Osteopontin promotes protective antigenic tolerance against experimental allergic airway disease

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Running title: Opn boosts antigenic tolerance via IFN-β-producing pDCs.

Abstract

In the context of inflammation, osteopontin (Opn) is known to promote effector responses facilitating a pro-inflammatory environment. However, its role during antigenic tolerance induction is unknown. Using a mouse model of asthma, we investigated the role of Opn during antigenic tolerance induction and its effects on associated regulatory cellular populations prior to disease initiation. Our experiments demonstrate that Opn drives protective antigenic tolerance by inducing accumulation of interferon (IFN)-β-producing plasmacytoid dendritic cells (pDCs), as well as regulatory T cells in mediastinal lymph nodes. We also show that recombinant Opn, and particularly its SLAYGLR motif, in the absence of TLR triggers, directly induces IFN-β expression in antigen-primed pDCs which renders them extra protective against induction of allergic airway disease upon transfer into recipient mice. Lastly, we show that blockade of Type I IFN receptor prevents antigenic tolerance induction against experimental allergic asthma. Overall, we unveil a new role for Opn in setting up a tolerogenic milieu boosting antigenic tolerance induction, and thus leading to prevention of allergic airway inflammation. Our results provide insight for future design of immunotherapies against allergic asthma.

Introduction

Mechanisms of central and peripheral tolerance are crucial for maintaining immune system homeostasis and preventing exaggerated immune responses to intrinsically harmless self or foreign antigens. Failure of this mechanism could lead to the development of chronic inflammation such as allergic asthma and autoimmune diseases.

As the incidence of allergic disease has risen dramatically, much effort has been put in determining the control mechanisms of peripheral tolerance to allergens, trying to find a treatment or prevention strategy for allergic disease. Allergic asthma is a disease caused by aberrant T helper cell 2 (T_H2) immune responses to inhaled allergens leading to eosinophilic airway inflammation, mucus hyper-secretion and variable airway obstruction (1). Regulatory T (Treg) cells are important suppressors of dysregulated T_H2 responses to inhaled antigens, as constitutive or induced deficiency of these cells leads to severe asthmatic reactions(2, 3). Likewise, several groups have demonstrated that both conventional (c) as well as plasmacytoid (p) dendritic cells (DCs) are key regulators of T_H2 responses in allergic airway inflammation (4-7). As in many processes of immunoregulation, cytokines like transforming growth factor-beta 1 (TGF-β1) and interleukin-10 (IL-10) are also important regulators of tolerance to inhaled antigens (3, 8, 9).

Osteopontin (Opn) is a cytokine expressed by immune cells, such as activated T cells and DCs, as well as by non-immune cells including tumor cells and stromal cells (10-12). In inflammatory conditions, Opn affects DC function (5, 13-18) and can drive T_H1, T_H2 and T_H17 effector immune responses (5, 11, 13, 14, 19, 20). On the other hand, Opn is constitutively expressed by a great variety of cells under non-inflammatory conditions (12, 21-23), but its physiological significance is largely unknown. For example, secreted Opn (sOpn) is expressed in the bone marrow (BM) and also upon inflammatory conditions mainly in the form of thrombin-cleaved fragments (24, 25). Opn fragments have binding motifs for several integrins: the SLAYGLR motif specifically interacts with integrins α4β1, α9β1 and α4β7, whereas the RGD motif interacts with the αvβ3, αvβ5, αvβ1 and α5β1 integrins (12, 26). In addition, the carboxy-terminal half of Opn interacts with certain CD44 variants (12,

27). Thrombin cleavage of Opn reveals the otherwise cryptic SLAYGLR domain and this modification is vital for its interaction with α9β1 integrin (28).

Recent reports show that Opn is expressed in Foxp3^{+} Treg cells (29, 30) suggesting its possible role in immune regulation. In the current report, we test whether Opn affects tolerance induction during intranasal administration of endotoxin-free antigen. Our results unveil a novel role for Opn as a tolerance enhancer against allergic airway disease, setting up an immunoregulatory milieu and potentiating CCR7-expressing pDC recruitment to the draining lymph nodes (dLNs). In addition, we reveal that, in the absence of PAMPs, sOpn, and specifically its integrin-binding SLAYGLR motif, induces low levels of IFN-β expression in antigen-primed pDCs. SLAYGLR-treated pDCs are highly efficient at suppressing allergic airway inflammation via type I IFN. Finally, we show that Type I interferons are crucial during antigenic tolerance induction against allergic airway disease.

Materials and methods

Mice. BALB/c, C57BL/6J (designated B6), ovalbumin (OVA)-specific T cell receptor transgenic C.Cg-Tg (DO11.10)10Dlo/J (designated DO11.10), C.129P2(B6)-IL-10^{tm1Cgn}/J (designated *Il10*$^{-/-}$), B6(Cg)-$Il10^{tm1.1Karp}$ (designated *Il10*^{GFP}), B6.129-*Ifnb1*^{tm1Lky}/J (designated *Ifnb*^{EYFP}), B6.129S2-*Ifnar1*^{tm1Agt}/Mmjax (designated *Ifnar1*$^{-/-}$), B6-Tg (C-type lectin domain family 4, member C, CLEC4C- heparin binding EGF like growth factor, HBEGF) 956Cln/J, designated plasmacytoid dendritic cell-specific type II C-type lectin (BDCA2)-diphtheria toxin receptor–(DTR) were purchased from the Jackson Laboratory (Bar Harbor, ME). C57BL/6-Tg Foxp3-DTR/enhanced green fluorescent protein (EGFP), designated depletion of regulatory T cell (DEREG) mice were provided by Dr. Sparwasser. B6.129S6(Cg)- secreted phosphoprotein 1 (Spp1)^{tm1Blh}/J, designated *Spp1*$^{-/-}$ mice were kindly provided by Dr. Lucy Liaw (Maine Medical Center Research Institute, Scarborough ME). All mice used in this study were 8- to10-wk old females. Mice were housed at the Animal Facility of the Biomedical Research Foundation of the Academy of Athens (BRFAA) and at the University Hospital Gent (Gent, Belgium). Use of mice in this study was reviewed and approved by the Bioethics Committee of BRFAA, the Veterinarian Office of Attica and the Animal Ethics Committee of Gent University. All procedures were in accordance with the US National Institutes of Health Statement of Compliance (Assurance) with Standards for Humane Care and Use of Laboratory Animals (A5736–01) and with the European Union Directive 86/609/EEC for the protection of animals used for experimental purposes.

***In vivo* experimental protocols.** For tolerance induction, mice received 200 μg of endograde OVA (Hyglos GmbH) (i.n.) in the presence of either 2.5 μg recombinant Opn protein (rOpn) (R&D Systems) or 72 ng synthetic secreted $Opn_{134-153}$ fragments (IVPTVDVPNGRGDSLAYGLR, frOpn) or PBS for control. The RGD domain (Arg-Gly-Asp) of frOpn1 is scrambled to RAA (Arg-Ala-Ala). The SLAYGLR (Ser-Leu-Ala-Tyr-Gly-Leu-Arg) of frOpn2 is scrambled to LRAGLRS (Leu-Arg-Ala-Gly-Leu-Arg-Ser). The frOpn3 has both the RGD and SLAYGLR scrambled to RAA and LRAGLRS, respectively (Caslo Laboratory ApS). Opn oligopeptide fragments have been previously described(31). Myelin oligodendrocyte glycoprotein peptide $(MOG)_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK) (Caslo Laboratory ApS)

was also used for tolerance induction (250 μg/mouse i.n.). Mice were euthanized 36-40 hrs later and analyzed. In certain experiments, tolerance was induced prior to allergic airway disease induction: mice received 200 μg of endograde OVA (i.n.) on days -2, -1 and 0 in the presence of either 2.5 μg of rOpn or 72 ng of frOpn1-3 synthetic fragments (i.n.). Control mice received PBS. Allergic airway disease (asthma) was subsequently induced: on day 10, mice were immunized with chicken OVA Grade V (Sigma-Aldrich) (50 μg) in 0.2 ml aluminium hydroxide (alum) (Serva) followed by 3 challenges with 5% aerosolized OVA between days 16-18 as described(5, 15). Mice were euthanized 2 d after the last aerosol (on day 20). DEREG mice received 1μg of diphtheria toxin (DT, Sigma-Aldrich) [35] or PBS, i.p. on days -3 and -2 (6 hrs prior to i.n. OVA administration). For DT control group, non Tg littermates were administered DT. For pDC depletion, mice received 225 μg of 120G8 pDC-depleting Ab (IgG2a-Dendritics, Lyon, France) (32) or Ig an isotype control Ab i.p., daily, on days -6, -5, -4 and -3. As in Fig. 4A**,** on days -2, -1 and 0, mice received i.n. 200 μg of OVA endograde along with 2.5 μg of rOpn and euthanized 7 days later. For pDC depletion in *BDCA2-DTR* mice 120 ng/mouse of DT was administered i.p. on days -4 and -3 in the 7-day tolerance induction protocol (as in Fig. 4A) (33). Efficient pDC depletion from dLNs in both approaches was determined by FACS analysis as ≥95% (data not shown). For neutralization of IFNAR1 mice received 20 μg i.p. of a polyclonal affinity-purified neutralizing Ab to mouse IFNAR1 (clone MAR1-5A3) (eBioscience) or goat IgG isotype control (R&D Systems) 2 hrs before tolerance induction. After IFNAR1 neutralization and tolerance, for allergy induction mice were immunized with chicken OVA on day 15, and OVA challenges were performed between days 21-23.

Penh. Lung function was measured in mice 24 hrs after the final OVA challenge (day 19) by whole body plethysmography (Buxco Technologies) in order to calculate enhanced pause (Penh). Responses to inhaled methacholine (Sigma Aldrich) at concentrations of 3–100 mg/ml were measured for 1 min, as previously described(5).

Analysis of Bronchoalveolar lavage (BAL) and lung histology. BAL harvesting and analysis was performed as previously described (34, 35). For histological analysis, paraffin-embedded (4μm) lung sections were stained with haematoxylin & eosin (H&E) and quantified as previously described (34). Goblet cells were quantified on Periodic-Acid-Schiff (PAS)-stained lung sections (5). A semi-quantitative scoring

system was used to grade the size of lung infiltrated as previously described (36). Goblet cells were counted on PAS stained sections using an arbitrary scoring system as previously described(36).

pDC generation from BM, cultures and adoptive transfer. For pDC generation, bone marrow (BM) cells were isolated and cultured with rhFlt3L as described(37). On day 11, $7AAD^{-}CD3^{-}CD19^{-}CD11c^{+}B220^{+}PDCA1^{+}Siglec\text{-}H^{+}$ pDCs were sorted using FACS ARIAIII to a purity ≥98% after enrichment with CD11c microbead kit (Miltenyi Biotec). Sorted pDCs from BM-cultures cultured were conditioned for 16 hrs with 100 μg/ml of endograde OVA or LoTox^{TM} *Dermatophagoides pteronyssinus* allergen 1 (Derp1) (Indoor Biotechnologies) in the presence of 250 ng/ml rOpn or 18.2 ng/ml frOpn1 or frOpn3 or PBS. After the culture with OVA, pDCs were washed and analysed or transferred intravenously (i.v.) via the tail vein (10^6/mouse). Allergic asthma was induced in mice 7 days later. Mice were euthanized 2 d after the last OVA challenge (Fig. 7B, 10A).

Cell culture, proliferation and cytokine analysis. Isolated dLN cells ($2x10^5$ to 10^6) were cultured with 125 μg/ml OVA (Sigma-Aldrich) for 48 h. We performed proliferation assays with thymidine incorporation, as previously described(38). For certain experiments, proliferation of cells was measured as % of Edu^{+} cells by FACS, using a Molecular Probes kit. For cytokine measurements, we used ELISA kits for IL-5 and IFN-γ (BD Biosciences); IL-4, IL-13 (R&D Systems) and IFN-β (BioLegend).

Flow cytometry. Freshly isolated live ($7AAD^{-}$, BD Biosciences) dLN cells and *in vitro* BM-derived cells were stained with combinations of fluorochrome-conjugated Abs to CD4-Pacific Blue or -PE/Cy5 (clone GK 1.5), CD3-PE/Cy7 or -PE/Cy5 or -PE or-FITC (17A2), CD11c-PE/Cy7 or -FITC or Orange 605 (N418), CD11b-PE/Cy7 or -FITC (M1/70), B220-PE or -BV 510 (RA3-6B2), CCR7-PE (4B12), Siglec-H-Pacific Blue or -FITC (551), PDCA-1-PE or -FITC (927), CD19-PE/Cy7 or -PE/Cy5 (6D5), CD25-PE (PC61.5) (BioLegend) and T1ST2 (DIH9) (-FITC, MD Biosciences or -PE BioLegend). For intra-nuclear staining of Foxp3, permeabilization kit and antibody (-Pacific Blue or -PE/CyC5, clone FJK-16s) were used (eBioscience). The flow cytometric measurements were performed using Attune Acoustic Focusing Cytometer (Applied Biosystems) and FACS ARIAIII (BD). FACS sorting of pDCs

was performed using FACS ARIAIII. Data analysis was performed by Flow-Jo Software (Tree Star).

Chemotaxis assay. Sorted pDCs from LNs and spleen of BALB/c mice, were treated with rOpn (500 ng/ml) for 18-20 h and assayed for migration in response to chemokines CCL19 or CCL21 (200 ng/mL) (R&D Systems). The lower chambers of Transwell plates (QCM^{TM} 5.0-μm chemotaxis assay 24-well-Colorimetric, Millipore) were filled with 500 μl serum-free medium in the presence or absence of chemokines. DCs (10^5 cells per 200 μl) resuspended in serum-free medium were deposited in the upper chambers of the Transwell plates and allowed to migrate for 4 h at 37°C in 5% CO2. For each experiment pooled total cells from spleen, inguinal and mesenteric lymph nodes of mice (n=8) were used and pDCs were isolated by sorting.

Suppression assay, Treg cell induction *in vitro*. BM-derived pDCs pre-treated with ±OVA and ±rOpn were cultured with naive DO11.10 $CD4^+$ T cells for 3 d at a 1:5 ratio. T cells were harvested and cultured without OVA in the presence of 1 ng/ml recombinant mouse IL-2 (R&D) for additional 7 d. Suppressive activity was assayed on 10^5 freshly purified CFSE (Invitrogen) labelled DO11.10 $CD4^+$ T cells stimulated with 10^4 irradiated BALB/c splenocytes, with 1 μg/ml $OVA_{323-339}$ peptide (Caslo Laboratory ApS), in the presence or absence of 10^5 DC-stimulated T cells. CFSE uptake was assayed 7 d later (Fig. 7A).

Quantitative RT-PCR analysis. Total RNA was extracted from cells isolated from dLNs with anti-mPDCA-1 and CD11c MicroBeads (Miltenyi Biotec) and further purified with FACS sorting. cDNA synthesis described(31). Primers were designed using the Primer3 program and are depicted in **Supplemental Table 1** (MWG Eurofins). Hypoxanthine-guanine phosphoribosyl transferase (*Hprt)*, *Foxp3*, *Il-10*, *Il27p28* and *Spp1* primers were previously described (31). Real-time PCR was performed and analyzed as previously described (31). The reference gene used for real-time PCR analysis is *Hprt*.

Statistical Analysis. Data were analyzed using Prism 7 Software (GraphPad). The two-tailed Student's t-test was used for statistical analyses of two-group comparisons. Multigroup comparisons were performed by a two-way analysis of variance (ANOVA) followed by the Bonferroni correction for the multiplicity of tests. All results are presented as mean ± standard error of the mean (SEM). In all experiments,

217 statistical significance was defined as: *P≤0.0332; **P≤0.0021; ***P≤0.0002,
218 ****P< 0.0001.

Results

Opn boosts antigenic tolerance leading to increased protection from allergic airway disease

To address whether Opn plays a role in tolerance induction, mice received i.n. endotoxin-free chicken OVA together with endotoxin-free rOpn or PBS for control, and protection against disease was assessed by utilizing a well-established model of allergic asthma (39) (**Fig. 1A**). OVA-tolerized mice showed a significant decrease in BAL, total cells and eosinophils, as well as lung tissue inflammatory scores and mucus secretion (**Fig. 1B-C**), compared to non-tolerized mice. In addition, enhanced pause (Penh) in OVA-tolerized mice was significantly reduced (**Fig. 1D**). Importantly, in OVA/rOpn-tolerized mice, numbers of eosinophils in BAL (**Fig. 1B**), airway hyperresponsiveness (AHR) (**Fig. 1D**), lung leukocytic infiltration and mucus secretion (**Fig. 1C**) were further reduced compared to OVA-tolerized mice. Furthermore, OVA/rOpn tolerization resulted in significantly reduced levels of IL-4, IL-13 and IFN-γ in the BAL, as well as in reduction of OVA-specific responses in mediastinal lymph node (mLN) cell cultures, and in T_H cell proliferation compared to OVA-tolerization (**Fig. 1E-G**). Studies from our group and others have shown that there are high levels of IFN-γ production in allergic airway disease (5, 15, 40-42). The above findings indicated that administration of Opn along with OVA promotes enhanced tolerance, conferring significant protection from disease development.

We next investigated whether Opn deficiency had any effect on tolerance induction. Tolerance induction in *Spp1*$^{-/-}$ mice was not as effective as in *Spp1*$^{+/+}$, since we noted no significant change in neither number of eosinophils nor total cell numbers in BAL (**Fig. 2A**). In accordance, tolerogenic i.n. OVA administration in *Spp1*$^{-/-}$ mice could not efficiently dampen the inflammation and mucus secretion in the lung, whereas it was very efficient in *Spp1*$^{+/+}$ mice (**Fig. 2B**). T_H2 cytokine production by OVA–stimulated mLN cells was also lower in OVA-tolerized *Spp1*$^{-/-}$ compared to PBS-treated *Spp1*$^{-/-}$ mice (**Fig. 2C**). OVA–stimulated mLN cells from OVA-tolerized *Spp1*$^{+/+}$ mice produced approximately 50% lower levels of T_H2 cytokines, compared to cells from PBS-treated *Spp1*$^{+/+}$ mice. Whereas, OVA-tolerized *Spp1*$^{-/-}$ mice had a lower reduction in cytokine levels compared to *Spp1*$^{+/+}$ (**Fig. 2C**). The reduction in IFN-γ levels was similar among groups (**Fig. 2C**). Finally, OVA tolerization of *Spp1*$^{-}$

$^{-/-}$ mice showed less decrease in the percentages of $CD3^+$ T proliferating cells in cultures of mLN cells, compared to those from *Spp1*$^{+/+}$ OVA-treated mice (**Fig. 2D**). The above results strongly support that in the presence of Opn, tolerance induction is more effective.

Opn administration along with antigen increases accumulation of tolerogenic pDCs

Migratory non-lymphoid tissue DCs transporting antigens to LNs are involved in promoting tolerance to self-antigens at steady state. As pDCs constitute a tolerogenic DC subset(43-47) and Opn has a dual role in the recruitment of DC subsets(5), we analyzed pDC numbers in the dLNs of OVA-tolerized mice and PBS-treated controls (**Fig. 3A**). Numbers of dLN pDCs were increased in OVA-treated group compared to the PBS control group (**Fig. 3B**). Mice treated with OVA/rOpn had increased percentages and significantly higher total numbers of pDCs in the dLNs at 36-40 hrs following treatment compared to OVA treatment (**Fig. 3B**). On the other hand, cDC numbers in the dLNs of OVA/rOpn mice were significantly reduced and percentages were lower compared to OVA treatment alone (**Fig. 3B**). Therefore, administration of Opn during tolerance induction affects the balance of DC subsets in the dLNs by increasing the numbers of pDCs and reducing the numbers of cDCs.

The importance of Opn for DC subset recruitment in tolerance was also demonstrated using Opn deficient mice. Antigenic tolerance induction in *Spp1*$^{-/-}$ mice resulted in significant reduction in pDC numbers in their dLNs, compared to *Spp1*$^{+/+}$ mice (**Supplemental Fig. 1**). At the same time, cDC numbers were significantly increased in the dLNs of *Spp1*$^{-/-}$ mice (**Supplemental Fig. 1**). Conclusively, these results show that tolerogenic administration of OVA in an Opn efficient microenvironment enhances pDC accumulation in the dLNs.

Administration of Opn during tolerance induction regulates CCR7 expression affecting DC subset homing to dLNs

To explore the reason for the increased numbers of pDCs in the dLNs of Opn treated mice, we investigated the effect of Opn on $CCR7^+$ pDCs. CCR7 is a chemokine receptor responsible for homing of DCs to dLNs (48-50). We quantified $CCR7^+$ DC subsets in peripheral blood, 36 hrs following OVA/rOpn tolerogenic administration

(**Fig. 3A**). Percentages of $CCR7^+$ pDCs per total pDC numbers were significantly elevated in peripheral blood of mice treated with rOpn during tolerance induction compared to PBS treated mice (**Fig. 3C**). Percentages of $CCR7^+$ cDC per total cDC numbers originating from peripheral blood of mice that had been administered OVA/rOpn were significantly decreased, compared to OVA-administered ones (**Fig. 3C**). Our results demonstrate that Opn leads to enhanced migratory $CCR7^+$ pDC percentages per total pDCs in the blood.

We also found that OVA/rOpn-administered mice had significantly higher levels of *Ccl19* and *Ccl21* (encoding CCL19 and CCL21 chemokines that bind to CCR7) expression in their dLNs compared to OVA-administered mice (**Fig. 3D**), possibly attracting the increased numbers of $CCR7^+$ pDCs to dLNs. Indeed, *in vitro* transmigration assays showed that sorted pDCs from naïve mice, pre-treated with rOpn had a 2-fold increase in CCL19-induced chemotaxis compared to PBS-treated pDCs (**Fig. 3E**). The above findings demonstrate that during tolerance induction, Opn can differentially regulate percentages of $CCR7^+$ DC subsets, therefore affecting their chemotactic migration to the dLNs in favor of pDCs. Moreover, the observed increased gene expression of the CCR7 ligands, CCL19 and CCL21, provide an extra explanation for the rOpn-mediated migration of pDCs to the dLNs.

Opn enhances pDC-dependent $Foxp3^+$ Treg cell accumulation and promotes an immunoregulatory milieu in the dLNs

As antigen administration for tolerance induction leads to generation of Treg cells (51), we investigated whether Opn had an effect early on this process. We administered i.n. OVA along either rOpn (OVA/rOpn) or PBS in mice for three consecutive days. One week later, mice were analyzed (**Fig. 4A**). OVA/rOpn treated mice showed almost a 2-fold increase in the percentages, as well as in total numbers of $CD4^+Foxp3^+$ Treg cells in the dLNs, compared to OVA-treated mice (**Fig. 4B**). Increased numbers of $Foxp3^+$ Treg cells in OVA/rOpn tolerized mice, were accompanied by significantly enhanced *Foxp3* expression in dLN cells (**Fig. 4C**). In addition, OVA/rOpn treatment also induced significant increase in the expression levels of immunoregulatory molecules such as *Il10, Ido, Tgfb1, Fgl2* and *Il27* in the dLNs compared to OVA treatment (**Fig. 4C**). Conclusively, Opn administration along

with antigen promotes Foxp3$^+$ Treg cell accumulation resulting in a highly tolerogenic microenvironment in the dLN.

To directly test whether increased numbers of Foxp3$^+$ Treg cells mediate the observed rOpn-promotion of tolerance, we depleted Foxp3$^+$ Treg cells prior to tolerance induction using DEREG mice (52) (**Fig. 5A**). Induction of tolerance with rOpn could not protect Treg-depleted mice which exhibited increased allergic responses as demonstrated by increased eosinophilia and lymphocytosis in the BAL, increased T cell proliferation, lung inflammation with mucus production and OVA specific cytokine responses, compared to Treg efficient mice (**Fig. 5B-E**).

To address whether the Opn-mediated increase in pDC numbers was responsible for the observed accumulation of Foxp3$^+$ Treg cells, we depleted pDCs by administering 120G8 pDC-depleting antibody(32) prior to induction of tolerance and examined the accumulation of Foxp3$^+$ Treg cells in the dLNs. This depletion led to a significant reduction in CD4$^+$Foxp3$^+$ Treg cell numbers versus OVA/rOpn administration in non-pDC-depleted mice (**Fig. 6A**), resulting in exacerbated allergic airway disease (**Fig. 6B**). We observed similar effects of rOpn on Treg cellularity in LNs after *in vivo* depletion of pDCs in *BDCA2-DTR* transgenic mice (33) (**Supplemental Fig. 2**). Thus, Opn-mediated pDC accumulation is at least partially responsible for the higher numbers of Treg cells in dLNs. In addition, dLN cells from OVA/rOpn-treated pDC-depleted mice were significantly less capable of suppressing OVA-specific T cell proliferation (**Fig. 6C**), compared to dLN cells from OVA/rOpn-treated non-pDC-depleted mice, reaching even lower suppressive ability than that of OVA-treated pDC-depleted dLN cells. Thus, OVA/rOpn treatment increased numbers of pDCs favoring accumulation of Foxp3$^+$ Treg cells that are crucial for tolerance maintenance.

Opn treatment of pDCs increases their suppressive activity against allergic airway disease

We tested the suppressive function of *in vitro* OVA/rOpn-treated BM-derived pDCs by co-culturing them with CD4$^+$ T cells. T cells obtained from OVA/rOpn-treated pDC cultures significantly suppressed responses of DO11.10 T cells to OVA compared to T cells obtained from OVA-treated pDC cultures (**Fig. 7A**).

We also adoptively transferred BM-derived pDCs, pre-conditioned *in vitro* with OVA or OVA/rOpn, into recipient mice prior to induction of allergic airway inflammation (**Fig. 7B**). Control mice received PBS-treated pDCs. Total and eosinophil cell numbers, as well as lung tissue inflammatory scores and mucus secretion were lower in BAL of OVA/rOpn-treated pDC recipient mice compared to OVA-treated pDC recipient mice (**Fig. 7B-C**). Furthermore, OVA/rOpn-treated pDC recipients had significantly reduced OVA-specific responses compared to OVA-treated pDC recipients (**Fig. 7D**). Thus, OVA/rOpn treatment of pDCs rendered them more regulatory, indicating that Opn affects the intrinsic tolerogenic function of pDCs.

Opn SLAYGLR motif is responsible for pDC recruitment and effective protection from allergic disease

RNA expression analysis revealed that, upon stimulation, mLN pDCs elevate expression of integrins αvβ3, α4β1 and α9β1 (data not shown). As the SLAYGLR motif of Opn interacts with α4β1, α4β7 and α9β1 integrins (12), and the RGD motif interacts with the αvβ3 integrin (11, 12), we asked which Opn domain is responsible for the observed effects on pDCs during tolerance induction. Thus, the synthetic frOpn$_{134\text{-}153}$, containing the RGD and the SLAYGLR integrin binding motifs either intact or scrambled (31), were used with OVA to induce tolerance in mice, allowing us to unveil the involvement of the different integrin-binding motifs of Opn in pDC recruitment (**Fig. 8A**). The frOpn$_{134\text{-}153}$ represents the thrombin cleaved fragment of Opn that reveals the otherwise cryptic domain SLAYGLR (28). OVA/frOpn1, containing an intact SLAYGLR motif but a scrambled RGD motif, induced accumulation of higher numbers of pDCs in the LNs compared to OVA, and OVA/rOpn **(Fig. 8A).** Conversely, frOpn2, which contains an intact RGD, but a scrambled SLAYGLR motif induced a reduction in pDC cell numbers in dLNs compared to all other treatments (**Fig. 8A**). When both motifs were scrambled as in the case of frOpn3, the numbers and percentages of pDCs were not significantly altered compared to OVA **(Fig. 8A, Supplemental Fig. 3A**). OVA/frOpn1 induced higher percentages of pDCs in the dLNs compared to OVA and OVA/frOpn3 (**Supplemental Fig. 3A**). These data revealed that the Opn SLAYGLR motif is responsible for pDC accumulation into dLNs. These data also reveal that the RGD motif suppresses this accumulation.

To examine the *in vivo* tolerogenic potential of frOpn1 in allergic disease, mice were treated with OVA together with either frOpn1 or frOpn3 for control before the induction of allergic airway inflammation (**Fig. 8B**). OVA/frOpn1 was more successful than OVA/frOpn3 in promoting tolerance and thus protecting mice against allergic airway inflammation as demonstrated by lower Penh values (**Fig. 8C**). BAL eosinophilia, as well as inflammatory score and mucus staining were also dampened after OVA/frOpn1 treatment (**Fig. 8D**). In addition, treatment with OVA/frOpn1 led to reduced levels of OVA-specific responses (**Fig. 8E**) and numbers of $T1ST2^{+}CD4^{+}$ T cells in the dLNs (**Fig. 8D**). Collectively, this dataset indicates that the Opn SLAYGLR motif significantly boosts tolerance.

Opn SLAYGLR motif induces IFN-β expression in pDCs

We further asked whether OVA/frOpn1 treatment affects *in vivo* pDC immune profile 36-40 hrs after tolerance induction (**Fig. 3A**). *In vitro*, OVA/frOpn1 conditioning of pDCs promoted a 2-fold increase in *Ifnb* mRNA expression at 16 hrs (**Fig. 9A**). The observed difference in *Ifnb* expression was also reflected in the amount of IFN-β secreted, that was 5-fold higher in OVA/frOpn1-conditioned pDCs compared to OVA/frOpn3 (**Fig. 9B**). House dust mite endotoxin-free Derp1 was also used to test whether the increased IFN-β response could be observed using another allergen. Indeed, Derp1/frOpn1 *in vitro* conditioning of pDCs resulted in higher *Ifnb* expression compared to Derp1/frOpn3 (**Fig. 9C**). We also measured higher levels of *Ifnb* expression in OVA/rOpn-conditioned pDCs compared to OVA/PBS (**Supplemental Fig. 3B**).

As RNA expression analysis revealed that mLN pDCs from *in vivo* OVA/frOpn1 treatment had elevated *Ifnb* (data not shown), we found that indeed *Ifnb* expression was upregulated in these cells (**Fig. 9E**). To confirm the changes in IFN-β expression *in vivo*, we used IFN-β^{EYFP} reporter mice. OVA/frOpn1-tolerized mice had significantly increased numbers of IFN-β^{EYFP+} pDCs and higher IFN-β expression per pDC (MFI) compared to OVA/frOpn3 (**Fig. 9D**). To test whether this effect is relevant to the antigen used, we tolerized IFN-β^{EYFP} mice with a self-antigen, the MOG_{35-55}, with frOpn1 or frOpn3. MOG_{35-55}/frOpn1 administration in IFN-β^{EYFP} mice resulted in significantly increased accumulation of IFN-β^{EYFP+} pDCs, as well as IFN-β expression per pDC (MFI) in LNs (**Fig. 9F**). However, the increase here was

modest compared to Opn/OVA, possibly because MOG is a peptide. These results suggest that under tolerogenic conditions, Opn SLAYGLR motif can boost *Ifnb* expression in pDCs through a mechanism independent of the antigen used.

Opn/SLAYGLR-induced IFN-β in pDCs is protective against allergic disease

pDCs were primed *in vitro* with endotoxin-free OVA and frOpn1 or frOpn3 and transferred into mice prior to induction of allergic airway inflammation (**Fig. 10A**). Recipient mice were *Ifnar1*$^{+/+}$ or *Ifnar1*$^{-/-}$. pDCs treated with frOpn1 were significantly more potent in protecting *Ifnar1*$^{+/+}$ mice against allergic airway inflammation, as demonstrated by reduced lung airway inflammation, BAL eosinophilia and goblet cell hyperplasia compared to control and OVA group (**Fig. 10A**). Similar enhanced protection was also observed when pDCs were treated with rOpn and adoptively transferred into *Ifnar1*$^{+/+}$ mice (**Fig. 7B-D**). Actually, type I IFN produced by pDCs was crucial in maintaining this protective effect, as *Ifnar1*$^{-/-}$ recipient mice were not protected against allergic asthma (**Fig. 10A**). These results demonstrate that the integrin-binding SLAYGLR domain of Opn renders pDCs more regulatory through type I IFN production in the context of allergic airway inflammation. In fact, pDCs cannot preserve their regulatory function in recipients that lack expression of type I IFN receptor. Finally, neutralization of IFNAR1 during the OVA tolerance induction phase (**Fig. 10B**) resulted in reduced protection from allergic airway disease concomitant with increased eosinophilia in BAL (**Fig. 10C**) and increased OVA-specific responses (**Fig. 10D**). This indicates that type I IFN is absolutely necessary for effective antigenic tolerance induction in this context.

Discussion

In the current report, we unveil a new role for Opn, and particularly its SLAYGLR motif, in setting up a tolerogenic milieu driving antigenic tolerance induction, and thus leading to prevention from allergic airway inflammation.

The respiratory mucosa is constantly being exposed to a myriad of non-pathogenic environmental antigens. To protect against the immunopathological consequences of this constant stimulus, a default low non-inflammatory T_H2 mechanism and/or a T cell-mediated tolerance mechanism is activated (53). The mechanism underlying these processes is not fully understood. In the present study, we initially demonstrate that Opn induces tolerance by tilting the pDC/cDC balance in favor of the anti-inflammatory pDCs, via differentially regulating their CCR7 expression. CCR7-dependent homing of DCs into the dLNs is required for the induction of tolerance(50).

Opn promotes Th2 effector responses when administered during the allergen sensitization phase (5). In contrast, we have previously shown that Opn inhibits Th2 responses during allergen challenge and administration of rOpn during that phase is suppressive for experimental allergic asthma (5). Here, we explore the role of Opn during a different phase, which is when antigen is administered during tolerance induction, prior to sensitization. Thus, Opn is administered, prior to sensitization phase, along with the allergen (antigen) intranasally, in order to test its effects upon antigenic tolerance induction. Together, our results in this manuscript as well as in previous studies (5) show that administration of Opn during (a) antigenic tolerance induction, and (b) antigenic challenge, is protective and can thus be used as either a preventive, or a therapeutic agent against allergic airway disease.

Regulatory cytokines such as *Tgfb1, Il10, Fgl2,* and molecules such as *Ido* (54) were substantially up-regulated in our Opn-driven tolerance model, creating an immunoregulatory microenvironment in the dLN. This tolerogenic milieu was accompanied by accumulation of $Foxp3^+$ Treg cells, which are very important for induction of tolerance (51). Mice that had enhanced Treg cell numbers, due to rOpn administration, showed suppressed effector responses and were protected from allergic disease. When $Foxp3^+$ Treg cells were depleted, Opn-mediated tolerance boosting was abolished. In light of its well-established pro-inflammatory function (10-12), it was unexpected that Opn could be an inducer of Treg cell accumulation *in*

vivo. In the absence of pDCs, rOpn administration was no longer capable to induce increased numbers of $Foxp3^+$ Treg cells in the dLNs, demonstrating that rOpn affects Treg numbers mainly through its impact on pDCs. On the other hand, after tolerance induction without addition of Opn, Treg cell numbers do not appear to depend on pDCs, as pDC depletion did not reduce their numbers. These results show that rOpn conditions pDCs to enhance Treg cell accumulation. Functional flexibility and fostering of regulatory T cell responses are typical features of pDC involvement in tolerance (4, 46), as also revealed by our studies.

Thrombin cleavage of Opn occurs during both homeostatic and inflammatory conditions (24, 25) and conformational changes after thrombin cleavage result in higher affinity binding to certain receptors (26). In fact, the cryptic SLAYGLR motif is revealed upon thrombin cleavage of Opn which is vital for its interaction with α9β1 integrin (28). As the SLAYGLR motif of Opn protein interacts with α4β1, α4β7 and α9β1 integrins (12), and the RGD motif interacts with the αvβ3 integrin (11, 12), we used the synthetic $frOpn_{134\text{-}153}$, containing both integrin binding motifs either intact or scrambled (31). Thrombin cleavage of Opn also produces a fragment containing the carboxy-terminal half of Opn, which interacts with certain CD44 variants (12, 27). In our settings, as well as in other settings (31, 55, 56), $frOpn_{134\text{-}153}$ has a greater efficiency compared to full length Opn. It is thus possible that interaction of Opn with CD44 interferes with certain Opn effects, such as pDC recruitment. Future investigation will elucidate the role of Opn-CD44 interaction in tolerance induction.

Under endotoxin-free conditions, we demonstrated that the SLAYGLR domain of Opn (frOpn1) enhances IFN-β expression in pDCs exposed to protein or peptide antigen. Accordingly, we showed that adoptively transferred OVA/frOpn1-treated pDCs were more efficient at suppressing allergic airway inflammation in recipient mice. This regulatory function was indeed mediated by the observed upregulated production of IFN-β by pDCs, as *Ifnar1*$^{-/-}$ recipient mice were not protected against allergic asthma. During the course of allergic airway inflammation, pDCs as well as their type I IFN production are suppressive for disease (4, 57-59). Our studies reveal that pDCs expressing higher IFN-β levels due to exposure to Opn are important for protective tolerance prior to disease induction. Importantly, our experiments point to a crucial role of type I IFNs during the induction of efficient anti-allergic antigenic

tolerance. Accordingly, IFNAR signalling promotes Treg cell development and function under stress conditions (60).

Opn administered during tolerance also resulted in a remarkable up-regulation of the ligands of CCR7, CCL19 and CCL21 in dLNs, reminiscent of that observed in tumors(61). Specifically, CCL21 high expression by melanoma in mice was associated with an immunotolerant microenvironment, which included the induction of lymphoid-like reticular stromal networks, an altered cytokine milieu, and most importantly the recruitment of regulatory leukocyte populations(61). High Opn expression is prevalent in many types of malignancy(62), and although Opn is considered pro-inflammatory, most of these tumors escape immune surveillance. Thus, it is possible that Opn and its mediated induction of CCL21 could also be involved in suppression of anti-tumor immunity.

In this report, we demonstrate that the SLAYGLR motif of sOpn is enhancing regulatory mechanisms when administered together with endotoxin-free antigen in a tolerogenic context. In this respect, Opn has a unique effect on immunity, differing substantially from its effects in the presence of danger signals. The SLAYGLR motif of Opn interacts with integrins(12). Addressing which integrin mediates the tolerogenic effects of the SLAYGLR motif of Opn on Ag-loaded pDCs, and mainly the induction of IFN-β, will assist in the design of therapies targeting tolerance in allergy. Finally, our results point to novel effects of Opn on $Foxp3^{+}$ Treg cells that remain to be explored.

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Author Contributions

T.A., B.N.L. and V.P. designed research; T.A., E.K., D.C.M.S., M.A., N.P. and H.H. performed research; L.B. and T.S. contributed new reagents; T.A., E.K., D.C.M.S. and V.P. analyzed data; T.A., E.K., B.N.L. and V.P. wrote the paper. B.N.L. and V.P. supervised the study.

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Footnotes

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Conflict of Interest:

The authors declared no conflict of interest.

Figure legends

Figure 1. Opn administration boosts antigenic tolerance leading to increased protection from allergic airway disease. (A) Experimental protocol utilized for endotoxin free-tolerance (OVA administration) induction followed by allergic asthma induction. Controls were mice pre-treated with PBS (no tolerance) and subsequent allergic airway disease induction (white bars). Controls for allergic airway disease were mice pre-treated and sensitized with PBS. **(B)** Total cell counts in (BAL) and eosinophils. **(C)** Representative photomicrograph of H&E and PAS stained lung sections and histological scores. Scale bar, 100 μm. **(D)** AHR responses depicted as (Penh) in day 19. **(E)** Levels of IL-4, IL-13 and IFN-γ in BAL and **(F)** in the supernatant of mLN cells stimulated ex-vivo with OVA. **(G)** [^{3}H]-thymidine incorporation of mLN cells stimulated ex-vivo with OVA. Different wells of pooled mLN cells cultured in E, F, G. Values are expressed as mean ± standard error of the mean (SEM, n=8 mice per group), one representative of three independent experiments.

Figure 2. *Spp1*$^{-/-}$ mice are more resistant to tolerance induction. *Spp1*$^{-/-}$ or *Spp1*$^{+/+}$ mice received three doses of endotoxin-free OVA or PBS i.n. on days -2, -1 and 0 as in Fig. 1A. On day 10, mice were sensitized i.p. with OVA in alum and were subsequently challenged through the airways with aerosolized OVA between days 16-18. Analysis was performed on day 20. **(A)** Differential eosinophil and total cell counts in BAL, **(B)** lung inflammation shown in H&E stained sections and mucus secretion shown in PAS stained sections from PBS and OVA-treated *Spp1*$^{+/+}$ and *Spp1*$^{-/-}$ mice are shown. Scale bar, 100 μm. **(C)** Levels of IL-4, IL-5, IL-13 and IFN-γ in supernatants of OVA-stimulated dLN cells and **(D)** percentages of Edu$^+$CD3$^+$CD4$^+$ T in OVA-stimulated dLN cells of both mice groups. Different wells of pooled mLN cells cultured in C and D. Values are expressed as mean ± SEM (n=6 mice per group), one representative of three independent experiments.

Figure 3. Opn-induced accumulation of pDCs in the dLN and differential CCR7 expression. (A) Treatment of mice with endotoxin free OVA (tolerance induction) together with endotoxin free rOpn, or PBS for B-D. Controls (without tolerance) were the PBS-treated mice (white bars). **(B-D)** 7AAD$^-$CD3$^-$CD19$^-$CD11c$^+$PDCA-1$^+$CCR7$^+$ pDCs and 7AAD$^-$CD3$^-$CD19$^-$CD11c$^+$CD11b$^+$CCR7$^+$ cDCs quantified in dLNs by

flow cytometry. **(B)** Representative percentages in flow cytometric plots and numbers of dLN pDCs (upper panel) and cDCs (lower panel). **(C)** Representative flow cytometric plots and percentages of peripheral blood $CCR7^+$ pDCs and cDCs among total pDCs and cDCs respectively and **(D)** relative expression to *Hprt* of *Ccl19* and *Ccl21* in dLNs of tolerized mice. Values are expressed as mean ± SEM (n=10 mice per group and cDNAs were pooled from three separate experiments). **(E)** Sorted pDCs from LNs and spleens of naïve BALB/c mice were pulsed with vehicle or rOpn for 24 hrs and were then subjected to transmigrate in response to CCL19. Numbers of migrating PBS (white bars) or rOpn treated-pDCs (black bars) are depicted. Triplicate wells of pooled pDCs sorted from spleens and LNs (n=8). Values are expressed as mean ± SEM (n=5-8 mice per group), one representative of five independent experiments in A-C and from three in E.

Figure 4. Opn administration with antigen promotes accumulation of Treg cells and immunoregulatory gene expression in dLNs. (A) Tolerance induction in mice received 3 doses of endotoxin free OVA along with rOpn or PBS. Controls (without tolerance) were PBS-treated mice (white bars). **(B)** Percentages and numbers of $CD3^+CD4^+Foxp3^+$ T cells quantified in dLNs by flow cytometry at day 7. **(C)** Relative expression to *Hprt* of immunoregulatory genes in dLNs. cDNAs were pooled from three separate experiments. Values are expressed as mean ± SEM (n=6 mice per group), one representative of three independent experiments.

Figure 5. Opn-induced tolerance is Treg cell-dependent. (A) Tolerance induction in diphtheria toxin (DT)-treated DEREG and control DEREG mice without DT injections. Controls (without tolerance) were DEREG mice pre-treated with PBS and subsequent allergy induction (white bars). **(B)** Eosinophil and total cell counts in BAL. **(C)** Numbers of $Edu^+CD3^+CD4^+$ T cells per 25 x 10^4 OVA-stimulated dLN cells. **(D)** Lung inflammation and mucus secretion depicted in H&E (top) and PAS stained slides (bottom). Scale bar, 100 μm. **(E)** Levels of IL-5 and IL-13 in supernatants of OVA-stimulated dLN cells. Different wells of pooled mLN cells cultured in C and E. Values are expressed as mean ± SEM (n=6 mice per group), one representative of three independent experiments.

Figure 6. Opn-induced tolerance increases pDC numbers favoring Treg cell accumulation. Mice received four doses of 120G8 pDC-depleting antibody or Ig

control i.p. on days -6, -5, -4 and -3, followed by tolerance induction for Treg cell generation with or without rOpn administration (for A). Also, allergic airway disease was subsequently induced on day 10 (for B and C). Control mice were pre-treated with PBS (in A) and sensitized with PBS (in B and C). **(A)** Representative percentages in flow cytometric plots and numbers of Treg cell ($CD3^+CD4^+Foxp3^+$) accumulation in dLNs. **(B)** Eosinophil counts in BAL of allergic mice. **(C)** [^{3}H]-thymidine incorporation in OVA stimulated dLN cells. Quadruplicate wells of pooled mLN cells. Values are expressed as mean ± SEM (n=5 mice per group), one representative of three independent experiments.

Figure 7. rOpn treatment of pDCs increases their suppressive activity against allergic airway disease. (A) [^{3}H]-thymidine incorporation in co-cultures of DO11.10 T cells pre-conditioned with $OVA_{323-339}$ and rOpn treated pDCs with responder DO11.10 T cells Different wells of cultured cells. Values are expressed as mean ± SEM, one representative of three independent experiments. **(B)** Sorted pDCs pre-conditioned *in vitro* with PBS or OVA or OVA/rOpn were adoptively transferred to recipient mice before the induction of allergic airway inflammation. Total and eosinophil cell count in BAL were evaluated (day 20). **(C)** Histological assessment of lung inflammation (H&E scoring) and lung mucus production (PAS score) and **(D)** levels of IL-4 and IL-13 in supernatants of OVA-stimulated mLN cells. Different wells of pooled mLN cells. Values are expressed as mean ± SEM (n=6-8 mice per group), one representative of three independent experiments.

Figure 8. Opn SLAYGR motif boost tolerance by enhancing pDC recruitment to protect from allergy. (A) Numbers of dLN $7AAD^-CD11c^+B220^+PDCA1^+SiglecH^+$ pDCs after 40 hrs of tolerance induction with endograde-OVA i.n. along with rOpn or frOpn1, or frOpn2, or frOpn3, or PBS as in Fig. 3A. **(B)** OVA/frOpn-tolerance induction before allergic asthma for c-e. Control mice were pre-treated with PBS and allergy was subsequently induced (white bars). **(C)** AHR responses depicted as Penh in day 19. **(D)** Eosinophil cell count in BAL, histological assessment of lung inflammation (H&E scoring) and lung mucus production (PAS score) and numbers of $T1ST2^+CD4^+$ T_H2 cells in mLNs. **(E)** Levels of IL-4, IL-5 and IL-13 in supernatants of OVA-stimulated mLN cells. Different wells of pooled mLN cells. Values are expressed as mean ± SEM (n=5, mice per group), one representative of three independent experiments.

Figure 9. Opn SLAYGR motif induces IFN-β production from pDCs. (A) Relative *Ifnb* expression to *Hprt* in pDCs conditioned *in vitro* with OVA/frOpn1 or frOpn3. **(B)** Levels of IFN-β in the supernatants of the same cultures. **(C)** Relative *Ifnb* expression to *Hprt* in pDCs *in vitro* conditioned with Derp1/frOpn1 compared to Derp1/frOpn3. **(D)** Representative percentages in flow cytometric plots and numbers of $7AAD^{-}CD3^{-}CD19^{-}CD11c^{+}PDCA\text{-}1^{+}Siglec\text{-}H^{+}IFN\text{-}\beta^{EYFP+}$ pDCs in dLNs of PBS-treated or OVA-tolerized mice, and geometrical mean fluorescence intensity (gMFI) of IFN-β-expressing pDCs. **(E)** Relative *Ifnb* expression to *Hprt* in pDCs isolated from dLNs of PBS-treated or OVA/frOpn3- and OVA/frOpn1-tolerized mice. **(F)** Representative percentages in flow cytometric plots and numbers of total $7AAD^{-}$ $CD3^{-}CD19^{-}CD11c^{+}PDCA\text{-}1^{+}Siglec\text{-}H^{+}$ $IFN\text{-}\beta^{EYFP+}$, and gMFI of IFN-β-expressing pDCs in dLNs of mice treated with PBS or tolerized with $MOG_{35\text{-}55}$/frOpn3 or $MOG_{35\text{-}55}$/frOpn1. Mice were tolerized as in Fig. 3A. Values are expressed as mean ± SEM (n=6-8 mice per group), pooled data from three independent experiments.

Figure 10. Opn/SLAYGLR induced IFN-β in pDCs renders them protective against allergic disease. (A) Isolated $Ifnar1^{+/+}$ pDCs were primed *in vitro* with OVA and frOpn1 or frOpn3 or with PBS and adoptively transferred to either $Ifnar1^{+/+}$ or $Ifnar^{-/-}$ mice. Histological scores for airway inflammation and goblet cell hyperplasia in lung sections stained with H&E and PAS respectively and number of eosinophils present in BAL. **(B)** OVA-tolerance induction with blocking of IFNAR1 before allergic asthma (for C and D). Control mice were pre-treated with PBS and allergy was subsequently induced (white bars). **(C)** Numbers of eosinophils in BAL and **(D)** levels of IL-4 and IL-13 in supernatants of OVA-stimulated dLN cells. Different wells of pooled mLN cells. Values are expressed as mean ± SEM (n=4-5 in A and n=6 in B-D mice per group), one representative of three independent experiments.