Multiple Inflammatory Cytokines Converge To Regulate CD8⁺ T Cell Expansion and Function during Tuberculosis

Matthew G. Booty, Cláudio Nunes-Alves, Stephen M. Carpenter, Pushpa Jayaraman and Samuel M. Behar

_J Immunol_ published online 11 January 2016
http://www.jimmunol.org/content/early/2016/01/09/jimmunol.1502206

Supplementary Material
http://www.jimmunol.org/content/suppl/2016/01/09/jimmunol.1502206
6.DCSupplemental

Why _The JI_?

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

Subscription
Information about subscribing to _The Journal of Immunology_ is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The _Journal of Immunology_ is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2016 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
The Journal of Immunity

Multiple Inflammatory Cytokines Converge To Regulate CD8+ T Cell Expansion and Function during Tuberculosis

Matthew G. Booty,*† Cláudio Nunes-Alves,* Stephen M. Carpenter,* Pushpa Jayaraman,* and Samuel M. Behar*†

The differentiation of effector CD8+ T cells is a dynamically regulated process that varies during different infections and is influenced by the inflammatory milieu of the host. In this study, we define three signals regulating CD8+ T cell responses during tuberculosis by focusing on cytokines known to affect disease outcome: IL-12, type I IFN, and IL-27. Using mixed bone marrow chimeras, we compared wild-type and cytokine receptor knockout CD8+ T cells within the same mouse following aerosol infection with Mycobacterium tuberculosis. Four weeks postinfection, IL-12, type I IFN, and IL-27 were all required for efficient CD8+ T cell expansion in the lungs. We next determined if these cytokines directly promote CD8+ T cell priming or are required only for expansion in the lungs. Using retrogenic CD8+ T cells specific for the M. tuberculosis Ag TB10.4 (EsxH), we observed that IL-12 is the dominant cytokine driving both CD8+ T cell priming in the lymph node and expansion in the lungs; however, type I IFN and IL-27 have nonredundant roles supporting pulmonary CD8+ T cell expansion. Thus, IL-12 is a major signal promoting priming in the lymph node, but a multitude of inflammatory signals converge in the lung to promote continued expansion. Furthermore, these cytokines regulate the differentiation and function of CD8+ T cells during tuberculosis. These data demonstrate distinct and overlapping roles for each of the cytokines examined and underscore the complexity of CD8+ T cell regulation during tuberculosis. The Journal of Immunology, 2016, 196: 000–000.

M. tuberculosis infection elicits IL-12, type I IFN, and IL-27, all of which have profound effects on disease outcome and host resistance. IL-12 is required for host resistance in mice and humans and has an essential role in promoting CD4+ T cell responses (1, 2). In contrast, IL-27 acts as an immunoregulatory cytokine and can dampen CD4+ T cell responses. During tuberculosis, IL-27 limits the control of bacterial growth but is necessary to prevent immunopathology during chronic disease (3, 4). Type I IFN has a variety of effects during infection, and its overproduction is detrimental to host resistance (5). The increased resistance of IFNAR knockout (−/−) mice to M. tuberculosis infection underscores this fact (6–9). A similar association exists in humans, in whom type I IFN signaling is linked to active disease (10).

In other infections, all of these cytokines are key regulators of CD8+ T cells and can act as essential signals promoting CD8+ T cell expansion and effector function. In particular, IL-12 and type I IFN can provide a necessary signal for priming naïve CD8+ T cells. This signal works in conjunction with TCR stimulation (signal 1) and costimulation (signal 2), and these “signal 3” cytokines influence CD8+ T cell expansion, differentiation, effector functions, and memory formation (11, 12). In the absence of 3 cytokines, primed CD8+ T cells can proliferate but fail to develop effector functions and become tolerant to Ag stimulation (13). The relative importance of IL-12 or type 1 IFN varies between different infections and is dictated by the inflammatory response elicited by the pathogen (14, 15). Currently, the signal 3 requirements for CD8+ T cell responses during tuberculosis are uncharacterized. IL-27 can also affect CD8+ T cell function in ways similar to IL-12 and type I IFN, though it has never been formally examined as a signal 3 cytokine. In certain vaccination strategies, CD8+ T cells require IL-27 for both primary expansion and recall responses (16). During vesicular stomatitis virus infection, IL-27 influences differentiation by promoting the accumulation of terminally differentiated short-lived effector cells (SLECs) (17). IL-27 is also associated with promoting CD8+ T cell function and required for IFN-γ expression during both Toxoplasma gondii and influenza virus infection (18).

Although M. tuberculosis infection elicits CD8+ T cell responses with similar kinetics and magnitude as CD4+ T cell responses, protection mediated by CD8+ T cells has been more difficult to demonstrate in vivo and in vitro (19, 20). In this study, we consider whether inflammatory signals augment or potentially inhibit CD8+ T cell function and begin by addressing the roles of IL-12, type I IFN, and IL-27. These cytokines were selected because of their impact on disease outcome and because previous studies have focused on their effects on CD4+ T cells. Given that IL-12, type 1 IFN, and IL-27 have distinct effects on CD8+ T cells in other infections, it is imperative to understand their role in CD8+ T cell responses during tuberculosis. Specifically, we are interested in defining the signal 3 cytokine requirements for CD8+ T cells responding to infection with M. tuberculosis.
Using 1:1 mixed bone marrow chimeras (MBMCs), we demonstrate that IL-12 is essential to promote CD8\(^+\) T cell expansion and the acquisition of effector functions. Type I IFN and IL-27 also augment the expansion of effector cells in this system. These findings support a model in which each cytokine influences CD8\(^+\) T cell expansion in a nonredundant way. In additional experiments with BM chimeras, we interrogate the cytolytic ability of CD8\(^+\) T cells incapable of responding to IL-12, type I IFN, or IL-27 in vivo. Overall specific killing is reduced in the absence of IL-12; however, CD8\(^+\) T cells remain highly cytolytic without IL-12 signaling. This surprising finding indicates that cytosis is a robust effector function during tuberculosis and is likely promoted and executed through redundant mechanisms (21).

Using the adoptive transfer of retrogenic (Rg) Ag-specific CD8\(^+\) T cells, we directly examine priming following low-dose aerosol infection. These studies reveal that IL-12 is necessary to prime CD8\(^+\) T cells in the lymph node (LN) and to continue their expansion in the lungs. For this reason, IL-12 is the dominant signal 3 cytokine during tuberculosis. In total, IL-12 promotes CD8\(^+\) T cell priming, expansion, SLEC differentiation, and IFN-\(\gamma\) production, whereas type I IFN and IL-27 support expansion in the lungs. To date, this is the most comprehensive study of CD8\(^+\) T cell regulation during tuberculosis. We believe such understanding has implications for rational vaccine design and the development of immunotherapies.

Materials and Methods

**Ethics Statement**

The animal studies were approved by the Institutional Animal Care and Use Committee at the Dana-Farber Cancer Institute (DFCI) or the University of Massachusetts Medical School (UMMS; Animal Welfare Assurance no. A3023-01 [DFCI] or A3306-01 [UMMS]), using the recommendations from the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the Office of Laboratory Animal Welfare.

**Mice**

C57BL/6 (wild-type [WT]), CD45.1 (B6.SJL-Ptprc\(^{Pepc}\)/BoyJ), CD90.1 (B6.PL-\(Tly1\)\(^{1}\)/CyJ), TCR\(^{a\sim\sim}\)/– (B6.129S2-Tcra\(^{mii\sim\sim\sim\sim}\)/mJ), CD80\(^{a\sim\sim}\)/– (B6.129S2-CD80\(^{mii\sim\sim\sim\sim}\))\//J), IL-12R\(^{a\sim\sim}\)–/– (IL-12R–/–: B6.129S1-Ii12r2\(^{m\sim\sim\sim\sim\sim\sim\sim\sim\sim\sim\sim}\)/J), and IL-27Ra–/– (IL-27Ra–/–: B6N.129P2-H27ra\(^{m\sim\sim\sim\sim\sim\sim\sim\sim\sim\sim\sim}\))/mJ) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IFN-\(\gamma\) receptor-deficient mice (IFNAR\(^{a\sim\sim}\)–/–) were obtained from Dr. Raymond M. Welsh (Department of Pathology, UMMS) and previously described (10, 22, 23). Mice were 8–10 wk old at the start of all experiments. Mice infected with *M. tuberculosis* were housed in a bio-safety level 3 facility under specific pathogen-free conditions at DFCI or UMMS.

**Generation of mouse BM chimeras**

MBMCs were made by lethally irradiating CD90.1\(^+\) recipients (two doses of 600 rad separated by 3 h). BM was flushed from the femurs, tibia, and humeri of donor mice and RBC lysed. BM cells were then enumerated, and FACS analysis was performed on an FACS Canto (BD Biosciences) or on an intracellular cytokine staining

A total of 5 \times 10^7–1 \times 10^8 cells was plated in each well of a round-bottom 96-well plate and incubated in the presence of TB10.4\(_{4,1}\) peptide (10 \(\mu\)M; New England Peptide). Cells incubated in the presence of anti-CD3/CD28 (1 \(\mu\)g/ml; BioLegend) or in the absence of stimuli were used as positive and negative controls, respectively. Cells were incubated for 1 h at 37 \({}^\circ\)C, at which point GolgiPlug solution (BD Pharmingen) was added to each well for the remaining 4 h. Cells were collected after the 5-h stimulation and then surface stained with the Abs described above, followed by intracellular staining for IFN-\(\gamma\) (clone XMG1.2), TNF (clone MPX6-T22), or granzyme B (clone gb11) using the BD PermWash Kit (BD Pharmingen) as per the manufacturer’s instructions.

**In vivo cytotoxicity assay**

In vivo cytotoxicity was determined using peptide-coated splenocytes, differentially labeled with the fluorescent dyes CFSE and eFluor 450 (eBioscience) as previously described (21). All target cells were obtained from the spleens of uninfected mice. Target cells were labeled in PBS for either 20 min (eFluor 450) or 10 min (CFSE) at room temperature, followed by extensive washing. Target cell populations were pulsed with 100 nmol TB10.4\(_{4,1}\) peptide at 37 \({}^\circ\)C for 1 h in complete medium or left unpulsed. Labeled populations were mixed at an equal cell ratio and injected i.v. into age-matched uninfected and infected recipient mice (2.5 \times 10^7 each labeled population per mouse). After 20 h, recipient spleens and lungs were harvested, and single-cell suspensions were made as described. Ratios of recovered CFSE- and eFluor 450-labeled target lymphocyte...
populations were determined by flow cytometry. Percent specific killing was determined by the following formula: percent specific killing = 100 – (100 – [ratio in infected mice]/ratio in uninfected mice]), where ratio is the percent peptide-pulsed target cells divided by percent un pulsed target cells.

**Adaptive T cell transfer**

Single-cell suspensions of pools of spleens and LNs from uninfected Rg mice (6–12 wk postreconstitution) were prepared. Naïve CD8+ T cells were purified from each suspension using the CD8+ T cell isolation kit and magnetic separation (Stemcell Technologies). After purification, cells were counted and transferred via the tail vein into congenically marked recipient. WT Rg CD8+ T cells survived in infected mice and were detectable in the LNs, lungs, and spleen for at least 4 wk after transfer.

**Measurement of cell proliferation**

For analysis of cell proliferation of Rg cells after adoptive transfer, bead-purified naïve Rg cells (see above) were labeled with 5 μmol cell pro liferation dye eFlour 450 (eBioscience) in PBS for 20 min at room temperature, followed by extensive washing.

**Cell isolation and microarray analysis**

Female C57BL/6 mice were infected with M. tuberculosis Erdman as described above. At the indicated time points, mice were euthanized by cervical dislocation, and lungs were harvested after perfusion with collagenase-containing media. Lungs were allowed to digest in collagenase-containing media for 15 min before being homogenized into single-cell suspensions. At this point, the lungs from three individual mice were combined into a single sample. T cells were then purified by negative magnetic bead selection (Miltenyi Biotec). Purified cells were stained to distinguish CD4+ and CD8+ T cells (CD19, CD3, CD4, and CD8). For cell sorting, stained cells were suspended in MACS buffer (Miltenyi Biotec) and deposited in collection tubes using a BD FACs Canto flow cytometer (BD Biosciences). A total of 50,000 CD19−CD3+CD8+ cells was sorted directly into TRIzol Reagent (Life Technologies) and immediately frozen. RNA extraction, microarray hybridization (Affymetrix Mouse Gene 1.0ST array; Affymetrix), and data processing were done at the ImmGen Project processing center. Details of the data analysis and quality control can be found at (http://www.immgen.org).

**Statistical analysis**

All data are represented as mean with SEM. Comparisons of two groups within 1:1 MBMCs were done with a paired Student t test. All other comparisons were done with an unpaired Student t test and are indicated in the figure legends. Comparisons of more than two groups were done using Holm–Šidák multiple comparisons testing following two-way ANOVA. Significance was represented by the following symbols: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, †p < 0.0001.

**Results**

CD8+ T cells express the IL-12, type I IFN, and IL-27 receptors throughout M. tuberculosis infection

IL-12, IFN-β, and IL-27 are all expressed in the lungs of M. tuberculosis–infected mice, as previously reported by multiple groups (2, 3, 26, 27). To determine the extent to which the receptors for IL-12, type I IFN, and IL-27 are expressed by CD8+ T cells during M. tuberculosis infection, we performed gene expression profiling on highly purified CD8+ T cells from the lungs of uninfected or M. tuberculosis–infected mice. Briefly, CD8+ T cells were enriched from the lungs of uninfected or infected mice using immunomagnetic beads and then flow sorted to >99% pure CD3+CD8+ cells. Microarray gene expression profiling was performed, and expression values were normalized across all of the samples and time points in the experiment. In this study, we focus on the relative expression of the subunits for the IL-12R (IIfnar1 and IIfnar2), and the IL-27R (Il27ra and Il6st) (Fig. 1). IIfnar1 transcripts were marginally detectable in CD8+ T cells from the lungs of uninfected mice (time 0), and IIfnar2b was undetectable (Fig. 1). Following infection, transcription of both IL-12R subunits increased and remained detectable throughout the infection (Fig. 1). CD8+ T cells from both uninfected and infected mice constitutively expressed the IFNAR and IL-27R subunits, which persisted at relatively high levels during the course of infection (Fig. 1). Together, these findings suggest that CD8+ T cells are capable of responding to all three cytokines throughout infection.

IL-12, type I IFN, and IL-27 augment the magnitude of CD8+ T cell responses during tuberculosis

The inflammatory response and degree of susceptibility to M. tuberculosis varies substantially between the IL-12 p40, IFNAR, and IL-27R KO mice, which confounds the elucidation of how these cytokine receptors regulate T cell function in an intact host (1–4, 6–9). To avoid this pitfall as we determined the effects of IL-12, type I IFN, and IL-27 on CD8+ T cell responses during tuberculosis, we used 1:1 MBMCs in an aerosol infection model, which allows for the direct comparison of WT and cytokine receptor KO CD8+ T cells within the same host mouse. This experimental system has the key advantage of exposing both WT and KO CD8+ T cells to the same inflammatory environment and bacterial burden throughout the infection.

To generate chimeric mice, congenically marked (CD90.1+) recipients were lethally irradiated and reconstituted with equal ratios of WT (CD45.1+) BM and BM from one of the receptor KO strains (CD45.2+) (Fig. 2A). In control experiments, we generated MBMCs with a mixture of WT CD45.1+ and WT CD45.2+ BM. After reconstitution, these control mice maintained equal ratios and numbers of CD45.1+ and CD45.2+ CD8+ T cells in the lungs before and 4 wk after M. tuberculosis infection (Fig. 2B, 2C), which confirms that both groups of donor-derived cells have the potential to respond similarly to M. tuberculosis infection in this model. Our experimental conditions used donor mice lacking one of following cytokine receptors: IL-12Rβ2 (IL-12R−/−), IFN-α/β receptor 1 (IFNAR−/−), or IL-27Rα (IL-27R−/−). Following reconstitution, the resulting chimeras had equivalent ratios of WT and KO CD8+ T cells in the peripheral blood and lungs (Fig. 3A, 3B). This indicates that the absence of the individual cytokine receptors did not significantly alter T cell development and homeostasis in uninfected mice. Once baseline reconstitution was assessed, the chimeras were infected via the aerosol route and examined 4 wk later, which is the peak of the T cell response to M. tuberculosis (28).

By determining the ratio of WT and KO CD8+ T cells in the blood, we tracked the proportion of WT and KO CD8+ T cells in the same mice before and after infection with M. tuberculosis. Four weeks postinfection, IL-12R−/−, IFNAR−/−, and IL-27R−/− CD8+ T cells were underrepresented in blood relative to WT cells (Fig. 3A, 3B). This was also true in the lungs of infected mice, in which the percentage of IL-12R−/−, IFNAR−/−, and IL-27R−/− CD8+ T cells were reduced relative to their WT counterparts in the same mouse (Fig. 3C). Importantly, the dramatic increase in the number of CD8+ T cells in the lungs of M. tuberculosis–infected mice was dependent upon all three cytokine receptors (Fig. 3D). Although CD8+ T cell expansion was suboptimal in the absence of IFNAR and IL-27R, it was nearly completely abrogated in the absence of IL-12. Tracking CD8+ T cells specific for the immunodominant epitope TB10.4−11 (Rv0288; EsxH) also revealed similar reductions in the number of Ag-specific CD8+ T cells in the lungs (Fig. 3E). Though the number of Ag-specific receptor KO CD8+ T cells is reduced, the percentages of TB10.4−11–specific cells within the KO populations are comparable to the percentages within the WT populations (see below). Thus, the reduced number of Ag-specific cells reflects the global reduction in receptor KO CD8+ T cells. Overall, these data show that
all three cytokine receptors (IL-12R, IFNAR, and IL-27R) are necessary for the expansion and accumulation of CD8+ T cells in the lungs during tuberculosis. Of these cytokines, IL-12 appears to have the greatest impact, as in the absence of IL-12, CD8+ T cells underwent little expansion; however, type I IFN and IL-27 clearly have crucial roles in regulating the magnitude of CD8+ T cell responses.

**IL-12 and IL-27 influence the differentiation of effector CD8+ T cells during tuberculosis**

Following priming, CD8+ T cells can differentiate into several effector subpopulations that are distinguishable by their expression of cell-surface markers KLRG1 and IL-7 receptor (CD127) (29, 30). Recently, primed effector cells lack expression of both KLRG1 and CD127 and are known as early effector cells (EECs) (17). These cells can give rise to two effector subpopulations: short-lived effector cells (SLEC; KLRG1hiCD127lo) and memory precursor effector cells (MPEC; KLRG1loCD127hi) (31). MPECs are the population with the greatest potential to generate long-lived memory cells (29, 30, 32). A fourth subpopulation expresses both KLRG1 and CD127 (double-positive effector cell [DPEC]); however, the functional relevance of these cells remains unclear.

The inflammatory environment elicited by each pathogen influences these cell fate decisions, and IL-12, type I IFN, and IL-27 can influence CD8+ T cell differentiation during different infections (15, 17, 33). The effects of these cytokines on CD8+ T cell differentiation were determined during tuberculosis using the MBMC model. Four weeks postinfection, we measured the cell-surface expression of KLRG1 and CD127 on WT and cytokine receptor KO Ag-specific CD8+ T cells, which were identified using TB10.4a1/Kb tetramers. Following *M. tuberculosis* infection, WT CD8+ T cells primarily adopt an SLEC (∼53–60%) and EEC (∼26–32%) phenotype with fewer cells expressing CD127 (Fig. 4A, top panel). Loss of IL-12 signaling severely reduced the population of SLECs, whereas the frequency of MPECs increased, as manifested by a loss KLRG1+ cells and increased expression of CD127 (Fig. 4A, 4B). In contrast, loss of type I IFN signaling had no impact on the relative proportion of effector subpopulations (Fig. 4A, 4B). Although IL-27 signaling had no effect on SLEC differentiation, it had a small but significant inhibitory effect on CD127 expression, leading to an increased proportion of MPECs in the absence of IL-27 signaling (Fig. 4A, 4B). Thus, of these three proinflammatory cytokines, IL-12 is the main driver of CD8+ T cell terminal differentiation during tuberculosis; in its absence, not only is there a failure of CD8+ T cell expansion (Fig. 3D), but also a substantial skewing of effector subpopulations that leads to a loss of SLECs.

**IL-12, type I IFN, and IL-27 have distinct and overlapping effects on CD8+ T cell function during tuberculosis**

IL-12, type I IFN, and IL-27 can all impact the acquisition of effector functions by CD8+ T cells; however, their direct effects on CD8+ T cell function during tuberculosis have not been examined. IFN-γ is a particularly important cytokine during tuberculosis, and CD8+ T cells are a source of this protective cytokine (24, 34). We stimulated lung cells from MBMCs ex vivo with the TB10.4a1/Kb minimal peptide epitope and performed intracellular cytokine staining to measure the percentage of Ag-specific CD8+ T cells that can produce IFN-γ. As controls, lung cells were also left unstimulated.
mulated or stimulated with anti-CD3 and anti-CD28 mAbs. Significant levels of cytokine production were not observed in the unstimulated samples. Of the three cytokines, only IL-12 signaling was essential for IFN-γ production by lung CD8+ T cells after M. tuberculosis infection (Fig. 5A, 5C). Though the number of Ag-specific receptor KO CD8+ T cells is diminished in the MBMCs (Fig. 3), KO CD8+ T cells are still elicited in response to infection, and the percentages of TB10.44–11-specific cells within the KO populations is comparable to the percentages within the WT populations (Fig. 5B). Thus, the reduced percentage of IL-12Rβ2/CD8+ T cells producing IFN-γ is the result of a functional defect and is not caused by a diminished percentage of Ag-specific cells within the KO population.

In addition to cytokine production, IL-12, type I IFN, and IL-27 can alter the cytolytic activity of CD8+ T cells. To address the role of these cytokines in regulating cytolytic function, we analyzed intracellular granzyme B levels in CD8+ T cells from the lungs of the indicated MBMCs 4 wk postinfection. (D) Total number of WT (CD45.1+) or KO (CD45.2+) CD8+ T cells in the lungs of MBMCs either uninfected or 4 wk after M. tuberculosis infection. (E) The number of TB10.44–11/Kb tetramer+ (Tetramer+) CD8+ T cells that are WT or KO in the lungs of infected 1:1 chimeras. Each bar represents the mean ± SEM (n = 4 to 5 mice/group for the uninfected groups and n = 8–10 mice/group for the infected groups). Data are representative of three independent experiments for the infected groups and two independent experiments for the uninfected groups. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (paired Student t test). Uninf., uninfected.

The Journal of Immunology 5

**FIGURE 3.** IL-12, type 1 IFN, and IL-27 promote CD8+ T cell expansion following M. tuberculosis (Mtb) infection. (A) Representative flow cytometry plots of CD3+CD8+ T cells from the blood of the same MBMC before and 4 wk postinfection with M. tuberculosis. (B) The ratio of WT to KO CD3+ CD8+ T cells in the blood of the indicated MBMCs before and postinfection. (C) Representative flow cytometry plots of CD3+ CD8+ T cells from the lungs of the indicated MBMCs 4 wk postinfection. (D) Total number of WT (CD45.1+) or KO (CD45.2+) CD8+ T cells in the lungs of MBMCs either uninfected or 4 wk after M. tuberculosis infection. (E) The number of TB10.44–11/Kb tetramer+ (Tetramer+) CD8+ T cells that are WT or KO in the lungs of infected 1:1 chimeras. Each bar represents the mean ± SEM (n = 4 to 5 mice/group for the uninfected groups and n = 8–10 mice/group for the infected groups). Data are representative of three independent experiments for the infected groups and two independent experiments for the uninfected groups. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (paired Student t test). Uninf., uninfected.

With the goal of examining cytolytic function, we used a second strategy to generate BM chimeras in which all the CD8+ T cells lacked the cytokine receptors of interest (Fig. 6A). Briefly, TCRαβ-/- mice were lethally irradiated and reconstituted with a mixture of donor cells consisting of 80% CD8αβ+ BM and 20% of either WT or receptor KO BM (IL-12Rβ2/+, IFNARβ2/+, or IL-27Rβ2/−). In the resulting 4:1 chimeric mice, CD8+ T cells can only be derived from either the donor WT or cytokine receptor KO BM, depending on the experimental group (Fig. 6A). Thus, these experiments differ from the MBMCs, because the WT and KO groups are separate sets of mice. CD8+ T cells developed normally in these mice and responded robustly to aerosol infection with granzyme B (Fig. 5D). These findings suggest that all three cytokines are required for optimal cytolytic function.

Following M. tuberculosis infection, elicited CD8+ T cells remain cytolytic in the absence of IL-12, type I IFN, or IL-27 signaling.
M. tuberculosis. After 6 wk of infection, the chimeras with IL-12R<sup>−/−</sup>, IFNAR<sup>−/−</sup>, or IL-27R<sup>−/−</sup> CD8<sup>+</sup> T cells had a similar bacterial burden as control chimeras with WT CD8<sup>+</sup> T cells, and all chimeras survived. These observations were anticipated given that mice completely lacking CD8<sup>+</sup> T cells only exhibit increased susceptibility to tuberculosis at very late time points (35).

An in vivo cytotoxicity assay was performed with the 4:1 chimeras by assessing the specific killing of fluorescently labeled target splenocytes loaded with TB10.4<sub>4-11</sub> as previously published (21). Diminished specific killing of targets was observed only in 4:1 chimeras with IL-12R<sup>−/−</sup> CD8<sup>+</sup> T cells; otherwise, efficient killing of target cells was detected in the both the lungs and spleens of infected mice (Fig. 6B, 6C). Because IL-12R<sup>−/−</sup> CD8<sup>+</sup> T cells have the most profound defect in the expansion of Ag-specific cells (Fig. 6D), we wished to determine whether the reduced specific killing resulted from fewer Ag-specific CD8<sup>+</sup> T cells or actually a dysfunction of IL-12R<sup>−/−</sup> CTL. By plotting the absolute number of TB10.4<sub>4-11</sub>-specific CD8<sup>+</sup> T cells in the spleen versus specific killing, it was apparent that cell number had a great impact on target cell lysis (Fig. 6E). This correlation was particularly robust in the spleens in which a higher number of target cells were recovered, but similar results were observed in the lungs (data not shown). These findings indicate that CTL are generated even in the absence of IL-12, type I IFN, or IL-27 signaling, and, despite an overall reduction in granzyme B levels, the cells function as effective CTL. Multiple molecules and several different pathways contribute to cytolytic activity (19, 21); thus, it is likely that, individually, these cytokines do not have a substantial impact on cytolytic function.

CD8<sup>+</sup> T cell priming in the lung draining LN requires IL-12, whereas CD8<sup>+</sup> T cell expansion in the lung depends on IL-12, type I IFN, and IL-27

IL-12 and type I IFN, or their combination, provide a necessary third signal for priming CD8<sup>+</sup> T cells postinfection with different pathogens, but their role as "signal 3" cytokines during tuberculosis is unknown. Although IL-27 is not usually considered a signal 3 cytokine, it has several of the same attributes as IL-12 and type I IFN. Because IL-12, type I IFN, and IL-27 all influence the magnitude of the CD8<sup>+</sup> T cell response during tuberculosis, we determined whether these effects are mediated during T cell priming in the LN or only during T cell expansion in the lungs.

To address this question, we used an Rg mouse model in which a high percentage of CD8<sup>+</sup> T cells express a TCR specific for the immunodominant Ag TB10.4<sub>4-11</sub> (TB10Rg) (24). A key advantage of TB10Rg mice is that naive Ag-specific CD8<sup>+</sup> T cells can be generated on nearly any genetic background, thus providing a source of naive TB10.4<sub>4-11</sub>-specific IL-12R<sup>−/−</sup>, IFNAR<sup>−/−</sup>, and IL-27R<sup>−/−</sup> CD8<sup>+</sup> T cells. After adoptive transfer of naive TB10Rg CD8<sup>+</sup> T cells, priming is detected in the draining LN ~11 d following aerosol infection (24). This delay in T cell priming results from the delayed transfer of M. tuberculosis and its Ags from the lung to the draining LN (36–38). To determine the role of IL-12R, IFNAR, and IL-27R signaling in priming, equal numbers of congenically marked WT and receptor KO TB10Rg CD8<sup>+</sup> T cells were transferred into recipient mice 7 d after low-dose aerosol M. tuberculosis infection, and the ratio of WT and KO TB10Rg CD8<sup>+</sup> T cells was measured on days 8, 11, 13, and 15 postinfection.

On day 8, the ratio of WT and receptor KO Rg CD8<sup>+</sup> T cells was unaltered from the ratio of cells injected at day 7 (Fig. 7A). By day 11 in the mediastinal LN, IL-12R<sup>−/−</sup> TB10Rg CD8<sup>+</sup> T cells were underrepresented relative to WT cells and continued to lag behind through days 13 and 15 (Fig. 7A). Throughout the experiment, IFNAR<sup>−/−</sup> and IL-27R<sup>−/−</sup> TB10Rg CD8<sup>+</sup> T cells maintained a consistent ratio with WT cells in the LN, indicating that type I IFN and IL-27 are dispensable for CD8<sup>+</sup> T cell priming following M. tuberculosis infection (Fig. 7A). Surprisingly, IL-12R<sup>−/−</sup> TB10Rg CD8<sup>+</sup> T cells still expanded significantly in the LN, though they did lag behind WT cells. The number of IL-12R<sup>−/−</sup> TB10Rg

![Figure 4](http://www.jimmunol.org/)
CD8+ T cells continued to increase in the LN throughout the time course of this experiment (Supplemental Fig. 1). Furthermore, IL-12Rβ2/- and IL-27RαβTB10Rg CD8+ T cells diluted their proliferation dye by day 11 posttransfer, though not as efficiently as WT cells (Fig. 7B). These data indicate that signals other than IL-12 in the LN contribute to CD8+ T cell priming and suggest that additional signal 3 cytokines may exist. Additionally, type I IFN or IL-27 may support priming in the absence of IL-12. IFNARβ2/- and IL-27RαβTB10Rg CD8+ T cells continued to expand over the course of the experiment; however, they underperformed relative to WT cells (Fig. 7C, 7D). This suggests that IL-12 is necessary for the efficient priming of CD8+ T cells in the LN as well as their continued expansion in the lungs. Though type I IFN and IL-27 are dispensable for CD8+ T cell priming, their influence on CD8+ T cell expansion in the lungs could be observed 13 d postinfection, as the percentage of

FIGURE 5. Signal 3 cytokines affect IFN-γ production and granzyme B expression by CD8+ T cells during tuberculosis. (A) Representative flow cytometry plots of lung CD8+ T cells of the indicated genotype 4 wk postinfection. Single-cell lung preparations were stimulated ex vivo with TB10.44–11 peptide prior to IFN-γ analysis. (B) The percentage of TB10.44–11-specific CD8+ T cells within the WT (CD45.1+) or KO (CD45.2+) populations based on positive staining with TB10.44–11/Kb tetramers (Tetramer*). Though the overall number of Ag-specific KO CD8+ T cells is reduced in each group of MBMCs (Fig. 3), the percentage of Tetramer* cells remains comparable within the WT and KO populations. (C) The percentage of WT and KO CD8+ T cells positive for intracellular IFN-γ after ex vivo stimulation with TB10.44–11 peptide. (D) The percentage of WT and KO CD8+ T cells positive for intracellular granzyme B staining. Each bar represents the mean ± SEM (n = 9 to 10 mice/group). Data are representative of three independent experiments. **p < 0.01, ****p < 0.0001 (paired Student t test).

CD8+ T cells continued to increase in the LN throughout the time course of this experiment (Supplemental Fig. 1). Furthermore, IL-12Rβ2/- TB10Rg CD8+ T cells in the LN substantially diluted their proliferation dye by day 11 posttransfer, though not as efficiently as WT cells (Fig. 7B). These data indicate that signals other than IL-12 in the LN contribute to CD8+ T cell priming and suggest that additional signal 3 cytokines may exist. Additionally, type I IFN or IL-27 may support priming in the absence of IL-12. IFNARβ2/- and IL-27RαβTB10Rg CD8+ T cells diluted their proliferation dye equivalent to WT cells by day 11, reinforcing that these signals are not needed to prime cells in the LN when IL-12 is present (Fig. 7B).

In the same priming experiments, the expansion of the transferred cells was monitored in the lungs of recipient mice. Similar to the observations in the LN, IL-12Rβ2/- TB10Rg CD8+ T cells continued to expand over the course of the experiment; however, they underperformed relative to WT cells (Fig. 7C, 7D). This suggests that IL-12 is necessary for the efficient priming of CD8+ T cells in the LN as well as their continued expansion in the lungs. Though type I IFN and IL-27 are dispensable for CD8+ T cell priming, their influence on CD8+ T cell expansion in the lungs could be observed 13 d postinfection, as the percentage of
**FIGURE 7.** CD8+ T cell priming in the lung draining LN requires IL-12, whereas CD8+ T cell expansion in the lung depends on IL-12, type I IFN, and IL-27. Equal numbers of Rg TB10.4–11-specific (TB10Rg) CD8+ T cells were transferred into mice 7 d after low-dose aerosol infection with *M. tuberculosis*. (A) The percentage of total Rg cells that were WT or KO for the indicated cytokine receptor in the mediastinal LN on days 8, 11, 13, and 15 following infection. (B) Histograms depicting the dilution of the proliferation dye eFluor 450 in TB10Rg cells in the LN at day 11. Each group of samples (WT or KO) was concatenated into a single histogram. (C) The percentage of total Rg cells that were WT or KO for the indicated cytokine receptor in the lungs at days 8, 11, 13, and 15 following infection. (D) Total number of WT and KO Rg cells detected in the lungs at the indicated time points. Each bar or point represents the mean ± SEM (*n = 4 to 5 mice/group). Data are representative of two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001 (Holm-Sidak multiple comparisons testing following two-way ANOVA).

IFIAR−/− or IL-27R−/− TB10Rg CD8+ T cells decreased relative to their WT counterparts on days 13 and 15 (Fig. 7C). These data indicate that, whereas IL-12 promotes CD8+ T cell expansion during priming in the LN, type I IFN and IL-27 primarily act after priming has occurred to expand CD8+ T cells in the lungs during *M. tuberculosis* infection.

**Discussion**

Numerous T cell subsets participate in the immune response following *M. tuberculosis* infection. T cell responses have different kinetics and magnitudes; they differ in respect to their function and where in the lung they localize. These differences complicate efforts to understand the relative importance of each T cell subset in mediating protection against tuberculosis. In this respect, the role of CD8+ T cells has been enigmatic. CD8+ T cells were first recognized to be essential for host resistance in the murine model, but are now recognized to be crucial for protection in nonhuman primates as well (19, 39–41). Mapping of the *M. tuberculosis* epitopes recognized by murine and human CD8+ T cells from infected individuals has characterized many of the Ags that elicit CD8+ T cells during infection and demonstrated dramatic clonal expansions that appear to be driven by TCR affinity (24). CD8+ T cells express potent effector functions, and there is potential for vaccine-elicited CD8+ T cells to mediate protection against tuberculosis. However, CD8+ T cells appear to be less effective than CD4+ T cells in mediating resistance, despite similar kinetics (28, 35, 42). There are numerous potential signals that modify T cell responses in a diseased lung—soluble mediators and cell-surface signals can have both positive and negative effects on T cell expansion and function (43). Although it is commonly accepted that optimal CD8+ T cell responses require a third signal, usually mediated by an inflammatory cytokine, how CD8+ T cells are regulated during chronic inflammation is less well understood (13). Infection with *M. tuberculosis* elicits a complex innate inflammatory response that shapes T cell immunity. We considered the possibility that IL-12, IL-27, and type I IFN, three cytokines produced by infected cells and known to influence host resistance, may have differential effects on CD8+ T cell function. In particular, we sought to determine if any of these cytokines directly limits CD8+ T cell responses during tuberculosis.

Using MBMCs and the adoptive transfer of naive TB10.4–11-specific CD8+ T cells, we show that IL-12 is a major positive regulator of the CD8+ T cell response during tuberculosis. Following aerosol infection, IL-12 is essential for efficient CD8+ T cell priming in the LN and subsequent expansion in the lung. IL-12 also promotes the terminal differentiation of SLECs and enhances IFN-γ production. Based on these results, we conclude that IL-12 is the dominant signal 3 cytokine during tuberculosis.

IL-12 does not act alone in the infected host. Instead of seeing inhibition, we demonstrate a direct positive supporting role for both type I IFN and IL-27 in CD8+ T cell expansion. These effects are not observed during the priming of naive CD8+ T cells in the LN and only become evident once activated CD8+ T cells are recruited to the infected lung. Because they are dispensable for priming, we argue that type I IFN and IL-27 are not acting as signal 3 cytokines. Nevertheless, type I IFN and IL-27 each have a nonredundant role augmenting the magnitude of pulmonary CD8+ T cell responses. This is most evident in the MBMCs, in which WT and KO cells are in direct competition within the same inflammatory environment. This complex involvement of multiple inflammatory cytokines is similar to other infections, in which IL-12 and type I IFN both support CD8+ T cell expansion (15). In this way, CD8+ T cells
reflect the inflammatory environment of the host, responding in different degrees to each cytokine induced by the pathogen.

Type I IFN negatively affects host immunity to *M. tuberculosis* through a number of mechanisms, but few studies have directly addressed the impact of type I IFN on T cell function during tuberculosis. Dorhöi et al. (9) examined T cell responses in *M. tuberculosis*-infected IFNAR−/− mice on the 129S2 background and noted no alteration in IFN-γ production by CD4+ or CD8+ T cells. In our MBMCs, type I IFN supports CD8+ T cell expansion while having no major effect on the expansion of CD4+ T cells (Fig. 3 and data not shown). We conclude that type I IFN does not directly inhibit T cell function, but rather promotes the expansion of CD8+ T cells in the lungs. Beyond this effect, we did not observe type I IFN to affect CD8+ T cell differentiation or function.

Recently, Torrado et al. (44) examined the expansion of TB10.44–11-specific CD8+ T cells in the lungs of intact IL-27−/− mice and did not observe a defect in expansion compared with WT controls. In our experiments, the effect of IL-27 on CD8+ T cell expansion was far less dramatic than that of IL-12 and only observable when IL-27R−/− CD8+ T cells were in direct competition with WT cells in the lungs of the same mouse. This was evident in both the MBMC model (Fig. 3) and the cotransfer of WT and IL-27R−/− TB10Rg cells (Fig. 7). We believe these experimental systems are more sensitive at detecting subtle defects in T cell expansion and bypass any confounding differences in the inflammatory response of WT and KO mice.

We were surprised to discover IL-27 did not affect basic CD8+ T cell effector functions. IL-27 is a critical promoter of IFN-γ production by CD8+ T cells in other infections (18), but we did not observe this effect in our model of tuberculosis. It is possible that the dominant role of IL-12 during tuberculosis masks any potential effects of IL-27. However, this hypothesis seems unlikely, because during *T. gondii* infection, IL-12 promotes the expansion of effector CD8+ T cells, whereas IL-27 is necessary for IFN-γ production (18, 45–47). Thus, IL-27 can be observed to drive IFN-γ production even in the presence of high IL-12 levels in other infections. As IL-27 is clearly dispensable for IFN-γ production by CD8+ T cells during tuberculosis, the effects of IL-27 on CD8+ T cell effector function are likely pathogen specific. The loss of IL-27 signaling does not influence CD8+ T cell SLEC differentiation. Instead, IL-27−/− mice were more likely to adopt a DPEC and MPEC phenotype, from which we infer that IL-27 signaling limits CD127 expression. Although it is tempting to speculate that IL-27 limits CD8+ T cell memory formation during tuberculosis, this possibility cannot be addressed with our current data.

IL-12, type I IFN, and IL-27 all promote granocyte B production following *M. tuberculosis* infection, but no substantial loss in in vivo cytolytic activity is observed. Mice with only IL-12−/− or CD8+ T cells show reduced specific killing; however, our data indicate this results from reduced CD8+ T cell numbers, not from a reduction in cytolytic function on a per-cell basis. In fact, IL-12−/− CD8+ T cells efficiently lysed targets in vivo. The cytolytic activity of IL-12−/− CD8+ T cells highlights the redundancy of cytokines that regulate cytolytic effector pathways. With multiple cytokines promoting cytolytic activity, the loss of a single cytokine fails to perturb specific killing. These data also suggest that granocyte B levels may not be the best proxy for CTL, because a moderate decrease in granocyte B levels (Fig. 5C) did not correlate with reduced in vivo killing (Fig. 6). Multiple molecules and mechanisms enable cytolyis during tuberculosis including granule exocytosis, Fas/FasL, and TNF (19, 21). During tuberculosis, in vivo cytolytic activity is only marginally affected by the absence of perforin; however, perforin is crucial for host immunity during tuberculosis (21). This indicates that although multiple molecular pathways may be similarly potent at lysing target cells, there may exist differences with respect to their ability to kill pathogens. We were unable to assess perforin expression in these experiments, as a reliable method for intracellular perforin staining in murine cells has been elusive until recently (48). Nonetheless, CD8+ T cells retain a high degree of cytolytic activity even in the absence of IL-12, indicating that cytolyis is a robust effector mechanism found even in dysfunctional cells, such as IL-12−/− CD8+ T cells.

Using IL-12R, IFNAR, and IL-27R G mice as a source for naive Ag-specific CD8+ T cells, we were able to determine how these cytokines affect T cell priming. In contrast to many infections, T cell priming following *M. tuberculosis* infection is delayed until approximately days 11–13 following aerosol infection. Furthermore, T cell priming appears to occur only after bacterial dissemination to the draining LN, which occurs approximately days 9–11 postinfection (24, 36, 38). Thus, there is a window of ~48 h for the bacteria to stimulate the production of soluble mediators including cytokines that can influence T cell priming. We tracked the fate of naive TB10Rg CD8+ T cells to determine how IL-12, type I IFN, and IL-27 affect T cell priming during *M. tuberculosis* infection. IL-12 was critical for supporting CD8+ T cell priming; however, naive IL-12R−/−/− CD8+ T cells were primed and expanded in all of our experiments. Thus, other signals support CD8+ T cell priming in the absence of IL-12. During tuberculosis, IL-23 can compensate for the absence of IL-12 to promote CD4+ T cell responses (2, 49). As we used IL-12Rβ2−/− cells in all of our experiments, IL-23 signaling remained intact. Although it is possible that IL-23 supports CD8+ T cell expansion, in vitro experiments have failed to associate IL-23 with signal 3 activity, making it an unlikely candidate (50). During vaccinia virus infection, neither IL-12 nor type I IFN are required to generate CD8+ T cell responses, raising the possibility that additional sources of signal 3 exist (15).

Our data illuminate a portion of the cytokine network regulating CD8+ T cells and illustrate the complex ways in which inflammation shapes adaptive immunity. CD8+ T cells are similar to CD4+ T cells in their requirement for IL-12, but have the opposite addition to CD4+ T cell help, the subtleties of signal 3 cytokines to optimally elicit a protective T cell response by vaccination. In this way, IL-27 can possibly achieve a balance of IFN-γ–producing cells in the lungs. These issues are crucial not only for understanding how CD8+ T cells function during infection but also how to optimally elicit a protective T cell response by vaccination. In addition to CD4+ T cell help, the subtleties of signal 3 cytokines on durable T cell memory will be important to understand if we hope to design better vaccination strategies in the future.

Acknowledgments

We thank G. Cottle, K. Stebienko, and B. Stowell for expert technical assistance with the mouse experiments. We also thank S. Urban for thoughtful input on the project and R. Welsh for generously providing the IFNAR−/− mice.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Though they lag behind WT cells, IL-12R−/− CD8+ T cells are still primed and can expand in the LN. Total number of WT and receptor KO TB10Rg CD8+ T cells detected in the lymph node at days 8, 11, 13, and 15 after aerosol infection.