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CpG DNA Induces IgG Class Switch DNA Recombination by Activating Human B Cells through an Innate Pathway That Requires TLR9 and Cooperates with IL-101

Bing He,* Xugang Qiao,* and Andrea Cerutti2*†

TLRs are pattern recognition receptors that initiate innate immune responses. TLR9 detects microbial DNA with hypomethylated CpG motifs and in humans is preferentially expressed by IFN-α-producing plasmacytoid dendritic cells and B cells. In addition to favoring IFN-α release, TLR9 signals B cell activation, proliferation, and IgM production. Recent findings suggest that CpG DNA-TLR9 interaction plays a key role in systemic lupus erythematosus and rheumatoid arthritis, two autoimmune disorders characterized by dysregulated production of DNA-reactive IgG. We show that CpG DNA initiates germline C1, C2, and C3 gene transcription by activating B cells through a TLR9-mediated NF-κB-Rel-dependent innate pathway that cooperates with IL-10 through STAT proteins and IFN-responsive factors. This pathway is inhibited by chloroquine, a drug that attenuates the clinical manifestations of IgG-mediated autoimmune disorders. Germline Cγ gene transcription is associated with up-regulation of activation-induced cytidine deaminase, a key element of the B cell class switch-inducing machinery, and is followed by class switch DNA recombination from Cμ to C1, C2, and C3. Subsequent IgG production requires additional signals from BCR and a B cell-activating factor of the TNF family (BAFF), produced by dendritic cells upon exposure to IFN-α. Our findings suggest that CpG DNA-TLR9 interaction may be important to initiate or amplify early T cell-independent IgG responses against pathogens. This implies that CpG DNA released during infections may exacerbate autoimmunity by stimulating autoreactive B cells to switch from an IgM to a more pathogenic IgG isotype. The Journal of Immunology, 2004, 173: 4479–4491.

Abbreviations used in this paper: CSR, class switch DNA recombination; AID, activation-induced deaminase; BAFF, B cell-activating factor of the TNF family; B cell, plasmacytoid dendritic cell; DN, dominant negative; ECS, evolutionarily conserved sequence; GAS, IFN-γ-induced sequence; IKK, IκB kinase; IRAK, IL-1R-associated kinase; IRF, IFN-responsive factor; ISRE, IFN-stimulated responsive element; Luc, luciferase reporter plasmid; NIK, NF-κB-inducing kinase; ODN, oligodeoxynucleotide; p, phospho; PAMP, pathogen-associated molecular pattern; RA, rheumatoid arthritis; RF, rheumatoid factor; SLE, systemic lupus erythematosus; TD, T cell-dependent; TI, T cell-independent; TIR, TLR/IL-1R domain; TOLLIP, Toll-interacting protein; TRAF, TNFR-associated factor.
innate cytokine released by circulating plasmacytoid DCs upon TLR9 engagement by microbial products, including DNA with unmethylated CpG motifs (18–21). Unlike TLR4, which is expressed on the cell surface (10), TLR9 is expressed in the endoplasmic reticulum and signals from an endosomal compartment upon CpG DNA internalization (22, 23). Not only do PAMPs stimulate B cells through BAFF, but they also directly activate B cells via TLRs. This is exemplified by LPS, which triggers mouse B cell CSR and Ab production by engaging TLR4 (1, 10). Human B cells lack TLR4, but express TLR9, and undergo proliferation and IgM production in response to CpG DNA (24, 25). The role of CpG DNA in human B cell CSR remains elusive.

Unmethylated microbial CpG DNA is similar to certain CpG DNA islands within the mammalian genome (22, 26, 27). Hypomethylated genomic CpG DNA is increased in subjects with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (28), two autoimmune disorders characterized by abnormal B cell reactivity to a relatively restricted set of self-Ags, including DNA and DNA-bound proteins (29–31). Recent studies suggest that dual TLR9 and BCR engagement by endogenous CpG DNA released from dying cells and CpG DNA-IgG complexes stimulates autoreactive B cells to produce DNA-reactive IgM and IgM rheumatoid factors (RFs), which recognize DNA-bound IgG (32, 33). Considering that anti-DNA IgG is usually more abundant than anti-DNA IgM and that RF-expressing B cells produce IgG in addition to IgM (29, 30), it is conceivable that CpG DNA stimulates autoreactive IgG CSR in addition to IgM production.

CD40L and BAFF induce CSR through p50, p52, p65, c-Rel, and RelB (1, 16). These NF-κB-Rel proteins are retained in a cytoplasmic inactive form by IκBα, an inhibitor of NF-κB (34, 35). By recruiting TNF receptor-associated factors (TRAFs) to their receptors, CD40L and, to a lesser extent, BAFF activate IκB kinase β (IKKβ) (4, 17), which is part of an IKK complex that includes two catalytic α and β subunits and a regulatory γ subunit (34, 35). Phosphorylation of IκBα by IKKβ is followed by IκBα degradation and p50, p65, and c-Rel nuclear translocation (34, 35). CD40L and, to a greater extent, BAFF also activate IKKα through NF-κB-inducing kinase (NIK) (34, 35). Phosphorylation of p100 by IKKα is followed by p100 processing to p52, which then translocates to the nucleus in association with RelB (34, 35). Similarly to CD40 and BAFF receptors, TLRs activate p50, p52, p65, c-Rel, and RelB through NIK and IKK (10). These kinases are turned on upon recruitment of MyD88, IL-1R-associated kinases (IRAKs), and TRAF6 to a cytoplasmic TLR/IL-1R (TIR) domain (36–38). This prompted us to hypothesize that TLR9 signaling initiates IgG CSR through NF-κB-Rel.

We show that CpG-containing oligodeoxynucleotides (ODNs) and bacterial DNA initiate germline Cα gene transcription in human B cells by turning on a TLR9 pathway that activates NF-κB-Rel, STATs, and IFN-responsive factors (IRFs) in cooperation with IL-10. CpG DNA-induced germline Cα gene transcription is inhibited by chloroquine, a compound used to treat patients with SLE and RA (39), and ultimately leads to CSR from C4μ to C1J, C2, and C3. Subsequent IgG production requires additional B cell stimulation through BCR engagement, BAFF, or CD40L.

**Materials and Methods**

**Cells and reagents**

Peripheral blood IgD+ IgM+ naive B cells were obtained as previously described (16). 2E2 B cells are characterized by high TLR9 expression levels and were obtained by subcloning CL-01, an IgD+ IgM+ B cell line that undergoes CSR upon in vitro exposure to appropriate stimuli (40). CL-01 subclones were obtained by limiting dilution and tested for TLR9 expression through semi-quantitative RT-PCR. Purified phosphochoate-modified ODNs (Operon Technologies, Alameda, CA; lowercase letters represent nuclease-resistant phosphorochoate linkage, uppercase letters represent phosphorodiestere linkage 3’ of the base, and underlined letters represent CpG dinucleotides), including 5′-CCTGTTGATTGAC-3′ ODN-2216 (type-A CpG DNA, were at 5 μg/ml. Control 5′-CCTGTTGATTGAC-3′ CpG DNA and 5′-CCTGTTGATTGAC-3′ ODN-2216/type-A CpG DNA were at 5 μg/ml. Recombinant BAFF (Alexis Biochemicals, San Diego, CA), CD40L (Immunex, Seattle, WA), IL-2 (from Dr. K. A. Smith, Cornell University, New York, NY), IL-4 (Schering-Plough, Kenilworth, NJ), IL-10, IL-15, LPS (Sigma-Aldrich, St. Louis, MO), IL-6, and IL-13 (R&D Systems, Minneapolis, MN) were used at 500 ng/ml, 500 ng/ml, 50 U/ml, 200 ng/ml, 100 ng/ml, 1 μg/ml, 5 ng/ml, and 10 ng/ml, respectively, unless otherwise indicated. Immuneobead reagent (InvivoGen, San Diego, CA) is a mixture of human IgGs that are used at 2 μg/ml to mimic extensive BCR cross-linking by a Ig Ag, Bafllumycin A and chloroquine (Sigma-Aldrich) were used at 10 nM and 20 ng/ml, respectively.

**Flow cytometry and ELISAs**

B cell surface CD19 and IgG were labeled with FITC- and PE-conjugated Abs (BD Pharmingen, San Diego, CA). Cells (10⁶) were acquired using a FACSCalibur analyzer. IgG ELISA was performed as previously described (16, 40).

**RT-PCRs**

cDNA was reverse transcribed from 3 μg of total RNA. TLR9 was RT-PCR amplified for 25 cycles using forward primer 5′-ACACAAACATTCACACGCCAATGTCTC-3′ and reverse primer 5′-AAGGCACGGTAATGTCACGGAG-3′. 1, C1–C3 (603 bp), 1, C2–C2 (597 bp), 1, C3–C3 (670 bp), 1, C4–C4 (411 bp), 1, C5–C5 (409 bp), 1, C6–C6 (537 bp), 1, C7–C7 (557 bp), 1, C8–C8 (608 bp), 1, C9–C9 (382 bp), and 1, C10–C10 (593 bp) transcripts were amplified for 25 cycles as previously described (16).

**Genomic DNA PCRs**

Genomic DNA was extracted by using the QiAmp DNA Mini Kit (Qiagen, Valencia, California). Total S-Sw circle switch circles and β-actin transcript were amplified from 500 ng of genomic DNA as previously described (16). The conditions were denaturation 1 min at 94°C, annealing 1 min at 68°C, and extension 4 min at 72°C for two rounds of 30 cycles each. Before each PCR, DNA was denatured for 5 min at 94°C. The identity of PCR products with switch circles was confirmed by DNA sequencing.

**Southern blots**

PCR products were fractionated on agarose gels, transferred overnight to nylon membranes, and hybridized with radiolabeled probes as described. Switch circles were hybridized with a probe recognizing the recombined S-Sw circle region (16). 1, C1–C1 transcripts were hybridized with a probe encompassing nt 1–250 of the first Cα exon. 1, C1–C1 transcripts were hybridized with a 5′-CAGGGGGAAGACCCATGT-3′ oligonucleotide recognizing a consensus Cα sequence. Similarly, 1, C1–C1 transcripts were hybridized with a 5′-TCCA CACAGAGCCCATC-3′ oligonucleotide recognizing Cα.

**Plasmids**

Genomic DNA fragments encompassing 1, 1, 1, 1, 1, 1, 1, and 1, promoters were inserted into a promoterless pGL3-Basic luciferase (Luc) reporter vector (Promega, Madison, WI) as previously reported (40–42). A DNA 5′-CAGGGGGAAGACCCATGT-3′ oligonucleotide was annealed with 1 ng of genomic DNA. GAS 1, 3, and 3, were generated and cloned into the KpnI/BglII-digested pGL3-Basic vector. Reporter vectors were transfected into 293T cells by the transfection method of Dr. H.-C. Liou, Cornell University, New York, NY, three GAS sites from the Ly-6/E gene (from Dr. J. J. Zhang, Cornell University), four GAS sites from the GAS site restriction site (40). Briefly, 5′ and 3′ DNA fragments were amplified using appropriate primers with 5′ overhangs containing KpnI (sense)/SacI (antisense) and SacI (sense)/BglII (antisense) sites, respectively. Then, DNA fragments were digested with KpnI/SacI and SacI/BglII, respectively, and cloned into the KpnI/BglII-digested pGL3-Basic vector.
296), DN-IRAK1 (residues 1–208; Tularik, San Francisco, CA), DN-TRAF2 (residues 248–501), DN-TRAF6 (residues 301–530; Science Reagents, El Cajon, CA), DN-IKKα (K44M), DN-IKKβ (K49A), DN-NIK (K429/430A; from Dr. J.-D. Li, University of South California, Los Angeles, CA), DN-STAT1 (Y701F), DN-STAT3 (Y705F; from Drs. J. E. Darnell Jr., and J. Bromberg, Rockefeller University, New York, NY), and DN-IRF4 (resides 1–150; from Dr. A. B. Pernis, Columbia University) were described previously (18, 20, 36, 44, 46). ΔTIR–TLR9 (residues 1–870) was PCR-amplified from a TLR9 plasmid (InvivoGen) using forward primer 5′-GGGGATCTACATGATTTATCAAAAAGAG-3′ and reverse primer 5′-GGGGGTACCTAGGCGAGCGGACATCTC-3′.

Luciferase reporter assays

2E2 B cells (20 × 10^6/ml) were transfected with 40 µl of plasmid DNA-TE solution containing 10 µg of L-Luc, J-Luc, ISRE-Luc, or STAT-Luc plasmid expressing firefly luciferase and 200 ng of control pRL-TK plasmid expressing Renilla luciferase under control of the thymidine kinase promoter (Promega). Similar transfections were performed in the presence or absence of 10 µg of empty expression plasmid or 10 µg of DN-MyD88, DN-IRAK1, DN-TRAF6, DN-TRAF2, Toll-interacting protein (TOLLIP), IκBα, DN-IKKα, DN-IKKβ, DN-NIK, DN-STAT1, DN-STAT3, or DN-IRF4 expression plasmid. Electroporations were performed at 625 V/cm and 950 µF using a Gene Pulser II apparatus (Bio-Rad, Hercules, CA). After electroporation, B cells were resuspended in complete medium (2 × 10^6/ml), split into aliquots, and cultured with or without stimuli. After 48 h, firefly and Renilla luciferase activities were measured using the dual luciferase reporter system (Promega) to assess promoter activity and transfection efficiency, respectively. Luciferase activity was expressed as relative light units normalized to a cotransfected pRL-TK control plasmid or as fold induction, i.e., normalized luciferase activity of extracts from stimulated B cells/normalized luciferase activity of extracts from unstimulated B cells.

Immunoblots

Equal amounts of total, cytoplasmic, or nuclear proteins (10 µg) were fractionated onto a 10% SDS-polyacrylamide gel and transferred to nylon membranes (Bio-Rad). After blocking, membranes were probed with primary Abs to TLR9 (InvivoGen), p50, p52, p65, c-Rel, RelB, IκBα, STAT1, STAT2, STAT3, STAT6, IRF1, IRF9, actin, Oct1 (Santa Cruz Biotechnology), or IRF4 (from Dr. A. B. Pernis) for 15 min at room temperature before adding the secondary Abs (Santa Cruz Biotechnology), or IRF4 (from Dr. A. B. Pernis; Santa Cruz Biotechnology)). Membranes were then washed and incubated with an appropriate secondary Ab (Santa Cruz Biotechnology). Proteins were detected with an ECL detection system (Amersham Biosciences, Little Chalfont, U.K.).

EMSAs

Nuclear proteins were extracted from 5 × 10^6 cells (40). Double-stranded oligonucleotide probes (consensus sequences are underlined or, when partially overlapping, underlined and in bold) encompassing 5′-CGTGGATCTGGCAATGCAGAAAACCAACCAAGAAG-3′, ISRE-x3 cis-I-3 (residues −216 to −192, 5′-CCCTGACACAGAACAACCCACCCCGAGAAG-3′), ISRE-x3 cis-I-3 (residues −198 to −176, 5′-CCAGAAAGAAAAAGGGAACCTCC-3′) and GAS cis-I-3 (residues −96 to −69, 5′-GAGCTGTGATTTCCTAGGAAGA-3′) sites were end-labeled with [γ-32P]ATP by T4 kinase and used at ~20,000 cpm in each EMSA reaction. Oct1-binding oligonucleotide (TGTCGAATGCAAATCAGTTAAGGAACACCACTGGAACAATACTAGAA; Santa Cruz Biotechnologies) was labeled in a similar fashion. Reaction samples were prepared as described, incubated at room temperature for 15 min, and electrophoresed through a 6% nondenaturing polyacrylamide gel. The compositions of DNA-bound protein complexes were determined by incubating reaction mixtures with 1 µl of a DNA-binding inhibiting/supershifting Ab to p65, c-Rel, Rel-B, p50, Stat1, Stat2, Stat3, Stat6, IRF1, IRF9 (Santa Cruz Biotechnology), or IRF4 (from Dr. A. B. Pernis) for 15 min at room temperature before adding the probe.

Results

B cells up-regulate TLR9 expression upon exposure to IL-10

IL-10 and IL-4 are two major CSR-inducing cytokines (1, 16). Recent studies indicate that CpG DNA cooperates with autocrine IL-10 to enhance IgG production in B cells (25, 49). Additional reports show that CpG DNA inhibits IgG and IgE production in B cells exposed to IL-4 (49, 50). These observations prompted us to verify whether TLR9 expression differs in B cells exposed to IL-10 or IL-4. Human IgD−IgM+ naive B cells up-regulated TLR9 transcripts and proteins as early as 1 h after incubation with IL-10 (Fig. 1A). This up-regulation peaked after 48 h and progressively increased upon B cell exposure to growing amounts of IL-10 (Fig. 1B). Conversely, naive B cells down-regulated TLR9 mRNA expression upon cytokine exposure.

FIGURE 1. IL-10 up-regulates TLR9 expression in human B cells. A, TLR9 and actin (loading control) transcripts and proteins in naive B cells incubated with IL-10 or IL-4 for different time points. Transcripts were RT-PCR amplified for 30 cycles. B, TLR9 and β-actin transcripts in naive B cells incubated with different doses of IL-10 or IL-4 for 2 days. Transcripts were RT-PCR amplified for 25 cycles. Data in A and B depict one of three experiments yielding similar results.
transcripts and proteins as early as 1 h after exposure to IL-4. This down-regulation was more evident after 48 h and progressively increased upon B cell exposure to growing amounts of IL-4. Thus, IL-10 and IL-4 may control B cell responses to CpG DNA by up- and down-modulating TLR9 expression, respectively.

CpG DNA up-regulates germline C1, C3, and C3 transcription in B cells by cooperating with IL-10

CSR from Cμ to a targeted downstream Cγ gene is preceded by germline Cμ-Cγ transcription (1). This early CSR event requires activation of the Cμ promoter S of the targeted Cγ gene and yields a germline Cμ-Cγ transcript encompassing a noncoding Cμ exon (3). To verify whether CpG DNA initiates germline Cμ-Cγ and IgM gene transcription, primary IgM”IgM” naive B cells or IgD”IgM” 2E2 B cells, a subclone of the CL-01 B cell line (40), were incubated for 48 h with a CpG DNA-containing ODN (ODN-2006). Cultures were conducted in the presence or the absence of IL-10, which facilitates switching to IgG1, IgG2, and IgG3, and with or without IL-4, which favors switching to IgG1, IgG2, IgG3, IgG4, and IgE (1, 51). When exposed to CpG ODN-2006, naive B cells up-regulated germline I1-C1, I2-C2, and I3-C3, but not I4-C4 and I4-C4 transcripts (Fig. 2), whereas 2E2 B cells activated I1, I2, and I3, but not I4 and I4 promoters (Fig. 3). The combination of CpG ODN-2006 and IL-10 up-regulated I1-C1, I2-C2, and I3-C3 and activated I1, I2, and I3 more effectively than CpG ODN-2006 or IL-10 alone. Control GpC ODN-2006 with inverted CpG motifs did not up-regulate I1-C1, I2-C2, and I3-C3, nor did it enhance the IL-10-induced up-regulation of I1-C1, I2-C2, and I3-C3. Similarly, GpC ODN-2006 did not activate I1, I2, and I3 or enhance IL-10-mediated activation of I1, I2, and I3.

Unlike IL-10, IL-4 up-regulated I1-C1, I2-C2, I3-C3, I4-C4, and I4-C4 transcripts and activated I1, I2, I3, I4, and I4 promoters. These effects were attenuated by CpG ODN-2006, but not by GpC ODN-2006, suggesting that CpG DNA cooperate with some, but not all, cytokines to initiate CSR. Consistent with this, CpG ODN-2006 did not activate I3 when combined with IL-2, IL-6, IL-12, IL-13, or IL-15 (Fig. 4A). The I3-inducing activity of type B CpG ODN-2006 was comparable with that of CD40L, but was higher than that of type A CpG ODN-2216, and extended to bacterial CpG DNA. In addition to inducing I3, bacterial DNA activated NF-κB-Rel, which is crucial for initiation of germline Cγ gene transcription (1). These effects were specific, because a non-CpG oligo, GpC ODN-2006, DNase I-treated bacterial DNA, and LPS did not activate NF-κB-Rel and I3 (Fig. 4B). Our findings indicate that CpG DNA preferentially cooperates with IL-10 to activate B cells and initiate germline C1, C2, and C3 transcription. They also suggest that CpG DNA inhibits IL-4-induced Cγ and Cγ transcription.

CpG DNA and IL-10 up-regulate AID and induce CSR from Cμ to C1, C2, and C3 in B cells

Additional experiments verified whether CpG DNA triggers IgG CSR in B cells. CSR requires AID and generates a circular DNA upon looping-out deletion of the IgH locus lying between Sγ and the targeted downstream S region, such as Sγ (Fig. 5A). The resulting Sγ-Sγ switch circle transcribes a chimeric Iγ-Cγ circle transcript, which includes the Iγ promoter S of the targeted Cγ gene, a noncoding Iγ exon, and the Cγ exon. Both switch circles and circle transcripts constitute specific markers of ongoing CSR (5). Naive B cells exposed to IL-10 and/or control CpG ODN-2006 for 4 days did not contain Sγ-Sγ switch circles (Fig. 5B), a byproduct of IgG CSR, nor did they express I1/12-Cγ and I3-Cγ circle transcripts, a byproduct of IgG1/IgG2 CSR and IgG3 CSR, respectively. Similarly treated B cells lacked AID transcripts. In contrast, naive B cells induced Sγ-Sγ, I1/13-Cγ, I1/13-Cγ, and AID upon

![FIGURE 3. CpG DNA cooperates with IL-10 to induce germline C1, C3, and C3 transcription in human B cells. Induction of I1-Luc, I2-Luc, I3-Luc, I4-Luc, and I4-Luc in 2E2 B cells cultured with or without CpG DNA ODN-2006, GpC ODN-2006, IL-10, and/or IL-4 for 2 days. Data correspond to one of three experiments yielding similar results (bars represent the SD of triplicate determinations).](image-url)

![FIGURE 4. CpG DNA induces germline Cγ, C3 gene transcription and NF-κB-Rel activation in human B cells through a highly specific mechanism. A. Induction of I3-Luc in 2E2 B cells cultured with or without CpG DNA ODN-2006, IL-2, IL-6, IL-10, IL-12, IL-13, and IL-15 for 2 days. B. Induction of I3-Luc and κB-Luc in 2E2 B cells cultured with or without non-CpG DNA. GpC ODN-2006, CpG ODN-2216, CpG ODN-2006, bacterial DNA, DNase I-treated bacterial DNA, LPS, or hCD40L for 2 days. Data correspond to one of three experiments yielding similar results (bars represent the SD of triplicate determinations).](image-url)
CpG DNA cooperates with IL-10 to induce CSR from C\(_3\) to C\(_{\gamma}J\), C\(_{\gamma}2\), and C\(_{\gamma}3\) in human B cells. A, Diagram of CSR from C\(_3\) to a downstream C\(_{\gamma}\) gene. Ovals indicate S regions; rectangles are V\(_{H}\)-D\(_{H}\), IgC, and C\(_{\mu}\) exons; V-shaped lines indicate splicing. B, Sy-SuScs, genomic \(\beta\)-actin, L\(_{1/2-C}\_\mu\), and L\(_{3-C}\_\mu\), and AID and \(\beta\)-actin transcripts in naive B cells cultured with or without CpG DNA ODN-2006, GpC ODN-2006, IL-10, and/or IL-4 for 4 days. C and D, Surface IgG expression and IgG secretion by naive B cells cultured with or without CpG DNA ODN-2006, GpC ODN-2006, IL-10, IL-4, anti-BCR, BAFF, and/or CD40L for 8 days. n.d., not determined. Data in B–D represent one of three independent experiments yielding similar results (bars represent the SD).

exposure to CpG ODN-2006, and this induction further increased when CpG ODN-2006 was combined with IL-10. Conversely, IL-4 attenuated the expression of S\(_3\)-\(\gamma\_\mu\), L\(_{1/2-C}\_\mu\), L\(_{3-C}\_\mu\), and AID in B cells exposed to CpG ODN-2006 (not shown). These findings indicate that CpG DNA triggers CSR to C\(_{\gamma}J\), C\(_{\gamma}2\), and C\(_{\gamma}3\) by cooperating with IL-10.

BCR engagement, BAFF, and CD40L enhance IgG production in B cells exposed to CpG DNA and IL-10

To verify whether CpG DNA up-regulates IgG production, naive B cells were exposed to CpG ODN-2006 and IL-10 in the presence or the absence of known CpG DNA-costimulating molecules, including anti-BCR and CD40L (24, 25, 49). Cultures also included BAFF, which is produced by myeloid DCs upon exposure to CpG DNA-induced cytokines, including IFN-\(\gamma\) (16, 19). Naive B cells up-regulated surface IgG upon exposure to CpG ODN-2006 (but not GpC ODN-2006) and IL-10, anti-BCR, BAFF, or CD40L for 5 days (Fig. 5C). The proportion of IgG-expressing B cells further increased when CpG ODN-2006 was combined with either IL-10 and anti-BCR or IL-10 and BAFF, or IL-10 and CD40L. This increase was incremental over that induced by either IL-10 and anti-BCR or IL-10 and BAFF, or IL-10 and CD40L. Finally, CpG ODN-2006 and IL-10 elicited IgG secretion only in B cells concomitantly exposed to anti-BCR, BAFF, or CD40L for 8 days (Fig. 5D). This suggests that CpG DNA requires IL-10 as well as additional TI or TD stimuli, including BAFF or CD40L, to stimulate IgG production in Ag-activated B cells.

CpG DNA requires TLR9, MyD88, IRAK1, and TRAF6 to induce germline C\(_{\gamma}3\) gene transcription

CD40 activates initiates NF-\(\kappa\)B-Rel-dependent germline C\(_{\gamma}\) transcription by recruiting TRAFs, including TRAF2 and TRAF6 (47, 52). Because TLR9 activates NF-\(\kappa\)B-Rel by recruiting MyD88, IRAK, and TRAF6 through a cytoplasmic TIR domain (10), it was hypothesized that the TLR9-MyD88-IRAK-TRAF6 axis is crucial for the initiation of IgG CSR by CpG DNA. Consistent with this, enforced expression of TIR-less TLR9 (\(\Delta TIR\)-TLR9), DN-MyD88, DN-IRAK1, or TOLLIP, an adapter protein that negatively regulates TLR signaling (53), inhibited the activation of C\(_{\gamma}3\)-Luc in 2E2 B cells exposed to CpG ODN-2006 for 48 h (Fig. 6A). In these cells, \(\Delta TIR\)-TLR9, DN-MyD88, DN-IRAK1, or TOLLIP inhibited NF-\(\kappa\)B-Rel-dependent activation of a minimal \(\kappa\)B-Luc reporter vector. As expected, DN-MyD88, DN-IRAK1, or TOLLIP did not inhibit activation of C\(_{\gamma}3\)-Luc and \(\kappa\)B-Luc by CD40L. Furthermore, a DN form of TRAF6 attenuated the activation of C\(_{\gamma}3\)-Luc and \(\kappa\)B-Luc by either CpG ODN-2006 or CD40L. These findings suggest that CpG DNA activates I\(\gamma\_\mu\) promoters as well as NF-\(\kappa\)B-Rel through an innate pathway that requires TLR9, MyD88, IRAK1, and TRAF6.

CpG DNA triggers germline C\(_{\gamma}3\) gene transcription through a chloroquine-sensitive pathway

Innate immune cells internalize CpG DNA and initiate TLR9 signaling through a mechanism that requires endosomal maturation and acidification (23). Consistent with this, inhibitors of endosomal maturation and acidification, such as bafilomycin-A and chloroquine, attenuate cell activation by CpG DNA (32, 39). Chloroquine and its derivatives might also block CpG DNA signaling by preventing CpG DNA binding to as yet elusive surface receptors (22, 26). Bafilomycin-A and chloroquine attenuated the activation of both C\(_{\gamma}3\)-Luc and \(\kappa\)B-Luc in 2E2 B cells exposed to CpG ODN-2006 (Fig. 6B). In contrast, bafilomycin-A and chloroquine did not affect the activation of C\(_{\gamma}3\)-Luc and \(\kappa\)B-Luc in 2E2 B cells exposed
FIGURE 6. CpG DNA requires TLR9, MyD88, IRAK, and TRAF6 to activate the I\(_3\) promoter in human B cells. A, Induction of I\(_3\)-Luc and \(\kappa\)B-Luc in 2E2 B cells transfected with empty vector (control), \(\Delta\)TIR-TLR9, DN-MyD88, DN-IRAK1, DN-TRAF6, or TOLLIP and subsequently incubated with CpG DNA ODN-2006 or CD40L for 2 days. Data are presented as a percentage of the yield (fold activation with expression vector/fold activation without expression vector × 100). B, Induction of I\(_3\)-Luc and \(\kappa\)B-Luc in 2E2 B cells incubated for 48 h with CpG DNA ODN-2006 or CD40L in the presence or the absence of control H\(_2\)O, bafilomycin A (BFLM) or chloroquine (CHLQ). H\(_2\)O, BFLM or CHLQ were added 6 h before CpG DNA ODN-2006 or CD40L. Data are presented as a percentage of the yield (fold activation with H\(_2\)O, BFLM or CHLQ/fold activation without H\(_2\)O, BFLM or CHLQ × 100). Data in A and B correspond to one of three experiments yielding similar results (bars represent the SD of triplicate determinations).

to CD40L. These data suggest that CpG DNA activates I\(_3\) promoters and NF-\(\kappa\)B-Rel through a chloroquine-sensitive B cell pathway.

CpG DNA and IL-10 trans-activate the I\(_3\) promoter through \(\kappa\)B, ISRE, and GAS cis-acting elements

Germline C\(_3\) gene transcription requires key cis-acting DNA regulatory elements located within the evolutionarily conserved sequence (ECS) of I\(_3\) promoters (Fig. 7A). The human ECS includes three \(\kappa\)B sites (\(\kappa\)B1, \(\kappa\)B2, and \(\kappa\)B3), a BSAP, and a STAT-binding GAS (1, 40). CD40L-induced C\(_3\) gene transcription involves binding of NF-\(\kappa\)B-Rel and BSAP to \(\kappa\)B1, \(\kappa\)B2, and BSAP sites, whereas IL-4-induced C\(_3\) gene transcription requires binding of STAT6 to the GAS site. This latter can bind STAT1, STAT2, STAT3, and IRF4 in addition to STAT6 (44, 46, 54–56). Interestingly, the ECS contains also an ISRE site, which partially overlaps with \(\kappa\)B3 (ISRE-\(\kappa\)B3) (40). In general, ISRE binds the STAT1-STAT2-IRF9 complex as well as IRFs, including IRF1 and IRF4 (44, 56). STATs and IRFs are induced by several receptors, including TLRs and IL-10Rs, and cooperate with NF-\(\kappa\)B-Rel proteins to modulate B cell activation, differentiation, and IgG production (44, 46, 50, 54–59). Thus, it was postulated that CpG DNA and IL-10 initiate C\(_3\) transcription upon NF-\(\kappa\)B-Rel, STAT, and IRF binding to cooperative \(\kappa\)B, ISRE, and GAS cis-I\(_3\) sites. To verify this, 2E2 B cells were transfected with reporter vectors carrying wild-type I\(_3\) or mutated I\(_3\) with targeted disruptions of \(\kappa\)B1, \(\kappa\)B2, ISRE-\(\kappa\)B3, BSAP, and GAS (43). In 2E2 B cells incubated with medium alone, wild-type and mutated I\(_3\) promoters displayed similar transcriptional activity (Fig. 7B). Disruption of \(\kappa\)B1 or BSAP did not affect activation of I\(_3\) by CpG ODN-2006 and/or IL-10. In contrast, disruption of \(\kappa\)B2, \(\kappa\)B3-ISRE, or GAS impaired activation of I\(_3\) by CpG ODN-2006 and/or IL-10. These findings suggest that CpG DNA and IL-10 require \(\kappa\)B2, \(\kappa\)B3, ISRE, and GAS, but not \(\kappa\)B1 and BSAP, cis-acting motifs to initiate germline C\(_3\) transcription.

CpG DNA and IL-10 activate NF-\(\kappa\)B-Rel

Given the key role of \(\kappa\)B2 and ISRE-\(\kappa\)B3 in the activation of I\(_3\) by CpG ODN-2006 and IL-10, it was postulated that CpG DNA and IL-10 synergistically induce C\(_3\) gene transcription by cooperatively activating NF-\(\kappa\)B-Rel. Initial experiments tested the induction of transcriptionally active NF-\(\kappa\)B-Rel. 2E2 B cells activated a minimal \(\kappa\)B-Luc reporter vector upon exposure to CpG ODN-2006, but not CpG ODN-2006, for 48 h (Figs. 3B and 8A). When combined, CpG ODN-2006 and IL-10 induced \(\kappa\)B-Luc more than either CpG ODN-2006 or IL-10 alone (Fig. 8A). Additional studies evaluated NF-\(\kappa\)B-Rel nuclear translocation. When exposed to CpG ODN-2006 for 3 h, naive B cells up-regulated I\(_{\kappa}\)B\(_{\alpha}\) phosphorylation and down-regulated total I\(_{\kappa}\)B\(_{\alpha}\), a hallmark of increased I\(_{\kappa}\)B\(_{\alpha}\) degradation (Fig. 8B). After 6 h, CpG ODN-2006 up-regulated the expression of nuclear p65, p50, c-Rel, p52, and RelB (Fig. 8C). Also, IL-10 activated \(\kappa\)B-Luc and up-regulated I\(_{\kappa}\)B\(_{\alpha}\) phosphorylation, I\(_{\kappa}\)B\(_{\alpha}\) degradation, and NF-\(\kappa\)B-Rel nuclear translocation, although less than CpG ODN-2006. When combined, CpG ODN-2006 and IL-10 induced more...
IκBα phosphorylation, IκBα degradation, and NF-κB-Rel nuclear translocation than CpG ODN-2006 or IL-10 alone. Thus, CpG DNA and IL-10 cooperatively activate p65, p50, c-Rel, p52, and RelB in B cells.

IL-4 activated κB-Luc and up-regulated IκBα phosphorylation, IκBα degradation, and NF-κB-Rel nuclear translocation. Compared with IL-4 or CpG ODN-2006 alone, CpG ODN-2006 and IL-4 did not significantly increase κB-Luc activation, IκBα phosphorylation, IκBα

FIGURE 8. CpG DNA and IL-10 induce transcriptionally active NF-κB-Rel and up-regulate NF-κB-Rel binding to κB2 cis-1,3 in human B cells. A, κB-Luc activity in 2E2 B cells incubated with or without CpG ODN-2006, IL-10, and/or IL-4 for 2 days. B and C, Cytoplasmic pIκBα, IκBα, and actin proteins as well as nuclear p65, p50, c-Rel, p52, and RelB proteins in naive B cells cultured as in A for 1 h. The ubiquitous nuclear protein octamer 1 (Oct1) was used as loading control. D, Aligned −232/−176 1,3, 1,1, and 1,2 DNA sequences. The boxed sequence corresponds to the κB2 cis-1,3 motif. Left gels show nuclear protein binding to an oligonucleotide encompassing κB2 cis-1,3 or a consensus Oct1-binding motif. Nuclear extracts were from naive B cells cultured for 6 h as described in A. Italic letters indicate protein-DNA complexes. Right gel shows protein-DNA complexes from naive B cells activated with CpG DNA after nuclear protein incubation with a cold κB2 cis-1,3 probe or Abs to p50, p52, p65, c-Rel, and RelB. Asterisks indicate lanes in which complexes are attenuated or supershifted by the Ab. Data in A–D correspond to one of three experiments yielding similar results.

FIGURE 9. CpG DNA and IL-10 up-regulate NF-κB-Rel, STAT, and IRF binding to ISRE-κB3 cis-1,3 in human B cells. A, Aligned −232/−176 1,3, 1,1, and 1,2 DNA sequences. The boxed sequence corresponds to the ISRE-κB3 motif. Gels show nuclear protein binding to a radiolabeled oligonucleotide encompassing ISRE-κB3 cis-1,3 or a consensus Oct1-binding motif (loading control). Nuclear extracts were from naive B cells cultured with or without CpG ODN-2006, IL-10, and/or IL-4 for 6 h. Italic letters indicate shifted protein-DNA complexes. B, ISRE-κB3 cis-1,3-binding protein-DNA complexes from naive B cells activated with CpG DNA after nuclear protein incubation with a cold probe or Abs to p50, p52, p65, c-Rel, and RelB. Asterisks indicate lanes in which complexes are attenuated or supershifted by the Ab. C, ISRE-κB3 cis-1,3-binding protein complexes from naive B cells activated with CpG DNA after nuclear protein incubation with a cold probe or Abs to STAT1, STAT2, STAT3, STAT6, IRF1, IRF4, or IRF9. Asterisks indicate lanes in which complexes are attenuated or supershifted by the Ab. Data in A–C correspond to one of three experiments yielding similar results.
degradation, or NF-κB-Rel nuclear translocation. These findings indicate that IL-4 does not cooperate with CpG DNA to activate NF-κB-Rel.

CpG DNA and IL-10 up-regulate NF-κB-Rel binding to κB2 and ISRE-κB3 cis-I,3

We next evaluated whether CpG DNA and IL-10 up-regulate the binding of nuclear NF-κB-Rel to κB2 cis-I,3. Naïve B cells up-regulated the binding of complexes a, b, c, d, e, f, and g to κB2 cis-I,3 upon incubation with CpG ODN-2006 for 6 h (Fig. 8D). These complexes were attenuated or supershifted by preincubating nuclear proteins with Abs to p65, p50 and p65, p50 and c-Rel, p52 and RelB, p50 and RelB, p50 and p52, and p50, respectively. IL-10 up-regulated complexes a, b, c, d, e, f, and g to κB2 less than CpG ODN-2006, whereas CpG ODN-2006 and IL-10 up-regulated complexes a, b, c, d, e, f, and g more than CpG ODN-2006 or IL-10 alone. Additional experiments evaluated whether CpG DNA and IL-10 up-regulate the binding of nuclear NF-κB-Rel to ISRE-κB3 cis-I,3. Naïve B cells up-regulated the binding of nuclear complexes a, b, and c to ISRE-κB3 cis-I,3 upon incubation with CpG ODN-2006 for 6 h (Fig. 9A). These complexes were attenuated or supershifted by preincubation to p50, p52, p65, c-Rel, and RelB; p50, p56, and c-Rel; and p50, respectively (Fig. 9B). IL-10 up-regulated complexes a, b, and c less than CpG ODN-2006, whereas CpG ODN-2006 and IL-10 up-regulated complexes a, b, and c more than CpG ODN-2006 or IL-10 alone. Thus, CpG DNA cooperates with IL-10 to increase p50, p52, p65, c-Rel, and RelB binding to I,3.

IL-4 up-regulated the binding of complexes a, b, c, d, e, f, and g to κB2 (Fig. 8D) as well as the binding of complex c to ISRE-κB3, but did not affect the binding of complexes a and b to ISRE-κB3 (Fig. 9A). Compared with IL-4 or CpG ODN-2006 alone, CpG ODN-2006 and IL-4 attenuated the binding of complexes d and e to κB2, but did not affect or even increased the binding of complexes a, b, c, f, and g to κB2 as well as the binding of complex c, b, and c to ISRE-κB3. Finally, CpG ODN-2006 and IL-4 up-regulated the binding of complexes a, b, c, d, and e to κB2 less efficiently than CpG ODN-2006 and IL-10. These findings suggest that IL-4 does not cooperate with IL-10 to up-regulate p50, p52, p65, c-Rel, and RelB binding to I,3.

CpG DNA and IL-10 activate STAT1, STAT3, IRF1, and IRF4

Considering the key role of ISRE-κB3 and GAS sites in I,3 transcription, it was hypothesized that CpG DNA and IL-10 synergistically activate the C,3 gene by cooperatively activating STATs and IRFs. Reporter vectors carrying multiple ISRE sites (ISRE-Luc), STAT1/3-binding GAS sites (STAT1/3-Luc), and STAT6-specific GAS sites (STAT6-Luc) were used to test the induction of transcriptionally active STATs and IRFs. 2E2 B cells activated ISRE-Luc, STAT1/3-Luc, and, to a lesser extent, STAT6-Luc upon exposure to CpG ODN-2006 or IL-10, but not CpG ODN-

FIGURE 10. CpG DNA and IL-10 induce transcriptionally active STAT1, STAT3, IRF1, and IRF4 in human B cells. A, Activation of ISRE-Luc, GAS-STAT1/3-Luc, or GAS-STAT6-Luc in 2E2 B cells incubated with or without with or without CpG ODN-2006, GpC ODN-2006, IL-10, and/or IL-4 for 2 days. B, Cytoplasmic pSTAT1 and total STAT1 (91- and 84-kDa isoforms), pSTAT2, total STAT2, pSTAT3, and total STAT3 (88- and 83-kDa isoforms), pSTAT6, and total STAT6 in naive B cells cultured with or without CpG ODN-2006, IL-10, and/or IL-4 for 1 h. Nuclear IRF1, IRF4, and IRF9 are also shown. NS, nonspecific band. Data in A and B correspond to one of three experiments yielding similar results.
2006, for 48 h (Fig. 10A). When combined, CpG ODN-2006 and IL-10 activated ISRE-Luc and STAT1/3-Luc more efficiently than CpG ODN-2006 or IL-10 alone. Although unable to significantly activate ISRE-Luc and STAT1/3-Luc, IL-4 activated STAT6-Luc more efficiently than CpG ODN-2006 and IL-10. This activation was attenuated by CpG ODN-2006, but not by GpC ODN-2006. These findings indicate that CpG DNA cooperates with IL-10, but not IL-4, to induce transcriptionally active STATs and IRFs. They also suggest that CpG DNA attenuates IL-4-induced, STAT6-dependent gene transcription.

STATs undergo nuclear translocation upon phosphorylation by Jaks (44), whereas IRFs undergo nuclear translocation upon phosphorylation by as yet elusive kinases, including IKKe and TANK-binding kinase 1 (TBK1) (56, 59). Naive B cells up-regulated STAT1 and STAT3 phosphorylation and induced IRF1 and IRF4 nuclear translocation upon exposure to CpG ODN-2006 or IL-10 for 1 h (Fig. 10B). In contrast, CpG ODN-2006 or IL-10 did not affect STAT2 and STAT6 phosphorylation or IRF9 nuclear translocation. When combined with IL-10, CpG ODN-2006 elicited more STAT1 and STAT3 phosphorylation and more IRF1 and IRF4 nuclear translocation than CpG ODN-2006 or IL-10 alone. In addition to inducing IRF1 and IRF4 nuclear translocation, IL-4 elicited STAT2 and STAT6 phosphorylation. Notably, CpG ODN-2006 attenuated STAT6 (but not STAT2) phosphorylation and IRF4 (but not IRF1) nuclear translocation in B cells exposed to IL-4. Conversely, IL-4 attenuated STAT1 and STAT3 phosphorylation as well as IRF1 and IRF4 nuclear translocation in B cells exposed to CpG ODN-2006. Collectively, these findings indicate that CpG DNA cooperates with IL-10, but not IL-4, to activate STAT1, STAT3, IRF1, and IRF4 in B cells. They also suggest that CpG DNA interferes with the activation of STAT6 and IRF4 by IL-4.

CpG DNA and IL-10 up-regulate STAT1, STAT3, IRF1, and IRF4 binding to ISRE and GAS cis-I3

Having shown that CpG DNA cooperates with IL-10 to increase the binding of NF-κB-Rel-containing complexes a and b to ISRE-κB3 cis-I3 (Fig. 9, A and B), it was determined whether these complexes include IRFs and STATs (Fig. 10). Consistent with this, the binding of complexes a and b to ISRE-κB3 was attenuated by incubating nuclear proteins from CpG ODN-2006-stimulated B cells with Abs to STAT1, IRF1, and IRF4 (Fig. 9C). Further assays evaluated STAT and IRF binding to the GAS element of I3. Naive B cells up-regulated the binding of complexes a, f, g, h, and i to GAS cis-I3 upon exposure to CpG ODN-2006 for 6 h (Fig. 11A). These complexes were attenuated or supershifted by Abs to STAT1, STAT3, IRF1, and IRF4; STAT1, STAT3, and IRF1; STAT1, IRF1, and IRF4; STAT1 and IRF4; and IRF4, respectively (Fig. 11B). Finally, IL-10 alone or combined with CpG ODN-2006 induced more binding of complexes a, f, g, h, and i to GAS cis-I3 than did CpG ODN-2006 alone. These findings indicate that CpG DNA and IL-10 cooperatively increase STAT and IRF binding to I3.

IL-4 up-regulated the binding of complexes b, c, d, e, and i, but not a, f, g, and h, to GAS cis-I3 (Fig. 11A). Complexes b, c, d, e, and i were attenuated or supershifted by preincubating nuclear proteins with Abs to STAT6 and IRF4 (not shown), STAT6, STAT2 and STAT6, STAT2, and IRF4, respectively (Fig. 11C). Compared with IL-4 alone, IL-4 and CpG ODN-2006 induced less binding of complexes b, c, and i to GAS cis-I3, whereas the binding of complexes d and e remained unchanged. Finally, CpG ODN-2006 and IL-4 induced less binding of complexes f, g, h, and i to GAS cis-I3 than CpG ODN-2006 alone. Thus, CpG DNA impairs IL-4-induced binding of STAT6 and IRF4 to I3; conversely, IL-4 prevents CpG DNA-induced IRF4 binding to I3.

**FIGURE 11.** CpG DNA and IL-10 up-regulate STAT and IRF binding to GAS cis-I3 in human B cells. A, Aligned −93 to −70 I3, I1, and I2 DNA sequences. The boxed sequence corresponds to the GAS motif. Gel shows binding of nuclear proteins to a radiolabeled oligonucleotide encompassing GAS cis-I3 or a consensus Oct1 motif (loading control). Nuclear proteins were from naive B cells incubated with or without CpG ODN-2006, IL-10, and/or IL-4 for 6 h. Italic letters indicate shifted protein-DNA complexes. B, GAS cis-I3-binding protein-DNA complexes from naive B cells activated with CpG DNA after nuclear protein incubation with a cold probe or Abs to STAT1, STAT2, STAT3, STAT6, IRF1, IRF4, or IRF9. Asterisks indicate lanes in which complexes are attenuated or supershifted by the Ab. C, GAS cis-I3-binding protein complexes from naive B cells activated with IL-4 after nuclear protein incubation with a cold probe or Abs to STAT1, STAT2, STAT3, STAT6, IRF1, IRF4, or IRF9. Asterisks indicate lanes in which complexes are attenuated or supershifted by the Ab. Data in A–C correspond to one of three experiments yielding similar results.
CpG DNA and IL-10 require NF-κB-Rel, STAT1, STAT3, and IRF4 to activate IκB

The involvement of NF-κB-Rel, STAT, and IRF proteins in CpG DNA-induced germline Cκ gene transcription was further evaluated in 2E2 B cells transfected with DNA plasmids inhibiting the activation and/or DNA-binding activity of endogenous NF-κB-Rel, STAT1, STAT3, and IRF4. Enforced expression of DN-IκBα, DN-IKKα, DN-IKKβ, or, to a lesser extent, DN-NIK attenuated the activation of both IκB-Luc and IκB-Luc in 2E2 B cells exposed to CpG ODN-2006 and IL-10 for 48 h (Fig. 12). In similar B cells, enforced expression of DN-STAT1, DN-IRF4, or, to a lesser extent, DN-STAT3 attenuated activation of IκB-Luc but not that of κB-Luc. These findings provide additional evidence that CpG DNA and IL-10 initiate IgG CSR by activating NF-κB-Rel, STAT, and IRF proteins.

Discussion

Marginal zone B cells and mucosal CD5+ B-1 cells generate a massive wave of extrafollicular Ab-producing plasmablasts in the initial 3 days of a primary Ab response through a mechanism that does not require T cell help (7). Although unable to induce affinity maturation and immune memory, early T B cell responses lead to the production of IgM, IgG, and IgA, which play important roles in the initial control of infections by quickly replicating pathogens (7, 8). Previous evidence indicates that PAMPs initiate T Ab responses by inducing extensive BCR cross-linking (9). Consistent with this, marginal zone and B-1 B cells recognize PAMPs through semiconserved BCRs encoded by a restricted set of Ig V(D)J genes carrying no or few mutations (11). In addition to cross-linking poorly diversified BCRs, PAMPs activate innate Ag receptors on B cells.

This is exemplified by LPS, which stimulates TI IgM production and IgG CSR by engaging TLR4 on mouse B cells (1). Not only does LPS stimulate mouse B cells to produce bacteria-specific IgM and IgG upon dual BCR and TLR4 engagement, but it also elicits polyclonal Ab production through TLR4 only (1). LPS-induced IgG CSR and Ab production would be further enhanced by BAFF, a B cell-stimulating TNF family member produced by LPS-stimulated myeloid DCs (14, 16). Like BCR and TLR4 (10, 60), BAFF receptors on B cells activate NF-κB-Rel proteins (61), which are essential for both CSR and Ab production (1). This implies that early TI IgM and IgG production results from the stimulation of both germline and somatically recombined Ag receptors with intersecting NF-κB-Rel pathways.

Human B cells lack TLR4 but express TLR9 (22), an intracellular pattern recognition receptor that detects CpG DNA from viruses and bacteria (18). Previous studies show that CpG DNA triggers B cell proliferation and TI IgM production (20, 24, 25), but do not clarify the role of CpG DNA in IgG CSR. By showing that CpG DNA elicits CSR to C2, C2a, C2h, and C3, our findings extend to human B cells recent data demonstrating that CpG DNA stimulates CSR to C2, C2a, C2h, and C3 in mouse B cells (62). Unlike LPS (1), CpG DNA does not elicit significant IgG production in the absence of additional stimuli. This might stem from the fact that TLR9 signals through a pathway distinct from that emanating from TLR4 (10, 22). In line with studies showing that CpG DNA induces IL-10 (22, 49) and synergizes with both IL-10 and BCR to activate B cells (24, 25), we found that CpG DNA cooperates with IL-10 and BCR cross-linking to up-regulate IgG. The expression of IgG is also enhanced by BAFF, which is released by myeloid DCs upon exposure to CpG DNA-inducible cytokines, such as IFN-α and IL-10 (14, 16, 19, 49).

Low concentrations of microbial CpG DNA are thought to favor the activation of DNA-specific B cells through dual TLR9 and BCR engagement (27, 32, 33). This response would involve B-1 and marginal zone B cells expressing poorly diversified BCRs and might be important to facilitate rapid removal of immunogenic CpG DNA released by invading pathogens. Higher concentrations of microbial CpG DNA would trigger polyclonal B cell activation mainly through TLR9 (25). Although less specific, this response might be important to amplify IgG class switching and production in Ag-primed B cells. Not only would CpG DNA favor the initiation of early TI IgG responses, but it would also amplify later-appearing TD IgG responses. Consistent with this, our results extend previous findings indicating that CpG DNA enhances IgG production in B cells exposed to CD40L (49), a TNF family member expressed by CD4+ T cells upon activation by APCs (4). CpG DNA would further enhance TD IgG production by favoring the release of IFN-α (19, 22), a powerful inducer of key T cell-co-stimulating molecules on APCs (21).

By showing that enforced B cell expression of ATIR-TLR9, DN-MyD88, DN-IRAK1, or DN-TRAF6 attenuates CpG DNA-induced, but not CD40L-induced, IκB-Rel activation, our data suggest that CpG DNA requires TLR9, MyD88, IRAK1, and TRAF6 to initiate IgG CSR. These findings are consistent with recently published data showing that TLR9 and MyD88 are required for the induction of IgG CSR in mouse B cells (62). That TLR9 accounts for CpG DNA-induced IgG CSR is also indicated by our observation that enforced B cell expression of TOLLIP, an adaptor protein that negatively regulates TLR signaling (53), or B cell exposure to chloroquine, an endosomal maturation inhibitor that interferes with CpG DNA-TLR9 interaction (39), attenuates the induction of IκB-Rel activation by CpG DNA. This inhibitory effect is specific, because TOLLIP and chloroquine do not affect IκB-Rel activation by CpG DNA. This inhibitory effect could account for the inhibition of IgG CSR observed in our experiments, which were conducted with concentrations of chloroquine lower than those required to specifically inhibit the endocytic pathway. In agreement with the idea that CD40 signals through both TRAF2 and TRAF6, whereas TLR9 requires TRAF6, but not TRAF2 (10, 36, 47, 52), overexpression of DN-TRAF6...
imparts Ι₃ and NF-κB-Rel activation in B cells exposed to either CpG DNA or Cd40L. Conversely, overexpression of DN-TRAF2 dampens Ι₃ and NF-κB-Rel activation in B cells exposed to Cd40L, but not in those exposed to CpG DNA. These findings imply that CpG DNA and Cd40L trigger CpG DNA-induced IgG CSR through partially overlapping signaling pathways.

NF-κB-Rel activation is essential for CpG DNA-induced IgG CSR, because overexpression of DN-IκBκκ and DN-Iκκ attenuation of Ι₃ and NF-κB-Rel activation. Nevertheless, the ‘κB and κB3 cis-I₃ sites impairs C₃,3 gene transcription in B cells exposed to CpG DNA. In agreement with previous studies showing that CpG DNA cooperates with IL-10, but not IL-4, to activate B cells (51, 52), we found that CpG DNA-induced C₄ transcription is selectively increased by IL-10. That CpG DNA preferentially cooperates with IL-10 to initiate IgG CSR is also indicated by our observation that IL-2, IL-6, IL-12, IL-13, and IL-15 fail to increase CpG DNA-induced C₄ gene transcription even though they are involved in various CpG DNA-mediated immune responses (22, 25, 49).

Our findings suggest that IL-10 amplifies CpG DNA-induced CSR by increasing TLR9 expression and subsequent TLR9-mediated NF-κB-Rel activation. It is also likely that B cells exposed to CpG DNA and IL-10 rapidly up-regulate autocrine NF-κB-Rel-activating factors, including BAFF (14, 61, 63).

In addition to inducing NF-κB-Rel, CpG DNA and IL-10 cooperatively activate STAT1, STAT3, IRF1, and IRF4, which are all implicated in the regulation of IgG CSR and Ab production (46, 54–58). IL-10 would activate STATs and IRFs through Jak1 (44), whereas CpG DNA would activate IRFs through IKKκ and TBK1 (59). CpG DNA would also activate STATs by inducing autocrine release of IL-10 (22, 49). Our findings suggest that binding of STAT1, STAT3, IRF1, and IRF4 to ISRE and GAS cis-I₃ sites proximal to or partially overlapping with κB2 and κB3 enhances NF-κB-Rel-mediated germline C₄ gene transcription. This effect might be associated with STAT and IRF interaction with NF-κB-Rel. Consistent with this, STATs and IRFs enhance the transcription of several Ig and non-Ig gene promoters by forming heterocomplexes with NF-κB-Rel (1, 44, 54, 64). These heterocomplexes would stabilize NF-κB-Rel binding to the κB cis-acting site, thereby enhancing NF-κB-Rel-dependent gene trans-activation. That STATs and IRFs play a key role in CpG DNA-induced IgG CSR is further indicated by our observation that disruption of ISRE-κB3 and GAS cis-I₃ sites or enforced DN-STAT-1, DN-STAT3, or DN-IRF4 expression inhibits germline C₃,3 gene transcription in B cells exposed to CpG DNA and IL-10.

By showing that CpG DNA impairs the IL-4-induced activation of Ι₃ and I₄ promoters, our results extend previous findings indicating that CpG DNA inhibits IgG1 and IgE production in B cells exposed to IL-4 (49, 50). The mechanism underlying this inhibition remains unclear. IL-4 initiates CSR by eliciting phosphorylation-dependent activation of Jak3, an IL-4R-associated kinase that triggers STAT6 phosphorylation and nuclear translocation (65). Once in the nucleus, STAT6 triggers germline C₄ and C₅ gene transcription upon binding to cis-acting GAS sites on Ι₃ and I₄ promoters (1, 41, 64). This early CSR-associated event would be further amplified by IRF4 (57), an IL-4-inducible protein that binds GAS sites in association with STAT6 and I₄ promoters (46, 56). Our experiments suggest that CpG DNA inhibits IL-4-dependent germline C₄ and C₅ gene transcription by attenuating IL-4-induced phosphorylation and nuclear translocation of STAT6 and IRF4. This effect might derive from the activation of Jak3 phosphatases, including CD45, a transmembrane receptor with Jak phosphatase activity (66). Consistent with this, recent studies indicate that CD45 engagement attenuates IL-4-induced IgE CSR in B cells by preventing the phosphorylation of STAT6 by Jak3 (67).

Notably, CpG DNA does not attenuate the IL-4-induced activation and nuclear translocation of STAT2. This transcription factor is activated by IFN-α (44), a known IL-4 signaling inhibitor (68), and would negatively regulate IL-4-induced C₄ and C₅ gene transcription by limiting the GAS-binding and/or the transcriptional activity of STAT6 (55). CpG DNA might also attenuate IL-4-induced C₄ and C₅ gene transcription by up-regulating the transcriptional repressor Bcl-6 (69). A similar function has been recently attributed to T-bet, a T-box transcriptional regulator induced by CpG DNA (50). Yet, the fact that T-bet is heavily involved in TI IgG production (70) suggests that T-bet may induce, rather than inhibit, IgG CSR at least in B cells exposed to CpG DNA and IL-10. Notably, not only is IL-4 signaling inhibited by CpG DNA, but it also inhibits CpG DNA-induced STAT1, STAT3, and IRF4 activation, possibly through a mechanism involving down-regulation of TLR9 expression. This implies that CpG DNA and IL-4 reciprocally turn off their signaling pathways.

Marginal zone and B-1 B cells express poorly diversified Abs that target microbial molecular patterns, such as CpG DNA, often shared by highly conserved intracellular self-Ags (11). This would explain the facility with which marginal zone and B-1 clones are recruited into autoreactive responses (11, 71). If tightly controlled, the natural self-reactivity of marginal zone and B-1 B cells would be important to optimize the removal of immune-stimulating products released by dying cells. This is exemplified by natural anti-DNA Abs, which facilitate the removal of apoptotic CpG DNA in addition to neutralizing microbial CpG DNA. The clearance of CpG DNA would be further enhanced by Rfs, which target IgG-bound DNA (11). Thus, autoreactive marginal zone and B-1 B cells might be useful to prevent untoward autoimmune responses initiated by close-reactive TI Ags. Yet in the presence of persistent or repetitive TI activation, the natural autoantibody repertoire would become pathogenic. This would entail both quantitative and qualitative changes, including increased switching from IgM to IgG. Previous studies indicate that dual TLR9 and BCR engagement by endogenous hypomethylated CpG stimulates autoreactive B cells to produce anti-DNA IgM and IgM-RFs (32, 33). Our findings suggest that a similar TI pathway might induce the production of autoreactive IgG. Consistent with this, CpG DNA administration augments the production of pathogenic DNA-reactive IgG in lupus-prone mice (72).

In SLE and RA, tissue damage stems from deposition of pathogenic IgG targeting a relatively limited set of self-Ags, including DNA (31). Unlike IgM, IgG activates powerful FcγRs on proinflammatory immune cells, including neutrophils, and therefore are highly pathogenic (29, 30). Although IgG CSR is known to play a key role in the pathogenesis of SLE and RA, the mechanisms leading to its dysregulation remain unclear. By showing that TLR9 signaling initiates IgG CSR in B cells, our findings suggest that factors increasing the availability of hypomethylated CpG DNA may facilitate the production of pathogenic IgG in predisposed subjects. Consistent with this, microbial infections, extensive cell death, or drugs inhibiting DNA methylation, such as procainamide, often initiate or exacerbate anti-DNA IgG responses as well as SLE and RA manifestations (29, 30, 71, 73).

Low concentrations of CpG DNA and CpG DNA-IgG immune complexes would induce anti-DNA IgG and RF-IgG by stimulating B cells through both TLR9 and BCR (26, 27, 32, 33), whereas high concentrations of CpG DNA would elicit polyclonal IgG production by stimulating B cells through TLR9 only (25). The IgG-inducing activity of self and microbial CpG DNA would be further enhanced by IFN-α and IL-10 (16, 70, 74), two CpG DNA-inducible cytokines abnormally increased in patients with SLE and RA (22, 31, 49, 75, 76). CpG DNA, IFN-α, and IL-10 would
further amplify autoreactive IgG production by up-regulating BAFF (14, 16, 77), a key innate player in the pathogenesis of IgG-mediated autoimmune disorders (17, 31, 78). Thus, it is conceivable that chloroquine attenuates SLA and RA clinical manifestations because of its ability to inhibit multiple CpG DNA-inducible pathways, leading autoreactive B cells to switch from IgM to a more pathogenic IgG isotype.

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