Original Article
NF-κB and STAT6 signaling transduction pathways in immunoglobulin E production of U266 cell

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Abstract: Immunoglobulin E (IgE) mediates type I hypersensitivity reactions in several allergic disorders. Thus, understanding the signaling pathways regulating IgE production may provide novel therapeutic targets for allergic disorders. In the present study, the roles of the transcription factors NF-κB and signal transducer and activator of transcription 6 (STAT6) in the production of IgE were investigated in vitro. Using quantitative real-time polymerase chain reaction, Western blotting, and enzyme-linked immunosorbent assays, we found that the U266 cell stimulated with the cytokine interleukin 4 (IL-4) or an antibody to cluster of differentiation 40 (CD40), a protein located on antigen-presenting cells that is required for their activation, promoted the production of IgE. The IgE production was markedly reduced by exposure to the STAT6 inhibitor A177-1726 or to the NF-κB-specific inhibitor JSH-23. In conclusion, we found that the anti-CD40/CD40 pathway and IL-4/IL-4 receptor signaling played important roles in the inducible production of IgE. These results may provide a new direction and targets for the treatment and prevention of allergy.

Keywords: IgE, IL-4, anti-CD40, B cell, signaling pathway

Introduction
The prevalence of allergy disorders has markedly increased in recent decades and is increasingly reaching near epidemic proportions across the globe [1]. Despite this high prevalence, clinical therapy for allergic diseases involves merely controlling the symptoms, rather than treating the disease, and recent research is devoted more to therapy than to prevention. For instance, nutritional factors, such as fatty acids, have been demonstrated to inhibit immunoglobulin E (IgE) production by directly interfering with signal transducer and activator of transcription 6 (STAT6) and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway in human B cells [2]. Moreover, another compound, omalizumab, effectively neutralizes IgE in the treatment of some allergic disorders, such as urticaria and allergic asthma [3].

It is well known that IgE, as the dominant effector Ig, mediates anaphylaxis in type I allergy. The production of IgE requires class-switch recombination, and this isotype switching to IgE production can be measured by determining the levels of epsilon-germline transcription (εGLT) and of activation-induced deaminase (AID) expression. Two different paths induce class switching to IgE, with the signaling pathways of IgE class switching involving cytokines, such as interleukin (IL)-4 and IL-13, and the tumor necrosis factor receptor family member cluster of differentiation (CD) 40 [4]. The binding of IL-4 to the IL-4 receptor (IL-4R) activates the cytoplasmic protein tyrosine kinases JAK1/JAK3, and after that, STAT6 is phosphorylated. The interaction of T-cell-expressed CD40 ligand (CD40L) with the CD40 expressed by B cells can accelerate the activation of a mitogen-activated protein kinase, which activates inhibitor of κB (IkB) kinase, resulting in the phosphorylation of IkB protein. This phosphorylation results in the dissociation of IkB from NF-κB, which can now migrate into the nucleus, bind the relevant DNA loci, and regulate the transcription of many genes. The stimulation of IL-4 and CD40 promotes the synergistic enhancement of εGLT and the expression of AID.
Although much is currently known, further elucidation of the signaling pathways mediating allergy may provide novel therapeutic targets for allergic diseases. Thus, the present study investigated the main signaling pathways in B cells.

**Materials and methods**

**Cell culture**

The IgE-producing human U266 myeloma cell line has been used for screening pharmaceutical agents for IgE stimulation and inhibitory effects. The U266 cell line was cultured at 37°C under 5% CO₂ in RPMI1640 culture medium containing 1% penicillin-streptomycin and 10% fetal bovine serum (Gibco, USA). Cells were grown at an initial concentration of 1 × 10⁶ cells/mL. Unless otherwise stated, cells were stimulated with an anti-CD40 antibody (Bio-Legend, USA) at a concentration of 2 μg/mL and IL-4 (PeproTech, USA) at 50 ng/mL to induce IgE synthesis.

**Chemical inhibitors, western blotting, and antibodies**

The STAT6 inhibitor A177-1726 and the NF-κB-specific inhibitor JSH-23 were purchased from Selleckchem and used at various concentrations. The antibodies used in Western blotting included anti-STAT6 (Abcam, USA), anti-p-STAT6 (Cell Signaling Technology), and anti-p65 (Abcam, USA).

**Enzyme-linked immunosorbent assay**

The U266 cells were stimulated with various concentrations of IL-4 and anti-CD40 antibody. Three days later, the supernatants were harvested, and IgE levels were determined using an enzyme-linked immunosorbent assay kit (Abcam, USA). Each experiment was repeated at least three times.

**Western blotting**

Cell lists were extracted using RIPA buffer containing protease inhibitors. After measuring the concentrations of the extracted proteins, equal amounts of protein in each group were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidenedifluoride membranes. Blocking was conducted through a 2-h incubation with 5% non-fat dry milk or bovine serum albumin in TBS with 0.1% Tween 20 at room temperature. The membranes were incubated overnight with primary antibodies against p-STAT6 (diluted 1:1,000), p65 (diluted 1:1,0000), and GAPDH (diluted 1:1,0000) at 4°C. After incubating with appropriate secondary antibodies conjugated with horseradish peroxidase (diluted 1:1,0000), the bands were visualized using enhanced chemiluminescence substrate (ECL). Each experiment was performed at least three times.

**Real-time polymerase chain reaction (RT-PCR)**

Quantitative RT-PCR was used to detect εGLT mRNA expression. The U266 cells (1 × 10⁶ cells/mL) were stimulated with IL-4 and an anti-CD40 antibody at 37°C under 5% CO₂ for 3 days. The cells were harvested, and total RNA was isolated using an RNA extraction kit (Magen, R4130-02) in accordance with the manufacturer’s instructions of the kit. The following primer sequences for εGLT were used: forward, CCCTCAACGGGACAACATCTG; reverse, TCTGTGGACGATGAGTGTG. Each experiment was repeated at least three times. The primer sequences for GAPDH were used: forward, CAGGAGCATTGCTGATGAT; reverse, GAAGGCTGGGGGTTCGATT.

**Cell viability**

Cell viability was evaluated using the CCK8 assay (Dojindo Laboratories, Tokyo, Japan). For CCK8 assay, U266 cells were seeded in 96-well plates at 1 × 10⁴ cells/ml/well and were allowed to grow to 70%-80% confluency and the cells were treated with different concentrations of inhibitor for 24 h. Treated cells were incubated with CCK8 for 4h at 37°C. The absorbance was measured at 450 nm. The relative amount of inhibition of cell growth was calculated as follows: cell viability (%) = (A sample - A blank)/(A control - A blank) × 100%.

**Statistical analysis**

The statistical analyses of all results obtained in the present study were performed using SPSS version 16.0. Comparisons among all groups were conducted with one-way analysis of variance (ANOVA). When statistical significance was found, Student’s t tests were performed. Values of P less than 0.05 were regarded as statistically significant.
**Results**

*IL-4/anti-CD40 induces IgE production in the U266 cell line*

To determine the optimal concentrations of IL-4 and anti-CD40 antibody for IgE production, U266 B cells were incubated with or without IL-4 (0, 10, 20, 50, and 100 ng/mL) and the anti-CD40 antibody (0, 0.5, 1.0, and 2.0 µg/mL) for 3 days. The levels of IgE in the supernatant and mRNA expression of εGLT were high following stimulation with IL-4 at 50 ng/mL or 100 ng/mL in the presence of anti-CD40 at 2 µg/mL (P<0.05; **Figure 1**). Therefore, we selected concentrations of 50 ng/mL IL-4 and 2 µg/mL anti-CD40 for the following experiments to determine the key roles of the IL-4 and CD40 signaling pathways in the production of IgE.

*STAT6-mediated production of IgE*

To investigate the effects of STAT6 on IgE production, we selected the p-STAT6 inhibitor A177-1726, which inhibits the activity of tyrosine kinase and pyrimidine nucleotide biosynthesis as well as the tyrosine phosphorylation of JAK and STAT6. As described previously, cell viability was evaluated using the CCK8 assay (Dojindo Laboratories, Tokyo, Japan). We deter-
NF-κB and STAT6 associated with IgE

mined that the cell viability decreased to some extent when the concentration of the inhibitor increased. The inhibitor was tested at concentrations of 0, 10, 15, 20, and 25 µM, and we found that when the concentration of A177-1726 was at 10 µM, the IgE production and εGLT mRNA expression in B cells was suppressed (P<0.05) and the cell viability was about 80%. Besides, as the concentration of the inhibitor increased, the inhibitory was as well as increased (Figure 2).

**NF-κB-mediated production of IgE**

We suppressed another signaling pathway in the production of IgE with the NF-κB-specific inhibitor JSH-23, which obstructs the nuclear localization of NF-κB-p65. JSH-23 at a concentration of 20 µM had no significant inhibitory effect on cell growth (Figure 3A). Thus, we selected 20 µM as the concentration in the next experiment. In this study, we determined that exposure to 6 and 12 h of JSH-23 significantly decreased IgE production and εGLT mRNA expression, and that exposure to 12 h markedly reduced NF-κB-p65 protein expression (all P<0.05; Figure 3).

**Discussion**

Generally, class switching at the C region of the IgH locus is determined by the yields of germ-line transcription [4]. Compared with plasma levels of IgG and IgM isotypes, those for the IgE isotype are generally lower [5, 6]. However, in some allergic disorders and in some parasitic diseases, plasma IgE levels markedly increase, indicating that class switching is tightly regulated. Therefore, our understanding of class switching regulation is critical for the treatment and prevention of such allergic disorders [4].

The production of IgE is regulated by the interaction of T and B cells via CD40L/CD40 and cytokines, such as IL-4 or IL-13. Previous studies have shown that IL-4 and anti-CD40 antibodies induce IgE production in peripheral blood mononuclear cells in patients with several allergic disorders [7, 8]. However, the optimal concentrations of anti-CD40 and IL-4 varied among these studies. We observed here that the production of IgE and the mRNA expression of εGLT were highest when the cells were stimulated with IL-4 at 100 ng/mL plus anti-CD40 antibody at 2 µg/mL.
The production of IgE requires class-switch recombination, which involves a series of molecular mechanisms [9]. CD40L activates the transcription factors of the NF-κB family, and then IκB kinase is activated, leading to the degradation of IκBα and IκBβ. This degradation leads to the release of NF-κB-p50 and NF-κB-p65, which translocate to the nucleus to induce the transcription of target genes [10, 11]. The cytokine IL-4 is indispensable for IgE transcription and synthesis [12]. Binding of IL-4 to its receptor IL-4R recruits the Janus kinases (JAK1/JAK3), which promote the phosphorylation and nuclear localization of STAT6 [13, 14]. The process involves two transcription factors, NF-κB and STAT6, which bind to the Cε and Cy1 promoters, driving the expression of Cε and Cy1 GLT, and synergize to drive εGLT expression in both human and mouse B cells [15, 16].

A177-1726, the active metabolite of leflunomide, inhibits the activity of tyrosine kinase and pyrimidine nucleotide biosynthesis and inhibits the tyrosine phosphorylation of JAK and STAT6 [17]. Consistent with these actions, we found that A177-1726 was effective in suppressing IgE production in U266 cells, with an IC50 of 22.48 µM. We also found that when the concentration of A177-1726 reached to 10 µM, the IgE production and εGLT mRNA expression in B cells was suppressed (P<0.05) and the cell viability was about 80% at this time; Otherwise, we found that IgE production was markedly suppressed at 25 µM of A177-1726, which may attribute to the cytotoxicity of the inhibitor.

Using RT-qPCR, we also found that A177-1726 specifically blocked IgE class switching by disrupting epsilon germline gene transcription. Clinically, leflunomide is used to treat rheumatoid arthritis. However, it may also reduce the level of IgE in the plasma of some patients with allergic disorders or diseases. JSH-23, an inhibitor of the NF-κB signaling pathway, inhibits the nuclear translocation of NF-κB-p65. In the present study, adding JSH-23 to U266 B cells for 1 h followed by stimulation with anti-CD40 plus IL-4 for 0, 6, and 12 h markedly reduced...
NF-κB and STAT6 associated with IgE

IgE production. Together, these findings indicated that activation of NF-κB was selective for IgE production. The RT-PCR results showed that the inhibitor reduced εGLT expression, indicating that the inhibition of the anti-CD40- and IL-4-induced IgE production was attributable to altered εGLT expression.

Some reports have indicated that IgE isotype switching requires sustained exposure of B cells to IL-4 for at least 72 h, which suggests that sustained activation of STAT6 may also be necessary for transcription at the Cε locus. We stimulated the cells using anti-CD40 plus IL-4 for at least 3 days. The required sustained activation of NF-κB and STAT6 may indicate that a lag period is needed for transcription at the Cε locus [18].

In conclusion, we sought herein to understand the regulation of IgE production by investigating the molecular and cellular pathways that generate IgE to provide future directions for exploring novel treatments in allergic disorders.

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Disclosure of conflict of interest

None.

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References

NF-κB and STAT6 associated with IgE
