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The TLR7 agonist imiquimod selectively inhibits IL-4-induced IgE production by suppressing IgG1/IgE class switching and germline ε transcription through the induction of BCL6 expression in B cells

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ABSTRACT

Imiquimod (IMQ) is a selective toll-like receptor 7 (TLR7) agonist. TLR7 activation leads to the production of IFN-γ and pro-inflammatory cytokines by innate immune cells. However, the role of TLR7 in B cells is not fully understood. In this study, we investigated the direct effect of in vitro stimulation with IMQ on Ab production and isotype switching in B cells. IMQ selectively diminished IL-4-induced IgE and IgG1 production in anti-CD40-activated mouse B cells. IMQ also inhibited germline ε transcripts (GLTε)/GLTγ1 and post-switch ε transcripts (PSTε)/PSTγ1 expression, while enhancing GLTγ2c and PSTγ2c expression in anti-CD40/IL-4-stimulated B cells. Interestingly, IMQ abrogated IL-4-induced circle transcripts ε-γ1 (CTε-γ1) expression, indicative of sequential switching from IgG1 to IgE. Furthermore, IMQ repressed IL-4-induced surface IgE/IgG1 expression while increasing surface IgG2c expression. The selective inhibition of IgE synthesis was not due to IMQ-induced production of IFN-γ or IL-12 in the same culture. IMQ also enhanced BCL6 expression, a transcriptional repressor for the GLTε promoter, in anti-CD40/IL-4-stimulated B cells. In addition, BCL6 siRNA restored IMQ-mediated suppression of GLTε transcription. Therefore, these results indicate that TLR7 engagement by IMQ inhibits IL-4-induced GLTε transcription by enhancing BCL6 expression and inhibits IL-4-induced sequential switching from IgM to IgE via IgG1, thus resulting in the downregulation of IgE production by B cells.

1. Introduction

Toll-like receptor 7 (TLR7), an intracellular endosomal TLR, is predominantly expressed in plasmacytoid dendritic cells but is also expressed in other myeloid and lymphoid cells including B cells [1–3]. TLR7 recognizes synthetic ssRNA derived from RNA viruses and can be also triggered by imiquimod (IMQ, R837), a nucleoside analogue of the imidazoquinoline family [4]. TLR7 engagement by its ligand in immune cells leads to the induction of type I IFN, IL-12, TNF-α, IL-6, and the Th1 cytokine IFN-γ and the inhibition of the Th2 cytokines IL-4, IL-5, and IL-13 [5]. TLR8 is highly homologous to TLR7 and also binds to viral ssRNA [6]. Another nucleoside analogue of imidazoquinoline, resiquimod (RSQ, R848), triggers TLR7 and TLR8 [7]. IMQ and RSQ are known to have potent antiviral and antitumor activities in animals [8,9]; IMQ has been approved for the treatment of external genital warts caused by human papillomavirus infection in humans [10]. Thus, IMQ and RSQ act as immune response modifiers, and their activities are mediated through the stimulation of innate and adaptive immune responses [5,11–13].

IMQ and RSQ have been shown to act as adjuvants, enhancing Ag-specific IgG2a (Th1 Ig) levels, while suppressing IgE and IgG1 (Th2 Igs) production [13,14]. These adjuvant activities may be linked to the induction of IFN-γ and the inhibition of IL-4/IL-13 production [5]. Systemic application of RSQ during the sensitization phase has been shown to prevent the production of OVA-specific IgE and IgG1 Abs and subsequently abolish all features of experimental asthma, including airway hyper-responsiveness and allergic airway inflammation [15]. IMQ treatment in vivo decreases serum IgE levels in antigen-induced pulmonary inflammation in a rat model for asthma [16]. Thus, TLR7 can be a drug target in IgE-mediated allergic diseases, including asthma, and the TLR7 agonists IMQ and RSQ have the therapeutic potential for treatment in humans [17]. In vitro, RSQ inhibits IgE production in anti-

Abbreviations: IMQ, imiquimod; GLT, germline transcripts; PST, post-switch transcripts; CT, circle transcripts; CSR, class switch recombination; RSQ, resiquimod

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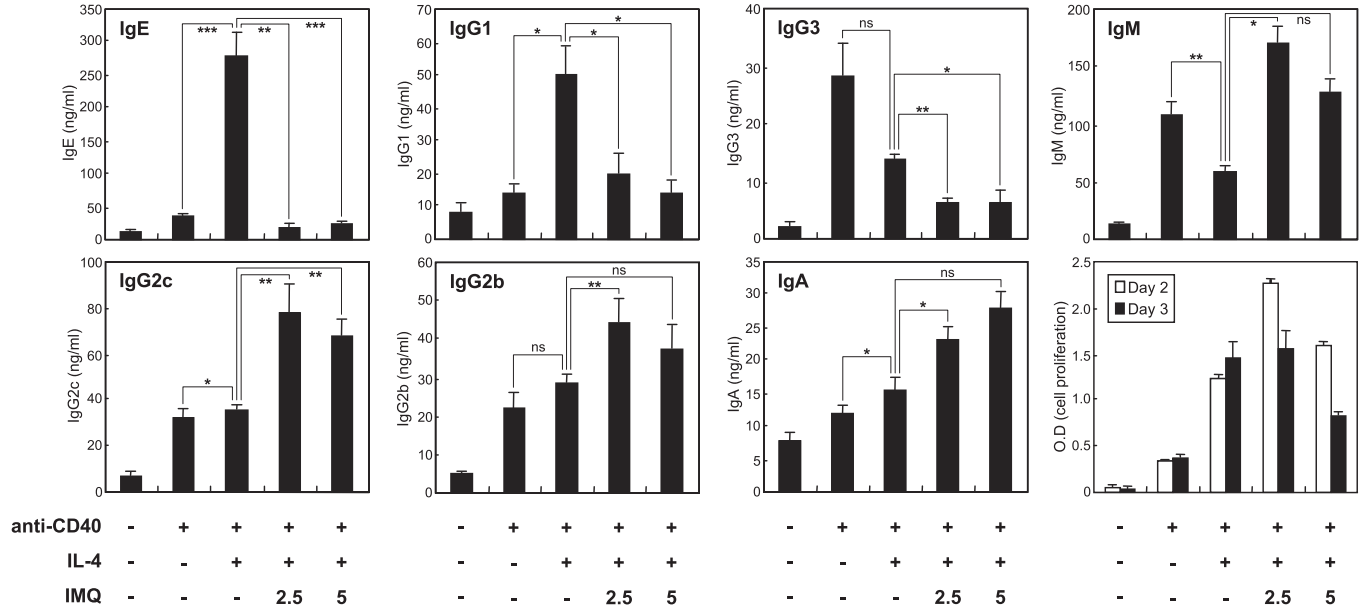

Fig. 1. Effect of IMQ on Ab production by anti-CD40/IL-4-stimulated mouse B cells. Purified resting B cells were stimulated with anti-CD40 Ab (2 μg/ml), IL-4 (10 ng/ml), and IMQ (2.5 or 5 μg/ml). After 7 days of culture, supernatants were harvested, and levels of Igs production were determined by isotype-specific ELISAs. Data presented are the means ± SEM from three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ns: not significant. After 2 and 3 days of culture, cell proliferation (O.D) was measured. Data presented are means of duplicate samples with ranges (bars).

CD40/IL-4-stimulated human PBMCs [18] and IgE and IgG1 production in anti-CD40/IL-4-stimulated mouse splenocytes [19].

Mature IgM^+IgD^+ B cells can be activated and differentiated into plasma cells to produce Abs through BCR cross-linking, CD40 ligation, pattern recognition receptor engagement, and cytokine stimulation. During this process in mice, each cytokine selectively induces Ig class switching in B cells: IL-4 to IgE and IgG1; IFN-γ to IgG2a (IgG2c) and IgG3; TGF-β1 to IgA and IgG2b. This selective switching to certain isotypes is caused by the transcription of germline transcripts (GLTs) in each switch region of the Ig heavy chain gene in mature B cells [20]. For instance, selective induction of GLTε by IL-4 initiates IgE class switching by increasing the accessibility of activation-induced cytidine deaminase (AID), an essential enzyme for Ig class switch recombination (CSR) [21], to the non-transcribed DNA strand of the switch region.

Although there are many reports on the negative regulation of IMQ/RSQ for IgE production in vivo and in vitro, the cellular and molecular mechanisms by which direct TLR7 stimulation of B cells affects IgE class switching have not been fully elucidated. In this study, we evaluated the in vitro effect of the TLR7 agonist IMQ on various processes, including the production of Igs, transcription of GLTs, and cell surface Ig expression in anti-CD40- and IL-4-stimulated mouse B cells. Our results indicate that IMQ selectively inhibits IgE production through the suppression of IgG1/IgE class switching in anti-CD40/IL-4-activated B cells, and this inhibition is caused by IMQ-induced BCL6 expression, a known repressor of IgE class switching.

2. Materials and methods

2.1. Experimental animals

C57BL/6 mice were purchased from Damool Science (Daejeon, Korea) and maintained on an 8:16 h light:dark cycle in an animal environmental control chamber. Eight- to twelve-week-old mice were used. The animal care was in accordance with the guidelines of the Institutional Animal Care and Use Committee of Konyang University.

2.2. Cell culture and reagents

Mouse resting B cells were purified as previously described [22]. The purity of resting B cells ($CD43^-B220^+$, ≥98%) was assessed by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA, USA), followed by staining of the cells with anti-CD43 FITC (eBioscience, San Diego, CA, USA) and anti-B220 PE (BD Biosciences). The mouse B cell line L10A6.2 was provided by Dr. J. Stavnezer (University of Massachusetts Medical School, Worcester, MA, USA). Cells were cultured at 37 °C in a humidified CO_2 incubator (Forma Scientific, Marietta, OH, USA) in RPMI-1640 medium (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (Welgene). Purified B cells were stimulated with anti-CD40 Ab (2 μg/ml, eBioscience), rmIL-4 (10 ng/ml, R&D Systems, Minneapolis, MN, USA), and IMQ (2.5 or 5 μg/ml, InvivoGen, San Diego, CA, USA). Neutralizing IFN-γ Ab (anti-mouse IFN-γ Ab) was purchased from BD Biosciences. 2E2 was stimulated with rhIL-4 (10 ng/ml, R&D Systems) and IMQ (5 or 10 μg/ml, InvivoGen). Anti-mouse IgE-PE, anti-mouse IgG1-PE, and anti-mouse IgM-PE were purchased from eBioscience. Anti-mouse IgG2c-FITC was obtained from Southern Biotech (Birmingham, AL, USA). Negative control siRNA and predesigned mouse BCL6 siRNA (siRNA number: 1330474) were purchased from Bioneer (Daejeon, Korea).

2.3. ELISAs

Antibodies produced in B cell cultures were detected using isotype-specific ELISAs as previously described [23]. ELISAs for IFN-γ and IL-12 were performed according to the manufacturer's instructions using BD OptEIA™ Set (BD Biosciences).

2.4. Cell proliferation assay

Cell proliferation was determined by EZ-Cytox cell viability assay kit (Daeil Lab Service Co, Seoul, Korea) as previously described [23].

2.5. RNA isolation, RT-PCR, and real-time quantitative PCR

RNA isolation, RT-PCR, and real-time quantitative PCR were

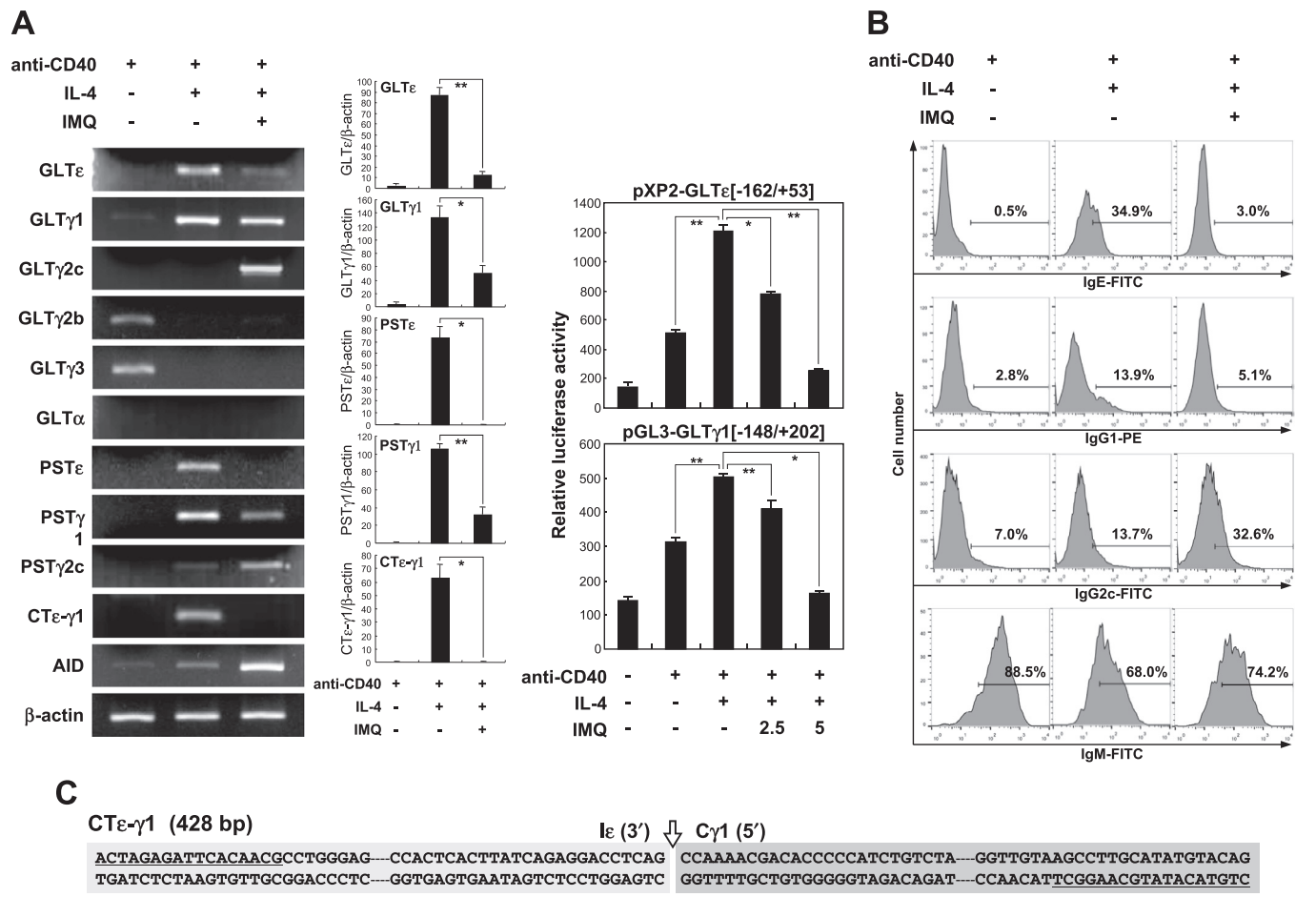

Fig. 2. Effect of IMQ on Ig isotype switching in anti-CD40/IL-4-stimulated mouse B cells. (A) Purified resting B cells were stimulated with anti-CD40 Ab (2 μg/ml), IL-4 (10 ng/ml), and IMQ (2.5 μg/ml). After 2.5 days of culture, mRNAs were isolated, and levels of GLTs, PSTs, CTε-γ1, and AID mRNA were measured by RT-PCR. Data shown are representative of three independent experiments (left panel). $*p < 0.05$, $**p < 0.01$. The mouse B cell line L10A6.2 was transfected with pXP2-GLTε[−162/+53] or pGL3-GLTγ1[−148/+202] (15 μg). Cells were then stimulated with anti-CD40 Ab (2 μg/ml), IL-4 (10 ng/ml), and IMQ (2.5 or 5 μg/ml), and luciferase activities were assayed 16 h later. Data represent mean ± SEM luciferase activities from three independent transfections (right panel). $*p < 0.05$, $**p < 0.01$. (B) Purified B cells were stimulated as in (A). After 3.5 days of culture, cells were stained with anti-IgE-FITC, anti-IgG1-PE, anti-IgG2c-FITC, or anti-IgM-FITC, and surface Ig expression was analyzed by flow cytometry. Data shown are representative of two independent experiments. (C) Junctional sequences of the CTε-γ1. CTε-γ1 PCR product was purified and sequenced using the ABI 3730XL DNA Analyzer. Light and dark gray boxes indicate the Iε and Cγ1 exons, respectively. Underlines indicate the primers for PCR and sequencing analysis. Arrow indicates the junction between two exons. Sequencing information is given in the Gene Bank Accession No.: Iε exon, M31133.1; Cγ1 exon, D78344.1.

performed as previously described [23]. The PCR primers (Suppl. Table 1) were synthesized by Bioneer. PCR for β-actin was performed in parallel to normalize cDNA concentrations within each set of samples. PCR products were resolved by electrophoresis on 2% agarose gels. Semi-quantitative RT-PCR analysis was performed using cDNA dilutions.

2.6. Sequence analysis

PCR product for circle transcripts ε-γ1 (CTε-γ1) was purified using Expin™ Gel SV (GeneAll, Seoul, Korea), and the sequence determined by BIOFACT (Daejeon, Korea). Primers for sequencing analysis were the same as the PCR primers as described in Suppl. Table 1.

2.7. Reporter plasmids

Luciferase reporter plasmids pXP2-GLTε[−162/+53] and pGL3-GLTγ1[−148/+202], which contain, respectively, mouse GLTε and GLTγ1 promoter, were provided by Dr. J. Stavnezer.

2.8. Transfection and luciferase reporter assays

Transfection was performed by electroporation with a Gene Pulser II electroporation system (Bio-Rad Laboratories, Hercules, CA) as described previously [24]. Reporter plasmids were co-transfected with pCMVβgal (Stratagene, La Jolla, CA), and luciferase and β-gal assays were performed as described previously [24].

2.9. Flow cytometric analysis

Surface staining was performed with anti-mouse IgE-PE, anti-mouse IgG1-PE, anti-mouse IgG2c-FITC, or anti-mouse IgM-PE in the dark for 30 min at 4 °C, and surface Igs-expressing B cells were analyzed by flow cytometry (FACSCalibur).

2.10. Statistical analysis

Statistical differences between experimental groups were determined by analysis of variances. All *p*-values were calculated by the unpaired two-tailed Student's *t* test to consider statistical significances.

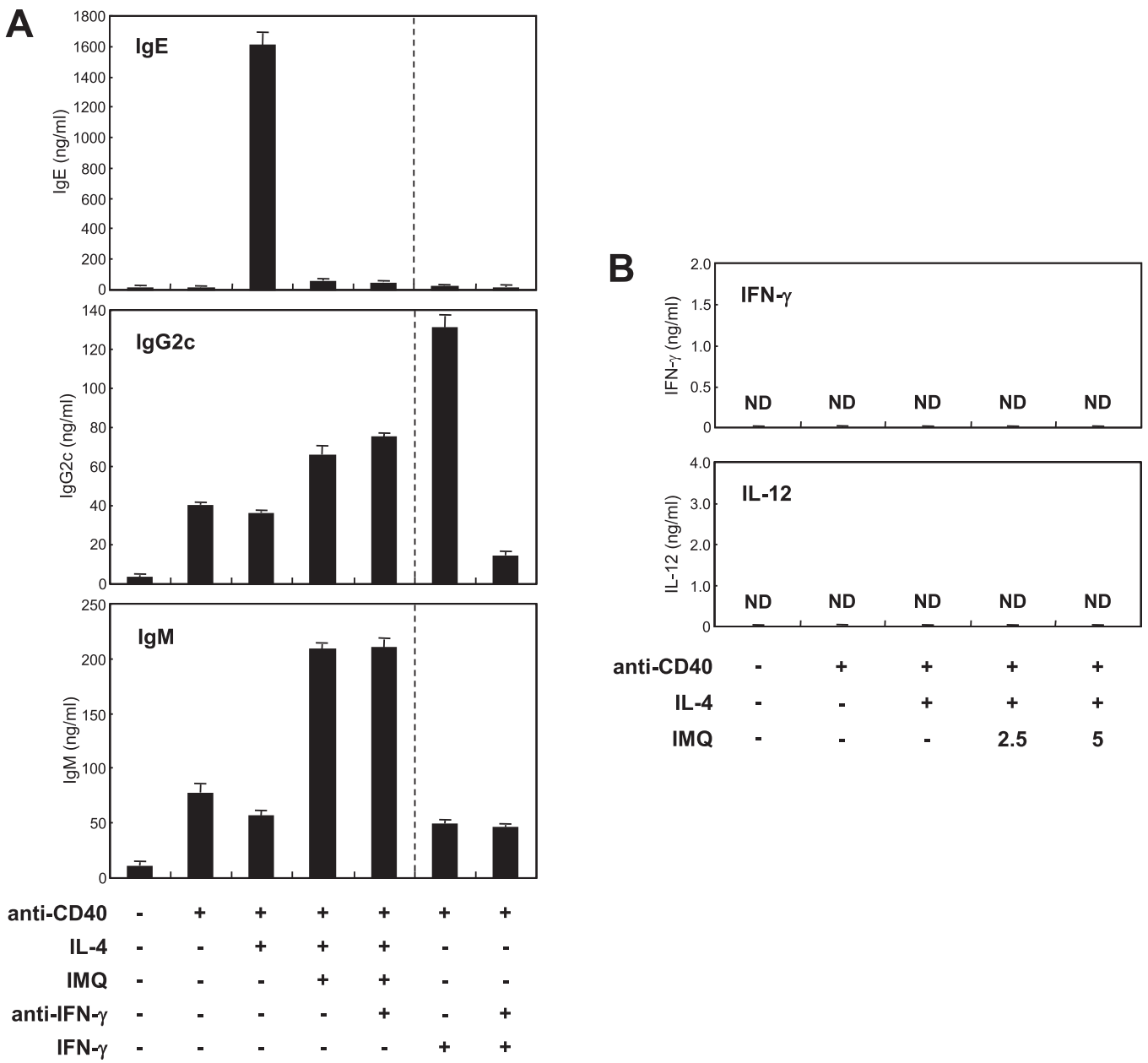

Fig. 3. IMQ does not induce IFN-γ and IL-12 production to inhibit IgE production by anti-CD40/IL-4-stimulated B cells. Purified resting B cells were stimulated with anti-CD40 Ab (2 μg/ml), IL-4 (10 ng/ml), IMQ (2.5 μg/ml), and IFN-γ (10 ng/ml) after pre-treatment with anti-IFN-γ neutralizing Ab (2 μg/ml) for 1 hr. After 7 days of culture, supernatants were harvested, and levels of Igs (A) and cytokines (IFN-γ and IL-12) (B) were determined by ELISAs. Data presented are means ± SEM from two independent experiments.

3. Results and discussion

3.1. IMQ selectively inhibits IL-4-induced IgE and IgG1 production in anti-CD40-stimulated mouse B cells

To determine the direct effect of IMQ on IgE production by B cells, we treated IMQ with the anti-CD40 Ab and IL-4 in purified mouse resting B cells. As shown in Fig. 1, IMQ completely abrogated IL-4-induced IgE production in anti-CD40-stimulated B cells. Furthermore, IMQ inhibited IgG1 induction by IL-4. IgG3 production was inhibited by IL-4 and further decreased with IMQ treatment. However, IMQ significantly increased the production of IgM, IgG2c, IgG2b, and IgA in anti-CD40/IL-4-stimulated B cells. In addition, we observed that IMQ enhanced B cell proliferation in the same culture. IMQ alone did not affect IgE and IgG1 production, while it increased IgG2c and IgM (Suppl. Fig. 1). Furthermore, the treatment with IMQ alone was insufficient for the induction of IgE and IgG1 production by IL-4. These results indicate that IMQ selectively suppresses IL-4-induced IgE and IgG1 production in anti-CD40-activated B cells.

3.2. IMQ inhibits IgE class switching via the suppression of sequential switching from IgG1 to IgE in anti-CD40/IL-4-stimulated mouse B cells

To verify whether the selective suppression of IgE/IgG1 production by IMQ is caused by the inhibition of IgE/IgG1 class switching, we first determined the expression levels of GLT and post-switch transcripts (PST). Transcription of GLT is a prerequisite for Ig CSR, and PST is generated after CSR [25] (Suppl. Fig. 1); thus, expression of these two transcripts is indicative of active Ig class switching. As shown in Fig. 2A (left and central panel), IMQ decreased IL-4-induced GLTε/GLTγ1 and

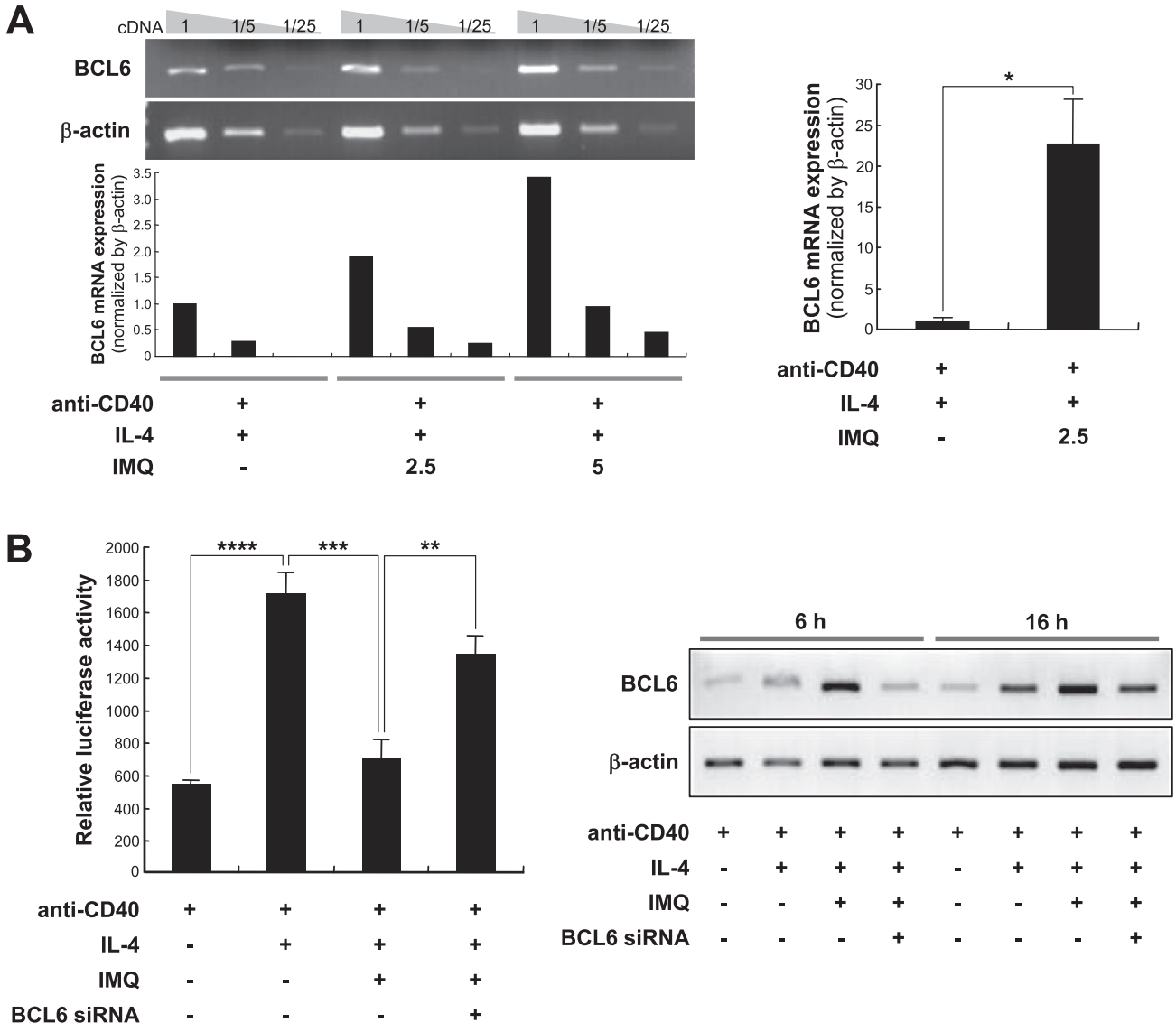

Fig. 4. Germline ε transcription is suppressed by IMQ-induced BCL6 expression in anti-CD40/IL-4-stimulated B cells. (A) Purified B cells were stimulated as in Fig. 1. After 6 h of culture, mRNA was isolated, and BCL6 mRNA expression was analyzed by semi-quantitative RT-PCR (left panel. The graph shows relative BCL6 cDNA levels normalized to β-actin cDNA expression using densitometric analysis) and real-time quantitative RT-PCR (right panel). Data represent means ± SEM of three independent experiments. *$p < 0.05$. (B) The mouse B cell line L10A6.2 was transfected with pXP2-GLTε[−162/+53] (10 μg) and BCL6 siRNA (1 μM). Cells were then stimulated with anti-CD40 Ab (2 μg/ml), IL-4 (10 ng/ml), and IMQ (10 μg/ml), and luciferase activity was assayed 16 h later. Data represent mean ± SEM luciferase activity from three independent transfections (left panel). **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. After 6 h and 16 h of culture, mRNA was isolated, and BCL6 mRNA expression was analyzed by RT-PCR. Data shown are representative of three independent experiments (right panel).

PSTε/PSTγ1 expressions in anti-CD40-stimulated B cells. Interestingly, GLTγ2c and PSTγ2c were induced by IMQ in the cells, while other GLTs (i.e., GLTγ2b, GLTγ3, and GLTα) were not induced. In addition, we observed that IMQ decreased IL-4-induced GLTε and GLTγ1 promoter activity in a dose-dependent manner (Fig. 2A, right panel). Moreover, IMQ abrogated the proportion of IL-4-induced IgE^+ and $IgG1^+$ B cells while increasing the proportion of $IgG2c^+$ cells (Fig. 2B). These results suggest that IMQ suppresses IL-4-induced IgE/IgG1 production through the selective inhibition of IgE/IgG1 class switching, and IMQ induces IgG2c class switching in anti-CD40/IL-4-stimulated B cells, resulting in IgG2c production. However, increased production of IgM, IgG2b, and IgA by IMQ in B cells (Fig. 1) may be caused by increased cell proliferation, as opposed to class switching. IL-4 induces direct switching to IgE (IgM → IgE) as well as sequential switching to IgE through successive IgM → IgG1 → IgE CSR [26] (Suppl. Fig. 2). The DNA sequences between S regions are looped out of the chromosome as switch circles during Ig CSR; another type of transcript, termed circle transcripts (CT), is then transcribed from the switch circles due to active I promoters [27]. Since IMQ suppressed both IgE and IgG1 class switching by IL-4, we hypothesized that IMQ may inhibit the sequential switching to IgE through IgG1 CSR by IL-4. During the sequential switching to IgE, CTε-γ1 was exclusively generated by B cells (Suppl. Fig. 2). Indeed, we found that IMQ diminishes IL-4-induced CTε-γ1 expression in anti-CD40-stimulated B cells (Fig. 2A, left and central panel). We confirmed the sequences of CTε-γ1 using sequencing analysis (Fig. 2C). Additionally, IMQ enhanced anti-CD40/IL-4-induced AID expression, which is consistent with previous studies demonstrating that another TLR7 agonist, RSQ, induces AID expression in human and mouse B cells [28,29]. Taken together, our results suggest that IMQ inhibits IL-4-induced IgE and IgG1 production by the selective suppression of IgE and IgG1 class switching, respectively, and also suppresses sequential switching to IgE through IgG1 CSR.

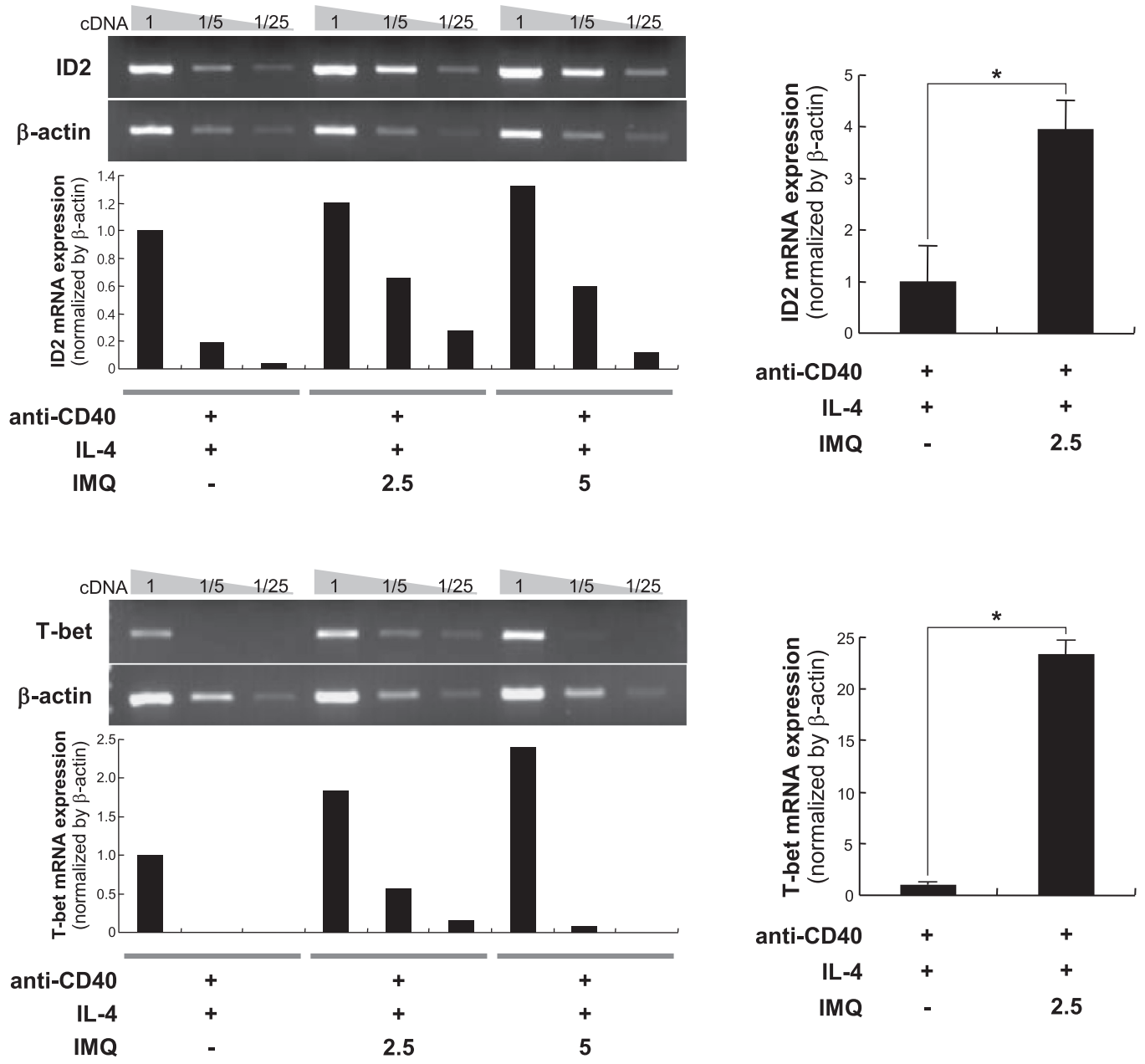

Fig. 5. Effect of IMQ on ID2 and T-bet expressions. Purified resting B cells were stimulated with anti-CD40 Ab (2 μg/ml), IL-4 (10 ng/ml), and IMQ (2.5 or 5 μg/ml). After 6 h of culture, mRNA was isolated, and ID2 and T-bet mRNA expression was analyzed by semi-quantitative RT-PCR (left panel. The graphs show relative ID2 and T-bet cDNA levels normalized to β-actin cDNA expression using ImageJ software [NIH, Bethesda, MD, USA] densitometric analysis) and real-time quantitative RT-PCR (right panel). Data represent means ± SEM of three independent experiments. *$p < 0.05$.

3.3. IMQ-induced suppression of IgE production is not caused by the production of IFN-γ and IL-12

The IgG2a (IgG2c) switching cytokine IFN-γ specifically inhibits IgE and IgG1 production in mouse B cells [30]. IL-12 also suppresses IgE production in IL-4-stimulated human PBMCs [31] while enhancing IgG2a levels in mice [32]. In addition, IMQ induces production of IFN-γ and IL-12 by mouse spleen cells and human PBMCs [5]. To determine if the selective inhibition of IgE production caused by IMQ is related to IFN-γ and IL-12 production in anti-CD40/IL-4-stimulated B cells, we examined Igs production after treatment with neutralizing IFN-γ Ab and determined the levels of IFN-γ and IL-12 (Fig. 3). The anti-IFN-γ neutralizing Ab effectively blocked IFN-γ-induced IgG2c production in anti-CD40-stimulated B cells (Fig. 3A, middle panel). Anti-IFN-γ did not restore the IgE suppression by IMQ and did not block enhanced production of IgG2c by IMQ (Fig. 3A). In addition, IMQ did not induce IFN-γ and IL-12 under the same culture conditions (Fig. 3B). Collectively, these data indicate that IMQ does not induce B cells to produce IFN-γ and IL-12, which would influence IgE and IgG2c production.

3.4. IMQ induces BCL6 expression and represses germline ε transcription in anti-CD40/IL-4-activated B cells

Various transcription factors regulate GLTε and GLTγ1 transcription in B cells [30,33]. Among them, STAT6, NF-κB, E2A, PAX5, AP-1, C/EBPβ, NFIL3, and SWAP-70 positively regulate mouse GLTε promoter activity. On the other hand, BCL6 and ID2 negatively regulate mouse GLTε promoter [34,35]. In particular, BCL6 competes with IL-4-activated STAT6 for binding to the STAT6 sites of the GLTε promoter and specifically represses IL-4-induced GLTε transcription. ID2 binds to E2A

and PAX5 and inhibits their binding to the GLTε promoter. TGF-β1 induces ID2 expression and is involved in the suppression of IgE class switching [35]. Since IMQ suppressed IL-4-induced IgE class switching, we preferentially focused on the role of IMQ in the induction of BCL6 expression. Purified resting B cells were stimulated with IMQ in the presence of anti-CD40 Ab and IL-4, and levels of BCL6 expression were measured by both semi-quantitative RT-PCR and real-time quantitative PCR. As shown in Fig. 4A, IMQ enhanced BCL6 expression in anti-CD40/IL-4-stimulated B cells in a dose-dependent manner. To determine whether IMQ-induced BCL expression is involved in the suppression of GLTε transcription, we tested the effect of BCL6 siRNA transfection on IMQ-mediated suppression of GLTε promoter activity in anti-CD40/IL-4-stimulated B cells. BCL6 siRNA significantly restored the IMQ-mediated suppression of GLTε promoter activity by 63.4% (Fig. 4B, left panel). Under the same transfection and culture conditions, we confirmed that the introduction of BCL6 siRNA effectively abrogated IMQ-induced BCL6 expression in anti-CD40/IL-4-activated B cells (Fig. 4B, right panel). In addition, we observed that IMQ dose-dependently enhanced BCL6 expression and suppresses GLTε expression induced by IL-4 in the human B cell line 2E2 (Suppl. Fig. 3A). IMQ also abrogated IL-4-induced human GLTε promoter activities in a dose-dependent manner (Suppl. Fig. 3B). These results indicate that IMQ inhibits IL-4-induced GLTε transcription though the induction of BCL6 expression in B cells. ID2, another well-known negative regulator of GLTε promoter activity, may also be involved in IMQ-mediated suppression of GLTε transcription. Indeed, IMQ enhanced ID2 expression in anti-CD40/IL-4-stimulated B cells (Fig. 5, upper panel). IMQ-induced upregulation of ID2 inversely correlated with GLTε expression and IgE production, although it remains to be determined if IgE inhibition is dependent on IMQ-induced ID2. The transcription factor T-bet positively regulates GLTγ2a expression/IgG2a class switching and is essential for IgG2a production in mouse B cells [36,37]. Conversely, T-bet-deficient B cells produce excess amounts of IgE and IgG1 [36]. T-bet expression in B cells has been shown to be induced by various stimuli including IFN-γ, IL-12, IL-27, CD40L, CpG, and RSQ [37–40]. As shown in Fig. 5 (lower panel), IMQ enhanced T-bet expression in anti-CD40/IL-4-stimulated B cells. Thus, IMQ-induced T-bet expression may cause both upregulation of IgG2c and downregulation of IgE/IgG1. However, this possibility remains to be elucidated, and other positive IgE regulators (i.e., STAT6, NF-κB, E2A, PAX5, AP-1, C/EBPβ, NFIL3, and SWAP-70) should be evaluated.

In conclusion, our observations show that TLR7 engagement by IMQ suppresses IL-4-induced GLTε transcription through the induction of BCL6 expression, and this, in turn, inhibits IgE class switching and IgE production in anti-CD40-activated B cells. In addition, IMQ inhibits IL-4-induced IgE class switching through the suppression of sequential switching from IgM to IgE via IgG1. ID2 and T-bet are also induced by IMQ and may be involved in IgE suppression and IgG2c induction, respectively, in anti-CD40/IL-4-activated B cells. Consequently, our findings serve as the basis for effectively treating IL-4-induced, IgE-mediated allergic diseases with IMQ, with the B cell TLR7 as a therapeutic target.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2019.02.006.

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