



BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Pharmacologic Inhibition of I κ B Kinase Activates Immediate Hypersensitivity Reactions in Mice

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Pharmacologic inhibitors of I κ B kinase (IKK), especially IKK- β , have been developed to treat inflammatory diseases. However, their interactions with components of the NF- κ B pathways are not fully known in allergic diseases. To examine whether IKK is involved in immediate hypersensitivity reactions and to determine whether counterregulatory mechanisms in the NF- κ B activation system were active, we examined the role played by IKK components on mast cell degranulation using a murine ocular immediate hypersensitivity reaction model. Pharmacologic inhibition of IKK in mice caused paradoxical aggravation of the mast cell-mediated immediate hypersensitivity reaction and up-regulation in the expression of inflammatory cytokines. Downstream analyses showed that B-cell deficiency or treatment by IL-1 receptor antagonist corrected the aberrant activation of tissue-resident mast cells, which would indicate contribution by activated B cells. Analyses of co-cultures of tissue-resident mast cells showed the contribution of activated B cells to activation of mast cells and secretion of inflammatory cytokines. Aberrant activation of the NF- κ B promoter in isolated B cells was induced exclusively by IKK- β inhibition and was negated by ablating IKK- α . Aggravated mast cell degranulation by pharmacologic IKK inhibition in the murine immediate hypersensitivity reaction was corrected by B-cell-targeted inhibition of IKK- α . Thus, IKK- β limits B-cell-mediated mast cell activation and inflammatory cytokine induction in immediate hypersensitivity by counterbalancing the activity of IKK- α . (*Am J Pathol* 2013, 183: 96–107; <http://dx.doi.org/10.1016/j.ajpath.2013.04.006>)

The NF- κ B cascade canonically organizes and executes proinflammatory transcriptional programs. Therefore, strategies that target the inactivation of NF- κ B have been gaining considerable interest in treating inflammatory diseases. The damage to tissues in various models of inflammatory diseases, including murine autoimmune encephalomyelitis, inflammatory colitis, and ischemia-induced neuronal death, has been shown to be less when NF- κ B was blocked.^{1–5} Thus, the inhibition of NF- κ B is believed to protect tissues against the damages induced by inflammatory diseases.^{6–8} However, the results of recent studies have been contradictory, which may be because of the multifaceted roles of NF- κ B signaling.^{9–11}

The NF- κ B family consists of five transcription factors—p50, p52, p65, c-REL, and RelB—that are all regulated by I κ B proteins. In unstimulated cells, NF- κ B is associated with I κ B proteins and is sequestered in the cytoplasm. When stimulated, I κ B is phosphorylated, ubiquitinated, and degraded

in the proteasome. NF- κ B is then released and translocated into the nucleus, where it can activate promoters.

The activation of NF- κ B is controlled by the I κ B kinase (IKK) complex, which mediates the phosphorylation of I κ B. The IKK complex is the centerpiece of the NF- κ B pathway and is composed of two catalytic subunits, IKK- α and IKK- β , and a regulatory subunit, IKK- γ . The major activity of IKK is mediated by IKK- β , which coordinates the classical arms of the two distinct NF- κ B activation pathways.

Many IKK- β inhibitors have been discovered or synthesized that alleviate inflammatory diseases. In contrast, the alternative NF- κ B pathway, which is mediated by the activation of p52/RelB target genes by IKK- α , has generated little interest as a target for pharmacologic intervention. The

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alternative NF- κ B pathway is typically activated by lymphotoxin, CD40, or B-cell activating factor (BAFF), which function as homeostasis factors in lymphogenesis and survival of B cells.

The recent findings of NF- κ B-related responses have shown significantly different outcomes. NF- κ B activates immune cells in response to environmental stresses, infection, and injury, and a blockade of NF- κ B seems to protect tissues from damage.³ In nonimmune cells, such as epithelial cells, parenchymal cells, and stromal cells, the classical NF- κ B pathway protects the tissue integrity against environmental stresses and plays a homeostatic role.^{12–14} Depending on the responding cells or tissues, the output of the NF- κ B pathway is very different, and the potential adverse effects of NF- κ B inhibition can be damaging.^{15–17}

Processes of immediate hypersensitivity reaction are involved in a variety of allergic diseases and can be manifested as an anaphylactic shock syndrome in the extreme. The ocular form of immediate hypersensitivity reaction is manifested as allergic conjunctivitis, and it can be vision threatening in the severe forms.¹⁸ The disease symptoms depend on IgE-mediated degranulation of mast cells in the tissues.^{19,20} Its vision-threatening severe form, vernal keratoconjunctivitis, requires strong immune suppressant therapy for management, such as tacrolimus or a systemic corticosteroid.¹⁸

Fc ϵ RI stimulation of mast cells activates kinases, phosphatases, and GTPase, which leads to the degranulation and production of cytokines and chemokines.²¹ Besides classical Fc ϵ RI activation, mast cells require costimulation signals. For example, there has been accumulating evidence to show crosstalk between Fc ϵ RI and CC chemokine receptor signaling.^{21,22} Without proper input from CCR1, CCR2, and CCR3, mast cell activation is severely impaired.^{19,23–25} Their ligands or other putative activators are supplied by mast cells themselves or by accumulated leukocytes in allergic inflammatory tissues. Because targeting of these mediators can be the basis of novel therapeutic approaches for allergic inflammatory diseases, we analyzed the transcriptome of ocular immediate hypersensitivity reaction activated by *CCR3 in vivo*.²³ Bioinformatic analysis suggested significant involvement of IKK and the NF- κ B cascade in the inflammatory network.

Thus, the purpose of this study was to analyze the effects of the pharmacologic inhibition of IKK on mast cell-mediated immediate hypersensitivity reactions in a murine allergic conjunctivitis model. The present results show that inhibition unexpectedly resulted in significant exacerbation of the immediate hypersensitivity reaction and massive expression of systemic inflammatory cytokines.

Materials and Methods

Animals

C57BL/6 background mice homozygous for the *Ighm*^{tm1Cgn} targeted mutation were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used as B-cell-deficient

mice. SWR/J mice were also obtained from The Jackson Laboratory. The procedures used conformed to all the regulations for laboratory animal research outlined in the Animal Welfare Act, the NIH guideline, and the Association for Research in Vision and Ophthalmology statement on the experimental use of animals.

Induction of Acute Allergic Inflammation in Mouse Eyes

For active immunization, mice were sensitized with ragweed pollen allergen using a protocol previously reported in detail.^{24–26} Briefly, a suspension of 50 μ g of ragweed pollen (ICN, Aurora, OH) and 1 mg of aluminum hydroxide (Sigma-Aldrich, St. Louis, MO) was injected into the left hind footpad of mice under general anesthesia. Acute allergic inflammation was induced 6 weeks later by topical application of 1.5 mg of ragweed suspended in 10 μ L of PBS. Control mice were mock sensitized and challenged identically with the ragweed suspension. For passive immunization, mice were injected i.v. with 3.5 μ g per mouse of monoclonal ragweed-specific IgE.²⁷

The allergen-induced immediate hypersensitivity reaction was histologically evaluated by counting the number of degranulated mast cells under the eyelid skin 90 minutes after the allergen challenge. The mice were sacrificed, and the tissues were fixed in 4% paraformaldehyde and then embedded in paraffin. Three-micrometer-thick serial sagittal sections were cut and stained with Giemsa. Three consecutive sections were examined, and the numbers of intact and degranulated mast cells under the eyelid skin were counted under a $\times 200$ microscope field by a masked observer.

Inhibition of IKK Activity in Allergic Inflammation

An IKK inhibitor peptide (Merck, Darmstadt, Germany)²⁸ was used to block the IKK activity. The IKK inhibitor peptide contained a sequence corresponding to the active I κ B α phosphorylation recognition sequence. An IKK inactive control peptide (Merck) containing a mutated recognition sequence of I κ B α was used as control. To inhibit IKK activity, mice were injected i.v. with 20 μ g per mouse of IKK inhibitor peptide 1 day before the allergen challenge. For the inhibition of I κ B α degradation, mice were injected i.p. with 40 μ g per mouse of isohelenin (Merck).

To block IL-1, 700 μ g per mouse of IL-1 receptor antagonist (IL-1RA), BB-loop Mimic hydrocinnamoyl-L-valyl pyrrolidine TIR domain mimic (Merck) was injected i.p. 1 day before and the day of the allergen challenge. To selectively inhibit IKK- β , an IKK- β inhibitor, VIII (2-Amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-(4-piperidinyl)-3-pyridinecarbonitrile; Merck), was used.

For *in vivo* B-cell transfection by siRNA, IKK- α (SI00951328; Qiagen, Valencia, CA) or control siRNA was conjugated with anti-B220 antibody (Clone RA3-6B2; R&D Systems, Minneapolis, MN) using a T3 conjugation

kit (Bioo Scientific, Austin, TX). The immunized mice were i.v. injected with siRNA-conjugated anti-B220 antibody on day 1, treated with IKK inhibitor peptide on day 2, and challenged with the allergen on day 3.

ELISA

The levels of IL-1 β and CCL2 (MCP-1) were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (ELISA Max Set; BioLegend, San Diego, CA), according to the manufacturer's instructions. The levels of caspase 1 and IL-13 were measured using commercial ELISA kits (AdipoGen, San Diego, CA, and eBioscience, San Diego, CA, respectively). To screen for the induced cytokines, a cytokine array analysis kit (Q-Plex array; Quansys Biosciences, Logan, UT) was used.

Isolation of Tissue-Resident Mast Cells and β -Hexosaminidase Assay

The eyelid and skin of 8- to 12-week-old mice were enzymatically digested in RPMI 1640 medium supplemented with 10% fetal bovine serum, 0.5 mg/mL of hyaluronidase (Sigma-Aldrich), 1.5 mg/mL of collagenase (Nitta Gelatin, Osaka, Japan), and 0.5 mg/mL of DNase I (Sigma-Aldrich) for 3 hours at 37°C as reported.^{20,24,25} The dispersed cells were layered onto Percoll density medium (density 1.041; Amersham Pharmacia Biotech, Piscataway, NJ) and were centrifuged at $800 \times g$ for 20 minutes. The collected cell pellet was cultured in RPMI 1640 medium with 10 ng/mL of stem cell factor (PeproTech, Rocky Hill, NJ), 10 ng/mL of IL-3 (PeproTech), and 5% serum for 6 days. Lineage marker-positive cells and plasmacytoid dendritic cells were depleted using rat anti-CD4, rat anti-CD8a, rat anti-B220, and rat anti-CD11b (all from eBioscience) and rat anti-PDCA-1 (Miltenyi Biotec, Auburn, CA).^{20,24,25}

β -Hexosaminidase Assay

Isolated tissue-resident mast cells were sensitized by incubating them overnight in anti-2,4-dinitrophenol (DNP) IgE (SPE-7; Sigma-Aldrich) in 10 ng/mL of RPMI 1640 medium containing recombinant IL-3, 10 ng/mL of stem cell factor, and 5% murine serum.^{20,24,25} The cells were washed and stimulated with DNP-albumin (Sigma-Aldrich).

The β -hexosaminidase activity of the culture supernatant was determined after 45 minutes using a published protocol.^{20,24,25} Briefly, supernatants were mixed with 2.5 mmol/L p-nitrophenyl-N-acetyl- β -D-glucosaminide (Sigma-Aldrich) in 0.04 mol/L citrate buffer (pH 4.5) at 37°C. The reactions were terminated by the addition of 0.4 mol/L glycine (pH 10.7). The density of the colored product was measured at 405 nm with a reference filter of 570 nm. The relative release of β -hexosaminidase was defined as the activity in the supernatant divided by the activity in the positive control cell supernatant multiplied by 100. Compound 48/80 (Sigma-Aldrich) stimulus was used for the assay control.

ChIP Assay

Chromatin immunoprecipitation (ChIP) assays were performed using a ChIP assay kit (Cell Signaling Technology Inc., Danvers, MA). In brief, chromatin from cross-linked B cells was digested with micrococcal nuclease and fragmented by sonication. The fragmented chromatin was incubated overnight with anti-IKK- α (Santa Cruz Biotechnology, Santa Cruz, CA), IKK- β antibodies (Cell Signaling Technology Inc.), or control IgG, followed by incubation with protein G magnet beads. After reversing the protein-DNA cross-links, the DNA was purified using a DNA purification spin column. Precipitated DNAs were analyzed for their association with IKK- α and IKK- β with *I κ B α* promoter by real-time PCR. The following promoter-specific primers and probe were used to amplify a 66-bp region in the mouse *I κ B α* promoter: the primers 5'-CTCGTCCTCCACTGAAGC-3' and 5'-GCTCATCGCAGGGAGTTTC-3' and the FAM-labeled probe 5'-CCAGGGCC-3' (Universal Probe Library; Roche, Basel, Switzerland).

Transient Transfection and Luciferase Assays

To measure NF- κ B promoter activity, B cells were separated by negative selection using a B-cell isolation kit (Miltenyi Biotec). The cells were transfected with 2 μ g of NF- κ B reporter plasmid, a mixture of inducible NF- κ B-responsive firefly luciferase constructs that constitutively express

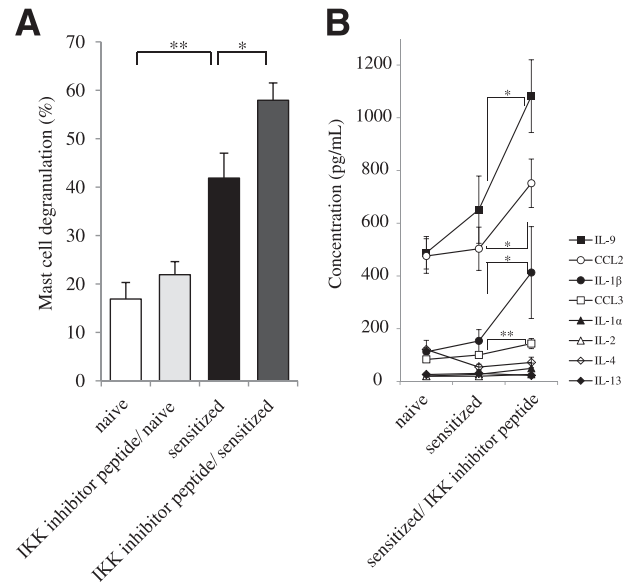


Figure 1 Aggravation of allergen-induced mast cell degranulation and systemic surge of inflammatory cytokines by IKK inhibition. **A:** Allergen-sensitized mice were injected i.v. with IKK inhibitor peptide 1 day before the challenge by allergen. Allergen-induced mast cell degranulation was significantly exacerbated by the injection of 20 μ g per mouse of IKK inhibitor peptide. * $P < 0.01$, ** $P < 0.001$. $n = 12$ per group. **B:** Surge of systemic inflammatory cytokines after IKK inhibition. Serum samples from allergen-challenged mice were analyzed for inflammatory cytokine induction by cytokine array analysis. IKK inhibitor treatment induced an array of inflammatory cytokines, including IL-1 β , IL-9, CCL2, and CCL3. * $P < 0.05$, ** $P < 0.01$. $n = 9$ per group. Data are expressed as means $\pm$ SEM.

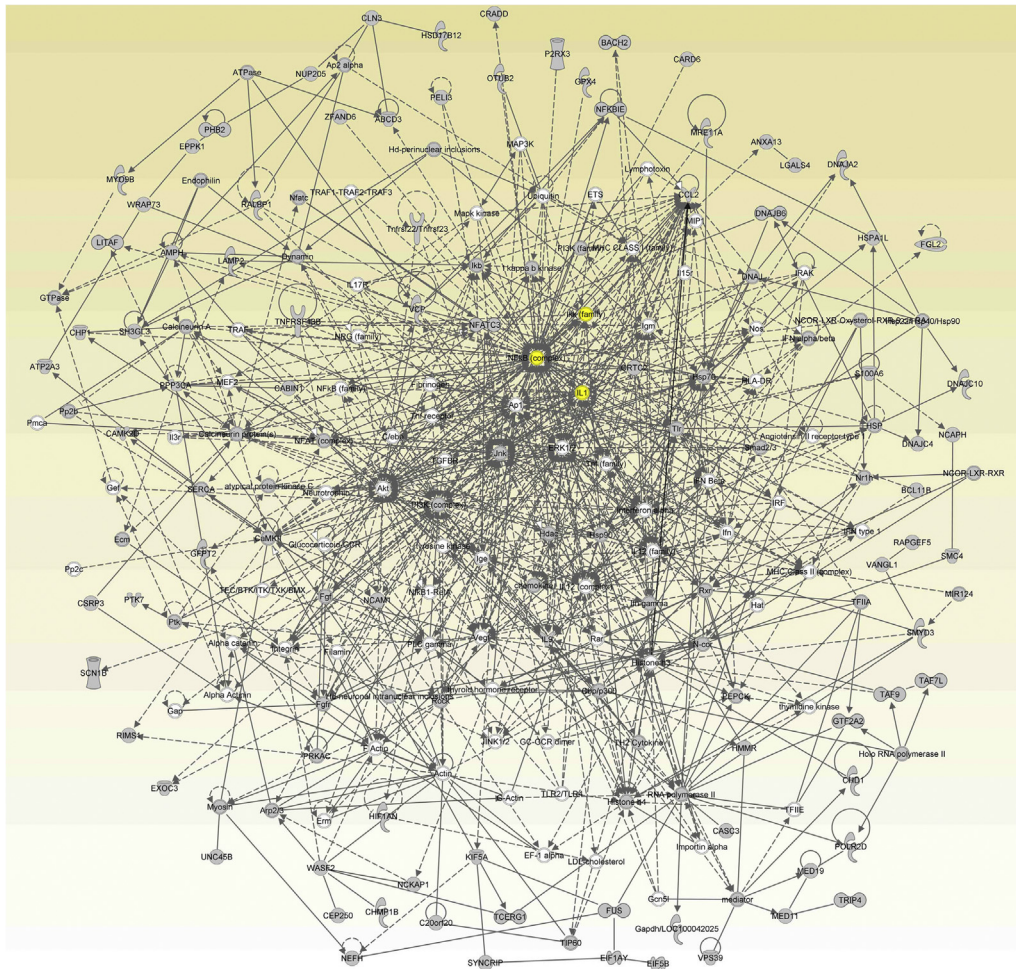


Figure 2 Network analysis of allergen-induced ocular immediate hypersensitivity reactions. A set of 509 allergen-induced genes (fold induction >1.4) were complemented with IL-9 and CCL2 and were analyzed for network generation of biological processes. The highest significant six networks ($P < 1 \times 10^{-29}$) are summarized as merged networks. NF κ B (complex), IKK family, and IL-1 are positioned in the center of the network and are highlighted in yellow. Nodes of IL-9 and CCL2 are positioned downstream of IL-1.

a *Renilla* luciferase construct (SABiosciences, Frederick, MD), using a mouse B-cell nucleofector kit (Lonza Inc., Walkersville, MD). After transfection, the B cells were plated in 3 mL of culture medium and were allowed to recover for 24 hours and were sensitized with anti-DNP IgE. Subsequently, cells were stimulated with DNP-albumin and were lysed in passive lysis buffer (Promega, Madison, WI). Luciferase activity was determined using the Dual-Luciferase reporter assay kit (Promega). The relative luciferase unit was calculated by dividing the firefly luciferase activity by the corresponding relative *Renilla* luciferase activity. For RNA interference of IKK- α , siRNA (SI00951328; Qiagen) was transfected with a nucleofection device.

Measurement of NF- κ B Subunit Activation

Nuclear protein extracts were obtained using the nuclear and cytoplasmic extraction kit (Thermo Scientific, Waltham, MA), and the binding activity of the NF- κ B subunits p50, p65, p52, and RelB was quantitatively

measured using the TransAM NF- κ B family kit (Active Motif, Carlsbad, CA). To do this, nuclear extracts were shaken for 1 hour at room temperature to allow their binding to the NF- κ B consensus sequence, followed by incubation with subunit-specific primary antibody. The secondary antibody was conjugated to horseradish peroxidase, and the specific binding was estimated by spectrophotometry at 450 nm.

Real-Time PCR

Total RNA prepared from B cells using the RNeasy kit (Qiagen) was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen). The cDNAs were amplified and quantified by LightCycler (Roche, Mannheim, Germany) using the QuantiTect SYBR Green PCR kit. The sequences of the real-time PCR primer pairs were as follows: IkB α forward 5'-CAGCAGACTCCACTCCACTT-3' and reverse 5'-GAGGGGTATTTCTCGAAAG-3'; and IKK- α forward 5'-GCCAGGGAGACTTGATGG-3' and reverse 5'-GAGGTCTGTGCTTTAGCTGCTT-3'.

Western Blot Analysis

Anti-DNP IgE-sensitized cells treated with IKK inhibitor peptide or IKK- β inhibitor VIII were stimulated with DNP-albumin, and the proteins were extracted with complete lysis buffer (Roche, Indianapolis, IN). The antibody used in the Western blot analysis was anti-I κ B α (Cell Signaling Technology Inc.).

Network Analyses of the Ocular Immediate Hypersensitivity Reaction

A set of allergen-induced genes in the ocular immediate hypersensitivity reaction that met the criterion of a 1.4-fold difference ($P < 0.05$)²³ was used to identify the pathways from the Ingenuity Pathways Analysis library of canonical pathways using the Ingenuity Pathways Analysis 2012 computer program (Ingenuity Systems, Redwood, CA).²⁹ Fisher's exact test was used to determine the possibility that each biological function assigned to that data set was due to chance alone.

The set of genes was analyzed for transcriptional networks using the Ingenuity Pathways Analysis 2012 computer program. The resulting networks were evaluated by the P value, which indicated the likelihood that the assembly of a set of focus genes in a network could be explained by random chance alone.

Statistical Analysis

Data are presented as means $\pm$ SEM. Statistical analyses were performed using unpaired Student's t -tests (two-tailed), U -tests, or analysis of variance.

Results

Aggravated Immediate Hypersensitivity Reaction and Induction of Strong Systemic Cytokine Expression by Inhibition of IKK

To evaluate the potential therapeutic effects of inhibiting IKK activity on allergic diseases, we tested whether the immediate hypersensitivity reaction can be suppressed by inhibiting IKK activity using a murine allergic conjunctivitis model. Because IKK activates the NF- κ B cascade by phosphorylating I κ B α , we first tested whether inhibition of IKK phosphorylation by a consensus sequence inhibitor peptide would suppress allergen-induced mast cell activation. An IKK inhibitor peptide that contains the sequence corresponding to the active I κ B α phosphorylation recognition sequence and that is known to be an anti-inflammatory compound exerting its effect by inhibiting I κ B α phosphorylation was used.

Allergen-immunized mice were injected systemically with the IKK inhibitor peptide and then were challenged by allergen exposure to the eyelid. Contrary to our expectation, the allergen-induced immediate hypersensitivity reaction, which should be manifested as mast cell degranulation, was not

lessened but instead was significantly worsened in treated mice (Figure 1A). IKK inactive control peptide treatment did not affect levels of mast cell degranulation (data not shown).

To understand the systemic effects of IKK inhibitor peptide treatment, the sera of allergen-sensitized mice treated with IKK inhibitor peptide were evaluated for representative inflammatory cytokines. In the sera of these mice, many inflammatory cytokines were induced (Figure 1B). This indicated that the inhibition of the IKK activity provoked systemic immune responses in the treated animal. A significant increase was observed in the NF- κ B-responsive cytokines, including IL-9, CCL2, IL-1 β , and CCL3 in descending order (Figure 1B). IL-1 α , IL-2, IL-4, and IL-13 were not induced. IL-1 β , which is a potent inducer of IL-9, had the highest ratio of increase relative to that of naive mice (3.7 fold).

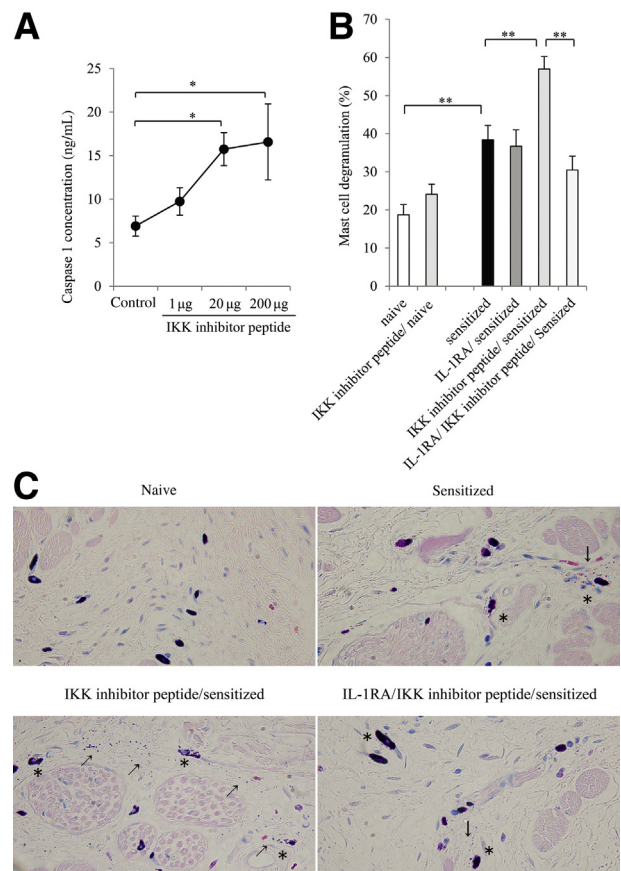


Figure 3 Role of IL-1 in the aggravation of allergen-induced mast cell degranulation by IKK inhibition. **A:** Naive mice were injected i.v. with IKK inhibitor peptide of the indicated amount. Serum samples collected after 24 hours were tested for systemic levels of caspase 1 using ELISA. $*P < 0.05$. $n = 6$ per group. **B:** Role of IL-1 in aggravated mast cell activation by pharmacologic inhibition of IKK. Allergen-induced mast cell degranulation was significantly enhanced after 20 μ g per mouse of IKK inhibitor peptide treatment. This enhancement was blocked by the administration of IL-1RA. $***P < 0.001$. $n = 12$ per group. **C:** IL-1RA-treated mice after IKK inhibitor peptide administration were challenged with allergen, and eyelid skin was Giemsa stained after 90 minutes. Allergen-induced mast cell granulation (asterisks) was enhanced in releasing mast cell granules (arrows) by IKK inhibitor peptide treatment and was negated by IL-1RA treatment. Original magnification, $\times 1000$. Data are expressed as means $\pm$ SEM.

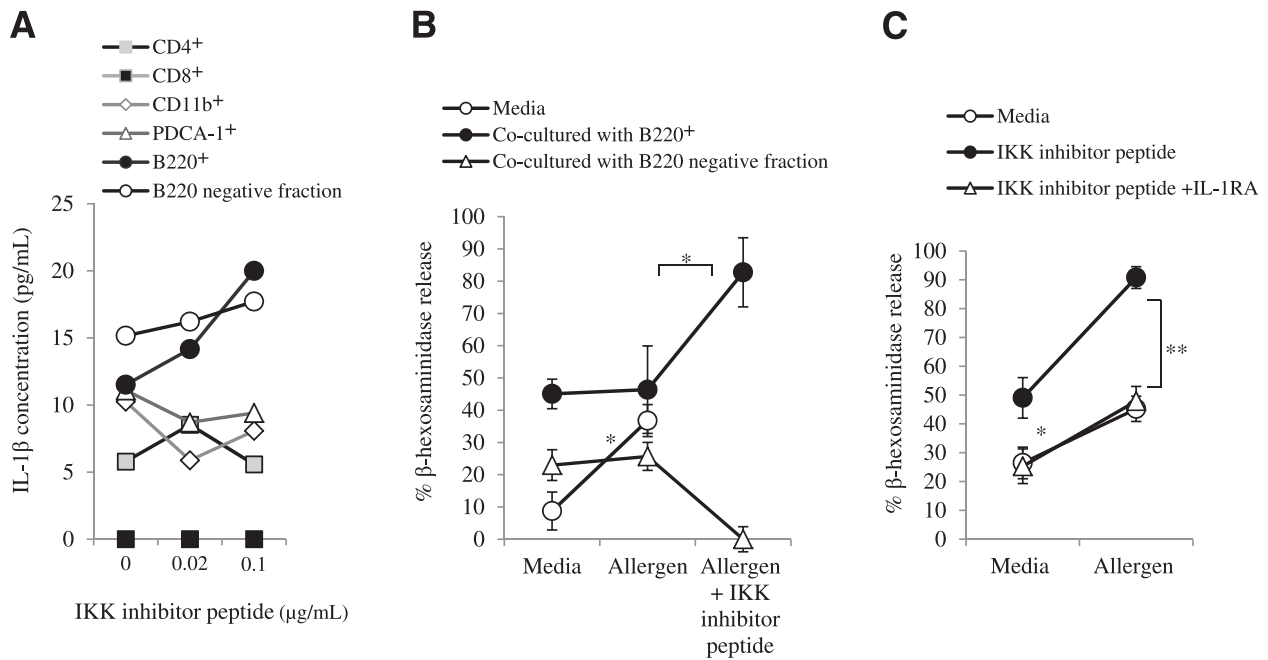


Figure 4 IL-1 β induction and exacerbation of allergen-induced mast cell degranulation by B220⁺ B cells after IKK inhibition. **A:** Digested tissue-derived cells were separated based on surface markers of CD4, CD8, CD11b, PDCA-1, or B220. The cells were screened for IL-1 β secretion after IKK inhibitor peptide treatment. Dose-responsive secretion of IL-1 β was observed for the B220⁺ B-cell fraction. $n = 4$ per group. **B:** IKK inhibitor peptide-treated B220⁺ B cells were co-cultured with IgE-sensitized mast cells, and allergen-induced mast cell degranulation was evaluated by determining β -hexosaminidase release. IKK inhibitor peptide-treated B220⁺ B cells significantly enhanced allergen-induced mast cell degranulation, whereas B220⁻ cell populations (including mixed subsets of CD4⁺ or CD8⁺ or CD11b⁺ or PDCA-1⁺) did not. $*P < 0.05$. $n = 6$ per group. **C:** Isolated tissue-resident mast cells were co-cultured in Transwells (Corning Inc., Corning, NY) with B cells treated with IKK inhibitor peptide. Allergen-induced β -hexosaminidase release from mast cells was significantly enhanced by co-culturing with IKK inhibitor-treated B cells and suppressed by IL-1RA. $*P < 0.01$, $**P < 0.0001$. $n = 6$ per group. Data are expressed as means $\pm$ SEM.

Prediction of Upstream Regulator in Aggravated Immediate Hypersensitivity

To identify candidate mediators responsible for the aggravated immediate hypersensitivity reaction, network analysis was used. Using previously reported acute phase transcriptome of murine allergic conjunctivitis,²³ 509 genes were extracted for network construction based on criteria of fold increase to naive animal > 1.4 ($P < 0.05$). Because IKK inhibitor treatment significantly induced large amounts of IL-9, CCL2, and IL-1 β (Figure 2), we analyzed how these molecules and IKK contributed to the networks by their inclusion. A set of the 509 genes was complemented with IL-9 and CCL2 and was analyzed for network generation. The resultant network was characterized by pathways relating to NF- κ B, as well as the JnK, PI3K, and ERK pathways (Figure 2). IL-1 was positioned adjacent to the NF- κ B node. To identify the upstream regulators in this network, upstream regulator analysis was conducted, and 664 molecules were detected. Of these, the highest significant upstream regulator was IL-1 ($P = 3.2 \times 10^{-7}$; data not shown). In this data set, the downstream target molecules of IL-1 were IL-9, CCL2, Alp, CaMKII, collagen type II, DDC, Fgf, Fgfr, Hsp70, IL1, NF κ B (complex), NR1H4, P38 MAPK, and pro-inflammatory cytokine.

Next, canonical pathway analysis was applied to the data set. The highest significant canonical pathways were assigned

as glucocorticoid receptor signaling, iCOS-iCOSL signaling in T-helper cells, PI3K signaling in B lymphocytes, April-mediated signaling, and BAFF signaling ($P = 3.75 \times 10^{-7}$, $P = 1.18 \times 10^{-6}$, $P = 4.29 \times 10^{-6}$, $P = 6.44 \times 10^{-6}$, and $P = 8.35 \times 10^{-6}$, respectively), and three of the five significant pathways were related to B-cell-related signaling.

Role of IL-1 β in Aggravated Mast Cell Activation and Immediate Hypersensitivity

Based on the outcome of the network analysis, we hypothesized that the inflammatory cytokine(s) induced by IKK inhibition will activate the allergen-induced immediate hypersensitivity reaction. Because IL-1 β detected by cytokine ELISA was supported as the most significant upstream regulator in the network, we then examined whether IKK inhibition may have induced the canonical pro-IL-1 β maturation enzyme caspase 1. Naive mice were injected with IKK inhibitor peptide and were examined for systemic caspase 1 induction. ELISA analysis confirmed significant induction of caspase 1 by the treatment (Figure 3A).

Of the many systemically induced inflammatory cytokines (Figure 1B), we evaluated the role of IL-1 β as the upstream regulator in aggravated immediate hypersensitivity. Sensitized mice that were systemically injected with IKK inhibitor peptide were treated with IL-1RA and were challenged with the allergen. The IKK inhibitor peptide

significantly enhanced allergen-induced mast cell degranulation (Figure 3, B and C). This enhancement was reduced by a previous administration of IL-1RA. In addition, systemic elevation of IL-9 or CCL2 by IKK inhibition was not observed when mice were treated with IL-1RA (data not shown). Thus, IL-1 β is most likely involved in the unexpected enhanced immediate hypersensitivity reaction induced by the inhibition of IKK.

Role of B Cells in Stimulating Tissue-Resident Mast Cells after IKK Inhibition

Next, we sought to determine how IKK inhibition caused the systemic release of inflammatory cytokines and aggravated the allergen-induced activation of tissue-resident mast cells. We first tested whether tissue-resident mast cells secreted IL-1 β . We found that isolated mast cells did not secrete appreciable amounts of IL-1 β after the inhibition of IKK (data not shown).

Based on the outcome of canonical pathway analysis, we then searched for the cell fraction(s) that secreted IL-1 β using a magnetic beads–based separation method. Using surface markers of CD4, CD8, CD11b, PDCA-1, and B220, various populations of pathway analysis–predicted lymphoid cells and other various populations were isolated from eyelid and skin tissue. The cell fractions were screened for IL-1 β secretion after the inhibition of IKK. Consistent with enrichment of the B-cell–related pathways in the canonical pathway analysis, the most responsive fraction was identified as B220⁺ B cells (Figure 4A). The other populations, including the CD4⁺, CD8⁺, CD11b⁺, and PDCA-1⁺ subsets, did not appreciably respond.

Next, we examined how B220⁺ B cells affected mast cell degranulation. For this, we evaluated mast cell degranulation by β -hexosaminidase release using co-cultures with the isolated subsets of B220⁺ B cells or B220[−] cells. When IKK inhibitor–treated B cells were co-cultured with IgE-sensitized mast cells, the allergen-induced mast cell degranulation measured by β -hexosaminidase release was significantly enhanced (Figure 4B). In contrast, other B220[−] cell fractions, including mixed subsets of CD4⁺ or CD8⁺ or CD11b⁺ or PDCA-1⁺, did not enhance degranulation. These findings indicated that B220⁺ B cells were responsible for the aggravated mast cell degranulation after IKK inhibition.

We then tested whether the activation of isolated tissue-resident mast cells by co-culturing them with tissue-resident B cells treated with IKK inhibitor peptide can be blocked by IL-1R blockade, as was observed *in vivo* (Figure 4C). β -Hexosaminidase release assays showed that increased allergen-induced mast cell degranulation was completely suppressed after IL-1RA treatment (Figure 4C). This indicated that IKK inhibitor–treated B cells can activate mast cells through IL-1/IL-1R.

Because the major systemic sources of B cells are the secondary lymphoid tissues, we assumed that the spleen-derived B cells may also serve and contribute to the systemic

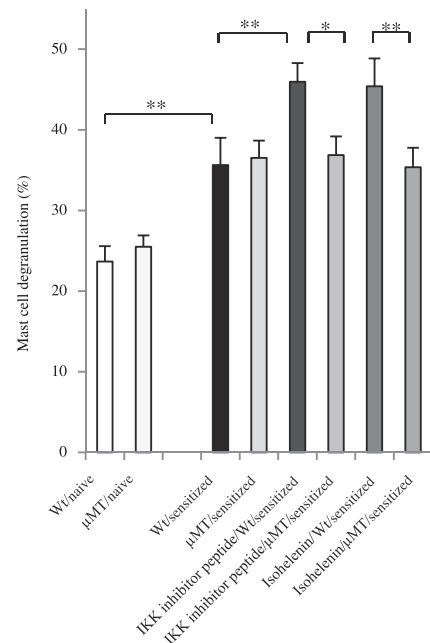


Figure 5 Role of B cells in aggravated mast cell activation by pharmacologic inhibition of IKK. Mice were passively sensitized with ragweed-specific IgE and induced for allergic conjunctivitis by allergen instillation. Allergen-induced mast cell degranulation was significantly enhanced after treatment with IKK inhibitor peptide or isohelenin. This enhancement was not observed in B-cell–deficient mice [Ighm^{tm1Cgn} targeted mutation (μMT)]. Wt, wild type. * $P < 0.05$, ** $P < 0.01$. $n = 14$ per group. Data are expressed as means $\pm$ SEM.

cytokine levels. When we tested spleen-derived B220⁺ B cells in co-cultures with tissue-resident mast cells, spleen-derived B cells after IKK inhibitor peptide treatment similarly exacerbated the allergen-induced mast cell degranulation (data not shown).

Requirement of B Cells for the Aggravated Immediate Hypersensitivity Reaction by Inhibition of IKK

To assess the contribution of B cells to the aggravated immediate hypersensitivity reaction provoked by IKK inhibition *in vivo*, we tested whether B-cell–deficient mice become unresponsive to the treatment of IKK inhibition. To exclude the possible involvement of primed T cells after the immunization protocol, mice were passively sensitized by an i.v. injection of allergen-specific IgE. Consistent with the previously noted observations, allergen-induced mast cell degranulation was significantly increased after IKK inhibitor peptide treatment (Figure 5). This effect by IKK inhibitor peptide was not observed in B-cell–deficient mice (Ighm^{tm1Cgn} targeted mutation). Without the treatment, B-cell deficiency did not affect allergen-induced mast cell degranulation.

Because the classical IKK activity mediates I κ B α degradation, the effect of blocking I κ B α degradation by isohelenin was tested next. When the mice were treated with isohelenin, allergen-induced mast cell degranulation was again increased significantly. In contrast, this effect by isohelenin was not observed in B-cell–deficient mice.

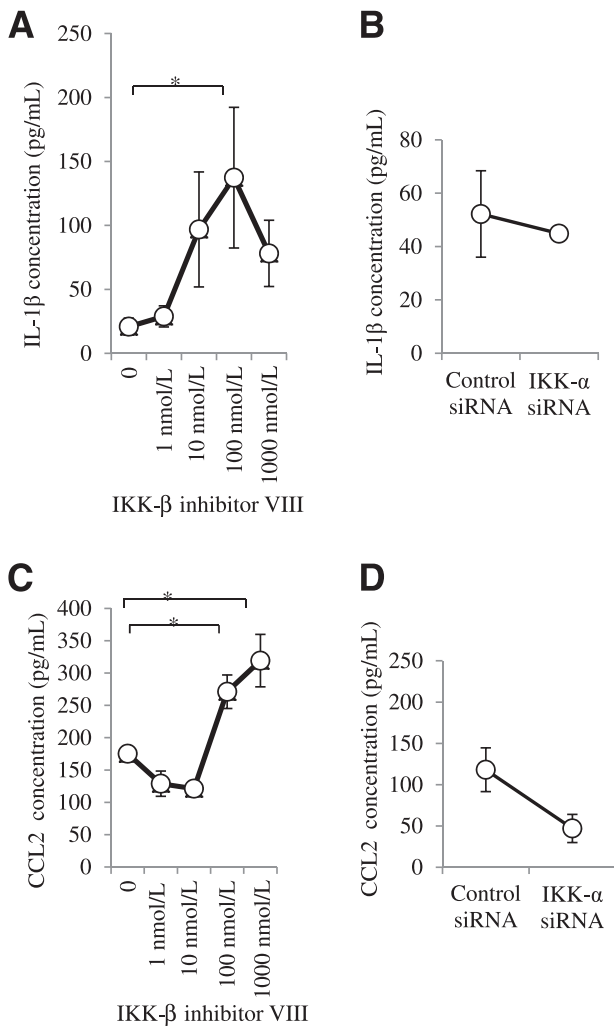


Figure 6 Secretion of IL-1 β in B cells after IKK- β inhibition. IKK- β inhibition by IKK- β inhibitor VIII resulted in the secretion of IL-1 β (A) and CCL2 (C) from B cells isolated from the spleen. In contrast, IKK- α inhibition by siRNA transfection did not appreciably induce IL-1 β (B) or CCL2 (D). * P < 0.05. n = 6 per group. Data are expressed as means $\pm$ SEM.

Stimulated Induction of IL-1 β by IKK- β Inhibition in B Cells

To determine which kinase activity is responsible for the aggravated inflammatory responses after IKK inhibition, B cells isolated from the spleen were inhibited for either IKK- α or IKK- β activities and were evaluated for IL-1 β secretion. B cells treated with IKK- β selective inhibitor VIII had significantly elevated levels of IL-1 β in a dose-dependent manner (Figure 6A). In contrast, IKK- α inhibition did not have such effects as was shown by IKK- α siRNA transfection (Figure 6B). The inhibitory effect of siRNA on IKK- α mRNA levels was 76.5% as measured by real-time PCR 48 hours after transfection (data not shown).

B cells treated with IKK- β selective inhibitor VIII also induced CCL2 secretion; however, it required more stringent inhibitory conditions (Figure 6C). IKK- α inhibition by siRNA transfection did not have such an effect (Figure 6D).

Limiting the Role of IKK- β in NF- κ B Promoter Activation in B Cells

To confirm that the inhibition of IKK activity can inhibit the degradation of I κ B α , IgE-sensitized B cells were treated with IKK- β selective inhibitor VIII or IKK inhibitor peptide, and the I κ B α levels were determined by Western blot analysis. As expected, IKK inhibition by IKK- β selective inhibitor VIII or IKK inhibitor peptide significantly elevated I κ B α levels before allergen exposure (Figure 7A).

To explain how inhibition of IKK activity enhanced cytokine secretion by B cells despite the reduction in I κ B α degradation, we examined whether there was activation of the NF- κ B promoter due to IKK inhibition. First, we evaluated the NF- κ B promoter binding activity of splenic B cells after IKK inhibition. Using nuclear extracts, we determined the binding activity of the NF- κ B family subunits p50, p65, p52, and RelB. Stimulation by the allergen significantly increased the binding activity of p52 and RelB (Figure 7B), which constituted an alternative NF- κ B cascade contributing to B-cell maturation or lymphogenesis. Exposure to the selective IKK- β inhibitor VIII

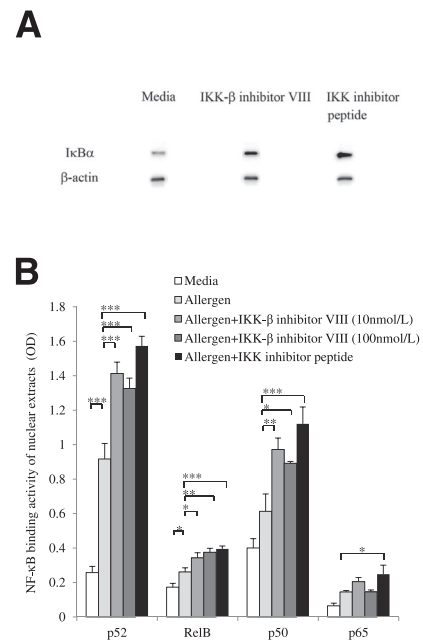


Figure 7 Enhanced NF- κ B binding activity of NF- κ B family subunits in B cells after IKK inhibitor treatment. **A:** Reduced degradation of I κ B α by IKK inhibition. I κ B α degradation of B cells isolated from the spleen was inhibited by 0.02 μ g/mL IKK inhibitor peptide or 10 nmol/L IKK- β inhibitor VIII. Whole cell extracts of B cells were assayed for I κ B α by Western blot analysis. Treatment by IKK inhibitor peptide or IKK- β inhibitor VIII significantly elevated the I κ B α levels. **B:** Nuclear extract from B cells were profiled for binding activity of subunits of the NF- κ B family, p52, RelB, p50, and p65 to NF- κ B-responsive consensus sequence. IKK inhibitor peptide, 0.02 μ g/mL, significantly elevated the binding activity of p52, RelB, p50, and p65. IKK- β inhibitor VIII (10 or 100 nmol/L) elevated the binding activities of p52, RelB, and p50. * P < 0.05, ** P < 0.01, and *** P < 0.001. n = 4 per group. Data are expressed as means $\pm$ SEM.

significantly increased the binding activity of the alternative cascade subunits p52 and RelB as well as the classical cascade subunit p50. IKK inhibitor peptide similarly elevated their binding activities.

We then examined whether IKK inhibition induced the transcriptional activation of the NF- κ B-responsive gene. As a representative NF- κ B-responsive gene, the mRNA of *I κ B α* was induced after the inhibition of IKK- β by IKK- β inhibitor VIII or IKK inhibitor peptide (Figure 8A). IKK- α has been shown to enter the nucleus and directly activate the NF- κ B-responsive promoter.^{30,31} Therefore, we tested whether IKK- α was recruited to the *I κ B α* promoter using the ChIP assay. In response to IKK- β inhibition, IKK- α was found to be significantly recruited to the *I κ B α* promoter (Figure 8B).

We next tested whether the observed activation of NF- κ B binding by IKK- β inhibition was translated into promoter activation. B cells that were transiently transfected with NF- κ B reporter plasmid were treated with IKK- β inhibitor VIII, and the promoter activity was determined by a luciferase assay (Figure 8C). Consistent with the elevated NF- κ B binding activity by IKK- β inhibition, the IKK- β selective inhibitor significantly increased the NF- κ B promoter activity. When B cells were cotransfected with IKK- α siRNA to inhibit IKK- α , this enhanced promoter activity by

IKK- β inhibition was not observed. This indicated that the NF- κ B promoter activation induced by IKK- β inhibition required IKK- α .

Role of IKK- α in the Aggravated Immediate Hypersensitivity Reaction

These results indicated that IKK inhibition of B-cell populations induced reciprocal NF- κ B promoter activation and inflammatory responses. To confirm that IKK- α activation is, indeed, responsible for aggravating the immediate hypersensitivity reaction in mice, an *in vivo* transfection method was used. To exclude the possible involvement of primed T cells after the immunization protocol, mice were passively sensitized by an intravenous injection of allergen-specific IgE. Then, IKK- α siRNA was selectively transfected into B cells *in vivo* by an injection of IKK- α siRNA-conjugated anti-B220 antibody. This *in vivo* B220-targeted transfection specifically reduced IKK-mRNA levels by 39% in the B-cell fraction 48 hours after transfection, whereas no inhibition was observed in the non-B-cell fraction (data not shown).

As expected, allergen-induced mast cell degranulation was significantly increased after IKK inhibitor peptide treatment (Figure 9). This increased mast cell degranulation

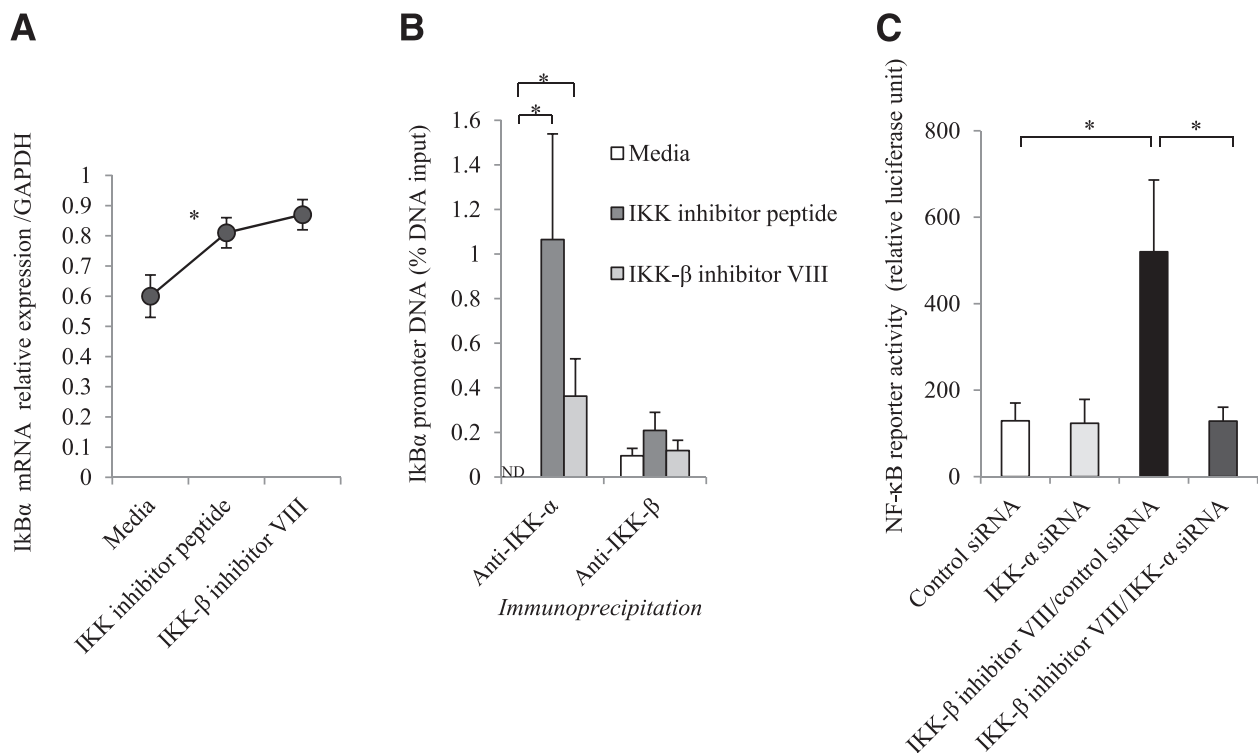


Figure 8 Role of IKK- α in NF- κ B promoter activation and enhanced recruitment of IKK- α to NF- κ B-responsive *I κ B α* promoter in B cells after IKK/IKK- β inhibition. B cells isolated from the spleen were inhibited for IKK/IKK- β activity by 0.02 μ g/mL IKK inhibitor peptide or 10 nmol/L IKK- β inhibitor VIII. **A:** IKK/IKK- β inhibition induced *I κ B α* mRNA in B cells by real-time PCR. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **B:** ChIP assay showed an enhanced recruitment of IKK- α but not IKK- β to NF- κ B-responsive *I κ B α* promoter in splenic B cells after IKK/IKK- β inhibition. **C:** B cells were transiently transfected with NF- κ B reporter plasmid and treated with 10 nmol/L IKK- β inhibitor VIII. IKK- β inhibitor VIII significantly activated NF- κ B promoter as detected by luciferase assay. The enhanced promoter activity was suppressed by IKK- α inhibition by cotransfection of IKK- α siRNA. * P < 0.05. n = 4 per group. Data are expressed as means $\pm$ SEM.

was significantly reduced by IKK- α siRNA transfection targeted for B cells. In contrast, the effect of IKK- α siRNA transfection for B cells without IKK inhibitor treatment was negligible.

Discussion

NF- κ B signaling mediates immune responses and contributes to the pathogenesis of many inflammatory diseases, including innate immune- and acquired immune-mediated diseases. Therefore, many pharmacologic inhibitors targeting the key elements of this cascade have been developed. These inhibitors were expected to be a new method to treat different inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, and atopic dermatitis.^{1,6,7} However, the present unexpected results raise serious questions about the translational application of pharmacologic inhibitors of the NF- κ B signaling pathways. These findings showed that inhibition of IKK can cause an aberrant activation of B cells and mast cells, whereas systemic B cells release inflammatory cytokines and activate mast cells. Thus, systemic B cells have another important role in mediating unregulated cytokine release when their signaling components are disrupted. For example, splenic B cells are strategically located to filter and present allergens, and they can also receive signals from follicular dendritic cells or marginal reticular cells. The responses of the aberrant B cells can become very significant.

NF- κ B signaling is also required for B-cell homeostasis. Signals from B-cell receptors and BAFF are essential determinants of B-cell differentiation and survival.³² For example, a B-cell receptor signal is required from the late pro-B-cell stage onward,³³ whereas BAFF receptor signaling mediates the transition to maturation stages.^{34–36} These signals are complementary and converged at the NF- κ B cascade. Earlier mutation studies showed that specific components of the classical NF- κ B pathway mediated B-cell receptor signaling,^{37–39} whereas BAFF receptor signaling preferentially activated the alternative NF- κ B pathway.^{40,41} Thus, both NF- κ B pathways are necessary for B-cell survival. Moreover, these receptors are cross-regulated and mutually dependent for the downstream NF- κ B cascade.³⁵ The present bioinformatic analysis of the immediate hypersensitivity reaction indeed showed that the NF- κ B pathway is centrally positioned in the transcriptome, which was characterized by B-cell-related cascades, PI3K, April, or BAFF by annotation of canonical pathway analysis. Thus, the essential requirements of the NF- κ B pathways may explain the unregulated activation of B cells induced by the inhibition of one critical component of the classical cascade.

Earlier, IKK- α was shown to activate the NF- κ B-responsive genes.^{30,31} In the nucleus, IKK- α interacts with the CREB-binding protein in conjunction with p65 and is recruited to NF- κ B-responsive promoters. Then, IKK- α phosphorylates histone H3 and mediates the subsequent

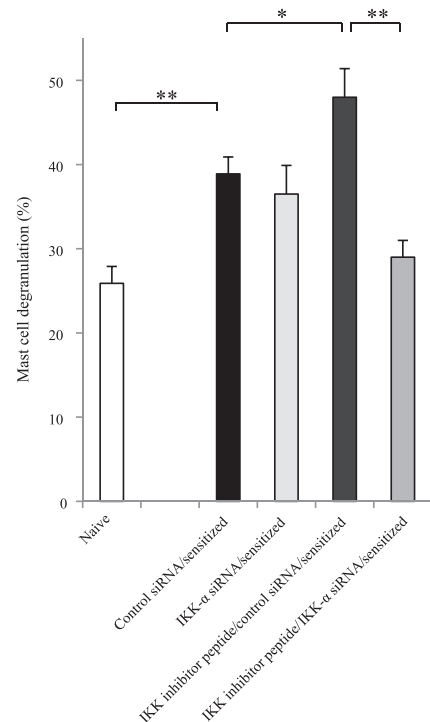


Figure 9 Role of IKK- α and B cells in the aggravated immediate hypersensitivity reaction after IKK inhibition. Mice were passively immunized with ragweed-specific IgE and challenged with ragweed allergen. Allergen-induced mast cell degranulation was significantly enhanced after IKK inhibitor treatment. To specifically target IKK- α in B cells, mice were systemically injected with IKK- α siRNA-conjugated anti-B220 antibody. B-cell-targeted inhibition of IKK- α negated the aggravation of mast cell activation by IKK inhibition. * $P < 0.05$, ** $P < 0.01$. $n = 12$ per group. Data are expressed as means $\pm$ SEM.

acetylation.³⁰ The chromatin-modifying activity of IKK- α is independent of the IKK phosphorylation consensus sequence and I κ B, which is consistent with the present observations with IKK phosphorylation consensus sequence inhibitor (IKK inhibitor peptide) or the I κ B degradation inhibitor isohelenin.

Several studies have confirmed that IKK- β inhibition has anti-inflammatory effects in various disease models, including autoimmune encephalomyelitis.^{5,42,43} Thus, an inhibition of IKK- β reduced the inflammation and promoted regeneration. In contrast, recent findings showed that a blockade of crucial elements in the NF- κ B pathway in innate immune cells can lead to disastrous outcomes.^{9,39} Moreover, in macrophages or myeloid cells that bridge the innate and adaptive immune system, IKK was shown to regulate the inflammatory cytokine induction after bacterial infection. However, the roles of IKK in these cells seem to be distinct from those of B cells. For example, IKK- β deficiency or prolonged pharmacologic inhibition of myeloid cells or macrophages causes an increase in their susceptibility to endotoxin-induced shock with the induction of IL-1 β .¹⁶ This was shown to be mediated by pro-IL-1 β processing by caspase 1, whose activation was limited by an NF- κ B-dependent gene product. In IKK- α deficiency in macrophages, the NF- κ B cascade is activated, which results

in enhancement of phagocytotic clearance and antigen-presenting capacity and in induction of tumor necrosis factor α , CCL2, and CCL3.¹⁵ These effects were related to the prolonged degradation of I κ B α due to uncontrolled activation of IKK- β . Collectively, an upset in the balance of the IKKs can cause paradoxical NF- κ B activation on pathogen encounter by the innate immune system.

When acquired immunity-mediated allergic models are examined for the effect of IKK inhibition, the general outcome is amelioration of the disease, including allergen-induced airway remodeling.⁴⁴ Moreover, in atopic dermatitis, an NF- κ B decoy oligodeoxynucleotide has been developed that effectively resolves the disease symptoms.⁶ Consistent with this, we also observed that blockade of p65 reduced the activation of tissue-resident mast cells (data not shown). These data led us to believe that targeting the NF- κ B cascade is a simple and effective way to treat these allergic diseases. However, one caveat of this strategy is that the outcome of general or lineage-specific inhibition of components of this cascade has not been fully explored.

Disease symptoms of allergic conjunctivitis are dependent on IgE-mediated degranulation of tissue-resident mast cells.^{19,20} Proper activation of IgE-mediated mast cell degranulation requires a co-stimulatory axis that is provided by CC chemokines, including CCL2, CCL3, and IL-1 β .^{19,25,45} In addition, IL-9 is a potent mast cell growth factor.⁴⁶ IKK inhibition results in a systemic induction of CCL2, CCL3, IL-1 β , and IL-9. This bioinformatic analysis of the transcriptome indeed showed pivotal and significant interactions of IL-1 with NF- κ B and related nodes (Figure 2). In addition, our and other laboratories have shown that IL-1/IL-1R is important for mast cell activation or mast cell-mediated allergic conjunctivitis.^{45,47} IL-1 β can induce all these inflammatory cytokines to target inflammatory cells.^{48–50} For example, IL-1 β can stimulate monocyte/macrophage to augment the surge of inflammatory cytokine production. For example, a major source of CCL2 in tissue was the monocyte/macrophage population in the allergic conjunctivitis model.²⁵

The resultant aberrant systemic activation of mast cells could cause an anaphylactic shock-like syndrome, especially when B cells are pathologically activated or physiologically not under control. Thus, systemic application of pharmacologic inhibitors of IKK needs to be carefully evaluated, especially for allergic individuals.

To summarize, pharmacologic inhibition of IKK- β aggravates the immediate hypersensitivity reaction by activated B cells. Strategies that target the NF- κ B pathway have great potential; however, this should be evaluated judiciously because alterations in the balance of IKK- α and IKK- β in mature B cells can lead to adverse reactions.

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References

1. Neurath MF, Pettersson S, Meyer zum Buschenfelde KH, Strober W: Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-kappa B abrogates established experimental colitis in mice. *Nat Med* 1996, 2:998–1004
2. Shibata W, Maeda S, Hikiba Y, Yanai A, Ohmae T, Sakamoto K, Nakagawa H, Ogura K, Omata M: Cutting edge: the IkappaB kinase (IKK) inhibitor, NEMO-binding domain peptide, blocks inflammatory injury in murine colitis. *J Immunol* 2007, 179:2681–2685
3. Dave SH, Tilstra JS, Matsuoka K, Li F, Karrasch T, Uno JK, Sepulveda AR, Jobin C, Baldwin AS, Robbins PD, Plevy SE: Amelioration of chronic murine colitis by peptide-mediated transduction of the IkappaB kinase inhibitor NEMO binding domain peptide. *J Immunol* 2007, 179:7852–7859
4. Herrmann O, Baumann B, de Lorenzi R, Muhammad S, Zhang W, Kleesiek J, Malfertheiner M, Kohrmann M, Potrovita I, Maegele I, Beyer C, Burke JR, Hasan MT, Bujard H, Wirth T, Pasparakis M, Schwaninger M: IKK mediates ischemia-induced neuronal death. *Nat Med* 2005, 11:1322–1329
5. van Loo G, De Lorenzi R, Schmidt H, Huth M, Mildner A, Schmidt-Suppran M, Lassmann H, Prinz MR, Pasparakis M: Inhibition of transcription factor NF-kappaB in the central nervous system ameliorates autoimmune encephalomyelitis in mice. *Nat Immunol* 2006, 7:954–961
6. Nakamura H, Aoki M, Tamai K, Oishi M, Ogihara T, Kaneda Y, Morishita R: Prevention and regression of atopic dermatitis by ointment containing NF-kB decoy oligodeoxynucleotides in NC/Nga atopic mouse model. *Gene Ther* 2002, 9:1221–1229
7. Jun-Ichi S, Hiroshi I, Ryo G, Ryuichi M, Kensuke E, Mitsuaki I: Initial clinical cases of the use of a NF-kappaB decoy at the site of coronary stenting for the prevention of restenosis. *Circ J* 2004, 68:270–271
8. Sunami Y, Leithauser F, Gul S, Fiedler K, Guldiken N, Espenlaub S, Holzmann KH, Hipp N, Sindrilari A, Luedde T, Baumann B, Wissel S, Kreppel F, Schneider M, Scharffetter-Kochanek K, Kochanek S, Strnad P, Wirth T: Hepatic activation of IKK/NF-kappaB signaling induces liver fibrosis via macrophage-mediated chronic inflammation. *Hepatology* 2012, 56:1117–1128
9. Pasparakis M: Regulation of tissue homeostasis by NF-kappaB signalling: implications for inflammatory diseases. *Nat Rev Immunol* 2009, 9:778–788
10. Pelzer C, Thome M: IKKalpha takes control of canonical NF-kappaB activation. *Nat Immunol* 2011, 12:815–816
11. Shembade N, Pujari R, Harhaj NS, Abbott DW, Harhaj EW: The kinase IKKalpha inhibits activation of the transcription factor NF-kappaB by phosphorylating the regulatory molecule TAX1BP1. *Nat Immunol* 2011, 12:834–843
12. Pasparakis M, Courtois G, Hafner M, Schmidt-Suppran M, Nenci A, Toksoy A, Krampert M, Goebeler M, Gillitzer R, Israel A, Krieg T, Rajewsky K, Haase I: TNF-mediated inflammatory skin disease in mice with epidermis-specific deletion of IKK2. *Nature* 2002, 417:861–866
13. Omori E, Matsumoto K, Sanjo H, Sato S, Akira S, Smart RC, Ninomiya-Tsuji J: TAK1 is a master regulator of epidermal homeostasis involving skin inflammation and apoptosis. *J Biol Chem* 2006, 281:19610–19617
14. Nenci A, Becker C, Wullaert A, Gareus R, van Loo G, Danese S, Huth M, Nikolaev A, Neufert C, Madison B, Gumucio D, Neurath MF, Pasparakis M: Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature* 2007, 446:557–561
15. Li Q, Lu Q, Bottero V, Estepa G, Morrison L, Mercurio F, Verma IM: Enhanced NF-kappaB activation and cellular function in macrophages lacking IkappaB kinase 1 (IKK1). *Proc Natl Acad Sci U S A* 2005, 102:12425–12430
16. Greten FR, Arkan MC, Bollrath J, Hsu LC, Goode J, Miething C, Goktuna SI, Neuenhahn M, Fierer J, Paxian S, Van Rooijen N, Xu Y,

- O'Cain T, Jaffee BB, Busch DH, Duyster J, Schmid RM, Eckmann L, Karin M: NF-kappaB is a negative regulator of IL-1beta secretion as revealed by genetic and pharmacological inhibition of IKKbeta. *Cell* 2007, 130:918–931
17. Zaph C, Troy AE, Taylor BC, Berman-Booty LD, Guild KJ, Du Y, Yost EA, Gruber AD, May MJ, Greten FR, Eckmann L, Karin M, Artis D: Epithelial-cell-intrinsic IKK-beta expression regulates intestinal immune homeostasis. *Nature* 2007, 446:552–556
 18. Miyazaki D, Tominaga T, Kakimaru-Hasegawa A, Nagata Y, Hasegawa J, Inoue Y: Therapeutic effects of tacrolimus ointment for refractory ocular surface inflammatory diseases. *Ophthalmology* 2008, 115:988–992
 19. Miyazaki D, Nakamura T, Toda M, Cheung-Chau K-W, Richardson RM, Ono SJ: Macrophage inflammatory protein-1a as a costimulatory signal for mast cell-mediated immediate hypersensitivity reactions. *J Clin Invest* 2005, 115:434–442
 20. Miyazaki D, Tominaga T, Yakura K, Kuo CH, Komatsu N, Inoue Y, Ono SJ: Conjunctival mast cell as a mediator of eosinophilic response in ocular allergy. *Mol Vis* 2008, 14:1525–1532
 21. Toda M, Kuo CH, Borman SK, Richardson RM, Inoko A, Inagaki M, Collins A, Schneider K, Ono SJ: Evidence that formation of vimentin mitogen-activated protein kinase (MAPK) complex mediates mast cell activation following FcepsilonRI/CC chemokine receptor 1 cross-talk. *J Biol Chem* 2012, 287:24516–24524
 22. Toda M, Dawson M, Nakamura T, Munro PM, Richardson RM, Bailly M, Ono SJ: Impact of engagement of FcepsilonRI and CC chemokine receptor 1 on mast cell activation and motility. *J Biol Chem* 2004, 279:48443–48448
 23. Komatsu N, Miyazaki D, Tominaga T, Kuo CH, Namba S, Takeda S, Higashi H, Inoue Y: Transcriptional analyses before and after suppression of immediate hypersensitivity reactions by CCR3 blockade in eyes with experimental allergic conjunctivitis. *Invest Ophthalmol Vis Sci* 2008, 49:5307–5313
 24. Miyazaki D, Nakamura T, Ohbayashi M, Kuo CH, Komatsu N, Yakura K, Tominaga T, Inoue Y, Higashi H, Murata M, Takeda S, Fukushima A, Liu FT, Rothenberg ME, Ono SJ: Ablation of type I hypersensitivity in experimental allergic conjunctivitis by eotaxin-1/CCR3 blockade. *Int Immunol* 2009, 21:187–201
 25. Tominaga T, Miyazaki D, Sasaki S, Mihara S, Komatsu N, Yakura K, Inoue Y: Blocking mast cell-mediated type I hypersensitivity in experimental allergic conjunctivitis by monocyte chemoattractant protein-1/CCR2. *Invest Ophthalmol Vis Sci* 2009, 50:5181–5188
 26. Miyazaki D, Kuo CH, Tominaga T, Inoue Y, Ono SJ: Regulatory function of CpG-activated B cells in late-phase experimental allergic conjunctivitis. *Invest Ophthalmol Vis Sci* 2009, 50:1626–1635
 27. Liu FT, Katz DH: Mouse monoclonal IgE antibodies specific for ragweed pollen antigens. *Hybridoma* 1984, 3:277–285
 28. Traenckner EB, Pahl HL, Henkel T, Schmidt KN, Wilk S, Baeuerle PA: Phosphorylation of human I kappa B-alpha on serines 32 and 36 controls I kappa B-alpha proteolysis and NF-kappa B activation in response to diverse stimuli. *EMBO J* 1995, 14:2876–2883
 29. Miyazaki D, Haruki T, Takeda S, Sasaki S, Yakura K, Terasaka Y, Komatsu N, Yamagami S, Touge H, Touge C, Inoue Y: Herpes simplex virus type 1-induced transcriptional networks of corneal endothelial cells indicate antigen presentation function. *Invest Ophthalmol Vis Sci* 2011, 52:4282–4293
 30. Yamamoto Y, Verma UN, Prajapati S, Kwak YT, Gaynor RB: Histone H3 phosphorylation by IKK-alpha is critical for cytokine-induced gene expression. *Nature* 2003, 423:655–659
 31. Anest V, Hanson JL, Cogswell PC, Steinbrecher KA, Strahl BD, Baldwin AS: A nucleosomal function for IkappaB kinase-alpha in NF-kappaB-dependent gene expression. *Nature* 2003, 423:659–663
 32. Cancro MP: Signalling crosstalk in B cells: managing worth and need. *Nat Rev Immunol* 2009, 9:657–661
 33. Torres RM, Flaswinkel H, Reth M, Rajewsky K: Aberrant B cell development and immune response in mice with a compromised BCR complex. *Science* 1996, 272:1804–1808
 34. Hsu BL, Harless SM, Lindsley RC, Hilbert DM, Cancro MP: Cutting edge: bLyS enables survival of transitional and mature B cells through distinct mediators. *J Immunol* 2002, 168:5993–5996
 35. Stadanlick JE, Kaileh M, Karnell FG, Scholz JL, Miller JP, Quinn WJ III, Brezski RJ, Trembl LS, Jordan KA, Monroe JG, Sen R, Cancro MP: Tonic B cell antigen receptor signals supply an NF-kappaB substrate for prosurvival BLyS signaling. *Nat Immunol* 2008, 9:1379–1387
 36. Meyer-Bahlburg A, Andrews SF, Yu KO, Porcelli SA, Rawlings DJ: Characterization of a late transitional B cell population highly sensitive to BAFF-mediated homeostatic proliferation. *J Exp Med* 2008, 205:155–168
 37. Pasparakis M, Schmidt-Supprian M, Rajewsky K: IkappaB kinase signaling is essential for maintenance of mature B cells. *J Exp Med* 2002, 196:743–752
 38. Xue L, Morris SW, Orihuela C, Tuomanen E, Cui X, Wen R, Wang D: Defective development and function of Bcl10-deficient follicular, marginal zone and B1 B cells. *Nat Immunol* 2003, 4:857–865
 39. Baldwin AS: Regulation of cell death and autophagy by IKK and NF-kappaB: critical mechanisms in immune function and cancer. *Immunol Rev* 2012, 246:327–345
 40. Yamada T, Mitani T, Yorita K, Uchida D, Matsushima A, Iwamasa K, Fujita S, Matsumoto M: Abnormal immune function of hemopoietic cells from alymphoplasia (aly) mice, a natural strain with mutant NF-kappa B-inducing kinase. *J Immunol* 2000, 165:804–812
 41. Claudio E, Brown K, Park S, Wang H, Siebenlist U: BAFF-induced NEMO-independent processing of NF-kappa B2 in maturing B cells. *Nat Immunol* 2002, 3:958–965
 42. Mourikioti F, Kratsios P, Luedde T, Song YH, Delafontaine P, Adami R, Parente V, Bottinelli R, Pasparakis M, Rosenthal N: Targeted ablation of IKK2 improves skeletal muscle strength, maintains mass, and promotes regeneration. *J Clin Invest* 2006, 116:2945–2954
 43. Acharyya S, Villalta SA, Bakkar N, Bupha-Intr T, Janssen PM, Carathers M, Li ZW, Beg AA, Ghosh S, Sahenk Z, Weinstein M, Gardner KL, Rafael-Fortney JA, Karin M, Tidball JG, Baldwin AS, Guttridge DC: Interplay of IKK/NF-kappaB signaling in macrophages and myofibers promotes muscle degeneration in Duchenne muscular dystrophy. *J Clin Invest* 2007, 117:889–901
 44. Broide DH, Lawrence T, Doherty T, Cho JY, Miller M, McElwain K, McElwain S, Karin M: Allergen-induced peribronchial fibrosis and mucus production mediated by IkappaB kinase beta-dependent genes in airway epithelium. *Proc Natl Acad Sci U S A* 2005, 102:17723–17728
 45. Keane-Myers AM, Miyazaki D, Liu G, Dekaris I, Ono S, Dana MR: Prevention of allergic eye disease by treatment with IL-1 receptor antagonist. *Invest Ophthalmol Vis Sci* 1999, 40:3041–3046
 46. Matsuzawa S, Sakashita K, Kinoshita T, Ito S, Yamashita T, Koike K: IL-9 enhances the growth of human mast cell progenitors under stimulation with stem cell factor. *J Immunol* 2003, 170:3461–3467
 47. Subramanian N, Bray MA: Interleukin 1 releases histamine from human basophils and mast cells in vitro. *J Immunol* 1987, 138:271–275
 48. Dinarello CA: Biologic basis for interleukin-1 in disease. *Blood* 1996, 87:2095–2147
 49. Stephens GL, Swerdlow B, Benjamin E, Coyle AJ, Humbles A, Kolbeck R, Fung M: IL-9 is a Th17-derived cytokine that limits pathogenic activity in organ-specific autoimmune disease. *Eur J Immunol* 2011, 41:952–962
 50. Burke SJ, Goff MR, Updegraff BL, Lu D, Brown PL, Minkin SC Jr, Biggerstaff JP, Zhao L, Karlstad MD, Collier JJ: Regulation of the CCL2 gene in pancreatic beta-cells by IL-1beta and glucocorticoids: role of MKP-1. *PLoS One* 2012, 7:e46986