

Intranasal Administration of CpG Induces a Rapid and Transient Cytokine Response Followed by Dendritic and Natural Killer Cell Activation and Recruitment in the Mouse Lung

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Key Words

Adjuvants • Lung • Dendritic cells • Cytokines • Mucosal vaccines • Natural killer cells

Abstract

CpG-containing oligodeoxynucleotides are potent mucosal adjuvants and effective as stand-alone treatment of respiratory infections in mice. Although CpG is also used as a type 1 helper immunomodulator in the treatment of asthma and allergic disease, immune modulation following intranasal application has not been fully characterized yet. Using a B-type CpG, we monitored RNA expression profiles, cytokine production and cellular activation in lung tissue and bronchoalveolar lavages ex vivo and cytokine production of purified cell populations in vitro. CpG triggered the upregulation of many transcripts, including interferon response genes and proinflammatory cytokine genes, between 3 h and 4 days. Overlapping subsets of these cytokine proteins were induced in vitro in purified CD11c+ cells, B cells and alveolar

macrophages from the lung, thus identifying these cells as direct targets of CpG. While lung B cells strongly respond to CpG in vitro, less activation is found ex vivo, suggesting efficient CpG sequestering or rapid B cell migration after activation. In contrast, a type II alveolar epithelial cell line did not respond to CpG in vitro. We noted selective recruitment of plasmacytoid dendritic cells (DCs) into the lung tissue, and of conventional DCs and natural killer (NK) cells into the lung tissue and bronchoalveolar space. Furthermore, CpG induced activation of intrapulmonary DCs, NK and T cells. We hypothesize that CpG-linked adjuvanticity and clearance of respiratory pathogens are mediated by two major mechanisms: transient induction of the interferon pathway limiting microbial survival and selective recruitment of DCs and NK cells, which allows for better adaptive responses.

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I.P. and E.M. share first, E.D.G. and A.W. share last authorship of this work.

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Introduction

Synthetic oligodeoxynucleotides containing unmethylated CpG sequences are promising vaccine adjuvants and immunotherapeutics [1]. CpG is able to mimic the immunostimulatory activity of bacterial DNA and to trigger host defense mechanisms through direct activation of cells expressing the DNA sensor Toll-like receptor (TLR) 9 [2]. In the mouse, TLR9 expression has been reported on immune cells of the myeloid lineage, such as monocytes, macrophages and conventional dendritic cells (cDCs), on plasmacytoid dendritic cells (pDCs) and on B cells [3]. In bone marrow-derived DCs, CpG induces the upregulation of major histocompatibility complex (MHC) class II molecules and of costimulatory molecules, such as CD40 and CD86, and triggers the production of proinflammatory cytokines, such as interleukin (IL)-12, IL-6 and tumor necrosis factor (TNF)- α [4]. Similarly, in murine macrophage cell lines, CpG induces the expression of activation markers and the secretion of interferon (IFN)- α , TNF- α , IL-1 β , IL-12, IFN- γ and IL-6 [5, 6]. These cytokines are important for T cell and natural killer (NK) cell activation and the development of adaptive immune responses. In addition, CpG induces potent B cell activation and proliferation and immunoglobulin secretion.

Systemic administration of CpG has a potent vaccine adjuvant effect leading to enhanced antigen-specific humoral and cellular responses to a wide variety of antigens [7, 8]. In particular, CpG increases IgG antibody titers (mainly IgG_{2a}) and the frequencies of IFN- γ -producing CD4⁺ T cells. In addition to antigen-specific immune responses, systemic administration of CpG can induce non-specific protective immune responses to several intracellular pathogens, including *Listeria monocytogenes*, *Francisella tularensis*, *Leishmania major* and *Plasmodium sp.* [9–11].

CpG has been used as stand-alone treatment or as vaccine adjuvant also by mucosal administration. The addition of CpG to inactivated influenza virus markedly increases antibody levels in the serum, saliva and genital tract of mice in an intranasal immunization model. Similarly, intranasal delivery of CpG with the hepatitis B surface antigen or β -galactosidase stimulates strong antigen-specific IgA responses throughout the mucosal immune system and in the serum [12]. CpG provides effective mucosal adjuvant activity in models of *Chlamydia trachomatis* mouse pneumonitis and invasive pulmonary aspergillosis [13, 14]. Mucosal administration of CpG can also have therapeutic effects in the

absence of antigen in the lung. In particular, CpG inhibits allergic lung inflammation in a murine asthma model by reducing type 2 helper (Th2) responses through the induction of IL-12 eventually leading to strongly reduced Th2 cytokines as well as reduced airway eosinophilia, systemic levels of IgE and bronchial hyperreactivity [15, 16]. Moreover, CpG induces generic protection in different animal models of infection. In a murine model of tuberculosis, CpG reduces growth of *Mycobacterium tuberculosis* and associated lung inflammation [17]. Furthermore, pulmonary administration of CpG protects from *Cryptococcus neoformans* challenge in an IL-12-dependent manner and induces protective immune responses against *Klebsiella pneumoniae* [18, 19]. In addition to the therapeutic potential in the lung, CpG stimulates innate immune responses in the murine female genital tract and in the gastrointestinal mucosa, eliciting protective immunity against genital herpes infection and *Helicobacter pylori*, respectively [20, 21].

In summary, although a wealth of reports document that CpG is a potent mucosal adjuvant and induces generic protection against airway pathogens, little is known about the molecular and cellular effects of CpG in mouse lung. In order to analyze immunological events induced in the lung by CpG, we monitored RNA levels, cytokine expression profiles and the cellular composition of lung tissue and bronchoalveolar lavage (BAL) for 2 weeks following intranasal administration. In addition, we tested the direct effects of CpG on alveolar macrophages (AMs), CD11c⁺ cells, B cells and epithelial cells, showing CpG-induced transient stimulation of host defense pathways and selective recruitment of DCs and NK cells into the lung.

Materials and Methods

Animals

BALB/c mice were purchased from Charles River Laboratories. 8- to 12-week-old female mice were used for all experiments. Animals were housed and treated according to the internal animal ethical committee and institutional guidelines.

Type II Alveolar Epithelial Cell Line

The mouse type II alveolar epithelial cell line T7 (European Collection of Cell cultures) was maintained at 33°C in the presence of 100 U/ml IFN- γ (Sigma, St. Louis, Mo., USA) and differentiated as described previously [22]. Differentiated cells were cultured with or without 10 μ g/ml CpG. After culture, cytokine levels were determined in cell supernatants.

Formulation of Oligonucleotides and Immunization Protocol

The CpG oligonucleotide (ODN) used in this study was 1826, a class B ODN (5'-TCC ATG ACG TTC CTG ACG TT-3'), with

the whole backbone having phosphorothioate linkages. The control ODN has the same sequence except for the CpG motif (underlined) which was inverted to GpC (5'-TCC ATG AGC TTC CTG AGC TT-3'). All ODNs were purchased from InvivoGen (San Diego, Calif., USA). The ODNs were tested for endotoxin using the *Limulus* ameocyte lysate assay (BioWhittaker/Cambrex Bio Science, Verviers, Belgium). All dilutions were conducted with endotoxin-free PBS (Sigma). PBS alone or CpG (10 µg) in PBS was administered intranasally in a total volume of 50 µl (25 µl/nostril) to anesthetized BALB/c mice [23]. Anesthesia was induced by intraperitoneal injection of 50 mg/kg ketamine plus 2.6 mg/kg xylazine in a 100-µl solution.

RNA Extraction and Purification

RNA was extracted from the lung as previously described [23]. In brief, lungs were homogenized in 5 ml of TRIzol (Invitrogen Life Technologies, Paisley, UK), and the total RNA was extracted following the manufacturer's protocol and purified using RNeasy RNA purification columns following the manufacturer's protocol (Qiagen, Milan, Italy). The quality of RNA was checked using the automated Experion electrophoresis system (Bio-Rad, Hercules, Calif., USA) coupled with the RNA StdSens analysis kit (Bio-Rad) following the manufacturer's protocol. For microarray analysis, lung tissue was sampled from 3 mice per time point.

RNA Labeling, Microarray Hybridization and Data Acquisition

Twenty-five micrograms of purified total RNA were retrotranscribed and labeled as described previously [23]. The labeled cDNA was purified using the QIAquick PCR purification kit (Qiagen) following the manufacturer's protocol. Total RNA obtained from a single mouse lung treated with CpG or PBS was labeled using dCTP-Cy5. A pool of total RNA extracted from the lung of 6 naïve mice was labeled with dCTP-Cy3 and used as reference control in all microarray hybridizations. The efficiency of incorporation of the Cy5 or Cy3 dye was measured by NanoDrop analysis. Equal amounts of labeled Cy5 and Cy3 cDNA were hybridized onto the Agilent Technologies 44K whole mouse genome microarray (Agilent Technologies, Palo Alto, Calif., USA), detecting over 40,000 transcripts, for 17 h at 60°C following the Agilent protocol. Images were acquired using the ScanArray Express microarray scanner from PerkinElmer (Boston, Mass., USA).

Microarray Data Analysis

Raw images of each microarray slide were initially analyzed using GenePix 6.0 software (Molecular Devices, Sunnyvale, Calif., USA). The data were then transferred to the BASE 1.2 database/analysis software [24]. For each spot, local background was subtracted from the mean fluorescence intensity of the Cy3 and Cy5 dyes. Spot intensities were then normalized by the global mean. Spots with a signal-to-noise ratio ≤ 3 in both channels were filtered. Four additional hybridizations using the same reference RNA labeled with Cy5 and Cy3 were processed in the same way to determine the dye incorporation bias and to correct for the baseline value of each spot. The average intensity ratio of repeated spots from three experimental replicates was estimated by geometric mean. The accuracy and statistical significance of the observed ratios were determined using Student's *t* test. Spots with less than two values passing previous filters in the same time point were considered 'not found'. Only genes having *t* test *p* val-

ues ≤ 0.05 and average intensity ratios ≥ 4 ($\log_2$ ratio ≥ 2) in at least one time point were selected for further analysis. In order to identify genes with a differential response to PBS and CpG, we used the significance analysis for microarray (SAM) algorithm for two-class, paired experiments on the $\log_2$ -transformed data set. The parameters used for the SAM to determine significant differences in the expression of genes were a change of at least twofold, a delta value of 1 and default s_0 , as defined by Tusher et al. [25]. Hierarchical clustering was performed applying the Euclidean distance matrix and the average linkage clustering method. Both SAM and hierarchical clustering were performed using Multiexperiment Viewer (MeV) 3.1 software (TIGR, Rockville, Md., USA) [26]. The complete set of microarray data has been submitted to the Array Express database EMBL-EBI (<http://www.ebi.ac.uk/arrayexpress/>) with accession number E-TABM-823.

Real-Time PCR Analysis of Cxcl10 Expression

For each sample, 5 µg of RNA extracted from the lungs was retrotranscribed into cDNA with the SuperScript reverse transcriptase enzyme (200 U/ml; Invitrogen Life Technologies) using the manufacturer's protocol. Real-time PCR to detect and measure Cxcl10 gene transcripts was conducted with the Brilliant SYBR Green qPCR master mix (Stratagene, La Jolla, Calif., USA) using the ODNs 5'-AGTCCTAATTGCCCTTGGTCTTC-3' (forward primer) and 5'-CGTCGCACCTCCACATAGC-3' (reverse primer). As a control, the housekeeping ribosomal protein 10 (RSP10) was amplified in parallel using the oligonucleotides 5'-GGCTGACAGAGACACCTAC-3' (forward primer) and 5'-ATACAACATAAACTCCAACCTTCAC-3' (reverse primer).

Preparation of Lung Homogenates to Determine Intra-Organ Cytokines

For cytokine analysis, lungs were taken from 3 mice/group at different time points after treatment with CpG or PBS, or from untreated mice (naïve). Lungs were snap-frozen in liquid nitrogen and stored at -80°C. To prepare homogenates, the snap-frozen lungs were homogenized at 4°C with an Ultra-Turrax T252 in 2 ml of tissue protein extraction reagent (T-PER; Pierce, Rockford, Ill., USA) containing Complete Mini Protease Inhibitor Cocktail tablets (Roche Diagnostics, Indianapolis, Ind., USA). Lung homogenates were subsequently centrifuged at 10,000 *g* for 10 min at 4°C, and supernatants were stored at -80°C. To normalize for protein content, the total protein concentration of each lung lysate was determined in the supernatants using a BCA protein assay kit (Pierce). Lung tissue homogenates were diluted with T-PER reagent to a protein concentration of 2 mg/ml. Then samples were diluted with 75% standard diluent, provided in the Bio-Plex Mouse Cytokine 23-Plex kit (Bio-Rad), to a final protein concentration of 500 µg/ml for cytokine measurements.

Cytokine Quantification

Cytokine levels in lung homogenates, in supernatants of total lung cell culture, AM cell culture, lung CD11c+ cell culture, lung B cell and type II alveolar epithelial cell line culture were determined using the Bio-Plex Mouse Cytokine 23-Plex kit (Bio-Rad) according to the manufacturer's instructions. The assay was read on a Bio-Plex array reader (Bio-Rad), and cytokine concentrations were calculated based on standard curve data using the Bio-Plex Manager Software (Bio-Rad). The panel included the following cytokines: IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-

10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), IFN- γ , CXCL1/keratinocyte-derived cytokine (KC), CCL2/monocyte chemotactic protein 1 (MCP-1), CCL3/macrophage inflammatory protein (MIP)-1 α , CCL4/MIP-1 β , CCL5/regulated upon activation normal T cell expressed and secreted (RANTES) and TNF- α . The level of IFN- α in the homogenates was separately measured by ELISA (PBL Bio-medical Laboratories, Piscataway, N.J., USA).

BAL of Mice and Stimulation of AM

AMs were isolated by BAL from naïve mice anesthetized as described above. BAL was performed by intratracheally instilling three times 500 μ l PBS containing 1 mM EDTA followed by gentle aspiration. AMs were isolated by adherence of BAL cells for 2 h in culture medium at 37°C, 5% CO₂, and were >97% pure by flow-cytometric analysis (data not shown). As culture medium, RPMI 1640 (GIBCO Invitrogen, Paisley, UK) supplemented with 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 50 μ M 2-mercaptoethanol and 10% heat-inactivated and filtered FBS (Euroclone, Life Sciences Division, Pero, Italy) was used. AMs were cultured for 18 h with CpG (10 μ g/ml), control GpC (10 μ g/ml) or PBS, and cell culture supernatants were collected to measure cytokine content as described above.

Enzymatic Digestion of Lung and Spleen for Cellular Assays

At the indicated time points after intranasal CpG administration, mice were sacrificed and lungs and spleens were harvested. As controls, the same organs were removed from naïve mice and from mice that had been treated with a control GpC ODN. Lungs were dissected from the trachea, main stem bronchi and surrounding tissue, and spleens were excised as a whole. Organs were cut into small pieces in cold HBSS (GIBCO Invitrogen), and the dissected tissue was incubated in HBSS containing 5% FBS (Euroclone), 20 μ g/ml Dnase I (Roche Diagnostics) and 200 U/ml collagenase type I (GIBCO Invitrogen) for 45 min at 37°C with constant gentle swirling. The digest mixture was then agitated, centrifuged and incubated in calcium- and magnesium-free HBSS containing 10 mM EDTA for 5 min at room temperature. Digested organ fragments were further disrupted by gently pushing the tissue through a 70- μ m cell strainer (BD Biosciences, San Diego, Calif., USA), and the single cell suspension was subjected to RBC lysis. Cells were then washed, and the viability was determined by trypan blue dye exclusion.

Total Lung Cell, CD11c+ and B Cell in vitro Stimulation

Lung cell suspensions were prepared from naïve mice. CD11c+ cells were isolated using CD11c microbeads (Miltenyi Biotec, Bologna, Italy) and B cells using the B cell isolation kit (Miltenyi Biotec). Total lung cells, CD11c+ and B cells were cultured for 18 h with CpG (10 μ g/ml), control GpC (10 μ g/ml) or PBS, and cytokine levels were determined in cell culture supernatants.

Flow-Cytometric Analysis of Lung and Spleen Cell Suspensions

For surface staining, lung and spleen cells were incubated with the Fc block reagent (anti-CD16/CD32 monoclonal antibodies, mAbs; BD Pharmingen, San Diego, Calif., USA) to reduce non-specific binding 10 min before Ab supplementation. The following anti-mouse Abs were used to define the lymphocyte subsets:

CD4-PE, CD8-biotin, DX5-FITC, B220-allophycocyanin and CD3-PerCP-Cy5.5. To determine the activation status of T and NK cells, cells were stained with anti-CD4-PE, anti-CD8-biotin, DX5-FITC, anti-CD44-allophycocyanin and anti-CD69-PerCP-Cy5.5; to determine B cell activation, cells were stained with anti-CD19-allophycocyanin, anti-MHC-II-biotin, anti-CD69-FITC, anti-CD80-PE and anti-CD86-PE. DC subsets and the levels of costimulatory molecules were defined using anti-MHC-II-biotin, anti-CD11c-allophycocyanin, anti-CD11b-PerCP-Cy5.5, anti-B220-Alexa700, anti-PDCA-1-PE, anti-CD80-FITC, anti-CD86-FITC and anti-CD40-FITC. Cells were stained for surface markers and then fixed with 2% paraformaldehyde. CD8 and MHC-II binding were revealed by secondary staining with Streptavidin-Pacific Blue. Isotype-matched mAbs (PE, Pacific Blue, FITC, allophycocyanin and PerCP-Cy5.5 conjugated) were used to determine the background of fluorescence. All Abs were purchased from BD Pharmingen except for the anti-plasmacytoid DC Ag-1 (PDCA-1; Miltenyi Biotec). Sample acquisition was performed on an LSRII flow cytometer (BD Biosciences) and data were analyzed using the Diva Software (BD Biosciences). The different cell types were identified on the basis of their forward and side scatter profile and by their phenotype as follows: mature cDCs (CD11c+ MHC-II^{high} CD11b+, R2 cells), the mixed population of AMs and immature DCs were gated as CD11c+ MHC-II^{int} cells (R1 cells), pDCs (PDCA-1+ B220+ CD11c^{int} MHC-II^{int}), T lymphocytes (CD3+ and CD4 or CD8+), B lymphocytes (CD19+ MHC-II+) and NK cells (CD3- DX5+). T and NK cell activation was determined by cell surface expression of the activation markers CD69 and CD44 on gated cells, B cell activation by expression of CD69, CD80 and CD86 and DC activation by expression of CD80 and CD86.

FACS Analysis on BAL Cells

BAL for FACS analysis was performed as described above. Cells from 3 mice per group were collected at the time points indicated in the figures. BAL from GpC-treated mice was collected 2 and 4 days after treatment. Cells were washed with PBS plus 5% FCS and incubated for 5 min with 20% rabbit serum before the addition of Abs. To identify the lymphocyte subset, cells were stained as described for FACS analysis on lung cell suspension. In addition, the following Ab mix was used to define macrophages and cDCs: anti-MHC-II-FITC, anti-CD11b-PE Cy7 and anti-CD11c-allophycocyanin.

BAL cells were diluted in equal volumes of PBS and the same amount of Liquid Counting Beads (BD Pharmingen) was added to normalize the data. Data acquisition was stopped after counting 10,000 beads for each sample. Macrophages were identified as MHC class II^{high}CD11b^{int}CD11c^{high} whereas cDCs were identified as MHC class II^{high}CD11b^{high}CD11c^{high} [27]. NK cells were identified as described above.

In vitro Activation of Lung Cells

Single cell suspensions prepared as described above were cultured for 1 or 2 days in culture medium as indicated above for AM activation in the presence of CpG at the indicated amounts. Cells were subsequently stained with the appropriate combinations of mAbs, and expression levels of CD69, CD86 and CD80 were determined on B cells gated as CD19+ MHC-II+ cells or on cDCs in R2 gated as CD11c+ MHC-II^{high} cells.

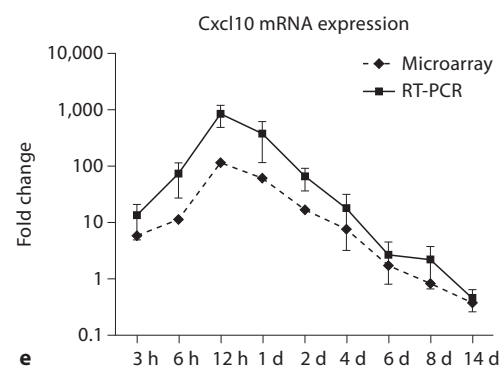
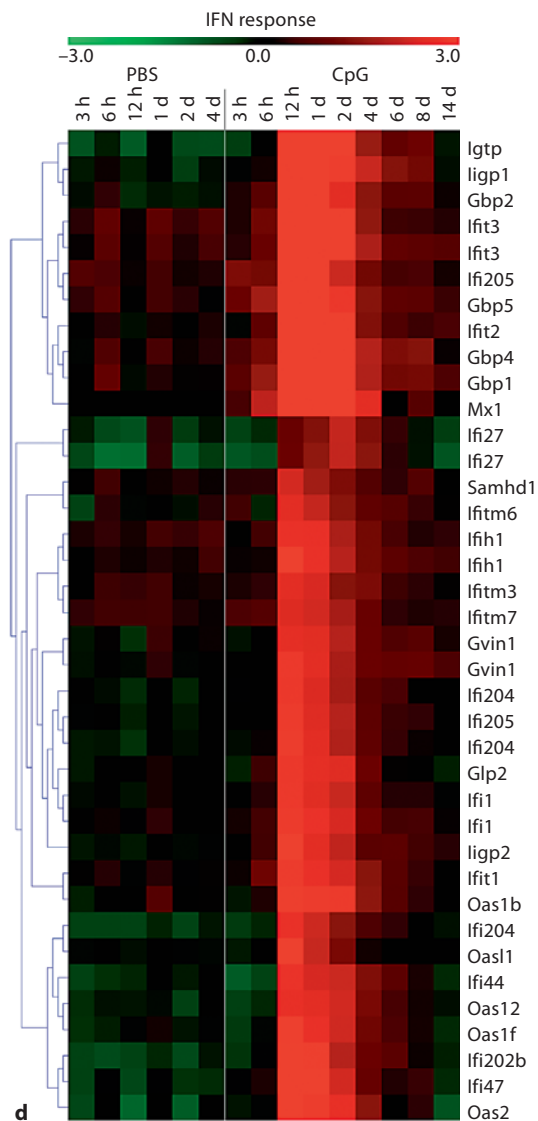
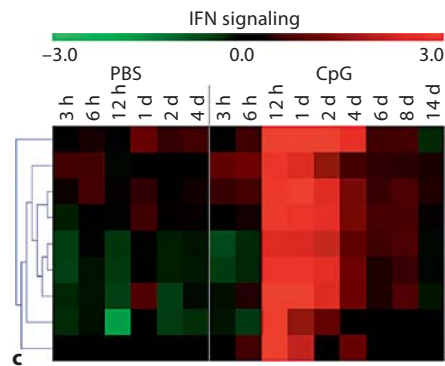
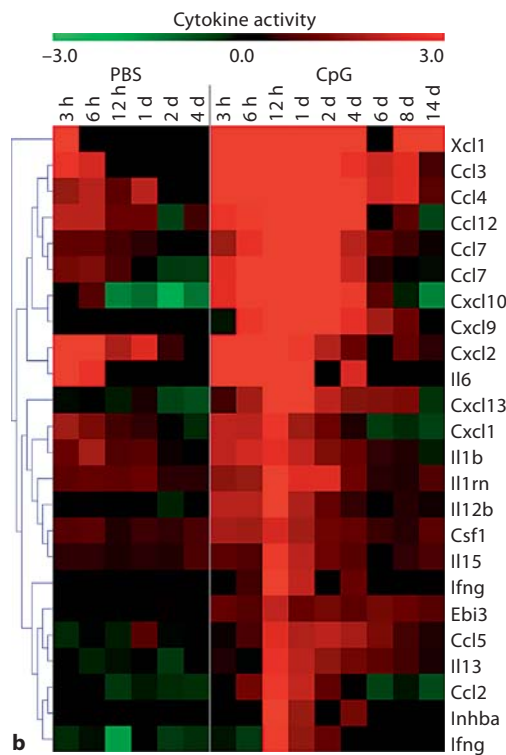
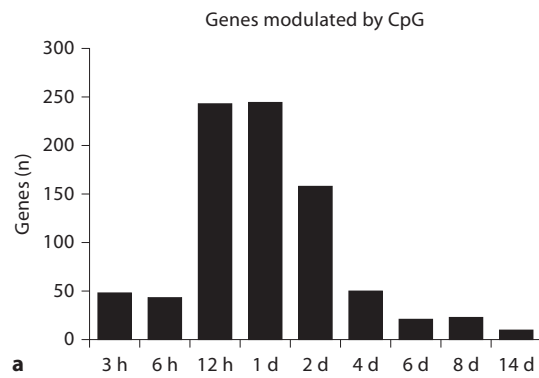


Table 1. DCs, macrophages and B cells produce inflammatory and chemoattractive chemokines in the lung

	Lung ex vivo	Total lung cells in vitro	Total CD11c+	AMs	Lung B cells	Epithe- lial cells
IL-1 α	8	+				
IL-1 β	9	+	+			
IL-6	10	+	+	+	+	
IL-10		+	+			
IL-12 p40	63	+	+	+		
IL-12 p70			+			
Eotaxin			+		+	
G-CSF	50		+			
IFN- γ		+	+			
KC	22			+		
MCP-1	17	+	+			
MIP-1 α	5	+	+	+	+	
MIP-1 β	16	+	+	+	+	
RANTES	16	+	+	+		
TNF- α		+	+			

Summary of cytokines detected in lysates of CpG-treated lungs ex vivo (numbers indicate fold increase over background at peak levels) and by the indicated purified cell subsets stimulated with CpG in vitro for 24 h (+ indicates at least threefold increase over background and at least 100 pg/ml in the supernatant, empty spaces indicate values below these inclusion criteria). The following cytokines were tested and not found sufficiently increased in any sample: IL-2, IL-3, IL-4, IL-5, IL-9, IL-13, IL-17 and GM-CSF.

Fig. 1. Microarray analysis of CpG-responsive genes: 841 genes have been selected with an average fold change $\geq |4|$ and $p \leq 0.05$. SAM algorithm comparing CpG and PBS responses has been used to identify 372 CpG-responsive genes. **a** Number of CpG-responsive genes between 3 h and 14 days after treatment. **b–d** Hierarchical clusters of genes encoding proteins with cytokine activity (**b**), genes involved in the IFN signaling (**c**) and IFN-responsive genes (**d**). Each column represents one time point. Each row represents the average expression kinetics of one gene. Some genes, such as Ccl7, appear more than once in the cluster because they are represented by multiple unrelated probes in the Agilent 44K whole mouse genome array. The expression values are shown as log2 fold change compared to untreated muscle and the color scale ranges from -3 (green, downregulation) to 3 (red, upregulation). **e** The Cxcl10 mRNA expression level, relative to ribosomal protein RPS10, was measured by qPCR (■) and compared with microarray data (◆). Data are expressed as average fold change in CpG-treated compared to untreated lung from three independent experiments.

Results

Microarray Analysis of the Gene Expression Profile in the Lung following Intranasal Administration of CpG

In order to investigate the local effect of CpG in mouse lung, we administered 10 μ g of CpG intranasally and monitored the lung gene expression profile from 3 h to 4 days using whole genome ODN microarrays as described in the Materials and Methods. As a control, we monitored the expression profile induced by an equal volume of PBS. For each time point, we performed three independent experiments and calculated the mean expression ratio. We selected 841 genes having an average fold change $\geq |4|$ compared to untreated mice and $p \leq 0.05$; 554 (66%) genes were upregulated, 285 (34%) were downregulated and 2 displayed a biphasic profile. To select the genes that were specific for CpG we performed SAM as described previously. We identified 372 CpG-specific genes: 366 (98%) were upregulated, 1 had a biphasic profile and 5 (1.3%) were downregulated (online suppl. table S1, www.karger.com/doi/10.1159/000254948). PBS treatment resulted in a short response between 3 and 24 h for a small subset of these genes. By contrast, most CpG-responsive genes were still regulated on day 4. To better characterize the kinetics of the gene expression profile in the lung in response to CpG, we extended microarray analysis to 6, 8 and 14 days. CpG modulated the largest number of genes between 12 and 48 h, while only a modest number was still modulated after 14 days (fig. 1a). Next we performed function-based analysis of CpG-responsive genes using several databases including Gene Ontology and Ingenuity Pathway Analysis. CpG induced the activation of several cytokines which were clustered based on their expression profile (fig. 1b). A group of early response cytokines, including Ccl4, Ccl12 and Ccl7, Cxcl1, Cxcl0 and Il1b, were upregulated only by CpG at 3 h, while other cytokines, such as Xcl1, Ccl3, Cxcl2 and Il6 were also weakly upregulated by PBS. The Th1-related cytokines Ifng, and Il12b, as well as Ccl5, Ccl2, Cxcl13, Il1r2, Il13 and Il15, were induced by CpG 12 h after treatment (fig. 1b). In agreement with the proposed role for CpG as a Th1 immunomodulator, we did not detect any upregulation of the Th2 cytokines Il4 and Il5, whereas Il13 was strongly upregulated at 12 h. Despite the Il13 gene upregulation, IL-13 protein was not detected in mouse lung or serum, or in supernatants of cells stimulated with CpG in vitro (table 1 and data not shown), consistent with a weak Th2 response.

Even if no genes encoding type I IFN were selected by microarray analysis, a cluster of genes involved in IFN

signaling (Irf1, Irf7, Stat1, Stat2 and Ifng) were upregulated by CpG 12 h to 4 days after treatment (fig. 1c). In addition, CpG modulated a cluster of IFN-inducible proteins (Oasl1, Oasl2, Oas2, Oasl1f, Gbp1, Gbp2 and Gbp4; fig. 1d) and IFN-responsive cytokines such as Cxcl10, Cxcl9 and Ccl2 (fig. 1b). Within the CpG-responsive genes, we also identified other host defense genes (Fc receptors, matrix metalloproteases, pentraxin-3, C-type lectins and scavengers receptors; online suppl. table S1) that may be involved in CpG-mediated generic protection from airway infection. The IFN responses induced by CpG in the lung peaked after 12 h and returned to basal levels 6 days after treatment (fig. 1c, d). To validate the expression profile of IFN-responsive genes we performed RT-PCR on Cxcl10 (fig. 1e). The results obtained by microarray and RT-PCR are comparable.

Rapid Induction of Inflammatory Cytokines in the Lung after CpG Administration

In order to study lung cytokine and chemokine expression at the protein level, we prepared lung homogenates at various time intervals after intranasal CpG administration and measured the production of cytokines by ELISA-based multiplex analysis. Already 3 h after CpG treatment, the proinflammatory cytokines CCL5 (RANTES), CCL4 (MIP-1 β), IL-12 (p40) and CCL2 (MCP-1; fig. 2a) as well as CCL3 (MIP-1 α), IL-1 α , IL-1 β , IL-6, G-CSF and CXCL1 (KC) (table 1 and online suppl. fig. S1) were induced in the lung, peaking after 6 h to 1 day. Most cytokines returned to baseline concentrations after 1–2 days (fig. 2a), whereas CCL5 and CCL4 concentrations remained at levels over background until at least day 4 (fig. 2a). A high degree of correlation was found between cytokine concentrations and RNA fold increase measured by microarray (fig. 2a). In lungs from mice treated with PBS (fig. 2a) and GpG control ODN (not shown), none of the cytokines tested were increased.

CpG-Induced Cytokine Release from Potential Target Cell Populations in vitro

In order to identify the direct target cell populations of CpG in the lung, we purified cell subsets from the lung and stimulated them in vitro. Results are summarized in table 1. Cell populations were stimulated with CpG for 18 h, and cytokines were scored as positive if the induction over PBS-treated samples was at least threefold. We excluded cytokines if the absolute amount in stimulated samples remained <100 pg/ml.

First, total lung cells were activated in vitro and cytokines produced were compared to those found ex vivo. Most cytokines detected in the lung were also observed in vitro, with the notable exception of G-CSF and KC. These two were however found in the CD11c⁺ and in the AM subsets, respectively, indicating that the two subsets are the main source of these cytokines and that only purified cells produced these cytokines at detectable levels.

A range of TLR9-expressing cells are described in the lung, e.g. B cells, DCs and interstitial macrophages and AMs. When we activated lung CD11c⁺ cells comprising AMs and interstitial lung macrophages and DCs, we detected largely the same set of cytokines that is also found in lung extracts ex vivo and in total lung cells stimulated in vitro (table 1). The only cytokine not found here is IL-1 α , suggesting that lung cells other than CD11c⁺ or B cells are the main producers of this cytokine. In addition, only here IL-12 (p70) was detected, indicating that the bioactive form of IL-12 is mainly produced by lung DCs and not by lung macrophages.

AMs constitute the first line of defense in the airways. They produced IL-6, IL-12 (p40), CXCL1 (KC), CCL3 (MIP-1 α), CCL4 (MIP-1 β) and CCL5 (RANTES) in response to CpG ODNs (fig. 2b; table 1). No changes were found for the other cytokines tested. AMs also appear to be the main producers of KC, since it was found only in this subset. Purified lung B cells produce a restricted set of cytokines upon CpG stimulation, comprising IL-6, eotaxin and MIP-1 α and - β . In contrast, there was no cytokine production by a type II alveolar epithelial cell line in response to CpG. This comprehensive analysis indicates that CpG specifically activates AMs, DCs, interstitial macrophages and B cells in vitro, inducing widely overlapping sets of cytokines. Taken together, these data suggest that except for the type II alveolar epithelial cell line all cell populations tested here are potential contributors to the cytokine microenvironment generated by CpG administration in the lung.

CpG-Dependent Increase in Number and State of Activation of NK Cells, cDCs and pDCs in the Lung

Lung DCs are ideally positioned to sample inhaled agents at alveolar surfaces within and below the epithelium and thus are likely to play an important role in the induction of immune responses to pulmonary infections. Therefore, we investigated if CpG administration in vivo altered the number and activation state of lung cDCs and pDCs. Mature lung cDCs at steady state were identified as CD11c⁺MHC-II^{high} (gate R2), while CD11c⁺MHC-II^{int} cells (gate R1) represented a mixture of AMs and imma-

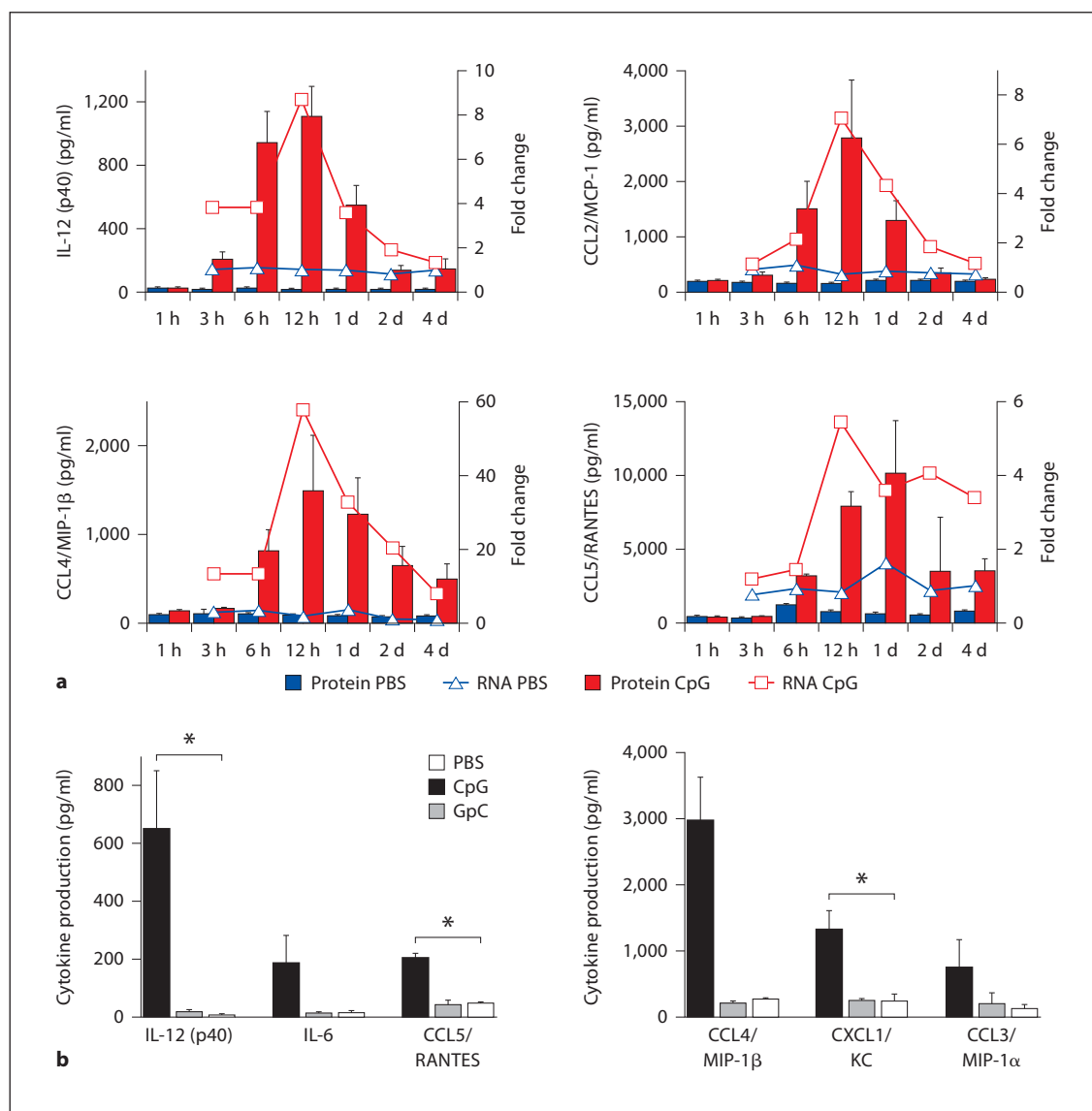


Fig. 2. Kinetics of CpG-induced cytokine genes and proteins detected in the lung and cytokine release by AMs in culture. **a** Protein concentrations (columns) and relative RNA levels (lines) of the indicated representative cytokines were determined in lung homogenates after CpG (□) or PBS (△) treatment (1 h to 4 days, d) by multiplex ELISA or expression profiling. Cytokine concentrations are expressed as means $\pm$ SEM (pg/ml) of three animals

per group (left y-axis) and RNA levels as fold increase (right y-axis). **b** AMs from naïve BALB/c mice were cultured with CpG (10 μ g/ml), control GpC (10 μ g/ml) or PBS for 18 h, and cytokine release was measured in culture supernatants by multiplex analysis. Data represent the mean cytokine production $\pm$ SEM in three experiments conducted in the same manner. * $p < 0.05$ (unpaired Student's t test).

ture cDCs (fig. 3a). Two days after CpG treatment, the number of mature cDCs was increased (fig. 3a, b), while the cell number in the R1 gate remained stable (not shown). The increased cell number in R2 may be caused by upregulation of MHC class II on immature cDCs or AMs upon activation. Alternatively, it could result from the recruitment of new DCs to the lung. Interestingly,

CpG induced also the rapid upregulation of CD86 (fig. 3c) and CD80 (not shown) in the R2 cell population, which starts after 12 h, peaks after 1–2 days and returns to background levels on day 6. Treatment with the control ODN GpC did not have any effect (data not shown).

Since pDCs strongly express TLR9 and are directly activated by CpG [3], the number and phenotype of pDCs

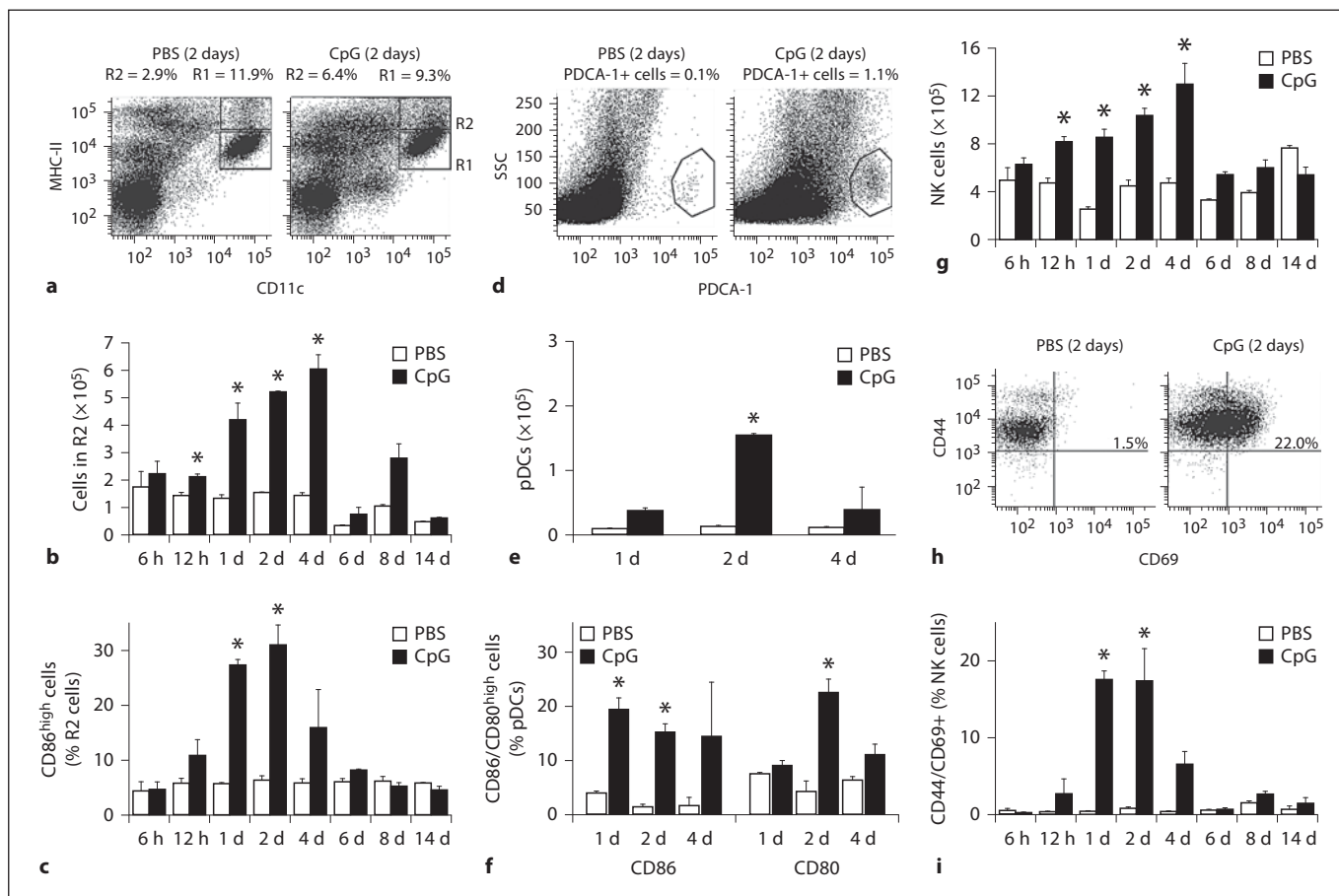


Fig. 3. CpG leads to an increase in both number and activation level of DCs and NK cells in the lung. **a** The number of lung DCs increases after 2 days of CpG treatment. Lung cDC (R2) and AM and immature cDC (R1) populations were identified on the basis of CD11c and MHC-II expression. **b** Absolute numbers of cells in R2 at different time points after CpG or PBS treatment. **c** CpG induces CD86 upregulation on the surface of lung cDCs. Percentage of cells in R2 expressing CD86 at high levels after treatments. **d** Lung pDCs were identified as PDCA-1-positive cells. **e** Absolute numbers of pDCs at different time points were calculated using percentages as in **d** times total viable lung cells. **f** Percentage of

pDCs expressing CD86 and CD80 at high levels over time. **g** Absolute number of NK cells (CD3-DX5+) after different periods of CpG or PBS treatment. **h** Activated NK cells were identified as CD3-DX5+ CD44+CD69+. **i** Quantification of the percentage of NK cells positive for CD44 and CD69 over time. The absolute number of these cell types was calculated by multiplying the respective percentage of cells by total viable cell number. All data are expressed as means $\pm$ SE of 3 mice/group and are representative of two independent experiments. * $p < 0.05$ vs. PBS-treated (unpaired Student *t* test).

were investigated at various time points after treatment. Two days after intranasal administration of CpG, the absolute number of lung pDCs increased 12-fold compared to control mice (fig. 3d, e). Moreover, lung pDCs rapidly acquired an activated phenotype, as determined by up-regulation of the costimulatory molecules CD86, CD40 and MHC-II on day 1 and CD80 on day 2 (fig. 3f and data not shown). Administration of control GpC did not lead to changes in the numbers or state of activation of lung pDCs (data not shown).

Another important cell type in the first line of host defense are NK cells. We therefore determined the effect of intranasal CpG administration on lung NK cells. The absolute number of NK cells increased from 12 h to 4 days after treatment (fig. 3g). CpG also induced NK cell activation at the same time points, determined by the up-regulation of CD44 and CD69 (fig. 3h, i). GpC control did not have any effect (data not shown).

In conclusion, CpG administration led to a transient increase in the number and state of activation of mature

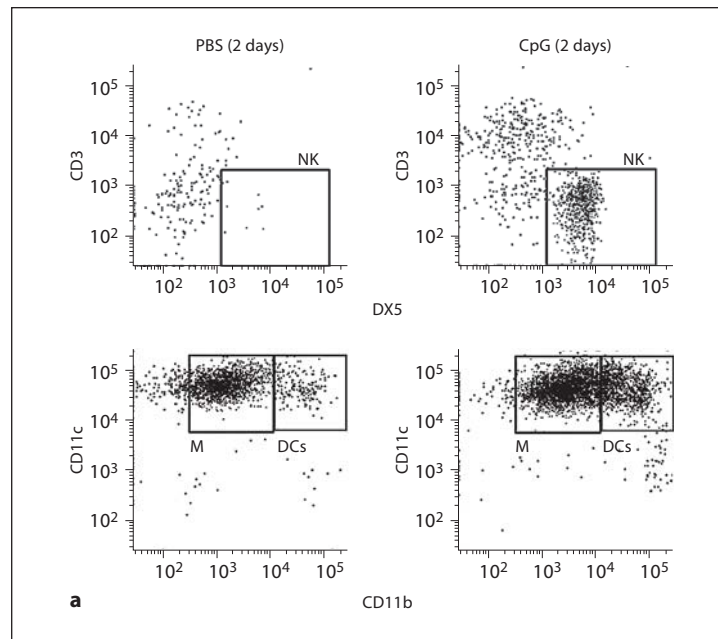
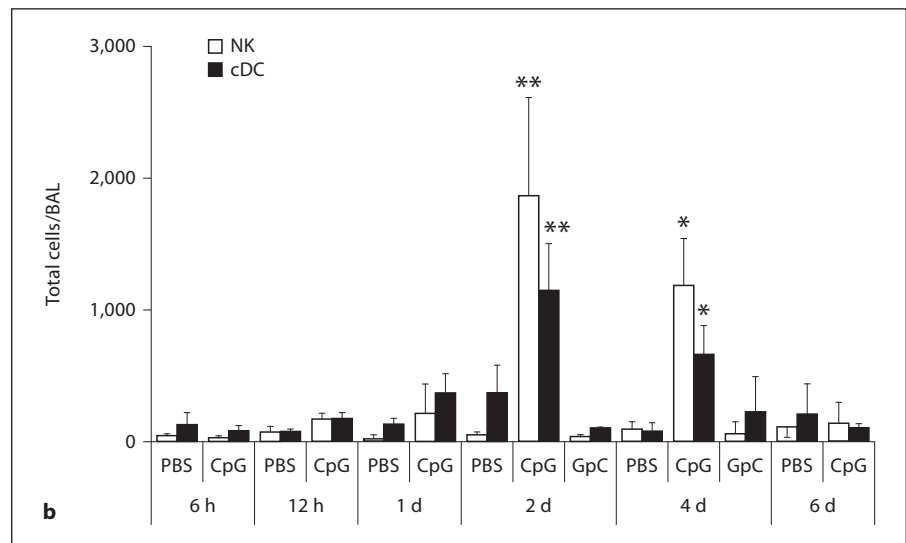


Fig. 4. CpG induces recruitment of NK cells and cDCs into the bronchoalveolar spaces. **a** Flow-cytometric analysis of the cell populations present in the BAL 2 days after treatment with CpG (right panels) or PBS (left panels) as control. NK cells were defined as CD3⁺DX5⁺, cDCs as CD11c^{high}CD11b^{high} and macrophages (M) as CD11c^{high}CD11b^{int}. **b** Kinetics of CpG-dependent recruitment of NK (□) and cDCs (■) in the BAL. BALs from mice immunized intranasally with CpG or PBS were collected between 6 h and 6 days and analyzed by FACS. In addition, control mice were immunized intranasally with GpC, and BALs were collected 2–4 days after treatment. ** $p < 0.01$; * $p < 0.05$, vs. PBS-treated (two-tailed, unpaired Student *t* test).



cDCs, pDCs and NK cells in the lung, starting 12 h after treatment, which returned to background levels 4–6 days after treatment.

CpG-Induced Selective Recruitment of NK Cells and cDCs in the Bronchoalveolar Space

As both numbers and activation state of innate immune cells are increased in the lung tissue by CpG administration, we determined whether these cells were also recruited to the airways, where the first encounter with inhaled pathogens occurs. To this end, cells ob-

tained from the bronchoalveolar spaces of mice treated with CpG and PBS or GpC as control were analyzed by flow cytometry. As shown in figure 4a, CpG induced the recruitment of NK cells (DX5⁺ and CD3⁺) and cDCs (CD11c^{high}CD11b^{high}) 2 days after treatment (fig. 4a, right panel), while no significant increase was triggered by PBS or GpC (fig. 4a, left panel, and 4b). Kinetic analysis of cell recruitment showed that the CpG-induced increment in NK cells and cDCs in the BAL was reversed by 4 days (fig. 4b). Changes in the number of AMs, neutrophils, eosinophils, T cells and B cells (data

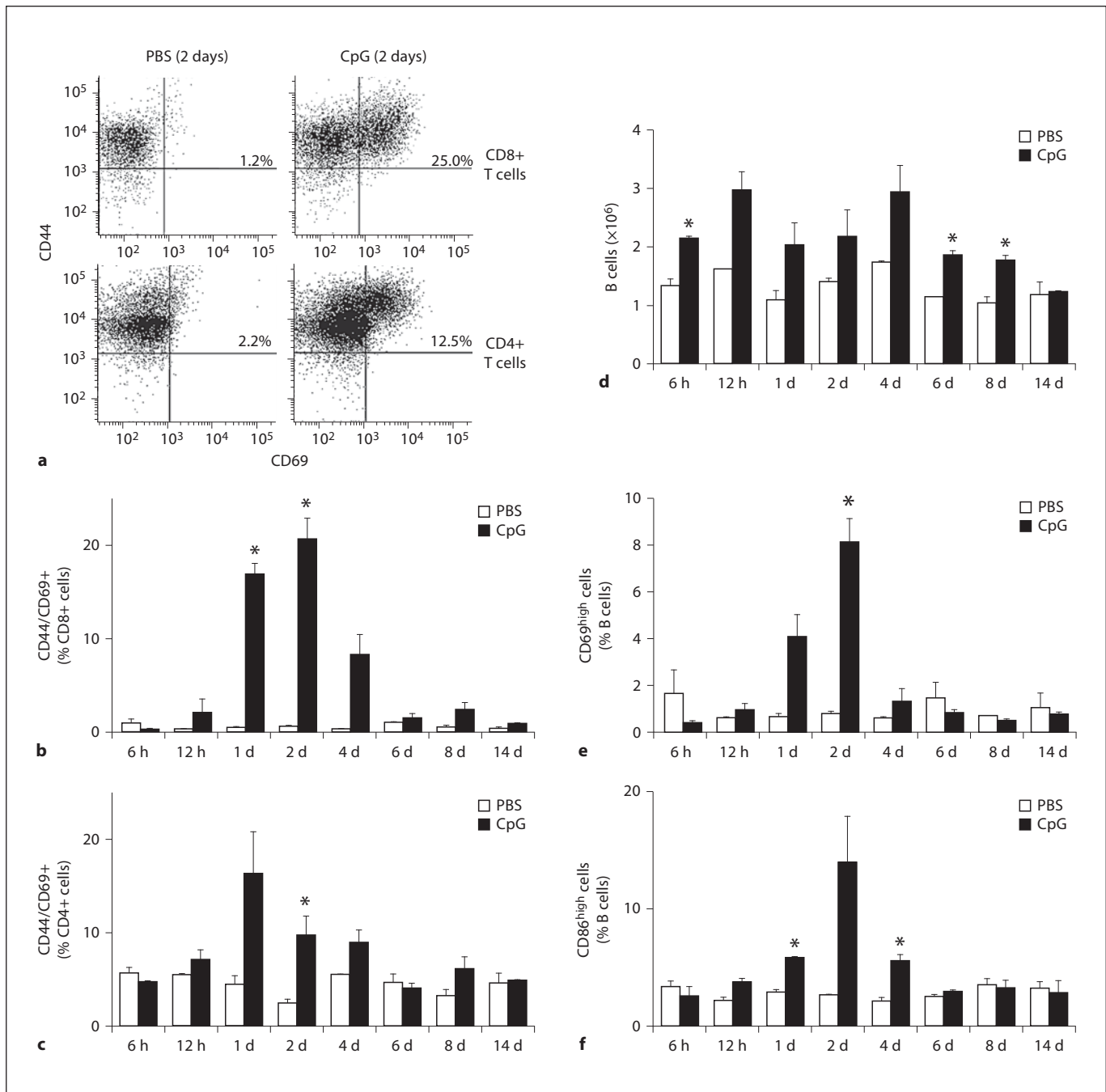
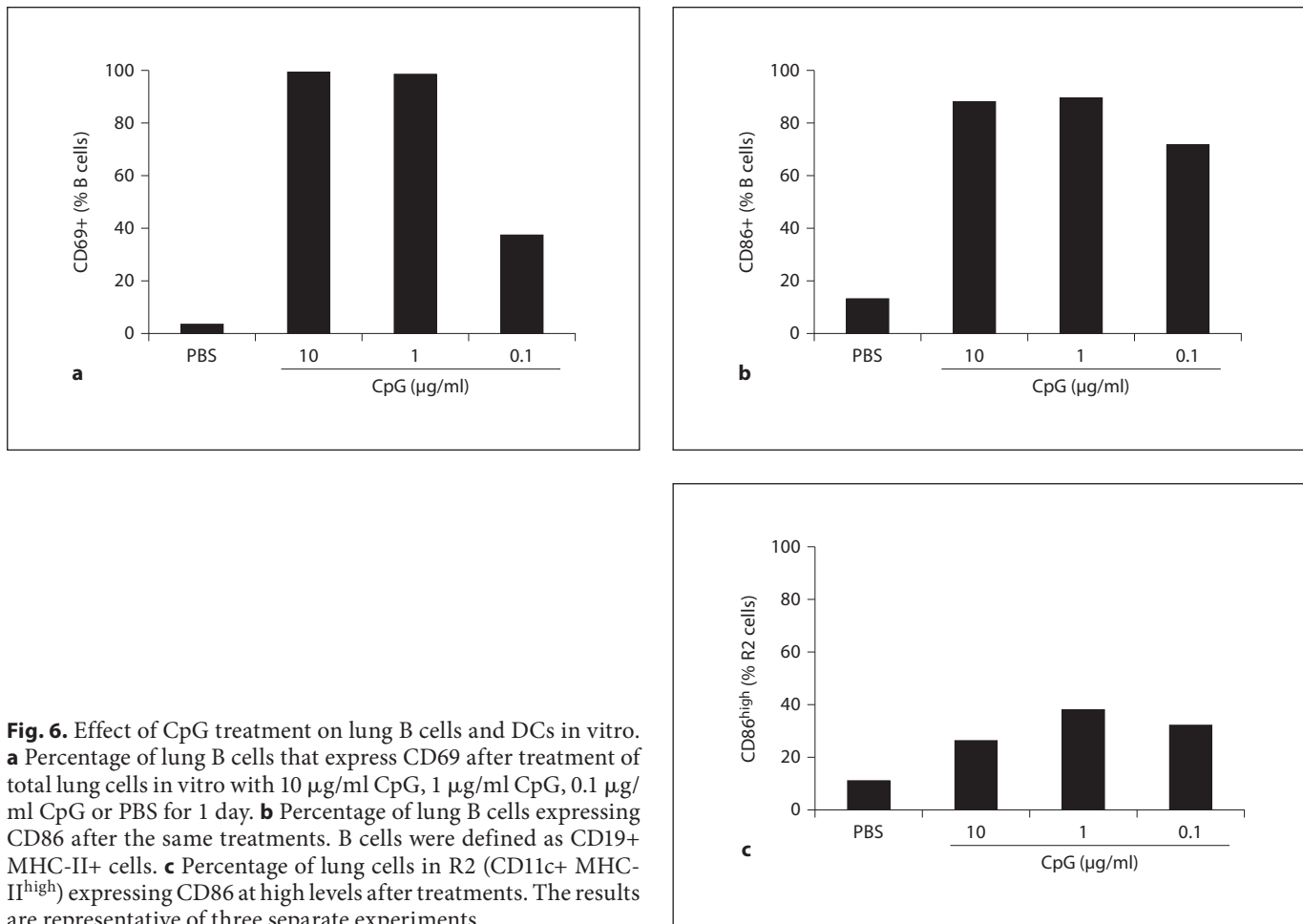


Fig. 5. CpG activates CD4+ and CD8+ T cells and induces moderate activation of B cells in the lung. **a** CD69 upregulation of lung CD8+ and CD4+ T cells after 2 days of CpG treatment. Lung cells were stained with anti-CD4 or -CD8, anti-CD44 and anti-CD69; activated T cells were identified as CD44+CD69+ among CD8+ or CD4+ cells. **b, c** Quantification of the percentage of CD8+ and CD4+ T cells positive for CD44 and CD69 at different time points of CpG or PBS treatment (6 h to 14 days). **d** Moderate increase in

B cell numbers after CpG administration. B cells were identified as CD19+ MHC-II+ cells. Absolute numbers of B cells at different periods of CpG or PBS treatment. **e, f** Percentage of B cells expressing CD69 (**e**) and CD86 (**f**) at high levels over time. Data are expressed as means $\pm$ SEM from 3 mice per group and are representative of two independent experiments. * $p < 0.05$ vs. PBS-treated at the same time point (unpaired Student t test).



no shown) were absent, highlighting that the CpG-dependent recruitment of cDCs and NK cells was highly selective.

CpG-Dependent Activation of T and B Cells in the Lung

Given the rapid induction of an inflammatory cytokine microenvironment in the lung and activation of DCs and NK cells after CpG treatment (described above), we investigated whether this was also reflected by lung tissue T cells. CD8+ and CD4+ T cell subsets found in the lung tissue 1–2 days after CpG administration showed an activated phenotype, as determined by cell surface expression of the activation markers CD69 and CD44 (fig. 5a–c). CD4+ and CD8+ T cells returned to resting state 6 days after treatment. In contrast to the lung, CpG-induced changes were absent on spleen T cells (data not shown). Since B cells are described as direct target cells of

CpG, the number and state of activation of these cells was also assessed in the lung, but only a moderate increase in cell number as well as slightly increased percentages of cells expressing the activation markers CD69 and CD86 (fig. 5d–f) were noted. To determine whether lung B cells are generally unresponsive to CpG or whether the in vivo conditions restricted activation, total lung cells were cultured with CpG and the same activation markers were tested on B cells present in culture. After in vitro exposure to CpG, B cells strongly upregulated CD69 and CD86 but not CD80 at doses >100 times lower than those given intranasally after 1 (fig. 6a, b) or 2 days of culture (data not shown). Thus, CpG-induced B cell activation in vitro clearly exceeds that in vivo (fig. 5e, f). In contrast, CD86 upregulation measured on MHC-II^{high} cDCs (R2 cells in fig. 3a) after in vitro culture is much lower than that of B cells (fig. 6c) and is comparable to the values found for cDCs in vivo (fig. 3c). Therefore, while lung B cells are

TLR9-positive and respond vigorously in vitro to B-type CpGs, recruitment and activation in the lung are neither faster nor stronger than those of T and NK cells which are TLR9-negative and therefore activated only indirectly.

Discussion

In this study, the immunomodulatory effects triggered by CpG treatment were analyzed in the lung by combining various cellular and molecular techniques. The consistent picture emerging from all approaches is that CpG triggers a rapid and transient induction of immunological events starting 3 h after treatment and peaking between 12 and 48 h, with all parameters returning to background levels 2–6 days after treatment. Global expression profiling showed that CpG-induced genes were associated with response to IFN and other host defense pathways and with a large number of cytokine and chemokine genes related to cell recruitment processes. Consistent with the lung gene expression profile, the same cytokine and chemokine proteins rapidly increased in the lung tissue. Many of these cytokines were also produced by in vitro stimulated AMs, B cells, and CD11c+ cells comprising DCs and most lung macrophages, suggesting that all these cells are potential contributors to the cytokines produced in CpG-treated lung. AMs are at the front line and therefore represent a prime CpG target in the lung. In contrast to our data, previous studies have shown that AMs do not release cytokines in response to CpG [28]. The use of different CpG ODNs might explain these discrepancies. Indeed, B-type CpG stimulation induced a similar set of cytokines as the one detected in this study in mouse peritoneal and bone marrow-derived macrophages [28].

The early upregulation of CpG-selective chemokines (Ccl4, Ccl12, Ccl7, Cxcl1 and Cxcl10) and Il1b may promote the recruitment of antigen-presenting cells including DCs in the lung. The subsequent upregulation of the Th1-inducing cytokine Il12 and of Ifng indicates that lung antigen-presenting cells are activated following recruitment. IL-12 production by cDCs is among the best-described pathways of CpG-B-triggered immune activation [29]. In addition, the overwhelming upregulation of IFN-regulated genes suggests an important role of the encoded proteins, in spite of the undetectability of IFN- α messengers or protein. Also in other experimental settings, a range of IFN-responsive genes were upregulated, but no type 1 IFN was found [30, 31], suggesting that under certain circumstances, type 1 IFN may not be required to induce these genes. Alternatively, type 1 IFN amounts be-

low our detection limit may be present and suffice to induce this gene cluster. In conclusion, we speculate that early chemoattractants for innate cells and DC-derived cytokines are the central components of the CpG effect, since increased numbers of activated DCs able to ingest the antigen migrate to lymph nodes, and priming of specific CD4 T cells is crucial for mucosal adjuvanticity.

Combining our ex vivo and in vitro data, lung DCs represent as the main if not sole source of IL-1 β , G-CSF and CCL2 (MCP-1), since they were produced by CD11c+ cells which comprise AMs and lung DCs but not by AMs alone. IL-12 (p70) is also produced by DCs only, while KC is secreted by AMs only. Other cytokines are produced by more than one cell type. The emerging picture is that most responsive cells produce an overlapping 'core' set of cytokines, while secretion of some others is a more specialized function of distinct subsets. We have also tested cytokine production by a mouse type II alveolar epithelial cell line and did not find any response to CpG, thus excluding this cell type as a direct target of CpG. In addition, the expression of TLR9 on lung DCs has been challenged. Some reports observed TLR9 expression in mouse lung cDCs and lung CD11c+ cells [28, 32], while other studies demonstrated that TLR9 is absent in cDCs and pDCs in lung-draining lymph nodes and that these cells do not respond to CpG [32]. In our study, multiparameter flow cytometry disclosed upregulation of activation markers on DCs, but we cannot exclude that this is a secondary effect.

B cells are TLR9-positive and produce cytokines in response to CpG in vitro. To our surprise, as assessed by flow cytometry ex vivo, B cell activation was moderate over the whole time range of this study for a number of different markers. In fact, the activation observed is slower and smaller compared to that of the indirect target cells, T and NK cells. In in vitro experiments, lung B cells per se were highly responsive to CpG, but activation observed in the lung was relatively weak. One possible explanation is that most CpG may be efficiently sequestered by cells having access to the bronchoalveolar space, e.g. AMs and DCs, which were also activated in vivo. Thereby, the local spread and/or half-life of CpG in the lung may be low, and the activation of B cells in the lung tissue would be limited by their restricted access to CpG. In agreement with our observations, a recent study described that TLR4 and TLR9 agonists are able to induce differentiation of memory B cells into plasma cells in vitro but not in vivo [33]. A second possibility is that CpG-activated B cells rapidly emigrate from the lung and therefore are not readily found ex vivo.

The cytokine/cytokine receptor network induced by CpG administration in the lung may play a central role in the recruitment and activation of NK cells and DCs in this organ. Among the cytokines detected in the lung, MCP-1 was shown to attract monocytes [34]. Furthermore, MIP-1 α , MIP-1 β and RANTES, upon engagement of their common receptor CCR5, are involved in the recruitment and activation of immature DCs, T and NK cells [35]. Microarray analysis showed a significantly higher CCR5 signal which parallels cell recruitment (online suppl. table S1). Both cell types were also selectively sequestered in the bronchoalveolar space after CpG treatment. NK cells and cDCs are components of innate defense mechanisms, and, in addition, DCs are responsible for the initiation of an adaptive immune response. Reciprocal activation between these cell types has been extensively reported [36, 37]. NK cells are recruited by DCs into lymph nodes, where they represent an important early source of IFN- γ for the induction of Th1 responses [38]. Cooperation between cDCs and NK cells has also been demonstrated in infection models in vivo. For instance, visceral *Leishmania* infection leads to induction of NK cell cytotoxicity only when cDCs are present and activated in a process dependent on IL-12 production [39]. Concomitant with NK cell activation, IL-12 levels were consistently increased in the lung.

Additionally, pDCs are able to activate NK cells via type I IFN secretion. Although we measured increased numbers of activated pDCs in the lung, we failed to detect a corresponding increase in IFN- α . These data may be explained by the use of B-type CpG, known to be poor IFN- α inducers in pDCs. These findings suggest that in our model, IL-12 secretion by cDCs is the main trigger of NK cell activation.

Besides DC/NK cell cross talk, cooperation between cDCs and pDCs was also reported [40]. pDCs are essential for the induction of IL-15 in cDCs following CpG administration [40], a process required for sustained induction of IL-12 and Th1 responses. In agreement with this, the recruitment of cDCs and pDCs was associated with an increase in IL-15 and IL-12 expression. It is conceivable that the proposed cross talk may occur in the lung as the cellular and molecular players are all present. Consistently, IL-15-induced protein granzyme B was upregulated, which plays a crucial role for the cytotoxic function of NK and CD8 $^{+}$ T cells [41, 42].

Findings of CpG-induced cell recruitment and cytokine production were previously reported. In a *Listeria* infection model, protection induced by systemic CpG administration was associated with an increase in splen-

ic DCs with a particular phenotype differing from both pDCs and cDCs [10]. Local cytokine production and cell recruitment were also found after intragastric and intravaginal administration of CpG, even though the precise composition of cell types recruited and their state of activation were not analyzed [20, 21]. Our findings suggest that the recruitment and activation of DCs and NK cells associated with a transient cytokine induction in response to intranasal CpG administration constitute the condition for an efficient generic protection from airborne pathogens and for mucosal adjuvanticity.

Another interesting feature of CpG-mediated lung immune modulation is the strong activation of T cells in the absence of protein antigens. CpG ODNs are not considered direct stimulators of T cells [3]. However, bystander activation of both naïve and memory T cells has been described [43]. The cytokines involved in these processes are IL-6, IL-1, TNF- α , IL-15 and IL-7, most of which are upregulated by CpG in the lung. The increased frequencies of activated CD4 $^{+}$ and CD8 $^{+}$ T cells induced by CpG may contribute to heterologous protection, as described before [44, 45].

In conclusion, we propose that CpG-induced generic protection and adjuvanticity are mediated by a multilayered response characterized by rapid upregulation of IFN pathway genes, induction of overlapping sets of proinflammatory and chemoattractive cytokines in alveolar and interstitial macrophages, DCs and B cells, and consequent recruitment, activation and bronchoalveolar sequestration of DCs and NK cells. Each of these cell types has multiple host defense functions: both produce antiviral and antibacterial cytokines, DCs ingest, process and present antigen, and NK cells are capable of killing infected cells. Additionally, NK-DC cross talk allows sustained reciprocal activation leading to highly efficient immune responses. B cells are moderately activated in the lung tissue. The increased frequency of activated T cells is likely due to cytokine-mediated bystander activation and potentially contributes to non-specific protection through cytokine production. The concerted action of all these components may be the key for the efficiency of CpG as mucosal adjuvant and for generic protection.

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