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Plasmacytoid DC promote priming of autoimmune Th17 cells and EAE

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EAE, an animal model for MS, is a Th17 and Th1-cell-mediated autoimmune disease, but the mechanisms leading to priming of encephalitogenic T cells in autoimmune neuroinflammation are poorly understood. To investigate the role of plasmacytoid DC (pDC) in the initiation of autoimmune Th17- and Th1-cell responses and EAE, we depleted pDC with anti-pDC Ag-1 (anti-PDCA1) mAb prior to immunization of C57BL/6 mice with myelin oligodendrocyte glycoprotein (MOG). pDC-depleted mice developed less severe clinical and histopathological signs of EAE than control mice, which demonstrates a promoting role for pDC in the initiation of EAE. The levels of type I IFN were much lower in the sera from anti-PDCA1-treated mice. However, neutralization of type I IFN ameliorated the early phase of EAE but did not alter the severity of disease. Thus, only a minor part of the EAE-promoting effect of pDC appears to be mediated by IFN-α/β secretion. The numbers of MOG-specific Th17 cells, but not Th1 cells, were lower in spleen from anti-PDCA1-treated mice compared with controls. In contrast, pDC depletion a week after MOG immunization resulted in more severe clinical signs of EAE. In conclusion, we demonstrate that pDC promote initiation of MOG-induced Th17-cell responses and EAE.

Key words: Autoimmunity · DC · EAE/MS · T cells · Type I IFN

Supporting Information available online

Introduction

EAE is an animal model for the human autoimmune demyelinating disease MS [1]. The pathological changes in the CNS during EAE are very similar to MS with perivascular infiltrates of T cells, B cells and macrophages [2]. Murine EAE is induced by injection of myelin autoantigens such as myelin oligodendrocyte glycoprotein (MOG) in CFA containing killed *Mycobacterium tuberculosis* and pertussis toxin. EAE was previously thought to be a purely IL-12-driven Th1-mediated autoimmune disease [3]. However Langrish *et al*. demonstrated that proinflammatory IL-17-producing Th17 cells mediate EAE [4]. Murine-naïve $CD4^+$ T cells differentiate into Th17 cells in the presence of soluble TGF-β or $CD4^+CD25^+$ Treg and IL-6 [5, 6]. Recently it was shown that different epitopes of MOG predominately induce a Th1 or Th17 response, which influences the lesion distribution and clinical symptoms of EAE and suggests a promoting role for both Th17 and Th1 cells in MOG-induced EAE [7].

DC are key actors when an adaptive immune response is initiated [8]. There are two major DC subsets in mice, which are characterized by differential expression of cell surface markers [9]. Myeloid DC (mDC) express $CD11c^{hi}B220^-Gr\text{-}1^-$, myeloid markers, and are highly specialized in priming naïve T cells [10]. Plasmacytoid DC (pDC) express $CD11c^{lo}B220^+Gr\text{-}1^+$ and secrete

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large amount of type I IFN upon viral challenge [11]. pDC also have a powerful ability to modify the adaptive immune response, *e.g.* T-cell differentiation [11, 12]. The expression of costimulatory molecules differs between mDC and pDC, *e.g.* maturation leads to upregulation of B7 molecules on mDC, but downregulation of these molecules on pDC [13, 14]. Moreover, mDC and pDC differ in their expression of TLR and response to TLR ligation. TLR7 and TLR9 ligation results in type I IFN production in pDC because of constitutive expression of IFN regulatory factor-7, whereas ligation of these receptors leads to IL-12 production in mDC [15, 16].

We have previously shown that TLR9 activation supresses EAE by unknown mechanisms [17], and because pDC express TLR9 it suggests a downregulatory role for pDC. Recently it was shown that depletion of pDC during the acute or relapsing phase of relapsing-remitting proteolipid protein-induced EAE exacerberate the clinical signs of EAE [18]. However, the role for pDC in the initiation of encephalolitogenic T-cell responses has never been investigated. Therefore, we depleted pDC by injection of anti-pDC Ag-1 (PDCA1) mAb prior to MOG immunization in order to study the effect of pDC on disease outcome. Depletion of pDC significantly ameliorated the clinical and histopathological signs of EAE compared with mice injected with normal rat IgG. Type I IFN levels in sera were lower in pDC-depleted mice than in controls. Neutralization of type I IFN ameliorated the early phase of EAE but did not alter the severity of disease. Importantly, the MOG-induced Th17-cell responses, but not the Th1-cell responses, were reduced following pDC depletion. In sharp contrast, pDC depletion a week after MOG immunization exacerbated the clinical signs of EAE. Thus, here we demonstrate a novel EAE-promoting role for pDC during the initiation of disease, although pDC appear to have a protective role later in the time course of EAE.

Results

pDC promote EAE

In order to investigate the role of pDC during the initiation of EAE, C57BL/6 mice were treated with either the pDC-depleting anti-PDCA1 mAb or normal rat IgG 1 day before and on the day of MOG immunization as previously described [19]. Injection of anti-PDCA1 mAb resulted in efficient, specific depletion of pDC (Supporting Information Fig. 1). Previous studies, using several-fold higher doses of anti-PDCA1 mAb, have demonstrated that pDC numbers are normalized 3–5 days following anti-PDCA1 treatment [18, 20]. The incidence of EAE was 62% and the maximum mean EAE score of 1.2 ± 0.4 ($p<0.05$) for anti-PDCA1-treated mice, compared with 92% incidence and a maximum mean EAE score of 2.8 ± 0.5 for normal IgG-treated mice (Fig. 1 and Table 1). In a second experiment, the incidence reached 100% for both groups, but the maximum mean EAE score was significantly lower after anti-PDCA1 treatment (Table 1). We therefore conclude that pDC depletion affects disease severity rather than incidence. Thus, pDC are important for the severity of EAE.

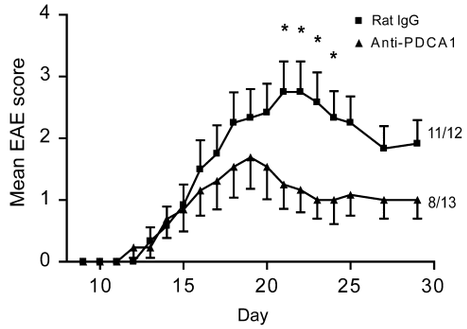

Figure 1. Mice treated with anti-PDCA1 before MOG immunization develop less severe EAE than control mice treated with normal rat IgG. Mean daily EAE scores $\pm$ SEM for mice treated with rat IgG (squares) or anti-PDCA1 mAb (triangles) ($n = 12$–13/group). Numbers in the graph represent affected/total mice. Data are representative of two independent experiments with the same results. *$p<0.05$ (Mann–Whitney's *U* test).

pDC promote inflammation and demyelination in the CNS

To assess the degree of inflammation and demyelination of the brain and spinal cord of mice treated with anti-PDCA1 mAb, a histopathological evaluation of the CNS was performed as previously described [21]. In the control mice there were profund EAE lesions in the spinal cord with inflammation, demyelination, axonal loss, T-cell and macrophage infiltration (Fig. 2A). In contrast, spinal cords from anti-PDCA1-treated mice only showed small lesions with much less inflammation, demyelination, axonal loss and fewer infiltrating T cells and macrophages (Fig. 2A). The mean inflammatory index in spinal cords from control IgG-treated mice was 1.5 ± 0.4 compared with 0.6 ± 0.4 in spinal cords from anti-PDCA1-treated mice ($p = 0.1$; Fig. 2B). Similarly, the mean demyelination score was 1.5 ± 0.3 in spinal cords from control IgG-treated mice compared with 0.6 ± 0.3 in spinal cords from anti-PDCA1-treated mice ($p = 0.08$; Fig. 2C). Even though the degree of inflammation and demyelination differed, the *p* value did not reach significance, which was probably due to small sample size. In addition, mild inflammation in the brain was noted in a few individuals from each group (data not included). Our data demonstrate that presence of pDC during initiation of EAE promote subsequent inflammation and demyelination in the spinal cord.

Reduced serum levels of IFN-α and IFN-β after pDC depletion

Since pDC are an important source of type I IFN [11], we asked whether type I IFN production after MOG immunization would be reduced in pDC-depleted mice compared with controls. The serum levels of IFN-β and IFN-α were measured 10 days after immunization. The mean serum level of IFN-β was 68% lower ($p<0.05$) in pDC-depleted mice compared with mice injected with control IgG, and the mean level of IFN-α was 46% lower ($p = 0.06$) in pDC-depleted mice compared with controls (Fig. 3).

Table 1. Effect of pDC depletion on clinical signs of MOG-induced EAE

Experiment	Treatment	Incidence	$p^{a)}$	Maximum mean score ± SEM	$p^{b)}$	Maximum mean score for affected mice ± SEM	$p^{b)}$
1	Anti-PDCA1	8/13 (62%)	0.16	1.2 ± 0.4	0.02	2.5 ± 0.2	0.07
	Rat IgG	11/12 (92%)		2.8 ± 0.5		3.3 ± 0.4	
2	Anti-PDCA1	11/11 (100%)	N/A[c)]	2.3 ± 0.1	0.02	N/A[c)]	
	Rat IgG	8/8 (100%)		3.1 ± 0.3			

[a)] Fisher's exact test.
[b)] Mann–Whitney's *U*-test.
[c)] N/A; not applicable.

The levels of IFN-β and -α were higher ($p<0.01$) in MOG-immunized mice compared with unimmunized mice (Fig. 3).

Because pDC can produce IL-6 [22] and IL-6 promotes differentiation of Th17 cells [6], we measured the amount of IL-6 in sera from IgG-treated or anti-PDCA1-treated mice 10 days after MOG administration. The mean IL-6 levels did not differ between the two treatment groups (data not included). We conclude that pDC do not contribute significantly to the total levels of IL-6 in sera, although local levels of IL-6 may be altered.

Depletion of IFN-α and IFN-β ameliorates the clinical course of EAE

The reduced EAE score together with reduced serum levels of type I IFN after pDC depletion suggested that pDC *via* type I IFN production promote EAE. In order to more directly investigate the role of type I IFN in the disease process IFN-α and IFN-β were neutralized in mice on the day of MOG immunization or 8 days after immunization with polyclonal sheep anti-IFN-α/β-specific Ab [23]. Control mice were treated with normal sheep Ab. The maximum mean EAE score did not differ between anti-IFN-α/β-treated and control mice (Table 2 and Fig. 4 A and B). However, depletion of IFN-α/β resulted in less severe disease during the early phase of EAE (Fig. 4A and B). These results suggests that pDC to some extent can promote the development of EAE *via* type I IFN production, but that other disease mechanism(s) must exist, which may be more important.

Effect of pDC depletion on mDC maturation and activation

In the next step we therefore investigated how pDC depletion affected mDC maturation and activation since mDC prime naïve Th cells *in vivo*. The expression of the maturation markers [24] MHC II, CD80 and CD86 on $CD11c^{hi}$ mDC from spleens of anti-PDCA1-treated or IgG-treated mice was examined by flow cytometry 10 days after MOG immunization. The mean expression of MHC II, CD80 and CD86 on mDC as measured by MFI did not differ between the two treatment groups (Fig. 5A and B).

Among the proinflammatory cytokines produced by activated mDC [25], IL-6 is essential for priming of encephalitogenic Th17 cells, which are maintained by IL-23 [6]. To assess the IL-6 and IL-23 expression by mDC, $CD11c^{hi}CD3^{-}$ mDC from pDC-depleted or normal IgG-treated control mice were sorted by flow cytometry 10 days after MOG immunization. The mRNA expression of IL-6 and IL-23 by mDC did not differ between the two treatment groups (Fig. 5C). Taken together, our data indicate that pDC influence neither maturation nor activation of mDC.

Fewer MOG-induced Th17 cells in anti-PDCA1–treated mice

To examine the effect of pDC depletion on the Th17-cell responses, the absolute numbers of Th17 cells were measured in the spleen 10 days after MOG immunization in anti-PDCA1-treated or normal IgG-treated mice after subsequent restimulation with or without MOG *ex vivo*. Anti-PDCA1-treated mice exhibited 50% lower numbers of MOG-induced Th17 cells *per* spleen ($p<0.01$) than IgG-treated control mice (Fig. 6A and B). In addition, the frequency of splenic Th17 cells was significantly lower ($p<0.05$) in anti-PDCA1-treated mice than in IgG-treated controls (data not shown). To confirm the flow cytometry data, we also measured the mRNA expression of IL-17 from MOG-immunized mice treated with anti-PDCA1 or normal IgG with quantitative RT-PCR (Q-PCR). Splenocytes were collected 10 days after immunization and were restimulated with or without MOG *ex vivo*. Similar to the reduced numbers of Th17 cells, MOG-induced IL-17 mRNA expression was 55% lower ($p<0.05$) in splenocytes from anti-PDCA1-treated mice than control mice (Fig. 6C). The experiment was repeated with the same results.

We conclude that the reduced numbers of MOG-induced Th17 cells in spleen may explain the observed ameliorated clinical signs of EAE after anti-PDCA1-treatment.

Th1-cell and IL-10 responses after pDC depletion

To examine the effect of pDC depletion on the Th1 cells, the absolute numbers of Th1 cells were measured in the spleen 10 days after MOG immunization in anti-PDCA1-treated or normal IgG-treated mice after subsequent restimulation with or without MOG *ex vivo*. Anti-PDCA1-treated mice exhibited higher numbers of MOG-induced Th1 cells *per* spleen ($p<0.05$) than IgG-treated

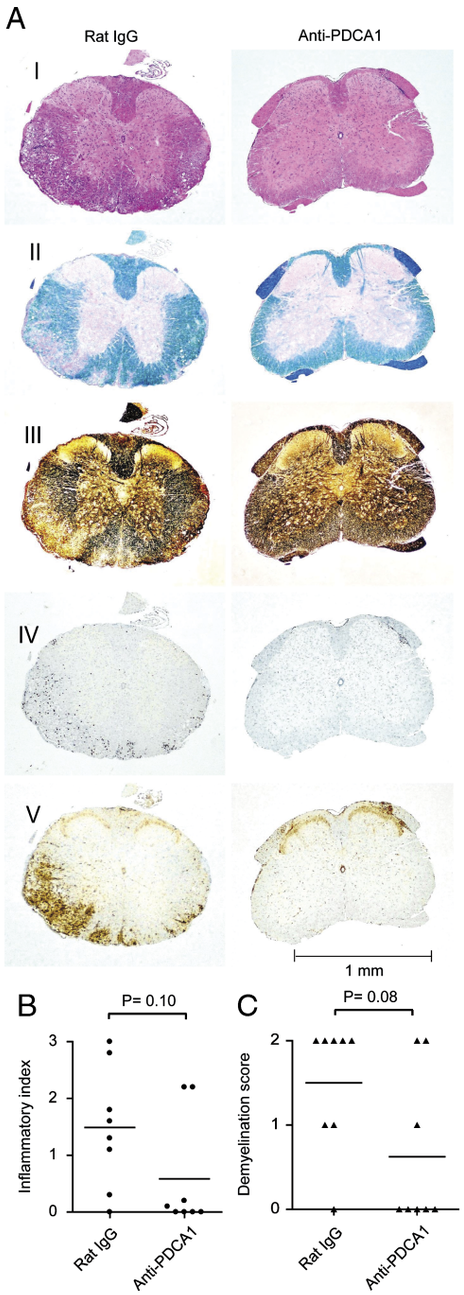

Figure 2. Strongly reduced inflammation and demyelination of the spinal cord after pDC depletion. (A) Histological analysis of the spinal cord from an IgG-treated control mouse (*left column*) showed profound inflammation (I), demyelination (II), axonal damage (III) and infiltration (IV and V), as visualized by H&E staining (I), Luxol fast blue staining (II), Bielschowsky silver impregnation (III) and immunohistochemistry using anti-CD3 (IV) and anti-Mac-3 (V) Ab. In contrast, the spinal cord from an anti-PDCA1-treated mice (*right column*) showed little inflammatory infiltration and no demyelination. Magnification: ×25. Scale bar = 1 mm. The degree of (B) inflammation and (C) demyelination in the spinal cord of mice 19 days after MOG immunization and treatment with rat IgG or anti-PDCA1 mAb ($n = 8$/group). Horizontal bars represent mean values (Mann–Whitney's *U*-test).

control mice (Fig. 6D). This difference was only detected for absolute numbers of Th1 cells, and not for the frequency of Th1 cells. The mean frequency was 2.5 % of Th1 cells for IgG-treated *versus* 2.6 % of Th1 cells for anti-PDCA1-treated mice. Moreover, Q-PCR analysis revealed no difference in IFN-γ-mRNA expression in splenocytes from pDC-depleted or control mice (Fig. 6E). Finally, we detected similar absolute numbers of IFN-γ-secreting cells *per* spleen in an ELISPOT assay (Fig. 6F). Taken together, our data suggest that the MOG-induced Th1-cell responses are not reduced by the pDC depletion.

pDC have been implicated in the activation of IL-10-producing T cells [26] and therefore we measured the mRNA expression of IL-10 in the spleen 10 days after MOG immunization in anti-PDCA1-treated or normal IgG-treated mice and subsequent restimulation with or without MOG *ex vivo*. Q-PCR analysis revealed no difference in IL-10 mRNA expression in splenocytes from pDC-depleted or control mice (data not included). Thus, our data suggest that IL-10 production is not promoted by pDC during the induction of EAE.

Foxp3$^+$ Treg responses in spleen after anti-PDCA1 treatment

Because pDC have been implicated in priming of Ag-induced Foxp3$^+$ Treg [27], we asked whether the Foxp3 and TGF-β1 expression in splenocytes was affected by anti-PDCA1 treatment. We observed that the mRNA expression of Foxp3 was three times higher ($p<0.05$) in splenocytes from anti-PDCA1-treated compared with control mice (Fig. 7A) after MOG immunization. However, TGF-β1 mRNA expression did not differ significantly between the groups (Fig. 7B). Next, the absolute numbers of Foxp3$^+$ Treg were measured in the spleen 10 days after MOG immunization in anti-PDCA1-treated or normal IgG-treated mice and subsequent restimulation with or without MOG *ex vivo*. The numbers of Foxp3$^+$ Treg *per* spleen did not differ between IgG- and anti-PDCA1-treated mice (Fig. 7C and D). These data suggest that pDC do not alter the Foxp3$^+$ Treg responses *in vivo*.

ICOS signaling in T cells is not affected by the pDC depletion

pDC upregulate the expression of ICOS ligand upon maturation and ICOS signaling has been implicated to be involved in autoimmunity [28, 29]. To test if pDC act on the T cells *via* ICOS ligand–ICOS interactions in EAE, we investigated whether ICOS signaling in T cells was affected by the pDC depletion. We isolated splenic T cells from anti-PDCA1-treated and normal IgG-treated mice 10 days after MOG immunization and measured the mRNA expression of several genes controlled by ICOS signaling pathways [30]: Il9, ICOS, Myo1F, Mal and Il2. The mRNA expression of these genes did not differ between the groups (data not included). Thus, we could not observe any influence of pDC on ICOS signaling in T cells in our system.

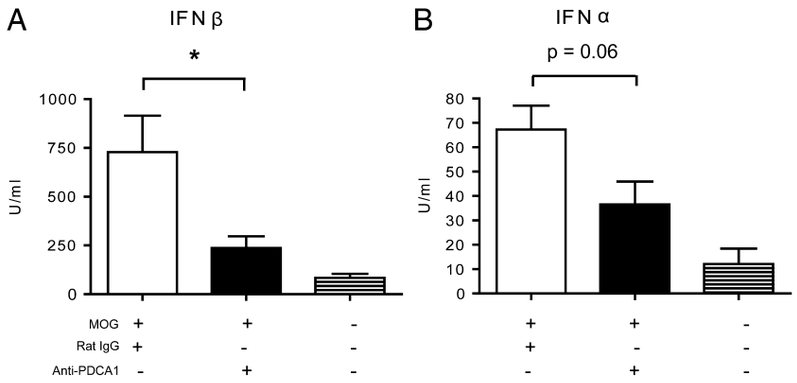

Figure 3. Lower amounts of serum IFN-β and IFN-α after pDC depletion. Mean serum (A) IFN-β and (B) IFN-α levels in sera from rat IgG-treated (white bars), anti-PDCA1-treated (black bars), respectively, 10 days after MOG immunization ($n = 4$–5/group). Data show mean ± SEM (Mann–Whitney's *U*-test).

Table 2. Clinical signs of EAE induced by MOG in anti-IFN-α/β-treated mice

Experiment	Treatment	Incidence	Mean max EAE score	$p^{a)}$
1[b)]	Anti-IFN-α/β Ab	9/9 (100%)	2.1 ± 0.4	0.24
	Sheep Ab	8/8 (100%)	3.0 ± 0.5	
2[c)]	Anti-IFN-α/β Ab	9/9 (100%)	2.6 ± 0.3	0.4
	Sheep Ab	9/9 (100%)	2.9 ± 0.3	

[a)] Mann–Whitney's *U*-test.
[b)] Mice treated with anti-IFN-α/β Ab or sheep Ab on the day of MOG immunization.
[c)] Mice treated with anti-IFN-α/β Ab or sheep Ab on day 8 after MOG immunization.

Anti-PDCA1-treatment of ongoing EAE exacerbates the clinical signs of disease

In a recent study, the clinical signs of EAE exacerbated considerably if pDC were depleted during the peak of the disease process [18]. To reproduce the results of this study in MOG-immunized C57BL/6 mice, we depleted pDC 8 days after immunization and then every other day for a total of four treatments (Fig. 8). The incidence of EAE was 100% and the mean accumulated EAE score on day 22 after immunization 27.3 ± 2.2 for anti-PDCA1-treated mice, compared with 83% incidence and a mean accumulated EAE score of 14.7 ± 4.6 for normal IgG-treated mice ($p<0.02$). The maximum mean EAE score was 3.5 ± 0.3 for anti-PDCA1-treated mice compared with 2.5 ± 0.8 for IgG-treated mice (NS) (Fig. 8).

Thus pDC depletion during peak of disease exacerbates EAE to some extent, whereas depletion at the time of immunization ameliorates the disease.

Discussion

Herein we show that pDC promote the induction of EAE, because depletion of pDC with anti-PDCA1 mAb before MOG immunization ameliorated the clinical and histopathological signs of the disease. This indicates an important role for pDC in the early induction of EAE. In contrast, EAE was exacerbated to some extent if pDC were depleted 8 days after MOG immunization. Thus, we could reproduce the findings by Bailey-Bucktrout *et al,* where SJL/J mice were treated with anti-PDCA1 mAb during the acute or relapsing phase of proteolipid protein-induced EAE [18]. Our results argue for different roles of pDC in the initiation of EAE and later during the acute phase of the disease.

Importantly, milder inflammation and demyelination of the spinal cord were observed in mice treated with anti-PDCA1 before EAE induction. Since pDC numbers return to normal after 3–5 days after depletion [18], it is unlikely that this is a direct effect of pDC on demyelination in the CNS during peak of disease. Instead, our data suggest that pDC promote activation of encephalitogenic Th cells, which results in increased T-cell infiltration of the CNS and subsequent inflammation and demyelination.

This study demonstrates reduced MOG-induced Th17-cell responses in mice depleted of pDC before EAE induction. The Th1-cell responses were not reduced by the pDC depletion, which argues against a general role for pDC on activation of Th cells. Thus pDC appears to drive the early development of Th17 cells, which are essential for the pathogenesis of EAE [4]. There are at least four plausible explanations for how pDC activate Th17 cells: (i) It could be a direct effect of type I IFN acting on Th cells during Th cell differentiation, because pDC are important producers of type I IFN in the blood [11, 31, 32]. pDC-produced IFN-α and IFN-β were

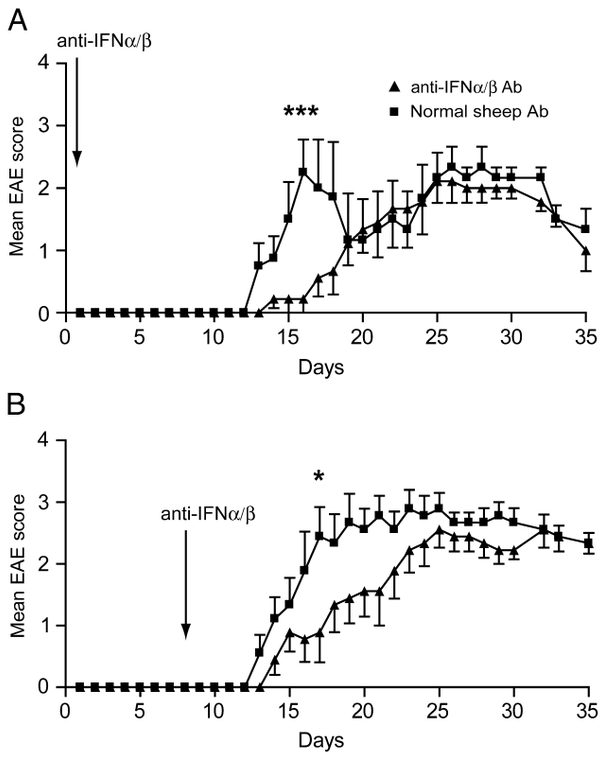

Figure 4. Ablation of IFN-α/β reduces the early clinical signs, but not the severity, of EAE. Mean daily EAE scores ± SEM for mice treated with normal sheep Ab or with 50 000 U of neutralizing polyclonal sheep anti-IFN-α/β Ab on the day of (A) or 8 days after (B) MOG immunization ($n = 8$–9/group). ($n = 9$/group). $^{***}p<0.001$, $^{*}p<0.05$ (Mann–Whitney's *U*-test).

upregulated in the blood after MOG immunization compared with unimmunized mice, and depletion of type I IFN after MOG immunization ameliorated the early phase of EAE but did not affect the severity of the disease. This suggests that pDC to some extent promote the development of EAE *via* type I IFN production, but that other immune mechanism(s) exist. pDC depletion did not completely abolish the type I IFN levels in the blood. This could either be explained by the incomplete depletion of pDC by anti-PDCA1 treatment or that other cell types, such as monocytes, also produce type I IFN after MOG immunization. (ii) mDC are implicated to prime autoimmune Th17 cells in EAE [17]. The expression of maturation markers or proinflammatory cytokines by mDC did not differ between pDC-depleted mice and controls, which suggests that mDC maturation and activation are not affected by pDC during the initiation of EAE. (iii) pDC-induced ICOS signaling have been implicated to be involved in autoimmunity [28, 29]. We could not find any signs of altered ICOS signaling in the T cells after pDC depletion and we conclude that ICOS ligand–ICOS interactions between pDC and Th cells are unlikely to cause the observed phenotype. (iv) It is believed that pDC has limited capacity to present exogenous Ag *via* MHC class II [11], although pDC can internalize exogenous Ag–Ab complexes *via* CD32 and subsequently present Ag on MHC class II [33]. However, at this stage we can neither confirm nor exclude a role for pDC in Ag-presentation in EAE. Further studies of the EAE-promoting mechanisms of pDC are warranted and ongoing in our laboratory.

IFN-β is used as a therapeutic agent against human MS [34–36] and both IFN-β- and TIR domain-containing adapter inducing IFN-β-deficient mice exhibit more severe symptoms of EAE than WT mice, suggesting a downregulatory role for IFN-β during the development of EAE [37, 38]. Moreover, the role of the type I IFN receptor was recently examined by Prinz *et al* in EAE [39]. Despite being broadly expressed, the type I IFN receptor has a unique downregulatory role on myeloid cells in the CNS during EAE [39]. In light of our own data where we observed exacerbated EAE after pDC depletion during peak of disease, we speculate that pDC may protect from EAE by providing type I IFN to these myeloid cells in the CNS.

In conclusion, we demonstrate that pDC are important for the pathogenesis of EAE and that these cells can both promote and suppress EAE during different phases of the disease. We observed that type I IFN mediate this effect to some extent, but the interaction between pDC and Th17 cells appears to be central. Thus, our results strengthen the view that pDC can act as a bridge between the innate and adaptive immune systems, but the exact mechanisms by which these cells promote and regulate EAE remains to be elucidated.

Materials and methods

Antigen

Escherichia coli-derived rat $MOG_{1–125}$ was produced as previously described [40]. MOG consists of aa 1–125 of the extracellular part of native MOG and a histidin tag at the C terminus.

Mice

C57BL/6 female mice were obtained from Taconic and kept at the animal house at Rudbeck laboratories, Uppsala University, at specific pathogen-free conditions. All studies have been reviewed and approved by the local ethical committee.

EAE

Age- and sex-matched 9–17 wk-old female mice were immunized with 100–260 μg of MOG in CFA containing 0.5 mg *M. tuberculosis* H37RA (Difco, BD Diagnostic systems, Sparks, MD, USA) in IFA (Sigma-Aldrich, St. Louis, MO, USA) s.c. At the day of immunization and 2 days after, mice were injected with 200 ng of pertussis toxin (Sigma-Aldrich) in 200 μL PBS i.p. Clinical symptoms of EAE were scored daily as follows: 1, tail weakness or tail paralysis; 2, hind leg paraparesis; 3, partial hind leg paralysis; 4, complete hind leg paralysis; 5, tetraplegia, moribund state or death caused by EAE.

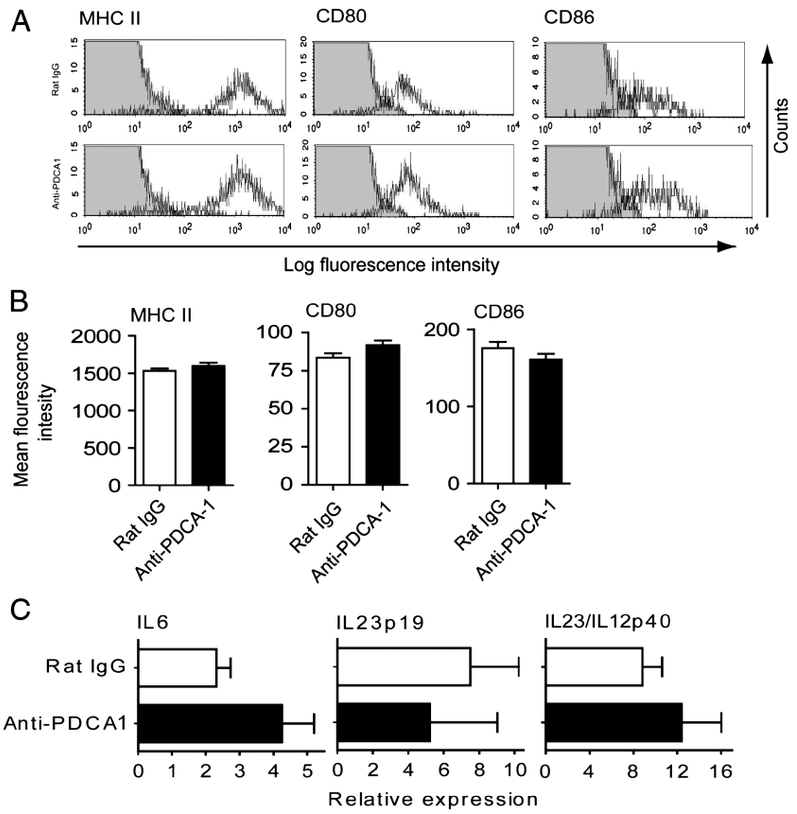

Figure 5. pDC are not required for maturation or proinflammatory cytokine expression by mDC. (A) Expression of MHC II, CD80 and CD86 on splenic CD11c^{hi} mDC from IgG- or anti-PDCA1-treated mice, respectively, 10 days after MOG immunization. (B) MFI of MHC II, CD80 and CD86 on CD11c^{hi} mDC ($n = 10$/group). Data show mean+SEM (Mann–Whitney's *U*-test). (C) CD11c^{hi}CD3^{-} splenic mDC were sorted by flow cytometry ($n = 6$/group). Q-PCR analysis of the mRNA expression of proinflammatory cytokines in mDC from normal IgG-treated (white bars) or anti-PDCA1-treated (black bars) mice, respectively. All values are normalized to 18s RNA. Data show mean+SEM (Mann–Whitney's *U*-test).

pDC depletion

To deplete the pDC fraction, mice were treated with 100 μg anti-PDCA1 mAb functional grade (Miltenyi Biotech, Bergisch Gladbach, Germany) or 100 μg control Ab (rat IgG; Sigma-Aldrich) injected i.p. with 200 μL PBS 1 day before and at the time of immunization as previously described [19]. The efficiency of the depletion was controlled by staining for CD11c, Gr-1 and B220 and subsequent flow cytometry analysis. The percentage of pDC in spleen (gated on a CD11c^{lo}Gr-1^{+}B220^{+} fraction) was measured 24 h after treatment with normal rat IgG or anti-PDCA1 mAb.

In addition, pDC were depleted during peak of disease in one experiment. Mice were injected with either 100 μg i.p. of anti-PDCA1 mAb or rat IgG on days 8, 10, 12 and 14 after MOG immunization.

Histological analysis

Brain and spinal cord from anti-PDCA1- or IgG-treated and MOG-immunized mice fixed by perfusion with 4% phosphate buffered paraformaldehyde were embedded in paraffin. Paraffin sections were stained with H&E, with Luxol fast blue for myelin and by Bielschowsky silver impregnation for visualization of neurons and axons. Demyelination scores: 0: no demyelination; 1: perivenous demyelination; 2: confluent demyelination. Adjacent serial sections were stained by immunocytochemistry, using primary Ab directed against CD3 or Mac-3. Immunocytochemistry was performed with a biotin avidin technique. Inflammatory index is the average number of inflammatory infiltrates *per* spinal cord cross section.

IFN-α/β depletion

To deplete IFN-α and -β *in vivo*, mice were injected s.c. with 50 000 neutralizing units of polyclonal sheep anti-mouse IFN-α/β Ab in 2 × 100 μL volume as previously described [23]. Normal sheep Ab served as control. Mice were treated with the anti-mouse IFN-α/β Ab or normal sheep Ab, either an hour before MOG immunization or 8 days after MOG immunization. To measure the efficiency of the ablation, the levels of IFN-α and -β were measured in sera by two dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) 2 or 10 days after injection.

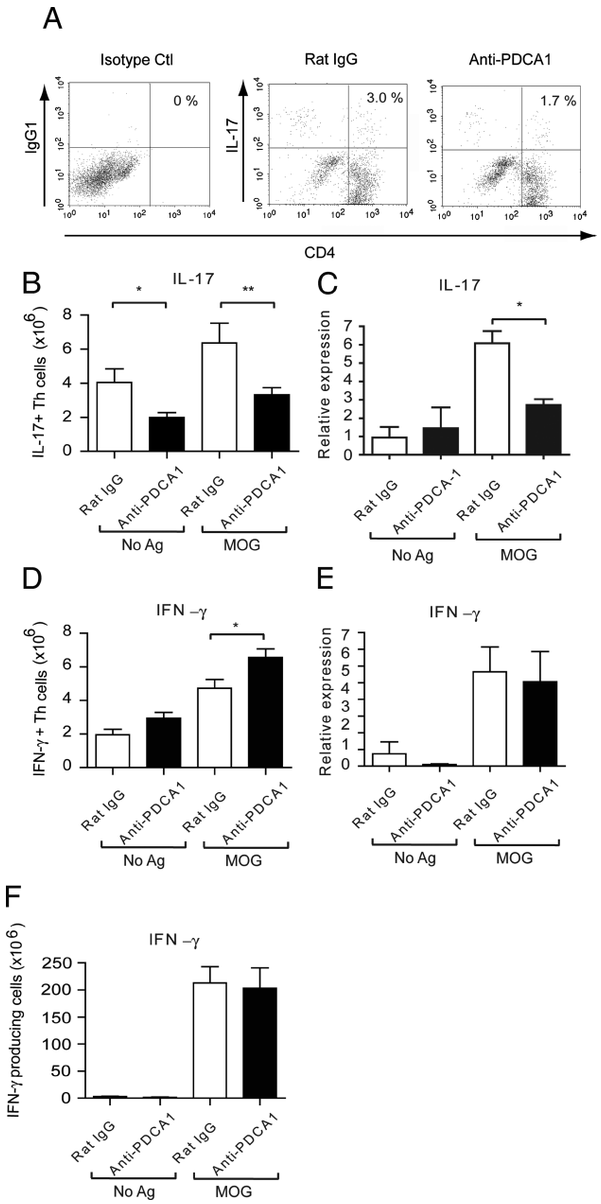

Figure 6. pDC enhances the MOG-specific Th17 responses in EAE. Splenocytes from rat IgG- (white bars) or anti-PDCA1-treated (black bars) mice were isolated 10 days after MOG immunization and restimulated for 48 h with (MOG) or without (No Ag) MOG. (A) Intracellular cytokine staining for IL-17. Percentage of IL-17$^+$ CD4$^+$CD3$^+$ Th17 cells of total Th cells in spleen. (B) Intracellular cytokine staining for IL-17. Mean absolute numbers of IL-17$^+$CD4$^+$CD3$^+$ Th17 cells *per* spleen ($n = 9$/group). (C) Q-PCR analysis of the mRNA expression of IL-17 in splenocytes ($n = 4$–5/group). All values are normalized to 18s RNA. Data are representative of two independent experiments. (D) Intracellular cytokine staining for IFN-γ. Mean absolute numbers of IFN-γ$^+$CD4$^+$CD3$^+$ Th1 cells *per* spleen ($n = 11$/group). (E) Q-PCR analysis of the mRNA expression of IFN-γ in splenocytes ($n = 13$/group). All values are normalized to 18s RNA. (F) ELISPOT analysis. Mean absolute numbers of IFN-γ-secreting cells *per* spleen ($n = 11$/group). Data show mean+SEM. $^*p<0.05$, $^{**}p<0.01$ (Mann–Whitney's *U*-test).

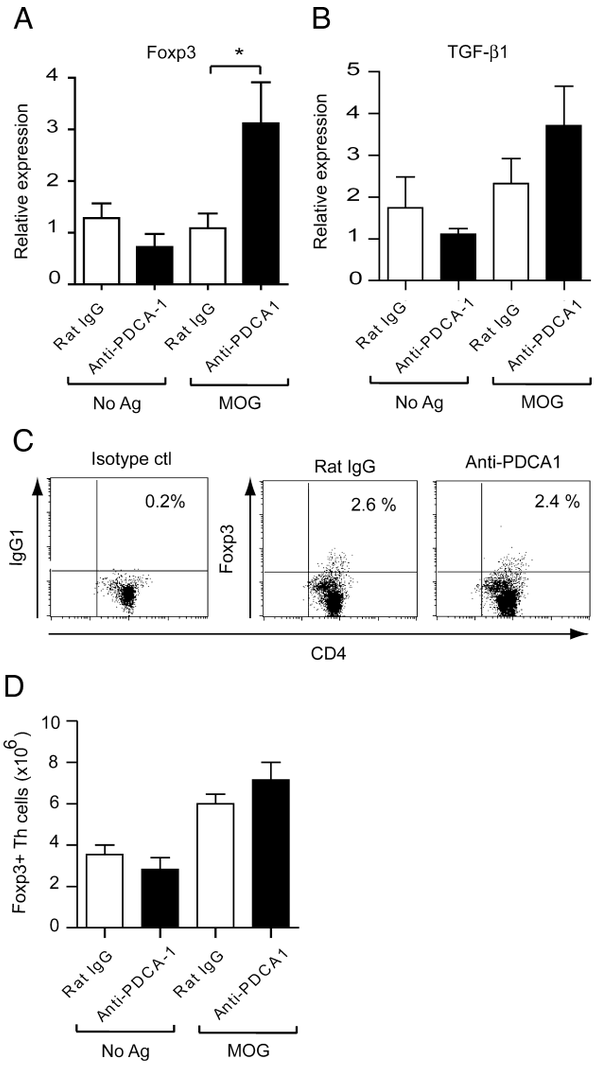

Figure 7. Amounts of Treg after pDC depletion. (A) Foxp3 and (B) TGF-β1 mRNA expression in splenocytes from IgG- or anti-PDCA1-treated mice. Q-PCR analysis of the mRNA expression of Foxp3 in splenocytes, from rat IgG- (white bars) or anti-PDCA1-treated (black bars) mice, isolated 10 days after MOG immunization and restimulated for 48 h with MOG (MOG) or without MOG (No Ag) ($n = 4$–5/group). All values are normalized to 18s RNA. Data show mean+SEM. $^*p<0.05$ (Mann–Whitney's *U*-test). (C) Percentage of Foxp3$^+$ CD4$^+$CD3$^+$ Treg of total Th cells in spleen 10 days after MOG immunization. (D) Mean absolute numbers of Foxp3$^+$CD4$^+$CD3$^+$ Treg *per* spleen from mice treated with rat IgG (white bars) or anti-mPDCA1 mAb (black bars), respectively ($n = 10$/group). Splenocytes were isolated on day 10 after MOG immunization and restimulated for 48 h with (MOG) or without (No Ag) MOG. Data show mean+SEM. (Mann–Whitney's *U*-test).

Isolation of splenocytes

Spleens were harvested 10 days after MOG immunization, cells were resuspended in DMEM (SVA, Uppsala, Sweden) and filtered through a 40 μm cellstrainer (Falcon BD)

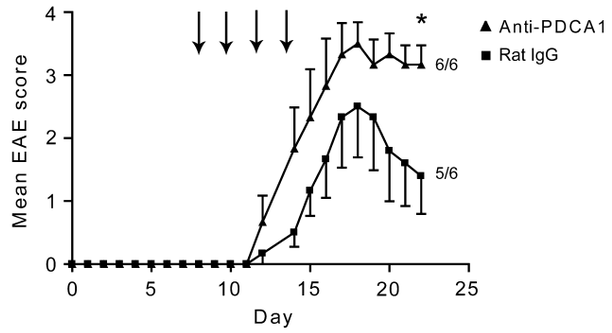

Figure 8. pDC depletion during peak of disease exacerbates EAE. Mean daily EAE scores ± SEM for mice treated with rat IgG or anti-PDCA1 mAb on days 8, 10, 12 and 14 (arrows) ($n = 6$/group). Numbers in the graph represent affected/total mice (Mann–Whitney's *U*-test).

Cell cultures

Splenocytes were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 292 μg/mL L-glutamine (DMEM complete) (all from Invitrogen, Carlsbad, CA, USA) with or without 5 μg/mL MOG for 48 h at 37°C and 5% CO_2.

Flow cytometry

Before staining, the cells were preincubated with rat anti-mouse CD16/CD32 (BD Pharmingen) to block binding of specific Ab to Fc receptors. The following rat anti-mouse Ab were used for staining from BD Biosciences (San Jose, CA, USA): CD3-FITC (anti-CD3), CD8b-PE (anti-CD8b), CD4-allophycocyanin (anti-CD4), IL-17-PE (anti-IL17), IFN-γ-PE (anti-IFN-γ), isotype control-PE (rat isotype control IgG1), CD11c-PE, MHC II-FITC, CD80-Alexa647, CD86-Alexa647 and from Biolegend (CA); Foxp3-PE.

Cultured splenocytes were prepared for intracellular staining of IL-17 or IFN-γ by stimulation with 500 ng/mL phorboldibutyrate (Sigma-Aldrich) and 500 ng/mL ionomycin (Sigma-Aldrich) for 3 h in the presence of Golgiplug™ containing brefeldin A (BD Biosciences) and subsequentially fixed and permeabilized using Cytofix/Cytoperm fixation/permeabilization kit (BD Biosciences) according to the manufacturer's instructions. Intracellular staining for Foxp3 was performed on cultured splenocytes after fixation and permeabilization using Cytofix/Cytoperm fixation/permeabilization kit (BD Biosciences). Flow cytometry analysis was performed on a FACSCalibur cytometer (BD Biosciences). The frequency of Th17, Th1 and Treg were subsequently multiplied with the total number of cells *per* spleen to get the total number of Th cell subset *per* spleen.

ELISPOT

To assess the number of IFN-γ-secreting splenocytes from IgG- or anti-PDCA1-treated and MOG-immunized mice, an ELISPOT method was used according to manufacturer's instructions (Mabtech, Nacka, Sweden). A total of 4×10^5 cells in 200 μL were cultured for 48 h in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 292 μg/mL L-glutamine (DMEM complete) (all from Invitrogen) with or without 5 μg/mL MOG for 48 h at 37 C and 5% CO_2. Spots were counted manually in a dissection microscope. The number of IFN-γ-secreting cells were then multiplied with the number of cells *per* spleen for the total number of cells *per* spleen.

Sorting of cells

Freshly isolated splenocytes were stained with the following Ab: anti-CD11c-PE, anti-B220-Cychrome and anti-CD3-FITC (BD Biosciences). $CD3^+$ T cells or $CD11c^{high}CD3^-$ mDC were sorted to greater than 94% purity using a FACSDiva cytometer (BD Biosciences).

Isolation of total RNA and cDNA synthesis

Total RNA was isolated from splenocytes using RNeasy mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Total RNA from sorted cells was isolated with RNeasy micro kit according to the manufacturer's instructions (Qiagen). cDNA synthesis was performed using Superscript III (Invitrogen).

Gene expression analysis

The expression of cytokine mRNA was measured by (Q-PCR) using SYBR green PCR kit (Qiagen) and amplification performed with MyiQ Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The relative amounts of the endogenous control 18s RNA and target mRNA in each sample could be deduced from the 18s RNA and target mRNA standard curves, respectively. The relative expression of each sample was calculated as the ratio between the relative amount of target and the relative amount of the corresponding endogenous control, 18s RNA.

DELFIA immunoassay

Sera from mice treated with normal IgG (Sigma) or anti-PDCA1 (Miltenyi Biotech) was collected at day 10 after immunization. The amount of IFN-α and IFN-β was measured with DELFIA as previously described [41]. The detection limit was ≥ 6 U/mL for IFN-α and ≥ 20 U/mL for IFN-β.

Serum IL-6 ELISA

Sera were collected 10 days after immunization. IL-6 was measured by ELISA according to the manufacturer's instructions (R&D Systems, England, UK). The detection limit was 1.6 pg/mL.

Statistical analysis

Differences between mean daily EAE scores for individual Ab-treated mice, gene expression and cytokine levels were analyzed with Mann–Whitney's *U*-test. *p*-Values lower than 0.05 were considered significant. To measure differences in mRNA expression of IFN-γ levels in the spleen (Fig. 6E), we pooled the data from two independent experiments. Samples belonging to experiment "1" or "2" were used as a factor in a two-way ANOVA analysis as previously described [17], and the analysis revealed that it was possible to pool the samples. All analyses were performed using Graphpad Prism™ 4.0 software.

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Abbreviations: **DELFIA:** dissociation-enhanced lanthanide fluoroimmunoassay · **mDC:** myeloid DC · **MOG:** myelin oligodendrocyte glycoprotein · **pDC:** plasmacytoid DC · **PDCA1:** pDC Ag-1 · **Q-PCR:** quantitative RT-PCR

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