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# *Mycobacterium tuberculosis* tRNA induces IL-12p70 via synergistic activation of pattern recognition receptors within a cell network

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## Abstract

Upon recognition of a microbial pathogen, the innate and adaptive immune systems are linked to generate a cell-mediated immune response against the foreign invader. The culture filtrate of *Mycobacterium tuberculosis* contains ligands such as *M. tuberculosis* tRNA that activate the innate immune response and secreted antigens recognized by T cells to drive adaptive immune responses. Here, bioinformatics analysis of gene expression profiles derived from human peripheral blood mononuclear cells treated with distinct microbial ligands identified a mycobacterial tRNA-induced

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innate immune network resulting in the robust production of IL-12p70, a cytokine required to instruct an adaptive Th1 response for host defense against intracellular bacteria. As validated by functional studies, this pathway comprised a feed-forward loop whereby the early production of IL-18, Type I interferons (IFNs) and IL-12p70, primed NK cells to respond to IL-18 and produce IFN-γ, enhancing further production of IL-12p70. Mechanistically, tRNA activates both TLR3 and TLR8, and this synergistic induction of IL-12p70 was recapitulated by the co-addition of a specific TLR8 agonist with a TLR3 ligand to PBMC. These data indicate that *M. tuberculosis* tRNA activates a gene network involving the integration of multiple innate signals including both Type I and Type II IFNs, as well as distinct cell types to induce IL-12p70.

## Introduction

The identification of the components of *M. tuberculosis* that trigger host immune responses has long been a goal of scientists studying the disease. In the 19th century, Robert Koch, after identifying *M. tuberculosis* as the cause of tuberculosis, went on to show that the bacilli induced protective immunity, which he ascribed to a culture filtrate preparation called "tuberculin" (1). Although tuberculin was later disproved to be a cure for tuberculosis, Koch had discovered what would become a standard diagnostic test for exposure to *M. tuberculosis*. The protocol for purifying tuberculin from the bacterial culture filtrate was refined by Frances Seibert, who demonstrated that the preparations confer antigenicity when injected into skin (2–7). This work led to the identification of the protein antigens from *M. tuberculosis* culture filtrate that are recognized by, and can elicit adaptive responses by T cells (8–10). The proteins purified from the culture filtrate elicit robust delayed type hypersensitivity responses in patients previously exposed to *M. tuberculosis* and individuals vaccinated with BCG. Yet protein purification largely hinders their ability to generate adaptive immune responses by themselves, suggesting that an immune adjuvant is present in the culture filtrate.

To combat the intracellular pathogen, *M. tuberculosis*, the innate and adaptive immune responses interact, with the innate response responsible for instructing the type of the adaptive T cell response. The innate immune system utilizes pattern recognition receptors (PRRs) to recognize a diverse array of microbial ligands known as pathogen associated molecular patterns (PAMPs). The host has deployed PRRs in a variety of subcellular locations recognizing distinct microbial ligands resulting in the activation of both common and specific innate immune responses. For example, distinct dendritic cell (DC) differentiation programs are induced by muramyl dipeptide of mycobacteria via the cytoplasmic receptor NOD2, vs. triacylated lipopeptide, a ligand for the cell surface receptor TLR2/1 (11). Furthermore, mycobacterial DNA, released from bacteria that reside in the host phagosome, gains access to the cytoplasm to trigger nucleotide sensor pathways such as STING, leading to a Type I IFN response (12–15). In chronic bacterial infections such as tuberculosis, the induction of Type I IFNs opposes the action of the Type II IFN, IFN-γ, required for an effective antimicrobial response against the causative pathogen (16, 17).

While the *M. tuberculosis* culture filtrate contains several protein antigens that elicit T cell responses, the culture filtrate also contains one or more microbial ligands, i.e. PAMPs,

which trigger innate instruction of the adaptive T cell response, that are largely removed during the purification of the purified protein derivative. In addition to proteins, *M. tuberculosis* culture filtrate contains nucleic acids (7), with tRNA being an abundant form of RNA (18). Treatment of human monocytes with tRNA purified from *M. tuberculosis* culture filtrate induced their apoptosis, which is thought to contribute to the pathogenesis of tuberculosis (18). However, the extent and specificity of the immune response triggered by *M. tuberculosis* tRNA remains unknown. Therefore, we investigated whether *M. tuberculosis* tRNA triggers a distinct innate immune response for instruction of the adaptive T cell response.

## Materials and Methods

### Cell purification and culture

Whole blood was obtained from healthy donors who provided written informed consent (UCLA Institutional Review Board). PBMC were isolated by Ficoll-hypaque (GE Healthcare) density gradient centrifugation and cultured in RPMI (Gibco) supplemented with 10% FCS (Hyclone) and 1% Pen/Strep glutamine (Gibco) at a density of $2\times10^6$/ml in 96-well flat bottomed plates (Corning) at 37 °C with 4% $CO_2$.

### Reagents for Cell Stimulation

TLR2/1L, a synthetic lipopeptide derived from the 19 kDa mycobacterial lipoprotein was obtained from EMC Microcollections and used at 10 μg/ml. PolyI:C (HMW) and TLR-506 were from Invivogen and used at 2 μg/ml and 500 nM respectively. ssRNA40 (phosphothioate backbone, HPLC purified) was synthesized by IDT and used at 500 ng/ml.

Total RNA was isolated from *M. tuberculosis* H37Rv that was grown to OD 0.8–1 ($2\times10^8$ cells/mL) and lysed using Trizol in bead-beating tubes, in the presence of antioxidants (19). The cells were agitated with 4 cycles of beating, each followed by a 5-minute rest period on ice. Chloroform (0.2 mL per mL of Trizol) was added followed by incubation at ambient temperature for 5 min. The samples were shaken and then centrifuged at 12,000 × g for 15 min at 4 °C. The aqueous phase was removed for further tRNA purifications using the Purelink miRNA Isolation Kit (Invitrogen) according to the manufacturer's instructions. Ethanol (100%) was added to the lysate to give a 35% concentration. The mixture was loaded onto the Purelink column and centrifuged at 12,000 × g for 1 min. The flow through was then mixed with 100% ethanol to give a final concentration of 70% and the mixture was loaded onto a Purelink column and centrifuged at 12,000 × g for 1 min to yield small RNAs (<200 nt). The columns were washed using wash buffer (Invitrogen) twice. The small RNAs were eluted by adding RNase-free water and centrifuging again. tRNA was finally purified by size-exclusion chromatography with an Agilent SEC-3 column (3 μm, 300 A, 7.8 × 300 mm) eluted with 100% 8 mM ammonium acetate at 65 °C to remove contaminating miRNA and other size-resolvable RNA fragments (19). tRNA eluted between 11 and 13 minutes for each sample. Fractions containing tRNA were collected and tRNA quantity and quality were checked using an Agilent Bioanalyzer (19). The RNA purity and quality were assessed using an Agilent Bioanalyzer (19). Using endotoxin-free reagents, endotoxin levels were <2 pg per μg of tRNA.

Nucleic acid ligands (1 μg/ml) were complexed with DOTAP (Roche) according to manufacturer's instructions to facilitate delivery to the endosome. DOTAP alone did not induce cytokine release. Reagents were determined to be endotoxin-free by Limulus amoebocyte lysate assay (Lonza).

### Cytokine Quantification

Primary cells were stimulated on the same day as isolation. Cell supernatants were harvested at 24h unless otherwise noted. Cytokines measured by sandwich ELISA using antibody pairs were as follows: IL-18 (MBL Intl.), IFN-γ (BD), IL-6 and IL-12p40 (Invitrogen). IFN-α, IL-1β, IL-10, IL-12p70 and TNF-α were measured by CBA (BD, Flex Sets).

### Blocking Antibodies and TLR inhibitors

PBMC were treated with the following monoclonal neutralizing antibodies compounds for 30 min before stimulation. IL-18 10 μg/ml (MBL Intl), IFN-γ 10 μg/ml (BD), IgG1 10 μg/ml from corresponding manufacturer was used as a control. The specific TLR8-inhibitor VTX-3119 and related control compound VTX-764 were gifts from VentiRx Pharmaceuticals and were used at a concentration of 100 nM which was determined by dose titration to provide optimal inhibition without off-target effects.

### CD56 depletion

PBMC were depleted of CD56+ cells using CD56 MicroBeads (Miltenyi Biotech) as directed by manufacturer's protocol. Depletion was confirmed at >99% purity by flow cytometry. CD56 depleted PBMC were cultured at $1.8\times10^6$/ml to reflect the loss of CD56+ cells, estimated to comprise 10% of PBMC.

### Flow cytometry

PBMC were labeled with antibodies to CD3 (CD3-FITC Invitrogen) and CD56 (CD56-PE eBioscience), or isotype control. For intracellular detection of IFN-γ, PBMC were treated with GolgiPlug (BD) 4 h prior to harvest. Following surface staining and fixation, cells were treated with Perm/Wash Buffer (BD) and stained with IFNγ-APC (Invitrogen) or isotype control. Flow cytometry was performed on a LSRII (BD Biosciences) in the UCLA Jonsson Comprehensive Cancer Center (JCCC) and Center for AIDS Research Flow Cytometry Core Facility that is supported by National Institutes of Health awards P30 CA016042 and 5P30 AI028697. Analysis was performed in FlowJo (Tree Star Inc.).

### RNA sequencing

PBMC were stimulated as described. RNA was harvested at 1, 6 and 24 h and isolated with RNeasy micro kit (Qiagen) according to manufacturer's directions, including on-column DNase digestion. RNA was quantified by Nanodrop and quality assessed by Agilent 2100 Bioanalyzer. Libraries were created from high quality RNA using TruSeq RNA Library Prep Kit v2 (Illumina). Libraries were quantified by Qubit, pooled by donor in groups of 12, and sequenced in duplicate on a HiSeq 2500 Sequencing System (Illumina).

### Differential Expression Analysis

Sequenced reads were aligned to the human reference genome (build hg19 UCSC) using TopHat and Bowtie2. Raw counts were calculated with HTseq using the hg19 Ensembl annotation. Normalization and differential expression analysis was performed using the DESeq2 package for R, which in all cases used the data for all three donors. FDR was controlled by applying the Benjamin-Hochberg correction to P-values. Differentially expressed genes were identified using the cutoffs FC > 2 vs Media and adjusted P-value < 0.05. Hierarchical clustering was performed with “hclust”, and principle component analysis via “prcomp” in R (version 3.2.4). Weighted Gene Correlation Network Analysis was performed using the WGCNA package as described (22). A network of relevant gene relationships as calculated by WGCNA was visualized using visANT.

### Functional Analysis

Functional analysis of differentially expressed genes was performed using Ingenuity Pathway Analysis (Qiagen). Gene ontology term analysis was performed using the ClueGO plugin (version 2.2.5) for Cytoscape (version 3.3.0) using the GO term database files from 08.06.2016. Significantly enriched terms were identified by right-sided hypergeometric test with Bonferroni P-value correction using a cutoff of $P < 0.05$. A network connecting canonical pathways and biological functions relevant to induction of Th1 responses derived from IPA, GO terms, and differentially expressed genes was visualized using Gephi (beta version 0.9.1). This was accomplished by the following strategy: 1) pathways chosen from ClueGO analysis of WGCNA turquoise module genes, 2) pathways chosen from IPA analysis of both turquoise module genes as well as similar pathways identified in parallel analysis of genes induced by activation of PBMC by tRNA, 3) select IPA biological functions tRNA relevant to cell types, 4) select genes of interest from tRNA genes differentially expressed vs. TLR2/1L, 5) Pearson correlations for IL18/IL12/IFNG calculated on rLog DESeq2 results matrix; and 6) Gephi used to illustrate connections between pathways and genes connected to IL18, IL12 and IFNG as well as significantly correlated genes.

### Statistics

Results are shown as mean ± SEM. Cytokine data was transformed using log10(x+1), and the Shapiro-Wilk test was used to test for normality. One-way ANOVA was performed for comparisons between three or more groups. The two-way repeated measures ANOVA was used to assess significance in experiments with multiple factors. Individual details of statistical analyses for individual experiments, such as post test for multiple comparisons, are explained in the figure legends. Statistical analyses were performed using GraphPad Prism 7 and R 3.4.1 (r-project.org).

## Results

### *M. tuberculosis* tRNA and bacterial lipopeptide induce distinct gene expression patterns

To investigate the innate immune response to *M. tuberculosis* tRNA, we measured the gene expression profiles induced by *M. tuberculosis* tRNA in human PBMC to include many of

the different immune cell types that are likely to be involved. *M. tuberculosis* tRNA was prepared from strain H37Rv as described (19) A ssRNA 30-mer derived from the HIV-1 long terminal repeat and known to activate TLR7 and TLR8 (20) served as a positive control for detecting innate immune responses to RNA. In addition, we compared the response to a mycobacterial 19-kDa triacylated lipopeptide, which activates cell surface TLR2/1 (TLR2/1 ligand, TLR2/1L) and is known to be a major activator of transcriptional pathways in response to *M. tuberculosis*. The optimal concentrations of these ligands were determined by dose titration or used as previously established in our laboratory (11).

PBMC from three donors were stimulated with the various ligands, and the cells collected after 1, 6 and 24 h. The mRNAs were isolated, libraries prepared and gene expression profiles obtained by RNA-seq, which after filtering out background expression, yielded a dataset of 14,637 genes (GEO accession GSE110325; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE110325). Principal component analysis (PCA) of the DESeq2 normalized counts was used to first identify samples displaying similar trends in gene expression (Fig. 1A) (5). At the 6 and 24 h timepoints, PCA indicated that gene expression for PBMC treated with *M. tuberculosis* tRNA and control ssRNA were similar, while gene expression for TLR2/1L-treated and untreated PBMC formed distinct groups. PCA of the gene expression data at 1 h formed a single group, with all three ligands inducing similar profiles as the control media.

A second unsupervised analysis, hierarchical clustering, was also performed to characterize the relationships between samples (Fig. 1B). Consistent with the PCA, gene expression profiles for both tRNA and ssRNA at the 6 and 24 h timepoints clustered together. The samples for TLR2/1L at 6 and 24 h formed their own group, as did the samples for the untreated controls at 6 and 24 h. All the 1h samples, regardless of the stimulus and including the media control, clustered on a separate branch from the 6 and 24 h samples, indicating that there was little difference in the transcriptional response at this early timepoint.

To determine the gene signatures induced in PBMC by each ligand vs. the media control, differential gene expression was calculated with DESeq2 (21). For each ligand we compared the two groups of samples (ligand vs media), with each group containing data from three donors. Differentially expressed genes for each timepoint were those that had a threshold of fold-change (FC) > 2 between the groups and a false discovery rate (FDR) < 0.05. We calculated a gene signature of significantly induced genes for each ligand by taking the union of the differentially expressed genes at each timepoint for a given ligand. Comparison of gene signatures overall showed a striking overlap [$P < 10^{-10}$] of the tRNA and ssRNA signatures, with 1,465 common genes out of a total of 1,692 genes for tRNA and 1,791 for ssRNA (Fig. 1C). Of these RNA-induced genes, 396 were also induced by TLR2/1L, leaving 1,069 induced by only tRNA and ssRNA. By contrast, TLR2/1L induced a distinct signature, with 720 significantly induced genes, of which 215 were unique to TLR2/1 activation.

To investigate the immune pathways induced by each ligand, functional analysis of the gene signatures was performed using Ingenuity. Analysis using the biologic functions tool showed common pathways between the tRNA and ssRNA gene signatures, identifying roles for NK cells, DC, monocytes, and differentiation of Th1 cells (Fig. 1D). By contrast, the biologic

functions of the TLR2/1L induced gene profile indicated monocytes, macrophages and neutrophil-associated pathways. Ingenuity analysis using the canonical pathways tool identified ‘DC maturation’, ‘NK-DC crosstalk’, ‘IFN signaling’, ‘PRR recognition of bacteria and viruses’, and ‘communication between innate and adaptive immune cells’, as all more significantly induced by tRNA as compared to TLR2/1L ($>10^{-5}$ difference in P-value, Fig. 1E). In comparison, the significance of canonical pathways identified for the tRNA and ssRNA signatures was similar. Together, these data suggest that tRNA and ssRNA, but not TLR2/1L, induced common gene signatures in PBMC indicative of an innate pathway for induction of Th1 cells.

## Identification of gene expression networks induced by M. tuberculosis tRNA

To further define the potential interaction between genes associated with tRNA/ssRNA induction and those associated with a Th1 response, we used weighted gene co-expression network analysis (WGCNA), an unbiased approach that defines modules of highly interconnected genes based on pairwise correlations (22). We tried to identify modules that were specifically induced by one of the ligands. This was accomplished by first encoding the ligands in a binary vector that was one for a specific ligand, and all its time points, and zero for all other ligands. The module expression levels were then correlated with these binary vectors to identify specific module/ligand associations.

WGCNA identified 31 gene modules, of which nine were positively correlated with at least one microbial ligand (Fig. 2A). In addition to the modules associated with tRNA and ssRNA activation of PBMC, TLR2/1L activation was associated with MEantiquewhite4, indicating significant enrichment of terms including ‘regulation of macroautophagy’ and ‘IL-1 signaling pathway’ (Fig. 2B). The MElightyellow module was also associated with TLR2/1L activation, and enriched for genes associated with ‘chemotaxis’ and ‘chemokine signaling pathway’. Several modules were associated with the media-treated cells. Also, there were modules that were negatively associated with a given stimulus, but were not further studied.

MEturquoise was the only module that significantly correlated with both tRNA and ssRNA treatment of PBMC. Gene ontology analysis (23) of MEturquoise revealed association with the terms ‘IFN gamma production’ and ‘IFN gamma signaling’ (Fig. 2B). This module contained IL18, IFNG, and IL12A, key cytokines involved in induction of a Th1 response, thereby identifying a correlated gene network for Th1 cell differentiation as revealed by the biologic function analysis of the tRNA induced genes. The other modules that were significantly associated with either TLR2/1L or ssRNA stimulation of PBMC were not associated with immune GO terms.

To map the gene network involved in Th1 differentiation, we filtered the tRNA associated MEturquoise module using the ‘immune system’ gene ontology term, identifying 339 genes which included the key genes involved in induction of Th1 cells. This gene set was further filtered by differential expression in tRNA vs. media control (FC > 2, FDR < 0.05, calculated by DESq2 using data from all three donors) identifying 241 genes. The correlation between these individual genes as calculated by WGCNA was visualized by a connectivity map (24) (Fig. 2C), centered on IL18, IFNG and IL12A with connections of

edge weight indicated (and requiring a pairwise correlation between genes ≥ 0.2). Highlighting the genes connected to IL18, IFNG and IL12A revealed a module of 120 genes, in which IFNG and IL12A were connected to each other and had multiple connections to other genes in the module. These included IL18R1, IL18RAP and IL12RB2, which encode relevant cytokine receptors, as well as STAT1, STAT2, STAT3 and STAT5A, which encode proteins involved in signaling pathways related to the induction or downstream effects of IL-18, IFNG and IL12A. Strikingly, both the Type II interferon, IFNG, and several Type I interferon genes, as well as interferon-induced downstream genes, were identified in the top 250 genes induced by tRNA (Table S1A and S1B). In fact, of the top 25 genes, 16 were Type I interferon or downstream genes, including ISG15, CASP4 and TLR3. These genes were strongly induced by tRNA and ssRNA but not by TLR2/1L. These data implicated both Type I IFN and the Type II IFN (IFNG) in the induction of a Th1 response, which was surprising to us, given that the induction of Type I IFN in tuberculosis is known to downregulate IFN-γ responses.

Integration of the bioinformatics analyses of the gene expression profiles for the tRNA treatment of PBMC and the WGCNA turquoise module was performed to link cell type-associated pathways with specific genes related to Th1 functional response pathways (Fig. 3). This analysis identified that *M. tuberculosis* tRNA directly or indirectly triggered pathways associated with monocytes, NK, DC and T cells, including Th1 cells, to induce a set of genes including IL18, IL18R1, IFNG, JAK2, STAT1, IL12A, IL12B, IL12RB2, and STAT4. These genes are contained within functional pathways including ‘Role of PRR recognition of Bacteria and Viruses’, ‘TLR signaling’, ‘IFN-γ signaling’, ‘DC maturation’, ‘T helper differentiation’ and ‘defense response’. In contrast, the top four TLR2/1L induced pathways were ‘Granulocyte Adhesion and Diapedesis’, ‘Agranulocyte Adhesion and Diapedesis, ‘Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F’, ‘Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F’. Together, these data suggest a model in which tRNA activates specific cell types that interact to establish a host-defense gene network involved in the innate instruction of an adaptive Th1 response.

## Analysis of genes characteristic of Th1 differentiation and validation in PBMC

Examination of the $\log_2$ normalized counts of key genes identified by the informatics analysis as part of the *M. tuberculosis* tRNA-induced Th1 differentiation pathway confirmed that, both tRNA and ssRNA, but not TLR2/1L, significantly induced expression of IL18 mRNA (Fig. 4A) and its receptor heterodimer IL18R1 and IL18RAP (Fig. S1A). IFNG and IL12A mRNAs were also upregulated by both tRNA and ssRNA, but not TLR2/1L. IL12B was upregulated by all stimuli, whereas IL23Awas not significantly induced by any of the treatments. IL6 mRNA was strongly induced by all three ligands. This analysis revealed that additional genes connected to the central genes in the Th1 differentiation pathway, including Type I IFN and downstream genes, as well as genes encoding TLR3, CASP4, and ISG15, were also upregulated, but again only in response to tRNA and ssRNA, not TLR2/1L (Fig. S1B).

To validate the gene expression profile, we determined whether the induction of the cytokine mRNAs leads to secretion of the encoded protein. PBMC were stimulated with *M. tuberculosis* tRNA, ssRNA40, and TLR2/1L, supernatants collected at 24 h and cytokine production measured. Consistent with the gene expression data, PBMC released IL-18, IFN-γ and IL-12p70 in response to tRNA (IL-18: 76±10 pg/ml; IFN-γ: 16586±2932 pg/ml; IL-12p70: 211±33 pg/ml) and ssRNA, but not media or TLR2/1L (Fig. 4B). IL-12p40 was secreted in response to all treatments, although tRNA and ssRNA induced approximately seven-times more IL-12p40 protein than TLR2/1L (not shown). IL-6 secretion served as a control to demonstrate cell activation by all treatments (Fig. 4B). Low levels of IL-23 were detected in supernatants of all three treatments (Fig. S1C). The differential production of IL-12p70 and IL-23 is consistent with the informatics analysis indicating a gene network for instruction of a Th1 immune response. In addition, IFN-α was induced by tRNA and ssRNA but not by TLR2/1L, consistent with the RNA-seq data.

### Interdependence of tRNA-induced cytokines in the induction of IL-12p70

Given that *M. tuberculosis* tRNA induced the robust production of IL-12p70, we next investigated the immune networks that led to production of this cytokine. The kinetic sequence of cytokine induction in response to the *M. tuberculosis* tRNA was determined by measuring the time-course of IL-18, IFN-γ and IL-12p70 protein production (Fig. 5A). The earliest detected response was the production of IL-18 at 6 h in response to tRNA and ssRNA (tRNA: 45±5 pg/ml, $P = 0.0001$). IL-18 protein increased two-fold by 24 h (tRNA: 76±2 pg/ml, $P = 0.0001$). In contrast, little IFN-α, IFN-γ and IL-12p70 was detected at 1h or 6h, but there was significant induction by 24 h (tRNA. IFN-α: 2351±481pg/ml, $P = 0.0001$; IFN-γ: 15751±4287 pg/ml, $P = 0.0001$; IL-12p70: 139±22 pg/ml, $P = 0.0001$). The temporal pattern of IL-6 secretion was similar to that of IL-18; protein was detected by 6h and the concentration doubled by 24 h (Fig. S1D). These data indicate that IL-18 secretion is triggered early, followed by secretion of IFN-α, IFN-γ and IL-12p70.

As IL-18 was detected early, i.e. at 6 h, we determined whether its secretion was required for downstream cytokine production. Pre-treatment of PBMC with anti-IL-18 neutralizing antibodies dramatically reduced tRNA-induced secretion of both IFN-γ (93% reduction, $P = 0.0001$) and IL-12p70 (70% reduction, $P = 0.0008$) (Fig. 5B). As a control, we measured induction of IL-6, which was not affected by anti-IL-18 treatment.

It has been suggested that the early production of Type I IFN can contribute to optimal induction of IL-12p70 (25). To determine the role of Type I IFN production in the *M. tuberculosis* tRNA induction of IL-12p70, we performed experiments using a neutralizing antibody to IFN α/β receptor A (IFNAR). Addition of anti-IFNAR mAbs had no effect on tRNA induction of IL-18, but blocked induction of IFN-γ by ~80% (Fig. 5C). In addition, anti-IFNAR mAbs blocked tRNA induction of IFN-α, consistent with the presence of a Type I IFN-driven positive feedback loop (26, 27). Anti-IFNAR antibody significantly blocked tRNA induction of IL-12p70, while the difference between the isotype control was not significant. There was no effect of anti-IFNAR treatment on tRNA induction of IL-10.

Previous studies have shown that in PBMC, TLR7/8 agonists induce $CD56^+$ (NK and NKT) cells to secrete IFN-γ, dependent on the production of IL-18, even with the concomitant

induction of Type I IFNs (28). In order to determine if $CD56^+$ cells were required for *M. tuberculosis* tRNA induction of IFN-γ, PBMC were depleted for this subset prior to stimulation, and induction of IFN-γ release was assessed. In PBMC depleted of $CD56^+$ cells, *M. tuberculosis* tRNA induction of IFN-γ was almost entirely inhibited (tRNA: 141±118 pg/ml CD56 depleted vs. 4557±4157 pg/ml PBMC, 97% reduction, $P = 0.0215$) (Fig. 6A). Although TLR3 ligands have been reported to directly activate NK cells (29), we did not find such activation here (data not shown).

We further demonstrated that $CD56^+$ cells were the major source of IFN-γ by intracellular cytokine labelling and flow cytometry. PBMC were stimulated as above, then stained with antibodies for IFN-γ, CD3 and CD56 to measure intracellular IFN-γ with the goal to differentiate NK, NKT and T cell populations. IFN-$\gamma^+$ cells were detectable above background in tRNA- and ssRNA– treated PBMC (Fig. S2). Of IFN-γ positive lymphocytes, the majority were $CD56^+CD3^-$ NK cells (tRNA: 62±9%; ssRNA 69±9%), followed by $CD56^-CD3^+$ T cells (tRNA: 28±10%; ssRNA: 22±8%), and a small number of $CD56^+CD3^+$ (tRNA: 2.8±1.5%; ssRNA: 2.4±1%) (Fig. 6B). Since the frequency of NK cells in PBMC is much lower than that of T cells (~10% vs. ~75%), this indicated that the populations were differentially predisposed to produce IFN-γ in response to the RNA stimuli. Although T cells are major producers of IFN-γ during the adaptive immune response, this early timepoint measures the innate immune response, in which NK cells produce the majority of IFN-γ.

Since IL-18 is known to trigger secretion of IFN-γ (30–32) and IFN-γ primes IL12A transcription (33), we hypothesized that IFN-γ contributed to the production of IL-12p70. The pre-treatment of PBMC with neutralizing anti-IFN-γ antibodies reduced the *M. tuberculosis* tRNA-induced secretion of IL-12p70 (62% reduction, $P < 0.0001$) (Fig. 6C). Secretion of IL-18 was unaffected by anti-IFN-γ treatment, consistent with the detection of IL-18 protein prior to the induction of IFN-γ. IL-6 production, which is IFN-γ independent, served a control.

The ability of TLR ligands to activate NK cells to produce IFN-γ not only involved the production of IL-18 but was also dependent on secretion of IL-12p70 (28). Purified NK cells did not produce IFN-γ in response to the same TLR ligands. We had detected the early production of IL-12p70 as soon as 6 h after stimulation with tRNA or ssRNA (Fig. 5A). Both tRNA and ssRNA induction of IFN-γ was blocked by anti-IL-12p70 neutralizing antibodies, with little effect on IL-18 or IL-6 secretion (Fig. 6D). Thus, we conclude that IL-12p70 and IL-18 are essential for the IFN-γ response to microbial RNA, and IFN-γ contributes to robust production of IL-12p70. In addition, a multicellular response to *M. tuberculosis* ligands involving monocytes and NK cells contributes to the induction of the Th1 pathway.

## tRNA induces cytokine responses via TLR8

The secondary structure of tRNA includes both single- and double-stranded RNA regions. Given that we detected upregulation of both TLR8, a PRR for single-stranded RNA, and TLR3, a PRR for double-stranded RNA, mRNAs by *M. tuberculosis* tRNA, we explored the role of these PRRs in triggering the Th1 cytokine network, starting with TLR8.

In order to test the requirement of TLR8 signaling for tRNA induction of the Th1 differentiation pathway, we utilized a specific TLR8 antagonist, VTX-3119 (34). A dose titration was performed to determine the optimal concentration for the antagonist and preclude off-target effects (Fig. S3). PBMC were pre-treated with the TLR8 antagonist VTX-3119, or control molecule VTX-764, and stimulated with TL8-506, a synthetic TLR8-specific agonist (35). Pre-treatment with the TLR8 antagonist suppressed cytokine response to the TLR8 agonist vs. pre-treatment with control compound (IL-18: 60% reduction $P < 0.0001$; IFN-γ: 67% reduction $P=0.024$; IL-12p70: 47% reduction $P = 0.0004$). The induction of IL-6 by all three TLR ligands was not affected by the TLR8 inhibitor.

Next, PBMC were pre-incubated with the TLR8 antagonist or control compound, then stimulated with *M. tuberculosis* tRNA, ssRNA or TLR2/1L (Fig. 7A). Treatment with the TLR8 antagonist reduced secretion of IL-18 by 50% for activation by *M. tuberculosis* tRNA (mTB tRNA: 12±6 pg/ml vs. 24±10 pg/ml, $P = 0.0106$) and 67% for ssRNA. IFN-γ secretion was 90% lower for *M. tuberculosis* tRNA (mTB tRNA: 695±224 pg/ml vs. 6778±2433 pg/ml, $P < 0.0001$) and 58% reduced for ssRNA. Secretion of IL-12p70 was diminished by 84% for *M. tuberculosis* tRNA (tRNA: 42±15 pg/ml vs. 264±108 pg/ml, $P < 0.0001$) and 73% for ssRNA. IL-6 secretion was not significantly affected for any treatment (data not shown).

To define further the role of TLR8 in the induction of IL-12p70, we surveyed the ability of various oligomers and base analogues targeting endosomal and cytosolic nucleic acid PRR for ability to induce secretion of this cytokine in PBMC (Fig. 7B). As before, *M. tuberculosis* tRNA and ssRNA induced IL-18, with induction also observed for synthetic TLR8 agonist. IFN-γ was strongly induced by ssRNA as well as, to a lesser extent, *M. tuberculosis* tRNA and the TLR8-specific ligand. High levels of IL-12p70 were secreted in response to ssRNA and *M. tuberculosis* tRNA. Despite their induction of IL-18 and IFN-γ, only low amounts of IL-12p70 were produced in response to synthetic agonists for R848, 3M-002 (TLR7/8), and TL8-506 (TLR8). By itself, poly I:C (dsRNA, TLR3) was a weak inducer of IL-18, IFN-γ and IL-12p70. Even lower levels of these cytokines were observed in response to Imiquimod (TLR7), CpG DNA (TLR9), cyclic-di-GMP (STING), dA:dT (STING/CDS) (36–38). These data suggest that TLR8 activation alone was not sufficient to explain the strong induction of IL-12p70 by *M. tuberculosis* tRNA.

We next addressed whether a combination of a specific TLR8 ligand plus a second ligand activating a different PRR could lead to IL-12p70 secretion. Given that tRNA has both single- and double-stranded regions, and has previously been shown to induce type I IFN via activation of TLR3 (39), it was logical to ask whether activation of both TLR8 and TLR3 could induce IL-12p70. We found that the TLR3 agonist weakly induced IL-18 to a greater extent than the TLR8 agonist, and, when combined, the induction of IL-18 by the TLR3 and TLR8 ligands was not synergistic. In contrast, the TLR3 and TLR8 ligands by themselves weakly induced IFN-γ and IL-12p70, but the combination of the two ligands resulted in the synergistic induction of these cytokines (Fig. 7C). Both TLR3 and TLR7, but not TLR8 ligands induced IFN-α, but the combinations were not synergistic. These data suggest that the mechanism by which TLR3 synergizes with TLR8 in the induction of the Th1 pathway likely includes, but is not limited to, the production of Type I IFN. Together, these data

identify an innate pathway requiring the recognition of the pathogen by distinct pattern recognition receptors and distinct cell types, involving the induction of both Type I and II IFNs as well as downstream genes, leading to the production of IL-12p70, a cytokine required for the induction of a Th1 response as part of a major host defense pathway.

## Discussion

The ability of the immune system to mount a cell-mediated immune response involving Th1 cells is critical for host defense against many intracellular bacteria, including *M. tuberculosis*. To generate cell-mediated immunity, the innate immune system must recognize the microbial invader and instruct the adaptive T cell response. Yet the classic PAMPs found in mycobacteria, lipoproteins that activate TLR2/1, and muramyl dipeptide that activates NOD2, only weakly induce IL-12p70, a cytokine that is required for induction of a Th1 response. Here, we found that *M. tuberculosis* tRNA, detected in the bacterial culture filtrate, is a potent inducer IL-12p70. Exploring tRNA-induced gene expression profiles in human PBMC, we identified an integrated cellular and molecular pathway, beginning with the production of IL-18, Type I interferons, and IL-12p70, resulting in NK cell secretion of IFN-γ, and the subsequent robust induction of IL-12p70. The ability of *M. tuberculosis* tRNA to trigger this gene network was dependent on TLR8, yet TLR8 activation by itself was not sufficient for induction. Instead, activation of both TLR3 and TLR8 synergized for the robust induction of IFN-γ and IL-12p70, suggesting a role for the concurrent activation of nucleotide receptors in mounting a cell-mediated immune response against intracellular mycobacteria.

Initially, studies revealed that the culture filtrate of *M. tuberculosis* was sufficient by itself to induce T cells responses in a naïve animal, suggesting the presence of an adjuvant to activate the innate immune response along with protein/antigen signals required to stimulate an adaptive immune response (2–7). Subsequently, a purified protein derivative (PPD) fraction of the culture filtrate was isolated and shown to be useful as a diagnostic test for tuberculosis exposure, identifying individuals that had developed a delayed type hypersensitivity response because of exposure to *M. tuberculosis* or vaccination with BCG. The component(s) of the culture filtrate that can act as an adjuvant to stimulate the innate immune response has not been evaluated extensively, but tRNA was previously found to be a major component of the nucleotide fraction of the filtrate, which was shown to induce apoptosis in monocytes (18). Our data demonstrate a mechanism by which mycobacterial tRNA can robustly induce IL-12p70, a key cytokine in the induction of a Th1 response, and hence could serve as an adjuvant for the adaptive T cell response.

It was unexpected that a single mycobacterial ligand simultaneously induced both the Type II IFN, IFN-γ, as well as the Type I IFNs, since in chronic mycobacterial infections including tuberculosis and leprosy, the Type I IFNs inhibit the production and action of IFN-γ (40, 41). Yet as part of the pathway to induce IL-12p70, both tRNA and ssRNA were found to be potent inducers of both types of IFN, as well as IFN downstream genes. IFN-α, at low levels. has been shown to augment the ability of TLR ligands to trigger IL-12p70 production in DC (42–44), consistent with our data that Type I IFNs, as part of the acute immune response to *M. tuberculosis* tRNA, are involved in the production of IL-12p70. The

findings presented here also demonstrate that Type I IFN was required for *M. tuberculosis* tRNA induction of IFN-γ by NK cells, thereby linking the production of Type I IFN to the production of Type II IFN. The induction of Type I IFNs, together with the early production of IFN-γ following tRNA activation of PBMC, is also likely to contribute to IL-12p70 production, as IFN-γ can augment the ability of other TLR ligands to induce IL-12p40 and IL-12p35 (45, 46). On the other hand, in chronic bacterial infection, Type I and Type II IFN have opposing functional roles resulting in the inhibition of IFN-γ-induced antimicrobial responses (41). The ability of Type I IFN to directly interfere with transcription of IL12B (47) leading to inhibition of IL-12p70 (48, 49) and blockade of Th1 responses (50, 51) can be overcome by the production of ISG15, which downregulates the Type I IFN response (52, 53). We note that both tRNA and ssRNA strongly induced ISG15. In viral infection, the initial induction of Type I IFN is required to clear the infection, but in chronic infections contributes to pathogenesis.

In determining the source of IFN-γ production, our data underscore the importance of NK cell activation in the robust induction of IL-12p70. We identified that *M. tuberculosis* tRNA, by triggering TLR8 to induce the production of IL-18, induces NK cell production of IFN-γ. The activation of NK cells to produce IFN-γ was dependent on the early production of IL-12p70, presumably through its ability to upregulate both subunits of the IL-18R on NK cells (54, 55), thereby enhancing responsiveness to IL-18. Consistent with this mechanism, tRNA upregulated IL18R1 and IL18RAP mRNA in PBMC, and the addition of neutralizing antibodies to IL-12p70 inhibited tRNA induction of IFN-γ. This early production of IFN-γ by NK cells was required for the later more robust induction of IL-12p70, since depletion of NK cells or the addition of anti-IFN-γ blocking antibodies reduced tRNA induction of IL-12p70. A role for NK cells in tuberculosis has been demonstrated in mouse models by their recruitment to the lung as early as seven days after infection (56) with a role in preventing tissue damage (57). In human tuberculosis, NK cells have been identified at the site of infection in patients, both in the pleural fluid (58) and in granulomas in pulmonary lesions (59). In addition, BCG, when used to revaccinate individuals, boosted IFN-γ producing $CD56^+$ cells *in vivo*, and when added to whole blood, upregulated $IFN^-\gamma$ producing $CD56^+$ cells *in vitro* via an IL-12 and IL-18 dependent mechanism (60).

The ability of *M. tuberculosis* tRNA to induce IL-12p70 is likely related to its natural location when released by *M. tuberculosis* in specific subcellular compartments. *M. tuberculosis* resides primarily in endosomes that contain the nucleotide-sensing TLRs including TLR3 (dsRNA), TLR7 (ssRNA), TLR8 (ssRNA) and TLR9 (dsDNA). TLR8 is primarily expressed in human monocytes and myeloid DC (61) and is localized to endosomes/phagosomes, allowing the innate immune system to distinguish self-RNA (nucleus, cytoplasm) from non-self RNA (endosome/phagosome). Our data indicate that the ability of *M. tuberculosis* tRNA to induce IL-18, IFN-γ and IL-12p70 was blocked by a specific TLR8 antagonist, consistent with previous studies (62) The mechanism by which TLR8 recognizes tRNA likely involves recognition of the ssRNA regions present in the tRNA stem loop sequences, for example in the anti-codon sequence (63). Of potential clinical relevance, a TLR8 gain-of-function polymorphism (TLR8 A1G), correlates with increased resistance to tuberculosis in humans (64–70). Presumably, the abundance of *M. tuberculosis* tRNA in the bacterial culture filtrate is due to a combination of accumulation

through bacterial lysis and resistance to degradation (18). Although TLR8 has been shown to recognize *Borrelia burgdorferi* RNA in infected human macrophages (71), experiments to demonstrate a role for TLR8 during an *in vitro* infection of monocytes/macrophages with *M. tuberculosis* have not been successful, perhaps because of the slow turnover of bacteria in the short term *in vitro* cultures.

Although *M. tuberculosis* tRNA was a potent inducer of IL-12p70, and did so in a TLR8-dependent fashion, a TLR8 agonist alone weakly induced IL-12p70. In addition to ssRNA regions, tRNA contains dsRNA regions and has been shown to trigger TLR3 (39), prompting us to measure the response to a combination of ligands. The combination of dsRNA which activates TLR3 and a synthetic ligand that activates TLR8 induced IL-18 in an additive manner, but were synergistic in the induction of IFN-γ and IL-12p70. We therefore propose a model for the ability of tRNA to potently induce IL-12p70 (Fig. 8), involving TLR8 induction of IL-18. TLR3 activation leads to production of Type I IFN, which can enhance induction of IL-18 (72), feedback to enhance TLR3 expression (73, 74), leading to induction of IL-12p35 via an IRF3 dependent pathway (75). This in combination with early TLR8 induction of IL-12p40 leads to formation of IL-12p70, which upregulates both subunits of the IL-18R on NK cells (54, 55), thus synergizing with IL-18 to induce secretion of IFN-γ from NK cells (76). We infer that since IL-12p70 is assembled by a single cell, that myeloid DC are involved, given that they express both TLR3 and TLR8 (61) and are key producers of this cytokine. The production of IFN-γ subsequently enhances production of IL-12p70, thereby amplifying its production.

The innate pathway we describe for induction of IL-12p70 is of disease relevance as there is evidence from both murine models and from genetic studies in humans that the induction of IFN-γ is required for protective immunity against tuberculosis. Mice lacking the gene for IFN-γ died from *M. tuberculosis* challenge 2–3 weeks from IV challenge and within a month from aerosol challenge (77). Activation of mouse macrophages by IFN-γ and TNF-α induced killing of intracellular bacteria through the induction of nitric oxide (78, 79). Individuals with genetic disorders leading to the decreased production of, or response to IFN-γ, are highly susceptible to tuberculosis and other mycobacterial diseases (80–82). One mechanism by which IFN-γ contributes to host defense against *M. tuberculosis* in humans is through activation of macrophage antimicrobial activity via the vitamin-D dependent induction of the antimicrobial peptides cathelicidin and DEFB4 (83).

Our data suggest that a single microbial ligand may trigger multiple PRRs, leading to upregulation of IL-12p35 and IL-12p40 to synergize in the production of IL-12p70, thereby inducing a distinct innate immune response critical to host defense. Combinatorial analysis of how multiple innate signals trigger gene expression identified synergistic and antagonistic interactions, suggesting a functional role in the adaptation to complex stimuli (84). As such, tRNA induced a gene network that was associated with innate instruction of an adaptive T cell response. In addition to IL18, IL12A, IL12B, and IFNG, which are known to polarize towards a Th1 adaptive response, we found upregulation of multiple T-cell costimulatory molecules, including CD40 and CD80 (42, 85). Also of note were chemoattractants, such as CXCL10, which recruit T cells, NK cells, DC, and monocytes to the site of infection (86). Several of the genes in this network have been shown to be critical for host defense against

mycobacterial infection in humans based on the enhanced susceptibility of individuals with genetic mutations, including IFNG, IFNGR1 and IL12RB1 (80–82). ISG15 is required to maintain IFN-γ production during mycobacterial infection (52, 53), such that genetic alteration of ISG15 increased the susceptibility of individuals to mycobacteria infection and abrogated the IFN-γ response to mycobacterial infection *in vitro* (87). As stated, the roles of IL-12, IL-18 and type I IFN in driving NK cell activation, as well as the ability of IFN-γ to amplify IL-12, have been established. Here, we have used both molecular and cellular approaches to identify a network by which a single microbial ligand triggers multiple PRRs leading to the production of both type I and type II IFNs, resulting in the type I and type II IFN dependent production of IL-12p70, as part of the innate immune response against many intracellular pathogens.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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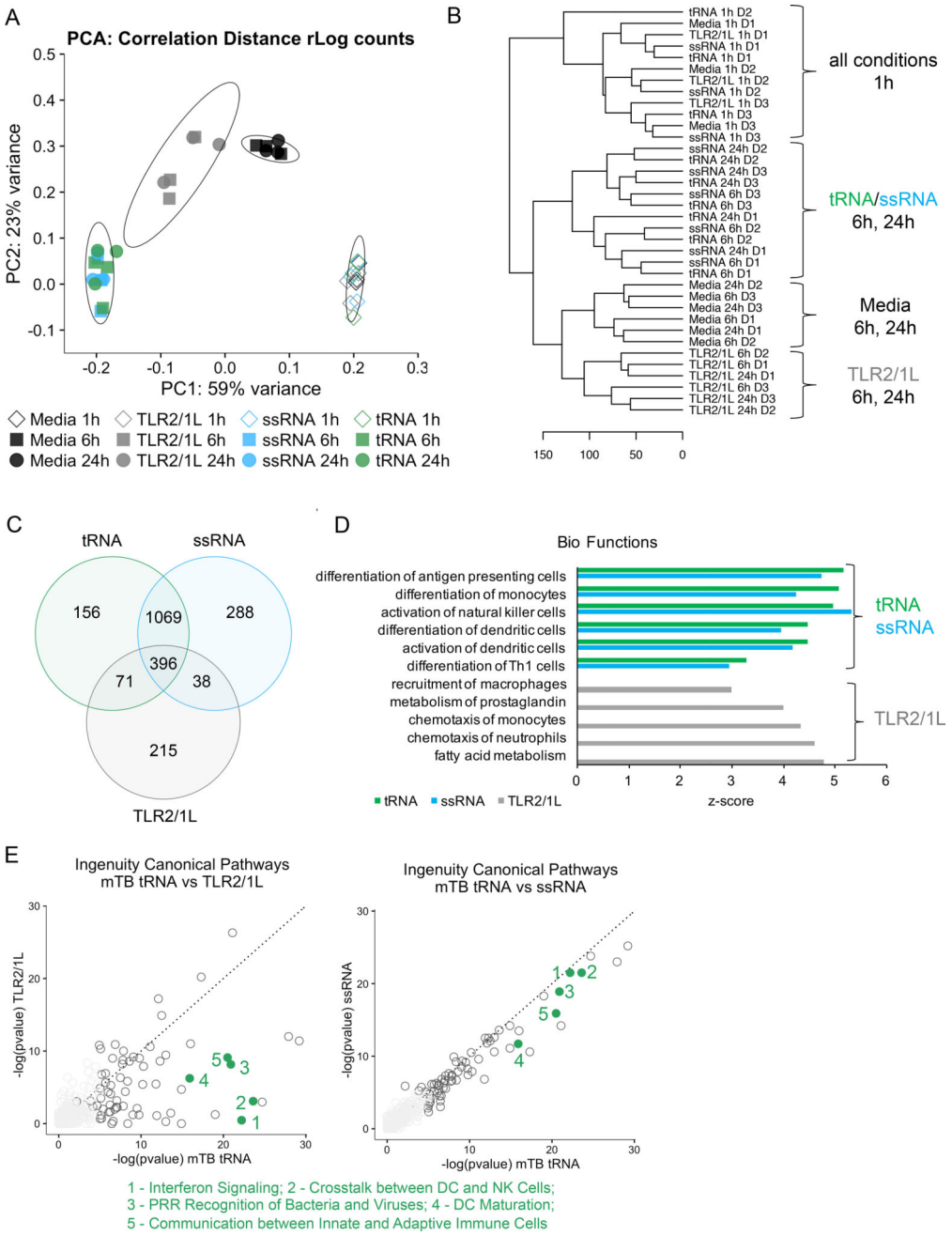


**Fig 1. Network analysis of *M. tuberculosis* tRNA, ssRNA, and TLR2/1L-induced gene expression profiles in PBMC**

(A) Principle component analysis of correlation of gene expression from RNAseq on rLog() matrix output from DESeq2. Ellipse denotes 95% confidence interval from k-means clustering of PC1 and PC2 variance. (B) Hierarchical clustering of rLog() transformed counts. Euclidean distance, complete clustering. (C) Overlap of significantly induced genes by mTB tRNA, ssRNA and TLR2/1L, defined by FC > 2 over Media and FDR < 0.05. Hypergeometric p-value calculated for overlaps excluding the 396 common genes. (D) Top Bio Functions identified by Ingenuity Pathway Analysis of significantly induced genes for

mTB tRNA, ssRNA or TLR2/1L. (E) Comparison of significantly induced functional pathways induced by mTB tRNA to ssRNA or TLR2/1L.

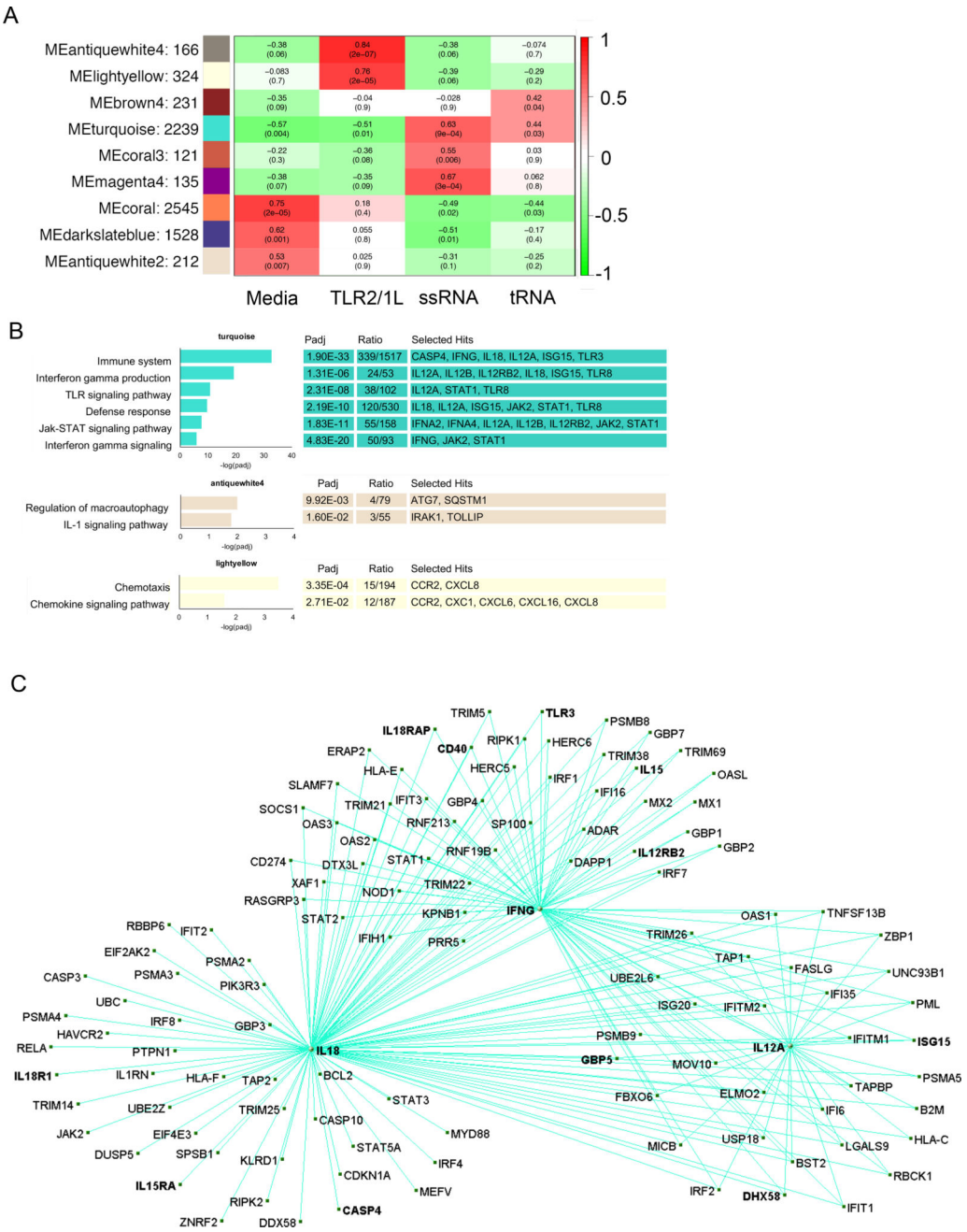


**Fig 2. Identification of RNA – correlated module and associated functional analysis**

(A) WGCNA eigengene modules correlated to at least one treatment condition $P < 0.05$. Red indicates positive correlation and green inverse correlation. (B) Top hits for functional term annotation of WGCNA modules positively correlated with mTB tRNA and ssRNA or TLR2/1L signatures. Padj is calculated by ClueGO as the P-value with Bonferroni correction for the association of the functional term with the gene expression data. Ratio represents the genes for a given functional term that are present in the module over the total number of genes for the term. (C) Visualization of the gene network derived from the WGCNA turquoise module. The module was filtered for genes exhibiting significant

differential expression during tRNA treatment and annotated with the GO term “immune pathway” to select genes associated response to mTB infection.

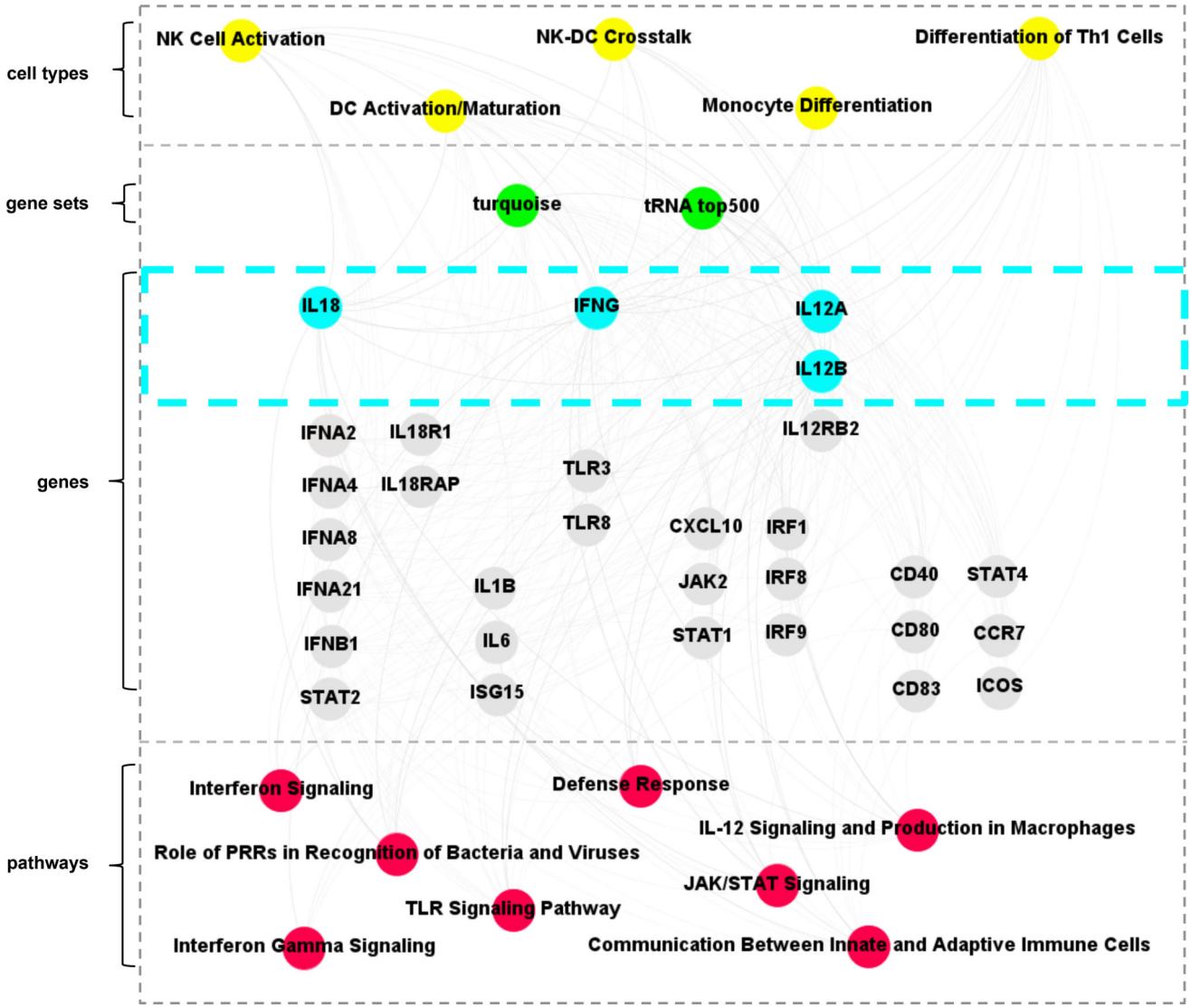


**Fig 3.**

Integrated network of gene expression and functional analysis terms. Gephi was used to create a functional annotation network, showing connections between significant gene ontology terms, IPA canonical pathways, WGCNA modules and significantly expressed genes.

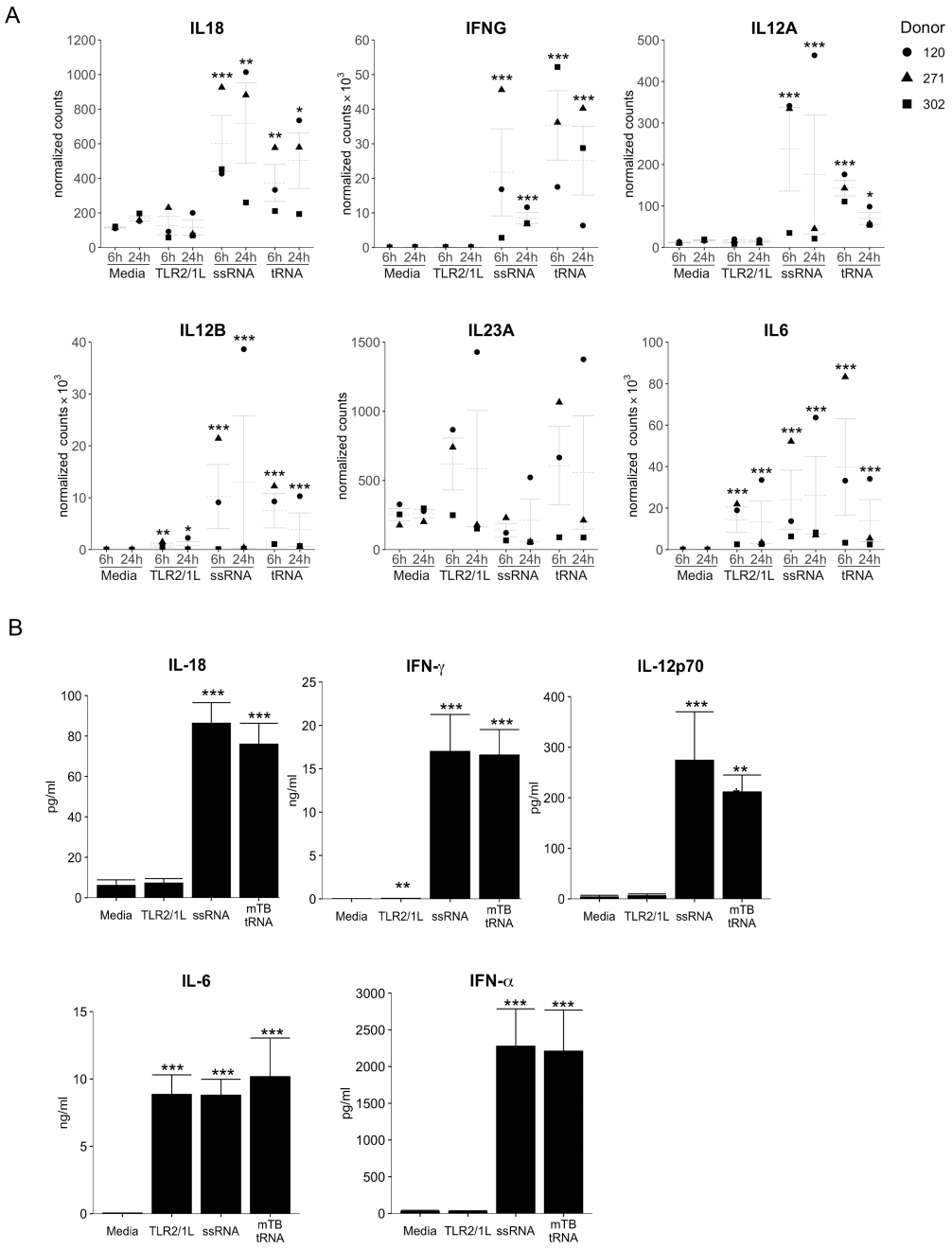


**Fig 4. Analysis of genes characteristic of Th1 differentiation and validation in PBMC**

(A) Log-transformed, normalized counts for select genes at 6h and 24h, mean ± SEM, statistical significance of differential expression (as compared to media at each timepoint) is reported as Benjamini-Hochberg adjusted p-value. (B) PBMC were stimulated with mTB tRNA, ssRNA or TLR2/1L and cytokine secretion measured at 24h. (n=20). Statistical significance of ligand-stimulated PBMC vs. media control calculated by one-way repeated measures ANOVA followed by Dunnett's multiple comparisons test. *p<= 0.05, ** p<=0.01, *** p<=0.001

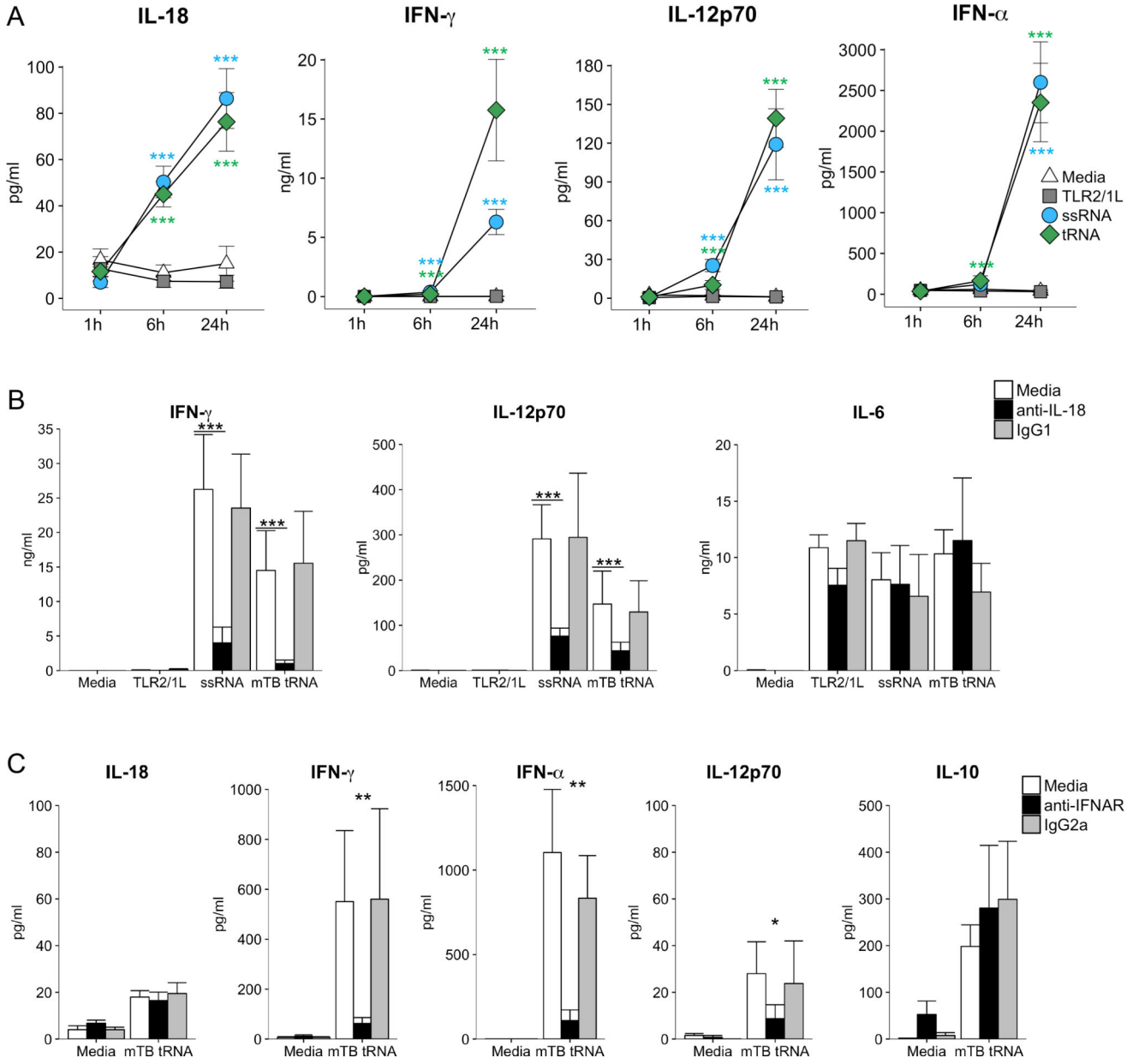


**Fig 5. Role of IL-18 and Type I IFN**

(A) PBMC were stimulated with mTB tRNA, ssRNA or TLR2/1L and cytokine secretion measured at 1 h, 6 h and 24 h from PBMC stimulated as above. (n=7). Statistical significance of ligand-stimulated PBMC at specific timepoints vs. media control calculated by two-way repeated measures ANOVA followed by Dunnett's multiple comparisons test. (B) PBMC were pre-treated with monoclonal anti-IL-18 neutralizing antibody or IgG1 isotype control (n=4) or (C) anti-IFNAR neutralizing antibody or IgG2a isotype control (n=3) for 30 m before stimulation with TLR2/1L, ssRNA or mTB tRNA. Supernatants were collected at 24 h and cytokine secretion measured. Statistical significance of ligand-stimulated PBMC vs. media control in the presence of neutralizing and isotype control antibodies calculated by one-way ANOVA followed by Tukey post-hoc test. *p<= 0.05, **

p<=0.01, *** p<=0.001. Data are represented as mean ± SEM. The data within each subfigure were derived from independent donors.

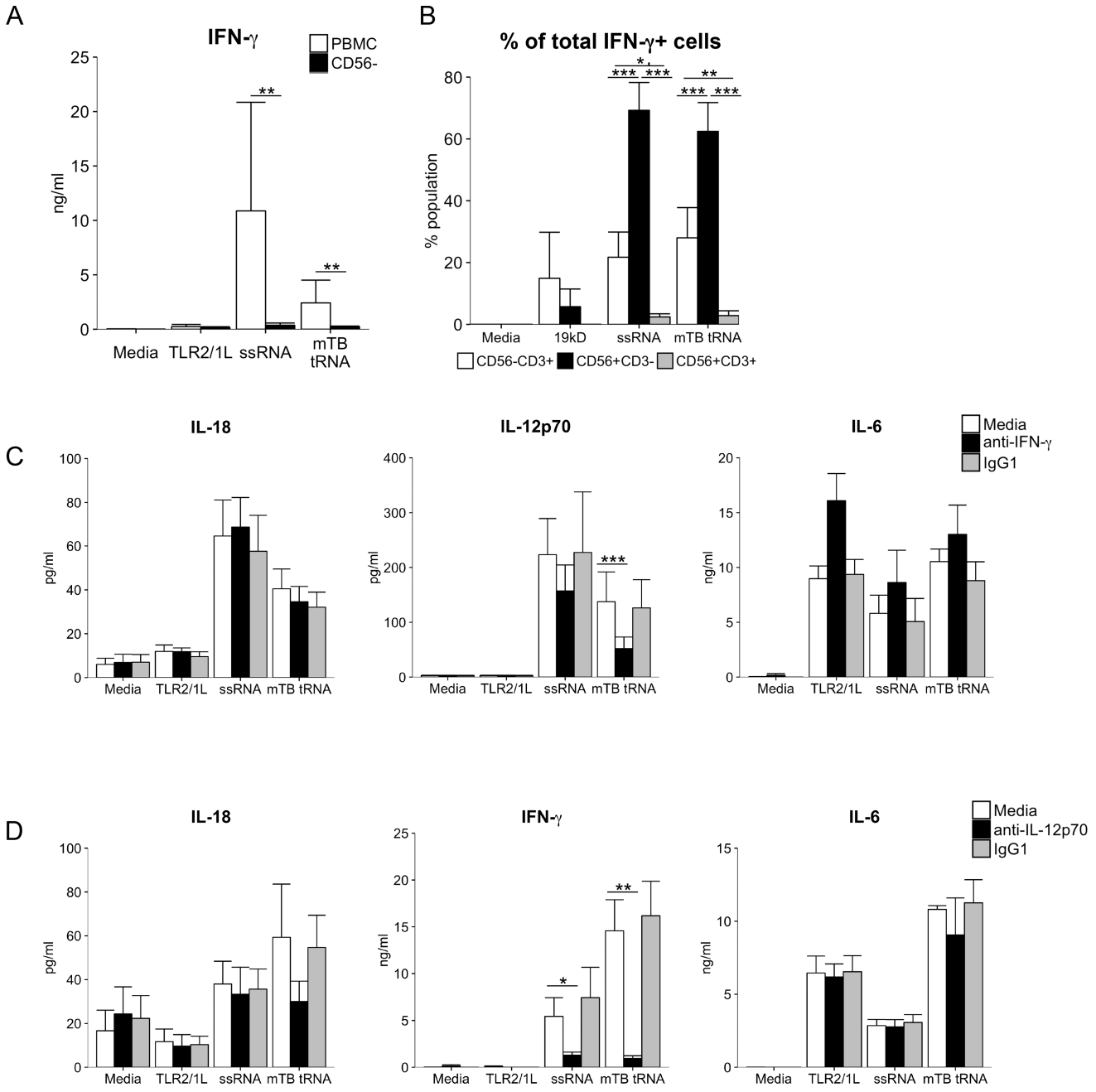


**Fig 6. Role for IFN-γ and NK cells**

(A) PBMC were depleted of $CD56^+$ cells, stimulated as shown and cytokine secretion measured at 24h (n=3). Statistical significance calculated by one-way repeated measures ANOVA and Šidák correction applied to the multiple comparison between PBMC and CD56 depleted populations. (B) PBMC were stimulated, and Brefeldin A added at 20h to arrest cytokine secretion. Cells were collected at 24h, stained with CD3-FITC, CD56-PE and IFN-γ-APC and measured by Flow cytometry. IFN-$\gamma^+$ lymphocytes were divided into subpopulations determined by CD3/CD56 staining and shown as percentage of parent (IFN-$\gamma^+$) population (n=3). (C) PBMC were pre-treated with monoclonal anti-IFN-γ neutralizing

antibody for 30m before stimulation as previous. Cytokine secretion was measured at 24h (n=6). (D) PBMC were pre-treated with monoclonal anti-IFN-γ neutralizing antibody for 30m before stimulation as previous. Cytokine secretion was measured at 24h (n=3). Data are represented as mean ± SEM. Statistical significance of ligand-stimulated PBMC in the presence of neutralizing or isotype control antibodies for B, C, and D was calculated by two-way ANOVA followed by Tukey post-hoc test. *p<= 0.05, ** p<=0.01, *** p<=0.001

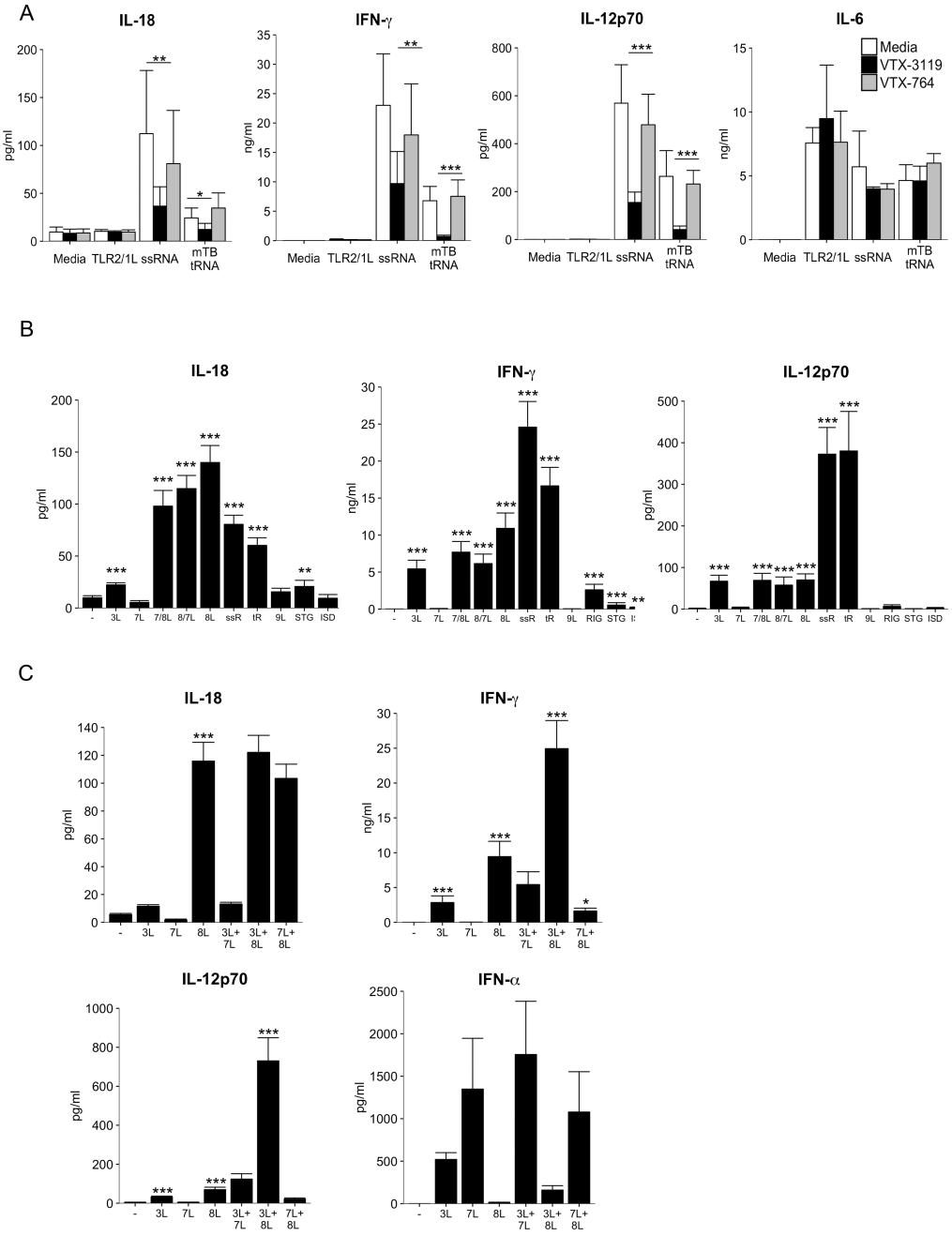


**Fig 7. Role of TLR8 and synergy with TLR3**

(A) PBMC were pre-treated with TLR8 antagonist or control for 30m before treatment with TLR2/1L, ssRNA and mTB tRNA. Cytokine secretion was measured at 24h. (IL-18 and IL-6: (n=3); IL-12p70 and IFN-γ (n=5). Statistical significance calculated by two-way repeated measures ANOVA and multiple comparisons by Tukey post-hoc test. * $P < 0.05$, ** $P < 0.01$. (B) PBMC were stimulated with nucleotide oligomers complexed with Dotap (endosomal PRR) or Lipofectamine2000 (cytosolic PRR) or synthetic agonists for 24h (n>=4). Statistical significance of ligand-stimulated PBMC vs. media calculated by one-way repeated measures ANOVA followed by Dunnett's multiple comparisons test. (C) PBMC

were stimulated with poly I:C, TL8-506 or combination and cytokine secretion measured at 24h (n=3). Data summarized using a mixed-effects model with a fixed effect for ligand and a random effect for subject. A two-way interaction term was fit to each ligand to test synergy. The likelihood ratio test was used to test for statistical significance. Data are represented as mean ± SEM. *p<= 0.05, ** p<=0.01, *** p<=0.001

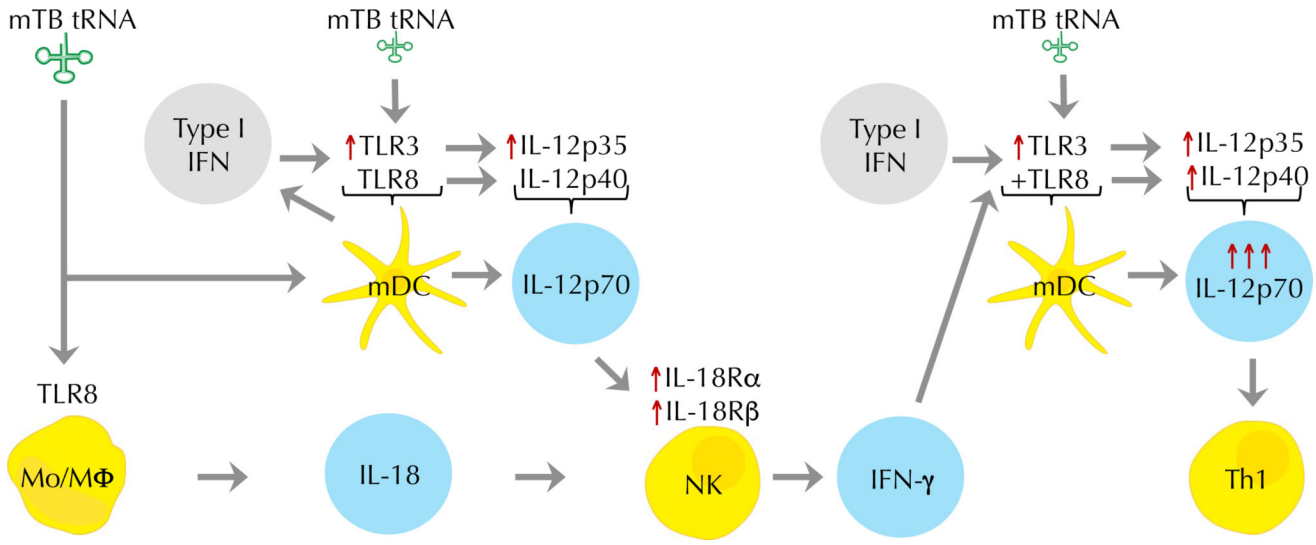


**Fig. 8. *M. tuberculosis* tRNA triggers the induction of IL-12p70**

This model, based on the experimental data in this manuscript as well as the literature, shows that mTB tRNA induces secretion of IL-18 via TLR8. Type I IFNs may contribute to enhanced induction of IL-12p35 via upregulation of TLR3, which in combination with IL-12p40 induction leads to secretion of bioactive IL-12p70. IL-12p70 upregulates IL-18 receptor on NK cells, facilitating the ability of IL-18 and IL-12p70 to synergize to induce secretion of IFN-γ. IFN-γ in turn enhances IL-12p70 secretion. The key cell types in this process are monocytes/macrophages, NK cells and myeloid DC.