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IL-12p70-Dependent Th1 Induction by Human B Cells Requires Combined Activation with CD40 Ligand and CpG DNA

Moritz Wagner,* Hendrik Poeck,2* Bernd Jahrsdoerfer,2† Simon Rothenfusser,* Domenik Prell,* Barbara Bohle,§ Evelyn Tuma,* Thomas Giese,§ Joachim W. Ellwart,¶ Stefan Endres,* and Gunther Hartmann3*

The detection of microbial molecules via Toll-like receptors (TLR) in B cells is not well characterized. In this study, we found that both naive and memory B cells lack TLR4 (receptor for LPS) but express TLR9 (receptor for CpG motifs) and produce IL-6, TNF-α, and IL-10 upon stimulation with CpG oligonucleotides (ODN), synthetic mimics of microbial DNA. Consistent with the lack of TLR4, purified B cells failed to respond to LPS. Similar to CpG ODN, CD40 ligand (CD40L) alone induced IL-6, TNF-α, and IL-10. Production of these cytokines as well as IgM synthesis was synergistically increased when both CpG ODN and CD40L were combined. Unlike IL-6, TNF-α, and IL-10, the Th1 cytokine IL-12p70 was detected only when both CpG ODN and CD40L were present, and its induction was independent of B cell receptor cross-linking. CpG ODN did not increase the capacity of CD40L-activated B cells to induce proliferation of naive T cells. However, B cells activated with CpG ODN and CD40L strongly enhanced IFN-γ production in developing CD4 T cells via IL-12. Together, these results demonstrate that IL-12p70 production in human B cells is under the dual control of microbial stimulation and T cell help. Our findings provide a molecular basis for the potent adjuvant activity of CpG ODN to support humoral immune responses observed in vivo, and for the limited value of LPS. The Journal of Immunology, 2004, 172: 954–963.

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The PDC is characterized by the production of extremely large amounts of type I IFN upon viral infection (7, 8). CpG ODN represent a unique microbial molecule recognized by PDC leading to activation and maturation of PDC (9–13). A certain type of CpG ODN was identified (CpG-A; prototype ODN 2216 (14)), which induces the production of large amounts of type I IFN. Despite its potent IFN-α induction in PDC, CpG-A is not recognized by human B cells (15).

In earlier studies, CpG ODN were developed based on activation of human B cells (CpG-B; prototype ODN 2006 (16)). This type of CpG ODN proved to be an excellent vaccine adjuvant in primates (17–20). In mice, a number of studies have shown that CpG ODN induces strong Th1 immune responses. This is based on the induction of IL-12 in murine B cells, myeloid dendritic cells, and macrophages, which all are sensitive to CpG ODN (5, 21). In contrast to mice, human myeloid dendritic cells lack TLR9 (11, 12, 22), and little information is available on the ability of CpG ODN to support Th1 responses.

Previously, we have shown that PDC stimulated with a combination of CpG ODN and CD40 ligand (CD40L) produce both IL-12p40 and IL-12p70 and induce IL-12-dependent Th1 differentiation of unprimed allogenic CD4 T cells (12). PDC are capable of presenting viral Ags following viral infection (23), but their ability to take up, process, and present soluble exogenous protein Ag remains elusive. Due to their low frequency (0.2–0.4% in PBMC), the amount of PDC-derived IL-12 in PBMC is low. In contrast, human B cells represent a large population in peripheral blood and in lymphoid tissues. CD40-dependent activation of B cells is well established (24). Recent data demonstrate that B cells upon CD40L-mediated stimulation can function as professional APCs in vitro (25, 26). To date, no information is available on CpG ODN-induced IL-12 production in purified human B cells.

In the mixed cell population of human PBMC stimulated with CpG ODN, both IL-12p35 and IL-12p40 mRNA were detectable by RT-PCR (27). Another group found that CpG ODN stimulated...
the production of marginal amounts of IL-12 in PBMC (28), and that CpG ODN-induced IFN-γ production in PBMC was both type I IFN and IL-12 dependent (29). In these studies, the cellular source of IL-12 and the contribution of costimulation with CD40L provided by other cell types within PBMC were not addressed.

In this study, we used purified human B cells including the naive and memory B cell subsets to analyze the regulation of B cell activity by CpG ODN. Based on the pattern of TLR expression we found that CpG ODN represent a unique microbial stimulus for B cells that licenses B cells to produce bioactive IL-12 upon encounter of preactivated Th cells providing CD40L.

Materials and Methods

Media and reagents

RPMI 1640 (PAA Laboratories, Linz, Austria) supplemented with 10% (v/v) heat-inactivated (56°C, 30 min) FCS (HyClone, Logan, UT), 1.5 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich, Munich, Germany) was used. All compounds were purchased endotoxin tested. ODN were provided by Coley Pharmaceutical Group (Wellesley, MA). The following ODN were used (phosphorothiate linkage; bold; CpG dinucleotides): CpG ODN, ODN 6006, 5′-TGTCGTGTTTGTTGTTGTTGTT-3′; ODN 2006, 5′-CCTCCCTCCCCCCCCCCCC-3′). No endotoxin was detected in ODN preparations using the Limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD); lower detection limit, 0.3 endotoxin U/ml. CpG ODN were added at a final concentration of 3 μg/ml rIFN-γ (Roche, Basel, Switzerland) and IL-4 (Promega, Madison, WI) were added at a final concentration of 1000 U/ml and 500 U/ml, respectively. IL-2 (Roche) and IL-7 (Strathmann Biotec, Hannover, Germany) were added at 100 U/ml and 10 ng/ml, respectively. F(ab′)2 of rabbit anti-human IgM Ab from Jackson ImmunoResearch Laboratories (West Grove, PA) were used at a final concentration of 25 μg/ml. Anti-human IL-12 Ab (purified mouse IgG 1; clone C8; 5 μg/ml; BD PharMingen, San Diego, CA) was added as indicated. LPS (Salmonella typhimurium; 1 μg/ml) and Escherichia coli DNA were from Sigma-Aldrich. E. coli DNA (Sigma-Aldrich) was purified from endotoxin by repeated extractions with Triton X-114 (Sigma-Aldrich) and tested for endotoxin content using the Limulus amebocyte lysate assay (BioWhittaker; lower detection limit, 0.1 endotoxin U/ml) as described earlier (30).

Isolation of cells

PBMC were obtained from buffy coats of healthy blood donors by Ficoll-Hypaque density gradient centrifugation (Biochrom, Berlin, Germany) as described (31). B cells were isolated by MACS using the CD19 B Cell Isolation kit from Miltenyi Biotec (Bergisch-Gladbach, Germany). The purity of isolated B cells was >95% as assessed by flow-cytometric analysis with no contaminating PDC detectable (<0.1%). Viability (>95%) was determined by trypan blue exclusion. For some experiments, CD19- B cells were further separated by cell sorting using a MoFlo high-speed FACSAria cytometer (BD, Fort Collins, CO). Cells were stained with directly labeled Abs against CD27 and IgD (BD PharMingen) to obtain naive (CD27+, IgD-) and memory (CD27+, IgD+) B cell subpopulations. Unconjugated Abs against CD27 and IgD (BD PharMingen) were used to assess the purity of isolated B cells. Naïve CD45RA+ T cells were prepared from PBMC using the Pan T Cell Isolation kit from CD45RO-labeled microbeads (all from Miltenyi Biotec). In a first step, non-T cells were depleted from PBMC using biotin-conjugated Abs to CD4, CD16, CD19, CD36, CD56, CD123, and glycoporphin A and anti-biotin-labeled magnetic beads. In a second step, CD45RA+ CD3+ T cells (>94% purity) were isolated from CD3+ T cells by depletion of CD45RO+ T cells. Unconjugated naive CD4+ T cells were prepared using the CD4+ T Cell Isolation kit and CD45RO microbeads from Miltenyi Biotec. In a first step, non-T cells were depleted from PBMC of adult healthy blood donors using hapten-coupled Abs to CD8, CD11b, CD16, CD19, CD36, and CD56, and anti-hapten magnetic beads. In a second step, CD45RA+ CD4+ T cells (>94% purity) were isolated from CD4+ T cells by depletion of CD45RO+ T cells.

Real time RT-PCR

The sorted B cell subpopulations were lysed and RNA was extracted using a total RNA isolation kit (High Pure; Roche). An aliquot of 8 μl of RNA was reverse transcribed using avian myeloblastosis virus-reverse transcriptase and oligo(dT) as primer (First Strand cDNA Synthesis Kit; Roche). The obtained cDNA was diluted 1/25 with water, and 10 μl was used for amplification. Parameter-specific primer sets optimized for the LightCycler (Roche) were developed by and purchased from Search-TC (Heidelberg, Germany). The PCR was performed with the LightCycler FastStart DNA SYBR Green I kit (Roche) as previously described (6). The copy number of the different transcripts was normalized with respect to the housekeeping gene β-actin and is presented as number of transcripts per 106 copies of β-actin.

Culture of B cells

Highly purified B cells were cultured in 96-well round-bottom plates (2 × 105 cells in 200 μl of medium per well). B cells were incubated as indicated with CD40L-transfected cells (transgenic BHK cells; hamster kidney cell line; mycoplasma negative; irradiated with 30 Gy; CD40L-B cell ratio, 1:10) or control transfectant BHK cells as indicated (transfected BHK cells were kindly provided by H. Engelmann (Institute of Immunology, University of Munich)). Throughout Results, incubation with CD40L-transfected cells is phrased as incubation with CD40L. At the indicated time points, supernatant was collected for cytokine detection by ELISA, and cells were harvested for flow-cytometric analysis. For IgM secretion, highly purified B cells were cultured at 1 × 106 cells/well in 24-well plates. At day 13 supernatant, was collected for IgM detection by ELISA (see Detection of cytokines and IgM by ELISA).

Flow cytometry

At the indicated time points, cells were harvested and surface Ag staining was performed as previously described (32). Fluorescein-labeled mAb against CD3, CD11c, CD19, CD20, CD27, CD86, CD123, IgD, and class II MHC were purchased from BD Biosciences (Heidelberg, Germany). Flow-cytometric data were acquired on a BD Biosciences FACSCalibur equipped with two lasers (excitation at 488- and 635-nm wavelength). Spectral overlap was corrected by appropriate compensation. Analysis was performed on viable cells. Data were analyzed using CellQuest software (BD Biosciences).

Detection of cytokines and IgM by ELISA

For the detection of cytokines, the human IL-6 OptEIA ELISA (detection range, 3–300 pg/ml), the human IL-10 OptEIA ELISA (detection range, 8–500 pg/ml), the human TNF-α OptEIA ELISA (detection range, 7.8–500 pg/ml), the human IL-12p40/p70 ELISA (detection range, 6.3–2000 pg/ml), and the human IL-12p70 OptEIA ELISA (detection range, 8–500 pg/ml) (all from BD PharMingen) were used. The IgM ELISA was performed as described previously (27).

T cell proliferation assay

Purified B cells were incubated with CpG ODN, CD40L, and IL-4 (200 U/ml) in 24-well flat-bottom plates (4 × 105 cells per well). At 3 days, cells were harvested, washed, irradiated (1500 rad) and counted. The MLR was performed as described previously (33). Briefly, 1 × 104 allogenic T cells were cocultured with the different B cell preparations in 96-well round-bottom tissue culture plates for 5–7 days at the indicated T cell to B cell ratios in triplicate. T cell proliferation was monitored by [3H]thymidine incorporation for the last 16 h. Results were corrected for [3H]thymidine incorporation of irradiated CD40L-activated B cells and of T cells. Alternatively, the MLR was performed using the CFSE (Molecular Probes, Eugene, OR) assay as described (16). Allogenic T cells were stained with CFSE and then cocultured with different B cell preparations in 96-well round-bottom tissue culture plates at the indicated T cell to B cell ratios in triplicate. After 5 days, the percentage of proliferating CD3+ T cells (CFSE-low) was measured by flow cytometry.

IFN-γ production by CD45RA+ CD4+ T cells

Human purified CD45RA+ CD4+ T cells were stimulated with anti-CD3/anti-CD28-coated microbeads (2.5 μl/106 cells; Dynabeads; Dynal, Hamburg, Germany) at 105 cells/ml in 100-μl final volume per well on 96-well plates in duplicate. B cell–derived supernatants were generated by incubating B cells for 3 days with CD40L or with CD40L in combination with CpG ODN. B cell–derived supernatants were neutralized using a neutralizing mAb for IL-12 (5 μg/ml; R&D Systems, Minneapolis, MN) was added from the beginning of the culture. After 3 days, supernatants from the T cell cultures were analyzed for IFN-γ production by ELISA.

Statistical analysis

Data are expressed as means ± SEM. Statistical significance of differences was determined by the paired two-tailed Student’s t test. Differences were considered statistically significant for p < 0.05 (*) and p < 0.005 (**).
Results

Both naive and memory B cells express TLR9 but lack TLR4 and are sensitive to CpG ODN but not LPS

In previous studies, we found that human B cells isolated from peripheral blood express TLR9 mRNA (6). However, there is no information whether the level of TLR9 expression differs between naive and memory B cells. A common feature of the somatically mutated B cell subsets is the expression of the CD27 cell surface Ag, which therefore represents a general marker for memory B cells in humans (34). Based on the differential expression of CD27 and IgD, the B cell pool in human peripheral blood consists of ~40% mutated memory B cells (IgD<sup>+</sup>CD27<sup>+</sup>) and 60% unmutated, naïve B cells (IgD<sup>+</sup>CD27<sup>−</sup>).

To study TLR expression in naive and memory B cells, total B cells were isolated from PBMC and stained with anti-CD27 and anti-IgD (Fig. 1A). Naïve (CD27<sup>+</sup>, IgD<sup>+</sup>) and memory B cells (CD27<sup>−</sup>, IgD<sup>−</sup>) were obtained by cell sorting. The expression of TLR4 and TLR9 was assessed by real time RT-PCR analysis (Fig. 1B): TLR9 was detected in both B cell subsets, albeit somewhat lower in naïve B cells; in contrast, TLR4 expression was at the detection limit in both naive and memory B cells. This was consistent with the observation that B cells were not sensitive to LPS (Fig. 1C). Both naïve and memory B cells produced comparable levels of IL-6 in response to the CpG-B ODN 2006 (Fig. 1D).

CpG ODN synergize with CD40L to induce IL-6, TNF-α, IL-10, and IgM synthesis in B cells

B cells are known to be activated by Th cells via CD40L. Sorted naïve and memory B cells were incubated with CpG ODN, with CD40L, or with a combination of CpG ODN and CD40L. After 72 h, IL-6 was determined in the supernatants. Although both naïve and memory B cells produced low amounts of IL-6 in response to CD40L alone, the additional presence of CpG ODN synergistically increased IL-6 synthesis in both B cell subsets (Fig. 1E). In addition to IL-6, synergy of CD40L and CpG ODN was observed for TNF-α and IL-10 production measured after 72 h, and IgM synthesis was analyzed after 13 days of incubation (Fig. 2). Although IL-6, TNF-α, and IL-10 all were induced by CpG ODN alone, CpG ODN alone was not sufficient to stimulate the production of IL-12p40 or IL-12p70 (Fig. 3). No synergy of CD40L and CpG ODN was found with regard to up-regulation of costimulatory molecules and MHC II. Furthermore, IL-2, IL-4, IL-5, and IFN-γ were not detected in B cells stimulated with CpG ODN or CD40L (data not shown).

Production of IL-12p70 by B cells requires both CD40L and CpG ODN, is negatively regulated by exogenous IL-4, and is independent of B cell receptor cross-linking

Unlike CpG ODN, CD40L was capable of inducing IL-12p40, but the amount produced was low, and no IL-12p70 could be detected. However, when CD40L was combined with CpG ODN, high amounts of IL-12p40 and considerable amounts of IL-12p70 were found (Fig. 3). These data indicated that the production of the

![FIGURE 1](https://example.com/image1.png)

**FIGURE 1.** Expression of TLR4 and TLR9 in naïve and memory B cells and response to CpG ODN and LPS. Human CD19<sup>+</sup> B cells were isolated from PBMC. A, Identification of naïve and memory B cells. Isolated B cells were stained with anti-CD27 and anti-IgD, and naïve (CD27<sup>+</sup>, IgD<sup>+</sup>) and memory B cells (CD27<sup>−</sup>, IgD<sup>−</sup>) were identified by two-color flow cytometry. B, Quantitative analysis of TLR4 and TLR9 mRNA in B cell subpopulations: naïve (CD27<sup>+</sup>, IgD<sup>−</sup>) and memory B cells (CD27<sup>−</sup>, IgD<sup>−</sup>) were sorted, and the expression of TLR4 and TLR9 mRNA was determined by real time RT-PCR. The number of transcripts per 10<sup>5</sup> copies of the housekeeping gene β-actin is depicted (n = 2). C, CD19<sup>+</sup> purified B cells (2 × 10<sup>5</sup> cells in 200 μl of medium per well) were incubated with CpG ODN or LPS. After 3 days, IL-6 was analyzed in the supernatants by ELISA (n = 3). D, Sorted naïve and memory B cells (1 × 10<sup>5</sup> cells in 200 μl of medium per well) were incubated with CpG ODN (3 μg/ml). After 3 days, IL-6 production was determined by ELISA (n = 3). E, Sorted naïve and memory B cells (1 × 10<sup>5</sup> cells in 200 μl of medium per well) were incubated with CpG ODN, with CD40L, or with a combination of CD40L and CpG ODN. After 3 days, IL-6 production was assessed by ELISA (n = 2). Data are shown as means ± SEM.
bioactive form of IL-12, IL-12p70, in B cells is restricted to situations where both CD40L and CpG ODN are present. A dose response revealed an optimal concentration of CpG ODN between 1 and 5 μg/ml (Fig. 4A). Next, we studied the kinetics of IL-12 production of B cells in response to CD40L and CpG ODN (Fig. 4, B and C). We found that cumulative IL-12p40 and IL-12p70 production increased until day 3. In our previous study, human PDC under the same conditions produced only ~4-fold higher amounts of IL-12, which peaked between 12 and 24 h (12), excluding the possibility that in a 95% pure B cell population IL-12 production is due to contaminating PDC.

T cell-derived cytokines may modulate IL-12 production in B cells. We found that the addition of exogenous IL-4 strongly inhibited IL-12p70 production by B cells stimulated with CD40L and CpG ODN (Fig. 4D; results from n = 3). CD40L and CpG ODN with and without IL-4: 24 h, 4.6 ± 1.8 vs 0.7 ± 0.5; 48 h, 14.6 ± 4.9 vs 3.2 ± 1.7; 72 h, 17.8 ± 4.7 vs 4.6 ± 1.7). A slight but not significant increase was found when IFN-γ was added (CD40L and CpG ODN with and without IFN-γ: 24 h, 4.6 ± 1.8 vs 7.4 ± 0.8; 48 h, 14.6 ± 4.9 vs 16.6 ± 4.4; 72 h, 17.8 ± 4.7 vs 20 ± 8.6; n = 3).

Besides Th cell-derived CD40L, cross-linking of the B cell receptor is known to trigger B cell activation. Indeed, we found that, similar to CD40L, anti-IgM mimicking B cell Ag strongly synergized with CpG ODN to stimulate IL-6 production in purified B cells (Fig. 5A). However, unlike CD40L, anti-IgM alone did not induce IL-12p40, and failed to induce IL-12p40 and IL-12p70 if combined with CpG ODN (Fig. 5, B and C). These results demonstrate that the detection of a microbial molecule and not the B cell receptor ligation is required to enable B cells to produce IL-12.

To exclude that B cells become sensitive to LPS upon stimulation with CD40L, we tested IL-12p40 and IL-12p70 production in the presence of CD40L with and without LPS (Fig. 5, B and C). LPS had no effect on CD40L-induced IL-12 production in B cells, confirming that LPS does not represent a microbial stimulus for human B cells.

Synthetic CpG ODN is thought to mimic microbial DNA. Therefore, we studied genomic E. coli DNA as a more physiological stimulus that is known to contain CpG motifs and to stimulate human B cells. In our previous studies, we found that unmodified DNA such as E. coli DNA requires repeated addition at relatively high concentrations to circumvent rapid degradation of DNA by nucleases (16). E. coli DNA was added to isolated B cells at 24 μg/ml after 0, 2, 4, and 16 h in the presence or absence of CD40L. Similar to CpG ODN, CD40L and E. coli DNA synergistically increased the production of IL-12p70 in B cells (Fig. 6A). A dose response revealed that, unlike for CpG ODN (Fig. 4A), the activity of E. coli DNA increased up to the concentration of 24 μg/ml (highest concentration tested) (Fig. 6B).

**Activated B cells induce proliferation of allogeneic naïve T cells**

It has been demonstrated that B cells stimulated with CD40L induce T cell proliferation (25, 35). We were interested whether stimulation of B cells with CpG ODN increases the ability of B cells to induce T cell proliferation. Purified B cells were incubated with CD40L, CpG ODN, or a combination of CD40L and CpG ODN or CD40L and IL-4. After 3 days, B cells were harvested, irradiated, and coincubated with allogeneic T cells isolated from peripheral blood of adult donors. B cells cultured with CD40L alone or in combination with CpG ODN or IL-4 induced considerable T cell proliferation (Fig. 7). B cells stimulated with CpG ODN alone were weak at inducing T cell proliferation.

The activity of stimulated B cells to drive T cell responses might depend on preactivation of T cells. We were interested whether activated B cells are capable of stimulating naïve T cells. Because the naïve phenotype of CD45RA⁺ T cells in adult donors is controversial (36), we isolated CD45RA⁺ naïve T cells from cord blood. Allogenic T cells were cocultured with prestimulated and irradiated B cells. We found that not only unselected CD3⁺ T cells from adult donors but also naïve T cells from cord blood showed high proliferative activity if coincubated with B cells that received CD40L-mediated stimulation (Fig. 8). Proliferation of naïve T cells was confirmed by using CFSE staining. No difference was found between B cells stimulated with CD40L alone or in combination with IL-4 or CpG ODN. The proliferative response of naïve cord blood T cells was in the same range or higher as the response of adult T cells (Fig. 8, A and B). The frequency of proliferating cells originating from the naïve T cell population (CFSE staining; Fig. 8, B and C) in the presence of CD40L-activated B cells was in the range of the T cell proliferation upon stimulation with CD3/CD28-coated microbeads, suggesting a high potential of CD40L-activated B cells to drive naïve T cell proliferation. Together, these results indicate that CD40L-stimulated B cells are capable to drive naïve T cell proliferation, and that CpG ODN does not further enhance this activity.

**CpG ODN and CD40L enable B cells to skew preactivated CD4⁺ T cells toward Th1**

Although CpG ODN did not increase the induction of T cell proliferation, CpG ODN may contribute to the Th1 vs Th2 bias of developing T cells. The IL-12p70 production of B cells stimulated by CD40L and CpG ODN prompted us to examine whether these B cells are able to induce IFN-γ production of preactivated CD4⁺ T cells. We used anti-CD3- plus anti-CD28-coated microbeads to
stimulated with CD40L alone did not enhance IFN- with or without CpG ODN for 72 h. The supernatant of B cells obtained by incubating isolated B cells in the presence of CD40L/H11005 (Results are presented as means IL-12p70 (C) ODN for up to 3 days. Total IL-12 (B) with the combination of CD40L and CpG ODN strongly supported IFN- production in CD4 T cells. The addition of anti-IL-12 Abs completely abolished this effect, demonstrating that the IFN- induction in CD4 T cells was due to B cell-derived IL-12. Together, these data are in agreement with the concept that CpG ODN are required for the production of bioactive IL-12 and the ability of B cells to skew preactivated CD4+ T cells toward Th1.

Discussion

B cells establish Ag-specific humoral immune responses which form a major part of acquired immunity. In addition, B cells display features of APCs being part of innate immunity. A general feature of APCs is that they become activated upon detection of microbial molecules via TLR. In this study, we made the surprising observation that, in humans, both naive and memory B cells lack TLR4, explaining why B cells are not capable of detecting LPS. In contrast, both B cell subsets expressed TLR9 and produced IL-6, TNF-α, and IL-10 in response to CpG ODN, but no IL-12. IL-12 production by B cells required stimulation with CD40L. CD40L alone induced only small amounts of IL-12p40 but no bioactive IL-12p70. The synthesis of IL-12p70 was seen only upon combined stimulation with both CpG ODN and CD40L. B cell receptor cross-linking was not involved in IL-12 production. CpG ODN did not enhance the ability of CD40L-stimulated B cells to induce proliferation of allogenic T cells, but strongly supported Th1 differentiation of CD4+ T cells via B cell-derived IL-12.

Similar to B cells in this study, PDC express TLR9 but lack TLR4; furthermore, consistent with TLR expression, PDC are sensitive to CpG but not to LPS (6, 12). It is interesting to note that, in humans, an immune cell coexpressing both TLR4 and TLR9 has not been identified. As a consequence, human immune cells are exclusively sensitive to LPS or CpG. Besides TLR9, TLR7 is expressed on B cells and PDC. TLR7 is the receptor for small antiviral imidazoquinoline compounds such as imiquimod, but the natural ligand of TLR7 is unknown (37). TLR6 and TLR10 are highly expressed on human B cells but not on PDC. So far, CpG DNA is the only well-defined microbial molecule recognized by human B cells as demonstrated in this study, but specific ligands of TLR6 and TLR7 might add other interesting B cell stimuli in the future. Of note, in contrast to human B cells, murine B cells express TLR4 and are sensitive to LPS (38, 39).

Based on our findings, another intriguing parallel between B cells and PDC is that the production of IL-12p70 in both cell types is under a strict dual control: T cell help (CD40L) and recognition of an appropriate microbial molecule (CpG ODN). In dendritic cells, the CD40 pathway has been thought to play a greater role than microbial stimulation in eliciting IL-12 production (40). However, there is evidence that the production of bioactive IL-12p70 heterodimer by dendritic cells in vivo requires both microbial and T cell-derived (CD40L) stimuli (41). In vitro, mechanical stress during isolation of cells or cell culture conditions may substitute for a second signal (41, 42). Similar events may be responsible for

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**FIGURE 3.** CD40L and CpG ODN synergize to induce IL-12p40 and IL-12p70 in B cells. Purified CD19+ B cells (2 × 10^7 cells in 200 μl of medium per well) were stimulated with CpG ODN, CD40L, and CD40L-negative transfectant cells (control) as indicated. After 3 days, total IL-12 and IL-12p70 by purifying isolated B cells in the presence of CD40L with or without CpG ODN for 72 h. The supernatant of B cells stimulated with CD40L alone did not enhance IFN-γ production in anti-CD3/anti-CD28-preactivated CD4+ T cells (Fig. 9). In contrast, the supernatants of B cells stimulated with CD40L and CpG ODN strongly supported IFN-γ synthesis of anti-CD3/anti-CD28-preactivated CD4+ T cells. The addition of anti-IL-12 Abs completely abolished this effect, demonstrating that the IFN-γ induction in CD4 T cells was due to B cell-derived IL-12. Together, these data are in agreement with the concept that CpG ODN are required for the production of bioactive IL-12 and the ability of B cells to skew preactivated CD4+ T cells toward Th1.

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Similar to B cells in this study, PDC express TLR9 but lack TLR4; furthermore, consistent with TLR expression, PDC are sensitive to CpG but not to LPS (6, 12). It is interesting to note that, in humans, an immune cell coexpressing both TLR4 and TLR9 has not been identified. As a consequence, human immune cells are exclusively sensitive to LPS or CpG. Besides TLR9, TLR7 is expressed on B cells and PDC. TLR7 is the receptor for small antiviral imidazoquinoline compounds such as imiquimod, but the natural ligand of TLR7 is unknown (37). TLR6 and TLR10 are highly expressed on human B cells but not on PDC. So far, CpG DNA is the only well-defined microbial molecule recognized by human B cells as demonstrated in this study, but specific ligands of TLR6 and TLR7 might add other interesting B cell stimuli in the future. Of note, in contrast to human B cells, murine B cells express TLR4 and are sensitive to LPS (38, 39).

Based on our findings, another intriguing parallel between B cells and PDC is that the production of IL-12p70 in both cell types is under a strict dual control: T cell help (CD40L) and recognition of an appropriate microbial molecule (CpG ODN). In dendritic cells, the CD40 pathway has been thought to play a greater role than microbial stimulation in eliciting IL-12 production (40). However, there is evidence that the production of bioactive IL-12p70 heterodimer by dendritic cells in vivo requires both microbial and T cell-derived (CD40L) stimuli (41). In vitro, mechanical stress during isolation of cells or cell culture conditions may substitute for a second signal (41, 42). Similar events may be responsible for

**FIGURE 4.** Expression of total IL-12 and IL-12p70 by purified CD19+ B cells. A, Purified CD19+ B cells (2 × 10^7 cells in 200 μl of medium per well) were cultured with different concentrations of CpG ODN with or without CD40L for 3 days, and IL-12p70 was measured by ELISA (n = 3). B and C, Kinetics of total IL-12 and IL-12p70 production by purified CD19+ B cells: purified CD19+ B cells (2 × 10^7 cells in 200 μl of medium per well) were incubated with CpG ODN or with the combination of CD40L and CpG ODN for up to 3 days. Total IL-12 (B) and IL-12p70 (C) were determined by ELISA. Results are presented as means ± SEM (n = 3). D, Purified CD19+ B cells incubated with CD40L and CpG ODN in the presence of IL-4 or IFN-γ. Supernatants were harvested after 1, 2 and 3 days and analyzed for IL-12p70 by ELISA. One representative experiment of three is shown.
the observation that tonsillar non-germinal center B cells produce IL-12p70 upon stimulation with CD40L alone (43). The preparation of surgical specimens of chronically infected tonsils may lead to mechanical stress and microbial costimulation that was found to be required for IL-12p70 production of peripheral blood B cells in our study. Furthermore, others found that murine B cells isolated from spleen produced high levels of IL-12 in response to CpG ODN alone (44). A CD40L-mediated preactivation of murine B cells isolated from spleen tissue or the different species may be responsible for the difference between both studies with regard to CD40L dependence of IL-12 production in B cells.

Although CD40L-activated B cells have been shown to activate naive T cells in vitro, the induction of a primary T cell response by B cells in vivo is controversial. It has been proposed that resting B cells are involved in the establishment of T cell tolerance (45–47). Although B cells are known to induce Th2 responses specific for the Ag recognized by its B cell receptor (48–50), a number of studies indicate that B cells upon CD40L-mediated stimulation in vitro acquire characteristics of dendritic cells, that is, to take up and present exogenous Ags to T cells independent of their specific B cell receptor (25, 35). In our study, CD40L-activated B cells strongly induced proliferation of allogenic naive T cells isolated from cord blood. Although additional stimulation with CpG ODN did not further increase T cell proliferation, it potently induced IFN-γ production in preactivated CD4 T cells via B cell-derived IL-12. The ability to support Th1 development is consistent with the phenotype of effector B cell 1 (Be1) that was defined by others by its functional activity to induce Th1 cells (51). In their study, Be1 cells were generated by coinoculation of naive B cells with pre-established Th1 cells. Our study demonstrates that appropriate microbial stimulation of B cells with CpG ODN together with CD40L is sufficient for the generation of Be1 cells independent of a pre-established Th1 response. Of note, although in the study by Harris et al. (51), IL-12 was hardly detectable in Be1 cells generated in the presence of Th1 cells, in their study, Be1 cells producing high amounts of IL-12 were found only in a Th1 infection model (Toxoplasma gondii) in vivo in which microbial stimulation via TLR9 might have been present.

Our observation that both CD40L and microbial stimulation are required for IL-12p70 production of human B cells adds to the model elaborated by Schultze et al. (43). In this model, originally presented by Janeway and colleague (52), B cells play an important role for the amplification and maintenance of a T cell response primarily initiated by dendritic cells. The concept of this model is that T cells primed by dendritic cells in the interfollicular area of lymphoid tissue migrate to the outer T cell zone (53) and subsequently to the B cell area (54) where they meet Ag-specific B cells. This interaction then leads to marked expansion of both T and B cells (55, 56). Based on Schultze’s data, B cells start to produce IL-12 if they interact with IFN-γ-producing T cells expressing surface CD40L. In this way, B cells would contribute to the maintenance of a pre-established Th1 bias of T cells. Conversely, if CD40L-expressing T cells produce IL-4 instead of IFN-γ, IL-12 production by B cells is inhibited, and the pre-established Th2 bias is maintained. In this model, B cells would play an indiscriminate role regarding the regulation of a Th1 vs Th2 bias of preactivated T cells. Our data confirm that B cells are capable of producing IL-12p70, that CD40L plays a pivotal role in regulating IL-12 production by B cells, and that the Th2 cytokine IL-4 inhibits
IL-12 production in B cells. However, based on our data, we propose to add the aspect of microbial stimulation of B cells to the above model. As a consequence, B cells do not indiscriminately react to the directive of Th-biased preactivated T cells, but rather make their own decision based on the detection of microbial molecules in their environment. Upon encounter of T cells, they produce IL-12p70 only if they simultaneously sense the appropriate microbial molecule. If the preactivated T cell produces IL-4, IL-12p70 production may be reduced, but it is not abolished. In that way, a two-level regulation of the Th bias of expanding CD4 T cells is achieved in that preactivated T cells (carrying the Th bias as a memory of previous microbial stimulation of dendritic cells) and B cells (sensing microbial molecules on their own) are equal partners regarding Th1 vs Th2 regulation.

It is interesting to note that, in our study, besides IL-12, CpG and CD40L synergistically induced IL-6 and IL-10. Because B cell-derived IL-10 has been reported to play a key role in controlling autoimmunity (57, 58), B cell-derived IL-10 may be involved in balancing the Th1-inducing activity of IL-12. In addition to the Th1/Th2 balance, IL-6, IL-10, and IL-12 are all known to support B cell differentiation (59–61). Our study suggests that not only exogenous IL-12 derived from dendritic cells, as shown by Dubois et al. (61), but also endogenous IL-12 of B cells could be involved in B cell differentiation in situations where B cells detect microbial DNA.

The expression of TLR9 and the TLR9-mediated sensitivity of naive and memory B cells to CpG ODN is intriguing. However, there is only limited information on the physiological role of TLR9 in B cells during bacterial or viral infections. Although CpG ODN are thought to mimic microbial DNA, there are some important differences of CpG ODN when compared with microbial DNA: 1) the frequency of CpG motifs in CpG ODN is higher, 2) the chemical backbone modification renders CpG ODN more stable against nucleases, and 3) the molecular mass of CpG ODN is much smaller.

FIGURE 6. *E. coli* DNA synergize with CD40L to induce IL-12p70 in B cells. A, Purified CD19+ B cells (2 × 10⁵ cells in 200 µl of medium per well) were stimulated with CpG ODN, *E. coli* DNA, and CD40L as indicated. *E. coli* DNA was added after 0, 2, 4, and 16 h at a concentration of 24 µg/ml. After 3 days, IL-12p70 was measured in the supernatants by ELISA. Results are shown as means ± SEM of three independent experiments. B, Purified CD19+ B cells (2 × 10⁵ cells in 200 µl of medium per well) were cultured with different concentrations of *E. coli* DNA (added after 0, 2, 4, and 16 h) with or without CD40L. After 3 days, IL-12p70 was measured in the supernatants by ELISA.

FIGURE 7. CD40L-activated B cells induce proliferation of allogenic CD3+ T cells. A, Purified B cells (4 × 10⁶ cells in 2 ml of medium per well) were cultured with different stimuli (CpG ODN; CD40L; CD40L and CpG ODN; CD40L and IL-4). After 3 days, B cells were harvested, irradiated (1500 rad), and counted. Purified allogenic adult CD3+ T cells (1 × 10⁵ cells in 200 µl of medium per well) were cocultured with these B cells for 7 days at the indicated ratios. [3H]Thymidine incorporation was assessed for the last 16 h in triplicate. One representative experiment of two is shown. B, B cells and T cells were cocultured as described above at a B cell:T cell ratio of 1:3. As a positive control, T cells were stimulated with anti-CD3- plus anti-CD28-coated microbeads. [3H]Thymidine incorporation was assessed for the last 16 h in triplicate.
facilitating internalization. In our study, we tested the activity of \( \text{E. coli} \) DNA as a more physiological stimulus. Like CpG ODN, \( \text{E. coli} \) DNA showed a strong synergy with CD40L to induce IL-12p70, supporting the concept that high local concentrations of genomic bacterial DNA indeed can represent a physiological microbial stimulus.

Exposure of B cells to such high concentrations of extracellular bacterial DNA may occur during serious systemic infections like sepsis. However, degradation of extracellular microbial DNA by nucleases will prevent polyclonal B cell activation during less severe infections. In our in vitro system, not only relatively high concentrations but also repeated addition of \( \text{E. coli} \) DNA were required to circumvent rapid degradation of unprotected bacterial DNA. DNA is more stable against nuclease degradation if complexed in nucleic acid-protein particles. There is recent evidence that DNA-containing chromatin-IgG complexes activate autoreactive B cells expressing an Ag receptor specific for self-IgG; in this study, activation of B cells was MyD88 dependent with inhibitor studies implicating TLR9 (62). Importantly, activation of B cells required dual engagement of the B cell receptor and the TLR. These results obtained with DNA-containing chromatin-IgG complexes suggest that immune complexes or similar particles that contain microbial DNA together with viral or bacterial Ags may be capable of stimulating B cells that carry the corresponding B cell receptor. Due to the requirement of dual engagement of B cell receptor and TLR9, B cells would be activated only by particles containing the B cell receptor-specific Ag, thereby avoiding polyclonal B cell activation under physiological circumstances.

Although larger nucleic acid-protein particles such as DNA-containing chromatin-IgG complexes require dual engagement of the B cell receptor and the TLR, it has been reported that small synthetic nuclease-stable CpG ODN conjugated to peptide Ag lead to polyclonal activation of B cells (44) similar to unconjugated CpG ODN. Interestingly, with these CpG ODN peptide conjugates, Shirota et al. (44) found that B cells gained characteristics of dendritic cells such as the ability of Ag-nonspecific capture and presentation, and the induction of Th1 differentiation from unprimed T cells. Although CpG ODN peptide conjugates comprise a promising class of compounds in vaccine development, like unconjugated CpG ODN they do not reflect the physiological situation in which polyclonal B cell activation may still be the exception.

In conclusion, our results demonstrate that the capacity of B cells to produce IL-12 and to modulate T cell responses is tightly regulated by two independent mechanisms, T cell help and the

![Image](image.png)

**FIGURE 8.** CD40L-activated B cells induce proliferation of allogenic naive cord blood T cells. **A**, Purified B cells (4 × 10^6 cells in 2 ml of medium per well) were cultured with different stimuli (CpG ODN; CD40L; CD40L and CpG ODN; CD40L and IL-4) as indicated. After 3 days, B cells were harvested, irradiated (1500 rad), and counted. Purified allogenic adult CD3^+ T cells or naive CD3^+ CD45RA^- T cells (1 × 10^5 cells in 200 µl of medium per well) obtained from cord blood were cocultured with B cells for 5 days at a B cell:T cell ratio of 1:3. As a positive control, adult T cells were stimulated with anti-CD3- plus anti-CD28-coated microbeads in the presence of IL-2. Addition of anti-CD3- plus anti-CD28-coated microbeads together with IL-2 and IL-7 served as a positive control for naive cord blood T cells. [³H]Thymidine was added for the last 16 h (assays in triplicate). **B**, Isolated T cells were stained with CFSE. B cells and CFSE-stained T cells were cocultured as described above. After 5 days, the frequency of proliferating T cells was determined by flow cytometry (triplicate). **C**, Representative histograms of B are shown.
FIGURE 9. B cells stimulated with CD40L and CpG ODN induce IFN-γ in preactivated CD4+ T cells. CD45RA+ CD4+ T cells were incubated with anti-CD3- plus anti-CD28-coated microbeads in the presence of supernatants (1:1) derived from B cells (n = 3) stimulated for 3 days with CD40L with or without CpG ODN. Neutralizing anti-IL-12 mAb was added at the beginning of the T cell cultures where indicated. After 3 days, supernatants were harvested and analyzed for IFN-γ production by ELISA. Results are presented as means ± SEM.

detection of microbial molecules, which both are required for B cell production of bioactive IL-12p70. Although CD40L-stimulated B cells induced proliferation of naive T cells in vitro, we like to emphasize that our studies provide no evidence that B cells are capable of inducing primary T cell responses in vivo. The identification of CpG DNA as a unique microbial molecule for human B cells opens new avenues in B cell biology. One example is the recent finding that CpG ODN but not LPS directly induce T-bet expression and inhibit IgG1 and IgE switching in B cells (63).

With respect to the use of CpG as vaccine adjuvant, our results stress the point that not the high toxicity of LPS per se but rather its failure to be recognized by human B cells due to the lack of TLR4 explains its limited value as a vaccine adjuvant in humans.

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