B-Cell Lymphomas Differ in their Responsiveness to CpG Oligodeoxynucleotides

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ABSTRACT

Human B cells detect CpG motifs within microbial DNA via TLR9. Synthetic CpG oligodeoxynucleotides are currently being tested in clinical trials for the therapy of different types of B cell non-Hodgkin's lymphoma. However, there is only limited information on the CpG oligodeoxynucleotide sensitivity of primary malignant B cells of different non-Hodgkin's lymphoma entities. Here we found that most B-cell malignancies except plasmacytoma respond to CpG oligodeoxynucleotides by up-regulating expression of costimulatory and antigen-presenting molecules, by increasing expression of CD20, and by proliferation. In an *in vitro* analysis of 41 individual patient-derived primary tumor samples, B-cell chronic lymphocytic leukemia (B-CLL) and marginal zone lymphoma showed the strongest activation upon stimulation with CpG oligodeoxynucleotides. Small lymphocytic lymphoma, follicular lymphoma, mantle cell lymphoma, and large cell lymphoma showed an intermediate response. Consistent with CpG oligodeoxynucleotides sensitivity, TLR9 mRNA was present in B-CLL but absent in plasmacytoma. Although CpG oligodeoxynucleotides induced proliferation in all CpG oligodeoxynucleotide–sensitive types of B-cell malignancies, proliferation was weaker than in normal B cells and at least for B-CLL was followed by increased apoptosis. In conclusion, B-cell malignancies show significant differences in their responsiveness to CpG oligodeoxynucleotides. Focusing clinical studies on patients with highly CpG oligodeoxynucleotide–sensitive B-cell malignancies may improve the clinical outcome of such trials.

INTRODUCTION

The vast majority of lymphoid neoplasms worldwide are derived from B lymphocytes at various stages of differentiation. Neoplasms originating from precursor B cells are rare. Neoplasms derived from mature naive B cells include B-cell chronic lymphocytic leukemia (B-CLL), B-cell small lymphocytic lymphoma (SLL), and mantle cell lymphoma (MCL). Follicular lymphoma (FL) and diffuse large B-cell lymphoma (LCL) are derived from germinal center B cells. Memory B cells can develop into marginal zone B-cell lymphoma (MZL) and B-CLL. Plasmacytoma is related to plasma cells (1). The most typical lymphomas are of diffuse large B-cell type (33%) followed by B-cell follicular lymphoma (22%). All other types have a frequency of <10% (2, 3).

Events of normal B-cell differentiation are relevant for understanding the biology of B-cell neoplasia. These include antigen receptor (immunoglobulin) gene rearrangement, somatic mutations of the immunoglobulin variable region genes, receptor editing, immunoglobulin heavy chain class switch, and differential expression of a variety of adhesion molecules and receptor proteins as the B cell progresses from a precursor B cell to a mature plasma cell. An algorithm can be used to establish diagnosis on the basis of gene expression, immunophenotype, morphology, homing patterns, and proliferation fraction (2).

Mainstays of treatment for non-Hodgkin lymphoma have been chemotherapy and radiotherapy. High toxicity associated with these treatments is based on the lack of selectivity for proliferating malignant B cells. Newer developments, such as the use of B cell–specific monoclonal antibodies, show reduced toxicity based on their ability to selectively target the B-cell compartment. Rituximab is a monoclonal antibody that binds specifically to the B-cell surface antigen CD20 (4). Enhanced clinical regression of lymphoma mediated by B cell–specific monoclonal antibody therapy can be achieved by linking radioisotopes to the anti-CD20 antibody (5) although the relative role of unlabeled versus radiolabeled anti-CD20 remains an important area of investigation.

Another strategy to target the B-cell compartment is based on the expression by B cells of TLR9. TLR9 belongs to the family of Toll-like receptors that evolved to recognize pathogen-derived microbial molecules leading to the activation of the corresponding cell. TLR9 detects CpG motifs within bacterial or viral DNA (6–9). CpG motifs are unmethylated CG dinucleotides within particular sequence contexts (10, 11). Synthetic oligonucleotides that contain such CpG motifs (CpG oligodeoxynucleotides) mimic microbial DNA. The CpG oligodeoxynucleotide 2006 (synonymous: oligodeoxynucleotide 7909) was originally developed based on its ability to activate normal

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human B cells (12). This oligodeoxynucleotide is currently being tested in clinical trials as a vaccine adjuvant and for immunotherapy of cancer including non-Hodgkin's lymphoma (13). In murine lymphoma models, CpG oligodeoxynucleotide was found to be effective as adjuvant for anti-idiotype vaccines (14), to enhance monoclonal antibody therapy (15, 16) and as monotherapy (17). In humans, the only two cell types known to express TLR9 are B cells and plasmacytoid dendritic cells (PDC; refs. 18, 19). The PDC is the major type I IFN producing cell. Due to the potent immunomodulatory role of PDC (20, 21), stimulation of PDC may contribute to the therapeutic activity of CpG oligodeoxynucleotides against B-cell malignancies.

Several studies have established that B-CLL cells respond to stimulation with CpG oligodeoxynucleotides (22–24). Whereas we know from clinical studies that non-Hodgkin's lymphoma are extremely heterogeneous, there is only limited information on CpG sensitivity of B-cell malignancies other than B-cell lymphocytic leukemia. In the present study, we show that B-cell malignancies differ in their response to CpG oligodeoxynucleotide 2006. Furthermore, we show that B-CLL cells express TLR9 suggesting that B-CLL cells directly respond to CpG oligodeoxynucleotides; in contrast, unresponsiveness of plasmacytoma cells was associated with a lack of TLR9. Finally, we answer the important question of whether CpG oligodeoxynucleotides support malignant B-cell proliferation and survival, or induce malignant B-cell death.

MATERIALS AND METHODS

Isolation of Cells and Cell Culture. Lymph nodes from patients with different types of B-cell lymphoma or with follicular hyperplasia (Table 1) were obtained from the operating

Table 1 Patient characteristics, laboratory findings, sample histology, and sample source

Sample code	Source	Purity (%)	Age (y)	Sex	WBC (4-10 × 10^3cells/μL)
FH-111298	lymph node	89	61	F	7.3
FH-250200	lymph node	36	24	M	9.3
FH-300300	lymph node	36	56	F	7.6
MCL-021199	lymph node	85	62	M	6.6
MCL-130898	lymph node	95	52	F	8.8
MCL-210600	peripheral blood	80	71	F	48.2
MCL-021299	lymph node	80	65	M	ND
SLL-300899	lymph node	98	72	M	4.2
SLL-190598	lymph node	90	63	M	11.1
SLL-231297	lymph node	84	64	F	8.9
SLL-220200	lymph node	32	35	M	7.0
SLL-260298	lymph node	88	53	M	48.8
CLL-070200	peripheral blood	92	57	M	76.0
CLL-140200	peripheral blood	85	60	M	61.0
CLL-150200	peripheral blood	96	76	M	113.0
CLL-280200	peripheral blood	95	54	M	108.3
CLL-060300	peripheral blood	88	69	M	34.8
CLL-100300	peripheral blood	39	72	F	22.0
CLL-170300	peripheral blood	77	60	M	12.5
CLL-230300	peripheral blood	94	68	M	83.2
CLL-280300	peripheral blood	57	77	F	124.9
CLL-230600	peripheral blood	83	86	M	ND
CLL-240600	peripheral blood	91	63	M	85.0
MZL-051199	lymph node	29	56	F	3.7
MZL-030300	lymph node	95	70	M	4.9
MZL-011299	lymph node	80	67	M	ND
FL-080200	lymph node	73	29	M	6.0
FL-030500	lymph node	71	55	M	18.3
FL-081298	lymph node	85	57	M	5.4
FL-300300	lymph node	38	68	F	7.4
FL-220200	lymph node	89	31	M	11.6
LCL-140198	bone marrow	59	75	F	5.2
LCL-230300	pleural fluid	12	83	M	5.2
LCL-100999	lymph node	7.4	57	M	11.9
MM-160501	bone marrow	<5	59	F	3.5
MM-310501	bone marrow	<5	70	M	10.6
MM-180601	bone marrow	>50	48	F	4.1
MM-050701	bone marrow	<5	48	F	3.8
MM-100901	bone marrow	<5	47	M	8.5
MM-181001	bone marrow	>15	54	M	1.4
MM-1311011	bone marrow	<5	81	F	8.7
MM-1311012	bone marrow	<5	61	M	4.7
PCL-210801	peripheral blood	74	54	M	124.0
PCL-260901	peripheral blood	>99	62	F	188.4

Abbreviations: FH, follicular hyperplasia; MCL, mantle cell lymphoma; SLL, small lymphocytic lymphoma; MZL, marginal zone lymphoma; FL, follicular lymphoma; LCL, large cell lymphoma; MM, multiple myeloma; PCL, plasma cell leukemia; ND, not determined.

room and minced with a scalpel under aseptic conditions. The resulting suspension was passed sequentially through a sterile sieve-tissue grinder containing a nylon mesh screen, a 150-μm mesh screen, and a 60-μm mesh screen. Mononuclear cells from lymph node samples, peripheral blood mononuclear cells or pleural fluid were prepared as described previously (22). For plasmacytoma, mononuclear cells were obtained from bone marrow aspirates. For analysis, cells were resuspended in RPMI 1640 containing 10% (v/v) heat-inactivated (56°C, 30 minutes) FCS (HyClone, Logan, UT), 1.5 mmol/L L-glutamine (all from Life Technologies, Grand Island, NY) and incubated on a 96-well plate (1×10^6 cells/mL).

For the analysis of TLR9 mRNA expression in normal B-cell subsets, specimens of nasopharyngeal tonsils were obtained from children (3-7 years) undergoing surgery of nasopharyngeal hyperplasia. Mononuclear cells were prepared as described previously (25). B cells were isolated by magnetically activated cell sorting 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 plasmacytoid dendritic cells detectable (<0.1%). Viability (>95%) was determined by trypan blue exclusion. $CD19^+$ B cells were stained with FITC-labeled anti-IgD (clone G18-158, BD PharMingen, San Diego, CA) and APC-labeled anti-CD38 (clone HIT2, BD PharMingen). Naive B cells (IgD^+ $CD38^-$), memory B cells (IgD^- $CD38^-$), and germinal center B cells (IgD^- $CD38^+$) were obtained by cell sorting using a MoFlo high speed fluorescence activated cell sorter (Cytomation, Inc., Fort Collins, CO). For the isolation of B cell subsets from peripheral blood, total B cells were stained with FITC-labeled anti-IgD (clone G18-158, BD PharMingen), and PE-labeled anti-CD27 (clone M-T271; BD PharMingen). The use of human tissues for research purposes was approved by the local ethics committee.

Oligonucleotides. Nuclease-stable phosphorothioate-modified oligonucleotides were provided by Coley Pharmaceutical Group, Inc. (Wellesley, MA). Endotoxin levels in all oligodeoxynucleotide were <0.075 EU/mL as determined by the *Limulus* amebocyte assay (LAL assay, Bio Whittaker, Walkersville, MD): CpG oligodeoxynucleotide 2006, 5′-TCGTCG-TTTTGTCGTTTTGTCGTT-3′; control oligodeoxynucleotide 2017, 5′-CCCCCCCCCCCCCCCCCCCC-3′. Oligonucleotides were added at a final concentration of 5 μg/mL.

Flow Cytometry. Surface antigens were stained as described previously (25). Monoclonal antibodies to CD5 (UCHT2), CD19 (SJ25C1), CD40 (5C3), CD54 (HA 58), CD56 (B159), CD64 (10.1), CD80 (L307.4), CD86 (IT2.2), CD95 (DX2), MHC-I (G46-2.6), MHC-II (TÜ39) as well as appropriate isotype controls were purchased from PharMingen. Monoclonal antibodies to CD38 (T16), CD138 (B-B4), IgGκ and IgGλ were purchased from Immunotech (Coulter, Marseille, France). C2B8, a monoclonal chimeric human/mouse anti-CD20 antibody, was purchased from IDEC Pharmaceuticals (San Diego, CA). C2B8 were labeled with FITC according to standard protocols (26). Malignant B cells were identified by surface CD19 staining and in B-CLL by additional staining against CD5. Malignant plasma cells were primarily identified by intracellular staining of IgGκ and IgGλ light chains using the Fix & Perm Kit from Caltag (Burlingame, CA). In subsequent experiments, the malignant plasma cell population was then identified by surface staining for either CD38, CD56, or CD138 as appropriate for the individual plasmacytoma sample. Flow cytometric data were acquired on a FACScan (Beckton Dickinson Immunocytometry Systems, San Jose, CA).

Carboxyfluorescein Diacetate Succinimidyl Ester Staining. Cells were resuspended in PBS (1×10^7 cells/mL) containing carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR) at a final concentration of 1 μmol/L, and incubated at 37°C for 10 minutes.

Viability Assay. At time points indicated, cells were harvested and stained with FITC-labeled Annexin V, with PE-labeled anti-CD19 and with propidium iodide using a commercially available apoptosis detection kit (all from BD Biosciences, San Diego, CA). To each sample calibration beads (Calibrite Beads, BD Biosciences) were added before flow cytometric analysis. Viable cells were defined as being negative for Annexin V and propidium iodide. To compare different samples at different time points, the ratios of the viable cell count and the bead count were calculated.

Real-time Reverse Transcription-PCR. For the analysis of TLR9 mRNA of normal B cell subsets (naive, germinal center, and memory B cells isolated from adenoid tissue) and of isolated plasmacytoma cells, cells were lysed and RNA was extracted using a total RNA isolation kit (High Pure, Roche, Mannheim, Germany). TLR9-specific primer sets optimized for the LightCycler (RAS) were developed by and purchased from Search-LC (Heidelberg, Germany). The PCR was done with the LightCycler FastStart DNA SYBR GreenI kit (RAS) as previously described (18). The copy number was normalized by the housekeeping gene cyclophilin-B and is presented as number of transcripts per 10^3 copies of cyclophilin-B.

For the analysis of TLR9 mRNA of B-CLL cells (>95% $CD19^+$ $CD5^+$), B cells and T cells isolated from peripheral blood, RNA was prepared using the RNeasy Mini kit from Qiagen (Qiagen, Valencia, CA); 500 ng of total RNA was reverse transcribed using SuperScript III RNase H^- Reverse Transcriptase (Invitrogen, Carlsbad, CA) in a 20-μL reaction volume. Three microliters of the generated cDNA were used per 25 μL reverse transcription-PCR reaction carried out with the Platinum Quantitative PCR SuperMix-UDG kit (Invitrogen). Primers and probes were purchased from IDT (Integrated DNA Technologies, Coralville, IA): TLR9 forward 5′-GCCAGACCCTCTGGAGAA-3′, TLR9 reverse 5′-AGACTTCAGGAACAGCCAGTTG-3′, TLR9 probe 5′-/56-FAM/TACCTTGCCTGCCTTCCTACCCTGTGA/3BHQ-1/-3′, β-actin forward 5′-CACACCTTCTACAATGAGCTGCGT-3′, β-actin reverse 5′-ACAGCCTGGATAGCAACGTACA-3′, β-actin probe: 5′-FAM/AACCGCGAGAAGATGACCCAGAT-CAT/BHQ-3′. The amplification efficiency of the primers was 2.0 ± 10%. TLR9 copy number is presented as number of transcripts per 10^3 copies of β-actin.

Statistics. Data are presented as means ± SE. Statistical significance of differences was determined by the paired two-tailed Student's *t* test for equal or unequal variance as appropriate or Wilcoxon test using the absolute values. Statistical analysis was done using the program StatView D-4.5 (Abacus Concepts, Berkeley, CA).

RESULTS

CpG Oligodeoxynucleotides Activate Different Types of B-Cell Lymphoma. We compared the response of primary malignant B cells of different histologies to CpG-B oligodeoxynucleotide 2006 (synonymous: oligodeoxynucleotide 7909). Based on the kinetics of B-CLL activation (data not shown), the time point 48 hours was selected for subsequent studies. Primary malignant B cells were obtained from patients with MCL, MZL, FL, SLL, LCL, CLL, and plasmacytoma. Primary B cells from patients with follicular hyperplasia served as a nonmalignant B cell control. A total of 41 individual B-cell samples were assessed (see Table 1). Plasmacytoma cells were identified by staining with CD138, CD56, or CD38 (depending on the phenotype of the individual plasmacytoma) in combination with intracellular immunoglobulin light chain staining; B-CLL cells by double staining with CD19 and CD5, and all other B-cell malignancies by staining with CD19. Activation of malignant B cells was assessed based on the expression of CD40, CD54, CD80, CD86, MHC I, MHC II, and the death receptor family member CD95/Fas. The Fc γ receptor 1 (CD64) served as a negative control. Stimulation with CpG oligodeoxynucleotide 2006 resulted in activation of all types of malignant B cells (Fig. 1; Table 2). However, the degree of activation differed between different types of malignant B cells. Based on the expression of CD54 and CD86, CLL cells showed the strongest activation upon stimulation with CpG oligodeoxynucleotide 2006 (CD54: $P = 0.02$ for SLL, $P = 0.02$ for MCL, $P = 0.006$ for FL, $P = 0.001$ for P; CD86: $P = 0.02$ for SLL, $P = 0.002$ for MCL, $P = 0.02$ for FL, $P = 0.001$ for plasmacytoma). Activation of MZL was as high as CLL, but with three samples available, statistical analysis could not be done. The lowest activation upon stimulation with CpG oligodeoxynucleotide 2006 was found in plasmacytoma (CD54: $P = 0.001$ for CLL, $P = 0.04$ for SLL, $P < 0.001$ for FL; CD86: $P = 0.001$ for CLL, $P = 0.02$ for SLL, $P = 0.03$ for FL). Of all markers tested, only MHC I showed a significant up-regulation in plasmacytoma ($P = 0.009$). The response of the three available samples of LCL was in the same range as for SLL and FL. Activation of B cells derived from samples of follicular hyperplasia showed a strong response that was comparable to B-CLL and MZL. Up-regulation of CD95/Fas in response to CpG oligodeoxynucleotides was seen in all types of lymphoma except plasmacytoma (Table 2). Similar up-regulation of CD95/Fas was also seen in normal B cells derived from follicular hyperplasia (see Table 2) and in normal peripheral blood B cells (data not shown).

B-CLL Cells but Not Plasmacytoma Cells Express TLR9 mRNA. We were interested in whether the marked CpG oligodeoxynucleotide response of B-CLL cells and the lack of CpG oligodeoxynucleotide response in plasmacytoma cells is associated with different levels of TLR9 mRNA expression. For B-CLL, patient samples were selected that contained >95% $CD19^+$ $CD5^+$ cells. Plasmacytoma cells were isolated from bone marrow of patients with multiple myeloma (>98% pure; Fig. 2*A*). TLR9 mRNA was essentially absent in isolated plasmacytoma cells (Fig. 2*C*), whereas considerable expression of TLR9 mRNA could be detected in B-CLL cells (Fig. 2*D*).

Three B-cell subsets were isolated from nasopharyngeal tissue of healthy patients with nasopharyngeal hyperplasia (Fig. 2*B*). We found that all three B-cell subsets representing

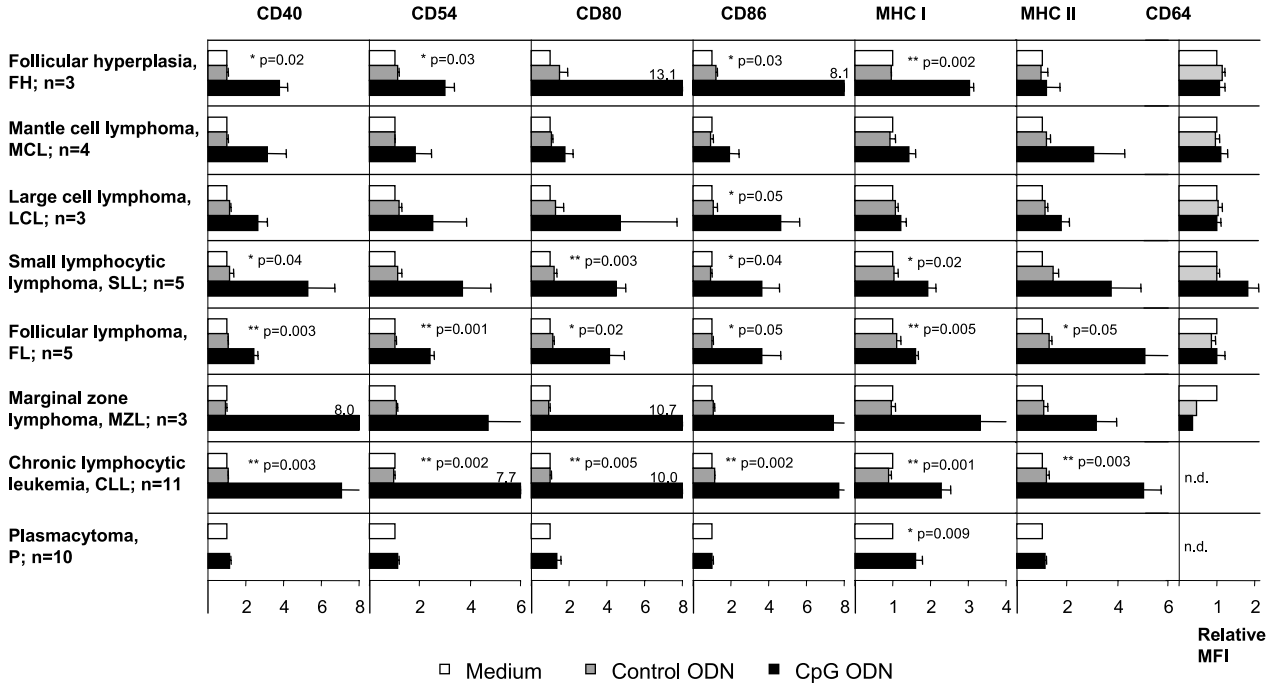

Fig. 1 CpG oligodeoxynucleotide (CpG ODN)–induced activation of different types of primary malignant B cells. Primary malignant B cells were incubated with medium alone (*open columns*) or with CpG ODN 2006 (*closed columns*), or the control ODN (*grey columns*). After 48 hours, the expression of CD40, CD54, CD80, CD86, MHC I, MHC II, and CD64 was measured by flow cytometry. Columns, means (n = no. samples) of the median fluorescence intensity of different surface molecules relative to the baseline expression on day 0 (set as 1); bars, ±SE. *, $P < 0.05$; **, $P < 0.005$.

Table 2 CpG oligodeoxynucleotide–induced proliferation and surface antigen expression in different entities of non-Hodgkin's lymphomas

Sample code	Medium, Proliferation (%)	Oligodeoxynucleotide 2006, Proliferation (%)	Medium, CD80 (MFI)	Oligodeoxynucleotide 2006, CD80 (MFI)	Medium, MHC I (MFI)	Oligodeoxynucleotide 2006, MHC I (MFI)	Medium, CD95 (MFI)	Oligodeoxynucleotide 2006, CD95 (MFI)
FH-111298	3.9	35.0	1.9	13.5	67.3	209	20.8	160
FH-250200	8.6	50.9	1.4	31.5	67.1	211	36.3	51.6
FH-300300	2.4	43.2	1.7	15.9	97	280	7.9	21.6
MCL-021199	12.0	13.1	35.3	43.1	64.2	70.3	112	150
MCL-130898	15.9	16.1	3.0	63.2	199	386	1.6	1.6
MCL-210600	2.8	13.1	1.3	1.9	62.4	88.5	2.97	9.8
MCL-021299	ND	ND	21.8	56.5	796	1,050	ND	ND
SLL-300899	5.2	8.8	21.1	117	119	202	9.3	8.6
SLL-190598	3.6	1.4	2.3	8.0	35.4	46.6	2.6	3.2
SLL-231297	3.1	4.7	2.9	8.8	60.9	105	15.3	51.5
SLL-220200	22.7	42.3	15.5	76	87.4	224	41.5	27.6
SLL-260298	1.9	13.5	1.0	5.6	26.7	63.3	8.1	10.3
CLL-070200	ND	ND	2.5	10.9	19.7	59.7	ND	ND
CLL-140200	3.7	2.5	1.1	9.3	91.1	150	ND	ND
CLL-150200	1.0	37	2.6	9.7	67.5	176	ND	ND
CLL-280200	5.5	21.9	2.0	22.4	160	494	ND	ND
CLL-060300	0.3	1.4	1.7	20.6	444	1,058	ND	ND
CLL-100300	0.9	11.9	5.9	92.6	95.1	244	ND	ND
CLL-170300	1.1	8.1	4.2	105	ND	ND	ND	ND
CLL-230300	3.5	21.7	2.4	14.5	392	477	ND	ND
CLL-280300	4.4	7.8	1.9	7.1	167	273	ND	ND
CLL-230600	0.4	8.2	ND	ND	ND	ND	ND	ND
CLL-240600	0.7	3.9	ND	ND	ND	ND	ND	ND
MZL-051199	4.8	47.5	2.2	9.76	95.8	191	241	522
MZL-030300	0.3	28.9	3.5	87.9	349	1,390	ND	ND
MZL-011299	ND	ND	4.9	11.3	114	458	ND	ND
FL-080200	0.9	30.0	3.4	10.8	60.1	109	58.8	86.8
FL-030500	3.4	7.6	7.0	31.9	84.2	151	228	310
FL-081298	11.3	10.4	35.2	130	85.1	111	138	216
FL-300300	6.0	32.5	10.6	25.4	203	284	68.6	137
FL-220200	ND	ND	3.7	25.5	162	266	12.4	26.3
LCL-140198	ND	ND	64.6	78.7	1.01	1.0	10.8	33.2
LCL-230300	6.1	17.0	37.4	80.4	446	536	176	274
LCL-100999	2.5	9.8	4.07	43.4	272	406	30.2	195
MM-160501	ND	ND	2.73	3.1	54.1	65.2	ND	ND
MM-310501	ND	ND	ND	ND	138	207	ND	ND
MM-180601	ND	ND	ND	ND	87.4	92	ND	ND
MM-050701	ND	ND	17.6	12.9	77.7	72.1	12.9	10.2
MM-100901	ND	ND	219	389	1,596	3,069	ND	ND
MM-181001	ND	ND	2.8	2.9	80.6	244	ND	ND
MM-1311011	ND	ND	249	925	25.3	29.2	ND	ND
MM-1311012	ND	ND	37	556	991	1,609	ND	ND
PCL-210801	2.07	2.0	6.2	5.7	92.8	125	ND	ND
PCL-260901	ND	ND	2.6	2.5	501	663	ND	ND

NOTE. For sample code see Table 1.
Abbreviations: MFI, median fluorescence intensity; ND, not done.

different stages of B-cell differentiation, naive B cells, memory B cells, and germinal center B cells, expressed considerable amounts of TLR9 mRNA albeit somewhat lower for germinal center B cells (Fig. 2*C*). The functional activity of TLR9 expression was confirmed by comparing CpG oligodeoxynucleotide–induced activation of naive B cells and memory B cells (Fig. 3). Except for CD86 which was higher in CpG oligodeoxynucleotide–activated naive B cells, both naive and memory B cells showed similar responses upon activation with CpG oligodeoxynucleotides. Together these data indicated that the response of B-CLL and plasmacytoma to CpG oligodeoxynucleotides is associated with the level of TLR9 expression. These studies also confirm that B cells express TLR9 at all stages of B cell differentiation up to germinal center B cells and memory B cells. From these data, responsiveness of different B-cell malignancies (naive B cells: B-CLL, SLL, and MCL; memory B cells: FL, MZL, and LCL) to CpG oligodeoxynucleotides is expected with the notable exception of plasmacytoma.

CpG Oligodeoxynucleotide Increases Surface Expression of the Rituximab Target CD20. The B cell marker CD20 is used as target for monoclonal antibody therapy (Rituximab) in different types of B-cell non-Hodgkin's lymphoma. Increased levels of CD20 may improve the clinical utility of such therapeutic antibodies. We evaluated whether CpG oligodeoxynucleotides can enhance CD20 expression in different types of B-cell malignancies (Fig. 4). Induction of CD20 expression was found to be most prominent in MZL ($n = 3$; $P = 0.05$ versus medium control) and SLL ($n = 5$; $P = 0.01$). CD20 was consistently up-regulated in B-CLL albeit to a lower extent

(n = 9; P = 0.009). Up-regulation of CD20 was less consistent for LCL and MCL, and was consistently low in FL (lower than in SLL: P = 0.01; lower than in CLL: P = 0.04), but still higher than in plasmacytoma (FL compared with plasmacytoma: P = 0.025), which did not up-regulate CD20 upon stimulation with CpG oligodeoxynucleotide 2006. Increased levels of CD20 were not only found in B-cell malignancies but also in nonmalignant B cells of follicular hyperplasia (Fig. 4).

Proliferation of Malignant B Cells in Response to CpG Oligodeoxynucleotide. Despite the advantages of activating malignant B cells and increasing CD20 expression, an unresolved question to date is whether the induction of malignant B-cell proliferation in response to CpG oligodeoxynucleotide limits its clinical utility. We analyzed CpG oligodeoxynucleotide–induced B cell proliferation on a per cell basis using the CFSE assay. Patient-derived samples were stained with CFSE. After 4 days of incubation in the presence of CpG oligodeoxynucleotide, the frequency of CD138+ (plasmacytoma) or $CD19^+$ (all other B cell types) $CFSE^{low}$ B cells (proliferating B cells) was determined by flow cytometry (Fig. 5*A*). In some samples, evaluation of the effect of CpG oligodeoxynucleotide on proliferation was limited by the number of primary cells available. The highest proliferative response to CpG oligodeoxynucleotide was seen in peripheral blood B cells from healthy donors (n = 8; P = 0.0001) and in nonmalignant B cells in samples of follicular hyperplasia (n = 3; P = 0.008; Fig. 5*B*). CpG oligodeoxynucleotide induced some proliferation of B-CLL cells (n = 6; P = 0.04). The highest induction of proliferation was seen in MZL; however, only two MZL samples were available for analysis. Enhanced proliferation was also seen for FL, SLL, and LCL, but with only three to five samples tested per histology, this increase did not reach statistical significance. Proliferation was not increased in three samples of MCL, nor in one sample of plasmacytoma. The lack of proliferative response of MCL and plasmacytoma is consistent with the weak or absent response of both B-cell malignancies with regard to up-regulation of activation markers (see Fig. 1). No proliferation was seen in any sample in response to the control oligodeoxynucleotide (data not shown).

CpG Oligodeoxynucleotide–Induced Apoptosis Outweighs Proliferation Leading to Decreased Number of Viable B-CLL Cells Over Time. Patients might still benefit from CpG oligodeoxynucleotide therapy if the observed increased proliferation of malignant B cells in the presence of CpG oligodeoxynucleotide described above is followed by enhanced cell death of cycling malignant B cells leading to a decreased overall malignant B cell survival. Absolute numbers of viable B cells were counted over time by using a defined number of calibration beads. Normal B cells derived from healthy donors (Fig. 6*A*) and B-CLL cells (Fig. 6*B*) were incubated for different periods of time in the presence of CpG oligodeoxynucleotide, the control oligodeoxynucleotide or with medium alone. Whereas the absolute number of viable (i.e., Annexin V/propidium iodide double negative) normal B cells markedly increased (Fig. 6*A*; n = 3; P < 01), the absolute number of viable B-CLL cells gradually decreased over time (Fig. 6*B*; n = 10; P < 0.01). Together, these data indicated that CpG oligodeoxynucleotides drive proliferation of normal B cells as well as malignant B cells, but at least for B-CLL cells, this increased proliferation was followed by an increased rate of cell death resulting in reduced B-CLL cell survival over time.

DISCUSSION

Ligation of Toll-like receptors signals the presence of microbial pathogens to the immune system. Activation of tumor cells via Toll-like receptors may render tumor cells

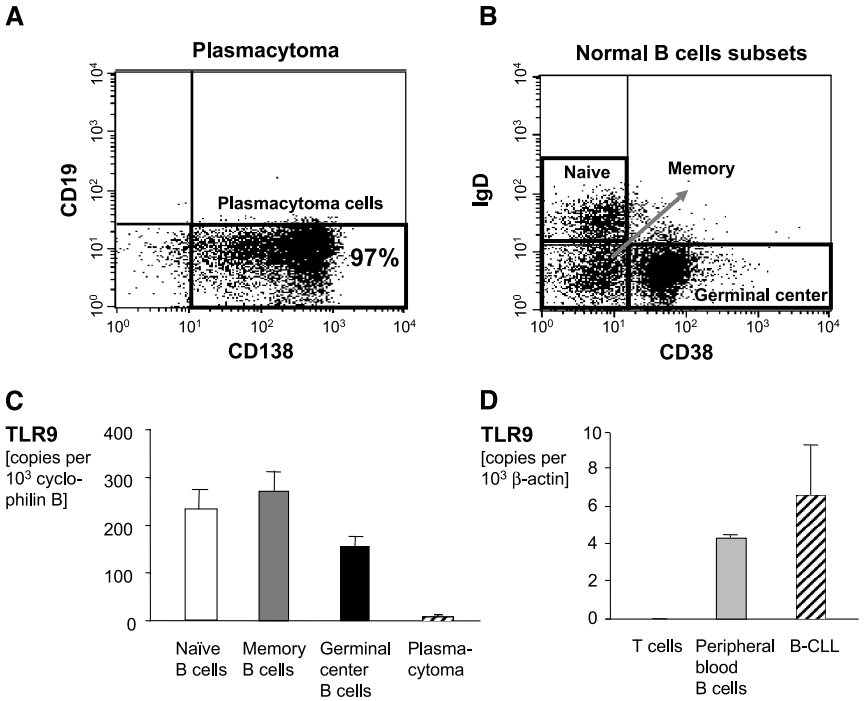

Fig. 2 TLR9 expression in plasmacytoma and B-CLL compared with normal B cell subsets and T cells. *A,* isolation of plasmacytoma cells (purity of $CD138^+$ cells >98%). *B,* isolation of normal B-cell subsets from adenoid tissue (naive B cells: IgD^+ $CD38^-$; germinal center B cells: IgD^- $CD38^+$; memory B cells: IgD^- $CD38^-$). *C,* TLR9 mRNA expression in isolated IgD^+ $CD38^-$ naive B cells, IgD^- $CD38^-$ memory B cells and IgD^- $CD38^+$ germinal center B cells (B-cell subsets from n = 4 individual adenoid tissue preparations), in $CD138^+$ plasmacytoma cells (n = 2 individual patients). Columns, means; bars, ±SE. *D,* TLR9 mRNA expression in B-CLL cells (% $CD19^+$ $CD5^+$ cells >95%; n = 4 individual patients with B-CLL), in peripheral blood CD19B cells (n = 2) and $CD3^+$ T cells (n = 3; *, P < 0.05). Columns, means; bars, ±SD.

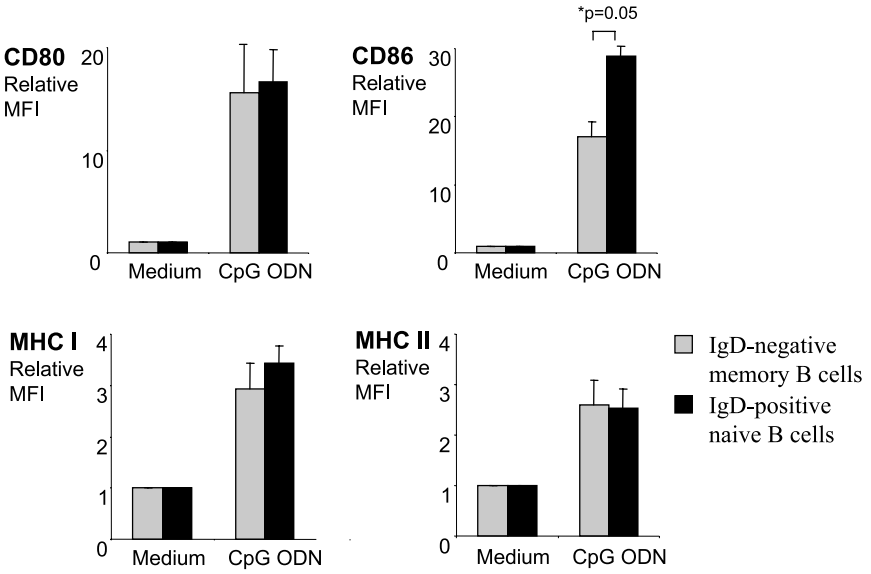

Fig. 3 Naive and memory B cells respond to CpG oligodeoxynucleotides (CpG ODN). Peripheral blood mononuclear cells from healthy donors were incubated in the presence or absence of CpG 2006. After 48 hours, the expression of CD80, CD86, MHC I, and MHC II was analyzed on IgD-negative (memory; *grey columns*) and IgD-positive (naive; *black columns*) B cells (CD19) by flow cytometry. Columns, means of the MFI of four independent experiments relative to the baseline expression on day 0 (set as 1); bars, ±SE. *, $P < 0.05$.

more visible for innate and acquired immunity. B-cell malignancies are unique in that they are derived from an antigen presenting cell that expresses a limited set of Toll-like receptors including TLR9, the receptor for CpG motifs within microbial DNA (18, 27, 28). Besides B cells, the only other human cell known to express TLR9 is the plasmacytoid dendritic cell. However, malignancies derived from plasmacytoid dendritic cells are extremely rare (29, 30). Therefore, human B-cell malignancies may represent the only human tumor type that is directly susceptible to CpG oligodeoxynucleotide–mediated activation.

Using primary cells from patient samples we show in the present study, that a broad spectrum of different histologies of B-cell malignancies is activated by CpG oligodeoxynucleotide including B-CLL, MZL, SLL, FL, LCL, and MCL. Plasmacytoma was the only malignant B cell type that showed virtually no response to CpG oligodeoxynucleotide. Susceptibility to CpG oligodeoxynucleotide–mediated activation was associated with the level of TLR9 expression as shown for B-CLL cells expressing high levels of TLR9 and plasmacytoma cells that lacked TLR9. Presumably, this applies for the other CpG oligodeoxynucleotide–responsive B-cell malignancies tested as well; however, TLR9 expression could not formally be shown due to the limited availability of primary malignant cells in samples from these patients.

Human B-cell malignancies originate from different stages of B-cell differentiation. We found that naive B cells, germinal center B cells and memory B cells from healthy donors express comparable levels of TLR9. TLR9 expression in different stages of B-cell differentiation is consistent with our finding of a broad spectrum of B-cell malignancies being responsive to CpG oligodeoxynucleotide. There seems no selective pressure on malignant B cells to down-regulate or to delete TLR9. Ongoing studies will clarify whether circulating clonotypic $CD19^+$ cells preceding the plasmacytoma cell (31) express TLR9. If these precursor cells in patients with plasmacytoma are responsive to CpG oligodeoxynucleotide, CpG oligodeoxynucleotides might be clinically useful in this tumor entity as well.

With different B-cell malignancies being sensitive to CpG-mediated stimulation, the key question is whether the malignant B-cell responses will be beneficial, or even

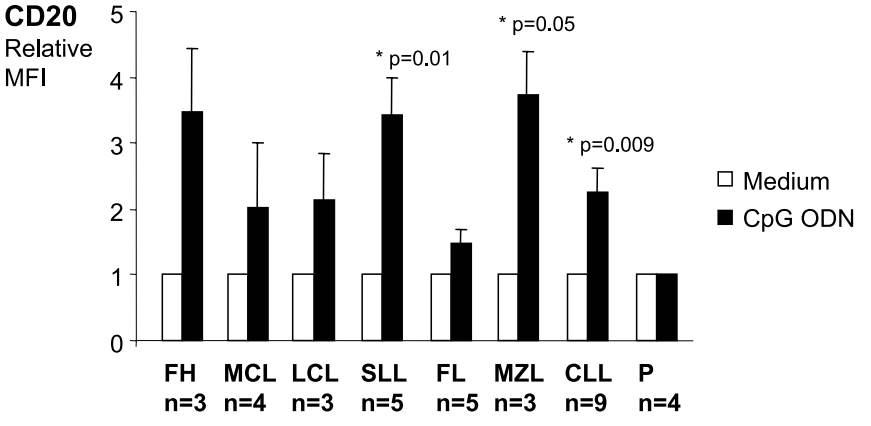

Fig. 4 CpG oligodeoxynucleotide (CpG ODN)–induced up-regulation of CD20 on different types of primary malignant B cells. Different types of primary malignant B cells and B cells derived from follicular hyperplasia were incubated with medium alone (*open columns*) or with CpG 2006 (*black columns*). After 48 hours, CD20 expression was determined by flow cytometry. Columns, means (n = number of samples) of the MFI of different surface molecules relative to the baseline expression on day 0 (set as 1); bars, ±SE. *, $P < 0.05$.

detrimental, for the patient. It is well established that CpG oligodeoxynucleotides inhibit apoptosis and strongly induce proliferation in B cells from healthy donors (32). CpG oligodeoxynucleotide–induced proliferation of malignant B cells has been shown for B-CLL (22, 33). In this study, we confirm that CpG oligodeoxynucleotide consistently induced proliferation of B-CLL cells. A trend towards increased proliferation rates was also found in MZL, FL, LCL, and SLL. However, whereas for normal B cells the absolute number of viable cells *in vitro* increased over time, the absolute number of viable B-CLL cells decreased in the presence of CpG oligodeoxynucleotides. These results show for the first time that for B-CLL an increased rate of apoptosis outweighs CpG oligodeoxynucleotide–induced proliferation leading to a lower overall survival of B-CLL cells in the presence of CpG oligodeoxynucleotide. These results extend our previous observation that CpG oligodeoxynucleotides induce early apoptosis within 2 days in a subset of patients with B-CLL (34). Here we show that CpG oligodeoxynucleotides enhanced apoptosis in all B-CLL samples examined as shown by absolute viable cell counts up to 7 days. The mechanism that leads to increased cell death in malignant B cells in the presence of CpG oligodeoxynucleotide is still unclear. Although we found that CD95/Fas is up-regulated by CpG oligodeoxynucleotide in most malignant B-cell samples tested, increased CD95/Fas expression in the presence of CpG oligodeoxynucleotide was also seen in normal B cells. Therefore, it is unlikely that up-regulation of CD95/Fas is a major mechanism by which CpG oligodeoxynucleotide selectively promote cell death of malignant B cells. Future studies will show whether activation of intrinsic cell death pathways by CpG oligodeoxynucleotides is involved.

Four aspects of the changes induced by CpG oligodeoxynucleotides could contribute to the potential clinical utility of CpG oligodeoxynucleotides in the treatment of human B-cell malignancies. First, activation of malignant B cells leads to increased expression of CD40, CD54, CD80, CD86, MHC I, and MHC II. Increased levels of CD40 render malignant B cells more sensitive for helper T cell–derived CD40L-mediated stimulation known to support B-cell differentiation. A combination of CD40L and CpG oligodeoxynucleotides for the treatment of B-cell malignancies may be promising. In this context, it is interesting to note that a strong synergy of CD40L and CpG oligodeoxynucleotides was found with regard to differentiation

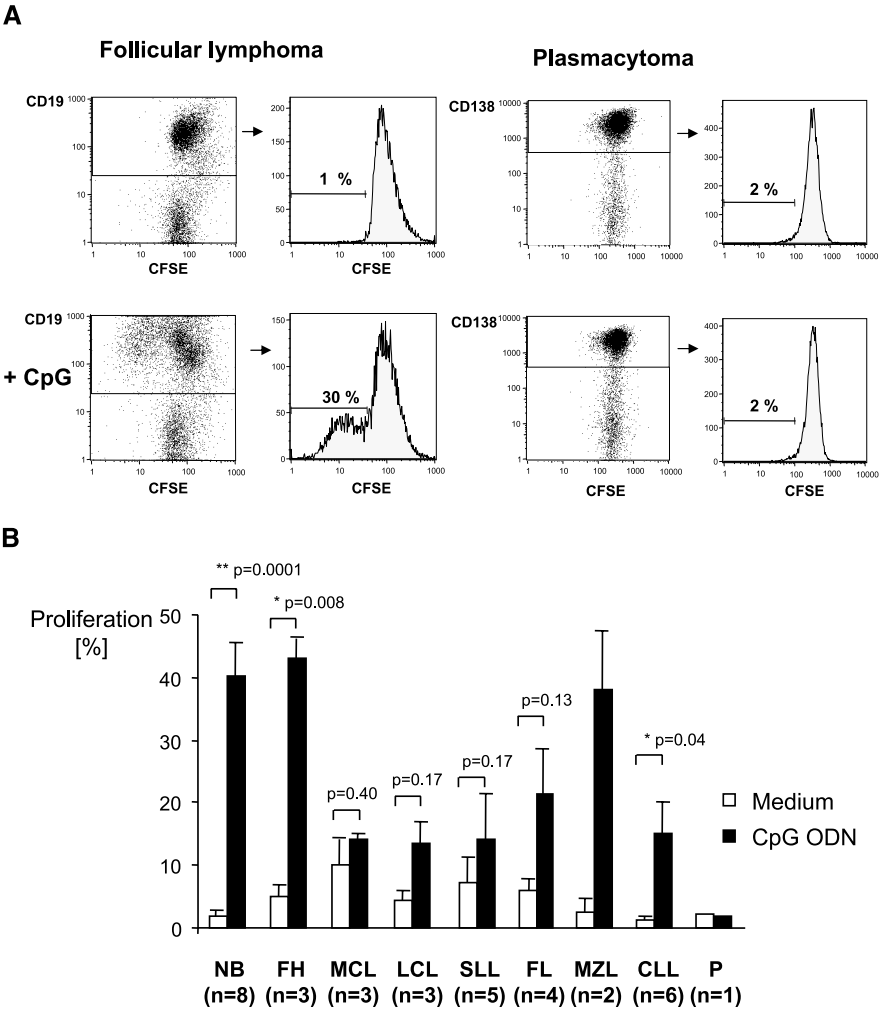

Fig. 5 CpG oligodeoxynucleotide (CpG ODN)–induced proliferation of different types of B cell lymphoma and of follicular hyperplasia. Peripheral blood mononuclear cells were stained with CFSE and incubated with or without CpG 2006. After 96 hours, cells were labeled with anti-CD19 or anti-CD138 (plasmacytoma) and analyzed by flow cytometry. Viable cells were gated according to morphologic criteria (FSC, SSC), and the percentage of $CFSE^{low}$ cells (proliferating cells) among viable cells was determined. *A,* percentage of $CD19^{+}$ $CFSE^{low}$ cells (proliferating malignant B cells in follicular lymphoma, *left*) and $CD138^{+}$ $CFSE^{low}$ cells (proliferating plasmacytoma cells, *right*) in response to CpG ODN (*bottom*). Columns, means of proliferating cells with (*black*) and without CpG ODN (*white*) are depicted (n = no. donors); bars, ±SE (*B*). *, $P < 0.05$; **, $P < 0.005$. NB, normal B cells.

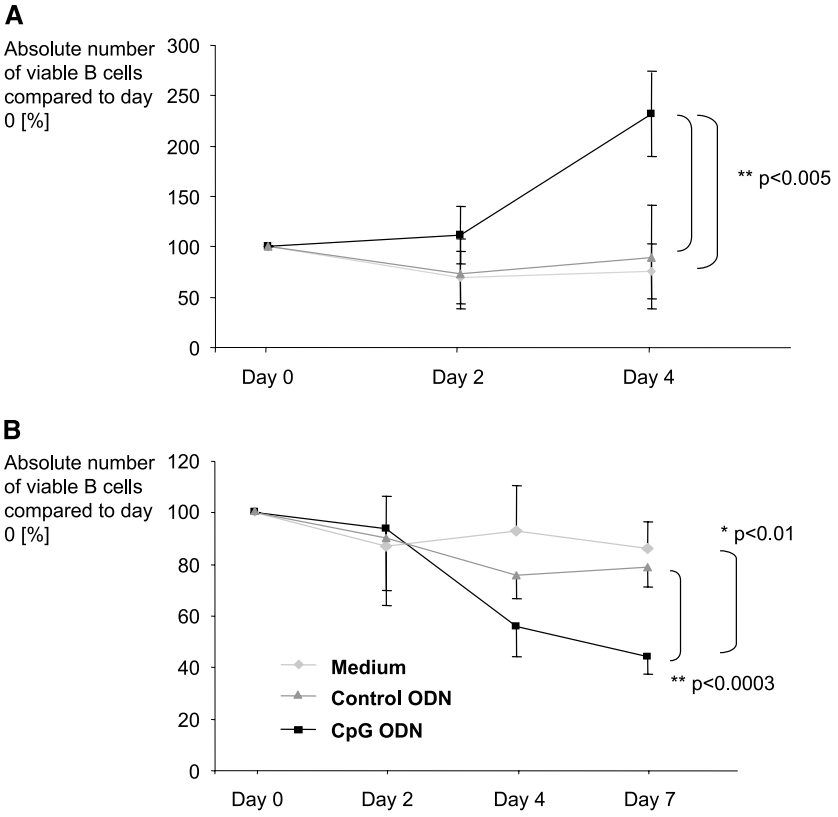

Fig. 6 Survival of B-CLL cells in the presence of CpG oligodeoxynucleotides (CpG ODN). PBMC from healthy volunteers (*A*) or patients with B-CLL (*B*) were incubated for different periods of time as indicated in the presence of CpG ODN (*squares*) or control ODN (*triangles*) or in the absence of ODN (*diamonds*). Cells were harvested and stained with anti-CD19 and anti-CD5 (B-CLL) or anti-CD19 (healthy donors). *A,* absolute number of viable B cells (n = 3 individual healthy donors; double negative for Annexin V and propidium iodide) was determined at different time points by using calibration beads and compared with day 0 (%). *B,* absolute number of viable B-CLL cells (n = 10 individual patients) at different time points compared with day 0 (%) is depicted. Points, means; bars, ±SE.

and IL-12 production in B cells from healthy donors (35). The adhesion molecule CD54 is thought to improve such cellular interactions of B cells and T cells. Increased levels of the costimulatory molecules CD80 and CD86 and of the antigen-presenting molecules MHC I and MHC II should facilitate idiotype-specific T-cell responses.

Second, stimulation of IFN-α production in PDC might contribute to immunotherapeutic activity of CpG oligodeoxynucleotides (36). Third, direct CpG oligodeoxynucleotide–mediated stimulation of malignant B cells can trigger cell death. Fourth, because CD20 is the target of many newer developments of antibody-based strategies to attack tumor cells, CpG oligodeoxynucleotides may improve the clinical activity of such compounds by up-regulating the expression of CD20. Because B-cell malignancies are systemic disorders and B cells need to be targeted with CpG oligodeoxynucleotides directly, the systemic availability of CpG oligodeoxynucleotides for malignant B-cell activation is a *conditio sine qua non* for successful treatment. In contrast, local injection is the preferred mode of administration of CpG oligodeoxynucleotides when used as vaccine adjuvant (37) or for experimental tumor therapy (38, 39).

In conclusion, B-cell malignancies are unique among human tumors in providing a direct target for TLR9-mediated therapy with CpG oligodeoxynucleotides. Our results identified B-cell malignancies that are particularly susceptible for CpG oligodeoxynucleotide–mediated stimulation. Focusing clinical trials on these CpG oligodeoxynucleotide–sensitive tumor types may help to advance the clinical development of CpG oligodeoxynucleotides in this area.

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