Expression of Constitutively Active STAT3 Can Replicate the Cytokine-suppressive Activity of Interleukin-10 in Human Primary Macrophages*

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There is general agreement that signal transducer and activation of transcription 3 (STAT3) is required to mediate the anti-inflammatory activities of interleukin (IL)-10. However, STAT3 is activated by multiple factors that do not share the anti-inflammatory activity of IL-10. The question remains whether STAT3 is sufficient for the anti-inflammatory effects or whether there are other signals required, as had been suggested previously. We set out to map the human IL-10 receptor and to identify the key elements involved in transducing the cytokine-suppressive effects of IL-10. We were able to show an absolute requirement for both of the tyrosine residues found within the YYQo-STAT3-docking site within the IL-10 receptor 1 and that no other signals appeared to be required. We used a constitutively active STAT3 to determine whether expression of this factor could suppress lipopolysaccharide-induced tumor necrosis factor and IL-6 production. Our data show that STAT3 activity can suppress both IL-6 and tumor necrosis factor production in lipopolysaccharide-stimulated macrophages. However, in synovial fibroblasts, STAT3 did not suppress IL-6 production, suggesting that the cellular environment plays an important role in dictating whether STAT3 drives a pro- or anti-inflammatory response.

Inflammation is a key aspect of the host defense against infection and injury. However, if prolonged the inflammatory response can have deleterious effects on the host as seen in chronic auto-inflammatory conditions. Therefore, multiple mechanisms have evolved to regulate and curtail inflammatory responses. One of the most potent is interleukin (IL)-10. The importance of IL-10 to immune homeostasis is clearly demonstrated in IL-10-deficient mice that spontaneously develop inflammatory bowel disease (1). The potency of the anti-inflammatory effects of IL-10 has been demonstrated in animal models of inflammation such as sepsis (2), collagen-induced arthritis (3), insulinitis (4), and in some models of experimental autoimmune encephalitis (5, 6). In a clinical setting, encouraging data have emerged from phase II trials of systemically administrated IL-10 in the treatment of psoriatic skin lesions (7), although similar data from trials in Crohn disease and rheumatoid arthritis produced only a mild amelioration of disease activity, although treatment was limited by toxicity (8, 9).

IL-10 has multiple effects on the immune response (10), the principal of which is inhibition of macrophage activation. IL-10 exerts its negative regulation on macrophage activation by inhibiting key activation processes, such as antigen presentation, by down-regulating major histocompatibility complex class II and co-stimulatory molecules CD80/86 (11–13), inhibiting the expression of cyclooxygenase 2 (COX2), and enhancing the release of anti-inflammatory factors such as soluble TNF receptors (R) and IL-1 receptor antagonist (14). One of the most potent anti-inflammatory effects of IL-10 is the suppression of pro-inflammatory cytokines and chemokines and in particular the key therapeutic target TNF.

However, despite extensive research, the intracellular molecular mechanism by which IL-10 inhibits TNF expression or mediates its other anti-inflammatory effects remains unclear. The IL-10R (15–17) is composed of two chain, IL-10R1 and CRF4/IL-10R2 (18, 19), that are members of the class II/interferon (IFN) subgroup of cytokine receptors. IL-10 activates Jak-1 and Tyk-2 (20) resulting in the activation of signal activator of transcription (STAT) 3; in addition the activation of STAT1 and STAT5 has also been reported (21–24). The numerous studies investigating how IL-10 suppresses cytokine expression have proved to be both controversial and often contradictory with a variety of transcriptionally, post-transcriptionally, and translationally mediated mechanisms being described (25–30). It is also unclear whether the effects of IL-10 on cytokine expression are direct or require de novo gene expression (31, 32). For instance, the induction of Bcl-3 expression has been proposed to be required, as murine macrophages deficient in this gene show no suppression of TNF production in response to IL-10 (33). Alternatively, it has been suggested that IL-10 simply antagonizes LPS-induced stabilization of mRNA as in the case of the chemokine KC (34). More recent data would suggest that the mechanism is indeed indirect as studies by Murray (35) have shown that IL-10 targets inflam-
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Adenoviral Vectors and Viral Infections—The murine/human IL-10R1 chimeric adenoviral vectors were constructed from full-length murine and human IL-10R1 cDNAs using the DNA in Escherichia coli, each clone was sequenced. p4xM67-tk-luci was generated as described previously (42). A 175-bp fragment containing the 4xM67 consensus sequence was excised using Sall/Xhol. The fragment was blunt-end cloned into pGL3, which had been opened using Xhol and phosphorylated. The constitutively activated STAT3C viral construct was a gift from Dr. Michitaka Ozaki (National Research Institute for Child Health and Development, Setagaya, Japan (43)). The NF-κB reporter adenovirus was provided by Dr. P. B. McCray, Jr. (University of Iowa, Iowa City) and is a modification of the pNF-κB reporter vector (Clontech) (44).

The adenoviral vectors were generated through homologous recombination in B cells, purified, and concentrated, based on the method devised by He et al. (7) and as described previously (41). Macrophages were routinely infected with the adenovirus at the stated multiplicity of infection (m.o.i.) for 1 h in

Experimental Procedures

Reagents—IL-10 was a kind gift from Schering Plough; macrophage colony-stimulating factor was a kind gift from Wyeth (Boston); Salmonella typhi LPS was purchased from Alexis (Dorset, UK); anti-HLA DR-CyChrom and Ig-conjugated CyChrom were purchased from BD Biosciences. TNF, IL-6, and IL-10 ELISA reagents were purchased from BD Biosciences. Cells—Monocytes were isolated by centrifugal elutriation as described previously (40). Macrophages were derived from monocytes by culturing the cells with macrophage colony-stimulating factor at 100 ng/ml (41). RA mononuclear cells and synovial fibroblasts were isolated from patients undergoing joint replacement surgery and were isolated as described previously (41).

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serum-free medium. Cells were washed and recultured in growth medium with 5% (v/v) fetal calf serum for 24 h.

Immunoprecipitation and Western Blot Analysis—Stimulated cells were lysed in 1% Triton X-100 lysis buffer (20 mM Tris, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM Na2VO4, 0.5 mM NaF, and protease inhibitor mixture (Sigma)) for 10 min. Lysates were centrifuged by precipitation and for immunoprecipitation purposes were pre-cleared with 20 μl of protein G-Sepharose (Amersham Biosciences). STAT3C was immunoprecipitated with 5 μg of anti-FLAG M2 (Sigma) for 2 h followed by the addition of 20 μl of protein G-Sepharose. Immune complexes were washed three times in 1 ml of Triton lysis buffer. Samples were separated by electrophoresis through 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore) that were blocked for 1 h with blocking buffer (5% w/v fat-free milk, 0.1% (v/v) Tween 20 in PBS) followed by 1 h of incubation with the antibodies, diluted 1:1000 in blocking buffer. Antibodies used were anti-Tyr-705 phospho-STAT3 and anti-STAT3 (New England Biolabs), anti-β-tubulin (Sigma), anti-FLAG M2 (Sigma), anti-HA (Covance, CA), anti-Bcl-3, and anti-SOCS3 (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Amersham Biosciences) was used as secondary antibodies at a dilution of 1:2000. Bound antibody was detected using the enhanced chemiluminescence kit (Amersham Biosciences) and visualized using Hyperfilm MP (Amersham Biosciences).

Quantitation of Gene Expression by Real Time TaqMan RT-PCR—RNA was isolated using a Qiagen RNA blood isolation kit (Qiagen Ltd., Crawley, UK). TaqMan RT-PCR core reagent kit, TNE, IL-6, and glyceraldehyde-3-phosphate dehydrogenase primer/probe mixtures were purchased from PE Biosystems (Warrington, Cheshire, UK). An ABI PRISM 7700 detector sequence was programmed for the initial step of 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Relative quantitation of gene expression was determined using the comparative Ct method. All calculations followed procedures outlined in ABI PRISM 7700 sequence detector system bulletin 2.

Luciferase Assays—After stimulation, cells were washed once in PBS and lysed with 100 μl of CAT lysis buffer (0.65% (v/v) Nonidet P-40, 10 mM Tris-HCl, pH 8, 0.1 mM EDTA, pH 8, 150 mM NaCl). 50 μl of cell lysate was transferred into the well of a luminometer cuvette strip containing 120 μl of luciferase assay buffer (1% Triton X-100, 25 mM Tris, pH 7.8, 8 mM MgCl2, 15% glycerol, 1 mM EDTA, 0.5 mM ATP, 1 mM dithiothreitol). Luciferase activity was measured with a Labsystem luminometer by dispensing 30 μl of luciferin (Bright-Glo luciferase assay system; Promega) per assay point. Cell lysates were assayed for protein concentration by the Bradford assay, and luciferase activity was adjusted accordingly.

FACS Analysis—Macrophages were seeded at 106 cells/well and infected at a m.o.i. of 100 with Ad GFP, Ad m/h-IL-10RI chimera, or the Ad IL-10RI mutants and left to express overnight. The cells were detached with Accutase (PAA Laboratories, Yeovil, UK) and were blocked in FACS wash buffer (PBS, 2% (v/v) fetal calf serum, 0.025% (v/v) NaN3, 2 mM EDTA). Cell surface expression of adeno-virally expressed m/hIL-10RI receptors was assessed using a monoclonal rat anti-mouse IL-10R1 antibody (clone 1B1.3a) kindly donated by Dr. Kevin Moore, DNAx Inc., Palo Alto, CA. 1B1.3a was biotinylated using the EZ-Link Sulfo-NHS-LC biotinylation kit (Pierce) following the manufacturer’s instructions. Cells were incubated with either 1B1.3a or biotinylated isotype control (Serotech, Kidlington, Oxon, UK) followed by incubation with streptavidin Per-CP (Pharminingen). Cells were analyzed on a BD Biosciences LSR FACS can.

RESULTS

Rationale and Design of Chimeric Murine/Human IL-10RI Adenoviral Constructs—This study was performed in primary human macrophages to ensure the greatest relevance to human physiology as possible. To identify functionally critical amino acid residues within the intracellular domain of the IL-10RI, we generated a number of chimeric receptors. These consisted of the murine IL-10RI extracellular domains that were fused with the human IL-10RI chain in an 8-amino acid sequence (QYFTVTVN), just proximal of the transmembrane region (n = amino acid residue 238 of the murine receptor), which is identical between species (Fig. 1a). The resulting chimeric receptor should be sensitive to stimulation by both murine and human IL-10, whereas the endogenously expressed human IL-10R is only sensitive to human IL-10. Therefore, by using murine IL-10 to stimulate cells, any effect of the endogenous human IL-10R would be avoided, allowing us to specifically analyze the effects of any mutations we care to introduce. Using this construct as a backbone, a series of mutations and deletions were generated to map the cytoplasmic domain of the human receptor (Fig. 1a). In particular, the (only) two tyrosine residues within a YXXX motif at 446 and 496 were mutated to phenylalanine (Y446F, Y496F, and Y446F/Y496F). These tyrosines correspond to Tyr-427 and Tyr-477 in the murine receptor which have been shown previously to serve as docking sites for the recruitment of STAT3 (24, 45). We also wanted to investigate whether the homologous four serine residues identified by Riley et al. (38) near the C terminus of the murine receptor were also important. A truncation was made to remove these from the human IL-10R. In addition, a serine → alanine mutant of these homologous four serine residues in the human IL-10RI was also constructed truncated after amino acid 579 (see “Experimental Procedures”). To achieve efficient expression of the murine/human IL-10R constructs in human macrophages, they were inserted into adenoviral vectors. As shown in Fig. 1b, using an anti-murine IL-10R1-specific antibody, we were able to detect expression of all chimeric receptors in the human macrophages.

IL-10-induced STAT3 Phosphorylation and SOCS3 Induction in m/h IL-10RI Chimera-infected Cells—To function as a signaling receptor, the IL-10R1 has to associate with the ubiquitously expressed IL-10R2. Because it was not possible to assess the assembly of such a complex with the m/h chimeras, we used the activation of signaling activity as an indicator of chimera function. Tyrosine phosphorylation of STAT3C is by far the strongest immediate effect of IL-10 signaling, and this was therefore used to test for m/h IL-10R function. In addition, a second readout of IL-10 signaling, the induction of SOCS3...
expression, was also assayed. Both the phosphorylation of STAT3 and the induction of SOCS3 were assayed by Western blot. Macrophages infected with a control virus (Ad GFP) (as expected) did not respond to murine IL-10 but did respond to human IL-10 as judged by phosphorylation of STAT3 or the induction of SOCS3 gene expression (Fig. 2, a and b). In contrast, infection with the construct containing the full-length m/h chimera resulted in cells becoming responsive to murine IL-10 as judged by the phosphorylation of STAT3 and SOCS3 expression (Fig. 2, a and b). Constructs containing single mutations at Tyr-446 or Tyr-496 and the serine-rich region were also still responsive to murine IL-10 as shown by the same criteria. However, the double mutation of both tyrosines Y446F/Y496F and the complete cytoplasmic deletion abolished STAT3 (Tyr-705) phosphorylation and the induction of SOCS3 expression (Fig. 2, a and b).

Tyr-446/496 Are Required to Mediate IL-10 Inhibition of LPS-induced TNF and IL-6 Production—The data presented in this study show that there is a redundancy between the two cytoplasmic tyrosine residues in their ability to induce STAT3 phosphorylation and a downstream effector gene SOCS3. It was also clear from the expression data in Fig. 1 that failure to induce STAT3/SOCS3 signaling is not related to a failure of the constructs to express. Next the ability of the various constructs to mediate the anti-inflammatory activity of IL-10 was evaluated by assessing the inhibition of LPS-induced TNF and IL-6 production. As shown in Fig. 2, in uninfected cells or those infected with Ad GFP control virus, LPS-induced TNF (d) and IL-6 (e) production was effectively inhibited by addition of human IL-10 but not by murine IL-10. Expression of m/h IL-10R1 rendered the cells sensitive to murine IL-10 with the expression of both TNF and IL-6 being inhibited. In parallel with the induction of STAT3/SOCS3 signaling (Fig. 2, a and b), both the single Y446F and Y496F mutants and the serine-rich domain mutant were also able to mediate the inhibitory activity of murine IL-10. Only the Y446F/Y496F and the cytoplasmic deletion failed to support a murine IL-10 response. In summary, these data would indicate that the ability to induce STAT3 activity strongly correlates with IL-10 anti-inflammatory activity. However, in contrast with the study of Riley et al. (38), we found no requirement for the conserved serine residues.

Generation of a Constitutively Active STAT3 (STAT3C)—The data support the view that STAT3 is necessary to mediate the anti-inflammatory effects of IL-10. The data also indicate that it is unlikely that another signal generated by the IL-10R (other than from the tyrosine residues) is required in addition to STAT3. There are two caveats to this supposition. There may be other signals that emanate from tyrosines 446 and 496 other than STAT3 and/or there are sequences in the membrane proximal region, in addition to the Box1 site for Jak1 association, that mediate the anti-inflammatory effect. Deletions of this
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STAT3C is critical to achieve maximal DNA binding affinity, which results in a slower off-rate and protects STAT3C from inactivation from phosphatases (47, 48). Fig. 3a shows high levels of expression of STAT3C in macrophages infected with increasing m.o.i., as detected by Western blotting of the FLAG-tagged STAT3C. To confirm that the STAT3C was constitutively active, cells were co-infected with a second virus encoding a STAT3-response element (M67 SIE) driving the expression of the luciferase gene. The STAT3-luciferase reporter construct responded to IL-10 and less so to IL-6, another STAT3-activating cytokine (Fig. 3b). This is in keeping with our previous observations that IL-10 induces a more sustained level of STAT3 phosphorylation than IL-6 (40). Expression of the STAT3C adenovirus produced a powerful dose-dependent activation of the STAT3 reporter gene with the maximal response occurring at an m.o.i. of 100 of the Ad STAT3C (Fig. 3c). The strength of the stimulus over that of IL-10 could be expected from the high level of overexpression of the STAT3C molecule. We observed very low level constitutive phosphorylation of STAT3C, which could be substantially enhanced by addition of either IL-10 (not shown) or LPS (Fig. 3, d and e). This was detectable within 60 min of post-LPS stimulation. To confirm the functional activity of STAT3C, we were also able to demonstrate that the expression of the molecule alone was able to induce the expression of the IL-10 inducible gene SOCS3 (Fig. 3f).

STAT3C Does Not Affect LPS-induced NF-κB or MAPK Activation but Does Induce Bcl-3 Expression—We were concerned that forced overexpression of STAT3C may have nonspecific effects on other luciferase-based reporter assays. To address this issue we investigated the effect of overexpression of STAT3C on an NF-κB luciferase reporter construct. This luciferase construct uses the NF-κB consensus site in a tandem repeat of four copies. Expression of STAT3C alone did not induce any activation of the NF-κB reporter gene. Also, STAT3C did not inhibit LPS-induced NF-κB activation (Fig. 4a). This is in agreement with our previous studies showing no effect of IL-10 on this NF-κB reporter gene system (27). In agreement with these data, STAT3C had no effect on LPS-induced IkBα degradation (Fig. 4b), although its ability to induce SOCS3 confirmed it was active in this assay. Another key signaling pathway induced by LPS is the activation of the mitogen-activated protein kinases (MAPK) (49, 50). Again, in agreement with our previous studies with IL-10 in primary human macrophages, STAT3C was unable to inhibit the LPS-induced phosphorylation of the MAP kinases p38, p42/44 ERK, or p54 JNK (Fig. 4c).

IL-10 induction of the NF-κB family member Bcl-3 has been proposed to play a key role in the regulation of TNF production, as demonstrated by the failure of macrophages from these Bcl-3–deficient mice to respond to IL-10, as IL-10 can no longer suppress TNF production (33). We were therefore interested to determine whether expression of STAT3 alone could induce Bcl-3 expression. As shown in Fig. 4d, expression of STAT3C alone induced very high levels of expression of Bcl-3, which could not be further enhanced with co-stimulation with IL-10.
Overexpression of STAT3C Inhibits LPS-induced TNF and IL-6 Protein and mRNA Production—Given that STAT3C alone was able to induce Bcl-3, a key regulator of TNF production, we next investigated the ability of STAT3C to regulate pro-inflammatory cytokine production. Infection of macrophages with Ad STAT3C resulted in a dose-dependent inhibition of LPS-induced TNF, IL-6 (Fig. 5, a and b) and IL-8 (data not shown) production. The level of inhibition was similar to that seen with 10 ng/ml of IL-10, although at the higher m.o.i. of virus, TNF expression was totally ablated. In contrast, the control virus, Ad GFP, or wild type Ad STAT3 (data not shown) had no effect on cytokine production at the highest m.o.i. used. Adding IL-10 to Ad STAT3C-infected cells had some additive effect at the lowest m.o.i., but this was not found at higher m.o.i. where Ad STAT3C alone completely inhibited cytokine production. These studies were extended to examine cytokine production induced by ligands for TLRs other than TLR4, e.g. TLR2 (LTA, Pam3Cys, MALP2), TLR5 (flagellin), and TLR7/8 (R848) with very similar results (data not shown). Previously, we have shown that a STAT3 dominant negative construct had no effect upon LPS-induced IL-10 production (40). Consistent with these findings, expression of STAT3C did not affect LPS-induced IL-10 production (Fig. 5c). This also demonstrates that expression of STAT3C does not globally suppress all LPS-induced cytokine synthesis but specifically targets a subset of LPS-induced cytokines.

We have previously shown that IL-10 can suppress TNF mRNA when added simultaneously with LPS (40). As shown in Fig. 5, d and e, expression of the STAT3C construct had no effect upon LPS-stimulated TNF and IL-6 mRNA to comparable levels as observed with IL-10 treatment. STAT3C only affected the LPS-stimulated increase in IL-6/TNF production as basal levels of these mRNAs were unaffected by expression of STAT3C.

So far we have studied the effects of STAT3-dependent cytokine suppression in a model system of TLR-induced cytokine production in human macrophages. However, from an inflammatory disease perspective, a more clinically relevant system to study would be an ongoing inflammatory reaction. We have previously used rheumatoid synovium membrane extracted from patients undergoing joint replacement as a system to investigate the ongoing inflammation-driven cytokine production. We used...
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**FIGURE 4.** Constitutively active STAT3 does not modify NF-κB and MAPK activity but does induce BCL3 expression. a, 1 × 10^6 macrophages were infected with Ad NF-κB-reporter virus (m.o.i. of 50) prior to infection with either Ad GFP or Ad STAT3C (m.o.i. of 50). The next day cells were stimulated with LPS (10 ng/ml) over a 60-min time course. Cell lysates were subjected to Western blotting and probed with anti-IκB, p42/p44 MAPK, and p38 MAPK. b, cells were infected with either Ad GFP or Ad STAT3C (m.o.i. 50). The next day the cells were stimulated with LPS (10 ng/ml) over a 60-min time course. Cell lysates were subjected to Western blotting and probed with anti-IκB, p42/p44 MAPK, and p38 MAPK. c, 2 × 10^6 cells were infected with either Ad GFP or Ad STAT3C (m.o.i. 50) for 1 h. After 18 h, cells were stimulated with IL-10 for 8 h. Cell lysates were subjected to Western blotting, and Bcl-3 expression was determined by probing with an anti-Bcl-3 antibody. The blot was stripped and reprobed for STAT3 and β-tubulin to ensure equal loading and STAT3C expression. These experiments are representative of three independent donors.

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The cytoplasmic tail of murine IL-10R1 contains tyrosine residues at positions 374, 396, 427, and 477. Only two of these, Tyr-446 and Tyr-496, are conserved in the human receptor and both are within the STAT3-binding YXXQ motif (16). It had been shown previously in murine macrophage cell lines that mutation of Tyr-427/446 and Tyr-477/496 resulted in a loss of IL-10 signaling as measured by a failure to induce STAT3 and the inhibitory effects on macrophage function (38, 53). However, the study of O’Farrell et al. (53) in the J774 murine macrophage cell line went on to show that STAT3 was not required for the inhibition of macrophage activation, i.e. cytokine production and CD86 expression. In contrast, Riley et al. (38) working in RAW264.7 cells concluded that an additional signal derived from a serine-rich region near the C terminus was

the Ad STAT3C in this system to see if we could modify the spontaneous production of TNF and IL-6. Fig. 6 shows the data generated from four patients for TNF (TNF was not detectable in two patient samples) and six patients for IL-6. Addition of IL-10 strongly inhibited TNF production in keeping with our previous findings (51), whereas IL-10 only had only a mild inhibitory effect on IL-6 production. Infection with the Ad GFP had no effect upon the spontaneous production of either IL-6 or TNF. However, expression of the STAT3C significantly inhibited TNF production, although not to the same degree as IL-10. This may be a reflection of the time lag between infection of the virus and expression of the STAT3C protein; there will be a window of time where the cells are releasing TNF, prior to expression of the STAT3C protein. Similarly, Ad STAT3C infection only caused a mild but still significant inhibition of IL-6 production.

STAT3 is present and is activated in many cell types by various stimuli that are not associated with anti-inflammatory activities. We were therefore interested to determine whether the effects of STAT3C were specific to cells of the myeloid lineages or whether STAT3C could display these profound cytokine-suppressive qualities in all cell types. To address this issue we cultured RA synovial cells over multiple passages (routinely 4) until they consisted of only synovial fibroblasts. We have shown previously (52) that these cells respond to LPS and produce pro-inflammatory cytokines. We infected these cells with either Ad GFP or Ad STAT3C, and we assessed their ability to respond to LPS-induced IL-6 production (as they do not produce significant quantities of TNF). In contrast to the inhibitory activities displayed in macrophages and RA synovial cells, STAT3C actually significantly enhanced production of both spontaneous and LPS-induced IL-6 production (Fig. 6c), suggesting that the cytokine suppressive activities of this molecule may indeed be limited to cells of the myeloid lineage. This was further supported by similar experiments performed in IL-1-stimulated HeLa cells. Again, expression of STAT3C enhanced IL-6 production (Fig. 6d) and IL-8 (data not shown).

**DISCUSSION**

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required in addition to STAT3 to provide the anti-inflammatory signal. The precise nature of this signal has never been elucidated. Moreover, the importance of STAT3 has been supported by studies in primary murine STAT3/−/− macrophages that showed impaired IL-10 function (37), suggesting that the data of O’Farrell et al. (53) could be unique to the J774 cell line. However, the lack of STAT3 does not preclude that other additional signals are required to mediate the anti-inflammatory activity of IL-10. A second signal, as proposed by Riley et al. (38), could have explained why STAT3, a signal common to numerous cytokines, appears to be anti-inflammatory only in the context of IL-10. However, this study shows that activated STAT3 alone can mimic many of the anti-inflammatory activities of IL-10, i.e. inhibition of LPS-induced TNF and IL-6 mRNA and protein production and the induction of Bcl-3. Given the data, we are only able to conclude that the difference between this study and that of Riley et al. (38) could be down to differences in the cell system/species used or the m/h IL-10R1 chimera versus the interferonγ/IL-10R used (38). Specifically, in our system the m/hIL-10R1 would be able to recruit the endogenous IL-10R2, whereas the chimeric receptors used by Riley et al. (38) would recruit the IFNGR2 chain. With respect to the difference between species, it is worth noting that the forced expression of STAT3C in murine dendritic cells was unable to inhibit TNF, contrary to our findings. However, this construct was able to inhibit IL-12 production (39). These data would support the requirement for an additional signal, other than STAT3, that is required to mediate the cytokine-suppressive effects of IL-10 in murine cells, but as our data show, this does not appear to be the case in human cells.

It is worth noting that STAT3C could not mimic all the activities of IL-10, as IL-10-induced expression of IkBα and B-ATF2 was not reproduced by STAT3C, and its overexpression resulted in decreased STAT1 expression (data not shown), which has been shown previously by others (54). STAT3C is predominantly localized within the nucleus, which is in contrast to the transient nature of IL-10-induced nuclear accumulation of STAT3. This persistent nuclear accumulation may well account for differences between IL-10-induced STAT3 activation and STAT3C action. STAT3C may be acting as a transrepressor by sequestering away co-factors/transcription factors from the promoters as these genes. For example, we observe STAT3C binding to the p65 subunit of the transcription factor NF-κB. This binding may sterically hinder association of the p65 with other cofactors required for the full transcription activity and thus inhibit transcription of NF-κB-driven genes such as TNF and IL-6.

It has been shown recently that STAT3C requires tyrosine phosphorylation for maximal DNA binding affinity (47, 48). In

FIGURE 5. Constitutively active STAT3 is sufficient for cytokine suppressive activity. 1 × 10⁵ macrophages were infected with Ad STAT3C m.o.i. of 50 or an Ad GFP control (50 m.o.i.) for 1 h; virus was removed and then left to express overnight. The next day, cells were left unstimulated (open bars) or stimulated with LPS (10 ng/ml) (solid bars) ± hIL-10 (10 ng/ml) (empty bars). After 18 h supernatants were harvested, and TNF (a) or IL-6 (b) and IL-10 (c) levels were determined by ELISA. Alternatively cells were stimulated for 2 h with LPS (10 ng/ml) (+) IL-10 (10 ng/ml); RNA was extracted, and mRNA analysis was performed using Taqman PCR to detect TNF (d) or IL-6 (e) mRNA. These experiments are representative of three independent donors.
unstimulated macrophages, we observed that STAT3C was only minimally tyrosine-phosphorylated, but this phosphorylation could be substantially enhanced by the addition of LPS. This basal level of tyrosine phosphorylation would appear to be sufficient to drive the transcription of Bcl-3; however, in the case of SOCS3, the increased level of tyrosine phosphorylation may significantly enhance the ability of STAT3C to give the optimal transcriptional response. Alternatively, as IL-10 induces other signaling pathways, distinct from the JAK/STAT3 pathway, these may contribute to the regulation of SOCS3. One of these is likely to be PI3K, and we have shown previously that IL-10-induced HO-1 expression requires both the STAT3 and PI3K pathways (55). Supporting this view, we have observed that inhibition of PI3K by either LY294002 or wortmannin partially inhibits IL-10-induced SOCS3 expression; however, this kinase is not required for IL-10 suppression of TNF and IL-6 production in human cells (56).

Given that many other cytokines activate STAT3, it might seem unlikely that this signal alone would be sufficient to mediate the anti-inflammatory response of IL-10. The answer to this may lie in quantitative rather than qualitative aspects of signaling. IL-10 is quite unique in its ability to induce a sustained level of STAT3 phosphorylation. Although other cytokines induce STAT3 phosphorylation, this activation is only transient. IL-6 is a potent activator of STAT3 in macrophages but only induces a burst of activation, lasting only a few hours, which is then rapidly deactivated by mechanisms such as SOCS3 induction. In SOCS3-deficient mice, a sustained activation of STAT3 by IL-6 was observed, and the cytokine displayed anti-inflammatory properties (57, 58). This may suggest that IL-10 is such a potent anti-inflammatory cytokine by virtue of its ability to deliver a sustained STAT3 activation, because SOCS3 is unable to deactivate the IL-10R system (57), which of course would be more than adequately mimicked by the STAT3C construct. However, we cannot rule out the possibility that the enhanced level of tyrosine phosphorylation on STAT3C we observed following LPS stimulation is responsible for mediating the effect of STAT3C on TNF and IL-6 production. As this phosphorylation is only evident after 60 min, it may suggest that it is not direct and rather is mediated from another LPS-induced cytokine. However, it does not detract from the central observation that STAT3 is mediating these cytokine-suppressive effects.

Another possibility is that the cellular context of STAT3 is important to its generation of the anti-inflammatory response. It is worth noting that another member of the IL-10-interferon family, IL-22, has been considered to be pro-inflammatory in hepatocytes. This cytokine also utilizes the IL-10R2 chain and is a strong activator of STAT3, but it actually induces the expression of pro-inflammatory mediators (59, 60). Our studies in synovial fibroblasts and HeLa cells support such a possibility, suggesting that the cellular environment is an important key to the fate of STAT3 activation.

Our studies have naturally led to the question as to how STAT3 might mediate the anti-inflammatory effects of IL-10. Much controversy exists within the IL-10 field, and various studies have proposed that IL-10 inhibits NF-κB (26, 30) or, alternatively, p38 MAPK activation (29, 61). However, consistent with previous studies in human macrophages, we found no effect of STAT3C on either pathway (27). There is much evidence to suggest that STAT3 is required to induce the expression of intermediate genes. HO-1 and Bcl-3 have both been proposed for this role. As mentioned, HO-1 is not induced by STAT3 alone but requires the PI3K pathway (55), but we were able to show that Bcl-3 is induced by STAT3C alone. Therefore, our data are consistent with a signaling pathway whereby IL-10 induces Bcl-3 via STAT3 to block TNF expression. However, it must be noted that macrophages deficient in Bcl3 are still

\[ \text{FIGURE 6. Constitutively active STAT3 suppresses TNF and IL-6 production in RA mononuclear cells but enhances IL-6 production in nonmyeloid cells.} \]

\[ \begin{align*}
\text{Uninfected} & \quad \text{Ad GFP} & \quad \text{Ad STAT3C} \\
\text{TNF (pg/ml)} & \quad \text{IL-6 (pg/ml)} & \quad \text{IL-6 (pg/ml)} \\
\text{unstimulated} & \quad \text{LPS} & \quad \text{IL-1} \\
\text{Uninfected} & \quad \text{Ad GFP} & \quad \text{Ad STAT3C} \\
\text{IL-6 (pg/ml)} & \quad \text{IL-6 (pg/ml)} & \quad \text{IL-6 (pg/ml)} \\
\text{unstimulated} & \quad \text{LPS} & \quad \text{IL-1} \\
\end{align*} \]

\[ \begin{align*}
\text{uns} & \quad \text{GFP} & \quad \text{STAT3C} & \quad \text{IL-10} \\
\text{TNF (pg/ml)} & \quad \text{IL-6 (pg/ml)} & \quad \text{IL-6 (pg/ml)} & \quad \text{IL-6 (pg/ml)} \\
\text{uns} & \quad \text{GFP} & \quad \text{STAT3C} & \quad \text{IL-10} \\
\end{align*} \]
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responsive to IL-10 suppression of IL-6 (33), suggesting that IL-10 utilizes multiple mechanisms, distal of STAT3, to suppress cytokine synthesis. To further elucidate the role of STAT3, we are currently profiling the array of genes driven by STAT3 expression to address this pivotal question.

In summary, this study has investigated a key aspect of the molecular mechanism by which the anti-inflammatory activity of IL-10 is mediated. Using the relatively homologous system of an m/h IL-10RI chimera expressed in human primary macrophages, this study showed that loss of the tyrosines 446/496 resulted in the loss of STAT3 activity, which correlated with loss of anti-inflammatory signaling. The study goes on to show that not only is STAT3 necessary but it is sufficient to replicate the cytokine suppressive activities of IL-10 in human macrophages. In addition, the study goes on to define the cellular environment as a critical factor in determining whether STAT3 can be considered to be an anti-inflammatory mediator.

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