IL-27 Signaling Compromises Control of Bacterial Growth in Mycobacteria-Infected Mice[1]

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Resistance to tuberculosis (TB) is dependent on the induction of Ag-specific CD4 Th1 T cells capable of expressing IFN-γ. Generation of these T cells is dependent upon IL-12p70, yet other cytokines have also been implicated in this process. One such cytokine, IL-27, augments differentiation of naive T cells toward an IFN-γ-producing phenotype by up-regulating the transcription factor T-bet and promoting expression of the IL-12Rβ2 chain allowing T cells to respond to IL-12p70. We show that the components of IL-27 are induced during TB and that the absence of IL-27 signaling results in an altered disease profile. In the absence of the IL-27R, there is reduced bacterial burden and an increased lymphocytic character to the TB granuloma. Although the number of Ag-specific CD4 IFN-γ-producing cells is unaffected by the absence of the IL-27R, there is a significant decrease in the level of mRNA for *IFN-γ* and *T-bet* within the lungs of infected IL-27R$^{-/-}$ mice. Ag-specific CD4 T cells in the lungs of *IL-27R*$^{-/-}$ also produce less IFN-γ protein per cell. The data show that expression of IL-27 during TB is detrimental to the control of bacteria and that although it does not affect the number of cells capable of producing IFN-γ it does reduce the ability of CD4 T cells to produce large amounts of IFN-γ. Because IFN-γ is detrimental to the survival of effector T cells, we hypothesize that the reduced IFN-γ within the IL-27R$^{-/-}$ lung is responsible for the increased accumulation of lymphocytes within the mycobacterial granuloma. *The Journal of Immunology,* **2004, 173: 7490–7496.**

Development of an appropriate T cell-mediated response to *Mycobacterium tuberculosis* infection is crucial to the containment of this pathogen, as is highlighted by the increased susceptibility of HIV-infected individuals to disease (1). The phenotype of this protective T cell response is predominantly Th1 in nature, with resistance to disease being dependent upon the production of IFN-γ and the subsequent activation of the primary host cell, the macrophage (reviewed in Ref. 2). Despite this apparent dependence, production of an Ag-specific IFN-γ response is not an absolute correlate of protection, with increased IFN-γ failing to correlate with increased protection (3–5). It has been shown that IFN-γ plays a regulatory role over the T cell response during a variety of mycobacterial infections (6–10) suggesting that while IFN-γ is protective it may also serve to limit cellular responses to these chronic pathogens.

With this issue in mind, we have endeavored to identify the factors controlling induction and regulation of IFN-γ-producing CD4 Th1 cells during tuberculosis (TB).[3] We have previously demonstrated that although the IL-12p40 subunit is critical for the induction and accumulation of Th1 cells within the TB-infected lung, the IL-12p35 subunit is dispensable (11); this disparity suggests that IL-12p70-independent induction of Th1 responses can occur during *M. tuberculosis* infection. One recently identified member of the IL-6/IL-12 family of cytokines, IL-27, has been implicated in the induction of Th1 responses to bacillus Calmette-Guérin (BCG) infection and thus may modulate Th1 responses during *M. tuberculosis* infection. IL-27 is a heterodimer of a p28 and an EBI3 molecule (12), which binds to a heteromeric signaling receptor consisting of gp130 and the T cell cytokine receptor (TCCR or WSX-1) (13). In the absence of the TCCR/WSX-1 molecule, decreased resistance to the intracellular pathogens, *Listeria monocytogenes* (14) and *Leishmania major* (15), has been correlated with delayed induction of Th1 responses. In addition however, data from WSX-1-deficient mice infected with *Toxoplasma gondii* (16) and *Trypanosoma cruzi* (17) show that absence of IL-27 signaling results in augmented cellular responses, thus indicating a regulatory role for this cytokine.

In its capacity as a Th1-promoting agent, IL-27 is released by APCs and induces proliferation and polarization of naive T cells undergoing signaling via the TCR (12). Specifically, IL-27 is thought to augment the polarization of Th1 T cells by inducing Th1 transcription factor T-bet via TCCR/WSX-1 receptor-mediated activation of STAT-1 and by inducing the expression of IL-12Rβ2 and thus responsiveness of the T cells to IL-12p70 signaling (18, 19). Indeed, in the absence of TCCR/WSX-1, in vitro-cultured cells exhibit reduced polarization which correlates with reduced IFN-γ production (14, 15, 18, 19). Although the TCCR/WSX-1 molecule is expressed preferentially on naive T cells, it is also expressed on differentiated human Th1 and Th2 cells and on monocytes (13); the ability of IL-27 to regulate cellular responses may therefore lie in an ability to modulate these cell types directly (reviewed in Ref. 20).

The absolute requirement for Th1 cells in the control of TB, combined with the chronicity of this disease presents a unique challenge for the immune response. The acknowledged function of IL-27 as both a promoter and regulator of Th1 responses suggested

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[3] Abbreviations used in this paper: TB, tuberculosis; TCCR, T cell cytokine receptor; BCG, bacillus Calmette-Guérin; iNOS, inducible NO synthase.

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to us that it might have a unique role in modulating Th1 cell development and disease progression during TB. We demonstrate here that in the absence of the IL-27R there is a reduced bacterial burden in all affected organs and an enhanced early proliferation and accumulation of CD4 T cells in the lung, thus supporting a regulatory role for IL-27 in TB. In addition however, we also show that although the absence of IL-27 does not alter the number of Ag-specific IFN-γ-producing CD4 T cells it does reduce the ability of these cells to produce IFN-γ on a per cell basis, which correlates with reduced levels of mRNA for IFN-γ in the lung. Thus, while IL-27 appears to be required for optimal polarization of Ag-specific Th1 cells during TB, its function as a regulating agent serves to limit the protective response to this infection.

Materials and Methods

Mice

Mice lacking a component of the IL-27R ($TCCR^{-/-}$) were generated as described previously (14), backcrossed onto the C57BL/6 background at Genentech (South San Francisco, CA), and bred from N12 founders at the Trudeau Institute animal facility (Saranac Lake, NY). C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used as controls throughout. Both male and female mice between the ages of 6–12 wk were used; experimental mice were age and sex matched.

Experimental infection

The H37Rv strain of *M. tuberculosis* was a kind gift from Dr. R. North (Trudeau Institute) and 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 ~75 bacteria, subject animals were placed in a Glas-Col (Terre Haute, IN) airborne infection system as described in detail elsewhere (21). Animals were monitored daily and neither the C57BL/6 nor $TCCR^{-/-}$ mice exhibited overt signs of disease during the period of infection.

Bacterial load determination

As previously described (21), infected mice were killed by CO_2 asphyxiation and target organs were aseptically excised. Each of these organs was individually homogenized in physiological saline and serial dilutions of the organ homogenate were plated on nutrient 7H11 agar. Bacterial colony formation was counted after 3 wk of incubation at 37°C.

Cell preparation and culture

A single-cell suspension was prepared from the spleen or draining lymph node by passing the organ through a 70-μm nylon cell strainer and lysing the RBC with Gey's solution (21). Lung cell suspensions were prepared by perfusing cold saline containing heparin through the heart until the lungs appeared white, whereupon they were removed and sectioned in ice-cold medium using sterile razor blades. Dissected lung tissue was then incubated in collagenase IX (0.7 mg/ml; Sigma-Aldrich, St. Louis, MO) and DNase (30 μg/ml; Sigma-Aldrich) at 37°C for 30 min (21). Digested lung tissue was gently disrupted by passage through a 70-μm nylon cell strainer; the resultant single-cell suspension was treated with Gey's solution to remove any residual RBC, washed twice, and counted. Cells prepared in this way were used for ELISPOT and flow cytometric analyses. For some experiments, single-cell suspensions were enriched for CD4 T cells by magnetic column (Miltenyi Biotec, Auburn, CA)-positive selection following the manufacturer's directions and as previously described (22).

ELISPOT and ELISA

For ELISPOT analysis, cells were resuspended to 5×10^6/ml (or 1×10^6/ml for CD4 T cells) in complete medium and serially diluted in Multiscreen HA 96-well plates coated with anti-IFN-γ Ab (clone R46A2; eBioscience, San Diego, CA). One million irradiated syngeneic splenocytes were added to each well along with 10 U/ml IL-2 and either 25 μg/ml culture filtrate protein from *M. tuberculosis* H37Rv (obtained from J. Belisle under National Institutes of Health Contract AI-75320) or 10 μg/ml 20-mer peptide of the relatively immunodominant (4% of CD4 T cells in infected lungs) Ag of *M. tuberculosis*, ESAT-6 (23, 24). Cells were cultured for 24 h at 37°C in 5% CO_2 whereupon they were washed from the wells and biotinylated anti-IFN-γ Ab (clone XMG 1.2) was added to the cleared wells. The presence of bound anti-IFN-γ was detected using substrate and the number of spots per well was determined using a dissecting microscope; wells containing 10–100 spots were counted. The frequency of responding cells was calculated and applied to the number of cells per sample to generate the total number of responding cells per organ. Neither samples cultured in the absence of Ag nor samples from uninfected mice produced detectable spots. The ability of cells to respond in the ELISPOT was confirmed by stimulation with Con A (5 μg/ml; Sigma-Aldrich) (data not shown).

For ELISA, CD4 T cells suspensions underwent stimulation in identical culture conditions as used for the ELISPOT, except that cells were cultured with the $ESAT\text{-}6_{1-20}$ peptide in flat-bottom, 96-well tissue culture plates for 4 days. Supernatant was then harvested and immediately analyzed by ELISA. The ELISA used the same Ab pair as the ELISPOT. The amount of IFN-γ produced for any one population of cells was accurately determined by performing both serial dilutions of the cell suspension before culture and analyzing serial dilutions of the harvested supernatant.

Isolation of mouse bone marrow-derived macrophages

Bone marrow-derived macrophages were cultured as described previously (25, 26), except that rGM-CSF was used as the differentiating agent. Six-well tissue culture plates were initially seeded with 4×10^6 cells/well and incubated at 37°C in a 5% CO_2 humidified air chamber for 24 h. The following day, the macrophages were supplemented with an additional 4 ml/well of complete DMEM. The cultures were incubated for a total of 7–10 days at 37°C in a 5% CO_2 humid air chamber with a change of fresh complete DMEM every 2 days. Twenty-four hours before infection, the macrophages were washed twice with PBS and then replenished with incomplete DMEM (complete DMEM, lacking GM-CSF and antibiotics). Macrophage cultures were infected at an multiplicity of infection of 10 using *M. tuberculosis* H37Rv and the RNA was harvested at specific time points thereafter.

Flow cytometry

Cells were cultured with anti-CD3 and anti-CD28 in the presence of monensin to inhibit protein secretion, as previously described (11). After 4 h, cells were stained with labeled Abs specific for CD4 (allophyocyanin clone GK1.5), CD3 (PerCP)-labeled clone 145-2C11), and CD44 (FITC-labeled clone IM7). Cells were then fixed and permeabilized and the presence of intracellular IFN-γ was determined using PE-labeled clone XMG 1.2 (11). Isotype Abs were also used in parallel samples to confirm specificity of binding. Cells were analyzed using CellQuest on a BD Biosciences FACSCalibur dual-laser flow cytometer with excitation at 488 nm and 633 nm. Lymphocytes were gated based on their forward and side scatter characteristics and then on CD4 and CD3 and the number of such lymphocytes per organ was determined. CD4-positive lymphocytes were then analyzed for their expression of IFN-γ and this frequency was applied to the total number of CD4 T cells per organ to determine the number of IFN-γ-producing cells per organ.

Delivery of BrdU and measurement of BrdU-positive cells ex vivo

Infected mice were given an i.p. injection of 0.8 μg of the nucleotide BrdU 18–24 h before harvest. Cells proliferating within the mouse take up the labeled nucleotide. Surface-stained single-cell suspensions generated as above were fixed and both the cell and nuclear membranes were permeabilized. The presence of BrdU within the nucleic acid of recently proliferated cells was then determined by fluorescently labeled Ab and flow cytometric analysis.

Real-time PCR analysis

Lung RNA was extracted from infected tissue by homogenizing the tissue in TRIZol (Invitrogen, Life Technologies, Carlsbad, CA). Macrophage RNA was extracted from infected monolayers by dissolving the monolayers in TRIzol. Total RNA was extracted from TRIzol according to the manufacturer's protocol. RNA samples ($n = 4$) from each group and each time point were reverse transcribed using TaqMan reagents (Applied Biosystems, Foster City, CA). cDNA was then amplified using TaqMan reagents on the ABI Prism 7700 sequence detection system (Applied Biosystems). Samples were also treated with DNase or run in the absence of the reverse transcription enzyme to confirm that signal was derived from RNA. Fold increase in signal over that derived from uninfected tissue or cells was determined using the ΔΔct calculation recommended by the ABI Prism 7700 manufacturer. The endogenous control used to normalize the samples was the *GAPDH* gene, the expression of which does not modulate in response to infection (data not shown). The primer and probe sequences for murine *p28* and *EBI3* (12), inducible NO synthase (*iNOS*), *TNF-α*, *IFN-γ*, and *IL-12p40* (27) were as published. The primer and probes for

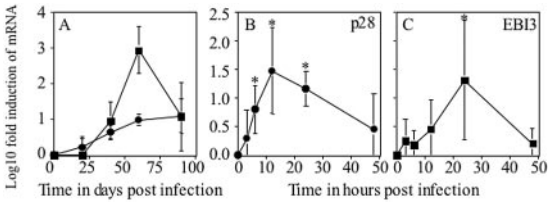

FIGURE 1. The induction of mRNA for the p28 (●) and EB13 (■) subunits of the IL-27 cytokine was measured in both the lungs of TB-infected C57BL/6 mice (*A*) and in vitro-cultured bone marrow-derived macrophages exposed to live *M. tuberculosis* (H37Rv) bacteria (*B* and *C*). *A*, The data points represent the mean fold increase in the specific mRNA compared with uninfected mice housed alongside the infected mice $n = 3–4$ for each measurement. *B* and *C*, The data points represent the mean fold increase in mRNA in macrophages exposed to bacteria compared with uninfected macrophages, $n = 2–3$ for each measurement. ∗, $p \leq 0.01$.

LRG-47 were obtained from Applied Biosystems. The primer and probe sequences for T-bet were designed and validated by the Molecular Biology Core Facility at the Trudeau Institute and are as follows: forward primer, CCTGTTGTGGTCCAAGTTCAAC; reverse primer, CACAAACATC CTGT-AATGGCTTGT, and probe, ATCATCACTAAGCAAGGACG GCGAATGTTCC. The primer and probe sequences for the *TCCR* were designed and validated at Genentech and are as follows: forward primer, TGGTCTCTCCTGGCAACAGC; reverse primer, AGCCAAGCACAC CAG-AGACA; and probe, CAGCTGGGTGCCTCCCACCA. All of the primer sets used were determined to be >95% efficient by the Trudeau Institute Molecular Biology Core Facility; efficiency was determined using naturally occurring mRNA derived from stimulated murine tissues.

Histology

The lower right lobe of each lung was inflated with 10% neutral-buffered saline and processed routinely for light microscopy. Sections were then stained with H&E. Slides were examined without knowledge of experimental group and subjectively graded for both quantity and quality of cellular accumulation as described previously (28).

Statistical analysis

Differences between the means of experimental groups were analyzed using two-tailed paired or unpaired *t* tests as appropriate. Differences were considered significant where $p \leq 0.05$.

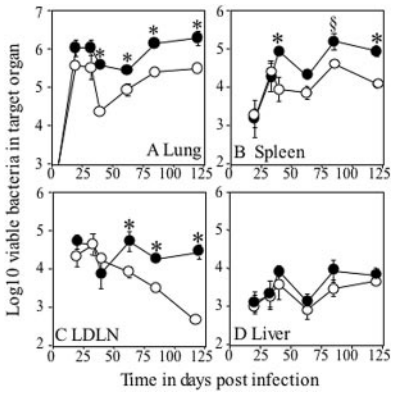

FIGURE 2. C57BL/6 (●) and IL-27R gene-deficient ($TCCR^{-/-}$) (○) mice were infected via the aerosol route with ~75 *M. tuberculosis* H37Rv bacteria and the bacterial burden determined in several organs over time. The data points represent the mean bacterial burden of four animals in all tissues examined. The graph shows one experiment representative of four total. ∗, $p \leq 0.05$ whereas § indicates $p < 0.055$. LDLN, Lung draining lymph node/mediastinal node.

Results

Aerosol infection of mice with M. tuberculosis *results in induction of both components of the heterodimeric cytokine IL-27*

To determine whether IL-27 was induced during infection, we performed real-time PCR analysis of lung mRNA derived from aerosol-infected C57BL/6 mice. We were able to detect increased mRNA levels for the class I cytokine receptor molecule EBI3 and the long-chain four-helix bundle cytokine p28, which comprise the IL-27 molecule, within the lungs of *M. tuberculosis*-infected mice (Fig. 1*A*). We also demonstrate here that in vitro-cultured murine macrophages are induced to make p28 (Fig. 1*B*) and EBI3 (Fig. 1*C*) mRNA following exposure to *M. tuberculosis*. In contrast, these same macrophage mRNA samples were examined for induction of mRNA for TCCR/WSX-1 and no significant induction was detected ($n = 3$; data not shown).

Absence of IL-27R-mediated signaling results in reduced bacterial burden in all infected organs

To determine whether IL-27 is able to moderate tuberculosis progression, we infected mice lacking the T cell cytokine receptor ($TCCR^{-/-}$) (14) recently demonstrated to bind to the IL-27 cytokine (15). We found that mice lacking the IL-27R ($TCCR^{-/-}$) were able to mediate improved control of bacterial growth in the lungs (Fig. 2*A*), spleen (Fig. 2*B*), and draining lymph node (Fig. 2*C*). Of particular interest was the fact that although the effect was observed by day 30 in the lung, it was maintained throughout infection and became marked in the nonlung organs by day 125 of infection (Fig. 2). This result indicates that not only could improved control of bacterial growth occur but that there was also improved reduction of the bacterial burden in the IL-27R-deficient ($TCCR^{-/-}$) mice.

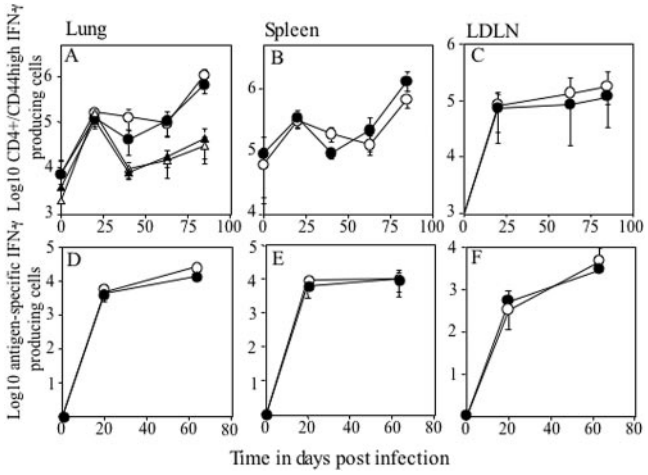

FIGURE 3. C57BL/6 (●) and $TCCR^{-/-}$ (○) mice were infected as for Fig. 2 and the lymphocytes isolated from the lung (*A* and *D*), spleen (*B* and *E*), and lung draining lymph node (*C* and *F*). Cells were cultured with anti-CD3 and anti-CD28 Ab in the presence of monensin (to inhibit protein secretion) and the presence of intracellular IFN-γ within $CD4^+$/CD44high cells (○ and ●, *A–C*) and $CD8^+/CD44^{high}$ (△ and ▲, *A*) determined by flow cytometric staining. Other cells were cultured with irradiated feeder cells and H37Rv culture filtrate protein (i.e., Ags for both CD4 and CD8 T cells) for 24 h and the number of IFN-γ producing cells determined by ELISPOT (*D–F*). The data points represent the mean (±SD) of values from four mice and show one experiment representative of four independent experiments (*A*, *B*, *D*, and *E*). The data points in *C* and *F* are the mean (±SD) values from four experiments where pooled lymph nodes from four mice were analyzed in each experiment. The data points obscure some SD.

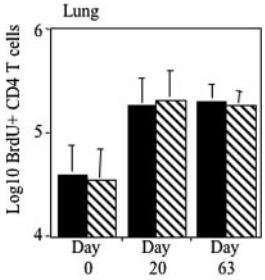

FIGURE 4. C57BL/6 (■) and TCCR$^{-/-}$ (▨) mice were infected as for Fig. 2 and the mice injected i.p. 24 h before harvest with the artificial nucleotide BrdU. Single cell suspensions were generated from the lung and the level of BrdU uptake by CD4 T cells measured by flow cytometry. Cells positive for BrdU were deemed to have proliferated within the previous 24 h. The bars represent the mean value for four mice (±SD) and one experiment representative of three experiments is shown.

Absence of the IL-27R does not significantly reduce the number of IFN-γ-producing cells within the M. tuberculosis*-infected lung*

IL-27 signaling via the TCCR (WSX-1) receptor increases T-bet expression (18, 19) and augments IL-12p70-mediated IFN-γ production by naive CD4 T cells (14, 15, 18, 19, 29). To determine whether IL-27 signaling is required to induce an IFN-γ-producing phenotype in Ag-specific CD4 T cells during TB, we assessed the number of these cells. We were unable to reproducibly detect any significant difference in the number of IFN-γ-producing CD4 or CD8 T cells in the lung (Fig. 3*A*) or IFN-γ-producing CD4 T cells in the draining lymph node or spleen by intracellular staining (Fig. 3, *B* and *C*). There was also no significant difference in the number of Ag-specific IFN-γ-producing T cells by ELISPOT responses to culture filtrate protein (Fig. 3, *D–F*). The lack of significant difference in the total number of IFN-γ-producing cells was a reflection of our failure to detect any reproducibly significant difference in the frequency of responding cells in both the intracellular staining and ELISPOT assays (data not shown) for the lung and spleen. In the draining node, there was a reproducibly lower frequency of IFN-γ-producing cells at day 20 (B6 2.05 ± 0.09% vs TCCR$^{-/-}$ 1.04 ± 0.39%, $p = 0.012$), and this is reflected in the fact that although there are more cells in the TCCR$^{-/-}$ lymph node there are not more IFN-γ-producing cells in this organ at day 20.

Absence of the IL-27R does not significantly alter the number of proliferating CD4 T cells during M. tuberculosis *infection*

IL-27 can induce proliferation of naive CD4$^+$ cells (12); however, in the absence of IL-27R signaling, hyperproliferation of T cells has also been demonstrated (14–16). With these observations in mind, we sought to determine whether absence of IL-27R-mediated signaling during *M. tuberculosis* infection significantly altered the accumulation of recently divided CD4 T cells in vivo. We were unable to discern any difference in the number of recently divided CD4 T cells in the lungs between the control and TCCR$^{-/-}$ mice (Fig. 4). Although this does not prove that the rate of proliferation is the same between the two groups of mice, it does indicate that there is no apparent defect in the accumulation of recently divided CD4 T cells into the lung. The proliferation of CD4 T cells begins in the lung draining lymph nodes, and in this organ we saw higher numbers of CD4 T cells and a higher number of CD4 T cells that had proliferated in the previous 24 h in the TCCR$^{-/-}$ mice (Table I). This increase in proliferation in the lymph node was mirrored by a significantly higher number of CD4 T cells in the lungs of the TCCR$^{-/-}$ mice at day 20 (1.67×10^6 in C57BL/6 lungs vs 2.47×10^6 in TCCR$^{-/-}$ lung, $n = 16$, $p = 0.033$). Despite this moderate difference in lymphocyte proliferation and accumulation at day 20 in the TCCR$^{-/-}$ mice lymph node and lungs, there was no significant difference in frequency of proliferating cells in any organ tested at any time point (data not shown). Similarly, although the total number (and frequency) of CD3$^+$CD4$^+$ cells that could be purified from the infected lungs was always slightly higher in the TCCR$^{-/-}$ mice (in four of four experiments, $n = 4$ group), this difference did not reach statistical significance at any time other than day 20 (data not shown).

Absence of the IL-27R allows increased lymphocyte accumulation within the mononuclear granulomata of TB-infected mice

To determine whether the granulomatous response was affected by the absence of the IL-27R, infected lungs were compared using standard histological techniques. The granulomata of the C57BL/6 and TCCR$^{-/-}$ mice developed with similar kinetics and were mononuclear in character (Fig. 5). There was no difference in size or number of granulomata between the two groups of mice. There was however a marked difference in the lymphocyte organization within the granulomata of the TCCR$^{-/-}$ mice with areas of lymphocyte accumulation being more pronounced throughout infection (Fig. 5). The total number of lymphocytes was determined by flow cytometry for both C57BL/6 and TCCR$^{-/-}$-infected lungs. We show in Fig. 5*A* that although the difference in lymphocyte numbers is not significant there are always more lymphocytes in the TCCR$^{-/-}$-infected lungs. Similarly, when a ratio of C57BL/6:TCCR$^{-/-}$ lymphocyte numbers in infected lungs is calculated, it is significantly less than the ratio for uninfected lungs which is ~1 (Fig. 5*B*).

Table I. *The number of lymphocytes, CD4 T cells, and recently divided CD4 T cells in the lung draining lymph nodes 20 days after aerosol infection with* M. tuberculosis

Parameter[a]	Mouse	Geometric Mean[b]	95% LCL[c]	95% UCL	Ratio[d]	95% LCL	95% UCL	p^e
Lymphocytes ($\times 10^7$)	C57BL/6	2.5	0.9	6.5				
	TCCR$^{-/-}$	4.6	1.7	12.7	1.87	0.82	4.29	0.094
CD4$^+$ T cells ($\times 10^6$)	C57BL/6	3.5	1.5	8.3				
	TCCR$^{-/-}$	8.6	2.7	27.5	2.44	0.97	6.14	0.054
CD4$^+$BrdU$^+$ T cells ($\times 10^4$)	C57BL/6	2.4	0.7	8.5				
	TCCR$^{-/-}$	5.6	1.4	22.3	2.33	1.00	5.48	0.051

[a] Mice were infected via aerosol as described for Fig. 2 and given an i.p. injection with BrdU 24 h prior to harvest. The draining lymph nodes were harvested at day 20 postinfection, disrupted, stained for surface markers, and intranuclear BrdU and analyzed by flow cytometry as described in *Material and Methods.*

[b] The data represent the geometric mean from four independent experiments within each of which lymph nodes from either four C57BL/6 or four TCCR$^{-/-}$ mice were pooled for analysis.

[c] LCL is lower confidence limit; UCL is upper confidence limit.

[d] The ratio of the mean number of TCCR$^{-/-}$ cells to that of the C57BL/6 number of cells.

[e] A paired two-tailed *t* test was carried out on log-transformed cell counts for the C57BL/6 and TCCR$^{-/-}$ mice.

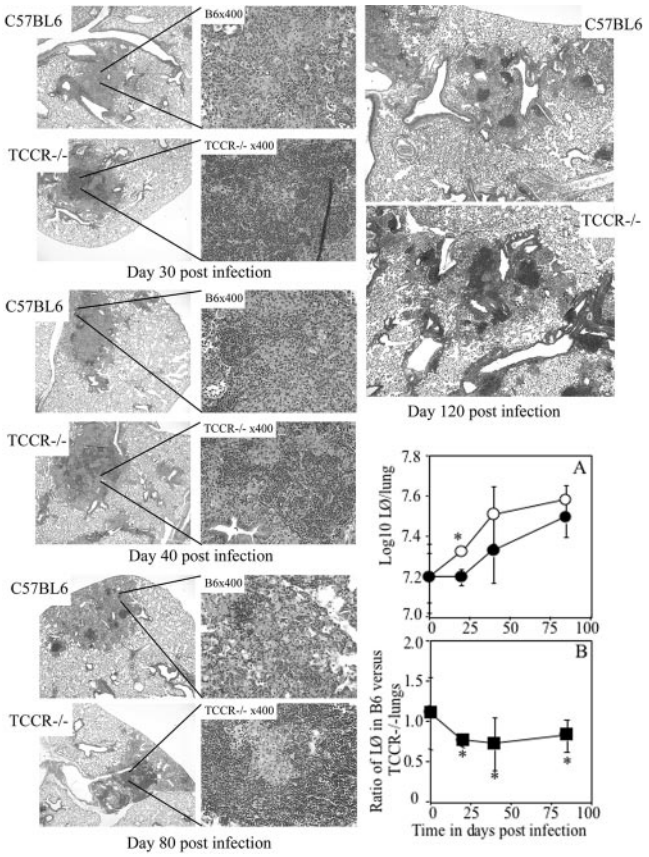

FIGURE 5. C57BL/6 (*A*) and TCCR$^{-/-}$ (*B*) mice were infected as described for Fig. 2. Upon harvest, the caudal lobe of the lung from each of four mice per group was perfused with 10% formal saline, embedded in paraffin, sectioned, and stained using H&E. Slides containing the sections were compared for granuloma composition between the infected C57BL/6 and TCCR$^{-/-}$ lungs. The panels in the *far-left column* show infected lungs ($\times$100) from both C57BL/6 and TCCR$^{-/-}$ mice throughout infection. The panels in the middle column show the cellular components of the granulomata in the *left column* ($\times$400) and demonstrate that the increased areas of darker staining in the TCCR$^{-/-}$ granulomata consist of lymphocytes as defined by morphology. The two larger images in the *left column* demonstrate that the increased lymphocytic nature of the TCCR$^{-/-}$ granulomata persists to day 120 of infection ($\times$100). Lung sections did not differ in any other parameter of granuloma structure. The individual panels show one lung section representative of four mice per group per time point; the presence of increased accumulations of lymphocytes was seen in two of two experiments. *Graph A* represents the number of lymphocytes as determined by flow cytometry for either C57BL/6 (●) or TCCR$^{-/-}$ (○) lungs. One experiment representative of three is shown, $n = 4$, $*$, $p < 0.05$. *Graph B* shows the change in the ratio of C57BL/6 lymphocyte numbers to TCCR$^{-/-}$ lymphocyte numbers over time of infection (■). At all three times the ratio is less than the ratio for uninfected lungs, $*$, $p < 0.01$, one experiment representative of three.

Absence of the IL-27R significantly reduces the expression of mRNA for IFN-γ during M. tuberculosis *infection in the lung*

The data from Figs. 3 and 4 clearly demonstrate that despite the lower bacterial burden in the absence of the IL-27R there were at least equivalent numbers of IFN-γ-producing T cells within the intact and gene-deleted mice. The above data did not however allow us to determine the level of IFN-γ present in the lung and so we compared the level of mRNA for this cytokine between the infected C57BL/6 and TCCR$^{-/-}$ mice (Fig. 6*A*). Interestingly, there was significantly lower induction of IFN-γ mRNA in the lungs of the TCCR$^{-/-}$ mice (Fig. 6*A*). This difference was detectable by day 20 in infection and was maintained throughout. As an indication that there may be lower levels of IFN-γ protein present in the infected TCCR$^{-/-}$ lungs, there was also reduced mRNA expression of the IFN-γ-induced gene *iNOS*, which is required to generate NO (Fig. 6*D*). The reduced induction of *IFN-γ* and *iNOS* gene expression was not seen for two other essential antimycobacterial agents, TNF-α and LRG-47 (Fig. 6, *E* and *F*), indicating that not all mediators of bacterial control were reduced in the absence of the IL-27R. Finally, to determine whether the reduced production of IFN-γ in the lung was related to decreased polarization of the Th1 response, we assessed the level of *T-bet* mRNA expression. Despite the rather crude nature of this type of analysis, we were able to detect a significant reduction in mRNA for this marker of Th1 polarization in the TCCR$^{-/-}$ mice throughout the infection (Fig. 6*B*). This reduced polarization could be due to reduced availability of IL-12p70; however, we did not see reduced expression of the inducible subunit IL-12p40 in the lungs of the TCCR$^{-/-}$ mice (Fig. 6*C*).

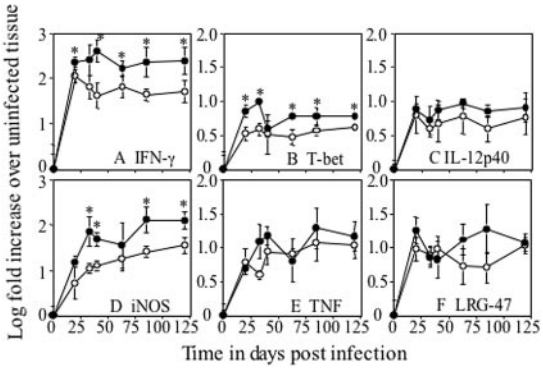

FIGURE 6. C57BL/6 (●) and TCCR$^{-/-}$ (○) mice were infected as described for Fig. 2 and at specific times postinfection lung tissue was harvested and processed to extract RNA. The presence of specific mRNA was determined by real-time PCR and the $\log_{10}$-fold-increase in mRNA determined for three to four infected mice vs four noninfected mice. The data points represent the mean and SE values for four mice for each time point. $*$, $p \leq 0.05$.

Absence of the IL-27R significantly reduces the ability of Ag-specific CD4 T cells to produce IFN-γ

The data from *A* and *B* in Fig. 6 is suggestive of reduced polarization of the Th1 response to TB in the lung. To ascertain whether indeed the Ag-specific CD4 T cell response was less polarized within the lung, we determined the amount of IFN-γ produced by these cells. In a first experiment, we determined that the amount of IFN-γ produced per lung-derived C57BL/6 CD4 T cell was greater than that produced per lung-derived TCCR$^{-/-}$ CD4 T cell (Fig. 7, *top left panel*). In contrast, the amount of IFN-γ produced per CD4 T cell derived from the spleens of infected C57BL/6 mice was much lower than that produced per lung-derived CD4 T cell. Additionally, there was no difference in the amount of IFN-γ produced per spleen-derived CD4 T cell from the C57BL/6 mice and the TCCR$^{-/-}$ mice (Fig. 7, *upper right panel*). In a separate experiment, we purified CD4 T cells from the lungs and spleens of infected mice and performed side-by-side ELISA and ELISPOT analysis on each population. We were thereby able to calculate the amount of IFN-γ produced per Ag-specific cell. This pair of assays demonstrated that the amount of IFN-γ produced per Ag-specific CD4 T cell was significantly different for cells derived from the lungs of C57BL/6 mice and TCCR$^{-/-}$ mice (Fig. 7, *lower left panel*). Again the amount of IFN-γ per Ag-specific cell in the

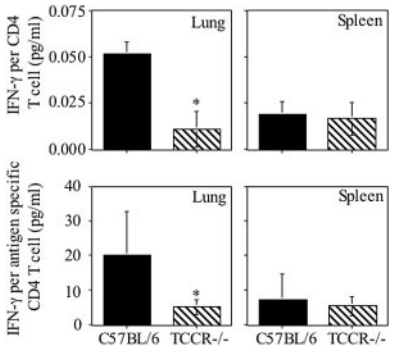

FIGURE 7. C57BL/6 (■) or TCCR$^{-/-}$ (▨) mice were infected as described for Fig. 2 and at day 20 (*upper panels*) or day 30 (*lower panels*) postinfection, CD4 T cells were purified from target organs and cultured with APC and Ag; ELISA was used to determine the amount of IFN-γ produced within each culture. The data is presented as the amount of IFN-γ per plated CD4 T cell in the *top two panels* or as IFN-γ per Ag-specific cell (as determined by ELISPOT on identical cultures) in the *bottom two panels*. The data points represent the mean and SE values for four mice for each time point. ∗, $p \leq 0.05$.

spleen was less than that seen for cells from the lungs and there was no difference in the amount between the C57BL/6 and TCCR$^{-/-}$ mice (Fig. 7, *lower right panel*).

Discussion

In this study, we demonstrate that in the absence of a component of the IL-27 receptor, the protective Th1 response to TB is altered. Specifically, there is an increased ability to reduce bacterial burden in all infected organs as disease progresses. This improved protection correlates with a minor increase in cellular responses early in disease and with an increased lymphocytic character to the granuloma. The Th1 response is uniquely affected by the absence of IL-27R signaling in that although there is no diminution in the number of Ag-specific IFN-γ producing CD4 T cells there is a reduction in the amount of IFN-γ that these cells can produce on a per cell basis. IL-27R signaling is therefore dispensable for the induction of IFN-γ producing Ag-specific CD4 T cells during TB, however, its absence results in reduced polarization of these cells and paradoxically improved protection against TB.

The inability of the immune host to eliminate *M. tuberculosis* results in chronic infection and the need for prolonged expression of a Th1 response to ensure survival (30); the factors which regulate this prolonged Th1 response are currently not defined. The recent observation that IL-27 is required to ensure survival by regulating the Th1 response during *T. gondii* infection (16) indicates that regulation of type 1 responses can be as important as the induction of these responses. Similar to the data from the *T. gondii* model, in the absence of IL-27 we see an early increase in proliferation of CD4 T cells in the draining lymph nodes, although it is much less pronounced than in the *T. gondii* model. We also observe an early increase in numbers of CD4 T cells in the lung and a tendency for increased lymphocytic accumulation in this organ, particularly within the granuloma. Unlike disease models such as *Trypanosoma cruzi* infections wherein absence of WSX-1 results in hepatic necrosis (17), the increased cellular response to TB does not result in any immunopathologic consequences nor does it associate with decreased survival. Thus, in contrast to these models the absence of IL-27 results in only a minor deregulation of the cellular response during TB.

Several lines of evidence support a role for IL-27 as a negative regulator of cellular responses: First, TCCR or WSX-1 negative T cells show enhanced proliferation in vitro (14–16). Second, in several infectious disease models, WSX-1 deficient mice showed exacerbated immune responses (16, 17). And lastly, WSX-1 mice display increased sensitivity to Concanavilin A induced hepatitis when compared with their wild-type counterparts (31). Together, the available data suggest that in many in vivo situations, IL-27 inhibits cellular responses, and thus it is plausible that the absence of IL-27 signaling during TB results in uninhibited early cellular responses, which thereby limit bacterial growth.

Although early improved control of bacterial growth may be due to uninhibited cellular responses, we do not see any significant increase in IFN-γ producing cells or in mRNA for this cytokine in the lung. Indeed, while numbers of CD4 and CD8 T cells producing IFN-γ in the lung remain equivalent between C57BL/6 and TCCR$^{-/-}$ mice the amount of *IFN-γ* mRNA is actually less in the TCCR$^{-/-}$ lungs. We attribute this reduced level of *IFN-γ* mRNA to the fact that Ag-specific CD4 T cells from TB-infected TCCR$^{-/-}$ lungs produce less IFN-γ than those from C57BL/6 mice on a per cell basis. Thus, the absence of IL-27 signaling during the polarization of TB-specific CD4 Th1 cells appears to reduce the ability of these cells to produce IFN-γ at the site of infection. This reduced ability to produce IFN-γ has been shown in vitro (14–16, 18, 19, 29) and in vivo for *Leishmania*, *Listeria*, and BCG infections (14, 15) but not during *T. gondii* infection where enhanced IFN-γ production was seen (16). Recently, the defect in IFN-γ-producing ability of IL-27R-deficient mice has been linked to the presence of IL-4 such that in the absence of this cytokine, IL-27R-deficient T cells produce normal amounts of IFN-γ (32). It is likely that IL-27 thus serves to suppress IL-4-induced expression of the Th2-specific zinc-finger transcription factor GATA-3 (18). It would appear that mycobacterial infection (BCG (15) and shown here) results in polarizing conditions which require the presence of IL-27 to counteract inhibition of Th1 polarization which may be mediated by IL-4 and GATA-3. This is further supported by the fact that *IL-4* mRNA is moderately induced following *M. tuberculosis* infection and that in its absence augmented levels of *IFN-γ* mRNA can be seen in the lung (33).

It is somewhat counterintuitive that the enhanced control of bacterial replication shown here is accompanied by reduced induction of *IFN-γ* and *iNOS*. However, this moderate induction may be sufficient to mediate control of bacterial growth and at the same time allow improved survival of the accumulating effector lymphocytes within the TB granuloma. We base this hypothesis on the fact that IFN-γ is a potent regulator of cell death for effector lymphocytes (34, 35) and that expression of IFN-γ and NO is detrimental to the accumulation of activated lymphocytes in mycobacterial disease (reviewed in Ref. 10). That there is an improved environment within the lung for lymphocyte survival is supported by the histological observations showing increased lymphocyte accumulation within TB granulomas in the infected TCCR$^{-/-}$ mice. Although the increased number of lymphocytes is clearly seen within the granulomata, we were only able to see a slight tendency to increased numbers of lymphocytes within the lungs suggesting that there are not a great deal more cells within the lung but that they are simply able to accumulate within the granuloma more readily. Whether the improved accumulation of lymphocytes is mediating the reduced bacterial burden in the absence of IL-27R is currently under investigation.

Very recently, it was shown that IL-27 can affect cells other than T cells, due to the presence of TCCR/WSX-1 and the second receptor chain, gp130, on a wide variety of immune cell types (13). Specifically, it was shown that IL-27 binds to and modulates the responses of human monocytes and mast cells (13) and enhanced myeloid responses are seen in WSX-1-deficient mice infected with

T. cruzi (17) and *Trichuris muris* (reported in Ref. 20). Although this suggests that IL-27 could affect the primary host cells for *M. tuberculosis*, we do not see altered expression of two key macrophage-dependent antibacterial agents, TNF-α (36, 37) and LRG-47 (38), in the lungs of the TCCR$^{-/-}$ mice. Furthermore, we do not see up-regulation of mRNA for the IL-27 receptor in murine macrophages exposed to *M. tuberculosis* bacteria. Hence, while enhanced myeloid function may mediate some of the protective mechanism against TB, we do not have data to support that hypothesis here.

Our results suggest that IL-27 limits the physiological immune response to TB. Consequently, it will be interesting to investigate whether polymorphisms in the IL-27R- or IL-27-induced pathways are associated with improved outcome in human TB. Furthermore, it will be important to determine to what extent the polarization of Ag-specific CD4 T cells is altered by the absence of IL-27 and also whether this affects the ability of the cells to become memory cells. If IL-27 does limit the potential protective efficacy of Ag-specific T cells then this will be an important consideration in the design of new vaccines.

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