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Impaired ICOSL in human myeloid dendritic cells promotes Th2 responses in patients with allergic rhinitis and asthma

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Running title: Impaired ICOSL in myeloid DCs promotes Th2 responses

Abstract

Background: Myeloid dendritic cells (mDCs) and costimulatory molecules such as ICOSL/B7H2 play a pivotal role in murine experimental asthma, while little is known in human allergic disease.

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The aim of this study was to characterize the phenotype and ICOSL expression of mDCs from allergic rhinitis patients (AR) and their functional correlates on mDC regulation of T cell responses.

Methods: Human blood myeloid, CD1c⁺ DCs were isolated from AR or healthy controls.

Expression of co-stimulatory molecules inducible costimulatory ligand (ICOSL) and programme death-ligand 1 (PD-L1) was analyzed in blood mDCs by flow cytometry and in nasal tissue biopsies by dual immunostaining. Blood mDCs were co-cultured with (allogeneic) CD4⁺ T cells before immunoassays for cytokine responses.

Results: mDCs from AR patients expressed a lower level of ICOSL, in both blood and nasal tissue.

mDCs from AR were constitutively primed to induce Th2 cytokines and TNF in allogeneic CD4⁺ T cells, while no difference was observed for IFN- γ or IL-10. Production of IL-10, IL-12 did not differ between AR and control mDCs. Blockade of ICOSL in control DCs upregulated IL-13 but not IFN- γ in co-cultures with T cells, while PD-L1 blockade upregulated both IL-13 and IFN- γ .

Conclusions: Our data show that mDCs from patients with AR display impaired expression of ICOSL, and this defect licenses mDCs to promote aberrant IL-13 and IL-5-producing Th2 cell responses.

Key points:

This study shows impaired expression of ICOSL by human myeloid dendritic cells in allergic rhinitis, which plays a critical role in DC-driven Th2 responses.

Keywords: Allergy, Costimulation, Human Dendritic cells, ICOSL (B7H2), Nasal Mucosa, Myeloid DC, PD-L1, Th1/Th2 cells, Tolerance, TSLP.

Abbreviations:

AR: allergic rhinitis; (m)DC(s): myeloid dendritic cell(s); RT: room temperature; ICOSL: Inducible co-stimulator ligand; PD-L1: Programmed cell death ligand 1; TSLP: Thymic stromal lymphopoietin; Treg: Regulatory T cells.

Introduction

Asthma and allergy are underlined by aberrant immune responses to environmental antigens that include allergen-specific IgE antibodies and effector Th2 cells. The respiratory tract is constantly exposed to inhaled microbes and allergens (1). Dendritic cells (DCs) are central to both innate and adaptive immune responses, and represent master regulators of allergic inflammation (2). Activation of DCs through microbial pathogen-associated molecular patterns plays a key role during inception of allergic sensitization (3, 4), and during regulation of T cell responses (5). DCs also play a pivotal role in balancing tolerance versus inflammatory responses to allergens (6, 7). Thus, DCs form a heterogeneous group of cells that are specialized in capture of antigens and their presentation to T-lymphocytes: they include myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) (8) and several subtypes according to localization, phenotype and/or function (9, 10). CD4⁺ Th2-driven hypersensitivity responses to allergens observed in atopic diseases, probably result from aberrant signals provided by mucosal epithelial cells and DCs (6). Allergic responses are initiated by T-cell recognition of major histocompatibility class II-peptide complexes at the surface of antigen-presenting cells (11). Activation of T cells requires a second nonspecific costimulatory signal, which is provided by interactions between various DC and T cell receptors. According to this second signal, as well as to the microenvironment (e.g. cytokines) as third signal, decision will be taken whether DC-T cell interactions lead to the development of an inflammatory allergic reaction or immune tolerance against a given allergen.

The B7:CD28 family of co-stimulatory molecules has a key role in regulating T cells (12). Recent studies point to inducible costimulatory ligand (ICOSL) /ICOS and programme death-ligand 1 (PD-L1)/PD-1 as major tolerogenic pathways (13, 14) which could potentially be exploited to treat autoimmune or allergic diseases. ICOSL has been detected on the surface of DCs and ICOS is upregulated on activated T cells (15). In mice lacking ICOS expression or function, marked reductions in Th2 responses were observed in experimental asthma (16, 17). In allergic rhinitis (AR), it has been shown that ICOS expression is up-regulated and associated with Th2 response (18). However, ICOS/ICOSL interaction was also shown as critical to the generation of regulatory T cells (Tregs) and suppression of experimental asthma (19). Another study showed that when plasmacytoid DCs (pDCs, in contrast to myeloid DCs, mDCs) are impaired in expressing ICOSL upon maturation (through TLR9), this results in defective generation of IL-10-producing Tregs (20). PD-1 on T cells interacts with PD-L1 or PD-L2 on DCs and also regulates Th1/Th2 bias, and is mainly reported as a regulatory molecule supporting DC-driven tolerance (21) through increased number and function of Tregs (22).

In contrast to experimental asthma, there is only limited evidence in human allergic disease that mDCs dysregulate the immune response to allergens. Hammad et al. (23) showed that monocyte-derived DCs from allergic patients promoted a Th2 response to mite allergen. Recently, we reported on aberrant features of DCs in both target tissues and in blood, including pro-Th2 activity of blood mDCs when pulsed with the allergen and co-cultured with CD4⁺ T cells (24). However, mechanisms of such dysfunction remain unknown. We hypothesized that abnormal expression and function of ICOSL and/or PD-L1 regulatory pathways in mDCs could be central to aberrant Th2 induction in allergic patients. To explore this hypothesis, the present study was designed to investigate critical co-stimulatory receptors such as ICOSL and PD-L1 in purified mDCs

from patients with allergic rhinitis and to correlate findings to mDC-driven regulation of T cell responses.

Methods

Patients

Atopic patients with a clinical history of allergic rhinitis (AR) and positive immediate skin (>3mm more than the negative diluent control) and serum specific IgE responses to at least house dust mite (*Dermatophagoides pteronyssinus*, Der p) were recruited for leukapheresis and subsequent purification of myeloid DCs (n= 20; Table 1). 10 patients were treated by intranasal steroids (total daily dose of mometasone, 200µg). 8 patients had concomitant, mild to moderate asthma; among these, 5 were treated with low-dose inhaled steroids ($\leq 400 \mu\text{g}$ beclomethasone equivalent, total daily dose). These topical treatments were withdrawn at least 48hrs before blood sampling. Non-atopic subjects with a negative history for allergic airway disease and negative skin prick tests to a battery of 17 common inhalant allergens served as controls (n= 23). Due to the low number of blood mDCs from each donor, FACS phenotyping and MLR assays were performed separately and included respectively at least n=8 (and up to n=14, according to cell numbers) and n=3 subjects in each group (allergic rhinitis, controls). Following initial validation, purity was also randomly checked in 8 experiments.

One additional series of asthma patients was included for FACS analysis of mDCs (Table 2).

Nasal tissue from a series of AR and control patients (Table 3) was used to study local mDCs (25).

Controls (n=13) and AR patients (n=11, most being sensitized to house dust mite) underwent surgery for anatomical obstruction (septoplasty). For all patients, a washout of oral and nasal corticosteroids or antibiotics was required for at least 3 weeks before surgery. Samples consisted

of biopsies of the inferior turbinate. AR patients were selected as for blood mDCs, while control patients consisted of subjects undergoing nasal surgery for a septal deviation.

The study was approved by the local Ethical Committee (Cliniques universitaires St-Luc, Brussels, Belgium) and was performed following approval and written informed consent.

Reagents and antibodies

Lymphoprep was from Axis Shield (Oslo, Norway), and Fluorescein isothiocyanate (FITC)-conjugated Lineage cocktail-1, PerCP-conjugated anti-HLA-DR (L243, IgG_{2a}, κ), APC-conjugated anti-CD11c (B-ly6, IgG₁, κ) and PE-Cy7-conjugated anti-human CD274/B7-H1/PD-L1 (MIH1, IgG₁, κ) were purchased from BD Biosciences (Erembodegem, Belgium). APC-conjugated anti-CD1c (L161, IgG₁, κ) and PE-conjugated anti-human CD275/B7-H2/ICOSL (MIH12, IgG₁, κ), blocking mAbs to human PD-L1 (MIH1, IgG₁, κ) and to ICOSL (MIH12, IgG₁, κ) and their isotype control (P3.6.2.8.1, IgG₁, κ) were from eBiosciences (Paris, France). Lipopolysaccharide (LPS) from *Escherichia coli*, serotype 0111:B4 was from Sigma Chemical Co. (St Louis, MO, USA).

Dendritic cell isolation and FACS staining

Peripheral blood mononuclear cells (PBMCs) were obtained after density-gradient centrifugation by Lymphoprep of buffy coats. mDCs and CD4⁺ T cells were purified from PBMCs using the BDCA-1⁺ DC and CD4⁺ T cell isolation kits, respectively (Miltenyi Biotech, Palo Alto, CA) according to manufacturer's instructions. Freshly isolated mDCs (CD1c⁺) were immediately stained to check for purity (in validation assays, and randomly during the study in 8 experiments), which was ≥ 90% based on Lin-1⁻ HLA-DR⁺ CD1c⁺ cells (Figure S1A-C and S1E). Contaminating cells were monocytes (4-5%) and B cells (2-3%). The purity of freshly isolated T cells was at least 98% (Figure S1D).

Figure S1F shows representative results of purified mDCs (after 18hrs-culture). Lin-1⁻ HLA-DR⁺ CD11c⁺ were further gated for surface phenotype analysis (Figure S1F).

Freshly isolated mDCs were adjusted to 1*10⁶/ml in supplemented RPMI-1640 and were stimulated or not with LPS (1 µg/ml) for 18 hrs (Figure S2 and S3). In separate experiments, mDCs were cultured for 18 hours with 50 ng/ml of human recombinant thymic stromal lymphopoietin (TSLP, Miltenyi Biotec, Bergisch Gladbach, Germany).

After culture, mDCs were first blocked for 30 minutes with decompemented human serum. Cells were washed and stained at 4°C with fluorescent antibodies in PBS for 30 min. Controls included corresponding isotype-matched control mouse IgGs. At least 10,000 events for mDCs were analyzed on a FACS Canto II (Becton Dickinson) flow cytometer.

Nasal biopsies and immunohistochemistry (IHC)

Nasal biopsies were processed for dual IHC to detect ICOSL- and PD-L1 expressing mDCs in the nasal mucosa. Turbinal biopsies were fixed in 4% formalin for 24 hours, embedded in paraffin and cut (5 µm thick) before IHC for CD1c and ICOSL or PD-L1. Sections were washed in Tris buffered saline (TBS) and incubated with 0.3% H₂O₂ for 30 minutes to block endogenous peroxidases.

Sections were then washed with TBS and incubated with 0.001% avidin and 0.001% biotin sequentially for 10 minutes respectively. After each step, sections were washed by PBS. Blocked sections were incubated with 2% BSA in TBS for 30 min. 16ug/ml CD1c antibodies in 1% BSA TBS (mouse anti-human CD1c mAb, clone 2F4, Abcam, Cambridge, UK) or isotype control were applied for overnight at 4° C. The next day after 3 TBST washes (tween 0.5% in TBS, pH 7.4), secondary antibody in 2% milk (goat anti-mouse IgG Biotin, Sigma, B-7401, 1:500 dilution) was applied to each section for 30 minutes. Then Streptavidin-Horseradish Peroxidase (1:500; BD

pharmingen, Erembodegem, Belgium) in 1% BSA TBS were applied for 30 mins. Reaction was developed using DAB for 5 min. Sections were further rinsed in distill water and again blocked by 2% BSA in TBS for 30 min at room temperature. PD-L1 antibody (20 µg/ml; rabbit anti-human polyclonal, ab58810, Abcam, Cambridge, UK) or ICOSL (rabbit anti-human polyclonal, B8286, LSBio, TE Huissen, The Netherlands) in 1% BSA TBS or isotype control were applied overnight at 4° C. The next day, 5ug/ml Alkaline phosphatase conjugated secondary antibody (donkey anti-rabbit IgG Biotin, pre-absorbed, ab 7084, Abcam, Cambridge, UK) in 1% BSA TBS was applied to each section for 30 minutes, before developing with Fast-Red (Sigma Chemical Co., St Louis, MO, USA). Sections were counterstained with hematoxylin, mounted, and observed under light microscope (Zeiss Axiocam). Images were acquired using Leica SCN400 slide scanner (Meyer Instruments, Houston, TX, USA), analyzed and counted with the slidepath software. The immunostained cells were counted in submucosal whole lamina propria area, and results were expressed as the number of positive cells/mm². The area of nasal tissue analyzed was 12.1± 1.5 mm² (mean ± SEM, 4.8–21.0 mm², range) in control and 13.0 ± 2.8 mm² (2.4–32.6 mm²) in AR respectively.

Co-cultures of DCs with CD4⁺ T cells

mDCs and T cells were co-cultured for MLR. In each experiment, mDCs from one AR patient and one control donor were incubated in parallel with the same CD4⁺ T cells from another (allogeneic) non-atopic control donor. After isolation and 18hrs-culture, mDCs were washed and co-cultured with freshly isolated CD4⁺ T cells at a ratio of 1:5 (DC:T) in 96 round-bottom plates in RPMI-1640 with 100µM β-mercaptoethanol, for 5 days. For blocking experiments, anti-ICOSL (10 µg/ml) or/and anti-PD-L1 (10 µg/ml) mAb was added.

Measurement of cytokines

Cytokines were assayed in culture supernatants from mDCs (18hrs-culture) or from DC/T cells (5-day co-cultures) by ELISA, using pairs of capture and biotin-coupled detection Abs to IL-10, IL-12p40, IL-12p70, TNF, IFN- γ , IL-13, IL-5 and TGF- β 1. The capture and biotinylated detection Abs (except otherwise indicated) were purchased from R&D Systems (Minneapolis, MN, USA). The following pairs were used: for IL-10 (BD554705 / BD554499, BD Biosciences), IL-12p40 (MAB609 / BAF219), IL-12p70 (MAB611 / BAF 219), TNF (MAB610 / BAF210), IFN- γ (MAB2852 / BAF285), IL-13 (MAB213 / BAF213) and IL-5 (MAB405 / BAM6051).

Plates were coated overnight with capture antibody, and then blocked for 1 hr with 1% bovine serum albumin in phosphate-buffered saline (PBS). Plates were washed and supernatants were incubated for 2 hrs at RT, and human recombinant cytokines were used as standards. After washing, detection Ab was added for 1 hr. After washing the plates were incubated with tetramethylbenzidine (TMB) solution containing hydrogen peroxide, and the reaction was stopped after 5-10 minutes with 1 N sulfuric acid and read at 450 nm. The detection limit for each cytokine were as follows: IL-10 and IL-12p70: 31.3 pg/ml; IL-12p40 and IL-5: 62.5 pg/ml; TNF: 7.8 pg/ml; IFN- γ : 125 pg/ml; IL-13: 156 pg/ml.

TGF- β 1 production was also measured by ELISA after sample acidification, to allow its release from latent complexes, by using a pair of a capture monoclonal antibody and a biotinylated detection antibody to human TGF- β 1 (TGF- β immunoassay kit, Biosource, Nivelles, Belgium). The sensitivity of TGF- β 1 immunoassay was 15.6 pg/ml.

Statistical analysis

Data were analyzed using GraphPad Software for statistics (San Diego, CA, USA). Between-group comparisons (AR vs control) were performed using the Mann-Whitney U-test. Within-group

comparisons (e.g. medium vs LPS) were performed using the Wilcoxon matched-pairs signed-rank test. All tests were two-tailed and p value <0.05 was considered statistically significant.

Results

Impaired ICOSL and PD-L1 expression in myeloid DCs from allergic rhinitis patients

Myeloid DCs from patients with AR expressed lower levels of ICOSL ($10.4 \pm 2.1\%$ in AR versus $42.9 \pm 9.9\%$ in controls, $P < 0.01$, Figure 1C). In addition, LPS down-regulated ICOSL in mDCs, in both AR and controls (Figure 1A and 1C, $P < 0.01$). This effect was not due to artefacts of the phenotyping performed after *in vitro* culture (Figure S2) or to cell death in short-term cultures, as LPS only had significant effects on mDC viability at 42hrs of culture (Figure S3).

PD-L1 was not significantly different between AR and control mDCs (Figure 1D), whereas LPS upregulated its expression ($P < 0.001$, Figure 1B and 1D) in a dose-dependent manner (Figure S4B). In parallel, upregulation of CD80 and CD86 was confirmed upon LPS stimulation (Figure S4C and S4D). Expression levels of the other co-stimulation receptors OX40L, ILT-3 and ILT-4 were not significantly affected in AR (Figure S5).

As a proportion of AR patients had concomitant asthma, we wondered whether ICOSL downregulation was observed in allergic rhinitis and/or asthma. First, ICOSL expression remained significantly downregulated when considering patients with AR only ($p < 0.05$). Secondly, we analysed ICOSL expression on mDCs from one additional series of patients with allergic asthma, which showed similar ICOSL downregulation (Figure S6A). In contrast, expression of PD-L1 was increased in asthma but not in AR (Figure S6B).

In nasal tissue, there was no significant difference in the numbers of $CD1c^+$ mDCs between AR and control patients (Figure 2A and 2B). Consistently with blood mDCs, expression of ICOSL by

nasal CD1c⁺ mDCs was reduced in AR ($15.6 \pm 2.3\%$, AR versus $31.9 \pm 4.8\%$, controls, $P < 0.05$, Figure 2C), while no difference was observed for PD-L1 (Figure 2D).

These data indicated that ICOSL was consistently reduced in patients with allergic rhinitis, both in blood and nasal tissue, as also observed in asthma, whilst PD-L1 was not significantly affected in rhinitis and even slightly upregulated in asthma.

Priming of Th2 responses by myeloid DCs from allergic rhinitis patients

To evaluate the functional correlates of our phenotypic findings, particularly on ICOSL, the model of alloMLR was used to assess T-cell regulation. Resting mDCs were co-cultured with allogeneic CD4⁺ T cells in a paired manner (mDCs from one AR patient and mDCs from one control co-cultured with the same CD4⁺ T cells from a non-atopic donor, *see Methods section*). In preliminary experiments, we confirmed that cytokine production in these co-cultures was DC-dependent, in particular (in resting conditions) for IL-13, IL-5 and IL-10 (Figure S7), and that alloMLR alone (in the absence e.g., of restimulation) did not trigger significant T cell proliferation (data not shown).

Production of IL-13, IL-5 and TNF was increased in T cells co-cultured with mDCs from AR patients, as compared to mDCs from controls (Figure 3A, B and D), without change in IFN- γ (Figure 3C). A trend for lower IL-10 induced by AR' mDCs was also observed (Figure 3E) but did not reach statistical significance. IL-4, IL-17 and the active form of TGF- β 1 were not detected in these co-cultures (data not shown). Of note, mDCs alone (either resting or LPS stimulated) did not release IL-5, IL-13 or IFN- γ whilst they could release IL-10 and TNF, but in marginal amounts when compared to levels in co-cultures (Figure S7).

Cytokine production by myeloid DCs

To further evaluate mDC dysfunction, the production of IL-10, IL-12 and TNF was assayed, initially up to 66 hrs of cultures with/without LPS (Figure S8). LPS dose-dependently upregulated IL-10 and TNF production, and to a lower extent (not significantly) IL-12p40. After 18hrs-culture, release of these cytokines by mDCs from AR patients was not significantly different from control mDCs, except a lower TNF response to LPS (Figure 4A-C). These data did not directly support a role for mDC cytokines in driving their proTh2 activity.

Upregulation of IL-13 by ICOSL and PD-L1 blockade in normal myeloid DCs

We then evaluated whether downregulation of ICOSL was involved in the aberrant capacity of mDCs from AR patients to trigger Th2 polarization in CD4⁺ T cells. To address this, mDCs from control subjects were first treated with anti-ICOSL or anti-PD-L1 blocking ab during the 5 day co-culture with T cells. Blocking ICOSL pathway in mDCs resulted in a significant upregulation of IL-13 production, when compared to isotype control (Figure 5A), without affecting IFN- γ (Figure 5C). Blockade ICOSL also showed a trend for IL-5 upregulation, but without reaching statistical significance (Figure 5B). In contrast, blocking PD-L1 upregulated both IL-13 and IFN- γ (Figure 5C), and the combination of ICOSL and PD-L1 blockade also selectively upregulated IL-13 and IFN- γ . IL-10 and TNF production were not significantly affected by either blockade (Figure 5D and 5E).

TSLP does not recapitulate ICOSL downregulation in mDCs

To evaluate whether altered expression of ICOSL in mDCs from allergic patients could relate to ongoing Th2 inflammation, we assessed the effects of the master epithelial proTh2 cytokine TSLP (26). TSLP only exerted a marginal effect on ICOSL expression, and had no effect on PD-L1 (Figure S9).

Discussion

We show here that myeloid DCs from allergic rhinitis patients display impaired expression of ICOSL/B7H2, both in blood and in the target mucosa, and this defect contributes to the pro allergic/Th2 activity of DCs from these patients. Thus, mDCs from these patients are able to drive in allogeneic CD4⁺ T cells, IL-13 and IL-5 as signature Th2 cytokines, as well as TNF. Blockade of ICOSL pathway in normal DCs resulted in upregulated IL-13 expression, without change in IFN- γ or TNF. In contrast, PD-L1 was not downregulated on mDCs from allergic patients was involved in the DC regulation of both IL-13 and IFN- γ production by T cells.

The ICOSL/ICOS and PD-L1/PD-1 pathways have been studied in details in the mouse, notably in experimental asthma, but their involvement in human allergic airway disease was almost lacking. The ICOSL/ICOS pathway has multiple effects for both Th1, Th2 and Treg generation (12). In this study of myeloid DCs from AR patients, we could link the aberrant pro-Th2 capacity (induction of IL-13 and IL-5) of DCs to ICOSL down-regulation. Previous studies in mice (16, 17) showed that ICOSL/ICOS interactions between DC and T cells lead to Th2 responses, and upregulated ICOS in PBMC from AR patients underlined Th2 responses (18). However, ICOS/ICOSL interaction also leads to Treg generation (17), as well as CD8 α ⁺ DCs expressing higher ICOSL and IL-10 (27). Although we did not observe a clear defect in IL-10 or TGF- β , Treg regulation could be specifically evaluated upon coculture with DCs expressing low levels of ICOSL. Altogether these data suggest that the final effect of ICOS/ICOSL activation depends on several factors including kinetics of activation, influence of ICOSL expression by other cell types (such as B cells and macrophages, 13) and microenvironmental cofactors. In addition, ICOS overexpression may also lead to ICOSL downregulation (28, 29). Nevertheless, our data indicate that ICOSL downregulation of mDCs is a key feature in AR and asthma, which contributes to mount Th2 immunity in these patients.

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In experimental asthma in mice, both PD-L1 and PD-L2 were markedly elevated on DCs, and PD-1 was upregulated on T cells (30), possibly following IL-4 expression (31). PD-L1 expression was not affected in mDCs from our allergic rhinitis patients. However, blockade of PD-L1 in DCs significantly enhanced IL-13 and IFN- γ production, suggesting an intrinsic suppressive role of PD-L1 on effector Th responses. Several studies indicated that PD-1 and PD-L1 provide inhibitory signals: accordingly blockade of PD-1 or PD-L1 exacerbated experimental autoimmune disorders (11); In murine experimental asthma, PD-1/PD-L1 interaction was shown to exacerbate Th2 responses and airway hyperresponsiveness (AHR), whereas PD-1/PD-L2 initiated a Th1/ IFN- γ response and subsequently reduced AHR (32). Similarly to PD-L1, we did not observe significant changes in the expression of OX40L and ILT3 / ILT4, as pro-Th2 and inhibitory receptors respectively (33, 34).

The main cytokine profile of mDCs did not differ substantially between allergic rhinitis patients and controls, except a lower TNF production upon LPS stimulation. This latter change was not due to a global default of responsiveness of allergic mDCs to the LPS/TLR4 axis, as other cytokine responses (e.g. IL-10) were preserved. TNF is rather overexpressed in asthma, and may amplify inflammatory reactions through activation of NF- κ B, AP-1 and other transcription factors (35). In allergic rhinitis, TNF is rapidly detected after allergen exposure, and induces expression of E-selectin adhesion receptor on endothelial cells that initiates local leuko-chemotaxis (36). Our study demonstrates that TNF, along with Th2 cytokines, is induced in CD4⁺ T cells upon co-culture with mDCs from allergic rhinitis patients. This was in contrast to TNF production by mDCs themselves, and was not affected by ICOSL or PD-L1 blockade. This discrepancy could be due to the experimental setting which involved stimulation by LPS for mDCs alone and MHC mismatch in alloMLR cocultures. Nevertheless, this increased capacity of mDCs to reprogramme T cells for TNF

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production probably contributes to amplify inflammatory responses in allergic patients, but appears independent from ICOSL or PD-L1 pathways. Reduction in TNF and IL-12 production was reported in DCs from atopic dermatitis patients, and these DCs were inducing increased IL-4⁺ T cells and fewer IFN- γ ⁺ cells (37). DCs from atopic dermatitis patients also underlie impaired IFN- γ responses and express lower levels of IFN- γ R (38). Thus, although short-term cultures might limit conclusions about production of bioactive IL-12 (p70), alterations in blood DC cytokines, including impaired Th1 immunity, could be more prominent in atopic dermatitis than in our allergic rhinitis patients. Our study presents some limitations. First, since ICOSL could also control T-cell proliferation, cytokine changes upon blockade of this receptor could relate to increased proliferation. However, it was shown (39) that blocking ICOS-ICOSL interactions does not affect T cell proliferation during alloMLR, although in a slightly different setting (alloMLR upon blockade of co-stimulation by anti-CD40/CD80/CD86). Therefore, it seems unlikely that changes in cytokine responses could be related to increased T-cell proliferation upon ICOSL blockade, although this could not be formally ruled out. Secondly, the master pro-Th2 cytokine IL-4 could not be detected in our co-cultures, but this is a frequent drawback of similar studies in allergy (40). Thirdly, DCs were not evaluated for other functions, such as antigen uptake or migration, which could be assessed in future studies to better characterize DC dysfunction in patients with airway allergy.

Whether abnormal programming of mDCs from patients with allergic airway disease results from the atopic genetic background or epigenetic conditioning, in the periphery and/or in the mucosal microenvironment, remains unanswered. TSLP represents a key epithelial-derived cytokine found in the skin and airways from allergic patients, which may programme DCs for Th2 responses (41). However, incubation of mDCs with TSLP did not clearly affect ICOSL (or PD-L1) expression. In addition, the consistency of our findings in blood and nasal mDCs from allergic patient further

suggests that ICOSL downregulation represent a systemic feature rather than a local reprogramming of mDCs within the inflamed nasal mucosa. It could however not be excluded that, *in vivo*, the combination of various factors of the local “immune milieu” could imprint DCs for reduced ICOSL expression. As allergen immunotherapy may notably promote DC expression of tolerogenic molecules (42, 43), whether immunotherapy and current therapies also impact on these mDC features remains unknown. Based on our findings, strategies aiming at restoring the expression of ICOSL in mDCs to inhibit Th2 cell responses to allergens, could represent an attractive option in allergic individuals.

Altogether, the present data demonstrate for the first time a defective expression of ICOSL in mDCs from patients with allergic rhinitis or asthma, which could underlie the dysfunction of mDCs that are primed to drive Th2 (24) and TNF responses. These data also provide evidence that ICOSL and PD-L1 pathways are differently involved in the regulation by human mDCs of T-cell responses to allergens. Thus restoring ICOSL in mDCs from allergic patients could be considered as a potential therapeutic strategy.

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Figure legends

Fig 1: ICOSL and PD-L1 expression in blood mDCs from allergic rhinitis patients and controls.

Purified mDCs were cultured for 18hrs with or without LPS (1µg/ml), and stained with anti-ICOSL or anti-PD-L1 or their isotype control. Histograms of ICOSL (A) and PD-L1 (B) expression in mDCs from a control donor are shown with or without stimulation (solid line). Numbers indicate in the gated area the percentage (%) of ICOSL⁺ or PD-L1⁺ mDCs after deduction of background (dashed line). ICOSL expression is shown in (C), from AR patients (n=8) versus controls (n=9). PD-L1 expression is shown in (D), in AR patients (n=13) versus controls (n=14). Data are mean values ± SEM (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

Fig 2: ICOSL and PD-L1 expression in nasal mDCs from allergic rhinitis patients and controls.

Nasal tissue sections from AR patients (n=11) and controls (n=13) were immunostained for CD1c (brown arrows) and ICOSL or PD-L1 (pink arrows) as described in *Methods* (A); double positive being CD1c⁺ ICOSL⁺ or CD1c⁺ PD-L1⁺ (black arrows). Isotype controls of each are shown in lower. CD1c⁺ mDCs were first counted (B), as well as the proportion of CD1c⁺ DCs expressing ICOSL (C) and PD-L1 (D). Bar represents the mean value (**P* < 0.05).

Fig 3: Regulation of CD4⁺ T cell responses by mDCs from allergic rhinitis patients and controls.

In each experiment, mDCs from one AR patient and one control donor were isolated in parallel, and after 18hrs were co-cultured for 5 days with the same freshly isolated allogeneic CD4⁺ T cells (from one non-atopic donor) at a ratio 1:5 (DC:T). Supernatants were assayed for IL-13 (A), IL-5 (B), IFN- γ (C), TNF (D), IL-10 (E) and TGF- β 1 (F). Data are mean values $\pm$ SEM. Each condition represents 3-5 experiments (**P* < 0.05).

Fig 4: Cytokine profile of mDCs from allergic rhinitis patients and controls.

Purified mDCs were cultured with LPS (1 μ g/ml) or not for 18hrs, and supernatants were frozen until assayed by ELISA for IL-12p40 (A), TNF (B) and IL-10 (C). Cytokines are shown from allergic rhinitis patients (n=17) versus controls (n=22). Data are mean values $\pm$ SEM. (***P* < 0.01, ****P* < 0.001).

Fig 5: IL-13 production driven by mDCs depends on ICOSL.

mDC-T cell co-cultures and cytokine assays were performed as described in Figure 3. Anti-ICOSL, anti-PD-L1 mAb or their isotype control were added at the first day of the 5-day co-culture, before analysis of IL-13 (A), IL-5 (B), IFN- γ (C), TNF (D) and IL-10 (E) production. Data are mean

values $\pm$ SEM. Each condition represents 4-7 experiments ($*P < 0.05$). mIgG control: non-immune mouse IgG; both mAbs: anti-ICOSL mAb *plus* anti-PD-L1 mAb.

Table 1.

Patient characteristics for blood DCs

	Allergic Rhinitis	Controls
N	20	23
Age (mean $\pm$ SD)	40 $\pm$ 13	48 $\pm$ 18
Sex	9M:11F	14M:9F
Concomitant asthma, n	8	0
Smoking, n	3	5
Topical steroids, n	10	0
Total IgE (U/ml), median (range)	172 (36 - 3,260)	Nd
Der p-IgE (U/ml), median (range)	10.3 (0.52 - 100)	Nd

Topical, intranasal (n=10) +/- inhaled (n=5), steroids were at low dose and withdrawn for at least 48hrs before blood sampling. Der p-IgE, *Dermatophagoides pteronyssinus*-specific IgE in serum.

Table 2.

Patient characteristics for blood DCs in allergic asthma

	Allergic Asthma	Controls
N	9	8
Age (mean $\pm$ SD)	46 $\pm$ 18	57 $\pm$ 16
Sex	6M:3F	4M:4F
Total IgE (U/ml), median (range)	30.3 (7.09 – 68.8)	8.44 (3.21 – 37.5)
Der p-IgE (U/ml), median (range)	2.60 (0.18 – 9.20)	0.06 (0.04 -0.07)

Table 3.

Patient characteristics for nasal DCs

	Allergic Rhinitis	Controls
N	11	13
Age (mean $\pm$ SD)	36 $\pm$ 9	38 $\pm$ 12
Sex	8M:3F	10M:3F
Concomitant asthma, n	1	1
Smoking, n	3	3
Total IgE (U/ml), median (range)	125 (9-523)	11 (2-47)
% sensitized to Der p	73 (8/11)	0
Der p-IgE (U/ml), median (range)	3.0 (0.8-10.4)	Nd

Figure 1

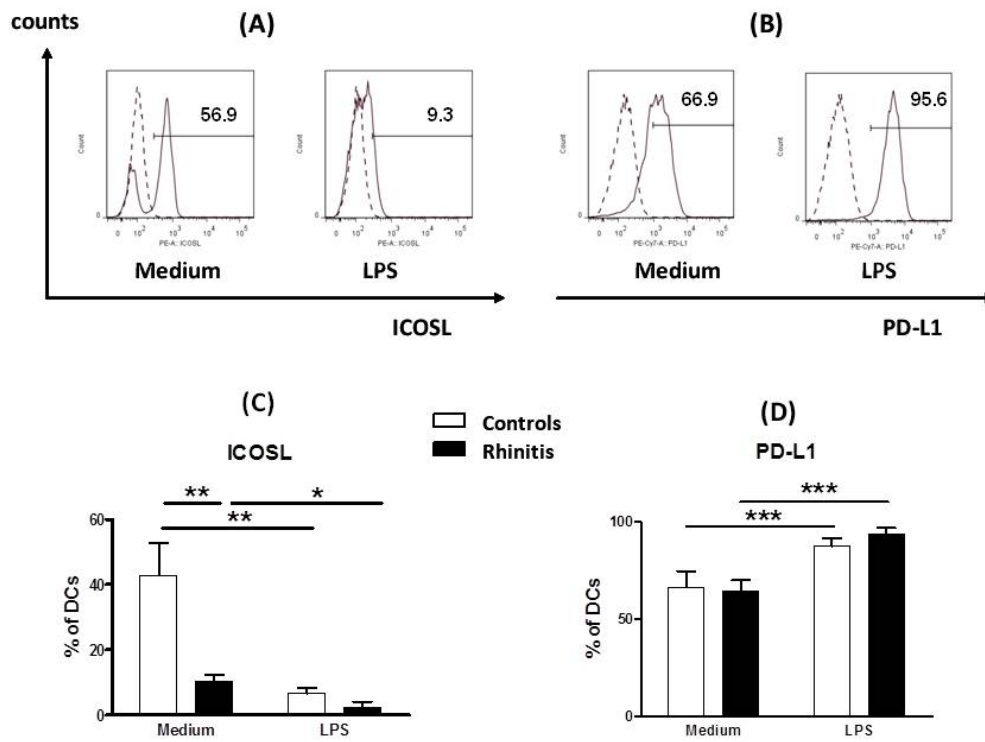


Figure 2

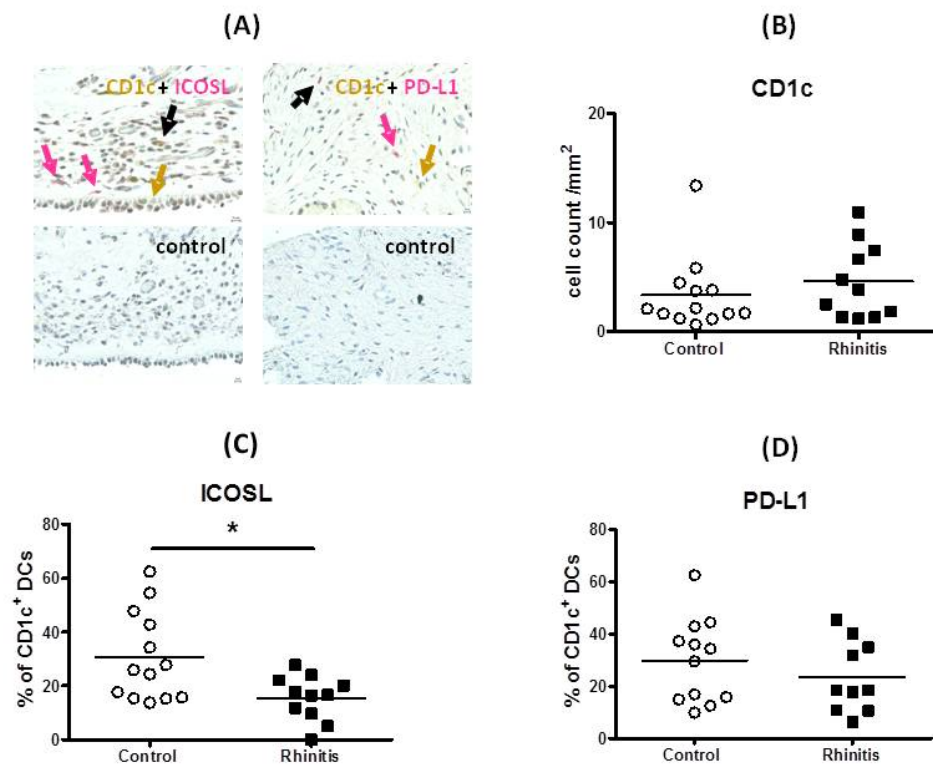


Figure 3

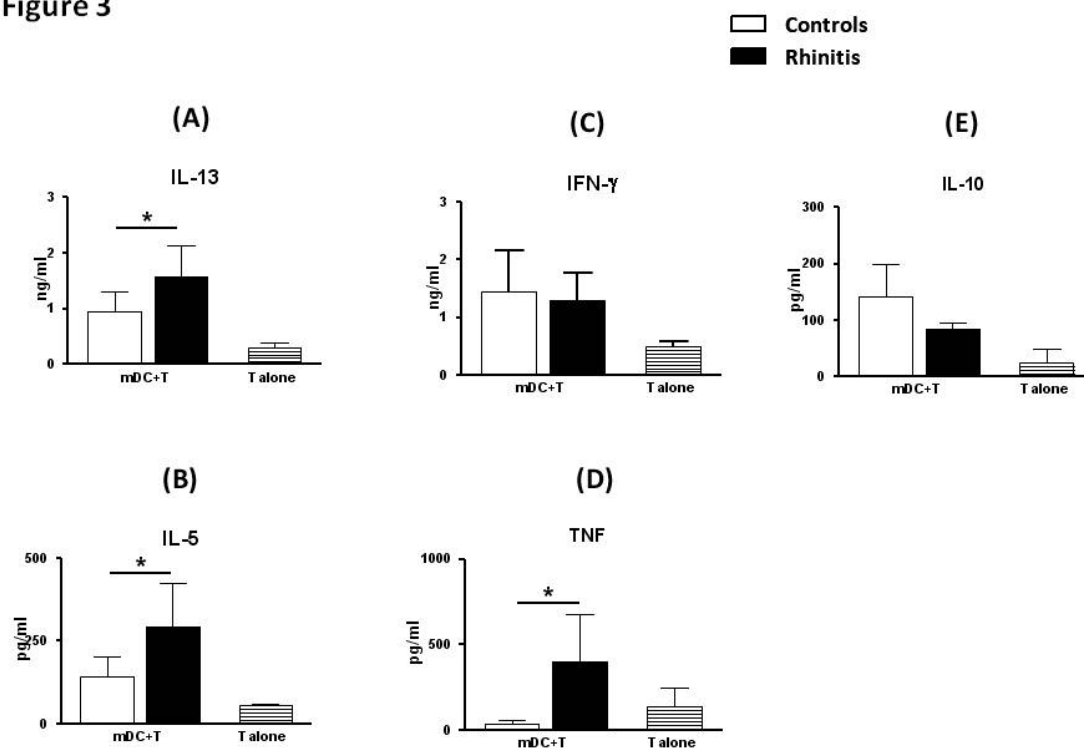


Figure 4

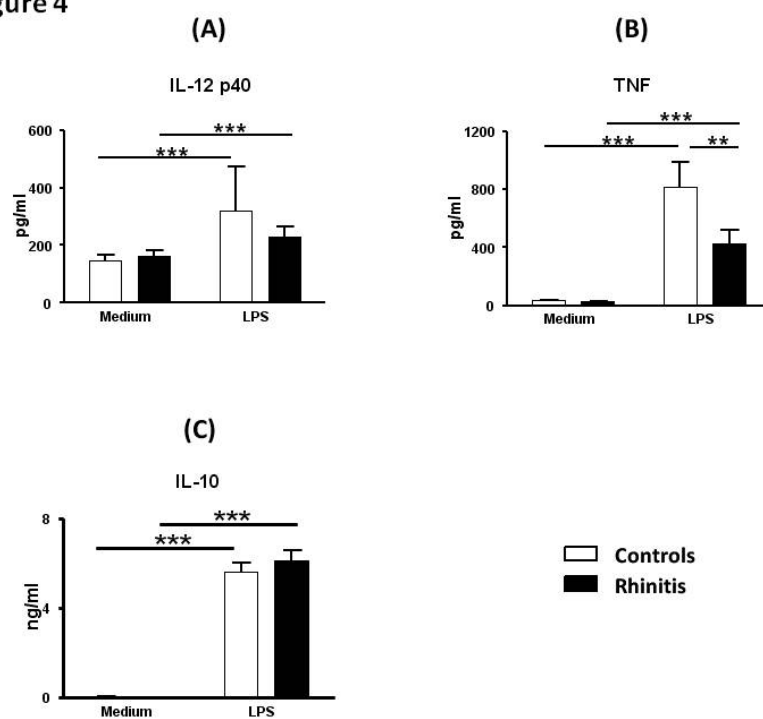


Figure 5

