

CpG inhibits IgE class switch recombination through suppression of NF κ B activity, but not through Id2 or Bcl6

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Abstract

The CpG motif in DNA plays a critical role in immunity via modulating the Th1/Th2 balance. In B cells, CpG-containing oligodeoxynucleotides (CpG ODNs) inhibit IL-4-mediated class switch recombination (CSR) to IgG1 and IgE through inhibition of the germline transcription (GLT) of these isotypes. However, the molecular mechanism of this inhibitory effect remains elusive. We showed here that Id2 and Bcl6, both of which inhibit IgE GLT and CSR, are not involved in this inhibitory pathway. We demonstrated that there is reduced activity of NF κ B binding to the IgE promoter and a reduction of Irf4 protein in CpG ODN-treated B cells. These data indicate the critical role of NF κ B and Irf4 in the regulation of IgE CSR through actions downstream of CpG signaling.

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Allergy is characterized by a predominant Th2 response to environmental allergens and the subsequent production of cytokines, such as IL-4, IL-5, and IL-13, that mediate the clinical features of this condition, including the production of IgE by B cells. Bacterial DNA sequences with adjuvant properties, known as CpG DNA, have been found to suppress Th2 responses by stimulating the innate immune system, and consequently to cause the release of abundant Th1-skewing cytokines, and thus are of therapeutic significance for allergic diseases [1–3]. In addition to their effects on

the innate immune system, Toll-like receptor 9 (TLR9), the receptor of CpC, is also expressed on mature B cells. It has recently been reported that CpG DNA directly inhibits B cell class switching of the Th2-associated immunoglobulin isotypes, IgE and IgG1, possibly through the induction of T-bet [4], which was originally identified as a transcriptional regulator of Th1 differentiation [5–8]. However, Peng et al. [9,10] used B cells from T-bet deficient mice and demonstrated that T-bet is dispensable for inhibition of IgE and IgG1 by CpG, although it is essential for IgG2a production. We also confirmed this conclusion by forced expression of T-bet in splenic B cells (data not shown).

The isotype specificity of CSR is essentially controlled by the induction of GLT of target isotypes [11–15]. In accordance with this notion, CpG-containing

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oligodeoxynucleotides (CpG ODNs) inhibit CSR to IgG1 or IgE through the suppression of $\gamma 1$ and ϵ GLT [4]. This suggests that CpG DNA might repress positive regulatory factors of $\gamma 1$ and ϵ GLT in activated B cells [16–22]. IL-4 is known to direct CSR to IgG1 and IgE by inducing $\gamma 1$ and ϵ GLT. In addition, signaling through CD40 or lipopolysaccharide (LPS) acts synergistically with IL-4 to further induce $\gamma 1$ and ϵ GLT [23–28]. Numerous previous studies have indicated that IL-4-responsive loci in B cells, including the $\gamma 1$ and ϵ germline promoters, and CD23 promoter, have similar configurations including binding sites for NF κ B, E-proteins, and the basic leucine zipper (bZIP) family proteins [29]. Among these factors, NF κ B is known to play a pivotal role in IL-4-responsive gene expression through synergistic action with Stat6 [16,17,19–22].

In this study, we first focused on the involvement of two factors that were previously suggested to play roles in the inhibitory effect of CpG in IgE CSR [9], namely, Id2, a negative regulator of E-proteins [30–34], and Bcl6, a negative regulator of STAT6 [35–40]. However, we found that these factors are not required for the inhibition of IgE CSR by CpG. We finally demonstrated reduced activities of NF κ B proteins and reduced amounts of IRF4 protein in CpG ODN-treated B cells. Unexpectedly, STAT6 activity was not markedly changed by CpG ODN treatment. These results indicate that CpG signals work in various ways to induce Th1-skewed immune responses involving B cells.

Materials and methods

Mice. Id2-deficient mice on the 129/Sv genetic background were maintained under specific pathogen-free conditions and used at 7–10 weeks of age. Bcl6-deficient mice on the C57BL/6 genetic background were maintained under specific pathogen-free conditions and used at 7–10 weeks of age. All animal procedures described in this study were performed in accordance with the guidelines for animal experiments of Kyoto University Graduate School of Medicine.

Reagents. LPS was purchased from Sigma, St. Louis, MO. Mouse IL-4, IL-12, and IFN- γ were purchased from Peprotech. Phosphorothionate CpG ODN (5'-TGACTGTGAACGTTTCGAG ATGA-3') or phosphorothionate mutant CpG ODN (mCpG) (5'-TGACTGTGAAGGTTAGAGATG-3') was purchased from Espec Oligo Service, Japan, and was used in the experiments described below.

Preparation of cells and in vitro cell culture. For the in vitro class switching study, purified splenic B cells were obtained and stimulated as described previously [30].

RT-PCR. Total RNAs were extracted from cultured splenic B cells with TRIzol (Gibco-BRL, Gaithersburg, MD), according to the manufacturer's instructions. Oligo(dT)-primed cDNAs were prepared with reverse transcriptase. Fifty nanograms of each cDNA was serially diluted and subjected to PCR amplification with the following primer pairs: 5' I ϵ , 5'-GCA GAA GAT GGC TTC GAA TAA GAA CAG T-3'; 3' C ϵ , 5'-TCG TTG AAT GAT GGA GGA TGT GTC ACG T-3'; 5' GAPDH, 5'-CCA TCA CAT CTT CCA GGA G-3'; 3' GAPDH, 5'-CCT GCT TCA CCA CCT TCT TG-3'. All the PCR products were resolved electrophoretically in 2% agarose gels and visualized by

ethidium bromide staining. All the PCR data shown are representative of three independent experiments. Quantification of intensity was performed using a Bio Imaging Analyzer MacBAS (Fujifilm).

Estimation of the levels of IgE in culture supernatants. Total IgE in culture supernatants was measured by means of ELISA as described previously [45].

Flow cytometric analysis of surface IgE expression on B cells. The following antibodies were used for staining: fluorescein isothiocyanate (FITC)-anti-mouse B220, biotin-anti-mouse IgE (both from Pharmingen, San Diego, CA), and allophycocyanin-streptavidin (Molecular Probes, Eugene, OR). The analyses were performed with a FACSCalibur (Becton-Dickinson, Mountain View, CA).

Electrophoretic mobility shift assay (EMSA). Nuclear extracts of splenic B cells were prepared after incubation with the indicated stimulus, and protein amounts were quantified with Micro BCA Protein Assay Reagent (PIERCE). Double-stranded oligoprobes were end-labeled using T4 polynucleotide kinase and purified using a Sepharose G50 column. Nuclear extracts and labeled oligoprobes were used in a gel-shift assay as described with slight modifications. For the super-shift experiment, nuclear extract was incubated with 5 μ g of a goat anti-mouse p50 antibody (C-19; Santa Cruz Biotechnology), or a rabbit anti-p65 antibody (H-286; Santa Cruz Biotechnology), or a rabbit anti-Stat6 antibody (S-20, Santa Cruz Biotechnology) for 15 min at room temperature before the labeled probe was added. The sequences of the oligoprobes were as follows: NF κ B: 5'-ATCAAAA GGGAACTTCCAAGGC-3' and 5'-CAGCCTTGGGAAGTTCCCTT TTG-3'; mut NF κ B: 5'-ATCAAAAAGGGCCTCGAGGAGGC-3' and 5'-CAGCCTCCTCGAGGCCCTTTTG-3'; STAT6: 5-AGTCAACTT CCCAAGAACAGAA-3' and 5'-GATTCTGTTCTTGGGAAGTT GA-3'; and mut Stat6: 5'-AGTCAACTAGCCAACCTACAGAA-3' and 5'-GATTCTGTAGTTGGCTAGTTGA-3'. Quantification of intensity was performed using a Bio Imaging Analyzer MacBAS (Fujifilm).

Immunoblot analysis. Cell extracts were prepared from primary B cells and protein amounts were quantified with Micro BCA Protein Assay Reagent (Pierce). For immunoblotting, we used anti-Irf4 antibody (sc-6059, Santa Cruz) and anti- β -actin antibody (AC74, Sigma). Quantification of intensity was performed using a Bio Imaging Analyzer MacBAS (Fujifilm).

Results

Inhibitory effect of CpG ODN on IgE CSR

We first checked the effect of CpG ODN on IgE production by B cells using wild-type mice. B cells were stimulated with LPS and IL-4 in the presence or absence of CpG ODN. After 7 days of culturing, the supernatants were obtained and the level of IgE was measured by ELISA. As shown in Fig. 1A, CpG ODN inhibited IgE production, while mutant CpG ODN had no effect on IgE production. This effect of CpG on IgE production reflects the inhibition of IgE CSR, as confirmed by FACS analysis (data not shown). Germline transcription from the isotype specific promoter proceeds and is required for CSR. To further examine the effect of CpG, we then isolated RNA from B cells on day 3 and examined the levels of ϵ GLT by semiquantitative RT-PCR. As expected, a fivefold reduction of the level of ϵ GLT was observed in the samples cultured with CpG ODN, but not mutant

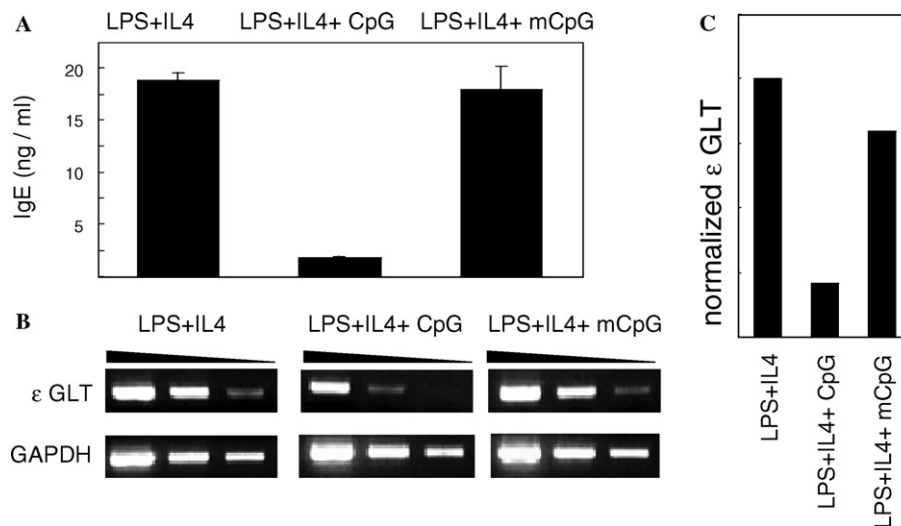


Fig. 1. Effect of CpG ODN on IgE class switching. Purified splenic B cells (5×10^6 /ml) from control mice were stimulated with LPS (10 μ g/ml) and IL-4 (10 ng/ml), with or without either CpG ODN or mutCpG ODN (3 μ M). (A) After 7 days of culture, supernatants were collected and the level of IgE in the supernatants was measured by ELISA. Data are shown as means $\pm$ SEM. (B) After 3 days of culturing, RNA was extracted from the cells and subjected to semi-quantitative RT-PCR. Fivefold serial dilutions of cDNAs were amplified for the indicated transcript. GAPDH was used as an internal control for RT-PCR. One of three independent experiments is shown. (C) The transcription levels of ϵ GLT are shown as relative expression levels to that of GAPDH.

CpG ODN (Figs. 1B and C). These data indicate that the inhibitory effect of CpG ODN on IgE CSR is caused by suppression of ϵ GLT.

Inhibition of IgE CSR by CpG ODN is independent of either Id2 or Bcl6

To identify the factors that act downstream of CpG-signaling and inhibit IgE CSR, we first focused on negative regulators of IgE CSR, such as Id2 [30] or Bcl6 [35]. Id2 suppresses IgE CSR by inhibiting the ϵ GLT-inducing activities of E2A and Pax5 [14,41]. Bcl6 also suppresses IgE CSR by inhibiting the ϵ GLT-inducing activity of Stat6 [35]. To determine whether the inhibition of IgE CSR by CpG ODN is dependent on Id2, B cells isolated from Id2-deficient and control mice were stimulated with LPS and IL4 with or without the addition of CpG ODN. After 7 days of culturing, the supernatants were obtained and the level of IgE was measured by ELISA. CpG ODN also inhibited IgE production in Id2-deficient B cells (Fig. 2A). However, the inhibitory effect is not so strong as that observed in wild-type B cells (Fig. 1A). These results suggest that Id2 might be involved in the regulation of IgE CSR, at least in part, acting downstream of CpG signaling. To further determine the possibility described above, RT-PCR was performed by using RNA prepared from B cells on day 3 to examine the expression of ϵ GLT. As shown in Figs. 2B and C, ϵ GLT in Id2-deficient B cells was inhibited by CpG ODN to almost the same degree as that in the control cells. In other words, CpG-ODN also cancelled the enhanced ϵ GLT caused by Id2 deficiency, indicating

that Id2 is not involved in the inhibitory effect of CpG ODN on IgE CSR. What, then, is the cause of increased IgE production in CpG-treated Id2-deficient B cells than in wild-type B cells? To address this, the surface expression of IgE was further assessed by FACS analysis after 5 days of culturing. As expected, CpG ODN significantly inhibited the surface expression of IgE in both control and Id2-deficient B cells (Fig. 2D). In addition, there are more IgE-positive cells in Id2-deficient B cells without stimulation than in control cells (Fig. 2D). This cell population might contribute to increased IgE production observed in CpG-treated Id2-deficient B cells. These results indicate that Id2 is dispensable for the inhibitory effect of CpG ODN on IgE CSR.

We next examined the role of Bcl6 function acting downstream of CpG-signaling by performing experiments like those shown in Figs. 2A and B, except that bcl6-deficient mice were used. Again, IgE production and ϵ GLT of both control and bcl6-deficient B cells were significantly inhibited by CpG ODN, indicating that the effect of CpG ODN on IgE CSR is independent of Bcl6 (Figs. 3A–C).

CpG inhibits NF κ B and IRF4 activities

Among the regulatory factors that contribute to inducing IL-4 responsive genes in B cells, in addition to Stat6, NF κ B plays a central role at various loci, including the ϵ and γ 1 promoter, and the CD23 promoter. At each locus, NF κ B binds near Stat6 binding sites, interacts directly with phosphorylated Stat6, and synergistically activates transcription of IL-4 responsive

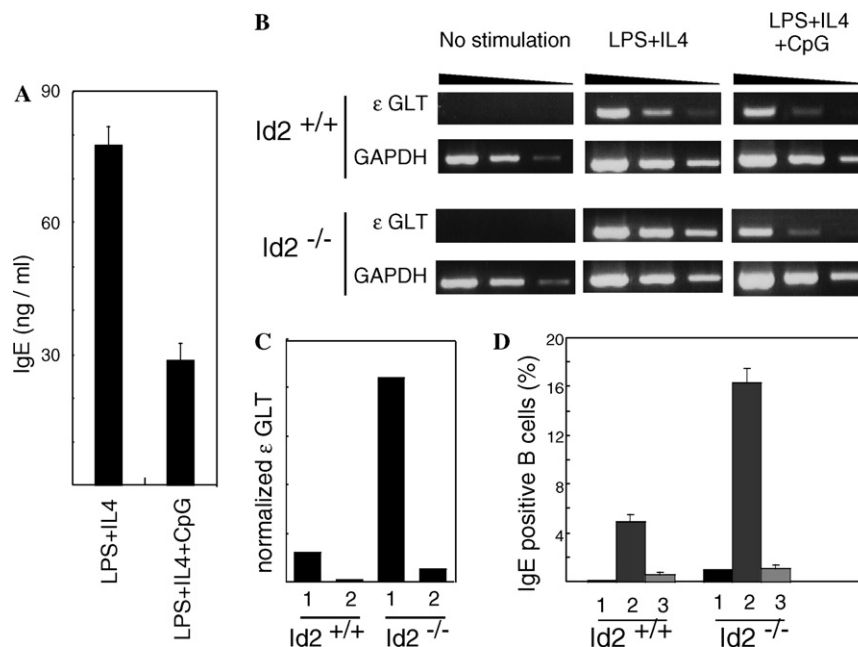


Fig. 2. Inhibition by CpG ODN of IgE class switch in $Id2^{-/-}$ B cells. (A) After 7 days of culturing of $Id2^{-/-}$ B cells with LPS (10 μ g/ml) and IL-4 (10 ng/ml) with or without CpG ODN (3 μ M), supernatants were collected and the level of IgE in the supernatants was measured by ELISA. Data are shown as means $\pm$ SEM. (B) RT-PCR of ϵ GLT in B cells after 3 days of culturing. One of three independent experiments is shown. GAPDH was used as an internal control for RT-PCR. Fivefold serial dilutions of cDNAs were amplified for the indicated transcripts. (C) The transcription levels of ϵ GLT are shown as relative expression levels to that of GAPDH. (1) LPS + IL-4; (2) LPS + IL-4 + CpG. (D) Class switch analysis by monitoring surface expression of IgE in $Id2^{-/-}$ or $Id2^{+/+}$ B cells cultured for 4 days. All assays were independently performed four times. Data are shown as means $\pm$ SEM. The various culture conditions used were indicated as follows: (1) non-stimulation; (2) LPS + IL-4; and (3) LPS + IL-4 + CpG.

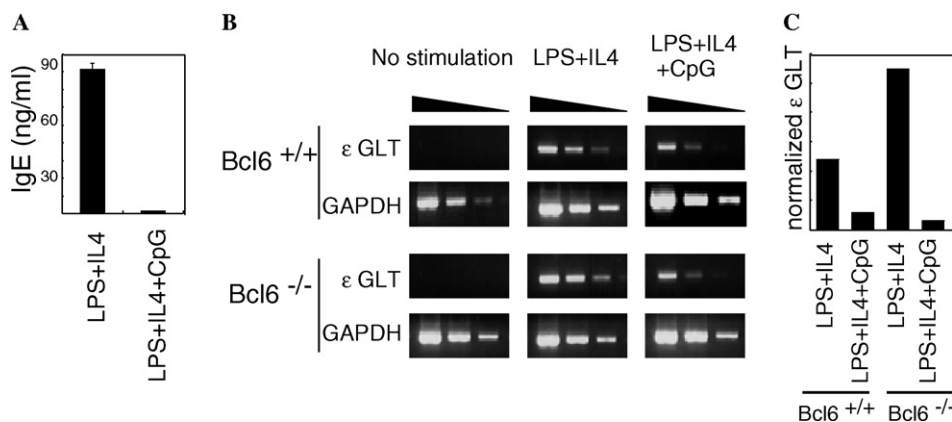
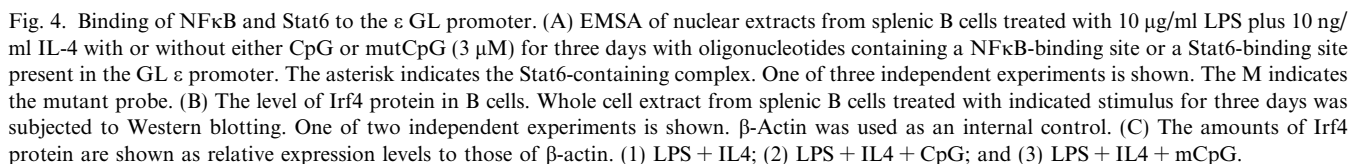


Fig. 3. Inhibition by CpG ODN of IgE class switch in $Bcl6^{-/-}$ B cells. (A) After 7 days of culturing of $Bcl6^{-/-}$ B cells with LPS (10 μ g/ml) and IL-4 (10 ng/ml) with or without CpG ODN (3 μ M), supernatants were collected and the level of IgE in the supernatants was measured by ELISA. Data are shown as means $\pm$ SEM. (B) RT-PCR of ϵ GLT in B cells after 3 days of culturing. One of three independent experiments is shown. GAPDH was used as an internal control for RT-PCR. Fivefold serial dilutions of cDNAs were amplified for the indicated transcripts. (C) The transcription levels of ϵ GLT are shown as relative expression levels to those of GAPDH.

genes [16,17,19–22,29]. We then concentrated our attention on these transcription factors. To determine whether these factors are involved in the inhibitory effect of CpG in IgE CSR, we compared the binding activities of these factors in nuclear extract from CpG ODN-treated and untreated cells. Nuclear extract prepared from B cells stimulated for 3 days was subjected to electrophoretic mobility shift assays (EMSAs) using binding sites

for NF κ B or Stat6 at the ϵ GL promoter as a probe. As shown in Fig. 4A left panel, two complexes were detected, and the presence of p50 and p65 in these complexes was verified by using specific antibodies against these factors (Fig. 4A left panel, lanes 4 and 5) and competitive oligonucleotides to which NF κ B binds (Fig. 4A left panel, lane 3). The binding activity was reduced when mutant oligonucleotide was used (Fig. 4A left



Although the significance of twofold reduction of Irf4 expression and STAT6 activity in the regulation of IgE CSR remains to be elucidated, loss of NFκB activities might contribute, at least in part, to the inhibition of IgE CSR by CpG ODN.

In this report, we confirmed that CpG, but not mutant CpG, specifically inhibits IgE CSR, as previously demonstrated. We initially speculated that Id2, which inhibits IgE CSR through the inhibition of E2A binding at the ϵ GL promoter [30,45], or Bcl6, which blocks IgE CSR via competing for Stat6 binding and represses transcription at the ϵ GL promoter [35,46], would be effector

molecules acting downstream of CpG signaling, as previously suggested. However, in purified splenic B cells from Id2^{-/-} or Bcl6^{-/-} mice, CpG ODN also inhibited CSR to IgE, as observed in control B cells. These results indicate that CpG ODN is a good candidate for therapeutic use as an anti-allergic drug. We then analyzed the factors involved in IL-4-responsive expression in activated B cells, such as Stat6, NFκB, and Irf4, and found that the activities of NFκB (p65 and p50) binding to the ε GL promoter and the amounts of Irf4 proteins were reduced by CpG ODN stimulation. Furthermore, the activity of Stat6 binding to the ε GL promoter was also reduced to a lesser extent. This finding is paradoxical to the previously demonstrated effect of CpG ODNs. Toll-like receptor 9 (TLR9), which recognizes CpG DNA in lysosomes [47–49], transduces its signals through the interaction of an adaptor protein, MyD88 [50]. Upon activation by CpG ODN, MyD88 recruits IL-1 receptor-associated kinase (IRAK) and TRAF6 to the receptors, resulting in activation of the IKK-NFκB, MAPK p38, and JNK-AP1 signaling pathways [47,51,52]. In this regard, we speculated that excessive signals to induce NFκB activation, such as combinatorial stimulation with C40L and CpG, or LPS and CpG, might induce negative feedback pathways more vigorously than one-pathway stimulation. However, we could not detect direct involvement of such negative regulatory pathways in CpG ODN-treated B cells, and further investigations will be required to clarify this point.

Recently, Lin et al. [53] showed that CpG DNA stimulates IgG2a, IgG2b, and IgG3 CSR, while inhibiting IgG1 and IgE CSR in IL-4-treated B cells. How can the differential effects of CpG DNA on various isotypes be explained? Numerous previous studies have indicated that various NFκB-deficient mice show defects in CSR [54–57] that affect various isotypes differentially: p50^{-/-} mice show reduced switching to IgG3, IgE, and IgA, while p65^{-/-} mice show reduced switching to IgG3 in vitro and IgG1 and IgA in vivo. Thus, differential regulation of several NFκB factors by CpG DNA does not provide a simple explanation of the effects observed in CpG ODN-treated B cells. In this regard, Irf4 is the most likely candidate to explain the effect of CpG DNA on CSR in IL4-dependent isotypes. Irf4 is induced by IL4 and synergistically activates CD23 expression in concert with Stat6 [58]. In addition, NFκB also enhances the expression of Irf4 [59], and under these conditions CSR usually takes place. Furthermore, Irf4 interacts with Stat6, but is inhibited by CD40-signaling via unknown mechanisms [58]. In addition, Stat6 also interacts with NFκB (p65 and p50) in a phosphorylation-dependent manner [17]. Thus, in B cells, a NFκB-Irf4-Stat6 containing complex seems to play a central role in IL-4-responsive gene expression. As expected, about a twofold reduction of Irf4 protein was observed in CpG ODN-

treated B cells. Although there is no direct evidence supporting the idea that Irf4 actually binds to the ε GL promoter and promotes ε GLT, Irf4 might contribute to the regulation of ε GLT expression, and CpG ODN would suppress such activity by reducing Irf4 expression. Clearly, a lot of missing pieces in the above scenario remain to be elucidated. Further evaluation of the molecular mechanisms of the effects of CpG DNA should help in the development of therapeutic strategies to prevent excess IgE production, and thus allergy.

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