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1 **Osteopontin promotes protective antigenic tolerance against experimental**  
2 **allergic airway disease**

3 Themis Alissafi<sup>1,2,\*</sup>, Evangelia Kourepini<sup>1,\*</sup>, Davina C. M. Simoes<sup>1,\*</sup>, Nikolaos  
4 Paschalidis<sup>1</sup>, Maria Aggelakopoulou<sup>1</sup>, Tim Sparwasser<sup>3</sup>, Louis Boon<sup>4</sup>, Hamida  
5 Hammad<sup>2</sup>, Bart N. Lambrecht<sup>2</sup> and Vily Panoutsakopoulou<sup>1</sup>

6 <sup>1</sup>Cellular Immunology Laboratory, Center for Basic Research, Biomedical Research  
7 Foundation of the Academy of Athens, Athens, Greece

8 <sup>2</sup>VIB Department for Molecular Biomedical Research, University of Gent, Gent,  
9 Belgium

10 <sup>3</sup>Institute of Infection Immunology, TWINCORE, Centre for Experimental and  
11 Clinical Infection Research, Hannover, Germany; a joint venture between the  
12 Helmholtz Centre for Infection Research (HZI), Braunschweig and the Hannover  
13 Medical School (MHH)

14 <sup>4</sup>Bioceros BV, Utrecht, Netherlands

15 Corresponding author:

16 Vily Panoutsakopoulou

17 Cellular Immunology Laboratory

18 Center for Basic Research

19 Biomedical Research Foundation of the Academy of Athens

20 4 Soranou Efessiou Street, 11527 Athens, Greece

21 Email: vpan@bioacademy.gr

22 \*equal contribution

23 **Running title: Opn boosts antigenic tolerance via IFN- $\beta$ -producing pDCs.**

24

25

26 **Abstract**

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

43

44

## 45 **Introduction**

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

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

64 Osteopontin (Opn) is a cytokine expressed by immune cells, such as activated T cells  
65 and DCs, as well as by non-immune cells including tumor cells and stromal cells (10-  
66 12). In inflammatory conditions, Opn affects DC function (5, 13-18) and can drive  
67 T<sub>H</sub>1, T<sub>H</sub>2 and T<sub>H</sub>17 effector immune responses (5, 11, 13, 14, 19, 20). On the other  
68 hand, Opn is constitutively expressed by a great variety of cells under non-  
69 inflammatory conditions (12, 21-23), but its physiological significance is largely  
70 unknown. For example, secreted Opn (sOpn) is expressed in the bone marrow (BM)  
71 and also upon inflammatory conditions mainly in the form of thrombin-cleaved  
72 fragments (24, 25). Opn fragments have binding motifs for several integrins: the  
73 SLAYGLR motif specifically interacts with integrins α4β1, α9β1 and α4β7, whereas  
74 the RGD motif interacts with the αvβ3, αvβ5, αvβ1 and α5β1 integrins (12, 26). In  
75 addition, the carboxy-terminal half of Opn interacts with certain CD44 variants (12,

76 27). Thrombin cleavage of Opn reveals the otherwise cryptic SLAYGLR domain and  
77 this modification is vital for its interaction with  $\alpha 9\beta 1$  integrin (28).

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

89 **Materials and methods**

90 **Mice.** BALB/c, C57BL/6J (designated B6), ovalbumin (OVA)-specific T cell receptor  
91 transgenic C.Cg-Tg (DO11.10)10Dlo/J (designated DO11.10), C.129P2(B6)-IL-  
92 10<sup>tm1Cgn</sup>/J (designated *Il10*<sup>-/-</sup>), B6(Cg)-Il10<sup>tm1.1Karp</sup> (designated *Il10*<sup>GFP</sup>), B6.129-  
93 *Ifnb1*<sup>tm1Lky</sup>/J (designated *Ifnb*<sup>EYFP</sup>), B6.129S2-*Ifnar1*<sup>tm1Agt</sup>/Mmjax (designated  
94 *Ifnar1*<sup>-/-</sup>), B6-Tg (C-type lectin domain family 4, member C, CLEC4C- heparin  
95 binding EGF like growth factor, HBEGF) 956Cln/J, designated plasmacytoid  
96 dendritic cell-specific type II C-type lectin (BDCA2)-diphtheria toxin receptor-(DTR)  
97 were purchased from the Jackson Laboratory (Bar Harbor, ME). C57BL/6-Tg Foxp3-  
98 DTR/enhanced green fluorescent protein (EGFP), designated depletion of regulatory  
99 T cell (DEREG) mice were provided by Dr. Sparwasser. B6.129S6(Cg)- secreted  
100 phosphoprotein 1 (*Spp1*)<sup>tm1Blh</sup>/J, designated *Spp1*<sup>-/-</sup> mice were kindly provided by Dr.  
101 Lucy Liaw (Maine Medical Center Research Institute, Scarborough ME). All mice  
102 used in this study were 8- to10-wk old females. Mice were housed at the Animal  
103 Facility of the Biomedical Research Foundation of the Academy of Athens (BRFAA)  
104 and at the University Hospital Gent (Gent, Belgium). Use of mice in this study was  
105 reviewed and approved by the Bioethics Committee of BRFAA, the Veterinarian  
106 Office of Attica and the Animal Ethics Committee of Gent University. All procedures  
107 were in accordance with the US National Institutes of Health Statement of  
108 Compliance (Assurance) with Standards for Humane Care and Use of Laboratory  
109 Animals (A5736-01) and with the European Union Directive 86/609/EEC for the  
110 protection of animals used for experimental purposes.

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

121 was also used for tolerance induction (250 µg/mouse i.n.). Mice were euthanized 36-  
122 40 hrs later and analyzed. In certain experiments, tolerance was induced prior to  
123 allergic airway disease induction: mice received 200 µg of endograde OVA (i.n.) on  
124 days -2, -1 and 0 in the presence of either 2.5 µg of rOpn or 72 ng of frOpn1-3  
125 synthetic fragments (i.n.). Control mice received PBS. Allergic airway disease  
126 (asthma) was subsequently induced: on day 10, mice were immunized with chicken  
127 OVA Grade V (Sigma-Aldrich) (50 µg) in 0.2 ml aluminium hydroxide (alum)  
128 (Serva) followed by 3 challenges with 5% aerosolized OVA between days 16-18 as  
129 described(5, 15). Mice were euthanized 2 d after the last aerosol (on day 20). DEREK  
130 mice received 1µg of diphtheria toxin (DT, Sigma-Aldrich)<sup>35</sup> or PBS, i.p. on days -3  
131 and -2 (6 hrs prior to i.n. OVA administration). For DT control group, non Tg  
132 littermates were administered DT. For pDC depletion, mice received 225 µg of 120G8  
133 pDC-depleting Ab (IgG2a-Dendritics, Lyon, France) (32) or Ig an isotype control Ab  
134 i.p., daily, on days -6, -5, -4 and -3. As in Fig. 4A, on days -2, -1 and 0, mice received  
135 i.n. 200 µg of OVA endograde along with 2.5 µg of rOpn and euthanized 7 days later.  
136 For pDC depletion in *BDCA2-DTR* mice 120 ng/mouse of DT was administered i.p.  
137 on days -4 and -3 in the 7-day tolerance induction protocol (as in Fig. 4A) (33).  
138 Efficient pDC depletion from dLNs in both approaches was determined by FACS  
139 analysis as  $\geq 95\%$  (data not shown). For neutralization of IFNAR1 mice received 20  
140 µg i.p. of a polyclonal affinity-purified neutralizing Ab to mouse IFNAR1 (clone  
141 MAR1-5A3) (eBioscience) or goat IgG isotype control (R&D Systems) 2 hrs before  
142 tolerance induction. After IFNAR1 neutralization and tolerance, for allergy induction  
143 mice were immunized with chicken OVA on day 15, and OVA challenges were  
144 performed between days 21-23.

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

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

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

157 **pDC generation from BM, cultures and adoptive transfer.** For pDC generation,  
158 bone marrow (BM) cells were isolated and cultured with rhFlt3L as described(37). On  
159 day 11, 7AAD<sup>-</sup>CD3<sup>-</sup>CD19<sup>-</sup>CD11c<sup>+</sup>B220<sup>+</sup>PDCA1<sup>+</sup>Siglec-H<sup>+</sup> pDCs were sorted using  
160 FACS ARIAM to a purity  $\geq 98\%$  after enrichment with CD11c microbead kit  
161 (Miltenyi Biotec). Sorted pDCs from BM-cultures cultured were conditioned for 16  
162 hrs with 100  $\mu\text{g/ml}$  of endograde OVA or LoTox<sup>TM</sup> *Dermatophagoides pteronyssinus*  
163 allergen 1 (Derp1) (Indoor Biotechnologies) in the presence of 250 ng/ml rOpn or  
164 18.2 ng/ml frOpn1 or frOpn3 or PBS. After the culture with OVA, pDCs were washed  
165 and analysed or transferred intravenously (i.v.) via the tail vein ( $10^6/\text{mouse}$ ). Allergic  
166 asthma was induced in mice 7 days later. Mice were euthanized 2 d after the last OVA  
167 challenge (Fig. 7B, 10A).

168 **Cell culture, proliferation and cytokine analysis.** Isolated dLN cells ( $2 \times 10^5$  to  $10^6$ )  
169 were cultured with 125  $\mu\text{g/ml}$  OVA (Sigma-Aldrich) for 48 h. We performed  
170 proliferation assays with thymidine incorporation, as previously described(38). For  
171 certain experiments, proliferation of cells was measured as % of Edu<sup>+</sup> cells by FACS,  
172 using a Molecular Probes kit. For cytokine measurements, we used ELISA kits for IL-  
173 5 and IFN- $\gamma$  (BD Biosciences); IL-4, IL-13 (R&D Systems) and IFN- $\beta$  (BioLegend).

174 **Flow cytometry.** Freshly isolated live (7AAD<sup>-</sup>, BD Biosciences) dLN cells and *in*  
175 *vitro* BM-derived cells were stained with combinations of fluorochrome-conjugated  
176 Abs to CD4-Pacific Blue or -PE/Cy5 (clone GK 1.5), CD3-PE/Cy7 or -PE/Cy5 or -PE  
177 or-FITC (17A2), CD11c-PE/Cy7 or -FITC or Orange 605 (N418), CD11b-PE/Cy7 or  
178 -FITC (M1/70), B220-PE or -BV 510 (RA3-6B2), CCR7-PE (4B12), Siglec-H-  
179 Pacific Blue or -FITC (551), PDCA-1-PE or -FITC (927), CD19-PE/Cy7 or -PE/Cy5  
180 (6D5), CD25-PE (PC61.5) (BioLegend) and T1ST2 (DIH9) (-FITC, MD Biosciences  
181 or -PE BioLegend). For intra-nuclear staining of Foxp3, permeabilization kit and  
182 antibody (-Pacific Blue or -PE/Cy5, clone FJK-16s) were used (eBioscience). The  
183 flow cytometric measurements were performed using Attune Acoustic Focusing  
184 Cytometer (Applied Biosystems) and FACS ARIAM (BD). FACS sorting of pDCs

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

187 **Chemotaxis assay.** Sorted pDCs from LNs and spleen of BALB/c mice, were treated  
188 with rOpn (500 ng/ml) for 18-20 h and assayed for migration in response to  
189 chemokines CCL19 or CCL21 (200 ng/mL) (R&D Systems). The lower chambers of  
190 Transwell plates (QCM™ 5.0-µm chemotaxis assay 24-well-Colorimetric, Millipore)  
191 were filled with 500 µl serum-free medium in the presence or absence of chemokines.  
192 DCs (10<sup>5</sup> cells per 200 µl) resuspended in serum-free medium were deposited in the  
193 upper chambers of the Transwell plates and allowed to migrate for 4 h at 37°C in 5%  
194 CO<sub>2</sub>. For each experiment pooled total cells from spleen, inguinal and mesenteric  
195 lymph nodes of mice (n=8) were used and pDCs were isolated by sorting.

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

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

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

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

219 **Results**

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

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

239 We next investigated whether Opn deficiency had any effect on tolerance induction.  
240 Tolerance induction in *Spp1*<sup>-/-</sup> mice was not as effective as in *Spp1*<sup>+/+</sup>, since we noted  
241 no significant change in neither number of eosinophils nor total cell numbers in BAL  
242 (**Fig. 2A**). In accordance, tolerogenic i.n. OVA administration in *Spp1*<sup>-/-</sup> mice could  
243 not efficiently dampen the inflammation and mucus secretion in the lung, whereas it  
244 was very efficient in *Spp1*<sup>+/+</sup> mice (**Fig. 2B**). T<sub>H</sub>2 cytokine production by OVA-  
245 stimulated mLN cells was also lower in OVA-tolerized *Spp1*<sup>-/-</sup> compared to PBS-  
246 treated *Spp1*<sup>-/-</sup> mice (**Fig. 2C**). OVA-stimulated mLN cells from OVA-tolerized  
247 *Spp1*<sup>+/+</sup> mice produced approximately 50% lower levels of T<sub>H</sub>2 cytokines, compared  
248 to cells from PBS-treated *Spp1*<sup>+/+</sup> mice. Whereas, OVA-tolerized *Spp1*<sup>-/-</sup> mice had a  
249 lower reduction in cytokine levels compared to *Spp1*<sup>+/+</sup> (**Fig. 2C**). The reduction in  
250 IFN- $\gamma$  levels was similar among groups (**Fig. 2C**). Finally, OVA tolerization of *Spp1*<sup>-</sup>

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

### 255 **Opn administration along with antigen increases accumulation of tolerogenic** 256 **pDCs**

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

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

### 276 **Administration of Opn during tolerance induction regulates CCR7 expression** 277 **affecting DC subset homing to dLNs**

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

282 (Fig. 3A). Percentages of CCR7<sup>+</sup> pDCs per total pDC numbers were significantly  
283 elevated in peripheral blood of mice treated with rOpn during tolerance induction  
284 compared to PBS treated mice (Fig. 3C). Percentages of CCR7<sup>+</sup> cDC per total cDC  
285 numbers originating from peripheral blood of mice that had been administered  
286 OVA/rOpn were significantly decreased, compared to OVA-administered ones (Fig.  
287 3C). Our results demonstrate that Opn leads to enhanced migratory CCR7<sup>+</sup> pDC  
288 percentages per total pDCs in the blood.

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

### 300 **Opn enhances pDC-dependent Foxp3<sup>+</sup> Treg cell accumulation and promotes an** 301 **immunoregulatory milieu in the dLNs**

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

313 with antigen promotes Foxp3<sup>+</sup> Treg cell accumulation resulting in a highly tolerogenic  
314 microenvironment in the dLN.

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

322 To address whether the Opn-mediated increase in pDC numbers was responsible for  
323 the observed accumulation of Foxp3<sup>+</sup> Treg cells, we depleted pDCs by administering  
324 120G8 pDC-depleting antibody(32) prior to induction of tolerance and examined the  
325 accumulation of Foxp3<sup>+</sup> Treg cells in the dLNs. This depletion led to a significant  
326 reduction in CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell numbers versus OVA/rOpn administration in non-  
327 pDC-depleted mice (**Fig. 6A**), resulting in exacerbated allergic airway disease (**Fig.**  
328 **6B**). We observed similar effects of rOpn on Treg cellularity in LNs after *in vivo*  
329 depletion of pDCs in *BDCA2-DTR* transgenic mice (33) (**Supplemental Fig. 2**). Thus,  
330 Opn-mediated pDC accumulation is at least partially responsible for the higher  
331 numbers of Treg cells in dLNs. In addition, dLN cells from OVA/rOpn-treated pDC-  
332 depleted mice were significantly less capable of suppressing OVA-specific T cell  
333 proliferation (**Fig. 6C**), compared to dLN cells from OVA/rOpn-treated non-pDC-  
334 depleted mice, reaching even lower suppressive ability than that of OVA-treated  
335 pDC-depleted dLN cells. Thus, OVA/rOpn treatment increased numbers of pDCs  
336 favoring accumulation of Foxp3<sup>+</sup> Treg cells that are crucial for tolerance maintenance.

### 337 **Opn treatment of pDCs increases their suppressive activity against allergic** 338 **airway disease**

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

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

### 352 **Opn SLAYGLR motif is responsible for pDC recruitment and effective** 353 **protection from allergic disease**

354 RNA expression analysis revealed that, upon stimulation, mLN pDCs elevate  
355 expression of integrins  $\alpha\beta3$ ,  $\alpha4\beta1$  and  $\alpha9\beta1$  (data not shown). As the SLAYGLR  
356 motif of Opn interacts with  $\alpha4\beta1$ ,  $\alpha4\beta7$  and  $\alpha9\beta1$  integrins (12), and the RGD motif  
357 interacts with the  $\alpha\beta3$  integrin (11, 12), we asked which Opn domain is responsible  
358 for the observed effects on pDCs during tolerance induction. Thus, the synthetic  
359 frOpn<sub>134-153</sub>, containing the RGD and the SLAYGLR integrin binding motifs either  
360 intact or scrambled (31), were used with OVA to induce tolerance in mice, allowing  
361 us to unveil the involvement of the different integrin-binding motifs of Opn in pDC  
362 recruitment (**Fig. 8A**). The frOpn<sub>134-153</sub> represents the thrombin cleaved fragment of  
363 Opn that reveals the otherwise cryptic domain SLAYGLR (28). OVA/frOpn1,  
364 containing an intact SLAYGLR motif but a scrambled RGD motif, induced  
365 accumulation of higher numbers of pDCs in the LNs compared to OVA, and  
366 OVA/rOpn (**Fig. 8A**). Conversely, frOpn2, which contains an intact RGD, but a  
367 scrambled SLAYGLR motif induced a reduction in pDC cell numbers in dLNs  
368 compared to all other treatments (**Fig. 8A**). When both motifs were scrambled as in  
369 the case of frOpn3, the numbers and percentages of pDCs were not significantly  
370 altered compared to OVA (**Fig. 8A, Supplemental Fig. 3A**). OVA/frOpn1 induced  
371 higher percentages of pDCs in the dLNs compared to OVA and OVA/frOpn3  
372 (**Supplemental Fig. 3A**). These data revealed that the Opn SLAYGLR motif is  
373 responsible for pDC accumulation into dLNs. These data also reveal that the RGD  
374 motif suppresses this accumulation.

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

### 385 **Opn SLAYGLR motif induces IFN- $\beta$ expression in pDCs**

386 We further asked whether OVA/frOpn1 treatment affects *in vivo* pDC immune profile  
387 36-40 hrs after tolerance induction (**Fig. 3A**). *In vitro*, OVA/frOpn1 conditioning of  
388 pDCs promoted a 2-fold increase in *Ifnb* mRNA expression at 16 hrs (**Fig. 9A**). The  
389 observed difference in *Ifnb* expression was also reflected in the amount of IFN- $\beta$   
390 secreted, that was 5-fold higher in OVA/frOpn1-conditioned pDCs compared to  
391 OVA/frOpn3 (**Fig. 9B**). House dust mite endotoxin-free Derp1 was also used to test  
392 whether the increased IFN- $\beta$  response could be observed using another allergen.  
393 Indeed, Derp1/frOpn1 *in vitro* conditioning of pDCs resulted in higher *Ifnb* expression  
394 compared to Derp1/frOpn3 (**Fig. 9C**). We also measured higher levels of *Ifnb*  
395 expression in OVA/rOpn-conditioned pDCs compared to OVA/PBS (**Supplemental**  
396 **Fig. 3B**).

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

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

#### 410 **Opn/SLAYGLR-induced IFN- $\beta$ in pDCs is protective against allergic disease**

411 pDCs were primed *in vitro* with endotoxin-free OVA and frOpn1 or frOpn3 and  
412 transferred into mice prior to induction of allergic airway inflammation (**Fig. 10A**).  
413 Recipient mice were *Ifnar1*<sup>+/+</sup> or *Ifnar1*<sup>-/-</sup>. pDCs treated with frOpn1 were  
414 significantly more potent in protecting *Ifnar1*<sup>+/+</sup> mice against allergic airway  
415 inflammation, as demonstrated by reduced lung airway inflammation, BAL  
416 eosinophilia and goblet cell hyperplasia compared to control and OVA group (**Fig.**  
417 **10A**). Similar enhanced protection was also observed when pDCs were treated with  
418 rOpn and adoptively transferred into *Ifnar1*<sup>+/+</sup> mice (**Fig. 7B-D**). Actually, type I IFN  
419 produced by pDCs was crucial in maintaining this protective effect, as *Ifnar1*<sup>-/-</sup>  
420 recipient mice were not protected against allergic asthma (**Fig. 10A**). These results  
421 demonstrate that the integrin-binding SLAYGLR domain of Opn renders pDCs more  
422 regulatory through type I IFN production in the context of allergic airway  
423 inflammation. In fact, pDCs cannot preserve their regulatory function in recipients  
424 that lack expression of type I IFN receptor. Finally, neutralization of IFNAR1 during  
425 the OVA tolerance induction phase (**Fig. 10B**) resulted in reduced protection from  
426 allergic airway disease concomitant with increased eosinophilia in BAL (**Fig. 10C**)  
427 and increased OVA-specific responses (**Fig. 10D**). This indicates that type I IFN is  
428 absolutely necessary for effective antigenic tolerance induction in this context.

429 **Discussion**

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

433 The respiratory mucosa is constantly being exposed to a myriad of non-pathogenic  
434 environmental antigens. To protect against the immunopathological consequences of  
435 this constant stimulus, a default low non-inflammatory T<sub>H</sub>2 mechanism and/or a T  
436 cell-mediated tolerance mechanism is activated (53). The mechanism underlying these  
437 processes is not fully understood. In the present study, we initially demonstrate that  
438 Opn induces tolerance by tilting the pDC/cDC balance in favor of the anti-  
439 inflammatory pDCs, via differentially regulating their CCR7 expression. CCR7-  
440 dependent homing of DCs into the dLNs is required for the induction of tolerance(50).

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

452 Regulatory cytokines such as *Tgfb1*, *Il10*, *Fgl2*, and molecules such as *Ido* (54) were  
453 substantially up-regulated in our Opn-driven tolerance model, creating an  
454 immunoregulatory microenvironment in the dLN. This tolerogenic milieu was  
455 accompanied by accumulation of Foxp3<sup>+</sup> Treg cells, which are very important for  
456 induction of tolerance (51). Mice that had enhanced Treg cell numbers, due to rOpn  
457 administration, showed suppressed effector responses and were protected from  
458 allergic disease. When Foxp3<sup>+</sup> Treg cells were depleted, Opn-mediated tolerance  
459 boosting was abolished. In light of its well-established pro-inflammatory function  
460 (10-12), it was unexpected that Opn could be an inducer of Treg cell accumulation *in*

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

469 Thrombin cleavage of Opn occurs during both homeostatic and inflammatory  
470 conditions (24, 25) and conformational changes after thrombin cleavage result in  
471 higher affinity binding to certain receptors (26). In fact, the cryptic SLAYGLR motif  
472 is revealed upon thrombin cleavage of Opn which is vital for its interaction with  $\alpha 9\beta 1$   
473 integrin (28). As the SLAYGLR motif of Opn protein interacts with  $\alpha 4\beta 1$ ,  $\alpha 4\beta 7$  and  
474  $\alpha 9\beta 1$  integrins (12), and the RGD motif interacts with the  $\alpha v\beta 3$  integrin (11, 12), we  
475 used the synthetic frOpn<sub>134-153</sub>, containing both integrin binding motifs either intact or  
476 scrambled (31). Thrombin cleavage of Opn also produces a fragment containing the  
477 carboxy-terminal half of Opn, which interacts with certain CD44 variants (12, 27). In  
478 our settings, as well as in other settings (31, 55, 56), frOpn<sub>134-153</sub> has a greater  
479 efficiency compared to full length Opn. It is thus possible that interaction of Opn with  
480 CD44 interferes with certain Opn effects, such as pDC recruitment. Future  
481 investigation will elucidate the role of Opn-CD44 interaction in tolerance induction.

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

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

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

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

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519 **Author Contributions**

520 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.  
521 performed research; L.B. and T.S. contributed new reagents; T.A., E.K., D.C.M.S.  
522 and V.P. analyzed data; T.A., E.K., B.N.L. and V.P. wrote the paper. B.N.L. and V.P.  
523 supervised the study.

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714 **Conflict of Interest:**

715 The authors declared no conflict of interest.

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718 **Figure legends**

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

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

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

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

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

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

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

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

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

803 **Figure 8. Opn SLAYGR motif boost tolerance by enhancing pDC recruitment to**  
804 **protect from allergy.** (A) Numbers of dLN 7AAD<sup>-</sup>CD11c<sup>+</sup>B220<sup>+</sup>PDCA1<sup>+</sup>SiglecH<sup>+</sup>  
805 pDCs after 40 hrs of tolerance induction with endograde-OVA i.n. along with rOpn or  
806 frOpn1, or frOpn2, or frOpn3, or PBS as in Fig. 3A. (B) OVA/frOpn-tolerance  
807 induction before allergic asthma for c-e. Control mice were pre-treated with PBS and  
808 allergy was subsequently induced (white bars). (C) AHR responses depicted as Penh  
809 in day 19. (D) Eosinophil cell count in BAL, histological assessment of lung  
810 inflammation (H&E scoring) and lung mucus production (PAS score) and numbers of  
811 T1ST2<sup>+</sup>CD4<sup>+</sup> Th2 cells in mLNs. (E) Levels of IL-4, IL-5 and IL-13 in supernatants  
812 of OVA-stimulated mLN cells. Different wells of pooled mLN cells. Values are  
813 expressed as mean ± SEM (n=5, mice per group), one representative of three  
814 independent experiments.

815 **Figure 9. Opn SLAYGR motif induces IFN- $\beta$  production from pDCs.** (A)  
816 Relative *Ifnb* expression to *Hprt* in pDCs conditioned *in vitro* with OVA/frOpn1 or  
817 frOpn3. (B) Levels of IFN- $\beta$  in the supernatants of the same cultures. (C) Relative  
818 *Ifnb* expression to *Hprt* in pDCs *in vitro* conditioned with Derp1/frOpn1 compared to  
819 Derp1/frOpn3. (D) Representative percentages in flow cytometric plots and numbers  
820 of 7AAD<sup>-</sup>CD3<sup>-</sup>CD19<sup>-</sup>CD11c<sup>+</sup>PDCA-1<sup>+</sup>Siglec-H<sup>+</sup>IFN- $\beta$ <sup>EYFP+</sup> pDCs in dLNs of PBS-  
821 treated or OVA-tolerized mice, and geometrical mean fluorescence intensity (gMFI)  
822 of IFN- $\beta$ -expressing pDCs. (E) Relative *Ifnb* expression to *Hprt* in pDCs isolated  
823 from dLNs of PBS-treated or OVA/frOpn3- and OVA/frOpn1-tolerized mice. (F)  
824 Representative percentages in flow cytometric plots and numbers of total 7AAD<sup>-</sup>  
825 CD3<sup>-</sup>CD19<sup>-</sup>CD11c<sup>+</sup>PDCA-1<sup>+</sup>Siglec-H<sup>+</sup> IFN- $\beta$ <sup>EYFP+</sup>, and gMFI of IFN- $\beta$ -expressing  
826 pDCs in dLNs of mice treated with PBS or tolerized with MOG<sub>35-55</sub>/frOpn3 or  
827 MOG<sub>35-55</sub>/frOpn1. Mice were tolerized as in Fig. 3A. Values are expressed as mean  $\pm$   
828 SEM (n=6-8 mice per group), pooled data from three independent experiments.

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