Lipopolysaccharide-Induced Suppression of Airway Th2 Responses Does Not Require IL-12 Production by Dendritic Cells[1]

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The prevalence of atopic asthma, a Th2-dependent disease, is reaching epidemic proportions partly due to improved hygiene in industrialized countries. There is an inverse correlation between the level of environmental endotoxin exposure and the prevalence of atopic sensitization. As dendritic cells (DC) have been implicated in causing sensitization to inhaled Ag, we studied the effect of endotoxin on Th2 development induced by bone marrow DC in vitro and by intratracheal injection in vivo, with particular emphasis on the role played by the polarizing cytokine IL-12. Bone marrow-derived DC stimulated with *Escherichia coli* O26:B6 LPS produced IL-12p70 for a limited period of time, after which production became refractory to further stimulation with CD40 ligand, a phenomenon previously called "exhaustion." The level of IL-12 production of DC did not correlate with Th1 development, as exhausted OVA-pulsed DC were still capable of shifting the cytokine pattern of responding OVA-specific Th cells toward Th1 in vitro and in vivo. When mice were first immunized by intratracheal injection of OVA-DC and subsequently challenged with OVA aerosol, prior in vitro stimulation of DC with LPS reduced the development of airway eosinophilia and Th2 cytokine production. Most surprisingly, the capacity of LPS to reduce Th2-dependent eosinophilic airway inflammation was IL-12-independent altogether, as IL-12p40 knockout DC had a similar reduced capacity to prime for Th2 responses. These results suggest that LPS reduces sensitization to inhaled Ag by reducing DC-driven Th2 development, but that IL-12 is not necessary for this effect. ***The Journal of Immunology*****, 2003, 171: 3645–3654.**

T helper type 1 (Th1) and type 2 (Th2) effector cells play a pivotal role in the adaptive immune response toward distinct classes of Ags (1). Dendritic cells (DC)[3] are generally considered to be the principal APCs involved in the generation of polarized effector cells (2). However, the exact mechanisms by which DC induce polarized Th responses are only incompletely understood, and many of these mechanisms are complementary. As such, the route of Ag encounter and the subtype of DC presenting the Ag can profoundly influence Th differentiation (3–6). The type of pathogen encountered or the adjuvant used for DC activation has an even greater impact on Th cell differentiation. DC-activating molecular patterns such as LPS (7), poly I:C (8), and CpG motifs (9, 10) induce Th1 polarization by signaling through pattern recognition receptors, while other products, like soluble egg Ags of the helminth *Schistosoma mansoni*, glycoproteins from the filarial nematode *Acanthocheilonema viteae*, or cholera toxin result in a skewing toward Th2 (8, 11). Recently, yet another model of DC-induced Th cell polarization in vitro was proposed (12). According to this "kinetics of activation" model, Th cell differentiation following DC encounter with microbial compounds is time-dependent, early in the response favoring Th1 activation while later on favoring Th2 polarization.

All these different models of T cell polarization share the paradigmatic view that the type of Th response that arises correlates closely with the capacity of DC to produce polarizing cytokines such as bioactive IL-12, a dominant Th1-promoting cytokine (2, 4, 13, 14). Indeed, subsets of DC have differential capacity to produce IL-12 and many of the Th1-prone molecular patterns in microbes induce IL-12 production in DC (7–9, 13). Along the same lines, the kinetics of activation model was explained by initial strong production of IL-12 by LPS-stimulated DC, after which prolonged stimulation would lead to exhaustion of IL-12 production, thus favoring Th2 development (12). According to some models, Th2 development indeed occurs as a default pathway when DC fail to produce polarizing IL-12 (4), although the relevance of this depends on the nature of the Ag and the experimental model used (15–17).

The question of how and under which microbial control mechanisms DC induce stable Th polarization is particularly relevant to atopic asthma. Atopic asthma is characterized by chronic eosinophilic airway inflammation, and occurs in individuals with strongly polarized Th2 recall responses to environmental allergen. The incidence of atopy and asthma has greatly increased over recent years, concomitant with an improved hygienic status in the industrialized world, suggesting some form of environmental control over Th2 development (18, 19). Interestingly, it has been reported that exposure to high levels of LPS endotoxins during early childhood are correlated with a lower incidence of asthma later in life

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Received for publication November 4, 2002. Accepted for publication July 17, 2003.

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[1] This work was supported by Netherlands Asthma Foundation Grant NAF 32.00.45 (to H.K. and B.N.L.). H.H. was supported by a European Union Marie Curie Foundation scholarship.

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[3] Abbreviations used in this paper: DC, dendritic cell; BM, bone marrow; CM, culture medium; sCD40L, soluble CD40 ligand; PRI, proliferation index; i.t., intratracheal; MLN, mediastinal lymph node; ALN, axillar lymph nodes; BAL, bronchoalveolar lavage; PI, propidium iodide; PAS, periodic acid-Schiff.

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0022-1767/03/$02.00

(20). The mechanisms by which this occurs are currently unknown but systemic exposure to LPS during sensitization to inhaled Ag decreased the severity of airway inflammation in animal models of asthma (21, 22), possibly by shifting the balance of immune responsiveness toward Th1. We have previously shown in a murine model of asthma, that airway DC are essential for inducing Th2 sensitization to inhaled allergen, leading to eosinophilic airway inflammation (6, 23). As DC are also responsive to LPS, we set out to study the effects of bacterial LPS on Th2 development induced by DC in vitro and in the airways in vivo, with a particular emphasis on the role played by the dynamic secretion of the polarizing cytokine IL-12. Our data show that LPS reduces DC-driven Th2 development in vitro and in vivo, through an IL-12-independent mechanism, resulting in reduced eosinophilic airway inflammation.

Materials and Methods

Mice

Female BALB/c mice (6- to 10-wk-old) were purchased from Harlan (Horst, The Netherlands). $OVA_{323–339}$-specific, MHC class II-restricted, TCR transgenic (DO11.10) (24), and IL-12p40$^{-/-}$ mice crossed back on a BALB/c background 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 University MC committee (Rotterdam, The Netherlands) for animal ethics.

Antigen

Chromatographically purified OVA was obtained from Worthington Biochemical (Lakewood, NJ). Endotoxin activity was 29 endotoxin units/mg, which corresponds to 2.9 ng of LPS per milligram of OVA, as determined by BioWhittaker Europe (Verviers, Belgium). This residual endotoxin activity did not result in IL-12p70 production when added to bone marrow (BM) DC cultures (data not shown). LPS (*Escherichia coli*, strain O26:B6) was purchased from Sigma-Aldrich (St. Louis, MO).

Generation and stimulation of BM-derived DC

BM-derived DC were generated as described (25). After RBC lysis, BM cells were resuspended at 2×10^5 per ml in DC culture medium (DC-CM; RPMI 1640 containing glutamax-I (Invitrogen, Carlsbad, CA) supplemented with 5% (v/v) FCS (Biocell, Rancho Dominguez, CA), 50 μM 2-ME (Sigma-Aldrich), 50 μg/ml gentamicin (Invitrogen), and 20 ng/ml recombinant mouse GM-CSF (a kind gift from Prof. K. Thielemans, Vrije Universiteit Brussel, Brussels, Belgium). Cells (2×10^6) were seeded in tissue-culture grade 100-mm Petri dishes (day 0). At day 3, 10 ml of fresh DC-CM were added. On days 6 and 8, 10 ml of each plate were centrifuged and resuspended in 10 ml of fresh DC-CM. At day 9, cells were pulsed for 24 h with either OVA (100 μg/ml; OVA-DC), LPS (100–500 ng/ml; LPS-DC), or a combination of these two (OVALPS-DC). In some experiments, unstimulated DC (control-DC) were used as a negative control. At day 10, mature DC were harvested by gentle pipetting. In some experiments, BM cells were cultured in six-well plates, with cell numbers and culture volumes adjusted to surface area. At day 9, LPS (25 ng/ml) was added for 8 or 24 h, cells were washed with DC-CM, and recultured for an additional 24 h either with or without soluble trimeric CD40L (sCD40L; a generous gift of Dr. C. Maliszewski, Immunex, Seattle, WA) at 2.5 μg/ml. After 24 h, cell recovery was determined and supernatants were kept at −20°C until further analysis.

Real-time quantitative RT-PCR

Cells were harvested at various time points and total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. cDNA was generated from total RNA using random hexamers and the Omniscript Reverse Transcriptase Kit (Qiagen). Relative expression levels were determined with an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Primers and probes for murine IL-12p40, IL-12p35, CCR7, and GAPDH were obtained from PerkinElmer (Foster City, CA). PCR conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. PCR amplification of the housekeeping gene GAPDH was performed to control for sample loading and to allow normalization between samples. Genomic DNA and water controls were included to ensure specificity. Data are expressed as the ratio between cytokine/chemokine transcript levels and GAPDH transcript levels.

CFSE labeling and analysis of T cell division

For fluorescent cell labeling, cells were washed twice with serum-free medium, labeled with 1 μM (DC) or 5 μM (T cells) CFSE (Molecular Probes, Oss, The Netherlands) in serum-free medium for 10 min at 37°C, and the reaction was stopped by adding excess ice-cold DC-CM (26). For quantification of cell division based on serial halving of CFSE intensity, algorithms provided by FlowJo software (Treestar, San Carlos, CA) were used. The number of events n under each CFSE fluorescence peak i was determined using a nonlinear least-squares fitting of a series of Gaussian functions. Once the data set had been fitted, the software derived two statistics to describe the proliferation. The proliferation index (PRI) has been defined as the average number of divisions of the cell fraction that divided (equation 1). The responder frequency (%D) has been defined as the percentage of input cells that responded to stimulation by dividing (equation 2).

$$PRI = \frac{\sum_{1}^{i} \frac{i \times n_i}{2^i}}{\sum_{1}^{i} i \times n_i} \quad (1)$$

$$\%D = \frac{\sum_{1}^{i} \frac{n_i}{2^i}}{\sum_{0}^{i} \frac{n_i}{2^i}} \quad (2)$$

In addition to cell division parameters, the original T cell pool that was necessary to generate the total number of daughter cells can also be calculated and compared between treatment groups (equation 3). Therefore, this so-called "CFSE content" is an indicator of cell death and/or recruitment of Ag-specific T cells within the lymph node.

$$CFSE\ content = \sum_{0}^{i} \frac{n_i}{2^i} \quad (3)$$

Th cell priming in vitro

Spleen and lymph node cells were isolated from DO11.10 mice and labeled with CFSE. These cells (10^6/well) were cocultured in a 24-well plate with OVA-DC (5×10^4/well), OVALPS-DC or control-DC. After 96 h, cells were harvested and secretion levels of IL-4 and IFN-γ were detected by flow cytometry.

DC migration and IL-12 staining in vivo

On day 0, mice ($n = 5$) were anesthetized with avertin and 2×10^6 CFSE-labeled OVA-DC or OVALPS-DC were injected intratracheally (i.t.) in a volume of 80 μl of PBS using the technique of Ho and Furst (27). After 36 h, the mediastinal lymph nodes (MLN) were isolated and pooled. As a control for specific migration, the axillar lymph nodes (ALN) were resected. Lymph nodes were incubated for 60 min at 37°C in digestion mixture (collagenase type II, 1 mg/ml; Worthington Biochemical; DNaseI, 2 U/ml, Sigma-Aldrich), supplemented with monensin (Golgi-Stop; BD Biosciences, Alphen a/d Rijn, The Netherlands). Frequency and IL-12p40 production of $CFSE^+$ DC were analyzed using flow cytometry.

Primary immune response following intratracheal injection of DC

On day −2, 10^7 CFSE-labeled DO11.10 TCR transgenic cells were adoptively transferred i.v. into naive BALB/c mice. On day 0, mice ($n = 9–10$ per group) were i.t. immunized with 10^6 OVA-DC or OVALPS-DC. On day 4, mice were sacrificed and MLN, ALN, and lungs were collected separately. Single cell suspensions of lymph nodes were prepared by mechanical disruption and lungs were homogenized using digestion mixture. Single cell suspensions were analyzed by flow cytometry or were restimulated (2×10^6 cells per ml) with 10 μg/ml OVA for 96 h, after which supernatants were harvested and assayed for IL-4, IL-5, IL-10, IL-13, and IFN-γ content.

Secondary immune response following i.t. injection of DC

On day 0, groups of mice ($n = 9–10$ per group) were immunized i.t. with 1×10^6 OVA-DC or OVALPS-DC. In some experiments, control animals received control-DC. From day 10 onward, mice were exposed to OVA

aerosols (1% (w/v) in PBS) for 3–4 consecutive days, 30 min daily. Twenty-four hours after the last exposure mice were sacrificed and bronchoalveolar lavage (BAL) was performed as described (6). MLN were isolated and single cell suspensions were cultured in vitro as described above. As a site-specific control, ALN of each group were pooled and treated identical to MLN.

Airway histology

After BAL, lungs were slowly inflated with 10% buffered formalin, the right-middle lobe was excised, and fixed in formalin overnight. Subsequently, lungs were embedded in paraffin, 3-μm sections were stained with hematoxylin and periodic acid-Schiff (PAS) reagent (Sigma-Aldrich) and photographed with a Leica DM-LB microscope (Rijswijk, The Netherlands).

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 manufacturer's instructions. IL-13 levels were measured using a commercially available kit from R&D Systems (Minneapolis, MN)

Flow cytometry

To reduce nonspecific Ab binding, anti-FcγRII Ab (2.4G2; American Type Culture Collection, Manassas, VA) was included in all cell surface stainings. To detect intracellular IL-12p40, single cell lymph node suspensions were incubated for 3 h in the presence of monensin, followed by cell surface staining. Subsequently, cells were fixed with 4% paraformaldehyde for 20 min at 4°C. (Cells were either used directly or stored overnight in FACSWash (PBS, 0.5% BSA, 0.05% NaN_3)). Cells were then resuspended in permeabilization buffer (Perm/Wash Buffer; BD Biosciences) and stained for 30 min at 4°C. The following Abs were used: anti-CD3ϵ-PE (145-2C11), anti-pan-NK-PE (DX5), anti-CD19-PE (1D3) in combination with anti-IL-12p40-APC (C15.6) or an isotype-matched control.

To assess Th cell priming in vitro, IL-4 and IFN-γ secretion levels were measured with cytokine secretion assays (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. Briefly, cell cultures were restimulated with plate-bound anti-CD3 (145-2C11; BD Biosciences, 1 μg/ml) and anti-CD28 (37.51, 4 μg/ml) for 4 h and subsequently labeled with the capture Ab for either IL-4 or IFN-γ. The cells were then diluted in medium and allowed to secrete cytokines for 45 min at 37°C, under continuous rotation. $CD4^+$ T cells secreting cytokines were identified by staining with PE-conjugated IL-4 or IFN-γ detection Ab and CD4-allophycocyanin (RM4-5). Before acquisition, propidium iodide (PI; 0.5 μg/ml) was added for discrimination of dead cells.

To study the primary immune response in CFSE adoptive transfer experiments, cells were labeled with anti-CD4-allophycocyanin in combination with the anti-clonotypic DO11.10 TCR Ab KJ1-26 (24), conjugated to PE. Dead cells were excluded by labeling with PI before acquisition.

Anti-CCR3-PE was used to detect eosinophils in the lung (28), together with anti-CD19-FITC (1D3), anti-CD8-PECy5 (53-6.7), and anti-CD4-APC (RM4-5) to determine the cellular composition in BAL. All fluorochrome-conjugated Abs 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 (5×10^4–1.5×10^6) were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software.

Statistical analysis

Reported values are expressed as mean ± SEM, unless indicated otherwise. Statistical analyses were performed with SPSS (Chicago, IL) using a Mann-Whitney U test. Values of $p < 0.05$ were considered significant.

Results

BM-derived DC express IL-12p70-encoding genes

IL-12p70 has been shown to be an important cytokine in the differentiation and polarization of $CD4^+$ Th cells toward type 1 (29, 30). To investigate the IL-12p70 expression kinetics of murine BM-derived DC in vitro, DC cultures were exposed to LPS (100 ng/ml) and relative mRNA expression levels of the subunit genes IL-12p35 and IL-12p40 were determined. Both subunit genes were up-regulated after LPS stimulation, reaching a maximum around 12 h after onset of stimulation (Fig. 1, *A* and *B*). After 24 h of stimulation, expression levels of both subunit genes dropped significantly and remained low thereafter. Although LPS-mediated apoptosis of DC could be a possible explanation for the sharp decrease in gene expression observed, the CCR7 mRNA levels increased consistently after addition of LPS (Fig. 1*C*), arguing against LPS-mediated cell death during culture.

The kinetics of IL-12 subunit gene expression suggested that no IL-12p70 was produced beyond 24 h of LPS stimulation. To test this hypothesis, cultured DC were stimulated for 8 or 24 h with LPS, extensively washed and recultured either in medium (to assess residual IL-12p70 production) or with sCD40L (to test IL-12p70 production capacity after secondary stimulation). As shown in Fig. 2, the residual release of IL-12p70 into fresh medium was greatly reduced after LPS stimulation for 8 and 24 h (▦). Of note was the decreased production after 8 h of LPS stimulation, despite the expression of the IL-12p35 and IL-12p40 genes (Fig. 1, *A* and *B*), still maximal at this point. However, secretion of IL-12p70 was restored by culturing the cells in a second stimulus provided by sCD40L, a surrogate marker for T cell contact. This restoration did not occur after prolonged LPS stimulation for 24 h (Fig. 2, □). Levels of the DC maturation markers CD40, CD80, and CD86 also increased after 24 h of LPS stimulation (data not shown). Altogether, these data show that with progression in time, LPS-activated DC acquire a higher costimulatory phenotype, but exhaust their IL-12 production capacity, in agreement with previous findings with human monocyte-derived DC (12, 31).

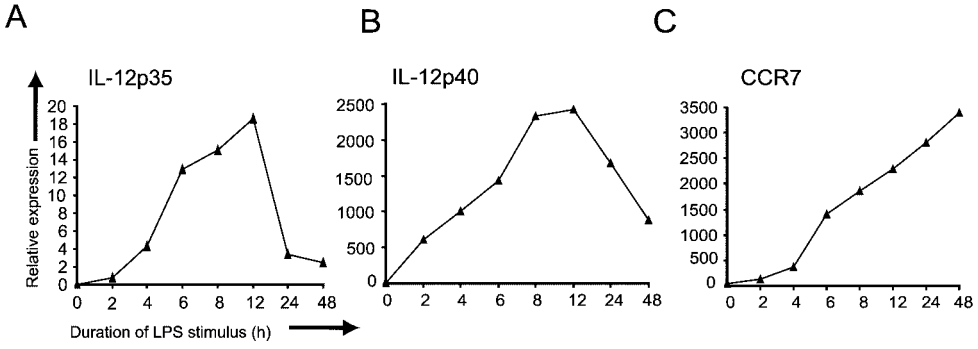

FIGURE 1. Murine DC produce IL-12 transiently after LPS stimulation. BM-derived DC cultures were stimulated with LPS (100 ng/ml) at day 9. Cells were harvested at indicated time points, total RNA was isolated and converted to cDNA. mRNA expression levels of IL-12p35 (*A*), IL-12p40 (*B*), and CCR7 (*C*) were determined by quantitative real-time RT-PCR. mRNA levels are displayed relative to GAPDH mRNA expression levels in each sample.

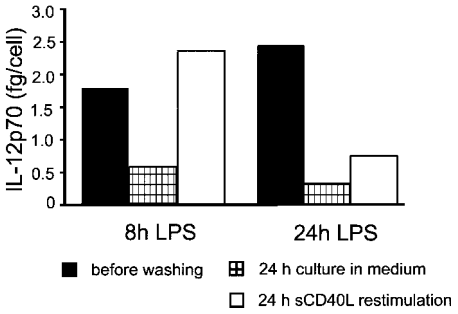

FIGURE 2. IL-12p70-producing capacity upon restimulation depends on duration of LPS stimulation. BM-derived DC were stimulated for 8 or 24 h with LPS (25 ng/ml). Supernatants were harvested (■) and cells were washed with DC-CM. DC were cultured for an additional 24 h either in DC-CM alone (▦) or in the presence of sCD40L (2.5 μg/ml; □). Supernatants were harvested and the cell number of each condition was determined. IL-12p70 cytokine levels were measured by ELISA. The IL-12p70 levels are expressed on a per cell basis to account for differences in cell numbers. The data are representative of duplicate experiments.

Th cell activation with LPS-stimulated DC result in a reduced Th2 profile in vitro

We next determined the functional consequences of LPS-induced DC maturation and exhaustion of IL-12 secretion, on Th cell polarization in vitro. OVA-pulsed-DC (OVA-DC) were pretreated or not for 24 h with LPS (OVALPS-DC) and were cocultured with naive CFSE-labeled lymphocytes from OVA-TCR transgenic mice. After 4 days, the cell division profile (Fig. 3*A*) as well as IL-4 and IFN-γ secretion levels as a function of cell division number (Fig. 3*B*) of resulting Th effector cells were determined. LPS stimulation had no significant effect on the stimulatory capacity of DC, as the division profile of OVALPS-DC-stimulated Th cells was almost similar to that of Th cells cultured with OVA-DC (Fig. 3*A*). This was confirmed by quantification of the cell proliferation, which revealed no major changes in the average number of divisions of the cell population that divided, hereafter referred to as PRI (OVA-DC: 2.52 $\pm$ 0.013, OVALPS-DC: 2.09 $\pm$ 0.023), and the responder frequency, defined as the cell population that participated in clonal expansion (OVA-DC: 68.9 $\pm$ 0.58, OVALPS-DC: 75.3 $\pm$ 2.12). In the OVA-DC group, secretion of IL-4 increased with every cell division, reaching a plateau after four divisions. The use of OVALPS-DC as APC resulted in significantly decreased levels of IL-4 secretion in each generation of dividing $CD4^+$ T cells compared with the OVA-DC group (Fig. 3*B*). No major difference in IFN-γ secretion was observed, with a large percentage of $CD4^+$ T cells secreting IFN-γ irrespective of whether the DC were previously stimulated with LPS or not (Fig. 3*B*). Consistent with these single cell secretion data, IL-4 and IFN-γ levels in the supernatant, as measured by ELISA, exhibited the same secretion profile (data not shown). The levels of the Th2-associated cytokines IL-5 and IL-13 were also decreased in the supernatant of OVALPS-DC-stimulated T cell cultures (data not shown). Thus, LPS stimulation of DC resulted in a selective decrease in Th2 cytokine secretion in responding T cells, without affecting Th1 cytokine secretion and despite the exhaustion of IL-12 production in vitro. It is unlikely that this reduced Th2 development was due to less stimulatory capacity or apoptosis of OVALPS-DC, because IFN-γ production and T cell division were similar compared with the OVA-DC group.

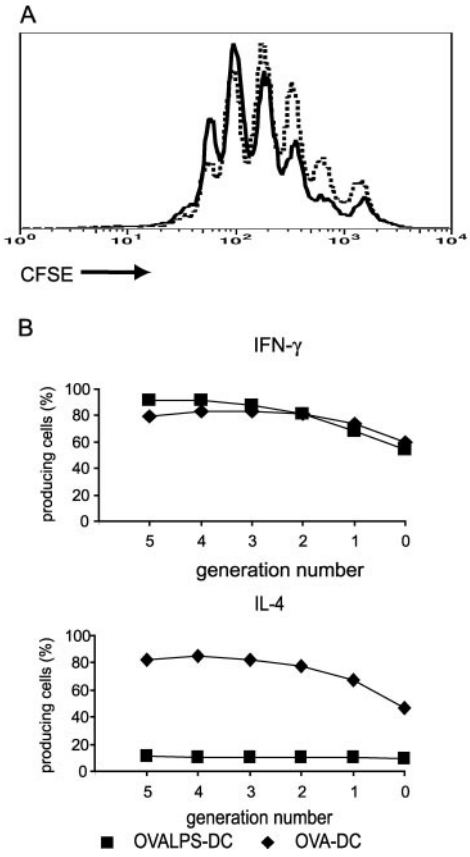

FIGURE 3. Stimulatory capacity of LPS stimulated DC on naive Th cells and the type of effector Th cell generated. BM-derived DC were pulsed with OVA protein (100 μg/ml) in the absence or presence of LPS (100 ng/ml). After a 24-h incubation, cells were thoroughly washed and used to stimulate CFSE-labeled Th cells. Four days later, cells were harvested and IL-4/IFN-γ secretion was measured, together with the division profile of CFSE-labeled cells. *A*, Division profile of living (PI^-), $CD4^+$ cells, stimulated with either OVA and LPS-pulsed DC (dashed line) or OVA alone (solid line). *B*, IL-4 and IFN-γ secretion per generation of PI^-, $CD4^+$ T cells stimulated with OVA-DC (▲) or OVALPS-DC (■). Data is depicted as percentage secretion above background. Background secretion levels were defined as the cytokine secretion measured without stimulation and were determined for each group and cytokine individually. Nonpulsed or DC pulsed with LPS do not result in cell division or cytokine secretion of Th cells (data not shown).

Effect of LPS stimulation of DC on proliferation and differentiation of Ag-specific $CD4^+$ T cell response in vivo

Next, we examined the effect of LPS on the stimulatory and polarizing capacity of DC in vivo. CFSE-labeled, OVA-TCR transgenic T cells were adoptively transferred to syngeneic recipient mice, which were subsequently immunized via the airways with OVA-pulsed DC, either stimulated or not with LPS. After 4 days, the frequency of OVA-specific T cells in the MLN, ALN, and lungs was determined. In the MLN, up to seven generations of divided cells could be distinguished (Fig. 4*A*). There was a slight decrease in frequency of OVA-specific T cells in the OVALPS-DC group compared with the OVA-DC group (0.48 $\pm$ 0.06% vs 0.36 $\pm$ 0.04%), respectively. Quantification of the cell proliferation revealed a small decrease in the PRI, as well as a decrease in the responder frequency (Table I). The quantification of cell division also enabled us to calculate the size of the original OVA-specific T cell population the progeny had arisen from (CFSE content), revealing differences in cell number at any given site as a

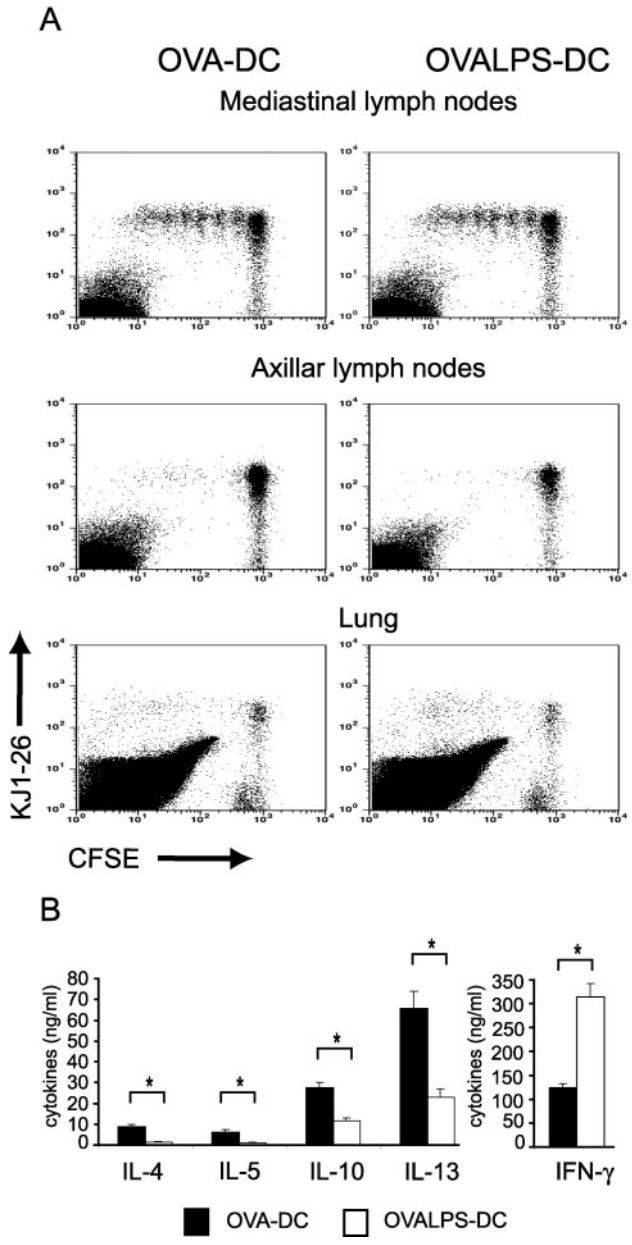

FIGURE 4. LPS-stimulated DC have equal stimulatory capacity but a distinct Th cell differentiation capacity. Naive BALB/c mice received a cohort DO11.10 T cells (KJ1-26$^+$) and were subsequently immunized with either OVA-pulsed DC ($n = 9$) or OVA + LPS-pulsed DC ($n = 10$). Four days later, MLN, ALN, and lungs were resected and analyzed for CFSE$^+$ T cells directly or cultured in vitro in the presence of OVA for 4 days. *A*, Frequency and cell division profile of OVA transgenic T cells in the MLN, ALN (PI$^-$, CD4$^+$), and lungs (PI$^-$) was assessed by flow cytometry. Dot plots shown are from representative mice of each group. *B*, 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 ± SEM from five mice per group. *, $p < 0.05$

result of recruitment, migration, or cell death, independent of cell division. There was no significant decrease in the original OVA-specific T cell pool size in the OVALPS-DC group compared with the OVA-DC group, indicating that LPS-stimulated DC were equally capable to recruit naive T cells (Table I). Due to the insufficient number of OVA-specific progeny T cells, we were unable to calculate the average division number of T cells in the nondraining ALN. However, as evident from Fig. 4*A*, a minor

Table I. *Quantification of OVA-specific CD4$^+$ T cell proliferation in the MLN*

Immunization	PRI[a]	Responder Frequency[b]	CFSE Content/ 10^5 PI$^-$ Cells[c]
OVA-DC	1.97 ± 0.04[d]	15.70 ± 0.67	237 ± 35
OVALPS-DC	1.82 ± 0.04	11.98 ± 1.30	211 ± 17

[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] Defined as the original T cell pool size necessary to generate the total number of daughter cells present, expressed per 10^5 living cells.
[d] Values represent the mean ± SEM of the data (n = 4–5 animals per group).

population of divided cells (more than three divisions) was present. No significant differences in frequencies of the PI$^-$, KJ1-26$^+$ T cell subset could be observed (OVA-DC: 0.47% ± 0.02%; OVALPS-DC: 0.48% ± 0.02%). OVA-specific T cells that underwent multiple divisions were also present in the lung but the number was insufficient to quantify the average cell division (Fig. 4*A*). There were also no differences in the frequencies of the PI$^-$, KJ1-26$^+$ T cell subset in the lung (0.117 ± 0.015% vs 0.107 ± 0.004% for OVA-DC and OVALPS-DC, respectively).

To determine the consequences of LPS stimulation on Th cell polarization in vivo, we also measured the cytokine levels of ex vivo cultures of lymph node cells taken at day 4 of the primary response after i.t. injection of DC (Fig. 4*B*). In the OVALPS-DC-immunized animals, a decrease in the production of all Th2 cytokines (IL-4, IL-5, IL-10, IL-13) with a concomitant increase in IFN-γ secretion was observed when compared with OVA-DC, indicating a clear shift in balance toward Th1 cytokine secretion.

Migration and IL-12 production of LPS-stimulated DC in vivo

Our in vitro experiments indicated that LPS stimulation of DC resulted in exhaustion of IL-12p70 production after 24 h, which would predict less Th1 development and more Th2 development in responding T cells. However, our in vitro and in vivo polarization experiments showed the opposite. To clarify this issue, we analyzed the expression of the IL-12p40 subunit by DC, previously used as a marker for IL-12p70 production by DC in vivo (32), after the migration of i.t. injected DC to the draining lymph nodes of the lung. After incubation with OVA and/or LPS, DC were labeled with CFSE and injected i.t. Thirty-six hours after instillation, there was a substantial increase in the percentage (Fig. 5*A*) and number (Fig. 5*B*) of OVALPS-DC migrating to the MLN compared with OVA-DC. These findings of enhanced migration are consistent with the up-regulation of CCR7 gene expression and down-regulation of CCR5 and CCR6 expression following LPS stimulation of BM-derived DC (Fig. 1*C* and data not shown). The same migration trend was observed when we looked at an earlier time point (24 h; data not shown), which makes it unlikely that there is a difference in migration kinetics between the two groups.

In contrast to the in vitro findings, OVALPS-DC produced more IL-12p40 following migration to the draining MLN compared with OVA-pulsed DC alone (Fig. 5*C*).

LPS stimulation of DC results in decreased development of eosinophilic airway inflammation

Despite the discrepancies between T cell polarization capacity and IL-12 secretion capacity of LPS-stimulated DC, the above data demonstrate that LPS-stimulated DC reduce Th2 development during the primary immune response. We next examined the effect of LPS activation of DC in a relevant Th2 dependent mouse model of

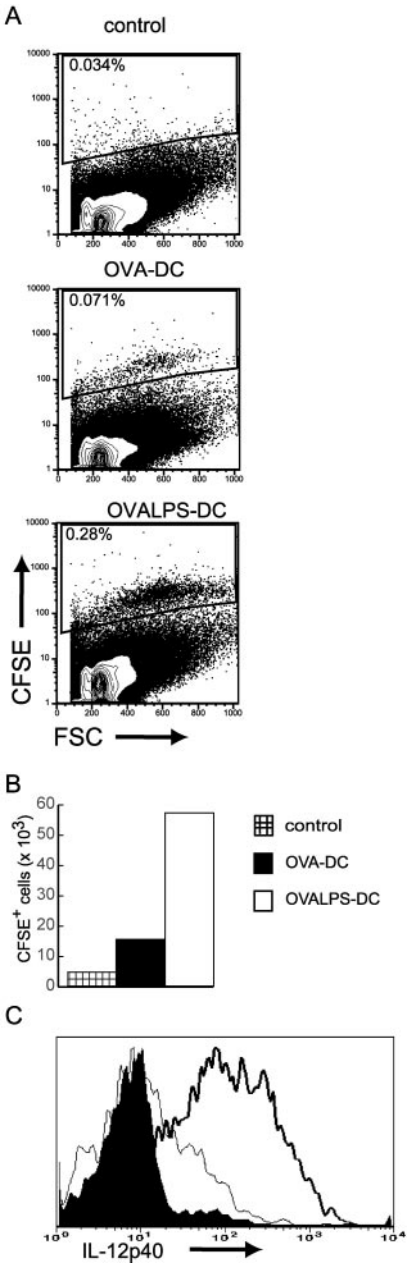

FIGURE 5. LPS increases migration and IL-12 production of DC in vivo. OVA-pulsed DC (OVA-DC) and OVA + LPS-pulsed DC (OVALPS-DC; both 2×10^6/mice) were CFSE-labeled and injected i.t. ($n = 5$ per group). Thirty-six hours later, MLNs from each group were isolated, pooled, and analyzed by flow cytometry for migration and IL-12 production of injected DC. As a control for specific migration, the ALNs of the OVALPS-DC were analyzed as well. Frequency (*A*) and total number (*B*) of $CFSE^+$ DC. *C*, IL-12p40 staining of $CFSE^+$ DC. Solid histogram, isotype-matched control; regular line, OVA-DC group; bold line, OVALPS-DC group. Cells (1.5×10^6) per sample were acquired. Data are representative of three independent experiments.

eosinophilic airway inflammation (6). In this model, sensitization to OVA is induced by i.t. injection of OVA-DC, followed by OVA aerosol challenge 10 days later, resulting in peribronchial and perivascular eosinophilic airway inflammation. When mice were immunized with control DC and subsequently challenged with OVA aerosol, there was no airway inflammation (data not shown and Ref. 6). When analyzing the degree of lung inflammation, immunization with OVALPS-DC revealed only a slight decrease in total cell number in BAL fluid compared with OVA-DC (Fig. 6*A*). However, the cellular composition of the BAL fluid was markedly different. A significant decrease in the frequency of eosinophils was observed, accompanied by an increase in frequency of alveolar macrophages in the mice immunized with OVALPS-DC (Fig. 6*A*). The frequency of T cells in BAL fluid was identical in both groups. Lung histology data were in concordance with BAL data, with lower but marked cellular infiltrate and mucus secretion in the OVALPS-DC group compared with the OVA-DC group (Fig. 6*D*). To explain why the development of airway eosinophilia was suppressed despite similar T cell recruitment, we also measured cytokine levels in lungs and MLN. In the BAL fluid there was a decrease in the levels of IL-5 and IL-13 after immunization with OVALPS-DC compared with OVA-DC (Fig. 6*B*). Th2-associated cytokine levels were also decreased in the MLN, in particular IL-4, IL-5, IL-10, and IL-13, while the levels of the Th1 cytokine IFN-γ did not significantly change following LPS stimulation of OVA-DC (Fig. 6*C*). These results suggest that LPS-stimulated DC, while still eliciting an immune response, have reduced capacity to prime for Th2 effector cells.

LPS-mediated suppression of Th2 development in vivo does not require IL-12

In an attempt to dissect the contribution of DC-derived IL-12 and recipient-derived IL-12 on Th2 effector cell generation in vivo, we first immunized wild-type mice with DC derived from IL-12p40 homozygous knockout mice. In the absence of LPS stimulation, BAL fluid analysis revealed that IL-12p40$^{-/-}$ OVA-DC induced a stronger Th2 response compared with wild-type OVA-DC, as the levels of BAL fluid eosinophilia were higher (Fig. 6*A*) and Th2 cytokine levels in BAL fluid revealed a significant increase in the Th2 cytokines IL-5 and IL-13 in the IL-12p40$^{-/-}$ group (Fig. 6*B*). Restimulation of lymph node cells in vitro also showed an increase for the Th2 cytokines in the IL-12p40$^{-/-}$ group (Fig. 6*C*). To our surprise, LPS stimulation of IL-12p40$^{-/-}$ OVA-DC had a similar suppressive effect compared with LPS stimulation of wild-type OVA-DC. In the IL-12p40$^{-/-}$ OVALPS-DC group, there was a slight decrease in total cell number accompanied by a statistical significant change in cellular composition, consisting of a decrease in the frequency of eosinophils and an increase in the frequency of alveolar macrophages compared with the IL-12p40$^{-/-}$ OVA-DC group (Fig. 6*A*). Additionally, the levels of IL-5 and IL-13 were significantly lower in BAL fluid of IL-12p40$^{-/-}$ OVALPS-DC mice compared with the IL-12p40$^{-/-}$ OVA-DC group (Fig. 6*B*). Finally, Ag-specific restimulation of MLN cells in vitro revealed lower levels of the Th2-specific cytokines measured, in particular IL-5 and IL-10, accompanied by a significant increase in the levels of IFN-γ after stimulation with LPS (Fig. 6*C*). To rule out any role for IL-12 in Th2 differentiation in this model, we next immunized IL-12p40$^{-/-}$ mice with IL-12p40$^{-/-}$-derived DC. Immunization with OVALPS-DC resulted in lower total cell number, frequency of eosinophils, and Th2-associated cytokines in the BAL compartment, compared with OVA-DC-immunized mice (Fig. 7). In summary, these data suggest that suppression of Th2 effector cell development by LPS stimulation of DC occurs independently of IL-12 secretion by either adoptively transferred DC or recipient-derived IL-12.

Discussion

Th cell polarization is influenced by the route of Ag exposure, the subtype of DC presenting the Ag, the dose of Ag, the genetic background of the host, and most importantly, by the nature of the Ag (2). It is increasingly clear that different molecular patterns

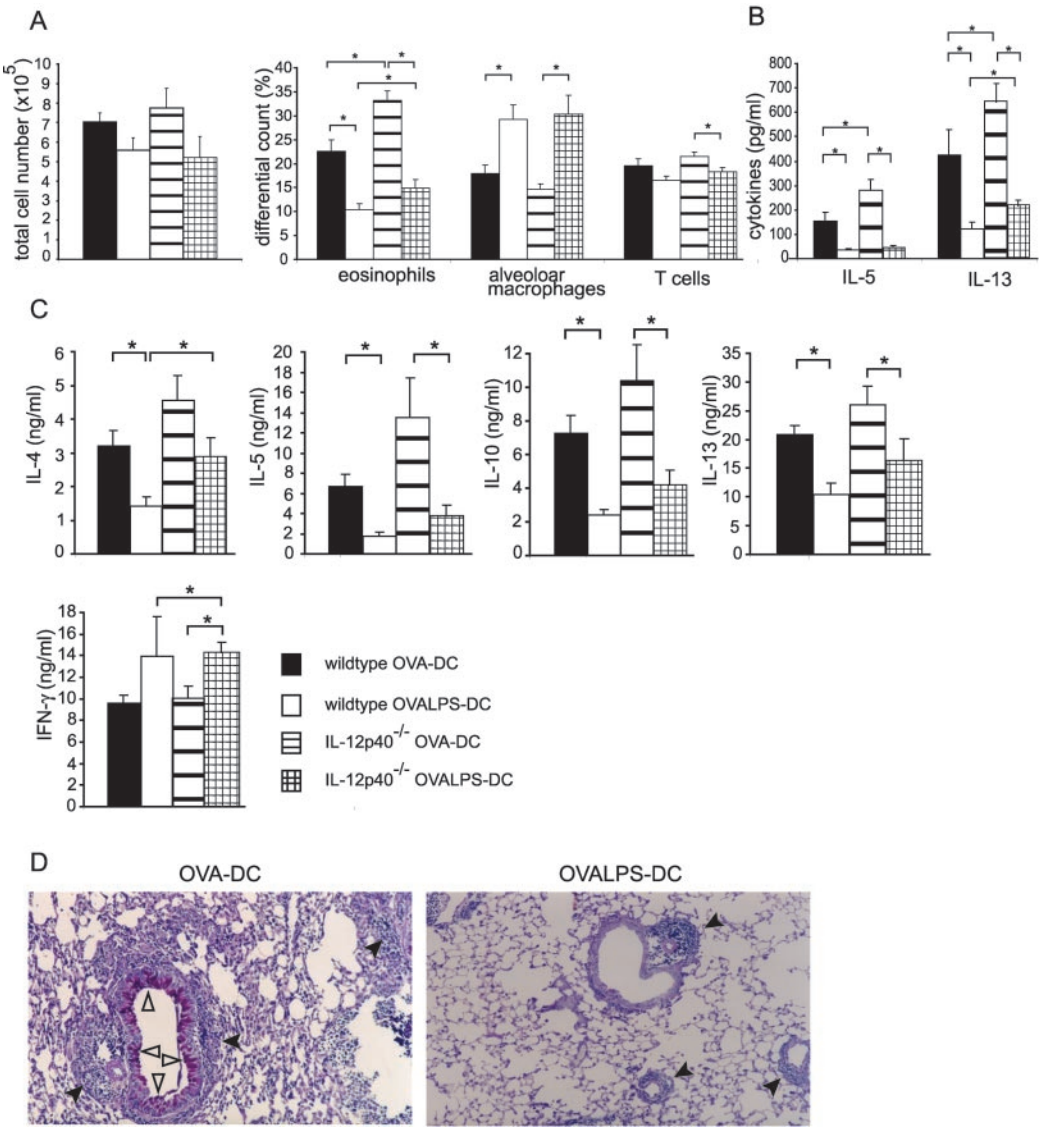

FIGURE 6. LPS-activated DC reduce inflammation in a mouse model of eosinophilic airway inflammation independent of DC-derived IL-12. On day 0, groups of mice were immunized by i.t. administration of 1×10^6 OVA-DC or OVALPS-DC, derived from either wild-type or IL-12p40$^{-/-}$ mice. On days 10–13, mice were exposed to OVA aerosols for 30 min daily. At 24 h after the last exposure mice were sacrificed, BAL was performed and lymph nodes were isolated as described in *Materials and Methods. A,* 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. *B,* Cytokine levels in BAL fluid. *C,* Cytokine levels after in vitro restimulation of MLN cells with OVA Ag. Note that ALN cells of each group were pooled and cultured in an identical manner. No cytokines could be detected in these cultures (data not shown). Results are expressed as means ± SEM from 8–10 mice per group. *, $p < 0.05$. Data are representative for one to three independent experiments. *D,* Leukocyte infiltration and PAS staining in lungs of wild-type OVA-DC or OVALPS-DC-immunized mice. Solid arrowheads indicate cellular infiltrate, open arrowheads indicate mucus accumulation (PAS$^+$)

expressed on pathogens can fundamentally influence Th differentiation by signaling through molecular pattern recognition receptors on DC (33, 34). The way by which these various factors are integrated by DC into a signal that can determine Th cell polarization is an area of intense research but involves the generation of a particular peptide-MHC density (signal 1), the provision of a particular combination of costimulatory molecules (signal 2), and the secretion of polarizing cytokines such as IL-12, IL-18, and IL-27 (2, 8, 35, 36). Many of the microbial patterns such as bacterial LPS, peptidoglycan, CpG motifs, and viral dsRNA, induce the secretion of IL-12 by signaling through the Toll-like receptors and the myeloid differentiation factor 88 signaling pathway, thus inducing polarization toward Th1 responses (16, 34).

We have examined the role of DC-derived IL-12 in LPS-mediated suppression of Th2 responses and took into account the recently proposed mechanism by which DC exert their influence on Th cell polarization by dynamic and time-dependent secretion of IL-12p70 (12, 31). We have shown that in vitro BM-derived DC stimulated with LPS express IL-12p35 and IL-12p40 mRNA only transiently, in line with other reports (37), while the expression of other maturation markers such as the chemokine receptor CCR7 gradually increased over time. Of note, the IL-12p40 and IL-12p35 peaked at around 12 h post-LPS activation, followed by a steep decline in relative expression. The capacity to produce IL-12p70 upon restimulation with a surrogate T cell contact provided by sCD40L also decreased with increasing duration of the LPS

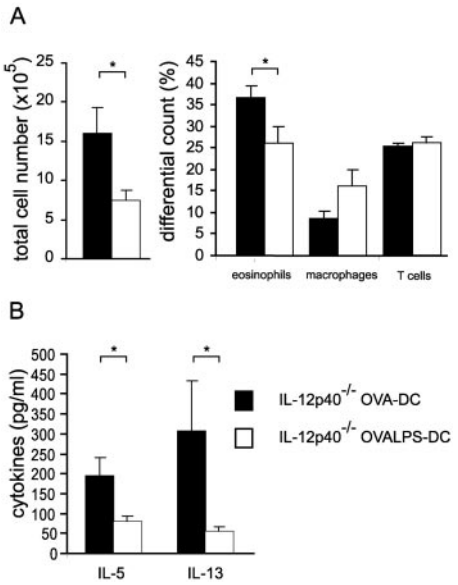

FIGURE 7. LPS-activated DC reduce airway inflammation independently of IL-12. A similar experiment as described in Fig. 6 was performed with IL-12p40$^{-/-}$ mice-derived DC transferred into IL-12p40$^{-/-}$ recipients. *A*, Cellular composition of BAL fluid. *B*, Cytokine levels in BAL fluid. Results are expressed as means ± SEM from eight mice per group. *, $p < 0.05$

stimulus, a phenomenon previously termed "IL-12 exhaustion." Surprisingly, despite exhaustion of IL-12 production, LPS-stimulated DC used as APC resulted in a more pronounced Th1 phenotype of Th cells in vitro, where individual T cells produced dramatically decreased levels of IL-4 and maintained levels of IFN-γ. Therefore, on a population level, the Th cell differentiation was skewed toward Th1, a finding also supported by the ELISA data. This was not due to a decreased strength of stimulation due to apoptosis of DC, as the overall degree of naive T cell activation was similar in both groups. Our findings are in contrast with a study of Langenkamp et al. (12), which concluded that LPS-induced IL-12 exhaustion in human monocyte-derived DC contributed to enhanced Th2 and nonpolarized central memory Th0 development. In this in vitro model, human naive T cells were stimulated by the TSST-1 superantigen on syngeneic human monocyte-derived DC, and reacting T cells were expanded for 3–9 days in neutral conditions using IL-2 before analysis of intracellular cytokine content, which might explain differences in outcome. To clarify this discrepancy further, we also examined the relevance of our findings in vivo, where DC interact with naive Ag-specific T cells in the T cell area, providing a physiologically relevant stimulus for terminal functional maturation of DC function, rather than the artificial CD40L stimulation in vitro (38, 39). To our surprise, we saw that DC that were pulsed with LPS for 24 h and subsequently injected into the lungs migrated to the draining lymph nodes and were still producing IL-12p40 36 h after injection. It is possible that in vivo, many other ligand/receptor interactions in addition to CD40/CD40L contribute to the rescue of IL-12 synthesis. As such, other members of the TNF/TNFR family have been shown to enhance the terminal differentiation of DC as they reach the T cell area (40). Not surprisingly, in vivo, the LPS-stimulated DC also induced the proliferation and differentiation of naive OVA-specific T cells toward Th1 cells producing mainly IFN-γ, whereas unstimulated DC induced T cells that made predominantly IL-4, IL-5, IL-10, and IL-13. Therefore, the in vivo data about Th polarization support the in vitro data and argue against the theory that IL-12 exhaustion would contribute to enhanced Th2 development (12). Based on the observed inconsistency between IL-12 production capacity and Th cell polarization by DC, we next studied the relevance of LPS-induced IL-12 production by DC using an established model of Th2 effector cell-driven eosinophilic airway inflammation that uses DC for immunization (6, 41). As DC can be manipulated in vitro before injection in vivo, this model is very useful to address the direct effects of LPS on sensitization via the airways, without avoiding the effects of LPS on other cells in the lungs. When DC were pretreated with LPS before injection into the trachea, in particular the allergen-induced influx of eosinophils was markedly decreased. Cytokines associated with eosinophilic airway inflammation, IL-5 and IL-13, were also decreased in the lung. Histological findings were in agreement with the BAL data, with decreased, but not totally absent, cellular infiltrates and mucus secretion. This reduced inflammation in the lung was accompanied by a shift in the cytokine profile in the draining MLN toward a Th1 type of response, with decreased levels of Th2-associated cytokines.

It has been shown that IL-12 has the capacity to down-regulate eosinophilic airway inflammation and airway hyperreactivity in mouse models of asthma, when given during the sensitization phase of the response (42–44). It has been hypothesized that IL-12 exerts its effect via induction of a Th1 type of response that reciprocally dampens the Th2 responses involved in asthma, analogous to what has been shown in models of parasitic infection (45). Moreover, immunization with DC that constitutively express high levels of IL-12 dramatically reduces eosinophilic airway inflammation (H. Kuipers, D. Hijdra, and B. N. Lambrecht, unpublished data). As LPS induced an increase in IL-12 production in DC following adoptive transfer in vivo we initially hypothesized that the development of eosinophilic airway inflammation was suppressed through the release of IL-12 in vivo. However, to our surprise, the experiments with IL-12p40 knockout mice indicated that the LPS-induced suppression of eosinophilic inflammation was independent of DC-derived IL-12. Moreover, when both the immunizing DC and the recipient mice could not produce IL-12, LPS was still capable to suppress airway inflammation. A possible explanation for this IL-12 independent effect of LPS might be that alternative pathways or cytokines were involved. Freudenberg et al. showed IL-12-independent IFN-γ production by Gram-negative bacteria via STAT4 activation with type I IFN and IL-18 (46). Other papers that reviewed the role of IL-12 in Th cell differentiation also found that Th1 cells did develop in the absence of IL-12 (16, 47). In both cases, however, IL-12 did amplify the Th1-dependent immune response. The recently described cytokine IL-27 may also be important as it induces IFN-γ production by naive T cells and is produced by APC. Importantly, its expression is up-regulated upon activation by LPS and mice deficient for its receptor TCCR have impaired Th1 responses (36, 48). Another novel candidate cytokine expressed by DC is IL-23, which shares the p40 subunit with IL-12 (49). Because the IL-12-deficient mice carry an inactivated p40 subunit, the Th1 polarizing capacity of the DC in these experiments cannot be attributed to this cytokine. One finding that also appeared from our studies using IL-12p40 knockout mice was the enhanced Th2 response that was induced in the absence of IL-12 production by DC. It is less-established how DC can polarize Th2 responses as there is very little if any evidence that they can produce the Th2 skewing cytokine IL-4 (2). Perhaps the simplest model for Th2 polarization would be one in which Th cells default to the Th2 pathway in the absence of IL-12 secretion by DC. Cholera toxin, IL-10, and PGE_2 indeed induce Th2 development by suppressing the production of IL-12 in DC in vitro (4, 50). However, in the absence of IL-12, $CD4^+$ T cell responses to

the intracellular pathogens *Toxoplasma gondii* and *Mycobacterium avium* and to the parasite *Trichinella spiralis* fail to default to the Th2 pathway in vivo (16, 17). Therefore, the exact contribution of DC-derived IL-12 to Th cell polarization is unclear and depends on the nature of the Ag and the experimental model used (4, 8, 16, 17). However, it has been suggested that deficient production of IL-12 by APC of allergic donors might be a key determinant of allergic sensitization (51, 52). In accordance with this, it was recently shown that polymorphisms in the IL-12p40 promoter gene, leading to lower levels of produced IL-12, are clearly associated with the development of atopic sensitization (53), a finding here supported by our data in the mouse model.

The findings in our study that LPS stimulation of DC during the priming alters the outcome of Th responses has implications for understanding the development of atopic sensitization. Studies of the effect of LPS on asthma appear to be complex. Epidemiological data has established a correlation between high endotoxin levels on farms and reduced likelihood of children living on these farms to develop asthma (20), while other studies found that endotoxins augment the severity of asthma once it is established (54, 55). The effect of LPS in animal models of asthma has also been conflicting, with most studies assigning a protective role to LPS (21, 22, 56), while others do not (57), probably depending on the time and route of Ag and/or LPS administration. One study implicated that the outcome of sensitization is dependent on the dose of LPS present during priming, with high doses favoring a Th1 response and low doses a Th2 response (58). However, the majority of studies that administer LPS before or during sensitization show a decrease in airway inflammation, although it is not exactly clear if this is due to an increase in Th1 effector function. Other bacterial factors that suppress development of Th2-associated sensitization and eosinophilic airway inflammation such as bacterial CpG motifs have also been shown to exert their effects independently of IL-12 and even IFN-γ, suggesting that they do not work by inducing a counterregulatory Th1 population (59). A recent epidemiological study in German children correlated current levels of LPS exposure in mattress covers with risk of atopic sensitization and diseases and found that increasing levels of LPS exposure led to reduced occurrence of atopic diseases, but also to lower levels of IL-12 production in polyclonally stimulated PBMCs, again arguing against a dominant role for IL-12 production in mediating the effects of LPS on sensitization (60). We are currently addressing whether LPS-stimulated DC reduce eosinophilic airway inflammation by inducing a particular population of regulatory T cells, which have been shown to be involved in tolerance induction in the lung (61). Indeed, other bacterial motifs such as those present in *Mycobacterium vaccae* suppress eosinophilia by such a mechanism (62).

To summarize, we have shown that LPS stimulation of DC suppresses the Th2-dependent development of eosinophilic airway inflammation independently of IL-12. In addition, the IL-12 expression levels of DC could not predict their polarizing capacity in vitro and in vivo, arguing against the recently proposed pathway of kinetics of activation, in which LPS-induced exhaustion of IL-12 secretion determines Th cell polarization.

Acknowledgments

We are grateful to K. Thielemans for providing recombinant mouse GM-CSF and C. Maliszewski for providing sCD40L. We also thank S. Manning for expert technical assistance with quantitative RT-PCR, and Nanda Vos and Monique Willart for histology work.

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