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IL-10 Induces IL-10 in Primary Human Monocyte-Derived Macrophages via the Transcription Factor Stat3[1]

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IL-10 is an important immunosuppressive cytokine that can down-regulate expression of other cytokines and has been shown to down-regulate itself. We show, in this study, that treatment of human monocyte-derived macrophages with IL-10 induces IL-10 mRNA in a dose- and time-dependent manner with an optimum induction at 100 ng/ml and at 6 h, whereas IL-10-induced IL-10 protein can be detected at 18 h. In the same cells, IL-10 can partially suppress IL-10 mRNA induced by LPS, but only down to the level of IL-10-induced IL-10. An adenoviral luciferase reporter construct driven by the −195 IL-10 promoter, which contains a Stat motif, was readily induced by both IL-10 and LPS. Mutation of this Stat motif ablated IL-10 activation of this promoter, but not the LPS activation. Finally, we show that overexpression of a dominant-negative Stat3 protein will prevent IL-10 induction, but not LPS induction, of IL-10 mRNA. These data show that IL-10 induces IL-10 in monocyte-derived macrophages in an autocrine manner via activation of the transcription factor Stat3. ***The Journal of Immunology,*** **2007, 178: 4779–4785.**

Interleukin 10 is an immunosuppressive cytokine produced by T cells, B cells, dendritic cells, and monocytes/macrophages (1–3). The immunosuppressive activity of IL-10 is highlighted by the findings that IL-10 knockout mice show increased autoimmune disease and increased resistance to infection (4, 5). As has been shown by the group of de Waal Malefyt et al. (6), IL-10 can suppress the immune response by decreasing cell surface expression of MHC class II and by down-regulating the expression of other cytokines. These early studies also noted that IL-10 could down-regulate expression of IL-10 induced by LPS.

IL-10 acts by binding to the IL-10R1/IL-10R2 receptor complex that recruits Jak1 and Tyk2, and these then phosphorylate and activate the transcription factor Stat3 (7, 8). Although IL-10 activates Stat3, we and others have provided evidence that IL-10 itself may be controlled by Stat3 binding to a cognate motif in the IL-10 promoter (9–11). These studies in human cells were supported by a report showing the absence of IL-10 production in peritoneal macrophages from animals that had a macrophage-specific knockout of the *Stat3* gene (12). These molecular studies would suggest that IL-10 is able to induce rather than suppress IL-10. In the present report, we demonstrate that this is, in fact, the case, and we show that IL-10 will induce IL-10 by transactivating the IL-10 promoter via Stat3 in primary human monocyte-derived macrophages (MDM).[4]

Materials and Methods

Materials

Salmonella abortus equii (*S.ae*) LPS was purchased from Axxora Life Sciences. Recombinant human IL-10 (rhIL-10) was obtained from R&D Systems and murine IL-10 was obtained from PeproTech. Polymyxin B (PMB) was purchased from Sigma-Aldrich.

Generation of MDM

PBMC were isolated from heparinized (10 U/ml) blood from healthy donors by centrifugation on Ficoll-Paque (Amersham Biosciences) according to the manufacturer's instructions. $CD14^+$ cells were positively selected from PBMC using MACS CD14 microbeads (Miltenyi Biotec) according to the manufacturer's instructions. Cells were resuspended at a density of 1×10^6 cells/ml in RPMI 1640 medium as previously described (10). Cells were cultured in 6-well Costar ultra-low attachment microplates (Costar) 3 days before any analyses or manipulation.

Plasmids

The four −195-bp IL-10-promoter fragments and the p4xM67-tk-luci (Stat reporter) were generated as previously described (10, 13). These promoter constructs were further subcloned into the pAdTrack vector (Qbiogene) to generate pAdT.IL10–195.wt-luc, pAdT.IL10–195.statm-luc, pAdT.IL10–195.irfm-luc, pAdT.IL10–195.stat/irfm-luc, and pAdT-4xM67-tk-luc.

The full-length murine human (m/h) IL-10RI chimeric construct was generated through the introduction of an *Ssp*I site in the membrane-proximal sequence of the 5′ section of the mIL-10RI and the 3′ section of the hIL-10RI using primers 5′Murine and 3′Human *Ssp*I. The PCR fragments were cloned into an intermediate blunt-end cloning vector using the pCR Blunt II vector and TOPO cloning kit (Invitrogen Life Technology). The 5′ primer contained a *Nhe*I, and all 3′ primers contained a *Hin*dII site to allow directional subcloning of the PCR fragment. All standard PCRs were conducted as 4 min at 92°C, then 35 cycles at 30 s 92°C, 30 s at 60°C, 2 min at 72°C per cycle, and then 10 min at 72°C. Primers used were 5′Murine 5′-GCT AGC ATG TTG TCG CGT TTG CTC CCA-3′; 3′ Murine *Ssp*I 5′-GCT AGC GTC GAC AAG CTT ACA GTG AAA TAT TGC TCC GTC GT-3′; 5′Human *Ssp*I 5′-CAC CAG GCA ATA TTT CAC CGT-3′;

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[4] Abbreviations used in this paper: MDM, monocyte-derived macrophage; rhIL-10, recombinant human IL-10; AdV, adenovirus; MOI, multiplicity of infection; PMB, polymyxin B; *S.ae*, *Salmonella abortus equii*; WT, wild type; IRF, IFN regulatory factor; dn, dominant negative; β_2m, β_2-microglobulin; m, murine; h, human.

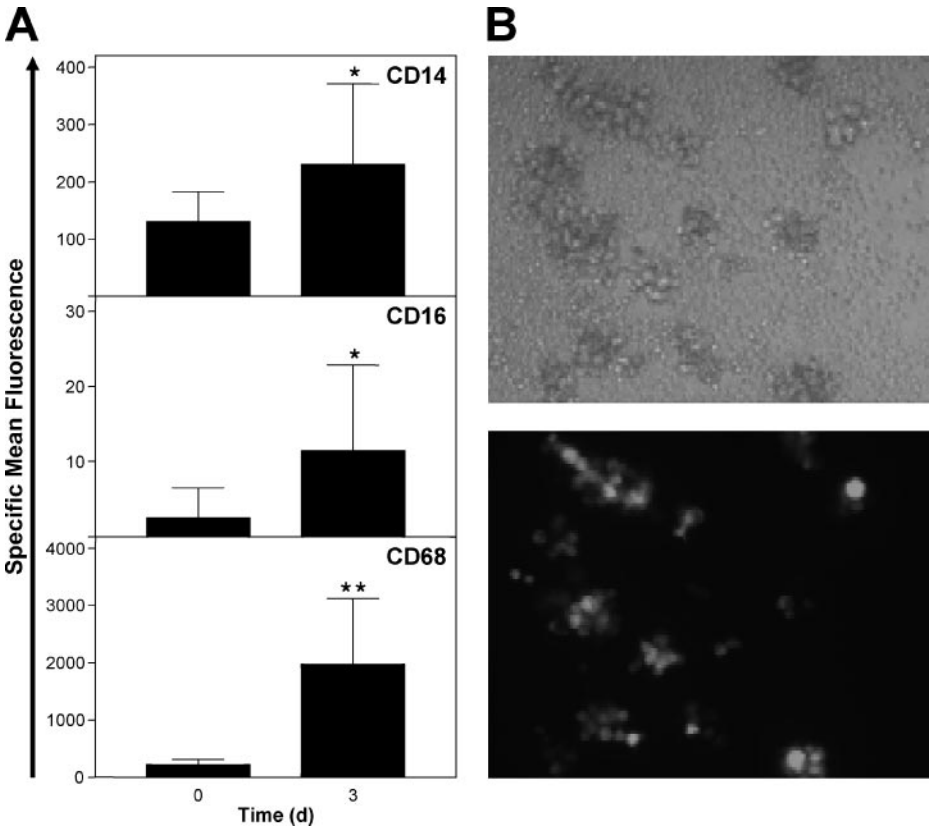

FIGURE 1. AdV specifically target MDM for infection. *A*, On day 0 or day 3 after incubation, PBMC were stained with PC5-labeled anti-CD14 and isotype control, PE-labeled anti-CD16 and isotype control, or PE-labeled anti-CD68 Ab and isotype control. Data are expressed as mean fluorescence intensity corrected for isotype control staining ± SD of six independent experiments. Results were considered significant if $p < 0.05$ (*) when compared with the day 0 sample. *B*, PBMC were cultured for 3 days before treatment with IL-10 AdV at a MOI of 100 for 2 h in serum-free medium. After addition of serum, cells were cultured overnight before analyzing on a Nikon Diaphot 300 fluorescent microscope using phase contrast (*top*) or fluorescent light using a FITC filter (*bottom*). Only macrophages are GFP^+.

and 3′Human FLAG 5′-AAG CTT TCA CTT GTC ATC GTC GTC CTT GTA GTC CTC ACT TGA CTG CAG CTA GA-3′. The full-length m/h IL-10RI chimeric construct was excised from TOPO using *Nhe*I/*Hin*dII and cloned into pAdT, which had also been opened with *Xba*I/*Hin*dII.

Adenoviral vectors and their propagation

The AdEasy Vector Kit (Qbiogene) was used to generate recombinant replication-deficient viruses according to the manufacturer's instructions and as previously described (14, 15). The Adeno-X Rapid Titer Kit (BD Biosciences) was used to determine viral titer. A recombinant, replication-deficient adenoviral vector encoding the dominant-negative human STAT-3 Tyr705→Phe (S3 DN) (16) was provided by Y. Fasjio (University of Osaka, Osaka, Japan). An identical construct lacking the insert (Ad0) was provided by A. Byrnes and M. Wood (University of Oxford, Oxford, U.K.).

Adenoviral infection of MDM

MDM were infected with luciferase reporter viruses and the Stat3DN adenovirus (AdV) for 2 h in serum-free medium in 24-well Costar ultra-low attachment plates at the multiplicity of infection (MOI) indicated. FCS was then added back to cultures at a concentration of 10% (v/v), and cells were incubated at standard conditions overnight. Cells were washed and resuspended in medium containing 10% serum. MDM were infected with the m/h IL-10RI chimera AdV at 1 MOI overnight in medium containing 5% FCS in 24-well Costar ultra-low attachment plates.

Luicferase assay

Infected cells were replated at 3.75×10^5 cells in 1 ml of medium onto a 24-well Costar ultra-low attachment plate before being subjected to stimulation for 6 h. Cells were then harvested and resuspended in 1 × reporter lysis buffer (Promega). Luciferase activity in cell lysates was determined using a Sirius model luminometer (Berthold Technologies) and the Luciferase Assay System from Promega. Protein concentrations were determined using Bio-Rad D_C Protein Assay reagents (Bio-Rad).

RNA and protein isolation

RNA was isolated from cells using TRI Reagent (Sigma-Aldrich) according to the manufacturer's instructions. Protein was isolated using either TRI Reagent or with cells being lysed with buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, and 1.5 mM $MgCl_2$) supplemented with 10 μg/ml aprotinin, 1 mM PMSF, 40 μg/ml leupeptin-propionyl, 20 μg/ml pepstatin A, and 2 mM DTT, as indicated.

Relative quantification of IL-10 mRNA expression using real-time RT-PCR

Reverse transcription of isolated RNA was conducted as previously described (17). Reverse transcription products (3 μl) were amplified in a 20-μl reaction mix containing 1× SYBR Green *Taq* ReadyMix Capillary Formulation (Sigma-Aldrich), 1.5 mM $MgCl_2$, and 0.375 μM of each primer. Primers for IL-10 were forward 5′-GCC TAA CAT GCT TCG AGA TC-3′ and reverse 5′-TGA TGT CTG GGT CTT GGT TC-3′. Primers for β_2-microglobulin (β_2m) were forward 5′-GGC TAT CCA GCG TAC TCC AAA G-3′ and reverse 5′-CAA CTT CAA TGT CGG ATG GAT G-3′. Amplifications were conducted on a Roche LightCycler using the following cycling parameters: preincubation at 95°C for 10 min, then 96°C for 10 s, 60°C for 10 s, and then 72°C for 25 s. All reactions were finished with a melting curve run to establish the specificity of the PCR. Data are expressed as a ratio to β_2m.

Western blot analysis

Ten micrograms of protein was resolved on a 4–12% Novex Tris-glycine gel (Invitrogen Life Technologies) and transferred to Hybond-N membranes (Amersham Biosciences) using a Novex X-Cell II Mini Cell. Blots were reacted with a pStat3 (Tyr705) Ab (SC 7993R; Santa Cruz Biotechnology) or polyclonal Stat3 Ab (SC 482; Santa Cruz Biotechnology) as indicated, followed by peroxidase-conjugated anti-rabbit IgG (A0545; Sigma-Aldrich). Blots were developed using the ECL kit (Amersham Biosciences) and were visualized on Kodak BioMax X-Omat AR film (Sigma-Aldrich).

Secretion assay for human IL-10 protein

After infection, cells were harvested and resuspended in 90 μl of medium containing 5% FCS, and 10 μl of human IL-10 catch reagent (Miltenyi Biotec) was then added. After a 5-min incubation on ice, 900 μl of serum-containing medium was added and the cells were stimulated as indicated. Cells were incubated at 37°C for ~18 h with rotation. Cells were then treated as per the manufacturer's instructions. They were then stained with anti-IL-10 and initially gated on the GFP^+ population. Positive signals in gate M1 were determined and expressed as specific mean fluorescence

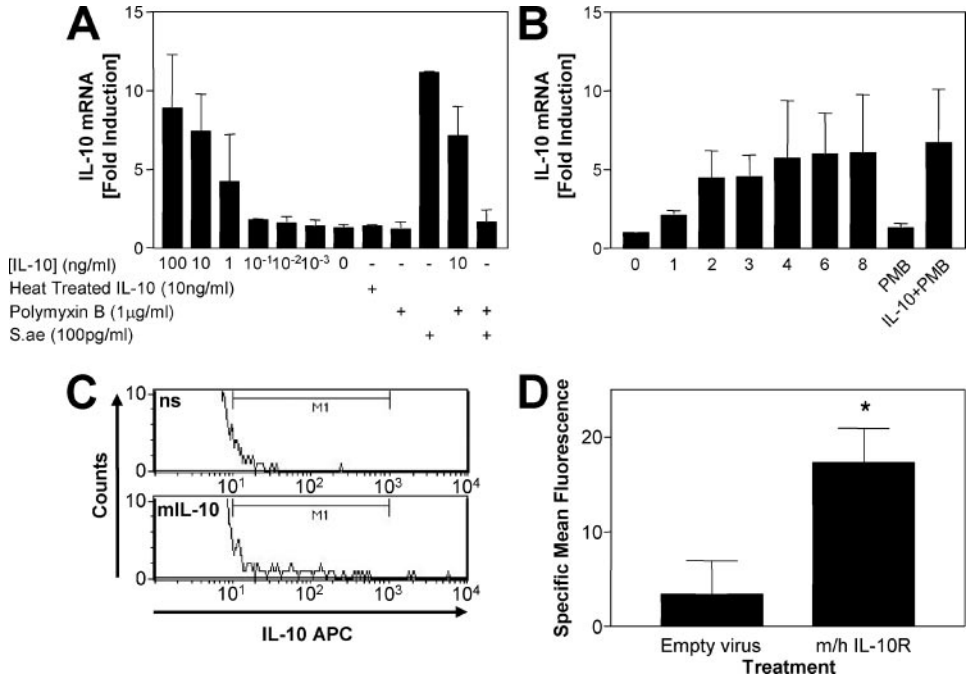

FIGURE 2. IL-10-stimulated MDM can produce IL-10. *A*, Dose-response analysis for mRNA. PBMC were cultured for 3 days before stimulation with IL-10 at the indicated concentrations, IL-10 (10 ng/ml) treated at 95°C for 5 min, PMB (1 μg/ml), or *S.ae* LPS (100 pg/ml). Cells were harvested after 6 h, and RNA was extracted and reverse transcribed. IL-10 and β_2m steady-state mRNA levels were measured using real-time PCR; data were normalized to β_2m and are expressed as fold induction over the untreated (0) sample. Data from three independent experiments are expressed as means ± SD, except heat-treated IL-10 and *S.ae*-treated cells where $n = 2$. *B*, Time-course analysis for mRNA. PBMC were cultured for 3 days, then washed and stimulated with IL-10 (10 ng/ml) and/or PMB (1 μg/ml for 6 h). Cells were harvested after the appropriate time points, RNA was extracted and reverse transcribed, and IL-10 mRNA was determined as described above. Data from at least three independent experiments are expressed as means ± SD. *C* and *D*, Induction of IL-10 protein. PBMC were cultured for 2 days before treatment with empty AdV or the m/h IL-10R chimera AdV at a titer of 1 MOI. Cells were cultured overnight before being resuspended in fresh serum-supplemented medium. After resuspension, cultures were treated with mIL-10 (100 ng/ml) for ~18 h in the presence of the Miltenyi Biotec catch reagent. Cells were then incubated with the allophycocyanin-labeled IL-10 detection reagent and were analyzed by flow cytometry. *C*, Example of staining for unstimulated (ns), and mIL-10-stimulated, chimera-infected cells. *D*, Specific mean fluorescence intensity for IL-10 protein expression was determined by calculating the Δ of unstimulated and stimulated cells for empty AdV-infected cells and for cells infected with m/h IL-10R chimera AdV. Average ± SD of three independent experiments, further analyzed using a paired *t* test. *, $p < 0.05$.

intensity. M1 was defined based on the signal obtained by adding exogenous human IL-10 as a stimulus, followed by staining with anti-IL-10.

Statistics

Analyses were performed using a paired *t* test. Results were considered significant if $p \leq 0.05$ (***, $p \leq 0.001$; **, $p \leq 0.01$; and *, $p \leq 0.05$).

Results

MDM: characteristics and infection by recombinant AdV

For generation of MDM, we isolated PBMCs and cultured these cells for 3 days in low-attachment plates. The MDM derived in this fashion expressed statistically significant greater amounts of CD14 (2-fold), CD16 (5-fold), and CD68 (6-fold) compared with freshly isolated PBMC as measured by FACS (Fig. 1*A*). The increase in expression in the latter two markers is consistent with maturation to macrophages. Such cells could be readily infected with AdV, which were added on day 3. This led to a robust infection rate with >80% of all $CD14^+$ MDM being GFP positive at 100 MOI, as measured by FACS. Positivity of macrophages, but not lymphocytes, is evident in fluorescence microscopy analysis (Fig. 1*B*).

IL-10 induces IL-10 mRNA in human MDM

Because IL-10 acts via activation of Stat3, and because we have shown that Stat3 is a crucial transcription factor controlling the IL-10 promoter (9, 10), we asked whether IL-10 might be able to induce its own gene. Addition of IL-10 protein (100 ng/ml) to human MDM, in fact, led to an 8-fold induction of IL-10 mRNA (Fig. 2*A*). At 1 ng IL-10/ml, there was still a 4-fold induction, but lower concentrations of the cytokine had no effect. To demonstrate that this was a genuine effect of IL-10 and not of contaminant LPS, we heat-treated IL-10 at 10 ng/ml, and this ablated all activity. Conversely, addition of 1 μg/ml PMB to IL-10 (10 ng/ml) had no effect on IL-10 mRNA production, whereas the same amount of PMB was able to block the induction caused by exogenous LPS from *S.ae* (Fig. 2*A*). Time-course analysis revealed a rapid induction of IL-10 mRNA by IL-10 in that a significant expression was already evident at 1 h with a plateau from 4 to 8 h. Again, PMB was unable to neutralize the action of IL-10 at 6 h (Fig. 2*B*).

IL-10 induces IL-10 protein in human MDM

The induction of IL-10 mRNA led us to investigate whether protein was also being induced. Cells cultured for 24 h with LPS (100 pg/ml) produced, on average, 120.4 pg/ml IL-10 protein (±SD, 80.2, $n = 3$) as measured by IL-10 ELISA. However, it was not possible to use the standard ELISA approach for analysis of IL-10-induced IL-10 protein, because of the problem of differentiating the exogenous human IL-10 used for stimulation and the endogenous human IL-10 produced in the culture. We therefore tried to detect IL-10-induced IL-10 protein using metabolic labeling, but could not induce a signal, possibly due to the low sensitivity of the system (data not shown). We therefore used a chimeric IL-10R construct consisting of a murine extracellular portion and a human

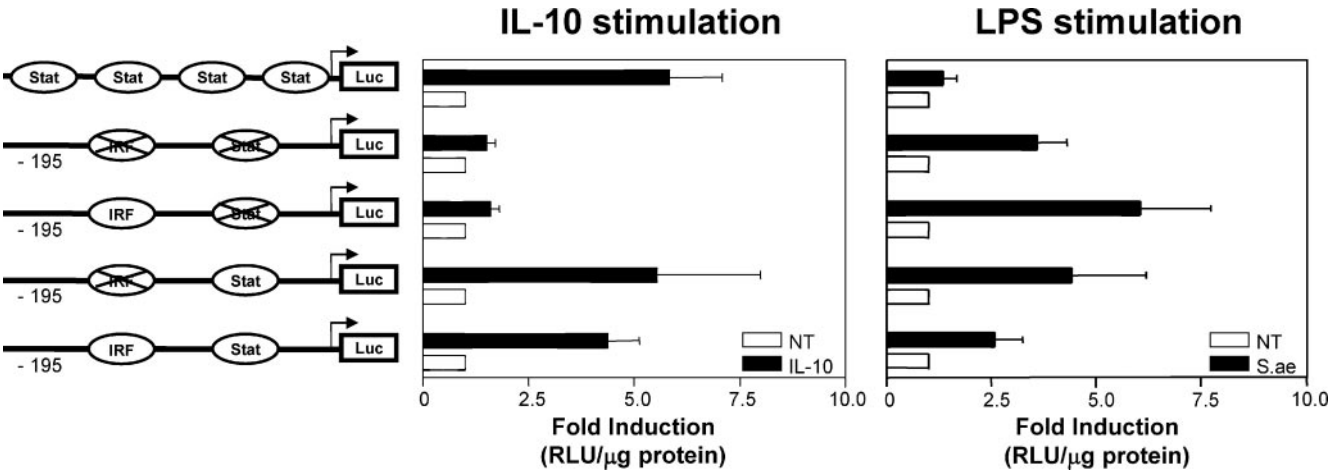

FIGURE 3. Stat3 is essential for IL-10 induction of the *IL-10* promoter, but dispensable for LPS induction in MDM. PBMC were cultured for 3 days before treatment with or without AdV, as indicated, at a MOI of 100 for 2 h in serum-free medium. After addition of serum, cells were cultured overnight before being resuspended in fresh serum-supplemented medium. After resuspension, cultures were not treated (NT, □) or treated (■) with rhIL-10 (10 ng/ml) or *S.ae* LPS (100 ng/ml), as indicated, for 6 h. After incubation, cells were harvested and luciferase (Luc) activity and protein concentration were measured. Data were normalized to protein concentration and are expressed as means ± SD of four independent experiments.

transmembrane and intracellular domain. This construct was delivered to MDM using adenoviral vectors and, unlike the endogenous human receptor, it responds to murine IL-10 by signaling via the human intracellular domain (18). Infected cells were stimulated overnight with recombinant murine IL-10, and the human IL-10 protein released was determined by cell surface staining using the secretion assay. As shown in Fig. 2*C*, treatment of cells expressing the m/h IL-10R with murine IL-10 induced detectable human IL-10 protein, unlike cells infected with an empty control virus (Fig. 2*D*). The levels of IL-10 were low but significant and show that IL-10 can induce its own protein in an autocrine fashion.

IL-10, but not LPS, induces IL-10 promoter activity via Stat3

In earlier studies in a B cell line, we demonstrated a crucial role for the −120 Stat motif of the human *IL-10* promoter in controlling expression of the gene (9, 10). To study the role of this promoter

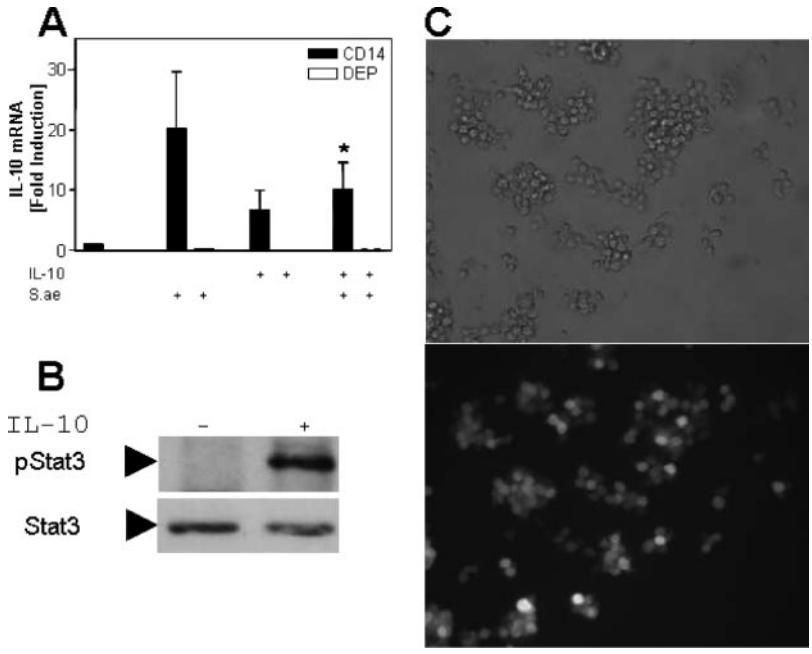

FIGURE 4. Induction of IL-10 by LPS and IL-10 is specific to MDM. *A*, PBMC separated by MACS into $CD14^+$ cells (■) or CD14-depleted cells (DEP, □) were cultured for 3 days before treatment with or without IL-10 (10 ng/ml) and/or *S.ae* LPS (100 pg/ml). Cells were harvested after 6 h, and RNA was extracted and reverse transcribed. IL-10 and β_2m steady-state mRNA levels were determined using real-time PCR. Data were normalized to β_2m and are expressed as fold induction over untreated sample. Data from four independent experiments are expressed as means ± SD and further analyzed using a paired *t* test. ∗, $p < 0.05$ when compared with the CD14, *S.ae*-treated sample. *B*, $CD14^+$ cells were cultured for 3 days before treatment with IL-10 (10 ng/ml). Cells were harvested after 1 h and cells were lysed with buffer A. Protein (10 μg) from each experiment were run on SDS-PAGE and immunoblotted with anti-phospho-Stat3 (*top*), then the membrane was stripped and reprobed with anti-Stat3 (*bottom*). Blots representative of three independent experiments are shown. *C*, $CD14^+$ cells were cultured for 3 days before treatment with IL-10 AdV at a MOI of 100 for 2 h in serum-free medium. After addition of serum, cells were cultured overnight before analyzing on a Zeiss Axiovert 135 fluorescent microscope using phase contrast (*top*) or fluorescent light with a FITC filter (*bottom*).

site in primary MDM, we have cloned wild-type (WT) and mutant *IL-10* promoter fragments into an adenoviral luciferase reporter virus. Infection of MDM with WT virus showed an average 4.5-fold induction of the promoter by human IL-10 and 2.6-fold by LPS (Fig. 3). Mutation of the Stat site led to an ablation of the induction by IL-10, whereas mutation of the IFN regulatory factor (IRF) motif had no effect. Also, mutation of both the Stat site and the IRF site ablated activity consistent with the ablation seen with mutation of the Stat site alone (Fig. 3). In contrast, none of the mutations ablated activity of the LPS-induced promoter; indeed, mutations of the promoter seemed to enhance the LPS-induced luciferase activity. Furthermore, when we infected the MDM with an adenoviral reporter construct containing a tetrameric Stat motif (4xM67), we observed an almost 6-fold induction by IL-10, but almost no induction (average 1.3-fold) by LPS (Fig. 3). These data are in line with the concept of tissue-specific and signal-specific expression of the *IL-10* gene in that Stat3 appears to be crucial for LPS-induced expression in a B cell line and not in primary macrophages, whereas upon stimulation with IL-10 in the same macrophages, Stat3 is used to control expression of the IL-10 gene.

IL-10 suppresses LPS-induced IL-10

The classical study on the biological activity of IL-10 reported that IL-10 can suppress its own expression (6). These studies were done with LPS stimulation with and without the addition of IL-10. We therefore analyzed the effect of IL-10 on LPS-induced IL-10 production in human MDM. As shown in Fig. 4*A*, addition of IL-10 to LPS did suppress the production of IL-10 mRNA by factor 2, confirming the earlier findings by de Waal Malefyt et al. (6). However, in the same experiments, we could confirm that IL-10 alone will induce IL-10 mRNA in these cells. Taken together, IL-10 will induce IL-10 in human MDM, but at the same time, it can reduce the LPS-induced activation. This suppression will not go below the level of IL-10-induced IL-10 (Fig. 4*A*). In addition, this production of IL-10 mRNA by rhIL-10 appears to be restricted to $CD14^+$ MDM, as CD14-depleted cell cultures (i.e., lymphocytes) were essentially negative for IL-10-induced IL-10 mRNA (Fig. 4*A*).

IL-10 induces IL-10 via Stat3

Since many of the previous experiments were conducted in 3-day PBMC cultures, to further study the possible role of Stat3 in IL-10-induced IL-10, we set out to confirm that IL-10 can activate the transcription factor Stat3 in $CD14^+$ MDM. As shown by Western blotting, tyrosine phosphorylation was clearly induced by IL-10 within 1 h in these cells (Fig. 4*B*). We could also show that $CD14^+$ MDM were still susceptible to infection by AdV, with a similar rate of infection (>80% at 100 MOI, Fig. 4*C*).

To confirm the importance of Stat3 in IL-10-induced expression of IL-10 in human MDM, we infected $CD14^+$ cells that had been cultured for 3 days with an AdV encoding a dominant-negative (dn) Stat3. When such cells were stimulated with rhIL-10, the induction of the endogenous *IL-10* gene was completely blocked. In contrast, uninfected cells and cells infected with empty virus showed a clear-cut induction of IL-10 mRNA (Fig. 5*A*). Furthermore, overexpression of the dn Stat3 had no effect on LPS-induced IL-10 mRNA expression (Fig. 5*B*). This observation suggests that the down-regulation seen for IL-10-induced IL-10 cannot be attributed solely to a global down-regulation of mRNA expression caused by the dn protein. Collectively, these data show that IL-10 induces IL-10 by activating the transcription factor Stat3, but that LPS acts via a different signaling mechanism in primary human MDM.

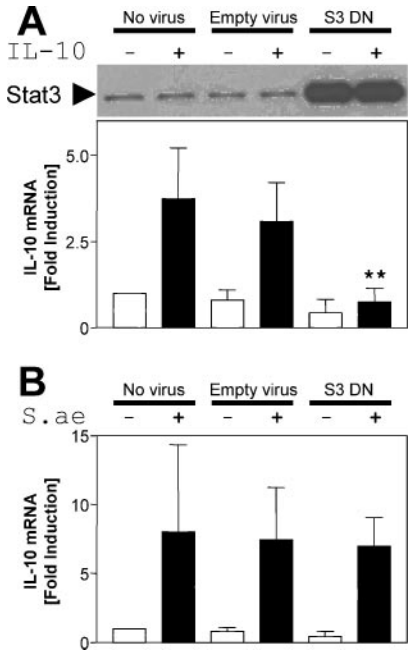

FIGURE 5. dn Stat3 AdV can inhibit IL-10 mRNA production from IL-10-activated $CD14^+$ MDM. *A*, $CD14^+$ cells were cultured for 3 days before treatment with or without AdV, as indicated, at a MOI of 200 for 2 h in serum-free medium. After addition of serum, cells were cultured overnight before being resuspended in fresh serum-supplemented medium. After resuspension, cultures were treated with IL-10 at 10 ng/ml for 6 h. Cells were then harvested, RNA and protein extracted, and the RNA reverse transcribed. IL-10 and β_2m steady-state mRNA levels were determined using real-time PCR. Data were normalized to β_2m and are expressed as fold induction over the noninfected, untreated sample. Data from three individual donors are expressed as means ± SD and further analyzed using a paired *t* test. Results were considered significant if $p < 0.05$ (*) when compared with the empty virus, IL-10-treated sample. Protein (10 μg) from each experiment was run on SDS-PAGE and immunoblotted with anti-Stat3, and a representative blot is shown. *B*, Same as *A*, except the cultures were treated with *S.ae* LPS (100 ng/ml) for 6 h.

Discussion

IL-10 was originally described as a cytokine synthesis inhibitory factor produced by murine Th2 cells (1). Along this line of IL-10 being a Th2 cytokine, it was also found to have some stimulatory activity for B cells (19). In humans, IL-10 was shown to be produced by both Th1 and Th2 cells (20), and IL-10 was found to suppress IgE production by B cells (21). Based on its ability to suppress cell-mediated and Ab-mediated responses, IL-10 is now considered to be a major immunosuppressive cytokine with potential as a therapy for various inflammatory diseases. We show in this study that IL-10 induces IL-10 in human primary MDM. This is in line with two studies that found an increase of IL-10 mRNA in leukocytes as part of an array approach (22, 23). Also, treatment of psoriasis patients with IL-10 led to expression of IL-10 mRNA in the skin (24). We show in this study in human macrophages that this induction is dose and time dependent, and that it leads to expression of both IL-10 mRNA and protein, and we analyzed the molecular mechanism involved. Although we have not systematically analyzed the effect of LPS-induced IL-10 in cells transduced with the adenoviral chimeric IL-10R, our preliminary data indicate that the level of IL-10 released is similar for LPS-stimulated and IL-10-stimulated macrophages under these conditions.

At first glance, these data could be considered to conflict with the original report by de Waal Malefyt et al. (6), who showed suppression of LPS-induced IL-10 by IL-10. We can, however,

confirm these findings and show a 2-fold suppression of LPS-induced IL-10 mRNA by IL-10 (Fig. 4*A*). Of note is the observation that IL-10 suppression of LPS-induced IL-10 reduces the level of IL-10 mRNA to the level of IL-10-induced IL-10 mRNA, but not any lower (i.e., the inductive effect of IL-10 on IL-10 is not overcome when IL-10 blocks LPS-induced IL-10). In experiments not shown, we have confirmed this finding with a higher dose of IL-10 (100 ng/ml) both for induction of IL-10 and suppression of LPS-induced IL-10.

Various transcription factors have been implicated in the regulation of the *IL-10* gene. Expression of IL-10 directed by the murine promoter was shown to depend on Sp1 (25, 26). For the human *IL-10* promoter, Sp1 was shown to be essential for expression in a monoblastic cell line (27), but in other studies, the same promoter element was shown to have a repressive function (28). A contribution of cAMP-responsive elements to catecholamine-driven transactivation has also been demonstrated (29), and, recently, it was shown that catecholamine action depends to a large extent on c/EBP-α (30). Finally, c-Maf was shown to transactivate the human promoter via a site at -196 bp (31). We have identified a Stat site at -120 bp in the human promoter and have provided evidence for the role of Stat3 in transactivation of the human promoter in B cells in response to both LPS and IFN-α (9, 10).

When it comes to IL-10 stimulation of cells, Stat3 is the main transcription factor invoked. IL-10 acts via the IL-10R, which consists of two chains, and ligand binding to the receptor will lead to phosphorylation of Jak1 and Tyk2 and then Stat3 (7, 8). We can confirm that IL-10 stimulation will lead to the phosphorylation of Stat3 in human MDM and that it will transactivate a tetrameric Stat reporter construct. Furthermore, we can demonstrate a role for Stat3 in IL-10-induced IL-10 production, based on the findings that IL-10 is inactive on a promoter with a mutated Stat site, and that overexpressing dn Stat3 blocks the induction of IL-10 mRNA expression.

Our data are in line with studies by Cheng et al. (12), who demonstrated a lack of IL-10 production in Stat3$^{-/-}$ mice, but they are at variance with the report of Takeda et al. (32), who showed increased IL-10 in such Stat3$^{-/-}$ animals. The discrepancy may be explained by the different approaches taken. In these knockout mice, Cheng et al. (12) have studied IL-10 production in LPS-stimulated peritoneal macrophages ex vivo. In contrast, Takeda et al. (32) have looked at serum IL-10 in animals injected with LPS. The latter approach may invoke additional, indirect mechanisms, which are independent of Stat3. For instance, one might speculate that increased induction of TNF in vivo in the Stat3$^{-/-}$ mice may induce IL-10 by a Stat3-independent mechanism. Still, in the system of human MDM as used in this study, LPS induction of IL-10 is not mediated by Stat3, because mutation of the Stat site does not prevent *IL-10* promoter activity. In addition, LPS cannot stimulate activity of a tetrameric Stat reporter construct and overexpression of a dn Stat3 had no effect on LPS-induced IL-10 mRNA expression. Previously, we have shown that LPS induction of IL-10 depends on Stat3 in a B cell line (9), whereas in the present study, we show no requirement for Stat proteins for IL-10 production in primary macrophages upon LPS stimulation. This discrepancy likely reflects tissue-specific gene expression, but analysis in different types of B cells and macrophages is required to address this issue.

The role of Stat3 in *IL-10* gene expression was, however, confirmed in several additional studies (33, 34). More recently, it was shown that SOCS3 deficiency in murine T cells led to increased Stat3 activity and IL-10 expression (35), and that constitutive IL-10 expression in T cell lines can be blocked by depletion of Stat3 by RNA interference (36).

Huang et al. (11) demonstrated that induction of IL-10 by LPS required Stat3 to be both tyrosine and serine phosphorylated. Tyrosine phosphorylation is a prerequisite for translocation of Stat1 into the nucleus (37), but for Stat3, nuclear transport can occur for unphosphorylated protein (38). Still, tyrosine phosphorylation is required for DNA binding and transactivation and, as shown in this study, IL-10 efficiently induces this phosphorylation in MDM (Fig. 4*B*).

Although it appears that Stat3 mediates the induction of IL-10 by IL-10, the mechanisms involved in IL-10-mediated down-regulation of LPS-induced IL-10 production remain unclear. The mechanism may be similar to the action of IL-4, which was shown to down-regulate IL-10 expression in murine dendritic cells in a process involving Stat6 (39). Additionally, Stat1 has been shown to be induced by IL-10 (40), and Stat1 was shown to mediate down-regulation of the *IL-10* promoter (10). However, because we see a robust induction of the luciferase from the -195 promoter by LPS, the relevant element(s) for LPS inducibility have to be within this 5′ fragment. For the human gene, transactivation by catecholamines appears to require the action of C/EBP, and the most important motif TTGCAAAA is located 44 bp downstream of the TATA box (30). Furthermore, there is evidence for a role of c-Maf in IL-10 gene expression, which is located directly next to the IRF sequence between -196 and -184 bp (31).

LPS has been shown to induce IFN-β followed by activation of Stat1 (41), and we have shown previously that Stat1 may act as a negative regulator of IL-10 expression (10). Hence, one might speculate that the slight increase in *IL-10* promoter activity with the mutation of the Stat site might be due to the lack of IFN-β-induced Stat1 action. In experiments not shown, we found, however, no effect of exogenous IFN-β or anti-IFN-β Ab on promoter activity. Thus, although IFN-β may well be important in regulation of IL-10 (42, 43), it appears not to operate in the primary MDM used in this study.

We have provided evidence for a role of Stat3 in IL-10-induced IL-10 in primary human macrophages (i.e., macrophages derived from blood monocytes in vitro). It remains unclear at this point whether the same mechanisms will operate in other cells like B and T cells. For instance, Stat3 might play a role in T cells where IL-10 was shown to be a positive autocrine factor in the development of IL-10-producing regulatory T cells (44).

Taken together, we show in this study that IL-10 can induce expression of IL-10 in an autocrine fashion, which involves mobilization of the transcription factor Stat3. This positive autocrine feedback will allow IL-10 to enhance its immunosuppressive action.

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Disclosures

The authors have no financial conflict of interest.

References

1. Fiorentino, D. F., M. W. Bond, and T. R. Mosmann. 1989. Two types of mouse T helper cell: IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.* 170: 2081–2095.
2. de Waal Malefyt, R., H. Yssel, M. G. Roncarolo, H. Spits, and J. E. de Vries. 1992. Interleukin-10. *Curr. Opin. Immunol.* 4: 314–320.
3. Chang, C. C., A. Wright, and J. Punnonen. 2000. Monocyte-derived CD1a$^+$ and CD1a$^-$ dendritic cell subsets differ in their cytokine production profiles, susceptibilities to transfection, and capacities to direct Th cell differentiation. *J. Immunol.* 165: 3584–3591.

4. Bettelli, E., M. P. Das, E. D. Howard, H. L. Weiner, R. A. Sobel, and V. K. Kuchroo. 1998. IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. *J. Immunol.* 161: 3299–3306.
5. Dai, W. J., G. Kohler, and F. Brombacher. 1997. Both innate and acquired immunity to *Listeria monocytes* infection are increased in IL-10-deficient mice. *J. Immunol.* 158: 2259–2267.
6. de Waal Malefyt, R., J. Abrams, B. Bennett, C. G. Figdor, and J. E. deVries. 1991. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J. Exp. Med.* 174: 1209–1220.
7. Donnelly, R. P., H. Dickensheets, and D. S. Finbloom. 1999. The interleukin-10 signal transduction pathway and regulation of gene expression in mononuclear phagocytes. *J. Interferon Cytokine Res.* 19: 563–573.
8. Williams, L. M., G. Ricchetti, U. Sarma, T. Smallie, and B. M. Foxwell. 2004. Interleukin-10 suppression of myeloid cell activation—a continuing puzzle. *Immunology* 113: 281–292.
9. Benkhart, E. M., M. Siedlar, A. Wedel, T. Werner, and H. W. Ziegler-Heitbrock. 2000. Role of Stat3 in lipopolysaccharide-induced IL-10 gene expression. *J. Immunol.* 165: 1612–1617.
10. Ziegler-Heitbrock, L., M. Lotzerich, A. Schaefer, T. Werner, M. Frankenberger, and E. Benkhart. 2003. IFN-α induces the human IL-10 gene by recruiting both IFN regulatory factor 1 and Stat3. *J. Immunol.* 171: 285–290.
11. Huang, Y., T. Li, D. C. Sane, and L. Li. 2004. IRAK1 serves as a novel regulator essential for lipopolysaccharide-induced interleukin-10 gene expression. *J. Biol. Chem.* 279: 51697–51703.
12. Cheng, F., H. W. Wang, A. Cuenca, M. Huang, T. Ghansah, J. Brayer, W. G. Kerr, K. Takeda, S. Akira, S. P. Schoenberger, et al. 2003. A critical role for Stat3 signaling in immune tolerance. *Immunity* 19: 425–436.
13. Besser, D., J. F. Bromberg, J. E. J. Darnell, and H. Hanafusa. 1999. A single amino acid substitution in the v-Eyk intracellular domain results in activation of Stat3 and enhances cellular transformation. *Mol. Cell. Biol.* 19: 1401–1409.
14. He, T. C., S. Zhou, L. T. da Costa, J. Yu, K. W. Kinzler, and B. Vogelstein. 1998. A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. USA* 95: 2509–2514.
15. Foxwell, B., K. Browne, J. Bondeson, C. Clarke, R. de Martin, F. Brennan, and M. Feldmann. 1998. Efficient adenoviral infection with IκB α reveals that macrophage tumor necrosis factor α production in rheumatoid arthritis is NF-κB dependent. *Proc. Natl. Acad. Sci. USA* 95: 8211–8215.
16. Kunisada, K., E. Tone, Y. Fujio, H. Matsui, K. Yamauchi-Takihara, and T. Kishimoto. 1998. Activation of gp130 transduces hypertrophic signals via STAT3 in cardiac myocytes. *Circulation* 98: 346–352.
17. Staples, K. J., M. Bergmann, P. J. Barnes, and R. Newton. 2000. Stimulus-specific inhibition of IL-5 by cAMP-elevating agents and IL-10 reveals differential mechanisms of action. *Biochem. Biophys. Res. Commun.* 273: 811–815.
18. Williams, L. M., U. Sarma, K. Willets, T. Smallie, F. Brennan, and B. M. J. Foxwell. 2007. Expression of constitutively active STAT3 can replicate the cytokine suppressive activity of IL-10 in human primary macrophages. *J. Biol. Chem.* 282: 6965–6975.
19. Rousset, F., E. Garcia, T. Defrance, C. Peronne, N. Vezzio, D. H. Hsu, R. Kastelein, K. W. Moore, and J. Banchereau. 1992. Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. *Proc. Natl. Acad. Sci. USA* 89: 1890–1893.
20. Del Prete, G., M. De Carli, F. Almerigogna, M. G. Giudizi, R. Biagiotti, and S. Romagnani. 1993. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *J. Immunol.* 150: 353–360.
21. Akdis, C. A., T. Blesken, M. Akdis, B. Wuthrich, and K. Blaser. 1998. Role of interleukin 10 in specific immunotherapy. *J. Clin. Invest.* 102: 98–106.
22. Lang, R., D. Patel, J. J. Morris, R. Rutschman, and P. J. Murray. 2002. Shaping gene expression in activated and resting primary macrophages by IL-10. *J. Immunol.* 169: 2253–2263.
23. Jung, M., R. Sabat, J. Kratzschmar, H. Seidel, K. Wolk, C. Schonbein, S. Schutt, M. Friedrich, W. D. Docke, K. Asadullah, et al. 2004. Expression profiling of IL-10-regulated genes in human monocytes and peripheral blood mononuclear cells from psoriatic patients during IL-10 therapy. *Eur. J. Immunol.* 34: 481–493.
24. Asadullah, K., W. Sterry, K. Stephanek, D. Jasulaitis, M. Leupold, H. Audring, H. D. Volk, and W. D. Docke. 1998. IL-10 is a key cytokine in psoriasis: proof of principle by IL-10 therapy: a new therapeutic approach. *J. Clin. Invest.* 101: 783–794.
25. Brightbill, H. D., S. E. Plevy, R. L. Modlin, and T. Smale. 2000. A prominent role for Sp1 during lipopolysaccharide-mediated induction of the IL-10 promoter in macrophages. *J. Immunol.* 164: 1940–1951.
26. Tone, M., M. J. Powell, Y. Tone, S. A. Thompson, and H. Waldmann. 2000. IL-10 gene expression is controlled by the transcription factors Sp1 and Sp3. *J. Immunol.* 165: 286–291.
27. Ma, W., W. Lim, K. Gee, S. Aucoin, D. Nandan, M. Kozlowski, F. Diaz-Mitoma, and A. Kumar. 2001. The p38 mitogen-activated kinase pathway regulates the human interleukin-10 promoter via the activation of Sp1 transcription factor in lipopolysaccharide-stimulated human macrophages. *J. Biol. Chem.* 276: 13664–13674.
28. Steinke, J. W., E. Barekzi, J. Hagman, and L. Borish. 2004. Functional analysis of −571 IL-10 promoter polymorphism reveals a repressor element controlled by Sp1. *J. Immunol.* 173: 3215–3222.
29. Platzer, C., E. Fritsch, T. Elsner, M. H. Lehmann, H.-D. Volk, and S. Prösch. 1999. Cyclic adenosine monophosphate-responsive elements are involved in the transcriptional activation of the human IL-10 gene in monocytic cells. *Eur. J. Immunol.* 29: 3098–3104.
30. Brenner, S., S. Prosch, K. Schenke-Layland, U. Reise, U. Gausmann, and C. Platzer. 2003. cAMP-induced interleukin-10 promoter activation depends on CCAAT/enhancer-binding protein expression and monocytic differentiation. *J. Biol. Chem.* 278: 5597–5604.
31. Cao, S., J. Liu, L. Song, and X. Ma. 2005. The protooncogene c-Maf is an essential transcription factor for IL-10 gene expression in macrophages. *J. Immunol.* 174: 3484–3492.
32. Takeda, K., B. E. Clausen, T. Kaisho, T. Tsujimura, N. Terada, I. Forster, and S. Akira. 1999. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity* 10: 39–49.
33. Herbeuval, J. P., E. Lelievre, C. Lambert, M. Dy, and C. Genin. 2004. Recruitment of STAT3 for production of IL-10 by colon carcinoma cells induced by macrophage-derived IL-6. *J. Immunol.* 172: 4630–4636.
34. Maritano, D., M. L. Sugrue, S. Tininini, S. Dewilde, B. Strobl, X. Fu, V. Murray-Tait, R. Chiarle, and V. Poli. 2004. The STAT3 isoforms α and β have unique and specific functions. *Nat. Immunol.* 5: 401–409.
35. Kinjyo, I., H. Inoue, S. Hamano, S. Fukuyama, T. Yoshimura, K. Koga, H. Takaki, K. Himeno, G. Takaesu, T. Kobayashi, and A. Yoshimura. 2006. Loss of SOCS3 in T helper cells resulted in reduced immune responses and hyperproduction of interleukin 10 and transforming growth factor-β 1. *J. Exp. Med.* 203: 1021–1031.
36. Kasprzycka, M., M. Marzec, X. Liu, Q. Zhang, and M. A. Wasik. 2006. Nucleophosmin/anaplastic lymphoma kinase (NPM/ALK) oncoprotein induces the T regulatory cell phenotype by activating STAT3. *Proc. Natl. Acad. Sci. USA* 103: 9964–9969.
37. Bromberg, J., and J. E. J. Darnell. 2000. The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* 19: 2468–2473.
38. Liu, L., K. M. McBride, and N. C. Reich. 2005. STAT3 nuclear import is independent of tyrosine phosphorylation and mediated by importin-α3. *Proc. Natl. Acad. Sci. USA* 102: 8150–8155.
39. Yao, Y., W. Li, M. H. Kaplan, and C. H. Chang. 2005. Interleukin-4 inhibits IL-10 to promote IL-12 production by dendritic cells. *J. Exp. Med.* 201: 1899–1903.
40. Finbloom, D. S., and K. D. Winestock. 1995. IL-10 induces the tyrosine phosphorylation of tyk2 and Jak1 and the differential assembly of STAT1 α and STAT3 complexes in human T cells and monocytes. *J. Immunol.* 155: 1079–1090.
41. Thomas, K. E., C. L. Galligan, R. D. Newman, E. N. Fish, and S. N. Vogel. 2006. Contribution of interferon-β to the murine macrophage response to the Toll-like receptor 4 agonist, lipopolysaccharide. *J. Biol. Chem.* 281: 31119–31130.
42. Rudick, R. A., R. M. Ransohoff, R. Peppler, M. S. VanderBrug, P. Lehmann, and J. Alam. 1996. Interferon β induces interleukin-10 expression: relevance to multiple sclerosis. *Ann. Neurol.* 40: 618–627.
43. Rep, M. H., H. M. Schrijver, T. van Lopik, R. Q. Hintzen, M. T. Roos, H. J. Ader, C. H. Polman, and R. A. van Lier. 1999. Interferon (IFN)-β treatment enhances CD95 and interleukin 10 expression but reduces interferon-γ producing T cells in MS patients. *J. Neuroimmunol.* 96: 92–100.
44. Barrat, F., D. J. Cua, A. Boonstra, D. F. Richards, C. Crain, H. F. Savelkoul, R. de Waal-Malefyt, R. L. Coffman, C. M. Hawrylowicz, and A. O'Garra. 2002. In vitro generation of interleukin 10-producing regulatory $CD4^+$ T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J. Exp. Med.* 195: 603–616.