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*J Immunol* 2007; 178:2661-2665; doi: 10.4049/jimmunol.178.5.2661  
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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/6-Rag1tm1Mom 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 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 (Treg) in control of immune responses to self-Ags and in immune homeostasis is well established, and there is increasing evidence for a role of Treg in the regulation of immunity to infection (11, 12). It is believed that these Treg 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.

Treg represent 5–10% of the CD4+ T cell population and are characterized by the expression of the transcription factor...
Foxp3. A large fraction of the T_{reg} population is CD25^{+}, and T_{reg} secret up to 90% of the CD4^{+}CD25^{+} T cell subset in naïve 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 Consumer Protection 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 (Glass-Col). Inocula were confirmed at day 1 postinfection by placing complete lung homogenates onto Middlebrook 7H11 ampicillin plates. CFU in the lung and spleen were determined after mechanical disruption of control in water supplemented with 1% skim milk 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 naïve C57BL/6 donor mice were incubated with purified rat IgG and anti-CD16/CD32 mAb (clone, 2.4G2). After 5–10 min, biotinylated anti-CD25 mAb (clone PC61) and magnetic anti-CD11b 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 >90% CD4^{+}, <5% CD25^{+}, and <4.0% Foxp3^{+}. Purified CD25^{−}, non-CD4^{+}CD25^{+}T cells (2 × 10^{5}) or CD4^{+}CD25^{+} T cells (1 × 10^{5}) or a combination of both cell populations were adoptively transferred (i.v.) into Rag1^{−/−} 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^{5} 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-IL-10 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 produced from hybridoma supernatants. The anti-CD25 and anti-Foxp3 mAbs 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.

Statistical analysis

Statistical significance of results was determined with 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 Treg into infected tissue (Fig. 1A). Rag1^{−/−} mice were infected with M. tuberculosis H37Rv using 15 pmol of each primer and 15 μl of 2X SYBR Green PCR Master mix (Applied Biosystems). Quantification was performed at least twice with independent cDNA samples and in triplicate for each cDNA and primer pair. Results 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^{\text{Ct}}). 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. Data analysis was performed using the Mann-Whitney U test for a nonparametric sample distribution ($p<0.05$).

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 at 94°C and 60 s at 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 CTT GGT GCA TCC ATG AAA C; β-actin-reverse (rev)), TAA ACG GCA GC TGT TAA CAG TCC G; GAPDH-fw, GAC AAT CCT ACT CCT CCA CCT TG; GAPDH-rev, CCT CTC TTTG CCT AGT GTC TTTG C; IL-10-fw, GAC GAA CAT ACT GCT AAC GTA CTC ATT; IL-10-rev, CTG CTC CAG TCG CTT GCT CTT AGT; IFN-γ-fw, AGG GAC CAG TCA TGG AAA GCC TA; IFN-γ-rev, CTG ACC ATC CTT TGG CCA GTC TTG CC; IL-10-fw, GGA GAA CAT ACT GCT AAC GTA CTC ATT; IL-10-rev, inducible NO synthase (iNOS)-fw, GAC GAG ACG AGT AGG CAG AGA TTG; iNOS-rev, CTT CCC AGG AGC AGA TGG ATG AG). Reaction mixtures were set up to a final volume of 30 μl using 15 pmol of each primer and 15 μl of 2X SYBR Green PCR Master mix (Applied Biosystems). Quantification was performed at least twice with independent cDNA samples and in triplicate for each cDNA and primer pair. Results 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^{\text{Ct}}). 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. Data analysis was performed using the Mann-Whitney U test for a nonparametric sample distribution ($p<0.05$).
LOSIS. C57BL/6 wt mice were infected with M. tuberculosis. C57BL/6 wt mice, and Rag1−/− mice reconstituted with either selected CD4+CD25+ T cells (Th) or CD4+CD25− T cells (Treg), or a combination of both (Th + Treg), 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+ CD25+ 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 Treg before infection using flow cytometry and intracellular cytokine staining (Fig. 2). Transfer of Th without Treg led to high numbers and frequencies of IFN-γ-producing CD4+ T cells, whereas cotransfer of Th and Treg not only prevented the development of IFN-γ-producing CD4+ T cells among the cotransferred Th cells, but also suppressed Th proliferation (Fig. 2B). 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).
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 Treg into Rag1−/− mice did not contribute to protection against M. tuberculosis (Fig. 1B). When cotransferred with Th, Treg even suppressed protection mediated by effector CD4+ T cells (Fig. 1B). To better understand the mechanisms of Treg-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 Treg on Th was not correlated by increasing numbers or frequencies of IL-10-producing CD4+ T cells, neither among the transferred Treg 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 Treg-reconstituted Rag1−/− mice (Fig. 3). Thus, our results reveal no correlation between IL-10 and probably also TGF-β (preliminary data) abundance and Treg-mediated suppression of protection against tuberculosis. Both cytokines represent critical mediators of Treg-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 Treg. 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 Treg. 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 Treg during natural infection. So far, it is unclear why Treg are activated during tuberculosis if they prevent sterile eradication and thus increase the risk of disease reactivation. It is possible that Treg are programmed by specific microbial stimuli, ensuring survival of tubercle bacilli as suggested for malaria parasites (23). Suppression by Treg 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 Treg. 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 Treg 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 Treg.

Disclosures
The authors have no financial conflict of interest.

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