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Inhibition of IL-10 Receptor Function in Alveolar Macrophages by Toll-Like Receptor Agonists

Stefan Fernandez,* Purnima Jose,† Margarita G. Avdiushko,* Alan M. Kaplan,*† and Donald A. Cohen2*†

Despite an immunosuppressive lung environment, alveolar macrophages (AM) retain the capacity to respond to microorganisms. This report demonstrates that IL-10, constitutively produced by normal alveolar epithelium, stimulates signal transduction through the IL-10R on AM and that IL-10R function can be inhibited by stimulation of Toll-like receptor (TLR) on AM. IL-10 mRNA and protein were constitutively expressed in normal alveolar epithelium of mice, and IL-10R were constitutively expressed on normal murine AM. Stimulation of AM through TLR2, TLR4, or TLR9 was sufficient to inhibit IL-10R signal transduction, including phosphorylation and nuclear translocation of STAT3 transcription factor. Inhibition of IL-10R function by TLRs was not associated with a decrease in IL-10R expression, but did require expression of the myeloid differentiation factor 88 adaptor protein. Continuous exposure of macrophages to IL-10 caused sustained expression of the chemokine receptors CCR1 and CCR5. However, the addition of TLR ligands inhibited IL-10-induced expression of CCR1 and CCR5. Finally, exposure of macrophages to TLR ligands blocked the ability of IL-10 to inhibit the induction of TNF-α by C2-ceramide. These findings demonstrate a novel regulatory mechanism that may allow AM to overcome inhibitory effects of constitutive IL-10 in the lungs that may permit a more effective response to pulmonary infections.

Interleukin-10 is a pleotropic cytokine recognized for its inhibitory activity on a variety of immune functions (1). IL-10 exerts anti-inflammatory effects on macrophages and dendritic cells by suppressing production of proinflammatory cytokines like TNF-α, IL-1, and IL-6 (2–4). IL-10 also can suppress the ability of APCs to initiate adaptive immune responses through inhibition of cell surface expression of stimulatory molecules like MHC class II, CD40, and B7 (5). IL-10 is often induced during both acute and chronic inflammatory conditions and deficiencies in IL-10 synthesis have been linked to several chronic inflammatory disorders, including Crohn’s disease, chronic enterocolitis, cystic fibrosis, and chronic hepatitis (6–11).

IL-10 has been shown to be present in the lungs of normal individuals (9), where it is presumably produced constitutively by bronchoalveolar epithelial cells. The role of constitutive IL-10 expression in the lungs is unknown, but the anti-inflammatory nature of IL-10 may serve to protect the delicate architecture of the lungs by inhibiting the elicitation of inflammatory processes in response to inhaled air that carries a spectrum of innocuous particles. However, macrophages in the lung retain the capacity to respond strongly to microbial pathogens, suggesting that alveolar macrophages (AM)3 may possess mechanisms to avoid the suppressive effects of constitutive IL-10 production when exposed to microorganisms. The response of macrophages to microbial infection is mediated by pattern recognition receptors, including Toll-like receptor (TLR), a highly conserved family of type I transmembrane receptors that recognize specific pathogen-associated molecular patterns on microbial structures. The 10 different TLRs (TLR1–10) that have been cloned in humans and mice are critical for the initiation of innate immunity and can recognize a diverse variety of pathogen-associated molecular patterns, ranging from nucleic acid moieties like viral dsRNA (TLR3) (12) and bacterial unmethylated DNA CpG oligodeoxynucleotides (ODN, TLR9) (13, 14) to surface structures like lipoteichoic acid (LTA) on Gram-positive bacteria (TLR2), LPS from Gram-negative bacteria (TLR4), and flagellin (TLR5) (15). Signaling through TLR requires homo- or heterodimerization of receptor molecules and uses common adaptor and signaling proteins, including myeloid differentiation factor (MyD88, IL-1R-associated kinase, and NF-κB (15–17).

This study tested the hypothesis that AM and peritoneal macrophage (PM) could alter their responsiveness to IL-10 following exposure to microbial substances, which bind to TLRs. We demonstrate that stimulation of macrophages through the TLRs inhibited macrophage responsiveness to IL-10. Specifically, LTA, which binds to TLR2 (18), Cpg ODN, which binds to TLR9, and Escherichia coli LPS, which binds to TLR4, all inhibited the ability of IL-10 to induce phosphorylation of STAT3 in macrophages. The inhibition of IL-10 responsiveness was not due to changes in IL-10R expression, but was dependent on expression of the MyD88 adaptor molecule. Binding of TLR ligands by macrophages inhibited IL-10-mediated translocation of STAT3 into nuclei, reduced the expression of CCR1 and CCR5 mRNA, which are genes that are selectively induced by IL-10 and also prevented IL-10 from suppressing TNF-α synthesis following exposure to C2-ceramide.

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Abbreviations used in this paper: AM, alveolar macrophage; PM, peritoneal macrophage; TLR, Toll-like receptor; LTA, lipoteichoic acid; MyD88, myeloid differentiation factor 88; BALF, bronchoalveolar lavage fluid; ODN, oligodeoxynucleotide; BALF, bronchoalveolar lavage fluid; MIP, macrophage-inflammatory protein; SOCS3, suppressor of cytokine signaling; MAPK, mitogen-activated protein kinase.
Materials and Methods

Mice and reagents

Normal C57BL/6 mice were purchased from the National Cancer Institute breeding facility at Charles River Breeding Laboratories (Wilmington, MA). MyD88-deficient C57BL/6 mice were obtained from Dr. S. Akira, (Osaka University, Osaka, Japan) and bred at the University of Kentucky (Lexington, KY). All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Kentucky in compliance with the Animal Welfare Act. LTA and LPS (0111:B4) and C2-ceramide were purchased from Sigma-Aldrich (St. Louis, MO). Unmeth-ylated CpG ODN (PD- CpG ODN; GAG AAC GCT CGA CCT TCG AT) was obtained from IDT Technologies (Santa Clara, CA). Components were tested for LPS contamination by inducible NO synthase induction in RAW 247 macrophage cell line (19). Anti-phospho-STAT3 Ab was purchased from Cell Signaling Technology (Beverly, MA) and anti-STAT3 Ab was obtained from BD Biosciences (Lexington, KY). IL-10 Fluorokine Cyto- metric Reagent was purchased from R&D Systems (Minneapolis, MN). AM and PM were obtained by lavage with PBS from normal, unnu- malated mice for all studies.

Immunohistochemistry

Lungs from normal mice were fixed in 10% buffered formalin and embed- ded in paraffin. Deparaffinized sections were mounted and stained with 200 µg/ml anti-IL-10 Ab (Santa Cruz Biotechnology, Santa Cruz, CA). Sec- ondary staining was performed with Vectastain Elite goat IgG (Vector Laboratories, Burlingame, CA), following the manufacturer’s protocol. As a control, tissues were also stained with the secondary Ab step alone. All tissues were counterstained with hematoxylin.

Western blot analysis

AM and PM from normal and MyD88−/− mice were seeded in 96-well plates at 2.0 × 10^3 cells/well. After overnight incubation, adherent cells were washed twice with medium and incubated for 1 h at 37°C before treatment. Cytological analysis of the adherent cells showed them to be at least 95% F4/80 and Mac-1 positive (data not shown). Cells were then stimulated with CpG ODN, LTA, LPS, or medium alone as described, followed by 5 ng/ml rIL-10 for 30 min. Cell lysates were prepared in the presence of sodium orthovanadate and the protease inhibitor mixture set III (Calbiochem, San Diego, CA). Protein determination was performed using bicinchoninic acid reagents (Bio-Rad, Hercules, CA) and up to 10 µg of protein from whole cell lysates were resolved on SDS 8% polyacrylamide gels and transferred to nitrocellulose membranes. Phospho-STAT3 was detected with anti-phospho-STAT3 polyclonal Ab, followed by HRP-conju- gated anti-rabbit IgG (Cell Signaling Technology). Stripped membranes were subsequently reprobed with anti-STAT3 Ab to detect total STAT3 levels.

Immunocytochemistry

AM and PM from normal mice were seeded into 16-well chamber slides (Lab-Tek, Naperville, IL) at 2.0 × 10^3 cells/well. After overnight incubation, washed adherent cells were treated as indicated. Treatment was stopped by gently washing cells twice with ice-cold PBS. Cells were fixed in cold (−20°C) methanol for 10 min, followed by 1 h blocking with 3% BSA, 0.1% Triton X-100 in TBS (50 mM Tris-HCl, 150 mM NaCl). Cells were rinsed with TBS and then incubated overnight at 4°C with anti-CD11b Ab (1/100 dilution) in 3% BSA in TBS. Cells were washed twice with 0.1% Triton X-100 TBS and then incubated with HRP-conju- gated goat anti-rabbit IgG using Vectastain ABC kit (Vector Laboratories) following the manufacturer’s instructions. Visualization of nuclear staining was obtained with diamobenzidine peroxidase substrate (Sigma-Aldrich) staining and counterstaining with methyl green (Vector Laboratories).

IL-10R staining

Peritoneal cells from normal mice were plated at 5 × 10^5 cells/well in 6-well plates. Washed adherent cells were stimulated for 12 h with LPS, LTA, CpG ODN, or control medium. After stimulation cells were rinsed once and then rested on ice for 15 min with ice-cold PBS-BSA-azide. Cells were scraped, pelleted, and resuspended in Fc Blocker (BD Pharmingen, San Diego, CA) for 15 min before staining with F4/80 Ab for 30 min. After washing once with PBS-BSA, cells were stained for IL-10R using Fluoro- kine Cytometry Reagents (R&D Systems) as recommended by the manufac- turer. Briefly, tubes were individually stained for 60 min on ice with biotinylated IL-10 or PBS-BSA. As a negative control, some cells were preincubated with excess unlabeled IL-10 before biotinylated IL-10. Avid- in-PE (10 µl) was added to the tubes without washing and incubated on ice for an additional 30 min. Cells were washed once with 2 ml of buffer, centrifuged, and resuspended in 0.5 ml of buffer for analysis by flow cytometry.

RT-PCR

Peritoneal cells from normal mice were plated at 3 × 10^5 cells/well in 12-well plates in 1 ml of RPMI 1640 medium supplemented with 10% FCS. After overnight incubation, nonadherent cells were removed by rins- ing with medium. Adherent cells were stimulated with rIL-10 (5 ng/ml) for 10 h at 37°C. CpG ODN, LTA, and LPS were added 2 h after IL-10 treatment began. Total RNA was extracted using TRIzol reagent following the manufacturer’s protocol. Total RNA (1 µg) was reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) for 50 min at 46°C. cDNA (2 µl) was used as a template for PCR using Taq DNA polymerase (Invitrogen). CCR1 and CCR5 PCR were performed at 35 cycles (95°C, 55°C, and 72°C) using the following primers: CCR1, for- ward AGC TCA CCC CAC AAC TAC A GA, and reverse CTT GTA GGG AAA ATG AGG GCT A; CCR5, forward ACT TGC GTG GTG GCT GTG GTT TTT, and reverse TTG TCT TGT TGG AAA ATT GAA. As a loading control, β-actin PCR was performed at 29 cycles (95°C, 55°C, and 72°C) using the following primers: β-actin, forward GTG GGC CGC TCT TGG TGG AGA A and reverse CGG TGG GCC TCA GAG TTC AGG GGG G. Control RT-PCR for DNA contamination was performed by reverse transcription in the absence of reverse transcriptase, followed by PCR. PCR products were electrophoresed in 1.2% agarose-containing ethidium bromide. For IL-10 RT-PCR, normal lungs were obtained from C57BL/6 mice. Left lungs were dissected and dropped into liquid nitrogen until RNA extraction. Homogenization was done using 1 ml of Trizol reagent in a tissue homogenizer. Reverse transcriptase was done as previously de- scribed. IL-10 PCR was done using the following primers: IL-10, forward CTG CTC CAC TGC CTT GCT CTT ATT, and reverse GTG AAG ACT TTC TTT CAA ACA AAG.

TNF-α induction by C2-ceramide

PM (4.5 × 10^5/well) were plated in 24-well plates and allowed to adhere for 2 h. After washing off the nonadherent cells, cultures were incubated in 0.25% FCS RPMI 1640 and treated with or without LPS (1 µg/ml) for 3 h. Cultures were washed again to remove the LPS and incubated in serum-free RPMI 1640 in the absence or presence of IL-10 (5 ng/ml) for 2 h, after which 5 µM C2-ceramide was added to some wells. Cultures were further incubated for 4 h. Medium was collected and stored at −20°C until assayed. Content of TNF-α protein in the medium was determined using OptEIA TNF-α ELISA kit by BD Pharmingen following manufacturer recommendations.

Results

IL-10 is constitutively present in bronchoalveolar lavage fluids (BALF) from normal mice

Studies by Bonfield et al. (9) have shown that BALF from normal individuals contain significant amounts of IL-10. To determine whether IL-10 was also present in the alveoli of normal mice, lungs from C57BL/6 mice were either lavaged with PBS and the cell-free supernatant was analyzed for IL-10 by ELISA or normal lung tissue was harvested and evaluated for IL-10 by RT-PCR or immunohistochemistry. Low but detectable levels of IL-10 were observed in BALF of normal mice at concentrations ranging from 800 to 1200 pg/ml (data not shown). Analysis of RNA from normal lung demonstrated the presence of mRNA for IL-10, suggest- ing that IL-10 was produced in situ within the lung (Fig. 1A). Immunohistochemical staining demonstrated substantial amounts of IL-10 in the alveolar tissues of normal mice (Fig. 1C) compared with lungs stained with secondary Ab alone (Fig. 1B). Staining was observed in both type I and type II alveolar epithelial cells, but was not detected in AM of normal mice. Constitutive IL-10 se- cretion into normal BALF was not unique to C57BL/6 mice be- cause IL-10 was also detected in BALF from normal A/J mice (data not shown).
CpG, LTA, and LPS independently inhibit IL-10 signaling

Preliminary studies demonstrated that exposure of AM and PM to recombinant murine IL-10 induced a time-dependent phosphorylation of STAT3, which was maximal 30 min after exposure (data not shown). Consequently, exposure to IL-10 for 30 min was chosen for all experiments involving immunoblotting. Prior exposure of AM to CpG ODN (1 μM), LPS (1 μg/ml), or LTA (1 μg/ml) for 1–24 h before the addition of IL-10 inhibited IL-10-mediated signal transduction as indicated by STAT3 phosphorylation (Fig. 2). CpG was observed to transiently prevent IL-10 signal transduction after 3 and 6 h of pretreatment. LPS was able to inhibit IL-10 signaling beginning at 6 h pretreatment and remained effective throughout 24 h of pretreatment. Finally, LTA showed moderate inhibitory effects on STAT3 phosphorylation by IL-10 at 9 and 12 h pretreatment and returned toward normal after 24 h. In multiple experiments, CpG was always shown to be the weakest inhibitor, and the effects of CpG and LTA were always transient. In contrast, the effects of LPS pretreatment were always the most inhibitory of the TLR ligands and the effect always persisted through 24 h of pretreatment. Signaling through TLR9 by CpG ODN is known to be dependent on the CpG motif (13). The ability of CpG ODN to inhibit IL-10R signaling was also related to the presence of an unmethylated CpG motif, because prior exposure to a scrambled ODN sequence not containing CpG motifs failed to alter IL-10 responsiveness (data not shown). It should be noted that neither the CpG nor LTA preparations contained any detectable contamination with LPS, as indicated by the failure of these preparations to induce NO in the RAW 264.7 macrophage cell line (19) (data not shown).

If TLR ligand binding inhibited IL-10R function, then translocation of phospho-STAT3 to the nucleus following IL-10 stimulation should also be diminished in TLR ligand-treated cells compared with IL-10 treatment alone. Whereas phosphorylation of STAT3 in the cytoplasm is required for translocation of STAT3 into the nucleus, it was important to confirm that TLR ligand binding inhibited IL-10-induced translocation of phospho-STAT3 into the nucleus. Fig. 3 shows that exposure of AM to IL-10 for 30 min led to intense nuclear staining with anti-phospho-STAT3 Ab (Fig. 3B), compared with untreated macrophages that display little nuclear staining (Fig. 3A). Pretreatment with CpG ODN for 6 and 12 h before IL-10 stimulation reduced the intensity of the nuclear staining only at 12 h, but not to untreated levels (Fig. 3, C and D). The failure of CpG ODN to completely inhibit IL-10-induced translocation of phospho-STAT3 is in agreement with Western blot analyses of STAT3 phosphorylation. LTA and LPS were much more effective at inhibiting IL-10-induced phospho-STAT3 translocation than was CpG ODN. LTA displayed strong inhibition at 12 h, with some of the phospho-STAT3 translocation returning toward untreated levels after 24 h (Fig. 3, E and F). LPS pretreatment inhibited phospho-STAT3 translocation totally at both 12

![Negative Control](image1.png)

**FIGURE 2.** CpG ODN, LPS, or LTA inhibit IL-10-induced STAT3 phosphorylation in AM. Normal AM were incubated with CpG ODN (1 μM), LTA (1 μg/ml), or LPS (1 μg/ml) for the indicated periods of time before the addition of IL-10 (5 ng/ml) for 30 min. Total protein extracts were obtained and 5 to 10 μg were analyzed by immunoblotting using anti-phospho-STAT3 or STAT3 Abs. Similar results were seen in three separate experiments.
and 24 h after pretreatment (Fig. 3, G and H). Similar data were observed when PM were evaluated for phospho-STAT3 translocation (Fig. 3, insets).

**Inhibition of STAT3 activation is dependent on the MyD88 adaptor molecule and not on altered IL-10R expression**

The binding of biotinylated murine IL-10 to TLR ligand-treated macrophages was used to determine whether the inhibition of IL-10 signaling was due to a decrease in IL-10R expression on macrophage plasma membranes. PM treated for 12 h with CpG ODN, LTA, or LPS bound the same level of biotinylated IL-10 as did untreated macrophages (Fig. 4). These data indicate that the capacity of IL-10R to bind IL-10 was not altered by TLR ligand binding.

Whereas these TLR ligands were shown to inhibit IL-10 signal transduction, it was important to confirm that this effect was due to signaling through TLR molecules rather than to nonspecific effects or alternative signaling pathways. A common adaptor molecule, MyD88, is required for proper signal transduction through all members of the TLR family (20). As another approach to confirm that inhibition of IL-10R function by LPS, LTA, or CpG ODN was due to signaling through TLRs, PM from MyD88−/− mice were compared with normal C57BL/6 mice for the ability of these TLR ligands to inhibit IL-10R signaling (Fig. 5). Western blot analysis of IL-10-mediated STAT3 phosphorylation demonstrated that macrophages from both wild-type and knockout mice responded similarly to IL-10 exposure. However, pretreatment of macrophages with CpG ODN, LTA, or LPS inhibited the phosphorylation of STAT3 by IL-10 when
macrophages were obtained from normal mice, but had no effect in macrophages from MyD88−/− mice. These data indicate that the inhibition of IL-10R function by TLR ligands is dependent on expression of the MyD88 adaptor molecule and suggest that signaling through the TLR is necessary for inhibition of IL-10 signal transduction.

**TLR signaling inhibits gene expression in macrophages continually exposed to IL-10**

Because the data obtained from bronchoalveolar lavage studies in Fig. 1 suggested that AM are continually exposed to IL-10 in normal lung and in vitro studies indicated that TLR ligands could inhibit IL-10R signal transduction, it was important to determine whether sustained IL-10-mediated gene transcription was also affected by exposure to TLR ligands. The expression of the CCR1 and CCR5 genes was evaluated by RT-PCR in IL-10-exposed macrophages because these genes have been shown to be induced by IL-10, but not by TLR ligand binding (21, 22). In preliminary studies, we determined that freshly isolated AM expressed CCR1 and CCR5 mRNA, consistent with IL-10-induced expression in normal lung. In addition, overnight culture of AM in the absence of IL-10 led to loss of expression as was seen in normal PM (data not shown). When normal PM were exposed to IL-10, CCR1 and CCR5 mRNA expression was induced and remained elevated for at least 10 h (Fig. 6). In contrast, the addition of TLR ligands 2 h after IL-10 rapidly inhibited CCR5 (Fig. 6, upper panel) and CCR1 mRNA expression (Fig. 6, lower panel). These results indicate that the expression of macrophage genes that are induced by IL-10 in the absence of infection can be inhibited when exposed to TLR ligands.

**LPS inhibits IL-10 immunosuppressive activity in ceramide-stimulated macrophages**

To test the effect of TLR ligand binding on IL-10-induced immunosuppression, we examined the ability of LPS to block the ability of IL-10 to suppress TNF-α production in C2-ceramide-stimulated PM. C2-ceramide, a membrane permeable intracellular second messenger, mimics the activity of endogenously synthesized ceramide, a proinflammatory molecule produced by the cleavage of membrane sphingolipids. The addition of C2-ceramide to macrophages in vitro has been shown by others (23) to result in the increase of inducible NO synthase activity and TNF-α production. PM from normal mice produced moderate quantities of TNF-α when treated with C2-ceramide for 4 h, compared with medium only (Fig. 7). Pretreatment of the cells for 2 h with IL-10 rendered the cells unresponsive to C2-ceramide, demonstrating the immunosuppressive effect of IL-10 in this system. However, pretreatment of macrophages with LPS for 3 h before the addition of IL-10 completely blocked the ability of IL-10 to inhibit C2-ceramide-induced TNF-α production. These findings illustrate that TLR ligand binding also inhibits the ability of IL-10 to mediate immunosuppression, at least at the level of TNF-α production.

**Discussion**

Results from this study demonstrate that IL-10 is synthesized constitutively within normal alveoli and that continuous exposure to IL-10 can cause prolonged signaling through the IL-10R. The consequence of constitutive IL-10 within alveoli is currently unknown, but given the potent anti-inflammatory properties of IL-10 it is possible that IL-10 is involved in maintenance of an immunosuppressive environment within normal lung. The respiratory
system is exposed to inhaled particulates that must be cleared constantly from the bronchoalveolar airways. The clearance of innocuous particles is conducted by AM in a manner that must avoid eliciting inflammatory reactions that may damage the structural integrity of alveolar tissue. An immunosuppressive environment within normal lung has been demonstrated by numerous investigators and it is clear that AM are critical mediators of this immune suppression (23). A variety of factors released by AM have been shown to suppress the proliferation and/or effector function of lymphocytes and the proinflammatory response of macrophages, including PGE2, TGFβ, NO, and IL-10 (23). However, the ability of these factors to regulate inflammation in normal lung depends on their presence and activity during homeostatic conditions. In this regard, the levels of PGE2 and TGFβ in normal lung are quite low and most of the TGFβ in normal lung is present in its latent form (24, 25). NO production by AM has been proposed to be largely responsible for the regulation of lymphocyte activity in the lung (23, 26). However, NO has also been shown to enhance the ability of macrophages to produce proinflammatory cytokines (27, 28), suggesting that constitutive NO production within normal alveoli could actually be proinflammatory during clearance of innocuous particles. In contrast, IL-10 is an effective inhibitor of proinflammatory cytokine synthesis by macrophages, including AM that represent the predominant cell type in normal alveoli (1). Interestingly, IL-10 suppresses proinflammatory cytokine release without affecting endocytic processes (1), which would allow effective clearance of innocuous particles without eliciting an inflammatory response. However, exposure of the lower respiratory tract to microorganisms is also known to induce proinflammatory responses in AM, a process that is dependent on TLR expression (29). Given these two divergent roles for AM, we hypothesized that TLR ligand binding may alter the response of AM to IL-10.

Data consistent with this hypothesis demonstrated that the activation of STAT3 by IL-10 was inhibited by prior exposure of AM and PM to microbial products that bind to TLRs. The inhibition of IL-10R signaling was induced by TLR ligands that bind to TLR2, TLR4, or TLR9. Whether ligands for other TLRs also inhibit IL-10 signaling is currently unknown; however, the dependency of this inhibitory effect on the MyD88 adaptor molecule suggests that other TLRs may mediate similar inhibition on IL-10R function. Western blot analyses and immunocytochemistry demonstrated that TLR-mediated inhibition was similar in both AM and PM; however, the strength and duration of the response varied depending upon the TLR agonist being tested. Unmethylated CpG ODN consistently inhibited IL-R signaling the least, compared with LPS or LTA. The reason for the weak response by CpG ODN is not yet understood, but potentially could be due to lower expression of TLR9, lower affinity of the CpG ODN for TLR9, or by unknown differences in TLR adaptor molecules or other signal transduction molecules among the different TLR family members. A likely consequence of infection by an intact microorganism is that AM would engage several different TLRs due to the presence of multiple microbial structures that are ligands for different TLRs. Thus, the ability of TLR signaling to inhibit IL-10R function may be modulated ultimately by the total number and types of TLRs that are engaged during infection. Of note is the observation that PM responded to TLR ligands in an identical manner as did AM. Peritoneal fluids from normal humans have been shown to contain IL-10 in the range from 6 to 10 pg/ml (30, 31), which is substantially lower than what we observed in normal murine BALF. However, PM are also not normally exposed to environmental particulates or microorganisms as are AM, so whether TLR-mediated IL-10R dysfunction in PM has a physiologic role in the peritoneum is not known.

In gene expression experiments, it was determined that engagement of TLRs down-regulated IL-10-induced transcription of the CCR1 and CCR5 chemokine receptor genes. Because the studies in this report have shown that AM are continually exposed to IL-10 under normal conditions, the observation that TLR ligands can rapidly inhibit IL-10R function suggests that AM in normal lung may also have the ability to escape the immunosuppressive effects of IL-10. As an indication of the effect of TLR ligands on IL-10-mediated immunosuppression, exposure of macrophages to

![FIGURE 5. Inhibition of STAT3 activation is dependent on MyD88−/−. Nonelicited PM from wild-type or MyD88−/− mice were treated with ODN CpG (1 μM for 6 h), LTA (1 μg/ml for 12 h), or LPS (1 μg/ml for 12 h), after which cells were treated with IL-10 (5 ng/ml) for 30 min. Total protein extracts were obtained and 5 to 10 μg were analyzed using anti-phospho-STAT3 or STAT3 Abs. One of three identical experiments is shown.](http://www.jimmunol.org/)

![FIGURE 6. TLR agonists inhibit CCR1 and CCR5 gene expression in macrophages continually exposed to IL-10. Peritoneal macrophages from normal mice were plated and pretreated with IL-10 (5 ng/ml) for 10 h. Two hours after initiation of the IL-10 stimulation, cells were treated with CpG ODN (1 μM), LTA (1 μg/ml), or LPS (1 μg/ml) or left alone. Total RNA was extracted using TRIzol reagent. Complementary DNA was amplified using primers for murine CCR1 (bottom panel and inset) and CCR5 (top panel and inset). As a control, PCR was also performed for β-actin. Data are represented as the ratio of mean OD of CCR1 and CCR5 to β-actin. One of three identical experiments is shown.](http://www.jimmunol.org/)
small signaling peptides, such as macrophage-inhibitory membrane molecules that mediate cell migration in response to cytokines in the lung. The response of the lung to microbial infection is not yet clear. CCR1 expression by TLR ligands may also be important to the initial inflammatory response and could allow a more multidirectional response to these ligands. Whether down-regulation of IL-10-mediated CCR1 and CCR5 expression by TLR ligands may also be important to the initial response of the lung to microbial infection is not yet clear. CCR1 and CCR5, C-C chemokine receptors found on macrophages and up-regulated by IL-10 (21, 22), are members of a larger family of membrane molecules that mediate cell migration in response to small signaling peptides, such as macrophage-inhibitory protein (MIP)-1α and MIP-1β (32, 33). Interestingly, the effect of IL-10 on the chemotactic response of macrophages appears to be multidirectional because studies in humans have shown that IL-10 up-regulates expression of CCR5, but inhibits the expression of CCR7 (21, 34). The regulation of CCR1 and CCR5 expression by CpG ODN, LTA, and LPS stimulation suggests possible modulation of macrophage chemotactic activity by TLR-derived signals. AM exposed to IL-10 in normal lungs should express C-C chemokine receptors like CCR1 and CCR5 and thus would likely be able to respond to chemokines produced at an infection site within the alveolus. In preliminary studies, we have shown that freshly isolated AM express both the CCR1 and CCR5, consistent with IL-10-induced expression in normal lung (data not shown). In addition, alveolar epithelial cells have been shown to express TLRs and to release chemokines, including MIP-1α, in response to TLR ligands and proinflammatory cytokines (35–38). In this context, exposure to a chemokine produced by an infected epithelial cell could promote AM migration toward the infected cell. Upon arrival at the infection site, AM would be exposed to TLR ligands themselves, which would down-regulate chemokine receptor expression, thus promoting macrophage retention at the infectious focus. Moreover, because IL-10R function is inhibited by TLR ligands, the ability of AM to produce proinflammatory cytokines in response to these ligands would likely be increased in an alveolus, even though normal levels of immunosuppressive IL-10 may be present, as shown by LPS inhibition of IL-10 immune suppression. Additional studies will be required to test the validity of this model.

How the activation of TLRs leads to inhibition of IL-10 signaling remains unknown, but two potential mechanisms can be envisioned. Suppressors of cytokine signaling (SOCS), a family of proteins that include SOCS1–7 and CIS (39, 40), are known to inhibit signal transduction by several cytokine receptors, including inhibition of the IL-10R (41). Preliminary studies in our lab (data not shown) have indicated that LPS, LTA, and CpG ODN each induce the expression of both SOCS1 and SOCS3 mRNA within 1–3 h in PM, which has also been observed in other studies (42, 43). The induction of SOCS1 and SOCS3 was consistent with the inhibition of IL-10-mediated CCR1 and CCR5 expression by these TLR ligands. Additional studies have shown that SOCS3 can associate with Janus kinase (Jak)2 through a Src homology 2 domain to inhibit Jak-Stat signaling (44), suggesting that a similar interaction with Jak1 of the IL-10R could also occur. However, recent publications have also suggested that SOCS3 may not be involved in the regulation of IL-10R signaling (45–47). Thus, the possible role of TLR-induced expression of SOCS proteins in the regulation of IL-10R function is unclear and additional studies are clearly needed to determine whether induction of SOCS1, SOCS3 or other members of the SOCS family of proteins by TLR ligands is required for inhibition of IL-10R function.

An alternative mechanism may involve TLR-mediated activation of the mitogen-activated protein kinase (MAPK) pathway. Two MAPKs, p38 and extracellular signal-related kinase 1/2, are rapidly induced by activation of a number of TLR family members (48, 49) and have been shown to interfere with the activation of STAT proteins via inhibition of Jak activity (50–52). Moreover, at least one of the MAPKs, p38, has been shown to inhibit IL-10R signaling via interference with STAT3 activation (53, 54).

In conclusion, we have identified two new features of immune regulation in normal mouse lungs. First, IL-10 is constitutively produced by alveolar epithelium, which can affect the function of normal AM. Secondly, exposure of normal AM to TLR ligands can inhibit IL-10R function on these cells. The data suggest that the distinction by normal AM between inhaled innocuous substances and potentially damaging infectious organisms via TLR engagement may permit AM to overcome the effects of alveolar IL-10, possibly allowing a more rapid and effective inflammatory response against an infection.

References


37. Agellon is mediated by Toll-like receptor 5.