Two distinct activation states of plasmacytoid dendritic cells induced by influenza virus and CpG 1826 oligonucleotide

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Abstract: There is growing evidence that plasmacytoid dendritic cells (pDC) are involved in the innate recognition of various microbes. However, the precise consequences of pathogen recognition on pDC activation and function are incompletely understood. Using a novel transgenic mouse model that facilitates the isolation of highly pure pDC populations, we found that influenza virus PR/8, a TLR7 ligand, and CpG 1826 oligonucleotide, a TLR9 ligand, induced surprisingly divergent activation programs in these cells. pDC stimulated with PR/8 produced large amounts of type I IFNs, and CpG 1826-stimulated pDC expressed higher levels of costimulatory molecules and proinflammatory cytokines and induced stronger proliferation of T cells. Transcriptome analysis uncovered the differential regulation in pDC of 178 and 1577 genes by PR/8 and CpG 1826, respectively. These differences may relate to the activation of discrete signaling pathways, as evidenced by distinct ERK1/2 and p38 MAPK phosphorylation kinetics. Finally, pDC isolated ex vivo during PR/8 infection or after i.v. CpG 1826 injection resembled their in vitro counterparts, corroborating that these cells can adopt specialized phenotypes in vivo. Thus, pDC display remarkable functional flexibility, which emphasizes their versatile functions in antimicrobial immunity and inflammatory processes. J. Leukoc. Biol. 83: 000–000; 2008.

Key Words: innate immune cells · Toll-like receptors · differentiation

INTRODUCTION

Plasmacytoid dendritic cells (pDC) are thought to exhibit potent regulatory functions during immune responses [1–3]. On the one hand, pDC participate in the immune defense against microbes in particular viruses. For example, pDC are the prime producers of type I IFNs during early murine cytomegalovirus infection [4]. Furthermore, depletion of pDC resulted in decreased viral clearance and increased pulmonary inflammation in a murine model of respiratory syncytial virus infection [5, 6], as well as an impaired induction of cytotoxic T cells during herpes simplex virus infection [7]. In contrast, pDC have been implicated in maintenance of tolerance. For instance, they prevent sensitization against harmless inhaled antigens, thereby reducing the likelihood of asthmatic reactions [8], and have been shown to enhance engraftment of hematopoietic stem cells and induce donor-specific tolerance to skin allografts [9, 10].

Nevertheless, besides their beneficial effects, pDC may also play a role in aberrant immune responses. Increased numbers of pDC have been observed in systemic lupus erythematosus (SLE), rheumatoid arthritis, and psoriasis, and pDC-derived IFNs may participate in the pathogenesis of these chronic inflammatory disorders [11–15]. Moreover, pDC may negatively impact anti-tumor immunity by inducing regulatory T cells (Tregs) and/or the production of negative immune regulators, such as indoleamine 2,3-dioxygenase [16]. This diversity implicates distinct functions/heterogeneity of pDC. However, although it has been speculated that distinct activation/differentiation states of pDC may exist [17], we have relatively little understanding of how they are regulated at the cellular and molecular level.

Similar to myeloid DC (mDC), pDC reside in an immature state in secondary lymphoid and the peripheral organs, including liver and lungs. There, they act as important sentinels of pathogen-associated molecular patterns (PAMPs), which are generally recognized by members of the TLR family. Upon recognition of PAMPs, pDC undergo a differentiation process that has been characterized by the production of proinflammatory mediators that act on other cells of the immune system. Thus, pDC have been shown to contribute to the differentiation of plasma cells, augment the activity of NK cells, attract T and NK cells during viral infections, and induce Tregs [4, 9, 18–24].

The response of pDC to pathogens and their components has been explored in vitro and in vivo by the use of model ligands.
for the two main TLRs expressed by pDC, TLR7 and TLR9 [25, 26]. Ligands for TLR7 include synthetic compounds of the family of imidazoquinolines [27] and viral ssRNA, including influenza virus [28, 29]. TLR9 on pDC can be triggered by unmethylated CpG-containing motifs common to bacterial and viral DNA [30, 31]. The traditional readout for evaluating the effects of such mediators on pDC has been the production of type I IFNs and a few select cytokines, such as IL-12. In addition, the induction of T cell proliferation and differentiation is commonly used to examine the effects of TLR ligands on pDC. This has led to the discovery that certain compounds induce different amounts of IFNs in pDC. For example, CpG-A oligonucleotides (ODN) are potent inducers of IFN-α, whereas CpG-B ODN elicit a weak release of IFN-α [30, 32]. However, the measurement of these select parameters may underestimate the complexity of changes occurring in pDC after ligand stimulation.

In the present study, we have defined the functional profile of pDC stimulated with two ligands for TLR7 and TLR9, influenza PR/8 virus (PR/8) and CpG 1826 ODN, respectively. We made use of a novel, transgenic mouse model, in which pDC are tagged by GFP, which allowed us to isolate highly pure pDC populations directly ex vivo. Our results demonstrate that pDC respond to PR/8 virus and CpG 1826 with surprising flexibility, resulting in two distinct activation states. Thus, the ability of pDC to be polarized into (at least) two functionally different populations may be used to selectively target their functions for the treatment of microbial and inflammatory diseases.

MATERIALS AND METHODS

Mice

DPEC5′/GFP (crossed to the C57BL/6 background for 10 generations) and DPEC5′/GFP mice crossed to RAG-1−/− (DPEC5′/GFP×RAG) mice were bred under specific, pathogen-free/viral antibody-free conditions at the Wistar Institute (Philadelphia, PA, USA) [33, 34]. C57BL/6 wild-type (WT) mice were purchased from Charles River (Portage, MI, USA). OT-I mice and 129Sv WT mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). IFNAR−/− (129Sv) [35] was obtained originally from L. Buckbaum (University of Pennsylvania, Philadelphia, PA, USA). All animal studies were performed according to Institutional Animal Care and Use Committee-approved protocols.

Fetal liver tyrosine kinase 3 ligand (Flt-3L) treatment

B16F10 tumor cells modified to express Flt-3L (B16-Flt-3L) were grown in RPMI-1640 media (Gibco, Grand Island, NY, USA) containing 10% FBS and antibiotics [36]. Tumor cells (3×10⁶) in 100 μL PBS were injected s.c. into the back skin of mice, and tumors were allowed to grow for 12–14 days.

Purification and stimulation of DC

For in vitro experiments, pDC and mDC were purified from the spleens of B16-Flt-3L-bearing mice. In brief, after RBC lysis, pDC from DPEC5′/GFP×RAG mice were sorted based on high GFP expression using a MoFlo cell sorter (DakoCytomation, Inc., Denmark). In the case of 129Sv WT and IFNAR−/− mice, pDC were identified as CD11c+pyridinedicarboxylic acid (PDCA)-1+ cells. mDC were sorted based on high CD11c and CD11b expression.

DC subsets were resuspended at 1×10⁶/mL in complete media [RPMI supplemented with 10% heat-inactivated FBS (Valley Biomedical, Inc., Winchester, VA, USA), penicillin/streptomycin (Gibco), 10 mM HEPES (Gibco), 2 mM L-glutamine (Gibco), 10 mM pyruvate (Gibco), 50 μM 2-ME (Fisher Scientific, Pittsburgh, PA, USA)]. pDC were incubated in complete media alone or in the presence of CpG 1826 (1–10 μg/mL; phosphorothioate-modified 5′-cgc atg tgc etg acg ttg tgc-3′) or UV-inactivated, sucrose gradient-purified influenza A/PR/8/34 [Mount Sinai School of Medicine, New York, NY, USA; 100–1000 haemagglutination units (HAU)/ml]. mDC were cultured in media with or without LPS (5 μg/mL). Cell pellets and supernatants were collected at the indicated time-points.

IFN-α ELISA

Supernatants from overnight cultures were collected and stored at −80°C. IFN-α ELISA was performed as described [37]. In brief, round-bottom plates (Costar, Corning, NY, USA) were coated with monoclonal anti-mouse IFN-α (HyCult Biotechnology, The Netherlands). Plates were then incubated with samples or a recombiant mouse IFN-α standard (HyCult Biotechnology) for 2 h at room temperature. Subsequently, a polyclonal rabbit anti-mouse IFN-α (PBL Biomedical Laboratories, New Brunswick, NJ, USA) was added for 1 h. Plates were developed with HRP-conjugated goat anti-rabbit IgG (Calbiochem, San Diego, CA, USA) for 1 h, which was followed by tetramethylbenzidine peroxidase substrate (Calbiochem) incubation for 20 min. Reaction was stopped with 250 mM HCl, and signals were measured based on their OD at 450 nm using a Microplate ELISA reader (BioTek Instruments, Winooski, VT, USA).

Flow cytometric analysis of DC and cytopsins

For phenotypic analysis, DC were collected after 24 h of culture, washed, and stained with PE-conjugated IgG2a, CD40, CD80, CD86, CD8α, IAα, and B220-PerCP and CD11c-APC (all from BD Biosciences, San Jose, CA, USA) or PDCA-1-APC (Millenyi Biotec, Auburn, CA, USA). Cytopsins of purified DC were performed using a Cytospin3 centrifuge (Thermo-Shandon, UK). Samples were fixed and stained using the Kwik-Diff stain kit (Thermo-Shandon). Images were taken on a Nikon E600 upright microscope.

T cell proliferation assay in vitro and in vivo

Sorted DC populations were stimulated for 4 h as described above. During the last hour, the SIINFEKL peptide (10 ng/mL, Alpha Diagnostic International, San Antonio, TX, USA) was added. The cells were then washed extensively and their viability was determined by trypan blue exclusion. DC were resuspended in complete media and plated in serial dilutions in 96-well round-bottom plates (Costar). CD8+ cells were isolated from the spleen and lymph nodes of OT-I mice by negative selection using magnetic beads (Miltenyi Biotec). T cells (5×10⁶) were added to each well. Seventy-two hours later, supernatants were collected and incubated for 16 h with 1 μg/mL [3H]thymidine (Perkin Elmer, Wellesley, MA, USA). Cells were transferred to a glass fiber filter (Packard, Dovers Grove, IL, USA), and [3H]thymidine incorporation was measured using a Matrix 96 β counter (Packard).

For the in vivo proliferation assay, C57BL/6 recipients received 5×10⁶ negatively selected CFSE (2 nM, Molecular Probes, Eugene, OR, USA)-labeled CD8+ OT-I T cells. Twenty-four hours later, mice were injected i.v. with 3–5×10⁵ peptide-pulsed or control DC subsets. CFSE dilution was determined in the spleen and lymph nodes 72 h later.

DNA microarrays and data analysis

Gene expression profile of pDC was studied by using mouse MOE430v2 GeneChip microarrays (Affymetrix, Santa Clara, CA, USA). Sorted pDC were incubated in media alone or stimulated with PR/8 (300 HAU/ml) or CpG 1826 (5 mg/ml) for 1 h and 4 h. Samples were prepared in two independent experiments; a third experiment included pDC in media and stimulated with CpG for 4 h. Subsequently, total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA, USA). RNAs were stored at −80°C until further processing. The quality and concentration of the RNA samples were determined on a bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The University of Pennsylvania Microarray Core Facility carried out RNA amplification, labeling, and hybridization procedures by using standard protocols. Data were processed using Affymetrix GeneChip Operating System v1.4 software, and probe intensity files (cel files) were imported into GeneSpring (Agilent Technologies). First, GeneChip Robust Multi-Array Average was used to summarize and normalize data. Principal Component Analysis ensured that

replicates were similar to each other. To identify statistically significant genes, a three-way mixed model ANOVA (time, treatment, and experiment, including an interaction between time and treatment) was calculated using Partek Discovery Suite v6.2 (Partek, Inc., St. Louis, MO, USA). In conjunction with the ANOVA, pair-wise contrasts of medium 1 h versus CPG 4 h and medium 1 h versus flu 4 h were performed. Post-hoc calculation of false discovery rate (using Benjamini Hochberg step-down, as implemented in Partek, Inc.) was applied to determine cutoff values for each P value generated by the ANOVA and the contrasts. Gene lists of pair-wise contrasts were divided into up- and down-regulated genes (compared with medium 1 h). Subsequently, greater than or equal to twofold differences were calculated on replicates to further increase the stringency of filtering the differentially expressed genes. Specificity of a gene for CPG at 4 h was defined as greater than or equal to twofold change as compared with flu 4 h and vice versa. Genes with a greater than or equal to twofold difference between CPG 4 h and flu 4 h, which were up- or down-regulated concomitantly under both conditions, were also defined as specific. Genes of interest were grouped based on their known immunological functions and gene ontology.

**Multianalyte profiling of pDC supernatants**

Supernatants were collected from pDC after 24 h culture under various stimulation conditions. Samples were then subjected to multianalyte profiling at Rules-Based Medicine (Austin, TX, USA).

**Immunoblotting**

Immunoblotting was carried out as described previously [38]. In brief, whole cell lysates were prepared from 10^6 cells using 0.1% Triton X-100, 20 mM HEPES, 10% glycerol, 150 mM NaCl buffer, supplemented with complete protease inhibitor (Roche Applied Sciences, Indianapolis, IN, USA) and phosphatase inhibitor cocktail 2 (Sigma-Aldrich, St. Louis, MO, USA). Primary antibodies for phospho-p38 and phospho-ERK1/2 were obtained from Cell Signaling Technology (Beverly, MA, USA), and anti-β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies were detected by incubation with anti-rabbit HRP-linked secondary antibodies from Pierce Biotechnology (Rockford, IL, USA), and immunoreactive bands were visualized using ECL (Amersham Pharmacia Biotech, UK). To determine total loading, immunoblots were stripped in a buffer containing 2 M Tris, 10% SDS, 14.4 M 2-ME for 30 min at 65°C, and then reprobed for total ERK, total p38, or β-actin (Cell Signaling Technology).

**In vivo stimulation of pDC**

For influenza virus infection, DPE<sup>GFP</sup> mice were anesthetized and inoculated intranasally with 500 TCID<sub>50</sub> influenza virus strain A/PR/8/34 (in 50 μl PBS). For stimulation with CpG 1826, DPE<sup>GFP</sup> mice were injected i.v. with a mixture of CpG 1826 (20 μg) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP; 15 μl, Roche Applied Sciences) in 200 μl PBS. At the indicated time-points, the phenotype of pDC in the lungs, spleens, and lymph nodes was assessed by flow cytometry.

**RESULTS**

**DPE<sup>GFP</sup> × RAG-1<sup>−/−</sup> mice harbor pDC with high levels of GFP expression**

We have recently developed a transgenic mouse strain, DPE<sup>GFP</sup>, in which GFP is expressed under the control of the murine CD4 promoter and two enhancer elements [33]. We observed that in these mice, besides T cells, a population of CD3<sup>+</sup> cells expressed high levels of GFP (Fig. 1, A and B, and data not shown). Extensive phenotypic and functional characterization revealed that these cells represented pDC. GFP<sup>hi</sup> cells were CD11<sup>hi</sup>CD20<sup>+</sup>PDCA-1<sup>+</sup>CD11b<sup>+</sup>NK1.1<sup>−</sup>, produced IFN-α in response to stimulation with TLR ligands, and could be expanded in vivo by Flt-3L, a key differentiation factor for pDC from hematopoietic progenitors (data not shown). Microscopic analysis of freshly isolated GFP<sup>hi</sup> cells demonstrated the typical round, plasma cell-like morphology characteristic of pDC (Fig. 1, C–E [37, 39, 40]). Of note, we found that CD11c<sup>hi</sup> mDC did not express GFP in DPE<sup>GFP</sup> mice, which may be a result of differential requirements for CD4 control elements in the myelomonocytic lineage [41]. Consequently, when we crossed DPE<sup>GFP</sup> mice to RAG-1<sup>−/−</sup> mice, pDC remained the only cells expressing high levels of GFP in secondary lymphoid organs (Fig. 1, A and B). When we compared the phenotype of pDC isolated from the spleens of DPE<sup>GFP</sup> and DPE<sup>GFP</sup> × RAG mice, no difference in the expression of costimulatory molecules, MHC class II, or adhesion molecules was discernable, indicating that pDC developed normally in the absence of B and T cells (data not shown). Thus, for most of the experiments presented here, we sorted GFP<sup>hi</sup> cells from the spleens of Flt-3L-treated DPE<sup>GFP</sup> × RAG. pDC can be expanded up to 100-fold under these conditions without changing their phenotype, which facilitates the purification of sufficient numbers for functional assays. Taken together, DPE<sup>GFP</sup> × RAG mice provide a source for the identification of pDC, simply based on high GFP expression.

**PR/8 virus and CpG 1826 induce differential expression of costimulatory molecules and IFN-α in pDC**

One of the hallmarks of pDC is their production of type I IFNs in response to TLR ligands. In the course of our functional
characterization of GFP<sup>hi</sup> cells in DPE<sub>GFP</sub> mice, we consistently observed that similar to studies with human cells [30, 32], pDC stimulated with PR/8 virus released markedly higher (approximately tenfold; \(P=0.0076\)) levels of IFN-\(\alpha\) into the culture supernatant as compared with CpG 1826-treated cells (Fig. 2A). However, although both stimuli led to increased expression levels of costimulatory molecules, such as CD40 and CD80, as well as MHC class II on pDC, the effect was significantly more pronounced with CpG 1826 (Fig. 2B). An exception was CD86, which was expressed to the same extent under the two conditions. Higher concentrations of virus (up to 5000 HAU/ml) or the use of live influenza virus did not increase the levels of costimulatory molecules or MHC class II on pDC (data not shown). When virus was added simultaneously with CpG 1826, pDC up-regulated costimulatory molecules similarly to CpG alone, suggesting that CpG 1826 had a dominant effect over PR/8 virus (data not shown). Together, there appeared to be a functional dichotomy of pDC after influenza virus and CpG 1826 stimulation that warranted further characterization at the cellular and molecular level.

**Differential role of autocrine IFN feedback in the induction of costimulatory molecules and type I IFNs in response to PR/8 virus and CpG 1826**

Previous work has suggested a positive feedback loop of type I IFN signaling on the up-regulation of costimulatory molecules in DC [42, 43]. Therefore, we sorted pDC from Flt-3L-treated 129/Sv WT and IFNR<sup>-/-</sup> animals and cultured them overnight under conditions as described above. As shown in Figure 2C, the production of IFN-\(\alpha\) in virus-stimulated pDC from IFNR<sup>-/-</sup> mice was significantly lower as compared with pDC from WT mice (\(P=0.006\)). In contrast, the low induction of

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**Fig. 2.** pDC activated with PR/8 virus and CpG 1826 exhibit phenotypic and functional differences. (A) Sorted GFP<sup>hi</sup> pDC were incubated for 24 h in media alone or with media containing 300 HAU/ml UV-treated PR/8 virus or 3 \(\mu\)g/ml CpG ODN 1826. Supernatants were analyzed for IFN-\(\alpha\) by ELISA after 24 h of culture. Results of two independent experiments are shown (bars represent mean \(\pm\) SEM). (B) GFP<sup>hi</sup> pDC (stimulated as in A) were analyzed for expression of F-A<sup>7</sup>, CD80, CD40, CD80, and CD86 by flow cytometry. Representative results of one out of five independent experiments are shown. (C) IFN-\(\alpha\) production and (D) expression level of costimulatory molecules in PDCA-1<sup>hi</sup> CD11c<sup>hi</sup> pDC sorted from spleens of Flt-3L-induced 129/Sv WT and IFNR<sup>-/-</sup> mice (stimulated as in A). Representative results of one out of two independent experiments are shown. ns, Not significant. **, .

IFN-α by CpG 1826 in WT mice was not influenced significantly by the deficiency in IFNR expression (P=n.s.). Consistent with the results described for GFP⁺ pDC, CpG 1826 induced higher levels of CD40 and MHC class II molecules in 129/Sv pDC than in PR/8 virus. The up-regulation of CD40 and CD86, but not class II molecules, was reduced significantly after PR/8 virus stimulation in IFNR-deficient pDC. In contrast, maturation of pDC induced by CpG 1826 was independent of type I IFNs (Fig. 2D). Together, these results suggested the involvement of different downstream signals after an encounter of PR/8 virus and CpG 1826 by pDC.

Peptide-pulsed pDC activated by CpG 1826 induce higher proliferation of naïve T cells in vitro and in vivo compared with PR/8-stimulated pDC

The capability of pDC to present antigen to naïve T cells has remained controversial, perhaps pertaining to difficulties in obtaining mDC-free pDC populations. Having observed that PR/8 and CpG 1826 induced differential expression of molecules involved in naïve T cells stimulation, we hypothesized that CpG-treated pDC may exhibit an enhanced ability to present antigen to T cells. Highly purified GFP⁺ pDC were stimulated with CpG 1826 or PR/8 virus, and untreated or LPS-treated GFP⁺ mDC served as a control. DC were then pulsed with the SIINFEKL peptide (10 ng/ml) and incubated at different ratios with purified CD8⁺ OT-I T cells. As shown in Figure 3A, CpG-activated pDC induced higher proliferation of T cells than pDC activated by influenza virus. However, this proliferation was considerably weaker as compared with T cells stimulated by mDC (Fig. 3A).

To further investigate the effects of pDC on naïve T cell priming in vivo, we adaptively transferred unstimulated or stimulated pDC into C57BL/6 mice that had received CFSE-labeled OT-I T cells 24 h earlier. T cell activation was assessed 72 h later in the spleens and lymph nodes of the mice. Consistent with the in vitro results, CpG-activated pDC induced moderately higher T cell proliferation than virus-activated pDC (Fig. 3B, upper row). Nevertheless, pDC were much less potent in inducing OT-I proliferation as compared with mDC (Fig. 3B, lower row), indicating that direct antigen presentation may not be their main function during immune responses.

Microarray analysis reveals distinct transcriptional programs in pDC induced by PR/8 virus and CpG 1826 ODN

Although the experiments described above pointed toward the possibility that pDC may enter distinct activation/differentiation states in response to CpG 1826 and PR/8 virus, our analyses thus far were limited to comparing a select panel of phenotypic markers and functional readouts. As it was conceivable that the response to either stimulus was much broader in nature, we next determined the change induced in pDC by PR/8 or CpG 1826 at the transcriptome level. To this end, RNA obtained from pDC after 1 and 4 h of culture in media alone or in the presence of stimuli was subjected to microarray analysis. The expression of genes in pDC after 1 h incubation in media served as the baseline. We applied a three-way mixed model ANOVA in conjunction with pair-wise contrasts (medium 1 h, CpG 4 h; medium 1 h, flu 4 h) and then extracted genes with a ≥2.0-fold difference in expression as compared with baseline. One hour after stimulation, a small number of genes, categorized as “response to biotic stimulus” [EASE score 2.73e-11, National Institutes of Health (NIH), Database for Annotation, Visualization and Integrated Discovery], were induced by CpG 1826 but not PR/8. Most changes were detected at 4 h after stimulation; hence, we focused our subsequent analyses on this time-point.

![Fig. 3.](image-url)
At the 4-h time-point, 5371 and 2377 genes changed in the CpG 1826 and PR/8 groups, respectively, as compared with baseline. After subsequent stringency filters (see Materials and Methods), we found 896 and 1387 genes up- or down-regulated, respectively, after CpG 1826 and PR/8 stimulation (Fig. 4A). However, we also identified genes that were regulated selectively in either group; i.e., CpG 1826 induced the change of 468 (up-) and 1109 (down-regulated) genes in pDC; and 133 up- and 45 down-regulated genes were specific for stimulation with PR/8 virus (Fig. 4A).

Taken together, CpG 1826 and PR/8 virus had overlapping yet distinct effects on the transcriptome of pDC, underscoring the flexibility of these cells to respond to external stimuli, which in the following, will be exemplified by well-known, immunoregulatory and signal-transduction molecules. More detailed information can be found in Supplementary Tables 1–6.

Differential regulation of proinflammatory cytokines and chemokines in pDC induced by PR/8 virus and CpG 1826 ODN

One of the most prominent features of pDC upon stimulation is their production of soluble mediators, such as chemokines and cytokines. However, the precise response to activation with distinct TLR ligands has not been investigated. Our microarray analyses revealed that consistent with the results obtained by ELISA (Fig. 2A), pDC up-regulated multiple genes of the IFN-α family after viral stimulation, which was up to 100-fold higher than in CpG 1826-treated pDC (Fig. 4B, left panels). In contrast, changes in IFN-β gene expression were slightly higher in CpG 1826-activated pDC, indicating differential regulation of these genes at the transcriptional level.

We further observed elevated transcription of CCL3, CCL4, and CXCL2 in CpG-treated pDC, but not virus-activated pDC, as early as 1 h after stimulation, which increased further after 4 h (see Fig. 5A). Whereas the transcriptional level of CCL2 in PR/8-activated pDC did not change over time, the RNA levels of CCL3, CCL4, and CCL5 increased after 4 h, albeit to a significantly lesser extent as in CpG 1826-stimulated cells. These results suggest kinetic differences in the regulation of these genes by the two stimuli. In addition, CpG-activated pDC up-regulated the expression of CCL5, XCL1, CXCL9, CXCL10, and CXCL11 after 4 h (Fig. 5A, and data not shown). The only chemokines induced to a similar extent in PR/8-stimulated pDC were XCL1 and the three known CXCR3 ligands (Fig. 5A, and data not shown).

**Fig. 4.** Microarray analysis of differentially induced genes in pDC activated by PR/8 virus and CpG 1826. (A) Venn diagrams of up- and down-regulated genes that are specific for CpG 1826 or PR/8 virus treatment or shared by both. Gene expression at 1 h incubation in medium served as the baseline. (B) Fold changes of transcripts for type I IFNs, costimulatory molecules, and genes involved in TLR signaling as well as transcription of cytokines for the time-points 1 h and 4 h. 4-1BBL, 4-1BB ligand; PD-L2, programmed death ligand 2; IRF7, IFN regulatory factor 7; SOCS1, suppressor of cytokine signaling 1; Trafl, TNFR-associated factor 1. (C) Cell lysates from pDC stimulated for the indicated times were probed by Western blot with the indicated antibodies. Representative data of one out of three independent experiments are shown. p-ERK1/2, Phosphorylated ERK1/2. U., (D) Intracellular staining for phosphorylated p38 in stimulated pDC shown as histograms or (E) mean fluorescence intensity (MFI).
CpG 1826 stimulation induced the transcription of TNF-α, IL-6, IL-12, and IFN-γ in a time-dependent manner (Fig. 5C). In contrast, the PR/8 virus had much lesser effects on these cytokines, with TNF-α the only one being expressed at significant levels at 4 h. We further observed an up-regulation of IL-10, a negative regulator of immune responses, only in CpG 1826-stimulated pDC. Moreover, Ebi3, a subunit of IL-27, which is important in limiting CD4+ T cell responses, was highly expressed in CpG 1826- but not PR/8-activated pDC (see Supplementary Table 5). Collectively, these data revealed considerable differences in the induction of proinflammatory cytokines at the mRNA level.

**mRNA expression of proinflammatory mediators correlates with protein levels**

Having determined the expression patterns of cytokines and chemokines, we next sought to verify our results at the protein level. To this end, the supernatants of CpG 1826- and PR/8-stimulated pDC were subjected to multianalyte profiling. As shown in Figure 5, the secretion of the tested mediators correlated well with the mRNA levels. An exception was IL-1β, which was highly expressed at the mRNA level but was not detected in the supernatants.

We further assessed the production of IL-12, TNF-α, and IFN-γ by intracellular cytokine staining of pDC, directly isolated ex vivo from a site of inflammation (Fig. 5, E and F). Thus, we harvested cells from the peritoneal cavity of DPE-GFP mice that were injected 72 h earlier with thioglycollate. Such a treatment leads to the accumulation of pDC, which remain in an immature differentiation state. PECs were then stimulated with PR/8 virus or CpG 1826 for 4 h in the presence of Brefeldin A and analyzed for the production of cytokines. In the absence of stimulation or after PR/8 activation, pDC did not produce IL-12, and a small subset of cells was TNF-α+. In contrast, after CpG 1826 stimulation, 30% and 64% of pDC stained positive for IL-12 and TNF-α, respectively. Although analysis of supernatant revealed the release of IFN-γ after overnight culture, this cytokine was undetectable in pDC by intracellular staining, which may relate to different sensitivities of these assays.

Taken together, our results indicate that pDC stimulated under the two conditions specialize with respect to the cytokines they secrete; i.e., virus-stimulated pDC prioritize to produce type IFNs and a few select chemokines that may attract inflammatory cells, and CpG 1826-stimulated cells release a large panel of anti-inflammatory mediators.

**Expression of costimulatory molecules**

Besides the production of soluble mediators that regulate the function of other immune cells, pDC can also express costimulatory molecules.
latory molecules that regulate the function of other immune cells upon cell–cell contact. We identified an early, robust up-regulation of transcripts for 4-1BBL, CD40, and CD83 in Cpg 1826-stimulated pDC and a further increase of CD40 expression at 4 h (Fig. 4B, middle panels). Consistent with our flow cytometry results (Fig. 2), CD86 was up-regulated to a similar extent in Cpg 1826- and PR/8-stimulated pDC, and CD80 was induced to a higher degree by Cpg 1826. Expression of PD-1, a negative immune regulator and its ligand PD-L2 did not change at either time-point. However, after 4 h, PD-L1 was up-regulated 40- and 20-fold by Cpg and PR/8, respectively. In addition, we identified a member of the semaphorin family, Sema6D, which is believed to play a role in costimulation [44], to be increased 100-fold in Cpg 1826- but only sevenfold in PR/8-activated pDC. Finally, in line with higher levels of class II molecule expression of Cpg 1826-activated pDC, we found higher up-regulation of the class II transactivator C2ta following Cpg treatment.

Differential regulation of transcription factors by Cpg 1826 and PR/8 in pDC

As PR/8 and Cpg 1826 induced markedly different panels of cytokines and costimulatory molecules in pDC, it seemed plausible to hypothesize that the two stimuli induced/activated different transcriptional programs in these cells, which can be regulated through modifications of transcription factors at the transcriptional or post-translational level. Consistent with our hypothesis, we found significantly higher induction of gene transcripts, such as IRF7 and STAT1, which are necessary for type I IFN production following viral stimulation (Fig. 4B, right panels). We further observed that Cpg 1826 stimulation resulted in the up-regulation of transcription factors c-Rel and I-kBα. It has been shown recently that Cpg-activated NF-κBα−/−/c-Rel−/− pDC produced normal levels of type I IFNs; however, IL-6 and IL-12 responses were deficient [45]. Moreover, Yamamoto et al. [46] demonstrated that I-kBα-deficient macrophages have impaired IL-6 production in response to TLR ligands. Together, these data indicate a role for the NF-κB pathway in the regulation of proinflammatory cytokines by pDC in response to Cpg 1826 stimulation.

We next investigated whether Cpg 1826 and PR/8 induced differences in kinase activation/phosphorylation, which are known to be downstream targets of TLR signaling pathways. To this end, highly purified pDC were stimulated with reagent for 15 h, 45 h, and 3 h and analyzed for I-kB degradation, as well as ERK1/2 and p38 phosphorylation. Degradation of I-kB followed similar kinetics in response to both stimuli. Although phosphorylated ERK1/2 was undetectable in freshly sorted, unstimulated pDC, ERK1 (p44) and to a lower extent, ERK2 (p42) were transiently phosphorylated (peak at 45 h) after Cpg 1826 activation (Fig. 4C). Interestingly, ERK1 phosphorylation followed a different time course in PR/8-stimulated pDC, with a peak at 3 h. We also determined p38 phosphorylation using a flow cytometry-based assay. As shown in Figure 4, D and E, Cpg 1826, but not PR/8 virus, induced p38 phosphorylation, starting after 45 h.

Taken together, our results show that Cpg 1826 and PR/8 virus induce distinct signaling pathways in pDC that may contribute to the observed differences in the production of cytokines and costimulatory molecules.

Discrete phenotypes of pDC during PR/8 virus infection and after Cpg 1826 stimulation in vivo

Having established and characterized two distinct differentiation states of pDC in response to PR/8 virus and Cpg 1826 in vitro, it was important to determine whether such phenotypes could also be observed during immune responses in vivo. We therefore infected DPE<sup>C57</sup> mice intranasally with PR/8 virus (500 TCID<sub>50</sub>) and assessed the expression of costimulatory molecules on pDC isolated from mediastinal lymph nodes, spleens, and lungs at various time-points. Starting 3 days postinfection, we observed that pDC in the infected lungs expressed high levels CD86. In contrast, the cells expressed only a low level of CD40 throughout the course of infection (Fig. 6A). pDC in the draining mediastinal lymph nodes as well as the spleen exhibited a CD40<sup>low</sup>/CD86<sup>low</sup> phenotype.

We next injected Cpg 1826 i.v. into DPE<sup>C57</sup> mice and analyzed the phenotype of pDC in the spleens 20 h later. As shown in Figure 6B, pDC up-regulated not only CD86 but also CD40, CD80, and class II molecules, thus differing from pDC activated during PR/8 virus infection. Taken together, our in vivo data are consistent with the observed phenotype of pDC after PR/8 and Cpg 1826 stimulation in vitro.

DISCUSSION

Although pDC have been identified as a distinct immune cell subset only a few years ago, they have already been implicated in the pathogenesis of eminent diseases, such as SLE and psoriasis or cancer as a result of their proinflammatory or
negative immune regulatory activities, respectively. On the other hand, pDC participate in the defense against pathogens, including viruses, bacteria, and parasites. These diverse activities raise the question as to how pDC exert such contrasting functions. It is clear from several other immune cell lineages that distinct, stimulus-dependent differentiation states can be adopted. Most prominently, CD4+ T cells can differentiate into Th1 and Th2 cells that participate in an opposing spectrum of immune responses and immunopathologies. In the present study, we have identified two functionally specialized activation states of pDC in response to two TLR ligands, i.e., influenza virus and CpG 1826, providing a rationale for their differential effects in a wide spectrum of immune responses.

By using model TLR7 and TLR9 agonists, i.e., ssRNA virus and unmethylated CpG B DNA, respectively, we have found a surprisingly large number of uniquely regulated genes in pDC. This suggests that different stimulatory agents can induce distinct transcriptional programs in pDC that may result in optimized functions in a given situation. The specificity of the pDC response may, on the one hand, depend on the engaged TLR and on the other hand, on the nature of the ligand. For example, A-type CpG ODN, which are strong inducers of IFN-α, localize to the endosomal compartment of pDC together with the MyD88-IRF7 complex [47]. In contrast, B-type CpG ODN, which are weak IFN-α inducers, are found within lysosomes, indicating a spatial dissociation of signaling pathways in these cells [48]. Given these differences, it is conceivable that the observed phenotypes in response to PR/8 and CpG 1826 stimulation are not specific for TLR7 and TLR9 signaling but are rather dependent on the individual agonist. Therefore, we would expect that the response of pDC to A-type CpGs resembles that of PR/8 virus. Ongoing studies in our laboratory aim to dissect the characteristics of pDC after stimulation with a variety of TLR7 and TLR9 ligands to determine whether PR/8 and CpG 1826 stimulation induces representative response patterns of pDC or whether each ligand induces unique phenotypic and functional profiles in these cells.

Consistent with previous reports, GFPpDC secreted large amounts of IFN-α in response to influenza virus and to a much lesser extent, to CpG 1826. Type I IFNs regulate the activation of other immune cells, including NK cells, T cells, and mDC, and also exert direct antiviral effects. Given these prominent functions and the fact that pDC can make large amounts of these cytokines, it has been proposed that the main purpose of pDC during immune responses is type I IFN production. However, our results emphasize that this may be an underestimation, as these cells induce mRNAs for a large variety of secreted factors, some of whose production we confirmed at the protein level. Thus, even when type I IFN secretion was high (after PR/8 stimulation), pDC produced copious amounts of chemokines implicated in effector T cell recruitment (XCL1, CXCR3 ligands) and proinflammatory mediators such as lymphotixin-α and TNF-α. The release of these factors was even more pronounced after CpG 1826 activation of pDC and included a broader spectrum of chemokines and cytokines than after viral stimulation. Interestingly, under the latter conditions, pDC also expressed molecules implicated in negative immunoregulation, such as IL-10 and PD-L1, indicating that pDC may be involved in limiting immune responses.

Many previous studies have reported that after activation, pDC differentiate into cells with antigen-presenting capabilities [4, 18, 49–51]. However, conflicting data have been generated with regard to their potency of T cell stimulation, which may relate to difficulties in generating a pure pDC population ex vivo. As sorted GFPpDC were devoid of mDC, we asked whether these cells could induce proliferation of naïve T cells in vitro and in vivo. As expected, based on the differential induction of costimulatory molecules, CpG 1826-stimulated pDC were superior in inducing proliferation of TCR-transgenic T cells in an antigen-dependent manner. Importantly, however, T cell activation in vivo was relatively poor when compared with mDC-induced T cell proliferation. For future studies, our model will facilitate visualizing GFP-tagged pDC directly within the microenvironment of lymph nodes using two-photon microscopy. Thus, we will be able to ask whether differentially activated pDC undergo direct physical interactions with T cells during immune responses.

A pertinent question arising from the two phenotypes following PR/8 and CpG 1826 stimulation of pDC is what molecular signals mediate such different responses. Common to TLR7 and -9 signaling is the recruitment of the adaptor molecule MyD88, which forms a complex with members of the IL-1R-associated kinase family and TRAF6 [52–56]. Downstream signals of this complex include IRF7, which is indispensable for the induction of IFN-α in pDC as well as members of the NF-κB transcription factor family, which are responsible for transcriptional activation of cytokine genes. Our functional studies revealed that the degradation of IκBα in pDC following PR/8 and CpG 1826 stimulation followed a similar time course, which indicates that the induction of downstream signaling cascades after TLR7 and -9 engagement may follow similar kinetics. Interestingly, however, we observed distinct differences in the kinetics of ERK1/2 phosphorylation after PR/8 and CpG 1826 stimulation. Moreover, p38 MAPK was phosphorylated only after CpG 1826 stimulation of the cells. This is consistent with earlier observations that CpG rapidly induces p38 and ERK phosphorylation in myeloid cells, such as DC and monocyes/macrophages [57–59]. Signals transmitted through stress-activated kinases are closely linked to the induction of proinflammatory cytokines/chemokines as well as IFN-α induction in pDC [56, 58], providing a mechanistic explanation for the observed differences. Of note, however, when we used a synthetic p38 inhibitor (SB202190), we found decreased up-regulation of costimulatory molecules in CpG 1826- and PR/8-activated pDC cultured overnight (data not shown). Although this result may appear counterintuitive, one needs to consider that type I IFNs also activate p38. Therefore, interpretation of inhibition studies is complicated by secondary effects on the secretion of mediators that influence the profile of signaling molecules in pDC.

Although we have learned a great deal about the functions of pDC from in vitro studies, arguably, their functional characterization during disease in vivo is important for understanding their contribution to immune defense and pathology. In vivo studies have been hampered by the fact that pDC-specific markers under steady-state conditions are also expressed on other immune cells during inflammation. Therefore, the unequivocal identification of pDC during infection or in autoim-

Iparraguirre et al. Activation of PDC 9
munity has been challenging. Not surprisingly, different phenotypic criteria have been used in the past, which has resulted in some contradictory results about pDC functions. The fact that CpG and virus-matured pDC remained GFP<sup>hi</sup> (as shown in cell culture) enabled us to identify these cells during influenza infection and after CpG 1826 application in vivo. Consistent with our in vitro results, pDC in the lungs of infected mice expressed high levels of CD86, but not CD40 and CD80. In contrast, CpG 1826 induced high levels of costimulatory molecules and class II on pDC. These results indicate that indeed, pDC can adopt the phenotypes described after stimulation in vitro also during disease in vivo.

What may be the functional significance of different differentiation states of pDC during disease in vivo? After influenza virus stimulation, pDC appear to be poised to produce antiviral mediators, including IFNs. In addition, they secrete chemokines CXCL9, CXCL10, and XCL1, which may be involved in attracting T cells and NK cells to the lung during influenza virus infection [20, 21]. The fact that these cells showed an incomplete induction of costimulatory molecules suggests that they may not be involved directly in T cell priming. In fact, pDC may have tolerizing functions and induce T cell anergy and/or generation of Tregs, and it will be important to determine whether the only partial induction of these molecules is causally involved in these phenomena. After injection of CpG 1826, pDC differentiated into cells with a more mature phenotype and released higher levels and a broader panel of proinflammatory cytokines. These data are of relevance, as ongoing clinical trials examine the efficacy of various CpG ODN as adjuvants to enhance immune responses [60]. In future studies, we will aim to dissect the effects of different CpGs on the differentiation of pDC and how this will influence downstream immune responses.

In summary, we undertook steps to decipher in detail the differences of pDC activated by two different TLR7 and TLR9 ligands. Based on our results, we propose that murine pDC can be polarized into separate populations with an overlapping panel of effector molecules together with distinct expression profiles of proinflammatory mediators, costimulatory molecules, and a variety of molecules whose function awaits further characterization. We expect that further elucidation of the molecular mechanisms of their generation and investigation of their precise roles during infections, autoimmunity, and cancer will contribute to our understanding of the pathogenesis of disease.

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