

9-*cis*-Retinoic acid induces a distinct regulatory dendritic cell phenotype that modulates murine delayed-type allergy

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doi:10.1111/cod.12868

Summary

Background. Hand eczema, which is frequently caused by delayed-type allergy, is treated with 9-*cis*-retinoic acid (9cisRA). However, knowledge on how 9cisRA modulates skin immunity is sparse.

Objective. As dendritic cells (DCs) are central in the pathogenesis of contact allergy, we investigated 9cisRA modulation of DC function in murine contact hypersensitivity (CHS).

Methods. 9cisRA-differentiated DCs (9cisRA-DCs) were analysed for phenotype and function. *In vivo* 9cisRA-DCs were tested in the CHS model.

Results. 9cisRA induces the differentiation of a distinct CD103[−] CD207[−] regulatory DC phenotype. CD11c⁺ DCs differentiated with 9cisRA have lower expression of major histocompatibility complex-II and costimulatory molecules, but conversely have higher expression of the inhibitory coreceptor PD1-L. 9cisRA-DC culture does not induce the expression of proinflammatory cytokines, but strongly enhances osteopontin (OPN) secretion. 9cisRA-DCs are compromised in the induction of T cell proliferation *in vitro*, but efficiently convert naive T cells into regulatory T cells (Tregs). Notably, OPN-deficient 9cisRA-DCs show a loss of Treg-inducing function, which is re-established by substituting OPN. *In vivo*, in allergic mice, allergen-primed 9cisRA-DCs suppress allergic inflammation and induce Treg accumulation in skin draining lymph nodes.

Conclusions. This study describes 9cisRA-mediated differentiation of a distinct DC phenotype that relies on OPN for Treg transformation and suppresses established CHS through Treg induction.

Key words: 9-*cis*-retinoic acid; contact hypersensitivity; dendritic cells; eczema; osteopontin; regulatory T cells; tolerance.

Retinoids have long been established for the treatment of immune-mediated skin diseases. Recently, 9-*cis*-retinoic acid (9cisRA) has been introduced to treat chronic hand

eczema, a typical manifestation of allergic contact dermatitis (ACD) (1). To date, little is understood about how retinoids modulate the skin immune system.

Retinoids are vitamin A derivatives that have pleiotropic effects regulating cell differentiation, proliferation and apoptosis through two families of retinoid nuclear receptors (2). The retinoic acid (RA) receptor (RAR) family is naturally activated by RA, and the retinoid X receptor (RXR) is naturally activated by 9cisRA (3). 9cisRA is a high-affinity pan-agonist for both RAR and RXR (3). In the intestinal immune system, dendritic cells (DCs) are able to express all-*trans*-retinoic acid, which interacts only with the RAR and promotes the

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Conflicts of interest: J. M. Weiss has received salaries for scientific lectures from Basilea Pharmaceuticals.

Accepted for publication 6 July 2017

differentiation of naive T cells into regulatory T cells (Tregs) for intestinal tolerance (2, 4).

DCs are essential for sensitization to an allergen. They carry haptens to lymph nodes and present them to naive T cells. Antigen-specific T helper (Th)1 and Th17 cells differentiate and recirculate to the skin (5). When an allergen penetrates the skin, these cells, together with resident DCs, orchestrate the specific inflammatory response against the allergen (5). However, as regulators, DCs are also essential for the termination of the inflammatory response (6, 7). DCs have been shown to express both RAR and RXR (8). Although phenotypic alterations of DCs by retinoids have been described, their functional potential is not known.

Tregs are relevant in several aspects of ACD, such as susceptibility, variation of response to an allergen, prevention of an exacerbated inflammatory response, termination of eczema, and tolerance (9). CD4⁺ CD25⁺ Foxp3⁺ Tregs are categorized into two major groups: naturally occurring Tregs and inducible Tregs (10). So far, transforming growth factor (TGF)- β is the most important factor known for the induction of inducible Tregs (10). Tregs fulfil their suppressive roles in part through cellular interactions and the production of cytokines, thereby modifying lymphocyte and DC functions (10). In ACD, the induction of specific tolerizing Tregs is a promising approach to modulate allergen sensitization (5).

Osteopontin (OPN) is expressed by various immune cells in a secreted form and an intracellular form (11). In the skin, OPN is produced by DCs, macrophages, mast cells, and activated T cells (12). We showed that, in ACD, OPN is highly expressed by infiltrating inflammatory cells, including DCs (13, 14). OPN has cytokine, chemotactic and cell signalling functions, enhancing Th1-mediated and Th17-mediated immunity. Also anti-inflammatory functions have been attributed to OPN (15–17).

Herein, we report that differentiation of DCs in the presence of 9cisRA generates a distinct tolerogenic DC phenotype [9cisRA-differentiated DCs (9cisRA-DCs)] that efficiently induces Tregs. OPN, which is highly secreted by such DCs, is central for the induction of Tregs. Furthermore, 9cisRA-DCs partially suppress the established T cell-mediated inflammatory response and support Treg accumulation in lymph nodes.

Materials and Methods

Mice

Mice lacking the functional OPN gene (*spp1*) have been described previously (18). For all experiments, C57BL/6J or BALB/c OPN mutant mice in the 10th generation

of backcrossing were used. All work was performed in accordance with EU Directive 2010/63/EU for animal experiments. Protocols were approved by the Committee of Animal Research (Regierungspräsidium Tübingen, Germany).

DC culture and 9cisRA treatment

Murine DCs were generated as described previously (14). Bone marrow was harvested, cells were cultured with granulocyte–macrophage colony-stimulating factor and interleukin (IL)-4 (both PeproTech, London, UK), and half of the supplemented medium was replaced on day (D) 3. Cells were harvested on D5 of culture by gradient centrifugation, with 15.5% Nycodenz (Axis-Shield, Oslo, Norway). 9cisRA was dissolved in ethanol (eth.) and added to DC cultures at concentrations of 3, 30 or 300 ng/ml throughout culture. Equal amounts of eth. were added as a control. 9cisRA or eth. were not toxic for DCs, as analysed by viability staining (data not shown). For activation experiments, DCs were generated as described above and stimulated with lipopolysaccharide (LPS) (Sigma-Aldrich, Schnellendorf, Germany) for 24 h.

Flow cytometry

Surface receptor expression on DCs was determined by use of a standard protocol. Cells were stained (4°C, 30 min) with fluorescein isothiocyanate-labelled, phycoerythrin (PE)-labelled, PEcy5-labelled or allophycocyanin-labelled monoclonal antibodies (mAbs): anti-major histocompatibility complex (MHC)-II (BD Pharmingen, Heidelberg, Germany; M5/114.15.2), anti-CD80 (Biolegend, Koblenz, Germany; 16-10A1), anti-CD86 (BD Pharmingen; GL1), anti-CD11c (eBioscience, Frankfurt, Germany; N418), anti-CD44 (BD Pharmingen; IM7), anti-PDL-1 (eBioscience; MIH5), and anti-ICOS-L (Biolegend; HK5.3). For viability analysis, 0.1 µg/ml propidium iodide (Sigma-Aldrich, Frickenhausen, Germany) was added. Samples were analysed with a FACSCanto II (Becton Dickinson), and mean fluorescence intensities were determined with FACS Diva[®] (Becton Dickinson, Heidelberg, Germany) or FlowJo[®] (Tree Star, Inc., Ashland, OR, USA) software.

Cytokine array and enzyme-linked immunosorbent assay (ELISA)

Supernatants from untreated or 9cisRA-cultured DCs were harvested on D5. Cytokines in supernatants were detected by use of the Proteome Profiler Array, Mouse Cytokine Array Panel A (R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer's

protocol. Profiles were analysed by quantifying the mean spot pixel densities from scanned images (Fusion FX7; Vilber Lourmat, Eberhardzell, Germany) with image analysis software (Adobe Photoshop; Adobe, San Jose, CA, USA).

Cytokines from DC cultures or mixed lymphocyte reactions (MLRs) were quantified by ELISA (Ready-Set-Go![®] ELISA Set; eBioscience) and measured at an extinction of 450 nm (Mithras LB-940 microplate reader and MICROWIN 2000 software; Berthold Technologies, Bad Wildbad, Germany) with OPN ELISA (R&D Systems) or IL-10 ELISA (Thermo Fisher Scientific, Freiburg, Germany). Cytokines in MLRs were measured with a Mouse Th1/Th2/Th17 Cytokines Multi-Analyte ELISArray (Qiagen, Hilden, Germany).

RNA isolation and quantitative real-time polymerase chain reaction (PCR)

DCs were generated from mouse bone marrow and cultured for 5 days in the presence of 9cisRA (3, 30 and 300 ng/ml). RNA was isolated by the use of peqGOLD RNAPure (Peqlab, Erlangen, Germany). To synthesize cDNA, reverse transcription was performed with a reverted First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The obtained cDNA was used for quantitative real-time PCR with FastStart Universal SYBR Green Master (Rox) (Roche Diagnostics, Mannheim, Germany) and an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, USA). Thermocycling conditions were as follows: hold at 95°C for 10 min, 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds, and an additional dissociation stage. The relative expression levels of the target gene in different samples were normalized to endogenous glyceraldehyde-3-phosphate dehydrogenase, and calculated with the $2^{-\Delta\Delta Ct}$ method. Mouse OPN primers were 5'-GGTGATAGCTTGGCTTATGGACTG-3' (forward) and 5'-GCTCTTCATGTGAGAGGTGAGGTC-3' (reverse). Mouse glyceraldehyde-3-phosphate dehydrogenase primers were 5'-TGGCCTTCCGTGTTCTTACC-3' (forward) and 5'-GGTCCTCAGTGTAGCCAAAGATG-3' (reverse) (13).

T cell proliferation assay and induction of Tregs

MLRs were performed as described previously (14). Enriched spleen T cells (CD4⁺ T cell kit; Miltenyi, Bergisch Gladbach, Germany) from OPN^{Wt} or OPN^{-/-} Balb/c mice were stained with carboxyfluorescein succinimidyl ester (CFSE) (5 μ M; Sigma-Aldrich, Munich, Germany) and cocultured with gradient-enriched irradiated (30 Gy) DCs from OPN^{Wt} or C57BL/6 OPN^{-/-} mice generated in the presence or absence of 9cisRA (3, 30 and

300 μ g/ml). Induction of Tregs was measured on D5 by fluorescence-activated cell sorting (FACS) (FACSCanto II) with a Mouse Regulatory T Cell Staining Kit (Affymetrix eBioscience, Frankfurt, Germany). For investigation of the role of OPN in the induction of Tregs, recombinant OPN (2 μ g/ml; R&D Systems) was added to cocultures.

Alternatively, to exclude an effect of DCs on Treg induction, 2×10^5 purified T cells were added to microplates coated with 10 μ g/ml purified NA/LE anti-mouse CD3e antibody (BD Pharmingen; 145-2C11) and 2 μ g/ml purified NA/LE anti-mouse CD28 antibody (BD Pharmingen; 37.51) with or without 9cisRA added to the culture medium. After 48 h, cells were analysed with a Regulatory T Cell Staining Kit. For IL-10 neutralization experiments, anti-IL-10 mAb (clone JES052A5; R&D Systems/Biotechne, Minneapolis, MN, USA) was added to cocultures at 15 ng/ml.

Autologous induction of Tregs

C57/BL6J DCs were differentiated from murine bone marrow for 5 days with or without 30 ng/ml 9cisRA (Ali30), and separated into two groups – one untreated, and the second loaded with 3 mM 2,4,6-trinitrobenzene sulfonic acid (TNBS) (Sigma-Aldrich, Schnellendorf, Germany) – and were then irradiated with 30 Gy (Fig. 4a). Naive T cells were purified by negative depletion with MACS from pooled lymph node and spleen cells of C57/BL6 mice. DCs and T cells were cocultured until D10, harvested, and resuspended. On D13, similarly treated DCs were added to appropriate previously primed T cells. FACS analysis of cocultured cells was performed on D18 with a Regulatory T Cell Staining Kit.

In vitro Treg suppression assay

Treg-mediated suppression was measured by CFSE dilution as described previously (19). In brief, conventional T cells (T_{conv}s) were labelled with 5 μ M CFSE, activated in anti-CD3/anti-CD28-coated wells, and cultured either alone or in the presence of cell suspensions from MLRs with untreated DCs or 9cisRA-DC at a 10:1 T_{conv}:Treg ratio. After 72 h, proliferation was determined by CFSE dilution with flow cytometry.

Modulation of contact hypersensitivity by 9cisRA-DCs

Contact hypersensitivity (CHS) was induced as described previously (20). C57BL/6 mice aged 8–9 weeks were painted on their abdominal skin with 100 μ l of 3% 2,4,6-trinitrochlorobenzene (TNCB) (VeZerf Laborsynthesen, Idar-Oberstein, Germany) in acetone on D0 (Fig. 6a). On D6, TNBS-loaded 9cisRA-DCs (30 ng/ml

9cisRA) or control DCs were injected subcutaneously into abdominal skin. Mice were challenged by painting 10 μ l of 1% TNCB on each side of the ears on D14. Ear thickness was measured with an engineer's micrometer (Mitutoyo, Neuss, Germany) before challenge and 24 and 48 h after challenge, and ear-swelling was calculated. In a second set of experiments, DCs were generated as above but injected on D6, D12, and D18 (Fig. 6c). To evaluate a lasting effect of Treg induction, challenge was performed on D25 and D48. Mice were killed on D50 to obtain skin draining lymph nodes (Fig. 6c). T cell suspensions were generated with a cell strainer, and analysed for the percentage of Tregs with the Mouse Regulatory T Cell Staining Kit.

Mesenteric lymph node (LN) cells and Langerhans cells (LCs)

Mesenteric LN cell suspensions and axillary LN cell suspensions from C57/BL6J mice were generated with a cell strainer and analysed for CD103 (Rat Anti-Mouse CD103, clone M290; BD Pharmingen) expression by FACS, with gating on CD11c⁺ cells.

For LC staining, epidermal cell suspensions were generated by limited trypsinization of trunk skin of C57/BL6J mice, as described previously (21). LCs were enriched by MACS separation (Epidermal Langerhans Cell MicroBead Kit; Miltenyi) and analysed by FACS with anti-CD207 mAb (929F3.01; Acris Antibodies, Herford, Germany).

Statistics

Student's *t*-test or all pairwise multiple comparison procedures (Holm–Sidak method) as implemented in SIGMASTAT (Systat Software, Erkrath, Germany) were used for the analyses presented in Figs. 1–5. One-way ANOVA was performed with GRAPHPAD PRISM (GraphPad Software, La Jolla, CA, USA), and the results are shown in Fig. 6. *p*-Values of ≤ 0.05 were regarded as significant.

Results

9cisRA modulates the phenotype of DCs

9cisRA was added to DC bone marrow cultures to investigate alterations in the DC phenotype (9cisRA-DCs). The CD11c-expressing 9cisRA-DCs dose-dependently had lower expression of the activation marker MHC-II and the costimulatory molecules CD80 and CD86 (Fig. 1a–e). The costimulatory receptor ICOS-L was not affected, whereas the inhibitory coreceptor PD1-L was strongly induced by 9cisRA (Fig. 1f, g). 9cisRA-DCs did not express CD103 or CD207 (Fig. 1h, i). To investigate their

plasticity, 9cisRA-DCs were treated with LPS (Fig. 1j). 9cisRA-DCs showed less upregulation of CD86 and CD80 than LPS-stimulated control DCs. LPS did not downmodulate ICOS-L in 9cisRA-DCs, but further stimulated expression of the inhibitory coreceptor PD-1L (Fig. 1j). Speculating that 9cisRA-DCs have a specific expression profile of inflammatory mediators, we used a cytokine profiling system to compare 9cisRA-treated DCs with controls. 9cisRA-DCs secreted significantly lower amounts of inflammatory cytokines and chemokines: IL-1 β , IL-12p70, IL-3, IL-7, CXCL9, CXCL10, and CCL-1. None of the tested cytokines or chemokines was upregulated by 9cisRA (Fig. 2a). In contrast, 9cisRA dose-dependently induced OPN secretion at the RNA and protein levels (Fig. 2b, c). These findings indicate that 9cisRA differentiates DCs towards a phenotype with regulatory potential.

9cisRA-cultured DCs induce Tregs

To investigate how 9cisRA influences DC antigen-presenting functions, allogeneic MLRs were performed. 9cisRA treatment of DCs downmodulated their allostimulatory effect on T cells (Fig. 3a). 9cisRA was not present during MLRs, thus excluding direct effects on T cells. When we analysed cytokine profiles in MLRs, we found that LPS-treated DCs strongly induced IL-2, IL-6, IL-13 and, especially, IL-17A secretion, indicating the induction of a Th17 phenotype (Fig. 3b). In contrast, in cocultures with 9cisRA-DCs, IL-2, IL-4, IL-5, IL-10, IL-13, IL-17A and interferon- γ secretion was inhibited, indicating that neither a Th17, a Th1 nor a Th2 phenotype was induced (Fig. 3b). As IL-10 is an important factor in Treg transformation (22), we additionally determined IL-10 secretion in supernatants from 9cisRA-DCs and MLRs (Fig. 3c). 9cisRA did not induce IL-10 secretion by DCs. In MLRs with 9cisRA-DCs, IL-10 secretion was downmodulated as compared with controls (Fig. 3c). Interestingly, OPN was highly secreted in MLR supernatants (Fig. 3d).

Previous work had shown that RA-synthesizing CD103⁺ DCs in the gut induce peripheral Tregs (4, 23). We therefore investigated whether 9cisRA treatment differentiates DCs with Treg-forming properties (Fig. 3e). 9cisRA-DCs were cultured with purified T cells of BALB/c mice, and the percentage of CD4⁺ Foxp3⁺ CD25⁺ cells was analysed by FACS after 5 days (Fig. 3e, f). 9cisRA-DCs, in contrast to controls, strongly induced the expansion of Tregs by up to >30% (Fig. 3g). Interestingly, 9cisRA-DCs were able to induce Tregs without upregulating TGF- β secretion, which was not inducible in cocultures (Fig. 3b). Blocking experiments with an anti-IL-10 mAb did not inhibit Treg transformation by 9cisRA-DCs (Fig. S1).

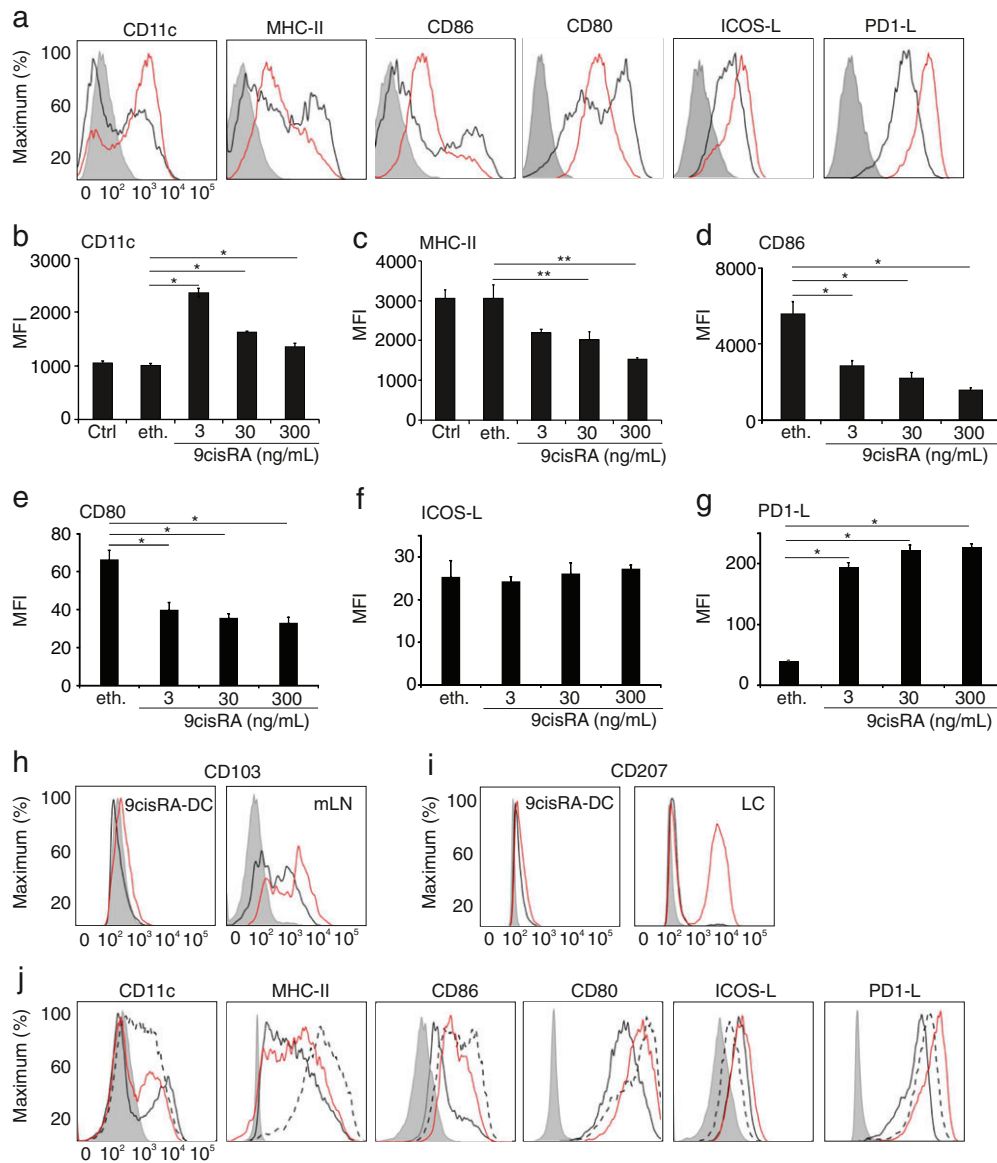


Fig. 1. 9-cis-Retinoic acid (9cisRA) affects the differentiation state of dendritic cells (DCs) in bone marrow cultures. DCs were generated from mouse bone marrow for 5 days in the presence or absence of the indicated 9cisRA concentrations. **(a)** Cell surface expression of the indicated molecules determined by fluorescence-activated cell sorting (FACS). Isotype controls: grey-shaded curves. Ethanol (eth.)-treated DCs: black curves. DCs treated with 30 ng/ml: red curves. **(b–g)** Expression of CD11c (b), major histocompatibility complex (MHC)-II (c), CD86 (d), CD80 (e), ICOS-L (f), and PD1-L (g). Data are shown as mean fluorescence intensity (MFI) $\pm$ standard error of the mean of surface marker expression of three individually treated DC cultures. **(h)** Control DCs and 9cisRA-differentiated DCs (9cisRA-DCs) were stained with anti-CD103 antibody (left box – isotype controls, grey-shaded curve; eth.-treated DCs, black curve; DCs treated with 30 ng/ml, red curve). As positive controls, mesenteric lymph node (mLN) cells were analysed (right box – isotype controls, grey-shaded curve; axillary lymph node cells, black curve; mLN cells, red curve). **(i)** Control DCs and 9cisRA-DCs were stained with anti-CD207 antibody (left box – isotype controls, grey-shaded curve; eth.-treated DCs, black curve; DCs treated with 30 ng/ml 9cisRA, red curve). For CD207 positive control staining, epidermal cell suspensions were stained with anti-CD207 antibody (right box – isotype control, grey-shaded curve; epidermal cell suspension, black curve; enriched Langerhans cell (LC) fraction: red curve). **(j)** DCs were generated for 5 days in the presence or absence of 9cisRA (30 ng/ml), and this was followed by treatment with lipopolysaccharide (LPS) for 24 h. Cell surface expression of the indicated molecules was analysed by FACS. Isotype controls: grey-shaded curves. LPS-treated DCs: dotted black curves. 9cisRA-treated DCs: black curves; DCs treated with 9cisRA and LPS: red curves. * $p \leq 0.05$; ** $p \leq 0.01$. Ctrl, control.

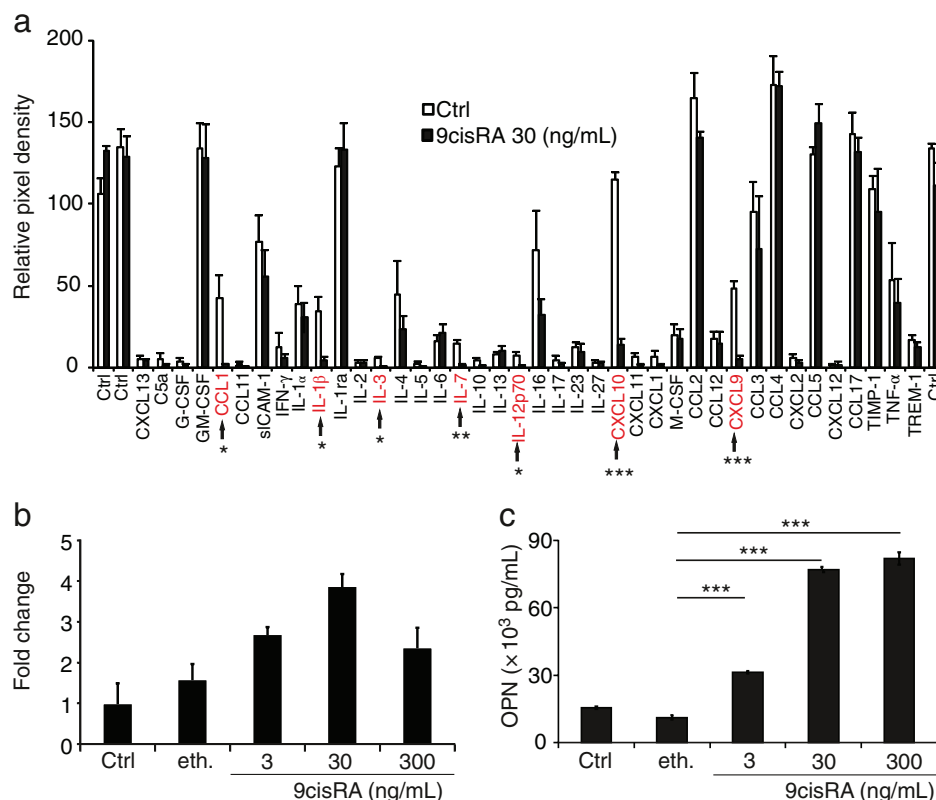


Fig. 2. Differential cytokine/chemokine secretion pattern in 9-*cis*-retinoic acid (9cisRA)-differentiated dendritic cells (DCs). DCs were generated from mouse bone marrow in the presence or absence of 9cisRA at the indicated concentrations. Ctrl, untreated DCs; eth., ethanol-cultured DCs. **(a)** Supernatants were analysed with PROTEOME PROFILER. Cells or supernatants were collected on day 5 of culture. The analytes were detected in pairs. Internal array positive controls for normalization are shown as control (Ctrl). Mean spot pixel densities from scanned images were analysed with image analysis software. Data are shown as relative pixel density. The average pixel density of pooled data from three independent experiments is shown. Significant changes are indicated by arrows. **(b)** Osteopontin (OPN) mRNA expression was determined by real-time polymerase chain reaction in DCs. Results are shown as fold change of OPN mRNA expression $\pm$ standard error of the mean (SEM) from three wells. Data are representative of two independent experiments. **(c)** DC supernatants were analysed with OPN-specific enzyme-linked immunosorbent assay. Data are shown as OPN concentration $\pm$ SEM. Data are representative of three independent experiments. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; TNF, tumour necrosis factor.

9cisRA-DC cultures routinely contained >75% CD11c⁺ cells. To show that CD11c⁺ DCs induced Tregs, 9cisRA-differentiated cells were sorted for CD11c⁺ and CD11c⁻ cells. Only CD11c⁺ 9cisRA-DCs induced Tregs (Fig. 3f). By the use of *in vitro* Treg suppression assays with cells from MLRs with control DCs or 9cisRA-DCs, we found significantly increased suppression by 9cisRA-DC-transformed Treg-containing suspensions (Fig. 3h). We further investigated whether 9cisRA may directly induce Tregs by stimulating naive T cells with CD3/CD28 in the presence of 9cisRA, and found no Treg induction (Fig. 3i). Our data indicate that 9cisRA-cultured DCs stimulate Treg conversion. In this setting, inflammatory cytokines are downmodulated. Whereas TGF- β and IL-10 are not upregulated, OPN is upregulated.

In an autologous, hapten-containing system, 9cisRA-DCs potentially induce Tregs

To determine whether 9cisRA influences the induction of Tregs in the presence of a strong hapten, autologous MLR was performed with TNBS. Control DCs and 9cisRA-DCs were cultured with autologous T cells in the presence or absence of TNBS (Fig. 4a). After 8 days, T cells were restimulated by control or TNBS-loaded untreated DCs or 9cisRA-DCs. Haptenized 9cisRA-DCs induced up to 50% of Tregs (Fig. 4b, c). On comparison of eth.-treated and 9cisRA-treated DCs, a seven-fold increase was observed for the control DCs and a three-fold increase for the haptenized cells. This indicates that 9cisRA-cultured DCs are stimulators of Tregs in the presence of a specific hapten.

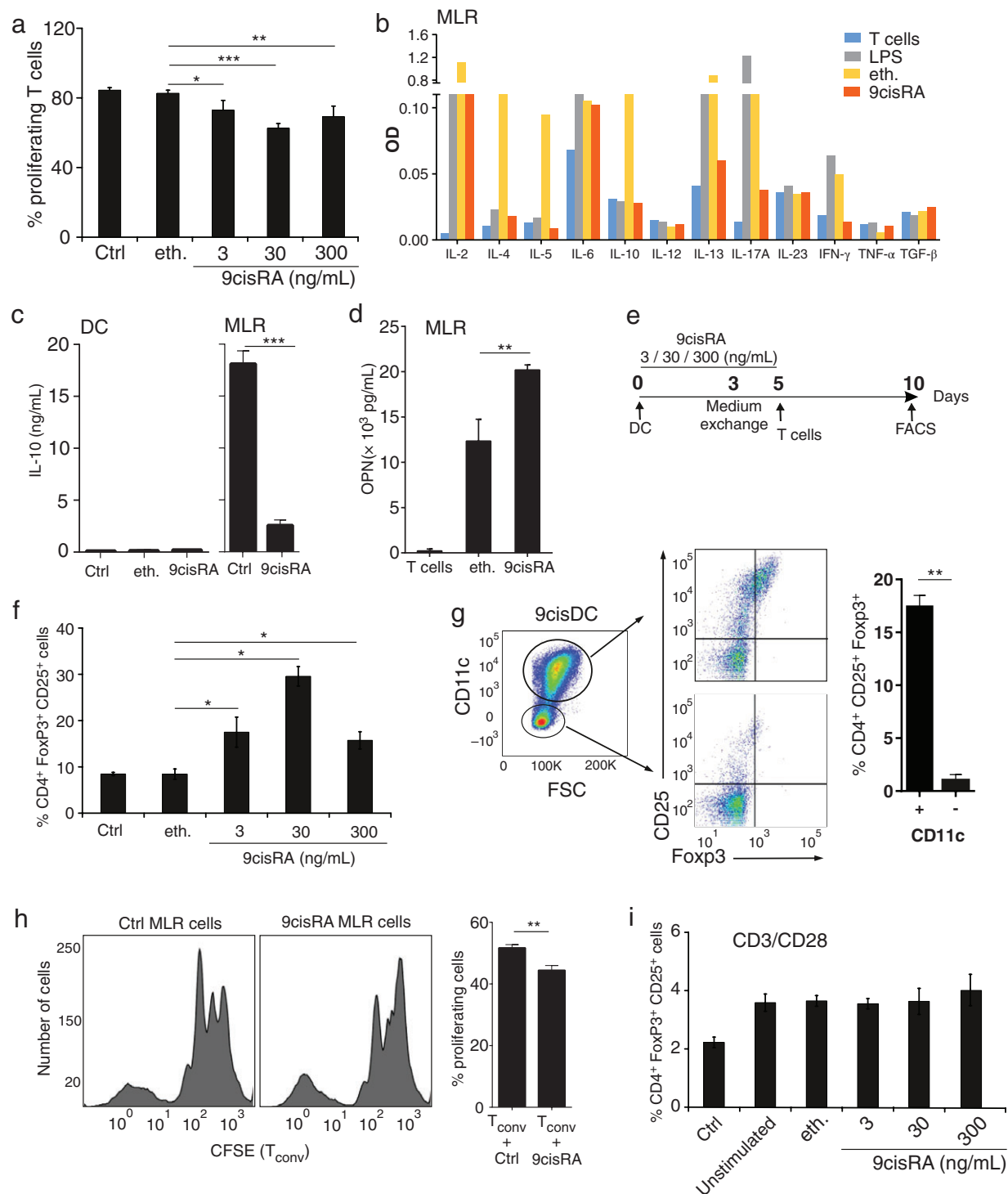


Fig. 3. Legend on next page.

OPN secretion by 9cisRA-DCs is important for transformation of naive T cells into Tregs

Because, in contrast to that of other cytokines, OPN secretion was induced in 9cisRA-DCs, we determined the role of OPN in Treg conversion. For this purpose, DC/T cell cocultures with cells from OPN wild-type (OPN^{Wt}) or OPN-deficient (*spp1*-deficient, OPN^{-/-}) mice on a C57Bl/6 or Balb/c background were performed (18) (Fig. 5). Untreated bone marrow-derived DCs from OPN^{Wt} and OPN^{-/-} mice induced comparable numbers of Tregs (average 8–12%) (Fig. 5a). However, 9cisRA-DCs (30 ng/ml) potentially converted naive T cells into Tregs (up to 30%), an effect that was achieved both with OPN^{Wt} and with OPN^{-/-} naive T cells by OPN^{Wt} DCs. However, OPN^{-/-} 9cisRA-DCs did not convert naive T cells into Tregs (Fig. 5a, b). Importantly, loss of OPN by DCs could be rescued by recombinant OPN (Fig. 5c). To exclude the possibility that this effect was attributable to decreased IL-10 secretion by OPN^{-/-} DCs, the levels of IL-10 secretion of 9cisRA DCs from OPN^{Wt} and OPN^{-/-} cells were compared. OPN^{-/-} 9cisRA-DCs had no significantly altered IL-10 secretion (Fig. S2). These results suggest that the enhanced secretion of OPN resulting from 9cisRA differentiation in concert with expression of coinhibitory molecules and reduced MHC-II expression on DCs is a central factor in the conversion of naive T cells into Tregs by 9cisRA-DCs.

9cisRA-DCs are able to inhibit established CHS

Currently, contact allergy memory in sensitized individuals is not erasable. The generation of tolerogenic DCs may constitute a tool with which to treat delayed-type allergy (6). To functionally test the modulatory potential of 9cisRA-DCs *in vivo*, we used the CHS model, which represents delayed-type allergy in humans (20) (Fig. 6a and c). Mice were sensitized, and were injected with TNBS-pulsed control DCs or 9cisRA-DCs 6 days after sensitization. When mice were challenged with TNCB on D14, the injection of TNBS-loaded DCs boosted ear swelling in comparison with sensitized, but DC-uninjected, mice. In contrast, antigen-loaded 9cisRA-DCs did not have the booster effect (Fig. 6b). To evaluate whether repeated injection of 9cisRA-DCs has stronger effects, 9cisRA-DCs or control DCs were injected three times (D6, D12, and D18) (Fig. 6c). Challenge was then performed on D25 and D48. When control DCs were repeatedly injected, they did not further boost established allergy, but supported continued allergic ear swelling (Fig. 6d). However, 9cisRA-DCs significantly inhibited the CHS response. This effect lasted even with repeated challenges up to D50. Importantly, skin draining lymph nodes, obtained after the final challenge in both experiments, either on D16 (data not shown) or D50 (Fig. 6e), contained significantly higher numbers of Tregs in 9cisRA-DC-injected mice. Our data indicate that, in allergic mice, hapten-primed

Fig. 3. Dendritic cells (DCs) cultured in the presence of 9-*cis*-retinoic acid (9cisRA) are partially inhibited in their allo-stimulatory capacity in mixed lymphocyte reactions (MLRs) but potentially induce CD4⁺ Foxp3⁺ CD25⁺ regulatory T cells. DCs from C57/Bl6 mice were cultured in the presence of the indicated 9cisRA concentrations, and were used in MLRs with pan-T cells selected by MACS purification from pooled lymph nodes and spleens of BALB/c mice. **(a)** T cell proliferation was measured by carboxyfluorescein succinimidyl ester (CFSE) staining on day (D) 6 of MLR. Data are shown as percentage of proliferating cells determined by CFSE staining $\pm$ standard error of the mean (SEM) for six independent MLR cultures. Representative data of two independent experiments are shown. Ctrl, untreated DCs; eth., eth.-cultured DCs; 3–30 ng/ml 9cisRA, DCs differentiated in the presence of 9cisRA until D5. **(b)** DCs were cultured until D6 with eth. or 30 ng/ml 9cisRA. For lipopolysaccharide (LPS) activation of DCs, LPS was added for the last 24 h. DCs were then used in MLRs as above. Supernatants were harvested, and the cytokine profile was analysed with the Mouse Cytokines Multi-Analyte ELISA Array. Results are representative of three independent experiments. **(c)** DCs were generated from mouse bone marrow in the presence or absence of 9cisRA (30 ng/ml), and interleukin (IL)-10 levels were determined in supernatants on D5 of culture. DCs were then used in MLR, and IL-10 levels were determined in supernatants. Data are shown as IL-10 concentration $\pm$ SEM. Data are representative of two independent experiments. **(d)** Supernatants from MLRs with eth.-treated DCs or 9cisRA-differentiated DCs (9cisRA-DCs) were analysed with osteopontin (OPN)-specific enzyme-linked immunosorbent assay (ELISA). Data are shown as OPN concentration $\pm$ SEM. Data are representative of two independent experiments. **(e)** Experimental design of MLR. DCs were generated from bone marrow of C57BL/6 mice, and cultured for 5 days in presence of three different 9cisRA concentrations, as indicated. CD4⁺ T cells were selected by MACS purification from pooled spleens and lymph nodes of BALB/c mice, and cocultured with the previously generated DCs until D10. Ctrl, untreated DCs; eth., eth.-cultured DCs. DCs were differentiated with 9cisRA at the indicated concentrations. **(f)** On D10 of coculture, Foxp3⁺ cells were analysed by fluorescence-activated cell sorting (FACS) with triple staining for CD4, Foxp3, and CD25. Data from one of four representative experiments are shown. Data are shown as the percentage of Foxp3⁺ cells $\pm$ SEM. **(g)** 9cisRA-DCs were sorted for CD11c expression on D5 of culture, and CD11c⁺ or CD11c⁻ DCs were cocultured with naive T cells to test for regulatory T cell (Treg) induction. On D10 of coculture, Foxp3⁺ cells were analysed by FACS. **(h)** Purified T cells from pooled spleens and lymph nodes of C57BL/6 mice were added to CD3/CD28-coated plastic wells. 9cisRA was added at the indicated concentrations. After 48 h, the percentage of CD4⁺ Foxp3⁺ CD25⁺ cells was analysed by triple FACS staining. Data are shown as the percentage of Foxp3⁺ cells $\pm$ SEM (not significant, Student's *t*-test). **(i)** Treg suppression assays were performed with cells from MLRs of control DCs and 9cisRA-DCs as described in Materials and Methods. The percentage of proliferating cells was determined by FACS gating. Data are shown as percentage proliferating cells $\pm$ SEM. **p* $\leq$ 0.05; ***p* $\leq$ 0.01; ****p* $\leq$ 0.001. OD, optical density; T_{conv}, conventional T cell; TGF, transforming growth factor; TNF, tumour necrosis factor.

9cisRA-DCs partially inhibit delayed-type allergy. This is accompanied by Treg accumulation in draining lymph nodes.

Discussion

In the intestinal immune system, RA synthesis by DCs is an important factor in Treg induction, mediating tolerance against nutritional allergens (2, 24). Like the intestinal immune system, the skin immune system protects an outer barrier through an elaborate DC system, suggesting that retinoids also contribute to the skin's immune homeostasis (25).

Reports on direct effects of different retinoids on DCs are sparse and contradictory (26). This is partially attributable to the fact that various stimuli contribute to the differentiation or activation of DCs in the context of retinoid treatment, such as cytokines or ligands for pattern recognition receptors (2, 27). Furthermore, the actions of different retinoids vary according to the receptors engaged or the type of DC tested. Most studies have been performed with RA that activates RAR. The 9cisRA studied here is a high-affinity pan-receptor agonist that ligates RAR and RXR (3), both of which are expressed by DCs (8, 28). Initially, Geissman et al. investigated the effect of retinoids on human Langerhans cell-like DCs by using, among other retinoids, 9cisRA (8). They found that treatment of immature DCs with 9cisRA resulted in their maturation (8), but functional capacities were not tested. Using human monocyte-derived DCs, Villablanca et al. found that 9cisRA inhibited CCR7 upregulation on maturing DCs, impeding their migration towards CCL19 (29). They also reported that RA synthesized by DCs from vitamin A induces its own synthesis in a positive feedback loop (30). In accordance with our findings, Hengesbach and Hoag reported that 9cisRA downmodulated CD80 and MHC-II in murine bone marrow-derived DCs. They suggested that these DCs favour Th2 development, but did not test their function (31). They also compared 9cisRA with RA, and found that the effects differed regarding costimulatory molecule expression. They suggested that the differences were attributable to RXR signalling of 9cisRA, which was not further analysed.

We used murine bone marrow-derived DCs that were differentiated with 9cisRA, and characterized their phenotype and functional abilities. 9cisRA-DCs expressed less MHC-II and secreted less IL-1 β and Th1-modulating IL-12p70, indicating their reduced Th1-shaping potential. 9cisRA suppresses the positive costimulators for T cell activation CD80 and CD86, but induces the inhibitory signal molecule PD1-L, giving 9cisRA-DCs a tolerogenic phenotype (10, 32). In fact, 9cisRA-DCs potently convert naive T cells into Tregs. *In vitro*, 9cisRA-DC-converted

cells had elevated suppressor activity as compared with cells generated in MLRs with untreated control DCs. Also, in an antigen-specific model, 9cisRA-DCs were efficient at expanding Tregs. This ability is also relevant *in vivo*, as 9cisRA-DCs partially inhibit established CHS by inducing Tregs in draining lymph nodes. Intestinal tolerogenic DCs express CD103 (33, 34). In the skin, tolerogenic functions have been attributed to CD207⁺ Langerhans cells (35–37). Neither marker is expressed by 9cisRA-DCs, underlining their distinct phenotype. To test their plasticity, 9cisRA-DCs were stimulated with LPS. As compared with control DCs, MHC-II and costimulatory molecules of 9cisRA-DCs were only partially upregulated by LPS. Interestingly, the coinhibitory PD1-L was further upregulated by LPS treatment of 9cisRA-DCs. These findings indicate that very strong DC-activating stimulators such as LPS do not completely reverse the specific 9cisRA-DC phenotype. Importantly, coinhibitory signals on 9cisRA-DC are even enhanced by LPS as an indicator of their coinhibitory stability.

In a setting with submitogenic doses of antigen and high levels of TGF- β , DCs that convert naive T cells into Tregs in secondary lymphoid organs are characterized by low costimulatory ability (38). The presence of RA further triggers Treg differentiation (39). Studies by Hill (39) have suggested that RA increases the production of Tregs by negatively regulating a population of CD4⁺ T cells with a CD44^{hi} memory and effector phenotype that suppress Treg expansion. Mucida et al. found that RA in combination with TGF- β directly expands Tregs (40). To date, the function of DCs in the process of Treg expansion caused by RA is not clearly defined. IL-10 and TGF- β are factors for Treg induction that are upregulated by tolerogenic DCs (6, 38). 9cisRA did not induce IL-10 secretion in DCs. In our 9cisRA-DC-Treg-inducing cocultures, IL-10 was downmodulated and TGF- β was detected in only small amounts. We cannot exclude the possibility that downmodulation in MLRs was attributable to IL-10 consumption. However, in blocking experiments with IL-10 antibody, the Treg-inducing capacity of 9cisRA-DCs was not suppressed. These findings indicate that 9cisRA-DCs do not predominantly rely on these cytokines for Treg transformation in this system. We found that 9cisRA-DCs had strongly upregulated OPN as the only cytokine of a large panel, suggesting that OPN is of relevance for Treg expansion.

In immune-mediated diseases, OPN is a multifunctional molecule with both proinflammatory and anti-inflammatory effects (11, 41). Various studies have described Th1-enhancing or Th17-enhancing functions (11, 42, 43). We have previously shown that OPN aggravates contact dermatitis through effects on DCs

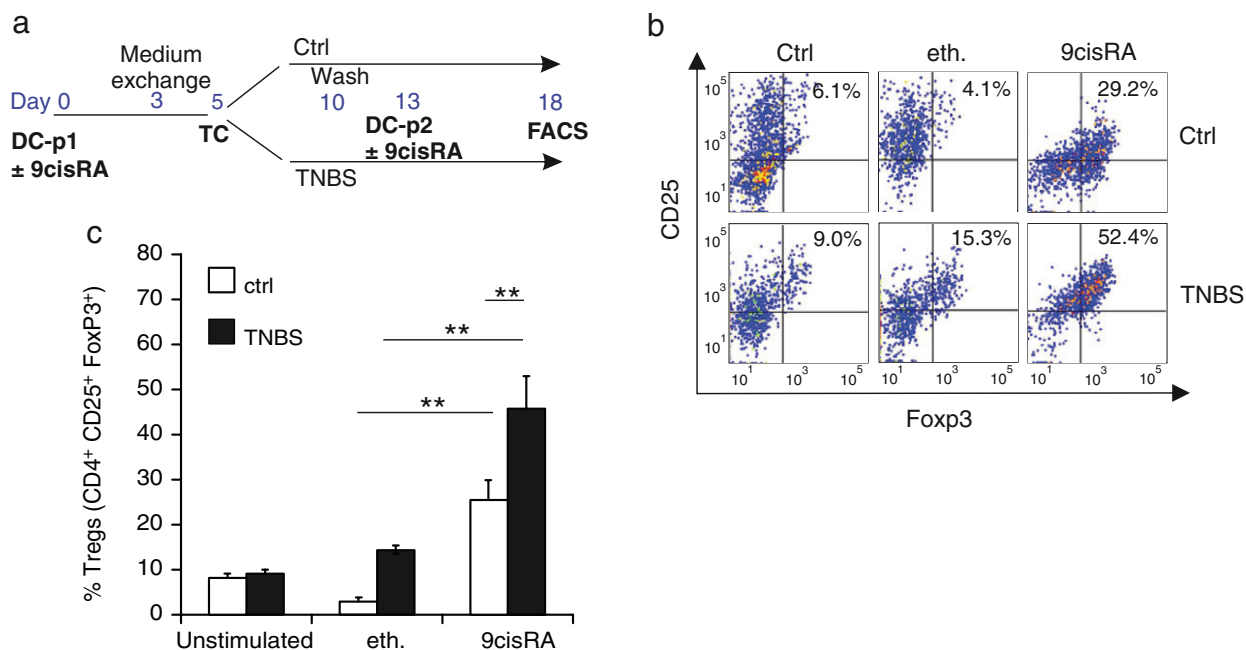


Fig. 4. 9-*cis*-Retinoic acid (9cisRA)-differentiated haptenized dendritic cells (DC) are highly effective in the induction of autologous regulatory T cells (Tregs). **(a)** Experimental design. DCs were differentiated from murine bone marrow of C57BL/6 mice with granulocyte-macrophage colony-stimulating factor and interleukin-4 (population 1, DC-p1; population 2, DC-p2) for 5 days without stimulus, with ethanol (eth.) or with 9cisRA 30 ng/ml. On day (D) 5, DC-p1s were separated into two groups: one remained untreated, and the second was loaded with 2,4,6-trinitrobenzene sulfonic acid (TNBS). DCs were irradiated with 30 Gy, and added to CD4⁺ T cells purified by MACS negative depletion from pooled lymph node and spleen cells of C57/BL6 mice. T cells were cocultured with DCs until D10, washed, and resuspended. On D13, 5-day-old DCs (DC-p2s, treated in the same way as DC-p1s) were added to the appropriate groups of previously primed T cells. Fluorescence-activated cell sorting (FACS) analysis of cocultured cells was performed on D18. **(b)** Cells from cocultures were triple-stained with antibodies against CD4, CD25, and FoxP3, and analysed by FACS on D18 of coculture, with gating on CD4⁺ cells. Data are shown as CD25/Foxp3 staining. The right upper quadrant contains the CD25/Foxp3 double-positive CD4⁺ T cells. **(c)** Average percentage of CD4⁺ CD25⁺ Foxp3⁺ cells in all coculture groups. Data represent the average from three cocultures ± standard error of the mean. Representative data from three independent experiments are shown. ***p* ≤ 0.01. Ctrl, control; eth., ethanol; TC, T cell.

and T cells, and that secreted OPN has Th1-polarizing effects on human DCs (13, 14). In contrast, in a murine asthma model, OPN had anti-inflammatory effects during secondary pulmonary antigenic challenge (41). Also, anti-inflammatory functions were seen in a murine inflammatory bowel disease model (16, 17). In this study, we found that 9cisRA OPN^{-/-} DCs were impaired in their Treg-inducing potential, and that this impairment was reversible by addition of OPN. OPN exists as an intracellular isoform and an extracellular isoform. The intracellular form in DCs is important for Th17-mediated immunity and follicular helper and regulatory T cell differentiation in germinal centres (11, 44, 45). The induction of secreted OPN by 9cisRA and our rescue experiments suggest that, in our system, secreted OPN is relevant for Treg conversion.

The mechanism through which RA-producing DCs in the gut immune system generate Tregs is controversial (27). Our findings that OPN is strongly regulated in DCs

by a retinoid and is a central mediator of Treg expansion suggest OPN as a missing link in RA-modulated Treg expansion. The findings that OPN may be an important factor in the proinflammatory versus anti-inflammatory balance is underlined by the observation that OPN null mice suffer exacerbated tissue destruction in a colitis model (16, 17). Possibly, in this model RA-expressing DC-mediated tolerance is eliminated through OPN deletion. Also in accordance with our findings, a recent study showed that myeloid cell-derived OPN has immunosuppressive functions in the response against breast cancer cells. Fewer Treg cells accumulate at sites of tumour metastasis in OPN^{-/-} mice (46). In a specific tissue context and the presence of a tolerogenic DC phenotype, OPN seems to be a central factor in Treg induction. Thus, in ACD, DC-derived OPN may help to prevent exaggerated allergic inflammation, eventually terminating the disease by Treg promotion. OPN induction to generate Tregs may be one further mechanism explaining the

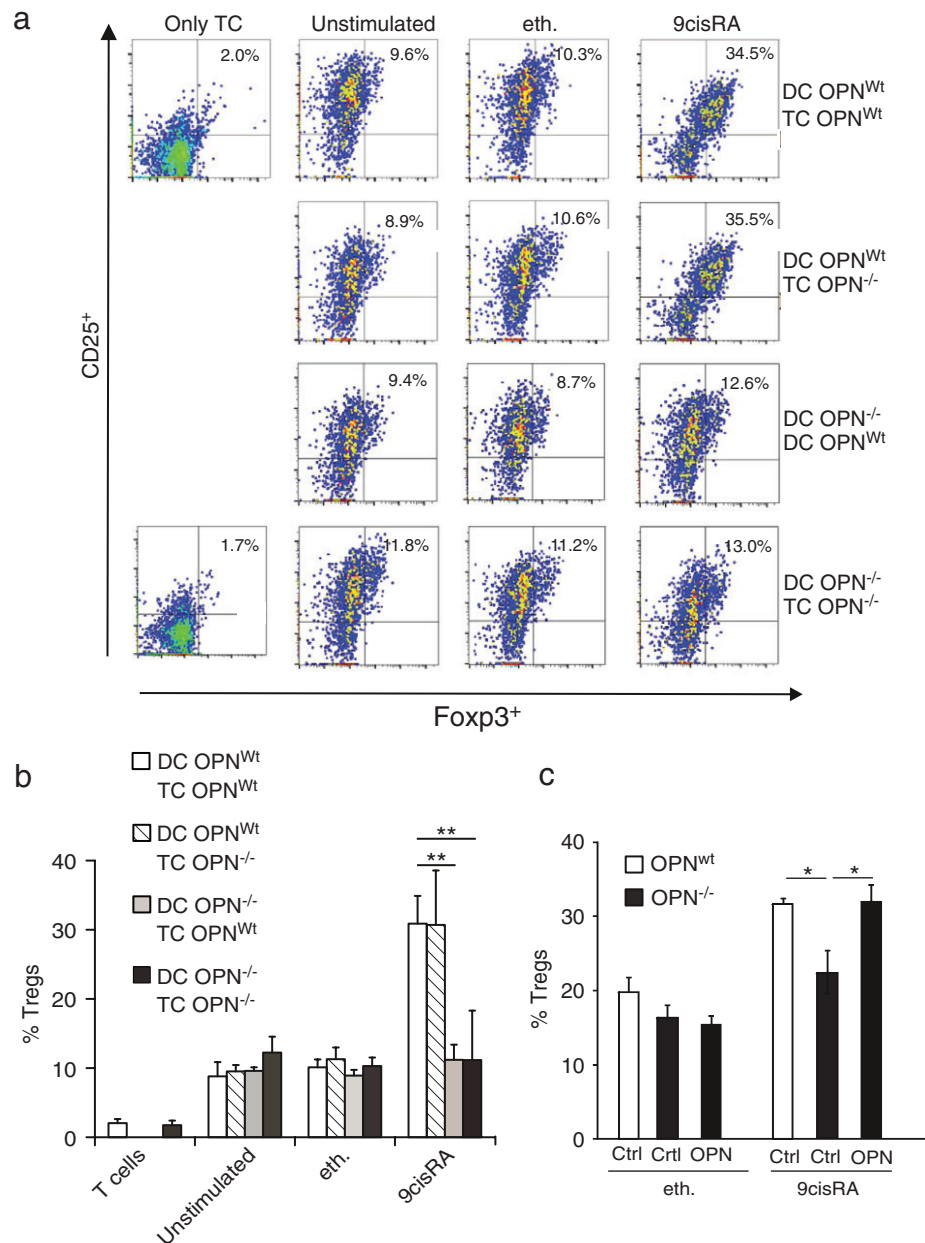


Fig. 5. Depletion of dendritic cell (DC)-secreted osteopontin (OPN) inhibits regulatory T cell (Treg) expansion. Murine bone marrow cells from OPN^{Wt} or OPN^{-/-} C57Bl/6 mice were generated. DCs were left untreated, or differentiated in the presence of ethanol (eth.) or 9-*cis*-retinoic acid (9cisRA) 30 ng/ml. On day (D) 5, DCs were washed and added to T cells purified from pooled spleen and lymph nodes from Balb/c OPN^{Wt} or Balb/c OPN^{-/-} mice. Cocultures were performed both with OPN^{Wt} or OPN^{-/-} DCs or OPN^{Wt} and OPN^{-/-} T cells as indicated. **(a)** Representative fluorescence-activated cell sorting (FACS) analysis of the indicated groups from one coculture experiment. Cells were triple-stained with antibodies against CD4, CD25, and Foxp3, and analyzed by FACS on D5 of coculture, with gating on CD4⁺ cells. Data are shown as CD25/Foxp3 staining. The right upper quadrant contains the CD25/Foxp3 double-positive CD4⁺ T cells. **(b)** Average percentage $\pm$ standard error of the mean of CD4⁺ CD25⁺ Foxp3⁺ cells in the indicated coculture groups. Representative data from three independent experiments are shown. **(c)** OPN^{Wt} or OPN^{-/-} DCs were generated in the presence of eth. or 9cisRA, and cocultured with Balb/c OPN^{Wt} T cells. Coculture was performed in the absence (Ctrl) or the presence of recombinant OPN. Representative data from three independent experiments are shown. * $p \leq 0.05$; ** $p \leq 0.01$. TC, T cell.

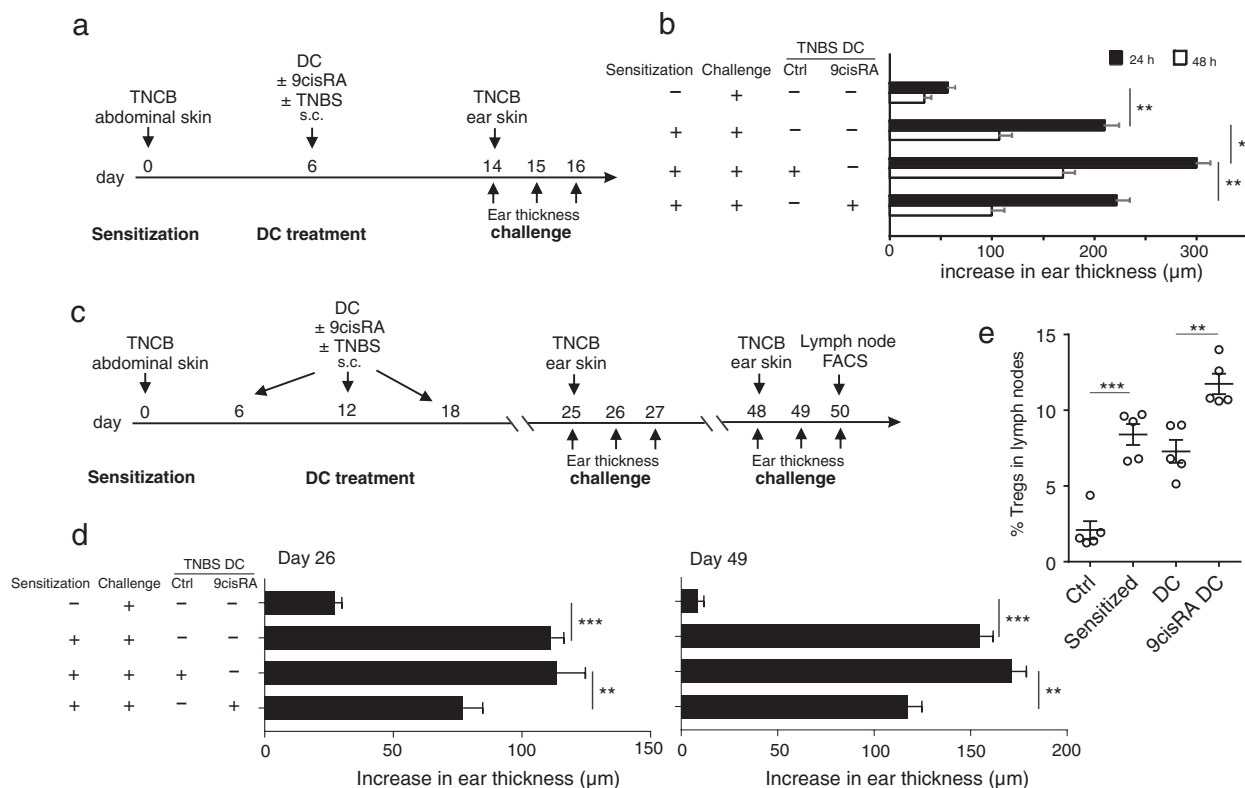


Fig. 6. 9-*cis*-Retinoic acid (9cisRA)-differentiated dendritic cells (DCs) suppress the contact hypersensitivity (CHS) response. **(a)** Experimental design. On day (D) 0, wild-type C57BL/6 mice were sensitized on the abdominal skin with 2,4,6-trinitrochlorobenzene (TNCB). On D6, mice were subcutaneously (s.c.) injected with DCs cultured with either 9cisRA or ethanol (eth.) that were 2,4,6-trinitrobenzene sulfonic acid (TNBS)-loaded or untreated. On D14, ear thickness was measured, and CHS was elicited on the ear with TNCB and ear swelling was measured on D15 and D16. **(b)** Increase in ear thickness $\pm$ standard error of the mean (SEM). **(c)** Experimental procedure. On D0, wild-type C57BL/6 mice were sensitized on the abdominal skin with TNCB. Mice were injected with 9cisRA-treated or eth.-treated DCs, either TNBS-loaded or untreated on D6, D12, and D18. Challenge was performed on D25 and D48, as indicated. **(d)** Increase in ear thickness $\pm$ SEM on D26 and D49. **(e)** Mice were killed on D50, and skin draining lymph nodes were obtained. T cell suspensions were analysed for the percentage $\pm$ SEM of CD4⁺ CD25⁺ FoxP3⁺ regulatory T cells by fluorescence-activated cell sorting. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. Ctrl, control.

therapeutic efficacy of 9cisRA in chronic hand eczema (47).

In our work, we have found that antigen-laden 9cisRA-DCs injected after sensitization partially inhibit CHS. Furthermore, they are able to stimulate Treg accumulation in lymph nodes. Importantly, TNCB is a strong obligatory hapten (20). A partial reduction in the inflammatory response through 9cisRA-DCs thus shows the efficacy of these regulatory DCs. Furthermore, upon repeated injection of antigen-primed 9cisRA-DCs, the partial inhibition was consistent, although, with repeated injections of haptenized DCs and reapplication of the antigen, upon challenge the overall amount of ear-swelling increased. Our findings may be of therapeutic relevance. It is intriguing to speculate that antigen-laden 9cisRA-DCs could be used to induce tolerance in established CHS.

In conclusion, 9cisRA-supplemented culture of DCs generates a distinct Treg-inducing DC phenotype. Functionally, such 9cisRA-DCs partially suppress established CHS and induce Treg accumulation in lymph nodes. Importantly, DC-derived OPN, which, in contrast to downmodulated inflammatory cytokines, is upregulated in 9cisRA-DCs, is a central factor in the induction of Tregs, because OPN^{-/-} 9cisRA-DCs lose their enhanced Treg-stimulating potential.

Acknowledgements

This work was supported by a grant from the German Research Foundation to J. M. Weiss (DFG WE 1919/5-1) and by a grant from Stiefel, a GSK company. We thank Lucy Liaw for the kind gift of the OPN-deficient mice. We thank Anja Camps for fruitful discussions, Kathrin Hauff

for proofreading of the manuscript, and Anca Sindrilaru for fruitful discussion and support with graphics.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Blockade by anti-interleukin (IL)-10 monoclonal antibody (mAb) does not inhibit the regulatory T cell (Treg)-inducing function of 9-*cis*-retinoic acid-differentiated dendritic cells (9cisRA-DCs). Murine bone marrow cells were differentiated in the presence of ethanol (EtOH-DCs) or 30 ng/ml 9cisRA (9cisRA-DCs). On D5, DCs were washed and added to T cells selected by MACS purification from pooled lymph nodes and spleens

of BALB/c mice. Where indicated, anti-IL-10 mAb (R&D Systems/Biotechne, Minneapolis; MN, USA; clone JES052A5) was added at 15 ng/ml. On D10 of coculture, Foxp3⁺ cells were analysed by fluorescence-activated cell sorting (FACS) with triple staining for CD4, Foxp3, and CD25. Data are shown as percentage $\pm$ standard error of the mean of Foxp3⁺ cells.

Figure S2. Osteopontin (OPN) deficiency does not affect interleukin (IL)-10 secretion from dendritic cells (DCs). Murine bone marrow cells from OPN^{Wt} or OPN^{-/-} C57Bl/6J mice were generated. DCs were differentiated in the presence of 9-*cis*-retinoic acid (9cisRA) 30 ng/ml. IL-10 secretion was measured in supernatants by enzyme-linked immunosorbent assay. Data are shown as IL-10 concentration $\pm$ standard error of the mean.

References

- Ruzicka T, Lynde C W, Jemec G B et al. Efficacy and safety of oral alitretinoin (9-*cis* retinoic acid) in patients with severe chronic hand eczema refractory to topical corticosteroids: results of a randomized, double-blind, placebo-controlled, multicentre trial. *Br J Dermatol* 2008; **158**: 808–817.
- Mora J R, Iwata M, von Andrian U H. Vitamin effects on the immune system: vitamins A and D take centre stage. *Nat Rev Immunol* 2008; **8**: 685–698.
- Kane M A. Analysis, occurrence, and function of 9-*cis*-retinoic acid. *Biochim Biophys Acta* 2012; **1821**: 10–20.
- Sun C M, Hall J A, Blank R B et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* 2007; **204**: 1775–1785.
- Honda T, Egawa G, Grabbe S, Kabashima K. Update of immune events in the murine contact hypersensitivity model: toward the understanding of allergic contact dermatitis. *J Invest Dermatol* 2013; **133**: 303–315.
- Raker V K, Domogalla M P, Steinbrink K. Tolerogenic dendritic cells for regulatory T cell induction in man. *Front Immunol* 2015; **6**: 569. doi: 10.3389/fimmu.2015.00569
- Kimber I, Travis M A, Martin S F, Dearman R J. Immunoregulation of skin sensitization and regulatory T cells. *Contact Dermatitis* 2012; **67**: 179–183.
- Geissmann F, Revy P, Brousse N et al. Retinoids regulate survival and antigen presentation by immature dendritic cells. *J Exp Med* 2003; **198**: 623–634.
- Tomura M, Honda T, Tanizaki H et al. Activated regulatory T cells are the major T cell type emigrating from the skin during a cutaneous immune response in mice. *J Clin Invest* 2010; **120**: 883–893.
- Josefowicz S Z, Lu L F, Rudensky A Y. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol* 2012; **30**: 531–564.
- Cantor H, Shinohara M L. Regulation of T-helper-cell lineage development by osteopontin: the inside story. *Nat Rev Immunol* 2008; **9**: 137–141.
- Frenzel D F, Weiss J M. Osteopontin and allergic disease: pathophysiology and implications for diagnostics and therapy. *Expert Rev Clin Immunol* 2011; **7**: 93–109.
- Seier A M, Renkl A C, Schulz G et al. Antigen-specific induction of osteopontin contributes to the chronification of allergic contact dermatitis. *Am J Pathol* 2010; **176**: 246–258.
- Renkl A C, Wussler J, Ahrens T et al. Osteopontin functionally activates dendritic cells and induces their differentiation towards a Th-1 polarizing phenotype. *Blood* 2005; **106**: 946–955.
- Salvi V, Scutera S, Rossi S et al. Dual regulation of osteopontin production by TLR stimulation in dendritic cells. *J Leukoc Biol* 2013; **94**: 147–158.
- Da Silva A P, Ellen R P, Sørensen E S et al. Osteopontin attenuation of dextran sulfate sodium-induced colitis in mice. *Lab Invest* 2009; **89**: 1169–1181.
- Da Silva A P, Pollett A, Rittling S R et al. Exacerbated tissue destruction in DSS-induced acute colitis of OPN-null mice is associated with downregulation of TNF- α expression and non-programmed cell death. *J Cell Physiol* 2006; **208**: 629–639.
- Liaw L, Birk D E, Ballas C B et al. Altered wound healing in mice lacking a functional osteopontin gene (spp1). *J Clin Invest* 1998; **101**: 1468–1478.
- Collison L W, Vignali D A. In vitro Treg suppression assays. *Methods Mol Biol* 2011; **707**: 21–37.
- Martin S F. Induction of contact hypersensitivity in the mouse model. *Methods Mol Biol* 2013; **961**: 325–335.
- Weiss J M, Renkl A C, Sleeman J et al. CD44 variant isoforms are essential for the function of epidermal Langerhans cells and dendritic cells. *Cell Adhes Commun* 1998; **6**: 157–160.
- Kryczanowsky F, Raker V, Graulich E et al. IL-10-modulated human dendritic cells for clinical use: identification of a stable and migratory subset with improved tolerogenic activity. *J Immunol* 2016; **197**: 3607–3617.
- Cassani B, Villablanca E J, De Calisto J et al. Vitamin A and immune regulation: role of retinoic acid in gut-associated dendritic cell education, immune protection and tolerance. *Mol Aspects Med* 2012; **33**: 63–76.
- Scott C L, Aumeunier A M, Mowat A M. Intestinal CD103⁺ dendritic cells: master regulators of tolerance? *Trends Immunol* 2011; **32**: 412–419.
- Nestle F O, Di Meglio P, Qin J Z, Nickoloff B J. Skin immune sentinels in health and disease. *Nat Rev Immunol* 2009; **9**: 679–691.
- Kiss M, Czimmerer Z, Nagy L. The role of lipid-activated nuclear receptors in shaping macrophage and dendritic cell function: from physiology to pathology.

- J Allergy Clin Immunol* 2013; **132**: 264–286.
- 27 Nagy L, Szanto A, Szatmari I, Szeles L. Nuclear hormone receptors enable macrophages and dendritic cells to sense their lipid environment and shape their immune response. *Physiol Rev* 2012; **92**: 739–789.
 - 28 Manicassamy S, Ravindran R, Deng J et al. Toll-like receptor 2-dependent induction of vitamin A-metabolizing enzymes in dendritic cells promotes T regulatory responses and inhibits autoimmunity. *Nat Med* 2009; **15**: 401–409.
 - 29 Villablanca E J, Zhou D, Valentini B et al. Selected natural and synthetic retinoids impair CCR7- and CXCR4-dependent cell migration in vitro and in vivo. *J Leukoc Biol* 2008; **84**: 871–879.
 - 30 Villablanca E J, Wang S, De Calisto J et al. MyD88 and retinoic acid signaling pathways interact to modulate gastrointestinal activities of dendritic cells. *Gastroenterology* 2011; **141**: 176–185.
 - 31 Hengesbach L M, Hoag K A. Physiological concentrations of retinoic acid favor myeloid dendritic cell development over granulocyte development in cultures of bone marrow cells from mice. *J Nutr* 2004; **134**: 2653–2659.
 - 32 Hall B M, Verma N D, Tran G T, Hodgkinson S J. Distinct regulatory CD4+T cell subsets; differences between naive and antigen specific T regulatory cells. *Curr Opin Immunol* 2011; **23**: 641–647.
 - 33 Hall J A, Grainger J R, Spencer S P, Belkaid Y. The role of retinoic acid in tolerance and immunity. *Immunity* 2011; **35**: 13–22.
 - 34 Johansson-Lindbom B, Svensson M, Pabst O et al. Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. *J Exp Med* 2005; **202**: 1063–1073.
 - 35 Kaplan D H, Igyarto B Z, Gaspari A A. Early immune events in the induction of allergic contact dermatitis. *Nat Rev Immunol* 2012; **12**: 114–124.
 - 36 Flacher V, Tripp C H, Mairhofer D G et al. Murine Langerin+ dermal dendritic cells prime CD8+ T cells while Langerhans cells induce cross-tolerance. *EMBO Mol Med* 2014; **6**: 1191–1204.
 - 37 Gomez D A, Vocanson M, Hacini-Rachinel F et al. Langerhans cells protect from allergic contact dermatitis in mice by tolerizing CD8(+) T cells and activating Foxp3(+) regulatory T cells. *J Clin Invest* 2012; **122**: 1700–1711.
 - 38 Liu J, Cao X. Regulatory dendritic cells in autoimmunity: a comprehensive review. *J Autoimmun* 2015; **63**: 1–12.
 - 39 Hill J A, Hall J A, Sun C M et al. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi cells. *Immunity* 2008; **29**: 758–770.
 - 40 Mucida D, Pino-Lagos K, Kim G et al. Retinoic acid can directly promote TGF-beta-mediated Foxp3(+) Treg cell conversion of naive T cells. *Immunity* 2009; **30**: 471–472.
 - 41 Xanthou G, Alissafi T, Semitekolou M et al. Osteopontin has a crucial role in allergic airway disease through regulation of dendritic cell subsets. *Nat Med* 2007; **13**: 570–578.
 - 42 Rittling S R, Singh R. Osteopontin in immune-mediated diseases. *J Dent Res* 2015; **94**: 1638–1645.
 - 43 Steinman L. A molecular trio in relapse and remission in multiple sclerosis. *Nat Rev Immunol* 2009; **9**: 440–447.
 - 44 Inoue M, Shinohara M L. Intracellular osteopontin (iOPN) and immunity. *Immunol Res* 2011; **49**: 160–172.
 - 45 Leavenworth J W, Verbinnen B, Yin J et al. A p85alpha-osteopontin axis couples the receptor ICOS to sustained Bcl-6 expression by follicular helper and regulatory T cells. *Nat Immunol* 2015; **16**: 96–106.
 - 46 Sangaletti S, Tripodo C, Sandri S et al. Osteopontin shapes immune suppression in the metastatic niche. *Cancer Res* 2014; **74**: 4706–4719.
 - 47 Schindler M, Drozdenko G, Kuhl A A, Worm M. Immunomodulation in patients with chronic hand eczema treated with oral alitretinoin. *Int Arch Allergy Immunol* 2014; **165**: 18–26.

Supplemental Figures

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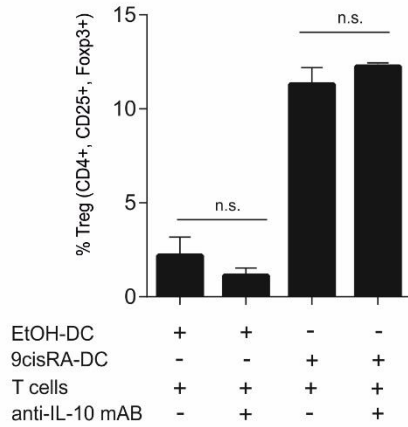


Fig. S1 Blockade by anti-IL-10 mAb does not inhibit Treg inducing function of 9cisRA-DC. Murine bone marrow cells were differentiated in the presence of ethanol (EtOH-DC) or 30 ng/ml of 9cisRA (9cisRA-DC). On day 5, DC were washed and added to T cells selected by MACS purification from pooled lymph nodes and spleen of BALB/c mice. Where indicated anti-IL-10 mAb (R&D Systems/Biotechne, Minneapolis, clone JES052A5) was added at 15 ng/ml. On day 10 of coculture, Foxp3⁺ cells were analyzed by FACS with triple staining for CD4, Foxp3, CD25. Data is shown as $\pm$ SEM of the percentage of Foxp3⁺ cells.

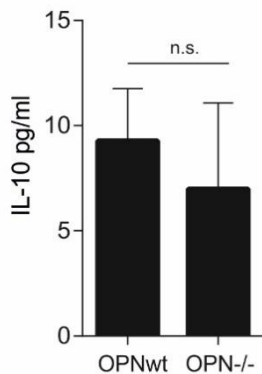


Fig. S2 OPN deficiency does not affect IL-10 secretion from DC. Murine bone marrow cells from Wt or OPN^{-/-} C57Bl/6J mice were generated. DC were differentiated in the presence of 9cisRA 30 ng/ml. IL-10 secretion was measured in supernatants by ELISA. Data is shown as IL-10 concentration $\pm$ SEM.