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CNS Plasmacytoid Dendritic Cells Regulate the Severity of Relapsing Experimental Autoimmune Encephalomyelitis¹

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Abstract

Plasmacytoid dendritic cells (pDC) have both stimulatory and regulatory effects on T cells. pDCs are a major CNS-infiltrating DC population during experimental autoimmune encephalomyelitis (EAE), but unlike myeloid DCs (mDC) have a minor role in T cell activation and epitope spreading. We show that depletion of pDCs during either the acute or relapse phases of EAE resulted in exacerbation of disease severity. pDC depletion significantly enhanced CNS but not peripheral CD4⁺ T cell activation, as well as IL-17 and IFN- γ production. Moreover, CNS pDCs suppressed CNS mDC-driven production of IL-17, IFN- γ and IL-10 in an IDO-independent manner. The data demonstrate that pDCs play a critical regulatory role in negatively regulating pathogenic CNS CD4⁺ T cell responses highlighting a new role for pDCs in inflammatory autoimmune disease.

Keywords

Autoimmune disease; T lymphocytes; dendritic cells; T cell activation; antigen presentation; immunoregulation

Introduction

Experimental autoimmune encephalomyelitis (EAE), is a widely employed model for multiple sclerosis (MS) (1) that is initiated and driven by myelin-specific CD4⁺ T cells producing IFN- γ and TNF (Th1) and IL-17 (by Th17) in the CNS (2,3). Disease progression in relapsing EAE (R-EAE) is characterized by ‘epitope spreading’ where new T cell responses develop to myelin epitopes distinct from the priming epitope (4). Our recent evidence indicates peripherally-derived myeloid DCs (mDCs) prime naïve CD4⁺ T cells in the CNS inducing a Th17 dominant phenotype (5,6). In contrast, CNS plasmacytoid DCs (pDCs) are inefficient at inducing the proliferation of and cytokine production by naïve and activated myelin specific CD4⁺ T cells, although the pDCs can ingest myelin proteins (6).

Enhanced numbers of activated pDCs have been described in MS, and activated pDCs associated with Sjögren's syndrome lupus and psoriasis (7-11). It was therefore important to

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understand the role of pDCs in MS/EAE pathogenesis. Using a monoclonal antibody (anti-mPDCA-1) to deplete pDCs from the CNS following R-EAE induction, we show that pDC depletion caused the rapid exacerbation of EAE severity in both the primary acute and relapse phases. Mechanistically, pDC depletion did not affect the frequency of myelin specific CD4⁺ T cells in peripheral lymphoid organs, but markedly enhanced CNS CD4⁺ T cell activation, as well as IL-17 and IFN- γ production. Moreover, CNS pDCs suppressed CNS mDC-driven production of IL-17, IFN- γ and IL-10 in an IDO-independent manner.

Materials and Methods

Mice

Female SJL/J mice were purchased from Harlan Sprague Dawley (SJL/JCrHsd) or Taconic (SJL/JCrNtac). Mice were housed and cared for according to Northwestern University ACUC approved protocols.

Induction of EAE

As previously reported using 50 μ g PLP₁₃₉₋₁₅₁ (6).

Peptides & Antibodies

PLP₁₃₉₋₁₅₁ (HSLGKWLGHDPKF) synthesized to > 95% purity by Genemed Synthesis, Inc. Conjugated antibodies were purchased from BD Pharmingen or eBioscience.

Depletion of plasmacytoid DCs

Mice received intraperitoneal injections of 250 μ g anti-mPDCA-1 (clone JF05-1C2, rat IgG_{2b}; Miltenyi Biotec), or purified rat IgG_{2b} (eBioscience) every other day for 4 treatments.

Isolation of cells from secondary lymphoid tissues and CNS

As previously reported (6).

Flow cytometric analysis and gating

For analysis of cytokines, CNS cells were cultured for 4 hours in R10 media (6) plus golgi-stopTM (BD) according to manufacturers recommendations. Cells were stained with 5-6 color antibody cocktails. Amine-reactive, fixable live/dead viability dye was used according to manufacturers instructions (Molecular Probes, Invitrogen) and dead cells excluded. Data was acquired on an LSR II cytometer (BD), and analyzed using FACS DIVA (BD) or Flow Jo (Treestar Inc.) software.

CD4⁺ T cell activation assay

Cell populations were flow sorted as described in (6) to > 98% purity. Sorted populations were >95% pure. 2 \times 10⁴ APCs were co-cultured with 10⁵ CD4⁺ T cells from the CNS or spleen for 96 h with R10 media, 5 μ g/ml antiCD3 and 200 μ M 1 methyl-D-tryptophan (Aldrich Chemical Company, Milwaukee), prepared according to (12). Carrier solution alone was added as a control. Cells were assessed for viability by flow cytometry as described above, and cytokines in the supernatants assessed by cytokine bead array for levels of IL-10, IL-17, and IFN- γ , according to manufacturers instructions (Upstate).

ELISPOT assays

ELISPOTS were performed as previously described (13), with 10⁶ cells plus 10 μ M PLP₁₃₉₋₁₅₁.

Immunohistochemistry

Immunohistochemistry was performed on 6 μm thick frozen cerebellar and lumbar spinal cord sections from PBS-perfused mice as previously described (13). Staining was analyzed using a Leica DM5000B fluorescent microscope and Advanced SPOT software.

Statistical Analysis

Differences between groups were determined using an unpaired Students t test.

Results and Discussion

Anti-mPDCA-1 mAb efficiently and specifically depletes CNS-infiltrating pDCs during EAE

pDCs are a minor subset of DC in the secondary lymphoid tissues of most mouse strains (14), comprising 23% of DCs, and 1.15% of cells in the LNs of SJL/J mice (data not shown). Strikingly, CNS infiltrates during EAE contain 37.7% pDCs, 5.4% of the total CNS mononuclear cells (6). To investigate the role of pDCs during EAE, anti-mPDCA-1 mAb (15) was used to deplete pDCs during EAE onset. One day after pDC depletion, pDCs were depleted from LNs (not shown) (15) and CNS ($92.4 \pm 0.9\%$ of CNS pDCs) (Fig. 1a,b). During the relapse phase of EAE, nine days following pDC depletion, the number of CNS pDCs returned to control levels in pDC depleted animals (Fig. 1a), and, importantly, anti-mPDCA-1 treatment did not affect the numbers of CNS mDCs or macrophages (Fig. 1b). Following pDC depletion a few mPDCA-1⁺ cells remained in the meningeal area of the CNS, however no mPDCA-1⁺ pDCs were detected in the parenchyma of the spinal cord or cerebellum (Fig. 1c). Inefficient clearing of pDCs from the blood vessel-rich areas of the CNS correlated with low level mPDCA-1 expression on blood CD11c⁺ B220⁺ pDCs (data not shown).

pDCs regulate the severity of EAE

The depletion of pDCs at the onset of R-EAE resulted in a significant exacerbation of peak clinical disease (Fig. 2a). Clinical scores in pDC-depleted mice returned to control levels 2-3 days after the last anti-mPDCA-1 mAb treatment, consistent with a report showing that pDC numbers recover 3-5 days following anti-mPDCA-1 depletion (15). That pDC depletion caused an immediate increase in clinical severity, with a rapid return to control levels with pDC reconstitution suggests that pDCs have a direct, acute regulatory effect on CNS autoimmune disease. pDCs were then depleted during the primary relapse of EAE, leading to enhanced EAE and the abrogation of secondary remission (Fig. 2b).

The depletion of pDCs could have affected the priming of pathogenic T cells in the periphery (16), explaining the clinical outcome of pDC depletion (Fig 2a,b). However, the frequency of IL-17, IFN- γ and IL-2-producing CD4⁺ T cells specific for the immunizing peptide in the LNs, one day following the final mPCDA-1 mAb injection was unchanged compared with controls (Fig. 2c). In fact, PLP₁₃₉₋₁₅₁-specific Th17 cells were significantly reduced in the spleens of pDC depleted mice, which may be reflective of reduced EAE severity (3), not enhanced severity as observed in the clinical experiments.

pDCs modulate the activation and frequency of Th1 and Th17 cells in the CNS

Clinical EAE generally correlates with the number and activation status of CNS-infiltrating effector CD4⁺ T cells, and inversely with Treg numbers (17). We found that pDC depletion did not significantly affect the numbers of CNS CD4⁺ T cells and Foxp3⁺ CD4⁺ Tregs ($p = 0.2$; Fig. 3a). We previously showed that CNS pDCs isolated during EAE poorly activate both naïve and activated myelin-specific T cells in comparison to mDCs (6). Because there was little change in the T cell numbers it is unlikely that the primary function of pDC during EAE is to prime and expand CD4⁺ T cells in the CNS.

Strikingly however, in contrast to peripheral responses, CNS CD4⁺ T cells were highly activated and produced more IL-17 and IFN- γ in the absence of pDCs. Following pDC depletion, CNS CD4⁺ T cells were significantly more activated than controls as assessed by down-regulation of CD45RB and upregulation of CD25 (Fig. 3b). Endogenous production of inflammatory cytokines by CNS CD4⁺ T cells was determined by incubating CNS isolates, which contain pathogenic T cells and DCs presenting endogenous myelin peptides (6), with golgi-stop for 4 h, then analyzing accumulated cells expressing IFN- γ and IL-17. The frequency of CNS Th17 cells was increased by an average of 1.6 ± 0.38 fold, and Th1 cells by 1.6 ± 0.27 fold, and more cytokine per cell (enhanced mean fluorescence intensity) was produced in pDC depleted mice (Fig. 3c). Thus, CNS pDCs promote the accumulation of CD4⁺ T cells and Tregs in the target organ, but strongly modulate the activation status of CD4⁺ T cells and, importantly, the frequency of CNS Th1 (IFN- γ) and Th17 (IL-17) cells.

CNS pDCs actively suppress IL-17, IFN- γ , and IL-10 production by CNS CD4⁺ T cells, in an IDO-independent manner

We next sought to determine the mechanism by which pDCs regulate CNS CD4⁺ T cell activation and cytokine production. pDCs have been implicated in inducing T cell anergy through IFN- α and IL-10 production (18), or TGF- β (19). Using quantitative PCR, we have previously shown that CNS pDCs expressed low levels of TGF- β transcripts (6), and similar levels of IFN- α 4 mRNA (not shown) compared with other CNS APCs. In addition, IL-10 levels were lower in CNS pDC - CNS T cell co-cultures (Fig. 4a) and CNS pDCs stimulated with CD40 ligand (not shown) compared with CNS mDCs. Based on the low production by CNS pDCs, it is unlikely that TGF- β , IL-10, or IFN- α 4 are a dominant pathway for pDC suppression of CD4⁺ T cells in the CNS.

pDCs are known to produce the T cell inhibitor indoleamine 2,3-dioxygenase (IDO) in response to interferons (12,20). To determine if CNS pDCs produce IDO that suppresses CD4⁺ T cells during EAE, CNS pDCs and mDCs isolated during acute R-EAE were co-cultured with CD4⁺ T cells from the CNS and spleen of the same animals +/- 1-MT. pDCs were poor stimulators of CNS CD4⁺ T cell survival and expansion *ex vivo* and addition of 1-MT had little effect on CNS T cell viability in the presence of pDCs or mDCs (not shown). In agreement with our previously published work (6), CNS mDCs supported the highest levels of IL-17, IFN- γ and IL-10 production by both splenic and CNS-derived T cells (Fig. 4.). The IDO inhibitor 1-MT enhanced mDC-induced CD4⁺ T cell IL-17 and IFN- γ secretion, and decreased IL-10 production. However, 1-MT had no effect on cytokine production in CD4⁺ T cells cultured with CNS pDCs. Most profoundly, when CNS pDCs were co-cultured with CNS mDC and CNS T cells, production of IL-17, IFN- γ and IL-10 was significantly suppressed (Fig. 4a). These results indicate that CNS pDCs regulate CD4⁺ T cell cytokines in an active manner that dominates that of mDCs-driving Th17 cells in the CNS (6), and regulation is via an IDO-independent pathway. IFN- β modulates IFN γ and IL-17 production by human PBMCs (21), thus pDC production of IFN- β (22) may play a primary role in pDC modulation of Th1/Th17 activation during R-EAE concordant with the observation that IFN- β treatment is therapeutic in both EAE and MS (23,24). We are currently investigating the role of IFN- β production by pDCs during EAE.

In summary, we demonstrate an acute, dominant regulatory role for pDCs in CNS autoimmune disease. pDC depletion results in exacerbated EAE, but once pDCs return to normal levels, relapse severity returns to control levels. Normal relapses suggest the priming of naïve T cells in the CNS is unaffected. pDCs suppress mDC-dependent induction/expansion of CNS Th17 and Th1 cells (Fig. 4a). pDC suppression of T cell cytokine production is IDO-independent, but not due to killing of T cells, because in CNS mDC/pDC co-cultures, CNS CD4⁺ T cells have enhanced viability (not shown). These data support a dominant regulatory role for pDCs

during EAE in that pDCs recruited to the CNS limit pathology by regulating T cell activation and cytokine production. Treatments that support and expand regulatory pDCs may therefore be attractive therapies for T cell-mediated autoimmune diseases.

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Abbreviations used in this paper

EAE	experimental autoimmune encephalomyelitis
MS	multiple sclerosis
mDC	myeloid dendritic cells
pDC	plasmacytoid dendritic cells
PLP	proteolipid protein

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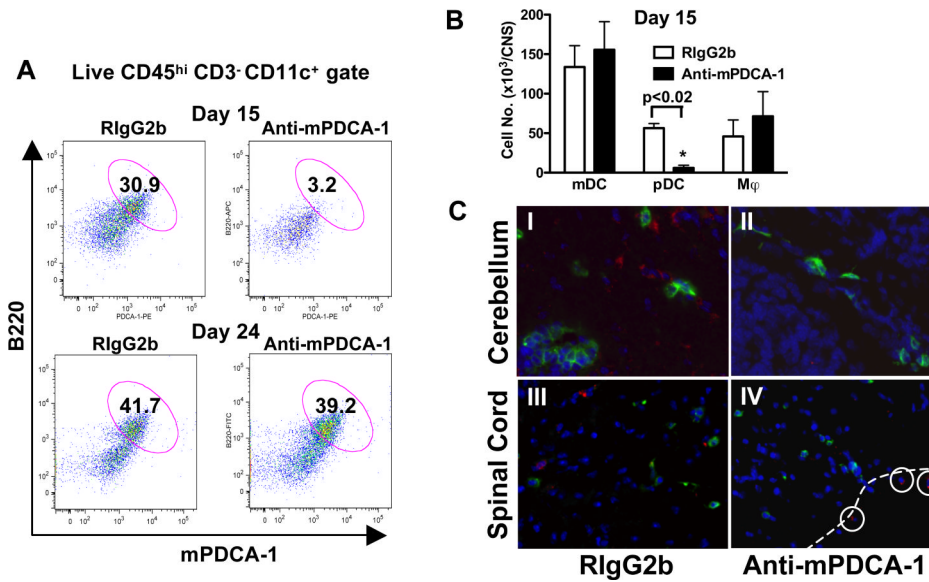


Figure 1. anti-mPDCA-1 efficiently and specifically depletes CNS pDCs during EAE
(a) SJL mice were treated with anti-mPDCA-1 or isotype-matched control (Rat IgG2b) mAb on days 8-14 after EAE induction. One day (day 15) and 9 days after pDC depletion (day 24), the effect of treatment on gated live CNS DCs (CD45^{high}CD3⁻CD11c⁺) was determined. **(b)** Day 15 analysis of CNS CD11b⁺ mDCs, CD11b⁻B220⁺ pDCs, and CD11b⁺CD11c⁻ macrophages (M ϕ). Data are mean \pm SEM of 4 animals per group representative of 5 experiments. p value determined by Mann-Whitney. **(c)** Day 15 immunohistochemical analysis of (a), cerebellum (I and II) and lumbar spinal cord (III and IV) stained for mPDCA-1 (red), CD4 (green) and DAPI (blue). Dotted line shows the meningeal edge of spinal cord tissue. mPDCA-1⁺ pDCs are circled. Magnifications $\times 200$ (I and II) and $\times 100$ (III and IV) are representative of 3 mice.

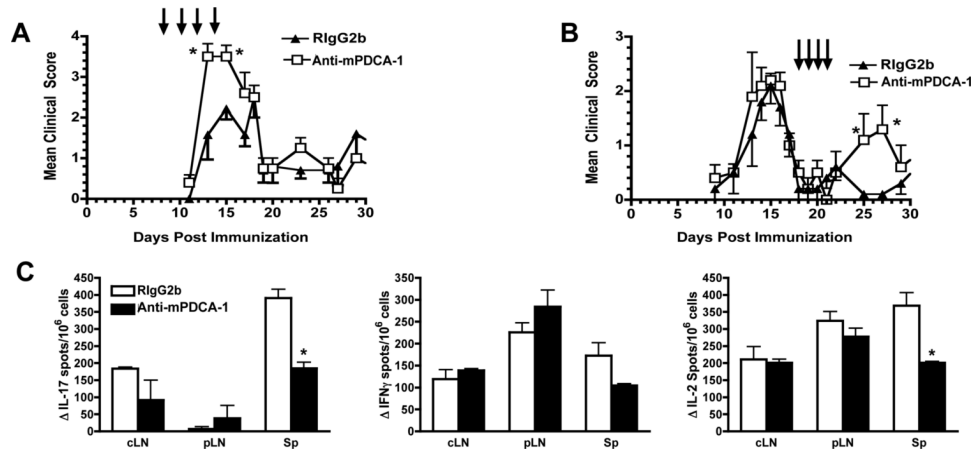


Figure 2. CNS pDC depletion increases the severity of acute and relapsing R-EAE
(a) Clinical EAE course following anti-mPDCA-1 or isotype control (RlgG2b) mAb treatment (arrows). Mean clinical scores \pm SEM of 5-7 mice per treatment group, 1 of 5 experiments.
(b) Treatment of EAE during remission (days 18-21). Mean clinical scores \pm SEM of 5 mice per treatment group, 1 of 2 experiments. *Mean clinical scores are significantly different from controls ($p < 0.05$; Mann-Whitney). **(c)** Day 15 ELISPOTS for IFN- γ , IL-17, and IL-2 in response to PLP₁₃₉₋₁₅₁ with cells from the cervical LN (cLN), inguinal LN (pLN) and spleen (Sp). Mean \pm SEM of 3 animals, 1 of 3 experiments. *Values significantly different, $p < 0.01$; un-paired Students t test.

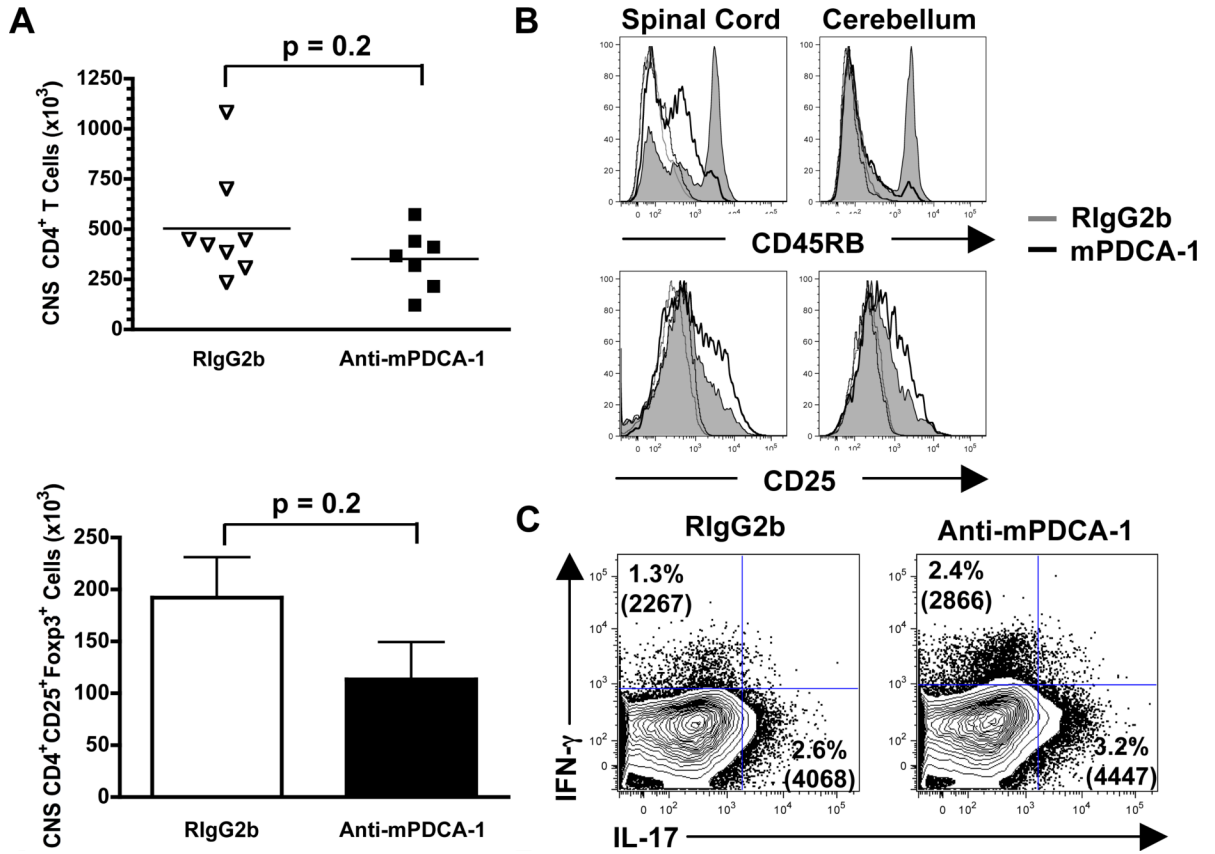
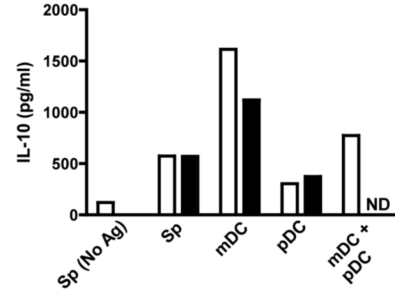
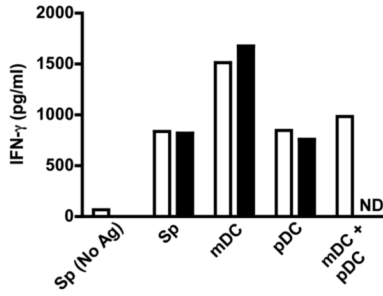
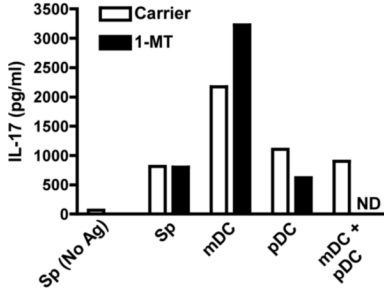


Figure 3. pDCs regulate CNS CD4⁺ T cell activation and IFN- γ and IL-17 production
(a) At day 15 after EAE induction, and treatment with RlgG2b or mPDCA-1 days 8-14, the numbers of CNS CD45⁺CD3⁺CD4⁺ T cells and CD4⁺FoxP3⁺CD25⁺ Tregs were enumerated. Data points for CD4⁺ T cells represents mice from 2 experiments, and the bars indicate the number of FoxP3⁺ T cells \pm SEM of 4 animals in 2 experiments. p values determined by Mann-Whitney. **(b)** CD45RB and CD25 expression by CNS CD4⁺ T cells at day 15. Isotype control staining is shown in dotted lines of corresponding colors. Representative of 4 animals from 2 experiments. **(c)** Intracellular cytokine analysis of CNS CD4⁺ T cells isolated from 2 mice at day 15. The percentage of IFN- γ and IL-17 staining cells within the viable CD45^{hi}CD3⁺CD4⁺ gate and the mean fluorescence intensities are indicated in parentheses. Results representative of 3 experiments.

A CNS CD4⁺ T Cells



B Splenic CD4⁺ T Cells

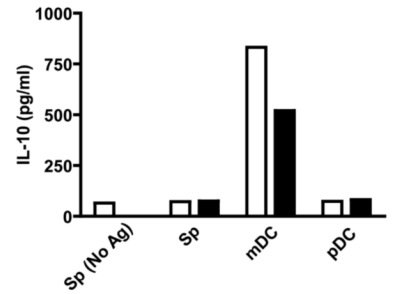
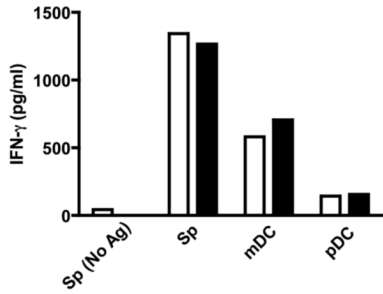
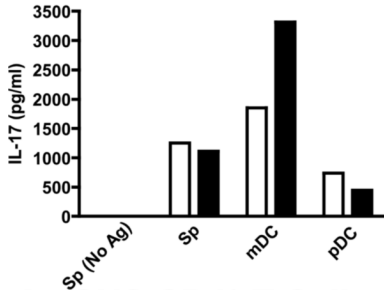


Figure 4. pDCs actively suppress mDC supported IFN-γ, IL-17 and IL-10 production by CNS CD4⁺ T cells

On day 14, 10⁵ CD4⁺ T cells from the CNS (a) or spleen (b) were cultured with 2x10⁴ irradiated splenic APCs (Sp), or sorted CNS pDCs, mDCs or a 1:1 mixture of pDCs and mDCs in the absence (white bars) or presence (black bars) of 1-MT. The amount of secreted IFN-γ, IL-17 and IL-10 in the culture supernatants was determined 4 days later. The results are representative of 3 experiments where 15-20 perfused mice were pooled. ND, not done.