Plasmacytoid dendritic cells have a cytokine-producing capacity to enhance ICOS ligand-mediated IL-10 production during T-cell priming

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Abstract

Plasmacytoid dendritic cells (pDCs) have the potential to prime CD4+ T-cells to differentiate into IL-10-producing T regulatory cells through preferential expression of inducible co-stimulatory ligand (ICOS-L). Although pDCs produce cytokines such as type-I IFNs, TNF-α, or IL-6 accompanying up-regulation of ICOS-L expression during activation in response to toll-like receptor (TLR)-ligands or IL-3, the roles of the pDC-derived cytokines in T-cell priming remain largely elusive. Therefore, we investigated the functional involvement of these cytokines in generating IL-10-producing T regulatory cells. We found that either IFN-α or IL-6 enhanced the pDC- or ICOS-L-driven generation of IL-10-producing T-cells from naive CD4+ T-cells and their regulatory functions. However, IFN-α stimulation in the absence of ICOS-L showed only a marginal tendency to increase the T-cell production of IL-10 and thus pDC-derived type-I IFNs in response to CpG could function together with ICOS-L. In addition, IL-6 functioned to generate IL-10-producing T-cells only on T-cell priming by pDCs activated by IL-3 or under IL-4-mediated Th2 conditions. Thus, type-I IFNs and IL-6 act as supplementary factors for the ICOS-L-dependent IL-10-producing T-cell differentiation in pDCs activated along the TLR-dependent and IL-3-dependent pathways, respectively. We also showed that pDC-derived TNF-α induced ICOS-L expression on pDCs in an autocrine manner and that IL-6 promoted ICOS expression on T-cells, contributing to the ICOS/ICOS-L-mediated T-cell response. Our results suggest that the ICOS-L-mediated tolerogenic pDC function in adaptive immunity is backed up by the elaborate cytokine-producing ability of pDCs.

Keywords: adaptive immune response, IFN-α, IL-6, TNF-α

Introduction

In general, recognition of microbial pathogens triggers immature dendritic cells (DCs) to develop into mature DCs as antigen-presenting cells (APCs) to initiate adaptive immune responses. In humans, CD11c+ myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) represent two major subsets of DCs. They play distinct roles in innate and adaptive immune responses by the expression of their specialized cytokines and molecules (1). In innate immunity, pDCs behave as type-I IFN-producers due to their ability to produce robust amounts of type-I IFNs, essential cytokines in anti-viral immunity, through the selective expression of toll-like receptor 7 (TLR7) and TLR9, which, respectively, recognize viral RNA and DNA (2–4). Indeed, in response to viruses or unmethylated cytosine phosphate guanosine (CpG)-DNA, pDCs can produce a wide repertoire of type-I IFNs including 13 subtypes of IFN-α, IFN-β, IFN-ω and three subtypes of IFN-λ and IFN-τ (2), suggesting that pDCs dedicate a large proportion of their transcription machinery to making type-I IFNs and thereby play a key role in anti-viral immune responses. In contrast to the capacity to produce the wide varieties of type-I IFNs, pDCs have only a limited ability to produce other cytokines, such as moderate amounts of TNF-α and IL-6, but not IL-1α, IL-1β, IL-3, IL-10, IL-12, IL-15, IL-18, IFN-γ, lymphotixin-α or granulocyte-macrophage colony-stimulating factor (GM-CSF) proteins following...
viral stimulation (5). pDC-derived TNF-α not only contributes to the common inflammatory response but also accelerates the maturation of pDCs themselves in an autocrine manner (5). Meanwhile, IL-6 secretion by pDCs mediates plasma-cell differentiation and immunoglobulin secretion in cooperation with the type-I IFN secretion (6), contributing to anti-viral humoral immune responses. Thus, the pDCs also act as immune modulators by their cytokine secretion in the innate response phase.

pDCs can switch their functional properties following the innate response phase from cytokine producers to mature DCs as important initiators or controllers of adaptive immunity by priming naive CD4+ T-cells (5, 7). Although mDCs show functional plasticity in determining the quality of T-cell responses depending on the type of maturational signals (4), a series of recent studies have suggested that pDCs have a tolerogenic function in the adaptive immunity; pDCs suppress inflammatory responses against pathogens (8), allergens (9) or tumors (10, 11) and promote oral tolerance (12), and engraftment of hematopoietic stem cells (13) or vascularized grafts (14). Signaling through TLR7/9 by viruses or synthetic CpG-ODN can trigger immature pDCs to rapidly develop mature APCs (15, 16), which instruct human naïve CD4+ allogeneic T-cells to differentiate into T regulatory type 1 (Tr1) cells that secrete high levels of IL-10 and IFN-γ (5, 17). Meanwhile, IL-3 can stimulate pDCs to develop into mature DCs, which preferentially drive conventional T2 cells to produce IL-4, IL-5, IL-13 and IL-10 (5, 7, 18). Although the biological significance of pDC differentiation in the presence of IL-3 is still not clear, it is possible that basophils, eosinophils and mast-cells produce IL-3 during parasite presence of IL-3 is still not clear, it is possible that basophils, eosinophils and mast-cells produce IL-3 during parasite presence of IL-3 suggests that basophils, eosinophils and mast-cells produce IL-3 during parasite presence of IL-3 is still not clear, it is possible that basophils, eosinophils and mast-cells produce IL-3 during parasite presence of IL-3.

Both types of effector CD4+ T-cells induced by the pDCs activated through both TLR- and IL-3-dependent pathways contain a T-cell population producing IL-10 as a global inhibitor of immune cell functions, indicating that the intrinsic machinery in pDCs can generate IL-10-producing T-cells with regulatory function. It has recently been demonstrated that this is owing to a member of the B7 family inducible co-stimulatory ligand (ICOS-L) expression on pDCs, which has been implicated in both induction of IL-10 production in T-cells and T regulatory (Treg) cell-mediated peripheral tolerance (19–22). Human mDCs have the ability to produce large amounts of IL-12, which is an decisive factor for T1 cell differentiation, whereas human pDCs, which have less capacity to produce IL-12 (2), instead have a unique functional property to rapidly up-regulate ICOS-L expression in response to either IL-3 or TLR-ligands (23). Thus, in pDC-mediated CD4+ T-cell priming, the key molecule dictating tolerogenic adaptive immune response is ICOS-L and the mature pDCs use ICOS-L to differentiate T-cells into IL-10-producing Treg cells.

Despite the above, it is still unclear whether the pDC-derived cytokines play a role in the adaptive response phase to differentiate IL-10-producing CD4+ T-cells through ICOS-L. Here, we investigated the role of IFN-α, IL-6 or TNF-α in the ICOS-L-mediated IL-10 production during T-cell priming to better understand the functional basis of pDCs in adaptive immunity. We showed that either IFN-α or IL-6 functions directly on T-cells to support the ICOS-L-mediated generation of IL-10-producing Treg cells and that TNF-α up-regulates ICOS-L expression on pDCs in an autocrine manner, indicating a synergistic role for supporting the ICOS-L-mediated adaptive immune response of pDCs. Thus, pDCs have a cytokine-producing capacity linking to the tolerogenic DC functions in adaptive immunity.

Methods

Media and reagents

RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 ng/ml streptomycin and heat-inactivated 10% fetal bovine serum (Biosource International) was used for cell cultures throughout the experiments. For human cell stimulation, we used the following reagents for cell stimulation: 5 µM CpG-ODNs 2216 (Invivogen), 10 ng/ml IL-3 (R&D Systems), 20 ng/ml IL-6 (R&D Systems), 10 ng/ml TNF-α (R&D Systems), 500 IU/ml IFN-αβ (PBL Biomedical Laboratories), 10 ng/ml IL-12 (R&D Systems), 25 ng/ml IL-4 (R&D Systems) and 1 µg/ml R848 (Invivogen). For CD40 stimulation, we used irradiated CD40L-transfected L cells as previously described (7). We used the following reagents for culture conditions: neutralizing anti-ICOS-L mAb (eBioscience 50 µg/ml), anti-IL-6 mAb (R&D Systems: 5 µg/ml), anti-TNF-α Ab (R&D Systems: 1 µg/ml), a mixture of neutralizing anti-human IFN-α Ab (2000 neutralization units/ml), neutralizing anti-human IFN-β Ab (1000 neutralization units/ml), anti-human IFN-α/β R mAb (20 µg/ml; PBL Biomedical Laboratories) and a mixture of neutralizing anti-IL-10 mAb (200 ng/ml) plus anti-IL-10R Ab (10 ng/ml) (R&D Systems). Mouse IgG2a and Goat IgG (R&D Systems) and PBS were used as controls.

Isolation and culture of blood DCs

Human blood DCs were isolated from healthy adult volunteers as described (24). Briefly, the DC-enriched population (CD14+/CD3-/CD14+ cells) was obtained from PBMCs by negative and subsequent positive immunoselections. The CD11c+/lin−/BDCA4+/CD4+ fraction as pDCs and CD11c−/lin−/BDCA4−/CD4− as mDCs were sorted by a FACS Aria® (BD Biosciences) using PE-labelled or allophycocyanin (APC)-labelled anti-CD11c (BD Biosciences), APC-labelled or PE-labelled anti-CD304 (BDCA-4/Neuropilin-1) (Miltenyi Biotec), a mixture of FITC-labelled mAbs against lineage markers, CD3 (M2AB: Exalpha), CD14 (M5E2: BD Biosciences), CD15 (M5E2: BD Biosciences), CD16 (J5511: Exalpha), CD19 (HIB19: BD Biosciences) and CD56 (NCAM16.2: BD Biosciences), and PE-Cy5.5-labelled anti-CD4 (CALTAG) to reach >99% purity. Cells were seeded and stimulated with CpG, IL-3 plus pre-coated CD40L-transfected L cells, or R848 in flat-bottomed 96-well plates at 5 × 10^6 cells in 200 µl of medium/well for 24h.

Analyses of DCs

To analyse the expression of surface ICOS-L, the DCs were stained with PE-labelled anti-ICOS-L mAbs (eBioscience) or isotype-matched control mAbs and then analysed by a FACScalibur® (BD Biosciences). The production of cytokines in the culture supernatants after 24 h was determined.
CD4+ naive T-cells (purity >99%) were isolated from PBMCs using the CD4+ T-cell Isolation Kit II (Miltenyi Biotec) followed by cell sorting (CD4+CD45RA+CD45RO– fraction as naive T-cells) by staining with FITC-labelled lineage cocktail (CD8, CD14, CD16, CD19, CD56, BDCA2, TCRI/β and Glycophorin A), PE-labelled CD45RO, PE-Cy5.5-labelled anti-CD4 and APC-labelled anti CD45RA (BD Biosciences). Maturing DCs after culture with different stimuli for 24 h were collected and then co-cultured with 2×10^5 freshly purified allogeneic naive CD4+ T-cells (DC to T ratio, 1:4) in 96-well round-bottomed culture plates in the presence or absence of the neutralizing Abs.

Analyses of T-cell cytokine production and ICOS expression

After 7 days of primary DC–T-cell co-culture, the primed CD4+ T-cells were collected and washed. For detection of cytokine production in the culture supernatants, the T-cells were re-stimulated with immobilized anti-CD3 (OKT3, 5 µg/ml) and soluble anti-CD28 (1 µg/ml). In some experiments, recombinant IL-6, TNF-α or IFN-γ was added in the T-cell cultures, or recombinant IL-12 and IL-4 were added for the T₁,₁ and T₂,₂ conditions, respectively, in the presence or absence of IL-6.

For analysis of ability to produce cytokines, purified CD4+ T-cells were cultured for 7 days in the presence of IL-2 (R&D Systems: 50 U/ml) on irradiated CD32/ICOS-L cells (23, 25) or parental CD32-L cells, which had been pre-coated with anti-CD3 mAb (OKT3, 0.2 µg/ml) in 48-well culture plates (T-cell-to-L-cell ratio 2.5:1). For analysis of surface ICOS expression, purified naive CD4+ T-cells were cultured for 4 days in the presence of IL-2 on immobilized anti-CD3 (OKT3, 5 µg/ml) and soluble anti-CD28 (1 µg/ml). In some experiments, recombinant IL-6, TNF-α or IFN-γ was added in the T-cell cultures, or recombinant IL-12 and IL-4 were added for the T₁,₁ and T₂,₂ conditions, respectively, in the presence or absence of IL-6.

Results

pDCs have the ability to induce ICOS-L expression and to generate IL-10-producing T-cells regardless of the activation pathways

To investigate the characteristics of functional pDC plasticity induced by activation pathways, we first compared the differences between pDCs in response to the two types of stimuli: TLR-mediated and IL-3-mediated activation. As previously reported (2, 3, 15, 16), we here investigated that in response to CpG-ODN, but not IL-3 plus CD40L, pDCs produced a vast amount of IFN-α, whereas mDCs produced bioactive IL-12 p70 but not IFN-α in response to R848 (Fig. 1A). All stimuli we tested induced both TNF-α and IL-6 production comparably from both pDCs and mDCs. We also confirmed that pDCs appear to have the intrinsic ability to up-regulate ICOS-L with both activation pathways (23), as shown by the flow cytometry data indicating that, in response to CpG or to IL-3/CD40L, maturing pDCs rapidly expressed ICOS-L during 24 h but maturing mDCs generated by R848 could not up-regulate ICOS-L (Fig. 1B). The R848-stimulated mDCs (R848-mDCs) primed allogeneic naive CD4+ T-cells to secrete very high levels of IFN-γ with no IL-4 and less IL-10, showing a T₁,₁-like response (Fig. 1C). By contrast, T-cells primed by CpG-stimulated pDCs (CpG-pDCs) simultaneously produced moderate amounts of IFN-γ and low amounts of IL-4, whereas T-cells primed by IL-3/CD40L-stimulated pDCs (IL-3-pDCs) produced large amounts of IL-4 but low amounts of IFN-γ. Notably, both types of maturing pDCs induced naive T-cells to produce a large amount of IL-10 (Fig. 1C). Further analysis with intracellular single-cell staining showed that CpG-pDCs generated IL-10-producing T-cells that co-expressed IFN-γ and that IL-3-pDCs induced the IL-10-expressing T-cells without IFN-γ expression (Fig. 1D). These cytokine profiles are consistent with Th1-like and T₂,₂-like responses, respectively, as previously reported (5, 26). Regardless of the functional plasticity of pDCs in determining the quality of T-cell responses, both types of maturing pDCs have the potential to induce the generation of IL-10-producing T-cells. Moreover, pDCs activated along the TLR-dependent pathway could provide an IFN-α-rich milieu, and pDCs activated along the IL-3-dependent pathway could provide a T₁,₁ milieu in the T-cell priming phase.
pDC-derived type-I IFNs induced by CpG promote the ICOS-L-driven T-cell production of IL-10

ICOS-L has been implicated in both induction of IL-10 production in T-cells and Treg cell-mediated peripheral tolerance (19–22). Therefore, to assess the roles of pDC-derived cytokines in the priming phase of T-cell responses through the ICOS-L expressed on pDCs, we first tested the functional involvement of type-I IFNs in the generation of...
Fig. 2. pDC-derived ICOS-L and type-I IFNs play a synergistic role for the generation of IL-10-producing T-cells. (A, B) Naive CD4+ T-cells were cultured for 7 days with pDCs pre-treated for 24 h with CpG-ODN (CpG-pDCs) in the presence of neutralizing anti-ICOS-L mAb, a mixture of pDC-derived cytokines support ICOS-L function.
IL-10-producing T-cells by the CpG-pDCs. We blocked the effects of type-I IFNs, as well as ICOS co-stimulation, using neutralizing Abs in the primary culture of CpG-pDCs with naive T-cells. A blockade of ICOS-L considerably inhibited the generation of IL-10-producing cells but did not have substantial effects on the IFN-γ production from T-cells primed by CpG-pDCs (Fig. 2A and B). Neutralizing type-I IFNs led to a strongly decreased production of IFN-γ and a concomitantly moderately decreased IL-10 production. Anti-type-I IFN Abs plus anti-ICOS-L mAb showed an additive effect on IL-10 production but not IFN-γ production. To further determine the potential of type-I IFNs in the induction of ICOS-L-dependent T-cell production of IL-10 by another way, we cultured naive CD4+ T-cells with anti-CD3 mAb in the presence of ICOS-L-transfected L cells with or without IFN-α. Although ICOS-L strongly primed naive T-cells to produce IL-10 but not IFN-γ, as previously reported (23), IFN-α exerted only a modest effect on the IL-10 production and significantly promoted IFN-γ production by T-cells (Fig. 2C). It is of note that the addition of IFN-α plus ICOS-L dramatically enhanced the ICOS-L-mediated production of IL-10. Intracellular single-cell staining with IFN-10 and IFN-γ showed that, although ICOS-L promoted the frequency of IL-10-single expressing T-cells, the addition of IFN-α further augmented the frequency of the ICOS-L-induced IL-10-expressing cells and endowed these cells with IFN-γ co-expression, leading to a Tr1-like expression profile (Fig. 2D). As might be inferred from our data, type-I IFNs most likely could function to generate IL-10-producing T-cells when ICOS-L is present.

**IL-6 functions to enhance the ICOS-L-dependent IL-10 production by T-cells primed by T₂ conditions or by IL-3-stimulated pDCs**

We next determined the potential involvement of pDC-derived IL-6 in the generation of IL-10-producing T-cells induced by the two types of maturing pDCs. A neutralizing mAb to ICOS-L significantly inhibited the ability of IL-3-pDC-primed T-cells to produce IL-10 (Fig. 3A), as described in a previous report (23), and anti-IL-6 mAb did this to a lesser, but significant, extent. The blocking of IL-6 concomitantly led to an increased production of IFN-γ, but the blocking of ICOS-L did not. These findings suggest that in the context of T-cell priming by IL-3-pDCs, IL-6 might contribute to the generation of IL-10-producing T-cells within T₂-type immune responses. This finding was also confirmed by the intracellular single-cell staining with IL-10 and IFN-γ (Fig. 3B). However, the blocking of IL-6 did not affect the production of IL-10 nor IFN-γ in the T-cell priming by CpG-pDCs (Fig. 3A). To further focus on the effect of IL-6 on the T-cell production of IL-10 in the ICOS-L-dependent pathway, we stimulated naive CD4+ T-cells using ICOS-L-transfected L cells with anti-CD3 mAb in the presence or absence of IL-6 in the context of T₁,1 and T₂ polarization. Interestingly, IL-6 functioned to promote T-cell production of IL-10 under the T₂-polarizing condition induced by IL-4 but not under the T₁,1-polarizing condition induced by IL-12 (Fig. 3C). Meanwhile, ICOS-L permitted the T-cells to significantly promote IL-10 production under either T₁,1- or T₂-polarizing conditions. Importantly, under the T₂-polarizing condition, the addition of IL-6 enhanced the increased IL-10 production in the T-cells activated along the ICOS-L-dependent pathway. Intracellular cytokine staining also showed that the addition of IL-6 increased the frequency with which cells produced IL-10 without IFN-γ under the T₂-polarizing condition even in the presence of ICOS-L (Fig. 3D). The T₁,1-polarizing condition induced a small fraction of IL-10- and IFN-γ-double expressing T-cells. ICOS-L increased the frequency of these double-producing cells, whereas IL-6 failed. Our results are consistent with the previous report showing that IL-6 can function to induce T-cells to produce IL-10 in the T₂,2 but not T₁,1 environment (27). In this context, our results provide a plausible explanation for how pDC-derived IL-6 might play a role in the generation of IL-10-producing T-cells in the priming of IL-3-pDCs, which make the T₂ milieu.

**TNF-α functions as an autocrine enhancer of ICOS-L expression in pDCs and IL-6 promotes ICOS expression on T-cells**

To assess the molecular basis of how pDC-derived cytokines enhance the ICOS-L-driven induction of IL-10-producing T-cells, we next tested the participation of IFN-α, IL-6 or TNF-α in the ICOS-L expression in pDCs as an autocrine effect and in the ICOS expression on T-cells as a paracrine effect by flow cytometry. TNF-α is known as a classical maturation-inducing factor of DCS and is produced either by pDCs in response to IL-3 plus CD40L or CpG, or by mDCs (Fig. 1A and ref. 2). Indeed, it functions as an autocrine enhancer of pDC maturation (5) by up-regulation of B7 costimulatory molecules such as CD80 and CD86. In the culture of pDCs, the addition of neutralizing anti-TNF-α Ab inhibited the ICOS-L up-regulation on pDCs when stimulated by IL-3 plus CD40L or CpG (Fig. 4A). However, neither anti-IFNs Abs nor anti-IL-6 mAb affected the ICOS-L expression during pDC maturation. These findings suggest that endogenous TNF-α, but not type-I IFNs nor IL-6 from pDCs, is an autocrine inducer of ICOS-L. To confirm these findings, we stimulated pDCs with recombinant IFN-α, which acts as a survival-maintaining factor of pDCs but is ineffective in inducing pDC maturation (5, 28). Although recombinant IFN-α alone or recombinant IL-6 plus IFN-α failed to up-regulate ICOS-L expression on pDCs, recombinant TNF-α plus IFN-α up-regulated its expression. These data indicate that pDC-derived TNF-α contributes to the induction of ICOS-L expression in an autocrine manner.
Fig. 3. pDC-derived ICOS-L and IL-6 play a synergistic role for the generation of IL-10-producing T-cells under Th2 conditions. (A, B) Naive CD4+ T-cells were cultured for 7 days with pDCs pre-treated for 24 h with CpG-ODN (CpG-pDCs) or with IL-3 plus CD40L (IL-3-pDCs) in the presence of neutralizing anti-ICOS-L mAb, anti-IL-6 mAb or control mAb. (C, D) Naive CD4+ T-cells were cultured for 7 days in the presence or absence of pDC-derived cytokines and ICOS-L support ICOS-L function.
Next, to assess the effect of pDC-derived cytokines on the ICOS expression on T-cells, we cultured naive CD4+ T-cells for 4 days in the presence or absence of the indicated recombinant cytokine on immobilized anti-CD3 and soluble anti-CD28. ICOS-L expression on DCs and ICOS expression on T-cells were analysed by flow cytometry. The staining profiles of the indicated mAb and isotype-matched control are indicated by shaded and open areas, respectively. Similar results were observed in four independent experiments and the results of a representative experiment are shown. rIFN-α; recombinant IFN-α, rTNF-α; recombinant TNF-α, rIL-6; recombinant IL-6, αIFNs; anti-type-I IFNs Abs, αTNF; anti-TNF-α mAb, αIL-6; anti-IL-6 mAb.

Fig. 4. pDC-derived TNF-α and IL-6 contribute to the up-regulation of ICOS-L expression on pDCs and ICOS expression on T-cells, respectively. (A) pDCs were cultured with the indicated stimuli for 24 h in the presence or absence of the indicated neutralizing antibodies. (B) Naive CD4+ T-cells were cultured for 4 days in the presence or absence of the indicated recombinant cytokine on immobilized anti-CD3 and soluble anti-CD28. ICOS-L expression on DCs and ICOS expression on T-cells were analysed by flow cytometry. The staining profiles of the indicated mAb and isotype-matched control are indicated by shaded and open areas, respectively. Similar results were observed in four independent experiments and the results of a representative experiment are shown. rIFN-α; recombinant IFN-α, rTNF-α; recombinant TNF-α, rIL-6; recombinant IL-6, αIFNs; anti-type-I IFNs Abs, αTNF; anti-TNF-α mAb, αIL-6; anti-IL-6 mAb.
in the regulatory function of the generated T-cells by priming with maturing pDCs. We added neutralizing anti-type-I IFNs Abs or anti-IL-6 mAb in the presence of anti-ICOS-L mAb into the primary culture of maturing pDCs and naive T-cells, and then analysed the suppressive function of these primed T-cells to the bystander naive T-cell response. The addition of anti-ICOS-L mAb during the primary culture remarkably counteracted the suppressive function of T-cells primed with CpG-pDCs or with IL-3-pDCs in the presence or absence of neutralizing anti-IL-10 Abs during re-stimulation with APCs. Ratios of suppressor to naive T-cells are shown. (B) Functional involvement of pDC-derived type-I IFNs or IL-6 in the regulatory function of the primed T-cells was tested by stimulating naive CD4+ T-cells with T-cells primed by CpG-pDCs or IL-3-pDCs in the presence or absence of the indicated neutralizing antibodies during primary culture of naive T-cells and maturing pDCs. The ratio of responder to suppressor was 2:1. Proliferation was assessed by [3H]thymidine incorporation and error bars represent the SEM of quadruplicate wells. Statistical significance was determined using Student’s t-test. αIFNs; anti-type-I IFNs Abs, αIL-6; anti-IL-6 mAb, αICOS-L; anti-ICOS-L mAb.

Discussion

pDCs have two unique functional attributes: as cells activating the innate immune system via type-I IFN production (5, 30) and as APCs inducing IL-10-producing Treg cells via ICOS-L in adaptive immunity (23). For example, in viral infection, immature pDCs trigger protective anti-viral inflammation through secretion of vast amounts of type-I IFNs, which not only directly inhibit viral replication but also activate an immune network of cytotoxic effector cells such as CTLs, NK cells, neutrophils and macrophages to induce the clearance of infected cells. These pDC/type-I IFN-mediated immune processes may contribute to the initial control of viral proliferation while concomitantly causing tissue damage of the infected host by excessive inflammation. In hepatitis B virus (HBV) infection, pDCs and type-I IFNs in fact most likely play an
important role in the protective response to the HBV through the activation of the innate immune system and subsequent adaptive CTL response (31–33). However, not only induced hepatitis B surface antigen (HBsAg)-specific CTLs kill the virus-infected hepatocytes but also Ag-non-specific activated macrophages and neutrophils induce liver damage, contributing to the immunopathology of fulminant hepatitis (31, 34). Indeed, IL-10-producing CD4+ T-cells are observed in inflammatory lesions of persistent infections with several pathogens (35–37), and the importance of these T-cells in protecting the host from the excessive inflammation was indicated in a report showing that an absence of IL-10 causes uncontrolled lethal immune response to infections (38). From these findings, we speculate that the biological relevance of the tolerogenic function of maturing pDCs is that they inhibit further damage to the host by preventing excessive inflammation during the adaptive response. Thus, the two distinct functional attributes of pDCs might constitute a fail-safe system against the invasion of pathogens.

There is substantial evidence that by inducing the IL-10-producing T-cells, pDCs have a specialized role in the induction of peripheral tolerance on the several immunopathological conditions (8–10, 13, 14, 17), and ICOS-L has been shown to function as the interface between pDCs and T-cells in the induction of IL-10 (19–22). Our present results newly indentify the elaborate system in pDCs to promote the generation of IL-10-producing Treg cells and suggest that pDCs are specialized in contributing to the peripheral tolerance in adaptive immunity.

The clinical impact of anti-TNF-α therapies in recent years on chronic inflammatory autoimmune disorders, such as rheumatoid arthritis, Behçet’s disease, Crohn’s disease, or psoriasis, helps us understand the critical role of TNF-α-dependent immunity. Indeed, TNF-α is a pivotal inflammatory cytokine that possesses an ability to induce DC maturation (39, 40), as shown by the up-regulation of B7 family members CD80 and CD86. With respect to expression of another B7 family member, ICOS-L, it has been demonstrated that TNF-α could induce ICOS-L in endothelial cells, fibroblasts and CD34+ hematopoietic progenitors (41–43). Our result confirmed this was the case also in pDCs. In addition to the endogenous TNF-α, which could function as an autocrine inducer of ICOS-L, pDC-derived IL-6 could function as a paracrine enhancer of ICOS expression on T-cells, indicating a contribution to the generation of IL-10-producing Treg cells via the ICOS/ICOS-L pathway. These might be an intrinsic feature of pDCs because TNF-α and IL-6 are produced and ICOS-L is dramatically upregulated in both types of pDC maturation that are induced by IL-3 and recognition of TLR (2, 23). Based on our findings, the pDC-derived type-I IFNs resulting from the TLR ligand-dependent maturation and the pDC-derived IL-6 resulting from the IL-3-dependent maturation, respectively, could both function as supportive factors for this intrinsic ability of pDCs to induce IL-10-producing Treg cells.

Although type-I IFNs are regarded as immune activators of the cell-mediated innate response against microbial infection, there is evidence, besides that from the current study, for an intimate link between type-I IFNs and T-cell production of IL-10 in adaptive immunity (44, 45). IFN-α, in combination with IL-10, has been described to have the ability to induce the generation of IL-10-producing Tr1 cells from naive CD4+ T-cells activated through T-cell receptor and CD28 (46). In addition, in support of our results, a role of pDC-derived type-I IFNs in immunosuppression has been determined by the finding that the regulatory function of T-cells primed with virus-stimulated pDCs could be blocked by neutralizing type-I IFNs during co-culture of the pDCs and naive T-cells (17). Accordingly, pDCs use vast amounts of type-I IFNs not only to eliminate invasive pathogens in the innate immune phase but also to support their own ability to prime IL-10-producing Treg cells through expressing ICOS-L in the adaptive immune phase.

IL-6 has a broad range of biological activities against a variety of immune cells and in turn regulates immune responses such as plasma-cell differentiation, T-cell differentiation and hematopoiesis. Indeed, it has been demonstrated that pDC-derived IL-6 contributes to the humoral immune response by plasma-cell differentiation and antibody production in cooperation with the concomitant secretion of type-I IFNs in response to host DNA-auto-antibody complexes in systemic lupus erythematosus pathogenesis (6). There is evidence indicating that IL-6 functions as a factor to induce conventional T,2 cells that produce IL-10 (47, 48). In addition, we newly identified that IL-6 functioned on T-cell priming as a pDC-derived factor that promotes the ICOS-L-driven induction of IL-10-producing Treg cells in the IL-3-dependent maturation pathway, which links to the T,2-polarizing condition. Our result showing the enhancing effect of IL-6 on T-cell expression of ICOS provides a possible explanation for a molecular basis underlying this phenomenon. However, IL-6 could hardly promote IL-10 production in the T,1 cells induced by recombinant IL-12, and blocking of IL-6 did not reduce the T-cell production of IL-10 primed by CpG-pDCs, which generate the T,1-like IFN-γ-producing T-cells through type-I IFN production. Because dominant T,1 responses sometimes conceal the effect of T,2-polarizing signals (49), this may be the case in the effect of IL-6. At the transcriptional level, although differential regulation of IL-10 production in T,1 and T,2 cells has been suggested (50, 51), signal transducer and activator of transcription 3 (STAT3) has been implicated in immune tolerance and induction of Treg cells and IL-10 (52). Moreover, several cytokines, such as IL-27, IL-10 and IL-2, as well as IFN-α or IL-6, which are capable of inducing T-cell production of IL-10 (46, 53–55), have been reported to be involved in the STAT3-mediated activation in T-cells (27, 56, 57). Although the STAT3-mediated signaling pathway underlying the ICOS-L-mediated IL-10 production in T-cells remains largely uncharacterized, it is possible that STAT3 is a key signaling pathway in the pDC-driven T-cell production of IL-10. Meanwhile, STAT4 is a key transcription factor that promotes T,1 cell development and their accompanying IL-10 production (58). Type-I IFNs also trigger the STAT4 activation during T-cell production of IFN-γ (59–61). The effect of the IL-6-mediated STAT3 pathway may not be dominant over or may not emerge in T-cells under the STAT4-dependent T,1 condition induced by IL-12 or type-I IFNs, but could function to induce IL-10 production by the T-cells primed with IL-3-pDCs, which do not have the ability to produce either IL-12 or IFN-α. In support of our results, a recent publication analysing the IL-6 effect on T-cell production of IL-10 in...
T₁- and T₂-polarizing conditions demonstrates that IL-6 induces STAT3-mediated IL-10 production and that the IL-6-dependent enhancement of IL-10 production is restricted in the T₂-polarizing condition (27). It is important that the transcriptional mechanism by which pDCs induce the generation of IL-10-producing Treg cells through ICOS-L, and whether the pDC-derived cytokines regulate ICOS-L-mediated STAT pathways, be clarified in future studies.

Collectively, pDC-derived molecules, type-I IFNs, IL-6, TNF-α and ICOS-L play a synergistic role in the adaptive response of pDCs. Namely, TNF-α induces ICOS-L expression on pDCs in an autocrine manner, IL-6 promotes ICOS expression on T-cells, and type-I IFNs or IL-6, respectively, support the ICOS-L-mediated generation of IL-10-producing Treg cells via the TLR-dependent or IL-3-dependent activation pathway. Thus, the pDC function through ICOS-L, which may lead to peripheral tolerance for controlling an excessive immune response, is backed up by the elaborate cytokine-producing ability of pDCs.

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References
pDC-derived cytokines support ICOS-L function


58 Saraiva, M., Christensen, J. R., Veldhoen, M., Murphy, T. L., Murphy, K. M. and O’Garra, A. 2009. Interleukin-10 production by Th1 cells requires interleukin-12-induced STAT4 transcription factor and ERK MAP kinase activation by high antigen dose. Immunity. 31:209.

