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TLR9-Activating DNA Up-Regulates ZAP70 via Sustained PKB Induction in IgM+ B Cells

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In the past, ZAP70 was considered a T cell-specific kinase, and its aberrant expression in B-CLL cells was interpreted as a sign of malignant transformation and dedifferentiation. It was only recently that ZAP70 was detected in normal human B cells. In this study, we show that TLR9-activated B cells resemble B-cell chronic lymphocytic leukemia cells with regard to CD5, CD23, CD25, and heat shock protein 90 expression. Furthermore, stimulatory CpG and GpC DNA oligonucleotides target CD27+ IgM+ and CD27+ IgM+ B cells (but not IgM− B cells) and enhance ZAP70 expression predominantly in the IgM+ CD27+ B cell subset. ZAP70 is induced via activation of TLR-7 or -9 in a MyD88-dependent manner, depends on protein kinase B (PKB)/mammalian target of rapamycin signaling, and is rapamycin sensitive. Furthermore, ZAP70 expression levels correlate with induction of cyclin A2, prolonged B cell proliferation, and sustained induction of PKB. These events are not observed upon CD40 ligation. However, this deficit can be overcome by the expression of constitutively active PKB, given that CD40 ligation of PKB-transgenic B cells induces B cell proliferation and ZAP70 expression. These results highlight a major difference between CD40- and TLR-7/-9-mediated B cell activation and suggest that ZAP70 expression levels in B cells give an estimate of the proliferative potential and the associated PKB availability. The Journal of Immunology, 2008, 181: 8267–8277.

ZAP70 is a syk-related tyrosine kinase of 70 kDa originally described in T cells and NK cells. It has been shown to be required in TCR signal transduction, in T cell apoptosis (1), and in positive and negative selection in the thymus (2). Until recently, expression of ZAP70 in human B cells was believed to be restricted to B cell chronic lymphatic leukemia (B-CLL)3 cells where it is associated with an unfavorable outcome, although its exact role remains unknown (3–7). Recently, ZAP70 expression was also observed in B cells from healthy donors (8–10). However, this issue remained controversial because other investigators failed to detect ZAP70 expression in mature human B cells (11). In marked contrast to T cells, ZAP70 is considered to be dispensable for mature B cell function in the presence of the closely related tyrosine kinase Syk (12, 13); thus far, only one study has described a specific role for ZAP70 in BCR-triggered pro- to pre-B cell development (14).

B-CLL cells phenotypically resemble activated B cells and typically express CD5, CD23, and CD25 among other activation markers (15). In an aggressive course of disease, B-CLL patients present with splenomegaly and lymph node engrossment. This coincides with high levels of soluble CD23, CD38, and ZAP70 expression that have been associated with an unfavorable prognosis (16, 17). Moreover, B-CLL cells express tumor-associated survival genes such as the anti-apoptotic molecules bcl-2 (18, 19), survivin (20), protein kinase B (PKB), also known as Akt (21), and heat shock protein 90 (Hsp-90; Ref. 22). Additionally, T-cell leukemia viral oncogene, enhances cell survival and proliferation by activating PKB signaling (23–27).

TLR9 ligands such as CpG DNA oligodeoxynucleotides (ODN) represent very potent activators of polyclonal B cell proliferation (28, 29). They induce a transient splenomegaly after in vivo administration (30) and have also been described as stimuli for B-CLL cells (21, 31–33). Because it is currently under debate whether infections can trigger the development or progression of B-CLL (15, 34–37) and B cell-active phosphorothioate-modified type B CpG DNA ODNs (PTO) are currently making their way into clinics (33, 38–40), we were prompted to investigate the effects and possible side effects of CpG DNA ODNs on normal human B cells.

This study shows that CpG DNA ODN-stimulated B cells closely resemble B-CLL cells, including ZAP70 expression. We further demonstrate that CpG DNA ODNs stimulate IgM+CD27+ and IgM−CD27− B cells, the B cell subsets thought to represent the cellular origin of B-CLL.

Materials and Methods

Cells, mice, cell isolation, and stimulation

The use of PBMCs from healthy volunteers was approved by the local ethics committee. For B cell isolation, PBMC were isolated from heparinized blood or from Buffy coats by density gradient centrifugation. B cells for Western blot analysis were always isolated fromuffy coats. CD19+ B
cells (purity, 98.5 ± 1%) were positively selected as previously described (41). CD27+ fractions were positively isolated with CD27+ microbeads from untouched resting B cells negatively isolated after depletion with CD3 microbeads followed by CD43 microbeads (purity, 98 ± 2%); CD43−CD27− naive cells remained untouched after CD27 depletion. For isolation of IgM+ and IgM− B cells, cells were first stained with an allophycocyanin-conjugated anti-human IgM mAb (BD Pharmingen). IgM+ B cells were first depleted from PBMCs with anti-allophycocyanin microbeads and then purified from the positive fraction (purity, 98 ± 1%); IgM− B cells were isolated from the IgM+ fraction with CD19+ beads as above (purity, 92 ± 5%). Isolated B cell fractions were cultured in RPMI 1640 (Biochrom) supplemented with 100 µl penicillin and 100 µg/ml streptomycin, 1% HEPES buffer (all from Sigma-Aldrich), and 10% heat-inactivated autologous serum and incubated overnight before stimulation. In all B cell fractions, the percentage of contaminating T cells was 0.5 ± 0.5% on the day of isolation; T cells were barely detectable on the day of analysis (Fig. 6d).

The experimental use of mice was approved by the local animal committee. MyD88 knockout mice and constitutively active myrPKB-transgenic (CD2 myrPKB tg) mice have been previously described (42, 43). myrPKB expression in B cells was demonstrated previously (44). Murine CD19+ B cells were isolated from single-cell suspensions from spleens of wild-type (WT; C57BL/6). MyD88 knockout (≥6 backcrosses) and myrPKB tg mice (≥8 backcrosses) with anti-CD19 microbeads. Purity was 92 ± 2%. Murine B cells were resuspended in RPMI 1640 supplemented with penicillin and streptomycin, 5 x 10^{-7} M 2-ME (Sigma-Aldrich), and 10% heat-inactivated FCS (Biochrom).

For stimulation, human and murine CD19+ B cells were plated at 1 x 10^5/well unless otherwise indicated. All media and reagents were tested for endotoxin as previously described (45). The stimulatory agents were titrated for optimal concentrations and used as follows: human CpG-B DNA ODN 2006-PTO (5′-ctgcgtttttgctttgtgttt-3′) and 2006-GpC DNA ODN-PTO (5′-cgctgcgtttttgtgtgttt-3′); murine CpG-B DNA ODN 1668-PTO (5′-ctcagcttttctttggcttt-3′), all at 0.5 µM (Western blots and experiments in Figs. 2 and 6) or 1 µM, full-length phosphorothioate linkage, all purchased from MWG Biotech; R848 (InvivoGen), 0.25 µg/ml, and IFN-γ 1000 U/ml (Prometheus); Pam3CysSerLys4 (EMC Microcollections), 1 µg/ml; highly purified LPS, 100 ng/ml (gift from U. Seidel, Research Center Borstel, Borstel, Germany); goat anti-human IgG (H+L) (AbD), 2 fragments, and goat anti-murine IgG (H+L) (AbD), 2 fragments, were purchased from Jackson ImmunoResearch and used at 5 µg/ml, unless otherwise indicated. BHK-CD40L and BHK-pTcF control cells (gift from H. Engelmann, Institute of Immunology, University of Munich, Munich, Germany) were UV irradiated (0.75 J/cm^2) and used at a ratio of 1:10 B cells. For murine CD40 activation, purified (no azide, low endotoxin) anti-IgG1 Ab (BD Pharmingen) was diluted in PBS (10 µg/ml). For murine CD40 activation, purified (no azide, low endotoxin) anti-CD40 Ab (BD Pharmingen) was diluted in PBS (10 µg/ml); cells were rarely detectable on the day of isolation; T cells were detectable on the day of analysis (Fig. 6d).

Flow cytometry and immunofluorescent microscopy

For flow cytometric analysis of cell surface markers, cells were stained in PBS/2% FCS according to standard procedures. The Abs used were purchased from BD Pharmingen: anti-human IgD (FITC); IgM (PE); IgG (allophycocyanin); IgG (FITC); CD5 (allophycocyanin); CD5 (FITC); CD20 (PerCP); CD19 (PE); CD25 (Cy-Chrome); CD225 (allophycocyanin); CD17-PE (FITC); CD38 (FITC); CD21 (FITC); CD138 (PE); anti-mouse B220 (PE). Anti-human CD23 (FITC) was purchased form Caltag Laboratories. Cells were analyzed on CASCANTO (BD Biosciences) with FACS Diva Software (BD Biosciences). Only live gated cells were subjected to analysis.

Intracellular staining for ZAP70 was performed after cell fixation in 4% paraformaldehyde with Alexa 488-conjugated anti-human IgG1 isotype control (Caltag Laboratories) in permeabilizing medium B (Caltag Laboratories). Mean fluorescence intensity for ZAP70 (AMFI) was calculated by subtracting the MFI (median) for the murine IgG1 isotype control from the MFI for the anti-ZAP70 mAb. Intracellular staining for IgM and IgG was performed as previously described (41). For BrdU incorporation assays, B cells were pulsed with 0.5 µM BrdU (Roche Biochemicals) on day 0. BrdU incorporation was quantified on day 5 using the allophtycocyanin BrdU flow kit from BD Biosciences following the protocol provided. CFSE staining was performed with 1 µM CFSE (Molecular Probes); CFSE dilution was analyzed on day 6. Annexin V-FITC and propidium iodide staining were performed according to the manufacturer’s protocol (BenderMedSystems).

For immunofluorescent staining, B cells were centrifuged onto poly-l-lysine (Sigma-Aldrich)-coated slides, air dried, and fixed with 4% paraformaldehyde-PBS. Cells were blocked in PBS, 0.1% saponin, 5% FCS, 2% nonfat dry milk and stained with Alexy 488-conjugated anti-ZAP70 mAb or murine IgG1 isotype control diluted 1:10. 4′, 6′-Diamidino-2-phenylindole (Molecular Probes) was added at 0.1 µM in PBS-lycorol mounting medium. Images were acquired on a Leica DMI 6000 B microscope.

Western blot analysis

Cell lysates were prepared in radioimmunoprecipitation assay lysis buffer from 2 or 5 x 10^6 B cells. Twelve percent polyacrylamide gels were blotted on nitrocellulose (ZAP70 and PKB blots) or polynylvilene diffu- oride (Hsp-90 blots) membranes. Membranes were blocked in 5% milk or 0.5% BSA and incubated with the respective Abs according to the manufacturers’ recommendations: rabbit anti-ZAP70 (Epitomics); rabbit anti-(pan-)Akt (Cell Signaling); mouse anti-GAPDH (Chemicon); anti-actin (Sigma-Aldrich); rabbit anti-(pan)-Akt (Cell Signaling).

Real-time RT-PCR

Total RNA isolation (High Pure RNA Isolation Kit; Roche) and cDNA synthesis with poly(T) primers (First Strand cDNA Kit; Fermentas) were performed according to the manufacturers’ protocols. Real-time PCR was performed with a quantitative PCR mix using SYBR-Green following the standard protocol (Eurogentech; ABI PRISM 7700; Applied Biosystems) on a Taqman (Applied Biosystems). RT-PCR was controlled by no template and no reverse transcriptase controls. Relative expression to β-actin was calculated as [1/2^Ct target gene - 2^Ct, actin)]. Primers and fragment size: β-actin, forward 5′AGACCTACGAGCTGTCGAC, reverse 5′AGCACTGGTTGGCCTGAC, 184 bp; cyclin D1, forward 5′AGGAGAGCTGTCGAC, reverse 5′GGCCATCCTCACCTCTTTG, 199 bp.

Statistics

Data are shown as means ± SEM. Statistical significance of differences was determined by the paired two-tailed Student’s t test using Microsoft Excel software. Statistically significant differences are indicated with * for p < 0.05 and ** for p ≤ 0.005. Whisker plots were created using Graph Pad Prism software.

Results

CpG DNA ODNs mediate B cell activation

CpG DNA ODN have been described as potent inducers of polyclonal B cell proliferation. Here we compared short-term proliferation over 72 h measured by [3H]Thy incorporation to long-term proliferation visualized with CFSE dilution over 5 days. When comparing CpG DNA ODN 2006 to BCR cross-linking with anti-human Ig or CD40L stimulation, one major difference became apparent: upon CD40 ligation, proliferation mainly resulting from CD27+ memory B cells was detectable with [3H]Thy incorporation (Fig. 1A) whereas absence of long-term proliferative activity was observed in CFSE dilution assays (Fig. 1B). Similar results were obtained with BCR cross-linking (Fig. 1, A and B); despite an early and comparably strong proliferative response to anti-Ig in [3H]Thy incorporation assays which was seen in both CD27+ and CD27− B cells, CFSE dilution was nearly absent on day 5. In marked contrast, TLR9 stimulation not only induced high proliferation rates at early time points (most prominent in the CD27+ B cell fraction; Fig. 1A), but additionally induced significant CFSE dilution resulting from ongoing cell division over several days (Fig. 1B). Synergistic activity of CD40L was observed when co-stimulating anti-Ig (Fig. 1, A and B) or CpG DNA (Fig. 1B), which...
synergistically increased proliferation rates and CFSE dilution, thus proving the integrity of the CD40L stimulus.

TLR9-induced prolonged B cell proliferation is linked to a B-CLL-like phenotype

B-CLL cells resemble activated B cells. Concomitant expression of CD5, CD23, and CD25 is used as a diagnostic criterion. Similarly, B cell stimulation with CpG DNA ODN induces long lasting up-regulation of activation markers, including those characteristic for B-CLL. This is shown in Fig. 1C which compares B cell expression of CD5, CD23, and CD25 in B cells stimulated with CD40L or CpG DNA ODN 2006 for 5 days. Marked up-regulation of expression of these markers is observed only upon CpG DNA treatment.

Another hallmark of B-CLL is overexpression of the chaperone molecule Hsp-90. Heat shock protein expression is associated with improved cell survival, and targeting Hsp-90 is therefore widely applied in tumor therapy. Here we corroborate previous reports (46–48) showing that B cell stimulation with CpG DNA ODN 2006 induces Hsp-90 expression on mRNA level (Fig. 1D, top) and on protein level (Fig. 1D, bottom). In summary, CpG DNA ODN-mediated prolonged proliferation, CD5, CD23, and CD25 expression along with Hsp-90 induction turns normal human B cells into B-CLL-resembling B cell blasts.

CpG DNA ODN activate both memory and naive B cell fractions

IgM⁺CD27⁺ B cells represent the human counterpart to murine marginal zone B cells (49, 50). These B cells are major players in the immune response against encapsulated bacteria such as pneumococci and Haemophilus. They are strongly reactive to T cell-independent Ags and are thought to represent the cellular origin for B-CLL. Because CpG DNA-stimulated B cells and B-CLL share many phenotypical features, we asked whether CpG DNA ODN target human IgM⁺ memory B cells. Currently, CpG DNA ODN have been described as polyclonal T cell-independent B cell stimuli that predominantly stimulate memory B cells. We therefore first wanted to confirm that TLR9 stimulation mainly activates memory (CD27⁺) B cells. To this end, resting (CD43⁻) B cells were isolated from human PBMCs and stimulated with CpG DNA ODN 2006 or CD40L. Activation marker expression, cell survival, and proliferation were monitored at different time points after stimulation. The main findings are summarized in Fig. 2A, which shows the results from a representative experiment on day 4 after CpG DNA stimulation. The forward/sideward scatter blots (Fig. 2A, left) show cell survival (live gate; P1) in both CD27⁺ and CD27⁻ cell fractions, albeit blast formation is predominant in CD27⁺ memory B cells. We further detected up-regulation of CD23 expression in CD27⁺ B cells.
expression in CD27−/H11002 naive B cells (Fig. 2A, middle) which was seen neither with CD27+/H11001 B cells nor after CD40L stimulation at this late time point (data not shown). In contrast, stimulation of CD27+/H11001 B cells with CpG DNA induced an up-regulation of CD27 expression that was completely absent in naive B cells (Fig. 2A, middle) and was not observed after CD40 ligation (data not shown). These differences confirm the purity of our cell fractions. Furthermore, CD71 expression which was used as a surrogate marker for cell proliferation was brighter in memory B cells (Fig. 2A, right panels), as expected from the results shown in Fig. 1A. Moreover, CD71 expression was highest in an intracellular IgM+/H11001 CD27+/H11001 B cell subset. We concluded that CpG DNA ODNs target both memory and naive B cells although proliferation is, indeed, most pronounced in IgM+/H11001 CD27+/H11001 B cells.

CpG DNA ODN target IgM+/H11001 B cells

To further assess which B cell subset represents the main target for CpG DNA ODN, we stimulated total CD19+/H11001 B cells with CpG DNA ODN 2006 or CD40L and analyzed intracellular IgM and IgG expression on day 0 and at different time points after stimulation. Fig. 2B shows the data obtained from three donors, thus summarizing the main result of these experiments: CpG DNA stimulation shifts Ig expression to IgM which is neither present on day 0 nor visible after CD40 stimulation. Moreover, CpG DNA-induced IgG expression is absent (Fig. 2B, donor 1) or negligible (Fig. 2B, donors 2 and 3). On the basis of these experiments, we

FIGURE 2. Cellular target of CpG DNA 2006. A, CD27−/H11002 (bottom row) and CD27+ B cells (top row) were isolated from resting (CD43+/H11002) peripheral blood B cells and stimulated with CpG DNA ODN 2006 (0.5 μM). Cells were analyzed on day 4 post stimulation for FSC/SSC properties (left), CD23 and CD27 expression (middle), and intracellular IgM and CD71 surface expression (right). One representative experiment of four is shown. B, CD19+/H11001 B cells were isolated form PBMC and analyzed for IgM and IgG expression on days 0 and 5 after stimulation with CpG DNA ODN 2006 or CD40L. Results are for three representative experiments of five independent experiments. C, IgM+ and IgM−/CD19+ B cell fractions were isolated from PBMCs. Cells were analyzed after 4 days of stimulation for FSC/SSC properties (left), CD23 and CD27 expression (middle), and intracellular IgM and CD71 surface expression (right). Results are for one representative donor of six.

FIGURE 3. TLR9-mediated up-regulation of ZAP70 in peripheral B cells. A, Human CD19+/H11001 peripheral blood B cells were stimulated with CpG DNA ODN 2006. On day 6, cells were stained with Alexa 488-conjugated anti-ZAP70 mAb or the corresponding isotype. 4′,6-Diamidino-2-phenylindole (DAPI) was used for counterstaining of the nuclei. The images show one representative experiment of ≥4. B, ZAP70 expression was quantified by intracellular flow cytometric analysis on days 0 and 6 in CD40L (40L) or CpG DNA ODN 2006-stimulated CD19+/H11001 B cells. Results are summarized frp from three experiments as mean values ± SEM for MFI (ΔMFI), *p = 0.04, CD40L:CpG. C, Expression of ZAP70 in B cell lysates generated on day 4. The results of two independent donors from 6 donors are shown. GAPDH was used as a loading control.

CpG DNA ODN target IgM+/H11001 B cells

To further assess which B cell subset represents the main target for CpG DNA ODN, we stimulated total CD19+/H11001 B cells with CpG DNA ODN 2006 or CD40L and analyzed intracellular IgM and IgG expression on day 0 and at different time points after stimulation. Fig. 2B shows the data obtained from three donors, thus summarizing the main result of these experiments: CpG DNA stimulation shifts Ig expression to IgM which is neither present on day 0 nor visible after CD40 stimulation. Moreover, CpG DNA-induced IgG expression is absent (Fig. 2B, donor 1) or negligible (Fig. 2B, donors 2 and 3). On the basis of these experiments,
up-regulated in the IgM

results obtained in these experiments are shown in Fig. 2 with CpG DNA ODN 2006 or CD40L the next morning. The key protocol, B cells were left unstimulated overnight and stimulated should be found in this fraction (Fig. 2)

Because B-CLL cells are thought to originate from IgM

TLR9-mediated B cell stimulation induces ZAP70 expression

FIGURE 4. Role of MyD88 and Hsp-90 in CpG DNA-stimulated ZAP70 expression. A, Splenic CD19+ B cells were isolated from WT and MyD88+/− mice. Cells were stimulated with CpG DNA ODN 1668 (CG) or anti-mouse Ig at 20 μg/ml. ZAP70 expression was measured on day 3 by flow cytometry. The results are given as mean values ± SEM of ΔMFI of four independent experiments. **, p = 0.003, WT/CpG:MyD88−/−/CpG. B, CD43+ CD27+ and CD43+ CD27− human B cells were isolated from PBMCs and stimulated with CpG DNA ODN 2006. ZAP70 expression was assessed on day 4. Costaining was performed with anti-CD20 (left) and anti-IgM (right). Results are for one representative experiment of four. C, CD19+ B cells were stimulated with CpG DNA 2006 ODN. On day 6 anti-ZAP70 staining (bottom) was compared with the isotype control (top). Costaining was performed with anti-CD38 to stain plasmablasts. D–F, Effect of Hsp-90-inhibitor 17-AAG on CpG DNA ODN 2006-stimulated CD19+ B cells. Analysis on day 3. D, ZAP70 expression. Results summarize the mean values ± SEM of six experiments, *, p = 0.04, CpG DMSO:CpG+ 17-AAG). E, Proliferation was assessed by ['H]TdR incorporation. Mean values ± SEM of four experiments are given. *, p = 0.017, CpG+DMSO:CpG+ 17AAG 1 μM. F, Cell viability was measured as a percentage of propidium iodide and annexin V- cells. The mean values ± SEM of six experiments are shown. **, p = 0.006, CpG+DMSO:CpG+ 17AAG 1 μM.

decided to compare IgM+ to IgM− B cells to assess whether CpG DNA ODN would predominantly activate IgM− B cells.

To address this question, we isolated IgM− B cells. After depletion of residual IgM+ cells from PBMC, we isolated the IgM− fraction of B cells using CD19+ microbeads. As in our routine protocol, B cells were left unstimulated overnight and stimulated with CpG DNA ODN 2006 or CD40L the next morning. The key results obtained in these experiments are shown in Fig. 2C; cell survival and blast formation were predominant in the IgM+ B cell fraction (Fig. 2C, left), CD23 induction was observed only in the IgM− B cells. This is well suitable with the fact that all naïve B cells should be found in this fraction (Fig. 2C, middle); CD27 was strongly up-regulated in the IgM+ population, whereas only a small fraction of B cells in the IgM− fraction became CD27+ (2.5 ± 1.5%). The latter cells may correspond to a residual IgM+CD27+ population (Fig. 2C, middle). This was supported by the finding that intracellular IgM expression correlated with CD71 expression in IgM+ B cells and CD71 was up-regulated only in a very small B cell subset in the IgM− fraction that stained IgM+ (Fig. 2C, right). We concluded that, indeed, the effects of B cell-active CpG DNA ODN are due to activation and expansion of IgM+ B cells.

TLR9-mediated B cell stimulation induces ZAP70 expression

Because B-CLL cells are thought to originate from IgM+ B cells and microbial stimuli are currently believed to drive progression of B-CLL (15, 34–37), we wanted to examine the TLR9-activated B cell phenotype more closely. We therefore asked whether ZAP70 that has been associated with an unfavorable prognosis could be induced with TLR9 ligands in B cells from healthy donors: Indeed, when CD19+ human peripheral blood B cells were stimulated with CpG DNA ODN 2006 for 6 days, ZAP70 was detected in individual, mostly large B cell blasts, with a cytoplasmic staining pattern (Fig. 3A).

Next, we wanted to know whether ZAP70 expression in human B cells represents an exclusive hallmark of TLR9-mediated B cell activation. To this end, we compared human CD19+ peripheral blood B cells stimulated with CpG DNA ODN 2006 with B cells stimulated with CD40L. Flow cytometric analysis revealed that despite detection of basal (low) ZAP70 expression in unstimulated and CD40L-treated cells, only CpG DNA ODN induced a strong and long-lasting increase in ZAP70 expression (Fig. 3, B and C). Similarly, stimulation with TLR2-active lipopolysaccharides and anti-human Ig (5, 10, and 20 μg/ml) failed to induce relevant increases in ZAP70 expression measured by flow cytometry and immunofluorescent microscopy (data not shown).

The immunofluorescence data were confirmed by Western blot analysis (Fig. 3C). Here, ZAP70 was detectable in both CpG DNA- and CD40L-stimulated B cells which is well explainable by the flow cytometric finding of basal ZAP70 levels in all B cells (Fig. 3, B and C). These basal levels are conserved with CD40L stimulation. Cellular hypertrophy visualized in the forward light scatter (FSC)-side light scatter (SSC) blots in Fig. 2 and by the increase in GAPDH expression as well as total protein content accompanies the elevated ZAP70 levels in CpG DNA ODN 2006-stimulated B cells when compared with CD40L stimulation. Donor variability after CD40 ligation may be explained by donor-dependent differences in the percentual representation of naïve and memory B cell populations or cellular preactivation (compare Fig. 1A).

Next, we tested whether ZAP70 expression after CpG DNA ODN stimulation would depend on MyD88, the key signaling molecule in TLR signaling. Splenic CD19+ B cells from
MyD88-deficient and WT control mice were stimulated with CpG DNA ODN 1668-PTO or anti-mouse Ig. As seen in Fig. 4A, CpG DNA ODN also induced ZAP70 expression in murine B cells; this was dependent on the presence of MyD88. As previously described (14), and in marked contrast to our findings in human B cells, ZAP70 expression in murine B cells was also inducible via BCR cross-linking, which was independent of MyD88 (Fig. 4A). These data indicated that in human B cells ZAP70 is expressed in response to a specific signal delivered by TLR9 agonists.

**TLR9-induced ZAP70 expression is highest in memory B cells and does not require terminal B cell differentiation**

Next we assessed which TLR9-responsive B cell subset would be prone to express higher levels of ZAP70. To this end, resting (CD43<sup>−</sup>), CD27<sup>+</sup>, and CD27<sup>−</sup> B cells were isolated and stimulated with CpG DNA ODN 2006 or CD40L. ZAP70 expression was analyzed on day 4. Fig. 4B summarizes the results from a representative experiment. All isolated cells stained positive for CD20, confirming the purity of the isolated fractions (Fig. 4B, left). ZAP70 expression was higher in CD27<sup>+</sup> B cells than in CD27<sup>−</sup> B cells. IgM<sup>−</sup> memory B cells expressed the comparably highest levels of ZAP70 (Fig. 4B, right). However, the isolation of CD43<sup>−</sup> resting B cells resulted in generally lower ZAP70 levels than those measured in CD19<sup>+</sup> B cells. This indicates that cellular preactivation, e.g., by positive selection or CD19-induced PI3K activation (51), may positively influence ZAP70 expression.

Because previous reports have associated B cell expression of ZAP70 with activation, terminal differentiation, and CD38 expression (8–10) and because CpG DNA ODNs have been demonstrated to induce terminal B cell differentiation, we argued that the detection of ZAP70 in TLR9-stimulated B cells could arise from plasmablasts. We therefore compared ZAP70 expression in CD138<sup>−</sup> B cells and CD138<sup>+</sup> (syndecan-1<sup>−</sup>) plasmablasts and in CD38<sup>−</sup> and CD38<sup>+</sup> B cell populations. ZAP70 MFI was enhanced in gated CD138<sup>−</sup> B cells and CD138<sup>+</sup> (syndecan-1<sup>−</sup>) plasmablasts and in CD38<sup>−</sup> and CD38<sup>+</sup> B cell populations. ZAP70 MFI was enhanced in gated CD138<sup>−</sup> B cells and CD138<sup>+</sup> (syndecan-1<sup>−</sup>) plasmablasts and in CD38<sup>−</sup> and CD38<sup>+</sup> B cell populations. Moreover, CD38 and ZAP70 expression were nearly absent in freshly isolated B cells from the majority of healthy donors but were detected in B cell blasts when B cells were isolated from buffy coats which were mainly used for the Western blot analyses (Fig. 6B). This indicated that ZAP70 expression could, indeed, be linked to the degree of cellular preactivation.

**Inhibition of Hsp-90 correlates with reduced ZAP70 expression**

Geldanamycin and its derivatives interfere with cell survival by blocking the interaction of the chaperone molecule Hsp-90 with its client proteins, e.g., ZAP70 (22, 52) and PKB (53–56), thus exposing them to ubiquitinylation and degradation (57). Because we found that ZAP70 mRNA levels were regulated only to the extent of variations in housekeeping gene expression (data not shown), we asked whether inhibition of the Hsp-90 chaperone function would affect ZAP70 protein expression. Indeed, treatment with the geldanamycin derivative 17-AAG decreased ZAP70 protein expression (Fig. 4C). Therefore, intact chaperone function of Hsp-90 is required for the induction of stable ZAP70 protein expression in human B cells.

Moreover, titrative analyses demonstrated that 17-AAG-mediated inhibition of ZAP70 expression was observed only at concentrations, e.g., ≥1 μM, that interfered with B cell proliferation and survival (Fig. 4, D and E). This prompted us to investigate whether ZAP70 expression could represent an indicator of cell viability or proliferative potential.

**ZAP70 expression is associated with B cell proliferation**

To test this hypothesis, ZAP70 expression in B cells stimulated in the presence of BrdU was compared in BrdU<sup>+</sup> and BrdU<sup>−</sup> B cell populations (Fig. 5A). The data obtained revealed that ZAP70 expression in response to TLR9 stimulation is highest in the proliferating BrdU<sup>+</sup> subset. Similar results were observed after stimulation with R848, a synthetic TLR7 agonist, with and without sensitization of B cells for TLR7 ligands with IFN-α (Fig. 5B). These data proved that ZAP70 expression accompanies B cell proliferation.

These results were further corroborated by the finding that TLR9 stimulation but not CD40 ligation induced expression of cyclin A2 mRNA, a molecule expressed in the S phase of the cell cycle. Interestingly enough, an increase of cyclin A2 mRNA was also observed after stimulation with the GpC DNA control ODN (Fig. 5B).

**PKB signaling is involved in the induction of ZAP70 expression**

Because targeting Hsp-90 also interferes with protein stability of PKB, an enzyme that plays a central role in cell cycle entry
GpC control ODNs stimulate ZAP70-expressing IgM+ B cells in a TLR9-dependent manner

Because up-regulation of cyclin A2 was a rather surprising finding, we investigated whether B cells stimulated with GpC control ODN expressed ZAP70 (Fig. 6, A and B). Interestingly enough, kinetics revealed that in the majority of donors GpC ODN-induced ZAP70 levels were comparable with those of CpG DNA until day 4 or 5 of stimulation. Differences in ZAP70 expression in most donors only became evident starting with day 5 or 6. This indicated that GpC ODN stimulation of B cells closely resembles that triggered by CpG DNA ODN.

Moreover, extensive Western blot studies revealed that, similar to CpG DNA, GpC ODN up-regulate PKB. In the majority of donors, PKB elevation is sustained until day 4 or 5, as already observed for ZAP70, but declines afterward (Fig. 6B). On day 6, ZAP70 and PKB expression are lower in GpC DNA ODN-stimulated B cells than with CpG DNA ODN treatment. ZAP70 and PKB levels are elevated after 6 days of CpG DNA stimulation in comparison with unstimulated (day 0) levels and are well compatible with cellular hypertrophy, e.g., elevated actin levels per cell. Detectable ZAP70 on day 0 indicates cellular preactivation as explained previously.

Most importantly, these observations were completed by an induction of Hsp-90 protein expression (data not shown) and B cell proliferation in response to GpC ODN (Fig. 6C). Thus, GpC ODN stimulated B cells in a manner similar to that of CpG DNA ODN, albeit over a shorter time span with markedly lower cell cycling. GpC DNA-induced B cell proliferation was further found to be dependent on TLR9 given that GpC and CpG DNA ODN failed to promote CFSE dilution in TLR9<sup>−/−</sup> B cells whereas TLR2-active lipopeptides did (Fig. 6C). Moreover, in analogy to CpG DNA ODN, GpC ODN stimulated IgM<sup>+</sup> B cells (Fig. 6D).

**TLR9-induced PKB signaling is a prerequisite for ZAP70 expression**

To test whether inhibition of PKB signaling interferes with ZAP70 expression, we stimulated human B cells with CpG DNA in the presence and absence of wortmannin, a PI3K inhibitor acting upstream of PKB (62), and with rapamycin, an inhibitor of mammalian target of rapamycin, an enzyme involved in the execution of PKB-mediated G1- to S-phase transition (63). These treatments lead to partial (wortmannin) or complete loss (rapamycin) of CpG DNA-induced B cell blasts (Fig. 7A, top), which were phenotyped as CD27<sup>+</sup>CD71<sup>+</sup> (proliferating) B cells (Fig. 7A, bottom). Loss of B cell blasts was accompanied by a reduction in CpG DNA-induced ZAP70 MFI (Fig. 7B, left). As an alternate approach, ZAP70 expression was assessed in Ramos B cells cultured in the presence or absence of rapamycin, wortmannin, or the NF-κB inhibitor (Bay-11-7082) (Fig. 7B, right). Because Ramos B cells represent drug-resistant tumor cells, this experimental setup enabled us to study the effects of the inhibitors on ZAP70 expression while avoiding some of their toxic...
effects on cellular proliferation and survival that affect primary B cells. These experiments revealed that ZAP70 expression was inhibited by rapamycin but was not affected by wortmannin or Bay-11-7082, indicating that ZAP70 expression in Ramos B cells occurred independently of NFκB or PI3K activation, the latter most likely being circumvented by constitutive activation of PKB downstream of PI3K as frequently encountered in B-CLL and other malignancies (64, 65). We can only speculate that the incomplete block in blast formation in wortmannin-treated primary B cells is due to cellular preactivation, e.g., possibly of PI3K, that counteracts the effects of wortmannin.

Expression of myrPKB restores ZAP70 expression and proliferation after CD40 ligation

Because our data indicated that in B cells ZAP70 expression and long-term proliferation are linked to the duration of PKB induction, we reasoned that expression of a constitutively active form of PKB may facilitate CD40-mediated B cell proliferation and ZAP70 expression. To test this hypothesis, we compared B cell proliferation and ZAP70 expression from WT and myrPKB tg mice. Although no difference in ZAP70 expression of WT and myrPKB tg B cells could be detected before stimulation (data not shown) myrPKB tg B cells displayed enhanced proliferation (Fig. 7A) and ZAP70 expression (Fig. 7C) compared with WT B cells. However, proliferation rates and ZAP70 expression levels after CD40 stimulation with the 3/23 mAb were similar in WT and myrPKB tg mice. Proliferation was assessed by BrdU incorporation (relative light units; RLU). Results were obtained from six independent mice. Because our data indicated that in B cells ZAP70 expression and proliferation after CD40 ligation are the effects of wortmannin.

FIGURE 7. Role of PKB in ZAP70 induction. A: CD19+ B cells or Ramos B cells (submitted to 4 h of serum starvation) were treated with rapamycin (Rap), wortmannin (Wort), Bay-11, or the vehicle control (DMSO; D) as indicated. Primary B cells were stimulated with CpG DNA ODN 2006. A, Flow cytometric analysis on day 3. Top row, B cell blasts in the FSC/SSC analysis are marked by arrows. Bottom row, Percentages of activated (CD27+) and proliferating (CD71+) B cells are indicated. Results are for one representative experiment of three. B, ΔMFI for ZAP70. Left: CD19+ B cells on day 3. Results are the mean values ± SEM from three independent experiments. *, p = 0.035, CpG/DMSO:CpG/rapamycin. Right, Ramos B cells treated with the inhibitors for 4 days. Results are ΔMFIs ± SEM for ZAP70. Results are summarized from three independent experiments. **, p = 0.01, DMSO: rapamycin.

Expression of myrPKB restores ZAP70 expression and proliferation from WT and myrPKB tg mice. Although no difference in ZAP70 expression of WT and myrPKB tg B cells could be detected before stimulation (data not shown) myrPKB tg B cells displayed enhanced proliferation (Fig. 7A) and ZAP70 expression (Fig. 7C) compared with WT B cells. However, proliferation rates and ZAP70 expression levels after CD40 stimulation with the 3/23 mAb were similar in WT and myrPKB tg mice. Proliferation was assessed by BrdU incorporation (relative light units; RLU). Results were obtained from six independent mice. D, ZAP70 expression in murine B cells on day 3 after anti (a)-CD40 or CpG DNA ODN 1668 treatment in WT and myrPKB tg mice (n = 6 mice each).

Discussion

The study presented here demonstrates that TLR9-stimulated B cells acquire a phenotype similar to B-CLL cells, including ZAP70 expression (Figs. 1 and 2). We provide evidence that ZAP70 expression is linked to sustained proliferation (Fig. 4) and to TLR-mediated long-lasting up-regulation of PKB (Figs. 4 and 5). We further show that both CpG and GpC DNA ODNs target IgM+ B cells (Figs. 6 and 7), the precursor cells for B-CLL. We conclude that B cell expression of ZAP70 is not only is a hallmark of B-CLL but is also connected to specific proliferation and differentiation processes in normal IgM+ B cells.

TLR7 and TLR9 agonists represent very potent stimuli for B cell activation and subsequent polyclonal human B cell expansion and Ig secretion (Fig. 1 and Refs. 41, 58, and 66–69). In addition, CpG DNA stimulation has been demonstrated to enhance B cell survival by promoting the expression of antiapoptotic molecules such as Bcl-xL and Bcl-2 and of heat shock proteins that stabilize protein expression of prosurvival molecules such as PKB (46, 47, 70, 71). Surprisingly, only a few studies have reported activity of GpC control ODN (72). Here we show that these ODN potentiate IgM+ B cells via TLR9 and that although activation by GpC DNA ODN displays a briefer kinetic they potentiate ZAP70 expression.

Thus far, TLR9-stimulatory CpG DNA ODN have been described as polyclonal B cell activators promoting the expansion of memory B cells. However, the activation of naive B cells in the absence of a BCR stimulus has remained a controversial issue (58, 66, 73, 74). Here we demonstrate that CpG DNA ODN stimulate both naive and memory peripheral blood B cells in the absence of
BCR cross-linking. CpG DNA stimulation results in the up-regulation of distinct activation markers, e.g., CD23 in naïve B cells and high expression of CD27 in memory B cells (Fig. 2). Moreover, CD27 up-regulation in memory B cells is associated with CD5 and CD38 up-regulation (data not shown). Therefore, CpG DNA-activated B cells differ from B-CLL cells that characteristically display concomitant CD5 and CD23 expression. In contrast to a recent report (73), we did not observe transitional (CD38+CD5− and CD38−CD23+) B cells in our naïve B cell fractions and subsequently did not detect CD27 up-regulation in the CD27− fraction. This may reflect differences in the cell isolation procedure, stimulatory conditions, and time points of analysis. Interestingly enough, a recent report described surface expression of TLR9 on two B cell subsets (CD27+CD80+ and a CD27−CD23−) that resemble the phenotype that in our hands is induced upon CpG DNA stimulation (75).

Although TLR-induced proliferation and cell survival is tightly linked to activation of NFκB (76–78), lately several groups focused on other TLR-mediated cellular activation pathways including PKB signaling (79–84). PKB induction is generally associated with cell survival and induction of cell cycling (60, 85–88), the two features accompanying TLR-induced ZAP70 expression. Pathological activation of PKB has been demonstrated in neoplasias including B-CLL where it promotes drug resistance (27, 89) by inactivating proapoptotic molecules (90), mediating cytoplasmic sequestration of transcription factors involved in promoting cell death (91) and nuclear translocation of NFκB with subsequent transcription of NFκB-dependent survival genes (92, 93) as well as supporting cell cycle entry (60, 61). Western blot analysis of PKB expression in activated normal B cells revealed that PKB expression is prolonged in B cells stimulated via endosomally located TLR9 when compared with B cells stimulated via extracellular CD40 ligation (Fig. 5C). In support of this finding, engagement of the cell surface receptors such as CD40 and the LPS receptor TLR4 has been shown to provoke a transient activation of PKB in different cell types but fails to induce long-lasting B cell proliferation (Refs. 74 and 94–98, Fig. 1, and data not shown). Furthermore, isolation of B cell blasts from 2-day CpG DNA-stimulated B cell cultures by density gradient centrifugation did not affect prolonged cell survival despite the absence of CpG DNA in the fresh culture medium (I. Bekeredjian-Ding, unpublished data). Endosomal retention of CpG DNA ODN along with a comparably long half-time life (99) may therefore be responsible for prolonged PKB activation. Duration of PKB signaling may represent a major determinant regulating the extent of cellular turnover and associated ZAP70 expression. Additionally, the resulting cellular hyperactivity and increased protein content facilitate the detection of ZAP70. This is evident when comparing the increase in sensitivity from fluorescent microscopy and Western blotting to flow cytometry.

Infections are increasingly being discussed in the context of B-CLL pathogenesis (15, 34–37, 100). Thus, both microbial Ag recognition via the BCR or by pattern recognition receptors such as TLRs might play a role in promoting B-CLL cell survival and sustained proliferation. Indeed, B-CLL cells have been shown to be responsive to TLR ligands, especially to CpG DNA ODN (21, 31–33). In a recent publication, Longo et al. (21) demonstrated that ZAP70+ B-CLL cells respond to CpG DNA stimulation with proliferation, whereas ZAP70 low to absent B-CLL cells were driven into cell cycle arrest and apoptosis. These two different outcomes reflected differences in PKB activation. PKB overexpression in noncycling B-CLL cells shifted the response to survival and proliferation. In our study, B cells from healthy donors responded to TLR9 stimulation by proliferation and enhanced ZAP70 expression, thus resembling the ZAP70+ B-CLL subset. Strong B cell stimuli such as CpG DNA ODN-triggered activation of TLR9 enhanced PKB expression and induced B cell cycling independently of myrPKB expression (Fig. 7C). In marked contrast, weaker stimuli such as CD40 ligation lacked strong proliferative activity (Figs. 1–7), despite their positive effects on B cell survival, which have been attributed to a transient activation of PKB (74, 94, 95, 98). In fact, expression of constitutively active PKB enabled CD40-mediated induction of B cell proliferation and up-regulation of ZAP70 expression (Fig. 7, C and D). Thus, pathologically elevated PKB levels may lower the threshold for recognition of weak endogenous or microbial stimuli, which may then be sufficient to drive continuous cellular proliferation in B-CLL.

From a clinical point of view, our results emphasize the potency of CpG DNA ODN as a B cell stimulus. In our hands, CpG DNA ODN stimulated IgM+ B cells only (Fig. 2C). ZAP70 expression was highest in memory B cells (Fig. 4B), a finding that is well compatible with the higher proliferation rates observed in Fig. 1A. Because IgM+ CD27− B cells are thought to represent the cellular origin for B-CLL, treatment with CpG DNA ODN over prolonged periods of time should be considered a potential risk factor for the development of an monoclonal lymphocytosis of unknown significance (101–103) or a clinically relevant B-CLL until refuted.

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Disclosures

The authors have no financial conflict of interest.

References

TLR9-INDUCED B CELL EXPRESSION OF ZAP70


