Interleukin 10 (IL-10)-mediated Immunosuppression

MARCH-I INDUCTION REGULATES ANTIGEN PRESENTATION BY MACROPHAGES BUT NOT DENDRITIC CELLS

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Background: IL-10 suppresses antigen presentation by macrophages (MΦ) and dendritic cells (DCs).

Results: IL-10 promotes expression of the MHC class II and CD86 E3 ubiquitin ligase March-I and suppresses antigen presentation in activated MΦ but not in DCs.

Conclusion: IL-10 suppression of antigen presentation is March-I-dependent in MΦ but March-I-independent in DCs.

Significance: IL-10 suppresses antigen presentation in MΦ and DCs by distinct molecular mechanisms.

Efficient immune responses require regulated antigen presentation to CD4 T cells. IL-10 inhibits the ability of dendritic cells (DCs) and macrophages to stimulate antigen-specific CD4 T cells; however, the mechanisms by which IL-10 suppresses antigen presentation remain poorly understood. We now report that IL-10 stimulates expression of the E3 ubiquitin ligase March-I in activated macrophages, thereby down-regulating MHC-II, CD86, and antigen presentation to CD4 T cells. By contrast, IL-10 does not stimulate March-I expression in DCs, does not suppress MHC-II or CD86 expression on either resting or activated DCs, and does not affect antigen presentation by activated DCs. IL-10 does, however, inhibit the process of DC activation itself, thereby reducing the efficiency of antigen presentation in a March-I-independent manner. Thus, IL-10 suppression of antigen presenting cell function in macrophages is March-I-dependent, whereas in DCs, suppression is March-I-independent.

Pathogen-specific CD4 T cells can be stimulated by a variety of antigen-presenting cells (APCs)† that express MHC class II molecules (MHC-II) and co-stimulatory proteins including CD80, CD86, and CD40 (1). MHC-II is constitutively expressed in DCs, and exposure of DCs to pathogens stimulates TLR signaling pathways that “activate” DCs to express large amounts of surface peptide-MHC-II complexes (pMHC-II) and co-stimulatory proteins essential for antigen-specific naive CD4 T cell activation (2, 3). Unlike DCs, monocytes and MΦ express very little MHC-II in the resting state. It is only after stimulation with IFN-γ that monocytes or MΦ express large amounts of MHC-II and co-stimulatory molecules necessary for these cells to be effective stimulators of pathogen-specific CD4 T cells (4).

Enhanced expression of MHC-II and co-stimulatory molecules promotes the ability of APCs to stimulate CD4 T cells, whereas reduced expression of these proteins on the surface of these cells suppresses this function. MHC-II and CD86 protein expression levels in APCs are controlled by the E3 ubiquitin ligase March-I (5, 6). March-I ubiquitinates MHC-II and CD86 (but not CD80 or CD40) and targets these proteins for lysosomal degradation (7). Maturation of DCs with a variety of activating signals terminates March-I expression (8, 9), a change in the DC that prolongs the half-life of pMHC-II and CD86 (8, 10, 11). It has been proposed that selective expression of March-I in immature APCs serves to promote the turnover of diverse pMHC-II complexes until the cells receive an activation signal (12) at which time the cells terminate March-I expression, reduce pMHC-II degradation, and enhance the stability of pMHC-II complexes derived from activating pathogens.

IL-10 is an immunosuppressive cytokine that is secreted by a wide variety of cells during the course of an immune response (13). IL-10 suppresses the ability of DCs and MΦ to stimulate the proliferation of antigen-specific CD4 T cells; however, the molecular mechanisms by which IL-10 suppresses APC function are not completely understood. IL-10 gene products are known to antagonize both IFN-γ and TLR-dependent signaling pathways that are critical for MΦ and DC activation, respectively (14). Curiously, IL-10 also directly stimulates expression of March-I in activated MΦ and monocytes (15, 16), thereby resulting in reduced surface expression of MHC-II and CD86. Given the well-documented ability of IL-10 to suppress immune responses by both DCs and MΦ (17, 18), it has been proposed that the immunosuppressive activity of IL-10 resides in the ability of IL-10 to stimulate March-I expression in APCs (16, 19). However, because IL-10 suppresses DCs and MΦ activation directly, the precise role of March-I in the IL-10-dependent suppression of APC function in these cells remains to be determined.

In the current study, we investigated the role of March-I in IL-10-mediated immunosuppression of APC function in MΦ and DCs. We found that IL-10 induces March-I expression in
IL-10 Suppression of APC Function

IFN-γ activated MΦ, thereby reducing surface MHC-II and CD86 expression and suppressing the ability of the MΦ to stimulate antigen-specific CD4 T cells. Studies using March-I-deficient mice confirmed that IL-10-mediated suppression of MHC-II/CD86 expression and macrophage APC function is March-I-dependent. Unlike in MΦ, IL-10 does not induce March-I expression in DCs but instead results in March-I-independent suppression of DC activation and T cell stimulatory function. Thus, IL-10 suppresses APC function in MΦ and DCs by distinct March-I-dependent and March-I-independent mechanisms.

Experimental Procedures

Mice and Cell Culture—Age- and sex-matched C57BL/6 and March-I−/− mice (on a C57BL/6 background) were bred and maintained in house at the NCI-Fredrick animal colony. All mice were cared for in accordance with National Institutes of Health guidelines with the approval of the National Cancer Institute Animal Care and Use Committee. BMDCs were generated by culturing bone marrow cells in the presence of GM-CSF (20 ng/ml) for 6 days as described previously (20). These cultures routinely contain >85% CD11chigh cells. MΦ were generated by culturing bone marrow cells in medium containing M-CSF (10 ng/ml) for 6 days in Petri dishes before transfer to tissue culture plates for the activation assay as described previously (21). These cultures routinely contain >90% CD11b+ and F4/80+ cells. DCs were isolated from mouse spleens using a MACS mouse DC isolation kit (Miltenyi Biotech). Cells were isolated quickly and maintained on ice to limit spontaneous DC activation. Peritoneal MΦ were harvested by peritoneal lavage from mice injected 4 days previously with 10% Brewer’s thioglycollate broth. BMDCs were activated (or not) using LPS (1 µg/ml) for 24 h and then cultured in the absence or presence of IL-10 (20 ng/ml) for the indicated times. In some experiments, BMDCs were activated using LPS (1 µg/ml) in the absence or presence of recombinant IL-10 (20 ng/ml) for the indicated times. MΦ were activated (or not) using either IFN-γ (100 ng/ml) or LPS (10 ng/ml) for 24 h and then cultured in the absence or presence of IL-10 (20 ng/ml) for the indicated times.

T Cell Proliferation Assays—Naive CD4 T cells from OT-II mice were isolated from spleen and lymph nodes using a MACS mouse CD4 T cell isolation kit (Miltenyi Biotech). Purified naive CD4 T cells were labeled with CFSE (5 µM) in PBS for 8 min at room temperature and washed with complete medium prior to incubation with APCs. In experiments using MΦ as APCs, we used a previously established protocol for MΦ-stimulated T cell proliferation (22). Briefly, MΦ were activated with IFN-γ for 24 h, washed, and cultured an additional 18 h in the presence or absence of IL-10. OVA(329–334) peptide (1 µM) was present during the 42-h MΦ culture and removed prior to the addition of CD4 T cells. Purified naive CD4 T cells were cultured with MΦ at a 1:2 MΦ:T cell ratio. The CD4 T cells were isolated from the co-culture after 36 h and cultured separately for an additional 5 days. In experiments using BMDCs as APCs, OVA(329–334) peptide (1 µM) was added to immature DCs for 8 h, the cells were washed, and the cells were then incubated overnight alone, with LPS, or with LPS and IL-10 together. After the indicated treatment, DCs were washed and co-cultured with CFSE-labeled T cells for 3 days at various DC:T cell ratios. CFSE dye dilution was analyzed by FACS using a FACSCalibur flow cytometer. The extent of T cell proliferation was calculated by FlowJo software and is shown as a division index.

Flow Cytometry—Cells were stained on ice using fluoro- chrome-conjugated antibody and appropriate isotype control antibody in FACS buffer (Hanks’ balanced salt solution containing 2% FBS). Antibodies recognizing mouse MHC-II (clone M5/114), CD86, CD40, CD11c, CD11b, and F4/80 were purchased from BD Biosciences. The stained cells were analyzed using a FACSCalibur flow cytometer and FlowJo software. Cells were initially gated by forward scatter/side scatter analysis (to gate out dead cells or cell debris), further gated on CD11c+ cells (DCs) or CD11b+ F4/80+ cells (MΦ), and analyzed for the expression of the indicated surface protein.

Quantitative RT-PCR—Total RNA was extracted using the TRIzol-chloroform (Invitrogen) method. A SuperScript III First Strand Synthesis kit (Invitrogen) was used to generate cDNA using random hexamers primers according to the manufacturer’s instructions. Real time PCR was performed using an ABI Prism 7900HT sequence detection system and the Quantitect SYBR Green PCR kit (Qiagen). Primers for GAPDH, CD86, CD40, IL-12, and TNF-α were purchased from Qiagen. Sequences for the mouse March-I and MHC-II primers have been described previously (23). GAPDH expression was used to normalize for the amount of mRNA present in each sample (mean of triplicate). Results are expressed relative to the values obtained after IFN-γ or LPS treatment of MΦ or DCs, respectively.

Immunofluorescence Microscopy—Cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature and permeabilized with 0.01% saponin in PBS at room temperature. Primary antibody were applied to the cells for 30 min on ice, and the cells were thoroughly washed in Hanks’ balanced salt solution before incubation with Alexa Fluor 488- or Alexa Fluor 555-conjugated secondary antibodies (Invitrogen) in the same buffer. Cells were mounted on polylysine-coated glass slides by incubation for 30 min at room temperature. Cells were imaged using a Zeiss LSM 510 META confocal microscope with a 63× oil immersion objective lens (numerical aperture, 1.4). Co-localization of MHC-II with intracellular LAMP-1 was scored by a blinded observer, and the percentage of cells with significant late endosomal/lysosomal MHC-II was expressed as a fraction of the total number of cells analyzed. Statistical analysis of the data was performed using a Z-test.

Immunoprecipitation and Immunoblotting—Cells were solubilized in lysis buffer (10 mM Tris–HCl, 150 mM NaCl, pH 7.4 containing 1% Triton X-100, 1 mg/ml BSA, 50 mM PMSF, 0.1 mM N-tosyl-L-lysine chloromethyl ketone HCl, and 25 mM N-ethylmaleimide) and immunoprecipitated by using protein A-agarose beads coated with the anti-pMHC-II mAb Y3P. The samples were analyzed by non-reducing SDS-PAGE and transferred to PVDF membranes. Immunoblots were probed for ubiquitinated proteins using biotinylated anti-ubiquitin mAb P4D1 and HRP-streptavidin. Blots were reprobed with a rabbit anti-MHC-II β-chain-specific serum to normalize for the
IL-10 Suppression of APC Function

amount of total MHC-II present in each immunoprecipitate. Immunoblots were analyzed by laser densitometry using protocols described previously (24).

Statistical Analysis—All results are presented as mean ± S.D. For microscopy studies, p values were calculated using a Z-test; in all other studies, p values were calculated using a two-tailed paired Student’s t test. p values < 0.05 were considered significant. p values are indicated by * (p < 0.05), and non-significant differences are indicated by ns.

Results

IL-10 Reduces MHC-II and CD86 Expression on Activated MΦ—To begin to examine the mechanism(s) by which IL-10 suppressed the ability of MΦ to activate CD4 T cells, bone marrow-derived MΦ were activated by treatment with IFN-γ for 24 h and then cultured alone or with IL-10 for an additional 18 h. Unstimulated MΦ did not express MHC-II and had very low expression of CD86 and CD40 (Fig. 1A). As expected, IFN-γ activation greatly enhanced the expression of MHC-II, CD86, and CD40 on the surface of these MΦ. IL-10 treatment of activated MΦ resulted in the down-regulation of surface MHC-II and CD86 but not CD40. IL-10 treatment did not affect steady-state levels of MHC-II, CD86, or CD40 mRNA in activated MΦ (Fig. 1B), demonstrating that down-regulation of MHC-II and CD86 protein expression is not due to IL-10-mediated alterations in MΦ activation. As in human monocytes (16), IL-10 treatment dramatically increased expression of the MHC-II and CD86 E3 ubiquitin ligase March-I in activated (but not unstimulated) MΦ (Fig. 1C). IL-10 increased expression of March-I in both IFN-γ-activated MΦ and LPS-activated MΦ, showing that the ability of IL-10 to induce March-I expression was not restricted to IFN-γ-activated cells (Fig. 1D). These results demonstrate that IL-10 induces expression of March-I and down-regulates MHC-II and CD86 protein expression on activated MΦ without affecting MHC-II or CD86 mRNA expression.

IL-10-mediated Down-regulation of MHC-II and CD86 on Activated MΦ Is March-I-dependent—Both MHC-II and CD86 are known substrates of March-I (7), and the selective loss of MHC-II and CD86 (but not CD40) on activated MΦ prompted us to ask whether the IL-10-mediated reduction in MHC-II and CD86 protein expression observed in activated MΦ was March-I-dependent. We therefore compared the effects of IL-10 on activated MΦ generated from wild-type and March-I-deficient mice (Fig. 2, A and B). Unlike the profound reduction of MHC-II and CD86 surface expression in wild-type MΦ, IL-10-mediated down-regulation of MHC-II and CD86 was completely absent in March-I-deficient MΦ. These data conclusively show that IL-10-mediated reduction in MHC-II and
CD86 protein expression in activated MΦ can be attributed to IL-10-induced March-I.

To confirm that the results obtained using bone marrow-derived MΦ could be generalized to MΦ present in an intact mouse, we repeated these studies using peritoneal MΦ isolated from wild-type and March-I-deficient mice (Fig. 2, C and D). As we observed in bone marrow-derived MΦ, IL-10 treatment diminished expression of MHC-II and CD86 on IFN-γ-activated peritoneal MΦ but had no effect on expression of CD40. These effects were also March-I-dependent in peritoneal MΦ as we did not observe any suppressive effect when IL-10 was added to activated peritoneal MΦ isolated from March-I-deficient mice. Collectively, these results demonstrate that IL-10-mediated down-regulation of MHC-II and CD86 on activated MΦ, whether generated from bone marrow precursors or isolated from mice, is March-I-dependent.

**IL-10-mediated Intracellular Accumulation of MHC-II in MΦ Is March-I-dependent**—One of the earliest reports of the effects of IL-10 on monocytes demonstrated that IL-10 treatment of human monocytes resulted in the intracellular accumulation of MHC-II in lysosomes (25). Given the known role of March-I in promoting lysosomal degradation of MHC-II in APCs (10), we next asked whether the IL-10-dependent alteration in MHC-II localization in MΦ was March-I dependent. Whereas acute administration of IL-10 resulted in the redistribution of MHC-II from the plasma membrane to lysosomes in wild-type MΦ, this change was not observed in MΦ generated from March-I-deficient mice (Fig. 3, A and B). These results support our finding that induction of March-I by IL-10 reduces surface MHC-II expression and demonstrate that IL-10 increases lysosomal MHC-II accumulation in activated MΦ.

**IL-10-mediated Suppression of CD4 T Cell Activation by MΦ Is March-I dependent**—Our findings that IL-10 stimulated March-I expression on activated MΦ and that the IL-10-dependent reduction in MHC-II and CD86 expression was March-I-dependent led us to predict that the ability of MΦ to activate CD4 T cells would be suppressed by IL-10 and that suppression would be entirely March-I-dependent. OVA(323–339)-loaded activated MΦ were cultured alone or with IL-10 for 18 h. The cells were then washed and incubated with CFSE-labeled OVA(323–339)-specific CD4 T cells. Antigen-specific CD4 T cell proliferation by wild-type activated MΦ was profoundly suppressed by IL-10 (Fig. 4). Most importantly, suppression of T cell activation by IL-10 was completely absent when MΦ from March-I-deficient mice were used as APCs. These data show that the ability of MΦ to stimulate antigen-specific CD4 T cells is suppressed by IL-10 and that this is due to IL-10-induced expression of March-I in activated MΦ.

* FIGURE 2. IL-10-mediated down-regulation of MHC-II and CD86 in activated MΦ is March-I-dependent. MΦ were generated from bone marrow cells (A and B) or from the peritoneal cavity (C and D) from either wild-type mice (A and C) or March-I KO mice (B and D). The cells were left untreated or were activated with IFN-γ for 1 day, washed, and recultured in the absence or presence of IL-10 for an additional 18 h. CD11b+ F4/80+ MΦ were analyzed for cell surface expression of MHC-II, CD86, and CD40 by FACS analysis using the indicated antibodies: isotype controls (shaded), untreated (dotted line), IFN-γ and then medium alone (solid line), or IFN-γ and then IL-10 (dashed line). The geometric mean fluorescence intensity of the indicated protein was expressed relative to that in cells treated with IFN-γ alone. The data shown are the mean ± S.D. (error bars) from three independent experiments. *p < 0.05; ns, non-significant differences.
**IL-10 Suppression of APC Function**

**A** - IL-10- and March-I KO-mediated lysosomal accumulation of MHC-II in MΦ is March-I-dependent. MΦ were generated from bone marrow cells isolated from either wild-type mice or March-I KO mice. The cells were activated by culture for 1 day in the presence of IFN-γ and then cultured in the absence or presence of IL-10 for an additional 18 h. The cells were harvested and fixed/permeabilized for analysis by confocal immunofluorescence microscopy. A, cells were stained with mAb recognizing pMHC-II (green) and the late endosome/lysosome marker LAMP-1 (red). Confocal images of a representative single 0.8-μm-thick optical section taken in the midplane of the cells are shown. B, co-localization of MHC-II with intracellular LAMP-1 was scored by a blinded observer, and the percentage of all cells with significant amounts of lysosomal MHC-II was calculated. The number of cells analyzed in each condition is indicated. Statistical analysis of the data was performed using a Z-test. *, p < 0.05; ns, non-significant differences.

**FIGURE 3. IL-10-mediated lysosomal accumulation of MHC-II in MΦ is March-I-dependent.** MΦ were generated from bone marrow cells isolated from either wild-type mice or March-I KO mice. The cells were activated by culture for 1 day in the presence of IFN-γ and then cultured in the absence or presence of IL-10 for an additional 18 h. The cells were harvested and fixed/permeabilized for analysis by confocal immunofluorescence microscopy. A, cells were stained with mAb recognizing pMHC-II (green) and the late endosome/lysosome marker LAMP-1 (red). Confocal images of a representative single 0.8-μm-thick optical section taken in the midplane of the cells are shown. B, co-localization of MHC-II with intracellular LAMP-1 was scored by a blinded observer, and the percentage of all cells with significant amounts of lysosomal MHC-II was calculated. The number of cells analyzed in each condition is indicated. Statistical analysis of the data was performed using a Z-test. *, p < 0.05; ns, non-significant differences.

**IL-10 Does Not Suppress Expression of Activation Markers on Immature or Mature BMDCs—**Given our findings that IL-10 directly stimulates expression of March-I in activated (but not unstimulated) MΦ and that suppression of MΦ APC function was March-I-dependent, we set out to determine whether IL-10 had a similar mechanism of action in DCs. We generated DCs from the same bone marrow precursors used to generate MΦ and cultured the DCs overnight in the absence or presence of LPS to generate immature or mature DC populations, respectively. Immature BMDCs have low expression of MHC-II, CD86, and CD40, and culturing these immature DCs with IL-10 for 18 h had no effect on expression of these proteins (Fig. 5A). Mature DCs express much more MHC-II, CD86, and CD40 on their surface; however, unlike in activated MΦ, IL-10 treatment did not alter expression of any of these activation markers on the mature DC surface (Fig. 5B). We know that IL-10 was biologically active in our assays as IL-12 mRNA levels in mature DCs were suppressed by IL-10 (data not shown). To directly examine the effect of IL-10 on DC function, we cultured OVA(323–339) peptide-pulsed LPS-matured DCs alone or with IL-10 for 18 h, washed the cells, and incubated the DCs with CFSE-labeled OVA(323–339)-specific CD4 T cells. The ability of mature DCs to stimulate CD4 T cell proliferation was completely unaffected by pretreatment of mature DCs with IL-10 (Fig. 5C). These data show that, whereas IL-10 suppresses CD4 T cell stimulatory function of activated MΦ, IL-10 does not affect MHC-II or CD86 expression on immature or mature BMDCs and does not affect the ability of mature DCs to stimulate antigen-specific CD4 T cells.

**IL-10 Suppresses LPS-induced Activation of BMDCs—**It is well known that IL-10 suppresses DC function (for a review, see Ref. 14), and given our inability to identify either immature or mature DCs as targets of IL-10 suppression, we next asked whether IL-10 affected the process of DC maturation and whether March-I played any role in the effect of IL-10 on DC maturation and/or function. BMDCs obtained from wild-type mice or March-I-deficient mice were cultured alone, with LPS only, or with LPS and IL-10 together for 18 h, and expression of various DC activation markers was quantitated by FACS analysis. IL-10 inhibited (but did not completely prevent) the LPS-induced up-regulation of surface MHC II, CD86, and CD40 expression on wild-type BMDCs (Fig. 6A), confirming previous studies demonstrating that IL-10 antagonizes TLR signaling required for DC activation (14). Despite the fact that BMDCs from March-I-deficient mice have enhanced expression of MHC-II and CD86 even in the immature state, IL-10 was also able to suppress LPS-induced up-regulation of MHC-II, CD86, and CD40 on March-I-deficient DCs (Fig. 6B).

We also examined the effects of IL-10 on the maturation of mouse spleen DCs *ex vivo*. Spleen DCs activate spontaneously in culture (26), and we found that addition of IL-10 to an overnight “activating” culture of purified spleen DCs resulted in diminished expression of the DC activation markers CD86 and CD40 (Fig. 6C), demonstrating that even spontaneous activation of spleen DCs was markedly suppressed by IL-10.

The ability of IL-10 to suppress the process of DC maturation was confirmed by RT-PCR analysis. LPS-dependent increases in CD86, CD40, and IL-12 were profoundly reduced by inclu-
**IL-10 Suppression of APC Function**

**IL-10 Suppresses Activation-induced March-I Down-regulation in Maturing DCs**—It has recently been suggested that IL-10 is capable of stimulating March-I expression in mouse DCs cultured with LPS (19). However, because IL-10 suppresses the activation process in DCs and because March-I expression is down-regulated during the process of DC activation (8, 9), it is possible that the apparent increase in March-I expression in cells treated with LPS and IL-10 together is due to IL-10-mediated suppression of the program of terminating March-I expression that accompanies DC activation. To address this question, we cultured BMDCs for 2 h in medium alone, with LPS only, with LPS and IL-10 together, or with IL-10 only and monitored changes in March-I mRNA expression by RT-PCR. In these same experiments, we examined the effect of IL-10 on March-I expression in resting and activated macrophage (Fig. 7A). By contrast, IL-10 does not enhance March-I expression in either immature or mature DCs. Furthermore, IL-10 completely blocks the LPS-dependent down-regulation of March-I expression in activating DC cultures. The dramatic down-regulation of March-I expression in spontaneously activating spleen DCs was also markedly reduced by the presence of IL-10 in the culture.

The ability of IL-10 to suppress activation-induced reduction in March-I mRNA was also assessed using a functional assay. MHC-II is oligo-ubiquitinated in immature DCs, and stimulation of immature DCs with LPS for as little as 4 h results in a dramatic reduction in MHC-II ubiquitination (Fig. 7B). In excellent agreement with our March-I mRNA analysis, activation-dependent suppression of MHC-II ubiquitination was partially blocked by the inclusion of IL-10 in the activating DC culture, and addition of IL-10 to immature DCs did not alter MHC-II ubiquitination. These data demonstrate that, whereas IL-10 induces March-I expression in activated macrophages, IL-10 does not promote March-I expression in DCs.

**IL-10 Suppression of T Cell Activation by DCs Is March-I-independent**—Based on the ability of IL-10 to affect the DC activation process equally in wild-type and March-I-deficient DCs, we predicted that IL-10 would suppress the ability of DCs to stimulate antigen-specific CD4 T cells in a March-I-independent manner. To test this hypothesis, we measured the ability of OVA(323–339) peptide-loaded immature DCs or DCs activated in the presence (or absence) of IL-10 to stimulate CFSE-labeled OVA-specific CD4 T cells. LPS-enhanced activation of OVA-specific T cells was suppressed by IL-10 to levels that were similar to those of immature DCs (Fig. 7, C and D). In agreement with the hypothesis that IL-10 effects on DC function are unrelated to March-I expression, essentially identical results were obtained when using DCs from March-I-deficient mice. Taken together, our data show that IL-10 suppresses the

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**FIGURE 5.** IL-10 does not affect MHC-II or CD86 expression in immature or mature DCs. BMDCs were cultured in medium alone (immature) or activated with LPS for 1 day (mature) and then cultured in the absence or presence of IL-10 for an additional 18 h. Expression of MHC-II, CD86, and CD40 was determined by FACS analysis using the indicated antibodies. Isotype control antibody staining is shown in the shaded histogram. A, immature DCs incubated in the absence (dotted line) or presence (dashed line) of IL-10. B, LPS-matured DCs incubated in the absence (solid line) or presence (dashed line) of IL-10. The geometric mean fluorescence intensity of the indicated protein was expressed relative to that of LPS-treated DCs in the absence of IL-10 at a DCT of 1:10. The data shown are the mean ± S.D. (error bars) from three independent experiments. ns, non-significant differences.
**IL-10 Suppression of APC Function**

**A**

| MHC-II | CD86 | CD40 |
|-------|------|------|
| wild-type DCs | None | LPS | LPS + IL-10 |
| MHC-II expression (relative to LPS) | 1.0 | 1.0 | 1.0 |
| CD86 expression (relative to LPS) | 1.0 | 1.0 | 1.0 |
| CD40 expression (relative to LPS) | 1.0 | 1.0 | 1.0 |

**B**

| MHC-II | CD86 | CD40 |
|-------|------|------|
| March-I KO DCs | None | LPS | LPS + IL-10 |
| MHC-II expression (relative to LPS) | 1.0 | 1.0 | 1.0 |
| CD86 expression (relative to LPS) | 1.0 | 1.0 | 1.0 |
| CD40 expression (relative to LPS) | 1.0 | 1.0 | 1.0 |

**C**

| CD86 | CD40 |
|------|------|
| wild-type spleen DCs | 37°C | 37°C |
| expression of CD86 and CD40 | 1.0 | 1.0 |
| expression (relative to 37°C) | 1.0 | 1.0 |

**D**

| CD86 | CD40 | IL-12 |
|------|------|------|
| wild-type | None | LPS | LPS-IL-10 |
| mRNA expression (relative to LPS) | 1.0 | 1.0 | 1.0 |
| March-I KO | None | LPS | LPS-IL-10 |
| mRNA expression (relative to LPS) | 1.0 | 1.0 | 1.0 |

**FIGURE 6.** IL-10 suppression of LPS-induced MHC-II, CD86, and CD40 expression is March-I independent in DCs. DCs generated from bone marrow cells from wild-type mice (A) or March-I KO mice (B) were cultured in medium alone (dotted line) or in medium containing LPS alone (solid line) or LPS and IL-10 together (dashed line). Expression of MHC-II, CD86, and CD40 was determined by FACS analysis using the indicated antibodies. Isotype control antibody staining is shown in the shaded histogram. The geometric mean fluorescence intensity of the indicated protein was expressed relative to that in cells treated with LPS alone. The data shown are the mean ± S.D. (error bars) from three independent experiments. The dashed line represents the baseline expression of each marker in untreated cells. *p < 0.05.

Discussion

The anti-inflammatory cytokine IL-10 suppresses the ability of DCs, monocytes, and MΦ to stimulate antigen-specific CD4 T cells (14); however, precisely how IL-10 suppresses various aspects of APC function remains to be determined. In this study, we examined the mechanisms underlying the immunosuppressive effect of IL-10 on the antigen presenting function of MΦ and DCs. We found that IL-10 directly stimulates expression of the E3 ubiquitin ligase March-I on activated MΦ, thereby down-regulating MHC-II and CD86 on the MΦ surface and suppressing the ability of activated MΦ to stimulate antigen-specific CD4 T cells. By contrast, IL-10 had no effect on the expression of MHC-II, CD86, or CD40 on either resting or activated DCs and did not affect the ability of these cells to function as APCs. In agreement with previous studies (14, 17, 27), we found that IL-10 directly suppressed TLR-dependent activation of BMDCs and the spontaneous activation of spleen DCs, thereby resulting in phenotypically and functionally immature APCs that have weak T cell stimulatory function. Unlike the March-I-dependent inhibitory effects of IL-10 on activated MΦ function, IL-10-mediated suppression of the ability of DCs to stimulate antigen-specific CD4 T cells was March-I-independent. Our study therefore reveals both March-I-dependent and March-I-independent mechanisms of suppression of APC function by IL-10.

IFN-γ treatment of MΦ promotes expression of the class II transactivator CIITA that converts the cells from MHC-II-negative MΦ to MHC-II-expressing APCs (4) and enhances expression of CD86 and CD40, key co-stimulatory molecules required for efficient APC function (16, 28, 29). In this study, we now show that IL-10 down-regulates MHC-II and CD86 expression in IFN-γ-treated MΦ, and it does so without affecting their transcription. How IL-10 accomplishes this is by stimulating expression of the E3 ubiquitin ligase March-I in IFN-γ-activated MΦ, thereby targeting MHC-II and CD86 for degradation. The net effect of IL-10 treatment therefore is reduced expression of MHC-II and CD86 on IFN-γ-activated MΦ that are no longer able to effectively activate antigen-specific CD4 T cells. IL-10 also increases expression of March-I in LPS-activated MΦ; however, because LPS-activated MΦ express little MHC-II (30) and are inefficient APCs, we did not examine the effect of IL-10 on antigen presentation by LPS-activated MΦ. Our studies in MΦ isolated from March-I-deficient mice confirmed that IL-10-induced intracellular sequestration of MHC-II in lysosomes, observed previously in human DCs, monocytes, and MΦ, requires March-I (27). Our study reveals both March-I-dependent and March-I-independent mechanisms of suppression of APC function by IL-10.
FIGURE 7. IL-10 suppresses activation-induced down-regulation of March-I expression, MHC-II ubiquitination, and activation-induced antigen presentation by DCs. A, bone marrow-derived macrophages were cultured in the absence or presence of IFN-γ for 1 day, and then IL-10 (or PBS) was added for an additional 4 h of culture. Bone marrow-derived DCs were cultured for 2 h in medium alone (None), with IL-10 alone (IL-10), with LPS in the absence or presence of IL-10 (Activating DCs), or with LPS overnight and then cultured for an additional 2 h in the absence or presence of IL-10 (Activated DCs). Spleen DCs were cultured for 1 h on ice or at 37 °C in the absence or presence of IL-10 as indicated. Expression of March-I mRNA was analyzed by quantitative RT-PCR. Data are shown as the 2^(-ΔΔCt) (ΔΔCt = GAPDH Ct - March-I Ct) value for each condition. The data shown are the mean ± S.D. (error bars) from three independent experiments. *, p < 0.05; ns, not significant. B, bone marrow-derived DCs were cultured in medium alone, with LPS alone, with LPS and IL-10 together, or with IL-10 alone. After 4 h, cells were harvested and solubilized in Triton X-100 lysis buffer, and pMHC-II was immunoprecipitated using mAb Y3P. The immunoprecipitates were analyzed by immunoblotting with antibodies recognizing ubiquitin or total MHC-II β-chain. A representative anti-ubiquitin blot and total MHC-II β-chain blot are shown. The relative amount of pMHC-II ubiquitination in each condition is expressed as a percentage of that observed in BMDCs cultured in medium alone. The data shown are the mean ± S.D. obtained from three independent experiments. *, p < 0.05; ns, not significant. C and D, DCs generated from bone marrow cells from wild-type or March-I KO mice were incubated with OVA(323–339) peptide for 8 h, washed, and cultured for 18 h in medium alone (dotted line), with LPS alone (solid line), or with LPS and IL-10 together (dashed line). The cells were incubated with CFSE-labeled OT-II CD4 T cells for 72 h at DC:T cell ratios of 1:20 (C) or 1:40 (D). CFSE dye dilution was measured by FACS analysis. DCs were unable to stimulate OT-II T cells if the cells were not exposed to OVA(323–339) peptide during the culture (shaded histogram). Data shown are the CD4 T cell division index relative to that using DCs cultured with LPS alone at a DC:T of 1:20. The mean ± S.D. (error bars) from three independent experiments is shown. *, p < 0.05.
IL-10 Suppression of APC Function

monocytes (25), is March-I-dependent. These results are also in excellent agreement with previous studies showing IL-10-induced expression of March-I and down-regulation of MHC-II in human monocytes (16) and mouse MΦ (15).

Unlike activated MΦ, IL-10 does not affect expression of MHC-II, CD86, or CD40 and does not suppress APC function in activated DCs. Instead, IL-10 suppresses the transition of immature DCs to mature DCs that possess efficient APC function. IL-10 directly interferes with TLR-dependent DC activation by a variety of mechanisms (14). For example, IL-10 promotes ubiquitin-mediated degradation of numerous components of the MyD88-dependent TLR signaling pathway in DCs (27). IL-10 signaling also promotes expression of the microRNAs miR-146b (31) and miR-155 (32) that ultimately leads to suppression of both MyD88-dependent (33) and MyD88-independent (34) activation of DCs.

In addition to suppressing DC activation, IL-10 also suppresses IFN-γ-dependent activation of monocytes and MΦ (18, 35). In this case, IL-10 signaling promotes expression of SOCS1 and SOCS3, suppressors of IFN-γ-mediated STAT1 signaling (36). We have confirmed that IL-10 suppresses the process of macrophage activation when added simultaneously with IFN-γ (data not shown). However, interpreting the suppressive effects of IL-10 on IFN-γ-induced expression of MHC-II and CD86 on activating MΦ is confounded by the simultaneous induction of March-I by IL-10 in these cells. Although we have therefore restricted our analysis to the role of March-I in mediating immunosuppression by fully activated MΦ, we must emphasize that the major point of our study is that March-I contributes to IL-10-mediated suppression of CD4 T cell activation by activated MΦ but not by DCs.

Given the findings that IL-10 dramatically increases March-I expression in IFN-γ-activated monocytes and MΦ, it has been generally assumed that IL-10 promotes March-I expression in other APCs. In fact, two recent reports have suggested that IL-10-stimulated expression of March-I in DCs and that the immunosuppressive effects of IL-10 on DC function could be attributed to enhanced expression of March-I (19, 37). Unlike the profound effect of IL-10 in promoting March-I expression in activated MΦ, we found no evidence that IL-10 dramatically alters expression of March-I in either immature DCs or mature DCs. In agreement with our findings, others have also observed that mature DCs are refractory to the effects of IL-10 (38, 39). Maturation of DCs from an immature cell to a fully mature APC leads to the well-documented (rapid) reduction in March-I expression and cessation of MHC-II ubiquitination (9). Whereas the addition of IL-10 gives the appearance that IL-10 stimulates March-I expression in LPS-treated DCs, in reality, IL-10 simply suppresses the well-documented process of DC activation that leads to March-I down-regulation. Our finding that down-regulation of MHC-II ubiquitination by LPS was partially reversed by IL-10 provides functional biochemical evidence for the lack of complete DC activation by IL-10.

In conclusion, our study reveals fundamental differences in the molecular mechanisms by which IL-10 mediates immunosuppression in DCs and MΦ. Whereas suppression of antigen presentation by MΦ is March-I-dependent, suppression of antigen presentation by DCs is not. Our study also suggests that March-I may be an important target for modulating the immunosuppressive effects of IL-10 in MΦ but not DCs, and therefore for the development of maximally effective DC-based vaccines, other (non-March-I) targets must be identified.

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