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CUTTING EDGE

Cutting Edge: Regulatory T Cells Prevent Efficient Clearance of *Mycobacterium tuberculosis*

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Mycobacterium tuberculosis ***remains one of the top microbial killers of humans causing ~2 million deaths annually. More than 90% of the 2 billion individuals infected never develop active disease, indicating that the immune system is able to generate mechanisms that control infection. However, the immune response generally fails to achieve sterile clearance of bacilli. Using adoptive cell transfer into* C57BL/6J-Rag1^{tm1Mom} *mice (Rag1$^{-/-}$), we show that regulatory T cells prevent eradication of tubercle bacilli by suppressing an otherwise efficient CD4$^+$ T cell response. This protective CD4$^+$ T cell response was not correlated with increased numbers of IFN-γ- or TNF-α-expressing cells or general expression levels of IFN-γ or inducible NO synthase in infected organs compared with wild-type C57BL/6 animals. Furthermore, suppression of protection by cotransferred regulatory T cells was neither accompanied by a general increase of IL-10 expression nor by higher numbers of IL-10-producing CD4$^+$ T cells. The Journal of Immunology,* 2007, 178: 2661–2665.**

Tuberculosis remains one of the most threatening bacterial infections, which is responsible for high incidences of morbidity and mortality, reaching ~2 million deaths annually (1). However, mortality figures are only one facet of this disease. Approximately one-third of the world's population is latently infected with *Mycobacterium tuberculosis*, and 90% of these individuals will never develop active disease, indicating that the human immune system is capable of controlling *M. tuberculosis* infection effectively. Generally, host immunity, however, fails to eradicate tubercle bacilli successfully. In the vast majority of cases, a chronic infection develops and a balance between pathogen persistence and immune response is maintained, perpetuating the risk of uncontrolled reactivation. The mechanisms underlying this fine-tuned balance, which is a likely consequence of the coevolution of both organisms, are incompletely understood. Probably, they involve sophisticated survival and immune escape strategies of *M. tuberculosis* and aberrant immunoregulatory host mechanisms, which intervene during chronic infection to prevent exacerbated immunopathology.

M. tuberculosis is generally transmitted via inhalation of mycobacteria-containing droplets. Within the lung alveolar space, bacilli are engulfed by alveolar macrophages and are either killed immediately or end up in deeper lung tissue where they reside within local macrophages. In an attempt to isolate the tissue site of infection, the immune response induces granuloma formation. Although various cells contribute to immunity against *M. tuberculosis*, T cells, notably effector CD4$^+$ T cells of the Th1 type, dominate protective immunity (2–4). Upon activation, CD4$^+$ T cells secrete IFN-γ and TNF-α, which in turn induce antimycobacterial mechanisms in macrophages. These include hydrogen peroxide and other reactive oxygen intermediates generated via the oxidative burst, as well as NO and related reactive nitrogen intermediates, produced by the inducible form of NO synthase (NOS2) (2, 5, 6). These and additional macrophage effector mechanisms effectively control *M. tuberculosis* in vitro, and studies using gene knockout mice indicate that reactive nitrogen intermediates and reactive oxygen intermediates are critical in vivo (7–10). Thus, it is unclear why the immune system only restricts mycobacterial growth and fails to achieve sterile eradication of this pathogen.

Critical involvement of regulatory T cells (T_{reg})[3] in control of immune responses to self-Ags and in immune homeostasis is well established, and there is increasing evidence for a role of T_{reg} in the regulation of immunity to infection (11, 12). It is believed that these T_{reg} down-modulate immune responses after pathogen eradication to avoid exacerbated pathology. Although this mechanism is generally to the benefit of the host in acute infections, it poses problems in chronic infections, notably when pathogen persistence is sustained in the face of an active immune response.

T_{reg} represent 5–10% of the CD4$^+$ T cell population and are characterized by the expression of the transcription factor

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[3] Abbreviations used in this paper: T_{reg}, regulatory T cell; fw, forward; rev, reverse; iNOS, inducible NO synthase; wt, wild type.

Foxp3. A large fraction of the T_{reg} population is $CD25^+$, and T_{reg} represent up to 90% of the $CD4^+CD25^+$ T cell subset in naive animals (13). The mechanisms by which T_{reg} control immune responses are incompletely understood, but there is evidence for a central role of the inhibitory cytokines IL-10 and TGF-β and the surface molecule CTLA-4 (12, 14). The role of T_{reg} in tuberculosis is thus far unknown and is central in the current study.

Materials and Methods

Mice and M. tuberculosis *infection*

C57BL/6 and $Rag1^{-/-}$ mice on a C57BL/6 background were purchased from the Federal Institute for Health Protection of Consumers and Veterinary Medicine in Berlin and bred in our facilities. All animal experiments were conducted according to German animal protection law.

Mice were infected by aerosol with 200 CFU of *M. tuberculosis* H37Rv using an aerosol chamber (Glas-Col). Inocula were confirmed at day 1 postinfection by plating complete lung homogenates onto Middlebrook 7H11 ampicillin plates. CFU in the lung and spleen were determined after mechanical disruption of organs in water supplemented with 1% albumin and 0.5% Tween 80, and plating serial dilutions onto Middlebrook 7H11 ampicillin agar at day 30 postinfection.

Purification of cells and reconstitution of $Rag1^{-/-}$ mice

Cells from spleen and peripheral lymph nodes of naive C57BL/6 donor mice were incubated with purified rat Ig and anti-CD16/CD32 mAb (clone, 2.4G2). After 5–10 min, biotinylated anti-CD25 mAb (clone PC61) and magnetic antibiotin microbeads (Miltenyi Biotec) were added, and $CD25^+$ T cells were purified with a magnetic column (autoMACS; Miltenyi Biotec). T cells bound and recovered from the column were >75% $CD25^+$, >90% $CD4^+$, and >80% $Foxp3^+$. CD25-depleted cells were incubated with FITC-conjugated anti-CD4 mAb (GK1.5) and subsequently with anti-FITC mAb-coated magnetic microbeads (Miltenyi Biotec). $CD4^+$ T cells were isolated using the autoMACS and were >98% $CD4^+$, <0.5% $CD25^+$, and <4.0% $Foxp3^+$. Purified $CD4^+CD25^-$ T cells (2×10^6) or $CD4^+CD25^+$ T cells (1×10^6) or a combination of both cell populations were adoptively transferred (i.v.) into Rag $1^{-/-}$ recipient mice. At day 1 after cell transfer, mice were infected with *M. tuberculosis* H37Rv by aerosol.

Cell separation, in vitro restimulation of cells, and flow cytometry

Spleens were removed, and single-cell suspensions were prepared using an iron mesh sieve. RBC were lysed, and spleen cells were washed twice with RPMI 1640 medium supplemented with glutamine, Na-pyruvate, 2-ME, penicillin, streptomycin, and 10% heat-inactivated FCS (complete RPMI 1640). Lungs were cut into small pieces and incubated for 30 min with complete RPMI 1640 supplemented with Collagenase D (Roche) and collagenase type VIII (Sigma-Aldrich). Single-cell suspensions of lungs were then prepared in a manner similar to those of spleens, using an iron mesh sieve and RBC lysis. Foxp3 staining was performed using the PE anti-Foxp3 staining kit (eBioscience) according to the manufacturer's recommendations. For the determination of cytokine expression, 4×10^6 cells were cultured for 6 h in a volume of 1-ml complete RPMI 1640. Cells were stimulated by adding 5 μg/ml anti-CD3 mAb and 5 μg/ml anti-CD28 mAb or as a negative control, with no supplementary additives. During the final 5 h of culture, 10 μg/ml brefeldin A were added. Cultured cells were washed and incubated for 10 min with rat IgG Abs and anti-CD16/CD32 mAb to block nonspecific Ab binding. Subsequently, cells were stained with PE-Cy5-conjugated anti-CD4 mAb and FITC-conjugated anti-Thy1.2. After 20 min on ice, cells were washed with PBS and fixed for 20 min at room temperature with PBS 2% paraformaldehyde. Cells were washed with PBS/0.2% BSA, permeabilized with PBS/0.1% BSA/0.5% saponin, and incubated in this buffer with rat IgG Abs and anti-CD16/CD32 mAb. After 10 min, Cy5-conjugated anti-IFN-γ or TNF-α mAb and PE-conjugated anti-IL10 mAb or isotype control mAb were added. After a further 20 min at room temperature, cells were washed with PBS and fixed with PBS/1% paraformaldehyde. Cells were analyzed using a FACS-Canto II and Diva software (BD Biosciences).

Antibodies

Rat IgG Abs, anti-CD16/CD32 mAb, anti-IFN-γ mAb, and anti-TNF-α mAb were purified from rat serum or hybridoma supernatants with protein G Sepharose. Abs were Cy5-conjugated according to standard protocols. Rat IgG1 isotype control mAb was purchased from BD Pharmingen. The anti-CD25 and anti-Foxp3 mAb were obtained from eBioscience, and PE-Cy5-conjugated anti-CD4 (RM4-5), FITC-conjugated anti-Thy-1.2, and PE-conjugated anti-IL-10 mAb were obtained from BD Biosciences.

Real-time RT-PCR

Total RNA was extracted from tissues using TRIzol reagent (Invitrogen Life Technologies) as recommended by the manufacturer. RNA from animals of similarly treated groups was pooled, and samples were treated with DNase I (Invitrogen Life Technologies) to eliminate genomic DNA contamination. For semiquantitative real-time RT-PCR, 5 μg of DNase I-digested RNA-pools were used for reverse transcription using 200 ng of random hexamers as primers for Superscript II (Invitrogen Life Technologies) according to the manufacturer's recommendation. All real-time PCR were run for 45 cycles with 20 s 94°C and 60 s 60°C in the ABI Prism 7000 Sequence Detection System (Applied Biosystems) using ABI PRISM optical 96-well plates (Applied Biosystems). Primers were designed to span large introns and to produce product sizes between 100 and 200 bp (β-*actin*-forward (fw), TGG AAT CCT GTG GCA TCC ATG AAA C; β-*actin*-reverse (rev), TAA AAC GCA GCT CAG TAA CAG TCC G; GAPDH-fw, GCA ACT CCC ACT CTT CCA CCT TC; *GAPDH*-rev, CCT CTC TTG CTC AGT GTC CTT GCT; *Foxp3*-fw, GCC TCT CCA GAG AGA AGT GGT G; *Foxp3*-rev, CCC TGA GTA CTG GTG GCT ACG ATG; *IFN*-γ-fw, ACG GCA CAG TCA TTG AAA GCC TA; *IFN*-γ-rev, CTC ACC ATC CTT TTG CCA GTT CC; *IL-10*-fw, GGA CAA CAT ACT GCT AAC CGA CTC CT; IL-10-rev, CTG CTC CAC TGC CTT GCT CTT ATT; *inducible NO synthase* (*iNOS*)-fw, GAC GAG ACG GAT AGG CAG AGA TTG; *iNOS*-rev, CCT GGG AGG AGC TGA TGG AGT AG). Reaction mixtures were set up to a final volume of 30 μl using 15 pmol of each primer and 15 μl of 2× SYBR Green PCR Master mix (Applied Biosystems). Quantification was performed at least two times with independent cDNA samples and in triplicates for each cDNA and primer pair. Data analyses were performed using the ABI Prism 7000 SDS Software (Applied Biosystems) and Excel (Microsoft). The threshold cycles were determined for each sample, and fold differences relative to the expression level in one of the analyzed cDNA samples was calculated for each cDNA sample and primer pair (fold-difference $= 2^{-\Delta C_T}$). Resulting fold differences for *Foxp3*, *IFN*-γ, *IL-10*, and *iNOS* expression levels were corrected for different amounts of cDNA by multiplication with the average fold difference of *GAPDH* and β-*actin* expression within the same sample. DNase I-digested, not reverse-transcribed RNA, was used as a template in separate reactions, to control for genomic DNA contamination.

Statistical analysis

Statistical significance of results was determined with the statistics program included in the GraphPad Prism software (GraphPad). Bacterial titers were analyzed using the Mann-Whitney *U* test for a nonparametric sample distribution ($p < 0.05$).

Results and Discussion

In the first set of experiments, we determined the number of Foxp3-expressing $CD4^+$ T cells in lung and spleen at different time points after *M. tuberculosis* infection. During the first 60 days postaerosol *M. tuberculosis* infection, a consistent increase of FoxP3-expressing $CD4^+$ T cells was detected in spleen as well as in lung, indicating infiltration of T_{reg} into infected tissue sites (Fig. 1*A*).

To evaluate the impact of T_{reg} in protection against *M. tuberculosis*, we purified $CD4^+$ T cells from naive C57BL/6 mice, separated $CD4^+CD25^-$ cells (Th) from $CD4^+CD25^+$ cells (T_{reg}), and adoptively transferred these T cell subsets into mice lacking T and B cells ($Rag1^{-/-}$ mice). At day 30 postaerosol infection with *M. tuberculosis*, we analyzed the mycobacterial burden in lungs and spleens (Fig. 1*B*). $Rag1^{-/-}$ mice reconstituted with selected Th controlled *M. tuberculosis* more efficiently than $Rag1^{-/-}$ controls. Notably, $Rag1^{-/-}$ mice reconstituted with Th harbored markedly lower bacterial titers in the order of several magnitudes as compared with C57BL/6 wild-type (wt) mice. In some Th-reconstituted $Rag1^{-/-}$ mice, the bacterial burden was below the detection limit of our assay, suggesting sterile eradication. In contrast, protection was reduced to that seen in C57BL/6 wt mice when Th and T_{reg} were cotransferred into $Rag1^{-/-}$ animals. Hence, Th can effectively control *M. tuberculosis* but are severely restricted in their function by T_{reg}.

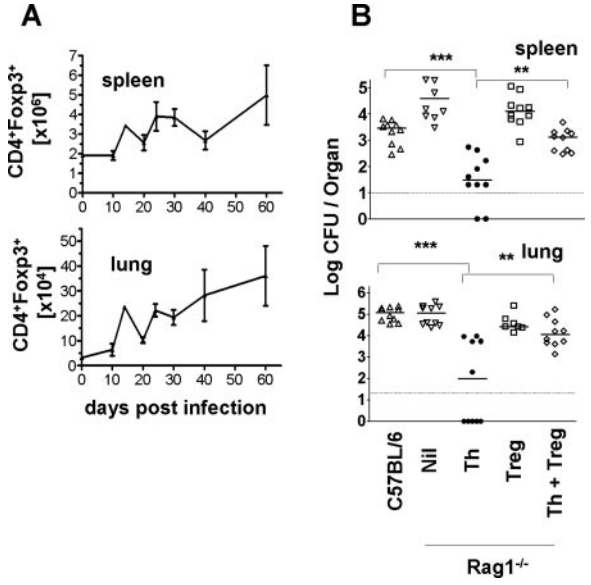

FIGURE 1. T_{reg} prevent eradication of *M. tuberculosis* by suppressing $CD4^+$ T cells. *A*, Increasing numbers of T_{reg} after infection with *M. tuberculosis*. C57BL/6 wt mice were infected with *M. tuberculosis* H37Rv (200 CFU; aerosol). At the indicated time points postinfection, numbers of $Foxp3^+CD4^+$ T cells were determined in spleens and lungs. Arithmetic averages of two independent experiments with two pools of seven mice are shown. Error-bars represent SD. *B*, T_{reg} suppress protection mediated by $CD4^+$ T cells. C57BL/6 wt mice, $Rag1^{-/-}$ mice lacking B and T cells (Nil), and $Rag1^{-/-}$ mice reconstituted with either selected $CD4^+CD25^-$ T cells (Th) or $CD4^+CD25^+$ T cells (T_{reg}), or a combination of both (Th + T_{reg}), were infected with *M. tuberculosis* H37Rv (200 CFU; aerosol). At day 30 postinfection, bacterial titers in spleens and lungs were determined. Data are represented as scatterplots with each spot representing one animal. Each figure combines results from two independent experiments with at least four mice per group. Significant differences between two groups are indicated by asterisks (Mann-Whitney *U* test: **, $p < 0.01$ and ***, $p < 0.001$).

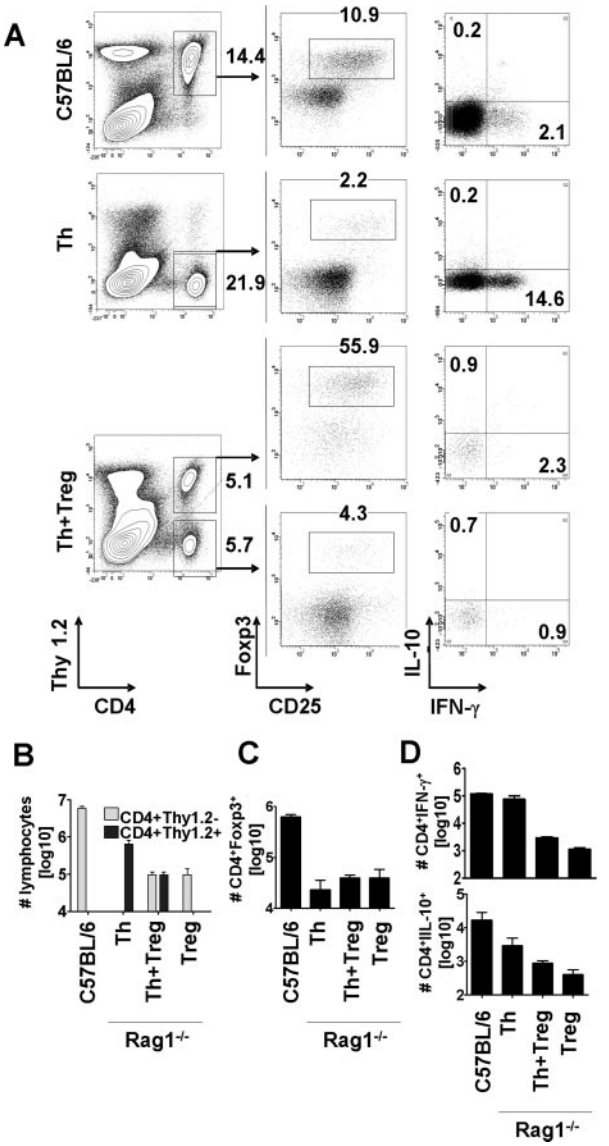

FIGURE 2. Frequencies and numbers of Th, T_{reg}, and cytokine producers in T cell-reconstituted $Rag1^{-/-}$ mice. C57BL/6 wt mice, and $Rag1^{-/-}$ mice reconstituted with either selected $CD4^+CD25^-$ T cells (Th; isolated from Thy 1.1 congenic C57BL/6 donors) or $CD4^+CD25^+$ T cells (T_{reg}), or a combination of both (Th + T_{reg}), were infected with *M. tuberculosis* H37Rv (200 CFU; aerosol). At day 30 postinfection, lung lymphocytes and splenocytes were isolated and analyzed by flow-cytometric means. *A*, Representative staining and gating illustrated on splenocytes isolated from C57BL/6 mice, Th and Th + Treg-reconstituted $Rag1^{-/-}$ mice. *Left column*, Contour blots of lymphocytes stained for Thy 1.2 and CD4 expression. Percentage of $CD4^+$ T cells positive or negative for Thy1.2 are indicated adjacent to the respective population. *Middle column*, FoxP3 vs CD25 expression among $CD4^+$ T cell populations gated in the *left column*. Percentage of $Foxp3^+$ among $CD4^+$ T cells are given as numbers in the dot blots. *Right column*, IFN-γ vs IL-10 producers among CD4 T cell populations gated in the *left column*. Numbers indicate percentages of cytokine-positive cells within the indicated quadrant. *B–D*, Bar graphs showing total numbers of $CD4^+Thy\,1.2^+$ or $CD4^+Thy\,1.2^-$ T cells (*B*), total numbers of $CD4^+Foxp3^+$ T cells (*C*), and total numbers of cytokine-positive T cells (*D*) in C57BL/6 wt mice and reconstituted $Rag1^{-/-}$ mice. Results depicted are representative of two independent experiments with at least three mice in each experimental group.

To gain insights into the mechanism underlying protection, we analyzed the composition of the T cell compartment at day 30 after *M. tuberculosis* infection in C57BL/6 mice and $Rag1^{-/-}$ mice that received either Th alone or Th and T_{reg} before infection using flow cytometry and intracellular cytokine staining (Fig. 2). Transfer of Th without T_{reg} led to high numbers and frequencies of IFN-γ-producing $CD4^+$ T cells, whereas cotransfer of Th and T_{reg} not only prevented the development of IFN-γ-producing $CD4^+$ T cells among the cotransferred Th cells, but also suppressed Th proliferation (Fig. 2*B*). However, despite higher frequencies, the total number of IFN-γ-producing $CD4^+$ T cells was still higher in less well-protected wt mice as compared with $Rag1^{-/-}$ mice that received selected Th. Notably, the level of protection correlated with the frequency, but not with the number of IFN-γ producers among $CD4^+$ T cells.

Because IFN-γ is a critical mediator of the protective immune response against *M. tuberculosis* (15, 16), we aimed to determine whether enhanced protection in $Rag1^{-/-}$ mice that received selected Th was due to increased IFN-γ production despite lower numbers of IFN-γ-producing $CD4^+$ T cells. To compare total *IFN-γ* mRNA levels, we performed real-time RT-PCR analyses of lung and spleen tissue from naive C57BL/6 and infected C57BL/6 mice, and T cell reconstituted $Rag1^{-/-}$ mice (Fig. 3). Notably, *IFN-γ* mRNA levels did not correlate with protection against *M. tuberculosis*. Spleen and lung levels of *IFN-γ* mRNA were equally enhanced in infected Th recipient $Rag1^{-/-}$ mice and C57BL/6 mice. Expression of *iNOS* is induced by IFN-γ and represents one of the key effector mechanisms of macrophages against *M. tuberculosis* (9, 10).

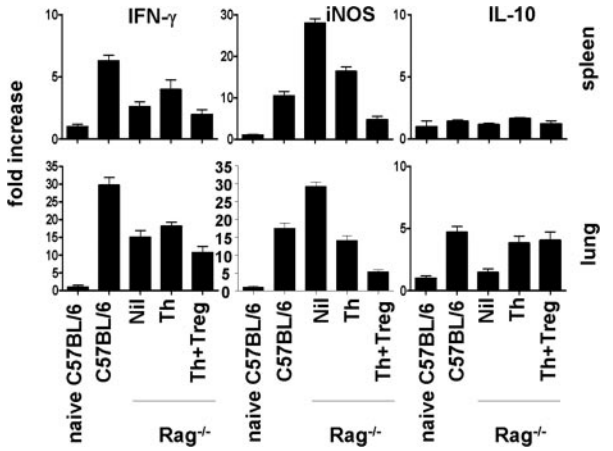

FIGURE 3. Cytokine mRNA expression in *M. tuberculosis*-infected mice. C57BL/6 wt mice, $Rag1^{-/-}$ (Nil), and $Rag1^{-/-}$ mice reconstituted with either purified $CD4^+CD25^-$ T cells (Th) or $CD4^+CD25^+$ T cells (T_{reg}), or a combination of both (Th + T_{reg}), were infected with *M. tuberculosis* H37Rv (200 CFU; aerosol). At day 30 postinfection, mice were killed and lungs and spleens of each group were pooled. mRNA expression levels of different cytokines were determined by quantitative real-time RT-PCR. Results represent two independent experiments with at least three mice in each experimental group.

We thus analyzed *iNOS* expression in the spleen and lung. Similar to *IFN-γ* expression, we observed strong induction of iNOS in infected C57BL/6 wt mice as well as in Th-reconstituted $Rag1^{-/-}$ mice. However, after infection, $Rag1^{-/-}$ mice without T cell reconstitution showed enhanced *IFN-γ* and *iNOS* expression as well. Therefore, our results are inconsistent with a direct correlation between strength of protection and abundance of *IFN-γ* and *iNOS*, particularly after Th reconstitution of $Rag1^{-/-}$ mice. This observation is in accordance with results previously described by Scanga et al. (17), who found that $CD4^+$ T cell-depleted mice were more susceptible to *M. tuberculosis* infection but did not show decreased IFN-γ and iNOS expression.

Transfer of T_{reg} into $Rag1^{-/-}$ mice did not contribute to protection against *M. tuberculosis* (Fig. 1*B*). When cotransferred with Th, T_{reg} even suppressed protection mediated by effector $CD4^+$ T cells (Fig. 1*B*). To better understand the mechanisms of T_{reg}-mediated suppression of Th, we performed intracellular IL-10 staining of $CD4^+$ T cells recovered from *M. tuberculosis*-infected wt and T cell-reconstituted $Rag1^{-/-}$ mice as well as real-time RT-PCR analyses of *IL-10* mRNA in tissue samples from these mice. Flow cytometric analysis revealed that the suppressive effect of T_{reg} on Th was not correlated by increasing numbers or frequencies of IL-10-producing $CD4^+$ T cells, neither among the transferred T_{reg} nor among the Th (Fig. 2, *A* and *D*). Moreover, total IL-10 expression did not differ significantly between infected C57BL/6 wt and Th- or Th- plus T_{reg}-reconstituted $Rag1^{-/-}$ mice (Fig. 3). Thus, our results reveal no correlation between IL-10 and probably also TGF-β (preliminary data) abundance and T_{reg}-mediated suppression of protection against tuberculosis. Both cytokines represent critical mediators of T_{reg}-controlled immune responses (12). In contrast to our results, reactivation of *M. tuberculosis* in mice was found to correlate with increased IL-10 expression after treatment with TNF-α-neutralizing Abs (18). However, reactivation of *M. tuberculosis* due to TNF-α neutralization probably encompasses different mechanisms of regulation as compared with our T cell transfer model. In humans, active tuberculosis correlates with increased IL-10 blood serum levels (19). Although, at first sight, this observation might seem to contradict our findings, it is essentially in line with our results, given the fact that murine tuberculosis represents a chronic disease, rather than latent infection, and that IL-10 expression was observed in all *M. tuberculosis*-infected mice harboring T cells. More precisely, we observed a correlation between IL-10 and the presence of T cells, although not specifically with T_{reg}. A possible explanation for this observation could be that other cells, like *M. tuberculosis*-infected dendritic cells and macrophages, express IL-10, particularly in the presence of effector T cells (20, 21).

Despite significant differences in protection and T cell responses, histological analyses revealed similar granuloma shape, size, and numbers between infected C57BL/6 wt and T cell-reconstituted $Rag1^{-/-}$ mice (data not shown).

In conclusion, our experiments focused on protective immunity conferred by $CD4^+$ Th. In this adoptive transfer system, $CD4^+$ Th were capable of controlling *M. tuberculosis* with high efficiency only in the absence of T_{reg}. We do not exclude the contribution of other cells to protection such as $CD8^+$ T cells, which have been found to participate in control of tuberculosis, particularly at later stages of disease (22). It remains to be established whether $CD8^+$ T cells are also under the control of T_{reg} during natural infection. So far, it is unclear why T_{reg} are activated during tuberculosis if they prevent sterile eradication and thus increase the risk of disease reactivation. It is possible that T_{reg} are programmed by specific microbial stimuli, ensuring survival of tubercle bacilli as suggested for malaria parasites (23). Suppression by T_{reg} could also serve as a means of the immune system to hedge a constant antigenic load within the host, thus maintaining a constant pool of mycobacteria-specific T cells ready to defend the host against reinfection. Such a mechanism has been described for infection with *Leishmania major* (11, 24–26). It is likely that latent infection provides a constant stimulus for Th as well as T_{reg}. A balanced response of both T cell populations can curtail both active disease and extensive pathology. This immune response is sufficiently fine-tuned for the majority of *M. tuberculosis*-infected individuals and only a minority experiences disease outbreak. However, in the face of HIV, latent *M. tuberculosis* infection has become a more serious issue. Coinfection with HIV debilitates the immune response, which controls *M. tuberculosis*, thus increasing the risk of disease reactivation by several 100-fold (4). A better understanding of the mechanisms by which T_{reg} suppress protective immunity against tuberculosis could promote the design of novel intervention strategies aimed at generating highly efficacious T cell responses unimpaired by concomitant generation of T_{reg}.

Disclosures

The authors have no financial conflict of interest.

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