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Macrophages and Myeloid Dendritic Cells, but Not Plasmacytoid Dendritic Cells, Produce IL-10 in Response to MyD88- and TRIF-Dependent TLR Signals, and TLR-Independent Signals¹

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We have previously reported that mouse plasmacytoid dendritic cells (DC) produce high levels of IL-12p70, whereas bone marrow-derived myeloid DC and splenic DC produce substantially lower levels of this cytokine when activated with the TLR-9 ligand CpG. We now show that in response to CpG stimulation, high levels of IL-10 are secreted by macrophages, intermediate levels by myeloid DC, but no detectable IL-10 is secreted by plasmacytoid DC. MyD88-dependent TLR signals (TLR4, 7, 9 ligation), Toll/IL-1 receptor domain-containing adaptor-dependent TLR signals (TLR3, 4 ligation) as well as non-TLR signals (CD40 ligation) induced macrophages and myeloid DC to produce IL-10 in addition to proinflammatory cytokines. IL-12p70 expression in response to CpG was suppressed by endogenous IL-10 in macrophages, in myeloid DC, and to an even greater extent in splenic CD8 α^- and CD8 α^+ DC. Although plasmacytoid DC did not produce IL-10 upon stimulation, addition of this cytokine exogenously suppressed their production of IL-12, TNF, and IFN- α , showing *trans* but not autocrine regulation of these cytokines by IL-10 in plasmacytoid DC. *The Journal of Immunology*, 2006, 177: 7551–7558.

Microbial products activate the innate immune response and stimulate the production of cytokines, thereby determining the class of adaptive response mounted against a particular pathogen (1–4). Dendritic cells (DC)³ originate in the bone marrow (BM) and migrate through blood to secondary lymphoid organs and tissue. DC continuously sense pathogens and take up, process, and present Ag to T lymphocytes, thereby initiating the adaptive immune response (1–4). Macrophages play a role in the early phases of the immune response by producing cytokines and killing pathogens (5).

Th1 cell responses can be promoted through the activation of different DC subsets by a wide range of pathogens or their products, largely through the secretion of proinflammatory molecules such as bioactive IL-12p70 (consisting of the IL-12p40 and IL-

12p35 subunits) and in some cases type I IFN (6–16). Activation of DC and macrophages by pathogens involves the specific interaction between pattern recognition receptors, such as the members of the TLR family, and pathogen-derived products (17, 18). Upon TLR ligation, signaling cascades are activated via Toll/IL-1 receptor domain-containing adaptors, such as MyD88 and Toll/IL-1 receptor domain-containing adaptor (TRIF), leading to cytokine production. All TLR, except TLR3, use MyD88, whereas TRIF is involved only in TLR3 and TLR4 signaling, resulting after binding of their specific ligands, dsRNA (or poly(I:C)) and LPS, respectively (17). The TRIF adaptor has been shown to be responsible for a MyD88-independent signaling pathway giving rise to IFN- β (19–21). Distinct DC subpopulations in both mice and humans (reviewed in Ref. 2) have been shown to express different TLR, and consequently to respond to distinct microbial products (22–26). For example, TLR4 is expressed by macrophages, human monocyte-derived DC, and mouse myeloid DC, but not by plasmacytoid DC (22, 23). In contrast, TLR9, specific for unmethylated DNA containing CpG motifs, abundantly present in microbial genomes, is expressed by both human and mouse plasmacytoid DC (22, 23, 27). In contrast to the limited expression of TLR9 mRNA by human plasmacytoid DC and human B cells, in the mouse TLR9 mRNA is expressed not only by these cells but also by mouse macrophages, myeloid DC, and splenic CD8 α^+ and CD8 α^- DC (23). Collectively, these findings explain 1) the ability of mouse myeloid DC, macrophages, and splenic DC, but not plasmacytoid DC, to drive Th1 cell development in response to LPS (23, 28), and 2) the ability of plasmacytoid DC, and to a lesser extent myeloid DC, to respond to CpG and drive Th1 development, as we have previously shown (23).

Surprisingly, although splenic CD8 α^+ DC, CD8 α^- DC, and myeloid DC express similar levels of TLR9 mRNA, CpG stimulation induced only low levels of IL-12p70 (23). Thus, in addition

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³ Abbreviations used in this paper: DC, dendritic cell; pDC, plasmacytoid dendritic cell; BM, bone marrow; KO, knockout; TRIF, Toll/IL-1 receptor domain-containing adaptor.

to differential TLR expression, other factors may play a role in the production of bioactive IL-12p70 (29). For example, T cells have been shown to enhance the production of IL-12p70 by APCs through cell-cell contact involving at least the interaction of CD40L on activated T cells with CD40 on the APC (30–34). In contrast, negative regulation exerted by anti-inflammatory cytokines such as IL-10 has been shown to suppress IL-12p70 production by DC and macrophages, resulting in the inhibition of Th1 cell development (12, 35–37).

It has been suggested that the induction of the anti-inflammatory cytokine IL-10 after stimulation with lectins derived from pathogens can be mediated by a TLR-independent signaling pathway (38, 39). Furthermore, it has been suggested that TLR2 agonists are specialized to induce IL-10, as shown by stimulation with lipopeptides or the LcrV Ag of *Yersinia pestis* (40, 41).

In this study, we show that significant levels of IL-10 are secreted by macrophages and myeloid DC upon stimulation with MyD88- and TRIF-dependent TLR ligands, as well as non-TLR signals. Endogenous IL-10 suppressed IL-12p70 production by myeloid DC, macrophages, and splenic DC. In contrast, plasmacytoid DC did not produce IL-10 upon stimulation and showed unrestrained IL-12p70 production. Nevertheless, plasmacytoid DC were responsive to the inhibitory effects of exogenous IL-10.

Materials and Methods

Mice

BALB/c, C57BL/6, MyD88-deficient, and TRIF-deficient mice were used to provide macrophages and DC. Breeding pairs of MyD88-deficient and TRIF-deficient mice were provided by S. Akira (Osaka University, Osaka, Japan) (21, 42). All mice were bred at the National Institute for Medical Research (London, U.K.) and housed under specific pathogen-free conditions. Female mice were used between 8 and 12 wk of age.

Reagents and cell lines

Culture medium was RPMI 1640 with 5% heat-inactivated FCS, 0.05 mM 2-ME, 10 mM HEPES buffer, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate. DC were stimulated with *Salmonella minnesota* LPS (Alexis), poly(I:C) (InvivoGen Life Technologies), or phosphorothioate CpG DNA (CpG1668: TCCATGACGTTCC TGATGCT; InvivoGen Life Technologies). Mouse Flt3 ligand was produced at DNAX, and mouse GM-CSF was obtained from Schering Plough. mAb used for isolation of DC subsets were anti-B220-FITC, anti-CD11c-PE, anti-CD11b-allophycocyanin, anti-CD8α-allophycocyanin (all BD Pharmingen or eBioscience), or 120G8-Alexa⁴⁸⁸ (43). The anti-IL-10R mAb (1B1.3) was provided by DNAX. The cell lines 3T3-CD40L and 3T3-vector cell (control) were a gift from Dr. P. Hwu (National Cancer Institute, Bethesda, MD) and were derived from National Institutes of Health 3T3 cells by stable transduction with murine CD40L or empty vector.

Generation of BM-derived macrophages

BM-derived macrophages were generated in the presence of L cell-conditioned medium containing M-CSF as described by Warren et al. (44). Briefly, BM cells were isolated by flushing femurs and tibia with culture medium, and RBC lysed. Cells were plated at 0.5×10^6 cells/ml in petri dishes (Barloworld Scientific; volume, 8 ml). At day 4, 10 ml of fresh L cell-conditioned medium were added. At day 7, adherent cells were harvested by gentle flushing. The purity was always >95% as determined by staining for F4/80⁺ cells by flow cytometry.

Generation of BM-derived DC

BM cells were isolated by flushing femurs and tibia with culture medium. RBC were lysed using 0.83% ammonium chloride. GM-CSF-derived myeloid DC were generated as described by Inaba et al. (45). In brief, BM cells were plated at 10^6 cells/ml in medium supplemented with 10 ng/ml GM-CSF in 12-well plates in a volume of 2 ml. At days 2 and 4, supernatant containing nonadherent cells was removed, the wells were washed gently, and fresh medium containing GM-CSF (10 ng/ml) was added. At day 6, nonadherent cells were collected, centrifuged, resuspended in fresh

medium with GM-CSF (10 ng/ml), and cultured overnight in petri dishes (Nunc). Cells were purified by flow cytometry as CD11c⁺ cells using a MoFlo cytometer (DakoCytomation). Plasmacytoid DC were generated by culturing BM cells in culture medium containing 100 ng/ml Flt3 ligand for 10 days at 10^6 cells/ml in 12-well plates in a volume of 2 ml. At day 5, 1 ml of medium was replaced by 1 ml of fresh medium containing Flt3 ligand (46). The resulting plasmacytoid DC were purified by flow cytometry as CD11c⁺CD11b⁻B220⁺ using a MoFlo cytometer (DakoCytomation). The purity was always $\geq 98\%$.

Preparation of splenic DC subsets

For the purification of splenic DC, spleens were treated for 30 min at 37°C with 0.4 mg/ml Liberase Cl (Boehringer Mannheim), followed by RBC lysis as above. Cells were maintained throughout the procedure in cold PBS, 5% FCS, and 0.5 mM EDTA. Spleen cell suspensions were enriched for DC using anti-CD11c microbeads using an AutoMACS (Miltenyi Biotec) according to the manufacturer's instructions. The enriched DC were stained with CD11c-PE, CD8α-allophycocyanin, and 120G8-Alexa⁴⁸⁸ and purified using a MoFlo cytometer (DakoCytomation) as the CD11c⁺CD8α⁺, CD11c⁺CD8α⁻, and the CD11c^{du11}20G8⁺ plasmacytoid DC (43). The purity was consistently $\geq 98\%$.

In vitro stimulation of DC and macrophages, and quantitation of cytokine production

For in vitro stimulations, 10^5 sorted DC or macrophages were cultured in 200 µl in 96-well flat-bottom culture plates (Nunc) and stimulated with medium alone, LPS (100 ng/ml), poly(I:C) (50 µg/ml), or CpG1668 DNA (1 µM) either alone, or on a monolayer of CD40L-transfected 3T3 cells or control empty vector-transfected cells. To some cultures anti-IL-10R mAb (1B1.3; 10 µg/ml) or IL-10 (10 ng/ml) was added. After culture for 24 h, supernatants were collected, and the cytokine concentration was determined by immunoassay. Commercially available ELISA kits were used for the detection of IL-12p70, TNF, IL-10 (eBioscience; Ready-Set-Go), and IFN-β (PBL supplier). IFN-α was measured by a sandwich ELISA with an anti-IFN-α capture mAb (F18; Hycult), and a rabbit anti-IFN-α polyclonal Ab (PBL supplier) followed by goat anti-rabbit HRP (Sigma-Aldrich). IL-12p40 was detected using C15.6.7 as capture mAb and biotinylated C17.8 as detection mAb.

Statistical analysis

Data from multiple experiments were analyzed by comparison to a defined control value using Dunnett's test. Analysis was performed using GraphPad Prism software (GraphPad). In defined cases, pairwise comparison was by Student's paired *t* test. Values of $p < 0.05$ were considered significant.

Results

CpG induces IL-10 production from macrophages and myeloid DC, but not from plasmacytoid DC

Although macrophages, myeloid DC, splenic DC, and plasmacytoid DC all express TLR9 (23, 25) and respond to CpG to produce IL-12p40 and TNF, we show in this study that they differ substantially with respect to the production of IL-10 and IL-12p70 (Fig. 1). We postulated that this differential IL-10 production may account for reduced production of IL-12p70 by splenic DC, myeloid DC, and macrophages.

Macrophages produced high levels of IL-10 (2260 pg/ml) upon stimulation via TLR9, whereas no IL-12p70 was detected (Fig. 1). Myeloid DC also produced IL-10 upon CpG stimulation, albeit at lower levels than by macrophages, and only low levels of IL-12p70 were detected (~200 pg/ml). In contrast, plasmacytoid DC produced no detectable IL-10 upon TLR9 ligation with CpG, whereas high levels of IL-12p70 were produced in response to CpG (1080 and 1610 pg/ml for BM-derived and splenic plasmacytoid DC, respectively). The production of IL-12p40 and TNF did not mirror that of IL-10 or IL-12p70. The ratio of IL-12p40/IL-12p70 was not identical in macrophages and the different DC populations. TNF was produced at relatively high levels by macrophages, myeloid DC, and plasmacytoid DC, and at low levels by splenic CD11c⁺ DC (Fig. 1). A similar cell type-specific pattern of

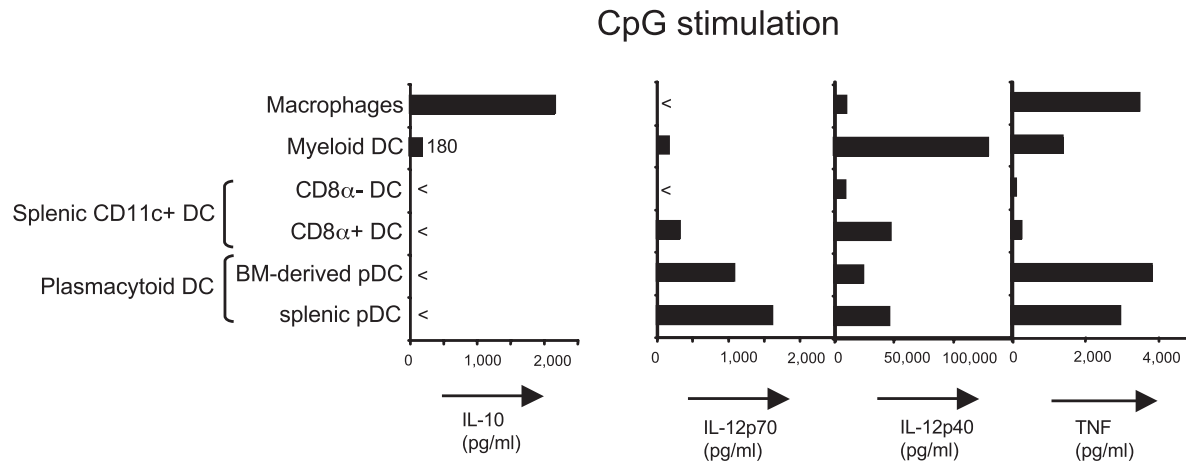


FIGURE 1. CpG induces high levels of IL-10 in macrophages, intermediate levels in myeloid DC, and no detectable IL-10 in plasmacytoid DC. Macrophages or sorted DC (10^5) obtained from BALB/c mice were stimulated in a volume of 200 μ l with 1 μ M CpG DNA for 24 h. The differences between IL-10 production by macrophages and myeloid DC, and between the other DC populations were significant ($p < 0.05$ by Dunnett's test). Cytokine levels were determined by immunoassay. The sensitivities of the ELISA were: IL-10, 50 pg/ml; IL-12p70, 50 pg/ml; IL-12p40, 1 ng/ml; and TNF, 100 pg/ml. Data are representative of four to eight experiments for each population.

IL-10, IL-12, and TNF production was observed upon stimulation with the TLR7 ligand R-848 (data not shown). Thus, macrophages, myeloid DC, and plasmacytoid DC have distinct intrinsic properties that affect their capacity to produce IL-10, IL-12p70, IL-12p40, and TNF that are not dictated by their TLR expression.

IL-10 production is induced by TLR ligands via MyD88-dependent and TRIF-dependent TLR signals in macrophages and myeloid DC

Although it is well documented that the production of proinflammatory cytokines upon TLR ligation is MyD88 dependent (21, 47, 48), it has been suggested that the production of the suppressive cytokine IL-10 is favored by usage of pathways involving triggering via C-type lectins, such as dectin-1 and DC-SIGN (38, 39), or TLR2 (40, 41). In this study, we show clearly that the induction of IL-10 by macrophages is entirely MyD88 dependent in response to CpG and partially MyD88-dependent in response to LPS, because IL-10 production by MyD88-deficient macrophages was absent or severely impaired upon stimulation with these TLR ligands (Fig. 2). IL-10 was also induced upon stimulation of wild-type macrophages with poly(I:C). Macrophages from TRIF knockout (KO) mice showed almost complete abrogation of IL-10 production induced by poly(I:C), whereas IL-10 production by MyD88 KO macrophages stimulated with poly(I:C) was not affected (Fig. 2). Residual IL-10 production in response to poly(I:C) in TRIF KO macrophages may be due to signaling via a TRIF-independent pathway using Mda-5, recently reported as inducing other proinflammatory cytokines (49). The production of IL-10 upon stimulation with LPS depended not only on MyD88 but also on TRIF-dependent signaling pathways. This indicates that IL-10 production in macrophages can be achieved via MyD88-dependent as well as TRIF-dependent TLR signals. Recently, it was shown that the production of IL-10 and IFN- β are both regulated by TNFR-associated factor 3, downstream of MyD88 and TRIF, implying coordinate expression (50). Indeed, similar to IL-10, the production of IFN- β by macrophages in response to CpG was MyD88 dependent and was completely dependent on TRIF signaling in response to poly(I:C). In contrast to IL-10 production, which is dependent on TRIF and MyD88 in response to LPS, IFN- β pro-

duction in macrophages upon LPS stimulation was completely dependent on TRIF and not MyD88 (Fig. 2).

Similar to macrophages, stimulation of myeloid DC with CpG and LPS induced production of IL-10 and was entirely MyD88 dependent in case of TLR9 triggering and dependent on MyD88 and TRIF upon TLR4 triggering (Fig. 2). In contrast to macrophages, myeloid DC did not or only weakly responded to poly(I:C) to produce IL-10, IL-12p40, IL-12p70, or TNF (Fig. 2 and data not shown).

IL-10 production is induced in macrophages, myeloid DC, and splenic DC subsets, but not in plasmacytoid DC upon CD40 ligation

TLR ligation of DC induces both cytokine production and the expression of costimulators like CD40, and differential regulation by T cell-derived signals, including CD40L, determines the different levels of IL-12p70 induced in different cell populations (31–33). Macrophages and DC were stimulated via CD40 using a CD40L-transfected cell line and a control empty vector-transfected cell line as a negative control. Interestingly, high levels of IL-10 were produced upon CD40 signaling in macrophages, myeloid DC, and splenic CD8 α^+ and CD8 α^- DC. IL-10 again was not secreted by plasmacytoid DC upon CD40 triggering (Fig. 3). In keeping with previous studies in DC (51) upon ligation of CD40 in the absence of TLR ligands, all mouse DC subsets analyzed produced significant levels of IL-12p70. This was not the case for macrophages (Fig. 3). Similar to the findings with TLR ligands, the ratio of IL-12p40/IL-12p70 was not identical in the different DC subsets, and TNF was detected only by CD40L-stimulated macrophages (Fig. 3).

In summary, stimulation of macrophages and myeloid DC resulted in IL-10 production in response to MyD88- and TRIF-dependent TLR ligands. These cells, and additionally splenic CD8 α^+ and CD8 α^- DC, produced IL-10 when stimulated via CD40 (Fig. 3). In contrast, no IL-10 was produced by plasmacytoid DC upon triggering of CD40, CpG, or CpG in the context of CD40 (data not shown), whereas IL-12p70 was secreted at very high levels by plasmacytoid DC upon ligation of TLR9 and/or CD40 (Figs. 1 and 3).

Macrophages

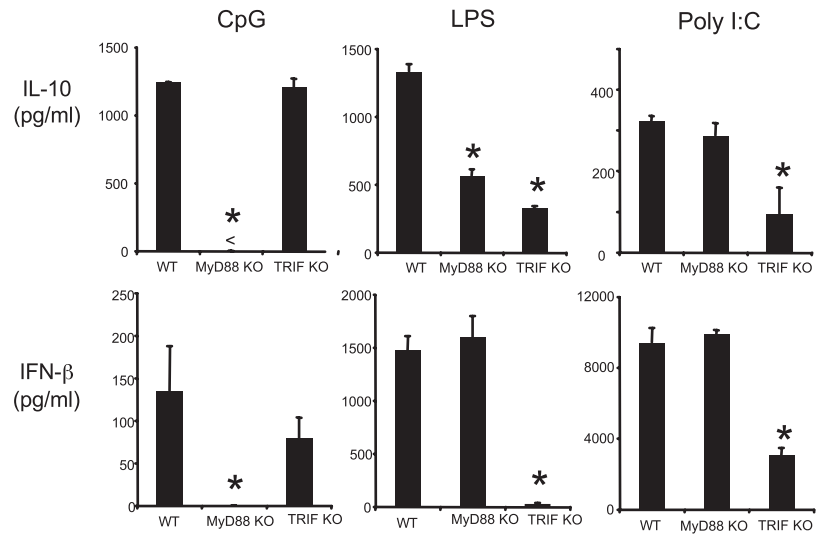
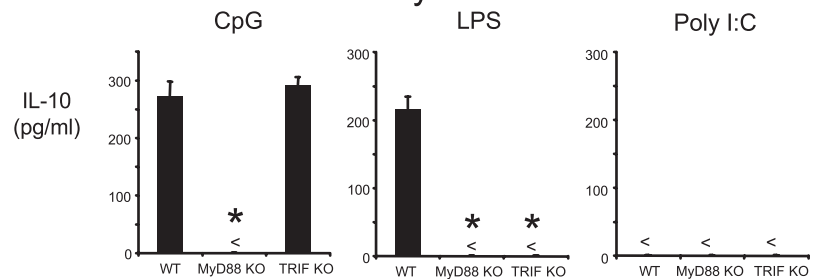


FIGURE 2. IL-10 production is induced by TLR ligands via MyD88- or TRIF-dependent pathways. BM-derived macrophages or myeloid DC (10^5) from wild-type (WT) C57BL/6, MyD88-deficient, and TRIF-deficient mice were stimulated in a volume of 200 μ l with 1 μ M CpG DNA, 100 ng/ml LPS, or 50 μ g/ml poly(I:C) for 24 h. Cytokine levels were determined by immunoassay. The sensitivities of the ELISA were 40 pg/ml for IL-10, and 15 pg/ml for IFN- β ELISA. Upon LPS stimulation, myeloid DC from MyD88-deficient mice produce \sim 500 pg/ml TNF, as compared 2500 pg/ml for wild-type myeloid DC (data not shown). Data are representative of three experiments. *, Statistical significance by *t* test, comparing cytokine responses by wild-type cells with KO cells ($p < 0.05$).

Myeloid DC



IL-12 production induced by simultaneous TLR and CD40 ligation is suppressed by endogenous IL-10 in macrophages, myeloid DC, and splenic CD8 α^+ and CD8 α^- DC populations, but not in plasmacytoid DC

To address the role of endogenous IL-10 in regulating cytokine production, macrophages, myeloid DC, splenic CD8 α^+ and CD8 α^- DC and plasmacytoid DC were stimulated with CpG and CD40L in the presence or absence of a neutralizing anti-IL-10R mAb.

IL-12p70 production upon CpG stimulation by macrophages was weakly enhanced when IL-10R signaling was blocked, but not

when triggered through CD40 alone (Fig. 4A). However, upon dual stimulation via TLR9 and CD40 in the absence of IL-10R signaling, substantial amounts of IL-12p70 were secreted by macrophages (780 pg/ml). Upon CpG stimulation, TNF and IL-12p40 production by macrophages was significantly enhanced upon neutralization of IL-10 activity, whereas CD40 triggering did not further increase TNF and IL-12p40 production (Fig. 4A and data not shown).

The low level of IL-12p70 induced in myeloid DC by CpG (128 pg/ml) was only slightly enhanced in the absence of endogenous

CD40L stimulation

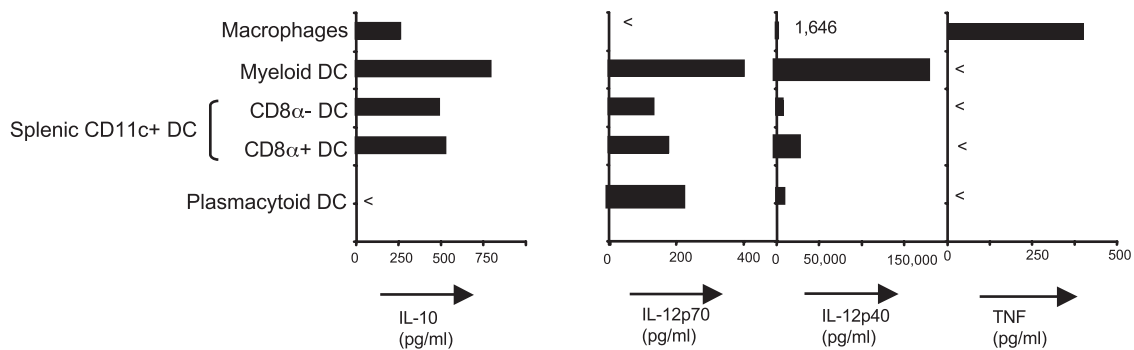


FIGURE 3. CD40 ligation of macrophages, myeloid DC, and splenic DC populations induces IL-10, whereas no IL-10 production is induced in plasmacytoid DC. Macrophages or DC (10^5) obtained from BALB/c mice were stimulated for 24 h with control-transfected or CD40L-transfected 3T3 cells in a volume of 200 μ l. IL-10, IL-12p40, IL-12p70, and TNF levels were determined by immunoassay. The lack of IL-10 production by plasmacytoid DC in response to CD40L stimulation was significantly different from IL-10 production by macrophages, myeloid DC, and splenic DC (Dunnett's test, $p < 0.05$). The sensitivities of the ELISAs were as described in the legend of Fig. 1. Data are representative of four to eight experiments for each DC population.

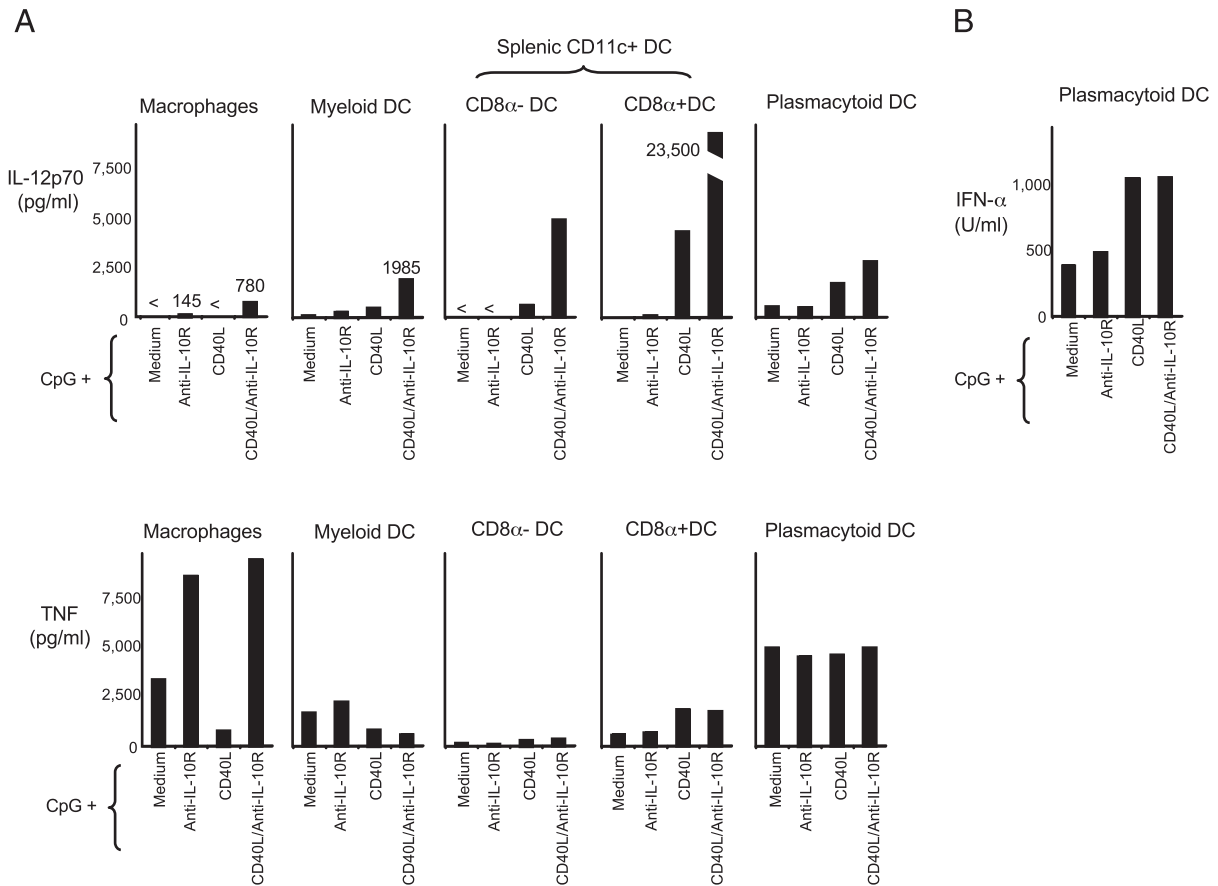


FIGURE 4. Upon ligation of TLR in the context of CD40 triggering, IL-10 profoundly suppresses IL-12p70 production by myeloid DC and splenic CD8 α^+ and CD8 α^- DC, but not or only weakly by plasmacytoid DC. Macrophages, myeloid DC, and splenic CD8 α^+ and CD8 α^- DC or plasmacytoid DC (10^5) were stimulated for 24 h in a volume of 200 μ l with medium, anti-IL-10R (10 μ g/ml), CD40L-transfected 3T3 cells, or CD40L-transfected 3T3 cells plus anti-IL-10R mAb in the presence of CpG. IL-12p70, TNF (A), and IFN- α (B) levels were determined by immunoassay. The sensitivity of the IFN- α ELISA is 50 U/ml. The sensitivity of the IL-12p70 and TNF ELISA is as described in the legend of Fig. 1. Significant increase of IL-12p70 was observed when comparing CpG/CD40L vs CpG/CD40L/anti-IL10R stimulation of myeloid DC, splenic CD8 α^+ DC and splenic CD8 α^- DC, but not macrophages or plasmacytoid DC ($p < 0.05$ by Dunnett's test). Data are representative of four to eight experiments for each population.

IL-10 signaling. Dual stimulation of myeloid DC with CpG plus CD40L resulted in augmentation of IL-12p70 production (496 pg/ml), compared with CD40L alone (181 pg/ml). Most significantly, however, when myeloid DC were stimulated with CpG and CD40L, abrogation of IL-10R signaling resulted in a high level of IL-12p70 production (1 or 2 ng/ml) (Fig. 4A). Significant enhancement of IL-12p70 production by myeloid DC when IL-10R signaling was abrogated was also observed upon TLR2 and TLR4 ligation together with costimulation through CD40 (data not shown).

Similarly, splenic CD8 α^+ and CD8 α^- DC produced substantially enhanced IL-12p70 when IL-10R signaling was neutralized upon triggering of TLR9 and CD40. This was most striking for CpG-stimulated splenic CD8 α^+ DC, which produced up to 23.5 ng/ml IL-12p70. It was consistently found that splenic CD8 α^+ DC produced higher levels of IL-12p70 than splenic CD8 α^- DC, which is in agreement with previous studies (51–53). However, CD8 α^- DC produce significant levels of IL-12p70 (~3000 pg/ml) when stimulated with CpG and CD40L upon neutralization of endogenous IL-10 activity. Although blocking IL-10R signaling had such a striking effect on the induction of IL-12p70 from myeloid DC and splenic CD8 α^+ and CD8 α^- DC, the effect on TNF levels was less pronounced in contrast to the effect on macrophages. Furthermore, myeloid DC did not respond to CD40 ligation to the

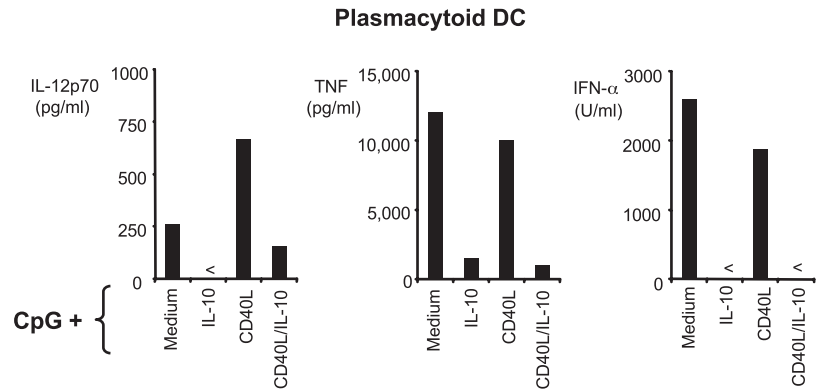
extent that splenic CD8 α^+ and CD8 α^- DC did, suggesting that the latter may be more dependent on T cell derived signals.

Plasmacytoid DC stimulated with CpG alone produced significant amounts of IL-12p70 (571 pg/ml), which was even further enhanced when simultaneously stimulated via CD40 (Fig. 4A). However, neutralization of IL-10R signaling had little effect on IL-12p70 and no effect on TNF production upon dual activation of plasmacytoid DC with CpG plus CD40L in contrast to the effects on myeloid DC and splenic CD8 α^+ and CD8 α^- DC. Similarly, CpG-induced IFN- α and IL-12p40 production by plasmacytoid DC was augmented by additional CD40 triggering, but, like IL-12p70 and TNF production, was not enhanced by blocking IL-10R signaling (Fig. 4B and data not shown).

Exogenous IL-10 suppresses the induction of proinflammatory cytokines by plasmacytoid DC

The absence of endogenous IL-10 production by plasmacytoid DC does not exclude that IL-10 produced by other cells can regulate IL-12p70 production by plasmacytoid DC. Addition of exogenous IL-10 to plasmacytoid DC showed suppression of CpG- and CpG/CD40L-induced production of IL-12p70, TNF, and IFN- α (Fig. 5). This indicates that although plasmacytoid DC do not produce

FIGURE 5. Exogenous IL-10 suppresses CpG-induced cytokine production by plasmacytoid DC. Splenic plasmacytoid DC (10^5) were stimulated for 24 h in a volume of 200 μ l with medium, IL-10 (10 ng/ml), CD40L-transfected 3T3 cells, or CD40L-transfected 3T3 cells plus IL-10 in the presence of CpG. IL-12p70, TNF, and IFN- α levels were determined by immunoassay. The sensitivity of the ELISA is as described in the legends of Figs. 1 and 4. Data are representative of two experiments.



IL-10 and are thus not subject to autocrine regulation of proinflammatory cytokines by this suppressive cytokine, they are responsive to IL-10 produced by other cells in their microenvironment.

Discussion

The present study shows that macrophages, myeloid DC and plasmacytoid DC have a differential capacity to produce IL-10 and IL-12 in response to TLR signaling. TLR-mediated MyD88-dependent and TRIF-dependent as well as non-TLR signals induce high levels of IL-10 by macrophages and intermediate levels by myeloid DC, whereas no detectable IL-10 is produced by plasmacytoid DC. Furthermore, we show that the differential IL-10 production partly explains the superior capacity of plasmacytoid DC to produce high levels of IL-12p70 as compared with macrophages or myeloid DC.

We observed striking differences in the cytokine profile of macrophages, myeloid DC, and splenic DC. CpG stimulation of macrophages resulted in high levels of IL-10, undetectable IL-12p70, relatively low IL-12p40, and relatively high levels of TNF. Myeloid DC produced intermediate levels of IL-10, low IL-12p70, very high levels of IL-12p40, and moderate amounts of TNF. In contrast, splenic DC produced moderate amounts of IL-12p40, but undetectable or very low amounts of IL-10, IL-12p70, and TNF. The limited ability of splenic DC to produce cytokines upon CpG stimulation was overcome by additional triggering via CD40, which resulted in substantial IL-12p70, IL-12p40, and TNF production, which surprisingly also induced IL-10. Interestingly, the synergy between TLR and CD40 triggering was much stronger for splenic DC than for macrophages and myeloid DC, showing their strong dependence on T cell-derived factors to induce cytokine production. Endogenous IL-10 was a crucial factor that strongly suppressed IL-12p70 production by macrophages, myeloid DC, and splenic CD8 α^+ and CD8 α^- DC upon CpG and CD40 ligation. With respect to IL-12p70 production, splenic DC, and especially splenic CD8 α^+ DC, were again most highly regulated by endogenous IL-10. In good agreement with this, it was reported that neutralization of IL-10, induced by *Schizosaccharomyces pombe* in splenic DC, resulted in enhanced Th1 cell development in vitro (54). Moreover, we previously showed in vivo that Ag-specific Th1 responses in the context of TLR ligation are suppressed by IL-10, and strong Th1 responses in vivo, induced by TLR ligation, are observed only when IL-10R signaling is abrogated (55, 56).

IL-10 production by macrophages was induced via TLR mediated MyD88- or TRIF-dependent pathways, as well as via non-TLR signals. Triggering through TLR9 (and TLR7; data not shown) showed that the induction of high levels of IL-10 was completely MyD88 dependent in macrophages and myeloid DC (Fig. 2); this was also the case for induction of IFN- β (data not

shown). Conversely, IL-10 induction via the TLR3 ligand poly(I:C) was dependent on TRIF and not MyD88 in macrophages (Fig. 2), which was also the case for the induction IFN- β (Fig. 2). LPS induction of IL-10 via TLR4 was dependent on MyD88 and TRIF (Fig. 2), contrasting with IFN- β induction by LPS, which was dependent only on TRIF (Fig. 2). Recently, it was shown that TNFR-associated factor 3, an adaptor molecule downstream of MyD88 and TRIF, regulates the production of both IL-10 and type I IFN, which is in keeping with some of our findings (50). However, we show in this study that whereas MyD88 and TRIF regulate the production of IL-10 by macrophages in response to LPS, the production of IFN- β via TLR4 is dependent on TRIF and not MyD88, suggesting that there may be independent signaling mechanisms regulating the production of IL-10 and IFN- β . Besides stimulation via TLR, CD40 triggering also resulted in IL-10 production. This indicates that induction of IL-10 can be achieved by a broad spectrum of stimuli including multiple TLR ligands, and not, as previously implied, preferentially as a result of ligation of TLR2- and MyD88-independent signals (38–41, 57). Thus, although it has been shown that triggering with pathogens of the C-type lectin DC-SIGN can induce IL-10 (38) and that dectin-1 stimulation with zymosan induces IL-10 via syk signaling (39), we show in this study that these are not exclusive signals to induce the expression of IL-10 in macrophages or DC.

We show that plasmacytoid DC do not produce IL-10 and display unrestrained IL-12p70 production upon TLR ligation, which is in keeping with their ability to induce strong Th1 development upon CpG stimulation in vitro (23). In addition, plasmacytoid DC produce relatively high levels of TNF and IFN- α upon CpG stimulation alone. Additional triggering via CD40 further enhances their production of IL-12p70, IL-12p40, and IFN- α , but not TNF.

Mouse plasmacytoid DC, however, are responsive to the inhibitory effect of IL-10 on cytokine production, as are human IFN-producing cells or plasmacytoid DC (58, 59), indicating that other IL-10-producing cells in their microenvironment may suppress their activity if colocalized. Plasmacytoid DC clusters, 6 h after CpG treatment in vivo, are mostly present in the marginal zone, separated from T cells and conventional DC (60). At later time points, plasmacytoid DC partially colocalize with T cells and conventional DC in the T cell area of the spleen. The specific localization of DC during the inflammatory response may be important for IL-10, produced by myeloid DC and/or macrophages, to act in *trans* on plasmacytoid DC, resulting in restrained IL-12p70 production and consequently suboptimal Th1 responses.

The differential production of IL-10 suggests that macrophages, myeloid DC, and plasmacytoid DC are intrinsically different and may possess distinct intracellular signaling mechanisms to activate different sets of cytokine genes. An alternative explanation could

be that plasmacytoid DC produce other endogenous cytokines such as IFN- α or IFN- γ , which may suppress IL-10 production by plasmacytoid DC. Interestingly, although endogenous IL-10 suppresses IL-12p70 production by macrophages, myeloid DC, and splenic DC, the suppressive effect of IL-10 on TNF production is cell type specific. In this, the induction of TNF by macrophages is highly suppressed by endogenous IL-10, but TNF production by myeloid and splenic DC does not appear to be regulated by endogenous IL-10. The underlying mechanisms for this are currently being studied.

Triggering via TLR induces not only IL-12, thereby promoting Th1 immunity, but also IL-10 that counteracts the effects of IL-12 and suppresses the development of an immune response to pathogens. By doing so, IL-10 prevents excessive activation of the immune response, which limits immune pathology, but alternatively may prevent the complete eradication of pathogens by inhibiting Th1 immunity. Our findings that macrophages and DC have cell-intrinsic properties controlling their production of IL-10, which subsequently affects their production of IL-12, provide important insight into the mechanisms underlying the complex balance of IL-10 vs IL-12 induction, which is fundamental for the understanding of the dynamics of the immune response to pathogens.

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Disclosures

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