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Activation of Marginal Zone B Cells from Lupus Mice with Type A(D) CpG-Oligodeoxynucleotides

Rachel Brummel and Petar Lenert

Several types of CpG-oligodeoxynucleotides (ODN) have been recently characterized. In mice, type A(D) CpG-ODNs primarily stimulate macrophages and dendritic cells, but fail to stimulate B cells. On the contrary, type B(K) CpG-ODNs are excellent B cell activators. Type C CpG-ODNs combine features of both types A(D) and B(K) CpG-ODNs. Despite cell type preferences, all CpG-ODNs require the presence of TLR9 for activation. In this study, we show that a subset of B cells from lupus mice responds to type A(D) CpG-ODN stimulation vigorously and directly with increased CD25 and CD86 expression and IL-10 secretion. Furthermore, these CpG-ODNs induce high surface IgM expression and promote 50- to 100-fold higher IgM and IgG3 secretion in lupus B cells than in controls. This response is similar to that seen with bacterial DNA stimulation of B cells. Type A(D)-responsive cells are enriched within lupus B cells with the marginal zone (MZ) phenotype. These cells are at least twice more numerous in lupus mice than in controls. The ability of lupus B cells to respond to type A(D) CpG-ODN stimulation is not due to differential TLR9 expression. Therefore, type A(D) CpG-ODNs may contribute to the lupus pathogenesis by inducing MZ-B cell activation, costimulatory molecule expression, and polyclonal Ig secretion. Through increased IL-10 secretion, MZ-B cells may also modify the activity of other cell types, particularly dendritic cells and macrophages. The Journal of Immunology, 2005, 174: 2429–2434.

Division of Rheumatology, Department of Internal Medicine, Carver College of Medicine, University of Iowa, Iowa City, IA 52242

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1 This study was supported by National Institutes of Health Grant AI047374-01A2.
2 Address correspondence and reprint requests to Dr. Petar Lenert, Department of Internal Medicine, C312 GH, University of Iowa, Iowa City, IA 52242. E-mail address: petar-lenert@uiowa.edu
3 Abbreviations used in this paper: ODN, oligodeoxynucleotide; MZ, marginal zone; NZB, New Zealand Black; NZW, New Zealand White; PN, Palmerston North.

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he engagement of TLR receptors, particularly TLR9 on B cells and dendritic cells, seems to play an important role in the pathogenesis of systemic autoimmunity (1–4). B cells respond to TLR9 activation by proliferating, ceasing a spontaneous cell death program, up-regulating MHC class II and B7 family members, and secreting IgM and cytokines (e.g., IL-6, IL-12p40, and IL-10) (5–7). Chromatin/IgG complexes trigger the proliferation of autoimmune B cell clones with rheumatoid factor specificity, and this response requires TLR9 (3). Moreover, when autoimmune B cells are activated through TLR9 by bacterial DNA or CpG-oligodeoxynucleotides (ODNs), 3 but not through the BCR or CD40 receptor, they can surprisingly in an IL-10-dependent manner (8). Interestingly, optimal concentrations of type A(D) CpG-ODNs may contribute to the lupus pathogenesis by inducing MZ-B cell activation, costimulatory molecule expression, and polyclonal Ig secretion. Through increased IL-10 secretion, MZ-B cells may also modify the activity of other cell types, particularly dendritic cells and macrophages.

Materials and Methods

Animals

We used female prediseased lupus-prone Palmerston North (PN) mice (kindly provided by B. Handwerger, University of Maryland, Bethesda, MD), lupus-prone New Zealand Black/New Zealand White (NZB/NZW) (F1), and control mice DBA/1, BALB/c, C57BL/6, and 129/Sv (The Jackson Laboratory). Lupus-prone MRL-Fas+/− mice were used in some experiments and showed results comparable to PN and NZB/NZW (F1) strains. All mice were between 5 and 8 wk of age when sacrificed for in vitro experiments. Animal protocols were approved by the University of Iowa Animal Care and Use Committee.

Purification of mouse B cell subsets

Mice were killed by CO2 asphyxiation. Spleens were removed aseptically, and RBCs were lysed with ammonium-chloride. All cultures were performed in 10% FCS-RPMI 1640 medium with supplements. Conventional B cells (follicular and MZ-B cells) were obtained by negative selection using anti-CD43-coated magnetic beads and passage over the Midi-MACS magnet (Miltenyi Biotec). The pass-through fraction reproducibly contained between 97 and 99% of B220+ CD19+ cells. Such B cells were used for purification of either MZ-B cells or IgD+ B cell subsets by sorting.
on FACSDiva cell sorter. The CD21<sup>bright</sup>CD23<sup>−</sup> subset (MZ-B cells) was obtained by staining purified total B cells from lupus mice with FITC anti-CD21 (clone 7E9) and PE anti-CD23 (clone B3B4), and by gating on CD23<sup>−</sup>CD21<sup>bright</sup> (MZ-B cells IgD<sup>+</sup>IgM<sup>−</sup>) and CD23<sup>+</sup>CD21<sup>−</sup> (follicular B cells IgD<sup>−</sup>IgM<sup>+</sup>) B cells. In some experiments, total B cells were labeled with PE anti-IgD Ab (clone 11.26) and FACS sorted into IgD<sup>bright</sup> (non-switched B cells) and IgD<sup>−</sup> populations. Purified B cell subsets were cultured at 0.5–0.7 × 10<sup>6</sup>/ml in 10% FCS-RPMI 1640 at 37°C, 5% CO<sub>2</sub> for 18 h or 6 days.

**Reagents**

**Oligonucleotides.** Type B(K) CpG-ODN, stimulatory ODN-1826, 5′-T*C*C*A*T*G*A*C*G*T*C*T*C*T*C*A*G*G*T*T–3′<sup>4</sup> (asterisk denotes phosphorothioate linkages); type A(D) CpG-ODN, stimulatory ODN-1855, 5′-G*G*GGTCAAGCTTGAG*G*G*G*G*G-3′<sup>4</sup>; stimulatory ODN-2216, 5′-G*G*GGACGATCGTCG*G*G*G*G*G*G-3′<sup>4</sup>; and control ODN-1982, 5′-T*C*C*A*G*G*A*C*T*T*C*T*C*T*C*A*G*G*T*T–3′. All ODNs used in this study were endotoxin free and were provided by the Coley Pharmaceutical Group. Highly purified low-endotoxin bacterial DNA (from *Escherichia coli*, Ultrapure DNA) was from Sigma-Aldrich.

**Abs.** PE-, HRP-, or biotin-labeled polyclonal Abs against IgG2a, IgG2b, IgG3, and IgA were from Southern Biotechnology Associates. Purified and biotin-labeled mAbs against IgE (R35-72 and R35-118), IgG1 (A85-3 and A85-1), and IgG2a (R11-89 and R19-95) were from BD Pharmingen. Polyclonal Abs (purified and HRP labeled) against IgM were from Bethyl Laboratories; PE-labeled anti-IgD Ab (clone 11.26) was from Southern Biotechnology Associates. FITC-labeled Ab against CD21 (7E9) was a gift from L. Tygrett, University of Iowa. PE-labeled Abs against CD45R (B220; clone RA3-6B2), CD23 (clone B3B4), CD40 (clone IC10), and FITC-labeled anti-CD86 (clone GL1) and anti-CD25 (clone PC61.5) were all from eBioscience. Isotype-matched controls were from different sources.

**Flow cytometry**

Highly purified B cells/B cell subsets were harvested after 18–24 h for CD40, CD25, and CD86 staining, or after 72–96 h for surface IgM staining. B cells were washed with PBS-1% FCS, pH 7.2 buffer, and Fc receptors were blocked with Ab against CD16/32 (clone 93; BioLegend). Stained cells were analyzed on a FACScan (BD Biosciences). Data were processed using CellQuest software (BD Biosciences).

**ELISA**

The concentration of IL-10 in culture supernatants was determined in an ELISA using JES5-2A5 as a capture Ab (2 µg/ml) in a phosphate buffer, pH 6.5, and biotin-labeled JES5-2A5E3 as a detection Ab (BioLegend). To determine the concentration of Ig isotypes, ELISA plates were coated with isotype-specific polyclonal Abs (IgM, IgG1, IgG2b, and IgG3; Southern Biotechnology Associates and Bethyl Laboratories) in carbonate buffer, pH 9.5. HRP-conjugated isotype-specific polyclonal Abs were used for detection of each particular isotype (Southern Biotechnology Associates and Bethyl Laboratories). Concentrations of IgE, IgG1, and IgG2a were determined using OptEIA IgE kit and with pairs of IgG1- or IgG2a-specific mAbs (BD Pharmingen). Tetramethylbenzidine was used as a substrate (KPG Laboratories). Absorbance at 450 nM was determined in an ELISA reader (Molecular Devices), at a sensitivity of ~16 pg/ml for IL-10.

**RT-PCR analysis of TLR9 expression**

RNA was purified from freshly isolated total B cells or B cell subsets (2 × 10<sup>6</sup> cells each) obtained from DBA/1 and PN mice. Total RNA was isolated using RNAasy mini kit (Qiagen), following the manufacturer’s instructions. cDNA was synthesized from 0.4 µg of total RNA using Omniscript and Sensiscript reverse transcriptases for 30 min at 50°C (Qiagen One-Step RT-PCR kit). For PCR, the following TLR9-specific primers were used: upstream primer, GCA CAG GAG CCG TGA AGG T, and downstream primer, GCA GGG GTG CCT ACG GGA G (19). PCR conditions were the following: 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s (PTC-200; MJ Research). TLR9 cDNA fragment was amplified for 30 cycles in a final volume of 50 µl containing 2.5 mM magnesium dichloride, dNTP mix (400 µM of each dNTP), HotStarTaq polymerase (2 µl per PCR.
condition, initially activated by incubation at 95°C for 15 min), and 0.6 μM each primer. PCR products were resolved by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining. A single band of 838 bp was identified. Identical results were obtained with mRNA diluted 5× or 25×, and with PCR performed for 25 and 28 cycles (data not shown). Saturation of TLR9 occurred when PCR was performed for >35 cycles. As a control for equal RNA loading, RT-PCR was performed for 30 cycles with specific primers for GADPH (0.6 μM each).

TLR9 expression was further analyzed by quantitative real-time PCR. Total RNA (400 ng) purified by using RNAqueous-4PCR Kit from Ambion was reverse transcribed using a High Capacity cDNA Archive Kit from Applied Biosciences on a PTC-200 Thermal Cycler (MJ Research). RNA samples were obtained from total DBA/1 and PN B cells and from FACS-sorted follicular, MZ, and T1-B cell subsets from PN spleens. Specific primers for TLR9 (Applied Biosciences; product number Mm00446193_m1) and Taqman chemistry were used for real-time PCR on cDNA corresponding to either 1 or 50 ng of mRNA for 40 cycles. Analysis was conducted on ABI PRISM 7700 Sequence Detection System (Applied Biosystems) in the DNA facility at the University of Iowa. TaqMan Rodent GAPDH Control Reagent Kit was used to normalize the TLR9 results to the relative level of GAPDH in each sample. Results are indicative of six independent measurements.

Statistical analysis
All results are expressed as means ± SEM. Student’s t-test (two tailed) was used to compare differences between the PN and age- and sex-matched DBA/1 mice. Values of p < 0.05 were considered significant.

Results
Type A(D) CpG-ODNs up-regulate CD86 and CD25, but not CD40 in lupus B cells
It is well established that optimal concentrations of type A(D) CpG-ODNs (~33–330 nM) fail to directly stimulate highly purified human or mouse B cells (12, 13, 18, 20). Higher concentrations may induce backbone-dependent CpG-independent stimulation of B cells.

As predicted, in contrast to the type B(K) CpG-ODN-1826 (Fig. 1, left two columns), we found that type A(D) ODN-1585 was unable to induce CD86 (B7.2), CD25, or CD40 expression in control DBA/1 B cells. However, ODN-1585 induced significant CD86 and CD25, but not CD40 expression, in a subset of autoimmune B cells obtained from young prediseased PN lupus mice (Fig. 1, PN, far right column; ODN-1585 treated, thick line; control ODN treated, thin line; medium treated, shaded area). Type A(D) CpG-ODNs failed to induce CD80 expression on B cells.

Type A(D) CpG-ODNs induce high IL-10 secretion in lupus B cells
High serum IL-10 levels are typically found in patients with systemic lupus erythematosus, as well as in several animal strains that spontaneously develop lupus-like disease (21). We recently showed that mixed spleen cells from lupus-prone PN mice produce
high levels of IL-10 in response to both type A(D) and B(K) CpG-ODNs (8). In this study, we show that lupus B cells, but not control B cells, respond to type A(D) CpG-ODN-1585 stimulation with high IL-10 production (Fig. 2).

Type A(D) CpG-ODNs induce polyclonal Ig secretion from lupus B cells

Polyclonal hypergammaglobulinemia and anti-nuclear Ab formation are immunologic hallmarks of systemic lupus. When control DBA/1 B cells were stimulated with optimal concentrations of type A(D) CpG-ODNs, only trace amounts of IgM and IgG3 could be detected even when B cells were cultured over extended periods of time (up to 10 days) (Fig. 3, A and B). A similar lack of induction of IgM and IgG3 with type A(D) CpG-ODNs, but excellent response to type B(K) CpG-ODN stimulation and LPS stimulation, was observed in several other control strains, e.g., C57BL/6 and 129/Sv (Fig. 3, C and D). Moreover, neither IgG1 nor IgE was detectable when type A(D) CpG-ODN stimulation was combined with IL-4 (data not shown). Similarly, no IgG2a secretion could be detected in response to type A(D) CpG-ODN stimulation when IgG-depleted B cells were used (data not shown). In contrast, lupus B cells from PN mice responded to type A(D) stimulation with significantly higher IgM (Fig. 3A, p < 0.001) and IgG3 secretion (B, p < 0.001) closely resembling their response to a natural TLR9 ligand, bacterial DNA (Fig. 4). We also observed induction of a high IgM surface phenotype in lupus B cells, although not in control B cells, when stimulated with the ODN-1585 (Fig. 5, right panels). In contrast, type B(K) CpG-ODNs enhanced surface IgM expression in both control and lupus B cells (Fig. 5, left panels).

**MZ-B cells primarily account for type A(D) CpG-ODN stimulatory response**

We further tested whether differential responsiveness to type A(D) CpG-ODNs in lupus B cells could be attributed to a particular B cell subset by investigating the response of MZ-B cells vs follicular B cells. We first tested the response of highly purified non-switched IgD^{+} B cells. As shown in Fig. 6. IgD^{+} B cells from lupus mice failed to produce IgM (A) and IgG3 (C) when stimulated with ODN-1585. In contrast, IgD^{-} B cells (Fig. 6, A and C) as well as B cells with the MZ phenotype (CD23^{-} CD21^{+}) (B and D) primarily responded to type A(D) CpG-ODN stimulation. In addition, MZ-B cells were at least twice as numerous in lupus mice as in controls (Fig. 6, E and F).

Figs. 7 and 8 demonstrate that the abnormal response of lupus B

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**FIGURE 7.** In vitro responsiveness of total B cells from lupus-prone NZB/NZW (F1) mice to stimulation with type A(D) CpG-ODNs. Total B cells from young lupus-prone NZB/NZW (F1) mice or control BALB/c mice were left untreated (E and F) or stimulated with type A(D) CpG-ODNs (dark shaded area) or control ODN (light gray shaded area) for 18 h. CD86 (A and B), CD40 expression (C and D), and percentages of MZ-B cells (FITC-CD21^{hi}, PE-CD23^{lo}; E and F region) were determined by flow cytometry. The dotted line represents isotype control. ELISA was used to measure IgM and IgG3 concentration in culture supernatants of total B cells stimulated with type A(D) CpG-ODNs or control ODNs for 6 days (mean ± SEM, n = 4; *, p < 0.05; G and H region).

**FIGURE 8.** MZ-B cells from lupus-prone NZB/NZW (F1) mice respond to stimulation with bacterial DNA with high IgM secretion. Total B cells from NZB/NZW (F1), total B cells from control BALB/c mice, and FACS-sorted follicular B cells and MZ-B cells from NZB/NZW (F1) mice (0.5 × 10^6/ml) were stimulated for 6 days with 5 μg/ml bacterial DNA, and IgM concentration was determined in ELISA (mean ± SEM, n = 3; *, p < 0.05).

**FIGURE 9.** TLR9 mRNA expression in lupus and control B cells. A, RNA from total DBA/1 and PN B cells or from PN B cell subsets was reverse transcribed and PCR amplified with primers specific for the mouse TLR9. Results obtained after 30 cycles of PCR are shown. The number of cycles chosen was within the linear range of amplification. RT-PCR of GADPH was used as a control for equal loading of RNA. B, TLR9 expression relative to GADPH expression in total splenic B cells from DBA/1 and PN mice, and in FACS-sorted B cell subsets from lupus-prone PN mice, as determined by real-time PCR.
cells to type A(D) CpG-ODN stimulation is not limited to PN strain. Similarly to PN mice, NZB/NZW (F1) mice up-regulated CD86 expression in a subset of B cells, and secreted more IgM and IgG3. CD40 expression was minimally changed in both control and lupus B cells. The MZ-B cell compartment was 2- to 3-fold enlarged in NZB/NZW (F1) mice compared with control BALB/c mice, and MZ-B cells were the primary source of IgM following stimulation with bacterial DNA (Fig. 8).

Finally, we compared the TLR9 expression at the mRNA level between total B cells obtained from control and lupus strains, as well as between the different B cell subsets from lupus mice (Fig. 9). Similar levels of TLR9 expression were detected independent of the B cell type or strain of mice. Results were confirmed with real-time PCR analysis.

Therefore, lupus B cells may have a very low threshold of activation when stimulated with bacterial DNA or unusual CpG-ligands; this abnormality may be specific to MZ-B cells and does not depend on TLR9 expression.

**Discussion**

Two different classes of CpG-ODN have been identified based on their distinct activities on plasmocytoid dendritic cells and B cells: type A(D) and type B(K). The prototype mouse type A(D) CpG-ODN sequence is exemplified by ODN-1585 (22). ODN-2216 is the best type A(D) CpG-ODN in the human system, but it is also active in mice (12, 20). Poly(G) tails and central phosphodiester palindromic sequences with unmethylated CpG motifs structurally characterize this type of CpG-ODNs. Human plasmocytoid dendritic cells produce large amounts of IFN-γ and poly(G) tails in type A(D) CpG-ODNs may fail to interact with the CpG motifs.

**Dendritic cell functions** (8, 41). IL-10 may be particularly responsive for decreased IL-12 secretion, and therefore may cause skewing of the immune response from a Th1 pattern to probably more favorable Th2 pattern. However, in view of the detrimental role of IFN-γ in animal models of lupus (28), and the increased pathology of lupus mice with the deleted IL-10 gene, B cell-derived IL-10 may also be of benefit by preventing or down-regulating ongoing T cell-mediated tissue destruction (29).

We have identified MZ-B cells as major responders to bacterial DNA and type A(D) CpG-ODNs in lupus mice. These nonrecirculating long-lived B cells are highly enriched in the MZ of the spleen, where they perform important function in the initiation of the immune response in particular to T-independent Ags (30). These cells have a lower threshold for activation and differentiation into Ab-secreting cells, which allows them to rapidly respond to low-affinity Ags, and to limiting amounts of Ag (30). MZ-B cells have immediate access to blood-borne particulate Ags, and are responsible for an early IgM production (31). In newborns, the MZ is severely underdeveloped, which explains the inability of infants to mount a strong response to T-independent Ags, e.g., pneumococcal polysaccharide (32, 33).

Additionally, MZ-B cells may function as excellent APCs as they can process and present Ags, and deliver costimulatory signals to T cells much more efficiently than other B cells (30). Indeed, within a few hours of exposure to an Ag, MZ-B cells with anti-hen egg lysozyme specificity could induce costimulatory molecules and present Ags to T cells (30). This directly relates to a possible role of MZ-B cells in autoimmunity. Self-reactive clones are enriched within the MZ compartment (34–36). Estrogens may rescue autoreactive B cell precursors from deletion, and most of these cells will differentiate along the MZ pathway, e.g., like B cells from transgenic mice spontaneously secreting anti-DNA (37). Enlargement of MZ-B cell population in NZB/NZW (F1) mice (and in PN mice; this work) occurs before signs of disease (38) and pathogenic MZ-like B cell lines have been found in salivary glands in mice with Sjogren’s syndrome (39).

The primary defect responsible for the low threshold of MZ lupus B cells to CpG stimulation has yet to be discovered. This may either be an intrinsic B cell abnormality, or may be secondary to abnormal B cell activating factor belonging to the TNF family (BAFF)/BAFF-receptor stimulation, programmed death-1 dysfunction, CD40 engagement, IFN priming, or continuous TLR9 signaling in vivo. There is also the possibility of an imbalance between the TLR9 expression and low expression of the putative inhibitory DNA receptor (yet to be characterized) in MZ lupus B cells, preventing G-rich tails from exerting their negative influence, and allowing type A(D) CpG-ODNs to stimulate B cells. However, our finding that TLR9 expression in lupus B cells is not different from controls speaks against this possibility. One can envision that in mice or humans with adequate genetic background, strong hormonal (estrogenic) influence may initially lead to an expanded MZ-B cell pool. Such B cells may respond to very low concentrations of TLR9 ligands frequently present in the bloodstream, including less favorable CpG sequences, like those found in hypomethylated mammalian DNA (4, 40). The dual engagement of BCR and TLR9 by these TLR9 ligands may preferentially expand low-affinity B cell clones specific for DNA, or clones with the rheumatoid factor specificity that recognize chromatin/IgG immune complexes. Through T cell help and somatic hypermutation, these clones may give rise to higher avidity autoreactive B cells, resulting in autoantibody production, complement activation, and immune complex-mediated pathology. Therefore, attempts to pharmacologically block TLR9 signaling at an early step may still allow MZ-B cells to mount productive T-independent responses,
but yet prevent unwanted activation and expansion of autoreactive B cells by unmethylated CpG-containing DNA.

In conclusion, lupus B cells, but not control B cells, respond to stimulation with type A(D) CpG-ODNs by up-regulating CD86 and CD25. In addition, lupus B cells produce 50- to 100-fold more IgM and IgG3 and high amounts of the regulatory cytokine IL-10. Interestingly, requirements for CD40 up-regulation may be more stringent than those for CD86 or CD25, as type A(D) CpG-ODN fail to up-regulate CD40 expression in B cells. Therefore, increased ligand/receptor interactions may be required for A(D) CpG-ODNs, as compared with type B(K) CpG-ODNs, to induce expression of some B cell surface molecules. Type A(D) CpG-ODNs may contribute to the lupus pathogenesis primarily through increased Ig secretion.

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