Responsive to IL-10

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Up-Regulation of IL-10R1 Expression Is Required to Render Human Neutrophils Fully Responsive to IL-10

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We have recently shown that IL-10 fails to trigger Stat3 and Stat1 tyrosine phosphorylation in freshly isolated human neutrophils. In this study, we report that IL-10 can nonetheless induce Stat3 tyrosine phosphorylation and the binding of Stat1 and Stat3 to the IFN-γ response region or the high-affinity synthetic derivative of the c-sis-inducible element in neutrophils that have been cultured for at least 3 h with LPS. Similarly, the ability of IL-10 to up-regulate suppressor of cytokine signaling (SOCS)-3 mRNA was dramatically enhanced in cultured neutrophils and, as a result, translated into the SOCS-3 protein. Since neutrophils’ acquisition of responsiveness to IL-10 required de novo protein synthesis, we assessed whether expression of IL-10R1 or IL-10R2 was modulated in cultured neutrophils. We detected constitutive IL-10R1 mRNA and protein expression in circulating neutrophils, at levels which were much lower than those observed in autologous monocytes or lymphocytes. In contrast, IL-10R2 expression was comparable in both cell types. However, IL-10R1 (but not IL-10R2) mRNA and protein expression was substantially increased in neutrophils stimulated by LPS. The ability of IL-10 to activate Stat3 tyrosine phosphorylation and SOCS-3 synthesis and to regulate IL-1 receptor antagonist and macrophage-inflammatory protein 1β release in LPS-treated neutrophils correlated with this increased IL-10R1 expression, and was abolished by neutralizing anti-IL-10R1 and anti-IL-10R2 Abs. Our results demonstrate that the capacity of neutrophils to respond to IL-10, as assessed by Stat3 tyrosine phosphorylation, SOCS-3 expression, and modulation of cytokine production, is very dependent on the level of expression of IL-10R1. The Journal of Immunology, 2001, 167: 2312–2322.

Interleukin 10 is a 18-kDa nonglycosylated polypeptide secreted by monocytes/macrophages, B lymphocytes, keratinocytes, and subclasses of CD4+ T lymphocytes, whose physiologic function is to inhibit inflammatory responses and Th type 1 cell-mediated immune responses (1). For instance, IL-10 is known to block the activation of proinflammatory cytokine synthesis by many cells, including Th1 and NK cells, and to act as a costimulator of the growth of B cells, thymocytes, and mast cells (1). IL-10 substantially inhibits accessory functions of monocytes/macrophages by down-regulating constitutive and IFN-γ-induced class II MHC expression, reactive oxygen intermediates, and NO production, and suppressing the synthesis of proinflammatory cytokines as well (1). In the murine system, IL-10 inhibits the development of Th1 effector cells from naive CD4+ T lymphocytes, thereby enhancing development of a Th2 dominant population (1). Furthermore, because of its capacity to inhibit the production and release of TNF-α and other proinflammatory cytokines by phagocytes in response to LPS, IL-10 reduces the lethality of experimental septic shock in mice (2, 3).

The intracellular mechanisms by which IL-10 mediates its biological effects remain largely unknown. IL-10 is known to bind to a multicomponent structure composed of at least two subunits: the IL-10R1 (4–6) and of the recently identified CRFB4/CRF2-4 molecule (7–9), now called IL-10R2, both members of the class II family of cytokine receptors. The primary ligand-binding component, IL-10R1, binds IL-10 with high affinity and in the presence of IL-10 associates with the accessory subunit, IL-10R2 (8). Both chains are required for signal transduction (8, 9) and interact with members of the Janus kinase (Jak)† family of tyrosine kinases in a ligand-independent manner: IL-10R1 associates with Jak1 (8), while IL-10R2 associates with Tyk2 (8, 10). IL-10 binding leads to receptor heterodimerization which, in turn, leads to the activation of the Jak kinases and phosphorylation of IL-10R1 on cytoplasmic tyrosine residues (11) followed by direct Stat3 recruitment and tyrosine phosphorylation. Stat1 and Stat5 may be also tyrosine phosphorylated in response to IL-10 (6, 12–14), but the mechanisms of their recruitment to the IL-10R complex and activation remain unclear. Upon phosphorylation, Stat1 and Stat3 (and Stat5) homo/heterodimerize and translocate to the nucleus where they bind to specific promoter sequences to modulate transcription. Moreover, IL-10 has also been shown to inhibit the p56lck tyrosine kinase activation and other subsequent events in this pathway, including Ras activation (15), and to activate both phosphatidylinositol 3-kinase and p70 S6 kinase as well (16).

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Recent studies performed in our laboratory, as well as by other groups, have shown that IL-10 can also regulate a number of poly-morphonuclear neutrophil (PMN) functional responses, including cytokine production, reactive oxygen intermediates and prostanoid production, platelet-activating factor synthesis and release, phagocytosis, apoptosis, and membrane Ag expression (17). Among these responses, the modulation of cytokine/chemokine production by IL-10 is certainly the most widely reported and has been the focus of intensive investigation (18). In contrast, the ability of IL-10 to directly trigger or modulate other responses is somewhat controversial since initial findings suggesting a given action mediated by IL-10 often could not be independently confirmed (17, 18). Whether this situation simply reflects the different experimental approaches used by various investigators, or whether it hints to unknown biological mechanisms is an issue that needs to be clarified. In this context, our recent observations on the ability of IL-10 to up-regulate suppressor of cytokine signaling (SOCS) 3 mRNA expression in the absence of detectable Stat3/Stat1 tyrosine phosphorylation and Stat-dependent DNA-binding activities in freshly isolated neutrophils were very intriguing (19, 20). Indeed, while these data have provided a possible explanation for the inability of IL-10 to induce CD64/FcYRi expression in neutrophils (19), as opposed to monocytes (12, 19, 21), they have also suggested that, at least in human neutrophils, the activation of Stat1 and/or Stat3 is not required for the regulatory effects of IL-10 on cytokine production. The latter notion is however in sharp contrast with other studies performed with monocytes from Jak1-deficient mouse embryos (22), and with neutrophils and macrophages derived from mice engineered to express a generic Stat3 deficiency (23), which have highlighted an obligatory role for Jak1 and Stat3 in mediating the effects of IL-10 on LPS-induced cytokine release.

The results reported in the current study reconcile the above-mentioned conflicting reports insofar as we show herein that human neutrophils can acquire the capacity to respond to IL-10 in terms of Stat activation if they are cultured with LPS for a few hours. This occurs because the gene and surface expression of the IL-10R1, which is only present at low levels in circulating or freshly isolated neutrophils, is significantly up-regulated in cultured neutrophils, reaching sufficient levels to confer IL-10 inducibility of responses such as Stat activation, modulation of proinflammatory cytokine production, and enhanced SOCS-3 expression.

Materials and Methods

Anti-human(h) IL-10R1 and -hIL-10R2 mAbs

A neutralizing mouse anti-hIL-10R1 mAb (3B6, IgG1) was prepared by immunization of BALB/c mice with soluble hIL-10R1-FLAG-His, as previously described (5). Mouse anti-hIL-10R2 mAbs (all IgG1) were prepared by immunizing BALB/c mice with a recombinant protein containing the extracellular domain of hIL-10R2 fused to the Fc portion of human IgG1. This protein was expressed transiently in COS cells (pCDM8 vector; Invitrogen, Carlsbad, CA) and purified by HiTrapA affinity chromatography (Pharmacia Biotech, Uppsala, Sweden). Candidate mAbs were identified first by ELISA, then by their ability to detect recombinant hIL-10R2 expressed in transfected Ba/F3 cells (5). The neutralizing anti-hIL-10R2 mAb 1A8.3 was identified by its ability to block responses to hIL-10 of human TF1 cells expressing recombinant hIL-10R1 (TF1-hIL-10R) and human PBMC (5). Anti-hIL-10R2 mAb 4B2.1 is a non-neutralizing mAb and was used for FACS staining.

Cell purification and culture

Highly purified granulocytes (>98.5%) and PBMC were isolated under endotoxin-free conditions from buffy coats of healthy donors as previously described (24). The granulocyte populations contained usually <4% eosinophils (n = 30) as revealed by May-Grünwald-Giemsa staining. Culture conditions slightly differed depending on the type of assay performed. For the in vitro and EMSA experiments, leukocytes were suspended in standard culture medium (RPMI 1640 medium; BioWhittaker, Walkersville, MD) supplemented with 10% low-endotoxin FCS (<0.05 endotoxin units/ml; Euroclone, Paington, U.K.), treated with the various stimuli, distributed in either 6/12 tissue culture well plates, or in polystyrene flasks (BioWhittaker), and then cultured for 20 min at 37°C in a 5% CO2 atmosphere before cell disruption (see below). Alternatively, neutrophils or PBMC were cultured in the absence or presence of 100 ng/ml LPS (from Esche- richia coli serotype 026:B6; Sigma, St. Louis, MO) for the times indicated in either standard culture medium or in serum-free medium on polyc(2-hydroxyethyl methacrylate (polyHEMA)-covered surfaces (25, 26) to achieve nonadherent conditions, before stimulation for 20 min (unless differently specified) and cell lysis. Stimuli used were: 200 U/ml IL-10 (from DNAX and Schering-Plough, Palo Alto, CA, or from PeproTech, Piscataway, NJ) (19, 20), 100 U/ml IFN-γ (Hoffmann-LaRoche, Basel, Switzerland) (19, 1000 U/ml IFN-α (Roferon; Roche Laboratories, Nutley, NJ), 1000 U/ml GM-CSF (Granulocyte; Hoffmann-LaRoche), or 10 ng/ml GM-CSF (PeproTech). In selected experiments, neutrophils were cultured in standard or serum-free medium for 4 h with or without LPS in the presence or absence of 20 μg/ml cycloheximide (CHX; Sigma) before stimulation with IL-10 or G-CSF and cell lysis for protein or RNA extraction.

In other experiments, LPS was preincubated with 10 μg/ml polymyxins, or freshly isolated neutrophils, is significantly up-regulated in cultured neutrophils, reaching sufficient levels to confer IL-10 inducibility of responses such as Stat activation, modulation of proinflammatory cytokine production, and enhanced SOCS-3 expression.

ImmunoBlots

After stimulation, neutrophils and PBMC (2.5–5 × 106/culture) were cultured in standard or serum-free medium for 4 h with or without 200 U/ml IL-10, stimulated with 100 ng/ml LPS 15–20 min later, and then cultured for up to 21 h in 24-well tissue culture wells. At the times indicated in the results, cell-free supernatants were harvested and stored at −20°C. In some of these latter experiments, 5 μg/ml anti-IL-10R1 or anti-IL-10R2 and appropriate control antibodies were added to cultures, either before or at various times after IL-10 and LPS. All reagents used were of the highest available grade and were dissolved in clinical grade pyrogen-free water (19, 20, 24).

Anti-human(h) IL-10R1 and -hIL-10R2 mAbs

A neutralizing mouse anti-hIL-10R1 mAb (3B6, IgG1) was prepared by immunization of BALB/c mice with soluble hIL-10R1-FLAG-His, as previously described (5). Mouse anti-hIL-10R2 mAbs (all IgG1) were prepared by immunizing BALB/c mice with a recombinant protein containing the extracellular domain of hIL-10R2 fused to the Fc portion of human IgG1. This protein was expressed transiently in COS cells (pCDM8 vector; Invitrogen, Carlsbad, CA) and purified by HiTrapA affinity chromatography (Pharmacia Biotech, Uppsala, Sweden). Candidate mAbs were identified first by ELISA, then by their ability to detect recombinant hIL-10R2 expressed in transfected Ba/F3 cells (5). The neutralizing anti-hIL-10R2 mAb 1A8.3 was identified by its ability to block responses to hIL-10 of human TF1 cells expressing recombinant hIL-10R1 (TF1-hIL-10R) and human PBMC (5). Anti-hIL-10R2 mAb 4B2.1 is a non-neutralizing mAb and was used for FACS staining.

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In other experiments, LPS was preincubated with 10 μg/ml polymyxins, or freshly isolated neutrophils, is significantly up-regulated in cultured neutrophils, reaching sufficient levels to confer IL-10 inducibility of responses such as Stat activation, modulation of proinflammatory cytokine production, and enhanced SOCS-3 expression.
double-stranded oligonucleotide probe corresponding to the IFN-γ response region (GRR) element located within the promoter of the FCγRIIgene (29) (5′-GCT TTC GGA AAA CAT CTC AAA TCC TGG AAA CAT GCT-3′) or the high-affinity synthetic derivative of the c-sis-inducible element (SIE), hSIE (5′-gtc gAT TCC CGT AAA TCG-3′) for 15 min (30). Supershift experiments were performed by incubating the extracts with 0.5 μg of anti-Stat3 and/or anti-Stat1 Abs (C20 and E23, respectively, from Santa Cruz Biotechnology) for 30 min at room temperature before adding the labeled probe.

**Northern blot analyses**

Total RNA was extracted from PMN using the guanidinium isothiocyanate method and processed for Northern blot analysis as already described (24). Individual mRNA species in human cells were detected by autoradiography after hybridization of nylon filters with cDNA probes labeled with 32p using Ready-to-go kits (Amer sham Pharmacia Biotech). The probes used consisted of full-length cDNA fragments encoding IL-10R2 (kindly provided by Dr. G. Uzé, Centre National de la Recherche Scientifique, Montpellier, France) (7), SOCS-3 (kindly provided by Dr. A. Yoshimura, Institute of Life Science, Kurume University, Japan) (31), as well as actin (kindly provided by Dr. G. Trinchieri, Schering-Plough, Dardilly, France). The IL-10R1 cDNA fragment was prepared by RT-PCR of mRNA purified from LPS-stimulated monocytes by using oligonucleotides specific for the IL-10R1 (5′-CGG TCT GTG TGG TTT GAA GCA GAA, 3′-GAT GAT GAC GTT GGT CAC GGT GAA, based on accession no. U00672), kindly provided by Dr. G. Trinchieri, Schering-Plough, Dardilly, France.

**Extracellular staining of IL-10R**

Surface expression of IL-10R1 and IL-10R2 in neutrophils, monocytes, and lymphocytes was performed by flow cytometry analysis as previously described (19). Cell staining was performed using predetermined optimal concentrations of mAbs (5 μg/ml) followed by a biotin-conjugated affinity-purified Ab (goat F(ab')2, anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) preadsorbed with human serum) and streptavidin-PE (BD Biosciences, Minneapolis, MN) for MIP-1β. The detection limits of these ELISA were 50 pg/ml for IL-1ra and 30 pg/ml for MIP-1β (32).

**Cytokine measurements**

Antigenic IL-1 receptor antagonist (IL-1ra) and macrophage-inflammatory protein 1β (MIP-1β) were measured in the cell-free-supernatants by using specific ELISA developed with Abs purchased from commercial sources: Biosource International (Camarillo, CA) for IL-1ra and R&D Systems (Minneapolis, MN) for MIP-1β. The detection limits of these ELISA were 50 pg/ml for IL-1ra and 30 pg/ml for MIP-1β (32).

**FIGURE 1.** IL-10-mediated Stat3 tyrosine phosphorylation in LPS-cultured neutrophils. A, Neutrophils and autologous PBMC, freshly isolated or cultured for 4 h in standard medium with or without 100 ng/ml LPS, were stimulated in the presence or absence of 200 U/ml IL-10 or 1000 U/ml IFN-α for 20 min before lysis. B and C, Neutrophils, freshly isolated or cultured for 4 h in standard medium with or without 100 ng/ml LPS, were stimulated with 200 U/ml IL-10 (B and C) or 1000 U/ml IFN-γ (B) for 20 min before lysis. D, Neutrophils cultured for 4 h in serum-free medium in the presence or absence of various concentrations of LPS (previously pretreated with 10 μg/ml PMX) were stimulated with 200 U/ml IL-10 for 20 min before lysis. Conditions of cell lysis are described in Materials and Methods. Thirty micrograms of PMN or PBMC lysate was usually loaded on the gels and immunoblots were performed using Abs specific for tyrosine-phosphorylated forms of Stat3. Subsequent reblotting with the indicated anti-Stat3 Abs was conducted to ensure that similar amounts of material were deposited in each lane. The data for each panel are representative of at least four independent experiments.
Data are expressed as means ± SEM. Statistical evaluation was performed using Student’s $t$ test and considered to be significant if $p < 0.05$.

**Results**

**Stat3 is massively phosphorylated on tyrosine residues by IL-10 stimulation in LPS-cultured but not in freshly isolated human neutrophils**

We have previously reported that IL-10 usually fails to induce detectable tyrosine phosphorylation of Stat3 in human neutrophils (Ref. 20 and Fig. 1A). In this regard, no detectable induction of Stat3 tyrosine phosphorylation by IL-10 was observed in freshly isolated neutrophils whether they were stimulated in the absence of serum or in polypropylene tubes instead of tissue culture well plates. Furthermore, Stat3 was found constitutively phosphorylated on tyrosine residues in freshly isolated neutrophils of a number of donors (Fig. 1, B and C), but, despite this, IL-10 was either completely ineffective (Fig. 1B) or only weakly stimulatory (Fig. 1C). In these latter particular cases, however, tyrosine-phosphorylated Stat3 was detected only in purified cytoplasmic fractions and not in nuclear fractions (data not shown). By contrast, Stat3 became highly tyrosine phosphorylated if neutrophils were stimulated with IFN-γ (Fig. 1A) or G-CSF (33), indicating that the lack of responsiveness of freshly isolated PMN to IL-10 cannot be attributed to a general inability of the cells to activate the Jak1/Stat3 signaling pathway. In addition, Stat3 was highly phosphorylated on tyrosine residues in IL-10-treated autologous PBMC (Fig. 1A). Unexpectedly, in all donors, IL-10 proved to be a very potent inducer of Stat3 tyrosine phosphorylation if neutrophils are first cultured for 4 h with LPS in standard medium (RPMI 1640 medium supplemented with 10% low-endotoxin FCS) (Fig. 1). Under the latter conditions, a strong tyrosine phosphorylation of Stat3 in response to IL-10 (100–500 U/ml) is already evident by 5 min, reaches a maximum at 15 min, and is still detectable at 45 min (data not shown). Culture in LPS-free standard medium also renders the neutrophils responsive to IL-10 in terms of Stat3 tyrosine phosphorylation, but less markedly so than in LPS-treated cells (Fig. 1). However, the ability of standard medium to render neutrophils responsive to IL-10 was not abrogated by PMX (data not shown), thereby excluding an eventual role of contaminating endotoxin in the medium or in the FCS. Importantly, the ability of neutrophils to strongly respond to IL-10 after a 4-h period of culture was essentially unaffected by the presence of serum in the culture medium (Fig. 1D). As expected, the effect of LPS in neutrophils cultured under serum-free conditions was completely abrogated by PMX (Fig. 1D) and required LPS concentrations above 10 ng/ml (Fig. 1D), with no significant difference between 100 and 1000 ng/ml (data not shown). Finally, time course experiments revealed that neutrophils acquire the capacity to phosphorylate Stat3 on tyrosine residues upon IL-10 stimulation only if cultured with LPS for at least 3 h (data not shown), regardless of the presence of serum in the medium. Taken together, these experiments indicate that LPS renders neutrophils responsive to IL-10 in terms of Stat3 tyrosine phosphorylation, but that simple in vitro culture may also be effective, albeit to a lesser extent.

**Induction of Stat-binding complexes by IL-10 in cultured but not in freshly isolated neutrophils**

We next determined whether IL-10-mediated tyrosine phosphorylation of Stat proteins in cultured neutrophils was paralleled by their functional abilities to exert DNA-binding activities. Fig. 2A shows that although no DNA-binding activities are detectable in whole-cell extracts of freshly isolated PMN stimulated with IL-10, GRR-binding activities (and hSIE-binding activities as well, see below) are consistently detected in preparations from neutrophils stimulated with IL-10 after a 4-h culture with or without LPS, and contain both Stat3 and Stat1 (Fig. 2B).

**Acquisition of the IL-10-induced tyrosine phosphorylation of Stat3 by cultured neutrophils is dependent on de novo protein synthesis**

To start elucidating the mechanisms whereby neutrophils acquire the capacity to respond to IL-10 in terms of Stat3 tyrosine phosphorylation, we examined the effect of the protein synthesis inhibitor CHX. Fig. 3A shows that Stat3 tyrosine phosphorylation in response to IL-10 is abrogated if neutrophils are cultured in the presence of CHX. However, under similar experimental conditions, tyrosine phosphorylation of Stat3 in preparations from G-CSF-stimulated neutrophils is consistently detectable at almost identical levels (Fig. 3A), thus excluding any eventual aspecific toxicity of CHX. Identical findings were obtained in experiments performed under serum-free conditions (data not shown) or by testing the functional ability of phosphorylated Stats to bind target sequences (hSIE) in EMSA (Fig. 3B). Furthermore, highlighting the specificity of the up-regulatory effect of LPS on IL-10 responsiveness, G-CSF-induced Stat3 tyrosine phosphorylation was greatly attenuated in LPS-treated neutrophils (Fig. 3A), in keeping with...
with the reported ability of LPS to down-regulate the G-CSF receptors in cultured neutrophils (34, 35). Taken together, these data suggest that novel protein synthesis is required to render cultured neutrophils fully responsive to IL-10 in terms of Stat3 tyrosine phosphorylation and activation. Accordingly, previous findings (36) also demonstrated that new protein synthesis is necessary for the IL-10-mediated cytokine modulation in human neutrophils. Based on the pattern of Stat3 protein expression revealed by our Western blot analyses (Fig. 1), we would tend to exclude that this newly synthesized protein is Stat3 itself. We would also exclude Jak1 or Tyk2 since, as shown in Fig. 1A, the effect of IFN-α (which like IL-10 utilizes Tyk2 and Jak1 to transmit its intracellular signal) is very potent in freshly isolated neutrophils, but decreases after cell culture, especially with LPS.

LPS strongly up-regulates IL-10R1 mRNA and protein expression in neutrophils

Previous studies using biotinylated IL-10 have demonstrated that freshly isolated neutrophils possess detectable IL-10 binding sites, albeit to a much lesser extent than peripheral blood monocytes or lymphocytes (19, 37). Because the receptor for IL-10 consists of two components (IL-10R1 and IL-10R2) whose expression and regulation in neutrophils has never been characterized, we investigated whether a modulation of the IL-10R mRNA and surface expression might be involved in LPS-mediated induction of IL-10 responsiveness observed herein. Initial studies revealed that although both IL-10R1 and IL-10R2 transcripts are constitutively expressed in freshly isolated human neutrophils, IL-10R1 mRNA expression is consistently lower than in autologous PBMC (data not shown). However, a 90-min culture of neutrophils in RPMI 1640 medium (with or without FCS) significantly increased the gene expression of IL-10R1; this effect was even more pronounced in the presence of LPS (Fig. 4A). By contrast, IL-10R2 mRNA levels showed only minimal variation between freshly isolated neutrophils and cultured cells (Fig. 4), suggesting that also the IL-10R2 gene has its own set of regulators. The increase in IL-10R1 mRNA steady-state levels in response to LPS was not inhibited by CHX (data not shown) and was time dependent, peaking at 3 h and gradually decreasing thereafter (Fig. 4B).

Anti-hIL-10R1 and -hIL-10R2 mAbs enabled us to subsequently analyze the surface expression of both IL-10R subunits in leukocytes. In line with RNA blot data, indirect immunofluorescence flow cytometry (FACS) analysis revealed that circulating neutrophils and mononuclear cells have both IL-10R1 and IL-10R2 on their surface, with IL-10R1 being poorly expressed in neutrophils compared with other leukocytes (Fig. 5). Identical results were obtained if cells were analyzed for IL-10R expression right after isolation (data not shown). However, surface expression of IL-10R1 was significantly up-regulated if neutrophils were cultured for 4 h in culture medium with or without serum or LPS.

**FIGURE 3.** Acquisition of the IL-10-induced Stat activation is dependent on de novo protein synthesis. A, Neutrophils cultured for 4 h in standard medium with or without 20 μg/ml CHX in the presence or absence of 100 ng/ml LPS were stimulated with 200 U/ml IL-10 or 1000 U/ml G-CSF for 20 min before lysis. Conditions of cell lysis and blotting are described in the legend to Fig. 1. B, Neutrophils cultured for 4 h in standard medium with or without 20 μg/ml CHX in the presence or absence of 100 ng/ml LPS were stimulated with 200 U/ml IL-10 for 20 min. Whole-cell extracts were then prepared and analyzed in EMSA using 32P-labeled hSIE oligonucleotide. A total of 12 μg of protein from PMN extracts was used in the binding reactions. The data for each panel are representative of at least three independent experiments.

**FIGURE 4.** LPS up-regulates IL-10R1 mRNA expression in neutrophils. A, Neutrophils, freshly isolated or cultured for 90 min in standard or serum-free medium in the presence or absence of 100 ng/ml LPS, were lysed for total RNA extraction. This experiment is representative of two. B, Neutrophils cultured for the times indicated in standard medium with or without 100 ng/ml LPS were lysed for total RNA extraction. This experiment is representative of four. Ten micrograms of total RNA was loaded on each gel lane. IL-10R1, IL-10R2, and actin mRNA expression were analyzed by Northern blot.
The up-regulation of neutrophil IL-10R1 surface expression was always more evident if LPS was present in standard medium (Figs. 6 and 7), and was completely blocked by CHX (Fig. 7). By comparison, the surface expression of CR3 (CD11b/CD18) was not significantly affected by CHX under the same culture conditions (Fig. 7). Taken together, these data show that IL-10R1 receptor expression is markedly up-regulated in LPS-cultured neutrophils, and that this phenomenon relies on de novo protein synthesis.

**Up-regulation of IL-10R1 expression is essential for IL-10-mediated cytokine modulation in neutrophils**

Since IL-10 is a potent modulator of cytokine/chemokine release in neutrophils cultured overnight with LPS (17, 18), including those of IL-1ra (up-regulatory effect, Fig. 8 A) (38) and MIP-1β (inhibitory effect, Fig. 8 C) (36), we analyzed the functional role of IL-10R1 and IL-10R2 on IL-1ra and MIP-1β production using specific neutralizing anti-IL-10R Abs. As shown in B and D of Fig. 8, the modulatory action of IL-10 on LPS-induced IL-1ra and MIP-1β secretion is mostly abrogated if neutrophils are cultured in the presence of anti-IL-10R1- or anti-IL-10R2-neutralizing Abs, indicating that both IL-10R chains are essential for mediating the effect of IL-10 (1, 39). Importantly, Fig. 8 also makes it clear that a characteristic feature of the IL-10-mediated modulation of cytokine/chemokine production by LPS-stimulated neutrophils is that it starts to occur only after 3–4 h of cell culture, as we and others have reported (36, 38, 40, 41). Similarly, IL-10 only starts to modulate the LPS-induced accumulation of cytokine/chemokine mRNA after 2–3 h in culture (36, 38, 40, 41). Remarkably, the effect of neutralizing anti-IL-10R1 or anti-IL-10R2 mAbs (measured after an overnight culture) does not appear to be affected by the time of their addition to the neutrophil cultures (Fig. 9), i.e., whether it is concurrently with or up to 3–4 h after LPS and IL-10. The latter findings are consistent with the requirement of a lag time necessary to induce the synthesis and expression of IL-10R1 by LPS-treated neutrophils, as shown above.

**Massive induction of SOCS-3 mRNA by IL-10 in cultured but not in freshly isolated neutrophils**

We have recently reported that SOCS-3 mRNA steady-state levels are specifically up-regulated by IL-10 in freshly isolated neutrophils (20). In the light of the findings described above, we wanted to determine whether this function of IL-10 was modulated in cultured neutrophils. The experiment shown in Fig. 10A demonstrates that this is indeed the case, as induction of SOCS-3 mRNA expression by IL-10 resulted dramatically stronger in cultured than in freshly isolated neutrophils (by an average of ~6- to 7-fold). Similarly to the acquisition of Stat3 activation (Fig. 3), the enhanced accumulation of SOCS-3 mRNA in response to IL-10 was almost completely abrogated if neutrophils were cultured in the presence of CHX (Fig. 10A). CHX itself superinduced SOCS-3 mRNA expression in either freshly isolated or in cultured PMN, without however influencing the effect of IL-10 in freshly isolated PMN (Fig. 10A). Taken together, these data demonstrate that the increased ability of IL-10 to up-regulate SOCS-3 mRNA expression in cultured neutrophils relies on de novo protein synthesis. Once again, the results are consistent with the notion that the up-regulation of IL-10R1 expression in neutrophils is required to render them completely responsive to IL-10.

**Effect of neutralizing anti-IL-10R Abs on the ability of IL-10 to activate SOCS-3 synthesis and Stat3 tyrosine phosphorylation in cultured neutrophils**

The availability of specific anti-SOCS-3 Abs (28) enabled us to demonstrate, for the first time, that neutrophils are able to synthesize SOCS-3 protein in response to IL-10 (Fig. 10B). However,
induction of SOCS-3 Ag was consistently detected only in lysates prepared from neutrophils stimulated with IL-10 after culture (with or without LPS), but not right after isolation (Fig. 10B), correlating with the higher levels of SOCS-3 mRNA expression induced by IL-10 in cultured cells (Fig. 10A). By contrast, GM-CSF, a cytokine known to potently modulate expression of SOCS members in neutrophils and other myeloid cells (20, 31), was found to potently induce SOCS-3 protein in freshly isolated as well as in cultured neutrophils (Fig. 10B), thus excluding a general inability of freshly isolated PMN to synthesize SOCS-3. Importantly, in addition to reversing the effects of IL-10 on cytokine production (Figs. 8 and 9), both anti-IL-10R1- and anti-IL-10R2-neutralizing Abs completely suppressed the induction of SOCS-3 protein (Fig. 10C) and Stat3 tyrosine phosphorylation (Fig. 10D) by IL-10 in cultured neutrophils, suggesting a causal relationship among these events.

FIGURE 8. Effect of neutralizing anti-IL-10R Abs on the ability of IL-10 to modulate LPS-induced release of IL-1ra and MIP-1β by neutrophils. A and C, PMN (5 x 10^6/ml) were preincubated with or without 200 U/ml IL-10 for 20 min and then cultured for up to 21 h after the addition of 100 ng/ml LPS. The cell-free supernatants were collected and the levels of IL-1ra and MIP-1β were determined by ELISA. The experiment depicted is representative of seven. B and D, Neutrophils were preincubated with neutralizing anti-IL-10R1 (3B6) and anti-IL-10R2 (1A8.3) mAbs before the addition of 200 U/ml IL-10. After 20 min, 100 ng/ml LPS was added to the cells and cultures were conducted for up to 21 h. The panels report the mean values ± SEM (n = 4) of the percentage of inhibition exerted by the neutralizing anti-IL-10R Abs on the modulatory effects of IL-10 on, respectively, IL-1ra and MIP-1β extracellular production induced by LPS.
Discussion

We have recently shown that IL-10 fails to induce phosphorylation of Stat1 and Stat3 on tyrosine residues (20) and binding of multimeric complexes containing both Stat1 and Stat3 to the GRR of Stat1 and Stat3 on tyrosine residues (20) and binding of multiple complexes containing both Stat1 and Stat3 to the GRR sequence of the FcγRI gene promoter in human neutrophils freshly isolated from the blood of normal donors (19, 20). These characteristics are in sharp contrast with the effect of IL-10 on the same responses in other cell types, such as monocytes and PBMC (12, 13, 19, 20, 42, 43). Since IL-10 is an established regulator of cytokine production in LPS-treated neutrophils (17, 18), our data raised the possibility that the modulation of cytokine production by IL-10 in human neutrophils might occur independently of Stat3 protein activation. In conflict with this notion, however, were some studies that, directly or indirectly, suggested an obligatory role for Stat3 in mediating this action of IL-10 (22, 23). In particular, neutrophils and 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 TNF-α upon stimulation with IL-10 plus LPS (23). We observed that contrary to its effect on freshly isolated human neutrophils, IL-10 is a very effective inducer of Stat3 tyrosine phosphorylation in bone marrow-derived murine neutrophils (S. Gasperini and M. A. Cassatella, unpublished data). Together, these various observations encouraged us to further study the molecular mechanisms by which IL-10 signals in human neutrophils, also in consideration of other peculiar features concerning the action of IL-10. One of these features is that the molecular mechanism whereby IL-10 inhibits LPS-inducible gene expression in human neutrophils has not been defined at all. We know, for instance, that IL-10 does not inhibit activation of NF-κB in freshly isolated human neutrophils activated by LPS or TNF-α (P. P. McDonald and M. A. Cassatella, unpublished data), in line with some studies performed in human monocytic cells (44, 45). Another characteristic feature is that we (38, 40) and others (36, 41) have often highlighted that, particularly in human neutrophils, the extracellular release of cytokines/chemokines, as well as the LPS-induced accumulation of mRNA encoding these cytokines/chemokines, are significantly modulated by IL-10 only after 4 h of cell culture and not at early time points of incubation. The latter observations suggest that IL-10 requires some time to be able to act on cultured neutrophils. In agreement with this necessity, it is well known that inhibition of cytokine gene expression by IL-10 in neutrophils and monocytes requires new protein synthesis (36, 46). Therefore, we reasoned that in the particular case of human neutrophils, correlating early events, such as the undetectable Stat3 phosphorylation, with late events, such as the modulation of cytokine production, although substantially acceptable, might have been perhaps too speculative.

The findings presented in the current study establish that also in human neutrophils IL-10 may activate a strong tyrosine phosphorylation of Stat3- and Stat-binding activities to the GRR and hSIE probes, but this activation occurs only if IL-10R1 expression is up-regulated. This latter event is obtained when neutrophils are appropriately stimulated, for instance with LPS, and as a result synthesize more IL-10R1. Interestingly, responsiveness to IL-10 in terms of Stat3 tyrosine phosphorylation was observed also in neutrophils cultured for 4 h in the absence of LPS. Although in the latter experimental conditions IL-10-mediated activation of Stat3 was usually lower than that observed with LPS-containing medium, the data suggest that factors other than endotoxin may "prime" neutrophils for IL-10 responsiveness. We also show that responsiveness to IL-10 by cultured neutrophils is acquired through a molecular mechanism dependent on new protein synthesis. A series of evidence indicate that this (or maybe one of these) newly synthesized protein(s) is the IL-10R1, as opposed to the classical components of the Jak-Stat signaling pathway that can be activated by IL-10 (namely, Jak1, Tyk2, Stat1, or Stat3). IL-10R1 is in fact expressed on the membrane of circulating or freshly isolated neutrophils at minimal but sufficient levels to bind IL-10 (19, 37), but dramatically augments on the cell surface after incubation with LPS (Figs. 3, 5, and 6). This increased surface expression of IL-10R1 correlates well with the capacity of IL-10 to either activate Stat3 tyrosine phosphorylation and Stat3-dependent DNA-binding activities to target sequences or to modulate IL-1ra and MIP-1β release by LPS-stimulated neutrophils. Consistent with the fact that a fully functional receptor complex is not present in freshly isolated neutrophils, suppression of the effects of IL-10 (modulation of cytokine production) was also obtained if neutralizing anti-IL-10R1 (and anti-IL-10R2 as well) Abs were added to the neutrophil culture before and after IL-10 at the times indicated. IL-1ra and MIP-1β release into the cell-free supernatants were measured by specific ELISA. Mean values of duplicate assays from a single representative experiment of three performed with similar results are shown.

![FIGURE 9.](http://www.jimmunol.org/) Effect of delayed anti-IL-10R Abs addition on the ability of IL-10 to modulate LPS-induced cytokine release by neutrophils. Neutrophils (5 x 10⁶/ml) were preincubated with or without 200 U/ml IL-10 for 20 min and then cultured for 21 h after the addition of 100 ng/ml LPS. Neutralizing anti-IL-10R1 (3B6), anti-IL-10R2 (1A8.3), and control IgG1 were added to the neutrophil culture before and after IL-10 at the times indicated. IL-1ra and MIP-1β release into the cell-free supernatants were measured by specific ELISA. Mean values of duplicate assays from a single representative experiment of three performed with similar results are shown.
and did not appear to be significantly influenced by the culture conditions. Furthermore, and in agreement with previous findings obtained by using biotinylated IL-10 (19) or a different anti-IL-10R1 Ab (47), we confirm that whole blood or freshly isolated monocytes and lymphocytes (PBMC) express surface levels of both IL-10R1 and IL-10R2 that are consistently higher than those found in autologous neutrophils. This might explain why IL-10 rapidly activates Stat tyrosine phosphorylation in freshly isolated PBMC, as opposed to neutrophils (20). However, the relevance of IL-10R2 in mediating IL-10 signaling (in association with the IL-10R1 chain) was demonstrated in neutrophils by the capacity of neutralizing anti-IL-10R2 mAbs to suppress the effects of IL-10 as much as the neutralizing anti-IL-10R1. In this regard, LPS was previously shown to selectively induce IL-10R1 expression in murine L929 fibroblasts, which do not constitutively express IL-10R1 (48), but despite this, the cells remained unresponsive to IL-10 with respect to the activation of Stat(s). Similarly, transfection of the murine IL-10R1 chain into L929 fibroblasts conferred to these cells the ability to bind murine IL-10, but again, IL-10 failed to induce Stat activation in these cells (48), suggesting the lack of a component critical for the generation of the IL-10 signaling. The data presented herein demonstrate that in human neutrophils IL-10R2 is already present at sufficient levels whereas IL-10R1 needs to be up-regulated, at least for inducing optimal activation of Stat3. Thus, both chains of the IL-10R complex appear to be critical for Stat signaling (39). By contrast, the Jak/Stat signaling pathway may not be required for the activation of other selective actions induced by IL-10 (16, 20, 49). We note recent findings suggesting that IL-10R2 is a shared receptor chain for IL-10 and the IL-10-related T cell-derived inducible factor (IL-T cell-derived inducible factor/IL-22) (50, 51) and possibly other IL-10 homologues (1, 51). Whether neutrophils express the ligand binding chains for these proteins (e.g., CRF2–9 for IL-T cell-derived inducible factor/IL-22) remains to be determined.

In the present work, we also demonstrate that the ability of IL-10 to up-regulate the expression of SOCS-3 mRNA in neutrophils (20) was strongly enhanced if PMN are stimulated with IL-10 after a 3- to 4-h period of culture. This augmented responsiveness to IL-10 was inhibited by CHX, likely as a result of the inhibitory effect of the drug on the IL-10R1 expression. Very importantly, induction of SOCS-3 mRNA by IL-10 in cultured neutrophils was also followed by the synthesis of antigenic SOCS-3 protein (20). No intracellular SOCS-3 protein was in fact detected in freshly isolated neutrophils (20). Together with previous observations (20), our results suggest that, in human neutrophils, SOCS-3 protein is synthesized in response to IL-10, despite a significant accumulation of SOCS-3 mRNA (20). Furthermore, activation of Stat3 tyrosine phosphorylation and synthesis of SOCS-3 Protein were both prevented if cultured neutrophils were stimulated with IL-10 in the presence of neutralizing anti-IL-10R1 and anti-IL-10R2 mAbs. Together with previous observations (20), our results suggest that, in human neutrophils, SOCS-3 protein is synthesized in response to IL-10 only in coincidence of a strong induction of Stat3 tyrosine phosphorylation and only when SOCS-3 mRNA is accumulated at very high levels.

Based on the current findings, we cannot exclude that other unidentified polypeptides are also synthesized de novo and contribute to render neutrophils fully responsive to IL-10. Certainly, the capacity of IL-10 to induce a strong Stat3 tyrosine phosphorylation and to modulate cytokine gene expression and release in LPS-activated neutrophils seem to correlate, supporting previous studies demonstrating a critical role of the Jak1/Stat3 pathway in the

FIGURE 10. SOCS-3 mRNA and protein expression in freshly isolated and cultured neutrophils stimulated with IL-10. A, Neutrophils, freshly isolated or cultured for 3 h in standard medium, were stimulated with 200 U/ml IL-10 for 90 min before total RNA extraction. Analysis for SOCS-3 mRNA expression was then performed by Northern blot. This experiment is representative of three. B, Neutrophils, freshly isolated or cultured for 4 h in standard medium, were stimulated with 200 U/ml IL-10 or 10 ng/ml GM-CSF for 2 h before lysis. PMN lysates (100 µg) were loaded on the gels, and immunoblots were performed using Abs specific for SOCS-3. This experiment is representative of three. C, Neutrophils cultured for 3 h with LPS, were treated for 20 min with 200 U/ml IL-10 in the presence of neutralizing anti-IL-10R1, anti-IL-10R2, and isotype control mAbs before lysis for characterization of SOCS-3 protein expression by immunoblotting. This experiment is representative of three. D, Neutrophils, freshly isolated or cultured for 4 h with LPS, were treated for 20 min with 200 U/ml IL-10 in the presence of neutralizing anti-IL-10R1, anti-IL-10R2, and isotype control mAbs before lysis for characterization of Stat3 tyrosine phosphorylation. This experiment is representative of two.
IL-10-mediated deactivation of phagocytes (22, 23). Our findings also provide some indications helping to clarify the reasons explaining the controversial data existing in the literature on the ability of IL-10 to directly trigger or modulate specific effector functions in human neutrophils (17). Based on the present results, it can be reasonably speculated that if the conditions or the materials and reagents used for neutrophil isolation and culture are not strictly controlled (for instance by a careful monitoring of endotoxin content/contamination of solutions, buffers, and culture media), the cells can easily become “primed” during the isolation procedure and, as a consequence, be rendered more responsive to IL-10 through up-regulation of IL-10R1. Finally, our findings suggest that LPS not only activates neutrophils to produce and release a host of proinflammatory mediators, but also prepares these cells to be subsequently able to respond immediately to anti-inflammatory signals such as IL-10, presumably to help limit the extent of inflammatory reactions.

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References
4. Wehinger, J., F. Gouilleux, B. Groner, J. Finke, R. Mertelsmann, and
5. Geng, Y., E. Gulbins, A. Altman, and M. Lotz. 1994. Monocyte deactivation by
K. W. Moore, and J. Xu. 1997. The Epstein-Barr virus interleukin-10 (IL-10)

citation and functional characterization of a second chain of the interleukin-10 receptor gene family maps on chromosome 21 at less than 35 kb from IFNAR. Mol.

affinity receptor for IgG (FcγRUCD64) gene and STAT5 and their distinct combinatorial assembly in the promoters of selected genes. FEBS Lett. 594:365.


14. Wehinger, J., F. Gouilleux, B. Groner, J. Finke, R. Mertelsmann, and
15. Wehinger, J., F. Gouilleux, B. Groner, J. Finke, R. Mertelsmann, and


affinity receptor for IgG (FcγRUCD64) gene and STAT5 and their distinct combinatorial assembly in the promoters of selected genes. FEBS Lett. 594:365.


45. Dokter, W. H. A., S. B. Koopmans, and E. Vellenga. 1996. Effects of IL-10 and IL-4 on LPS-induced transcription factors (AP-1, NF-IL6 and NF-κB) which are involved in IL-6 regulation. Leukemia 10:1308.


