

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Ursodeoxycholic acid suppresses eosinophilic airway inflammation by inhibiting the function of dendritic cells through the nuclear farnesoid X receptor

M. A. M. Willart^{1,2}, M. van Nimwegen³, A. Grefhorst⁴, H. Hammad^{1,2}, L. Moons⁵,
H. C. Hoogsteden³, B. N. Lambrecht^{1,2,3,*} & A. KleinJan^{3,*}

¹Laboratory of Immunoregulation and Mucosal Immunology, Department of Molecular Biomedical Research, Flemish Interuniversity Institute of Biotechnology (VIB), Ghent; ²Department of Pulmonary Medicine, Ghent University, Ghent, Belgium; ³Department of Pulmonary Medicine, Erasmus MC, Rotterdam; ⁴Department of Internal Medicine, Erasmus MC, Rotterdam; ⁵Departments of Gastroenterology and Hepatology, Erasmus MC, Rotterdam, The Netherlands

To cite this article: Willart MAM, van Nimwegen M, Grefhorst A, Hammad H, Moons L, Hoogsteden HC, Lambrecht BN, KleinJan A. Ursodeoxycholic acid suppresses eosinophilic airway inflammation by inhibiting the function of dendritic cells through the nuclear farnesoid X receptor. *Allergy* 2012; DOI: 10.1111/all.12019.

Keywords

dendritic cell; eosinophilic airway inflammation; farnesoid X receptor; gastrointestinal tract; ursodeoxycholic acid.

Correspondence

Alex KleinJan, Department of Pulmonary Medicine, Erasmus MC, Room Ee2257b, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands.
Tel.: +31 10 4087701
Fax: +31 10 4089453
E-mail: a.kleinjan@erasmusmc.nl

*These authors shared supervision over the work.

Accepted for publication 28 July 2012

DOI:10.1111/all.12019

Edited by: Angela Haczku

Abstract

Background: Ursodeoxycholic acid (UDCA) is the only known beneficial bile acid with immunomodulatory properties. Ursodeoxycholic acid prevents eosinophilic degranulation and reduces eosinophil counts in primary biliary cirrhosis. It is unknown whether UDCA would also modulate eosinophilic inflammation outside the gastrointestinal (GI) tract, such as eosinophilic airway inflammation seen in asthma. The working mechanism for its immunomodulatory effect is unknown.

Methods: The immunosuppressive features of UDCA were studied *in vivo*, in mice, in an ovalbumin (OVA)-driven eosinophilic airway inflammation model. To study the mechanism of action of UDCA, we analyzed the effect of UDCA on eosinophils, T cells, and dendritic cell (DCs). DC function was studied in greater detail, focussing on migration and T-cell stimulatory strength *in vivo* and interaction with T cells *in vitro* as measured by time-lapse image analysis. Finally, we studied the capacity of UDCA to influence DC/T cell interaction.

Results: Ursodeoxycholic acid treatment of OVA-sensitized mice prior to OVA aerosol challenge significantly reduced eosinophilic airway inflammation compared with control animals. DCs expressed the farnesoid X receptor for UDCA. Ursodeoxycholic acid strongly promoted interleukin (IL)-12 production and enhanced the migration in DCs. The time of interaction between DCs and T cells was sharply reduced *in vitro* by UDCA treatment of the DCs resulting in a remarkable T-cell cytokine production. Ursodeoxycholic acid-treated DCs have less capacity than saline-treated DCs to induce eosinophilic inflammation *in vivo* in Balb/c mice.

Conclusion: Ursodeoxycholic acid has the potency to suppress eosinophilic inflammation outside the GI tract. This potential comprises to alter critical function of DCs, in essence, the effect of UDCA on DCs through the modulation of the DC/T cell interaction.

Abbreviations

UDCA ursodeoxycholic acid; OVA ovalbumin; PBS phosphate-buffered saline; DC dendritic cell; IL interleukin; rmGM-CSF recombinant murine granulocyte macrophage-colony-stimulating factor; MCH metacholine.

Bile acids are end products of cholesterol metabolism and have essential functions in controlling the secretion of bile fluid, regulating cholesterol elimination by the liver, and promoting absorption of lipids and fat-soluble vitamins, through detergent properties in the gut. Ursodeoxycholic acid (UDCA) is a naturally occurring dihydroxy bile acid that is

only found in trace amounts (1–3% of bile acids) in human bile, but has strong choleretic activity and thus prevents cholestasis. Although UDCA is commonly used in the treatment for primary biliary cirrhosis (PBC) an autoimmune disease, the mechanisms behind this beneficial effect are not fully understood. In addition to promoting bile secretion, it has been suggested that UDCA has immunomodulatory properties (1–5), such as the suppression of immunoglobulin production; suppression of cytokine synthesis by lymphocytes; downregulation of MHC I expression on hepatocytes; inhibition of mast cell activation (6); inhibition of gall bladder inflammation (7, 8); and suppression of eosinophil activation and degranulation, typical of PBC (9, 10). The immunosuppressive effects of UDCA are, at least in part, mediated through the activation of the glucocorticoid receptor (GR) and inhibition of NF- κ B-dependent transcription through GR-p65 interaction (11–13).

The purpose of this study was to observe whether UDCA also has immunomodulatory effects outside the gastrointestinal (GI) tract. To study this, we turned to the lung because this organ is also exposed to the outside world, lined with mucosal epithelial cells, and has been shown to develop eosinophilic inflammation when triggered with allergens. In this model, we report that intratracheal administration of UDCA strongly suppresses the development of airway inflammation induced by allergen challenge.

Methods

Mice

Six- to 8-week-old Balb/c and C57bl/6 female mice were obtained from Harlan (Zeist, the Netherlands). Bone marrow was harvested from farnesoid X receptor (FXR)-deficient (14) mice, and OTII lymphocytes (all C57bl/6 background) were obtained from Erasmus MC or UMC Groningen. The mice were housed under specific pathogen free (SPF) conditions at the animal care facility of the Erasmus MC Rotterdam with the approval of the local animal ethics and welfare committee. The bronchial hyperreactivity (BHR) experiments were performed at the VIB, Ghent, Belgium, with the approval of the local animal ethics and welfare committee.

A mouse model of airway inflammation

To induce airway inflammation, we immunized Balb/c mice with ovalbumin (OVA) (10 μ g OVA, grade V from Sigma Aldrich, Zwijndrecht, the Netherlands) emulsified in 2 mg AL(OH)₃ (OVA/alum) or as a control with phosphate-buffered saline (PBS) in alum. Six days later on three consecutive days, mice anesthetized with isoflurane were treated with UDCA 30 μ g/animal or diluent intratracheally (i.t.). After recovery, they were challenged by exposure to OVA aerosols 10 mg/ml or PBS during 30 min.

Twenty-four hours after the last aerosols, the mice were killed using an anesthetic overdose followed by bleeding from the vena inguinalis and bronchoalveolar lavage (BAL). The lungs were snap-frozen in OCT freezing solution (TissueTek,

Zoeterwoude, the Netherlands). The mediastinal lymph nodes were collected and stimulated with OVA. Bronchial hyperactivity was analyzed 24 h after the last aerosols using Flexi-vent invasive measurement of dynamic resistance (SCIREQ). Mice were anesthetized with urethane, paralyzed using d-tubocurarine, and tracheotomized, followed by mechanical ventilation. Increasing concentrations of methacholine (MCH) (0–100 mg/ml) were nebulized using Aeroneb (SCIREQ). Dynamic resistance and compliance were recorded after a standardized inhalation maneuver given every 10 s for 2 min. Baseline resistance was restored before administering the subsequent doses of MCH.

In some experiments, we performed an adoptive transfer of dendritic cell (DCs) to induce asthma. In brief, myeloid DCs were grown for 8 days from the bone marrow (Balb/c or in case of WT and FxR-KO C57bl/6) in recombinant murine granulocyte macrophage-colony-stimulating factor (rmGM-CSF) 20 ng/ml, were subsequently pulsed with OVA or OVA in the presence of UDCA or PBS, and then 1×10^6 DCs were injected (i.t.). After this treatment, mice were given three additional OVA aerosol challenges.

Bronchoalveolar lavage fluid (BALF)

Bronchoalveolar lavage was performed, and cells were collected for cellular differentiation by flow cytometry using a method described elsewhere (15). Antibodies for CD3 labeled with PeCy5 (clone 145-2C11; BD Biosciences, Breda, the Netherlands), CD19 labeled with PeCy5 (clone 1D3; eBiosciences, Vienna, Austria), CD11c labeled with PeCy7 (clone N418; eBiosciences), MHCII labeled with Fitc (M5/114.15.2; eBiosciences), and CCR3 labeled with APC (clone FAB729A; R and D systems, Abingdon, UK). In some experiments, when C57bl/6 animals were used, half of the BAL cells were stimulated with ionomycin (Sigma), phorbol 12-myristate 13-acetate (PMA; Sigma), and Golgiplug (BD Biosciences); incubated at 37°C for 4 h; and subsequently stained with the following antibodies: CD3 APC cy7 (clone 145-2C11; BD Biosciences), CD4 PeCy5 RM4-5 (eBiosciences), interleukin (IL)-4 (clone 11B11; BD Biosciences), and interferon (IFN)- γ (clone XMG1.2; BD Biosciences). Fixable Aqua Dead Cell Stain kit for 405 nm (Invitrogen, Molecular Probes, Life Technologies Europe BV, Bleiswijk, the Netherlands) was used as a live-dead marker. Cells were measured on a Flowcytometer LSRII (BD Biosciences) and analyzed with FlowJo software.

Bronchoalveolar lavage fluid and supernatants of the OVA-stimulated lymph nodes (LN) cell suspensions were assayed with enzyme-linked immunosorbent assay (ELISA) for the quantification of IL-4, IL-5, IL-10, IL-13, and IFN- γ .

T-cell stimulation and eosinophil culture

Lymphocytes obtained from animals that were sensitized against OVA were stimulated *in vitro* in a nonspecific way with culture plate-coated antibodies against CD3 (overnight 1 μ g/ml) and antibodies against CD28 in solution (2.5 μ g/ml) for 4 days. Ursodeoxycholic acid was present during the whole culture period.

To generate eosinophils, bone marrow cells (0.2×10^6 /well) were cultured in 30% fetal bovine serum (FBS) and IL-5 25 ng/ml, as described (15). Ursodeoxycholic acid was added on day 5. Twenty-four hours later, cells were counted and stained for the eosinophil-specific marker CCR3 (eotaxin receptor) and for live–dead with PI.

Bone-marrow-derived myeloid DCs

To generate mDCs, bone marrow cells were collected from naive mice and cultured in 5% FBS and rmGM-CSF (kindly provided by Dr. K. Thielemans, Belgium) for 9 days as described (16). At day 9, they were exposed to a different concentration of UDCA and pulsed *in vitro* overnight with 100 µg/ml OVA or sham-pulsed with PBS. Supernatants of DC medium were stored for IL-10 and IL-12 ELISA (BD Biosciences).

Visualization of DC/T cell interaction

For visualizing dynamic interactions between DCs and specific T cells, OVA-specific T cells obtained from OTII TCR Tg (H-2b) mice were cocultured with unpulsed bone marrow derived dendritic cells (BMDCs), OVA protein-pulsed BMDCs (100 µg/ml), or OVA peptide (323–339)-pulsed BMDCs (1 µg/ml) treated or not treated with 10 µM UDCA overnight. Prior to incubation, DCs were stained with an fluorescein isothiocyanate (FITC)-labeled antibody to I-Ak,b (M5/114), and T cells were stained with biotin-tagged TCR Vα2 antibodies, followed by Qdot 655 streptavidin conjugates (Invitrogen) essentially as described (17). Stained DCs adhere in a poly-L-lysine (PLL)-coated imaging chamber at 37°C in an atmosphere of 5% CO₂. After 1 h, T cells were added in a ratio of 1 DC to 10 T cells. Interactions were visualized with an LSM 510 confocal microscope (Zeiss, Slidrecht, the Netherlands) equipped with 488- and 633-nm lasers. Time-lapse images were collected every 20 s for 40 min. Interactions were quantified using IMARIS software (version 5.0; Bitplane AG, Zurich, Switzerland) (17).

Statistical analysis

For statistical analysis, Kruskal–Wallis one-way ANOVA was used to calculate the overall *P*-value. A *P*-value of <0.05 was considered a significant difference between groups. The non-parametric Mann–Whitney *U*-test was performed to analyze each group with respect to each other. The Pearson correlation test was used to indicate a significant concentration dependency.

Results

Ursodeoxycholic acid treatment reduces eosinophilic airway inflammation and IL-13 production

Ursodeoxycholic acid was used as a topical therapeutic drug in a mouse model of acute allergic inflammation in which we administered 30 µg UDCA in diluent (=PBS) (1 mM 80 µl) or diluent approximately 30 min before each OVA aerosol challenge during 30 min in OVA-immunized mice. Twenty-

four hours later, animals were killed, and UDCA-treated animals (OVA–alum/UDCA/OVA group) demonstrated a significant reduction in the percentage and the number of BAL eosinophils compared with the controls (OVA–alum/PBS/OVA group) (Fig. 1A,E). Matching the reduction in eosinophils, we found that the number and percentage of DCs and T cells (Fig. 1B,C,F,G) were also lower in the UDCA-treated group compared with the control (OVA–alum/PBS/OVA) group. Next, we measured BHR. A significant less response to MCH was observed in the UDCA-treated animals (OVA–alum/UDCA/OVA group) as compared to PBS-treated animals (OVA–alum/PBS/OVA group). Phosphate-buffered saline–alum controls showed no signs of BHR (Fig. 1I).

To address the effect of UDCA treatment on cytokine production, the mediastinal LNs draining the lungs were stimulated with OVA *in vitro*. After 4 days of OVA restimulation, levels of IL-13 (Fig. 2B) were markedly reduced, while other measured cytokines like IL-4 (Fig. 2A), IL-5 (Fig. 2C), and IL-10 (Fig. 2D) remained unchanged in OVA–alum/UDCA/OVA group compared with OVA–alum/PBS/OVA group.

In line with the reduced eosinophilic BAL inflammation, significantly lower levels of IL-13 in the BAL were observed in OVA–alum/UDCA/OVA group (104 ± 3.7 pg/ml vs 140 ± 7.1 pg/ml, compared with OVA–alum/PBS/OVA group, *P* = 0.006). The controls, PBS–alum/UDCA/OVA group, and PBS–alum/PBS/OVA group had much lower IL-13 concentrations that fell in the same range as the OVA–alum/UDCA/OVA group (Fig. 2E).

Next, we measured intracellular IFNγ in BAL and MLN and observed a trend (*P* = 0.06) of increased numbers of IFNγ-positive CD4 T cells in BAL and significantly (*P* = 0.03) more IFN γ-positive CD4 T cells in MLN in the UDCA-treated animals (OVA–alum/UDCA/OVA group) when compared with PBS-treated animals (OVA–alum/PBS/OVA group) (Fig. 2F–G).

Ursodeoxycholic acid induce IL-12 production in DCs and has no toxic effect on eosinophil, DC survival, and T-cell cytokine production

A detailed *in vitro* characterization of UDCA-treated eosinophils, DCs, and T cells is shown in Fig. S1 with the conclusion that UDCA is not harmful for eosinophils and DCs (Fig. S1A,B) and does not influence T-cell cytokine production (Fig. S1C–E).

As we found that UDCA enhances migration of bone-marrow-derived DCs after i.t. injection (Fig. S1F), yet at the same time suppresses the potential of these cells to prime for Th2-dependent eosinophilic airway inflammation, we sought to further identify the mechanism by which Th2 development was abolished. Previous studies in our group have identified that high-level production of IL-12 by GM-CSF-cultured DCs abolished their potential to induce Th2 immunity, via promotion of a counterregulatory Th1 response (18, 19). IL-12 is known to be a key inducer of cell-mediated immunity. In addition, IL-12 stimulates the differentiation of CD4-positive Th cells in IFNγ-producing Th1 cells (18). Incubation of DCs with increasing UDCA concentrations revealed a

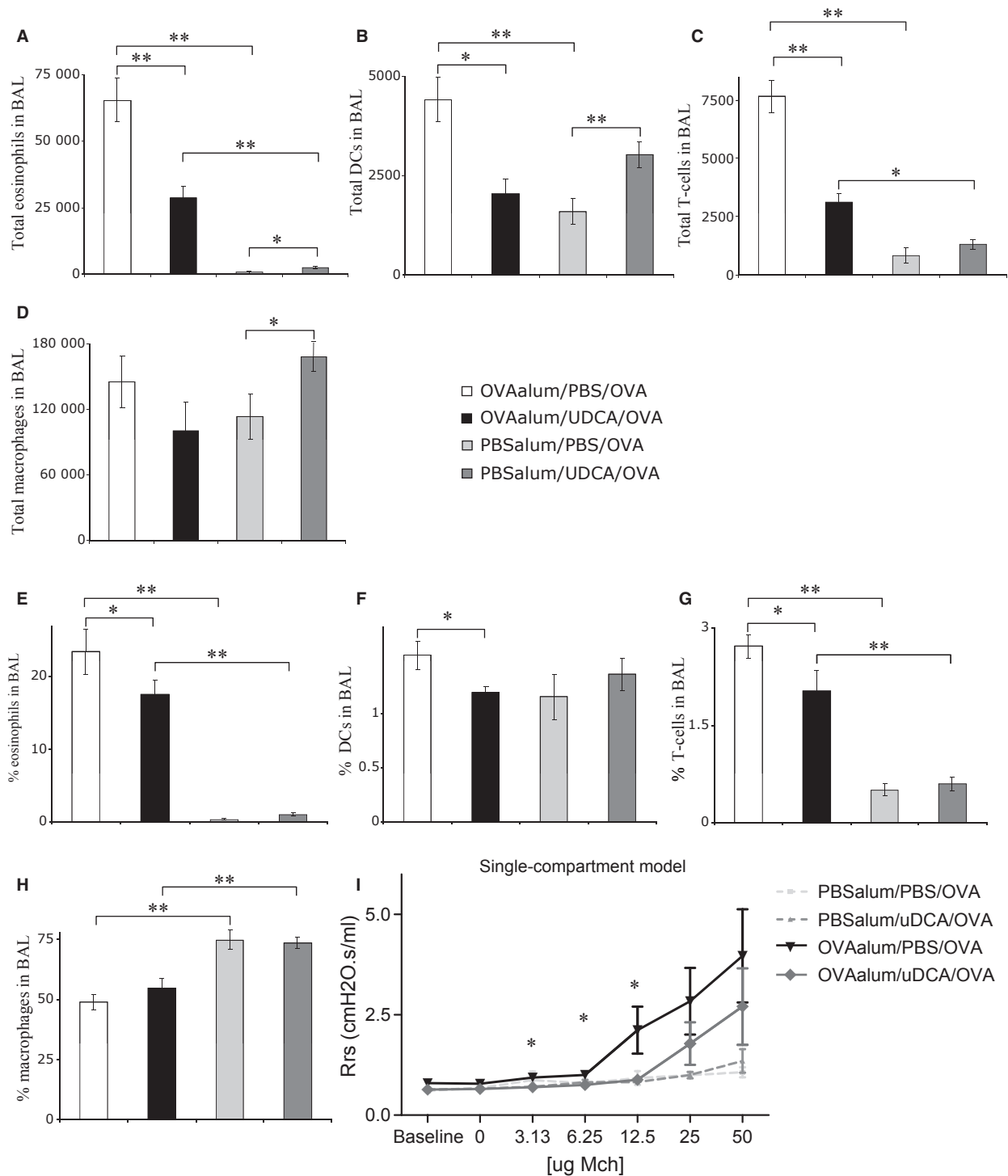


Figure 1 Impaired eosinophilic airway inflammation after ursodeoxycholic acid treatment. Model of allergic airway inflammation in ovalbumin-sensitized mice. Bronchoalveolar lavage cells were stained for eosinophils (CCR3-positive cells) and T cells (CD3-positive cells), as well as for B cells (data not shown) and macrophages and DCs. (A and E) eosinophils (cell numbers and percentage); (B

and F) DCs (cell numbers and percentage); (C and G) T cells (cell numbers and percentage); (D and H) macrophages (cell numbers and percentage); (** $P < 0.01$ and * $P < 0.05$ $n = 6$ animals per group); (I) bronchial hyperreactivity measurements (* $P < 0.05$ $n = 4$ –6 animals per group).

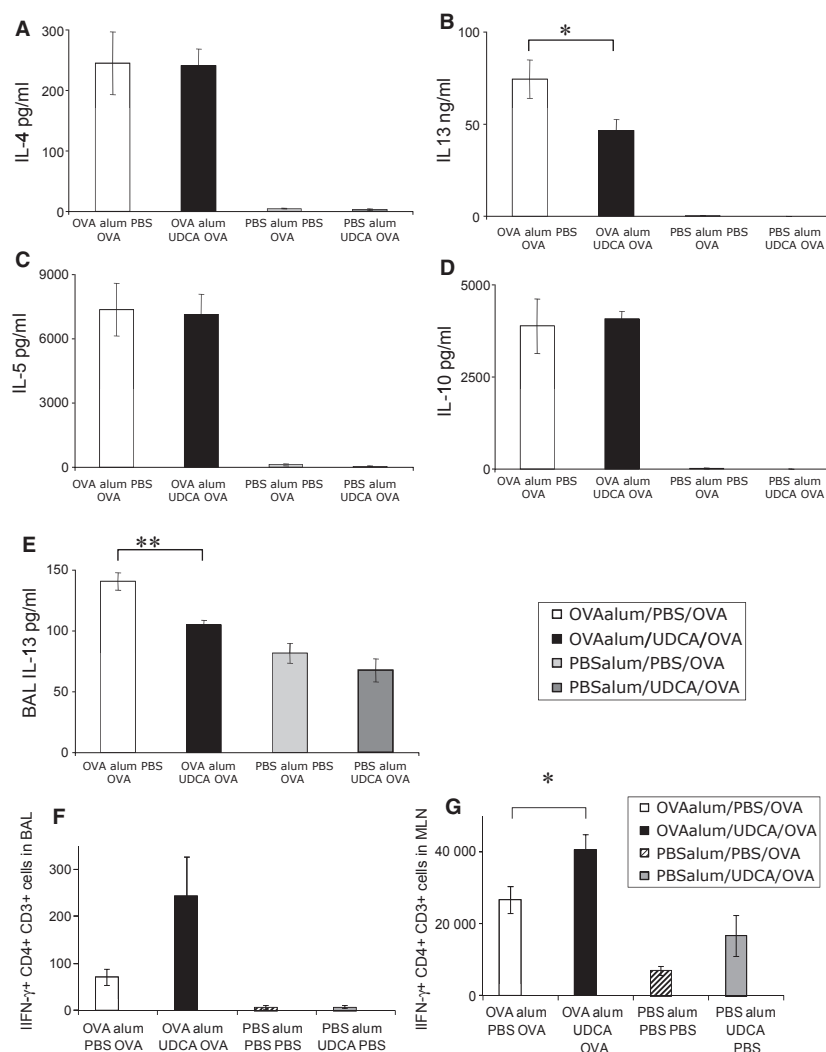


Figure 2 Reduced interleukin (IL)-13 production after ursodeoxycholic acid (UDCA) treatment. Mediastinal lymph nodes (LN) cell suspensions were restimulated *in vitro* for 4 days with ovalbumin and assayed for IL-4 (A), IL-13 (B), IL-5 (C), IL-10 (D) production using enzyme-linked immunosorbent assay. The level of the IL-13 promoting Th2 cytokines was significantly reduced in mice treated

with UDCA. Levels of other cytokines seem to be unchanged in the UDCA-treated group. IL-13 levels (E) in bronchoalveolar lavage (BAL) fluid were lower in UDCA-treated animals compared with the diluent-treated animals (F) and (G) intracellular interferon- γ in CD4 T cells in, respectively, BAL cells and MLN cells (** $P < 0.01$ and * $P < 0.05$).

concentration-dependent increase in IL-12 production (Fig. 3A), promoting the induction of IFN γ in responding T cells (Fig. 3C), while suppressing induction of Th2 cytokine IL-13 (Fig. 3B), and supports the migration of DCs (Fig. S1F).

Ursodeoxycholic acid-treated DCs were less effective in inducing asthma

We employed experiments demonstrating that intratracheal administration of UDCA reduces the number of DCs in allergen-challenged lungs. In previous studies, we identified lung DCs as key proinflammatory cells that are necessary and sufficient for Th2 cell stimulation during ongoing airway inflammation (20).

To test whether UDCA interferes with the potential of DCs to induce eosinophilic airway inflammation, we used an adoptive transfer model of DCs to immunize the animals, bypassing OVA-alum immunization (21). Myeloid DCs were grown for 8 days from the bone marrow in GM-CSF, were subsequently pulsed with OVA or OVA in the presence of UDCA or PBS, and then 1×10^6 DCs were injected into the trachea of mice. After this treatment, mice were given three additional OVA aerosol challenges.

As previously reported (22), the adoptive i.t. transfer of OVA-pulsed mDCs leads to Th2 priming, and eosinophilic airway inflammation subsequently develops upon OVA aerosol challenge 10 days later. As expected, in mice receiving unpulsed PBS DCs or UDCA DCs, only a few inflammatory

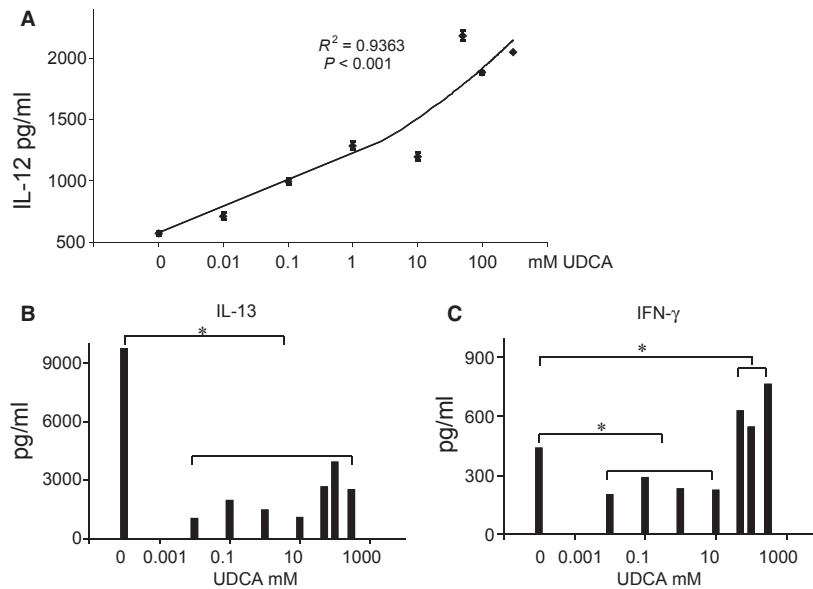


Figure 3 Ursodeoxycholic acid (UDCA) promotes the interleukin (IL)-12 production of DCs in a concentration-dependent manner (A). The effect of UDCA treatment on DCs was studied in the context of the interaction between T cells and DCs. Levels of T-cell cytokines were reduced when UDCA-treated DCs were used. Reduced levels in the production of IL-13 (B) were shown for concentrations

up to 300 μ M UDCA. This concentration is not toxic for DCs. Low doses of up to 10 μ M of UDCA-treated DCs reduced the production of T-cell cytokine interferon (IFN)- γ (C). Ursodeoxycholic acid concentrations of 30 μ M and more induce a stronger IFN- γ response (* $P < 0.05$).

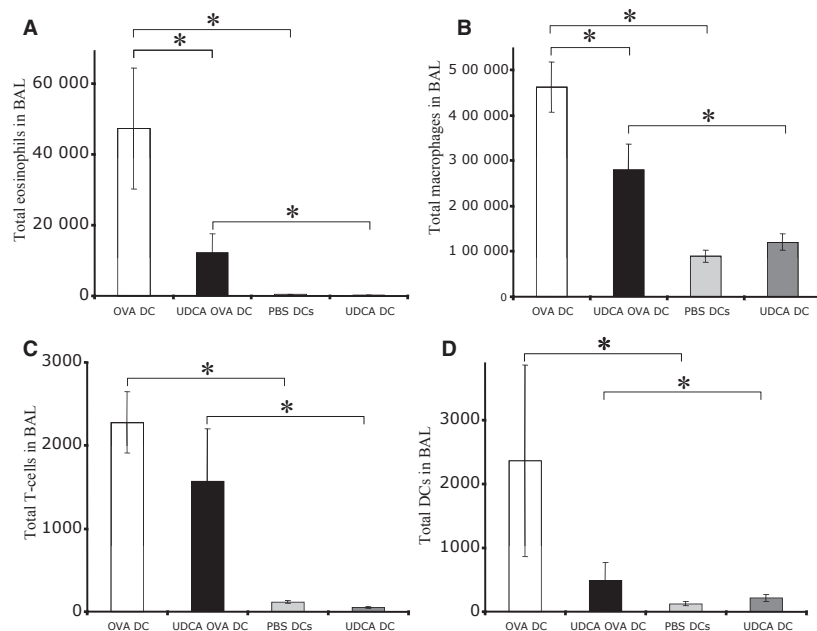


Figure 4 Ursodeoxycholic acid (UDCA)-treated dendritic cells (DCs) are less effective in inducing a Th2 response. Effects of *in vitro* UDCA treatment on the potential of mDCs to induce eosinophilic airway inflammation. On day 0, mice received an i.t. injection of diluents ovalbumin (OVA)-DCs, UDCA OVA-DCs, diluent DCs, or

cells were observed in the BALF (Fig. 4A). Mice immunized with OVA-pulsed DCs demonstrated a strong cellular recruitment of eosinophils and macrophages in the BALF (Fig. 4A,

UDCA DCs. From days 10–13 onwards, mice were OVA aerosol-challenged. Total number of eosinophils in bronchoalveolar lavage (BAL) (A); total number of macrophages in BAL (B); total number of T cells (C); and total number of DCs (D). Data indicate mean $\pm$ SEM. * $P < 0.05$ $n = 6$ animals per group.

B). Ursodeoxycholic acid treatment of OVA-pulsed DCs abolished their potential to induce airway eosinophilia and macrophage influx into the lung. There was also a trend

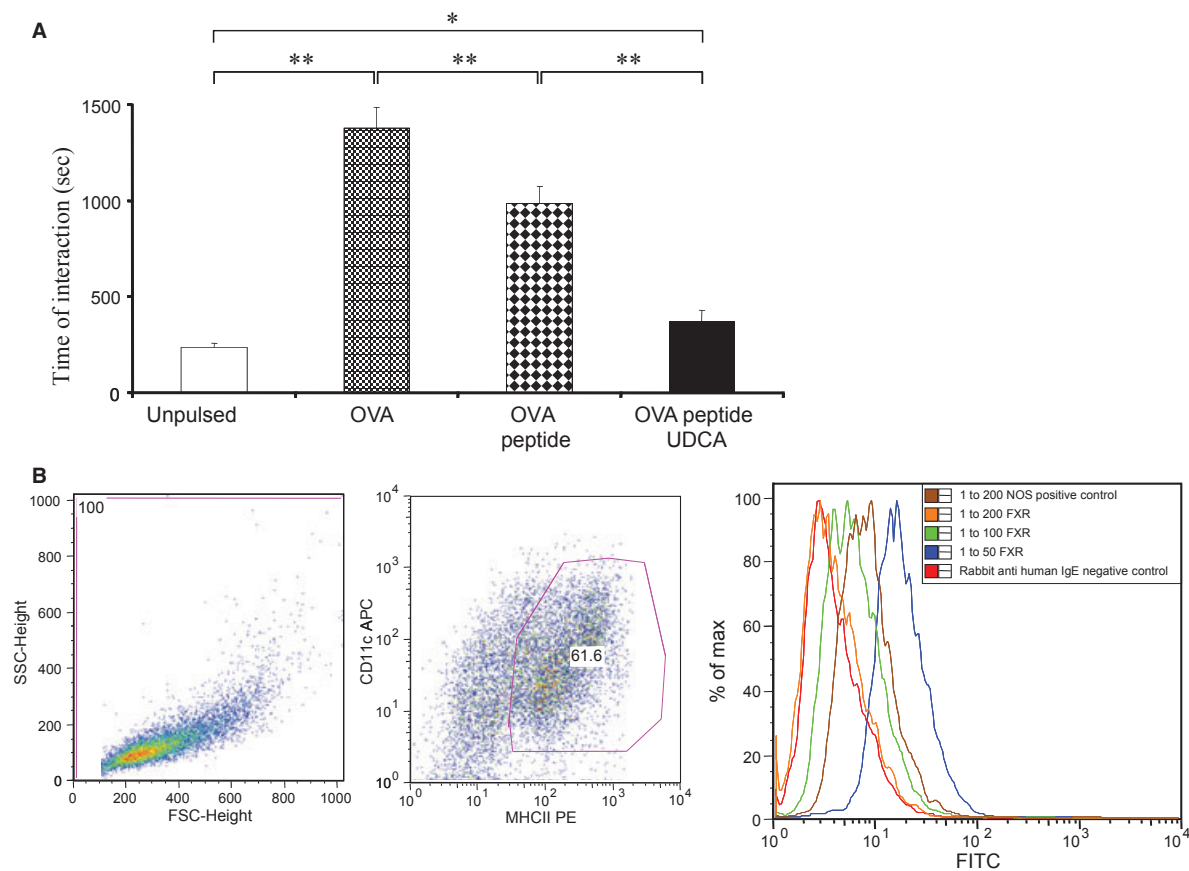


Figure 5 The duration of interaction between T cells and dendritic cells (DCs) is strongly reduced if DCs are treated with ursodeoxycholic acid (UDCA), and DCs express farnesoid X receptor (FXR). The duration of interaction between T cells and DCs is strongly reduced if DCs are treated with UDCA. (A). Bile acid acts as a

ligand on the transcription factor FXR, a nuclear receptor for bile acid. GM-CSF-cultured bone-marrow-cell-derived DCs stained positive for FXR receptor, as indicated by FACS analyses with rabbit anti FXR antibodies. As a negative control, we used rabbit anti human IgE, and as a positive control, we used NOS antibodies (B).

toward reduced T cell (Fig. 4C) and DC (Fig. 4D) influx, but this failed to reach statistical significance.

Shorter interaction time between DCs and T cells when DCs were treated with UDCA

Ursodeoxycholic acid has been shown to induce higher levels of IL-12 production in an UDCA concentration-dependent way. The migration of bone-marrow-derived DCs after i.t. injection was increased. These phenomena, and the immunosuppressive action of UDCA in an allergic airway model, suggest that more factors are involved in affecting the DC/T cell interaction. It has become possible to study the duration of interaction as a marker for synapse formation between DCs (C57bl/6) and antigen-specific T cells *in vitro* (17). Untouched naive CD4-positive T cells were isolated from LNs obtained from OTII (OVA-specific TCR transgenic) mice carrying the V α 2 TCR chain, were cultured with MHC-II-positive DCs for 40 min and formed cellular interaction.

Unpulsed DCs showed short interactions between DCs and T cells, while OVA protein (100 μ g/ml)-pulsed DCs showed long interactions between DCs and T cells. This was used as a method control. A long interaction time was also seen when DCs were pulsed with preprocessed OVA peptide. However, significantly shorter interactions were observed when OVA peptide (1 μ g/ml)-pulsed DCs were treated with UDCA (10 μ M) compared with saline (Fig. 5A).

Dendritic cells express transcription factor farnesiod X receptor

Bile acids are ligands of the FXR, a nuclear receptor that mediates, among others, hepatic bile acid synthesis (23, 24). Fluorescence activated cell sorting (FACS) analysis indicated that the FXR is expressed on DCs grown in GM-CSF (Fig. 5B), suggesting the possibility that the suppressive effects of UDCA on DCs are partly mediated via FXR.

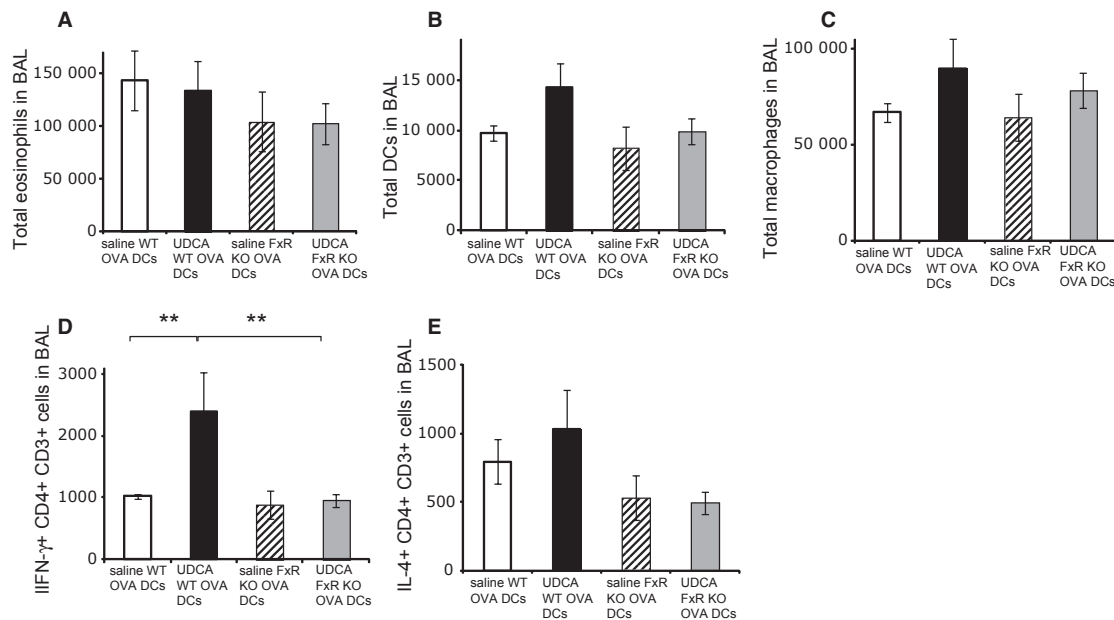


Figure 6 Higher interferon (IFN)- γ -positive T-cell numbers in eosinophilic airway inflammation after ursodeoxycholic acid (UDCA) treatment. A model of asthma in ovalbumin (OVA)-sensitized mice. Bronchoalveolar lavage (BAL) cells were stained for eosinophils (CCR3-positive cells) and macrophages and DCs (A; eosinophils) (B; DCs) (C: macrophages). Bronchoalveolar lavage cells were stimulated with phorbol 12-myristate 13-acetate and ionomycin in the presence of Golgistop for 4 h. T-cell cytokine production as mea-

sured by intracellular cytokine staining showed significantly ($P = 0.006$) higher IFN- γ -positive T-cell numbers in the UDCA-OVA-WT-DC group compared with saline-OVA-WT-DC group and compared with saline-OVA-farnesoid X receptor-KO-DC group (D). No other differences were observed in the number of IFN- γ -positive T cells. Interleukin-4-positive T cells showed no differences between the groups (E) (** $P < 0.01$ $n = 6$ animals per group).

Ursodeoxycholic acid promotes DC-driven Th1 development by acting on the farnesoid X receptor

We finally wanted to find the molecular mechanism by which UDCA promotes Th1 induction and suppresses airway eosinophilia.

To study whether FXR is involved in mediating the effects of UDCA on DC function, we grew myeloid DCs from FXR knockout mice and their wild-type littermates (C57Bl/6). The DCs were stimulated with OVA (100 μ g/ml) only, OVA and UDCA (10 μ M), or PBS, and UDCA. Subsequently, 1×10^6 DCs were injected intratracheally to sensitize C57Bl/6 mice. Ten days later, the mice were challenged with OVA aerosols on three consecutive days. Twenty-four hours later, animals were killed, and UDCA-OVA-WT-DC-treated animals demonstrated no significant reduction in the number of BAL eosinophils, macrophages, and DCs compared with the WT-OVA-DC-treated control animals. Moreover, UDCA-OVA-FXR-KO-DC-treated animals demonstrated no significant differences in the number of BAL eosinophils, macrophages, and DCs compared with the saline-OVA-FXR-KO-DC-treated control animals (Fig. 6B–D).

To check for Th polarization, BAL T-cell cytokine production was estimated. Intracellular cytokine staining showed significantly ($P = 0.006$) higher IFN- γ -positive T cell numbers in the UDCA-OVA-WT-DC group compared with saline-OVA-WT-DC group and compared with saline-OVA-

FXR-KO-DC group (Fig. 6E). No differences were observed in the number of IFN- γ -positive T cells between saline-OVA-WT-DC group and saline-OVA-FXR-KO-DC group and between saline-OVA-FXR-KO-DC group and UDCA-OVA-FXR-KO-DC group. *In vivo*, there was no difference in the number of IL-4-positive T cells between the groups (Fig. 6F).

Discussion

Ursodeoxycholic acid modulates cellular and immunological processes *in vivo* and *in vitro*. Here, we have shown that UDCA treatment lowers the production of T-cell cytokines following DC-driven activation *in vitro*. Moreover, UDCA treatment *in vivo* in the lung reduces tissue eosinophilia in mice in an allergic airway inflammation model. Ursodeoxycholic acid affects DCs and supports the production of IL-12 (Fig. 3A), suggesting that it is responsible for a pro-Th1 T-cell cytokine induction, in a process requiring the FXR (25).

Previous studies in our group have identified that high-level production of IL-12 by GM-CSF-cultured DCs abolished their potential to induce Th2 immunity, via promotion of a counterregulatory Th1 response (18, 19).

Higher numbers of intracellular IFN- γ -positive CD4 T cells in MLN suggest a change in favor of Th1 balance because of the UDCA treatment. This is supported by *in vitro* data that DCs treated with UDCA are less capable to induce IL-13

(Th2) response and that low concentrations of UDCA had similar effects on IFN γ but higher concentrations induce and support IFN γ . Intratracheal administration of UDCA could also act on structural cells of the airways that are responsible for the production of IL-25 and IL-33, both important for the recruitment of eosinophils (26) independent of T cells, and these cytokines are as well important for the recruitment of type 2 innate lymphoid cells (ILC2) (27) and may also act indirectly to suppress T-cell activation. However, in the *in vitro* experiments, we observed lower levels of Th2 (IL-13) and increasing Th1 (IFN- γ) cytokines, suggesting a direct immunosuppressive effect of UDCA on DC-driven activation of T cells. This is in line with previous observations (6). Somewhat surprisingly, there was a change in the balance of polarizing cytokine production in favor of IL-12, because the levels of IL-10 measured in DC supernatants were at the detection limit of the ELISA. The most likely explanation for the observed reduction in Th cytokine production was that the duration of the interaction between T cells and DCs was significantly reduced when DCs were treated with UDCA. This supports the idea that UDCA targets the DC. The outcome of an immune response after T cell/DC interaction is dependent on its duration, Ag levels, and T-cell numbers as recently shown using drugs that block the interaction between DCs and T cells (28).

The effect of UDCA on suppressing allergic eosinophilic inflammation could be very well explained by the effects on DCs. Allergic airway inflammation is highly DC dependent, DCs playing an important role in the induction and maintenance of allergic airway inflammation (22, 29, 30). Another good target candidate for UDCA could be the eosinophils. In humans, there is strong evidence that UDCA prevents the degranulation of eosinophils in patients with PBC after 4 weeks of UDCA (10). Evidence from mouse eosinophils suggests that they do not degranulate in OVA-driven allergic airway inflammation models (31). We failed to see an effect of UDCA on eosinophil degranulation (data not shown). The effect of UDCA on DCs required the nuclear FXR. This receptor has been described in peripheral blood mononuclear cells (PBMCs), including T cells and monocytes at the protein level and mRNA level, and it is inhibited by proinflammatory cytokines such as tumor necrosis factor (TNF)- α and IL-1 release during the acute phase response (32). Farnesoid X receptor is known as a receptor for bile acid metabolism, and our finding of FXR expression on bone-marrow-derived DCs strongly supports the idea that DCs are targets for UDCA. Ursodeoxycholic acid activates the FXR and may be either an agonist or an antagonist (23, 24). To prove the functionality of FXR, we used FXR-deficient bmDCs or WT bmDCs and showed that sensitization with either FXR-KO bmDCs or WT bmDCs has the capacity to induce eosinophilic inflammation

in C57bl/6 animals. However, the UDCA-induced promotion of Th1 responses by DCs required the FXR. We failed, however, to observe a direct immunosuppressive effect of UDCA on eosinophil counts in WT DCs in C57bl/6 mice. This could be because of strain differences between C57bl/6 and Balb/c (33). Other ligands of the FXR are chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA). However, we found that these compounds were cytotoxic for DCs and for T cells (our unpublished observations). This cytotoxic effect was already reached at concentrations of 10 and 50 μ M (data not shown).

Our present results are another example of an unexpected orphan nuclear receptor–ligand interaction that has the capacity to suppress allergic inflammation. Other examples are the PPAR-gamma receptor (34), the vitamin D receptor, and the aryl hydrocarbon receptor (35). As these nuclear receptors often form heterodimers with each other and other nuclear transcription factors, it will be very interesting to see how the FXR is regulated in DCs.

Although UDCA has been shown to reduce eosinophilic functions in humans with PBC, our data suggest that UDCA might also affect DC function in the gut. Particularly in disease states of the GI tract that are dominated by eosinophils, such as eosinophilic gastritis and esophagitis, it will be interesting to study whether UDCA has the same potential to suppress tissue eosinophilia as reported in our lung inflammation model. This approach might be more amenable to therapeutic intervention as UDCA is available in an oral formulation.

In conclusion, our study shows that UDCA has immunomodulatory capacities to suppress eosinophilic inflammation outside the GI tract, via modulation of DC function.

Conflict of interest

None.

Supporting Information

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

Data S1. UDCA is not cytotoxic for eosinophils or DCs neither it influences T-cells directly. UDCA-treated DCs have an enhance migratory activity *in vivo*.

Figure S1. UDCA has no toxic effect on eosinophil and DC survival T-cell cytokine production.

Figure S2. UDCA doesn't effect DC phenotype.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

- Combes B, Emerson SS, Flye NL, Munoz SJ, Luketic VA, Mayo MJ, et al. Methotrexate (MTX) plus ursodeoxycholic acid (UDCA) in the treatment of primary biliary cirrhosis. *Hepatology* 2005;**42**:1184–1193.
- Combes B, Carithers RL Jr, Maddrey WC, Munoz S, Garcia-Tsao G, Bonner GF, et al. Biliary bile acids in primary biliary cirrhosis: effect of ursodeoxycholic acid. *Hepatology* 1999;**29**:1649–1654.
- ter Borg PC, Schalm SW, Hansen BE, van Buuren HR. Prognosis of ursodeoxycholic acid-treated patients with primary biliary cirrhosis. Results of a 10-yr cohort study

- involving 297 patients. *Am J Gastroenterol* 2006;**101**:2044–2050.
4. Ikegami T, Matsuzaki Y, Fukushima S, Shoda J, Olivier JL, Bouscarel B, et al. Suppressive effect of ursodeoxycholic acid on type IIA phospholipase A2 expression in HepG2 cells. *Hepatology* 2005;**41**:896–905.
 5. Ikegami T, Matsuzaki Y, Shoda J, Kano M, Hirabayashi N, Tanaka N. The chemopreventive role of ursodeoxycholic acid in azoxymethane-treated rats: suppressive effects on enhanced group II phospholipase A2 expression in colonic tissue. *Cancer Lett* 1998;**134**:129–139.
 6. Yoshikawa M, Tsujii T, Matsumura K, Yamao J, Matsumura Y, Kubo R, et al. Immunomodulatory effects of ursodeoxycholic acid on immune responses. *Hepatology* 1992;**16**:358–364.
 7. Guarino MP, Cong P, Cicala M, Alloni R, Carotti S, Behar J, Inventors. Ursodeoxycholic acid improves muscle contractility and inflammation in symptomatic gallbladders with cholesterol gallstones. *Gut* 2007;**56**:815–820.
 8. Omata M, Yoshida H, Toyota J, Tomita E, Nishiguchi S, Hayashi N, et al. A large-scale, multicentre, double-blind trial of ursodeoxycholic acid in patients with chronic hepatitis C. *Gut* 2007;**56**:1747–1753.
 9. Yamazaki K, Gleich GJ, Kita H. Bile acids induce eosinophil degranulation by two different mechanisms. *Hepatology* 2001;**33**:582–590.
 10. Yamazaki K, Suzuki K, Nakamura A, Sato S, Lindor KD, Batts KP, et al. Ursodeoxycholic acid inhibits eosinophil degranulation in patients with primary biliary cirrhosis. *Hepatology* 1999;**30**:71–78.
 11. Wagner M, Zollner G, Trauner M. Nuclear bile acid receptor farnesoid X receptor meets nuclear factor-kappaB: new insights into hepatic inflammation. *Hepatology* 2008;**48**:1383–1386.
 12. Wang YD, Chen WD, Wang M, Yu D, Forman BM, Huang W. Farnesoid X receptor antagonizes nuclear factor kappaB in hepatic inflammatory response. *Hepatology* 2008;**48**:1632–1643.
 13. Miura T, Ouchida R, Yoshikawa N, Okamoto K, Makino Y, Nakamura T, et al. Functional modulation of the glucocorticoid receptor and suppression of NF-kappaB-dependent transcription by ursodeoxycholic acid. *J Biol Chem* 2001;**276**:47371–47378.
 14. Kok T, Hulzebos CV, Wolters H, Havinga R, Agellon LB, Stellaard F, et al. Enterohepatic circulation of bile salts in farnesoid X receptor-deficient mice: efficient intestinal bile salt absorption in the absence of ileal bile acid-binding protein. *J Biol Chem* 2003;**278**:41930–41937.
 15. van Rijt LS, Prins JB, Leenen PJ, Thielemans K, de Vries VC, Hoogsteden HC, et al. Allergen-induced accumulation of airway dendritic cells is supported by an increase in CD31(hi)Ly-6C(neg) bone marrow precursors in a mouse model of asthma. *Blood* 2002;**100**:3663–3671.
 16. Lambrecht BN, De Veerman M, Coyle AJ, Gutierrez-Ramos JC, Thielemans K, Pauwels RA. Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *J Clin Invest* 2000;**106**:551–559.
 17. Idzko M, Hammad H, van Nimwegen M, Kool M, Muller T, Soullie T, et al. Local application of FTY720 to the lung abrogates experimental asthma by altering dendritic cell function. *J Clin Invest* 2006;**116**:2935–2944.
 18. Kuipers H, Heirman C, Hijdra D, Muskens F, Willart M, van Meirvenne S, et al. Dendritic cells retrovirally overexpressing IL-12 induce strong Th1 responses to inhaled antigen in the lung but fail to revert established Th2 sensitization. *J Leukoc Biol* 2004;**76**:1028–1038.
 19. Kuipers H, Hijdra D, De Vries VC, Hammad H, Prins JB, Coyle AJ, et al. Lipopolysaccharide-induced suppression of airway Th2 responses does not require IL-12 production by dendritic cells. *J Immunol* 2003;**171**:3645–3654.
 20. Hammad H, Lambrecht BN. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat Rev Immunol* 2008;**8**:193–204.
 21. Kool M, Soullie T, van Nimwegen M, Willart MA, Muskens F, Jung S, et al. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J Exp Med* 2008;**205**:869–882.
 22. van Rijt LS, Jung S, Kleinjan A, Vos N, Willart M, Duez C, et al. In vivo depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J Exp Med* 2005;**201**:981–991.
 23. Hofmann AF, Hagey LR. Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics. *Cell Mol Life Sci* 2008;**65**:2461–2483.
 24. Wang H, Chen J, Hollister K, Sowers LC, Forman BM. Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. *Mol Cell* 1999;**3**:543–553.
 25. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. *Annu Rev Immunol* 2000;**18**:767–811.
 26. Bulek K, Swaidani S, Aronica M, Li X. Epithelium: the interplay between innate and Th2 immunity. *Immunol Cell Biol* 2010;**88**(2):257–268.
 27. Klein Wolterink RG, Kleinjan A, van Nimwegen M, Bergen I, de Bruijn M, Levan Y, et al. Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. *Eur J Immunol* 2012;**42**:1106–1116.
 28. Garcia Z, Pradelli E, Celli S, Beuneu H, Simon A, Bousso P. Competition for antigen determines the stability of T cell-dendritic cell interactions during clonal expansion. *Proc Natl Acad Sci USA* 2007;**104**:4553–4558.
 29. Lambrecht BN, Salomon B, Klatzmann D, Pauwels RA. Dendritic cells are required for the development of chronic eosinophilic airway inflammation in response to inhaled antigen in sensitized mice. *J Immunol* 1998;**160**:4090–4097.
 30. van Rijt LS, Vos N, Willart M, Kleinjan A, Coyle AJ, Hoogsteden HC, et al. Essential role of dendritic cell CD80/CD86 costimulation in the induction, but not reactivation, of TH2 effector responses in a mouse model of asthma. *J Allergy Clin Immunol* 2004;**114**:166–173.
 31. Denzler KL, Borchers MT, Crosby JR, Cieslewicz G, Hines EM, Justice JP, et al. Extensive eosinophil degranulation and peroxidase-mediated oxidation of airway proteins do not occur in a mouse ovalbumin-challenge model of pulmonary inflammation. *J Immunol* 2001;**167**:1672–1682.
 32. Schote AB, Turner JD, Schiltz J, Muller CP. Nuclear receptors in human immune cells: expression and correlations. *Mol Immunol* 2007;**44**:1436–1445.
 33. Gueders MM, Paulissen G, Crahay C, Quesada-Calvo F, Hacha J, Van Hove C, et al. Mouse models of asthma: a comparison between C57BL/6 and BALB/c strains regarding bronchial responsiveness, inflammation, and cytokine production. *Inflamm Res* 2009;**58**:845–854.
 34. Hammad H, de Heer HJ, Soullie T, Angeli V, Trottein F, Hoogsteden HC, et al. Activation of peroxisome proliferator-activated receptor-gamma in dendritic cells inhibits the development of eosinophilic airway inflammation in a mouse model of asthma. *Am J Pathol* 2004;**164**:263–271.
 35. Lawrence BP, Denison MS, Novak H, Vorderstrasse BA, Harrer N, Neruda W, et al. Activation of the aryl hydrocarbon receptor is essential for mediating the anti-inflammatory effects of a novel low-molecular-weight compound. *Blood* 2008;**112**:1158–1165.

1 Ursodeoxycholic acid suppresses eosinophilic airway inflammation by inhibiting the
2 function of dendritic cells through the nuclear Farnesoid X Receptor.

3
4 Alex KleinJan¹, Monique A.M. Willart², Aldo Grefhorst³, Hamida Hammad², Leon
5 Moons⁴, Menno van Nimwegen¹, Henk C. Hoogsteden¹ and Bart N. Lambrecht^{1,2}

6
7 1 Department of Pulmonary Medicine, Erasmus MC, Rotterdam, Netherlands

8 2 Laboratory of Immunoregulation and Mucosal Immunology Department of
9 Respiratory Medicine, University Hospital Ghent, Belgium

10 3 Department of Pediatrics, Center for Liver Digestive and Metabolic Diseases,
11 University Medical Center Groningen, University of Groningen, Netherlands

12 4 Departments of Gastroenterology and Hepatology Erasmus MC, Rotterdam,
13 Netherlands

14
15 Corresponding author:

16 Alex KleinJan, PhD

17 Department of Pulmonary Medicine

18 Erasmus MC

19 Room Ee2257b

20 Dr. Molewaterplein 50

21 3015 GE Rotterdam

22 Netherlands

23 Telephone 31 10 4087701

24 Fax 31 10 4089453

25 Email: a.kleinjan@erasmusmc.nl

26

27

UDCA is not cytotoxic for eosinophils

UDCA treatment reduces eosinophilic airway inflammation, however without affecting the major eosinotrophic cytokine IL-5 or the production of the eosinophil specific chemokine eotaxin. To exclude the possibility of a direct cytotoxic effect of UDCA on eosinophils, that could cause the reduction in eosinophils in UDCA-treated group, we grew eosinophils *in vitro* and exposed them to varying concentrations of UDCA. Bone marrow cells were isolated from mice and cultured in the presence of IL-5 to promote eosinophil differentiation. On day 5, these cells were treated with UDCA and analyzed the next day. The viability of eosinophils, identified as CCR3-positive cells with high scatter characteristics, was analyzed using propidium iodide (PI) exclusion staining. No toxic effect was measured in eosinophils up to a concentration of 1000 μ M UDCA (390 μ g/ml) (supplementary figure 1a, indicating the percentage of total live eosinophils in culture), which is the dose that was given i.t. in the previous *in vivo* experiments. Higher UDCA concentrations induced eosinophil death.

Thus, we applied non-toxic UDCA concentrations in our *in vivo* experiments excluding direct cytotoxic effects of UDCA on eosinophils.

UDCA does not influence T-cells directly

UDCA treatment of OVA-immunized mice during OVA challenge also resulted in a reduced cytokine production that is classically associated with Th2 responses, e.g. the increased IL-13 concentration. To address whether UDCA directly suppresses the cytokine production of T-cells, we stimulated T-cells from OVA-immunized mice and challenged these cells non-specifically with plate bound anti-CD3 and with anti-CD28 in suspension. Supplementary figures 1b-d show that no differences in T-cell cytokine IL-2, IL-4 and IFN- γ production were observed upon increasing UDCA concentrations. Thus, the cytokine production via non-specific T-cell stimulation was not abrogated by UDCA.

No cytotoxic effect of UDCA on DCs

To exclude the possibility of a direct cytotoxic effect of UDCA on DCs, that could cause the reduction in DCs in UDCA-treated group, we grew DCs *in vitro* and exposed them to UDCA. CD11c-positive GM-CSF-cultured bone marrow cells (bone-

marrow-derived DCs) were exposed to various UDCA concentrations on day 8 of culture and were subsequently exposed to OVA on day 9 and analyzed on day 10. There was no cytotoxic effect of UDCA on bone marrow DCs (Supplementary figure 1e).

UDCA-treated DCs have an enhance migratory activity in vivo

Prior overnight incubation with UDCA did not abolish the potential of DCs to migrate from the trachea to the draining mediastinal lymph nodes, yet even enhanced it (Supplementary figure 1f), showing the number of labeled DCs reaching the mediastinal nodes 24h after intratracheal injection).

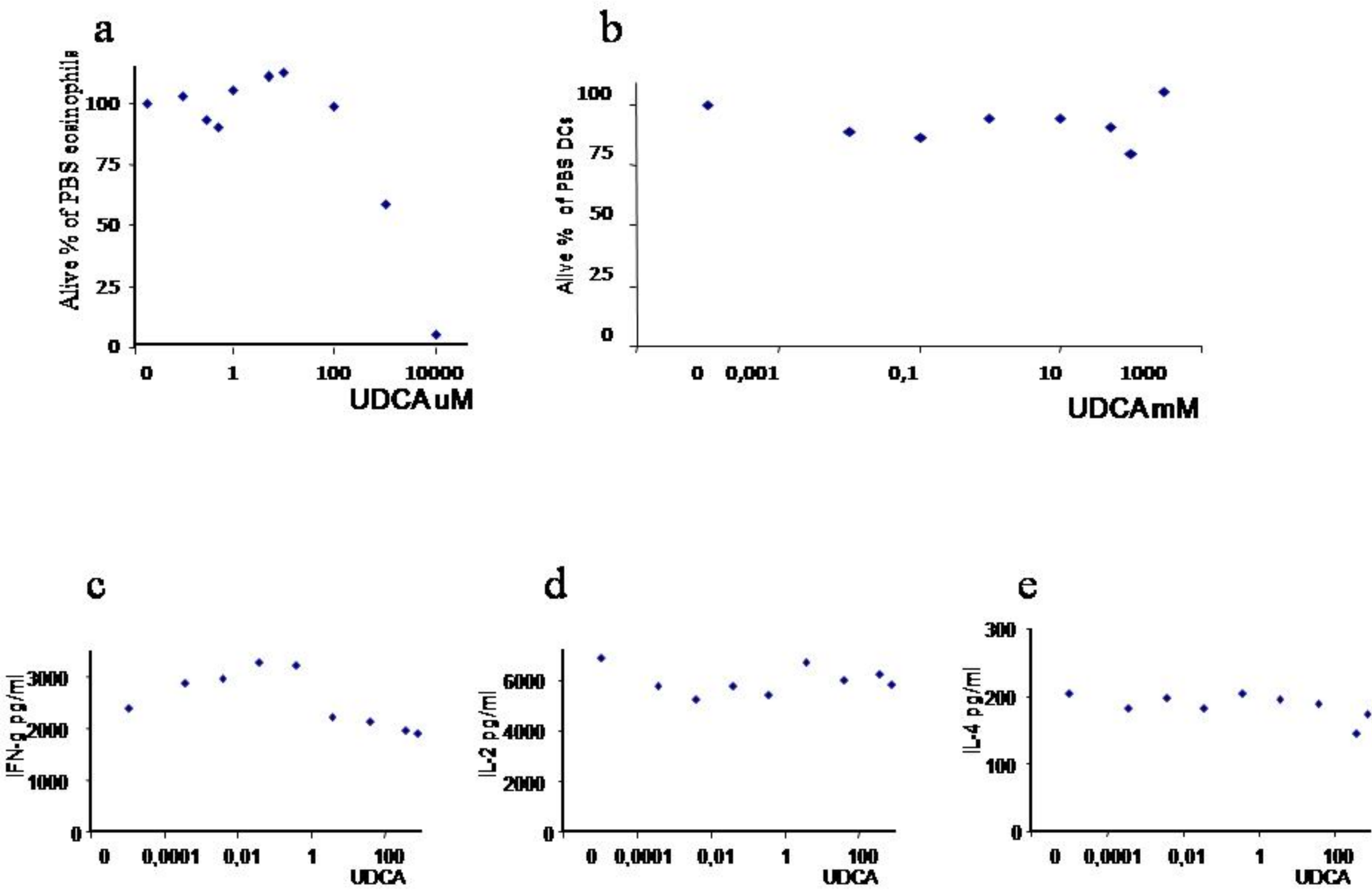
UDCA induces an increased IL-12 production by DCs, promotes induction of IFN γ in responding T cells while suppressing induction of Th2 cytokines

The experiments employing UDCA demonstrated that intratracheal administration of UDCA reduces the number of DCs in allergen challenged lungs. In previous studies, we identified lung DCs as key proinflammatory cells that are necessary and sufficient for Th2 cell stimulation during ongoing airway inflammation (1).

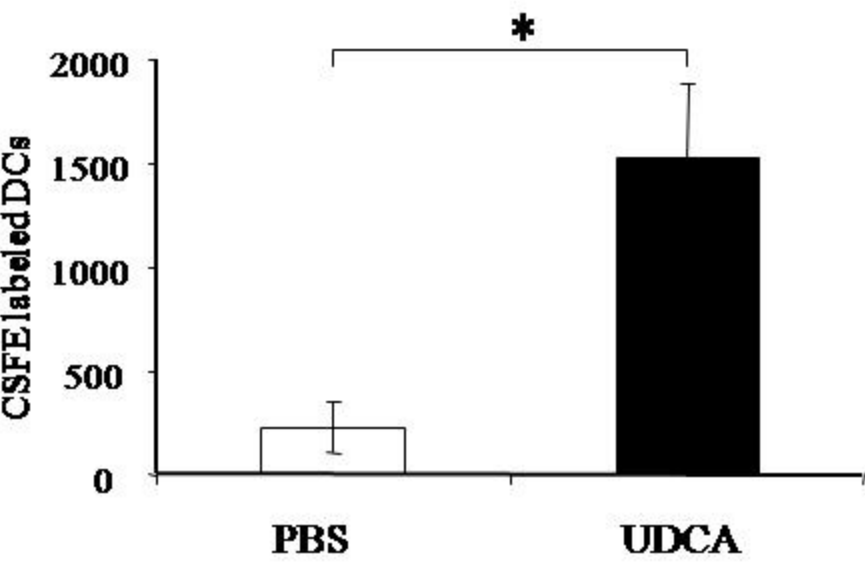
CD11c-positive cells (DCs) showed slightly less MHC II staining for UDCA (highest dosis) treated DCs than DCs treated with PBS. The phenotype of DCs exposed to UDCA was only slightly less mature (Supplementary figure 2a). No significant alteration in costimulatory molecules was observed at different UDCA concentrations (Supplementary figure 2b). Activation of bone-marrow-derived DCs from MyD88 deficient mice, e.g., mice that lack Toll-like receptor signalling, and their wild-type littermates WT controls with UDCA showed similar results (Supplementary figure 2c), confirming that the effects of UDCA are mediated via Toll-like receptor independent mechanism.

1. Hammad H, Lambrecht BN. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat Rev Immunol* 2008;**8**(3):193-204.

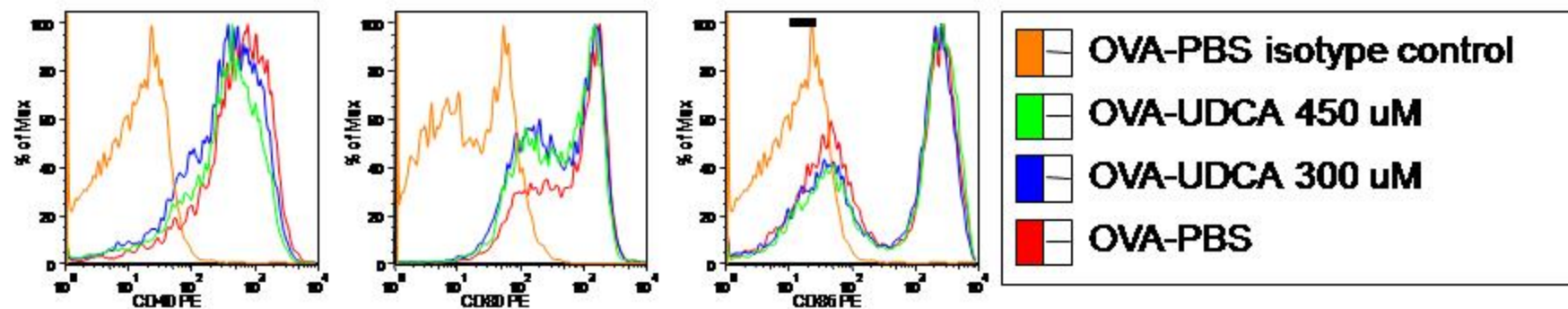
Supplementary figure 1



Supplementary figure 1f



Supplementary figure 2a



Supplementary figure 2b

UDCA conc

0

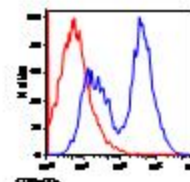
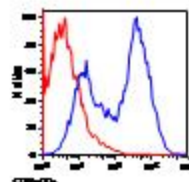
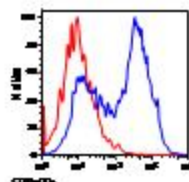
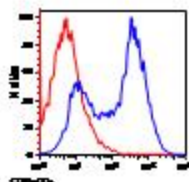
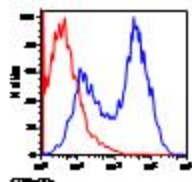
0.5

5

10

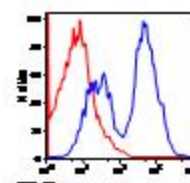
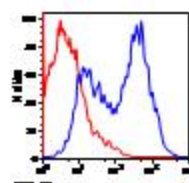
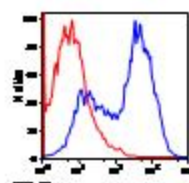
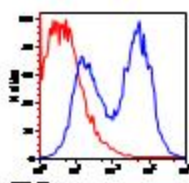
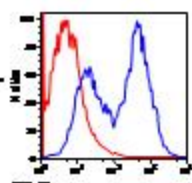
100 μ M

CD86
WT

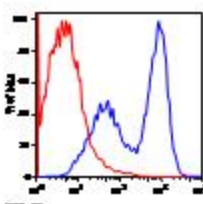
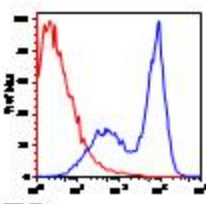
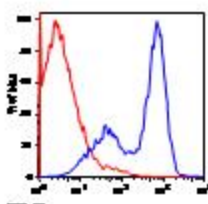
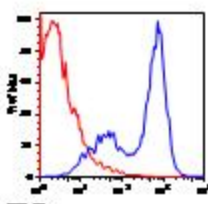
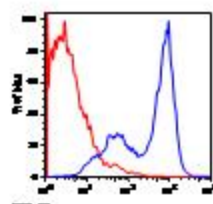


CD86
Isotype control

MyD88

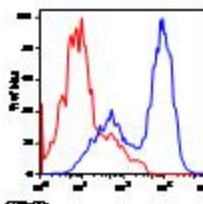
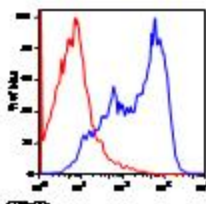
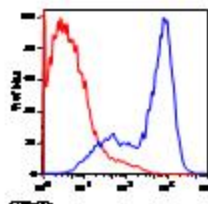
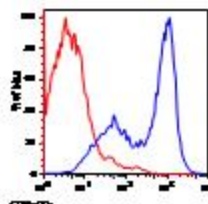
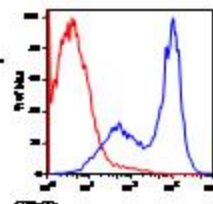


MHC II
WT



MHCII
Isotype control

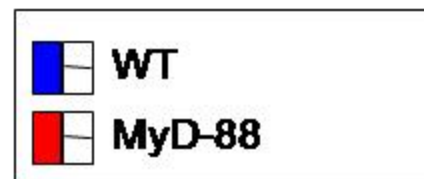
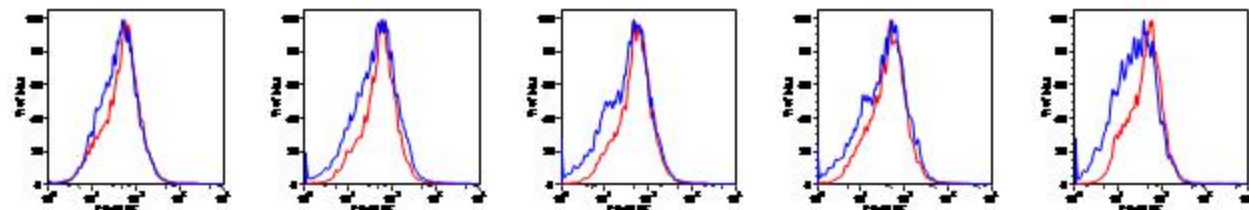
MyD88



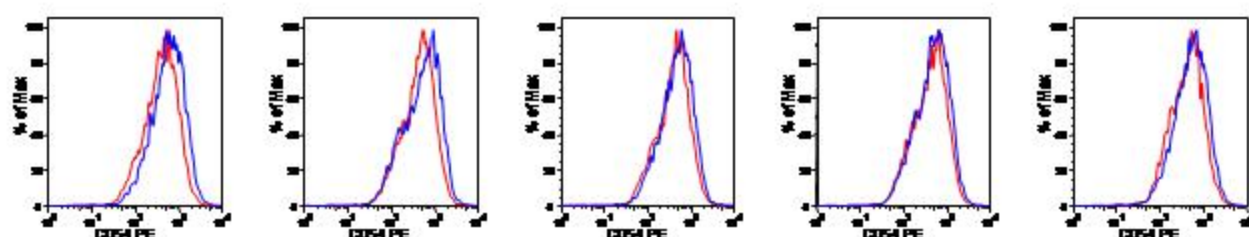
Supplementary figure 2c

UDCA 0 0.5 5 10 100 μ M

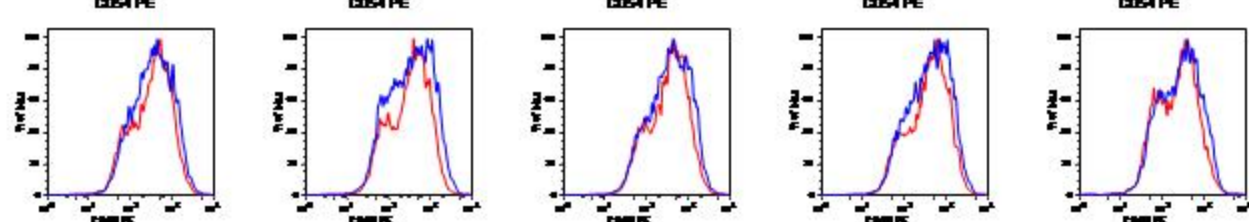
CD40



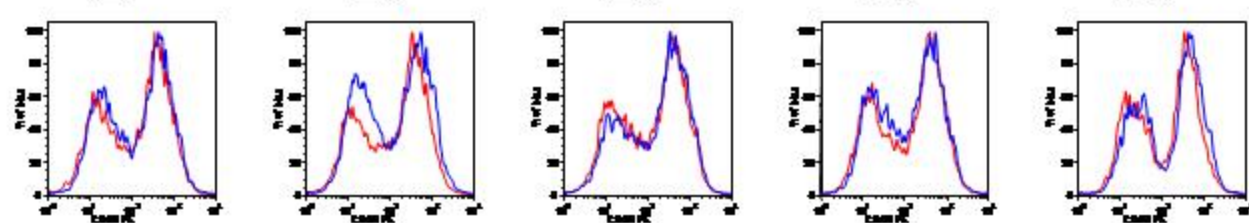
CD54



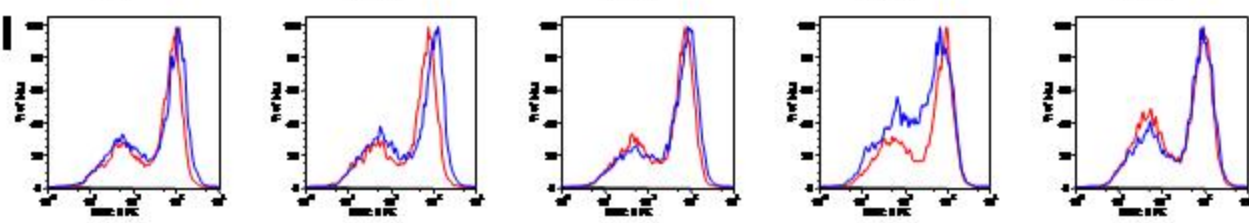
CD80



CD86



MHC II



Supplementary figure 1. **UDCA has no toxic effect on eosinophil and DC survival**
T-cell cytokine production

UDCA has no toxic effects on eosinophils (a) or DCs (b) at concentrations of less than 1000uM. UDCA has no direct effect on cytokine production of IFN- γ (c), IL-2 (d) and IL-4 (e) of non-specifically stimulated (with anti-CD28 and anti-CD3) lymphocytes obtained from OVA-sensitized mice. The migratory capacity of UDCA-treated DCs is elevated compared to saline-treated DCs (d).

Supplementary figure 2 UDCA doesn't effect DC phenotype. CD11c-positive cells (DCs) showed slightly less (but not significantly less) MHC II staining (MFI and percentages) for UDCA-treated OVA-pulsed DCs than OVA-pulsed DCs treated with PBS. DCs showed only a slightly less mature phenotype subsequent to exposure to UDCA. The expression of CD40, CD80 and CD86 does not differ between OVA-pulsed DCs that are sham treated or those treated with 300uM or 450uM of UDCA (a). No significant alteration in costimulatory molecules was observed at different UDCA concentrations. Bone-marrow-derived DCs from MyD88 (in the absence of signalling from Toll-like receptors) and WT controls showed similar results for MHCII and isotype and CD86 and isotype (b), which confirms that the effect of UDCA is independent of Toll-like receptors. There is no difference between WT and MyD88 bone-marrow-derived DCs regarding costimulatory molecules (c).