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Autocrine Deactivation of Macrophages in Transgenic Mice Constitutively Overexpressing IL-10 Under Control of the Human CD68 Promoter

Roland Lang,* Robert L. Rutschman,* David R. Greaves,† and Peter J. Murray2*

IL-10 plays an essential role in blocking cytokine production by activated macrophages. To analyze the consequences of enforced expression of IL-10 by macrophages on innate and adaptive immune responses, we generated transgenic mice (macIL-10tg mice) expressing an epitope-tagged IL-10 (Flag-IL-10) under control of the human CD68 promoter. Expression of Flag-IL-10 was constitutive and restricted to macrophages, as shown by sorting splenocyte cell populations and intracellular staining for IL-10. Transgenic macrophages displayed suppressed production of TNF-α and IL-12 upon stimulation with LPS. When macIL-10tg mice were challenged with LPS, serum levels of proinflammatory cytokines were attenuated compared with controls. Infection with Mycobacterium bovis bacille Calmette-Guérin resulted in ∼10-fold-higher bacterial loads than in wild-type mice. Normal T and B cell responses were observed in macIL-10tg mice, suggesting that macrophage-specific overexpression of IL-10 predominantly acts in an autocrine/paracrine manner, resulting in chronically deactivated macrophages that manifest an impaired ability to control pathogens. The Journal of Immunology, 2002, 168: 3402–3411.

 interleukin-10 is produced by macrophages, T cells, B cells, and a variety of other cell types including mast cells, keratinocytes, and some tumor cell lines. The effects of IL-10 on immune responses are mostly inhibitory (1). In macrophages, IL-10 inhibits production of proinflammatory cytokines such as TNF-α, IL-6, and IL-12 (2–4). Inhibition of IL-12 production by IL-10 (4) may be a mechanism by which IL-10 weakens the development of Th1-type T cell responses (5). However, IL-10 can also have immunostimulatory effects by inducing the proliferation of mast cells (6) and CD8 T cells (7). As demonstrated in IL-10 gene-deficient mice, the absence of IL-10 causes overproduction of inflammatory cytokines after LPS challenge (8) and development of chronic inflammatory bowel disease (9). However, IL-10-deficient mice also show increased resistance to intracellular pathogens as diverse as Leishmania major (10), Listeria monocytogenes (11), Chlamydia trachomatis (12), Mycobacterium avium (13), and Mycobacterium bovis bacille Calmette-Guérin (BCG) (14, 15).

Thus, IL-10 is required to prevent immunopathology, but it can also delay or impair protective responses against pathogens.

We previously found that overexpression of IL-10 from T cells in a transgenic mouse line impairs clearance of BCG (16). T cells from these mice produced abundant IFN-γ and IL-2 in response to BCG infection, indicating that excess IL-10 inhibited killing of BCG at the level of the infected macrophage without affecting T cell responses. The use of T cell IL-10-transgenic mice has also provided important information on the role of T cell-derived IL-10 in models of autoimmune diseases (17, 18). However, because IL-10 is only overexpressed in these mice when T cells become activated, effects of overproduction of IL-10 by innate immune cells cannot be studied. The phenotype of mice with a macrophage-specific disruption of STAT3 (19) closely resembles that of IL-10-deficient mice, suggesting that macrophages are the major target of IL-10 in vivo. For these reasons, we were interested in creating a mouse model to analyze the consequences of overexpression of IL-10 from macrophages themselves. To direct transgene expression to macrophages, promoter fragments of the CD11b (20), c-fms (21), scavenger receptor A (SR-A) (22), lysozyme (23), and MHC class II (MHC-II) (24) genes have been used. None of these constructs is ideal for macrophage-specific expression of transgenes, because they are either not restricted to macrophages or only active in a subset of these cells. Human CD68 and its murine homolog macrosialin are expressed in the endosomal compartment of cells of the mononuclear phagocyte lineage (25–27). To achieve macrophage-specific overexpression of murine IL-10 tagged with a Flag epitope in vivo, we have used an expression cassette that combines 2.9 kb of the CD68 5′ flanking region with the 83-bp first intron of the CD68 gene, which has been shown to contain a macrophage-specific enhancer (28). This combination of human CD68 gene sequences directed macrophage-specific expression of a type III human SR-A in transgenic mouse lines (29).

In this study, we show that human CD68 sequences can be used to direct constitutive expression of a Flag-IL-10 transgene in vivo specifically in macrophages. Transgenic IL-10 acted on macrophages in an autocrine manner, resulting in deactivated macrophages with impaired capacity to produce inflammatory cytokines when challenged with LPS. Although adaptive immune responses
were largely intact, macIL-10tg mice were unable to clear mycobacteria efficiently.

Materials and Methods

Construction of macIL-10g mice

The murine IL-10 cDNA was amplified by PCR to lack the endogenous sequence encoding the signal peptide. The PCR product was digested with MluI and NheI (introduced in the amplifying oligonucleotides) and cloned into the same sites in pEF-BOS/Flag (gift of Dr. D. Hilton, Walter and Eliza Hall Institute, Melbourne, Australia). The IL-10 coding region is fused in frame to DNA encoding the Flag epitope. To express Flag-IL-10 under control of the CD68 promoter, Flag was used. Construction of Flag-IL-10 expressing the organs through a nylon mesh (Falcon, Mountain View, CA) and suspensions of spleens, lymph nodes, and thymus were prepared by strain-

Cell culture

Bone marrow-derived macrophages (BMDM) were generated as described previously (16, 30) by differentiation in 15% L cell-conditioned medium as a source of M-CSF for 5–7 days. To obtain peritoneal-derived macrophages (PDM), mice were injected with 3 ml of 4% Brewer’s thioglycolate and were sacrificed after 3 days, and the peritoneal cavity was flushed with 10 ml ice-cold PBS. Cell culture medium for macrophage cultures was DMEM supplemented with 10% FBS, 2-ME (50 μM), and antibiotics. Cell suspensions of spleens, lymph nodes, and thymus were prepared. OVA-specific Abs and reagents for flow cytometry were from BD PharMingen. For staining of cell surface proteins, cells were resuspended in 2% FBS and then incubated for 30 min at 4°C with the respective combinations of fluorescently labeled Abs. For analytical purposes, cells were analyzed on a FACSCalibur (BD Biosciences, San Jose, CA) and were gated on the cell population of interest. For intracellular cytokines, after the staining procedure for cell surface proteins was completed, cells were fixed in 2% paraformaldehyde for at least 20 min at room temperature. Cells were then washed twice in staining buffer (PBS with 0.5% saponin, 0.5% BSA, and 0.05% azide), followed by a 30-min incubation with PE-labeled anti-TNF-α or anti-IL-10 Ab in staining buffer. After washing again three times, cells were taken up in PBS and analyzed.

Real-time quantitative RT-PCR

Total RNA from cells and mouse tissues was prepared using the Absolutely RNA kit (Stratagene, La Jolla, CA), which includes a DNase I digestion step to remove contaminating genomic DNA. One microgram of RNA was reverse-transcribed using Superscript II (Life Technologies, Rockville, MD) and random primers. Real-time quantitative PCR was performed on a SDS7700 instrument (PE Applied Biosystems, Foster City, CA). Primers and probes were designed using PrimerExpress software and had the following sequences: β-actin (sense 5′-ACCCACACTGTGCCCATC-3′; antisense 5′-AGGCAAGTTGCACGACGG-3′); probe 5′-AGGGGA TATGTCTTCCCTACGACA-3′. IL-10 (antisense 5′-CCCAAGTACACTTAAAGCTTCGC-3′; sense 5′-TCCAACCTTTAAGAGTG-3′); GC-3, sense 5′-ATAATGCACCCACTTCCCAGTC-3′). Fluorogenic probes were labeled with FAM at the 5′ and with TAMRA at the 3′ end and synthesized by PE Applied Biosystems. Because primers for β-actin and Flag-IL-10 amplified genomic DNA as well, control (no template) samples were included to control the efficiency of the PCR. To generate standard curves, PCR products were cloned into pCR2.1 (Invitrogen, Carlsbad, CA), and mini-prep DNA was digested with EcoRI and, after estimation of DNA quantity on a agarose gel, diluted serially down from 10^4 to 1 copies/μl. DNA samples and standard dilutions were analyzed in duplicate.

Immunization with OVA

Mice were immunized with 100 μg/footpad OVA (Sigma-Aldrich) in CFA (Pierce, Rockford, IL) followed by a booster injection with 100 μg/footpad OVA in IFA 10 days later. Twenty days after the first injection, mice were sacrificed, blood was collected, and the popliteal lymph nodes were prepared. OVA-specific Ab titers were determined by ELISA. Briefly, plates were coated with OVA (2 μg/ml) and blocked with PBS containing 10% FBS. Sera were diluted down 3-fold serially and allowed to bind. After extensive washing with PBS 0.05% Tween, OVA-specific Ig isotypes were detected by incubation with AP-conjugated isotype-specific mouse Abs. After extensive washing, substrate was added and the OD492 was determined. The last serum dilution yielding an OD492 value of 0.1 over background was recorded as the endpoint titer for each sample.

LPS challenge

Escherichia coli LPS was purchased from Sigma-Aldrich (catalog no. L4130), dissolved at 5 mg/ml in PBS, and diluted before injection, and a volume of 200 μl was injected i.p. At the indicated time points, mice were sacrificed, blood was collected, and the sera was separated by centrifugation.

Infection with BCG

BCG Pasteur was grown in 40 ml Middlebrook 7H9 broth for 3 days, washed twice with PBS with 0.02% Tween 80, taken up in 20 ml and diluted 1/20 in PBS with 0.02% Tween 80, and sonicated in a water bath sonicator for 15 s to break up clumps. Mice were injected in a lateral tail vein with 200 μl of this solution, which corresponded to 10^9 viable bacteria, as determined by plating of the inoculum. After 2, 5, and 8 wk, groups of mice were sacrificed and tail DNA was prepared for confirmatory genotyping. The left lung, upper half of the spleen, and the upper lobe of the liver were used for determination of CFU. A lobe of the right lung, a piece of the spleen, and the liver were placed in 4% formaldehyde for histology. The rest of the spleen was taken for preparation of splenocytes for restimulation in vitro.

Isolation of CD4 T cells from splenocytes

Splenocytes from groups of six BCG-infected or control mice were prepared as described above and pooled. A total of 2.5 × 10^8 splenocytes were resuspended in 1.8 ml of ice-cold PBS containing 2% FBS (wash buffer) followed by addition of 200 μl mouse CD4 (L3T4) MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Samples were incubated for 30 min at 4°C followed by magnetic separation with a LS positive selection column (Miltenyi Biotec). After washing the column with 3 ml wash buffer, the cell suspension was applied to the column and negative cells were allowed to pass through. The column was washed three times with 3 ml
A sample buffer was added. A total of 40 taste activity. After 30 min on ice, lysates were cleared from debris and SDS/HEPES/H9253 (31), and revealed promoter fragments are not drawn to scale.

**Table I. Results of pronucleus injections of CD68-Flag-IL-10 constructs**

<table>
<thead>
<tr>
<th>Construct Injected and Founder Mouse</th>
<th>Copy No.</th>
<th>Serum Flag-IL-10 (ng/ml)</th>
<th>Outcome</th>
<th>Pathological Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.9-kbp CD68-Flag-IL-10</td>
<td>TG445 (male)</td>
<td>1–2</td>
<td>Up to 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Healthy and fertile with multiple offspring</td>
</tr>
<tr>
<td>423-bp CD68-Flag-IL-10</td>
<td>TG462 (male)</td>
<td>Not done</td>
<td>Not done</td>
<td>Died after 10 wk</td>
</tr>
<tr>
<td>432-bp CD68-Flag-IL-10</td>
<td>TG469 (female)</td>
<td>20</td>
<td>Not done</td>
<td>Died after 3 mo</td>
</tr>
<tr>
<td>432-bp CD68-Flag-IL-10</td>
<td>TG520 (male)</td>
<td>Not done</td>
<td>Not done</td>
<td>Died after 4 mo</td>
</tr>
<tr>
<td>432-bp CD68-Flag-IL-10</td>
<td>TG552 (male)</td>
<td>40</td>
<td>1189</td>
<td>Normal</td>
</tr>
</tbody>
</table>

<sup>a</sup> Similar levels of Flag-IL-10 and IL-10 were detected by ELISA in the serum of TG445 and its descendants. IL-10 was not detectable in the serum of untreated FVB mice (see also Fig. 2).
that the amount of IL-10 produced in macIL-10tg under normal conditions is similar to levels observed when FVB mice were injected with LPS (Fig. 2A). At the mRNA level, real-time quantitative RT-PCR showed that in spleens of untreated macIL-10tg mice IL-10 mRNA is ∼100-fold more abundant than in FVB control spleen (Fig. 2B). At the protein and at the mRNA level this difference is entirely due to transgenic Flag-IL-10 (Fig. 2A and B). When cells from different tissues were cultured without stimulation, IL-10 could be detected in the supernatants of macIL-10tg splenocyte and bone marrow cell cultures and, to a much lesser extent, of lymph node cells but not thymocytes (Fig. 2C). To define the source of IL-10 more directly, adherent splenocytes were cultured in the presence of brefeldin A to block secretion of cytokines and were stained for expression of the macrophage marker F4/80 and intracellular IL-10. Numbers indicate the percentages in the F4/80-PE quadrants (of all cells). E, Sorting for Mac1+GR1+ splenocytes enriches for IL-10 producers. Splenocytes of FVB or macIL-10tg mice were stained with CD3-PE/B220-FITC or Mac1-allophycocyanin/GR1-FITC, followed by sorting for the indicated populations. A total of 10^5 cells were cultured in 96-well U-bottom plates in medium alone or stimulated with LPS, and supernatants were collected after 24 h for ELISA determination of IL-10 content. F, BMDM were stimulated with increasing amounts of LPS for 24 h.

FIGURE 2. Expression of IL-10/Flag-IL-10 in FVB and macIL-10tg mice. A, Mice were injected i.p. with 200 μg LPS or not, followed after 30 min by i.v. injection of 10 μg biotinylated anti-IL-10 Ab or PBS. Twenty hours later, mice were killed and the serum was analyzed by ELISA using either anti-mouse IL-10 or anti-Flag M2 Ab to capture IL-10 and Flag-IL-10. Results are mean ± SD of two mice per group. The limit for detection of IL-10 was 150 pg/ml serum. B, mRNA expression of transgenic Flag-IL-10, endogenous murine IL-10, and total IL-10 in spleens of FVB and macIL-10tg mice. No Flag-IL-10 mRNA was detectable in FVB spleen. Data are mean ± SD of four mice per genotype. C, IL-10 secretion from suspension cultures derived from various organs. After preparation of single cell suspensions by passing the organs through a cell strainer, 3 × 10^6 cells were cultured for 24 h in complete RPMI. Supernatants were analyzed for IL-10 by ELISA. D, Detection of intracellular IL-10 in F4/80+ adherent splenocytes cultured in the presence of brefeldin A. Spleens were digested with collagenase for 1 h at 37°C, and the cell suspension was passed through a 70-μm nylon cell strainer and allowed to adhere to plastic for 4 h. Nonadherent cells were gently washed away and fresh medium was added containing brefeldin A. After 4 h, the cells were harvested and stained for expression of the macrophage marker F4/80 and intracellular IL-10. Numbers indicate the percentages in the F4/80-PE quadrants (of all cells). E, Sorting for Mac1+GR1+ splenocytes enriches for IL-10 producers. Splenocytes of FVB or macIL-10tg mice were stained with CD3-PE/B220-FITC or Mac1-allophycocyanin/GR1-FITC, followed by sorting for the indicated populations. A total of 10^5 cells were cultured in 96-well U-bottom plates in medium alone or stimulated with LPS, and supernatants were collected after 24 h for ELISA determination of IL-10 content. F, BMDM were stimulated with increasing amounts of LPS for 24 h.
cell, B cell, granulocyte, and macrophage lineage cells, enrichment for macrophages (Mac1<sup>+</sup>GR1<sup>−</sup>) strongly increased the amount of IL-10 produced from macIL-10tg cells (Fig. 2E). Finally, BMDM from macIL-10tg mice produced high levels of IL-10 that slightly increased after stimulation with LPS (Fig. 2F), whereas FVB BMDM produced IL-10 only after stimulation with LPS. Taken together, these experiments show that Flag-IL-10 is expressed in a macrophage-specific and constitutive manner in macIL-10tg mice.

macIL-10tg mice have normal cell numbers in the peripheral lymphoid and myeloid compartments and show normal Ag-specific T and B cell responses

When compared with FVB littermates, macIL-10tg mice showed no major differences in the distribution of T cells and B lymphocytes in spleen and mesenteric lymph node, except for a slightly reduced frequency of CD8<sup>+</sup> T cells (Table II). The percentage of Mac1<sup>+</sup> cells was slightly but significantly increased in macIL-10tg spleens. Overall, lymphoid development and the cellular composition of the peripheral lymphoid organs appeared normal in macIL-10tg mice. When splenocytes were stimulated with anti-CD3, proliferation as measured by incorporation of [3H]thymidine was indistinguishable between FVB and macIL-10tg splenocytes (Fig. 3A). The ability of macIL-10tg mice to mount an Ag-specific immune response was tested by immunization with OVA in CFA (Fig. 3B). The intensity and isotype distribution of OVA-specific Ab production did not differ between FVB and macIL-10tg mice, as determined by endpoint titers. In addition to an unaltered B cell response, macIL-10tg mice developed strong T cell memory, because restimulation of popliteal lymph node cells with OVA in vitro resulted in strong production of IFN-γ (data not shown).

Macrophages from macIL-10tg mice fail to produce TNF-α and IL-12 when stimulated with LPS in vitro

Consequences of overexpression of IL-10 on macrophage function were first evaluated in vitro using both PDM and BMDM. Compared with FVB macrophages, stimulation of macIL-10tg macrophages with LPS resulted in strongly reduced amounts of both TNF-α (reduced by 83.6%) and IL-12p40 (71.4%) in the culture supernatants (Fig. 4A). As expected, addition of exogenous IL-10 strongly reduced the levels of both cytokines in FVB macrophages but had much less effect on macIL-10tg macrophages. Importantly, addition of a neutralizing anti-IL-10 Ab to the cultures restored cytokine production from macIL-10tg macrophages, demonstrating that the inhibition observed is due to transgenic Flag-IL-10. Compared with the strong inhibition of TNF-α and IL-12p40, production of NO by macrophages stimulated with LPS was less affected in macIL-10tg (30.2% reduction)- and IL-10-treated FVB macrophages (Fig. 4A). In the case of TNF-α, the kinetics of production after LPS stimulation were analyzed by intracellular FACS staining (Fig. 4B). In FVB macrophages, LPS treatment induced a sharp increase in the percentage of TNF-α<sup>+</sup> cells (45.1% after 30 min and 63.1% after 75 min). After 150 min, TNF-α synthesis had begun to decline, returning to 16% positive cells by 300 min. The course of TNF-α staining in macIL-10tg macrophages stimulated with LPS was very similar to that of FVB macrophages exposed to IL-10 plus LPS. There was an increase in the percentage of TNF-α<sup>+</sup> cells, but transgenic and exogenous IL-10 blunted the response by ~40% at the 30- and 75-min time points. Furthermore, IL-10 induced a faster down-regulation of TNF-α production, with TNF-α staining approaching baseline levels after 150 min and a >70% inhibition relative to FVB macrophages.

macIL-10tg mice are hyporesponsive to LPS challenge in vivo

We studied whether macIL-10tg mice also produce less TNF-α and IL-12p40 when challenged with LPS in vivo. After i.p. injection of 200 μg LPS, the serum levels of IL-12p40, TNF-α, and IFN-γ were measured (Fig. 5). In macIL-10tg mice the response to LPS was dampened with a significant reduction in the levels of TNF-α after 1.5 h, IFN-γ after 6 h, and both time points in the case of IL-12p40. Inhibition of cytokine production was not as strong as in the in vitro culture system (Fig. 4), which is probably due to the higher relative concentrations of Flag-IL-10 in pure macrophage cultures compared with in vivo, where the Flag-IL-10 produced will be taken up and removed continuously.

Table II. Lymphoid compartments of macIL-10tg mice

<table>
<thead>
<tr>
<th>Positive Cells</th>
<th>FVB&lt;sup&gt;a&lt;/sup&gt;</th>
<th>macIL-10tg&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>36.8</td>
<td>5.4</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>28.0</td>
<td>3.3</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt;</td>
<td>9.0</td>
<td>1.0</td>
</tr>
<tr>
<td>B220&lt;sup&gt;+&lt;/sup&gt;</td>
<td>41.7</td>
<td>6.2</td>
</tr>
<tr>
<td>Mac1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>12.5</td>
<td>1.9</td>
</tr>
<tr>
<td>Mesenteric lymph node</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>80.3</td>
<td>2.2</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>57.3</td>
<td>4.2</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt;</td>
<td>22.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Thymus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;−&lt;/sup&gt;</td>
<td>81.2</td>
<td>5.8</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3.5</td>
<td>1.5</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>12.5</td>
<td>3.5</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2.8</td>
<td>1.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data shown are from five mice per group.
<sup>b</sup> Data were analyzed by Student’s t test.

FIGURE 3. A. A total of 5 × 10<sup>5</sup> splenocytes of FVB (○) or macIL-10tg (●) were stimulated in 96-well plates with anti-CD3 mAb at the indicated concentrations and from 40 to 48 h were pulsed with [3H]thymidine. Data shown are mean ± SEM of quadruplicate wells. B. OVA-specific IgM and IgG subtypes were determined in sera of mice immunized with OVA in CFA as described in Materials and Methods. Results are mean ± SD of log<sub>10</sub> dilution of sera from four mice per genotype.
macIL-10tg mice develop higher bacterial loads after infection with BCG

To investigate the role of macrophage IL-10 production in mycobacterial infection, macIL-10tg and FVB mice were infected i.v. with BCG. macIL-10tg mice, as well as FVB mice, controlled the infection and appeared outwardly healthy. At 2, 5, and 8 wk after infection, mice were killed and tissue homogenates of spleen, liver, and lung were plated onto 7H10 plates and the number of CFU per organ was determined (Fig. 6A). A difference in CFU was apparent after 2 wk in all three organs but was stronger at the 5-wk time point. This higher bacterial load was maintained after 8 wk of infection in liver and spleen but not in the lung. Histopathological analysis of spleen and liver sections showed that the number of granulomas in the livers was roughly equal between macIL-10tg mice and FVB controls at all three time points. Staining for the presence of acid-fast bacilli revealed that the number of mycobacteria in the granulomas was higher in macIL-10tg mice than in FVB mice (Fig. 6B). The development of a BCG-specific T cell response after infection was assessed by measurement of IFN-γ in culture supernatants of splenocytes from infected FVB and

FIGURE 5. Serum cytokine levels in FVB (●) and macIL-10tg mice (∆) challenged with LPS. Groups of four mice were injected i.p. with 200 μg LPS. After 1.5 and 6 h, mice were killed and the serum was analyzed for cytokines by ELISA. Data are mean ± SD; p values from Student’s t test are indicated.
macIL-10tg mice restimulated in vitro with heat-killed BCG (Fig. 7). IFN-γ production from macIL-10tg splenocytes was significantly weaker than from FVB cultures 5 wk after infection, but by 8 wk after infection this difference was no longer observed and macIL-10tg splenocytes produced even more IFN-γ than controls. To dissect the contributions of the T cell and APC components of the IFN-γ response, we purified CD4 T cells from BCG-infected mice as well as control FVB and macIL-10tg mice. We used wild-type and transgenic BMDM as APC for restimulation in vitro (Fig. 8). As expected, CD4 T cells from uninfected FVB and macIL-10tg mice did not produce detectable IFN-γ when exposed to BMDM pulsed with purified protein derivative (PPD) or infected with BCG (data not shown). IFN-γ levels induced by anti-CD3/Con A in control CD4 T cells were highest for the combination of FVB CD4 T cells with FVB BMDM and lowest when both cell types came from macIL-10tg mice. Infection with BCG resulted in strongly increased IFN-γ production upon stimulation with anti-CD3/Con A for all combinations of T cells and APC. Purified CD4 T cells from BCG-infected macIL-10tg mice showed higher PPD- and BCG-specific IFN-γ production than their FVB counterparts. In contrast, macIL-10tg BMDM induced a much weaker IFN-γ response than those from FVB mice. This difference was also evident after stimulation with anti-CD3/Con A but more pronounced for PPD- or BCG-specific IFN-γ. Thus, the use of purified CD4 T cells and defined APC showed that BCG-specific Th1 T cell development is not impaired in macIL-10tg mice. Together with normal development of granulomas in macIL-10tg mice, the main reason for the impaired clearance of BCG appeared to be an inhibition of intracellular killing by infected macrophages.

Alterations in STAT activation by IFN-γ in macIL-10tg macrophages

Increased survival of mycobacteria in macIL-10tg macrophages may be due to a disturbed response to IFN-γ. To address this issue,
we chose to analyze the phosphorylation of STAT1, an early event in IFN-γ signal transduction, in BMDM from wild-type FVB and macIL-10tg mice. Macrophages from macIL-10tg mice showed attenuated phosphorylation of STAT1 when stimulated with titrated amounts of IFN-γ (Fig. 9A). Because inhibition of STAT1 activation by IL-10 has been correlated with expression of SOCS3 (32), protein levels of SOCS3 were determined in BMDM (Fig. 9B). SOCS3 was absent in unstimulated FVB macrophages but was induced 3 h after addition of IFN-γ. In contrast, macIL-10tg macrophages contained constitutively expressed SOCS3, which was further increased by treatment with IFN-γ, while neutralization of IL-10 greatly reduced the level of SOCS3. Overexpression of IL-10 in macIL-10tg macrophages also resulted in constitutive phosphorylation of STAT3, which was transiently activated by IFN-γ in FVB macrophages.

Discussion

This study describes a new transgenic mouse model where an epitope-tagged IL-10 is constitutively overexpressed by macrophages in vivo under the control of the CD68 promoter. Macrophage-specific overexpression of IL-10 induced deactivation of macrophages but had little impact on development of Ag-specific T and B cell responses. Self-deactivation of macrophages in macIL-10tg mice significantly affected the inflammatory response to LPS challenge and the ability to clear infection with the intracellular pathogen BCG.

Macrophage specificity of CD68 promoter-driven Flag-IL-10 expression

Several lines of evidence indicate that expression of the Flag-IL-10 transgene by the CD68 promoter was restricted to macrophages. First, when cells from different tissues were assayed for Flag-IL-10 production, significantly more IL-10 was detected in supernatants of cultures enriched in macrophages. Second, sorting of splenocytes showed strong enrichment of Flag-IL-10 secretion in the Mac1+/GR1− population. Finally, intracellular staining detected IL-10 only in the F4/80+ population within adherent splenocytes of macIL-10tg mice. Overall, our findings confirm the usefulness of the CD68 promoter for macrophage-specific expression in transgenic mice (29). Expression of Flag-IL-10 in macrophages was constitutive and at least as strong as IL-10 secretion induced by treatment with LPS, at both the mRNA and protein levels. In the serum of macIL-10tg mice, IL-10 was present at concentrations comparable to what was induced by LPS in FVB mice.

Several founder mice derived from injection with different fragments of the CD68 promoter controlling Flag-IL-10 died prematurely without producing viable offspring. The copy number of integrated transgene was estimated to be between 20 and 40 in these animals, and very high levels of Flag-IL-10 were detected in the serum (Table I). Although there are no published data on toxic effects of IL-10, it cannot be ruled out that chronic exposure to excessive amounts of IL-10 can have direct devastating effects on multiple organ systems. In contrast, the pathological findings we obtained are compatible with systemic infection, in one case ascending from the genito-urinary tract, in the other spread from a large abscess. Thus, high chronic IL-10 levels in these founder mice may have caused paralysis of the immune system leading to terminal infection.

Comparison of macIL-10tg mice to other models of overexpression of IL-10 in vivo

Overexpression of Flag-IL-10 in macIL-10tg mice did not obviously interfere with peripheral T and B cell homeostasis. This is consistent with other transgenic mouse lines overexpressing IL-10 either from activated T cells (16, 33) or under control of the MHC-II promoter (24). The induction of Ag-specific adaptive immunity appeared unaffected despite elevated levels of IL-10 in macIL-10tg mice (Fig. 3). This is at variance with the recently reported inhibition of Ag-specific responses by T and B cells in a transgenic mouse line expressing human IL-10 under the control of the MHC-II promoter (hIL-10 TG) (Ref. 24 and this study). This discrepancy is somewhat surprising given that IL-10 is expressed from APCs in both models, reaching similar steady-state levels in the serum of mice (Ref. 24 and this study). The cellular source of IL-10 production directed by the different promoters may be relevant here. While macIL-10tg B cells did not make Flag-IL-10, B cells from hIL-10 TG strongly secreted transgenic IL-10 upon stimulation (24). Also, the expression of IL-10 by dendritic cells can be expected to differ between the transgenic mouse lines, although Flag-IL-10 expression by dendritic cells was not tested directly in macIL-10tg. In addition to spatial differences in IL-10 production, the temporal context of constitutive or inducible over-expression of IL-10 may explain the differential effects on adaptive immune responses in macIL-10tg and hIL-10 TG mouse lines.

Macrophage deactivation in macIL-10tg mice affects inflammatory and antimycobacterial responses

Stimulation of macIL-10tg macrophages in vitro with LPS resulted in similarly down-regulated levels of TNF-α and IL-12p40 as were observed in control macrophages treated with IL-10, and neutralization of IL-10 increased cytokine production by macIL-10tg macrophages. Importantly, upon challenge with LPS in vivo, a similar reduction in the output of TNF-α and IL-12p40 was observed. In addition, IFN-γ was significantly lower in macIL-10tg, probably reflecting a secondary effect of decreased IL-12 levels in the transgenic mice. These effects on the innate response to LPS are consistent with work by others using exogenous administration of IL-10 (34–36) or IL-10 gene transfer (37).
Although down-regulation of cytokine production by macrophages is desirable and beneficial in autoimmune diseases and septic shock, it may negatively influence the capacity to fight infections. Infection of macrophages with Lipopolysaccharide (LPS) results in the production of tumor necrosis factor-alpha (TNF-α), which triggers the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). These species can cause cellular damage and death, leading to the activation of necroptosis, a form of cell death that is characterized by the release of intracellular contents and the activation of death receptors on the cell surface. The activation of death receptors triggers the activation of the caspase family of enzymes, which leads to cell death.

Despite the potential benefits of down-regulating cytokine production, the suppression of TNF-α may have negative consequences. For example, the suppression of TNF-α may impair the activation of the innate immune system, which is crucial for the clearance of pathogens. Additionally, the suppression of TNF-α may impair the activation of the adaptive immune system, which is responsible for the generation of memory T cells and the production of antibodies.

It is crucial to understand the mechanisms that regulate cytokine production and to develop strategies that can modulate these pathways in a targeted and safe manner. The identification of cytokine regulatory mechanisms may provide insights into the pathogenesis of various diseases, such as autoimmune diseases, septic shock, and cancer.

References


