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Dynamics of Dendritic Cell Phenotype and Interactions with CD4⁺ T Cells in Airway Inflammation and Tolerance¹

Timothy B. Oriss,* Marina Ostroukhova,* Carole Seguin-Devaux,* Barbara Dixon-McCarthy,* Donna B. Stolz,[†] Simon C. Watkins,[†] Brendan Pillemer,* Prabir Ray,* and Anuradha Ray^{2*}

An emerging concept is that different types of dendritic cells (DCs) initiate different immune outcomes, such as tolerance vs inflammation. In this study, we have characterized the DCs from the lung draining lymph nodes of mice immunized for allergic airway inflammation or tolerance and examined their interactions with CD4⁺ T cells. The DC population derived from tolerized mice was predominantly CD11c⁺, B220⁺, Gr-1⁺, CD11b⁻, and MHC class II^{low}, which resembled plasmacytoid-type DCs whereas DCs from the inflammatory condition were largely CD11c⁺, B220⁻, Gr-1⁻, CD11b⁺, and MHC class II^{high} resembling myeloid-type DCs. The DCs from the tolerogenic condition were poor inducers of T cell proliferation. DCs from both conditions induced T cell IL-4 production but the T cells cultured with tolerogenic DCs were unresponsive to IL-4 as indicated by inhibition of STAT6 activation and expression of growth factor-independent 1, which has been recently shown to be important for STAT6-activated Th2 cell expansion. Our data suggest that airway tolerance vs inflammation is determined by the DC phenotype in lung draining lymph nodes. *The Journal of Immunology*, 2005, 174: 854–863.

Peripheral T cell tolerance is considered to be an important mechanism that prevents unwanted immune responses to both self- and non-self-Ags. However, tolerance is breached in disease that can manifest as autoimmune or allergic disease. Various lines of evidence indicate that Th2-type cells play a central role in orchestrating the responses seen in allergic airway inflammation (1, 2). We and others identified GATA-3 to be a Th2-specific transcription factor (3, 4) and we also reported increased expression of GATA-3 in human asthma and demonstrated its importance in the development of allergic airway inflammation in mice (1, 5). In contrast to our knowledge about factors that drive Th2 differentiation and, in turn, promote allergic inflammation, much less is known about mechanisms that induce tolerance to Ags and limit unwarranted immune responses in the respiratory tract.

Whereas the role of T cells has been fairly well described, much less information is available concerning the role of dendritic cells (DCs)³ in airway inflammation. DCs are an important cell type to consider in airway inflammation since they are now commonly accepted to be the cell type that initiates priming of naive T cells (6, 7). DCs are not only considered to be crucial for initiating inflammatory immune responses, but are also thought to be inti-

mately involved in the establishment and maintenance of peripheral tolerance. Therefore, since allergic asthma in humans essentially represents a failure of immune tolerance to Ags that are common in the environment, it makes sense to carefully describe the functions of all DC subtypes that might be involved in various airway immune responses.

In the most general terms, DCs are thought to be “inflammatory” or “tolerogenic” based on their state of maturation. So-called “immature” DCs have a high capacity for endocytosing Ag, but are poor inducers of naive CD4 T cell stimulation due to relatively low expression of MHC class II and costimulatory molecules such as CD40, CD80, and CD86 (8). For this reason, immature DCs are thought to be generally tolerogenic (9). In contrast, upon maturation, DCs reduce their ability to endocytose Ags in favor of increasing their stimulatory capacity for T cells. “Mature” DCs express high levels of MHC class II and costimulatory molecules (8). This paradigm has certainly been demonstrated in the lungs since lung parenchymal DCs have an immature phenotype, while those in the draining lymph nodes under conditions of inflammation have a mature phenotype (10, 11).

In addition to the relative maturation state affecting DC function, subtypes of DCs have been described that may deliver different stimulatory signals to T cells. For example, a class of DCs expressing B220 and Gr-1 but not other lineage markers, termed plasmacytoid DCs, have been shown to possess tolerogenic qualities in both murine and human systems (12–14). Other DC subsets with tolerogenic properties such as a CD8α⁻CD4^{low} subset and a CD11c^{low}CD45RB^{high} population have been identified in lymph nodes and/or spleens of mice (15, 16). The topic of tolerogenic DCs has been recently reviewed in detail by Steinman et al. (17).

In this study, we have taken a novel approach to the induction of airway inflammation that has allowed for direct comparison of DCs from inflammatory vs tolerogenic conditions. We have identified lung lymph node DCs that are phenotypically and functionally distinct under the two conditions, suggesting that they may play crucial roles in the maintenance of airway tolerance and the induction of inflammation.

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³ Abbreviations used in this paper: DC, dendritic cell; CT, cholera toxin; Gfi-1, growth factor-independent 1.

Materials and Methods

Experimental animals

Male BALB/cByJ mice were purchased from The Jackson Laboratory. The animals were used for experiments at 8–12 wk of age. DO11.10 T cell TCR-transgenic mice were bred and maintained in the Department of Laboratory Animal Resources at the University of Pittsburgh.

Intranasal instillation and aerosol challenge

For intranasal instillation of OVA (Sigma-Aldrich), mice were lightly anesthetized with Metofane (methoxyfluorane; Medical Developments). The OVA contained 46.5 ng of LPS/mg OVA by chromogenic *Limulus* amoebocyte lysate assay (BioWhittaker), but the dose received by individual animals (4.7 ng) was substantially below the 100 ng reported to induce airway inflammation (18). One hundred micrograms of OVA was administered with (1 μ g) or without cholera toxin (CT; List Biological Laboratories). The intranasal treatments were performed daily for 3 consecutive days. Analysis of lymph node cells was performed 24 h following the final intranasal administration unless otherwise noted. To test for development of airway inflammation, the three daily intranasal treatments were followed by 7 consecutive daily, 20-min aerosol challenges with 1% w/v OVA in sterile PBS using an ultrasonic nebulizer (Omron Healthcare) beginning 5 days after the last intranasal treatment.

Assessment of airway inflammation

The development of airway inflammation was assessed by common criteria as previously described (5).

Cell purification

Whole lung draining lymph nodes were harvested from animals using a dissecting microscope. The nodes collected included the anterior mediastinal, posterior mediastinal, and parathymic. Cells obtained from these nodes were routinely examined by flow cytometry for absence of CD4/CD8 double-positive cells as a control for accidental inclusion of thymic tissue (data not shown). The lymph nodes were physically disrupted by pressing through a 70- μ m nylon mesh.

Lung lymph node DCs were prepared by first removing macrophages and most lymphocytes by centrifugation on 14.5% metrizamide (Accurate Chemical & Scientific) gradients (12). Residual cell contamination consisted primarily of T and B cells as determined by flow cytometry (data not shown). These were eliminated by positive selection of DCs using anti-CD11c magnetic beads (Miltenyi Biotec) with multiple rounds of purification using an AutoMACS automated magnetic cell sorter (Miltenyi Biotec).

Splenic CD4 T cells from DO11.10 TCR-transgenic mice were prepared by negative selection using a CD4 isolation kit (Miltenyi Biotec) and multiple rounds of magnetic sorting using AutoMACS (Miltenyi Biotec). Anti-MHC class II magnetic beads (Miltenyi Biotec) were included in the purification procedure to guarantee that all APCs were removed from the T cell preparations. Purified DO11.10 T cells were routinely cultured with specific peptide alone to ensure that all APC activity had been removed (data not shown). Lymph node T cells were prepared from pellets of 14.5% metrizamide gradients after physical disruption of lung draining lymph nodes as described above.

Naive spleen DCs were prepared using lymphocyte separation medium centrifugation followed by positive selection of CD11c-expressing cells, as a generic source of APC for restimulation of lymph node T cells.

Cell culture

Cells were cultured in six-well plates in RPMI 1640 medium (Invitrogen Life Technologies/Invitrogen) supplemented with 10% heat-inactivated (60°C, 30 min) FBS and penicillin/streptomycin. Purified DO11.10 TCR-transgenic T cells ($2\text{--}3 \times 10^6$ /well) were cultured with purified DCs (5×10^4 cells/well) in a total culture volume of 2 ml. Specific antigenic peptide (OVA aa 323–339) was included at a concentration of 5 μ g/ml. In experiments using lymph node T cells and naive spleen DCs, whole OVA Ag was used at 100 μ g/ml and the DCs were incubated with the OVA for 2 h before adding T cells.

Proliferation assays were performed in 96-well plates with 3×10^4 T cells/well. OVA peptide was used at 5 μ g/ml and the number of DCs/well was varied. These assays were incubated for 5 days with 1 μ Ci/well [3 H]thymidine (PerkinElmer) added during the final 18 h of culture, at which time the cells were harvested onto filters for scintillation counting.

Flow cytometry

Analysis of cell surface molecules was performed by flow cytometry. Samples ($1 \times 10^4\text{--}1 \times 10^5$ cells/tube) were stained with appropriately diluted mAb in 100 μ l of FACS buffer (PBS, 2% FBS, and 0.02% sodium azide) for 10–15 min. In cases where biotinylated Abs were used, the cells were washed with FACS buffer and then incubated with streptavidin-Tricolor secondary reagent (Caltag Laboratories). Appropriate isotype control Abs were used to set the position of cursors on dot plots and background staining was subtracted in cases where percentages are presented.

The Abs used in these studies were grouped according to species; fluorochrome and isotype were as follows: biotinylated hamster IgG1 and biotinylated anti-CD11c (BD Pharmingen); rat IgG2a-FITC (BD Pharmingen) and anti-MHC class II-FITC (BD Pharmingen); rat IgG2a-PE, anti-CD40-PE, anti-CD80-PE, anti-CD86-PE, anti-B220-PE, and anti-CD19-PE (BD Pharmingen); rat IgG2b-PE, anti-CD11b-PE, anti-F4/80-PE, and anti-Gr-1-PE (BD Pharmingen); and rat IgG1-PE and anti-CD14-PE (BD Pharmingen).

Preparation of nuclear extracts and immunoblot analysis

Nuclear and cytoplasmic extracts were prepared at the indicated time points and analyzed by immunoblotting techniques essentially as previously described (3, 5, 19). The purity of nuclear extracts was verified by lack of expression of β -tubulin (Santa Cruz Biotechnology) which is reported to be expressed exclusively in the cytoplasm (20, 21). Densitometry was performed using a Kodak Imaging Station and 1D software.

RT-PCR

To assess cytokine expression in DCs, RT-PCR were performed with primers specific for murine IFN- γ , IL-6, IL-10, and GAPDH used at 50 pmol. Reactions were amplified for 35 cycles.

The primers (5'–3'), annealing temperatures, and product sizes for the RT-PCR were as follows: IFN- γ , TGCATCTTGGCT-TTGCAGCTCTTCCTCATGGC and TGGACCTGTGGGTTGACCTCAAACCTTGGC, annealing temperature 59.9°C, product size 365 bp; IL-6, CTGCAAGAGACTTCATCCAGTT and GAAGTAGGGAAGGCCGTGG, annealing temperature 51.9°C, product size 70 bp; IL-10, ATGCAGGACTTTAAGGGTTACTTGGGTT and ATTTCCGGAGAGAGGTACAAACGAGGTTT, annealing temperature 58°C, product size 465 bp; growth factor-independent 1 (Gfi-1), CTGCTACAAGAGGAGGCATCACCTCTTA and GGAAGCACAGAACA CAGGCTCTAGCTAT, annealing temperature 58.3°C, product size 429 bp; and GAPDH, AGGTTGTCTCCTGCGACTTC and CTTGCTCAGTGT CTTGTGCTG, annealing temperature 67°C, product size 211 bp.

The PCR products were run on 1% agarose gels, visualized by ethidium bromide staining, and were verified to consist of a single band of the expected size.

Measurement of cell culture supernatant cytokines

Cell culture supernatants to be assayed for cytokines were stored at -70°C before use. Cytokine concentrations for IL-4, IL-5, IL-10, and IL-13 were measured using commercially available ELISA kits (R&D Systems).

Measurement of OVA-specific serum IgE

Peripheral blood was collected from animals at the time of sacrifice following intranasal treatments and aerosol challenge as described above. Serum was isolated by centrifugation and was stored at -70°C until assay for OVA-specific IgE by ELISA. The IgE ELISA was performed as follows: First, Immulon 2 microplates (Dynatech) were coated overnight with purified rat anti-mouse IgE (4 μ g/ml; Southern Biotechnology Associates). Serum samples were serially diluted in blocking buffer and incubated overnight at 4°C. Biotinylated OVA (0.25 mg/ml; biotinylated using a Biotin-X-NHS kit; Calbiochem) was added and incubated for 1 h at room temperature. The plates were incubated with avidin peroxidase (1/20,000; Sigma-Aldrich) for 30 min at room temperature. The plates were extensively washed between steps. The substrate peroxidase substrate (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; Sigma-Aldrich) was added and after a 30-min incubation at room temperature, color development was stopped using 1% SDS in PBS. Color development was measured using a microplate reader at 405 nm.

Scanning electron microscopy and cytospins

For scanning electron microscopy, cells were allowed to settle onto glass coverslips in PBS for 1 h at 37°C. The cells were then fixed for 10 min with glutaraldehyde, washed with PBS, and then stored at 4°C. Further processing and analysis was performed by the Center for Biologic Imaging at the University of Pittsburgh.

Cytospins were performed using a Cytospin3 centrifuge (Thermo Shandon). Cells were stained with Hema-3 reagents (Fisher Scientific) according to the manufacturer's recommendations.

Results

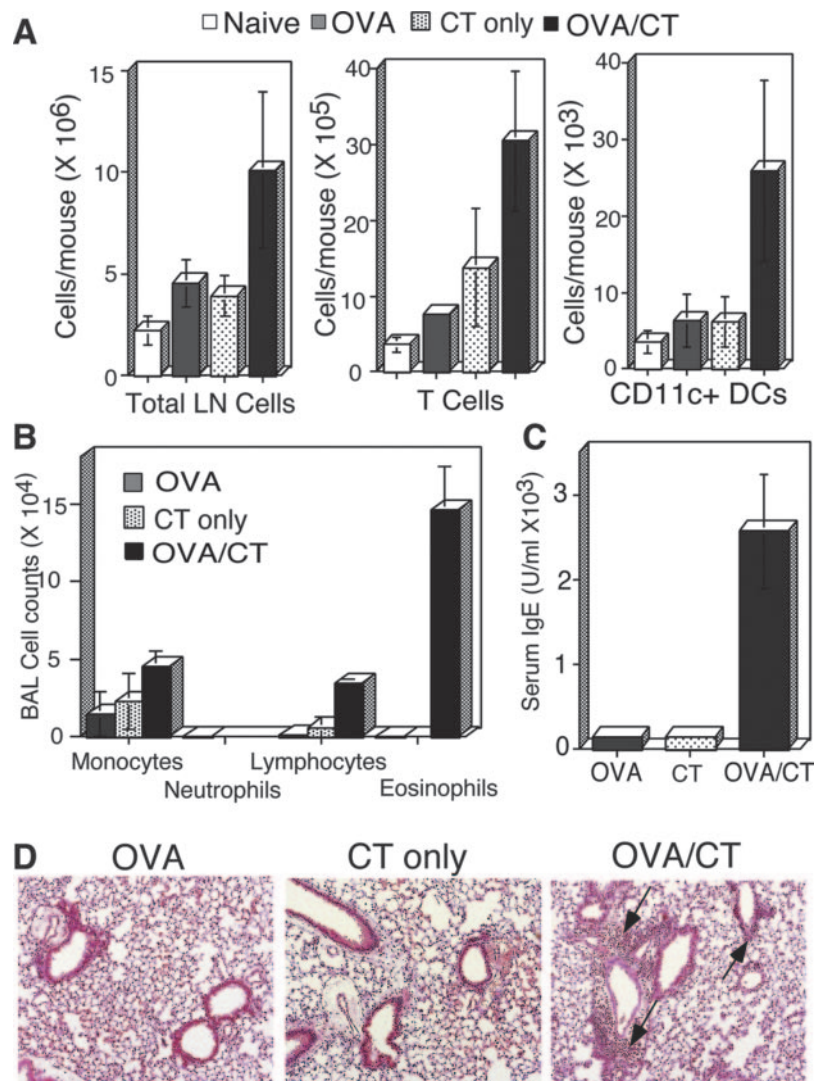
Inclusion of CT in an intranasal immunization protocol induces airway inflammation

It has been reported that three daily intranasal administrations of a high dose of OVA leads to Ag-specific tolerance (22), and further that this tolerance may be mediated by the induction of IL-10-producing regulatory T cells (23). Using an identical protocol, we have observed similar induction of tolerance to OVA in the lungs. Numerous attempts using booster immunizations (additional intranasal treatments with OVA alone) at various time intervals failed to induce any evidence of an immune response or airway inflammation, indicating the strong and stable nature of this tolerance. It has been previously reported that bacterial LPS-containing OVA results in airway inflammation in the absence of any other adjuvant(s) delivered via the intranasal route at a dose of 100 ng (18, 24). The OVA used in our studies contained an LPS dose of only 4.7 ng; therefore, it is apparent that this amount of LPS is functionally irrelevant in our hands, as it likely was in the hands of Umetsu and coworkers (22, 23) since we both describe tolerance induction in response to intranasal OVA. Therefore, we have used

this intranasal protocol as the model of tolerance induction throughout the remainder of this article.

To induce airway inflammation, we intranasally delivered the Ag OVA mixed with the mucosal adjuvant CT that has been shown to promote Th2 responses (25, 26). CT has been used in other intranasal systems to induce Th2 responses (27–29) and it may act to enhance the Th2-promoting capacity of DCs (30–32), possibly via its interaction with DC cell surface GM1-ganglioside (33). After three daily administrations of the OVA/CT mixture, exposure of the animals to inhaled OVA for 20 min/day for 7 consecutive days caused all of the hallmarks of allergic airway inflammation, including a dramatic increase in the number of total lymph node cells, CD4⁺ T cells, and CD11c⁺ DCs (Fig. 1A). OVA alone and CT alone did result in some expansion of lymph node cells compared with naive animals, suggesting that some degree of active response did occur (Fig. 1A) but only OVA/CT resulted in eosinophilic airway inflammation. After the three consecutive intranasal treatments, when mice were challenged with aerosolized OVA daily for 1 wk, animals that were immunized with OVA and CT alone showed minimal lymphocytic and no eosinophilic infiltration in the airways as observed by sampling of the bronchoalveolar lavage fluid (Fig. 1B). Serum OVA-specific IgE levels were also low (Fig. 1C) and tissue histology failed to

FIGURE 1. CT combined with OVA induces airway inflammation. Animals were given three daily consecutive intranasal treatments with OVA alone or combined with CT. These treatments were followed after 5 days by seven daily treatments with aerosolized OVA. **A**, Lymph node cell counts 1 day after the three intranasal OVA, CT alone, or OVA/CT instillation treatments. Naive animals were age-matched but received no treatments. Data shown are the mean \pm SD of at least three experiments. **B**, Differential cell counts in the bronchoalveolar lavage of animals following intranasal treatments and aerosol challenge. Three hundred total cells were counted per sample, and the proportions obtained were used to calculate the number of cells per animal. Data shown are the mean \pm SD of at least three to five animals per group. **C**, OVA-specific serum IgE as determined by ELISA. Data shown are the mean \pm SD of at least three to five animals per group. **D**, Histology showing inflammation around bronchovascular bundles in OVA/CT-treated (arrows) but not in OVA- or CT-treated animals. Data are representative of at least three independent experiments.



show any evidence of inflammation (Fig. 1*D*). In comparison, mice that were immunized with OVA/CT displayed all of the features of allergic inflammation, including increases in airway eosinophilic and lymphocytic infiltration, serum IgE levels, and inflammation around bronchovascular bundles (Fig. 1, *B–D*). No evidence of airway inflammation was seen in animals given aerosol treatments alone (data not shown). Therefore, as cited in numerous publications, CT is an effective Th2-inducing adjuvant in the lungs when delivered intranasally along with OVA.

Since we have previously shown that a Th2 response characterized by increased GATA-3 expression and production of Th2-specific cytokines is important for the initiation and orchestration of the allergic response (5), we next investigated whether T cells isolated from the lung draining lymph nodes of the three groups of animals, naive, OVA, and OVA/CT, differed with respect to the markers of a Th2 phenotype. Toward this end, on days 1, 3, and 5 after the last OVA or OVA/CT intranasal instillation, lymph node T cells were isolated and cultured *in vitro* with naive spleen DCs and whole OVA protein to stimulate recall responses from previously activated T cells and thus to amplify cytokine concentrations. At the end of the 5-day culture period, nuclear extracts were prepared to investigate GATA-3 expression and IL-5 production was assessed in the culture supernatant. As shown in Fig. 2, neither GATA-3 expression nor IL-5 production was detected in T cells derived from the lymph nodes of naive animals, indicating (as expected) absence of any recall response to Ag for T cells from naive animals (Fig. 2). Animals that had received OVA only to induce tolerance showed an initial low level of GATA-3 expression that was subsequently down-regulated (Fig. 2*A*). No IL-5 production was detected from this group of T cells (Fig. 2*B*). In the T cells derived from the OVA/CT group, GATA-3 expression and IL-5 production were found to steadily increase with time (Fig. 2). IFN- γ levels were similar under both conditions (data not shown), again demonstrating the Th2-inducing potential of CT as an adjuvant in this mode. The OVA/CT combination that we have used in this study is therefore a robust protocol to study DC-T cell interactions in the respiratory tract. Since DCs are known to initiate primary T cell responses, we hypothesized that there would be quantitative and/or qualitative differences in lung lymph node DCs between the tolerogenic (OVA) and immunogenic (OVA/CT) conditions.

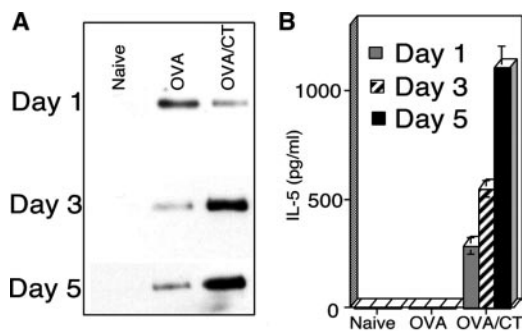


FIGURE 2. CT induces Th cells in the lung draining lymph nodes with a Th2 phenotype. Animals were given three daily consecutive intranasal treatments with OVA alone or combined with CT. Naive animals were age-matched but received no treatments. One, 3, and 5 days after the last treatment, the lymph nodes were harvested and CD4 T cells were purified. The cells were stimulated *in vitro* with whole OVA presented by naive spleen DCs for 5 days, at which time the cells were harvested for examination of GATA-3 expression by immunoblotting (*A*) and analysis of IL-5 expression in the culture supernatant by ELISA (*B*). Data are representative of at least three independent experiments.

Phenotype of lung draining lymph node DCs under tolerogenic and inflammatory conditions

Lung draining lymph node DCs were isolated from mice by a two-step purification process. Typically, DCs are isolated based on their expression of the cell surface marker CD11c following enzymatic digestion of lymph nodes. We found that simple physical disruption of lymph nodes yielded populations of cells with much higher viability and provided higher quality cells for flow cytometry staining compared with enzymatic digestion. Also, since CD11c is also strongly expressed on alveolar macrophages (34) and these cells reportedly can migrate to the lymph nodes (35), we took the precautionary step of centrifugation on metrizamide gradients to minimize macrophage contamination before performing CD11c selection. When isolating DCs from lung tissue or lung draining lymph nodes, we found that metrizamide gradient centrifugation, which has been used to fractionate DCs (12, 13, 36), efficiently separates macrophage contamination in the pellet fraction with DCs fractionating at the interphase as revealed by scanning electron microscopy. We viewed this as an important step in our studies for various reasons. First, alveolar macrophages have been shown to inhibit the Ag-presenting function of DCs (37, 38). Second, in addition to CD11c, DCs and macrophages can share other markers as well, including those previously thought to be lineage specific (34, 39). The cells resulting from this two-step purification were routinely highly purified for CD11c (>95%, see also Fig. 4*A*) with morphologic characteristics typical of DCs as determined by cyospin (Fig. 3, *A* and *B*) and scanning electron microscopy (Fig. 3, *C* and *D*). DCs from tolerized animals tended to be smaller (approximately one-half as big; cf 1- μ m bars in Fig. 3, *C* and *D*), somewhat rounder, and with fewer large dendritic processes relative to OVA/CT DCs. The more “dendritic” appearance of OVA/CT DCs was an indication of either increased activation/maturation of DCs or the influx of a different DC population into the lymph nodes as a result of the CT treatment. Importantly, scanning electron microscopic analysis failed to reveal cells that morphologically resembled monocytes/macrophages in DC preparations from either group of animals.

RNA was isolated from purified DCs from the two groups of animals for semiquantitative RT-PCR analysis of cytokine expression. The DCs from the two groups expressed similar levels of several cytokine RNAs (shown is IFN- α). DCs from the inflammation group appeared to express more IL-6 (Fig. 3*E*), and neither DC preparation expressed detectable IL-10 (Fig. 3*E*). Akbari et al. (22, 23) reported IL-10 production by a CD11c⁺ population of APCs in OVA-tolerant animals that mediated the generation of regulatory T cells. As noted above, their purification procedure did not provide for removal of macrophages that could produce IL-10. Even if there were no macrophages present in their preparations, these authors do mention that IL-10 production was transient, and it is apparent from their data that it can vary greatly between experiments (22). It would be interesting to determine whether macrophage-derived IL-10 is involved in tolerance induction, whether certain conditions are necessary for DC IL-10 production, and/or whether there are IL-10-independent, DC-mediated mechanisms of regulatory T cell generation in the lung draining lymph nodes. No IL-4 expression has been described in DCs and we similarly found none in the DCs from either group. It is interesting that IL-6 levels were higher in DCs from the inflammation group given that IL-6 has been shown to be a Th2-promoting cytokine (40) and that IL-6 produced by DCs has been shown to overcome inhibition by regulatory T cells (41).

Next, the stimulatory capacity of the DCs was assessed by their ability to induce proliferation of CD4⁺ T cells from DO11.10

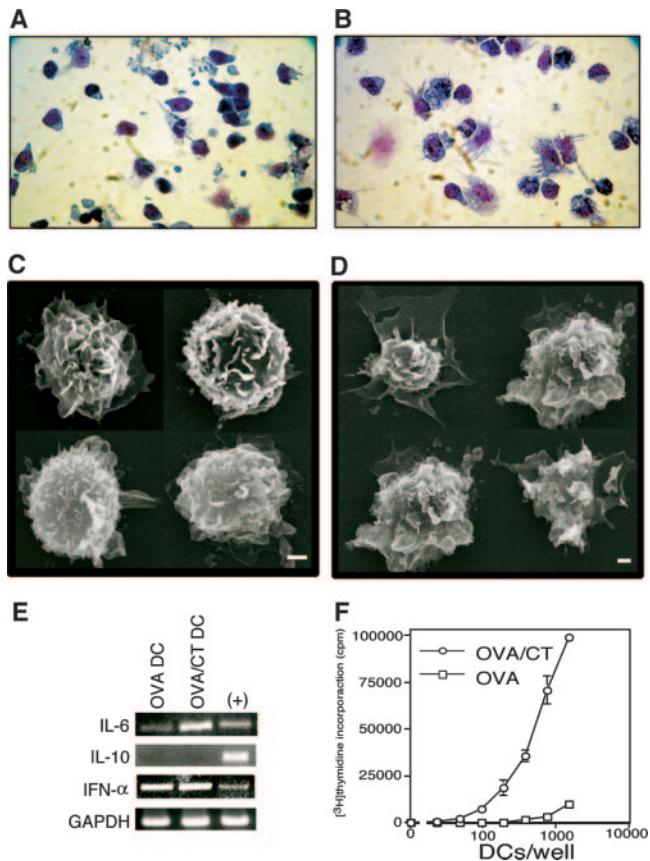


FIGURE 3. Phenotype of lung draining lymph node DCs from OVA- and OVA/CT-treated animals. DCs were evaluated by several criteria: Cytopins and light microscopy of OVA (A) and OVA/CT DCs (B); scanning electron microscopy of OVA (C) and OVA/CT (D) DCs (bar, 1 μ m); and RT-PCR for IL-6, IL-10, and IFN- α (E) using RNA isolated from purified DCs. The positive control is RNA isolated from T cells stimulated under neutral conditions in vitro. F, Proliferation of DO11.10 TCR-transgenic T cells induced by OVA and OVA/CT DCs. Data are representative of at least three independent experiments.

TCR-transgenic animals. In this assay, the DCs were loaded with the specific peptide for the DO11.10 TCR instead of the whole OVA Ag to avoid differences due to uptake, processing and presentation of Ag. The intention was to evaluate the DCs in their immediate ex vivo state. DCs from OVA/CT-treated animals had a much greater ability to induce proliferation of DO11.10 T cells compared with those obtained from OVA-treated mice (Fig. 3F).

The cell surface phenotype of DCs was evaluated by flow cytometry, which revealed at least two populations of DCs in the lung draining lymph nodes based upon CD11c and MHC class II staining in both groups of mice (Fig. 4A). Each population had similar levels of CD11c expression but differed in the level of expression of MHC class II. The two populations were present, albeit in different proportions, in the two conditions. The phenotype of the cells with lower MHC class II expression was consistent with plasmacytoid-type DCs: Gr-1⁺, B220⁺, and CD19⁻ (Fig. 4B). The other population with higher MHC class II expression was consistent with myeloid-type DCs (Fig. 4B). Both populations were essentially negative for the monocyte/macrophage markers F4/80 and CD14 (it should be noted that F4/80 has been detected on some DC subsets from specific locations). The light scatter properties of the two DC populations revealed that the plasmacytoid DCs were generally smaller than the myeloid DCs (data not shown), which is also consistent with the scanning electron

microscopic data showing that OVA DCs tended to be smaller than OVA/CT DCs (Fig. 3). The cell surface phenotype of the two populations of the DCs that are shown in Fig. 4B are stains of DCs from the lymph nodes of OVA/CT-treated animals but the profiles were essentially identical for cells from the other conditions (data not shown).

Although both DC populations, plasmacytoid and myeloid, were present under conditions of inflammation and tolerance, the real difference between the two groups was in the proportions of these populations. As shown in Fig. 4A, mice in the tolerance group had a higher proportion of plasmacytoid-type DCs (69.3% vs 30.7%), and the opposite was true with mice treated for inflammation (28.5% vs 71.5%). Naive animals and those treated with CT alone had proportions of plasmacytoid to myeloid DCs that were similar to OVA-treated mice. This suggests that inflammation-promoting conditions provided with the Ag/adjuvant combination (but not with adjuvant alone) induce an influx of myeloid-type DCs bearing costimulatory molecules into the lung draining lymph nodes, which could explain the inflammatory nature of the lymph nodes under this condition.

The percentages obtained by flow cytometry shown in Fig. 4A were combined with absolute cell counts of purified DCs to arrive at the data presented below the respective dot plots in Fig. 4A. These data show that treatment of animals with CT or OVA alone appears to result in a small influx of both plasmacytoid and myeloid DCs, although neither treatment results in airway inflammation upon challenge (Fig. 1). Inclusion of CT with OVA results in a modest increase in the number of plasmacytoid DCs (1.7-fold higher than OVA alone), but a substantial increase in myeloid DCs (8.5-fold higher than OVA alone).

The expression of costimulatory molecules on plasmacytoid and myeloid DCs was then assessed (Fig. 5). Neither population expressed appreciable amounts of CD80, CD40 and CD86 were expressed on a higher percentage of myeloid DCs compared with plasmacytoid DCs regardless of whether the cells were isolated from OVA- or OVA/CT-treated animals. This was consistent with the proposed function of the more immature plasmacytoid DCs as tolerance-inducing APCs (13) and that, in our assays, much lower T cell proliferation was observed when OVA DCs were used as APCs (Fig. 3F). It appears therefore that the surface phenotype of both types of DC is similar in the lymph nodes under conditions of tolerance or inflammation. However, the most important finding in the phenotypic analyses was a predominance of plasmacytoid DCs in the lung draining lymph nodes of animals that received OVA alone compared with an abundance of myeloid DCs in the lymph nodes of OVA/CT-treated animals. This difference between inflammation and tolerance seemed logical since the higher level of expression of costimulatory molecules on myeloid DCs can be expected to confer greater T cell stimulatory capacity to this population.

Functional differences in DCs isolated from the lung draining lymph nodes of mice treated for inflammation or tolerance

Given the low level of T cell proliferation induced by lung draining lymph node DCs from mice treated with OVA only (Fig. 3F), we sought to determine whether lack of expansion of T cells was simply a failure of the DCs to stimulate the cells or whether another mechanism(s) was involved. Toward this end, we examined the expression of nuclear transcription factors associated with the development and maintenance of Th2 cells as well as Th2 cytokine production after stimulation with DCs obtained from both tolerance (OVA) and inflammation (OVA/CT) conditions.

Lung draining lymph node DCs from the two conditions were used to stimulate splenic CD4⁺ T cells isolated from naive

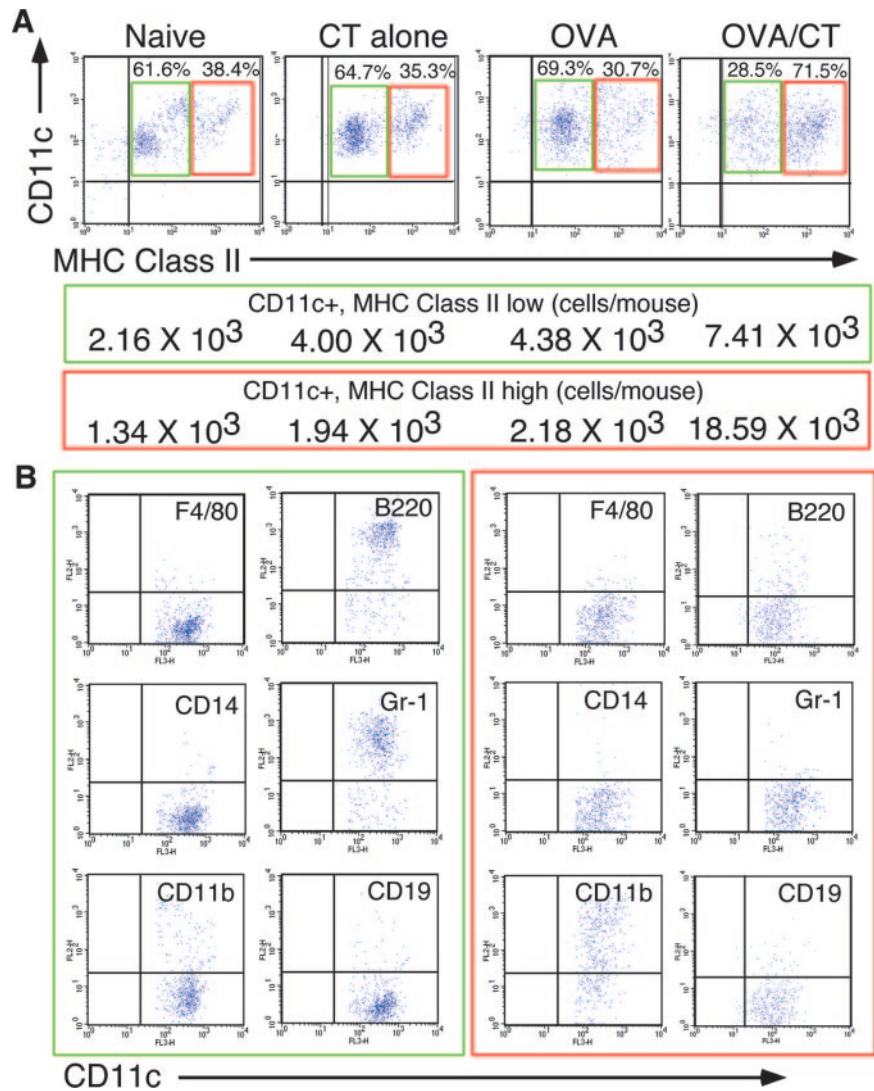


FIGURE 4. Flow cytometric analysis of lung draining lymph node DCs. DCs were isolated from naive animals and those treated with CT alone, OVA, or OVA plus CT. *A*, Expression of MHC class II and CD11c revealing two populations of DCs (MHC class II^{high} and MHC class II^{low}) with the proportions of each indicated. Each dot plot contains ~1000 events. Using total DC cell counts and these percentages, the number of each DC type was calculated in the four groups of animals and the numbers are presented under the corresponding dot plot. They are divided into two groups which are CD11c⁺MHC class II^{low} (green box) and CD11c⁺MHC class II^{high} (red box). *B*, Expression of the indicated cell surface markers on CD11c⁺MHC class II^{low} DCs (green box) and CD11c⁺MHC class II^{high} DCs (red box). In all cases, the positioning of the quadrants was determined using staining with appropriate isotype control Abs. Data are representative of at least three independent experiments.

DO11.10 mice. Peptide specific for the DO11.10 TCR was used to minimize the chance of altering the phenotype of DCs (e.g., inducing maturation) that could occur during uptake, processing and presentation of whole Ag in *in vitro* culture (42). Thus, this experiment was designed to assess the stimulatory capacity of DCs immediately *ex vivo* with regard to expression of MHC class II, costimulatory molecules, and any other factors (e.g., cytokines) that might be involved in T cell activation. The T cells were subsequently examined for the expression of GATA-3, which is critical for Th2 development (3, 4, 43), and STAT6, which is a major component of the IL-4 signaling pathway in Th2 cells (44–46). Expression of STAT6 and GATA-3 was somewhat higher in response to OVA/CT DCs 48 h after primary stimulation (Fig. 6A). As observed with lymph node T cells (Fig. 2A), DCs from naive animals were not able to up-regulate GATA-3 (Fig. 6A). To ensure the purity of the nuclear extracts and thus to conclude that the STAT6 expression observed was attributable to activation and nuclear translocation, we reprobated the blots for β -tubulin which is expressed exclusively in the cytoplasm (20, 21). Used as a negative control, incubation of any DC type with T cells in the absence of antigenic peptide routinely gave no detectable responses (data not shown). We next subjected the cells to a second round of stimulation to determine the status of transcription factor expression in the two sets in more committed cells. After 5 days of primary stimulation, equal numbers of T cells were taken from the

two groups (far more cells were detected in the inflammation group due to rapid proliferation of cells which required adjustment of cell numbers in the secondary stimulation) and restimulated with the same type of freshly isolated DC population as in the primary stimulation. As shown in Fig. 6C, enhanced differences were noticed between the transcription factor profiles of the two sets with GATA-3 and STAT6 being expressed at higher levels after T cell stimulation with OVA/CT DCs. The opposite profile of STAT6 levels in nuclear vs cytoplasmic fractions in T cells stimulated with OVA vs OVA/CT DCs highlights a blockade in STAT6 signaling by OVA DCs.

The cytokine levels in the supernatants of the secondary cultures produced from equal numbers of T cells were comparable between OVA and OVA/CT DC stimulations. In the particular experiment shown, the level of IL-4 was higher with OVA/DC stimulation (Fig. 6B). Rather than reflecting lower IL-4 production, it is likely that this was the result of more IL-4 consumption in the inflammation group which would be consistent with a much higher rate of T cell proliferation (Fig. 3F) and its well-known role in Th2 expansion. Regardless, it appears that lack of T cell proliferation in response to OVA DCs cannot be explained merely by a failure to actively engage the cells. Some degree of stimulation occurs, leading to cytokine production, but it appears there is little STAT6 signaling when T cells are stimulated by OVA DCs. Therefore,

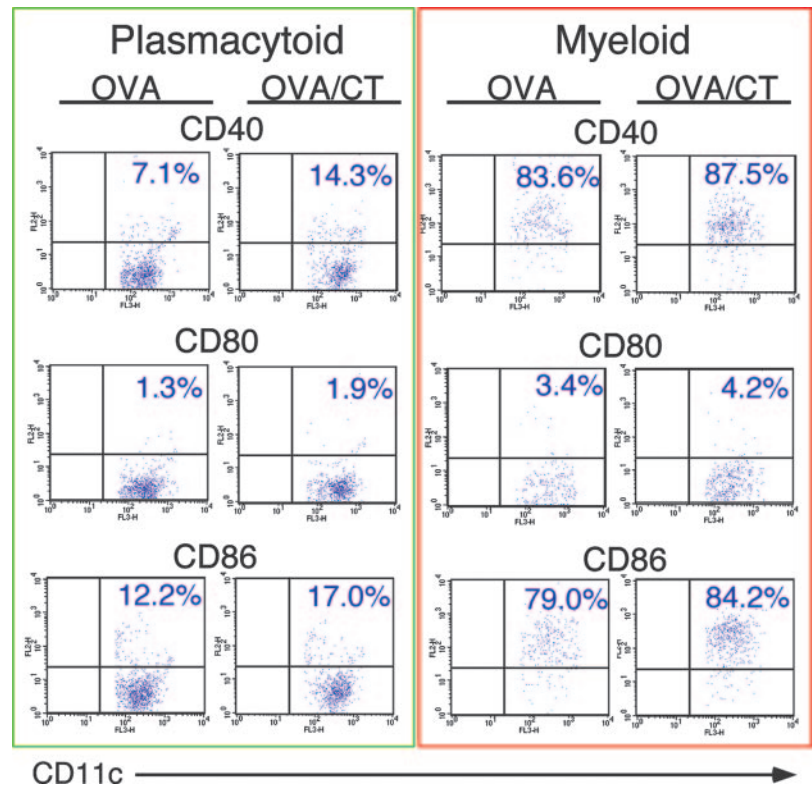


FIGURE 5. Costimulatory molecule expression on lung draining lymph node DC subtypes. Animals were treated and DCs were purified as described in the legends to Figs. 3 and 4. The cells were stained with anti-MHC class II and anti-CD11c Abs to identify the MHC class II^{low} (plasmacytoid) and MHC class II^{high} (myeloid) DC subtypes described in Fig. 4. After gating on these two populations, the expression of costimulatory molecules CD40, CD80, and CD86 was assessed on each (plasmacytoid, green box; myeloid, red box). The percentages indicated were obtained after subtracting background staining obtained using appropriate isotype control Abs. Data are representative of at least three independent experiments.

even though IL-4 is produced, there may be a block in the signaling pathway for this cytokine resulting in the observed minimal T cell proliferation. Whether this represents generation of T regulatory cells is a question for further investigation.

Although STAT6 is an important factor for Th2 differentiation, recent reports have demonstrated its importance in cell proliferation as well (47–49). To further analyze the apparent lack of signaling through the IL-4/STAT6 pathway in T cells stimulated with OVA DCs, despite the presence of adequate IL-4 in culture supernatants, we evaluated expression of Gfi-1 in the DC-stimulated T cells. Gfi-1 has been reported to strongly promote Th2 proliferation and is also a STAT6-dependent transcription factor (48, 49). Therefore, if the STAT6 pathway was truly dysfunctional, it would be expected that Gfi-1 expression would be low as well since it is downstream of STAT6. This is precisely what was found by RT-PCR analysis of Gfi-1 expression. At 48 h after primary stimulation, Gfi-1 expression was low in cells stimulated with OVA/CT DCs and not detected in those stimulated with OVA DCs (Fig. 7). After restimulation, OVA/CT DCs induced markedly more expression of Gfi-1 RNA in the T cells than did OVA DCs (Fig. 7), which closely paralleled the differences in STAT6 expression (Fig. 6C). These data all demonstrate that there are quantitative and qualitative differences in DCs isolated from OVA- and OVA/CT-treated animals and suggest that these differences may be intimately involved in maintenance of tolerance or the induction of inflammation.

Discussion

Previous studies have examined the functions of various types of DCs in the lung parenchyma and/or in the airways (11, 50–53). Clearly the functions of these DCs are important in the effector/inflammatory phase of lung and airway inflammation (52, 53). What we focused on in the present study were the cells that are likely candidates to initiate the process of inflammation or tolerance in the lung draining lymph nodes, namely, DCs. Although

one recent report suggested that T cell priming can occur in the lung parenchyma (51), more than ample evidence exists to suggest that DCs migrate to the draining lymph nodes to initiate T cell priming (10, 54, 55). Also, activation of memory/long-lived effector cells was recently shown to occur in the lung parenchyma (53). This is not to suggest that priming may not also occur in the lung itself to some extent, but we chose to examine the lung draining lymph nodes to have a higher degree of confidence that the cells we examined were those involved primarily in tolerance and inflammation initiation, rather than in the effector phase of these processes.

Flow cytometric analysis revealed two major DC subtypes in the lung draining lymph nodes, namely, plasmacytoid-type DCs (CD11c⁺, MHC class II^{low}, B220⁺, Gr-1⁺, CD19⁻) and myeloid-type DCs (CD11c⁺, MHC class II^{high}, CD11b⁺). We report herein the first description of altered proportions of the two DC subtypes in the same lung draining lymph nodes under different immunological conditions. In our studies, examination of the absolute number of plasmacytoid and myeloid DCs in lung draining lymph nodes showed a greater increase in the number of myeloid DCs in OVA/CT-treated animals compared with plasmacytoid DCs (9- to 10-fold and 2-fold with respect to DCs from OVA-immunized mice, respectively). Thus, in the specific models that we have used, the plasmacytoid DCs in the lung lymph nodes are MHC II^{low} and express low levels of costimulatory molecules regardless of tolerance- or inflammation-inducing conditions.

We hypothesized that DC populations with altered proportions of DC subsets would have different effects on T cell stimulation. Plasmacytoid DCs have been associated with the establishment of tolerance and mature myeloid DCs are known to stimulate inflammatory T cell activation (13, 56–58). When viewed in combination with other studies on plasmacytoid DCs, it appears that in general plasmacytoid DCs serve to maintain Ag unresponsiveness (13) unless stimulated with specific stimuli such as viruses or CpG oligonucleotides that can prompt these DCs to initiate an adaptive

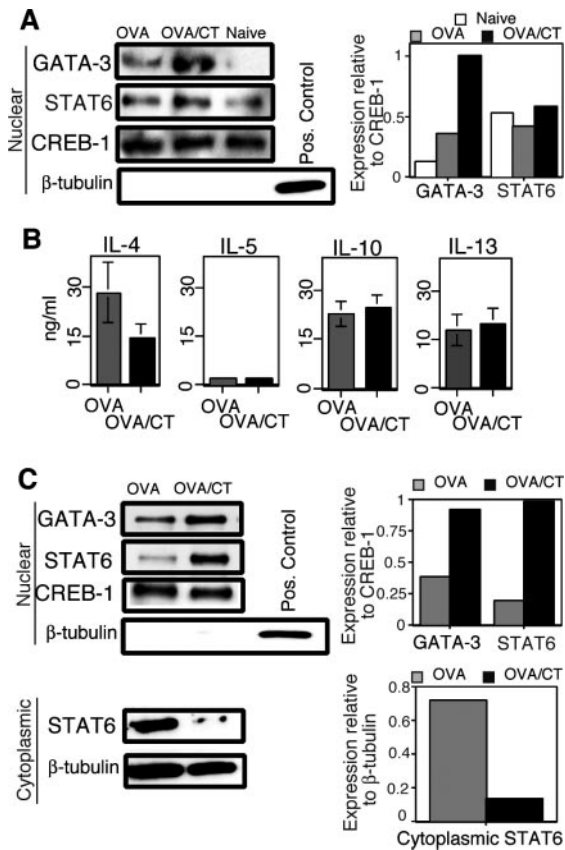


FIGURE 6. Functional characteristics of lung draining lymph node DCs from OVA- and OVA/CT-treated animals. DCs were cultured with naive DO11.10 T cells and specific peptide. *A*, Western blot analysis of nuclear extracts from primary stimulation (48 h) of T cells with OVA, OVA/CT, or naive DCs. The expression of the exclusively cytoplasmic protein β -tubulin is shown to be absent, thus verifying the purity of the nuclear extracts. The positive control for β -tubulin is a whole cell extract from purified murine T cells. Each blot was scanned by densitometry and the ratio to CREB-1 was calculated and is presented. *B*, Cytokines in supernatants at the end (5 days) of secondary T cell cultures (using equal numbers of T cells from the primary cultures) as determined by ELISA. *C*, Western blot analysis of nuclear extracts from secondary stimulation of equal numbers of T cells with fresh OVA or OVA/CT DCs. Also shown are a blot and corresponding densitometric data of cytoplasmic extracts from the same cells.

response (59–61). The plasmacytoid DCs we have observed appear to have remained in an immature state since expression of MHC class II and costimulatory molecules is generally low and similar between OVA- and OVA/CT-treated animals. Since the lung is a generally “tolerant” environment, it makes logical sense that DCs such as plasmacytoid DCs, which respond to limited stimuli to induce immunogenic responses, would be the predominant population used to maintain a state of tolerance in the tissue. It then follows that to override the state of tolerance, an inflammatory signal such as a combination of Ag and adjuvant might trigger the activation and migration of stimulatory DCs such as myeloid DCs that are known to express high levels of MHC II and costimulatory molecules in response to many stimuli.

We have shown that DCs from tolerized animals (OVA DCs, predominantly plasmacytoid) stimulate minimal T cell proliferation, relative to DCs from mice immunized for inflammation (OVA/CT, predominantly myeloid) (Fig. 3*F*). Interestingly, the OVA DCs were not without stimulatory function. Expression of GATA-3 was higher in T cells stimulated with OVA DCs com-

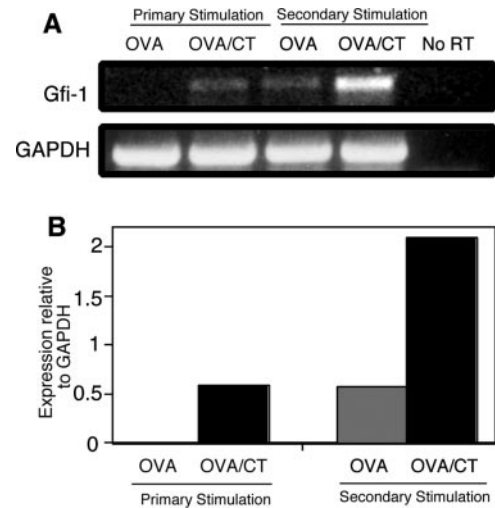


FIGURE 7. Expression of the STAT6-dependent transcription factor Gfi-1 is deficient in OVA-treated animals. *A*, RT-PCR analysis of RNA extracted from DC-stimulated T cells. The “No RT” control lane represents RT-PCR conducted in the absence of reverse transcriptase (RT) using RNA from OVA/CT DC-stimulated T cells. *B*, Densitometry data for the Gfi-1 RT-PCR relative to expression of GAPDH. Data are representative of at least three independent experiments.

pared with that in T cells stimulated with naive DCs (Fig. 6*A*). In the secondary cultures, when equal numbers of T cells from the primary cultures were stimulated with fresh DCs isolated from OVA- or OVA/CT-treated mice, the difference in GATA-3 expression between the groups of T cells was even more marked (Fig. 6*C*). Interestingly, the level of STAT6 in the nuclei of the T cells stimulated with OVA DCs was considerably lower than that in the nuclei of T cells stimulated with OVA/CT DCs (Fig. 6*C*), despite the presence of ample IL-4 (almost 30 ng/ml for OVA DCs; Fig. 6*B*) in the culture supernatant. All the more intriguing is the fact that STAT6 expression has been reported to rapidly diminish only when IL-4 is removed (62). Consistent with the finding of decreased STAT6 presence in the nucleus, the Th2 proliferation-associated, STAT6-dependent molecule Gfi-1 (48, 49) was expressed at much higher levels in cells stimulated with OVA/CT DCs (Fig. 7), which is also in line with these cells’ ability to induce enhanced T cell proliferation (Fig. 3*F*). These data suggest that differentiation toward Th2 can be initiated by DCs from both tolerizing or inflammatory conditions to induce the production of IL-4 and other Th2 cytokines. However, the DCs derived from tolerizing conditions appear unable to stimulate the T cells to utilize IL-4 such that STAT6 is not activated, and downstream events such as Gfi-1 expression and proliferation are not initiated and GATA-3 expression is not induced at high levels.

The low level of GATA-3 expression observed with OVA/DCs in the absence of appreciable STAT6 activation could be due to STAT6-independent autoactivating potential of GATA-3 (63) in cooperation with additional transcription factors such as NF- κ B and NFAT activated by TCR stimulation that, as we have shown, are important for GATA-3 expression (19, 64). The expression of GATA-3 may be sufficient to maintain cytokine expression, but the lack of cell expansion in the absence of STAT6 aborts the Th2 commitment. Taken together, it appears that Ag stimulation in a low IL-12 environment can initiate Th2-type development. However, without strong costimulatory signals, mechanisms are induced that down-regulate IL-4-induced STAT6 signaling, which precludes the up-regulation of Gfi-1 and ultimately does not allow for the proliferative expansion of the Th2 cells.

Several possibilities exist that might explain the blockade of STAT6 activation in OVA DC-stimulated T cells. These include down-regulation of the IL-4R or its phosphorylation status through the activity of specific phosphatases (such as Src homology region 2 domain-containing phosphatase 1) and/or modulation of JAK1/JAK3, suppressor of cytokine signaling proteins, and various kinases and proteases. An additional but not mutually exclusive possibility is the induction of regulatory T cells in tolerance that might also regulate STAT6 phosphorylation in activated T cells.

In conclusion, tolerizing and inflammation-inducing conditions induce distinct microenvironments in lung lymph nodes characterized by DCs with very different functionalities. Future studies with purified preparations of plasmacytoid and myeloid DCs will determine whether they have independent, complementary, or competing activities in these models and whether particular DC subsets mediate tolerance and inflammation *in vivo*.

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