

Dendritic cells retrovirally overexpressing IL-12 induce strong Th1 responses to inhaled antigen in the lung but fail to revert established Th2 sensitization

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Abstract: It has been postulated that low-level interleukin (IL)-12 production of antigen-presenting cells is associated with the risk of developing atopic asthma. To study the relationship between IL-12 production capacity of dendritic cells (DCs) and development of T helper type 2 (Th2) responses in the lung, we genetically engineered DCs to constitutively overexpress bioactive IL-12. Retrovirally mediated overexpression of IL-12 in DCs strongly polarized naïve ovalbumin (OVA)-specific CD4⁺ T cells toward Th1 effector cells *in vitro*. After intratracheal injection, OVA-pulsed IL-12-overexpressing DCs failed to induce Th2 responses *in vivo* and no longer primed mice for Th2-dependent eosinophilic airway inflammation upon OVA aerosol challenge, readily observed in mice immunized with sham-transfected, OVA-pulsed DCs. Analysis of a panel of cytokines and chemokines in the lung demonstrated that the lack of Th2 sensitization was accompanied by increased production of the Th1 cytokine interferon- γ (IFN- γ), chemokines induced by IFN- γ , and the immunoregulatory cytokine IL-10. When Th2 priming was induced using OVA/alum prior to intratracheal DC administration, DCs constitutively expressing IL-12 were no longer capable of preventing eosinophilic airway inflammation and even enhanced it. These data show directly that high-level expression of IL-12 in DCs prevents the development of Th2 sensitization. Enhancing IL-12 production in DCs should be seen as a primary prevention strategy for atopic disorders. Enhancing IL-12 production in DCs is less likely to be of benefit in already Th2-sensitized individuals. *J. Leukoc. Biol.* 76: 1028–1038; 2004.

Key Words: gene therapy · inflammation

INTRODUCTION

Asthma is an inflammatory disease of the airways leading to significant morbidity and mortality. With advances in the understanding of the molecular and cellular mechanisms involved in the asthmatic response, researchers have identified specific

mediators that may be targeted to control the inflammatory state of asthma. The T helper cell type 2 (Th2) hypothesis proposes that the inflammation in asthma arises from an imbalance between the two CD4⁺ T lymphocyte subsets, Th1 and Th2. Th2 cells release many cytokines that have been shown to regulate the inflammatory response, and it has been suggested that the Th1 cytokines counteract this response [1]. As Th2 cells are the main orchestrators of allergic airway inflammation, elucidating how these cells arise from their precursors is an area of intense research [2]. Airway dendritic cells (DCs) play a crucial role in this process, as they pick up allergen in the lung and transport it to the draining lymph nodes (LN), where they prime naïve CD4⁺ T cells to differentiate into Th1, Th2, or regulatory T (Treg) cells [3–9]. The precise mechanisms by which DCs determine Th polarization in the lung are currently unknown. It was suggested that the lung tissue microenvironment is Th2-prone, although the exact explanation for this observation is unclear [10–12]. Several factors besides tissue environment influence Th cell differentiation through effects on DCs, in particular, the type and dose of antigen and the presence of polarizing microbial motifs acting through pattern recognition receptors [13–16]. Integration of these different stimuli in DCs leads to the expression of major histocompatibility complex (MHC)-peptide and costimulatory molecules and to the release of cytokines and chemokines, resulting in a polarization signal for naïve CD4⁺ T cells [2, 13]. A key cytokine produced by DCs involved in Th polarization is interleukin (IL)-12, which polarizes naïve CD4 T cells toward the Th1 end of the spectrum [17, 18]. It has been suggested that lung DCs, in a resting state, produce little bioactive IL-12p70, which might explain the Th2 prone state of the lung [5, 12].

Based on several observations, a reduced capacity of DCs to provide pro-Th1 signals, such as IL-12, has been implicated in the development of Th2 responses to inhaled allergens. First, DCs from atopic individuals and their immediate blood monocyte precursors produce less IL-12p70 compared with nonatopic controls [19, 20]. Second, sensitization to inhaled aller-

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Received June 8, 2004; revised July 8, 2004; accepted July 19, 2004; doi: 10.1189/jlb.0604325.

gens occurs predominantly before the age of 12, when the capacity to produce IL-12 is severely reduced compared with adults, potentially explaining the Th2 bias at a young age [21, 22]. In children at risk for allergic diseases, low IL-12 production by circulating DCs in the neonatal period is associated with stronger Th2 responses to inhaled and food allergens [23]. Third, polymorphisms in the IL-12B gene promoter, leading to lower levels of IL-12 production, are associated with an enhanced severity of atopic asthma in children [24]. Finally, certain diseases characterized by high-level production of IL-12, such as multiple sclerosis, are associated with a reduced risk of developing allergic diseases [25, 26].

Together, these papers suggest that low IL-12 levels in DCs are associated with the risk of developing Th2 immunity to allergens, whereas high levels of IL-12 production seem to offer protection from allergic diseases. In this paper, we have addressed this issue directly by retrovirally overexpressing IL-12 p35 and p40 in DCs, which were subsequently used to sensitize mice to inhaled antigens. For this purpose, we used a previously established model of eosinophilic airway inflammation that uses ex vivo-generated bone marrow (BM) DCs injected in the airways to prime naïve CD4⁺ T cells [4, 27]. Overexpression of IL-12 in DCs (IL12-DC) strongly reduced Th2 sensitization to inhaled antigen and abolished subsequent eosinophilic airway inflammation by skewing the response toward strong Th1 immunity. However, DCs overexpressing IL-12 were not capable of preventing eosinophilic airway inflammation in animals sensitized prior to IL12-DC instillation and even enhanced eosinophilic airway inflammation. These data show directly that high-level expression of IL-12 in DCs can prevent the development of Th2 sensitization and therefore, should be seen as a primary prevention strategy for atopic disorders. Enhancing IL-12 production in DCs is less likely to be beneficial in already sensitized individuals.

MATERIALS AND METHODS

Mice

Female BALB/c mice (6–10 weeks old) were purchased from Harlan (Horst, The Netherlands). Ovalbumin (OVA)_{323–339}-specific, MHCII-restricted, T cell receptor (TCR)-transgenic (DO11.10) mice [28] were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in microisolators under specified, pathogen-free conditions, and experiments were performed under approval of the Erasmus MC (Rotterdam, The Netherlands) committee for animal ethics.

Retroviral vectors and generation of retroviral particles

The retroviral construct secreting bioactive IL-12p70 (pMFG-IL-12) was generated by cloning the murine IL-12 subunits p35 and p40 into the Moloney murine leukemia virus-derived backbone MFG [29]. The p35 gene was cloned by polymerase chain reaction (PCR) from pcDNA1Amp-p35 (a kind gift from Dr. Thomas Gajewski) with the forward primer 5'-cccatgggtcaatcacgtacctcc-3' and the reverse primer 5'-cccctcaggcgagctcagatagccc-3'. This PCR product was cloned in pCR2.1 and completely sequenced. The p35 fragment was digested with *Nco*I and *Eco*RV and cloned into *Nco*I-*Sma*I-restricted pBlue-internal ribosome entry site (IRES; obtained by transfer of the *Eco*RI-*Bam*HI IRES fragment). The *Hind*III-*Xho*I p40 fragment from pcDNA1Amp-p40 (also a gift from Dr. T. Gajewski) and the *Xho*I-*Bam*HI IRES-p35 fragment were cloned in a three-fragment ligation in pEE14 *Hind*III-*Bam*HI. The entire

IL-12 (p40-IRES-p35) gene was excised with *Bam*HI and cloned in pMFG (a gift from Dr. Olivier Danos). A retroviral vector not expressing a gene was used to control for virus-specific effects (pMFG-S). Retroviral particles were produced by transient transfection as described [30], with the minor modification that the medium consisted of DC culture medium (DC-CM) RPMI 1640 containing glutamax-I (Invitrogen, Carlsbad, CA), supplemented with 5% (v/v) fetal calf serum (Biocell, Rancho Dominguez, CA), 50 μ M β -mercaptoethanol (Sigma Chemical Co., St. Louis, MO), and 50 μ g/ml gentamycin (Invitrogen).

Generation and retroviral transduction of BM-derived DCs

BM-derived DCs were generated as described [30] with some minor modifications. After red blood cell lysis, BM cells were incubated for 30 min with a panel of monoclonal antibodies (Ab) consisting of anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD45R (RA3-6B2), anti-Ly6-G (RB6.8C5), and anti-I-A^{b,d,q}/I-E^{d,k} (M5/114) to deplete lineage-positive cells. All the Ab used were purchased from BD Biosciences (San Diego, CA), except anti-I-A^{b,d,q}/I-E^{d,k}, which was produced in-house. Ab-labeled cells were incubated with sheep anti-rat Ab-coated magnetic beads (Dyna, Oslo, Norway) at a cell-to-bead ratio of 1:4. Lineage-negative cells were plated in 24-well culture plates (10⁶ cells/ml; 1 ml/well) in DC-CM supplemented with 20 ng/ml recombinant mouse granulocyte macrophage-colony stimulating factor (GM-CSF; produced in-house) and 10 ng/ml recombinant human fetal liver tyrosine kinase 3 ligand (Flt3-L; a generous gift of Dr. Charlie Maliszewski, Amgen, Seattle, WA). After overnight incubation, the cells were replated in 24-well culture plates (5 $\times$ 10⁵ cells/well in 1 ml). On days 2, 3, and 4, the medium was removed and replaced with 1 ml viral transfection supernatant containing 8 μ g/ml polybrene (Sigma Chemical Co.). DCs transduced with MFG-IL-12 containing supernatant are hereafter designated IL12-DC; MFG-S-transduced DCs are hereafter named control-DC. The cells were transduced during centrifugation of the 24-well plates for 2 h at 2500 rpm at 32°C, after which the supernatant was replaced with DC-CM supplemented with 10 ng/ml recombinant human Flt3-L and 20 ng/ml recombinant mouse GM-CSF. At days 7 and 9, the medium was refreshed with DC-CM supplemented with 20 ng/ml recombinant mouse GM-CSF. At day 10, cells were pulsed with OVA protein (100 μ g/ml). This batch of OVA contained low levels of lipopolysaccharide (LPS; 29 endotoxin units per mg; Worthington Biochemical Corp., Lakewood, NJ) and did not induce IL-12 production in purified BM-DCs (data not shown). After 24 h, DCs were harvested by gentle pipetting and washed three times with phosphate-buffered saline (PBS). Expression and secretion of IL-12p70 were confirmed with a commercially available enzyme-linked immunosorbent assay (BD Biosciences).

Effect of IL-12 overexpression in DCs on T cell polarization in vitro and in vivo

Spleen and LN cells were obtained from DO11.10 mice, and untouched CD4⁺ T cells were isolated by negative depletion with a commercially available panel of biotin-conjugated antibodies, followed by labeling with anti-biotin magnetic cell sorter beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The resulting population was typically >95% CD4⁺. Cells were labeled with 5,6-carboxy-fluorescein diacetate succinimidyl ester (CFSE), as described previously [8, 27]. Cells (5 $\times$ 10⁵) were cultured with OVA-pulsed MFG-IL-12 or control virus-transduced DCs, starting at a DC-to-T cell ratio of 1:10 up to 1:160 in 48-well plates. After 96 h, cells and supernatant were harvested, and OVA-specific T cell proliferation and cytokine levels were determined.

To examine the primary immune response in vivo, 10⁷ CFSE-labeled, transgenic CD4⁺ T cells specific for OVA_{323–339} were adoptively transferred intravenously into naive BALB/c mice on day -2. On day 0, mice (n=8–10 per group) were intratracheally (i.t.) immunized with 10⁶ OVA-pulsed IL12-DC or OVA-pulsed control-DC. On day 4, mice were killed, and mediastinal LN (MLN) and axillary LN (ALN) were collected separately. Single-cell suspensions of LN were prepared by mechanical disruption and analyzed by flow cytometry or were restimulated (2 $\times$ 10⁶ cells per ml) with 10 μ g/ml OVA for 96 h, after which supernatants were harvested and assayed for IL-4, IL-5, IL-10, IL-13, and interferon- γ (IFN- γ) content.

Effect of IL-12 overexpression in DCs on the potential to induce asthma

Groups of mice ($n=4-10$ per group) were immunized i.t. on day 0 with 1×10^6 IL12-DC or control-DC, pulsed overnight with OVA. From day 10 onward, mice were exposed to OVA aerosols [1% (w/v) in PBS generated through jet nebulizers] for 3–4 consecutive days, 30 min daily, as previously mentioned [8]. Twenty-four hours after the last exposure, mice were killed, and bronchoalveolar lavage (BAL) was performed as described [31]. In one experiment analyzing gene expression levels of cytokines, chemokines, and chemokine receptors, lungs were excised, snap-frozen in liquid nitrogen, and stored at -80°C until processing for RNA analysis.

In a second experiment, the effect of IL-12 overexpressing DCs on the development of asthma in already sensitized mice was studied. Therefore, groups of mice ($n=5-10$ per group) were first immunized intraperitoneally (i.p.) with 10 μg OVA emulsified in 1 mg aluminum hydroxide (Sigma Chemical Co.), a protocol previously shown to induce strong Th2 priming for OVA [31]. Ten days later, control-DC and IL12-DC, pulsed or not with OVA, were i.t.-injected. At days 20, 21, and 22, mice were challenged with OVA aerosol as described above, and after 24 h, the degree of airway inflammation was analyzed.

Airway histology

In some experiments, after BAL lungs were slowly inflated with a 1:1 (v/v) mixture of PBS and optical cutting temperature compound, excised, snap-frozen in liquid nitrogen, and stored at -80°C until further processing. 7 μm sections were cut, subsequently stained with hematoxylin and periodic acid-Schiff reagent (PAS; Sigma Chemical Co.), and photographed with a Leica DM-LB microscope (Leica Microsystems, Rijswijk, The Netherlands).

Flow cytometry

To reduce nonspecific Ab binding, anti-CD16/CD32 (2.4G2, American Type Culture Collection, Manassas, VA) was included in all cell-surface stainings. To study T cell priming in vitro or the primary immune response in adoptive transfer experiments, T cells were labeled with CFSE and with the anticolonotypic DO11.10 TCR Ab KJ1-26 [28]. For the in vivo experiments, anti-CD4 (RM4-5) was also included. Dead cells were excluded by labeling with TO-PRO-3 (Molecular Probes, Leiden, The Netherlands) or propidium iodide (PI) prior to acquisition. Cell divisions were quantified as described [8]. Briefly, the CFSE dataset was fitted with algorithms provided by the analysis program FlowJo (Treestar, San Carlos, CA), resulting in two parameters that describe the proliferation. The proliferation index (PRI) has been defined as the average number of divisions of the cell fraction that divided and the responder frequency, as the percentage of input cells that responded to stimulation by dividing.

Anti-CC chemokine receptor 3-phycoerythrin (CCR3-PE) was used to detect eosinophils in the lung [32], together with anti-CD8-PECy5 (53-6.7) and

anti-CD4-APC to determine the cellular composition in BAL. All fluorochrome-conjugated antibodies were purchased from BD Biosciences, except anti-CCR3-PE, which was from R&D Systems (Minneapolis, MN), and anti-clonotypic-TCR-PE (KJ1-26), which was from Caltag Laboratories (Burlingame, CA). Events were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software.

Cytokine measurements

Levels of IL-12p70, IL-4, IL-5, IL-10, and IFN- γ in culture supernatants or BAL were measured using OptEIA kits (BD Biosciences) according to the manufacturer's instructions. IL-13 levels were measured using a commercially available kit from R&D Systems.

Real-time quantitative reverse transcriptase (RT)-PCR

Frozen lung tissue was homogenized, RNA-isolated with RNeasy midi-prep columns (Qiagen, Hilden, Germany), and treated on-column with DNase I, according to the manufacturer's protocol. RNA (1 μg) was reverse-transcribed using SuperscriptIII (Invitrogen) and random hexamers (Amersham Biosciences, Rosendaal, The Netherlands) for 120 min at 42°C . Primer sequences are listed in **Table 1**. PCR conditions were 2 min at 50°C and 10 min at 95°C , followed by 40 cycles of 15 s at 95°C and 60°C for 1 min using an ABI PRISM 7900 HT (Applied Biosystems, Foster City, CA) and SYBR Green mastermix (Stratagene, La Jolla, CA). Water controls were included to ensure specificity, and primer pairs were evaluated for integrity by analysis of the amplification plot, dissociation curves, and efficiency of PCR amplification. PCR amplification of the housekeeping gene ubiquitin C, was performed during each run for each sample to allow normalization between samples.

Statistical analysis

Reported values are expressed as mean $\pm$ SEM, unless indicated otherwise. Statistical analyses were performed with SPSS (SPSS Inc., Chicago, IL) using a Mann-Whitney U-test. *P* values less than 0.05 were considered significant.

RESULTS

Genetically engineered DCs expressing high levels of IL-12 polarize naïve CD4⁺ T cells toward Th1 in vitro

Following transduction of DCs with a retroviral vector encoding for IL-12p70 (IL12-DC), we detected significantly higher levels of IL-12 p70 in the supernatant at day 11 (median 183 ng/ml,

TABLE 1. Primer sequences used for real-time quantitative RT-PCR

Gene	Forward primer	Reverse primer	Amplicon (bp)
Ubiquitin C ^a	AGGTCAAACAGGAAGACAGACGTA	TCACACCCAAGAACAAGCACAA	80
IL-4 ^b	CTCATGGAGCTGCAGAGACTCTT	CATTTCATGGTGCAGCTTATCGA	70
IL-5 ^b	CTCACCGAGCTCTGTTGACAAAG	CCATATGCATAGCTGGTGATTTTAT	79
IL-6 ^b	ACACATGTTCTCTGGAATTCGT	AAGTGCATCATCGTTGTTATACA	84
IL-10 ^a	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT	191
IL-13 ^b	TGACCAACATCTCCAATTGCA	TTGTTATAAAGTGGGCTACTTTCGATTT	132
IFN- γ ^a	TCAAGTGGCATAGATGTGGAAGAA	TGGCTCTGCAGGATTTTCATG	92
CCL2 ^a	CTTCTGGGCTGCTGTTCA	CCAGCCTACTCATTTGGGATCA	127
CCL7 ^b	GGGAAGCTGTTATCTTCAAGACAAA	CTCCTCGACCACTTCTGATG	74
CCL11 ^b	CCAGGCTCATCCCAACTT	TGGTGATTTCTTTGTAGCTCTTCAGT	88
CCL17 ^b	GGATGCCATCGTGTCTTCTGA	GCCTTCTTCACATGTTTGTCTTTG	75
CXCL5 ^c	TCACACTATCGGAAATTGTGATACTTTA	AATGCAATAGTCACCCCTCAGTTCA	96
CXCL9 ^b	TGCACGATGCTCCTGCA	AGGTCTTTGAGGGATTTGTAGTGG	63
CXCL10 ^b	GACGGTCCGCTGCAACTG	GCTTCCCTATGGCCCTCATT	65

References: ^a [33]; ^b [34]; ^c Stephen Manning, Millenium Pharmaceuticals (Cambridge, MA). CCL2, CC chemokine ligand 2; CXCL5, CXC chemokine ligand 5.

range 73–919 ng/ml, $n=8$ transductions) compared with DCs transduced with the control constructs (control-DC; median 0 ng/ml, range 0–2.2 ng/ml, $n=8$ transductions). Phenotypic analysis at day 11 by fluorescence-activated cell sorter (FACS) revealed that the cells exhibited cell-surface expression of markers characteristic for DCs (CD11c, MHCII, CD40, CD80, CD86; data not shown), which has been shown by us and others previously [30, 35]. It should be noted that no differences could be observed in the levels of maturation markers between control and IL-12-transduced DCs as determined by FACS analysis (data not shown and ref. [30]).

As DC-derived IL-12 is a potent inducer of Th1 responses in vitro as well as in vivo [18], we first established the T cell-polarizing capacity of IL12-DC in vitro. OVA-specific, CFSE-labeled CD4⁺ T cells were cultured with OVA-pulsed IL12-DC or control-DC at various ratios of DC and T cells. After 4 days, the proliferation of OVA-specific T cells and the levels of cytokines in the culture supernatants were assayed. There was a major difference in cytokine secretion pattern, with increased levels of the Th1 prototype cytokine IFN- γ as well as IL-10 when IL12-DCs were used as APC at a high DC-T cell ratio, and priming with control-DC resulted in higher levels of the Th2-associated cytokines IL-4, IL-5, and IL-13 compared with IL12-DC (**Fig. 1A**). As cytokine production was not quantified on the single-cell level but on the whole population, and it has been reported that cytokine production may be dependent on the number of T cell divisions [36], we also verified whether the observed differences in cytokine production were attributable to differences in division profiles of divided T cells. The division profiles of T cells stimulated with IL12-DC or control-DCs were identical, except for the highest DC-to-T cell ratio, when IL12-DC-driven T cell proliferation was slightly lower compared with the control-DC group (Fig. 1B). Thus, the observed differences in cytokine expression patterns are likely to be caused by differences in polarization of primed T cells and not in differences in total CD4⁺ T cell number or division history. Moreover, the efficient proliferation of naïve CD4⁺ T cell demonstrated that retrovirally transduced DCs are functional APC.

Genetically engineered DCs expressing high levels of IL-12 migrate efficiently to lung-draining LN but do not strongly polarize naïve CD4⁺ T cells in vivo

Next, we investigated the polarizing capacity of IL12-DC in the draining LN of the lung. OVA-specific T cells were adoptively transferred into syngeneic recipient mice, which were subsequently immunized with OVA-pulsed IL12-DC or control-DC. Four days later, the MLN and ALN were removed, lymphocytes cultured ex vivo, and cytokines in the supernatant determined (**Fig. 2A**). In contrast to the in vitro studies, the Th2-type cytokines IL-4 and IL-5 were similar when IL12-DCs were used as APC, only the IL-13 levels were statistically, significantly decreased compared with control-DC. High levels of IFN- γ were produced in mice immunized with control-DC and IL12-DC. Also, IL-10 levels were decreased in the IL12-DC-immunized group, contradictorily to the in vitro studies.

To rule out the possibility that the priming of T cell was weaker or altered as a result of decreased migration or in-

creased cell death of i.t.-injected DCs, we injected mice with a cohort of CFSE-labeled, OVA-TCR CD4⁺ T cells, which we subsequently immunized with OVA-pulsed IL12-DC or control-DC. Four days later, MLN and ALN were dissected, and the division profile of OVA-specific T cells was analyzed. The division profile in the MLN was identical in control-DC and IL12-DC-immunized animals (Fig. 2B, upper panels). This was confirmed by quantification of the cell proliferation, which revealed a slight increase in the average number of divisions of divided T cells (PRI) and the responder frequency, defined as the cell population that participated in clonal expansion (**Table 2**). In the ALN representing the nondraining LN, some OVA-specific T cells that underwent multiple divisions (>5) were present (Fig. 2B, lower panels). As no cells that had divided one to two times were present at this nondraining site, these cells represent recirculating effector cells, as previously shown [27]. Thus, these data show that IL-12-transduced DCs have a similar, stimulatory capacity in vivo compared with control-DC and induce a T cell response characterized by a decreased IL-13 production.

Genetically engineered DCs expressing high levels of IL-12 fail to induce eosinophilic airway inflammation

We have previously shown that OVA-pulsed BM-DCs injected into the trachea of naïve BALB/c mice induce sensitization to inhaled OVA and to Th2-dependent, eosinophilic airway inflammation upon OVA aerosol challenge [4]. This model was therefore well-suited to study the effect of IL-12 overexpression on the potential of these cells to induce Th2 sensitization. As a measure of airway inflammation, we measured the total number of BAL fluid cells 24 h after a series of OVA aerosol challenges and observed that cellularity was lower when mice were immunized with OVA-pulsed IL12-DC compared with control-DCs (**Fig. 3A**). Unpulsed DCs did not induce airway inflammation, as previously reported (data not shown and ref. [4]). Further characterization revealed that the remaining cell population in the IL12-DC-immunized group was almost devoid of eosinophils, in contrast to control-DC-immunized mice. However, both groups contained identical frequencies of T lymphocytes, well above the level seen in mice immunized with unpulsed DCs (3–5%; ref. [4]; Fig. 3B). The levels of the Th2-associated cytokines IL-5 and IL-13 in the BAL fluid were also significantly decreased in the IL12-DC group compared with the control-DC group (Fig. 3C). As reported, histological examination of the lungs demonstrated large peribronchial infiltrates rich in eosinophils, as well as PAS-positive goblet cell hyperplasia in the OVA-pulsed, control-DC-immunized group [4]. Animals immunized with IL12-DC, however, showed only small, peribronchial infiltrates devoid of eosinophils and almost no mucus production (Fig. 3D), which is consistent with the BAL fluid data.

Altogether, these data demonstrated that immunization with IL-12-producing DCs did not lead to eosinophilic airway inflammation, although the presence of peribronchial infiltrates in the lung and increased numbers of T cells in the BAL fluid indicated that mild inflammation was present. To further characterize the type of inflammation induced, we measured an array of cytokines and chemokines, which have been shown to

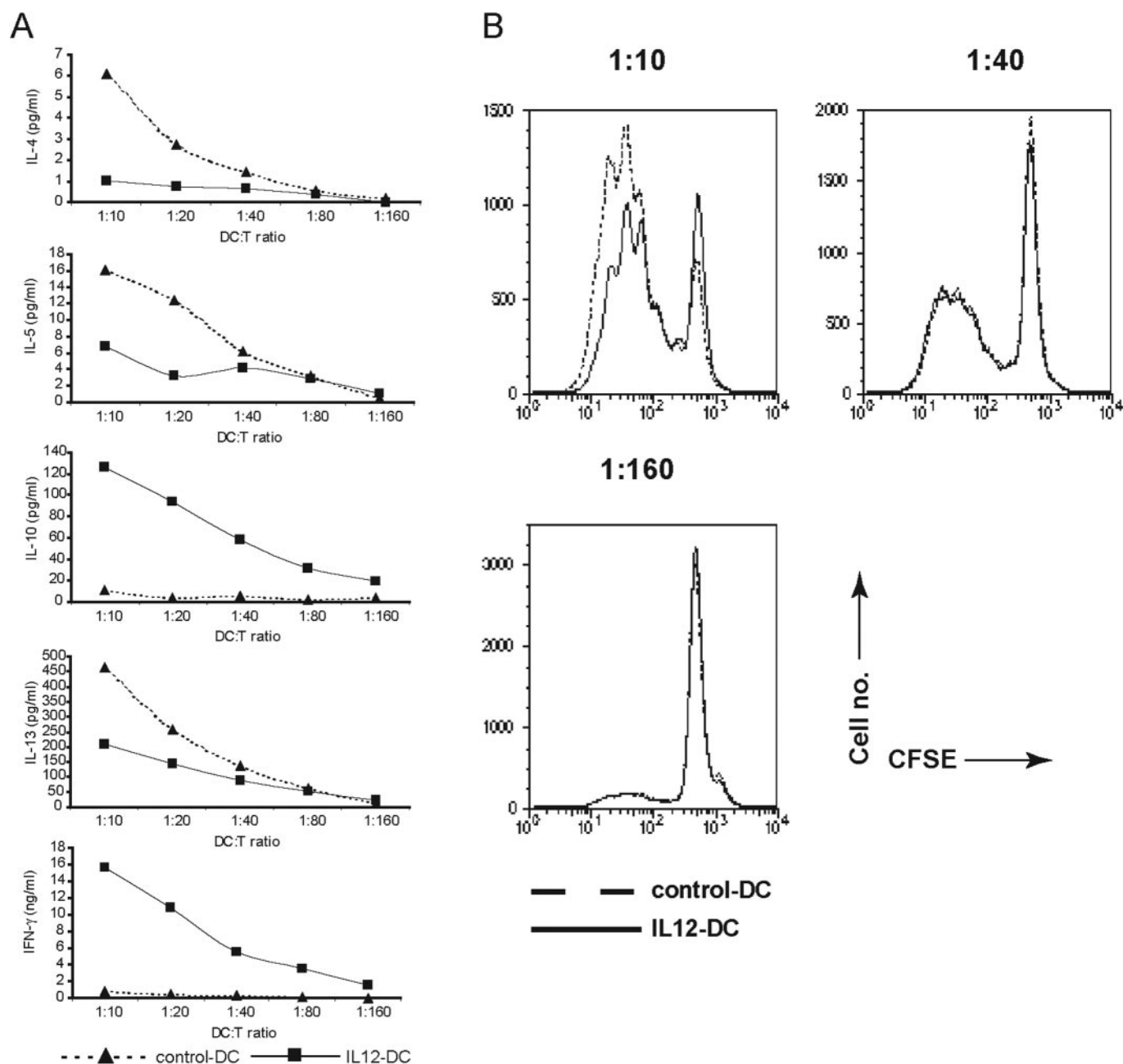


Fig. 1. IL-12-producing DCs have equal stimulatory capacity but skew naïve CD4⁺ T cells toward Th1 in vitro. BM-derived DCs were retrovirally transduced with a vector encoding IL-12p70 (IL12-DC) or a control construct (control-DC) and pulsed with OVA (100 μ g/ml). After 24 h, cells were harvested, washed, and put into culture with purified, CFSE-labeled, OVA-specific (DO11.10) CD4⁺ T cells (5×10^5) at various ratios. Four days later, cells and supernatant were harvested, and the division profile of CFSE-labeled cells as well as cytokine levels in the supernatant were determined. (A) Levels of cytokines in the supernatant at various DC-to-T cell ratios. Results are representative of two independent experiments. Note the different scale of the graph depicting IFN- γ levels. (B) Division profile of living (TOPRO-3⁺), OVA-specific (KJ1-26⁺) CD4⁺ cells stimulated with control-DC (dashed line) or IL-12-DC (solid line). The various ratios of DC to T cells are indicated above each histogram. Of note, there was no cell division when unpulsed DCs were used as APC (data not shown).

be highly informative of the type of induced Th response in the lung [34]. Mice were immunized with OVA-pulsed IL12-DC or control-DC and subsequently challenged via the airways. As a control, for the effect of OVA exposure per se, mice that did not receive DCs were challenged as well. Twenty-four hours after the final aerosolization, lungs were harvested, snap-frozen, and processed for RNA. Gene expression levels were determined with real-time, quantitative RT-PCR. Compared with control-DC and IL12-DC-immunized animals, almost no cytokine

and chemokine expression was observed in the lungs of mice that were solely challenged with OVA aerosol (**Fig. 4A**, no DC group). The cytokine expression pattern observed in the lung when animals were immunized with IL12-DC consisted of a Th1-like response, with significantly elevated levels of IFN- γ mRNA and a trend toward a lower expression of the Th2-type cytokines IL-5 and IL-13 (Fig. 4A). Of note is the concurrent, increased expression of IL-10 in IL12-DC-immunized mice, also observed during in vitro CD4⁺ T cell priming by these

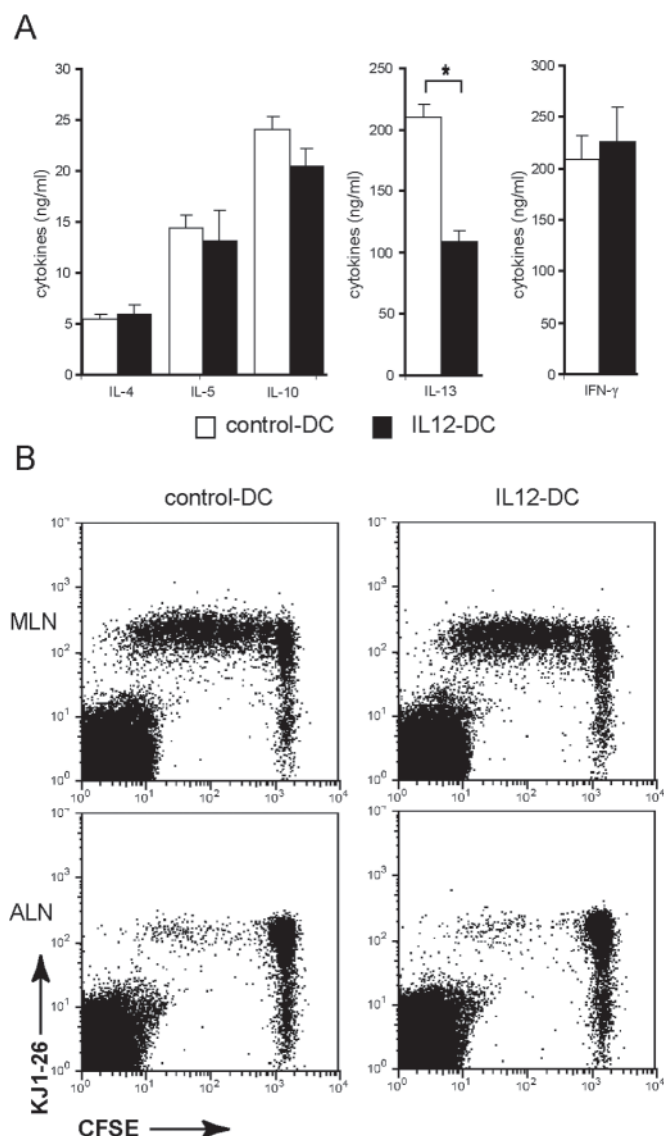


Fig. 2. IL12-DC have equal stimulatory capacity but a distinct Th cell differentiation capacity in vivo. Naïve BALB/c mice received a cohort of DO11.10 T cells and were subsequently immunized with OVA-pulsed IL12-DC ($n=8-10$) or OVA-pulsed control-DC ($n=8-9$). Four days later, MLN and ALN were resected and analyzed for the presence of OVA-specific CFSE⁺ CD4⁺ T cells directly or stimulated with OVA in vitro for 4 days. (A) Cytokine levels after in vitro restimulation with OVA. No cytokines could be detected in cultures of ALN (data not shown). Results are expressed as mean $\pm$ SEM from seven to eight mice per group. *, $P < 0.05$. (B) Cell division profile of OVA-transgenic T cells (KJ1-26⁺, PI⁻, CD4⁺) in the MLN and ALN was assessed by flow cytometry. Dot plots shown are from representative mice of each group. Results are representative for one (cytokines) or two (division profile) independent experiments.

DCs (Fig. 1A). The chemokine expression pattern in the lung of these mice correlated highly with the Th1-type cytokine expression pattern, as the IFN- γ -inducible chemokines IFN-inducible protein 10 (IP-10)/CXCL10 and monokine induced by IFN- γ (MIG)/CXCL9 were strongly increased compared with control-DC-immunized mice. Also, the levels of monocyte chemoattractant protein-1 (MCP-1)/CCL2 mRNA were increased. However, gene expression of the Th2-associated chemokines eotaxin/CCL3, thymus and activation-regulated che-

mokine (TARC)/CCL17, and LPS-induced chemokine (LIX)/CCL5 was similar or slightly decreased when compared with control-DC-immunized mice (Fig. 4B). Taken together, it can be concluded that the overall gene expression pattern of cytokines and chemokines of IL-12-DC-immunized mice suggested a Th1-type of response in the lung, which is in agreement with the cellular composition and cytokine levels in the BAL fluid (Fig. 3, B and C).

Genetically engineered DCs expressing IL-12 fail to revert Th2 sensitization and exacerbate Th2-dependent lung inflammation in sensitized mice

In view of the potential of Th1 cells to suppress the development of Th2 responses, it has been suggested that Th1 cells might be of potential therapeutic benefit for Th2-mediated diseases. Therefore, we next investigated if IL12-DC could suppress or revert a developing Th2 response (secondary prevention). Mice were first sensitized to OVA in the Th2-adjuvant alum and subsequently treated with OVA-pulsed IL12-DC or OVA-pulsed control-DC 10 days later. Another 10 days later, mice were challenged with three OVA aerosols. As a control, mice received unpulsed IL12-DC, control-DC, or no DCs. As shown in **Figure 5**, administration of unpulsed IL12-DC or control-DC after priming did not affect eosinophilic airway inflammation and Th2 cytokine in the BAL fluid. Exposure of mice to OVA-pulsed DCs, irrespective of their IL-12 production capacity, led to severely enhanced eosinophilic airway inflammation, with enhanced total cell recovery, increase in the frequency of eosinophils (Fig. 5A), and increase of IL-5 and IL-13 in the BAL fluid (Fig. 5B). Thus, IL12-DCs are not capable of suppressing a developing Th2-dependent airway response but rather enhance it when presenting OVA to previously sensitized mice.

DISCUSSION

In this paper, we have directly addressed whether high-level expression and secretion of IL-12 in DCs are sufficient to reduce sensitization to inhaled antigens. For this, we first overexpressed the IL-12 p35 and p40 subunit using a retroviral vector that minimally affected the expression of costimulatory molecules on DCs and studied the effect on Th differentiation in vitro and in vivo by injecting DCs into the Th2-prone lung compartment [10]. In vitro, we observed a profound polarization of the cytokine production of naïve CD4⁺ T cells toward a Th1 phenotype when IL-12-producing DCs were used as APC.

TABLE 2. Quantification of OVA-specific CD4⁺ T cell proliferation in the MLN

<i>n</i>	Proliferation index (PRI) ^a	Responder frequency ^b
IL12-DC	2.34 $\pm$ 0.05 ^c	24.4 $\pm$ 3.5
Control-DC	2.19 $\pm$ 0.04	19.9 $\pm$ 2.5

^a Defined as the average number of divisions of the CD4⁺, KJ1-26⁺, PI⁻ cell population that divided. ^b Defined as the percentage of input CD4⁺, KJ1-26⁺, PI⁻ cells that responded to stimulation by dividing. ^c Values represent the mean $\pm$ SEM of the data ($n=8$ animals per group).

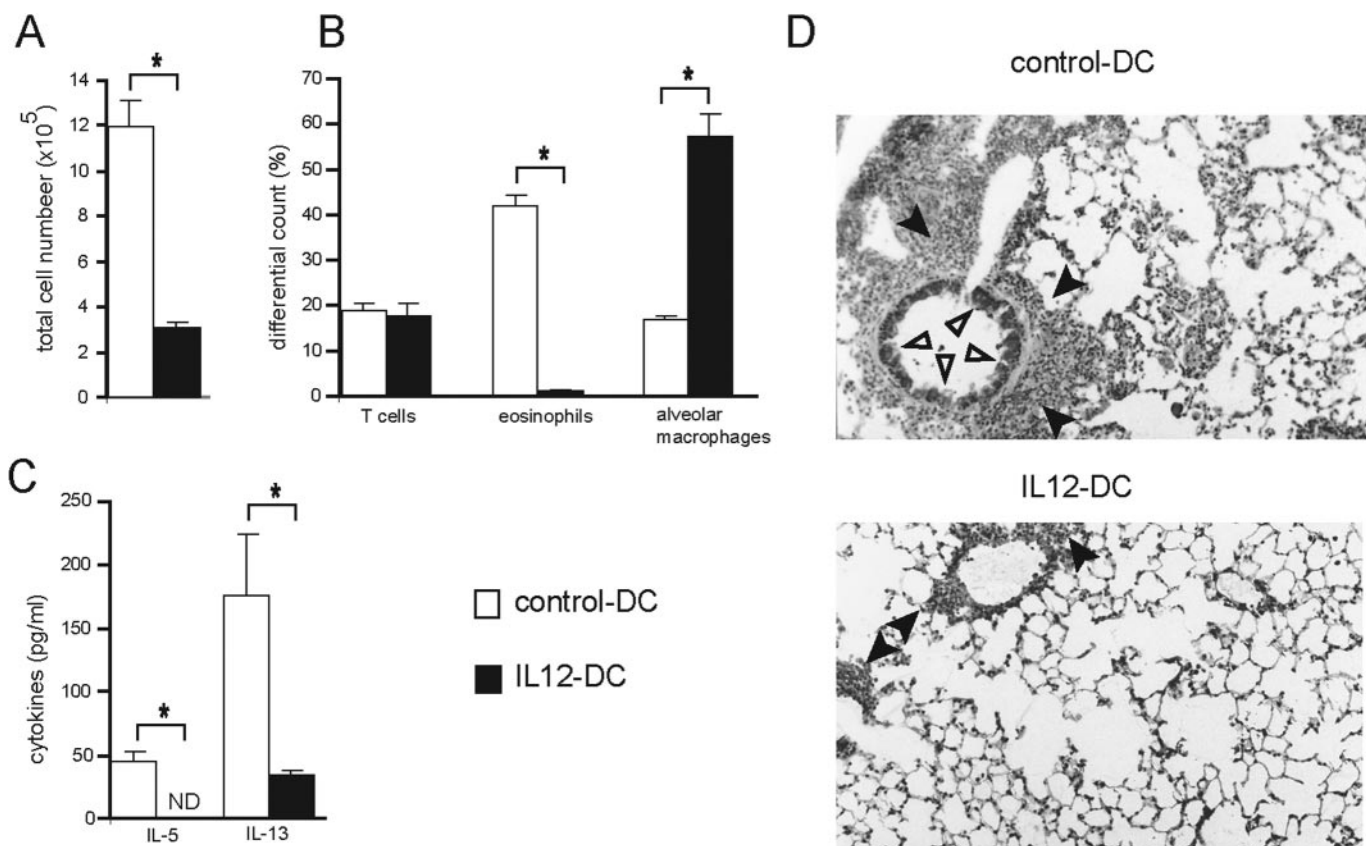


Fig. 3. IL12-DCs fail to induce eosinophilic airway inflammation. On day 0, groups of mice were immunized by i.t. administration of 1×10^6 IL12-DC or control-DC. On days 10–13, mice were exposed to OVA aerosols for 30 min daily. At 24 h after the last exposure, mice were killed, BAL and lung histology performed, and LN isolated as described in Materials and Methods. (A) Total cell number in BAL fluid. (B) Cellular composition of BAL fluid. Alveolar macrophages were characterized by their light-scatter and autofluorescence properties. Eosinophils are defined by their CCR3⁺, CD4⁺, CD8⁺ staining pattern. The T cell fraction consists of CD4⁺ and CD8⁺ cells within the appropriate light-scatter gate. (C) Cytokine levels in BAL fluid. (D) Leukocyte infiltration and PAS staining in lungs of control-DC or IL12-DC-immunized mice. Solid arrowheads indicate cellular infiltrate, open arrowheads indicate mucus accumulation (PAS⁺). Results are expressed as means $\pm$ SEM. *, $P < 0.05$. Data shown are representative of three independent experiments with four to ten mice per group. ND, None detected.

This was reflected by decreased levels of IL-4, IL-5, and IL-13 and increased levels of IFN- γ compared with control-DC. A striking observation was the concurrent increase of IL-10 with IFN- γ when IL12-DCs were used to prime CD4⁺ T cells. This was shown before for human T cells derived from cell cultures

primed in the presence of IL-12 [37, 38]. As we examined IL-10 gene expression at the population level but not at the single-cell level, we cannot conclude that IL-10 and IFN- γ are produced by the same cell. An appropriate candidate would be a subset of Treg cells, which produce IL-10 and IFN- γ , and

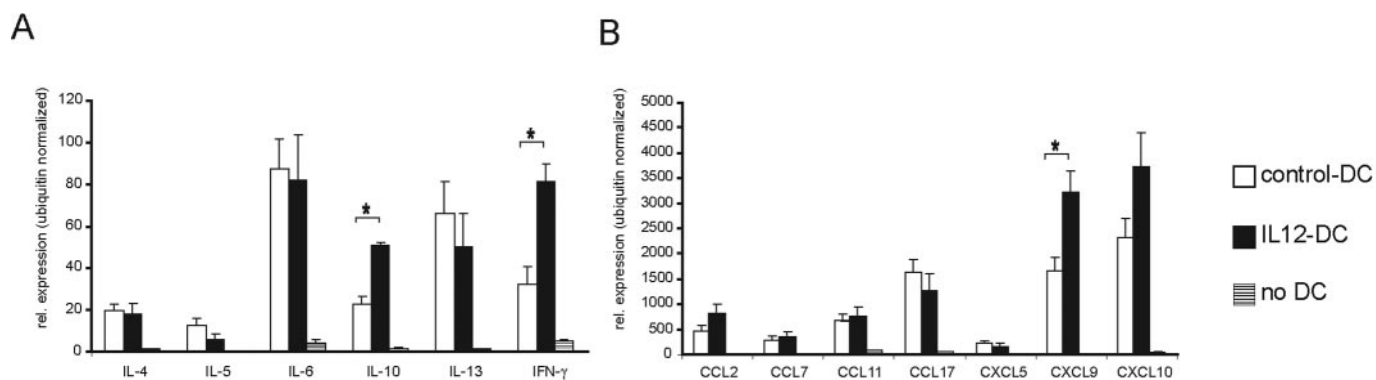


Fig. 4. Gene expression pattern in the lungs of IL12-DC-immunized mice suggests a Th1 response. Mice were immunized (four to five per group) as described in Figure 3. To account for OVA aerosol-induced effects, a control group of nonimmunized mice receiving only OVA aerosols was included. At day 14, lungs were excised, snap-frozen, and RNA-isolated, and gene expression of selected cytokines and chemokines was analyzed with real-time, quantitative RT-PCR. (A) Expression levels of cytokine transcripts. (B) Expression levels of chemokine transcripts. Data shown are the ubiquitin-normalized values of transcript RNAs. Results are expressed as mean $\pm$ SEM. *, $P < 0.05$.

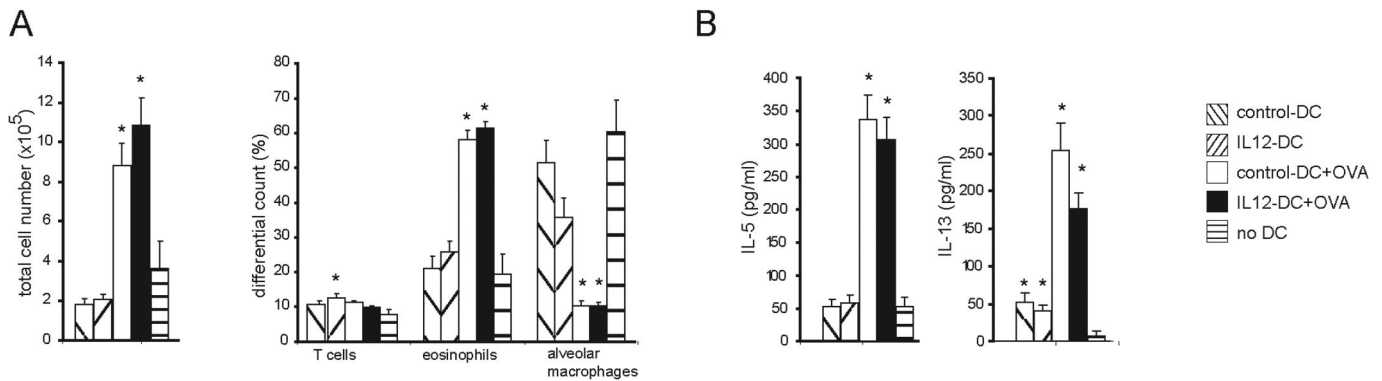


Fig. 5. IL12-DCs are unable to suppress a developing eosinophilic airway inflammation. Mice were immunized by i.p. injection of OVA/alum. Ten days later, mice were injected i.t. with IL12-DC and control-DC not pulsed with OVA. To control for the effect of i.p. immunization alone, one group did not receive DCs (no DC). From day 20 onwards, mice were challenged with OVA aerosol for 3 consecutive days. Airway inflammation was analyzed at day 24, as described for Figure 3. Results are expressed as mean $\pm$ SEM from nine to 10 mice per group. *, $P < 0.05$, versus no DC group.

have been shown to be generated in the lungs of mice upon challenge with microbial pathogens that induce IL-12 [39]. At a high DC-to-T cell ratio (1:10), T cell proliferation was slightly lower when IL12-DCs were used as APC. Grogan et al. [40] reported a similar phenomenon, when they stimulated naïve CD4⁺ T cells with anti-CD3 and anti-CD28 antibodies under strong Th1-polarizing conditions, implicating that lower proliferation is intrinsic to strongly polarized Th1 cells. At lower DC-to-T cell ratios, proliferation was identical between both groups, indicating that Th1 polarization was a direct effect of IL-12 overexpression and was not a result of the fact that IL12-DCs were more mature APC and thus provided a stronger strength of stimulus to T cells known to influence Th polarization [41–43]. Ex vivo LN-recall responses to OVA antigen from mice immunized with IL12-DC were less clearly polarized. As only a small percentage of i.t.-injected DCs migrate to the MLN [8], a possible explanation might be that naïve T cells became less polarized in vivo as a result of a lower DC-to-T cell ratio, as supported by the in vitro data.

The Th2 hypothesis of asthma proposes that airway inflammation arises from an imbalance between Th1 and Th2 CD4⁺ T lymphocyte subsets. Th2 cells release many cytokines that have been shown to regulate the inflammatory response, and it has been postulated that Th1 cytokines may counteract this response [1]. Not surprisingly, systemic or lung administration of the Th1-polarizing cytokines IFN- γ or IL-12, during sensitization to inhaled allergen, was able to inhibit all the cardinal features of asthma through induction of a counter-regulatory Th1 subset [44–47]. It was therefore of interest to study whether IL-12 overexpression in DCs also abolished the potential of these cells to prime for eosinophilic airway inflammation, a defining characteristic of asthma. DCs overexpressing IL-12p70 no longer induced eosinophilic airway inflammation or goblet cell hyperplasia, and concomitantly, a reduction was seen in IL-5 and IL-13, known to cause eosinophilic airway inflammation and goblet cell hyperplasia [48, 49]. In contrast, untransfected DCs, producing low levels of bioactive IL-12, caused all the salient features of asthma, as previously reported [4, 8]. These observations have important implications for understanding the mechanisms underlying Th2 sensitiza-

tion and development of allergic diseases. Several authors have suggested that low-level production of bioactive IL-12 in APC underlies the propensity of atopic individuals to develop Th2 responses to commonly inhaled allergens [19, 20, 24]. Conversely, the level of exposure to microbial stimuli in early life, known to induce high-level production of IL-12 in APCs, is inversely correlated with the development of atopic sensitization [50]. Several animal models have shown that bacterial stimuli, such as heat-killed *Listeria monocytogenes*, bacterial LPS, and mycobacterium Bacille Calmette Guérin, reduce the onset of Th2 sensitization and eosinophilic airway inflammation typical of asthma and at the same time, strongly enhance the production of IL-12 in vivo [7, 8, 51–54]. Our data suggest that the induction of IL-12 by these microbial patterns is more than just a marker of the Th1 bias induced by microbes but is causally related to the inhibition of sensitization.

Although airway inflammation was strongly reduced in IL12-DC-immunized mice compared with control-DC-immunized mice, there was still induction of BAL fluid lymphocytosis (18%, Fig. 3A) and mild lymphocytic infiltration around blood vessels (Fig. 3D), well above the levels of 1–5% normally seen in naïve or sham-sensitized mice [4]. Although this could be the reflection of a Th1 response to OVA aerosol, it was striking to see no associated neutrophilic inflammation, classically associated with Th1 responses to inhaled OVA [55–57]. It was therefore of interest to study the cytokine and chemokine expression pattern in the lungs of IL12-DC-immunized mice [34, 58]. By quantitating the mRNA expression level of a panel of cytokines and chemokines, we obtained evidence that immunization with IL12-DC resulted in a Th1-dominated lung pathology, with significantly increased levels of IFN- γ mRNA in the lung. The chemokine expression pattern was in accordance with a predominant Th1 response, as increased expression of CXCL9/MIG and CXCL10/IP-10 was seen [40, 58, 59]. CXCL9 and CXCL10 are ligands for CXCR3, a CXC chemokine receptor highly expressed on Th1 cells and believed to be important for migration of these cells to the lung [59–61]. The mRNA levels of the macrophage-derived CCL2/MCP-1 chemokine were also slightly increased as a reflection of Th1-mediated pathology, the most likely source being the increased

numbers of alveolar macrophages [34]. Strikingly, BAL fluid lymphocytosis and monocytosis are characteristic of many Th1-mediated lung diseases such as sarcoidosis, hypersensitivity pneumonitis, and early transplant rejection. In these diseases, there is an increased production of IL-12, eventually leading to expression of CXCL10/IP-10 chemokine within the lung [62, 63]. Our data suggest that IL-12 overproduction in DCs in response to recognition of the known or unknown antigens might be the cause of the deranged Th1 response leading to lymphocytary and monocytary alveolitis.

Although these findings suggest a predominant Th1 response, we did not see a decrease in the expression of Th2-associated chemokine CCL11/eotaxin mRNA nor in levels of IL-5 and IL-13 transcripts between the IL12-DC and control-DC groups. As these chemokines and cytokines are known to attract eosinophils to the lung [64], it was striking that there was a dramatic reduction in airway eosinophilia in IL12-DC-immunized mice. This might reflect a discrepancy between mRNA levels and protein levels, as for IL-5 and IL-13, it is known that protein levels in the BAL are significantly decreased (Fig. 3C). Another explanation could be the induction of IL-10 and IFN- γ production by IL12-DC. These cytokines have been shown to directly inhibit airway eosinophilia by reducing the recruitment of eosinophils or by inducing their apoptosis [65, 66]. Moreover, it was very recently shown that CXCL9/MIG can act as a direct antagonist of eotaxin-mediated eosinophil recruitment to the lungs [67]. Therefore, the high levels of CXCL9/MIG in IL12-DC mice might explain the absence of airway eosinophilia despite high levels of eotaxin production in the lung.

In view of the induction of a polarized Th1 response by IL12-DC in the lung, we questioned whether IL12 DCs were also able to revert or suppress a developing Th2 response to OVA antigen. In contrast to the strong inhibition of Th2 responses seen in the primary immune response to OVA, IL12-DC were unable to suppress eosinophilic airway inflammation in OVA/alum Th2-sensitized mice. Rather, DCs given after Th2 sensitization strongly enhanced airway inflammation and Th2 cytokine production, irrespective of IL-12 production. It is known that polarization of primed Th2 cells is hard to revert, as a result of loss of IL-12 receptor expression on these cells [68]. It has also been shown that antigen-specific Th1 lymphocytes can enhance the potential of Th2 lymphocytes to cause all the cardinal features of asthma, and therefore, IL12 DC might exacerbate disease via Th1 induction [69, 70]. Despite this, exogenous administration of recombinant IL-12 can inhibit the salient features of asthma, even when given during the challenge phase in Th2-sensitized mice [44, 45, 71]. It is likely that these treatments lead to effects of IL-12 that are not mediated directly on T cell polarization. It has been shown that high systemic levels of IL-12 suppress the BM output of eosinophil precursors and can directly induce the apoptosis of eosinophils in the lungs [72]. Moreover, IL-12 directly suppresses the formation of eotaxin by lung epithelial cells, independently of IFN- γ [73]. As overexpression of IL-12 was limited to injected DCs in our system, high, local levels of IL-12 are only thought to occur in the draining MLN during interaction with T cells, not leading to high systemic or lung concentrations, which might explain the differences between

recombinant IL-12 administration and IL-12 overexpression in DCs.

In summary, we show in this report that high-level expression of IL-12 in DCs renders these cells incapable of inducing Th2 sensitization to inhaled antigen. Finding strategies that enhance IL-12 production in endogenous lung DCs might lead to novel forms of prevention of allergic sensitization.

ACKNOWLEDGMENTS

This work was supported by a grant from The Netherlands Asthma Foundation (NAF 32.00.45) to H. K. and B. N. L. We are grateful to C. Maliszewski for providing recombinant human Flt3-L. We thank S. Hurst and S. Manning for providing primer sequences. The plasmids that were used in the construction of pMFG-moIL-12 were kindly provided by Dr. O. Danos (pMFG) and Dr. T. Gajewski (pcDNA1Amp-p35 and pcDNA1Amp-p40).

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