

Regulatory Activity of Autocrine IL-10 on Dendritic Cell Functions¹

Silvia Corinti,² Cristina Albanesi, Andrea la Sala, Saveria Pastore, and Giampiero Girolomoni

IL-10 is a critical cytokine that blocks the maturation of dendritic cells (DCs), but the relevance of autocrine IL-10 on DC functions has not been investigated. In this study, we found that immature monocyte-derived DCs released low but sizeable amounts of IL-10. After stimulation with bacteria, LPS, lipoteichoic acid, or soluble CD40 ligand, DCs secreted high levels of IL-10. Addition of an anti-IL-10-neutralizing Ab to immature DCs as well as to soluble CD40 ligand- or LPS-maturing DCs led to enhanced expression of surface CD83, CD80, CD86, and MHC molecules and markedly augmented release of TNF- α and IL-12, but diminished IL-10 mRNA expression. Moreover, DCs treated with anti-IL-10 Ab showed an increased capacity to activate allogeneic T cells and primed naive T cells to a more prominent Th1 polarization. DC maturation and IL-10 neutralization were associated with enhanced accumulation of the IL-10 receptor binding chain (IL-10R1) mRNA and intracellular IL-10R1 protein. In contrast, surface IL-10R1 and IL-10 binding activity diminished in mature DCs. These results indicate that autocrine IL-10 prevents spontaneous maturation of DCs in vitro, limits LPS- and CD40-mediated maturation, and increases IL-10 production by DCs. Moreover, IL-10R expression appears to be regulated by both transcriptional and posttranscriptional mechanisms. Endogenous IL-10 and IL-10R can be relevant targets for the manipulation of DC functions. *The Journal of Immunology*, 2001, 166: 4312–4318.

Dendritic cells (DCs)³ are essential elements in the initiation of immune responses (1). DCs reside in unperturbed tissues in an immature form, where they are adapted for capturing and accumulating Ags. Indeed, DCs possess a wide spectrum of recognition systems for an efficient screening of the tissue environment. A variety of danger signals, including microorganisms, dying cells, or proinflammatory cytokines, induce the terminal differentiation, also known as maturation, of DCs (1–5). Mature DCs migrate to lymph nodes, acquire potent Ag-presenting capacity, and stimulate T cell responses vigorously. Moreover, maturation of DCs is strengthened during interactions with T cells by signals such as CD40 ligand (CD40L) provided by T cells themselves (6–8). Mature DCs express high levels of Ag-presenting and costimulatory molecules, and release large amounts of IL-12, thereby stimulating preferentially Th1 responses. Thus, DC maturation is a key checkpoint in the initiation of immunity and has important consequences also on the quality of the immune response.

To prevent exaggerated and unwanted immune responses, DC maturation and functions are tightly regulated. IL-10 has been identified as a major factor that can prevent the differentiation of DCs from monocytes and strongly inhibit DC maturation induced

by different stimuli (9–14). In particular, IL-10 blocks the up-regulation of costimulatory molecules and IL-12 production and thus impairs the ability of DCs to generate Th1 responses (15). IL-10-treated DCs are not only less efficient at stimulating T cell responses but can induce a state of Ag-specific tolerance (9, 16, 17). IL-10 can be secreted by different cell types, including monocytes, mast cells, T regulatory cells, and tumor cells, and has an important role in limiting allergic and autoimmune reactions or in mediating tumor escape from immune surveillance (17, 18). In contrast, several reports have shown that mature DCs are no longer sensitive to the inhibitory effects of IL-10 (14, 15, 17, 19–21), but the molecular bases of this phenomenon are as yet unknown. IL-10 exerts its actions through a heterodimeric membrane receptor formed by a binding chain (IL-10R1) and a transducing chain (IL-10R2, also known as CFR2–4), whose mutual interaction activates a series of intracellular signaling molecules, including STAT proteins (22–29).

Although some DC subsets can produce IL-10 (3, 13, 30–32), the impact of endogenous IL-10 on DC biology has been only marginally investigated (13, 33). In this work, we provide evidence that autocrine IL-10 serves as a potent mechanism for limiting the maturation of monocyte-derived DCs and their capacity to initiate Th1 responses. Moreover, we show that mature DCs accumulate higher amounts of IL-10R1 mRNA and intracellular IL-10R1 protein but reduce surface IL-10R1 expression and IL-10 binding activity. Thus, endogenous IL-10 and IL-10R appear to be important regulators of DC biology and can represent relevant targets for the manipulation of DC functions.

Materials and Methods

Reagents and Abs

Streptococcus gordonii strain GP1221 was a gift from Dr. D. Medaglini (University of Siena, Siena, Italy), and *Salmonella typhi* (Neotyf) was provided by Chiron Italia (Siena, Italy). LPS (from *Salmonella typhimurium*), lipoteichoic acid (LTA; from *Staphylococcus aureus*), and poly I:C were purchased from Sigma-Aldrich (Milan, Italy). Soluble CD40L (sCD40L) was obtained from Alexis (San Diego, CA). It is composed of the extracellular domain of human CD40L fused to the N terminus of a linker

Laboratory of Immunology, Istituto Dermatologico dell'Immacolata, Istituto di Ricovero e Cura a Carattere Scientifico, Rome, Italy

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² Address correspondence and reprint requests to Dr. Silvia Corinti, Laboratory of Immunology, Istituto Dermatologico dell'Immacolata, Via Monti di Creta 104, 00167 Roma, Italy. E-mail address: s.corinti@idi.it

³ Abbreviations used in this paper: DC, dendritic cell; CD40L, CD40 ligand; sCD40L, soluble CD40L; LTA, lipoteichoic acid.

peptide and a FLAG-tag and was used together with an enhancer that increases the biological activity of sCD40L. The mAbs FITC-conjugated and pure anti-HLA-DR (L243), FITC-conjugated anti-CD14 (MΦP9), anti-CD3 (SK7), anti-CD4 (SK3), and anti-CD45RA (L48) were obtained from Becton Dickinson (San Jose, CA). FITC-conjugated anti-CD1a (HI149), anti-CD86 (2331), anti-CD40 (5C3), anti-IFN- γ (4S.B3), pure anti-CD28 (CD28.2), PE-conjugated anti-IL-4 (MP4-25D2, rat), and pure anti-CD45RO (UCHL1) were obtained from BD PharMingen (San Diego, CA). FITC-conjugated anti-CD54 (84H10) and anti-CD80 (MAB104), and pure anti-CD83 (HB15A) and anti-CD3 (UCHT1) came from Immunotech (Marseille, France). Anti-MHC class I (W6/32) was obtained from Dako (Glostrup, Denmark). Control mouse or rat Ig were obtained from Becton Dickinson or BD PharMingen. The mouse mAb anti-human IL-10 (23738.11) came from R&D Systems (Minneapolis, MN), and the rat anti-human IL-10R1 mAb 3F9 was obtained from BD PharMingen. Anti-IL-10 mAb and sCD40L had undetectable endotoxin levels (<10 pg/mg) by the *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD).

DC preparation and stimulation

DCs were prepared from PBMC of healthy individuals as described previously (3). Briefly, PBMC were separated on multistep Percoll gradients (Pharmacia, Uppsala, Sweden), and cells from the light density fraction (42.5–50%; $>90\%$ CD14 $^{+}$) were cultured at 1×10^6 cells/ml in RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 μ g/ml streptomycin (all obtained from Life Technologies), and 0.05 mM 2-ME (Merck, Darmstadt, Germany) (complete RPMI), and supplemented with 10% FBS (HyClone, Logan, UT), 100 ng/ml human rGM-CSF (Mielogen, Schering-Plough, Milan, Italy), and 200 U/ml human rIL-4 (R&D Systems) at 37°C with 5% CO $_2$. Medium was changed after 3 days, and at day 6 of culture, cells were recovered and depleted of CD2 $^{+}$ and CD19 $^{+}$ cells by means of immunomagnetic beads coated with specific mAbs (Dyna, Oslo, Norway). This procedure gave $>97\%$ pure CD14 $^{+}$ and CD14 $^{-}$ DC preparations. DCs were incubated in 6-well plates (1×10^6 cells/ml) at 37°C with *S. gordonii* (bacteria-to-DCs ratio, 50:1), *S. typhi* (bacteria-to-DCs ratio, 1:1), LPS (10 μ g/ml), LTA (10 μ g/ml), poly I:C (100 ng/ml), or sCD40L (1 μ g/ml). Where indicated, DCs were treated with anti-HLA-DR mAb (10 μ g/ml) for 1 h at 4°C, washed, and cultured at 37°C in the presence or absence of sCD40L. To block endogenous IL-10, the neutralizing anti-IL-10 mAb or mouse Ig were added to unstimulated DC cultures or at the time of stimulation with LPS or sCD40L, and cells were analyzed after 18 h. Preliminary dose-response experiments indicated that the optimal neutralizing dose of anti-IL-10 mAb was 10 μ g/ml.

ELISA

Measurement of IL-12 (p70) and TNF- α in DC supernatants was performed by using OptEIA kits from BD PharMingen, as per the manufacturer's protocol. IL-10R1 was determined in DC supernatants and cell lysates by using the Ab pair mouse mAb 370607.11 for coating and goat polyclonal biotinylated AF-274-NA for detection (R&D Systems).

Flow cytometry analysis of DCs

DCs either untreated or stimulated for 18 h with LPS or sCD40L in the presence of anti-IL-10 mAb or control IgG were washed and then incubated in PBS containing 2% FBS and 0.01% Na $_2$ S $_2$ O $_3$ with FITC-conjugated mAbs for 40 min at 4°C. When pure mAbs were used, a second incubation with a FITC-coupled goat (Fab') $_2$ anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) was performed. Matched isotype mouse Ig were used in control samples. Binding of biotinylated IL-10 was performed on DCs fixed with 2% paraformaldehyde by using the Fluorokine kit (R&D Systems), as per the manufacturers' protocol. To detect intracellular IL-10R and IL-10 binding activity, DCs were fixed and permeabilized with 0.5% saponin prior the staining. Cells were analyzed in a FACScan equipped with CellQuest software (Becton Dickinson, Mountain View, CA).

DC-T cell cocultures

For the primary MLR assay, T lymphocytes were purified ($>95\%$ CD3 $^{+}$) from the heavy-density fraction (50–60%) of Percoll gradients followed by immunomagnetic depletion with a mixture of anti-MHC class II- and anti-CD19 mAb-conjugated beads (Dyna). DCs were washed and then cultured in 96-well microculture plates in serial dilutions (78 to 5×10^3 cells/well) together with purified allogeneic T lymphocytes (1.5×10^5 cells/well) in complete RPMI supplemented with 5% human serum (Sigma). Cocultures were pulsed at day 3 with 1 μ Ci/well [3 H]thymidine (Amersham, Little

Chalfont, U.K.) for ~ 16 h at 37°C and then harvested onto fiber-coated 96-well plates (Packard Instruments, Groningen, The Netherlands). Radioactivity was measured in a β -counter (Topcount; Packard Instruments). Results are given as mean cpm \pm SD of triplicate cultures. Where indicated, allogeneic naive T cells were purified ($>95\%$ CD45RA $^{+}$) by incubation of CD3 $^{+}$ T cells with anti-CD45RO mAb followed by a goat anti-mouse Ig coupled to immunomagnetic beads (Dyna), and then cocultured (10^6 cells/well) with DCs (5×10^4 cells/well) in 24-well plates in complete RPMI plus 5% human serum. After 6 days, T cells were restimulated with plate-coated anti-CD3 and soluble anti-CD28 mAbs (both at 1 μ g/ml) and examined for IFN- γ and IL-4 production. Two-color intracellular staining for IFN- γ and IL-4 was performed 6 h after stimulation. Monensin (10 μ M; Sigma) and brefeldin A (10 μ g/ml; Sigma) were added into the cultures before the staining to prevent cytokine secretion. T cells then were fixed with 2% paraformaldehyde, permeabilized with 0.5% saponin, stained with FITC-conjugated mouse anti-IFN- γ and PE-conjugated rat anti-IL-4, and finally analyzed with a FACScan. In control samples, staining was performed by using isotype-matched control Ig. In parallel samples, cytokines were measured in the T cell supernatants 48 h after activation by ELISA by using matched pairs of mAbs (for IL-4 and IFN- γ) and OptEIA kit (for IL-5) from BD PharMingen. T cells that were not restimulated at day 6 did not show any lymphokine production (data not shown).

Northern blot analysis

The probes specific for human IL-10, IL-10R1, and IL-10R2 were obtained by RT-PCR on RNA isolated from purified DCs. IL-10-specific synthetic oligonucleotides were 5'-GAA GGA TCA GCT GGA CAA CTT GTTG-3' and 5'-CTC ATG GCT TTG TAG ATG CCT TTC TC-3'. IL-10R1- and IL-10R2-specific primer pairs were 5'-CCG TCT GTG TGG TTT GAA GCA GAA-3' and 5'-GAT GAT GAC GTT GGT CAC GGT GAA-3' (22), and 5'-GAG TCA CCT GCT TTT GCC AAA GGG-3' and 5'-CAT GGG TTG TTT GCT CAC AGA CAG-3' (24), respectively. Amplificates were cloned into pCR-TOPO vector (Invitrogen, Carlsbad, CA), and then subjected to an automated sequence analysis with a Perkin-Elmer sequencer (model ABI Prism 377 XL; Perkin-Elmer, Norwalk, CT). Total RNA was extracted from DCs by using the TRIzol solution (Life Technologies), fractionated on denaturing-agarose gels, and blotted to nylon membranes (Amersham Pharmacia Biotech, Milan, Italy). After UV fixation, the membranes were hybridized with 32 P-radiolabeled probes for 1 h at 68°C in Quickhyb solution (Stratagene, La Jolla, CA). The filters were washed under high stringency conditions and exposed at -80°C to Kodak Biomax MS-1 films (Kodak, Rochester, NY). Before blotting, 28S and 18S rRNA were stained on gels with ethidium bromide and photographed with a UV transilluminator using Polaroid positive/negative films (Polaroid, Cambridge, MA). Films were subjected to densitometry by an Imaging Densitometer model GS-670 (Bio-Rad, Richmond, CA) supported by the Quantity One software, and densitometric values were calculated by dividing the values of specific bands by the values of 28S rRNA.

Western blot analysis

Western blot analysis of tyrosine-phosphorylated STAT-1 and STAT-3 was determined in DC whole-cell lysates. In brief, DCs (2×10^7 cells/ml) were resuspended in RIPA buffer containing 1% Nonidet P-40, 0.5% sodium deoxycolate, 0.1% SDS, 1 mM sodium orthovanadate, and protease inhibitors and sheared through a 28-gauge needle. After a 30-min incubation on ice, lysates were centrifuged at $13,500 \times g$ for 15 min and supernatants were collected. A total of 5–10 μ g of proteins were subjected to 6.5% SDS-PAGE under reducing conditions and then transferred to polyvinylidene difluoride membranes (Hybond-P; Amersham). Membranes were probed with anti-phosphotyrosine STAT-1 and STAT-3 rabbit polyclonal Abs (New England Biolabs, Beverly, MA), and developed with HRP-conjugated anti-rabbit Ig (Santa Cruz Biotechnology, Santa Cruz, CA) by using the ECL-plus detection system (Amersham).

Statistics

The unpaired two-tailed Student's *t* test was used to compare differences in DC membrane markers expression, cytokines release, and T cell proliferation, and $p \leq 0.05$ were considered significant.

Results

DCs release IL-10 in response to different maturation signals

In the first series of experiments, we tested the capacity of DCs to release IL-10 in response to different maturation signals. Fig. 1 shows that immature DCs secreted low but sizeable amounts of IL-10 (20–50 pg/ml/ 10^6 cells). DC stimulated with Gram-negative

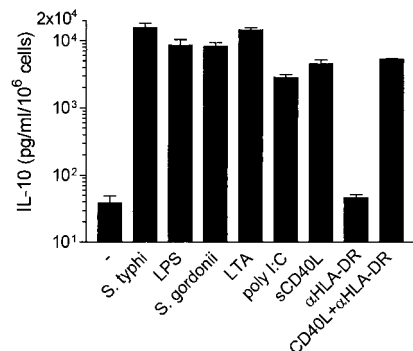


FIGURE 1. DCs secrete IL-10 in response to different maturation signals. DCs were generated from purified peripheral blood CD14⁺ cells cultured for 6 days with GM-CSF and IL-4. Thereafter, CD2⁺ and CD19⁺ cells were removed by immunomagnetic beads and DCs stimulated with *S. gordonii* (bacteria-to-DCs ratio, 50:1), *S. typhi* (bacteria-to-DCs ratio, 1:1), LPS (10 μ g/ml), LTA (10 μ g/ml), poly I:C (100 ng/ml), or sCD40L (1 μ g/ml). DCs also were treated with mAb anti-HLA-DR (10 μ g/ml) for 1 h at 4°C, washed, and cultured at 37°C in the presence or absence of sCD40L. After 18 h, IL-10 was measured in the supernatants by ELISA. Results are expressed as mean \pm SD of triplicate cultures. One of four experiments from different donors is shown.

or Gram-positive bacteria or bacterial cell wall constituents released high levels of IL-10 (8–16 ng/ml), whereas exposure of DCs to poly I:C led to moderate IL-10 release (2–4 ng/ml). CD40 triggering with either sCD40L or CD40L-transfected L cells (not shown) was also an efficient stimulus for IL-10 secretion (4–5 ng/ml), although less potent than bacteria. Treatment with anti-HLA-DR mAb had no effect on IL-10 release and did not significantly modify cytokine secretion induced by CD40 ligation.

IL-10 neutralization increases TNF- α and IL-12 release, reduces IL-10 production, and augments DC maturation induced by LPS or sCD40L

To evaluate the role of endogenous IL-10 in DC cytokine production, a neutralizing anti-IL-10 mAb was added to immature DC cultures as well as to DCs concomitantly treated with LPS or sCD40L, paradigmatic of the noncognate and cognate maturation signals, respectively. Incubation of immature DCs with anti-IL-10 mAb but not with control mouse IgG for 18 h induced a higher secretion of TNF- α (Fig. 2A). More strikingly, a prominent augmented secretion of TNF- α and IL-12 was measured in DCs stimulated with LPS or sCD40L and concomitantly treated with anti-IL-10 mAb, indicating that endogenous IL-10 has a strong inhibitory effect on IL-12 and TNF- α production by maturing DCs. In contrast, IL-10 neutralization diminished by 1.5- to 2-fold the IL-10 mRNA accumulation induced by LPS or CD40 triggering as shown by Northern blot analysis (Fig. 2C). Thus, these results suggested that autocrine IL-10 increased its own production, extending previously published data at the protein level (13, 14). IL-10 protein detection could not be performed by ELISA because the presence of the neutralizing anti-IL-10 mAb interfered with a precise estimation of the cytokine.

Maturation of DCs is associated with profound changes in the surface phenotype, including an up-regulated expression of MHC and costimulatory molecules, and de novo expression of the DC-specific marker CD83. Table I shows that immature DCs incubated for 18 h with anti-IL-10 blocking mAb displayed enhanced membrane levels of CD80, CD86, and CD83, suggesting that DCs deprived of endogenous IL-10 undergo spontaneous maturation in vitro. In addition, IL-10 neutralization further augmented the up-

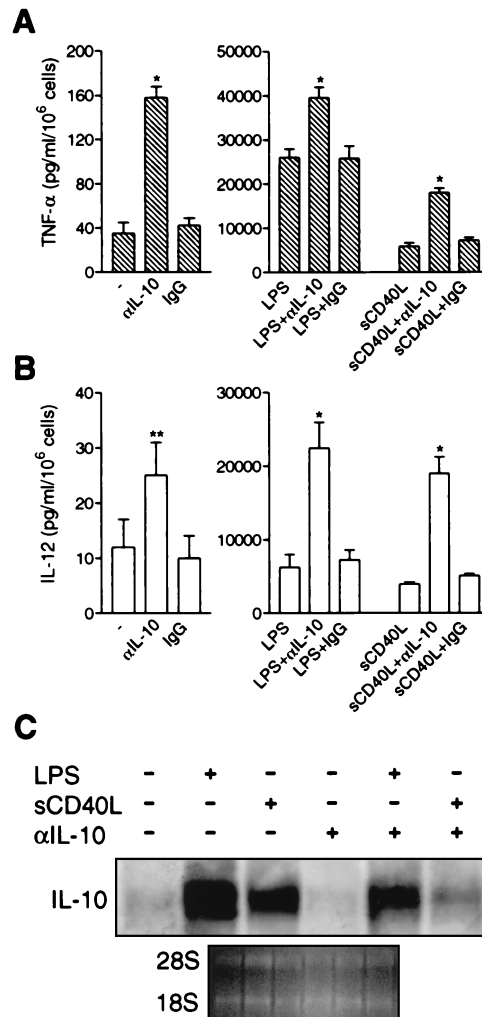


FIGURE 2. IL-10 neutralization increases the release of TNF- α and IL-12, but diminishes IL-10 production. DCs were treated with neutralizing anti-IL-10 mAb or mouse IgG in the absence or presence of LPS (10 μ g/ml) or sCD40L (1 μ g/ml). After 18 h, TNF- α (A) and IL-12 (B) were measured in the supernatants by ELISA. Results are expressed as mean \pm SD of triplicate cultures. *, $p < 0.05$ vs untreated or mouse Ig-treated DCs; **, $p > 0.05$ vs mouse Ig-treated DCs. (C) IL-10 production was determined by Northern blot analysis on total RNA derived from purified DCs. Shown is one of three or four experiments performed.

regulation of CD80, CD86, CD83, and MHC molecules induced by either LPS or sCD40L. Although IL-10 neutralization augmented membrane HLA-DR expression in most experiments, in some cases no changes or, less frequently, slight reduction was observed, as noted previously (11, 14, 15).

Endogenous IL-10 limits the Ag-presenting functions of DCs and reduces the capacity of DCs to initiate Th1 responses

The functional consequences of IL-10 neutralization initially were tested in the primary MLR assay. Blocking endogenous IL-10 did not change the capacity of immature DCs to activate allogeneic T cells (Fig. 3A). However, DCs matured with LPS or sCD40L in the presence of anti-IL-10 mAb exhibited enhanced allostimulatory capacity (Fig. 3, B and C). Next, we studied the ability of DCs treated with anti-IL-10 to direct the differentiation of naive T cells. To this end, purified naive CD45RA⁺ allogeneic T cells were activated with immature or mature DCs treated with anti-IL-10 or control IgG. After 6 days, T cells were restimulated with plate-coated anti-CD3 and

Table I. Endogenous IL-10 limits the membrane maturation of DCs

Treatment ^a	IgG	CD40	CD80	CD86	CD83	MHC-I	HLA-DR
IgG	5 ± 1	32 ± 6	46 ± 5	53 ± 9	24 ± 8	363 ± 28	245 ± 21
Anti-IL-10	4 ± 0.5	39 ± 5	76 ± 8*	138 ± 16*	80 ± 10*	365 ± 45	275 ± 29
LPS + IgG	4 ± 0.3	43 ± 5	67 ± 5	174 ± 11	99 ± 7	416 ± 32	379 ± 34
LPS + anti-IL-10	5 ± 0.5	40 ± 8	97 ± 12 [‡]	304 ± 25 [‡]	147 ± 15 [‡]	976 ± 88 [‡]	590 ± 50 [‡]
sCD40L + IgG	4 ± 0.4	59 ± 7	56 ± 6	95 ± 10	91 ± 9	440 ± 35	324 ± 42
sCD40L + anti-IL-10	4 ± 0.8	54 ± 6	76 ± 8 [‡]	166 ± 13 [‡]	123 ± 13 [‡]	663 ± 55 [‡]	662 ± 58 [‡]

^a DCs were treated with neutralizing anti-IL-10 mAb or mouse IgG in the absence or presence of LPS or sCD40L for 18 h and then analyzed for surface marker expression by flow cytometry. Results are expressed as the average (± SD) of the net mean fluorescence from seven independent experiments.

*, $p < 0.001$ vs IgG-treated DCs; †, $p < 0.001$ vs DCs stimulated with LPS + IgG; ‡, $p < 0.001$ vs DCs stimulated with CD40L + IgG.

soluble anti-CD28 and analyzed for lymphokine production. T cells stimulated with immature DCs differentiated into both Th1 and Th2 cells, whereas LPS- or sCD40L-matured DCs induced the preferential development of Th1 cells (Fig. 4). Addition of anti-IL-10 Ab augmented the percentage of IFN- γ -positive cells induced by both immature or mature DCs. In parallel, the percentage of IL-4-positive cells generated with immature DCs was reduced by anti-IL-10 treatment. Similar results were observed by measuring lymphokines secreted at 48 h, where immature or mature DCs stimulated T cells to

release higher IFN- γ but lower IL-4 and IL-5 after endogenous IL-10 neutralization (Fig. 5).

Immature but not mature DCs phosphorylate STAT-1 and STAT-3 in response to IL-10

Several reports have demonstrated that mature DCs become resistant to the effects of exogenous IL-10 (14, 15, 17, 19–21). Consistent with this notion, addition of anti-IL-10 mAb to already matured DCs had no influence on cytokine release and surface phenotype (data not shown). To test the sensitivity of mature DCs to IL-10, the activation of molecules involved in the signal transduction to IL-10 was investigated. Ligation of IL-10 to its receptor leads to the activation of Janus kinase 1 and tyrosine kinase 2, and then to the recruitment and phosphorylation of STAT-1 and STAT-3 (25–27). Fig. 6 shows that immature DCs exposed to IL-10 for 20 min displayed high amounts of tyrosine-phosphorylated STAT-3 and low levels of phosphoSTAT-1 (lane 3 vs lane 1). In contrast, LPS-matured DCs exhibited high basal-phosphorylated STAT-3 and limited STAT-1 that were only slightly increased by IL-10 (lane 4 vs lane 2). DCs that received LPS together with anti-IL-10 mAb did not reduce STAT-3 phosphorylation, possibly because LPS promoted the secretion of other cytokines (e.g., IL-6, IL-12) which also activate STAT-3 (data not shown).

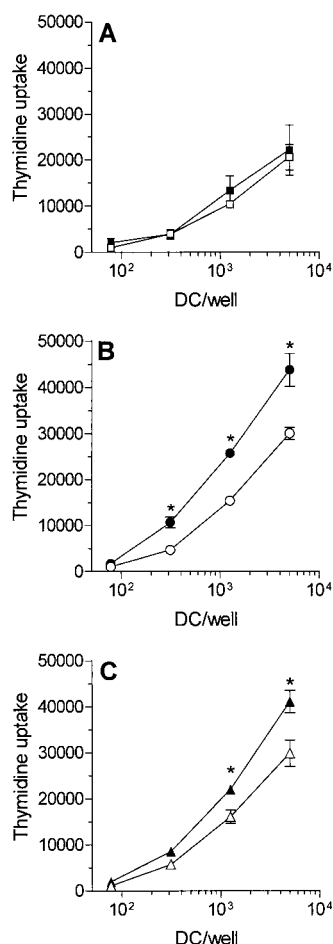


FIGURE 3. Autocrine IL-10 decreases the Ag-presenting functions of DCs. DCs were treated with neutralizing anti-IL-10 mAb (filled symbols) or mouse IgG (open symbols) in the absence (A) or presence of LPS (B) or sCD40L (C). After 18 h, DCs were extensively washed and then cocultured in graded numbers with purified allogeneic CD3⁺ T cells. [³H]Thymidine uptake was measured after 3 days. Results are the mean cpm ± SD of triplicate cultures. *, $p < 0.05$ vs control IgG-treated DCs. One experiment of four performed is shown.

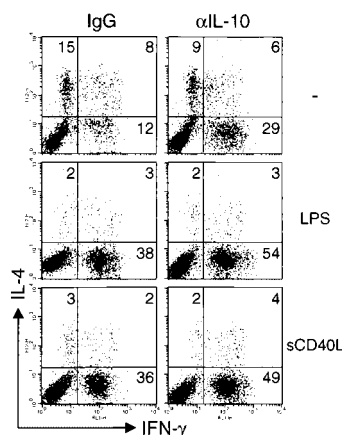


FIGURE 4. Endogenous IL-10 inhibits the capacity of DCs to initiate Th1 responses. DCs were treated with neutralizing anti-IL-10 mAb or mouse IgG in the presence or absence of LPS or sCD40L for 18 h. DCs then were washed and used to activate purified naive (>95% CD45RA⁺) allogeneic T cells. After 6 days, T cells were restimulated with immobilized anti-CD3 and soluble anti-CD28 mAbs (both at 1 μ g/ml) for 6 h, and examined for intracellular IFN- γ and IL-4 by flow cytometry. The numbers indicate the percentage of positive cells in each quadrant. Three additional experiments gave comparable results.

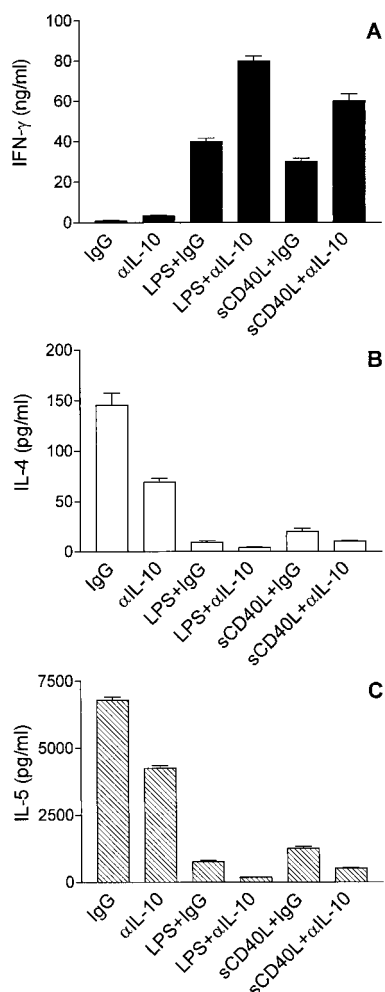


FIGURE 5. T cells primed with DCs treated with anti-IL-10 release higher levels of IFN- γ and lower amounts of IL-4 and IL-5. DCs were treated with neutralizing anti-IL-10 mAb or mouse IgG in the presence or absence of LPS or sCD40L for 18 h and then washed and cocultured with naive CD45RA⁺ allogeneic T cells. After 6 days, T cells were restimulated with plate-coated anti-CD3 and soluble anti-CD28 mAbs for 48 h, and examined for IFN- γ (A), IL-4 (B), and IL-5 (C) release. Lymphokines were detected in the supernatants by ELISA. Differences in lymphokine levels between T cell lines generated with control IgG- and anti-IL-10-treated DCs were statistically significant ($p < 0.05$). Data are expressed as mean nanograms or picograms per milliliter \pm SD of triplicate cultures for each condition. Three additional experiments showed similar results.

Mature DCs show enhanced accumulation of IL-10R1 mRNA and intracellular IL-10R protein but reduced membrane IL-10R and IL-10 binding activity

To gain insight into the mechanisms underlying the unresponsiveness of mature DCs to IL-10, we examined the expression of IL-10R during DC maturation. Immature DCs expressed similar amounts of the mRNA for both IL-10R1 and IL-10R2 subunits. After activation with LPS or sCD40L, the mRNA specific for the IL-10R1 but not for IL-10R2 was markedly up-regulated, with a 3- to 5-fold increase as determined by densitometric analysis (Fig. 7A). Treatment with anti-IL-10 increased the mRNA signals for IL-10R1 but not for IL-10R2 in both immature and in LPS- or sCD40L-matured DCs (from 1.5- to 3-fold increase), suggesting that endogenous IL-10 can reduce IL-10R1 mRNA accumulation. Total IL-10R1 protein expression and IL-10 binding activity both were enhanced in mature DCs or cells treated with anti-IL-10

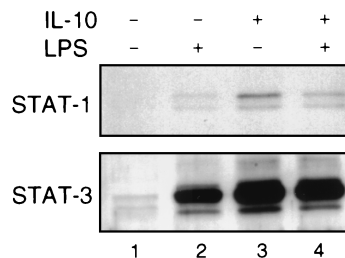


FIGURE 6. Immature but not mature DCs activate STAT-1 and STAT-3 in response to IL-10. Immature DCs and DCs stimulated with LPS for 18 h were exposed to IL-10 for 20 min and then Western blot analysis for tyrosine-phosphorylated STAT-1 and STAT-3 was performed by using specific mAbs. Both p91 and p84 forms of STAT-1 were detected. One of three experiments performed is shown.

mAb, paralleling the mRNA data (Fig. 7B). In contrast, surface IL-10R1 and IL-10 binding capacity decreased in the same conditions. ELISA confirmed that higher levels of IL-10R1 were present in whole cell lysates from LPS-matured DCs compared with immature DCs (930 ± 110 vs 600 ± 54 pg/ml; mean \pm SD of three experiments; $p < 0.02$). Finally, IL-10R1 could not be

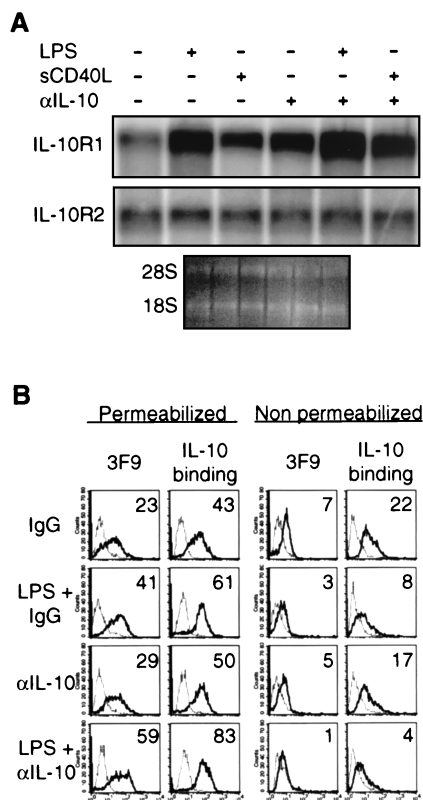


FIGURE 7. Mature DCs show enhanced accumulation of IL-10R1 mRNA and intracellular IL-10R1 protein, but reduced surface IL-10R expression and IL-10 binding activity. A, Purified DCs were treated with neutralizing anti-IL-10 mAb or mouse IgG in the presence or absence of LPS or sCD40L for 18 h. Total RNA was extracted and subjected to Northern blot analysis by using probes specific for the IL-10R1 (binding chain) and IL-10R2 (transducing chain). B, DCs were analyzed for IL-10R1 expression (3F9 mAb) and IL-10 binding activity after fixation and permeabilization, or fixation alone. IL-10 binding was determined by using biotinylated IL-10. The numbers indicate the net mean fluorescence intensity, with dotted histograms representing the staining with control Ig. Similar results were obtained in two different experiments.

detected by ELISA in supernatants from either immature or mature DCs, indicating that the receptor was not shed from the membrane.

Discussion

The functions of DCs are tightly regulated in such a way that protective immune responses are elicited and unwanted immune responses are prevented. Several levels of regulation have been uncovered, including DC accumulation into tissues and their migration from tissues to lymph nodes (34, 35), DC survival during encounter with T cells (8), and T cell-dependent elimination of DCs (36). However, a crucial point of regulation is the maturation process during which DCs switch from an Ag-capturing to an Ag-presenting mode, and that allows an efficient activation of naive and resting T lymphocytes (1). In this study, we show that signals that recapitulate the noncognate and cognate maturation promote high IL-10 release from DCs, and that endogenous IL-10 provides an important mechanism for limiting this process. Although immature DCs secreted low levels of IL-10, its neutralization was sufficient to promote some DC maturation as indicated by the higher expression of CD83 and CD86 and increased release of TNF- α . These findings suggest that DCs can undergo spontaneous maturation *in vitro* even in the absence of exogenous signals and that autocrine IL-10 is important in maintaining DC in an immature state. IL-10 neutralization also was able to reinforce DC maturation induced by LPS or CD40 triggering in terms of phenotypic changes and cytokine production as well as alloantigen-presenting capacity. Moreover, DCs matured in the presence of anti-IL-10 mAb were more potent at directing Th1 differentiation of naive T cells, clearly indicating that autocrine IL-10 also can efficiently limit the capacity of DCs to initiate Th1 responses. Although IL-10 neutralization increased the release of IL-12 and TNF- α , it diminished IL-10 synthesis, suggesting that autocrine IL-10 also provides a positive feedback mechanism for its own production. Interestingly enough, primary DCs undergo spontaneous maturation *in vitro* on isolation from tissues such as the skin or the spleen (37, 38), but the mechanisms responsible have not been defined yet. However, both human epidermal Langerhans cells and mouse spleen DCs do not express IL-10 mRNA (39, 40), and this may explain, at least in part, their spontaneous maturation during short-term culture. Also, DCs generated from CD1a⁺ progenitors (30) and Langerhans cells differentiated from monocytes or CD34⁺ progenitors in the presence of TGF- β fail to synthesize IL-10 (41, 42), whereas peripheral blood CD83⁺ DCs express IL-10 mRNA (43). Therefore, it appears that IL-10 production is a property of some DC subsets. In particular, IL-10 is abundantly produced by monocyte-derived DCs, which are paradigmatic of DCs that accumulate at sites of chronic inflammation (44). Hence, it is possible that these DCs are equipped with IL-10 as a potent mechanism to prevent exaggerated or distorted (e.g., against self-Ags) immune responses. By contrast, the lack of IL-10 in DCs that usually reside in and patrol unperturbed tissues may be important for not rendering them insensitive to potential danger signals and ultimately favors the induction of protective immune responses against pathogens (45). Alternatively, distinct DC subsets may rely on different mechanisms to refrain from uncontrolled maturation (41). A regulatory role for autocrine IL-10 has been described previously for monocytes and macrophages (46, 47). Similarly to DCs, IL-10 has been shown to inhibit the production of proinflammatory cytokines in monocytes. However, in sharp contrast to DCs, IL-10 (both exogenous and autocrine) also inhibited its own production in monocytes (46). IL-10 derived from DCs can also affect DCs in a paracrine fashion and alter the functions of other cell types. For example, it may affect the differentiation of T lymphocytes and

promote the development of T regulatory cells or increase the effector functions of CD8⁺ T cells (45).

Mature DCs lose sensitivity to IL-10, but the mechanisms of this unresponsiveness have not been investigated. Here we found that on exposure to IL-10, immature DCs showed high levels of tyrosine-phosphorylated STAT-3 and low STAT-1. In contrast, mature DCs displayed high basal-phosphorylated STAT-3 and some STAT-1 that were only minimally increased by IL-10, suggesting an impaired early signal transduction defect to IL-10 in mature DCs. IL-10 acts through a heterodimeric membrane receptor formed by a binding (IL-10R1) and a signaling subunit (IL-10R2). Both chains are required for optimal signal transduction (22, 23). Here, we found that immature DCs expressed comparable amounts of IL-10R1 and IL-10R2 mRNA. After maturation, DCs up-regulated selectively the IL-10R1 mRNA, with IL-10R1 and IL-10 binding activity increased intracellularly but markedly reduced on the surface. Similar findings were observed in immature DCs after neutralization of endogenous IL-10, and addition of anti-IL-10 to maturing DCs further amplified the difference between intracellular and surface IL-10R1 expression, suggesting that endogenous IL-10 can modulate the expression and function of its own receptor. In agreement with our findings, a recent report showed that rheumatoid synovial DCs, representative of mature DCs, expressed IL-10R1 protein predominantly intracellular (21). The reduced membrane IL-10R1 expression may be one mechanism by which mature DCs become resistant to IL-10. Mature DCs also are unresponsive to IFN- γ and down-regulate membrane IFN- γ R1 expression (48). Both the IFN- γ R1 and IL-10R1 belong to the class II cytokine receptor family, and share many similarities in their structure (22–24). At the moment, it is difficult to reconcile the finding that during maturation DCs increased IL-10R1 mRNA and intracellular protein but reduced surface IL-10R1 and IL-10 binding activity. Loss of membrane IL-10R1 was not attributable to receptor shedding, as no soluble IL-10R1 could be measured in cell culture supernatant. It is instead possible that membrane IL-10R1 down-regulation is secondary to posttranslational events such as altered receptor trafficking and/or recycling from intracellular stores to the cell membrane, as suggested for the chemokine receptor CCR5 in LPS-stimulated monocytes (49).

In conclusion, our study provides evidence that autocrine IL-10 and IL-10R serve as a relevant modulatory loop for the regulation of DC maturation, with important consequences on the outcome of the immune response. Blocking IL-10 production by DCs or DC responsiveness to IL-10 may add to DC-based therapeutic strategies aimed at inducing or amplifying type 1 immunity, as recently shown in mouse models (50, 51). In contrast, an unrestricted production of IL-10 by DCs may be exploited to dampen unwanted type 1 immune responses, and indeed drugs such as corticosteroids and vitamin D3 with the ability to suppress these reactions inhibit the maturation of DCs, including IL-12 release, but do not affect or even stimulate IL-10 production (52–55).

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