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Quantitative Expression of Toll-Like Receptor 1–10 mRNA in Cellular Subsets of Human Peripheral Blood Mononuclear Cells and Sensitivity to CpG Oligodeoxynucleotides[1]

Veit Hornung,[2]* Simon Rothenfusser,[2]* Stefanie Britsch,* Anne Krug,* Bernd Jahrsdörfer,* Thomas Giese,† Stefan Endres,* and Gunther Hartmann[3]*

The Toll-like receptor (TLR)9 is critical for the recognition of immunostimulatory CpG motifs but may cooperate with other TLRs. We analyzed TLR1–10 mRNA expression by using quantitative real-time PCR in highly purified subsets of human PBMC and determined the sensitivity of these subsets to CpG oligodeoxynucleotides (ODN). TLR1 and TLR6 were expressed in all cell types examined. TLR10 was highly expressed in B cells and weakly expressed in plasmacytoid dendritic cells (PDC). High expression of TLR2 was characteristic for monocytes. PDC and B cells expressed marked levels of TLR7 and TLR9 and were directly sensitive to CpG ODN. In CpG ODN-stimulated PDC and B cells, TLR9 expression rapidly decreased, as opposed to TLR7, which was up-regulated in PDC and decreased in B cells. In monocytes, NK cells, and T cells, TLR7 was absent. Despite low expression of TLR9, monocytes, NK cells, and T cells did not respond to CpG ODN in the absence of PDC but were activated in the presence of PDC. In conclusion, our studies provide evidence that PDC and B cells, but not monocytes, NK cells, or T cells, are primary targets of CpG ODN in peripheral blood. The characteristic expression pattern of TLR1–10 in cellular subsets of human PBMC is consistent with the concept that TLR9 is essential in the recognition of CpG ODN in PDC and B cells. In addition, selective regulation of TLR7 expression in PDC and B cells by CpG ODN revealed TLR7 as a candidate TLR potentially involved in modulating the recognition of CpG motifs. ***The Journal of Immunology*****, 2002, 168: 4531–4537.**

Recognition of bacterial DNA by the vertebrate immune system is based on the presence of unmethylated CG dinucleotides in particular sequence contexts (CpG motifs) (1). Synthetic oligodeoxynucleotides (ODN)[4] that contain such CpG motifs mimic bacterial DNA and have been shown to induce a coordinated set of immune responses that comprise innate immunity and acquired Th1-biased cellular and humoral immunity (2, 3). The set of immune responses elicited by a microbial molecule such as CpG ODN is based on the activation of immunocompetent cells primarily involved in the recognition of this specific molecule. The plasmacytoid dendritic cell (PDC) has been identified as a primary target cell for CpG ODN (4–9). Besides the function as a dendritic cell (DC), the characteristic feature of PDC is to produce large amounts of type I IFN (IFN-α and IFN-β) upon viral infection (10, 11). Based on their distinct activity on PDC, two different types of CpG ODN have been defined: CpG type A (prototype ODN 2216) (7), which induces large amounts of IFN-α in PDC; and CpG type B (prototype ODN 2006) (7, 12), which is weak at inducing IFN-α but promotes survival, activation, and maturation of PDC. Besides PDC, B cells are primary target cells for CpG ODN (13–15). Direct activation of other human cell types is controversial to date. CpG ODN activates monocytes in the context of PBMC (16), but there are also reports that isolated monocytes respond to CpG ODN (5, 17). Both direct and indirect actions of CpG ODN have been proposed for human NK cells and T cells (18–20).

Although the CpG motifs differ between mice and humans (6, 13), in both species Toll-like receptor (TLR)9 seems to be involved in the recognition of CpG motifs (6, 9, 21). TLR9 belongs to the family of TLR, which established a combinatorial repertoire to discriminate among a wide spectrum of pathogen-associated microbial molecules (for a detailed review see Ref. 22). So far, 10 members of the TLR family (TLR1 to TLR10) have been reported (23–27). It has been demonstrated that the cytoplasmatic domains of TLR1, TLR2, and TLR6 form functional pairs to recognize a variety of microbial molecules (28). TLR cooperation not only extends the spectrum of ligands but also modulates the response toward a specific ligand. For example, TLR2-mediated response to phenol-soluble modulin is enhanced by TLR6 but inhibited by TLR1, indicating a functional interaction between these receptors (29). Although TLR9 seems to be essential for the recognition of CpG ODN in mice (21), other TLRs might be involved in modulating its activity.

In this study we used quantitative real-time PCR to examine the expression of TLR1 to TLR10 in subsets of human immunocompetent cells. In addition, we evaluated the sensitivity of these cell

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[4] Abbreviations used in this paper: ODN, oligodeoxynucleotide; TLR, Toll-like receptor; RT, reverse transcriptase; DC, dendritic cell; PDC, plasmacytoid DC; BDCA, blood DC Ag.

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populations to CpG ODN and examined the modulation of TLR expression in CpG ODN-sensitive cells. The quantitative level of different TLRs in conjunction with sensitivity to CpG ODN allowed us to identify candidate TLRs potentially involved in specific recognition of CpG motifs or in its modulation. Furthermore, our study reveals PDC as a key sensor of CpG motifs in the human immune system, which regulates the activity of other cell types such as monocytes, NK cells, and T cells via PDC-derived cytokines.

Materials and Methods

Oligodeoxynucleotides

The following completely and partially phosphorothioate-modified ODN were provided by Coley Pharmaceutical Group (Wellesley, MA) and used at a final concentration of 6 μg/ml (small letters represent phosphorothioate linkage, capital letters represent phosphodiester linkage 3′ of the base, and boldface represents CpG dinucleotides): ODN 2006, 5′-t**cg**t**cg**ttttgt**cg**tttt gt**cg**tt-3′ (12); and ODN 2216, 5′-ggGGGA**CG**AT**CG**TCgggggG-3′ (7). ODN were tested for endotoxin using the *Limulus* amebocyte lysate assay (lower detection limit, 0.1 EU/ml; BioWhittaker, Walkersville, MD).

Preparation, isolation, and culture of cells

Human PBMC were isolated from buffy coats provided by the blood bank of the University of Greifswald (Greifswald, Germany). Blood donors were 18- to 65-year-old healthy men and women who were tested to be negative for HIV, hepatitis B virus, and hepatitis C virus. Further exclusion criteria are manifest infections during the last 4 wk, fever, symptomatic allergies, abnormal blood cell counts, increased liver enzymes, or medication of any kind except vitamins and oral contraceptives. PBMC were prepared from buffy coats by Ficoll-Hypaque density gradient centrifugation (Biochrom, Berlin, Germany). PDC were positively isolated using an anti-blood DC Ag (BDCA)-4 Ab according to the manufacturer's protocol (BDCA-4 cell isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany). Untouched primary B cells were prepared by an indirect magnetic labeling system using Abs against CD2, anti-IgE, CD4, CD11b, CD16, and CD36 to deplete T cells, NK cells, monocytes, granulocytes, platelets, and erythroid precursor cells from PBMC (B cell isolation kit; Miltenyi Biotec) (purity > 95% and no PDC detectable). Monocytes were isolated by depletion of non-monocytes (T cells, granulocytes, NK cells, B cells, DCs, and basophils) by using a mixture of Abs against CD3, CD7, CD19, CD45RA, CD56, and anti-IgE (monocyte isolation kit; Miltenyi Biotec) (purity > 97%). Untouched T cells were prepared from PBMC with a mixture of CD11b, CD16, CD19, CD36, and CD56 Abs (Pan T Cell Isolation kit; Miltenyi Biotec) (purity > 96%; depletion of non-T cells). Depletion of non-NK cells (T cells, B cells, and myeloid cells) was used to isolate untouched NK cells (CD3, CD14, CD19, CD36, and anti-IgE Abs; purity > 95%) (NK cell isolation kit; Miltenyi Biotec). When necessary, PDC were depleted before isolation of the other cell types by using the anti-BDCA-4 Ab (Miltenyi Biotec). $CD8^+$ memory T cells were isolated by positive sorting of $CD8^+CD45R0^+$ double-positive cells on a FACS sorter (FACSVantage SE-DIVA; BD Biosciences, Heidelberg, Germany) (purity > 99%). Cells were resuspended in IMDM supplemented with 8% human AB serum (BioWhittaker), 1.5 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all Sigma, Munich, Germany). All compounds purchased were endotoxin tested. Viability of cells was determined by trypan blue exclusion. In some experiments a transwell culture system was used for coculture of either purified NK cells or T cells with PBMC (0.2-μm Anapore membrane device; Nunc, Roskilde, Denmark). For the generation of PDC-derived supernatant, purified PDC (200,000 cells/ml) were stimulated with 3 μg/ml ODN 2006 or ODN 2216. Cell-free supernatant was harvested after 48 h and added to purified T cells and NK cells at a final concentration of 5%.

Generation of peptide-specific $CD8^+$ T cell clones

Melan A_{26-35} A27L peptide-specific $CD8^+$ T cell clones were generated from PBMCs of HLA-A*0201-positive healthy volunteers. PBMCs were stimulated in vitro for 14 days with Melan A_{26-35} A27L peptide to increase the frequency of Ag-specific cells. Melan A_{26-35} A27L peptide-specific cells were labeled after restimulation using the IFN-γ secretion assay (Miltenyi Biotec), subsequently sorted directly into 96-well plates using a $FACStar^{Plus}$ flow cytometer (BD Biosciences) at a frequency of 1 cell per well, and expanded as previously described (30). Peptide-specific clones were simultaneously stimulated at a ratio of 10:1 with TAP-deficient T2 cells (lymphoblast cell line ATCC CRL-1992 (American Type Culture Collection, Manassas, VA); T2 cells present only exogenous peptides) loaded with their cognate peptide and CpG ODN (6 μg/ml) either in the presence or absence of PBMC separated by a transwell cell culture device (0.2-μm Anapore membrane; Nunc).

Flow cytometry

At the indicated time points, cells were harvested and surface Ag staining was performed as previously described (31). Anti-human CD3 (UCHT19), CD8 (RPA-T8), CD14 (3E2), CD19 (HIB19), CD69 (FN50), CD80 (L307.4), CD86 (IT2.2), CDw123 (7G3), and HLA DR (L243) were purchased from BD PharMingen (Heidelberg, Germany). Flow cytometric data were acquired on a FACSCalibur equipped with two lasers (BD Biosciences). Analysis was performed on viable cells. Data were analyzed using CellQuest software (BD Biosciences).

RT-PCR

Purified cell populations were cultured for 3 or 15 h, respectively, in RPMI 1640 with 10% FCS. Cells were lysed and RNA was extracted using the total RNA isolation kit (High Pure; RAS, Mannheim, Germany). An aliquot of 8.2 μl RNA was reverse transcribed using avian myeloblastosis virus-reverse transcriptase (RT) and oligo(dT) as primer (First Strand cDNA Synthesis kit; Roche, Mannheim, Germany). The obtained cDNA was diluted 1/25 with water and 10 μl were used for amplification. Parameter-specific primer sets optimized for the LightCycler (RAS) were developed by and purchased from Search-LC (Heidelberg, Germany). The primer positions and amplification efficiency are shown in Table I. The PCR was performed with the LightCycler FastStart DNA SYBR GreenI kit (RAS) according to the protocol provided in the parameter-specific kits. To control for specificity of the amplification products, a melting curve analysis was performed. No amplification of unspecific products was observed. The copy number was calculated from a standard curve, obtained by plotting known input concentrations of four different plasmids at log dilutions to the PCR cycle number (CP) at which the detected fluorescence intensity

Table I. *Characteristics of primer sets used to detect TLRs and IL-8*

Target Gene	Accession Code[a]	Amplicon Position	Amplification Efficiency	Genomic DNA (5 ng/ml)	Transcripts/2 × 10^6 PBMC (n = 3)	HEK293 300 cells
Cyclophilin-B	M60857	105–358	1.95	n/a[b]	1.6 × 10^6	12,396
IL-8	XM_003501	101–366	1.90	n/a	ND	ND
TLR1	NM_003263	1919–2254	1.86	886	415,704	1
TLR2	NM_003264	1549–1819	1.90	864	323,971	0
TLR3	NM_003265	1505–1717	1.93	858	16,134	12
TLR4	NM_003266	439–767	1.91	1130	317,530	0
TLR5	U88881	733–982	1.93	695	37,757	22
TLR6	NM_003268	2378–2650	1.93	1791	117,269	4
TLR7	AF245702	774–1012	1.98	563	23,972	0
TLR8	AF245703	2296–2590	1.95	831	60,790	0
TLR9	AF245704	2741–2941	1.95	853	66,799	2
TLR10	AF296673	1767–2054	1.90	785	53,267	0

[a] GenBank, National Center for Biotechnology Information.
[b] n/a, Not applicable, because primers were designed to be intron over-spanning.

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reaches a fixed value. Using >300 data points, the actual copy number per microliter of cDNA was calculated as follows: $X = e^{(-0.6553 \times CP + 20.62)}$. This approach dramatically reduced variations due to dilution errors over several logarithmic dilution steps. The amplification efficiency of the PCR was determined by running log dilutions of standards. The slope of the standard curve was converted to the amplification efficiency E by the following algorithm: $E = 10^{-1/slope}$. All used primer sets had an efficiency >1.86 (Table I). The data of two independent analyses for each sample and parameter were averaged. The copy number of the different TLRs and of IL-8 was normalized by the housekeeping gene cyclophilin-B and is presented as number of transcripts per 10^3 copies of cyclophilin-B.

The PCR primer sets used for the amplification of TLR cDNA also recognize genomic DNA. To confirm that the primer sets for different TLRs have comparable amplification characteristics, we tested each TLR primer set on 5 ng genomic DNA as a quality control. Five nanograms of genomic DNA is known to contain ~1500 genomic copies. As shown in Table I, the copy number detected by the TLR primer sets is within the range of 563-1791 copies (Table I). This range is close to the expected value of 1500 genomic copies, considering a 16 million-fold amplification demonstrating how precisely our assay performed. To exclude contamination of cDNA with genomic DNA in randomly selected samples we performed controls in which the RT was replaced by water during the cDNA synthesis. Such "minus RT" controls did not contain genomic DNA, as shown by a lack of PCR amplification. As a positive control for the amplification of TLR we measured the number of TLR transcripts in the cDNA prepared from 2×10^6 PBMC (Table I). As TLR^{low} expressing control we used HEK293 cells (Table I).

Statistical analysis

Data are expressed as mean values ± SEM. Statistical significance of differences was determined by the paired or unpaired two-tailed Student *t* test. Differences were considered statistically significant for $p < 0.05$. Statistical analyses were performed using StatView 4.51 software (Abacus Concepts, Calabasas, CA). In Figs. 3–5, an asterisk indicates values of $p < 0.05$ between medium control and stimulation with CpG ODN.

Results

Quantitative analysis of TLR1–10 mRNA expression in PDC, B cells, monocytes, NK cells, and T cells

We applied quantitative real-time PCR to study expression of TLR transcripts in cellular subsets of human PBMC. A detailed description of the technique is provided in *Materials and Methods*. The characteristics of primer sets and controls are provided in Table I. Human PDC, B cells, NK cells, T cells, and monocytes were purified from freshly isolated PBMC, and TLR1 through TLR10 were measured after 3 h of cell culture in the absence of stimulation (Fig. 1).

In PDC expression of TLRs was restricted to TLR1, TLR6, TLR7, TLR9, and low levels of TLR10 (Fig. 1). Among these, TLR9 was most prominent. B cells expressed a broad range of TLRs, which included the TLR expressed in PDC, namely TLR1, TLR6, TLR7, TLR9, and TLR10 (Fig. 1). Compared with PDC, TLR1 and TLR6 were higher and TLR9 was lower in B cells. In addition to PDC, B cells expressed considerable levels of TLR2 and high levels of TLR10, while expression of TLR4 was relatively low. Monocytes were characterized by an extremely high expression of TLR2 (Fig. 1), with intermediate levels of TLR1 and TLR4, moderate levels of TLR5, TLR6, and TLR8, and weak but detectable TLR9 expression. In NK cells expression levels of TLR1 were highest, followed by moderate levels of TLR2, TLR3, TLR5, and TLR6 (Fig. 1). TLR expression in T cells in general was low, with TLR5 being the only TLR that was expressed at higher levels (Fig. 1). Low levels of TLR9 could be detected in both NK cells and T cells.

Comparison of the expression levels of single TLRs in different cell types (Fig. 1, vertical comparison) revealed the following characteristic and statistically significant differences ($p < 0.05$) between the cell subsets: *TLR1* was higher in B cells, monocytes, and NK cells as compared with PDC and T cells, while between PDC and T cells, as well as among monocytes, NK cells, and T cells, no significant difference was found. *TLR2* was higher in monocytes than in all other subsets. Expression of *TLR3* was higher in NK as compared with other cell types. Regarding *TLR4*, monocytes expressed higher levels than other cell types. In B cells, TLR4 expression seemed to be more prominent than in PDC, NK cells, and T cells, but this difference was not significant. Marked levels of *TLR5* were found in monocytes, NK cells, and T cells, all of which were significantly higher than in PDC and B cells lacking TLR5. *TLR6* was highest in B cells as compared with all other cell types. Moreover, monocytes and NK cells expressed higher TLR6 levels than did T cells. *TLR7* showed the opposite expression pattern from TLR5; TLR7 was significantly higher in PDC and B cells as compared with monocytes, NK cells, and T cells, which showed only marginal levels of TLR7. Furthermore, TLR7 was significantly higher in PDC than in B cells. Considerable levels of *TLR8* were only expressed in monocytes, which were higher than in PDC, B cells, and T cells. The difference of TLR8 expression between monocytes and NK cells did not reach statistical significance ($p = 0.06$). *TLR9* showed a very similar expression pattern among cell subsets as TLR7, but on a higher level. As for TLR7,

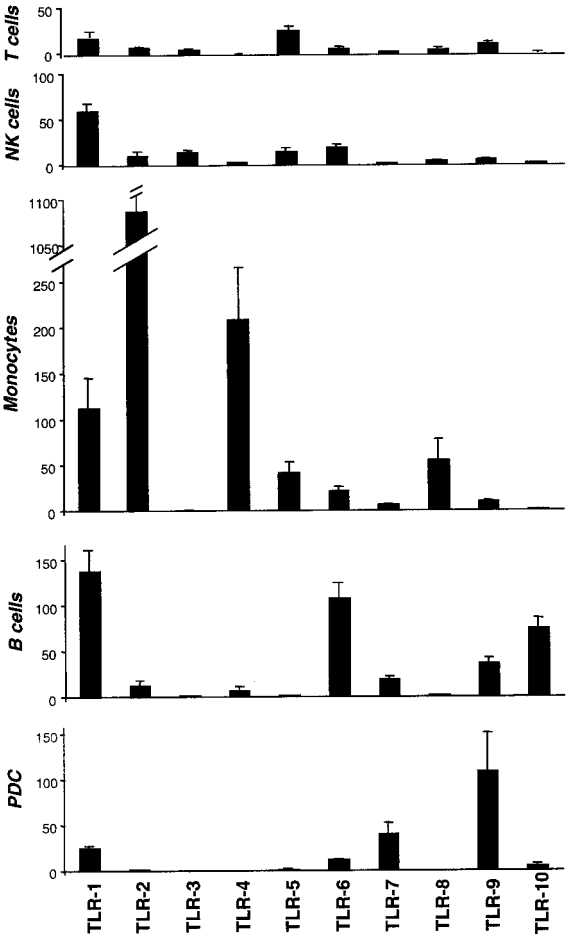

FIGURE 1. Quantitative analysis of TLR1–10 mRNA expression in PDC, B cells, monocytes, NK cells, and T cells. Isolated PDC (50,000–100,000 cells), B cells, monocytes, NK cells, and T cells (2×10^6 cells each) were incubated without stimulus for 3 h. Expression of TLR1–10 mRNA was determined by quantitative real-time RT-PCR and is depicted as the number of transcripts per 10^3 copies of the housekeeping gene cyclophilin-B. Data from experiments with cells from different individual donors are summarized (PDC, $n = 6$; B cells, $n = 10$; monocytes, $n = 8$; NK cells, $n = 6$; T cells, $n = 9$) and presented as mean values ± SEM (SEM for TLR2 in monocytes is 198).

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PDC expressed higher levels of TLR9 than did B cells; both PDC and B cells showed higher TLR9 expression than did monocytes, NK cells, and T cells, which only expressed marginal levels of TLR9 and among themselves showed no significant differences. *TLR10* was prominent on B cells as compared with all other cell types ($p < 0.001$). Besides B cells, only PDC consistently expressed low levels of TLR10, which were significantly higher than in monocytes, NK cells, and T cells, which lacked TLR10.

In the absence of PDC, purified monocytes are not sensitive to CpG ODN-mediated activation

Sensitivity of different purified immune cell subsets to CpG ODN in conjunction with the expression pattern of TLRs may allow the identification of TLRs potentially involved in recognition of CpG ODN. In previous studies we and others demonstrated that both purified PDC and B cells are directly sensitive to CpG ODN-mediated activation (4–7, 9, 13–15). We were interested in whether monocytes are directly or indirectly activated by CpG ODN. Two CpG ODN with distinct biological properties were used for these studies. ODN 2006 is the prototype of a CpG ODN which potently activates human B cells and PDC (5, 7, 12). The specific characteristic of ODN 2216 is to induce high amounts of type I IFN in PDC (7).

Monocytes were purified from freshly isolated PBMC by depletion of other cell types using magnetic beads (purity > 95%; see *Materials and Methods*). We found that this procedure led to an enrichment of PDC within the resulting monocyte population (increase of PDC from 0.2 to 0.4% in PBMC up to >1% in isolated monocytes). B cells were not detected within isolated monocytes. To avoid contamination with PDC, PDC were depleted by a magnetically labeled PDC-specific Ab (anti-BDCA-4 Ab) before the standard monocyte isolation protocol was applied to PBMC. The PDC-containing monocyte population (standard protocol) as well as the PDC-free monocyte population were incubated with CpG ODN.

After 48 h of culture, expression of CD80, CD86, and MHC class II was assessed by flow cytometry (Fig. 2). In the monocyte preparation without PDC, monocytes did not respond to stimulation by CpG ODN (Fig. 2, open bars). In contrast, in the presence of PDC (Fig. 2, filled bars), CpG ODN induced a marked increase of CD80 (ODN 2006 MFI, 64 vs 11; $n = 3$), CD86 (ODN 2006 MFI, 106 vs 75; $p < 0.05$; $n = 3$) and MHC class II (ODN 2006 MFI, 130 vs 74; $n = 3$) (Fig. 2, filled bars). These results indicated that purified monocytes were not sensitive to CpG ODN unless PDC were present.

Purified NK cells and memory T cells are not activated by CpG ODN unless reconstituted with PDC

Previous studies demonstrated that, within PBMC, NK cells are activated by CpG ODN (12, 18, 20). However, there has been controversy over whether this activation is direct (20) or indirect (12, 18). We separated NK cells from PBMC and confirmed that, to the limits of detection, isolated NK cells did not contain PDC or B cells. NK cells were incubated with ODN 2006 and ODN 2216 in a transwell system in the presence or absence of PBMC. Expression of CD69 was examined after 24 h of culture. In the absence of PBMC none of the CpG ODN up-regulated CD69 expression in NK cells (Fig. 3*A*, open bars), demonstrating that purified NK cells are not sensitive to CpG ODN. In contrast, NK cells cocultivated with PBMC were strongly activated by ODN 2216 (Fig. 3*A*, filled bars). ODN 2006 was less effective. Because the transwell system inhibited cell to cell contact, PBMC-derived

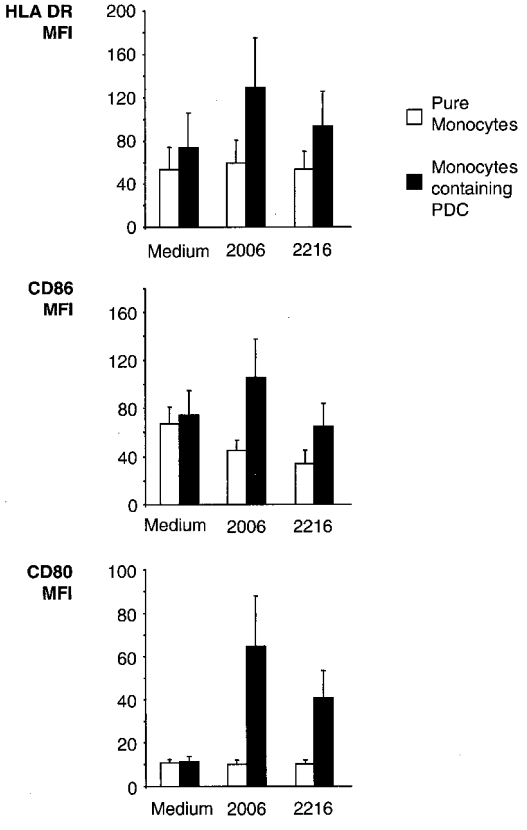

FIGURE 2. In the absence of PDC, purified monocytes are not sensitive to CpG ODN-mediated stimulation. Monocytes were isolated from PBMC by depletion of other cell types. Before isolation of monocytes, PBMC were either depleted of PDC (open bars) or not depleted of PDC (filled bars). After 2 days of stimulation with CpG ODN, monocytes were harvested and the expression of HLA DR, CD86, and CD80 was assessed by flow cytometry. Data from three experiments with cells from different individual donors are summarized and presented as mean values ± SEM.

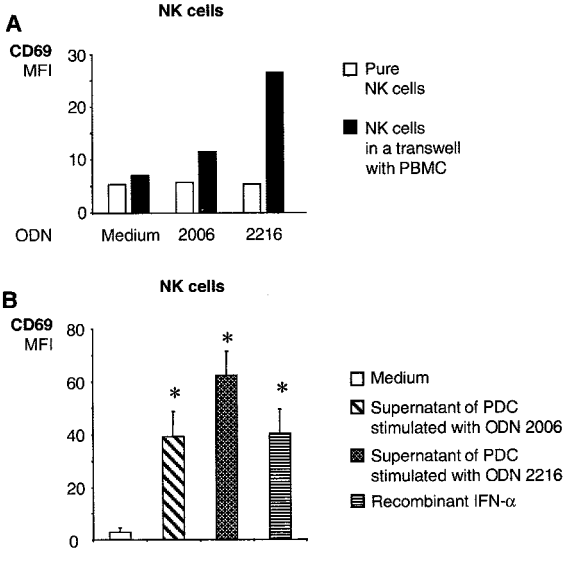

FIGURE 3. CpG ODN activate purified NK cells not directly but via PDC-derived cytokines. *A*, Purified NK cells were stimulated with CpG ODN either in the presence of PBMC (filled bars) or in the absence of PBMC (open bars) separated by a transwell system. After 24 h of culture, NK cells were harvested and the activation marker CD69 was assessed by flow cytometry. Results are representative of three experiments with similar results. *B*, Purified NK cells were incubated with supernatants of CpG-stimulated PDC or with 5000 IU/ml rIFN-α. After 48 h of culture, NK cells were harvested and the activation marker CD69 was assessed by flow cytometry (data are shown as means ± SEM; $n = 3$ individual donors).

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soluble factors were responsible for indirect activation of NK cells with CpG ODN. Next we tested whether PDC-derived cytokines contribute to activation of NK cells. Supernatants were collected from purified PDC, which were stimulated for 2 days with ODN 2006 or ODN 2216. In the presence of these supernatants containing PDC-derived cytokines, isolated NK cells strongly up-regulated CD69 expression within 2 days (Fig. 3*B*). Similar activation of NK cells was found in the presence of rIFN-α (Fig. 3*B*, *right bar*), which is known to be produced by PDC in response to CpG ODN (4, 5, 7).

For CpG ODN-mediated activation of T cells, direct (20, 32) and indirect (19, 33) mechanisms have been proposed. To address this question we studied the effects of ODN 2006 and ODN 2216 on memory T cells and on Ag-specific activation of peptide-specific $CD8^+$ T cell clones. $CD45R0^+CD8^+$ memory T cells were isolated from PBMC by fluorescence-activated cell sorting and were stimulated with ODN 2006, ODN 2216, the supernatants of CpG ODN-activated PDC, or rIFN-α (5000 IU/ml). After 48 h of cell culture, expression of CD69 was assessed by flow cytometry. Although no stimulation of purified T cells was observed in the presence of CpG ODN, both the supernatants of CpG-activated PDC and rIFN-α strongly activated memory T cells, as indicated by up-regulation of CD69 (Fig. 4*A*).

To test the ability of CpG ODN to promote Ag-specific T cell responses, $CD8^+$ T cell clones with specificity for the Melan A_{26-35} A27L peptide were generated and used as a model system. Clones were restimulated in the presence or absence of PBMC in a transwell cell culture. As expected, restimulation of T cell clones with their cognate peptide (loaded on T2 cells) for 24 h led to an increased expression of CD69 both with and without PBMC (Fig. 4*B*). In the absence of PBMC none of the CpG ODN up-regulated CD69 on $CD8^+$ T cells (Fig. 4*B*, open bars). However, when PBMC were present in the transwell system CD69 expression on $CD8^+$ T cells was markedly increased in the presence of ODN 2216. The effect of ODN 2006 again was lower. Soluble factors were responsible for indirect activation in the presence of PBMC, as cell to cell contact was inhibited by the transwell system. Together, these results indicated that purified NK cells as well as $CD8^+$ T cells are not sensitive to CpG ODN but are activated by CpG ODN-induced PDC-derived cytokines. ODN 2216 was more potent than ODN 2006 to indirectly stimulate NK cells, memory $CD8^+$ T cells, and CD8 T cell clones.

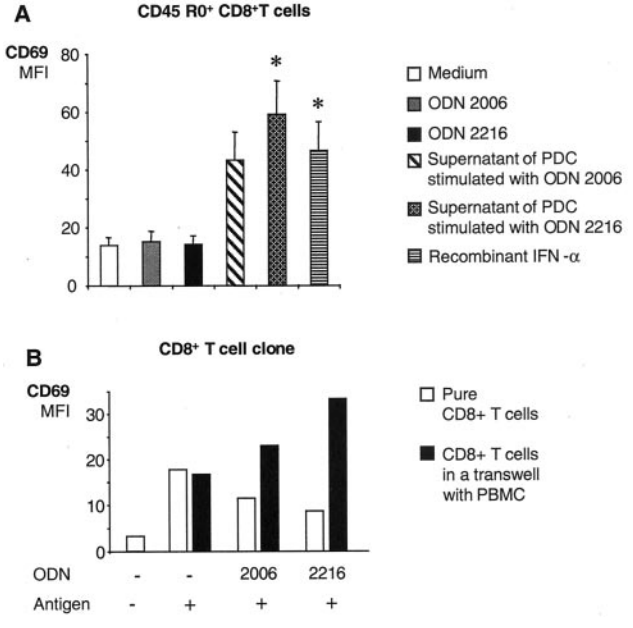

FIGURE 4. CpG ODN activate purified memory T cells and costimulate Ag-triggered T cell clones via PDC-derived cytokines. *A*, $CD45R0^+CD8^+$ memory T cells were sorted from PBMC and were stimulated with ODN 2006, ODN 2216, the supernatants of CpG ODN-activated PDC, or rIFN-α (5000 IU/ml). After 48 h of cell culture, T cells were harvested and CD69 was assessed by flow cytometry (mean $\pm$ SEM; $n = 3$ individual donors). *B*, $CD8^+$ T cell clones specific for Melan A_{26-35} A27L peptide were restimulated with their cognate peptide presented by T2 cells both separated from PBMC by a transwell system (filled bars) or without PBMC (open bars). After 24 h, $CD8^+$ T cells were harvested and CD69 was analyzed by flow cytometry. The experiment shown is representative of three experiments with similar results.

Comparison of sensitivity to CpG ODN and the TLR expression pattern

To confirm that both PDC and B cells are directly activated by CpG ODN in our system, we examined whether IL-8 as one representative indicator of activation is up-regulated in response to CpG ODN. We incubated purified PDC and B cells with or without CpG ODN. After 3 h and again after 15 h, cells were harvested and mRNA was prepared. Real-time PCR analysis revealed that IL-8 mRNA was rapidly up-regulated in B cells (Fig. 5*A*, *left panel*) and PDC (Fig. 5*B*, *left panel*) within 3 h. These results indicated that PDC and B cells are able to recognize CpG motifs.

To identify TLRs possibly involved in recognition of CpG motifs, we were interested in TLRs that were expressed in both PDC and B cells but not in monocytes, NK cells, and T cells. According to Fig. 1, only TLR7 and TLR9 match these criteria. Because TLR1 and TLR6 are expressed in both PDC and B cells, but are also expressed in other cell types, they must not be sufficient for the recognition of CpG motifs but are potential candidates involved in modulating the recognition of CpG motifs.

Modulation of TLR9 and TLR7 expression in B cells and PDC in response to CpG ODN

It has been described that microbial stimuli affect the expression of their cognate TLR (34–37). We hypothesized that the expression of a TLR involved in the recognition of CpG ODN would be modulated upon stimulation with CpG ODN. We quantified TLR1, TLR6, TLR7, and TLR9 mRNA after 3 h and again after 15 h of stimulation of B cells and PDC with CpG ODN. Because ODN 2216 is weak at stimulating B cells (our unpublished observation), B cells were stimulated with ODN 2006, while PDC were stimulated with both ODN 2006 and ODN 2216. In B cells both TLR9 and TLR7 were down-regulated within 15 h after exposure to ODN 2006 (Fig. 5*A*, *middle* and *right panels*). In PDC, almost a complete loss of TLR9 expression was seen after stimulation with ODN 2006 as well as ODN 2216 (Fig. 5*B*, *middle panel*, hatched bars). A similar decrease of TLR9 was found in the presence of the growth factor IL-3, which, like CpG ODN, is known to induce differentiation of PDC (Fig. 5*B*, *middle panel*, open bars). IL-3 also decreased TLR7 (Fig. 5*B*, *right panel*, open bars). However, unlike for TLR9, both CpG ODN increased the expression of TLR7 (Fig. 5*B*, hatched bars). The mRNA levels of TLR1 and TLR6 did not show significant changes upon stimulation with CpG ODN (data not shown).

Discussion

The identification of combinatorial sets of TLRs which are associated with the recognition of specific microbial molecules is critical to the understanding of pathogen-host interactions. The immunological repertoire elicited by a particular microbial molecule comprises the effector functions of immune cells equipped with the corresponding set of TLR, as well as the regulatory role of these

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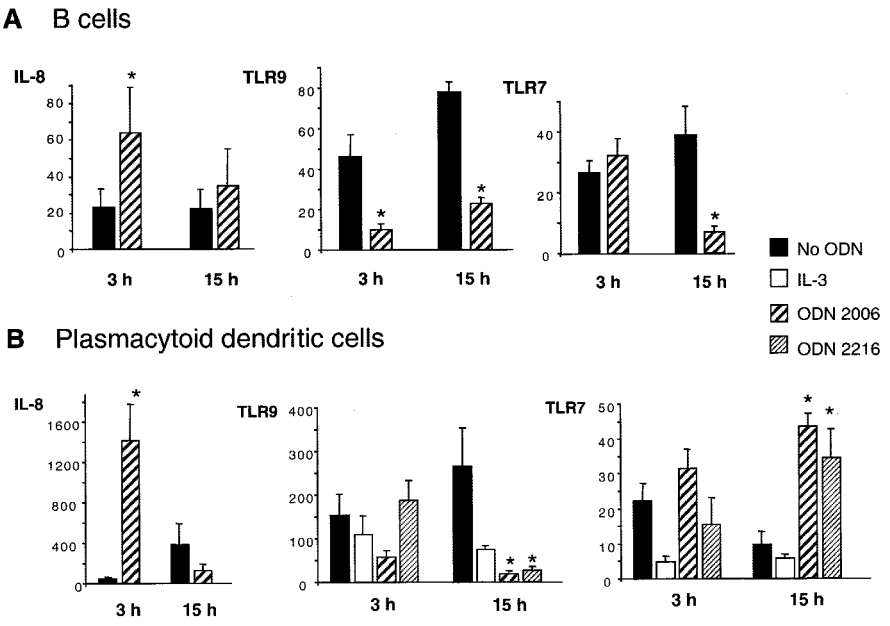

FIGURE 5. Direct CpG-mediated activation of purified B cells and PDC, and modulation of TLR9 and TLR7 mRNA expression. Purified B cells (2×10^6 cells) (*A*) were incubated with (hatched bars) or without (filled bars) ODN 2006. Purified PDC (50,000–100,000 cells) (*B*) were incubated with (filled bars) or without (hatched bars) ODN 2006 and ODN 2216, or with IL-3 (10 ng/ml; open bars). After 3 h and again after 15 h, cells were harvested and expression of IL-8, TLR7, and TLR9 transcripts was quantified by real-time RT-PCR. The number of transcripts indicated is normalized to 10^3 copies of the housekeeping gene cyclophilin-B. Data represent the means ± SEM of independent experiments with different individual donors (B cells, $n = 5$ for TLR7 and TLR9 and $n = 6$ for IL-8; PDC, $n = 4$). *, Significant differences ($p < 0.05$).

primary target cells on other immunocompetent cells. The exact separation of primary vs secondary effects of a microbial molecule requires the preparation of highly purified immune cell subsets to avoid indirect effects mediated by the presence of other immune cells. Using purified subsets of human PBMC we made the observation that PDC and B cells, but not monocytes, NK cells, and T cells, were sensitive to CpG ODN. Of note, low numbers of PDC or PDC-derived cytokines were sufficient to cause CpG ODN-mediated indirect activation of otherwise nonresponding cell types. These results emphasize the potent regulatory role of PDC in the human immune system and highlight the pivotal role of PDC in the functional profile of CpG ODN.

In our study, the CpG sensitivity in conjunction with the TLR expression pattern of purified subsets of PBMC was consistent with the concept that TLR9 is primarily involved in recognition of CpG motifs in B cells and PDC. Interestingly, we found that CpG ODN rapidly down-regulated the expression of TLR9 in PDC. However, a similar decrease of TLR9 was observed during differentiation of PDC with IL-3 in the absence of CpG ODN. Consequently, the CpG-induced decrease in TLR9 cannot be separated from the reduction of TLR9 as a general aspect of PDC differentiation and thus provides no additional evidence that TLR9 is involved in recognition of CpG motifs. Similarly, it has been described that monocyte-derived immature DCs down-regulate TLR4 upon maturation with the corresponding cognate ligand LPS, but also in response to the unrelated maturation stimulus TNF (35).

It has been reported that TLR9 is essential for recognition of CpG ODN in mice (21) and confers responsiveness to CpG ODN in human cell lines (6). According to the model of combinatorial recognition of microbial molecules by TLR (28), other members of the TLR family might exist which modulate the activity of TLR9. Besides TLR9, we found that marked levels of TLR7 were expressed in CpG-sensitive cell types (PDC and B cells) but not in the other cell types, which are not directly sensitive to CpG ODN. Of note, in PDC TLR7 was up-regulated in response to CpG ODN as opposed to decreased levels of TLR7 in the presence of IL-3. In contrast to PDC, in B cells TLR7 was down-regulated in response to CpG ODN. One might speculate that CpG-induced regulation of TLR7 expression may be involved in positively or negatively modulating the recognition of CpG motifs by TLR9. Furthermore, other TLRs such as TLR1 and TLR6 may still be involved in modulating the recognition of CpG motifs despite not being regulated upon CpG-mediated stimulation. Cotransfection experiments are currently being performed to study a cooperative role of TLR7 and TLR9 in the context of recognition of CpG motifs. It has been reported that transfection of HEK293 cells with TLR9 conferred responsiveness to CpG motif containing DNA (6). We found that the relative lack of TLR1–10 expression in HEK293 cells (see Table I) provides a valuable transfection model to examine cooperation between different TLRs.

Our study represents the first analysis of TLR expression using real-time PCR, which is both a sensitive and a quantitative method to assess the number of transcripts of the target mRNA. We demonstrate that each cell type examined displays a characteristic profile of TLRs that was consistent between the different donors as demonstrated by statistical analysis. These results support the concept that different types of infections induce distinct types of immune responses based on activation of the subsets of immune cells that express the corresponding profile of TLRs. Because our studies were performed with cells from healthy blood donors, it will be interesting to study whether conditions such as a history of allergies or infectious disease affect the TLR profile.

In a previous study Muzio et al. (36) examined expression of TLR1 through TLR5 in human leukocyte subsets by Northern blot analysis. We confirm their results and, by using a more sensitive method, extend their study, demonstrating that TLR2 and TLR5 are not restricted to myelomonocytic cells. In our study, TLR2 was also present in B cells, NK cells, and T cells, and TLR5 was present in T cells and NK cells. TLR3 was also expressed in NK cells and is absent in PDC. Consequently, expression of TLR3 seems not to be a general feature of DCs.

Consistent with an earlier study (28), we found that TLR1 and TLR6 are expressed in monocytes. In addition, we detected high levels of TLR1 and TLR6 in B cells, suggesting a particular role of these two TLRs in B cells. TLR10, which is phylogenetically closely related to TLR1 and TLR6 (27), was present in B cells at similarly high levels.

In earlier studies we and others demonstrated by using semiquantitative RT-PCR that PDC express TLR9, while monocytes expressed TLR2 and TLR4 (6, 9). This study confirms and complements these results on a quantitative level. It has been suggested that isolated human monocytes are activated by CpG ODN and

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bacterial DNA (5, 17). In contrast, in the present study, evaluation of PDC within the monocyte population revealed that, in the absence of PDC, monocytes were nonresponsive to both CpG ODN tested, and that very low numbers of PDC were sufficient to activate monocytes via PDC-derived cytokines. Therefore, our early results of monocyte activation within human PBMC were likely due to secondary effects of CpG ODN mediated by PDC (16).

Of note, there seem to be significant differences between mice and humans regarding sensitivity of myeloid cells to CpG ODN. In mice macrophages and myeloid DC are directly activated upon recognition of CpG motifs (38–41). In humans, besides monocytes as discussed above, monocyte-derived DC and peripheral blood myeloid DC are not activated by CpG ODN (6–8). However, the detection of baseline levels of TLR9 in human monocytes in this study suggests that monocytes might be able to modulate TLR9 expression along distinct differentiation pathways they enter and the cytokine milieu and the microbial molecules they encounter.

In conclusion, quantitative assessment of TLR expression in immunocompetent cells at different stages of differentiation and activation will open new avenues in the field of TLR research. As new members join the growing list of TLRs, the approach presented in this study will help to assign the large variety of microbial molecules to their cognate innate receptors.

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