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Bacterial CpG-DNA Triggers Activation and Maturation of Human CD11c$^-$, CD123$^+$ Dendritic Cells[1]

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Human plasmacytoid precursor dendritic cells (ppDC) are a major source of type I IFN upon exposure to virus and bacteria, yet the stimulus causing their maturation into DCs is unknown. After PBMC activation with immunostimulatory bacterial DNA sequences (CpG-DNA) we found that ppDC are the primary source of IFN-α. In fact, either CpG-DNA or dsRNA (poly(I:C)) induced IFN-α from purified ppDC. Surprisingly, only CpG-DNA triggered purified ppDC survival, maturation, and production of TNF, GM-CSF, IL-6, and IL-8, but not IL-10 or IL-12. Known DC activators such as CD40 ligation triggered ppDC maturation, but only IL-8 production, while bacterial LPS was negative for all activation criteria. An additional finding was that only CpG-DNA could counteract IL-4-induced apoptosis in ppDC. Therefore, CpG-DNA represents a pathogen-associated molecular pattern for ppDC. In contrast to these finding, CpG-DNA, like LPS, caused TNF, IL-6, and IL-12 release from PBMC and purified monocytes; however, differentiation of monocytes into DCs with GM-CSF and IL-4 unexpectedly resulted in refractoriness to CpG-DNA, but not LPS. Taken together, these results suggest that within a DC subset a multiplicity of responses can be generated by distinct environmental stimuli and that responses to a given stimulus may be dissimilar between DC subsets. ***The Journal of Immunology,*** **2001, 166: 5000–5007.**

Dendritic cells (DCs)[3] in their naive or so-called immature state act as environmental sentinels detecting pathogen presence and sampling interstitial fluids from which they take up and process Ag (1). Maturation of DCs to professional APCs can be initiated by T cells expressing CD40 ligand (CD40L) or directly via engagement of pathogen constituents displaying conserved molecular patterns, also termed pathogen-associated molecular patterns (PAMP) (2–7). Maturing DCs express T cell-costimulating molecules on their surface, such as CD80, CD86, and CD40, and release soluble mediators, such as cytokines and chemokines. DCs then efficiently interact with peripheral T cells to initiate adaptive immune responses and dictate the Th polarization toward either Th1 or Th2 (1).

DCs have been subdivided into lineage subsets based on surface marker phenotype. Functional characterization of human DCs has established myeloid-like DCs as Th1-inducing precursor DC type 1 (pDC1) and lymphoid-like DCs as Th2-inducing precursor DC type 2 (pDC2) (8). pDC1 are generated from peripheral blood monocytes by treatment with GM-CSF and IL-4 and are also known as monocyte-derived DCs (MDDCs). These DCs express CD11c, CD13, CD33, and GM-CSF-Rα (CD116), but not CD4, and become mature after stimulation with CD40L or PAMP. pDC1 production of IL-12 upon stimulation is a likely explanation for Th1 polarization. pDC2 are plasmacytoid cells isolated from the tonsil, termed here plasmacytoid precursor DC (ppDC) (8). These cells are CD4$^+$ CD11c$^-$ CD13$^-$, CD33$^-$, CD45RA$^+$, IL-3Rα^+ (CD123$^+$) and use IL-3 as a survival factor (9–11). DCs of this phenotype can also be found circulating in the peripheral blood or resident in lymphoid organs (9, 10, 12–16). CD4$^+$/CD11$^-$ DCs from the blood have also been termed plasmacytoid cells, IFN-producing cells, natural IFN-$\alpha\beta$-producing cells, IL-3Rα^{high} DCs, or pDC2 (8–10, 17, 18). CD40 ligation matures ppDC, but does not induce IL-12; however, they do produce type I IFNs if stimulated with UV-irradiated HSV (8, 18). Type I IFNs (IFN-α and IFN-β) are involved in antiviral defense, cell growth regulation, immune activation, and Th1 polarization. ppDC have been implicated as the major source of type I IFNs after viral or bacterial stimulation (17, 19).

Viruses and bacteria probably activate MDDC and ppDC through engagement of pattern recognition receptors (e.g., Toll-like receptor (TLR) or dsRNA-responsive protein kinase). Well-documented PAMP are endotoxins (LPS), dsRNA, and immunostimulatory bacterial CpG-DNA sequences (CpG-DNA) (7). LPS, a prototypic PAMP, matures and induces cytokine production from murine bone marrow-derived DCs and human MDDC (2). A LPS binding and signaling complex assembles when TLR4 interacts with LPS bound to CD14, thus initiating the IL-1R/TLR receptor transduction pathway (20–22). CpG-DNA-driven activation of APCs also acts through the IL-1R/TLR-like signal transduction pathway; however, cellular uptake and translocation into early endosomes are required (23–25). It has been recently determined that CpG-DNA signals via TLR9 (26). TNF-associated factor-6 is a critical element in the IL-1R/TLR as well as CD40 signaling pathways (27). Subsequent to TNF-associated factor-6 both IκB kinase

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[3] Abbreviations used in this paper: DC, dendritic cell; MDDC, monocyte-derived DC; CD40L, CD40 ligand; PAMP, pathogen-associated molecular patterns; ppDC, plasmacytoid precursor DC; pDC1, precursor DC type 1; pDC2, precursor DC type 2; CpG-DNA, immunostimulatory bacterial CpG-DNA sequences; TLR, Toll-like receptor; ODN, oligodeoxynucleotide.

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and Jun kinase are activated. Interestingly, dsRNA activation of dsRNA-responsive protein kinase also results in IκB kinase and Jun kinase activation (28). The convergence of these multiple stimuli may explain how they are all able to activate and mature DCs.

Bacteria and virus stimulate the release of IFNs from plasmacytoid cells; however, the PAMPs involved remain unidentified with the possible exception of dsRNA. Bacterial CpG-DNA was originally recognized for its ability to induce IFNs from both murine spleen cells and human peripheral blood cells. Given that bacterial stimuli activate DCs (29–31), we attempted to characterize the effects of CpG-DNA and other stimuli on human MDDC and ppDC. We describe that in contrast to LPS, bacterial CpG-DNA activates human lymphoid $CD4^+$, $CD11c^-$, ppDC cells to produce IFN-α and subsequently to mature into phenotypic DCs that display dendritic morphology, express high levels of costimulatory molecules, and produce cytokines. Conversely, LPS, but not CpG-DNA, activated myeloid MDDC/pDC1. Additionally, the effects of dsRNA and CD40 ligation were examined.

Materials and Methods

Reagents

Escherichia coli DNA and poly(I:C) (Sigma, Deisenhofen, Germany) were used at a concentration of 50 μg/ml (32). When needed as a control, *E. coli* DNA was digested by DNase I (Roche, Mannheim, Germany) overnight and checked for complete digestion by gel electrophoresis. LPS (Sigma, Germany) was used at a concentration of 100 ng/ml. All cytokines were purchased from PharMingen (San Diego, CA). The following oligodeoxynucleotides (ODNs) were used in their phosphorothioate form: CpG-ODN 2006, 5′-TCGTCGTTTTGTCGTTTTGTCGTT-3′; the nonstimulatory oligonucleotide GpC-ODN, 5′-TGCTGCTTTTGTGCTTTTGTGCTT-3′; and the control oligonucleotide C-ODN, 5′-GCTTGATGACTCAGCCGGAA-3′ (32). All ODN were used at 2 μM, which yielded maximal activity (data not shown) (32).

Cell culture

Cells were cultivated in RPMI 1640 supplemented with 50 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, 10 mM HEPES, and 10% FCS (Seromed, Berlin, Germany). IL-3 was used at a concentration of 500 U/ml, GM-CSF at 1000 U/ml, and IL-4 at 800 U/ml. The fibroblast cell line 3T3 stably transfected with human CD40L was described previously (33).

Cell preparation and purification

PBMC were isolated from citrate-stabilized buffy coats by centrifugation over Ficoll-Hypaque gradient. Briefly, 15 ml of the buffy coat was diluted 1/1 with PBS, underlayed with 15 ml of Ficoll-Hypaque solution, 1.077 g/l (Biochrom, Berlin, Germany), and centrifuged for 30 min at 1000 × *g*. Cells at the interface were harvested and washed four times with HBSS. Depletion of $CD123^+$ DCs from PBMC was performed by MACS separation (Miltenyi Biotec, Bergisch Gladbach, Germany) on the basis of CD123 expression.

Monocytes were purified by plastic adherence. Briefly, PBMC (10×10^6/well) were incubated in RPMI 1640/10% FCS in six-well plates (Falcon, Heidelberg, Germany) for 2 h at 37°C. After decanting the medium, the cells were washed with PBS/2% FCS and incubated for an additional hour. The washing step was repeated, and the adherent cells were harvested with a cell scraper. The monocytes were >90% pure as analyzed by flow cytometry.

Myeloid DC were generated from monocytes (MDDC/pDC1). Monocytes were isolated from PBMC by positive selection using a MACS separation kit according to the manufacturer's instruction (Miltenyi Biotec). The purity was determined by FACS analysis using a Coulter EPICS XL (Coulter, Krefeld, Germany) and was >95%. The monocytes were cultured for 7 days in 800 U/ml GM-CSF and 500 U/ml IL-4. Every second day 500 U/ml GM-CSF and 300 U/ml IL-4 were added.

ppDC/pDC2 were isolated from tonsils by a combination of elutriation and FACS sorting or MACS separation followed by FACS sorting. Fresh tonsils were cut into small fragments and pushed through a mesh. The cell suspension was fractionated by countercurrent centrifugation in an elutriation rotor (JE-6B with Sandersonchamber; Beckman, Krefeld, Germany) at constant speed (1800 rpm) with increasing flow rate (8–24 ml/min). Fractions enriched for $CD123^+$ cells, as accessed by FACS analysis, were pooled. For further purification, the enriched $CD123^+$ cell fractions were sorted by FACS according to $CD123^+$, $HLA\text{-}DR^+$ expression. The purity was determined by flow cytometry and was >98%. All steps were performed with the addition of DNase I (Roche) to prevent clumping of the cells. The anti-CD123 mAb, clone 9F5, is a nonblocking Ab, so signaling by IL-3 via the receptor was possible after sorting. For MACS separation, a tonsilar single-cell suspension was stained with PE-conjugated mAb against CD123 (PharMingen) and counterstained with anti-PE microbeads. $CD123^+$ cells were positively selected on a column. Further purification was performed by FACS as stated above.

Cytology analysis

Cells were cytocentrifuged onto slides and fixed with methanol for 5 min. Dried slides were stained for 20 min with May-Giemsa solution (Merck, Darmstadt, Germany) and rinsed with distilled water. The slides were analyzed by confocal microscopy on an LSM 510 (Zeiss, Heidelberg, Germany) and imaged digitally.

Determination of cell survival and proliferation

ppDC were cultured at a concentration of 20×10^3/well at 37°C in 96-well U-bottom plates with different stimuli. After 24 h cells were harvested and incubated with propidium iodide at an end concentration of 1 μg/ml for 10 min. Staining of the DNA in dead cells was determined by FACS and presented as the percentage of living cells. In parallel experiments proliferation was determined by seeding ppDC (20×10^3/well) in triplicate 96-well U-bottom plates (Falcon) followed by culture for 3 days at 37°C with or without stimulation. For the last 16 h cells were pulsed with 1 μCi of [^{3}H]thymidine (6.7 Ci/mM). Lysed cells were harvested onto filter papers, the filters were washed, and the [^{3}H]thymidine incorporation into the DNA was measured and expressed as counts per minute. When CD40L transgenic 3T3 cells were used as stimulus, they were irradiated with a dose of 4000 rad.

Measurement of cytokine release

PBMC (5×10^6/ml) or MDDC/pDC1 (1×10^6/ml) were incubated at 37°C in 24-well plates in the presence or the absence of stimuli. The supernatants were harvested after 12 h for TNF-α or after 24 h for all other cytokines. ppDC were cultured at a concentration of 0.2×10^6/ml at 37°C in 24-well plates, and supernatants were taken after 12 h for TNF-α or after 36 h for all other cytokines. The samples were analyzed in duplicate, and the ELISAs were performed according to the manufacturer's instruction. ELISA kits for IL-6, IL-10, and TNF-α were purchased from PharMingen; those for IL-8, GM-CSF, and total IL-12 were obtained from R&D Systems (Minneapolis, MN); and those for IFN-α were obtained from PBL Biomedical Laboratories (New Brunswick, ME). Streptavidin-peroxidase conjugate, as enzyme (Sigma), and *o*-phenylenediamine, as substrate (Sigma), were used for development when not included in the kits.

Expression of surface markers

PBMC (5×10^6/ml) and MDDC/pDC1 (1×10^6/ml) were cultured for 24 h at 37°C in 24-well plates in the presence or the absence of stimuli. ppDC (0.2×10^6/ml) were cultured for 36 h. Cells were harvested, washed, and preincubated with human IgG (Miltenyi) for FcR blockade. Abs used for specific staining or isotype controls were FITC- or PE-conjugated anti-CD1a, anti-CD3, anti-CD4, anti-CD11c, anti-CD13, anti-CD14, anti-CD16, anti-CD19, anti-CD33, anti-CD40, anti-CD56, anti-CD80, anti-CD86, anti-CD116, anti-CD123, anti-HLA-DR, and anti-HLA-ABC (PharMingen). The staining was performed on ice for 30 min; cells were washed twice and then fixed with 1% paraformaldehyde solution (Sigma). FACS analysis was performed on a Coulter EPICS XL (Coulter).

Th1 vs Th2 induction

ppDC (2×10^5) were cultured in 24-well plates in the presence or the absence of the stimuli at 37°C. After 24-h incubation 10^6 allogenic naive T cells were added. Naive T cells were obtained from PBMC depleted of $CD11b^+$, $CD16^+$, $CD19^+$, $CD36^+$, and $CD56^+$ (Pan T Cell Isolation Kit) and additionally of $CD8^+$ and $CD\ 45RO^+$ cells on a MACS column (Miltenyi). Naive T cells and ppDC were cocultured in supplemented RPMI 1640 with added IL-3 (500 U/ml) and sodium pyruvate (1 mM) for 6 days. The T cells were then washed, counted, and restimulated at a concentration of 5×10^5 cells/ml with PMA (50 ng/ml) and ionomycin (0.5 μg/ml) for 24 h. The secretion of IL-4, IL-5, and IFN-γ was determined by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Results

CpG-DNA, but not LPS, induces type I IFN from PBMC

In studies designed to determine APC reactivity to PAMPs, we found that bacterial CpG-DNA and LPS stimulated human monocytes to produce cytokines, such as IL-12, IL-6, and TNF-α (Fig. 1, *A* and *B*) (32). Monocyte activation through *E. coli* DNA was DNA dependent, because DNase digestion destroyed cytokine release. CpG-DNA also caused IFN-α release from PBMCs in a CpG-dependent manner; in contrast, however, LPS did not induce IFN-α (Fig. 1*C*). Poly(I:C), a dsRNA known to induce IFN-α from PBMCs, was used as a positive control (Fig. 1*C*). Interestingly, purified monocytes did not produce IFN-α upon CpG-DNA stimulation, although poly(I:C) remained effective (Fig. 1*D*). These data implied that IFN-producing cells within PBMCs were lost during the monocyte preparation. Additionally, CpG-DNA, LPS, and poly(I:C) were at variance with regard to IFN production pattern.

MDDCs respond to LPS, but not CpG-DNA

DC are a known source of various cytokines and IFNs (34–36). Most contemporary studies rely on MDDC as a source of DCs. MDDC generated by culturing monocytes in GM-CSF and IL-4 are myeloid in origin and have been termed pDC1 based on functional analysis (8). We tested MDDC/pDC1 for their responsiveness toward CpG-DNA or LPS. In agreement with others (2, 37, 38), LPS induced human MDDC to up-regulate the expression of costimulatory molecules such as CD40 and CD86 and to produce cytokines such as TNF-α, IL-12, IL-6, and IL-8 (Fig. 2 and Table I). MHC class I and II molecules were also up-regulated 2-fold (data not shown). However, CpG-DNA was negative for up-regulation of activation markers and cytokine production (Fig. 2 and Table I). Additionally, LPS and CpG-DNA were both negative for the production of IFN-α (Table I), although these cells have been shown to release type I IFNs upon poly(I:C) stimulation (34, 35). Given that ex vivo-prepared human monocytes are sensitive to immunostimulatory CpG-DNA, (Fig. 1*A*) (32), these results implied that the conversion of monocytes to MDDC is associated with a CpG-DNA refractory state.

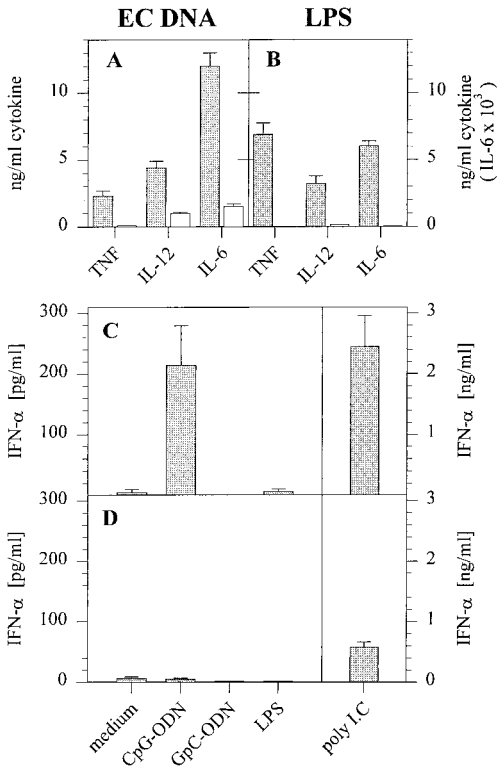

FIGURE 1. CpG-DNA induces cytokine release from PBMC and monocytes, but not IFN-α release from monocytes. *A*, Human monocytes (5×10^5/well) were cultured with 50 μg/ml *E. coli* (EC) DNA (■) or DNase-digested 50 μg/ml EC DNA (□), and cytokine release was measured by ELISA. *B*, Human monocytes were cultured with 1 μg/ml LPS (■) or medium only (□), and cytokine release was measured. Note that the IL-6 value should be multiplied by 1×10^3. *C*, PBMC (10×10^6/well) were cultured with medium only, 2 μM CpG-ODN, 2 μM GpC-ODN, 1 μg/ml LPS, or 50 μg/ml poly(I:C), and IFN-α was measured by ELISA. *D*, Human monocytes (5×10^5/well) were cultured as described in *C*. All supernatants were collected for ELISA at 24 h, with the exception of TNF at 12 h. The data are reported as the mean ± SD of three independent experiments using different donors.

The ppDC/pDC2 respond to CpG-DNA, but not LPS

Plamacytoid $CD4^+$, $CD11c^-$, $CD123^+$ (IL-3Rα^+) ppDC, functionally described as pDC2, acutely respond to microbial stimuli with the production of type I IFN (18, 19). Upon depletion of $CD123^+$ cells from PBMCs, bacterial CpG-DNA failed to trigger IFN-α from the remaining PBMCs, yet poly(I:C) was still effective (Table II). In contrast, IFN-α producer cells within PBMCs were nonresponsive to LPS (Table II). This contrasted with IL-12 production, which was induced in PBMCs or CD123-depleted PBMCs by LPS, CpG-ODN, and *E. coli* DNA, but not by poly(I:C) (Table II). Furthermore, CpG-DNA, but not LPS, up-regulated the expression of CD86 and CD40 costimulatory molecules on $CD123^+$, $CD4^+$ cells within PBMCs (Fig. 3). These data implied that $CD123^+$, $CD4^+$ cells are responsive to bacterial DNA and thus focussed our attention on the purification and characterization of ppDC.

CpG-DNA, but not LPS or poly(I:C), promotes the survival of purified ppDC

The tonsil is a rich source of ppDC (9). Upon purification (>98%; see *Materials and Methods*) the phenotype of these cells was lin^-,

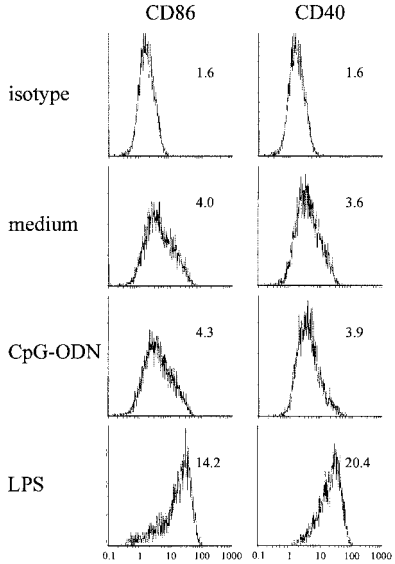

FIGURE 2. LPS, but not CpG-DNA, activates MDDC to up-regulate costimulatory molecules. MDDC (1×10^6/ml) were cultured with medium alone, 2 μM CpG-ODN, or 1 μg/ml LPS. After 24 h cells were stained with FITC-coupled Abs against CD86, CD40, or corresponding isotype controls. The corresponding FACS histograms are shown with mean fluorescence values given adjacent to the histogram. Data shown are representative of three experiments with cells from different donors.

Table I. *CpG-DNA does not activate MDDC to produce cytokines*[a]

Stimulation	Cytokines (ng/ml)				
	IL-12	TNF	IL-6	IL-8	IFN-α
Medium	0.02	0.00	0.00	0.03	0.01
E. coli DNA	0.00	0.00	0.03	0.03	0.00
CpG-ODN	0.02	0.00	0.00	0.06	0.01
LPS	0.36	23.16	15.1	99.24	0.01

[a] Immature MDDC (1×10^6/ml) were cultured with medium alone, 2 μM CpG-ODN, or 1 μg/ml LPS. Supernatants were collected and assayed for TNF, IL-12, IL-6, IL-8, and IFN-α by ELISA. Data shown are representatives of three experiments with cells from different donors.

$CD1a^-$, $CD11c^-$, $CD13^-$, $CD33^-$, $CD45RO^-$, $CD45RA^+$, $CD123^+$, $CD4^+$ (data not shown), as demonstrated previously (9, 10). Consistent with previous reports, nearly all freshly isolated ppDC apoptosed during overnight culture in medium alone; however, 70% survived if cultured with IL-3 (Fig. 4*A*). CD40 ligation in the absence of IL-3 promoted some survival over a 24-h period (Fig. 4*A*); however, over extended periods cells continued to die (data not shown). In our hands, GM-CSF also promoted survival (Fig. 4*A*), consistent with data derived from blood-borne $CD123^+$ DCs (11). In agreement with others TNF-α alone did not promote survival (data not shown) (11). The most effective survival stimuli were *E. coli* DNA and CpG-ODN, which supported 80% survival of ppDC (Fig. 4*A*). Control DNAs were ineffective, with the possible exception of a minimal effect from the GpC-ODN. LPS was an ineffective anti-apoptotic agent (Fig. 4*A*), consistent with $CD123^+$, $CD4^+$ cell nonresponsiveness to LPS (Fig. 3). Poly(I:C) was also ineffective in blocking ppDC apoptosis (Fig. 4*A*).

ppDC are believed to be Th2 polarizing, and IL-4 was demonstrated to abolish IL-3-driven survival (8, 11). Indeed, a combination of IL-3 and IL-4 did not allow survival from apoptosis (Fig. 4*B*). Activation via CD40 cross-linking plus IL-3 was also inefficient in promoting survival when IL-4 was added simultaneously (Fig. 4*B*). In contrast, CpG-DNA stimulation was able to overcome the negative effects of IL-4 (Fig. 4*B*). Additionally, ppDC proliferated upon culture with IL-3 (Fig. 4*C*). In contrast, neither CD40 cross-linking nor CpG-DNA promoted proliferation, implying that maturation/differentiation (see below) in these DCs curtailed proliferation (11).

The ppDC display activated DC morphology upon CpG-DNA stimulation

The cell morphology of ppDC was consistent with published descriptions of plasmacytoid cells that reside in T cell-rich areas of the tonsil and lymph nodes, and $CD11c^-$, $CD4^+$ DCs from the blood (Fig. 5*A*) (11, 18). Although IL-3 was anti-apoptotic, the overall plasmacytoid morphology was maintained for 36 h with perhaps the appearance of a few small dendrites and a subtle increase in size (Fig. 5*E*). In contrast, CpG-DNA promoted long dendrite formation, a strong increase in size, an alteration in nuclear structure, and increased vacuolization, seemingly clustered around a dense focal point (Fig. 5, *B–D*). This was similar in nature to IL-3/CD40 activation (Fig. 5*F*), which has been documented previously (10). IL-3/LPS or IL-3/poly(I:C) treatment did not differ from the mild maturational effects of IL-3 alone (compare Fig.

Table II. *IL-12 and IFN-α release by PBMC and PBMC depleted of $CD123^+$ cells*[a]

	Medium	CpG ODN	*E. coli* DNA	pI:C	LPS
IL-12 (ng/ml)					
PBMC	0.01	0.68	1.04	0.00	3.53
PBMC, depleted	0.00	0.73	1.07	0.00	3.48
IFN-α (pg/ml)					
PBMC	3.0	129.6	207.7	1989.0	2.3
PBMC, depleted	4.5	3.3	2.0	1328.0	2.2

[a] PBMC and PBMC depleted of $CD123^+$ cells (5×10^6/ml) were cultured with medium alone, 2 μM CpG-ODN, 50 μg/ml *E. coli*-DNA, 50 μg/ml poly(I:C), or 1 μg/ml LPS. After 24 h, supernatants were analyzed for total IL-12 and IFN-α. Data shown are representatives of three experiments with cells from different donors.

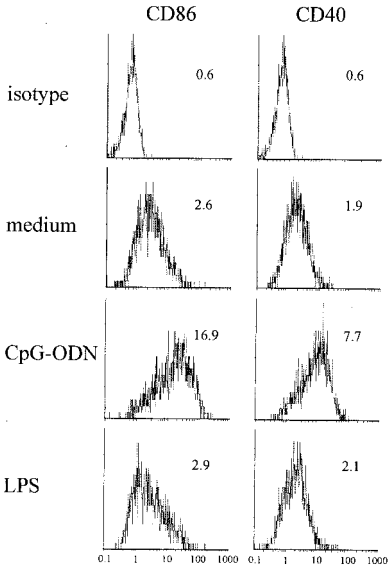

FIGURE 3. CpG-DNA stimulates $CD123^+$, $CD4^+$ DCs in PBMC. PBMC (10×10^6/ml) were cultured with medium alone, 2 μM CpG-ODN, or 1 μg/ml LPS. After 24 h cells were stained with CyChrome-labeled anti-CD4, PE-labeled anti-CD123, and FITC-coupled Abs against CD86, CD40, or the corresponding isotype controls. The corresponding FACS histograms for CD86 and CD40 expression gated on $CD4^+$, $CD123^+$ cells are shown, and mean fluorescence values are given adjacent to the histogram. Data shown are representative of three experiments with cells from different donors.

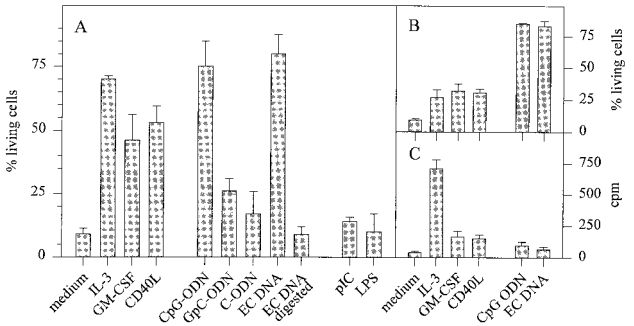

FIGURE 4. CpG-DNA rescues purified ppDC from apoptosis. DCs (20×10^3/well) were incubated in 96-well round-bottom plates with medium alone; 500 U/ml IL-3; 1000 U/ml GM-CSF; 20×10^3 CD40L transgenic 3T3 cells; 2 μM CpG-ODN, GpC-ODN, or C-ODN; 50 μg/ml *E. coli* (EC) DNA; 50 μg/ml digested EC DNA; 50 μg/ml poly(I:C); or 1 μg/ml LPS. *A*, After 24 h the cells were stained with propidium iodide, and the percentage of living cells was determined by FACS analysis. *B*, IL-4 (800 U/ml) was added simultaneously with the indicated stimuli, and living cells were counted at 24 h as described above. *C*, Cells were cultured with the indicated stimuli for 72 h and pulsed with [^{3}H]thymidine for the last 16 h. The mean ± SD are given for three (*A*) or two (*B* and *C*) independent experiments using different donors.

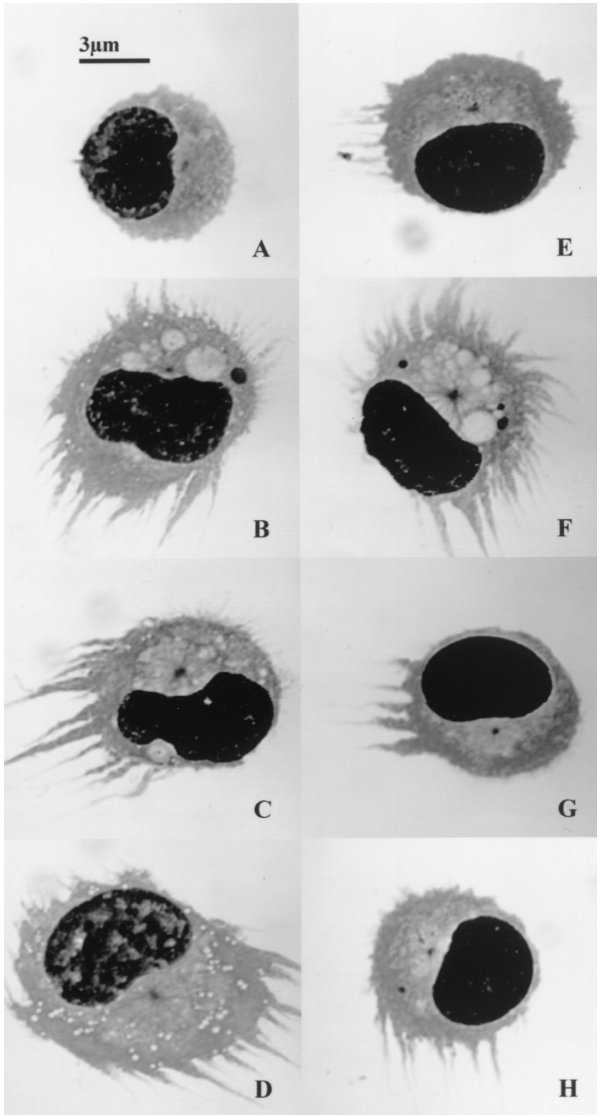

FIGURE 5. CpG-DNA induces morphologic changes in ppDC. Cells were used fresh (*A*) or were cultured for 36 h with the indicated stimuli (*B–H*). The stimuli were *E. coli* DNA (*B*), CpG-ODN (*C* and *D*), IL-3 (*E*), CD40L transgenic 3T3 cells plus IL-3 (*F*), poly(I:C) plus IL-3 (*G*), or LPS plus IL-3 (*H*). Cytospins were prepared, stained with May-Giemsa solution, and analyzed by confocal microscopy.

5, *G* and *H*, with Fig. 5*E*). These data implied that CpG-DNA is a strong activating agent for ppDC and confirmed the lack of activation by LPS.

The ppDC become activated by bacterial DNA

Up-regulation of costimulatory molecules such as CD86 and CD40 are a hallmark of DC activation (1). IL-3 was inefficient in activating ppDC (Fig. 6). In contrast, CpG-DNA induced strong CD86 and CD40 expression in a CpG-dependent fashion, although some activation was seen with the GpC-ODN (Fig. 6). CD40 ligation induced CD86 up-regulation, but not that of CD40, presumably due to CD40 ligation-driven receptor down-regulation (Fig. 6). CD80 and MHC classes I and II were also up-modulated by CpG-DNA and CD40 ligation (data not shown). In contrast, poly(I:C) and LPS were ineffective (Fig. 6). Thus, CpG-DNA activates/matures ppDC, but LPS and poly(I:C) are ineffective.

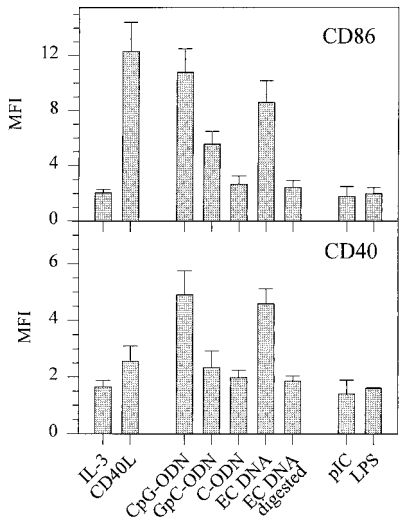

FIGURE 6. Up-regulation of CD86 and CD40 by CpG-DNA. Purified ppDC (0.2×10^6/ml) were stimulated for 36 h with 0.2×10^6 CD40L transgenic 3T3 cells; 2 μM CpG-ODN, GpC-ODN, or C-ODN (control); 50 μg/ml *E. coli* (EC) DNA or digested EC DNA; 50 μg/ml poly(I:C); or 1 μg/ml LPS. All cultures were supplemented with 500 U/ml IL-3 to support cell survival. Cells were stained with FITC-labeled Abs against CD86, CD40, or the corresponding Ab isotype controls. The mean fluorescence intensity values were determined by FACS analysis as described in Figs. 2 and 3. Data are the mean ± SEM of three experiments with cells from different donors.

CpG-DNA-activated ppDC produce cytokines

IFN-producing cells/pDC2 produce IL-8 upon CD40 cross-linking, but in acute response to enveloped viruses and bacteria they produce type I IFNs (8, 18, 19, 36, 39). Given the strong stimulatory effects of bacterial CpG-DNA, we tested the cytokine release pattern of ppDC after CpG-DNA exposure. Confirming previous reports, CD40 ligation induced only IL-8 release (Fig. 7) (8). Poly(I:C) induced only IFN-α, while LPS was nonstimulatory (Fig. 7). However, CpG-DNA sequence specifically induced not only IFN-α and IL-8, but also TNF and GM-CSF (Fig. 7). IL-6 was weakly induced, but, interestingly, neither IL-10 nor IL-12 was induced (data not shown). The control GpC-ODN induced significant TNF and GM-CSF; however, this was not true for the control unrelated sequence, C-ODN. This implies that the activation of ppDC by GpC-ODN was not due to phosphorothioate modification, but perhaps was due to flanking sequences outside the GpC held in common with the CpG-ODN. Overall, these data implied that the type of stimuli used dictates the response pattern triggered in purified ppDC. For example, poly(I:C) triggered only IFN-α production, but not DC maturation. CD40 ligation induced maturation, but only IL-8 production. In contrast, CpG-DNA triggered phenotypic maturation into DCs and the production of a variety of cytokines.

Allogenic ppDC induce T cell differentiation

Although ppDC did not produce IL-12, both CpG-DNA and poly(I:C) induced IFN type I production, which may influence T cell differentiation. We stimulated ppDC with either IL-3 or IL-3 plus CpG-DNA, LPS, CD40L, or poly(I:C) and used these DC to differentiate naive $CD4^+$ T cells (Fig. 8). IL-3-cultured ppDC differentiated T cells toward a mixed response, producing both Th1 and Th2 cytokines, IFN-γ, or IL-4 and IL-5, respectively (similar to published data) (36). LPS or CD40L did not significantly alter this response; however, LPS depressed IL-4 production, while

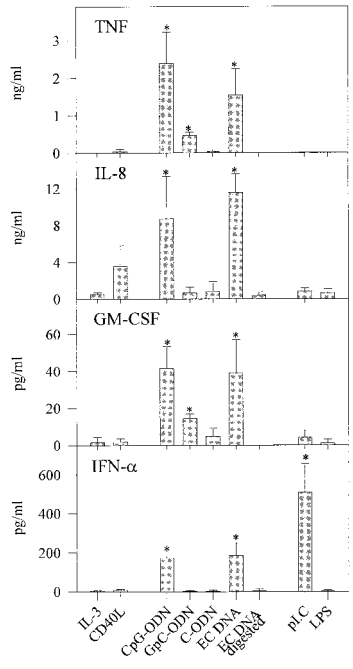

FIGURE 7. Cytokine release by purified ppDC. Cells (0.2×10^6/ml) were cultured in 24-well plates with 0.2×10^6 CD40L transgenic 3T3 cells, 2 μM CpG-ODN, GpC-ODN or C-ODN, 50 μg/ml *E. coli* (EC) DNA or digested EC DNA, 50 μg/ml poly(I:C), or 1 μg/ml LPS. All cultures were supplemented with 500 U/ml IL-3. Supernatants were collected and assayed in duplicate for TNF, IL-8, GM-CSF, or IFN-α by ELISA. The data are reported as the mean ± SD of three independent experiments with different donors. Significance differences between the IL-3 treatment and test treatments are denoted in terms of *t* test values (∗, $p \leq 0.05$).

CD40L depressed both IL-4 and IL-5. Poly(I:C) behaved similarly to viral stimulation, that is, it enhanced IFN-γ production and significantly reduced IL-4 and IL-5 production. CpG-DNA, although strongly trending toward a poly(I:C)-like induced response, did not prove to be significantly different from IL-3 alone.

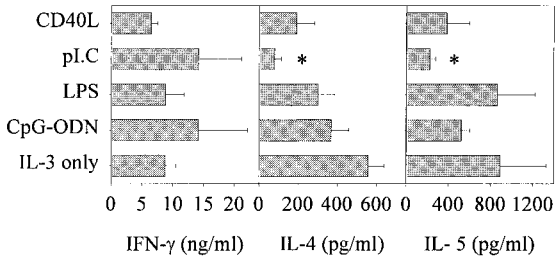

FIGURE 8. Allogenic ppDC induce T cell differentiation. The ppDC (2×10^5) were cultured in 24-well plates in the presence of 500 U/ml of IL-3 only or in combination with 2 μM CpG-ODN, 50 μg/ml poly(I:C), CD40L transgenic 3T3 cells, or 1 μg/ml LPS. After 24-h incubation 10^6 allogenic naive $CD4^+$ T cells were added and cultured for an additional 6 days. After priming, T cells were restimulated at a concentration of 5×10^5 cells/ml with PMA (50 ng/ml) and ionomycin (0.5 μg/ml) for 24 h. Supernatants were collected and assayed in duplicate for IL-4, IL-5, and IFN-γ by ELISA. The data are reported as the mean ± SD of four independent experiments with different donors. Significance differences between the IL-3 treatment and test treatments are denoted in terms of *t* test values (∗, $p \leq 0.05$).

Discussion

Bacterial CpG-DNA appears to be a component of infection that acutely triggers nonself pattern recognition by innate immunocytes and thus initiation of adaptive immune responses (for review, see Refs. 40 and 41). In the mouse these responses are Th1 polarized, which can be partly explained by the ability of CpG-DNA to initiate immature DC transit to professional APCs that secrete high levels of IL-12 (24, 29, 42). Here two prototypic human DCs were selected to test for CpG-DNA responsiveness: myeloid-like DCs differentiated in vitro from monocytes (MDDC/pDC1) and lymphoid-like DCs (ppDC/pDC2). Strikingly, MDDC responded to LPS, but not CpG-DNA, while ppDC responded to CpG-DNA, but not LPS.

Human monocytes responded to CpG-DNA and LPS, as determined by activation marker up-regulation and cytokine production (Fig. 1, *A* and *B*) (32). Unexpectedly, MDDC were refractory to CpG-DNA (Table I), but responsive to LPS, confirming previous reports (2, 43). The protocol used to compel in vitro transformation of human monocytes into immature MDDC cells uses culture in GM-CSF and IL-4 (44). During the culture period the cells loose their expression of CD14, an LPS coreceptor (44). However, the resultant MDDC remain responsive to LPS, because the signaling complex is reconstituted by serum-borne soluble CD14 (43). Interestingly, culture of murine macrophages or primary bone marrow-derived DCs in IL-4 also renders these cells refractory to CpG-DNA (our unpublished observations). Human DCs grown from $CD34^+$ bone marrow progenitors in the absence of IL-4, in contrast, are CpG-DNA responsive (C. Meyer zum Büschenfelde, unpublished observations). Whether IL-4 suppresses the response of MDDC to CpG-DNA via mechanisms such as receptor loss or signal interference needs to be analyzed. However, CpG-DNA uses a nearly identical signal transduction pathway to LPS, namely, the IL-1R/TLR pathway (25). This signaling pathway was apparently fully active, as judged by LPS activation/maturation of MDDC, implying that the CpG-DNA receptor may be absent in MDDC. Very recently, Hemmi et al. reported that murine TLR9-deficient mice do not respond to CpG-DNA (26). We determined that MDDC do not express human TLR9, but do express the LPS receptor TLR4 by semiquantitative PCR. Conversely, ppDC express TLR9, but not TLR4 or TLR2.[4]

Type I IFNs produced by a variety of cells in response to viruses, bacteria, and mycoplasma confer cellular resistance to virus, affect cell growth and differentiation, and modulate the immune system. It has been observed that PBMC produce type I IFNs in response to bacteria, but not to bacterial LPS (45). We document here that PBMC produce type I IFNs in response to bacterial CpG-DNA and not LPS (Fig. 1 and Table II), extending earlier studies that used CpG-DNA transfection with lipofectin (46, 47). Within PBMC, plasmacytoid cells (lin^- $CD123^+$ $CD11C^-$ $CD4^+$) that bear characteristic DC surface markers were recently identified as the major source of type I IFN (18). These precursor DCs were stimulated to produce IFN-α by both live and UV-irradiated enveloped viruses and Gram-negative and Gram-positive bacteria (8, 18, 19, 39). We show here that ppDC produce IFN-α in response to CpG-DNA and poly(I:C), but not LPS. Nucleic acid-based PAMPs could thus be the common link between viral and bacterial pathogen-derived stimuli and induction of IFN responses. Furthermore, nucleic acid structures should be considered a potential immunostimulatory component within crude pathogen mixtures.

[4] S. Bauer, C. Kirschning, V. Redecke, H. Hacker, S. Akira, H. Wagner, and G. B. Lipford. Human TLR9 expression correlates with responsiveness to bacterial DNA. *Submitted for publication.*

Here we show that the nature of the stimulus dictates the quality of ppDC activation and maturation. For example, poly(I:C), a mimic of viral infection, triggered IFN-α production, but no obvious cellular activation, as defined by DC morphology, up-regulation of costimulatory molecules, and production of proinflammatory cytokines such as TNF-α. In contrast, bacterial CpG-DNA not only caused IFN-α production, but acted as a survival factor and also effectively brought about maturation into DCs expressing high amounts of costimulatory molecules. Furthermore, the maturing DCs, in addition to IFN-α, produced TNF-α, GM-CSF, IL-6, and IL-8, but no IL-10 or IL-12. CD40 ligation, used as a mimic for DC-activating T cells, seemed intermediate to poly(I:C) or CpG-DNA, in that DCs matured, but only produced IL-8. Thus, bacterial CpG-DNA induced in ppDC a breath of responses not seen by other stimuli, including CD40 ligation (8, 11). Therefore, given the proper stimulus, these cells demonstrate a repertoire of responses commonly ascribed to myeloid DCs, with the exception of IL-12 production. The induction of Th polarization by CpG-DNA was noninformative, although consistent enhancement of IFN-γ and diminished IL-4 and IL-5 were observed (Fig. 8). In allogenic mixed lymphocyte reactions none of the tested stimuli enhanced allogenic T cell proliferation over IL-3 only (data not shown), confirming observations made with HSV- vs IL-3-stimulated ppDC (36). Overall, these results demonstrate that ppDC act as environmental sentinels, converting to effector DCs in response to inflammatory pathogen stimuli. This implies that ppDC are not simply intermediate stage DCs in transition to more differentiated forms (16) or that they are only responsible for peripheral T cell tolerance (1).

Purified human ppDC succumbed within 24 h to apoptosis unless cultured together with IL-3 (Fig. 4*A*) (10). The survival factor IL-3 promoted long term proliferation of phenotypically immature ppDC (Figs. 4*C* and 5*E*), and the Th2 cytokine IL-4 abolished IL-3-driven survival (Fig. 4*B*). It has been postulated that IL-4 may self-limit, via apoptosis, any potential recruitment of immature pDC2 within repopulating Th2 biased lymph nodes or sites of infection (8). Interestingly, CpG-DNA acted as a survival factor for ppDC (Fig. 4*A*) and was anti-apoptotic in the presence of IL-4 (Fig. 4*B*). Anti-apoptotic effects of CpG-DNA have been observed in B cells due to activation of NF-κB and prevention of mitochondrial membrane potential disruption via a chloroquine-sensitive pathway (48). However, cross-linking of CD40 on ppDC failed to counteract the apoptotic effect of IL-4, suggesting that CpG-DNA induces additional signaling events compared with CD40 (Fig. 4*B*). This is in agreement with the findings of others and implies that activation/maturation alone is insufficient to promote survival (11). Rissoan et al. demonstrated that IFN-γ was able to rescue ppDC from IL-4-induced apoptosis (8). They concluded that the induction of Th1 differentiation by pDC1 cells would promote the survival of Th2, supporting pDC2 as a reciprocal control mechanism. Although not formally demonstrated in the human, CpG-DNA is a strong Th1-inducing adjuvant in murine models (47, 49, 50). Therefore, it is intriguing that only CpG-DNA counteracted IL-4-induced apoptosis of purified ppDC (see below).

It has been postulated that DC lineage may determine the type of Th cell differentiation (1, 51, 52), although the potential roles of various DC culture conditions and activation protocols have not been considered. DC-like cells have been expanded in vitro from various sources, but whether in vitro-generated DCs are equivalent to their in vivo counterparts is a question of debate (44, 53–56). Here we focused on ex vivo-purified ppDC. These cells produced IFN-α, but not IL-12, when stimulated with CpG-DNA (Fig. 7). During productive T cell responses, IFN-α functions in vitro as a T cell survival factor and in humans as a Th1-polarizing cytokine (57–59). We have previously noted that CpG-DNA induced Th1-polarized responses in PBMC via the induction of IFN-α and IL-12 (60). Here we show that CD123$^+$ cells produce IFN-α, while IL-12 originates from a CD123$^-$ cell population (Table II). Others have reported that ppDC induced Th2 responses after CD40 cross-linking (8), a stimulus unable to trigger Th1 promoting IFN-α production (Fig. 7). Additional studies challenged the classification of plamacytoid/pDC-2 cells as Th2-polarizing DCs because blood-borne lin$^-$/ILT3$^+$/ILT1$^-$ DCs with a plasmacytoid DC phenotype (CD4$^+$/CD123$^+$/CD11c$^-$), which responded to virus, CD40 ligation, and LPS, induced a nonpolarized Th1 and Th2 differentiation (38). Here we show that CpG-DNA stimulation of ppDC trended toward enhanced Th1 polarization, although it was not statistically significant (Fig. 8). In our hands, while CD40L and LPS were not neutral, poly(I:C) was a significant inducer of Th1 polarization (Fig. 8). Stimulating ppDC with HSV can drive a very strong type I IFN-dependent Th1 differentiation (36). The apparent difference in Th1 promotion among CpG-DNA, poly(I:C), and HSV seems partly related to type I IFN output by ppDC. Although CpG-DNA induces full maturation of ppDC, the type I IFN production was low relative to HSV stimulation (Fig. 7) (36). Overall, these data suggest that within a given DC subset it is the quality of the stimulus encountered that dictates the respective response pattern and that the respective DC stimulus needs to be considered when accessing T cell differentiation.

Currently it is believed that infection is sensed through pattern recognition receptors driving Th cell-independent DC activation to professional APCs. The idea of DCs directing either Th1 or Th2 responses has been expressed in the context of different DC lineage, such as myeloid vs lymphoid or pDC1 vs pDC2, but not via integration of signal within one subset of DCs. Our data show that ppDC responded to LPS, dsRNA, CpG-DNA, or CD40 ligation with a broad range of distinct responses. This implies that DCs interpret environmental stimuli, rendering the appropriate response, and that the nature of environmental stimuli determines the DC response pattern. It follows that the propensity of ex vivo DCs to direct Th1 or Th2 responses needs to be evaluated in the context of various activation schemes.

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