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***IL12B* expression is sustained by a heterogenous population of myeloid lineages during tuberculosis**

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Summary

IL12B is required for resistance to *Mycobacterium tuberculosis* (Mtb) infection, promoting the initiation and maintenance of Mtb-specific effector responses. While this makes the IL12-pathway an attractive target for experimental tuberculosis (TB) therapies, data regarding what lineages express *IL12B* after infection is established are limited. This is not obvious in the lung, an organ in which both hematopoietic and non-hematopoietic lineages produce IL12p40 upon pathogen encounter. Here, we use radiation bone marrow chimeras and *Yet40* reporter mice to determine what lineages produce IL12p40 during experimental TB. We observed that hematopoietic IL12p40-production was sufficient to control Mtb, with no contribution by non-hematopoietic lineages. Furthermore, rather than being produced by a single subset, IL12p40 was produced by cells that were heterogenous in their size, granularity, autofluorescence and expression of CD11c, CD11b and CD8 α . While depending on the timepoint and tissue examined, the surface phenotype of IL12p40-producers most closely resembled macrophages based on previous surveys of lung myeloid lineages. Importantly, depletion of CD11c^{hi} cells during infection had no affect on lung IL12p40-concentrations. Collectively, our data demonstrate that IL12p40 production is sustained by a heterogenous population of myeloid lineages during experimental TB, and that redundant mechanisms of IL12p40-production exist when CD11c^{hi} lineages are absent.

Keywords

IL12 production; IL12p40; Macrophages; Dendritic cells; Innate immunity; Experimental tuberculosis

1. Introduction

It is now well established that the IL12p40-containing cytokines IL12 and IL23 serve host-protective roles during infection with *Mycobacterium tuberculosis* (Mtb).¹ IL12 contributes to the establishment of T_H1 -immunity, restricts bacterial proliferation during early stages of

experimental infection, and limits pulmonary pathology.²⁻⁴ IL23, on the other hand, is required for long-term bacterial control and promotes primary T_H17-responses.^{5,6} While these mechanistic insights have largely been gained using the mouse model of tuberculosis (TB), studies in other experimental systems support these models of IL12/IL23 function. In the guinea pig, for example, expression of IL12p40 increases after aerosol infection^{7,8} and is preferentially expressed in different parts of the granuloma depending on previous exposure.⁹ In the rabbit, expression of IFN γ — an important effector lymphokine produced downstream of IL12 signaling — is dynamic in the aerosol model¹⁰ and accelerates lesion healing and bacterial control in the skin model of TB.¹¹ Collectively, these data provide a mechanistic basis for the characteristics of TB as it occurs in individuals lacking the IL12/IL23 signaling pathway^{12,13} and provides a framework for exploring modulators of the IL12-response as an experimental vaccine strategy.¹⁴⁻¹⁶

Given the importance of IL12p40 to regulating TB - as well many other infectious diseases - much attention has been devoted to understanding the interaction between IL12p40-producing cells and adaptive lymphocytes during the initiation of pathogen immunity.¹⁷ For example, it was recently demonstrated that dendritic cells (DCs) in the draining lymph nodes are the primary source of IL12p40 during the initiation of *Toxoplasma gondii*-specific immunity.¹⁸ Emigrant DCs producing IL12 after subcutaneous exposure to *Listeria monocytogenes* reach the draining lymph node more efficiently than those not producing IL12,¹⁹ where they are capable of secreting IL12 via preloaded membranous-vesicles.²⁰ Activation of DCs with the viral dsRNA analog Poly (I:C) results in IL12 secretion near the immunological synapse,²¹ where it signals through IL12R β 1 and IL12R β 2 to promote T_H1 differentiation.²² IL23 is likewise produced by DCs^{23,24} and signals through IL12R β 31/IL23R to promote T_H17 differentiation.^{5,6,25} The cytokine milieu present during the initiation of Mtb-immunity is important, as it can significantly alter the course of disease at later stages.²⁶

While the studies mentioned above examine IL12p40-production during the initiating stages of immunity, information regarding which cell lineages continue to produce this cytokine during chronic stages of Mtb-infection is limited. This is important, as continual production of IL12 during later stages of infection is required to maintain control of Mtb in the lung.²⁷ The most extensive analysis to date is that of Rothfuchs et al.,²⁸ who demonstrated that following systemic *Mycobacterium bovis* infection, CD11b^{hi}EhCD8 α ^{neg}CD11c^{high} cells in the spleen are the primary providers of IL12p40.²⁸ This same lineage was identified as producing IL12p40 in the lung during Mtb-infection; however, it was acknowledged by the authors that a more extensive phenotypic analysis of those cells producing IL12p40 during experimental TB was still needed.²⁸ This is important in the context of lung infection since multiple cell types — including non-hematopoietic lineages²⁹ — are capable of producing IL12p40 in this organ.

Here we take advantage of both bone marrow chimera systems and the IL12 *Yet40* reporter mouse strain¹⁹ to characterize which cells produce IL12 during later stages of experimental Mtb-infection. As we will demonstrate, these data have led us to conclusions that differ from those of Rothfuchs et al.²⁸ Namely, we conclude that CD11b^{high}CD8 α ^{neg}CD11c^{high} cells are actually a minor population of IL12p40 producers in the chronic stages of Mtb infection,

with the bulk of IL12p40 produced by a more heterogenous population. The significance of this distinction to our understanding of TB is discussed.

2. Materials and methods

2.1. Mice

Mice were bred at the Medical College of Wisconsin (MCW) in the MCW Biomedical Resource Center and were treated according to National Institutes of Health and MCW Institute Animal Care and Use Committee (IACUC) guidelines. C57BL/6 and B6.FVB-Tg (Itgax-DTR/EGFP)57Lan/J (i.e. CD11c-DTR³⁰) strains were originally purchased from the Jackson Laboratory (Bar Harbor, ME). B6.129S1-*M2b^{tm1Jm}* (i.e. *il12b*^{-/-} mice³¹) and B6.129-*Il12b^{tm1Lky}*/J (i.e. *yet40* mice¹⁹) were kindly provided by Drs. Andrea M. Cooper (Trudeau Institute, Saranac Lake, NY) and Richard Locksley (Howard Hughes Medical Institute, University of California San Francisco, San Francisco, CA), respectively.

2.2. Radiation bone-marrow chimeras

Bone marrow cells from either C57BL/6, *il12b*^{-/-} or CD11c-DTR donors were harvested via perfusion of the femur and tibia medullary cavities with complete DMEM. Marrow suspensions were pelleted and subsequently resuspended in RBC lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃) to remove red blood cells; following RBC lysis, marrow preparations were washed and resuspended at 5×10^7 cells/mL in sterile PBS. Recipient mice received a lethal dose of whole-body irradiation (2×5 Gy) 3 h apart in a Gammacell Irradiator (1000 rad total). Immediately following the second dose, mice were given 200 μ l i.v. of indicated marrow preparations (i.e. 1×10^7 total bone marrow cells). Mice were allowed 4 weeks to reconstitute prior to their use in experiments.

2.3. Experimental infection

The H37Rv strain of Mtb (generously provided by Dr. Andrea Cooper of the Trudeau Institute) was grown in Proskauer Beck medium containing 0.05% Tween 80 to mid-log phase and frozen in 1-ml aliquots at -70 °C. For aerosol delivery of ~80 bacteria, animals were placed in a Glas-Col Inhalation Exposure System (Glas-Col, Terre Haute, IN) at a maximum 20 mice per sector. After loading the nebulizer (Glas-Col) with 10 mL of diluted H37Rv (5×10^6 CFU/mL in deionized water), mice were infected using the following exposure settings: 900 s warm-up, 3600 s nebulize, 1800 s cloud decay, 900 s UV exposure (Vacuum Pressure Setting: 50; Comp Air Pressure Setting: 15). Immediately after infection, mice were placed in microisolator cages; during the entire period after infection, mice were monitored daily for outward signs of distress per IACUC oversight. Lungs from a group of control mice were plated at day 1 post-infection to confirm the delivery ~ 80 CFU.

2.4. Bacterial load determination

Infected mice were euthanized by CO₂ asphyxiation; lungs and spleen were aseptically removed and individually homogenized in sterile normal saline using the Gentle Macs, program RNA 2.01 (Miltenyi, Bergisch Gladbach, Germany). The Gentle Macs system was used to increase containment of infectious aerosols generated during the homogenization process; efficient homogenization using RNA 2.01 was ensured by comparing our Mtb CFU

counts to those of others using the traditional PTFE Pestle/Borosilicate glass tube system (personal communication, Dr. Robert North of the Trudeau Institute in Saranac Lake, NY). Serial dilutions of the organ homogenate were plated on nutrient 7H11 agar. The number of mycobacterial CFU was determined after incubating plates for two weeks at 37 °C in 7% CO₂.

2.5. Histological analysis

Lungs were inflated with 10% neutral-buffered formalin, followed by resection and fixation in the same formalin solution. Serial sections of paraffin-embedded tissues were then stained with either carbofuchsin and methylene blue (i.e. Ziehl-Neelsen stain) or hematoxylin and eosin (i.e. H&E stain). Once slides were generated, a histopathological analysis of each lobe was performed. Images of both Ziehl-Neelsen and H&E stains were taken with a Labophot-2 upright microscope (Nikon, Tokyo, Japan) using a Retiga 2000R camera (QImaging, Surrey, British Columbia, Canada), and were analyzed using NIS Elements software (Nikon).

2.6. Cell preparations

Lung cell suspensions were prepared by perfusing EDTA-containing PBS through the mouse heart until the lungs appeared white, whereupon they were removed (along with the draining mediastinal lymph node, MLN) and placed in ice-cold incomplete DMEM. After this point, lungs and MLNs were identically treated in order to directly compare lung and MLN IL12p40-producers' expression of select phenotypic markers (e.g. integrins CD11b and CD11c). Specifically, both organs were incubated in DMEM containing collagenase IX(0.7 mg/ml) and DNase(30 µg/ml) at 37 °C for 30 min. Digested tissues were gently homogenized using the Miltenyi Gentle Macs system and passed through a 40-µm nylon tissue strainer; the resultant single-cell suspension was treated with RBC lysis solution, washed and counted. Cells prepared in this way were subsequently used for staining and flow cytometric analyzes.

2.7. Flow cytometry

All antibodies used for flow cytometric analysis were purchased from BD Pharmingen (San Diego, CA, USA). Lung cell preparations were washed with FACS buffer (2% FCS in PBS) and stained as indicated with antibodies recognizing either CD11c, CD11b, CD8α, I-A^b or Thy1. Since preliminary experiments demonstrated that fixation quenched the YFP signal in *Yet40* cell preparations (data not shown), all data were acquired from unfixed samples using a BSC-contained Guava easyCyte 8HT flow cytometer. At each time point, the integrity of the lasers/detectors was verified using standardized fluorescent beads (Guava Easy Check Kit). Over the course of each experiment, no drift in detector sensitivity was noted (data not shown), allowing us to directly compare MFI data from different time points. Acquired data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

2.8. Quantitative PCR analysis

Lung RNA was extracted from infected, snap-frozen tissue by homogenizing the tissue in RLT lysis buffer (Qiagen, Germantown, MD) using the Gentle Macs, program RNA 2.01

(Milenyi). Total RNA was extracted from the lysis solution according to the manufacturer's protocol. RNA samples from each group/time point were reverse transcribed using Fermentas reagents (Thermo Scientific, Glen Burnie, MD). cDNA was then amplified using SYBR Green reagents (Fermentas) on the BioRad iQ5 detection system; C_t values were determined using the BioRad iQ5 bundled software (Bio-Rad, Hercules, CA). The expression of select mRNAs (i.e. *il12a* and *il12b*) relative to that of *gapdh* was determined using the ΔC_t calculation recommended by the manufacturer. The forward (F) and reverse (R) primers used for real time amplification of cDNA samples are listed here (5'—3'): *gapdh*, F: CATGGCCTTCCGTGTTCTTA, R: GCGGCACGT-CAGATCCA; *il12a*, F: CAATCACGCTACCTCCTCTTTT, R: CAGCAGTG-CAGGAATAATGTTTC; *il12b*, F: ATGGAGTCATAGGCTCTGGAAA, R: CCGGAGTAATTTGGTGCTTCAC; *il123a*, F: AATAATGTGCCCCGTATC-CAGT, R: GCTCCCCTTTGAAGATGTCAG.

2.9. Diphtheria toxin treatment

For continual depletion of CD11c⁺ cells over the course of a 40-day infection, CD11c-DTR → *il12b*^{-/-} chimeric mice received an i.p. injection of 4 ng DT/g of body mass; this treatment began one day prior to infection, and was repeated every three days until the time mice were euthanized for lung IL12p40 measurement.

2.10. Lung IL12p40 measurement

The apical lobe of each lung was resected and immediately homogenized in NP40 lysis buffer (50 mM Tris [pH 7.4], 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na₃VO₄, 1% NP40, 0.02% NaN₃) containing a cocktail of mammalian protease inhibitors (Roche). Lysates were kept on ice until all groups were processed in this manner, and then clarified by centrifugation and filter sterilized. For ELISA determination of lung IL12p40 concentration, we used the BD OptEIA Mouse IL-12 (p40) ELISA Set system per the manufacturer's instructions (BD Biosciences).

2.11. Statistical analysis

All graphs were prepared using GraphPad Prism version 5.0a; statistical analyses were performed using software bundled to GraphPad. Error bars in all figures show mean ± SD. Asterisks shown between data points represent significant *p*-values (i.e. *p* < 0.05) for the comparison indicated. Statistical comparisons involving more than two experimental groups used ANOVA analysis. All other statistical comparisons used Student's *t*-test.

3. Results

3.1. Hematopoietic *il12b*-expression is required to limit *Mtb*-burdens in the lung and spleen

IL12B expression by hematopoietic lineages is widely considered the most immunologically important source of IL12p40 during pathogen infection.¹⁸⁻²¹ However, non-hematopoietic lineages in the lung are also capable of producing host-protective IL12p40.²⁹ To determine if non-hematopoietic expression of *il12b* (the mouse homolog of human *IL12B*) impacts experimental TB outcome, we aerogenically infected the following groups of radiation bone marrow chimeras with *Mtb* strain H37Rv: C57BL/6 → C57BL/6, *il12b*^{-/-} → C57BL/6, C57BL/6 → *il12b*^{-/-}, and *il12b*^{-/-} → *il12b*^{-/-}. On select days after infection, bacterial

burdens were measured in the lungs and spleen. Beginning 40-days post-infection, *il12b*^{-/-} → C57BL/6 chimeras exhibited lung burdens that were both significantly higher than those in C57BL/6 → C57BL/6 mice and equal to those levels found in *il12b*^{-/-} → *il12b*^{-/-} mice (Figure 1A). In the spleen, Mtb burdens in *il12b* → C57BL/6 chimeras were also above that of C57BL/6 → C57BL/6 controls and equal to those observed in *il12b*^{-/-} → *il12b*^{-/-} mice (Figure 1B). In both organs, control C57BL/6 → *il12b*^{-/-} mice exhibited Mtb burdens levels equal to that of C57BL/6 → C57BL/6 mice (Figure 1A, B). From these data, we conclude that non-hematopoietic expression of *il12b* has no obvious impact on the course of experimental TB, and that the primary source of host-protective IL12p40 is a hematopoietic lineage.

3.2. Hematopoietic *il12b*-expression restricts intracellular Mtb-growth

In addition to examining bacterial burden at a whole lung level, we visually determined whether acid fast bacilli (AFB) were more numerous in the *il12b*^{-/-} → C57BL/6 chimeras relative to C57BL/6 → C57BL/6 controls. Lung sections from each mouse (3 sections/mouse, 3 mice/group) were stained for AFB and counter stained with methylene blue (i.e. Ziehl—Neelsen stain). Serial sections adjacent to those stained for AFB were stained with hematoxylin and eosin (H&E) to allow for a qualitative assessment of the cellular response surrounding AFB. The results of this analysis are shown in Figure 2. Consistent with the thorough survey of Rhoades et al.,³² AFB in control mice (i.e. C57BL/6 → C57BL/6) at day-20 were difficult to locate, but when found were primarily associated with alveolar phagocytes (Figure 2A). Surrounding these phagocytes were the mononuclear and polymorphonuclear participants known to associate with Mtb-induced alveolitis (Figure 2C, E). In *il12b*^{-/-} → C57BL/6 mice, AFB were also difficult to locate at this time (day-20); however, when found, the number of AFB per cell was higher compared to C57BL/6 → C57BL/6 controls (Figure 2B). Higher densities of AFB were also observed at later time points (data not shown). Consistent with the flow cytometric analysis of Cooper et al.,³³ H&E staining of areas immediately adjacent to that shown in Figure 2B demonstrated that pulmonary accumulations in *il12b*^{-/-} → C57BL/6 chimeras comprised more polymorphonuclear cells than C57BL/6 → C57BL/6 controls (Figure 2D, F). Based on these data, we conclude that hematopoietic IL12p40-production is required to limit intracellular Mtb division, even at a time when differences are not discernable by total lung CFU counts alone.

3.3. IL12p40-producers in the lung and MLN exhibit distinct cytometric characteristics

Given that sustained production of IL12p40 is required to maintain TB control,²⁷ we wished to identify which hematopoietic lineage(s) expressed *il12b* after the immune response to Mtb was established. To address this question, we aerogenically infected *il12b* reporter mice (i.e. *Yet40* mice) to determine via flow cytometry which lineage(s) produced IL12p40 in MLN and lungs on days-27, -42 and -56 post-infection. The wild type *il12b* allele in *Yet40* mice is replaced with an *il12b*-IRES-YFP reporter construct, resulting in yellow fluorescent protein (YFP) expression whenever *il12b* is transcribed.¹⁹ This reporter allele does not affect secretion of IL12p40-containing cytokines.¹⁹ We examined days-27, -42 and -56 post-infection given previous reports that Mtb-specific T-cell priming begins ~7 days after infection in the C57BL/6 model,³⁴⁻³⁶ and markedly declines after 30 days of infection in the

same system.³⁷ Uninfected *Yet40* mice were also examined in this manner, with Mtb-infected C57BL/6 mice used as non-reporter controls for establishing gates for flow cytometric analysis of MLN (Figure 3A—D) and lung (Figure 3E—H) cell preparations. This is particularly important for analysis of the lungs, which contain a larger number of autofluorescent cells relative to secondary lymphoid organs.³⁸ Regarding the comparability of experimental TB in the *Yet40* and C57BL/6 strains, Mtb burdens in lungs and spleen of both strains were similar 52-days after infection with the same aerosol (Figure 3I).

As an initial assessment of IL12p40-producers' cytometric characteristics, we visually and quantitatively examined the forward and side scatter of YFP⁺ events in the MLN and lung at select times post-infection. Forward scatter (FSC) and side scatter (SSC) are general measurements of size and granularity, respectively. The results of this analysis are shown in Figure 4. After 27 days of infection, the FSC and SSC characteristics of YFP⁺ cells in the MLN (represented as a contour plot in Figure 4A) were distinct from those of Thy1⁺ lymphocytes (represented by the blue dot plot overlay in Figure 4A) in the same cell preparation. This was not surprising, given the reports from other infectious model systems that IL12p40 is primarily produced by non-lymphocytic sources.¹⁸⁻²¹ Lung YFP⁺ cells, however, exhibited FSC and SSC characteristics distinct from their MLN counterparts (compare contour plots of MLN cells [Figure 4A, C, E] to those of lung cells [Figure 4B, D, F]). Specifically, both the FSC and SSC values of lung YFP⁺ cells were greater than YFP⁺ cells of the MLN on each day examined. Quantitation of the mean FSC and SSC values from multiple *Yet40* mice per time point confirmed this pattern to be statistically significant (Figure 4G—H). Analysis of the median FSC and SSC values gave identical results (data not shown). We conclude from this analysis that *il12b*-expressing cells in the Mtb-infected lung and MLN are cytometrically distinct, with those in the lung being, on average, larger and more granular than their MLN counterparts.

3.4. Lung and MLN IL12p40-producers display distinct autofluorescence, CD11c, CD11b and CD8α expression patterns

Rothfuchs et al.²⁸ identified CD11b^{low} CD8α^{pos} CD11c^{high} dendritic cells as providing an initial burst of IL12p40 after systemic *M. bovis* infection, with CD11b^{high} CD8α^{neg} CD11c^{high} providing this cytokine during later stages of infection.²⁸ For this reason, in addition to examining size and granularity differences after aerogenic *M. tuberculosis* infection (Figure 4), we compared MLN and lung IL12p40-producers' relative expression of the following: autofluorescence (Red2 channel), CD11c, CD11b, CD8α and I-A^b. The results of this analysis are shown in Figure 5. Compared to YFP⁺ cells in the MLN, lung YFP⁺ cells expressed a significantly higher degree of autofluorescence at days-27 and -42 (Figure 5A, B); lung YFP⁺ cells also expressed higher levels of CD11c (Figure 5A, C), a difference that was significant at day 56 post-infection (Figure 5C). These differences could be seen via overlay of the CD11c/auto-fluorescence contour plots (Figure 5A) and quantitation of autofluorescence and CD11c mean fluorescence intensity (MFI) values (Figure 5B and C, respectively). Identical results were observed using median fluorescence values (data not shown). Classification of YFP⁺ cells as either “CD11c^{lo}” or “CD11c^{hi}” demonstrated that lung and MLN IL12p40⁺ cells also comprised different percentages of CD11c^{lo} and CD11c^{hi} IL12p40-producers, with the later making up higher percentage of

IL12p40-producers in the lung relative to the MLN (Figure 6A–C). Subsequent analysis of CD11b and CD8 α expression used this relatively high autofluorescence (10^3 fluorescent units) as the background against which expression of both markers was assessed. We observed that both CD11b (Figure 5D–E) and CD8 α (Figure 5F–G) expression by MLN YFP⁺ cells was significantly higher than those of lung YFP⁺ cells at each time point examined. In both the lung and MLN there was also a gradual increase in the representation of CD8 α ^{pos} YFP⁺ cells over time (Fig 5F and Figure 6D–F). As a percentage of IL12p40⁺ cells, CD11b⁺ cells did not change significantly over the time points examined (Fig 5D and Figure 6G–I). All YFP⁺ cells in the MLN and lung expressed high levels of IA^b (data not shown). Collectively, we conclude from these data that lung and MLN IL12p40-producers display distinct autofluorescence, CD11c, CD11b and CD8 α expression patterns, with CD11b^{pos} CD8 α ^{pos}CD11c^{high} becoming more predominant over time in both organs.

3.5. IL12p40-production by CD11c^{low} lineages can compensate in the absence of CD11c^{high} lineages

Finally, we wished to determine if CD11c^{high} cells' role as primary IL12p40-producer was non-redundant. In other words, could IL12p40 production by other lineages compensate the host in the absence of CD11c^{high} lineages. To test this, we turned to the CD11c-DTR system³⁰ to test if CD11c^{high} depletion lowered lung IL12p40 levels during Mtb-infection. Specifically, bone marrow from

CD11c-DTR mice was transferred into lethally irradiated *il12b*^{-/-} mice so as to both (1) eliminate any contribution of radioresistant CD11c cells to IL12p40 production and (2) allow for continual CD11c depletion over the course of a 40-day Mtb infection (repeated treatment of non-chimeric CD11c-DTR mice with DT is lethal³⁰). After a 40-day DT treatment period, lung IL12p40 concentrations were assessed and compared to those of appropriate controls (Figure 7). Flow cytometric analysis confirmed DT-mediated depletion of ~80% of lung CD11c^{hi} cells using this protocol (Figure 7A, B); this approximates the extent of CD11c-depletion observed by others using the DT system.³⁹ Day-40 post-infection was chosen as our endpoint since *il12b* and *il12a* mRNA levels plateau near this time in C57BL/6 mice (Figure 7C). We observed that, compared to all control groups (i.e. PBS-treated C57BL/6 $\rightarrow$ *il12b*^{-/-}, DT-treated C57BL/6 $\rightarrow$ *il12b*^{-/-}, and PBS-treated CD11cDTR $\rightarrow$ *il12b*^{-/-} mice), IL12p40 levels in DT-treated C57BL/6 $\rightarrow$ *il12b*^{-/-} lungs were unchanged (Figure 7D). Mtb burdens were similarly unaffected by DT treatment (Figure 7E). We conclude from this analysis that IL12p40-production by CD11c^{low} lineages can compensate in the absence of most CD11c^{high} lineages, maintaining levels of host protection identical to that observed when all CD11c^{high} cells are present.

4. Discussion

IL12 and IL23 are members of a cytokine family that also include IL27 and IL35.⁴⁰ Despite being structurally similar due to their shared incorporation of IL12p40,^{41,42} IL12 and IL23 have immunological properties that are distinct from one another.²⁵ Initially identified as NK cell stimulatory factor,⁴³ IL12 is now established as possessing anti-angiogenic properties⁴⁴ and being host-protective in the context of intracellular bacterial infections.⁴⁵ As it pertains to tuberculosis, IL12 is expressed in the pleura of actively infected

individuals,⁴⁶⁻⁴⁸ where its concentrations exceed those in the circulation of the same individuals.⁴⁸ This suggests that IL12 production and catabolism occur locally in the human lung, where its sustained production is required to limit Mtb-associated pathology in the mouse model.²⁷ Like IL12, IL23 is also elevated in the pleura of Mtb-infected individuals relative to uninfected controls⁴⁹; however, its effects on TB outcome are distinct. Specifically, *il23*-deficient animals exhibit an impaired ability to limit Mtb only during chronic stages of experimental infection, whereas *il12*-deficient animals succumb much sooner.^{5,6} The impact of IL23 deficiency is more profound in the context of vaccination. Specifically, ESAT6₁₋₂₀/MPL/TDIVI/DDA-driven T_H17 differentiation is limited in the absence of IL23 — a consequence of which is delayed recruitment of protective T_H1 cells to the lung upon subsequent Mtb-infection.⁵⁰ The positive role of IL23 during vaccination has also been observed in the BCG-immunization system.⁵¹ Collectively, the abundant literature on IL12 and IL23 demonstrate that these cytokines serve protective roles in the context of TB.

Here we have demonstrated that production of IL12p40 during experimental TB is sustained by myeloid lineages that are heterogeneous in size, granularity, autofluorescence and surface expression of CD11c, CD11b and CD8α. While IL12p40-producers' cytometric properties varied depending on the tissue and time-point examined, overall IL12p40-producers at the site of infection (i.e. the lung) resembled macrophages based on studies of Vermaelen and Pauwels³⁸ and Gonzalez-Juarrero et al.⁵² These lung macrophage-associated properties include expression of CD11c, CD8α, high autofluorescence,³⁸ and mixed expression of CD11b during Mtb-infection.⁵² This conclusion differs somewhat from that of Rothfuchs et al.²⁸ Their well-performed study concluded from experiments primarily using *M. bovis* BCG that IL12p40 production is sustained by CD8α^{neg}CD11b^{hi} CD11c^{hi} DCs during chronic mycobacterial infection. While direct comparisons of flow cytometry data generated in separate labs is difficult (e.g. due to different settings, analysis gates, etc), we observed that IL12p40-producers at day 56 post-infection - which is past the point that new T-cell responses are initiated in the C57BL/6 model³⁷ — were primarily CD8α^{pos} myeloid lineages (Figure 5F). This distinction is important, as different myeloid lineages have different functional properties^{53,54} and interact with Mtb via distinct mechanisms^{55,56}; defining these interactions impacts both our basic understanding of TB and our ability to predict how macrophages/DCs might be targeted for therapeutic purposes. Specifically, the identification of CD11c⁺ lineages as being the primary producer of IL12p40-containing cytokines (IL12 and/or IL23) justifies the design of experimental therapies that target negative regulators of CD11c IL12p40 production. CD11c-targeted therapies have been used successfully in tumor models, wherein siRNA-containing complexes directed at CD11c + cells silence immunosuppressive protein expression.⁵⁷ This can be done via siRNA-containing liposomes that take advantage CD11c⁺ cells' phagocytic properties,^{57,58} or via conjugation of siRNA complexes directly to antibodies targeting the CD11c integrin.^{59,60} As it applies to targeting CD11c⁺ cells in pulmonary TB, similar approaches could be taken to target negative regulators of IL12p40. An example of such a target would be the miR-21, which regulates IL12 production following mycobacterial exposure by targeting *il12a* mRNA transcripts for degradation.^{61,62} Although such a therapy would require modification for treating lungs rather than tumor (inhalation therapy as opposed to simple injection,⁶³).

Aerosol delivery of siRNAs can affectively alter pulmonary gene expression during experimental TB.^{64,65}

The events that give rise to IL12p40 production in the lung are both host- and Mtb-driven. On the host side, Gonzalez-Juarrero et al.⁵² demonstrated that multiple myeloid lineages immigrate into the lung following Mtb infection, where they express pattern recognition receptors (PRRs) known to be triggered by Mtb. These PRRs include TLR2,^{66–68} TLR4,⁶⁹ TLR6,⁷⁰ TLR9,⁷¹ NOD2,⁷² mannose receptor,⁷³ DC-SIGN,⁷⁴ MINCLE⁷⁵ and MARCO.⁷⁶ Rather than hiding from these innate immigrants, Mtb produces several molecules that strongly elicit the production of IL12p40 via the PRRs listed above. These include lipomannan, mannosylated phosphatidylinositol, 19-kDa lipoprotein, unmethylated CpG dinucleotides, muramyl dipeptide, mannose-capped lipoarabinomannan and trehalose6,6'-dimycolate.^{66–77} Depending on the stage of infection, myeloid lineages may encounter these inflammatory molecules both in the form of free bacteria, which can be observed in both select animal models and in human tissues,⁷⁸ or as membranous material that has trafficked out of Mtb-containing vacuoles.⁷⁹ Recently it was demonstrated that mycolic acids themselves — principal components of the Mtb cell wall that gives rise to colonies' characteristic waxy appearance⁸⁰ — were subject to *mmaA4*-dependent modifications that significantly affect macrophage IL12 production.⁸¹ In contrast to the immune evasion strategies used by other pathogens, we believe these data support a model wherein Mtb targets host immune activation as a part of its life cycle and survival strategy. This model has been well-described by Ernst,⁸² and is further supported by a large body of historical data demonstrating that patients with active TB generate and maintain high levels of cellular immunity to Mtb antigens.⁸³

Due to the variety of immunogens produced by Mtb, as well as the variety of PRRs that respond to these molecules, we view the dynamic nature of which myeloid lineages produces IL12p40 as a reflection of Mtb's variable immunogenicity over time. For example, the increased representation of CD8 α ⁺CD11c^{high} cells among YFP⁺ cells over time likely reflects changes in Mtb expression of CD8 α ⁺-stimulants over time. These changes could reflect different types of CD8 α ⁺-stimulants, or simply different levels of CD8 α ⁺-stimulants, as CD8 α ⁺ myeloid lineages in the lung are more sensitive to TLR-activation than CD8 α [−] myeloid lineages.⁸⁴ While we cannot exclude the possibility that changes in cellular composition of the lung “granuloma” over time account for variable IL12p40-production, Dannenberg et al. demonstrated - albeit in a different model system - that macrophages within tuberculous lesions undergo constant turnover, with near-continuous entry of recent bone marrow emigrants.⁸⁵ The variable representation of CD8 α ⁺ and CD8 α [−] cells among IL12p40-producers may have an important impact on the phenotype of those T cells activated downstream of IL12p40-producers' activation.⁸⁶ While our experiments cannot discriminate whether Mtb-infected cells are among IL12p40-producers, Rothfuchs et al.²⁸ demonstrated that following i.v. BCG infection, those cells producing IL12p40 and those that were infected were distinct from one another. However, Mtb-infection of macrophages and DCs does affect other properties of these lineages, most notably class II MHC-dependent antigen presentation.⁵⁵

After having established that CD11c^{hi} cells are the principal source of IL12p40 during Mtb-infection, we were surprised that depletion of CD11c^{hi} cells had no impact on either the total lung IL12p40 levels or Mtb-burden. We interpret this as evidence that the CD11c^{lo} and/or CD11c^{neg} IL12p40-producers can compensate in the absence of CD11c^{hi} cells, and is reminiscent of the redundant mechanisms that exist to produce IFN γ .⁸⁷ Nevertheless, Tian et al.⁸⁸ demonstrated that CD11c-depletion immediately prior to i.v. Mtb-infection negatively affects subsequent T_H1 cell differentiation, and consequently, CFU burdens at days-14 and -21 postinfection. Reconciling our data with those Tian et al.⁸⁸ likely lies in the different time-points, tissues and route-of-infection used in their study and ours. We interpret the lack of difference between DT-treated and control CD11c-DTR mice (Figure 7E) as indicating that despite 40 days of continuous CD11c-depletion, a sufficiently protective T cell response is capable of forming. This is most likely due to the presence of other types of antigen presenting cells in the lung and draining lymph node (e.g. B cells). Whether it is IL12 or IL23 being produced by IL12p40⁺ CD11c⁺ cells at given time is also remaining question, given the different immunological effects that IL12 and IL23 carry. The expression of *il12a* (encoding the p35 subunit of IL12) is higher than that of *il23a* (encoding the p19 subunit of IL23) during the time points we've examined. Nevertheless, it is possible that distinct CD11c⁺ populations produce IL12, while others produce IL23, or both. Answering this important question would require either clonal analysis of IL12p40⁺ cells or utilization of a second fluorescence reporter (e.g. *il12a*-IRES-RFP).

The mechanisms through which IL12 and IL23 protect the Mtb-infected host have been recently and expertly reviewed.¹⁷ An integral component of these cytokines' protectiveness is *IL12RB1*, a gene that encodes — among other protein isoforms⁸⁹ — the low-affinity receptor for both cytokines.⁹⁰⁻⁹² Like IL12 and IL23, *IL12RB1* is also expressed by pleural cells of tuberculous individuals.^{93,94} Reflecting the importance of its expression, *IL12RB1*^{null} are susceptible to disseminated forms of disease caused by members of the *M. tuberculosis* complex (i.e., *M. tuberculosis*¹² and *M. bovis* BCG⁹⁵⁻⁹⁷) as well as non-tuberculous mycobacteria (i.e. *Mycobacterium avium*,⁹⁸⁻¹⁰⁰ *Mycobacterium intracellulare*⁹⁹ and *Mycobacterium abscessus*¹⁰¹). Building on the observation that T-cell differentiation is defective in *IL12RB1*^{null} individuals,^{102,103} we recently demonstrated that it is *rag1*-dependent lineages that must express *il12b1* (the mouse homolog of human *IL12RB1*) to control experimental Mtb-infection.²² Interestingly, as we also observed in *il12rb1*^{-/-} mice,²² *il12b*^{-/-} lungs exhibit many more acid-fast bacilli (AFB) per phagocyte than wild type controls at day-20 post-infection. This is a time in the mouse-model when differences are not observable by total lung burden measurements. Since this phenomenon is observed independent of homogenization method utilized (data not shown), there are several potential technical or biological explanations for this observation. Technically, these data may indicate the need for detergent when homogenizing infected tissues from the lungs of immunocompromised mice (so as to better disrupt such hydrophobic clumps of bacilli). Biologically, this observation may reflect an IL12-dependent host response that either negatively regulates Mtb expression of *kasB* (the gene that confers acid-fastness^{104,105}) or alters Mtb metabolism in the lung.¹⁰⁶ If the former model is correct, Mtb in wild type lungs are present at the same AFB per phagocyte ratio as those in *il12b*^{-/-} lung, but are just not visible by acid-fast staining. As anecdotal support for IL12's influence on Mtb metabolism,

we have observed that Mtb CFUs from *il12b*^{-/-} lung homogenate plated on 7H11 appear sooner than those from C57BL/6 controls (data not shown). Future work in our lab will be directed at teasing apart these possibilities and understanding how *il12b*-deficiency influences both Mtb metabolism and potential infectivity.

Finally, it is worth noting that while our study and that of Rothfuchs et al.²⁸ lead to somewhat different conclusions regarding which lineages produce IL12p40 during Mtb, both demonstrate that IL12p40 producing cells are relatively rare in the Mtb-infected lung. This is surprising given the profound importance of IL12p40 to TB control as first reported by Flynn et al.³ and Cooper et al.² The rarity of IL12p40 producers may be a reason why experimental therapies designed to protect the host from intracellular pathogens are more effective if given in combination with either recombinant IL12 or IL12-encoding plasmids that augment hosts' natural IL12 response.^{16,107-109} Grahmann and Braun¹¹⁰ recently demonstrated that treating individuals with aerosolized IFN γ , a lymphokine downstream of IL12-signaling, served as an effective adjunct to chemotherapy in four cases of MDR-TB. Although treating animals with rIL12,³ IL12-encoding vaccines¹⁶ — or even IL12-transgenic fruits¹¹¹ - effectively reduces Mtb-burdens in animal models, it remains to be determined if recombinant IL12 would be an effective adjunct to chemotherapy. While careful attention must be given to maintaining an appropriate balance of IL12 in the Mtb-infected lung,¹¹² as well as to preventing IL12-associated toxicity,¹¹³ adjunctive IL12 therapy may possibly be more effective than adjunctive IFN γ therapy since IL12 can function through IFN γ -in-dependent mechanisms to control Mtb.²²

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Figure 1.

Hematopoietic *il12b* expression is required to limit Mtb burden. Four groups of radiation bone marrow chimeras (C57BL/6 → C57BL/6, *il12b*^{-/-} → C57BL/6, C57BL/6 → *il12b*^{-/-}, and *il12b*^{-/-} → *il12b*^{-/-} mice) were simultaneously infected via aerosol with ~80 CFU of *M. tuberculosis* H37Rv. At select times post-infection, Mtb burden in the (A) lungs and (B) spleen of each group was assessed by plating serial dilutions of homogenized organs on 7H11. Shown for each time is the mean number of CFU (Log₁₀) present in three mice per group per time point. Error bars represent ±SD; asterisks indicate times at which a significant difference existed between groups with *il12b*-competent hematopoietic cells (i.e. C57BL/6 → C57BL/6 and C57BL/6 → *il12b*^{-/-} mice) and *il12b*-deficient hematopoietic cells (*il12b*^{-/-} → C57BL/6 and *il12b*^{-/-} → *il12b*^{-/-} mice). A difference was considered significant if $p < 0.05$ as determined using Student's T-test. This experiment was repeated two separate times, each with similar results.

Figure 2.

Hematopoietic *il12b*-expression restricts intracellular Mtb-growth. Serial sections of lungs collected at 20-days post-infection from either (A, C, E) C57BL/6 → C57BL/6 or (B,D,F) *il12b*^{-/-} C57BL/6 lungs were either stained (A—B) with Ziehl—Neelsen to identify acid-fast bacilli or (C—F) with hematoxylin and eosin to broadly discriminate which immune lineages localized near Mtb-infected cells. For this reason, the section shown in (A) from C57BL/6 → C57BL/6 mice was immediately adjacent to that shown in (C, E). Likewise, the section shown in (B) from *il12b*^{-/-} C57BL/6 was immediately adjacent to that shown in (D,

F). The micrographs shown in (A, B) were taken at 40×, while those of adjacent section in (C, D) were taken at 10×. Features of particular interest in (C) and (D) are magnified in (E) and (F), respectively. These features include (E) in C57BL/6 → C57BL/6, the presence of numerous small mononuclear cells (thin arrows) proximal to larger mononuclear cells (large arrow with asterisk) and (F) in *il12b*^{-/-} → C57BL/6, an alveolitis predominated by large polymorphonuclear cells (thin arrows) and picnotic debris (large arrow with asterisk). These sections are representative of two separate experiments with three mice per group at day-20 post infection. Images shown are also representative of several microscopic fields per lung.

Figure 3.

Gating strategy to identify YFP^{pos} events in Mtb-infected *Yet40* mice. In order to identify and directly compare YFP^{pos} events in the (A—D) MLN and (E—H) lung, cell preparations from these two organs were identically prepared from (B, F) Infected C57BL/6, (C, G) Uninfected *Yet40* and (D, H) Infected *Yet40* mice. After gating based on their (A, E) FSC and SSC characteristics, the YFP-channel fluorescence and FSC of cells from each group of mice were analyzed. Our gate for YFP^{pos} events (the gray inset in each dot plot) was set for both the MLN (top panel) and lung (lower panel) so as to exclude the majority of autofluorescent cells from our analysis. Given the abundance of autofluorescent cells in the lung compared to the MLN (compare dot plots B and F), it was necessary to reduce the size of our analysis gate for analyzing lung populations (compare gate size in B—D to that of F—H). The dot plots shown in (A—H) were collected from tissues at day 27 post-infection; however, the gates shown are identical to those used at all other time points. (I) As a measure of the comparability of experimental TB in C57BL/6 and *Yet40* mice, Mtb burdens (day 52 post infection) were determined for both the lung and spleen of both strains (closed bars, C57BL/6; open bars, *Yet40*). No significant difference in Mtb burdens existed determined by Student's T-test ($p = 0.32$ for lung; $p = 0.18$ for spleen).

Figure 4.

IL12p40-expressing cells in the MLN and lung display distinct forward and side scatter patterns. *Yet40* mice were infected with ~80 CFU of *M. tuberculosis* H37Rv. At (A, B) day-27, (C, D) day-42 and (E, F) day-56 post-infection, the forward and side scatter of YFP^{pos} cells in the (A, C, E) MLN and (B, D, F) Lungs of the same animal were examined. Forward and side scatter plots of YFP^{pos} cells are represented as contour plots in (A–F), while that of Thy1⁺ cells in the same cell preparation is depicted as a blue dot plot overlaid onto (A). (G, H) the mean $\pm$ SD of (G) forward and (H) side scatter values for 3–4 mice per group per time point are displayed for each time point. Closed circles represent those values for lung YFP^{pos} cells, while those for MLN YFP^{pos} cells are represented by open circles. Asterisks indicate those times at which a significant difference in either forward or side scatter existed between MLN and lung YFP^{pos} cells ($p < 0.05$ as determined using Student's T-test). This experiment was repeated twice, with each experiment providing similar results.

Figure 5.

IL12p40-expressing cells in the MLN and lung exhibit dynamic patterns of autofluorescence, CD11c, CD11b, and CD8α expression. *Yet40* mice were Mtb-infected as previously indicated, followed by flow cytometric analysis of (A–C) Red2 channel autofluorescence and CD11c expression patterns on lung and MLN YFP^{pos} cells. Shown in (A) is an overlay of the CD11c/autofluorescence contour plots for lung YFP^{pos} cells (blue) and MLN YFP^{pos} cells (red) at day-27 post-infection. (B) Mean autofluorescence and (C) CD11c expression were followed over time and quantitated. Prior to each timepoint, fluorescent beads were used to establish that our cytometer's lasers and channels had not drifted in intensity and sensitivity, respectively. (D, E) gating off of YFP^{pos} cells, we examined expression of the integrins CD11c and CD11b. Shown are representative contour

plots of these markers' expression among both lung (top panel) and MLN (lower panel) YFP^{POS} cells. The hatched line (at 10^3 fluorescence units) indicates the threshold below which we considered values to be due to intrinsic autofluorescence, rather than being due to CD11b staining. This threshold was required based on the autofluorescence data shown in (A) and was also used for analysis of (F) CD8 α expression among lung and MLN YFP^{POS} cells. (E, G) For both CD11b and CD8 α , we quantitated the mean fluorescence intensity (MFI) of both markers above the autofluorescence threshold. (E) CD11b and (G) CD8 α MFI values were determined for three mice per timepoint; the average MFI ($\pm$ SD) of YFP^{POS} cells from the lungs and MLN are indicated in blue and red, respectively. As indicated by asterisk, at all times examined the MFI of both CD11b and CD8 α expression differed significantly between lung and MLN YFP^{POS} cells ($p < 0.05$ as determined using Student's *T*-test).

Figure 6.

CD11c^{hi} and CD11c^{lo} subsets comprise different percentages of IL12p40-producers depending on the tissue examined. (A) Dot plots of surface CD11c expression (gated off of YFP⁺ cells) were used to classify IL12p40-producers in the lung (top panel) or MLN (bottom panel) as either CD11c^{lo} (highlighted green, CD11c levels that range between 10¹ and 10² fluorescence units) or CD11c^{hi} (highlighted pink, CD11c levels that are greater than 10²). Shown are the gates as drawn on the data from day-27 post-infection (Figure 5D); however, the same criteria were used for all other times and tissues. (B, C) the percentage of

IL12p40-producers that were either CD11c^{lo} or CD11c^{hi} in both the (B) lung and (C) MLN was then compared across three time points (days -27, -42, and -56 post-infection). An analysis of the percentage of (D—F) CD8α⁺ and CD8α⁻ cells similarly performed, with CD8α⁺ cells (highlighted blue) being defined as those whose CD8α-staining is above the background fluorescence (indicated by the hatched line). Likewise, CD8α⁻ cells (highlighted orange) were defined as those whose CD8α-staining was at or below background fluorescence values. (G—I) an analysis of CD11b-staining — performed in a manner identical to that of CD8α analysis - was also performed to quantitate the percentage of CD11b⁻ and CD11b⁺ cells among IL12p40-producers in the lung and MLN. With the exception (H) and (I), all data represented by solid squares significantly differed from data represented by open squares at each time ($p < 0.05$ as determined using Student's T-test of data from each timepoint).

Figure 7.

IL12p40-production by CD11c^{lo} lineages can compensate in the absence of CD11c^{hi} lineages. CD11c-DTR $\rightarrow$ *il12b*^{-/-} bone marrow chimeras were aerogenically infected with ~ 80 CFU of *M. tuberculosis* H37Rv. Beginning one day prior to infection, half of the mice were given i.p. DT every three days until the end of the experiment. The other half received PBS only as a control. Control C57BL/6 $\rightarrow$ *il12b*^{-/-} bone marrow chimeras were identically treated. At day 40-post infection, mice were euthanized and the (A, B) degree of CD11c depletion examined in either the presence of (A) PBS or (B) DT. (C) Lung *il12b*, *il12a* and *il23a* expression levels in C57BL/6 mice were measured at select times post-infection and normalized to that of *gapdh* in the same sample. Each data point represents the mean $\pm$ SD at that time relative to uninfected controls. This experiment (representative of two separate trials) was performed in order to gauge when to assess (D) IL12p40-concentrations in the apical lobe of both PBS- and DT-treated C57BL/6 $\rightarrow$ *il12b*^{-/-} and CD11cDTR $\rightarrow$ *il12b*^{-/-} chimeras. (E) Mtb-burdens in the same mice were assessed and compared to those

of control DT-treated $il12b^{-/-} \rightarrow il12b^{-/-}$ chimeras. As indicated by an asterisk, a statistical difference was observed between lung CFUs in CD11cDTR $\rightarrow il12b^{-/-}$ and $il12b^{-/-} \rightarrow il12b^{-/-}$ chimeras ($p < 0.05$ as determined using ANOVA of all groups); this experiment was repeated twice, with each experiment providing similar results.

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