

# IL-6 Production by Pulmonary Dendritic Cells Impedes Th1 Immune Responses<sup>1</sup>

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Mucosal tissues, such as the lung, are continually exposed to both foreign and environmental Ags. To counter the potential inflammatory tissue injury of chronic Th1-mediated responses against these Ags, mucosal sites may skew toward Th2 immune responses. However, the mechanism by which this occurs is unknown. Dendritic cells (DC), as orchestrators of the immune response, skew Th1/Th2 differentiation by cytokine secretion and expression of specific cell surface markers. We compared DC from mucosal and systemic locations. In this study, we show that the lung lacks a CD8 $\alpha^+$  DC subpopulation and contains DC that appear less mature than splenic DC. Furthermore, we demonstrate that pulmonary DC produce significant levels of IL-6 and fail to produce the Th1-polarizing cytokine IL-12. Importantly, we demonstrate that IL-6 negatively regulates IL-12 production, as pulmonary DC from IL-6<sup>-/-</sup> mice produce significant levels of IL-12 and induce Th1 polarization of naive CD4<sup>+</sup> T cells. Furthermore, we demonstrate that IL-6 is sufficient to explain the differential polarizing abilities of pulmonary and splenic DC, as splenic DC cocultures supplemented with IL-6 polarize naive T cells toward Th2, and pulmonary DC cultures in which IL-6 was removed with neutralizing Ab resulted in more Th1 polarization, pointing to IL-6 as the mechanism of Th2 polarization in the lung. We propose that the Th2 response seen in the lung is due to DC-mediated inhibition of Th1 responses via IL-6 production, rather than enhanced Th2 responses, and that this regulation decreases the likelihood of chronic inflammatory pathology in the lung. *The Journal of Immunology*, 2003, 170: 4457–4464.

Dendritic cells (DC)<sup>4</sup> are highly specialized APCs, central to the initiation of Ag-specific responses by naive T cells. DC also play a critical role in mediating the differentiation of CD4<sup>+</sup> Th cells into Th1- and Th2-polarized subsets. DC in peripheral tissues, such as the lung, gut, skin, or genitourinary tract, are continually recruited from the bone marrow and exposed to Ag in these peripheral sites, where they act as sentinels, sampling the surface portals of the body. After uptake of Ag in peripheral sites, DC migrate via the afferent lymph into lymphoid organs (1), where they localize in T cell zones and interact with naive T cells to initiate Ag-specific responses (2–4). In the lung, inhalation of bacteria, virus, or protein Ags induces a wave of DC recruitment from the blood into the respiratory tract mucosa (5). DC maturation occurs following Ag uptake and results in the increased expression of the Ag-presenting molecule, MHC class II, and accessory molecules such as CD80 (B7-1), CD86 (B7-2), CD54 (ICAM-1), and CD40, and is often associated with a diminished capacity for further Ag uptake.

Current evidence suggests that several distinct DC subsets exist (6–9). Although the majority of DC in mice express CD11c (in-

tegrin  $\alpha_X\beta_2$ ), two main CD11c<sup>+</sup> DC subtypes have been suggested, termed lymphoid-related and myeloid-related DC based on the expression of the lymphoid and myeloid markers CD8 $\alpha^+$  and CD11b (Mac-1,  $\alpha_M\beta_2$ ), respectively (6, 10). The CD8 $\alpha^+$ , CD11b<sup>low</sup> DC and CD8 $\alpha^-$ , CD11b<sup>high</sup> DC are both found in lymphoid organs, such as spleen, lymph node, and thymus (7, 11); however, important functional differences that correlate with DC phenotype are emerging. CD8 $\alpha^-$  murine DC have been found to induce the activation of T cells that secrete high levels of the Th2 cytokines IL-4, IL-5, and IL-10 in vitro, while CD8 $\alpha^+$  DC-sensitized T cells have been found to produce mainly the Th1 cytokines, IFN- $\gamma$ , and IL-2 (8, 12, 13). Th1 responses to infections, such as viruses, are characterized by the production of IFN- $\gamma$ , IL-2, and delayed-type hypersensitivity, while Th2 responses to parasites and allergens are characterized by the production of IL-4, IL-5, IL-10, and IL-13, and strong IgE production (14).

Although balanced cytokine and cellular responses are the rule, chronic Th1 responses are associated with inflammatory tissue injury, and chronic Th2-polarized responses are associated with allergic disorders. A number of factors influence the Th1/Th2 differentiation of naive T cells, including the dose of Ag and the strength of interaction between the APC and the naive T cells. However, the most clearly defined factor influencing naive T cell polarization is the cytokine milieu experienced by the T cell during primary activation (14–16). IL-12 produced by APC together with IFN- $\gamma$  from memory T cells and/or NK cells has been shown to drive polarization toward Th1. In contrast, IL-4 directs the development of Th2 cells, and correspondingly inhibits the development of Th1 cells. The inflammatory cytokine IL-6 has also been implicated in Th2 polarization (17). As well as the cytokine milieu, the site of Ag exposure also influences the outcome of naive T cell polarization.

In the lung, Ag uptake leads to preferential Th2 differentiation of pulmonary T cells. This is in contrast to activation of T cells in the spleen, which typically leads to differentiation of both Th1 and Th2 effector cells in response to Ag. As the lung, in contrast to the

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<sup>4</sup> Abbreviations used in this paper: DC, dendritic cell; MFI, mean fluorescence intensity.

spleen, is continually exposed to both infectious and harmless Ags, the Th2 propensity of the lung may have evolved to counterbalance negative inflammatory effects resulting from prolonged Th1 activation. One mechanism that could explain the fine balance of pulmonary Th1 vs Th2 polarization may be the presence of a unique population of DC within the lung. We hypothesize that pulmonary DC may be innately programmed to induce Th2 polarization either by influencing the overall cytokine milieu in the lung or due to expression of a unique array of Th2-biasing cell surface markers. In this study, we contrast DC populations found in the lung and spleen, define IL-6 as a mechanism underlying the propensity of pulmonary DC to induce Th2 polarization of naive T cells, and propose a system by which the immune system regulates the adverse effects of chronic Ag exposure in the lung.

## Materials and Methods

### Mice

BALB/c mice were obtained from Taconic Farms (Germantown, NY) or bred in house at the Dana-Farber Cancer Institute (Boston, MA). IL-6<sup>-/-</sup> and B6/129sF2/J background-matched control mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed under specific pathogen-free conditions and were generally used at 8–10 wk of age.

### Monoclonal Abs

The following Abs were used as controls: rat IgG2a PE (R35-95), rat IgG2b PE (A95-1), hamster IgG PE (G235-2356), rat IgG2b biotin (A95-1), and hamster IgG biotin (G235-2356), all purchased from BD Pharmingen (San Diego, CA). The following experimental mAbs were also purchased from BD Pharmingen: anti-CD11c biotin (HL3), anti-CD11b PE (M1/70), anti-CD8 $\alpha$  PE (53-6.72), anti-CD4 PE (RM4-5), anti-CD80 PE (B7-1; 16-10A1), anti-CD86 PE (B7-2; GL1), anti-CD40 PE (3/23), anti-CD54 PE (ICAM-1; 3E2), anti-CD1d PE (1B1), MHC class II PE (M5/114.15.2), anti-CD95 ligand PE (*f*as ligand; MFL3), anti-CD3 $\epsilon$  PE (145-2C11), anti-CD19 (1D3), anti-CD45RB FITC (16A), and anti-CD28 (37.51). F4/80 PE (A3-1) was obtained from Serotec (Oxford, U.K.). Hybridomas for the following Abs were obtained from American Type Culture Collection (Manassas, VA) and grown and purified in our laboratory using ultralow bovine IgG serum: anti-CD3 $\epsilon$  (145-2C11) and anti-Fc $\gamma$ II/III (2.4G2). Streptavidin-conjugated Alexa-488 was obtained from Molecular Probes (Eugene, OR).

### Isolation of CD11c<sup>+</sup> cells from spleen and lung

Spleens were excised, injected three times with 1 ml of 100 U/ml collagenase IV in RPMI/5% FBS (Worthington Biochemical, Lakewood, NJ), and incubated for 15 min at 37°C. After incubation, spleens were transferred to new dishes containing 400 U/ml collagenase/RPMI/FBS and dissociated. The dishes containing cells in 100 U/ml collagenase were scraped, and the contents retained on ice. Dissociated spleens were subjected to two 20-min incubations at 37°C, with pipetting in between. After digestion, RBC were removed by 0.15 M NH<sub>4</sub>Cl, 0.1 mM EDTA, pH 7.4 (ACK) lysis.

To isolate pulmonary CD11c<sup>+</sup> cells, lungs were perfused with PBS to remove RBC, excised, minced, and incubated with 200 U/ml collagenase IV in RPMI/FBS for 90 min with shaking at 37°C. After digestion into single cell suspensions, cells were passed serially through a 60-mesh cup sieve and a 100- $\mu$ m nylon cell sieve.

The single cell suspensions from spleen and lung were then spun through a 44/67% Percoll gradient to remove epithelial cells and debris. CD11c<sup>+</sup> cells collected from the Percoll interface were positively selected on a MACS magnetic column per the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). The resulting splenic DC preparations were >95% CD11c<sup>+</sup> and contained <2% F4/80<sup>+</sup> cells and <3% CD19<sup>+</sup> cells. The resulting lung DC preparations were >90% CD11c<sup>+</sup> and contained <1% F4/80<sup>+</sup> cells and <3% CD19<sup>+</sup> cells, the contaminating cells morphologically resembling epithelial cells. The reagents used for isolation and culture of DC were endotoxin free, as determined by *Limulus* amoebocyte lysis assay.

To isolate CD8-depleted splenic DC, CD11c-enriched DC were negatively selected using CD8 DynaBeads (Dyna, Oslo, Norway), according to the manufacturer's instructions.

### Isolation of CD4<sup>+</sup> CD45RB<sup>high</sup> cells

Single cell splenocyte suspensions were achieved by passing spleens through a 70- $\mu$ m plastic mesh screen. After RBC lysis, CD4<sup>+</sup> cells were positively selected using CD4 DynaBeads and Detach-a-bead (Dyna) on a rocker for 20 min at 4°C, according to the manufacturer's instructions. Detached CD4<sup>+</sup> cells were stained with anti-CD4 PE and anti-CD45RB FITC, and CD4<sup>+</sup>CD45RB<sup>high</sup> cells were isolated by FACS sorting (Moflo; Cytomation, Fort Collins, CO).

### Coculture of CD11c<sup>+</sup> cells and CD4<sup>+</sup> CD45RB<sup>high</sup> cells

CD11c<sup>+</sup> cells from lung and spleen and splenic CD4<sup>+</sup>CD45RB<sup>high</sup> cells were isolated, as described above. A total of  $1 \times 10^4$  CD11c<sup>+</sup> cells/well and  $5 \times 10^4$  CD4<sup>+</sup>CD45RB<sup>high</sup> cells/well was cocultured in cRPMI (RPMI with 10% FBS, 1% HEPES, 1% L-glutamine, 1% penicillin-streptomycin, 0.1% 2-ME, and 2 nM IL-2 (Proleukin; Chiron, Emeryville, CA)) in 96-well flat-bottom plates precoated overnight with 10  $\mu$ g/ml anti-CD3. Cocultures were incubated for 7 days at 37°C, 5% CO<sub>2</sub>. After 7 days, cocultured cells were Ficolled to remove dead cells, and  $5 \times 10^4$  live cells were restimulated on plate-bound anti-CD3 (10  $\mu$ g/ml) with soluble anti-CD28 (1  $\mu$ g/well) for 48 h in 96-well flat-bottom plates, and supernatants were harvested for analysis. In the IL-6 reconstitution experiments, murine rIL-6 (1 ng/ml; BD Pharmingen) was added on days 0, 2, 4, and 6 of the initial coculture, day 0 representing the initial day of culture. For the IL-6 depletion experiments, neutralizing anti-IL-6 (20  $\mu$ g/ml; catalogue 554398; BD Pharmingen) or control Ab (catalogue 554682; BD Pharmingen) was added on days 0, 2, 4, and 6 of the initial coculture, day 0 representing the initial day of coculture.

### Direct stimulation of CD11c<sup>+</sup> cells

CD11c<sup>+</sup> cells were isolated, as described above. Cells were plated at  $1 \times 10^5$  per well (96-well flat bottom) in cRPMI (without the added IL-2) and stimulated with 0–20  $\mu$ g/ml LPS (*Escherichia coli* 026:B6; Sigma-Aldrich, St. Louis, MO) for 6 or 18 h at 37°C, and supernatants were harvested for analysis.

### Cytokine ELISAs

Cytokine levels in culture supernatants were determined using sandwich ELISAs, with Ab pairs for IFN- $\gamma$ , IL-4, IL-12p40, and IL-6, according to manufacturer's instructions (BD Pharmingen or Endogen, Woburn, MA). Plates were developed with ABTS (Sigma-Aldrich) and read on a microplate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 405 nm. Amount of cytokine was determined by comparing sample values to the linear portion of the standard curve as calculated using a four-parameter analysis. To detect IL-10, supernatants were analyzed using an IL-10 ELISA kit (R&D Systems, Minneapolis, MN). For each condition, three to six culture samples were analyzed in duplicate.

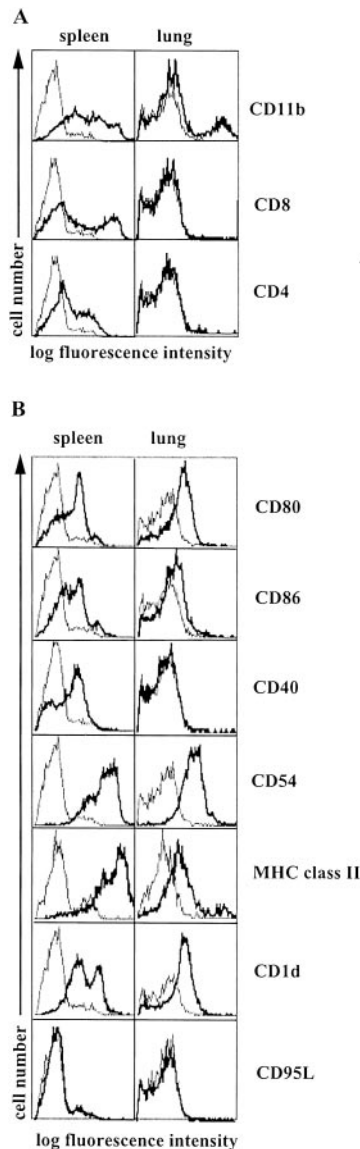
### FACS analysis of CD11c-enriched DC

Samples were analyzed using two-color staining. Briefly, CD11c-enriched cells were preincubated with 40  $\mu$ g/ml of 2.4G2 mAb with 10% FCS and 10% normal mouse serum to block FcRs and then stained with both biotin- and PE-conjugated mAbs. The cells were washed, incubated with streptavidin Alexa-488, and then analyzed by flow cytometry using a FACScan and CellQuest software (BD Biosciences, San Diego, CA). All steps were performed at 4°C to prevent receptor internalization.

## Results

### Distinct phenotypes of splenic vs pulmonary DC

Differential T cell polarization can result from stimulation by distinct DC populations, which express CD11b, CD8, or CD4, or from the activation status of the DC, defined by the expression of costimulatory, adhesion, and Ag-presenting molecules (18). Thus, to elucidate the mechanisms by which DC from distinct tissue sites mediate differential T cell polarization and, in particular, the mechanism by which pulmonary DC dampen a Th1 response, we examined DC subsets in the lung and spleen. Flow cytometric analysis of cell surface markers revealed that splenic and pulmonary DC expressed the subpopulation markers CD11b, CD8, and CD4 in dramatically distinct patterns (Fig. 1a). The integrin CD11b (Mac-1), which is often used as a marker for myeloid-type DC, was expressed at broad, intermediate levels by the majority of



**FIGURE 1.** Spleen and lung DC are phenotypically distinct. DC were analyzed by two-color flow cytometry for subpopulation markers (A) or costimulatory/activation markers (B). Cells were gated on CD11c<sup>+</sup> cells, and histograms of the second marker are shown. Thin lines represent staining with isotype-matched control Ab, thick lines represent specific Ab staining. One representative experiment of five is shown.

splenic DC (mean fluorescence intensity (MFI) = 391). In contrast, CD11b expression by the majority (85%) of pulmonary DC was very low (MFI = 27), only slightly above the negative control Ab staining. The remaining 15% of the pulmonary DC expressed very high levels of CD11b (MFI = 1300). Interestingly, the CD11b<sup>high</sup> pulmonary DC were noted to express CD11c at slightly lower levels than the CD11b<sup>low</sup> DC (data not shown), and this population was absent in splenic DC. The significance of this expression pattern is not clear, although it suggests that these CD11b<sup>high</sup>CD11c<sup>low</sup> pulmonary DC might predominantly use the CD11b adhesion molecule. Less than 1% of pulmonary DC stained for the macrophage marker F4/80, which suggests that these cells are myeloid-like DC and not contaminating macrophages.

The coreceptor CD8 $\alpha$ , which is often used to identify a lymphoid-related DC subpopulation, was also differentially expressed in pulmonary and splenic DC. The spleen contains both CD8 $\alpha$ <sup>+</sup> and CD8 $\alpha$ <sup>-</sup> DC subpopulations, with 45% of the splenic DC ex-

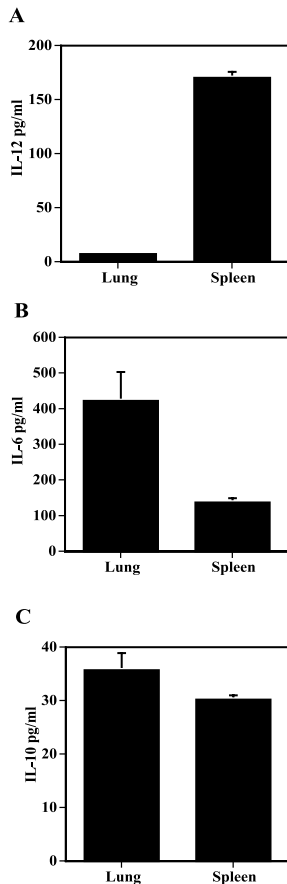
pressing CD8 $\alpha$ . In contrast, >99% of pulmonary DC were CD8 $\alpha$ <sup>-</sup> (Fig. 1a). Finally, one-third of the splenic DC population expressed CD4, which was expressed by less than 0.5% of the pulmonary DC. CD4 has recently been described as a subpopulation marker on splenic CD11b<sup>+</sup> DC, although the functional significance of this expression remains to be determined (19). Thus, the spleen and lung display strikingly distinct DC subpopulations, as defined by the expression of CD11b, CD8 $\alpha$ , and CD4.

Analysis of costimulatory molecules expressed on splenic and pulmonary DC revealed that both DC populations express similar levels of the costimulatory molecules CD80 and CD86 (MFI CD80 = 35 vs 53; CD86 = 40 vs 19, respectively) (Fig. 1b). In contrast, splenic DC display enhanced expression of several other costimulatory and adhesion molecules when compared with pulmonary DC. Expression of the costimulatory molecule, CD40, was notable, with two-thirds of the splenic DC expressing a moderate level of CD40 (MFI = 50). In contrast, pulmonary DC failed to express detectable levels of CD40 (Fig. 1b). Levels of the integrin counterreceptor CD54 (ICAM-1) were also elevated on splenic compared with pulmonary DC (MFI = 757 vs 223). In addition, the Ag presentation molecules MHC class II and CD1d were expressed at higher levels on splenic than on pulmonary DC (MFI MHC class II = 1860 vs 163; CD1d = 133 vs 57). Interestingly, all splenic DC expressed high levels of MHC class II, but only 10% of pulmonary DC expressed comparably high levels, with the majority of pulmonary DC expressing MHC class II at low levels. This suggests that only a small subpopulation of pulmonary DC is significantly activated, while the majority are not activated. Finally, neither DC population expressed CD95 ligand (Fas ligand), although this marker has been reported to be expressed on CD8 $\alpha$ <sup>+</sup> DC (20, 21). The phenotype of pulmonary DC described in this work is consistent with previously published studies that show moderate levels of costimulatory and accessory molecules, and these levels were not affected by positive selection on CD11c beads (22, 23) (data not shown). In summary, pulmonary DC are essentially devoid of CD8 $\alpha$ <sup>+</sup> DC and contain a subpopulation of cells that express high levels of CD11b. In contrast, splenic DC consistently expressed high levels of costimulatory and activation markers and contain both CD8 $\alpha$ <sup>+</sup> and CD8 $\alpha$ <sup>-</sup> subpopulations. These striking differences in DC phenotype could contribute to the differential polarization of naive T cells by splenic and pulmonary DC.

#### *LPS stimulation of splenic and pulmonary DC*

It is known that CD8 $\alpha$ <sup>+</sup> and CD8 $\alpha$ <sup>-</sup> DC can secrete differing amounts of cytokines that influence the polarization of naive T cells. Therefore, we next determined whether the phenotypic differences observed between splenic and pulmonary DC correlated with differential cytokine secretion in response to stimulation. CD11c-enriched DC from the spleen and lung were cultured for 6 or 18 h in the presence of LPS, a Gram-negative bacterial product known to activate DC via Toll-like receptor 4 (24–26). DC supernatants were collected and assayed for three DC cytokines: IL-12, which polarizes naive T cells to a Th1 phenotype (27–30); IL-6, a multifunctional inflammatory cytokine that has been implicated in Th2 polarization (17, 31); and IL-10, which down-modulates Th1 responses and has been implicated in the induction of tolerance (32, 33). The cytokines produced by splenic and pulmonary DC are strikingly different. Splenic DC secrete IL-12 at levels greater than 20-fold higher than pulmonary DC (170 vs 7 pg/ml,  $p < 0.0001$ ; Fig. 2a). In contrast, pulmonary DC, while secreting very little IL-12, secrete significant levels of IL-6, 3-fold higher than that produced by splenic DC (425 vs 130 pg/ml,  $p = 0.0254$ ; Fig. 2b). DC from the lung and the spleen were found to produce similar levels of IL-10 ( $p > 0.1$ ; Fig. 2c). Similar patterns were also



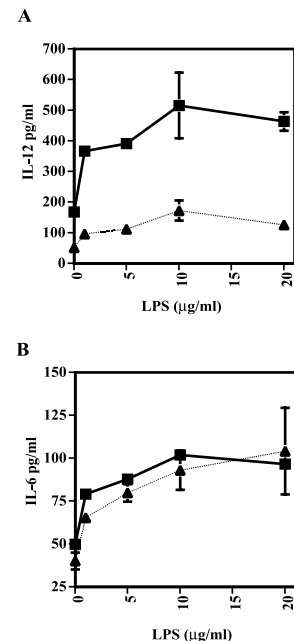


**FIGURE 2.** LPS stimulation of lung and spleen DC results in differential cytokine production. DC were isolated and cultured with LPS (10 µg/ml), as described in *Materials and Methods*. Six-hour culture supernatants were collected and assayed by ELISA for IL-12 (A), IL-6 (B), and IL-10 (C). The experiment shown is representative of four experiments performed. Error bars show the SEM of triplicate culture wells, each assayed in duplicate.

observed when lipoteichoic acid was used as a stimulus rather than LPS (data not shown). DC supernatants analyzed following 18 h of stimulation showed similar results to the 6-h stimulation, and an LPS dose-response curve indicated maximal cytokine responses at 10 µg/ml (data not shown). These data suggest that the differential IL-12 production by splenic DC and IL-6 production by pulmonary DC might selectively impact the outcome of naive T cell polarization by each of these two DC populations.

#### *Effect of CD8 $\alpha$ depletion on cytokine production by splenic DC*

Because the splenic DC population contains both CD8 $\alpha$ <sup>+</sup> and CD8 $\alpha$ <sup>-</sup> DC and the pulmonary DC population lacks the CD8 $\alpha$ <sup>+</sup> subpopulation, we sought to determine the contribution of CD8 $\alpha$ <sup>+</sup> DC to the polarization differences noted between splenic and pulmonary DC. CD8 $\alpha$ <sup>+</sup> DC are known to be potent producers of IL-12. Furthermore, CD8 $\alpha$ <sup>+</sup> DC in the spleen might impact the ability of splenic DC to produce high levels of IL-6. To examine this, CD11c-enriched splenic DC were isolated and CD8 $\alpha$ <sup>+</sup> DC were depleted by negative selection from the population. The resulting CD11c<sup>+</sup> and CD8 $\alpha$ <sup>-</sup> populations were cultured with increasing amounts of LPS, and levels of IL-12 and IL-6 produced after 6 h of stimulation were determined (Fig. 3). As expected, depletion of the CD8 $\alpha$ <sup>+</sup> DC subset led to a marked decrease in the level of IL-12 produced by splenic DC. However, depletion of CD8 $\alpha$ <sup>+</sup> splenic DC failed to enhance the production of IL-6. Thus,

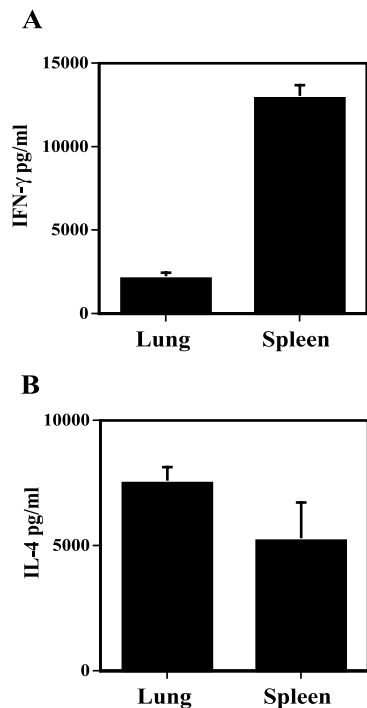


**FIGURE 3.** Depletion of CD8<sup>+</sup> cells from the splenic DC population reduces IL-12 production, but fails to enhance IL-6 production. Purified CD11c<sup>+</sup> splenic DC were isolated, and CD8<sup>+</sup> cells were depleted by negative selection. CD11c<sup>+</sup> (squares) and the CD8 depleted DC (triangles) were cultured for 6 h with 0–20 µg/ml of LPS, and the supernatants were assayed by ELISA for the production of IL-12 (A) and IL-6 (B). The experiment shown is representative of three experiments performed. Error bars show the SEM of triplicate culture wells, each assayed in duplicate.

the elevated level of IL-6 produced by lung DC is not simply due to the lack of a controlling CD8 $\alpha$ <sup>+</sup> DC population. This suggests that production of IL-6 is inherent to pulmonary DC.

#### *Polarization of naive CD4<sup>+</sup> T cells by splenic or pulmonary DC*

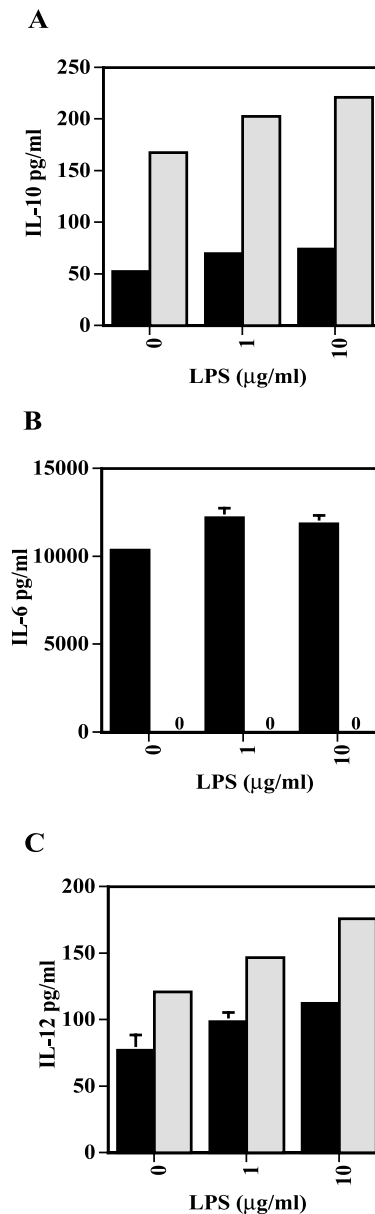
To determine whether the differences in splenic vs pulmonary DC phenotype and cytokine secretion lead to differences in naive T cell polarization, we cocultured naive CD4<sup>+</sup> T cells with splenic or pulmonary DC and assayed their Th cytokine production upon restimulation. Single cell suspensions of splenic or pulmonary DC from BALB/c mice were isolated by positive selection using CD11c beads. These DC were cocultured with naive CD45RB<sup>high</sup> CD4<sup>+</sup> splenic T cells to assess the differential impact of mucosal (pulmonary) vs systemic (splenic) DC on the cytokine polarization of naive T cells. T cells and DC were cocultured on anti-CD3-coated microtiter plates for 7 days, followed by T cell restimulation in the absence of DC with anti-CD3 plus anti-CD28 for 48 h. Supernatants from the restimulated cultures were assayed for the Th1 cytokine, IFN- $\gamma$ , or the Th2 cytokine, IL-4. Splenic DC induced naive T cells to secrete significant levels of IFN- $\gamma$ , at levels ~6-fold higher than those induced by coculture with DC from the lung (13,000 vs 2,100 pg/ml,  $p < 0.0001$ ; Fig. 4a). Pulmonary DC induced naive T cells to produce significantly more IL-4 than splenic DC ( $p = 0.0048$ ), although splenic DC also produced high IL-4 (Fig. 4b). Thus, splenic DC appear to polarize naive T cells toward a mixed Th1 and Th2 response, while pulmonary DC polarize toward a strong Th2 response, but fail to polarize efficiently toward Th1. These results are consistent with reports demonstrating the propensity of pulmonary T cells to exhibit a Th2 phenotype, but the mechanisms responsible for this phenomenon are not known (34–37).



**FIGURE 4.** Differential polarization of naive CD45RB<sup>high</sup> CD4<sup>+</sup> splenic T cells by DC isolated from lung or spleen. T cells and DC were cocultured, as described in *Materials and Methods*, and supernatants from restimulated cultures were collected and assayed by ELISA for the Th1 cytokine IFN- $\gamma$  (A) and the Th2 cytokine IL-4 (B). The figure shows one representative experiment of four performed. Error bars indicate the SEM from triplicate cultures, each assayed in duplicate.

#### *Role of IL-6 in pulmonary DC cytokine production and polarization of naive T cells*

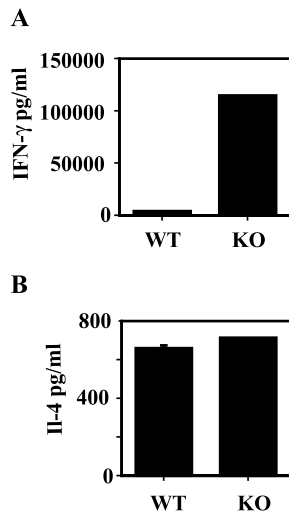
We observed that IL-6 is produced at a significantly higher level by pulmonary DC compared with splenic DC. We therefore wished to determine whether IL-6 is a mechanism of polarization away from Th1 in the lung. Using mice deficient for the IL-6 gene, we sought to determine whether IL-6 directly influences the ability of pulmonary DC to induce a Th2, but not a Th1, response from naive T cells. CD11c-enriched pulmonary DC from IL-6<sup>-/-</sup> and control B6/129 mice were isolated and stimulated for 6 h with LPS. As expected, DC from IL-6<sup>-/-</sup> mice failed to produce IL-6, while control mice produced significant levels (Fig. 5*b*). DC from IL-6<sup>-/-</sup> mice produced somewhat elevated levels of IL-10 compared with the B6/129 controls ( $p = 0.0131$ ; Fig. 5*a*), but importantly, DC from IL-6<sup>-/-</sup> mice produced significantly elevated levels of IL-12 compared with pulmonary DC from both the background-matched B6/129 and BALB/c mice ( $p = 0.0015$ ; Figs. 5*c* and 3*a*). Flow cytometric analysis of pulmonary DC from the IL-6<sup>-/-</sup> and B6/129 control mice showed that, like BALB/c, these mice do not possess a pulmonary CD8 $\alpha$ <sup>+</sup> DC subset and pulmonary DC from these mice express minimal levels of CD40 and are thus phenotypically similar to pulmonary DC from BALB/c mice (data not shown). Thus, phenotypic differences in pulmonary DC from these varying strains of mice do not account for the increased IL-12 production by IL-6<sup>-/-</sup> pulmonary DC. This finding suggests that the presence of high levels of IL-6 in the lung may act to down-modulate IL-12 production by pulmonary DC, and points to the possibility that an increased level of IL-6 in the lung may contribute to the reduced ability of pulmonary DC to



**FIGURE 5.** Effect of IL-6 on LPS-induced cytokine secretion by pulmonary DC. DC from lungs of IL-6<sup>-/-</sup> mice (□) and from WT mice (■) were isolated and cultured for 6 h with 0–10  $\mu$ g/ml of LPS. The collected supernatants were assayed by ELISA for the production of IL-10 (A), IL-6 (B), and IL-12 (C). The experiment shown is representative of three experiments performed. Error bars show the SEM of triplicate culture wells, each assayed in duplicate.

induce Th1 polarization. Without IL-6-mediated suppression, pulmonary DC produce IL-12 and may polarize naive T cells toward Th1.

To confirm this hypothesis, pulmonary DC isolated from IL-6<sup>-/-</sup> and B6/129 control mice were cocultured with naive CD45RB<sup>high</sup> CD4<sup>+</sup> T cells from B6/129, as described above. Although the absence of IL-6 had little impact on the ability of IL-6<sup>-/-</sup> pulmonary DC to influence Th2 polarization, the absence of IL-6 significantly enhanced the ability of IL-6<sup>-/-</sup> pulmonary DC to induce a Th1 response (Fig. 6). In fact, IFN- $\gamma$  induction by IL-6<sup>-/-</sup> pulmonary DC is comparable to the induction seen by splenic DC.

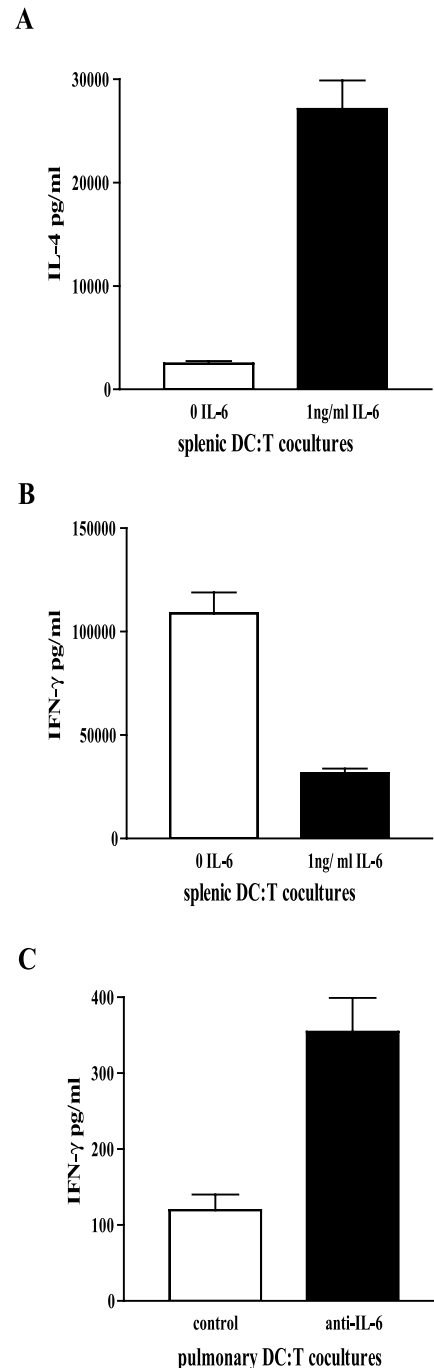


**FIGURE 6.** Effect of IL-6 on the ability of pulmonary DC to polarize naive T cells. Naive CD45RB<sup>high</sup> CD4<sup>+</sup> splenic T cells were isolated from B6/129 background controls and cultured with pulmonary DC isolated from IL-6<sup>-/-</sup> or B6/129 control mice, as described in *Materials and Methods*. Supernatants from restimulated cultures were collected and assayed by ELISA for the Th1 cytokine IFN-γ (A) and the Th2 cytokine IL-4 (B). The figure shows one representative experiment of four performed. Error bars indicate the SEM from triplicate cultures, each assayed in duplicate.

*IL-6 reconstitution of splenic DC cocultures results in a pulmonary phenotype, and IL-6 depletion of pulmonary DC cocultures results in a splenic phenotype*

To confirm that IL-6 production by pulmonary DC is the mechanism by which pulmonary DC polarize naive T cells toward Th2, we performed two related experiments. First, we cocultured naive CD45RB<sup>high</sup> CD4<sup>+</sup> splenic T cells from BALB/c mice with splenic DC, as described above, with the addition of 1 ng/ml IL-6 at days 0, 2, 4, and 6 during the primary coculture. This amount of IL-6 was within the range of IL-6 that was produced by pulmonary DC after 6 h of LPS stimulation (Fig. 2 and data not shown). In the presence of exogenous IL-6, splenic DC induced naive T cells to produce significantly more IL-4 ( $p < 0.0001$ ; Fig. 7a), and significantly less IFN-γ ( $p < 0.0001$ ; Fig. 7b) than naive T cells cocultured with splenic DC without exogenous IL-6. This result demonstrates that IL-6 is sufficient to promote Th2 polarization of naive T cells in the context of splenic DC, thus converting the splenic DC to a more pulmonary phenotype.

We also wished to determine whether we could convert pulmonary DC to a more splenic DC phenotype by removing IL-6 from the pulmonary DC cocultures with neutralizing Ab. This experiment complements the IL-6<sup>-/-</sup> pulmonary DC experiment described above, but avoids the complication of possible compensation by pulmonary DC during development in the IL-6<sup>-/-</sup> animals. Pulmonary DC were cocultured with naive CD45RB<sup>high</sup> CD4<sup>+</sup> splenic T cells, as described above, with the addition of either 20 μg/ml neutralizing anti-IL-6 Ab or control Ab at days 0, 2, 4, and 6 of the primary coculture. T cells were isolated and restimulated, as described above, and 48-h culture supernatants were assayed for IFN-γ production. Strikingly, IFN-γ production was significantly enhanced when IL-6 was neutralized in the pulmonary DC cocultures ( $p = 0.0014$ ; Fig. 7c). This implies that IL-6 is necessary to inhibit IFN-γ production in the pulmonary DC cocultures, and that IL-6 is the mechanism for this inhibition. Together, these results point to a critical role for IL-6 in controlling



**FIGURE 7.** Effect of IL-6 addition/depletion on naive T cell polarization. A and B, Naive CD45RB<sup>high</sup> CD4<sup>+</sup> splenic T cells were isolated from BALB/c mice and cultured with splenic DC, as described in *Materials and Methods*, with or without the addition of 1 ng/ml murine rIL-6 on days 0, 2, 4, and 6 of the primary coculture. Supernatants from restimulated cultures were collected and assayed by ELISA for the Th2 cytokine IL-4 (A) and the Th1 cytokine IFN-γ (B). C, Naive CD45RB<sup>high</sup> CD4<sup>+</sup> splenic T cells were isolated from BALB/c mice and cultured with pulmonary DC, as described in *Materials and Methods*, with the addition of 20 μg/ml control or neutralizing anti-IL-6 Ab on days 0, 2, 4, and 6 of the primary coculture. Supernatants from restimulated cultures were collected and assayed by ELISA for the Th1 cytokine IFN-γ. Error bars indicate the SEM from triplicate cultures, each assayed in duplicate.

the ability of pulmonary DC to promote Th2 polarization of naive T cells, and provide a mechanism for the observed Th2 skewing of pulmonary T cells observed in vivo.

## Discussion

DC play a key role in directing the differentiation of naive T cells into Th1 or Th2 effector cells. The impact of DC on T cell polarization is regulated by many factors, including the DC subset, activation status, and profile of secreted cytokines. In this study, we have examined the effect of the tissue source of DC on T cell polarization, and have found that the tissue origin of DC has a significant impact on both DC cell surface marker expression and cytokine production. We have also clearly demonstrated that IL-6 production by pulmonary DC is both necessary and sufficient for polarizing naive T cells away from inflammatory Th1 responses *in vitro*, which may provide an explanation for the Th2-biased profile of pulmonary T cells observed *in vivo*.

Mucosal sites, including the gut and the lung, are commonly referred to as Th2-promoting environments (38, 39). In the case of the lung, Constant et al. (35) found that intranasal administration of the Th1-biased Ag *Leishmania major* to a Th1-biased mouse strain, C57BL/6, resulted not in the expected Th1 response, but in a Th2 response. This result is further strengthened by studies in both rats and mice, in which respiratory tract DC were found to stimulate Th2 responses upon Ag challenge both *in vitro* and *in vivo* (34, 40). More recently, pulmonary DC have been identified as a potent source of IL-10, a cytokine crucial for the inhibition of Th1 responses and the maintenance of tolerance within the lung (33). The results presented in this work expand upon these published findings and demonstrate that the Th2-promoting potential of pulmonary DC may not be due to the promotion of Th2 differentiation *per se*, but rather due to the inhibition of Th1 differentiation caused by IL-6 production and the concomitant reduction of IL-12 production.

Several factors are likely to contribute to the limited production of IL-12 by pulmonary DC. First, the increased production of IL-6 by pulmonary DC appears to directly inhibit IL-12 production. This conclusion is supported by the finding that wild-type pulmonary DC produce minimal levels of IL-12, while pulmonary DC from IL-6<sup>-/-</sup> mice produce levels similar to those of splenic DC. IL-6, itself, is a multifunctional cytokine involved in both pro- and anti-inflammatory responses. Although IL-6 has been implicated in the differentiation of naive T cells into Th2 effectors (17), recent reports have shown that the absence of IL-6 does not abolish production of Th2 cytokines nor affect the development of Ag-specific Th2 cells (41, 42). At the same time, IL-6 has also been implicated in the direct down-modulation of IL-12, and subsequently, the Th1 immune response. IL-6 was found to inhibit T cell-independent production of IL-12 when added to LPS/IFN- $\gamma$ -stimulated DC cultures (43), and was further found to negatively regulate IL-12 levels in response to *Schistosoma mansoni* in a model using IL-6<sup>-/-</sup> mice (44). Finally, IL-6 has been found to inhibit Th1 differentiation directly, in an IL-12-independent manner, by up-regulating suppressor of cytokine signaling 1 expression in activated CD4<sup>+</sup> T cells (31). These results, together with our present findings, point to a critical role for IL-6 in regulating Th1/Th2 differentiation. This is particularly true in the case of the lung, in which airway epithelial cells are a significant additional source of IL-6 (45, 46), leading to a IL-6-rich environment in the lung.

In addition to the difference in IL-6 production, several phenotypic differences between pulmonary and splenic DC most likely contribute to the decreased Th1 polarization induced by pulmonary DC. First, the pulmonary DC population lacks CD8 $\alpha$ <sup>+</sup> DC, a DC subset known to secrete high levels of IL-12 (12, 13). Intriguingly, our data suggest that the CD8 $\alpha$ <sup>-</sup> DC present in the lung maintain an ability to produce IL-12. This is demonstrated by IL-6<sup>-/-</sup> pulmonary DC, which produce IL-12, but fail to express a significant

CD8 $\alpha$ <sup>+</sup> population. Second, pulmonary DC fail to express significant levels of the costimulatory molecule CD40. Engagement of CD40 by its counterreceptor, CD40L/CD154, expressed on T cells, is a major stimulus of IL-12 production by DC (47, 48); in the absence of this stimulation, decreased IL-12 production would be predicted. Finally, the expression of other DC costimulatory and activation markers is reduced on pulmonary DC relative to splenic DC; this suggests that DC from the lung are less mature than splenic DC. In general, less mature DC would be unlikely to polarize to Th1, as weaker, low affinity interactions between DC and T cell are thought to induce Th2, rather than Th1, differentiation (16, 49).

Clearly, Th1 immune responses are mounted in the lung in response to numerous infectious challenges such as influenza virus and tuberculosis. However, these strong Th1 responses may occur as a result of Th1 polarization at various peripheral lymphoid sites with subsequent trafficking of Th1-polarized cells to the lung. In addition, the nature of the Ag (and Toll-like receptor stimulation), uptake by macrophages rather than DC, as well as the strength of the antigenic stimulation may all impact the profile of cytokines secreted by pulmonary DC and, thus, the polarization of naive T cells (50, 51) in pulmonary lymph nodes. Given the constant antigenic exposure faced by the lung, both infectious and environmental, the balance of the immune response in the lung is critically important and must be finely regulated to avoid unwanted inflammatory or allergic responses. Our data suggest that enhanced pulmonary DC secretion of IL-6, with its pro- and anti-inflammatory properties, provides the mechanism for maintaining this fine balance in the lung.

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