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Functional Outcome of B Cell Activation by Chromatin Immune Complex Engagement of the B Cell Receptor and TLR9

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We have previously shown that rheumatoid factors produced by Fas-deficient autoimmune-prone mice typically bind autologous IgG2a with remarkably low affinity. Nevertheless, B cells representative of this rheumatoid factor population proliferate vigorously in response to IgG2a/chromatin immune complexes through a mechanism dependent on the sequential engagement of the BCR and TLR9. To more precisely address the role of both receptors in this response, we analyzed the signaling pathways activated in AM14 B cells stimulated with these complexes. We found that the BCR not only serves to direct the chromatin complex to an internal compartment where it can engage TLR9 but also transmits a suboptimal signal that in combination with the signals emanating from TLR9 leads to NF-κB activation and proliferation. Importantly, engagement of both receptors leads to the up-regulation of a group of gene products, not induced by the BCR or TLR9 alone, that include IL-2. These data indicate that autoreactive B cells, stimulated by a combination of BCR and TLR9 ligands, acquire functional properties that may contribute to the activation of additional cells involved in the autoimmune disease process. The Journal of Immunology, 2007, 179: 7397–7405.

Despite the multitude of self-constituents that potentially serve as targets in systemic autoimmune diseases, the autoantibody repertoire is remarkably limited. In this context, DNA, chromatin-associated proteins, RNA, and ribonucleoprotein macromolecules stand out as particularly common autoantibody targets (1, 2). Very often, such anti-nuclear Abs are the first indication of autoimmune disease in both patients and in animal models of systemic lupus erythematosus. We have proposed that the frequent occurrence of anti-nuclear Abs is due to the capacity of nucleic acid-associated autoantigens to engage both the BCR and a member of the TLR family gene, either TLR9 or TLR7 (3, 4).

This activation process can be best examined in B cells representative of the actual autoantibody repertoire. Our laboratory has used the BCR-transgenic mouse line, AM14, to better understand the relationship between nucleic acid autoantigens and the development of autoimmune diseases. The AM14 B receptor is a rheumatoid factor derived from an autoimmune Fas-deficient mouse that binds IgG2a with relatively low affinity (5). AM14 B cells proliferate robustly in response to IgG2a immune complexes (ICs) in which IgG2a is bound to chromatin (chromatin ICs), CpG-enriched dsDNA fragments (DNA ICs), RNA, or snRNP but no activation takes place when the IC contains IgG2a bound to a protein Ag (6).

The proliferative response to both chromatin ICs and DNA-fragment ICs is suppressed by agents that interfere with endosome acidification as well as by oligodeoxynucleotide (ODN) inhibitors of TLR9. Moreover, AM14 B cells that either fail to express the adaptor protein MyD88 or express the 3d mutation (7), and cannot signal through TLR9 (or TLR7), do not respond to chromatin ICs, even though their response to anti-IgM F(ab’)2 remains comparable to nontransgenic B cells (3, 8). Collectively, these data indicate that the AM14 BCR recognizes IgG2a bound to chromatin and then shuttles these ICs to an endosomal or lysosomal compartment where components of the complex engage TLR9 (or TLR7). These studies have not addressed the effect of IC engagement on the various branches of the BCR-signaling cascade or the capacity of such signals to modulate the functional properties of TLR9-activated AM14 B cells.

To more precisely address the functional outcome of BCR/TLR9 coengagement on the activation of AM14 B cells, we asked whether chromatin ICs could elicit the expression of gene products not induced by either the BCR or TLR9 alone. One notable effect was a shift in the pattern of cytokine production. This observation led us to examine in more detail the signal transduction pathways activated by chromatin ICs in wild-type and TLR-deficient AM14 responder populations. The current studies demonstrate that BCR/TLR engagement results in unique functional properties. Moreover, the BCR plays an active role in signal transduction but TLR9

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Abbreviations used in this paper: IC, immune complex; ODN, oligodeoxynucleotide; PLC, phospholipase C; CsA, cyclosporin A; TNP, trinitrophenol.

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B CELL ACTIVATION BY CHROMATIN ICs

Materials and Methods

Mice
AM14, AM14 MyD88−/−, AM14 TLR9−/−, and AM14 3d mice have been described previously (3, 8). MyD88−/− and TLR9−/− mice were provided by Dr. S. Akira (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan) and the 3d (Unc93B−/−) were provided by Dr. B. Berzofsky (Scripps Research Institute, La Jolla, CA). The microarray analysis used B cells from MRL/10 mice. Mice were bred and maintained in microisolator cages at the Boston University School of Medicine Laboratory Animal Science Center. Procedures were approved by the Institutional Animal Care and Use Committee of Boston University Medical Center.

B cell preparation and in vitro stimulation
Splenic B cells were isolated by positive selection with anti-B220-coupled magnetic beads (Miltenyi Biotec). B cells were also isolated by negative selection using a combination of anti-CD43+, anti-CD4−, and anti-CD8−coupled magnetic beads. For MAPK experiments, B cells were rested overnight at 37°C in culture medium before the addition of ligands. For all experiments, the cells were stimulated immediately after isolation. Proliferation was quantitated as previously described (9). F(ab′)2 goat anti-mouse IgM (Jackson ImmunoResearch Laboratories), CpG phosphorothioate ODN 1826, 5′-TCCATgACgTTCCTgACgTT-3′ (Trilink BioTechnologies), R848 (InvivoGen) were used as ligands. Hybridoma cell lines producing monoclonal IgG2a Abs specific for nucleosomes (PL2–3) (10) and trinitrophenol (TNP) (Hy1.2) (11) were provided by Drs. M. Monestier (Temple University, Philadelphia, PA) and M. Shlomchik (Yale University, New Haven, CT). For proliferation assays, PL2–3 was added directly to AM14 B cells. For the signaling studies, PL2–3 was preincubated for 2 h at 37°C with spent culture fluid; these preformed chromatin ICs were then added to B cell cultures at concentrations of 5 or 20 µg/ml PL2–3. DNA fragment ICs were prepared as described previously (9). Some experiments included the inhibitors cyster-closporin A (BioMol International), U0126 (Calbiochem), cycloheximide. B cells were stimulated with preformed chromatin ICs in the presence or absence of chloroquine (2 µg/ml) for varying periods of time and lysed in Laemmli buffer. Cell lysates were resolved on 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. Immuneblots were probed with anti-IgM F(ab′)2, anti-IgG2a and anti-IgG1 (Trilink Biotechnologies), R848 (InvivoGen) were used as ligands. Hybridoma cell lines producing monoclonal IgG2a Abs specific for nucleosomes (PL2–3) (10) and trinitrophenol (TNP) (Hy1.2) (11) were provided by Drs. M. Monestier (Temple University, Philadelphia, PA) and M. Shlomchik (Yale University, New Haven, CT). For proliferation assays, PL2–3 was added directly to AM14 B cells. For the signaling studies, PL2–3 was preincubated for 2 h at 37°C with spent culture fluid; these preformed chromatin ICs were then added to B cell cultures at concentrations of 5 or 20 µg/ml PL2–3. DNA fragment ICs were prepared as described previously (9). Some experiments included the inhibitors cyster-closporin A (BioMol International), U0126 (Calbiochem), cycloheximide or chloroquine (Sigma-Aldrich). All reagents were endotoxin-free (<0.1 U/ml) as determined by the Limulus amebocyte lysate assay (BioWhittaker).

Microarray gene chip analysis
Cells (3 × 10⁶) were stimulated with different ligands for 6 h. Total RNA was converted into biotin-labeled cRNA (MessageAmp arNA kit; Ambion) and hybridized to murine U430_2.0 probe arrays (Affymetrix). The microarray analysis used B cells from MRL/10 mice. Mice were bred and maintained in microisolator cages at the Boston University School of Medicine Laboratory Animal Science Center. Procedures were approved by the Institutional Animal Care and Use Committee of Boston University Medical Center.

Cytokine assays
Cell culture supernatants were recovered 24 h after stimulation. Cytokine levels were measured using Luminex-based multiplex cytometric bead arrays (13) or the OptEIA ELISA kit (BD Biosciences).

Western blot analysis
A total of 1.5 × 10⁶ cells were stimulated for varying periods of time and lysed in Laemmli buffer. The lysates were resolved on 10% (w/v) SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. The following Abs were used: anti-phospho-Syk (Tyr525/526), anti-phospho-Src family (Tyr416), anti-phospho-phospholipase Cy2 (PLCy2) (Tyr177), anti-phospho-p44/42 MAPK (Thr202/Tyr204), anti-phospho-stress-activated protein kinase/JNK (Thr183/Tyr185), anti-phospho-p38 MAPK (Thr180/Tyr182), anti-β-actin (Cell Signaling Technology), anti-tubulin and goat anti-mouse IgG-HRP (Sigma-Aldrich), anti-c-rel, anti-lamin B, goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology), anti-ERK1–2 and anti-phosphotyrosine 4G10) biotin-conjugated (Upstate Biotechnology/Cell Signaling Technology), donkey anti-goat IgG-HRP (Jackson ImmunoResearch Laboratories). The polyclonal antisera specific for T-bet was provided by Dr. L. Glimcher (Harvard School of Public Health, Boston, MA). Blots were stripped using the Re-blot Western Blot Recycling kit (Chemicon International Inc) and reprobed up to four times.

Calcium flux
AM14 MyD88-deficient B cells were loaded with Indo1-AM (Molecular Probes) at 1 µM for 30 min at 37°C, washed, and resuspended to a final concentration of 5 × 10⁶ cells/ml. After 20-min incubation at room temperature, cells were stimulated and intracellular Ca²⁺ was evaluated by measuring fluorescence at 405 and 485 nm after excitation at 355 nm with a Mo-Flo flow cytometer (DakoCytomation). Data analysis was performed using FlowJo software (Tree Star).

EMSA
AM14 B cells were stimulated for 24 h. The preparation of nuclear extracts, EMSA, and supershift analysis was done as described previously (14). Samples were electrophoresed in 4.5% polyacrylamide/TBE gel and analyzed by autoradiography. Abs to c-rel, RelB, p50, p52, and p65 (Santa Cruz Biotechnology) were used in supershift analysis.

Nuclear translocation of c-Rel
A total of 5 × 10⁶ AM14 or AM14 3d B cells were stimulated for 3.5 h with the specified ligands. Nuclear extracts were prepared as described previously (14). Samples were resolved on 10% (w/v) SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. Immunoblots were probed with anti-c-rel and anti-lamin B Abs. In addition, AM14 or AM14 3d B cells were placed into 8-well CC2-treated chamber slides (Labtek) and stimulated with the specified ligands for 3–4 h at 37°C. Cells were washed, fixed with 3% paraformaldehyde, permeabilized for 30 min with 0.5% Triton X-100, and stained with TRITC-conjugated anti-c-Rel (Santa Cruz Biotechnology) for 1 h. The nuclei were stained with 4′,6′-diamidino-2-phenylindole. Cells were imaged with a Zeiss Axiovert 200M inverted microscope equipped with the Apotome system and viewed with Axioview software (bar 20 µm; Carl Zeiss).

IκB-α degradation
A total of 2.5 × 10⁶ AM14 B cells were preincubated for 1 h with 50 µg/ml cycloheximide. B cells were stimulated with preformed chromatin ICs in the presence or absence of chloroquine (2 µg/ml) for varying periods of time and lysed in Laemmli buffer. Cell lysates were resolved on 10% (w/v) SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. Immunoblots were probed with anti-IκB-α and anti-a-tubulin Abs and analyzed by densitometry.

Results

Chromatin IC activation of AM14 B cells leads to IL-2 production
Chromatin ICs engage both the BCR and TLR9. To determine whether cross-talk between the two pathways induces a program of gene expression unique to chromatin IC stimulation, we compared the overall gene expression profile of AM14 B cells stimulated for 6 h with PL2–3 (chromatin ICs), anti-IgM F(ab′)2, CpG, and a combination of anti-IgM F(ab′)2 plus CpG as an experimental condition presumably equivalent to full engagement of both the BCR and TLR9. In three independent experiments, chromatin ICs selectively increased the expression level of 44 transcripts and reduced the expression level of 65 genes by 1.5-fold or more as compared with cells treated with either anti-IgM F(ab′)2 or CpG alone (Fig. 1). A subset of these genes was similarly regulated by the combination of anti-IgM F(ab′)2 plus CpG. These data suggest that chromatin IC activation of AM14 B cells results in a distinct

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genetic program. For example, IL-2 expression increased only after stimulation with PL2–3 or a combination of anti-IgM F(ab’)_2 plus CpG (Fig. 1A).

To confirm that the RNA data reflected actual cytokine production, culture supernatants collected 24 h after stimulation were assayed for IL-2. Consistent with the mRNA data, AM14 B cells, isolated by our standard positive selection procedure, produced IL-2 in response to PL2–3 and anti-IgM F(ab’)_2 plus CpG, but not in response to anti-IgM F(ab’)_2 or CpG alone (Fig. 2A). To prove that the IL-2 was B cell-derived, B cells isolated by a rigorous negative selection were also analyzed. The negatively selected population was completely unresponsive to Con A or anti-CD3 (Table I), and was therefore devoid of T cells, but still produced IL-2 in response to chromatin ICs as well as the combination of anti-IgM F(ab’)_2 plus CpG (Fig. 2A). Moreover, nontransgenic B cells still produced IL-2 in response to anti-IgM F(ab’)_2 plus CpG but did not produce IL-2 in response to PL2–3, further eliminating a non-B cell population as the source of IL-2 (Fig. 2B).

We next addressed the role of BCR and TLR9 in IL-2 secretion. As shown previously (9), cyclosporin A (CsA), an inhibitor of BCR signaling, blocks proliferation of AM14 B cells stimulated with anti-IgM F(ab’)_2 or PL2–3, but not CpG. CsA also blocked IL-2 production in AM14 B cells stimulated with PL2–3 or the combination of anti-IgM F(ab’)_2 plus CpG (Fig. 2C). PL2–3 also failed to stimulate IL-2 production in AM14 TLR9-deficient B cells (Fig. 2D). Collectively, these data demonstrate that IL-2 secretion depends on signals emanating from both the BCR and TLR9.
Chromatin IC activation of AM14 B cells does not induce expression of cytokines normally induced by CpG

B cell stimulation by CpG alone, as well as by other TLR ligands) routinely leads to the production of TNF-α, IL-6, IL-10, and IL-12 (13, 15). Despite the documented requirement for TLR9 in the PL2–3 response, the microarray data indicated minimal production of these cytokines in PL2–3-stimulated B cells. This trend was confirmed by ELISA; both AM14 and nontransgenic B cells stimulated with CpG, or the combination of anti-IgM F(ab′)2 plus CpG, secreted high amounts of these cytokines, whereas these cytokines were not produced by PL2–3-stimulated cells (Fig. 3). These data demonstrate that the signaling cascades activated by chromatin ICs do not simply recapitulate the sum of optimal TLR9 and BCR ligands.

Chromatin ICs induce T-bet expression

One possible explanation for the inability of PL2–3 to induce these cytokines is that mammalian DNA ligands are not able to fully activate the TLR9-signaling cascades. To examine signaling events induced downstream of TLR9, we monitored the expression of T-bet, a member of the T-box family of transcription factors that is induced in B cells by engagement of TLR9, but not TLR4 or the BCR. Consistent with published reports (16), CpG, but not anti-IgM F(ab′)2, markedly augmented T-bet expression, as determined by Western blot analysis of cell lysates collected at 20 h postactivation. Moreover, both chromatin ICs and dsDNA fragment ICs induced high levels of T-bet expression (Fig. 4). dsDNA ICs consisted of a 600-bp fragment of haptenated CpG-rich dsDNA bound the anti-TNP IgG2a mAb Hy1.2 (9). These dsDNA fragment ICs

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Chromatin IC activation of AM14 B cells does not induce the cytokines normally produced in response to CpG. AM14 (left panel) or nontransgenic (right panel) B cells were stimulated with 15 μg/ml anti-IgM F(ab′)2, 1 μg/ml CpG, 5 μg/ml chromatin ICs (PL2–3), or a combination of anti-IgM F(ab′)2 plus CpG. Culture fluids were collected at 20–24 h. The data in A represent mean ± SEM of four different experiments.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Chromatin ICs induce T-bet. B cells isolated from AM14 mice and AM14 TLR9−/− were stimulated with 15 μg/ml anti-IgM F(ab′)2, 3 μg/ml CpG, 5 μg/ml PL2–3 (chromatin ICs), DNA ICs (25 μg/ml Hy1.2, 250 ng/ml CG50-TNP2), or 150 ng/ml R848 for 20 h. The responses were analyzed by Western blot of cell lysates using a polyclonal T-bet-specific antiserum. The blot was stripped and reprobed with anti-β-actin as a loading control.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Chromatin IC-induced tyrosine phosphorylation does not require activation of TLRs. A, Proliferation of AM14 MyD88-sufficient and AM14 MyD88−/−-deficient B cells stimulated with increasing concentrations of PL2–3. Data are representative of three experiments with similar results. B, Upper panel, B cells from AM14 MyD88-deficient mice were stimulated with 15 μg/ml anti-IgM F(ab′)2, 3 μg/ml CpG, or two concentrations of preformed chromatin ICs (5 and 20 μg/ml) for the indicated periods of time. The lysates were electrophoresed in SDS-PAGE and immunoblotted with anti phosphotyrosine (4G10). B, lower panel, Replicate membranes were probed using Abs against p-Syk, p-Src family protein tyrosine kinase, p-PLCγ2, and β-actin (loading control). Similar data were obtained in two additional experiments.
effectively stimulate AM14 B cell proliferation even though they bind less avidly to AM14 B cells than chromatin ICs (9).

T-bet induction by PL2–3 depended on the presence of an active TLR9-signaling pathway, as it was markedly reduced in AM14 B cells obtained from TLR9-deficient AM14 mice. T-bet expression could still be induced in TLR9-deficient cells by R848, a synthetic TLR7 ligand. These data demonstrate that mammalian DNA contained in the chromatin ICs was able to up-regulate T-bet through effectve engagement of TLR9.

**Chromatin IC-induced tyrosine phosphorylation does not require activation of TLRs**

In response to the more common polyclonal BCR ligand, anti-IgM F(ab′)2, the BCR transduces a signal that leads to the rapid tyrosine phosphorylation of intracellular molecules such as Src family kinases, Syk and PLCγ2 (17), and the proliferation of both wild-type and MyD88-deficient B cells. To further examine the role of the BCR in chromatin IC-mediated activation of the AM14 receptor, we used AM14 MyD88-deficient B cells. These cells allowed us to analyze BCR signaling in the absence of an active TLR9-signaling cascade. Chromatin ICs were formed by premixing the mAb PL2–3 with spent culture fluid and initially used at a concentration of 5 μg/ml PL2–3. This concentration of PL2–3 routinely elicited the maximum level of proliferation of AM14 MyD88-sufficient B cells but fails to induce proliferation in AM14 MyD88-deficient cells (Fig. 5A).

Stimulation of AM14 MyD88-deficient B cells with chromatin ICs induced a marked increase in overall protein tyrosine phosphorylation, as determined by Western blot using anti phospho-Tyr Ab (Fig. 5B, upper panel). To examine specific proteins, replicate membranes were probed with Abs against p-Syk, p-Src family members, and p-PLCγ2 (Fig. 5B, lower panel). Chromatin ICs at 5 μg/ml, similarly to anti-IgM F(ab′)2, induced a rapid activation of the Src protein tyrosine kinase family, but elicited very weak Syk phosphorylation and less intense and delayed PLCγ2 activation. However, the proliferative response of MyD88-sufficient AM14 B cells to both 5 and 20 μg/ml PL2–3 was comparable and neither concentration was able to drive the proliferation of AM14 MyD88-deficient cells (3). Overall, these results support the notion that chromatin ICs suboptimally trigger components of the BCR signaling cascade in the absence of TLR engagement.

**Chromatin ICs induce a diminished calcium response**

Intracellular Ca2+ is a key second messenger in B cells following BCR engagement. We therefore examined calcium mobilization
after chromatin IC stimulation of AM14 MyD88-deficient B cells. As observed for tyrosine phosphorylation, 5 μg/ml PL2–3 evoked less efficient response than cells stimulated with chromatin ICs at 20 μg/ml (Fig. 6, upper panel). Although chromatin ICs at 20 μg/ml triggered a greater level of calcium flux with faster kinetics, the levels of intracellular calcium rapidly decayed to that elicited by the lower chromatin IC concentration. Stimulation with anti-IgM F(ab’)2 induced a rapid surge and a more sustained elevation...
of Ca\(^{2+}\) (Fig. 6, upper panel). As expected, CpG failed to trigger Ca\(^{2+}\) flux even in MyD88-sufficient cells (Fig. 6, lower panel). Taken together, these results indicate that AM14 B cells are not anergic and respond normally to anti-IgM F(ab\(^{\prime}\))\(_2\) but that chromatin ICs induce suboptimal engagement of the BCR.

**Chromatin ICs activate MAPKs through engagement of BCR and TLR9**

To further characterize the signaling pathways activated by chromatin ICs, we evaluated chromatin IC-induced MAPK activation. As previously reported for B cell lines (18), stimulation of AM14 B cells with anti-IgM F(ab\(^{\prime}\))\(_2\), but not with CpG, led to rapid and sustained ERK activation (Fig. 7A). Both ligands induced phosphorylation of JNK and p38, but they elicited different overall phosphorylation patterns and distinct activation kinetics. Three phosphorylated isoforms of JNK were detected in extracts from anti-IgM F(ab\(^{\prime}\))\(_2\)-treated cells. All three bands detected by the anti-p-JNK Ab corresponded to JNK isoforms as their phosphorylation could be blocked by the JNK inhibitor SP600125 (data not shown). Although the fastest migrating form was not detected after CpG treatment, chromatin IC stimulation induced phosphorylation of all three MAPK family members, further indicating a contribution of the BCR to the signaling response.

We also stimulated AM14 B cells with the dsDNA fragment ICs. The JNK phosphorylation pattern elicited by TNP\(_2\)-CG50 ICs was very similar to that of the CpG-stimulated cells, although ERK phosphorylation was lower than in the chromatin ICs-stimulated cells (Fig. 7A). These results indicate that both chromatin ICs and the dsDNA fragment ICs activate MAPKs through both the BCR and TLR9, but that the contribution of the BCR can vary depending on the valency of the Ag.

Although CpG did not induce ERK phosphorylation in B cells, the ERK cascade can be activated by TLR9 ligands in other cell types (18, 19). To formally rule out a role of TLR9 in the ERK pathway of PL2–3-stimulated cells, we examined AM14 MyD88-deficient and AM14 TLR9-deficient cells. The pattern of ERK phosphorylation was similar in AM14 wild-type, MyD88\(^{-/-}\), and TLR9\(^{-/-}\) cells, confirming that TLR9 did not contribute to the observed ERK activation (Fig. 7B). To address the contribution of ERK to the chromatin IC-induced proliferative response, we added U0126, an inhibitor of MEK, the kinase upstream of ERK, to the cultures. U0126 markedly reduced the activation of AM14 B cells stimulated with anti-IgM F(ab\(^{\prime}\))\(_2\) and chromatin ICs but, as expected, had no effect on the response to CpG (Fig. 7C). This result further supports a significant contribution of the BCR to the chromatin IC-induced proliferative response.

**Chromatin ICs require an active TLR9 to induce NF-κB activation**

NF-κB plays an important role in the proliferative response of B cells following either BCR (anti-IgM F(ab\(^{\prime}\))\(_2\)) or TLR9 (CpG) engagement. As expected, anti-IgM F(ab\(^{\prime}\))\(_2\), CpG, and PL2–3 all induced NF-κB activation in AM14 B cells, as shown by the ability of nuclear extracts to bind NF-κB target sequences (Fig. 8A). Ab supershift analysis revealed that all three treatments induced NF-κB dimers containing c-rel and/or p-50 (Fig. 8A) (20). To further examine the role of the BCR and TLR9 in AM14 B cell activation, we examined c-rel translocation to the nucleus in response to anti-IgM F(ab\(^{\prime}\))\(_2\), CpG, or PL2–3 in AM14 wild-type and AM14 3d mutant cells (Fig. 5B). 3d AM14 B cells fail to signal through TLR9, TLRL, or TLR3 (7). In all cases, stimulation with anti-IgM F(ab\(^{\prime}\))\(_2\) induced c-rel translocation to the nucleus. In contrast, CpG and PL2–3 induced c-rel translocation in WT AM14 cells but not in 3d AM14 B cells. Similar results were obtained by assessing c-rel translocation in AM14 and AM14 3d B cells by immunofluorescence (Fig. 8C). We confirmed the previous results by analyzing IkB degradation in wild-type AM14 B cells stimulated with PL2–3 in the presence or absence of chloroquine, a reagent known to increase the endosomal pH and specifically block TLR3/7/9 signaling (Fig. 8D). These results indicate that TLR9 is essential for NF-κB activation.

**Discussion**

The current report examined the functional outcome of chromatin IC activation of AM14 B cells and identified unique patterns of gene expression that included unanticipated effects on cytokine production. For example, both chromatin ICs and the combination of anti-IgM F(ab\(^{\prime}\))\(_2\) plus CpG induced IL-2 expression. Although IL-2 is commonly considered a T cell cytokine, production of IL-2 has been noted previously in B cells activated by a combination of the BCR and CD40 (21) or by activated Th2 cells and Ag (22). The requirement for dual receptor engagement is similar to the situation in T cells where engagement of both the TCR and costimulatory receptors are necessary for optimal IL-2 production (13). In T cells, members of at least four unrelated families of transcription factors, NFAT, Oct, NF-κB, and AP-1, have been implicated in IL-2 production (23), consistent with the need for convergence of multiple signal transduction pathways for maximal gene expression. IL-2 can serve as a growth factor for both B cells and T cells and therefore the selective production of IL-2 by BCR/TLR9 engagement may play an important role in the prolonged survival of autoreactive lymphocytes.

In contrast to the IL-2 response, IL-6, IL-10, and IL-12p40 were induced by CpG (or other TLR ligands) (13), either alone or in combination with anti-IgM F(ab\(^{\prime}\))\(_2\), but not by PL2–3. The lack of production of these cytokines by PL2–3-stimulated cells may be the consequence of multiple factors, including active suppression through the BCR or suboptimal stimulation of TLR9 by PL2–3. The high level of T-bet expression in TLR9-sufficient (but not TLR9-deficient) PL2–3-stimulated cells indicated that at least the pathway leading to the expression of this transcription factor was comparably activated by CpG, and chromatin ICs. Nevertheless, it is possible that other pathways downstream of TLR9 may be differentially triggered by the two types of ligand.

In plasmacytoid dendritic cells, the nature of the TLR9 response depends on the intracellular compartment in which the ligand and TLR9 interact and the site of interaction depends on the size of the ligand (24, 25). Thus, CpG-A ODNs form multimers and localize predominantly in early endosomes where TLR9 engagement leads to IFN-α production while CpG-B ODNs do not multimerize and are more likely to engage TLR9 in a late endosome and trigger IL-6 production. It is possible that chromatin ICs are delivered to one type of endosomal compartment where receptor interaction with other proteins might preferentially lead to IL-2 production, while the CpG used in this study (a CpG-B type) preferentially localizes to a different compartment where it triggers IL-6 production. We had previously noted that PL2–3 activation of AM14 B cells was much more sensitive to low concentrations of bafilomycin A (an agent that blocks endosome acidification) than was CpG activation; whether the differential response to subtle changes in pH reflects the site of ligand/TLR9 interaction remains to be determined.

BCR signaling in response to anti-IgM F(ab\(^{\prime}\))\(_2\) and other high-affinity ligands has been extensively investigated, but the signaling components activated in response to lower affinity ligands are incompletely understood. We separated the BCR-signaling events triggered by chromatin ICs from TLR9/7-dependent signaling events by using AM14 B cells that lacked a functional TLR9 (or...
TLR9/7) signaling cascade. These studies included AM14 mice with targeted mutations in TLR9, MyD88, or a spontaneous mutation in Unc98 (8). The quality of the signal induced by chromatin ICs was inherently different from that induced by cross-linking the receptor with anti-IgM F(ab′)2, a conventional, but nonphysiological, ligand. Although the overall pattern of tyrosine-phosphorylated proteins was similar, stimulation with 5 μg/ml PL2–3, the optimal mitogenic concentration, routinely resulted in delayed kinetics of phosphorylation and a less robust response than seen with anti-IgM F(ab′)2. More rapid and extensive levels of tyrosine phosphorylation could be obtained with a higher concentration (20 μg/ml) of PL2–3, but even this concentration fails to induce proliferation, as measured by [3H]thymidine incorporation, in MyD88−/− deficient B cells as well as in 3d B cells.

The level of protein phosphorylation is the result of a balance between the action of kinases and phosphatases. We cannot rule out the possibility that the less extensive tyrosine phosphorylation and reduced calcium flux might be a consequence of higher phosphatase activity. B cells express multiple inhibitory receptors, including FcyRIBB (26). Coligation of BCR and FcyRIBB can lead to negative regulation of the BCR-signaling cascade through recruitment of the inositol phosphatase SHIP (27). Chromatin ICs potentially engage FcyRIBB receptor. However, IgG2a is a weak ligand for FcyRIBB and we have found that AM14 and AM14/FcyRIBB-deficient B cells proliferate comparably in response to PL2–3 through a TLR9-dependent mechanism and did not show major differences in the pattern of tyrosine phosphorylation (A. Avalos and A. Marshak-Rothstein, manuscript in preparation).

BCR engagement drives the assembly of a multiprotein scaffold, referred to as an “early signalosome,” that is sufficient to trigger limited Ca2+ mobilization and activate NFAT and ERK (28), similar to the outcome observed in AM14 B cells in response to PL2–3. The current report demonstrates that BCR-dependent ERK phosphorylation is essential for the subsequent BCR/TLR9-dependent cell cycle entry of chromatin IC-activated AM14 B cells. In this context, ERK phosphorylation does not actively contribute to the form of anergy observed with higher affinity BCR ligands (29).

Formation of the early signalosome is not sufficient to drive B cell proliferation. NF-κB activation requires further assembly of adaptor proteins to form an “immunosome.” Immunosome assembly is purportedly driven by extensive BCR cross-linking or, when there is less extensive cross-linking of the BCR, by the engagement of a costimulatory receptor (28). In the case of AM14 B cells stimulated by chromatin IC, the costimulus is likely to be TLR9. How the BCR and TLR9 intersect to form the functional equivalent of an immunosome is unclear. Some receptors, such as the epidermal growth factor receptor, have been found to activate the ERK pathway after sequestration to endosomal compartments (30, 31). Although BCR signaling and internalization have been considered mutually exclusive events (32), in our system, ERK activation is greatly reduced in the presence of inhibitors that block receptor internalization (L. Busconi, unpublished data). It is tempting to speculate that BCR-mediated internalization of early signalosome/immunosome components could lead to TLR9 colocalization in an endosomal compartment.

PL2–3 activation of AM14 B cells was suboptimal as determined by induced changes in intracellular Ca2+. PLCγ2 generates two messengers, diacylglycerol and inositol (1, 4, 5)-trisphosphate (IP3). IP3 then induces Ca2+ release from intracellular stores. However, PLCγ2 is not only responsible for the transient initial phase of Ca2+ released from internal stores of the endoplasmic reticulum but also plays a role in the sustained second phase of Ca2+ entry from the extracellular space which is important for the amplification of BCR signaling (33). Because PLCγ2 catalyzes the step immediately upstream of the increase in intracellular calcium, the weak PLCγ2 activation that we observed may account for the suboptimal level of calcium flux and the lack of a sustained response (34).

In summary, chromatin ICs engage both the BCR and TLR9, although the overall strength of the each event may not be optimal. Nevertheless, this type of BCR/TLR9 dual receptor engagement leads to unique functional outcomes. A similar level of BCR/TLR9 cross-talk is likely to occur in any autoreactive B cell with a receptor specific for DNA or DNA-associated proteins or B cells reactive with RNA or RNA-associated proteins. The critical question to be addressed in future studies will be whether genes specifically induced by BCR/TLR9 coengagement will serve as useful therapeutic targets for the treatment of autoimmune diseases.

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Disclosures

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