Reversal of Proinflammatory Responses by Ligating the Macrophage Fcγ Receptor Type I

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Summary

Macrophages can respond to a variety of infectious and/or inflammatory stimuli by secreting an array of proinflammatory cytokines, the overproduction of which can result in shock or even death. In this report, we demonstrate that ligation of macrophage Fcγ receptors (FcγR) can lead to a reversal of macrophage proinflammatory responses by inducing an upregulation of interleukin (IL)-10, with a reciprocal inhibition of IL-12 production. IL-10 upregulation was specific to FcγR ligation, since the ligation of the Mac-1 receptor did not alter IL-10 production. The identification of the specific FcγR subtype responsible for IL-10 upregulation was determined in gene knockout mice. Macrophages from mice lacking the FcR γ chain, which is required for assembly and signaling by FcγRI and FcγRIII, failed to upregulate IL-10 in response to immune complexes. However, mice lacking either the FcγRII or the FcγRIII were fully capable of upregulating IL-10 production, implicating FcγRI in this process. The biological consequences of FcγRI ligation were determined in both in vitro and in vivo models of inflammation and sepsis. In all of the models tested, the ligation of FcγR promoted the production of IL-10 and inhibited the secretion of IL-12. This reciprocal alteration in the pattern of macrophage cytokine production illustrates a potentially important role for FcγR-mediated clearance in suppressing macrophage proinflammatory responses.

Key words: CD64 • macrophage • interleukin 10 • inflammation • Fc receptors

Macrophages are prodigious secretory cells which can produce a number of molecules that can either potentiate or dampen immune responses (1). In response to infectious or inflammatory stimuli, macrophages can produce several proinflammatory molecules, including IL-12, TNF-α, IL-6, and IL-1 (1, 2). These proinflammatory molecules are important for host defense, because experimentally infected animals deficient in these cytokines are more susceptible to acute bacterial infections than are normal animals (3, 4). However, the production of proinflammatory cytokines must be tightly regulated, since their production is also correlated with many of the pathologies associated with acute sepsis or with autoimmune diseases. Macrophages themselves can participate in this regulation by the production of antiinflammatory molecules. The secretion of prostaglandins, TGF-β, and IL-10 by macrophages has been associated with antiinflammatory responses. Thus, the balance between the secretion of pro- and antiinflammatory molecules by macrophages is a critical component of the acute phase response and has the potential to affect the adaptive immune response that subsequently develops.

IL-10 has been associated with the inhibition of Th1-type immune responses. IL-10 has been shown to inhibit the production of Th1 cytokines and to decrease the proliferation of Th1 cells to antigen (5, 6). The administration of exogenous IL-10 can diminish the toxicity of LPS (7). IL-10 has macrophage-deactivating effects and can inhibit the production of IL-12 by macrophages (8, 9). It is now well established that IL-12 plays an important role in the development of Th1-type immune responses (2). This cytokine is a potent inducer of IFN-γ from T and NK cells, and has been shown to play a crucial role in the development of immunity to intracellular pathogens (10, 11).

In this study, we examine the production of IL-10 and IL-12 by macrophages and the influence that phagocytic receptor ligation can exert on this production. We demonstrate that the ligation of FcγRI can enhance the production of IL-10, reversing the proinflammatory response of macrophages to stimuli such as bacteria or bacterial products.

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Materials and Methods

Mice and Macrophages. 6–8-wk-old BALB/c and C57BL/6 mice were obtained from Taconic Farms, Inc. (Germantown, NY). FcR γ chain–deficient (FcR$\gamma^{-/-}$) and FcγRII$^{-/-}$ mice (12, 13) were provided by Dr. Jeffrey Ravetch (The Rockefeller University, New York). FcγRIII$^{-/-}$ mice (14) were provided by Dr. J. Sjef Verbeek (University Hospital Utrecht, Utrecht, The Netherlands). Bone marrow–derived macrophages (BMMϕ) were established as described previously (15).

Opsonized Erythrocytes. IgG-opsonized sheep erythrocytes (E-IgG) were generated by incubating SRBC (Lampire Biological Laboratories, Pipersville, PA) with rabbit anti-SRBC IgG (Organon Teknika-Cappel, Durham, NC) at nonagglutinating titers for 40 min at room temperature. E-IgG were washed and resuspended in HBSS (GIBCO BRL, Gaithersburg, MD) before their addition to macrophages. Complement-opsonized erythrocytes (E-C3bi) were generated by incubating SRBC with culture supernatants of hybridoma S-S.3 (anti-SRBC IgM/κ; American Type Culture Collection, Rockville, MD) at nonagglutinating titers for 40 min at room temperature. IgM-opsonized erythrocytes were washed twice with HBSS and resuspended at 10^8 cells/ml in HBSS with 10% murine C5–deficient serum. After a 15-min incubation at 37°C, E-C3bi were washed and resuspended in HBSS before their addition to macrophages. Erythrocytes were added to macrophage monolayers at a ratio of 20:1.

Macrophage Stimulation. BMMϕ monolayers were stimulated with LPS (*Escherichia coli* 0127:B8; Sigma Chemical Co., St. Louis, MO) at a final concentration of 100 ng/ml, in the presence or absence of opsonized erythrocytes. Cytokine levels in cell supernatants were measured by ELISA 24 h after the addition of stimuli. For mRNA analysis, cells were harvested 6 h after the addition of stimuli, and cytokine mRNA levels were determined by reverse transcription (RT)-PCR, as described previously (15). In some instances, macrophages were stimulated with heat-killed bacteria. The *Eagan* clinical isolate of type b *Haemophilus influenzae* has been described and characterized previously (16). Organisms were grown for 3 h at 37°C in brain–heart infusion broth (Difco Laboratories Inc., Detroit, MI) supplemented with NAD and hemin and then washed twice in HBSS. Bacteria were heat killed by incubating at 60°C for 15 min. Bacteria were opsonized by incubation with anti–*H. influenzae* polyserotype antiserum (Difco Laboratories Inc.) at a 1:25 dilution for 15 min at room temperature. IgG-opsonized or unopsonized bacteria were added to monolayers of BMMϕ, at a ratio of 130 bacteria per macrophage. Cytokine levels in cell supernatants were measured by ELISA 24 h after the addition of bacteria. In some studies, cytokine production induced by LPS or IgG-LPS was examined. IgG-LPS was generated by incubating LPS (*E. coli* 0128:B12, 100 μg/ml; Sigma Chemical Co.) with rabbit anti-LPS polyclonal antiserum (Calbiochem-Novabiochem, San Diego, CA) at a 1:1 dilution for 15 min at 4°C. For in vitro studies, LPS or IgG-LPS was added to monolayers of BMMϕ at a final LPS concentration of 100 ng/ml. For in vivo challenge studies, recombinase-activating gene (RAG)-1$^{-/-}$ mice (The Jackson Laboratory, Bar Harbor, ME) received either IgG-LPS or LPS intravenously (tail vein) at a final LPS dose of 4 μg per mouse. Control LPS was incubated with an equal volume of HBSS. Mice were bled by retroorbital puncture at the indicated time intervals, and serum cytokine levels were determined by ELISA.

Cytokine ELISAs. Levels of murine cytokines were measured by ELISA using appropriately diluted culture supernatants or serum. IL-10 concentrations were determined with a mouse IL-10 ELISA kit (Genzyme Corp., Cambridge, MA, or Biosource International, Camarillo, CA) according to the manufacturer's instructions. Murine IL-12(p40) levels were measured with a mouse IL-12 ELISA kit (Biosource International) according to the manufacturer's instructions. Murine IL-12(p70) levels were measured by ELISA using mAbs C18.2 (anti–murine IL-12 p35) and C17.15 (biotinylated anti–murine IL-12 p40) as ELISA capture and detection antibodies, respectively, according to protocols provided by PharMingen (San Diego, CA). Recombinant murine IL-12 (Genzyme Corp.) was used as a standard. mAbs C18.2 and C17.15 were purified from ascitic fluid provided by Dr. Giorgio Trinchieri (The Wistar Institute, Philadelphia, PA).

Results

Effect of FcγR Ligation on Macrophage IL-10 Production. The production of IL-10 by BMMϕ was examined after specific receptor ligation. BMMϕ were stimulated either with LPS alone, or with LPS in the presence of erythrocytes opsonized with either IgG or complement. The addition of LPS to monolayers of BMMϕ induced a modest but significant production of IL-10 by macrophages. However, the ligation of FcγR simultaneously with the addition of LPS enhanced markedly the production of IL-10. This enhancement was observed at both the mRNA (Fig. 1 *A*) and protein (Fig. 1 *C*) levels. IL-10 mRNA was increased by four- to eightfold (Fig. 1 *B*), and protein secretion was increased by greater than sixfold after FcγR ligation (Fig. 1 *C*). The induction of IL-10 was specific to the FcγR, because ligation of macrophage complement receptors did not significantly alter IL-10 mRNA (Fig. 1 *A*) or protein (Fig. 1 *C*) production. The ligation of macrophage FcγR or complement receptors in the absence of LPS was not sufficient to induce the production of notable levels of IL-10 (Fig. 1 *C*, *inset*).

Effect of FcγR Ligation on IL-10 Production in Macrophages from Gene Knockout Mice. To determine the FcγR subtype responsible for IL-10 upregulation, BMMϕ from gene knockout mice were studied. The FcR γ chain is an essential component of both the FcγRI and FcγRIII, and is required for both receptor assembly and signaling (12). Macrophages from mice lacking the common γ chain (FcR$\gamma^{-/-}$) failed to upregulate IL-10 production in response to E-IgG (Fig. 2), implicating one of these two receptors in this phenomenon. Macrophages derived from mice lacking either the FcγRII or the FcγRIII were fully capable of upregulating IL-10 production in response to E-IgG (Fig. 2). These results are consistent with the high affinity FcγRI being the mediator of IL-10 induction.

Macrophage-derived IL-10 Can Suppress the Production of IL-12. Studies were undertaken to determine whether the amount of IL-10 produced by macrophages in response to FcγR ligation was adequate to suppress IL-12 production. Macrophages were stimulated with LPS in the presence of FcγR ligation for 24 h. Supernatants from these monolayers were collected and assayed for their ability to inhibit IL-12 production. Monolayers of BMMϕ were primed with IFN-γ and then stimulated with LPS in the

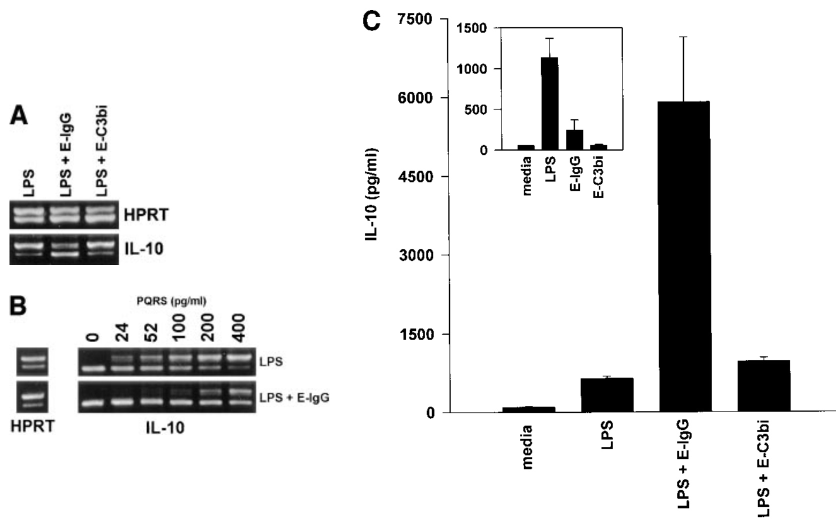

Figure 1. FcγR ligation enhances LPS-induced IL-10 production. (*A*) BMMϕ were exposed to either LPS alone or LPS in combination with either E-IgG or E-C3bi. 6 h after the addition of stimuli, total RNA was isolated and used to carry out competitive RT-PCR. Input cDNAs were adjusted to yield comparable ratios of competitor (*upper band* in each reaction) to wild-type (*lower band* in each reaction) intensities for the amplification reaction for hypoxanthine-guanine phosphoribosyltransferase (HPRT), as resolved on a 2% ethidium-stained agarose gel. The adjusted input cDNAs were then used in subsequent RT-PCR reactions using primers for IL-10. Results are representative of two separate experiments. (*B*) cDNA generated from BMMϕ exposed to LPS or LPS in combination with E-IgG, were first normalized for HPRT levels. Constant volumes of normalized cDNAs were then amplified in the presence of increasing concentrations of competitor (*PQRS*), using primers for IL-10. The concentration of the experimental cDNA is represented by the equivalent intensities of competitor and wild-type bands. The fold increase in IL-10 levels between BMMϕ exposed to LPS or LPS in combination with E-IgG can be determined by taking the ratio of their equivalence points. (*C*) BMMϕ were exposed to either media, LPS, E-IgG, or E-C3bi (*inset*), or LPS alone or LPS in combination with either E-IgG or E-C3bi. After 24 h, the supernatant was harvested, and IL-10 levels were determined by ELISA. Values represent the mean of three independent experiments, each performed in triplicate, ±SE.

presence or absence of a 33% supernatant from LPS/FcγR-stimulated macrophages. 24 h after this stimulation, the production of IL-12(p70) was measured by ELISA. The supernatants from LPS/FcγR-stimulated BMMϕ reduced IL-12(p70) secretion to background levels (Fig. 3). Treating these inhibitory supernatants with a neutralizing mAb to IL-10 partially restored IL-12(p70) production. These results indicate that the IL-10 produced by macrophages after LPS/FcγR stimulation is adequate to inhibit the production of IL-12 by IFN-γ–primed macrophages.

Modulating Macrophage Proinflammatory Responses by Ligating FcγR. Cytokine production by macrophages in response to potential proinflammatory stimuli was examined after FcγR ligation. IL-10 and IL-12(p40) levels were measured by ELISA 24 h after the addition of either LPS or IgG-opsonized LPS to BMMϕ. As expected, LPS induced a potent proinflammatory response by macrophages, characterized by moderate levels of IL-10 (Fig. 4 *A*) and high levels of IL-12(p40) (Fig. 4 *B*). In contrast to this, IgG-opsonized LPS induced higher levels of IL-10 and only modest levels of IL-12(p40). Similar studies were performed using the Gram-negative bacterium, *H. influenzae*. Cytokine production by macrophages in response to unopsonized heat-killed type b *H. influenzae* was compared with that induced in response to IgG-opsonized heat-killed bac-

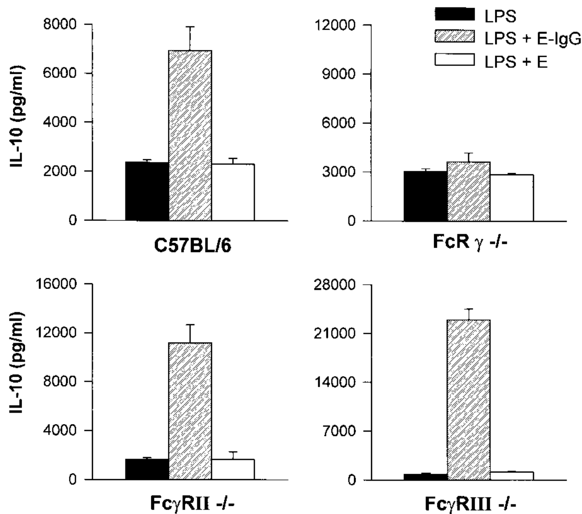

Figure 2. FcγRI is responsible for the FcγR-mediated enhancement of IL-10 production. BMMϕ from C57BL/6, $FcR\gamma^{-/-}$, $Fc\gamma RII^{-/-}$, or $Fc\gamma RIII^{-/-}$ mice were exposed to LPS alone or LPS in combination with either E-IgG or unopsonized erythrocytes (*E*). After 24 h, the supernatant was harvested, and IL-10 levels were determined by ELISA. Determinations were performed in triplicate, and values are expressed as the means ± SD. Results are representative of three separate experiments.

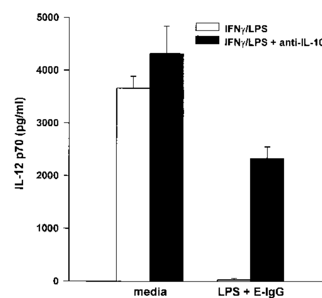

Figure 3. IL-10 produced by macrophages stimulated with LPS/FcγR can suppress IL-12 production. Supernatants from BMMϕ exposed to either media alone or LPS in combination with E-IgG for 24 h were harvested and filtered through a 0.2-μm filter. Supernatants were diluted 1:3 with media and incubated for 15 min at 4°C in either the presence or absence of a neutralizing mAb to IL-10 (JESS-2A5; 20 μg/ml). Diluted supernatants were then added to BMMϕ that had been primed with IFN-γ (100 U/ml) for 8 h, and immediately treated with LPS. After 24 h, the supernatant was harvested, and IL-12(p70) levels were determined by ELISA. Values represent the mean of three independent experiments, each performed in triplicate, ±SE.

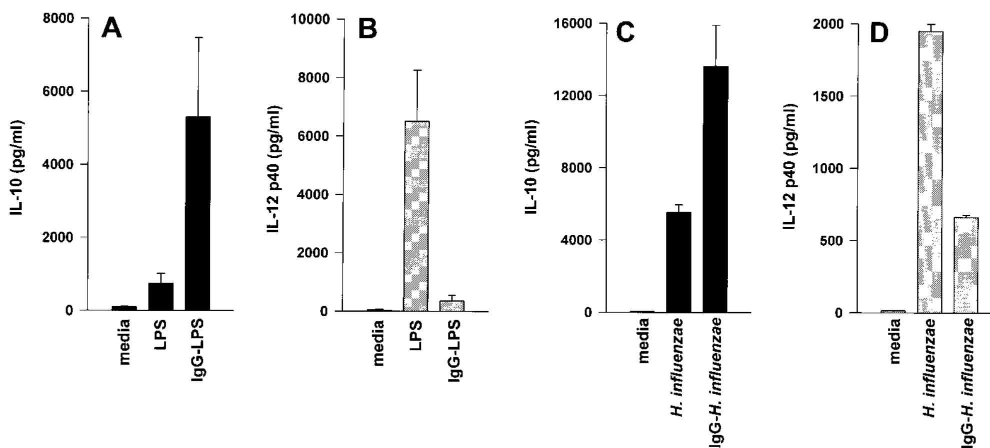

Figure 4. The modulation of inflammatory response by FcγR ligation. BMMϕ were exposed to either media, LPS, or IgG-LPS (*A* and *B*). After 24 h, the supernatant was harvested, and IL-10 (*A*) and IL-12(p40) (*B*) levels were determined by ELISA. Determinations were performed in triplicate, and values are expressed as the means ± SD. Results are representative of four separate experiments. BMMϕ were incubated with media alone or with equal numbers of either unopsonized or IgG-opsonized *H. influenzae* (*C* and *D*). After 24 h, the supernatant was harvested, and IL-10 (*C*) and IL-12(p40) (*D*) levels were determined by ELISA. Determinations were performed in triplicate, and values are expressed as the means ± SD. Results are representative of three separate experiments.

teria. Unopsonized *H. influenzae* induced the production of relatively high levels of both IL-10 (Fig. 4 *C*) and IL-12(p40) (Fig. 4 *D*). However, IgG-opsonized bacteria induced a significant decrease in the production of IL-12(p40) protein and an increase in the production of IL-10. Thus, in both in vitro models, the ligation of FcγR by opsonization with IgG resulted in a reduction in macrophage proinflammatory responses.

Modulation of In Vivo Responses to Bacterial Endotoxin. Studies similar to the in vitro studies performed above were repeated in experimental animals. Several groups have demonstrated that the administration of LPS to experimental animals results in the rapid production of proinflammatory cytokines (17). Given our in vitro observations, we sought to determine whether IgG opsonization of LPS could reverse the inflammatory cytokine response to LPS in vivo. These studies were performed in RAG-$1^{-/-}$ mice, since recent studies have demonstrated that normal mice have naturally occurring antibodies to LPS (18). Mice were injected with either LPS or IgG-LPS, and the generation of cytokines in serum was analyzed over the ensuing 24 h. The injection of low levels (4 μg) of LPS into RAG-$1^{-/-}$ mice induced the transient production of relatively high levels of serum IL-12(p40) (Fig. 5 *A*) and only modest levels of IL-10 (Fig. 5 *B*). The observation that RAG-$1^{-/-}$ mice make high amounts of IL-12 in response to low levels of LPS is consistent with previous observations that antibody-deficient mice are hypersusceptible to LPS (18). The injection of IgG-opsonized LPS into these mice induced an alteration in the cytokine profile. RAG-$1^{-/-}$ mice injected with IgG-LPS made only modest levels of IL-12(p40) (Fig. 5 *A*), but they more than doubled their production of IL-10 (Fig. 5 *B*). This reciprocal alteration in the pattern of cytokine production suggests that IgG opsonization of LPS not only increases the rate of LPS clearance through FcγR, but in doing so also mediates a desirable effect by dampening the proinflammatory response to LPS.

Discussion

Monocytes and macrophages are a primary source of IL-12. IL-12 is a potent inducer of cell-mediated immune responses, and animals lacking IL-12 are invariably more susceptible to infections with intracellular pathogens (2). Because IL-12 plays such a central role in the development of Th1-type immune responses, we have begun to examine the regulation of IL-12 production in macrophages. We have described previously a mechanism whereby receptor ligation can downmodulate IL-12 production by macro-

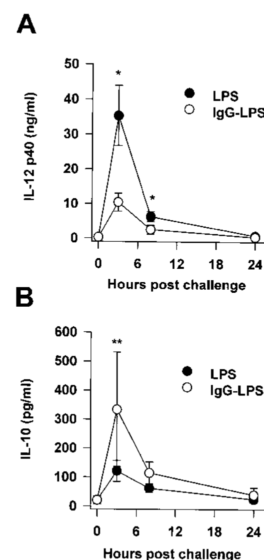

Figure 5. Production of IL-12(p40) and IL-10 in a murine model of septic shock. RAG-$1^{-/-}$ mice received either LPS or IgG-LPS intravenously at a final LPS dose of 4 μg per mouse. Serum levels of IL-12(p40) (*A*) and IL-10 (*B*) were measured at the indicated times after challenge. Data show the mean ± SD of groups of four separately handled mice. $^{*}P < 0.01$, and $^{**}P < 0.08$ (significant by rank–sum analysis) versus the LPS-treated group as determined by Student's *t* test.

phages (15). In this work, we describe a second novel mechanism of downregulating IL-12. This mechanism is distinct from the previously described mechanism in several important ways. First, the present mechanism is not a direct regulation of IL-12 transcription, but rather depends on the production of the inhibitory cytokine IL-10. Second, this regulation is specific to a single receptor class on macrophages, the FcγRI. We show that ligating the macrophage FcγRI increases IL-10 mRNA, resulting in a substantial increase in IL-10 secretion. This macrophage-derived IL-10 is a potent inhibitor of IL-12 production by macrophages. Even IFN-γ–primed macrophages fail to make IL-12 in response to LPS when exposed to macrophage supernatants containing IL-10. Thus, the ligation of the macrophage FcγRI can downmodulate IL-12 production via a mechanism that is dependent on macrophage-derived IL-10.

In identifying the FcγRI as the macrophage receptor that upregulates IL-10 production, we can now associate distinct biological activities with each of the three FcγR classes. CD16, the FcγRIII, is the prototypical proinflammatory Fcγ receptor. Ligating FcγRIII has been associated with the production of proinflammatory cytokines (19), and mice lacking FcγRIII undergo diminished Arthus reactions (14). CD32, the FcγRII, is a negative regulator of immune complex–triggered immune responses, and mice lacking FcγRII have augmented anaphylactic responses to IgG (13). Our studies would classify the FcγRI (CD64) as another inhibitory FcγR, but by a different mechanism than that observed for FcγRII. Whereas FcγRII inhibits signaling (20), FcγRI actively promotes the transcription of an inhibitory cytokine, IL-10. Thus, by two distinct mechanisms, both the FcγRI and FcγRII can inhibit inflammatory responses to immune complexes. Previous observations that immune complexes can inhibit both the in vivo clearance of *Listeria monocytogenes* (21) and the in vitro macrophage tumoricidal and cytotoxic activity (22, 23) are consistent with FcγR ligation leading to an inhibition of immune responses.

The in vitro studies presented here indicate that FcγRI ligation has the potential to dampen the acute response to inflammatory stimuli such as LPS or Gram-negative bacteria. In both cases, opsonization with IgG increased macrophage IL-10 production and diminished IL-12 production. The prediction from these studies is that bacterial clearance in an immune animal may be associated with a diminished inflammatory response relative to nonimmune animals. Furthermore, targeting LPS specifically to FcγRI might be a practical way of eliminating endotoxin without the consequent proinflammatory sequelae. The reciprocal alteration of IL-10 and IL-12 after FcγR ligation also has the potential to exert an impact on the acquired immune response, biasing it towards a Th2-type response. The implication from these studies is that IgG itself may be an important promoter of the Th2-type immune response.

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