

# Impaired Lung Dendritic Cell Migration and T Cell Stimulation Induced by Immunostimulatory Oligonucleotides Contribute to Reduced Allergic Airway Inflammation<sup>1</sup>

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CpG-containing oligonucleotides (CpG) have been shown to reduce key features of allergic airway inflammation in mouse models. Given the inhibitory effects of CpG treatment on Ag presentation of subsequently encountered Ags via MHC class I and II molecules by dendritic cells (DC), we hypothesized that intranasal CpG treatment would lead to reduced Ag-specific T cell stimulation in the lung-draining lymph nodes, thereby reducing the inflammatory response in sensitized mice. Intranasal CpG administration led to phenotypic maturation of lung and mediastinal lymph node DC as determined by expression of MHC class II, CD80, and CD86. This was accompanied by a significant reduction in the proliferation of adoptively transferred Ag-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells in mediastinal lymph nodes, when CpG was given before inhalative OVA challenges. DC obtained from mediastinal lymph nodes of CpG-treated mice before OVA inhalation led to reduced T cell stimulation via MHC class I and II molecules. In addition, CpG diminished airway eosinophilia and pulmonary infiltration after sensitization or following adoptive transfer of Ag-specific Th2 cells. These results were explained by reduced CCL21 expression and inhibition of lung DC migration following CpG administration, which could be restored by transfer of bone marrow-derived DC, because CpG had no major impact on the constitutive MHC class II Ag presentation of protein-derived Ag by lung tissue-derived DC. We conclude that CpG treatment can effectively impair the DC-mediated Ag transport from the lungs to the lymph nodes, resulting in reduced T cell activation and blunted airway inflammation. *The Journal of Immunology*, 2009, 183: 3443–3453.

Asthma is a chronic disease of the lungs, and its characteristic features include symptoms such as recurrent episodes of increased airway inflammation, enhanced mucus production, and constriction of the airways (1). Environmental factors have been suggested to be important contributing factors for raising prevalence of allergic diseases. The so-called hygiene hypothesis proposes that a major source of microbial, Th1-like immune stimulations has been lost with the decreased incidence of infectious diseases due to vaccinations, the use of antibiotics, and improved health conditions (2, 3). This had led to an increase in Th2-biased immune responses toward environmental allergens, and consequently to an increase in allergic asthma.

TLRs are expressed mainly on macrophages and dendritic cells (DC)<sup>3</sup> and recognize signature microbial products. The interaction of TLRs with their microbial ligands activates innate immunity to mount a defense mechanism, i.e., up-regulation of costimulatory molecules and the production of chemokines and cytokines (4, 5).

CpG-containing immunostimulatory DNA sequences (CpG) activate the TLR9 pathway (6) and facilitate the development of a Th1 cell response in vivo (7, 8). CpG has been shown to reduce key features of allergic asthma and airway inflammation, such as airway hyperresponsiveness, mucus production, and airway eosinophil infiltration in mouse models (9–16). CpG was effective when given before allergen challenge (11, 13, 14) and has even been demonstrated to reverse established disease (12, 17, 18). Given that CpG activates the innate immune system, thereby inducing release of cytokines to generate Th1 cells, such as TNF- $\alpha$ , IL-12, IFN- $\alpha$ , and indirectly, IFN- $\gamma$  (7, 8), it was postulated that long-term treatment with CpG would lead to a rebalancing of Th1/Th2 responses (19). This, however, would not explain the immediate inhibitory effects of CpG on allergen-induced Th2 response. Studies investigating these immediate effects demonstrated that the impact of CpG on allergen-induced eosinophil infiltration is partially dependent on IFN- $\gamma$  (12, 20), whereas others provide evidence that cytokines, such as IL-12 or IFN- $\gamma$ , are of minor importance (21–24). More recently, a study showed that CpG inhibits the Th2 response by rendering lung APCs unable to effectively present Ag to Th2 cells, but not to Th1 cells (19). In addition, CpG inhibited the IgE-dependent release of Th2 cytokines, especially IL-4, from basophils and/or mast cells in the airways of Th2-primed mice. Based on previous observations about the inhibitory effects of CpG on MHC class I- and II-mediated Ag presentation of subsequently encountered Ags by DC (25, 26), we aimed to investigate the impact of CpG on lung DC when given before inhalative Ag challenge. We hypothesized that down-regulation of MHC class II Ag presentation would lead to impaired CD4<sup>+</sup> T cell proliferation, thereby reducing major features of pulmonary hypersensitivity and inflammation. In this study, we show that instead impaired migration of pulmonary DC leads to reduced T cell stimulation and

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Received for publication December 17, 2008. Accepted for publication July 6, 2009.

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<sup>1</sup> This work was supported by the German Research Council (SFB 587, B5) and partially by a grant from the Excellence Cluster "From Regenerative Biology to Reconstructive Therapy" (German Research Foundation; to G.M.N.B.).

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<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell; BAL, bronchoalveolar lavage; BMDC, bone marrow-derived DC; HPRT, hypoxanthine guanine phosphoribosyl-transferase 1.

contributes to allergic airway inflammation. Our results thus demonstrate a previously unrecognized functional action of pulmonary CpG administration.

## Materials and Methods

### Animals

Eight- to 10-wk-old female C57BL/6 (B6) mice, female BALB/c mice (Charles River Laboratories), DO11.10 mice (The Jackson Laboratory), and OT-II and OT-I mice were used throughout the study. The OT-I and OT-II mice express MHC class I- and II-restricted TCR ( $V\alpha 2/V\beta 5.1^+$ ) specific for the H-2K<sup>b</sup>-restricted OVA peptides OVA<sub>257–264</sub> and OVA<sub>323–339</sub>, respectively. These mice were provided by B. Heath (Walter Eliza Hall Institute of Medical Research, Melbourne, Australia). CCR7-deficient mice were provided by R. Förster (Hannover Medical School, Hannover, Germany). Mice were fed with OVA-free laboratory food and tap water ad libitum, and kept in a regular 12-h dark/light cycle at a temperature of  $21 \pm 2^\circ\text{C}$ . All experimental procedures were performed according to a protocol approved by the appropriate governmental authority and ethics committees.

### Sensitization and allergen challenge of mice

The mice were sensitized with OVA (10  $\mu\text{g}$ , grade VI; Sigma-Aldrich) absorbed to aluminum hydroxide (1.5 mg; Pierce Biotechnology) or PBS as diluent control by i.p. injection on days 1, 14, and 21. On days 28 and 29, all mice were challenged with 1% OVA dissolved in PBS for 20 min. Allergen exposition was performed by dispersing of the relevant agent using a jet nebulizer, LC Star, 2.8  $\mu\text{m}$  mass median aerodynamic diameter (Pari) in a closed plexiglass box, in which mice could move freely. For treatment with CpG (TIB MolBio), mice received 20 nmol CpG (dissolved in PBS) via intranasal administration under ketamine/rompun anesthesia the day before the first challenge. To generate Ag-specific Th2-biased DO11.10 or OT-II cells, single-cell suspensions obtained from cervical, brachial, axillary, inguinal, and mesenteric lymph nodes were treated with mAbs against MAC-1, F4/80, erythroid cells, GR-1, MHC-II, and CD8 $\alpha$ . The Ab-coated cells were incubated with anti-rat IgG-coupled magnetic beads (Biomag), following the manufacturer's protocols, and enriched T cells cocultured with purified DC from BALB/c or B6 mice, respectively, in complete medium containing IL-4, IL-2, anti-IFN- $\gamma$ , and OVA<sub>323–339</sub> peptide (Biosyntan). Four days later, Th2-biased DO11.10 or OT-II cells were quantified, and 2 (BALB/c) or 4 (B6)  $\times 10^6$  lymphocytes were adoptively transferred i.v. into recipient mice (27). Induction of an effective Th2 cytokine pattern by measuring IL-4 production in DO11.10 cells was confirmed using an ELISPOT assay (ELISPOT<sup>PLUS</sup> for mouse IL-4; Mabtech), with a mean of 7 (0–10) spot-forming units per  $2.5 \times 10^5$  T cells, when naive DO11.10 cells were used, as compared with a mean of 909 (856–962) spot-forming units, when Th2-biased DO11.10 cells were used. On 5 (B6 mice) or 2–3 consecutive days (BALB/c mice), respectively, recipient mice were challenged with 1% OVA in PBS via inhalation. Control animals received adoptively transferred cells, but were challenged with PBS only. Twenty-four hours after the last challenge, mice were dissected for further analysis.

### Cell differentiation of the bronchoalveolar lavage (BAL) fluid

Total cell counts in BAL fluid were scored using a Neubauer chamber (Brand). Leukocyte subsets (eosinophils, neutrophils, macrophages, or lymphocytes) were counted in BAL fluid using cytopsins (centrifuged preparations) stained with Diff-Quik (Medion Diagnostics). A total of 400 cells was counted in each sample.

### Histology

Twenty-four hours after the last airway challenge, lungs were fixed with 4% formalin and embedded in paraffin. The paraffin blocks were cut into 4- $\mu\text{m}$  slices and stained with H&E (Merck). From each mouse lung, four sections (containing hilar structures and periphery) of the right and left lung were evaluated. Microphotographs were performed using a Laborlux K microscope (LEITZ) with an Olympus DP71 camera (Olympus). Peribronchial and perivascular inflammation of two to four mice from each group were analyzed and scored as follows (28): 0, normal; 1, few cells; 2, a ring of inflammatory cells one cell layer deep; 3, a ring of inflammatory cells two to four cells deep; and 4, a ring of inflammatory cells of more than four cells deep.

### DC isolation and flow cytometric analysis

DC were isolated from mouse lungs and mediastinal lymph nodes, as previously described (29–32). In brief, the organs were cut into small frag-

ments and digested with collagenase and DNase I (Sigma-Aldrich), and lung DC enriched by gradient centrifugation using Nycodenz reagents (Axis-Shield) with a density of 1.074. Mediastinal lymph node DC were directly enriched by FACS after digestion. To prevent DC maturation during the isolation protocol, the procedure was conducted on ice, with the exception of the initial 20-min digestion with collagenase/DNase, which was performed at room temperature. Lung and mediastinal DC were stained for CD11c (HL3) and MHC class II (2G9), CD11b (M1-70), CD103 (M290) (all BD Pharmingen), and CCR7 (4B12; BioLegend), and for gating of lung DC, special emphasis was given to exclude autofluorescent cells such as macrophages. In some cases, unstained samples were analyzed in the FL-1 and FL-2 channel first to identify autofluorescent cells, which then were excluded from further analysis. If necessary, autofluorescent cells were removed by an initial sort of unstained cells, followed by Ab labeling of the remaining cell populations for specific surface marker expression analysis. Analytical and preparative FACS was done on a FACSAria (BD Biosciences), or a Mo-Flo (DakoCytomation) instrument, and sorts were usually 95–98% pure.

### T cell isolation and in vitro T cell proliferation assay

For stimulation of OT-II or DO11.10 cells, single-cell suspensions from the lymph nodes were enriched using magnetic beads, as described above. OT-I cells were enriched using a commercial kit (BD CD8 Enrichment Set), according to the manufacturer's instructions. Enriched T cells were labeled with CFSE, as described elsewhere (25). For T cell stimulation, DC were plated in U-bottom 96-well plates ( $0.5\text{--}1 \times 10^4$  cells/well in RPMI 1640 supplemented with 10% FCS and 25 mM HEPES) with 100  $\mu\text{g}/\text{ml}$  OVA for 45 min at  $37^\circ\text{C}$  in complete medium, unless otherwise indicated. The DC were washed three times and cocultured in 200  $\mu\text{l}$  of complete medium containing  $5 \times 10^4$  CFSE-labeled OT-I, OT-II, or DO11.10 cells. For proliferation analysis after 60 h of culture, OT-II or DO11.10 cells were stained with CD4-allophycocyanin (GK1.5) and OT-I cells with CD8-allophycocyanin (53-6.7) (all BD Biosciences), and samples were analyzed by flow cytometry. The total number of dividing ( $\text{CD4}^+$  or  $\text{CD8}^+$  propidium iodide<sup>negative</sup>CFSE<sup>low</sup>) cells was determined in duplicate. For in vivo T cell proliferation assays,  $2 \times 10^6$  CFSE-labeled OT-I or OT-II cells were adoptively transferred, mice were challenged with OVA as indicated, and mediastinal lymph nodes 60 h after the last challenge assessed for T cell proliferation after staining, as described above. For intracellular IFN- $\gamma$  staining, single-cell suspension of mediastinal lymph nodes from mice that had received CFSE-labeled OT-I cells and intranasal OVA challenges was analyzed using the BD Cytotfix/Cytosperm Kit BD (BD Biosciences), according to the manufacturer's instruction, and measured by flow cytometry.

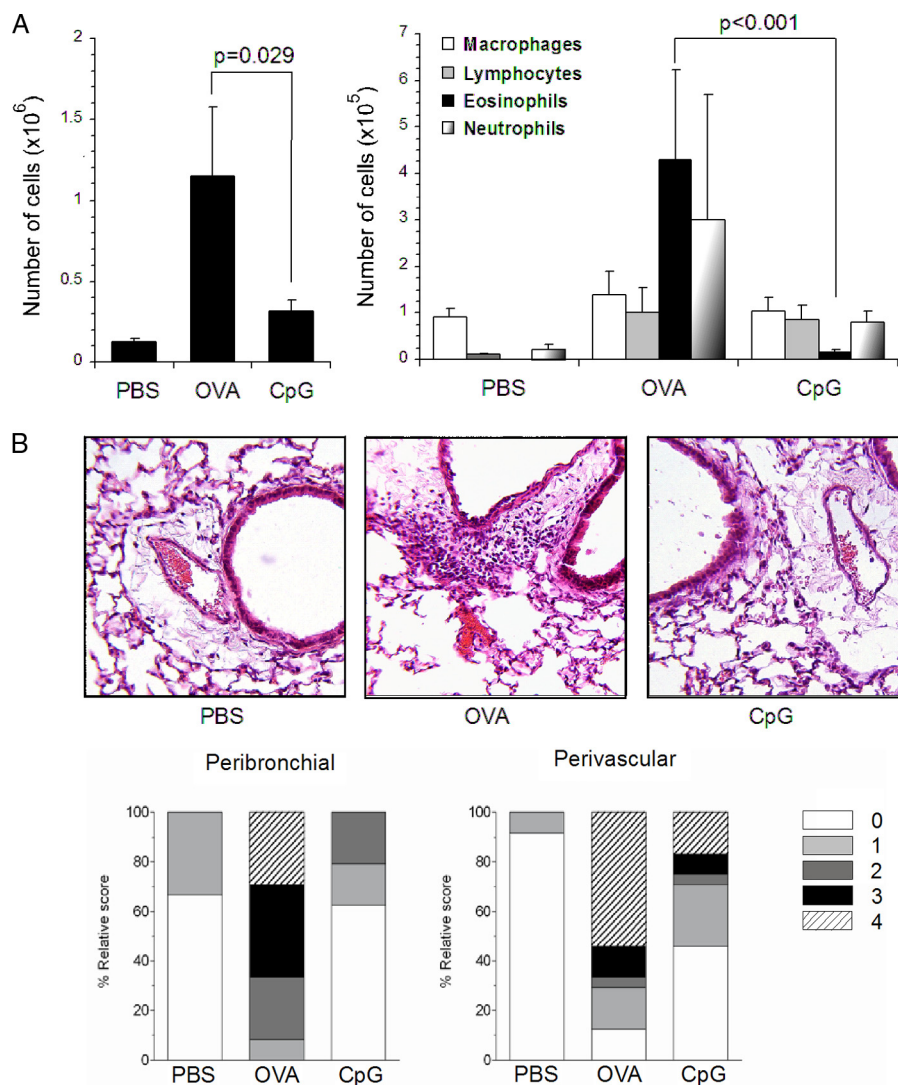
### Migration assays

Mice were treated with PBS or 20 nmol CpG, and 24 h later received 40  $\mu\text{l}$  of 5 mM CFSE intranasally. The next day, DC were enriched from the mediastinal lymph nodes and stained for CD11c, and  $\text{CD11c}^+$ CFSE<sup>+</sup> cells were quantified by flow cytometry. For alternative analysis of DC migration,  $5 \times 10^{12}$  Fluoresbrite YG Microspheres (0.05  $\mu\text{m}$ ; Polysciences) were administered intranasally in untreated or CpG-treated mice. After 24 h, mediastinal lymph node DC or lung DC were stained after enrichment for CD11c and MHC class II (lung DC), and uptake of beads (lung DC) or transport into the mediastinal lymph node was assessed by flow cytometry. For further migration experiments, bone marrow-derived DC (BMDC) were generated by flushing out the bone marrow from tibia and fibula of B6 mice. RBC were lysed by RBC lysis buffer containing ammonium chloride. The cells were filtered through a 70- $\mu\text{m}$  nylon mesh, counted, and plated at a concentration of  $1 \times 10^6$  cells/ml complete IMDM (Biochrom) supplemented with 20 ng/ml rGM-CSF. Medium was changed on days 3 and 5, and usually 7-day-old cultures were used for experiments. Mice received  $4\text{--}5 \times 10^5$  CFSE-labeled unsorted BMDC intranasally, and 18 h later mediastinal lymph nodes were dissected, DC stained with CD11c, and the entire cells of the lymph nodes analyzed for  $\text{CD11c}^{\text{high}}$ CFSE<sup>high</sup> cells. For the functional assays, mice that had received  $2\text{--}3 \times 10^6$  CFSE-labeled OT-II cells by tail vein injection received FACS-sorted  $4\text{--}5 \times 10^5$  BMDC intranasally 30 min before the inhalative challenge with 1% OVA for 20 min. Sixty hours later, OT-II proliferation was analyzed in the mediastinal lymph nodes by flow cytometry.

### RNA preparation and real-time quantitative PCR

Total RNA extraction was done using RNeasy Protect Mini Kit (Qiagen). Samples were treated with DNase I (Qiagen). cDNA was synthesized from total RNA in 20  $\mu\text{l}$  using random nonamers, oligo(dT) primers, and Omniscript RT Kit (Qiagen). Real-time quantitative PCR analyses for the genes encoding CXCL13, CCL19, CCL21, and hypoxanthine guanine

**FIGURE 1.** CpG treatment inhibits allergic airway inflammation in mice. **A**, B6 mice were sensitized with 10  $\mu$ g of OVA adsorbed to 1.5 mg of Alum and challenged by inhalation with 1% OVA on 2 consecutive days 1 wk after the last sensitization. CpG (20 nmol CpG) was administered intranasally the day before the first challenge, and control mice (OVA) were left untreated. Twenty-four hours after the second challenge, mice were sacrificed, and total numbers (*top left panel*) as well as leukocyte subpopulations (*top right panel*) of BAL fluid were analyzed. Data represent the mean  $\pm$  SEM obtained in three independent experiments with at least three mice per group. **B**, Lung histology (magnification  $\times 100$ , H&E staining) and score for the respective mouse groups. 0, Normal; 1, few cells; 2, a ring of inflammatory cells one cell layer deep; 3, a ring of inflammatory cells two to four cells deep; 4, a ring of inflammatory cells of more than four cells deep.



phosphoribosyltransferase 1 (HPRT) were performed in a final volume of 25  $\mu$ l using QuantiTect SYBR Green PCR Kit (Qiagen) in an iCycler. The primers for CXCL13, CCL19, CCL21, and HPRT (housekeeping gene) were as recently published (33). Amplifications were performed in specifically designed optical 96-well plates using a spectrofluorometric thermal cycler (iCycler; Bio-Rad). Genes of interest and housekeeping gene products were amplified separately, using identical cycling conditions. An initial cycle at 95°C for 15-min incubation was performed for activation of HotStarTaq DNA polymerase, and then 42 cycles of one step at 95°C (30 s) for denaturation, one step at 58°C (30 s) for annealing, and one step at 72°C (30 s) for extension. Products were also analyzed by melting curve analysis and on ethidium bromide-stained agarose gel to ensure that a single amplicon of the expected size was indeed obtained. To measure PCR efficiency, serial dilutions of reverse-transcribed RNA (1, 1/5, and 1/25) were amplified, and a standard curve was obtained by plotting cycle threshold values as a function of starting reverse-transcribed RNA, the slope of which was used for efficiency calculation using the iCycler software. The relative quantification for any given gene was calculated after dividing the standard curve value of the given gene A (CXCL13, CCL19, or CCL21) by that of the calibrator gene B (HPRT) in CpG-treated or untreated control B6 mice. The effect of CpG was calculated by comparing mean values obtained from two independent experiments. In each of the experiments, RNA from the mediastinal lymph nodes, lung tissue, inguinal lymph nodes, and spleen was isolated. The RNA was reverse transcribed, and cDNA was obtained by three separate reactions. This cDNA was pooled and measured in a real-time PCR in triplicates.

#### Statistical analyses

All values are expressed as mean plus SEM. Statistical analysis was performed with Student's *t* test using PRISM 4 (GraphPad). Where necessary,

data were log transformed to obtain a normal distribution. A *p* value  $<0.05$  was considered as statistically significant.

## Results

### CpG leads to phenotypic maturation of lung DC and inhibits allergic airway inflammation in mice

To confirm that administration of CpG previous to inhalative Ag challenge reduces the pulmonary hypersensitivity response, B6 mice were OVA sensitized and received 20 nmol CpG intranasally or diluent control 7 days following the last sensitization. Mice were then challenged on 2 consecutive days by inhalation. After this, mice were sacrificed, and the BAL fluid and the lungs were examined. CpG-pretreated mice had reduced total cell numbers in the BAL fluid ( $p = 0.029$ ) and also presented with a significant 90% reduction ( $p < 0.001$ ) in eosinophil counts as compared with control mice (Fig. 1B). Numbers of neutrophils, macrophages, and lymphocytes were not different among the groups. Similar results were obtained from the histological sections of the lungs, revealing a nearly absent peribronchiolar and perivascular infiltration of immune cells in CpG-pretreated animals as compared with sensitized, PBS-pretreated, and OVA-challenged mice (Fig. 1B). Taken together, CpG pretreatment was able to significantly reduce the inflammatory response in sensitized mice when given intranasally before the allergen challenge.

To test a prerequisite of our hypothesis, we also examined whether the administration of CpG would lead to DC maturation,

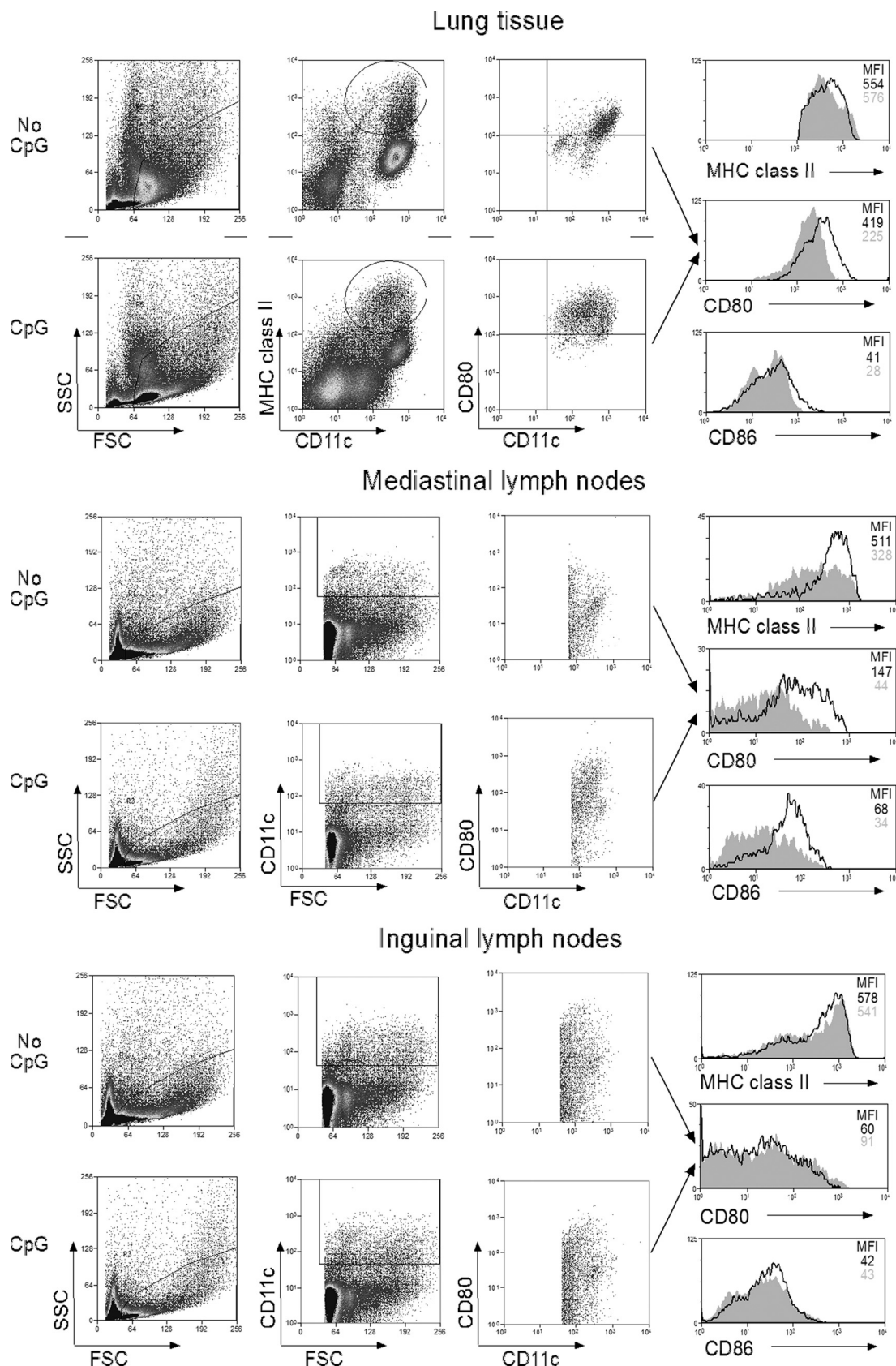


**FIGURE 2.** Up-regulation of MHC class II and costimulatory molecules of lung and mediastinal lymph node DC after administration of CpG. B6 mice were given 20 nmol CpG (black line) or PBS (gray histogram) intranasally, and 24 h later, DC were enriched from the lungs and mediastinal and inguinal lymph nodes. Cells were gated as depicted, and lung DC were classified according to their MHC class II<sup>+</sup> CD11c<sup>+</sup> expression. In lymph nodes, CD11c<sup>+</sup> DC were identified and DC were assessed for their surface expression of MHC class II and costimulatory molecules as depicted for CD80. Histograms represent expression analysis for MHC class II, CD80, and CD86 molecules on 5000 (lung), 2000 (mediastinal), and 4000–6000 (inguinal) cells of the respective DC population. The mean fluorescence intensities (MFI) are depicted in the histograms in black (CpG-treated animals) or gray (PBS-treated animals). The results are representative for three independent experiments.

as determined by up-regulation of MHC class II molecules and costimulatory molecules such as CD80 and CD86. To this end, mice were treated with CpG, and the phenotypic changes of lung DC, DC in mediastinal lymph nodes, and, as a control, DC from inguinal lymph nodes were assessed by flow cytometry. We observed a clear increase of MHC class II, CD80, and CD86 surface expression on DC obtained from the lungs or mediastinal lymph nodes following intranasal CpG administration, as depicted in Fig. 2. According to the mean fluorescence intensity, MHC class II expression increased by 55% in mediastinal lymph node DC, CD80 expression by 86 and 243%, and CD86 expression by 46 and 50% on lung DC and mediastinal lymph node DC, respectively. This up-regulation appeared to be nonsystemic, because there was no increase in expression of these molecules in nondraining lymph nodes (Fig. 2). We conclude that intranasal CpG administration leads to a phenotypic maturation of lung and mediastinal lymph node DC as determined by expression of MHC class II and costimulatory molecules.

#### *CpG inhibits naive CD4 and CD8 T cell proliferation in mediastinal lymph nodes*

Next, we wished to assess whether intranasal CpG application to mice before Ag exposure by inhalation would reduce MHC class I- and II-mediated Ag presentation by DC in the mediastinal lymph nodes, eventually leading to less proliferation of adoptively transferred Ag-specific T cells. For this, nonsensitized mice were treated with CpG intranasally, received  $2-3 \times 10^6$  CFSE-labeled OT-I or OT-II cells, and were challenged with OVA on 2 consecutive days. Sixty hours after the first challenge, proliferation of transferred T cells in the mediastinal lymph nodes was measured by flow cytometry. This revealed a significant reduction in the proliferation of OT-II ( $p = 0.003$ ) or OT-I cells ( $p < 0.001$ ) by a factor of 3 or 10, respectively, in mice that were given CpG before the Ag challenge, as compared with diluent control (Fig. 3). Analysis of T cell function in OT-I cells confirmed that the lower T cell proliferation was directly and linearly correlated to a reduced intracellular IFN- $\gamma$  production of transferred T cells after CpG treatment (supplemental Fig. 1).<sup>4</sup>

#### *Airway eosinophilia after adoptive transfer of Ag-specific Th2 cells is reduced by CpG*

To better demonstrate that the impact of CpG on pulmonary hypersensitivity is mediated through impaired Ag presentation and T cell proliferation, we made use of an adoptive transfer model (27). T cells of OT-II or DO11.10 mice were enriched and stimulated with peptide Ag and DC in vitro in medium containing IL-4, IL-2, and anti-IFN- $\gamma$ . After 4 days of culture, these Th2-biased cells were transferred by tail vein injection into B6 or BALB/c mice, which had been treated with CpG 2 h previously and which, from the next day on, were then challenged five times (B6) or twice (BALB/c) with OVA or PBS. Analysis of the BAL fluid confirmed that exposure to the Ag had led to a significant increase of total cells in the BAL fluid, and that these mice developed marked eosinophilia (Fig. 4). CpG-treated mice, however, had lower total cell numbers ( $p = 0.01$ ) and a significant reduction in eosinophil counts ( $p = 0.01$ ) in BAL fluid. Similarly, BALB/c mice demonstrated a reduced inflammatory infiltration in the peribronchial and perivascular regions upon histological examination, as compared with mice that did not receive CpG (Fig. 4). Taken together, we conclude that these observations would be consistent with a CpG-mediated impaired Ag presentation by DC, leading

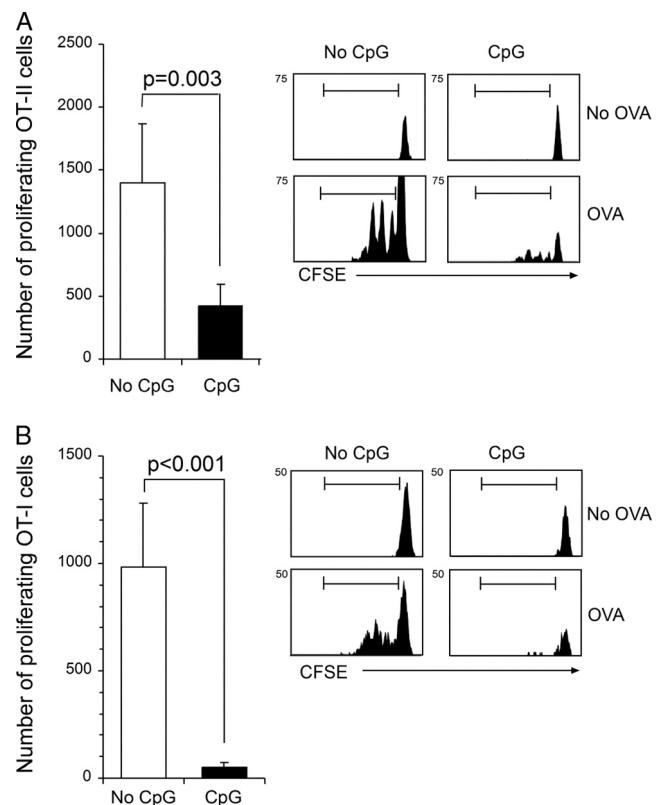


**FIGURE 3.** Proliferation of adoptively transferred naive T cells in mediastinal lymph nodes is reduced after intranasal application of CpG. A, A total of  $2-3 \times 10^6$  CFSE-labeled OT-II or OT-I cells (B) was injected i.v. into B6 mice. Two hours later, mice were challenged by inhalation with 1% OVA in PBS, followed by a second inhalative challenge the next day. Sixty hours after the first challenge, transferred T cells were recovered from inguinal and mediastinal lymph nodes for analysis by flow cytometry. Intranasal application of 20 nmol CpG (■) or no treatment (□) was performed the day before the first Ag exposure. The total numbers of proliferated (CFSE<sup>low</sup>) T cells depicted in the graphs are mean (+SEM) of four (OT-II) and two (OT-I) independent experiments with at least three mice per group. Representative T cell proliferation is shown in the histograms.

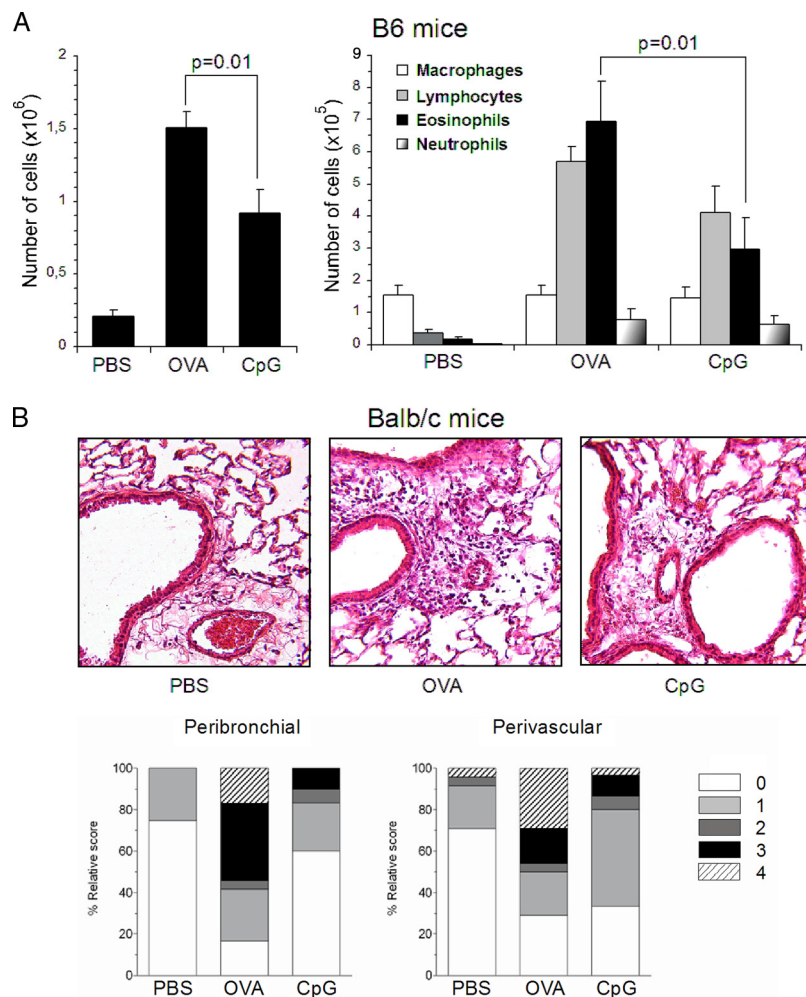
to reduced T cell proliferation, and eventually diminished eosinophilia and reduced pulmonary infiltration.

#### *Impaired Ag presentation of DC obtained from mediastinal lymph nodes after treatment with CpG*

To more accurately assess the impact of CpG on the Ag presentation via MHC class I and II molecules by DC in the mediastinal lymph nodes, B6 mice were treated with OVA intranasally after they had received CpG or PBS. The next day, DC were enriched from the mediastinal lymph nodes and used to stimulate CFSE-labeled OT-I and OT-II cells in vitro. Under these conditions, we observed a low, but readily detectable Ag-specific proliferation of both CD4<sup>+</sup> as well as CD8<sup>+</sup> T cells, when DC of OVA-treated mice were used in the cultures (Fig. 5). In contrast, DC from mediastinal lymph nodes of CpG-treated mice led to significantly reduced T cell proliferative response of both OT-II ( $p = 0.013$ ) as well as OT-I cells ( $p = 0.02$ ) to the in vivo inoculated Ag comparable to levels of control-treated mice. To exclude an intrinsic defect in Ag presentation after CpG administration, DC were pulsed in vitro with either the MHC class I- or II-restricted OVA peptide and cocultured with OT-I or OT-II cells, respectively. This

<sup>4</sup> The online version of this article contains supplemental material.

**FIGURE 4.** CpG treatment reduces airway eosinophilia in an adoptive Th2 cell transfer model. BAL fluid of B6 mice (A) and representative histology sections (magnification  $\times 100$ ) and inflammatory score of BALB/c mice (B) after transfer of Th2-biased cells and inhalative OVA challenge. Th2-biased T cells for B6 or BALB/c mice were generated by cocultivation of OT-II or DO11.10 cells, respectively, with OVA peptide and splenic DC for 4 days in medium containing IL-4, IL-2, and anti-IFN- $\gamma$ . A total of 2 (BALB/c) or 4 (B6)  $\times 10^6$  Th2-biased cells per mouse was injected i.v. after intranasal CpG application in the treated group. Mice of the positive control and treated group were challenged by inhalation with 1% OVA on the following 2 (BALB/c) or 5 (B6) days; mice of the negative control group inhaled PBS. One day after the last challenge, mice were sacrificed, and BAL fluid and lung histology were analyzed (for score, see Fig. 1). Data are the mean  $\pm$  SEM of two to four independent experiments with at least three mice per group.



confirmed the overall comparable Ag presentation and T cell stimulation capacity of all DC with a trend toward higher T cell proliferation of OT-II or OT-I cells, when CpG-treated and OVA peptide-pulsed DC were used (Fig. 5).

#### Inhibition of lung DC migration by CpG administration

Although the results to date would suggest that down-regulation of Ag presentation by lung DC following CpG administration could contribute to impaired T cell responses, we reasoned that reduced migration of DC from the lungs to the draining lymph nodes could be an alternative explanation and in agreement with our observations to date. To address this question, mice were given CpG or left untreated the day previous to an intranasal application of CFSE to label lung DC in vivo (32). After 18 h, the mice were sacrificed and the mediastinal lymph nodes were assessed for migratory CFSE<sup>+</sup> CD11c<sup>+</sup> DC. In mice that had received CpG intranasally, the number of CFSE<sup>+</sup> DC was markedly reduced as compared with untreated animals (Fig. 6A). In mice that were treated with CpG and CFSE simultaneously, however, the numbers of migratory DC from the periphery to the draining lymph node were unaffected. As a control, and to further quantify the degree of impaired DC migration, we used CCR7-deficient mice, which are deficient of constitutive migration of DC from peripheral organs to the draining lymph nodes. The numbers of CFSE<sup>+</sup> CD11c<sup>+</sup> DC in these animals were within the range of the results obtained from CpG-treated mice, confirming the major impact of CpG on DC migration. Similarly, we observed a significant reduction of DC migration ( $p = 0.001$ ), when the mice, instead of soluble CFSE,

received fluorescent-labeled beads into their lungs, to determine the uptake and transport of Ag from the peripheral tissue to the draining lymph nodes (Fig. 6B). We excluded the possibility that CpG treatment had reduced the phagocytic uptake of beads by lung DC, because analysis of lung DC after CpG treatment showed comparable and sufficient uptake of fluorescent-labeled beads in mice irrespective of whether they had received CpG or not. There was minimal or no uptake of beads by lung-derived macrophages (Fig. 6C).

To test whether CpG or CpG-induced events would act directly on the DC or predominantly generate an environment that prevents DC from migration, CpG-treated mice were given CFSE-labeled BMDC intranasally and the migration of these cells to the draining lymph nodes was determined by flow cytometry. The results shown in Fig. 7A indicate that the adoptively transferred DC were readily detectable in the draining lymph nodes of CpG-treated mice with even increased migratory DC numbers. To confirm the functional capacity of the transferred BMDC and to exclude that CpG administration leads to conditions that prevent effective T cell proliferation in vivo, we tested whether the transfer of BMDC would reconstitute the proliferation of Ag-specific T cells after CpG treatment. As shown in Fig. 7B, intranasally administered BMDC before giving the Ag led to efficient proliferation of OT-II cells in CpG-treated mice comparable to levels obtained from untreated control animals.

To investigate the impact on CpG on chemokine homeostasis in lung tissue, which may significantly influence DC migration, we assessed the expression of CCL21, CCL19, and CXCL13 1 day

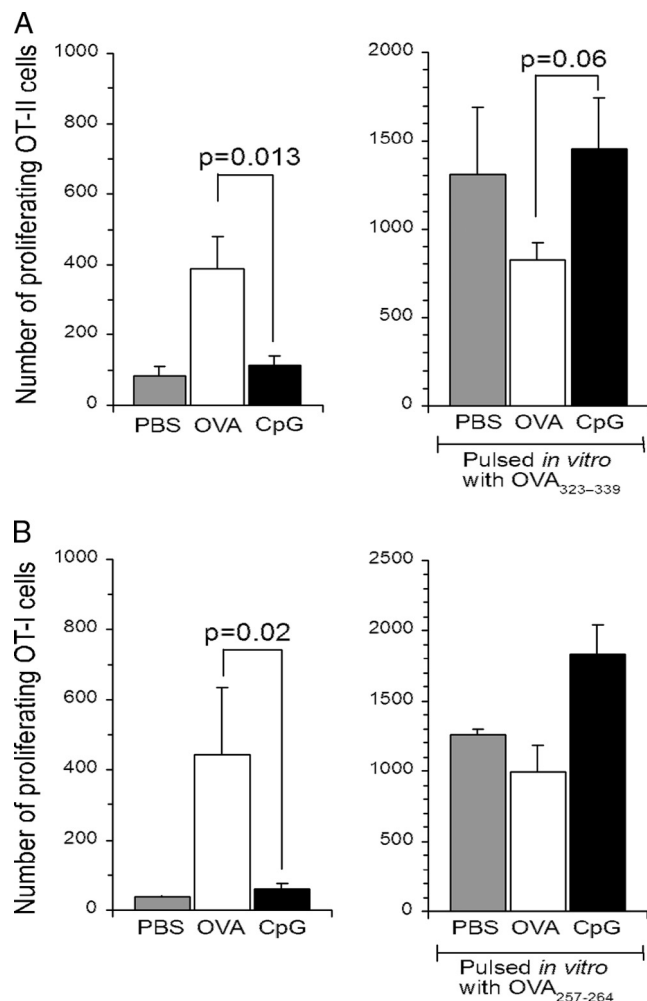


**FIGURE 5.** Ag presentation of DC obtained from mediastinal LN is impaired after CpG treatment. B6 mice were challenged intranasally with 25  $\mu$ g of OVA (OVA) or PBS (PBS) as control. One day before intranasal OVA application, the treatment group received CpG intranasally (CpG). Twenty-four hours after Ag challenge,  $1 \times 10^4$  DC from the mediastinal lymph nodes were isolated and used *in vitro* to stimulate CFSE-labeled OT-II (A, left column) and OT-I cells (B, left column). DC of the respective animal groups were pulsed with the MHC class II (OVA<sub>323-332</sub>) or MHC class I (OVA<sub>257-264</sub>) restricted, and then used to stimulate the corresponding T cells (right columns). Data represent the mean  $\pm$  SEM of results obtained in three (OT-II cells) and two (OT-I cells) independent experiments.

following intranasal CpG administration. This revealed a highly significant reduction in CCL21 expression with only little changes of the other chemokines at this early time point (Fig. 8A). These alterations were paralleled by raised numbers of CCR7<sup>+</sup> DC in mediastinal lymph nodes, but not in inguinal lymph nodes (Fig. 8B). Specifically, a recently characterized migratory lung-DC population (CD11c<sup>high</sup> MHC class II<sup>+</sup> CD103<sup>+</sup> CD11b<sup>-</sup>) supposed to be involved in Ag transport to the lung-draining lymph nodes was markedly reduced in the lung tissue 16 h after CpG administration (supplemental Fig. 2).

Collectively, we conclude from these results that CpG or secondary events impact significantly on lung-resident DC and the chemokine homeostasis, and result in a significant reduction of lung DC migration to the lymph nodes after at least 18 h, leading to declined T cell stimulation and pulmonary infiltration. This effect appears to dominate following initial lung-DC activation and an early phase of increased migration of CCR7<sup>+</sup> DC toward the draining lymph nodes after CpG intranasal application.

#### Impact of CpG on lung DC Ag presentation

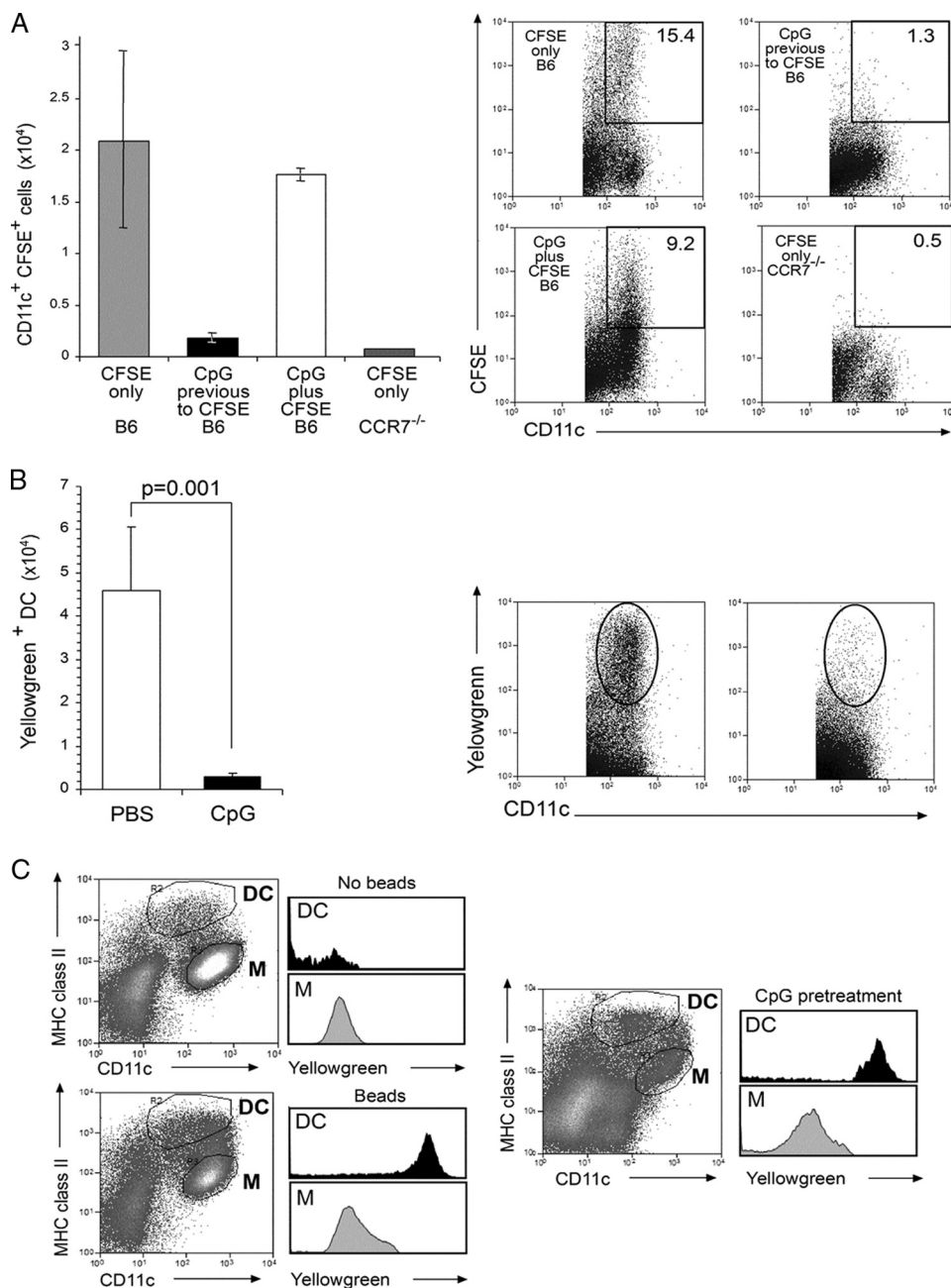
To directly test the effect of CpG on the MHC class II Ag presentation of subsequently encountered Ags by lung DC, we treated B6 mice with CpG or left them untreated and 18 h later, enriched lung DC by cells sorting, and either pulsed the DC with OVA protein or OVA class II peptide. These cells were then used to stimulate CFSE-labeled OT-II cells *in vitro*. Lung DC of untreated mice showed comparable T cell stimulation when pulsed with OVA or OVA peptide. In contrast, lung DC from CpG-treated mice led to 4-fold increase in OT-II cell proliferation ( $p = 0.004$ ) when pulsed with peptide, and the OT-II proliferation, resulting from uptake and presentation of protein Ags, was also slightly, but significantly ( $p = 0.01$ ) increased as compared with untreated mice (Fig. 9). This led us to conclude that CpG has no major impact on the constitutive MHC class II Ag presentation of protein-derived Ag by lung tissue-derived DC, but that MHC class II-mediated T cell stimulation of peptide Ags is enhanced.

#### Discussion

Our experiments have led us to identify a new mechanism on how CpG treatment previous to allergen exposure contributes to the prevention of pulmonary inflammation in a murine model for allergic pulmonary hypersensitivity. In this study, we have shown that intranasal administration of the TLR9 ligand CpG leads to reduced transport of newly encountered Ag by lung DC to the draining lymph node. This results in impaired T cell stimulation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells and contributes to the reduced pulmonary inflammatory response to inhaled Ags. The expression of TLR9 and the response of lung DC to CpG have been previously demonstrated by others (34, 35). In contrast to studies using splenic or peripheral lymph node DC after systemic CpG injection (26), CpG-induced maturation of lung DC did not down-regulate their constitutive MHC class II-mediated Ag presentation.

DC have a high capacity to endocytose Ags and present them on their MHC class II molecules (36). In their immature state, DC are highly endocytic and express low amounts of MHC class II molecules on their surface. Upon stimulation with TLR ligands, DC undergo phenotypic changes resulting in a mature phenotype. Mature DC down-regulate macropinocytosis and phagocytosis, but retain their micropinocytic activity (37, 38). Mature DC accumulate long-lived surface MHC II-peptide complexes, derived from exogenous and endogenous Ags contained within the endosomal compartments at the time of activation (39–45). Down-regulation of MHC II-peptide complex turnover allows long-term presentation of Ags captured, or synthesized, at the time of activation (46). We had hypothesized that CpG-induced DC maturation would reduce the uptake of exogenous Ags and/or the processing and presentation of subsequently encountered Ags via MHC class II molecules to CD4<sup>+</sup> T cells. These effects have been described for DC obtained from the spleen or various peripheral lymph nodes, but were not studied in DC derived from peripheral organs. We chose a local application in our model and speculated that CpG can directly access the draining lymph node via the lymphatics to stimulate mediastinal DC or, in addition, activate migratory lung DC, which accumulate in the draining lymph nodes, contributing to the phenotypically mature DC in mediastinal lymph nodes. We have previously shown that systemic DC preactivation impaired cross-presentation and the stimulation of CD8<sup>+</sup> T cell responses against HSV and influenza virus, which are proposed to be induced by cross-priming (25, 47). Impairment of cross-presentation of viral Ags was attributed chiefly to down-regulation of phagocytosis, which prevented mature DC from capturing infected cells, and this is also the primary cause of impairment of MHC II presentation of

**FIGURE 6.** Migration of lung DC is inhibited by intranasal CpG administration. **A**, B6 or control mice (CCR7-deficient mice, CCR7<sup>-/-</sup>) received CFSE intranasally to assess DC migration from the lungs to the mediastinal lymph nodes. Similarly, two other groups of mice received CFSE intranasally, but were also treated with CpG 24 h previously or simultaneously, as indicated. Mediastinal lymph nodes were examined 18 h after the CFSE application. For analysis by flow cytometry, cells isolated from the lymph nodes of the respective mouse groups (at least three mice per group) were pooled and gated on CD11c<sup>+</sup> cells. The data depicted are the mean  $\pm$  range obtained in two independent experiments and represent the percentage (histograms) and absolute numbers (bars) of cells of each group. **B**, Intranasal administration of  $5 \times 10^{12}$  0.05  $\mu$ m yellow-green microsphere beads per mouse ( $n = 3$  per group) was performed 1 day after intranasal treatment with CpG or PBS. Twenty-four hours after the bead application, mediastinal lymph node DC were obtained. CD11c<sup>+</sup> lymph node DC of individual mice were analyzed. The graph represents the mean  $\pm$  SEM of pooled data from two independent experiments. CD11c<sup>+</sup> DC displayed in the dot plots are representative examples of each group. **C**, B6 mice were left untreated or received yellow-green microsphere beads, as described above. The next day, lung DC (CD11c<sup>+</sup> MHC class II<sup>high</sup>) and macrophages (M, CD11c<sup>+</sup> MHC class II<sup>low</sup>) were analyzed for bead uptake by flow cytometry.



cell-associated Ags by mature DC (25). Indeed, mature DC could present endogenous viral Ags via MHC I if infected (25), indicating that their endogenous MHC I presentation pathway was operative. In contrast, MHC class II presentation of newly encountered endogenous viral Ags is reduced in mature DC (26), showing that additional factors downstream of Ag uptake, such as down-regulated MHC class II synthesis, also contribute to poor presentation of new Ags after systemic CpG administration (26). Our data presented in this work are consistent with a different regulation of Ag presentation after TLR9 signaling of lung DC vs lymph node and splenic DC in that CpG-treated lung DC had unchanged constitutive MHC class II Ag presentation of endocytosed and processed Ag, revealed no major reduction in phagocytosis of fluorescent-labeled beads, but had increased presentation of peptides via MHC class II molecules in agreement with their phenotypic maturation. Because we used the fluorescent-labeled beads to monitor DC migration and analyzed uptake of beads by lung DC only to rule out significant

effects on the interpretation of the migration analysis, further studies with different DC to bead ratios might be necessary to fully characterize the impact of CpG on the phagocytosis of lung DC.

Despite the lack of impact on pulmonary DC, we observed that in animals, in which CpG had caused pulmonary DC maturation, the Ag presentation of new Ags, such as OVA to CD4<sup>+</sup> T cells and the resulting T cell proliferation in the draining lymph node, was markedly reduced. Our results using CFSE labeling of DC or monitoring the transport of fluorescent-labeled beads in vivo indicate a strong reduction in Ag transport from the periphery to the mediastinal lymph nodes as explanation for this. In line with this, the constitutive number of peripheral lung tissue DC expressing CCR7 was low and comparable to numbers found in CpG-treated animals, consistent with the view that migratory DC increase their CCR7 expression upon activation and subsequently start migration toward the lymphatics and draining lymph nodes. Consequently, the number of CCR7 expressing DC in the mediastinal lymph

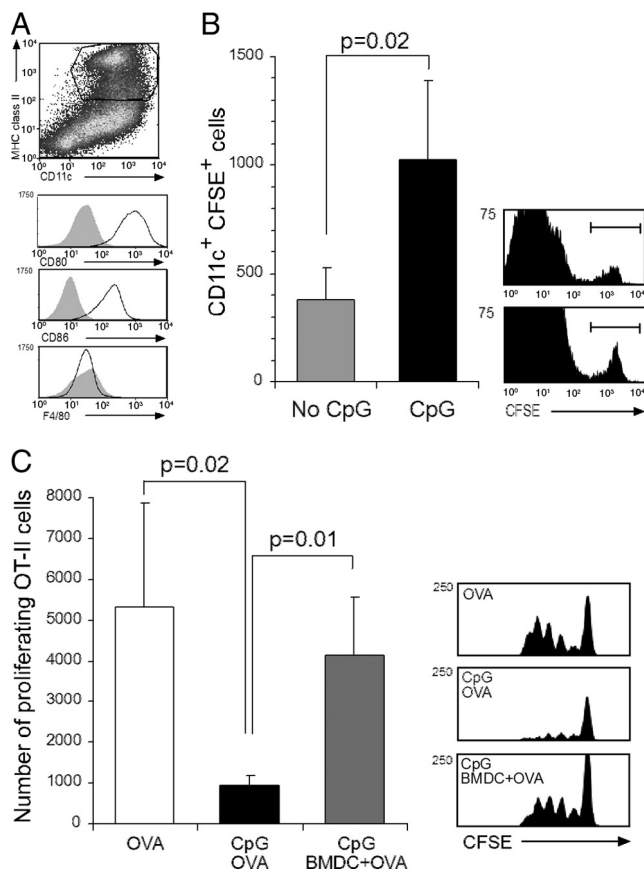


**FIGURE 7.** Impaired migration of lung DC and T cell proliferation following CpG treatment can be rescued by intranasal application of BMDC. *A*, CD11c<sup>+</sup> MHC class II<sup>+</sup> BMDC express costimulatory molecules such as CD80 and CD86 after culture, but are negative for F4/80 (gray, isotype control). *B*, CFSE-labeled BMDC ( $4\text{--}5 \times 10^5$ ) were intranasally transferred into untreated (□) or CpG-treated (■) B6 mice. One day later, CFSE<sup>+</sup> BMDC were recovered from mediastinal lymph nodes and quantified by flow cytometry. *C*, Groups of three B6 mice were left untreated (□) or received 20 nmol CpG intranasally (■ and ▨). The next day, the mice received  $2\text{--}3 \times 10^6$  CFSE-labeled OT-II cells and were challenged with OVA by inhalation (■) or treated with  $4\text{--}5 \times 10^5$  sorted BMDC intranasally before OVA challenge (▨). After 60 h, mediastinal lymph nodes were analyzed for proliferation of adoptively transferred OT-II cells. Data are mean + SEM of two to four independent experiments. The histograms depict the representative T cell proliferation.

nodes of CpG-treated mice was consistently higher as compared with control-treated mice. These findings suggest an early increase in migration of pulmonary DC from the periphery after CpG treatment, followed by a reduced DC-mediated Ag transport 18 h after CpG application. Efficient T cell proliferation in mediastinal lymph nodes of CpG-pretreated mice could be restored after the mice had received BMDC previous to Ag administration, indicating that CpG had not caused overt suppression of CD4<sup>+</sup> T cell activation in the mediastinal lymph nodes of these mice. Thus, immunosuppressive effects on T cell activation, such as induced by IDO after TLR9 stimulation (48), appear not to be dominating mechanisms during the conditions studied in this work. This is further supported by our observation that CpG-treated DC induced rather than increased T cell proliferation, when pulsed with peptide Ag. Finally, both the proliferation as well as IFN- $\gamma$  production in T cells were almost identically reduced, suggesting that proliferation and effector cell functions such as cytokine production were closely and directly correlated in both groups and not altered by CpG.

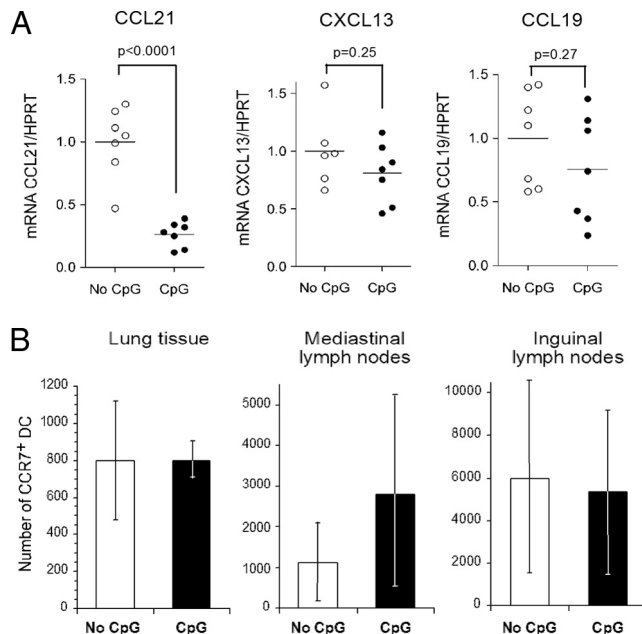


**FIGURE 8.** Changes in lymphoid chemokines and CCR7<sup>+</sup> DC distribution after intranasal CpG application. *A*, Real-time PCR analysis of CCL21, CXCL13, and CCL19 chemokine expression in mediastinal lymph nodes from PBS-treated (○) or CpG-treated (●) B6 mice 1 day after intranasal CpG application. Circles represent individual mice of two independent experiments with at least three to four mice per group. *B*, Numbers of CCR7<sup>+</sup> DC in the lung tissue and mediastinal and inguinal lymph nodes 1 day after PBS (□) or CpG administration (■). Data represent mean and range of two independent experiments with three mice per group.

Based on our CCL21 expression analysis, we speculate that disturbed regulation of homeostatic chemokine expression, chemokine receptor expression, and cell trafficking after CpG administration may contribute to impaired DC migration. This would be consistent with reports showing that CCL21, CCL19, and CXCL13 are transiently down-regulated within lymphoid tissues during immune responses after viral infection or TLR9 stimulation (33). This modulation was found to alter the localization of lymphocytes and DC within responding lymphoid tissue. In agreement

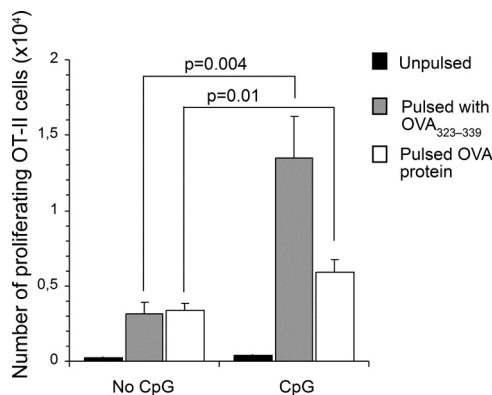


**FIGURE 9.** Constitutive Ag presentation and T cell stimulation of lung DC are unaffected following CpG treatment. B6 mice were left untreated or received 20 nmol CpG intranasally. The next day, lung DC ( $1 \times 10^4$  per well) of the mice were enriched, left unpulsed (■), pulsed with MHC class II-restricted OVA<sub>329-332</sub> (▨) or OVA protein (□), and cocultured with  $5 \times 10^4$  CFSE-labeled OT-II cells. Sixty hours later, T cell proliferation was quantified by flow cytometry. Data represent the mean + SEM obtained in three independent experiments with at least three mice per group.

with these observations, studies have demonstrated that pulmonary influenza virus infection results in a transient acceleration of respiratory DC to the regional lymph nodes during the first 24 h after infection, and the DC remained refractory to further migration thereafter (49). Previous intranasal CpG administration in this study abrogated the virus-induced increased pulmonary DC migration, and we suggest that a similar effect of CpG contributes to the reduced lung DC migration, when given before inhalative Ag challenge. Further research is required to delineate the specific effects and kinetics of CpG and other TLR ligands on the expression of chemokines and chemokine receptors in the lungs and lung-draining lymphatics.

CpG-containing oligonucleotides have shown significant promise for preventing allergic responses to pulmonary allergen challenge (9–18, 50, 51) both following systemic (9, 10, 12–14) or local administration into the respiratory tract (9, 11, 14). Given that CpG not only inhibited acute responses to allergen, but also chronic manifestations, such as airway remodelling (18, 50–52), and considering the complex cascade of cellular events, the number of different cell types, and the cellular interactions involved during the response to a pulmonary allergen, it is difficult to dissect the CpG-induced events that lead to reduced airway inflammation. In this regard, inhibited Ag transport by lung DC to the draining lymph nodes is likely to be only one among several additional CpG-mediated effects that might act synergistically or have a different relevance, depending on the pathogenetic phase of pulmonary inflammatory response.

In contrast to our findings, Hessel et al. (19) described a drastic reduction in the overall expression of costimulatory molecules on CD11c<sup>+</sup> DC from the airways of CpG-treated mice, including PD-L1, PD-L2, CD40, ICOS, CD80, and CD86, accompanied by a clear reduction in the expression of MHC class II molecules, creating an overall picture of a much less competent DC. We speculate that these differences as compared with our observations may result from different isolation protocols for lung DC, and that the studies by Hessel et al. (19) studied cytokine production of Th2 cells instead of T cell proliferation. In any case, our phenotypic characterization of increased MHC class II together with the enhanced T cell proliferation after peptide pulsing of CpG-treated mediastinal DC clearly provide no evidence for a negative impact on the overall MHC class Ag presentation capacity of these cells. We cannot exclude, however, that both mechanisms, reduced Ag transport and impaired stimulation of Th2 cells, coexist at the same time.

In summary, our data implicate that CpG treatment prevents pulmonary hypersensitivity by reduced pulmonary DC migration, but may also lead to a reduced DC-mediated transport of other inhalative Ags (e.g., viral particles) to the draining lymph nodes. This could have negative impact on the effective adaptive antiviral immunity due to reduced helper cell priming and cytotoxic T cell responses. Alternatively, it could be beneficial, because it might prevent the collateral priming against inhaled Ags during pulmonary hypersensitivity reactions (53), and thereby limit sensitization against further allergens. Thus, our findings on reduced Ag transport and T cell stimulation reveal some exciting new mechanism of action of pulmonary CpG administration, but also raise some important caveats for its therapeutic application in patients.

## Acknowledgments

We acknowledge the excellent technical assistance of the members of the Hannover Medical School Core Facility for Cell Sorting. We thank Marion Hitzigrahn, Mannan Abdul Baru, Adan Chari Jirmo, and C. K. Prajeeth for technical help and animal care. We thank Karin Westermann and Damaris Leemhuis for help with BAL and histology analysis.

## Disclosures

The authors have no financial conflict of interest.

## References

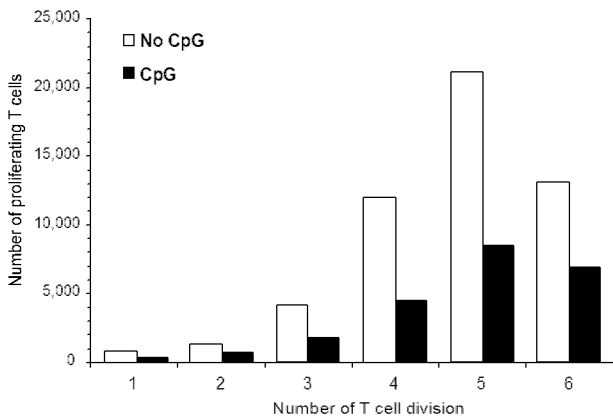
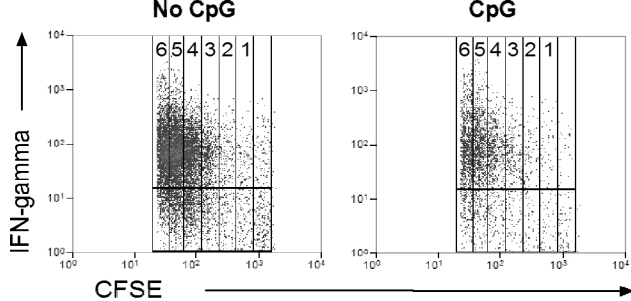
- Lambrecht, B. N., and H. Hammad. 2003. Taking our breath away: dendritic cells in the pathogenesis of asthma. *Nat. Rev. Immunol.* 3: 994–1003.
- Wills-Karp, M., J. Santeliz, and C. L. Karp. 2001. The germless theory of allergic disease: revisiting the hygiene hypothesis. *Nat. Rev. Immunol.* 1: 69–75.
- Kline, J. N. 2007. Eat dirt: CpG DNA and immunomodulation of asthma. *Proc. Am. Thorac. Soc.* 4: 283–288.
- Akira, S., K. Takeda, and T. Kaisho. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* 2: 675–680.
- Iwasaki, A., and R. Medzhitov. 2004. Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* 5: 987–995.
- Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408: 740–745.
- Krieg, A. M. 2002. CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* 20: 709–760.
- Klinman, D. M. 2004. Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat. Rev. Immunol.* 4: 249–258.
- Broide, D., J. Schwarze, H. Tighe, T. Gifford, M. D. Nguyen, S. Malek, J. Van Uden, E. Martin-Orozco, E. W. Gelfand, and E. Raz. 1998. Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J. Immunol.* 161: 7054–7062.
- Kline, J. N., T. J. Waldschmidt, T. R. Businga, J. E. Lemish, J. V. Weinstock, P. S. Thorne, and A. M. Krieg. 1998. Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J. Immunol.* 160: 2555–2559.
- Sur, S., J. S. Wild, B. K. Choudhury, N. Sur, R. Alam, and D. M. Klinman. 1999. Long term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J. Immunol.* 162: 6284–6293.
- Serebrisky, D., A. A. Teper, C. K. Huang, S. Y. Lee, T. F. Zhang, B. H. Schofield, M. Kattan, H. A. Sampson, and X. M. Li. 2000. CpG oligodeoxynucleotides can reverse Th2-associated allergic airway responses and alter the B7.1/B7.2 expression in a murine model of asthma. *J. Immunol.* 165: 5906–5912.
- Broide, D. H., G. Stachnick, D. Castaneda, J. Nayar, M. Miller, J. Y. Cho, M. Roman, J. Zubeldia, T. Hayashi, and E. Raz. 2001. Systemic administration of immunostimulatory DNA sequences mediates reversible inhibition of Th2 responses in a mouse model of asthma. *J. Clin. Immunol.* 21: 175–182.
- Jahn-Schmid, B., U. Wiedermann, B. Bohle, A. Repa, D. Kraft, and C. Ebner. 1999. Oligodeoxynucleotides containing CpG motifs modulate the allergic TH2 response of BALB/c mice to Bet v 1, the major birch pollen allergen. *J. Allergy Clin. Immunol.* 104: 1015–1023.
- Kohama, Y., O. Akizuki, K. Hagihara, E. Yamada, and H. Yamamoto. 1999. Immunostimulatory oligodeoxynucleotide induces TH1 immune response and inhibition of IgE antibody production to cedar pollen allergens in mice. *J. Allergy Clin. Immunol.* 104: 1231–1238.
- Hirose, I., H. Tanaka, G. Takahashi, K. Wakahara, M. Tamari, T. Sakamoto, S. Kojima, N. Inagaki, and H. Nagai. 2008. Immunomodulatory effects of CpG oligodeoxynucleotides on house dust mite-induced airway inflammation in mice. *Int. Arch. Allergy Immunol.* 147: 6–16.
- Kline, J. N., K. Kitagaki, T. R. Businga, and V. V. Jain. 2002. Treatment of established asthma in a murine model using CpG oligodeoxynucleotides. *Am. J. Physiol.* 283: L170–L179.
- Jain, V. V., K. Kitagaki, T. Businga, I. Hussain, C. George, P. O'Shaughnessy, and J. N. Kline. 2002. CpG-oligodeoxynucleotides inhibit airway remodeling in a murine model of chronic asthma. *J. Allergy Clin. Immunol.* 110: 867–872.
- Hessel, E. M., M. Chu, J. O. Lizcano, B. Chang, N. Herman, S. A. Kell, M. Wills-Karp, and R. L. Coffman. 2005. Immunostimulatory oligonucleotides block allergic airway inflammation by inhibiting Th2 cell activation and IgE-mediated cytokine induction. *J. Exp. Med.* 202: 1563–1573.
- Kitagaki, K., V. V. Jain, T. R. Businga, I. Hussain, and J. N. Kline. 2002. Immunomodulatory effects of CpG oligodeoxynucleotides on established Th2 responses. *Clin. Diagn. Lab. Immunol.* 9: 1260–1269.
- Kline, J. N., A. M. Krieg, T. J. Waldschmidt, Z. K. Ballas, V. Jain, and T. R. Businga. 1999. CpG oligodeoxynucleotides do not require TH1 cytokines to prevent eosinophilic airway inflammation in a murine model of asthma. *J. Allergy Clin. Immunol.* 104: 1258–1264.
- Chiaromonte, M. G., M. Hesse, A. W. Cheever, and T. A. Wynn. 2000. CpG oligonucleotides can prophylactically immunize against Th2-mediated schistosoma egg-induced pathology by an IL-12-independent mechanism. *J. Immunol.* 164: 973–985.
- Broide, D. H., G. Stachnick, D. Castaneda, J. Nayar, M. Miller, J. Cho, M. Rodriguez, M. Roman, and E. Raz. 2001. Immunostimulatory DNA mediates inhibition of eosinophilic inflammation and airway hyperreactivity independent of natural killer cells in vivo. *J. Allergy Clin. Immunol.* 108: 759–763.
- Ashino, S., D. Wakita, Y. Zhang, K. Chamoto, H. Kitamura, and T. Nishimura. 2008. CpG-ODN inhibits airway inflammation at effector phase through down-regulation of antigen-specific Th2-cell migration into lung. *Int. Immunol.* 20: 259–266.
- Wilson, N. S., G. M. Behrens, R. J. Lundie, C. M. Smith, J. Waithman, L. Young, S. P. Forehan, A. Mount, R. J. Steptoe, K. D. Shortman, et al. 2006. Systemic activation of dendritic cells by Toll-like receptor ligands or malaria infection impairs cross-presentation and antiviral immunity. *Nat. Immunol.* 7: 165–172.

26. Young, L. J., N. S. Wilson, P. Schnorrer, A. Mount, R. J. Lundie, N. L. La Gruta, B. S. Crabb, G. T. Belz, W. R. Heath, and J. A. Villadangos. 2007. Dendritic cell preactivation impairs MHC class II presentation of vaccines and endogenous viral antigens. *Proc. Natl. Acad. Sci. USA* 104: 17753–17758.
27. Cohn, L., R. J. Homer, A. Marinov, J. Rankin, and K. Bottomly. 1997. Induction of airway mucus production by T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. *J. Exp. Med.* 186: 1737–1747.
28. Bandukwala, H. S., B. S. Clay, J. Tong, P. D. Mody, J. L. Cannon, R. A. Shilling, J. S. Verbeek, J. V. Weinstock, J. Solway, and A. I. Sperling. 2007. Signaling through Fc $\gamma$ RIII is required for optimal T helper type (Th)2 responses and Th2-mediated airway inflammation. *J. Exp. Med.* 204: 1875–1889.
29. Henri, S., D. Vremec, A. Kamath, J. Waithman, S. Williams, C. Benoist, K. Burnham, S. Saeland, E. Handman, and K. Shortman. 2001. The dendritic cell populations of mouse lymph nodes. *J. Immunol.* 167: 741–748.
30. Vremec, D., J. Pooley, H. Hochrein, L. Wu, and K. Shortman. 2000. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J. Immunol.* 164: 2978–2986.
31. Wilson, N. S., D. El Sukkari, G. T. Belz, C. M. Smith, R. J. Steptoe, W. R. Heath, K. Shortman, and J. A. Villadangos. 2003. Most lymphoid organ dendritic cell types are phenotypically and functionally immature. *Blood* 102: 2187–2194.
32. Belz, G. T., C. M. Smith, L. Kleinert, P. Reading, A. Brooks, K. Shortman, F. R. Carbone, and W. R. Heath. 2004. Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus. *Proc. Natl. Acad. Sci. USA* 101: 8670–8675.
33. Mueller, S. N., K. A. Hosiawa-Meagher, B. T. Konieczny, B. M. Sullivan, M. F. Bachmann, R. M. Locksley, R. Ahmed, and M. Matloubian. 2007. Regulation of homeostatic chemokine expression and cell trafficking during immune responses. *Science* 317: 670–674.
34. Suzuki, K., T. Suda, T. Naito, K. Ide, K. Chida, and H. Nakamura. 2005. Impaired Toll-like receptor 9 expression in alveolar macrophages with no sensitivity to CpG DNA. *Am. J. Respir. Crit. Care Med.* 171: 707–713.
35. Chen, L., M. Arora, M. Yarlaga, T. B. Oriss, N. Krishnamoorthy, A. Ray, and P. Ray. 2006. Distinct responses of lung and spleen dendritic cells to the TLR9 agonist CpG oligodeoxynucleotide. *J. Immunol.* 177: 2373–2383.
36. Wilson, N. S., and J. A. Villadangos. 2005. Regulation of antigen presentation and cross-presentation in the dendritic cell network: facts, hypothesis, and immunological implications. *Adv. Immunol.* 86: 241–305.
37. Garrett, W. S., L. M. Chen, R. Kroschewski, M. Ebersold, S. Turley, S. Trombetta, J. E. Galan, and I. Mellman. 2000. Developmental control of endocytosis in dendritic cells by Cdc42. *Cell* 102: 325–334.
38. West, M. A., A. R. Prescott, E. L. Eskelinen, A. J. Ridley, and C. Watts. 2000. Rac is required for constitutive macropinocytosis by dendritic cells but does not control its down-regulation. *Curr. Biol.* 10: 839–848.
39. Cella, M., A. Engering, V. Pinet, J. Pieters, and A. Lanzavecchia. 1997. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* 388: 782–787.
40. Askew, D., R. S. Chu, A. M. Krieg, and C. V. Harding. 2000. CpG DNA induces maturation of dendritic cells with distinct effects on nascent and recycling MHC-II antigen-processing mechanisms. *J. Immunol.* 165: 6889–6895.
41. Villadangos, J. A., M. Cardoso, R. J. Steptoe, D. van Berkel, J. Pooley, F. R. Carbone, and K. Shortman. 2001. MHC class II expression is regulated in dendritic cells independently of invariant chain degradation. *Immunity* 14: 739–749.
42. Wilson, N. S., D. El Sukkari, and J. A. Villadangos. 2004. Dendritic cells constitutively present self antigens in their immature state in vivo and regulate antigen presentation by controlling the rates of MHC class II synthesis and endocytosis. *Blood* 103: 2187–2195.
43. Shin, J. S., M. Ebersold, M. Pypaert, L. Delamarre, A. Hartley, and I. Mellman. 2006. Surface expression of MHC class II in dendritic cells is controlled by regulated ubiquitination. *Nature* 444: 115–118.
44. Van Niel, G., R. Wubbolts, T. Ten Broeke, S. I. Buschow, F. A. Ossendorp, C. J. Melief, G. Raposo, B. W. van Balkom, and W. Stoorvogel. 2006. Dendritic cells regulate exposure of MHC class II at their plasma membrane by oligoubiquitination. *Immunity* 25: 885–894.
45. Ohmura-Hoshino, M., Y. Matsuki, M. Aoki, E. Goto, M. Mito, M. Uematsu, T. Kakiuchi, H. Hotta, and S. Ishido. 2006. Inhibition of MHC class II expression and immune responses by c-MIR. *J. Immunol.* 177: 341–354.
46. Villadangos, J. A., P. Schnorrer, and N. S. Wilson. 2005. Control of MHC class II antigen presentation in dendritic cells: a balance between creative and destructive forces. *Immunol. Rev.* 207: 191–205.
47. Jirmo, A. C., C. H. Nagel, C. Bohnen, B. Sodeik, and G. M. Behrens. 2009. Contribution of direct and cross-presentation to CTL immunity against herpes simplex virus 1. *J. Immunol.* 182: 283–292.
48. Hayashi, T., L. Beck, C. Rossetto, X. Gong, O. Takikawa, K. Takabayashi, D. H. Broide, D. A. Carson, and E. Raz. 2004. Inhibition of experimental asthma by indoleamine 2,3-dioxygenase. *J. Clin. Invest.* 114: 270–279.
49. Legge, K. L., and T. J. Braciale. 2003. Accelerated migration of respiratory dendritic cells to the regional lymph nodes is limited to the early phase of pulmonary infection. *Immunity* 18: 265–277.
50. Youn, C. J., M. Miller, K. J. Baek, J. W. Han, J. Nayar, S. Y. Lee, K. McElwain, S. McElwain, E. Raz, and D. H. Broide. 2004. Immunostimulatory DNA reverses established allergen-induced airway remodeling. *J. Immunol.* 173: 7556–7564.
51. Cho, J. Y., M. Miller, K. J. Baek, J. W. Han, J. Nayar, M. Rodriguez, S. Y. Lee, K. McElwain, S. McElwain, E. Raz, and D. H. Broide. 2004. Immunostimulatory DNA inhibits transforming growth factor- $\beta$  expression and airway remodeling. *Am. J. Respir. Cell Mol. Biol.* 30: 651–661.
52. Fanucchi, M. V., E. S. Schelegle, G. L. Baker, M. J. Evans, R. J. McDonald, L. J. Gershwin, E. Raz, D. M. Hyde, C. G. Plopper, and L. A. Miller. 2004. Immunostimulatory oligonucleotides attenuate airways remodeling in allergic monkeys. *Am. J. Respir. Crit. Care Med.* 170: 1153–1157.
53. Eisenbarth, S. C., A. Zhadkevich, P. Ranney, C. A. Herrick, and K. Bottomly. 2004. IL-4-dependent Th2 collateral priming to inhaled antigens independent of Toll-like receptor 4 and myeloid differentiation factor 88. *J. Immunol.* 172: 4527–4534.

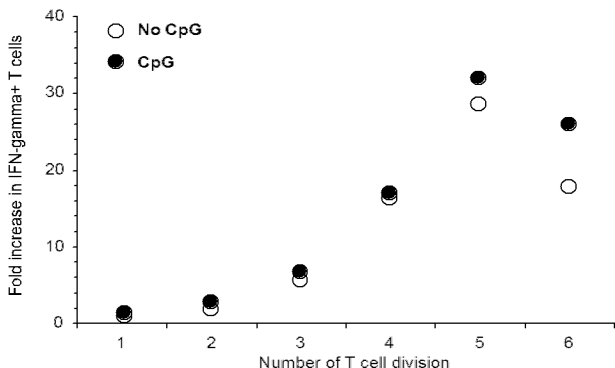
**Supplementary Figure 1. Intracellular cytokine production of adoptively transferred T cells after CpG-administration.** **A**, Dot plot of CFSE-labelled OT-I cell proliferation and corresponding intracellular IFN $\gamma$ -production after transfer of cells ( $2 \times 10^6$ ) into B6 mice, which were PBS- or CpG-pretreated and then challenged intranasally with 25 $\mu$ g OVA on two consecutive days. The middle panel depicts the absolute number of OT-I cells per cell division in the respective groups of recipient mice. **B**, Fold increase of IFN $\gamma^+$  T cells per cell division of adoptively transferred OT-I cells in PBS- or CpG-pretreated mice normalized for the number IFN $\gamma^+$  non-divided cells.

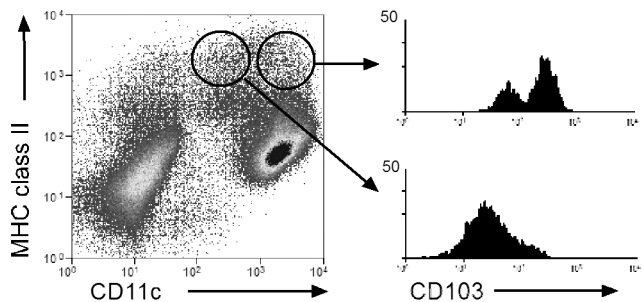
**Supplementary Figure 2. CpG-associated changes in composition of lung tissue DC populations.** **A**, In lungs of B6 mice, at least two distinct DC populations can be identified. In addition to their different CD11c and MHC class II expression, CD11c<sup>high</sup> MHC class II<sup>high</sup> DC also express CD103 whereas CD11c<sup>+</sup> MHC class II<sup>high</sup> are negative for CD103. **B**, One day after i.n. CpG-application, the population of CD11c<sup>high</sup> MHC class II<sup>high</sup> CD103<sup>+</sup> DC is reduced in the lung tissues of B6 mice. **C**, Quantification of the changes after CpG-application revealed reduced number of CD103<sup>+</sup>CD11b<sup>-</sup> DC and increased numbers of CD103<sup>-</sup>CD11b<sup>+</sup> DC. The dot plots depict cells that are gated on CD11c<sup>+</sup> MHC class II<sup>high</sup> cells from the lung tissues and then divided in CD103<sup>+</sup>CD11b<sup>-</sup> and CD103<sup>-</sup>CD11b<sup>+</sup> DC populations.

A



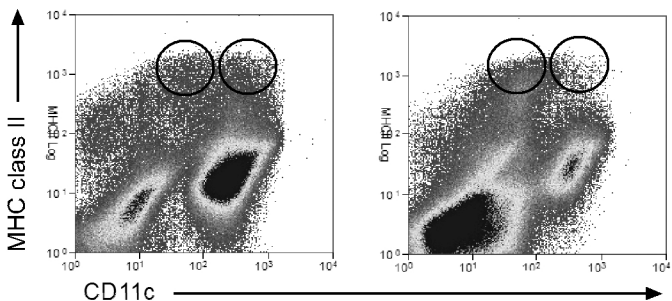
B



**A****B**

No CpG

CpG

**C**

No CpG

CpG

