

CD40-mediated p38 mitogen-activated protein kinase activation is required for immunoglobulin class switch recombination to IgE

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Background: Signaling through CD40 activates multiple kinases and signal pathways that drive diverse CD40-mediated biologic functions. The specific pathways activated by CD40 signaling involving CD40-dependent Ig class switch recombination (CSR) have not been defined.

Objective: We sought to dissect CD40-activated signaling required for CD40-mediated Ig CSR by using the specific signal pathway inhibitors, with the emphasis on CD40-activated p38 mitogen-activated protein kinase (p38 MAPK) signaling in CD40-mediated CSR to IgE.

Methods: Human B cells were costimulated with IL-4 plus anti-CD40 in the presence or absence of specific signal pathway inhibitors. Ig production, kinase phosphorylation, IgH ϵ germline transcripts and S μ -Se recombination were examined, and their relationships were analyzed.

Results: CD40-dependent IgE induction was inhibited by the specific p38 MAPK inhibitor SB203580 but not by the extracellular signal-regulated protein kinase-specific inhibitor PD98059 or the phosphatidylinositol 3-kinase-specific inhibitor LY294002. CD40 activation of p38 MAPK correlated with CD40-dependent IgE production, and IgE suppression by SB203580 correlated with the inhibition of CD40-activated p38 MAPK phosphorylation. Suppression of IgE production by SB203580 was not due to inhibition of cell proliferation because SB203580 did not suppress IL-4 plus α -CD40-induced cell proliferation. SB203580, but neither PD98059 nor LY294002, inhibited CD40-dependent S μ -Se recombination, as determined by using a digestion circularization PCR assay. The inhibitory effects of SB203580 on IgE production and S μ -Se recombination were directly related to its ability to suppress production of Ig ϵ germline transcripts.

Conclusion: These results demonstrate that p38 MAPK is required for CD40-mediated class switching to IgE. (*J Allergy Clin Immunol* 2002;110:421-8.)

Key words: B lymphocyte, antibody, gene rearrangement, signal transduction

Abbreviations used

AID:	Activation-induced cytidine deaminase
CSR:	Class switch recombination
DC-PCR:	Digestion circularization PCR
ϵ GT:	Ig ϵ germline transcript
ERK:	Extracellular signal-regulated protein kinase
JNK:	C-Jun-N-terminal kinase
NF:	Nuclear factor
p38 MAPK:	p38 Mitogen-activated protein kinase
PI-3K:	Phosphatidylinositol 3-kinase
TGF:	Transforming growth factor
TRAF:	Tumor necrosis factor receptor-associated factors

CD40 plays a central role in B-cell biology, including aspects of proliferation, differentiation, apoptosis, germinal center formation, memory, VH hypermutation, affinity maturation, and Ig class switching.¹⁻³ Triggering of CD40 results in the recruitment of multiple signal transduction adapter components, most notably the tumor necrosis factor receptor-associated factors (TRAF) 2, 3, 5, and 6,⁴⁻⁷ to the cytoplasmic tail of CD40. Association of these molecules with CD40 recruits additional proteins to form the complex that mediates CD40 signaling. Such signaling results in activation of multiple downstream pathways, including C-Jun-N-terminal kinase (JNK/SAPK1),⁸ p38 mitogen-activated protein kinase (p38 MAPK/SAPK2),⁹ Janus kinase/signal transducers and activators of transcription,¹⁰ phosphatidylinositol 3-kinase (PI-3K),¹¹ Ras¹² and nuclear factor (NF) κ B,¹³ and extracellular signal-regulated protein kinase (ERK).¹⁴ However, the differential involvement of specific CD40-activated signal pathways in distinctive CD40-mediated responses have not been well characterized.

CD40 signaling is required for T cell-dependent Ig class switching.¹⁻³ The importance of CD40 involvement in Ig class switching is seen in CD40 and CD40 ligand mutant mice and patients with hyper-IgM syndrome who have mutations in CD40 ligand and therefore can not switch.¹⁵⁻¹⁷ CD40 could potentially affect Ig class switching at 3 different levels: (1) IgH germline promoter activity^{18,19}; (2) putative switch recombinase activity²⁰; and (3) 3' α -enhancer-mediated activity.²¹ Among these processes, CD40-activated NF- κ B is the only CD40-activated signal pathway demonstrated to be required for CD40-dependent Ig class switch recombination (CSR)²²; the roles of other CD40-activated signal pathways in CSR remain to be determined.

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p38 MAPK is one of the signal pathways driven by CD40 activation.^{5,9,23} CD40-induced gene expression in human B cells is p38 MAPK dependent, including upregulation of intracellular adhesion molecule 1, IL-1 β , and IL-10 production^{5,9,23} and inhibition of DNA synthesis.²⁴ In contrast, CD40-induced CD95/Fas, CD40, cIAP2, TRAF1, TRAF4, and DR3 expression are p38 MAPK independent.²³ This differential involvement indicates that CD40-activated p38 MAPK pathway selectively mediates some CD40-directed biologic functions. Because Ig CSR is CD40 dependent, we used the p38 MAPK-specific inhibitor SB203580²⁵ to block CD40-mediated p38 MAPK activation to test whether p38 MAPK is indeed directly involved in CD40-driven Ig CSR. We demonstrate that induction of IgE is significantly suppressed by SB203580 but not by the ERK-specific inhibitor PD98059 or the PI-3K-specific inhibitor LY294002. IL-4 plus α -CD40-induced Ig ϵ germline transcripts (ϵ GTs) are inhibited by SB203580. The suppression of IgE production by SB203580 is directly correlated with its inhibition of switch recombination. These results indicate that signaling through p38 MAPK pathway activated by CD40 plays an important role in CSR to IgE.

METHODS

Reagents

Recombinant human IL-4, IL-10, and transforming growth factor (TGF) β 1 were purchased from R&D systems (Minneapolis, Minn). Anti-human CD40 mAb G28-5²⁶ was generated and purified from ascites. Affinity-purified rabbit anti-phospho-p38 MAPK (Thr180/Tyr182), JNK, and ERK and anti-p38 MAPK, JNK, and ERK antibodies and horseradish peroxidase-labeled goat anti-rabbit IgG were purchased from New England Biolabs (Beverly, Mass). Anti-human IgD was purchased from Pharmingen (San Diego, Calif). Antibodies for ELISAs were described previously.²⁶ The specific kinase inhibitors SB203580, PD59058, and LY294002 were purchased from Calbiochem Inc (San Diego, Calif).

Cells and cell culture

Human B cells were isolated from healthy blood donors and discarded tonsillectomy tissue with appropriate consent. Isolation and purification of human B cells were performed as described previously.²⁶ Cells were pretreated with SB203580, PD98059, and LY294002 for 30 minutes before stimulation with IL-4 (10 ng/mL), IL-10 (50 ng/mL), α -CD40 (1 μ g/mL), or TGF- β 1 (10 ng/mL). The supernatants were harvested at day 12 for ELISA to measure IgE levels.²⁶ Thymidine incorporation assay to measure cell proliferation-DNA synthesis was performed as previously described.²⁷

Western blot analysis of p38 MAPK phosphorylation in human tonsillar B cells

Human tonsillar B cells were suspended at 1×10^7 /mL and pretreated with either SB203580 (10 μ mol/L), PD98059 (20 μ mol/L), or dimethyl sulfoxide (1%) for 30 minutes at 37°C, followed by stimulation with IL-4, α -CD40, or both for 30 minutes. The cell pellets were lysed with 200 μ L of lysis buffer and sonicated for 3 seconds, and the protein-containing supernatants were collected. Eighty micrograms of protein were loaded for SDS-PAGE and blotted to nylon membrane, as described previously.²⁸ The blots were incubated with 1:1000 anti-phospho-p38 MAPK for 2 hours. After 3 washes, blots were incubated with 1:5000 diluted anti-rabbit

IgG-horseradish peroxidase for 1 hour. An enhanced chemiluminescence (Amersham, Piscataway, NJ) Western blot kit was used to detect the signals, as previously described.²⁸

RT-PCR

Total RNA was isolated from the stimulated cells by using Trizol reagent (Life Technologies, Gaithersburg, Md), according to the manufacturer's instructions. Two micrograms of total RNA was used for reverse transcription in 20- μ L reactions. Three microliters of cDNA were used as DNA templates for PCR amplification of ϵ GTs, unless otherwise stated, with the primers GM3 (5'-AGCT-GTCCAGGAACCCGACAGGGAG-3') and C ϵ 2M (5'-TGGA-CAAGTCCACGTCCATGACC-3'). This pair of primers would amplify a 557-bp fragment for ϵ GTs. PCR was carried out at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute for 30 cycles. Reduced glyceraldehyde-phosphate dehydrogenase was amplified for 25 cycles with 1.5 μ L of cDNA as input DNA templates (986 bp).

Digestion circularization PCR for human S μ -S ϵ recombination

A digestion circularization PCR (DC-PCR) assay²⁹ was established to quantify S μ -S ϵ recombination levels. Genomic DNA was digested with *Bgl*II, followed by a ligation under conditions favoring self-ligation (2 μ g/mL DNA). The resultant ligated DNA was used as a template for PCR amplification with the primers E μ 4 (5'-GATATGCTGTTTGCACAACTAG-3') and DC6 (5'-AACAAACCTC ATGACCACCAGCT-3') at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute for 40 cycles. The size of the PCR product is expected to be 222 bp.

The 222-bp DC-PCR product was cloned into the PCR 2.1 vector (Invitrogen, San Diego, Calif) to create an internal standard for measuring S μ -S ϵ recombination. A 241-bp *Bgl*II-*Bam*HI fragment was inserted into the *Bgl*II site. The resulting plasmid was used as internal standard for quantifying S μ -S ϵ switch frequency because it would simultaneously be amplified by the primers used to amplify S μ -S ϵ switch events but would yield a larger product (463 bp).

A modified DC-PCR protocol was also developed to enhance the sensitivity so that S μ -S ϵ recombination was detectable in donors with low-IgE-producing ability (<5 ng/mL) with the primers E μ 3 (5'-AGAAGTTGATATGCTGTTTGCAC-3') and DC5 (5'-GCAAGAGGAGG GCACACAGAGCTCAGAGA-3') at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute for 20 cycles for first-round PCR. Two microliters of first-round PCR products was used as a DNA template for second-round PCR with the primers E μ 4 and DC6 for 25 cycles with the same PCR amplification protocol as for first-round PCR.

The human activation-induced cytidine deaminase (*AID*) gene³⁰ was used as an unrelated control gene for the DC-PCR assay. *Bgl*II digestion would generate a 4478-bp fragment from the human *AID* gene (EMBL/Genbank accession no. AB040431). Primer pairs AID5 (5'-CCATGGTACAAATCTCAGGACGAATC-3') and AID6 (5'-AGATGGTGAAACCCGCTCTATTAA-3') were used. This pair of primers would amplify a 238-bp product. PCR was conducted with 20 ng of ligated DNA as a template at 94°C for 1 minute, 62°C for 1 minute, and 72°C for 1 minute for 40 cycles.

RESULTS

IL-4 plus CD40-induced IgE production is suppressed by SB203580, a p38 MAPK-specific inhibitor

CD40-activated p38 MAPK signaling is required for some CD40-induced gene expression in B cells.²³ To investigate the role of p38 MAPK in CD40-dependent Ig

CSR in human B cells, we first tested whether CD40-activated p38 MAPK was involved in CD40-dependent CSR to IgE by using the cell-permeable p38 MAPK-specific inhibitor SB203580.²⁵ As summarized in Fig 1, A, IL-4 plus α -CD40-induced IgE production was significantly suppressed by SB203580 at 10 μ mol/L in all 4 donor's PBMCs, with average inhibition rates of over 85%, but not by the ERK-specific inhibitor PD98059³¹ or the PI-3K-specific inhibitor LY294002³² (Fig 1, A). In fact, IL-4 plus α -CD40-induced IgE levels were slightly enhanced by PD98059 at 20 μ mol/L and by LY294002 at 10 μ mol/L (Fig 1, A). As a positive control for IgE inhibition, TGF- β strongly suppressed induced IgE production (Fig 1, A). Titration showed that IgE suppression was SB203580 dose dependent (Fig 1, C), with significant ($P < .001$) IgE suppression achieved at 2.5 μ mol/L. Similar effects on IgE production from the purified human IgD⁺ B cells were also observed (Fig 1, B).

SB203580-mediated Ig suppression does not correlate with inhibition of cell proliferation and DNA synthesis

To determine whether the suppression of SB203580 on induced Ig production was due to the suppression of induced cell growth, DNA synthesis, or both, we analyzed the number of viable cells and DNA synthesis under various culture conditions. As shown in Fig 2, IL-4 plus α -CD40-induced cell proliferation was not significantly inhibited by SB203580 at 10 μ mol/L at days 5 and 12 (Fig 2, A). DNA synthesis at day 5, as determined by means of thymidine incorporation, was also not significantly inhibited in IL-4 plus α -CD40-treated cultures (Fig 2, B), even though the same dose of SB203580 moderately inhibited α -CD40-induced cell growth and DNA synthesis (Fig 2, B, and data not shown). Thus inhibition of IL-4 plus α -CD40-induced cell growth and DNA synthesis could not account for SB203580-driven suppression of the induced Ig production from human B cells.

IgE suppression by SB203580 correlates with the inhibition of CD40-activated p38 MAPK

To test whether the effect of SB203580 on IgE production correlated with its suppression of CD40-driven p38 MAPK activation, we simultaneously assayed the effects of SB203580 on CD40-activated p38 MAPK phosphorylation and IgE production from the same sets of tonsillar B cells. As presented in Fig 3, p38 MAPK was weakly phosphorylated in unstimulated tonsillar B cells (Fig 3, lane 1). α -CD40, but not IL-4, significantly increased phosphorylation of p38 MAPK (Fig 3, lanes 2 and 3). α -CD40-activated p38 MAPK phosphorylation was inhibited by SB203580, with α -CD40-activated phosphorylation decreasing about 4-fold (compare Fig 3, lanes 3 and 7). The basal level of p38 MAPK activity was also inhibited by SB203580 (compare Fig 3, lanes 1 and 2 with 4 and 5). Thus both spontaneous and CD40-activated p38 MAPK activities were sensitive to SB203580 inhibition. α -CD40-induced p38 MAPK phosphoryla-

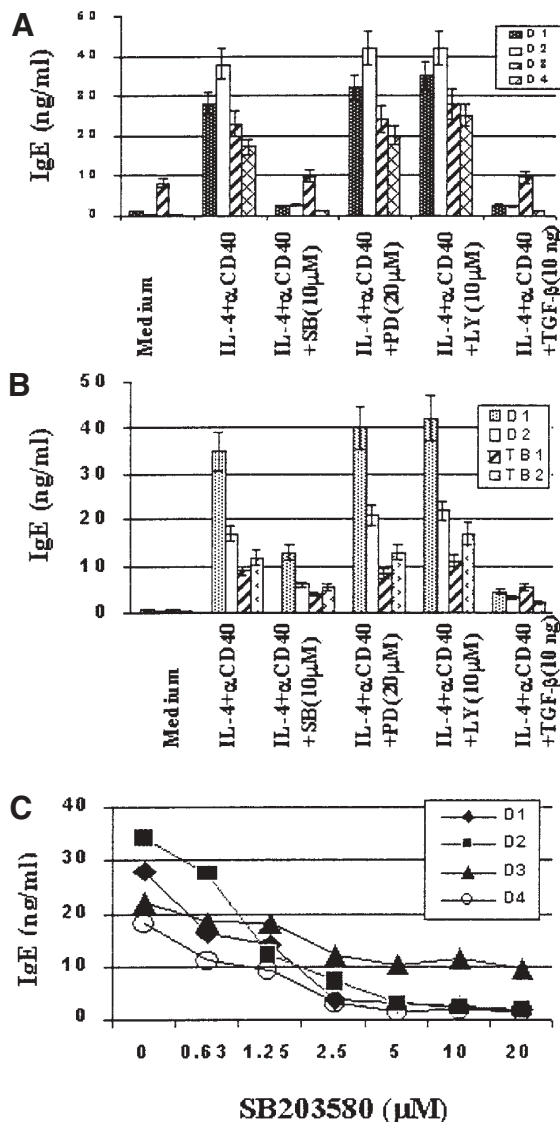


FIG 1. Suppression of IgE by the specific, cell-permeable p38 MAPK inhibitor SB203580. **A**, Suppression of IgE by SB203580 in human PBMCs. PBMCs (1×10^6 /mL) from 4 donors were cultured with 10 μ mol/L SB203580, 20 μ mol/L PD98059, and 10 μ mol/L LY294002 for 30 minutes, followed by culture and stimulation with IL-4 (10 ng/mL) plus α -CD40 (1 μ g/mL) or human TGF- β 1 (10 ng/mL) for 12 days. The supernatants were stored at -20°C , and a group of samples were assayed at same time. The data presented average values from triplicate wells. **B**, Suppression of IgE from the purified human B cells by SB203580. **C**, Dose response of SB203580 on IgE production from PBMCs. Culture conditions were the same as in Fig 1, A, except that SB203580 concentration was different, as indicated. SB, SB203580; PD, PD98059; LY, LY294002.

tion was not inhibited by the ERK-specific inhibitor PD98059 (Fig 3, lanes 9-12). IL-4 plus α -CD40-induced IgE levels (Fig 3, lower panel, lane 4) were inhibited by SB203580 (Fig 3, lane 8) but not by PD98059 (Fig 3, lane 12). JNK was already activated in this tonsil, and SB203580 did not significantly alter the JNK phosphorylation levels (data not shown). Activation

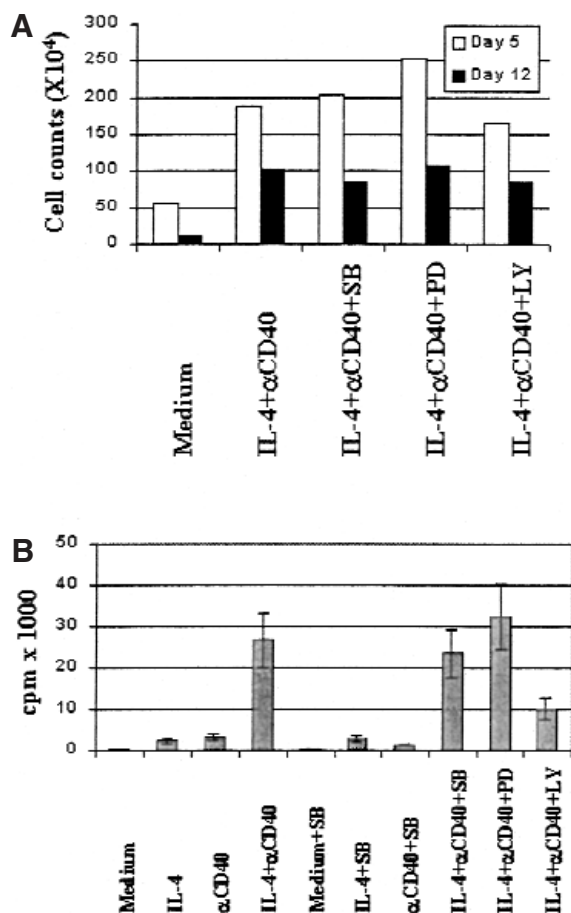


FIG 2. Effects of SB203580 on cell growth and DNA synthesis. **A**, Effects of SB203580 on cell growth. B cells cultured for 5 and 12 days were harvested, and the viable cells were determined by means of Trypan blue exclusion. The numbers represent cell counts from duplicate cultures from one of 3 donors examined at day 5 and day 12. **B**, Effects of SB203580 on DNA synthesis. B cells (1×10^6 /mL) were plated in flat-bottom, 96-well plates (200 μ L/well) in triplicate for 4 days in the appropriate stimulation, as indicated. The cells were pulsed with 1 μ Ci of tritiated thymidine 16 hours before the cells were harvested, and the incorporated radioactivity was counted by means of liquid scintillation, as previously described.²⁷ SB, SB203580; PD, PD98059; LY, LY294002.

of ERK by IL-4, α -CD40, or both was initially undetectable but was inducible by PD98059 in tonsillar B cells (data not shown). These results indicated that CD40-dependent IgE induction correlated with the CD40-activated p38 MAPK phosphorylation, and suppression of IgE correlated with the specific inhibition of CD40-activated p38 MAPK activity by SB203580.

Establishment of a DC-PCR assay for quantification of S μ -Se recombination

To quantitatively measure S μ -Se recombination in human B cells, we established a DC-PCR assay.²⁹ A *Bam*HI-*Bgl*III fragment was inserted into the *Bgl*III site in the cloned DC-PCR products to create a DC-PCR standard. The standard could be simultaneously amplified with the S μ -Se product, yielding a larger product (463

bp; Fig 4, A). No significant competition between the standard and DC-PCR products was observed when the standard was kept at less than 300 copies per reaction (Fig 4, A). However, competition between sample and standard plasmid DNA became significant when the input standard DNA was greater than 300 copies (Fig 4, A). DNA heteroduplex was formed under conditions in which both standard plasmid and S μ -Se products were in relatively higher levels (Fig 4, A). For this reason, the standard plasmid and the DNA samples to be detected were amplified in separate tubes rather than in the same tubes. To monitor the digestion, ligation efficiencies, and amounts of input DNA samples, we used the *AID* gene (EMBL/Genbank accession no. AB040430) as a control.³⁰ *Bgl*III digestion generated a 4478-bp fragment from the human *AID* gene, and the digested and self-ligated *AID* gene yields a 238-bp DC-PCR fragment (Fig 4, B, lower panel).

Preliminary experiments revealed that S μ -Se recombination could not be reproducibly detected by using the PCR protocol when cultured IgE protein levels were less than 5 ng/mL (data not shown). Because SB203580 frequently inhibited IgE to levels less than 5 ng/mL (Fig 1), we further developed an alternative approach to enhance the sensitivity by using a nested DC-PCR with lower amplification cycles for each round of amplification (20 and 25 cycles for first- and second-round PCR, respectively). Those modifications enhanced sensitivity while preserving the quantitative ability of the assay (data not shown) and therefore were used as an alternate when the sensitivity of the regular DC-PCR was not enough for some low-IgE-producing samples.

S μ -Se recombination is blocked by p38 MAPK inhibition

To determine whether the suppression of IgE by SB203580 occurred at the level of DNA switch recombination, we measured S μ -Se recombination by using the DC-PCR assay described above. As shown in Fig 4, B, S μ -Se recombination induced by IL-4 plus α -CD40 was profoundly inhibited by SB203580 but was not significantly altered by PD98059 or LY294002. As a control, S μ -Se recombination was also blocked by TGF- β . The inhibition of S μ -Se recombination by SB203580 was correlated with suppression of IgE (Fig 4, B, lower panel). These results indicate that SB203580 blocks IL-4 plus α -CD40-induced S μ -Se recombination.

SB203580 inhibits IL-4 plus α -CD40-induced ϵ germline promoter-directed IgH ϵ germline transcription

To determine whether α -CD40-activated p38 MAPK is involved in IL-4 plus α -CD40-induced activation of the Ig ϵ germline promoter and resulting ϵ GT production, we used semiquantitative RT-PCR to investigate the effect of SB203580 on IL-4 plus α -CD40-induced ϵ GT production. As shown in Fig 5, A, IL-4 plus α -CD40 induction of ϵ GTs (Fig 5, A, lane 2) was markedly inhib-

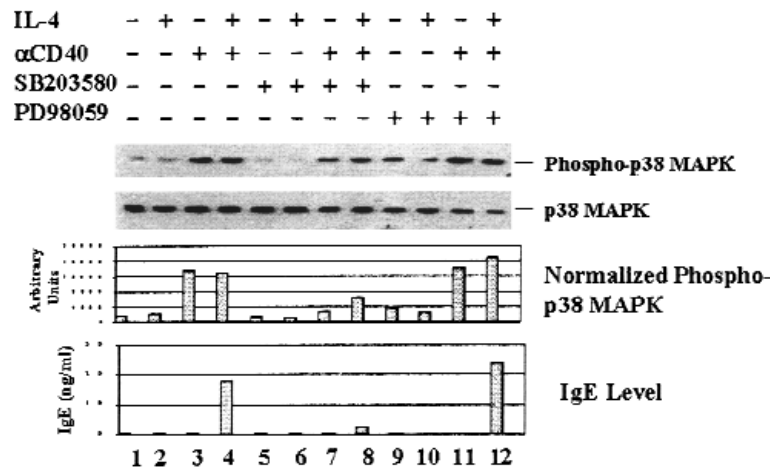


FIG 3. Correlation between the inhibition of induction of p38 MAPK phosphorylation and IgE production by SB203580. Tonsillar B cells ($1 \times 10^7/\text{mL}$) were pretreated with 1% dimethyl sulfoxide and 10 $\mu\text{mol/L}$ SB203580 or 20 $\mu\text{mol/L}$ PD98059 for 30 minutes at 37°C, followed by stimulation with IL-4, α -CD40, or both for 30 minutes. Cell lysates were loaded for detection of phospho-p38 MAPK. The same blot was stripped and reprobed with anti-p38 MAPK as a protein loading control for normalization of the α -CD40-induced p38 MAPK phosphorylation. The IgE from the same donor's B cells was provided for correlation comparison of the effects of SB203580 on p38 MAPK phosphorylation and IgE production.

ited by SB203580 (10- to 30-fold decrease) in tonsillar B cells (Fig 5), B cells from PBMCs, and the B-cell line Ramos 2G6 (Fig 5, A). Neither PD98058 nor LY294002 suppressed ϵ GTs (Fig 5, lanes 4 and 5). As a positive control, TGF- β showed inhibitory effect on ϵ GTs (Fig 5, lane 6). These results demonstrate that IL-4 plus α -CD40-induced ϵ GTs were blocked specifically by SB203580, indicating that CD40-activated p38 MAPK, but not ERK and PI-3K, signaling was required for ϵ germline promoter activation and ϵ GT induction in human B cells.

Suppression of ϵ GTs by SB203580 correlates with $\text{S}\mu$ - $\text{S}\epsilon$ recombination and IgE production

To investigate whether suppression of the IL-4 plus α -CD40-induced ϵ GTs by SB203580 was correlated with the inhibition of $\text{S}\mu$ - $\text{S}\epsilon$ recombination and IgE production, we assayed the effects of SB203580 on ϵ GTs, $\text{S}\mu$ - $\text{S}\epsilon$ recombination, and IgE levels from the same donor samples. As shown in Fig 5, B, induction of ϵ GTs was significantly inhibited by SB203580, which is consistent with the results shown in Fig 5, A. Correspondingly, $\text{S}\mu$ - $\text{S}\epsilon$ recombination and IgE were inhibited by SB203580 but not PD98059 or LY294002. Taken together, these results show that suppression of ϵ GTs by SB203580 was directly correlated with the subsequent inhibition of $\text{S}\mu$ - $\text{S}\epsilon$ recombination and IgE production in human B cells.

DISCUSSION

Signaling through CD40 activates multiple signaling pathways that eventually result in diverse CD40-driven biologic functions. CD40 engagement simultaneously activates multiple kinase activities and signaling path-

ways, including Lyn, Ras, p38 MAPK, JNK, PI-3K, germinal center kinase family member GCK-related kinase, and NF- κ B.^{8-14,33,34} However, the roles of the individual signaling pathway or pathways mediated by CD40 in specific biologic functions remain not well characterized. We used specific kinase inhibitors and assayed progressive steps in IgE isotype switching so as to dissect the signaling pathway or pathways involved in CD40-mediated IgE CSR.

CD40-dependent IgE induction was strongly inhibited by the p38 MAPK-specific inhibitor SB203580 but was not inhibited by the ERK-specific inhibitor PD98059 or the PI-3K-specific inhibitor LY294002. The CD40 activation of p38 MAPK was correlated with CD40-dependent IgE production, and suppression of IgE production by SB203580 correlated with the inhibition of CD40-activated p38 MAPK phosphorylation. By using a DC-PCR assay, SB203580 was shown to potently inhibit CD40-dependent $\text{S}\mu$ - $\text{S}\epsilon$ recombination. These inhibitory effects of SB203580 on IgE production and $\text{S}\mu$ - $\text{S}\epsilon$ recombination were correlated with the ability of SB203580 to suppress ϵ GTs. Taken together, these results strongly support the proposal that the CD40-activated p38 MAPK pathway is directly involved in CD40-mediated CSR to IgE. However, demonstration that CD40-activated p38 MAPK is absolutely required for IgE class switching will need to be proven by the reagents with absolute specificity for p38 MAPK, such as a dominant negative p38 MAPK, because SB203580 could potentially inhibit other signal pathways that are required for IgE CSR.

The fact that IL-4 plus α -CD40-induced ϵ GTs were strongly suppressed by SB203580 indicates that p38 MAPK is required for CD40-dependent ϵ germline transcription. It is reasonable to posit that the inhibition of the CD40-dependent ϵ germline transcription and ϵ GTs is related to the subsequent suppression of $\text{S}\mu$ - $\text{S}\epsilon$ recom-

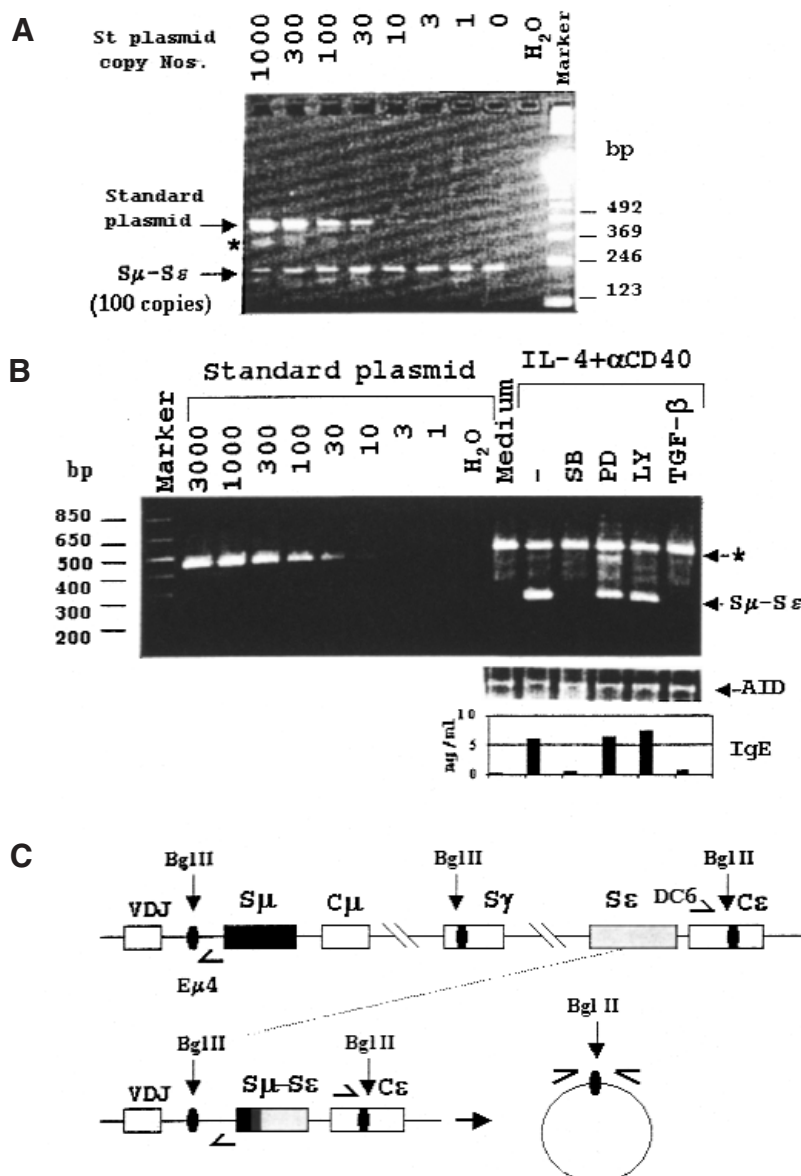


FIG 4. Quantification of Sμ-Sε recombination by means of DC-PCR. **A**, DC-PCR standardization with standard plasmid for quantification of Sμ-Sε recombination. The serially diluted standard plasmid was mixed with a fixed-copy number of Sμ-Sε DC-PCR product (100 copies) for simultaneous amplification. Heteroduplex formation is indicated by an asterisk. **B**, Effects of SB203580 on Sμ-Sε recombination, as determined by means of DC-PCR. The standard plasmid is shown at left, whereas the DNA samples are shown at right. The asterisk identifies nonspecific amplification products that occurred with the nested PCR. In this case a nested DC-PCR was used with 100 ng of ligated DNA as an input template, with the primers Eμ3 and DC5 for first-round PCR (20 cycles), followed by second-round PCR (25 cycles) with the primers Eμ4 and DC6. The ligated AID DNA (20 ng) was amplified as DNA input control. The IgE levels from the same donors' B cells were also included for comparison. SB, SB203580; PD, PD98059; LY, LY294002. **C**, Diagram of the DC-PCR strategy for quantifying human Sμ-Sε recombination.

ination and IgE production because both germline transcription process and GTs were required for Ig CSR.³⁵ A critical role of p38 MAPK in activation of the ε germline promoter also has been recently reported.³⁶

The role of p38 MAPK in Ig CSR might well go beyond regulating germline transcription. CD40 engagement activates multiple events required for Ig CSR, including IgH germline transcription and GTs,^{18,19} the

putative switch recombinase,²⁰ and even the 3' Ig α-enhancer activity.²¹ p38 MAPK might well also be involved in these activities. Thus inhibition of Ig CSR through blocking p38 MAPK might also be contributed to by inhibition of the switch recombinase-mediated function, 3' Ig α-enhancer-mediated function, or both. p38 MAPK-dependent NF-κB should play a role in IgE CSR because NF-κB is required at least for ε germline

transcription and 3' Ig α -enhancer activities.^{36,37}

Suppression of Ig production, ϵ GTs, and S μ -S ϵ recombination by SB203580 all correlated with inhibition of p38 MAPK activity but not with inhibition of cell proliferation. Our results demonstrated that under the IL-4 plus α -CD40 stimulation, cell proliferation was not significantly suppressed by SB203580 at concentrations that strongly inhibited IgE CSR. SB203580 suppressed CD40-enhanced B-cell proliferation but potentiated anti-IgM-activated p38 MAPK-mediated cell proliferation.²³ The distinction between SB203580 effects on α -CD40-versus IL-4 plus α -CD40-mediated cell proliferation suggested that either IL-4 plus α -CD40-induced cell proliferation was not susceptible to the inhibition of SB203580 or the suppression of the α -CD40-promoted cell proliferation was offset by IL-4-promoted cell growth.

Overall, by using the specific inhibitors of p38 MAPK and PI-3K to quantitatively test steps in isotype switching, we demonstrated that CD40-activated p38 MAPK, but not PI-3K, was directly involved in and required for IgE class switching. The role of other CD40-activated kinases or signaling pathways in IgE class switching (eg, JNK, germinal center kinase family member GCK-related kinase, or both), however, remains to be tested when the specific inhibitors for these pathways become available.

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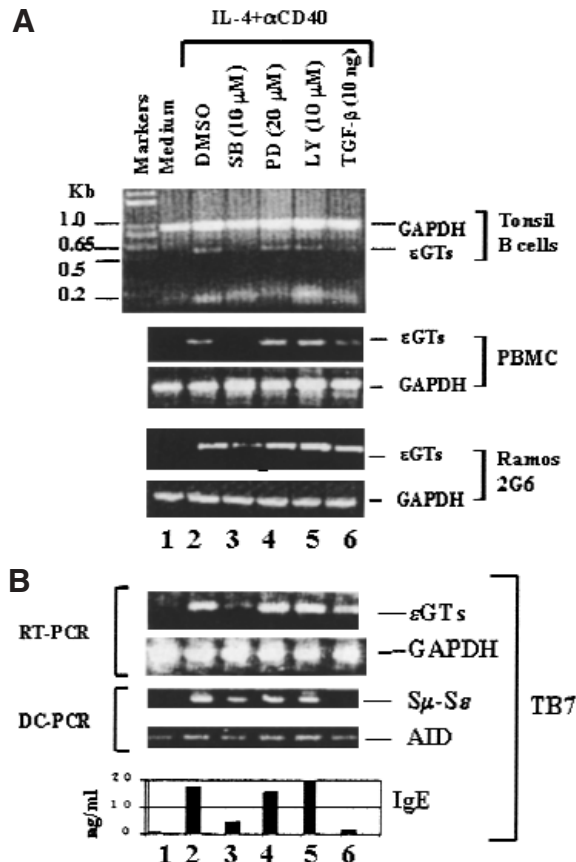


FIG 5. Effects of SB203580 on ϵ GTs. **A**, Inhibition of the induced ϵ GTs from human B cells by SB203580. Human tonsillar B cells, PBMCs, and the B-cell line Ramos 2G6 were cultured with SB203580 (10 μ M/L), PD98059, LY294002, or TGF- β 1 for 30 minutes at 37°C, followed by stimulation with IL-4 plus α -CD40 for 48 hours. **B**, Correlation between inhibition of ϵ GTs by SB203580 with S μ -S ϵ recombination and IgE production from primary B cells. RNA from the B cells stimulated as indicated for 48 hours was used for RT-PCR with the conditions described in Fig 5, A, above. DNA from cells stimulated for 5 days was used for DC-PCR. An aliquot of cells from the same donors under the same conditions was cultured for 12 days for measurement of IgE. Data are representative of 3 independent experiments with 3 donors. SB, SB203580; PD, PD98059; LY, LY294002; GAPDH, reduced glyceraldehyde-phosphate dehydrogenase.

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