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MAPK Kinase 3 Specifically Regulates FcεRI-Mediated IL-4 Production by Mast Cells

Adam J. MacNeil,^{*,†} Yong Jun Yang,[‡] and Tong-Jun Lin^{*,†}

Mast cells play a central role in allergic inflammation and are activated through cross-linking of FcεRI receptor-bound IgE, initiating a signaling cascade resulting in production of biologically potent mediators. Signaling pathways in the regulation of specific mediators remain incompletely defined. In this study, we examined the role of MAPK kinase 3 (MKK3) in IgE-dependent mast cell activation. In an in vivo model of passive cutaneous anaphylaxis, MKK3-deficient mice showed a deficit in late-phase IgE-dependent inflammation. To characterize the mechanism of this deficiency, we cultured bone marrow-derived mast cells (BMMCs) from wild-type and MKK3-deficient mice. We found that FcεRI-mediated mast cell activation induced rapid MKK3 phosphorylation by 5 min, diminishing slowly after 6 h. In MKK3-deficient BMMCs, phosphorylation of p38 was reduced at early and later time points. Among 40 cytokines tested using a protein array, IL-4 was the only cytokine specifically downregulated in MKK3-deficient BMMCs. Reduced IL-4 expression was seen in the local skin of MKK3-deficient mice following passive cutaneous allergic reaction. Furthermore, early growth response-1 (Egr1) bound to the promoter of *IL-4* in FcεRI-activated mast cells, and Egr1 transcription factor activity was diminished in MKK3-deficient BMMCs. Finally, mast cell-deficient mice reconstituted with MKK3-deficient BMMCs displayed a significantly impaired late-phase allergic inflammatory response. Thus, mast cell MKK3 signaling contributes to IgE-dependent allergic inflammation and is a specific regulator of FcεRI-induced IL-4 production. *The Journal of Immunology*, 2011, 187: 000–000.

Mast cells play a central role in IgE-dependent allergic diseases and are important immune effector cells. Through constitutive expression of the high-affinity receptor, FcεRI, mast cells are sensitive to, and activated by, Ag-dependent cross-linking of IgE on the cell membrane (1). Activation through FcεRI induces an immediate signaling cascade that culminates in the release of various proinflammatory mediators, including potent granule-bound preformed components (e.g., histamine), as well as in the activation of transcription factors that direct gene expression of key cytokines and chemokines (2, 3). Allergic diseases are the manifestation of excessive release and production of these mast cell-derived inflammatory mediators (3). Mast cell-signaling mechanisms that drive the production of specific mediators remain incompletely defined. Elucidation of the signaling mechanisms regulating specific mediator release is the focus of this study.

FcεRI is a multisubunit receptor that includes an IgE-binding α-chain, a signal-amplifying β-chain, and two signal-initiating γ-chains (4). Upon FcεRI cross-linking, Src family protein tyrosine kinases Lyn and Fyn are activated, which then cooperatively

activate several important pathways, including the MAPK-signaling pathway, as well as PI3K, IκB-NF-κB and NFAT, resulting in mast cell degranulation and the production of inflammatory cytokines (5–8). The MAPK family includes subgroups p38, ERK, and JNK. Specific MAPK activation is controlled by upstream MAPK kinases (MKKs), which generally act on specific MAPKs. The p38 subfamily is activated by MKK3 and MKK6, with MKK4 also having some specificity for this subfamily (9).

The impact of activation of many of these signaling pathways, including p38 MAPK, is generally at the gene-expression level, because downstream transcription factors are activated (10). These transcription factors then bind to the promoter of inflammatory cytokine genes and drive gene expression. Mast cells secrete many important cytokines following IgE-dependent signaling, including IL-6, IL-13, IL-4, and TNF. We recently described the role of the de novo-expressed transcription factor early growth response-1 (Egr1) in the full responsiveness of mast cell cytokine production following FcεRI signaling (11). The concerted activity of inducible transcription factors like Egr1, with others, including NF-κB and NFAT, can direct mast cell mediator production and shape the cytokine milieu at the site of inflammation (12, 13).

The significance of the MKK3–p38 MAPK pathway in mast cell function has not been examined. In this study, we identified MKK3 as a proinflammatory mediator of allergic inflammation in vivo and characterized the mechanism of MKK3-dependent events in activated mast cells. MKK3 deficiency led to impaired inflammatory response in an in vivo model of cutaneous allergic reaction. Mast cells from MKK3-deficient mice displayed no defect in maturation or degranulation, but they displayed impaired production of proinflammatory cytokine IL-4 in vitro and in vivo and produced an impaired IgE-dependent allergic inflammatory response in vivo in reconstituted mast cell-deficient mice. MKK3 was activated rapidly (5 min) following IgE-dependent signaling in mast cells and persisted for >6 h. MKK3 was required for full p38 activity, as well as for full activation of Egr1. Furthermore,

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Abbreviations used in this article: BMMC, bone marrow-derived mast cell; DNFB, dinitrofluorobenzene; Egr1, early growth response-1; MKK, MAPK kinase; TNP, trinitrophenyl.

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Egr1 bound to the IL-4 promoter in activated mast cells, demonstrating that this MKK3-dependent transcription factor can directly drive *IL-4* gene transcription. Thus, MKK3 is an important component of FcεRI signaling and a specific mediator of IL-4 production in allergic inflammation.

Materials and Methods

Animals

MKK3 knockout mice were generated, as previously described (14), and obtained from The Jackson Laboratory (B6.129S1-Map2k3tm1Flv/J). Mast cell-deficient *W-sh* mice were also obtained from The Jackson Laboratory (B6Cg-kit W-sh/HNihJacBmJ NistltF4). The protocols were approved by the University Committee on Laboratory Animals, Dalhousie University, in accordance with the guidelines of the Canadian Council on Animal Care.

Abs

Abs to phospho-MKK3/6, phospho-p38 MAPK (Thr 180/Tyr 182), phospho-JNK (Thr 183/Tyr 185), JNK, phospho-ERK1/2, and ERK were purchased from Cell Signaling Technology. Abs to MKK3, p38 MAPK, Egr1, Oct-1, and actin and HRP-linked secondary Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-conjugated rat anti-mouse c-Kit mAb and FITC-rat IgG2a were purchased from Cedarlane Laboratories (Hornby, ON, Canada). FITC-conjugated rat anti-mouse IgE (IgG1) and FITC-rat IgG1 were purchased from BD Biosciences (San Jose, CA). Alexa Fluor 594 goat and rabbit secondary Ab was from Invitrogen (Eugene, OR). Abs for IL-4 (clone 11B11) and isotype control rat IgG1κ, as well as DyLight 594 goat anti-rat secondary Ab were from BioLegend (San Diego, CA).

Mast cell culture, activation, and degranulation

Mouse bone marrow-derived mast cells (BMMCs) were cultured, as previously described (11). Mast cells were confirmed by toluidine blue staining and flow cytometry analysis for c-Kit and IgE receptor expression (FACSARIA). Following 5–6 wk in culture, mast cell purity was >98%. BMMCs were passively sensitized with IgE from TIB-141 cells (American Type Culture Collection). Cells were then activated by stimulation with 10 ng/ml trinitrophenyl (TNP)-BSA (Biosearch Technologies, Novato, CA). Mast cell degranulation was determined by measuring β-hexosaminidase release.

Western blotting

BMMCs were lysed in radioimmunoprecipitation assay buffer supplemented with a mixture of protease and phosphatase inhibitors. Cleared lysates (30 μg protein) were subjected to electrophoresis in 10% SDS-polyacrylamide gels. Gels were transferred to polyvinylidene difluoride membrane, blocked with 5% nonfat milk powder, probed with primary and secondary Abs, and detected by an ECL-detection system (Western Lightning Plus-ECL; PerkinElmer) on BioMax film (Kodak). Blots were scanned and quantified using ImageJ software.

Cytokine protein array and ELISA

Protein arrays (Mouse Inflammation Array 1; RayBiotech, Norcross, GA) were conducted according to the manufacturer's protocol. Mast cells were either unstimulated or stimulated with 10 ng/ml TNP-BSA for 6 h. ELISAs were used to measure cytokine concentrations in cell culture supernatant from mast cells stimulated with TNP-BSA and were conducted according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Abs and standards (DuoSet) were from R&D Systems (Minneapolis, MN). For serum IgE, whole blood was collected from MKK3^{+/+} and MKK3^{-/-} mice, and cleared serum was assayed for total IgE using a mouse Total IgE ELISA kit, according to the manufacturer's protocol (MD Biosciences, St. Paul, MN).

Intracellular calcium

Mast cells were incubated for 30 min with 2 μM fura 2-AM. After washing, mast cells were resuspended in phosphate buffer with 1.5 mM CaCl₂ at a concentration of 1 × 10⁶ cells/ml. Fluorescence was measured by placing 2 ml mast cell suspension in a 37°C thermostated quartz cuvette with magnetic stirring in an RF-1501 spectrofluorophotometer (Shimadzu, Tokyo, Japan).

IgE-dependent late-phase cutaneous reactions

Mice were passively sensitized by i.v. injection of 2 μg anti-DNP IgE mAb (Sigma-Aldrich, St. Louis, MO). After 24 h, a cutaneous reaction was

elicited by the application of 20 μl dinitrofluorobenzene (DNFB, 0.3% w/v; Sigma-Aldrich) in acetone/olive oil (4:1) to both sides of the right hind paw or right ear and 20 μl of acetone/olive oil to the left hind paw or left ear as a control. The thickness of the footpad or ear was measured using a digital micrometer after 24 h. The weight of the hind paw or ear punch (8 mm) was also determined. The thickness and weight of the right ear or right hind paw (treated with acetone/olive oil only) were used as baseline values. The DNFB-induced change in tissue thickness and weight was expressed as a percentage of the baseline values.

IgE-mediated passive cutaneous anaphylaxis

Mice were sensitized by intradermal injection of 20 ng anti-DNP IgE mAb (Sigma-Aldrich) into ear tissue. After 24 h, mice were challenged by i.v. injection of 100 μg DNP-BSA in 200 μl Evan's blue dye (1% w/v; Sigma-Aldrich). Thirty minutes later, ear tissue was collected in 300 μl formamide and incubated at 80°C for 2 h in a water bath to extract the Evan's blue dye. The absorbance was determined at 620 nm.

Reconstitution of mast cell-deficient *W-sh* mice

W-sh mice were reconstituted by injection of 20 μl BMMCs at a density of 25 × 10⁶ cells/ml in RPMI 1640 (5 × 10⁵ cells/injection) into the ear and footpad. MKK3^{+/+} BMMCs were injected into the left side ear and footpad, whereas MKK3^{-/-} BMMCs were injected into the right side ear and footpad. Five weeks later, mice received 2 μg anti-DNP IgE mAb via tail vein, and late-phase cutaneous reactions were elicited as described above, with both the left and right side receiving the DNFB application.

Immunohistochemistry

Ear tissues from late-phase cutaneous reactions in MKK3^{+/+} and MKK3^{-/-} mice were collected and fixed in 10% formalin. Specimens were embedded in paraffin and sectioned onto microscope slides. Briefly, specimens were deparaffinized in xylene, rehydrated in decreasing concentrations of alcohol, and incubated in 10 mM citrate-Tween buffer at 95°C for 30 min for Ag unmasking. Specimens were blocked in 2% goat serum/5% BSA in phosphate buffer, incubated overnight with primary Ab (rat anti-mouse IL-4, clone 11B11, 20 μg/ml) or isotype control (rat IgG1κ) diluted in Perm/Wash buffer (BD Biosciences), washed in Perm/Wash buffer, incubated in secondary DyLight 594 goat anti-rat IgG (1:1000), and mounted in DAPI (VECTASHIELD). All images were taken using equivalent settings on

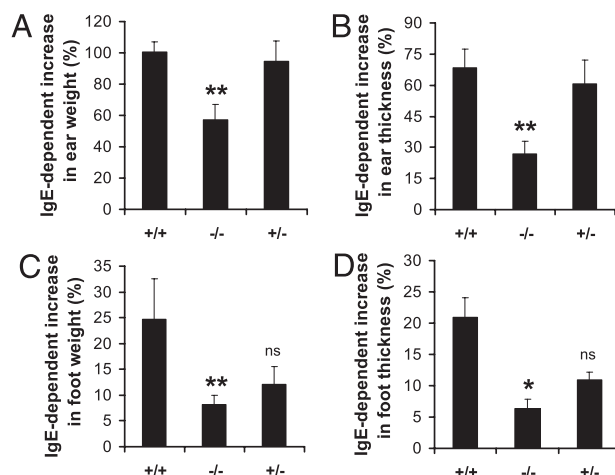


FIGURE 1. MKK3 deficiency leads to a deficit in late-phase cutaneous anaphylaxis. A–D, A model of late-phase cutaneous reactions in wild-type (+/+), MKK3 knockout (−/−), and heterozygous (+/−) mice. Mice were passively sensitized by i.v. injection of 2 μg of anti-DNP IgE mAb. Twenty-four hours later, 0.3% DNFB in acetone/olive oil (4:1) was applied to the right ear and hind paw, whereas acetone/olive oil was applied on the left. Twenty-four hours later, ear and foot thickness was measured using a digital micrometer. The weight of the foot and an 8-mm ear punch was also measured. The DNFB-induced increase in thickness and/or weight is expressed as the percentage change over the baseline values. A deficit in IgE-dependent swelling in knockout mice was observed for ear thickness (A) and weight (B), as well as for foot thickness (C) and weight (D). *n* = 10 MKK3^{+/+}, 11 MKK3^{-/-}, and 6 MKK3^{+/-} mice. Data are mean ± SEM. **p* < 0.05, ***p* < 0.005. ns, not significant.

a Nikon E600 microscope equipped with a 40 $\times$ objective lens using ACT-1 software (Nikon).

Immunofluorescence

BMMCs from wild-type and MKK3^{-/-} mice were treated or not with TNP-BSA (10 ng/ml) for 1 h, fixed, and permeabilized using Cytofix/Cytoperm solution (BD Biosciences, San Diego, CA) for 20 min on ice. Cells were then blocked with 5% goat serum (Cedarlane Laboratories) for 1 h, incubated with rabbit polyclonal anti-Egr1 Ab for 3 h, washed, and stained for 1 h with Alexa Fluor 594-conjugated secondary Ab. Fluorescence-labeled mast cells were cytocentrifuged (Cytospin 4; Thermo Shandon, Cheshire, U.K.) onto slides and mounted with DAPI (VECTA-SHIELD). All images were taken using equivalent settings on a Nikon E600 fluorescence microscope equipped with a 100 $\times$ objective lens (Nikon, Tokyo, Japan).

EMSA

Nuclear protein extracts were isolated using a nuclear extract kit (Active Motif, Carlsbad, CA), according to the manufacturer's protocol. EMSA was performed as previously described (11). Briefly, samples (8 μ g) were electrophoresed on native polyacrylamide gels with radiolabeled (³²P) dsDNA probe for Egr (Santa Cruz Biotechnology), dried on filter paper for 2 h at 80°C, and exposed to BioMax film (Kodak). Results were scanned and quantified using ImageJ software.

Chromatin immunoprecipitation

Assays were performed using the ChIP-IT Express Enzymatic kit (Active Motif), according to the manufacturer's protocol. Briefly, BMMCs were left unstimulated or were stimulated for 1 h with TNP-BSA, fixed with 1% formaldehyde, and the nuclei were subjected to an enzymatic digestion with 5 U enzymatic shearing mixture solution for 25 min at 37°C. Sheared chromatin was immunoprecipitated with 4 μ g Egr1, Oct-1 (Santa Cruz Biotechnology), or control IgG (Active Motif). Input DNA and 5% of the precipitated DNA were used as templates for each PCR, consisting of 36 cycles of 20 s at 94°C, 30 s at 54°C, and 30 s at 72°C. PCR products were

separated by a 2% agarose gel. Primers for amplification of the *IL-4* promoter region were 5'-GAGGGGTGTTTCATTTTCCA-3' (forward) and 5'-TGCTGGCAGAGGTCTCTCTAT-3' (reverse); for *IL-13*, 5'-GCCTTCTGCTTGTCTTGAGG-3' (forward) and 5'-CCAGTCCCTTCCACTG-3' (reverse); and for *TNF*, 5'-GGGGAGGAGATTCCTTGATG-3' (forward) and 5'-TCGCTGAGGGAGCTTCTG-3' (reverse).

Statistical analysis

The paired Student *t* test was used for statistical evaluation of data. Results were considered significant when *p* < 0.05. Data are expressed as mean $\pm$ SEM.

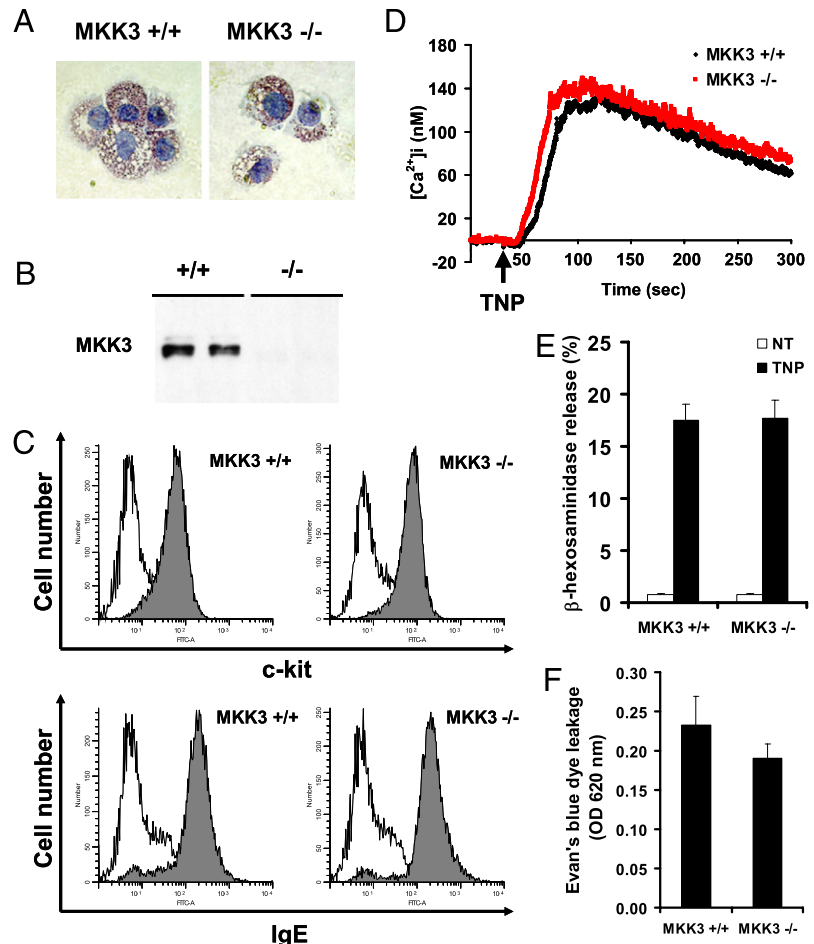
Results

MKK3 deficiency leads to a deficit in *Fc ϵ RI*-mediated late-phase cutaneous anaphylaxis

Activated mast cells play a role in the development of late-phase allergic reactions through the production and release of cytokines (15). To examine the role of MKK3 in late-phase allergic inflammation, MKK3-deficient and wild-type mice were sensitized with anti-DNP IgE i.v. 24 h prior to epicutaneous application of Ag, a 0.3% solution of DNFB, or a control solution on the ear and foot for 24 h. MKK3-deficient mice displayed a deficit in IgE/Ag-dependent inflammation, as assessed by ear and foot weight (Fig. 1A, 1C). This inflammatory deficit was also evident when measured by tissue thickness for the ear and foot (Fig. 1B, 1D), suggesting a regulatory role for MKK3 in allergic reaction. We also examined MKK3-deficient mice for any defect in basal serum IgE levels and found no difference compared with wild-type mice (1.04 ± 1.03 versus 1.00 ± 0.64 μ g/ml, respectively; *n* = 3 mice).

The response of MKK3^{+/-} mice resembled that of MKK3^{+/+} mice at the ear, yet it seemed to be more compromised at the foot,

FIGURE 2. BMMCs from MKK3 knockout mice display no deficit in maturation in vitro. *A*, BMMCs were cultured in conditioned media with IL-3 and PGE₂ for 4–5 wk. Cells were stained with toluidine blue to determine mast cell purity. A representative photomicrograph is shown (original magnification $\times 100$). *B*, Western blot analysis of total-protein lysates for MKK3 expression confirms the absence of MKK3 expression in knockout BMMCs. *C*, BMMCs were sensitized with anti-TNP IgE, stained with FITC-conjugated anti-*c*-Kit or anti-IgE, and examined by flow cytometry. *D*, Intracellular calcium ion flux analysis of MKK3^{+/+} and MKK3^{-/-} BMMCs following IgE-mediated mast cell activation. *E*, β -Hexosaminidase release by stimulated MKK3^{+/+} and MKK3^{-/-} BMMCs shows no difference in mast cell degranulation in vitro. *F*, Passive cutaneous anaphylaxis in wild-type (MKK3^{+/+}) and MKK3 knockout (^{-/-}) mice by Evan's blue dye leakage in ear tissue. Mice were intradermally injected with 20 ng of anti-DNP IgE in the ear tissue. After 24 h, mice received i.v. injection of 100 mg DNP-BSA in 1% w/v Evan's blue dye. Ear punches (8 mm) were collected 1 h later, and Evan's blue dye was extracted from ear tissue. Samples were measured for dye leakage into tissue at 620 nm (*n* = 6 mice).



falling between the response for $MKK3^{+/+}$ and $MKK3^{-/-}$ mice, although no statistical significance was observed (Fig. 1). To examine MKK3 expression in $MKK3^{+/-}$ mice, skin tissue and splenic tissue were analyzed by Western blotting for MKK3. $MKK3^{+/-}$ mice were found to have partially impaired MKK3 expression (Supplemental Fig. 1A).

These results indicated that MKK3 is an important regulator in the development of IgE-dependent late-phase cutaneous anaphylaxis. This finding prompted us to investigate the mechanism through which MKK3 regulates FcεRI-mediated mast cell activation.

Development of mast cells in the absence of MKK3

To exclude the possibility that the inflammatory deficiency in MKK3-deficient mice is the result of abnormal mast cell development in vivo (e.g., reduced mast cell numbers), ear, back skin, and tongue tissue were collected and analyzed for mast cell numbers by Alcian blue histological staining. Mast cells were present in similar number and morphology in MKK3-deficient mice compared with a wild-type control (Supplemental Fig. 1B, 1C).

To examine the role of MKK3 in FcεRI-mediated mast cell activation, we cultured BMMCs from MKK3-deficient mice. Bone marrow was obtained from wild-type and MKK3-deficient mice and cultured in IL-3 and PGE₂-conditioned media for 5 wk. Toluidine blue staining showed similar metachromatic staining and morphology of mast cells from wild-type and MKK3-deficient mice (Fig. 2A). MKK3-deficient mice have a targeted disruption removing exons 8 and 9 from the *MKK3* gene, including the dual-phosphorylation site required for MKK3 protein activation (14). To confirm the absence of MKK3 protein in BMMCs, Western blotting was performed on total-protein lysates from MKK3-deficient and wild-type BMMCs. MKK3 protein was not detected in MKK3-deficient BMMCs (Fig. 2B). To further examine the development of MKK3-deficient BMMCs in vitro, BMMCs were analyzed by flow cytometry for c-Kit and IgE receptor expression; no difference was observed (Fig. 2C). These results suggested that mast cells develop normally in vivo and in vitro in the absence of MKK3.

MKK3 deficiency does not affect calcium mobilization or mast cell degranulation

Calcium influx following FcεRI-mediated signaling enhances and sustains mast cell-activation signals (16). To examine whether MKK3 plays a role in mast cell calcium mobilization, MKK3-deficient BMMCs were sensitized with Ag-specific anti-TNP IgE and preloaded with fura 2-AM, followed by stimulation with TNP-BSA. Calcium influx following FcεRI-mediated signaling was unaffected in MKK3-deficient BMMCs compared with wild-type control BMMCs (Fig. 2D).

To examine whether MKK3 plays a role in FcεRI-mediated mast cell degranulation, wild-type and MKK3-deficient BMMCs were sensitized with anti-TNP IgE and stimulated with TNP-BSA for 20 min. Mast cell degranulation was assessed by β-hexosaminidase release. No effect on mast cell degranulation in vitro was detected in MKK3-deficient BMMCs (Fig. 2E). This finding was consistent with an in vivo model of mast cell degranulation in which MKK3-deficient and wild-type mice were sensitized with anti-DNP IgE intradermally in ear tissue for 24 h, followed by i.v. challenge with DNP-BSA in a 1% solution of Evan's blue dye at the tail. Thirty minutes later, ear tissue was collected, and Evan's blue dye was extracted to assess vascular permeability by spectrophotometry. MKK3-deficient mice did not incur a significant difference in vascular permeability in ear tissue compared with wild-type mice (Fig. 2F). These results indicated that MKK3 does not play a role in FcεRI-mediated mast cell degranulation in vitro or in vivo.

MKK3 is rapidly activated by phosphorylation following mast cell activation through FcεRI

To examine the activation of MKK3 following FcεRI-mediated mast cell activation, wild-type BMMCs were sensitized with anti-TNP IgE and stimulated with TNP-BSA. Cell lysates were analyzed by Western blotting to examine the dynamics of MKK3 and p38 MAPK activation. MKK3 was rapidly activated by phosphorylation (by 5 min) following FcεRI-mediated activation. This activation persisted for up to 3 h, followed by a reduction to near basal levels by 6 h (Fig. 3). This early activation of MKK3 was consistent with p38 (p-p38) activation, but although p38 activity was diminished by 1 h, followed by a rebound in phosphorylation, MKK3 activity was not diminished (Fig. 3A). These findings indicated that MKK3 is a signaling target downstream of FcεRI in activated mast cells.

MKK3 deficiency leads to reduced p38 activation, but does not affect activation of MAPKs ERK and JNK following FcεRI-mediated mast cell activation

To examine the impact of MKK3 deficiency on the MAPK pathway, wild-type and MKK3-deficient BMMCs were sensitized with anti-TNP IgE and stimulated with TNP-BSA. Cell lysates were analyzed by Western blotting for activation of MAPKs p38, ERK, and JNK. Activation of p38 was specifically reduced at both early (5 min) and later (3–6 h) time points in MKK3-deficient BMMCs (Fig. 4A), whereas ERK and JNK activation was consistent with

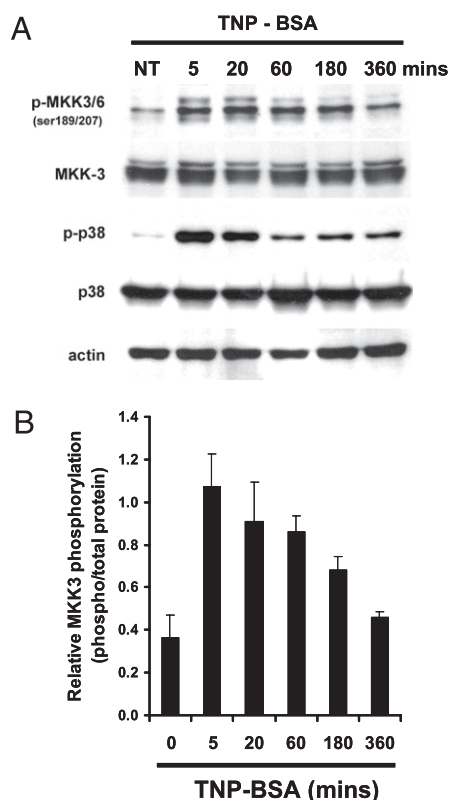


FIGURE 3. MKK3 phosphorylation occurs rapidly and persists following IgE signaling in BMMCs. BMMCs from wild-type mice were sensitized with anti-TNP IgE and stimulated for various lengths of time with TNP-BSA in vitro. Total lysates were analyzed by Western blot for phosphorylated (p-) and total MKK3 and p38 protein. An appreciable increase in MKK3 phosphorylation was observed following 5 min of activation, consistent with early p38 MAPK phosphorylation. Actin served as a loading control. A, Representative blots. B, Relative MKK3 phosphorylation ($n = 3$ separate experiments).

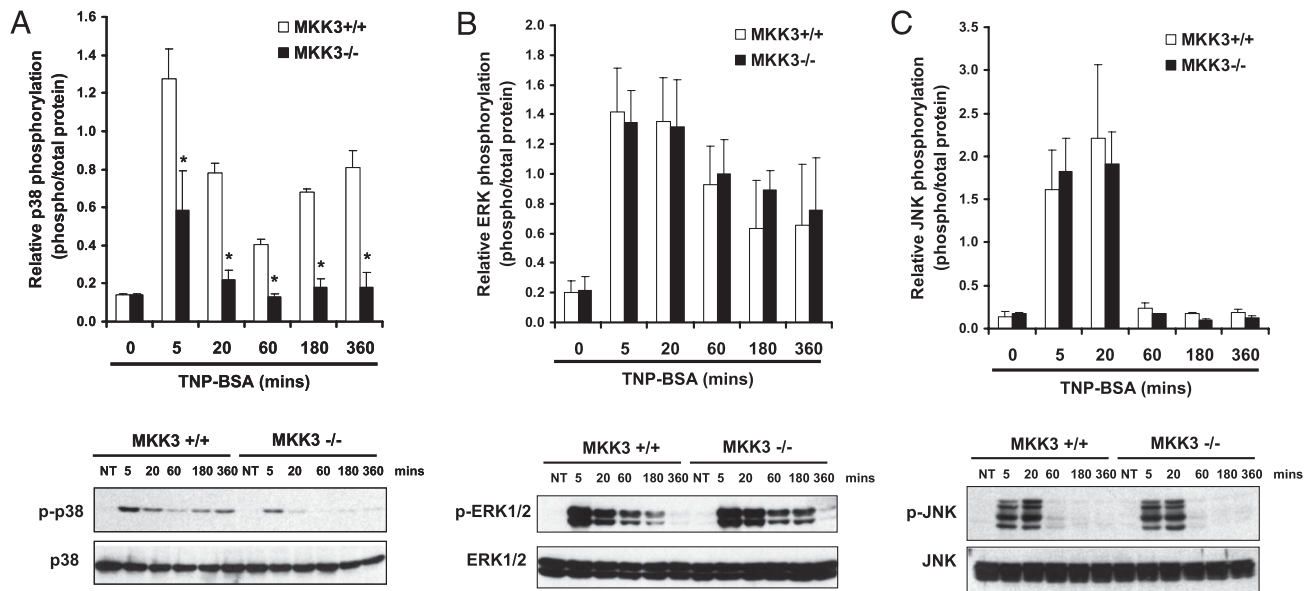


FIGURE 4. MKK3-deficient BMMCs have reduced p38 MAPK phosphorylation. BMMCs from wild-type ($^{+/+}$) and MKK3 knockout ($^{-/-}$) mice were analyzed by Western blot for phosphorylation of MAPK proteins following IgE-mediated signaling. A deficit in p38 phosphorylation (A) was observed in MKK3 knockout BMMCs, whereas MAPKs ERK (B) and JNK (C) phosphorylation remained unaffected. Relative phosphorylation was quantified ($n = 3$ separate experiments). Data are mean $\pm$ SEM. Representative images are shown in the bottom panel. $*p < 0.05$.

that of wild-type BMMCs (Fig. 4B, 4C). This finding demonstrated that MKK3 activity is specific to p38 in mast cells activated through Fc ϵ RI and that MKK3 is required for full activation of p38.

MKK3 deficiency leads to impaired IL-4 production in response to Fc ϵ RI-dependent mast cell activation

Mast cell-derived cytokines contribute to late-phase allergic reactions. To determine whether MKK3 signaling plays a role in Fc ϵ RI-dependent mast cell cytokine production, wild-type and MKK3-deficient BMMCs were sensitized with anti-TNP IgE and stimulated with TNP-BSA for 6 h. Cell-free supernatants were collected and analyzed for cytokine content using a protein array to assess the cytokine profile of MKK3-deficient BMMCs. Of 40 inflammatory cytokines tested, only IL-4 production was markedly reduced in MKK3-deficient BMMCs compared with wild-type BMMCs (Supplemental Fig. 2A, 2B). To more specifically examine the dynamics of mast cell-derived cytokine production in MKK-deficient BMMCs, cell-free supernatants were prepared for various time points and analyzed by ELISA for IL-4, IL-6, IL-13, TNF, and CCL3 production. Production of IL-4 was significantly reduced at 1, 3, and 6 h following TNP-BSA stimulation (Fig. 5A), with production levels at 45, 40, and 38% of wild-type levels, respectively. IL-6, IL-13, TNF (Fig. 5B–D), and CCL3 (Supplemental Fig. 2C) production was not significantly altered in MKK3-deficient BMMCs. These data indicated that MKK3 activity in activated mast cells contributes to specific signals that promote the production of the proinflammatory cytokine IL-4 and are consistent with MKK3 playing a proinflammatory role in late-phase allergic reactions in vivo (Fig. 1).

MKK3 deficiency leads to impaired IL-4 production in IgE-mediated late-phase cutaneous anaphylaxis

To determine whether the in vitro deficit in BMMC IL-4 production following Fc ϵ RI-dependent signaling was consistent with in vivo IL-4 production during IgE-dependent late-phase reactions, we examined mouse ear tissues for IL-4 expression by immunohistochemistry. Treatment with Ag (DNFB) displayed an inducible expression of IL-4 in the tissue of wild-type ($^{+/+}$) mice (Fig. 6). However, a markedly impaired induction of IL-4 expression was

observed in the tissue from MKK3-deficient mice compared with that from wild-type mice (Fig. 6). These data supported a role for MKK3 in IgE-dependent IL-4 production in vivo and are consistent with our in vitro data (Fig. 5A) demonstrating impaired IL-4 production in BMMCs stimulated through Fc ϵ RI.

Mast cell-deficient mice reconstituted with MKK3-deficient BMMCs have impaired IgE-dependent late-phase cutaneous anaphylaxis

To determine the significance of mast cell MKK3 signaling in IgE-dependent late-phase cutaneous anaphylaxis, mast cell-deficient (W-sh) mice were reconstituted at the ear and footpad with either wild-type (left side) or MKK3-deficient (right side) BMMCs. Reconstituted W-sh mice then received IgE via tail vein and were

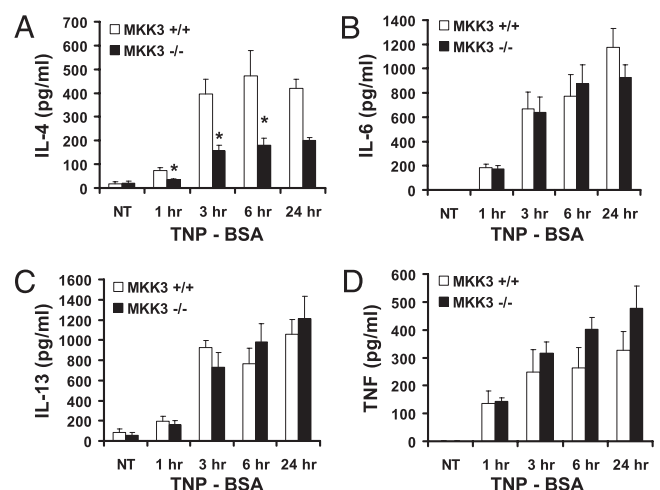


FIGURE 5. MKK3-deficient BMMCs have reduced IgE-dependent IL-4 production. IL-4 (A), IL-6 (B), IL-13 (C), and TNF (D) production by wild-type (MKK3 $^{+/+}$) and MKK3-deficient ($^{-/-}$) Fc ϵ RI-stimulated BMMCs was assessed by ELISA in cell-free culture media following stimulation. IL-4 production was significantly impaired in MKK3-deficient ($^{-/-}$) BMMCs. Data are expressed as mean $\pm$ SEM ($n = 6$ separate experiments). $*p < 0.05$, MKK3 $^{+/+}$ versus MKK3 $^{-/-}$.

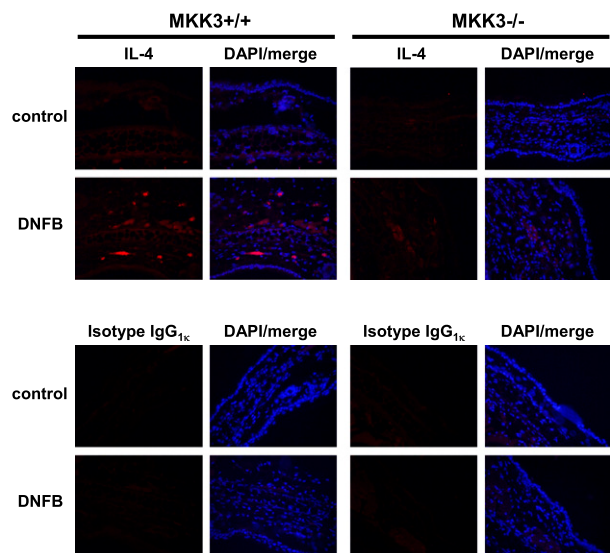


FIGURE 6. MKK3-deficient mice have impaired IL-4 expression in the dermis following IgE-dependent late-phase cutaneous anaphylaxis. Wild-type ($^{+/+}$) and MKK3-deficient ($^{-/-}$) mice were passively sensitized by i.v. injection of 2 μ g of anti-DNP IgE mAb. Twenty-four hours later, 0.3% DNFB in acetone/olive oil (4:1) was applied to the right ear, whereas acetone/olive oil (control) was applied to the left. The next day, ear tissues were collected and processed for immunohistochemistry. IL-4 expression was induced in MKK3 $^{+/+}$ ear tissue treated with DNFB, whereas MKK3-deficient mice showed an impaired expression of IL-4 following DNFB treatment. Nuclei were stained with DAPI. Rat IgG_{1k} was used as an isotype control for the IL-4 Ab. Images are representative of two separate experiments (original magnification $\times 40$).

analyzed for responsiveness in IgE-dependent late-phase cutaneous anaphylaxis with a solution of 0.3% DNFB applied to both sides. Ear and foot swelling was significantly impaired in tissues reconstituted with MKK3 $^{-/-}$ BMMCs compared with tissues reconstituted with MKK3 $^{+/+}$ BMMCs (Fig. 7). This finding underscores the significance of MKK3 signaling in mast cells following IgE-dependent late-phase reactions in vivo.

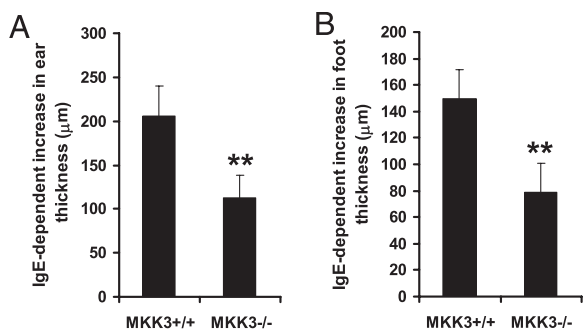


FIGURE 7. Mast cell-deficient mice reconstituted with MKK3-deficient mast cells have impaired IgE-dependent late-phase cutaneous anaphylaxis. Mast cell-deficient (W-sh) mice were reconstituted with wild-type (*left*) and MKK3-deficient (*right*) BMMCs in the ear and footpad 5 wk prior to analysis for IgE-dependent late-phase cutaneous reactions. Reconstituted mice were sensitized by i.v. injection of 2 μ g of anti-DNP IgE mAb. Twenty-four hours later, 0.3% DNFB was applied to both the left and right ears and hind feet. The following day, ear (A) and foot (B) thickness was measured. Ear and foot tissues reconstituted with MKK3 $^{-/-}$ BMMCs displayed impaired swelling compared with tissues reconstituted with MKK3 $^{+/+}$ BMMCs. Data are expressed as mean $\pm$ SEM ($n = 10$ mice). ** $p < 0.01$.

MKK3 deficiency leads to reduced activity of candidate IL-4 transcription factor Egr1

Among the downstream targets of the MAPK-signaling pathway are various transcription factors that differ based on the activating cell stimuli and cell type. To examine potential transcription factors that might regulate IL-4 transcription in mast cells, the IL-4 gene promoter was analyzed for predicted transcription factor binding sites. Among those candidate transcription factors were NFAT and Egr1. To examine transcription factor activity, nuclear protein extracts from TNP-BSA-stimulated wild-type and MKK3-deficient BMMCs were analyzed by EMSA using ^{32} P-labeled DNA probes based on transcription factor consensus binding sites. Although NFAT activation was relatively unaffected in MKK3-deficient BMMCs (Supplemental Fig. 3), Egr1 activation was significantly reduced from 20 min to 6 h following Fc ϵ RI-mediated mast cell activation (Fig. 8A, 8B). The specificity of the Egr probe was confirmed using unlabeled or mutant probes as competitors for the labeled probe. Supershift assay was also performed to further verify specificity, and nuclear Egr protein was partially blocked by Egr1-specific Ab (Fig. 8C). Egr1 expression was also examined by immunofluorescence and found to be partially impaired in MKK3 $^{-/-}$ BMMCs activated through Fc ϵ RI for 1 h (Supplemental Fig. 4), suggesting a role for MKK3 signaling in the de novo production of Egr1 protein. These data indicated that Egr1 is specifically expressed and activated through an MKK3-dependent signaling mechanism in Fc ϵ RI-activated mast cells.

MKK3 deficiency results in impaired Egr1 binding at the promoters of IL-4, IL-13, and TNF upon induction of Fc ϵ RI in mast cells

We previously documented impaired mast cell IL-13, TNF, and IL-4 production using Egr1-deficient mice (11, 17). A chromatin immunoprecipitation assay was performed to determine whether Egr1 interacts with the promoters of IL-4, IL-13, and TNF following Fc ϵ RI-mediated mast cell activation and to examine the impact of partial Egr-1 impairment through MKK3 deficiency. Nuclei from wild-type BMMCs either untreated or activated through Fc ϵ RI for 1 h (maximal for Egr1 activity) were enzymatically digested, followed by immunoprecipitation with anti-Egr1 Abs. Immunoprecipitates were analyzed by PCR using primers specific to the gene promoters (Fig. 9A). As shown in Fig. 9B, TNP stimulation specifically induced Egr1 binding to the promoters of all three cytokine genes. In MKK3-deficient cells, relative detection of binding to all three was clearly impaired (Fig. 9B). These results demonstrated that Egr1 is recruited to the IL-4 promoter, as well as the promoters of IL-13 and TNF following Fc ϵ RI-dependent signaling in mast cells. However, production of IL-4, but not IL-13 or TNF, was impaired in MKK3-deficient BMMCs (Fig. 5A, 5C, 5D) suggesting that, by comparison, IL-4 gene transcription may be more sensitive to partially impaired Egr1 activity.

Discussion

The current study elucidated a previously unknown role for MKK3 as a regulator of Fc ϵ RI-mediated allergic inflammation and demonstrated that MKK3 can specifically regulate IL-4 production by mast cells. MKK3 is rapidly activated following Fc ϵ RI signaling in mast cells, and its activity is required for full p38 MAPK activation. MKK3 is required for the induction of an important mast cell transcription factor, Egr1, following Fc ϵ RI activation. Mast cell activation through Fc ϵ RI induces binding of Egr1 to the promoter of IL-4, suggesting a mechanism linking MKK3 signaling as a specific regulator of IL-4 production in activated mast cells via Egr1 activity (Fig. 9C).

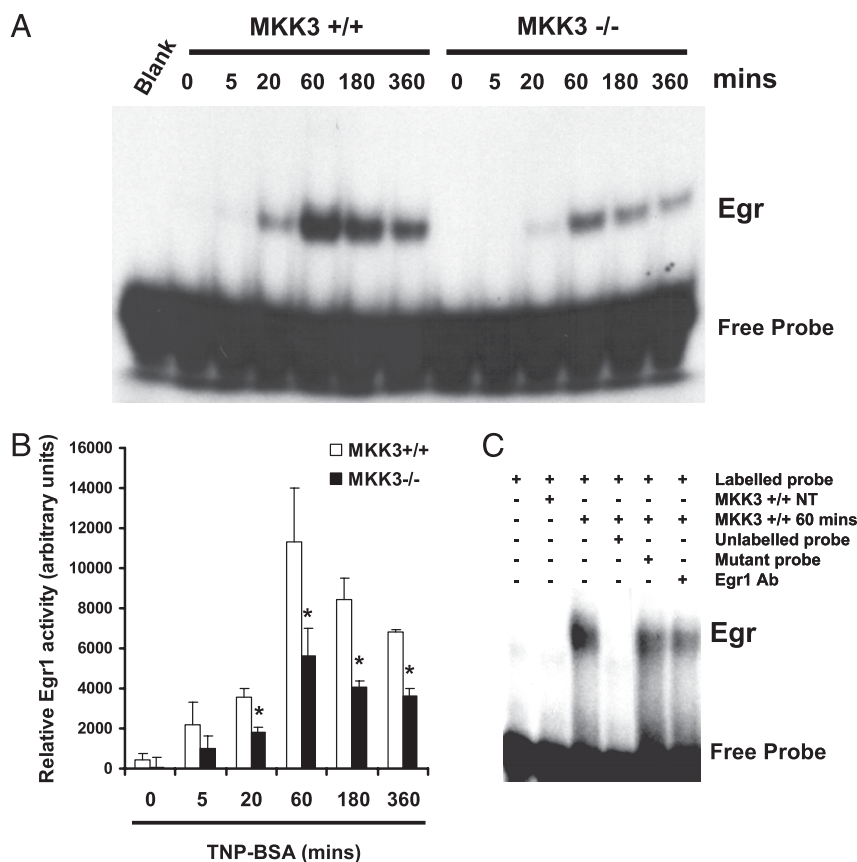


FIGURE 8. Egr1 activity is reduced following IgE-mediated mast cell stimulation. *A*, Nuclear proteins from IgE-sensitized and untreated or TNP-BSA-treated BMMCs (MKK3^{+/+} and MKK3^{-/-}) were analyzed by EMSA for Egr transcription factor activity at 5, 20, 60, 180, and 360 min poststimulation. A deficit in Egr transcription factor activity was observed from 20 to 360 min. A representative from three independent experiments is shown. *B*, Results from three independent experiments were quantified. *C*, Competition and supershift assay demonstrate the specificity of the Egr probe. **p* < 0.05.

Cytokine expression must be strictly controlled, because inappropriate expression is associated with many diseases (18). Mast cells secrete a multitude of factors that can contribute to important immune functions; however, when misregulated, they can also contribute to inflammatory diseases, such as allergy. IL-4, in particular, is associated with allergic diseases and autoimmunity (19, 20). Immune cell types of several distinct lineages can express IL-4, including CD4⁺ and CD8⁺ T cells, NKT cells, eosinophils, basophils, and mast cells (21). The most extensively studied functions for IL-4 are its role in directing Th2 cell differentiation and promotion of B cell Ab isotype class switch to IgE, a contribution that has significant implications for allergy (19). Mast cell-derived IL-4 contributes to the promotion of IgE synthesis by B cells (22). IL-4Rs are expressed in various cell types, including T cells, B cells, monocytes, epithelial cells, endothelial cells, mast cells, and synovial fibroblasts (19, 20). Thus, mast cell-derived IL-4 may have multiple targets in allergy. Mechanisms involved in FcεRI-mediated mast cell IL-4 production are unclear. It is likely that distinct mechanisms are involved in the regulation of specific mast cell mediators. In this study, we identified MKK3 as a specific regulator of mast cell-derived IL-4 in vitro and in vivo and showed that MKK3 is a proinflammatory mediator of allergic inflammation. We determined a mechanism for a mast cell-derived contribution of IL-4 in allergic inflammation, dependent on MKK3 signaling, in an IgE-dependent inflammatory response. Furthermore, we established that MKK3 signaling, even when only impaired in mast cells, plays a significant role in shaping the phenotype of proinflammatory late-phase allergic reactions (Fig. 7).

The proximal signaling pathways that regulate mast cell function following FcεRI-dependent signaling are reasonably well defined, but divergent downstream signaling events in the regulation of specific mast cell mediators warrant further study. It is notable that

of a panel containing 40 proinflammatory cytokines, only IL-4 production was impaired in MKK3-deficient mast cells. This deficiency was not a general feature of MKK3-deficient cells, because calcium ionophore (A23187)-treated BMMC IL-4 production was not impaired (data not shown) (23). Therefore, MKK3-dependent IL-4 production in mast cells is specific to the FcεRI-signaling pathway and is not required for alternative mechanisms promoting IL-4 production. This finding underscores the specificity of MKK3-dependent events in FcεRI-activated mast cells. Furthermore, MKK3 was shown to play a role in IL-6 expression in synoviocytes in a model of rheumatoid arthritis (24). By contrast, IL-6 production was not affected in MKK3-deficient mast cells, further highlighting the cell-type-specific nature of MKK3 signaling in mast cells.

In a model of rheumatoid arthritis, Inoue et al. (24) characterized an MKK3-dependent defect in TNF signaling in fibroblast-like synoviocytes. The MKK3-deficient synoviocytes are deficient in IL-6 and IL-1β production following TNF treatment. MKK3-dependent TNF signaling at the joint synovium is an example of a potential secondary inflammatory deficiency in our IgE-dependent model using the footpad. IgE-dependent mast cell-produced TNF could activate synoviocytes and further perpetuate the inflammatory response through the secretion of IL-6 and IL-1β. In an MKK3-deficient mouse, the response to secreted TNF from the IgE-mediated inflammatory response is very likely compromised at the foot joint. Thus, MKK3 could play a role in IgE-mediated allergic inflammation in vivo through direct regulation of mast cell function and through regulation of the secondary response of local tissue cells to mast cell-produced cytokines.

MKK3^{+/-} mice seem to have reduced expression of MKK3. These results suggested that partial expression of MKK3 is sufficient to initiate an allergic response. A trend of reduced inflammation in the footpad was observed in MKK3^{+/-} mice, which

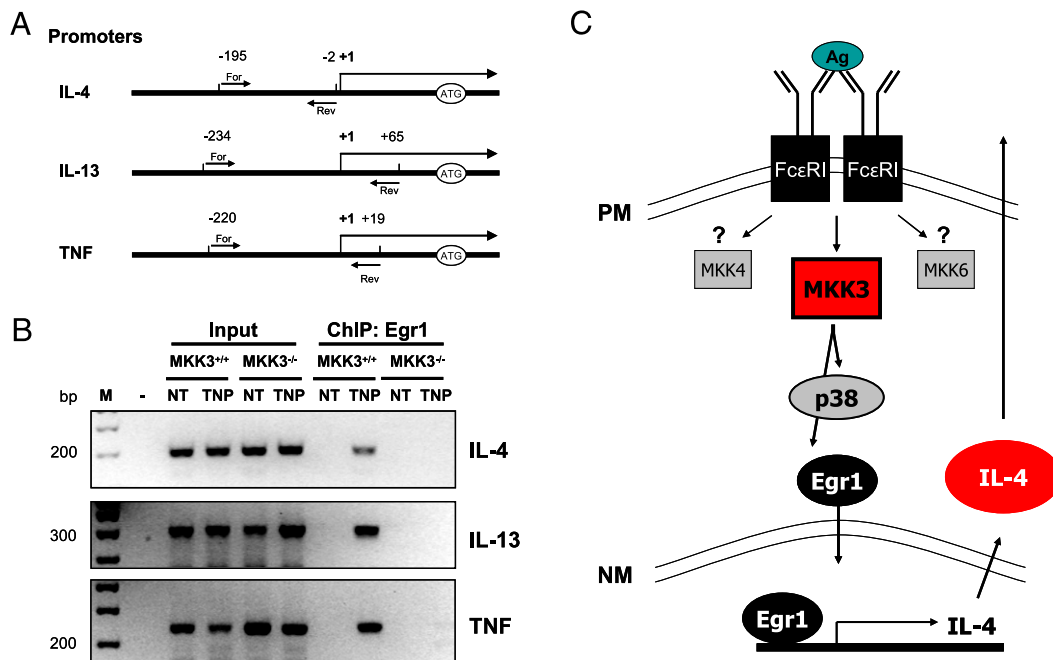


FIGURE 9. Egr1 binding to the promoters of IL-4, IL-13, and TNF following IgE-mediated signaling is impaired in MKK3-deficient BMMCs. **A**, Schematic drawing of the IL-4, IL-13, and TNF promoters and the location of primers used in chromatin immunoprecipitation analysis. **B**, BMMCs were sensitized with anti-TNP IgE, followed by stimulation with TNP-BSA for 60 min or were left untreated (NT). Protein–DNA complexes were precipitated with anti-Egr1 Abs. PCR was performed using primers specific to the IL-4, IL-13, and TNF gene promoters and separated on 2% agarose gel. Inducible Egr1 binding at promoters of all three genes was detected in cells activated through IgE (TNP) but not when untreated (NT). Egr1 promoter binding was significantly impaired in MKK3^{-/-} BMMCs stimulated through the IgE. Results are representative of four independent experiments. **C**, A working model of MKK3-dependent downstream activation of IL-4 by Egr1 in mast cells. Mast cells are activated by IgE cross-linking of FcεRI on the plasma membrane (PM). Subsequent rapid phosphorylation of MKK3 is required for p38 activation and downstream Egr1 activity in the nucleus. Full Egr1 expression and activity are required for appropriate binding at the *IL-4* gene promoter and for full activation of IL-4 production in allergic inflammation. NM, nuclear membrane.

may be potentially reflective of the more complex structure of the foot compared with that of the ear. Additional cell types that can participate in the immune response present in the foot (e.g., synovocytes at the joints) might contribute to the secondary inflammatory response following IgE-dependent cutaneous reactions.

The specificity of MKK3 for the p38 MAPK pathway is well described (9, 25, 26). It is clear from the current study that MKK3 plays a role in p38 activation downstream of FcεRI in mast cells, but it is worth noting that MKK3 deficiency does not abrogate p38 activation completely (Fig. 4A). This suggests that other kinases with substrate specificity for p38 play a role in FcεRI signaling and raises the questions of what role MKK6 and/or MKK4 may play in mast cell activation and whether interruption of their function may exacerbate the phenotype seen in late-phase allergic reactions in MKK3-deficient mice (Fig. 9C).

Our findings defined a critical role for full Egr1 expression in the regulation of FcεRI-dependent IL-4 production by mast cells. We showed that Egr1 can bind to the promoter of the *IL-4* gene in mast cells and that binding is inducible upon signaling through FcεRI (Fig. 9). This finding is consistent with a recent report that Egr1 contributes to *IL-4* transcription in TCR-stimulated Th2 cells through synergistic binding activity with NFAT and NF-κB (27). Furthermore, we found that MKK3 deficiency significantly reduced Egr1 expression and activity in the nucleus of activated mast cells (Fig. 8). Thus, MKK3 likely regulates FcεRI-dependent *IL-4* transcription through Egr1 (Fig. 9C).

Interestingly, FcεRI-dependent Egr1 binding to the promoters of *IL-13* and *TNF* was also impaired in MKK3-deficient mast cells, whereas IL-13 and TNF expression was not affected, suggesting that IL-4 expression is more sensitive to Egr1 regulation. It is possible that Egr1 may interact with other transcription factors at

the *IL-4* promoter in a way that is distinct from that at *IL-13* and *TNF* promoters. Alternatively, MKK3 may regulate additional transcription factors that may play a role in the regulation of IL-4 expression. We analyzed NFAT and NF-κB activation in MKK3^{-/-} BMMCs and found no major deficiency in these transcription factors following IgE activation. We also analyzed the *IL-4* promoter for further impairment of potential transcription factors, including Oct-1, SRF, and the GATA family, but found no deficiency by EMSA and Western blot analysis (data not shown). Thus, the exact mechanism of MKK3–Egr1 pathway on the specific regulation of IL-4 expression requires further study.

Given that Egr1 is expressed de novo (11, 28), it stands to reason that MKK3 signaling likely plays a role in a mechanism supporting *Egr1* gene transcription. It seems probable that MKK3 may direct downstream signaling, most likely through p38 (10, 29), which acts on the *Egr1* promoter itself, which may then lead to IL-4 production through de novo-expressed Egr1 activity in the nucleus of activated mast cells. Without full Egr1 expression, *IL-4* transcription is downregulated, likely as a result of this missing key transcription factor. However, the mechanism of MKK3-dependent signaling on the regulation of Egr1 expression remains to be determined.

Collectively, we demonstrated a novel finding that MKK3 signaling contributes to allergic inflammation via mast cell IL-4 production in vitro and in vivo. Given the significance of IL-4 in allergic inflammatory disorders, MKK3 offers a highly specific and unique pathway affecting mast cell IL-4 production. This finding offers a new signaling mechanism to target in the development of therapies for allergic disease. IL-4 is a strong candidate target in the development of therapeutics for allergy, as evidenced by current clinical trials testing the effectiveness of

soluble IL-4R in the treatment of patients with allergic diseases (20, 30). The p38 MAPK pathway has drawn attention in the development of therapies for inflammatory conditions, such as rheumatoid arthritis, but inhibitors of p38 have been hampered by organ toxicity and other undesirable side effects (31). Targeting upstream proteins with specificity for the p38 subfamily may offer improved therapies for inflammatory disorders, including allergic diseases.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References

- Kawakami, T., and S. J. Galli. 2002. Regulation of mast-cell and basophil function and survival by IgE. *Nat. Rev. Immunol.* 2: 773–786.
- Gilfillan, A. M., and C. Tkaczuk. 2006. Integrated signalling pathways for mast-cell activation. *Nat. Rev. Immunol.* 6: 218–230.
- Galli, S. J., M. Tsai, and A. M. Piliponsky. 2008. The development of allergic inflammation. *Nature* 454: 445–454.
- Kinet, J. P. 1999. The high-affinity IgE receptor (Fc epsilon RI): from physiology to pathology. *Annu. Rev. Immunol.* 17: 931–972.
- Eiseman, E., and J. B. Bolen. 1992. Engagement of the high-affinity IgE receptor activates src protein-related tyrosine kinases. *Nature* 355: 78–80.
- Parravicini, V., M. Gadina, M. Kovarova, S. Odom, C. Gonzalez-Espinosa, Y. Furumoto, S. Saitoh, L. E. Samelson, J. J. O'Shea, and J. Rivera. 2002. Fyn kinase initiates complementary signals required for IgE-dependent mast cell degranulation. *Nat. Immunol.* 3: 741–748.
- Hutchinson, L. E., and M. A. McCloskey. 1995. Fc epsilon RI-mediated induction of nuclear factor of activated T-cells. *J. Biol. Chem.* 270: 16333–16338.
- Marquardt, D. L., and L. L. Walker. 2000. Dependence of mast cell IgE-mediated cytokine production on nuclear factor-kappaB activity. *J. Allergy Clin. Immunol.* 105: 500–505.
- Brancho, D., N. Tanaka, A. Jaeschke, J. J. Ventura, N. Kelkar, Y. Tanaka, M. Kyuma, T. Takeshita, R. A. Flavell, and R. J. Davis. 2003. Mechanism of p38 MAP kinase activation in vivo. *Genes Dev.* 17: 1969–1978.
- Cook, R., C. C. Wu, Y. J. Kang, and J. Han. 2007. The role of the p38 pathway in adaptive immunity. *Cell. Mol. Immunol.* 4: 253–259.
- Li, B., M. R. Power, and T. J. Lin. 2006. De novo synthesis of early growth response factor-1 is required for the full responsiveness of mast cells to produce TNF and IL-13 by IgE and antigen stimulation. *Blood* 107: 2814–2820.
- Decker, E. L., N. Nehmann, E. Kampen, H. Eibel, P. F. Zipfel, and C. Skerka. 2003. Early growth response proteins (EGR) and nuclear factors of activated T cells (NFAT) form heterodimers and regulate proinflammatory cytokine gene expression. *Nucleic Acids Res.* 31: 911–921.
- Chapman, N. R., and N. D. Perkins. 2000. Inhibition of the RelA(p65) NF-kappaB subunit by Egr-1. *J. Biol. Chem.* 275: 4719–4725.
- Lu, H. T., D. D. Yang, M. Wysz, E. Gatti, I. Mellman, R. J. Davis, and R. A. Flavell. 1999. Defective IL-12 production in mitogen-activated protein (MAP) kinase kinase 3 (Mkk3)-deficient mice. *EMBO J.* 18: 1845–1857.
- Nagai, H., T. Abe, I. Yamaguchi, K. Mito, M. Tsunematsu, M. Kimata, and N. Inagaki. 2000. Role of mast cells in the onset of IgE-mediated late-phase cutaneous response in mice. *J. Allergy Clin. Immunol.* 106: S91–S98.
- Ng, S. W., J. di Capite, K. Singaravelu, and A. B. Parekh. 2008. Sustained activation of the tyrosine kinase Syk by antigen in mast cells requires local Ca²⁺ influx through Ca²⁺ release-activated Ca²⁺ channels. *J. Biol. Chem.* 283: 31348–31355.
- Li, B., J. Berman, J. T. Tang, and T. J. Lin. 2007. The early growth response factor-1 is involved in stem cell factor (SCF)-induced interleukin 13 production by mast cells, but is dispensable for SCF-dependent mast cell growth. *J. Biol. Chem.* 282: 22573–22581.
- Feldmann, M. 2008. Many cytokines are very useful therapeutic targets in disease. *J. Clin. Invest.* 118: 3533–3536.
- Brown, M. A., and J. Hural. 1997. Functions of IL-4 and control of its expression. *Crit. Rev. Immunol.* 17: 1–32.
- Mohapatra, S. S., M. Qazi, and G. Hellermann. 2010. Immunotherapy for allergies and asthma: present and future. *Curr. Opin. Pharmacol.* 10: 276–288.
- Weiss, D. L., and M. A. Brown. 2001. Regulation of IL-4 production in mast cells: a paradigm for cell-type-specific gene expression. *Immunol. Rev.* 179: 35–47.
- Gauchat, J. F., S. Henchoz, G. Mazzei, J. P. Aubry, T. Brunner, H. Blasey, P. Life, D. Talabot, L. Flores-Romo, J. Thompson, et al. 1993. Induction of human IgE synthesis in B cells by mast cells and basophils. *Nature* 365: 340–343.
- Plaut, M., J. H. Pierce, C. J. Watson, J. Hanley-Hyde, R. P. Nordan, and W. E. Paul. 1989. Mast cell lines produce lymphokines in response to cross-linkage of Fc epsilon RI or to calcium ionophores. *Nature* 339: 64–67.
- Inoue, T., D. L. Boyle, M. Corr, D. Hammaker, R. J. Davis, R. A. Flavell, and G. S. Firestein. 2006. Mitogen-activated protein kinase kinase 3 is a pivotal pathway regulating p38 activation in inflammatory arthritis. *Proc. Natl. Acad. Sci. USA* 103: 5484–5489.
- Remy, G., A. M. Risco, F. A. Iñesta-Vaquera, B. González-Terán, G. Sabio, R. J. Davis, and A. Cuenda. 2010. Differential activation of p38MAPK isoforms by MKK6 and MKK3. *Cell. Signal.* 22: 660–667.
- Enslin, H., J. Raingeaud, and R. J. Davis. 1998. Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6. *J. Biol. Chem.* 273: 1741–1748.
- Lohoff, M., M. Giaisi, R. Köhler, B. Casper, P. H. Krammer, and M. Li-Weber. 2010. Early growth response protein-1 (Egr-1) is preferentially expressed in T helper type 2 (Th2) cells and is involved in acute transcription of the Th2 cytokine interleukin-4. *J. Biol. Chem.* 285: 1643–1652.
- Yang, Y. J., W. Chen, A. Edgar, B. Li, J. D. Molkentin, J. N. Berman, and T. J. Lin. 2009. Rcan1 negatively regulates Fc epsilonRI-mediated signaling and mast cell function. *J. Exp. Med.* 206: 195–207.
- Tamma, S. M., K. W. Chung, T. Patel, S. P. Balan, and S. Pahwa. 2006. p38 MAPK plays a role in IL-4 synthesis in jacalin plus CD28-stimulated CD4+ T cells—II. *J. Leukoc. Biol.* 79: 1339–1347.
- Borish, L. C., H. S. Nelson, J. Corren, G. Bensch, W. W. Busse, J. B. Whitmore, J. M. Agosti; IL-4R Asthma Study Group. 2001. Efficacy of soluble IL-4 receptor for the treatment of adults with asthma. *J. Allergy Clin. Immunol.* 107: 963–970.
- Kumar, S., J. Boehm, and J. C. Lee. 2003. p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. *Nat. Rev. Drug Discov.* 2: 717–726.

Supplemental Figure Legends

Figure S1. *MKK3* deficiency affects *MKK3* expression, but no mast cell production *in vivo*.

A. Heterozygous (*MKK3*^{+/-}) mice have partially impaired *MKK3* expression. Skin tissue from the ear and tissue from the spleen were collected from two *MKK3*^{+/+}, two ^{+/-}, and two ^{-/-} mice (M1, mouse 1; M2, mouse 2). Tissues were homogenized and lysates were prepared in RIPA buffer with protease inhibitors. Cleared protein samples (40 µg) were analyzed by Western blotting for total *MKK3*. Samples were also probed for actin as a loading control. **B and C.** *MKK3*-deficient mice have normal mast cell numbers. Back skin, tongue, and ear tissue from wild-type and *MKK3*-deficient mice was fixed, sectioned, and stained with alcian blue. Similar staining and mast cell numbers were observed for wild-type and *MKK3*-deficient mice (**B**), a representative image from tongue tissue is shown (**C**). Specimens were viewed on a microscope (Eclipse E600; Nikon) equipped with a camera (DMX1200; Nikon) and a DDL 40 × /0.75 objective lens. Acquisition was performed with Nikon ACT-1 software (version 2.20). Images were processed using Adobe Photoshop software (version 5.0). Data are representative of sections taken from 2-5 mice per group. The number of mast cells was enumerated by counting 5 randomly selected fields. Data are means ± SEM (*n* = 5 fields). Bars = 5 µm.

Figure S2. *MKK3* deficiency specifically affects IL-4 production by mast cells. **A.** Cytokines found on the protein arrays by position. **B.** Cell-free culture supernatants from *MKK3*^{+/+} and *MKK3*^{-/-} BMMCs were analyzed for production of 40 cytokines by protein array. Samples were collected at 6 hours following IgE-dependent mast cell activation. Out of 40 cytokines tested, a specific reduction in IL-4 production was observed. **C.** CCL3 production by wild-type (*MKK3*^{+/+}) and *MKK3*-deficient (^{-/-}) FcεRI-stimulated BMMCs was assessed by ELISA in cell-free culture media following a time course of stimulation.

Figure S3. NFAT activity in FcεRI-mediated mast cell activation. **A.** NFAT activity was assessed in wild-type (^{+/+}) and *MKK3*-deficient (^{-/-}) BMMCs over a time course of TNP-BSA stimulation through FcεRI by EMSA. **B.** Competition and supershift assay demonstrates specificity of the NFAT probe.

Figure S4. Egr1 protein expression is impaired in MKK3-deficient BMMCs. MKK3^{+/+} and MKK3^{-/-} BMMCs were analyzed for Egr1 protein expression by immunofluorescence. IgE-sensitized MKK3^{-/-} BMMCs displayed a partially inhibited induction of Egr1 expression following stimulation with TNP-BSA. Cells were stained with rabbit anti-Egr1 or rabbit serum as a control and cell nuclei were stained with DAPI. Images are representative of $n = 4$ separate experiments.

Fig S1

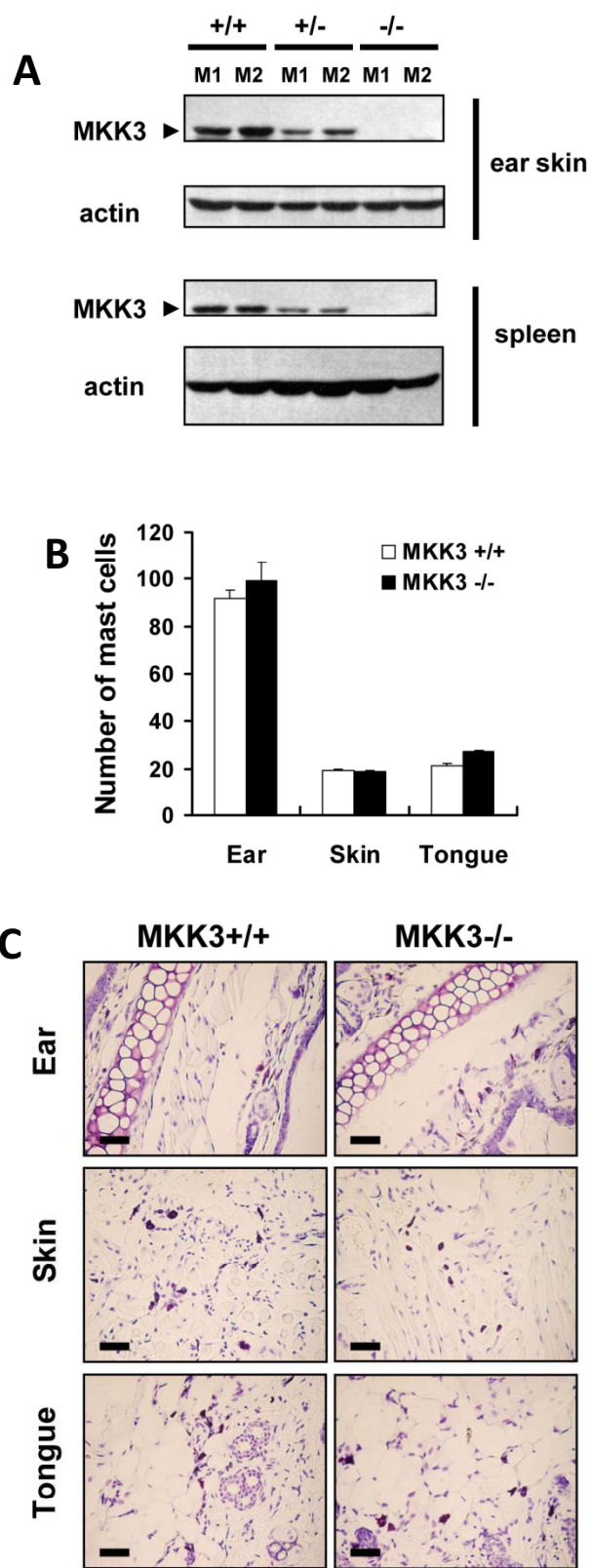


Fig S2

A

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1	POS	POS	NEG	NEG	Blank	BLC	CD30L	Eotaxin	Eotaxin-2	Fas Ligand	Fractalkine	GCSF
2	POS	POS	NEG	NEG	Blank	BLC	CD30L	Eotaxin	Eotaxin-2	Fas Ligand	Fractalkine	GCSF
3	GM-SCF	IFN γ	IL-1a	IL-1b	IL-2	IL-3	IL-4	IL-6	IL-9	IL-10	IL-12p40p70	IL-12p70
4	GM-SCF	IFN γ	IL-1a	IL-1b	IL-2	IL-3	IL-4	IL-6	IL-9	IL-10	IL-12p40p70	IL-12p70
5	IL-13	IL-17	I-TAC	KC	Leptin	LIX	Lymphotactin	MCP-1	MCSF	MIG	Mip-1a	Mip-1g
6	IL-13	IL-17	I-TAC	KC	Leptin	LIX	Lymphotactin	MCP-1	MCSF	MIG	Mip-1a	Mip-1g
7	RANTES	SDF-1	TCA-1	TECK	TIMP-1	TIMP-2	TNF α	sTNF RI	sTNF RII	Blank	Blank	POS
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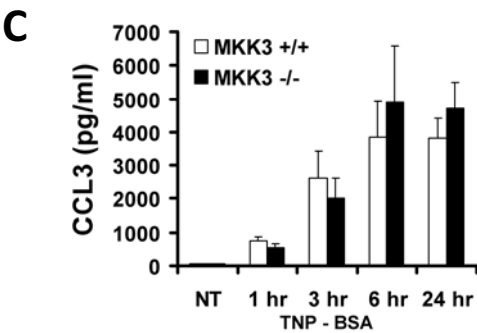
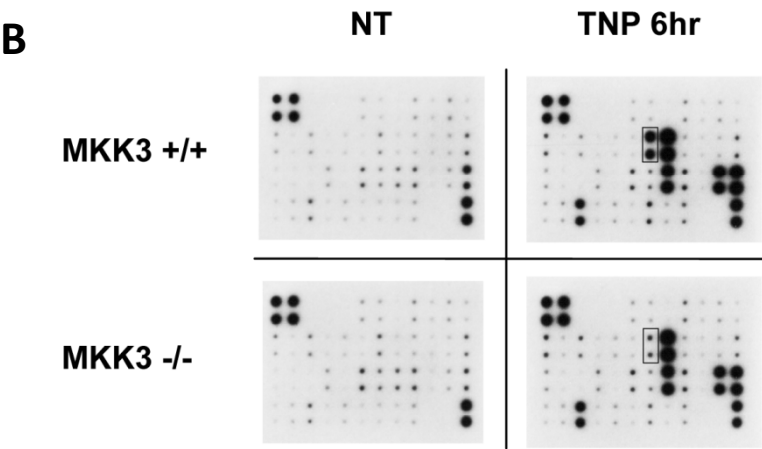
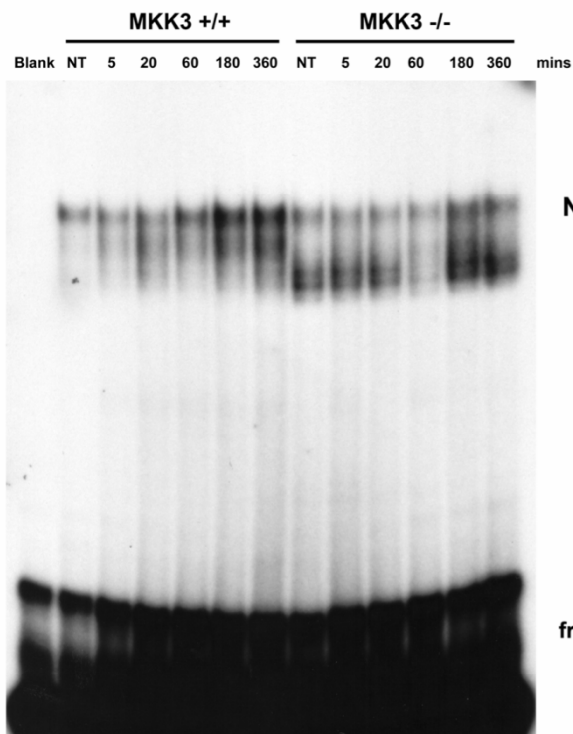
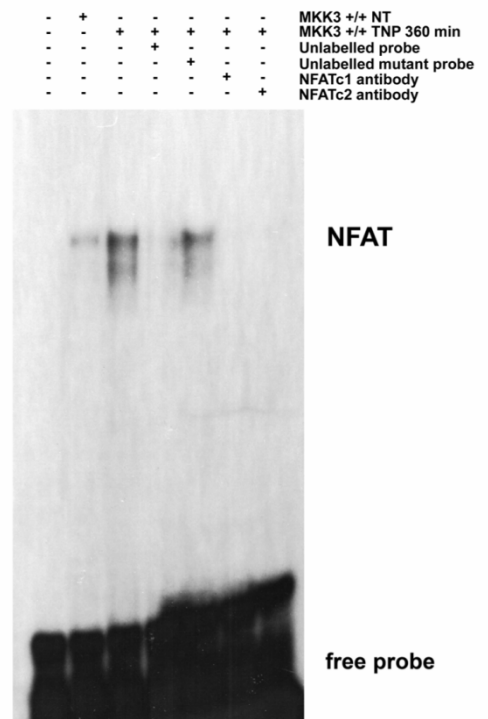


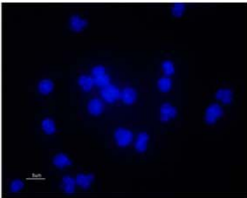
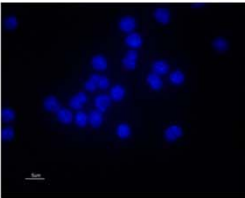
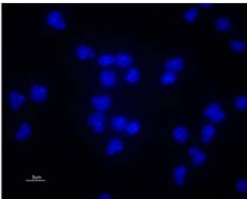
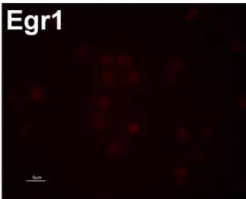
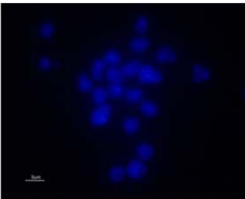
Fig S3

A



B



		MKK3 $+/+$		MKK3 $-/-$	
NT					
					
TNP 1hr		