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# Plasmacytoid Dendritic Cells Control TLR7 Sensitivity of Naive B Cells via Type I IFN<sup>1</sup>

Isabelle Béatrice Bekerédjian-Ding,\* Moritz Wagner,\* Veit Hornung,\* Thomas Giese,† Max Schnurr,\* Stefan Endres,\* and Gunther Hartmann<sup>2\*</sup>

Detailed information of human B cell activation via TLR may lead to a better understanding of B cell involvement in autoimmunity and malignancy. In this study we identified a fundamental difference in the regulation of TLR7- and TLR9-mediated B cell stimulation: whereas the induction of polyclonal naive B cell proliferation by the TLR7 ligands resiquimod (R848) and loxoribine required the presence of plasmacytoid dendritic cells (PDCs), activation via the TLR9 ligand CpG was independent of PDCs. We found that PDC-derived type I IFN enhanced TLR7 sensitivity of B cells by selectively up-regulating TLR7 expression. In contrast the expression levels of TLR9 and of other TLRs studied remained unchanged. In the presence of type I IFN, TLR7 ligation triggered polyclonal B cell expansion and B cell differentiation toward Ig-producing plasma cells; notably, this occurred independently of T cell help and B cell Ag. Human B cells did not respond to ligands of other TLRs including TLR2, TLR4 and TLR6 with and without type I IFN. In conclusion, our results reveal a distinct regulation of TLR7 and TLR9 function in human B cells and highlight TLR7 and TLR9 as unique targets for therapeutic intervention in B cell-mediated immunity and disease. *The Journal of Immunology*, 2005, 174: 4043–4050.

**B** cells are involved in autoimmune disorders such as lupus erythematosus and in B cell malignancies. Furthermore, most vaccines in use today aim at the establishment of pathogen-specific Ab responses to protect the individual from infection. A better understanding of the mechanisms that regulate B cell activity may help to improve therapies for B cell-mediated diseases (1), and may lead to the design of vaccines for clinical settings in which their efficacy is limited to date, such as tuberculosis, HIV or other immunosuppressive diseases. In general, B cell production of protein Ag-specific Abs is thought to require 1) binding of the protein Ag to an Ag-specific B cell surface Ig and 2) costimulation by Ag-specific T cells through CD40-CD40L interaction and T cell-derived cytokines; activated B cells proliferate and differentiate into Ig-producing plasma cells or long-lived memory cells.

Recent understanding of B cell biology indicates that B1 cells found in peritoneal and pleural cavities (2) as well as conventional (B2) B cells can be regulated in a T cell-independent manner (3, 4). As other APCs, B cells express TLRs, a receptor family that provides the combinatorial repertoire to discriminate among a wide spectrum of pathogen-associated molecules (5). The TLR family belongs to innate immunity, initiating both immediate pro-

TECTIVE responses against pathogens and instructing the adaptive immune response through activation and maturation of APCs.

To date, 10 members of the TLR family have been identified in humans (5), and 11 in mice (6). The type of immune response triggered by a specific TLR member depends on the specific expression pattern of TLRs in different immune cell subsets and on the specific type of signaling pathway elicited. The best known TLR member expressed in human B cells is TLR9 (7). The natural ligand for TLR9 is microbial DNA containing CpG motifs (8–11). Activation of murine and human B cells by CpG motif containing DNA (CpG DNA) is well established (12–14). In fact, CpG motifs were discovered based on B cell stimulation (15), and B cells were the first immune cell subset in humans identified to respond to CpG DNA in a CpG-specific fashion (16, 17). CpG oligodeoxynucleotides (ODN)<sup>3</sup> license human CD40L-primed B cells to produce high amounts of IL-12 and to support IFN- $\gamma$  production in CD4 T cells (18). CpG ODN have been tested as humoral vaccine adjuvants in nonhuman primates (17, 19–22) and show promising results in initial clinical trials (23).

Besides TLR9, human peripheral blood B cells express high levels of TLR1, TLR6, and TLR10, intermediate levels of TLR7, and low levels of TLR2 and TLR4 (7). TLR1 and TLR6 both dimerize with TLR2 and discriminate between different bacterial lipoproteins (5). A ligand for TLR10 has not been identified. TLR7 belongs to the TLR9 subfamily that is thought to participate in the discrimination of nucleic acid-like structures from microorganisms (24). The natural ligand of human TLR7 has not yet been defined. TLR7 recognizes several synthetic compounds, which are structurally related to nucleic acids. These include imidazoquinolines (imiquimod and R848), loxoribine (7-allyl-7,8-dihydro-8-oxoguanosine), and bropirimine. Topical application of imiquimod has been approved for the treatment of genital warts caused by infection with human papillomavirus (5). Despite good evidence

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<sup>3</sup> Abbreviations used in this paper: ODN, oligodeoxynucleotide; PGN, peptidoglycan; PDC, plasmacytoid dendritic cell.

that TLR7 ligands activate murine B cells (25–27), little is known about their ability to activate human B cells.

TLR7 and TLR9 are the only TLR members expressed in another leukocyte subset, the plasmacytoid dendritic cell (PDC). PDCs represent the major type I IFN-producing cells in the immune system (28, 29). They produce large amounts of type I IFN (IFN- $\alpha$  and IFN- $\beta$ ) upon stimulation with different viruses (30–35). The TLR9 ligand CpG-A ODN 2216 was the first synthetic molecule capable of stimulating the production of large amounts of IFN- $\alpha$  in PDCs thereby mimicking the presence of virus (36). Similarly, the TLR7 ligands imiquimod and resiquimod were reported to be potent inducers of IFN- $\alpha$  in PDCs (37).

In the present study we made the surprising observation that highly purified human naive B cells did not respond to TLR7 ligands unless cocultured with PDCs.

## Materials and Methods

### Media and reagents

RPMI 1640 (Biochrom) supplemented with 10% (v/v) heat-inactivated FCS (Invitrogen Life Technologies), 3 mM L-glutamine, 0.01 M HEPES, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (all from Sigma-Aldrich) was used. CpG ODNs (Coley Pharmaceutical Group) show small letters, phosphorothioate linkage and capital letters, phosphodiester linkage 3' of the base: CpG-B ODN 2006 5'-tcgtcgtttttgctgtttgctgtt-3' (16); CpG-A ODN 2216 5'-ggGGGACGATCGTCgggggG-3' (36). R848, lipoteichoic acid from *Staphylococcus aureus*, peptidoglycan (PGN) from *Bacillus subtilis*, zymosan, and Pam<sub>3</sub>CSK<sub>4</sub> were purchased from InvivoGen. Loxoribine and LPS from *Escherichia coli* were obtained from Sigma-Aldrich.

### Isolation of cells and cell culture

PBMC were isolated from healthy donors by Ficoll gradient centrifugation. PDCs were isolated with the anti-blood DC Ag 4 isolation kit (Miltenyi Biotec). For PBMC experiments with PDC-depleted PBMC, the PBMC negative fraction was incubated with another 20  $\mu$ l of anti-blood DC Ag 4 MicroBeads per 150–200  $\times$  10<sup>6</sup> PBMC and the remaining PDCs were depleted from PBMC on an LD column (Miltenyi Biotec) (PDCs in PDC-depleted PBMC <0.005%).

B cells were isolated after PDC depletion. CD19<sup>+</sup> B cells were positively selected with anti-CD19 MicroBeads (Miltenyi Biotec) on an LS+ column. For naive and memory B cell isolation T cells were depleted with anti-CD3 MicroBeads (Miltenyi Biotec) on a CS column and memory B cells were positively selected from the negative fraction with anti-CD27 MicroBeads on an LS+ column. Naive B cells were isolated from the remaining CD27 negative fraction by positive selection with anti-CD19 MicroBeads on an LS+ column. B cell purity was 97–98% for CD19<sup>+</sup> total B cells and >97% for both naive and memory B cells. Naive B cells contained 10–15% CD27<sup>+</sup> B cells, and memory B cells contained <10% IgD<sup>+</sup> B cells. B cells were cultured in complete medium overnight before stimulation.

### B cell proliferation assay

B cells were incubated in 96-well U-bottom plates (BD Biosciences) at a concentration of 2.5  $\times$  10<sup>4</sup> cells/200  $\mu$ l. B cells were stimulated with the indicated stimuli in triplicates for 72 h. [<sup>3</sup>H]Thymidine (Amersham) was added for the last 18 h (1  $\mu$ Ci/well). For cytokine measurements cells were stimulated at a density of 5  $\times$  10<sup>4</sup> cells/200  $\mu$ l and supernatants were harvested after 72 h. In some experiments, B cell proliferation was determined by the CFSE dilution method as previously described (16).

The following stimuli were used for B cell stimulation: activated PDCs (overnight stimulation with 0.125  $\mu$ g/ml R848 or 2  $\mu$ g/ml (or 3  $\mu$ g/ml when indicated) CpG ODN 2216) were added at a ratio of 1:20. Loxoribine was used at 500  $\mu$ M. PDC supernatant was added at a dilution of 1/8; for the generation of PDC supernatants, freshly isolated PDCs (1  $\times$  10<sup>5</sup>/ml) were stimulated with CpG ODN 2216 (6  $\mu$ g/ml) for 48 h. Recombinant IFN- $\alpha$  (Strathmann Biotec) was used at 1000 U/ml, TNF- $\alpha$  (100 U/ml; R&D Systems), IFN- $\beta$  (100 U/ml; PeproTech), IFN- $\gamma$  (100 U/ml; Roche), and IL-6 (10 ng/ml; Strathmann Biotec). RNA transfection was performed with DOTAP (N-[1–2,3-dioleoyloxy]propyl]-N,N,N-trimethylammonium methylsulfate; Roche) at concentrations ranging from 10 to 30  $\mu$ g/ml, with poly-L-lysine (Sigma-Aldrich) at 1, 2, and 5  $\mu$ g/ml and with lipofectamine (Invitrogen Life Technologies) at 2.5  $\mu$ g/ml. The following RNA molecules were used: RNA40 (33) with and without a phosphorothioate backbone (synthesized from CureVac) and poly(U) and poly(G) ssRNA (Sigma-

Aldrich) at concentrations from 1 to 10  $\mu$ g/ml. The functional activity of TLR ligands and of the transfected RNA sequences was confirmed by stimulating PBMC and assessing TNF- $\alpha$  in the supernatants.

For the blocking experiments, the neutralizing anti-IFN- $\alpha$  receptor chain 2 (CD118) Ab (no. 21385-1; PBL Biomedical Laboratories) was used. B cells were preincubated with this Ab at 20  $\mu$ g/ml for 1.5 h at 37°C and 5% CO<sub>2</sub> before they were added to stimulated PDCs.

### Flow cytometry

Cells were washed and resuspended in 50  $\mu$ l of PBS/1% FCS/0.5 mM EDTA and incubated with directly conjugated Abs for 6 min at room temperature or 20 min at 4°C. The following Abs were used: anti-CD19 PE-Cy5.5 (Caltag Laboratories), anti-CD20 allophycocyanin, anti-CD27 PE, anti-IgD FITC, anti-CD123 PE, anti-CD11c allophycocyanin, anti-HLA-DR PerCP, and lineage FITC (all from BD Pharmingen). Analysis was performed on a FACSCalibur (BD Biosciences).

### RNA isolation and quantitative real-time RT-PCR

Cells were washed in 1 $\times$  TBS and cell pellets were lysed and frozen in 300  $\mu$ l of lysis buffer from the MagnaPure mRNA isolation kit I supplemented with 1% DTT (Roche Diagnostics). Preparation of mRNA was performed with the MagnaPure-LC device using the mRNA-I standard protocol. An aliquot of 8.2  $\mu$ l of RNA was reverse-transcribed using avian myeloblastosis virus-reverse transcriptase and oligo(dT) as primer (First Strand cDNA synthesis kit; Roche) according to the manufacturer's protocol in a thermocycler. The reaction mix was diluted to a final volume of 500  $\mu$ l and stored at –20°C until PCR analysis.

Parameter specific primer sets optimized for the LightCycler (Roche Applied Science) were developed and purchased from SEARCH-LC. The PCR was performed with the LightCycler FastStart DNA Sybr GreenI kit (Roche Applied Science) according to the protocol provided in the parameter specific kits. The calculated copy numbers were normalized according to the expression of cyclophilin B.

### Analysis of cytokines and Igs

The following ELISAs were used: IL-6, IL-12p40 (both from BD Biosciences), TNF- $\alpha$  (BioSource International), IgM and IgG (Bethyl Laboratories), IFN- $\alpha$  (Bender MedSystems), and IFN- $\gamma$  (BD Biosciences).

### Statistical analysis

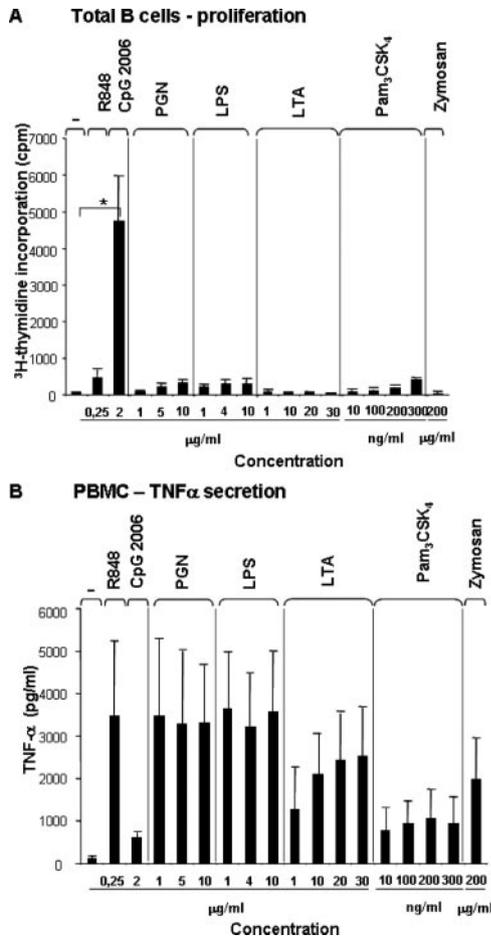
Data are depicted as mean  $\pm$  SEM. Statistical significance of differences was determined by the paired two-tailed Student's *t* test. Statistically significant differences are indicated for *p* < 0.05 and *p* < 0.005.

## Results

### Peripheral blood B cells show strong polyclonal proliferation in response to TLR9 but not to other TLR ligands

In previous studies we demonstrated that total peripheral blood B cells express considerable levels of mRNA for TLR1, TLR6, TLR9, and TLR10, intermediate levels for TLR7 and low levels for TLR2 and TLR4, whereas TLR3, TLR5, and TLR8 expression was below the detection limit (7). TLR9 ligation through CpG-B (ODN 2006) is known to directly activate purified human B cells (7, 18). Little is known about the functional activity of other TLRs in B cells. We therefore compared the responsiveness of human B cells upon ligation of different TLRs expressed in human B cells (TLR2, TLR4, TLR6, TLR7, TLR9).

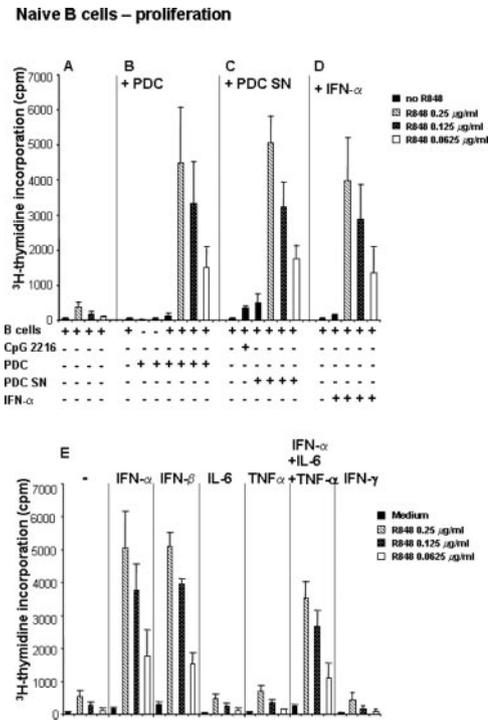
Peripheral blood B cells isolated from PBMC of healthy donors were incubated with different concentrations of PGN from *B. subtilis* (TLR2), LPS from *E. coli* (TLR4), lipoteichoic acid from *S. aureus* (TLR4), Pam<sub>3</sub>CSK<sub>4</sub> (TLR2), zymosan (TLR2 and TLR6), CpG ODN 2006 (TLR9), and resiquimod (R848, TLR7, and TLR8). In these experiments CpG ODN 2006 was the only stimulus capable of inducing a strong polyclonal B cell proliferation, whereas the effect of ligands for TLR2, TLR4, TLR6, TLR7, and TLR8 was weak or absent (Fig. 1A). Immunologic activity of all TLR ligands was confirmed by TNF- $\alpha$  induction in whole PBMC (Fig. 1B). In contrast to TLR2, TLR4, TLR6, and TLR7/8 ligands, the TLR9 ligand CpG ODN 2006 was weak at inducing TNF- $\alpha$  in PBMC (Fig. 1B) as previously described (38).



**FIGURE 1.** B cell proliferation is selectively induced by ligation of TLR9 but not of TLR2 and TLR4. CD19<sup>+</sup> human peripheral blood B cells isolated from PBMC ( $2.5 \times 10^4$  cells/well) or whole PBMC ( $8.3 \times 10^4$  cells/well) were incubated in the presence of the following TLR ligands at the indicated concentrations: peptidoglycan (PGN) from *B. subtilis* (TLR2), LPS from *E. coli* (TLR4), lipoteichoic acid from *S. aureus* (TLR4), Pam<sub>3</sub>CSK<sub>4</sub> (TLR2), zymosan (TLR2 and TLR6), CpG DNA ODN 2006 (TLR9), and R848 (TLR7 and TLR8). *A*, After 72 h, B cell proliferation was determined by [<sup>3</sup>H]thymidine uptake. The mean values  $\pm$  SEM of three individual donors are shown. \*,  $p = 0.040$ . *B*, After 24 h, TNF- $\alpha$  was measured in the supernatants of PBMC. The mean values  $\pm$  SEM of four individual donors are shown.

*TLR7 sensitivity of naive B cells is controlled by PDC type I IFN secretion*

Because R848-induced activation of murine B cells has been described in the literature (27), the weak activity of R848 on isolated human B cells in our study was surprising. A considerable proliferative response of human B cells upon stimulation with R848 was only observed in experiments with either a low B cell purity or a high fraction of memory B cells (>30%) (data not shown). Because PDCs are known to express TLR7 and to synthesize IFN- $\alpha$  upon stimulation with R848 (37) we hypothesized that stimulated PDCs within B cell preparations could have mediated B cell responsiveness to R848. To exclude a contribution of either memory B cells or PDCs to TLR7-triggered B cell proliferation we prepared naive B cells from PDC-depleted PBMC. Indeed, the proliferative response of these PDC-free naive B cells to R848 was very low (Fig. 2A), whereas the addition of small numbers of PDCs dramatically increased the proliferation rates of naive B cells upon R848 stimulation (Fig. 2B). A similar increase was found when naive B cells were incubated in the presence of con-



**FIGURE 2.** PDCs render naive B cells responsive to the TLR7/8 ligand resiquimod. Naive peripheral blood B cells (CD27<sup>+</sup>CD19<sup>+</sup>,  $2.5 \times 10^4$  cells/well) isolated from PBMC were stimulated with three different concentrations of the TLR7/8 ligand R848 as indicated. After 72 h, B cell proliferation was determined by [<sup>3</sup>H]thymidine uptake. *A*, B cells were incubated in the absence of PDCs ( $n = 6$ ). *B*, B cells were incubated in the presence of autologous PDCs (PDCs to B cells, 1:20) ( $n = 6$ ). R848 0.25  $\mu$ g/ml  $\pm$  PDCs.  $p = 0.005$ . *C*, B cells were incubated in the presence of PDC supernatant (SN; allogeneic PDCs,  $1 \times 10^5$  cells/well, stimulated with 6  $\mu$ g/ml CpG ODN 2216 for 48 h) ( $n = 4$ ). R848 0.25  $\mu$ g/ml  $\pm$  PDC supernatant.  $p = 0.003$ . *D*, B cells were incubated in the presence of recombinant IFN- $\alpha$  ( $n = 6$ ). R848 0.25  $\mu$ g/ml  $\pm$  IFN- $\alpha$ .  $p = 0.002$ . *E*, B cells were stimulated in the presence of IFN- $\alpha$ , IFN- $\beta$ , IL-6, TNF- $\alpha$ , a combination of IFN- $\alpha$  and IL-6 and TNF- $\alpha$ , or IFN- $\gamma$  as indicated ( $n = 3$ ). Mean values  $\pm$  SEM with individual donors are shown.

ditioned medium derived from CpG ODN-stimulated PDCs (Fig. 2C), indicating that soluble factors mediate increased TLR7 sensitivity. For these studies, the CpG-A ODN 2216 was used which is known to stimulate maximal IFN- $\alpha$  production in PDCs without showing direct activity on B cells (4). We speculated that IFN- $\alpha$  released by PDCs is responsible for the increased TLR7 sensitivity in naive B cells. Indeed, the addition of recombinant IFN- $\alpha$  was sufficient to mimic increased TLR7 sensitivity of B cells in the presence of PDCs (Fig. 2D). These results indicated that TLR7 responsiveness of naive B cells is controlled by PDC-derived IFN- $\alpha$ .

To determine whether other PDC-derived cytokines have similar activities we tested IFN- $\beta$ , TNF- $\alpha$ , IL-6 or a combination of these cytokines (Fig. 2E). Only IFN- $\beta$  but not TNF- $\alpha$  or IL-6 increased TLR7 sensitivity of naive B cells. Because IFN- $\gamma$  has been reported to increase TLR7 in eosinophils (39) we examined IFN- $\gamma$  in B cells. In contrast to IFN- $\alpha$  and IFN- $\beta$ , IFN- $\gamma$  did not up-regulate TLR7 sensitivity in naive B cells (Fig. 2E). These data suggested that PDCs control TLR7 sensitivity of naive B cells through type I IFNs (IFN- $\alpha$  and IFN- $\beta$ ) but not through the PDC-derived proinflammatory cytokines TNF- $\alpha$  and IL-6; furthermore, the data indicated that IFN- $\gamma$  lacks this activity.

To study whether activation of the type I IFN receptor via IFN- $\alpha$  and IFN- $\beta$  is the only mechanism by which PDCs enhance TLR7

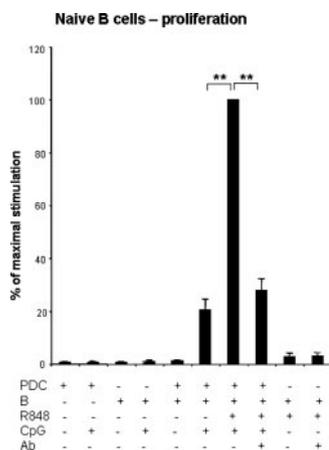
sensitivity in B cells, we blocked the type I IFN receptor with a neutralizing Ab. As shown in Fig. 3, in the presence of the neutralizing Ab (anti-IFN- $\alpha$  receptor chain 2 Ab) the increased activity of the TLR7/8 ligand R848 returned to the control level without R848, indicating that the activity of R848 on naive B cells requires ligation of the type I IFN receptor.

#### IFN- $\alpha$ induces the expression of TLR7 and MyD88 in naive B cells

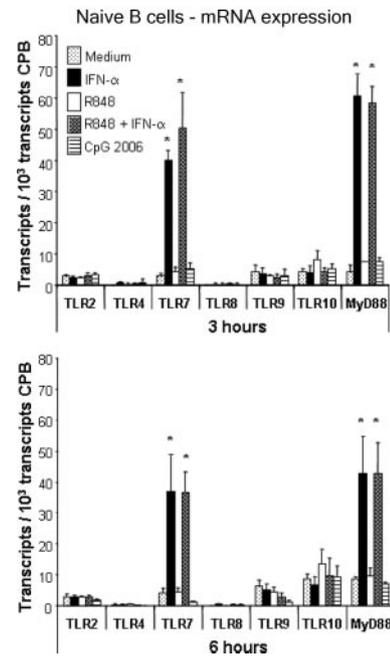
It has been shown that TLR7 signaling requires the adaptor molecule MyD88 (40). To study the impact of IFN- $\alpha$  and TLR ligation on the expression levels of TLR7 and MyD88, naive peripheral blood B cells were incubated in the presence of IFN- $\alpha$ , R848, a combination of IFN- $\alpha$  and R848, or CpG ODN 2006. The mRNA expression of TLR2, TLR4, TLR7, TLR8, TLR9, TLR10, and MyD88 was analyzed by quantitative real-time RT-PCR. Interestingly, IFN- $\alpha$  strongly up-regulated the expression of TLR7 and MyD88 within 3 h, whereas the expression levels of other TLRs (TLR2, TLR4, TLR8, TLR9, and TLR10) were not affected (Fig. 4). Up-regulation of TLR7 mRNA by IFN- $\alpha$  was comparable in the presence or absence of R848. In addition, R848 alone did not alter TLR7 expression levels (Fig. 4). These results suggest that IFN- $\alpha$  enhances TLR7 sensitivity of naive B cells by selectively up-regulating the expression of TLR7 and its key adaptor molecule MyD88.

#### IFN- $\alpha$ selectively increases sensitivity of naive B cells to TLR7 ligands

MyD88 is not only involved in TLR7 but also in TLR2, TLR4, and TLR9 signaling (41, 42). We examined the impact of IFN- $\alpha$  on B cells stimulated with CpG ODN 2006 (TLR9) (Fig. 5A), PGN (TLR2), Pam<sub>3</sub>CSK<sub>4</sub> (TLR2) or LPS (TLR4) (Fig. 5B). With IFN- $\alpha$  stimulation, the ability of CpG ODN 2006 to stimulate naive B cell proliferation was increased by 30% (Fig. 5A). On a much lower level, the activities of PGN, Pam<sub>3</sub>CSK<sub>4</sub>, and LPS to induce naive



**FIGURE 3.** PDCs mediate R848 sensitivity of naive B cells through type I IFN secretion. A total of 1250 PDCs/well were stimulated with CpG 2216 (3  $\mu$ g/ml) overnight. B cells ( $2.5 \times 10^4/100 \mu$ l) were incubated with or without the anti-IFN- $\alpha$  receptor Ab (Ab) 21385-1 (neutralizing) at 20  $\mu$ g/ml for 1.5 h, then added to the prestimulated PDCs and stimulated with R848 (0.25  $\mu$ g/ml). Proliferation was assessed by [<sup>3</sup>H]thymidine incorporation after a 72-h stimulation. Data shown were normalized to the maximal stimulus (stimulated PDCs + stimulated B cells) at 100% (100% corresponds to  $4488 \pm 1648$  cpm) and represent the mean of three independent experiments from independent donors with the SEM given. \*\*,  $p = 0.001$ , PDC (CpG 2216) + B cell  $\pm$  R848; \*\*,  $p = 0.002$ , PDC (CpG 2216) + B cell + R848  $\pm$  Ab no. 21385-1.



**FIGURE 4.** IFN- $\alpha$  selectively induces TLR7 and MyD88 mRNA in naive B cells. Naive peripheral blood B cells were stimulated with R848 (0.25  $\mu$ g/ml) alone, with IFN- $\alpha$  alone, with R848 in combination with IFN- $\alpha$  or with CpG ODN 2006 (3  $\mu$ g/ml). After 3 h (top panel) or 6 h (bottom panel) mRNA was prepared and TLR2, TLR4, TLR7, TLR8, TLR9, and MyD88 mRNA expression was analyzed using quantitative real-time RT-PCR. Results are shown as mean copy numbers per  $10^3$  copies of the housekeeping gene cyclophilin B (CPB)  $\pm$  SEM. \*,  $p < 0.05$  compared with the controls without IFN- $\alpha$ .

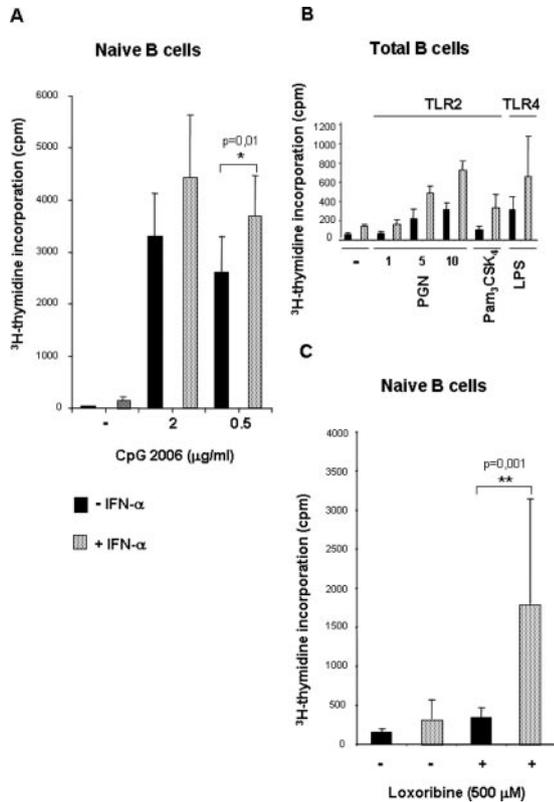
B cell proliferation were also increased (Fig. 5B, note different scale). However, these IFN- $\alpha$ -mediated changes for TLR2, TLR4, and TLR9 ligands were low compared with a more than 10-fold increase in R848-induced B cell proliferation in the presence of IFN- $\alpha$  (see Fig. 2).

R848 is a ligand for both TLR7 and TLR8. According to our data naive B cells lack TLR8 both in the presence and absence of IFN- $\alpha$  (see Fig. 4). Therefore, the activity of R848 on B cells is likely mediated by TLR7. To confirm the contribution of TLR7, we used the guanosine analog loxoribine, a selective TLR7 agonist (43). Similar to R848, the ability of loxoribine to induce naive B cell proliferation was strongly increased in the presence of IFN- $\alpha$  (Fig. 5C).

Because ssRNA has been proposed as natural ligand for TLR7 in mice and for TLR8 in humans (32, 33), we tested poly(U) ssRNA and the ssRNA ODN RNA40 (33) in our experimental setting. In contrast to the synthetic TLR7 agonists, IFN- $\alpha$ -stimulated naive B cells did not respond to RNA40 or poly(U) ssRNA with and without transfection with lipofectamine, DOTAP, or poly-L-lysine (data not shown).

#### TLR7 ligand sensitivity of B cells depends on the presence of PDCs within PBMC

The studies discussed indicate that PDCs have the potential to increase the TLR7 sensitivity of naive B cells. To investigate whether other leukocyte subsets within PBMC could directly respond to TLR7 ligation and thus contribute to increased B cell TLR7 sensitivity we examined TLR7-induced B cell proliferation in PBMC before and after depletion of PDC. To this end PBMC were labeled with CFSE, and B cell proliferation was quantified by CFSE dilution. Both the selective TLR7 ligand loxoribine and the

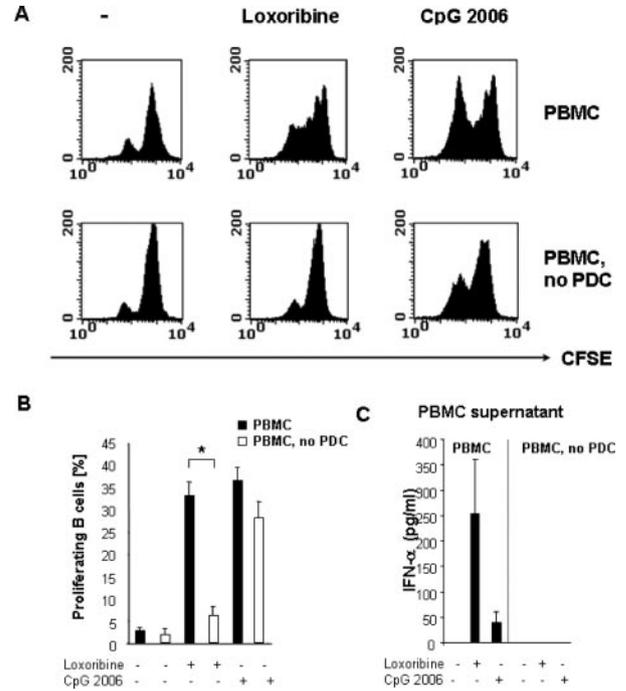


**FIGURE 5.** IFN- $\alpha$  exclusively enhances TLR7 sensitivity of naive B cells. Naive peripheral blood B cells ( $25 \times 10^3$  cells/well) were stimulated with different TLR ligands in the presence or absence of IFN- $\alpha$  as indicated. After 72 h B cell proliferation was determined by [ $^3$ H]thymidine incorporation. *A*, Naive B cells were stimulated with the TLR9 ligand CpG ODN 2006 (0.5 or 2  $\mu$ g/ml) and IFN- $\alpha$  ( $n = 4$ ). *B*, Total CD19 $^+$  B cells were stimulated with peptidoglycan (PGN) from *B. subtilis* (1, 5, or 10  $\mu$ g/ml), Pam<sub>3</sub>CSK<sub>4</sub> (100 ng/ml) and LPS from *E. coli* (10  $\mu$ g/ml) and IFN- $\alpha$  ( $n = 3$ ). *C*, Naive B cells were stimulated with the TLR7 ligand loxoribine (500  $\mu$ M) and IFN- $\alpha$  ( $n = 7$ ). Data are shown as mean values  $\pm$  SEM with B cells isolated from independent donors.

TLR9 ligand CpG ODN 2006 induced a vigorous B cell proliferation within PBMC (Fig. 6, *A* and *B*). However, when PDCs were depleted from PBMC, loxoribine, in contrast to CpG ODN 2006, failed to induce B cell proliferation (Fig. 6, *A* and *B*). Consistent with complete depletion of PDCs (PDCs < 0.005% of total PBMC) the induction of IFN- $\alpha$  by loxoribine or CpG ODN 2006 was completely abolished (Fig. 6*C*). These data demonstrate an exclusive role for PDCs in the control of TLR7 sensitivity of B cells.

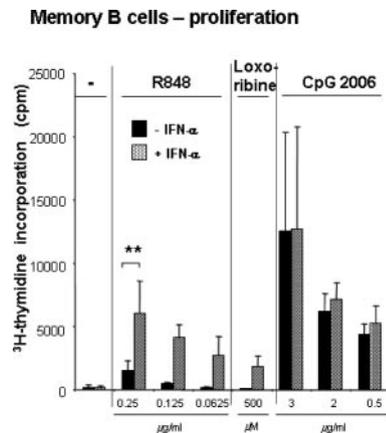
*TLR7-induced proliferation of memory B cells is further increased in the presence of IFN- $\alpha$*

In preliminary studies we observed a considerable R848-induced proliferative response of total human B cells in samples with a high proportion of memory B cells (>30% of B cells) (data not shown). To address the question whether memory B cells, in contrast to naive B cells, are directly sensitive toward stimulation via TLR7, memory B cells (CD3 $^-$ CD27 $^+$  cells) were isolated from PBMC and incubated with IFN- $\alpha$ , R848, loxoribine or with a combination of IFN- $\alpha$  and R848 or loxoribine. CpG 2006 was used as a positive control. We found that R848 stimulated the proliferation of memory B cells in the absence of IFN- $\alpha$  to some extent, whereas CpG 2006 stimulated a vigorous proliferative response (Fig. 7). The addition of IFN- $\alpha$  alone had no effect on the proliferative



**FIGURE 6.** Depletion of PDCs strongly decreases TLR7-induced B cell proliferation in PBMC. PBMC with and without depletion of PDCs were stained with 1  $\mu$ M CFSE and were stimulated for 5 days with the TLR7 ligand loxoribine (500  $\mu$ M) or CpG ODN 2006 (3  $\mu$ g/ml). *A*, Proliferation of gated CD19 $^+$  B cells without stimulation (*left*), after stimulation with loxoribine (*middle*) or with CpG ODN 2006 (*right*) in the presence or absence of PDCs. *B*, Mean percentages  $\pm$  SEM of proliferating B cells from three independent experiments are depicted. PBMC (■) and PBMC depleted of PDCs (□) are presented. \*,  $p = 0.03$ . *C*, Mean values  $\pm$  SEM of IFN- $\alpha$  in the supernatants of PBMC of three independent experiments on day 4. PBMC (*left half*) and PBMC depleted of PDCs (*right half*) are shown. \*,  $p < 0.05$ .

activity of memory B cells but further increased R848-induced memory B cell proliferation up to a similar level as that induced by the TLR9 ligand CpG ODN 2006 (with and without



**FIGURE 7.** IFN- $\alpha$  increases TLR7 sensitivity of memory B cells. Memory B cells (CD3 $^-$ CD27 $^+$ ,  $2.5 \times 10^4$ /well) were isolated from PBMC and stimulated with R848 in three different concentrations as indicated, 500  $\mu$ M loxoribine and three different concentrations of CpG 2006 as indicated in the presence or absence of recombinant IFN- $\alpha$ . The data show the mean values  $\pm$  SEM. Number of experiments from left to right (-IFN- $\alpha$ /+IFN- $\alpha$ ): 15/15, 12/12, 3/3, 3/3, 3/3, 3/3, 3/3, 3/3, 3/3. \*\*,  $p = 0.0008$ , R848 0.25  $\mu$ g/ml  $\pm$  IFN- $\alpha$ ; \*,  $p = 0.04$ , R848 0.125  $\mu$ g/ml  $\pm$  IFN- $\alpha$ .

IFN- $\alpha$ ). Loxoribine stimulated memory B cells only in the presence of IFN- $\alpha$  (Fig. 7). These results indicate that memory B cells are more susceptible to TLR7-mediated stimulation than naive B cells, and that this baseline TLR7 sensitivity of memory B cells is strongly increased in the presence of IFN- $\alpha$ . In contrast to TLR7, TLR9 sensitivity of memory B cells in the absence of IFN- $\alpha$  is already high, and shows almost no further increase in the presence of IFN- $\alpha$ .

#### IFN- $\alpha$ synergistically enhances TLR7-induced IL-6 and Ig-production in naive and memory B cells

It has been demonstrated that endogenous IL-6 in B cells contributes to B cell differentiation (44). Therefore, we investigated whether IFN- $\alpha$  could not only increase TLR7-induced polyclonal proliferation of naive and memory B cells but also enhance the production of IL-6. Naive and memory B cells isolated from PBMC were stimulated with R848 in the presence or absence of IFN- $\alpha$  with CpG ODN 2006 serving as a positive control (18). Memory B cells showed higher spontaneous and higher R848-induced IL-6 production than naive B cells (Fig. 8A). In the presence of IFN- $\alpha$ , naive and memory B cells produced similar levels of IL-6 in response to R848 that were in the same range as CpG ODN 2006-induced IL-6 production in the absence of IFN- $\alpha$  (Fig.

8A). TNF- $\alpha$  and IL-12p40 could not be detected (data not shown). Increased levels of IL-6 suggested that IFN- $\alpha$  not only enhanced polyclonal B cell proliferation but also B cell differentiation. An indicator of B cell differentiation is the production of Igs: IFN- $\alpha$  synergistically increased IgM (Fig. 8B, left) and IgG (Fig. 8B, right) production in R848-stimulated B cells derived from naive and memory B cell preparations (note different scales). IgG production in B cells derived from naive B cells was very low compared with B cells derived from memory B cells. Together these data indicated that IFN- $\alpha$  not only controls the activity of TLR7 ligands to induce polyclonal B cell proliferation but also to induce B cell differentiation toward Ig-producing plasma cells.

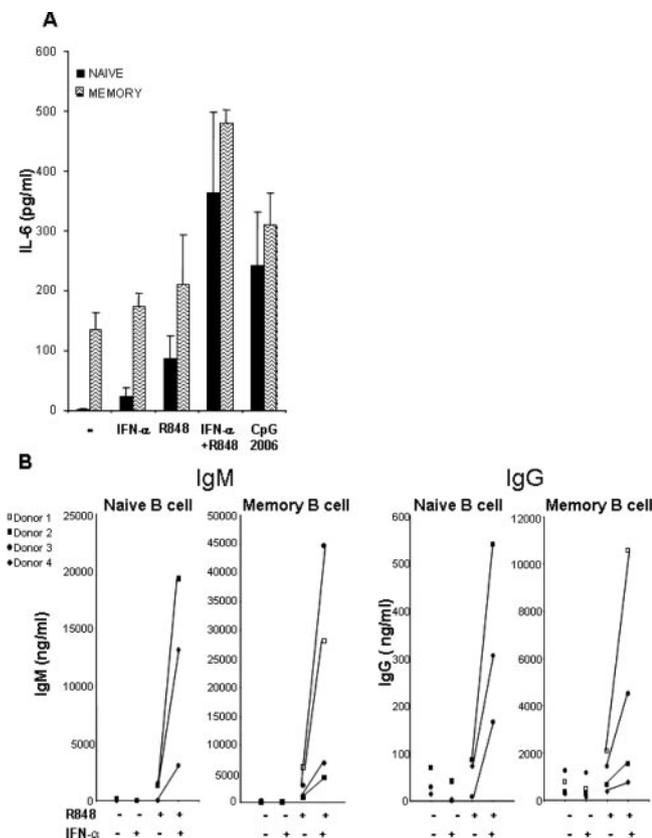
## Discussion

In this study we demonstrate that among all TLRs expressed in freshly isolated human peripheral blood B cells and for which specific ligands are available, only ligation of TLR9 induced strong polyclonal B cell proliferation. However, in cocultures of B cells and PDCs, TLR7 ligands but none of the other TLR ligands gained similar potency to stimulate B cells than the TLR9 ligand CpG-B. IFN- $\alpha$  released by PDCs upon stimulation with TLR7 or TLR9 ligands was found to be responsible for increased TLR7 sensitivity of B cells; IFN- $\alpha$  selectively up-regulated the mRNA expression of TLR7 thereby licensing B cells to respond to TLR7 ligands by polyclonal expansion and differentiation into Ig-producing plasma cells.

Although memory B cells showed a higher baseline TLR7 sensitivity than naive B cells, the TLR7 sensitivity of both naive and memory B cells was strongly increased in the presence of PDCs or type I IFN. In contrast, both naive and memory B cells showed a vigorous response to TLR9 ligand without the requirement of PDCs or type I IFN. Our results demonstrate a fundamental difference between TLR7 and TLR9 in B cells: the dependence of TLR7 and the independence of TLR9 function from the presence of type I IFN.

Although the activity of TLR9 in B cells was largely independent of type I IFN, some increase was seen in the presence of type I IFN. We found that not TLR9 but the key adaptor molecule MyD88 involved in signaling of many TLRs including TLR9 and TLR7 was strongly up-regulated in B cells by type I IFN. High TLR9 sensitivity of B cells in the absence of type I IFN suggests that the expression level of the adaptor molecule MyD88 is not limiting for B cell activation. However, the presence of other not yet defined adaptor molecules (other than MyD88) might be limiting for TLR7 signaling but not TLR9 signaling because both TLR7 and TLR9 showed similar baseline levels of mRNA expression in naive B cells despite different levels of functional activity. Alternatively, the distinct baseline sensitivity of TLR7 and TLR9 could be due to quantitative differences at the protein level or to different cellular localizations of the TLRs. This yet undefined deficiency that limits TLR7 sensitivity in naive B cells seems to be less limiting in memory B cells. As a consequence, in the absence of PDCs the activity of TLR7 ligands will preferentially support a memory B cell response, a situation that is much safer with regard to autoimmunity than initiating a B cell response against new Ags.

Our study identifies the unique ability of type I IFN to selectively up-regulate TLR7 mRNA in B cells; none of the other TLRs examined was induced by type I IFN. Our results demonstrate that depletion of PDC from mixed cell cultures (PBMC) leads to a complete loss of TLR7 ligand-induced B cell proliferation; furthermore, none of the other cytokines tested (TNF- $\alpha$ , IL-6, and IFN- $\gamma$ ) up-regulated TLR7 sensitivity. These data reveal a key role for PDC-derived type I IFN in controlling TLR7 sensitivity in B cells. It will be interesting to study whether the TLR7 sensitivity of



**FIGURE 8.** IFN- $\alpha$  and R848 synergistically induce proliferation and IL-6 and Ig secretion in naive and memory B cells. Naive and memory B cells ( $5 \times 10^4$  cells/well) isolated from PBMC were incubated in the presence of IFN- $\alpha$ , R848 (0.25  $\mu$ g/ml) or a combination of IFN- $\alpha$  and R848 as indicated. A, After 72 h, IL-6 concentrations were measured in the supernatants. Stimulation with CpG ODN 2006 (3  $\mu$ g/ml) was used as a positive control. Mean values  $\pm$  SEM of three experiments are shown. B, IgM (left) and IgG (right) secretion was quantified on day 13 of cell culture. The results of three (naive B cells) and four (memory B cells) independent experiments with B cells isolated from individual donors (donors 1–4 as indicated) are shown.

other immune cell subsets such as myeloid cells also depends on PDC-derived type I IFN. Of note, type I IFNs were shown to induce TLR7 in macrophages (45), and only myeloid dendritic cells generated in the presence but not in the absence of IFN- $\alpha$  were reported to express TLR7 (46).

In the literature there is little information on the activity of TLR7 and TLR8 ligands in human B cells. It has been mentioned (data not shown) that human B cells isolated from PBMC based on CD19 expression (90% purity) proliferated in response to the TLR7/8 ligand resiquimod (27). A different group found no activation of CD19<sup>+</sup> human peripheral blood B cells in response to imiquimod or resiquimod (47). Another study found that resiquimod induced NF- $\kappa$ B activation in the human B cell line Ramos, but the response was weaker and delayed (60 min) as compared with the mouse B cell lines (5 min) tested (25). The same group found that Ramos cells did not up-regulate the activation marker CD80 upon stimulation with resiquimod unless costimulated via CD40 (26). These findings are consistent with our results. Similar to the memory B cells in our study, the B cell line Ramos may show an elevated baseline TLR7 sensitivity when compared with naive B cells. This elevated baseline TLR7 sensitivity of memory B cells may have contributed to the increased B cell proliferation of total CD19<sup>+</sup> B cells found by Tomai et al. (27). Naive B cells and the role of PDCs and type I IFN have not been examined in their studies. In our hands, depletion of PDCs before isolation of B cells was necessary to avoid the TLR7 priming effect of even small numbers of PDCs in the B cell preparations.

Most information on TLR7/8-mediated B cell activation was obtained from studies in mice (27). However, murine TLR expression patterns differ from those in humans. Although it is well established that murine B cells respond to the TLR4 ligand LPS (48, 49), in the present study and in our earlier studies (7, 18) TLR4 expression in human B cells was found to be weak, and human B cells were not responsive to LPS with and without type I IFN priming. It is interesting to note that neither TLR2 nor TLR4 or TLR6 ligands were able to induce considerable polyclonal proliferation of human B cells, although all of them were strong inducers of TNF- $\alpha$  production in PBMC most likely due to monocyte activation. Besides low expression of TLR2 and TLR4, the absence of transmembrane adaptor molecules such as CD14 might contribute to the lack of activity of these TLR ligands in B cells. Therefore, the limitations of TLR2 and TLR4 agonists when used as humoral vaccine adjuvants in humans may be due to their inability to directly activate human B cells and due to monocyte-mediated toxicity.

Based on TLR-transfected cell lines it has been demonstrated that the imidazoquinoline resiquimod (R848) represents a ligand for both human TLR7 and TLR8 (50). In this study and in our earlier studies (7) TLR8 mRNA was absent in human B cells and was not up-regulated by type I IFN priming. The TLR7-specific ligand loxoribine (43) showed similar results than the TLR7/8 ligand R848 confirming that TLR7 is not only expressed in human B cells but that it is functionally active.

The lack of TLR8 expression in B cells and PDCs together with the potent TLR8-mediated induction of TNF- $\alpha$  and other proinflammatory cytokines in primary human monocytes limits the clinical utility of TLR8 ligands as humoral vaccine adjuvants. Based on these considerations we predict that selective TLR7 ligands may very likely represent more promising humoral vaccine adjuvants than TLR8 ligands.

In an earlier study we found that stimulated PDC can substitute for CD4 T cell help (4) with regard to the induction of B cell differentiation. In this study we demonstrate that simultaneous ac-

tivation of cocultured PDCs and B cells cannot only be achieved with TLR9 (51) but also with TLR7 ligands. Stimulation with IFN- $\alpha$  was sufficient for R848 to induce both polyclonal B cell proliferation and the production of large quantities of Igs. Of note, neither PDC and B cell cell-to-cell contact, nor CD4 T cell-derived costimulation via CD40L or T cell-derived cytokines, nor B cell Ag was required for R848-induced Ig-production in B cells. As a consequence, in the presence of IFN- $\alpha$  R848-induced Ig-production is unleashed from the controls normally provided by CD4 T cells (restricted by pathogen-derived Ag presentation on MHC class II) and by the B cell surface Ig. It is possible that without these critical checkpoints for B cell activity the risk for autoreactivity may be increased. In fact, autoreactivity is a well-known side effect of IFN- $\alpha$  therapy. Based on our results autoimmune disorders may be strongly aggravated by natural or exogenously administered TLR7 ligands.

In our study, R848 is a strong inducer of IgM production in both naive and memory B cells, whereas the induction of IgG was largely restricted to memory B cells. This indicates that R848, even in the presence of IFN- $\alpha$ , is unable to support differentiation of naive B cells to isotype-switched IgG producing plasma cells. This may limit the negative consequences of TLR7-induced autoreactivity. It will be important to define additional stimuli required for TLR7-induced isotype switching.

In conclusion, our studies emphasize the unique ability of the TLR9 ligand CpG ODN to directly activate purified human B cells in vitro. Although the presence of PDC is required for TLR7 sensitivity of B cells, in vivo TLR7 and TLR9 ligands may still be equally potent. PDC were found at multiple compartments relevant for immune responses including blood, lymphoid tissues, mucosa (52), inflamed skin (53, 54), and even in solid tumor tissue (55–57). For the development of novel humoral vaccine adjuvants TLR7 and TLR9 ligands so far represent the only potent TLR stimuli for human B cells, whereas TLR2, TLR4, and TLR6 ligands will not be useful. An important advantage of selective TLR7 ligands over TLR9 ligands as vaccine adjuvants may be their potential of directly targeting TLR7 expressing myeloid dendritic cells thereby facilitating the effective priming of T cell responses.

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## Disclosures

The authors have no financial conflict of interest.

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# CORRECTIONS

Niels Schaft, Jan Dörrie, Peter Thumann, Verena E. Beck, Ina Müller, Erwin S. Schultz, Eckhart Kämpgen, Detlef Dieckmann, and Gerold Schuler. Generation of an optimized polyvalent monocyte-derived dendritic cell vaccine by transfecting defined RNAs after rather than before maturation. *The Journal of Immunology*, 2005, 174: 3087–3097.

An error was made in the grant information. The correct footnote is shown below.

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Michelle A. Hurchla, John R. Sedy, Maya Gavrieli, Charles G. Drake, Theresa L. Murphy, and Kenneth M. Murphy. B and T lymphocyte attenuator exhibits structural and expression polymorphisms and is highly induced in anergic CD4<sup>+</sup> T cells. *The Journal of Immunology*, 2005, 174: 3377–3385.

The third author's last name is misspelled. The correct name is Maya Gavrieli.

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Isabelle Béatrice Berkeredjian-Ding, Moritz Wagner, Veit Hornung, Thomas Giese, Max Schnurr, Stefan Endres, and Gunther Hartmann. Plasmacytoid dendritic cells control TLR7 sensitivity of naive B cells via type I IFN. *The Journal of Immunology*, 2005, 174: 4043–4050.

The first author's last name is misspelled. The correct name is Isabelle Béatrice Bekeredjian-Ding. The error has been corrected in the online version, which now differs from the print version as originally published.

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Gerard L. Bannenberg, Nan Chiang, Amiram Ariel, Makoto Arita, Eric Tjonahen, Katherine H. Gotlinger, Song Hong, and Charles N. Serhan. Molecular circuits of resolution: formation and actions of resolvins and protectins. *The Journal of Immunology*, 2005, 174: 4345–4355.

In *References*, an author's name was omitted from Reference 17. The correct citation is shown below.

17. Marcheselli, V. L., S. Hong, W. J. Lukiw, X. H. Tian, K. Gronert, A. Musto, M. Hardy, J. M. Gimenez, N. Chiang, C. N. Serhan, and N. G. Bazan. 2003. Novel docosanoids inhibit brain ischemia-reperfusion-mediated leukocyte infiltration and pro-inflammatory gene expression. *J. Biol. Chem.* 278:43807.

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