

I κ B Kinase 2 Is Essential for IgE-Induced Mast Cell De Novo Cytokine Production but Not for Degranulation

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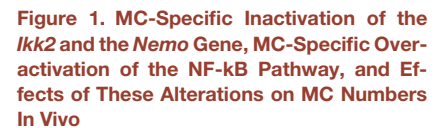
SUMMARY

The immunoglobulin E (IgE)-mediated mast cell (MC) response is central to the pathogenesis of type I allergy and asthma. I κ B kinase 2 (IKK2) was reported to couple IgE-induced signals to MC degranulation by phosphorylating the SNARE protein SNAP23. We investigated MC responses in mice with MC-specific inactivation of IKK2 or NF- κ B essential modulator (NEMO), or animals with MC-specific expression of a mutant, constitutively active IKK2. We show that the IgE-induced late-phase cytokine response is reduced in mice lacking IKK2 or NEMO in MCs. However, anaphylactic in vivo responses of these animals are not different from those of control mice, and in vitro IKK2-deficient MCs readily phosphorylate SNAP23 and degranulate similarly to control cells in response to allergen or calcium ionophore. Constitutive overactivation of the NF- κ B pathway has only slight effects on allergen-triggered MC responses. Thus, IKK2 is dispensable for MC degranulation, and the important question how IgE-induced signals trigger MC vesicle fusion remains open.

INTRODUCTION

Mast cells (MCs) are the most important effector cells in immunoglobulin E (IgE)-mediated allergic immune responses (Galli and Tsai, 2012). IgE binds to the high-affinity receptor for IgE (Fc ϵ RI) on MCs, and crosslinking of Fc ϵ RI-bound IgE by multivalent antigen triggers immediate release of preformed proinflammatory mediators stored in the prominent cytoplasmic granules, including histamine and tumor necrosis factor (TNF). Several hours after IgE-mediated exocytosis of secretory granules (“degranulation”), the MC secretes a range of cytokines, chemokines, and lipid mediators synthesized de novo in response to the stimulus (“late-phase response”). Both phases of the MC

IgE response are key events in the pathogenesis of allergic disease (Galli and Tsai, 2012). Crosslinking of MC-bound IgE triggers a signaling cascade (reviewed in Kambayashi and Koretzky, 2007) resulting in IP₃- and DAG-triggered Ca²⁺ mobilization and activation of protein kinase (PK) C, which leads to degranulation and induction of genes encoding proinflammatory mediators. Fc ϵ RI-induced gene induction is controlled among others via NF- κ B transcription factors (Jeong et al., 2002; Klemm et al., 2006; Lorentz et al., 2003; Peng et al., 2005). These dimeric factors are sequestered in the cytosol by inhibitor of NF- κ B (I κ B) proteins in the steady state. NF- κ B activity is controlled by the IKK complex, which phosphorylates I κ B proteins, targeting them for proteasomal degradation thereby releasing NF- κ B to activate transcription. The IKK complex consists of two kinases, IKK1 and IKK2, and the essential scaffolding protein NEMO. Downstream of PKC activated by Fc ϵ RI-induced second messenger signals, Bcl10 and Malt1 transduce a signal activating the IKK complex and thereby elicit NF- κ B translocation and MC late-phase cytokine production (Klemm et al., 2006). In contrast to de novo mediator synthesis, Fc ϵ RI-induced MC degranulation does not depend on NF- κ B (Klemm et al., 2006; Marquardt and Walker, 2000). Calcium sensors activated by proximal Fc ϵ RI signaling activate the soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE) protein machinery, which triggers fusion of secretory vesicles with the plasma membrane (Lorentz et al., 2012). An important MC SNARE protein is synaptosome-associated protein of 23 kDa (SNAP23) (Lorentz et al., 2012). Although phosphorylation of SNAP23 on residues 95 and 120 is essential for MC degranulation (Hepp et al., 2005), little was known about events coupling the second messenger signals to SNARE protein activation. IKK2 was proposed to phosphorylate MC SNAP23 in response to IgE signals in an NF- κ B-independent fashion (Suzuki and Verma, 2008). Given that complete loss of IKK2 causes embryonic lethality, this study relied on MCs differentiated from *Ikk2*^{−/−} fetal liver cells (FLMCs). These cells featured a defect in IgE-mediated SNAP23 phosphorylation and degranulation in vitro. Moreover, anaphylactic responses of MC-deficient Kit mutant mice were restored by reconstitution with wild-type but not *Ikk2*^{−/−} FLMCs.



of the *Ilk2* and the (X-chromosomal) *Nemo* gene in *Ilk2^{FL/FL}Cre⁺* mice and *Nemo^{FL}Cre⁺* mice (male *Nemo^{FL/Y}* and female *Nemo^{FL/FL}*), respectively, was demonstrated by nested single target amplification of the mutated locus from single MCs and non-MCs purified by fluorescence-activated cell sorting (FACS)

from peritoneal lavage or skin cell suspensions (Figures 1A, S1A, S1B, S1D, and S1E; Table S1). Absence of IKK2 and NEMO protein in peritoneal MCs expanded in vitro (PCMCs) was verified by western blot (Figures S1C and S1F). Cell type specificity and efficiency of Cre-mediated activation of the *ROSA26 Ikk2ca^{sFL}* knockin allele was determined by monitoring expression of the eGFP marker coexpressed with IKK2ca (Figures S1G–S1I). Spontaneous degradation of I κ B α was demonstrated in PCMCs (Figure 1B). Loss of IKK2 or NEMO or expression of constitutively active IKK2 did not affect numbers (Figure 1C) and phenotype of MCs in peritoneal lavage or skin except for a slight reduction of CD117 expression (data not shown).

Complete Loss or Constitutive Gain of IKK2 Activity or IKK Disruption through NEMO Ablation Specifically in Mast Cells Do Not Influence IgE-Induced Anaphylaxis In Vivo

The *Mcpt5-Cre* transgene is active in CTMCs but not in mucosal MCs (Dudek et al., 2011). We first determined the contribution of CTMCs in the anaphylactic response to intraperitoneally (i.p.) injected allergen and induced passive systemic anaphylaxis (PSA) in *R-DTA⁺Mcpt5-Cre⁺* mice. These mice are characterized by profound CTMC deficiency (Dudek et al., 2011). Other hematopoietic cell types, including basophils, were not altered in numbers (Figure S2A). Although body temperature (determined

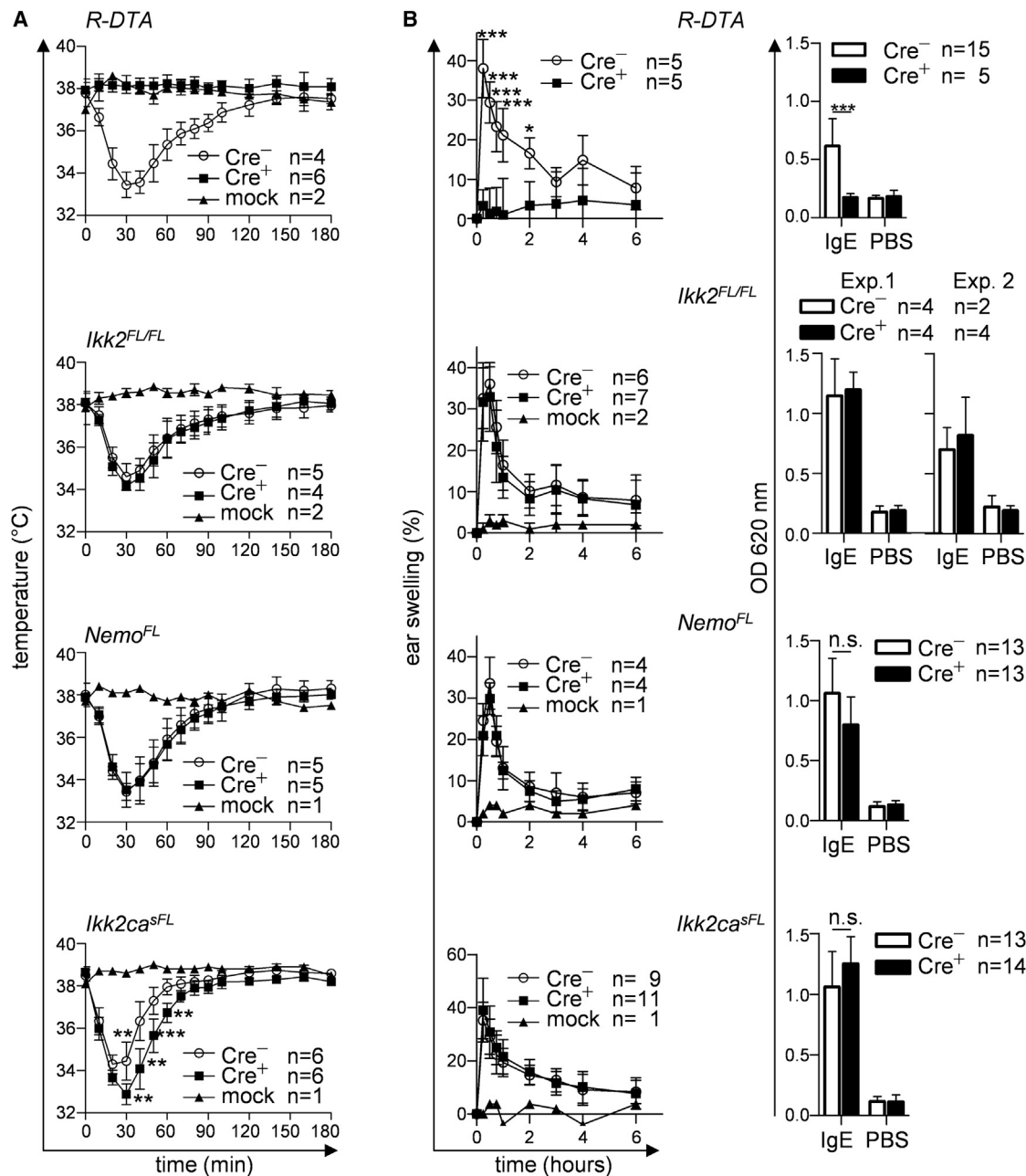


Figure 2. Neither MC IKK2 nor NEMO Is Required for IgE-Induced Anaphylaxis, and Constitutive Activity of IKK2 Has Little Effect on Anaphylaxis In Vivo

(A) PSA in *R-DTA⁺Mcpt5-Cre⁺* mice (constitutively MC-deficient, see also Figure S2), *Ikk2^{FL/FL}Mcpt5-Cre⁺*, *Nemo^{FL}Mcpt5-Cre⁺*, *Ikk2ca^{SFL}Mcpt5-Cre⁺* mice, and the respective Cre-negative littermate controls. Drop of body temperature was measured using a rectal probe. Mock controls were sensitized but received saline instead of allergen challenge. The slight increase of amplitude and duration of the response of *Ikk2ca^{SFL}Mcpt5-Cre⁺* compared to control mice was not reproduced in a second experiment based on infrared temperature monitoring (Figure S2B).

(B) PCA in *R-DTA⁺Mcpt5-Cre⁺* mice, *Ikk2^{FL/FL}Mcpt5-Cre⁺* and *Nemo^{FL}Mcpt5-Cre⁺* mice, *Ikk2ca^{SFL}Mcpt5-Cre⁺* mice, and the respective Cre-negative controls. Mock controls were not sensitized but challenged. *R-DTA⁺Cre⁻* mock controls gave results similar to all other genotypes (not shown for graphical clarity). Mean ear swelling $\pm$ SD is displayed. Vascular leak was quantified by measuring OD of Evans blue extracted from ear tissue. PBS-injected ears served as control. Mean $\pm$ SD is displayed in (A) and (B). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (comparison between *Cre⁺* and *Cre⁻* in A and B).

by rectal probe) dropped by 4°C in *R-DTA⁺Cre*-negative control mice, no response was detectable in MC-deficient *R-DTA⁺Mcpt5-Cre⁺* animals (Figure 2A).

We also analyzed *R-DTA⁺Mcpt5-Cre⁺* for their capacity to mount immediate phase passive cutaneous anaphylaxis (PCA) responses. Animals were locally sensitized with anti-DNP-IgE

by injections into the ear pinna. Following intravenous (i.v.) challenge with HSA-DNP 24 hr later, we monitored ear thickness and vascular leakage (Figure 2B). Allergen-induced ear swelling and vascular leakage were reduced to control levels in the MC-deficient group. These results showed that CTMCs are the major effector cells in the PSA response to i.p.-injected allergen as well as the PCA response to i.v.-injected allergen.

To test whether deletion of IKK2 or NEMO in CTMCs or MC-specific expression of constitutively active IKK2 affected IgE-induced in vivo responses, PSA was induced in the mutant mouse lines. MC-specific loss of IKK2 or NEMO had no effect on the response to the allergen (Figure 2A). We performed independent experiments in which skin temperature was measured using an infrared camera (Figure S2B) and obtained similar results. Expression of constitutively active IKK2 in connective tissue MCs resulted in enhanced and prolonged PSA in the first experiment (Figure 2A), which, however, was not reproduced in a second experiment based on infrared skin temperature recording (Figure S2B). Confounding effects of the *Mcpt5-Cre* transgene itself were excluded by analysis of *Mcpt5-Cre*⁺ mice devoid of *loxP*-flanked genes, which behaved indistinguishably from wild-type mice (Figure S2B). Thus, MC IKK2 and NEMO are dispensable for systemic anaphylaxis and constitutive IKK2 activity in MCs enhances anaphylaxis only moderately, if at all.

We next investigated the PCA response of mice of the three mutant lines. The ear swelling response of the MC-specific IKK2 and NEMO mutant mice was not different from that of controls (Figure 2B). Likewise, allergen-induced extravasation of Evans blue was not different between mutant and control animals (Figure 2B). *Ikk2ca^{FL}Mcpt5-Cre*⁺ mice did not show significantly different responses in ear swelling or Evans blue extravasation compared to control mice (Figure 2B). *Mcpt5-Cre*⁺ mice without *loxP*-flanked genes were analyzed to control for effects of the transgene alone (Figure S2C). Thus, MC-specific inactivation of the *Ikk2* or the *Nemo* gene does not affect IgE-mediated anaphylactic responses in vivo. Selective expression of constitutively active IKK2 in MCs resulted in significantly enhanced responses in only one of three independent assays (PSA, PCA, and Evans blue extravasation) and in the PSA assay only in one of two independent experiments. We therefore conclude that inhibition or constitutive activation of IKK2 does not affect anaphylactic responses in vivo.

Loss of IKK2 or NEMO or Constitutive IKK2 Activity Do Not Affect MC Degranulation or SNARE Protein Phosphorylation In Vitro

Suzuki and Verma described impaired in vitro degranulation of MCs differentiated from the fetal liver of *Ikk2*^{−/−} mice and an essential function of IKK2 in the phosphorylation of SNAP23 (Suzuki and Verma, 2008). We triggered degranulation of PCMCs derived from *Ikk2^{FL/FL}Mcpt5-Cre*⁺ and *Nemo^{FL}Mcpt5-Cre*⁺ mice. Absence of the respective proteins in these cells was demonstrated by western blot (Figures S1C and S1F). Degranulation of IKK2-deficient PCMCs and of NEMO-deficient PCMCs was similar to the response of control cells as determined by quantification of surface phosphatidylserine. This molecule translocates to the outer leaflet of the plasma membrane upon MC degranulation and can be detected by annexin V staining

(Figures 3A and 3B). Allergen-triggered IKK2-deficient and NEMO-deficient PCMCs released similar amounts of β -hexosaminidase as control cells (Figure 3A). Also, the early release of TNF, which is stored in the MC granules, is a measure of degranulation (before onset of IgE-induced de novo synthesis of this cytokine). IKK2- and NEMO-deficient MCs did not differ from control cells in TNF release 30 min after allergen challenge (Figure 3C; data not shown). In addition to triggering degranulation by IgE crosslinking, we treated IKK2- or NEMO-deficient PCMCs with the calcium ionophore A23187 and observed similar β -hexosaminidase release compared to control cells (Figure 3A). Moreover, we found unimpaired allergen-induced degranulation of IKK2-deficient bone marrow-derived MCs (BMMCs) (Figures S3A–S3D). Allergen-induced phosphorylation of SNAP23 on Ser95 and Ser120 was not affected by IKK2-deficiency as demonstrated by western blot (Figures 3D, 3E, and S3E). Expression of constitutively active IKK2 in PCMCs from *Ikk2ca^{FL}Mcpt5-Cre*⁺ mice did not affect allergen-induced degranulation (Figure 3A). In summary, we show that IKK2 and NEMO are dispensable for IgE-induced SNAP23 phosphorylation and MC degranulation in vitro.

IKK2 and NEMO Are Essential for the MC Late-Phase Response to Allergen

Hours after IgE crosslinking, MCs de novo synthesize TNF, interleukin 6 (IL-6), pro-IL-1 β , and other mediators. In order to test whether this late-phase response is dependent on IKK complex components, we sensitized mice with MC-specific deficiency for IKK2 or NEMO, or animals with MC-specific expression of constitutively active IKK2 by i.v. injection of anti-DNP-IgE. The animals were challenged 24 hr later by epicutaneous administration of DNFB, and the resulting ear swelling was monitored. The response was severely impaired in mice lacking IKK2 or NEMO in MCs (Figures 4A, 4B, and S4A). *Ikk2ca^{FL}Mcpt5-Cre*⁺ animals showed slightly enhanced responses compared to controls; however, the difference was significant only at one time point (Figure 4C).

In order to assess IgE-induced de novo expression of proinflammatory cytokines, we stimulated IKK2-deficient BMMCs (Figure S4B), NEMO-deficient PCMCs, and IKK2ca-overexpressing BMMCs by Fc ϵ RI crosslinking (Figures 4D–4F). The cytokine responses of IKK2- and NEMO-deficient cells were significantly reduced as determined by RT-PCR-based quantification of mRNAs and by determination of cytokine concentrations in the supernatant. In accordance with the results shown in Figure 3, the same cultures were not impaired in their degranulation response (Figure S4C; data not shown). In accordance with the results obtained from IKK2-deficient cells, the cytokine response of cells overexpressing the constitutively active IKK2ca protein featured a significantly enhanced cytokine response (Figure 4F). Thus, IKK2 and NEMO are essential for normal IgE-induced MC cytokine responses and constitutive IKK2 activity enhances these responses.

DISCUSSION

Fc ϵ RI-induced MC degranulation is a key event in the pathogenesis of allergic diseases. Induction of second messengers and

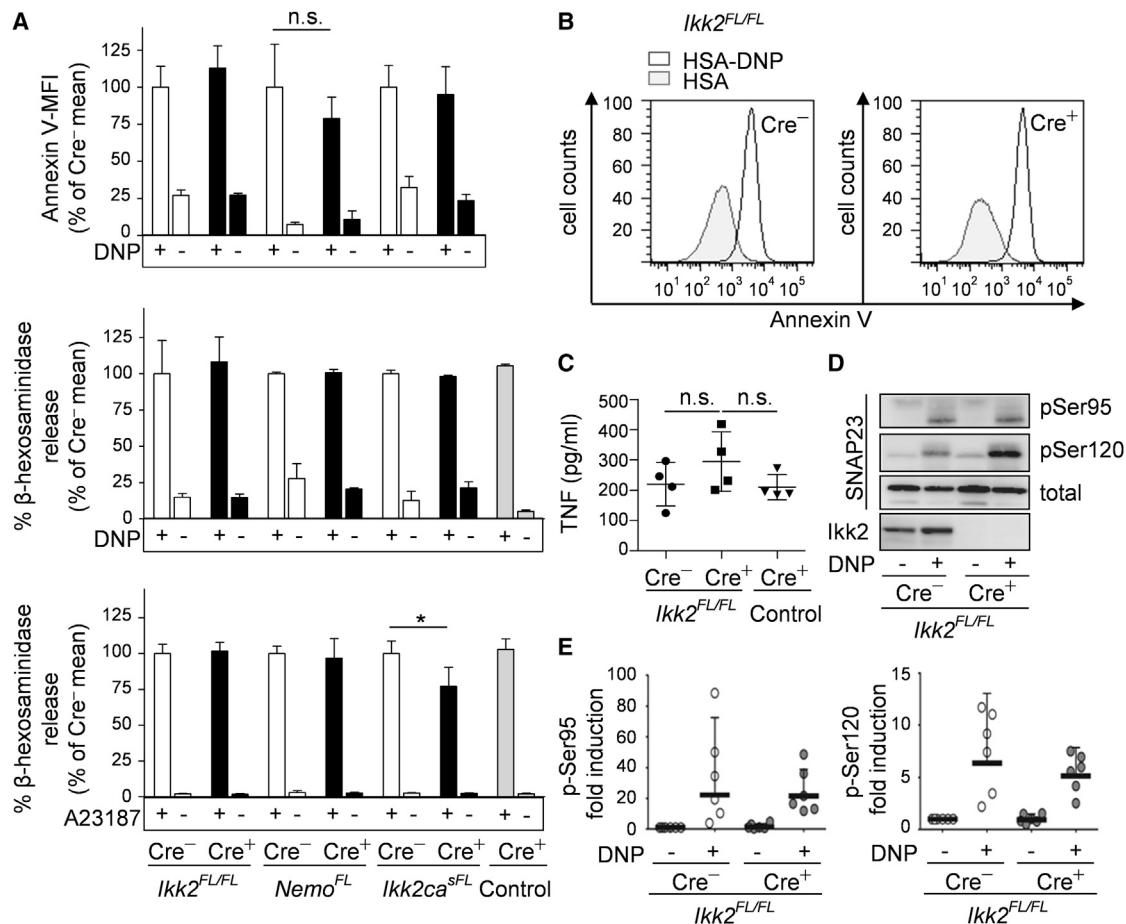


Figure 3. Deficiency for IKK Complex Components or Expression of Constitutively Active IKK2 Has No Effect on MC Degranulation In Vitro Peritoneal lavage cells were cultivated in the presence of stem cell factor and IL-3. The cells were loaded overnight with anti-DNP-IgE and then stimulated with HSA-DNP ("+" DNP"). Control cell samples were exposed to HSA instead of allergen ("– DNP").

(A) Degranulation of PCMCs from *Ikk2^{FL/FL}Mcpt5-Cre⁺* as well as *Nemo^{FL}Mcpt5-Cre⁺* and *Ikk2ca^{sFL}Mcpt5-Cre⁺* mice and the respective Cre-negative littermates (n = 3–7 per group). In two independent experiments, degranulation was quantified by annexin V staining (upper panel) and by determination of β-hexosaminidase release (middle panel). The latter experiment included a "Mcpt5-Cre only" group to exclude confounding effects of the transgene itself. Degranulation was also induced by the ionophore A23187 (lower panel). Means obtained for allergen- or ionophore-treated Cre-negative control cells were set to 100%. Mean ± SD is shown. *p < 0.001, n.s., not significant. IKK2-deficient BMMCs yielded similar results as obtained for IKK2-deficient PCMCs (see Figure S3).

(B) Example of degranulation of *Ikk2^{FL/FL}Mcpt5-Cre⁺* and *Ikk2^{FL/FL}* Cre-negative control PCMCs as determined by flow cytometric quantification of surface annexin V (from the data set represented in A).

(C) TNF release from *Ikk2^{FL/FL}Mcpt5-Cre⁺* as well as Cre-negative and "Mcpt5-Cre only" control PCMCs allergen-stimulated as in (B). Supernatant was harvested for ELISA 30 min after allergen challenge. Mean ± SD is shown.

(D) Phosphorylation of SNAP23 in PCMCs from one *Ikk2^{FL/FL}Mcpt5-Cre⁺* and one Cre-negative control mouse sensitized and triggered as described above was assessed by phospho-specific western blot analysis. See Figure S3 for results obtained for four additional mutant and five additional control samples.

(E) Quantification of phospho-SNAP23 induction in *Ikk2^{FL/FL}Mcpt5-Cre⁺* and control cells by densitometric analysis of all western blot results. Shown are geometric mean and 95% confidence interval of six values per genotype and stimulation condition.

PKC activation by proximal FcεRI signaling has been intensely studied, whereas coupling of these signals to exocytosis of MC granules is incompletely understood. Suzuki and Verma reported that IKK2 serves an essential function in MC degranulation by phosphorylating the SNARE protein SNAP23 (Suzuki and Verma, 2008). We generated mice with selective knockouts of the *Ikk2* or the *Nemo* gene in MCs and mice with MC-specific expression of a constitutively active IKK2 protein. Surprisingly, we found that loss of IKK2 in MCs did not affect their allergen-induced degranulation. Given the tremendous importance of

molecular mechanisms of MC degranulation for human diseases, we decided to use our models to clarify the role of IKK complex components in MC responses. We confirmed that a functional IKK complex is required for the IgE-induced MC late-phase cytokine response. We demonstrate, however, that IKK2 is dispensable for SNARE protein phosphorylation and for degranulation in vitro and in vivo.

Suzuki and Verma differentiated MCs from fetal liver of *Ikk2^{-/-}* embryos (E11.5–12.5) to circumvent lethality of this mutation. These cells were compromised in their degranulation response

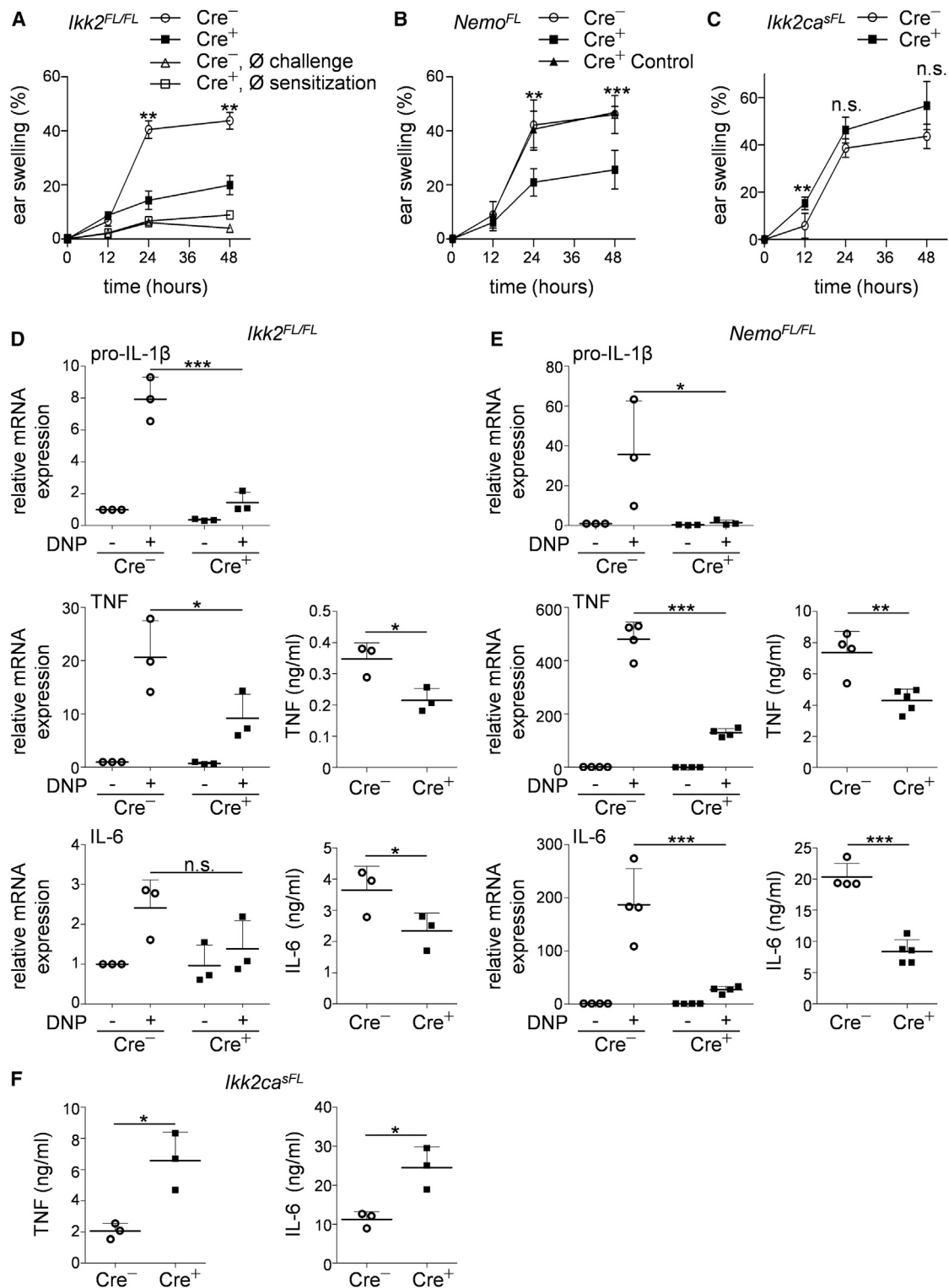


Figure 4. The Late-Phase MC Response Is Dependent on a Functional IKK Complex

LP-PCA was induced by i.v. sensitization with anti-DNP-IgE and challenge by epicutaneous administration of DNFB followed by monitoring of ear thickness. (A) Five *Ikk2^{FL/FL}Mcpt5-Cre⁺* and four Cre-negative littermate controls were analyzed. A Cre-negative control animal was sensitized but treated with vehicle (acetone/olive oil) alone, instead of challenge (Ø challenge). One *Ikk2^{FL/FL}Mcpt5-Cre⁺* was not sensitized but challenged with DNFB (Ø sensitization). The latter two animals served as controls also for data in (B) and (C), which were generated in parallel with data in (A). (See Figure S4 for a second experiment.)

(legend continued on next page)

to Fc ϵ R1 crosslinking. Moreover, upon transfer into MC-deficient WBB6F1-Kit^W/Kit^{W-v} mice, wild-type but not *Ikk2*^{-/-} FLMCs repaired defective anaphylaxis of the recipients. An important difference between the experimental models used is that the entire MC differentiation from *Ikk2*^{-/-} fetal-liver-derived precursors proceeds in the absence of IKK2. In contrast, *Ikk2*^{FL/FL}*Mcpt5*-Cre⁺ mice represent normal animals in which MCs differentiate in the presence of IKK2. Only during the last steps of MC maturation, the *Mcpt5* promoter is activated resulting in Cre-mediated inactivation of the *Ikk2* gene. We hypothesize that IKK2 may serve an important function in earlier steps of MC differentiation (occurring before induction of the *Mcpt5*-promoter), loss of which results in defective MCs characterized by impaired degranulation.

We show that phosphorylation of SNAP23 on Ser95 and Ser120 is not compromised in IKK2-deficient PCMCs. In MCs differentiated under physiological conditions, i.e., in the presence of IKK2, SNAP23 phosphorylation induced by Fc ϵ R1 activation may depend on kinases other than IKK2 or may represent a redundant function of different kinases, including IKK2. MCs lacking IKK2 throughout differentiation from fetal liver cells may be deficient in these pathways. An argument against this concept is that we found normal degranulation in BMMCs differentiated from IKK2-deficient bone marrow cells. Also, these BMMCs lacked IKK2 throughout their development but were not compromised in granule exocytosis. However, abnormal differentiation may be a distinctive feature of MCs differentiated in vitro from fetal liver cells.

Local responses of skin MCs were not changed in mice with MC-specific loss of IKK2, and in vitro degranulation of IKK2-deficient MCs was not different from the response of control cells. Constitutive IKK2 activity in MCs slightly enhanced systemic anaphylaxis in one experiment. This, however, was not reproduced in a second experiment. Therefore, our experiments demonstrate that even overexpression of a constitutively active IKK2 has only a minor, if any, impact on MC degranulation. Collectively, our results rule out an essential role for IKK2 in the signal transduction events leading to MC degranulation.

In conclusion, the question of how Fc ϵ R1-triggered second messenger signals are coupled to MC secretory granule exocytosis remains open. The identification of kinases responsible for allergen-induced SNARE-protein phosphorylation remains an important task because they might represent a target for therapeutic suppression of the MC response in allergic diseases.

EXPERIMENTAL PROCEDURES

Mice

Mcpt5-Cre (Scholten et al., 2008), *Mx*-Cre (Kühn et al., 1995), *R-DTA* (Voehringer et al., 2008), *R26Stop*^{FL}*Ikk2ca* (*Ikk2ca*^{FL}; Sasaki et al., 2006), *Ikk2*^{FL}

(Pasparakis et al., 2002), and *Nemo*^{FL} mice (Schmidt-Suppran et al., 2000), all C57BL/6, were bred and housed at the Experimental Center, Medical Faculty Carl-Gustav Carus, TU Dresden, under specific pathogen-free conditions. In all experiments, littermates were used as controls. All animal experimentation was performed according to institutional guidelines and was approved by the Landesdirektion Dresden.

Passive Systemic Anaphylaxis

Mice were sensitized by i.p. injection of 12.5 μ g SPE-7 anti-DNP-IgE (Eshhar et al., 1980). Twenty-four hours later, mice were challenged by i.p. injection of 200 μ g human serum albumin (HSA)-DNP (Sigma-Aldrich) in 0.9% NaCl or NaCl alone. Temperature drop was assessed using a rectal thermometer (Bio-TK 9882, Bioseb).

Passive Cutaneous Anaphylaxis

Mice were locally sensitized by intradermal injection of 20 ng anti-DNP-IgE (Sigma-Aldrich) in 20 μ l PBS into the left ear pinna. Twenty-four hours later, mice were challenged by i.v. injection of 100 μ g HSA-DNP (Sigma-Aldrich) in 0.9% NaCl. Ear thickness was monitored thereafter using an engineer's micrometer (Mitutoyo). Ear swelling was determined as percentage increase compared to prechallenge ear thickness. Additional mice were sensitized on the left ear as described above but also received an injection of 20 μ l PBS into the right ear. The next day, mice were challenged with 100 μ g HSA-DNP in 140 μ l 2% Evans blue. After 30 min, mice were sacrificed, and ear tissue was incubated in 500 μ l formamide at 50°C for 4–5 days. Extracted dye was quantified photometrically at 620 nm.

For quantification of the late-phase anaphylactic response, mice were sensitized by i.v. injection of 5 μ g SPE-7 anti-DNP-IgE on 2 consecutive days. On day 3, mice were challenged by epicutaneous administration of 10 μ l 0.2% (w/v) DNFB (Sigma-Aldrich) in acetone/olive oil (4:1) onto both sides of the ear. Ear thickness was measured before and 12, 24, and 48 hr after challenge.

Induction and Quantification of MC Degranulation

PCMCs were incubated with 1 μ g/ml SPE-7 anti-DNP-IgE and stimulated with 100 ng/ml HSA-DNP or HSA alone. In some experiments, cells were left unsensitized and were stimulated with 500 ng/ml A23187 (Sigma-Aldrich) for 30 min. β -hexosaminidase release was determined as percentage release of total content (percentage release = 100 $\times$ amount supernatant/ (amount in supernatant + cell lysate)). To quantify surface phosphatidylserin, cells were stained with FITC- or Pacific blue-annexin V (BioLegend) and propidium iodide. See the [Supplemental Experimental Procedures](#) for details.

Real-Time RT-PCR

RNA was reverse transcribed by Revert Aid H Minus RT (Thermo Fisher Scientific). Specific mRNAs were quantified using SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific), and the primers are listed in the [Supplemental Experimental Procedures](#).

ELISA

TNF and IL-6 protein levels in supernatants of stimulated PCMC or BMMC cultures were determined by ELISA (eBiosciences) as recommended by the manufacturer's protocol.

(B) LP-PCA in ten male *Nemo*^{FL/Y}*Mcpt5*-Cre⁺, six Cre-negative controls (five male *Nemo*^{FL/Y}, one female *Nemo*^{FL/FL}, collectively addressed as *Nemo*^{FL}) and four female *Nemo*^{FL/WT}*Mcpt5*-Cre⁺ control mice.

(C) LP-PCA in six *Ikk2ca*^{FL}*Mcpt5*-Cre⁺ and seven Cre⁻ controls.

(D–F) (D) BMMCs from poly I:C-induced *Ikk2*^{FL/FL}*Mx*-Cre⁺ mice, or (E) PCMCs from *Nemo*^{FL}*Mcpt5*-Cre⁺ mice, or (F) BMMCs from poly I:C-induced *Ikk2ca*^{FL}*Mx*-Cre⁺ mice and the respective Cre-negative littermate controls were sensitized and allergen-stimulated as described above (Figure 3). Cytokine mRNA levels were assessed 1 hr after antigen challenge by quantitative RT-PCR. Ct values were normalized to TBP and relative changes in transcript levels compared to HSA-exposed Cre-negative control cells are displayed.

All samples were assayed in triplicates. Concentrations of TNF and IL-6 in the culture supernatant were determined 6 hr after antigen challenge by ELISA. Mean $\pm$ SD is displayed. *p < 0.05, **p < 0.01, ***p < 0.001; n.s., not significant.

Statistical Analysis

Data are shown as means $\pm$ SD if not stated otherwise. Statistical analysis was performed using Student's *t* test or two-way ANOVA. Significance levels are indicated in each figure.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.07.046>.

AUTHOR CONTRIBUTIONS

K.P. designed and performed experiments, A.P., K. Heger, R.B., A.D., and N.S. performed experiments, J.S. generated Mcpt5-Cre mice and performed experiments, D.V. developed R-DTA mice, A.D. and K. Hartmann contributed to the design of the study, M.S.-S. and A.R. conceived and supervised the study, and M.S.-S. and A.R. wrote the paper.

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REFERENCES

- Dudeck, A., Dudeck, J., Scholten, J., Petzold, A., Surianarayanan, S., Köhler, A., Peschke, K., Vöhringer, D., Waskow, C., Krieg, T., et al. (2011). Mast cells are key promoters of contact allergy that mediate the adjuvant effects of haptens. *Immunity* 34, 973–984.
- Eshhar, Z., Ofarim, M., and Waks, T. (1980). Generation of hybridomas secreting murine reagenic antibodies of anti-DNP specificity. *J. Immunol.* 124, 775–780.
- Galli, S.J., and Tsai, M. (2012). IgE and mast cells in allergic disease. *Nat. Med.* 18, 693–704.
- Hepp, R., Puri, N., Hohenstein, A.C., Crawford, G.L., Whiteheart, S.W., and Roche, P.A. (2005). Phosphorylation of SNAP-23 regulates exocytosis from mast cells. *J. Biol. Chem.* 280, 6610–6620.
- Jeong, H.J., Koo, H.N., Na, H.J., Kim, M.S., Hong, S.H., Eom, J.W., Kim, K.S., Shin, T.Y., and Kim, H.M. (2002). Inhibition of TNF- α and IL-6 production by Aucubin through blockade of NF- κ B activation RBL-2H3 mast cells. *Cytokine* 18, 252–259.
- Kambayashi, T., and Koretzky, G.A. (2007). Proximal signaling events in Fc epsilon RI-mediated mast cell activation. *J. Allergy Clin. Immunol.* 119, 544–552, quiz 553–554.
- Klemm, S., Gutermuth, J., Hültner, L., Sparwasser, T., Behrendt, H., Peschel, C., Mak, T.W., Jakob, T., and Ruland, J. (2006). The Bcl10-Malt1 complex segregates Fc epsilon RI-mediated nuclear factor kappa B activation and cytokine production from mast cell degranulation. *J. Exp. Med.* 203, 337–347.
- Kühn, R., Schwenk, F., Aguet, M., and Rajewsky, K. (1995). Inducible gene targeting in mice. *Science* 269, 1427–1429.
- Lorentz, A., Klopp, I., Gebhardt, T., Manns, M.P., and Bischoff, S.C. (2003). Role of activator protein 1, nuclear factor- κ B, and nuclear factor of activated T cells in IgE receptor-mediated cytokine expression in mature human mast cells. *J. Allergy Clin. Immunol.* 111, 1062–1068.
- Lorentz, A., Baumann, A., Vitte, J., and Blank, U. (2012). The SNARE Machinery in Mast Cell Secretion. *Front. Immunol.* 3, 143.
- Marquardt, D.L., and Walker, L.L. (2000). Dependence of mast cell IgE-mediated cytokine production on nuclear factor- κ B activity. *J. Allergy Clin. Immunol.* 105, 500–505.
- Pasparakis, M., Courtois, G., Hafner, M., Schmidt-Supprian, M., Nenci, A., Toksoy, A., Krampert, M., Goebeler, M., Gillitzer, R., Israel, A., et al. (2002). TNF-mediated inflammatory skin disease in mice with epidermis-specific deletion of IKK2. *Nature* 417, 861–866.
- Peng, Y., Power, M.R., Li, B., and Lin, T.J. (2005). Inhibition of IKK down-regulates antigen + IgE-induced TNF production by mast cells: a role for the IKK-I κ B-NF- κ B pathway in IgE-dependent mast cell activation. *J. Leukoc. Biol.* 77, 975–983.
- Sasaki, Y., Derudder, E., Hobeika, E., Pelanda, R., Reth, M., Rajewsky, K., and Schmidt-Supprian, M. (2006). Canonical NF- κ B activity, dispensable for B cell development, replaces BAFF-receptor signals and promotes B cell proliferation upon activation. *Immunity* 24, 729–739.
- Schmidt-Supprian, M., Bloch, W., Courtois, G., Addicks, K., Israël, A., Rajewsky, K., and Pasparakis, M. (2000). NEMO/IKK gamma-deficient mice model incontinentia pigmenti. *Mol. Cell* 5, 981–992.
- Scholten, J., Hartmann, K., Gerbaulet, A., Krieg, T., Müller, W., Testa, G., and Roers, A. (2008). Mast cell-specific Cre/loxP-mediated recombination in vivo. *Transgenic Res.* 17, 307–315.
- Suzuki, K., and Verma, I.M. (2008). Phosphorylation of SNAP-23 by I κ B kinase 2 regulates mast cell degranulation. *Cell* 134, 485–495.
- Vöhringer, D., Liang, H.E., and Locksley, R.M. (2008). Homeostasis and effector function of lymphopenia-induced “memory-like” T cells in constitutively T cell-depleted mice. *J. Immunol.* 180, 4742–4753.

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Supplemental Information

I κ B Kinase 2 Is Essential for IgE-Induced Mast Cell De Novo Cytokine Production but Not for Degranulation

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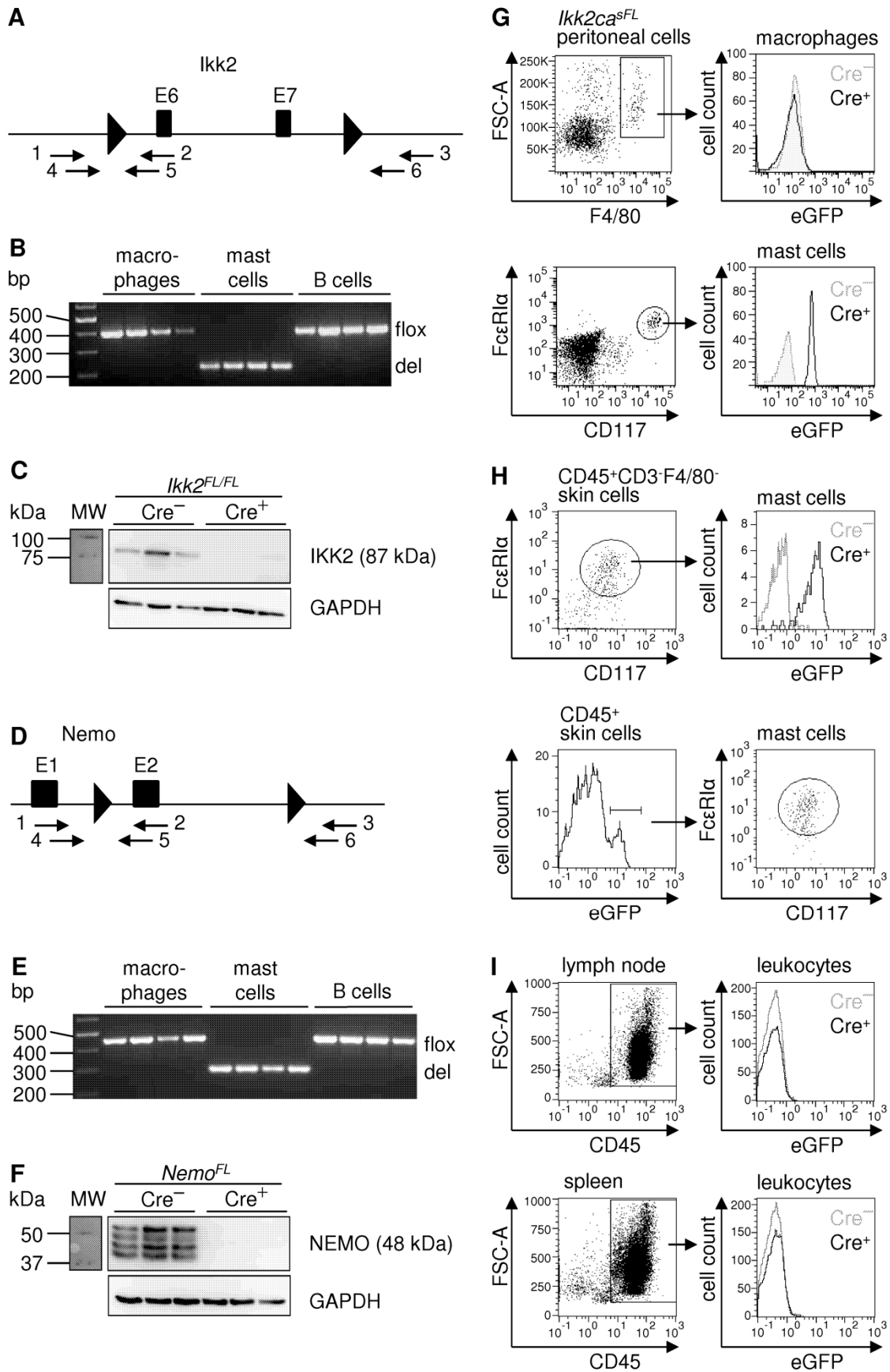


Figure S1. MC-specific inactivation of the *Ikk2* and *Nemo* genes and MC-specific de-repression of the *Ikk2ca^{SFL}* knock in allele, related to Figure 1.

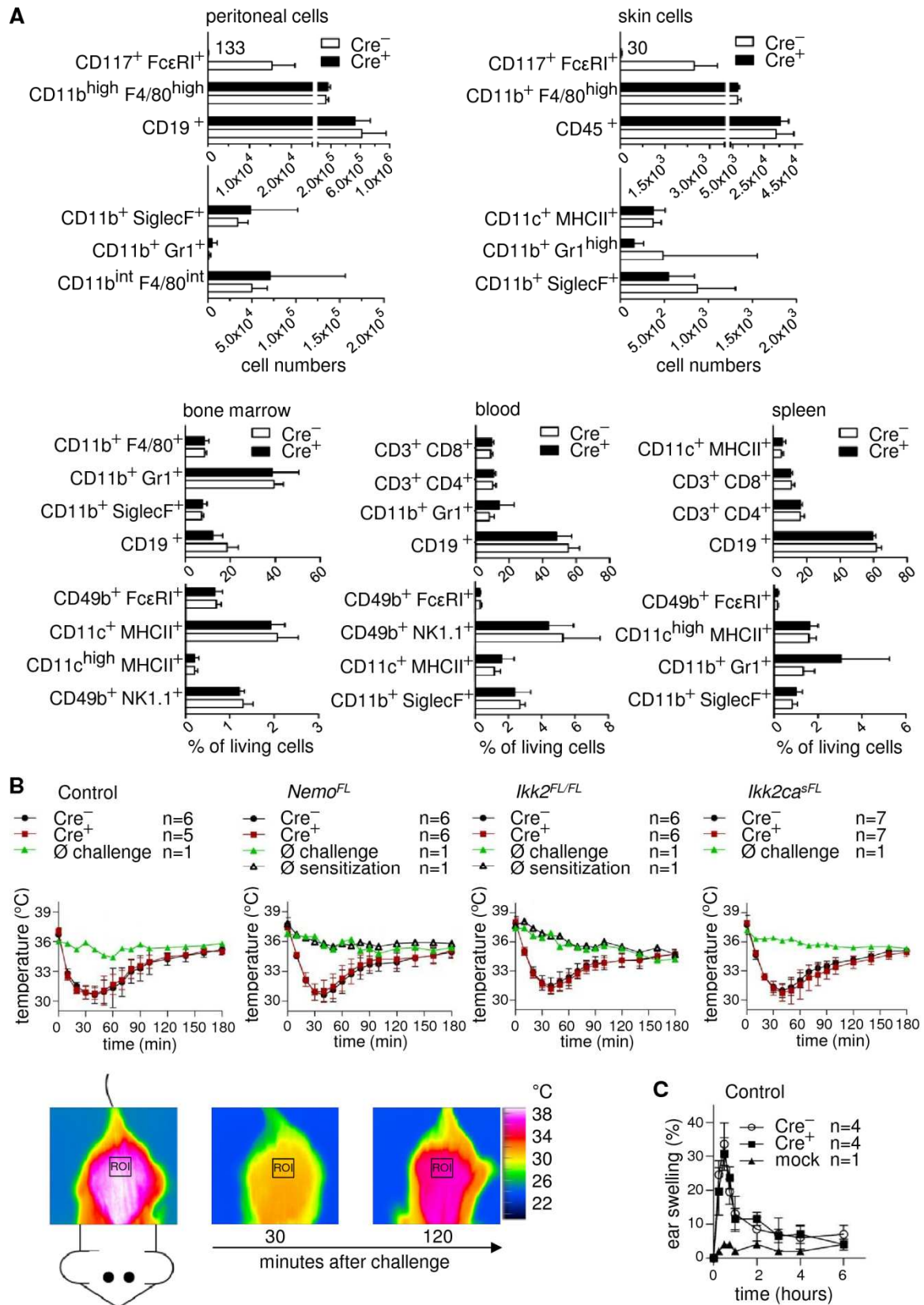


Figure S2. Quantification of different types of non-MCs in MC-deficient *Mcpt5-Cre⁺R-DTA⁺* mice and allergen-induced anaphylaxis in animals with MC-specific inactivation or over-activation of the IKK complex, related to Figure 2.

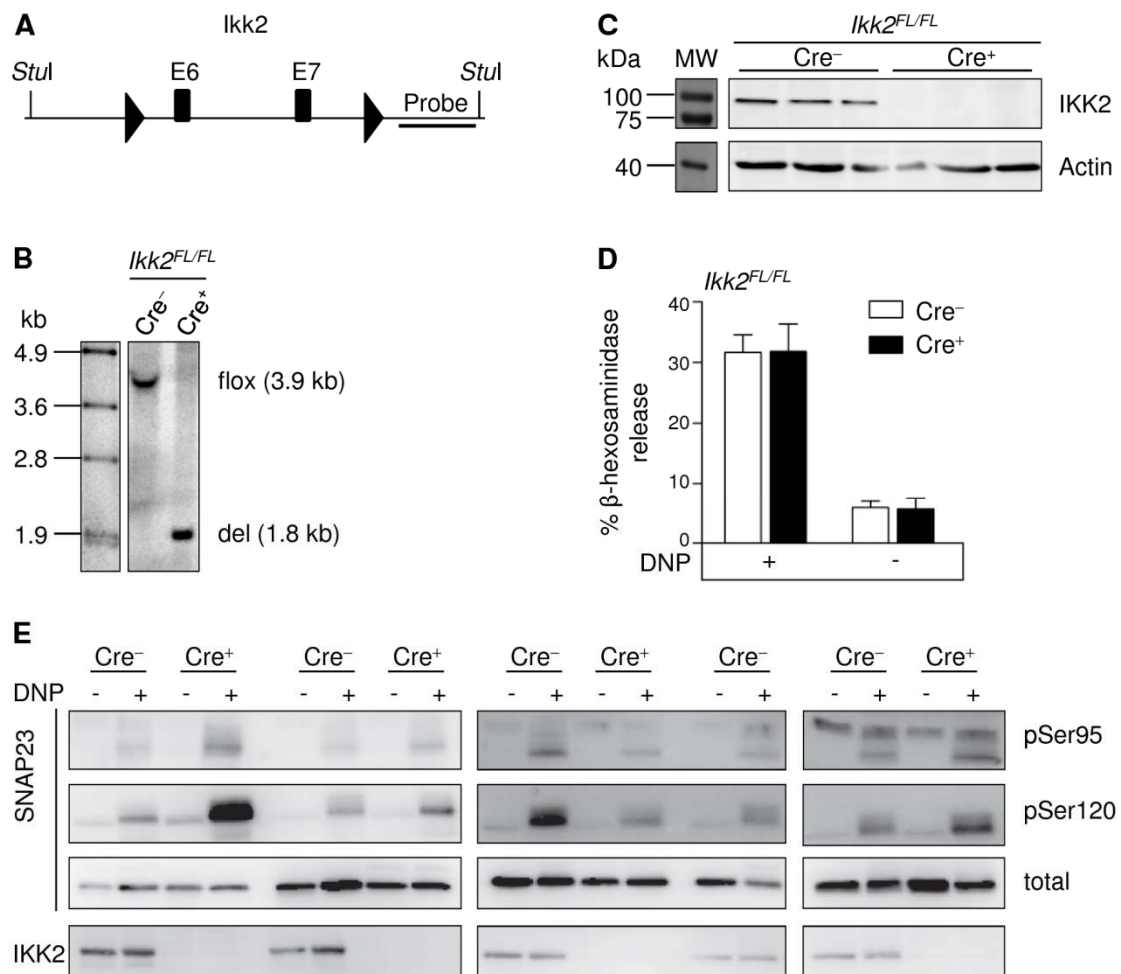


Figure S3. *In vitro* degranulation of bone marrow-derived MCs and Fc ϵ RI-induced phosphorylation of SNAP-23 in MCs cultivated from peritoneal lavage do not depend on IKK2, related to Figure 3.

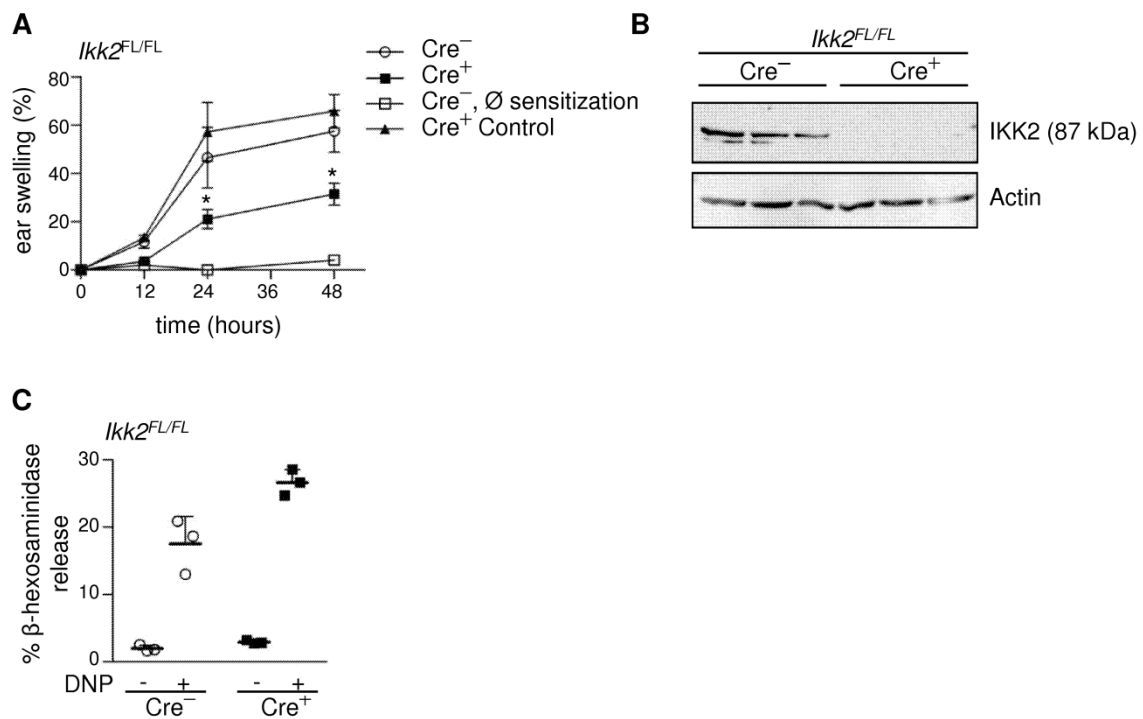


Figure S4. The late phase MC response is dependent on IKK activity, related to Figure 4.

Supplemental Figure Legends

Figure S1. MC-specific inactivation of the *Ikk2* and *Nemo* genes and MC-specific de-repression of the *Ikk2ca^{SFL}* knock in allele, related to Figure 1.

A, D Primer positions for nested single target amplification of the *loxP*-flanked (“floxed”) or deleted *Ikk2* and *Nemo* alleles. Primers 1, 2 and 3 were used in the first, primers 4, 5 and 6 in the second round of amplification (Second round products: *Ikk2* floxed 391bp, *Ikk2* deleted 218bp; *Nemo* floxed 454bp, *Nemo* deleted 290bp) **B, E** Examples of nested amplification of both loci from single *Ikk2^{FL/FL}Mcpt5-Cre⁺* (B) and *Nemo^{FL}Mcpt5-Cre⁺* (E) peritoneal lavage cells deposited into PCR tubes (one cell per tube) by FACS (directly *ex vivo* without prior cultivation). Products representing the *loxP*-flanked alleles were obtained from non-MCs, while MCs yielded products representing the deleted alleles (see also Table S1). **C, F** Western blot demonstrating absence of IKK2 and NEMO in *Ikk2^{FL/FL}Mcpt5-Cre⁺* and *Nemo^{FL}Mcpt5-Cre⁺* MCs, respectively, cultivated from peritoneal lavage (PCMCs). Each lane represents cells from a different animal. **G, H, I** Efficient and MC-specific de-repression of the *Ikk2ca^{SFL}* knock in allele in *Ikk2ca^{SFL}Mcpt5-Cre⁺* mice as determined by FACS analysis of expression of reporter eGFP co-expressed with IKK2ca upon deletion of the stop element. **G** MCs and macrophages were identified in peritoneal lavage fluid as FcεRIα⁺CD117⁺ and F4/80⁺ cells, respectively. (representative of 22 mice) **H** Single cell suspensions of skin cells were generated by enzymatic digest of ear skin and MCs were identified in the CD45⁺CD3⁻F4/80⁻ fraction as FcεRIα⁺CD117⁺ cells (upper panels). Conversely, eGFP-expressing CD45⁺ skin cells were analyzed for expression of MC markers (representative of eight mice). **I** No reporter fluorescence was detected in CD45⁺ lymph node or spleen cells from *Ikk2ca^{SFL}Mcpt5-Cre⁺* mice (representative of four mice each).

Figure S2. Quantification of different types of non-MCs in MC-deficient *Mcpt5-Cre⁺R-DTA⁺* mice and allergen-induced anaphylaxis in animals with MC-specific inactivation or over-activation of the IKK complex, related to Figure 2.

A No significant changes in the numbers of other hematopoietic cell types in MC-deficient *Mcpt5-Cre⁺R-DTA⁺* mice. Various hematopoietic cell populations in the peritoneal cavity, ear skin, bone marrow, blood and spleen of six *Mcpt5-Cre⁺R-DTA⁺* and seven Cre-negative controls were analyzed by flow cytometry using the markers indicated. Data represented as mean $\pm$ SD. **B** Monitoring of anaphylaxis by infra-red temperature measurement. Passive systemic anaphylaxis (PSA) was induced by i.p. sensitization with anti-DNP IgE. 24 hours later, the animals were challenged by i.p. injection of HSA-DNP. Drop of body temperature was measured by infra-red imaging of the shaved skin of the lower back. Upper panels, PSA response of control *Mcpt5-Cre⁺* mice without floxed alleles (control excluding effects of the *Cre* transgene alone) of *Ikk2^{FL/FL}Mcpt5-Cre⁺*, *Nemo^{FL}Mcpt5-Cre⁺* (the “*Nemo^{FL}*” groups include male *Nemo^{FL/Y}* and female *Nemo^{FL/FL}* mice) and *Ikk2ca^{sFL}Mcpt5-Cre⁺* mice and the respective Cre-negative controls. Additional control mice were either not sensitized (“ $\emptyset$ sensitization”) or not challenged (“ $\emptyset$ challenge”). Lower panel, example of temperature measurements before and after induction of anaphylaxis. **C** Passive cutaneous anaphylaxis (PCA) in *Mcpt5-Cre⁺* control mice excluding effects of the *Cre* transgene alone. Mean $\pm$ SD is displayed.

Figure S3. *In vitro* degranulation of bone marrow-derived MCs and Fc ϵ RI-induced phosphorylation of SNAP23 in MCs cultivated from peritoneal lavage do not depend on IKK2, related to Figure 3.

A-D MCs were differentiated from bone marrow of poly I:C-induced *Ikk2^{FL/FL}Mx-Cre⁺* and Cre-negative littermate control mice by cultivation in the presence of IL-3 and

SCF. **A, B** Efficient deletion of the *loxP*-flanked fragment of the *Ikk2* gene in BMMCs demonstrated by Southern blot analysis of *StuI*-digested DNA extracted from bulk cultures (representative of cultures from three different mutant animals). **C** Absence of IKK2 protein in BMMCs was demonstrated by Western blot analysis. **D** IgE-induced degranulation of IKK2-deficient and IKK2-proficient BMMCs was assessed by loading the cells overnight with anti-DNP IgE followed by stimulation with DNP-HSA (“+ DNP”) and quantification of β -hexosaminidase release. Samples exposed to HSA instead of the DNP-HSA served as negative controls (“- DNP”). Mean $\pm$ SD is displayed. **E** IgE-induced phosphorylation of MC SNAP23 does not depend on IKK2. Phosphorylation of SNAP23 on Ser95 and Ser120 in PCMCs from four *Ikk2*^{FL/FL}*Mcpt5-Cre*⁺ and five Cre-negative control mice sensitized and triggered as described above was assessed by phospho-specific Western blot analysis.

Figure S4. The late phase MC response is dependent on IKK activity, related to Figure 4.

A LP-PCA was induced in 5 *Ikk2*^{FL/FL}*Mcpt5-Cre*⁺, 6 Cre-negative littermate controls and 2 *Ikk2*^{wt/FL}*Mcpt5-Cre*⁺ controls (*Cre*⁺ Control) by i.v. sensitization with anti-DNP IgE and challenge by epicutaneous administration of DNFB on both sides of one ear 24 hours later. One Cre-negative animal was not sensitized but challenged with DNFB ($\emptyset$ sensitization). Ear thickness was monitored for 48 hours. Mean ear swelling $\pm$ SD is displayed. **p*<0.01 (comparison between *Cre*⁺ and *Cre*-negative). **B** Western Blot analysis demonstrates efficient *Ikk2* gene inactivation in the BMMC cultures that showed impaired IgE-induced cytokine responses as shown in Figure 4D. **C** β -hexosaminidase release assay demonstrates unimpaired degranulation of the *Ikk2*-deficient BMMC cultures that showed impaired IgE-induced cytokine responses as shown in Figure 4D. Mean $\pm$ SD is displayed.

Supplemental Tables

A MC-specific inactivation of the *Ikk2* gene

peritoneal cavity

	tubes which yielded...				
cell type and number of cells/tube	1 product representing loxP-flanked locus	1 product representing deleted locus	2 products representing loxP-flanked and deleted locus	no product	total number of tubes
	<i>Ikk2^{FL/FL} Mcpt5-Cre⁺</i>				
1 mast cell	-	92	-	16	108
1 macrophage	17	-	-	3	20
1 B cell	19	-	-	1	20
10 mast cells	-	20	-	-	20
8 mast cells + 2 macrophages	-	2	18	-	20
water control	-	-	-	48	48
	<i>Ikk2^{FL/FL} Mcpt5-Cre⁻</i>				
1 mast cell	65	-	-	7	72
1 macrophage	17	-	-	3	20
1 B cell	18	-	-	2	20
water control	-	-	-	28	28

skin cell suspension

	tubes which yielded...				
cell type and number of cells/tube	1 product representing loxP-flanked locus	1 product representing deleted locus	2 products representing loxP-flanked and deleted locus	no product	total number of tubes
	<i>Ikk2^{FL/FL} Mcpt5-Cre⁺</i>				
1 mast cell	-	70	1	9	80
1 macrophage	14	-	-	6	20
1 T cell	19	-	-	1	20
10 mast cells	-	18	2	-	20
8 mast cells + 2 macrophages	-	2	18	-	20
water control	-	-	-	20	20
	<i>Ikk2^{FL/FL} Mcpt5-Cre⁻</i>				
1 mast cell	17	-	1	2	20
1 macrophage	18	-	-	2	20
1 T cell	19	-	-	1	20
water control	-	-	-	10	10

B MC-specific inactivation of the *Nemo* gene

peritoneal cavity

	tubes which yielded...				
cell type and number of cells/tube	1 product representing loxP-flanked locus	1 product representing deleted locus	2 products representing loxP-flanked and deleted locus	no product	total number of tubes
	<i>Nemo</i> ^{FL} <i>Mcpt5-Cre</i> ⁺				
1 mast cell	-	36	-	-	36
1 macrophage	30	1	-	5	36
1 B cell	13	-	-	7	20
10 mast cells	-	20	-	-	20
8 mast cells + 2 macrophages	-	-	20	-	20
water control	-	-	-	20	20
	<i>Nemo</i> ^{FL} <i>Mcpt5-Cre</i> ⁻				
1 mast cell	19	-	-	1	20
1 macrophage	13	-	1	6	20
1 B cell	14	-	-	6	20
water control	-	-	-	12	12
skin cell suspension					
	tubes which yielded...				
cell type and number of cells/tube	1 product representing loxP-flanked locus	1 product representing deleted locus	2 products representing loxP-flanked and deleted locus	no product	total number of tubes
	<i>Nemo</i> ^{FL} <i>Mcpt5-Cre</i> ⁺				
1 mast cell	-	34	-	6	40
1 macrophage	19	-	-	1	20
1 T cell	19	-	-	1	20
10 mast cells	-	11	-	-	11
8 mast cells + 2 macrophages	-	-	5	-	5
water control	-	-	-	20	20
	<i>Nemo</i> ^{FL} <i>Mcpt5-Cre</i> ⁻				
1 mast cell	19	-	-	1	20
1 macrophage	19	-	-	1	20
1 T cell	20	-	-	-	20
water control	-	-	-	12	12

Table S1. Efficient MC-specific inactivation of the *Ik2* and the *Nemo* gene *in vivo* as demonstrated by single cell PCR, related to Figure 1.

Overview of products of the single target amplification of the loxP-flanked *Ik2* (A) and *Nemo* (B) locus from FACS-sorted single MCs and non-MCs from the peritoneal

cavity or skin. Single cells or the indicated numbers of cells were deposited into individual PCR tubes. The numbers of tubes which yielded a product representing the *loxP*-flanked (undelated) or the deleted locus, or both, are listed.

A Only products representing the deleted *Ikk2* locus were amplified from 112 tubes containing in total 292 *Ikk2^{FL/FL}Mcpt5-Cre⁺* peritoneal MCs, while non-MCs yielded only products representing the undelated, *loxP*-flanked (“floxed”) locus. 91 tubes containing in total 271 *Ikk2^{FL/FL}Mcpt5-Cre⁺* MCs sorted from skin cell suspensions all yielded “deleted products” except for three, which gave both a deleted and a floxed product. These three floxed products could result from imperfect Cre-mediated deletion but also from rare erroneous deposition of a non-MC together with a MC by the cell sorter or from rare contamination with genomic DNA or PCR products. A fraction of tubes did not yield a product, which was most likely due to either failure of single cell deposition or to damaged template molecules. PCR tubes into which no cell was sorted but which were otherwise handled exactly as the tubes containing cells served as negative (“water”) controls. Collectively, these data show that the *Ikk2* gene was deleted in close to 100% of peritoneal and skin MCs in *Ikk2^{FL/FL}Mcpt5-Cre⁺* mice. **B** Similar results were obtained for MC-specific inactivation of the *Nemo* gene in *Nemo^{FL}Mcpt5-Cre⁺* mice.

Supplemental Experimental Procedures

Stainings for flow cytometric analysis and sorting

Cells were resuspended in PBS/0.5% BSA and stained for 30 min at 4°C with antibodies against the following markers (from eBiosciences, if not indicated otherwise): CD117 (clone 2B8), FcεRIα (clone MAR-1), CD45 (clone 30-F11), F4/80 (clone BM8), CD11b (clone M1/70), CD4 (clone GK1.5), CD19 (clone eBio1D3), Ly-6G (clone RB6-8C5), CD49b (clone DX5), NK1.1 (clone PK136), CD3e (clone 145-2C11), CD8a (clone 53-6.7), SiglecF (clone E50-2440, BD Pharmingen), CD11c (clone N418), MHCII (clone DX-3, Serotec). Cell suspensions were washed twice and were resuspended in PBS/0.5% BSA. To determine cell viability, 1 µg/ml propidium iodide was added. Analysis was done using the Miltenyi MacsQuant flow cytometer with MacsQuantTM Analysis or FlowJo Software.

Single cell sorting and single target amplification of the *Ikk2* and *Nemo* loci

Single cell suspensions containing MCs were generated by lavaging the peritoneal cavity or by digesting ear tissue. Ears were cut into small pieces and digested in 1 ml DMEM containing 20 mM Hepes, 0.025 mg/ml Liberase TM (Roche), 396 U/ml DNase I (Roche), and 0.5 mg/ml Hyaluronidase (Sigma) at 37°C with shaking at 1400 rpm for 1 h. Samples were passed through a 40 µm sieve and the cell suspension was washed twice with PBS. Single MCs (CD19⁻ F4/80⁻ CD117⁺ FcεRIα⁺), B cells (CD117⁻ F4/80⁻ CD19⁺ B220⁺) and macrophages (CD117⁻ CD19⁻ B220⁻ F4/80⁺) from peritoneal lavage and T cells (CD45⁺ F4/80⁻ CD4⁺), macrophages (CD45⁺ CD4⁻ F4/80⁺), and MCs (CD45⁺ CD4⁻ F4/80⁻ CD117⁺ FcεRIα⁺) from ear skin cell suspension were sorted into PCR tubes containing 15 µl water using a FACS Aria II cell sorter (BD Biosciences). Water control tubes were placed in the cell sorter but no cell was deposited into these tubes. Tubes containing single cells were stored at -80°C.

After thawing, cells were digested in 1 x DreamTaq PCR buffer (Fermentas) containing Proteinase K (Fermentas, 2 mg/ml final concentration) in a total volume of 20 µl. Cells were incubated at 50°C for 1.5 h followed by inactivation of Proteinase K by heating to 95°C for 10 min. Single target PCR was performed in two rounds of amplification (see Figure S1 for primer positions; the primer sequences were as follows: *Ikk2*-1 5'- ACT-GGG-AGA-ACA-CCA-TAC-TAG-CTG-A-3', *Ikk2*-2 5'- TTT-CGT-GAA-GGT-ATC-TAA-GAG-CCG-AT-3', *Ikk2*-3 5'- ATT-CCT-CTC-CTC-GTC-ATC-CTT-CGA-G-3', *Ikk2*-4 5'- GGA-GAA-CAC-CAT-ACT-AGC-TGA-ACT-G-3', *Ikk2*-5 5'- AAG-AGC-CGA-TGC-TGA-GGA-AGA-G-3', *Ikk2*-6 5'- TCT-GCC-ACC-CGA-CTA-CAT-CTT-C-3', *Nemo*-1 5'-TGT-GTC-CTA-TTC-TTG-GAA-GGG-TAT-G-3', *Nemo*-2 5'-AGC-ATC-TGA-TTG-CTC-TGC-CGG-ATA-3', *Nemo*-3 5'-ACT-GAA-CAA-ATT-CTA-TTC TCG-TAG-CTC-TA-3', *Nemo*-4 5'-GTC-CTA-TTC-TTG-GAA-GGG-TAT-GGC-C-3', *Nemo*-5 5'-GAT-AGC-GTC-TAG-GGA-CAG-GGA-AG-3', *Nemo*-6 5'- TCT-CGT-AGC-TCT-AAT-AAC-TGT-CTA-CCT-3'). For the first round, 30 µl of "single cell mix" (1x DreamTaq PCR buffer (2 mM MgCl₂, Fermentas), 200 µM each of dATP, dTTP, dGTP and dCTP, 0.2 µM each HPLC-purified primer *Nemo*-1, -2 and -3 or *Ikk2*-1, -2 and -3 and 2 U DreamTaq DNA Polymerase (Fermentas)) was added to each tube. Thermal cycling was performed with initial denaturation at 95°C for 2 min followed by 35 cycles at 95°C for 20 sec, 30 sec annealing at 52°C for *Nemo* or 53°C for *Ikk2* and 72°C for 1 min. Finally the reaction was incubated at 72°C for 10 min. 1 µl of the first round of amplification was used as template for the second round PCR, which was performed in 1 x DreamTaq buffer, 200 µM each dNTP, 0.2 µM each primer *Nemo*-4, -5 and -6 or *Ikk2*-4, -5 and -6, 1.5 U DreamTaq DNA Polymerase in a total volume of 50 µl. Initial denaturation at 95°C for 2 min was followed by 35 cycles at 95°C for 20 seconds, 30 sec annealing at 61°C for *Nemo* or

54°C for *Ikk2*, 72°C for 1 min and a final 10 min incubation at 72 °C. PCR products were analyzed by agarose gel electrophoresis and verified by sequencing.

Cell culture

Peritoneal cell-derived MCs (PCMCs) were obtained by cultivating peritoneal cell suspensions in DMEM+GlutaMAX (Gibco) containing 10% heat inactivated FCS, 50 µM β-mercaptoethanol, 100 U/ml penicillin, 100 U/ml streptomycin, 2% culture supernatant of the mIL3-transformed cell line X63Ag8-653 and 0.5% supernatant of CHO transfectants secreting murine SCF (generated by S. Lyman, Immunex, Seattle, and kindly provided by P. Dubreuil, Marseille). After 5 weeks more than 98% of the cells expressed high levels of CD117 and FcεRIα. For the generation of bone marrow derived MCs (BMMCs) *Ikk2^{F/F}Mx-Cre⁺*, *Nemo^FMx-Cre⁺* or *Ikk2ca^{sF}Mx-Cre⁺* mice and Cre-negative littermate controls received 400 µg poly I:C. After three days, bone marrow was flushed out of long bones and cultured as described above except that 0.5% supernatant of CHO transfectants secreting murine SCF was added only after three weeks of culture. More than 97% of cells expressed high levels of CD117 after 6 weeks.

Induction and quantification of MC degranulation

PCMCs were incubated with 1 µg/ml SPE-7 anti-DNP-IgE over night. The next day, the same amount of anti-DNP-IgE was added again. After 2 h, cells were washed twice in Tyrode's Buffer (135 mM NaCl, 5 mM KCl, 5.6 mM glucose, 1.8 mM CaCl₂, 1 mM MgCl₂, 0.5 mg/ml BSA Fraction V, 20 mM HEPES, pH 7.3). After washing, 1 x 10⁵ PCMCs or 2 x 10⁶ BMMCs were stimulated with 100 ng/ml HSA-DNP (Sigma-Aldrich) or HSA alone for 30 min at 37°C. In some experiments, cells were left unsensitized and were stimulated with 500 ng/ml A23187 (Sigma-Aldrich) for 30 min.

To quantify β -hexosaminidase release, stimulated cells were sedimented by centrifugation (5 min, 1600 rpm, 4°C) the supernatant was collected and the cell pellet was lysed in 200 μ l 0.5% Triton X-100 in Tyrode's Buffer. 25 μ l of supernatants and cell lysate were incubated with 25 μ l 1 mM p-nitrophenyl-N-acetyl β -D-glucosamine in citrate buffer (0.1 M, pH 4.5) for 1 h at 37°C. The reaction was stopped with 200 μ l carbonate buffer (0.1 M Na_2CO_3 , 0.1 M NaHCO_3 , pH 9) and enzyme activity was determined by measuring OD at 405 nm. β -hexosaminidase release was determined as percent release of total content (percent release = $100 \times \text{amount supernatant} / (\text{amount in supernatant} + \text{cell lysate})$). To quantify surface phosphatidylserin by annexin V staining, stimulation of MCs was stopped by adding ice cold annexin V staining buffer (10 mM Hepes, NaOH, pH 7.3, 150 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1.8 mM CaCl_2). Cells were washed and stained with FITC- or Pacific blue-annexin V (BioLegend) in 100 μ l annexin V buffer for 15 min at room temperature. After staining, 400 μ l annexin V buffer and 1 μ g/ml propidium iodide were added. Analysis was done using a MacsQuant flow cytometer (Miltenyi).

Western Blot

Cells were lysed in RIPA buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1% NP40, 1 mM EDTA, 0.25% Na-deoxycholate) supplemented with 10 μ g/ml Aprotinin, 10 μ g/ml Leupeptin, 0.1 mM Na_3VO_4 , 1 mM PMSF, 10 mM NaF, 1 mM DTT and 8 mM β -glycerophosphate. After standard SDS-PAGE and blotting, PVDF membranes were probed with the following antibodies: anti-NEMO (rabbit polyclonal serum, gift from Ralph Gareus, Cologne), anti-IKK2 (clone 10AG2, Millipore), anti-I κ B α (clone 44D4, Cell Signaling), anti-actin (polyclonal, Sigma-Aldrich) anti-GAPDH (Calbiochem), anti-SNAP23 (Santa Cruz), anti-phospho-SNAP23 Ser95 and Ser120 (both polyclonal (Yasuda et al., 2011), gift from Makoto Itakura, Kitasato, Japan). Western blot bands

were quantified using image-J software. Phospho-SNAP23 (P-SNAP23) pixel counts were divided by SNAP23 pixel counts. The P-SNAP23/SNAP23 pixel counts were then normalized to the value for unstimulated control mast cells („- DNP“ *IKK2^{FL/FL}* Cre-negative). To average the resulting fold-changes we calculated the geometric means and 95% confidence interval.

Primers for RT-PCR analysis of the MC cytokine response

pro-IL-1 β (IL-1 β -Fwd 5'-GCC-ACC-TTT-TGA-CAG-TGA-TGA-G, IL-1 β -Rev 5'-AGC-TTC-TCC-ACA-GCC-ACA-AT), TNF (TNF-Fwd 5'-ACT-GAA-CTT-CGG-GGT-GAT-CG, TNF-Rev 5'-CTT-GGT-GGT-TTG-CTA-CGA-CG), IL-6 (IL-6-Fwd 5'-TTC-CAT-CCA-GTT-GCC-TTC-TT, IL-6-Rev 5'-CTG-CAA-GTG-CAT-CAT-CGT-TG) and TATA box-binding protein (TBP) (TBP-Fwd 5'-TCT-ACC-GTG-AAT-CTT-GGC-TGT-AAA, TBP-Rev 5'-TTC-TCA-TGA-TGA-CTG-CAG-CAA-A)

Quantification of skin MCs

Biopsies of back skin were fixed in 4% (v/v) buffered formalin. 5 μ m paraffin sections were Giemsa-stained and MCs located between epidermis and panniculus carnosus were counted along 3 mm of skin at a magnification of 400x.

Quantification of PSA responses by thermal imaging

Drop in body temperature upon induction of passive systemic anaphylaxis (PSA) was quantified by thermal imaging on the shaved back using a VarioCAM high resolution instrument (Jenoptik) and IRBIS remote 3.0 software. Mean temperature of a rectangular region of interest was calculated.

I κ B Kinase 2 Is Essential for IgE-Induced Mast Cell De Novo Cytokine Production but Not for Degranulation

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SUMMARY

The immunoglobulin E (IgE)-mediated mast cell (MC) response is central to the pathogenesis of type I allergy and asthma. I κ B kinase 2 (IKK2) was reported to couple IgE-induced signals to MC degranulation by phosphorylating the SNARE protein SNAP23. We investigated MC responses in mice with MC-specific inactivation of IKK2 or NF- κ B essential modulator (NEMO), or animals with MC-specific expression of a mutant, constitutively active IKK2. We show that the IgE-induced late-phase cytokine response is reduced in mice lacking IKK2 or NEMO in MCs. However, anaphylactic in vivo responses of these animals are not different from those of control mice, and in vitro IKK2-deficient MCs readily phosphorylate SNAP23 and degranulate similarly to control cells in response to allergen or calcium ionophore. Constitutive overactivation of the NF- κ B pathway has only slight effects on allergen-triggered MC responses. Thus, IKK2 is dispensable for MC degranulation, and the important question how IgE-induced signals trigger MC vesicle fusion remains open.

INTRODUCTION

Mast cells (MCs) are the most important effector cells in immunoglobulin E (IgE)-mediated allergic immune responses (Galli and Tsai, 2012). IgE binds to the high-affinity receptor for IgE (Fc ϵ RI) on MCs, and crosslinking of Fc ϵ RI-bound IgE by multivalent antigen triggers immediate release of preformed proinflammatory mediators stored in the prominent cytoplasmic granules, including histamine and tumor necrosis factor (TNF). Several hours after IgE-mediated exocytosis of secretory granules ("degranulation"), the MC secretes a range of cytokines, chemokines, and lipid mediators synthesized de novo in response to the stimulus ("late-phase response"). Both phases of the MC

IgE response are key events in the pathogenesis of allergic disease (Galli and Tsai, 2012). Crosslinking of MC-bound IgE triggers a signaling cascade (reviewed in Kambayashi and Koretzky, 2007) resulting in IP₃- and DAG-triggered Ca²⁺ mobilization and activation of protein kinase (PK) C, which leads to degranulation and induction of genes encoding proinflammatory mediators. Fc ϵ RI-induced gene induction is controlled among others via NF- κ B transcription factors (Jeong et al., 2002; Klemm et al., 2006; Lorentz et al., 2003; Peng et al., 2005). These dimeric factors are sequestered in the cytosol by inhibitor of NF- κ B (I κ B) proteins in the steady state. NF- κ B activity is controlled by the IKK complex, which phosphorylates I κ B proteins, targeting them for proteasomal degradation thereby releasing NF- κ B to activate transcription. The IKK complex consists of two kinases, IKK1 and IKK2, and the essential scaffolding protein NEMO. Downstream of PKC activated by Fc ϵ RI-induced second messenger signals, Bcl10 and Malt1 transduce a signal activating the IKK complex and thereby elicit NF- κ B translocation and MC late-phase cytokine production (Klemm et al., 2006). In contrast to de novo mediator synthesis, Fc ϵ RI-induced MC degranulation does not depend on NF- κ B (Klemm et al., 2006; Marquardt and Walker, 2000). Calcium sensors activated by proximal Fc ϵ RI signaling activate the soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE) protein machinery, which triggers fusion of secretory vesicles with the plasma membrane (Lorentz et al., 2012). An important MC SNARE protein is synaptosome-associated protein of 23 kDa (SNAP23) (Lorentz et al., 2012). Although phosphorylation of SNAP23 on residues 95 and 120 is essential for MC degranulation (Hepp et al., 2005), little was known about events coupling the second messenger signals to SNARE protein activation. IKK2 was proposed to phosphorylate MC SNAP23 in response to IgE signals in an NF- κ B-independent fashion (Suzuki and Verma, 2008). Given that complete loss of IKK2 causes embryonic lethality, this study relied on MCs differentiated from *Ikk2*^{-/-} fetal liver cells (FLMCs). These cells featured a defect in IgE-mediated SNAP23 phosphorylation and degranulation in vitro. Moreover, anaphylactic responses of MC-deficient Kit mutant mice were restored by reconstitution with wild-type but not *Ikk2*^{-/-} FLMCs.

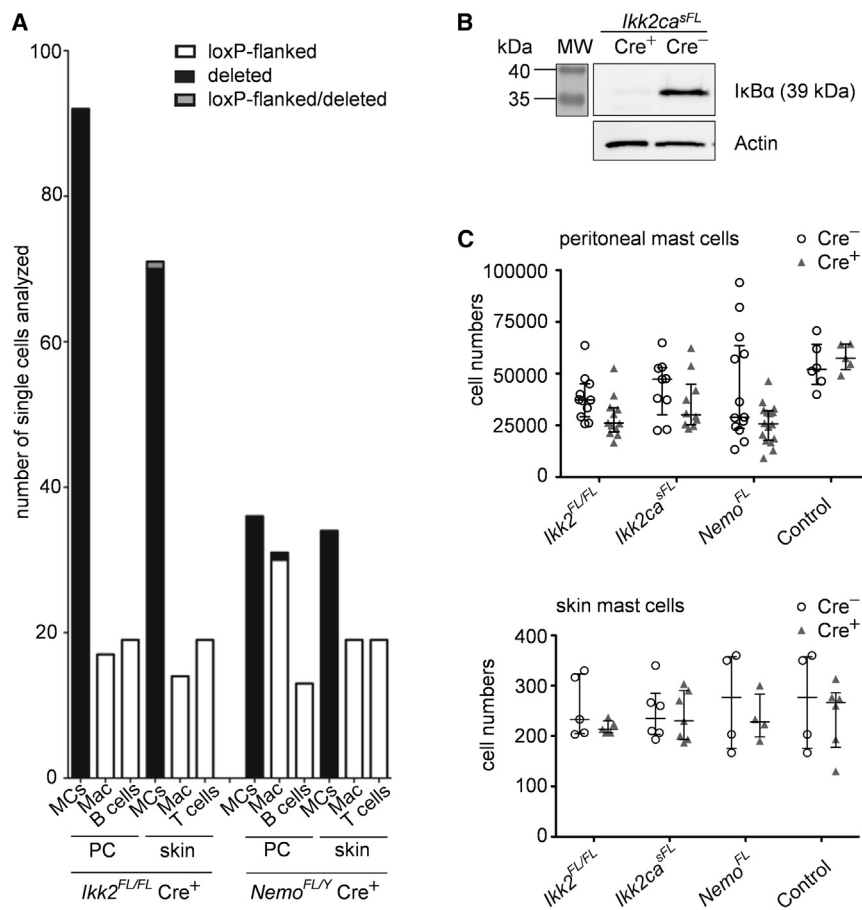


Figure 1. MC-Specific Inactivation of the *Ikk2* and the *Nemo* Gene, MC-Specific Overactivation of the NF- κ B Pathway, and Effects of These Alterations on MC Numbers In Vivo

(A) Single-cell PCR assay demonstrating efficient inactivation of the *Ikk2* and the *Nemo* gene in MCs of *Ikk2^{FL/FL}Mcpt5-Cre⁺* and *Nemo^{FL}Mcpt5-Cre⁺* mice, respectively. Single MCs and non-MCs (macrophages [Mac] and B and T cells) from peritoneal cell (PC) or skin cell suspensions were deposited into PCR tubes by FACS, and the locus of interest was amplified from each cell by nested PCR to assess whether the *loxP*-flanked fragment was deleted by Cre-mediated recombination or not. (See Figure S1 for PCR strategy and examples of PCR results and Table S1 for the entire data set.) (B) Western blot analysis of I κ B α in *Ikk2ca^{sFL}Mcpt5-Cre⁺* PCMCs FACS-sorted as CD117⁺ Fc ϵ RI α ⁺eGFP⁺ cells (representative of four mice). (C) MCs were quantified in peritoneal lavage fluid and skin cell suspensions from animals of the indicated genotypes by flow cytometry. Median and interquartile ranges are indicated.

of the *Ikk2* and the (X-chromosomal) *Nemo* gene in *Ikk2^{FL/FL}Cre⁺* mice and *Nemo^{FL}Cre⁺* mice (male *Nemo^{FL/Y}* and female *Nemo^{FL/FL}*), respectively, was demonstrated by nested single target amplification of the mutated locus from single MCs and non-MCs purified by fluorescence-activated cell sorting (FACS)

We intended to investigate the role of IKK complex components in MCs and, in particular, IKK2-mediated MC degranulation in various MC in vivo responses. For this purpose, we employed MC-specific gene targeting using *Mcpt5-Cre* mice, which allows the analysis of gene function in MCs in situ. We generated mouse lines in which we ablated IKK2 or NEMO specifically in MCs, and mice with MC-specific expression of a mutant, constitutively active IKK2 protein. Unexpectedly, we found that degranulation of IKK2-deficient MCs was not impaired in vitro or in vivo compared to control MCs.

RESULTS

Mice with MC-Specific Inactivation or Overactivation of the IKK Complex

We recently described efficient Cre-mediated recombination in connective tissue MCs (CTMCs) in *Mcpt5-Cre* transgenic mice (Dudeck et al., 2011; Scholten et al., 2008). In order to inactivate or to constitutively activate the IKK complex in CTMCs in vivo, *Mcpt5-Cre* mice were crossed to *Ikk2^{FL}* (Pasparakis et al., 2002) and *Nemo^{FL}* mice (Schmidt-Suprian et al., 2000) or the *Ikk2ca^{sFL}* mouse strain, respectively. The latter mice express a mutant, constitutively active IKK2 protein (IKK2ca) in cells that excised a *loxP*-flanked stop element by Cre-mediated recombination (Sasaki et al., 2006). Highly efficient and MC-specific inactivation

from peritoneal lavage or skin cell suspensions (Figures 1A, S1A, S1B, S1D, and S1E; Table S1). Absence of IKK2 and NEMO protein in peritoneal MCs expanded in vitro (PCMCs) was verified by western blot (Figures S1C and S1F). Cell type specificity and efficiency of Cre-mediated activation of the *ROSA26 Ikk2ca^{sFL}* knockin allele was determined by monitoring expression of the eGFP marker coexpressed with IKK2ca (Figures S1G–S1I). Spontaneous degradation of I κ B α was demonstrated in PCMCs (Figure 1B). Loss of IKK2 or NEMO or expression of constitutively active IKK2 did not affect numbers (Figure 1C) and phenotype of MCs in peritoneal lavage or skin except for a slight reduction of CD117 expression (data not shown).

Complete Loss or Constitutive Gain of IKK2 Activity or IKK Disruption through NEMO Ablation Specifically in Mast Cells Do Not Influence IgE-Induced Anaphylaxis In Vivo

The *Mcpt5-Cre* transgene is active in CTMCs but not in mucosal MCs (Dudeck et al., 2011). We first determined the contribution of CTMCs in the anaphylactic response to intraperitoneally (i.p.) injected allergen and induced passive systemic anaphylaxis (PSA) in *R-DTA⁺Mcpt5-Cre⁺* mice. These mice are characterized by profound CTMC deficiency (Dudeck et al., 2011). Other hematopoietic cell types, including basophils, were not altered in numbers (Figure S2A). Although body temperature (determined

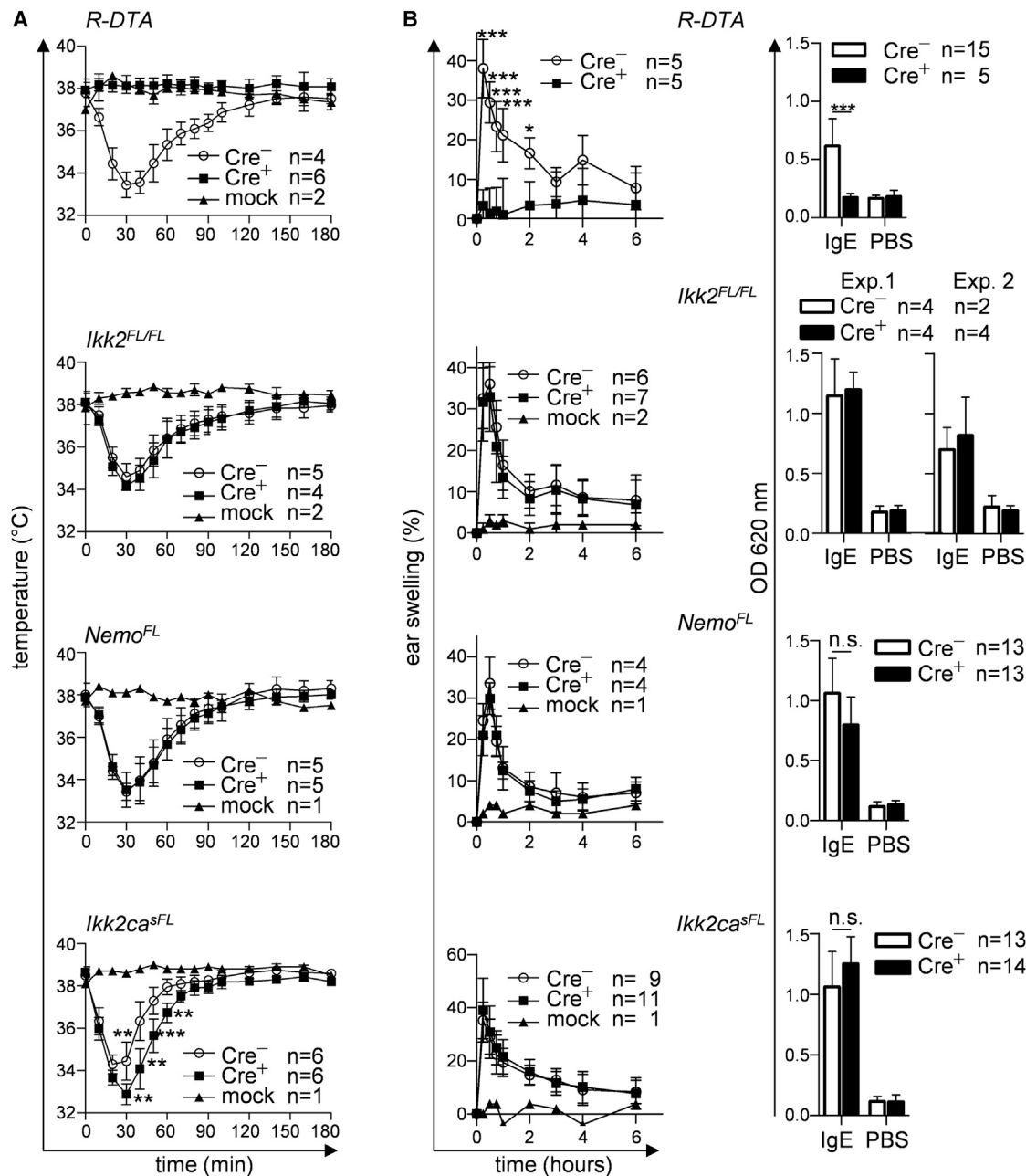


Figure 2. Neither MC IKK2 nor NEMO Is Required for IgE-Induced Anaphylaxis, and Constitutive Activity of IKK2 Has Little Effect on Anaphylaxis In Vivo

(A) PSA in $R\text{-DTA}^+ \text{Mcpt5-Cre}^+$ mice (constitutively MC-deficient, see also Figure S2), $Ikk2^{FL/FL} \text{Mcpt5-Cre}^+$, $Nemo^{FL} \text{Mcpt5-Cre}^+$, $Ikk2ca^{SFL} \text{Mcpt5-Cre}^+$ mice, and the respective Cre-negative littermate controls. Drop of body temperature was measured using a rectal probe. Mock controls were sensitized but received saline instead of allergen challenge. The slight increase of amplitude and duration of the response of $Ikk2ca^{SFL} \text{Mcpt5-Cre}^+$ compared to control mice was not reproduced in a second experiment based on infrared temperature monitoring (Figure S2B).

(B) PCA in $R\text{-DTA}^+ \text{Mcpt5-Cre}^+$ mice, $Ikk2^{FL/FL} \text{Mcpt5-Cre}^+$ and $Nemo^{FL} \text{Mcpt5-Cre}^+$ mice, $Ikk2ca^{SFL} \text{Mcpt5-Cre}^+$ mice, and the respective Cre-negative controls. Mock controls were not sensitized but challenged. $R\text{-DTA}^+ \text{Cre}^-$ mock controls gave results similar to all other genotypes (not shown for graphical clarity). Mean ear swelling $\pm$ SD is displayed. Vascular leak was quantified by measuring OD of Evans blue extracted from ear tissue. PBS-injected ears served as control. Mean $\pm$ SD is displayed in (A) and (B). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (comparison between Cre^+ and Cre^- in A and B).

by rectal probe) dropped by 4°C in $R\text{-DTA}^+ \text{Cre}^-$ control mice, no response was detectable in MC-deficient $R\text{-DTA}^+ \text{Mcpt5-Cre}^+$ animals (Figure 2A).

We also analyzed $R\text{-DTA}^+ \text{Mcpt5-Cre}^+$ for their capacity to mount immediate phase passive cutaneous anaphylaxis (PCA) responses. Animals were locally sensitized with anti-DNP-IgE

by injections into the ear pinna. Following intravenous (i.v.) challenge with HSA-DNP 24 hr later, we monitored ear thickness and vascular leakage (Figure 2B). Allergen-induced ear swelling and vascular leakage were reduced to control levels in the MC-deficient group. These results showed that CTMCs are the major effector cells in the PSA response to i.p.-injected allergen as well as the PCA response to i.v.-injected allergen.

To test whether deletion of IKK2 or NEMO in CTMCs or MC-specific expression of constitutively active IKK2 affected IgE-induced in vivo responses, PSA was induced in the mutant mouse lines. MC-specific loss of IKK2 or NEMO had no effect on the response to the allergen (Figure 2A). We performed independent experiments in which skin temperature was measured using an infrared camera (Figure S2B) and obtained similar results. Expression of constitutively active IKK2 in connective tissue MCs resulted in enhanced and prolonged PSA in the first experiment (Figure 2A), which, however, was not reproduced in a second experiment based on infrared skin temperature recording (Figure S2B). Confounding effects of the *Mcpt5-Cre* transgene itself were excluded by analysis of *Mcpt5-Cre*⁺ mice devoid of *loxP*-flanked genes, which behaved indistinguishably from wild-type mice (Figure S2B). Thus, MC IKK2 and NEMO are dispensable for systemic anaphylaxis and constitutive IKK2 activity in MCs enhances anaphylaxis only moderately, if at all.

We next investigated the PCA response of mice of the three mutant lines. The ear swelling response of the MC-specific IKK2 and NEMO mutant mice was not different from that of controls (Figure 2B). Likewise, allergen-induced extravasation of Evans blue was not different between mutant and control animals (Figure 2B). *Ikk2ca^{FL}Mcpt5-Cre*⁺ mice did not show significantly different responses in ear swelling or Evans blue extravasation compared to control mice (Figure 2B). *Mcpt5-Cre*⁺ mice without *loxP*-flanked genes were analyzed to control for effects of the transgene alone (Figure S2C). Thus, MC-specific inactivation of the *Ikk2* or the *Nemo* gene does not affect IgE-mediated anaphylactic responses in vivo. Selective expression of constitutively active IKK2 in MCs resulted in significantly enhanced responses in only one of three independent assays (PSA, PCA, and Evans blue extravasation) and in the PSA assay only in one of two independent experiments. We therefore conclude that inhibition or constitutive activation of IKK2 does not affect anaphylactic responses in vivo.

Loss of IKK2 or NEMO or Constitutive IKK2 Activity Do Not Affect MC Degranulation or SNARE Protein Phosphorylation In Vitro

Suzuki and Verma described impaired in vitro degranulation of MCs differentiated from the fetal liver of *Ikk2*^{−/−} mice and an essential function of IKK2 in the phosphorylation of SNAP23 (Suzuki and Verma, 2008). We triggered degranulation of PCMCs derived from *Ikk2^{FL/FL}Mcpt5-Cre*⁺ and *Nemo^{FL}Mcpt5-Cre*⁺ mice. Absence of the respective proteins in these cells was demonstrated by western blot (Figures S1C and S1F). Degranulation of IKK2-deficient PCMCs and of NEMO-deficient PCMCs was similar to the response of control cells as determined by quantification of surface phosphatidylserine. This molecule translocates to the outer leaflet of the plasma membrane upon MC degranulation and can be detected by annexin V staining

(Figures 3A and 3B). Allergen-triggered IKK2-deficient and NEMO-deficient PCMCs released similar amounts of β -hexosaminidase as control cells (Figure 3A). Also, the early release of TNF, which is stored in the MC granules, is a measure of degranulation (before onset of IgE-induced de novo synthesis of this cytokine). IKK2- and NEMO-deficient MCs did not differ from control cells in TNF release 30 min after allergen challenge (Figure 3C; data not shown). In addition to triggering degranulation by IgE crosslinking, we treated IKK2- or NEMO-deficient PCMCs with the calcium ionophore A23187 and observed similar β -hexosaminidase release compared to control cells (Figure 3A). Moreover, we found unimpaired allergen-induced degranulation of IKK2-deficient bone marrow-derived MCs (BMMCs) (Figures S3A–S3D). Allergen-induced phosphorylation of SNAP23 on Ser95 and Ser120 was not affected by IKK2-deficiency as demonstrated by western blot (Figures 3D, 3E, and S3E). Expression of constitutively active IKK2 in PCMCs from *Ikk2ca^{FL}Mcpt5-Cre*⁺ mice did not affect allergen-induced degranulation (Figure 3A). In summary, we show that IKK2 and NEMO are dispensable for IgE-induced SNAP23 phosphorylation and MC degranulation in vitro.

IKK2 and NEMO Are Essential for the MC Late-Phase Response to Allergen

Hours after IgE crosslinking, MCs de novo synthesize TNF, interleukin 6 (IL-6), pro-IL-1 β , and other mediators. In order to test whether this late-phase response is dependent on IKK complex components, we sensitized mice with MC-specific deficiency for IKK2 or NEMO, or animals with MC-specific expression of constitutively active IKK2 by i.v. injection of anti-DNP-IgE. The animals were challenged 24 hr later by epicutaneous administration of DNFB, and the resulting ear swelling was monitored. The response was severely impaired in mice lacking IKK2 or NEMO in MCs (Figures 4A, 4B, and S4A). *Ikk2ca^{FL}Mcpt5-Cre*⁺ animals showed slightly enhanced responses compared to controls; however, the difference was significant only at one time point (Figure 4C).

In order to assess IgE-induced de novo expression of proinflammatory cytokines, we stimulated IKK2-deficient BMMCs (Figure S4B), NEMO-deficient PCMCs, and IKK2ca-overexpressing BMMCs by Fc ϵ RI crosslinking (Figures 4D–4F). The cytokine responses of IKK2- and NEMO-deficient cells were significantly reduced as determined by RT-PCR-based quantification of mRNAs and by determination of cytokine concentrations in the supernatant. In accordance with the results shown in Figure 3, the same cultures were not impaired in their degranulation response (Figure S4C; data not shown). In accordance with the results obtained from IKK2-deficient cells, the cytokine response of cells overexpressing the constitutively active IKK2ca protein featured a significantly enhanced cytokine response (Figure 4F). Thus, IKK2 and NEMO are essential for normal IgE-induced MC cytokine responses and constitutive IKK2 activity enhances these responses.

DISCUSSION

Fc ϵ RI-induced MC degranulation is a key event in the pathogenesis of allergic diseases. Induction of second messengers and

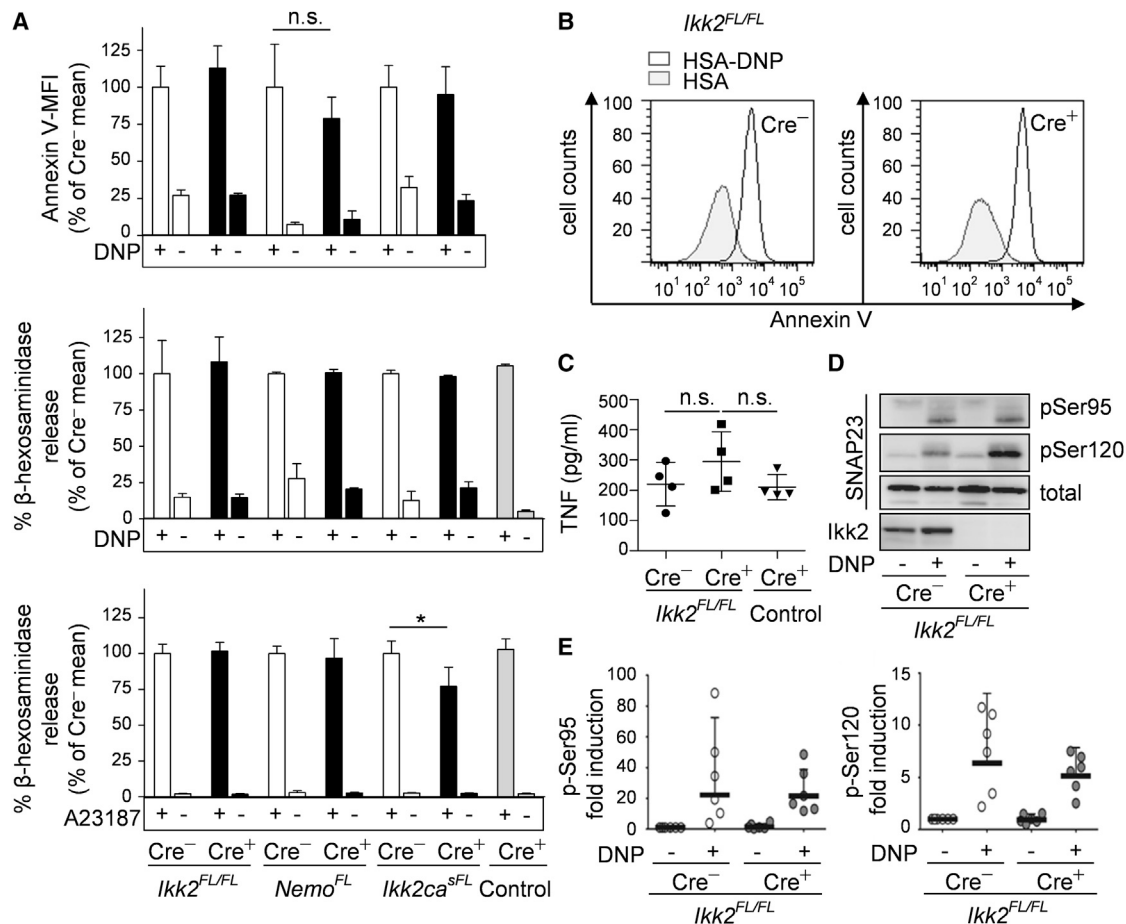


Figure 3. Deficiency for IKK Complex Components or Expression of Constitutively Active IKK2 Has No Effect on MC Degranulation In Vitro
Peritoneal lavage cells were cultivated in the presence of stem cell factor and IL-3. The cells were loaded overnight with anti-DNP-IgE and then stimulated with HSA-DNP (“+ DNP”). Control cell samples were exposed to HSA instead of allergen (“– DNP”).

(A) Degranulation of PCMCs from *Ikk2*^{FL/FL}*Mcpt5*-Cre⁺ as well as *Nemo*^{FL}*Mcpt5*-Cre⁺ and *Ikk2ca*^{sFL}*Mcpt5*-Cre⁺ mice and the respective Cre-negative littermates (n = 3–7 per group). In two independent experiments, degranulation was quantified by annexin V staining (upper panel) and by determination of β-hexosaminidase release (middle panel). The latter experiment included a “*Mcpt5*-Cre only” group to exclude confounding effects of the transgene itself. Degranulation was also induced by the ionophore A23187 (lower panel). Means obtained for allergen- or ionophore-treated Cre-negative control cells were set to 100%. Mean ± SD is shown. *p < 0.001, n.s., not significant. IKK2-deficient BMMCs yielded similar results as obtained for IKK2-deficient PCMCs (see Figure S3).

(B) Example of degranulation of *Ikk2*^{FL/FL}*Mcpt5*-Cre⁺ and *Ikk2*^{FL/FL}Cre-negative control PCMCs as determined by flow cytometric quantification of surface annexin V (from the data set represented in A).

(C) TNF release from *Ikk2*^{FL/FL}*Mcpt5*-Cre⁺ as well as Cre-negative and “*Mcpt5*-Cre only” control PCMCs allergen-stimulated as in (B). Supernatant was harvested for ELISA 30 min after allergen challenge. Mean ± SD is shown.

(D) Phosphorylation of SNAP23 in PCMCs from one *Ikk2*^{FL/FL}*Mcpt5*-Cre⁺ and one Cre-negative control mouse sensitized and triggered as described above was assessed by phospho-specific western blot analysis. See Figure S3 for results obtained for four additional mutant and five additional control samples.

(E) Quantification of phospho-SNAP23 induction in *Ikk2*^{FL/FL}*Mcpt5*-Cre⁺ and control cells by densitometric analysis of all western blot results. Shown are geometric mean and 95% confidence interval of six values per genotype and stimulation condition.

PKC activation by proximal FcεRI signaling has been intensely studied, whereas coupling of these signals to exocytosis of MC granules is incompletely understood. Suzuki and Verma reported that IKK2 serves an essential function in MC degranulation by phosphorylating the SNARE protein SNAP23 (Suzuki and Verma, 2008). We generated mice with selective knockouts of the *Ikk2* or the *Nemo* gene in MCs and mice with MC-specific expression of a constitutively active IKK2 protein. Surprisingly, we found that loss of IKK2 in MCs did not affect their allergen-induced degranulation. Given the tremendous importance of

molecular mechanisms of MC degranulation for human diseases, we decided to use our models to clarify the role of IKK complex components in MC responses. We confirmed that a functional IKK complex is required for the IgE-induced MC late-phase cytokine response. We demonstrate, however, that IKK2 is dispensable for SNARE protein phosphorylation and for degranulation in vitro and in vivo.

Suzuki and Verma differentiated MCs from fetal liver of *Ikk2*^{-/-} embryos (E11.5–12.5) to circumvent lethality of this mutation. These cells were compromised in their degranulation response

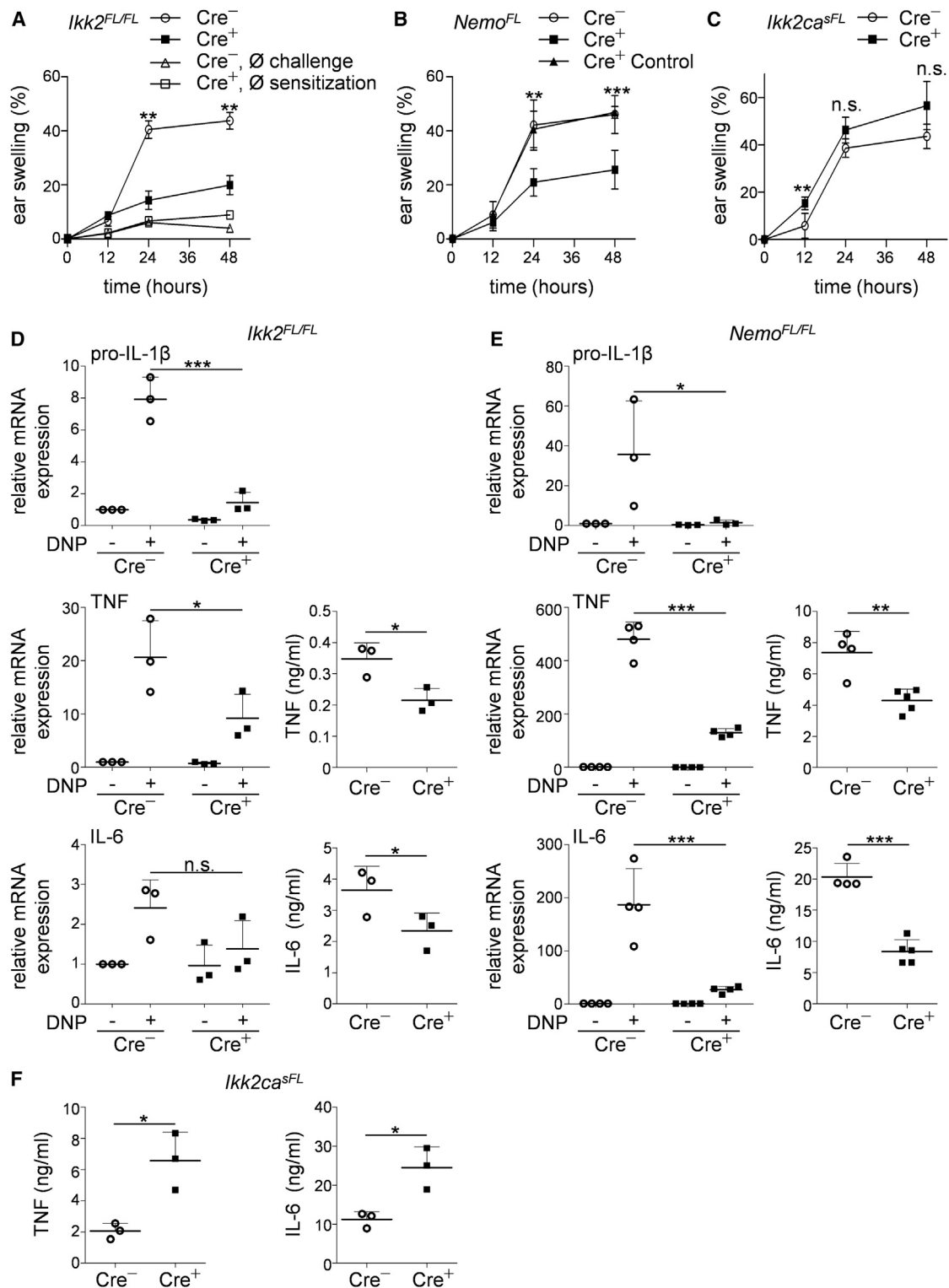


Figure 4. The Late-Phase MC Response Is Dependent on a Functional IKK Complex

LP-PCA was induced by i.v. sensitization with anti-DNP-IgE and challenge by epicutaneous administration of DNFB followed by monitoring of ear thickness. (A) Five *Ikk2^{FL/FL}Mcpt5-Cre⁺* and four Cre-negative littermate controls were analyzed. A Cre-negative control animal was sensitized but treated with vehicle (acetone/olive oil) alone, instead of challenge (Ø challenge). One *Ikk2^{FL/FL}Mcpt5-Cre⁺* was not sensitized but challenged with DNFB (Ø sensitization). The latter two animals served as controls also for data in (B) and (C), which were generated in parallel with data in (A). (See Figure S4 for a second experiment.)

(legend continued on next page)

to Fc ϵ R1 crosslinking. Moreover, upon transfer into MC-deficient WBB6F1-Kit^W/Kit^{W-v} mice, wild-type but not *Ikk2*^{-/-} FLMCs repaired defective anaphylaxis of the recipients. An important difference between the experimental models used is that the entire MC differentiation from *Ikk2*^{-/-} fetal-liver-derived precursors proceeds in the absence of IKK2. In contrast, *Ikk2*^{FL/FL}*Mcpt5*-Cre⁺ mice represent normal animals in which MCs differentiate in the presence of IKK2. Only during the last steps of MC maturation, the *Mcpt5* promoter is activated resulting in Cre-mediated inactivation of the *Ikk2* gene. We hypothesize that IKK2 may serve an important function in earlier steps of MC differentiation (occurring before induction of the *Mcpt5*-promoter), loss of which results in defective MCs characterized by impaired degranulation.

We show that phosphorylation of SNAP23 on Ser95 and Ser120 is not compromised in IKK2-deficient PCMCs. In MCs differentiated under physiological conditions, i.e., in the presence of IKK2, SNAP23 phosphorylation induced by Fc ϵ R1 activation may depend on kinases other than IKK2 or may represent a redundant function of different kinases, including IKK2. MCs lacking IKK2 throughout differentiation from fetal liver cells may be deficient in these pathways. An argument against this concept is that we found normal degranulation in BMMCs differentiated from IKK2-deficient bone marrow cells. Also, these BMMCs lacked IKK2 throughout their development but were not compromised in granule exocytosis. However, abnormal differentiation may be a distinctive feature of MCs differentiated in vitro from fetal liver cells.

Local responses of skin MCs were not changed in mice with MC-specific loss of IKK2, and in vitro degranulation of IKK2-deficient MCs was not different from the response of control cells. Constitutive IKK2 activity in MCs slightly enhanced systemic anaphylaxis in one experiment. This, however, was not reproduced in a second experiment. Therefore, our experiments demonstrate that even overexpression of a constitutively active IKK2 has only a minor, if any, impact on MC degranulation. Collectively, our results rule out an essential role for IKK2 in the signal transduction events leading to MC degranulation.

In conclusion, the question of how Fc ϵ R1-triggered second messenger signals are coupled to MC secretory granule exocytosis remains open. The identification of kinases responsible for allergen-induced SNARE-protein phosphorylation remains an important task because they might represent a target for therapeutic suppression of the MC response in allergic diseases.

EXPERIMENTAL PROCEDURES

Mice

Mcpt5-Cre (Scholten et al., 2008), *Mx*-Cre (Kühn et al., 1995), *R-DTA* (Voehringer et al., 2008), *R26Stop*^{FL}*Ikk2ca* (*Ikk2ca*^{FL}; Sasaki et al., 2006), *Ikk2*^{FL}

(Pasparakis et al., 2002), and *Nemo*^{FL} mice (Schmidt-Suppran et al., 2000), all C57BL/6, were bred and housed at the Experimental Center, Medical Faculty Carl-Gustav Carus, TU Dresden, under specific pathogen-free conditions. In all experiments, littermates were used as controls. All animal experimentation was performed according to institutional guidelines and was approved by the Landesdirektion Dresden.

Passive Systemic Anaphylaxis

Mice were sensitized by i.p. injection of 12.5 μ g SPE-7 anti-DNP-IgE (Eshhar et al., 1980). Twenty-four hours later, mice were challenged by i.p. injection of 200 μ g human serum albumin (HSA)-DNP (Sigma-Aldrich) in 0.9% NaCl or NaCl alone. Temperature drop was assessed using a rectal thermometer (Bio-TK 9882, Bioseb).

Passive Cutaneous Anaphylaxis

Mice were locally sensitized by intradermal injection of 20 ng anti-DNP-IgE (Sigma-Aldrich) in 20 μ l PBS into the left ear pinna. Twenty-four hours later, mice were challenged by i.v. injection of 100 μ g HSA-DNP (Sigma-Aldrich) in 0.9% NaCl. Ear thickness was monitored thereafter using an engineer's micrometer (Mitutoyo). Ear swelling was determined as percentage increase compared to prechallenge ear thickness. Additional mice were sensitized on the left ear as described above but also received an injection of 20 μ l PBS into the right ear. The next day, mice were challenged with 100 μ g HSA-DNP in 140 μ l 2% Evans blue. After 30 min, mice were sacrificed, and ear tissue was incubated in 500 μ l formamide at 50°C for 4–5 days. Extracted dye was quantified photometrically at 620 nm.

For quantification of the late-phase anaphylactic response, mice were sensitized by i.v. injection of 5 μ g SPE-7 anti-DNP-IgE on 2 consecutive days. On day 3, mice were challenged by epicutaneous administration of 10 μ l 0.2% (w/v) DNFB (Sigma-Aldrich) in acetone/olive oil (4:1) onto both sides of the ear. Ear thickness was measured before and 12, 24, and 48 hr after challenge.

Induction and Quantification of MC Degranulation

PCMCs were incubated with 1 μ g/ml SPE-7 anti-DNP-IgE and stimulated with 100 ng/ml HSA-DNP or HSA alone. In some experiments, cells were left unsensitized and were stimulated with 500 ng/ml A23187 (Sigma-Aldrich) for 30 min. β -hexosaminidase release was determined as percentage release of total content (percentage release = 100 $\times$ amount supernatant/ (amount in supernatant + cell lysate)). To quantify surface phosphatidylserin, cells were stained with FITC- or Pacific blue-annexin V (BioLegend) and propidium iodide. See the [Supplemental Experimental Procedures](#) for details.

Real-Time RT-PCR

RNA was reverse transcribed by Revert Aid H Minus RT (Thermo Fisher Scientific). Specific mRNAs were quantified using SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific), and the primers are listed in the [Supplemental Experimental Procedures](#).

ELISA

TNF and IL-6 protein levels in supernatants of stimulated PCMC or BMMC cultures were determined by ELISA (eBiosciences) as recommended by the manufacturer's protocol.

(B) LP-PCA in ten male *Nemo*^{FL/Y}*Mcpt5*-Cre⁺, six Cre-negative controls (five male *Nemo*^{FL/Y}, one female *Nemo*^{FL/FL}, collectively addressed as *Nemo*^{FL}) and four female *Nemo*^{FL/WT}*Mcpt5*-Cre⁺ control mice.

(C) LP-PCA in six *Ikk2ca*^{FL}*Mcpt5*-Cre⁺ and seven Cre⁻ controls.

(D–F) (D) BMMCs from poly I:C-induced *Ikk2*^{FL/FL}*Mx*-Cre⁺ mice, or (E) PCMCs from *Nemo*^{FL}*Mcpt5*-Cre⁺ mice, or (F) BMMCs from poly I:C-induced *Ikk2ca*^{FL}*Mx*-Cre⁺ mice and the respective Cre-negative littermate controls were sensitized and allergen-stimulated as described above (Figure 3). Cytokine mRNA levels were assessed 1 hr after antigen challenge by quantitative RT-PCR. Ct values were normalized to TBP and relative changes in transcript levels compared to HSA-exposed Cre-negative control cells are displayed.

All samples were assayed in triplicates. Concentrations of TNF and IL-6 in the culture supernatant were determined 6 hr after antigen challenge by ELISA. Mean $\pm$ SD is displayed. *p < 0.05, **p < 0.01, ***p < 0.001; n.s., not significant.

Statistical Analysis

Data are shown as means $\pm$ SD if not stated otherwise. Statistical analysis was performed using Student's *t* test or two-way ANOVA. Significance levels are indicated in each figure.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.07.046>.

AUTHOR CONTRIBUTIONS

K.P. designed and performed experiments, A.P., K. Heger, R.B., A.D., and N.S. performed experiments, J.S. generated Mcpt5-Cre mice and performed experiments, D.V. developed R-DTA mice, A.D. and K. Hartmann contributed to the design of the study, M.S.-S. and A.R. conceived and supervised the study, and M.S.-S. and A.R. wrote the paper.

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REFERENCES

- Dudeck, A., Dudeck, J., Scholten, J., Petzold, A., Surianarayanan, S., Köhler, A., Peschke, K., Vöhringer, D., Waskow, C., Krieg, T., et al. (2011). Mast cells are key promoters of contact allergy that mediate the adjuvant effects of haptens. *Immunity* 34, 973–984.
- Eshhar, Z., Ofarim, M., and Waks, T. (1980). Generation of hybridomas secreting murine reagenic antibodies of anti-DNP specificity. *J. Immunol.* 124, 775–780.
- Galli, S.J., and Tsai, M. (2012). IgE and mast cells in allergic disease. *Nat. Med.* 18, 693–704.
- Hepp, R., Puri, N., Hohenstein, A.C., Crawford, G.L., Whiteheart, S.W., and Roche, P.A. (2005). Phosphorylation of SNAP-23 regulates exocytosis from mast cells. *J. Biol. Chem.* 280, 6610–6620.
- Jeong, H.J., Koo, H.N., Na, H.J., Kim, M.S., Hong, S.H., Eom, J.W., Kim, K.S., Shin, T.Y., and Kim, H.M. (2002). Inhibition of TNF- α and IL-6 production by Aucubin through blockade of NF- κ B activation RBL-2H3 mast cells. *Cytokine* 18, 252–259.
- Kambayashi, T., and Koretzky, G.A. (2007). Proximal signaling events in Fc epsilon RI-mediated mast cell activation. *J. Allergy Clin. Immunol.* 119, 544–552, quiz 553–554.
- Klemm, S., Gutermuth, J., Hültner, L., Sparwasser, T., Behrendt, H., Peschel, C., Mak, T.W., Jakob, T., and Ruland, J. (2006). The Bcl10-Malt1 complex segregates Fc epsilon RI-mediated nuclear factor kappa B activation and cytokine production from mast cell degranulation. *J. Exp. Med.* 203, 337–347.
- Kühn, R., Schwenk, F., Aguet, M., and Rajewsky, K. (1995). Inducible gene targeting in mice. *Science* 269, 1427–1429.
- Lorentz, A., Klopp, I., Gebhardt, T., Manns, M.P., and Bischoff, S.C. (2003). Role of activator protein 1, nuclear factor- κ B, and nuclear factor of activated T cells in IgE receptor-mediated cytokine expression in mature human mast cells. *J. Allergy Clin. Immunol.* 111, 1062–1068.
- Lorentz, A., Baumann, A., Vitte, J., and Blank, U. (2012). The SNARE Machinery in Mast Cell Secretion. *Front. Immunol.* 3, 143.
- Marquardt, D.L., and Walker, L.L. (2000). Dependence of mast cell IgE-mediated cytokine production on nuclear factor- κ B activity. *J. Allergy Clin. Immunol.* 105, 500–505.
- Pasparakis, M., Courtois, G., Hafner, M., Schmidt-Supprian, M., Nenci, A., Toksoy, A., Krampert, M., Goebeler, M., Gillitzer, R., Israel, A., et al. (2002). TNF-mediated inflammatory skin disease in mice with epidermis-specific deletion of IKK2. *Nature* 417, 861–866.
- Peng, Y., Power, M.R., Li, B., and Lin, T.J. (2005). Inhibition of IKK down-regulates antigen + IgE-induced TNF production by mast cells: a role for the IKK-I κ B-NF- κ B pathway in IgE-dependent mast cell activation. *J. Leukoc. Biol.* 77, 975–983.
- Sasaki, Y., Derudder, E., Hobeika, E., Pelanda, R., Reth, M., Rajewsky, K., and Schmidt-Supprian, M. (2006). Canonical NF- κ B activity, dispensable for B cell development, replaces BAFF-receptor signals and promotes B cell proliferation upon activation. *Immunity* 24, 729–739.
- Schmidt-Supprian, M., Bloch, W., Courtois, G., Addicks, K., Israël, A., Rajewsky, K., and Pasparakis, M. (2000). NEMO/IKK gamma-deficient mice model incontinentia pigmenti. *Mol. Cell* 5, 981–992.
- Scholten, J., Hartmann, K., Gerbaulet, A., Krieg, T., Müller, W., Testa, G., and Roers, A. (2008). Mast cell-specific Cre/loxP-mediated recombination in vivo. *Transgenic Res.* 17, 307–315.
- Suzuki, K., and Verma, I.M. (2008). Phosphorylation of SNAP-23 by I κ B kinase 2 regulates mast cell degranulation. *Cell* 134, 485–495.
- Vöhringer, D., Liang, H.E., and Locksley, R.M. (2008). Homeostasis and effector function of lymphopenia-induced “memory-like” T cells in constitutively T cell-depleted mice. *J. Immunol.* 180, 4742–4753.

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Supplemental Information

I κ B Kinase 2 Is Essential for IgE-Induced Mast Cell De Novo Cytokine Production but Not for Degranulation

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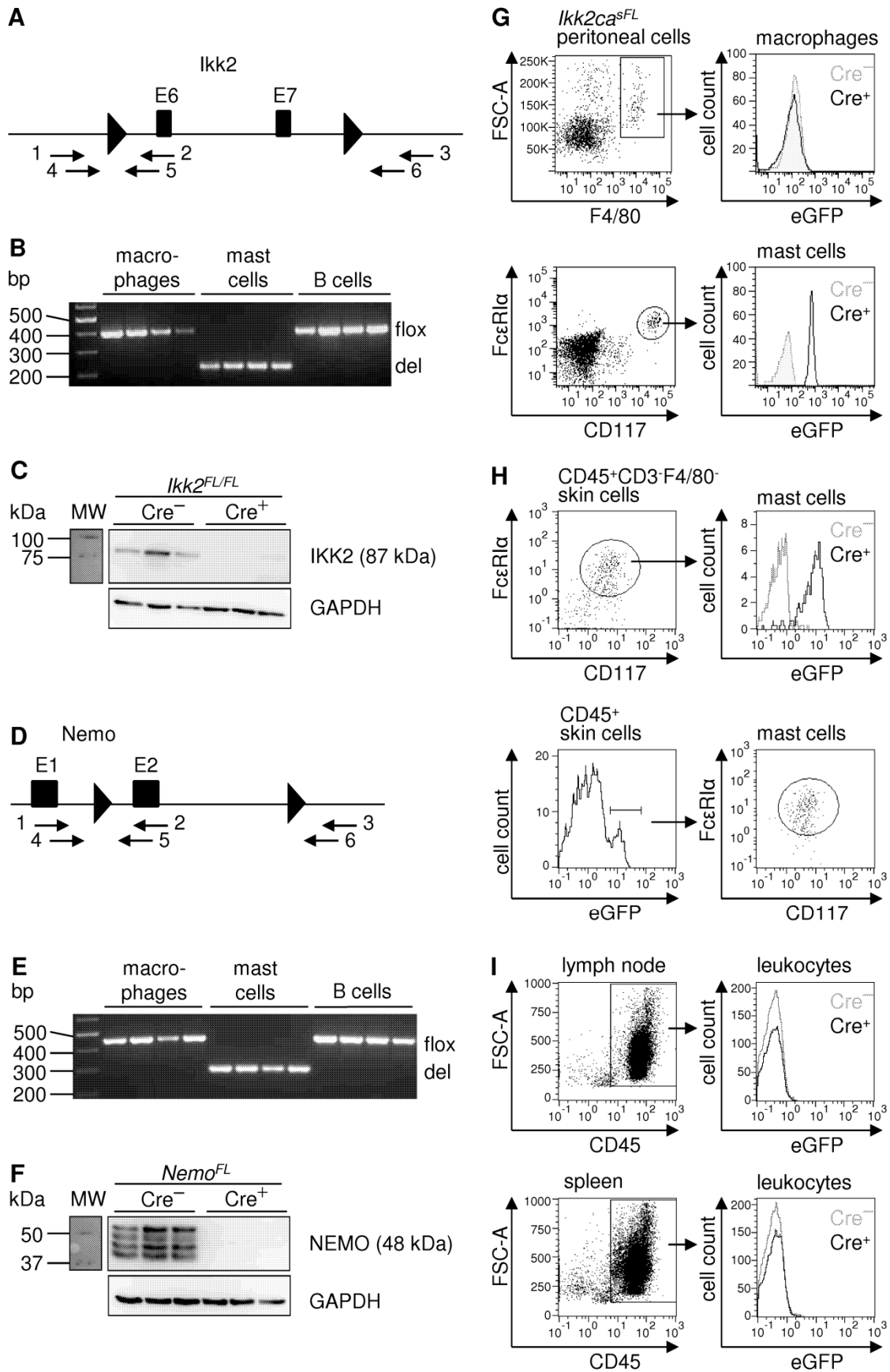


Figure S1. MC-specific inactivation of the *Ikk2* and *Nemo* genes and MC-specific de-repression of the *Ikk2ca^{SFL}* knock in allele, related to Figure 1.

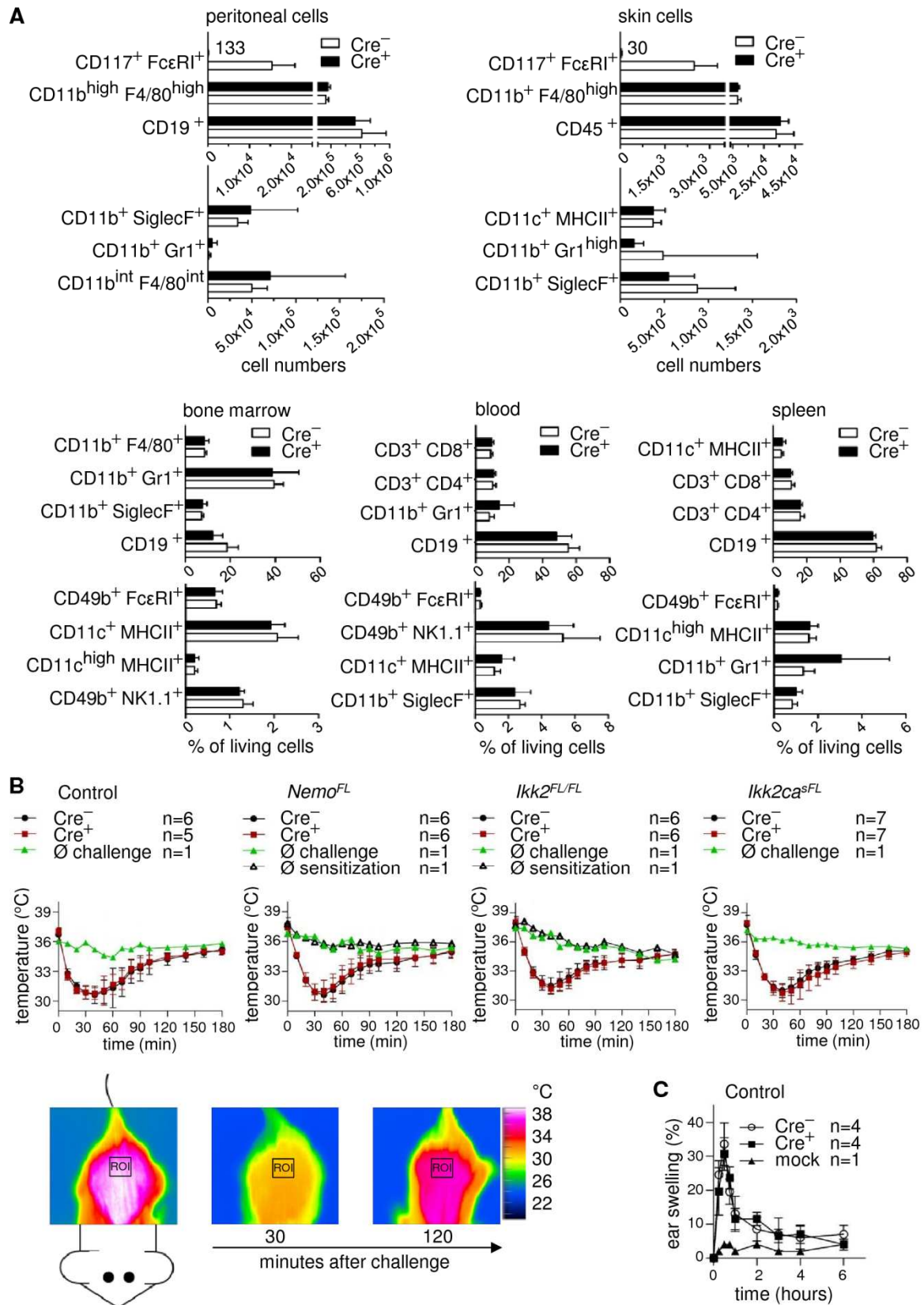


Figure S2. Quantification of different types of non-MCs in MC-deficient *Mcpt5-Cre⁺R-DTA⁺* mice and allergen-induced anaphylaxis in animals with MC-specific inactivation or over-activation of the IKK complex, related to Figure 2.

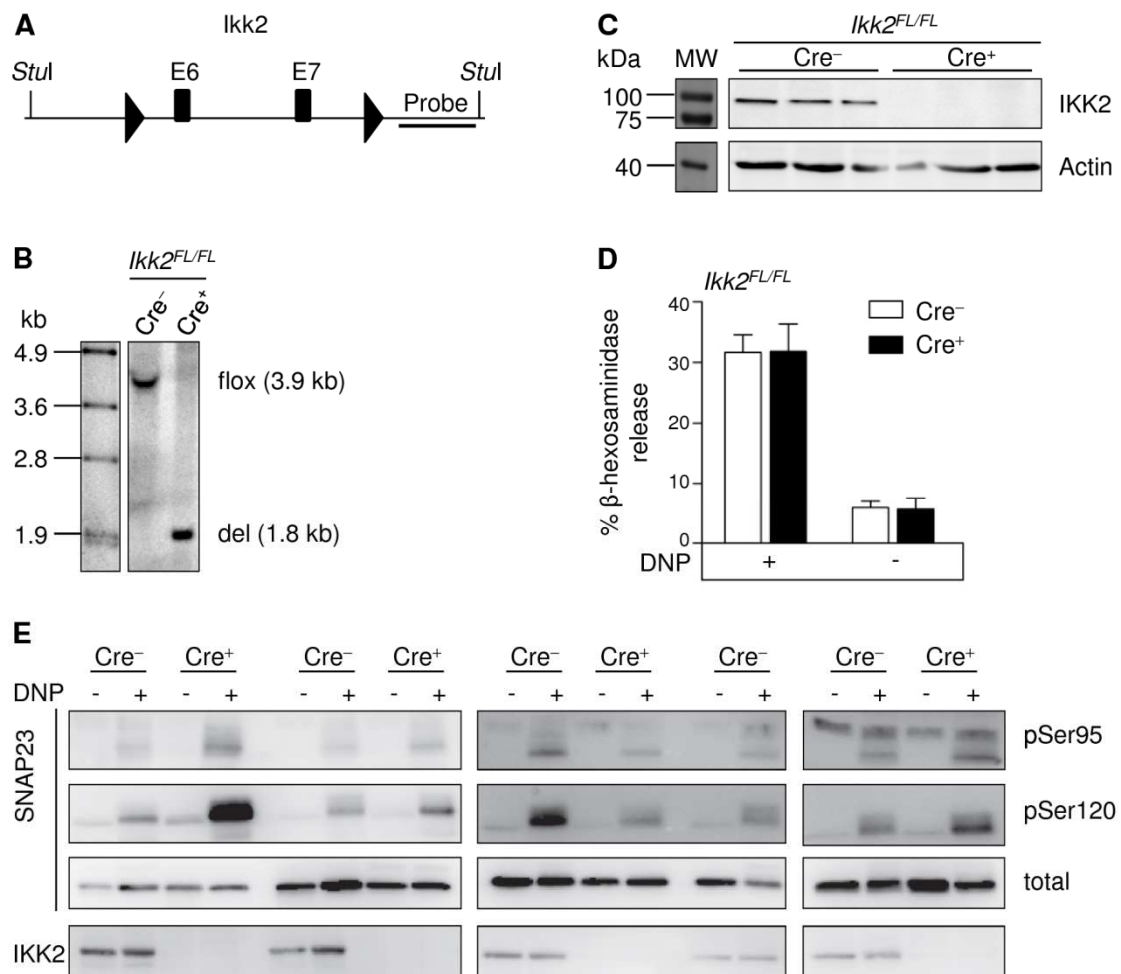


Figure S3. *In vitro* degranulation of bone marrow-derived MCs and Fc ϵ RI-induced phosphorylation of SNAP-23 in MCs cultivated from peritoneal lavage do not depend on IKK2, related to Figure 3.

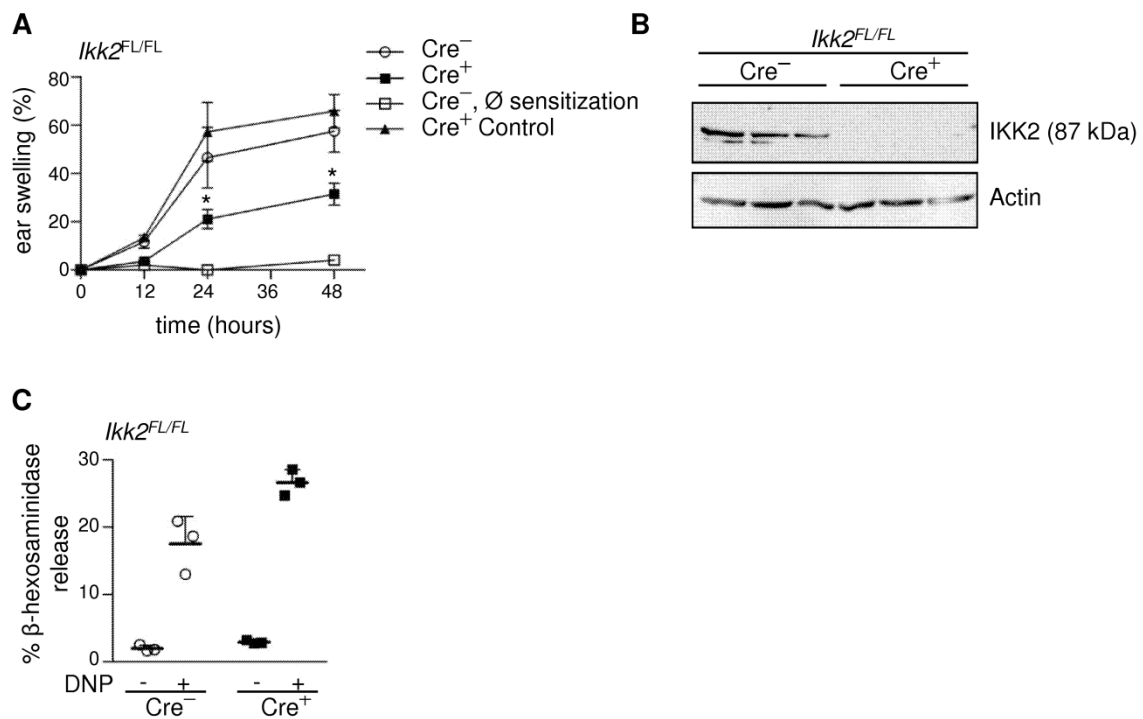


Figure S4. The late phase MC response is dependent on IKK activity, related to Figure 4.

Supplemental Figure Legends

Figure S1. MC-specific inactivation of the *Ikk2* and *Nemo* genes and MC-specific de-repression of the *Ikk2ca^{SFL}* knock in allele, related to Figure 1.

A, D Primer positions for nested single target amplification of the *loxP*-flanked (“floxed”) or deleted *Ikk2* and *Nemo* alleles. Primers 1, 2 and 3 were used in the first, primers 4, 5 and 6 in the second round of amplification (Second round products: *Ikk2* floxed 391bp, *Ikk2* deleted 218bp; *Nemo* floxed 454bp, *Nemo* deleted 290bp) **B, E** Examples of nested amplification of both loci from single *Ikk2^{FL/FL}Mcpt5-Cre⁺* (B) and *Nemo^{FL}Mcpt5-Cre⁺* (E) peritoneal lavage cells deposited into PCR tubes (one cell per tube) by FACS (directly *ex vivo* without prior cultivation). Products representing the *loxP*-flanked alleles were obtained from non-MCs, while MCs yielded products representing the deleted alleles (see also Table S1). **C, F** Western blot demonstrating absence of IKK2 and NEMO in *Ikk2^{FL/FL}Mcpt5-Cre⁺* and *Nemo^{FL}Mcpt5-Cre⁺* MCs, respectively, cultivated from peritoneal lavage (PCMCs). Each lane represents cells from a different animal. **G, H, I** Efficient and MC-specific de-repression of the *Ikk2ca^{SFL}* knock in allele in *Ikk2ca^{SFL}Mcpt5-Cre⁺* mice as determined by FACS analysis of expression of reporter eGFP co-expressed with IKK2ca upon deletion of the stop element. **G** MCs and macrophages were identified in peritoneal lavage fluid as FcεRIα⁺CD117⁺ and F4/80⁺ cells, respectively. (representative of 22 mice) **H** Single cell suspensions of skin cells were generated by enzymatic digest of ear skin and MCs were identified in the CD45⁺CD3⁻F4/80⁻ fraction as FcεRIα⁺CD117⁺ cells (upper panels). Conversely, eGFP-expressing CD45⁺ skin cells were analyzed for expression of MC markers (representative of eight mice). **I** No reporter fluorescence was detected in CD45⁺ lymph node or spleen cells from *Ikk2ca^{SFL}Mcpt5-Cre⁺* mice (representative of four mice each).

Figure S2. Quantification of different types of non-MCs in MC-deficient *Mcpt5-Cre⁺R-DTA⁺* mice and allergen-induced anaphylaxis in animals with MC-specific inactivation or over-activation of the IKK complex, related to Figure 2.

A No significant changes in the numbers of other hematopoietic cell types in MC-deficient *Mcpt5-Cre⁺R-DTA⁺* mice. Various hematopoietic cell populations in the peritoneal cavity, ear skin, bone marrow, blood and spleen of six *Mcpt5-Cre⁺R-DTA⁺* and seven Cre-negative controls were analyzed by flow cytometry using the markers indicated. Data represented as mean $\pm$ SD. **B** Monitoring of anaphylaxis by infra-red temperature measurement. Passive systemic anaphylaxis (PSA) was induced by i.p. sensitization with anti-DNP IgE. 24 hours later, the animals were challenged by i.p. injection of HSA-DNP. Drop of body temperature was measured by infra-red imaging of the shaved skin of the lower back. Upper panels, PSA response of control *Mcpt5-Cre⁺* mice without floxed alleles (control excluding effects of the *Cre* transgene alone) of *Ikk2^{FL/FL}Mcpt5-Cre⁺*, *Nemo^{FL}Mcpt5-Cre⁺* (the “*Nemo^{FL}*” groups include male *Nemo^{FL/Y}* and female *Nemo^{FL/FL}* mice) and *Ikk2ca^{sFL}Mcpt5-Cre⁺* mice and the respective Cre-negative controls. Additional control mice were either not sensitized (“ $\emptyset$ sensitization”) or not challenged (“ $\emptyset$ challenge”). Lower panel, example of temperature measurements before and after induction of anaphylaxis. **C** Passive cutaneous anaphylaxis (PCA) in *Mcpt5-Cre⁺* control mice excluding effects of the *Cre* transgene alone. Mean $\pm$ SD is displayed.

Figure S3. *In vitro* degranulation of bone marrow-derived MCs and Fc ϵ RI-induced phosphorylation of SNAP23 in MCs cultivated from peritoneal lavage do not depend on IKK2, related to Figure 3.

A-D MCs were differentiated from bone marrow of poly I:C-induced *Ikk2^{FL/FL}Mx-Cre⁺* and Cre-negative littermate control mice by cultivation in the presence of IL-3 and

SCF. **A, B** Efficient deletion of the *loxP*-flanked fragment of the *Ikk2* gene in BMMCs demonstrated by Southern blot analysis of *StuI*-digested DNA extracted from bulk cultures (representative of cultures from three different mutant animals). **C** Absence of IKK2 protein in BMMCs was demonstrated by Western blot analysis. **D** IgE-induced degranulation of IKK2-deficient and IKK2-proficient BMMCs was assessed by loading the cells overnight with anti-DNP IgE followed by stimulation with DNP-HSA (“+ DNP”) and quantification of β -hexosaminidase release. Samples exposed to HSA instead of the DNP-HSA served as negative controls (“- DNP”). Mean $\pm$ SD is displayed. **E** IgE-induced phosphorylation of MC SNAP23 does not depend on IKK2. Phosphorylation of SNAP23 on Ser95 and Ser120 in PCMCs from four *Ikk2*^{FL/FL}*Mcpt5-Cre*⁺ and five Cre-negative control mice sensitized and triggered as described above was assessed by phospho-specific Western blot analysis.

Figure S4. The late phase MC response is dependent on IKK activity, related to Figure 4.

A LP-PCA was induced in 5 *Ikk2*^{FL/FL}*Mcpt5-Cre*⁺, 6 Cre-negative littermate controls and 2 *Ikk2*^{wt/FL}*Mcpt5-Cre*⁺ controls (*Cre*⁺ Control) by i.v. sensitization with anti-DNP IgE and challenge by epicutaneous administration of DNFB on both sides of one ear 24 hours later. One Cre-negative animal was not sensitized but challenged with DNFB ($\emptyset$ sensitization). Ear thickness was monitored for 48 hours. Mean ear swelling $\pm$ SD is displayed. * $p < 0.01$ (comparison between *Cre*⁺ and *Cre*-negative). **B** Western Blot analysis demonstrates efficient *Ikk2* gene inactivation in the BMMC cultures that showed impaired IgE-induced cytokine responses as shown in Figure 4D. **C** β -hexosaminidase release assay demonstrates unimpaired degranulation of the *Ikk2*-deficient BMMC cultures that showed impaired IgE-induced cytokine responses as shown in Figure 4D. Mean $\pm$ SD is displayed.

Supplemental Tables

A MC-specific inactivation of the *Ikk2* gene

peritoneal cavity

	tubes which yielded...				
cell type and number of cells/tube	1 product representing loxP-flanked locus	1 product representing deleted locus	2 products representing loxP-flanked and deleted locus	no product	total number of tubes
	<i>Ikk2^{FL/FL} Mcpt5-Cre⁺</i>				
1 mast cell	-	92	-	16	108
1 macrophage	17	-	-	3	20
1 B cell	19	-	-	1	20
10 mast cells	-	20	-	-	20
8 mast cells + 2 macrophages	-	2	18	-	20
water control	-	-	-	48	48
	<i>Ikk2^{FL/FL} Mcpt5-Cre⁻</i>				
1 mast cell	65	-	-	7	72
1 macrophage	17	-	-	3	20
1 B cell	18	-	-	2	20
water control	-	-	-	28	28

skin cell suspension

	tubes which yielded...				
cell type and number of cells/tube	1 product representing loxP-flanked locus	1 product representing deleted locus	2 products representing loxP-flanked and deleted locus	no product	total number of tubes
	<i>Ikk2^{FL/FL} Mcpt5-Cre⁺</i>				
1 mast cell	-	70	1	9	80
1 macrophage	14	-	-	6	20
1 T cell	19	-	-	1	20
10 mast cells	-	18	2	-	20
8 mast cells + 2 macrophages	-	2	18	-	20
water control	-	-	-	20	20
	<i>Ikk2^{FL/FL} Mcpt5-Cre⁻</i>				
1 mast cell	17	-	1	2	20
1 macrophage	18	-	-	2	20
1 T cell	19	-	-	1	20
water control	-	-	-	10	10

B MC-specific inactivation of the *Nemo* gene

peritoneal cavity

	tubes which yielded...				
cell type and number of cells/tube	1 product representing loxP-flanked locus	1 product representing deleted locus	2 products representing loxP-flanked and deleted locus	no product	total number of tubes
	<i>Nemo</i> ^{FL} <i>Mcpt5</i> -Cre ⁺				
1 mast cell	-	36	-	-	36
1 macrophage	30	1	-	5	36
1 B cell	13	-	-	7	20
10 mast cells	-	20	-	-	20
8 mast cells + 2 macrophages	-	-	20	-	20
water control	-	-	-	20	20
	<i>Nemo</i> ^{FL} <i>Mcpt5</i> -Cre ⁻				
1 mast cell	19	-	-	1	20
1 macrophage	13	-	1	6	20
1 B cell	14	-	-	6	20
water control	-	-	-	12	12
skin cell suspension					
	tubes which yielded...				
cell type and number of cells/tube	1 product representing loxP-flanked locus	1 product representing deleted locus	2 products representing loxP-flanked and deleted locus	no product	total number of tubes
	<i>Nemo</i> ^{FL} <i>Mcpt5</i> -Cre ⁺				
1 mast cell	-	34	-	6	40
1 macrophage	19	-	-	1	20
1 T cell	19	-	-	1	20
10 mast cells	-	11	-	-	11
8 mast cells + 2 macrophages	-	-	5	-	5
water control	-	-	-	20	20
	<i>Nemo</i> ^{FL} <i>Mcpt5</i> -Cre ⁻				
1 mast cell	19	-	-	1	20
1 macrophage	19	-	-	1	20
1 T cell	20	-	-	-	20
water control	-	-	-	12	12

Table S1. Efficient MC-specific inactivation of the *Ik2* and the *Nemo* gene *in vivo* as demonstrated by single cell PCR, related to Figure 1.

Overview of products of the single target amplification of the loxP-flanked *Ik2* (A) and *Nemo* (B) locus from FACS-sorted single MCs and non-MCs from the peritoneal

cavity or skin. Single cells or the indicated numbers of cells were deposited into individual PCR tubes. The numbers of tubes which yielded a product representing the *loxP*-flanked (undelated) or the deleted locus, or both, are listed.

A Only products representing the deleted *Ikk2* locus were amplified from 112 tubes containing in total 292 *Ikk2^{FL/FL}Mcpt5-Cre⁺* peritoneal MCs, while non-MCs yielded only products representing the undelated, *loxP*-flanked (“floxed”) locus. 91 tubes containing in total 271 *Ikk2^{FL/FL}Mcpt5-Cre⁺* MCs sorted from skin cell suspensions all yielded “deleted products” except for three, which gave both a deleted and a floxed product. These three floxed products could result from imperfect Cre-mediated deletion but also from rare erroneous deposition of a non-MC together with a MC by the cell sorter or from rare contamination with genomic DNA or PCR products. A fraction of tubes did not yield a product, which was most likely due to either failure of single cell deposition or to damaged template molecules. PCR tubes into which no cell was sorted but which were otherwise handled exactly as the tubes containing cells served as negative (“water”) controls. Collectively, these data show that the *Ikk2* gene was deleted in close to 100% of peritoneal and skin MCs in *Ikk2^{FL/FL}Mcpt5-Cre⁺* mice. **B** Similar results were obtained for MC-specific inactivation of the *Nemo* gene in *Nemo^{FL}Mcpt5-Cre⁺* mice.

Supplemental Experimental Procedures

Stainings for flow cytometric analysis and sorting

Cells were resuspended in PBS/0.5% BSA and stained for 30 min at 4°C with antibodies against the following markers (from eBiosciences, if not indicated otherwise): CD117 (clone 2B8), FcεRIα (clone MAR-1), CD45 (clone 30-F11), F4/80 (clone BM8), CD11b (clone M1/70), CD4 (clone GK1.5), CD19 (clone eBio1D3), Ly-6G (clone RB6-8C5), CD49b (clone DX5), NK1.1 (clone PK136), CD3e (clone 145-2C11), CD8a (clone 53-6.7), SiglecF (clone E50-2440, BD Pharmingen), CD11c (clone N418), MHCII (clone DX-3, Serotec). Cell suspensions were washed twice and were resuspended in PBS/0.5% BSA. To determine cell viability, 1 µg/ml propidium iodide was added. Analysis was done using the Miltenyi MacsQuant flow cytometer with MacsQuantTM Analysis or FlowJo Software.

Single cell sorting and single target amplification of the *Ikk2* and *Nemo* loci

Single cell suspensions containing MCs were generated by lavaging the peritoneal cavity or by digesting ear tissue. Ears were cut into small pieces and digested in 1 ml DMEM containing 20 mM Hepes, 0.025 mg/ml Liberase TM (Roche), 396 U/ml DNase I (Roche), and 0.5 mg/ml Hyaluronidase (Sigma) at 37°C with shaking at 1400 rpm for 1 h. Samples were passed through a 40 µm sieve and the cell suspension was washed twice with PBS. Single MCs (CD19⁻ F4/80⁻ CD117⁺ FcεRIα⁺), B cells (CD117⁻ F4/80⁻ CD19⁺ B220⁺) and macrophages (CD117⁻ CD19⁻ B220⁻ F4/80⁺) from peritoneal lavage and T cells (CD45⁺ F4/80⁻ CD4⁺), macrophages (CD45⁺ CD4⁻ F4/80⁺), and MCs (CD45⁺ CD4⁻ F4/80⁻ CD117⁺ FcεRIα⁺) from ear skin cell suspension were sorted into PCR tubes containing 15 µl water using a FACS Aria II cell sorter (BD Biosciences). Water control tubes were placed in the cell sorter but no cell was deposited into these tubes. Tubes containing single cells were stored at -80°C.

After thawing, cells were digested in 1 x DreamTaq PCR buffer (Fermentas) containing Proteinase K (Fermentas, 2 mg/ml final concentration) in a total volume of 20 µl. Cells were incubated at 50°C for 1.5 h followed by inactivation of Proteinase K by heating to 95°C for 10 min. Single target PCR was performed in two rounds of amplification (see Figure S1 for primer positions; the primer sequences were as follows: *Ikk2*-1 5'- ACT-GGG-AGA-ACA-CCA-TAC-TAG-CTG-A-3', *Ikk2*-2 5'- TTT-CGT-GAA-GGT-ATC-TAA-GAG-CCG-AT-3', *Ikk2*-3 5'- ATT-CCT-CTC-CTC-GTC-ATC-CTT-CGA-G-3', *Ikk2*-4 5'- GGA-GAA-CAC-CAT-ACT-AGC-TGA-ACT-G-3', *Ikk2*-5 5'- AAG-AGC-CGA-TGC-TGA-GGA-AGA-G-3', *Ikk2*-6 5'- TCT-GCC-ACC-CGA-CTA-CAT-CTT-C-3', *Nemo*-1 5'-TGT-GTC-CTA-TTC-TTG-GAA-GGG-TAT-G-3', *Nemo*-2 5'-AGC-ATC-TGA-TTG-CTC-TGC-CGG-ATA-3', *Nemo*-3 5'-ACT-GAA-CAA-ATT-CTA-TTC TCG-TAG-CTC-TA-3', *Nemo*-4 5'-GTC-CTA-TTC-TTG-GAA-GGG-TAT-GGC-C-3', *Nemo*-5 5'-GAT-AGC-GTC-TAG-GGA-CAG-GGA-AG-3', *Nemo*-6 5'- TCT-CGT-AGC-TCT-AAT-AAC-TGT-CTA-CCT-3'). For the first round, 30 µl of "single cell mix" (1x DreamTaq PCR buffer (2 mM MgCl₂, Fermentas), 200 µM each of dATP, dTTP, dGTP and dCTP, 0.2 µM each HPLC-purified primer *Nemo*-1, -2 and -3 or *Ikk2*-1, -2 and -3 and 2 U DreamTaq DNA Polymerase (Fermentas)) was added to each tube. Thermal cycling was performed with initial denaturation at 95°C for 2 min followed by 35 cycles at 95°C for 20 sec, 30 sec annealing at 52°C for *Nemo* or 53°C for *Ikk2* and 72°C for 1 min. Finally the reaction was incubated at 72°C for 10 min. 1 µl of the first round of amplification was used as template for the second round PCR, which was performed in 1 x DreamTaq buffer, 200 µM each dNTP, 0.2 µM each primer *Nemo*-4, -5 and -6 or *Ikk2*-4, -5 and -6, 1.5 U DreamTaq DNA Polymerase in a total volume of 50 µl. Initial denaturation at 95°C for 2 min was followed by 35 cycles at 95°C for 20 seconds, 30 sec annealing at 61°C for *Nemo* or

54°C for *Ikk2*, 72°C for 1 min and a final 10 min incubation at 72 °C. PCR products were analyzed by agarose gel electrophoresis and verified by sequencing.

Cell culture

Peritoneal cell-derived MCs (PCMCs) were obtained by cultivating peritoneal cell suspensions in DMEM+GlutaMAX (Gibco) containing 10% heat inactivated FCS, 50 µM β-mercaptoethanol, 100 U/ml penicillin, 100 U/ml streptomycin, 2% culture supernatant of the mIL3-transformed cell line X63Ag8-653 and 0.5% supernatant of CHO transfectants secreting murine SCF (generated by S. Lyman, Immunex, Seattle, and kindly provided by P. Dubreuil, Marseille). After 5 weeks more than 98% of the cells expressed high levels of CD117 and FcεRIα. For the generation of bone marrow derived MCs (BMMCs) *Ikk2^{F/F}Mx-Cre⁺*, *Nemo^FMx-Cre⁺* or *Ikk2ca^{sF}Mx-Cre⁺* mice and Cre-negative littermate controls received 400 µg poly I:C. After three days, bone marrow was flushed out of long bones and cultured as described above except that 0.5% supernatant of CHO transfectants secreting murine SCF was added only after three weeks of culture. More than 97% of cells expressed high levels of CD117 after 6 weeks.

Induction and quantification of MC degranulation

PCMCs were incubated with 1 µg/ml SPE-7 anti-DNP-IgE over night. The next day, the same amount of anti-DNP-IgE was added again. After 2 h, cells were washed twice in Tyrode's Buffer (135 mM NaCl, 5 mM KCl, 5.6 mM glucose, 1.8 mM CaCl₂, 1 mM MgCl₂, 0.5 mg/ml BSA Fraction V, 20 mM HEPES, pH 7.3). After washing, 1 x 10⁵ PCMCs or 2 x 10⁶ BMMCs were stimulated with 100 ng/ml HSA-DNP (Sigma-Aldrich) or HSA alone for 30 min at 37°C. In some experiments, cells were left unsensitized and were stimulated with 500 ng/ml A23187 (Sigma-Aldrich) for 30 min.

To quantify β -hexosaminidase release, stimulated cells were sedimented by centrifugation (5 min, 1600 rpm, 4°C) the supernatant was collected and the cell pellet was lysed in 200 μ l 0.5% Triton X-100 in Tyrode's Buffer. 25 μ l of supernatants and cell lysate were incubated with 25 μ l 1 mM p-nitrophenyl-N-acetyl β -D-glucosamine in citrate buffer (0.1 M, pH 4.5) for 1 h at 37°C. The reaction was stopped with 200 μ l carbonate buffer (0.1 M Na₂CO₃, 0.1 M NaHCO₃, pH 9) and enzyme activity was determined by measuring OD at 405 nm. β -hexosaminidase release was determined as percent release of total content (percent release = 100 x amount supernatant/ (amount in supernatant + cell lysate)). To quantify surface phosphatidylserin by annexin V staining, stimulation of MCs was stopped by adding ice cold annexin V staining buffer (10 mM Hepes, NaOH, pH 7.3, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂). Cells were washed and stained with FITC- or Pacific blue-annexin V (BioLegend) in 100 μ l annexin V buffer for 15 min at room temperature. After staining, 400 μ l annexin V buffer and 1 μ g/ml propidium iodide were added. Analysis was done using a MacsQuant flow cytometer (Miltenyi).

Western Blot

Cells were lysed in RIPA buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1% NP40, 1 mM EDTA, 0.25% Na-deoxycholate) supplemented with 10 μ g/ml Aprotinin, 10 μ g/ml Leupeptin, 0.1 mM Na₃VO₄, 1 mM PMSF, 10 mM NaF, 1 mM DTT and 8 mM β -glycerophosphate. After standard SDS-PAGE and blotting, PVDF membranes were probed with the following antibodies: anti-NEMO (rabbit polyclonal serum, gift from Ralph Gareus, Cologne), anti-IKK2 (clone 10AG2, Millipore), anti-IkBa (clone 44D4, Cell Signaling), anti-actin (polyclonal, Sigma-Aldrich) anti-GAPDH (Calbiochem), anti-SNAP23 (Santa Cruz), anti-phospho-SNAP23 Ser95 and Ser120 (both polyclonal (Yasuda et al., 2011), gift from Makoto Itakura, Kitasato, Japan). Western blot bands

were quantified using image-J software. Phospho-SNAP23 (P-SNAP23) pixel counts were divided by SNAP23 pixel counts. The P-SNAP23/SNAP23 pixel counts were then normalized to the value for unstimulated control mast cells („- DNP“ *IKK2^{FL/FL}* Cre-negative). To average the resulting fold-changes we calculated the geometric means and 95% confidence interval.

Primers for RT-PCR analysis of the MC cytokine response

pro-IL-1 β (IL-1 β -Fwd 5'-GCC-ACC-TTT-TGA-CAG-TGA-TGA-G, IL-1 β -Rev 5'-AGC-TTC-TCC-ACA-GCC-ACA-AT), TNF (TNF-Fwd 5'-ACT-GAA-CTT-CGG-GGT-GAT-CG, TNF-Rev 5'-CTT-GGT-GGT-TTG-CTA-CGA-CG), IL-6 (IL-6-Fwd 5'-TTC-CAT-CCA-GTT-GCC-TTC-TT, IL-6-Rev 5'-CTG-CAA-GTG-CAT-CAT-CGT-TG) and TATA box-binding protein (TBP) (TBP-Fwd 5'-TCT-ACC-GTG-AAT-CTT-GGC-TGT-AAA, TBP-Rev 5'-TTC-TCA-TGA-TGA-CTG-CAG-CAA-A)

Quantification of skin MCs

Biopsies of back skin were fixed in 4% (v/v) buffered formalin. 5 μ m paraffin sections were Giemsa-stained and MCs located between epidermis and panniculus carnosus were counted along 3 mm of skin at a magnification of 400x.

Quantification of PSA responses by thermal imaging

Drop in body temperature upon induction of passive systemic anaphylaxis (PSA) was quantified by thermal imaging on the shaved back using a VarioCAM high resolution instrument (Jenoptik) and IRBIS remote 3.0 software. Mean temperature of a rectangular region of interest was calculated.