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Autocrine IL-10 Induces Hallmarks of Alternative Activation in Macrophages and Suppresses Antituberculosis Effector Mechanisms without Compromising T Cell Immunity

Tanja Schreiber,²* Stefan Ehlers,†‡ Lisa Heitmann,*, Alexandra Rausch,³* Jörg Mages,§ Peter J. Murray,⁷ Roland Lang,⁸ and Christoph Hölscher⁴*

Elevated IL-10 has been implicated in reactivation tuberculosis (TB). Since macrophages rather than T cells were reported to be the major source of IL-10 in TB, we analyzed the consequences of a macrophage-specific overexpression of IL-10 in transgenic mice (macIL-10-transgenic) after aerosol infection with Mycobacterium tuberculosis (Mtb). MacIL-10 transgenic mice were more susceptible to chronic Mtb infection than nontransgenic littermates, exhibiting higher bacterial loads in the lung after 12 wk of infection and dying significantly earlier than controls. The differentiation, recruitment, and activation of Th1 cells as well as the induction of IFN-γ-dependent effector genes against Mtb were not affected by macrophage-derived IL-10. However, microarray analysis of pulmonary gene expression revealed patterns characteristic of alternative macrophage activation that were overrepresented in Mtb-infected macIL-10 transgenic mice. Importantly, arginase-1 gene expression and activity were strikingly enhanced in transgenic mice accompanied by a reduced production of reactive nitrogen intermediates. Moreover, IL-10-dependent arginase-1 induction diminished antimycobacterial effector mechanisms in macrophages. Taken together, macrophage-derived IL-10 triggers aspects of alternative macrophage activation and promotes Mtb recrudescence independent of overt effects on anti-TB T cell immunity. The Journal of Immunology, 2009, 183: 1301–1312.

Tuberculosis (TB) is one of the most prevalent bacterial infections worldwide and constitutes a leading global health threat (1). Human TB caused by Mycobacterium tuberculosis (Mtb) is responsible for 8 million new cases and ~2 million deaths annually. Primary TB is thought to depend on a combination of genetic susceptibility of the host and virulence of the infecting Mtb strain, whereas postprimary TB is due to a temporal dysregulation of otherwise intact immune defenses. More detailed elucidation of the balance between protective and inflammatory immune responses to Mtb may accelerate the development of more effective vaccines and therapies targeting both or either form of TB.

The cell-mediated immune response is known to be critical in the host defense against infection with intracellular pathogens such as mycobacteria. Th1 lymphocytes play an important role in granuloma formation by secreting type 1 cytokines, primarily IFN-γ and TNF (2, 3). These cytokines stimulate the antimicrobial activity of infected macrophages, allowing intracellular bacterial killing through reactive nitrogen intermediates (RNI) and LRG-47 (4, 5). IL-12 indirectly promotes an effective cell-mediated immune response by inducing IFN-γ production from T and NK cells. On the other hand, these protective T cell-mediated immune responses also play a causative role in the development of the pathology of this type of chronic disease (6). Hence, down-regulation of the inflammatory response by endogenous factors that limit tissue damage can promote establishment of chronic infection and may also lead to reactivating TB. In fact, increased levels of Th2-type anti-inflammatory cytokines such as IL-4 and IL-10 were detected in patients suffering from postprimary disease (7).

IL-10 is produced by macrophages, T cells, B cells, and a variety of other cell types. The effects of IL-10 on immune responses are mostly inhibitory (8). In macrophages, IL-10 inhibits antimicrobial effector mechanisms, the expression of costimulatory molecules, and the production of proinflammatory cytokines (9, 10). Inhibition of IL-12 production by IL-10 (11) may be one mechanism by which IL-10 blocks the development of protective Th1 immune responses (12). The absence of IL-10 causes overproduction of inflammatory cytokines (13) and the development of chronic inflammatory bowel disease (14). On the other hand, IL-10-deficient (−/−) mice also show increased resistance to intracellular pathogens such as Listeria monocytogenes (15), Leishmania major (16), and Trypanosoma cruzi (17). Therefore, IL-10 is...
required to prevent immunopathology, but it can also impair protective responses against several pathogens. The immunoregulatory function of IL-10 and the fact that elevated levels of IL-10 have been detected in TB patients (18, 19) suggest a role for IL-10 in susceptibility to TB. However, one report of Mtb infection in IL-10−/− mice found neither increased protective immune responses nor exacerbated immunopathology (20).

By contrast, mice lacking IL-10 were shown to clear Mycobacterium bovis bacillus Calmette-Guérin infection more efficiently (21). Taken together, further investigation of Mtb infection in different IL-10 genetic models differing in the level or mates. To generate BMMo, bone marrow cells were cultivated in L-929 conditioned medium as source for M-CSF activity for 9 days (27).

Materials and Methods

Mice and macrophages

Transgenic mice (macIL-10g mice) specifically expressing IL-10 in macrophages under control of the human CD68 promoter were bred under specific pathogen-free conditions at the Technical University of Munich (25). macIL-10g mice were on an FVB genetic background, and transgene-negative littermates were used as controls (FVB). Myeloid-specific arginase-1-deficient Tie2crv.Arg1flox/flox mice were housed at St. Jude Children’s Research Hospital (26). In any given experiment, mice were matched for age and sex. For experiments, mice were maintained under barrier conditions in the BSL 3 facility at the Research Center Borstel. Before infection of experimental animals, stock solutions of mycobacteria were diluted in sterile distilled water, and pulmonary infection was performed using an inhalation exposure system (Glas-Col). To infect mice with 100 CFU of Mtb or 105 M. avium per lung, animals were exposed for 40 min to an aerosol generated by nebulizing ~5.5 ml of a suspension containing 2 × 106 or 1 × 107 live bacteria/ml, respectively. Inoculum size was checked 24 h after infection by determining the bacterial load in the lung of infected mice. Mice were regularly weighed before and after infection. In accordance with the Animal Research Ethics Board of the Ministry of Environment, mice that lost 25% of their original weight during the course of infection had to be sacrificed.

Colonies were harvested, aliquoted, and frozen at −80°C. After thawing, viable cell counts were determined by plating serial dilutions of the cultures on Middlebrook 7H10 agar plates followed by incubation at 37°C. All experiments were performed in the BSL 3 laboratories at the Research Center Borstel. Before infection of experimental animals, stock solutions of mycobacteria were diluted in sterile distilled water, and pulmonary infection was performed using an inhalation exposure system (Glas-Col). To infect mice with 100 CFU of Mtb or 105 M. avium per lung, animals were exposed for 40 min to an aerosol generated by nebulizing ~5.5 ml of a suspension containing 2 × 106 or 1 × 107 live bacteria/ml, respectively. Inoculum size was checked 24 h after infection by determining the bacterial load in the lung of infected mice. Mice were regularly weighed before and after infection. In accordance with the Animal Research Ethics Board of the Ministry of Environment, mice that lost 25% of their original weight during the course of infection had to be sacrificed.

RNA isolation and microarray analysis of pulmonary gene expression

Before and at different time points after aerosol infection with Mtb, weighed lung samples were homogenized in 5 ml of 4 M guanidinium-acid-albumin-dextrose-catalase (OADC) enrichment medium (Life Technologies), 0.002% glycylglycine, and 0.05% Tween 80. Mid-log-phase cultures were harvested, aliquoted, and frozen at −80°C. After thawing, viable cell counts were determined by plating serial dilutions of the cultures on Middlebrook 7H10 agar plates followed by incubation at 37°C. All experiments were performed in the BSL 3 laboratories at the Research Center Borstel. Before infection of experimental animals, stock solutions of mycobacteria were diluted in sterile distilled water, and pulmonary infection was performed using an inhalation exposure system (Glas-Col). To infect mice with 100 CFU of Mtb or 105 M. avium per lung, animals were exposed for 40 min to an aerosol generated by nebulizing ~5.5 ml of a suspension containing 2 × 106 or 1 × 107 live bacteria/ml, respectively. Inoculum size was checked 24 h after infection by determining the bacterial load in the lung of infected mice. Mice were regularly weighed before and after infection. In accordance with the Animal Research Ethics Board of the Ministry of Environment, mice that lost 25% of their original weight during the course of infection had to be sacrificed.

Bacterial loads in lung, liver, and spleen were evaluated at different time points after infection with mycobacteria to follow the course of infection. Organs from sacrificed animals were removed aseptically, weighed, and homogenized in PBS containing a proteinase inhibitor mixture (Roche Diagnostics) for subsequent protein extraction. cDNA synthesis and amplification were performed according to the manufacturer’s instructions. Ten-fold serial dilutions of organ homogenates were plated in duplicates onto Middlebrook 7H10 agar plates containing 10% OADC and incubated at 37°C for 19–21 days. Colonies on plates were enumerated, and results were expressed as log10 CFU per organ.

Immunohistology

One lung lobe per mouse was fixed in 4% formalin-PBS, set in paraffin blocks, and sectioned (2–3 μm). For immunohistolological detection of NO2, tissue sections were deparaffinized, placed in 10-nm sodium citrate buffer (pH 6.0), and then pressure-cooked for exactly 1 min. After blocking for 20 min in 1% H2O2 solution, slides were incubated with appropriately diluted polyclonal rabbit anti-mouse inducible NO synthase (NOS2) (BIOMOL) in TBS/10% FCS for 30 min in a humid chamber. Appropriately diluted goat anti-rabbit IgG-peroxidase conjugate (tertiary Ab) (Dianova) were used in sequential incubations of 30 min each. For immunohistolological detection of Bacterial loads in lung, liver, and spleen were evaluated at different time points after infection with mycobacteria to follow the course of infection. Organs from sacrificed animals were removed aseptically, weighed, and homogenized in PBS containing a proteinase inhibitor mixture (Roche Diagnostics) for subsequent protein extraction. cDNA synthesis and amplification were performed according to the manufacturer’s instructions. Ten-fold serial dilutions of organ homogenates were plated in duplicates onto Middlebrook 7H10 agar plates containing 10% OADC and incubated at 37°C for 19–21 days. Colonies on plates were enumerated, and results were expressed as log10 CFU per organ.

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Bone marrow-derived macrophages (BMMo) were obtained after flushing of femora from Tie2−/−pos Arg1+/-mice and cre-negative littermates. To generate BMMo, bone marrow cells were cultivated in L-929 conditioned medium as source for M-CSF activity for 9 days (27).
Benjamini-Hochberg (28). The 4188 probe sets passing the filtering criteria were then subjected to hierarchical clustering and visualization procedures using the Spotfire DecisionSite Functional Genomics software. Data mining of differentially regulated gene lists for enrichment of coassociations with cells and tissues was performed using Genomatix Bibliosphere software (Genomatix).

**RT-PCR**

From extracted RNA, CDNA was obtained using murine moloney leukemia virus reverse transcriptase (Invitrogen) and oligo(dT) (12–18-mer; Sigma-Aldrich) as a primer. Quantitative PCR was performed on a LightCycler (Roche Diagnostics) as described previously (29). Data were analyzed using the “Fit Points” and “Standard Curve Method” using hprt as housekeeping gene to calculate the level of gene expression normalized for hprt expression. The following Abs, CD4-allophycocyanin, CD3-PerCP, CD44-FITC, mouse and rat serum to block nonspecific binding to FcR. Cells were then incubated with a mixture containing anti-Fc

**Flow cytometric analysis of surface markers and intracellular cytokines**

For flow cytometric analysis of surface markers, cells were washed and incubated with a mixture containing anti-FcyRII/III mAb (clone 2.4G2), mouse and rat serum to block nonspecific binding to FcR. Cells were then incubated in consecutive steps for 20 min with optimal concentrations of the Abs, 4°C-allophycocyanin, CD3-PerCP, CD4-PE, CD62L-PE, IA4-PE, CD124-PE (IL-4RA) (all from BD Biosciences), F4/80-AlexaFluor647 (CalTag Laboratories), and CD206 (MMR) (Serotec). For detection of intracellular IFN-γ, an intracellular cytokine staining kit was used (BD Biosciences). Briefly, single-cell suspensions were prepared at 19 days after infection, and 2 × 10^6 cells were stimulated with plate-bound anti-CD3/CD28 mAb (clone 2C11 and clone 37/51 at 10 μg/ml, respectively) for 4 h in the presence of GolgiPlug (BD Biosciences). Non-specific Ab binding was blocked by incubation with a mixture containing anti-FcyRII/III mAb (clone 2.4G2), mouse and rat serum. Cells were washed and incubated with optimal concentrations of anti-CD4-FITC (BD Biosciences). After staining, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), and intracellularly accumulated IFN-γ was labeled with PElabeled anti-IFN-γ mAb (BD Biosciences). For arginase activity in lung homogenates

To determine arginase activity in murine tissue, weighed pieces of organs were homogenized in 100 μl of 0.1% Triton X-100 (Sigma-Aldrich) containing a protease inhibitor mixture (Roche). Fifty microliters of 10 mM MnCl2 (Merck) and 50 mM Tris-HCl (Merck) was added to all samples, and the enzyme was activated by heating for 10 min at 55°C. Arginine hydrolysis was conducted by incubating 25 μl of the activated lysate with 25 μl of 0.5 μL-arginine (Merck) at 37°C for 60 min. The reaction was stopped with 400 μl of H2SO4 (96%/H3PO4, 85%/H2O (1/37, v/v/v). As a degree of arginase activity, the urea concentration was measured at 540 nm after addition of 25 μl of α-isonicotinopropiophenone (Sigma-Aldrich; dissolved in 100% ethanol) followed by heating at 95°C for 45 min. One unit of arginase activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol urea/min.

**Detection of RNI**

To detect RNI in uninfected and infected mice, blood was collected at different time points. Using a commercial nitrate reductase kit (Cayman Chemical), NOx was converted into NO2 and added. After incubation with HRP coupled to avidin and developing with tetramethylbenzidine substrate reagent, the absorbance was read on a microplate reader (Sunrise; Tecan). Using a test wavelength of 450 nm and a reference wavelength of 630 nm, samples were compared with appropriate recombiant cytokine standards. The detection limit for all cytokines were 5 pg/ml.

**IFN-γ-induced bacterial growth inhibition in macrophages**

To analyze the antibacterial activity of macrophages, BBM6 were cultured 48 h before infection with Mtb in DMEM supplemented with 10% FCS (Biologhrom), 2 mM l-glutamine, 1 mM sodium pyruvate, and 10 mM HEPES (all from PAA) in the presence of medium or infected with 10 ng/ml IL-4 (BD Biosciences) and 10 ng/ml IL-10 (BD Biosciences). One day before infection, cells were incubated with medium or stimulated with 100 μM IFN-γ (ProtopTech). Cells were infected with Mtb H37Rv at a multiplicity of 5. To determine bacterial uptake at different time points, macrophages were lysed by addition of 0.5% Triton X-100 (Serva). Lysates were serially diluted in 0.05% Tween 80 (Merck), plated on Middlebrook 7H10 agar containing 10% OADC, and incubated at 37°C for 19–21 days. Colonies on plates were enumerated, and results were expressed as log_{10} CFU per culture.
test defining different error probabilities (*, \( p \leq 0.05; **, p \leq 0.01; ***, p \leq 0.001 \)). ANOVA was performed using the Dunnett multiple comparison test, correcting for multiple testing, which defines different error probabilities between untreated cells and macrophages that have been incubated 21 days after plating of serial diluted organ homogenates. Data represent means and SDs of four mice. One experiment representative of three performed is shown. Statistical analysis was performed using the unpaired Student’s \( t \) test defining differences between FVB and macIL-10\(^\alpha\) mice as significant (*, \( p \leq 0.05 \)), C, during the course of infection, survival of 10 infected mice per group was monitored. Animals that lost >25% of their original body weight were sacrificed. Statistical analysis of the resulting survival curve was performed using the log-rank test. Differences in survival kinetics between FVB and macIL-10\(^\alpha\) mice were highly significant \( (p = 0.001) \). Therefore, the experiment was terminated, and remaining wild-type animals were euthanized in compliance with ethical guidelines for animal experimentation.

Results

macIL-10\(^\alpha\) mice are highly susceptible to Mtb infection

To determine the influence of increased levels of macrophage-derived IL-10 on the outcome of experimental TB, mice overexpressing IL-10 under control of the macrophage-specific CD68 promoter were infected with 100 CFU of Mtb via the aerosol route. Whereas FVB control mice were able to control mycobacterial infection in the lungs of both FVB and macIL-10\(^\alpha\) mice during the course of infection with Mtb, downstream effector mechanisms could have been directly impaired by the macrophage-specific overexpression of IL-10. Quantitative real-time PCR revealed that during Mtb infection transcripts for Nos2 were increasingly induced in lungs from both FVB and macIL-10\(^\alpha\) mice to the same extent (Fig. 3a). In fact, at day 90 postinfection, Nos2 gene expression was found to be even higher in transgenic animals. It should be noted that IL-10 has been shown to induce Nos2 expression in some settings, suggesting that Nos2 is not a canonical target of the IL-10 anti-inflammatory response (32). The kinetics of gene expression of Lrg-47 (Irgm1) was comparable in the lungs of both FVB and macIL-10\(^\alpha\) mice peaking on day 21 of Mtb infection (Fig. 3b). Six weeks after infection, immunohistochemical detection of Nos2 in lung sections verified these findings on the protein level, showing no differences in Nos2 expression between FVB and macIL-10\(^\alpha\) mice (Fig. 3c).

Macrophage-derived IL-10 specifically suppresses IL-12p40 in infected tissue but not in draining lymph nodes

So far, the increased susceptibility of macIL-10\(^\alpha\) mice to experimental TB could neither be attributed to an impaired function of IFN-\(\gamma\)-producing T cells nor to defective IFN-\(\gamma\)-dependent downstream mechanisms in macrophages. IL-10 specifically suppresses the production of proinflammatory cytokines in activated macrophages and dendritic cells (9, 33). To evaluate the suppressive effect of macrophage-derived IL-10 on anti-TB effector cytokines during the course of infection with Mtb, we compared the expression of TNF (produced by T cells and macrophages/dendritic cells) and IL-12p40 (produced by macrophages/dendritic cells) in the lungs of FVB wild-type and IL-10\(^\alpha\) mice. Until day 90 after infection, increasing amounts of Tnf transcripts were found in the lungs of FVB mice (Fig. 4a). In the lungs of macIL-10\(^\alpha\) mice, however, gene expression was found to be even higher on days 49 and 90 postinfection. In contrast, 49 and 90 days after Mtb infection, gene expression of IL-12p40 (I12b) was found to be significantly reduced in lungs from macIL-10\(^\alpha\) mice when compared with FVB mice (Fig. 4b).

After Mtb infection, IL-12 is essential for driving protective Th1 immune responses (34). In lymphoid organs, IL-10 inhibits IL-12-driven Th1 development, whereas it has no suppressive effect on already differentiated Th1 cells in infected tissue (35). Because Mtb-infected macIL-10\(^\alpha\) mice displayed a normal Th1 development, we speculated that the magnitude of transgenic IL-10 expression would be different in draining lymph nodes and lung tissue. In contrast to dendritic cells, only a few macrophages are
wild-type and macIL-10tg mice represent two performed is shown. Mtb shapes the host response to infection. To investigate how macrophage-specific overexpression of IL-10 in macrophages impacts the host response to Mtb infection, at the early time point day 25, of all probe sets induced by infection, 158 showed higher expression in macIL-10tg, whereas 68 only one probe set (C3ar1) had significantly increased expression in macIL-10tg mice, whereas Igh-VJ588 was higher in FVB control animals. Of the 970 probe sets induced by Mtb 42 days after infection, 158 showed higher expression in macIL-10tg, whereas 68 were significantly higher in lungs from wild-type mice. Therefore, the majority of IL-10 effects was apparent at the later stages of infection.

Impact of Mtb infection on pulmonary gene expression in wild-type and macIL-10tg mice

To investigate how macrophage-specific overexpression of IL-10 shapes the host response to Mtb at the transcriptome level, genome-wide expression analysis was performed on tissue RNA isolated from the lungs of FVB and macIL-10tg mice at baseline as well as 25 and 42 days after infection. Excluding variability for the infection itself, only 219 probe sets were different between FVB and macIL-10tg, indicating a comparatively small effect of IL-10 on the transcriptional changes. The effect of IL-10 on the number of up- and down-regulated genes is summarized in Fig. 5a. Before infection, only 36 probe sets were up-regulated in lungs from macIL-10tg mice compared with FVB animals (data not shown). These included some known IL-10-induced genes (Msr1, Ccr2, Ccr5, Fcgr2b, and Saa3). Only four probe sets, all detecting Igg, gave significantly higher signals in lungs from FVB mice. After infection, at the early time point day 25, of all probe sets induced >3-fold in either lung RNA from wild-type or macIL-10tg mice, only one probe set (C3ar1) had significantly increased expression in macIL-10tg mice, whereas Igh-VJ588 was higher in FVB control animals. Among the 970 probe sets induced by Mtb 42 days after infection, 158 showed higher expression in macIL-10tg, whereas 68 were significantly higher in lungs from wild-type mice. Therefore, the majority of IL-10 effects was apparent at the later stages of infection.

Hierarchical clustering of the probe sets regulated by IL-10 (Fig. 5b; data are listed in supplemental Table I) revealed groups of genes overexpressed in the lungs of macIL-10tg mice already un-
and cytokines (Il1b and Il6) were also expressed at higher levels in the lungs of macIL-10<sup>tg</sup> mice compared with wild-type animals, indicating that the inhibitory effect of IL-10 on proinflammatory gene expression is likely affected by the increased bacterial load in macIL-10<sup>tg</sup> mice.

**Data mining: changes in cellular composition**

The presence of various transcripts encoding cell type-specific surface proteins, such as the dendritic cell marker Cd207 in cluster C and the monocyte/macrophage marker Msr1 in cluster B, suggested an impact of IL-10 on the infection-induced changes in the cellular composition of the lung tissue. We used a literature-based data mining tool, Genomatix Bibliosphere, to search for overrepresentation of anatomical terms in lists of genes up-regulated in macIL-10<sup>tg</sup> or wild-type mice after Mtb infection (Fig. 6a). Although transcriptional evidence for NK cells, dendritic cells, and T lymphocytes was similar in both genotypes, in the lungs of macIL-10<sup>tg</sup> mice, a strong enrichment for probe sets associated with macrophages was evident. In contrast, probe sets up-regulated to higher levels in FVB lungs at day 42 were connected to B lymphocytes. Immunohistological analysis also revealed a moderately reduced infiltration of B cells in lungs from macIL-10<sup>tg</sup> mice (supplemental Fig. 1).<sup>6</sup> Taken together, IL-10 appears to regulate leukocyte subset migration to the lung, or local proliferation of the cells, during Mtb infection.

**Signs of alternative macrophage activation in the lung transcriptome after Mtb infection**

Among the group of genes induced to high levels in infected macIL-10<sup>tg</sup> mice was Arg1 (Fig. 6b), encoding the enzyme arginase-1, whose extrahepatic expression is typically associated with IL-4/IL-13-induced alternative macrophage activation, indicating that after Mtb infection IL-10 may promote the development of alternatively activated macrophages. Although Il4 and Il13 mRNA was not detectably expressed in the lung data set, small amounts of IL-4 or IL-13 could promote alternative activation of lung macrophages in macIL-10<sup>tg</sup> mice. IL-10 may enhance the sensitivity to IL-4 because it increases Il4ra expression on macrophages in vitro (32), an effect that was also observed in the lung data set after Mtb infection (Fig. 6b). In addition to arginase-1, many other markers have been described to define IL-4Ra-mediated alternatively activated macrophages, especially during pulmonary inflammation such as Fizz1, various chitinases, and MMR (29). Among these genes, only Fizz1 (encoded by Retnla) and the chitinase Chi3l1 were clearly expressed at higher levels in macIL-10<sup>tg</sup> lungs (Fig. 6b), whereas the chitinases Ym1 (= Chi3l3) and Ym2 (= Chi3l4) as well as the Mmr (encoded by Mrc1) were down-regulated after infection to similar levels in both genotypes. The differential induction of markers for alternatively activated macrophages in Mtb-infected macIL-10<sup>tg</sup> mice reflects also some opposite biological effects of IL-10 to IL-4 and IL-13, e.g., the regulation of MMR expression and function (37). In contrast to this rather inhibitory effect, IL-10 has been clearly shown to contribute to alternative macrophage activation during TLR ligation by a strong synergistic induction of arginase-1 (32). Taken together,
our genome-wide expression analysis revealed that after infection with Mtb, macrophage-derived IL-10 induces several features of alternatively activated macrophages.

After Mtb-infection, macIL-10<sup>tg</sup> mice develop alternatively activated macrophages.

To verify our findings revealed by genome-wide expression analysis, we next performed quantitative RT-PCR analysis to test the model that macrophage-derived IL-10 promotes alternative macrophage activation after Mtb infection (Fig. 7a). Six weeks after infection, we found significantly elevated gene expression of Fizz1 and Arg1. In contrast, transcripts for Mmr and the chitinase Ym1 were not elevated. In whole tissue, Il4ra gene expression in macIL-10<sup>tg</sup> mice was elevated but not significantly different from gene expression in lungs from FVB wild-type mice. To determine the activation state of macrophages in Mtb-infected macIL-10<sup>tg</sup> mice, we analyzed macrophages from perfused lungs by flow cytometry. Six weeks after infection, IAq, CD80, CD86, and the MMR were expressed to the same extent on propidium iodide-negative F4/80-positive cells from FVB and macIL-10<sup>tg</sup> mice. Whereas only a subpopulation of macrophages from infected FVB mice expressed the IL-4Rα, all macrophages from macIL-10<sup>tg</sup> mice were found to be positive for this receptor subunit. Taken together, we found that after Mtb infection IL-10 secretion by macrophages induces an alternatively activated program characterized by an enhanced expression of Fizz1, Arg1, and the Il4ra.
In Mtb-infected macIL-10tg mice, increased arginase-1 activity is concomitant with reduced production of RNI.

Classical macrophage activation plays an important role in combating infection with Mtb through IFN-γ-induced expression of effector molecules such as NOS2-dependent RNI (4). This induction may be counterregulated in alternatively activated macrophages. Because in alternatively activated macrophages arginase-1 hydrolyzes L-arginine to urea and L-ornithine, this pathway is also discussed to regulate RNI production in macrophages through depletion of L-arginine as the substrate for NOS2 (39, 40). Therefore, we speculated that in macIL-10tg mice an increased arginase-1 activity modulates the production of RNI, thereby affecting effector mechanisms against Mtb usually exerted by classically activated macrophages. A kinetic quantification of gene expression revealed that Arg1 is not induced in lungs from FVB mice during the course of Mtb infection (Fig. 8a). In contrast, Arg1 expression in macIL-10tg mice was found to be significantly induced during the late phase of Mtb infection. Accordingly, arginase activity in lungs from macIL-10tg mice was found to be elevated already 49 days postinfection (Fig. 8b). In contrast, arginase activity was detectable in the lungs of FVB mice but did not increase after infection (Fig. 8b). Concomitant with the increased arginase-1 activity in infected macIL-10tg mice, RNI were only moderately produced in these animals (Fig. 8c). In distinction, significantly increasing amounts of RNI were found in sera from Mtb-infected FVB mice (Fig. 8c). Hence, the increased susceptibility in macIL-10tg mice after infection with Mtb appeared to be caused by an elevated induction of arginase-1 in alternatively activated macrophages, which in turn led to an inefficient production of RNI. To examine whether IL-10-induced arginase-1 expression in macrophages diminishes antimycobacterial effector mechanisms in macrophages, we performed a Mtb growth inhibition assay in arginase-1-deficient BMM/H9278. Incubation of BMM/H9278 from Tie2cre-neg Arg-1flox/flox arginase-1-competent and Tie2cre-pos Arg-1flox/flox arginase-1-deficient mice lead to a significant reduction of mycobacterial growth 48 and 72 h after infection with Mtb when compared with infected macrophages that have not been activated with IFN-γ. Preincubation with IL-4 and IL-10, however, diminished IFN-γ-dependent mycobacterial growth inhibition in arginase-1-competent BMMφ. In contrast, IL-4/IL-10 treatment of IFN-γ-activated arginase-1-deficient macrophages did not lead to reduced antimycobacterial effector mechanisms, indicating that induction of arginase-1 in macrophages reduces the capability of controlling Mtb growth.

macIL-10tg mice control bacterial growth after M. avium infection

To test our hypothesis that macrophage-derived IL-10 mostly subverts NOS2-dependent effector mechanisms, we infected macIL-10tg mice aerogenically with M. avium, a pathogen that is insensitive to...
The Journal of Immunology

1309

NOS2-derived RNI production (6, 41), and determined bacterial loads in different organs 105 days after infection (Fig. 9). Bacterial loads in macIL-10<sup>tg</sup> mice were found to be only moderately increased in lung and liver and comparable to FVB mice in spleens. However, infection of NOS2<sup>−/−</sup> mice with <i>M. avium</i> revealed that bacterial loads rather decrease in the absence of NOS2 (40), corroborating the contention that after infection with RNI-insensitive mycobacteria the suppressive effect of NO on T cells appears (6). Therefore, the inability of macIL-10<sup>tg</sup> mice to efficiently control bacterial growth after infection with RNI-sensitive and, to some extent, also -insensitive mycobacteria indicates that autocrine IL-10 operates partially by reducing RNI production via arginase-1 induction.

**Discussion**

TB is a systemic disease that becomes manifest most prominently in the lung (24). Progressive primary TB occurs when the initial inflammatory focus is unable to contain mycobacterial replication, and the disease spreads to other parts of the lung. Postprimary disease develops when a lesion containing a previously “dormant”, i.e., growth-arrested organism resumes growth after a period of clinical latency. This organism may have been implanted years before during a primary infection that was successfully contained by a protective Th1-type immune response. Reactivation is often a consequence of a perturbed immune status of the host, although the cause of deregulation may be multifactorial.

Elevated levels of the Th2 cytokine IL-10 were detected in individuals with active TB (18, 19). Importantly, macrophages rather than T cells were reported to be the major source of IL-10 in these patients (18). However, whether and how macrophage-derived IL-10 promotes exacerbation of primary infection or reactivation of chronic latent infection is not clear. Our study revealed that in macIL-10<sup>tg</sup> mice an increased production of IL-10 by macrophages resulted in enhanced <i>Mtb</i> replication, particularly during the chronic phase of infection, and thus bears some resemblance to reactivation TB in humans.

Humans infected with <i>Mtb</i> typically have a strong DTH response, as measured by the purified protein derivative skin test (24). This holds also true for patients suffering from postprimary TB. Thus, despite an apparently adequate initial differentiation of cell-mediated immune responses, <i>Mtb</i> is capable of persisting and can even resume replication within macrophages. In the draining lymph nodes of <i>Mtb</i>-infected macIL-10<sup>tg</sup> mice, T cells efficiently differentiated into Th1 cells and migrated to the site of infection. Despite the presence of macrophage-derived IL-10 in the lungs of infected transgenic mice, Th1 cells efficiently expressed effector functions and secreted sufficient IFN-γ to induce a classical macrophage activation characterized by expression of Nos2 and Lrg-47. However, bacteria survived and successfully proliferated within IFN-γ-activated IL-10<sup>−/−</sup> macrophages. It appears, therefore, that during experimental TB macrophage-derived IL-10 does not affect initiation and maintenance of Th1 immune responses but specifically acts on macrophage functions in a partially dominant manner over IFN-γ at the functional level.

It is important to note that overexpression of IL-10 specifically in T cells resulted in completely different immunosuppressive mechanisms after <i>Mtb</i> infection (22). rIL-10<sup>tg</sup> mice failed to efficiently prime naive CD4<sup>+</sup> T cells in draining lymph nodes, resulting in decreased numbers of activated T cells in the blood circulation and lung tissue; this defective Th1 response eventually led to an increased bacterial burden, due to a failure of classical macrophage activation. In contrast, IL-10 expression in macrophages at the site of infection induces an alternative activation program specifically in macrophages that counteracts concomitant classical macrophage activation. Since T cell-mediated immune responses remain largely unaffected (as is the case in human postprimary TB), the outcome of experimental TB in macIL-10<sup>tg</sup> mice may therefore better reflect the situation in patients with reactivating TB.
We could link the increased susceptibility of *Mtb*-infected macIL-10<sup>tg</sup> mice neither to an impaired function of IFN-γ-producing T cells nor to defective IFN-γ-dependent downstream mechanisms in macrophages. Transcriptome analysis revealed a strong enrichment for probe sets associated with macrophage function in the lungs of *Mtb*-infected macIL-10<sup>tg</sup> mice. IL-10 has previously been shown to act primarily on activated macrophages, where IL-10R expression is highest. The IL-10R activates STAT3, and loss of this transcription factor in macrophages mimics loss of IL-10 itself (42). Hence, because STAT3 in myeloid cells is both necessary and sufficient for the effects of IL-10, macrophages are major targets of IL-10 in vivo (42–45). On a functional level, IL-10 has been shown to negatively regulate macrophage function in a variety of experimental systems (17, 30, 31, 46). In vitro, IL-10 antagonizes the IFN-γ-dependent induction of NOS2 (30, 31). Our finding that macIL-10<sup>tg</sup> mice fail to efficiently control bacterial growth especially after infection with RNI-sensitive mycobacteria corroborates our contention that the mode of action of autocrine IL-10 proceeds mainly via arginase-1-mediated reduction of RNI production in macrophages. However, we found NOS2 normally expressed in *Mtb*-infected macIL-10<sup>tg</sup> mice, whereas the production of RNI was reduced during the course of infection. Hence, other pathways must be affected in macrophages of macIL-10<sup>tg</sup> mice that lead to an impaired expression of effector molecules such as RNI.

Among the group of genes induced to high levels in *Mtb*-infected macIL-10<sup>tg</sup> mice, some were associated with alternative macrophage activation such as *Il4ra*, *Fizz1*, and *Arg1* (29). Alternative macrophage activation is typically induced by IL-4Rα ligation. Although *Il4* and *Il13* mRNA was not detectably expressed in the lung data set, small amounts of IL-4 may still drive alternative activation of lung macrophages in macIL-10<sup>tg</sup> mice. IL-10 may enhance the sensitivity to IL-4 because it increases IL-4 receptor expression on macrophages in vitro (32), an effect that was also observed in the lung data set after *Mtb* infection. The differential induction of markers for alternatively activated macrophages in *Mtb*-infected macIL-10<sup>tg</sup> mice reflects also some opposite biological effects of IL-10 to IL-4 and IL-13, e.g., the regulation of MMR expression and function (37). In contrast to this rather inhibitory effect, IL-10 has been clearly shown to contribute to alternative macrophage activation during TLR ligation by a strong synergistic induction of arginase-1 (32). Hence, during experimental TB, *Arg1* expression may be directly induced by IL-10 or indirectly by IL-10-dependent up-regulation of the high-affinity IL-4Rα. Alternative activation of macrophages via the IL-4Rα has been shown to differentially regulate protective and immunopathological immune responses (29). Importantly, analysis of macrophage-specific IL-4Rα-deficient mice clearly demonstrated immunomodulation by macrophages to be a central regulatory mechanism during inflammatory immune responses in vivo (47). Because alternative macrophage activation reduces RNI production (40, 48, 49) and proinflammatory cytokine release (50), protective (39, 51, 52) and pathological (50) immune responses are significantly regulated by these types of macrophages.

In the context of TB and alternatively activated macrophages, one genome-wide expression analysis in IL-4-stimulated murine bone marrow-derived macrophages infected with *Mtb* has been published so far (51). A specific reanalysis of our lung transcriptome data for differences in the expression of the 15 most highly increased genes in *Mtb*-infected IL-4-stimulated macrophages revealed that 5 genes were expressed at significantly higher levels in macIL-10<sup>tg</sup> lungs than in FVB mice after infection (*Fcr2b, Fn1, Cish, Ccl9, and *Mrcl*), and *Cdhl* was increased under basal conditions in macIL-10<sup>tg</sup> lungs (data not shown). *Mmp12* (encoding matrix metalloproteinase-12, MMP-12) and *Tfr* (encoding the transferrin receptor, TFR) were found to be significantly up-regulated in *Mtb*-infected alternatively activated macrophages in vitro (51). The induction of the TFR appears to be of major importance in the context of experimental TB because intracellular *Mtb* exploit the transferrin-transferring receptor complex for iron utilization (53). However, neither our genome-wide expression analysis nor quantitative RT-PCR revealed a differential regulation of *Tfr* in *Mtb*-infected macIL-10<sup>tg</sup> mice in vivo (data not shown). Additionally, Kahnert et al. (51) suggested MMP-12 induced in alternatively activated macrophages to be involved in the pathogenesis of TB. Although our genome-wide expression analysis in *Mtb*-infected macIL-10<sup>tg</sup> mice revealed an increased expression of *Mmp12* in macIL-10<sup>tg</sup> mice at later time points of infection, the difference was not significant (data not shown). Moreover, quantitative RT-PCR of *Mmp12* did not confirm a difference between wild-type and macIL-10<sup>tg</sup> mice (data not shown). Taken together, genome-wide expression analysis in *Mtb*-infected and IL-4-induced alternatively activated macrophages in vitro and in *Mtb*-infected macIL-10<sup>tg</sup> mice in vivo revealed an overlapping set of regulated genes, consistent with our interpretation that IL-10 supports a macrophage phenotype in vivo that is phenotypically related to alternatively activated macrophages.

Until recently, little was known about the impact of alternative macrophage activation and arginase-1 on the outcome of *Mtb* infection. Classical macrophage activation plays a central role in combating infection with *Mtb* through IFN-γ-induced expression of effector molecules such as the NOS2-dependent production of RNI (4, 54). Importantly, inhibition of endogenous NOS2 reacti-vates latent infection in *Mtb*-infected mice, indicating that NOS2-dependent mechanisms in macrophages are crucial for the control of *Mtb* persisting in immunocompetent hosts (55). The production of RNI might be counterregulated by IL-4Rα and/or IL-10-dependent mechanisms, leading to arginase-1 expressing alternatively activated macrophages. In fact, RNI production in *Mtb*-infected alternatively activated macrophages was found to be reduced (51). In line with these findings, we here show the induc-tion of arginase-1 in macrophages subverts IFN-γ-induced antmycobacterial effector mechanisms in macrophages. Since NOS2 shares l-arginine as a substrate with arginase-1, substrate depletion by either enzyme is a key regulatory mechanism in macrophages, and differential expression of NOS2 and arginase-1 is important for regulating macrophage effector functions (40, 49). Very recently, El Kasmi et al. (26) showed that specific deletion of arginase-1 in macrophages reduced lung bacterial loads during *Mtb* infection, supporting our contention that arginase-1 induction in macrophages undermines NOS2-dependent effector mechanisms against *Mtb* by depletion of the common substrate l-arginine. In addition, arginase-dependent production of polyamines was shown to promote elevated growth of *L. major* in macrophages (52) and may also be beneficial for the proliferation of *Mtb*. Importantly, in human pulmonary TB, high production of IL-10 also parallels arginase activity in PBMC (56). Hence, elevated IL-10-dependent arginase activity during the chronic phase of infection may promote recrudescence of *Mtb* growth. We do not exclude that macrophage-derived IL-10 suppresses other antimycobacterial effector mechanisms in addition to regulating RNI synthesis. In contrast to infection with *Mtb*, macIL-10<sup>tg</sup> mice were indeed able to control bacterial growth after infection with *M. avium*, which has been shown to be insensitive to the effects of RNI (6, 41). However, because bacterial loads have been described to be further reduced after infection with *M. avium* in the complete absence of RNI in NOS2<sup>−/−</sup> mice, it appears that although autocrine IL-10 subverts...
The Journal of Immunology

RNI synthesis in macrophages other suppressive mechanisms may also be induced by IL-10.

We conclude that during Mtb infection macrophage-derived IL-10 drives reactivation during the chronic stage of the disease by acting primarily at the level of the macrophage. Macrophage-derived IL-10 appears to override IFN-γ-dependent classical macrophage activation and effector mechanisms against Mtb by inducing an alternatively activated phenotype. Specifically, Arg1 expression in these macrophages may provide better growth conditions for Mtb. Th2 cytokines that potently drive alternative macrophage activation have been associated with the development of postprimary TB in patients (7, 57). Thus, reactivating TB may, in part, be mediated by a Th2-type immune reaction characterized by an alternatively activated state of macrophages that eventually subverts protective immune mechanisms. It is possible that the identification of an increased Th2 cytokine immune response may identify infected individuals at risk to reactivate a latent Mtb infection. Additionally, prophylactic treatment of these individuals with immunomodulating drugs aimed at inhibiting the production of type 2 cytokines or downstream immune mechanisms may prevent or ameliorate reactivation TB.

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Disclosures

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References


