Interleukin-10 Receptor Signaling through the JAK-STAT Pathway

REQUIREMENT FOR TWO DISTINCT RECEPTOR-DERIVED SIGNALS FOR ANTI-INFLAMMATORY ACTION

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Interleukin-10 (IL-10) is a cytokine that has pleiotropic effects on a variety of different cell types. Although many of the biologic responses induced by IL-10 are also induced by other cytokines, such as IL-6, IL-10 is relatively unique in its ability to potently inhibit production of pro-inflammatory cytokines in macrophages. In this study, we have used gain-of-function and loss-of-function genetic approaches to define the intracellular components involved in the different biologic actions of IL-10. Herein, we demonstrate that the ability of IL-10 to inhibit tumor necrosis factor α (TNFα) production in lipopolysaccharide-stimulated macrophages requires the presence of Stat3, Jak1, and two distinct regions of the IL-10 receptor intracellular domain. Macrophages deficient in Stat3 or Jak1 were unable to inhibit lipopolysaccharide-induced TNFα production following treatment with murine IL-10. Structure-function analysis of the intracellular domain of the IL-10 receptor α chain showed that whereas two redundant Stat3 recruitment sites (427YQK430 and 477YLK480) were required for all IL-10-dependent effects on either B cells or macrophages, expression of IL-10-dependent anti-inflammatory function required the presence on the intracellular domain of the IL-10 receptor of a carboxyl-terminal sequence containing at least one functionally critical serine. These results thus demonstrate that IL-10-induced inhibition of TNFα production requires two distinct regions of the IL-10 receptor intracellular domain and thereby establish a distinctive molecular basis for the developmental versus the anti-inflammatory actions of IL-10.

Interleukin-10 (IL-10) is a cytokine produced by Th0 and Th2 CD4⁺ T cells, CD5⁺ B cells, thymocytes, keratinocytes, and macrophages (1–5) that regulates the function and/or development of both lymphoid and myeloid cells (2, 6, 7). One of the most unique actions of this cytokine is its ability to inhibit production of pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα), IL-1, and IL-12, which are synthesized by macrophages in response to bacterial products, such as lipopolysaccharide (LPS). This activity results in decreased IFNγ production and inhibition of cell-mediated immune responses while concomitantly enhancing humoral immunity (6–10).

IL-10 exerts its biologic effects on cells by interacting with a specific cell surface receptor (1, 11). Functionally active IL-10 receptors are composed of two distinct subunits. Both subunits belong to the class II cytokine receptor family that also contains the receptors for IFNα and IFNγ (12). The IL-10 receptor α chain is a 110-kDa polypeptide that plays the dominant role in mediating high affinity ligand binding and signal transduction (1, 11). The IL-10 receptor β subunit (also known as CRF2–4) is predicted to be a 40-kDa polypeptide that is largely required only for signaling (13, 14).

Engagement of the IL-10 receptor has been shown to activate the JAK-STAT signaling pathway. Specifically, IL-10 effects the activation of Jak1 (associated with the IL-10 receptor α chain) and Tyk2 (associated with the IL-10 receptor β chain) and induces the activation of Stat1, Stat3, and, in some cells, Stat5 (15–19). Previous work from our laboratory using cells from mice with disrupted genes for Jak1 and Stat1 has revealed that the characteristic ability of IL-10 to inhibit TNFα production in LPS-stimulated macrophages (i.e. anti-inflammatory actions of IL-10) displays an obligate dependence on Jak1 but does not require the presence of Stat1 (20, 21). Thus, whereas the Janus kinases are clearly required for promoting the anti-inflammatory effects of IL-10, it remains uncertain which STAT protein, if any, is involved in mediating these unique IL-10-induced responses.

Other studies have suggested that Stat3 participates in manifesting at least some of the biologic effects of IL-10 on B cells and macrophages. First, Stat3 is directly recruited to two redundant YXXQ sequences in the intracellular domain of the IL-10 receptor following ligand binding (18, 22). Receptor mutants lacking these two sequences fail to activate Stat3 and fail to promote a variety of IL-10-dependent responses when expressed in the Ba/F3 pro-B cell line (16, 18). Second, overexpression of a dominant-negative Stat3 mutant protein in the J774 murine macrophage cell line inhibited IL-10-dependent antiproliferative responses and partially blocked IL-10-induced expression of the CD32/16 Fcγ receptor (23). Importantly, these latter developmental responses are also effected by other cytokines that activate Stat3, such as IL-6. However, these same studies failed to find a blocking effect of mutant Stat3 proteins on the anti-inflammatory actions of IL-10. Thus, the existing data suggest either that the IL-10 receptor utilizes different signaling mechanisms to manifest anti-inflammatory versus developmental effects or that different IL-10-induced biologic responses in cells display differential requirements for Stat3 activation.

Herein we demonstrate that macrophages derived from mice engineered to express a genetic Stat3 deficiency in the myeloid cell compartment fail to respond to IL-10 and secrete high
levels of TNFα upon stimulation with IL-10 plus LPS. These results thus unequivocally establish the requirement of Stat3 for the anti-inflammatory functions of IL-10 in primary cells. Using gain-of-function and loss-of-function receptor mutants, we also define the functionally critical regions on the IL-10 receptor that regulate developmental versus anti-inflammatory functions of this cytokine. The developmental functions map to IL-10 receptor regions that are critical for Stat3 recruitment and that are shared by receptors for other cytokines that activate Stat3, such as IL-6. In contrast, the anti-inflammatory function of IL-10 displays the additional requirement for a carboxyl-terminal 30-amino acid sequence in the intracellular domain of the receptor that contains at least one functionally important serine residue. Thus, the unique anti-inflammatory functions of IL-10 can be explained by distinctive receptor intracellular domain sequences that are not shared by Stat3 activating cytokine receptors.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant murine IL-10 was generated as described previously (24). Purified recombinant human and murine IFNγ were generated as described previously by Genentech (South San Francisco, CA). Purified recombinant murine IL-3 and IL-6 were purchased from Genzyme (Cambridge, MA). M-CSF were obtained from R&D Systems (Minneapolis, MN). Lipopolysaccharide (LPS) derived from *Esherichia coli* 0127:B8 was purchased from Difco (Detroit, MI).

**Antibodies**—GIR-205 is a murine IgG1 monoclonal antibody specific for the human IFNγ receptor (25), and 9E10 is a murine IgG1 monoclonal antibody specific for a 13-amino acid peptide tag derived from the human c-Myc protein (SMEQKLISEEDLN) (26). These two antibodies were purified and conjugated to biotin using the Enzo biotinylation reagent (Enzo Biochem, Inc.) as described (25).

**Primers**—The primers listed below were synthesized on an oligo 1000 DNA synthesizer (Beckman, Fullerton, CA) and were based on the nucleotide sequence of either the human IFNγ cDNA (27) or the murine IL-10 receptor cDNA (11): primer 100741, 5′-CCTGAATTCACATGACGATTTTTTCTTGCAGAC-3′; primer 100341, 5′-CTCTTTCAAGGAGTCTAGG-3′; primer 100242, 5′-CTCACAACCTATGGACCCAC-3′; primer 100243, 5′-GAGACCTTATGCTGCTGCAATGGTCGAGCC-3′; primer 100244, 5′-GAGAGGTTCGATATTTATTTATGAACTGCTGCTGCAATGGTCGAGCC-3′; primer 38436, 5′-GCTTCTAGATTAAGAGCCAAGGCTATCCAGG-3′; primer 38437, 5′-GCTGTCGTAATGATTTATTATATGAACTGCTGCTGCAATGGTCGAGCC-3′; primer 38438, 5′-GCTGTCGTAATGATTTATTATATGAACTGCTGCTGCAATGGTCGAGCC-3′; primer 38439, 5′-GCTGTCGTAATGATTTATTATATGAACTGCTGCTGCAATGGTCGAGCC-3′; primer 5393, 5′-GCTTCTAGATTAAGAGCCAAGGCTATCCAGG-3′; primer 5394, 5′-GCTTCTAGATTAAGAGCCAAGGCTATCCAGG-3′; primer 5395, 5′-GCTTCTAGATTAAGAGCCAAGGCTATCCAGG-3′; primer 5396, 5′-GCTTCTAGATTAAGAGCCAAGGCTATCCAGG-3′; primer 5397, 5′-GCTTCTAGATTAAGAGCCAAGGCTATCCAGG-3′; primer 5398, 5′-GCTTCTAGATTAAGAGCCAAGGCTATCCAGG-3′; primer 5399, 5′-GCTTCTAGATTAAGAGCCAAGGCTATCCAGG-3′; primer 5400, 5′-GCTTCTAGATTAAGAGCCAAGGCTATCCAGG-3′; primer 5401, 5′-GCTTCTAGATTAAGAGCCAAGGCTATCCAGG-3′; primer 5402, 5′-GCTTCTAGATTAAGAGCCAAGGCTATCCAGG-3′.

**DNA Transfection**—Cells (1 × 10⁵) were transfected with 50 μg of plasmid DNA using electroporation at 320 V and 960 μF (Bio-Rad Gene Pulser). Cells were then plated at 5 × 10⁶ cells per 100 mm dish for 48 h before transfection. Selection with 400 V and 960 μF (Ba/F3 cells) on a Bio-Rad gene pulser. Cotransfections were carried out using 5 μg of the pMon1118 plasmid (hygromycin resistance) and 45 μg of the expression plasmid. Selection with G418 and hygromycin B was begun 48 h after transfection. After selection was completed, cells were sorted based on their expression of both the hgr/IL-10 receptor and the human IFNγ receptor β chain.

**Demonstration of Receptor Expression in Transfected Cell Lines**—Flow cytometry for the hgr/IL-10 receptor chain was used to verify the expression of the transfected constructs. The hgr/IL-10 receptor chain was detected using antibodies (1:1000 dilution) and a biotinylated form of 9E10 (anti-human IFNγ receptor) and 9E10 (anti-c-Myc peptide) respectively and streptavidin-phyceroerythrin conjugate (Flowtech, Redwood City, CA) as described (31). Cells were analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA).

**Electrophoretic Mobility Shift Assay**—Cells (5 × 10⁶) were washed once and then resuspended in 500 μl of medium. 1000 units/ml murine or human IFNγ or 200 ng/ml muIL-10 was added to the cells and incubated for 7 min at 37 °C. The cells were analyzed by electrophoretic mobility shift assay as described previously (32) using an 18-base pair oligonucleotide probe that contained the gamma response region of the FcyRI gene.

**Ba/F3 Proliferation Assay**—Ba/F3 cells were washed twice in supplemented RPMI 1640 medium that lacked muIL-3. The cells were seeded in a 96-well plate at a density of 2 × 10⁵ cells/well and rested for 3 h in the absence of muIL-3. The cells were then incubated with varying amounts of huIFNγ or muIL-10 in a total volume of 150 μl. Cells were incubated for 48 h at 37 °C and an 3-15-dimethyl thiourea 2-yl-2,5-diphenyl tetrazolium bromide assay was performed as described previously (33). Experiments were performed at least three times using multiple clones of each transfected cell line. Similar results were obtained using transfected bulk populations.

**RAW264.7 Bioassay**—RAW264.7 cells were washed once, seeded in 96-well plates at a density of 1 × 10⁵ cells/well and incubated with varying amounts of huIFNγ, muIL-6, or muIL-10 in a total volume of 150 μl supplemented Dulbecco’s modified Eagle’s medium. Cells were incubated for 1 h at 37 °C and then treated with 10 ng of LPS/well. The cells were placed at 37 °C for 24 h, at which point the culture supernatants were harvested, and TNFα levels were quantitated via a TNFα ELISA (34). Experiments were performed at least three times using multiple clones of each transfected cell line. Similar results were obtained using transfected bulk populations.
IL-10 Signaling for Inhibition of TNFα Production

RESULTS

Stat3 Is Necessary but Not Sufficient for IL-10-dependent Inhibition of TNFα Production by Macrophages—To determine whether Stat3 is required for the anti-inflammatory actions of IL-10, we monitored the ability of this cytokine to inhibit LPS-induced TNFα production in macrophages derived from mice with a genetic deficiency of Stat3 targeted to myeloid cells (35). IL-10 treatment of wild type macrophages resulted in a dose-dependent inhibition of LPS-induced TNFα production that reached maximal levels at a 1 ng/ml dose of IL-10 (Fig. 1). In contrast, macrophages derived from mice with a myeloid cell Stat3 deficiency were unable to inhibit LPS-induced TNFα production at any dose of IL-10 used. As additional controls, macrophages from Jak1−/− and Stat1−/− mice were also tested in these experiments for IL-10 sensitivity (21, 36). In agreement with our previous reports (20, 21), Jak1−/− macrophages were unresponsive to IL-10, whereas the IL-10 response in Stat1−/− macrophages was indistinguishable from that of wild type mice. Thus, Stat3, together with Jak1, is obligatorily required for IL-10-dependent inhibition of TNFα production in LPS-stimulated macrophages.

Whereas these results demonstrated that Stat3 was required for mediating the anti-inflammatory actions of IL-10, they did not reveal whether it was sufficient to induce these functions. To address this issue, we compared the anti-inflammatory action of IL-10 to that of IL-6, a cytokine that utilizes a distinct receptor system but that also activates Stat3 and Jak1. Pretreatment of RAW264.7 cells with increasing doses of IL-10 resulted in a dose-dependent inhibition of TNFα production, reaching a maximal level of 75% at a dose of 200 ng of the cytokine (Fig. 2A). In contrast, RAW264.7 cells pretreated with IL-6 were only slightly inhibited in their ability to produce TNFα (maximal level of inhibition, 30% at a 200-ng dose of IL-6). We also compared the anti-inflammatory effects of IL-10 and IL-6 on primary murine macrophages. Here again, whereas IL-10 displayed potent inhibitory activity on TNFα production, IL-6 did not (Fig. 2B). These functional differences could not be ascribed to different levels of Stat3 activation because the kinetics and ultimate magnitude of Stat3 activation by IL-10 and IL-6 in either RAW264.7 cells or primary macrophages was identical (data not shown). Thus, the JAK-STAT pathway is required but not sufficient for IL-10-dependent inhibition of LPS-induced TNFα production.

Demonstration That IL-10-dependent Biologic Responses in Macrophages and B Cells Can Be Manifest by a Gain of Function hgro/IL-10Ra Chimeric Receptor—To identify functionally critical amino acid residues within the intracellular domain of the muIL-10 receptor ligand binding chain involved in mediating the anti-inflammatory versus developmental effects

FIG. 1. Stat3 and Jak1 but not Stat1 are required for IL-10-dependent inhibition of TNFα production by macrophages. Macrophages derived from wild type, Stat1−/−, Stat3−/−, and Jak1−/− mice were pretreated with varying concentrations of muIL-10 for 12 h. The cells were treated with 2 μg/ml LPS and incubated for an additional 24 h. Supernatants were harvested, and TNFα levels were quantitated via ELISA. Percentage of inhibition of TNFα production upon IL-10 stimulation is indicated.

FIG. 2. The JAK/STAT pathway is necessary but not sufficient for the anti-inflammatory actions of IL-10. A. RAW264.7 cells were pretreated with varying doses of either muIL-10 or muIL-6 for 1 h. Subsequently, the cells were treated with 60 ng/ml LPS and incubated for 24 h. Supernatants were harvested, and TNFα levels were quantitated by ELISA. The graph indicates percentage of inhibition of TNFα production. B. peritoneal exudate cells were isolated from naive female BALB/c ByJ mice. Cells were plated and allowed to adhere for 4 h. The cells were then washed once and pretreated for 1 h with various doses of either muIL-10 or muIL-6. The cells were stimulated with LPS (60 ng/ml) for 24 h. TNFα levels in the supernatant were quantitated by ELISA.
of IL-10, we generated a chimeric receptor that consisted of the human IFNγ receptor α chain extracellular and transmembrane domains and the first 184 amino acids of the intracellular domain (i.e. truncated just above the Stat1 docking site) attached to a 140-amino acid region of the IL-10 receptor consisting of residues 420–559, which contains the Stat3 recruitment sites. Thus, the resulting chimeric receptor retains the Jak1 binding site, lacks the Stat1 docking site, and now contains the two redundant Stat3 docking sites from the muIL-10 receptor. All cells transfected with the chimeric receptor were cotransfected with the human IFNγ receptor β chain (IFNGR2).

Engagement of the endogenous IL-10 receptor on RAW264.7 cells with muIL-10 led to a dose-dependent inhibition of TNFα production in LPS-treated cells (Fig. 4A). HuIFNγ has no effect on these cells because of the strict species specificity that governs the interaction of IFNγ with its receptor. Treatment of RAW264.7 cells expressing the hgro/IL-10Rα chimeric receptor with human IFNγ induced a dose-dependent inhibition of LPS-dependent TNFα production in a manner that was quantitatively identical to that induced by the activated, endogenous IL-10 receptor. In contrast, treatment of wild type or transfected RAW264.7 cells with murine IFNγ caused a dose-dependent increase of TNFα production (data not shown). In a similar manner, Ba/F3 engineered to express the wild type muIL-10 receptor proliferated in the presence of muIL-10 (Fig. 4B) but did not respond to human IFNγ. In contrast, Ba/F3 cells bearing the hgro/IL-10Rα chimeric receptor proliferated upon exposure to human IFNγ. Thus, transfer of IL-10 receptor sequences containing the Stat3 recruitment sites to the IFNγ receptor results in the generation of a modified IFNγ receptor...
that promotes IL-10-dependent rather than IFNγ-dependent responses in murine macrophages and B cells exposed to human IFNγ.

Tyrosine Residues 427 and 477 within the Intracellular Domain of the Chimeric hgr/IL-10Ra Are Redundantly Required for the Development of IL-10-like Effects on Macrophages and B Cells—In a previous report, we showed that tyrosine residues at positions 427 and 477 in the intact murine IL-10 receptor played redundant roles in recruiting Stat3 to the activated receptor (18). In addition, we found that Stat3 recruitment was required for development of IL-10-induced proliferative responses (i.e. developmental responses) in B cells. To examine whether the anti-inflammatory functions of IL-10 also show the same requirement for these Stat3 recruitment sites and to test whether the functionally redundant tyrosines act in a similar manner in the chimeric receptor, we generated a set of tyrosine mutations behaving in a manner that was analogous to the intact receptor (18). In an attempt to identify specific amino acid residues within the carboxyl-terminal 60 amino acids of the chimeric receptor, we expressed them in macrophages and B cells (Table I) and examined their capacity to promote IL-10 like biologic effects on the transfected cells.

**Table I**

| Chimeric receptor | Subunit expression (mean channel shift) | RAW264.7 | Ba/F3 |
|------------------|----------------------------------------|----------|-------|
|                  | α chain | β chain | α chain | β chain |
| hgr/IL-10Ra      | 27.1    | 9.92    | 16.5    | 8.0     |
| hgr/IL-10RaY427F | 23.2    | 37.2    | 22.7    | 12.2    |
| hgr/IL-10RaY477F | 47.5    | 5.7     | 17.1    | 10.3    |
| hgr/IL-10RaY427F/Y477F | 22.2 | 30.6    | 16.2    | 16.9    |
| hgr/IL-10RaΔ1    | 23.6    | 27.6    | 19.7    | 17.2    |
| hgr/IL-10RaΔ2    | 15.0    | 38.4    | 16.8    | 11.3    |
| hgr/IL-10RaΔ3    | 19.0    | 25.7    | 14.6    | 10.0    |
| hgr/IL-10RaΔ4    | 19.3    | 33.4    | 21.2    | 10.0    |
| hgr/IL-10RaS541A/S544A/S553A/S554A | 16.2 | 25.5    |

The data presented this far show that the JAK-STAT signaling pathway and specifically Stat3 and Jak1 are required for induction of IL-10-dependent anti-inflammatory and developmental responses in macrophages and B cells. However, they did not reveal a mechanism by which IL-10 could uniquely manifest its anti-inflammatory effects. Thus, at least one signal in addition to IL-10 must arise from the activated IL-10 receptor in stimulated macrophages. To explore this possibility, we generated a family of progressive deletion mutants of the hgr/IL-10Ra chimeric receptor such that each sequential mutant was 15 amino acids shorter at the carboxyl terminus compared with its predecessor. Each receptor was expressed in RAW264.7 and Ba/F3 cells (Table I and Fig. 3). Expression of each receptor in the resulting stably transfected cell populations was confirmed by FACS analysis, and several clones of each transfected cell line were chosen for further analysis based on their expression of comparable levels of receptor. HuIFNγ treatment of RAW264.7 cells bearing either intact hgr/IL-10Ra or the chimeric receptor lacking the most carboxyl-terminal 15 residues (hgr/IL-10RaΔ1) produced a dose-dependent inhibition of LPS-stimulated TNFα production (Fig. 5A). However, RAW264.7 cells bearing receptor mutants lacking additional carboxyl-terminal sequence (hgr/IL-10RaΔ2-hgr/IL-10RaΔ4) were unable to mediate this response. In contrast, all of the receptor carboxyl-terminal deletion mutants were able to support IL-10-dependent proliferative responses in Ba/F3 cells (Fig. 5B). Thus, additional residues within the carboxyl terminus are used by the IL-10 receptor to inhibit TNFα production in macrophages but not proliferative responses in B cells.

The basis for the activity differences observed with the various deletion mutants in RAW264.7 versus Ba/F3 cells was not due to differences in Stat3 activation. As determined by electrophoretic mobility shift assay analysis, huIFNγ induced comparable levels of Stat3 homodimers (slower migrating band) and Stat1:Stat3 heterodimers (faster migrating band) in each cell type regardless of the presence of the carboxyl-terminal 60 amino acids of the chimeric receptor (Fig. 6, A and B).

Serine Residues within the Carboxyl Terminus of the muIL-10 Receptor Are Required for Inhibition of TNFα Production by Macrophages—In an attempt to identify specific amino acids in the carboxyl-terminal region of the IL-10 receptor that are required for inhibition of TNFα production, we generated one final hgr/IL-10Ra chimeric receptor mutant in which all of the carboxyl-terminal serine residues (i.e. those residing at positions 541, 544, 553, and 554) were mutated to alanines and stably expressed it in RAW264.7 cells (Table I). HuIFNγ treatment of RAW264.7 cells bearing this mutant failed to inhibit LPS-mediated TNFα production. The minimal levels of TNFα inhibition manifest by this mutant were indistinguishable from those produced by IL-6 (Fig. 7A). Electrophoretic mobility shift assay analysis showed comparable Stat3 activation by huIFNγ treated RAW264.7 cells bearing either full-length chimeric receptor or the receptor lacking the carboxyl-terminal serines (Fig. 7B). Thus, IL-10-dependent inhibition of LPS-induced TNFα production in macrophages requires the concomitant presence of Stat3 docking sites on the receptor and a restricted carboxyl-terminal portion of the receptor that contains at least one functionally important serine residue.

**DISCUSSION**

The focus of this study was to characterize the mechanism that underlies the selective ability of IL-10 to inhibit TNFα production by LPS-stimulated macrophages. Using cells from mice with a genetic Stat3 deficiency in the myeloid cell compartment, we demonstrated that Stat3 is obligatorily required for expression of the inhibitory functions of IL-10 on TNFα.
Taking into account our previous study (21), which showed an obligate role for Jak1 in this process, we can now unequivocally conclude that the JAK-STAT signaling pathway is required for expression of the anti-inflammatory actions of IL-10. In addition, we find that the developmental versus anti-inflammatory actions of IL-10 are distinguishable by their requirements for different regions of the IL-10 receptor α chain intracellular domain. For the former, the functionally critical receptor region maps selectively to a redundant set of Stat3 docking sites residing at positions 427–430 and 477–480. In contrast, for the latter, the additional presence on the receptor of a carboxyl-terminal sequence that contains at least one.

**FIG. 4.** Demonstration that IL-10-dependent biologic responses in macrophages and B cells can be manifest by a gain of function hgr/IL-10Rα chimeric receptor. A, RAW264.7 cells bearing the wild type IL-10 receptor, the intact chimeric receptor, or distinct chimeric receptor intracellular domain mutants were pretreated with various doses of IL-10 or huIFNγ for 1 h and subsequently treated with 60 ng/ml of LPS for 24 h. Again, TNFα concentrations were determined by ELISA. The graph depicts percentage of inhibition of LPS-induced TNFα production. B, Ba/F3 cells bearing the wild type IL-10 receptor, the intact chimeric receptor, or distinct chimeric receptor intracellular domain mutants were rested in medium lacking muIL-3 for 3 h. The cells were then treated with increasing doses of either muIL-10 or huIFNγ, respectively. The cells were incubated for 48 h, and subsequently, 50 μl of 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide at a concentration of 2.5 mg/ml was added to the cells. The cells were then incubated at 37 °C for 2 h. The dark blue formazan crystals were dissolved by adding 100 μl of lysis solution. The figure represents viable cells as determined by A570.

**FIG. 5.** Distinct residues within the carboxyl terminus of the IL-10 receptor are required to inhibit LPS-induced TNFα production but not for the induction of an IL-10 proliferative response. A, RAW264.7 cells bearing the wild type muIL-10 receptor, the intact hgr/IL-10Rα chimeric receptor, or the chimeric receptor carboxyl-terminal truncation mutants were pretreated with various doses of muIL-10 or huIFNγ for 1 h. The cells were then treated with 60 ng/ml LPS and incubated for an additional 24 h. TNFα concentrations were quantified via ELISA. This panel depicts percentage of inhibition of LPS-induced TNFα production. B, Ba/F3 cells bearing the wild type muIL-10 receptor, the intact hgr/IL-10Rα chimeric receptor, or the chimeric receptor carboxyl-terminal truncation mutants were rested in medium lacking muIL-3 for 3 h. The cells were then treated with increasing doses of either muIL-10 or huIFNγ. The cells were incubated for 48 h, and subsequently, 50 μl of 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide at a concentration of 2.5 mg/ml was added to the cells. The cells were then incubated at 37 °C for 2 h. The dark blue formazan crystals were dissolved by adding 100 μl of lysis solution. The figure represents viable cells as determined by A570.
The cells were then lysed, and the nuclear extracts were generated. The latter possibility has been ruled out by the current study, which shows that the ability of IL-10 to inhibit LPS-induced TNFα production in macrophages requires the concomitant presence in cells of Stat3 and Jak1. Moreover, as presented elsewhere (35), mice lacking Stat3 in the myeloid compartment display enhanced spontaneous inflammatory responses similar to those observed in mice lacking either IL-10 (43) or the CRF2–4 component of the IL-10 receptor (13). These mice develop chronic enterocolitis and exhibit exaggerated Th1 responses characterized by a 4–5-fold increase in IFNγ production as compared with control animals. Thus, taken together, these results show that Stat3 is required for expression of IL-10 anti-inflammatory functions both in vitro and in vivo. In addition, the data presented in the current manuscript directly support the possibility that an accessory signaling pathway in addition to the JAK-STAT pathway is required for expression of the anti-inflammatory actions of IL-10. Specifically we show that a second region of the IL-10 receptor intracellular domain is required in addition to the Stat3 recruitment sites on the receptor to mediate the capacity of IL-10 to inhibit LPS-induced TNFα production in macrophages.

This conclusion is thus in conflict with that of O’Farrell et al. (25), who reported that IL-10-dependent inhibition of TNFα production occurs in a Stat3-independent manner. The conclusions reached in the latter study were derived from two types of experiments. First, macrophages were engineered to express a tagged form of Stat3 containing the bacterial gyrB protein (gyrB-Stat3) that could be inducibly dimerized within the cell following addition of the antibiotic coumermycin (44). Whereas this manipulation led to inhibition of macrophage proliferation, it did not effect LPS-induced TNFα production. However, based on the data presented in our study, we now know that Stat3 is required but not sufficient for induction of the anti-inflammatory actions of IL-10, and thus the coumermycin experiments delivered only one of the two signals needed to inhibit TNFα production. Second, J774 macrophages were engineered to overexpress a dominant negative mutant form of Stat3 lacking the carboxyl terminus. Whereas these cells became either totally or partially insensitive to many of the actions of IL-10, they still displayed IL-10-induced inhibition of LPS-dependent TNFα production. However, the cells that overexpressed mutant Stat3 were not completely blocked in their capacity to activate (i.e. phosphorylate) their own endogenously expressed Stat3. Thus, the lack of effect of the dominant negative Stat3 mutant on the anti-inflammatory functions of IL-10 may reflect the incomplete blockade of Stat3 activation and different threshold requirements for activated Stat3 for induction of various biologic responses. It is also possible that for induction of IL-10 anti-inflammatory responses, Stat3 may function as an adapter protein that facilitates recruitment of another signaling component to the activated receptor. In this case, a Stat3 mutant that could not be tyrosine-phosphorylated maintained a functional SH2 domain but could, through its ability to bind to the activated IL-10 receptor, still be able to fulfill a role as an adapter protein.

A key approach used in our present study was the employment of a gain-of-function chimeric cytokine receptor in which the murine IL-10 receptor Stat3 recruitment sites and carboxyl-terminal region were substituted for the Stat1 recruitment site on the human IFNγ receptor. We specifically chose the human IFNγ receptor α chain as the recipient of the IL-10 for expression of IL-10-dependent anti-inflammatory actions and that at least one additional signal must be concomitantly delivered to the cell through the ligated IL-10 receptor. The second is that the anti-inflammatory actions of this cytokine are induced through a different signaling pathway.
IL-10 Signaling for Inhibition of TNFα Production

Fig. 7. Serine residues within the carboxyl terminus of the muIL-10 receptor are required for inhibition of TNFα production by macrophages. A. RAW264.7 cells bearing the wild type murine IL-10 receptor, the intact chimeric receptor, or chimeric receptor serine to alanine mutant were pretreated with various doses of muIL-10, muIL-6, or huIFNγ. The cells were then treated with 60 ng/ml LPS and incubated for an additional 24 h. TNFα concentrations were quantified via ELISA. This panel depicts percentage of inhibition of LPS-induced TNFα production. B, an electrophoretic mobility shift assay was performed on these cells. 5 μg of extract was incubated with the gamma response region of the FcyRI gene probe for 30 min. The protein-DNA binding complexes were imaged by autoradiography.

receptor sequence for three reasons. First, the α chains of the IFNγ receptor and the IL-10 receptor belong to the same class II cytokine receptor family. Second, although the polypeptides belong to the same receptor family, they differ not only in the STAs they recruit but also in the biologic responses they induce. Third, the use of the huIFNγ receptor extracellular domain allowed us to take advantage of the strict species-specific receptor binding activity exhibited by human and murine IFNγ, thus providing us with a mechanism to selectively monitor the activity of the chimeric receptor expressed on murine cells through the addition of huIFNγ.

Based on the data presented herein, we envision two possible models by which the different regions of the IL-10 receptor may function. In the first, the receptor may deliver two distinct signals that arise independently through interaction of different proteins with the two functionally important regions of the receptor intracellular domain. In this model, one of these signals is Stat3, which binds to the Stat3 docking sites on the IL-10 receptor, becomes tyrosine-phosphorylated, dimerizes, and then translocates to the nucleus, where it functions as a transcriptional activator. The second signal is then derived from an additional protein that docks at the carboxyl terminus of the IL-10 receptor, possibly by binding to serine or phosphoserine residues. In the second model, the association of Stat3 with the ligand-induced Stat3 docking site on the receptor may result in the formation of a binding site for another protein that also requires interaction with the carboxyl-terminal sequence of the IL-10 receptor. In this model, Stat3 would function as an adapter protein. An adapter role for Stat3 has been previously reported for signaling by the IFNα/β receptor, where Stat3 binding to the IFNAR1 provides a binding site on the receptor for phosphotidylinositol 3-kinase. Our current efforts are focused on defining the nature of the second signal needed for induction of IL-10-dependent anti-inflammatory responses and in determining the mechanism by which it is recruited to the activated IL-10 receptor.

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