ABSTRACT

Title of Document: THE ROLE OF INTERLEUKIN-19 IN INTERLEUKIN-10 PRODUCTION BY REGULATORY MACROPHAGES.

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Interleukin-19 (IL-19) is a recently discovered member of the IL-10 family of Class II cytokines. Although it is known to be secreted by monocytes and has been associated with various models of disease, the biological function of IL-19 remains largely unknown. IL-19 does share many important characteristics with IL-10. Because of this, we hypothesized that IL-19 may be regulated in a manner similar to IL-10, and may provide insight into the molecular mechanism of IL-10 regulation. In addition, IL-19 has been reported to increase IL-10 production in monocytes, and we theorized that it may be able to do the same in macrophages. Like IL-10, IL-19 is expressed in regulatory macrophages. Also, IL-19 is itself able to increase IL-10 production in regulatory macrophages, and the mechanism is independent of ERK phosphorylation. This work suggests that IL-19 can play a central role in the anti-inflammatory processes of IL-10.

THE ROLE OF INTERLEUKIN-19 IN INTERLEUKIN-10 PRODUCTION BY REGULATORY MACROPHAGES.

By

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Dedication

This thesis is dedicated to the two most important women in my life; my girlfriend Dr. Cameron Braswell, and my mother, Dr. Jane Yahil, for their invaluable love, support, guidance, and advice throughout my graduate school career. I could not have done this without either of you, and I will be forever grateful!

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Chapter 1: Introduction

The immune system

Immunology is the study of the methods by which an organism gets rid of foreign or otherwise unwanted material from the body. The immune system has the remarkable ability to recognize and destroy any substance found within the body that is identified as being foreign, or "non-self". The failure of the immune system to function properly can result in any number of diseases or disorders, ranging from common illnesses to serious autoimmune disorders, or even cancer. The immune system itself consists of two main components; innate and adaptive immunity. Innate immunity provides the body's first line of defense against invading pathogens. It consists of not only physical barriers, such as the epithelia and mucosal linings of the gut and airways, but also phagocytic cells like neutrophils and macrophages (Mϕs), and cytotoxic effector cells such as natural killer (NK) cells. There are also soluble chemical components, such as complement and cytokines. Innate immunity identifies pathogens with a relatively limited specificity. It does this by identifying general patterns on the surface of invading organisms via pattern recognition receptors. The adaptive immune response, however, exhibits a rather high level of specificity, and generally develops after an innate immune response. In fact, the innate system must activate the adaptive system through antigen presentation and co-stimulation. The adaptive immune system is composed of B and T lymphocytes (and their products) which identify and respond to each pathogen separately. Each naïve lymphocyte is specific to a different antigen through recombination of receptor genes, allowing for a

high level of specificity to a wide range of possible pathogens. B cells produce immunoglobulins (Ig), also known as antibodies, to neutralize and eliminate extracellular microbes. T cells mature and differentiate into two major groups; CD4+ and CD8+. Naïve CD4+ T helper (Th) cells can differentiate into one of several types of T effector cells (Th1, Th2, TH17, etc) depending on the influence of cytokines secreted from innate immune cells. Put simply, Th1 cells produce interferon-γ (IFN-γ) and interleukin-12 (IL-12) to promote cell-mediated immunity, whereas Th2 cells produce interleukin-4 (IL-4), which induces class-switching of Ig genes in B cells and promotes humoral (antibody-mediated) immunity. Th17 cells produce IL-17, IL-17F, and IL-22. These cells promote both neutrophilia and production of antimicrobial proteins as well as tissue remodeling and repair. CD8+ cytotoxic T cells can recognize and destroy host cells that are infected with intracellular microorganisms (e.g. viruses, some species of bacteria, etc.) by lysing the infected cells.

States of macrophage activation

Mϕs play a central role in both the innate and adaptive immune responses. They are derived from monocytes, but are strongly influenced by their tissue microenvironment to produce distinct subpopulations that are heterogeneous in both their phenotype and function[1]. The classically activated Mϕ is defined as a Mϕ activated in response to stimulation with IFN-γ and/or LPS. Once activated, it becomes an antigen presenting cell (APC) due to induced expression of surface molecules like major histocompatibility complex (MHC) class II and B7. Classically activated Mϕs produce high levels of nitric oxide (NO), and also produce large

amounts of the inflammatory cytokines IL-12 and IL-23, which in turn induce naïve CD4+ Th cells to differentiate into Th1 effector cells. However, it is important to note that excessive or uncontrolled inflammation caused by classically activated Mϕs can lead to injury to the host (e.g. tissue damage, autoimmune disease, granuloma formation, etc.).

The alternatively activated Mϕs are activated by either IL-4 and/or interleukin-13 (IL-13), and are involved in tissue repair processes. These cells are not good at antigen presentation, and are also ineffective at killing microbes since they do not produce NO.

The regulatory Mϕs are a third distinct population of macrophages that differ from the other two in several ways. The first distinguishing characteristic of these cells is their ability to induce Th2 effector immune responses. The second is the fact that two distinct signals are necessary for their activation; (1) a stimulatory signal through any of the toll-like receptors (TLRs), CD40, or CD44, and (2) cross-linkage of Fcγ receptors (FcγR), which is accomplished by the binding of these receptors to antigens that have been opsonized with IgG. These cells are able to produce large amounts of the anti-inflammatory cytokine interleukin-10 (IL-10), while also exhibiting a diminished capacity to produce the pro-inflammatory cytokine IL-12. Note that classically activated Mϕs exhibit the exact opposite phenotype with respect to these two cytokines; they produce high levels of IL-12 and little IL-10. However, like classically activated Mϕs, regulatory Mϕs produce high levels of MHC class II and B7 and therefore are effective APCs. Although they can make NO, the high levels of IL-10 produced by these cells will inhibit nearby cells from responding to

IFN-γ and effectively making reactive nitrogen species. Both classically and regulatory Mϕs can function as APCs, but they influence Th cell differentiation differently. IL-12 produced by classically activated Mϕs stimulates T cells to primarily produce IFN-γ in response to antigen, whereas IL-10 secreted by regulatory Mϕs causes T cells to produce IL-4, which in turn induces B cells to produce IgG1 against that antigen[2]. Although both classically activated and regulatory Mϕs express high levels of CD86 and exhibit low arginase activity, regulatory Mϕs can also potentially be identified in tissue by two markers, namely sphingosine kinase-1 and LIGHT[3].

IL-10 vs. IL-12

As previously mentioned, IL-12 and IL-10 are two important cytokines with opposing roles in maintaining homeostasis of the immune system. IL-12 is primarily produced by APCs, and most often induces an inflammatory cell-mediated immune response. IL-10, however, is an anti-inflammatory cytokine. IL-12 is transcribed and translated as two different subunits called p40 and p35. Most of the biological activity of IL-12 occurs when these two subunits are linked, via disulfide bonding, into a heterodimer called IL-12p70. IL-12 stimulates T cells and NK cells to produce IFN-γ, which in turn activates Mϕs. These activated Mϕs are then able to increase their production of inflammatory cytokines, including IL-12, which results in an escalating feedback loop of inflammation. These Mϕs that have been activated by IL-12-induced IFN-γ are much more efficient at killing microbes than their resting counterparts.

IL-10 is produced by macrophages, as well as Th2 cells, Treg cells, and B cells. IL-10 is produced as a non-covalent homodimer with a molecular weight of 37 kDa. Originally identified as an inhibitory factor for cytokine synthesis [4], IL-10 can inhibit the transcription and translation of inflammatory cytokines by Th1 cells by directly acting on APCs, inhibiting the expression of costimulatory molecules like MHC class II and B7 as well as other cytokines needed for optimal T cell activation [5, 6]. Since IL-10 reduces antigen presentation by APCs, it biases T cells towards a Th2 response [7]. IL-10's role as an anti-inflammatory cytokine is critical in maintaining the delicate balance between a normal immune response intended to eliminate a pathogen from a host, and damage caused by excessive inflammation [8, 9]. For example, IL-10 has been shown to rescue BALB/c mice from lipopolysaccharide (LPS)-induced endotoxemia [2, 10]. IL-10 can also upregulate the expression of FcγRs on Mϕs, which is correlated with enhanced phagocytosis of opsonized particles [11].

Toll-like receptors

One of the basic strategies that the innate immune system uses to fight infection is to identify certain molecular structures, called "pathogen-associated molecular patterns" (PAMPs) that are common to infectious organisms. PAMPs are loosely defined as highly conserved microbial components that have distinct features that are not associated with host molecules (e.g. bacterial LPS, CpG DNA, flagellin, etc.). The innate immune system has the ability to recognize and respond to PAMPs through the use of certain germline-encoded signaling receptors and, by activating downstream intracellular signaling pathways, can initiate and control the major aspects of the adaptive immune system response. Mϕs express several different

pattern recognition receptors, including a family of receptors that were first identified in fruit flies known as the Toll-like receptors (TLRs) which are also expressed on dendritic cells, B cells, and even on non-immune cells such as epithelial cells. TLRs play an important role in innate immunity, as well as in the development of an adaptive immune response. Activation of TLRs leads to signal transduction cascades that cause cells to produce inflammatory cytokines and transcribe antimicrobial genes. It also induces the upregulation of co-stimulatory molecules that in turn induce an adaptive immune response. To date, there have been 11 TLRs identified in humans and 13 in mice, although the function of some of these TLRs are not fully understood [12]. What distinguishes TLRs from other receptors is a conserved extracellular region consisting of several tandem leucine-rich repeat motifs, and a cytoplasmic tail consisting of a sequence that is homologous to the interleukin 1 receptor (IL-1R) domain, known as the Toll/IL-1R homology (TIR) domain. TIR domains are crucial to TLR functioning since they bind to adaptor molecules that mediate the downstream TLR signaling pathways. These activating adaptors include myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM), and others [13]. Most of the TLRs are expressed on the cell surface, while TLR's 3, 7, 8, and 9 are found almost exclusively inside intracellular compartments such as endosomes. MyD88 is the key adaptor protein in almost all of the TLR signaling pathways except for TLR3, and is required for innate immune responses to a variety of pathogenic organisms, as evidenced by numerous studies with MyD88 $^{-/-}$ mice [14]. Although each of the known PAMPs is

recognized by a specific TLR, there is actually a great deal of diversity as to which ligands a given TLR can recognize[15]. TLR2, for instance, will not only bind peptidoglycan (found on Gram positive bacteria), but also other structurally unrelated molecules like LAM (*Mycobacteria*), hemagglutinin (Measles virus), and phospholipomannan (*Candida*). Genetically engineered mice that are deficient in a given TLR will easily succumb to infection by the organisms that the TLR recognizes in normal mice. Hence, TLR2 $^{-/-}$ mice are more susceptible than wild-type mice to infection by any Gram positive bacteria of the family *Streptococcus* or *Staphylococcus* [16].

Fcγ receptors

Antibody-mediated, or humoral, immune responses also play a critical role in both innate and adaptive immunity. One method by which antibodies regulate immune responses is through interactions with Fc receptors. IgG antibodies specifically interact with the Fc (gamma) receptor (FcγR), which is expressed on various immune cells, and plays a central role in phagocytosis, immune complex clearance, and cytokine production [17]. The FcγR recognizes and binds to opsonized particles coated with IgG, which allows the Mϕ to ingest and destroy pathogens. Structurally, the FcγR is composed of an α chain that binds to the Fc portion of IgG, and a γ chain dimer that is associated with signal transduction. There are four known types of Fc-receptors; FcγRI, FcγRII, FcγRIII, and FcγRIV, which can be further classified as either inhibitory (FcγRIIB) or activating (all other FcγRs) receptors. FcγRI is the high-affinity receptor for IgG, whereas (in mice) the FcγRIIb, FcγRIII,

and FcγRIV have about a 100-1000 fold lower affinity for monomeric IgG [18]. FcγRII and FcγRIII, however, are capable of binding IgG immune complexes [19]. Of the activating FcγRs (with the exception of human FcγRIIa and FcγRIIC), all activating FcγRs contain an immunoreceptor tyrosine-based activating motif (ITAM) in their cytoplasmic domain. The FcγRIIB, on the other hand, contains an immunoreceptor tyrosine-based inhibitory motif (ITIM), and indeed activation of inhibitory FcγRIIB serves to negatively regulate activation of the other FcγRs [19].

Cross-linking of FcγRs by immune complexes leads to the phosphorylation of the ITAMs of the γ-chain by Src family kinases. This in turn leads directly to the recruitment and activation of the tyrosine kinase Syk. Syk contains two N-terminal Src homology 2 (SH2) domains and a C-terminal kinase domain with a flexible linker between them. Syk is activated in a three-fold process; binding of its SH2 domain to the phosphorylated ITAMs of the FcγR γ chain, phosphorylation by Src family kinases, and finally autophosphorylation [20]. Once activated, Syk can then induce various signal transduction molecules including phosphoinositide 3-kinase (PI3K), and regulators of Ras and other G proteins. This leads to activation of mitogen-activated protein kinases (MAPKs) such as p38 and ERK, and calcium-dependent signaling pathways [18]. In Mϕs, IL-10 production is dependent on signaling mediated through the γ chain of FcγR following cross-linking and activation of this receptor [21].

Mitogen-activated protein kinase (MAPK) signaling pathways

MAPK signaling pathways are essential in a variety of cellular processes, including growth, metabolism, differentiation, motility, apoptosis, and the immune

response. Unsurprisingly, they are very highly evolutionarily conserved in eukaryotes, from yeast all the way to humans. Currently, there are four preeminent MAPK pathways identified in mammals; the extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway, the p38 pathway, the c-Jun NH2-terminal kinase (JNK) pathway, and the ERK3, 4, and 5 pathway. The key feature of the MAPKs is that activation requires phosphorylation at both a threonine and a tyrosine residue in a conserved Thr-X-Tyr (TXY) motif. This type of phosphorylation is much longer-lived than the tyrosine phosphorylation associated with FcγRs and other membrane-bound receptors, which allows the MAPKs to convert these short signals into sustained ones that can be relayed downstream to the nucleus and can alter the pattern of gene expression. The protein kinase that catalyzes phosphorylation of the TXY motif is called a MAP kinase kinase (MKK), which in turn is activated through phosphorylation of their Ser/Thr residues by a MAP kinase kinase kinase (MKKK). Activation of all MAPKs is achieved via this kind of triple kinase cascade; MKKK to MKK to MAPK [22].

ERK 1 and 2 are ubiquitously expressed in all cell types, and are involved in cell growth and differentiation. Typically, the ERK 1/2 pathway is activated by receptor tyrosine kinases and G protein-coupled receptors that transmit signals to the Raf/MEK/ERK cascade. Briefly, the GTP-binding protein Ras is activated via signaling from a membrane receptor responding to the presence of growth factors, carcinogens, or transforming agents. This allows Ras to interact with and activate Raf, although the exact mechanism is not well understood [23]. Activated Raf can then phosphorylate the MKK MEK1/2, which in turn phosphorylates ERK 1/2 at a

conserved Thr-Glu-Tyr (TEY) motif [24]. Once it has been phosphorylated, ERK 1/2 accumulates in the nucleus, where it is known to interact with several nuclear proteins like NF-AT, c-Fos, c-Myc, and STAT3, as well as the nuclear proteins MSKs, MNKs, and RSKs [24].

The p38 pathway is heavily influenced by inflammatory cytokines (e.g. IL-1, TNF-α, etc.) and environmental or physical stresses (e.g. oxidative stress, UV radiation, etc.). There are several MKKKs in this pathway, including MEKK's 1 through 4, MLK2 and 3, and Tpl2, among others. The MKK's MEK3 and MEK6 are also in this pathway, as are the four known isoforms of p38 (α, β, γ, and δ). p38γ is found in skeletal muscle, and p38δ is expressed in lung, kidneys, and the small intestine. However, p38α and β are ubiquitously expressed in all cell types. MEK3 and 6 show a high degree of specificity for p38, and will not activate ERK1/2 or JNK. Specifically, activation of p38 by MEK3/6 is accomplished by phosphorylation of a conserved Thr-Gly-Tyr (TGY) motif in the activation loop of p38. Once activated, p38 can translocate from the cytoplasm to the nucleus and activate transcription factors such as ATF 1/2, NF-κB, and p53 [25].

It is well established that the JNK pathway is activated in response to cytokines, but it is also activated by environmental stresses like UV radiation and growth factor deprivation. In fact, the JNK pathway was first identified in studies where rat liver cells were injected with the protein synthesis inhibitor cyclohexamide [26]. Regulation of the JNK pathway is extremely complex, involving over 13 MKKKs. Like p38 and ERK1/2, JNK activation requires dual phosphorylation on a conserved Thr-Pro-Tyr (TPY) motif, which is catalyzed by MEK4 and 7. In addition, JNK

activation allows it to translocate to the nucleus following stimulation. The most well-known substrate for JNK is the transcription factor c-Jun, which is part of the AP-1 transcription complex that controls the expression of many cytokine genes [27].

MAPKs are central mediators of gene expression in eukaryotes in response to extracellular signals, including cytokine stimulation. As previously mentioned, MAPKs can easily phosphorylate transcription factors, but they can also activate co-regulatory proteins and are involved in chromatin remodeling. Thus, MAPKs can influence protein stability, localization, and transcription, as well as DNA binding and nucleosome accessibility [28, 29].

Regulation of IL-10

Expression of IL-10 is regulated at the level of gene transcription [30], mRNA stability [31], and via chromatin remodeling at the IL-10 locus [32]. Numerous transcription factor binding sites are known to exist within the IL-10 promoter region, and are associated with IL-10 regulation in Mϕs. For example, the specificity binding protein 1 (Sp1) binding site in the murine IL-10 promoter (-89/-78) is a central mediator for IL-10 production, and is necessary for LPS-induced IL-10 synthesis [33]. Although Sp1 is known to be activated via phosphorylation by Sp1 kinase, it has been shown in human Mϕs that Sp1 phosphorylation is dependent on p38 MAPK [34]. Signal transducer and activator of transcription-3 (STAT3) also binds to the IL-10 promoter at two sites (-629/-621 and -479/-471), and in fact IL-10 can induce its own expression in an autocrine function via activation of STAT3 [35]. However, numerous studies in STAT3 conditional knockout mice have shown that, while STAT3 clearly plays a role in IL-10 production, it is not required for gene expression [36]. Recent

studies in mice have also shown that the NF-κB family member p50 forms homodimers and binds to a site (-130/-100, -124/-103) in the IL-10 promoter [37].

In addition to transcription factor mediated-regulation, IL-10 is also regulated by epigenetic modifications to the promoter site. In Mϕs, transcription factor binding is actually preceded by a transient phosphorylation of chromatin around the IL-10 promoter, which is dependent on ERK activity [32, 38]. This evidence therefore provides a mechanistic explanation for the fact that optimal IL-10 production seen in the activated regulatory Mϕs upon stimulation with two distinct signals; a TLR ligand (LPS) and immune complexes. As with most cytokines, the signal provided by TLR activation induces transcription factor activity within the nucleus. However, in the case of IL-10, this signal is not sufficient to activate transcription of the *il-10* gene because the promoter site itself remains physically inaccessible. The second signal, provided by cross-linking of the FcγRs by immune complexes, activates the tyrosine kinase Syk. This, in turn, activates ERK which then phosphorylates histone H3 associated with the IL-10 promoter. This modification of the histone is rapid and transient, peaking only 30 minutes post activation after which histone phosphorylation rapidly declines [32, 38].

IL-19 and the IL-10 family of cytokines

A number of Class II cytokines with sequence homology to IL-10 have been discovered either by data mining of EST databases, or by other means and were later found to be IL-10 homologues [30]. The IL-10 family now consists of six members; IL-10, IL-19, IL-20, IL-22, IL-24 (MDA-7), and IL-26. The genes for IL-19, IL-20, and IL-24 are located on chromosome 1 within the same gene locus as IL-10 (1q32). The

gene for IL-19 is closest in proximity to IL-10, although the two genes run antisense with respect to each other. Like IL-10, the gene for IL-19 has five exons and four introns, and the length of each exon is virtually identical to the respective exons of IL-10 [39]. IL-19 has a putative molecular weight of ~21 kDa, although there are two glycosylation sites on the protein that could explain some reports of multiple IL-19 bands in the 35-40 kDa range in SDS-PAGE analysis [30]. The crystal structure of IL-19 has been solved. Although there is only ~20% sequence homology with IL-10, IL-19 does share a very similar three-dimensional structure to IL-10, the main differences being that 1) IL-19 has six α-helices whereas IL-10 has seven, 2) IL-19 exists as a monomer and IL-10 as a dimer, and 3) the N-terminal region of both cytokines differ dramatically [40].

Each of the IL-10 family members binds to one or more of the Class II cytokine receptors (Fig. 1), with some receptor subunits being present in more than one receptor, suggesting an overlap in function [41, 42]. There is also commonality in the downstream signaling of these cytokines, since all of the Class II cytokine receptors activate Janus kinase (JAK) and/or STAT signaling pathways [43]. IL-19 in particular binds to the IL-20R1/IL-20R2 receptor complex and activates STAT3, as do IL-20 and IL-22 [41, 44]. It is therefore likely that the biological function of IL-19, as with all the IL-10 family members, is largely dependent on the cells secreting and responding to it, as well as the microenvironment in which it is released [43].

IL-19 is known to be secreted by monocytes, and to a lesser extent, by B cells [45]. LPS stimulation of monocytes induces IL-19 expression after 4hr, compared with only 2hr for IL-10 [39]. Pretreatment of monocytes with IL-4 or IL-13 results in a

significant increase in LPS-induced IL-19 mRNA expression, but inflammatory cytokines such as IFN-γ and TNF-α do not have this affect. Monocytes treated with IL-19 release IL-6 and TNF-α, as well as reactive oxygen species. In addition, IL-19 can induce apoptosis [46].

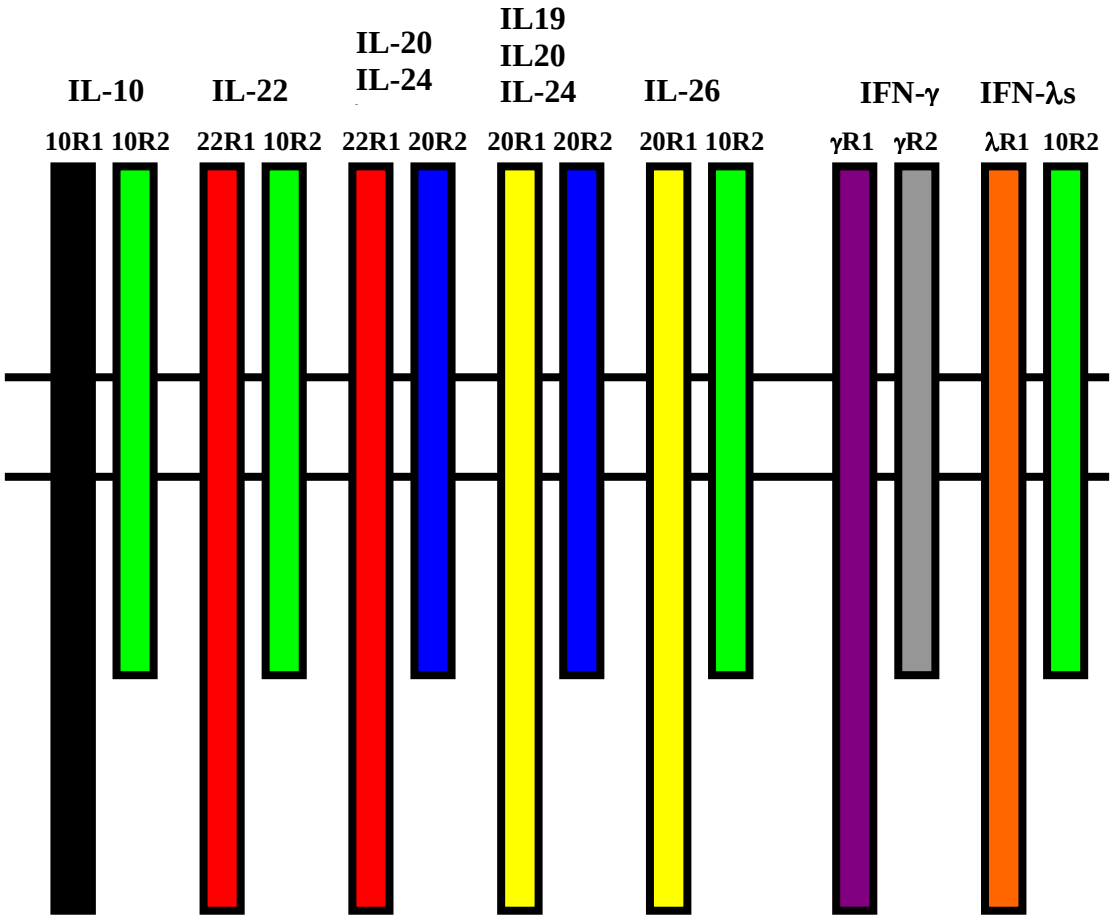


Fig.1. The IL-10 family of cytokine receptors.
IL-10 family members all bind to structurally related receptors, some of which share receptor subunits and some of which bind to more than one cytokine. All members of this family signal through STAT and JAK kinase pathways.

Since IL-19 can bind to the IL-20 receptor complex, it is involved in the pathogenesis of certain chronic inflammatory diseases, like psoriasis and asthma. It is known that Th1 cells producing IFN-γ are a central feature of psoriatic skin lesions.

Treatment with IL-4 can induce Th2 differentiation in naïve and memory T cells, suppressing IFN-γ production and alleviating psoriasis. Studies have shown that this kind of treatment has no effect on levels of IL-20 or its receptor, but it significantly reduces the amount of IL-19 mRNA [47]. IL-19 is also known to be elevated in the serum of asthma patients in both human and mouse models [48]. It can not only induce the production of Th2-associated cytokines like IL-10, IL-4, and IL-13 (and even itself in an autocrine fashion [49]) in multiple cell types, but can also bias T cell development towards a Th2 response [50].

Although the human IL-19 promoter has been cloned [46], the mouse IL-19 promoter has not, and it is not yet known what transcription factors are necessary for IL-19 production in either model. The purpose of this study is to determine whether IL-19 is expressed by regulatory Mϕs, and if so what similarities and/or differences there are in the expression and regulation of this novel cytokine and IL-10.

Chapter 2: Materials and Methods

Mice

BALB/c mice were purchased from Taconic Farms (Germantown, NY). IL-$10^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, Maine). All mice were used at 6-10 weeks of age as a source of bone marrow-derived macrophages (BMMϕs). Mice were maintained in HEPA-filtered Thoren units (Thoren Caging Systems, Hazelton, PA) at the University of Maryland (College Park, MD). All procedures using animals were reviewed and approved by the University of Maryland Institutional Animal Care and Use Committee.

Cell culture

BMMϕs were prepared by removing the tibias and femurs of 6-10 week old mice and flushing the bone marrow out with PBS + penicillin/streptomycin. The cells were plated in Petri dishes in DMEM/F12 (Fisher Scientific, Pittsburgh, PA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), 2 mM glutamine (Fisher Scientific), 100 U/mL penicillin (Fisher Scientific), 100 μg/mL streptomycin (Fisher Scientific), and 10% L929 conditioned medium obtained from the supernatants of M-CSF secreting L929 (LC14) fibroblasts. The cells were fed on day 2, and complete medium was replaced on day 6. All BMMϕs were used at 7-10 days for experiments. BMMϕs were removed from the Petri dishes using Cellstripper (Mediatech, Manassas, VA) and were plated onto 48-well plates for cytokine measurements ($2x10^5$ Mϕs/well), or 6-well plates for RNA isolation and

Western blotting ($2x10^6$ Mϕs/well). All cells were cultured overnight in complete medium without L929 before experimentation.

RAW264.7 macrophages were obtained from the American Type Culture Collection (Manassas, VA), and were grown in RPMI (Fisher Scientific) supplemented with 10% FBS, 2 mM glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin. Cells were plated at $2x10^5$ cells per well of a 48-well plate for transient transfections and luciferase assays.

Macrophage activation

Either BMMϕs or RAW264.7 cells were used in all experiments. Cells were grown and plated as described above, then washed and stimulated with 10 ng/mL of Ultra-Pure LPS (*Escherichia coli* K12, Invivogen, San Diego, CA), or with LPS plus immune complexes consisting of IgG-opsonized ovalbumin (OVA). Immune complexes were made by mixing a tenfold molar excess of rabbit anti-OVA IgG antibody (Cappel, Durham, NC) to OVA (Worthington, Lakewood, NJ) for 30 min at room temperature. Where indicated, cells were washed and primed overnight with either 100 U/mL recombinant IFN-γ or 10 U/mL recombinant IL-4 (R&D Systems, Minneapolis, MN) before being washed and stimulated. Some experiments also involved priming with recombinant IL-19 (Biosource International Inc., Camarillo, CA) at the indicated concentrations and for the indicated time periods, before cells were stimulated. Where indicated, the ERK inhibitor U0126 (5 μM) or the p38 inhibitor SB203580 (5 μM) were added 30 min prior to stimulation.

ELISA

Cells were stimulated overnight as described above. Supernatants were collected, and cytokine concentrations were measured by sandwich ELISA using the following pairs of capture and detection (biotinylated) antibodies (BD Pharmingen, San Jose, CA); IL-10, JES5-2A5 and JES5-16E3; IL-12/23p40, C15.6 and C17.8; TNF-α, G281-2626 and MP6-XT3. Antibody concentrations used were according to the manufacturer's instructions. Cytokine concentrations were compared to a standard curve (4000-0 pg/mL) generated by serial dilution of recombinant protein standards (BD Pharmingen). Streptavidin alkaline phosphatase (AP) and p-nitrophenyl phosphate (PNPP) substrate (Southern Biotechnology, Birmingham, AL) were used to develop samples according to the manufacture's instructions. All samples were placed in 96-well flat bottom plates, and measured at 405 nm.

RT-PCR and quantitative real-time PCR

RNA was extracted from BMMϕs using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). Any contaminating DNA was removed via treatment with DNase I. RNA concentrations were quantified using spectrophotometry. cDNA was generated from total RNA using the ThermoScript RT-PCR system (Invitrogen Life Technologies) with oligo$(dT)_{20}$ primers as per the manufacturer's instructions. Standard PCR analysis was performed using Platinum PCR Supermix (Invitrogen Life Technologies). Electrophoresis of the final PCR products was performed after amplification to ensure that a single product had been obtained. Real-time PCR analysis was conducted on the Roche LightCycler 480 Real-Time PCR System using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) as per the

manufacturers instructions. The C_T value for GAPDH was used to normalize variations in loading amount. A $\Delta\Delta C_T$ value was obtained by subtracting control ΔC_T values from the corresponding experimental ΔC_T. Data was then represented as a fold difference compared with the control by raising 2 to the $\Delta\Delta C_T$ power. The primers used for all RT-PCR and Real-Time PCR experiments are listed below in Table 1.

Gene	Ascension Number	Primer Sequence
IL-10	NM_010548	5'-CCAGTTTTACCTGGTAGAAGTGATG-3' 5'-TGTCTAGGTCCTGGAGTCCAGCAGACTCAA-3'
IL-10 (For Real-Time PCR)	NM_010548	5'-AAGGACCAGCTGGACAACAT-3' 5'-TCTCACCCAGGGAATTCAAA-3'
IL-12/23p40	NM_008352	5'-ATGGCCATGTGGGAGCTGGAGAAAG-3' 5'-GTGGAGCAGCAGATGTGAGTGGCT-3'
IL-19	NM_001009940	5'-TCTGCTCTCTGATCCCACCT-3' 5'-TGCACTACAGCACACCACAA-3"
GAPDH	NM_001001303	5'-GCACTTGGCAAAATGGAGAT-3' 5'-CCAGCATCACCCCATTAGAT-3'

Table 1. Primer pairs used in both standard and quantitative Real-Time PCR.

Western blotting

BMMϕs were plated at $2x10^6$ cells/well in 6-well plates overnight as described above. Cells were then lysed in ice-cold lysis buffer (100 mM Tris (pH 8), 2 mM EDTA, 100 mM NaCl, 1% Triton X-100, and a Complete EDTA-free Protease Inhibitor Cocktail Tablet (Roche Diagnostics, Indianapolis, IN)), 5 mM sodium vanadate, 10 mM sodium fluoride, 10 mM glycerophosphate sodium, and 5 mM sodium pyrophosphate, and left on ice for 30 min. Lysates were cleared by centrifugation (13,000 rpm, 10 min. 4^{o}C). Pre-cast 10% SDS-polyacrylamide gels (Bio-Rad) were loaded with equal amounts of protein per well, which were then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes

were then incubated with primary antibodies (1/1000 in 5% BSA, TBS-Tween 20) overnight at 4^{o}C. The membranes were then washed and incubated with HRP-conjugated secondary antibody (1/5000) for 1 hr at room temperature, and were developed using Lumi-Light PLUS Western Blotting Substrate (Roche Diagnostics) according to the manufacturer's instructions.

Generation of pGL4.19-IL-19 promoter plasmid

Approximately 3 kb of the mouse IL-19 promoter was cloned from murine genomic DNA by PCR using the Expand Long Template PCR System (Roche Diagnostics) and the following primers: forward- 5'-GATATCTCCGCACCAAGCCTACATAGT-3' and reverse- 5'-TCTAAGATCTAAGGTCAGACAACCCATGCTC-3' (Fig. 2). The PCR product was ligated into the pCRII-TOPO vector (Invitrogen Life Technologies) to generate usable quantities. The cloned promoter fragment (-3000/+200 relative to the transcriptional start site) was removed using EcoRV and BglII restriction enzymes, then gel purified and ligated into the pGL4.19[*luc2CP*/Neo] luciferase reporter vector (Promega Corporation, Madison, WI). The sequence of the resulting ~3 kb promoter fragment was verified by GENEWIZ Inc. (Germantown, MD). The -2000/+200 promoter construct was made by SacI digestion of the -3000/+200 construct, while the -1000/+200 promoter construct was made by digestion of the -3000/+200 construct with KpnI. In both cases the larger fragment was then gel purified, and the sticky ends were filled in using Klenow enzyme. The resultant fragment was then blunt-end ligated.

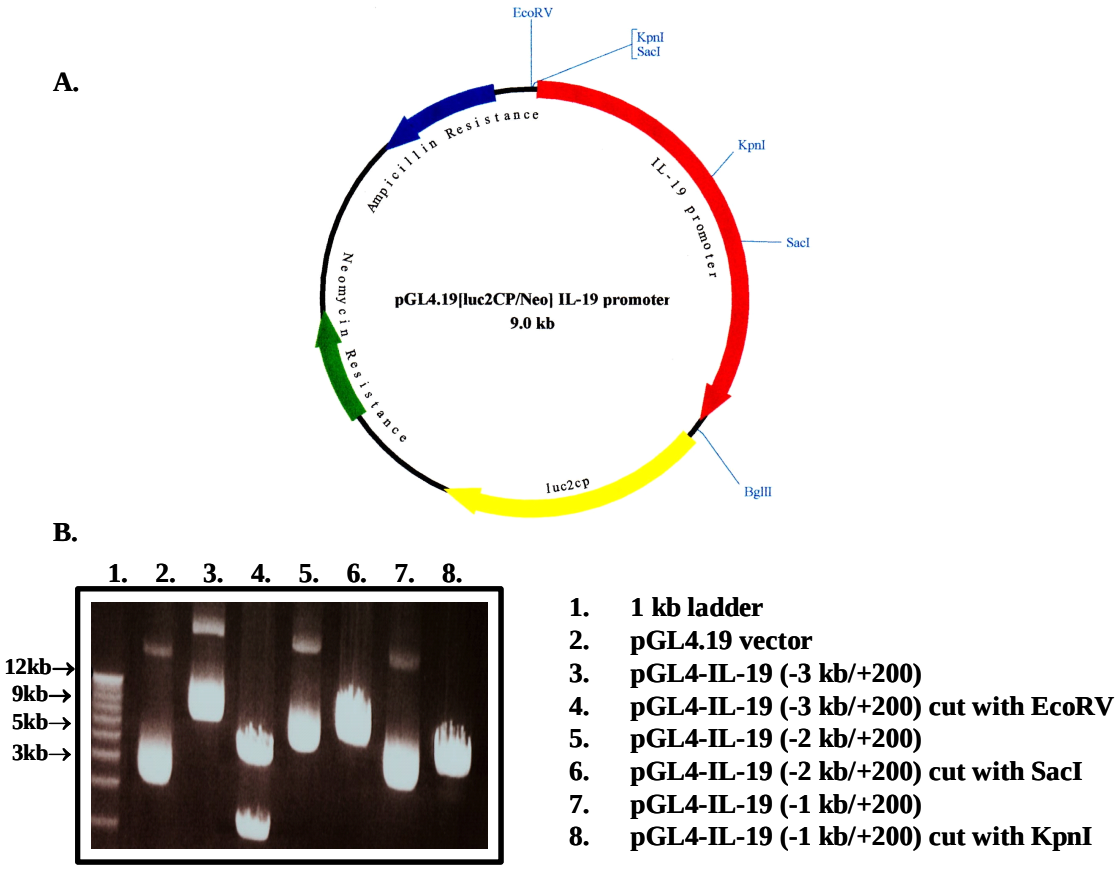


Fig. 2. IL-19 promoter plasmid constructs.
(A) Plasmid map of the 3 kb pGL4.19[*luc2CP*/Neo] IL-19 promoter construct used in the construction and validation of the 2 kb and 1 kb reporter plasmids. Relevant restriction enzyme sites are indicated. The 2 kb promoter construct was created by SacI digestion, the 1 kb construct by digestion with KpnI. Both constructs were then filled in with Klenow and blunt-end ligated.
(B) Restriction enzyme analysis for validation of IL-19 promoter constructs. Plasmids were either uncut, or cut with restriction enzymes as indicated.

Transient transfections and luciferase assays

RAW264.7 cells were transiently transfected using the FuGENE HD transfection reagent (Roche Diagnostics). 2 μg of plasmid was added to 3 μg of FuGENE diluted in 100 μL of RPMI and incubated at room temperature for 30 min.

20 μL of the resulting transfection complex was added per well of a 48-well plate. Each reporter construct was co-transfected with the pRL-null *Renilla* luciferase plasmid (Promega) at a ratio of 40:1 and incubated at 37^{o}C overnight. Media was replaced the following morning, and cells were stimulated as indicated in figures. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase activity was measured for each IL-19 promoter construct and normalized to *Renilla* luciferase activity.

Chapter 3: IL-19 Production in Regulatory Macrophages

The published literature on IL-19 demonstrates that this cytokine, as well as other IL-10 family members, shares many important characteristics with IL-10. It has led many researchers to speculate that IL-10 may in fact be the evolutionary descendent of IL-19 and other members of the extended family[51]. The similarity in three-dimensional structure of the two cytokines suggests that both proteins might be able to bind to the other's respective receptor, suggesting an overlap in function. The redundant nature of signaling within the IL-10 family of cytokine receptors is further evidence to support this theory. Finally, the close proximity of the respective promoter sites for these two genes suggests that it is possible they share transcription factor binding sites, implying that not only do the two cytokines have similar functions but that they are themselves regulated in a similar manner. Due to the unique role that IL-10 has in regulatory macrophages, we hypothesized that IL-19 might have a similar role for the reasons stated above. Furthermore, we hypothesized that any differences that occur between IL-19 production or activity and IL-10 might provide further insights into the complex molecular regulatory mechanism of IL-10.

IL-19 mRNA is upregulated in regulatory macrophages, but is negatively regulated by IL-10

We first examined whether IL-19 was produced in our model of BMMϕs. Bone marrow cells were grown in culture from 6-10 week old BALB/c mice as described in Materials and Methods to produce BMMϕs. Cells were stimulated either with LPS or with LPS in conjunction with IgG-OVA immune complexes (LPS/IC).

Where indicated, cells were also pretreated overnight with either IFN-γ or IL-4 in order to produce classically activated or alternatively activated macrophages respectively. RNA was extracted from the cells at the designated time points (Fig. 3, left side) and reverse transcribed into cDNA. PCR analysis was then performed using primers specific for mature IL-19 transcript. A basal level of IL-19 mRNA was expressed in LPS-stimulated macrophages after 4 hours, but a higher level of mRNA was expressed in cells treated with LPS/IC. The kinetics of mRNA expression is also different in LPS/IC-treated cells. Detectable levels of IL-19 mRNA were seen as early as 1 hour post-stimulation. Priming with IFN-γ did not alter either the kinetics or level of mRNA expression in response to any stimulation. However, IL-4 priming did alter the kinetics of IL-19 expression in that IL-19 mRNA production dramatically tapered off by 4 hours post-stimulation with LPS/IC. IL-19 therefore does share a characteristic with IL-10, namely that cytokine mRNA expression is upregulated and faster in regulatory macrophages as compared to cells treated with LPS alone.

It has also been reported in human monocytes that IL-19 can itself induce IL-10 expression and that IL-10 can downregulate production of IL-19[49]. To determine whether or not this is also the case in murine macrophages, this experiment was repeated using macrophages from IL-10 $^{-/-}$ mice (Fig. 3, right side). IL-19 was basally expressed at detectable levels even without stimulation in these cells, and a higher overall level of mRNA was expressed in response to LPS stimulation as compared to wild-type cells. The kinetics of LPS-induced IL-19 mRNA production was also altered in these cells, with product being formed as early as 1 hour post-

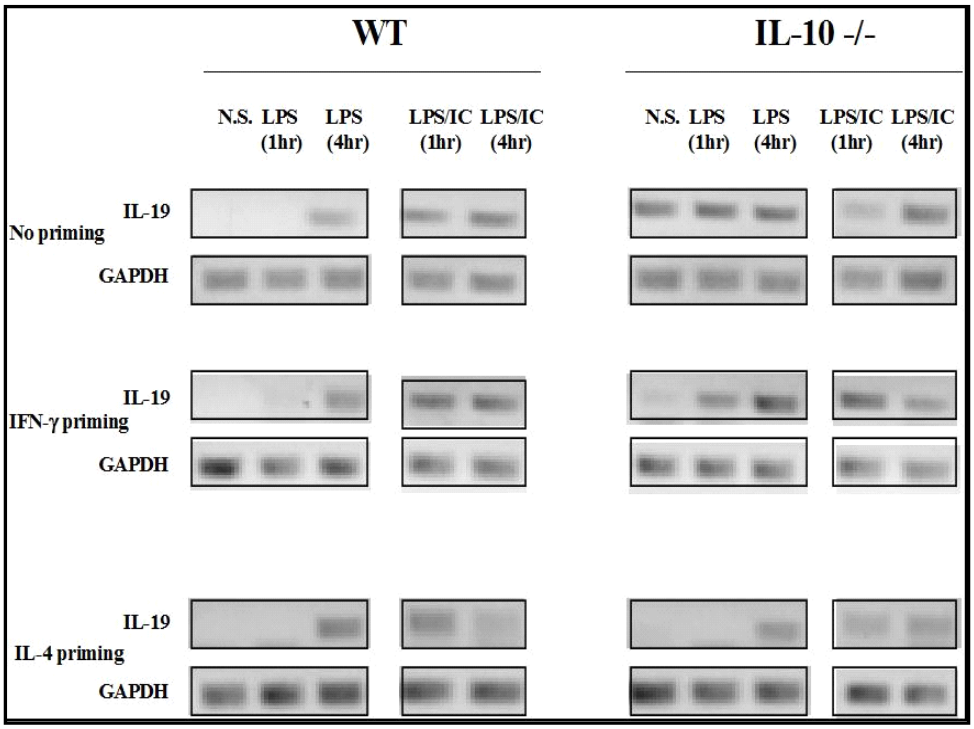


Fig. 3. IL-19 expression in classically activated, alternatively activated, and regulatory macrophages.
RT-PCR was performed to examine IL-19 expression in different populations of BMMϕs isolated from wild-type BALB/c (left) and IL-10 $^{-/-}$ mice (right). Cells were treated overnight either with media alone (top group), 100 U/mL IFN-γ (center group) or 10 U/mL IL-4 (bottom group). RNA was collected and reverse transcribed into cDNA after stimulation with either LPS alone, or with LPS plus immune complexes (LPS/IC) for the indicated time periods. GAPDH RNA was used as a loading control. Data are representative of three independent experiments.

stimulation. A similar pattern was seen in response to cells primed with IFN-γ. LPS stimulation of IFN-γ primed IL-10 $^{-/-}$ cells produced overall levels of IL-19 mRNA that were higher compared to wild-type cells, and mRNA transcription occurred earlier at 1 hour post-stimulation. No significant difference was seen in knockout cells primed with IL-4 as compared to wild-type controls. These data indicate that, as in monocytes, IL-10 plays a regulatory role in IL-19 production.

Activity of IL-19 promoter in response to stimulation

To further investigate the production of IL-19 in macrophage populations, we examined luciferase activity in RAW 264.7 cells that had been transfected with IL-19 promoter constructs of various lengths. Cells were transfected with either the full length promoter construct (-3 kb/+200, Fig. 2), or with truncated promoters (-2 kb/+200, -1 kb/+200). RAW cells that had been transfected with either of the two truncated promoters showed only modest levels of luciferase activity as compared to the empty pGL4.19 vector under any stimulation conditions. However, the full length promoter exhibited a dramatic increase in luciferase activity upon stimulation with LPS (Fig. 4A), indicating that the primary transcription factor binding sites are most likely located within the region from -3 kb to -2 kb upstream from the transcription start site. LPS stimulation of the full-length promoter in classically activated cells (Fig 4B) exhibited an even higher level of luciferase activity, whereas alternatively activated cells (Fig. 4C) exhibited an activity level lower than that of classically activated cells, but still higher than unprimed cells. Interestingly, stimulation with LPS/IC did not induce significant levels of IL-19 promoter activity under any priming

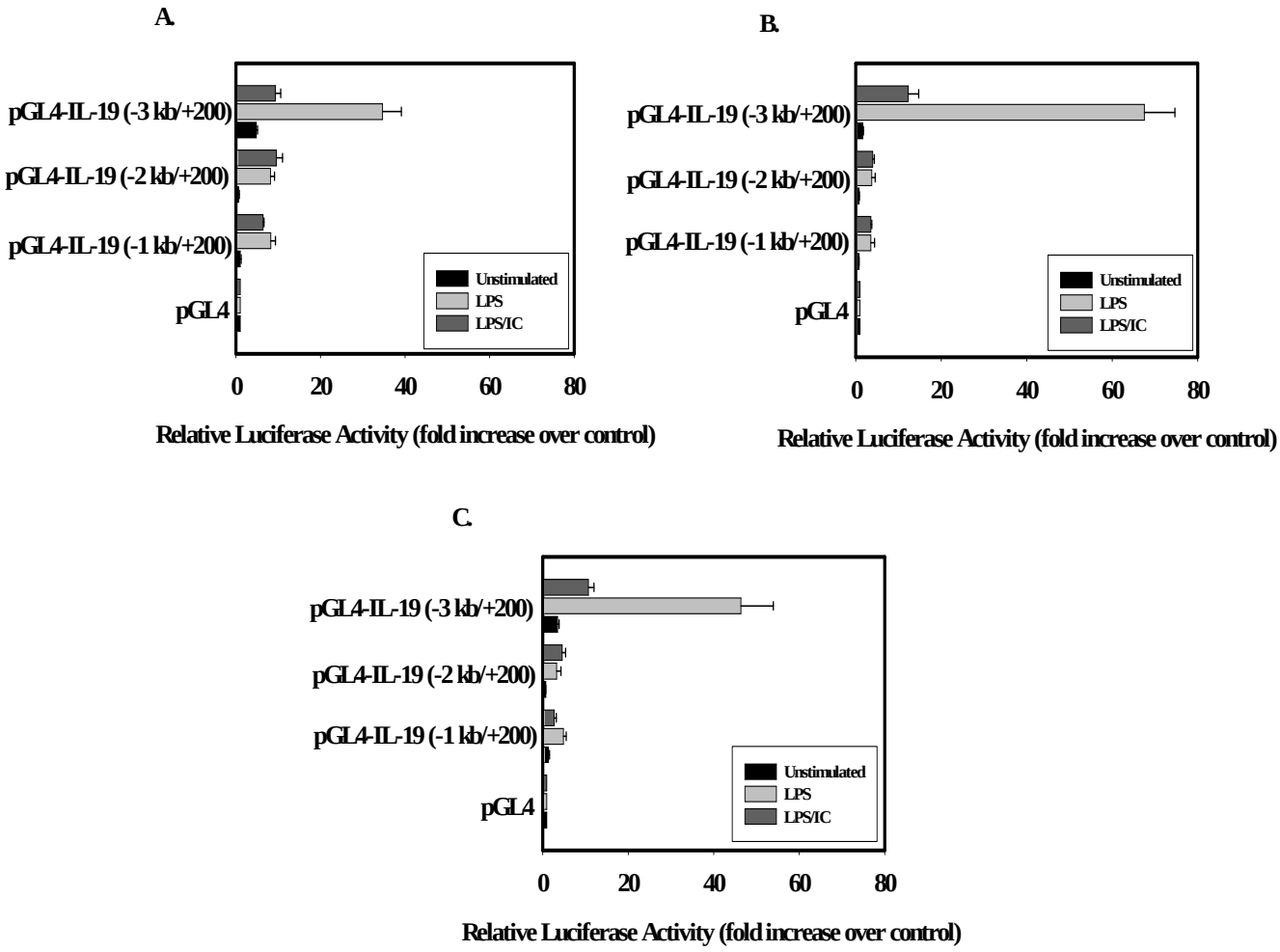


Fig. 4. Luciferase activity of RAW 264.7 cells transfected with either full-length or truncated IL-19 promoter vectors after stimulation with LPS or LPS/IC in the presence or absence of IFN-γ or IL-4.

pGL4.19 luciferase reporter constructs containing either the full-length (-3 kb/+200) or truncated promoter constructs (-2 kb/+200, -1 kb/+200) were transfected into RAW 264.7 cells, then grown overnight either in the presence of media alone (A), 100 U/mL IFN-γ (B), or 10 U/mL IL-4 (C). Cells were then stimulated with either LPS or LPS/IC for 8hrs. Each sample was measured for firefly luciferase activity and normalized to *Renilla* luciferase activity (transfected at a ratio of 40:1). Values are represented as fold changes relative to unstimulated cells transfected with the empty vector. Figures shown are representative of three independent experiments. Error bars are ± standard deviation for experiment done in triplicate.

conditions, in contrast to what was seen in IL-19 mRNA experiments.

Discussion

We have shown here that IL-19 is in fact expressed in macrophages, and that it does indeed share many important similarities with IL-10. Like IL-10, IL-19 expression is upregulated in the presence of immune complexes and the classical activation signals IFN-γ and LPS[1]. Interestingly, IL-19 expression in response to LPS stimulation is not enhanced by priming with IL-4. This is in stark contrast to what has been reported in human monocytes, where IL-19 production is upregulated by IL-4 and downregulated by IFN-γ[39]. IL-19 has also been associated in the literature with various inflammatory processes (e.g. atherosclerosis, asthma[48]), and its exact role in inflammatory processes is unclear due to the fact that it is secreted at different time points and by different cells types depending on the model of inflammation being studied. Our evidence, taken in conjunction with what has been reported in human monocytes, supports the idea that IL-19 production, like IL-10, is highly dependent on the cell type involved and the microenvironment in which the cell is found.

As has been reported previously in human monocytes, IL-19 is itself inhibited by IL-10 production in macrophages[49]. We showed that IL-19 is expressed at basal levels in IL-10 $^{-/-}$ mice even without stimulation, and that IL-19 production is increased in response to stimulation with LPS/IC. Oddly, these findings did not correlate with studies of the IL-19 promoter, which only showed activity in response to stimulation with LPS. Faced with a similar problem with regard to the IL-10 promoter, previous work in our lab has demonstrated that the superinduction of IL-10

seen in regulatory macrophages is dependent on histone modifications and chromatin remodeling, both of which would not have been present on the plasmid vector used in a luciferase assay[32]. It is possible that the same is true for the IL-19 promoter. Future studies using chromatin immunoprecipitation (ChIP) assays should determine whether histone modifications occur at distinct sites along the IL-19 promoter, and whether these can be correlated with transcription factor binding sites.

Chapter 4: Role of MAPKs in IL-19 expression

Inhibition of ERK decreases IL-19 promoter activity in response to LPS stimulation

Previous work by our lab has shown that the MAPK ERK plays a central role in IL-10 production in regulatory macrophages, specifically translating signals from cross-linked FcγRs into dynamic and transient remodeling of chromatin at the IL-10 promoter. This results in the superinduction of IL-10 seen in this macrophage population[3, 32, 38]. To determine if IL-19 promoter activity might also be dependent on ERK, we tested whether or not IL-19 promoter constructs still exhibited luciferase activity in the presence of the ERK inhibitor U0126. RAW 264.7 cells were transfected with the IL-19 promoter constructs as described previously. Cells were then pretreated with 5 μM U0126 or vehicle alone, before being stimulated with LPS (Fig. 5A) or LPS/IC (Fig. 5B). As before, only the full length 3 kb construct exhibited any significant luciferase activity in response to LPS stimulation. This response was completely erased by the presence of U0126, bringing luciferase activity back down to basal levels seen in unstimulated control samples. As before, none of the constructs showed any significant luciferase activity in response to stimulation with LPS/IC. Interestingly, when the p38 inhibitor SB203580 was used instead of U0126, the luciferase activity of the full-length 3 kb promoter construct was inhibited by approximately half in response to LPS stimulation instead of being completely erased as it was with the ERK inhibitor. Cells stimulated with LPS/IC again failed to illicit any appreciable level of promoter activity above that of

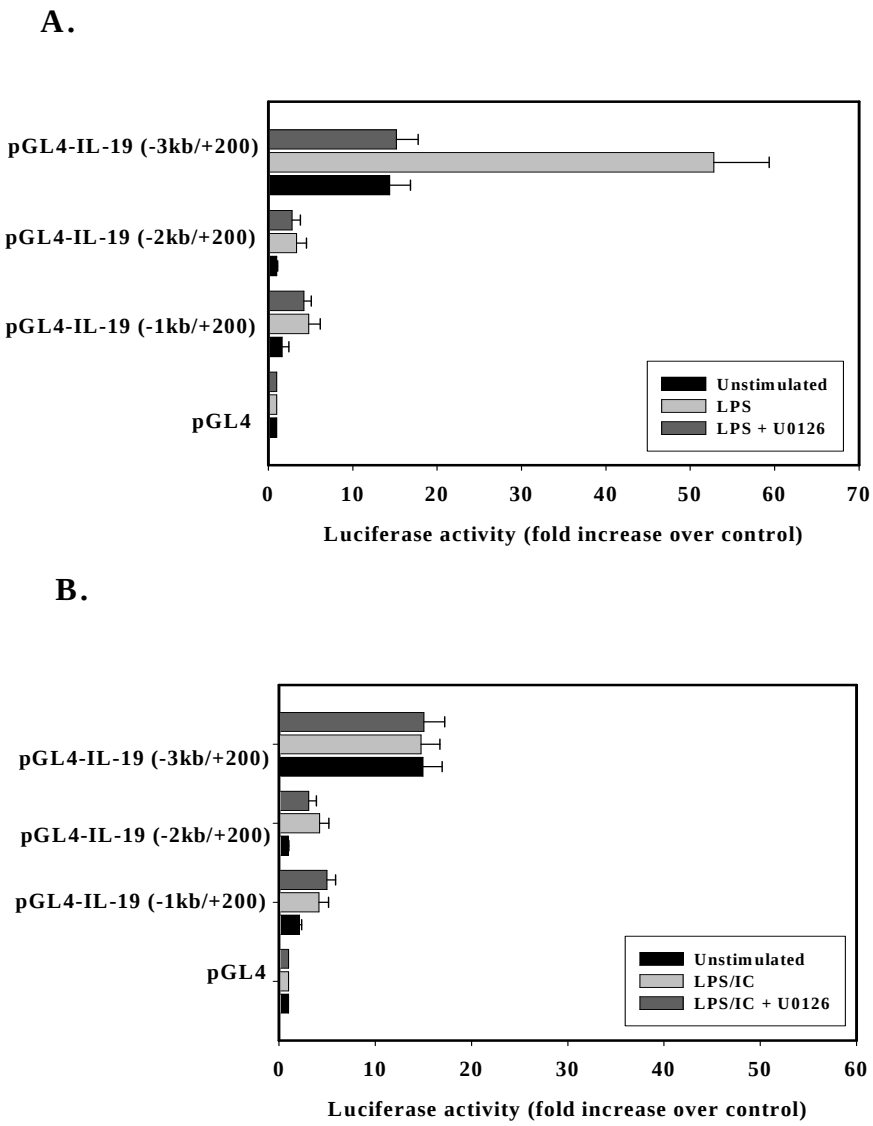
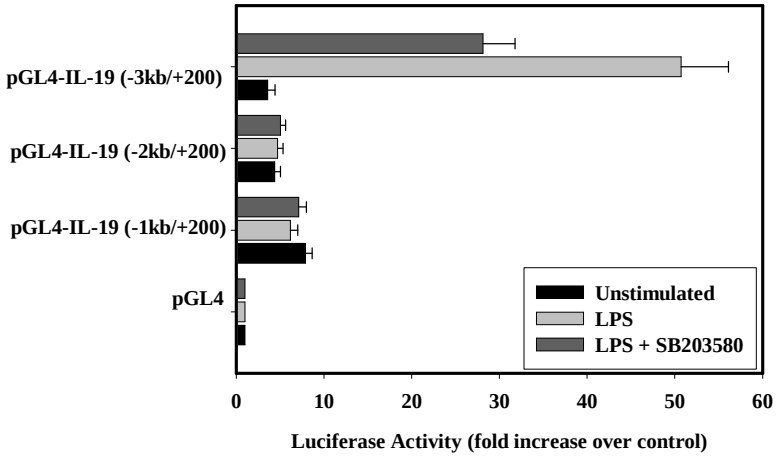


Fig. 5. Luciferase activity of RAW 264.7 cells transfected with either full-length or truncated IL-19 promoter vectors after stimulation with LPS or LPS/IC in the presence or absence of U0126.

pGL4.19 luciferase reporter constructs containing either the full-length (-3 kb/+200) or truncated promoter constructs (-2 kb/+200, -1 kb/+200) were transfected into RAW 264.7 cells. Cells were pretreated with 5 μM U0126 or vehicle alone, then stimulated with either LPS (A) or LPS/IC (B) for 8hrs. Each sample was measured for firefly luciferase activity and normalized to *Renilla* luciferase activity (transfected at a ratio of 40:1). Values are represented as fold changes relative to unstimulated cells transfected with the empty vector. Figures shown are representative of three independent experiments. Error bars are ± standard deviation for experiment done in triplicate.

A.



B.

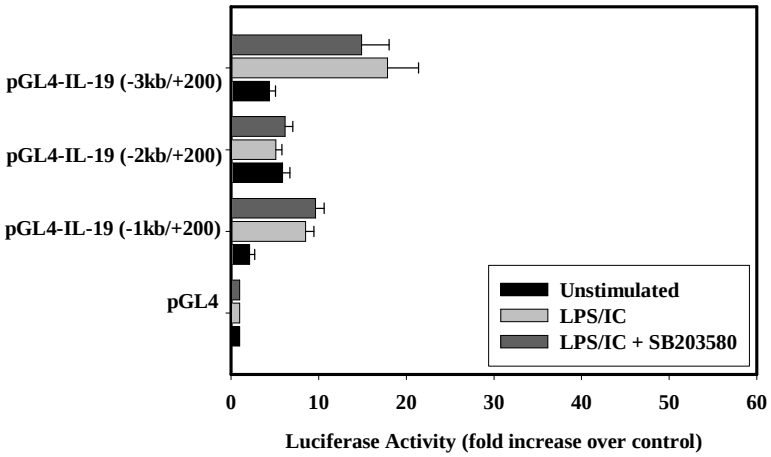


Fig. 6. Luciferase activity of RAW 264.7 cells transfected with either full-length or truncated IL-19 promoter vectors after stimulation with LPS or LPS/IC in the presence or absence of SB203580.
pGL4.19 luciferase reporter constructs containing either the full-length (-3 kb/+200) or truncated promoter constructs (-2 kb/+200, -1 kb/+200) were transfected into RAW 264.7 cells. Cells were pretreated with 5 μM SB203580 or vehicle alone, then stimulated with either LPS (A) or LPS/IC (B) for 8hrs. Each sample was measured for firefly luciferase activity and normalized to *Renilla* luciferase activity (transfected at a ration of 40:1). Values are represented as fold changes relative to unstimulated cells transfected with the empty vector. Figures shown are representative of three independent experiments. Error bars are ± standard deviation for experiment done in triplicate.

unstimulated controls. These results are an interesting contrast to the aforementioned previous data obtained by our lab. We have shown that modest amounts of phosphorylated ERK and p38 are produced in unprimed macrophages in response to LPS. However, these levels are always moderate in comparison to those produced by stimulation with LPS/IC, and we have never shown any absolute requirement of MAPK activity to produce IL-10 in macrophages stimulated with LPS alone[38]. Further investigation into the mechanism behind this data is warranted.

Chapter 5: Regulation of IL-10 Production by IL-19

Priming with IL-19 upregulates IL-10 expression in a dose-dependent manner

Previous data has demonstrated that, in human monocytes, IL-19 can induce IL-10 production, but is also negatively regulated by IL-10 in the manner of a negative feedback loop[49]. To determine if IL-19 could increase production of IL-10 in regulatory macrophages, we measured cytokine production by ELISA in BMMϕs that had been primed for 6 hours with 100 pg/mL of recombinant IL-19. IL-19 priming did indeed cause an increase in IL-10 production in macrophages that had been stimulated with LPS/IC as opposed to cells treated with LPS/IC alone (Fig. 7A). This effect was limited to IL-10 production, since there was no statistically significant decrease in either IL-12/23 p40 (Fig. 7A) or TNF-α (Fig. 7B) production.

Further analysis indicated that this effect of IL-19-induced overproduction of IL-10 was a dose-dependent phenomenon. BMMϕs were primed with increasing doses of recombinant IL-19 for 6 hours prior to stimulation. IL-19 caused a dose-dependent increase in IL-10 production in macrophages in response to stimulation with LPS/IC (Fig. 8A). The effect was again specific to IL-10 production, since no similar dose-dependent effect was observed with either IL-12/23 p40 (Fig. 8B) or TNF-α (Fig. 8C).

Priming with IL-19 increases IL-10 mRNA expression

We next wanted to know whether IL-19 regulated IL-10 at the level of mRNA transcription, or merely at the level of protein expression. To address this question,

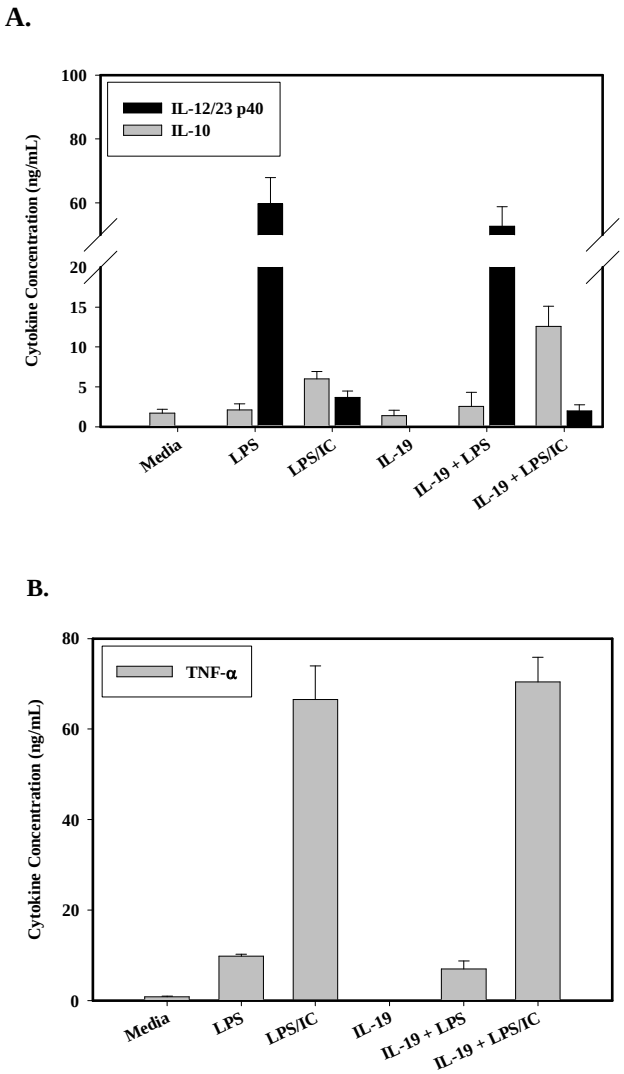


Fig. 7. IL-19 priming enhances IL-10 production in regulatory macrophages. BMMϕs were primed for 6 hours with either media or 100 pg/mL of recombinant IL-19, then stimulated with either LPS or LPS/IC as indicated. Supernatants were collected after 8 hours, the concentration of IL-10 and IL-12/23 p40 (A), and TNF-α (B) were measured by ELISA. Data shown is representative of three separate experiments. Error bars indicate ± standard deviation of experiments done in triplicate.

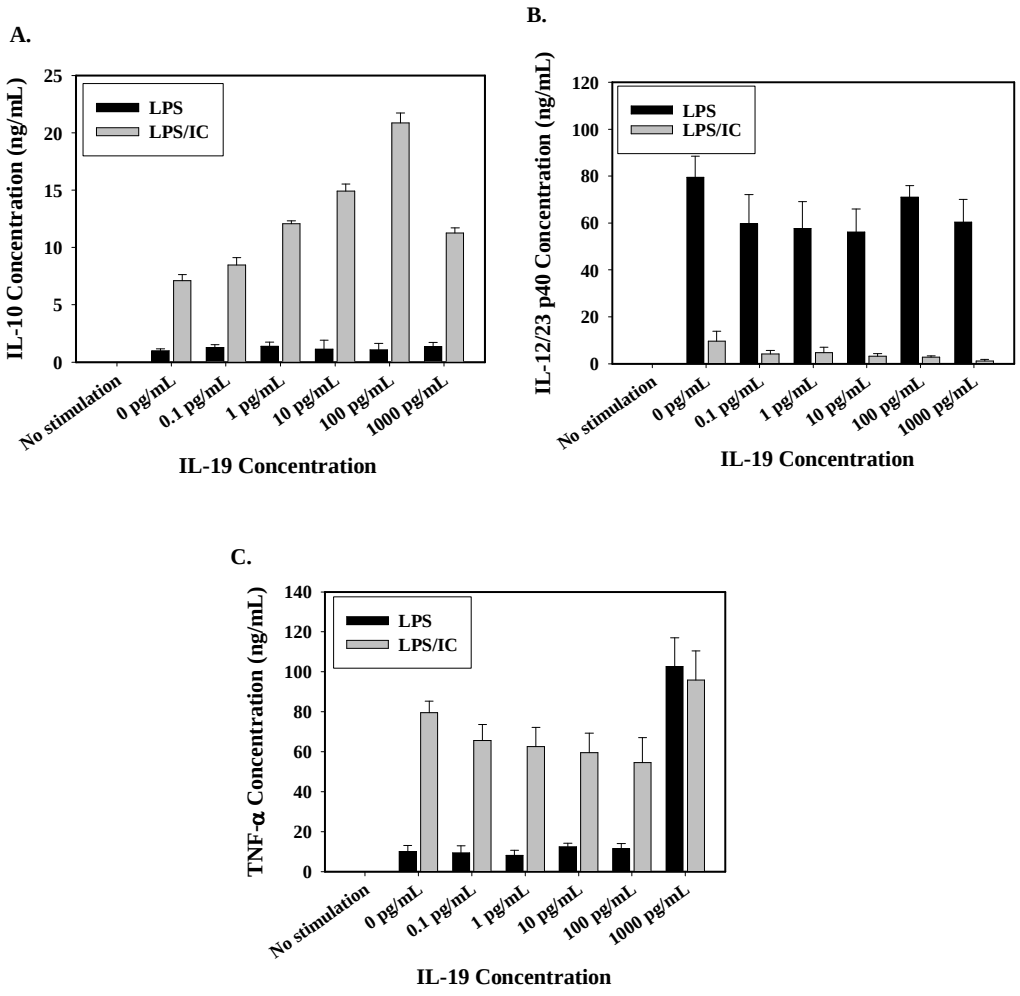


Fig. 8. IL-19 enhances IL-10 production in a dose-dependent manner. BMMϕs were primed for 6 hours with increasing doses of recombinant IL-19, then stimulated with either LPS or LPS/IC as indicated. Supernatants were collected after 8 hours, the concentration of IL-10 (A), IL-12/23 p40 (B), and TNF-α (C) were measured by ELISA. Data shown is representative of three separate experiments. Error bars indicate ± standard deviation of experiments done in triplicate.

we primed BMMϕs for 6 hours with increasing doses of recombinant IL-19. After 4 hours of stimulation with either LPS or LPS/IC, RNA was extracted and converted to cDNA. PCR was then performed to detect total mRNA levels of IL-10, IL-12/23 p40, and IL-19 (Fig. 9). Surprisingly, IL-10 mRNA levels did not rise above that seen in the control sample. In fact, IL-10 mRNA levels decreased in a dose-dependent fashion with increased concentrations of IL-19, which is the exact opposite result of what was seen on the protein level measured by ELISA (Fig. 8A). In addition, IL-19 induced IL-10 mRNA transcription in samples stimulated with LPS alone, although these levels did not increase with higher concentrations of IL-19. IL-19 also increased its own expression in cells stimulated with LPS, a fact that has also been observed in human monocytes[49]. This observation was also reversed in cells stimulated with LPS/IC, where IL-19 priming caused a decrease in IL-19 mRNA production in a dose-dependent manner.

We theorized that a possible explanation for the discrepancy between our observations at the protein and mRNA levels might be due to the fact that we were observing total mRNA product, and might instead need to look specifically at mature RNA being made in response to our experimental conditions. The next step therefore was to use quantitative RT-PCR (QRT-PCR) to look for mature RNA transcript made in regulatory macrophages in response to IL-19 priming. BMMϕs were primed with either 100 pg/mL IL-19 or vehicle control (Fig. 10). Cells were then stimulated with LPS/IC for the indicated time points. RNA was then isolated and converted to cDNA, and quantitative real-time PCR was performed using primers

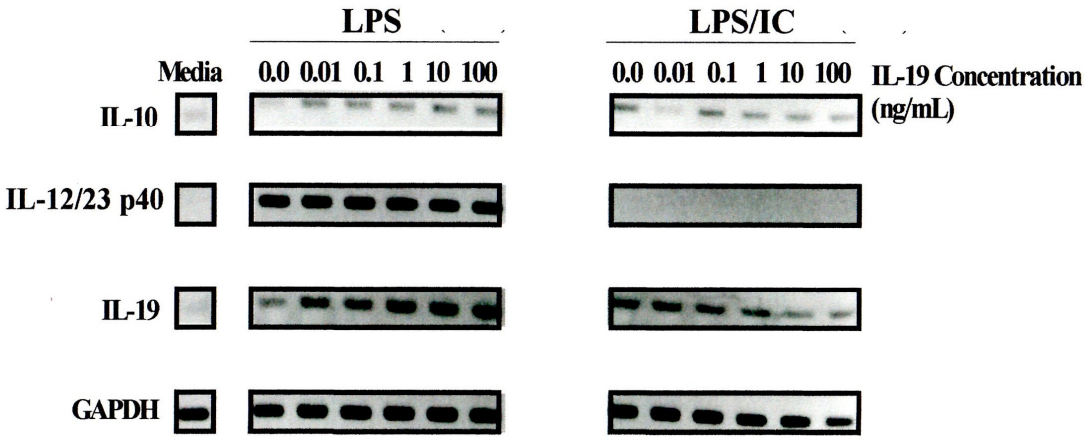


Fig. 9. Effect of IL-19 priming on total mRNA expression.
RT-PCR was performed to examine IL-10 expression in BMMϕs primed with increasing concentrations of recombinant IL-19 for 6 hours. Cells were stimulated with either LPS (left side) or LPS/IC (right side). RNA was collected and reverse transcribed into cDNA, followed by PCR using primers specific for IL-10, IL-12/23 p40, and IL-19. GAPDH RNA was used as a loading control. Data are representative of three independent experiments.

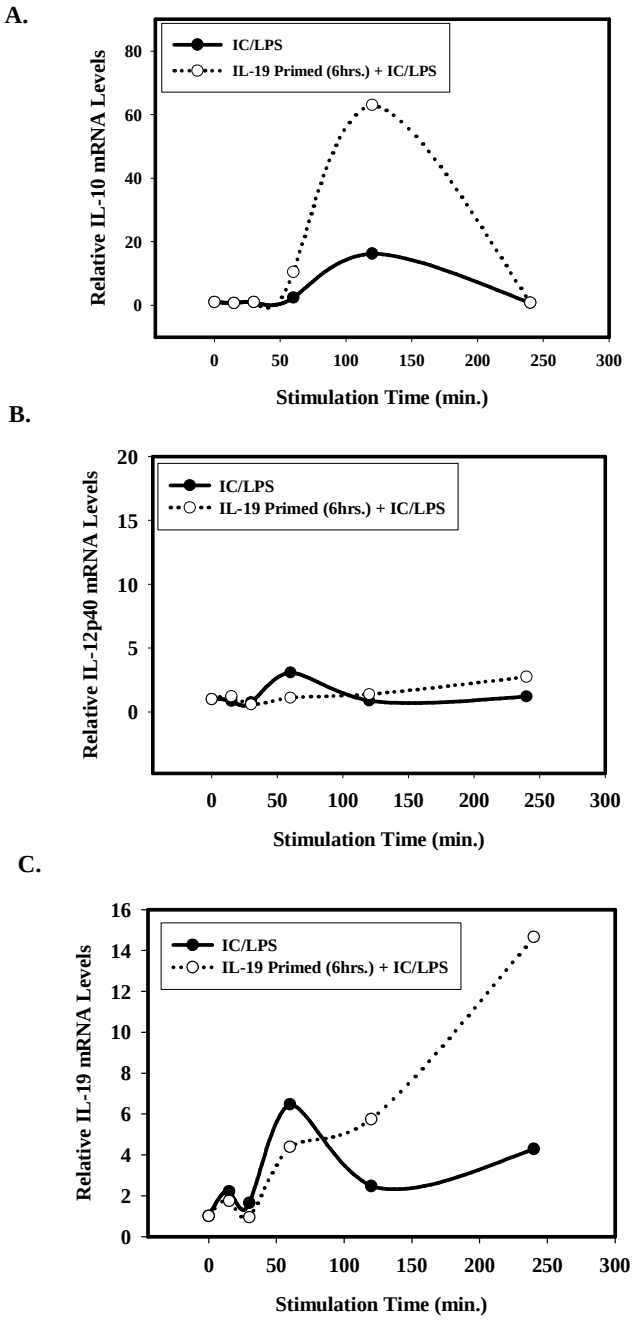


Fig. 10. Regulatory macrophages upregulate IL-10 mRNA in response to IL-19 priming.

(A) IL-10 mRNA was measured by QRT-PCR after priming with media plus vehicle control (closed circles) or with 100 pg/mL recombinant IL-19 (open circles), followed by stimulation with LPS plus immune complexes for the indicated time periods. QRT-PCR was also used to measure IL-12/23 p40 (B) and IL-19 (C) mRNA. Fold values are relative to unstimulated control. Figures are representative of at least three separate experiments.

specific for IL-10, IL-12/23 p40, and IL-19. A robust increase in IL-10 mRNA levels was seen in regulatory macrophages primed with IL-19 compared to unprimed controls, with the peak expression appearing at 120 minutes post stimulation with LPS/IC (Fig. 10A). This effect was not unique to IL-10, since IL-19 mRNA expression also increased in response to IL-19 priming in regulatory macrophages (Fig. 10C). IL-12/23 p40 mRNA expression was not significantly affected (Fig. 10B), indicating that IL-19 priming affects expression of only itself and IL-10. This data is more in line with our previous experiments (Fig. 7, 8) and what has been reported in human monocytes[49].

IL-19 priming decreases ERK phosphorylation in regulatory macrophages

Since our lab has already established that IL-10 superinduction in regulatory macrophages is an ERK-dependent phenomenon [3, 32, 38], we theorized that IL-19 priming would increase ERK activity in response to stimulation with LPS/IC. To test this theory, BMMϕs were cultured in the presence or absence of 100 pg/mL of recombinant IL-19 for 6 hrs. Cells were then stimulated with either LPS or LPS/IC, then lysed and the protein analyzed by Western blotting. Membranes were probed with antibodies specific for phosphorylated ERK, p38, and JNK (Fig. 11). Surprisingly, ERK phosphorylation was dramatically decreased in IL-19-primed macrophages that had been stimulated with LPS/IC as compared to cells stimulated with LPS/IC alone. ERK phosphorylation peaked at only 5 minutes post stimulation, then decreased rapidly thereafter. JNK and p38 activity remained the same in IL-19 primed regulatory macrophages as it did in unprimed cells. IL-19 priming therefore

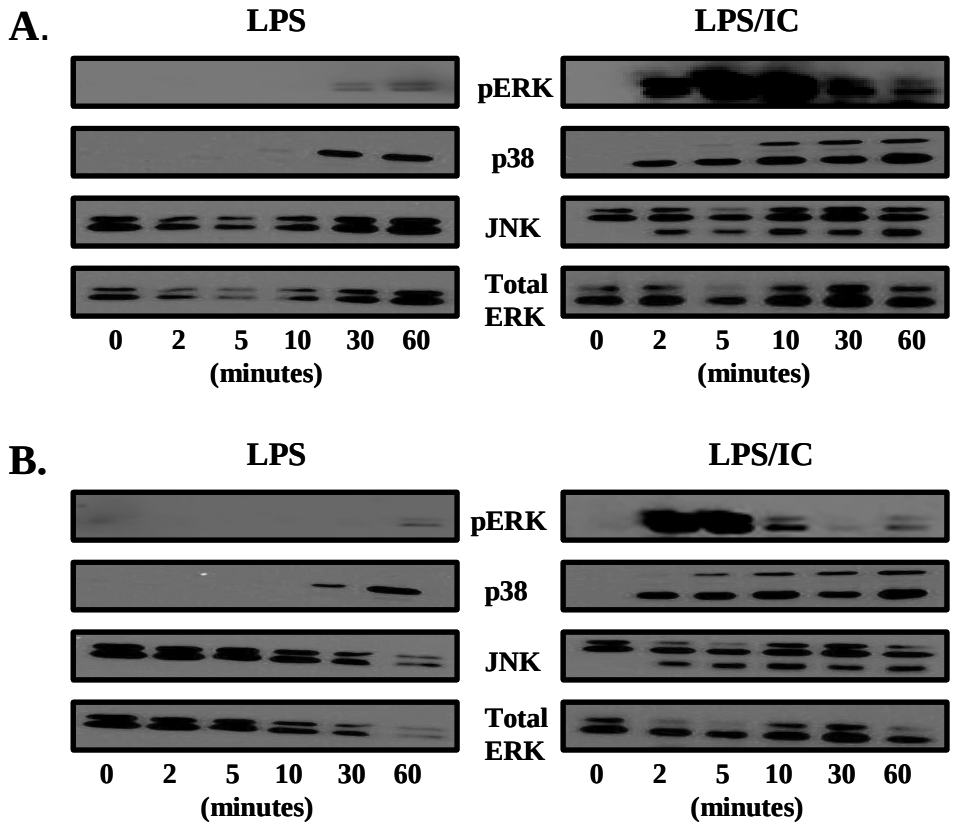


Fig. 11. ERK phosphorylation decreases in regulatory macrophages primed with IL-19.

BMMϕs were primed with either vehicle control (A) or 100 pg/mL of recombinant IL-19 for 6 hours (B), then stimulated with either LPS or LPS/IC as indicated. Cells were then lysed in the presence of protease and phosphotase inhibitors at the indicated time periods. Samples were subjected to SDS-PAGE and Western blotting with antibodies specific for phosphorylated ERK, p38 and JNK. Antibodies specific for total ERK was used as a loading control. Figures are representative of at least three independent experiments.

specifically inhibits the robust ERK phosphorylation normally seen in regulatory macrophages while still producing a higher level of IL-10 then is seen with regulatory macrophages stimulated with LPS/IC alone (Fig. 8, 10).

Discussion

We have shown here that IL-19 can upregulate IL-10 expression in regulatory macrophages in a dose-dependent manner, both at the mRNA and protein level. Strangely, this increase in IL-10 production did not seem to be dependent on ERK. In fact, IL-19 significantly decreased ERK phosphorylation in cells stimulated with LPS plus immune complexes. The fact that IL-19 priming did not cause this effect in cells stimulated with LPS alone indicates that signaling through the FcγR is still required for IL-10 production in these cells, but that an ERK-independent mechanism of activation may be involved. Further studies should explore what the exact mechanism of IL-10 induction is in these cells. In particular, studies should focus on the possibility that IL-19 priming increases access to the IL-10 promoter through an as-yet unknown ERK-independent pathway. It would also be interesting to determent whether there are other differences in activity between regulatory macrophages and these same cells primed with IL-19, including whether or not the later cell type expresses the same markers (e.g. LIGHT, HB-EGF), can bias T cells towards a Th2 phenotype, or shows the same metabolic activity (e.g. arginine metabolism) as regulatory macrophages. Further studies should also be made into the role of IL-19 in various murine models of disease, such as atherosclerosis and asthma, to determine what role this cytokine plays in the overall inflammatory process.

Summary of Findings

We have shown here that IL-19 is produced in classically activated, alternatively activated, and regulatory macrophages. IL-19 is similar to IL-10 in that the quantity and kinetics of its production are increased in response to stimulation with LPS plus immune complexes as compared to stimulation with LPS alone. Studies of the IL-19 promoter, however, indicate that the major transcription factor(s) responsible for its production are located between 2 and 3 kb upstream from the transcription start site, and that LPS stimulation is sufficient to induce IL-19 production. Furthermore, this LPS induced response is dependent on ERK activity, and partially dependent on p38 activity. IL-19 is also capable, in a dose-dependent manner, of increasing IL-10 production in regulatory macrophages above the levels induced by stimulation with LPS/IC alone. This is the case at both the level of IL-10 mRNA and protein. In addition, ERK activity does not seem to play a significant role in IL-10 production as a result of IL-19 priming.

References

1. Mosser,D.M. The many faces of macrophage activation. *J Leukoc Biol* **73**, 209-212 (2003).

2. Anderson,C.F. & Mosser,D.M. Cutting Edge: Biasing Immune Responses by Directing Antigen to Macrophage Fc{gamma} Receptors. *J Immunol* **168**, 3697-3701 (2002).

3. Edwards,J.P., Zhang,X., Frauwirth,K.A., & Mosser,D.M. Biochemical and functional characterization of three activated macrophage populations. *J Leukoc Biol* **80**, 1298-1307 (2006).

4. Fiorentino,D.F., Bond,M.W., & Mosmann,T.R. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.* **170**, 2081-2095 (1989).

5. de Waal Malefyt,R. *et al.* Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J. Exp. Med.* **174**, 915-924 (1991).

6. Ding,L., Linsley,P.S., Huang,L.Y., Germain,R.N., & Shevach,E.M. IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. *J Immunol* **151**, 1224-1234 (1993).

7. Grutz,G. New insights into the molecular mechanism of interleukin-10-mediated immunosuppression. *J Leukoc Biol* **77**, 3-15 (2005).

8. Reed,S.G. *et al.* IL-10 mediates susceptibility to Trypanosoma cruzi infection. *J Immunol* **153**, 3135-3140 (1994).

9. Wagner,R.D., Maroushek,N.M., Brown,J.F., & Czuprynski,C.J. Treatment with anti-interleukin-10 monoclonal antibody enhances early resistance to but impairs complete clearance of Listeria monocytogenes infection in mice. *Infect. Immun.* **62**, 2345-2353 (1994).

10. Howard,M., Muchamuel,T., Andrade,S., & Menon,S. Interleukin 10 protects mice from lethal endotoxemia. *J. Exp. Med.* **177**, 1205-1208 (1993).

11. Caslzada-Wack,J.C., Frankenberger,M., & Ziegler-Heitbrock,H.W. Interleukin-10 Drives Human Monocytes to CD16 Positive Macrophages. *Journal of Inflammation* **46**, 78-85 (1996).

12. Kawai,T. & Akira,S. TLR signaling. *Cell Death Differ* **13**, 816-825 (2006).

13. Kenny,E.F. & O'Neill,L.A.J. Signalling adaptors used by Toll-like receptors: An update. *Cytokine* **43**, 342-349 (2008).

14. Kumar,H., Kawai,T., & Akira,S. Toll-like receptors and innate immunity. *Biochemical and Biophysical Research Communications* **388**, 621-625 (2009).

15. Akira,S., Uematsu,S., & Takeuchi,O. Pathogen Recognition and Innate Immunity. *Cell* **124**, 783-801 (2006).

16. Akira,S. Toll-like receptors: lessons from knockout mice. *Biochem. Soc. Trans.* **28**, 551-556 (2000).

17. Unkeless,J.C., Scigliano,E., & Freedman,V.H. Structure and Function of Human and Murine Receptors for IgG. *Annual Review of Immunology* **6**, 251-281 (1988).

18. Nimmerjahn,F. & Ravetch,J.V. Fc[gamma] receptors as regulators of immune responses. *Nat Rev Immunol* **8**, 34-47 (2008).

19. Nimmerjahn,F. & Ravetch,J.V. Fc[gamma] Receptors: Old Friends and New Family Members. *Immunity* **24**, 19-28 (2006).

20. Wossning,T. *et al.* Deregulated Syk inhibits differentiation and induces growth factor-independent proliferation of pre-B cells. *J. Exp. Med.* **203**, 2829-2840 (2006).

21. Sutterwala,F.S., Noel,G.J., Salgame,P., & Mosser,D.M. Reversal of Proinflammatory Responses by Ligating the Macrophage Fc{gamma} Receptor Type I. *J. Exp. Med.* **188**, 217-222 (1998).

22. Zhang,Y. & Dong,C. MAP Kinases in Immune Responses. *Cellular and Molecular Immunology* **2**, 20-27 (2005).

23. Chong,H., Vikis,H.G., & Guan,K.L. Mechanisms of regulating the Raf kinase family. *Cellular Signalling* **15**, 463-469 (2003).

24. Peyssonnaux,C. & Eychene,A. The Raf/MEK/ERK pathway: new concepts of activation. *Biol. Cell* **93**, 53-62 (2001).

25. Roux,P.P. & Blenis,J. ERK and p38 MAPK-Activated Protein Kinases: a Family of Protein Kinases with Diverse Biological Functions. *Microbiol. Mol. Biol. Rev.* **68**, 320-344 (2004).

26. Kyriakis,J.M. & Avruch,J. pp54 microtubule-associated protein 2 kinase. A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine. *Journal of Biological Chemistry* **265**, 17355-17363 (1990).

27. Chen,Z. *et al.* MAP Kinases. *Chemical Reviews* **101**, 2449-2476 (2001).

28. Whitmarsh,A.J. Regulation of gene transcription by mitogen-activated protein kinase signaling pathways. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1773**, 1285-1298 (2007).

29. Avruch,J. MAP kinase pathways: The first twenty years. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1773**, 1150-1160 (2007).

30. Pestka,S. *et al.* Interleukin-10 and Related Cytokines and Receptors. *Annual Review of Immunology* **22**, 929-979 (2004).

31. Powell,M.J., Thompson,S.A.J., Tone,Y., Waldmann,H., & Tone,M. Posttranscriptional Regulation of IL-10 Gene Expression Through Sequences in the 3'-Untranslated Region. *J Immunol* **165**, 292-296 (2000).

32. Zhang,X., Edwards,J.P., & Mosser,D.M. Dynamic and Transient Remodeling of the Macrophage IL-10 Promoter during Transcription. *J Immunol* **177**, 1282-1288 (2006).

33. Brightbill,H.D., Plevy,S.E., Modlin,R.L., & Smale,S.T. A Prominent Role for Sp1 During Lipopolysaccharide- Mediated Induction of the IL-10 Promoter in Macrophages. *J Immunol* **164**, 1940-1951 (2000).

34. Ma,W. *et al.* The p38 Mitogen-activated Kinase Pathway Regulates the Human Interleukin-10 Promoter via the Activation of Sp1 Transcription Factor in Lipopolysaccharide-stimulated Human Macrophages. *Journal of Biological Chemistry* **276**, 13664-13674 (2001).

35. Staples,K.J. *et al.* IL-10 Induces IL-10 in Primary Human Monocyte-Derived Macrophages via the Transcription Factor Stat3. *J Immunol* **178**, 4779-4785 (2007).

36. Matsukawa,A. *et al.* Aberrant Inflammation and Lethality to Septic Peritonitis in Mice Lacking STAT3 in Macrophages and Neutrophils. *J Immunol* **171**, 6198-6205 (2003).

37. Cao,S., Zhang,X., Edwards,J.P., & Mosser,D.M. NF-kB1 (p50) Homodimers Differentially Regulate Pro- and Anti-inflammatory Cytokines in Macrophages. *Journal of Biological Chemistry* **281**, 26041-26050 (2006).

38. Lucas,M., Zhang,X., Prasanna,V., & Mosser,D.M. ERK Activation Following Macrophage Fc{gamma}R Ligation Leads to Chromatin Modifications at the IL-10 Locus. *J Immunol* **175**, 469-477 (2005).

39. Gallagher,G. *et al.* Cloning, expression and initial characterisation of interleukin-19 (IL-19), a novel homologue of human interleukin-10 (IL-10). *Genes & Immunity* **1**, 442 (2000).

40. Chang,C. *et al.* Crystal Structure of Interleukin-19 Defines a New Subfamily of Helical Cytokines. *Journal of Biological Chemistry* **278**, 3308-3313 (2003).

41. Dumoutier,L., Leemans,C., Lejeune,D., Kotenko,S.V., & Renauld,J.C. Cutting Edge: STAT Activation By IL-19, IL-20 and mda-7 Through IL-20 Receptor Complexes of Two Types. *J Immunol* **167**, 3545-3549 (2001).

42. Donnelly,R.P., Sheikh,F., Kotenko,S.V., & Dickensheets,H. The expanded family of class II cytokines that share the IL-10 receptor-2 (IL-10R2) chain. *J Leukoc Biol* **76**, 314-321 (2004).

43. Mosser,D.M. & Zhang,X. Interleukin-10: new perspectives on an old cytokine. *Immunological Reviews* **226**, 205-218 (2008).

44. Parrish-Novak,J. *et al.* Interleukins 19, 20, and 24 Signal through Two Distinct Receptor Complexes. *Journal of Biological Chemistry* **277**, 47517-47523 (2002).

45. Wolk,K., Kunz,S., Asadullah,K., & Sabat,R. Cutting Edge: Immune Cells as Sources and Targets of the IL-10 Family Members? *J Immunol* **168**, 5397-5402 (2002).

46. Liao,Y.C. *et al.* IL-19 Induces Production of IL-6 and TNF-{alpha} and Results in Cell Apoptosis Through TNF-{alpha}. *J Immunol* **169**, 4288-4297 (2002).

47. Ghoreschi,K. *et al.* Interleukin-4 therapy of psoriasis induces Th2 responses and improves human autoimmune disease. *Nat Med* **9**, 40-46 (2003).

48. Liao,S.C. *et al.* IL-19 Induced Th2 Cytokines and Was Up-Regulated in Asthma Patients. *J Immunol* **173**, 6712-6718 (2004).

49. Jordan,W.J. *et al.* Human IL-19 Regulates Immunity Through Auto-Induction of IL-19 and Production of IL-10. *European Journal of Immunology* **35**, 1576-1582 (2005).

50. Gallagher,G. *et al.* Human interleukin-19 and its receptor: a potential role in the induction of Th2 responses. *International Immunopharmacology* **4**, 615-626 (2004).

51. Commins,S., Steinke,J.W., & Borish,L. The extended IL-10 superfamily: IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, and IL-29. *Journal of Allergy and Clinical Immunology* **121**, 1108-1111 (2008).