

CpG directly induces T-bet expression and inhibits IgG1 and IgE switching in B cells

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CpG DNA has immunomodulatory effects, such as the suppression of allergic responses mediated by type II T cell help (T_H2). Here we report that CpG, but not lipopolysaccharide (LPS), rapidly induces expression of T-bet mRNA in purified B cells. Up-regulation of T-bet by CpG is abrogated in mice deficient in Toll-like receptor 9 (TLR9) and MyD88, but remains intact in B cells deficient in STAT1 (signal transducer and activator of transcription 1). Interleukin 12 (IL-12) alone does not up-regulate T-bet mRNA, but greatly enhances CpG-induced T-bet expression. Furthermore, CpG inhibits immunoglobulin G1 (IgG1) and IgE switching induced by IL-4 and CD40 signaling in purified B cells, and this effect correlates with up-regulation of T-bet. Thus, CpG triggers anti-allergic immune responses by directly regulating T-bet expression via a signaling pathway in B cells that is dependent upon TLR9, independent of interferon- γ (IFN- γ)-STAT1 and synergistic with IL-12.

Innate immunity recognizes pathogen-associated molecular patterns that signal antigen-presenting cells (APCs) to express costimulatory molecules and secrete cytokines. These APC-derived signals can drive the polarization of naive CD4⁺ helper T cells toward the T helper type I (T_H1) or T_H2 phenotype^{1,2}. T_H1 and T_H2 cells express different sets of genes that are regulated by the transcription factors T-bet (T_H1 gene regulator) or GATA3 and STAT6 (T_H2 gene regulators)³. Thus, T_H1 cells produce interferon- γ (IFN- γ) and tumor necrosis factor β , and regulate B cells to produce antigen-specific IgG2a. T_H2 cells express interleukin 4 (IL-4), IL-5, IL-9 and IL-13, and induce allergic inflammation by promoting IgG1 and IgE class switching.

Dysregulated T_H1 or T_H2 responses are thought to be central to the pathology of diseases such as asthma, which is characterized by T_H2-dominated pulmonary inflammation. Thus, rebalancing immune responses may be an effective strategy in developing therapeutic reagents for such diseases. Bacterial DNA or CpG have immunomodulatory effects^{4,5}. At the cellular level, CpG promotes dendritic cells and monocyte and macrophage activation and maturation^{6–8}, activates natural killer cells⁹, and stimulates proliferation, the production of cytokines, and up-regulation of surface markers and immunoglobulin (Ig)M in B cells^{10–12}. The prophylactic and therapeutic effects of CpG in allergies have been demonstrated by the selective down-regulation of IgE and T_H2 cytokine production in mouse models of asthma and in human peripheral blood mononuclear cells (PBMCs) from atopic patients^{13–17}.

The discovery that Toll-like receptor 9 (TLR9) is involved in the recognition of CpG DNA sheds light on the CpG signaling pathway¹⁸. TLRs are single-pass transmembrane proteins sharing common structural features in their cytoplasmic portions. The adaptor protein MyD88 interacts with TLRs through the Toll/IL-1 receptor (TIR)

domain. Upon activation by microbial components, MyD88 recruits IL-1 receptor-associated kinase (IRAK) and TRAF6 to the receptors, resulting in the activation of the IKK-NF- κ B, MAPK p38 and JNK-AP-1 signaling pathways^{1,19–21}.

However, it is still largely unknown how the molecular and cellular signaling cascades of CpG-TLR9 elicit protective effects in allergic inflammation. NF- κ B and AP-1 are the target transcription factors identified for all the TLRs²². Given the similar expression patterns of TLRs in myeloid cells, it is difficult to attribute the unique protective effect of CpG solely to the activation of NF- κ B and/or AP-1. In addition, as T cells do not express TLR9, it is unclear how CpG achieves its regulatory effects on T helper cells. Furthermore, mechanisms underlying CpG-induced IgE production *in vivo* are still unclear. To address these issues, we investigated CpG-regulated gene expression. We show here that T-bet mRNA is increased after CpG treatment but not after lipopolysaccharide (LPS) stimulation. In addition, we demonstrate that CpG uses a pathway that is independent of IFN- γ and signal transducer and activator of transcription 1 (STAT1), but synergistic with IL-12, to induce T-bet expression. We also found that treatment of B cells with CpG inhibits IgE and IgG1 class switching induced by IL-4 and CD40 ligation, and that this effect correlates with the induction of T-bet expression. Thus, CpG might mediate anti-allergic responses via the direct regulation of T-bet and inhibition of IgE and IgG1 production in B cells.

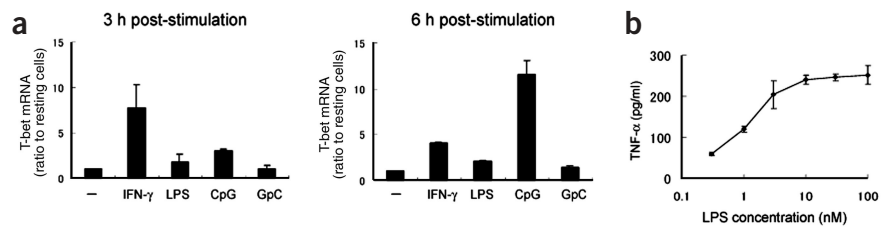
RESULTS

CpG induces T-bet transcription

To determine whether T-bet is regulated by CpG, we treated mouse splenocytes with 3 μ M CpG for 3 or 6 h and measured the expression of mRNA by quantitative reverse transcriptase-polymerase chain

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Figure 1 Induction of T-bet in mouse splenocytes. Spleen cells of C57BL/6 mice were cultured with or without the indicated stimuli. (a) The concentrations of IFN- γ , LPS, CpG and GpC are 50 ng/ml or 10 μ g/ml, 50 ng/ml, 3 μ M and 3 μ M, respectively. Cells were collected at 3 or 6 h after stimulation, and the T-bet mRNA was measured by real time LightCycler PCR. Data are representative of three independent experiments (mean $\pm$ s.d.). (b) Splenocytes were incubated with different concentrations of LPS. At 14 h after stimulation, secreted TNF- α was assessed by ELISA. Data are representative of two separate experiments.



reaction (RT-PCR). Cells treated with IFN- γ were used as a positive control. GpC (inactive oligonucleotide with a reversed CpG motif) and the TLR4 agonist *Escherichia coli* LPS were used to determine the specificity of oligonucleotides and signaling pathways, respectively. We found that the abundance of T-bet mRNA was slightly (threefold) increased in CpG-treated splenocytes at 3 h, and then showed a profound induction at 6 h after stimulation, surpassing the induction produced by IFN- γ treatment (Fig. 1a). The effect of CpG on T-bet transcription was specific, as the inactive GpC DNA did not up-regulate T-bet mRNA. LPS (50 ng/ml) did not induce T-bet transcription over the 6-h time course, although we saw a full induction of tumor necrosis factor α (TNF- α) production by LPS even at 10 ng/ml (Fig. 1b), consistent with previous data²³.

CpG induces STAT1 phosphorylation

STAT1 activity is required for the induction of T-bet in CD4⁺ T cells and myeloid cells^{23,24}. To investigate the possibility that CpG-induced T-bet expression is also mediated by STAT1, we assessed the phosphorylation of STAT1 α/β on tyrosine 701. IFN- γ induced phosphorylation of STAT1, quickly peaking at 2 h. CpG induced phosphorylation of STAT1 to a maximum extent at 5 h, where it was maintained over a long duration, up to 18 h (Fig. 2). Although LPS did not induce expression of T-bet mRNA (Fig. 1), we saw considerable phosphorylation of STAT1 in cells treated with LPS (Fig. 2)²⁵.

Regulation of T-bet and STAT1 by CpG requires TLR9

To clarify whether TLR9 itself, or a yet unknown CpG-responding receptor, transmits the signal leading to the transactivation of T-bet and phosphorylation of STAT1, we used TLR9- and MyD88-deficient mice to define the signal. CpG-induced T-bet mRNA expression and STAT1 phosphorylation were completely ablated in MyD88- or TLR9-deficient splenocytes, whereas the effect of IFN- γ was unchanged (Fig. 3a,b). Thus, the induction of T-bet and phosphorylation of STAT1 by

CpG requires both TLR9 and MyD88. In contrast to CpG, LPS-induced STAT1 phosphorylation is independent of MyD88 (Fig. 3b). This shows that T-bet induction is peculiar to CpG/TLR9 signaling, whereas STAT1 phosphorylation can be induced by both TLR9 and TLR4 agonists.

Cell types expressing T-bet in response to CpG

To further investigate the mechanism and functional consequences of CpG-induced up-regulation of T-bet, we sought to characterize cell types expressing T-bet in response to CpG. As some cellular effects of CpG are indirect (such as CpG-induced IFN- γ production in T cells

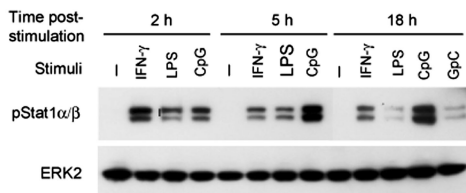


Figure 2 Stimulus-induced STAT1 α/β phosphorylation in splenocytes. Spleen cells of C57BL/6 mice were treated with the indicated stimuli (at the concentrations shown in Fig. 1), and cell lysates were made after incubation for 2, 5 and 18 h. STAT1 phosphorylation was assessed by immunoblotting with antibodies to phospho-STAT1 α/β (pY701). Data are representative of three separate experiments.

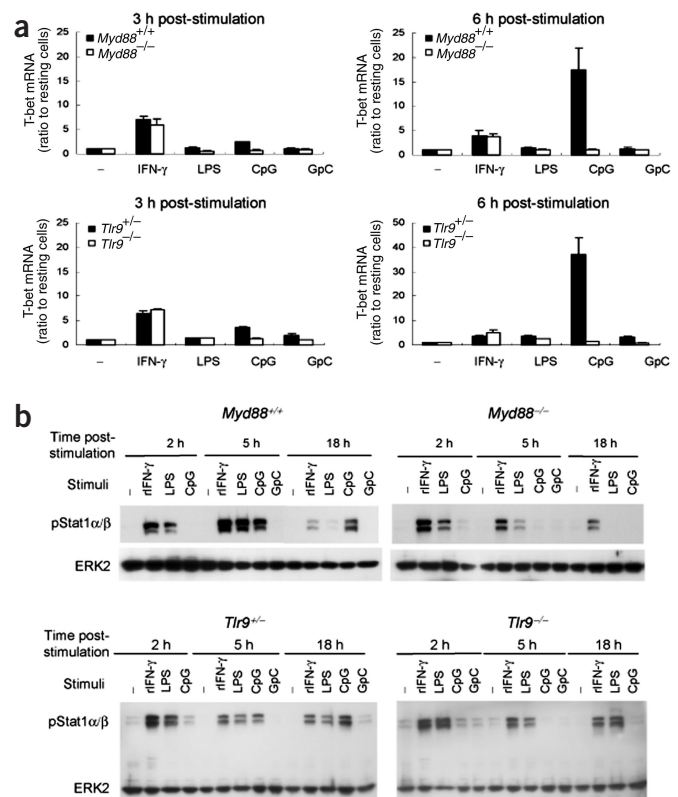


Figure 3 T-bet transactivation and STAT1 phosphorylation in MyD88- or TLR9-deficient mice. Splenocytes of MyD88^{-/-} and MyD88^{+/+}, Tlr9^{-/-} and Tlr9^{+/+} mice were isolated and treated with the indicated stimuli. (a) T-bet induction (ratio to the mRNA abundance in resting cells). (b) Phosphorylation of Y701 in STAT1 α/β . Immunoblotting was done with an antibody to phospho-STAT1 α/β . ERK2 antibodies were used at the same time as a control to detect total protein loading. Data are representative of three independent experiments (mean $\pm$ s.d., a) or a single experiment, representative of three (b).

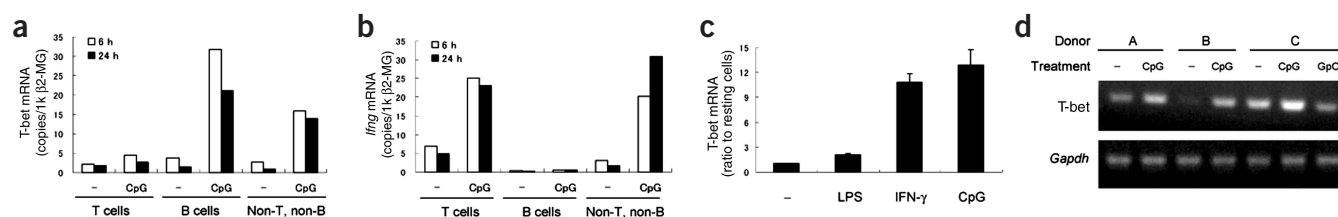


Figure 4 Cell types expressing T-bet in response to CpG. (a,b) Splenocytes were isolated from C57BL/6 mice and incubated in medium with or without CpG (3 μ M) for 6 h or 24 h. Thereafter, T cells and B cells were purified by positive and negative selection, respectively. 'Non-T, non-B' represents the remaining cells after T and B cell depletion. The expression of T-bet (a) and IFN- γ (b) was assessed by LightCycler RT-PCR and normalized with 1,000 copies of the housekeeping gene β_2 -microglobulin (1k β_2 -MG). Data are from one experiment, representative of three separate experiments. (c) T-bet expression in purified B cells in the absence or presence of LPS (10 μ g/ml), IFN- γ (50 ng/ml) or CpG (3 μ M) for 6 h. Data are the average of three independent experiments. (d) T-bet expression in human peripheral B cells in response to treatment with ODN 2006 (CpG) or a negative ODN 2006 (GpC) for 18 h.

and natural killer cells), we carried out the first set of experiments with splenocytes. Cells were treated with CpG for 6 h, the time point where T-bet expression reaches maximum, and for 24 h, where secondary effects could be observed. We then isolated the T cell and B cell fractions by sequential positive and negative selection and recovered the T cell- and B cell-depleted splenocytes (mainly natural killer cells and myeloid cells, designated as 'non-T, non-B'). When we assessed the abundance of T-bet mRNA in these three populations by quantitative RT-PCR, we found a substantial induction of T-bet in B cells and the non-T, non-B cell population (Fig. 4a). We did not observe increased T-bet expression in T cells, at least over a time course of 24 h. T cells are not expected to respond to CpG directly, owing to their lack of TLR9 expression. To preclude the possibility that CpG-induced signaling had not yet transmitted or failed to transmit to T cells, we measured IFN- γ mRNA abundance in the same samples. CpG-induced induction of IFN- γ mRNA was found in T cells with comparable to non-T, non-B splenocytes, even at 6 h after CpG stimulation (Fig. 4b). This result shows that even in mixed cultures, CpG does not up-regulate T-bet mRNA in T cells in a period of at least 24 h, despite driving T cells to express IFN- γ mRNA.

The rapid induction of T-bet in B cells led us to examine whether CpG acts on B cells directly or through other cells, such as dendritic cells, macrophages and monocytes. Splenic B cells were isolated (purity >99%) and then treated with CpG for 6 h. Given the absence of CD14 in B cells, we used 10 μ g/ml of LPS for stimulation of B cells (1 μ g/ml of LPS induces B cell proliferation; data not shown). We observed increased T-bet expression in highly purified B cells treated with CpG (Fig. 4c). However, incubation of B cells with LPS even at high concentration did not induce considerable expression of T-bet

mRNA, which confirmed and extended the results obtained with splenocytes (Fig. 1), monocytes and dendritic cells²³. We also examined T-bet induction in human B cells. Peripheral B cells were purified from three healthy donors and treated with ODN 2006 (2 μ M), a well-characterized human B cell-stimulatory CpG DNA²⁶. CpG-induced up-regulation of T-bet was clearly seen in the B cells of all three donors (Fig. 4d). These results show that CpG-TLR9 signaling could lead to a rapid induction of the mRNA encoding the key transcription factor T-bet in B cells, directly linking innate and adaptive immunity through the B cell compartment.

CpG regulates T-bet independently of IFN- γ and STAT1

As CpG can effectively induce STAT1 phosphorylation (Fig. 2), which was also confirmed in purified B cells (data not shown), we investigated the potential role of IFN- γ and STAT1 in CpG-induced T-bet

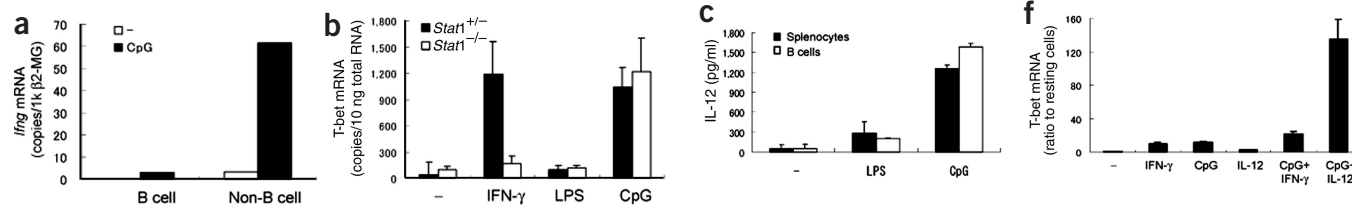


Figure 5 CpG-induced, IFN- γ -STAT1-independent expression of T-bet in purified B cells. (a) Splenic B cells (isolated by negative selection) and the remaining splenocytes (non-B) were incubated with or without CpG for 6 h. Expression of IFN- γ was then measured with LightCycler RT-PCR and normalized with 1,000 copies of β_2 -microglobulin (1k β_2 -MG). Data are from single experiments, representative of three. (b) B cells purified from spleens of *Stat1*^{+/+} and *Stat1*^{-/-} mice were treated with the indicated stimuli for 6 h. T-bet mRNA was measured thereafter (b). Data are the mean $\pm$ s.d. of three independent experiments.

Figure 6 Characterization of signal cascades involved in CpG-induced T-bet transactivation in purified B cells. (a,b) Effect of cycloheximide (3 μ g/ml; a) and inhibitors of kinase or NF- κ B (100 nM dexamethasone, 1 μ M MG 132, 10 μ M SB203580, and 10 nM wortmannin; b) on CpG-induced T-bet expression. (c) Production of IL-12 from cells stimulated with LPS or CpG for 6 h. (d) Effects of neutralizing antibodies on CpG-induced T-bet expression. (e) T-bet expression in wild-type and IL-12-deficient B cells in response to CpG and IFN- γ . (f) T-bet expression induced by the indicated stimuli. For b-f, $n = 3$; for a, data is from a single experiment, representative of three.

Table 1 Regulation of IL-4- and CD40-induced Ig production by CpG

Stimuli	Cell number ($\times 10^6/\text{ml}$) ^a	IgE (ng/ml) ^b	IgG1 (ng/ml)	IgG2a (ng/ml)
–	1.02 $\pm$ 0.14	ND ^c	3.6 $\pm$ 0.9	0.37 $\pm$ 0.10
IL-4 + anti-CD40	1.14 $\pm$ 0.13	3.6 $\pm$ 6.3	246.0 $\pm$ 30.9	0.49 $\pm$ 0.14
IL-4 + anti-CD40 + CpG	1.20 $\pm$ 0.08	ND	32.8 $\pm$ 6.0	0.69 $\pm$ 0.41

^aB cells were isolated from splenocytes by negative selection and treated as described. ^bIg production was determined by ELISA. ^cND, not detected. Data represent four animals in each group. Values are mean $\pm$ s.e.m.

up-regulation. The production of IFN- γ is unlikely to have a major effect on CpG-induced T-bet expression, as we did not see any substantial increase in IFN- γ transcript abundance (Fig. 5a) or in the amount of IFN- γ protein in the culture medium as measured by ELISA (below the detection limit of 15 pg/ml; data not shown) after CpG stimulation. If the phosphorylation of STAT1 by CpG was not caused by IFN- γ , but rather through type I IFNs, which have been reported to be rapidly induced by CpG²⁷, we wondered whether STAT1 phosphorylation was required for the T-bet induction by CpG. In B cells of *Stat1*^{–/–} mice, IFN- γ -induced expression of T-bet was greatly reduced (Fig. 5b), consistent with previous reports on myeloid cells and T cells^{23,25}. In contrast to IFN- γ , CpG-induced T-bet expression was not affected by STAT1 deficiency, indicating that CpG and IFN- γ use differential signaling pathways in the regulation of T-bet transcription.

T-bet induction by CpG requires NF- κ B, p38 and IL-12

To further trace the downstream signal pathways induced by CpG and TLR9 that lead to T-bet up-regulation, we tested several pharmacological inhibitors. All the inhibitors were confirmed to be nontoxic at the concentrations used here by water-soluble tetrazolium salt (WST-1) assay. When B cells were incubated with cycloheximide, an inhibitor of protein synthesis, CpG-induced T-bet expression in B cells was completely diminished (Fig. 6a), indicating a requirement for *de novo* protein synthesis. Incubation of B cells with the NF- κ B inhibitor dexamethasone or MG 132, or with a specific inhibitor for MAPK p38, SB203580, resulted in a profound reduction in CpG-triggered T-bet

expression (Fig. 6b), whereas wortmannin (a specific inhibitor of PI-3K) did not have any substantial effect. On the basis of these results and earlier data showing that p38 is important in CpG-induced IL-12 production *in vitro* and the anti-allergic effect of CpG *in vivo*^{28,29}, we investigated whether IL-12 might be involved in CpG-dependent T-bet up-regulation in B cells. We first determined

whether IL-12 was produced in CpG-treated B cells, with B cell-depleted splenocytes and LPS treatment as a reference. Six hours after stimulation (where a clear induction in T-bet mRNA could be detected), we assayed secreted IL-12 with ELISA. Biologically relevant amounts of IL-12 (1.6 ng/ml) were produced in CpG-treated B cells, whereas the production in cells treated with LPS was much lower (200 pg/ml; Fig. 6c). IFN- γ was not detected in the same culture medium (data not shown). To show that IL-12 is important for CpG-driven T-bet expression, we set up experiments with neutralizing antibodies to IL-12 and B cells from IL-12-deficient mice. CpG-induced T-bet expression was reduced by 66% in B cells treated with IL-12-blocking antibodies, and by 68% in IL-12-deficient B cells, whereas T-bet transcription was not affected either by an IFN- γ blocking antibody or in IFN- γ -treated, IL-12-deficient B cells (Fig. 6d,e). Even in IL-12-deficient B cells, CpG-induced T-bet transcription was not eliminated. To further address whether IL-12 functions additively with CpG, B cells were stimulated with CpG, IL-12 and IFN- γ , alone or in combination. IL-12 synergistically enhanced CpG-induced T-bet up-regulation, in contrast to IFN- γ , which appeared to affect T-bet expression only additively (Fig. 6f). Thus, IL-12 together with CpG stimulates a much greater induction of T-bet mRNA (130 times background) than does CpG alone (12 times) or CpG plus IFN- γ (20 times).

CpG directly inhibits IgG1 and IgE production in B cells

Although CpG can inhibit antigen-induced IgE production in mice and in PBMCs from atopic patients, such an effect has not been

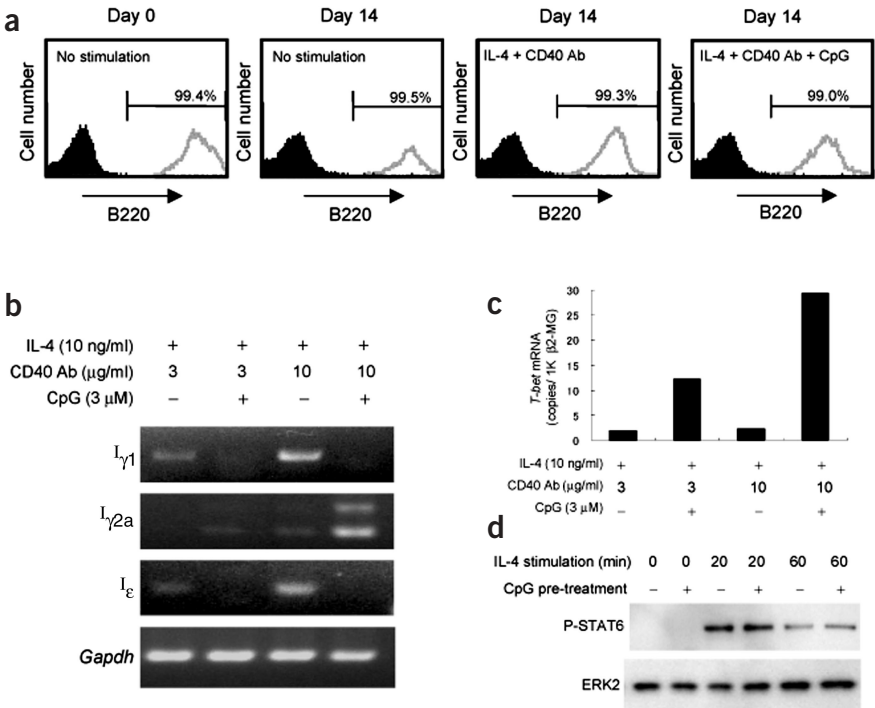


Figure 7 Inhibition of IL-4- and CD40-ligation-induced IgG1, IgG2a and IgE production. B cells were isolated from splenocytes by negative selection and treated as described. (a) Cell purity was checked by FACS with PE-labeled B220 antibody. Data represent four animals in each group. (b) Germline transcripts were measured by RT-PCR with primer sets corresponding to the sequences of the *I*_{H1}–*C*_{H1}, *I*_{H2a}–*C*_{H2a} and *I*_E–*C*_E exon-hinge region, respectively. The housekeeping gene *Gapdh* was used as a control. Shown is a single experiment, representative of three. (c) Samples from b were assessed for T-bet expression. (d) Effect of CpG treatment on IL-4-induced STAT6 phosphorylation. B cells were pretreated with CpG for 20 h and then stimulated with IL-4 for 20 or 60 min. Activation of STAT6 was assessed by immunoblotting with anti-phospho-STAT6. ERK2 antibodies were used as a control to detect total protein loading.

reported in cultured B cells. Given our finding that CpG can rapidly increase T-bet mRNA in B cells, and earlier work indicating that ectopic expression of T-bet or T-bet deficiency affects the production of IgG2a, IgG1 and IgE³⁰, we speculated that the induction of T-bet by CpG in B cells might influence Ig class switching. To determine this, we cultured splenic B cells (purity >99%) in a medium containing IL-4 (10 ng/ml) and CD40 agonistic antibodies (3 µg/ml) in the presence or absence of CpG (3 µM) for 10–14 d. We then assessed cell number, purity and the amounts of secreted IgG1, IgG2a and IgE. Secretion of IgE induced by IL-4 and CD40 ligation (36.1 ± 6.3 ng/ml, mean $\pm$ s.e.m.) was completely inhibited, and IgG1 production (246 ± 30.9 ng/ml) showed an 87% reduction after CpG treatment (Table 1). Because there were no substantial changes in either cell number (Table 1) or purity (Fig. 7a) during the cell culture, these data show that CpG effectively inhibits IL-4/CD40-induced, T_H2-related IgE and IgG1 production through a direct effect on B cells. This inhibition seemed to occur at the transcriptional level, because I_E and I_{γ1} germline transcripts were completely diminished in B cells cultured with IL-4 and agonistic antibodies to CD40 and CpG (Fig. 7b). To investigate the involvement of T-bet, we assessed mRNA by quantitative RT-PCR. As compared to cells treated with IL-4 and anti-CD40 alone, we saw a notable up-regulation of T-bet mRNA in cells treated with additional CpG. Thus, the expression of T-bet correlated with the inhibition of IgE and IgG1 class switching. As expected, IgG2a class switching showed a positive correlation with T-bet mRNA expression (Fig. 7b,c). Because activation of STAT6 is important in IL-4-induced IgE and IgG1 class switching, we wondered whether CpG treatment or T-bet expression could inhibit IL-4-induced phosphorylation of STAT6. B cells were pretreated with CpG for 20 h and then stimulated with IL-4 for 20 or 60 min. We then assessed the activation of STAT6 by immunoblotting with an antibody recognizing phospho-STAT6. CpG treatment did not interfere with IL-4-induced STAT6 phosphorylation (Fig. 7d), indicating that some other mechanism must be involved.

DISCUSSION

An increasing body of literature supports the view that T_H2 cells are important in the pathology of type I allergic diseases such as bronchial asthma, rhinitis or conjunctivitis. Consequently, shifting T cells towards a T_H0 or T_H1 phenotype may present a therapeutic strategy for the treatment of allergies. As CpG DNA is one reagent capable of such immunomodulation, we undertook the present study to investigate the critical mechanisms of the protective effects of CpG in allergic inflammation.

We provide evidence that CpG, but not LPS, can induce a rapid up-regulation of the key transcription factor T-bet mRNA in B cells. Previous work showed that LPS and IL-1 do not up-regulate T-bet in monocytes and dendritic cells, suggesting that T-bet induction is peculiar to CpG-TLR9 signaling but not to LPS-TLR4 and IL-1–IL-1R signaling. It is notable that both CpG and IFN-γ rapidly induce expression of T-bet mRNA in B cells, and neither CpG nor IFN-γ alone up-regulates T-bet expression in naive T cells. This emphasizes the functional importance of B cells in the early immunomodulatory cascade induced by CpG.

Induction of T-bet has been suggested to be mainly dependent upon IFN-γ signaling and STAT1 activation^{23,25,31}. We demonstrate here that CpG uses an IFN-γ-independent signaling pathway, because B cells do not express substantial amounts of IFN-γ at either the mRNA or protein level upon CpG stimulation, and an IFN-γ-blocking antibody does not affect CpG-induced T-bet expression. Furthermore, the lack of STAT1, which is necessary for IFN-γ-induced T-bet up-regulation, has no effect on CpG-induced T-bet expression in B cells. The difference in

the requirement for STAT1 between CpG and IFN-γ may be a result of the regulation of T-bet through GAS (IFN-γ-activated site) elements³². IFN-γ induces STAT1 phosphorylation through IFNGR, which results in the STAT1 homodimer binding to GAS, while CpG induces type I IFN expression²⁷, which subsequently causes the phosphorylation of both STAT1 and STAT2 and results in the STAT1-STAT2 heterodimer binding to IFN-stimulated response elements, which may not appear in the T-bet promoter. This hypothesis also could explain why type I IFNs and LPS (through production of IFN-β²⁴) stimulate STAT1 phosphorylation but not T-bet expression.

How is T-bet up-regulated in B cells upon CpG stimulation? We first noticed that T-bet induction requires MAPK p38- and NF-κB-driven *de novo* protein synthesis, and we found that large amounts of IL-12 (1.6 ng/ml) were produced. Whereas production of IL-10 and IFN-γ was not detectable. CpG-induced T-bet expression was reduced markedly (>65%) by treatment with an IL-12 neutralizing antibody and in IL-12-deficient cells. However, IL-12 seemed neither sufficient nor absolutely required for CpG-induced T-bet expression, as it alone did not induce a substantial amount of T-bet mRNA and CpG still stimulated a fivefold induction of T-bet mRNA in IL-12-deficient B cells. Notably, when B cells were treated with IL-12 and CpG, we saw an induction of T-bet mRNA that was 130-fold over background, compared to only a 10- to 30-fold induction by CpG alone. These results suggest a mechanism by which CpG-induced IL-12 further synergizes with other CpG-induced signals (for example, activated NF-κB) and leads to a marked induction of T-bet. Moreover, our finding that IL-12 is more important than IFN-γ in CpG-mediated T-bet expression is in agreement with recent work from several groups. Previous studies³⁰ showed that T-bet-mediated regulation of Ig switching was independent of IFN-γ. Intrapulmonary CpG DNA-mediated inhibition of allergic inflammation is ablated in *Il12*^{-/-} mice²⁹. Furthermore, CpG still inhibits ovalbumin-induced pulmonary inflammation and airway hyperactivity (AHR) in *Ifng*^{-/-} mice with adoptively transferred ovalbumin-specific T_H2 cells³³. Taken together, these findings indicate that IL-12, but not IFN-γ, may be a central mediator of CpG-induced anti-allergic effects through induction of T-bet.

The most important result obtained in this study is the insight into the role of B cells in the CpG-induced anti-allergy responses. Inhibition of IgE production *in vivo* or in PBMCs from atopic patients by CpG has previously been thought to occur via T_H1-type cytokine production from T_H1, natural killer and dendritic cells^{16,18}. Here we provide evidence that CpG effectively inhibits IgE and IgG1 production induced by IL-4–CD40 via a direct action on B cells. In addition, this inhibitory effect correlates with the induction of T-bet and IgG2a expression. Ectopic expression of T-bet alone is sufficient to induce IFN-γ-independent IgG2a production and to inhibit IL-4-induced activation of the I_E promoter³⁰. In addition, T-bet-deficient B cells do not produce IgG2a and produce excess amounts of IgG1 and IgE³⁰. Overall, these findings suggest that CpG-induced T-bet expression may account for the effect on Ig class switching. The final conclusion, however, awaits analysis in T-bet-deficient B cells.

Notably, the outcome of CpG-TLR9- and LPS-TLR4-mediated Ig switching in purified B cells is different. It has been shown that LPS promotes both T_H1- and T_H2-related Ig production, depending on the cytokine milieu. When B cells are treated with LPS and IFN-γ, IgG2a is produced. In the presence of IL-4, LPS augments IgE production³⁴. These differential properties of CpG and LPS may result from the difference in their abilities to induce T-bet expression. Thus, CpG, through induction of T-bet, increases the production of IgG2a and suppresses that of IgG1 and IgE. LPS does not up-regulate T-bet but

can enhance cell survival, promote proliferation or stimulate transcription in general, which results in the augmentation of both T_H1 - and T_H2 -related Ig production.

This study may influence the development of therapeutic CpGs currently being evaluated as drug candidates. In contrast to the CpGs that are effective in mice, there are two structurally distinct CpGs for humans^{35,36}. One is 'K' type CpG, which has a phosphorothioate backbone and stimulates human B cells to produce cytokines and secrete IgM. CpG ODN 2006, which up-regulates T-bet mRNA, belongs to this family. The 'D' type CpG containing G-rich and palindromic sequences preferentially stimulates IFN production by natural killer cells. Given our finding that CpG can achieve its inhibitory effects on IgE and IgG1 production through direct effects on B cells, and considering that B cells produce very low levels of inflammatory cytokines such as TNF- α ³⁷, 'K' type CpGs may be therapeutically preferable, given their side effect profile.

METHODS

Reagents. We obtained phosphorothioate CpG oligodeoxynucleotide (ODN) with sequences of 5'-TCCATGACGTTCTGACGTT-3' for mouse and 5'-TCGTCGTTTTGTGCGTTTTGTGCGTT-3' for human, and control GpC ODNs with sequences of 5'-TCCATGAGCTTCCTGAGTCT-3' for mouse and 5'-TCGTCGTTTTGTGCGTTTTGTGCGTT-3' for human, from Hokkaido System Science. Recombinant mouse IFN- γ , IL-12 and IL-4 were from Genzyme Techno. *E. coli* LPS was from Sigma. Anti-phospho-STAT1 and anti-phospho-STAT6 were from Zymed Laboratories and Cell Signaling, respectively. Anti-CD40 agonistic monoclonal antibody, anti-IL-12 and anti-IFN- γ blocking antibodies were from PharMingen. Inhibitors used in this study were dexamethasone (TCI), SB203580 (Calbiochem), and wortmannin (Alexis).

Mice. We obtained wild-type C57BL/6 mice from Charles River Laboratory. We generated 129/C57BL/6/Myd88^{+/+} and 129/Sv/C57BL/6/Myd88^{-/-}, 129/Sv/C57BL/6/Th9^{+/+} and 129/Sv/C57BL/6/Th9^{-/-}, 129/Sv/C57BL/6/Stat1^{+/+} and 129/Sv/C57BL/6/Stat1^{-/-}, and 129/Sv/C57BL/6/Il12^{+/+} and 129/Sv/C57BL/6/Il12^{-/-} mice as previously described^{21,38,39}. Mice used in this study were backcrossed against the C57BL/6 strain at least four times before use. We obtained Th9^{+/+} and Th9^{-/-} animals by mating Th9^{+/+} mice. Genotypes of TLR9-deficient mice were determined by PCR on tail DNA with the following primers: sense, 5'-CATGGCTGGTGGACTGCAA-3'; wild-type antisense, 5'-TGAAGAGAACGCGCAGG-CTGT-3'; and knockout antisense, 5'-ATCGCCTTCTATCGCCTTCTTGACGAG-3'. All the mice were housed in a pathogen-free animal facility. All experiments were done in accordance with Institutional Guidelines of Research Center Kyoto, Bayer Yakuhin Ltd.

Cell purification and culture conditions. Splenocytes were obtained from mice at 7–10 weeks. B cells were purified by negative selection with a B cell isolation kit containing biotin-conjugated monoclonal antibodies to CD43, CD4 and Ter-119 (Miltenyi Biotec). We obtained T cells by positive selection with CD90 MicroBeads. Cells were cultured at a density of 2.0×10^6 /ml in RPMI 1640 with 10% fetal bovine serum (RPMI–10% FBS; Gibco-BRL). We used the following conditions for different cell stimulation: 3 μ M CpG ODN; 3 μ M GpC ODN; 50 ng/ml IFN- γ ; and 50 ng/ml LPS for splenocyte and 10 μ g/ml LPS for purified B cells. For treatments with pharmacological inhibitors, we added 3 μ g/ml cycloheximide, 100 nM dexamethasone, 1 μ M MG 132, 10 μ M SB203580 or 10 nM wortmannin to the culture medium, and RPMI–10% FBS 30 min before stimulation with CpG. For the Ig isotype switch assay, purified B cells (>99% pure) were incubated in RPMI–10% FBS with 10 ng/ml IL-4 and 3 or 10 μ g/ml anti-CD40 antibodies in the presence or absence of CpG (3 μ M) for 10–14 d.

Assessment of mRNA expression. We prepared total RNA with TRIzol Reagent (Gibco-BRL) and then treated it with DNA-free (Ambion) to avoid genomic DNA contamination. First-strand cDNA was synthesized with the SuperScript first-strand synthesis system (Gibco-BRL). For real-time LightCycler PCR, we normalized samples with β_2 -microglobulin and presented data as copies per 1,000 β_2 -microglobulin. Primers and PCR conditions for $I_{\gamma 1}$, I_E and $I_{\gamma 2a}$

germline transcripts were described previously⁴⁰. Primers for T-bet expression were T-bet-L1, 5'-GCCAGGAACCGCTTATATG-3', and T-bet-R1, 5'-GACGATCATCTGGGTCACATTGT-3'.

Cytokine and Ig production. We evaluated cytokines and Ig production in culture supernatants by ELISA (TNF- α and IL-12 (Genzyme), IgE (PharMingen) and IgG1 and IgG2a (Bethyl Laboratory)).

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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