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**Author:** El Bannoudi, Hanane  
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DXS⁺ CD4⁺ T cells modulate CD4⁺ T cell response via inhibition of IL-12 production by DCs

Chapter 6

Hanane El Bannoudi¹, Wanda GH Han¹, Jeroen N Stoop², Pascale Louis-Plence², Tom WJ Huizinga¹ and René EM Toes¹

1 Department of Rheumatology, Leiden University Medical Centre, Leiden, The Netherlands
2 Inserm U844, Université Montpellier I, Montpellier, France

Abstract

DX5^CD4^ T cells have been shown to dampen collagen-induced arthritis and delayed type hypersensitivity reactions in mice. These cells are also potent modulators of T-helper cell responses through direct effects on CD4^ T cells in an IL-4 dependent manner. To further characterize this T cell population, we studied their effect on DCs and the potential consequences on T cell activation. Here, we show that mouse DX5^CD4^ T cells modulate DCs by robustly inhibiting IL-12 production. This modulation is IL-10 dependent and does not require cell contact. Furthermore, DX5^CD4^ T cells modulate the surface phenotype of LPS-matured DCs. DCs modulated by DX5^CD4^ T cell-supernatant express high levels of the coinhibitory molecules PD-L1 and PD-L2. OVA-specific CD4^ T cells primed with DCs exposed to DX5^CD4^ T cell supernatant produce less IFN-γ than CD4^ T cells primed by DCs exposed to either medium or DX5^CD4^ T cell supernatant. The addition of IL-12 to the coculture with DX5^ DCs restores IFN-γ production. When IL-10 present in the DX5^CD4^ T cell supernatant is blocked, DCs reestablish their ability to produce IL-12 and to efficiently prime CD4^ T cells. These data show that DX5^CD4^ T cells can indirectly affect the outcome of the T-cell response by inducing DCs that have poor Th1 stimulatory function.

Introduction

The immune system can protect the host against the detrimental effects of a broad range of pathogenic microorganisms and, at the same time, maintain the tolerance to self-antigens. Triggering an immune response to self-antigens can result in the induction of autoimmunity. The induction of autoimmunity and the damage it can cause is, among others, controlled by the presence and action of suppressor T cells [1-5].

Several populations of CD4^ T cells have been described that are involved in the maintenance of self-tolerance and prevention of autoimmunity and inflammation. The most prominent and well studied T-cell population with regulatory properties is characterized by the expression of the transcription factor Foxp3. These cells have been shown to possess the ability to influence different types of immune responses such as inhibiting the proliferation and/or cytokine production of effector T cells [6-11]. Likewise, they have also been reported to influence the differentiation of naïve CD4^ T cells into IL-10 or TGF-β-producing adaptive T reg cells [12]. Furthermore, these cells can alter the function of APCs through inhibition of their antigen presenting activity, pro-inflammatory chemokine production, and expression of co-stimulatory molecules [13-20].

Other T cell subsets also have the ability to influence the outcome of immune responses that affect the integrity of the body. For example, a population of T cells characterized by the expression of CD49b [21] that we will call DX5^CD4^ T cells, has been shown to alleviate diabetes, as well as collagen-
induced arthritis (CIA) and delayed-type hypersensitivity reactions in mice [21-23]. CD49b is a β-2 integrin and is not only expressed by a subpopulation of CD4+ and CD8+ T cells, but also on NKT cells. In contrast to NKT cells, DX5+CD4+ T cells are MHC class II restricted and exhibit a diverse TCR repertoire [19, 22].

We have shown that DX5+CD4+ T cells can have suppressive effects on CD8+ T cells and can change the outcome of CD4+ T cell responses in vitro [24, 25]. Upon antigen-specific triggering of naïve OVA-specific CD4+ T cells in the presence of DX5+CD4+ T cells, a striking difference in cytokine production was observed. An IL-10-producing CD4+ T cell response was induced instead of the predominant IFN-γ-producing Th1 reactions normally seen in mice on a C57BL/6 background. This modulation did not require cell-cell contact. Instead, IL-4 produced by DX5+CD4+ T cells was primarily responsible for the inhibition of IFN-γ and promotion of IL-10 production by responding CD4+ T cells. These data therefore indicate that DX5+CD4+ T cells can directly modulate the outcome of Th responses by diverting potentially pathogenic Th1 induction into Th responses characterized by the production of IL-10.

The studies described above demonstrate that DX5+CD4+ T cells can modulate the outcome of Th responses by directly acting on the responding CD4+ T cells but do not exclude the possibility that DX5+CD4+ T cells also have an impact on DCs. Modulation of DCs could represent another strategy by which DX5+ CD4+ T cells influence the outcome of immune responses. DCs are professional APCs that play a major role in determining whether pro-inflammatory or regulatory Th cells are induced [23].

Depending on the type of pathogen they encounter, DCs are able to direct the development of naïve CD4+ T cells to several distinct Th cell subsets. For example, IL-12 produced by DCs after TLR-4 triggering biases the CD4+ T cell response toward the differentiation of a Th1 response that is characterized by the production of IFN-γ [26-28].

Costimulatory molecules expressed on DCs are also playing a central role in maintaining the balance between immunity and tolerance. Molecules, such as CD80 and CD86, can promote T cell activation [29, 30], whereas molecules such as programmed death ligand-1 (PDL-1, B7 H1) and PDL-2 (B7-DC) can inhibit T-cell responses [31-33]. The latter molecules are therefore instrumental in the induction of T-cell tolerance and prevention of autoimmunity [34-37]. The interaction between programmed death (PD) ligands and their receptor PD-1 is involved in T-cell exhaustion and failure of viral control during chronic infection [38].

This pathway is also involved in the attenuation of protective immune response against tumors [39-41] and has been shown to regulate the development, maintenance, and function of T reg cells. In this study, we have analyzed the potency of the DX5+CD4+ T-cell population to modulate DC function. Our results indicate that DX5+CD4+ T cells can inhibit the production of IL-12 by DCs resulting in the
inhibition of Th1-cell responses. These results therefore add to our understanding of the immunomodulatory potential of DX5⁺CD4⁺ T cells.

Results

DX5⁺CD4⁺ T cells inhibit IL-12 production by DCs and alter the phenotype of DCs

In previous work, we have shown that DX5⁺CD4⁺ T cells can influence the outcome of CD4⁺ T-cell responses via the production of IL-4 [21]. To further investigate the immunomodulatory potential of DX5⁺CD4⁺ T cells, we now examined their effects on DC maturation and their ability to instruct DCs to modulate the outcome of T-cell responses. To this end, we first incubated DX5⁺CD4⁺ T cells, which were isolated from mice that have received three injections with immature DCs [18, 19, 21, 22] with fresh bone marrow derived DCs from naive animals. Interestingly, we observed that DCs matured with LPS for 2 days in the presence of DX5⁺CD4⁺ T cells produced significantly less IL-12 p40 as compared to DCs cultured in the absence of these T cells. In contrast, DCs cultured in the presence of DX5⁻CD4⁺ T cells maintained their IL-12 production (Figure 1A). These data indicate that DX5⁺ CD4⁺ T cells can modulate the activation of DCs by inhibiting their IL-12 production.

To assess whether cell-cell contact or a soluble factor is responsible for the suppression of IL-12 production, we next collected supernatant of either DX5⁺CD4⁺ or DX5⁻CD4⁺ T cells stimulated with anti-CD3 and anti-CD28 for 3 days. Addition of this supernatant to fresh DCs cultures revealed that DX5⁺CD4⁺ T cell-supernatant, but not supernatant from DX5⁻CD4⁺ T cells, reduced the production of IL-12 by DCs (Figure 1B). Together, these data indicate that a soluble factor derived from DX5⁺ CD4⁺ T cells can functionally modulate DCs by inhibiting IL-12 production.

To explore the possibility that DX5⁺CD4⁺ T cells also modulate the cell-surface expression of molecules involved in T cell activation, we next analyzed the expression of various surface molecules (PDL-1, PDL-2, CD80, CD86, CD40, and MHC class II) on DCs after culture with the supernatant of DX5⁺CD4⁺ T cells. The data show that the supernatant of DX5⁺CD4⁺ T cell cultures is able to enhance the expression levels of the inhibitory molecules PDL-1 and PDL-2 on the surface of DCs.

Likewise, the expression of CD80, CD86, CD40, and MHC class II was also increased after incubation of DCs with DX5⁺CD4⁺ T cell supernatant (Figure 2). These effects were not observed when DCs were cultured with DX5⁻CD4⁺ T cell supernatant or were left in medium alone. These data show that phenotypic changes of DCs installed by DX5⁺CD4⁺ T cells are caused by (a) soluble factor(s) secreted by DX5⁺CD4⁺ T cells. Together, these data demonstrate the ability of DX5⁺CD4⁺ T cells to modulate the expression of cell surface molecules on DCs and cytokine production by DCs that are involved in setting the outcome of T cell responses.
CD49b^+CD4^+ T cells modulate dendritic cells

Figure 1. DX5^+CD4^+ T cells inhibit the production of IL-12 by DCs. DCs were cultured with (A) DX5^+CD4^+ or DX5^-CD4^+ T cells, or (B) with supernatants of DX5^+CD4^+ or DX5^-CD4^+ T cell cultures for 3 days. After one day of culture, 0.01 μg/mL LPS was added. The supernatants were collected and the levels of IL-12 p40 and IL-12 p70 were measured by ELISA. (A) Data of two independent experiments are shown individually; (B) data are shown as mean ± SEM of results pooled from five independent experiments. *p < 0.05, Mann-Whitney test.
Figure 2. DX5<sup>+</sup>CD4<sup>+</sup> T cells induce upregulation of the expression levels of PDL-1, PDL-2, and other surface molecules. Bone marrow-derived DCs were cultured with the supernatants of DX5<sup>+</sup>CD4<sup>+</sup>, DX5<sup>-</sup>CD4<sup>+</sup>, or medium only. LPS (0.01 μg/mL) was added the next day. After 3 days, the expression of the indicated surface molecules (PDL-1, PDL-2, CD80, CD86, CD40, and MHC class II) was assessed by flow cytometry. Solid line histograms, expression on DCs exposed to DX5<sup>+</sup>CD4<sup>+</sup> supernatant; dashed line histograms, expression on DCs exposed to DX5<sup>-</sup>CD4<sup>+</sup> supernatant; dotted line histograms, expression on DCs cultured in medium only. Results shown are from one experiment representative of six performed.

**IL-10 secreted by DX5<sup>+</sup>CD4<sup>+</sup> T cells is responsible for the suppression of IL-12 production**

We next wished to identify the soluble factor responsible for the suppression of IL-12 production. To this end, we used the results of the analysis of cytokine production of DX5<sup>+</sup>CD4<sup>+</sup> T cells as published recently [22, 24]. Of the cytokines produced by DX5<sup>+</sup>CD4<sup>+</sup> T cells, especially IL-4 and IL-10 [22, 24] gained our interest as these cytokines have been implicated in the inhibition of IL-12 production.
DCs were cultured together with DX5^CD4^+ or DX5^CD4^- T cell supernatant in the presence of blocking antibodies against IL-4 or IL-10. Our results show that inhibition of IL-10 present in the DX5^CD4^+ T cell supernatant restored the ability of DCs to produce IL-12. In contrast, neutralization of IL-4 did not result in the restoration of IL-12 production by DCs (Figure 3). Together, these findings indicate that IL-10 but not IL-4 secreted by DX5^CD4^+ T cells is responsible for the suppression of IL-12 production.

**Figure 3.** IL-12 production by DCs is restored upon blocking of IL-10 secreted by DX5^CD4^+ T cells. Bone marrow-derived DCs were cultured together with the supernatants of DX5^CD4^+ or DX5^CD4^- T-cell cultures in the presence of anti-IL-4 (100 μg/mL), or anti-IL-10 (150 μg/mL). After 3 days, the levels of IL-12p70 were measured by ELISA. Data are shown as mean ± SEM of results pooled from three independent experiments. *p < 0.05, unpaired t-test.

**DCs modulated by DX5^CD4^+ T cells inhibit IFN-γ after antigen-specific T-cell stimulation.**

The results presented above indicate that DX5^CD4^+ T cells can modulate the expression and secretion of various molecules involved in T-cell activation and skewing. To analyze whether DX5^CD4^+ T cell-modulated DCs display altered abilities to activate naïve CD4^+ T cell, we next investigated the impact of DC modulation by DX5^CD4^+ T cells on the outcome of T-cell responses.

To this end, we incubated DCs with supernatants of DX5^CD4^+ or DX5^CD4^- T cell cultures. After extensive washing, the DCs exposed to supernatant from DX5^+ (DX5^DCs) or DX5^- (DX5^-DCs) T-cell cultures were co-cultured with OVA specific CD4^+ D0.11.10 T cells and OVA peptide. After 3 days, IFN-γ production by OVA-specific CD4^+ T cells was analyzed by flow cytometry. Interestingly, OVA-specific CD4^+ T cells primed with DX5^DCs produced less IFN-γ as compared with CD4^+ T cells primed with either DX5^-DCs or DCs exposed to medium only (medium DCs) (Figure 4A and B). These data indicate
that DCs exposed to the action of DX5\textsuperscript{CD4\textsuperscript{+}} T cells are affected in their ability to prime CD4\textsuperscript{+} T cells for IFN-γ production.

As DX5\textsuperscript{CD4\textsuperscript{+}} T cells produced factors that inhibited IL-12 production by DCs and as IL-12 is a prominent cytokine capable of inducing IFN-γ production, we next determined whether the reduced IL-12 production was responsible for the effects observed. To this end, we supplemented cultures of naïve OVA-specific T cells and OVA-peptide-loaded DX5\textsuperscript{DC} with exogenous IL-12. Addition of IL-12 was sufficient to restore the potential of DX5\textsuperscript{DC} primed CD4\textsuperscript{+} T cells to secrete IFN-γ (Figure 4C and D).

As inhibition of IL-12 production was dependent on IL-10 present in the DX5\textsuperscript{CD4\textsuperscript{+}} T-cell supernatants, we next blocked IL-10 in the supernatant of DX5\textsuperscript{CD4\textsuperscript{+}} T cell cultures upon addition to DCs. These DCs were subsequently used to prime OVA-specific D0.11.10 cells as described above. DCs exposed to anti-IL-10-treated DX5\textsuperscript{+} supernatant regained their capacity to prime CD4\textsuperscript{+} T cells for IFN-γ production, as OVA-specific CD4\textsuperscript{+} T cells were able to produce IFN-γ at levels comparable with (or higher than) that produced by T cells primed by DX5\textsuperscript{DCs} or medium DCs. Conversely, IFN-γ-production by responding CD4\textsuperscript{+} T cells was not restored after treatment of DX5\textsuperscript{DCs} with anti-IL-4 (Figure 5A and B). Together, our results show that DX5\textsuperscript{CD4\textsuperscript{+}} T cells have the capacity to control the outcome of T-cell responses through modulation of DCs via their secretion of IL-10.
Figure 4. DX5^+DCs modulate CD4^+ T-cell response in vitro. Bone marrow-derived DCs were exposed to either medium (medium DCs), supernatants of DX5^+CD4^+ (DX5^+DCs), or DX5^+CD4^+ (DX5^+DCs) T-cell cultures. These DCs were cultured with OVA-specific CD4^+ D011.10 T cells in the presence of 1 μg/mL OVA. After 3 days, IFN-γ production by OVA-specific CD4^+ T cells was determined by flow cytometry. Dot plots are gated on CD4^+ KJ1-26^+ (OVA-specific) T cells. (A) Flow cytometric analysis of IFN-γ production by OVA-specific CD4^+ T cells cultured with medium DCs, DX5^+DCs, or DX5^+DCs is shown. Values in the flow cytometry plots represent the percentage of CD4^+ KJ1-26^+ IFN-γ producing cells. (B) Percentage of IFN-γ-producing OVA-specific CD4^+ T cells cultured in the presence of medium DCs, DX5^+DCs, or DX5^+DCs is shown as mean + SEM of results pooled from eight independent experiments. (C) Representative flow cytometry analysis of IFN-γ production by OVA-specific CD4^+ T cells cultured in the presence of DX5^+ DCs after addition of exogenous IL-12. (D) Percentage of IFN-γ production by OVA-specific CD4^+ T cells stimulated with DX5^+ DCs and exogenous IL-12 is shown as mean + SEM of results pooled from four independent experiments. *p < 0.05, unpaired t-test.
Figure 5. Inhibition of IL-10 present in the DX5^+CD4^+ T-cell cultures restores the ability of DCs to activate OVA-specific CD4^+ T cells. Bone marrow-derived DCs were exposed to either medium, supernatants of DX5^+CD4^+ T-cell cultures in the presence of anti-IL-10 (150 μg/mL), or anti-IL-4 (100 μg/mL). These DCs were cultured with OVA-specific CD4^+ D011.10 T cells and 1 μg/mL OVA. After 3 days, IFN-γ production by OVA-specific CD4^+ T cells was analyzed by flow cytometry. Dot plots are gated on CD4^+ KJ1-26^+ (OVA-specific) T cells. (A) Flow cytometric analysis of IFN-γ production by OVA-specific CD4^+ T cells is shown. Values in the flow cytometry plots indicate percentage of CD4^+ KJ1-26^+ IFN-γ-producing cells. (B) Percentage of IFN-γ-producing OVA-specific CD4^+ T cells activated either with anti-IL-10-treated DX5^+ DCs or anti-IL-4 treated DX5^+ DCs. Results from two out of three independent experiments are shown.
Discussion

Our data show that DX5+CD4+ T cells are excellent modulators of DC function and phenotype. DX5+CD4+ T cells cause a substantial reduction of IL-12 production in IL-10-dependent manner and a significant upregulation of the coinhibitory ligands PDL-1 and PDL-2. In previous studies, DX5+CD4+ T cells were demonstrated to have both protective and therapeutic potential in a murine arthritis model. The capacity of DX5+CD4+ T cells to dampen inflammatory reactions has also been shown in delayed-type hypersensitivity reactions [21, 22]. Additional evidence that these cells have immunomodulatory properties comes from a study in a murine diabetes model where these cells were found to be protective [23].

We previously analyzed the cytokine secretion profile of DX5+CD4+ T cells. DX5+CD4+ T cells secrete large amount of Th-2-associated cytokines such as IL-10 and IL-4 [21]. Indeed, studies where the role of adoptively transferred DX5+CD4+ T cells in suppression of inflammatory related diseases is examined indicate the involvement of the suppressive cytokines IL-10 and/or IL-4. The protective role of DX5+CD4+ T cells in murine diabetes was associated with the production of both IL-10 and IL-4 [20]. In CIA, IL-10 was also indicated in the protective effect conferred by DX5+CD4+ T cells [18, 19].

To understand the underlying mechanism involved in DX5+CD4+ T cell-mediated immunosuppressive effects, we have also demonstrated that DX5+CD4+ T cells strongly modulate the outcome of a primary CD4+ Th1 response via production of IL-4 [21]. The effect was directly targeted to the responding T cells as DX5+CD4+ T cells were also able to affect the outcome of CD4+ T cell responses in the absence of DCs. Now we show that DX5+CD4+ T cells can also modulate the outcome of CD4+ T cell response indirectly via modulation of DCs. In this case, not IL-4 but IL-10 produced by DX5+CD4+ T cells was the cytokine primarily responsible for the effects observed. DX5+CD4+ T cells induced a strong increase in the expression levels of the inhibitory molecules PDL-1 and PDL-2 on DCs.

Interaction of these ligands with the PD-1 receptor expressed on T cells has been implicated in the negative regulation of T-cell responses and maintenance of tolerance [31-33]. Both PD-1- and PDL-1-deficient mice exhibit hyperactivation of the immune system that subsequently leads to the development of autoimmune diseases [42]. In a murine model for diabetes, PD-1 blockade was shown to accelerate the onset of the disease that is associated with an increased production of IFN-γ [43, 44].

PD-1 is also involved in the regulation of T cell exhaustion during chronic infection and tumor immunity [36-38]. The expression of PD-1 is upregulated on exhausted CD8+ T cells, which are characterized by impaired cytokine production (e.g. IFN-γ) and defective cytotoxicity. Blockade of PD-1 pathway ameliorates the effector functions of CD8+ T cells that leads to enhanced control of viral...
infection [38]. Likewise blockade of PD-1 signaling reverses tumor-induced T-cell exhaustion and enhances the functions of CD8+ T cells [37, 38].

In the present work, PDL-1 and PDL-2 do not seem to be involved in the regulation of CD4+ T cells as we could not observe an effect on responding CD4+ T cells after neutralizing of these ligands on DX5+CD4+ T cell-modulated DCs (data not shown).

Nonetheless, their high expression levels on DCs after modulation with DX5+CD4+ T cell supernatants, combined with the phenomena described above, also point to the multiple pathways implemented DX5+CD4+ T cells to steer the outcome of T-cell responses. These pathways do not only involve the modulation of cytokine secretion but also the expression of molecules known to affect T cell responses. Whether the action of DX5+CD4+ T cells on DC function and phenotype is responsible for the effects observed in disease models is not known. Nonetheless, our findings are in line with data obtained in vivo indicating a preferential reduction of Th-1-associated IgG2a against collagen type II in CIA model following adoptive transfer of DX5+CD4+ T cells [19].

Likewise, some of our findings resemble the findings observed in studies focusing on FoxP3+CD25+CD4+ T reg-cell-mediated immune regulation. Like DX5+CD4+ T cells, CD25+CD4+ T reg cells can exert their suppressive effect directly and indirectly via suppressing T-cell responses and altering the phenotype and the function of DCs, respectively. In addition, human T reg cells inhibit the maturation and antigen presentation of monocyte-derived DCs to become poor APCs [7, 10, 13-17]. Together our results point to the plethora of pathways affected by DX5+CD4+ T cells that could be involved in the control of autoimmune responses. Understanding of these pathways might be instrumental to further define potential immune modulating strategies with the aim to counteract autoimmune diseases.

Materials and methods

Mice

D011.10 (OVA-specific TCR Tg) mice were used for the generation of bone marrow DCs and for the isolation of CD4+ T cells. C57BL/6 mice were used for the generation of DX5+CD4+ T cells and DX5-CD4+ T cells. D011.10 mice were housed and bred in the animal facility of the Leiden University Medical Center. C57BL/6 mice were purchased from Charles River. Experiments were performed in accordance with our institutional guidelines on animal use in research.

DX5+CD4+ and DX5-CD4+ T cell isolation

Splenocytes were isolated from spleens of mice that were injected three times (7, 5, and 3 days before purification of DX5+CD4+ T cells) intraperitoneally with 1 × 10^7 immature DCs in PBS. RBCs were lysed...
and CD4+ T cells were purified by positive selection using Dynal CD4 positive isolation kit (Invitrogen). Afterwards, DX5- cells and DX5+ cells were isolated from CD4+ T cells derived from the same mice using CD49 (DX5) MicroBeads (Miltenyi Biotec). The purity was 80–90%. DX5-CD4+ and DX5-CD4+ T cells were isolated from the same mice. The purified cells were incubated in the ratio of 1:1 with anti-CD3/CD28 beads (Invitrogen), after 3 days the supernatants were collected and stored at −80°C for future use.

**DC generation and culture with DX5 supernatants**

Bone marrow cells were harvested from the femur and tibiae of DO11.10 mice. Subsequently, the erythrocytes were lysed. After washing with 1% FCS supplemented RPMI 1640 medium, T and B cells were depleted using mouse pans T and B dynabeads (Invitrogen). T- and B-depleted cells were incubated at 37°C. After 4 h, nonadherent cells were harvested and cultured at 5 × 10⁶/mL in 24-well plate in complete medium (RPMI 1640 supplemented with 8% FCS, 2 mM L-glutamin, 5 × 10⁻⁵ M β-mercaptoethanol, streptomycin, nonessential amino acids (Gibco) and 1mM sodium pyruvate (Sigma-Aldrich)) with 1000 IU/mL of rmGM-CSF (R&D systems), and 1000 IU/mL of rmIL-4 (R&D systems). The medium was refreshed every other day for 1 week.

After 1 week culturing, bone marrow-derived DCs were harvested and cultured with DX5-CD4+, DX5-CD4+ T cells or their supernatants or medium for 3 days. LPS (0.01 μg/mL; Sigma-Aldrich) was added after 1 day. The DCs obtained were cultured at 1 × 10⁶/mL with OVA323-339 peptide and OVA-specific CD4+ T cells in total volume of 150 μL for 3 days. After 3 days, cytokine production was determined by flow cytometry. IL-12 (20 ng/mL) that was added to the cocultures of CD4+ T cells and DCs was purchased from eBioscience. The concentrations of anti-IL-4 and anti-IL-10 antibodies used for blocking studies were chosen on the basis of titration experiments where known concentrations of cytokine were effectively inhibited in a bioassay [45].

**Determination of cytokine concentration**

Cytokine levels in DCs cell culture supernatants were measured by ELISA using IL-12p70 kit ELISA Ready-set-Go (eBioscience) according to the manufacturer’s instructions. Matched pairs of antibodies to measure IL-12p40 were purchased from BD.

**Flow cytometry**

The expression of the surface molecules was examined using fluorescence-labeled antibodies against B7-H1 (MIH5) and B7-DC (TY25) from eBioscience and CD80 (16-10A-1), CD86 (GL-1), CD40 (3/23), and MHC class II from BD. CD4+ T cells were visualized by staining with anti-CD4-PerCP-Cy5.5 (L3T4/RM4-5; BD Pharmingen). KJ1-26-PE (Invitrogen) was used to detect OVA specific T cells. Anti-IFN-γ-FITC (XMG1.2; BD Pharmingen) was used to detect IFN-γ-producing cells.
The staining reactions were performed according to manufacturer’s protocol. In brief, the cells were first washed in the staining buffer (PBS containing 0.5% BSA); subsequently, the cells were incubated with antibodies for surface markers for 20 min at 4°C. For intracellular cytokine staining, Brefeldin A (10 μg/mL; Sigma-Aldrich) was added to co-culture of CD4+ T cells and DCs for 4 h. After washing, the cells were fixed using Cytofix/Cytoperm (BD Bioscience) followed by washing with Perm/wash (BD Bioscience). For determination of cytokine production, the cells were stained for intracellular cytokines in Perm/wash for 20 min. Following washing, the cells were analyzed on a flow cytometer (FACS Calibur; Becton Dickinson). FACS data analysis was performed using Flowjo software.

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