Type III interferons are expressed in tuberculosis granulomas and can enhance anti-mycobacterial activity of macrophages

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Type III interferons are expressed in tuberculosis granulomas and can enhance anti-mycobacterial activity of macrophages

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Humans and non-human primates express four subtypes of type 3 interferons (IFNλs; IFNλ1-IFNλ4). Unlike type I interferons, which have been extensively investigated in tuberculosis (TB), the role of IFNλs and their effects on immunity in TB remain unknown. Here we examined expression of IFNλ1 and IFNλ4 in *Mycobacterium tuberculosis* infected cynomolgus macaque granulomas and investigated the effects of IFNλ1, IFNλ4 and IFNα signaling on macaque macrophages. We identified differential IFNλ1 and IFNλ4 expression in granuloma macrophages and neutrophils, including IFNλ4 localization in the nuclei of epithelioid and alveolar macrophages. Further, we found that macrophages from granulomas from long term *M. tuberculosis* infection have a higher concentration of IFNλ1 as compared to those from acute infections. To measure IFNλ1 and IFNλ4’s effect on macrophage gene expression and compare these cytokines against type 1 interferons (IFN1), we performed transcriptional profiling and analysis on cytokine-stimulated macrophages to identify differentially regulated pathways. We found that IFN1 upregulated the greatest number of interferon stimulated genes (ISGs), followed by IFNλ1, whereas IFNλ4 stimulation had minimal effect on gene expression. Pro-inflammatory genes including *IL-1β, IL-8, NFkB1*, and *NFkB2* were upregulated by IFNλ1 while they were downregulated by IFN1. To determine the effect of IFNλ signaling on anti-mycobacterial macrophage responses, we used a reporter Mtb strain to determine how IFNλ1 and IFNλ4 affect the viability of *M. tuberculosis*. There was a reduction in mycobacterial transcriptional activity, as
indicated by reduced GFP expression, when macrophages were activated with IFNλ1 prior to infection. Furthermore, we identified that pre-treatment with IFNλ1 enhanced acidification of macrophage phagolysosomes. Our data suggest that IFNλs have non-redundant properties with type 1 interferons that may promote macrophage activation, inflammation, and antibacterial activity in TB.
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1.0 Introduction

1.1 Tuberculosis: a global health burden

Tuberculosis (TB) was the leading infectious disease killer globally until the year 2020, when it was supplanted by SARS-CoV-2. Currently, TB is the 13th leading cause of death globally, and among infectious diseases it is only second to COVID-19 [1]. Around one-quarter of the world’s population (~2 billion people) have been infected by *M. tuberculosis* (Mtb) and people who are infected experience a 5-10% risk that they will develop active (symptomatic) disease [1, 2]. People living with HIV are 15-21 times more likely to develop active TB in comparison to people who are HIV negative, and both these diseases speed each other’s progression [1]. In 2020, approximately 10 million people were infected with Mtb worldwide, resulting in 1.5 million deaths [1]. The highest percentage of new cases reported in 2020 were from the WHO South-East Asian regions, accounting for 43% of new cases, followed by the WHO African region, which accounted for 25% of the new cases, and the WHO Western Pacific region, which accounted for 18% of the new cases [1]. The COVID-19 pandemic led to a reversal in the progress of efforts at reducing global TB burden, with a 18% drop in the diagnosis and reporting of new TB cases in 2020 [3]. Due to the ongoing pandemic, reduced access to diagnostics and treatments has increased the number of TB related deaths in 2020 and these number are predicted to be even higher for the coming years [3].
1.2 Mycobacterium tuberculosis

*Mycobacterium tuberculosis* (Mtb), discovered by Robert Koch in the year 1882 [4], is a pathogenic bacterial species, belonging to Mycobacteriaceae family and is the causative agent of most cases of tuberculosis. Mtb forms a complex with other highly related bacteria called the *Mycobacterium tuberculosis* complex that consists of 6 members: *M. tuberculosis* and *M. africanum* which infect humans, *M. bovis*, which infects multiple mammalian species including humans, BCG (bacille-Calmette-Guérin) an attenuated form of *M. bovis*, *M. microti* which infects vole and *M. canetti* which can also cause disease in humans [5]. Mtb H37Rv is the best-characterized and the most studied laboratory strain. It has a circular genome that consists of 4,411,529 bp and contains approximately 4,000 genes, with a G+C content of 65.6%. This is the second largest genome sequence after *E.coli* [6, 7].

Mtb is approximately 2-3μm in length and 0.3-0.5μm in diameter and is a rod-shaped bacilli with a curved center, often looking like a ‘comma’. The bacterium is non-motile, non-flagellated and can appear as clumps in sputum or clinical specimens. Its characteristic features include slow growth, complex cell envelope, intracellular pathogenicity, dormancy, and genetic homogeneity. The bacterium does not produce virulence factors such as capsules and fimbriae. Instead, some unique properties contribute to the virulence of Mtb [6].

Mtb differs markedly from other organisms in that a large portion of its genome is involved in the synthesis of enzymes associated with lipogenesis and lipolysis [6]. The cell envelope contains several unique lipids and glycolipids like lipoarabinomannan, mycolic acids, trehalose dimycolate and phthiocerol dimycocerosate [7-9]. Not only are these compounds toxic to eukaryotic cells, but they also create a hydrophobic barrier around the bacterium that facilitates
impermeability and resistance to antimicrobial agents, resistance to lysozyme, resistance to killing by alkaline and acidic compounds, resistance to osmotic lysis, etc. The cell wall glycolipids also associate with mannose giving Mtb control over entry into macrophages, exploiting the process of phagosome-lysosome fusion by altering the phagosome membrane [10]. Due to the cell wall’s high lipid content, gram staining is ineffective for mycobacteria and instead other staining procedures like Ziehl-Neelsen staining in which the bacilli retain the carbol fuschin stain after acid-alcohol washes is used to detect the bacterium [11, 12]. Therefore, mycobacteria are also known as acid-fast bacilli.

Protein secretion systems are major virulence factors for pathogenic bacteria and Mtb contains five type VII secretion systems (ESX1-5), of which ESX1 is the best characterized system [13]. Although controversial, some studies suggest ESX1 contributes to the virulence of Mtb by promoting the escape of the bacilli from the acidic phagosome to the cytosol of infected macrophages [14-16]. Among the many proteins included in ESX1, two of the highly immunogenic proteins secreted by ESX1 are early secretory antigenic target (ESAT-6) and culture filtrate protein (CFP)-10 which form the basis of immunological detection of Mtb by interferon-gamma release assay (IGRA) [17]. Bacille Calmette-Guerin (BCG), an attenuated form of M. bovis [18] lacks the region of difference 1 (RD1) genomic region that encodes part of the ESX1 secretion system [6, 19, 20]. Due to the lack of this virulence system, BCG is attenuated and is the only currently licensed vaccine for TB. The genome of Mtb also codes many potential resistance determinants, like hydrolytic or drug-modifying enzymes including β-lactamases and aminoglycoside acetyl transferase, potential drug-efflux systems including fourteen members of the major facilitator family and numerous ABC transporters. Some of the other virulence factors include catalase-peroxidase that provides protection against reactive oxygen species (ROS), mce
that codes for macrophage-colonizing factor and a sigma factor sigA (*rpoV*), which if mutated leads to attenuation of the bacilli [21].

1.3 Clinical aspects of TB: transmission, diagnosis and treatment

A person gets infected with tubercle bacilli after inhalation of the Mtb-laden aerosolized droplets that are released when an actively infected person coughs or sneezes [22]. Most Mtb-infected individuals are not highly contagious and on average, an infected individual might infect 3-10 people per year [23]. Around 5-15% of infected individuals will develop active (symptomatic) TB disease [24], while the rest have a persistent risk of developing active disease throughout their lifetime by the process of reactivation [25]. The concept of long-term infection, latency, and reactivation of existing bacterial populations in the absence of reinfection is controversial, however, and recent work suggests that much of the reactivation seen is attributable to recent (new) infections rather than persistent infection [26]. Certain conditions are associated with increased rates of reactivation and primary TB including comorbidities such as HIV co-infection [27], chronic renal failure [28], immunosuppression due to transplantation [29] and diabetes [30] among many of the conditions that can elevate the risk of reactivation.

Diagnosis of active TB depends on radiographic findings and bacteriologic studies [31, 32], with sputum or bronchoalveolar lavage examination and culture still considered to be the gold standard [33]. However, culture of Mtb is a time consuming and can further delay the diagnosis of infection. Sputum smear microscopy (SSM) for the detection of acid-fast bacilli using Ziehl-Neelsen staining is relatively inexpensive that does not require sophisticated laboratory
infrastructure and is still the most commonly used diagnostic test in countries with high TB infection rates [34, 35]. Despite having high specificity, the sensitivity of SSM is low with chances of false negative results in children and false positive results in people infected with non-tuberculous bacilli and people with HIV infections [36]. Further, it requires more than $10^4$ bacilli/ml of sputum which affects the sensitivity of the test results especially in children [37]. In comparison to the light-microscopy based Ziehl-Neelsen staining method that is widely used in low- and middle-income countries, auramine rhodamine or auramine O staining method based on fluorescent microscopy is used in higher income countries as this test has greater sensitivity but requires a fluorescence microscope [34]. Sputum culture in contrast yields greater sensitivity (80-96%) than sputum smears (50%) and is essential for species identification and drug susceptibility testing [37]. Diagnosis of asymptomatic TB is often made with the tuberculin skin test (TST) which relies on the principal of delayed-type hypersensitivity (DTH) to purified protein derivative (PPD) of Mtb, that is injected intracutaneously into the forearm and induration at the site is measured 48 to 72 hours after injection. However, TST can yield false positive results due to poor specificity in people who have been BCG vaccinated, people who live in countries with high TB prevalence, or people infected with nontuberculous mycobacteria [38]. Moreover, false negative results due to waning DTH which could be due to prolonged interval between infection and testing, or immunosuppression also add to the inaccuracy of this test [37]. An alternative to TST is the blood-based in vitro interferon-\(\gamma\) release assay (IGRA) developed in recent years. The assay is based on the stimulation of peripheral blood T cells with Mtb specific Gene Xpert Mtb/Rif (Xpert) (Cepheid Inc.) assay that can detect the bacilli as well as rifampicin resistance in less than 2 hours, has a high sensitivity in sputum-negative pulmonary TB cases and is a potential tool for detection of extrapulmonary samples [39, 40].
Following exposure to the bacillus, approximately 30% of the exposed individuals show evidence of infection by TST [41]. An estimated 5-10% of those infected within 2 years of exposure, will develop clinical manifestations of active TB, also called primary TB [42]. The symptoms of active TB usually include a cough that lasts more than 3 weeks, coughing up blood or sputum, signs of disease on chest x-ray or CT, fever, chills, night sweat, loss of appetite, weight loss, TST or IGRA positivity. The remaining 90-95% of infected individuals develop latent infection (LTBI), which is characterized by the absence of clinical signs or symptoms of TB, normal chest radiographs, but positive evidence of Mtb infection via TST or IGRA. Some of these individuals with subclinical TB may represent a reservoir for potential future transmission or be at risk of developing active TB [43, 44], especially for people with immunocompromising conditions including HIV co-infection where the risk of experiencing active TB is estimated to be up to 10% per year [44, 45].

However, with the recent paradigm of LTBI [42, 46-48], the dogma of the binary nature of Mtb infection i.e., active vs latent is now considered obsolete. Mtb infection can result in a spectrum of clinical outcomes and manifestations [42, 46, 47] and from humans and animal models, it is now known that there is substantial heterogeneity within the classical LTBI classification, that can influence reactivation to active disease [42, 46]. As shown in Fig. 1 [49], on one end of the spectrum are individuals who after exposure to Mtb can eradicate the bacteria with innate or acquired immune responses where there is no T cell priming or memory generation and these individuals are TST and IGRA negative; or those who developed a memory immune response and are TST and IGRA positive. These individuals have no symptoms and have negative sputum smear results. Some of these individuals are “resisters”, who despite repeated exposure to a confirmed TB case (index case), appear to be resistant and are TST and IGRA negative [50-52],
or “reverters”, who initially develop a positive TST and IGRA and then revert back to being negative within 2 years [53-55]. In some individuals the bacteria can persist in a quiescent state, and thus they do not exhibit any symptoms and remain sputum smear negative but are positive for TST and IGRA. Next on the spectrum are individuals with subclinical TB disease, who display mild to no symptoms, are intermittently sputum culture positive but smear negative due to low bacterial load and are TST and IGRA positive [49, 56, 57]. At the end of the spectrum are individuals with active TB who show mild to severe symptoms and range of diseases by chest x-rays, are TST and IGRA positive, sputum culture positive and smear positive or negative due to anergy induced by comorbidity related immunosuppression [49, 56, 57].

*M. bovis* bacille Calmette-Guérin (BCG) is currently the only available vaccine and has variable efficacy in providing protection against pulmonary TB in adults [58]. BCG is derived from a virulent *M. bovis* strain that was attenuated by continuous *in vitro* passage for more than 13 years. Currently, the WHO recommends all babies to be vaccinated with BCG shortly after they are born in all countries with high risk of TB infection. However, variations in BCG strains in different countries, along with differences in immunization policies, have made it challenging to test the efficacy of this vaccine [59]. While most reports agree that BCG protects children against severe TB manifestations like miliary TB and TB meningitis [60-64], in adults BCG vaccination offers 0-80% protection against pulmonary TB based on clinical trials [58, 60, 65-68]. Some of the underlying reasons for the inconsistency in its protective efficacy could be due to variation in the immunogenicity of the different BCG strains, molecular and phenotypic differences owing to the different manufacturing methods, demographic and genetic factors [69-71]. Further, previous exposure to environmental mycobacterial has been reported to generate cross-reactive immune response that can severely compromise the efficacy of BCG [72, 73].

Some of the novel vaccine strategies target TB at three different stages of the infection/disease – preexposure vaccines that are administered to infants soon after birth and include the current BCG vaccine, recombinant live vaccines and subunit vaccines that are considered as boosters for priming with BCG [74, 75]. Examples of the live vaccines are rBCG and rMtbc deletion mutant [76-78]. The rBCG VPM1002 has a gene encoding for listeriolysin from *Listeria monocytogenes*, with a deletion of the urease C gene that allows stronger acidification of VPM1002 containing phagosomes [79]. MTBVAC is the other live vaccine candidate that is a clinical isolate of Mtb with deletion of the genes *PhoP* and *Fad26*. These genes encodes a transcription factor associated with expression of virulence factors and synthesis of phthiocerol
dimycocerosates [76], respectively. Second is the postexposure vaccine strategy that targets adolescents and individuals with LTBI. Some of the vaccines for this strategy are subunit vaccines that have been formulated with antigens associated with Mtb latency [80], and are considered as boosters for the BCG prime that is administered after birth [81]. An example of a subunit vaccine that is currently in clinical trial includes a protein fusion H56, which is a combination of Ag85B, ESTA-6 and Rv660c and results in stimulation of immune response to antigens expressed at different stages of Mtb infection [82, 83]. In a phase 2b clinical trial, the GSK vaccine M72/AS01E composed of two Mtb antigens (Rv1196 and Rv0125) with the adjuvant QS21 and monophosphoryl lipid A, showed an efficacy of 54% in preventing pulmonary TB disease in adults already infected with Mtb [84]. A phase 2 clinical trial was conducted in BCG vaccinated, IGRA negative adolescents from high-risk TB setting, to evaluate the efficacy of H4:IC31 vaccine and BCG revaccination in preventing Mtb infection (i.e., conversion to IGRA positive) and perturbing sustained Mtb infection (i.e., reversion of positive IGRA to negative), in comparison to placebo [85]. While the study showed that none of the vaccines effectively prevented initial Mtb infection, BCG revaccination reduced sustained IGRA conversion rate with an efficacy of 45.4%, whereas H4:IC31 had an efficacy of 30.5% in comparison to placebo [85]. This trial led to a renewed interest in BCG revaccination. Last are the therapeutic vaccines designed for active TB patients in adjunct to or to minimize the length of chemotherapy or for extensively or totally drug-resistant TB cases (XDR and TDR, respectively) [74, 86]. An example for this is RUTI, an inactivated Mtb-based vaccine composed of detoxified, fragmented Mtb cells delivered in liposomes and this vaccine has finished phase II clinical trial assessment in HIV infected or uninfected individuals with LTBI [87]. Another example is that of killed *M. indicus pranii* that was originally designed
for leprosy, but is currently undergoing phase III clinical trial assessment in India due to its potential efficacy against TB [88].

TB treatment can take 4, 6 or 9 months depending on the regimen. Treatment for drug-susceptible TB can include either a 4-month rifapentine-moxifloxacin regimen or a 6-9 month RIPE (Rifampin, Isoniazid, Pyrazinamide and Ethambutol) treatment regimen [89]. The 4-month Rifapentine-moxifloxacin regimen is composed of an 8-week intensive regimen of high dose rifapentine (RPT) with moxifloxacin (MOX), isoniazid (INH) and pyrazinamide (PZA), followed by a 9-week continuation regimen of all the drugs except PZA [89]. The RIPE regimen is composed of a 2-month intensive regimen of rifampin (RIF), INH, PZA and ethambutol (EMB), followed by a continuation phase of 4 or 7 months of RIF and INH.

Anti-microbial treatments for bacterial infections became a reality with the discovery of penicillin and sulfonamides in 1930s. The first drug identified to be effective against Mtb, was streptomycin (SM) discovered by Selman Wakman in 1944. Jorgen Lehman in the same year synthesized the para-amino salt of salicylic acid (PAS) and both SM and PAS were effective against Mtb. Due to a shortage of SM, the British Medical Research Council (BMRC) performed the first randomized clinical trial comparing the efficacy of SM or PAS alone with that of a combination therapy, and published for the first time in 1950s demonstrating the greater effectiveness of a combination therapy at cure and prevention of acquired drug resistance in comparison to using PAS or SM alone [90].

Discovery of the sulfonamides in 1930s facilitated the discovery of the antimycobacterial activity of isonicotinic acid hydrazide or INH in 1952. INH is a prodrug that needs to be activated by the catalase-peroxidase enzyme, KatG expressed by the bacilli [91]. The drug then blocks fatty acid synthase and inhibits the synthesis of mycolic acids which are the building blocks of
mycobacterial cell wall component [92, 93] and also disrupts nucleic acid synthesis [94], thereby killing actively diving bacteria [95]. Thus, the introduction of INH into PAS and SM generated a ‘triple therapy’, which led to predictable cures for 90-95% of patients. However, the triple therapy needed to be continued for 24 months to achieve these results [96] due to the persistence of viable Mtb in tissues. In 1960s, this 24-month regimen was shortened to a 18-month regimen with the replacement of PAS with EMB which was also better tolerated [97]. Similar to INH, EMB also inhibits mycobacterial cell wall synthesis by inhibiting arabinosyltransferases (embA, embB, embC) which is required for the synthesis of cell wall components like arabinogalactan and lipomannan, and thus prevents cell division [98-100]. A major advance in the field of TB treatment was the introduction of rifampicin (RIF) which was derived from Streptomyces mediterranei. Studies performed by BMRC in Hong Kong [101] and East Africa [102], demonstrated that the combination of RIF, INH, EMB and SM achieved predictable cures greater than 95% in 8-9 months. RIF inhibits bacterial DNA-dependent RNA polymerase by binding to the DNA/RNA channel within the polymerase subunit and directly blocking the elongating RNA [103, 104]. The final step was the inclusion of PZA which resulted in culture negativity and cure rates greater than 95% when used in combination with RIF and INH in 6 months [101, 105, 106]. PZA is a prodrug and is converted to its active form, pyrazinoic acid (POA) by the bacterial enzyme nicotaminidase/pyrazinaminidase encoded by pncA gene, mutations in which results in PZA resistance [107, 108]. The exact mechanism of action of PZA remains unknown, due to its inactivity against Mtb grown in vitro in normal media [109]. PZA can kill non-replicating persistent bacilli that are not killed by other TB drugs and inhibits different targets like energy generation, fatty acid synthetase I, trans-translation, membrane transport function and pantothenate/coenzyme A important for survival of dormant bacilli [109-111].
Although the efficacy of antituberculosis regimen is up to 95%, nonadherence to treatment is a major reason for this reduced efficacy [112, 113]. To improve adherence and treatment outcomes, since the early 1990s WHO has recommended the adoption of Directly Observed Treatment, Short-Course (DOTS) strategy [114]. The DOTS strategy focuses on five main components- government commitment, case detection based on sputum-smear microscopy tests performed on patients reporting active TB symptoms, standardized treatment regimen of 6 to 9 months with direct observation by a healthcare or community health worker at least for the first 2 months, drug supply and standardized reporting and recording of cases to assess treatment results [115].

1.4 Granulomas- the pathological hallmark of TB

The histologic hallmark of Mtb infection is the formation of lesions known as granulomas, which are compact and highly organized aggregates of different types of immune cells. Once inhaled, Mtb is ingested by antigen presenting cells (APCs) including alveolar macrophages and dendritic cells (DCs) which transport the bacteria across the alveolar epithelium into the deeper tissues [116]. Unless in a highly activated state, APCs do not kill the bacilli, which then replicates until it bursts out of these cells, allowing the bacteria to be phagocytosed by other APCs and initiating the development of small aggregates of immune cells [117]. At around 8-12 days post-infection, DCs traffic Mtb antigens from the lung to lung draining lymph nodes, resulting in T cell priming and activation [117]. Approximately 2-3 weeks post-infection, activated T cells migrate via the blood to the lungs where they form the granuloma structure and also activate other cells
like macrophages to kill the intracellular Mtb [118, 119]. As shown in Fig.2 a classical granuloma’s architecture is composed of a central acellular core containing caseous necrosis, surrounded by a layer of epithelioid macrophages that can be interspersed with neutrophils, giant cells and foamy macrophages. This inner macrophage layer is surrounded by a lymphocyte cuff that is primarily composed of T cells, B cells, NK cells but also contains macrophages and neutrophils [120, 121].

The function of these highly structured lesions is to contain or kill Mtb, but Mtb has evolved strategies to counteract the host responses and granulomas can serve as a niche where the bacilli can persist. Granulomas that fail to control the bacteria can facilitate dissemination, seeding new granulomas in adjacent lung tissues, progress to TB pneumonia, or lead to formation of lung cavities and result in transmission to new hosts [122]. Multiple granulomas can arise after the initial infection and each of these granulomas are heterogeneous and can have variable bacterial burden, killing ability, histopathology and follow diverse trajectories which contribute to the spectrum of disease in the host [46, 121, 123, 124]. A single host can have granulomas ranging from sterile to disseminating granulomas, and this variation depends on a wide range of bacterial and host factors [121]. Cynomolgus macaques with active TB develop contain more lesions and their lesions show increased metabolic activity at 3-6 weeks post-infection than animals that progress to develop latent TB [125]. PET/CT studies have also revealed associations between larger granulomas in early infection (4-5 weeks) and an increased risk of dissemination and generation of new of culture-positive lesion [122]. Granulomas can be necrotic or caseous and this is the most common type of lesion observed in active TB. This type of granuloma has a central necrotic core whereas non-necrotic granulomas have epithelioid macrophages and giant cells at their centers and suppurative granulomas are characterized by substantial neutrophilic infiltration into their central regions. All of the previous granuloma ‘types’ differ from fibrocalcific granulomas where tissue fibrosis surrounds a mineralized center [126]. Importantly, heterogeneity is a feature within an individual granuloma and granulomas contain microenvironments with differences in cytokine milieu, bacterial antigen concentration, oxygen availability etc. [127-130]. This heterogeneity extends to macrophage differentiation and functional states in different granuloma regions where epithelioid macrophages in central regions express more pro-
inflammatory factors while alveolar macrophages in outer and granuloma-adjacent regions express more pro-healing anti-inflammatory markers [129]. Cytokines play a key role in determining the outcome of the infection at the granuloma level since a granuloma’s ability to restrict the growth of Mtb is determined by the qualitative balance between pro-inflammatory and anti-inflammatory cytokines [128], making it necessary to explore the functions of different cytokines expressed in TB granulomas.

1.5 Non-human primate models of TB

Different animal models have been used for research in TB. Mice are the most common experimental model due to the availability of inbred, outbred and transgenic strains [131] and this model has contributed significantly to our understanding of this disease. BALB/c and C57BL/6 are the popular mouse models and develop inflammatory but non-necrotic lung lesions [132]. Even though they have similar immune responses as humans after Mtb infection, mouse models do not have disease presentations that are similar to human TB [133], as they fail to develop organized caseating granulomas and lung cavitations like humans, they do not develop latent TB and carry relatively high bacterial burden in lungs and spleen without showing signs of disease and survive up to a year [134, 135].

Non-human primates (NHPs) are an excellent model of human TB as they recapitulate the full spectrum of pathology and infection outcomes including clinical latency as seen in humans [136, 137]. Cynomolgus macaques infected with a low dose inoculum (<25 CFU) of Mtb Erdman strain represent the full spectrum of human infection, where 50-60% of the animals develop active-
chronic infections and 40% develop latent TB infection with no clinical signs of disease [124]. Furthermore, they show a wide variety of granuloma morphologies that are similar to the range of histopathology seen in human TB, including caseous, cellular non-necrotic and fibrocalcific granulomas [137]. Among the different NHP species that are available, rhesus macaques and Mauritius cynomolgus macaques are more susceptible to disease progression, whereas Indochinese cynomolgus macaques are more resistant to TB disease [136, 138, 139]. In addition to recapitulating the wide spectrum of Mtb infection outcomes, NHP models of TB also allow the tracking of disease progression with the use of serial positron emission tomography and computed tomography (PET-CT) [140-142]. PET-CT imaging provides the ability to track individual granulomas, including their time of establishment, location and distribution and changes in size or metabolic activity (inflammation) [143].

Among the different experimental animal models, NHPs have a genetic makeup closest to that of humans. NHPs represent a superior model for the study of IFNλs, as they express all the four IFNλs in functional forms, unlike mouse models where both IFNλ1 and IFNλ4 are pseudogenes. IFNλ4 is expressed in its functional form (encoded by ΔG transcript) in certain human populations, whereas in others the TT allele introduces a frameshift mutation and renders it a pseudogene [144]. In contrast, NHPs encode only the functional form of IFNλ4 and not the pseudogene [144, 145], thereby making it an unique model for the study of the functionalities of the different IFNλ genes.
1.6 Host immune response to \textit{M. tuberculosis} infection

Following inhalation with Mtb, the bacteria first come in contact with airway epithelial cells (AECs), which detect the pathogen via pattern recognition receptors (PRRs) like toll-like receptors (TLRs). Among the many PRRs expressed by AECs, TLR2 recognizes Mtb’s 19 kDa lipoprotein and lipoarabinomannan (LAM) \[146\]. Downstream signaling mediated via TLR-2 activates downstream signaling molecules including NF-κB, leading to production of pro-inflammatory cytokines including IL-8 and expression of the anti-microbial peptide (AMP) human β-defensin-2 (HBD2) that can recruit other immune cells to the infection site \[147, 148\]. AECs can also produce reactive oxygen species (ROS), nitric oxide (NO), enzymes and other major antimicrobial peptides like LL-37, lysozyme, lactoferrin etc. \[147\]. Further, PRR activation also activates mucosal-associated invariant T cells that can be early producers of TNF-α and IFNγ \[149\].

Macrophages are the first immune cells to encounter Mtb. Macrophages identify Mtb by ligation of pathogen associated molecular patterns (PAMPs) on the surface of the bacilli with TLRs, C-type lectin receptors (CLR/CTL), Fc receptors (FcR), cytosolic DNA sensors, scavenger receptors (SR), mannose receptors, CD14, surfactant protein A receptors, complement receptors and immunoglobulin receptors \[150-152\]. TLR-2 recognizes Mtb by interacting with LAM, lipomannans (LMs), phosphatidyl-myoinositolmannoside (PIM) and the 19kDa lipoprotein \[153\]. The signaling pathways triggered by ligation of these receptors facilitates phagocytosis of Mtb and this initiates a series of events that initiate the host response against Mtb. The host response includes cytokine expression, with IL-18, IL-12 and IL-23 secreted by macrophages and dendritic cells, and activation of IFNγ-expressing Th1 cells and subsequent macrophage activation and
enhanced TFN expression [154]. Activated macrophages express proteins involved in antigen presentation and T cell co-stimulation including MHC I, II, CD86, CD80 and lymphocyte IFNγ expression promote phagosomal maturation and formation of phagolysosomes in macrophages that restrict intracellular Mtb [154-156]. Moreover, activated macrophages use inducible nitric oxide synthase (iNOS) to generate nitric oxide (NO) [157, 158] and undergo activation of GTPases that recruit NOX2 to mycobacteria-containing phagosomes for generation of reactive oxygen species (ROS) [159, 160]. TNF also stimulates ROS generation from mitochondria in macrophages [154]. Along with PRR signals, lymphocyte-expressed IFNγ and/or TNF are important for macrophage activation and other factors such as antimicrobial peptides including cathelicidin antimicrobial peptide (CAMP) and LL-37 are upregulated under vitamin D-regulated pathways. The hormonal form of vitamin D, 1,25-dihydroxyvitamin D (1,25D) bound to vitamin D receptor (VDR) has been reported to directly induce transcription of AMPs like human β-defensin 2 (HBD2) and CAMP [161-163]. Further, 1,25D can also stimulate the transcription of IL-1β in macrophages, a critical cytokine for defense against Mtb infection [164]. IL-1β belongs to the IL-1 family of cytokines that also includes IL-1α. Absence of both IL-1β and IL-1α in mice leads to increased bacterial burden and exacerbated lung inflammation [165]. IL-1β promotes the expression of ROS, reactive nitrogen species (RNS), stimulates the generation of proinflammatory cytokines and leads to Mtb killing via activation of TNF and caspase-3 pathways as reported in a mouse model of Mtb infection [166, 167]. Mice with IL-1β knockouts are acutely susceptible to Mtb infection [168]. Mtb can manipulate this system, however; the Mtb gene zmp1 that encodes for a Zn²⁺ metalloprotease and can suppress inflammasome assembly and IL-1β production [164].

TNF plays a critical control in restricting Mtb as a macrophage-activating cytokine [169]. Additionally, TNF mediates apoptosis of infected cells and this can promote CD8⁺ T cell cross
priming [170, 171]. TNF also promotes phagosome-lysosome maturation [172], thus enhancing antigen presentation and CD4+ T cell function [173]. Absence of TNF impairs Mtb control and proper lung granuloma formation in mice [174, 175]. Neutralization of TNF in mice disrupts the aggregation of cells in lungs and prevents cells recruited to the lungs from getting the signals needed for aggregation and granuloma formation [176]. TNF is important for controlling reactivation of persistent TB as evidenced from a low dose persistent murine TB model, where neutralization of the cytokine resulted in fatal reactivation of TB [176]. In humans, the use of anti-TNF therapies for the treatment of inflammatory disease like rheumatoid arthritis and Crohn’s diseases are associated with increased risk of reactivation in individuals with latent TB [177].

Neutrophils are another subset of immune cells that are implicated in killing Mtb during infection early. Neutrophils phagocytose Mtb and it has been noted that ROS production and the arsenal of peptides and enzymes expressed by neutrophils are important for eliminating Mtb in phagolysosomes [178, 179]. Neutrophil enzymes including elastase and cathepsin G limit replication of mycobacteria in early infection [180] while human neutrophil peptides (HNPs) like α-defensins modulate cytokine production and act as chemotactic factors or opsonins [181]. In vitro studies report HNPs can restrict Mtb growth [182] and macrophages taking free HNPs have enhanced ability to control Mtb [183]. Neutrophil extracellular traps (NETs) can trap Mtb although the role of this process in TB is controversial and it has not been found to eliminate Mtb [184]. Although neutrophils may contribute to Mtb control, as has been shown ex vivo in human blood [182], an association has been found between delayed Mtb clearance in sputum and neutrophilia during TB diagnosis [185]. Also, in human whole blood, neutrophil driven interferon-inducible gene profiles correlate with clinical severity [186]. In active TB patients, neutrophils represent the predominant Mtb-infected immune cells in the airways and can serve as permissive host cells for
Mtb replication before transmission [187]. Infected neutrophils may also serve as a ‘Trojan horse’ and facilitate Mtb trafficking to distal sites [187, 188]. During Mtb infection, anti-IL-17 treatment in mice, a process that reduces granulocyte recruitment, has been found produce a 100-fold reduction in Mtb numbers in spleen [189]. Neutrophils have been identified to interact with macrophages and T cells in infected non-human primate lung granulomas and express cytokines including TNF, IFNγ, IL-4, and IL-10 that can potentially influence different cell types [190].

DCs are perhaps the most potent antigen presenting cells for priming naive T cells. Immature DCs play an important role in antigen uptake and processing, following which they undergo maturation and migration to prime naive T cells and secrete immunoregulatory cytokines such as IL-12 [191]. Mtb infected DCs can produce high levels of chemokines like CXCL8, CXCL9, CCL3, CCL4 and CCR7 chemokine receptor that are important for T and NK cell migration [192]. Depletion of DCs in mice delayed initiation of CD4⁺ T cell responses and led to high bacterial loads in lungs and spleen [193] and a different study noted that genetic mutation in the IRF8 gene in DCs led to early onset of disseminated BCG disease [194]. Mtb can promote this and work has shown that Mtb can inhibit DC maturation and T cell activation [195, 196]. Other studies have shown that the fate of DCs is determined by the receptors with which they interact with Mtb [197] where TLR mediated interaction with Mtb activates DC function and IL-12 production while interaction via DC-SIGN can lead to DC inactivation and IL-10 production [197]. A study in aerosol-infected mice reported that myeloid DCs represent a major cell population that is infected by Mtb in lungs and lymph nodes and this impairs their ability to stimulate CD4⁺ T cells [198]. Mtb infection can also lead to IL-10 production and this can inhibit DC migration to the lymph nodes [73].
NK cells may also contribute to control of Mtb and have been noted in granulomas from individuals with active TB [199]. IL-12 secreted by macrophages, DCs and neutrophils promotes activation and cytolytic activity of NK cells and secretion of IFNγ, thereby promoting macrophage activation and reactive oxygen and nitrogen species production [200-202]. NK cells also secrete IL-22 and this may play an important role in promoting the chronic disease caused by infection with hyper-virulent Mtb strains [203]. NK cells can also directly kill Mtb in a contact dependent manner by releasing the cytolytic proteins perforin and granulysin [204]. NK cells when activated with Mtb stimulated monocytes have been reported to lyse expanded CD4^+CD25^+ Tregs in humans [205]. The protective role for NK cells has not been completely defined but depletion of NK cells in healthy tuberculin reactors reduced the frequency of Mtb-specific CD8^+IFNγ^+ T cells and limited their ability to lyse Mtb infected macrophages [206], suggesting they may have important protective functions.

An adaptive immune response is usually detectable within 3-8 weeks of infection and plays a critical role in determining the infection outcome [207]. CD4 T cell depletion can exacerbate Mtb infection and lead to increased granuloma formation, extra thoracic dissemination events and reduced CD8+ T cell activation and promote reactivation during latent infection [208, 209]. CD4 T cell depleted macaques have undetectable levels of pulmonary T cells that constitutively produce IFNγ, TNF, IL-22, IL-17 and perforin, but have IL4^+ effector T cells [209]. Polyfunctional T cells producing a combination of IFNγ^+TNF^+IL-2^+ are considered more efficient at mediating Mtb control due to their proliferative and effector functions [210]. Active TB disease has been found to be associated with higher frequencies of Mtb specific CD4^+ T cells that produce one or dual cytokines like TNF^+ or IFNγ^+TNF^+. In contrast, in cases of latent TB, polyfunctional Mtb specific CD4^+ T cells producing a combination of IFNγ, TNF and IL-2 have been reported to be present at
a higher level [211-214]. However, some studies have also reported the opposite where active TB was associated with an increased frequency of trifunctional T cells than latent infection [215-217]. The role of CD8 T cell in anti-TB immunity is less well understood [218]. CD8 T cells can mediate apoptosis of Mtb infected cells via Fas mediated pathways, degranulation of perforin, granzyme and granulysin, expression of the T cell-stimulatory cytokine IL-2 or macrophage activating cytokines IFNγ and TNF [219, 220]. Mtb-specific CD8+ T cells in circulation [221] as well as in active TB disease sites [222] show reduced cytotoxic activity in comparison to latently infected patients. CD8 T cell depletion in BCG vaccinated rhesus macaques can reduce the protective benefit provided by BCG vaccination [218].

The role of IL-17 producing (Th17) during Mtb infection has mostly been studied in murine TB model. During early infection, IL-17 is primarily produced by γδ T cells rather than CD4+ T cells [223] and has been found to play an important role in granuloma formation in high dose intratracheal murine infection model [224, 225]. IL-17 signaling is important for proper neutrophil targeting to the site of infection early after infection [226] and dysregulation in IL-17 production led to excess neutrophil driven immunopathology in murine lung during Mtb infection [227, 228]. IL-17 has been reported to play a key protective role during infection with the hypervirulent Mtb HN878 strain, where IL-17 signaling mediated induction of CXCL13 was found to be required for T cell localization in lymphoid follicles [229]. Studies in humans have shown that latent TB is associated with higher IL-17 production and/or higher frequencies of IL-17+ CD4+ T cells than people with active TB [230-232].

CD4+ regulatory T cells (Tregs) express the transcription factor Foxp3 and can suppress effector functions in a contact-independent manner via IL-10 production or in a contact-dependent way [233, 234]. Active TB patients have higher frequencies of CD4+ Tregs in blood and
granulomas and the Tregs can inhibit Mtb specific IFNγ production by Th1 cells \textit{ex vivo} [235-238]. In mice, Mtb specific CD4+ Tregs have been reported to expand alongside Mtb specific Th1 cells in pulmonary lymph nodes and have been linked with delayed recruitment of CD4+ and CD8+ T cells in lungs during early infection [239]. IL-10 derived from Tregs and other cells can suppress macrophage activation by IFNγ, inhibit phagosomal uptake of Mtb and antigen presentation by macrophages and also diminishes production of TNF, IL-12, IL-1α/β by myeloid cells [240-242]. In macaques, depletion of IL-10 diminished lung inflammation and increased cytokine production at 3-4 weeks after infection, but did not affect bacterial burden during the early phase of infection [243]. While Th1 cytokines are important for protection in TB, granulomas producing both pro- and anti-inflammatory cytokines like IL-17 and IL-10, have been associated with Mtb clearance, highlighting that a qualitative balance between these two opposing groups of cytokines is crucial for protection at the granuloma level [128].

1.7 Interferons and tuberculosis

Interferons (IFNs) are a large family of proteins that are secreted in an autocrine and paracrine manner that activate intracellular and intercellular networks that regulate anti-viral responses, modulate survival and death of normal and tumor cells, and augment innate and acquired immune response [244]. There are three distinct groups of IFNs that have been identified (type I, type II and type III) based on their structural characteristics, receptor preferences and biologic activities [245]. IFNs are induced following the binding of microbial products to Toll-like receptors (TLRs) or through chemical inducers. After binding to their respective receptors, IFNs
initiate a downstream signaling cascades that induce expression of IFN-stimulated genes (ISGs) that mediate antiviral, antitumor and immunoregulatory effects [244].

1.7.1 Type I IFN and TB

The role of type I IFNs in immune response against TB remains controversial. There have been numerous studies suggesting that type I IFNs have a potential deleterious role in active TB. A transcriptomic study of active and latent TB patients as well as healthy individuals found that active TB patients have peripheral blood transcriptional profiles that are dominated by type I IFN inducible transcripts, primarily in neutrophils and monocytes, that correlated with radiographic disease and these transcriptional signatures diminished after treatment [186]. In clinical case reports, several instances were identified that patients undergoing IFNα-based therapy for chronic hepatitis experienced reactivated TB [246, 247]. A study in Chinese populations showed that patients with a genetic mutation in IFNAR1 gene that decreased IFNAR’s affinity to IFNβ found increased resistance to TB in this population [248]. Moreover, patients with a deficiency in ISG15 who display signs of enhanced type 1 IFN responses had increased susceptibility to mycobacterial infections [249-251]. Infection of mice with hypervirulent clinical Mtb isolates induced more type I IFNs and had diminished expression of TNF-α, IL-12, reduced T cell activation and decreased mice survival in comparison to infection with less virulent strain [252]. Further, intranasal instillation of purified IFNα/β in HN878 infected immune competent mice resulted in increased lung bacterial load and reduced survival in the mice [253]. Numerous studies have also reported that abrogation of negative regulators of type 1 IFN signaling resulting in increased type I IFN response led to increased bacillary load and impaired Mtb clearance [254, 255]. Even though the
mechanism behind the deleterious effects of type I IFN in TB remains unknown, several studies have reported the role of type I IFNs in downregulating cytokines that are essential for promoting protective responses in TB. Both human [256-258] and mouse-based [257, 259, 260] studies have shown that type I IFN have an antagonistic effect on production of IL-1α, IL-1β and prostaglandin E2 (PGE2) [261], the latter being important for promoting macrophage apoptosis [262, 263] and for mediating IL-1-dependent host-protective functions [261]. Type I IFN can also induce expression of immunosuppressive IL-10 in vitro in macrophages [257, 260] and in vivo in CD4+ T cells [264], and this has been linked to increased susceptibility to Mtb infection [265] and may play a role in inhibiting TNF and IL-12 production in infected macrophages [260]. Further, type I IFN also inhibits IFNγ-mediated antibacterial effects in macrophages [256, 257, 260, 266].

In contrast, there are certain conditions where type I IFNs may have potential protective roles in TB. Patients who fail to respond to conventional treatment and have recurrent TB have experienced improved clinical outcomes when IFNα was co-administered with antimycobacterial chemotherapy [267-270]. Mechanistically, type I IFN’s protective role in patients with complete or partial IFNγR deficiencies [271, 272] and in mouse models with Ifnγr−/− deletion [273, 274] is associated with generation of activated macrophages in the absence of IFNγ signaling related to overlap between the signaling pathways induced by these cytokines [274].

1.7.2 Type II IFN and TB

Type II IFN or IFNγ is a Th1 cytokine that plays a pivotal role in defense against intracellular pathogens like Mtb and is a principal mediator of macrophage activation [275-277]. CD4+ and CD8+ T cells are the primary source of IFNγ, whereas innate lymphocytes including natural killer
(NK) T, γδ T cells and NK cells serve as secondary source of IFNγ in mycobacterial infection. These innate sources of IFNγ are important, particularly during HIV infection-related T cell depletion [278]. IFNγ induces the production of reactive nitrogen intermediates (RNI) and reactive oxygen species (ROS) in infected macrophages [279], that in turn result in upregulation of receptors for TNF and NRAMP-1 molecules. Nitric oxide (NO) expression induced by IFNγ has been identified to be important for macrophages to kill Mtb particularly in mouse models of infection [280] and can induce apoptosis in IFNγ-activated macrophages [281]. IFNγ promotes differentiation of CD4+ T cells into Th1 cells and induces expression of class I and II MHC molecules which enhances antigen presentation [282, 283]. IFNγ revokes Mtb imposed blockage of phagosome-lysosome fusion, possibly via the induction of autophagy-related pathway, which exposes Mtb to the acidic compartment of phagolysosome and antimicrobial effectors including antimicrobial peptides (AMPs), RNI, and ROS [284-286]. Although IFNγ is a proinflammatory cytokine, it can also limit neutrophilic inflammation by limiting T cell IL-17 production [278]. IFNγ plays an essential role in mediating protective cellular immunity during TB infection [275], as mice with genetic disruptions to IFNγ cannot control even a sublethal dose of the bacteria [287]. In humans, mutations in the IFNγ receptor gene increase susceptibility to TB [288] whereas complete absence of the receptor results in increased severe risk of developing TB, poor granuloma formation, and multibacillary infection [289]. Mutation in the IFNγR1 gene are also associated with fatal disseminated BCG infection [290]. Recently, IFNγR adaptor, Mal signaling has been associated with protective immunity as IFNγ signaling through Mal leads to phosphorylation of MAPK-p38, induction of autophagy, phagosome maturation, and intracellular killing of Mtb [291]. Mutations in this adaptor have been associated with impaired immune responses to Mtb [291].
However, Mtb has developed counterstrategies to evade the deleterious effects of IFNγ-mediated immunity. Mycobacterial cell wall components can subvert IFNγ-mediated macrophage activation in a TLR-dependent manner. The 19 kDa lipoprotein of Mtb can inhibit antigen processing and class II MHC expression, primarily due to TLR2 and MAPK-mediated inhibition of class II transactivator (CIITA) of chromatin remodeling [292, 293]. Prolonged TLR signaling mediated by Mtb has also been identified to downregulate macrophage genes involved in class II MHC antigen processing and presentation and T cell recruitment genes [294].

### 1.8 Type III IFNs or IFNλs

Type III IFNs, also designated as IFNλs [295] include IL28/29 [296], are the latest addition to the IFN family. Discovered in 2002-2003 by two independent groups, IFNλs belong to the class II family of cytokines and have structural similarities to the IL-10 family of cytokines but have functional similarities to type I IFNs [295-297]. This family of IFNs consist of four cytokines: IFNλ1 (IL-29), IFNλ2 (IL-28A), IFNλ3 (IL-28B) [295, 296] and the recently discovered IFNλ4 [144], and are encoded by genes located on chromosome 19 (19q13.13 region) in humans (Fig.3) [245]. Among these four members, IFNλ1 shares 81% amino acid sequence similarity with IFNλ2/3, whereas IFNλ2 and IFNλ3 share 96% amino acid sequence similarity [295]. In contrast, IFNλ4 shares only 28% amino acid sequence similarity with the other members of IFNλ family. In mice, the IFNλ genes are present on chromosome 7 (7A3 region) and only IFNλ2 and IFNλ3 are functional, with IFNλ1 being a pseudogene and IFNλ4 is absent in mice [298]. Humans encode functional forms of IFNλ1-3, whereas IFNλ4 is a pseudogene in some populations due to TT/ΔG
polymorphism [144]. The TT allele causes a frameshift mutation introducing a premature stop codon that suppresses IFNλ4 expression and the ΔG allele of a genetic variant rs368243815 encodes for the functional form [144, 299]. Like humans, non-human primates (NHPs) encode functional forms of IFNλ1-3, and IFNλ4 is expressed in most NHPs including macaques, orangutan and chimpanzee [144]. Interestingly, chimpanzees and human African Congo rainforest hunter-gatherers are reported to encode a more functional form of IFNλ4 that contains glutamic acid at position 154 (E154) that has higher ISG induction and anti-viral potential. In contrast, most humans encode an attenuated variant of IFNλ4 due to mutation of glutamic acid to lysine at position 154 (K154E) [300, 301]. IFNλs signal via the heterodimeric receptor that consists of IL-10Rβ subunit and the cytokine specific IFNλR1 (IL28RA) subunit [296, 297]. The role of IFNλs have been extensively studied in different viral infections, with some studies extending IFNλ mediated immunity to other pathogens like bacteria, fungi, and parasites.

Figure 3. Organization of IFNλ genes. Figure modified from “Interferon-Lambda: A New Addition to an Old Family”, Copyright 2010, and used with permission by Mary Ann Liebert, Inc.

1.8.1 IFNλ expression and signaling

IFNλ expression is induced after host cell detection of pathogen associated molecular patterns (PAMPs) via different pattern recognition receptors (PRRs). Following recognition of
PAMPs, RIG-I like receptors (RLRs) recruit mitochondrial anti-viral signaling protein (MAVS) to mitochondrial membranes or peroxisomes, which result in activation of transcription factors like NF-κB and IRFs, that result in the induction of IFNλs and also type I IFNs [302]. Other PRRs involved in IFNλ expression include Toll-like receptors and cytosolic DNA sensor like Ku70 [303-305]. Most of the PRRs that induce IFNλ also activate IFNαβ expression, except for Ku70 and peroxisome-localized MAVS that uniquely trigger IFNλ expression [302, 305]. Transcription factors like IRF1, IRF3, IRF7 and NF-κB are important for the expression of IFNλ genes with their synergistic action mediating maximum induction of IFNλ [306]. Similar to IFNα, IFNλ2 and IFNλ3 expression are regulated by IRF7 and NF-κB, whereas IFNλ1 like IFNβ is regulated by the combination of IRF3, IRF7 and NF-κB [307].

IFNλs are predominantly expressed at mucosal surfaces by epithelial and myeloid cells during viral infection [308] and high levels are reportedly expressed in lung and liver tissues [298, 309, 310]. A reason for the preferential induction of IFNλ by epithelial cells could be due to greater abundance of MAVS localization to peroxisomes [302]. Further, tissue-specific undefined factors could also lead to this preferential induction of IFNλ over type I IFNs at the epithelial barrier [311]. IFNλ expression has been identified in keratinocytes, hepatocytes, DCs, primary neuronal cells and respiratory epithelial cells [312]. However, type 2 myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) are major producers of IFNλ [313-318], while monocytes, monocyte-derived DCs (MDDCs) and monocyte-derived macrophages (MDMs) have been observed to produce IFNλ in response to TLR agonists [318]. IFNλ has also been reported to mediate autocrine signaling in pDCs that strengthens anti-viral response by promoting IFNλ and IFNα expression which results in increased pDC survival [316].
Both IFNλ and type I IFNs have a similar downstream signaling pathway even though each signals via their distinct heterodimeric receptor complex (Fig.4) [297, 309, 319-321]. The heterodimeric IFNλR is comprised of IFNλR1 (also known as IL-28RA, CRF2-12 or LICR) and IL-10Rβ (also known as IL-10R2 or CRF2-4) which also is another subunit of the IL-10 receptor and is present in other IL-10 cytokine family receptor complexes [322-324]. IFNλ binding to its receptor results in activation of receptor-associated Jak kinases, JAK1 and TYK2, which leads to phosphorylation of tyrosine residues of the IFNλR intracellular domains and downstream activation of STAT family of transcription factors namely STAT1 and STAT2, however other STAT proteins (STAT3-STAT5) have also been reported to be activated by IFNλ [295, 296, 325, 326]. Phosphorylated STAT1 and STAT2 form a heterodimeric complex, and interacts with DNA-binding protein IRF9, thereby forming the trimeric complex IFN-stimulated gene factor 3 (ISGF3), which translocates to the nucleus and binds to the IFN-stimulated response element (ISRE) in the promoters of ISGs like OAS1, MX1, IRF7 and results in their induction. Additionally, IFNλ can specifically mediate phosphorylation of JAK2 which suggests that IFNλ mediated upstream signaling events could be distinct from that of IFNαβ [302, 327].
1.8.2 IFNλ.R expression

Unlike the receptors for type I (IFNαβ) and type II IFNs (IFNγ) that are ubiquitously expressed by nearly all cell types, IFNλR has a very restricted cellular distribution [328]. Mucous membranes in the gastrointestinal and respiratory tracts are main targets of IFNλ [329] and IFNλR1 has been identified in liver, lungs, intestines and upper epidermis [312]. IFNλ.R1 is strongly expressed in epithelial cells in both mice [329] and humans [295, 296]. Hepatocytes express
*IFNλR1* transcripts in both mice and humans [330-332], however *IFNλR1* transcript is barely detected in murine livers [329, 330] and studies suggest there could be differences in IFNλ responsiveness in mice and humans [333]. *IFNλR1* transcript has been detected in naïve and memory B cells from human blood, both of which are also responsive to IFNλ [334, 335], however the transcript is expressed at extremely low levels in mice [336, 337]. Mouse neutrophils express *IFNλR1* mRNA and respond to IFNλ stimulation [336-338], and even though neutrophils isolated from human blood express *IFNλR1* mRNA [338, 339], it is not known if these cells respond to the cytokine. A recent study on human immune cell subsets, identified that epithelial cells and B cells have the highest levels of *IFNλR1* transcript, with CD4+ and CD8+ T cells having lesser levels of *IFNλR1*, whereas monocytes, neutrophils and NK cells barely show any transcript expression [340]. This study also reported that CD8+ T cells have higher levels of IFNλR1 than CD4+ T cells. Human pDCs express IFNλR1 and also respond to it [316, 334], however IFNλR1 expression has not been identified in DCs derived from mouse bone marrow or lungs [336, 337, 341] with some studies suggesting that only mouse spleen pDCs respond to IFNλ [342]. Whether monocytes and T cells can express functional IFNλR1 is subject to ongoing debate [330, 336, 337, 343-346]. NK cells do not directly respond to IFNλ but can be indirectly activated by macrophages [347-349]. Human MDMs have been noted to express IFNλR1 transcript and express ISGs and TLR-mediated cytokines after IFNλ stimulation [349-353].

Interestingly, IFNλ.R1 can exist in two different variants: a membrane bound form (mIFNλR1) and a soluble form (sIFNλR1) which lacks the transmembrane domain and does not lead to downstream signaling [340, 346]. Human immune cells have a low ratio of the membrane/soluble forms, whereas liver and lung epithelial cells have a high ratio, explaining why epithelial cells expressing the same level of IFNλR1 transcript as B cells show a comparatively
higher ISG induction with IFN\(\lambda\)3 stimulation [340]. This soluble variant of IFN\(\lambda\)R1 is present in apes including humans and old-world monkeys like rhesus macaques, baboons, and green monkeys but not in new-world monkeys like marmoset and other mammals including mice, rats, dogs, cows with the exception of guinea pigs, which also express the soluble receptor variant [340].

### 1.8.3 IFN\(\lambda\) and macrophages

Human MDMs are a dominant group of myeloid immune cells that can respond to IFN\(\lambda\) and orchestrate tissue inflammation [350]. IFN\(\lambda\)R1 expression increases as monocytes differentiate into macrophages, with the latter expressing high levels of ISG mRNAs encoding ISG15 and viperin in comparison to NK and T cells after IFN\(\lambda\)3 stimulation [350]. Interestingly, macrophages differentiated in the presence of GM-CSF had comparatively higher expression of IFN\(\lambda\)R1 and greater responsiveness to IFN\(\lambda\) stimulation than M-CSF differentiated macrophages [350]. An increase in Th1 chemokine expression profile (CCL3-5 and CXCL9-11) and immune cell activation markers (CD86, CD80 and IL15) at the transcriptional level has been identified in macrophages that are differentiated in the presence of IFN\(\lambda\)3. IFN\(\lambda\)3-treated GM-CSF-differentiated macrophages also stimulate lymphocyte migration, NK cell IFN\(\gamma\) production and degranulation, and increased macrophage phagocytosis and cytotoxicity [350].

IFN\(\lambda\)1 and IFN\(\lambda\)2 can inhibit HIV-1 infection of macrophages by increasing expression of CC chemokines that restrict the virus entry into macrophages and anti-viral factors like type I IFN, APOBEC3G and APOBEC3F [354]. Interestingly, IFN\(\lambda\)3 has been identified to be the most potent at inhibiting HIV infection in macrophages, in comparison to IFN\(\lambda\)1 and IFN\(\lambda\)2 [352]. IFN\(\lambda\)1 enhances expression of TNF and IL-10 in monocyte-derived macrophages in response to TLR7/8
stimulation and similar to IFN\(\gamma\), IFN\(\lambda\)1 can augment IL-12p40 expression of monocyte-derived of macrophage after TLR7/8 stimulation [351]. IFN\(\lambda\)2 and IFN\(\lambda\)3 can also enhance TNF and IL-10 expression in monocyte-derived macrophages in response to TLR7/8 and lipopolysaccharide (LPS) stimulation but to a lesser extent as compared to IFN\(\lambda\)1 [351]. IFN\(\lambda\)1 has been identified to increase IFN\(\gamma\)R1 expression on macrophages that promotes IFN\(\gamma\) induced IL-12p40 expression of monocyte-derived macrophages [351]. IFN\(\lambda\)3 has been reported to inhibit replication of PRRSV (porcine reproductive and respiratory syndrome virus) in primary porcine alveolar macrophages, where it induced different anti-viral ISGs like MX1, OAS1, IFITM3 and ISG15 [355]. A recent study reported the importance of IFN\(\lambda\) signaling in human lung macrophages for the detection and response to viral infections [356]. IFN\(\lambda\)1 is the most abundant IFN secreted by GM-CSF differentiated macrophages in response to influenza virus PA8 and CA09 infections, and pre-treatment of GM-CSF differentiated as well as human alveolar macrophages with IFN\(\lambda\)1 was found to restrict influenza virus replication [356]. Moreover, knock-out of IFN\(\lambda\)R1 on PMA differentiated Thp1 cells abrogated influenza virus infection induced ISG expression. Overall, these data highlight the importance of IFN\(\lambda\) in activation of macrophage and their ability to confer an antiviral immune response.

1.8.4 Immunomodulatory roles of IFN\(\lambda\).

IFN\(\lambda\) can modulate immune responses either directly or indirectly. Many reports have highlighted the modulatory effects of these cytokines on neutrophils [336, 338, 357], which have been identified to express IFN\(\lambda\)R and respond to IFN\(\lambda\)s as well [336]. Reportedly, IFN\(\lambda\) can modulate neutrophil function independent of the canonical JAK-STAT pathway [336]. IFN\(\lambda\) has
been reported to regulate neutrophil function in a non-translational, JAK2 dependent pathway that
controls AKT signaling and impairs neutrophil degranulation and ROS production, resulting in
suppression of intestinal inflammation [336, 358]. In contrast, in A. fumigatus infection, IFNλ
signaling via STAT1 promotes NADPH-dependent ROS production which is important for the
control of A. fumigatus infection [339]. IFNλ2 treatment also impairs recruitment of IL-1β
expressing neutrophils [338] and neutrophil extracellular trap (NET) release [359]. IFNλ2
treatment of mouse lung CD11c+ DCs can upregulate T-bet expression and promote IL-12
production in response to LPS stimulation, thereby favoring a Th1 skewing effect [360]. IFNλ
triggers the production of thymic stromal lymphopoietin (TSLP) by upper airway M cells that
activates migratory DCs. In the draining lymph nodes, these stimulated migratory DCs then boost
antigen-dependent germinal center reactions resulting in increased production of IgA and IgG1as
well as generation of efficient CD8+ T cells [361]. A study in mice comparing the immunoadjuvant
effects of IFNλ2 and IL-12 for HIV vaccination, showed that unlike IL-12, IFNλ2 can reduce the
population of regulatory T cells and increase the level of antigen-specific IgG2a and splenic CD8+
T cells that have higher antigen-specific cytolytic degranulation [362]. IFNλ can also modulate T
cell responses indirectly; studies have shown that stimulation of human PBMCs with IFNλ and
concanavalin A promotes Th1 (IFNγ) and suppresses Th2 (IL-13, IL-4, IL-5) cytokine expression
[343, 344, 363].
1.9 IFNλ and diseases

1.9.1 Role of IFNλ in viral infections

IFNλ plays an important role in mediating immunity against a wide range of viral infections. It can exert direct anti-viral effects as well as mediate long-term immunomodulatory effects on T and B cells [364]. The anti-viral effects of IFNλ are targeted against viruses that invade the gastrointestinal tract, liver, respiratory tract and urogenital tract [309].

Much work has been done in in vitro cell culture systems where IFNλ treatment can control replication of different viruses like hepatitis B and C viruses (HBV, HCV) [308, 331, 332, 365, 366], HIV [352, 354, 367], influenza virus [368-370], herpes simplex virus type 1 and 2 [371, 372], human and murine CMV [373, 374], dengue virus [375, 376], respiratory syncytial virus [377-379], norovirus [380], encephalomyocarditis virus [295, 296, 381], lymphocytic choriomeningitis virus (LCMV) [382], Sendai virus [303, 307, 311], and vesicular stomatitis virus [383-385]. Epithelial cells in the respiratory tract predominantly express IFNλ during respiratory virus infections [312, 368, 386, 387]. The importance of IFNλ in controlling viral infections have been demonstrated in vivo, primarily with IL-28RA knockout mouse models. IFNλ has been reported to play a non-redundant role in upper airways for limiting transmission of respiratory viruses [388]. IFNLR1 knockout mice experience significantly higher disease burdens in SARS-coronavirus and influenza infections [369, 370, 389, 390]. Administration of pegylated IFNλ1 to BALB/c mice resulted in reduced lung SARS-COV2 titers [391]. IFNλR is expressed at high levels in mouse stomach and intestinal epithelial cells and IFNλ exclusively controls norovirus, rotavirus and reovirus infections in epithelial cells. Due to the more restricted expression of IFNλR, IFNλ
may have advantages over IFNα treating chronic HCV infection since IFNα therapy has been associated with lymphopenia and neutropenia [392]. Phase I clinical trial studies show that pegylated IFNλ treatment is highly effective in controlling the virus level and does not induce any significant hematologic toxicities [393-395]. A clear link has been established between SNPs in the IFNλ genes and HCV treatment outcome and spontaneous clearance in patients [396]. The rs368234815(TT/ΔG) polymorphism results in a frameshift upstream of IFNλ3 gene, leading to the expression of IFNλ4 gene [144, 397, 398]. The IFNλ4-ΔG variant is associated with increased ISGs levels that desensitizes the liver to IFNα/RBV therapy and hence results in failure to clear HCV [399-401].

1.9.2 Role of IFNλ in bacterial infections

In contrast to the studies on viral infections, reports of IFNλ in bacterial infections are comparatively limited. Bacterial antigens such as LPS, TLR4 and TLR9 stimulations have been reported to induce IFNλ genes in MDDCs [303, 402, 403]. Gram-positive pathogens including *Listeria monocytogenes, Staphylococcus aureus, S. epidermis, Enterococcus faecalis* have been reported to induce IFNλ genes in human intestinal and placental cells, whereas gram-negative microbes like *Shigella flexneri, Salmonella enterica* serovar Typhimurium and *Chlamydia trachomatis* do not induce substantial IFNλ expression in those cells [364, 404]. *S. enterica* serovar Typhimurium has also been reported to increase *IFNλ1* and *IFNλ2/λ3* transcripts in human DCs [402]. IFNλ1 treatment of polarized T84 cells increased transepithelial electrical resistance (measure of epithelial barrier integrity) and reduced transmigration of *S. enterica* serovar Typhimurium and *S. flexneri* across the monolayer [405], whereas IFNλ2 administration improved
clinical outcomes in *Pseudomonas aeruginosa* pneumonia mice model, by inhibiting neutrophil recruitment [406]. Another study has reported that IFNλ treatment during influenza infection can decrease bacterial uptake by neutrophils and thereby impair the clearance of bacteria during influenza superinfection [407].

### 1.9.2.1 IFNλ and *M. tuberculosis* infection

*M. tuberculosis* infection has been reported to upregulate *IFNλ1* and *IFNλ2* genes in A549 lung epithelial cells [404]. A later study to this report identified an increased level of IFNλ2 protein in the sputum of active pulmonary TB patients, in comparison to healthy individuals or those with latent infections [408]. Interestingly, the study also reported that patients who were on antituberculosis therapy for longer period (>7 days) had significantly reduced sputum IFNλ2 levels compared to patients either before or early in their therapy [408]. A study conducted in elderly individuals reported that BCG vaccination leads to increased plasma IFNλ1 and IFNλ2 levels and decreased type I IFN levels a month post vaccination, and IFNλ1 levels positively correlated with frequencies of pDCs [409]. Another study identified a panel of cytokines that included IFNλ1 along with 14 other inflammatory proteins to discriminate between healthy versus latently infected children, and of these factors, IFNλ1 was one of the 7 predictors that was informative for the LTBI group [410]. In contrast, IFNλ2 belonged to a panel of 15 other inflammatory proteins that discriminated between healthy versus active TB groups and IFNλ2 was one of the 13 predictors informative for the active TB group [410]. This study also reported that the median concentrations of IFNλ2 was significantly higher in the sera of TST-negative children than TST-positive children from the LTBI group, and IFNλ1 along with 11 other inflammation associated proteins were informative of the TST size [410]. Overall, these studies indicate that IFNλs are produced in
response to Mtb infection and that IFN\(\lambda\) expression is dynamic and may have relevance to infection outcomes.

1.10 Gaps in knowledge and specific aims

Despite the availability of the BCG vaccine and effective chemotherapy for over 60 years, 1.5 million people continue to die every year from TB, making it one of the world’s top sources of infectious disease-related mortality [3]. Moreover, a lack of validated immune correlates of protection represents an obstacle for the development of new TB therapies. Many of these challenges are caused by the pathologic characteristic of TB where immune responses in granulomas are highly regulated and the outcome of the infection depends on a balance between pro- and anti-inflammatory cytokines that cannot be ascertained by sampling the blood [128]. When appropriately balanced, this strategy leads to control of bacterial burden, while deviation from this equilibrium promotes bacterial dissemination and increasingly severe disease. Considering this paradigm, understanding what constitutes ‘protective’ and ‘detrimental’ inflammation in TB is important for vaccine design, development of new therapeutics, and for improving current treatments.

While IFN\(\lambda\) has been detected to be present at higher levels in chronic TB patients, the relationship between IFN\(\lambda\) expression and Mtb burden remains unknown. Cytokines are firmly established as factors that play a major role in outcome of TB since they coordinate immune cell effector functions to mount immunity against Mtb [411]. The disease is presumed to manifest when the protective balance between pro- and anti-inflammatory cytokines gets dysregulated. Few
cytokine families better demonstrate this paradigm than the cytokines in the IFN family where

type II IFN is crucial for controlling TB while type I IFNs are associated with disease pathology
[259, 275]. In the case of IFNλ, the protective or pathogenic capacity of these cytokines remain
undefined in TB. Furthermore, the source and signaling effects of IFNλ within the context of TB
remains unknown. We have developed two aims to investigate the source and expression pattern
of IFNλ in TB granulomas and determine their impact on macrophage functions-

AIM I: Quantify IFNλs expression in nonhuman primate granulomas and identify
IFNλ-expressing cells and signaling dynamics. IFNλ expression in granulomas remains
unknown but may have implications for granuloma function. We will quantify IFNλ content in
granulomas and non-diseased lung tissue to compare IFNλ expression in infected and uninfected
tissue. Next, we will identify cells in granulomas that express IFNλ1 and IFNλ4, two dissimilar
members of the IFNλ family, and compare expression of these IFNλs in different granuloma
microenvironments. Finally, we will characterize IFNλR1 expression in granulomas and determine
how IFNλR1 ligation and Mtb antigen stimulation affects IFNλ signaling in vitro. These studies
will identify basic aspects of IFNλ biology in granulomas from an experimental system that
mimics the pathobiology of human TB.

AIM II: Compare the effects of IFNλ1, IFNλ4, and type I IFN on macrophage
transcriptional profiles, phenotype, and antimycobacterial activity to identify how these
cytokines affect macrophage function in TB. IFNλs and type I IFNs (IFN1) may have similar
properties because of overlap in their signaling pathways, but this has not been assessed in
macrophages. Moreover, the effect of IFNλ signaling on macrophage function and
antimycobacterial activity have not been investigated. To address these knowledge gaps, we will
perform transcriptional analysis on monocyte-derived macrophages that were stimulated with IFNλ1, IFNλ4, and type 1 interferon to identify differences and similarities in IFNλ- and IFN1-regulated genes. We will follow these studies up by investigating protein-level responses in IFNλ- and IFN1-stimulated macrophages. The consequences of these signaling events on macrophage antimicrobial activity are not well understood, and we will address this by using fluorescent protein-expressing Mtb reporter strains and fluorescent probes to assess macrophage how these cytokines affect macrophage activity against Mtb.
2.0 Macrophages and neutrophils express IFNλ genes in granulomas from *Mycobacterium tuberculosis* infected-nonhuman primates

This section is adapted from the publication:

Priyanka Talukdar, Beth F. Junecko, Daniel S. Lane, Pauline Maiello, Joshua T. Mattila,

Macrophages and neutrophils express IFNλs in granulomas from *Mycobacterium tuberculosis* infected-nonhuman primates, submitted to Frontiers in Immunology, 2022

2.1 Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (Mtb) and claims approximately 4,400 lives per day [45], leading to at least 1.5 million deaths per year [412]. TB is associated with granuloma formation and immune cells in granulomas engage in coordinated activities that limit Mtb replication and dissemination [120]. Immune responses in granulomas are highly regulated and infection outcomes depend on the balance between pro- and anti-inflammatory cytokines [128]. This balance is maintained across heterogenous granuloma microenvironments where different regions vary by oxygen tension, cytokine milieu, necrotic cell abundance, and Mtb antigen concentration, all of which affect cellular activation states and functions [128-130, 190]. When appropriately balanced, granulomas generate sterilizing immunity [123], but deviation from this equilibrium promotes bacterial dissemination, leading to increasingly severe disease [128]. The equilibrium defining these outcomes is not well understood.
but identification of factors that differentiate protective and detrimental outcomes is critically important for understanding TB pathogenesis.

Cytokine-mediated communication regulates granuloma function [128, 190, 411, 413]. Type III IFNs (IFNλs) have important antiviral roles [295, 296, 308, 370] but their function in bacterial infections is not well characterized. The human genome encodes four IFNλ proteins that are sometimes identified by their interleukin identifier including IFNλ1 (IL-29), IFNλ2 (IL-28A), IFNλ3 (IL-28B), and IFNλ4 [144, 295, 296]. IFNλ1-3 have 80-96% amino acid sequence identity [295], whereas IFNλ4 is 28% identical to the other IFNλs. In humans, IFNλ4 is encoded by transcripts with a ΔG allele of a genetic variant rs368243815, while TT allele introduces a frameshift and creates a pseudogene that does not encode IFNλ4 [144, 414]. In contrast, due to the invariant presence of the rs368243815 ΔG allele, non-human primate (NHP) genomes encode only the functional IFNλ4 and not the pseudogene [144, 145]. Chimpanzees and human African hunter gatherer pygmies encode a more active IFNλ4 (E154) that has higher ISG induction and antiviral potentials, whereas majority of humans encode an attenuated version of IFNλ4 due to mutation of a highly conserved amino acid residue (E154K) [300, 301]. IFNλs signal through IFNλR, a heterodimeric receptor consisting of IL28Rα (IFNλR1) and IL-10Rβ [295]. Ligation of the IFNλR1/IL-10Rβ receptor complex induces STAT1/2 phosphorylation and expression of IFNλ-regulated genes, many of which overlap with type 1 IFN (IFNα/β)-regulated genes [295, 297].

Surprisingly little is known about how IFNλs affect immune function during TB. Mtb-infection induces IFNλ2 gene expression in the human lung epithelium-like cell line A549, suggesting that mycobacterial antigens or infection may upregulate IFNλ expression by lung epithelia during TB [404]. Consistent with this, elevated IFNλ2 concentrations are present in sputum from individuals with active TB, whereas lower amounts are present in Mtb-negative or
latently infected individuals [408]. Interestingly, after individuals with active TB were treated with anti-mycobacterial drugs, sputum IFNλ2 concentrations decreased to be equivalent to concentrations seen in healthy individuals, a phenomenon noted as early as 7 days post-treatment [408]. These studies suggest that IFNλ is upregulated in Mtb infection, but they do not identify which cells express IFNλ in the lungs of infected people, if IFNλ is expressed in granulomas, or if granuloma cells respond to IFNλ.

Here, we investigate unanswered questions of IFNλ biology in TB using granulomas from Mtb-infected cynomolgus macaques. This NHP is a well-established model of human TB and has been used to generate critical insights into TB pathogenesis and disease [137, 415]. Like humans, macaques express all four IFNλ proteins, but unlike humans, macaques do not have the rs368243815-TT allele and thus produce IFNλ4 and not the pseudogene [144], thus giving us the ability to investigate this cytokine without being limited by host genotype. We found that granulomas express more IFNλ than uninvolved lung and identified IFNλ1 and IFNλ4 were expressed by macrophages and neutrophils, with variation in expression patterns across different granuloma microenvironments. Interestingly, IFNλ4 was expressed by numerous cells and was unique in being localized in the nuclei of macrophages. IFNλ stimulation induced IFNλR1 localization to the nuclei of human cell lines, monocyte-derived macrophages from macaques, and epithelial cells and other cells in granulomas, suggesting a relationship between receptor nuclear translocation and signaling in vitro and in vivo. Our results provide new insight into IFNλ biology in TB and suggest that IFNλs may have unappreciated roles in anti-mycobacterial immunity.
2.2 Materials and methods

2.2.1 Animal ethics statement and sourcing of macaque tissue samples

Animal procedures and husbandry practices were performed according to protocols approved by University of Pittsburgh’s Institutional Animal Use and Care Committee (IACUC) which adheres to guidelines established in the Animal Welfare Act, Guide for the Care and Use of Laboratory Animals, and Weatherall report (eighth edition). The University of Pittsburgh is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The tissue sections and samples included in this study originated from animals that were necropsied as part of other studies and made available as convenience samples. Briefly, cynomolgus macaques (Macaca fascicularis) were infected with 4-415 CFU of Erdman-strain Mtb via intra-tracheal instillation or aerosol inhalation [136, 137]. At the end of the study, animals were humanely euthanized and necropsied as described previously [124, 136] and tissues were excised and fixed in 10% neutral buffered saline for histology and immunohistochemistry. Fixed samples were paraffin embedded, cut into 5 μm-thick sections and mounted on SuperFrost Plus slides (Thermo Fisher Scientific, Waltham, MA) by the University of Pittsburgh Medical Center’s in situ histology lab. Information on each animal by involvement in this work is included in Tables 1 and 2.
Table 1. Information on animals from which samples originates

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Table 2. Characteristics of samples used for ELISA and IHC based assays

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2.2.2 Immunohistochemistry and fluorescence imaging

A cyclic IHC process, like that described by Lin et al [416], was used for multiple rounds of staining on the same formalin-fixed paraffin-embedded (FFPE) tissue section. FFPE sections were deparaffinized in xylenes and 100% ethanol and then antigen retrieval was performed in a buffer containing 20 mM Tris/820 μM EDTA/0.0001% Tween 20 [pH 9.0] using a Retriever (Pick Cell, Waltham, MA) as previously indicated [190]. Sections were blocked in 1% BSA/PBS for 30 minutes at room temperature before addition of primary antibodies that were diluted in blocking buffer. The slides were washed 3-4 times with 1xPBS and then incubated for 1 hour with species-specific secondaries, or where multiple antibodies from the same species were used, isotype-specific secondary antibodies conjugated with AF488, AF594, or AF647 (Thermo Fisher Scientific, or Jackson ImmunoResearch Laboratories, West Grove, PA). In all cases, antibodies were diluted in blocking buffer. Following incubation in secondary antibodies, slides were washed with 1xPBS and coverslips were applied using ProLong Gold mounting medium containing DAPI (Thermo Fisher Scientific). The mounting medium was cured for 1-2 hours and then the slides were stored at -20°C until they were imaged. After imaging, the slides were incubated in Copland jars containing Milli-Q water until the coverslip fell off and then washed for 20 minutes under gentle shaking at room temperature. Antibodies were stripped off the tissue sections by repeating the process of antigen retrieval (incubation under pressure in antigen retrieval buffer at 121°C for 20 minutes) and stripping was validated by re-mounting a coverslip and reexamining the slide by microscopy. After stripping, the slides were incubated with blocking buffer and a second round of staining with a different combination of primary and secondary antibodies were applied to the
tissue section before a coverslip was mounted with DAPI ProLong Gold and the slide was reimaged.

Tissue sections were first stained to detect macrophage and neutrophil IFN\(\lambda\)1 expression and then stripped to visualize IFN\(\lambda\)4 expression in macrophages and neutrophils. Staining was performed as previously described [129]. To ensure that our results did not include crosstalk between different rounds of staining for cytokine expression, we used different fluorochromes to visualize and quantify IFN\(\lambda\)1 (AF594) and IFN\(\lambda\)4 (AF488) expression. Moreover, the success of stripping the previous round of anti-calprotectin staining (AF488-stained neutrophils) was confirmed visually before beginning analysis of the sections in the second round of staining. The differential localization of these cytokines was also compared and the results of these analyses are included in the Results section.

Antibodies used for staining tissues included CD11c (clone 5D11, 1:30 dilution; Leica Microsystems, Buffalo Grove, IL), calprotectin/S100A9 (clone MAC387, 1:30 dilution; Thermo Fisher Scientific), polyclonal IFN\(\lambda\)1 (1:30 dilution; R&D Systems, Minneapolis, MN), monoclonal IFN\(\lambda\)4 (clone 4G1, 1:50 dilution; EMD Millipore, Burlington, MA) and IFN\(\lambda\)R1 (1:50 dilution; Sigma Aldrich, St. Louis, MO). Human and non-human primate IFN\(\lambda\)1, IFN\(\lambda\)4 and IFN\(\lambda\)R1 transcripts share greater than 90% nucleotide sequence similarity with each other and therefore we expected the anti-human IFN\(\lambda\)1, IFN\(\lambda\)4 and IFN\(\lambda\)R1 antibodies to work in non-human primates. For IFN\(\lambda\)4 staining, a directly labeled conjugate of calprotectin-AF594 was used because both anti-calprotectin and anti-IFN\(\lambda\)4 antibodies were mouse IgG1 antibodies. Zenon direct labeling kit (Thermo Fisher Scientific) was used to conjugate calprotectin with AF594. Granulomas were imaged with a Nikon Eclipse E1000 epifluorescence microscope (Nikon Instruments, Melville, NY) at 20x magnification with illumination provided by SOLA light engine (Lumencor, Beaverton, OR) and images captured with a DS-Qi2 camera (Nikon.
Instruments). NIS-Elements AR 4.50 software (Nikon Instruments) was used for image capture and setting imaging parameters which were fixed across all the granuloma images. Four color channels, with DAPI as the fourth channel, were acquired for all images. Animals used in IHC are mentioned in Table 2.

2.2.3 Image analysis

QuPath version 0.2.1 software [417] was used to measure IFNλ expression and fluorescence intensity in granulomas. For quantifying these metrics, whole granuloma images were loaded into QuPath and the cells were classified as neutrophils and macrophages based on calprotectin and CD11c expression, respectively, using a high threshold to eliminate non-specific background signal and ensure only cells that truly expressed these antigens were being analyzed. The threshold intensity for defining IFNλ signal was based on the isotype control and background staining of each tissue section. After classification of positive and negative signal for each channel, the cells were segmented by QuPath based on DAPI signal and the channel intensity measurements for each cell were recorded. Since our measurements are using mean pixel intensity per cell, which normalizes fluorescence per unit area per cell type, we do not expect the different sizes of cell types to impact the interpretation of the intensity data. For analysis of region-based IFNλ intensities, manual segmentation yielded the most accurate results. For these analyses, at least 100-300 neutrophils at the caseum-epithelioid macrophage interface or in the lymphocyte cuff, and macrophages in the epithelioid macrophage region adjacent to caseum or in the lymphocyte cuff were chosen. After all the annotations were selected, the detection measurements were exported which contained mean measurements of individual channels for each cell, as well as for cell nuclei.
and cytoplasm. QuPath detections were used in CytoMAP version 1.4.7 [418] to generate the spatial map of IFNλ expression in granulomas.

2.2.4 BCA protein quantification and ELISA

Protein levels in supernatants from homogenized granulomas and non-diseased lung lacking bacterial loads and without granulomas (Tables 1 and 2) was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. Samples with detectable protein levels were selected for IFNλ level detection by ELISA using a human IL-29/IL-28B (IFN-lambda 1/3) DuoSet ELISA kit (R&D Systems), and the assay was performed according to the manufacturer’s protocol. For reporting data, the IFNλ content was normalized to micrograms of total input protein.

2.2.5 Flow cytometry

Non-diseased lung was obtained from Mtb-infected macaques (Table 1) being necropsied as part of ongoing studies. These tissues were mechanically disaggregated with a Medimachine tissue processor (BD Biosciences, San Jose, CA) and single cell suspensions were stained to detect IFNλR1 expression. Samples were stained for viability (Aqua viability dye, Thermo Fisher Scientific) and surface and intracellular markers according to standardized protocols. The antibody panel for IFNλR1 detection in lung tissue included surface marker staining for IL28RA (an alternate name for IFNlR1; Clone MHLICR2a, BioLegend, San Diego, CA), CD45 (Clone D058-1283, BD Biosciences), CD206 (Clone 19.2, BD Biosciences), CD3 (Clone SP34-2, BD

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Biosciences, CD20 (Clone 2H7, BD Biosciences), CD14 (Clone MφP9, BD Biosciences), CD11b (Clone ICRF44, BD Biosciences), and intracellular staining for calprotectin (Clone MAC387, Thermo Fisher Scientific) labeled by Zenon labeling was used to identify neutrophils. The gating strategy for tissue cells is shown in Figure 5. As a gating control and to compare IFNλR1 expression in peripheral blood cells and lung tissue, erythrocytes in an aliquot of autologous peripheral blood were lysed using RBC lysing buffer (BD Biosciences) and the nucleated cells were stained at the same time as the tissue cells with the same antibody cocktail. Data were acquired with a LSRFortessa flow cytometer (BD Biosciences) and analyzed with FlowJo v10 (BD Biosciences).

Figure 5. Gating strategy to detect IFNλR1 in lung tissue cells.
2.2.6 Differentiation of monocyte derived macrophages (MDMs) and cell culture

Monocytes were isolated from macaque peripheral blood mononuclear cells (PBMCs) (Supplementary table 1) and cryopreserved using CellBanker II freezing medium (Amsbio, Cambridge, MA). After thawing, cells were labeled with NHP-specific anti-CD14 beads (Miltenyi Biotec, Auburn, CA) according to manufacturer’s instructions. Isolated monocytes were plated in 12-well flat bottom plates that were coated with Anti-Adherence Rinsing Solution (STEMCELL technologies, Cambridge, MA), at a density of 1-1.5x10^6 cells/well in RPMI 1640 media (Lonza, Walkersville, MD) supplemented with 20% FBS (Gibco, Grand Island, NY), 1% L-glutamine (Sigma-Aldrich St. Louis, MO), 0.1 mM sodium pyruvate (Gibco), 50 μM 2-mercaptoethanol (Gibco), 0.006 μg/ml GM-CSF (Sigma-Aldrich), 0.01 μg/ml M-CSF (Sigma-Aldrich) and 100 U/ml penicillin-streptomycin (Gibco). Media was changed to RPMI 1640 media supplemented with 10% FBS, 1% HEPES (HyClone, Logan, UT), 1% L-glutamine (hereafter referred to as R10) and 1mg/ml penicillin (Alfa Aesar, Haverhill, MA). Monocytes were cultured for 7-10 days for differentiation into macrophages with media change every 3-4 days. For studies using human cell lines, monocyte-like THP-1 and lung epithelium-like A549 cell lines were originally purchased from ATCC (Manassas, VA), and were cultured in RPMI/10% FBS supplemented with 100 U/ml penicillin-streptomycin and 50 μM 2-mercaptoethanol (only in THP-1 cell cultures) for 3-4 days before being subcultured for downstream assays.
2.2.7 IFNλ.R1 nuclear localization assay

A549 and MDMs were seeded into 12-well chamber slides (ibidi, Fitchburgh, WI) and stimulated with IFNλ1 (100 ng/ml, Peprotech, Cranbury, NJ), IFNλ4 (100 ng/ml, R&D Systems) and gamma-irradiated Mtb (BEI Resources, Manassas, VA) and incubated at 37°C with 5% CO₂ for 2 hours. After incubation, cells were fixed and permeabilized with the BD Cytofix/Cytoperm kit (BD Biosciences) and washed with 1xPerm-Wash buffer. Assays with THP-1s were done in round-bottom tubes (Corning, Glendale, Arizona). For the TLR1/2 and TLR4 blocking assays, cells were incubated with 2 µM CU CPT 22 (Tocris Bioscience, Minneapolis, MN) and 20 µM C34 (Tocris Bioscience), respectively, for 30 minutes, before addition of gamma-irradiated Mtb. After incubation with gamma-irradiated Mtb, the cells were fixed and cytospin was performed. Cells were then blocked in 1% BSA/PBS containing AF647-labeled phalloidin (1:40 dilution; Thermo Fisher Scientific) for 30 minutes at room temperature, prior to addition of primary and secondary antibodies diluted in 1xPerm-Wash buffer. Anti-IFNIR1 and fluorochrome-conjugated secondary antibody were used at the same dilution as for the IHC experiments described above. After staining, cells were washed in Perm-Wash buffer and coverslips were applied using Prolong Gold mounting medium containing DAPI (Thermo Fisher Scientific). Slides were imaged with an epifluorescence microscope (Nikon Eclipse E1000) at 40x magnification, and a Nikon camera (DS-Qi2) was used to capture the images as previously described.
2.2.8 Statistics

GraphPad Prism v9.1 (GraphPad Software, San Diego, CA) was used for statistical analyses. None of our analyses used cross-antibody (IFNλ1 vs IFNλ1) tests to avoid confounding factors induced by antibody affinity and avidity-related issues. The Shapiro-Wilk test was used to test the normality of all datasets before performing statistical analyses and parametric tests were used for normally-distributed data and non-parametric tests were used for data that did not fit a Gaussian (normal) distribution. P< 0.05 was considered to be statistically significant.

2.3 Results

2.3.1 IFNλ1/3 are expressed in lung granulomas from Mtb-infected macaques

*IFNλ1* and *IFNλ2* genes are upregulated by A549 lung epithelial cells after Mtb stimulation [404] and elevated IFNλ2 protein concentrations are present in sputum from TB patients [408]. To determine whether IFNλ is expressed in granulomas, we compared IFNλ1/3 protein concentrations in non-diseased lung (no bacteria or granuloma present) and lung granulomas from matched as well as unmatched animals and found significantly more IFNλ1/3 in granulomas than non-diseased lung (Fig. 6A). Further, a correlation analysis between IFNλ1/3 concentrations and CFU burden in the granulomas revealed a significant negative correlation between IFNλ1/3 concentration and CFU/granuloma (Fig. 6B), suggesting IFNλ may be associated with improved antibacterial activity.
Figure 6. IFNλ expression in lung granulomas negatively correlates with bacterial burden. 

(A) IFNλ1/3 concentrations in non-diseased uninvolved lung (n=8) and lung granulomas (n=10) were normalized to total protein per sample and compared by ELISA. Bars represent median values. Statistical comparison by Mann-Whitney test. (B) Correlation between log_{10} transformed bacterial burden per granuloma and IFNλ1/3 concentration per granuloma. Pearson correlation coefficient and corresponding p-value reported and simple linear regression line shown.

This led us to use IHC to identify cells expressing IFNλ1 in granulomas. We decided to stain for IFNλ1 as it shares greater than 90% similarity at the amino acid level with IFNλ2 and IFNλ3 and is well studied in humans. Importantly, in our preliminary experiments, we found that the commercially available reagents for IFNλ1 appeared to work better in NHPs than the reagents we tested for IFNλ2/3 and as a consequence, we continued our follow-up studies by investigating IFNλ1 expression. We randomly selected thirteen granulomas from nine animals, including five animals that had short-term infections (4 weeks; n=7 granulomas), and four that had long-term
infection (26-50 weeks, n=6 granulomas) to assess this. A classical granuloma structure is composed of a central necrotic (caseous) core of necrotic cell debris, surrounded by a layer of epithelioid macrophages, followed by an outer layer referred to as the lymphocyte cuff that contains T and B cells, but also contains macrophages (Fig. 7A). We used CD11c as a macrophage marker because it is expressed by alveolar and epithelioid macrophages [129, 419], and calprotectin as a neutrophil marker [129]. We found that IFNλ.1 was expressed by macrophages and neutrophils (Fig. 7B). We used image analysis to identify the frequency of IFNλ.1-expressing macrophages, neutrophils, and the other cells not labeled by our markers. We found that neutrophils were the cell subset most likely to express IFNλ.1, followed by macrophages (Fig. 7C). Further, we measured IFNλ.1 intensity/cell as a proxy for IFNλ.1 expression by cell type and found that neutrophils expressed significantly more IFNλ.1 than macrophages (Fig. 7D). Overall, these data show that granulomas express higher levels of IFNλ compared to uninvolved lung and that macrophages and neutrophils contribute to IFNλ.1 expression in granulomas.
Figure 7. IFNλ1 is expressed in macrophages and neutrophils in granulomas.

(A) A representative lung granuloma stained with hematoxylin and eosin (H&E; top left) to indicate the lymphocyte cuff (LC), epithelioid macrophage (EM), and caseous (C) regions and to detect IFNλ1 (red), CD11c+ macrophages (blue), and calprotectin+ neutrophils (green) (bottom left). Nuclei were stained with DAPI (grey). The white boxes in the immunofluorescence image indicate regions depicted in panel B. Scale bar represents 500 μm. (B) Region 1 shows IFNλ1 (red) expression in CD11c+ macrophages (blue). Region 2 shows IFNλ1 (red) expression in calprotectin+ neutrophils (green). Images acquired at 20x magnification, scale bars represent 50 μm. (C) Percentage of CD11+ macrophages, calprotectin+ neutrophils, and other cells expressing IFNλ1 in granulomas (n=13). Median values for granuloma are shown where each
marker color represents an animal. Statistical comparison by Tukey’s multiple comparisons test. (D) IFNλ1 expression, as measured by median fluorescence intensity, by CD11c+ macrophages and calprotectin+ neutrophils (n=13 granulomas). Each point depicts the median intensity values for macrophages or neutrophils per granuloma, with each marker’s color representing a different animal. Lines connect cells from the same granuloma. Statistical comparison by Wilcoxon matched-pairs signed rank test.

2.3.2 IFNλ1 expression differs by cell type and granuloma microenvironment

Granulomas contain unique microenvironments [129] and we performed spatial analyses to identify IFNλ1’s distribution by granuloma region. We found that IFNλ1 was expressed by lymphocyte cuff cells and adjacent to necrotic regions. When the cell types in each region were considered, we found that macrophages and neutrophils in the lymphocyte cuff and neutrophils in necrotic regions expressed IFNλ1 (Fig. 8A). To investigate differences in IFNλ1 expression by cell type between these regions, we quantified the intensity of IFNλ1 fluorescence by macrophages in the lymphocyte cuff and epithelioid macrophage region and neutrophils in lymphocyte cuff and necrotic regions as a proxy for IFNλ1 protein content (Fig. 8B). Pairwise comparisons revealed that lymphocyte cuff macrophages expressed significantly more IFNλ1 than epithelioid macrophages whereas neutrophils in lymphocyte cuff and necrotic regions expressed equivalent amounts (Fig. 8B). We then compared the IFNλ1 intensity across macrophages and neutrophils in these regions and found that epithelioid macrophages expressed less IFNλ1 than lymphocyte cuff neutrophils and macrophages (Fig. 8C). Since the animals involved in this study were infected for different durations, i.e., some necropsied during early infection (≤4 weeks p.i) and others during
late infection (26-50 weeks p.i), we assessed whether IFNλ1 expression differed in granuloma macrophages and neutrophils from animals with early or late infection. We found greater IFNλ1 expression in lymphocyte cuff macrophages relative to epithelioid macrophages in granulomas harvested later during infection but not early infection (Fig. 8D). In contrast, there were not significant differences between lymphocyte cuff and caseum neutrophils in granulomas from either infection stage (Fig. 8D). These data suggest that IFNλ1 expression varies in macrophages from different granuloma microenvironments, which may differentially influence the functions of neighboring cells in the granuloma.
Figure 8. IFNλ1 expression varies by granuloma microenvironment.

(A) A representative granuloma stained to identify IFNλ1 (red) expressed by CD11c+ macrophages (blue) and calprotectin+ neutrophils (green). Scale bar represents 500 μm. Spatial distribution of macrophages (blue) and neutrophils (green) in the granuloma, distribution of IFNλ1 (red), and distribution of IFNλ1+ macrophages (blue) and neutrophils (green). (B) Comparison of IFNλ1 expression, as measured by median fluorescence intensity for each cell subset per granuloma, for epithelioid and lymphocyte cuff macrophages (n=13) (left), and caseum...
and lymphocyte cuff neutrophils (n=10) (right). Statistical comparisons by paired t test. (C) Comparison of median IFNλ1 intensity in epithelioid macrophages, lymphocyte cuff macrophages, caseum neutrophils, and lymphocyte cuff neutrophils (n=13 granulomas). A mixed effect test used to account for repeated measures and pairwise groups compared using Tukey’s multiple comparisons test (Tukey adjusted p-values reported). (D) Comparison of IFNλ1 expression, as measured by median fluorescence intensity, between epithelioid and lymphocyte cuff macrophages (left) in granulomas harvested within 4 weeks post-infection (n=7) or 26-50 weeks post-infection (n=6). A similar comparison of IFNλ1 expression by caseum and lymphocyte cuff neutrophils (right) from granulomas harvested by 4 weeks post-infection (n=5) or between 26-50 weeks post-infection (n=5). Statistical comparisons by paired t test.

2.3.3 IFNλ4 is expressed in macaque granulomas

We also investigated IFNλ4 expression, the IFNλ with the greatest sequence divergence from the other IFNλs and found IFNλ4 expression by macrophages, neutrophils, and other granulomas cells (Fig. 9A, 9B). To better understand the distribution of IFNλ4+ cells within granuloma macrophages and neutrophils, we quantified the frequency of IFNλ4+ expressing cells in FFPE granulomas. We found that calprotectin+ neutrophils were more likely to express IFNλ4 than CD11c+ macrophages, and that macrophages were more likely to express IFNλ4 than non-neutrophil and non-macrophage subsets (Fig. 9C). Further, pairwise comparison of the intensity of IFNλ4 staining as a proxy for IFNλ4 expression revealed that neutrophils expressed more IFNλ4 than macrophages (Fig. 9D).
Figure 9. IFNλ4 is expressed in macrophages and neutrophils in granulomas.

(A) A representative lung granuloma stained with H&E (top left) to indicate the lymphocyte cuff (LC), epithelioid macrophage (EM), and caseous (C) regions and to detect IFNλ4 (red), CD11c+ macrophages (blue), and calprotectin+ neutrophils (green) (bottom left). Nuclei were stained with DAPI (grey). The white boxes in the immunofluorescence image indicate regions depicted in (B.) Scale bar represents 500 μm. (B) Region 1 shows IFNλ4 (red) expression in CD11c+ macrophages (blue). Region 2 shows IFNλ4 (red) expression in calprotectin+ neutrophils (green). Images acquired at 20x magnification, scale bars represent 50 μm. (C) Percentage of CD11+ macrophages, calprotectin+ neutrophils, and other cells expressing IFNλ4
in granulomas (n=13). Median values for granuloma are shown where each marker color represents an animal. Statistical comparison by Tukey’s multiple comparisons test. (D) IFNλ4 expression by CD11c+ macrophages and calprotectin+ neutrophils as measured by median fluorescence intensity by cell subset per granuloma (n=13 granulomas). Each point depicts the median values for macrophages or neutrophils per granuloma, with each marker’s color representing a different animal. Statistical comparison by Wilcoxon matched-pairs signed rank test.

In our pilot experiments, we were surprised by the abundance of IFNλ4 in different cell types, and to verify that our IHC-based staining was representative of the overall capacity to express IFNλ4, we used RNAscope with probes against IFNλ4 mRNA to detect this cytokine’s transcripts in situ (Fig. 10). We performed this assay in conjunction with IHC to detect CD163 as an alveolar macrophage marker and found that IFNλ4 mRNA was detectable in a broad range of cell types in non-diseased lung but was enriched in alveolar macrophages (Fig. 10). These data provided support that our antibody-based detection of IFNλ4 was representative for this protein’s expression, and to accommodate our sample set, we proceeded with IFNλ4 IHC-based staining and analysis of NHP granulomas.
Figure 10. IFNλ4 mRNA is expressed by cells in non-diseased lung, including alveolar macrophages.

*IFNλ4 mRNA are visible as green puncta in proximity to DAPI-stained nuclei (blue) and CD163 (red) counterstaining was used to visualize alveolar macrophages in non-diseased lung from an animal with TB.*

Next, we investigated the spatial distribution of IFNλ4 to determine where it was most likely to be expressed by macrophages and neutrophils in granulomas. We observed that IFNλ4 was widely expressed in granulomas, with prominent lymphocyte cuff expression and differences in IFNλ4+ macrophage and neutrophil localization (Fig. 11A). To identify whether macrophage and neutrophil IFNλ4 expression varied by microenvironment, we performed pairwise comparisons on IFNλ4 signal intensity (expression) between macrophages in lymphocyte cuff and epithelioid macrophage regions, and calprotectin+ neutrophils in the lymphocyte cuff and adjacent to caseum. We did not find differences in IFNλ4 expression between spatially-distinct macrophage
and neutrophil populations (Fig. 11B), but a comparison among these cell populations showed lymphocyte cuff neutrophils expressed more IFNλ4 than epithelioid macrophages (Fig. 11C).

After investigating relative IFNλ4 expression by macrophages and neutrophils in different granuloma regions, we stratified our granulomas by the time point post infection to determine whether duration of infection affects IFNλ4 expression. We did not find significant differences between macrophage populations in animals with early-stage disease, whereas lymphocyte cuff macrophages expressed more IFNλ4 than epithelioid macrophages from animals with long-term infections (Fig. 11D). On examining neutrophil IFNλ4 expression, we found that lymphocyte cuff neutrophils from animals with early-stage TB expressed more IFNλ4 than neutrophils in caseum, whereas differences were not observed in granulomas from animals with later-stage disease (Fig. 11D). These data indicate that IFNλ4 is expressed in granulomas, primarily by macrophages and neutrophils, and its expression in different cell types can be influenced by the duration of infection.
Figure 11. IFNλ4 expression varies by granuloma microenvironment.

(A) A representative granuloma stained to identify IFNλ4 (red) expressed by CD11c+ macrophages (blue) and calprotectin+ neutrophils (green) (right). Scale bar represents 500 μm. Spatial distribution of macrophages (blue) and neutrophils (green) in the granuloma, distribution of IFNλ4 (red), and distribution of IFNλ4+ macrophages (blue) and neutrophils (green). (B) Comparison of IFNλ4 expression, as measured by median fluorescence intensity per cell subset per granuloma, for epithelioid and lymphocyte cuff macrophages (n=13) (left), and caseum and
lymphocyte cuff neutrophils (n=10) (right). Statistical comparisons by paired t test. (C) Comparison of median IFNλ4 intensity in epithelioid macrophages, lymphocyte cuff macrophages, caseum neutrophils, and lymphocyte cuff neutrophils (n=13 granulomas). A mixed effect test used to account for repeated measures and pairwise groups compared using Tukey’s multiple comparisons test (Tukey adjusted p-values reported). (D) Comparison of IFNλ4 expression, as measured by fluorescence intensity, between epithelioid and lymphocyte cuff macrophages (left) in granulomas harvested within 4 weeks post-infection (n=7) or 26-50 weeks post-infection (n=6). A similar comparison of IFNλ4 expression by caseum and lymphocyte cuff neutrophils (right) from granulomas harvested by 4 weeks post-infection (n=5) and between 26-50 weeks post-infection (n=5). Statistical comparisons by paired t test.

2.3.4 IFNλ1 and IFNλ4 differ in their subcellular localization

We noted differences in IFNλ1 and IFNλ4 subcellular localization across cell types. When granulomas were stained with both antibodies simultaneously in conjunction with CD11c as a macrophage marker, we noted different patterns of IFNλ expression in different cell regions including strong localized IFNλ1 expression by infiltrating neutrophils (Fig. 12, region 1), pockets of alveolar macrophage-like cells in the lymphocyte cuff where cytoplasmic IFNλ1 was co-expressed with nuclear IFNλ4 (Fig. 12, region 2), and other clusters of macrophages that expressed low levels of cytoplasmic IFNλ1 but stained robustly for IFNλ4 (Fig. 12, region 3).
Figure 12. Co-staining for IFNλ1 and IFNλ4 reveals different patterns of expression for these cytokines in granulomas.

A granuloma was stained for both IFNλ1 and IFNλ4 and three patterns of IFNλ expression was highlighted including strong cytoplasmic IFNλ1 expression in a cluster of infiltrating neutrophils (region 1), cytoplasmic IFNλ1 and nuclear IFNλ4 expression in lymphocyte cuff macrophages (region 2) and limited cytoplasmic IFNλ1 expression and robust nuclear IFNλ4 expression in epithelioid macrophage-like cells (region 3).

IFNλ4 was primarily found in DAPI-negative euchromatic regions of macrophage nuclei, while it was more distributed in the cytoplasm of neutrophils (Fig. 13A). To compare subcellular localization of IFNλ1 and IFNλ4, we segmented the cells and measured each cytokine’s presence
in nuclei and cytoplasm. We did not find significant difference between subcellular compartments for IFNλ1 in macrophages from lymphocyte cuff or epithelioid macrophage regions (Fig. 13B). In contrast, IFNλ4 localized to nuclei rather than cytoplasm of macrophages in both microenvironments (Fig. 13C). For neutrophils, IFNλ1 and IFNλ4 were present at greater levels in the nucleus of lymphocyte cuff neutrophils, but this difference in subcellular signal intensities was not observed for either cytokine when the neutrophils were adjacent to caseum (Fig. 13D, 13E). We also noted that the difference between nuclear and cytoplasmic signal for IFNλ4 was higher in lymphocyte cuff macrophages (difference in medians = 181.5) and epithelioid macrophages (difference in medians = 116.0), than for lymphocyte cuff neutrophils (difference in medians = 86.6) and neutrophils in the caseum (difference in medians = 21.9). Overall, these data highlight that despite belonging to the same family, IFNλ1 and IFNλ4 have different subcellular localization in macrophages, suggesting they may regulate different cell functions or behaviors.
Figure 13. IFNλ1 and IFNλ4 differ in their subcellular localization in macrophages.

(A) IFNλ4 (red) localization in calprotectin+ neutrophils (green) and in the nuclei (grey) of CD11c+ macrophages (blue). 40x magnification, scale bars represent 50 mm. (B) Comparison of IFNλ1 intensity in the nuclei and cytoplasm of epithelioid (left) and lymphocyte cuff macrophages (right). (C) Comparison of IFNλ4 intensity in the nuclei and cytoplasm of epithelioid (left) and lymphocyte cuff macrophages (right). (D) Comparison of IFNλ1 intensity in the nuclei and cytoplasm of caseum (left) and lymphocyte cuff neutrophils (right). (E) Comparison of IFNλ4
intensity in the nuclei and cytoplasm of caseum (left) and lymphocyte cuff neutrophils (right). In
B-E, n=13 granulomas and statistical comparisons done by paired t test.

2.3.5 IFNλ is expressed in human TB granulomas

We stained a section of a human lung granuloma that was previously used to define
macrophage subsets in human TB [129] to determine whether IFNλ was detected in this lesion.
Similar to what we saw in NHP granulomas, we detected IFNλ1 expression in CD11c+
macrophages (Fig. 14A). We stained the same granuloma to investigate IFNλ4 expression and
found that IFNλ4 was expressed by macrophages and localized to the nuclei, as we saw previously
in NHP granulomas (Fig. 14B). Expression of IFNλ4 in this granuloma indicate that IFNλ4 was
expressed in its functional form and was not a pseudogene in this individual. Overall, these data,
on a very limited basis, suggest there are similarities in IFNλ expression between NHP and human
TB granulomas.
Figure 14. IFNλs are expressed in a human TB granuloma.

(A) IHC image of a human lung granuloma showing IFNλ1 localization in CD11c+ macrophages. (B) IHC image showing IFNλ4 localization in the nuclei of CD11c+ macrophages in the same human lung granuloma.
2.3.6 IFNλ.R1 is expressed in NHP lung granulomas

Since we identified IFNλ expression in granulomas, we next wanted to identify IFNλ.R1 expression to determine whether granuloma cells can respond to IFNλ. In preliminary work using flow cytometry to measure IFNλ.R1 in peripheral blood, we found that myeloid cells including monocytes and neutrophils were more likely to express IFNλ.R1 than T cells and B cells (Fig. 15A). To compare this pattern to lung tissue, we stained non-diseased lung tissues from the same animals and found that CD206+ alveolar macrophages were more likely to express IFNλ.R1 than other immune cells (Fig. 15B). To refine our understanding of granuloma IFNλ.R1 expression, we stained FFPE sections for IFNλ.R1, IFNλ.1, and CD163 as a macrophage and ciliated epithelium marker [129, 420]. In a section where a granuloma was invading an airway and was adjacent to ciliated epithelia, which would be anticipated to express IFNλ.R1, we noted strong IFNλ.R1 expression on the apical surface of ciliated epithelial cells (Fig. 15C). Interestingly, we also observed IFNλ.R1 localizing to the nuclei of some epithelial cells and macrophage-like cells (Fig. 15C) suggesting that IFNλ.R1 may translocate to the nucleus as has been observed for other IFN receptors [421-423].

To determine whether IFNλ. signaling is associated with IFNλ.R1 translocation to the nucleus, we performed in vitro experiments measuring IFNλ.R1 dynamics in human cell lines and macaque monocyte-derived macrophages (MDMs). Stimulation of A549 cells with IFNλ.1 induced IFNλ.R1 translocation from the membrane into the nucleus (Fig. 15D). While we observed only a trend of increased overall IFNλ.R1 signal in A549 after IFNλ.1 stimulation, the nuclear IFNλ.R1 signal intensity was significantly elevated (Fig. 15E). However, we did not observe significant
changes in IFNλR1 dynamics in IFNλ4-stimulated A549 cells (Fig. 15F). In MDMs, however, both IFNλ1 and IFNλ4 induced significant increases in both overall and nuclear IFNλR1 intensities (Fig. 15G, 15H, 15I) indicating this behavior occurs in response to diverse members of this cytokine family in macrophages.

Figure 15. IFNλR1 localizes to the nuclei of macrophages and epithelial cells in granulomas.

(A) IFNλR1 expression in different immune cell types from whole blood. (B) IFNλR1 expression in immune cells from macaque lung tissue (n=7). Friedman test was performed and pairwise groups compared using Dunn’s multiple comparisons test (Dunn’s adjusted p-values are
reported. (C) Detection of IFNλR1 (green), IFNλ1 (red) and CD163 (blue) in a lung granuloma. Arrows indicate instances of IFNλR1 localized to nuclei. (D) A549 lung epithelial cells stained for IFNλR1 (green), actin (red) and DAPI (blue) after IFNλ1 stimulation. (E), (F) Comparison of overall (left) and nuclear (right) IFNλR1 intensity in A549 epithelial cells, following IFNλ1 (E) and IFNλ4 (F) stimulations showing the mean value and standard deviation of 8 independent assays. Statistical comparisons by paired t test. (G) MDMs stained for IFNλR1 (green), actin (red) and DAPI (blue) after IFNλ1 stimulation. (H), (I) Comparison of overall (left) and nuclear (right) IFNλR1 intensity in monocyte-derived macrophages, following IFNλ1 (H) and IFNλ4 (I) stimulations (n=8). Each point depicts the median IFNλR1 value in macrophages, with each marker’s color representing a different animal. Statistical comparisons by paired t test (H) and paired t test for overall IFNλR1 intensity or Wilcoxon matched-pairs signed rank test (I).

We next wanted to investigate whether Mtb antigens induce IFNλR1 translocation as an indicator of IFNλ signaling. Stimulating A549 cells with gamma-irradiated Mtb did not significantly upregulate overall or nuclear IFNλR1 expression (Fig. 16A), whereas gamma-irradiated Mtb-stimulated MDMs had increased overall and nuclear IFNλR1 expression (Fig. 16B). We previously demonstrated that neutrophil cytokine expression could be antagonized by inhibiting toll like receptor (TLRs) signaling [190], so we sought to determine how antagonizing TLRs affect nuclear translocation of IFNλR1. Myeloid cells responded more strongly than A549 cells, so we used the human monocyte-like THP-1 cell line in our initial experiments and compared nuclear IFNλR1 localization after inhibition of TLR signaling by the TLR1/2 and TLR4 antagonists CU CPT22 and C34, respectively. We found that CU CPT22, but not C34, inhibited
Mtb-mediated nuclear IFNλR1 translocation in THP-1 cells (Fig. 16C). We observed a similar and significant decrease in nuclear IFNλR1 intensity when MDMs were treated with CU CPT22 (Fig. 16D), suggesting that IFNλ expression and signaling in myeloid cells is at least partially regulated by TLR1/2 signaling. Overall, our data suggest that like type I and II IFNs, IFNλ signaling can include nuclear translocation of IFNλR1 and that Mtb antigens can activate the TLR1/2 pathway in myeloid cells, potentially leading to IFNλ-mediated responses in granuloma cells.

Figure 16. TLR2 mediated signaling by gamma-irradiated Mtb regulates IFNλR1 expression and localization in myeloid cells.
(A) Comparison of overall (left) and nuclear (right) IFNλR1 intensity in gamma-irradiated Mtb-stimulated A549 epithelial cells. Bars and lines represent the mean value and standard deviation from 5 independent experiments with statistical comparisons by paired t test. (B) Overall (left) and nuclear (right) IFNλR1 intensity in gamma-irradiated Mtb-stimulated macaque monocyte derived macrophages (MDMs; n=6). Each point depicts the median IFNλR1 value in macrophages, with each marker’s color representing a different animal. Statistical comparisons by Wilcoxon matched-pairs signed rank test and paired t test, respectively. (C) Comparison of nuclear IFNλR1 intensity in gamma-irradiated Mtb stimulated THP-1 cells with or without CU CPT22 (TLR2 antagonist) and C34 (TLR4 antagonist). Bars and lines represent mean values and standard deviation of 7 independent experiments. RM one-way ANOVA used to account for repeated measures and pairwise groups compared using Tukey’s multiple comparisons test (Tukey’s adjusted p-values reported). (D) Comparison of nuclear IFNλR1 intensity in gamma-irradiated Mtb-stimulated macaque MDMs with or without CU CPT22 (TLR2 antagonist) and C34 (TLR4 antagonist) (n=6). Each point depicts the median IFNλR1 value for an animal’s MDMs, with each marker’s color representing a different animal. RM one-way ANOVA used to account for repeated measures and pairwise groups compared using Tukey’s multiple comparisons test (Tukey’s adjusted p-values reported).

2.4 Discussion

IFNλs are regulators of innate immunity in the lungs [309]. Many studies have focused on viral infections where IFNλ is expressed by epithelial and myeloid cells at mucosal surfaces [308,
Like the type 1 IFNs, IFNλ expression is triggered by detection of microbe-associated molecular patterns through pattern recognition receptors [424]. Bacterial ligands including lipopolysaccharide and agonists of TLR1/2, TLR4, TLR5 and TLR9 can also induce IFNλ expression [303, 402, 405]. IFNλs have received little attention in host responses to Mtb infection aside from data from Mtb-infected A549 lung epithelial cells [404] and the sputum from TB patients [408]. The presence and source of IFNλ expression in granulomas has remained undefined. Here, investigated two IFNλs, IFNλ1 and IFNλ4, to determine whether they contribute to a granuloma’s cytokine milieu. Of the four IFNλs, IFNλ1 is the best studied isoform in humans and shares a high degree of amino acid sequence similarity with IFNλ2 and IFNλ3, and so we selected it as a representative IFNλ family. In contrast, IFNλ4 is less conserved at the nucleotide and amino acid level, and expression has been selected against in both NHPs and humans [144, 425]. Our findings demonstrate that IFNλ1 and IFNλ4 are expressed in granulomas but differ in some aspects of their biology, suggesting they have previously unappreciated functions in TB.

Microenvironment-specific cytokine expression may influence a granuloma’s ability to control bacteria. We observed differences in IFNλ expression across granuloma microenvironments and showed that macrophage subsets in the lymphocyte cuff region were more likely to express IFNλ1 than epithelioid macrophages. IFNλ1 has Th1 skewing properties [344, 360, 426] and elevated IFNλ1 expression by lymphocyte cuff macrophages may promote Th1 polarization in T cells, thus promoting macrophage activation and control of bacilli. Relatedly, our ELISA data suggested a negative correlation between IFNλ and granuloma bacteria loads, supporting this possibility. We also observed that lymphocyte cuff macrophages expressed more IFNλ4 than epithelioid macrophages, primarily in granulomas from animals with long-term Mtb
infection. Less is known about IFNλ4’s function in immunity, and while specific polymorphic IFNλ4 genotypes are associated with liver fibrosis in chronic hepatitis C infection [427], it remains to be determined whether IFNλ4 promotes fibrosis in pulmonary TB. Taken together, we hypothesize that IFNλ expression is likely to be related to a cell’s activation state. The differences in region-specific macrophage IFNλ expression highlight variation in macrophage functional capacity across microenvironments characterized by different immunologic and microbiologic stimuli and suggest new routes by which macrophages may engage with neighboring cells.

Neutrophils are often found in granulomas where they are associated with poor outcomes [186, 189, 428] but are also linked to protection in some settings [429, 430]. We previously showed that neutrophils express cytokines in granulomas [190], and our work here extends that to expression of IFNλ1 and IFNλ4. Interestingly, neutrophils appeared to be a major population expressing IFNλ in granulomas, producing comparatively higher levels of IFNλs than macrophages. Unlike macrophages, neutrophils in different granuloma microenvironments expressed almost equivalent levels of IFNλs. IFNλs have been identified as critical regulators of neutrophil functions, since they can activate as well as inhibit neutrophil effector functions [337, 338]. However, IFNλ expression by neutrophils has not been thoroughly investigated and the protective or pathologic implications for neutrophil-produced IFNλ in TB remain unclear.

Our work highlights novel aspects of IFNλ biology in tissue including protein localization and receptor dynamics. We noted not just cytoplasmic presence of IFNλ, but also intranuclear localization. Intranuclear localization of IFNλ4 was particularly prominent, especially in macrophages, and is attributable to IFNλ4’s nuclear localization signal (NLS) [431]. The relevance of this feature is not well understood but intranuclear localization is reported for other IFNs
including IFNγ where nuclear translocation of complexed IFNγ-IFNγR enhances IFNγ’s biologic activities [422, 432]. Interestingly, IFNλ4 was abundant in neutrophil cytoplasm, which differed from other cells in granulomas. The reasons underlying this are unclear, but this distinction may have implications for a neutrophil’s ability to secrete and respond to IFNλ4. IFNλ1 was also noted in the nucleus of some cells, albeit at a lower frequency and abundance, further suggesting that this cytokine has different properties than IFNλ4. Not only do cells in granulomas express IFNλ, but some undergo IFNλ-regulated signaling as suggested by nuclear localization of IFNλ.R1 subunit in some granuloma cells. Nuclear translocation of type I and type II IFN receptor subunits has been reported previously [421-423]. The C-terminus of IFNγ contains an NLS that mediates the nuclear translocation of the α subunit of IFNγR, where the ligand-receptor complex acts as a nuclear chaperone for STAT1α transcription factor [422]. Similarly, the IFNαR1 subunit contains an NLS and is translocated to the nucleus upon ligand stimulation [423]. It needs to be further investigated whether IFNλ.R1 nuclear translocation leads to interaction with any transcription factors or how it affects IFNλ-regulated functions, but our work suggests it may be an important contributor to IFNλ signaling in granulomas.

Our work provides insight into IFNλ as a player in the granuloma cytokine milieu. We found a negative correlation between IFNλ concentration and granuloma bacterial burden, suggesting that IFNλ may be associated with protection in TB. Moreover, we found an unexpected distribution of IFNλ expression in different myeloid cells and future work investigating how IFNλ promotes macrophage anti-Mtb activity, or whether IFNλ expression correlates with a different protective factor, will help define the role of this cytokine family in granuloma function.
2.5 Limitations of the study

Our data provides insight into the expression of IFNλs in TB granulomas from NHPs. In the work presented here, we made significant use of IHC on convenience samples and future studies will include evaluation of a larger and more diverse sample set. Moreover, our ability to perform high-dimensional flow cytometry-based experiments was limited by a lack of commercially available anti-IFNλ antibodies for this application and the lack of mechanically homogenized granulomas with sufficient macrophage populations for analysis. Although we selected the best-available candidate antibodies for IHC, the anti-human anti-IFNλ1 and IFNλ4 antibodies have not been fully assessed in humans and could have enhanced non-specific binding in the context of macaque tissues. Future work on in-depth characterization of the binding properties of these antibodies to human/NHP proteome and development of better antibodies will improve the interpretation of our data. Moreover, although these antibodies against human proteins cross-reacted with NHP proteins, we recognize that there may be different levels of avidity and affinity for their target proteins. This limited our ability to directly compare IFNλ1 and IFNλ4 expression and we only made direct comparisons with the same antibody and did not make cross-antibody comparisons. Granuloma macrophages are diverse and here we grouped them into two broad categories based on their location in the granuloma, but there may be variations in IFNλ biology that our experimental design cannot capture because of the limitations we faced in the surface markers we chose. Relatedly, we used CD11c as a broadly-expressed macrophage marker but recognize that this antigen can be expressed by other cell types, including dendritic cells; thus, a subset of our CD11c results may include data from these cell types. Future experiments targeting better-defined populations of macrophages may lead to additional data on the role that IFNλs play...
in TB granulomas. Lastly, some of the animals represent a limitation to the interpretation of the data because the BCG vaccination might represent a confounding factor due to the unknown possible influence of pre-existing or trained immunity.

2.6 Acknowledgements

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3.0 Identification of transcriptional and phenotypic profiles that differentiate IFNλ1, IFNλ4 and IFNα stimulated macrophages

3.1 Introduction

IFNλs and type 1 interferons (IFN1s) have been thought to have redundant functions that can be attributed to overlap in the downstream signaling pathways they activate. However, recent studies have highlighted differences both in the pathways that lead to induction of these cytokines and the eventual downstream signaling initiated by members of these IFNs. For example, MAVS localized on mitochondria typically induce IFN1, whereas peroxisomal MAVS induce IFNλs [302]. Likewise, PAMP and TLR4 interaction in endosomes results in the induction of IFN1 [433], whereas TLR4 engagement at the plasma membrane in some settings induces IFNλ production. Similarly, the cytosolic DNA sensor Ku70 [305] and IRF1 [302] are associated with IFNλ but not IFN1 induction [434]. When it comes to their downstream signaling pathways, IFN1 needs JAK1 and TYK2 to mediate signaling whereas IFNλs signaling can occur in the absence of TYK2. Furthermore, the ubiquitin-specific protease USP18 can bind IFNAR1 and inhibit IFNAR1’s interaction with JAK1 to regulate downstream signaling [435] while IFNλR lacks the USP18 binding site indicating that IFNλ’s signaling is not regulated by USP18 [436]. In addition to differences in signaling pathway usage, the kinetics of IFN expression and signaling occur at different rates. For example, in human hepatoma Huh7 cells or primary human hepatocytes, IFNα and ISG expression are induced and peak quickly and then rapidly decline, whereas IFNλ and IFNβ show a longer-lasting effect [388, 437-439]. Overall, these studies reinforce that although
IFN1 and IFNλs use similar elements in their signaling pathways, subtle differences in their regulation, induction, and kinetics may ultimately lead to distinct variations in their activity on target cells.

IFNs are important cell-cell mediators that lead to anti-viral as well as anti-bacterial infections. This includes TB, where IFNγ plays a critical role for mediating protection whereas IFN1 is usually considered a negative regulator of protective immunity in TB. IFNλs are a recently discovered group of IFNs and have anti-viral effects [308, 309, 364]. Even though IFNλs have their own unique receptor complex which is comprised of one subunit of IL-28Rα and another subunit of IL-10Rβ [323, 324], this family of cytokines has a downstream signaling pathway that is very similar to the type 1 IFN (IFN1) signaling pathway [297, 309, 319, 321]. IFNλs are important for mediating anti-viral effects in the upper respiratory tract, and considering their use of similar signaling pathways, redundancy between IFNλs and IFN1s is often associated with their role mediating anti-viral effects in the lower respiratory tract [388]. Recent studies, however, suggest that IFNλs can also signal via different factors including JAK2 in certain cell types [302, 440], potentially leading to different properties than those seen by IFN1s. With this in mind, IFNλ1 have been found to have Th1 skewing effects and inhibit Th2 cytokine production by naïve or memory T cells [344, 426]. In contrast, IFN1 signaling can promote T cell priming when the signaling coincides with or follows T-cell receptor (TCR) stimulation whereas in the absence of TCR stimulation, IFN1 signaling inhibits T cell priming and proliferation [441].

IFN1 is often linked to poor outcomes in TB and active TB patients have a blood transcriptional profiles dominated by an IFN1 signature that correlates with the radiologic extent of the disease [187]. Treating mice with IFN1 after they have been infected with hypervirulent
Mtb strains increased their susceptibility to severe disease [252, 253]. Although the mechanism behind these effects are unknown, IFN1 can inhibit TNF-α and IL-12 production in macrophages and reduce IFNγ-mediated antibacterial effects on macrophages [260, 266]. IFN1 has also been reported to inhibit the production of IL-1 cytokines which are critical mediators of protection, by DCs and inflammatory monocyte-macrophage populations in mice [257]. Furthermore, virulent Mtb induces the expression of IFN1 in human macrophages that inhibits the production of IL-1β [258]. Also, IFN1 expressed by B cells in Mtb infected mice have been shown to induce a regulatory/anti-inflammatory phenotype in macrophages in vitro [442].

IFNλ1 and IFNλ2 genes have been identified to be upregulated during Mtb infection in the human epithelial-like cell line A549 [404] and has been detected to be present at a higher level in the sputum of active TB patients in comparison to latently infected or healthy individuals [408]. Thus IFNλ s are expressed during Mtb infection and therefore can potentially influence the function of host immune cells. Macrophages are an indispensable component of the innate immune response that play a key role in limiting the growth of Mtb. IFNλ3 can promote pro-inflammatory profile in macrophages and also enhance their antigen presentation and cytotoxic capacity [350]. Further, macrophages differentiated in the presence of IFNλ3 also promote chemotaxis in T and NK cells. These reports suggest that IFNλ s can activate macrophage function, which could be beneficial for mediating protection in TB, although it remains to be further determined.

Here, we sought to determine whether IFNλ and IFN1s differentially regulate gene expression in macrophages. We hypothesized that these two families would lead to distinct transcriptional responses in macrophages because of the differences between IFN1- and IFNλ-regulated signaling pathways. Furthermore, we also sought to determine whether two disparate
members of the IFNλ family, IFNλ1 and IFNλ4, would lead to different responses. For these studies, we used Nanostring’s NHP-specific immunology panel and downstream analyses including Ingenuity Pathway Analysis (IPA) to define differentially-regulated genes and pathways induced in macrophages by these cytokines. We further validated our gene expression data using flow cytometry to confirm the phenotypic changes that are modulated by IFNα and IFNλ signaling. Our work showed that there are differences in transcriptional profiles in macrophages that are stimulated with different IFNs, and even between different IFNλs, suggesting the biology of these cytokines is complex and deserving of additional study.

3.2 Materials and methods

3.2.1 Macrophage culture

Monocytes were isolated from PBMCs of non-human primates (Table 3) using anti-CD14 beads (Miltenyi Biotec, Auburn, CA) as per the manufacturer’s instructions. Isolated monocytes were suspended in RPMI 1640 media (Lonza, Walkersville, MD) supplemented with 20% FBS (Gibco, Grand Island, NY), 1% L-glutamine (Sigma-Aldrich St. Louis, MO), 50 μM 2-Mercaptoethanol (Gibco), 0.1 mM sodium pyruvate (Gibco), 0.01 μg/ml M-CSF (Sigma-Aldrich) and 0.006 μg/ml GM-CSF (Sigma-Aldrich) and 100 U/ml penicillin-streptomycin (Gibco) and plated in 12-chamber flat bottom well plates at a density of 1-1.5x10^6 cells/well as described previously. Media was changed every 3-4 days and fresh RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, 1% HEPES (HyClone, Logan, UT) (hereafter referred to as R10 media)
and 1 mg/ml penicillin (Alfa Aesar, Haverhill, MA) was added, and cells were cultured for 7-10 days for differentiation into macrophages.
Table 3. Information on animals involved in transcriptional and flow cytometry analyses

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3.2.2 Interferons and treatment conditions

Monocyte derived macrophages (MDMs) were stimulated with recombinant human IFNλ1 (100 ng/ml, Peprotech, Cranbury, NJ), recombinant human IFNλ4 (100 ng/ml, R&D Systems, Minneapolis, MN), IFNα hybrid protein (100 U/ml, PBL Assay Science, Piscataway, NJ) for 6 hours for the NanoString transcriptional study. Cytokine concentrations were selected based on review of literature and reports from other groups and a 6 hour stimulation time frame was expected to result in changes in mRNA expression patterns. For flow cytometry assay to detect changes in surface marker and intracellular cytokine expression, MDMs were stimulated with the cytokines for 22 hours before being processed for assays. For all the other Mtb infection assays, MDMs were stimulated with the cytokines 24 hours either prior to infection or after infection as mentioned further in the paper.

3.2.3 RNA isolation and transcriptional analyses

Total RNA from MDMs was extracted using the RNAeasy kit (Qiagen, Germantown, MD) following the manufacturer’s instructions. RNA concentration was then assessed using a NanoPhotometer (Implen, Westlake Village, CA) and stored at -80°C until NanoString analysis. NanoString transcriptional analysis was performed to determine the changes in transcript expression level of macrophages after stimulation with IFNλs and IFNα. Version 1 of NanoString’s macaque-specific kit (Nanostring, Seattle, WA) targeting 770 immunology-associated genes was used to profile macrophage transcriptional activity. 100 ng of isolated RNA was submitted to the University of Pittsburgh Genomics Research Core for Tapestation analysis to confirm the quality of RNA and the transcriptional assay was then performed as per
manufacturer’s instructions. Nanostring’s nSolver 4.0 software package was used to normalize and analyze the raw data to identify the differentially expressed genes. Briefly, raw transcript counts were normalized using the positive and negative controls provided with each codeset to account for technical variation and background noise, respectively. Normalization between samples was carried out by selecting 10 endogenous genes that had the least amount of variation (<16%) between samples. After normalization, the transcript counts were exported for further analysis.

Ingenuity pathway analysis version 81348237 (Qiagen) was used to identify significant canonical pathways and to perform upstream analysis. Canonical pathways with \(-\log(p\text{-value})\) greater than 1.3 (\(p\text{-value} <0.05\)) and absolute \(z\)-score greater than 2 (considered to be biologically significant) were chosen. Gene Set Enrichment Analysis (GSEA) was performed using the GSEA v4.1.0 software and hallmark gene set database (h.all.v7.2.symbols.gmt).

3.2.4 Analysis of IFN-regulated protein expression by flow cytometry

MDMs were lifted off of the plate by pipetting and stimulated with IFN\(\lambda\)1, IFN\(\lambda\)4 and IFN\(\alpha\) for 6 hours at 37°C with 5% \(\text{CO}_2\), prior to the addition of brefeldin A (BD Bioscience, San Jose, CA) and Monensin (BD Bioscience) and then the cells were incubated for an additional 16 hours. Cells were stained for viability (Aqua viability dye, Thermo Fisher Scientific) and surface and intracellular markers according to standardized protocols. The antibody panel for surface marker staining consisted of CD14 (Clone MφP9, BD Biosciences), CD86 (Clone 2331 (FUN-1), BD Biosciences), CD80 (Clone 2D10, 3BioLegend), CD40 (Clone 5C3, BD Biosciences), CD54 (Clone HA58, BioLegend), TLR1 (Clone GD2.F4, BD Biosciences), TLR2 (Clone TL2.1, Thermo Fisher Scientific) and intracellular staining panel consisted of CCL5 (Clone VL1, BioLegend), IFN\(\gamma\) (Clone B27 (RUO), BD Biosciences), IL-1\(\beta\) (Clone JK1B-1, BioLegend), IL-6 (Clone MQ2-
13A5 (RUO), BD Biosciences), IFNα2b (Clone 7N4-1 (RUO), BD Biosciences), IL-10 (Clone JES3-9D7, Thermo Fisher Scientific), IL-4 (Clone 8D4-8 (RUO), BD Biosciences) and Arginase-1 (Clone A1exF5, Thermo Fisher Scientific). Data acquisition was performed with LSRFortessa flow cytometer (BD Biosciences) and analyzed by FlowJo (BD Biosciences) version 10.7.1 for Windows.

3.2.5 Statistical analyses

Statistical analyses were performed in GraphPad prism v9.1.2 (GraphPad software, San Diego, CA). Paired t-test (normal [parametric] data) and Wilcoxon matched-pairs signed rank test (for nonparametric data) was performed for comparing two groups. Dunnett’s multiple comparisons test (parametric data) and Dunn’s multiple comparisons test (for nonparametric data) was performed for making multiple paired comparisons. P values less than <0.05 were considered significant.

3.3 Results

3.3.1 IFNλ1 promotes pro-inflammatory transcriptional profile in macrophages

Our first analysis was to identify genes that were upregulated or downregulated in macrophages after stimulation by the three different IFNs. We found that IFNλ1 treatment upregulated inflammation associated transcripts associated with multiple processes including platelet production and activation including MAFG and PTAFR, cytokines responsible for recruitment of neutrophils and monocytes including IL-8 and CCL2 (also known as monocyte
chemoattractant protein-1, MCP-1) [443, 444], and downregulation of SERPING1, a serine protease that inhibits the first component of the complement system (Fig. 18A) [445]. IFNλ1 stimulation also upregulated genes involved in costimulatory signaling including CD40 and CD40LG while downregulating IL-33 and TSLP, both which are involved in promoting synthesis of TH2 cytokines [446, 447].

In contrast to IFNλ1, stimulation by IFNλ4 changed the expression level of very few genes. Of the few genes that were upregulated, certain genes were involved in structural functions like RORC, a gene that encodes a Th17-associated transcription factor and is important for lymphoid organogenesis, thymopoiesis and [448, 449] expression of HSPB2, a heat shock protein involved in maintenance of muscle function [450], and TWIST2, a gene involved in fibrosis [451, 452] (Fig. 18B). The fact that IFNλ4 differentially regulated very few of the genes in our immunology focused NanoString panel suggests this cytokine is functioning differently from IFNλ1 and may be regulating other genes that are not part of our panel, including genes related to homeostasis or wound resolution.

Macrophages that were treated with IFNα experienced an upregulation of canonical interferon stimulated genes (ISGs) including IFIT1, IFIT2, IFIT3, IRF7, ISG20, OAS1, OAS2, MX1 (Fig. 18C). Members of the chemokine receptor CXCL family including CXCL9, CXCL10, CXCL11 and CXCL12 were also upregulated after IFNα stimulation. SERPING1, which was downregulated by IFNλ1 was upregulated in IFNα stimulated macrophages. In contrast, genes encoding IL-18, IFNγ, and IFNGR1, all of which promote Th1 activation [453], were downregulated. These data support the paradigm that IFN1-regulated signaling is counterproductive to immunity in Mtb infection, possibly by downregulating responses that are critical for macrophage anti-mycobacterial activity.
Overall, we found that stimulation with IFNα led to upregulation of the highest number of genes followed by IFNλ1 whereas IFNλ4 modulated the expression of very few genes (Fig. 18C). Likewise, IFNα treatment resulted in a more transcripts that had >2-log fold change in expression levels as compared to IFNλ treatment (Fig. 18C). Of all the genes that were differentially regulated above the 2-fold threshold, 49.3% (n= 66) of these genes were unique to IFNα, 23.1% (n=31) were unique to IFNλ1, and 1.5% (n=2) were unique to IFNλ4. In comparison, 24.6% of the genes (n=33) overlapped between IFNα and IFNλ1, while there was very little overlap between IFNλ1 and IFNλ4 (1.5% of genes, n=2) and no overlap was noted between IFNα and IFNλ4. In total, only 9 genes (6.7% of all the upregulated genes) were differentially regulated by all three cytokines. These data suggest that IFNα and IFNλ are acting through some common signaling pathways but are also activating different responses that may have downstream consequences for how these cytokines affect macrophage interactions with Mtb.
Figure 17. IFNλ.1 promotes pro-inflammatory profile in macrophages.
Volcano plots showing genes that are differentially regulated by macrophages stimulated by (A) IFNλ1, (B) IFNλ4 or (C) IFNα. The dotted line in each graph indicates a p-value of 0.05. (D) Venn-diagram showing the number of genes with at least 1-log fold change that were differentially regulated by IFNα, IFNλ1 and IFNλ4. (E) Heat map comparing expression patterns between untreated and IFNλ1 treated macrophages (left) and IFNλ1 and IFNα treated macrophages (right).

3.3.2 IFNλ1 and IFNα show opposite regulation patterns for genes associated with protection in TB

We noted that IFNλ1 and IFNα upregulated both unique and shared transcripts, and to better understand the differences in the magnitude of transcriptional activity, we plotted heatmaps of the differentially regulated genes that are associated with immune cell activation and protection in TB. When macrophages were treated with IFNλ1 alone, we observed increased expression of transcripts associated with T cell activation and co-stimulation including IL-12B, CD40, and CD80 (Fig. 18E, left). We also noted upregulation of IFNGR1 and IFNGR2, which suggests that IFNλ1 stimulation may make macrophages more receptive to IFNγ. Moreover, cytokines associated with protection in TB including IL-1B and CSF-2 (GM-CSF) were also upregulated. In contrast, when we compared IFNλ1- and IFNα-regulated expression of genes implicated in anti-mycobacterial immunity including IFNGR1, IFNGR2, TGFBR1, TGFBR2, IL1B, IL-18 [156, 167, 168, 454] we noted opposite expression patterns between these two cytokines (Fig. 18E, right). Importantly, these genes were downregulated in IFNα treated macrophages but upregulated following IFNλ1 stimulation, further suggesting that even though these two cytokines may have similarities in their
downstream signaling pathways, they may induce different responses in macrophages that respond to them.

Since genes do not have an independent effect, we performed gene set enrichment analysis (GSEA) to determine the combination of top gene sets that are enriched in macrophages post IFNλ1, IFNλ4 and IFNα stimulations. We found that in IFNλ1-treated macrophages gene sets involved in TNFα signaling via NFκB (normalized enrichment score, NES 1.97), followed by IFNγ response (NES 1.97) and IFNα response (NES 1.88) were upregulated. This indicate that IFNλ1 stimulation might be important for mediating protective response in mycobacterial infection (Fig. 19A). In IFNλ4 stimulated macrophages, IFNα response was the top enriched gene set (NES 1.63), followed by IFNγ response (NES 1.47) and PI3K AKT mTor signaling (NES 1.33) (Fig. 19B). In IFNα stimulated macrophages, the top gene set enriched was IFNγ response (NES 2.19), followed by IFNα response (NES 2.14) and IL-6 JAK STAT3 signaling (NES 1.61) (Fig. 19C). This indicates that IFNα stimulation in macrophages can upregulate expression of genes associated with IFNγ response, some of which could be ISGs that are induced by the two groups of IFNs.
A. Gene sets enriched in IFNλ1 stimulated macrophages

B. Gene sets enriched in IFNλ4 stimulated macrophages

C. Gene sets enriched in IFNα stimulated macrophages

Figure 18. Gene set enrichment analysis of macrophages stimulated with IFNλ1, IFNλ4 and IFNα.

(A-C) Top three gene sets enriched in macrophages post stimulation with (A) IFNλ1, (B) IFNλ4 and (C) IFNα.
3.3.3 Ingenuity pathway analysis shows IFNλ1 promotes microbial detection pathways in macrophages

To investigate the overall immunological pathways and processes that were differentially regulated following IFNλ1, IFNλ4 and IFNα treatments in macrophages, we used Ingenuity Pathway Analysis on immunologic pathways to identify the differentially-regulated immunologic pathways and signaling elements affected by these cytokines. This tool uses genomic and transcriptional data extracted from the literature to infer relationships between factors not directly included in the NanoString panel and can also identify transcriptional similarities between the condition being tested and other disease states and interactions between pathways. From the transcriptional profile of IFNλ1, IFNλ4 and IFNα stimulated macrophages, IPA analysis showed that IFNλ1 stimulation resulted in the greatest number of significantly differentially regulated pathways, followed by IFNα and IFNλ4 (Fig. 20A). This indicates that even though IFNλ1 differentially regulated fewer transcripts than IFNα, it significantly regulated a greater number of immunological pathways as compared to the latter.

In IFNλ1 stimulated macrophages, we identified 41 canonical pathways of which 21 pathways were significantly differentially regulated by IFNλ1 (Z-score>2 or <-2). Fig. 20B shows the top 10 significantly differentially regulated pathways in IFNλ1 stimulated macrophages. Pathways associated with role of hypercytokinemia/hyperchemokinemia, more commonly referred to as a ‘cytokine storm’ in the pathogenesis of influenza was identified as the topmost significantly upregulated pathway for all the three different IFNs. IFNλ1 treatment also significantly upregulated pathways associated with pattern recognition receptors (PRRs) recognition of bacteria and viruses in macrophages (Fig. 20B). TREM1-associated signaling, which is mediated by a receptor that is broadly expressed on myeloid cell subsets and involved in
promoting inflammatory responses and antigen-presentation [455-457], was also upregulated in IFNλ1-stimulated macrophages. In macrophages treated with IFNλ4, we found that interferon signaling was the only pathway with a significant activation Z-score apart from the from the previously mentioned hypercytokinemia/hyperchemonikemia pathway as mentioned before (Fig. 20C).

IFNα stimulated macrophages displayed patterns of gene expression that were associated with 52 canonical pathways, and of these, 13 pathways were significantly differentially regulated (Z-score>2 or<-2). Unsurprisingly, hypercytokinemia/hyperchemonikemia in influenza and role of Interferon signaling were the first and second most significantly enriched canonical pathways, respectively (Fig. 20D). Most of the other pathways like antiviral response and activation of IRFs were anticipated to be upregulated by IFNα. Interestingly, we found that IFNλ1 upregulated different immunological pathways including those related to PRR responses, Th1 responses, DC maturation, TREM1 signaling to a higher extent than IFNα (Fig. 21E). However, for IL-6 signaling, PPAR signaling and acute phase response pathways there was a opposite trend in regulation by these two IFNs. This again confirmed that IFNλ1 differs from IFNα, and IFNλ1 promotes upregulation of pathogen detection and Th1 pathways to a greater extent than IFNα, suggesting IFNλ1 could potentially have protective effects in TB.
Figure 19. IFNλ1 upregulates anti-microbial response pathways in macrophages.

(A) Numbers of pathways with z-scores >2 or < -2 that were differentially regulated in macrophages after IFNλ1, IFNλ4 and IFNα stimulation. (B, C) Top canonical pathways that were differentially regulated by IFNλ1, IFNλ4 and (D) and IFNα. (E) Comparison of activation Z-scores for pathways that were regulated by IFNλ1 and IFNα.

The overall summary of network interactions predicted by our transcriptional and pathway analysis indicate that in IFNλ1 stimulated macrophages, there was significant upregulation of biological processes associated with T cell activation, proinflammatory immune response, pattern recognition receptor response and antimicrobial activity (Fig. 21A) which resembles what we observed in our volcano plots in Fig. 18A. We observed increases in antiviral function and
inhibition of viral replication which would be expected from IFNs. We also detected upregulation of IL-1β, CD40LG and IFNγ pathways in IFNλ1 stimulated macrophages. The overall biological functions that were significantly upregulated in IFNλ4 stimulated macrophages mostly involved interferon signaling and upregulation of different entities associated with antiviral functions (Fig. 21B). Lastly, in IFNα stimulated macrophages, the major biological pathways that were upregulated were associated with interferon signaling and mediating antiviral response and inhibition of viral replication (Fig. 21C). We also noted upregulation of the IFNγ pathway, as was noted for IFNλ1.
Figure 20. Graphical summary of pathways regulated by IFNλs and IFNα.
(A-C) Overview of major biological themes in the transcriptional profiles of (A) IFNλ1-, (B) IFNλ4-, and (C) IFNα-stimulated macrophages. Orange nodes indicate activation (z-score ≥2) and blue nodes indicate inactivation (z-score ≤2). Blue lines and symbols represented downregulation and entities downregulated, respectively, whereas orange lines and symbols indicate upregulation and upregulated entities, respectively.

In addition to the analysis of the pathways defined by differentially expressed transcripts, we also looked at predicted upstream regulators which are identified in an unsupervised manner in IPA, to more comprehensively define the drivers of observed gene expression changes in IFN-stimulated macrophages. In IFNλ1 stimulated macrophages, we found 3224 upstream regulators with a p-value of overlap <0.05, with 392 regulators having activation Z-scores >2 and 498 having Z-scores <2. IFNγ was identified to be the top upstream regulator, with 44 of the 53 differentially expressed genes having expression direction consistent with activation by IFNγ (activation Z-score of 4.948, p-value of overlap 3.20E-51), even though IFNG transcript was not upregulated at the transcript level (Fig. 22A). The regulatory effects tool in IPA which identifies the impact of upstream regulators and potential mechanisms behind a phenotype identified P38 MAPK and IL1β as the top regulators regulating functions related to cell movement of granulocytes, recruitment of leukocytes and recruitment of T lymphocytes, respectively in IFNλ1 stimulated macrophages (Fig. 22B). These findings corroborated our GSEA analysis, where TNFα signaling via NFκB and IFNγ responses were the top two gene sets enriched in macrophages stimulated with IFNλ1 (normalized enrichment scores for both 1.97) (Fig. 19A).
Figure 21. Predicted upstream regulator and regulator effects for IFNλ1-stimulated macrophages.

(A) IPA identified ING as the top upstream regulator in IFNλ1 stimulated macrophages.

(B) Schematic showing regulatory effects mediated by P38 MAPK and IL-1B in IFNλ1 stimulated macrophages.
In IFNλ4 stimulated macrophages, IPA identified IFNλ1 as the top significantly activated upstream regulator, as 9 of the 9 differentially expressed genes had expression directions that were consistent with activation by IFNλ1 (activation Z-score 2.947, p-value of overlap 2.37E-19) (Fig. 23A). We identified 10 different regulators (EIF2AK2, Ifn, IFNA1/IFNA13, IFNL1, IRF1, KRAS, PML, RNY3, SMARCB1, SP110) with the regulator effects tool that identify factors that are responsible for inhibiting viral replication, which suggests that IFNλ1 and IFNλ4 functions can be regulated and driven by different upstream regulators (Fig. 23B).

Figure 22. Predicted upstream regulator and regulator effects for IFNλ4-stimulated macrophages.

(A) IPA identified IFNλ1 as the top upstream regulator in IFNλ4 stimulated macrophages.
(B) Schematic showing regulatory effects mediated by EIF2AK2, Ifn, IFNA1/IFNA13, IFNL1, IRF1, KRAS, PML, RNY3, SMARCB1, SP110 in IFNλ4 stimulated macrophages.

In IFNα-stimulated macrophages, we identified 3032 upstream regulators with IFNα as one of the top significantly-activated upstream regulators with 53 of 60 genes having measurement directions consistent with activation by IFNα (activation z-score of 6.805 and p-value of overlap
3.00E-76) (Fig. 24A). The regulator effects tool in IPA predicted IFNλ1 and IL-18 as the top regulators involved in inhibition of RNA virus replication and attraction of leukocytes (Fig. 24B). It is to be noted that *IL18* was inhibited at the transcript level in IFNα stimulated macrophages, however based on the expression pattern of its target molecules IPA predicted IL-18 as one the regulators mediating cellular migration and activation.
Figure 23. Predicted upstream regulator and regulator effects for IFNα-stimulated macrophages.
(A) IPA identified INα as the top upstream regulator in IFNλ4 stimulated macrophages.

(B) Schematic showing regulatory effects mediated by IFNλ1 in IFNα stimulated macrophages.

3.3.4 IFNλ upregulates expression of co-stimulatory molecules and pro-inflammatory cytokine and chemokine expression in macrophages

Once we had investigated the patterns of activation induced by the different IFNs at the transcriptional level, we sought to validate some of our observations at the protein level by performing flow cytometry on IFN-stimulated MDMs. Our transcriptional analyses showed that IFNλ stimulation upregulated expression of co-stimulatory molecules and pro-inflammatory cytokine expression thus we sought to determine whether these transcripts carried through to phenotypic changes at the protein level. We therefore determined the expression levels of different molecules associated with co-stimulation, adhesion, pathogen recognition (Table 4). We investigated the expression levels of pro-inflammatory cytokines that were differentially expressed in our transcriptional data set. Although IFNγ and IFNα did not show substantial differential expression in response to our stimulations, we wanted to determine whether IFNλ stimulation could induce production of either of these cytokines and so we included antibodies for these proteins in our panel. Likewise, although we did not note any significant differential regulation of IL-10, IL-4 and arg1, we wanted to investigate how IFNλ stimulation modulated expression of these factors because of their relationship to TB pathobiology. IL-10 is a suppressive cytokine expressed by M2 macrophages and can be an IFNα-regulated ISG [260, 458]. IL-4 is a Th2-polarizing cytokine for T cells that can be expressed by specific M2 macrophage subsets [459] and arg1 is indicative of M2 macrophage polarization and arginine catabolism can inhibit T cell function [129, 460, 461].
Table 4. Surface Marker and cytokine/chemokine panel for flow cytometry

<table>
<thead>
<tr>
<th>Surface antigens</th>
<th>Pro-inflammatory cytokine/chemokine</th>
<th>Anti-inflammatory cytokines and factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-stimulation: CD40, CD80, CD86</td>
<td>IL-1β, TNF, IL-6, IFNγ, IFNα, CCL5</td>
<td>IL-10, IL-4</td>
</tr>
<tr>
<td>Adhesion: CD54</td>
<td></td>
<td>M2 macrophage polarization: Arg1</td>
</tr>
<tr>
<td>Pathogen recognition: TLR1, TLR2</td>
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</table>

We found that both IFNλs, but not IFNα, upregulated expression of the co-stimulatory molecule CD86 (Fig. 25A). CD86 is classically identified as a M1 marker but is also expressed by M2b-polarized macrophages, which are a subtype of the M2 macrophages that contribute to Th2 T cell polarization [462-464]. CD86 binds CD28 or CTLA4 on T cells to provide co-stimulation for T cell activation and survival [465, 466], suggesting that IFNλ-activated macrophages may contribute to T cell regulation. IFNλ1 also upregulated macrophage CD40 expression, whereas IFNλ4 and IFNα did not (Fig. 25B). CD40 is a co-stimulatory molecule that interacts with CD154 (CD40 ligand) on activated T cells, that in turn results in the activation of macrophage and pro-inflammatory cytokine and chemokine production [467-469]. We did not see significant changes in expression of the adhesion molecule CD54 (ICAM-1), TLR1 and TLR2 on macrophages with either of the three IFN treatments (Fig. 26 A, B and C, respectively).
Figure 24. Expression of CD86 and CD40 by IFNλ1-, IFNλ4- and IFNα-stimulated macrophages.

(A) Macrophages expression of CD86 after IFNλ1, IFNλ4 and IFNα stimulation. Statistical comparisons by paired t-test unadjusted for multiple comparisons for the paired plots (left). Statistical comparisons by Dunnett’s multiple comparisons test adjusted for multiple comparisons (right). (B) CD40 expression by macrophages after IFNλ1, IFNλ4, and IFNα stimulation. Statistical comparisons by Wilcoxon matched-pairs signed rank test for untreated IFNλ stimulation and paired t-test for untreated vs. IFNα stimulation. Paired graphs (left) are unadjusted for multiple comparison. For comparison of multiple conditions against the untreated control (right), statistical comparisons were performed using Dunn’s multiple comparisons test adjusted for multiple comparisons.
Figure 25. Expression of CD80, CD54, TLR1 and TLR2 by IFNλ1-, IFNλ4- and IFNα-stimulated macrophages.

(A) Expression of CD80 on macrophages following IFNλ1, IFNλ4 and IFNα stimulations. Statistical comparisons between untreated and cytokine-treated cells (right) were performed by the Wilcoxon matched-pairs signed rank test for untreated vs. IFNλ1 or paired t-tests for untreated vs. IFNλ4 or IFNα treated cells. Paired graphs are unadjusted for multiple comparisons.
When we looked at intracellular cytokine and chemokine expression patterns, we observed that expression of the chemokine CCL-5 was upregulated in macrophages after IFNλ1 and IFNλ4 stimulation, but not IFNα stimulation (Fig. 27A). IFNγ expression was upregulated by all the three different IFNs (Fig. 27B). Lastly, we saw a trend of increased IL-1β expression in macrophages after IFNλ1 stimulation, but not with IFNλ4 or IFNα stimulation (Fig. 27C). We did not see significant changes in expression of pro-inflammatory cytokines like TNFα, IL-6, IFNα (Fig. 28 A, B and C, respectively) or anti-inflammatory cytokines like IL-10, IL-4, or the arginine-catabolizing M2-macrophage associated enzyme arginase 1 (Fig. 28 A, B and C, respectively). Overall, our data confirms that IFNλ1 promotes pro-inflammatory activation of macrophages to a
greater extent than IFNλ4 and differs from IFNα in regulating the expression of co-stimulatory molecules and pro-inflammatory cytokines and chemokines in macrophages.

Figure 26. Macrophage expression CCL-5, IFNγ and IL-1β after IFNλ1, IFNλ4 or IFNα stimulation.

(A) Macrophage expression of CCL-5 after IFNλ1, IFNλ4 or IFNα stimulation. Statistical comparisons by paired t-test for paired graphs (left). Paired graphs are unadjusted for multiple comparison. Statistical comparisons by Dunnett's multiple comparisons test adjusted for multiple comparisons (right). (B) Macrophage IFNγ expression after IFNλ1, IFNλ4 and IFNα stimulation.

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Statistical comparisons by paired t-test for untreated vs. IFNλ1 or IFNα stimulation and Wilcoxon matched-pairs signed rank test for untreated vs. IFNλ4 stimulation (left). Paired graphs are unadjusted for multiple comparison. Statistical comparisons by Dunnett’s multiple comparisons test adjusted for multiple comparisons (right). (C) Macrophage IL-1β expression after IFNλ1, IFNλ4 or IFNα stimulations. Statistical comparisons were performed as per (A).

Figure 27. Macrophage expression of TNFa, IL-6 or IFNa after IFNλ1, IFNλ4, or IFNα stimulation.

(A) Macrophage expression of TNF and (B) IL-6 expression after IFNλ1, IFNλ4 or IFNα stimulation. Statistical comparisons by paired t-test for untreated vs. IFNλ1 or IFNα stimulation.
and Wilcoxon matched-pairs signed rank test for untreated vs. IFNλ4 stimulation (left). Paired graphs are unadjusted for multiple comparison. Statistical comparisons by Dunn’s multiple comparisons test adjusted for multiple comparisons (right). (C) Macrophage expression of IFNα after IFNλ1, IFNλ4 or IFNα stimulation. Statistical comparisons Wilcoxon matched-pairs signed rank for the paired graphs (left) which are unadjusted for multiple comparison. Statistical comparisons by Dunn’s multiple comparisons test adjusted for multiple comparisons (right).

Figure 28. Macrophage expression of IL-10, IL-4 or Arg-1 after IFNλ1, IFNλ4 or IFNα stimulations.

(A) Macrophage IL-10 expression after IFNλ1, IFNλ4 or IFNα stimulation. Statistical comparisons by Wilcoxon matched-pairs signed rank test for untreated vs. IFNλ1 or IFNλ4
stimulations and paired-t test for untreated vs. IFNα stimulation graph (left). Paired graphs are unadjusted for multiple comparison. Statistical comparisons by Dunn’s multiple comparisons test adjusted for multiple comparisons (right). (B) Macrophage IL-4 expression after IFNλ1, IFNλ4 or IFNα stimulation. Statistical comparisons by paired t-test for untreated vs. IFNλ1 or IFNλ4 stimulation or Wilcoxon matched-pairs signed rank test for comparing untreated vs. IFNα stimulation (left). Paired graphs are unadjusted for multiple comparison. Statistical comparisons by Dunn’s multiple comparisons test adjusted for multiple comparisons (right). (C) Macrophage arginase 1 expression after IFNλ1, IFNλ4 or IFNα stimulation. Statistical comparisons by paired t-test for untreated vs. IFNλ1 or Wilcoxon matched-pairs signed rank test untreated vs. IFNλ4 or IFNα stimulation (left). Paired graphs on the left are unadjusted for multiple comparison. Statistical comparisons by Dunn’s multiple comparisons test adjusted for multiple comparisons (right).

3.4 Discussion

Macrophages are key players for defense against Mtb and cytokines play an important role in determining their functional behavior. IFN1s have mostly been associated with detrimental outcomes and can suppress IFNγ-mediated antimycobacterial responses in both human and mouse monocytes and macrophages [260, 266]. Exogenous addition of IFNβ to Mtb infected murine macrophages inhibits production of protective pro-inflammatory cytokines including TNF, IL-1β and IL-12, and increases production of IL-10, which is immunosuppressive [260] and decreases the responsiveness to IFNγ. IFN1 also inhibits IFNγ-driven inducible nitric oxide synthase (iNOS) and IL12/23 p40 in mouse lung macrophages during in vivo Mtb infection [257, 470]. Furthermore,
IFN1 is important for mediating the death of bone marrow derived macrophages that are infected with Mtb in mice [471]. However, the role of IFN1 in TB is ambiguous as other studies have shown that IFN1 can also have a protective role in some situations. For example, in the absence of IFNγ signaling, IFN1 has been reported to inhibit skewing of macrophage to alternatively activated phenotypes, induction of iNOS, and inhibition of arginase 1 [274].

In contrast to IFN1, IFNλs, particularly IFNλ3 can promote pro-inflammatory cytokine and chemokine production in GM-CSF treated macrophages and induce macrophage cytotoxicity and phagocytic activity [350]. Addition of IFNλ3 to GM-CSF differentiated macrophages promoted lymphocyte migration when these cells were co-cultured with macrophages and in NK cells, this combination of factors promotes NK cell degranulation [350]. Another study reported that IFNλ1 increased TLR induced IL-12p40 production of human monocyte derived macrophages [351]. IFNλ1 also enhanced IFNγ-induced TNF and IL-12p40 production by macrophages in response to TLR7/8 agonist R848 (Resiquimod) stimulation [472], thereby making the macrophages more responsive to IFNγ [351]. IFNλ1 promotes IFNγR1 expression on macrophages, whereas IFNα downregulated its expression, highlighting that although IFNλ and IFN1 have common elements in their downstream signaling pathways, they can differentially modulate macrophage function. IFNλ1 has been reported to mediate IL-12 production by macrophages which facilitates IFNγ production by NK cells [335].

We therefore performed a direct comparison among IFNλ1, IFNλ4 and IFNα to identify how they modulate macrophage transcriptional activity and protein expression. We chose IFNλ1 as a representative member of the IFNλ family and selected IFNλ4 because it shares only 28% amino acid sequence similarity with the rest of the IFNλ family members. Considering the difference between these cytokines, we also wanted to determine whether IFNλ4 had a different impact on macrophages than IFNλ1. In our transcriptional analysis, we found that when
macrophages were stimulated with IFNλ1, IFNλ4 or IFNα, IFNα differentially regulated the highest number of genes, followed by IFNλ1, and IFNλ4 differentially regulated the least number of genes. IFNα has been reported to have faster kinetics that peaks early, whereas IFNλ has a slow but longer-term effect which can account for the greater number of genes differentially regulated by IFNα [437, 438]. Many of the IFNα-regulated genes were canonical ISGs and included several IRFs and STAT1 as well as IFN1-associated pro-inflammatory chemokines including CXCL9, CXCL10 and CXCL11. We observed decreased IL-18 and IFNγR1 expression, which has been previously demonstrated in IFNα-stimulated human monocyte-derived macrophages [351]. This pattern of IFNα-stimulated genes, anti-viral responses elements, and chemokines gave us confidence that our assay was working as predicted and that IFNα-stimulated macrophages can be directed toward antiviral phenotypes.

In contrast to IFNα, IFNλ1 differentially regulated fewer genes and most of the genes were associated with lymphocyte co-stimulation and pro-inflammatory activation states. Reflecting this, we noted that cytokines associated with Th2 responses including IL33, TSLP, IL4 were downregulated at the transcript level. IFNλ4 differentially regulated very few genes in the NanoString immunology panel and some of the upregulated genes were associated with fibrosis and wound resolution. This indicates that IFNλ1 and IFNλ4 could regulate different genes in macrophages but future studies using RNAseq instead of Nanostring transcriptional profiling may lead to a better understanding of the different functions mediated by IFNλ1 and IFNλ4.

Our transcriptional analysis further highlights the differing effects of IFNλ1 and IFNα on macrophages. IFNλ1 stimulated upregulated expression of elements in Th1 response pathways and microbial defense responses in macrophages by upregulating pattern recognition receptor signaling pathways in comparison to IFNα treated macrophages. The responses noted in IFNλ1-
regulated macrophages overlapped with the responses predicted when IFNγ is an upstream regulator. This observation suggests that IFNλ1-activated macrophages have phenotypes that resemble macrophages activated by IFNγ at the transcriptional level. Further experiments should investigate how this influences macrophage functional responses including whether it modifies macrophage antimicrobial activity.

Our flow cytometry data further validates that IFNλ1 and IFNλ4 upregulate macrophage proteins involved in T cell co-stimulation including CD86 and CD40 (by only IFNλ1) at both the transcriptional and protein level, whereas these proteins were not significantly by IFNα stimulation. Moreover, IFNλ1-stimulated macrophages upregulated proinflammatory cytokines and chemokines including IL1β and CCL5, respectively. This was observed only in case of IFNλ1 but not IFNλ4 or IFNα stimulated macrophages, further suggesting that although these cytokines have similarities in their downstream signaling pathways, each can produce substantially different responses in stimulated macrophages.

We were surprised to see that all three IFN stimulations induced IFNγ expression in macrophages. Typically, IFNγ expression and secretion are viewed as part of T cell- and NK cell-mediated immunity. However, recent studies have reported that IFNγ is constitutively expressed by unstimulated peritoneal macrophages that have been freshly explanted from mice [473] and LPS stimulation upregulated peritoneal macrophage IFNγ expression [474]. Human alveolar macrophages that have been infected with Mtb in vitro have also been reported to express IFNγ, and IFNγ expression has been noted by uninfected alveolar macrophages after stimulation with IL-12 or by IFNγ itself [475].
3.5 Limitations of the study

Our study provides new insight into the differential regulation of macrophage function by IFNλ and IFNα. However, our transcriptional study involved macrophages from three animals and future studies involving a larger number of animals will be important to better define the range of functions mediated by these cytokines. Furthermore, in our transcriptional analysis, we used NanoString’s NHP immunology panel that includes probes for 770 immunologically relevant genes with controls, thus our transcriptional analysis was biased toward these pre-selected transcripts and may have lacked many transcripts that may be relevant to IFNλ- or IFNα-mediated responses. This may have contributed to the low number of differentially regulated transcripts we detected in IFNλ4-stimulated macrophages. Therefore, future studies using bulk RNAseq, or single cell RNAseq to profile intra-culture heterogeneity, would produce additional information and the more comprehensive transcriptional profile that is needed to fully describe the functions of IFNλs and IFNα. Future studies including the Type II IFN, IFNγ, would also yield valuable information, especially with regard to similarities and differences between IFNγ- and IFNλ1-mediated responses. For our flow cytometry data, some of the animals represent a limitation to the interpretation of the data because the BCG vaccination might represent a confounding factor due to the unknown possible influence of pre-existing or trained immunity.

3.6 Acknowledgements

We thank staff of the University of Pittsburgh Genomics Research Core for providing technical advice on RNA preparation for the NanoString assay. We also thank Jia Yao Phuah and
technical support at NanoString advice on how to perform the initial steps of normalization and data processing in the NanoString transcriptional analysis.
4.0 Determining the impact on IFNα and IFNλ treatments on anti-mycobacterial activity of macrophages

4.1 Introduction

Tuberculosis (TB) is caused by infection with Mycobacterium tuberculosis (Mtb) and is a global health problem that claimed at least 1.5 million lives in 2020 and is expected to claim even more lives in the subsequent years [3]. Unfortunately, a lack of understanding for how Mtb and host cells interact, and what constitutes the mechanistic basis for protection in TB, has limited the design and development of successful vaccination and host-directed treatment strategies. Overcoming these limitations and identifying how host cell relate to the Mtb and host factors that mediate protective effects will be crucial for improving the current treatment regimens.

Mtb is transmitted via aerosol route and after inhalation, alveolar macrophages are the first immune cells that encounter and phagocytose Mtb [476, 477]. As different immune cells aggregate to form the granuloma, cytokine mediated communication is essential for proper activation of host immune cells and restriction of Mtb growth, both before and after the onset of adaptive immune response. This protection is mediated primarily through IFNγ- and TNF-mediated activation of macrophages, which is important for the induction of vitamin-D dependent pathways that generate anti-microbial peptides, induce nitric oxide production, promote autophagy, and lead to phagosomal maturation [172, 478-480]. Mtb can subvert many of these mechanisms and resist macrophage antimicrobial activity [476, 481, 482]. This ability to persist in the host and the protracted nature of the anti-Mtb drug regimen make it necessary to identify alternate pathways for controlling Mtb infection.
While IFN\(\gamma\) and TNF have been identified as cytokines that are critical for macrophage activation in TB, [281, 282, 483-485], the role of certain cytokines in TB remains complicated. The function of type I interferons (IFN1) in TB is controversial, with some studies reporting that IFN1 is associated with deleterious outcomes. TB patients who progress to active TB have a blood transcriptional profile dominated by IFN1 inducible transcripts [186] and IFN1 has been reported to inhibit IL-1\(\beta\) which is associated with host-protective effects in TB [166, 167, 261]. However, IFN1 has also been found to improve antitycobacterial immunity in patients with partial or complete IFN\(\gamma\)R deficiencies and promote nitric oxide production in murine macrophages [271, 272].

While the role of adaptive immunity in TB has been investigated to some extent, the protective or pathologic roles of innate cytokines are ambiguous and need to be further investigated. The type III interferon (IFN) family (hereafter referred to as IFN\(\lambda\)s) are mostly known for their antiviral role but may have unappreciated antibacterial functions [405]. This cytokine family contains four members including IFN\(\lambda\)1 (IL-29), IFN\(\lambda\)2 (IL-28A), IFN\(\lambda\)3 (IL-28B) and IFN\(\lambda\)4 [144, 295, 296] where IFN\(\lambda\)1-3 are 80-96% similar to each other at the amino acid sequence level. In contrast, IFN\(\lambda\)4 shares only 28% amino acid homology with the other members of this family. Moreover, IFN\(\lambda\)4 is present in a functional form in a fraction of the human population but and in most people, IFN\(\lambda\)4 exists as a pseudogene due to a frameshift mutation. In contrast, non-human primates (NHPs) do not carry this frame-shift mutation and express functional IFN\(\lambda\)4. Even though IFN\(\lambda\)s and type I IFNs have downstream signaling elements that conserved [295], recent works have highlighted that there are differences between these two groups of IFNs in terms of their signaling pathways and kinetics [337, 439, 486-489].

There is a dearth of information regarding the role of IFN\(\lambda\) in TB but the available evidence supports that IFN\(\lambda\)s are expressed during Mtb infection. In cell culture, IFN\(\lambda\)1 and IFN\(\lambda\)2 mRNA
expression is upregulated in A549 lung epithelial cells after Mtb infection [404] suggesting that lung epithelial cells can respond to this bacillus by expressing IFNλs. Less is known about tissue level responses in Mtb-infected hosts but higher levels of IFNλ2 have been noted in the sputum of active TB patients than in latently infected or healthy people [408]. Moreover, BCG vaccination in elderly adults leads to increased plasma IFNλ1 and IFNλ2 levels and decreased IFN1 levels at one month post vaccination [409].

The cells that produce and respond to IFNλ in granulomas are not known but in response to bacterial antigenic stimulation and viral infection, macrophages are important producers of IFNλs [318]. IFNλ can have a Th1 skewing effect [343, 344, 360, 363] and promote Th1 chemokine production and cytotoxicity in monocyte-derived macrophages [350]. This raises the potential for IFNλs to drive antibacterial responses and in Salmonella typhimurium and Shigella flexneri infection, IFNλ mediates protection by enhancing intestinal epithelial barrier integrity [405]. That said, a complete understanding of IFNλ’s effect on bacterial infections remains ambiguous and other studies have reported that IFNλ compromise airway epithelial barrier integrity in response to bacterial infections [490-493].

Identifying how IFNλs influence macrophage antimicrobial activity in TB will improve our understanding of granuloma pathobiology. Previously, we showed that IFNλs are expressed in NHP lung TB granulomas by macrophages and neutrophils, and their protein levels negatively correlated with bacterial burden in the granulomas [494]. In Chapter 2 of my dissertation, we noted that IFNλ1, IFNλ4, and IFN1 activate different transcriptional and translational programs in macrophages, but also that IFNλ1 can promote an M1-like state in macrophages. Our objective here is to determine how IFNλ affect functional antimycobacterial responses in monocyte-derived macrophages from NHPs. We used fluorescent Mtb reporter strains to determine how these cytokines affect viability of intracellular Mtb and identified the mechanisms underlying the
outcomes we identified. We found that macrophages are primed for antimycobacterial activity by pre-treatment with IFNλ1 and this enhanced their ability to limit transcriptional activity in intracellular Mtb. In work identifying the factors underlying this change in mycobacterial viability, we found that IFNλ1 pre-treatment led to increased acidification of Mtb containing phagolysosomes but did not strongly affect oxidative or hypoxic stress generation by macrophages. Overall, our results indicate that IFNλ1 upregulates macrophage antimycobacterial activity and suggest that this cytokine family has previously unappreciated functions in TB.

4.2 Materials and methods

4.2.1 Macrophage culture

Percoll or Ficoll gradient isolated PBMCs were obtained from Mtb-infected cynomolgus, and rhesus macaques (Table 5) involved in studies being performed at the University of Pittsburgh. Monocytes were isolated from PBMCs using anti-CD14 beads that cross react with NHP CD14 (Miltenyi Biotec, Auburn, CA) as per the manufacturer’s instructions. Isolated monocytes were suspended in RPMI 1640 media (Lonza, Walkersville, MD) supplemented with 20% FBS (Gibco, Grand Island, NY), 1% L-glutamine (Sigma-Aldrich St. Louis, MO), 50 μM 2-Mercaptoethanol (Gibco), 0.1 mM sodium pyruvate (Gibco), 0.01 μg/ml M-CSF (Sigma-Aldrich) and 0.006 μg/ml GM-CSF (Sigma-Aldrich) and 100 U/ml penicillin-streptomycin (Gibco) and plated in 12-chamber flat bottom well plates at a density of 1-1.5x10^6 cells/well. Media was changed every 3-4 days and fresh RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, 1% HEPES (HyClone, Logan, UT) (hereafter referred to as R10 media) and 1 mg/ml penicillin (Alfa Aesar,
Haverhill, MA) was added, and cells were cultured for 7-10 days for differentiation into macrophages.
Table 5. Information on animals used for *in vitro* infection assays

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<th>Treatment</th>
<th>Infection Date</th>
<th>Necropsy Score</th>
<th>Total CFU</th>
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The table details information on animals used for *in vitro* infection assays, including NHP number, experiment details, age, sex, dose, days infected, treatment, infection date, necropsy score, total CFU, and lung CFU.
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4.2.2 Cell treatments

Monocyte-derived macrophages (MDMs) were stimulated with recombinant human IFNλ1 (100 ng/ml, Peprotech, Cranbury, NJ), recombinant human IFNλ4 (100 ng/ml, R&D Systems, Minneapolis, MN), IFNα hybrid protein (100 U/ml, PBL Assay Science, Piscataway, NJ). The IFNα hybrid protein was selected as a stimulator because it mimics all of the IFN1 isoforms and was shown in our previous studies to stimulate canonical IFN1-regulated responses. For Mtb infection assays, MDMs were stimulated with the cytokines for 24 hours prior to infection (pre-treatment) or after infection (post-treatment) as mentioned further in the paper.

For IFNλ.R1 neutralization experiments, macrophages were incubated with 10µg/ml of anti-human IFNλ.R1 neutralizing antibody (PBL Assay Science) for 1 hour at 37°C with 5% CO2 prior to Mtb infection. The concentration of antibody was based on the manufacturer’s data indicating that 10-60ng/ml was required to neutralize 100pg of IFNλ1 to a 50% endpoint. Isotype antibody was used at the same concentration as the anti-IFNλ.R1 antibody (10µg/ml of mouse IgG1 antibody (Thermo Fisher Scientific, Waltham, MA)) as a control.

For experiments where we blocked lysosomal acidification, 50nM bafilomycin A1 (Tocris Bioscience, Minneapolis, MN) was added to macrophage cultures with or without IFNλ1 and the cells were incubated overnight at 37°C with 5% CO2. Fresh media containing bafilomycin A1 was added to the cultures before Mtb infection and the cells were incubated in bafilomycin A1-containing medium until the endpoint of the experiment.
4.2.3 Mtb culture and infection

Aliquots of the bacterial reporter strains (*hspX*:GFP, *smyc*:mCherry, Live/Dead-H37Rv, mCherry-H37Rv) were stored in -80°C were thawed before being added to 5ml of 7H9 media (Sigma-Aldrich), supplemented with 0.2% glycerol (Sigma-Aldrich), 10% ADC+O, 0.05% Tween-80 (Sigma-Aldrich) and Hygromycin B (only for the transformed Mtb reporter strains) in a T25 flask (Thermo Fisher Scientific). Bacteria were cultured for 5-6 days at 37°C with 5% CO₂ until an OD₆₀₀nm of 0.5-0.6 was reached. Bacteria were then passed through a sterile 10-µm syringe filter (MilliporeSigma, Burlington, MA) to get single cell suspension for infections. For Mtb Erdman only, prior to this filtering step, bacterial clumps were broken by aspirating 25-30 times with a syringe and blunt-tip syringe.

MDMs were either added to 5 ml polystyrene round-bottom tubes (Corning, Glendale, AZ) for flow cytometry assays or seeded into 12 chamber slides (ibidi, Fitchburgh, WI) for microscope-based assays. Cells were treated with cytokines as previously described and infected at an MOI of 2 bacteria/cell. After 4 hours of incubation at 37°C with 5% CO₂, the cells were washed with 1x PBS and fresh R10 media with or without cytokines was added and infection was allowed to progress overnight. For the live/dead strain, anhydrotetracycline (100ng/ml, Cayman Chemical Company, Ann Arbor, MI) was added the following day and the cells were incubated for another 19-20 hours before fixation in 2% paraformaldehyde (PFA). For the nitric oxide detection assay, no anhydrotetracycline was added and cells were fixed in 2% PFA after the overnight infection period.
4.2.4 ELISA

MDMs were stimulated with gamma-irradiate Mtb or infected with Mtb Erdman for overnight period. IFNλ level in cell culture supernatants was detected by ELISA using a human IL-29 (IFN-lambda 1) ELISA kit (R&D Systems), and the assay was performed according to the manufacturer’s protocol.

4.2.5 Nitric oxide and superoxide detection

MDMs were seeded into 12-well chamber slides (ibidi) and treated with IFNλ1, IFNλ4 and IFNα for 24 hours before infection or after Mtb infection. At the end of the incubation, supernatants from the cells harvested and stored in -80°C for nitrite detection by the Griess assay. DAF-FM diacetate and CellROX Deep Red (Thermo Fisher Scientific, Waltham, MA) staining was done as per manufacturer’s instructions where the cells were incubated in R10 containing 1 μM DAF-FM diacetate (Thermo Fisher Scientific) and 5 μM CellROX Deep Red reagent (Thermo Fisher Scientific) for 30 mins at 37°C with 5% CO2. Following incubation, the cells were washed twice in PBS and then incubated in PBS for 20-30 mins before fixation in 2% PFA. The slides were imaged on the same day after they were transferred out of the BSL3, and coverslips were mounted ProLong Gold Mounting Medium (ThermoFisher Scientific). Images were acquired with a DS-Qi2 camera (Nikon Instruments) with filters for DAPI, TRITC, FITC, and Cy5. NIS-Elements AR version 4.50 (Nikon Instruments) was used for image capture and setting imaging parameters.

Nitrite and nitrate release from cytokine treated and Mtb infected MDMs were detected using a Nitrate/Nitrite colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI). Supernatants from the cells stored in -80°C under BSL3 conditions and were thawed and heated in water bath
at 65°C for 1 hr to kill Mtb and transferred to new Eppendorf tubes for further processing under BSL2+ conditions. Total nitrate and nitrite level was assessed according to the manufacturer’s instructions.

4.2.6 Hypoxia detection

Hypoxia levels in IFNλ1 pre-treated and Mtb-infected MDMs was assessed using the flow cytometry-based Hypoxia Green reagent (Thermo Fisher Scientific) as per manufacturer’s protocol.

4.2.7 LysoTracker assay

MDMs were pre-treated with IFNλ1 and infected with SYTO9 green (Thermo Fisher Scientific) labelled Mtb Erdman. Briefly, after syringe aspiration and 10μm filtration, bacteria were centrifuged at 3000 rpm for 10 minutes, resuspended in 0.005 mM SYTO9 containing 1ml of PBS and incubated for 15 mins. Bacteria were then washed thrice with PBS to remove excess SYTO-9 dye and OD<sub>600nm</sub> was measured to determine the final concentration of bacteria after washing. MDMs were infected at an MOI of 2 for 4 hours, washed with PBS and incubated in R10 media overnight. Cells were then stained with pre-warmed R10 containing 50 nm Lysotracker Red DND-99 dye (Thermo Fisher Scientific) for 1hr at 37°C with 5% CO<sub>2</sub>, washed with PBS and fixed with 2% PFA. Images were acquired in three color channels on the same day as the experiment was performed.
4.2.8 Image analysis

For the live/dead viability and LysoTracker assay images, analyses were done in ImageJ software [495]. Briefly, TIF images were loaded into the software and the different colors were split into individual channels. A merged image of red and green channels was generated by using the AND operation of the red and green channels in image calculator, so that we could determine the green (GFP) pixel intensity of only the red bacteria in the live/dead viability assay. A similar procedure was performed to determine the red LysoTracker pixel intensity that colocalized with the green SYTO9-labeled bacteria in the cells. Channels were stacked as necessary for different analyses and color intensities were measured and exported.

4.2.9 Statistics

All statistical analyses were performed in GraphPad Prism v9.1.2 (GraphPad Software, San Diego, CA). Prior to performing statistical analyses, normality of all datasets were checked with Shapiro-Wilk test and parametric tests were used for normally-distributed data and non-parametric tests were used for data that did not fit a Gaussian (normal) distribution. A p-value < 0.05 was considered to be statistically significant.
4.3 Results

4.3.1 Mtb antigens stimulate IFNλ1 production by macrophages

We previously showed that exposure to inactivated Mtb can induce IFNλ signaling in MDMs in a TLR2-dependent manner [494], suggesting that this process may induce macrophage IFNλ expression. With this in mind, we sought to determine whether Mtb infection upregulated IFNλ expression by macrophages, and because macrophages are the primary cell type infected by Mtb [496], whether this influenced macrophage antimycobacterial activity. We measured IFNλ concentration in supernatant from macrophages that were stimulated with gamma-irradiated Mtb and found increased concentrations of IFNλ1 in the supernatant (Fig. 30A, left). In contrast, we did not find that increased IFNλ1 concentrations in supernatant increased when macrophages were infected with viable Mtb (Fig. 30A, right). Next, we wanted to determine how intrinsic IFNλ signaling affects Mtb viability and therefore we neutralized IFNλR1 expression on macrophages prior to Mtb infection. We used a live-dead fluorescent reporter strain of Mtb that constitutively expresses mCherry protein under the control of the GroEL promoter [497, 498] and has inducible green fluorescent protein (GFP) expression under the control of a tetracycline-inducible promoter (Fig. 30B, left). Therefore, the GFP signal can be used to identify transcriptionally active bacteria as a proxy for bacterial viability. We observed that when IFNλR1 was blocked, there was a modest increase in the GFP/mCherry ratio, which is indicative of increased GFP transcription Mtb as a proxy for bacterial transcriptional activity and viability (Fig. 30C). This indicates that IFNλ expressed in response to Mtb antigens may play a modest role in restricting transcriptional activity the activity of intracellular Mtb.
Figure 29. IFNλ1 inhibits transcriptional activity of intracellular Mtb in vitro.

(A) IFNλ1 levels in supernatants of gamma-irradiated Mtb stimulated macrophages (left) and viable Mtb infected macrophages (right). Statistical comparisons by paired t test for untreated vs. gamma-irradiated Mtb (left) and Wilcoxon matched-pairs signed rank test for untreated vs. viable Mtb (right). (B) Live/Dead strain of Mtb as viewed under the microscope. (C) Graph comparing GFP/mCherry pixel intensity ratio of Live/Dead Mtb strain in macrophages treated with isotype vs. anti-IFNλRI antibody. Statistical comparison by paired t test. (D, E and F) Graphs comparing the GFP/mCherry pixel intensity ratios of Live/Dead Mtb strain in macrophages that were pre or post-stimulated with IFNλ1 (D), IFNλ4 (E) and IFNα (F). Statistical comparisons by Dunnett’s multiple comparisons test.
4.3.2 IFNλ1 pre-treatment enhances antimycobacterial activity of macrophages

Since the infection with viable Mtb did not significantly increase IFNλ1 expression in comparison to gamma-irradiated Mtb, we next wanted to determine how exogenous addition (supplementation) of IFNλ affected the viability of intracellular Mtb. Simultaneously, we also wanted to compare how activation by IFNλ and IFNα affected the viability of intracellular Mtb to determine how these cytokines, which share common signaling elements, affect macrophage antimicrobial activity. MDMs were treated with supplementary IFNλ1, IFNλ4 and IFNα either prior to Mtb infection (pre-treatment) or after infection (post-treatment). We observed modest reduction in the Mtb GFP/mCherry pixel intensity ratio when macrophages were pre-treated with IFNλ1 (Fig. 29D), indicating that IFNλ1 enhances macrophage anti-Mtb activity. This inhibitory effect was not observed when IFNλ1 was added post-infection suggesting that priming of macrophages with IFNλ1 is necessary to activate their Mtb-restrictive activity. Moreover, we also did not detect inhibition of Mtb’s transcriptional activity during pre- or post-treatment with IFNλ4 or IFNα (Fig. 29E, F). These data, for the first time, show the anti-mycobacterial capacity of IFNλ1 and also highlight that although IFNλ1 and IFNλ4 belong to the same family, they have different functional properties.

We plated aliquots of IFNλ1 pre-treated Mtb infected macrophages on 7H11 agar plates to determine whether the responses we noted equated to reduced Mtb viability (fewer colony-forming units (CFUs)). We did not observe a reduction in CFUs/culture when macrophages were treated with IFNλ1 prior to Mtb infection (Fig. 29G). Overall, our results indicate that IFNλ1 can potentially activate macrophage defense responses that restrict Mtb’s transcriptional activity but might not be directly bactericidal. Alternatively, it could also be possible that the antibacterial activity is below the sensitivity level of the CFU plating assay.
4.3.3 IFNλ1 mediated inhibition of Mtb’s activity is not dependent on oxidative stress generation by macrophages

We next sought to identify the antimicrobial mechanisms that IFNλ1 induces in macrophages that reduce transcriptional activity in intracellular Mtb. To determine whether IFNλ1 induced oxidative or hypoxic stress by pre-treated macrophages, we used an Mtb reporter strain that constitutively expresses mCherry and inducibly expresses GFP in response to hypoxic or oxidative mediated stress (Fig. 30A) [499, 500]. We found a trend of increased GFP expression in IFNλ1 pre-treated macrophages (Fig. 30B), and although not statistically significant, this suggested that IFNλ1 may promote macrophage nitric oxide or superoxide production or induce activation-related intracellular hypoxic conditions in macrophages.

We therefore investigated whether IFNλ1 stimulation activates reactive nitrogen species (RNS) or reactive oxygen species (ROS) production in Mtb-infected macrophages. We used DAF-FM diacetate (a green fluorescent stain for nitric oxide) to detect RNS production, CellROX Deep Red (a far-red fluorescent dye for reactive oxygen species) to detect ROS production (Fig. 30C) and a colorimetric assay to detect nitrate and nitrite levels (the end product of nitric oxide production) present in the cell culture supernatants. We observed a trend of increased DAF-FM diacetate fluorescence in Mtb-infected macrophages in comparison to uninfected cells (Fig. 30D, left). However, we did not find increased DAF-FM diacetate or CellROX fluorescence in macrophages that were treated with IFNλ1 before Mtb infection (Fig. 30D left and right, respectively). Similarly, we did not detect increases in nitrate and nitrite levels in the supernatant of IFNλ1 pre-treated after Mtb infection suggesting that nitric oxide was not being produced (Fig. 30E).
To determine whether macrophages were becoming hypoxic because of their activation state and this was restricting bacterial transcription, we used flow cytometry to detect changes in hypoxia level after Mtb infection with or without IFNλ1 stimulation before infection (Fig. 30F, left). We observed increased hypoxia green fluorescence in macrophages after Mtb infection, but we did not observe significant difference in the hypoxia levels of IFNλ1 pre-treated macrophages after Mtb infection (Fig. 30F, right). Overall, these data indicate that IFNλ1 is not inducing upregulation of reactive oxygen or nitrogen species by macrophages or hypoxic stress to reduce transcriptional activity in Mtb.

Figure 30. IFNλ1 pre-treatment does not promote oxidative stress generation in infected macrophages.
(A) hspX’ strain as viewed under the microscope (40X magnification). (B) Comparison of GFP/mCherry pixel intensity ratio of the hspX’ strain in untreated vs. pre- IFNλ1 treated macrophages. Statistical comparison by paired t test. (C) DAF-FM and CellROX deep red staining in mCherry infected macrophages as viewed under the microscope (40X magnification). (D, E) Graphs comparing DAF-FM (left) and CellROX (right) intensities and total nitrate and nitrite in cell supernatants among untreated, mCherry Mtb infected and pre-IFNλ1 treated, mCherry Mtb infected macrophages. Statistical comparisons by Dunnett’s multiple comparisons test. (F) Flow plots showing hypoxia green positively stained macrophages (left). Graph on the right showing the mean fluorescence intensity hypoxia green staining among untreated, mCherry Mtb infected and pre-IFNλ1, mCherry Mtb infected macrophages. Statistical comparisons by Dunnett’s multiple comparisons test.

4.3.4 IFNλ1 can increase acidification of Mtb containing phagolysosomes in macrophages

One of the major mechanisms that Mtb uses to evade the host immune responses is to prevent acidification of its phagosome so it can survive intracellularly [50, 51]. Considering this, we used LysoTracker Red dye to determine whether IFNλ1-stimulated macrophages can overcome Mtb’s ability to block phagosomal acidification. In these studies, we labeled Mtb Erdman with SYTO9 green, a fluorescent DNA stain, to visualize it after phagocytosis in combination with LysoTracker Red-stained macrophages (Fig. 31A). We observed that Mtb colocalized with stronger LysoTracker Red fluorescence in IFNλ1 pre-treated macrophages than phagosomes in macrophages that were not pre-treated with IFNλ1 (Fig. 31B). This increase in colocalization indicates that IFNλ1 improves a macrophage’s ability acidify Mtb-containing phagosomes after infection.
We used bafilomycin A1, a small molecule inhibitor of vacuolar H\(^+\)-ATPases \([501, 502]\), to verify that IFN\(\lambda\)1 priming improves a macrophage’s ability to acidify Mtb-containing phagosomes. In these studies, we predicted that bafilomycin A1-treated macrophages would lose their ability to restrict mycobacterial transcription, even after being primed by IFN\(\lambda\)1. We used the same approach as previously indicated for our studies using GFP-mCherry live/dead Mtb reporter strains and found that bafilomycin-treated macrophages did not reduce bacterial GFP expression (Fig. 31C) indicating that blocking lysosomal acidification abrogated IFN\(\lambda\)1’s antimycobacterial activity. When bafilomycin was used in combination with IFN\(\lambda\)1, we no longer observed the overall decrease in Mtb’s transcriptional activity (using GFP expression as a proxy) indicating that blocking lysosomal acidification abrogates IFN\(\lambda\)1’s ability to activate macrophages. We did observe, however, that macrophages from some animals (light pink and sky-blue colors) still reduced Mtb’s transcriptional activity after IFN\(\lambda\)1 pre-treatment in the presence of bafilomycin A1 (Fig. 31C), although this change was similar to that seen with bafilomycin A1-only samples suggesting off-target effects may limit the application of this reagent in this system. Taken together, these results indicate that IFN\(\lambda\)1 promotes phagolysosomal fusion and acidification in macrophages and may activate some other pathways that enhance macrophage activity against Mtb.
Figure 31. IFNλ1 pre-treatment of macrophages promotes a modest increase in acidification in Mtb containing phagolysosomes

(A) Microscopic images of LysoTracker red staining in Mtb containing phagolysosomes of macrophages (60X magnification). (B) Comparison of LysoTracker red intensity in Mtb containing phagolysosomes between untreated and IFNλ1 pre-treated macrophages. Statistical comparison by paired t test. (C) Fold change in GFP/mCherry pixel intensity ratio in bafilomycin treated only and IFNλ1 pre-treated macrophages in the absence or presence of bafilomycin, with respect to untreated condition (Mtb only). Statistical comparisons by Dunnett’s multiple comparisons test.
4.4 Discussion

Macrophages are one the first host immune cells that Mtb encounters after being inhaled and are a critical component of the innate immune system that prevents establishment of infection [476, 477]. Mtb can evade many of the antimicrobial pathways that kill other bacteria and instead use macrophages as a niche to survive intracellularly [151, 502]. Identifying factors that enhance anti-mycobacterial macrophage functions is crucial for developing new treatments against TB. We previously identified that IFNλ1 and IFNλ4 are expressed by macrophages in NHP granulomas (Chapter 1) and that macrophages undergo IFNλ-mediated signaling (Chapter 2). In Chapter 3, we explored the impact of IFNλ treatment on the activity of macrophages against Mtb. Moreover, IFNλs and IFNα have a similar downstream signaling pathway and IFNα is often associated with exacerbated TB, we also wanted to determine how these two groups of cytokines regulate macrophage activity against Mtb.

Cytokine mediated cell-cell communication plays a key role in regulating immune cell function against invading pathogens. In contrast to other infections, adaptive immunity is delayed in TB; thus, innate immunity during early infection plays a pivotal role in controlling bacterial replication before the onset of adaptive immune responses. Cytokines continue to be important later in infection where protective immune responses against Mtb are mediated by a qualitative balance of by pro- and anti-inflammatory cytokines in granulomas [128]. Understanding the complicated mix of which cytokine is protective on one situation and detrimental in another and linked to protection or pathology, respectively, is key for the producing the next generation of efficacious treatment strategies.

We found that macrophages that were stimulated by gamma-irradiated Mtb expressed IFNλ1, whereas macrophages that were infected with live Mtb Erdman did not appear to
upregulate IFNλ1 expression. This suggests that Mtb antigens can stimulate macrophage IFNλ1 expression, but viable Mtb might be able to inhibit production of this cytokine. Alternatively, another reason for this variation could be the potentially different antigen loads between out gamma-irradiated and viable Mtb Erdman stocks. We found that pre-treatment of macrophages with IFNλ1 reduced bacterial transcriptional activity, as indicated by less Mtb GFP expression. This did not occur when IFNλ1 was added after infection suggesting that IFNλ1 pre-stimulation activates macrophage Mtb-restrictive functions but once an infection is established, IFNλ1 stimulation cannot overcome Mtb’s ability to inhibit pathogen processing. This is consistent with recent work showing that pre-treatment of GM-CSF differentiated human MDMs or human alveolar macrophages with IFNλ1, restricted replication of influenza virus [356]. In contrast to our results with IFNλ1, we did not find that either IFNλ4 or IFNα were able to restrict Mtb when macrophages were treated before or after infection. This corresponds with our results in Chapter 2 where IFNλ1 induced an activated pro-inflammatory state in macrophages while IFNλ4 and IFN1 induced a different set of genes, although in those studies, the macrophages were not infected with Mtb.

Oxidative stress generation is important an important tool in the macrophage arsenal against Mtb, as demonstrated by increased susceptibility to Mtb in iNOS-deficient mice [503]. The role that NO plays in restricting Mtb in humans remains controversial for human macrophages [158, 504]. We were unable to identify upregulation of ROS and RNI in IFNλ1 pre-treated macrophages despite these cells being able to restrict Mtb, suggesting that this activity occurs through different mechanism. Hypoxic stress can enhance production of pro-inflammatory cytokines including IL-1β and TNF by human macrophages and a change in metabolic activity that promotes restriction of Mtb [505-508]. We found that IFNλ1 pre-treatment did not increase
hypoxia in infected macrophages. We did not investigate how IFNλ1 influences macrophage metabolic activity, and this factor may also play a role and deserves to be investigated further.

Mtb’s ability to block phagosomal maturation is critical to its ability to survive in macrophages [501], thus, our next set of experiments focused on this aspect of macrophage biology. Our results showed that pre-treating macrophages with IFNλ1 increased acidification of Mtb-containing phagosomes indicating this antimicrobial mechanism is modified by IFNλ1 treatment and contributes to macrophage restriction of Mtb. Our follow up studies blocking phagosomal acidification further suggested that this subcellular activity is important for mediating this effect on Mtb. Conversely, our observation that IFNλ1 post-treatment fails to generate Mtb-restrictive macrophages suggests that this cytokine cannot overcome the bacterial block on phagosomal maturation. We previously showed that IFNλ1 upregulates some of the same sets of genes as IFNγ (Chapter 2) but IFNλ1’s inability to overcome an Mtb’s block on phagosomal maturation sets it apart from this critically important cytokine and suggests the activity of IFNλ1 on macrophages is more modest than IFNγ’s effect.

Taken together, our results indicate that IFNλ1 may have novel functions in TB that have not been previously appreciated. IFNλ1’s ability to activate macrophages and restrict Mtb may help the innate immune response to keep the Mtb in check and ultimately augment the effects of the adaptive immune response in vivo. Future work identifying how IFNl1 levels change over the course of infection and how IFNλ1 affects responses in tissue cells, including differentiated macrophages from lung tissue or granulomas, will lead to new information on the role this cytokine plays in determining lesion- and host-level outcomes in TB.
4.5 Limitations

Some of the animals included in this study were drug treated and BCG vaccinated which might represent a limitation to the interpretation of the data because the BCG vaccination might represent a confounding factor due to the unknown possible influence of pre-existing or trained immunity. Bafilomycin A1 has off target effects that are not limited to V-ATPase and can extend into autophagy pathways that contribute to antimycobacterial immunity. Further experiments using alternative approaches including quantification of Rab5 to Rab7 conversion on phagosomes may clarify the role that phago-lysosomal fusion and acidification plays in promoting IFNλ1-mediated antimycobacterial activity.

4.6 Acknowledgements

We gratefully acknowledge JoAnne Flynn and Flynn lab technicians for providing the macaque PBMCs we used in these studies.
5.0 Summary and implications, public health significance, and future directions

5.1 Summary and implications

TB is the second leading infectious killer after COVID-19 and claimed the lives of 1.5 million people in 2020 [1]. TB is an ancient disease and despite it being preventable and curable, much of the world’s population is infected with Mtb and TB continues to be responsible for high morbidity and mortality [1, 509]. The only commercially-available vaccine against TB (BCG) is 70-80% effective against miliary TB, which is the most severe form of TB, but offers minimal protection against pulmonary TB in adults [510]. Mtb is an airborne pathogen that is transmitted via droplets produced by infected people when coughing, sneezing or talking. Following inhalation, Mtb is phagocytosed by lung resident macrophages and these cells release cytokines and chemokines that recruit other immune cells to the site of infection and this eventually leads granuloma formation [511-513]. Mtb infection can have a spectrum of outcomes ranging from subclinical to life threatening, as has been demonstrated in low dose NHP infection models [124, 136]. At the heart of all of this are granulomas, and these lesions are heterogenous and have variable potentials for limiting Mtb, and thus follow a distinct fate even within one individual [122, 123]. A major contributor to granuloma function is the qualitative balance between pro-inflammatory and anti-inflammatory cytokines that ultimately determines the activation states of the immune cells in granulomas. Furthermore, TB is characterized by a delayed onset of adaptive immune response, and the cytokine milieu in the initial stages of granuloma formation may play an important role in restricting the bacilli and also proper activation of lymphocytes as the granuloma matures. IFNs are important constituents of the cytokine milieu in granulomas, and
while there has been much research regarding the roles of IFN1 and IFNγ in TB disease, the source and function of IFNλs in TB remains unknown. Here we investigate the presence and source of IFNλ expression in lung TB granulomas of Mtb infected NHPs and determine their role in modulating the function of macrophages, which are the primary host cells for Mtb.

5.1.1 IFNλs are expressed in Mtb-infected NHP lung granulomas by neutrophils and macrophages

In our first study we wanted to determine whether IFNλs are expressed in lung granulomas and by which cells. Previous studies by other groups have shown that Mtb-infected A549 epithelial cells upregulated IFNλ2 transcript [404]. Similarly, active TB patients have high levels of IFNλ2 protein in their sputum compared to those with latent TB or healthy individuals [408]. This indicates that Mtb infection can induce IFNλ expression but it was unknown whether cells in granulomas express IFNλs and which cells may be responsible for this expression.

We measured IFNλ concentration in granulomas and uninvolved lung and found that granulomas contain significantly more IFNλ1/3 than uninvolved lung and granuloma IFNλ concentrations negatively correlated with granuloma bacterial burden. Although correlation is not equivalent to causation, the strong negative correlation indicates that IFNλs may be directly or indirectly associated with protection in TB. To follow up on these results, we next sought to determine which cells in the granuloma express IFNλs by focusing on IFNλ1 and IFNλ4, two disparate members of the IFNλ family. We found that neutrophils represented the highest frequency of cells expressing IFNλ1 and IFNλ4, followed by macrophages and that cells in different microenvironments expressed different levels of these cytokines. This observation may impact the activation status and functions of the nearby immune cells and affect overall granuloma
function [129]. For instance, lymphocyte cuff macrophages can have anti-inflammatory M2 phenotypes while epithelioid macrophages have features associated with pro-inflammatory M1 phenotypes [129]. We observed that macrophages in the lymphocyte cuff expressed more IFNλ1 than epithelioid macrophages and considering that IFNλ1 has been reported to have a Th1 skewing effect [344, 426], expression in the lymphocyte cuff may modulate the Th1/Th2 polarization of T cells in that region. We also noted that granulomas harvested from animals with longer-duration infections had lymphocyte cuff macrophages with greater IFNλ1 and IFNλ4, and lymphocyte cuff neutrophils with higher IFNλ4 expression than caseum neutrophils, suggesting intra-granuloma IFNλ expression may have a temporal dynamic. A reason for the variation in IFNλ expression in different granuloma microenvironments and infection timepoints could be due to exposure to different concentrations of bacterial antigens, DAMPs, and cytokines. The consequence of this level of heterogeneity is not fully understood but could lead to different cellular activation states and differential capacity to express IFNλs.

We also noted interesting results with regard to the subcellular localization of IFNλ4 and IFNλR1 in our study. We found that IFNλ4 in granuloma macrophages was primarily localized inside of the nuclei whereas IFNλ1 rarely showed a similar localization profile. A search of the literature indicated that IFNλ4 contains a nuclear localization sequence (NLS) and this is likely responsible for its nuclear localization [431]. Intracellular localization of cytokines has also been reported for IFNα, IFNγ and IL-1α [514, 515]. IFNλ4 was present in both the cytoplasm and nuclei suggesting that there are fundamental differences in how this cytokine behaves in these two myeloid cells, and also suggesting that IFNλ4 may have unique regulatory roles in macrophages that require further investigation. We also noted that IFNλR1, not just IFNλ4, was present in the nucleus after TLR2 ligation and in MDMs stimulated with gamma-irradiated Mtb. suggesting that
stimulation by Mtb antigens initiates a signaling pathway that leads to nuclear translocation of IFNλR1. A similar phenomenon has also been observed for IFNγR1 where its translocation to the nucleus and this behavior enhance the strength of IFNγ signaling [422, 432]. Overall, these observations add to our understanding of how cytokines function, how cytokine signaling occurs in vitro and in vivo, and how the biology of cytokines has a spatial component that is often overlooked when considering their function.

In summary, the first chapter of my thesis shows for the first time that IFNλs are expressed in TB granulomas, that myeloid cells are the major sources of IFNλ production, and that IFNλ concentrations negatively correlate with bacterial burden in granulomas. Furthermore, we also highlight subtle differences in the sub-cellular localization and signaling properties of IFNλ1 and IFNλ4 and demonstrate that TLR2 pathway and IFNλ signaling can mediate changes in IFNλR1 expression by myeloid cells. Although these studies did not identify a function for IFNλs, they offer new insights into this previously unappreciated cytokine family at the site of disease in TB.

5.1.2 IFNλ and IFNα have different macrophage activation properties

My second study addressed the impact of IFNλ stimulation on macrophage phenotype and function and compared these responses against IFNα-stimulated macrophages. Both IFNλ and IFN1 have a similar signaling pathway and IFN1 is often associated with exacerbated outcomes in TB [186, 246, 248]. Moreover, IFN1 can inhibit IFNγ mediated production of pro-inflammatory cytokines like IL-12, TNF by macrophages and promote expression of immunosuppressive cytokine like IL-10 [260, 266]. In my previous chapter, I found that IFNλs are expressed by myeloid cells in granulomas and these cells appear to be able to respond stimulation by these cytokines. Thus, it is critical to know how IFNλ might affect macrophage phenotypes and
functional behaviors and if responses to IFNλs stimulation differs from IFN1. Moreover, because there is substantial divergence in the IFNλ family members in terms of amino acid sequence and localization patterns, it is important to assess how responses mediated by these cytokines differ. Overall, we hypothesized that IFNλ stimulation would promote pro-inflammatory activation and upregulation of anti-mycobacterial effector genes in macrophages and these responses would differ from IFNα-induced responses. We investigated this with NanoString transcriptional profiling and analyses and then validated these mRNA-level results at the protein level with flow cytometry.

We found that macrophages were responsive to IFNλ1, IFNλ4 and IFNα and that each cytokine induced a distinct set of genes. IFNλ1 upregulated genes for co-stimulatory proteins including CD86, CD40LG, CD80 and ICOSLG suggesting that IFNλ1-polarized macrophages may contribute to T cell activation. We also observed upregulation of other pro-inflammatory factors including PTAFR, MAFG, cytokines like IL-8, chemokines like CCL2 and CCL5. IFNλ1 downregulated anti-inflammatory and type 2 immunity-related factors factors including TSLP, IL-33, IL-4 suggesting that IFNλ1-activated macrophages may have potent inflammation promoting and T cell activating properties. In contrast, IFNα upregulated canonical IFN1-regulated ISGs while downregulating macrophage activating genes including IL18 and IFNγR1, and macrophage-expressed cytokines that are important for anti-Mtb immunity including IL-B and IL-12. Interestingly, IFNλ4 differed from both IFNλ1 and IFNα both in terms of the number of genes it induced and the genes that were induced. IFNλ4 stimulated macrophages expressed relatively few genes and the genes that were induced were genes involved in fibrosis and immune homeostasis including TWIST2, HSPB2, and RORC.

At the protein level, both IFNλ1 and IFNλ4 increased expression of the co-stimulatory molecule CD86 on macrophages while IFNλ1 also increased expression of CD40 (a costimulatory molecule) and pro-inflammatory cytokines and chemokines including IL-1β and CCL5,
respectively. IL-1β promotes anti-bacterial activity in murine and human macrophages and can mediate apoptosis in Mtb-infected macrophages [167]. CCL5 is associated with protection in TB, and CCL5 knockout mice have altered immune cell recruitment and impaired T cell function and IFNγ production [516]. We did not observe IFNα-mediated upregulation of any of these proteins, further suggesting that these cytokines promote different functional responses in target cells, including macrophages.

Our most significant observation was the predicted similarity observed between IFNλ1 and IFNγ. This was noted both by gene set enrichment analysis where we observed that genes involved in IFNγ and TNF signaling, which are important for macrophage activation and proper granuloma formation [169, 172, 176], were enriched in IFNλ1-stimulated macrophages. This was also noted in our Ingenuity Pathway Analysis (IPA) which predicted IFNγ as the top upstream regulator in IFNλ1-stimulated macrophages. IL-1β was also noted as a potential regulator for mediating some of the genes expressed in our IFNλ1-stimulated macrophages. These observations highlight the potential for IFNλ1 to be an important player as a macrophage-acting cytokine that contributes to protection against Mtb.

Overall, chapter 2 of my thesis identifies distinct differences between IFNλs and IFNα, and even between different IFNλs, with respect to how they regulate macrophages. Moreover, I demonstrated that IFNλ activates antibacterial defense responses in macrophages whereas this did not occur in response to IFNα stimulation. This chapter further highlights the non-redundant roles of IFNλ and IFNα in modulation of macrophage phenotype, and considering this, each cytokine is likely to have an unexpectedly distinct role in granuloma function.
5.1.3 IFNλ1 priming enhances macrophage antimycobacterial activity by promoting phagolysosomal acidification

The third chapter of my thesis addressed the effect of IFNλ treatment on the antimycobacterial activity of macrophages. In chapter 1 we showed that IFNλ is expressed in TB granulomas, and it negatively correlates with the bacterial burden. In chapter 2 we identified that IFNλ1 promotes pro-inflammatory activation of macrophages and upregulates pathogen defense response pathways in these cells. Therefore, our next question was to determine whether IFNλs have protective or pathogenic roles in Mtb-infected macrophages. My objective was to identify the effect that IFNλ1, IFNλ4 and IFNα treatment had on Mtb using a variety of systems including fluorescent Mtb reporter strains and quantifying elements involved in antimicrobial macrophage activity. Since in our previous chapter, we showed that IFNλ1 upregulates antimycobacterial genes and cytokines in macrophages, we hypothesized that treatment of macrophages with IFNλ1 will have greater anti-Mtb activity than either IFNλ4- or IFNα-stimulated macrophages.

Our first objective was to determine the effect of stimulation with IFNλ1, IFNλ4, and IFNα, before or after Mtb infection, on macrophage antibacterial activity. We found that macrophages that were pre-stimulated (primed) with IFNλ1 had the ability to significantly reduce Mtb transcriptional activity. We did not see this reduction when IFNλ1 was added to macrophage cultures after infection, or in either situation for IFNλ4- or IFNα-stimulated macrophages. I followed up these results with studies to identify the anti-Mtb mechanisms that are being induced by IFNλ1 by investigating three macrophage-associated effector functions including reactive oxygen and nitrogen production, hypoxia induction, and phagosomal acidification.

We first used a hspX′::GFP reporter Mtb strain that is sensitive to oxidative stress to determine whether IFNλ1 pre-stimulation increased nitric oxide or superoxide production, or
induced hypoxic stress in macrophages [499, 500]. This reporter strain constitutively expresses mCherry and upregulates GFP expression after induction of the hspX gene and although we did not see a significant increase in the level of GFP expression by IFNλ1 pre-stimulated MDMs, we noted a trend of increased GFP expression. This suggested to us that IFNλ1 pre-stimulation may be inducing oxidative stress, but our follow up experiments did not show increased nitric oxide or superoxide production. Furthermore, we did not find that IFNλ1-primed MDMs were hypoxic, suggesting that IFNλ1 does not act by the nitric oxide or hypoxia generation pathways, and is likely to be enhancing some other anti-microbial defense response in macrophages.

One of the strategies by which Mtb evades the host immune response and persist intracellularly in macrophages, is by blocking the fusion of phagosome and lysosomes and inhibiting phagosome maturation. Cytokine stimulation can promote phagolysosomal fusion in Mtb-infected macrophages [497], therefore we sought to determine whether IFNλ1 priming MDMs will promote greater degree of phagosomal maturation. We found that this occurred in IFNλ1-primed macrophages where Mtb colocalized with acidified phagosomes in these cells, indicating that IFNλ1 promotes the phagosome maturation pathway. Moreover, the effect of IFNλ1 priming on Mtb viability was abrogated when lysosomal acidification was blocked, further supporting our hypothesis that IFNλ1 promotes phagosomal maturation in macrophages.

Our results in Chapter 3 demonstrate a diversity of functional outcomes can be induced in macrophages by stimulation with different IFNs. This was evident with the three cytokines we investigated here, where IFNλ1 induced macrophage antimicrobial activity whereas IFNλ4 and IFNα did not. The observation that IFNα did not decrease viability in Mtb corroborates the work of other groups who have shown that IFN1 does not promote antibacterial activity whereas our results with IFNλ4 show that there are differences in function even within members of the IFNλ
family. This behavior corresponded to changes we saw at the transcriptional and protein levels after IFNλ1 stimulation and supports our overall observation that this cytokine activates macrophage antimicrobial activity. Interestingly, IFNλ1’s antimicrobial activity was only observed in macrophages that were treated with cytokine before infection rather than after infection. A reason for not seeing reduction in Mtb’s transcriptional activity (GFP expression) when IFNλ1 was added after infection could be that Mtb had already begun suppressing the defense response pathways of macrophages and IFNλ1 could not overcome that block. This would distinguish IFNλ1 from IFNγ, which we were unable to test here, and suggest that on the spectrum of activation capacity, IFNλ1 can to provide mild stimulation to macrophages that enhances their antimicrobial functions. Not seeing a decrease in viability of Mtb in case of IFNα or IFNλ4 confirms our hypothesis that IFNλ1 and IFNα can have differential impact on the function of macrophages, and these differences can extend even within the members of IFNλ family.

5.1.4 Overall summary of project

The schematic (Fig. 32) shows a summary of our hypothesis, indicating that IFNλs are expressed at a higher level in NHP lung granulomas and have the potential to increase the expression of co-stimulatory molecules and pro-inflammatory cytokine and chemokine expression by macrophages. Furthermore, IFNλ1 can promote facilitate maturation of phagosomes that can inhibit the transcriptional activity of Mtb. Overall, the macrophage activating properties of IFNλs, particularly IFNλ1 might lead to the negative correlation between IFNλ levels and bacterial burden in granulomas.

Thus, to conclude my project, I hypothesize that IFNλ expressed in granulomas, particularly IFNλ1, may contribute to the pro-inflammatory activation of macrophages by
facilitating the expression of cytokines like IFNγ, IL-1β and chemokines like CCL5 and by promoting the expression of co-stimulatory molecules like CD86, CD40 (Fig. 32). This may increase the interaction between macrophages and T cells, thereby facilitating T cell activation and promotion of Th1 responses. A culmination of these interactions, coupled with the intrinsic ability of IFNλ1-activated macrophages to restrict Mtb transcriptional activity, may lead to reductions in the rate of bacterial replication and lower overall granuloma bacterial load.

Figure 32. Schematic showing overall hypothetical summary of IFNλ1's mode of action. Created with Biorender.com.
Overall, we see that IFNλs are present at a higher level in NHP TB granulomas and are expressed by macrophages and neutrophils. In our in vitro assays, we observe that IFNλs, particularly IFNλ1, promote macrophage activation by upregulating different co-stimulatory molecules and by inducing production of pro-inflammatory chemokine and cytokines by macrophages. IFNλ1 also upregulates antimycobacterial defense response by upregulating phagolysosomal acidification. We therefore propose that the combination of macrophage activation and antimicrobial properties of IFNλs, could result in the negative correlation between IFNλ levels and CFU burden in the granulomas.

### 5.2 Public health significance

Despite the existence of anti-Mtb drug regimen and the BCG vaccine, TB is the world’s second leading cause of infectious disease related mortality after COVID-19. The COVID-19 pandemic has reversed the progress at mitigating TB cases, and beginning in the year 2020, TB related deaths have increased for the first time in a decade [517]. Furthermore, the number of TB related deaths is predicted to rise in the next few years due to the pervasive effects of lower rates of detection, diagnosis, and treatment of TB amidst the pandemic [517]; hence, there is a dire need for the development of new and effective intervention strategies and drugs for TB. Although TB research has been progressing for more than a century, the long evolutionary history of Mtb as a human pathogen, coupled with the lack of knowledge on the immune correlates of protection, represents an obstacle to development of better treatment strategies.

Vaccine development process depends on identifying critical points in the cycle of host-pathogen interaction that can be interrupted. In TB, the outcome of Mtb infection depends on the
bacterium’s fate in granulomas, and as such, understanding the granuloma’s cytokine milieu may help identify the determinants of protection or pathology in TB. The work I presented in Chapter 1 demonstrates for the first time that IFNλs are expressed in TB granulomas while the work presented in Chapters 2 and 3 highlight the impact of IFNλ signaling on macrophage phenotypes and demonstrate for the first time that IFNλ1-activated macrophages restrict Mtb transcriptional activity. These results highlight the potential protective effects that IFNλ1 may have on TB at the granuloma-level and suggest that IFNλ1 therapy may have applications in TB treatment. Pegylated IFNλ1 has been investigated in humans for treatment of hepatitis and more recently for COVID-19 and considering our results, IFNλ1 may have application as a vaccine adjuvant that might enhance activation of immune cells. Alternatively, exogenous IFNλ1 may have applications as an adjunctive therapy that promotes macrophage antimicrobial activity and shortens traditional drug therapy. My work, done in a highly translational NHP model, provides preliminary evidence for testing these possibilities and moves the field forward by providing the first evidence for the protective effects of IFNλ1 in TB.

5.3 Future directions

While our studies included in this thesis provides new insights into the role of IFNλ for protection in TB, many questions remain unexplored which deserve further investigation.

Much of the work in chapter 1 focused on identifying the expression of IFNλ in macrophages and neutrophils present in granulomas. We were limited to the use of CD11c as a broadly-expressed macrophage marker and additional work investigating IFNλ expression in specific macrophage subsets may yield new information on the role of these cytokines in
granulomas. Another interesting aspect to study would be comparing the levels of IFNλ between granulomas harvested during early infection vs. granulomas harvested during late infection via ELISA, to determine if IFNλ concentrations change according to the time of infection. Similarly, it would be interesting to study IFNλ levels in peripheral blood of Mtb infected NHPs during early infection and chronic infection to determine the changes in the level of this cytokine with progression of infection.

Chapter 2 of my thesis focuses in understanding how IFNλ modulates the phenotype of macrophages. Although we observed that IFNλ1 promotes M1/Th1 phenotypes and upregulates co-stimulatory molecule expression, it would be interesting to determine whether IFNλ1 treated macrophages can promote T cell activation. This can be achieved by performing a co-culture assay of IFNλ1 pre-stimulated macrophages and T cells from Mtb infected animals and then utilizing flow-cytometry to look at T cell activation markers like CD69 and cytokine expression like IFNγ and TNF upon stimulation with Mtb antigens.

Chapter 3 of my thesis investigates the effect of IFNλ on the viability of intracellular Mtb. While the research sheds light on the antimycobacterial potential of IFNλ1 in vitro, it would be interesting to determine its effect during in vivo infection in NHPs to further validate the function of this cytokine. One interesting avenue to study would be to investigate the effectiveness of pegylated-IFNλ1 as an adjuvant to boost immune cell activation during early TB infection in vivo. IFNλ administration has been reported to enhance the production of thymic stromal lymphopoietin (TSLP) that can improve the performance of intranasal influenza vaccines [321]. Thus, it would be interesting to study whether IFNλ administration can enhance the effectiveness of BCG vaccination against Mtb infection.


74. Frick, M., The TB vaccines pipeline: where are we going, where have we been. Clayden P, Collins S, Daniels C, et al.; i-Base/Treatment Action Group, 2013.


89. Prevention, C.f.D.C.a., Treatment for TB Disease.


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