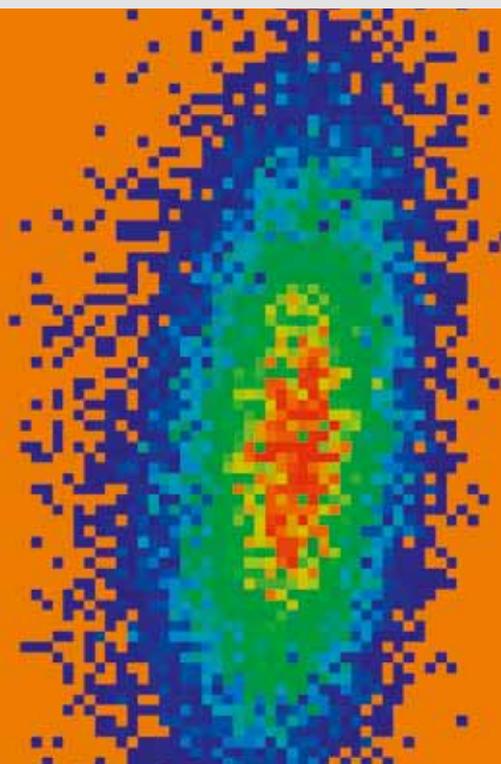


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Vasoactive intestinal peptide inhibits IFN- α secretion and modulates the immune function of plasmacytoid dendritic cells



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Introduction

Plasmacytoid dendritic cells (PDCs) are one of the major subpopulations of dendritic cells (DCs) contributing to viral defense and immune tolerance. PDCs are characterized by their ability to produce high levels of type 1 interferons, for example interferon alpha (IFN- α), which is the reason why they were formerly referred to as natural IFN- α -producing cells. Upon viral infections, they secrete large amounts of IFN- α which blocks viral replication and stimulates innate and adaptive immune responses¹. PDCs were also shown to be critically involved in the pathogenesis of autoimmune processes and may therefore play a major role in the maintenance of tolerance.²⁻⁴

Vasoactive intestinal peptide (VIP) is a 28-amino-acid peptide that was first isolated from the gastrointestinal tract and was shown to have vasodilatory capacity⁵. It was also identified in the central nervous system and peripheral nerves⁶ and has therefore been traditionally defined as a neuropeptide. Further on, VIP was shown to be involved in the modulation of innate and adaptive immune responses⁷⁻⁹. VIP can be considered a cytokine because it is produced and secreted by immune cells including lymphocytes and granulocytes in response to various immune signals⁸. VIP exhibits a broad spectrum of immunological functions and exerts them, in a paracrine and/or autocrine way, through

three different specific receptors, VPAC1, VPAC2, and PAC1^{10,11}. Although VIP has been classically considered an immunosuppressive agent, several studies suggest that this peptide is rather a modulator of the homeostasis of the immune system^{11,12}. The immunological effects of VIP have been studied in mice¹³, but its role in the human immune system is less clear and its effects on PDC function are unknown.

In this study we investigated whether human PDCs express VIP receptors and whether VIP has immunological effects on PDCs to elucidate the role of VIP in human PDC function¹⁴.

Material and methods

(for details see ref. 14)

PDC isolation and cell culture

This study was reviewed and approved by the University of Iowa Institutional Review Board. Buffy coats of 20 healthy subjects were received from the Blood Bank at the University of Iowa. PBMCs were separated by density gradient centrifugation and remaining erythrocytes were lysed with ammonium chloride-potassium lysing buffer. PDCs were isolated from PBMCs using the CD304 (BDCA-4/Neuropilin-1) MicroBead Kit for positive selection of PDCs or the Plasmacytoid Dendritic Cell Isolation Kit for

the isolation of untouched PDCs by depletion of unwanted cells. To determine the purity of the isolated PDCs, the cells were stained with CD303 (BDCA-2)-PE and with a cocktail of FITC-conjugated anti-lineage antibodies. The isolated cell fractions contained more than 90% CD303 (BDCA-2)⁺ cells and less than 5% CD3⁺ or CD14⁺ cells. For PCR and some additional experiments, the Diamond Plasmacytoid Dendritic Cell Isolation Kit was utilized. This kit enables to obtain almost pure PDC fractions, combining untouched isolation of PDCs and subsequent positive selection with CD304 (BDCA-4/Neuropilin-1) MicroBeads. After PDC isolation using the Diamond Plasmacytoid Dendritic Cell Isolation Kit, more than 98% of the cells were positive for CD303 (BDCA-2). PDCs were cultured for 2 days in AIM-V medium (Invitrogen), supplemented with 10 ng/mL recombinant human IL-3 (R&D Systems) in a flat-bottom 96-well plate (Corning) at a density of 1×10⁵ cells/well. Purity at day 2 was 99% PDCs. PDCs were treated with 1 μ g/mL class B CpG oligodeoxynucleotide (CpG ODN 2006; Coley Pharmaceutical Group) and 10⁻⁶ M VIP (Bachem) after testing various concentrations. As control cells served the human lymphoblast cell line Molt-4b and the human colon carcinoma cell line HT-29, cultured as described previously¹⁵.

Immunofluorescence

The PDC suspensions were fixed with paraformaldehyde and applied to poly-L-lysine-coated coverslips. Protein blocking was performed with either normal goat serum and BSA for VPAC1 staining or normal rabbit serum for VPAC2 staining. For staining of VPAC1, PDCs were labeled with mouse anti-human VIPR1 (Exalpha Biologicals) as primary antibody followed by incubation with the secondary antibody goat anti-rabbit Alexa Fluor 568 (Invitrogen). For VPAC2 staining, PDCs were incubated with goat anti-human VPAC2 (Santa Cruz Biotechnology) as primary antibody followed by labeling with the secondary antibody rabbit anti-goat Alexa Fluor 568 (Invitrogen). Staining with CD303 (BDCA-2)-FITC (Miltenyi Biotec) was performed to demonstrate specificity for PDCs; cell nuclei were counterstained with To-Pro-3 (Invitrogen). The cells were examined and imaged using a Bio-Rad MRC1024 laser scanning confocal microscope.

Real-time RT-PCR

RNA was isolated from purified PDCs (98% CD303 (BDCA-2)⁺CD3⁻CD14⁻) and the control cell lines Molt-4b and HT-29. The lymphoblast cell line Molt-4b served as negative control for VPAC1 and positive control for VPAC2; the colon carcinoma cell line HT-29 served as positive control for VPAC1 and negative control for VPAC2¹⁵. The RNA concentration was determined by spectrophotometry and RNA was reverse-transcribed. Generated cDNA was quantified in PCR conducted with the Platinum Quantitative PCR SuperMix-UDG kit (Invitrogen). Primers and probes were purchased from Integrated DNA Technologies (for details see ref. 14). Δ Ct (cycle threshold) was determined by subtracting the average rRNA Δ Ct value from the average VPAC Ct value. $\Delta\Delta$ Ct values were calculated by subtraction of Δ Ct values from controls Molt-4b (for VPAC1) and HT-29 (for VPAC2), and the final value was determined as $2^{-\Delta\Delta$ Ct} (PE Applied Biosystems).

Phenotypic determination by flow cytometry

We evaluated PDC expression profiles of surface molecules with significance for T cell–PDC interactions including the costimulatory molecules CD80 and CD86,

the antigen-presenting molecules MHC class I and II, the chemokine receptor CCR7, the immunological synapse-establishing molecule CD304 (BDCA-4/Neuropilin-1), and the DC activation marker CD83. Fluorescently labeled antibodies to CD80, CD83, CD86, HLA-ABC, HLA-DR, CCR7, CD304 (BDCA-4), and CD303 (BDCA-2) were used to stain the PDCs. CD3 and CD14 were used to exclude contaminating T cells and monocytes, respectively. PDCs were analyzed by flow cytometry; data were expressed as median fluorescence intensities (MFI) of treated cells relative to the MFI of non-treated cells.

ELISAs

PDCs were isolated as described previously and cultured in IL-3-containing AIM-V medium in the presence of CpG ODN and VIP. After 2 days, culture supernatants were harvested. IFN- α , TGF- β , and IL-10 concentrations were determined by sandwich immunoassays.

T cell/PDC-cocultures and CFDA-SE staining

CD4⁺ T cells from healthy donors were isolated by MACS[®] Technology via positive selection using CD4 MicroBeads and labeled with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Molecular Probes). The CFDA-SE-labeled T helper cells were then cocultured with allogeneic PDCs, which were pretreated with CpG ODN in the presence or

absence of VIP, for 5 days at different ratios. Cells were harvested and analyzed by flow cytometry.

Statistical analysis

Statistical significance was assessed by Student's *t*-test and mixed linear regression analysis as appropriate. Differences were considered significant at a *p*-value ≤ 0.05 and highly significant at a *p*-value ≤ 0.01 .

Results

VPAC1 and VPAC2 are expressed on human PDCs

Previously, we demonstrated that VPAC1 and VPAC2 receptors are expressed on human monocytes and T cells⁹. The present study was designed to delineate the significance of VIP for PDCs. Therefore, we first investigated whether or not PDCs express the VIP receptors VPAC1 or VPAC2. To test the expression of the respective receptor proteins, PDCs were isolated (fig. 1A), fixed, and stained with Abs to human VPAC1 and VPAC2 for analysis by confocal microscopy. As illustrated in figure 2, PDCs show expression of both VPAC1 and VPAC2 in a pattern characteristic of membrane-bound proteins. Human cell lines HT-29 and Molt-4b served as negative and positive controls.

To confirm results obtained by immunofluorescence microscopy, PDCs

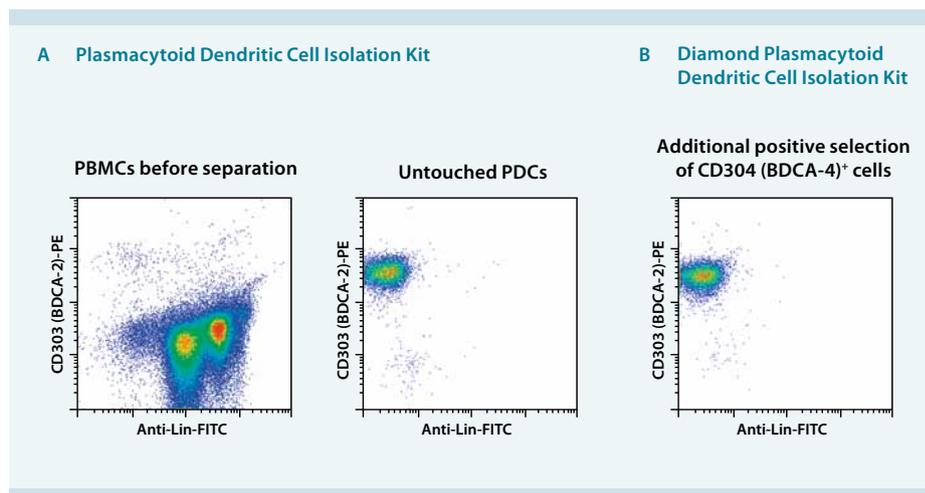


Figure 1 Isolation of PDCs from human PBMCs. Untouched PDCs were isolated by magnetic labeling and depletion of non-PDCs using a Biotin-Antibody Cocktail and Anti-Biotin MicroBeads (A). For increased purity, PDCs were additionally isolated by positive selection using CD304 (BDCA-4/Neuropilin-1) MicroBeads (B). Cells were stained with PDC-specific marker CD303 (BDCA-2) and a lineage cocktail.

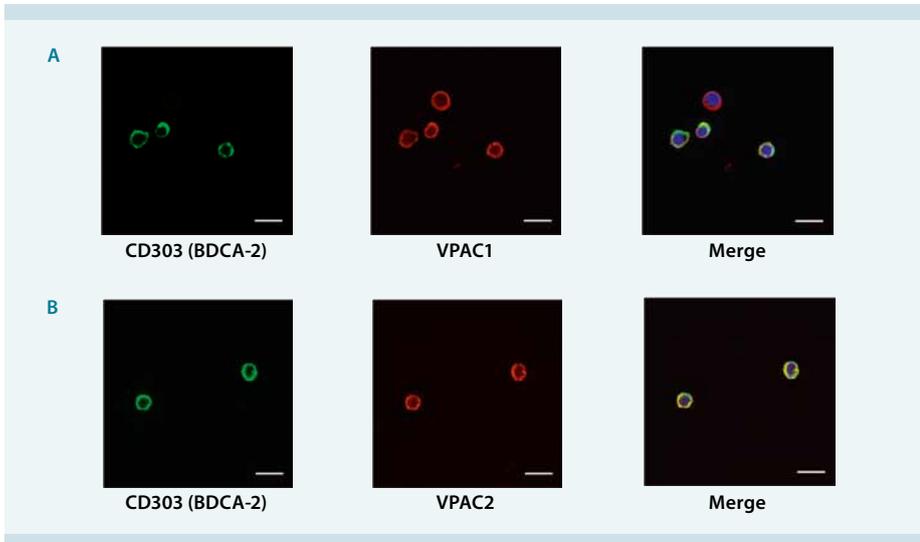


Figure 2 Expression of VIP receptors on PDCs. Isolated PDCs were stained for VIP receptor expression with (A) anti-VPAC1 (red stain) or (B) anti-VPAC2 (red stain) in combination with the PDC-specific marker CD303 (BDCA-2)-FITC (green stain). Analysis by confocal microscopy shows the expression of both VPAC1 and VPAC2 on the cell surface of human PDCs. Bar indicates 15 μ m.

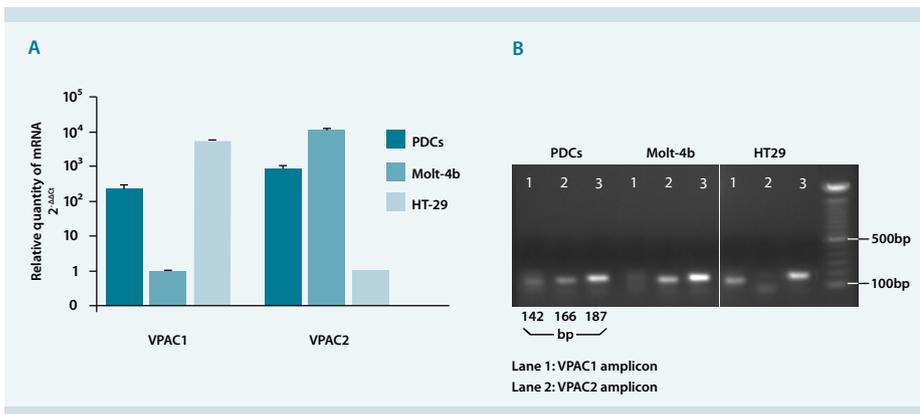
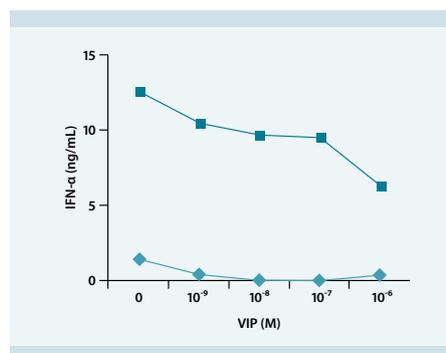


Figure 3 VIP receptor mRNA expression in PDCs. PDCs were isolated with the Diamond Plasmacytoid Dendritic Cell Isolation Kit and mRNA was isolated immediately. The relative quantity of VIP receptor VPAC1 and VPAC2 mRNA in PDCs was determined by real-time RT-PCR. The lymphoblast cell line Molt-4b served as negative control for VPAC1 and positive control for VPAC2, the colon carcinoma cell line HT-29 served as positive control for VPAC1 and negative control for VPAC2. (A) Bar graphs represent relative mRNA quantity. Error bars indicate SD. (B) The PCR products were fractionated in 1% agarose gels. The expected amplicon sizes were 142 bp for hVPAC1, 166 bp for hVPAC2, and 187 bp for 18S ribosomal RNA fragment.

Figure 4 VIP inhibits IFN- α secretion by PDCs. Isolated PDCs were cultured in the presence of IL-3 and various concentrations of VIP alone (diamonds) or in combination with class B CpG ODN (squares). After 48 hours, supernatants were harvested and IFN- α concentrations were determined by ELISA. VIP-dose response is shown as line graphs representative of two independent experiments.



were isolated with high purity (fig. 1B), and total RNA was immediately extracted for quantitative analysis of VPAC1 and VPAC2 transcripts by real-time RT-PCR. As shown in figure 3, PDCs express RNA for both VPAC1 and VPAC2. RNA extracted from cell lines HT-29 and Molt-4b served as control. These data demonstrate that VPAC1 and VPAC2 are co-expressed in freshly isolated PDCs, as has been shown for human T cells and monocytes¹¹.

VIP inhibits IFN- α secretion by PDCs

A main feature of PDCs is their capacity to secrete large amounts of IFN- α in response to viral stimuli as well as to synthetic CpG ODN¹. We therefore investigated the effect of VIP on IFN- α production in response to class B CpG ODN. CpG ODN are synthetic ODNs containing CpG motifs, which mimic viral or bacterial DNA and are potent PDC activators¹⁶. PDCs were incubated in IL-3-containing AIM-V medium for 48 h in the presence or absence of VIP alone or in combination with CpG ODN. The culture supernatants were harvested and IFN- α concentrations were tested using ELISA. VIP inhibited the secretion of IFN- α by PDCs in a dose-dependent manner with a maximum decrease at 10⁻⁶ M (fig. 4).

VIP modulates human PDC phenotype

The observation that PDCs express receptors for VIP prompted us to test the effects of VIP on the PDC phenotype. Human primary PDCs were matured *in vitro* via the IL-3-dependent pathway in the presence or absence of VIP (10⁻⁶ M) and CpG ODN. The control alone had no effect (data not shown). Expression of CD86, CD80, CD83, CD304 (BDCA-4), MHC class I, MHC class II, and CCR7 was determined by flow cytometric analysis (fig. 5). PDC viability was not affected at this concentration (data not shown). As has been shown by others¹⁷, CpG ODN class B strongly up-regulates CD80 and CD86 in PDCs. VIP oppositely regulated MHC class I and MHC class II on PDCs. Although VIP down-modulated MHC class I expression, it upregulated MHC class II expression. We also observed down-regulation of CD304 (BDCA-4) expression by VIP, both in the presence and the absence of CpG ODN. The chemokine receptor CCR7, previously shown to be up-regulated by CpG ODN¹⁸, is

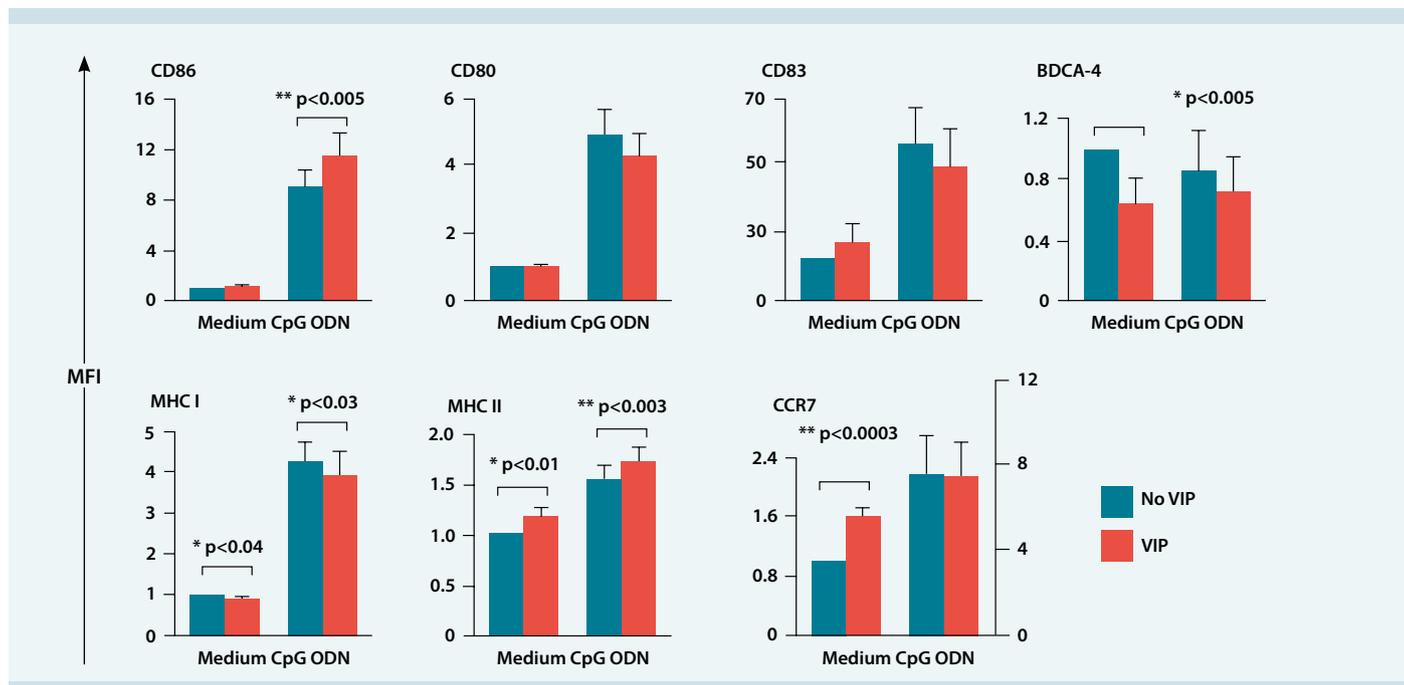


Figure 5 VIP induces phenotypic changes in PDCs. Isolated PDCs were incubated in the presence or absence of VIP (10^{-6} M) alone or in combination with CpG ODN. After 48 hours, the cells were harvested and the expression of CD86, CD80, CD83, CD304 (BDCA-4), MHC class I, MHC class II, and CCR7 was determined by flow cytometry. Bar graphs represent average MFI values of VIP-treated vs untreated cells relative to medium from 15 (CD86), 8 (CD80), 2 (CD83), 4 (BDCA-4), 11 (MHC I), 15 (MHCII), and 6 (CCR7) independent experiments. Error bars indicate SEM. Note the different axes for medium and CpG in the CCR7 panel.

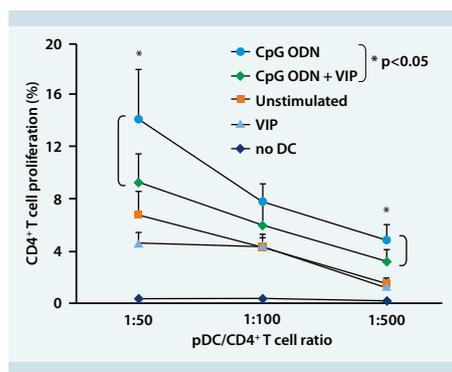


Figure 6 VIP inhibits the potential of PDCs to induce proliferation of allogeneic CD4⁺ T cells. Proliferation of allogeneic CD4⁺ T cells after 5-day coculture with PDCs pre-incubated with different stimuli for 48 hours is shown. PDCs of six different donors were isolated as described and cultured in IL-3-containing (10 ng/mL) AIM-V medium in the presence of CpG ODN (1 μ g/mL) and VIP (10^{-6} M) for 48 h. Allogeneic CD4⁺ T cells from healthy donors were isolated, stained with CFDA-SE, and co-incubated with pre-cultured PDCs at different ratios as indicated. After 5 days, cocultures were harvested, and the percentage of proliferated CFDA-SE^{low} T cells was determined by flow cytometry. The line graphs represent average T cell response of six different donors. Error bars indicate SEM. * p-values <0.05 indicate significant differences at a PDC/T cell ratio of 1:50 and 1:500.

also up-regulated in the presence of VIP, but VIP does not further stimulate CpG ODN induction of CCR7. CD83 is up-regulated by VIP in the absence but not further enhanced in the presence of CpG ODN.

VIP-primed PDCs have a decreased potential to induce allogeneic CD4⁺ T cell proliferation

To investigate the effect of VIP on PDC-T cell interactions, CD4⁺ T cells were isolated, stained with CFDA-SE, and co-incubated with PDCs of six different donors. Prior to coculture, the PDCs had been cultured in IL-3-containing AIM-V medium in the presence of CpG ODN and/or VIP for 48 hours. After 5 days, cocultures were harvested, and the percentage of proliferated T cells was determined by flow cytometry. Figure 6 demonstrates that PDCs matured in the presence of VIP, and that CpG ODN has a reduced capability to induce allogeneic CD4⁺ T cell proliferation as compared with PDCs matured with CpG ODN alone. The inhibitory effect of VIP pre-treatment was significant at PDC/T cell ratios of 1:50 and 1:500, whereas VIP alone had no

significant effect on PDC stimulation of CD4⁺ T cell proliferation.

Summary

In this study, we demonstrate for the first time that human PDCs express the VIP receptors VPAC1 and VPAC2 and that PDCs show biological responses to VIP. VIP significantly inhibits CpG ODN-induced secretion of IFN- α by PDCs. VIP modulates their immune phenotype enhancing their interaction with CD4⁺ T cells, whereas T cell proliferation is inhibited. VIP may therefore be involved in the modulation of immune responses to viral infections, thereby helping to prevent immunological overdrive.

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