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CpG-B Oligodeoxynucleotides Inhibit TLR-Dependent and -Independent Induction of Type I IFN in Dendritic Cells

Yi C. Liu,*1 Reginald C. Gray,*†,1 Gareth A. D. Hardy,* John Kuchtey,*2 Derek W. Abbott,* Steven N. Emancipator,* and Clifford V. Harding*†,‡

CpG oligodeoxynucleotides (ODNs) signal through TLR9 to induce type I IFN (IFN-αβ) in dendritic cells (DCs). CpG-A ODNs are more efficacious than CpG-B ODNs for induction of IFN-αβ. Because IFN-αβ may contribute to autoimmunity, it is important to identify mechanisms to inhibit induction of IFN-αβ. In our studies, CpG-B ODN inhibited induction of IFN-αβ by CpG-A ODN, whereas induction of TNF-α and IL-12p40 by CpG-A ODN was not affected. CpG-B inhibition of IFN-αβ was observed in FLT3 ligand-induced murine DCs, purified murine myeloid DCs, plasmacytoid DCs, and human PBMCs. CpG-B ODN inhibited induction of IFN-αβ by agonists of multiple receptors, including MyD88-dependent TLRs (CpG-A ODN signaling via TLR9, or R837 or Sendai virus signaling via TLR7) and MyD88-independent receptors (polynosinic:polycytidylic acid signaling via TLR3 or ds break-DNA signaling via a cytosolic pathway). CpG-B ODN did not inhibit the IFN-αβ positive feedback loop second-wave IFN-αβ, because IFN-αβ-induced expression of IFN-αβ was unaffected, and CpG-B inhibition of IFN-αβ was manifested in IFN-αβR−/− DCs, which lack the positive feedback mechanism. Rather, CpG-B ODN inhibited early TLR-induced first wave IFN-α4 and IFN-β. Chromatin immunoprecipitation revealed that association of IFN regulatory factor 1 with the IFN-α4 and IFN-β promoters was induced by CpG-A ODN but not CpG-B ODN. Moreover, CpG-A–induced association of IFN regulatory factor 1 with these promoters was inhibited by CpG-B ODN. Our studies demonstrate a novel mechanism of transcriptional regulation of first-wave IFN-αβ that selectively inhibits induction of IFN-αβ downstream of multiple receptors and may provide targets for future therapeutic inhibition of IFN-αβ expression in vivo.

To induce innate immunity and trigger adaptive immunity, ssDNA or CpG oligodeoxynucleotides (ODNs) signal through TLR9 (1–5). In mice, TLR9 is widely expressed by APCs, including myeloid dendritic cells (mDCs), plasmacytoid DCs (pDCs), macrophages, and B cells. In humans, TLR9 expression is restricted to pDCs and B cells (4–6).

There are three classes of CpG ODNs, class A (also known as D-type), class B (also known as K-type), and class C ODNs (1, 3, 4, 7, 8). CpG-B and CpG-C ODNs stimulate B cells efficiently to produce IgM and IL-6, whereas CpG-A ODNs are weak inducers of B cell responses. CpG-A and CpG-C ODNs are more efficacious than CpG-B ODNs for inducing type I IFN (IFN-αβ) (1, 7–9), particularly in pDCs (1, 3, 4, 7, 9–11), although CpG-B ODNs are as potent for the induction of low levels of IFN-αβ (9). All three classes of ODNs induce DCs to mature and produce TNF-α (1, 3, 10). Thus, CpG-A and CpG-B ODNs have distinct effects, and CpG-C ODNs combine properties of CpG-A and CpG-B ODNs.

Differences in CpG ODN induction of IFN-αβ may depend on differences in spatial localization of different ODNs within the cell as well as differences in signaling in different cell types. CpG-A ODNs localize to endosomes in pDCs and lysosomes in mDCs; CpG-B ODNs localize to lysosome-associated membranes in both pDCs and mDCs (12, 13). In pDCs, CpG-A induction of IFN-αβ is IFN regulatory factor (IRF) 7-dependent, whereas in mDCs, CpG-A and CpG-B induction of IFN-β is independent of IRF7, but dependent on IRF1 (14, 15).

IFN-αβ is induced by CpG-A ODNs, agonists that signal via other TLRs (dsRNA via TLR3, LPS via TLR4, and ssRNA and antiviral agents via TLR7) and agonists that signal via non-TLRs (dsRNA via melanoma differentiation-associated gene-5 or retinoic acid-inducing gene I [RIG-I], and ds break-DNA [dsB-DNA] via a cytosolic pathway) (16–19). IFN-αβ includes 13 functional isoforms of IFN-α and one of IFN-β that all signal through a single transmembrane receptor (IFN-αβR) to induce key immunoregulatory functions that affect both innate and adaptive immunity. Signaling by TLRs and other receptors initially leads to induction of IFN-β and IFN-α4, which induce autocrine or paracrine signaling to trigger a positive feedback loop in which IFN-β and IFN-α4 induce more IFN-αβ, including other forms of IFN-α, amplying the IFN-αβ response and ultimately inducing a number of IFN-αβ–stimulated genes.

Currently, CpG ODNs demonstrate potential in preclinical and clinical studies as vaccine adjuvants and as therapies for allergies and cancers. Autoimmune diseases, such as systemic lupus erythematosus and multiple sclerosis, however, may be exacerbated by CpG-induced IFN-αβ (20). In addition, host DNA may contribute to pathogenesis of autoimmune diseases, including rheumatoid arthritis (20). Thus, further studies are needed to examine the mechanisms by which CpG

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Abbreviations used in this paper: CHP; chromatin immunoprecipitation; CIITA, class II MHC transactivator; DC, dendritic cell; dsB-DNA, ds break-DNA; FLT3L, FLT3 ligand; IFN-αβ, type I IFN; IRF, IFN regulatory factor; ISGF3, IFN-stimulated gene factor 3; mDC, myeloid dendritic cell; mPDCA-1, murine plasmacytoid dendritic cell Ag-1; NC, normalized copy number; ODN, oligodeoxynucleotide; PBST, PBS with 0.1% Tween 20; pDC, plasmacytoid dendritic cell; qRT-PCR, quantitative real-time PCR; RIG-I, retinoic acid-inducing gene I; SeV, Sendai virus.

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ODNs induce IFN-α and to develop agents to inhibit induction of IFN-αβ by self- and nonself-DNA.

Our studies describe selective inhibition of IFN-αβ induction by CpG-B ODNs. High concentrations of CpG-B ODNs specifically inhibited the induction of IFN-αβ by agonists of TLR9 and other receptors without inhibiting other responses (e.g., induction of TNF-α and IL-12p40). This inhibition was evident as early as 3 h and for a period of at least 48 h in all cell types tested: FLT3 ligand (FLT3L)-induced murine bone marrow-derived DCs, purified murine mDCs, purified murine pDCs, and human PBMCs. This mechanism inhibited induction of IFN-αβ by agonists of a range of receptors, including MyD88-dependent TLRs (TLR9, TLR7), a MyD88-independent TLR (TLR3), and non-TLR signals (e.g., a cytosolic pathway for signaling by dsB-DNA and perhaps RIG-I-like proteins) (19), suggesting that the mechanism affected downstream signaling at a point where signaling from these receptors converges for the induction of IFN-αβ. On the other hand, inhibition was upstream of mechanisms involved in IFN-αβ positive feedback signaling (second-wave IFN-αβ). Furthermore, chromatin immunoprecipitation (ChIP) demonstrated that CpG-B ODN inhibited association of IRF1 with IFN-αβ and IRF-ε promoters. Our results indicate a novel path for transcriptional regulation that results in inhibition of first-wave IFN-αβ.

Materials and Methods

Abs and reagents

CpG ODNs included CpG ODN-A2216 (5′-ggg GGA CGATGTC CGG ggg G-3′), CpG ODN-A2336 (5′-ggg GGC GAC GAC GTG ggg ggg-3′), CpG ODN-B2006 (5′-Agc tcg ttg tgt gtt gtt gtt-3′), CpG ODN-B1668 (5′-ttc agt ccg ccg ccg agc ctg agc-3′), CpG ODN-B1826 (5′-ttc agt ccg ccg ccg agc ctg agc-3′), and non-CpG ODN-B2138 (5′-ttc agt ccg ccg ccg agc ctg agc-3′). Lowercase letters in ODN sequences refer to nucleotides for which the 3′ internucleotide linkage is phosphorothioate-modified, and uppercase letters refer to standard phosphodiester-linked nucleotides. ODNs contained phosphorothioate-modified linkages at the 5′ and 3′ ends (A class) or throughout (B class) to resist nuclease degradation. ODNs were provided by Coley Pharmaceutical Group (Wellesley, MA), purchased from InvivoGen (San Diego, CA) or described elsewhere (21, 22), and synthesized by MWG-Biotech (High Point, NC). Sendai viruses (SeVs) (ATCC strain 52-SeV) were purchased (from American Type Culture Collection, Manassas, VA, and Charles River Laboratories, Wilmington, MA). Sendai viruses (SeVs) (ATCC strain 52-SeV) were purchased (from American Type Culture Collection, Manassas, VA, and Charles River Laboratories, Wilmington, MA, respectively), grown in chicken eggs, and inactivated with β-propiolactone as previously detailed (23, 24). LFS (ultra pure Escherichia coli), iriquimod (R-3873), sRNA 40, and dsDNA were purchased from InvivoGen. ODNs and other agonists were dissolved in endotoxin-tested (≤0.05 units) PBS (Cambrex, East Rutherford, NJ) or sterile cell-culture water (Sigma-Aldrich, St. Louis, MO). Recombinant mouse IFN-αa (rIFN-α) and IFN-β (rIFN-β) were purchased from PBL InterferonSource (Piscataway, NJ). Abs to IRF1 (sc-640), IRF3 (sc-9082), IRF7 (sc-9083), α-tubulin (sc-5286), and Ku-70 (sc-9033) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Marine cell culture and media

Standard medium was RPMI 1640 with 1-glutamine and glucose (HyClone Laboratories, Logan, UT) with 10% heat-inactivated FCS (HyClone), 50 μM-2-ME, 100 U/ml penicillin, and 100 μg/ml sodium pyruvate (HyClone), and penicillin-streptomycin (HyClone). Mice were housed under specific pathogen-free conditions. DCs were prepared from femur and tibia bone marrow cells of C57BL/6 mice (generous gifts from S. Akira (2, 11) on a C57BL/6 background]. IFN-α2b, mouse IFN-β2b, IL-12p70, and 10 μM penicillin and streptomycin at 5 × 105 cells/well in 48-well flat-bottom non-tissue culture-treated plates with or without CpG ODN-A2336 and/or CpG ODN-B2006. Supernatants were harvested 4 h after addition of ODN and frozen for subsequent IFN-αβ quantification by ELISA.

ELISA and quantitative real-time PCR

DCs were plated at 7.5–10 × 105 cells/well in flat-bottom 96-well plates or 2 × 106 cells/well in 48-well non-tissue culture-treated plates in standard medium and incubated for 3–48 h with or without CpG ODN and/or other agonists. Plates were centrifuged to pellet cells, and supernatants were removed and either assessed immediately or stored at −80°C. Supernatants were diluted and tested by ELISA for mouse IFN-α or IFN-β (PBL InterferonSource) or mouse TNF-α or IL-12p40/BD Biosciences, San Jose, CA). For quantitative real-time PCR (qRT-PCR), RNA was isolated from DCs using a RNeasy Plus mini kit (Qiagen, Valencia, CA). Cells were pelleted at 4°C and resuspended in RLT lysis buffer (Qiagen). Total RNA was extracted following on-column DNase digestion using RNeasy Plus mini columns (Qiagen) and collected in RNase-free water. Yield was determined by OD. Oligo(dT)-primed reverse transcription of RNA into cDNA was performed with SuperScript II First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA), and 5% of the product was used for each qRT-PCR sample using PCR buffer with hot start Invitrogen Taq polymerase, Bio-Rad SYBR green detection, and the Bio-Rad iCycler fluorescence detection system (Bio-Rad, Hercules, CA). All conditions were tested in triplicate. Primer pairs from murine gene sequences were as follows: for total IFN-α (sense, 5′-ATG GCT AGT TCC GTC TCT CTG CT-3′ and antisense, 5′-AGG GCT TCT CAG AYT TCT TCT-3′), IFN-β (sense, 5′-CAT CAA CTA AAG CCA GCT CCA-3′ and antisense, 5′-TCC AAG TGA AGG GCA GGT GAG-3′), GAPDH (sense, 5′-AAG GCC CCC TTC ATT GAC-3′ and antisense, 5′-TCC AGC ACA TAC TCA GCA C-3′), CD40 (sense, 5′-ACGT AGC AAG CCA CTG CAC AG-3′ and antisense, 5′-CTT CTT GGT GCA GCT TTG TC-3′), CD80 (sense, 5′-TCG GCG CAG TAA TAA CAG-3′), CD86 (sense, 5′-CTC TTT CAT TCA CGG ATG TTG-3′ and antisense, 5′-CTA GCC GTA TCC GCT TTT G-3′), and class II MHC transactivator (CIITA) (sense, 5′-AGC CTT TCT GGC TGG ATT GT-3′ and antisense, 5′-TCA ACG CCA TCT TGA CAA AGG-3′). Primers were previously described (9, 25, 26) or designed using Clone Manager Suite v7.11 and Primers Designers v5.11 (Scientific & Educational Software, Cary, NC). A BLAST search was then performed to verify specificity.

Western blot analysis of whole cell lysates and nuclear and cytoplasmic fractions

DCs were incubated in 12-well tissue culture-treated dishes with or without CpG ODN for various periods at 37°C. Subsequent steps were performed at 4°C unless otherwise stated. Cells were washed in PBS with protease inhibitor mixture (P8340, Sigma-Aldrich, 1:200), 1 mM NaF, 1 mM PMSF, and 10 μM calcium ion. To prepare whole cell lysates, the cells were pelleted and resuspended in 160 μl RIPA lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.5% Na-deoxycholate, and 0.1% SDS [pH 7.4]) with protease inhibitors and mixed on a rotator for 1 h. A 10-μl sample was centrifuged at 16,000 × g for 10 min, and 100 μl supernatant was collected as whole cell lysate. Alternatively, to isolate nuclear and cytoplasmic fractions, the cells were pelleted and resuspended for 1 min in 1 ml Nuclear EZ Prep Lysis Buffer (N3408, Sigma-Aldrich) with proteinase inhibitors. After centrifugation at 500 × g for 5 min, supernatants were removed as cytoplasmic fractions. Pellets were resuspended in 1 ml Nuclear EZ Prep Lysis Buffer (Sigma-Aldrich) with protease inhibitors for 10 min to wash cell debris and nuclei. The nuclear and cytosolic fractions were collected by centrifugation at 500 × g for 5 min. For SDS-PAGE, aliquots of whole cell lysates or nuclear and cytoplasmic fractions containing equal quantities of total protein were added to reducing sample buffer (Thermo Fisher Scientific, Waltham, MA; 30000), subjected to electrophoresis on 8% SDS polyacrylamide gels, and transferred onto polyvinylidene difluoride membranes. Membranes were washed in PBS with 0.1% Tween 20 (PBST), incubated for 1 h at room temperature in 5% nonfat milk in PBST, incubated overnight at 4°C with primary Ab in 3% BSA or 5% nonfat milk in PBST, washed, incubated for 1 h at room temperature with HRP-labeled secondary Ab (Amersham Biosciences, Piscataway, NJ), and developed with the ECL detection kit (Amersham Biosciences, Amersham, U.K.).
ChIP assays

DCs were plated at 8 × 10^5/well in six-well plates in standard medium and incubated for 3 h with or without CpG ODN. ChIP assays were performed as previously described (27). Cells were fixed in 1% paraformaldehyde for 10 min at room temperature. Nuclei were isolated using Nuclei EZ Prep Lysis Buffer (Sigma-Aldrich). Nuclei were then incubated in nuclear lysis buffer (50 mM Tris-Cl [pH 8.1], 1% Triton X-100, 10 mM EDTA, and 1% SDS) on ice for 10 min, and chromatin DNA was sheared to ~500 bp average size with a sonicator (Misonion X-3000, Cup horn 431B, Misonion, Farmingdale, NY). Sheared lysates were spun at 16,000 × g for 10 min at 4°C. The supernatant was harvested, diluted 10-fold with immunoprecipitation dilution buffer (0.01% SDS, 1.1% Triton X-100, 2 mM EDTA, 16.7 mM Tris-Cl, and 167 mM NaCl [pH 8.1]), preclarified with salmon sperm DNA/protein A agarose beads (Millipore, Billerica, MA; 16–157), and incubated with anti-IRF1 (Santa Cruz, sc-64947x or anti-acetyl histone H4 (Millipore, 06-866) Ab for 4 h at 4°C. Ab-bound chromatinwas precipitated with salmon sperm DNA/protein A agarose beads. Beads were washed five times with immunoprecipitation washing buffer (100 mM Tris-Cl, 500 mM LiCl, 1% Nonidet P-40, and 1% deoxycholic acid [pH 8]) and eluted with elution buffer (50 mM NaHCO3, with the exception of triplicate samples with 1% SDS). Crosslinking was reversed by incubation in 300 mM NaCl overnight at 67°C, and chromatin DNA was purified via phenol/chloroform extraction. Chromatin recovery was quantified by qPCR with primers for murine IFN-α or β promoter (sense 5′-GGG AGG GTA TTC CGA AGG A-G-3′; antisense 5′-TCT GGG CTG TGG GGT GTG A-G-3′) or murine IFN-β promoter (sense 5′-GCT CCA GCA ATT GGT GAA AC-3′; antisense 5′-CAA AGG CAG CAG GTA GAA TG-3′). Primers were designed using Clone Manager Suite v9.0 (Scientific & Educational Software). A BLAST search was then performed to verify specificity.

Statistical analysis

Data for qRT-PCR were analyzed based on normalized copy number (NC), defined as 1000 × (NC of mRNA of interest)/(NC of GAPDH). The fold induction was computed as (NC of treated cells)/(NC of untreated cells). Unless otherwise stated, data points show the means of triplicate samples with SD, and p values were calculated with two-tailed Student t test. ELISA figures show pooled data from three independent experiments, each of which included independent duplicate samples for each condition, resulting in six values for calculation of mean and SD. Statistical analysis was performed with SigmaStat version 3.1 (Systat Software, Chicago, IL) for two- or three-group comparisons.

Results

High concentrations of CpG-B ODNs inhibit CpG-A–induced production of IFN-α/β by murine DCs

Although CpG-A ODNs and CpG-B ODNs have similar potency for induction of IFN-α/β, CpG-A ODNs are much more efficacious in this regard (9). CpG-B ODNs demonstrate a biphasic dose-response curve in which the induction of IFN-α/β declines with increasing CpG-B ODN concentrations above 0.1 μM (9, 10). This biphasic response is unique to the induction of IFN-α/β, because high concentrations of CpG-B ODNs effectively induce TNF-α and IL-12p40 in both splenic DCs and FLT3L–induced DCs (10). It has been unclear whether CpG-B ODNs simply lack activity possessed by CpG-A ODNs that is required for efficacious induction of IFN-α/β or if higher concentrations of CpG-B ODNs actively induce a mechanism that inhibits induction of IFN-α/β.

Our studies addressed the hypothesis that high concentrations of CpG-B ODNs may specifically inhibit induction of IFN-α/β. DCs were incubated with CpG-A ODN and/or CpG-B ODN. Because our previous studies established consistent patterns of activity for the different ODN classes with multiple CpG-A and CpG-B ODNs ([9] and data not shown), our current studies focused on representative ODNs, including CpG ODN-A2336, CpG ODN-A2216, CpG ODN-B1668, CpG ODN-B1827, CpG ODN-B2006, and non-CpG ODN-B2138.

DCs from FLT3L–induced mouse bone marrow cultures (containing pDCs and mDCs) were stimulated for 24 h with CpG ODN-A2216 or CpG ODN-B1827 at varying concentrations (Fig. 1A). In the absence of CpG-A ODN, CpG-B ODN was more efficacious than CpG-B ODN for induction of IFN-α/β. CpG ODN-B1827 induced IFN-α/β expression only at lower ODN concentrations (0.01–0.3 μM) with a biphasic dose-response curve and decreasing induction of IFN-α/β at higher ODN concentrations. These results confirm that CpG-A ODN and CpG-B ODN have similar potency, but CpG-A ODN has much higher efficacy, consistent with prior observations (9).

To explore the impact of CpG-B ODN on induction of IFN-α/β, FLT3L–induced DCs were incubated with a maximal dose (3 μM) of CpG ODN-A2336 and varying concentrations of CpG ODN-B1668 (Fig. 1A). Interestingly, induction of IFN-α by CpG-A ODN was inhibited by CpG-B ODN at concentrations as low as 0.03 μM, and strong inhibition was observed in the range of 0.1–0.3 μM, mirroring the concentrations of CpG-B ODN at which the downturn occurs in the biphasic CpG-B ODN dose curve for induction of IFN-α. In subsequent experiments, DCs were incubated with CpG ODN-A2336 (3 μM), CpG ODN-B1668 (30 nM or 3 μM), non-CpG ODN-B2138 (30 nM), or mixtures containing both CpG ODN-A2336 and CpG ODN-B1668 (both ODNs at 3 μM). Supernatants were assessed for IFN-α, IFN-β, and IL-12p40 by ELISA (Fig. 1B–D). Consistent with Fig. 1A, CpG ODN-B1668 induced a low level of IFN-α/β at 30 nM, but at 3 μM, it induced little or no IFN-α/β, whereas 3 μM CpG ODN-A2336 induced IFN-α/β effectively (Fig. 1B, 1C). As observed in Fig. 1A, when DCs were exposed to both CpG-A ODN and CpG-B ODN at 3 μM, the presence of CpG-B ODN inhibited the induction of IFN-α/β by CpG-A ODN (Fig. 1B, 1C). Importantly, this effect was CpG sequence–specific, as non-CpG-B ODN exerted significantly less inhibition of IFN-α/β induction by CpG-A ODN than the CpG-B ODN. In contrast, induction of IL-12p40 (Fig. 1D) and DC maturation (assessed by induction of mRNA for CD80, CD86, and reduced expression of mRNA for CIITA) (Fig. 2) were effectively induced by CpG-A ODN, CpG-B ODN, or the combination of both ODNs at 3 μM. These data suggest that the inhibitory mechanism does not occur at the level of TLR-ligand binding or signaling components that are common to allTLR9 responses and that the inhibitory mechanism selectively involves induction of IFN-α/β. MyD88−/− DCs failed to produce either IFN-α/β or IL-12p40 in response to CpG ODN (Fig. 1B–D), consistent with the dependence of TLR9 signaling on MyD88. Purified mDCs and pDCs showed similar responses, where induction of IFN-α/β by CpG ODN-A2336 was inhibited by 3 μM CpG ODN-B1668 (Fig. 1E, 1F), despite some possible differences in the mechanisms of IFN-α/β induction in the two cell types (28). In conclusion, CpG-B ODN (3 μM) induces a novel inhibitory mechanism that inhibits the induction of IFN-α/β by CpG-A ODN.

CpG-B ODN inhibits induction of IFN-α by CpG-A ODN in human PBMCs

Although mice and humans respond similarly to CpG ODNs, species to species variability exists (5, 6). Therefore, studies were performed to confirm that CpG-B ODNs also inhibit induction of IFN-α/β in human cells. We tested the effect of 3 μM CpG ODN-B2006 (a CpG-B ODN that is active for human cells) on the ability of CpG ODN-A2336 to induce IFN-α expression by human PBMCs. PBMCs were incubated for 48 h with 3 μM CpG-A ODN, 3 μM CpG-B ODN, or a combination of both ODNs at 3 μM. Supernatants were assessed for IFN-α by ELISA. CpG ODN-B2006 failed to induce IFN-α and inhibited induction of IFN-α by CpG ODN-A2336 (Fig. 3). These data indicate that CpG-B ODN can inhibit the induction of IFN-α in human cells as well as mouse cells.

CpG-B ODNs inhibit induction of IFN-α mRNA within 3 h

To elucidate the mechanisms involved in CpG-B–mediated inhibition of IFN-α/β expression, we used qRT-PCR to assess transcription of IFN-β and IFN-α genes at an early time point after the addition

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of CpG ODN. FLT3L-DCs were incubated with CpG-A and/or CpG-B ODNs (3 μM). By 3 h, CpG-A ODN induced transcription of IFN-β and IFN-α genes, and this induction was inhibited by CpG-B ODN (Fig. 4). CpG-A also induced IFN-β and IFN-α mRNA at 6, 12, 24, and 48 h, and this induction was inhibited by CpG-B ODN at all of these time points (data not shown). These data indicate that CpG-B ODN induces a mechanism to inhibit induction of IFN-β and IFN-α gene transcription within 3 h of exposure to ODN.

CpG-B ODN inhibits induction of IFN-αβ by multiple TLRs and other receptors, including MyD88-dependent and MyD88-independent receptors

Inhibition of IFN-αβ induction by CpG-B ODN could occur at different points along the signaling pathway for induction of IFN-αβ by CpG-A ODN. In principle, CpG-B ODNs could compete with CpG-A ODNs for binding to TLR9, affect signaling mechanisms upstream of initial induction of first-wave IFN-β and IFN-α, or inhibit the autocrine/paracrine IFN-αβ positive feedback loop that produces second-wave IFN-αβ. To investigate these possibilities, we tested the effect of CpG-B ODN on induction of IFN-αβ by agonists of several different receptors with varying downstream signal transduction molecules, including TLRs other than TLR9 (some MyD88-dependent, some MyD88-independent) and non-TLRs (MyD88-independent).

FLT3L-Induced DCs or purified pDCs or mDCs were incubated for 24 h with agonists of various receptors with or without 3 μM CpG ODN-B1668. CpG-B ODN inhibited induction of IFN-αβ by infectious (Fig. 5A) or inactivated (data not shown) strain 52-SeV; infectious SeV signals through the cytosolic receptors RIG-I or melanoma differentiation-associated gene-5 as well as TLR3 and TLR7, whereas inactivated SeV signals only through TLR7. Moreover, CpG-B ODN inhibited responses to the MyD88-dependent TLR7 agonist R837 (Fig. 5D), a MyD88-independent TLR3 agonist (polyinosinic:polycytidylic acid; Fig. 5E, 5F), and a TLR-independent, MyD88-independent agonist that signals through a putative cytosolic receptor [dsB-DNA (16–19); Fig. 5E, 5F]. Finally, CpG-B ODN inhibited LPS-induced IFN-α protein in FLT3L-DCs (Fig. 5G); LPS signals through TLR4 via MyD88-dependent and -independent pathways. Non–CpG-B negative control ODN did not
inhibit induction of IFN-αβ (Fig. 5C). Thus, CpG-B ODN inhibited the induction of IFN-αβ by agonists of multiple receptors, including TLRs and non-TLRs that signal through both MyD88-dependent and MyD88-independent pathways. These findings were reproduced with purified pDCs and mDCs and confirmed for both IFN-α and IFN-β (Fig. 5H, 5I). Two important conclusions derive from these findings. First, the inhibitory mechanism does not depend on competition of CpG-B ODN with other agonists for TLR9, because induction of IFN-αβ by agonists of many other receptors is inhibited by CpG-B ODN. Second, the inhibitory mechanism may lie downstream of initial signal transduction molecules (e.g., MyD88) that differ between these receptors and likely involves downstream signaling steps that are common to all of these receptors.

Comparison with earlier data (Fig. 1D) (10) suggests that CpG-B-induced inhibition may be restricted to the induction of IFN-αβ without inhibition of other cytokines, so we also tested the effect of CpG-B ODN on induction of IL-12p40 by strain 52-SeV, an agonist of TLR7 (MyD88-dependent). Strain 52-SeV–induced IL-12p40 was not inhibited by CpG-B ODN (Fig. 5B), further indicating that CpG-B–induced inhibition is selective to the IFN-αβ signaling pathway. Similarly, TNF-α protein expression induced by other TLR agonists was not inhibited by CpG-B ODN (data not shown). Collectively, these data demonstrate a novel mechanism induced by CpG-B ODN that selectively inhibits induction of IFN-αβ (at a point of convergence downstream of a variety of receptors) without inhibiting other cellular responses to activation of these receptors (Figs. 1D, 2, 5B, and data on TNF-α that are not shown).

CpG-B ODN does not inhibit the IFN-induced positive feedback loop and induction of second-wave IFN-αβ in DCs

TLR signaling induces first-wave IFN-αβ, which is restricted to IFN-β and IFN-α4, and the expression of these and other IFN-αβ expression. C57BL/6 Fli3L–induced DCs were incubated for 3 h with or without 3 μM CpG ODN-A2336, 3 μM CpG ODN-B2006, or both ODNs at 3 μM (A3/B3). IFN-α was measured by ELISA. Data represent five different donors (each assessed in duplicate assays). Statistical analysis was done to compare the mean response for the five different donors under each condition, and significance was assessed with one-way ANOVA and Student-Newman-Keuls method. Results reveal statistically significant (p < 0.05) induction of IFN-α by CpG-A ODN (comparison of PBMCs with CpG ODN-A2336 to PBMCs with medium alone), higher induction of IFN-α by CpG ODN-A2336 than CpG ODN-B2006 (p < 0.05), and statistically significant (p < 0.05) inhibition of CpG-A–induced IFN-α production by CpG-B ODN (comparison of PBMCs with CpG ODN-A2336 to A3/B3).

FIGURE 2. CpG-A ODN and CpG-B ODN both induced DC maturation. FLt3L–induced DCs were incubated for 3 h with or without 3 μM CpG ODN-A2336, CpG ODN-B1668, or both ODNs at 3 μM (A3/B3). Expression of mRNA for CD40, CD80, CD86, and CIITA was determined by qRT-PCR with normalization to GAPDH. A, Results for CD40, CD80, and CD86, for which mRNA is increased by treatment with CpG ODN, are expressed as fold-change relative to expression with medium alone. B, Results for CIITA mRNA, which is decreased by treatment with CpG ODN, are expressed as percent of control expression in medium alone. Data points represent mean ± SD for triplicate samples in a representative experiment. Three independent experiments produced similar results. CpG ODN-A2336, CpG ODN-B1668, or both ODNs all induced statistically significant (p < 0.01 for comparison with DCs with medium alone) and significant repression of IFN-αβ (at a point of convergence downstream of a variety of receptors) without inhibiting other cellular responses to activation of these receptors (Figs. 1D, 2, 5B, and data on TNF-α that are not shown).

CpG-B ODN does not inhibit the IFN-induced positive feedback loop and induction of second-wave IFN-αβ in DCs

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FIGURE 3. CpG-B ODN inhibits CpG-A ODN-induced expression of IFN-α by human PBMCs. PBMCs were cultured for 48 h with or without 3 μM CpG ODN-A2336, 3 μM CpG ODN-B2006, or both ODNs at 3 μM (A3/B3). IFN-α was measured by ELISA. Data represent five different donors (each assessed in duplicate assays). Statistical analysis was done to compare the mean response for the five different donors under each condition, and significance was assessed with one-way ANOVA and Student-Newman-Keuls method. Results reveal statistically significant (p < 0.05) induction of IFN-α by CpG-A ODN (comparison of PBMCs with CpG ODN-A2336 to PBMCs with medium alone), higher induction of IFN-α by CpG ODN-A2336 than CpG ODN-B2006 (p < 0.05), and statistically significant (p < 0.05) inhibition of CpG-A–induced IFN-α production by CpG-B ODN (comparison of PBMCs with CpG ODN-A2336 to A3/B3).

FIGURE 4. CpG-B ODN inhibits CpG-A ODN-induced IFN-α mRNA expression. C57BL/6 Fli3L–induced DCs were incubated for 3 h with or without 3 μM CpG ODN-A2336, CpG ODN-B1668, or both ODNs at 3 μM (A3/B3). Expression of mRNA for IFN-α and IFN-β was determined by qRT-PCR with normalization to GAPDH and is expressed as fold-change relative to expression with medium alone. Data represent mean and SD for triplicate samples in a representative experiment. Three independent experiments produced similar results. CpG ODN-A2336 induced statistically significant production of IFN-α and IFN-β (p < 0.01 for comparison with DCs with medium alone), and CpG ODN-B1668 caused statistically significant inhibition of CpG ODN-A2336-induced IFN-α or IFN-β. p < 0.01 for comparison with DCs with CpG ODN-A2336 alone.
FIGURE 5. CpG-B ODN inhibits induction of IFN-αβ by multiple TLRs and other receptors, including MyD88-dependent and MyD88-independent receptors. DCs were incubated for 24 h with receptor agonists, including infectious strain 52-SeV at multiplicity of infection 2.5 (SeV-2.5), inactivated strain 52-SeV at 5 μg/ml (In-Sev-5), R837 (0.3 μg/ml), polymyxin:bolycytidylic acid (50 μg/ml), dsB-DNA (10 μg/ml), LPS (10 ng/ml), CpG ODN-A2336 (3 μM), CpG ODN-B1668 (3 μM), or non-CpG ODN-B2138 (3 μM). The agonists other than CpG-B ODN were added either alone or in combination with 3 μM CpG ODN-B1668 (B3) or non-CpG ODN-B2138 (non-B2138, also at 3 μM). ELISA was used to measure IFN-α (A, C, D, F–I) or IL-12p40 (B). Expression of mRNA for IFN-α and IFN-β was determined by qRT-PCR with normalization to GAPDH and is expressed as fold-change compared with expression with medium alone (E). FLT3L-induced DCs were obtained from wild-type C57BL/6 mice (A–G) or MyD88−/− mice (A, B). Purified pDCs (H) or mDCs (f) were from wild-type C57BL/6 mice. Data points represent mean ± SD of pooled data from three independent experiments, each of which included duplicate ELISA or triplicate qRT-PCR assays for each condition. All panels show statistically significant agonist-induced production of IFN-α (p < 0.05 for comparison with DCs with medium alone) and statistically significant inhibition of agonist-induced IFN-α or IFN-β by CpG ODN-B1668 (B3) or non-CpG ODN-B2138 (non-B2138, also at 3 μM). The expression and localization of IRF1, IRF3, and IRF7 were determined by Western blot of whole cell lysates (Fig. 7A) and nuclear and cytoplasmic fractions (Fig. 7B, 7C). IRF7 was induced by CpG-A ODN, whereas IRF1 and IRF3 were of medium level or undetectable. These data indicate that the IFN-αβ positive feedback loop is not inhibited by CpG-B ODN and suggest that the inhibition occurs at earlier steps in the initial induction of first-wave IFN-αβ by TLR signaling.

CpG ODNs induce IRF1, but CpG-B ODN does not inhibit IRF1 induction or nuclear localization

IFN-αβ gene induction is dependent on a number of regulatory and transcription factors, particularly the IRFs, including IRF1 (15), IRF3 (28, 31, 32), and IRF7 (14, 28). The dependence of IFN-αβ gene induction on different IRFs varies with cell type and stimulus. Induction of IFN-β by TLR9 signaling in mDCs and macrophages requires IRF1 but not IRF7, whereas IRF7 functions in IFN-β induction by CpG-A ODN in pDCs (15). These considerations led us to examine the effects of CpG-A and CpG-B ODNs on IRF expression and localization (localization of IRFs to the nucleus results from their activation).

The expression and localization of IRF1, IRF3, and IRF7 were determined by Western blot of whole cell lysates (Fig. 7A) and nuclear and cytoplasmic fractions (Fig. 7B, 7C). IRF7 was induced by CpG-A
after ~7 h (slightly at 3.5 h), whereas IRF1 was induced earlier at 3.5 h (slightly at 1 h) (Fig. 7A). IRF3 was constitutively expressed and was not upregulated by CpG stimulation. Subcellular fractionation was used to separate nuclear and cytoplasmic fractions (Fig. 7B, 7C); effective separation was confirmed by detection of α-tubulin (cytoplasmic marker) and Ku-70 (nuclear marker). IRF1 appeared in both the cytoplasm and nuclear fraction of CpG-activated DCs at both 4 h (Fig. 7B) and 12 h (Fig. 7C), whereas IRF7 was not detected at 4 h and appeared only in the cytoplasm at 12 h (the possibility of low levels of nuclear IRF7 below the limit of detection in this assay or translocation of IRF7 after 12 h cannot be excluded). IRF3 was localized to the cytoplasm and not detected in the nucleus at both 4 h and 12 h (Fig. 7C). The addition of CpG-B to the stimulation condition (along with CpG-A) did not change the expression or localization of IRF1, IRF3, or IRF7. Tubulin was used as a cytoplasmic marker, and Ku-70 was used as a nuclear marker. Because IRF7 was not detected in B, an IRF7-positive control was added in the rightmost lane of the gel; this sample was derived from whole cell lysate of the CpG-A2336 24 h condition analyzed in A. Data are representative of at least three independent experiments.
CpG-B ODN inhibits IRF1 binding and histone H4 acetylation at IFN-α4 and IFN-β promoter sequences

Although IRF1 expression and nuclear localization were not inhibited by CpG-B ODN, these events do not establish effective induction of IFN-αβ by IRF1. Accordingly, we assessed the hypothesis that CpG-B ODN inhibits binding of IRF1 to IFN-α4 and IFN-β promoter sequences, resulting in inhibition of IFN-αβ promoter activation. ChIP assays used anti-IRF1 Ab to precipitate IRF1 along with promoter sequences to which it was bound, and qPCR was performed using primers specific for IFN-α4 or IFN-β promoter sequences to determine association of IRF1 with these promoters. Incubation of DCs with CpG-A ODN for 3 h induced IRF1 binding to both IFN-α4 and IFN-β promoters (Fig. 8A, 8B). CpG-B ODN was much less effective at inducing IRF1 binding to these promoters. Furthermore, CpG-B ODN inhibited induction of IRF1 binding by CpG-A ODN (A3/B3 condition). As predicted by kinetic studies of IRF7 expression (Fig. 7A), ChIP studies with anti-IRF7 Ab revealed no specific association of IRF7 with the IFN-α4 and IFN-β promoters at 3 h; at this time point, IRF7 expression is not yet induced, yet the CpG-B inhibitory effect is already present. Thus, inhibition of IRF1 binding to IFN-α4 and IFN-β promoters may contribute to CpG-B ODN inhibition of IFN-αβ induction.

Changes in transcription factor binding can be associated with altered local chromatin modification. Histone H4 acetylation was previously reported to be associated with IFN-β transcriptional activation (33). Accordingly, we assessed the effect of CpG ODNs on acetylation of histone H4 at the IFN-α4 and IFN-β promoters. ChIP assays using anti-acetyl histone H4 indicated that CpG-A ODN induced histone H4 acetylation at both IFN-α4 and IFN-β promoters (Fig. 8C, 8D), whereas CpG-B ODN did not. Furthermore, CpG-B ODN inhibited CpG-A ODN-induced histone H4 acetylation when both ODNs were added together. This correlated with lower IRF1 binding with CpG-B ODN and is consistent with decreased transcriptional activity at the IFN-α4 and IFN-β promoters.

To demonstrate the promoter specificity of changes in histone H4 acetylation revealed by ChIP, we compared the results for the IFN-α4 and IFN-β promoters to the results for CIITA promoter I, which is rapidly repressed via histone deacetylation upon DC maturation (34). ChIP with antiacetyl histone H4 and qPCR with CIITA promoter I-specific primers (35) showed a profound decrease in acetylation of histone H4 at CIITA promoter I posttreatment with 3 μM CpG ODN-A2336, 3 μM CpG ODN-B1668, or both ODNs at 3 μM (three independent experiments, data not shown). These results are consistent with the CIITA mRNA expression data shown in Fig. 2 and indicate that the ChIP assays reveal promoter-specific increases or decreases in association of acetylated histone H4.

Discussion

Previous studies showed that CpG-B ODNs induce low levels of IFN-αβ at low ODN concentrations but fail to induce IFN-αβ at higher ODN concentrations (9, 10). Previously, however, the mechanism for this biphasic response was unknown, and it was unclear whether CpG-B ODNs simply lacked activity possessed by CpG-A ODNs that is required for efficacious induction of IFN-αβ or if higher concentrations of CpG-B ODNs actively induce a mechanism that inhibits induction of IFN-αβ. Our studies reveal that CpG-B ODNs actively induce a mechanism that inhibits expression of IFN-αβ mRNA downstream of signaling by a wide variety of distinct receptors, including TLR9, TLR7, TLR3, TLR4, and a putative cytosolic receptor for dsB-DNA that has been linked to RIG-I (19). Moreover, the IFN-αβ response to SeV, potentially mediated by several membrane-bound and cytosolic receptors, was also blunted by CpG-B ODNs. The inhibitory mechanism appears to be selective for induction of IFN-αβ, because TNF-α and IL-12p40 expression are unaffected. In addition to FLTL3-induced DCs, which contain both mDCs and pDCs, we observed the CpG-B–induced inhibitory effect in purified mDCs and pDCs. These data suggest that mDCs and pDCs, although mechanistically different in some aspects of CpG ODN responses and signaling (28), are both affected by CpG-B inhibition. CpG-B–induced inhibition of IFN-αβ is apparent at the mRNA level as early as 3 h posttreatment with CpG-B ODN and is sustained for at least 48 h posttreatment. We conclude that CpG-B ODNs induce a novel mechanism that selectively inhibits the induction of IFN-αβ in DCs by multiple pattern recognition receptors.

Examination of different receptors and signaling pathways provides some insight into the components of TLR9 and IFN-αβ signaling pathways that may be affected by CpG-B ODN. Because TNF-α and IL-12p40 expression are not affected by CpG-B ODN, the inhibition does not appear to occur at the level of TLR9 binding or initial signal transduction molecule(s) (e.g., MyD88) that are common to all signaling downstream of TLR9. Furthermore, because CpG-B ODN inhibited IFN-αβ induction through both MyD88-dependent and MyD88-independent receptors, the inhibitory mechanism must involve components at or downstream of the convergence of these diverse signaling pathways. Our finding that IFN-αβ–induced second-wave IFN-αβ production was not inhibited by CpG-B ODN indicates that the mechanism involves inhibition of first-wave IFN-β and IFN-α4, possibly by regulation of the activity of transcription factors that bind and regulate the IFN-α4 and IFN-β promoters.
Waibler et al. (36) recently described a mechanism by which induction of IL-10 expression by mDCs inhibits IFN-αβ expression by pDCs. Although such a mechanism could contribute to regulation of IFN-αβ, our findings indicate some differences from this model. First, we observed inhibition of IFN-αβ induction by CpG-B ODN in purified pDCs as well as mDCs; an mDC-mediated IL-10 paracrine mechanism is excluded in the purified pDC system. Similarly, we recapitulated the same inhibitory effect in human PBMCs, even though human mDCs (and monocytes) do not express TLR9. In addition, our mRNA data suggest a profound inhibitory effect as early as 3 h; although additional studies are needed to assess possible contributions of paracrine mechanisms, the mechanism must explain rapid regulation of IFN-αβ expression, consistent with direct effects of TLR signaling or the involvement of rapidly induced paracrine mediators.

Our results suggest that CpG-B ODNs inhibit IFN-αβ induction at a stage that is after IFR activation and nuclear translocation and that affects the induction of first-wave IFN-αβ (IFN-α4 and IFN-β), not the IFN-αβ positive feedback loop. Although IFR7 is known to contribute to IFN-αβ regulation at some time points in pDCs, IFR1 is more likely to contribute to the CpG-induced inhibitory mechanism, because IFR1 was induced and translocated into the nucleus rapidly, within 1–3 h, when the CpG-induced inhibition becomes apparent. In contrast, IFR7 was not induced until ∼7 h, and IFR3 was not regulated by CpG-A or CpG-B ODN. Moreover, ChIP at 3 h postexposure to CpG-A ODN showed association of IFR1 with the IFN-α4 and IFN-β promoters that was inhibited by CpG-B ODN. In contrast, ChIP revealed no specific association of IFR7 with these promoters at 3 h poststimulation with CpG-A ODN. Consistent with the implied regulation of first-wave IFN-αβ in Fig. 6, the IFR1 and acetyl histone H4 ChIP results suggest that CpG-B ODN inhibits first-wave IFN-αβ mRNA by transcriptional regulation. Although IFR1 expression and nuclear localization were not inhibited by CpG-B ODN, CpG-B ODN inhibited binding of IFR1 to IFN-α4 and IFN-β promoter sequences, resulting in inhibition of promoter activation, inhibition of histone H4 acetylation, and decreased expression of IFN-αβ mRNA.

CpG ODNs modulate immune responses in mice and humans, but the two species differ in some aspects of responses to CpG ODNs. In mice, mDCs, pDCs, B cells, and macrophages express TLR9, but in humans, only pDCs and B cells express TLR9. There are also differences in the ODN sequences that are optimally recognized by human TLR9 versus mouse TLR9. Nonetheless, the CpG-B–induced inhibition of IFN-αβ expression was demonstrated in human PBMCs as well as mouse DCs. Thus, these data have relevance to the effects of two distinct CpG ODNs on dendritic cell subsets. J. Immunol. 170: 3059–3064.


References


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Disclosures

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