NK cells is primarily surveillance for the presence of pathogens or cancer cells. The primary cytokine produced by NK cells upon activation is IFNγ and it is believed that NK cells are the earliest producers of IFNγ in many diseases. NK cells also produce TNFα, an important cytokine for the clearance of many viral and bacterial infections. In addition to cytokine

production, NK cells are armed with granules containing several cytolytic mediators including perforin and granzyme B. These molecules are released into target cells by pores formed by perforin. NK cells also play a role in influenceing the adaptive immune response. In addition to killing tumor cells, NK cells can fine tune the adaptive immune response by killing antigen presenting cells and activated T cells (104, 105).

1.10.2 NK Cells in HIV Infection

NK cells are activated during HIV infection and display alterations in the frequency of the CD56 subsets. HIV associated immune activation leads to the down regulation of NK cell CD56 expression and an increase in $CD56^{negative}$ cells (106). The exact role of $CD56^{negative}$ cells in HIV is not entirely understood, although the population of $CD56^{negative}$ NK cells diminishes in individuals on antiretroviral therapy (ART), suggesting a link with viral load (107). Also during HIV infection, numerous NK cell receptors become downregulated, decreasing NK cell diversity. HIV disease outcomes are associated with different polymorphisms of KIR genes, highlighting the importance of NK cell responses in this disease (108). In a prospective cohort study, NK cell diversity was identified as a correlate of protection against acquisition of HIV infection (109). NK cells also regulate infected CD4 T cells during infection. In vitro, NKG2A+ NK cells recognize and kill HIV infected CD4 T cells (110). Overall, it is apparent that NK cells play a major role in regulating the immune response during HIV infection and that NK cell phenotypic diversity plays a role in HIV susceptibility and disease course.

1.10.3 NK Cells in Bacterial Infection

Several bacteria species, including mycobacteria, are recognized by NK cells through binding of NKp44 (111). TLR2 is highly expressed on NK cells and binds directly to *M. bovis* (112). In this study blocking TLR2 did alter the response to *M. bovis* suggesting direct interaction between mycobacteria and TLRs. In response to bacterial pathogens, NK cells primarily produce TNFα and IFNγ (112). NK cell are the primary source of IFNγ in several infections including *Francisella tularensis*, *Listeria monocytogenes*, *Chlamydia pneumoniae* (113-115). The importance of IFNγ production by NK cells can be seen in infections of mice with *Legionella pneumophila*. In this study, researchers showed that mice depleted of NK cells were not capable of clearing infection and that this clearance was dependent on IFNγ production (116). NK cells are also capable of producing the antimicrobial molecules alpha-defensins and cathelicidin (117). Other than direct recognition, NK cells can become indirectly activated by bacterium. Monocytes and DCs are the most well studied accessory cell involved in indirect NK cell activation (118). These antigen presenting cells become activated after sensing pathogen and produce IL-12 and IL-18 to induce activation of NK cells (103). Through direct and indirect activation, NK cells are capable of providing a crucial immune response towards bacterial pathogens.

1.10.4 Adaptive NK Cells

NK cells have traditionally been considered exclusive members of the innate immune system. Although these cells lack of receptor diversity found in T and B cells recent studies suggest that NK cells are capable of developing a memory like response after re-exposure to a stimulus. Most of this work has been conducted in cytomegalovirus (CMV) infection of mice and humans. In mice, NK cells have a more robust response upon re-exposure to murine CMV, similar

to other memory responses (119, 120). This phenomenon has also been seen in other infections such as simian immunodeficiency virus (SIV). In this study researchers showed that NK cells killed Gag and Env-pulsed DCs in an antigen dependent manner (121). Phenotypically, adaptive NK cells in humans are identified by increased expression of CD57 and NKG2C and decreased expression of NKp30 and NKp46 (122). Interestingly, long lasting NK cells can also be generated in vivo with stimulation with IL-12, IL-15 and IL-18 (123). Induction of memory-like NK cells has also been demonstrated in BCG-vaccinated mice, which show increased NK cell responses to Mtb challenge but responses to other bacterial species was not enhanced (124). In humans, long-lived NK cells have also been identified after BCG vaccination (125). The exact role that NK adaptation may play in Mtb is still to be determined.

Thesis Overview

Mtb infects nearly a quarter of the global population and is responsible for over 10 million deaths each year (126). The majority of people infected with Mtb will never develop active TB disease, thus indicating that the host immune response is usually capable of containing the infection. Despite years of research, the immune correlates of protection in human Mtb infection and TB disease have not been well defined. One of the greatest known risk factors for developing active TB is HIV infection, thus highlighting a particularly important role for CD4 T cells in controlling Mtb infection. In addition to CD4 T cells, many other immune cell populations are involved in the host immune response, including CD8 T cells, B cells, γδ T cells, MAIT cells, DCs, macrophages, neutrophils, and NK cells. NK cells are a major source of IFNγ production, which is necessary for containment of Mtb, and recent cohort studies indicate that a decline in the frequency of NK cells precedes development of active TB disease (127). Although increasing evidence points to a role of NK cells in the host immune response to Mtb, no previous studies have comprehensively evaluated the phenotype and function of NK cells in individuals across a spectrum of diverse Mtb exposure and infection states, including co-infection with Mtb and HIV.

The overall hypothesis of this thesis is that the phenotypic and functional profiles of NK cells are modified in people with persistent Mtb infection and with HIV/Mtb co-infection. To address this hypothesis, we analyzed NK cells in peripheral blood samples collected from cohorts of adults recruited in the U.S. and a TB-endemic region in western Kenya. Using multi-parameter flow cytometry, we performed a rigorous and robust analysis of the phenotype and function of NK cells in healthy control adults, adults with latent Mtb infection, and adults with HIV and Mtb co-infection. We evaluated NK cell responses across all cohorts to generic NK cell stimuli,

including tumor cell lines and antibody-coated cells; moreover, we evaluated NK cell responses to stimulation with Mtb antigens in vitro to determine how Mtb and HIV infection may impact NK cell reactivity to Mtb. These data provide novel insights into how infection with Mtb and/or HIV modifies NK cell phenotypic and functional profiles. Moreover, these novel data lay the foundation for future studies aimed at defining NK cell signatures that may be associated with either protection against acquisition of Mtb infection or progression to active TB disease.

Chapter 2. Distinct human NK cell phenotypes and functional responses to *Mycobacterium tuberculosis* in adults from TB endemic and non-endemic regions

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2.1 Abstract

Mycobacterium tuberculosis (Mtb) is the causative agent of tuberculosis (TB), which leads to an estimated 1.5 million deaths worldwide each year. Although the immune correlates of protection against Mtb infection and TB disease have not been well defined, natural killer (NK) cells are increasingly recognized as a key component of the innate immune response to Mtb and as a link between innate and adaptive immunity. In this study, we evaluated NK cell phenotypic and functional profiles in QuantiFERON-TB (QFT)+ and QFT− adults in a TB endemic setting in Kisumu, Kenya, and compared their NK cell responses to those of Mtb-naïve healthy adult controls in the U.S. We used flow cytometry to define the phenotypic profile of NK cells and identified distinct $CD56^{dim}$ NK cell phenotypes that differentiated the Kenyan and U.S. groups. Additionally, among Kenyan participants, NK cells from QFT+ individuals with latent Mtb infection (LTBI) were characterized by significant downregulation of the natural cytotoxicity receptor NKp46 and the inhibitory receptor TIGIT, compared with QFT– 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 U.S. healthy adult controls. Taken together, these data provide evidence that the phenotypic and functional profiles of NK cells are modified in TB

endemic settings and will inform future studies aimed at defining NK cell-mediated immune correlates that may be protective against acquisition of Mtb infection and progression to TB disease.

2.2 Introduction

Infection with *Mycobacterium tuberculosis* (Mtb) can lead to development of active tuberculosis (TB) disease, which is currently the leading cause of death in the world due to a single infectious agent (128). The vast majority of individuals infected with Mtb remain asymptomatic and are considered to have latent Mtb infection (LTBI). Approximately one quarter of the global population is estimated to harbor Mtb infection (126), with 10 million individuals developing active TB disease each year (128). The only currently licensed TB vaccine, *Mycobacterium bovis* bacille Calmette-Guérin (BCG), provides variable efficacy, ranging from 0 – 80%, against pulmonary TB disease in adults (129).

Both innate and adaptive immunity, including Mtb-specific T cell and antibody (Ab) responses, are clearly important in maintaining control of Mtb (130, 131), although the precise immune correlates of protection to Mtb infection have not been well defined. Natural killer (NK) cells are increasingly recognized as a key component of the innate immune response to Mtb and as a link between innate and adaptive immunity (132, 133). IFN-γ production by NK cells activates antimicrobial effector functions of macrophages, which is essential for control of Mtb; furthermore, secretion of granulysin by NK cells can kill intracellular Mtb when delivered by perforin (79, 134). Studies of Mtb infection in T cell-deficient mice indicated that NK cell-mediated IFN-γ production contributes significantly to inhibition of bacterial replication (135). Mtb can bind directly to TLR2 on NK cells (136) as well as the natural cytotoxicity receptor (NCR) NKp44 (111). Human NK cells can lyse Mtb-infected macrophages *in vitro* via interactions with c-type lectins and NCRs expressed on NK cells (76), and suppress growth of Mtb in infected monocytes (77, 78). IL-22 production by NK cells inhibits intracellular growth of Mtb *in vitro* by

enhancing phagolysosomal fusion (137). NK cells are recruited to the lung in patients with active TB disease (80). However, NK cells circulating in peripheral blood of patients with pulmonary TB disease exhibit decreased IFN-γ production capacity (138, 139), which is partially restored following anti-TB treatment (140), thus suggesting an association between NK cell functional capacity and bacterial load. Moreover, longitudinal cohort studies have indicated that progression to active TB disease is preceded by a decline in the frequency of circulating NK cells, which is restored following successful treatment for active TB (127), thus providing further evidence of an important role for NK cells in Mtb infection and TB disease in humans.

NK cell activity is tightly regulated through a sophisticated network of numerous germline-encoded activating and inhibitory receptors (141), the variegated expression of which generates heterogenous populations of NK cells with high diversity (142, 143). Moreover, NK cell surface marker expression changes in the settings of inflammation, infection and cancer, and increasing evidence indicates NK cells can differentiate into distinct subsets with specialized functions, referred to as 'adaptive' NK cells (144, 145). Adaptive NK cells in humans have been defined most clearly in the context of human cytomegalovirus (HCMV) infection, which has been associated with expansion of distinct NK cell subsets and enhanced responsiveness to virally-infected cells in an antibody-dependent manner (146-149). Antigen-specific NK cells have also been described in simian immunodeficiency virus (SIV)-infected and vaccinated rhesus macaques (121). In humans, infection with HCMV and other viruses leads to expansion of NK cell subsets with adaptive features expressing CD57 and the activating receptor NKG2C (122, 150-152). Adaptive NK cells in HCMV infection are characterized by downregulation of receptors such as NKp30 and NKp46 (122), downregulation of the transcription factors PLZF and IKZF2 and loss of

intracellular adaptor signaling molecules (146, 147). Downregulation of these molecules has been associated with pronounced changes in DNA methylation patterns (146, 147), thus clearly demonstrating pathogen-induced epigenetic reprogramming as a mechanism driving the generation of adaptive NK cells.

In addition to HCMV, NK cells with adaptive features have been described in other chronic human infections, including human immunodeficiency virus (HIV), hepatitis C virus (HCV) and Epstein-Barr virus (EBV) (153). The potential for persistent bacterial infections, such as Mtb, to promote adaptive diversification of NK cell is less clear. In a mouse model of TB, vaccination with BCG induces memory-like NK cells producing IFN-γ, which provide protection against challenge with Mtb (124). In humans, recent studies in South Africa indicate that BCG revaccination of individuals with LTBI boosts BCG-reactive NK cell responses for at least one year after revaccination (125). NK cells from patients with active TB disease exhibit decreased expression of the activating NCRs NKp30 and NKp46 (138), a phenotype consistent with changes in adaptive NK cell phenotypic profiles in HCMV infection (122).

While increasing evidence from animal models and humans indicates that NK cells can differentiate into distinct subsets with specialized functions, it currently remains unclear if Mtb exposure and infection modifies NK cell phenotypic and functional signatures, and if so, how Mtb-associated changes in the NK cell repertoire may impact acquisition of Mtb infection and/or progression to active TB disease. To test the hypothesis that Mtb exposure and infection are associated with distinct NK cell phenotypic and functional profiles, we recruited a cohort of HIV-negative, Mtb-infected and uninfected adults in a TB endemic setting in Kisumu, Kenya and a cohort of Mtb-naïve, healthy adults in the U.S., a non-TB endemic setting. We performed a

comprehensive analysis of NK cells from individuals in these cohorts and identified distinct $CD56^{dim}$ NK cell phenotypic profiles that differentiated Kenyan adults from U.S. adults. Moreover, we demonstrated that $CD56^{dim}$ phenotypic profiles correlated with dampened NK cell responses to MHC class I-devoid cells and diminished reactivity to Mtb antigen stimulation in Kenyan adults.

2.3 Materials and Methods

Study participants: *Kenya cohort:* Blood samples were collected from individuals ≥18 years of age enrolled at the Kenya Medical Research Institute Clinical Research Center in Kisumu, Kenya. Study participants included adults with a normal chest x-ray and no symptoms of TB disease and no previous history of diagnosis or treatment for active TB disease. Mtb infection status was evaluated by QuantiFERON®-TB Gold In-Tube (QFT; Qiagen). Individuals with a positive QFT result (TB Antigen-Nil ≥0.35 IU/ml) were defined as having LTBI. Individuals with a TB Antigen-Nil response <0.35 IU/ml were defined as healthy controls (QFT^-). Serologic testing for HIV antibodies was done for all individuals using the Diagnostic Kit for HIV (1+2) Antibody V2 (*KHB*® Shanghai Kehua Bio-engineering Co., Ltd). All participants enrolled for the study were seronegative for HIV. HCMV seropositivity in healthy Kenyan adults is 97% (154), thus participants enrolled in Kisumu are presumed to be HCMV seropositive. *U.S. healthy controls*: Blood samples were collected from healthy adults enrolled at the Emory Vaccine Center in Atlanta, GA. U.S. healthy adult controls were U.S.-born, had not been vaccinated with BCG, and had no history of exposure to TB. All U.S. healthy controls were seropositive for HCMV IgG antibodies, as measured using the Cytomegalovirus IgG ELISA kit (Abnova).

Ethics statement: This study was conducted in accordance with the principles expressed in the Declaration of Helsinki. All subjects provided written informed consent for participation in the study, which was approved by the Kenya Medical Research Institute Scientific and Ethics Review Unit and the Emory University Institutional Review Board.

PBMC isolation: Blood samples from all participants were collected in sodium heparin tubes for isolation of peripheral blood mononuclear cells (PBMCs). PBMCs were isolated via density gradient centrifugation, cryopreserved, and stored in LN_2 until use. Cryopreserved PBMCs were thawed in a 37°C water bath and resuspended in 10 ml RPMI 1640 (Corning) with deoxyribonuclease I (DNase, 10 μg/ml, Sigma-Aldrich) and washed twice with RPMI 1640. Cells were then suspended in R10 (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum [FCS], 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine) and used in phenotypic and functional NK cell assays described below.

NK cell phenotyping: Thawed PBMCs were washed in PBS and stained with Zombie NIR™ Fixable Viability Dye (BioLegend) for 15 minutes at room temperature. Cells were washed with PBS and surface stained for 30 minutes in the dark at room temperature with anti-CD3 Alexa Fluor 700 (BioLegend; UCHT1), anti-CD14 Alexa Fluor 700 (BioLegend; HCD14), anti-CD19 Alexa Fluor 700 (BioLegend; HIB19), anti-CD56 Brilliant Violet (BV) 711 (BioLegend; HCD56), anti-CD16 BV 605 (BD; 3G8), anti-NKG2A PE (Beckman Coulter; IM329IU), anti-NKG2D BV 421 (BioLegend; 1D11), anti-NKp30 Alexa Fluor 647 (BioLegend; P30-15), anti-NKp46 PE-Cy7 (BioLegend; 9E2), and anti-CD57 FITC (BioLegend; HCD57). After incubation with conjugated antibodies, cells were washed with PBS and fixed with 2% paraformaldehyde (PFA).

A second phenotyping panel was designed to measure the NK cell expression of intracellular markers. PBMCs were stained with Zombie NIR™ Fixable Viability Dye and surface stained for 30 minutes at room temperature with anti-CD56 BV 711 (BioLegend; HCD56), anti-CD16 BV 605 (BD; 3G8), anti-CD3 Alexa Fluor 700 (BioLegend; UCHT1), anti-CD14 Alexa Fluor 700

(BioLegend; HCD14), anti-CD19 Alexa Fluor 700 (BioLegend; HIB19), and anti-TIGIT PE (BioLegend; A15153G). Cells were washed in PBS and suspended in FoxP3 Fixation Buffer (eBioscience) for 30 minutes on ice. After fixation, cells were washed with FoxP3 Permeabilization Buffer (eBioscience) and stained with anti-granzyme B PE-CF594 (BD; GB11), anti-perforin PE-Cy7 (BD; BD48), and anti-granulysin Alexa Fluor 488 (BD; RB1) for 30 minutes in the dark at room temperature. Finally, cells were washed with FoxP3 Staining Buffer and resuspended in PBS.

Stimulation and staining of NK cells: NK cell responses to target cells were evaluated using K562 and p815 tumor cell lines (ATCC). Just prior to use in NK cell stimulation experiments, p815 cells were incubated with rabbit anti-mouse polyclonal lymphocyte serum (Cedarlane) for 30 minutes to coat the cells with Ab. Ab-coated p815 cells were then washed with R10 before being added to PBMCs. Donor PBMCs were incubated with K562 or Ab-coated p815 cells at an effector to target ratio of 10:1. PBMCs incubated in R10 media alone served as a negative control. CD107a PE-Cy7 (BioLegend; H4A3), brefeldin A (5 μg/ml; Sigma-Aldrich), and monensin (5 μg/ml; BioLegend) were added to each sample at the beginning of stimulation. Cells were incubated at 37°C degrees for 5 hours.

NK cell responses to Mtb antigens were evaluated by stimulation of PBMCs with 10 μg/ml of *Mycobacterium tuberculosis* H37RV derived cell wall, cell membrane and whole cell lysate (obtained from BEI Resources, NIAID, NIH; catalog numbers NR14828, NR14831 and NR14822, respectively). PBMCs were incubated in R10 with anti-CD107a PE-Cy7 (BioLegend; H4A3), recombinant human IL-2 (100U/ml; NIH AIDS Research and Reference Reagent Program, Catalog #136), and Mtb antigens for 24 hours at 37°C and 5% CO_2. Brefeldin A (5 μg/ml; Sigma-Aldrich)

and monensin (5 μg/ml; BioLegend) were added at the final 5 hours of incubation. PBMCs incubated in R10 media with anti-CD107a and IL-2 alone served as a negative control. For cytokine neutralization experiments, PBMCs were incubated with purified NA/LE mouse anti-human IL-12 (p40/p70) (BD; C8.6), purified mouse anti-human IL-18 (R&D Systems; 125-2H), or purified NA/LE mouse IgG1 κ isotype control (BD; 107.3) for 15 minutes prior to addition of Mtb antigens, as described above.

Following stimulation of PBMCs with either target cell lines or Mtb antigens, cells were washed with PBS and stained with Zombie NIR™ Fixable Viability Dye (BioLegend) for 15 minutes. Cells were washed with PBS and surface stained with anti-CD56 BV 711 (BioLegend; HCD56), anti-CD16 BV 605 (BD; 3G8), anti-CD3 Alexa Fluor 700 (BioLegend; UCHT1), anti-CD14 Alexa Fluor 700 (BioLegend; HCD14), anti-CD19 Alexa Fluor 700 (BioLegend; HIB19), anti-CD158a FITC (BioLegend; HP-MA4), anti-CD158b FITC (BioLegend; DX27), and anti-CD158e1 FITC (BioLegend; DX9) for 30 minutes at room temperature in the dark. Stained cells were washed with PBS and fixed with FoxP3 Fixation Buffer (eBioscience) for 30 minutes on ice. Cells were washed with FoxP3 Permeabilization Buffer (eBioscience) and stained intracellularly with anti-CD69 PerCP-Cy5.5 (BioLegend; FN50), anti-IFN-γ BV 480 (BD; B27), anti-TNFα Alexa Fluor 647 (BioLegend; MAB11) and anti-IL-22 PE (BioLegend; 2G12A41) for 30 minutes at room temperature in the dark. Cells were washed in PBS and prior to acquisition on a BD LSRII flow cytometer.

Flow cytometry and data analysis: Cells were acquired on a BD LSRII flow cytometer with BD FACSDiva software (v8.0) and analyzed with FlowJo software (v9.6; BD). Compensation was performed using single-stained anti-mouse Ig,κ beads (BD Bioscience). Single cells were

identified by plotting forward scatter height and forward scatter area. Lymphocytes were identified by plotting forward scatter height and side scatter height. Viable lymphocytes were identified by low expression of NIR viability dye. NK cells were identified as live lymphocytes negative for CD3, CD14, CD19 and positive for the NK lineage markers CD56 and/or CD16. NK cells were further stratified based on intensity of the expression of CD56 ($CD56^{dim}$, $CD56^{bright}$ and $CD56^{neg}$ NK cells).

Data analysis and statistics: A minimum of 1,000 NK cells was acquired in each panel for each individual. Expression of functional markers by NK cells (CD69, IFN-γ, CD107a, TNF-α, and IL-22) were analyzed after subtraction of background expression in the negative control condition. A non-parametric Mann-Whitney test was used to compare differences between two groups. Differences between 3 groups were evaluated using a non-parametric Kruskal-Wallis test, with *p*-values adjusted for multiple comparisons using Dunn's post-test. Correlations were evaluated using Spearman's rank-order correlation. *P*-values of less than 0.05 were considered significant.

2.4 Results

Study participants

Blood samples were collected from 61 participants in Kisumu, Kenya and from 9 Mtb-naïve healthy adult controls in Atlanta, GA (U.S.) (Table 1). Participants enrolled in Kenya were stratified by QFT result: QFT+ (considered to have LTBI, n=31) and QFT– (n=30). The majority of participants from Kisumu were household contacts of an active TB patient within two years prior to study enrollment (20/31 QFT+ individuals [64.5%] and 21/30 QFT– individuals [70%]). All three groups were similar with regard to age and sex characteristics, with the exception that U.S. healthy controls were older than Kenya QFT– participants.

NK cells in QFT+ and QFT– Kenyan adults are characterized by increased proportions of CD56neg NK cells

Redistribution of NK cell subsets, including expansion of the $CD56^{neg}$ subset, has been described in the setting of chronic viral infections (106). To determine if the frequency and distribution of NK cell subsets are modified in the setting of Mtb infection, we used flow cytometry to measure NK cells directly ex vivo in PBMCs from all three participant groups (Figure 2-1A). The frequency of total NK cells in the lymphocyte population was similar between the three participant groups (Figure 2-1B). As expected, $CD56^{dim}$ NK cells constituted the dominant subset of NK cells in all participant groups (Figure 2-1C). There were no significant differences in the proportions of CD56 subsets between QFT+ and QFT– Kenyan groups, thus indicating that Mtb infection does not significantly modify the frequency and distribution of NK cells subsets in peripheral blood. However, both QFT+ and QFT– Kenyan adults had significantly higher

proportions of CD56^{neg} NK cells, compared with Mtb-naïve healthy adult controls in the U.S. (Figure 2-1C).

CD56^{dim} NK cells exhibit distinct phenotypic profiles in Kenyan and U.S. adults

Given that NK cell surface marker expression can change in the setting of infection (144), we performed flow cytometry to evaluate expression of 10 phenotypic markers expressed by NK cells in each of the three participant groups (Figure 2-2A). Since CD56 subsets have distinct transcriptional profiles (155), we determined the phenotypic profiles of CD56^{dim} and CD56^{bright} NK cells subsets separately. There was a progressive increase in expression of the differentiation marker CD57 by CD56^{dim} NK cells from U.S. healthy controls to QFT– and QFT+ Kenyan individuals. By contrast, NKG2A, NKp30 and NKp46 were progressively decreased on CD56^{dim} NK cells from U.S. healthy controls to QFT– and QFT+ Kenyan individuals (Figure 2-2B). While the cytotoxic molecules perforin and granulysin were expressed at similar levels among the three groups, expression of granzyme B was markedly increased in both groups of Kenyan participants, compared with U.S. healthy controls. Moreover, expression of the inhibitory receptor TIGIT was significantly higher on CD56^{dim} NK cells from Kenyan participants, compared with U.S. healthy controls. Similar phenotypic differences were also found when evaluating the phenotype of the total NK cell population in the three participant groups (Figure 2-S1 in Supplementary Material).

While there were substantial differences in the phenotypic profile of CD56^{dim} NK cells between Kenyan and U.S. adults, we also identified phenotypic markers that were expressed at significantly different levels between QFT+ and QFT– Kenyan adults, with lower expression of both NKp46 and TIGIT on CD56^{dim} NK cells from QFT+ individuals, compared with QFT– individuals

(Figure 2-2B). These data indicate that human Mtb infection may be associated with downregulation of specific receptors expressed by circulating $CD56^{dim}$ NK cells.

By contrast with $CD56^{dim}$ NK cells, fewer differences were found among the three participant groups when evaluating the phenotypic profiles of $CD56^{bright}$ NK cells. Although expression of NKG2D was similar among the groups on $CD56^{dim}$ NK cells, NKG2D expression was significantly higher on $CD56^{bright}$ NK cells from QFT+ and QFT− Kenyan adults, compared with U.S. healthy controls (Figure 2-2C). Importantly, similar to $CD56^{dim}$ cells, expression of NKp46 was also significantly lower on $CD56^{bright}$ NK cells from Kenyan QFT^{+} individuals, compared with QFT− individuals. PCA of expression of the 10 phenotypic markers by $CD56^{dim}$ cells from the three groups indicated that U.S. healthy controls can be clearly differentiated from Kenyan individuals. By contrast, PCA of the same 10 phenotypic markers by $CD56^{bright}$ NK cells in the same individuals does not clearly distinguish the participant groups (Figure 2-2D).

Taken together, these data indicate substantial differences in $CD56^{dim}$ NK cell phenotypic profiles distinguish Kenyan and U.S. adults. Moreover, these data also indicate that among Kenyan adults residing in a TB endemic environment, expression levels of the natural cytotoxicity receptor NKp46 and the inhibitory receptor TIGIT are further modified in QFT+ adults with LTBI, compared with QFT− adults.

$CD56^{dim}$ NK cells from Kenyan adults have dampened responses to tumor cells, compared with U.S. adults

Through receptor dependent mechanisms, NK cells recognize tumor and virus infected cells that downregulate MHC class I expression as an immune escape mechanism (156). Given

the substantial differences in CD56^{dim} NK cell phenotype in the three participant groups, we next evaluated the functional capacity of CD56^{dim} NK cells to respond to the generic NK cell targets of MHC class I-devoid K562 tumor cells and Ab-coated p815 cells (Figure 2-3A). CD56^{dim} NK cells from U.S. healthy adults had a generally more robust response to stimulation with K562 cells, as measured by CD69, CD107a and IFN-γ expression (Figure 2-3B), compared with Kenyan adults. In addition, Boolean analysis of all three markers indicated increased co-expression of two or three markers by CD56^{dim} NK cells from U.S. healthy controls, compared with Kenyan adults (Figure 2-3C). Interestingly, among Kenyan adults, CD56^{dim} NK cells from QFT+ individuals with LTBI co-expressed CD69 and CD107a at higher levels following stimulation with K562 cells, compared with QFT− individuals, although this did not maintain statistical significance after correction for multiple comparisons (Figure 2-3C). No significant differences in CD69, CD107a, or IFN-γ expression were observed by CD56^{dim} NK cells from the three groups following stimulation with Ab-coated p815 target cells (Figure 2-3D, E). Taken together, these data indicate that a high pathogen burden environment in Kenya is associated with reduced NK cell reactivity to MHC class I-devoid cells, whereas Ab-mediated activation of NK cells is maintained at similar levels to those seen by NK cells from U.S. healthy controls.

Differential reactivity of CD56^{dim} NK cells to Mtb antigen stimulation in Kenyan and U.S. adults

The above data indicated that NK cell phenotype and functional responses to generic stimuli are impacted by Mtb infection status, as well as high (Kenya) versus low (U.S.) pathogen burden settings. We next sought to determine if Mtb infection modifies NK cell reactivity to Mtb antigen stimulation. Thus, we stimulated PBMCs from each of the participant groups with Mtb

cell wall antigen for 24 hrs in the presence of IL-2, followed by flow cytometry for expression of CD69, CD107a, IFN-γ, TNF-α, and IL-22 (Figure 2-4A and data not shown). $CD56^{dim}$ NK cells expressed very low levels of TNF-α and IL-22 following stimulation of PBMCs with Mtb cell wall antigens (median <0.1% of $CD56^{dim}$ NK cells in each group; data not shown). However, Mtb antigen stimulation induced upregulation of CD69 and IFN-γ expression by $CD56^{dim}$ NK cells, as well as degranulation, as measured by surface expression of CD107a. Of note, there were marked differences in $CD56^{dim}$ NK cell reactivity to Mtb antigens in healthy adults from the U.S. versus Kenya, with U.S. healthy controls expressing significantly higher levels of CD69 and CD107a following stimulation with Mtb antigens, compared with QFT+ and QFT− individuals from Kenya (Figure 2-4B).

We next evaluated Mtb cell wall-induced co-expression of CD69, CD107a and IFN-γ by $CD56^{dim}$ NK cells. The frequency of $CD56^{dim}$ NK cells expressing CD69, either alone or in combination with CD107a and/or IFN-γ, was consistently higher in U.S. healthy controls, compared with Kenyan adults (Figure 2-4C). These data suggest a heightened level of Mtb antigen-induced activation, as measured by upregulation of CD69, by NK cells from Mtb-naïve, healthy adults in the U.S., compared with Kenyan adults in a TB-endemic environment. Interestingly, among Kenyan adults, the frequency of Mtb antigen-induced CD69 single-positive $CD56^{dim}$ NK cells was higher in QFT− individuals, compared with QFT+ individuals, although this difference did not remain statistically significant following correction for multiple comparisons (Figure 2-4C).

To determine if our results of NK cell reactivity to Mtb cell wall were reproducible with other Mtb antigen preparations, we also stimulated PBMCs with Mtb cell membrane and Mtb

whole cell lysate. Similar to stimulation with Mtb cell wall, stimulation of PBMCs with Mtb cell membrane and whole cell lysate antigens induced higher expression of CD69 and CD107a by $CD56^{dim}$ NK cells from Mtb-naïve U.S. healthy adults, compared with Kenyan adults (Figure 2-S2 in Supplementary Material). Similar to our findings with Mtb cell wall, among Kenyan adults, the frequency of Mtb cell membrane induced $CD56^{dim}$ NK cells co-expressing CD69 and CD107a was higher in QFT– individuals, compared with QFT+ individuals (Figure 2-S2D in Supplementary Material), although this did not maintain statistical significance following correction for multiple comparisons. Overall, these data indicate that $CD56^{dim}$ NK cell responses to Mtb antigens are characterized predominately by upregulation of CD69 and CD107a expression, and that $CD56^{dim}$ NK cells from Mtb-naïve adults express significantly higher frequencies of these markers, compared with Kenyan adults. Moreover, these data suggest that the capacity to restimulate NK cells with Mtb antigens in vitro may be further dampened in individuals with LTBI, compared with QFT– individuals from the same TB endemic environment.

Ex vivo phenotype of $CD56^{dim}$ NK cells correlates with functional reactivity to Mtb antigens

NK cells can directly recognize pathogens through antigen interaction with NK cell receptors (111, 157). To better define the relationship between NK cell receptor expression ex vivo and functional responses to Mtb antigens, we generated a correlation matrix of $CD56^{dim}$ NK cell receptor expression ex vivo and the frequency of $CD56^{dim}$ NK cells expressing CD69, CD107a, and IFN-γ following stimulation with Mtb cell wall in vitro (Figure 2-5). We focused our analysis of phenotypic markers to those markers that were expressed at significantly different levels among the participant groups (see Figure 2-2A): NKG2A, NKp30, NKp46, granzyme B, and TIGIT.

Among the phenotypic receptors, there was a strong positive correlation between expression of granzyme B and TIGIT, and between NKG2A, NKp30 and NKp46. There were also strong inverse correlations between granzyme B and NKG2A, NKp30, and NKp46 (Figure 2-5A). By contrast, analysis of $CD56^{bright}$ NK cells by a similar correlation matrix approach revealed no relationship between granzyme B expression and NKG2A, NKp30, and NKp46 on $CD56^{bright}$ cells (Figure 2-5B).

As expected, the frequencies of Mtb-induced NK cells expressing CD69, CD107a and IFN-γ correlated positively with each other (Figure 2-5A, B). The frequency of $CD69^{+}$ $CD56^{dim}$ cells following stimulation with Mtb antigen correlated positively with ex vivo frequencies of NKG2A, NKp30, and NKp46, and inversely with ex vivo frequencies of granzyme B and TIGIT (Figure 2-5A). Furthermore, Mtb-induced expression of CD107a by $CD56^{dim}$ cells correlated inversely with ex vivo granzyme B expression, while Mtb-induced induction of IFN-γ correlated positively with NKp46 (Figure 2-5A). No significant correlations were found between ex vivo NK cell expression of CD57, KIRs, or granulysin and the NK cell responses to Mtb antigens in vitro (data not shown).

By contrast with $CD56^{dim}$ NK cells, significant correlations between ex vivo $CD56^{bright}$ phenotype and Mtb antigen-induced CD69, CD107a and IFN-γ expression were limited to TIGIT, with ex vivo expression of TIGIT correlating inversely with all three effector molecules by $CD56^{bright}$ cells following Mtb antigen stimulation (Figure 2-5B). Unlike $CD56^{dim}$ cells, there were no positive correlations between ex vivo receptor expression by $CD56^{bright}$ NK cells and $CD56^{bright}$ reactivity to Mtb antigen stimulation. Taken together, these data identify NK cell phenotypic markers that correlate with functional NK cell responses to Mtb antigens in vitro. In addition, these results highlight the differences in reactivity by $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets to Mtb antigen stimulation.

NK cell reactivity to Mtb antigens is partially dependent on IL-12 and IL-18

NK cells express a number of cytokine receptors that allow them to become activated in proinflammatory environments. It has been well described that NK cells become activated through signaling mediated by IL-12 and IL-18 produced by activated monocytes and dendritic cells (DCs) (158-163). To better define the mechanism of NK cell activation by Mtb antigens, we stimulated PBMCs from each participant group with Mtb antigens in the presence of neutralizing Abs to either IL-12, IL-18, a combination of IL-12 and IL-18 together, or an isotype control (Figure 2-6A). Neutralization of IL-12 alone did not significantly impact Mtb-induced expression of CD69 or CD107a (Figure 2-6B, C), although Mtb-induced expression of IFN-γ was significantly reduced by IL-12 neutralization (Figure 2-6D). Neutralization of IL-18 alone resulted in a significant reduction in the frequencies of $CD56^{dim}$ NK cells expressing CD69, CD107a, and IFN-γ following Mtb antigen stimulation (Figure 2-6 B-D). $CD56^{dim}$ NK cell responses to Mtb antigens were further diminished by simultaneous neutralization of IL-12 and IL-18 together (Figure 2-6B-D). Overall, these data suggest that $CD56^{dim}$ NK cell reactivity to Mtb antigens, regardless of Mtb exposure or infection status, is at least partially mediated by indirect mechanisms in which Mtb antigens induce cytokine production by other cells present within PBMCs, which in turn induce expression of CD69, CD107a and IFN-γ by NK cells.

2.5 Discussion

In this study we evaluated the phenotypic and functional profiles of NK cells from QFT+ and QFT− adults residing in a TB-endemic region in western Kenya, and compared those with NK cell profiles in Mtb-naïve, healthy adults from the U.S. We demonstrated that $CD56^{dim}$ NK cells from Kenyan adults have distinct phenotypic profiles and attenuated responses following stimulation with MHC class I-devoid target cells and Mtb antigens, compared with U.S. healthy adult controls. Furthermore, within Kenyan adults, we found evidence of significant downregulation of NKp46 and TIGIT expression on $CD56^{dim}$ cells from QFT+ individuals with LTBI, compared with QFT− individuals. Moreover, we demonstrated that NK cell responses to Mtb antigen stimulation are inversely associated with ex vivo expression of granzyme B and TIGIT. We further characterized NK cell reactivity to Mtb antigen stimulation and determined that NK cell reactivity to Mtb is dependent, at least in part, on IL-12 and IL-18.

NK cells in the blood of humans can be divided into three distinct populations based on expression of CD56 (164). While $CD56^{dim}$ NK cells are highly cytolytic and $CD56^{bright}$ cells secrete large amounts of cytokines, $CD56^{neg}$ NK cells have diminished cytolytic activity and cytokine production capacity (106). Previous studies have indicated that $CD56^{neg}$ NK cells are expanded in individuals with chronic viral infections such as HIV and hepatitis C virus (165-168). Despite similar frequencies of total NK cells within the lymphocyte population, we found that QFT^{+} and QFT^{-} Kenyan adults have increased proportions of $CD56^{neg}$ cells within the total NK cell population, compared with U.S. healthy adult controls. The increased proportion of $CD56^{neg}$ NK cells observed in adults from Kenya, a high pathogen burden setting that is endemic for malaria, helminths and

TB (128, 169, 170), could be an indication of NK cell activation resulting from an accumulation of pathogen exposures over time.

NK cell activity is regulated through expression of various activating and inhibitory receptors (141), the varied expression of which generates heterogenous populations of NK cells with high diversity (142, 143). The most compelling evidence for pathogen-induced differentiation of NK cells with unique phenotypic and functional properties has come from studies of CMV infection (153). The prevalence of HCMV infection in healthy Kenyan adults approaches 100% (154), thus it was not feasible to evaluate NK cell profiles in our Kenyan groups according to HCMV status. Given the near universal prevalence of HCMV infection in Kenyan adults, we selectively enrolled HCVM^{+} U.S. adults as controls, to minimize potential differences in NK cell profiles between groups that may be attributed to HCMV infection. Thus, although all study participants were HCMV^{+}, we still observed marked differences in NK cell phenotype between Kenyan and U.S. adults, particularly in the CD56^{dim} subset. CD57, a marker of differentiation on NK cells (171), was expressed at higher levels on CD56^{dim} cells from Kenyan adults, compared with U.S. adults, thus suggesting an increased level of NK cell differentiation in adults residing in a TB-endemic region in Kenya. Expression of CD57 and NKG2C has been proposed as a marker of adaptive NK cells (151). Unfortunately, we did not evaluate expression of NKG2C in our study participants, thus we are unable to determine if NKG2C^{+} cells are also expanded within this CD57^{+} subset of CD56^{dim} NK cells in Mtb-infected and exposed Kenyan adults.

We also observed marked downregulation of NKp30 and NKp46 expression by CD56^{dim} NK cells in QFT+ and QFT− Kenyan adults, compared with U.S. adults. Downregulation of both

NKp30 and NKp46 has been described in HCMV infection (122), yet here, among our HCMV$^+$ participant groups, we observed progressive downregulation of NKp46 from the highest expression levels in Mtb-naïve U.S. adults, to intermediate levels in QFT– Kenyan adults and finally the lowest NKp46 expression in QFT+ Kenyan adults. In addition, we observed significant downregulation of NKp46 expression on CD56^{bright} cells in QFT+ individuals with LTBI, compared with QFT– Kenyan adults, thus providing further evidence of Mtb infection-associated changes in NKp46 expression across NK cell subsets. A previous study also reported significant downregulation of NKp30 and NKp46 expression by NK cells from patients with active TB disease, compared with Mtb-uninfected healthy adults (138). NKp46 has been implicated as a receptor mediating NK cell lysis of Mtb-infected monocytes (172-174), thus downregulation of NKp46 could lead to impaired NK cell lysis of Mtb-infected cells. Although we did not evaluate NK cells from patients with active TB disease in the present study, future studies directly comparing NKp46 expression levels across a spectrum of Mtb exposure, infection and disease are warranted to further define the relationship between NKp46 expression and control of Mtb.

In addition to NKp30 and NKp46, significant differences in expression of NKG2A, granzyme B, and TIGIT were also observed by CD56^{dim} NK cells from Kenyan adults, compared with U.S. adults. Among Kenyan adults, TIGIT expression was downregulated on CD56^{dim} cells from QFT$^+$ individuals, compared with QFT– individuals. Although no previously published studies have comprehensively evaluated TIGIT on NK cells in the setting of human Mtb infection, increased TIGIT expression on NK cells has been reported in studies of HIV-infected individuals, compared with HIV-uninfected individuals (175-177), indicating that TIGIT expression on NK cells may be modulated in the setting of chronic infection. Moreover, the level of expression of TIGIT on

human NK cells has been associated with NK cell functional heterogeneity in healthy adults (178), and blockade of TIGIT has been reported to increase NK cell effector functions (178-180), thus providing rationale for exploring TIGIT as a potential immunotherapeutic target. Future studies will be required to more clearly define the role of TIGIT expression on NK cells in human Mtb infection.

Our analysis of NK cell functional capacity revealed dampened IFN-γ production to K562 tumor cells in QFT+ and QFT− Kenyan adults, compared with U.S. healthy adults. These results are consistent with the functional profile of adaptive NK cells, which have been reported to exhibit diminished IFN-γ production to tumor cells, compared with conventional NK cells (150, 181). By contrast with dampened responses to tumor cells, adaptive NK cells have been reported to have enhanced Ab-dependent responses, compared with conventional NK cells (146, 147). We measured Ab-dependent NK cell responses in our participant groups using Ab-coated p815 cells and did not find statistically significant differences in $CD56^{dim}$ NK cell responses, as measured by expression of CD69, CD107a and IFN-γ. Using a cytotoxicity assay, a previous study of adolescents in South Africa indicated enhanced NK cell killing of Ab-coated p815 cells in QFT+ adolescents, compared with QFT− adolescents (127). Although we observed a trend of higher frequencies of $CD69^{+}CD107a^{+}$ NK cells following stimulation with Ab-coated p815 cells in QFT+ adults, compared with QFT− Kenyan adults and Mtb-naïve controls, this difference did not reach statistical significance. We did not measure actual killing of Ab-coated target cells in our study and it is currently unclear whether or not QFT+ Kenyan adults have enhanced Ab-dependent cellular cytotoxicity (ADCC) responses, compared with QFT− Kenyan adults.

Although adoptive transfer studies in mouse cytomegalovirus (MCMV) infection have demonstrated the capacity of adaptive NK cells to mediate protective immunity against viral challenge (120), definitive evidence for Ag-specific adaptive NK cells in humans has been challenging. A previous study in South Africa reported that BCG revaccination of individuals with LTBI boosts BCG-reactive NK cell responses after revaccination (125). We initially hypothesized that QFT+ adults with LTBI would display enhanced NK cell reactivity to Mtb antigen stimulation, compared with QFT− and Mtb-naïve adults. Contrary to our initial hypothesis, we observed the lowest reactivity to Mtb antigen stimulation by NK cells from QFT+ Kenyan adults, a result consistent across multiple Mtb antigen preparations (cell wall, whole cell lysate, and cell membrane). These data suggest that NK cells from QFT+ individuals with LTBI may either be more tolerized to Mtb or have a higher threshold by which they become activated.

A previous study of healthy adults in the U.K. reported that heterogeneity in NK cell responses to BCG was associated with KIR haplotype (80), thus suggesting a potential contribution of host genetics to NK cell reactivity to mycobacteria. Our analysis of KIR expression was limited to CD158 (KIR2DL1/S1/S3/S5), CD158b (KIR2DL2/L3), and CD158e1 (KIR3DL1); furthermore, we did not perform KIR genotyping of the participants in our cohort, thus the potential contribution of KIR haplotype to NK cell responses to Mtb in our cohorts remains uncertain.

To better understand the relationship between NK cell phenotype ex vivo and functional reactivity to Mtb antigens, we performed a correlation matrix analysis and found that $CD56^{dim}$ NK cell activation to Mtb antigens, as measured by CD69 upregulation, is inversely correlated with ex vivo expression of granzyme B and TIGIT, and positively correlated with expression of

NKG2A, NKp30 and NKp46. These data suggest that elevated $CD56^{dim}$ expression of granzyme B and TIGIT ex vivo could be indicative of attenuated NK cell responses to mycobacteria. Interestingly, the only receptor that was found to correlate positively with Mtb antigen-induced NK cell IFN-γ production was NKp46, which is significantly downregulated on both $CD56^{dim}$ and $CD56^{bright}$ subsets in QFT+ individuals. Of note, only NKp46 expression by $CD56^{dim}$ cells, and not $CD56^{bright}$ cells, correlated positively with IFN-γ production following stimulation with Mtb antigen. Downregulation of NKp46 by NK cells in QFT+ individuals with LTBI could be a potential mechanism contributing to dampened reactivity of NK cells to Mtb antigen stimulation, although it remains to be determined what role NKp46 may play in mediating NK cell activation to Mtb.

The positive correlation between NK cell reactivity to Mtb antigen stimulation and ex vivo expression of NKG2A, NKp30 and NKp46 by $CD56^{dim}$ cells, and not $CD56^{bright}$ cells, could be reflective of the higher proportions of $CD56^{dim}$ NK cells, compared with $CD56^{bright}$ NK cells, thus facilitating more robust detection of NK cell responses to Mtb antigens due to the higher starting number of $CD56^{dim}$ cells than $CD56^{bright}$ cells in PBMCs. Given the distinct transcriptional programs of $CD56^{dim}$ versus $CD56^{bright}$ NK cells (155), it is also likely that there are other receptors on $CD56^{bright}$ cells that we did not evaluate in this study that may be associated with NK cell reactivity to Mtb. Future studies in which $CD56^{dim}$ and $CD56^{bright}$ NK cells are sorted from PBMCs prior to Mtb antigen stimulation, as well as receptor blockade studies, will help further elucidate the contribution of each NK cell subset and receptors that are directly involved in NK cell reactivity to Mtb.

The precise mechanisms by which NK cells recognize Mtb remains an open area of investigation. We evaluated NK cell responses by stimulating PBMCs with Mtb antigens in the

presence of IL-2, thus both direct and indirect mechanisms could potentially contribute to NK cell activation. Monocytes are also present in PBMCs and can recognize bacterial antigens through pathogen-associated molecular pattern molecules and produce several inflammatory mediators including IL-12 and IL-18, which are potent stimulators of NK cells (8, 159, 160). Our experiments with IL-12 and IL-18 neutralizing Abs indicated that the combined blockade of IL-12 and IL-18 signaling substantially inhibited NK cell IFN-γ production to Mtb antigens. NK cell degranulation, as measured by CD107a expression, and activation, as measured by CD69 upregulation, are also significantly reduced by combined IL-12 and IL-18 blockade, although not entirely abolished. These results suggest that at least part of the NK cell reactivity measured in these assays may be due to indirect mechanisms of NK cell activation whereby stimulation of PBMCs with Mtb antigens induces cytokine production by other cell populations, such as monocytes and DCs, which in turn activates NK cells. In addition, stimulation with Mtb antigens can induce cytokine production by γδ T cells and CD1-restricted T cells (182-186), which could also influence NK cell activation. In addition to cytokines, direct interaction with monocytes and DCs can regulate NK cell activity (78, 187-189). NK cells from QFT+, QFT−, and Mtb-naïve controls reacted in a similar manner to neutralization of IL-12 and IL-18, thus suggesting a common mechanism of NK cell activation by Mtb antigens, regardless of Mtb infection or exposure status. Due to limited cell availability, we were not able to conduct Mtb stimulation experiments on purified populations of NK cells to better define the capacity of Mtb antigens to directly activate NK cells. Further studies are warranted to stimulate purified NK cells with Mtb, or deplete distinct subsets from Mtb-stimulated PBMCs, such as monocytes, DCs or T cells, to inform the receptors and pathways by which Mtb activates NK cells.

There are several limitations to our study, including the use of flow cytometry to evaluate a discrete number of NK cell phenotypic markers. Future studies conducting transcriptional profiling by RNA sequencing of purified populations of NK cells from individuals across a spectrum of Mtb infection and disease states will be necessary to more comprehensively define NK cell signatures associated with human Mtb infection. Another limitation in this study was that we analyzed NK cells circulating in the peripheral blood, and it remains unknown whether distinct populations of NK cells with unique phenotypic and functional profiles are present in the lungs of Mtb-infected individuals. Additionally, we stimulated PBMCs with complex Mtb antigen preparations, rather than individual Mtb antigens, and it is unknown which Mtb antigens mediate direct or indirect activation of NK cells. It is also important to note that while Kenya is a TB-endemic country, it is also endemic for other infections, including malaria and helminths (190), which are not endemic in the U.S. While our Kenyan subjects did not have concurrent malaria or helminth infection at the time of blood sample collection for our study, it is possible that they had previous malaria and/or helminth infections, which may have contributed to shaping the NK cell repertoire. Thus, markedly different pathogen burdens and environmental exposures likely contribute to intrinsic differences in NK cell profiles between Kenyan and U.S. adults. Lastly, we enrolled Mtb-naïve healthy adult controls in the U.S. who are seropositive for HCMV to more closely match the HCMV prevalence in the adult population in Kenya. However, we do not know the duration of HCMV infection in the U.S. and Kenya participant groups; it is possible that HCMV was acquired at a younger age in the Kenyan participants, compared with U.S. controls, which could potentially contribute to shaping the phenotypic and functional profiles of circulating NK cells due to longer duration of infection. Given the low prevalence of LTBI among U.S. born

individuals (191), we were not able to compare NK cell profiles between QFT+ and QFT− U.S. born adults, as we did among Kenyan adults. Future studies comparing NK cell profiles between QFT+ and QFT− individuals in a low pathogen burden, non-TB endemic environment, as well as longitudinal studies of the same individual before and after Mtb infection, will be important to better define the direct contribution of Mtb infection to NK cell phenotypic and functional profiles.

In conclusion, we performed a comprehensive analysis of phenotypic and functional profiles of NK cells from QFT+ and QFT− adults in a TB-endemic region in Kenya and found distinct phenotypes of $CD56^{dim}$ NK cells in these individuals, compared with Mtb-naïve healthy adult controls. Furthermore, the ex vivo expression of specific markers by $CD56^{dim}$ NK cells correlated with NK cell reactivity to Mtb antigen stimulation. There is growing appreciation that a subset of individuals who are highly exposed to infectious TB do not develop LTBI and remain persistently TST− and/or QFT− (192-195), thus suggesting the innate immune response may be capable of clearing the bacteria in some individuals (131). Our results identify specific differences in $CD56^{dim}$ NK cell phenotype and function in QFT+, QFT− and Mtb-naïve individuals and inform future studies aimed at defining NK cell correlates that may be protective against acquisition of Mtb infection and progression to TB disease.

2.6 Tables and Figures

Table 2-1 Characteristics of Study Participants

Participant Group	*n*	Age, y[a] (IQR)	Sex (% male)	QFT, IFN-γ IU/ml[b] (IQR)
Kenya QFT+	31	32 (23–53)	26	8.84 (3.22 – 10.00)
Kenya QFT−	30	24 (21–33)	37	0.00 (0.00 – 0.05)
U.S. Healthy Controls	9	43 (33–60)[c]	22	N/A

[a] value denotes median age in years
[b] value denotes median
[c] $p<0.05$, compared with Kenya QFT−
IQR, interquartile range

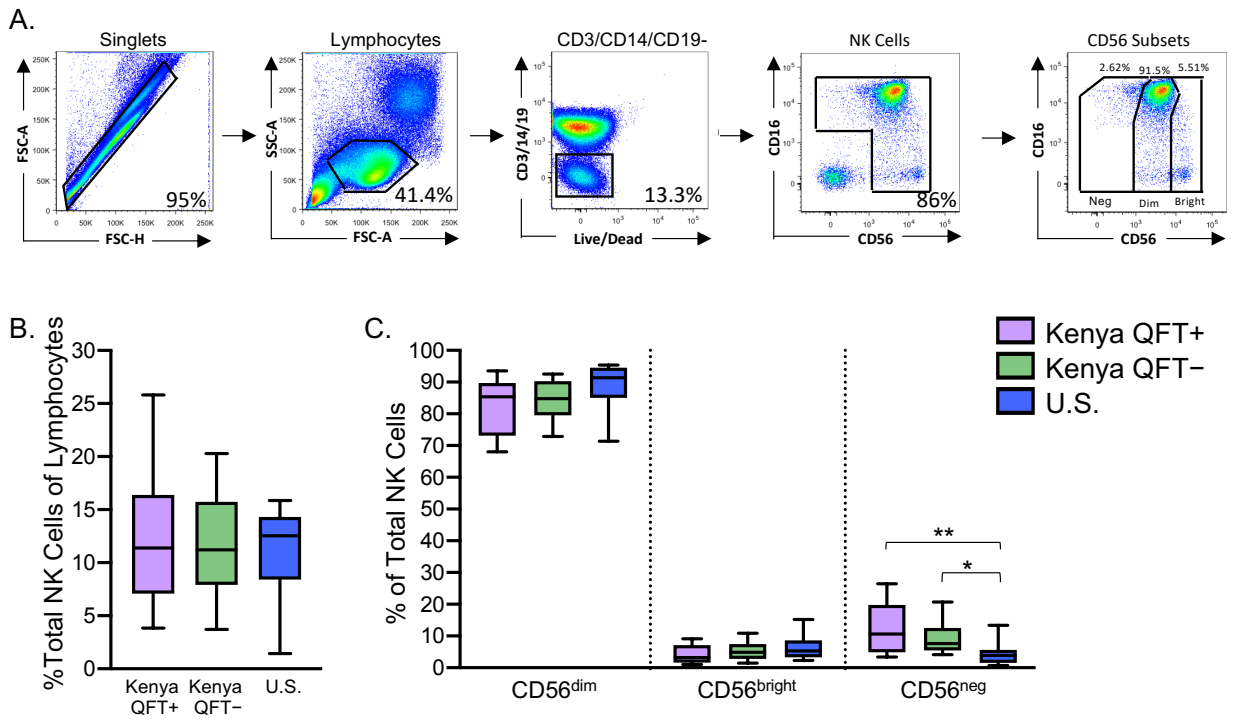


Figure 2-1. Kenyan adults exhibit higher proportions of CD56^{neg} NK cells, compared with U.S. adults.

Flow cytometry was used to identify NK cell subsets in PBMCs from Kenyan adults (n=31 QFT+; n=30 QFT−) and U.S. adult healthy controls (U.S., n=9). (**A**) Flow cytometry gating strategy for NK cells and CD56 subsets (CD56^{neg}, CD56^{dim}, CD56^{bright}). (**B**) Frequency of total NK cells as a percentage of lymphocytes. (**C**) Frequency of CD56 subsets as a proportion of total NK cells. Boxes in panels B and C represent the median and interquartile ranges; whiskers represent the 10th and 90th percentiles. Differences among groups were assessed using a Kruskal-Wallis test, with *p*-values adjusted for multiple comparisons using Dunn's post-test. *p<0.05; **p<0.01.

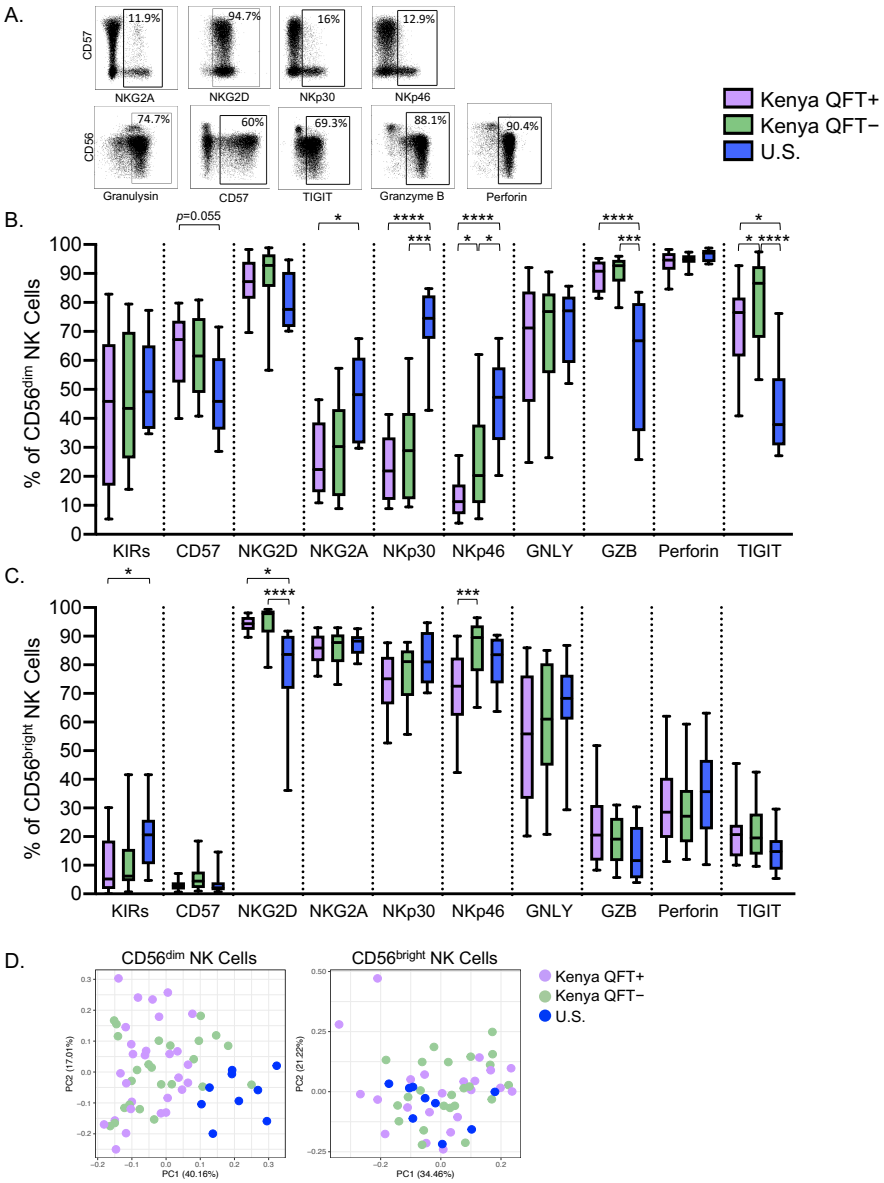


Figure 2-2. CD56^{dim} NK cells exhibit distinct phenotypes in Kenyan and U.S. adults.

Flow cytometry was used to evaluate the phenotype of NK cells in PBMCs from each of three participant groups. (**A**) Representative flow plots from a Kenyan QFT+ individual demonstrating expression of the indicated phenotypic markers; plots are shown gated on total NK cells. (**B**) Frequency of CD56^{dim} NK cells expressing each phenotypic marker. (**C**) Frequency of CD56^{bright} NK cells expressing each phenotypic marker. GNLY, granulysin; GZB, granzyme B. (**D**) Principal component analysis (PCA) of expression of the 10 phenotypic markers on CD56^{dim} NK cells (left panel) and CD56^{bright} NK cells (right panel) from each of the three participant groups. Boxes in panels B and C represent the median and interquartile ranges; whiskers represent the 10th and 90th percentiles. Differences among groups in panels B and C were assessed using a Kruskal-Wallis test, with *p*-values adjusted for multiple comparisons using Dunn's post-test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

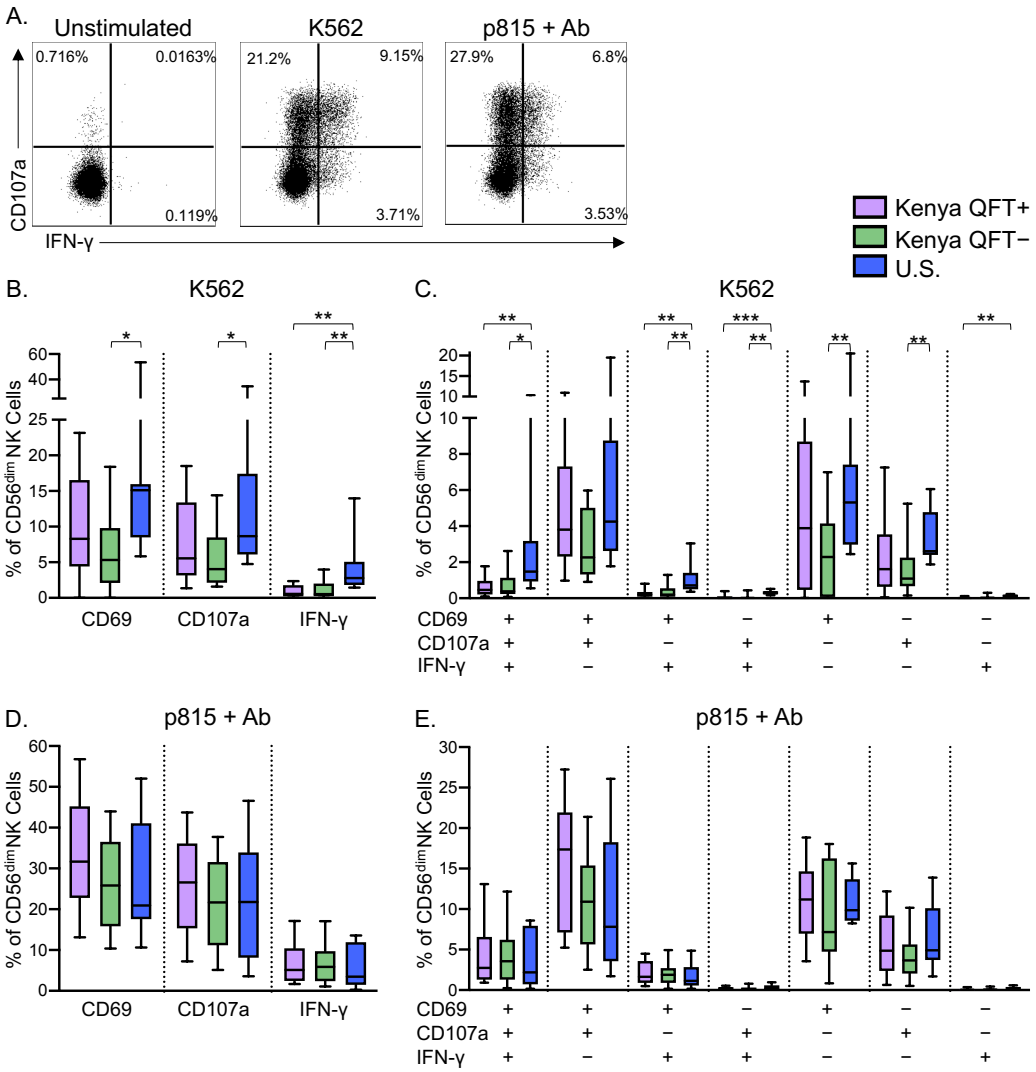


Figure 2-3. NK cells from Kenyan adults have dampened responses to MHC class I-devoid target cells, but similar ADCC responses, compared with U.S. adults.

PBMCs were stimulated for 5 hrs with either MHC class I-devoid K562 cells or with Ab-coated p815 cells. NK cells were evaluated by flow cytometry for expression of CD69, CD107a, and IFN-γ. (**A**) Representative flow plots from a U.S. healthy control of CD107a and IFN-γ production by $CD56^{dim}$ NK cells following stimulation with K562 cells or Ab-coated p815 cells. (**B, D**) Expression of CD69, CD107a and IFN-γ by $CD56^{dim}$ NK cells after stimulation with K562 cells (B) or Ab-coated p815 cells (D). (**C, E**) Boolean analysis of co-expression patterns of CD69, CD107a and IFN-γ by $CD56^{dim}$ NK cells after stimulation with K562 cells (C) or Ab-coated p815 cells (E). Data in panels (B – E) are shown from 53 Kenyan individuals (n=26 QFT+ and n=27 QFT−) and 9 U.S. healthy controls. Frequencies in panels B – E are shown after subtraction of background expression in the unstimulated control condition. Boxes in panels B – E represent the median and interquartile ranges; whiskers represent the 10^{th} and 90^{th} percentiles. Differences among groups were assessed using a Kruskal-Wallis test, with *p*-values adjusted for multiple comparisons using Dunn's post-test. **p*<0.05; ***p*<0.01; ****p*<0.001

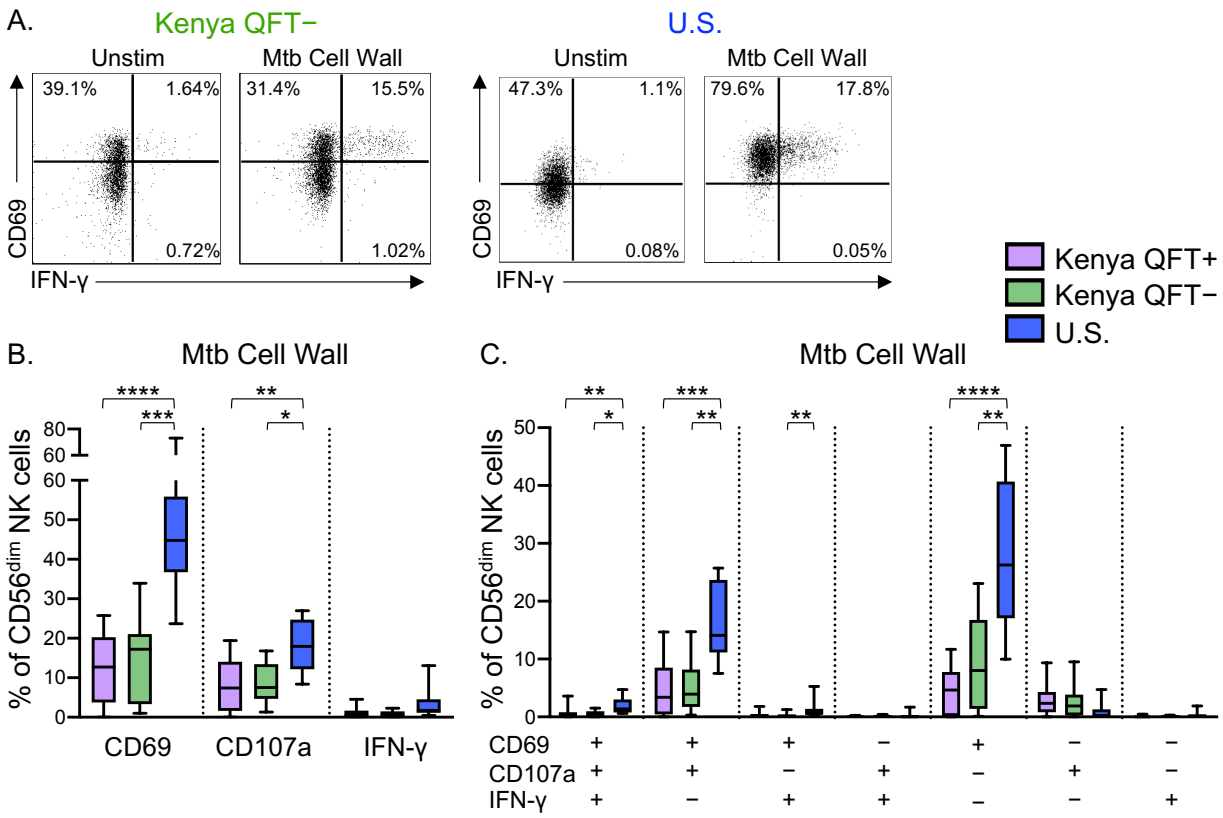


Figure 2-4. Differential reactivity of CD56^{dim} NK cells to Mtb antigen stimulation in Kenyan adults and U.S. healthy controls.

PBMCs were stimulated with Mtb cell wall antigen for 24 hrs in the presence of 100U/ml IL-2; PBMCs incubated with IL-2 alone, in the absence of Mtb antigen, served as a negative control (Unstim). (**A**) Representative flow plots of CD69 and IFN-γ expression by CD56^{dim} NK cells from a QFT− Kenyan donor and a U.S. healthy donor. (**B**) Expression of CD69, CD107a and IFN-γ measured on CD56^{dim} NK cells after stimulation with Mtb cell wall antigen. (**C**) Boolean analysis of co-expression patterns of CD69, CD107a and IFN-γ expression by CD56^{dim} NK cells after stimulation of PBMCs with Mtb cell wall. Data in panels B and C are shown from 51 Kenyan donors (n=26 QFT+, n=25 QFT−) and 9 U.S. healthy controls. Frequencies in panels B and C are shown after subtraction of background expression by CD56^{dim} NK cells in the presence of media with IL-2 alone. Boxes in panels B and C represent the median and interquartile ranges; whiskers represent the 10th and 90th percentiles. Differences among groups were assessed using a Kruskal-Wallis test, with *p*-values adjusted for multiple comparisons using Dunn's post-test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

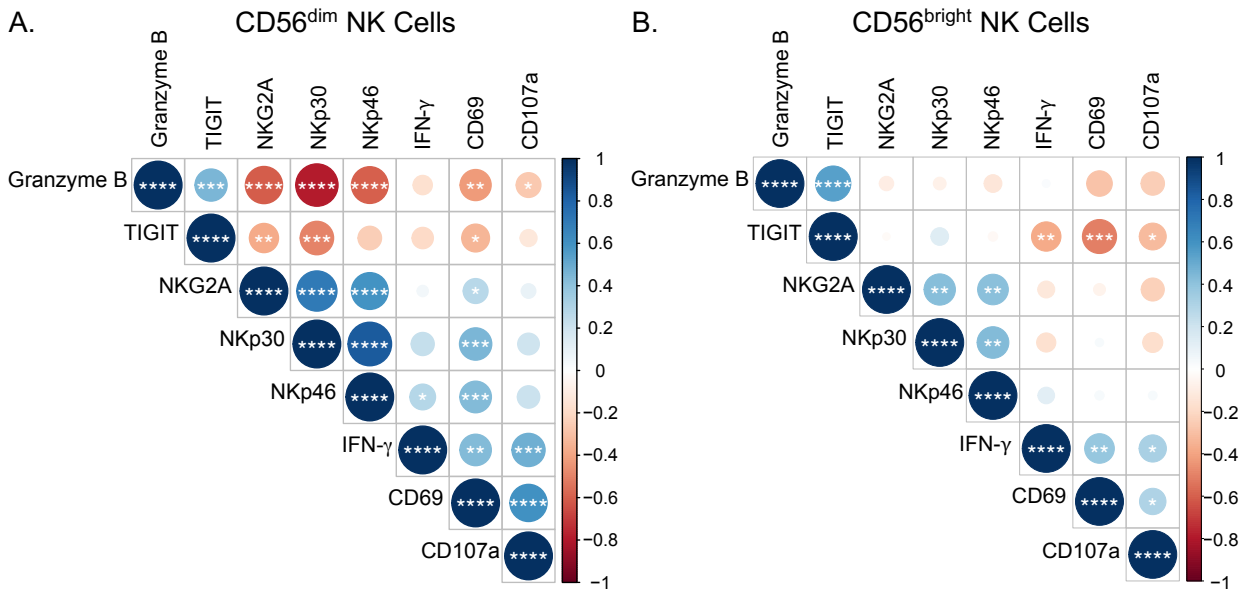


Figure 2-5. Ex vivo phenotype of CD56^{dim} NK cells correlates with reactivity to Mtb cell wall.

The phenotype of CD56^{dim} and CD56^{bright} NK cells in PBMCs ex vivo was determined as described in Figure 2-1. Expression of CD69, CD107a, and IFN-γ by CD56^{dim} and CD56^{bright} NK cells following stimulation with Mtb cell wall was determined as described in Figure 4. Correlations between CD56^{dim} (A) and CD56^{bright} (B) NK cell phenotype and Mtb cell wall-induced effector function were evaluated using a non-parametric Spearman rank correlation. Correlograms were generated using the corrplot function in R. Positive correlations are displayed in blue and negative correlations in red. Color intensity and the size of the circle are proportional to the correlation coefficients. Phenotypic markers and effector molecules within the CD56^{dim} plot were ordered using the Ward method of hierarchical clustering. The order was then applied to the CD56^{bright} data. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

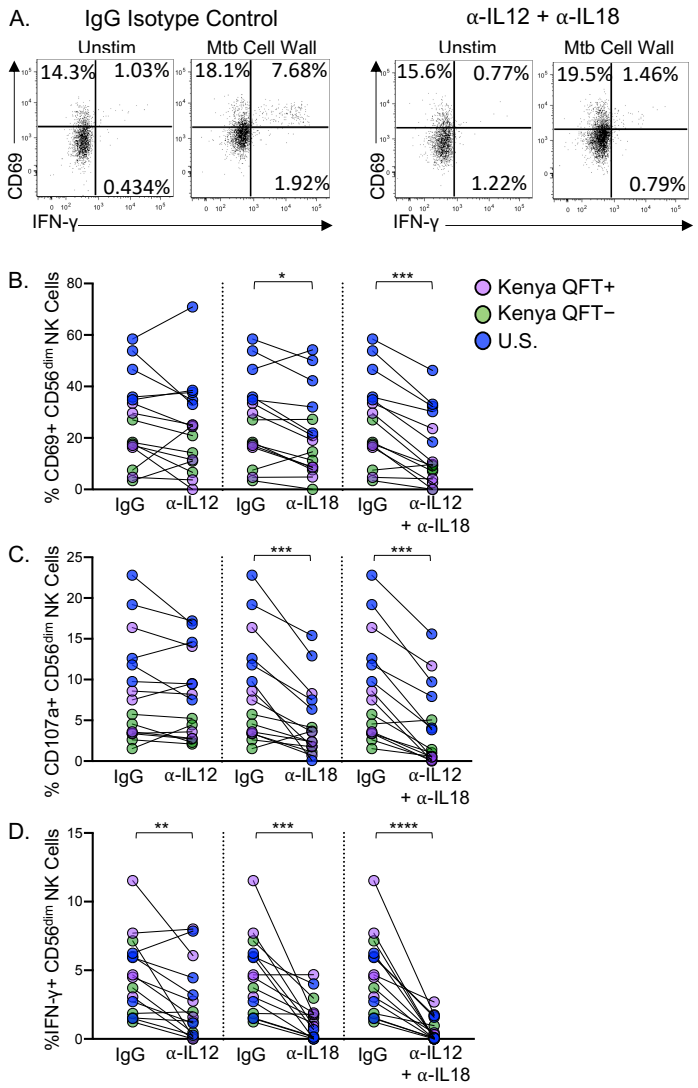
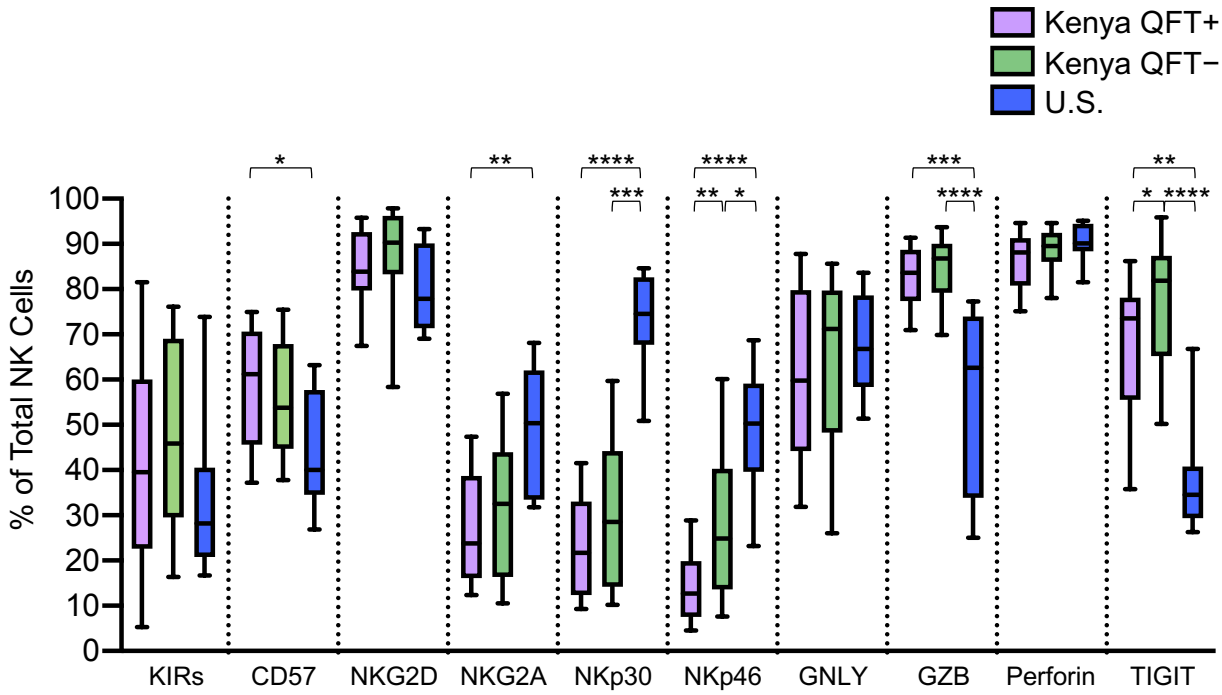


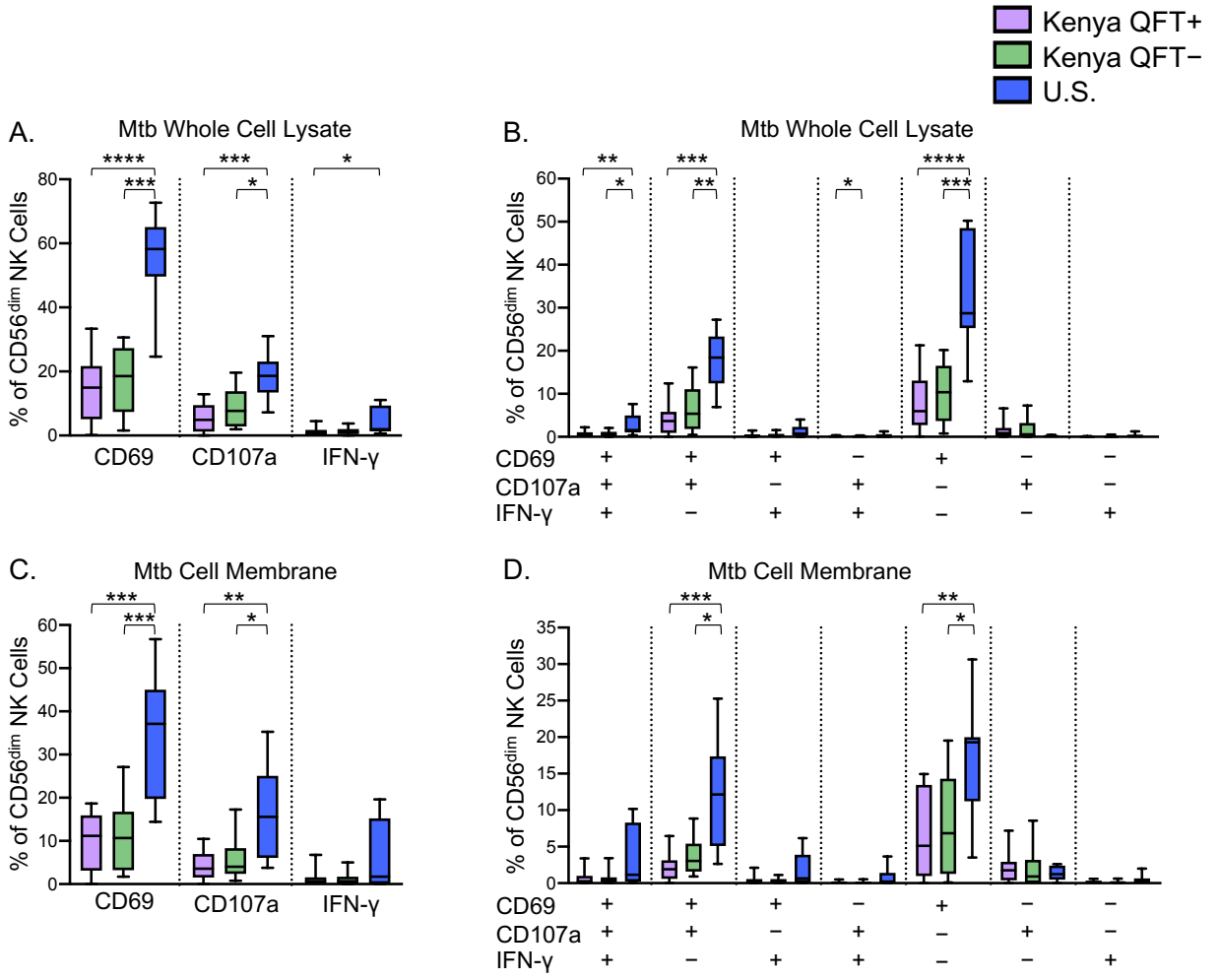
Figure 2- 6. NK cell reactivity to Mtb antigen stimulation is partially dependent on IL-12 and IL-18.

PBMCs were stimulated with Mtb cell wall, as described in Figure 2-4, in the presence of anti-human IL-12 Ab, anti-human IL-18 Ab, anti-IL-12 and anti-IL-18 Abs together, or an IgG1 isotype control. (**A**) Representative flow plots of PBMCs from a Kenya QFT^{+} individual stimulated with Mtb cell wall in the presence of either anti-IL-12 + anti-IL-18 Abs or an IgG1 isotype control. **(B – D)** CD69, CD107a, and IFN-γ expression, respectively, by CD56^{dim} NK cells in PBMCs stimulated with Mtb cell wall in the presence of anti-IL12, anti-IL-18, or anti-IL-12 + anti-IL-18 together. Data from 15 individuals are shown in panels B – D (n=5 Kenya QFT+, n=5 Kenya QFT–, n=5 U.S. healthy controls). Frequencies of Mtb cell wall-stimulated CD56^{dim} NK cells in panels B – D are shown after subtraction of background expression in the unstimulated negative control condition. Differences between the IgG1 isotype control and anti-IL-12/IL-18 neutralizing Abs were determined using a Wilcoxon matched-paired signed rank test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.



Suplemental Figure 2-1. Phenotypic profile of total NK cells from QFT+ and QFT- Kenyan adults is distinct from U.S. adult healthy controls.

Cryopreserved PBMCs were thawed and stained for surface and intracellular phenotypic markers. Total NK cells were gated according to gating strategy described in Figure 2-1A. Data are shown from 30 QFT– Kenyan adults, 31 LTBI Kenyan adults, and 9 U.S. healthy adult controls. Boxes represent the median and interquartile ranges; whiskers represent the 10th and 90th percentiles. Differences were assessed using the Kruskal-Wallis nonparametric one-way ANOVA, with p-values adjusted for multiple comparisons using Dunn's post-test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.



Suplemental Figure 2-2. QFT+ and QFT– Kenyan adults have attenuated NK cell responses to Mtb whole cell lysate and cell membrane antigens.

PBMCs were stimulated with Mtb whole cell lysate and cell embrane antigens for 24 hrs in the presence of 1000U/ml IL-2; PBMCs incubated with IL-2 alone, in the absence of antigen, served as a negative control. (A,C) Single marker expression of CD69, CD107 and IFN-γ measured on $CD56^{dim}$ NK cells after stimulation with Mtb whole cell lysate (A) and cell membrane (C). (B,D) Polyfunctional analysis of CD69, CD107a and IFN-γ co-expresion by $CD56^{dim}$ NK cells after stimulation of PBMCs with Mtb whole cell lysate (B) and cell membrane (D). Data in panels A-D are shown from 26 QFT+ Kenyan adults, 25 QFT– Kenyan adults, and 9 U.S. healthy adult donors. Frequencies are shown after subtraction of background expression of CD69, CD107a and IFN-γ expression in the negative control condition (PBMCs incubated in media with IL-2 alone). Boxes represent the median and interquartile ranges; whiskers represent the 10^{th} and 90^{th} percentiles. Differences were assessed using the Kruskal-Wallis non-parametric one-way ANOVA, with p-values adjusted for multiple comparisons using Dunn's post-test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Chapter 3. The effect of HIV co-infection on NK cell phenotypic and functional profiles in Kenyan adults with LTBI

3.1 Abstract

Mycobacterium tuberculosis (Mtb) is one of the most lethal pathogens in human history and is currently responsible for over 10 million new cases of active tuberculosis (TB) disease every year. Many infected individuals establish latent Mtb infection (LTBI) and will not develop active disease throughout their lifetime. LTBI is maintained, in part, by a competent immune response from CD4 T cells. Infection with HIV leads to depletion of CD4 T cells and is associated with increased risk of reactivation in individuals with LTBI. The importance of CD4 T cells in latent infection have been well described, but the effect of HIV on the phenotype and function of other immune cell populations responding to Mtb have not been fully described. NK cells are a subset of innate immune cells that are found in the granuloma and are capable of responding to Mtb. In this study we performed flow cytometry to evaluate the phenotypic and functional profiles of NK cells in a cohort of HIV-infected (HIV+) and HIV-uninfected (HIV–) Kenyan adults. QuantiFERON (QFT) was used to define LTBI status. We evaluated and compared the phenotype and function of NK cells between HIV+ QFT+ and HIV– QFT+ individuals. We found that HIV co-infection is associated with expansion of $CD56^{neg}$ NK cells, along with increased expression of NKG2D by $CD56^{dim}$ cells, and increased expression of the inhibitory receptor TIGIT and maturation marker CD57 by $CD56^{bright}$ cells, compared with HIV– QFT+ individuals. NK cells from HIV co-infected individuals also had diminished degranulation and cytokine production to antibody-coated target cells. However, NK cells from co-infected individuals maintained functional response to Mtb antigens at levels similar to that of HIV– QFT+ individuals. Following one year of antiretroviral therapy (ART) in HIV+ QFT+ individuals, the proportion of $CD56^{neg}$ NK cells decreased significantly, as did CD57 and TIGIT expression by $CD56^{bright}$ NK cells.

Taken together, these data indicate that although HIV infection modulates NK cell phenotype and function, the capacity of NK cells to respond to Mtb is not significantly impaired in HIV co-infected individuals.

3.2 Introduction

Mycobacterium tuberculosis (Mtb) infection represents one of the largest infectious disease burdens in the world and is the most lethal infectious disease in human history. In 2018 there were 10 million new cases and 1.5 million deaths due to TB worldwide with over 250,000 of these cases in HIV-infected individuals. It is also estimated that a quarter of the world is latently infected with Mtb (9). The exact immune correlates of protection to Mtb are not fully understood but it is known that HIV infection significantly increases the risk of reactivation in individuals with latent Mtb infection (LTBI). Indeed, HIV infection is the greatest risk factor for Mtb reactivation (9). A better understanding of how HIV compromises immune protection in LTBI individuals is crucial for understanding the immune response to Mtb and also aiding in the development of new therapeutic and vaccine targets.

HIV causes immune suppression of several subsets of cells from the adaptive and innate immune systems. CD4 T cells, in addition to being depleted during HIV infection, have been shown to be functionally altered. Previous studies have shown that HIV-specific memory CD4 T cells have decreased proliferative capacity and decreased IL-2 production (196, 197). In addition, HIV gp120 is capable of inhibiting activation of CD4 T cells by blocking the upregulation of CD40L (198). Another mechanism leading to suppression of CD4 T cells in HIV infection is the upregulation of inhibitory receptors such as Tim-3, PD1 and CTLA-4 (199). HIV infection has also been shown to be associated with depletion of Mtb-specific CD4 T cells, with some evidence that HIV may preferentially infect Mtb-specific CD4 T cells, compared with CD4 T cells specific for other pathogen such as CMV (200). Overall, HIV infections leads to a decreased capacity of CD4

T cells to produce effector molecules and to become activated, which has detrimental consequences in the setting of Mtb infection.

HIV has also been shown to dysregulate cells in the innate immune system. Dendritic cells (DCs) play a major role in recognition of HIV and priming the adaptive immune response (201). Previous studies suggest that infected DCs are inhibited by accumulation of viral particles leading to decreased production of effector molecules (202). Another important cytokine produced by activated DCs is IL-12. This cytokine is important for Th1 polarization and its production is suppressed by HIV Vpr and gp120 (203). Suppression of IL-12 delays the differentiation of Th1 CD4 T cell responses, allowing for viral escape and replication. Macrophages can be infected by HIV and are believed to be a reservoir for virus (204). Macrophages and monocytes from infected patients with undetectable viral loads have been shown to contain viral p24, a marker of active viral replication (205). Collectively, these perturbations to the immune system leads to increased susceptibility to opportunistic infections such as Mtb.

It is clear that many cell subsets important for the immune response in LTBI are perturbed by HIV. Individuals who are co-infected with HIV have increased risk of developing active TB compared to individuals with HIV alone (9). In general, the decreased activation and function of memory CD4 T cells and inhibition of macrophages by HIV diminishes the protection generated by the granuloma. Mtb also regulates the expression of CCR5 and CD4 receptor expression on CD4 T cells and HIV increases CD14 on macrophages (206). Thus, both infections potentiate one another in the host.

Although CD4 T cells and macrophages have been well studied in the context of co-infection, other cell populations, including NK cells, are important for Mtb immunity but the

effect of co-infection on these cells has not been well defined. NK cells are a group of innate immune cells that are regulated by a large number of activating and inhibitory receptors (100). The receptor expression of NK cells changes in the context of infection and disease (109). NK cells play a role in linking the adaptive and innate immune systems and in bacterial infections they are known to be primary producers of IFNγ leading to activation of macrophages that are better capable of killing intracellular bacteria (133, 207). NK cells are capable of recognizing Mtb through TLR2 and NKp44 (111). Upon activation, NK cells kill intracellular bacterium through a granulysin dependent mechanism (79). Furthermore, the capacity of NK cells to kill Mtb-infected macrophages has been demonstrated in vitro (76).

Previous studies have reported alterations in NK cells during HIV infection. CD56 populations in the peripheral blood of HIV+ individuals are known to be skewed during infection leading to an increase in $CD56^{neg}$ NK cells and a decrease in $CD56^{bright}$ cells (107). The inhibitory receptor Tim-3 is also upregulated on NK cells in HIV+ individuals, leading to loss of reactivity (208). Moreover, a population of NKG2A+ NK cells have been shown to lyse autologous HIV-infected CD4 T cells (110). From these examples it is apparent that NK cells are altered phenotypically and functionally in HIV. How NK cells may be further perturbed in HIV/Mtb co-infection have not been explored.

In a previous study we performed NK cell phenotypic and functional profiling in a cohort of Mtb-infected and uninfected adults from Kisumu, Kenya to determine if Mtb infection modified NK cell profiles. We found that LTBI was associated with a distinct phenotypic and functional profile compared to healthy adults. In the current study we evaluated the effect of HIV co-infection on NK cell phenotype and function by evaluating the following groups of adults from

Kisumu, Kenya: HIV+ QFT–, HIV+ QFT+, and HIV– QFT+. We performed flow cytometry experiments on PBMCs to evaluate the effect of HIV/Mtb co-infection and antiretroviral therapy (ART) on NK phenotypic markers and functional responses to generic NK cell stimuli as well as Mtb antigen stimulation. Overall, we show that HIV/Mtb co-infection is associated with alterations in CD56 subsets and expression of phenotypic markers, as well as functional responses to antibody (Ab)-coated target cells, although NK cell functional responses to Mtb antigen stimulation is maintained in HIV/Mtb co-infected individuals.

3.3 Materials and Methods

Study participants. Blood samples were collected from individuals ≥18 years of age enrolled at the Kenya Medical Research Institute Clinical Research Center in Kisumu, Kenya. Study participants included adults with a normal chest x-ray and no symptoms of TB disease and no previous history of diagnosis or treatment for active TB disease. Mtb infection status was evaluated by QuantiFERON®-TB Gold In-Tube (QFT; Qiagen). Individuals with a positive QFT result (TB Antigen-Nil ≥0.35 IU/ml) were defined as having LTBI. Serologic testing for HIV antibodies was done for all individuals using the Diagnostic Kit for HIV (1+2) Antibody V2 (KHB® Shanghai Kehua Bio-engineering Co., Ltd). HIV viral load and absolute CD4 T cell counts were measured in all HIV+ participants. HIV+ participants were antiretroviral therapy (ART)-naïve at the time of study enrollment and had ≥200 CD4 cells/µl. Blood samples were collected from a subset of HIV+ QFT+ participants 12 months after initiation of ART.

Ethics statement: This study was conducted in accordance with the principles expressed in the Declaration of Helsinki. All subjects provided written informed consent for participation in the study, which was approved by the Kenya Medical Research Institute Scientific and Ethics Review Unit and the Emory University Institutional Review Board.

PBMC isolation: Blood samples from all participants were collected in sodium heparin tubes for isolation of peripheral blood mononuclear cells (PBMCs). PBMCs were isolated via density gradient centrifugation, cryopreserved, and stored in LN_2 until use. Cryopreserved PBMCs were thawed in a 37°C water bath and resuspended in 10 ml RPMI 1640 (Corning) with

deoxyribonuclease I (DNase, 10 μg/ml, Sigma-Aldrich) and washed twice with RPMI 1640. Cells were then suspended in R10 (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum [FCS], 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine) and used in phenotypic and functional NK cell assays described below.

NK cell phenotyping: Thawed PBMCs were washed in PBS and stained with Zombie NIR™ Fixable Viability Dye (BioLegend) for 15 minutes at room temperature. Cells were washed with PBS and surface stained for 30 minutes in the dark at room temperature with anti-CD3 Alexa Fluor 700 (BioLegend; UCHT1), anti-CD14 Alexa Fluor 700 (BioLegend; HCD14), anti-CD19 Alexa Fluor 700 (BioLegend; HIB19), anti-CD56 Brilliant Violet (BV) 711 (BioLegend; HCD56), anti-CD16 BV 605 (BD; 3G8), anti-NKG2A PE (Beckman Coulter; IM329IU), anti-NKG2D BV 421 (BioLegend; 1D11), anti-NKp30 Alexa Fluor 647 (BioLegend; P30-15), anti-NKp46 PE-Cy7 (BioLegend; 9E2), and anti-CD57 FITC (BioLegend; HCD57). After incubation with conjugated antibodies, cells were washed with PBS and fixed with 2% paraformaldehyde (PFA).

A second phenotyping panel was designed to measure the NK cell expression of intracellular markers. PBMCs were stained with Zombie NIR™ Fixable Viability Dye and surface stained for 30 minutes at room temperature with anti-CD56 BV 711 (BioLegend; HCD56), anti-CD16 BV 605 (BD; 3G8), anti-CD3 Alexa Fluor 700 (BioLegend; UCHT1), anti-CD14 Alexa Fluor 700 (BioLegend; HCD14), anti-CD19 Alexa Fluor 700 (BioLegend; HIB19), and anti-TIGIT PE (BioLegend; A15153G). Cells were washed in PBS and suspended in FoxP3 Fixation Buffer (eBioscience) for 30 minutes on ice. After fixation, cells were washed with FoxP3 Permeabilization Buffer (eBioscience) and stained with anti-granzyme B PE-CF594 (BD; GB11), anti-perforin PE-Cy7 (BD;

BD48), and anti-granulysin Alexa Fluor 488 (BD; RB1) for 30 minutes in the dark at room temperature. Finally, cells were washed with FoxP3 Staining Buffer and resuspended in PBS.

Stimulation and staining of NK cells: NK cell responses to target cells were evaluated using K562 and p815 tumor cell lines (ATCC). Just prior to use in NK cell stimulation experiments, p815 cells were incubated with rabbit anti-mouse polyclonal lymphocyte serum (Cedarlane) for 30 minutes to coat the cells with Ab. Ab-coated p815 cells were then washed with R10 before being added to PBMCs. Donor PBMCs were incubated with K562 or Ab-coated p815 cells at an effector to target ratio of 10:1. PBMCs incubated in R10 media alone served as a negative control. CD107a PE-Cy7 (BioLegend; H4A3), brefeldin A (5 μg/ml; Sigma-Aldrich), and monensin (5 μg/ml; BioLegend) were added to each sample at the beginning of stimulation. Cells were incubated at 37°C degrees for 5 hours.

NK cell responses to Mtb antigens were evaluated by stimulation of PBMCs with 10 μg/ml of Mtb H37RV derived cell membrane (obtained from BEI Resources, NIAID, NIH; catalog number NR14831). PBMCs were incubated in R10 with anti-CD107a PE-Cy7 (BioLegend; H4A3), recombinant human IL-2 (100U/ml; NIH AIDS Research and Reference Reagent Program, Catalog #136), and Mtb antigen for 24 hours at 37°C and 5% CO_2. Brefeldin A (5 μg/ml; Sigma-Aldrich) and monensin (5 μg/ml; BioLegend) were added at the final 5 hours of incubation. PBMCs incubated in R10 media with anti-CD107a and IL-2 alone served as a negative control.

Following stimulation of PBMCs with either target cell lines or Mtb antigen, cells were washed with PBS and stained with Zombie NIR™ Fixable Viability Dye (BioLegend) for 15 minutes. Cells were washed with PBS and surface stained with anti-CD56 BV 711 (BioLegend; HCD56), anti-

CD16 BV 605 (BD; 3G8), anti-CD3 Alexa Fluor 700 (BioLegend; UCHT1), anti-CD14 Alexa Fluor 700 (BioLegend; HCD14), anti-CD19 Alexa Fluor 700 (BioLegend; HIB19), anti-CD159c (NKG2C) VioBright FITC (Miltenyi Biotec; REA205) for 30 minutes at room temperature in the dark. Stained cells were washed with PBS and fixed with FoxP3 Fixation Buffer (eBioscience) for 30 minutes on ice. Cells were washed with FoxP3 Permeabilization Buffer (eBioscience) and stained intracellularly with anti-CD69 PerCP-Cy5.5 (BioLegend; FN50) and anti-IFNγ BV 480 (BD; B27) for 30 minutes at room temperature in the dark. Cells were washed in PBS and prior to acquisition on a BD LSRII flow cytometer.

Flow cytometry and data analysis: Cells were acquired on a BD LSRII flow cytometer with BD FACSDiva software (v8.0) and analyzed with FlowJo software (v9.6; BD). Compensation was performed using single-stained anti-mouse Ig,κ beads (BD Bioscience). Single cells were identified by plotting forward scatter height and forward scatter area. Lymphocytes were identified by plotting forward scatter height and side scatter height. Viable lymphocytes were identified by low expression of NIR viability dye. NK cells were identified as live lymphocytes negative for CD3, CD14, CD19 and positive for the NK lineage markers CD56 and/or CD16. NK cells were further stratified based on intensity of the expression of CD56 ($CD56^{dim}$, $CD56^{bright}$ and $CD56^{neg}$ NK cells).

Data analysis and statistics: A minimum of 1,000 NK cells was acquired in each panel for each individual. Expression of functional markers by NK cells (CD69, IFNγ, and CD107a) were analyzed after subtraction of background expression in the negative control condition. A non-parametric

Mann-Whitney test was used to compare differences between two groups. HIV+ QFT+ individuals were compared with HIV+ QFT– individuals to determine the effect of Mtb infection on NK cell responses within HIV+ individuals. HIV+ QFT+ individuals were compared with HIV– QFT+ individuals to determine the effect of HIV infection on NK cell responses within individuals with LTBI. For comparison between time points in HIV+ individuals on ART, a non-parametric Wilcoxon matched pairs test was performed. Differences between 3 groups were evaluated using a non-parametric Kruskal-Wallis test, with p-values adjusted for multiple comparisons using Dunn's post-test. P-values of less than 0.05 were considered significant.

3.4 Results

Study Participants

Blood samples were collected from 95 participants in Kisumu, Kenya (Table 3-1). Participants were stratified by QFT and HIV status: HIV+ QFT– (considered to be HIV-infected and Mtb-uninfected, n=33); HIV+ QFT+ (considered to be HIV-infected with LTBI, n=31), and HIV– QFT+ (considered to be HIV-uninfected with LTBI, n=31). All three groups were similar with regard to sex and age distribution.

Influence of HIV infection on IFNγ production in the QFT assay

We next evaluated HIV viral load and absolute CD4 T cell count in QFT– and QFT+ individuals. There was a trend towards lower viral loads and higher CD4 counts in HIV+ QFT+ individuals, compared with HIV+ QFT– individuals (Figure 3-1A, 1B). We also compared IFNγ responses to the TB Antigen and Mitogen conditions of the QFT assay in all three of our cohorts to determine if HIV infection was associated with a dampened QFT response compared to individuals who are HIV-uninfected. As expected, QFT+ individuals had significantly higher IFNγ responses to the TB Antigen condition when compared to the HIV+ QFT– cohort. Among individuals with a positive QFT response, HIV+ individuals had lower levels of IFNγ than HIV– individuals (Figure 3-1C). Next, we compared IFNγ responses to the mitogen condition (PHA) of the QFT assay. We found that all three cohorts had robust responses to mitogen stimulation (Figure 3-1D). Taken together, these results suggest that HIV infection may be associated with a dampened TB-specific IFNγ production in the QFT assay.

HIV co-infection is associated with increased CD56^{neg} and decreased CD56^{dim} NK cells

Chronic viral infections are associated with changes to the distribution of CD56 subpopulations of NK cells (209). We next evaluated whether the distribution of CD56 subsets was altered in individuals with HIV/Mtb co-infection. We performed flow cytometry to measure the frequency of CD56 subsets (CD56^{dim}, CD56^{bright}, and CD56^{neg}) from the PBMCs collected from all three of our cohorts (Figure 3-2A). CD56^{dim} cells were the major subset in all cohorts, as has been previously described (164). When we compared the frequency of CD56 subpopulations in all three groups, we found that CD56^{dim} cell frequency was decreased and CD56^{neg} cells were increased in HIV+ QFT+ individuals, compared with the HIV– QFT+ individuals (Figure 3-2B). We found no differences in CD56 subset distribution between the two HIV+ groups, suggesting that LTBI does not further perturb the distribution of NK cell subsets within HIV+ individuals. Overall, these data suggest that HIV infection, but not Mtb infection, has an appreciable effect on CD56 subset distribution.

Phenotypic profiles of CD56^{dim} and CD56^{bright} NK cells in HIV/Mtb co-infection

NK cell activating and inhibitory receptor expression is altered in the setting of inflammation and infection and has been associated with different disease outcomes (109). In order to determine the effect of Mtb and HIV infection on NK cell phenotypic profiles, we performed flow cytometry to evaluate expression of an array of commonly expressed NK cell markers (Figure 3-3A). Since CD56 subsets are transcriptionally distinct and have unique expression patterns (155), we performed analysis on CD56^{dim} and CD56^{bright} NK cells separately.

HIV+ and HIV– individuals with LTBI had similar expression of almost all markers evaluated on $CD56^{dim}$ cells, with the exception of NKG2D, which was significantly higher in HIV+ QFT+, compared with HIV– QFT+ individuals (Figure 3-3B). No significant differences in expression of any of the phenotypic markers were found between the two HIV+ groups (Figure 3-3B), indicating that the phenotypic profiles of $CD56^{dim}$ NK cells are not further altered by Mtb infection within HIV+ individuals.

Next, we compared the phenotypic profiles of $CD56^{bright}$ cells. Among individuals with LTBI, HIV+ individuals had increased expression of CD57 and TIGIT on $CD56^{bright}$ cells, compared with HIV– individuals. Among HIV+ individuals, the only significant difference we observed was an increase in NKp46 expression by $CD56^{bright}$ cells from QFT+ individuals, compared with QFT– individuals. Overall these data show that NKp46 expression may be modified in Mtb infection, and that HIV co-infection is further associated with increased expression of CD57 and TIGIT on $CD56^{bright}$ cells.

NK cell responses to Ab-coated target cells are dampened in HIV infection

NK cells, through a receptor dependent mechanism, recognize tumor cells that downregulate MHC class I receptors as an immune escape mechanism. NK cells are also activated through antibody-dependent cellular cytotoxicity that is dependent on CD16. We performed stimulations of PBMCs with either Ab-coated p815 cells or MHC class I-devoid K562 cells to measure NK cell functional responses. We used flow cytometry to measure CD69, CD107a and IFNγ expression from $CD56^{dim}$ NK cells from both stimulation conditions (Figure 3-4A). NK cells from HIV+ QFT+ individuals had decreased expression of CD69, CD107a, and IFNγ, compared with

HIV– QFT+ individuals (Figure 3-4B). Boolean analysis of these responses identified significant decreases in two distinct NK cell subsets: CD69+CD107a+IFNγ+ and CD69+CD107a–IFNγ+ cells (Figure 3-4C). No differences in NK cell responses to Ab-coated p815 cells were found between the two HIV+ groups. There were also no differences in NK cell functional responses by either Mtb or HIV infection status following stimulation with K562 cells (Figure 3-4D, E). Overall, these data suggest that $CD56^{dim}$ NK cell responses to Ab-coated target cells are dampened HIV co-infected individuals, and this decreased response is driven by HIV infection and not Mtb infection.

Degranulation and cytokine production to Mtb antigens is maintained in HIV-infected individuals

After evaluating the responses of $CD56^{dim}$ cells to generic NK cell stimuli, we next evaluated the effect of co-infection on NK cell responses to Mtb antigen. We performed a 24hr stimulation of PBMCs with Mtb cell membrane and used flow cytometry to measure expression of CD69, CD107a, and IFNγ responses by $CD56^{dim}$ cells (Figure 3-5A). Similar levels of Mtb antigen-induced expression of CD69, CD107a, and IFNγ were observed by $CD56^{dim}$ NK cells from all three groups (Figure 3-5B). Next, we evaluated co-expression patterns of these markers using a Boolean analysis strategy. When we compared individual subsets between QFT+ individuals, we found a significant decrease in the frequency of CD69+CD107–IFNγ– cells in HIV+ individuals, compared with the HIV– individuals (Figure 3-5C), suggesting the capacity to upregulate the activation marker CD69 in response to Mtb antigens may be dampened in HIV/Mtb co-infected individuals. Among HIV+ individuals, we did not find any differences in the frequencies of $CD56^{dim}$ NK cells responding to Mtb antigens by QFT status. Overall, the capacity of $CD56^{dim}$ NK cells to

degranulate (as measured by CD107a expression) and produce IFNγ in response to Mtb antigen stimulation is not significantly impaired in individuals with HIV/Mtb co-infection.

ART is associated with redistribution of NK cell subsets

HIV infection is associated with CD56 population alterations during the course of disease, which is modified following antiretroviral therapy (ART)-mediated suppression of viremia (210). To evaluate the effect of HIV viremia on the perturbation of NK cell subsets we observed in our HIV+ individuals (Figure 3-2B), we used flow cytometry to measure the distribution of NK cell subsets in a subset of 10 HIV+ QFT+ individuals who were followed longitudinally for 12 months after initiating ART. To confirm that each individual achieved suppression of viremia on ART, we measured viral load at study enrollment (pre-ART) and 12 months post-ART and confirmed that all individuals had undetectable viral loads (<20 copies/ml) by 12 months (Figure 3-6A). Absolute CD4 T cell counts were also measured at both time points and increased significantly after 12 months of ART (Figure 3-6B).

We next evaluated distribution of CD56 subsets within the total NK cell population before and 12 months after ART. There was a significant increase in both $CD56^{dim}$ and $CD56^{bright}$ cells at 12 months post-ART, compared with pre-ART levels (Figure 3-6C). This coincided with a significant decrease in $CD56^{neg}$ cells at 12 months post-ART, suggesting an association between viral load and expansion of the $CD56^{neg}$ NK cell subset.

ART is associated with decreased expression of CD57, TIGIT, and granulysin by $CD56^{bright}$ NK cells

In order to better understand the effect of viremia on NK cell phenotypic profiles in our HIV/Mtb-infected cohort, we performed flow cytometry on PBMCs from these 10 HIV+ QFT+ individuals at baseline and 12 months post-ART. We did not find any significant differences in expression of any of the phenotypic markers on $CD56^{dim}$ NK cells after 12 months of ART (Figure 3-7A). However, we found that ART-mediated suppression of viremia was associated with decreased expression of CD57, TIGIT, and granulysin by $CD56^{bright}$ NK cells (Figure 3-7B). These data suggest that the phenotype of $CD56^{bright}$ cells may be more sensitive to viremia than $CD56^{dim}$ NK cells, and that elevated expression of markers such as CD57, TIGIT, and granulysin by NK cells may be reflective of immune activation or inflammation that is characteristic of untreated HIV infection.

Finally, we were interested in evaluated the effect of HIV viremia on NK cell responses to Mtb antigens. We stimulated PBMCs from HIV+ QFT+ individuals before and after ART with Mtb cell membrane. Boolean analysis of CD69, CD107a and IFNγ revealed that NK cell responses to Mtb antigen stimulation did not change significantly after 12 months of ART (Figure 3-7C). These data suggest that NK cell responses to Mtb antigens in HIV-infected individuals are not directly related to HIV viral load.

3.4 Discussion

In this study we evaluated the phenotype and functional profiles of NK cells from HIV+ QFT–, HIV+ QFT+ and HIV– QFT+ individuals from a TB-endemic region in western Kenya. We have previously shown that HIV– QFT+ Kenyan adults have a unique phenotypic and functional profile compared to HIV– QFT– U.S. adults (211). The primary purpose of this study was to evaluate how co-infection with HIV modifies the phenotypic and functional profiles of NK cells among QFT+ Kenyan adults. We found that HIV/Mtb co-infection is associated with modification of NK cell phenotypic profiles, with increased NKG2D expression by $CD56^{dim}$ NK cells and increased CD57 and TIGIT expression by $CD56^{bright}$ NK cells, compared with HIV– individuals with LTBI. Functionally, HIV/Mtb co-infection is associated with a dampened response to Ab-coated target cells, compared with HIV– individuals with LTBI, although NK cell responses to Mtb antigens are largely preserved. Among HIV+ individuals, we found little evidence of differences in NK cell phenotype or function between QFT– and QFT+ individuals, with the exception of NKp46 expression by $CD56^{bright}$ cells. These data suggest that LTBI does not substantially modify NK cell profiles in HIV-infected individuals. Finally, by longitudinal analysis of HIV+ QFT+ individuals on ART, we show that the phenotype of $CD56^{bright}$ NK cells, but not functional responses to Mtb antigens, is associated with viremia.

NK cells are divided into 3 subsets based on the expression of CD56 (164). The major population of NK cells found in the blood are the $CD56^{dim}$ while the $CD56^{bright}$ cells are the minor subset found. HIV associated immune activation is associated with perturbations to CD56 subset frequency including a decrease in $CD56^{bright}$ and $CD56^{dim}$ cells and increased $CD56^{neg}$ population (168, 212). When we compared the frequency of CD56 subsets between HIV+ and HIV–

individuals, we found a significant decrease in $CD56^{dim}$ cells and a significant increase in $CD56^{neg}$ cells in HIV/Mtb co-infected individuals. There were no significant differences between the two HIV+ groups, suggesting the redistribution of CD56 subsets is driven by HIV infection and not Mtb infection. Our results in HIV/Mtb co-infected Kenyan adults are thus consistent with previous studies reporting expansion of $CD56^{neg}$ NK cells in HIV-infected adults from North American and European cohorts (206, 213-215).

NK cell responses are regulated by an array of inhibitory and activating receptor and it is the summation of signaling through these various receptors that dictates whether the NK cell becomes activated (100). NK cell receptors and NK cell diversity are known to be modulated in HIV infection (142). In the phenotypic analysis of $CD56^{dim}$ NK cells we found increased NKG2D expression in HIV/Mtb co-infected individuals compared to HIV– individuals. NKG2D is a highly conserved activating NK cell receptor that recognizes a large number of diverse ligands (216). Most of these ligands are upregulated on stressed cells, such as tumor cells or infected cells. In HIV infection, nef downregulates NKG2D ligands in an attempt to shield infected host cells from being detected and lysed by the immune system (217). Mechanisms leading to increased expression of NKG2D in HIV/Mtb co-infected individuals are currently unknown. Perhaps NKG2D is upregulated in response to decreased ligand sensing. Further studies would be needed to fully understand the regulation of NKG2D in HIV/Mtb co-infection.

In $CD56^{bright}$ cells we found higher expression of CD57 and TIGIT in HIV/Mtb co-infected individuals compared to HIV– individuals with LTBI. CD57 is a terminal differentiation marker and is increased as NK cells become more activated. In HIV infection NK cells are skewed towards higher proportion of CD57 expressing cells (218). It is interesting that we see an increase in CD57

expression on CD56^{bright} cells since this subset is described as being less differentiated compared to other CD56 subsets. These data suggest that co-infection is characterized by highly differentiated CD56^{bright} cells compared to HIV– individuals with LTBI.

TIGIT is an inhibitory receptor that is expressed on T cells and NK cells (180). Previous studies suggest that TIGIT expression is inversely correlated with NK cell function (178). In HIV infection there is an increase in TIGIT expression on NK cells, which is associated with decreased ability to produce IFNγ (177). The increase in TIGIT expression in HIV/Mtb co-infected individuals suggests that CD56^{bright} NK cells may be less functional compared to HIV– individuals with LTBI. Overall, these differences suggest that CD56^{bright} cells are more differentiated and may have decreased function in the context of co-infection. Additional studies evaluating the functional capacity of CD56^{bright} NK cells will be required to better understand the consequences of increased expression of TIGIT in the setting of HIV/Mtb co-infection.

In addition to NK cell phenotypic profiles, we were also interested in evaluating the influence of HIV on NK cell functional profiles. We used K562 MHC I-devoid and Ab-coated p815 cells to measure the general response of CD56^{dim} NK cells in all participants. We did not see any differences between our cohorts in response to K562 stimulation. The NK cell response to Ab-coated p815 cells was associated with decreased expression of all markers (CD69, CD107a and IFNγ). Ab-coated p815 cells are recognized by NK cells through a Fc-receptor (CD16) mediated mechanism. These data suggest that NK cells in co-infected individuals may have diminished capacity to mediate Ab-dependent cellular cytotoxicity (ADCC) responses.

Beyond non-specific stimuli, increasing evidence indicates that NK cells are capable of responding to pathogen antigens. A previous study in South Africa provided evidence that BCG

revaccination of individuals with LTBI boosts BCG-reactive NK cell responses for at least 1 year after revaccination (125). We found that HIV/Mtb co-infection was associated with a decrease in upregulation of the activation marker CD69 in response to Mtb cell membrane antigen, compared with HIV– individuals with LTBI. However, degranulation and cytokine production were not significantly impaired in HIV+ individuals. Our initial studies of NK cell response to Mtb antigen stimulation involved direct incubation of PBMCs with Mtb antigen preparations. Given that NK cell responses to Ab-coated target cells are dampened in HIV+ individuals, further studies are warranted to determine the role of Ab-mediated activation of NK cells in the immune response to Mtb.

HIV infection is associated with alterations in CD56 subset distribution and receptor expression (165). These perturbations are normalized following initiation of ART (210). Similar to previous reports, we found that there was an increase in the proportion of $CD56^{dim}$ cells after suppression of viremia on ART (219). $CD56^{neg}$ cells are significantly increased in HIV infection and we observed this population to decrease after 12 months of ART. These data also suggest that the perturbation in CD56 NK cell subsets we observed in HIV/Mtb co-infected individuals is associated with viral load and that these changes in NK cell subsets are reversible. Regarding NK cell phenotypic profiles, we observed a decrease in CD57, TIGIT, and granulysin expression by $CD56^{bright}$ NK cells following 12 months of ART. Both CD57 and TIGIT were increased on $CD56^{bright}$ NK cells in ART-naïve HIV+ QFT+ individuals, compared with HIV– QFT+ individuals, suggesting a relationship between expression of these markers and viral load.

There were a number of limitations in our study. We used flow cytometry to evaluate the diversity of NK cell receptor expression. This technology is limited in the number of receptors

that can be evaluated. It is possible that there are additional NK cell receptors that we did not evaluate here that better distinguish HIV and Mtb infection states in our cohorts. Furthermore, we performed studies on PBMCs and did not include any tissues for analysis. The phenotype and function of NK cells at the site of Mtb infection in the lung may not be fully appreciated by studying NK cells circulating in peripheral blood. Furthermore, we observed the most NK cell phenotypic alterations in $CD56^{bright}$ NK cells, which constitute a minor component of NK cells in blood but are more abundant in tissues. Mtb stimulations were conducted with whole bacteria antigen preparations instead of individual Mtb antigens. Future studies should use more specific antigens in order to identify specific receptors and pathways involved in recognition and activation of NK cells by Mtb.

In conclusion, we performed a robust analysis of phenotypic and functional profiles of NK cells from HIV+ QFT–, HIV+ QFT+, and HIV– QFT+ Kenyan adults. We found distinct changes in the phenotype of $CD56^{dim}$ and $CD56^{bright}$ cells in HIV+ QFT+ individuals, as well as dampened NK cell responses to Ab-coated target cells, compared with HIV– QFT+ individuals. Moreover, these phenotypic perturbations were reversed by ART, suggesting an association with viral load. Individuals with HIV/Mtb co-infection have a significantly increased risk of developing active TB disease, which has been attributed in part to loss of CD4 T cells. Our results identify HIV-associated phenotypic and functional changes in NK cell populations of HIV/Mtb co-infected individuals and warrant further studies to define specific NK cell populations that may contribute to successful immune control of Mtb infection.

3.5 Tables and Figures

Table 3-1. Characteristics of Study Participants

Participant Group	*n*	Age, y[a] (IQR)	Sex (% male)	QFT, IFN-γ IU/ml[b] (IQR)	CD4+ T Cell Count[b,e] (IQR)	HIV Viral Load (IQR)[b,f]
HIV+ QFT−	33	29 (25 - 41)	36	0.02 (0 - 0.13)[c]	522 (341 - 661)	53,212 (6,413 - 150,258)
HIV+ QFT+	31	33 (25 - 38)	39	4.44 (1.56 - 8.24)[d]	660 (417 - 812)	11,656 (1,718 - 62,532)
HIV− QFT+	31	32 (23 - 53)	26	8.84 (3.22 -10)	N/A	N/A

[a] value denotes median age in years
[b] value denotes median
[c] p<0.0001, compared with HIV+ QFT+
[d] p<0.01, compared with HIV− QFT+
[e] Units, Cells/μL
[f] Units, HIV-1 RNA/ml plasma
IQR, interquartile range

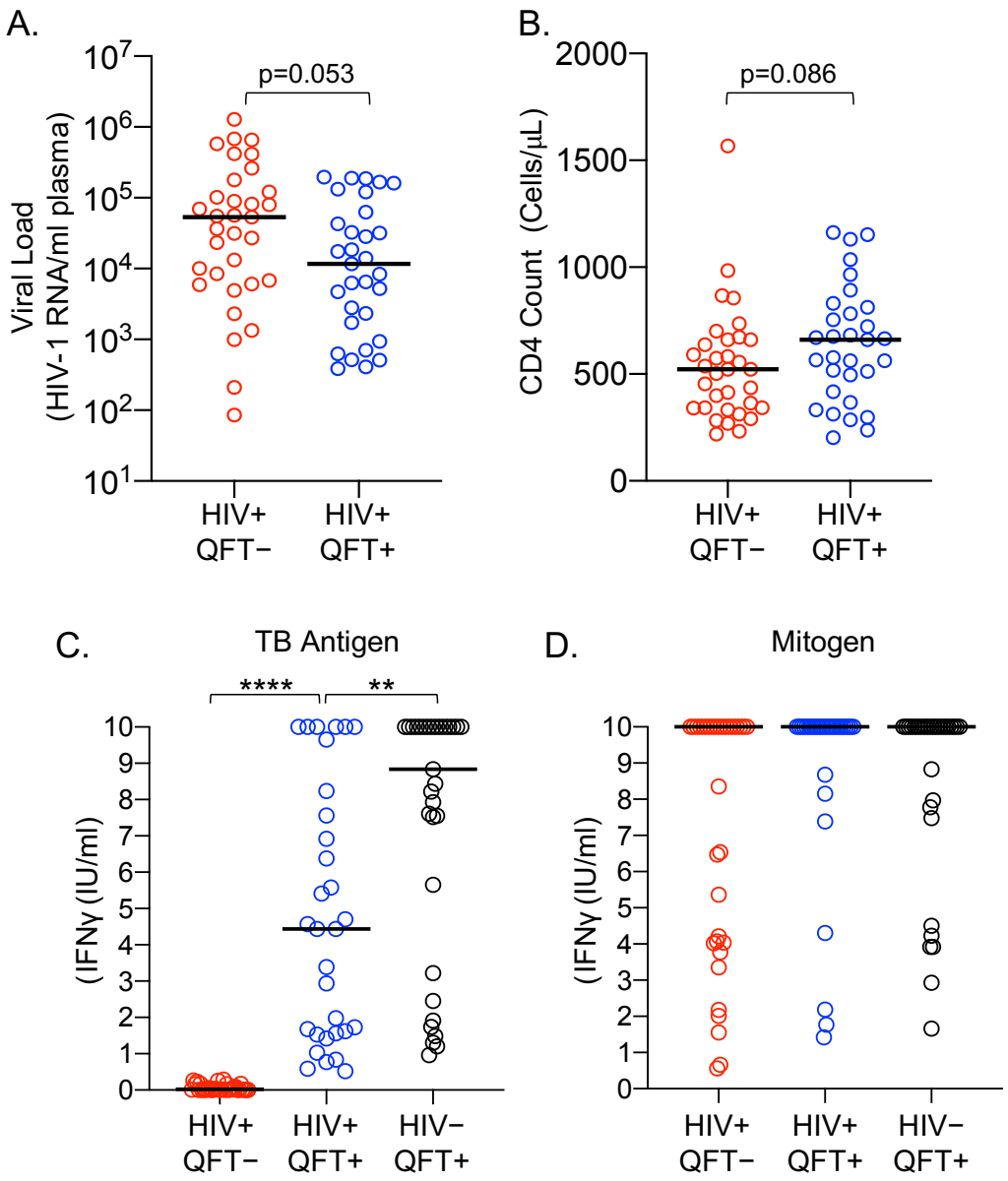


Figure 3-1. Parameters of HIV and Mtb infection.

CD4 counts and HIV viral loads were measured in individuals at the time of study enrollment (HIV+ QFT–, n=33; HIV+ QFT+, n=31). **(A)** Viral load of HIV infected individuals. **(B)** CD4 count of HIV infected individuals. TB Antigen **(C)** and Mitogen **(D)** responses from HIV+QFT–, HIV+ QFT+ and HIV– QFT+ individuals. TB Antigen and Mitogen responses in the QFT assay are shown after subtraction of background IFNγ the unstimulated (Nil) control tube. Differences between groups were assessed using Kruskal-Wallis test, with p-values adjusted for multiple comparisons using Dunn's post-test. ****p<0.0001; **p<0.01

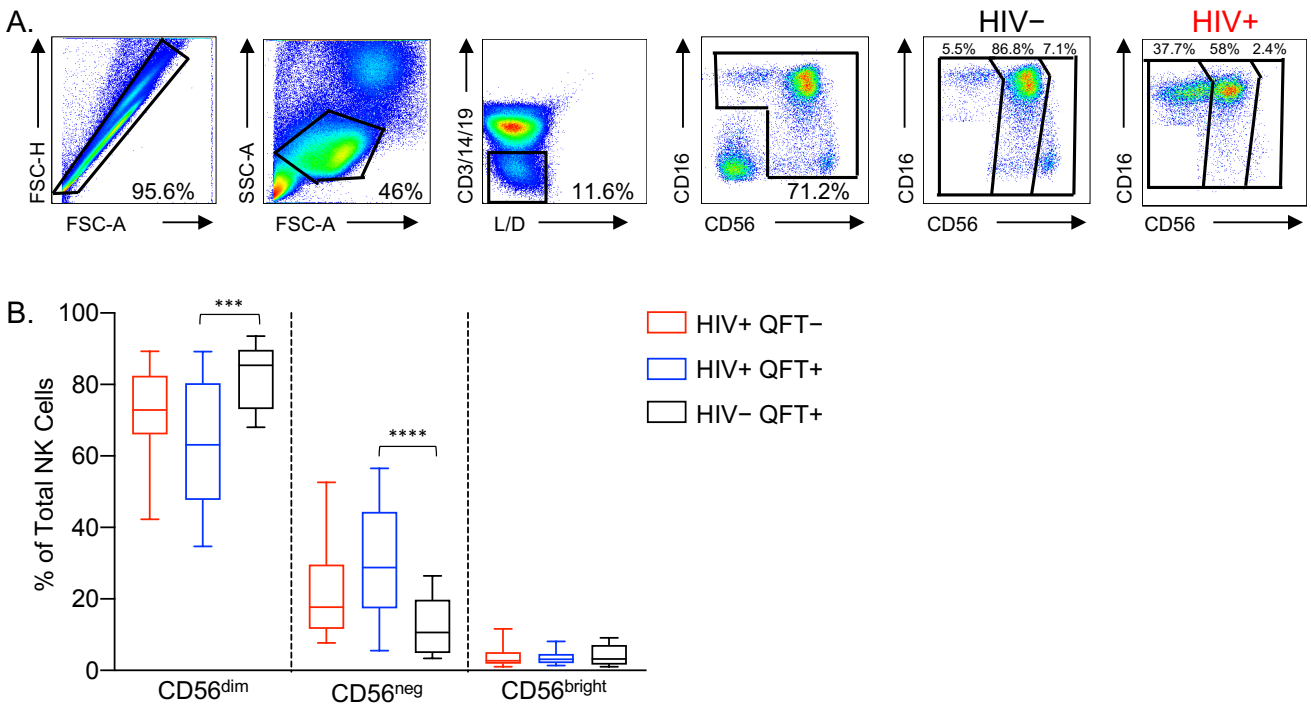


Figure 3-2. Distribution of NK cell subsets is modulated in HIV infection.

(A) Flow cytometry gating strategy for NK cell and CD56 subsets ($CD56^{dim}$, $CD56^{bright}$, and $CD56^{negative}$). **(B)** Frequency of CD56 subsets as a percentage of total NK cells. Differences between groups were assessed using Kruskal-Wallis test, with p-values adjusted for multiple comparisons using Dunn's post-test. ***p<0.001; ****p<0.0001

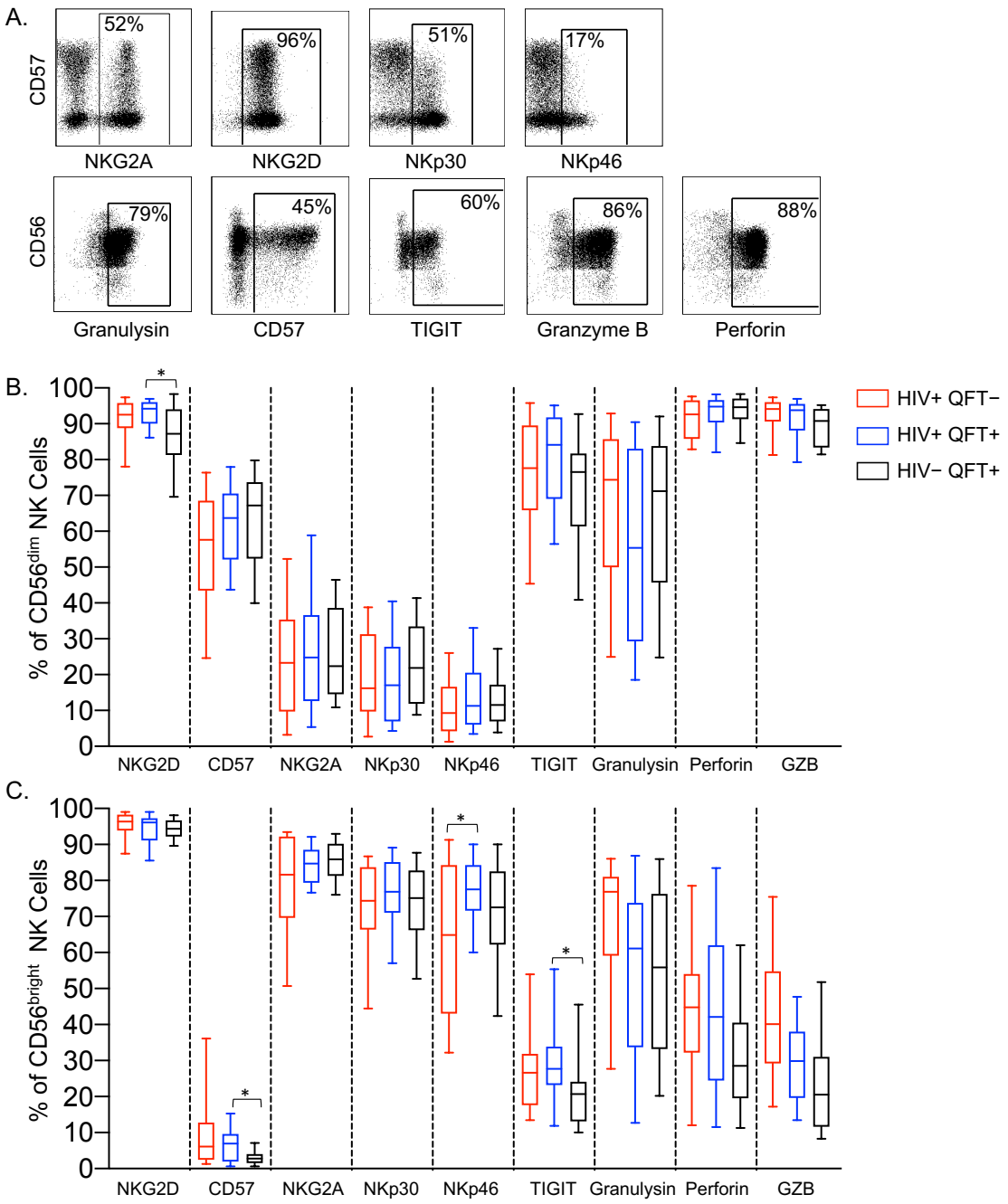


Figure 3-3. HIV infection in QFT+ individuals is associated with alterations in CD56^{dim} and CD56^{bright} marker expression.

Flow cytometry was performed on PBMCs from all cohorts to measure NK cell phenotypic markers. **(A)** Representative flow panels of NK cell marker expression from total NK cells of HIV+ QFT– individual. **(B)** CD56^{dim} NK cell expression of each phenotypic marker. **(C)** CD56^{bright} NK cell expression of each phenotypic marker. Differences between groups were assessed using Kruskal-Wallis test, with p-values adjusted for multiple comparisons using Dunn's post-test. *p< 0.05

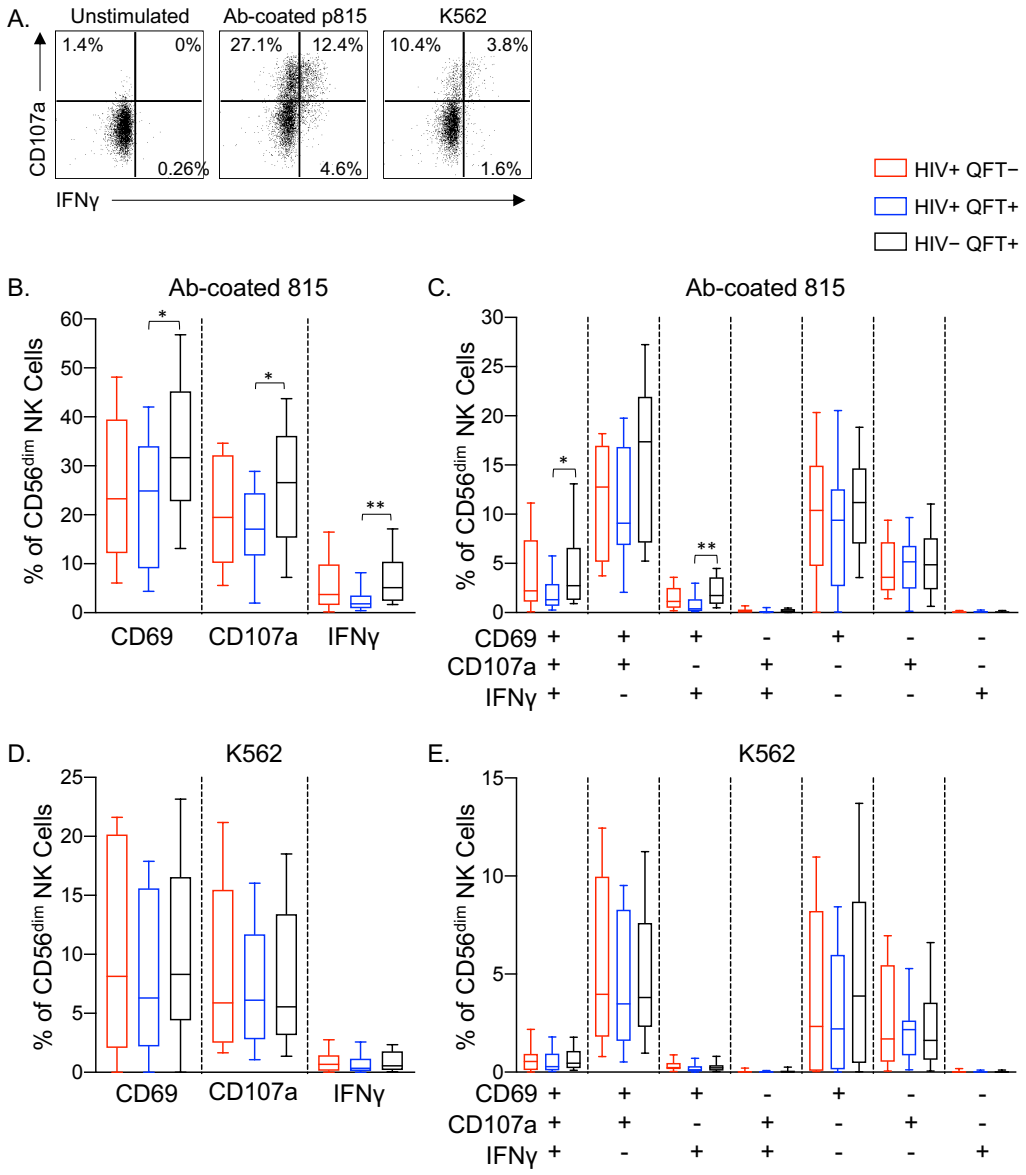


Figure 3-4. NK cell functional responses to Ab-coated target cells is decreased in individuals with HIV/Mtb co-infection.

PBMCs were incubated for 5hr with either Ab-coated p815 cells or MHC class-I devoid K562 cells. Flow cytometry was performed to measure NK cell expression of CD69, IFNγ and CD107a. Frequencies represent expression after background subtraction with unstimulated control. **(A)** Representative flow plots of CD107a and IFNγ expression by NK cells following stimulation with Ab-coated p815 cells and K562 cells in an HIV+ QFT– individual. **(B,D)** Single marker expression of CD69, CD107a and IFNγ expression of $CD56^{dim}$ NK cells stimulated with K562 cells (B) and Ab-coated p815 cells (D). **(C,E)** Boolean analysis of CD69, CD107a and IFNγ expression of $CD56^{dim}$ NK cells stimulated with Ab-coated p815 cells **(C)** or K562 cells **(E)**. Differences between groups were assessed using Kruskal-Wallis test, with p-values adjusted for multiple comparisons using Dunn's post-test.

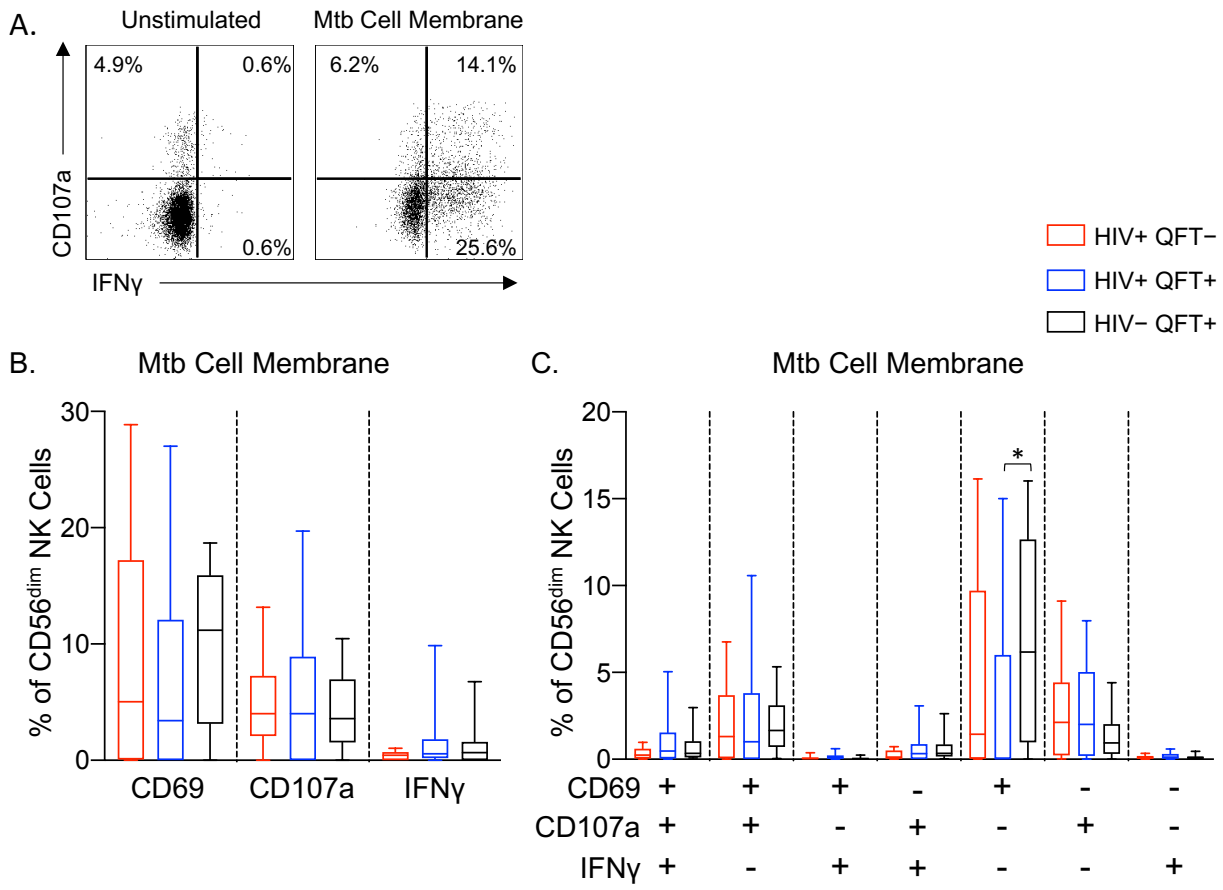


Figure 3-5. NK cells from HIV-infected individuals maintain functional responses to Mtb antigen stimulation.

PBMCs were incubated with Mtb cell membrane for 24 hrs in the presence of IL-2 and Monensin and BFA added at final 5 hours of stimulation. Flow cytometry was performed to measure the expression of CD69, IFNγ and CD107a. Frequencies represent expression after background subtraction with unstimulated control. **(A)** Representative flow plots indicating NK cell expression of CD107a and IFNγ after stimulation with Mtb cell membrane. **(B)** Single marker expression of CD69, CD107a and IFNγ from $CD56^{dim}$ NK cells after Mtb cell membrane stimulation. **(C)** Boolean analysis of CD69, CD107a and IFNγ expression by $CD56^{dim}$ NK cells following stimulation with Mtb cell membrane. Differences between groups were assessed using Kruskal-Wallis test, with p-values adjusted for multiple comparisons using Dunn's post-test. *p< 0.05

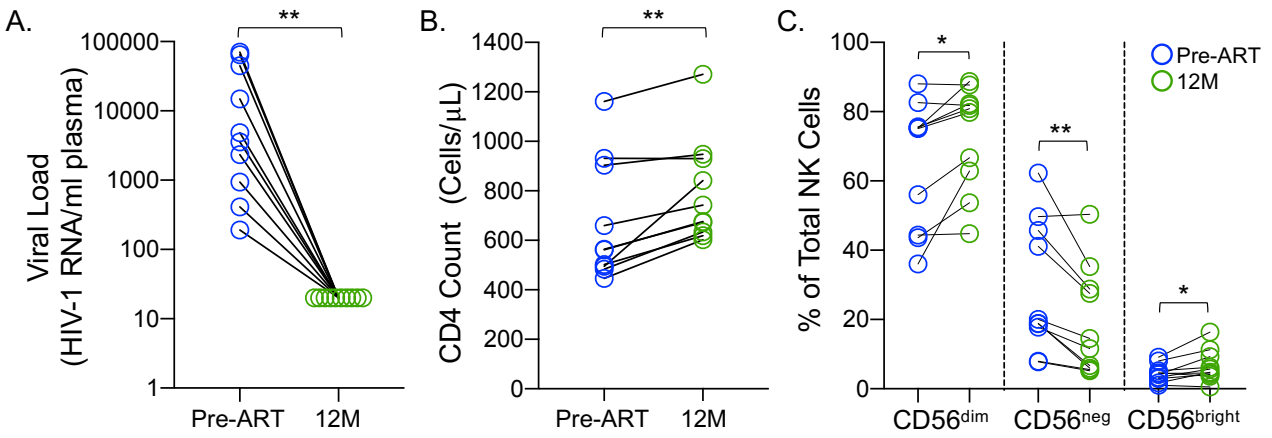


Figure 3-6. Redistribution of NK cell subsets following initiation of ART in HIV+ QFT+ individuals.

A subset of HIV+ QFT+ individuals (n=10) were followed longitudinally for 12 months after initiation of ART. Data represents matched pairs of individuals at baseline (pre-ART) and 12 months post-ART (12M). **(A)** Viral loads and (**B**) CD4 count pre- and post-ART. (**C**) Frequency of CD56 NK cell subsets as a percentage of total NK cells. Differences between time points were assessed using Wilcoxon matched-pairs test. *p< 0.05; **p<0.01

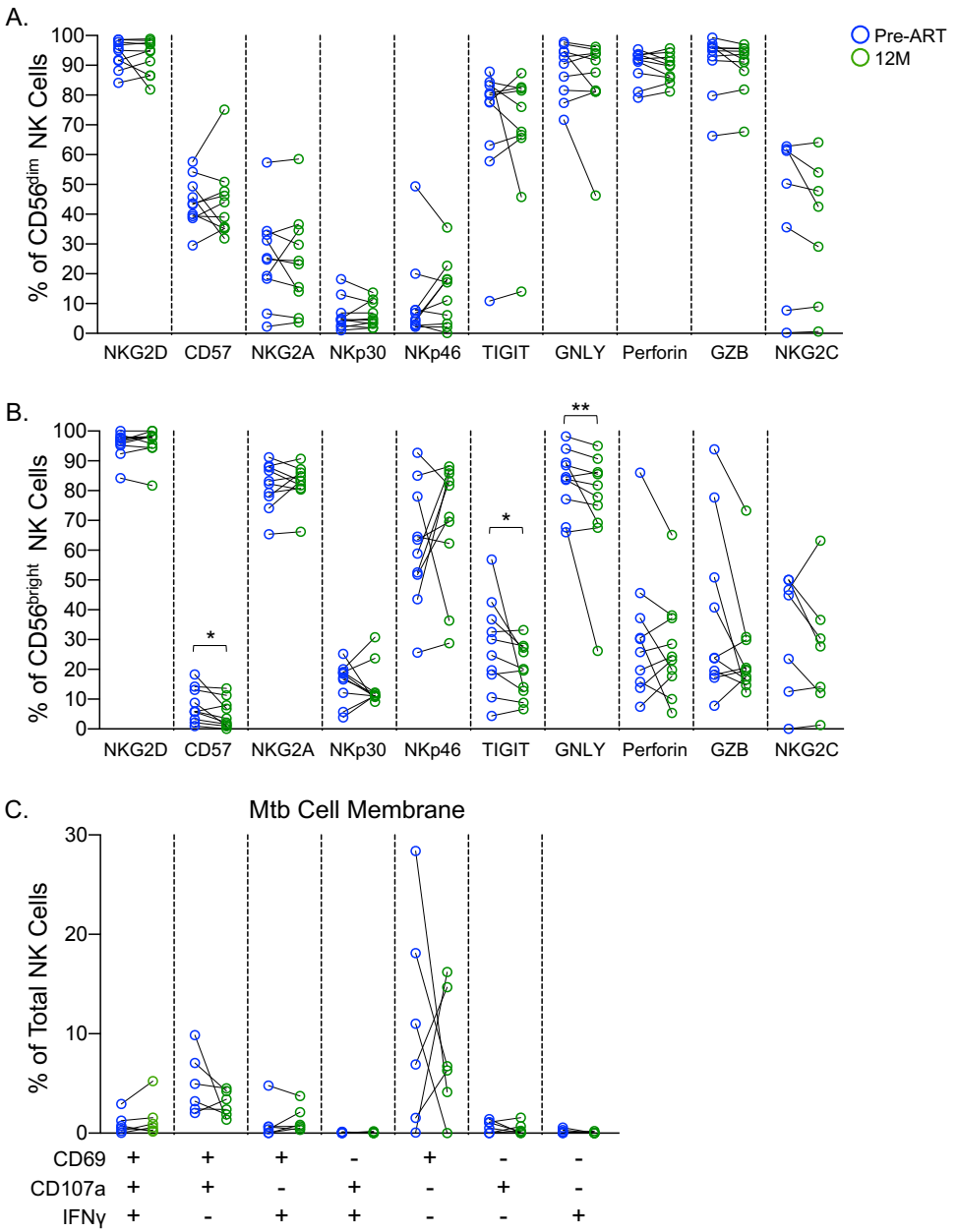


Figure 3-7. Effect of ART on NK cell phenotype and functional responses to Mtb antigen stimulation.

Flow cytometry was performed to measure the expression of phenotypic markers on $CD56^{dim}$ **(A)** and $CD56^{bright}$ **(B)** NK cells in PBMCs from HIV+ QFT+ individuals at the indicated time points (n=10). In a separate experiment, PBMCs from 6 HIV+ QFT+ individuals were incubated with Mtb cell membrane as described in Figure 5. Flow cytometry was performed to measure the expression of CD69, IFNγ and CD107a **(C)**. Frequencies in panel C represent expression of the indicated combinations of CD69, IFNγ and CD107a after subtraction of background levels in the negative control condition. Differences between time points were assessed using Wilcoxon matched-pairs test, *p< 0.05 and **p<0.01

Chapter 4. CMV infection in healthy US adults is associated with dampened NK cell degranulation capacity to *Mycobacterium tuberculosis*

4.1 Abstract

NK cells are a subset of lymphocytes that recognize and kill tumor cells and infected cells. Although long considered to be part of the innate immune response, recent work has suggested that NK cells are capable of forming adaptive-like subsets after infection or vaccination. Much of this work has been performed in the setting of infection with either murine or human cytomegalovirus, although less is known about how CMV-driven alterations in NK cell profiles impacts the functional capacity of NK cells to respond to other persistent infections such as *Mycobacterium tuberculosi*s (Mtb). CMV is associated with generation of adaptive NK cells that are transcriptionally distinct and have unique responses upon reinfection. CMV infection has also been implicated in dampening the response of NK cells toward multiple vaccines. In this study we were interested in determining if CMV infection modified NK cell responses to Mtb stimulation in vitro. We performed flow cytometry using PBMCs on a cohort of CMV seronegative (CMV–) and seropositive (CMV+) individuals residing in the U.S. to profile NK cell phenotype and functional responses to generic and Mtb stimuli. Our results show that CMV is associated with upregulation of CD57 and NKG2C and that CMV+ individuals had reduced NK cell degranulation capacity to Mtb antigens, compared with CMV– individuals. Given that CMV infection rates approach 100% in high TB burden countries, these results provide important insights into how CMV infection may modify host cellular immune responses to Mtb.

4.2 Introduction

Mycobacterium tuberculosis (Mtb) is currently responsible for 10 million new cases of active tuberculosis (TB) disease and 1.5 million deaths each year (9). Most infected individuals develop latent infection (LTBI) and a majority do not develop active disease for their entire lifetime. Many immune subsets of the innate and adaptive immune system are responsible for this response (220). While CD4 T cells have been well studied in the adaptive immune response to Mtb infection, there are multiple innate immune cells, including NK cells, that contribute to containment of Mtb. Being better informed about the role of these cells in Mtb infection will be crucial in defining immune correlates of protection against Mtb infection and TB disease.

NK cells are a group of highly diverse innate immune cells implicated in the immune response to an array of different pathogens including cancer, viruses and bacteria. NK cells recognize Mtb through TLR2 signaling and are capable of killing Mtb directly through a granulysin mediated process (111). Furthermore, NK cells lyse Mtb infected macrophages in vitro and these cells are also found in the granuloma (173). Although NK cells have been shown to interact with Mtb, their exact contribution to immunity against Mtb is not well understood.

Recent studies have provided evidence that NK cells differentiate into memory-like cells with adaptive properties after challenge with pathogens. These cells have unique transcriptional and phenotypic profiles compared to naïve NK cells (146, 147). Much of this work has been performed in the context of murine and human CMV infection (120). NK cells responding to infection with CMV in humans is associated with more differentiated cells that increase NKG2C and CD57 expression and decrease expression of the adaptor signaling molecule FcεR1γ and the transcription factor PLZF (147, 221, 222). These adaptive cells are long lived and undergo

transcriptional imprinting that is passed on to cell progeny (223). Beyond the generation of adaptive NK cells, CMV infection is also associated with altering NK cell responses to vaccines. Previous studies of CMV+ individuals have indicated that NK cells have reduced reactivity to Pertussis and H1N1 influenza vaccine antigens (224). Whether CMV status modulates NK cell response to Mtb antigen has not been explored.

In this study we evaluated whether CMV infection influenced the functional responses of NK cells to Mtb. We performed flow cytometry to analyze the phenotypic and functional profiles of NK cells from CMV– and CMV+ individuals residing in a low TB exposure setting in the U.S. We performed stimulations of NK cells with generic stimuli, including tumor cell lines and antibody (Ab)-coated target cells, as well with Mtb antigens. We found CMV+ individuals to have increased expression of both CD57 and NKG2C, with similar functional responses to generic NK cell stimuli, but decreased degranulation to Mtb antigen stimulation, compared with CMV– individuals.

4.3 Material Methods

Study participants: Healthy adults were enrolled at Yerkes National Primate Research Center at Emory University. Blood was collected from each individual and used for separation of peripheral blood mononuclear cells (PBMCs) and plasma. CMV status was designated based on detection of anti-CMV antibodies in plasma from Cytomegalovirus IgG ELISA Kit (Abnova). Individuals with positive CMV IgG response were classified as CMV+; those with a negative CMV IgG response were classified as CMV–.

Ethics statement: This study was conducted in accordance with the principles expressed in the Declaration of Helsinki. All subjects provided written informed consent for participation in the study, which was approved by the Emory University Institutional Review Board.

PBMC isolation: Blood samples from all participants were collected in sodium heparin tubes for isolation of peripheral blood mononuclear cells (PBMCs). PBMCs were isolated via density gradient centrifugation, cryopreserved, and stored in LN_2 until use. Cryopreserved PBMCs were thawed in a 37°C water bath and resuspended in 10 ml RPMI 1640 (Corning) with deoxyribonuclease I (DNase, 10 μg/ml, Sigma-Aldrich) and washed twice with RPMI 1640. Cells were then suspended in R10 (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum [FCS], 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine) and used in phenotypic and functional NK cell assays described below.

NK cell phenotyping: Thawed PBMCs were washed in PBS and stained with Zombie NIR™ Fixable Viability Dye (BioLegend) for 15 minutes at room temperature. Cells were washed with PBS and surface stained for 30 minutes in the dark at room temperature with anti-CD3 Alexa Fluor 700 (BioLegend; UCHT1), anti-CD14 Alexa Fluor 700 (BioLegend; HCD14), anti-CD19 Alexa Fluor 700 (BioLegend; HIB19), anti-CD56 Brilliant Violet (BV) 711 (BioLegend; HCD56), anti-CD16 BV 605 (BD; 3G8), anti-NKG2A PE (Beckman Coulter; IM329IU), anti-NKG2D BV 421 (BioLegend; 1D11), anti-NKp30 Alexa Fluor 647 (BioLegend; P30-15), anti-NKp46 PE-Cy7 (BioLegend; 9E2), and anti-CD57 FITC (BioLegend; HCD57). After incubation with conjugated antibodies, cells were washed with PBS and fixed with 2% paraformaldehyde (PFA).

A second phenotyping panel was designed to measure the NK cell expression of intracellular markers. PBMCs were stained with Zombie NIR™ Fixable Viability Dye and surface stained for 30 minutes at room temperature with anti-CD56 BV 711 (BioLegend; HCD56), anti-CD16 BV 605 (BD; 3G8), anti-CD3 Alexa Fluor 700 (BioLegend; UCHT1), anti-CD14 Alexa Fluor 700 (BioLegend; HCD14), anti-CD19 Alexa Fluor 700 (BioLegend; HIB19), and anti-TIGIT PE (BioLegend; A15153G). Cells were washed in PBS and suspended in FoxP3 Fixation Buffer (eBioscience) for 30 minutes on ice. After fixation, cells were washed with FoxP3 Permeabilization Buffer (eBioscience) and stained with anti-granzyme B PE-CF594 (BD; GB11), anti-perforin PE-Cy7 (BD; BD48), and anti-granulysin Alexa Fluor 488 (BD; RB1) for 30 minutes in the dark at room temperature. Finally, cells were washed with FoxP3 Staining Buffer and resuspended in PBS.

Stimulation and staining of NK cells: NK cell responses to target cells were evaluated using K562 and p815 tumor cell lines (ATCC). Just prior to use in NK cell stimulation experiments, p815 cells

were incubated with rabbit anti-mouse polyclonal lymphocyte serum (Cedarlane) for 30 minutes to coat the cells with Ab. Ab-coated p815 cells were then washed with R10 before being added to PBMCs. Donor PBMCs were incubated with K562 or Ab-coated p815 cells at an effector to target ratio of 10:1. PBMCs incubated in R10 media alone served as a negative control. CD107a PE-Cy7 (BioLegend; H4A3), brefeldin A (5 μg/ml; Sigma-Aldrich), and monensin (5 μg/ml; BioLegend) were added to each sample at the beginning of stimulation. Cells were incubated at 37°C degrees for 5 hours.

NK cell responses to Mtb antigens were evaluated by stimulation of PBMCs with 10 μg/ml of Mtb H37RV derived cell membrane (obtained from BEI Resources, NIAID, NIH; catalog number NR14831). PBMCs were incubated in R10 with anti-CD107a PE-Cy7 (BioLegend; H4A3), recombinant human IL-2 (100U/ml; NIH AIDS Research and Reference Reagent Program, Catalog #136), and Mtb antigens for 24 hours at 37°C and 5% CO_2. Brefeldin A (5 μg/ml; Sigma-Aldrich) and monensin (5 μg/ml; BioLegend) were added at the final 5 hours of incubation. PBMCs incubated in R10 media with anti-CD107a and IL-2 alone served as a negative control.

Following stimulation of PBMCs with either target cell lines or Mtb antigens, cells were washed with PBS and stained with Zombie NIR™ Fixable Viability Dye (BioLegend) for 15 minutes. Cells were washed with PBS and surface stained with anti-CD56 BV 711 (BioLegend; HCD56), anti-CD16 BV 605 (BD; 3G8), anti-CD3 Alexa Fluor 700 (BioLegend; UCHT1), anti-CD14 Alexa Fluor 700 (BioLegend; HCD14), anti-CD19 Alexa Fluor 700 (BioLegend; HIB19), anti-CD159c (NKG2C) VioBright FITC (Miltenyi Biotec; REA205) for 30 minutes at room temperature in the dark. Stained cells were washed with PBS and fixed with FoxP3 Fixation Buffer (eBioscience) for 30 minutes on ice. Cells were washed with FoxP3 Permeabilization Buffer (eBioscience) and stained

intracellularly with anti-CD69 PerCP-Cy5.5 (BioLegend; FN50) and anti-IFNγ BV 480 (BD; B27) for 30 minutes at room temperature in the dark. Cells were washed in PBS and prior to acquisition on a BD LSRII flow cytometer.

Flow cytometry and data analysis: Cells were acquired on a BD LSRII flow cytometer with BD FACSDiva software (v8.0) and analyzed with FlowJo software (v9.6; BD). Compensation was performed using single-stained anti-mouse Ig,κ beads (BD Bioscience). Single cells were identified by plotting forward scatter height and forward scatter area. Lymphocytes were identified by plotting forward scatter height and side scatter height. Viable lymphocytes were identified by low expression of NIR viability dye. NK cells were identified as live lymphocytes negative for CD3, CD14, CD19 and positive for the NK lineage markers CD56 and/or CD16. NK cells were further stratified based on intensity of the expression of CD56 ($CD56^{dim}$, $CD56^{bright}$ and $CD56^{neg}$ NK cells).

Data analysis and statistics: A minimum of 1,000 NK cells was acquired in each panel for each individual. Expression of functional markers by NK cells (CD69, CD107a, and IFNγ) were analyzed after subtraction of background expression in the negative control condition. A non-parametric Mann-Whitney test was used to compare differences between the groups. P-values of less than 0.05 were considered significant.

4.4 Results

Study participants

We screened 30 healthy donors for anti-CMV IgG antibodies to determine if individuals had previously been infected with CMV. Individuals with positive CMV IgG antibody detection were considered seropositive (CMV+, n=13) and individuals with negative IgG were considered seronegative (CMV–, n=17). The groups were similar with regard to sex distribution and percentage that were born in the U.S.; however, CMV+ individuals were older than CMV– individuals (Table 4-1). The percentage of individuals positive for CMV antibody (43%) was similar to previous studies detailing prevalence of CMV infection in U.S. adults (225).

CMV infection is associated with increased $CD56^{dim}$ and decreased $CD56^{bright}$ NK cell frequencies

NK cells can be subdivided based on CD56 expression into three sub populations ($CD56^{dim}$, $CD56^{bright}$, $CD56^{negative}$) (164). The distribution of these subsets is altered in chronic viral infections (209). We therefore used flow cytometry to evaluate the distribution of CD56 subsets within NK cells from CMV– and CMV+ individuals in our cohort (Figure 4-1A). There was a significant increase in the proportion of $CD56^{dim}$ cells and corresponding decrease in proportion of $CD56^{bright}$ cells in CMV+ individuals, compared with CMV– individuals (Figure 4-1B). There was no change in the proportion of $CD56^{neg}$ NK cells between the two groups. These data suggest that CMV infection is associated with a shift in the distribution of $CD56^{dim}$ and $CD56^{bright}$ NK cells.

CD57+NKG2C+ NK cell population is increased in CMV+ individuals

We next performed flow cytometry to evaluate phenotypic profiles of $CD56^{dim}$ and $CD56^{bright}$ NK cells in our cohort according to CMV status (Figure 4-2A). We did not find any significant differences in expression of the phenotypic markers evaluated on $CD56^{dim}$ NK cells (Figure 4-2B). However, $CD56^{bright}$ cells from CMV+ individuals had increased expression of CD57 and decreased expression of NKG2A, compared with CMV– individuals (Figure 4-2C).

In addition to our initial phenotypic analysis, we also performed flow cytometry on a subset of CMV– (n=8) and CMV+ (n=8) individuals to measure the expression of CD57 and NKG2C (Figure 4-3A), two receptors that have been implicated in differentiation of adaptive NK cells (122, 151). Expression of CD57 was significantly higher on the total NK cell population in CMV+ individuals, compared with CMV– individuals (Figure 4-3B). Analysis of combinations of these two markers revealed an increase in CD57+NKG2C+ and CD57+NKG2C– cell populations in CMV+ individuals, compared to those that are CMV– (Figure 4-3C). In addition, we analyzed co-expression of CD57 and NKG2C on $CD56^{dim}$ and $CD56^{bright}$ NK cells. Similar to our analysis of the total NK cell population, CD57+NKG2C+ cells were significantly higher within $CD56^{dim}$ cells from CMV+ individuals (Figure 4-3D). No significant differences between the two groups were observed for CD57 and NKG2C expression within $CD56^{bright}$ cells (Figure 4-3E). These data suggest that CMV infection is associated with increased frequencies of CD57+NKG2C+ $CD56^{dim}$ NK cells.

NK cell responses to tumor cell lines are similar between CMV+ and CMV– individuals

NK cells recognize tumor cells that downregulate MHC class I receptors as an immune escape mechanism (226). NK cells are also activated through activation of CD16 by Ab cross-

linking. We stimulated PBMCs with K562 MHC class I-devoid cells and Ab-coated p815 cells to measure NK cell functional capacity. Flow cytometry was used to measure CD69, CD107a and IFNγ expression from the $CD56^{dim}$ NK cells from both stimulation conditions. There were no significant differences in NK cell responses to K562 or Ab-coated p815 cells between CMV+ and CMV– individuals (Figure 4-4B-E).

CMV is associated with decreased NK cell degranulation capacity to Mtb antigen stimulation

NK cells recognize Mtb through TLR2 and NKp44 and are capable of killing Mtb directly through a granulysin dependent mechanism (79, 111, 173). Given the previous reports of dampened NK cell reactivity to vaccine antigens in CMV+ individuals (224), we were interested in evaluating the possible influence of CMV infection on the response of NK cells to Mtb antigens. We stimulated PBMCs with Mtb cell membrane in presence of IL-2, monensin and BFA for 24hr (Figure 4-5A). We performed flow cytometry on these stimulated cells to measure CD69, CD107a and IFNγ. CMV+ individuals had decreased Mtb-induced CD107a expression compared with CMV– individuals (Figure 4-5B). Analysis of co-expression of CD69, CD107a, and IFNγ indicated that this was due to a decrease in the CD69+CD107a+IFNγ– subset of $CD56^{dim}$ NK cells in CMV+ individuals (Figure 4-5C). Taken together, these data suggest cytokine production capacity to Mtb is maintained but NK cell degranulation may be diminished in CMV+ individuals.

4.5 Discussion

In this study we used flow cytometry to evaluate and compare the phenotypic and functional profiles of NK cells in a cohort of CMV– and CMV+ healthy adults from the U.S. We found that the distribution of CD56 NK cell subsets is altered in CMV+ individuals. Consistent with previous reports (122, 151), phenotypic analysis indicated that CMV+ individuals in our cohort have increased frequencies of CD57+NKG2C+ NK cells compared to CMV– individuals. Although we did not observe significant differences in NK cell functional responses to generic NK cell stimuli, we did see evidence for decreased NK cell degranulation capacity to Mtb antigens in CMV+ individuals.

NK cells are divided into 3 subsets based on CD56 expression (164). We found the proportion of $CD56^{dim}$ NK cells to be increased in CMV+ individuals compared to CMV– individuals. $CD56^{bright}$ cells constituted a lower proportion of NK cells in CMV+ individuals as well. $CD56^{bright}$ cells are considered to be naïve and as they become activated and mature, CD56 is downregulated and the cells become $CD56^{dim}$. The increased $CD56^{dim}$ and decrease in $CD56^{bright}$ cells may be due to increased maturation of the $CD56^{bright}$ subset in CMV+ individuals.

We used flow cytometry to measure the phenotypic profiles of $CD56^{dim}$ and $CD56^{bright}$ NK cells in both groups. While we did not see any evidence for a difference in expression of our initial markers in $CD56^{dim}$ cells, there was a decrease in NKG2A and an increase in CD57 in the $CD56^{bright}$ subset from CMV+ individuals. Previous studies in human CMV have shown that $CD56^{dim}$ NK cells have an increased population of NKG2C+CD57+ memory-like NK cells (120). These memory-like NK cells are also described as having lower expression of NKG2A (151). The decreased expression of NKG2A and increased expression of CD57 on $CD56^{bright}$ cells in CMV+ individuals may be early

evidence of these cells transitioning into memory-like NK cell subset. In a separate experiment, we stained eight individuals in each group for CD57 and NKG2C expression and found that CMV+ individuals had increased CD57 expression and increased frequencies of CD57+NKG2C+ cells. These data confirm previous reports of CMV infection being associated with generation of CD57+NKG2C+ NK cell population (151).

When we performed stimulation of NK cells with generic cell lines, we did not find any difference in responses in NK cell function between the two cohorts. Memory-like NK cells undergo epigenetic changes that lead to unique functional properties (146, 147, 227), although we were unable to detect significant differences in NK cell function to tumor cell lines in our assays. This could reflect the small sample size in our study and/or the functional readouts of our assays (expression of CD69, CD107a, and IFNγ). Previous studies suggest that CMV+ individuals have decreased responses to vaccines. We found Mtb cell membrane stimulation in CMV+ individuals to be associated with a decrease in degranulation (as measured by CD107a expression). Our observations of NK cell activity following Mtb antigen stimulation are similar to previous vaccine studies describing decreased NK cell expression of CD107a from CMV+ individuals after stimulation Pertussis and H1N1 vaccines (224).

There are some limitations of this study, including a relatively small sample size of 13 CMV+ individuals. This was essentially a pilot study to begin to address the impact of CMV infection on NK cell reactivity to Mtb antigen stimulation. Such studies are difficult to do in high TB burden settings, where CMV seropositivity is >95% (154, 228). We therefore conducted this pilot study in healthy U.S. adults, although additional studies including more CMV+ individuals would be required to confirm our results. All of the CMV+ individuals in this study were identified

by positive CMV-specific IgG antibodies, although the duration of CMV infection in these individuals is not known. Time from initial exposure may be important for the representation of the phenotypic and functional profiles. The CMV+ individuals enrolled were also older than the CMV– individuals, thus future studies will need to be conducted to better define the effect of age on NK cell phenotype and function. In addition, we measured a limited number of effector molecules in our functional assays (CD69, CD107a, and IFNγ), and it is likely that evaluation of a broader range of cytotoxic molecules, cytokines, and chemokines following stimulation with Mtb antigens would provide a more complete evaluation of how CMV infection may modify NK cell responses in the setting of Mtb exposure and infection.

In conclusion, we performed phenotypic and functional profiling of NK cells from a cohort of CMV– and CMV+ healthy adults in the U.S. We found distinct changes in the NK cell subset distribution and phenotypes between these two groups. We confirmed previous reports of an expansion of CD57+NKG2C+ NK cells, and provide novel evidence that CMV infection may impact NK cell reactivity, in particular degranulation, to Mtb. These data add to the growing body of evidence that CMV infection reprograms NK cell populations that may influence reactivity to other pathogens or to vaccination.

4.6 Tables and Figures

Table 4-1. Characteristics of Study Participants

Participant Group	*n*	Age, y[a] (IQR)	Sex (% male)	% U.S.-born	CMV IgG Titer (IQR)[c]
CMV−	17	29 (27 – 40)	18	76	2.01 (2.00 – 2.05)
CMV+	13	41 (31 – 54)[b]	38	69	163.6 (89.3 – 233.4)

[a] value denotes median
[b] $p<0.05$
[c] value denotes median artificial units (AU)/ml

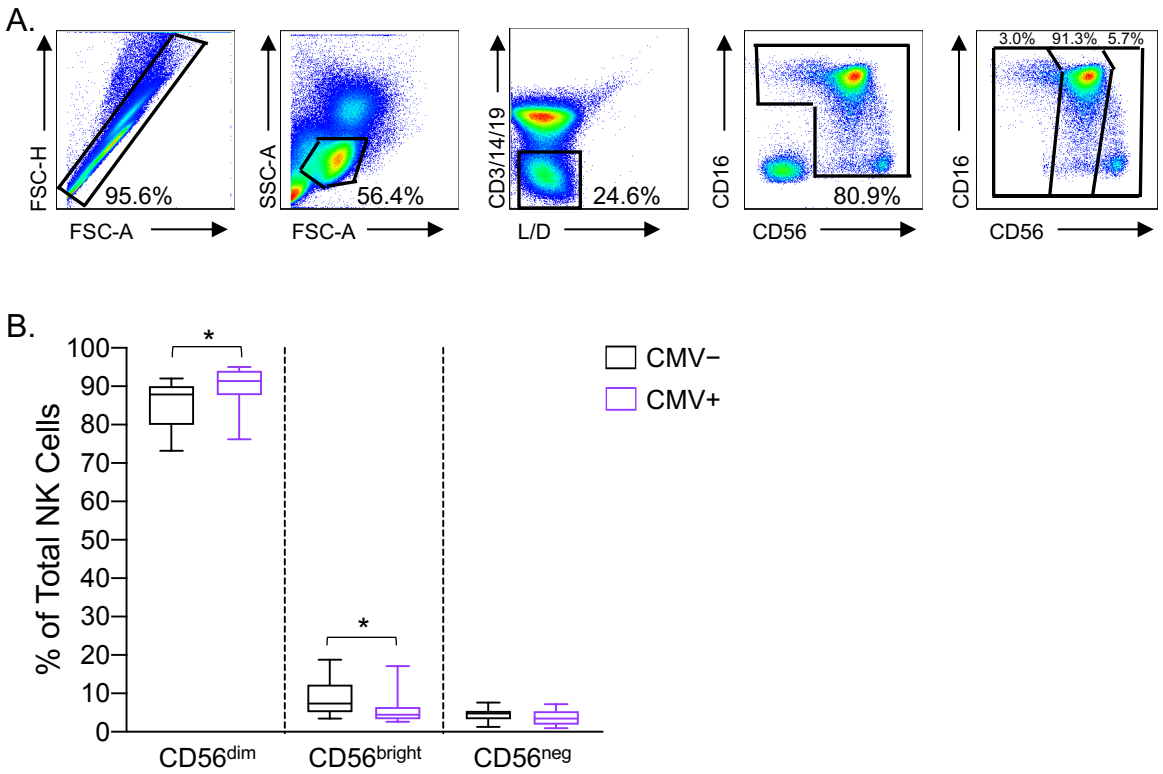


Figure 4-1. CMV infection is associated with a shift in the proportions of CD56^{dim} and CD56^{bright} NK cell subset.

(A) Flow cytometry gating strategy for NK cell and CD56 subsets (CD56^{dim}, CD56^{bright} and CD56^{negative}). **(B)** Frequency of CD56 subsets as percent of total NK cells for CMV– (n=17) and CMV+ (n=13) individuals. Differences between groups were assessed using the Mann Whitney test. *p< 0.05

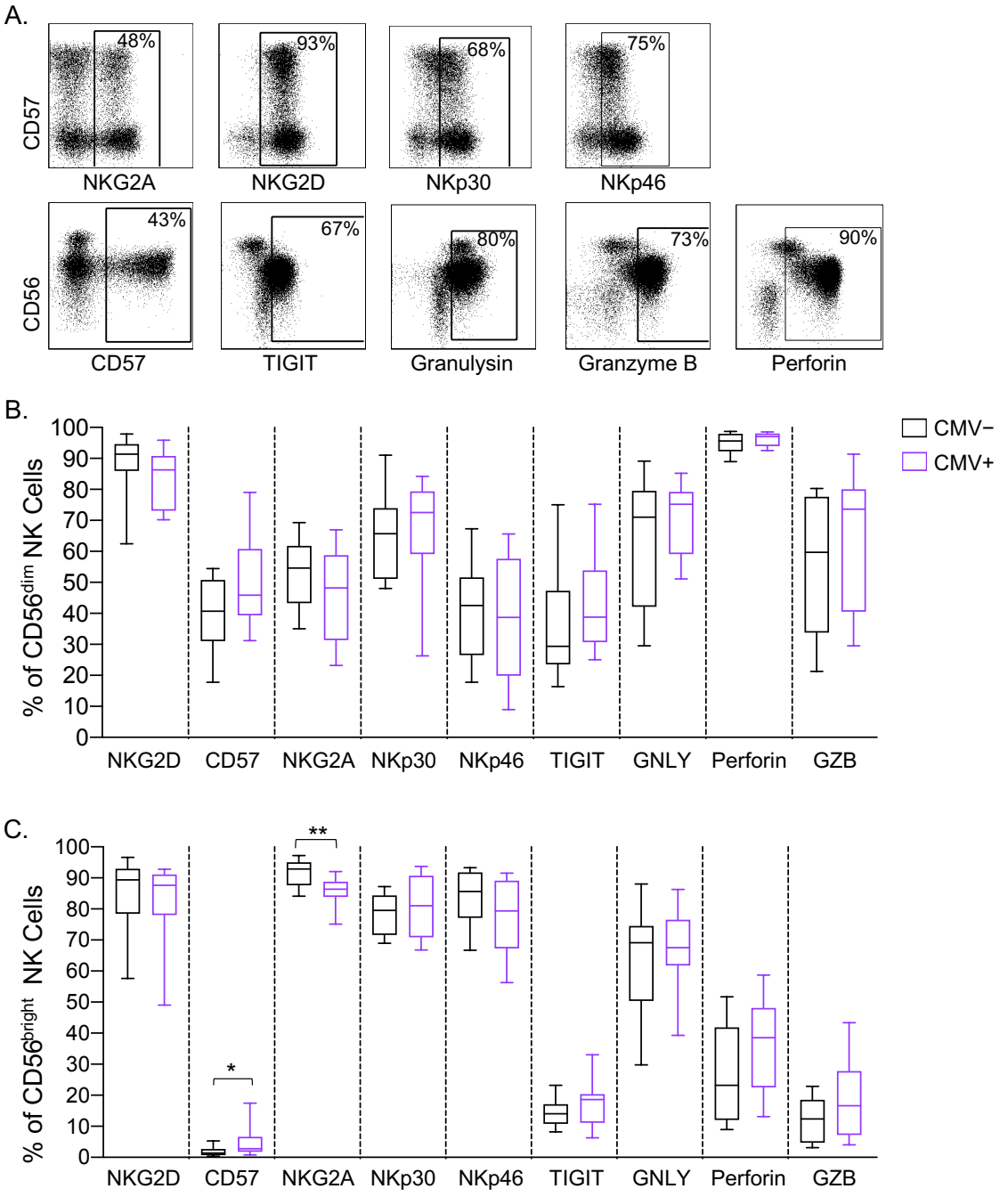


Figure 4- 2. CMV infection is associated with changes in CD57 and NKG2A expression on CD56^{bright} NK cells.

(A) Representative flow plots of NK cell marker expression on total NK cells from a CMV+ individual. **(B)** CD56^{dim} NK cell expression of each phenotypic marker. **(C)** CD56^{bright} NK cell expression of each phenotypic marker. GNLY, granulysin; GZB, granzyme B. Differences between groups were assessed using the Mann Whitney test. *p< 0.05; **p<0.01

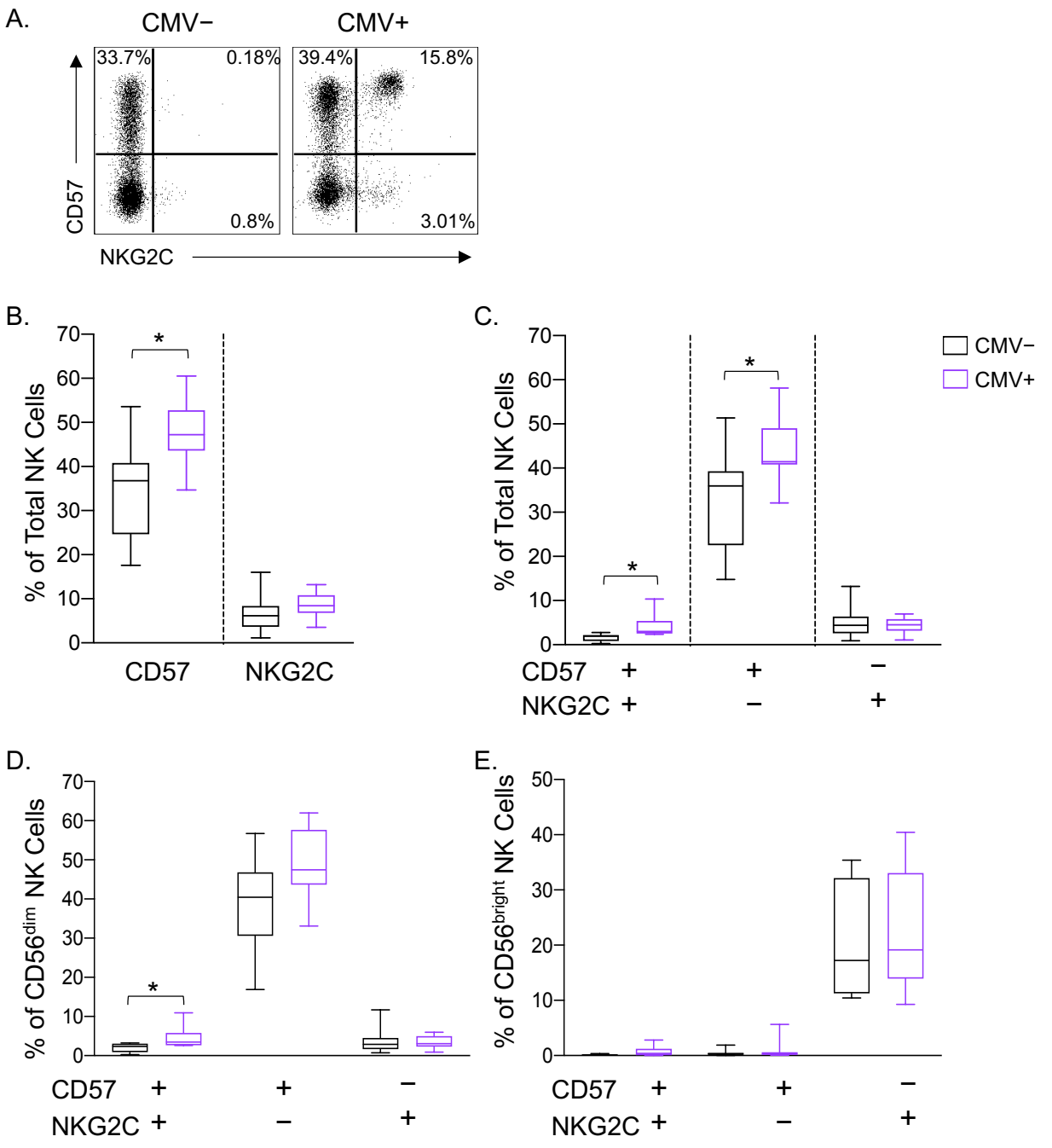


Figure 4-3. CMV infection is associated with expansion of CD57+NKG2C+ NK cells.

Flow cytometry was performed to measure expression of CD57 and NKG2C in CMV– (n=8) and CMV+ (n=8) individuals. **(A)** Flow plots of NKG2C and CD57 from total NK cells in a CMV– and a CMV+ individual. **(B)** Total NK cell single marker expression of NKG2C and CD57. **(C)** Total NK cell Boolean analysis of NKG2C and CD57. $CD56^{dim}$ **(D)** and $CD56^{bright}$ **(E)** cell Boolean analysis of CD57 and NKG2C expression. Differences between groups were assessed using the Mann Whitney test. *p< 0.05

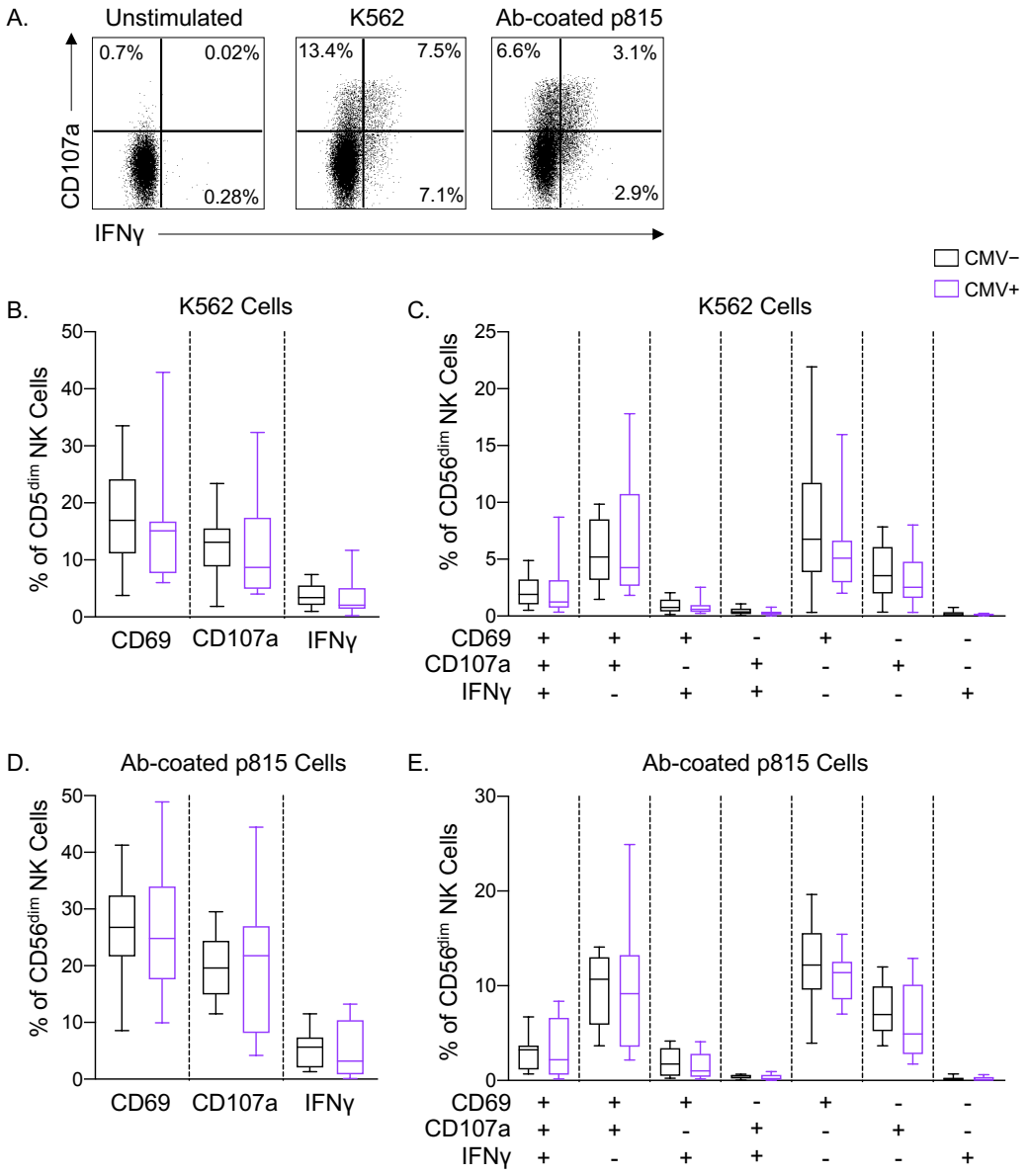


Figure 4- 4. CMV infection is not associated with alterations to NK cell response to tumor cell lines.

PBMCs were incubated for 5hr with either MHC class-I devoid cells K562 or Ab-coated p815 cell lines in the presence of BFA and Monensin. Flow cytometry was performed to measure the expression of CD69, CD107a, and IFNγ. Frequencies represent expression after background subtraction with unstimulated control. **(A)** Example flow plot of CD107 and IFNγ production from K562 and p815 stimulation in a CMV+ individual. **(B,D)** Single marker expression of CD69, CD107a and IFNγ expression of $CD56^{dim}$ NK cell following stimulation with K562 **(B)** and Ab-coated p815 cells **(D)**. **(C,E)** Boolean analysis of CD69, CD107a, and IFNγ expression in either K562 **(C)** or Ab-coated p815 **(E)** stimulation conditions. Differences between groups were assessed using the Mann Whitney test.

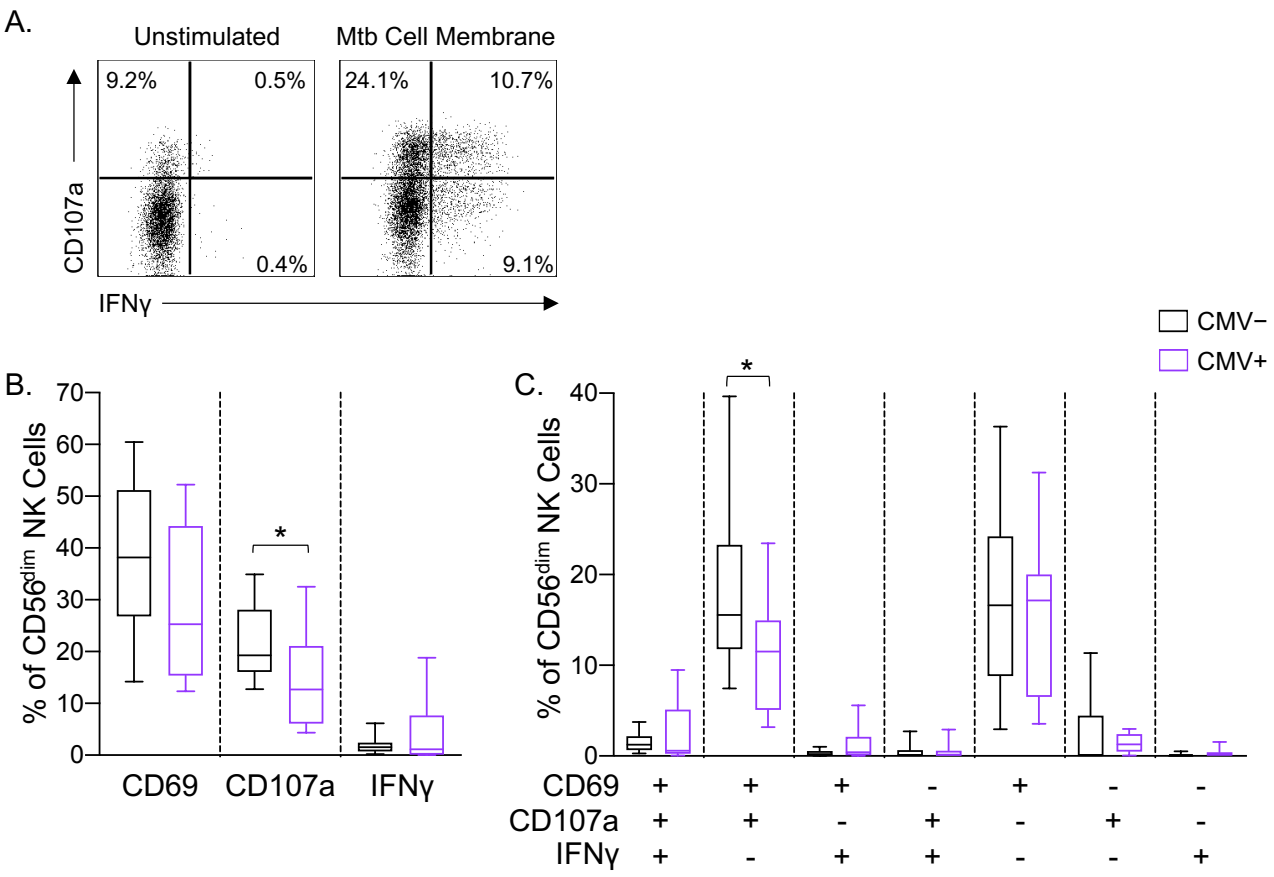


Figure 4-5. CMV infection is associated with lower NK cell degranulation capacity in response to Mtb antigens.

PBMCs were incubated with Mtb cell membrane for 24 hrs in the presence of IL-2. Monensin and BFA added at final 5 hrs of stimulation. Flow cytometry was performed to measure the expression of CD69, CD107a, and IFNγ. Frequencies represent expression after background subtraction with unstimulated control. **(A)** Sample flow plot displaying expression of CD107 and IFNγ after stimulation with Mtb cell membrane in a CMV+ individual. **(B)** Single marker expression of CD69, CD107a and IFNγ from $CD56^{dim}$ NK cells after Mtb cell membrane stimulation. **(C)** Boolean analysis of CD69, CD107a and IFNγ expression after Mtb cell membrane stimulation. Differences between groups were assessed using Mann Whitney test. *p<0.05.

Chapter 5. Discussion

5.1 Overview of findings

The main findings from this dissertation include:

1. NK cells from healthy, TB-unexposed adults in the US have markedly different expression of key receptors and effector molecules, including NKG2A, NKp30, NKp46, TIGIT, and granzyme B, compared with adults residing in western Kenya. Expression of these markers correlated with dampened NK cell reactivity to tumor cell lines and with diminished activation, degranulation, and cytokine production by NK cells from Kenyan adults following stimulation with Mtb antigens in vitro.
2. HIV/Mtb co-infection in Kenyan adults is associated with perturbations in NK cell subset distribution and phenotype, with diminished effector functions to antibody-coated target cells. Although T cell IFNγ production to immunodominant Mtb antigens is decreased in HIV-infected individuals with LTBI, we did not find evidence of significant impairment of NK cell degranulation and cytokine production capacity following stimulation with Mtb antigens in HIV-infected individuals with LTBI, compared with HIV-uninfected individuals with LTBI.
3. Consistent with previous reports, we found that memory-like CD57+NKG2C+ NK cells are expanded in cytomegalovirus (CMV)-seropositive U.S.-resident healthy adults. We also provide novel evidence that NK cells in CMV-seropositive individuals have diminished degranulation capacity following stimulation with Mtb antigens. These findings could have important implications in understanding mechanisms regulating host immunity in TB-endemic countries, where there is near universal CMV seroprevalence by early adulthood.

5.2 Conclusion

Mycobacterium tuberculosis is one of the deadliest infectious agents in human history. In 2018 Mtb was responsible for 10 million new cases and 1.5 million deaths, including 250,000 deaths in people living with HIV (9). Although the immune response is important in protection, the precise immune correlates of protection against Mtb infection and TB disease are unknown. While CD4 T cells have been shown to be important in protection against Mtb, many other immune cell populations also likely play important roles in mediating successful containment of Mtb infection. NK cells are a group of innate immune cells that have been studied less thoroughly than T cells in human TB immunology, although increasing evidence indicates that the frequency and function of NK cells may correlate with Mtb infection and progression to TB disease (127). The studies described in this dissertation were formulated to gain better insight into the role of NK cells in Mtb and HIV infection by performing experiments to thoroughly evaluate the phenotypic and functional signatures of NK cells across cohorts representing diverse Mtb exposure and infection states, including people living with HIV.

In the second chapter of this thesis we performed flow cytometry to measure the expression of phenotypes and functional responses of NK cells in Mtb-uninfected healthy adults (QFT–) and LTBI (QFT+) adults from Kenya. We also compared these two groups with the profiles of NK cells from Mtb-naïve US healthy adults. In this study we found distinct phenotypes of $CD56^{dim}$ NK cells from adults in Kenya that correlated with NK cell reactivity to Mtb antigen stimulation. These data will inform future designed to define NK cell correlates that may be protective against acquisition of Mtb infection and progression to TB disease.

In the third chapter of this thesis we describe the effect of HIV infection on the phenotypic and functional profiles of NK cells in Mtb-uninfected (QFT–) and Mtb-infected (QFT+) Kenyan adults. HIV/Mtb co-infection was associated with an expansion of $CD56^{neg}$ NK cells, which normalized after successful ART, suggesting an association with viral load. In addition to phenotypic perturbations in $CD56^{bright}$ NK cells in HIV+ individuals, we also observed dampened NK cell responses to Ab-coated target cells in HIV+ individuals, compared with HIV– individuals. Contrary to our initial hypothesis, we did not find compelling evidence that NK cell responses are significantly dampened following Mtb antigen stimulation in HIV+ individuals. Future studies will expand the readouts of NK cell functional activity to Mtb, including killing capacity and mycobacterial growth inhibition, to more comprehensively determine the effect of HIV infection on NK cell responses to Mtb.

In the fourth chapter, we describe results from our pilot study evaluating the effect of CMV infection on NK cell responses to Mtb in healthy adults in the US. A growing body of evidence indicates that CMV infection leads to epigenetic reprogramming of NK cells and adaptive diversification (146, 147) and may be associated with dampened NK cell responses to vaccine antigens (224). We confirmed previous reports of expansion of memory-like NK cells expressing CD57 and NKG2C; moreover, we also provide novel evidence that CMV infection may dampen NK cell degranulation capacity to Mtb. These data have important implications for understanding immune mechanisms of protection against Mtb infection and disease, particularly in high TB burden countries where there is near universal CMV seropositivity by early adulthood.

5.3 Future Implications

Currently it is estimated that one quarter of the world is latently infected with tuberculosis (126). A major goal of reducing the prevalence of Mtb is to develop a vaccine that is effective in preventing pulmonary TB disease in adults, which is largely responsible for transmission of TB. In order to make an effective vaccine, a better understanding of the precise immune correlates of Mtb infection and TB disease are needed. In this dissertation, we evaluated the phenotypic and functional profiles of NK cells in individuals with Mtb and HIV infection. Overall, we found distinct features of NK cells that are characteristic of Mtb infection and HIV/Mtb co-infection. These insights into NK cell signatures across different Mtb exposure and infection states will inform future studies designed to identify NK cell correlates of protection against acquisition of Mtb infection and progression to TB disease.

In Chapter Two of this dissertation we described the phenotypic and functional responses of NK cells in a cohort of QFT– and QFT+ Kenyan adults and in a cohort of healthy US adults. The goal of this study was to determine if NK cells were modulated in people with LTBI, as has been described in people with persistent viral infections such as CMV (229). One of the major findings from this study was the drastic differences in phenotype and function of NK cells in adults from TB-endemic and non-endemic regions. The decreased effector functions and receptor expression of NK cells in our Kenyan cohort is possibly a consequence of a pathogen exposure burden in Kenya as compared with the US, therefore resulting in higher activation of NK cells and overall dampening of the response.

The phenotypic markers that we found to be differentially expressed between QFT+ and QFT– Kenyan adults included the activating receptor NKp46 and the inhibitory receptor TIGIT. NKp46 is downregulated as a consequence of activation and loss of NKp46 expression is a

characteristic of memory-like or 'adaptive' NK cells (230). CD57 expression was also increased in both Kenyan groups relative to US healthy adults. CD57 is increased in memory-like NK cells and as a result of terminal differentiation of NK cells (151). These phenotypic changes seen in Kenyan donors may be the result of differentiation of memory-like NK cells in these cohorts and possible memory NK cells differentiated in response to Mtb infection in QFT+ individuals. Further follow up studies of potential differentiation of memory-like NK cell subsets in Mtb infection are warranted as these cells could be a valuable goal for novel vaccination strategies in the future. TIGIT is an inhibitory receptor expressed on T cells and NK cells. Previous studies have shown this receptor to be inversely proportional to the functional response of NK cells (178). Decreased expression of TIGIT on NK cells from QFT+ Kenyan adults, relative to QFT– Kenyan adults, suggests that these cells are more functional or easier to become activated. Recent studies in mice indicate that blockade of signaling through TIGIT enhances the functions of NK cells and T cells (180), thus providing rationale for future studies evaluating TIGIT blockade approaches to enhance NK cell reactivity to Mtb.

When we explored the functional profiles of NK cells from Kenyan and US adults, we found diminished responses from Kenyan donors compared to US donors following stimulation with MHC class I-devoid K562 cells. The difference in these responses may reflect increased differentiation of NK cells as memory-like NK cells are associated with epigenetic changes that allow these cells to produce higher quantities of effector molecules upon activation (227). All three cohorts had similar responses to Ab-coated p815 tumor cells, suggesting that Mtb exposure and infection do not substantially influence Fc-receptor mediated NK cell responses.

Our evaluation of NK cell responses to Mtb antigens indicated that Mtb-naïve US adults had significantly higher responses compared to both groups of Kenyan adults. Surprisingly, IFNγ was produced by a lower frequency of NK cells, as compared with the degranulation marker CD107a and the activation marker CD69. IFNγ is one of the major effector molecules produced by NK cells in response to pathogens and tumor cells. While surface expression of CD107a is a marker of degranulation, its expression does not indicate which cytotoxic molecules have been released by the cells. Further studies should be conducted to identify the cytotoxic molecules released in response to Mtb; moreover additional studies are warranted to evaluate the capacity of NK cells to directly kill Mtb-infected cells.

We concluded this study by performing experiments neutralizing IL-12 and IL-18 during our Mtb antigen stimulation assays. We were interested in determining whether activation of NK cells by Mtb was occurring via direct or indirect mechanisms. We show that neutralization of IL-12 and IL-18 led to significant decreases in Mtb-induced CD69, CD107a and IFNγ expression, suggesting that NK cells are activated by Mtb antigens at least in part by indirect mechanisms, such as cytokine production by other cells present in PBMCs. IL-12 and IL-18 are primarily produced by monocytes and are the most likely the source of IL-12 and IL-18 in our stimulation assay (231). The influence of these cells on the NK cell response to Mtb warrants further investigation as these cells may serve as a target to modulate the NK response in future studies. Additional studies that could be performed to address the mechanism of NK cell activation by Mtb include targeted blocking of individual receptors on NK cells as well as depletion of distinct cell subsets in PBMCs, such as monocytes, CD4 T cells and γδ T cells.

Overall, in this initial study we defined phenotypic and functional profiles of NK cells in Mtb-uninfected healthy US adults, Mtb-uninfected healthy Kenyan adults, and LTBI Kenyan adults. These finding help to define a baseline for future studies evaluating NK cells across a broader spectrum of human Mtb infection, including active TB disease and anti-TB treatment. We also show the diversity of NK cell profiles according to geographic region, which highlights the importance of participant characteristics and study setting in the interpretation of studies of human NK cell responses.

In Chapter Three of this thesis we explored the effect of HIV infection on NK cell signatures in Kenyan adults with LTBI. LTBI individuals co-infected with HIV represent an important population in the fight to eradicate TB due to the fact that these individuals are highly susceptible to developing active TB disease compared with HIV-uninfected individuals with LTBI (9). Thus, it is important to understand what influence HIV infection may have on the immune response to Mtb.

We found that HIV co-infection was associated with diminished T cell IFNγ responses in the QFT assay compared with HIV– QFT+ individuals, providing further evidence of dampened Mtb-specific T cell immunity in HIV+ individuals. $CD56^{negative}$ cells were also increased in co-infection compared with HIV– QFT+ individuals, coincident with a decrease in $CD56^{dim}$ frequency.

We performed a phenotypic analysis on both $CD56^{dim}$ and $CD56^{bright}$ NK cells. $CD56^{dim}$ cell in HIV co-infection were associated with increased expression of NKG2D. This receptor recognizes ligands that are upregulated on stressed cells and its increase may be a result of increased stressed cells due to chronic immune activation that is characteristic of HIV infection (217). In $CD56^{bright}$ cells we found an increase in TIGIT and CD57 in HIV+ QFT+ individuals. This observation

suggests that co-infected individuals have less functional $CD56^{bright}$ cells and that these cells are more differentiated due to increased expression of CD57. $CD56^{bright}$ cells are primary producers of cytokine and their increased maturation and increased inhibitory marker expression suggests that these cells may be less functional and succumb to immune activation in HIV co-infection compared to the cells in HIV– LTBI individuals. Further studies into the role of $CD56^{bright}$ cells in HIV infection are needed to further substantiate these observations.

We performed functional analysis on $CD56^{dim}$ NK cells and found that NK cells in co-infection are associated with decreased reactivity to Ab-coated p815 cells. This stimulation involves Ab ligation of Fc-gamma receptors on NK cells. We also performed Mtb cell membrane stimulations on $CD56^{dim}$ cells and found that HIV co-infection was associated with decreased frequencies of a subset of CD69+CD107a–IFNγ– NK cells. Surprisingly, we did not observe evidence for impairment of degranulation of cytokine production by NK cells to Mtb antigen stimulation in HIV+ individuals. However, further studies evaluating a broader range of cytokines and cytotoxic molecules are required to more comprehensively define the impact of HIV infection on NK cell reactivity to Mtb, as our initial studies were limited to production of IFNγ and surface expression of CD107a. Moreover, we did not measure direct killing of Mtb-infected cells, thus it is possible that the capacity of NK cells to suppress Mtb growth is dysregulated in HIV-infected individuals, which may contribute to their increased risk of progression to TB disease.

We concluded this study by looking at the effect of HIV viral load on NK cell responses by studying a subset of 10 HIV co-infected individuals before and 12 months after initiating ART. The subsequent decline in the proportion of $CD56^{neg}$ cells and increase in $CD56^{bright}$ and $CD56^{dim}$ subsets after suppression of viremia on ART suggests a link between HIV viral load and NK cell

subset distribution. Phenotypically we observed decreases in TIGIT, CD57 and granulysin expression by $CD56^{bright}$ cells after 12 months of ART. It is interesting to note that the most significant changes in phenotypic marker expression occurred within $CD56^{bright}$ NK cells and not $CD56^{dim}$ cells. It is possible that longer-term follow-up of HIV+ individuals on ART is required to detect significant changes in phenotypic profiles of $CD56^{dim}$ NK cells. Additionally, analysis of NK cells in sites other than peripheral blood, such as the lung, may be required to identify specific NK cell populations that correlate with different Mtb infection outcomes.

In Chapter Four of this dissertation we looked at the effect of CMV infection on NK cells in a pilot study of healthy adults in the US. Similar to previous studies (122, 151). We found an increase in the memory-like NK cell markers NKG2C and CD57 in CMV+ individuals, compared with CMV– individuals. We also found that NK cell degranulation capacity following stimulation with Mtb antigens is diminished in CMV+ individuals. These data demonstrate for the first time that there may be a link between CMV infection and NK cell functional responses to Mtb. Interestingly, recent studies in South African infants identified CMV infection is a risk factor for development of active TB disease (232). Increasing evidence indicates CMV infection imprints long-lasting effects on NK cell repertoires (229) and our preliminary findings provide rationale for further evaluation of the effect of CMV infection on NK cell responses to Mtb.

Overall this dissertation describes the phenotypic and functional profiles of NK cells in people across a spectrum of Mtb exposure and infection and in the setting of HIV infection. These studies serve as a platform to build future studies to better define the role of NK cells in the human immune response to Mtb and to define NK cell correlates that may eventually be useful in identifying individuals who are at high risk for developing active TB disease and target TB

preventive therapy to these individuals, thereby reducing the incidence of TB disease and decreasing the overall global burden of TB.

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