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INTERLEUKIN-10 RECEPTOR DYSFUNCTION IN PERITONEAL MACROPHAGES BY TOLL-LIKE RECEPTOR LIGANDS

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ABSTRACT OF THESIS

INTERLEUKIN-10 RECEPTOR DYSFUNCTION IN PERITONEAL MACROPHAGES BY TOLL-LIKE RECEPTOR LIGANDS

Interleukin-10 (IL-10) is a pleiotropic cytokine which limits inflammatory responses by balancing the host's immune response against infection. Mammalian Toll-like receptors (TLRs) are pattern recognition receptors that recognize specific molecular patterns on microbial pathogens and activate intracellular signaling via the transcription factors NF- κ B and IRF-3. In this study we evaluate the contribution of the TLR ligands Poly I:C, Pam₃CSK4, LPS and LTA to IL-10 receptor dysfunction in murine peritoneal macrophages (PM). We examine how these ligands are able to alter IL-10 mediated STAT3 phosphorylation and CCR5 gene expression in PM. The ability of Poly I:C and Pam₃CSK4 to alter the immunosuppressive activity of IL-10 in C2-ceramide stimulated PM is also examined. The results of our study indicate a delayed inhibition of IL-10 mediated activation of STAT3 by LPS, LTA, Poly I:C and Pam₃CSK4. The CCR5 gene expression experiments demonstrate that LPS was able to down-regulate IL-10 induced CCR5 mRNA expression in PM.

KEYWORDS: Interleukin-10, STAT3, Toll-like receptors, Poly I:C, Pam₃CSK4.

Surjya Bhattacharyya

5.30.05

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MACROPHAGES BY TOLL-LIKE RECEPTOR LIGANDS

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THESIS

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The Graduate School

University of Kentucky

2005

INTERLEUKIN-10 RECEPTOR DYSFUNCTION IN PERITONEAL
MACROPHAGES BY TOLL-LIKE RECEPTOR LIGANDS

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
Graduate Center for Toxicology
at the University of Kentucky

By

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2005

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ACKNOWLEDGEMENTS

I would like to sincerely thank my Thesis Chair, Dr. Donald Cohen for the insight, timely and instructive comments, and evaluation of this thesis. Next I wish to thank the complete Thesis Committee: Dr. Alan Kaplan, Dr. Subbarao Bondada, and Dr. Charlotte Kaetzel. Each individual provided insights that guided and challenged my thinking, substantially improving the finished product.

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Introduction

Interleukin-10 (IL-10) is a multifunctional cytokine which has diverse effects on various types of hematopoietic cells, including B and T lymphocytes, natural killer cells (NK) cells, mast cells, and eosinophils (1,2). IL-10 regulates growth and differentiation of B cells, NK cells, cytotoxic and helper T cells, mast cells, granulocytes, dendritic cells, keratinocytes, and endothelial cells (1). IL-10 is also crucial in the differentiation and function of T regulatory cells, which play a prominent role in the control of immune responses and tolerance in vivo (1). IL-10 can be produced by both CD4⁺ and CD8⁺ T cells, B cells, monocytes, macrophages, and dendritic cells (2). IL-10 can inhibit production of cytokines such as IL-2 and interferon- γ (IFN- γ) by Th1 type T cells (1). This inhibitory effect of IL-10 is mediated indirectly via effects on antigen-presenting cells (APCs), including monocytes, macrophages and dendritic cells (2). IL-10 can inhibit a variety of macrophage/monocyte functions, including IL-12 and NO production, and expression of class II MHC and costimulatory molecules like CD80/CD86 (2). IL-10 can also inhibit production of IL-1 α , IL-1 β , IL-6, IL-12, IL-18, GM-CSF, G-CSF, M-CSF, TNF, LIF, and PAF by activated macrophages (3,4,5,6).

The principal in vivo function of IL-10 appears to limit and ultimately terminate inflammatory responses (1). IL-1 and TNF synergistically activate inflammatory pathways and processes, and induce chemokines, prostaglandins, and PAF, which amplify the inflammatory responses (1). IL-10 inhibits both CC and CXC chemokines, which are involved in recruitment of monocytes, dendritic cells, neutrophils, and T cells during inflammation (7,8,9). The anti-inflammatory activity of IL-10 is mediated in part by its inhibitory effects on both IL-1 and TNF production. IL-10 deficient mice demonstrate exaggerated inflammatory responses like inflammatory bowel disease (IBD), which clearly demonstrates the importance of IL-10's anti-inflammatory function (10,11).

IL-10 plays a crucial role in striking a balance between immune-mediated pathology and protection in the tissues of an infected host, which tries to provide a sufficiently intense immune response against the infection, while minimizing nonspecific tissue injury. *In vitro* methods have been used to study some aspects of IL-10 biology by evaluating the influence of IL-10 on the response of individual cell types to microorganisms or microbial products (1). The shortcomings of these studies are that host-pathogen interactions are complex,

and vary according to the life-cycle of the pathogen and progress of the host's immune response (1). Animal models, principally mouse, of infectious disease have been used to evaluate the overall role of IL-10 in the recovery from infection. Aspects of both innate and adaptive immune responses are enhanced by experimental depletion of IL-10 *in vivo*, while the same responses are impaired by experimental elevation of IL-10 *in vivo*. Elevated IL-10 severely compromises innate immune responses to *Listeria* in *scid* mice (12), while there are enhanced immune responses to *Listeria* in IL-10 *-/-* mice (13). Similarly, there is rapid control of *Listeria* within the first few days of infection in anti-IL-10 treated mice (14).

IL-10 and IL-10 Receptor Structure

The bioactive form of IL-10 is a homodimer consisting two 18 kDa subunits which are noncovalently associated. In monocytes, the expression of the IL-10 gene can be induced by Gram-positive and Gram-negative bacteria, purified endotoxin, bacterial exotoxins, and certain viruses (2). In lipopolysaccharide (LPS)-stimulated monocytes, the expression of the IL-10 gene is delayed relative to other cytokine genes, such as tumor-necrosis factor- α (TNF- α) and IL-1 (3). In activated T cells, IL-10 gene expression also is delayed compared to other cytokine genes, such as IL-2 and IL-4 (15). IL-10 receptors (IL-10R) are expressed by hematopoietic cells such as B cells, T cells, NK cells, monocytes, macrophages, and dendritic cells. Non-hematopoietic cells such as fibroblasts or endothelial cells do not express IL-10Rs (2). However, other non-hematopoietic cells have been shown to express functional IL-10Rs. The human IL-10R is species specific, while the murine IL-10R can bind both murine and human IL-10 with comparable affinity. Functional IL-10R complexes are tetramers consisting of two IL-10R1 polypeptide chains and two IL-10R2 chains. The IL-10R1 chain is the ligand-binding chain of the IL-10R complex. Homodimeric IL-10 binds to the extracellular domains of the two adjoining IL-10 R1 molecules and activates phosphorylation of the receptor-associated Janus tyrosine kinases, JAK1 and Tyk2 (16,17). JAK1 associates with the IL-10R1 chain, while Tyk2 associates with the IL-10R2 chain. These kinases then phosphorylate specific tyrosine residues (Y446 and Y496) on the intracellular domain of the IL-10R1 chain (17,18,19). These phosphorylated Tyr residues and their flanking peptide sequences serve as docking sites for the latent transcription factor, STAT3 (Signal Transducer and Activator of Transcription 3). STAT3 binds to these docking sites by its SH2

domain, following which it is Tyr phosphorylated by the receptor associated JAKs. The phosphorylated STAT3 then homodimerizes and translocates to the nucleus where the dimer binds with high affinity to SBEs (STAT binding elements) in the promoters of various IL-10 responsive genes. One of these genes is SOCS3 (Suppressor of Cytokine signaling 3), which is known to inhibit JAK/STAT-dependent signaling, and acts to down-regulate IL-10R function. IL-10 activates both STAT1 α and STAT3 in most IL-10R positive cell types (16). Three distinct STAT complexes can be formed by activation of these STATs: STAT1 homodimers, STAT3 homodimers, and STAT1:STAT3 heterodimers (18). This variation in the assembly of STAT complexes may induce distinct patterns of gene expression in different cell types (2).

Functional Effects of IL-10 Signaling

IL-10 inhibits expression of several LPS-inducible genes in monocytes, including the cytokines TNF- α , IL-1, IL-6, IL-8, IL-12, G-CSF, and GM-CSF. The expression of other LPS-inducible genes like cyclooxygenase-2 (Cox-2) is also inhibited by IL-10 (2). IL-10 inhibits expression of several IFN- γ inducible genes, including MHC class II molecules, B7, and IP-10 (25,26,24). In monocytes, IL-10 also activates expression of the genes for CCR5, Fc γ R1, TIMP-1, TNF-R2, and SOCS-3 (20,21,22,23,24).

The critical role of IL-10 in regulating the immune response is seen in several chronic inflammatory diseases, where the function of IL-10 appears to be suppressed. It has been observed that IL-10 was not effective in suppressing cytokine production in monocytes or macrophages derived from patients with Systemic Lupus Erythematosus (SLE) or Rheumatoid Arthritis (RA) (27, 28). In SLE there appears to be a defect in the IL-10-induced suppression of IL-6 production. Mongan et al (27), examined recombinant human IL-10-mediated suppression of IL-6 production by monocytes and B cells from lupus patients, and compared the results with normal controls. IL-10 caused a concentration-dependent suppression of IL-6 production in normal monocytes and B cells, which was not observed in the same cells from lupus patients. Another study has demonstrated that ligation of Fc γ Rs by immune complexes inhibited IL-10 induced activation of the Jak-STAT pathway in IFN- γ exposed, synovial fluid macrophages, obtained from RA patients (29).

Regulation of IL-10 Receptor Function

One of the key regulators of the Jak-STAT signaling is a family of molecules known as suppressor of cytokine signaling (SOCS). Eight members of the SOCS protein family have been identified, SOCS1 through SOCS7, and CIS (cytokine inducible SH2 domain protein) (30). SOCS3 inhibits Jak2 kinase activity by binding to the SH2 domain to regulate Jak-STAT signaling (31). This suggests that a similar interaction of SOCS3 with Jak1 of the IL-10R could also occur. IL-10 also induces SOCS1 expression which negatively regulates IL-10 function and IL-10R signaling (32). Preliminary studies in our lab have indicated that the Toll-like receptor (TLR) ligands, LPS, LTA and CpG ODN each induce the expression of both SOCS1 and SOCS3 mRNA within 1-3 hours in peritoneal macrophages (data not shown), which has also been observed in other studies (33,34). However, some groups have suggested that SOCS3 may not be involved in the regulation of IL-10R signaling (35,36). It is still unclear if SOCS1 or SOCS3, or other members of the SOCS family of proteins induced by TLR ligands, are involved in the inhibition of IL-10R function in macrophages. Hart et al have shown that prior exposure of macrophages to GM-CSF inhibited subsequent responsiveness of macrophages to IL-10 (37). Previous studies by our lab suggest that pre-exposure of macrophages to TNF- α reduces their response to IL-10, which was not related to a change in receptor expression (38). The ability of IL-10 to inhibit LPS-induced production of IL-6 was significantly reduced in peritoneal macrophages pre-treated with TNF- α (38). We have also demonstrated that LPS, LTA, and CpG inhibited IL-10R signal transduction in alveolar and peritoneal macrophages and appeared to require the expression of the myeloid differentiation factor 88 (MyD88) adaptor protein without decreasing IL-10R expression. (39).

Recognition of Microbes by the Innate Immune System

The innate immune system utilizes germ-line encoded receptors to recognize pathogens that express highly-conserved structures present in a large group of microorganisms. These microbial structures are known as pathogen-associated molecular patterns (PAMPs) and are produced by microbial pathogens, but not by their hosts. For example, LPS and LTA are cell wall components that are synthesized only by bacteria. Usually PAMPs are essential for the pathogenicity or survival of the microorganisms. Bacterial lipopolysaccharide (LPS), lipoteichoic

acid (LTA), bacterial DNA, double-stranded RNA, peptidoglycan, mannans, and glucans are examples of some well known PAMPs. The receptors of the immune system that evolved to recognize PAMPs are collectively called pattern-recognition receptors (PRRs). PAMPs are usually invariant structures that are present in an entire class of pathogen. For example, LPS is present in all gram-negative bacteria and the specific PRR of the host recognizes virtually any gram-negative bacterial infection (40).

PRRs are expressed on antigen-presenting cells including macrophages, dendritic cells and B cells. These cells are immediately activated once PRRs bind PAMPs, accounting for the rapid kinetics of the innate immune response. PRRs can be classified based on their structural domains into receptors having either leucine-rich repeats, calcium-dependent lectin, or scavenger-receptor protein. Functionally, PRRs can be divided into three classes: secreted, endocytic, and signaling (40). Secreted PRRs function as opsonins which bind to the microbial cell walls, thus initiating recognition by the complement system and phagocytes. An example of this class is mannan-binding lectin, which is also a member of the calcium-dependent lectin family (40). This receptor binds to microbial carbohydrates to initiate the lectin pathway of complement activation. Endocytic PRRs occur on the surface of phagocytes, which internalize and deliver the pathogen to lysosomes once these receptors recognize PAMPs on a microbial cell. An example of this class of PRR is the macrophage mannose receptor, which also belongs to the calcium-dependent lectin family. It specifically recognizes carbohydrates with large number of terminal mannoses and mediates their phagocytosis by macrophages. Signaling receptors recognize PAMPs and activate signal-transduction pathways that induce the expression of genes of inflammatory cytokines and chemokines(40). Receptors of the Toll family belong to this class of PRRs and play a crucial role in *Drosophila* and Mammalian host defence. In mammals these receptors are called Toll-like Receptors (TLRs) and activate intracellular signaling via NF- κ B or IRF-3, which results in the induction of a variety of effector genes (41). Since PAMPs are also expressed in commensal microorganisms, the term immune adjuvant may be more appropriate and TLRs could be defined as adjuvant receptors (42). In mammalian species there are at least eleven TLRs, each having a distinct function in innate immune recognition (42,43). Some common themes that are emerging are that TLR ligands tend to be conserved microbial products, which may be structurally

unrelated and some TLRs require accessory proteins to recognize their specific ligands (43).

Based on their molecular characteristics TLR ligands can be divided into three categories: lipid, nucleic acid, and protein (42). The following are some examples of the above three categories of TLR ligands.

Lipid Ligands: Lipopolysaccharide (LPS) is a complex glycolipid which is an integral component of the Gram-negative bacterial cell wall. LPS is composed of a hydrophilic polysaccharide portion and a hydrophobic domain known as lipid A, which is responsible for its biological activity (44). TLR 4, the first characterized mammalian TLR, functions as the signal transducing receptor for LPS from Gram-negative bacteria (43,46). The recognition of LPS by TLR4 is complex and requires several accessory molecules. LPS first binds to a serum protein called LPS-binding protein (LBP), which then transfers LPS monomers to CD14. CD14 can either be secreted into serum or expressed as a glycosylphosphatidylinositol (GPI)-linked protein on the surface of macrophages. Another component of the TLR4 receptor complex is MD-2, which is a small protein lacking a transmembrane region that is expressed on the cell surface and associated with the ectodomain of TLR4 (43). The outer membrane of Gram-positive bacteria have a thick layer of peptidoglycan which also contains a variety of lipoproteins and lipopeptides. The cytoplasmic membranes of mycoplasmas also have several lipopeptides. TLR2 recognizes several of these components by associating as homo- or heterodimers with other TLR2 relatives. For example, both TLR2 and TLR6 are required by macrophages to recognize peptidoglycan. Diacetylated mycoplasmal lipopeptides are recognized by TLR2:TLR6 heterodimers. TLR2 and TLR1 cooperate in the recognition of bacterial lipopeptides (42). PAM₃CSK4 is a synthetic bacterial lipopeptide containing a triacetylated cysteinyl residue, which is recognized by the TLR2:TLR1 heterodimer (44).

Nucleic Acid Ligands: Bacterial DNA abundantly expresses unmethylated CpG motifs, while mammalian DNA rarely contains this motif. This structural difference is utilized by TLR9 in the host to alert the immune system of bacterial infections. TLR7 and TLR8 are highly homologous to each other and TLR9 (42). Imidazoquinoline derivatives, imiquimod and resiquimod (R848), are TLR7 ligands which exert antiviral activity. Anticancer synthetic chemicals, including loxoribine (7-allyl-8-oxoguanosine), and bropirimine (2-amin-5-bromo-6-phenyl-4(3)pyrimidinone) and certain guanosine analogs are also TLR7 ligands.

Single-stranded RNAs derived from influenza virus or human immunodeficiency virus-1 and non-viral RNAs like poly-uridine, are also TLR7 ligands. Human TLR8 recognizes R848 and certain ssRNAs. TLR7 and TLR9 recognize their ligands in a similar manner. TLR7 and TLR9 are both expressed in the endoplasmic reticulum and then move to the endosome, where they interact with their specific ligands. RNA virus infection leads to generation of double-stranded RNAs (dsRNAs), which are recognized by TLR3. Polyinosinic-polycytidylic acid (poly(I:C)), is a synthetic analogue of dsRNA which is effectively recognized by TLR3 (42).

Protein ligand: Flagellin, is a protein that forms bacterial flagella. The amino- and carboxy- termini of flagellin are extremely conserved and recognized by TLR5. TLR5 is expressed on the basolateral side, but not on the apical side of the intestinal epithelium. This polarized expression may account for discrimination between commensal and pathogenic bacteria, since only the latter cross the epithelial barriers (42, 43).

TLR Signaling Pathway

An intracytoplasmic domain of all TLRs is similar to each other and to the other IL-1R family members. This highly conserved domain is called the Toll/IL-1R homologous (TIR) domain. MyD88 is a cytoplasmic adaptor which also contains of a TIR domain and can interact with both TLRs and IL-1Rs, via interaction between the respective TIR domains (42). When activated, MyD88 recruits a death domain-containing serine/threonine kinase, the IL-1R-associated kinase (IRAK). IRAK is subsequently activated by phosphorylation and then associates with tumor necrosis factor-associated factor 6 (TRAF6), leading to NF- κ B activation. Under normal conditions, I κ B sequesters NF- κ B in the cytoplasm and regulates its activity. Phosphorylation of I κ B on serine residues by the I κ B kinase (IKK) complex leads to the dissociation and nuclear translocation of NF- κ B (45).

LPS is also able to activate NF- κ B with delayed kinetics in MyD88-deficient macrophages, indicating that there is also a MyD88-independent component in the LPS signaling pathway (45,46). It seems that this alternate pathway is responsible for the LPS-induced maturation of dendritic cells (45). LPS stimulation leads to the activation of the transcription factor IFN-Regulatory Factor-3 (IRF-3), which then induces IFN- β . Viral-derived dsRNA is also a potent activator of IRF-3 through the MyD88-independent pathway, which then also

initiates IFN- β induction. It seems that the IKKs Tank-binding kinase (TBK-1) and IKK ϵ /IKK ι regulate activation of IRF-3 by phosphorylation in response to TLR3 activation. However, it is unclear if these IKKs are involved in TLR4-mediated IRF-3 activation (46).

Studies in this dissertation tested the hypothesis that the IRF-3 pathway contributes to IL-10R dysfunction in peritoneal macrophages. Peritoneal macrophages were examined for altered responsiveness to IL-10 following exposure to the TLR ligands LPS (*E. coli*), Poly(I:C), and Pam₃CSK4. LPS is known to activate both the IRF-3 pathway and the MyD88 pathway, while Poly (I:C) activates the IRF-3 pathway only (46), and Pam₃CSK4 activates only the MyD88 pathway (44). The results of this study indicate a delayed inhibition of IL-10 mediated activation of STAT3 by TLR ligands LPS, LTA, Poly (I:C), and Pam₃CSK4. CCR5 gene expression experiments demonstrate that LPS was able to down-regulate IL-10 induced CCR5 mRNA expression in PM. Also, Poly (I:C) and Pam₃CSK4 seem to reduce IL-10 mediated suppression of TNF- α production by C2-ceramide.

Materials and Methods

Mice and Reagents

Normal C57BL/6 (6-8 weeks old) female mice were purchased from Jackson Laboratories. Mice were allowed to acclimate for 1-2 weeks in microisolator cages and were provided with sterile water and food *ad libitum* and maintained by the Department of Laboratory Animal Resources. *E. coli* 0111:B4 lipopolysaccharide (LPS), *Streptococcus aureus* lipoteichoic acid (LTA), and C2-ceramide (N-Acetyl-D-sphingosine), were purchased from Sigma-Aldrich (St. Louis, MO). Poly (I:C) was obtained as a gift from Dr. Maria Bruno in Dr. Charlotte Kaetzel's laboratory. Pam₃CSK4 was purchased from InvivoGen (San Diego, CA). Alexa Fluor 680 goat anti-rabbit IgG (H+L) Ab was purchased from Molecular Probes (Eugene, OR), and IR Dye 800CW goat anti-mouse IgG (H+L) Ab was purchased from Rockland (Gilbertsville, PA). Anti-phospho-STAT3 Ab was purchased from Cell Signaling Technology (Beverly, MA), and anti-STAT3 Ab was obtained from BD Biosciences (Lexington, KY). Odyssey blocking buffer was obtained from LI-COR Biosciences (Lincoln, NE). Recombinant murine IL-10 (rm IL-10) was purchased from Peprotech (Rocky Hill, NJ). Peritoneal macrophages (PM) were obtained by lavage with PBS from normal, unmanipulated mice for all studies.

Western Blot Analysis

PM from normal C57BL/6 (6-8 weeks old) female mice were seeded in 6-well plates at 1×10^6 cells per well in RPMI 1640 medium containing 0.2% fetal calf serum plus penicillin and streptomycin (complete medium). After overnight incubation, adherent cells were washed twice in complete medium before adding 2ml of complete medium to each well. Cells were then stimulated with LPS, LTA, Poly (I:C), Pam3CSK4, or medium alone as described, followed by 5ng/ml rmIL-10 for 30 minutes. At the end of the experiment the medium was aspirated and 1 ml of cold PBS was added to each well to terminate the reaction. The cells were then scraped off with a cell scraper and the suspension was then transferred to Eppendorf tubes. The samples were then centrifuged at 3000 rpm for 15 minutes after which the sample supernatants were aspirated. The cell pellets were then dissolved in 40 μ l of cell lysis buffer (0.5% Triton X-100, 300mM NaCl, 0.25mM EDTA, 50mM Tris, pH=7.5) in the presence of sodium orthovanadate and protease inhibitor mixture set III (Calbiochem, San Diego, CA). The samples

were then frozen at -20°C overnight, after which the samples were thawed and sonicated for 5 seconds. Samples were then centrifuged at 7000 rpm for 10 minutes after which the sample supernatants were transferred to separate tubes. Protein determination was performed on these supernatants using bicinchoninic acid reagents (Bio-Rad, Hercules, CA). Up to 5 µg of whole cell lysate protein was resolved on SDS 8% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). Blots were then blocked for 1 hour in Odyssey blocking buffer without Tween-20. Phospho-STAT3 and STAT3 were detected by coincubating membranes overnight at 4 degrees C with anti-phosphoSTAT3 polyclonal Ab (1:1000 dilution) and anti-STAT3 Ab (1:1000 dilution) in Odyssey blocking buffer containing 0.1% Tween-20. Membranes were then washed thrice (15 minutes each wash) in PBS with 0.1% Tween-20. Membranes were then incubated for 1 hour at room temperature with Alexa Fluor 680 goat anti-rabbit IgG (H+L) Ab and IRDye 800CW goat anti-mouse IgG (H+L) Ab (both at 1:10,000 dilution) in Odyssey blocking buffer containing 0.1% Tween-20. The membranes were washed four times (15 minutes each wash) in PBS with 0.1% Tween-20. The blots were analyzed on the LI-COR Odyssey imaging system.

RT-PCR

Peritoneal macrophages (PM) from normal C57BL/6 (6-8 weeks old) female mice were seeded in 6-well plates at 1×10^6 cells per well in complete RPMI medium. After overnight incubation, adherent cells were washed twice in the same medium before adding 2ml of complete medium to each well. Adherent cells were stimulated with rIL-10 (5ng/ml) for 26 hours at 37°C. LPS, Poly I: C, and PAM₃CSK4 were added 2 hours after the IL-10 treatment. Total RNA was extracted using TRIzol reagent following the manufacturer's protocol.

Total RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) for 45 min at 42°C. cDNA was used as a template for PCR using *Taq* DNA polymerase (Invitrogen). CCR1 and CCR5 PCRs were performed at 30-35 cycles. As a loading control, β-actin PCR was performed at 30 cycles. PCR products were electrophoresed in 1.2% agarose-containing ethidium bromide.

TNF- α induction by C2-ceramide

PM were plated in 24 well plates at 0.5×10^6 cells per well and allowed to adhere overnight in 0.2% FCS RPMI medium. After washing off the non-adherent cells, the cultures were incubated in the same medium and treated with or without Poly I:C (20 $\mu\text{g/ml}$) or Pam3CSK4 (200 ng/ml) for 24 hours. Cultures were then washed again to remove the ligand and incubated in serum free RPMI 1640 in the absence or presence of IL-10 (5ng/ml) for 2 hours. 1 μM of C2-ceramide was then added to some wells. Cultures were further incubated for 4 hours. Medium was collected and stored at -20°C until TNF- α protein content was evaluated using an OptEIA TNF- α ELISA kit by BD PharMingen, following the manufacturer's protocol.

Results

Preliminary studies demonstrated that exposure of PM to recombinant murine IL-10 induced a time dependent phosphorylation of STAT3, which was maximal at 30 minutes after exposure and began to decay 60 minutes after exposure to IL-10. Consequently, exposure to IL-10 for 30 minutes was chosen for all experiments involving immunoblotting. Previous studies in the laboratory have demonstrated that pre-exposure of alveolar macrophages to LPS and LTA transiently reduced IL-10 mediated STAT3 phosphorylation. LPS was observed to inhibit IL-10 mediated STAT3 phosphorylation from 6-24 hours pretreatment, while LTA showed moderate inhibitory effects at 12 hour pretreatment (39).

The goal of these studies was to evaluate the ability of other TLRs ligands to inhibit IL-10 signal transduction. We chose to evaluate the ligand for TLR3 (Poly I:C), since it signals via IRF-3, and demonstrates a delayed activation of NF- κ B independent of MyD88 (42,54). The TLR4 ligand LPS was also evaluated since it has the unique ability to activate NF- κ B using both MyD88-dependent and independent pathways, and also activate IRF-3 in a MyD88 independent manner (42). We also re-evaluated IL-10R dysfunction through TLR2 by using the synthetic ligand Pam₃CSK4, since LTA preparations are often contaminated by other microbial components. In addition, Pam₃CSK4 signals through a TLR2/TLR1 heterodimer (44), whereas LTA binds only to TLR2 and only signals through the MyD88 adaptor protein (53).

Inhibition of IL-10 Signaling by LPS

Previous studies by Fernandez et al (39) showed that prior incubation of normal alveolar macrophages with LPS for at least 9 hours caused a reduction in the ability of IL-10 to phosphorylate STAT3. While inhibition of IL-10 signaling was not observed with only 1 hour of LPS preincubation, we did not know if shorter exposures to LPS could cause IL-10R dysfunction. Studies by Ahmed and Ivashkiv demonstrate that preincubation of primary macrophages with LPS for 20 minutes inhibits IL-6 mediated STAT3 phosphorylation and that a 20 minute exposure to IL-1 could block IL-10 mediated STAT3 phosphorylation (52). To evaluate short exposure time, PM were treated with LPS (1 μ g/ml) for 10-60 minutes before the addition of 5 ng/ml of IL-10 for 30 minutes (Fig. 1). The addition of IL-10 to untreated PM induced a 3-fold increase in the level of STAT3 phosphorylation. Prior incubation with LPS caused a moderate inhibition of STAT3 at 30 minutes of LPS preincubation, which was not statistically significant.

We next evaluated whether longer exposures to LPS would alter IL-10 mediated STAT3 activation in PM. Preliminary experiments for this study failed to demonstrate any significant reduction in IL-10 mediated STAT3 phosphorylation in PMs pre-exposed to LPS for 1-10 hours (data not shown). Normal PM were pretreated with LPS (1 μ g/ml) for 12 and 34 hours before the addition of 5 ng/ml of IL-10 for 30 minutes (Fig. 2). The addition of IL-10 to untreated PM induced an 8-fold increase in the level of STAT3 phosphorylation (lane2 vs. lane1) ($p < 0.0001$). Also, there was a significant reduction in IL-10 mediated STAT3 phosphorylation at both 12 and 34 hours pretreatment with LPS compared to IL-10 treatment alone ($p < 0.0001$). Delayed reduction in IL-10 mediated STAT3 phosphorylation by LPS was not unique to PM from C57BL/6 mice, since it was also observed at 36 hours in PM from BALB/C, C3H/HeSnJ, and A/J mice (data not shown).

Delayed Inhibition of IL-10 Signaling by LTA

PM were preexposed to the TLR2 ligand *S. aureus* LTA (1 μ g/ml) for 12 and 34 hours before the addition of 5 ng/ml IL-10 for 30 minutes (Fig.3). The addition of IL-10 to untreated PM induced an 8-fold increase in the level of STAT3

phosphorylation compared to untreated cells ($p < 0.0001$). Also, there was a significant reduction in IL-10 mediated STAT3 phosphorylation at both 12 and 34 hours pretreatment with LTA, compared to IL-10 only treated cells ($p < 0.0001$). Delayed reduction in IL-10 mediated STAT3 phosphorylation by LTA was not unique to PM from C57BL/6 mice, since it was also observed at 36 hours in PM from BALB/C and C3H/HeSnJ mice (data not shown).

Delayed Inhibition of IL-10 Signaling by Poly I:C

PMs were pretreated with Poly (I:C) (20 $\mu\text{g/ml}$) for 12-36 hours before the addition of 5ng/ml of IL-10 for 30 minutes. The addition of IL-10 alone to untreated PM induced an 8-fold increase in the level of STAT3 phosphorylation (Fig. 4, lane2 vs. lane1) ($p < 0.05$). Poly (I:C) alone for 12-36 hours did not significantly alter the level of STAT3 phosphorylation (Fig. 4, lanes 3,4,5). In contrast, there was a significant reduction in IL-10 mediated STAT3 phosphorylation at 36 hours pretreatment with Poly (I:C), compared to IL-10 treatment alone ($p < 0.05$) (Fig. 4, lane 8). 24 hour Poly (I:C) pretreatment was also able to induce a fourfold decrease in STAT3 activation that approached significance ($p = 0.0617$) (Fig. 4, lane 7).

Delayed Inhibition of IL-10 Signaling by Pam₃CSK4

PMs were pretreated with Pam₃CSK4 (200 ng/ml) for 12-36 hours before the addition of 5ng/ml of IL-10 for 30 minutes (Fig. 5). The addition of IL-10 alone to untreated PM induced a significant increase in the level of STAT3 phosphorylation compared to untreated cells (Fig 5, lane 2 vs. lane 1) ($p < 0.05$). In contrast, Pam₃CSK4 treatment alone did not significantly alter STAT3 phosphorylation compared to untreated PM (Fig. 5, lanes 3,4,5 vs. lane 1). However, there were significant reductions in IL-10 mediated STAT3 phosphorylation at 12, 24, and 36 hours pretreatment with Pam₃CSK4 compared to IL-10 treatment alone ($p < 0.05$) (Fig. 5, lanes 6,7,8 vs. lane 2).

Gene expression in peritoneal macrophages continually exposed to IL-10 before TLR ligands

While the data from the previous experiments showed that TLR ligands could inhibit IL-10 signaling, it was important to determine if this inhibition had an effect on IL-10 induced genes. Consequently, the expression of the chemokine receptor gene, CCR5, was evaluated by RT-PCR in IL-10-exposed PMs, because this gene has been shown to be induced by IL-10, but not by TLR ligand binding (47,48). Previous studies in the laboratory have demonstrated that the TLR ligands LPS, LTA and CpG inhibit CCR5 gene expression in PMs pre-exposed to IL-10 (39). In this study, when PMs were exposed to IL-10 (5ng/ml), CCR5 mRNA expression was induced and remained elevated for 26 hours. LPS (1µg/ml) was added 2 hours after the IL-10 treatments. The addition of LPS 2 hours after IL-10 resulted in a 5-fold reduction in CCR5 mRNA expression (Fig. 6). However, these results proved not to be statistically significant. These results indicate that the IL-10 mediated expression of the PM gene CCR5 may be altered when exposed to LPS.

Similar experiments were conducted with Poly I:C (20 µg/ml) instead of LPS. Cells treated with IL-10 alone demonstrated a relatively low CCR5 activation (Fig.7, lane 2), which made it difficult to evaluate if Poly I:C could significantly reduce IL-10 mediated CCR5 expression in PM.

IL-10 immunosuppressive activity in ceramide-stimulated macrophages pre-exposed to TLR ligands

The ability of the TLR ligands Poly (I:C) and Pam₃CSK4 to block the IL-10 mediated suppression of TNF-α production by C2-ceramide was also evaluated. C2-ceramide is a membrane permeable intracellular second messenger, which 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 results in an increase of TNF-α production. Previous studies in the laboratory have demonstrated that LPS inhibits the ability of IL-10 to suppress TNF-α production by C2-ceramide in PMs (39). In this study, the ability of Poly (I:C) and Pam₃CSK4 to individually suppress TNF-α production by C2-ceramide in PM was evaluated. Figure 8

demonstrates the influence of Poly I:C on the ability of IL-10 to suppress TNF- α production by C2-ceramide. Figure 9 demonstrates the influence of Pam₃CSK4 on the ability of IL-10 to suppress TNF- α production by C2-ceramide. The trends were similar for both studies. Only Fig. 9 will be described since the trends could be more easily observed.

In contrast to previous studies in our lab by Fernandez et al (39) which used LPS, the addition of Pam₃CSK4 caused a 4-fold increase in TNF- α production (Fig. 9, lane 2 vs. lane1). As expected ceramide alone also induced TNF- α , but to relatively low level (Fig. 9, lane 3). The addition of IL-10 before ceramide inhibited the ability of ceramide to induce TNF- α (lane 5 vs. lane 3). The addition of ceramide to Pam₃CSK4-pretreated cultures failed to induce TNF- α synthesis above the Pam₃CSK4-induced level (lane 4 vs. lane 2). Consequently, the ability of Pam₃CSK4 to block the immunosuppressive effect of IL-10 could not be evaluated.

Figure 1. **LPS does not show any early inhibition of IL-10 induced STAT3 phosphorylation in PM.** Normal PM were incubated with LPS (1 μ g/ml) for the indicated time periods before the addition of IL-10 (5ng/ml) for 30 minutes. Total protein extracts were obtained and 2-5 μ g were analyzed by immunoblotting using anti-phospho-STAT3 or anti-STAT3 Abs. The average of three identical experiments \pm -S.E. is shown.

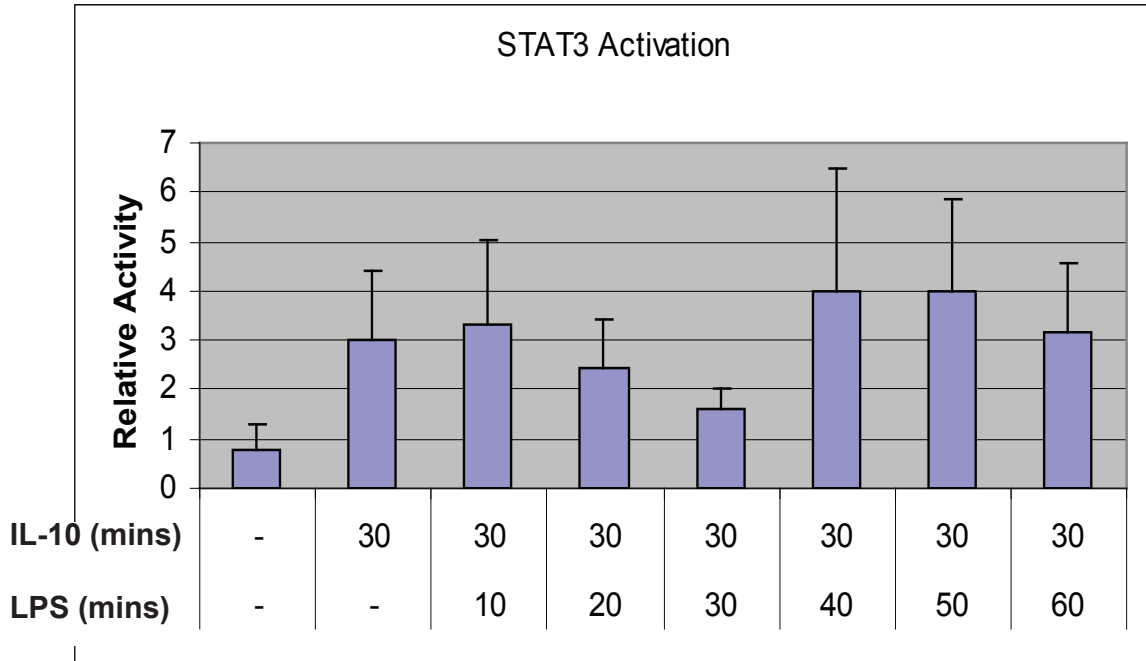


Figure 2. LPS shows late inhibition of IL-10 induced STAT3 phosphorylation in PM. Normal PM were incubated with LPS (1 μ g/ml) for either 12 or 34 hours before the addition of IL-10 (5ng/ml) for 30 minutes. Total protein extracts were obtained and 2-5 μ g were analyzed by immunoblotting using anti-phospho-STAT3 or anti-STAT3 Abs. The average of two identical experiments \pm S.E. is shown. ** indicates significantly different ($p < 0.0001$) from cells treated with IL-10 only. * indicates significantly different ($p < 0.0001$) from untreated cells.

STAT3 Activation

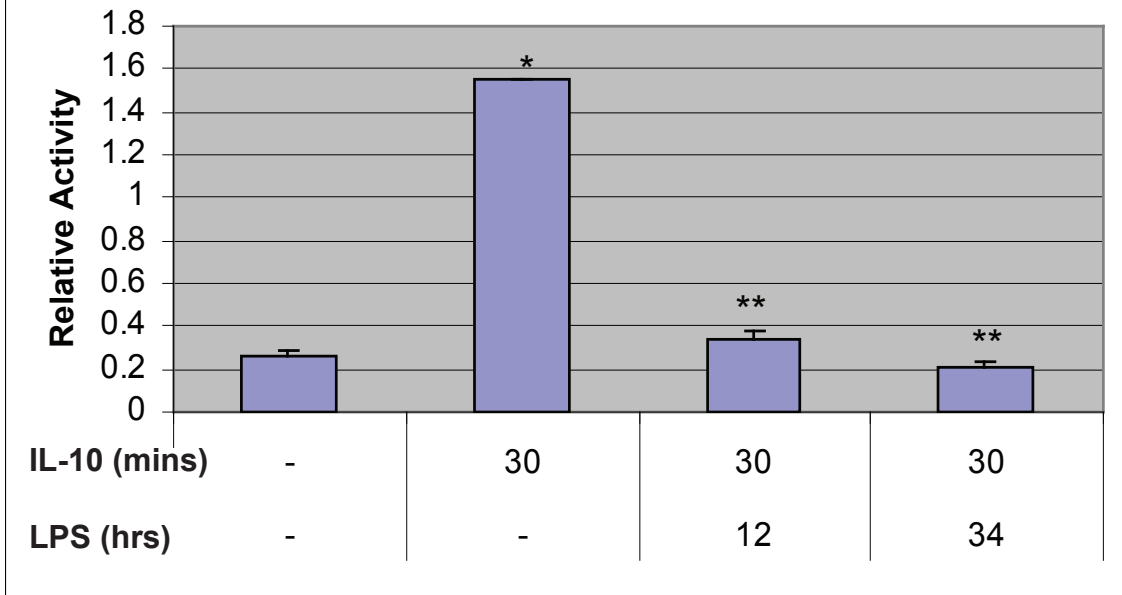


Figure 3. LTA shows late inhibition of IL-10 induced STAT3 phosphorylation in PM. Normal PM were incubated with LTA (1 μ g/ml) for either 12 or 34 hours before the addition of IL-10 (5ng/ml) for 30 minutes. Total protein extracts were obtained and 2-5 μ g were analyzed by immunoblotting using anti-phospho-STAT3 or anti-STAT3 Abs. The average of two identical experiments \pm S.E. is shown. ** indicates significantly different ($p < 0.0001$) from cells treated with IL-10 only. * indicates significantly different ($p < 0.0001$) from untreated cells.

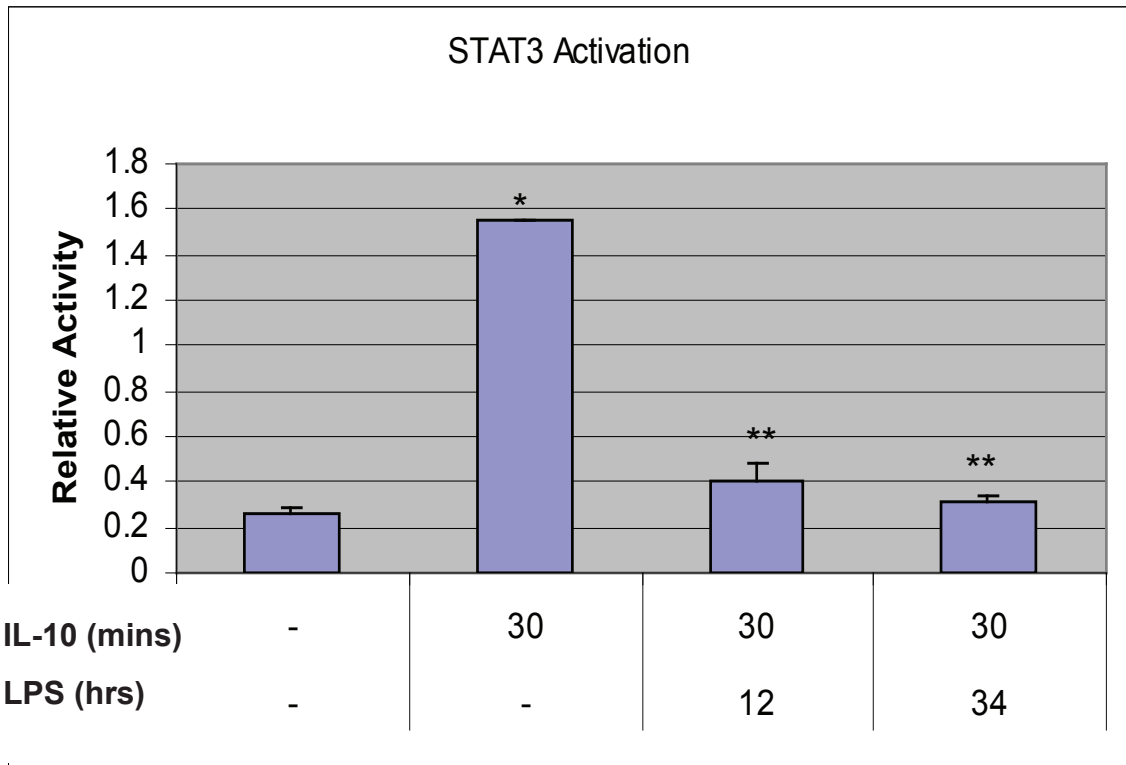
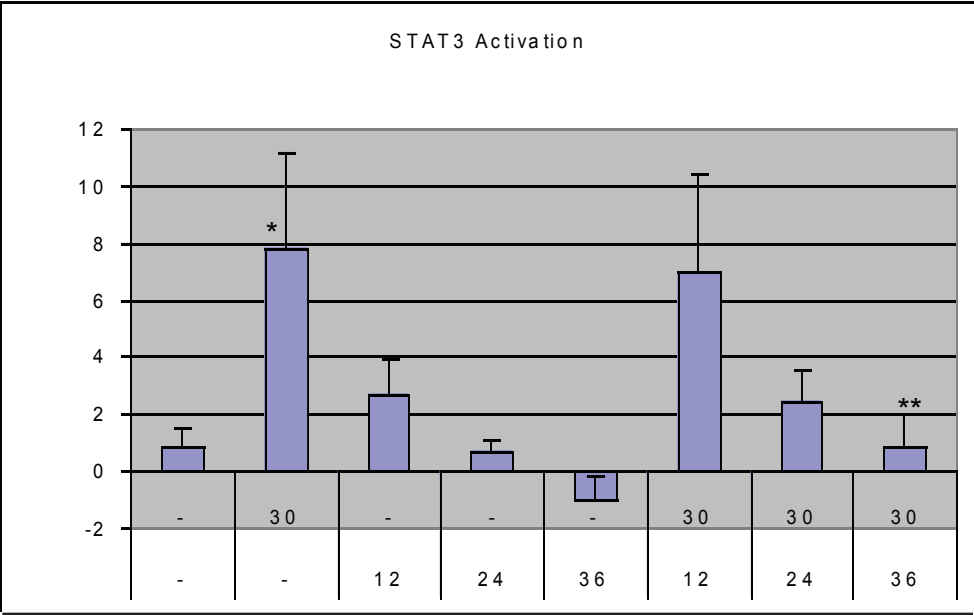


Figure 4. Poly (I:C) shows late inhibition of IL-10 induced STAT3 phosphorylation in PM. Normal PM were incubated with or without Poly (I:C) (20 $\mu\text{g/ml}$) for either 12, 24 or 36 hours, before the addition of IL-10 (5ng/ml) for 30 minutes. Total protein extracts were obtained and 2-5 μg were analyzed by immunoblotting using anti-phospho-STAT3 or anti-STAT3 Abs. The average of three identical experiments \pm S.E. is shown. ** indicates significantly different ($p<0.05$) from cells treated with IL-10 only. * indicates significantly different ($p<0.05$) from untreated cells.



IL-10 (mins)
 Poly I:C (hrs)

Fig. 5. Pam₃CSK4 shows late inhibition of IL-10 induced STAT3 phosphorylation in PM. Normal PMs were incubated with Pam₃CSK4 (200 ng/ml) for either 12, 24 or 36 hours, before the addition of IL-10 (5ng/ml) for 30 minutes. Total protein extracts were obtained and 2-5 µg were analyzed by immunoblotting using anti-phospho-STAT3 or anti-STAT3 Abs. The average of three identical experiments +-S.E. is shown. ** indicates significantly different (p<0.05) from cells treated with IL-10 only. * indicates significantly different (p<0.05) from untreated cells.

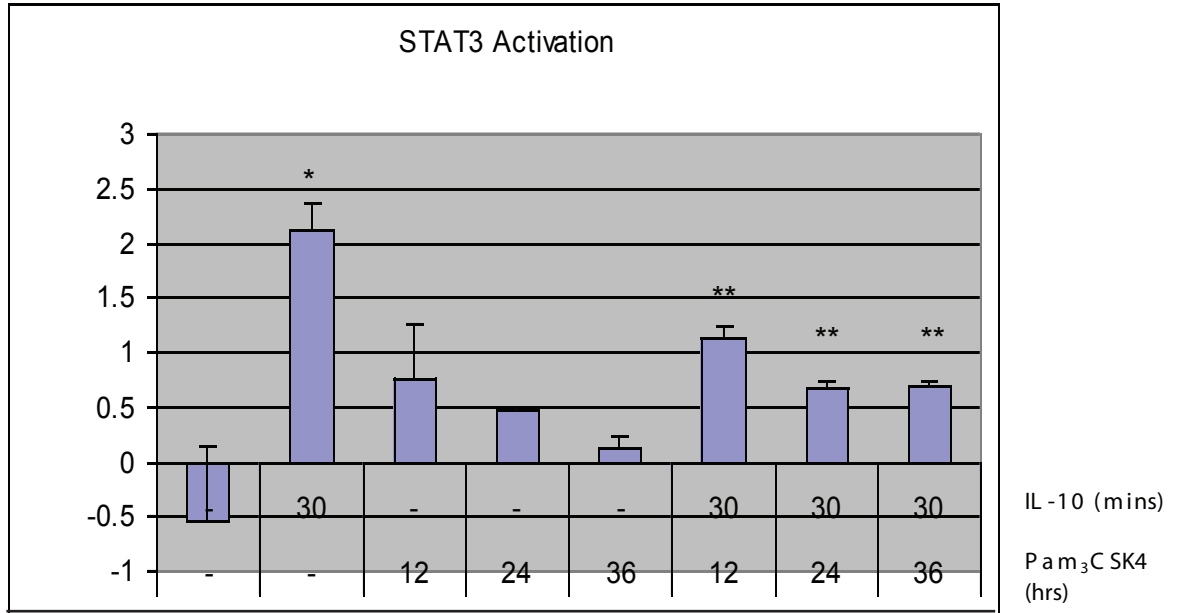


Fig. 6. CCR 5 gene expression in PM exposed to IL-10 before LPS. PM from normal mice were plated and pretreated with IL-10 (5ng/ml) for 26 hours. Two hours after the IL-10 stimulation, cells were treated with LPS or left untreated. Total RNA was extracted using TRIzol reagent. Complementary DNA was amplified using primers for murine CCR5. As a control, PCR was also performed for β -actin. One of three identical experiments, all with similar results, is shown.

CCR5 Induction

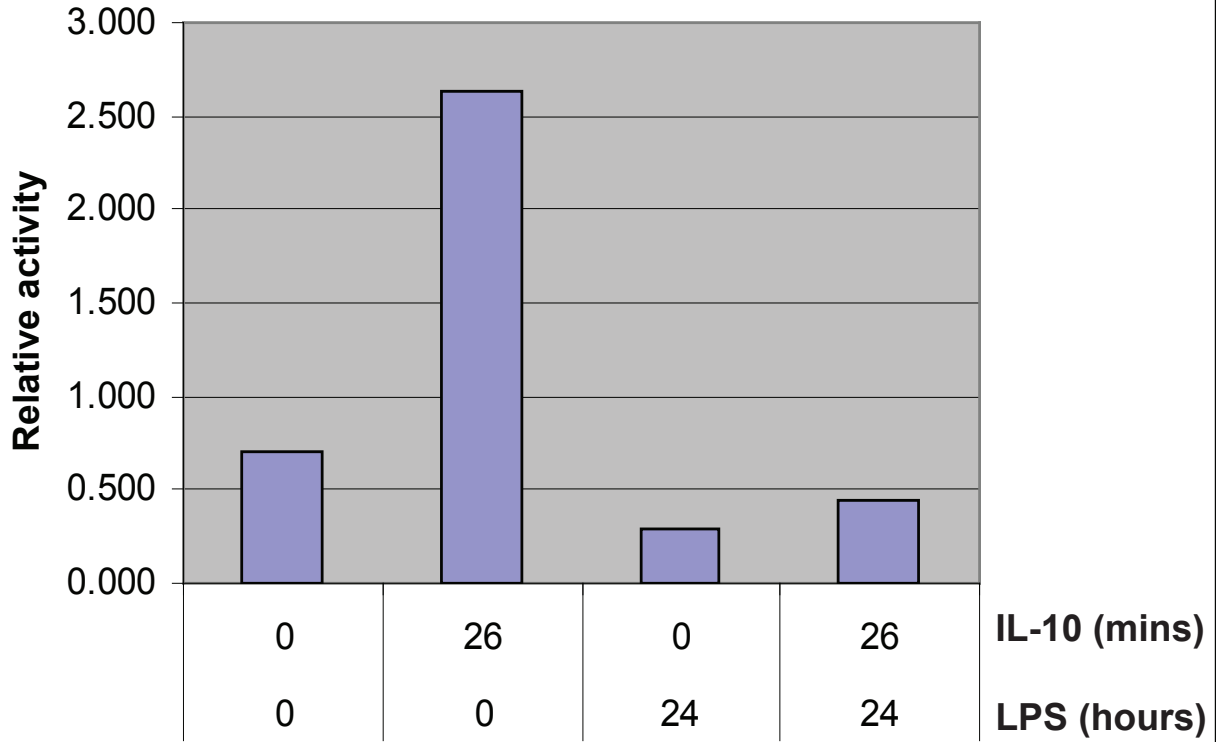


Fig. 7. CCR 5 gene expression in PM exposed to IL-10 before Poly I:C. PM from normal mice were plated and pretreated with IL-10 (5ng/ml) for 26 hours. Two hours after the IL-10 stimulation, cells were treated with Poly (I:C) or left untreated. Total RNA was extracted using TRIzol reagent. Complementary DNA was amplified using primers for murine CCR5. As a control, PCR was also performed on for β -actin. Two identical experiments, both with similar results, is shown.

CCR5 Induction

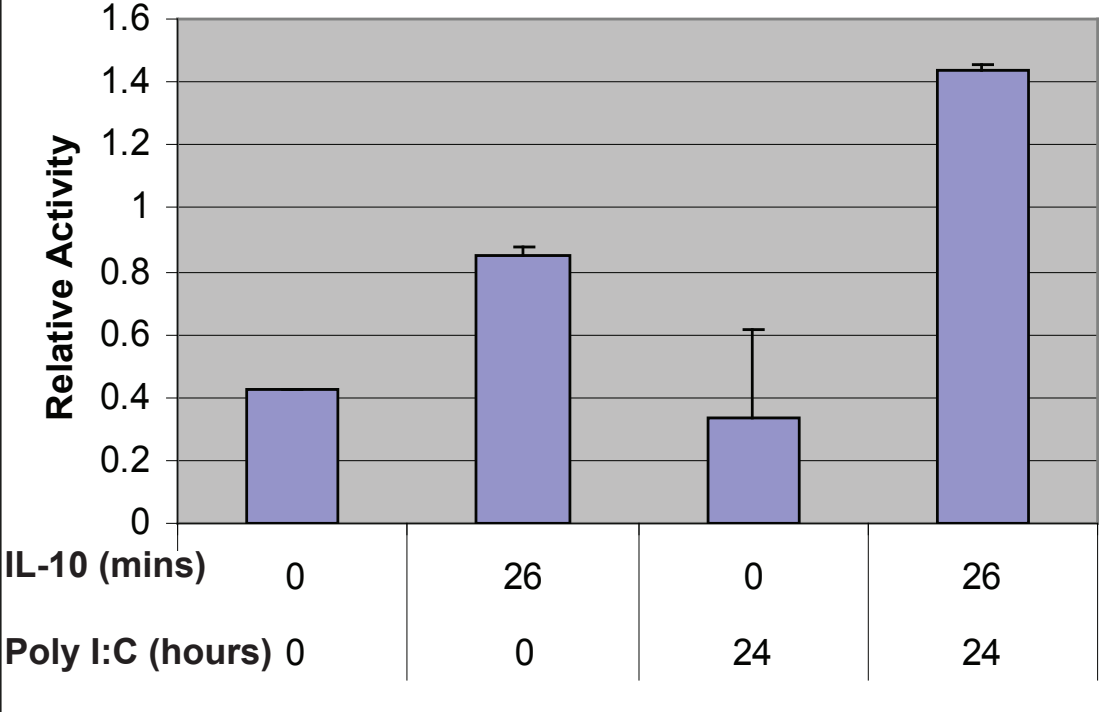


Fig. 8. IL-10 immunosuppressive activity in ceramide-stimulated macrophages pre-exposed to Poly (I:C). Normal PM were treated with or without Poly (I:C) (20 μ g/ml) for 24 hours. Poly (I:C) was then removed from the wells by rinsing and then cultured in serum-free medium for an additional 2 hours in the presence or absence of IL-10 (5ng/ml). C2-ceramide was then added to the culture for an additional 4 hours. Medium was collected and assayed for TNF- α . The average of three identical experiments (each in duplicate) \pm SE is shown.

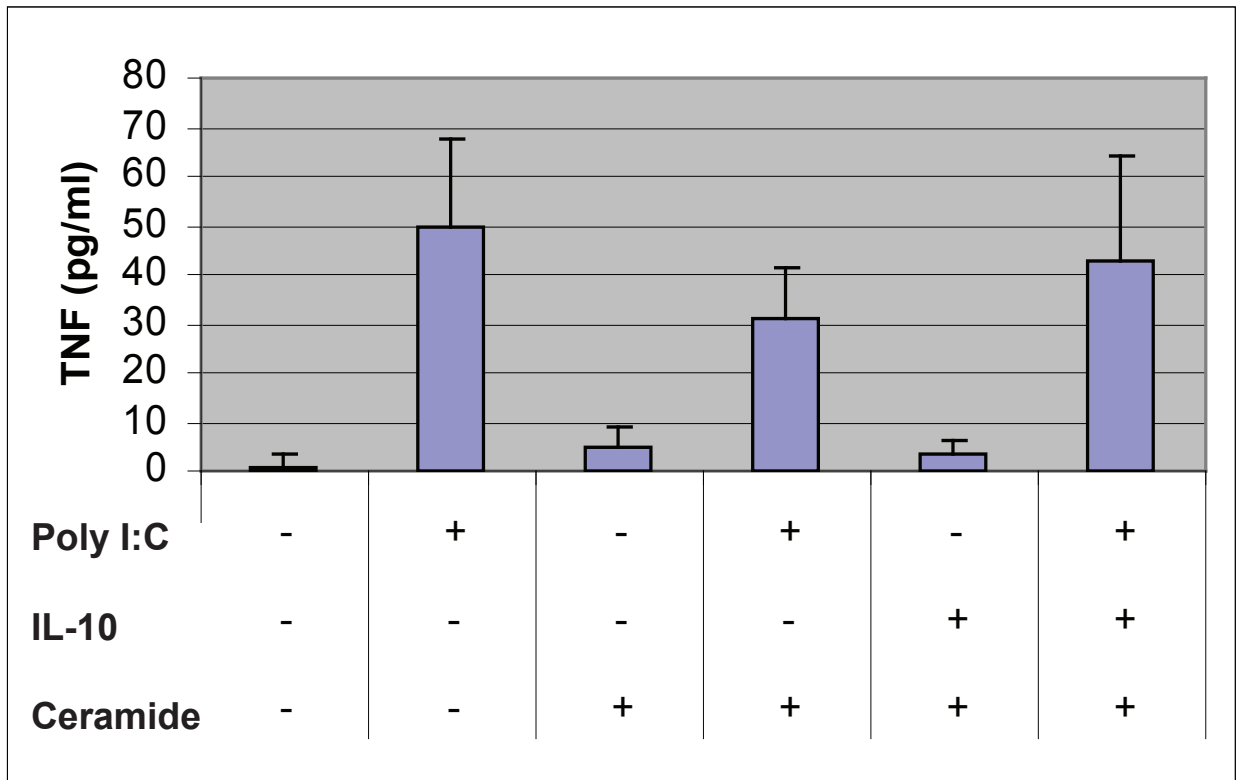
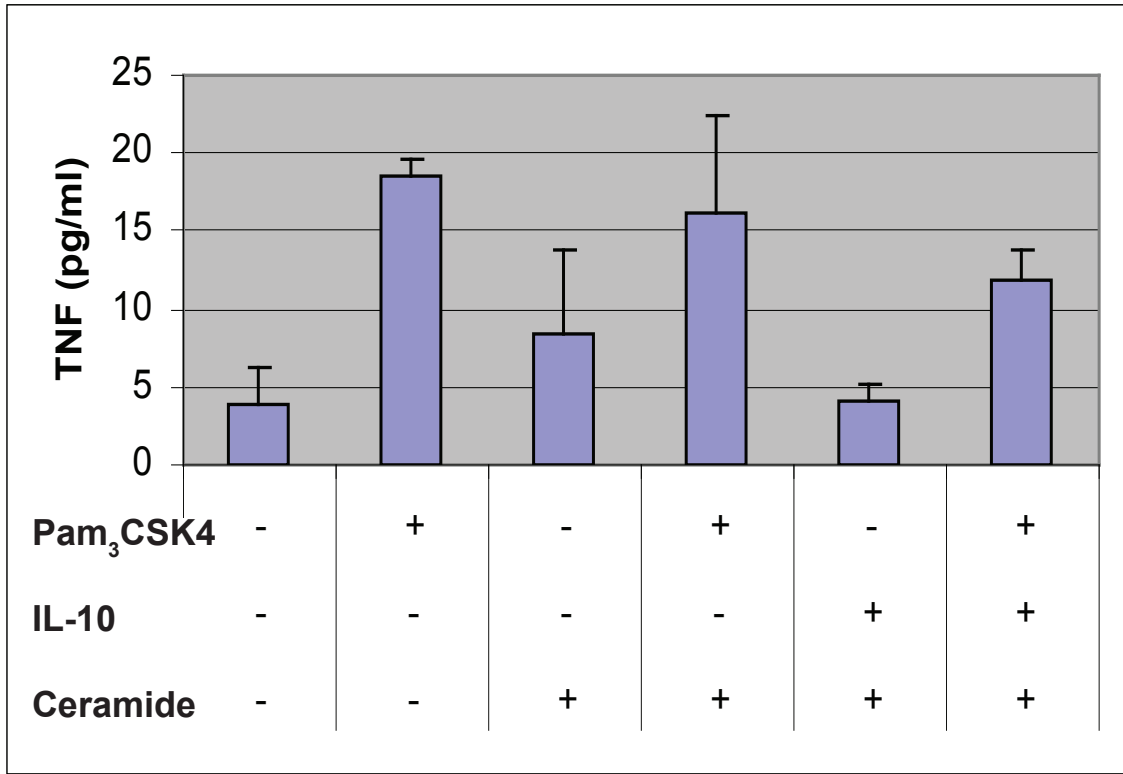


Fig. 9. IL-10 immunosuppressive activity in ceramide-stimulated macrophages pre-exposed to Pam₃CSK4. Normal PM were treated with or without Pam₃CSK4 (200 ng/ml) for 24 hours. Pam₃CSK4 was then removed from the wells by rinsing and then cultured in serum-free medium for an additional 2 hours in the presence or absence of IL-10 (5ng/ml). C2-ceramide was then added to the culture for an additional 4 hours. Medium was collected and assayed for TNF- α . The average of three identical experiments (each in duplicate) \pm SE is shown.



Discussion

Results from the western blot analyses in this study indicate that the activation of STAT3 by IL-10 was inhibited by prior exposure of PM to TLR ligands that bind to TLR2, TLR3, or TLR4. LPS, LTA, Poly I:C, and Pam₃CSK4, all demonstrated a delayed inhibition of activation of STAT3 by IL-10. LPS and LTA demonstrated significant inhibition of IL-10 mediated STAT3 activation at both 12 and 34 hours. Pam₃CSK4 demonstrated significant inhibition of IL-10 mediated STAT3 activation at 12, 24, and 36 hours, while Poly I:C showed a significant inhibition of the same only at 36 hours. Previous studies in the laboratory have observed that PM and AM respond identically to TLR ligands. From a single mouse PM can be isolated in much larger quantities than AM. Therefore, PM were chosen for this study on TLR-mediated IL-10R dysfunction, although the peritoneal fluid contains a hundredfold lower concentration of IL-10 compared to bronchioalveolar fluid. PM are not normally exposed to environmental particulates or microorganisms as frequently as AM. Thus, the physiological significance of IL-10R dysfunction in PM is unclear (39).

In the normal lung, AM in the bronchioalveolar fluid are continually exposed to IL-10. Our previous studies have also been able to demonstrate elevated expression of CCR1 and CCR5 mRNA in normal PM that were exposed to IL-10 for 10 hours. Subsequent addition of LPS two hours later inhibited expression of both CCR1 and CCR5 mRNA (39). Similarly, CCR5 gene expression experiments in this study demonstrated that LPS treatment 2 hours after a 24 hour IL-10 treatment down-regulated CCR5 mRNA expression in PM. However, this down-regulation only approached statistical significance (P=0.06). In similar experiments in which Poly I:C was added instead of LPS, there was no down-regulation in CCR5 expression. It would be interesting to examine if Pam₃CSK4 is capable of altering IL-10 induced CCR5 gene expression since Pam₃CSK4 only signals via the MyD88 pathway. IL-10 also induces the expression of the CCR1, but inhibits the expression of CCR7 in macrophages. Thus, the ability of both Poly (I:C) and Pam₃CSK4 to alter CCR1 and CCR7 expression in PM would also be worth examining.

We performed additional experiments in which we evaluated the ability of Poly (I:

C) and Pam₃CSK4 to alter the ability of IL-10 to inhibit TNF- α production by C2-ceramide. In these experiments, both Poly (I:C) and Pam₃CSK4 produced high background levels of TNF- α , which made it difficult to determine if there was any significant influence of any of the ligands on IL-10 mediated immunosuppression. Alexopoulou *et al* (54) have reported that Poly (I:C) can induce activation of NF- κ B, and Pam₃CSK4 is also known to induce NF- κ B through the MyD88 dependent signaling pathway (42); which may explain the high background levels of TNF- α observed in the experiments of this study. Also similar experiments conducted by Fernandez *et al* required several preliminary studies to optimize background levels of TNF- α production. The immunoblot experiments in Figures 4 and 5 indicate that both Poly (I:C) and Pam₃CSK4 are capable of reducing IL-10 receptor function in PM. This suggests that IL-10R dysfunction in PM could occur via both the IRF-3 dependent and MyD88 dependent TLR signaling pathways. Pam₃CSK4 mediates signaling through TLR2, which activates the MyD88 dependent pathway, inducing IL-6, TNF- α , and IL-12 via NF- κ B. Using MyD88^{-/-} mice, our lab has also demonstrated that inhibition of IL-10 mediated STAT3 activation by TLR ligands in PM is dependent on MyD88 (39).

The delayed inhibition observed in this study with all the TLR ligands indicates the possibility that inducible factors like cytokines may be involved in the observed IL-10R dysfunction. Previous studies in the laboratory have observed that TNF- α induces partial resistance to IL-10 in normal PM (38). Neutralizing antibodies against TNF- α could be used in future studies to evaluate the possible role of TNF- α in the IL-10R dysfunction observed in this study. Poly (I:C) signals through TLR3, which can mediate production of IFN- α and IFN- β . LPS signals through TLR4, which induces IFN- β , but not IFN- α production (42). This makes IFN- β a likely candidate in mediating the observed IL-10R dysfunction in PM. Neutralizing antibodies against IFN- β could also be used in future studies to evaluate the possible role of this cytokine in the IL-10R dysfunction observed in this study. It has been demonstrated in macrophages that IFN- γ redirects IL-10 signaling from the activation of STAT3 to STAT1 (55). STAT3 and STAT1 oppose each other in the regulation of several important cellular functions, including proliferation and inflammation (49). There is evidence which shows that individual cytokines can activate STATs which oppose each other's function (50). It has also been observed that IFN- γ sensitizes macrophages such that IL-10 signaling can be nearly completely blocked after Fc γ R ligation by immune complexes

(29). Pretreatment of mouse leukemia M1 cell line with IFN- γ has been shown to result in SOCS1 induction and a reduction in STAT3 activation by IL-10 (32). Other reports show that IL-10 signaling can be relatively resistant to inhibition by SOCS proteins, dependent on cell type, and suggest that SOCS3 may not be involved in the regulation of IL-10R signaling (35, 36). If expression of SOCS1 or SOCS3 is involved in IL-10R hyporesponsiveness, then overexpression of either of these proteins should also lead to the hyporesponsive state independently of TLR signaling. In future studies macrophage cell lines could be stably transfected with expression plasmids for murine SOCS1 and SOCS3. The ability of IL-10 to induce STAT3 phosphorylation in the transfected and untransfected cell lines could then be compared by incubating the cells in IL-10. If either of the SOCS proteins are involved in IL-10R hyporesponsiveness, overexpression of the specific SOCS would inhibit IL-10 signaling, whereas the empty plasmid would have no effect. If overexpression SOCS reduces IL-10 signaling, flow cytometry could be used to verify that the expression of IL-10 receptors is unaltered.

Studies by Ahmed and Ivashkiv (52) demonstrate that preincubation of primary macrophages with LPS for 20 minutes inhibits IL-6 mediated STAT3 phosphorylation, and that 20 minutes of exposure to IL-1 could block IL-10 mediated STAT3 phosphorylation (52). In our study, pretreatment of PM with LPS did not demonstrate any early or rapid inhibition of STAT3 activation by IL-10. However, preliminary experiments in this study have revealed that preincubation of PM with LPS for both 10 and 20 minutes was able to inhibit IL-6 induced STAT3 phosphorylation, at $P < 0.005$ and $P < 0.05$, respectively (data not shown). Future studies could evaluate the ability of Poly I:C to cause any early or rapid inhibition of IL-10R function, since Poly I:C is known to induce activation of the p38 mitogen-activated protein kinase (51). p38 has been shown to inhibit IL-6 signaling upstream of STAT3 activation (52).

In conclusion, this study demonstrates that Poly I:C, and Pam₃CSK4 are both capable of mediating a delayed inhibition of IL-10R function, at least at the level of STAT3 activation. The mechanism underlying this observation is yet to be resolved.

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5.30.05