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Dexamethasone/1α-25-dihydroxyvitamin D3-treated dendritic cells suppress colitis in the SCID T-cell transfer model

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Summary

Autoantigen-presenting immunomodulatory dendritic cells (DCs) that are used for adoptive transfer have been shown to be a promising therapy for a number of autoimmune diseases. We have previously demonstrated that enteroantigen-pulsed DCs treated with interleukin-10 (IL-10) can partly protect severe combined immunodeficient (SCID) mice adoptively transferred with $CD4^+$ $CD25^-$ T cells from the development of wasting disease and colitis. We therefore established an *in vitro* test that could predict the *in vivo* function of DCs and improve strategies for the preparation of immunomodulatory DCs in this model. Based on these *in vitro* findings, we here evaluate three methods for DC generation including short-term and long-term IL-10 exposure or DC exposure to dexamethasone in combination with vitamin D3 (Dex/D3). All DCs resulted in lower $CD4^+$ $CD25^-$ T-cell enteroantigen-specific responses *in vitro*, but Dex/D3 DCs had the most prominent effect on T-cell cytokine secretion. *In vivo*, Dex/D3 DCs most efficiently prevented weight loss and gut pathology upon $CD4^+$ $CD25^-$ T-cell transfer in SCID mice, although the effect on gut pathology was antigen independent. Our data in the SCID T-cell transfer model illustrate some correlation between *in vitro* and *in vivo* DC function and document that prevention of experimental inflammatory bowel disease by transfer of immunosuppressive DCs is possible.

Keywords: colitis; dendritic cells; dexamethasone; interleukin-10; severe combined immunodeficiency; tolerance

Introduction

The *in vitro* generation of immunomodulatory or tolerogenic dendritic cells (DCs) for therapeutic use is a major focus in autoimmunity research and transplantation immunology, and stimulation of immature DCs with interleukin-10 (IL-10), tumour necrosis factor-α or other immunomodulatory reagents[1–4] has proved successful in a number of murine disease models. These models include allotransplantation,[5–7] experimental autoimmune encephalomyelitis (EAE),[1,4] myasthenia gravis[3] and type 1 diabetes[8] and recently we have shown that short-term IL-10-treated DCs pulsed with enteroantigen can suppress the wasting disease and development of colitis in the severe combined immunodeficiency (SCID) T-cell transfer model.[9] In this model adoptively transferred $CD4^+$ T cells repopulate the spleen and the gut-associated lymphoid tissue and lead to the development of chronic colitis.[10] The transferred $CD4^+$ T cells have been shown to react in an antigen-specific, H-2-restricted way against enterobacterial antigens.[11] In this sense, the model resembles human inflammatory bowel disease (IBD).[12,13]

In our previous study,[9] we used bone-marrow-derived DCs (BM-DCs) that were exposed to a short 2-day IL-10 culture before being pulsed with enteroantigen. Injection of these DCs prevented both the weight loss observed in SCID mice upon $CD4^+$ $CD25^-$ T-cell transfer and, partly, the gut pathology. We aimed to improve strategies for

Abbreviations: APC, antigen-presenting cell; BM, bone marrow; BSA, bovine serum albumin; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; FCS, fetal calf serum; IBD, inflammatory bowel disease; IFN, interferon; IL, interleukin; i.p., intraperitoneally; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PE, phycoerythrin; SCID, severe combined immunodeficiency; SEM, standard error of the mean; TGF, transforming growth factor.

the generation of immunomodulatory DCs that would exert an improved effect in this model and to develop assays to predict *in vitro* the function of DCs *in vivo*. We established an *in vitro* assay that allowed for the screening of immunomodulatory DCs with an ability to reduce enteroantigen-specific $CD4^+$ $CD25^-$ T-cell responses and based on this, we aimed to compare DCs exposed to short-term IL-10 (s-IL-10 DC) with alternative protocols reported to be optimal for the generation of immunomodulatory DCs, in particular DCs exposed to long-term IL-10 (l-IL-10 DC)[2] or a combination of dexamethasone and vitamin D3 (Dex/D3 DC).[14] We compared phenotype, DC cytokine secretion, T-cell differentiation and cytokine secretion together with the *in vivo* effect in prophylactic experiments in the SCID T-cell transfer model of colitis. Based on these *in vitro* experiments, Dex/D3 DCs were shown to be potent candidates for the inhibition of enteroantigen responses. Here, we demonstrate that transfer of Dex/D3 DCs partly prevents weight loss in the SCID T-cell transfer model of colitis, whereas prevention of gut pathology is seen after transfer of Dex/D3 DCs unexposed to enteroantigen, demonstrating the need for further enteroantigen characterization to fully understand DC-mediated regulation in this model of IBD.

Materials and methods

Mice

Conventional 6- to 8-week-old female BALB/c mice and SCID mice were purchased from Taconic Europe (Ry, Denmark) and kept under controlled microbial conditions at the local animal facility of the Panum Institute. All animal experiments were performed in accordance with the permissions obtained from the Animal Ethical Committee, Copenhagen, The Department of Justice.

Generation of BM-DC

Dendritic cells were generated from BM cells derived from BALB/c mice. The BM cells obtained from femurs and tibias were washed and cultured overnight in six-well plates (TPP, Trasadingen, Schwitzerland) at 2×10^6 cells/ml in 3 ml culture medium/well. Culture medium (CM) was RPMI-1640 with Glutamax supplemented with 10% fetal calf serum (FCS; Harlan Sera-Lab Ltd, Hillcrest, UK) and antibiotics. The next day, non-adherent cells were harvested and resuspended in CM containing 10 ng/ml granulocyte–macrophage colony-stimulating factor plus 20 ng/ml IL-4 (both from Peprotech, Rocky Hill, NJ) and cultured at 1×10^6 cells/ml in 3 ml CM/well. Fresh cytokines and medium were added on day 3. For different subsets of DCs, 20 ng/ml IL-10 was added from day 1 or 40 ng/ml IL-10 (Peprotech) was added on day 6, 1 μM dexamethasone (Sigma-Aldrich, St Louis, MO) was added on day 6, 10 nM vitamin D3 (BIOMOL Research Labs, Butler Pike, PA) was added on day 7 as published previously[14] and 133 μg/ml enterobacterial extract on day 7 in different combinations. Cells were harvested on day 8 using phosphate-buffered saline (PBS)/trypsin/ethylenediaminetetraacetic acid solution (Gibco, Paisley, UK). Dendritic cells were typically 70% CD11c-positive, as published elsewhere.[9]

Induction of colitis

The $CD4^+$ T cells were isolated from mesenteric lymph nodes or spleens of BALB/c mice with anti-CD4 Dynabeads and Detach-a-Bead (Dynal, Oslo, Norway) according to the manufacturer's instructions. These cells were subsequently depleted of $CD25^+$ cells with phycoerythrin (PE)-conjugated anti-CD25 antibody (cat. no. 130-091-013) and Anti-PE microbeads (cat. no. 130-048-801) kit from Miltenyi Biotec (Bergisch Gladbach, Germany). The $CD4^+$ $CD25^-$ T cells were resuspended in PBS/1% bovine serum albumin (BSA) and transferred intraperitoneally (i.p.) to SCID mice, 3×10^5/mouse. Mice treated with DCs received 1×10^6 DCs i.p. 2–3 hr in advance. Mice were then monitored once or twice per week for weight loss, loose stools, bloody diarrhoea and rectal prolapse.

Immunohistochemistry

For histological examinations, the anal 2 cm of the gut were fixed in 4% paraformaldehyde. The samples were embedded in paraffin, cut by serial microtomy and stained with periodic acid Schiff/haematoxylin/eosin. The specimens were all examined in a blinded fashion by one pathologist. Histopathological changes were evaluated as described previously.[15] Score 0, no signs of inflammation; score 1, slight infiltrations in lamina propria by mononuclear cells (scores 0 to 1 are present in non-transplanted SCID mice); score 2, moderate mononuclear cell infiltration of lamina propria, increased mitotic activity in the epithelial layer; score 3, as in 2 but patchy mononuclear cell infiltration in the submucosal layer, elongation of the crypts with depletion of goblet cells; score 4, as in 3, but some crypt destruction and epithelial metaplasia; score 5, as in 4, but including increased crypt destruction and microabscesses, polymorphonuclear infiltration of epithelial layer and surrounding infiltration with mononuclear cells in the submucosal layer, epithelial ulcerative lesions.

Enterobacterial extract

The extract was prepared by removing the fecal content of colon and caecum into ice-cold PBS followed by sonication, centrifugation, filtration at 4° and protein concentration measurement as described previously.[11] The extract was kept at −80° until use.

Enteroantigen specific CD4$^+$ T-cell proliferation

For primary proliferation assays, titrated day 8 enteroantigen-pulsed or unpulsed DCs were cultured together with 1×10^5 CD4$^+$ CD25$^-$ T cells purified from mesenteric lymph nodes of BALB/c mice. The cells were cultured for 5 days including 18 hr incorporation of 0·5 μCi/well [^{3}H]thymidine (Amersham, Little Chalfont, UK) for the measurement of proliferation and supernatant was collected for analysis of secreted cytokines. For secondary proliferation assays, 1×10^5 total cells from primary cultures were harvested and cocultured with 1×10^5 enteroantigen-pulsed or unpulsed antigen-presenting cells (APCs; unfractionated spleen cells). These stimulator APCs are prepared from splenocytes (8×10^6 cells/well in 2 ml CM in 24-well plates pulsed overnight with 200 μg/ml enterobacterial extract, washed and irradiated) from BALB/c mice as previously described.[11] Unpulsed spleen cells from C57/BL6 mice served as the positive control in the secondary proliferation assay. The cells were cultured for 5 days, including 18 hr incorporation of 0·5 μCi/well [^{3}H]thymidine (Amersham) for the measurement of proliferation, and supernatant was collected from these secondary cultures for the analysis of cytokine secretion.

Measurement of cytokines

Murine interferon-γ (IFN-γ), IL-2, IL-4, IL-5, IL-10 and IL-12p40/p70, were analysed in supernatants of DCs and mixed lymphocyte reactions with a T helper type 1 (Th1)/Th2 cytokine 6-Plex and IL-17 with a singleplex for Luminex (Biosource Cat. No LMC0002 and LMC0171, respectively) according to the manufacturer's protocol. Measurement was carried out using the Luminex 100 IS (Luminexcorp, Austin, TX). More than 100 events were acquired per bead set. STARSTATION version 2·0 software (Applied Cytometry Systems, Sheffield, UK) was used for cytokine quantification analysis.

Expression of notch ligands measured with QuantiGene Plex

Levels of messenger RNA (mRNA) for Jagged 1, Jagged 2 and Delta 4 were compared with and expressed as % of mRNA of the housekeeping gene hypoxanthine guanine phosporibosyl transferase 1. Measurement was performed with a customized QuantiGene® Plex Reagent System (Panomics, Fremont, CA) from Panomics. In short, cell lysates were hybridized with mRNA target-specific probes conjugated to fluorescent microspheres. Amplification was then performed by hybridization with biotinylated label probes with the ability to bind streptavidin-conjugated R-PE, allowing determination of target bead and mRNA quantification simultaneously on a flow cytometer. Measurement was carried out using the Luminex 100 IS (Luminexcorp). Approximately 100 events were acquired per bead set. STARSTATION version 2·0 software (Applied Cytometry Systems) was used for cytokine quantification analysis.

Statistics

Significant differences between sample means were determined with the two-tailed Student's *t*-test for independent samples, and results were considered significant when $P < 0{\cdot}05$. A two-way analysis of variance (ANOVA) was used to determine the significant differences between weight curves of untreated and treated SCID mice. Significant differences between histopathology scores were determined with the Fisher's exact test.

Fluorescence-activated cell sorting analysis

The BM-DCs were harvested as described above and stained for 0·5 hr in PBS with 0·25% BSA, using allophycocyanin-, fluorescein isothiocyanate- or PE-coupled antibodies against CD11c, CD40, CD80, CD86, CD273 (PD-L1), CD274 (PD-L2) and I-A/E (all from BD Pharmingen, San Jose, CA). For the measurement of cytokine secretion, 4×10^5 cells/well were set up in round-bottom microculture plates at the concentration of 2×10^6/ml in complete CM together with 50 ng/ml phorbol 12-myristate 13-acetate and 750 ng/ml ionomycin to activate the cytokine-secreting T cells. Monensin was supplied and cells were incubated for 3 hr, incubated with Fc blocking antibodies, with antibodies against surface markers, fixated, permeabilized and stained with antibodies for intracellular cytokine staining, all according to the protocol of a BD Cytofix/Cytoperm Plus Fixation/Permeabilization kit (BD Bioscience cat. no. 554715). Antibodies against IFN-γ and IL-17 were used (all from BD Pharmingen). Subsequently, cells were washed once and cells were analysed on a FACSCalibur (BD) and data were analysed using BD CELLQUEST software. At least 10 000 cells were collected, using a live gate based on forward scatter/side scatter properties.

Results

In vitro reactivity of enteroantigen-specific CD4$^+$ CD25$^-$ T cells

We examined long-term and short-term exposure of DCs to IL-10 as well as Dex/D3 treatment in relation to proliferation of enteroantigen-specific CD4$^+$ CD25$^-$ T cells from normal mice *in vitro*. From these studies both l-IL-10 DCs and Dex/D3 DCs induced significantly lower responses of enteroantigen-specific CD4$^+$ T-cell proliferation [$P = 0{\cdot}004$ and $P < 0{\cdot}0001$ respectively (two-way ANOVA)] in particular at relevant DC numbers (7500 DC/well) whereas reduction observed with s-IL-10 DCs was

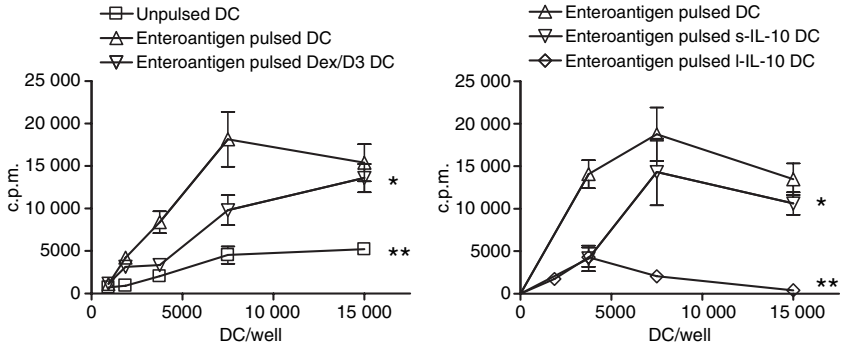

Figure 1. Dendritic cell (DC) -mediated suppression of enteroantigen-specific $CD4^+$ $CD25^-$ T cells *in vitro*; 1×10^5 $CD4^+$ $CD25^-$ from naïve BALB/c mice were cocultured with titrated number of unpulsed or enteroantigen-pulsed (133 μg/ml) bone-marrow-derived dendritic cells exposed to dexamethasone (1 μM) + vitamin D3 (10 nM) (Dex/D3 DC) or short-term interleukin-10 (40 ng/ml; sIL-10 DC) or long term IL-10 (20 ng/ml; l-IL-10 DC). Cultures were left for 5 days including 18 hr incorporation of [^{3}H]thymidine for the measurement of proliferation. (a) The effect of Dex/D3 DC; (b) the effect of s-IL-10 and l-IL-10 DCs. Data are shown as one selective experiment of three performed with similar findings. Curves that significantly different from curves for enteroantigen-pulsed DCs are indicated (*$P < 0{\cdot}05$, **$P < 0{\cdot}001$, two-way ANOVA).

less significant [$P = 0{\cdot}01$ (two-way ANOVA)] (Fig. 1). In accordance with these data, secretion of IL-4, IL-5, IL-10 and IFN-γ from DC–T-cell cultures was reduced when Dex/D3-treated or long-term IL-10-treated enteroantigen-exposed DCs were used as stimulator cells as compared with short-term IL-10-treated or untreated enteroantigen-exposed DCs (Fig. 2). In contrast, IL-12 was reduced in DC–T-cell cultures with enteroantigen-exposed DCs in response to pretreatment with Dex/D3 or IL-10 when compared with cultures using untreated enteroantigen-exposed DCs (Fig. 2). In addition, we tested the induction of Th17 cells in the primary DC–T-cell cocultures and observed a small enteroantigen-specific increase in the number of these cells from 5·8% to 8·4% (Fig. 3a), which was not reduced when enteroantigen-exposed Dex/D3-treated or IL-10-treated DCs were used as stimulator cells (data not shown). The antigen-specific IL-17 secretion was more pronounced when we measured IL-17 in secondary cultures where normal or treated enteroantigen-pulsed DCs were used as primary stimulators and cells from these cultures were re-exposed to enteroantigen-pulsed spleen cells in secondary cultures (Fig. 3b) where the number of enteroantigen-specific Th17 cells also increased from a background of 13–23%. Here, initial stimulation with enteroantigen-pulsed Dex/D3 DC resulted in lower IL-17 secretion in secondary cultures (Fig. 3b). In addition, secretion of IL-2, IL-4, IL-12 and IFN-γ was lower whereas IL-5 secretion was unchanged and IL-10 secretion was increased in similar secondary cultures. However, these changes were not antigen-specific and enteroantigen-specific proliferation was not suppressed in secondary cultures (data not shown).

DC phenotype

In accordance with previous results, untreated DCs had the phenotype of immature DCs with low expression of costimulatory molecules CD40, CD80 and CD86 as well as CD273, CD274 and major histocompatibility complex (MHC) class II. The expression of all these surface molecules was upregulated upon addition of enterobacterial extract (Fig. 4) as a consequence of DC maturation. The Dex/D3 DCs exhibited downregulation of CD40, CD86, CD273 and CD274 to levels comparable with or lower than unpulsed DCs whereas downregulation of MHC class II molecules was similar to the downregulation observed after long-term culture with IL-10 (i.e. l-IL-10 DC). Long-term culture with IL-10 resulted in downregulation of CD80, CD86 and CD273 and MHC class II to levels comparable with or lower than unpulsed DCs, whereas short-term culture with IL-10 (i.e. s-IL-10 DC) resulted in downregulation of CD80, CD86 and CD273 to levels comparable with or lower than unpulsed DCs (Fig. 4). Downregulation of CD40, CD86 and MHC class II was seen with both dexamethasone and vitamin D3 alone, whereas major downregulation of CD273 and CD274 was only seen with the two reagents in combination (data not shown).

In addition, we quantified mRNA levels for the Notch ligands Jagged 1, Jagged 2 and Delta 4 because the relative expression of these are important in determination of Th1 (Delta 4) or Th2 (Jagged 1) responses.[16,17] Only Jagged 1 expression was detectable (Fig. 5) and was upregulated after DC pulsing with enteroantigen. The Dex/D3 DC and both types of IL-10-exposed DCs did not upregulate Jagged 1 after enteroantigen pulsing.

DC secretion of IL-10 and IL-12

We next tested the levels of secreted of IL-10 and IL-12p40/p70 in DC supernatants. Dex/D3-treated DCs did not have an increased secretion of IL-10 compared with untreated DCs pulsed with enteroantigen (data not shown). However, Dex/D3-treated DCs and those DCs

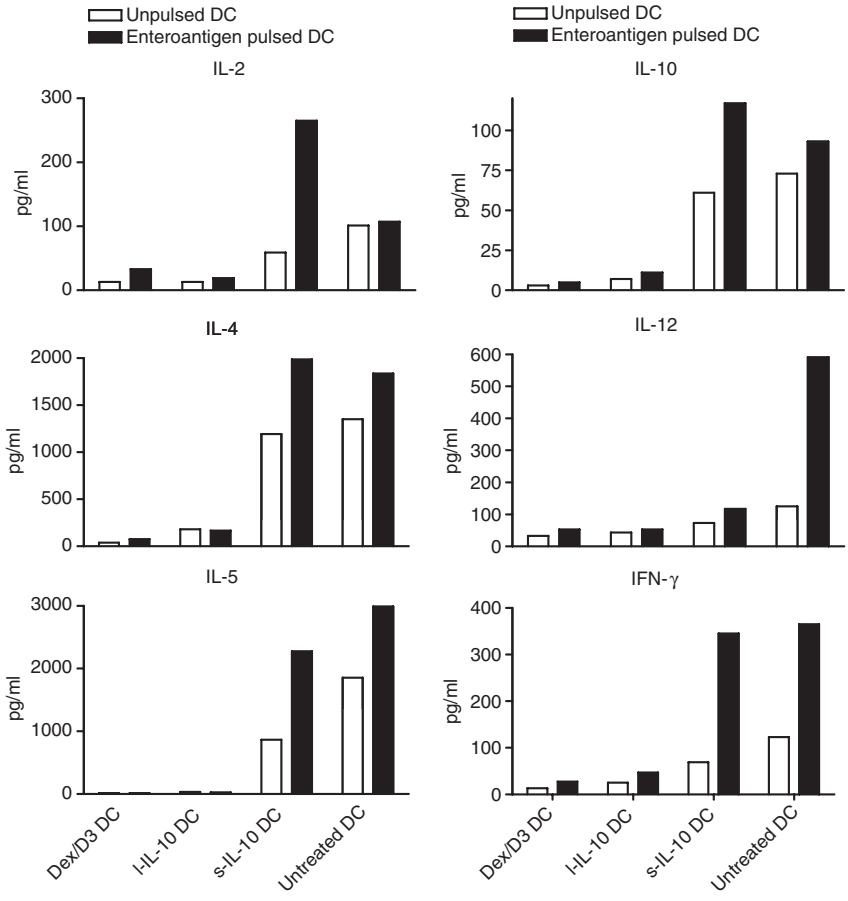

Figure 2. Dendritic cell (DC)-mediated suppression of cytokine secretion; 1×10^5 $CD4^+$ $CD25^-$ from naïve BALB/c mice were cocultured with 1×10^4 enteroantigen unpulsed or pulsed bone-marrow-derived DCs exposed to dexamethasone (1 μM) + vitamin D3 (10 nM) (Dex/D3 DC) or short-term interleukin-10 (40 ng/ml; sIL-10 DC) or long-term IL-10 (20 ng/ml; l-IL-10 DC). Cytokines were analysed from the supernatant after 5 days of DC–T-cell cultures. Data are shown as one selective experiment out of two performed with similar findings.

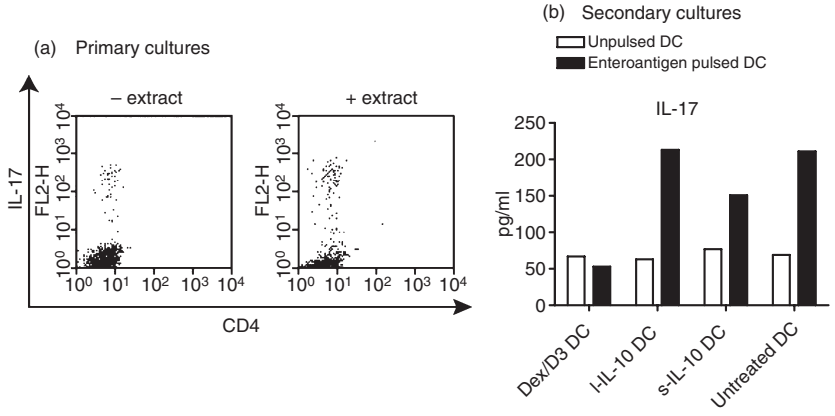

Figure 3. Induction of T helper type 17 (Th17) cells. (a) 1×10^5 $CD4^+$ $CD25^-$ from naïve BALB/c mice were cocultured with 1×10^4 enteroantigen-pulsed or unpulsed bone-marrow-derived dendritic cells and Th17 cells were analysed by flow cytometry after 5 days. (b) Alternatively, total cells from cocultures were re-exposed to enteroantigen-pulsed antigen-presenting cells (unfractionated spleen cells) and interleukin-17 (IL-17) secretion was analysed from supernatants from these secondary cultures. Data are shown as one selective experiment out of two performed with similar findings.

undergoing long-term and short-term treatments with IL-10 led to suppression of IL-12 as compared with untreated DCs pulsed with enteroantigen (Fig. 6). The suppressive effect of Dex/D3-treated DCs was exclusively caused by dexamethasone (data not shown).

Enteroantigen-pulsed Dex/D3 DC ameliorate colitis development

Adoptive transfer of $CD4^+$ $CD25^-$ cells into SCID mice leads to a chronic colitis with weight loss, loose stools,

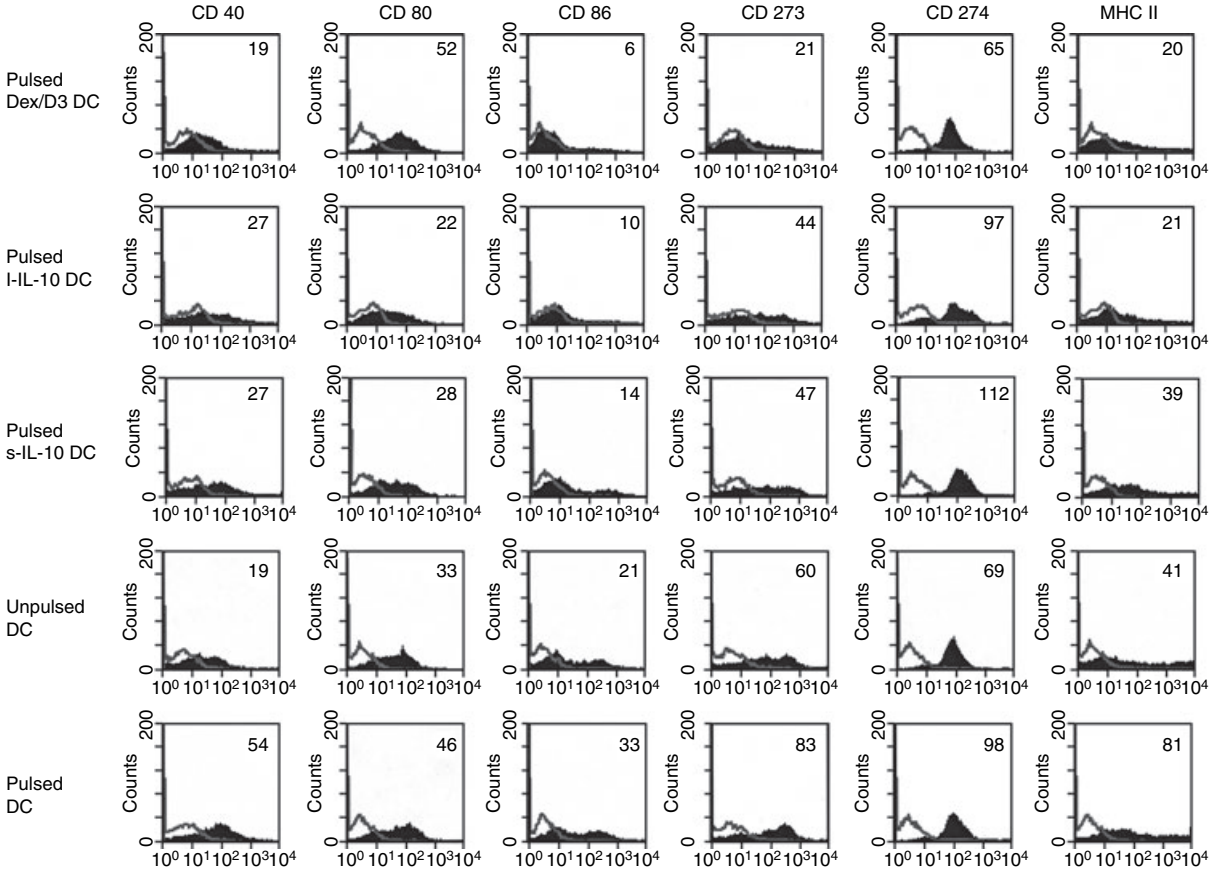

Figure 4. Dendritic cell (DC) phenotype. Bone-marrow-derived DCs (BM-DCs) were stimulated as indicated, harvested on day 8 and analysed by flow cytometry for expression of various surface molecules. Enteroantigen-unpulsed or pulsed BM-DCs and enteroantigen-pulsed Dex/D3 DC, short-term IL-10 DCs (s-IL-10 DC) and long-term IL-10 DCs (l-IL-10 DC) stained for CD40, CD80, CD86, CD273, CD274 and MHC class II molecules (filled curves) or isotype control antibodies (open line) (1×10^6 cells pr. sample) are shown. DCs are gated as large granular cells. Numbers indicate mean fluorescence intensity (MFI) values. Data are shown as one selective experiment of two performed with similar findings.

bloody diarrhoea and rectal prolapse.[10,11,18] We have previously shown that enteroantigen-pulsed s-IL-10 DCs injected i.p. into SCID mice 2–3 hr before adoptive transfer of $CD4^+$ $CD25^-$ T cells can prevent weight loss.[9] We here tested the effect of enteroantigen-pulsed Dex/D3 DCs and demonstrate that these DCs are also able to reduce the development of weight loss in colitic mice ($P < 0{\cdot}0001$, two-way ANOVA) whereas l-IL-10 DC were not effective (Fig. 7). The Dex/D3 DCs without enteroantigen exposure were without effect. Also, the observed effect was primarily mediated by dexamethasone (data not shown) and was dependent on DC generation in FCS. Finally, therapeutic experiments where DCs were injected after the establishment of colitis were generally unsuccessful (data not shown).

Histopathology

Figure 8 shows the colitis score after adoptive transfer of $CD4^+$ $CD25^-$ T cells with or without injection of enteroantigen-pulsed Dex/D3 DCs or l-IL-10 DCs. However, no significant reduction in histology score was observed in contrast to pretreatment with s-IL-10 DC, which was previously shown to induce a minor reduction.[9] However, suppression of the immunopathology was possible with Dex/D3 DCs in the absence of enteroantigen exposures [mean score 1·5, $P = 0{\cdot}04$ (Fisher's exact test)] (Fig. 8) which were superior to other combinations tested (data not shown).

T cells *in vivo*

Finally, we analysed the change in proinflammatory Th1 and Th17 and regulatory T cells *in vivo* in relation to DC treatment. Mesenteric lymph nodes from colitic animals contained a mixture of IFN-γ-producing Th1 and Th17 in mesenteric lymph nodes (Fig. 9d). Also, regulatory T cells identified as $CD4^+$ $CD25^+$ $Foxp3^+$ T cells were identified (Fig. 9a–c), and a similar distribution was observed in the spleen (data not shown). However, we did not

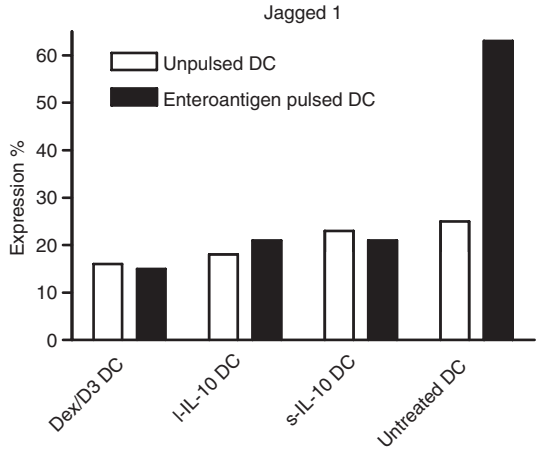

Figure 5. Dendritic cell (DC) expression of Jagged 1. Bone marrow-derived DCs (BM-DCs) were unpulsed or enteroantigen-pulsed exposed to dexamethasone (1 μM) + vitamin D3 (10 nM) (Dex/D3 DC) or short-term interleukin-10 (40 ng/ml; sIL-10 DC) or long-term IL-10 (20 ng/mll l-IL-10 DC) as indicated. At day 8, cell lysates were made from 4×10^5 cells/sample and messenger RNA was analysed with the QuantiGene Plex Reagent System. Relative expression as compared to expression of the housekeeping gene hypoxanthine guanine phosporibosyl transferase 1 is shown. Data are shown as one selective experiment out of two performed with similar findings.

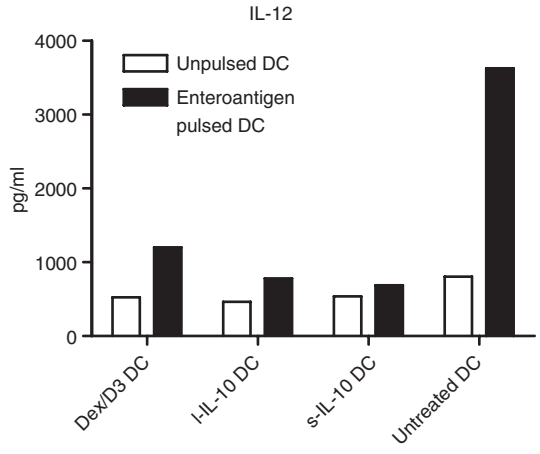

Figure 6. Dendritic cell (DC) -mediated interleukin-12 (IL-12) p40/p70 secretion. Bone marrow-derived DCs (BM-DCs) were stimulated as indicated and supernatants (from approximately 1×10^6 cells/sample) were harvested on day 8 for analysis of cytokine secretion. Unpulsed or enteroantigen-pulsed BM-DCs exposed to dexamethasone (1 μM) + vitamin D3 (10 nM) (DEX/D3 DC) or short-term IL-10 (40 ng/ml) (sIL-10 DC) or long-term IL-10 (20 ng/ml) (l-IL-10 DC) are indicated. Data are shown as one selective experiment out of two performed with similar findings.

observe any significant changes in levels of Th1, Th17 or $CD4^+$ $CD25^+$ $Foxp3^+$ T cells after treatment with immunomodulatory DCs (data not shown).

Discussion

DCs can be manipulated to induce either effector T-cell responses or T-cell anergy and regulatory T-cell activity depending on the maturation level.[1,5–7,19,20] Recently, immunomodulatory DCs have been generated that in allotransplantation settings and several murine models of autoimmune diseases have been shown to reduce graft-rejection or to ameliorate chronic inflammation.[1,3–8,19,20] Previously, we demonstrated for the first time that this therapy can also be used to modify the disease severity of experimental IBD.[9] Based on the SCID T-cell transfer model of colitis, we demonstrated that s-IL-10 DCs pulsed with enterobacterial extract were able to prevent the weight loss observed in SCID mice after $CD4^+$ $CD25^-$ T-cell transfer, and ameliorate the immunopathology of the gut wall.

Interleukin-10 pretreatment has been shown to induce immunosuppressive DCs, which can inhibit $CD4^+$ Th1, $CD4^+$ Th2 and $CD8^+$ T-cell responses in an antigen-specific manner, but different protocols for IL-10 exposure exist and can be separated into protocols that use either long-term or short-term exposure.[19–21] Also, immunosuppressive DCs have been established with the use of dexamethasone, vitamin D3 or both.[14,22,23] Here, based on the identification of DCs with the ability to induce low enteroantigen-specific $CD4^+$ $CD25^-$ T-cell responses *in vitro*, we compared s-IL-10 DCs, l-IL-10 DCs and Dex/D3 DCs in terms of immunomodulatory effect on colitogenic T-cell responses *in vitro* and on *in vivo* development of IBD in the SCID T-cell transfer model of colitis.

All three methods tested seemed to suppress DC secretion of proinflammatory cytokines and to inhibit CD40, CD86 and MHC class II expression upon exposure to enteroantigen. We also tested PD-L1 and PD-L2 expression of DCs. PD-1 ligation on T cells by PD-L1 or PD-L2 induces an inhibitory signal mediated by the immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM) and has shown to play a critical role in immunological tolerance.[24–27] Consequently, the downregulation of these surface molecules was unexpected, but not necessarily contradictive for immunosuppression of T cells which is likely to be a result of further modulation of additional costimulatory molecules, inhibitory molecules and cytokines. In addition we analysed the mRNA expression of Notch ligands Jagged 1, Jagged 2 and Delta 4. Here only Jagged 1 was expressed upon exposure to enteroantigen. Since Jagged 1 expression in contrast to Delta 4 expression has been shown to be involved in the induction of Th2 cells this is in conflict with the cytokine profile observed, demonstrating that additional DC–T-cell interactions other than the balance between Jagged 1 and Delta 4 determine the final outcome of Th1 and Th2 responses.[16,17,28]

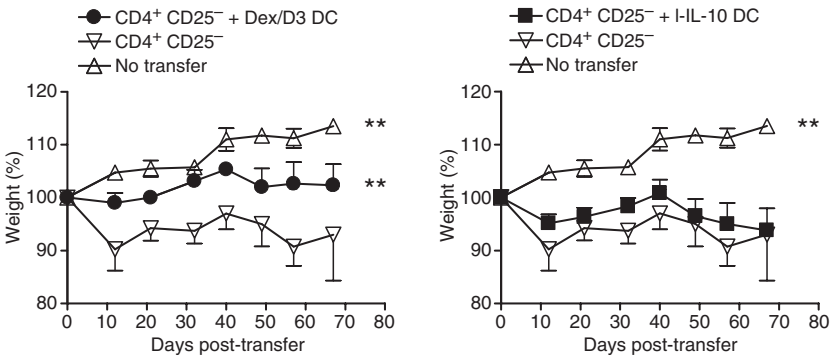

Figure 7. Enteroantigen-pulsed dexamethasone/vitamin D3 (Dex/D3) dendritic cells (DCs) ameliorate weight loss in severe combined immunodeficiency (SCID) mice adoptively transferred with $CD4^+$ $CD25^-$ T cells. Enteroantigen-pulsed BM-DCs were exposed to dexamethasone (1 μM) + vitamin D3 (10 nM) (Dex/D3 DC) or long-term IL-10 (20 ng/ml; l-IL-10 DC) as indicated, harvested on day 8 and injected intraperitoneally into SCID mice (1×10^6/mouse) before $CD4^+$ $CD25^-$ T-cell transfer (3×10^5/mouse). Mouse weight was registered regularly, and mice were killed around day 70. Data represent mean ± SEM of mice in each treatment group ($n = 6$). Curves that are significantly different from curves derived from SCID mice without transfer are indicated (**$P < 0{\cdot}001$, two-way ANOVA).

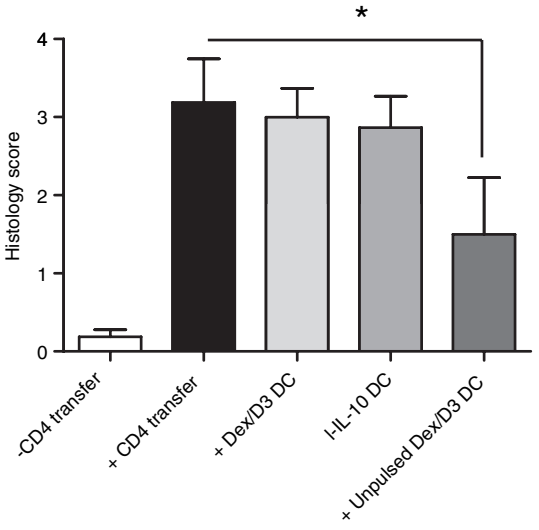

Figure 8. Histology score in severe combined immunodeficiency (SCID) mice. Enteroantigen-pulsed or unpulsed bone marrow-derived dendritic cells (BM-DCs) were exposed to dexamethasone (1 μM) + vitamin D3 (10 nM) (Dex/D3 DC) or short-term IL-10 (40 ng/ml; sIL-10 DC) or long-term IL-10 (20 ng/ml; ;l-IL-10 DC) as indicated, harvested on day 8 and injected intraperitoneally into SCID mice (1×10^6/mouse) before $CD4^+$ $CD25^-$ T-cell transfer (3×10^5/mouse). When mice were killed around day 70, the distal 2 cm of the gut was stained with haematoxylin & eosin and histopathology scoring was performed. Data represents mean ± SEM of mice in each treatment group ($n = 6$ to $n = 12$). (*$P < 0{\cdot}05$, Fischer's exact test).

The *in vitro* DC effect on $CD4^+$ CD25 T cells primarily correlated with reduction of clinical symptoms such as weight loss *in vivo*, which is an early symptom in colitic mice and which is related to the large cytokine production induced when mice are reconstituted with T cells. Long-term IL-10 culture or exposure to Dex/D3 was, compared with short-term IL-10 culture, in general more efficient in terms of *in vitro* reduction of $CD4^+$ $CD25^-$ T-cell responses against enteroantigen where lower Th1- and Th2-related cytokines were observed in primary cultures. We did not see DC-dependent changes in induction of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells which generally reached a level of 2–4% during culture (data not shown). However, although all three types showed potent *in vitro* reduction of T-cell responses, only s-IL-10 DCs and Dex/D3 DCs had an effect on weight loss *in vivo* whereas, l-IL-10 DCs had no effect. Also, no correlation was observed in terms of gut pathology, and the *in vitro* predictions must therefore be interpreted with caution.

The minor effect on the *in vivo* development of colitis, could be linked to the lack of true tolerance in responding T cells. Indeed, no suppression of the proliferative response was observed when cells from primary cultures were set up in secondary cultures against enteroantigen-pulsed spleen cells (data not shown). However, in these secondary cultures we did observe an enteroantigen-specific suppression of IL-2, IL-4, IL-12 and IFN-γ secretion whereas IL-5 secretion was unchanged and IL-10 secretion was increased when cells from primary cultures were cultured together with enteroantigen-pulsed APCs as compared with unpulsed APCs. However, this pattern was independent of the DC type used for primary stimulation (data not shown).

An alternative explanation for the minor effects *in vivo* could be that Th17 cells were only partly affected. The Th17 cells are a unique subset of $CD4^+$ T cells driven by the transcription factor RORγt after induction by the cytokines TGF-β and IL-6 or IL-21 and they are maintained by IL-23.[29–38] They are known to be important for a number of experimental autoimmune diseases such as EAE and collagen-induced arthritis. We here demonstrate that these cells are differentiated *in vitro* and react with

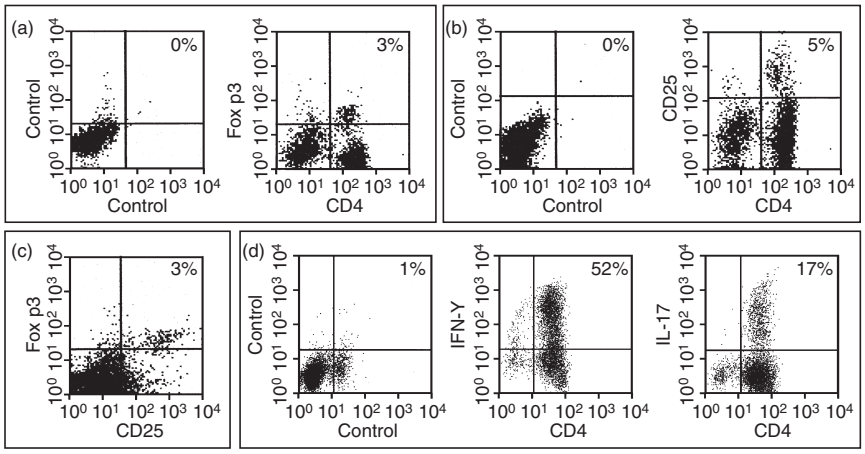

Figure 9. T-cell profile in colitic mice. Severe combined immunodeficiency (SCID) mice were injected intraperitoneally with 3×10^5 $CD4^+$ $CD25^-$ T cells. When mice were killed around day 70, mesenteric lymph nodes were harvested and analysed for the presence of $CD4^+$ $Foxp3^+$ cells (a), $CD4^+$ $CD25^+$ cells (b), $CD25^+$ $Foxp3^+$ cells (c), and interferon-γ (IFN-γ) or interleukin-17 (IL-17) -secreting $CD4^+$ T cells (d). Data are presented as one selective experiment out of two experiments performed with similar findings. Pools of lymphoid cells from four animals were used.

enteroantigen specificity after repeated stimulations (in particular when enteroantigen-pulsed DCs are used as primary stimulators followed by restimulation with enteroantigen-pulsed spleen cells) and could therefore be involved in the immunopathology induced against enteroantigens seen in experimental and clinical IBD. Interestingly, only Dex/D3 DCs induced full suppression of IL-17 secretion in secondary cultures, which might reflect the effect on clinical symptoms *in vivo* of these DCs as compared with the other DCs tested. Also, we demonstrate that they are induced *in vivo* after $CD4^+$ $CD25^-$ transfer into SCID mice, but in contrast to *in vitro* experiments, treatment with immunosuppressive DCs neither changed the number of Th17 nor the number of Th1 cells or regulatory T cells (Treg cells) present in relevant lymphoid organs *in vivo*. Also, immunomodulatory enteroantigen-pulsed DCs were unable to suppress *in vitro* the Th17 cells from mice with established colitis (data not shown). However, although Th17 cells might still be important for driving inflammation, the exact role of IL-17 cells in pathogenesis seems to be less important for the induction of experimental colitis[39,40] as compared with EAE or collagen-induced arthritis, since transfer of IL-$17a^{-/-}$ or co-treatment with neutralizing antibodies does not prevent colitis induction, but controversy on this still remains.

In the SCID transfer model of colitis, no single well-defined antigen has been discovered except for *Helicobacter* species, which in certain models have been associated with the immune pathology of colitis.[41,42] In the present study we therefore used an enterobacterial extract. Exposure to this extract clearly delivers a maturation signal to DCs, as shown by upregulation of CD80, CD86 as well as CD40, and MHC class II molecules, and the increased secretion of IL-1α, IL-6, IL-12 p40/70 and tumour necrosis factor-α.[9] It is likely that DCs exposed to our enterobacterial extract are more unstable as immunosuppressive cells *in vivo* and may be converted to proinflammatory DCs after injection i.p., which explains the lack of effect on gut pathology and Th17 and Treg T-cell numbers induced *in vivo*. Indeed, we did observe an effect of Dex/D3 DCs when these were not pulsed with enteroantigen extract. In contrast, a clinical effect was seen on weight loss observed in SCID mice after T-cell transfer when enteroantigen-pulsed Dex/D3 DCs were used. Therefore, the effect on weight loss is probably more sensitive to even small and temporary changes in T-cell function compared with gut pathology. However, the reduction in weight loss was dependent on DC generation in FCS which is known to induce a $CD4^+$ T-cell-dependent Th2 response to FCS-derived antigens including BSA and thereby enhances the systemic production of IL-4, IL-5 and IL-10 as an adjuvant effect.[21,43–45]

In conclusion, our data show that DCs pretreated with dexamethasone and vitamin D3 inhibit enteroantigen-specific T-cell differentiation, prevent the weight loss and ameliorate gut pathology observed in SCID mice after $CD4^+$ $CD25^-$ T-cell transfer with improved capacity compared with both long-term and short-term IL-10-treated DCs. However, in particular the effect on gut pathology was shown to be antigen-independent. If antigen-specific tolerance is to be induced, which would make DC-based therapy superior to conventional immunosuppressive treatment, the use of such immunomodulatory DCs in experimental and clinical IBD awaits the inclusion of appropriate enteroantigens without unspecific immunostimulatory functions which indeed seem to abolish the inhibitory function of the Dex/D3 DCs used in this study.

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