

IL-10 receptor dysfunction in macrophages during chronic inflammation

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Abstract: The immunosuppressive activity of interleukin-10 (IL-10) makes this cytokine a potentially important clinical tool to reduce inflammatory responses in various diseases. Its efficacy as a therapeutic modality is dependent on the responsiveness of immune cells. We report that macrophages from mice chronically infected with the LP-BM5 retrovirus had a reduced capacity to respond to IL-10 in vitro. The ability of IL-10 to inhibit lipopolysaccharide-induced production of tumor necrosis factor (TNF) α and IL-6 was significantly reduced in both alveolar and peritoneal macrophages from infected versus uninfected mice. IL-10 hyporesponsiveness was not related to direct infection by the retrovirus, because bone marrow-derived macrophages infected in vitro with LP-BM5 were as responsive to IL-10 as were uninfected bone marrow-derived macrophages. TNF- α appeared to contribute to development of IL-10 hyporesponsiveness, because exposure of normal macrophages to TNF- α but not interferon- γ reduced macrophage responsiveness to IL-10. Reverse transcriptase-PCR and flow cytometry demonstrated normal expression of the α and β chains of the IL-10 receptor in macrophages from infected mice, suggesting that IL-10 hyporesponsiveness is not related to a change in receptor expression. The potential role of reduced IL-10 responsiveness in the chronicity of inflammation in this and other diseases is discussed. *J. Leukoc. Biol.* 70: 624–632; 2001.

Key Words: cytokine · inflammation · retroviruses · TNF- α

INTRODUCTION

Interleukin (IL)-10 is a pleiotropic cytokine that displays suppressive activity toward a number of cell types in the immune system and appears to be important in regulating the severity and duration of inflammatory responses [1]. IL-10 has been shown to exert its immunosuppressive activity on macrophages, dendritic cells, neutrophils, eosinophils, and T helper 1 cells [2–4]. The inhibitory effect of IL-10 on macrophages has also been shown to suppress the production of the proinflammatory cytokines tumor necrosis factor (TNF) α , IL-1, IL-6, and IL-12 and the expression of major histocompatibility complex class II and costimulatory molecules, such as B7 and CD40 [1]. The

reduction of proinflammatory cytokine synthesis by IL-10 has been shown to correlate with enhanced survival in animal models of septic shock and immune complex alveolitis [5–9]. Given the protective responses induced by IL-10 administration in such animal models, the use of IL-10 as a possible therapeutic tool has been suggested for several clinical inflammatory conditions, such as chronic autoimmune diseases, septic shock, and adult respiratory distress syndromes [10–13].

Synthesis of IL-10 usually occurs as a consequence of acute and chronic inflammatory responses, and neutralization of IL-10 often exacerbates inflammatory lesions. However, a number of examples exist in which IL-10 is expressed during chronic inflammatory states in which the immunosuppressive effects of IL-10 appear to be minimal. In this regard, coexpression of interferon (IFN) γ and/or TNF- α with IL-10 has been shown to occur during autoimmune diseases, such as rheumatoid arthritis [14], systemic lupus erythematosus (SLE) [15], and multiple sclerosis [16], and in inflammatory conditions such as allograft rejection [17]. Simultaneous expression of IFN- γ , TNF- α , and IL-10 has also been reported to occur during chronic infections by HIV, *Borrelia*, and *Plasmodium* species [18–21]. Suggestions have been made by some investigators that coexpression of IL-10 with cytokines such as IFN- γ and TNF- α may indicate that the level of IL-10 in these conditions is not sufficient to completely suppress the inflammatory response. However, a corollary of this argument is that the in vivo expression or responsiveness of IL-10 receptors might be altered such that even high levels of IL-10 are incapable of down-regulating inflammation. We previously showed that C57Bl/6 mice infected with the LP-BM5 retrovirus develop a chronic progressive interstitial pneumonitis in which mRNAs for IFN- γ , TNF- α , IL-1, and IL-10 are coexpressed during the chronic phase of infection [22]. We demonstrate in this report that the elevated expression of proinflammatory cytokines in the face of chronic expression of IL-10 is caused in part by a decrease in the responsiveness of macrophages to IL-10 and that hyporesponsiveness is not caused by a change in expression of the IL-10 receptor.

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MATERIALS AND METHODS

Animals

Six-week-old C57Bl/6 female mice were purchased from Charles River Laboratories (Boston, MA). All animals were housed in sterile microisolator cages with sterile food and water provided ad libitum and were maintained by the Division of Laboratory Animal Resources at the University of Kentucky according to the guidelines of the Animal Welfare Act.

Viral infection

LP-BM5 virus is a mixture of murine leukemia viruses containing the disease-causing retrovirus Bm5 and an ecotropic helper virus and mink cell focus-forming virus that are constitutively produced by chronically infected SC-1 cells [23]. Chronically infected SC-1 cells (clone 6) were obtained from H. C. Morse III [National Cancer Institute, National Institutes of Health (NIH), Bethesda, MD], and stocks were prepared as previously described [22]. For infection, mice were inoculated intraperitoneally with 1 mL of LP-BM5 virus stock, which contains an effective dose sufficient to cause a 50% increase in the spleen weight in 1–2 weeks and immunodeficiency by 8 weeks [22]. All experiments in the current studies used mice infected for 10–12 weeks. All experiments were performed at least three times unless otherwise indicated.

Macrophage isolation and treatment in vitro

Alveolar cells were collected from normal mice by lavage of lungs with 10 mL of phosphate-buffered saline-EDTA. Cells from four mice per group were pooled, washed, and brought to a concentration of 10^6 cells/mL in RPMI 1640 medium containing 5% fetal calf serum (FCS). Normal peritoneal cells were collected by lavage of the peritoneum with 5 mL of phosphate-buffered saline. Isolated cells from four mice per group were pooled, washed, and brought to a concentration of 10^6 cells/mL in RPMI medium containing 5% FCS. All cells were cultured in 24-well plates at 10^6 cells per well for 2 h at 37°C in 5% CO₂.

Nonadherent cells were removed by rinsing plates twice with medium. Adherent cells were >90% macrophages as determined by flow cytometry with F4/80 antibody. The adherent cells were incubated with IL-10 (5 ng/mL) for 2 h at 37°C after which lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (5 µg/mL) (Difco, Inc., Detroit, MI) was added, and plates were cultured for an additional 24 h. Supernatants were collected and stored at –20°C.

Quantification of cytokine protein

Cell culture supernatants were analyzed for cytokine content by ELISA as previously described [22], using antibodies and standards purchased from PharMingen, Inc. (San Diego, CA). For the analysis of IL-6, the following reagents were used at concentrations recommended by the manufacturer: capture antibody, purified rat anti-mouse IL-6 (PharMingen catalog no. 18071D); detection antibody, biotin rat anti-mouse IL-6 (PharMingen catalog no. 18082); standard, recombinant mouse IL-6 (PharMingen catalog no. 19251V). For the analysis of TNF-α, the following reagents were used at concentrations recommended by the manufacturer: capture antibody, purified anti-mouse TNF-α (PharMingen catalog no. 18131D); detection antibody, biotin rat anti-mouse TNF-α (PharMingen catalog no. 18352D); standard, recombinant mouse TNF-α (PharMingen catalog no. 19321T).

Generation and infection of bone marrow-derived macrophages

Femurs from normal C57Bl/6 mice were flushed with RPMI 1640 medium containing 10% FCS. Single-cell suspensions of bone marrow cells were cultured in 100-mm-diameter tissue culture plates in RPMI 1640 containing 10% FCS and 50% culture supernatant from the LadMac cell line, which is transfected with the murine macrophage-colony stimulating factor (M-CSF) gene and constitutively produces M-CSF [24]. This cell line was generously provided by W. Walker, St. Jude Children's Hospital, Memphis, TN. Plates containing bone marrow cells were cultured at 37°C in 5% CO₂ to allow macrophage development. Twenty-four hours after culture, 10 mL of LP-BM5 virus-containing supernatant or 10 mL of medium (control) were added to the cultures. Plates were incubated an additional 6 days before analysis to allow complete macrophage development and virus infection to occur. Infection with

LP-BM5 retrovirus was verified by reverse transcriptase (RT)-PCR for expression of RNA for the disease-causing BM5 virus as described below.

RT-PCR for analysis of cytokines, cytokine receptor, and viral RNA

For analysis of IFN-γ, IL-10, and IL-10R mRNA expression, total RNA was extracted from lung lymphoid cells (IFN-γ and IL-10) or lung and peritoneal macrophages (IL-10R mRNA) and were pooled from four mice per group. Infected mice were used at 10–12 weeks postinfection unless otherwise indicated. Normal controls were age matched to the infected mice. For analysis of viral RNA, total RNA was extracted from cultured bone marrow-derived murine macrophages. One microgram of total RNA was reverse transcribed into cDNA with the Promega Reverse Transcription System (Promega Corp., Madison, WI) and then amplified with *Taq* polymerase for 30 cycles in a Perkin-Elmer thermal cycler (denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min, and primer extension at 72°C for 2 min). Primers were synthesized based on analysis of the DNA sequence in GenBank, using the on-line primer selection program from the Virtual Genome Center, University of Minnesota, Minneapolis (<http://alces.med.umn.edu/rawprimer.html>) or from previously published sequences. Primers for the disease-causing BM5 retrovirus were as previously published [25]. Sequences of these primers are indicated below.

- IL-10: forward, 5GTGAAGACTTTCTTTCAAACAAAG-3'; reverse, 5'-CTGCTCCACTGCCTTGCTCTTATT-3' (274 bp)
- IFNγ: forward, 5'-TACTGCCACGGCACAGTCATTGAA-3'; reverse, 5'-GCAGCGACTCCTTTCCGCTTCTT-3' (405 bp)
- IL-10Rα: forward, 5'-GCTGCCTTCAGACTTTC-3'; reverse, 5'-AAC-CCCTCTGTGATCGGA-3' (508 bp)
- IL-10Rβ: forward, 5'-AGGCAGAGCGAGCAGCCAGCAGAATGCT-3'; reverse, 5'-TGGAGCCTGGCTAGCTGGTACAGTAGGTCT-3' (298 bp)
- BM5 virus: forward, 5'-CCTTATCGACACTTCCCTT-3'; reverse, 5'-CCGCTCTTCTTAACTGGTC-3' (209 bp)
- β-actin: forward, 5'-CTGGGCCGCTCTAGGCACCA-3'; reverse, 5'-CG-GTTGCCCTTAGGGTTACGGGGG-3' (245 bp).

Preliminary studies have indicated that 30 is the number of cycles that is subsaturating for IFN-γ, IL-10, IL-10R chains, and BM5. β-actin RT-PCR was performed for 25 cycles. PCR products were separated by electrophoresis on 2% agarose gels and were visualized by ethidium bromide staining on a GelPrint 2000i imaging system (BioPhotonics Corp., Ann Arbor, MI). The PCR product images presented are negative images of otherwise unaltered band images. Integrated optical densities of negative-image bands were obtained using NIH Image software.

Flow-cytometric analysis of IL-10 receptor expression

Surface expression of IL-10 receptors was determined by the binding of fluorescein isothiocyanate (FITC)-labeled recombinant human IL-10 (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. Briefly, normal and infected alveolar and peritoneal cells were preincubated with anti-FcR to block Fc receptor binding and then incubated with RPE-Cy5-labeled F4/80 (Serotec, Inc., Raleigh, NC) and FITC-labeled IL-10. As a negative control, groups of cells were stained with F4/80 Ab and FITC-labeled IL-10 which had first been neutralized by preincubation with anti-IL-10 antibody. Flow cytometry was performed with a FACSCalibur cytometer (Becton-Dickinson, San Jose, CA). Cells were gated on F4/80⁺ macrophages and then analyzed for the capacity to bind FITC-labeled IL-10 or neutralized FITC-labeled IL-10.

Statistics

Significant differences between groups were analyzed using the SigmaPlot software program (SSPS Science, Chicago, IL). Data were analyzed using Student's *t*-test or one-way analysis of variance (ANOVA) as indicated in the figure legends. Multiple comparisons versus a control group were compared using the Bonferroni test.

RESULTS

Chronic pulmonary inflammation has been shown by our laboratory to develop in the lungs of mice infected with the LP-BM5 retrovirus [22]. Aberrant expression of a number of cytokines has been demonstrated in splenic and lymphoid tissues of LP-BM5-infected mice [26]. In particular, elevated mRNA expression of IFN- γ and IL-10 has been shown to occur early after infection and to persist throughout the infectious process [27]. In the current study, analysis of cytokine mRNA expression in the lungs of normal and infected mice demonstrated that beyond 8 weeks postinfection, both IFN- γ and IL-10 were expressed simultaneously (**Fig. 1**). IFN- γ expression was observed by 2 weeks after infection and peaked between weeks 4 and 8. In contrast, significant levels of IL-10 were not observed until 8 weeks after infection, and high levels of expression were maintained through 16 weeks of infection. As expected, expression of IL-10 was associated with reduced expression of IFN- γ at 12 and 16 weeks after infection, indicating the known inhibitory effect of IL-10 on IFN- γ expression [28]. However, we consistently observed that the expression of IFN- γ never returned to normal levels in spite of the strong expression of IL-10 in lung tissue. Moreover, we have shown that severe lung inflammation can persist through 16 weeks of infection in LP-BM5-infected mice [22], suggesting that persistent expression of IL-10 failed to control the inflammatory response in the lungs of infected mice.

To address whether the chronic expression of IFN- γ and persistent pulmonary inflammation are associated with a change in the cellular response to IL-10, alveolar macrophages were isolated from normal and 10-week-infected mice and evaluated for the ability of recombinant murine IL-10 to inhibit LPS-induced synthesis of TNF- α and IL-6. As seen in **Figure 2**, TNF- α production by normal alveolar macrophages stimulated with LPS for 24 h was inhibited nearly to background levels by preincubation for 2 h with recombinant-IL (rIL)-10 at 2.5 and 5 ng/mL. In contrast, TNF- α synthesis was only marginally reduced by IL-10 treatment of infected macro-

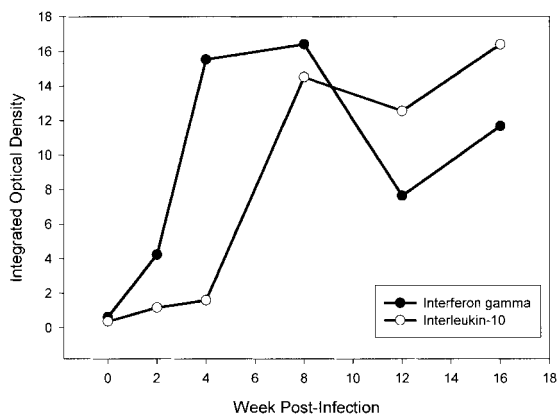


Fig. 1. Kinetics of cytokine expression in lungs during LP-BM5 infection. RNA was extracted from isolated interstitial lymphoid cells, which were collected and pooled from four mice per group at the indicated time after infection. RNA was analyzed by RT-PCR for expression of IFN- γ and IL-10. Data are expressed as integrated optical density of the PCR products. Data are representative of two separate experiments.

phages. Similarly, IL-6 production was inhibited 68 and 73% by preincubation of normal alveolar macrophages with 2.5 and 5 ng/mL of IL-10, respectively, whereas infected macrophages were inhibited by only 23 and 26% by the same respective concentrations of IL-10. Thus, alveolar macrophages from LP-BM5-infected mice displayed partial resistance to the immunosuppressive effects of IL-10.

To determine whether the resistance of infected macrophages is restricted to the lung, peritoneal macrophages were also evaluated for the ability of IL-10 to inhibit TNF- α and IL-6 production (**Fig. 3**). A similar pattern of IL-10 resistance was observed in peritoneal macrophages from normal versus infected mice. Synthesis of both TNF- α and IL-6 was low in both normal and infected macrophages in the absence of any *in vitro* stimulation. Culture for 24 h in the presence of LPS induced the synthesis of TNF- α and IL-6 in both normal and infected cells with the production of cytokines generally being greater in macrophages from infected mice. Prior incubation of normal macrophages in concentrations of mouse rIL-10 from 1.25 to 5 ng/mL reduced TNF- α and IL-6 synthesis to levels near those observed in unstimulated macrophages. In contrast, preincubation of infected macrophages with IL-10 also reduced cytokine production; however, substantial synthesis still occurred and often was above that seen in normal macrophages stimulated with LPS in the absence of IL-10. Thus, infection of mice with LP-BM5 retrovirus resulted in the systemic appearance of macrophages that showed pronounced resistance to the inhibitory effects of IL-10.

Several possible reasons exist for resistance to IL-10 in macrophages from retrovirus-infected mice. Macrophages are targets of the LP-BM5 retroviruses, and intracellular replication of the virus could affect either the expression or function of IL-10 receptors on these cells. Alternatively, macrophages in infected mice are chronically exposed to a variety of cytokines and mediators that could also alter expression or function of IL-10 receptors. To determine whether direct virus infection of macrophages affects IL-10 responsiveness, normal macrophages were infected *in vitro* with LP-BM5 retroviruses. Because efficient infection *in vitro* of mature peritoneal macrophages with LP-BM5 proved difficult, bone marrow-derived macrophages were used for these studies. Bone marrow cells were cultured for 24 h in recombinant murine M-CSF after which LP-BM5 retrovirus was added to the cultures. After an additional 6 days of incubation in M-CSF and virus, the cultures were shown by RT-PCR to express RNA of the disease-causing defective retrovirus, Bm5 (data not shown). Infected and normal bone marrow-derived macrophages were then evaluated for the ability of IL-10 to inhibit the production of LPS-induced IL-6 (**Fig. 4**). Both infected and normal macrophages responded equally well to preincubation with IL-10 in that synthesis of IL-6 was effectively inhibited in a dose-dependent manner. These studies suggested that direct infection of macrophages is not sufficient to cause resistance to IL-10.

Our previous studies and those of others demonstrated that IFN- γ and TNF- α are expressed persistently during infection with LP-BM5 [22, 26]. Preexposure of normal macrophages to IFN- γ was therefore evaluated for the ability to induce resistance to IL-10 (**Fig. 5**). Stimulation of normal peritoneal

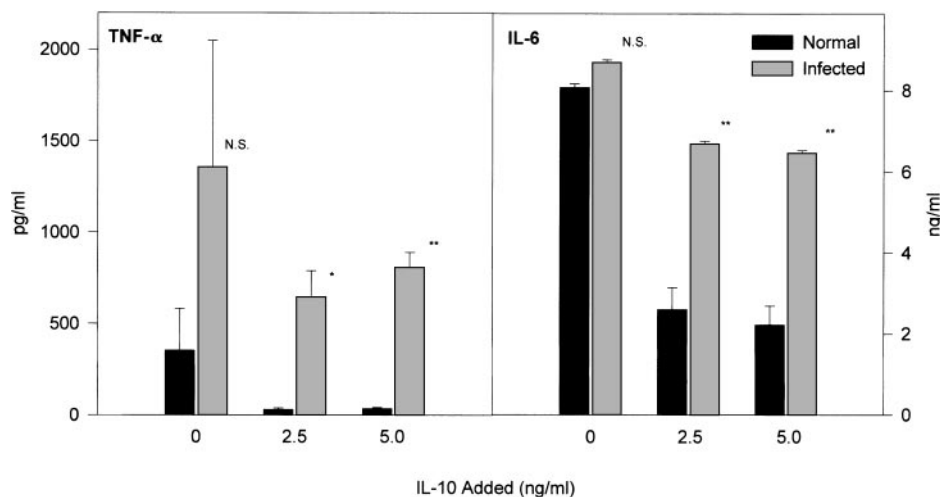


Fig. 2. Resistance of alveolar macrophages to IL-10. Alveolar macrophages from normal and 10-week-infected mice were incubated for 2 h in the indicated concentrations of murine rIL-10, after which LPS (10 μ g/mL) was added to the cultures. Twenty-four hours later, the culture supernatants were analyzed for TNF- α and IL-6 by ELISA. Data were analyzed by one-way ANOVA, and multiple comparisons versus a control were performed with Bonferroni's test. *, $P \leq 0.05$; **, $P \leq 0.01$; n.s., not significant. Data are representative of three separate experiments.

macrophages with LPS alone induced IL-6 synthesis, whereas 2-h preincubation with IL-10 inhibited LPS-induced IL-6 synthesis by approximately 50%. Neither IL-10 alone nor IFN- γ alone led to induction of IL-6 synthesis. Preexposure of macrophages to IFN- γ for 24 h at doses of 2, 4, and 8 ng/mL had no effect on LPS-induced IL-6 synthesis. More importantly, that preexposure to IFN- γ for 24 h also had no effect on the ability of IL-10 to inhibit IL-6 synthesis in LPS-stimulated macrophages. This suggests that the expression of IFN- γ in infected mice, which occurred prior to expression of IL-10 (Fig. 1), is not the likely reason for development of IL-10 resistance. Similar studies were performed to determine whether prior exposure to TNF- α could contribute to IL-10 resistance (Fig. 6). IL-10 (10 ng/mL) was able to inhibit IL-6 synthesis in response to LPS by 80.6%. Preexposure to TNF- α at concentrations of 1 ng/mL and 1 μ g/mL for 24 h partially reduced the inhibitory effect of IL-10 in that the same concentrations of IL-10 inhibited IL-6 synthesis by only 68.5 and 49.0%, respectively. The effect of TNF preexposure was not caused by any additive effect with LPS on IL-6 synthesis, because TNF preexposure had no effect on the level of IL-6 synthesis induced either by an optimal concentration (5 μ g/mL) of LPS alone (Fig. 6) or by a suboptimal concentration of LPS (1 μ g/mL) (data not shown). Studies were performed on the effect of preexposure to combined IFN- γ and TNF- α . The combined preexposure to IFN- γ and TNF- α neither enhanced nor dimin-

ished the effect of TNF- α on IL-10 receptor responsiveness (data not shown). The ability of TNF- α to induce resistance to IL-10 was usually less than that observed in macrophages from infected mice. These data suggest that exposure of macrophages to TNF- α might contribute to the develop of IL-10 resistance but that exposure to TNF- α alone might not be sufficient to achieve the level of resistance seen in infected macrophages.

We began to address the molecular mechanisms of IL-10 resistance in infected macrophages by evaluating expression of the IL-10 receptor in peritoneal and alveolar macrophages from normal and infected mice. The IL-10 receptor is a heterodimer composed of a 110-kDa α chain and a 40-kDa β chain [1, 29]. The receptor density on macrophages is low, with only a few hundred receptors per cell [1]. RT-PCR analysis of the IL-10 α and IL-10 β chains showed that both chains of the receptor are expressed in normal and infected peritoneal macrophages (Fig. 7, insert). Expression of IL-10 α and β chain mRNA was also observed in normal and infected alveolar macrophages, but at a lower level than that observed in peritoneal macrophages (data not shown). Surface expression of IL-10 receptors was evaluated by measuring the ability of alveolar and peritoneal macrophages to bind FITC-conjugated recombinant human IL-10 (Fluorokine; R&D Systems), compared with binding of a negative control which consisted of IL-10 that was first neutralized by preincubation with anti-human IL-10 (Fig. 7).

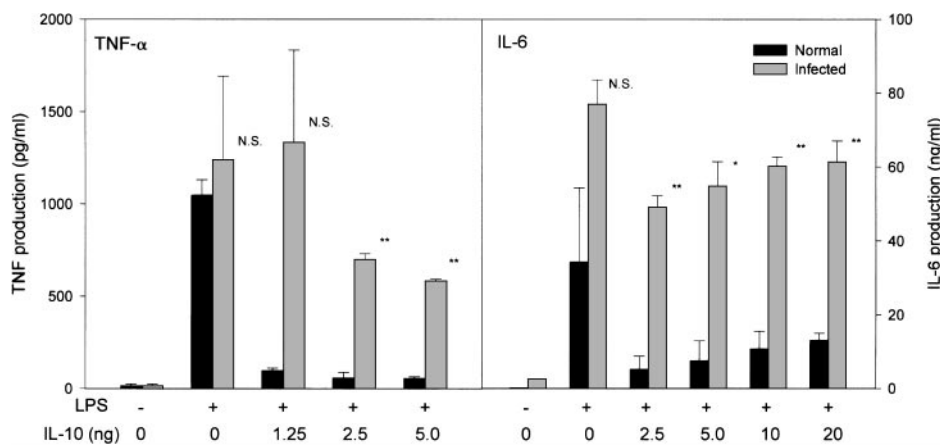


Fig. 3. Resistance of peritoneal macrophages to IL-10. Peritoneal macrophages from normal and 10-week-infected mice were incubated for 2 h in the indicated concentrations of murine rIL-10, after which LPS (10 μ g/mL) was added to the cultures. Twenty-four hours later the culture supernatants were analyzed for TNF- α and IL-6 by ELISA. Data were analyzed by one-way ANOVA, and multiple comparisons versus a control were performed with Bonferroni's test. *, $P \leq 0.05$; **, $P \leq 0.01$; n.s., not significant. Data are representative of three separate experiments.

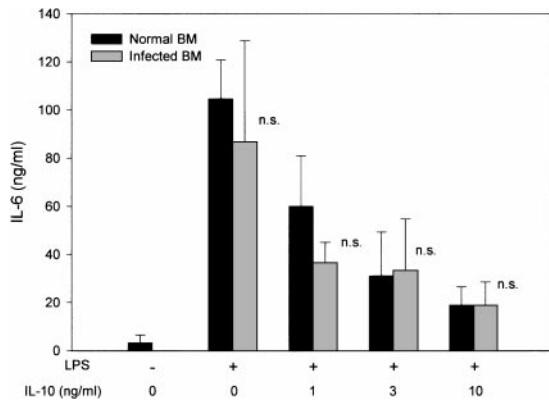


Fig. 4. Responsiveness of bone marrow-derived macrophages to IL-10. Normal and LP-BM5-infected bone marrow macrophages were incubated for 2 h in the indicated concentrations of murine rIL-10, after which LPS (10 μ g/mL) was added to the cultures. Twenty-four hours later, the culture supernatants were analyzed for TNF- α and IL-6 by ELISA. Data from infected versus normal groups were analyzed for each concentration of IL-10 using Student's *t*-test. *, $P \leq 0.05$; **, $P \leq 0.01$; n.s., not significant. Data are the means of three separate experiments.

Background autofluorescence (Fig. 7, shaded histogram) in normal F4/80⁺ peritoneal macrophages stained with antibody-neutralized IL-10 showed two populations, that were also seen in unstained macrophages (data not shown). Normal peritoneal macrophages (solid-line histogram) displayed about a 10-fold increase in fluorescence intensity, demonstrating the presence of IL-10-binding receptors on their plasma membranes. Peri-

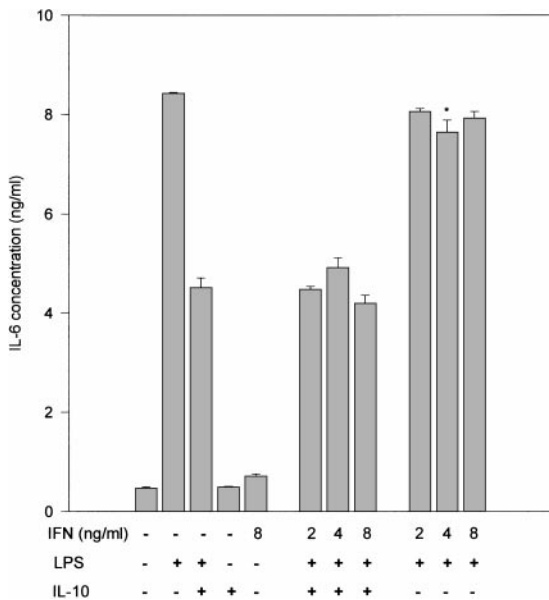


Fig. 5. IFN- γ exposure does not induce IL-10 resistance in macrophages. Normal peritoneal macrophages were cultured for 24 h in the presence of the indicated concentrations of recombinant murine IFN- γ . Cells were then exposed to murine rIL-10 (10 ng/mL) for 2 h, after which LPS (10 μ g/mL) was added to the cultures. Twenty-four hours later, the culture supernatants were analyzed for TNF- α and IL-6 by ELISA. Data were analyzed by one-way ANOVA, and multiple comparisons versus a control were performed with Bonferroni's test. The control for cells treated with LPS without IL-10 was bar 2; the control for cells treated with LPS plus IL-10 was bar 3. *, $P \leq 0.05$. Data are representative of three separate experiments.

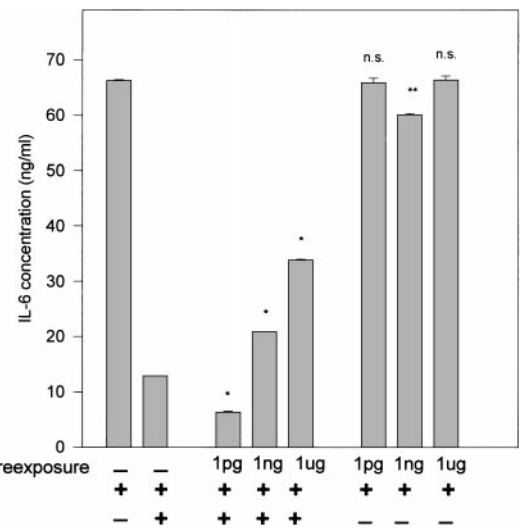


Fig. 6. TNF- α induces partial resistance to IL-10. Normal peritoneal macrophages were cultured for 24 h in the presence of the indicated concentrations of recombinant murine TNF- α . Cells were then exposed to murine rIL-10 (10 ng/mL) for 2 h after which LPS (10 μ g/mL) was added to the cultures. Twenty-four hours later the culture supernatants were analyzed for IL-6 by ELISA. Data were analyzed by one-way ANOVA and multiple comparisons versus a control were performed with Bonferroni's test. *, $P \leq 0.05$; **, $P \leq 0.01$; n.s., not significant. Data are representative of three separate experiments.

toneal macrophages from infected mice (dashed-line histogram) displayed an identical level of IL-10 binding to that of normal macrophages, indicating that the expression of IL-10 receptors was not changed in peritoneal macrophages from infected mice. Similar results were observed with alveolar macrophages from normal and infected mice (Fig. 7). The level of background autofluorescence was significantly higher in alveolar macrophages compared with peritoneal macrophages; however, this difference was not caused by nonspecific binding of neutralized IL-10, because the fluorescence pattern was identical to that of unstained alveolar macrophages (data not shown). Alveolar macrophages displayed binding of FITC-labeled IL-10 above background fluorescence, and infection did not appear to change the level of IL-10 receptor expression because both normal (solid-line histogram) and infected (dashed-line histogram) alveolar macrophages had similar patterns of fluorescence. Thus, resistance of infected macrophages to IL-10 is not associated with a change in either the mRNA expression or surface receptor expression of either the α or β chains of the IL-10 receptor.

DISCUSSION

IL-10 is a potent anti-inflammatory cytokine that has been proposed as a possible therapeutic tool for a variety of inflammatory conditions. Considerable experimental and clinical data indicate that IL-10 might ameliorate acute and chronic inflammation in clinical situations. For example, the addition of IL-10 to macrophages in vitro can potently inhibit a variety of macrophage functions, including expression of major histocompatibility complex class II, costimulatory B7 molecules,

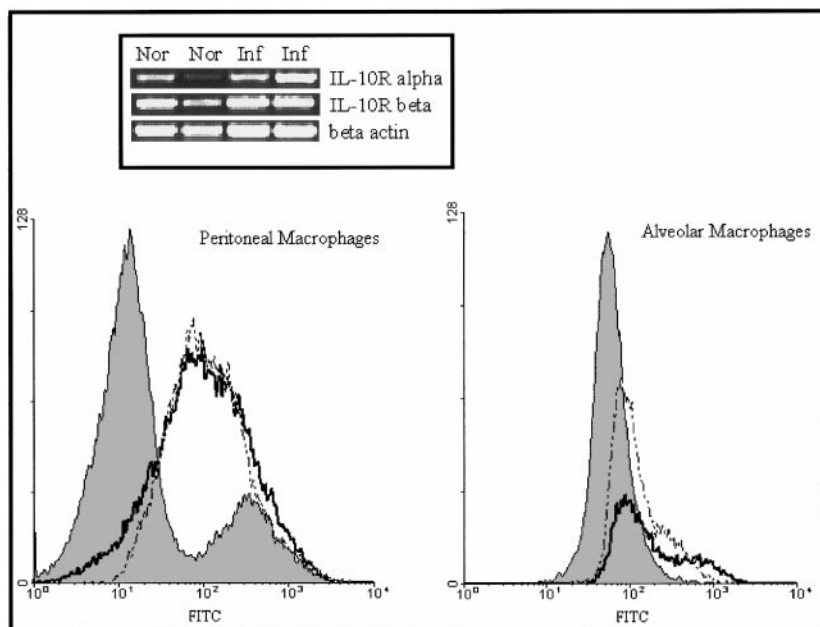


Fig. 7. Expression of IL-10 receptor expression. Total RNA was isolated from peritoneal macrophages from normal and 10-week-infected mice and was analyzed by RT-PCR for expression of the α and β chains of the IL-10 receptor. Expression of β -actin was analyzed as a positive control. Data are negative images of ethidium bromide-stained PCR products. Binding of FITC-labeled human rIL-10 to normal (solid-line) and infected (dashed-line) peritoneal and alveolar macrophages was analyzed by flow cytometry and compared with binding of anti-IL-10-neutralized IL-10 to normal macrophages as a negative control (shaded histogram). The analyzed cells were first gated on F4/80⁺ cells to identify macrophages in the alveolar- and peritoneal-cell populations. Data are representative of two separate experiments.

antigen presentation, and accessory cell function [1]. Moreover, several animal models of inflammation have been shown to be responsive to treatment with IL-10 [5–9]. Clinical trials with IL-10 in Crohn’s disease have been encouraging in that a significant number of patients have experienced a decrease in symptoms during treatment [30, 31]. However, the use of IL-10 in other inflammatory diseases has not shown the same degree of success. Smeets et al. showed that treatment of rheumatoid arthritis (RA) patients with IL-10 failed to have a significant clinical effect and did not alter the immunopathology in synovial tissue [32]. Analysis of synovial tissues from RA patients demonstrated high constitutive expression of IL-10 by synovial macrophages, and exogenous IL-10 was only marginally effective at reducing IL-6 production by these cells compared with blood monocytes from the same patients [33, 34]. In addition, synovial fluids from RA patients contain high levels of IL-10, IL-12, and IFN- γ , suggesting that endogenous production of IL-10 may be inefficient *in vivo* in down-regulating proinflammatory cytokine expression [14]. A recent study by MacDonald et al. [35] showed that synovial dendritic cells from RA patients were also partially resistant to the inhibitory effects of IL-10, compared with blood dendritic cells. Individuals with SLE also display elevated expression of IL-10, IFN- γ , and TNF- α compared with normal controls, suggesting an inability of IL-10 to effectively inhibit cytokine synthesis in this autoimmune disease [36]. Moreover, studies by Mongan et al. showed that recombinant IL-10 failed to inhibit production of IL-6 *in vitro* in monocytes from SLE patients compared with those in normal controls [15]. Thus, in at least two different chronic autoimmune diseases, endogenous production of IL-10 and exogenous treatment with IL-10 might be insufficient to ameliorate inflammation and tissue injury. Moreover, the inability of IL-10 to work appears to be related to expression and/or function of the IL-10 receptor.

Septic shock is thought to be mediated by massive production of proinflammatory cytokines by monocytes and macrophages, in particular TNF- α . A number of studies have shown

that IL-10 is quite effective in reducing proinflammatory secretion and mortality in endotoxin shock models in rodents [5, 6]. However, only marginal protection has been achieved with IL-10 in polymicrobial shock models [38, 39]. In patients with septic shock, coexpression of IL-10 with TNF- α and IL-6 has been reported, suggesting an inability of massive IL-10 synthesis in septic shock to control production of proinflammatory mediators [40, 41]. The reason for the difference in effectiveness of IL-10 for endotoxin versus polymicrobial shock is unknown. However, it has been shown that the time of exposure to IL-10 relative to endotoxin can affect the ability of IL-10 to down-regulate proinflammatory cytokine synthesis in that delayed exposure to IL-10 after endotoxin stimulation reduces the immunosuppressive ability of IL-10 [42]. In other studies by Hart et al. [43], prior exposure of macrophages to GM-CSF inhibited subsequent responsiveness of macrophages to IL-10. Thus, prior exposure of macrophages to cytokines generated during polymicrobial sepsis can contribute to the partial resistance of macrophages to IL-10. The failure of IL-10 to reduce proinflammatory cytokine synthesis during infection may not be unique for septic shock. Coexpression of high amounts of IL-10 with IFN- γ or TNF- α and the inability of IL-10 to inhibit production of these cytokines have also been observed during chronic infections with *Plasmodium*, *Borrelia*, *Brucella*, *Schistosoma*, and *Mycobacterium* species [18, 19, 44–47].

Results presented here are the first to our knowledge demonstrating that persistent coexpression of IL-10 with IFN- γ and TNF- α during a chronic viral infection is caused in part by resistance of macrophages to the immunosuppressive effects of IL-10. Other retrovirus infections have also been shown to display persistent expression of IL-10, IFN- γ , and TNF- α , including HIV, simian immunodeficiency virus, and feline immunodeficiency virus [20, 21, 48–52]. Whether cellular resistance to IL-10 is also a factor in cytokine expression patterns in these retroviral infections is unknown, but it could be affected by either direct virus infection of macrophages or other cytokine-producing cells

or, alternatively, by an effect of chronic exposure of these cells to other cytokines or inflammatory mediators during the course of infection. We were unable to demonstrate a role for direct viral infection in our model. LPS-induced cytokine production by bone marrow-derived macrophages infected with the LP-BM5 retrovirus was suppressed by IL-10 to the same extent as in uninfected cells. Thus, we suggest that in this murine model previous exposure to cytokines or inflammatory mediators rather than direct virus infection may alter the responsiveness of macrophages to IL-10. Cytokines expressed persistently after infection by LP-BM5 retrovirus are IFN- γ and TNF- α . Although prior in vitro exposure of peritoneal macrophages to IFN- γ had no effect on the response to IL-10, TNF- α preexposure induced partial resistance to IL-10. However, TNF- α was not usually able to induce resistance to IL-10 to the same degree seen in macrophages from infected mice. Therefore, although TNF- α can contribute to IL-10 resistance, other unidentified factors may synergize with TNF- α to further increase the resistance of macrophages to IL-10. It is interesting that transgenic mice overexpressing TNF- α also express very high levels of IL-10 [53]. In addition, a study by Bessis et al. [54] demonstrated that, in TNF- α -transgenic mice engrafted with fibroblasts expressing IL-4, IL-10, or IL-13, only IL-4 and IL-13 could effectively inhibit endogenous expression of TNF- α and IL-1, whereas IL-10 had a reduced capacity to inhibit these cytokines. Thus, similar to our observations, persistent exposure to TNF- α in vivo also appears to partially reduce the ability of IL-10 to inhibit proinflammatory cytokine expression.

The molecular mechanism by which chronic exposure of macrophages to cytokines or other inflammatory mediators during the course of LP-BM5 infection causes these cells to become resistant to the immunosuppressive effects of IL-10 is unknown. Macrophages from infected mice clearly express mRNA for both chains of the IL-10 receptor and display receptors on surface membranes, which are capable of binding IL-10. Given that IL-10 receptor expression remains intact after infection, it is probable that disruption of the IL-10 signal transduction pathway may account for the defective IL-10 responsiveness. Engagement of the IL-10 receptor activates a Jak-Stat signaling pathway involving Jak1 and Tyk2 kinases, which are associated with the α and β chains of the receptor, respectively [55–59]. IL-10 receptor engagement also leads to the binding of Stat3 to the receptor [59]. It is important that IL-10-mediated inhibition of TNF- α production by macrophages is also dependent on recruitment of Stat3 to the receptor complex [60]. A family of inhibitor proteins has been described, suppressors of cytokine signaling (SOCS), which can inhibit the responsiveness of several cytokine receptors to their ligands [61, 62]. SOCS-3 has been implicated in inhibition of IL-10 signaling [63, 64], and recent data by Bode et al. [65] demonstrate that exposure of macrophages to TNF- α induces the expression of SOCS-3, thereby mediating inhibition of Stat3-dependent cytokine receptors. Whether macrophages from LP-BM5-infected mice have elevated levels of SOCS-3 or display other defects in signal transduction pathways for the IL-10 receptor remains to be determined.

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