The EMBO Journal Vol.17 No.4 pp.1006–1018, 1998

IL-10 inhibits macrophage activation and proliferation by distinct signaling mechanisms: evidence for Stat3-dependent and -independent pathways

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Interleukin-10 (IL-10) limits inflammatory responses by inhibiting macrophage activation. In macrophages, IL-10 activates Stat1 and Stat3. We characterized IL-10 responses of the J774 mouse macrophage cell line, and of J774 cells expressing wild-type hIL-10R, mutant hIL-10R lacking two membrane-distal tyrosines involved in recruitment of Stat3 (hIL-10R-Tyr^{FF}), a truncated Stat3 (ΔStat3) which acts as a dominant negative, or an inducibly active Stat3–gyraseB chimera (Stat3–GyrB). A neutralizing anti-mIL-10R monoclonal antibody was generated to block the function of endogenous mIL-10R. IL-10 inhibited proliferation of J774 cells and of normal bone marrow-derived macrophages, but not J774 cells expressing hIL-10RTyr^{FF}. Dimerization of Stat3–GyrB by coumermycin mimicked the effect of IL-10, and expression of ΔStat3 blocked the anti-proliferative activity of IL-10. For macrophage de-activation responses, hIL10R-Tyr^{FF} could not mediate inhibition of lipopolysaccharide-induced TNFα, IL-1β or CD86 expression, while ΔStat3 did not interfere detectably with these IL-10 responses. Thus signals mediating both anti-proliferative and macrophage de-activation responses to IL-10 require the two membrane-distal tyrosines of IL-10R, but Stat3 appears to function only in the anti-proliferative response.

Keywords: IL-10/IL-10 receptor/macrophage deactivation/proliferation/Stat3

Introduction

The inflammatory response is a key component of host defense, but excessive inflammation such as occurs in arthritis or septic shock can be harmful or even fatal. Therefore, inflammation must be tightly regulated *in vivo*. Interleukin-10 (IL-10) is a key inhibitor of many aspects of the inflammatory response. In lipopolysaccharide (LPS)-activated macrophages, production of pro-inflammatory cytokines [IL-1β, IL-6, granulocyte–macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor α (TNFα)] and chemokines [IL-8, macrophage inflammatory peptide-1α (MIP-1α)], generation of nitric oxide and up-regulation of surface antigen expression (MHC class II, CD80, CD86) are all strongly inhibited by IL-10 (for review, see Moore *et al.*, 1993). The importance of IL-10 in regulating the inflammatory response is indicated by the phenotype of the IL-10-knockout mouse, which develops chronic enterocolitis and exhibits dysregulated inflammatory responses (Kuhn *et al.*, 1993; Berg *et al.*, 1995; Rennick *et al.*, 1995).

The IL-10 receptor (IL-10R) is a member of the class II interferon receptor (IFNR)-like cytokine receptor family (Ho and Moore, 1994; Liu *et al.*, 1994). The best understood signaling pathway activated by these receptors is the Jak/Stat pathway. Stats are transcription factors, which are normally resident in the cytoplasm in monomeric inactive forms (Sadowski *et al.*, 1993; Darnell *et al.*, 1994; Shuai *et al.*, 1994). Tyrosine phosphorylation by Jak kinases triggers dimerization of Stats, which translocate to the nucleus and transactivate target genes. Stimulation of IL-10R-expressing cells (including macrophages) with IL-10 leads to activation of Jak1 and Tyk2, and Stats 1 and 3 (Finbloom and Winestock, 1995; Ho *et al.*, 1995; Lai *et al.*, 1996; Wehinger *et al.*, 1996). Stat3 is recruited directly to sequences surrounding two membrane-distal tyrosines in the IL-10R cytoplasmic domain, and these tyrosines are redundantly required for Stat3 activation and receptor function in Ba/F3 cells expressing mIL-10R (Weber-Nordt *et al.*, 1996a). Stat5 can also be activated in response to IL-10 (Wehinger *et al.*, 1996), although this has not been observed in macrophages (Weber-Nordt *et al.*, 1996a; A.-M.O'Farrell, unpublished results).

To study IL-10 signal transduction in macrophages, we characterized the IL-10 responses of J774, a mouse monocyte/macrophage cell line. Addition of IL-10 alone to J774 cells revealed a previously undescribed activity for IL-10, inhibition of macrophage proliferation. Addition of IL-10 to J774 cells in the presence of LPS inhibited macrophage activation by LPS. To investigate the role of Stats in these responses, we dissected IL-10 signal transduction using J774 cells expressing mutant IL-10Rs and inducibly active Stat3 or dominant-negative Stat3. The results provide evidence for the existence of Stat3-dependent and -independent IL-10 signaling pathways in macrophages.

Results

Isolation of a neutralizing anti-mIL-10R mAb

Rat monoclonal antibodies (mAbs) raised against mIL-10R were generated as described for anti-hIL-10R mAb 3F9 by immunization with purified recombinant soluble mIL-10R-FLAG-His$_6$ (Liu *et al.*, 1997). mAb 1B1.2 was identified by enzyme-linked immunosorbent assay (ELISA) on soluble mIL-10R-FLAG-His$_6$-coated plates, then tested for its ability to stain Ba/F3 cells expressing mIL-10R (BaF-mIL-10R) and hIL-10R (Ho *et al.*, 1993; Liu *et al.*, 1994). mAb 1B1.2 stains BaF-mIL-10R (Figure 1A, filled histogram) but not BaF-hIL-10R cells

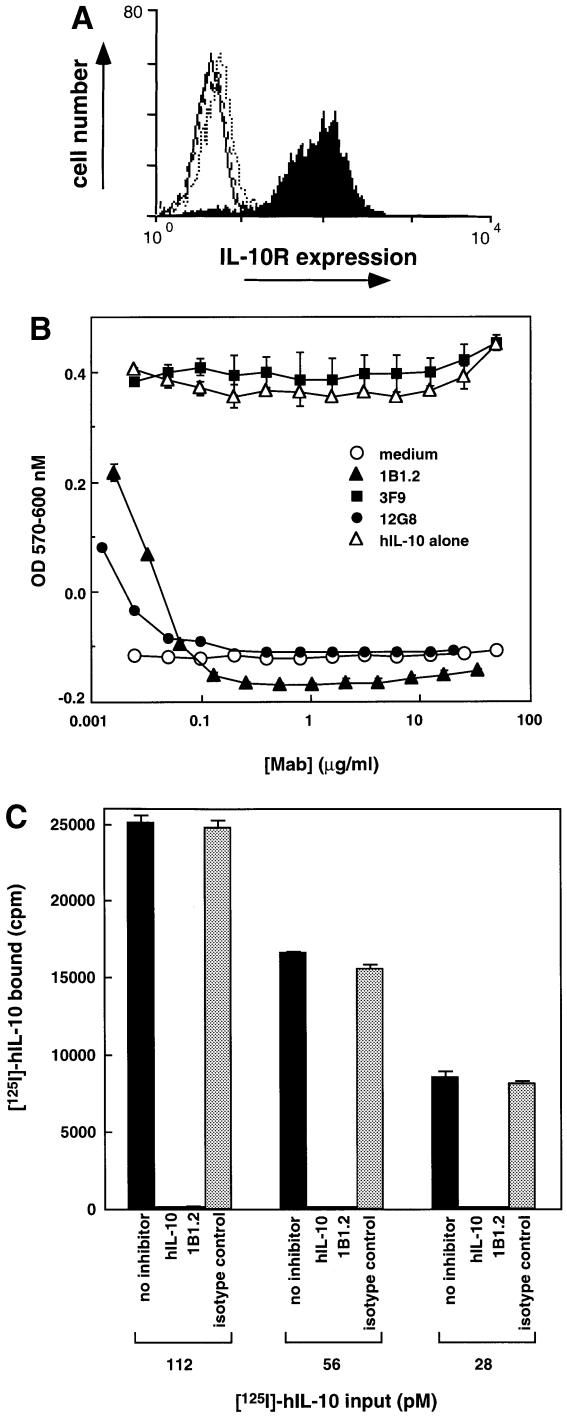


Fig. 1. Characterization of mAbs against mIL-10R. (**A**) Staining of BaF-mIL-10R cells with 1B1.2. BaF-mIL-10R cells were stained with isotype control (dotted histogram), mAb 3F9 (anti-hIL-10R) (dashed line histogram) or mAb 1B1.2 (anti-mIL-10R) (filled histogram). Baf-hIL-10R cells stained with mAb 1B1.2 are also shown (solid line histogram). (**B**) mAb 1B1.2 inhibits IL-10-induced short-term proliferation of BaF-mIL-10R cells. BaF-mIL-10R cells were cultured in the presence or absence of 6 ng/ml hIL-10 and the indicated amounts of mAb 1B1.2, mAb 12G8 (anti-hIL-10) or mAb 3F9 (anti-hIL-10R). After 48 h, Alamar Blue was added, and 8 h later the $A_{570-600}$ was determined. Error bars represent the range of duplicate samples. (**C**) mAb 1B1.2 inhibits binding of [^{125}I]hIL-10 to BaF-mIL-10R cells. BaF-mIL-10R cells were incubated with the indicated concentrations of [^{125}I]hIL-10, in the presence or absence of 200 nM hIL-10, 20 μg/ml mAb 1B1.2 or isotype control mAb (GL113) for 4.5 h at 4°C. Bound [^{125}I]hIL-10 was measured in a gamma counter. Error bars represent the standard deviation of triplicate samples.

(solid line histogram), indicating its specificity for mIL-10R. Furthermore, mAb 1B1.2 inhibits IL-10-stimulated short-term proliferation of BaF-mIL-10R cells (Figure 1B) but not BaF-hIL-10R cells (data not shown); as little as 0.1 μg/ml completely blocked the response of BaF-mIL-10R cells. mAb 3F9 had no effect on BaF-mIL-10R cells, as described (Liu *et al.*, 1997). Finally, mAb 1B1.2, but not isotype control mAb, blocked binding of [^{125}I]hIL-10 to mIL-10R (Figure 1C). We conclude that mAb 1B1.2 is a neutralizing anti-mIL-10R antibody.

IL-10 inhibits macrophage proliferation

J774 cells proliferate in the presence of serum and do not require exogenous cytokines for growth. IL-10 inhibited proliferation of J774 cells, as measured by [^{3}H]thymidine incorporation (Figure 2A). Inhibition of [^{3}H]thymidine incorporation was dose dependent, apparent at 1 ng/ml IL-10 with maximal inhibitory effect (50–80% over 15 independent experiments) observed at ~100 ng/ml mIL-10. Human (h) IL-10, which cross-reacts with mIL-10R, also inhibited proliferation (Figure 2A). IL-10 also inhibited proliferation of the RAW264.7 mouse monocyte/macrophage cell line (data not shown).

Prolonged culture in the presence of IL-10 caused further suppression of [^{3}H]thymidine incorporation with a maximum inhibition (~70–90% over independent experiments) obtained after 48 h. Trypan blue cell counts showed that viable cell number was maintained in the presence of IL-10 and cell death was not apparent, suggesting that IL-10 did not promote apoptosis of J774 cells. To clarify this possibility further, we assayed for apoptosis by the TUNEL method. J774 cells treated with IL-10 appeared similar to untreated cells (Figure 2B), suggesting that the reduction in [^{3}H]thymidine incorporation was due to cell cycle arrest and not to apoptosis. In contrast, apoptotic cells revealed by bright staining of fragmented DNA are clearly apparent in cells treated with staurosporin which is a positive control for apoptosis. Furthermore, the reduction in [^{3}H]thymidine incorporation in response to IL-10 was associated with an accumulation of cells in the G_1 phase of the cell cycle, suggesting that IL-10 caused a G_1 block (data not shown).

Conditioned medium from IL-10-treated J774 cells (CM^{IL-10}) did not inhibit proliferation of J774 cells in the presence of mAb 1B1.2, suggesting that IL-10 did not stimulate production of an autocrine inhibitory factor (Figure 2C, bars 1 and 2). Suppression of [^{3}H]thymidine incorporation by IL-10 was reversible, as removal of IL-10 or addition of IL-10R neutralizing mAb 1B1.2 led to increased [^{3}H]thymidine incorporation relative to that observed in the presence of IL-10 (Figure 2C, bars 3–5). Addition of CM from untreated J774 cells (Figure 2C, bar 6) did not rescue the growth inhibitory effect of IL-10 more efficiently than addition of fresh culture medium (bar 5), suggesting that J774 cells do not produce an autocrine growth factor. Furthermore, blocking antibodies against IL-1 receptor, GM-CSF receptor, transforming growth factor-β (TGF-β) or macrophage colony-stimulating factor (M-CSF) had no effect on serum-stimulated [^{3}H]thymidine incorporation in J774 cells (A.-M.O'Farrell, unpublished results).

To determine whether these results were peculiar to cell lines, we tested the ability of IL-10 to inhibit proliferation of macrophages derived from normal murine bone marrow

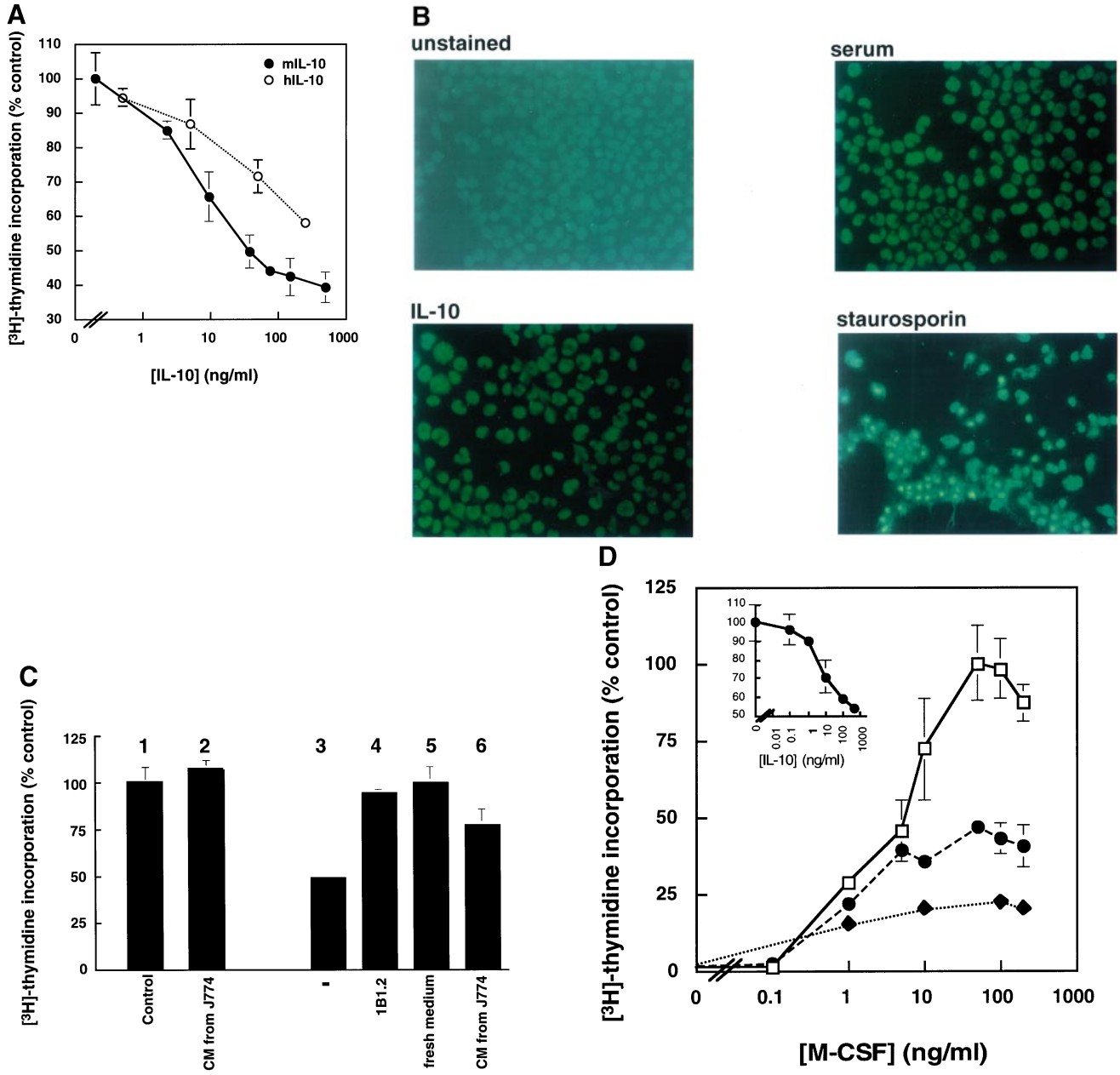


Fig. 2. IL-10 inhibits macrophage proliferation. (**A**) Effect of IL-10 on [^{3}H]thymidine incorporation in J774 cells. J774 cells were cultured in the absence or presence of mIL-10 (●) or hIL-10 (○). [^{3}H]Thymidine incorporation was measured after 30 h. Results are expressed as a percentage of control (cells incubated in the absence of IL-10, 151 173 c.p.m.). Error bars represent the standard deviation of triplicate samples. (**B**) TUNEL assay for apoptosis of J774 cells. J774 cells were cultured in the absence or presence of mIL-10 (200 ng/ml) for 48 h, or staurosporin (10 μM) for 8 h. Apoptotic cells were detected by the TUNEL assay, and immediately visualized by fluorescence microscopy. Unstained cells are overexposed relative to other samples. This experiment was performed three times with similar results. (**C**) Suppression of DNA synthesis by IL-10 is reversible and not an indirect effect. Bars 1 and 2: J774 cells were cultured in fresh medium (control, bar 1, 146 542 c.p.m.) or in J774-CM^{IL-10} (bar 2) for 48 h. Bars 3–6: J774 cells were cultured in the presence of mIL-10 (20 ng/ml) for 24 h, after which either IL-10 was maintained in culture (bar 3), mAb 1B1.2 (10 μg/ml) was added (bar 4), or IL-10 was removed by washing and 100 μl of fresh medium or CM from J774 cells (bars 5 and 6 respectively) was added, for an additional 24 h. [^{3}H]Thymidine incorporation was measured, and error bars represent the standard deviation of triplicate samples. (**D**) Effect of IL-10 on [^{3}H]thymidine incorporation in NBM-derived macrophages. NBM-derived macrophages were cultured in the presence of M-CSF at the concentrations indicated, in the absence (□) or presence of mIL-10 (200 ng/ml) added either simultaneously with (●) or 2 h prior (◆) to M-CSF addition. [^{3}H]Thymidine incorporation was measured after 30 h of culture. Results are expressed as a percentage of control (50 ng/ml M-CSF, 50 974 c.p.m.). Figure inset: NBM-derived macrophages were cultured in the presence of M-CSF (50 ng/ml, control), in the absence or presence of IL-10 at the concentrations indicated. [^{3}H]Thymidine incorporation was measured after 30 h of culture. Error bars represent the standard deviation of triplicate samples.

(NBM). M-CSF stimulated a dose-dependent increase of [^{3}H]thymidine incorporation in NBM-derived macrophages. IL-10 at concentrations above 1 ng/ml inhibited M-CSF-dependent proliferation (Figure 2D, inset) in a dose-dependent manner, similar to inhibition of J774 cell proliferation. Pre-treatment with IL-10 increased the magnitude of inhibition relative to that observed when IL-10 and M-CSF were added simultaneously (Figure 2D).

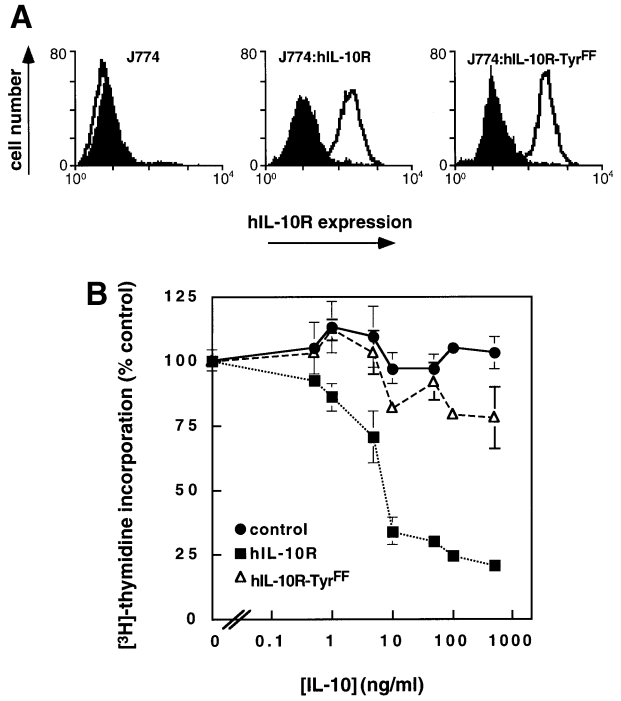


Fig. 3. Proliferation responses of J774:hIL-10R and J774:hIL-10R-Tyr^{FF} cells. (**A**) Expression of hIL-10R and hIL-10R-Tyr^{FF} on J774 cells. J774 cells were transduced with hIL-10R and hIL-10R-Tyr^{FF}. hIL-10R (open histogram) or isotype control (solid histogram) staining of puromycin-resistant clones used in the experiments described is shown. (**B**) Effect of IL-10 on [^{3}H]thymidine incorporation in J774:hIL-10R cells. J774 (●), J774:hIL-10R (■) and J774:hIL-10R-Tyr^{FF} (△) cells were cultured in the absence (control) or presence of hIL-10 at the concentrations indicated, in the presence of 1B1.2 (10 μg/ml). [^{3}H]Thymidine incorporation was measured after 30 h. Controls were 72 909, 41 270 and 70 003 c.p.m. for J774, J774:hIL-10R and J774:hIL-10R-Tyr^{FF} respectively.

IL-10 also inhibited proliferation of NBM-derived macrophages stimulated by GM-CSF or L-cell CM (data not shown).

Tyr446 and Tyr496 of hIL-10R, and Stat3 mediate the growth inhibitory effects of IL-10

hIL-10R is functional in mouse cells, and two of the four cytoplasmic tyrosine (Tyr) residues in mIL-10R (Tyr427 and Tyr477) are conserved in hIL-10R (Tyr446 and Tyr496) (Liu *et al.*, 1994, 1997). These residues are both involved in recruitment and activation of Stat3 (Weber-Nordt *et al.*, 1996a). To analyze the effect of specific mutations in IL-10R on macrophage responses to IL-10, we expressed either wild-type hIL-10R or a mutant hIL-10R in which Tyr446 and Tyr496 were mutated to phenylalanine (hIL-10R-Tyr^{FF}) in J774 cells. Similar levels of hIL-10R were expressed on J774:hIL-10R and J774:hIL10R-Tyr^{FF} cells (Figure 3A). hIL-10 was employed in all experiments using these cells and, since hIL-10 can bind to and activate mIL-10R (Ho *et al.*, 1993), mAb 1B1.2 (10 μg/ml) was used as a mIL-10R-specific blocking antibody.

hIL-10 dramatically inhibited proliferation of J774 cells expressing hIL-10R, whereas hIL-10R-Tyr^{FF} was unable to transmit the growth inhibitory signal (Figure 3B). Lack of inhibition in control J774 cells indicated that mAb 1B1.2 effectively blocked endogenous mIL-10R. These data suggested that the macrophage growth inhibitory effect of IL-10 requires Tyr446 and/or Tyr496.

Next we determined whether Stat3, which is directly recruited to these tyrosines, mediates the growth inhibitory effect of IL-10. Two mutant forms of Stat3 were generated and expressed in J774 cells; a truncated Stat3 (ΔStat3) which lacks the transactivation domain and acts as a dominant negative (Minami *et al.*, 1996; Mui *et al.*, 1996), or Stat3 fused to the dimerization domain of bacterial gyraseB protein (Stat3–GyrB), which is inducibly dimerized upon addition of coumermycin (Ali *et al.*, 1995; Farrar *et al.*, 1996; Gormley *et al.*, 1996). To verify that ΔStat3 and Stat3–GyrB acted as predicted, activation of these Stats and Stat3-responsive luciferase reporters was examined. In J774 cells expressing ΔStat3, tyrosine phosphorylation of endogenous Stat3 in response to IL-10 was dramatically reduced relative to control [Figure 4A (i), lanes 3 and 6]. In a transactivation assay, an important indicator of Stat activity, expression of ΔStat3 blocked β-casein promoter activity by 60%, and this activity was restored by co-expression of wild-type Stat3 [Figure 4A (ii)]. Similar results were obtained using a c-fos reporter (data not shown). These data suggest that ΔStat3 functions as a specific dominant-negative inhibitor of Stat3 activity.

Stat3–GyrB is predicted to dimerize in response to coumermycin. In J774 cells expressing Stat3–GyrB but not control cells, addition of coumermycin caused formation of high molecular weight complexes containing Stat3–GyrB, of molecular mass consistent with dimers [Figure 4B (i)]. Novobiocin, which has similar structure to coumermycin but lacks dimerization capacity and so binds GyrB as a 1:1 complex (Ali *et al.*, 1995), had little effect (lanes 1 and 2). The appearance of multiple bands in the coumermycin-treated J774:Stat3–GyrB cells may be due to cross-linking at different positions, as previously suggested for cross-linking of GyrB in the presence of coumermycin (Gormley *et al.*, 1996). Coumermycin had no effect on control cells. These results suggest that coumermycin specifically causes dimerization/oligomerization of Stat3–GyrB. Dimerization of Stat proteins confers the ability to bind specific DNA target sequences. In a traditional gel shift (EMSA) assay, we were unable to detect Stat3–GyrB binding to Stat-binding sequences, most probably due the inability of the coumermycin–Stat3–GyrB complex to withstand either the cell lysis or EMSA conditions. However, using plasmon resonance (BIAcore), which detects associations in real time, we observed coumermycin-induced association of Stat3–GyrB proteins with a Stat-binding (SIE), but not an irrelevant NF-κB oligonucleotide. Cell lysates prepared from control or 293T cells transfected with Stat3–GyrB were treated with 10 mM of either novobiocin or coumermycin, and supplemented with 100 μg/ml poly(dIdC) which suppressed non-specific DNA binding. The lysates from both untransfected cells and Stat3–GyrB-transfected cells treated with novobiocin resulted in ~1000 RU of binding to the SIE-coupled sensor chip [Figure 4B (ii) top panel]. This level of binding probably represents the endogenous Stat activity present in 293T cells grown in serum. However, treatment of Stat3–GyrB lysates, but not lysates from control cells, resulted in a significant enhancement of binding to the SIE-coated sensor chip [Figure 4B (ii) top and bottom panels]. As expected, coumermycin treatment did not

change the amount of NF-κB binding activity in Stat3–GyrB lysates (bottom panel).

Stat3–GyrB was shown to activate transcription from a c-fos reporter, upon addition of coumermycin [Figure 4B (iii)]. The magnitude of activation was similar to that induced by IL-10 which acts through endogenous Stat3, whereas novobiocin had no significant effect, and coumermycin did not induce reporter activity in control cells. Similarly, addition of coumermycin to BaF/3 cells expressing Stat3–GyrB or Stat5–GyrB activated β-casein promoter activity, but had no effect on control cells (data not shown). These data infer that Stat–GyrB chimeras can transactivate Stat reporter genes, and are specifically activated in response to coumermycin. Expression levels of ΔStat3 and Stat3–GyrB in independent J774 clones used in the experiments described are shown in Figure 4C.

Expression of ΔStat3 blocked the ability of IL-10 to inhibit [^{3}H]thymidine incorporation (Figure 4D), implying a role for Stat3 in mediating growth inhibition by IL-10. This effect could be rescued by overexpression of WT Stat3 in J774:ΔStat3 cells, restoring inhibition of proliferation by IL-10 (Figure 4D). In a complementary approach, activation of Stat3–GyrB in J774 cells by addition of coumermycin suppressed [^{3}H]thymidine incorporation by up to 40%, in the absence of IL-10 (Figure 4E), whereas novobiocin had no effect (data not shown). Coumermycin did not suppress [^{3}H]thymidine incorporation in control J774 cells or in J774 cells expressing Stat5–GyrB (Figure 4E). These data suggest that activation of Stat3 is both necessary and sufficient to elicit growth arrest.

Effects of hIL10R-Tyr^{FF} and ΔStat3 on CD86 and FcγR expression

Expression of FcγR, the receptor for IgG, is up-regulated by IL-10 in macrophages (te Velde *et al.*, 1992). Accordingly, IL-10 up-regulated cell surface expression of FcγR in J774 cells (Figure 5). In cells expressing hIL10R-Tyr^{FF}, IL-10 failed to induce FcγR cell surface expression (Figure 5), suggesting that the membrane-distal tyrosines are essential for this response. In J774:ΔStat3 cells, IL-10 up-regulated FcγR expression, although the extent of up-regulation was less than in J774 or J774:hIL-10R cells. Up-regulation of FcγR was IL-10 dose dependent. At lower IL-10 concentrations (0.2 or 2 ng/ml), up-regulation of FcγR in ΔStat3 cells was still apparent though consistently lower than that observed in parental cells (data not shown). In J774:ΔStat3 cells overexpressing wild-type Stat3, the fold induction of FcγR expression by IL-10 (20 ng/ml) was increased relative to cells expressing ΔStat3 alone. Therefore, it appears that Stat3 plays a role in induction of FcγR but is not the sole pathway(s) involved.

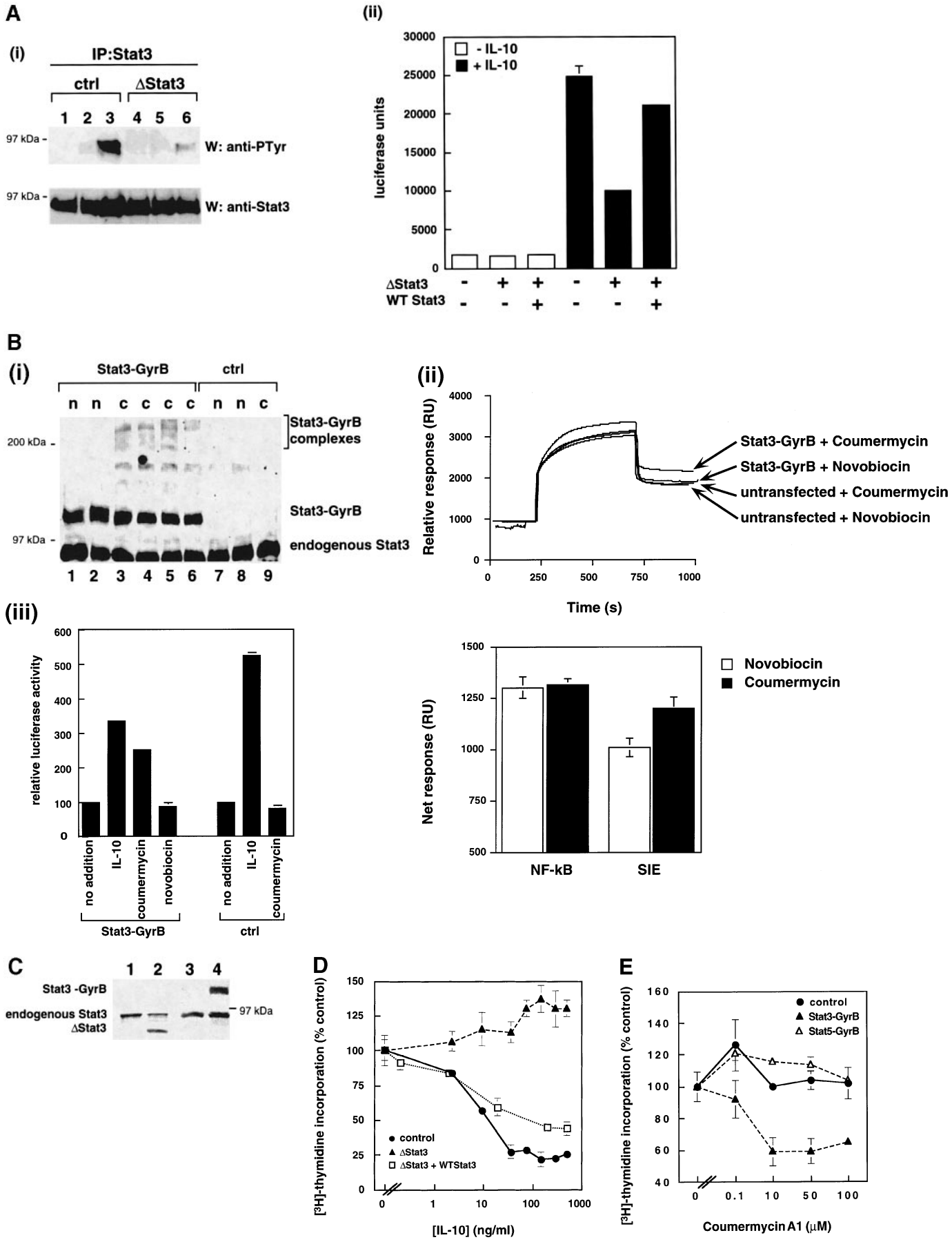
Inhibition of TNFα and IL-1β production by IL-10 requires Tyr446 and Tyr496 of hIL-10R but is not blocked by ΔStat3

The J774 cell line has been used as an *in vitro* model to study macrophage activation by LPS (Fujihara *et al.*, 1994; Briend *et al.*, 1995; Hattori *et al.*, 1996). Treatment with LPS promotes differentiation and activation, manifested by up-regulated expression of specific cell surface antigens, production of reactive nitrogen intermediates, chemokines and cytokines including TNFα and IL-1β.

LPS induced TNFα secretion from J774 cells, and mIL-10 potently inhibited TNFα production (Figure 6). Similarly, TNFα induction by LPS in J774:hIL10R cells was inhibited by hIL-10. In J774 cells expressing hIL10R-Tyr^{FF}, LPS-induced TNFα production was comparable in kinetics and concentration with J774:hIL10R cells, but hIL-10 had no inhibitory effect. Thus the two membrane-distal tyrosines of IL-10R are essential for inhibition of TNFα production by IL-10.

However, this response did not require Stat3: IL-10 still inhibited TNFα production in J774:ΔStat3 cells, with similar potency to control cells (Figure 6). Moreover, inducible dimerization of Stat3–GyrB in J774 cells did not detectably inhibit TNFα production (data not shown). These data suggest that suppression of LPS-induced TNFα

Fig. 4. Proliferation response of J774:ΔStat3 and J774:Stat3–GyrB cells. (**A**) Characterization of ΔStat3. (i) ΔStat3 suppresses tyrosine phosphorylation of endogenous Stat3. Total lysates from unstimulated or IL-10-treated J774 (lanes 1–3) or J774:ΔStat3 (lanes 4–6) cells were immunoprecipitated with anti-Stat3, and subjected to anti-phosphotyrosine Western analysis. Lanes 1 and 4, untreated cells; lanes 2 and 5, IL-10 (2 ng/ml); lanes 3 and 6, IL-10 (20 ng/ml). The bottom panel shows the control anti-Stat3 Western. (ii) ΔStat3 suppresses transactivation of a Stat3-responsive reporter. BaF-mIL-10R cells were co-transfected with the β-casein luciferase reporter plasmid and pME18S-ΔStat3 or pME18S-WT Stat3 as indicated. Medium (□) or IL-10 (■) was added to half of each sample 8 h after transfection, and luciferase activity was determined after a further 16 h. (**B**) Characterization of Stat3–GyrB. (i) Coumermycin treatment induces formation of protein complexes which contain Stat3–GyrB. Lysates from J774 or J774:Stat3–GyrB cells were treated with coumermycin or novobiocin at the concentrations indicated for 15 min at 25°C, cross-linked with BS3 (1 mM) and subjected to SDS–PAGE and anti-Stat3 Western analysis. Lanes 1 and 7, 10 nM novobiocin; lanes 2 and 8, 10 μM novobiocin; lanes 3–6, 1 nM, 10 nM, 100 nM and 100 μM coumermycin respectively; lane 9, 100 nM coumermycin. (ii) Coumermycin treatment induces association of Stat3–GyrB with SIE oligonucleotides. In the top panel, hypotonic lysates from control or Stat3–GyrB-transfected 293T cells were treated with 10 mM novobiocin or coumermycin for 15 min at 25°C and applied to SIE oligonucleotide-coupled sensor chips. The relative response is plotted in resonance units (RU). In the bottom panel, lysates from Stat3–GyrB-transfected 293T cells were incubated as above with either novobiocin or coumermycin before application to sensor chips coupled with either SIE or NF-κB oligonucleotides. The net response is plotted in RU. Error bars represent the standard deviation of triplicate samples for each condition. (iii) Coumermycin treatment stimulates transactivation of a Stat3-responsive reporter. NIH-3T3-mIL-10R cells were co-transfected with c-fos luciferase reporter plasmid, and pME18S:Stat3–GyrB or pME18S vector control, together with pMEβ-galactosidase. Coumermycin (10 μM), novobiocin (10 μM) or IL-10 (100 ng/ml) was added 12 h after transfection, and luciferase activity was determined in cell lysates 16 h later. Data are normalized for transfection efficiency with respect to β-galactosidase activity, and error bars represent the standard deviation of triplicate samples. (**C**) Expression of ΔStat3 and Stat3–GyrB in J774 cells. J774 cells were transduced with ΔStat3 or Stat3–GyrB. Lysates from drug-resistant clones used in the experiments described were subjected to Western analysis with anti-Stat3. Lanes 1 and 3, control J774 cells; lane 2, J774:ΔStat3; lane 4, J774:Stat3–GyrB. The molecular masses of ΔStat3 and Stat3–GyrB are ~75 and 120 kDa respectively. (**D**) Effect of IL-10 on [^{3}H]thymidine incorporation in J774:ΔStat3 cells. J774 cells were cultured in the absence (control) or presence of mIL-10 at the concentrations indicated, and [^{3}H]thymidine incorporation was measured after 30 h. Results for parental J774 (●), J774:ΔStat3 (▲) and ΔStat3 cells overexpressing wild-type Stat3 (□) are shown. Control c.p.m. were 46 720, 25 980 and 35 150, respectively. (**E**) Effect of coumermycin on [^{3}H]thymidine incorporation in J774:Stat–GyrB cells. J774 (●), J774:Stat3–GyrB (▲) or J774:Stat5–GyrB (△) cells were cultured in the absence (control, 68 213, 55 319 and 88 644 c.p.m. for J774, J774:Stat3–GyrB and J774:Stat5–GyrB respectively) or presence of coumermycin at the concentrations indicated, and [^{3}H]thymidine incorporation was measured after 36 h.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97 kDa
D
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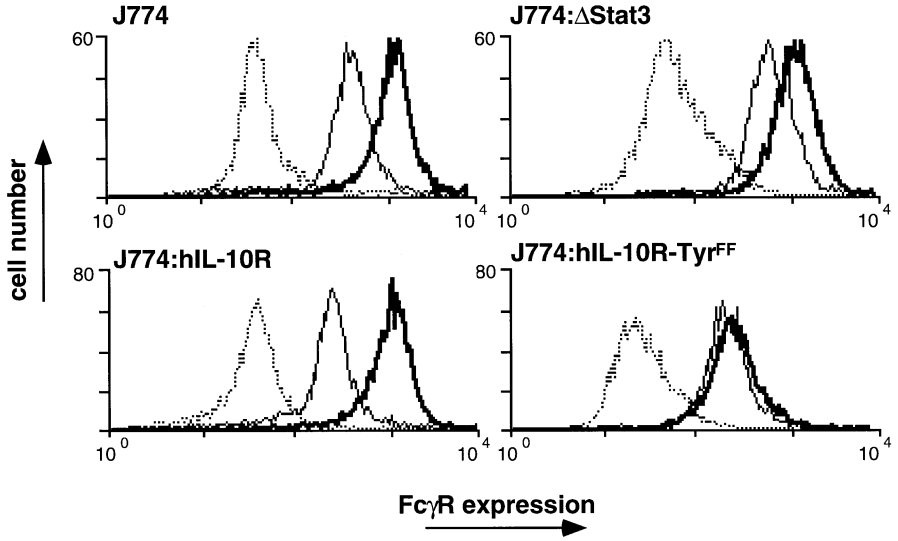

Fig. 5. Effect of IL-10 on expression of FcγR. J774 cells were cultured in the absence (thin line histogram) or presence (thick line histogram) of IL-10 (20 ng/ml) for 40 h [in the presence of IBI.2 (10 μg/ml) for hIL-10R and hIL-10R-Tyr^{FF} cells]. Cells were stained with FcγR–FITC, and isotype control staining (dotted line histogram) of IL-10-treated cells is also presented.

production by IL-10 occurs by a Stat3-independent signaling pathway(s), which still requires the two membrane-distal tyrosines of IL-10R.

Similar results were obtained when LPS-induced IL-1β secretion was analyzed in the presence and absence of IL-10 (Figure 7A and B). Again, cells expressing hIL10R-Tyr^{FF} were non-responsive to IL-10, as levels of IL-1β induced in response to LPS in the presence of IL-10 were indistinguishable from those produced in response to LPS alone. IL-10 completely inhibited LPS-induced IL-1β production in J774:ΔStat3 cells, suggesting that ΔStat3 could not block this response, as observed for TNFα production.

Inhibition of CD86 expression by IL-10 requires Tyr446 and Tyr496 of hIL-10R but is not blocked by ΔStat3

In J774 cells, we observed that LPS triggered up-regulation of CD86 (B7-2), a surface antigen involved in lymphocyte activation. IL-10 had no effect on basal levels of CD86 expression, but reduced LPS-induced CD86 expression (Figure 8). Likewise, hIL-10 inhibited this LPS response in J774:hIL-10R, but not in J774:hIL10R-Tyr^{FF} cells. In contrast to hIL10R-Tyr^{FF}, expression of ΔStat3 did not compromise the ability of IL-10 to suppress CD86 expression (Figure 8), similar to data described for cytokine production.

IL-10 inhibits NO production in J774 and J774:ΔStat3 cells

Expression of inducible nitric oxide synthase (iNOS) and production of reactive nitrogen intermediates such as nitric oxide (NO) are hallmarks of macrophage activation, and provide important mechanisms of macrophage cytotoxicity. Activation of J774 cells by LPS stimulated nitrite production, indicative of the presence of NO (Figure 9A). This response was inhibited by IL-10 in control J774 cells and also in J774:ΔStat3 cells, suggesting that Stat3 is not essential for this response. However, it was not possible

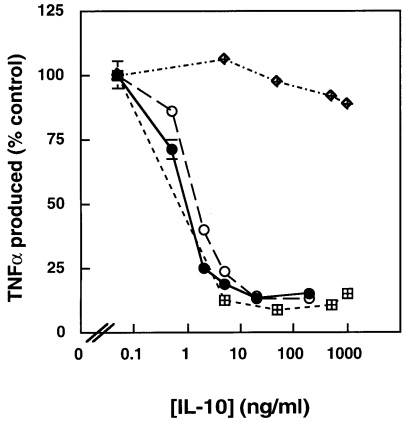

Fig. 6. Effect of IL-10 on TNFα production. J774 (●), J774:ΔStat3 (○), J774:hIL-10R (⊞) and J774:hIL-10R-Tyr^{FF}(◆) cells were cultured in the presence of LPS (1 μg/ml), IL-10 or LPS plus IL-10, for 8 h [in the presence of IBI.2 (10 μg/ml) for hIL-10R and hIL-10R-Tyr^{FF} cells]. The TNFα contents of culture supernatants were determined by ELISA, and are presented as the percentage of TNFα produced in response to LPS alone (12, 18, 11 and 14 ng/ml TNFα for J774, J774:ΔStat3, J774:hIL-10R and J774:hIL-10R-Tyr^{FF} respectively). IL-10 alone did not stimulate TNFα production. Error bars represent the standard deviation of triplicate samples.

to determine the requirement for Tyr446 and Tyr496 in this response since in J774:hIL-10R cells, hIL-10 was unable to inhibit nitrite production, and moreover enhanced this LPS response (Figure 9B). In contrast, mIL-10 inhibited LPS-stimulated nitrite production in J774:hIL-10R cells in the absence of mAb 1B1.2 (data not shown), indicating that the signaling pathway necessary for inhibition of NO production by IL-10 is intact. These data suggest that hIL-10R may not be completely functional in mouse cells, failing to interact with or activate a component(s) of the receptor complex or signal transduction pathway(s). This pathway is probably distinct from that which mediates inhibition of TNFα, IL-1β or CD86 expression.

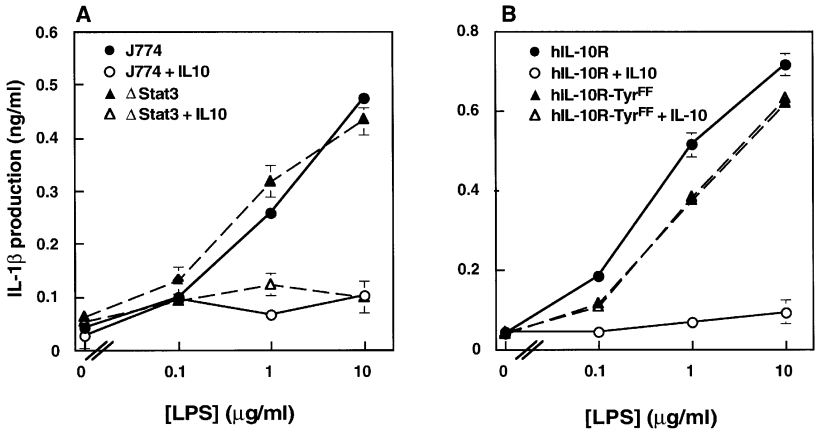

Fig. 7. Effect of IL-10 on IL-1β production. (**A**) J774 and J774:ΔStat3 or (**B**) J774 hIL-10R and hIL-10R-Tyr^{FF} cells were cultured in the presence of LPS (0.1, 1 or 10 μg/ml) with or without IL-10 (50 ng/ml), for 40 h [in the presence of IBI.2 (10 μg/ml) for hIL-10R and hIL-10R-Tyr^{FF} cells]. IL-1β concentrations in culture supernatants were determined by ELISA. Error bars represent the range of duplicate samples.

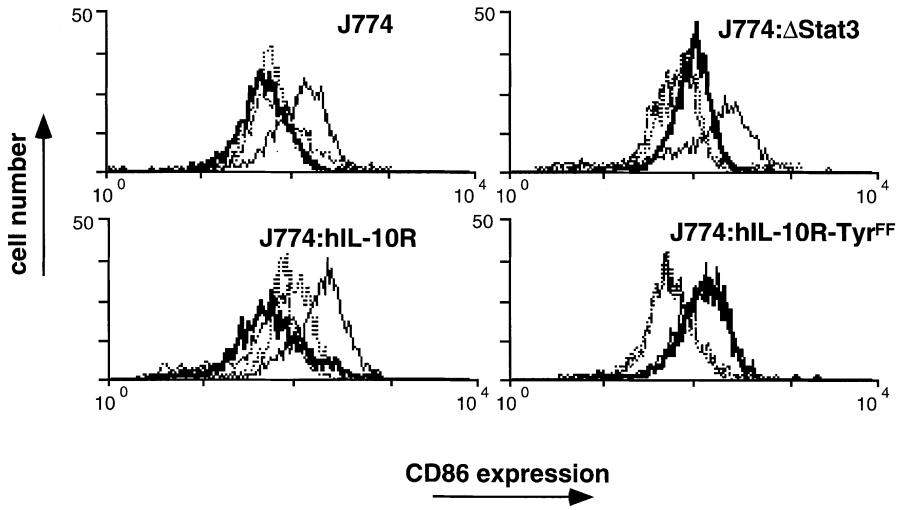

Fig. 8. Effect of IL-10 on expression of CD86. J774 cells were cultured in the presence of LPS (1 μg/ml), IL-10 (50 ng/ml) or LPS plus IL-10, for 40 h [in the presence of IBI.2 (10 μg/ml) for hIL-10R and hIL-10R-Tyr^{FF} cells]. Cells from each condition were stained with biotinylated anti-CD86, followed by streptavidin–FITC, and analyzed by flow cytometry. Dotted histogram, no cytokine added; dashed histogram, IL-10; line histogram, LPS; thick line histogram, LPS plus IL-10.

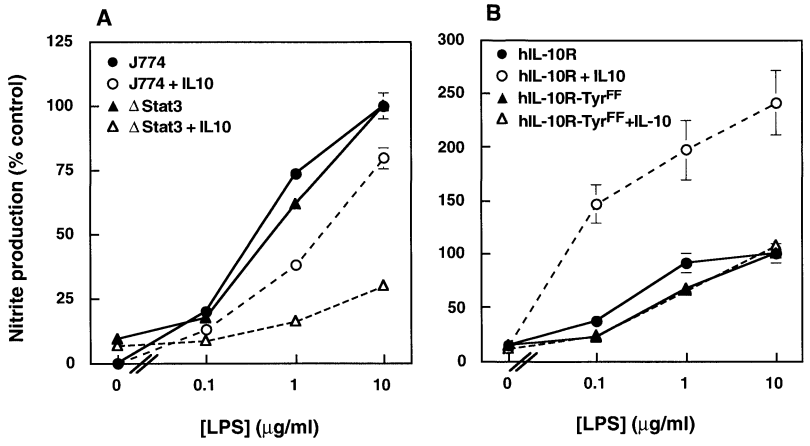

Fig. 9. Effect of IL-10 on NO production. (**A**) J774 and J774:ΔStat3 or (**B**) J774:hIL-10R and J774:hIL-10R-Tyr^{FF} cells were cultured in the presence of LPS (0.1, 1 or 10 μg/ml) with or without IL-10 (50 ng/ml) for 24 h [in the presence of IBI.2 (10 μg/ml) for hIL-10R and hIL-10R-Tyr^{FF} cells]. Nitrite concentrations in culture supernatants were determined, and are represented as the percentage of nitrite produced in response to LPS (10 μg/ml). Control values were 91, 96, 122 and 120 μM nitrite for J774, J774:ΔStat3, J774 hIL-10R and hIL-10R-Tyr^{FF} cells respectively. Error bars represent the range of duplicate samples.

Discussion

IL-10 inhibits macrophage proliferation

IL-10 has been well characterized as an inhibitor of monocyte/macrophage activation, inhibiting production of pro-inflammatory cytokines and chemokines as well as regulating expression of cell surface antigens (for review, see Ho and Moore, 1994). We show here that IL-10 inhibits the proliferation of the macrophage cell line J774 and M-CSF-dependent proliferation of NBM-derived macrophages. IL-10 previously has been shown to inhibit spontaneous proliferation of hemopoietic progenitors from peripheral and cord blood (Oehler *et al.*, 1997; Schibler *et al.*, 1994, respectively) and of myelogenous leukemic blast cells (Bruserud *et al.*, 1995; Geissler *et al.*, 1996). In these instances, and for suppression of T cell proliferation by IL-10 (Taga and Tosato, 1992; de Waal Malefyt *et al.*, 1993), the effect of IL-10 has been attributed to its inhibition of growth factor production (GM-CSF or IL-2 respectively). However, the effect of IL-10 that we report appears to be direct: we could find no evidence either for production of an IL-10-induced autocrine growth inhibitor or for inhibition of production of an autocrine growth factor (Figure 2C). Furthermore, the effect of IL-10 on J774 cells was not associated with enhanced apoptosis. This finding is consistent with IL-10's role as a viability-enhancing factor for murine B cells and mast cells (Go *et al.*, 1990; Thompson-Snipes *et al.*, 1991) and IL-10R-transfected mouse and human cell lines (Ho *et al.*, 1995; Liu *et al.*, 1997), as well as its ability to inhibit apoptosis in human myeloid progenitors (Weber-Nordt *et al.*, 1996b) and IL-2-starved T cells (Taga *et al.*, 1993), although it contrasts with reports of IL-10-induced or -accelerated apoptosis in B-CLL cells and mature Langerhans cells (Fluckiger *et al.*, 1994; Ludewig *et al.*, 1995, respectively). Since suppression of DNA synthesis by IL-10 was reversible, the possibility that IL-10 alters the differentiation status of J774 cells resulting in decreased proliferation potential is unlikely. Furthermore, we found no evidence for up-regulation of differentiation-associated antigens, such as CD80, CD86, CD54 or Mac1 in response to IL-10 (A.-M.O'Farrell, unpublished data).

These findings reveal a possible new aspect of regulation of the inflammatory response by IL-10. In addition to its inhibition of multiple effector functions of activated macrophages, IL-10 can regulate their proliferation and thus the numbers of inflammatory cells. Thus IL-10, which has shown short-term anti-inflammatory effects *in vivo* (Chernoff *et al.*, 1995; Fuchs *et al.*, 1996), may have a potential role in ameliorating chronic inflammatory conditions. In addition, these results along with others (Bruserud *et al.*, 1995; Geissler *et al.*, 1996; Oehler *et al.*, 1997) suggest the possible use of IL-10 in treatment of myeloid leukemias.

IL-10's inhibition of macrophage proliferation and activation contrasts with its role as a co-stimulator of growth of B cells, mast cells and thymocytes (MacNeil *et al.*, 1990; Thompson-Snipes *et al.*, 1991; Vieira *et al.*, 1991; Rousset *et al.*, 1992). Our results thus emphasize the fact that, despite utilization of a common receptor subunit (IL-10Rα) and similar signaling molecules, the effects of IL-10 can vary markedly according to the cell type.

Roles of IL-10R and Stat3 in inhibition of macrophage activation and proliferation

To explore the roles of IL-10R and Stat transcription factors in mediating IL-10's effects on macrophages, we expressed mutant hIL-10R where two conserved tyrosines in the cytoplasmic domain were mutated to phenylalanine (hIL-10RTyr^{FF}), and two different modified forms of Stat3 (ΔStat3 and Stat3–GyrB) in J774 cells. The two tyrosine residues near the C-terminus of IL-10R are required for IL-10R function in transfected Baf/3 cells and for recruitment of Stat3 to the activated receptor (Ho *et al.*, 1995; Lai *et al.*, 1996; Weber-Nordt *et al.*, 1996a). We found that hIL-10R:Tyr^{FF} was unable to mediate either the anti-proliferative or macrophage de-activation activities of IL-10. Thus, IL-10 signal transduction in macrophages requires the presence (and presumably phosphorylation) of Tyr446 and/or Tyr496 of hIL-10R.

The anti-proliferative effect of IL-10 on J774 cells was blocked by expression of ΔStat3, and coumermycin-induced dimerization of Stat3–GyrB inhibited DNA synthesis, similar to the effect of IL-10. Induction of FcγR expression by IL-10 was also partially inhibited by expression of ΔStat3, similar to previous observations (Minami *et al.*, 1996), implicating Stat3 in regulation of FcγR expression. In cells expressing hIL-10R:Tyr^{FF}, IL-10's induction of FcγR was completely ablated, suggesting that pathways in addition to Stat3 activation which couple to Tyr446 and/or Tyr496 are required for FcγR induction. This is reminiscent of the model recently proposed for the IL-2R, where two tyrosines which redundantly mediate Stat5 activation couple to additional signaling pathways, which together with Stat activation mediate induction of the IL-2Rα gene (Ascherman *et al.*, 1997).

Expression of ΔStat3 interfered with the functional activity of endogenous Stat3, as observed by reduction in tyrosine phosphorylation, DNA binding (not shown) and luciferase activity from Stat3-responsive reporter plasmids. This suggests that ΔStat3 (which lacks Tyr705 and thus does not become tyrosine phosphorylated) competes with endogenous Stat3 for binding to the IL-10R complex or binding to the kinase responsible for Stat3 phosphorylation. An important consideration when using dominant-negative molecules is the possibility of non-specific effects. However, several lines of evidence suggest that the effect of ΔStat3 on proliferation could be attributed specifically to suppression of endogenous Stat3 activity; First, overexpression of wild-type Stat3 rescued the proliferation defect of J774:ΔStat3 cells. Second, the role of Stat3 in inhibition of proliferation was shown independently using the inducibly active Stat3–GyrB. Third, ΔStat3 expression had no detectable effect on additional IL-10 responses such as inhibition of TNFα, IL-1β or CD86 expression, which also require Tyr446 and/or Tyr496 of IL-10R. It is possible that Stat3 plays distinct roles in different IL-10 responses, and ΔStat3 might not interfere with all Stat3 activities. However, we believe it is unlikely that a residual low level of functional Stat3 could be sufficient to mediate inhibition of cytokine production (but not sufficient to block proliferation) in J774:ΔStat3 cells, because coumermycin-induced dimerization of Stat3–GyrB had no inhibitory effect on LPS-induced cytokine production. Our data support the existence of both Stat3-dependent (proliferation; FcγR induction) and -independent (macrophage de-

activation) pathways of IL-10 signaling in macrophages, both of which couple to membrane-distal tyrosines of IL-10R.

Since IL-10 activates Stat1 in macrophages (Finbloom *et al.*, 1995), it is possible that the Stat3-independent effects of IL-10 could be mediated by Stat1. The mechanism of Stat1 recruitment to the IL-10R complex is not clear. Stat1 is not, however, directly recruited to the membrane-distal tyrosines, and a truncated receptor lacking the domain containing these tyrosines can still activate Stat1 DNA-binding activity (Weber-Nordt *et al.*, 1996a). Expression of ΔStat1, a C-terminus truncated Stat1 analogous to ΔStat3, in J774 cells had no effect on the IL-10 responses described (A.-M.O'Farrell, unpublished results), consistent with the observation that inhibition of LPS-induced TNFα expression by IL-10 remains intact in Stat1-knockout mice (Meraz *et al.*, 1996). Embryonic lethality in mice homozygous for targeted disruption of the Stat3 gene (Takede *et al.*, 1997) precludes similar analysis of IL-10 responses.

Other groups have shown the involvement of Stat3 in growth arrest. IL-6, a cytokine which signals via gp130 and activates Stat3 (Zhong *et al.*, 1994), promotes growth arrest, differentiation and apoptosis of the M1 cell line. Use of dominant-negative forms of Stat3 showed that activation of Stat3 is required for IL-6-mediated growth arrest of M1 cells (Minami *et al.*, 1996; Nakajima *et al.*, 1996). Our data with J774 cells similarly imply a role for Stat3 in growth arrest but, distinct from IL-6 action on M1 cells, IL-10 did not cause apoptosis of J774 cells (or NBM-derived macrophages). More recently, Stat3 was implicated in inhibition of apoptosis in Ba/F3 cells expressing chimeras of the granulocyte colony-stimulating factor (G-CSF) receptor and gp130, where G-CSF transmits a mitogenic signal (Fukuda *et al*., 1996). Since J774 cells proliferate in the presence of serum, which does not activate Stat3 (A.-M.O'Farrell, unpublished results), it appears that Stat3 activation is not necessary to provide an anti-apoptotic signal. To our knowledge, the action of IL-10 in J774 cells provides the first evidence of a purely anti-proliferative role for Stat3. An anti-proliferative role for Stat1 has been proposed in interferon responses (Bromberg *et al.*, 1996; Chin *et al.*, 1996), and growth arrest triggered by IFNγ is associated with induction of the cyclin-dependent kinase inhibitor p21 (Chin *et al.*, 1996). Although we do not have direct evidence for a role for Stat1 in IL-10′s anti-proliferative function, we cannot exclude the possibility that Stat1–Stat3 heterodimers formed in response to IL-10 play a role.

The use of Stat–GyrB chimeras provides an inducible and functional model to examine Stat activity. The ability of GyrB fusion proteins to undergo coumermycin-induced dimerization was shown by Farrar *et al.* (1996), who demonstrated that a Raf-1–GyrB fusion protein activated the downstream MAP kinase cascade in response to coumermycin. We found that both Stat3–GyrB and Stat5–GyrB could mediate coumermycin-dependent transactivation of Stat-responsive reporter plasmids. Furthermore, both constructs elicited biological responses indicative of activated Stats: Stat3–GyrB (but not Stat5–GyrB) dimerization acted in a manner similar to IL-10 with respect to inhibition of J774 proliferation, a function of IL-10 we propose as a Stat3-mediated pathway based on our ΔStat3 studies. Similarly, coumermycin-induced Stat5–GyrB (but not Stat3–GyrB) dimerization stimulated DNA synthesis in Ba/F3 cells (A.L.-F.Mui, unpublished results), consistent with the involvement of Stat5 in IL-3 driven Ba/F3 proliferation (Mui *et al.*, 1996). These data provide new evidence that dimerization/oligomerization of Stats, in the absence of tyrosine phosphorylation, is sufficient to mediate at least a subset of Stat functions.

Stat proteins can be modified by Ser/Thr phosphorylation, ubiquitination and phosphatase activity (Wen *et al.*, 1995; Haspel *et al.*, 1996; Kim and Maniatis, 1996; Sasse *et al.*, 1997). Stats can also interact with other transcription factors and co-activators (Look *et al*., 1995; Schaefer *et al.*, 1995; Bhattacharya *et al.*, 1996; Zhang *et al.*, 1996), and can act as enhancers of gene activation (Kordula and Travis, 1996; Stocklin *et al.*, 1996). Whether these additional levels of regulation are recapitulated by Stat–GyrB proteins is not yet clear. The efficacy of the Stat–GyrB action may be dependent on specific requirements for Stat binding and transactivation at each promoter. That coumermycin dimerization of Stat3–GyrB is able to inhibit J774 proliferation suggests that the Stat3–GyrB dimer can transactivate (or repress) the genes necessary for growth inhibition. Although it is possible that IL-10 and Stat3–GyrB inhibit growth via distinct mechanisms, the finding that ΔStat3 can block the anti-proliferative effect of IL-10 implicates the Stat3 pathway in mediating this action of IL-10. Further studies are underway to compare the transcriptional events elicited by IL-10 and Stat3–GyrB.

We have analyzed signal transduction events associated with two distinct IL-10 responses in macrophages: de-activation and suppression of proliferation. Using receptor mutants, and dominant-negative and inducibly active forms of Stat3, we have classified IL-10 responses into Stat3-dependent (inhibition of proliferation) and Stat3-independent (de-activation) pathways. We provide evidence that novel pathway(s) elicited from membrane-distal tyrosines of IL-10R mediate macrophage de-activation responses. This classification will enable dissection of the molecular requirements of each pathway and facilitate identification of additional signaling molecules activated by IL-10.

Materials and methods

Cell culture

J774 cells (American Type Tissue Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) on tissue culture grade plates, and passaged twice weekly. NBM-derived macrophages were generated by *in vitro* differentiation of bone marrow cells in the presence of M-CSF. Briefly, bone marrow was extracted from femurs of 8- to 12-week-old female BALB/c mice (Taconics, Germantown, NY), and cultured in RPMI1640 supplemented with 10% FBS and 50 ng/ml M-CSF (R&D Systems, Minneapolis, MN). After 4 days, non-adherent cells were removed, and adherent cells maintained in culture. Eight- to 15-day-old cultures, starved of M-CSF for ~4 h, were used in the experiments. Wright–Giemsa staining of cultures was performed to confirm macrophage morphology. Staining with anti-c-fms and analysis by flow cytometry showed high levels of expression of c-fms, the M-CSF receptor, indicative of macrophage phenotype.

Ba/F3 cells transfected with mIL-10R (BaF-mIL-10R) cells were maintained as described (Ho *et al.*, 1993).

Anti-mIL-10R mAb

A rat anti-mIL-10R mAb (1B1.2; IgG1) was produced as described for the anti-hIL-10R mAb 3F9 (Liu *et al.*, 1997) by immunization with the purified recombinant epitope-tagged ligand-binding domain of mIL-10R

(smIL-10R-FLAG-His_6). Hybridoma supernatants were screened by ELISA on plates coated with smIL-10R-FLAG-His_6 followed by FACS staining of BaF-mIL-10R cells. The neutralizing antibody 1B1.2 was identified by its ability to block the response of BaF-mIL-10R cells to IL-10, as described in the Results.

[^{125}I]hIL-10 binding assay

BaF-mIL-10R cells (6×10^5) were incubated with [^{125}I]hIL-10 (Dupont/NEN, North Billerica, MA), as described (Ho *et al.*, 1995; Liu *et al.*, 1997), in the presence or absence of 200 nM hIL-10 or 20 μg/ml mAb 1B1.2 or isotype control (GL113) mAb, in a total volume of 200 μl, for 4.5 h at 4°C. Cells were pelleted through silicone oil/paraffin oil, and bound [^{125}I]hIL-10 was measured in a gamma counter. The approximate K_d for [^{125}I]hIL-10 binding to mIL-10R is 40 pM (Ho *et al.*, 1995).

Plasmid construction

Construction of ΔStat3, Stat3–GyrB and hIL10R-Tyr^{FF}: to generate ΔStat3, wild-type Stat3 was truncated after Lys685 (leaving the SH2 domain intact), by replacing the *Xma*I–*Not*I fragment of wild-type Stat3 (cloned into the *Eco*RI–*Not*I sites of pME18S) with an *Xma*I–*Not*I digested PCR fragment generated with 5′-GAGACCCACTCCTTGGCAGTT (sense) and 5′-tagttatGCGGCCGCTACTTTCCAAATGCCTCCTCCTT (antisense) primers. Expression of ΔStat3 inhibited transactivation of Stat-responsive promoters, as described for Stat5 (Mui *et al.*, 1996). To construct Stat3–GyrB, the cDNA encoding the GyrB dimerization domain (kindly provided by Dr R.Perlmutter) was modified by addition of an *Mlu*I site to the 5′ end and a stop codon and *Not*I site to the 3′ end [the 700 bp GyrB sequence was amplified using *Mlu*I (5′-ttcgACGCGTAGCAATTCTTATGACTCC)- and *Not*I (5′-attaGCGGCCGCTTAGCCTTCATAGTGGAAGTG)-containing primers]. The stop codon in wild-type Stat3 was likewise replaced with a *Mlu*I site [the *Xma*I–*Not*I wild-type fragment was replaced with an *Xma*I–*Mlu*I-digested PCR fragment generated with 5′-GAGACCCACTCCTTGGCAGTT (sense) and 5′-tcagACGCGTCATGGGGGAGGTAGCACAC (antisense) primers]. The GyrB and Stat3 fragments were then co-ligated into the *Eco*RI–*Not*I sites of pME18S. Both ΔStat3 and Stat3–GyrB were also subcloned into pMX-puro (Onishi *et al.*, 1996) for retroviral transductions. To construct hIL10R:Tyr^{FF}, the *Bsm*I–*Bam*HI fragment of wild-type hIL-10R was replaced with a *Bsm*I–*Bam*HI-digested PCR fragment generated with PCR primers each containing the appropriate point mutation [5′-GTGGCATTCCAGGGTTTCCTGAGGCAGACC (sense) and 5′-GAGGATCCTGTTTCAAAAAGCCCTTGGCCAGGGC (antisense)]. The identity of all constructs was confirmed by sequencing.

Expression of cDNAs in J774 cells by retroviral transduction

cDNAs cloned into the retroviral vector pMXpuro were introduced into J774 cells by retroviral infection. Briefly, packaging cells (Bosc23) were transfected with pMX constructs using lipofectamine as described (Kitamura *et al.*, 1995; Onishi *et al.*, 1996), except that Bosc23 cells were irradiated (2000 rads) 24 h after transfection and co-cultured with J774 (1×10^5) cells in the presence of 8 μg/ml polybrene. Polybrene was removed after a futher 24 h, and cells were drug selected [5 μg/ml puromycin (Sigma Chemical Co., St Louis, MO)]. For ΔStat3- and Stat3–GyrB-expressing cells, independent puromycin-resistant colonies were isolated, and high-expressing clones identified by anti-Stat3 Western blot analysis. For rescue of ΔStat3, a wild-type Stat3 construct cloned into the pMXneo retroviral vector was introduced into ΔStat3 cells, as described above, and positive clones selected in neomycin (0.75 mg/ml). Western analysis confirmed overexpression of wild-type Stat3. For cells infected with hIL-10R retroviruses, expression levels of hIL-10R on puromycin-resistant clones were analyzed by flow cytometry, as described below. Dimerization of Stat3–GyrB was induced by addition of coumermycin A1 (coumermycin) (Sigma Chemical Co.), at concentrations of 0.1–100 μM. Multiple (at least two) single clones and bulk cultures of each cell line were assayed for all experiments described, and gave similar results.

Transient transfection assays

BaF-mIL-10R and NIH-3T3-mIL-10R cells were transfected by electroporation at 960 μF, 0.26 kV, with a total of 15 μg of plasmid DNA in RPMI1640 at room temperature. All cDNAs were cloned in the pME18S vector, and luciferase gene expression was under control of the minimal β-casein promoter as described (Mui *et al.*, 1995). For co-transfection experiments, equimolar ratios of each plasmid were used, with empty pME18S vector used where necessary. After an 8–12 h recovery period in RPMI1640/10% FBS, IL-10 or coumermycin was added for an additional 16 h, as indicated. Cytoplasmic extracts were prepared in reporter lysis buffer (Promega, Madison, WI), normalized for protein concentration (BCA assay, Pierce Chemical Co.), and assayed in triplicate for luciferase activity (Promega). Where β-galactosidase was used as a control for transfection efficiency, cells were co-transfected with 1 μg of pMEβ-Gal, and β-galactosidase assay (Tropix, Bedford, MA) was performed. For 293T cells, transient transfection with Stat3–GyrB was by the lipofectamine method, as described for Bosc 23 cells (Onishi *et al.*, 1996).

Immunoprecipitation and Western blot analysis

Cells were treated with IL-10 at 37°C, where indicated, washed twice with cold phosphate-buffered saline (PBS), and lysed at 4°C with 0.5% (v/v) Triton X-100 (Pierce Chemical Co., Rockford, IL) in lysis buffer [50 mM HEPES, 100 mM NaF, 10 mM NaPPi, 2 mM Na_3VO_4, 4 mM EDTA, 2 mM pefabloc (Boehringer Mannheim, IN), 10 μg/ml aprotinin and 2 μg/ml leupeptin] for 40 min. Cell lysates were collected following centrifugation at 15 000 r.p.m. for 15 min and stored at –70°C. Lysates were equalized for protein concentration as necessary. Immunoprecipitation experiments were performed as previously described (O'Farrell *et al.*, 1996), using a polyclonal anti-Stat3 antibody (UBI) (this antibody does not immunoprecipitate ΔStat3). Protein transfer and Western blot analysis were performed as described (O'Farrell *et al.*, 1996), using a Stat3 mAb (Transduction Labs, Lexington, KY) or anti-phosphotyrosine mAb 4G10 (UBI) followed by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL). For analysis of Stat3–GyrB oligomerization, hypotonic lysates were made by dounce homogenization and shearing of cells through a 25 gauge needle in lysis buffer (50 mM sodium phosphate pH 8.1, 5 mM EGTA, 5 mM $MgCl_2$), and lysates were collected as above. Lysates (10 μg) were treated with coumermycin at room temperature for 15 min and cross-linked with 1 mM BS3 (Pierce), followed by SDS–PAGE and Western analysis.

Plasmon resonance (BIAcore)

The BIAcore system, sensor chip SA and HBS (10 mM HEPES with 0.15 M NaCl, 3.4 mM EDTA and 0.05% surfactant P20, pH 7.4) were from Pharmacia Biosensor (San Diego, CA). 5′-Biotinylated oligonucleotides (Research Genetics, Huntsville, AL) consisting of three tandem copies either of the high affinity SIE (GATTTACGGGAAATG) or consensus NF-κB (GGGGACTTTCCC) sites were annealed to their complement in TE (10 mM Tris, pH 7.5, 0.1 mM EDTA) and diluted 50-fold into HBS. SA sensor chips were saturated with double-stranded oligonucleotides by flowing 10 μl of a 1 μM oligonucleotide solution over the surface and washed (1 min each of 0.5 M NaCl/HBS and 0.5% SDS/water) before re-equilibration in HBS. Lysates, prepared from either Stat3–GyrB-transfected or untransfected 293T cells, were incubated with 10 μM of either novobiocin or coumermycin for 15 min at 25°C. Samples were supplemented with 100 μg/ml poly(dIdC) before a 40 μl aliquot was applied to the sensor chip at 5 μl/min. The oligonucleotide surface was regenerated with two 1 min washes as above before application of the next sample.

Proliferation and apoptosis assays

[^{3}H]Thymidine incorporation by J774 cells and NBM-derived macrophages cultured on 96-well plates was measured as previously described (O'Farrell *et al.*, 1996), except that cultures were trypsinized prior to harvesting (Micro 96 Skatron Harvester). For all experiments, each condition was assayed in triplicate and, where transfected/transduced cells were used, similar results were obtained with multiple independent clones. At various times during incubations, cells were counted in the presence of trypan blue to confirm cell viability.

Apoptotic cells were detected by the TUNEL assay (TdT-mediated dUTP-X nick end labeling) (Boehringer Mannheim), which detects single- and double-stranded DNA breaks, indicative of apoptosis. Cells were grown on glass slides, fixed, permeabilized and stained according to the manufacturer's protocol prior to visualization by fluorescence microscopy (Zeiss Axioskop).

Assay for short-term proliferation of BaF-mIL-10R cells.

BaF-mIL-10R cells were cultured in the presence or absence of 6 ng/ml hIL-10 (Schering-Plough Research Institute, Kenilworth, NJ) and the indicated amounts of mAb 1B1.2, anti-hIL-10 mAb 12G8 (de Waal Malefyt *et al.*, 1991; Liu *et al.*, 1994) or anti-hIL-10R mAb 3F9 (Liu *et al.*, 1997) in 100 μl/well in 96-well plates. After 48 h, 10 μl of Alamar Blue (Accumed International, Westlake, Ohio) was added per well, and 8–12 h later the $A_{570–600}$ was determined.

Detection of cytokine and nitrite production

Cells were seeded at 1×10^5 cells /ml in DMEM containing 10% serum in 6- or 24-well tissue culture plates. mIL-10 (purified, recombinant, sp. act. 2×10^7 U/mg), hIL-10, LPS (*Escherichia coli* 0111:B4, Difco Laboratories, Detroit, MI) or LPS plus IL-10 were added at the concentrations indicated. Supernatants were removed after incubation times of 4–48 h, to determine concentrations of released cytokines and nitrite. Production of mTNFα and mIL-1β was measured by ELISA, using kits according to the manufacturer's protocols (Genzyme, Cambridge, MA). Culture supernatants were diluted as appropriate to obtain values in the linear range of standard curves. Duplicate or triplicate samples were assayed for each condition and, where transduced cells were used, similar results were obtained with multiple independent clones.

Nitrite production was measured by the Greiss method (Ding *et al.*, 1988). One hundred μl of culture supernatant was combined with 100 μl of Greiss reagent [1% (v/v) sulfanilamide, 0.1% (v/v) naphthylethylene diamine hydrochloride, 2.5% (v/v) phosphoric acid], and incubated at room temperature for 10 min. Absorbance at 570 nM was determined in a Titertek plate reader. Sodium nitrite (Sigma Chemical Co., St Louis, MO) (10 nM–10 μM) was used to prepare a standard curve. Duplicate or triplicate samples were assayed for each condition and, where transfected/infected cells were used, similar results were obtained with multiple independent clones.

Detection of cell surface antigen expression

Cells were grown in 6-well plates, as described for cytokine production. All antibody incubations were for 30 min on ice, followed by two washes with FACS buffer [PBS, 3% (v/v) FCS, 0.025% (v/v) sodium azide], and cells were analyzed on a FACSCAN (Becton-Dickinson, Milipitas, CA) in the presence of propidium iodide (3 μg/ml). For analysis of hIL-10R expression, J774 cells were stained with the anti-hIL-10R monoclonal antibody 3F9 and detected using a fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (10 μg/ml). For detection of Fcγ receptor (FcγR), a FITC-conjugated primary antibody (Pharmingen, San Diego, CA) which recognizes FcγRII and FcγRIII was used, with rat IgG2a–FITC as isotype control. For detection of CD86, it was necessary to block endogenous FcR by pre-incubation of cells with purified anti-FcγR (500 μg/ml) (Pharmingen) and purified isotype control (500 μg/ml) rat IgG2b for 10 min on ice prior to addition of biotinylated anti-CD86 (5 μg/ml), which was visualized by streptavidin–FITC (10 μg/ml) (Pharmingen) staining. All isotype controls gave negative staining profiles, which were not altered following treatment with IL-10 or LPS.

Acknowledgements

We gratefully acknowledge Michael Farrar and Roger Perlmutter for providing us with the GyrB cDNA, and Theo Sana for assistance with BIAcore experiments. We thank Jim Johnston, Ger Manning, Martin McMahon and Rene de Waal Malefyt for review of this manuscript. We are grateful to Atsushi Miyajima for helpful suggestions, Debra Liggett for oligonucleotide synthesis, Dan Gorman, Allison Helms and Connie Huffine for DNA sequencing, members of the FACS lab for cell sorting, and members of the Moore lab for useful discussion during this work. A.-M.O. was supported by a DNAX Research Institute postdoctoral fellowship. The DNAX Research Institute of Molecular and Cellular Biology is supported by Schering-Plough Corporation.

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Received May 29, 1997; revised November 12, 1997; accepted December 9, 1997