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Intravenous immunoglobulin skews macrophages to an anti-inflammatory, IL-10-producing activation state

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ABSTRACT

Intravenous Ig is used to treat autoimmune or autoinflammatory disorders, but the mechanism by which it exerts its immunosuppressive activity is not understood completely. To examine the impact of intravenous Ig on macrophages, we compared cytokine production by LPS-activated macrophages in the presence and absence of intravenous Ig. Intravenous Ig treatment induced robust production of IL-10 in response to LPS, relative to LPS stimulation alone, and reduced production of proinflammatory cytokines. This anti-inflammatory, intravenous Ig-induced activation was sustained for 24 h but could only be induced if intravenous Ig were provided within 1 h of LPS stimulation. Intravenous Ig activation led to enhanced and prolonged activation of MAPKs, Erk1/2, p38, and Erk5, and inhibition of each reduced intravenous Ig-induced IL-10 production and suppression of IL-12/23p40. IL-10 production occurred rapidly in response to intravenous Ig + LPS and was sufficient to reduce proinflammatory IL-12/23p40 production in response to LPS. IL-10 induction and reduced IL-12/23p40 production were transcriptionally regulated. IL-10 played a direct role in reducing proinflammatory cytokine production by macrophages treated with intravenous Ig + LPS, as macrophages from mice deficient in the IL-10R β chain or in IL-10 were compromised in their ability to reduce proinflammatory cytokine production. Finally, intraperitoneal injection of intravenous Ig or intravenous Ig + LPS into mice activated macrophages to produce high levels of IL-10 during subsequent or concurrent LPS challenge, respectively. These findings identify IL-10 as a key anti-inflammatory mediator produced by intravenous Ig-treated macrophages and provide insight into a novel mechanism by which intravenous Ig may dampen down inflammatory responses in patients with autoimmune or autoinflammatory diseases. *J. Leukoc. Biol.* 98: 983–994; 2015.

Abbreviations: BMK1 = big MAPK1, CIHR = Canadian Institutes of Health Research, DC-SIGN = dendritic cell-specific ICAM-3-grabbing nonintegrin, Ic = immune complexes, IVIG = intravenous Ig, M(Ic) = macrophages activated by the immune complex treatment, M(IFN-γ) = macrophages activated by IFN-γ treatment, M(IFN-γ + LPS) = macrophages activated by IFN-γ + LPS treatment, M(IL) = macrophages activated by the IL treatment, M(LPS) = macrophages activated by the LPS treatment, M(TGF-β), macrophages activated by the TGF-β treatment, p = phospho

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Introduction

Macrophages are innate immune cells that play a critical role in host defense against infectious diseases contributing to inflammation during the innate immune response and directing the subsequent acquired immune response. Importantly, they participate in all stages of the inflammatory response, which include recognition, response, and resolution phases. To do so, a hallmark of macrophage biology is their "plasticity," that is, their ability to respond to cues in their local microenvironment to mount an appropriate response. As such, macrophages are a highly heterogeneous cell type [1].

Macrophage phenotype and polarization are terms that have been used to characterize the activation state of macrophages treated with exogenous agents that affect their function [2]. This has permitted comparison of macrophage responses in experimental model systems, in which untreated macrophages are compared with macrophages that have been "reprogrammed" by addition of 1 or 2 exogenous agents to mimic an in vivo environment. The best-characterized macrophages are those that have been treated with IFN-γ [M(IFN-γ)] and LPS [M(LPS)], an outer membrane component of gram-negative bacteria, or M(IFN-γ + LPS), which have distinct proinflammatory functions that are critical in host defense against invading pathogens [3]. M(IL-4) have properties consistent with their role in wound healing and tissue restitution [4]. Macrophages with several different activation states have been described and can cross-regulate one another [2]. In addition, there are many instances of macrophages having

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overlapping activation states that do not fit within a single classification [5].

It is noteworthy that macrophages have been described, which can produce large amounts of the anti-inflammatory cytokine, IL-10. Anti-inflammatory, IL-10-producing macrophages require 2 external stimuli, 1 of them being proinflammatory [5]. They can be activated by macrophage-derived TGF-β, which is secreted by macrophages after phagocytosis of apoptotic cells and a proinflammatory stimulus, M(TGF-β) [6], or by IL-10 or Ic and a proinflammatory stimulus, M(IL-10) or M(Ic) [7, 8]. These macrophages play an important role in turning off the inflammatory response [9]. They are distinct from M(IL-4), in that they do not promote the production of extracellular matrix, and do not express M(IL-4) markers, such as arginase I or found in inflammatory zone 1 [10]. The best marker for these macrophages is their ability to produce very high levels of IL-10 and very low or no proinflammatory IL-12/23p40 [11]. IL-10 is an important cytokine involved in the restoration of tissue homeostasis, as it has the ability to stop intrinsic and extrinsic inflammatory signaling from innate and adaptive immune pathways [1, 12].

Ic activate macrophages by binding to the high-affinity, activating FcγRI [9]. This leads to activation of the MAPKs, Erk1/2 and p38, both of which are required for IL-10 production by M(Ic) [13]. Erk1/2 causes phosphorylation of serine 10 on histone 3 in the *il10* promoter, opening it up for transcription, and p38 drives the transcription of *il10* [13]. It is interesting to note that treatment of macrophages with antibodies has been reported to activate them to produce high amounts of IL-10 in response to what are normally considered proinflammatory stimuli. Human macrophages treated with anti-TNF-α antibodies have been shown to produce high amounts of IL-10 in response to LPS and suppress T cell proliferation [14]. Serum, which contains high amounts of antibody, has also been used to activate macrophages with T cell-suppressive capacity via induction of iNOS in mouse macrophages and IDO expression in human macrophages [15–17].

IVIG is polyvalent IgG pooled from the blood of >1000 donors. It is used therapeutically as an adjuvant immunotherapy to supplement antibody in patients who are immune compromised or to suppress immune responses in people with autoimmune diseases, such as idiopathic thrombocytopenic purpura, or inflammatory diseases, such as chronic inflammatory demyelinating polyneuropathy [18]. The use of IVIG for off-label indications is increasing, with few controlled clinical trials or mechanistic studies to support its use [18]. The mechanism by which IVIG works to suppress autoimmune and inflammatory responses is not understood completely. IVIG can reduce autoantibody-mediated autoinflammation, as a minor fraction of sialylated Fc fragments within the pooled IgGs binds to DC-SIGN receptors on myeloid cells [19], causing up-regulation of the inhibitory FcγRIIB, which then suppresses autoantibody-mediated inflammation of inflammatory macrophages [20]. It has also been suggested that the Fab portion of the antibody may block activating FcγRs, including the neonatal receptor FcRn [21], but this does not explain the requirement for the high doses of the drug given (1–2 g/kg) [22]. Based on the high dose of IVIG antibodies required to treat autoimmune and inflammatory diseases and examples of antibody activation of IL-10-producing macrophages, we asked whether IVIG may work, in part, by activating macrophages to produce high levels of IL-10 in response to an inflammatory stimulus.

Herein, we report a new mechanism by which IVIG reduces macrophage inflammatory responses. M(IVIG) produced high levels of anti-inflammatory IL-10 and little to no proinflammatory IL-12/23p40 in response to stimulation with LPS. Unlike M(Ic), the 2 signals for activation did not need to be given simultaneously for IL-10 production to occur. Similar to M(Ic), we demonstrate that pErk1/2 and pp38 are required for IL-10 production. We extend these findings, demonstrating an additional requirement for Erk5, also known as BMK1, in the production of IL-10. We show that IL-10 production by M(IVIG) occurs rapidly and that the amount of IL-10 produced is sufficient to reduce proinflammatory cytokine production. Increased IL-10 production and decreased production of IL-12/23p40 by LPS-stimulated M(IVIG) were transcriptionally regulated. We confirm the important role for IL-10 in dampening down macrophage proinflammatory cytokine production by use of macrophages derived from 2 gene knockout mice, mice deficient in the IL-10R β chain, and mice deficient in IL-10. Finally, peritoneal macrophages from mice treated by intraperitoneal injection of IVIG produced higher levels of IL-10 ex vivo in response to LPS, and macrophages from mice treated by intraperitoneal injection of IVIG + LPS produced high levels of IL-10 and low levels of IL-12/23p40 compared with PBS or PBS + LPS injection controls, respectively. Taken together, these results demonstrate that IVIG can skew macrophages to an anti-inflammatory, IL-10-producing activation state in vitro and in vivo.

MATERIALS AND METHODS

Mice

Wild-type C57BL/6 mice were used to prepare bone marrow-derived macrophages for the majority of experiments, except where indicated. Eight-week-old male and female mice were used. Wild-type C57BL/6 mice were housed at the Child & Family Research Institute (Vancouver, BC, Canada), a barrier facility that is *Helicobacter* free and specific pathogen free. Experiments were performed in accordance with institutional and Canadian Council on Animal Care guidelines. *Fcgr1*$^{-/-}$ and *Fcgr1*$^{+/+}$ mouse femura and tibiae were obtained from Dr. Sjef Verbeek at the Leiden University Medical Center (Leiden, The Netherlands). *Fcgr2b*$^{-/-}$ mice were on a B6129SF2/J background, and *Fcgr3*$^{-/-}$ and *Fcer1g*$^{-/-}$ mice were on a C57BL/6J background. Femura and tibiae from each of these knockout mice and control strains were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). *Il10rb*$^{-/-}$ mice on a C57BL/6 background were provided by Dr. Megan Levings at the University of British Columbia (Vancouver, BC, Canada), and C57BL/6 mice were used to derive *Il10rb*$^{+/+}$ macrophages for experimental controls. *Il10*$^{-/-}$ mice on a BALB/c background and BALB/c *Il10*$^{+/+}$ control mice were maintained at the University of Alberta Animal Care Facility (Edmonton, AB, Canada).

Macrophage derivation

Bone marrow macrophages were derived from bone marrow aspirates of femura and tibiae from all mice, as described previously [23]. Following adherence depletion, bone marrow aspirates were resuspended in IMDM,

10% FBS, and penicillin/streptomycin at a concentration of 0.5×10^6 cells/ml for 10 d in the presence of 5 ng/ml M-CSF (Stemcell Technologies, Vancouver, BC, Canada), with complete media changes at d 4 and 7.

Cell stimulations

Cells were plated at a density of 1.0×10^6 cells/ml (100 μl/well in 96-well plates) and stimulated with 10 ng/ml LPS (*Escherichia coli* serotype 127:B8; Sigma-Aldrich, St. Louis, MO, USA), 30 mg/ml IVIG (or indicated concentration; Gamunex Immune Globulin Intravenous 10% solution for infusion; Transfusion Medicine, BC Children's Hospital, Vancouver, BC, Canada), or both IVIG + LPS. Cells were incubated for 24 h. After incubation, cell supernatants were harvested and clarified by centrifugation for analysis. For IVIG pretreatment studies, macrophages treated with IVIG were washed 3 times with complete medium before they were stimulated with LPS. For IVIG posttreatment studies, LPS used to stimulate macrophages was not removed from cultures before the addition of IVIG. For inhibitor studies, inhibitors were added 1 h before stimulations at final concentrations of DMSO (vehicle control; 0.1%), SB203580 (10 μM; Cell Signaling Technology, Danvers, MA, USA), BIRB796 (180 nM; Cayman Chemical, Ann Arbor, MI, USA), PD98059 (50 μM; Cell Signaling Technology), SCH772984 (1 μM; MedchemExpress, Princeton, NJ, USA), XMD8-92 (5 μM; Axon Medchem, Groningen, The Netherlands), and BIX02189 (20 μM; Axon Medchem). Mouse rIL-10 was used at a final concentration of 5 ng/ml (Affymetrix eBioscience, San Diego, CA, USA).

Cytokine measurements

Cytokines were assayed by ELISA, according to the manufacturer's instructions. ELISA kits for mouse IL-10, IL-12/23p40, IL-6, and TNF were obtained from BD Biosciences (Mississauga, ON, Canada).

SDS-PAGE and Western blotting

Macrophages were stimulated for 0, 10, 40, and 120 min; 0, 20, and 80 min; or 0, 4, 8, and 24 h, as indicated. After stimulation, macrophages were placed on ice and rinsed twice with cold PBS. Whole-cell lysates were prepared for SDS-PAGE by lysing in 1× Laemmli's digestion mix, DNA was sheered by use of a 26-gauge needle, and samples were boiled for 1 min. Cell lysates were separated on a 12% (see Fig. 4A) or 10% polyacrylamide gel (see Fig. 4E and Supplemental Fig. 2), and Western blotting was carried out, as described previously [24]. Antibodies used for Western blot analyses were anti-pErk1/2 (Cell Signaling Technology), anti-pp38 (Cell Signaling Technology), anti-pErk5 (Cell Signaling Technology), and anti-GAPDH (Fitzgerald Industries International, Acton, MA, USA).

Gene-expression analyses

RNA was prepared from mouse cells by use of the RNeasy Plus Mini Kit with DNAse I digestion (Qiagen, Toronto, ON, Canada) and reverse transcribed by use of qScript cDNA SuperMix (Quanta BioSciences, Gaithersburg, MD, USA), according to the manufacturers' instructions. Gene expression was measured by quantitative PCR by use of the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Mississauga, ON, USA). IL-10 (*Il10*) and IL-12/23p40 (*Il12b*) gene expression was normalized to gene expression for GAPDH (*Gapdh*). PrimePCR SYBR Green assay primers were obtained from Bio-Rad Laboratories. The catalog number for primers is 100-25636, with the following unique identification numbers: qMmuCID0015452 (*Il10*), qMmuCID0022424 (*Il12b*), and qMmuCED0027497 (*Gapdh*).

In vivo mouse studies

Wild-type C57BL/6 mice, 8–10 wk of age, were injected intraperitoneally with IVIG (2.5 g/kg) or an equal volume of PBS as an injection control. Peritoneal macrophages were harvested 1 h postinjection by flushing the peritoneal cavity 3× with 5 ml PBS. Cells were resuspended in IMDM, 10% FBS, and penicillin/streptomycin. Macrophages were enriched by adherence to tissue-culture plastic for 1 h [25], resuspended, and plated at density of 1.0×10^6 cells/ml (100 μl/well in 96-well plates). Cells were unstimulated or stimulated with LPS (10 ng/ml) for 24 h, and clarified cell supernatants were harvested for cytokine analyses.

Wild-type C57BL/6 mice, 8–10 wk of age, were also injected intraperitoneally with IVIG (2.5 g/kg) or an equal volume of PBS as a control, together with LPS (0.2 μg/g body weight). Peritoneal macrophages were harvested 1 h postinjection; peritoneal lavage fluid, conditioned medium from 1 h adherence, and 24 h culture supernatants were harvested; and clarified supernatants were used for cytokine analyses.

Statistical analyses

Unpaired Student's *t* tests and 1- and 2-way ANOVAs with either Tukey's or Dunnett's corrections for multiple comparison were applied as indicated. Analyses were performed by use of GraphPad Prism software, version 6.03. Differences of $P < 0.05$ were considered significant.

RESULTS

IVIG-treated macrophages produce high levels of IL-10 and low levels of IL-12/23p40, IL-6, and TNF in response to LPS

IVIG has been reported to reduce proinflammatory cytokine production by dendritic cells and macrophages, but the mechanisms for reduced proinflammatory cytokine production are not fully understood. Antibody cross-linking has also been reported to activate macrophages, known as M(Ic), which produce high levels of anti-inflammatory IL-10 in response to the proinflammatory stimulus, LPS. Based on this, we asked whether IVIG-treated macrophages produce high levels of IL-10 in response to LPS. M-CSF-derived bone marrow macrophages were stimulated with LPS, IVIG, or IVIG + LPS. LPS-stimulated macrophages produced high levels of proinflammatory IL-12/23p40, whereas IVIG alone did not induce production of these cytokines (**Fig. 1A**). IVIG + LPS suppressed IL-12/23p40 production completely at a dose of 30 mg/ml, and the effect was dose dependent (Fig. 1A). LPS treatment induced 1 ng/ml IL-10, whereas IVIG treatment did not induce IL-10 (Fig. 1B). Intriguingly, concomitant treatment with IVIG + LPS induced a significant 3.5-fold increase in IL-10 production relative to treatment with LPS alone. The effect of IVIG cotreatment on IL-10 production was also dose dependent (Fig. 1B). IVIG + LPS treatment also decreased the production of proinflammatory cytokines, IL-6 and TNF, relative to treatment with LPS alone (Fig. 1C). In keeping with current recommendations for macrophage nomenclature, we will refer to these macrophages as M(IVIG) [2].

Concomitant treatment with IVIG and LPS is not required for M(IVIG) to produce high levels of IL-10 and low levels of IL-12/23p40

Induction of IL-10-producing M(Ic) required concomitant treatment with Ic and LPS. Thus, next, we asked if the impact of IVIG on LPS-induced cytokine production required concomitant signals. Bone marrow-derived macrophages were pretreated with IVIG (30 mg/ml) for 0, 0.5, 1, 2, 4, 8, or 24 h and washed before stimulation with LPS. Macrophages were left unstimulated or were stimulated with LPS for an additional 24 h. IL-10 production was significantly higher with IVIG pretreatment compared with stimulation with LPS alone at all time points (**Fig. 2A**). Moreover,

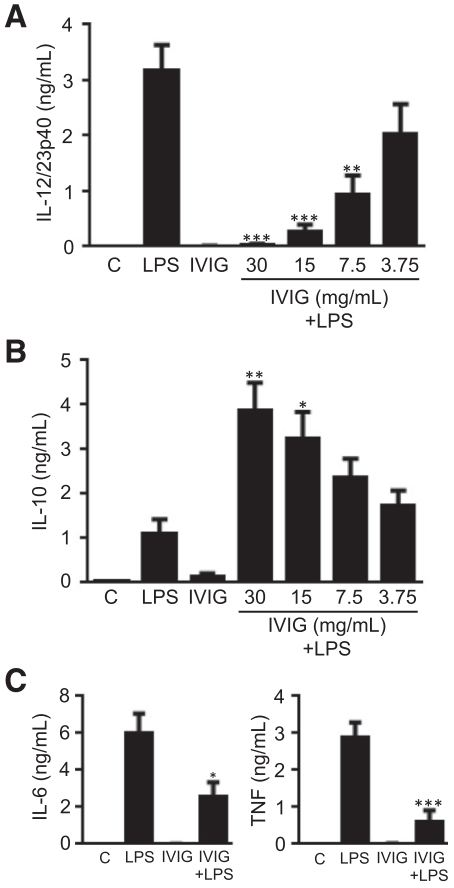

Figure 1. Macrophages costimulated with IVIG + LPS produce high levels of IL-10 and low levels of proinflammatory cytokines. M-CSF-derived bone marrow macrophages were unstimulated [control (C)] or stimulated with LPS (10 ng/ml) or IVIG (30 mg/ml) or costimulated with IVIG (at the dose indicated) and LPS (10 ng/ml) for 24 h. Clarified cell supernatants were assayed for (A) IL-12/23p40, (B) IL-10, and (C) IL-6 and TNF. Data are means ± SD of $n = 3$; macrophages were derived from 1 mouse for each of 3 independent experiments, and ELISAs were performed in duplicate. $*P < 0.05$, $**P < 0.01$, and $***P < 0.0001$ comparing IVIG + LPS-treated macrophages with macrophages treated with LPS alone. Statistical analyses were performed by use of 1-way ANOVA with Tukey's posttest for multiple comparisons.

there was no change in the IVIG-dependent increase in LPS-induced IL-10 production over time (Fig. 2A). IL-12/23p40 production in response to LPS was significantly lower at all pretreatment time points (Fig. 2B). However, the reduction of LPS-induced IL-12/23p40 production waned when IVIG treatment preceded LPS treatment by 24 h compared with other time points. We then asked if IL-10 production can be induced and IL-12/23p40 production reduced by IVIG treatment when macrophages were stimulated with LPS before IVIG treatment. Bone marrow-derived macrophages were stimulated with LPS (10 ng/ml) for 0, 0.5, 1, 2, 4, 8, or 24 h. LPS was not removed from cultures, and macrophages were left untreated or were treated with IVIG (30 mg/ml) for an additional 24 h. IL-10 production was significantly higher, and IL-12/23p40 production was reduced when IVIG treatment was provided within 1 h of LPS stimulation (Fig. 2C and D).

FcγRI, FcγIIb, or FcγIII alone is not sufficient for IVIG-induced IL-10 production or reduced IL-12/23p40 production in response to LPS

The anti-inflammatory activity of IVIG has been attributed to sialylated IgGs within IVIG binding to the DC-SIGN receptors and up-regulating FcγRIIb that binds autoimmune antibodies and inhibits immune responses [26], whereas the induction of IL-10 by M(Ic) is reported to act via FcγRI [9]. Thus, we next asked which FcγR(s) were involved in increased in IL-10 production and reduced IL-12/23p40 production by IVIG + LPS. We compared LPS and IVIG + LPS responses in macrophages deficient in FcγRI, FcγRIIb, FcγRIII, or the FcR γ chain used by FcγRI, FcγRIII, and FcγRIV and their wild-type counterparts. Wild-type *Fcgr1*$^{+/+}$ and deficient *Fcgr1*$^{-/-}$ macrophages produced similar levels and induction of IL-10 when stimulated with IVIG + LPS compared with LPS alone (**Fig. 3A**, left). IL-12/23p40 production in response to LPS was lower in *Fcgr1*$^{-/-}$ macrophages compared with their wild-type counterparts, but IL-12/23p40 production was ablated in both genotypes upon treatment with IVIG + LPS (Fig 3A, right). *Fcgr2b*$^{-/-}$ macrophages produced more IL-10 in response to LPS and IVIG + LPS than their wild-type counterparts, and correspondingly, *Fcgr2b*$^{-/-}$ macrophages produced dramatically less IL-12/23p40 in response to LPS (Fig. 3B). As a consequence, the fold induction of IL-10 in response to IVIG + LPS versus LPS alone was compromised in *Fcgr2b*$^{-/-}$ macrophages (Fig. 3B, left), but IVIG + LPS treatment effectively ablated IL-12/23p40 production by both genotypes (Fig. 3B, right). *Fcgr3*$^{+/+}$ and *Fcgr3*$^{-/-}$ macrophages produced similar levels IL-10 in response to LPS, which was induced in response to IVIG + LPS in both genotypes, although modestly higher in *Fcgr3*$^{-/-}$ macrophages (Fig. 3C, left). *Fcgr3*$^{+/+}$ and *Fcgr3*$^{-/-}$ macrophages produced similar levels of IL-12/23p40 in response to LPS, and it was reduced in IVIG + LPS-treated macrophages (Fig 3C, right). *Fcer1g*$^{+/+}$ and *Fcer1g*$^{-/-}$ macrophages produced similar levels of IL-10 in response to LPS, which was reduced in response to IVIG + LPS in both genotypes. IVIG + LPS was modestly less effective at reducing IL-12/23p40 production in *Fcer1g*$^{-/-}$ macrophages compared with their wild-type controls.

MAPK signaling is required for IVIG-induced IL-10 production in response to LPS

We next determined whether IL-10 production by IVIG + LPS-stimulated macrophages required MAPK signaling, as has been reported for M(Ic) [13]. Macrophages were unstimulated or stimulated with LPS, IVIG, or IVIG + LPS for 0, 10, 40, and 120 min; whole-cell lysates were separated by SDS-PAGE, Western blotted, and probed for pErk1/2, pp38, and GAPDH as a loading control (**Fig. 4A**). IVIG alone and macrophages stimulated with IVIG + LPS had earlier and prolonged pErk1/2 compared with LPS-stimulated macrophages, which were evident by 10 min and maintained through 120 min. LPS-stimulated macrophages had strong pp38, which peaked at 40 min, whereas macrophages stimulated with IVIG + LPS had earlier pp38, evident by 10 min and maintained through 120 min. IVIG alone induced only modest levels of pp38, whereas it induced pErk1/2 at levels similar to IVIG + LPS. No differences in pJNK were observed between macrophages stimulated with LPS or IVIG + LPS (data not shown). The impact

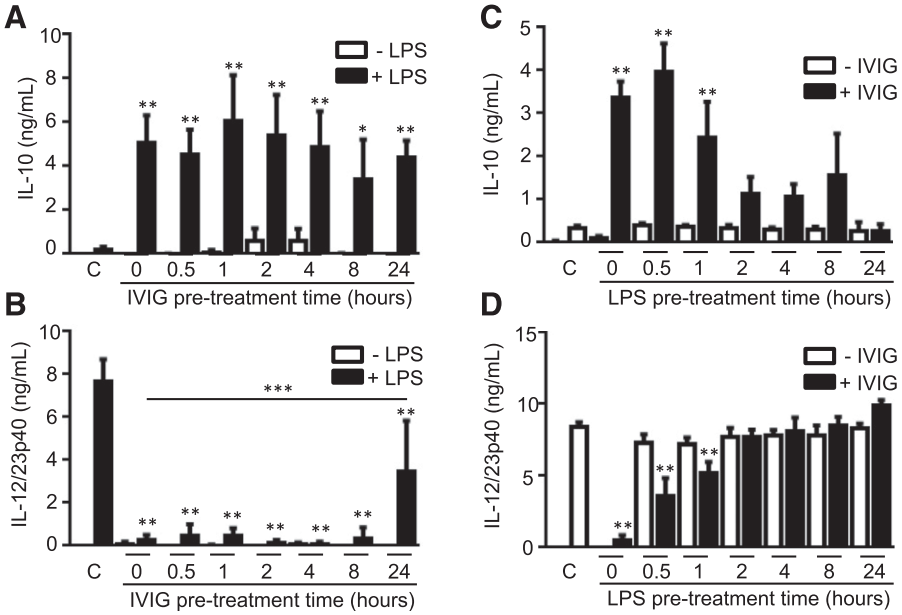

Figure 2. IVIG does not need to be provided at the same time as LPS to induce IL-10 or repress IL-12/23p40 production. M-CSF-derived bone marrow macrophages were treated with IVIG (30 mg/ml) for 0, 0.5, 1, 2, 4, 8, or 24 h; washed 3 times with complete medium after the time indicated; and then unstimulated or stimulated with LPS (10 ng/ml) for 24 h. Clarified cell supernatants were assayed for (A) IL-10 or (B) IL-12/23p40 by ELISA. M-CSF-derived bone marrow macrophages were stimulated with LPS for 0, 0.5, 1, 2, 4, 8, or 24 h and then were left untreated or treated with IVIG (30 mg/ml) for 24 h. Clarified cell supernatants were assayed for (C) IL-10 or (D) IL-12/23p40 by ELISA. Data are means ± SD for $n = 3$; macrophages were derived from 1 mouse for each of 3 independent experiments, and ELISAs were performed in duplicate. $*P < 0.001$, and $**P < 0.0001$ comparing macrophages treated with IVIG + LPS with macrophages treated with LPS alone, and $***P < 0.001$ comparing macrophages treated with IVIG + LPS with those treated with IVIG + LPS for $t = 0$ min. Statistical analyses were performed by use of 1-way ANOVA with Tukey's posttest for multiple comparisons.

of MAPK signaling on IL-10 and IL-12/23p40 production in response to IVIG + LPS was assessed by use of inhibitors. SCH772984 is a novel and specific Erk1/2 inhibitor, and PD98059 inhibits the activation of the Erk1/2 kinase, MEK1 [27]. IL-10 production was lower in macrophages stimulated with IVIG + LPS in the presence of PD98059 compared with vehicle control (Fig. 4B). The specific Erk1/2 inhibitor, SCH772984, also reduced IL-10 production in response to IVIG + LPS but was less effective (Fig. 4B). p38 inhibitors SB203580 (inhibits p38α and p38β) and BIRB796 (inhibits p38α) also significantly reduced IL-10 production in response to IVIG + LPS compared with solvent control (DMSO; Fig. 4C). The MEK1 inhibitor PD98059 was more effective in our assay, and it has been reported to inhibit Erk5 (also known as BMK1) [28]. Thus, to investigate whether the impact of PD98059 was a result of off-target effects on Erk5, we used the Erk5 inhibitor XMD8-92 and the MEK5 (Erk5 kinase) inhibitor BIX02189 in our assay. Both XMD8-92 and BIX02189 significantly decreased IL-10 production in response to IVIG + LPS relative to the vehicle control, DMSO (Fig. 4D). None of these inhibitors blocked the IVIG-induced suppression of LPS-induced IL-12/23p40 production (Supplemental Fig. 1). Finally, bone marrow-derived macrophages were stimulated with LPS or IVIG + LPS for 0, 20, and 80 min. Whole-cell lysates were separated by SDS-PAGE, Western blotted, and probed with pErk5, pErk1/2, pp38, and GAPDH as a loading control (Fig. 4E). As demonstrated previously, pErk1/2 was stronger at both 20 and 80 min, and pp38 was stronger at 20 min in IVIG + LPS-stimulated macrophages compared with those stimulated with LPS. Consistent with its role in IVIG + LPS macrophage activation, pErk5 was stronger in macrophages stimulated with IVIG + LPS compared with LPS-stimulated macrophages at 80 min. To determine whether these signaling events were maintained for 24 h, similar to the induction of IL-10 by IVIG-pretreated macrophages, bone marrow-derived macrophages were stimulated with LPS, IVIG, or IVIG + LPS for 0, 4, 8, and 24 h. Whole-cell lysates were separated by SDS-PAGE, Western blotted, and probed with pErk5, pErk1/2, pp38, and GAPDH as a loading control. pErk5, pErk1/2, and pp38 were stronger in macrophages treated with IVIG + LPS compared with LPS or IVIG stimulations alone at all time points and were still elevated above background levels at 24 h posttreatment (Supplemental Fig. 2).

IL-10 is transcriptionally up-regulated rapidly in response to IVIG + LPS and in sufficient amount to reduce IL-12/23p40 transcription and production

To determine whether IL-10 produced in response to IVIG + LPS may contribute to reduced proinflammatory cytokine production, we examined the kinetics of IL-10 and IL-12/23p40 production. Macrophages were stimulated with LPS (**Fig. 5A**, left) or IVIG + LPS (Fig. 5A, right) for 0, 0.5, 1, 2, 4, 8, or 24 h. LPS-stimulated macrophages produced high amounts of IL-12/23p40 (closed squares) that reached a maximum of 7 ng/ml by 8 h and low amounts of IL-10 that peaked at 4 h (open circles). In contrast, IVIG + LPS treatment caused a steep curve for IL-10 production that peaked at 8 h and was 7-fold higher than that produced by LPS treatment alone. Very low levels of IL-12/23p40 were produced relative to treatment with LPS alone. *Il10* and *Il12b* transcription showed similar kinetics. LPS stimulation caused induction of IL-12/23p40 mRNA in macrophages (closed squares) and very little induction of IL-10 mRNA (open circles; Fig. 5B, left). IVIG + LPS stimulation caused a dramatic spike in IL-10 mRNA levels (open circles) that peaked at 2 h after stimulation and very little induction of IL-12/23p40 mRNA (closed squares; Fig. 5B, right). To determine whether early and robust IL-10 production may contribute to reduced IL-12/23p40 production, we stimulated macrophages in the presence of mouse rIL-10. Macrophages were unstimulated or stimulated

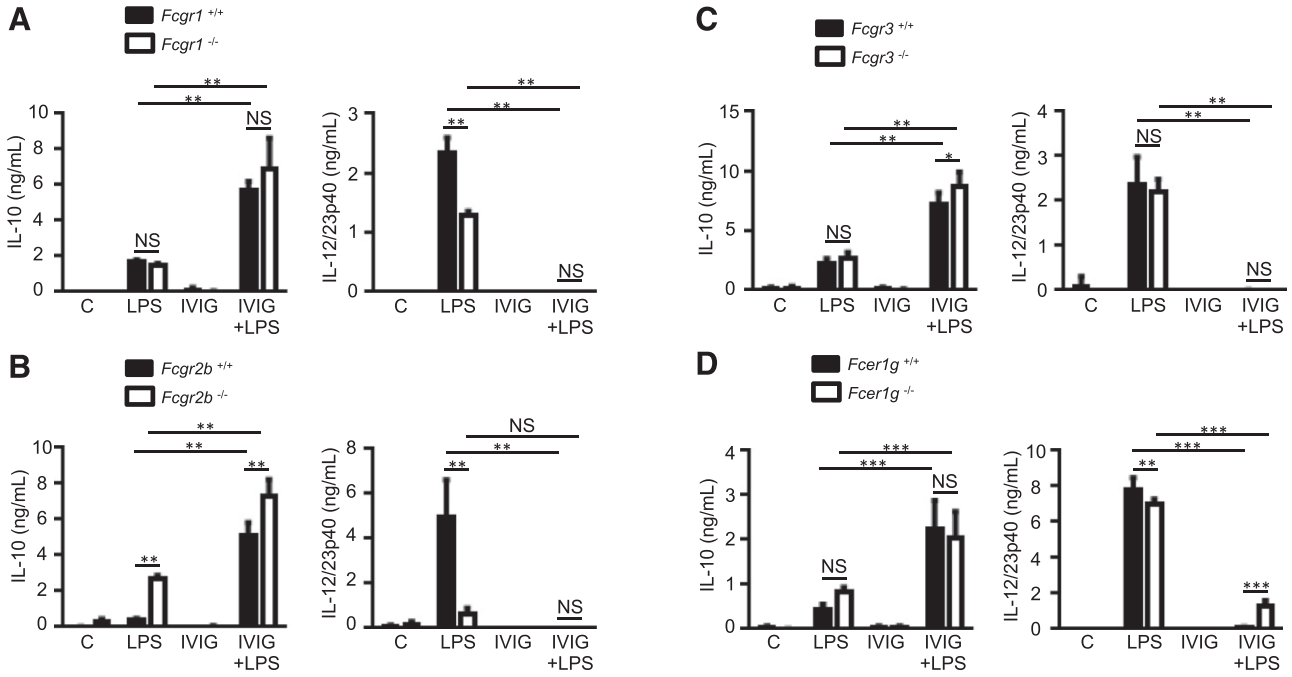

Figure 3. FcγRI, FcγIIb, or FcγIII alone is not sufficient for IVIG-induced IL-10 production or reduced IL-12/23p40 production in response to LPS. M-CSF-derived bone marrow macrophages were prepared from mice deficient in FcγR subunits and their wild-type counterparts. Macrophages were unstimulated or stimulated with LPS (10 ng/ml), IVIG (30 mg/ml), or IVIG + LPS for 24 h, and clarified cell supernatants were assayed for IL-10 and IL-12/23p40 by ELISA. (A) *Fcgr1*$^{+/+}$ and *Fcgr1*$^{-/-}$ macrophages, (B) *Fcgr2b*$^{+/+}$ and *Fcgr2b*$^{-/-}$ macrophages, (C) *Fcgr3*$^{+/+}$ and *Fcgr3*$^{-/-}$ macrophages, and (D) *Fcer1g*$^{+/+}$ and *Fcer1g*$^{-/-}$ macrophages. Data are means ± SD for $n = 3$; macrophages were derived from 1 pair of mice for each of 3 independent experiments, and ELISAs were performed in duplicate. *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$, and NS = not significantly different for comparisons as indicated. Statistical analyses were performed by use of 2-way ANOVA with Tukey's posttest for multiple comparisons.

with LPS in the presence of rIL-10 (5 ng/ml; an amount comparable with that produced by IVIG + LPS-stimulated macrophages). rIL-10 reduced IL-12/23p40 production in response to LPS (Fig. 5B).

IL-10 contributes to IVIG-induced suppression of proinflammatory cytokine production in response to LPS

To determine whether IL-10 contributes to reduced proinflammatory cytokine production in response to IVIG + LPS, we compared cytokine production in response to LPS, IVIG, or IVIG + LPS in wild-type macrophages (*Il10rb*$^{+/+}$) and macrophages deficient in the IL-10R β chain (*Il10rb*$^{-/-}$), which is required for IL-10R signaling. *Il10rb*$^{+/+}$ and *Il10rb*$^{-/-}$ bone marrow-derived macrophages produced similar levels of IL-10 in response to IVIG + LPS (**Fig. 6A**, top left), and *Il10rb*$^{-/-}$ macrophages produced more IL-12/23p40 in response to LPS (Fig. 6A, top right). Importantly, in *Il10rb*$^{-/-}$ macrophages deficient in IL-10 signaling, IVIG + LPS treatment was less effective at reducing IL-12/23p40 production compared with their wild-type counterparts (Fig. 6A, top right). IL-6 production was higher in *Il10rb*$^{-/-}$ compared with *Il10rb*$^{+/+}$ for LPS and macrophages stimulated with IVIG + LPS; however, IL-6 production was not significantly reduced by IVIG treatment in these mice (Fig. 6A, bottom left). TNF production was comparable in *Il10rb*$^{+/+}$ and *Il10rb*$^{-/-}$ macrophages in response to LPS and was reduced dramatically in wild-type macrophages but not *Il10rb*$^{-/-}$ macrophages (Fig. 6A, bottom right). To solidify a specific role for IL-10 in IVIG-induced suppression of LPS-induced proinflammatory cytokine production, we stimulated macrophages from *Il10*$^{+/+}$ and *Il10* $^{-/-}$ mice with LPS, IVIG, or LPS + IVIG. *Il10* $^{-/-}$ macrophages do not produce IL-10 (Fig. 6B, top left), whereas *Il10*$^{+/+}$ macrophages produced high levels of IL-10 in response to IVIG + LPS compared with treatment with LPS alone. IL-12/23p40 production did not differ between *Il10*$^{+/+}$ and *Il10*$^{-/-}$ macrophages in response to LPS, but it was abrogated completely in *Il10*$^{+/+}$ macrophages and only partially reduced (35%) in *Il10*$^{-/-}$ macrophages (Fig. 6B, top right). Likewise, *Il10*$^{+/+}$ macrophages had reduced IL-6 production in response to IVIG + LPS compared with LPS, but *Il10*$^{-/-}$ macrophages did not (Fig. 6B, bottom left). *Il10*$^{-/-}$ macrophages produced more TNF in response to LPS than *Il10*$^{+/+}$ macrophages. Importantly, TNF production was reduced dramatically by IVIG + LPS treatment relative to LPS treatment in *Il10*$^{+/+}$ macrophages, but IVIG-induced suppression of TNF production was compromised severely in *Il10*$^{-/-}$ macrophages (Fig. 6B, bottom right).

IVIG skews macrophages to an anti-inflammatory, IL-10-producing activation state in vivo

To determine whether IVIG could skew macrophages to an anti-inflammatory activation state in vivo, IVIG was given to mice intraperitoneally, and its effect on peritoneal macrophages was

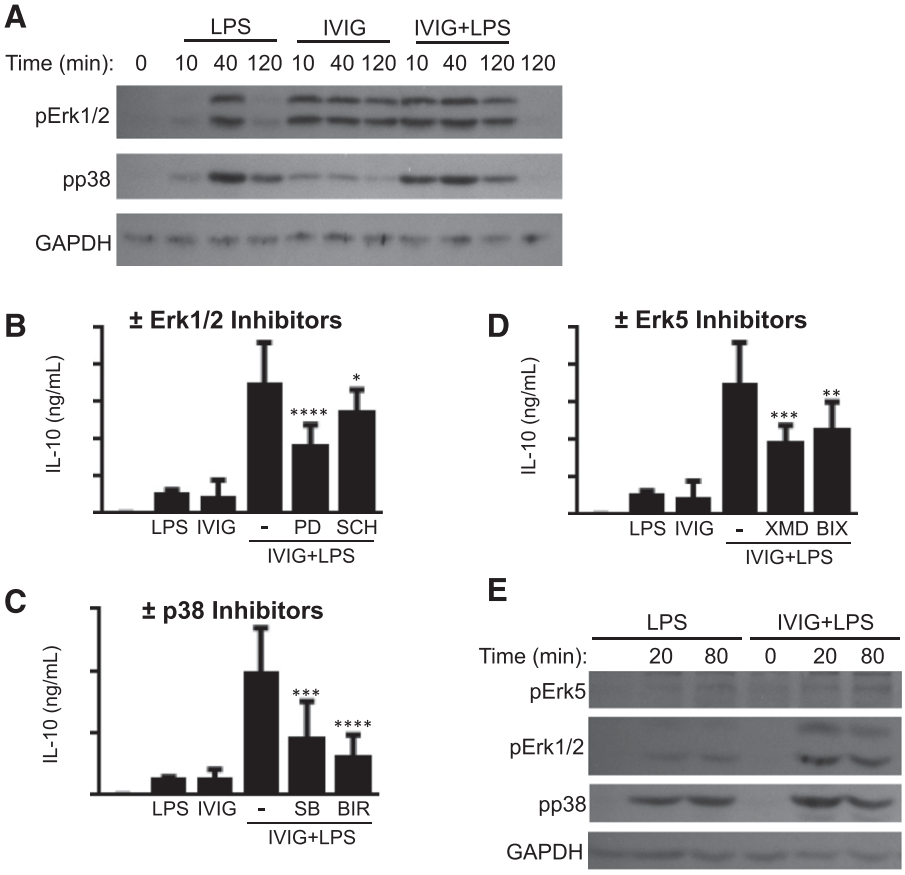

Figure 4. MAPKs are required for IVIG-induced IL-10 production in response to LPS. (A) M-CSF bone marrow-derived macrophages were unstimulated or stimulated with LPS (10 ng/ml), IVIG (30 mg/ml), or IVIG + LPS for 0, 10, 40, or 120 min. Cell lysates (1.0×10^6 cells/time point) were prepared at the indicated times. Lysates were separated by SDS-PAGE and analyzed by Western blotting by use of phosphospecific antibodies for Erk1/2, p38, and GAPDH as a loading control. Results shown are representative of $n = 3$ experiments; macrophages were derived from 1 mouse for each of 3 independent experiments. Macrophages were pretreated for 1 h with an appropriate volume of DMSO, as a vehicle control, or (B) the Erk1/2 inhibitors, PD98059 (PD) and SCH772984 (SCH); (C) p38 inhibitors, SB203580 (SB) and BIRB796 (BIR); or (D) Erk5 inhibitors, XMD8-92 (XMD) and BIX02189 (BIX), and then were left unstimulated or stimulated with LPS (10 ng/ml), IVIG (30 mg/ml), or IVIG + LPS for 24 h. Clarified cell supernatants were analyzed for IL-10 by ELISA. (B–D) Values reported are means ± SD for $n = 4$; macrophages were derived from 1 mouse for each of 4 independent experiments, and ELISAs were performed in duplicate. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ comparing macrophages treated with inhibitor and stimulated with IVIG + LPS with those treated with DMSO and stimulated with IVIG + LPS. Statistical analyses were performed by use of 1-way ANOVA with Dunnett's posttest for multiple comparisons. (E) M-CSF bone marrow-derived macrophages were unstimulated or stimulated with LPS or IVIG + LPS, Western blots were prepared as in A and probed with phosphospecific antibodies for Erk5, Erk1/2, p38, and GAPDH as a loading control. Results shown are representative of $n = 3$ experiments; macrophages were derived from 1 mouse for each of 3 independent experiments.

assessed ex vivo. First, we compared peritoneal macrophage responses with LPS in mice that were injected with IVIG or PBS. Peritoneal macrophages were harvested 1 h after injections. Peritoneal macrophages from IVIG-treated mice produced significantly more IL-10 in response to LPS than LPS-stimulated macrophages from mice treated with PBS as an injection control (**Fig. 7A**, left). Unstimulated peritoneal macrophages (no LPS) did not differ in the amount of IL-10 produced when treated in vivo with IVIG or PBS. Peritoneal macrophages from mice that received IVIG or PBS did not produce detectable levels of IL-12/23p40 (Fig. 7A, right).

In a second series of experiments, mice were injected intraperitoneally with IVIG + LPS or PBS + LPS, and peritoneal lavages were performed 1 h after injection. Peritoneal lavage fluid from mice treated with IVIG + LPS contained high levels of IL-10 compared with that harvested from mice treated with PBS + LPS (Fig. 7B, left). IL-12/23p40 levels were not significantly lower in the lavage fluid of mice treated with IVIG + LPS compared with those treated with PBS + LPS (Fig. 7B, right). Conditioned medium from peritoneal lavage cells cultured for 1 h to select macrophages by adherence was also assayed for IL-10 and IL-12/23p40. Conditioned medium from peritoneal cells harvested by lavage from mice treated with IVIG + LPS had higher levels of IL-10 compared with that from controls injected with PBS + LPS, with neither producing detectable levels of IL-12/23p40 (Fig. 7C). Peritoneal macrophages isolated from mice injected with IVIG + LPS produced 5-fold more IL-10 and significantly lower IL-12/23p40 after 24 h in culture compared with macrophages that were isolated from mice injected with PBS + LPS (Fig. 7D).

DISCUSSION

Herein, we report a novel mechanism by which IVIG inhibits inflammation. IVIG activates macrophages to produce large amounts of IL-10 in response to a proinflammatory stimulus, LPS. The IL-10 produced then acts in an autocrine fashion and contributes to reduced IL-12/23p40, IL-6, and TNF production by the macrophages. The 2 signals, IVIG and LPS, did not need to be given simultaneously. Increased IL-10 production required MAPKs: Erk1/2, p38, and Erk5 were all required for the induction of IL-10 downstream of IVIG. IL-10 production occurred rapidly in response to IVIG at a level that was sufficient to impact IL-12/23p40 production. Moreover, suppression of the production of proinflammatory cytokines IL-12/23p40, IL-6, and TNF by IVIG was compromised in macrophages from *Il10rb*$^{-/-}$ or *Il10*$^{-/-}$ mice relative to

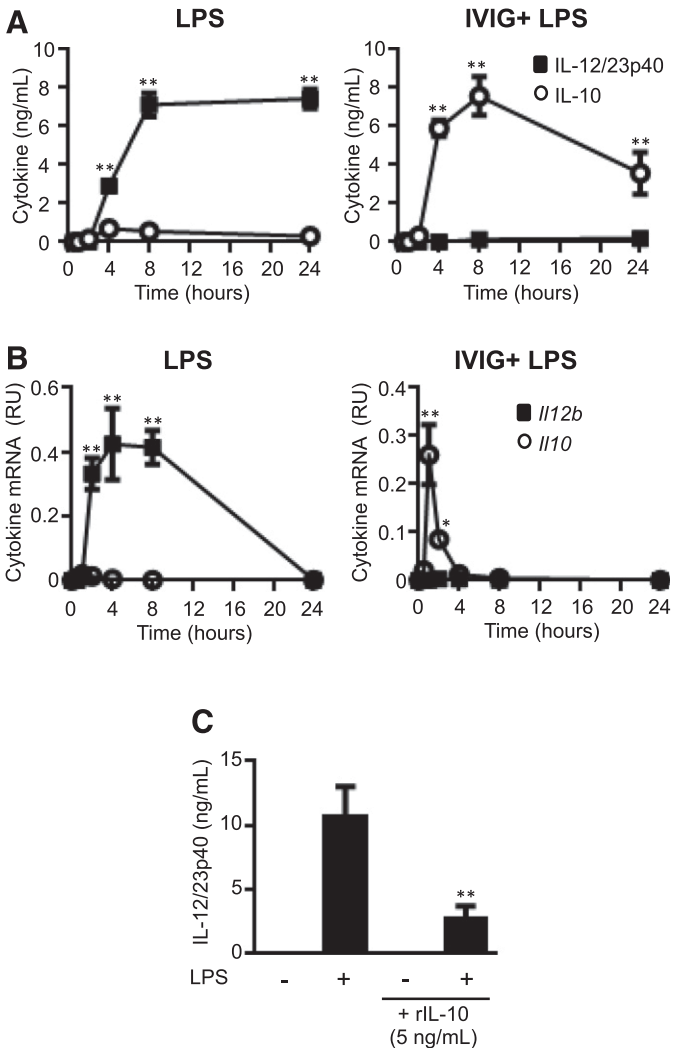

Figure 5. IL-10 is produced early in response to treatment with IVIG + LPS, and the amount produced is sufficient to inhibit IL-12/23p40 production in response to LPS stimulation. (A) M-CSF-derived bone marrow macrophages were stimulated with LPS (10 ng/ml) or IVIG (30 mg/ml) + LPS (10 ng/ml) for 0, 0.5, 1, 2, 4, 8, or 24 h. Clarified cell supernatants were collected and assayed at each time point for IL-10 and IL-12/23p40 by ELISA. (B) M-CSF-derived bone marrow macrophages were stimulated with LPS (10 ng/ml) or IVIG (30 mg/ml) + LPS (10 ng/ml) for 0, 0.5, 1, 2, 4, 8, or 24 h. Abundance of *Il10* or *Il12b* mRNA relative to *Gapdh* was analyzed by quantitative PCR. Data represent means ± SD for $n = 3$; macrophages were derived from 1 mouse for each of 3 independent experiments; ELISAs were assayed in duplicate (A), and quantitative PCR was assayed in triplicate (B). $*P < 0.001$, and $**P < 0.0001$ for treatment with IVIG + LPS compared with LPS alone. Statistical analyses were performed by use of 1-way ANOVA with Tukey's posttest for multiple comparisons. RU, Relative units. (C) Macrophages were unstimulated or stimulated with LPS (10 ng/ml), rIL-10 (5 ng/ml), or rIL-10 (5 ng/ml) + LPS (10 ng/ml) for 24 h. IL-12/23p40 production was assayed in clarified cell supernatants by ELISA. Data represent means ± SD for $n = 3$; macrophages were derived from 1 mouse for each of 3 independent experiments, and ELISAs were assayed in duplicate. $**P < 0.0001$ for treatment compared with LPS stimulation. Statistical analysis was performed by use of 1-way ANOVA.

macrophages from their wild-type counterparts, demonstrating a critical role of IL-10 and IL-10 signaling in the anti-inflammatory activity of IVIG. Tissue resident peritoneal macrophages primed with IVIG in vivo produced high levels of IL-10 when stimulated with LPS in vitro. Moreover, mice given IVIG + LPS by intraperitoneal injection produced high levels of IL-10 and low levels of IL-12/23p40, which was evident in lavage fluid and cultured peritoneal macrophages, demonstrating the potent effect of IVIG on macrophage activation in vivo.

Induction of IL-10 by macrophages treated with IVIG + LPS, M(IVIG), was dependent on the dose of IVIG used, with higher doses of IVIG leading to higher IL-10 production. This is consistent with a model, wherein if antibody produced by the acquired immune system is present in sufficient excess, then it can feed back and inactivate the innate immune response. Similar to our data, for macrophages activated by Ic + LPS (M(Ic)), i.e., SRBCs coated with IgG, a density threshold of IgG on SRBCs was required to permit sufficient crosslinking of FcγRs for robust IL-10 production [29]. IVIG may act in a similar fashion and require FcγR crosslinking for its activity. Receptor crosslinking may occur, as a portion of the IgGs within IVIG preparations exists as dimers or multimers. Alternatively, receptor crosslinking may be enabled at high doses of IVIG by saturation of cell-surface FcγRs, which is sufficient to induce cell signaling, as for IgE [30]. The molecular events that lead to IVIG-induced FcγR signaling remain to be determined, but in either case, our results do account for the high dose of IVIG (25–35 mg/ml) that is required to suppress inflammation in patients with autoimmune or inflammatory diseases [31, 32].

M(IVIG) also produced less IL-12/23p40, a cytokine subunit shared by both IL-12 and IL-23, as well as less TNF and IL-6. These cytokines are key inflammatory mediators: IL-12 is a potent inducer of proinflammatory IFN-γ, a mediator of antibacterial immunity [33]; IL-23 enhances the expansion of Th17 cells in autoimmune inflammatory diseases [34, 35]; TNF causes fever and inflammation and is elevated in inflammatory diseases, such as inflammatory bowel disease and rheumatoid arthritis [36]; and IL-6 is also proinflammatory, driving the differentiation of $CD4^+$ T cells toward Th2 and Th17 cells [37]. Reduction of proinflammatory cytokine production by M(IVIG) was profound and may be unique to IVIG treatment. M(Ic) produce low levels of IL-12/23p40, but TNF production is not affected by Ic activation [11]. Human macrophages treated with anti-TNF-α antibodies + LPS and cultured with activated allogeneic T cells also produce high amounts of IL-10, but their production of the proinflammatory cytokines TNF-α and IL-1β is not reduced [14].

Another important distinction between M(IVIG) and M(Ic) is that IVIG and LPS did not need to be given simultaneously to activate macrophages to produce high IL-10 and low IL-12/23p40 levels. M(Ic) are somewhat unique among macrophage activation states in that Ic do not reprogram macrophages, which are then assessed by an inflammatory insult. Rather, M(Ic) activation is achieved by providing Ic and LPS simultaneously [11]. To compare M(IVIG) with M(Ic), we provided IVIG to macrophages for various times up to 24 h before LPS

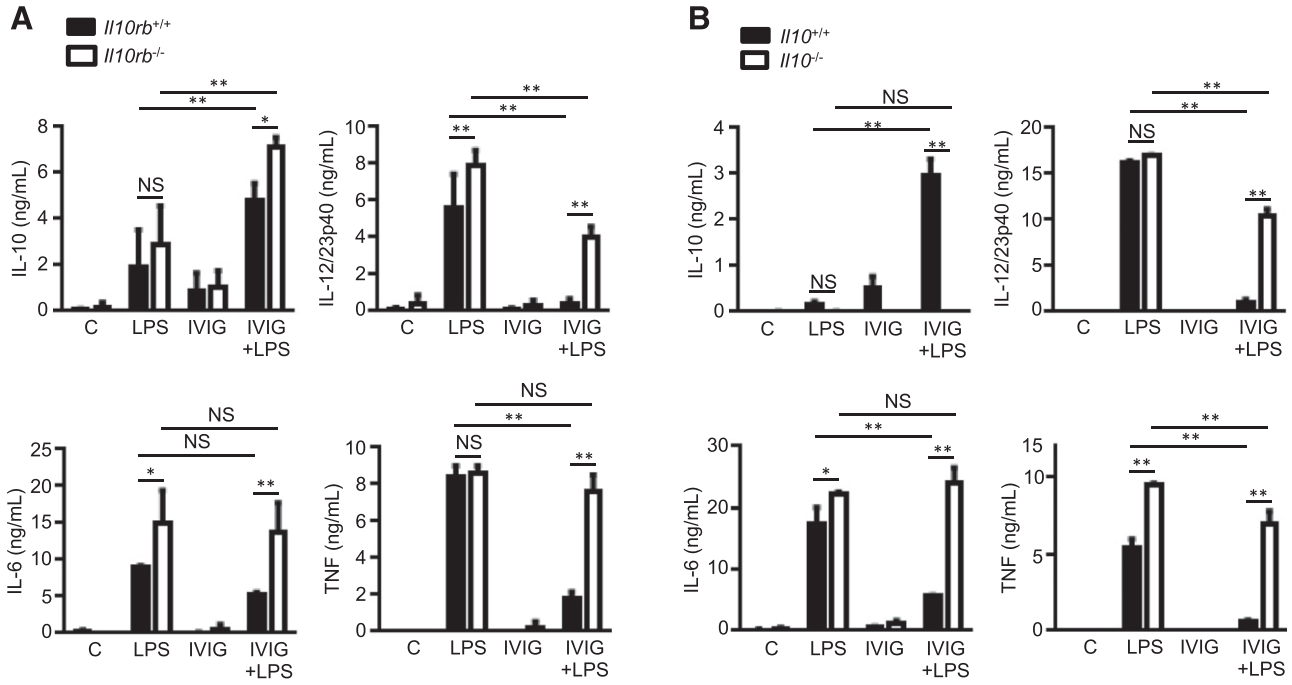

Figure 6. IL-10 produced in response to IVIG + LPS contributes to reduced proinflammatory cytokine production. M-CSF-derived bone marrow macrophages from *Il10rb*$^{+/+}$ and *Il10rb*$^{-/-}$ mice (A) or *Il10*$^{+/+}$ and *Il10*$^{-/-}$ mice (B) were unstimulated or stimulated with LPS (10 ng/ml), IVIG (30 mg/ml), or IVIG + LPS for 24 h. Clarified cell supernatants were assayed for IL-10, IL-12/23p40, IL-6, or TNF by ELISA. Data are means ± SD for $n = 3$; macrophages were derived from 1 pair of mice for each of 3 independent experiments, and ELISAs were assayed in duplicate. *$P < 0.01$, **$P < 0.001$, and NS = not significantly different for the comparisons indicated. Statistical analyses were performed by use of 2-way ANOVA with Tukey's posttest for multiple comparisons.

stimulation. We found that IL-10 production, in response to LPS, remained high even 24 h after IVIG treatment: reduced IL-12/23p40 was also evident, although the impact of IVIG waned at the 24 h time point. This is consistent with sustained elevation of phosphorylation of MAPKs, observed 24 h after IVIG + LPS treatment. We also found that IVIG induced high IL-10 production and prevented IL-12/23p40 production if provided within 1 h of LPS stimulation. This could have implications for the timing and use of IVIG for the treatment of septic shock, where it has been shown to reduce mortality rates [38]. To our knowledge, this is the first demonstration of a sustained, high IL-10 and low/no IL-12/23p40-producing activation state in macrophages.

The effect of IVIG on macrophages has been attributed to signaling through the inhibitory receptor FcγRIIb [39] or by the activating receptor FcγRIII [40]. In addition, FcγRI has been implicated in IL-10 production by M(Ic), as IL-10 production was lost in macrophages deficient in the FcR γ chain (required for signaling though FcγRI, FcγIII, and FcγIV) but not in FcγRII- or FcγIII-deficient macrophages [9]. Ours is the first direct test of the role of FcγRI in enhanced IL-10 production by macrophages, as FcγRI knockout mice were not available when M(Ic) were first described [9]. Our data suggest that FcγRI, FcγRIIb, or FcγRIII is not sufficient for IL-10 induction in response to IVIG + LPS or decreased IL-12/23p40 in response to IVIG + LPS. However, the induction of IL-10 in response to IVIG + LPS compared with IVIG was compromised in FcγRIIb$^{-/-}$ macrophages because of higher IL-10 production in response to LPS alone. This correlated with reduced IL-12/23p40 production in response to LPS stimulation, which was reduced further and inversely correlated with higher absolute levels of IL-10 produced in response to IVIG + LPS treatment. Our data demonstrating that FcR γ chain signaling was not sufficient for IVIG induction of IL-10 further support a role for FcγRIIb in this process. Alternatively, multiple FcγRs could be involved in IL-10 induction and IL-12/23p40 suppression in response to IVIG + LPS, as a result of overlapping activity, and/or there could compensatory effects of the FcγR deficiencies in the single-gene knockout mice. For example, *Fcgr2b*$^{-/-}$ and *Fcgr3*$^{-/-}$ mice have increased amounts of FcγRIVs on their cell surface [42].

We found that MAPKs were required for IL-10 production by M(IVIG). Erk1/2 and p38 activation occurs earlier, stronger, and is prolonged in macrophages activated by IVIG + LPS compared with LPS-activated macrophages. FcγRI signaling in M(Ic) requires Erk1/2 activation, which leads to chromatin modifications opening up the IL-10 promoter and p38 activation, which drives transcription of IL-10 [13, 29]. Our data support that model in that IVIG + LPS and IVIG are both strong activators of Erk1/2 (IVIG-priming cells for IL-10 production), and IVIG + LPS and LPS are strong activators of p38 (permitting promoter-dependent transcription of IL-10). Indeed, more IL-10 is produced by LPS-stimulated macrophages than dendritic cells, as a result of increased strength of

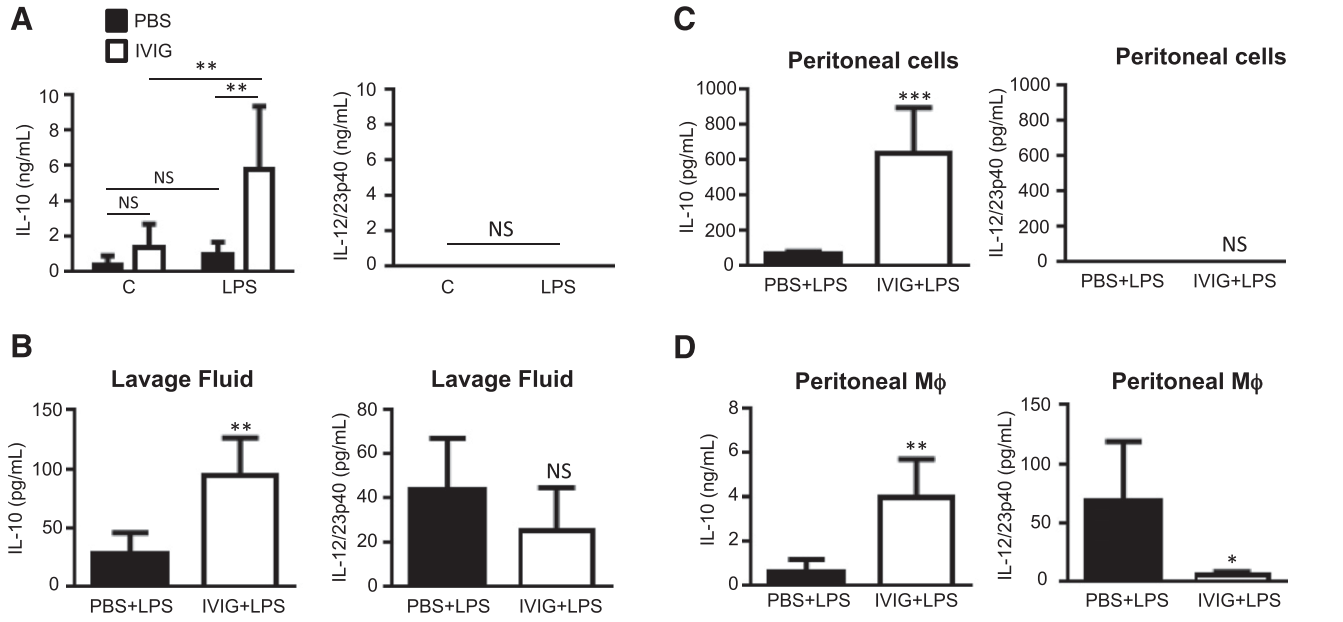

Figure 7. IVIG skews macrophages to an anti-inflammatory, IL-10-producing activation state in vivo. (A) Wild-type C57BL/6 mice were given IVIG (2.5 g/kg) or an equal volume of sterile PBS intraperitoneally, and peritoneal macrophages were isolated after 1 h. Macrophages enriched by adherence to tissue-culture plastic were unstimulated or stimulated with LPS (10 ng/ml) for 24 h. Clarified cell supernatants were assayed for IL-10 and IL-12/23p40 by ELISA. (B–D) Wild-type C57BL/6 mice were given IVIG (2.5 g/kg) + LPS (0.2 μg/g body weight) or an equal volume of PBS + LPS (0.2 μg/g body weight). Mice were euthanized for peritoneal lavage after 1 h and enriched for macrophages by adherence to tissue-culture plastic for 1 h. Clarified peritoneal lavage fluid (B), clarified conditioned medium from 1 h adherence step (C), and clarified conditioned medium from 24 h macrophage (Mϕ) cultures (D) were assayed for IL-10 and IL-12/23p40 by ELISA. (A) Data are means ± SD for $n = 5$ mice/group treated in 3 independent experiments with ELISAs assayed in duplicate. (B–D) Data are means ± SD for $n = 5$ mice/group treated in 3 independent experiments with ELISAs assayed in duplicate. (A) $^{**}P < 0.01$, and NS = not significantly different for the comparisons indicated. (B–D) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and NS = not significantly different for mice injected with IVIG + LPS compared with mice injected with PBS + LPS. Statistical analyses were performed by use of 2-way ANOVA with Sidak's posttest for multiple comparisons for (A) and by use of Student's *t* test for (B–D).

Erk activation [42]. Activation of MAPKs was still evident 24 h after IVIG + LPS stimulation, which may account for to the longevity of the effect. In addition, for M(Ic), pErk1/2 has been reported to lead to phosphorylation of the serine 10 residue on histone 3 of the *il10* promoter, which opens up the promoter for transcription and may also be a long-lived modification [13]. Erk1/2 inhibitors, SCH772984 and PD98059, and p38 inhibitors, SCH772984 and PD98059, reduced IL-10 production by M(IVIG). PD98059 reduced IL-10 more effectively than SCH772984, which could be attributed to its off-target effects on Erk5 [29]. The potent and selective Erk5 inhibitor, XMD8-92, and BIX02189, a MEK5 inhibitor [43], also effectively decreased IL-10 by M(IVIG). In macrophages, pErk5 was also stronger and prolonged after IVIG + LPS stimulation, mirroring the activation patterns of the other MAPKs. This provides evidence that Erk5 can have an anti-inflammatory role in macrophages, in addition to its inflammatory effects [44]. Interestingly, the combination of Erk1/2, p38, and Erk5 inhibition is able to reduce IL-10 to very low levels and results in production of IL-12/23p40 that is comparable with LPS-stimulated macrophages (data not shown).

Our results suggest that IL-10 production by M(IVIG) contributes to reduced proinflammatory cytokine production. IL-10 production by M(IVIG) occurs rapidly and at sufficient levels to reduce IL-12/23p40 production, and IL-10-induced reduction of proinflammatory cytokine production in response to proinflammatory stimuli, including IL-6, TNF-α, and IL-1β, has been reported previously [45]. Consistent with activation of MAPKs leading to increased transcription of *Il10*, IL-10 mRNA was rapidly up-regulated in macrophages stimulated with IVIG + LPS, 1 h poststimulation. Reduced IL-12/23p40 production also correlated with reduced transcription of *Il12b*, which is induced only after *Il10* transcription and may be dampened by production of IL-10 [14]. In addition, we have used 2 independent genetic models to demonstrate that IL-10 is required for reduced proinflammatory cytokine production by M(IVIG). First, macrophages from mice deficient in the IL-10R β subunit ($Il10rb^{-/-}$), which cannot signal in response to IL-10, produced more IL-12/23p40, IL-6, and TNF when stimulated with IVIG + LPS compared with their wild-type littermates. Interestingly, IL-10 production was also higher in $Il10rb^{-/-}$ versus $Il10rb^{+/+}$, which can be attributed to loss of the IL-10 feedback mechanism that IL-10 uses to regulate its own production [42]. Second, macrophages from mice deficient in IL-10 itself ($Il10^{-/-}$) also produced higher levels of IL12-23/p40, IL-6, and TNF in response to IVIG + LPS stimulation when compared with their wild-type littermates. It is interesting to note that loss of IL-10 or IL-10 signaling did not abrogate the effects of IVIG on macrophage proinflammatory cytokine production, suggesting that reduced proinflammatory

cytokine production by M(IVIG) also occurs by 1 or more IL-10-independent mechanisms. One mechanism may be a direct effect of IVIG on IL-12/23p40 production, reported to occur downstream of the inhibitory FcγRIIb, which is upregulated by specific sialylated antibodies within IVIG preparations binding to DC-SIGN [26, 39]. IL-10 is more effective at reducing IL-12/23p40 production than production of IL-6 or TNF. The potency of its efficacy may account for the failure to note significant differences for other proinflammatory cytokine production by M(Ic), which also produce IL-10. Conditioned medium from M(Ic) is able to reduce IL-12p70 production completely when added back to macrophages treated with IFN-γ + LPS, and anti-IL-10-blocking antibodies abrogate the effect of conditioned medium [9]. Alternatively, this may also indicate other soluble factors produced by M(Ic) and by M(IVIG), contribute to reduction of IL-12/23p40 production.

Our data also demonstrate the potent ability of IVIG to skew macrophages to an anti-inflammatory, IL-10-producing activation state in vivo. First, murine peritoneal macrophages primed with IVIG in vivo were harvested and stimulated with LPS ex vivo. IVIG-primed macrophages produced high amounts of IL-10 in response to LPS compared with those from mice primed with PBS as a control. This is consistent with the ability of cultured peritoneal macrophages activated with Ic, M(Ic), and stimulated with LPS to produce high levels of IL-10 [25]. However, we found that these peritoneal macrophages did not produce IL-12/23p40. Thus, in a second series of experiments, we injected mice intraperitoneally with IVIG + LPS or PBS + LPS as a control and measured IL-10 and IL-12/23p40 present in lavage fluid, 1 h conditioned medium from cells present in peritoneal lavage (while enriching for macrophages by adherence), and 24 h conditioned medium from peritoneal macrophages. IVIG + LPS injection caused higher production of IL-10 than PBS + LPS injection in peritoneal lavage fluid and in media conditioned by peritoneal cells and enriched macrophages. Peritoneal macrophages from mice injected with IVIG + LPS also produced significantly lower amounts of IL-12/23p40 in 24 h compared with those from mice injected with PBS + LPS. These data are consistent with our in vitro observations, in that IL-10 was produced rapidly in response to IVIG + LPS and reduced subsequent IL-12/23p40 production and demonstrate that IVIG can induce macrophage IL-10 production in vivo and dampen down macrophage inflammatory responses to LPS. This is consistent with a previous report that demonstrated that IgG + LPS increased IL-10 and reduced IL-12/23p40 levels in plasma of $RAG1^{-/-}$ mice (deficient in mature T and B cells) compared with mice treated with LPS alone [9]. Current thinking is that reduction of mortality rates in clinical studies when polyclonal IVIG is given therapeutically to treat sepsis is a result of the presence of antibodies directed against bacterial or cytokine antigens [46]. However, increased production of anti-inflammatory IL-10 and reduced proinflammatory cytokine production by macrophages could also contribute to reduced mortality in sepsis [38, 46].

In conclusion, macrophages treated with IVIG not only reduce production of proinflammatory cytokines in response to inflammatory stimuli but also produce high levels of the anti-inflammatory cytokine IL-10, which is critical for turning off the inflammatory signaling, both innate and adaptive immune responses, and restoring tissue homeostasis, particularly at mucosal sites. Despite that, IL-10 has not proven to be effective at reducing inflammation, likely because its half-life in vivo is not sufficient for it to be effectively delivered to inflamed tissues [47]. IVIG treatment may work as an effective strategy to block inflammation, as it can cause production of IL-10 at the site of inflammation, as IL-10 production actually requires a second proinflammatory signal, such as LPS. The data described herein show a completely novel mechanism, by which IVIG dampens down proinflammatory cytokine production by macrophages and which has the potential to affect acquired immune responses. IVIG signaling in mouse macrophages activates Erk1/2, p38, and Erk5 MAPKs to drive production of high amounts of IL-10 in response to LPS, which then reduces production of the proinflammatory cytokines IL-12/23p40, IL-6, and TNF. IVIG can induce high IL-10 production in peritoneal macrophages in vitro and in vivo, as well as block IL-12 production in response to LPS in vivo, which could explain its efficacy as an anti-inflammatory in humans. FcγRs are not identical in mice and humans, and ongoing studies in our laboratory are investigating IL-10 production by human M(IVIG). Thus, this work defines a novel mechanism of action for IVIG, which may contribute to its efficacy as a treatment for patients with autoimmune and autoinflammatory diseases.

AUTHORSHIP

L.K.K., G.S.D.R., K.W., J.H., K.L.M., and L.M.S. conceived of the study and the experiments. L.K.K., Z.Y.Z., S.C.M., M.F., N.H., and L.M.S. designed and performed the experiments. L.M.S. supervised all aspects of the study. L.K.K. and L.M.S. prepared and wrote the manuscript with contributions from all authors.

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DISCLOSURES

The authors declare no conflict of interest.

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Intravenous immunoglobulin skews macrophages to an anti-inflammatory, IL-10-producing activation state

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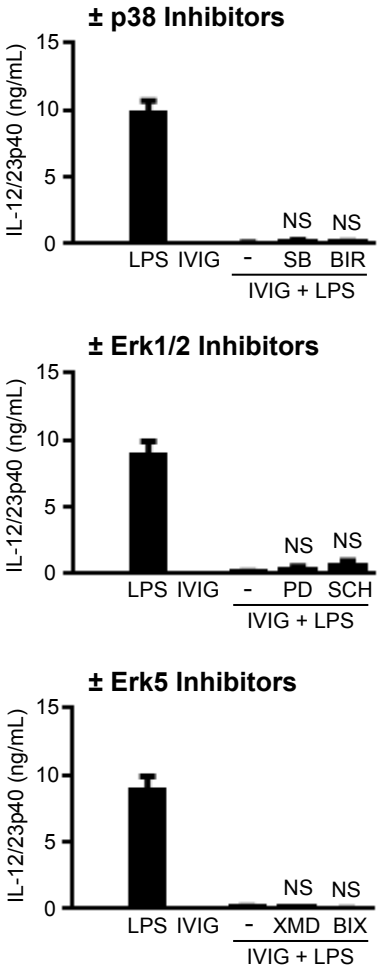
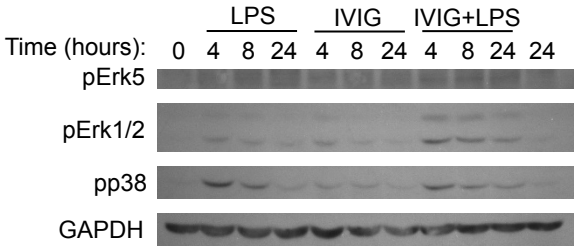


Supplemental Figure 1. MAPK inhibition did not block IVIG-induced suppression of IL-12/23p40 production in response to IVG+LPS. MCSF-derived bone marrow macrophages were treated for 1 h with DMSO, as a vehicle control, or p38 inhibitors, SB203580 (SB) and BIRB796 (BIR) (top graph); Erk1/2 inhibitors, PD98059 (PD) and SCH772984 (SCH) (middle graph); or Erk5 inhibitors, XMD8-92 (XMD) and BIX02189 (BIX) (bottom graph). Macrophages were unstimulated or stimulated with LPS (10 ng/mL), IVIG (30 mg/mL), or IVIG+LPS for 24 h. Clarified cell supernatants were assayed for IL-10 by ELISA. Data are means ± SD for n = 4; macrophages were derived from 1 mouse for each of 4 independent experiments and ELISAs were assayed in duplicate. NS = not significantly different, comparing inhibitor treatments to DMSO control. Statistical analyses were performed using one-way ANOVA with Dunnett's post-test for multiple comparisons.



Supplemental Figure 2. Activation of MAPKs by IVIG+LPS is sustained for 24 h. MCSF bone marrow-derived macrophages were either unstimulated or stimulated with LPS (10 ng/mL), IVIG (30 mg/mL), or IVIG+LPS for 0, 4, 8, or 24 h. Cell lysates (1.0×10^6 cells/time point) were prepared at the indicated times. Lysates were separated by SDS-PAGE and analyzed by Western blotting using phospho-specific antibodies for Erk5, Erk1/2, p38, and GAPDH, as a loading control. Results shown are representative of n = 3 experiments; macrophages were derived from 1 mouse for each of 3 independent experiments.