

Autocrine hemokinin-1 functions as an endogenous adjuvant for IgE-mediated mast cell inflammatory responses

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Background: Efficient development of atopic diseases requires interactions between allergen and adjuvant to initiate and amplify the underlying inflammatory responses. Substance P (SP) and hemokinin-1 (HK-1) are neuropeptides that signal through the neurokinin-1 receptor (NK1R) to promote inflammation. Mast cells initiate the symptoms and tissue effects of atopic disorders, secreting TNF and IL-6 after FcεRI cross-linking by antigen-IgE complexes (FcεRI-activated mast cells [FcεRI-MCs]). Additionally, MCs express the NK1R, suggesting an adjuvant role for NK1R agonists in FcεRI-MC-mediated pathologies; however, in-depth research addressing this relevant aspect of MC biology is lacking.

Objective: We sought to investigate the effect of NK1R signaling and the individual roles of SP and HK-1 as potential adjuvants for FcεRI-MC-mediated allergic disorders.

Methods: Bone marrow-derived mast cells (BMMCs) from C57BL/6 wild-type (WT) or NK1R^{-/-} mice were used to investigate the effects of NK1R signaling on FcεRI-MCs. BMMCs generated from *Tac1*^{-/-} mice or after culture with *Tac4* small interfering RNA were used to address the adjuvancy of SP and HK-1. WT, NK1R^{-/-}, and c-Kit^{W-sh/W-sh} mice reconstituted with WT or NK1R^{-/-} BMMCs were used to evaluate NK1R signaling on FcεRI-MC-mediated passive local and systemic anaphylaxis and on airway inflammation.

Results: FcεRI-activated MCs upregulated NK1R and HK-1 transcripts and protein synthesis, without modifying SP expression. In a positive signaling loop HK-1 promoted TNF and IL-6 secretion by MC degranulation and protein synthesis, the latter through the phosphoinositide 3-kinase/Akt/nuclear factor κB pathways. *In vivo* NK1R signaling was necessary for the development of passive local and systemic anaphylaxis and airway inflammation.

Conclusions: FcεRI stimulation of MCs promotes autocrine secretion of HK-1, which signals through NK1R to provide adjuvancy for efficient development of FcεRI-MC-mediated disorders. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: Hemokinin-1, substance P, neurokinin-1 receptor, mast cells, IgE, FcεRI, TNF, IL-6, passive anaphylaxis, airway inflammation

The immune system is designed to eliminate foreign antigens while maintaining tissue integrity. Innate and adaptive immune responses should effectively be resolved after the neutralization of foreign antigen to accomplish these functions. Adjuvant-mediated increases in the intensity or prolongation of inflammatory reactions result in fatal outcomes in the case of anaphylaxis and irreversible tissue damage with function loss. Inflammatory responses are amplified by neuropeptides from the tachykinin family, such as substance P (SP) and hemokinin-1 (HK-1).¹ Both neuropeptides exert their effects by signaling through the neurokinin-1 receptor (NK1R), a 7-transmembrane domain G protein-coupled receptor (GPCR).² NK1R signaling mediates pain, inflammation, and immune function, the latter through dendritic cell and monocyte activation,³⁻⁶ chemotaxis and cytotoxicity of natural killer cells,^{7,8} and differentiation and survival of pro-T and pro-B lymphocytes.⁹⁻¹² Previous reports on SP and HK-1 proinflammatory function and the observation that SP expression is increased in autoimmune disorders¹³⁻¹⁵ highly suggest an adjuvant role for NK1R agonists.

Peripheral tissue-resident mast cells (MCs) express the NK1R¹⁶ and are ideally positioned to respond to foreign antigens with innate and adaptive immune functions.^{17,18} Activation of FcεRI-activated mast cells (FcεRI-MCs) is central to their pathologic inflammatory function. Cross-linking of surface FcεRI in MCs initiates a biphasic inflammatory response comprising immediate degranulation with release of stored proinflammatory mediators and delayed secretion of *de novo*-synthesized proinflammatory cytokines. FcεRI-MCs release TNF and IL-6, which trigger anaphylaxis and mediate the symptoms and tissue effects of chronic atopic disorders.^{17,18}

Mechanistically, FcεRI activation recruits Src family kinases¹⁸ to activate phosphoinositide 3-kinase (PI3K) and phospholipase C cascades that interconnect with intracellular signaling pathways initiated by GPCR.^{2,18} Accordingly, interactions between FcεRI and NK1R signaling might regulate MC inflammatory functions. Although scarce reports have associated SP with IgE-independent MC functions,¹⁹⁻²¹ the mechanisms and individual roles of NK1R agonists in the biology and function of FcεRI-MCs remain unknown. Furthermore, to our knowledge, information regarding the contribution of HK-1 to MC inflammatory functions is lacking.

In the present work we demonstrate that signaling murine MCs through FcεRI upregulates (1) expression of the NK1R, (2) transcription of the HK-1 gene (*Tac4*), and (3) synthesis of HK-1, without modifying transcription of the SP gene (*Tac1*) or secretion of SP peptide. In an autocrine/paracrine positive signaling loop, binding the NK1R by HK-1 is critical for FcεRI-mediated

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Abbreviations used

BAL:	Bronchoalveolar lavage
BMMC:	Bone marrow–derived mast cell
DNP-HSA:	Dinitrophenyl–human serum albumin
EPO:	Eosinophil peroxidase
ERK:	Extracellular signal-regulated kinase
FcεRI-MC:	FcεRI-activated mast cell
FcεRI-BMMC:	FcεRI-activated bone marrow mast cell
GPCR:	G protein–coupled receptor
HK-1:	Hemokinin-1
JNK:	c-Jun N-terminal kinase
MC:	Mast cell
MPO:	Myeloperoxidase
NF-κB:	Nuclear factor κB
NK1R:	Neurokinin-1 receptor
OVA:	Ovalbumin
PCA:	Passive cutaneous anaphylaxis
PFA:	Paraformaldehyde
PSA:	Passive systemic anaphylaxis
PI3K:	Phosphoinositide 3-kinase
PMN:	Polymorphonuclear
SP:	Substance P
WT:	Wild-type

MC secretion of granule stored *de novo* synthesized TNF, leading to *in vivo* initiation of local and systemic anaphylaxis, as well as development or maintenance of airway inflammation.

METHODS

Supplemental information can be found in the [Methods section](#) in this article's Online Repository at www.jacionline.org.

Mice

Female wild-type (WT) C57BL/6 and B6.Cg Tac1^{tm1Bm/j} (*Tac1*^{−/−}) mice (8–12 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, Me) and rested for 1 week before use. C57BL/6-Kit^{W-sh/W-sh} mice were initially purchased from Jackson Laboratories and bred in the University of Pittsburgh's Animal Facility. NK1R^{−/−} mice, initially provided by Dr Christopher Paige, University of Toronto, have been backcrossed to homozygosity by breeding 8 generations before use. Studies were performed according to Institutional Animal Care and Use Committee approval of protocols and procedures (University of Pittsburgh).

Statistical analysis

Data were analyzed by using 1- or 2-way ANOVA with Bonferroni *post hoc* analysis with GraphPad Prism version 5.0 software (GraphPad Software, La Jolla, Calif). When only 2 groups were compared, significant differences were determined by using the 2-tailed Student *t* test. A *P* value of less than .05 was considered significant.

RESULTS**NK1R signaling enhances FcεRI-initiated MC functions**

We analyzed the presence of the functional NK1R through detection of its intracellular C-terminus motif² in IL-3–derived WT C57BL/6 bone marrow–derived mast cells (BMMCs). The basal level of *NK1R* expression was low in nonactivated BMMCs, as reported previously.¹⁶ However, FcεRI activation of BMMCs significantly increased NK1R mRNA and protein expression

(Fig 1, A and B). It has been shown that differentiation of BMMCs with high concentrations of IL-4 increases NK1R expression.¹⁶ Because FcεRI activation causes release of IL-4 from BMMCs (see Fig E1, A, in this article's Online Repository at www.jacionline.org),¹⁷ we hypothesized that autocrine IL-4 might play a role in the regulation of NK1R expression. Inhibition of autocrine IL-4 with neutralizing anti-IL-4 antibody inhibited FcεRI-driven NK1R expression (see Fig E1, B). In contrast, BMMCs cultured with exogenous IL-4 without FcεRI activation were unable to further increase NK1R expression (see Fig E1, C). Together, these results indicate that in our working conditions, NK1R expression in BMMCs is regulated by autocrine IL-4 secretion initiated by FcεRI signaling.

Then we investigated IL-4–independent signaling pathways regulating NK1R expression in FcεRI-BMMCs, including nuclear factor κB (NF-κB), c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK), which regulate NK1R expression in dendritic cells^{3,4} and other cell types.^{22,23} Inhibition of NF-κB and JNK, but not ERK, reduced *NK1R* mRNA induced by FcεRI ligation (Fig 1, B), demonstrating that FcεRI ligation of MCs promotes NK1R expression through common intracellular pathways described in other cell types.^{3,4,22,23}

Ca²⁺-dependent FcεRI-MC degranulation is potentiated by certain GPCR agonists.^{17,18} Because the NK1R is a GPCR, we analyzed whether it was involved in Ca²⁺ flux and MC degranulation by comparing these 2 functions in FcεRI-BMMCs generated from WT or NK1R^{−/−} mice. Both BMMC strains had comparable differentiation and maturation stages according to expression of c-Kit and FcεRI (see Fig E2 in this article's Online Repository at www.jacionline.org), and they displayed similar changes in intracellular Ca²⁺ levels (Fig 1, C). However, fewer NK1R^{−/−} BMMCs degranulated compared with WT BMMCs (Fig 1, D), irrespective of antigen concentration (Fig 1, E). Conversely, degranulation of MCs induced with compound 48/80, an independent GPCR signaling stimulus,²⁴ triggered robust degranulation in both WT and NK1R^{−/−} BMMCs. These data demonstrate that the NK1R belongs to the group of GPCR family members that potentiate FcεRI-mediated MC degranulation.

In line with deficient degranulation, NK1R^{−/−} FcεRI-BMMCs had reduced TNF and IL-6 secretion compared with WT FcεRI-BMMCs (Fig 1, F). This effect was receptor specific because it was prevented by pretreatment with the NK1R antagonists RP 67,580 (see Fig E3, A and B, in this article's Online Repository at www.jacionline.org) and L733,060 (see Fig E3, C and D). Collectively, these data demonstrate that NK1R stimulation represents a downstream component of the proinflammatory signaling cascade initiated by FcεRI activation of MCs.

MCs secrete HK-1

The previous results, obtained with a highly pure MC population and without addition of exogenous NK1R agonists, suggested that MCs are the source of NK1R agonists, which increase their inflammatory functions in a highly regulated fashion. Therefore we investigated the capacity of FcεRI-BMMCs to synthesize and secrete HK-1 and SP. We analyzed the regulation of *Tac4* and *Tac1* transcripts (encoding HK-1 and SP, respectively) and secretion of HK-1 and SP peptides in WT untreated BMMCs (control) and FcεRI-BMMCs. *Tac4* and *Tac1* mRNAs were detected in untreated BMMCs, but only *Tac4* transcripts increased in FcεRI-BMMCs (Fig 2, A). Accordingly, *Tac4*

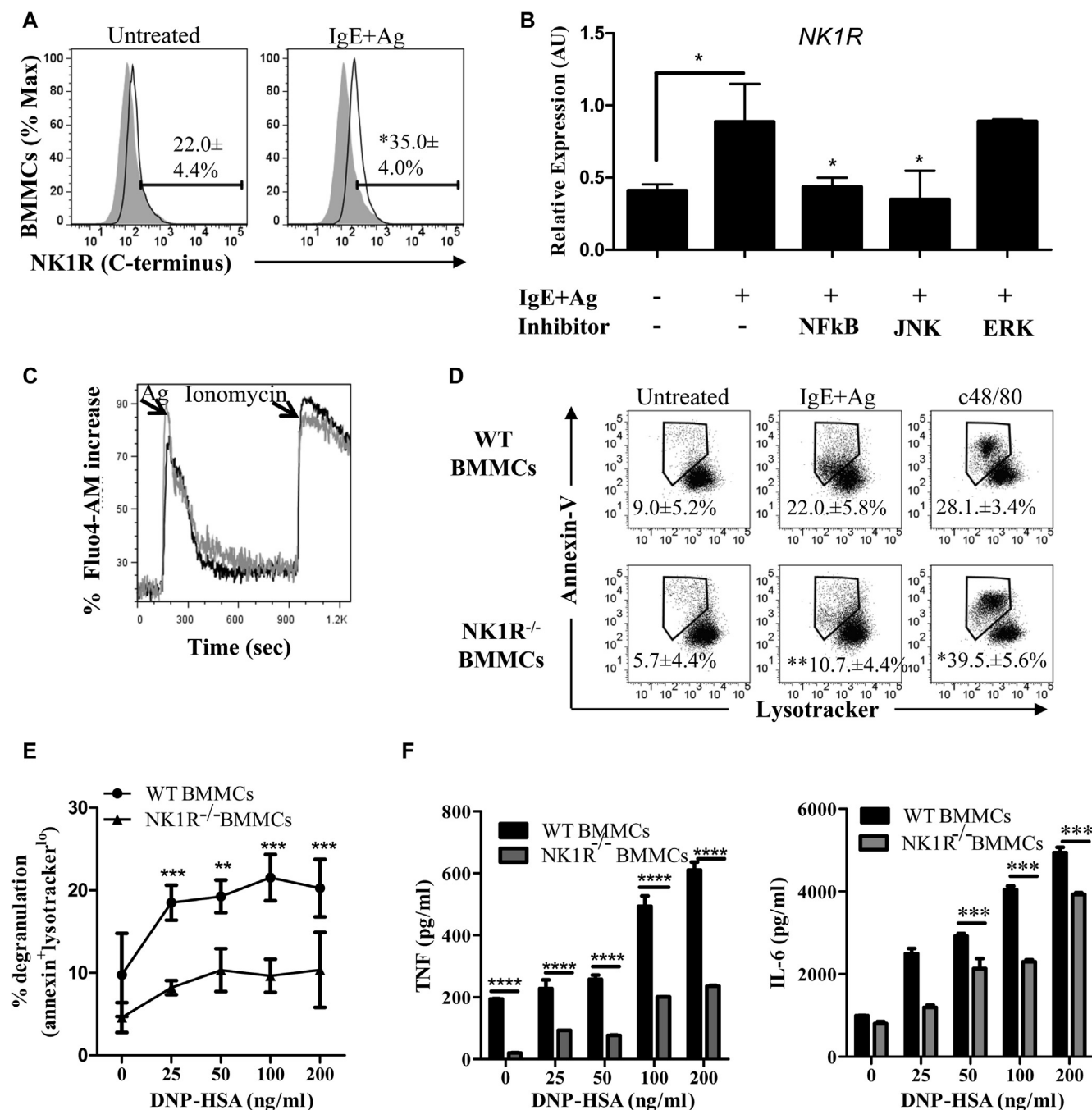


FIG 1. Role of the NK1R in FcεRI-BMMCs. **A**, NK1R expression in control BMMCs (shaded histogram) and FcεRI-BMMCs (open histogram); (loaded with IgE [SPE-07, 1.0 μg/mL] for 1 hour and then cross-linked with antigen [DNP-HSA, 200 ng/mL] for 18 hours). Means ± 1 SD of the percent positive from 3 experiments are shown. **B**, Signaling pathways involved in NK1R transcription in FcεRI-BMMCs 2 hours after FcεRI ligation with antigen in the presence of inhibitors specific for the indicated pathways. Means ± 1 SD from 3 experiments are shown. **C**, Calcium flux in WT (black line) or NK1R^{-/-} (gray line) BMMCs loaded with immunoglobulin and pulsed with antigen or ionomycin at the indicated time. One representative of 3 experiments is shown. **D** and **E**, Degranulation of WT and NK1R^{-/-} FcεRI-BMMCs 90 minutes after FcεRI ligation with antigen. **Fig 1, D**, Representative flow plot from 3 experiments. Values are means ± 1 SD of the percentage of degranulating BMMCs either loaded with IgE (1.0 μg/mL for 1 hour) and then cross-linked with antigen (200 ng/mL for 90 minutes) or activated with compound 48/80 (1.0 μg/mL). **Fig 1, E**, Data points depict the mean ± 1 SD of the percentage of degranulating BMMCs from 3 experiments. **F**, TNF and IL-6 release by FcεRI-BMMCs (18 hours, antigen). Means ± 1 SD of duplicate values from a representative of 3 experiments are shown. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001. Ag, Antigen.

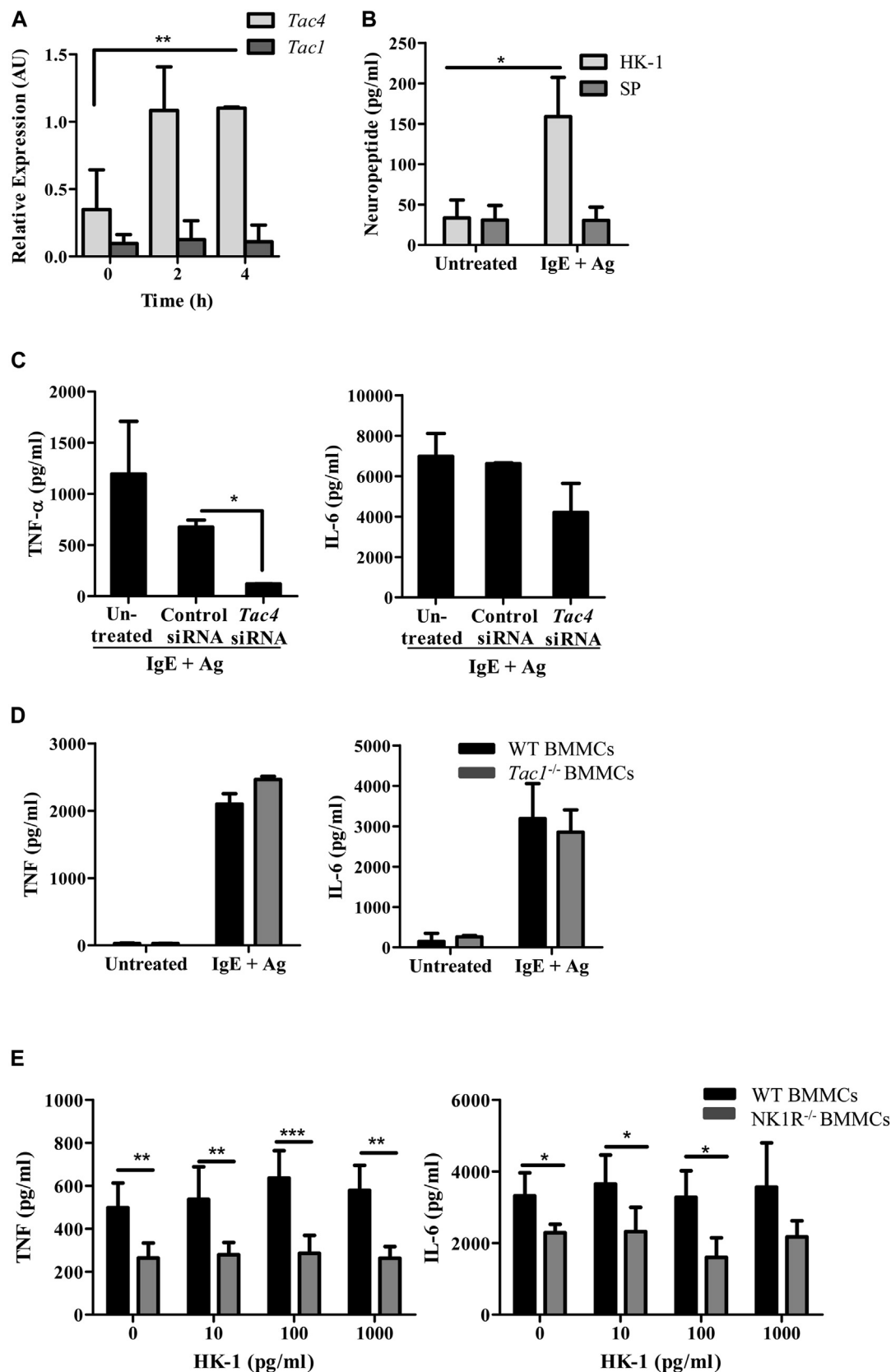


FIG 2. HK-1 is required for maximal TNF secretion from FcεRI-BMMCs. **A**, Quantification of *Tac4* or *Tac1* mRNAs in WT FcεRI-BMMCs. Means + 1 SD from 2 experiments are shown. **B**, HK-1 and SP secretion by WT FcεRI-BMMCs 18 hours after antigen cross-linking. Means + 1 SD from 3 experiments are shown. **C** and **D**, TNF and IL-6 secretion by *Tac4*-silenced WT FcεRI-BMMCs (Fig 2, C) or *Tac1*^{-/-} BMMCs (Fig 2, D). Means + 1 SD from 2 experiments are shown. **E**, TNF and IL-6 secreted by WT or NK1R^{-/-} FcεRI-BMMCs in which exogenous HK-1 was added at the time of antigen. Means + 1 SD from 3 independent experiments are shown. Cytokines were measured in supernatants 18 hours after antigen cross-linking. *P < .05, **P < .01, and ***P < .001. Ag, Antigen.

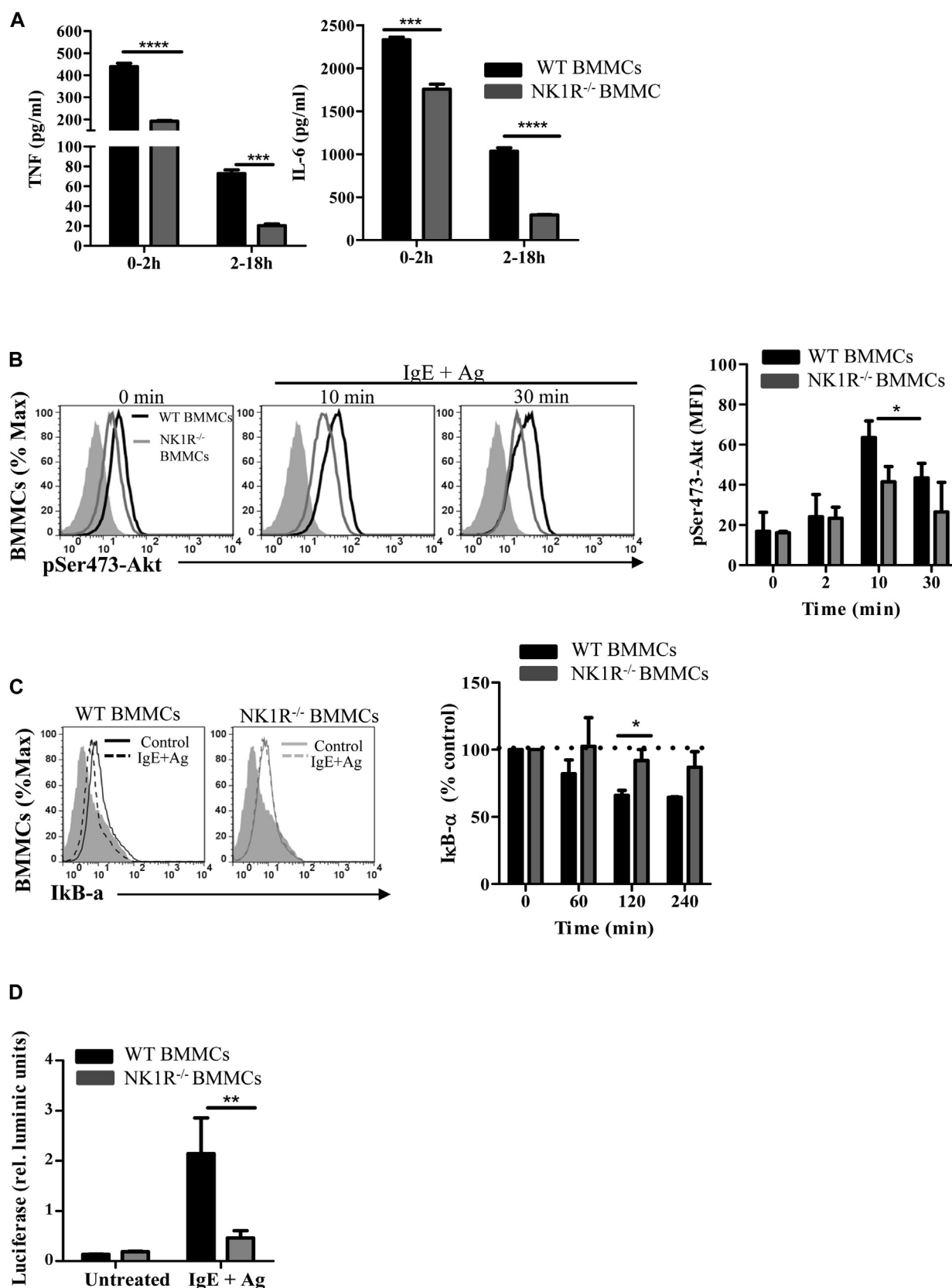


FIG 3. NK1R signaling potentiates PI3K/Akt/NF- κ B activation in Fc ϵ RI-BMMCs. **A**, TNF and IL-6 released by BMMCs. Means + 1 SD from 3 experiments are shown. **B**, *Left*, Histograms comparing intracellular phospho-Ser473Akt in WT Fc ϵ RI-BMMCs (black line) and NK1R^{-/-} BMMCs (gray line) loaded with IgE and stimulated with antigen for 10 minutes, as detected by means of flow cytometry. *Filled histograms*

mRNA levels and HK-1 secretion were induced to significantly higher levels than *Tac1* mRNA levels and SP secretion in FcεRI-BMMCs (Fig 2, A and B). The analysis of the signaling pathways regulating *Tac4* transcription in BMMCs demonstrated a role for NF-κB and JNK, but not ERK, after FcεRI ligation (data not shown). These pathways, which have been demonstrated to regulate *Tac4* levels in other cell types,^{25,26} also regulated NK1R transcription in FcεRI-BMMCs (Fig 1, B).

Next, we analyzed the role of autocrine/paracrine HK-1 and SP on MC proinflammatory functions. To address this question, we compared TNF and IL-6 secretion in culture supernatants of WT FcεRI-BMMCs transfected with *Tac4* small interfering RNA, which reduced *Tac4* mRNA expression by $30.5\% \pm 3.5\%$, or in *Tac1*^{-/-} FcεRI-BMMCs, which lack SP and neurokinin-A, a low affinity NK1R ligand. Inhibition of HK-1 significantly reduced TNF and showed a consistent reduction of IL-6 secretion, an effect that was not observed in the absence of SP (Fig 2, C and D).

Because autocrine HK-1 amplified the function of FcεRI-BMMCs, we also investigated whether exogenous HK-1 at concentrations within the range of that produced by MCs (Fig 2, B) could modify the proinflammatory effect of autocrine HK-1. However, exogenous HK-1 did not have any significant effect on degranulation (data not shown) or on TNF or IL-6 secretion by FcεRI-BMMCs (Fig 2, E). Moreover, exogenous HK-1 was unable to affect the threshold for IgE-antigen activation in BMMCs (data not shown). Additionally, secretion of TNF and IL-6 from NK1R^{-/-} FcεRI-BMMCs was not modified. This latter observation shows that the proinflammatory effects of HK-1 require signaling through the functional NK1R variant. Together, these results demonstrate that autocrine HK-1 is the MC-derived neuropeptide that signals through functional NK1R to provide adjuvancy to the proinflammatory functions of FcεRI-BMMCs.

NK1R autocrine signaling promotes TNF and IL-6 synthesis in FcεRI-BMMCs

The previous data indicate that the autocrine HK-1/NK1R positive loop increases the proinflammatory potential of FcεRI-BMMCs by promoting secretion of TNF and IL-6 through degranulation or *de novo* protein synthesis. To investigate this further, we analyzed cytokine secretion in culture supernatants of WT and NK1R^{-/-} BMMCs harvested 0 to 2 hours (degranulation) or 2 to 18 hours (*de novo* synthesis) after FcεRI cross-linking. WT FcεRI-BMMCs secreted higher levels of both cytokines than NK1R^{-/-} FcεRI-BMMCs at both time points (Fig 3, A). These results demonstrate that NK1R signaling by autocrine secreted agonists enhances TNF and IL-6 release by FcεRI-BMMCs during both the immediate and delayed phases of the MC inflammatory response.

To investigate the mechanisms integrating NK1R signaling pathways^{2,4} related to TNF and IL-6 synthesis in

FcεRI-BMMCs,^{27,28} we focused on the PI3K/Akt and IκB-α/NF-κB pathways. In BMMCs PI3K-dependent phosphorylation of Ser473-Akt occurs within 10 minutes of FcεRI activation, and Akt is restored to its inactive unphosphorylated state within 30 minutes.^{27,28} Accordingly, Ser473-Akt phosphorylation increased in WT FcεRI-BMMCs (Fig 3, B) 10 minutes after activation and returned to basal levels by 30 minutes. Ser473-Akt phosphorylation was reduced in NK1R^{-/-} BMMCs, and this effect was inhibited by culture with the NK1R antagonists RP 67,580 and L733,060 (not shown). The levels of IκB-α expression, analyzed with flow cytometry,²⁹ significantly decreased 2 hours after antigen cross-linking (Fig 3, C), and NF-κB nuclear translocation, as assessed in luciferase reporter assays (Fig 3, D) and ELISAs (data not shown), significantly increased in WT compared with NK1R^{-/-} FcεRI-BMMCs. Together, these findings demonstrate that the NK1R signals through the PI3K/Akt/NF-κB axis to regulate transcription and synthesis of TNF and IL-6 in FcεRI-BMMCs.

NK1R signaling is relevant for MC-dependent local and systemic anaphylaxis

The relevance of NK1R signaling in type I hypersensitivity reactions was analyzed in the context of FcεRI-MC-mediated models of passive cutaneous anaphylaxis (PCA) and passive systemic anaphylaxis (PSA). PCA, in response to IgE sensitization, occurs in 2 phases. During the acute phase of PCA, dermal MCs release histamine, leading to increased vascular permeability and edema. During the late phase, cutaneous MCs secrete TNF to recruit polymorphonuclear (PMN) leukocytes.³⁰ We assessed the role of NK1R signaling in the setting of PCA by comparing changes in ear thickness, edema, and leukocyte infiltrate in IgE-sensitized versus nonsensitized (vehicle control) ears of WT and NK1R^{-/-} mice. During the acute phase of PCA, the sensitized ears of WT mice, but not NK1R^{-/-} mice, showed a significant thickness increase caused by edema (Fig 4, A-C). To specifically confirm the role of NK1R signaling in MCs in this model, we reconstituted the ears of c-Kit^{W-sh/W-sh} mice with WT or NK1R^{-/-} BMMCs before PCA induction. Reconstitution with WT BMMCs, but not NK1R^{-/-} BMMCs, induced an early PCA response in c-Kit^{W-sh/W-sh} mice (Fig 4, D and E).

During late PCA, the ear thickness increase in IgE-sensitized and antigen-challenged WT mice was caused by PMN leukocyte infiltration, and this response was negligible in equally treated ears of NK1R^{-/-} mice (Fig 5, A and B). Given the effect of NK1R signaling on increasing TNF release (Fig 1, E), we hypothesized that the reduced late PCA response in NK1R^{-/-} mice was caused by deficient TNF secretion. Accordingly, TNF levels increased in IgE-sensitized WT mouse ears compared with those seen in vehicle-treated control ears. Importantly, TNF levels from IgE-sensitized NK1R^{-/-} mouse ears were consistently less than

are immunoglobulin isotype controls. *Right*, Mean + 1 SD of the median fluorescence intensity (MFI) for the kinetics of pSer473-Akt detected by means of intracellular flow cytometry in WT or NK1R^{-/-} FcεRI-BMMCs from 3 independent experiments. *C*, *Left*, IκB-α expression in FcεRI-BMMCs 2 hours after antigen cross-linking. *Right*, kinetics of IκB-α degradation in FcεRI-BMMCs expressed as the percentage of the median fluorescence intensity of untreated control cells. Means + 1 SD from 3 independent experiments are shown. *D*, Reporter assays of NF-κB activity in FcεRI-BMMCs transfected with pLuc-NF-κB measured after 18 hours of FcεRI ligation. Means + 1 SD of quadruplicates from a representative of 3 experiments are shown. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001. Ag, Antigen.

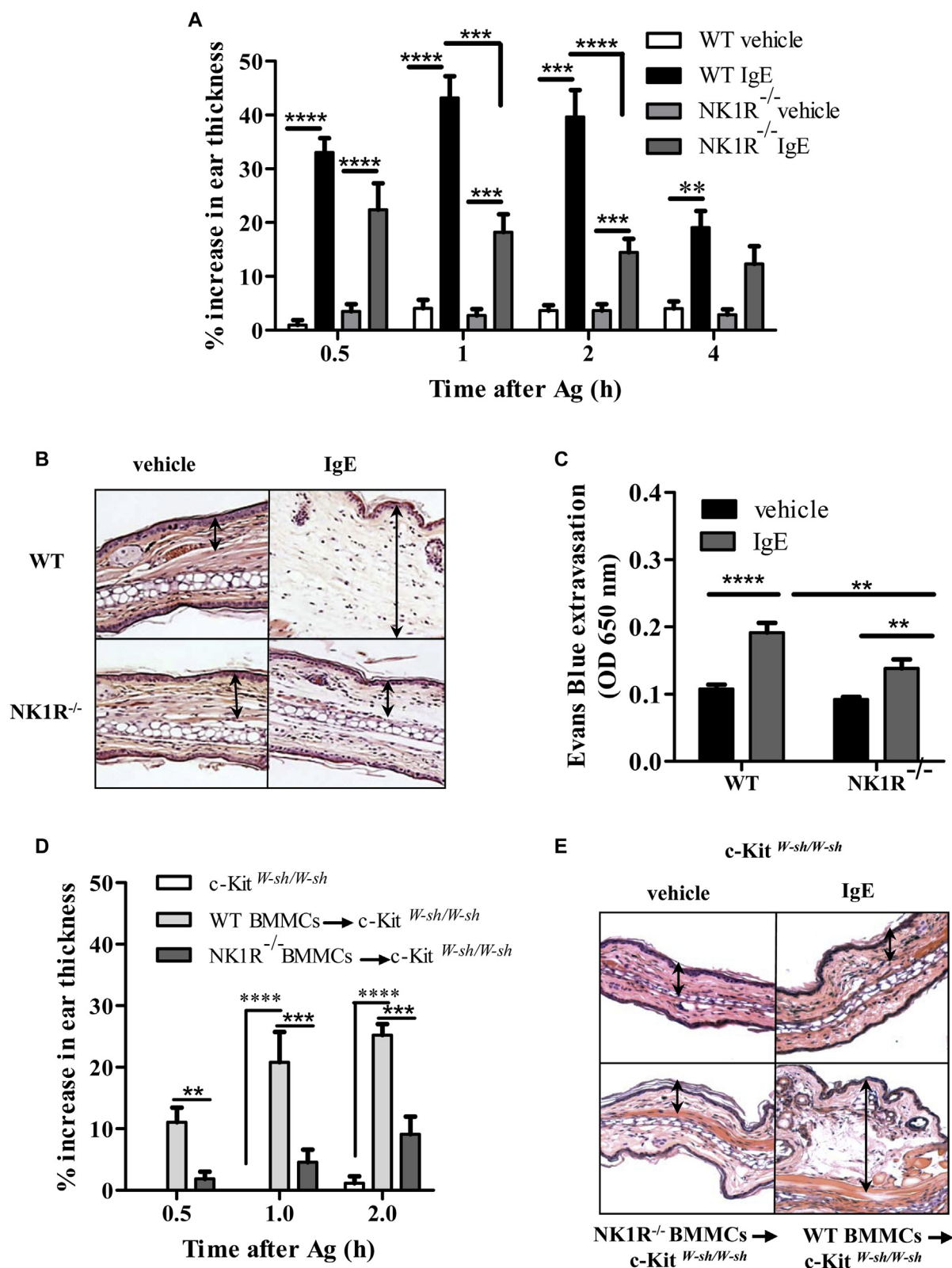


FIG 4. NK1R signaling amplifies the early phase of PCA. PCA was induced by injecting IgE (20 ng per ear administered intradermally). Twenty-four hours later, antigen (100 μ g of DNP-HSA) was intravenously administered. **A**, Increases in ear thickness after antigen injection. Means $\pm$ 1 SEM of 13 mice from 3 independent experiments are shown. **B**, Histology of skin sections comparing edema (arrows) in IgE-sensitized or control ears of WT and NK1R^{-/-} mice 2 hours after antigen challenge (magnification $\times$ 200). **C**, Evans Blue extravasation from ear tissue 2 hours after antigen challenge. Means $\pm$ 1 SEM from 2 experiments are shown. **D** and **E**, c-Kit^{W-sh/W-sh} mice were reconstituted with WT or NK1R^{-/-} BMMCs (1×10^6 per ear administered intradermally) 8 weeks before PCA induction. Fig 4, **D**, Increases in ear thickness after induction of PCA. Means $\pm$ 1 SEM from 4 to 5 mice per group are shown. Fig 4, **E**, Histology of skin sections comparing edema 2 hours after sensitization (magnification $\times$ 200). ** $P < .01$, *** $P < .005$, and **** $P < .001$. Ag, Antigen.

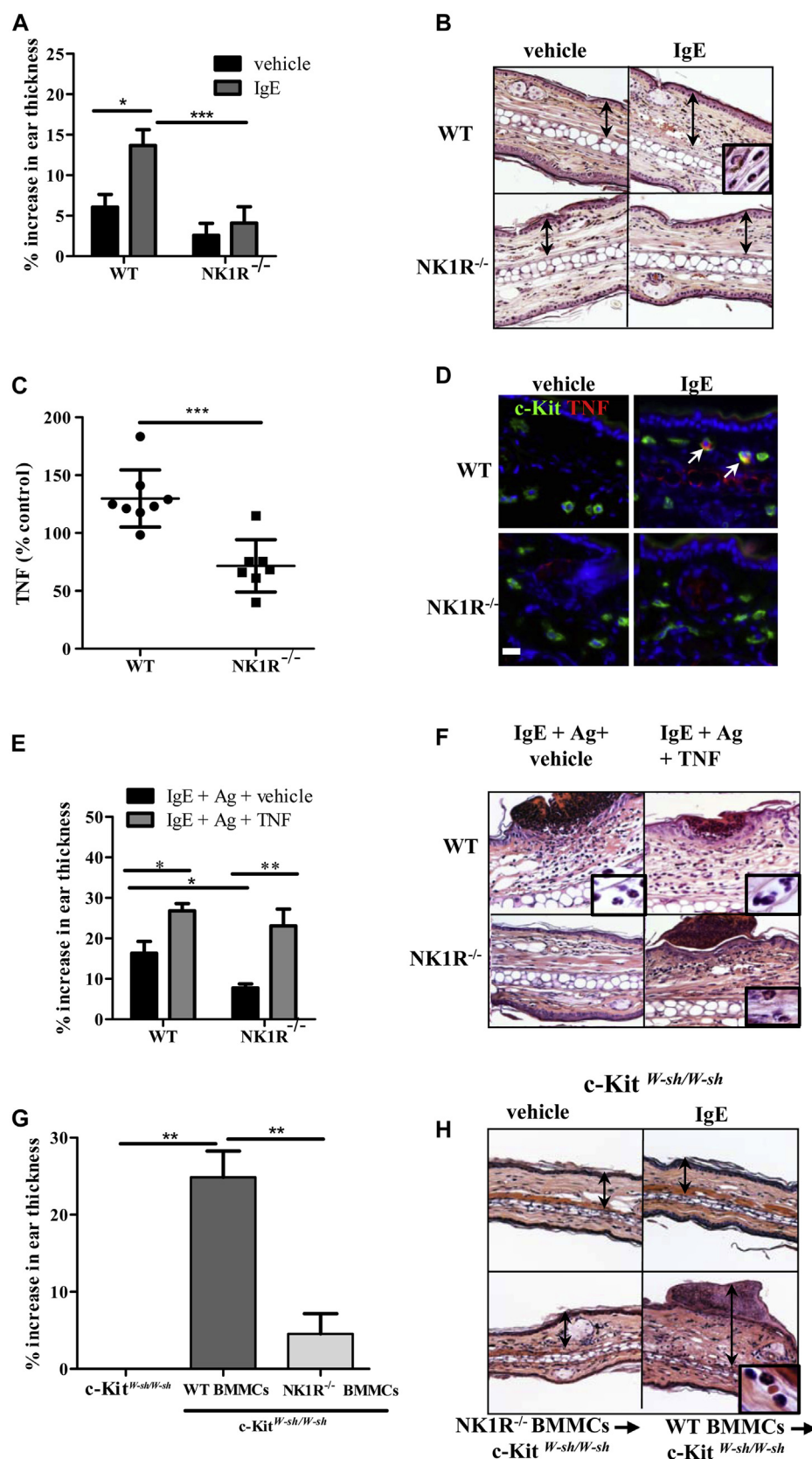


FIG 5. NK1R signaling is required for the TNF-dependent development of late-phase PCA. **A** and **B**, Increase in ear thickness (Fig 5, **A**) and histology of mouse ears (Fig 5, **B**) 24 hours after antigen challenge in late PCA. **C**, TNF levels from IgE-sensitized ears depicted as percentage of control ears. Means $\pm$ 1 SEM of 2 experiments are shown. Also shown is immunofluorescence microscopy of ears of WT mice 24 hours after

those observed in ears of equally treated WT mice (Fig 5, C). Using fluorescence microscopy, we further confirmed that cytoplasmic TNF was present in dermal c-Kit⁺ MCs of ear sections from sensitized WT mice but not in dermal MCs from equally treated NK1R^{-/-} mouse ears (Fig 5, D).

Deficient recruitment of PMN leukocytes depends on TNF-dependent³⁰ and TNF-independent³¹ mechanisms. To test whether under our experimental conditions the lack of PMN leukocytes in NK1R^{-/-} mouse ears was associated with a TNF deficit, we presensitized WT and NK1R^{-/-} mouse ears with IgE. After antigen challenge, we injected intradermally mouse TNF to 1 ear. The skin of NK1R^{-/-} mice locally treated with TNF had infiltrating PMN leukocytes to the same extent observed in WT mice (Fig 5, E and F).

Then we investigated the role of NK1R signaling in MCs during late-phase PCA, using c-Kit^{W-sh/W-sh} mice reconstituted with WT or NK1R^{-/-} BMMCs. Reconstitution with WT BMMCs, but not NK1R^{-/-} BMMCs, restored late-phase PCA induction in c-Kit^{W-sh/W-sh} mice, as demonstrated by increases in ear thickness and significant inflammatory infiltrate (Fig 5, G and H). These results demonstrate that NK1R signaling is required for elicitation of the late phase of PCA through a mechanism mediated by TNF secretion from cutaneous FcεRI-MCs.

Next, we analyzed the relevance of NK1R signaling in the onset and progression of FcεRI-MC-mediated PSA. We evaluated hypothermia, changes in pulmonary vascular permeability, and serum concentrations of TNF and IL-6. At early time points (10–20 minutes after antigen challenge), WT and NK1R^{-/-} mice had hypothermia to a similar extent (see Fig E4, A, in this article's Online Repository at www.jacionline.org). However, NK1R^{-/-} mice recovered faster and displayed signs of well-being (ie, alertness and normal activity) not observed in WT mice, and edema was significantly greater in the lungs of WT compared with NK1R^{-/-} mice (see Fig E4, B and C). Accelerated recovery from PSA is associated with low TNF concentrations in mouse serum.³² Likewise, in our working conditions TNF and IL-6 levels were significantly lower in NK1R^{-/-} mice compared with those in WT mice (see Fig E4, D). The role of NK1R-expressing MCs in PSA was investigated using c-Kit^{W-sh/W-sh} mice (which do not develop PSA) reconstituted with WT or NK1R^{-/-} BMMCs. Reconstitution with WT BMMCs, but not NK1R^{-/-} BMMCs, restored PSA in c-Kit^{W-sh/W-sh} mice, as demonstrated by induction of significant hypothermia (see Fig E4, E). Collectively, these findings demonstrate that NK1R signaling is required for FcεRI-MC-dependent immediate and delayed responses of local and systemic anaphylaxis caused by a mechanism requiring TNF.

NK1R signaling by HK-1 is necessary for FcεRI-MC-dependent experimental airway inflammation

The role of NK1R signaling in chronic atopic disorders was investigated in an FcεRI-MC-dependent model of chronic airway

inflammation induced in WT and NK1R^{-/-} mice with ovalbumin (OVA).³³ This protocol requires MC-produced TNF to initiate T_H2 bias and to recruit PMN leukocyte and eosinophil infiltration in the airways and lungs. We evaluated (1) leukocyte infiltration, (2) myeloperoxidase (MPO) and eosinophil peroxidase (EPO) activities, and (3) cytokine concentrations in bronchoalveolar lavage (BAL) fluid in the lungs of OVA-sensitized and OVA-challenged mice and compared these with values in vehicle-treated control animals.

The lungs of OVA-challenged WT mice exhibited severe PMN and eosinophil infiltration distributed in foci throughout the parenchyma and airways (Fig 6, A). The activities of MPO and EPO increased in the lungs of OVA-challenged WT mice compared with the activities of MPO and EPO in the lungs of vehicle-treated WT mice (Fig 6, B). Conversely, lungs from OVA-challenged NK1R^{-/-} mice exhibited negligible infiltration by lymphocytes and eosinophils (Fig 6, A) and lower increases in MPO and EPO activity than seen in the lungs of equally treated WT mice (Fig 6, B). Likewise, concentrations of TNF (Fig 6, C) and the T_H2 cytokines IL-4, IL-5, and IL-13 in the BAL fluid increased significantly in OVA-challenged WT mice but not in NK1R^{-/-} mice, whereas the low levels of IFN-γ detected remained similar between WT and NK1R^{-/-} mice (see Fig E5 in this article's Online Repository at www.jacionline.org). The impaired MC-dependent inflammatory and immune responses of NK1R^{-/-} mice were not due to deficient IgE secretion because both groups had similar concentrations of serum total and OVA-specific IgE (see Fig E6 in this article's Online Repository at www.jacionline.org).

The pathophysiologic role of NK1R signaling by HK-1 in the proinflammatory function of MCs relevant for chronic airway inflammation was further confirmed by comparing the levels of TNF in the BAL fluid and the inflammatory infiltrate in the lungs of MC-deficient (c-Kit^{W-sh/W-sh}) mice reconstituted with WT, NK1R^{-/-}, or *Tac1*^{-/-} BMMCs before OVA sensitization. After OVA challenge, Kit^{W-sh/W-sh} mice reconstituted with WT or *Tac1*^{-/-} BMMCs had severe eosinophil infiltrate in the lungs, whereas equally treated c-Kit^{W-sh/W-sh} mice reconstituted with NK1R^{-/-} BMMCs did not have inflammatory responses or other lung immune pathology (Fig 6, D). After OVA challenge, BAL fluid from c-Kit^{W-sh/W-sh} mice engrafted with WT or *Tac1*^{-/-} BMMCs had significantly higher TNF concentrations than those from mice reconstituted with NK1R^{-/-} BMMCs (Fig 6, E), correlating with eosinophilic infiltration. Collectively, these results demonstrate that NK1R signaling by autocrine HK-1 is critical for the proinflammatory function of FcεRI-MCs, accounting for the onset and maintenance of chronic airway inflammation.

DISCUSSION

Given the roles of neuroinflammation and MCs in type 1 hypersensitivity and T_H2-mediated chronic allergic diseases,

induction of PCA showing c-Kit⁺ MCs (green) containing TNF-α (red; magnification ×200). D, Increase in ear thickness in IgE-sensitized WT or NK1R^{-/-} mice injected with TNF-α (40 U per ear). Means ± 1 SEM of 5 mice per experimental group are shown. E, Histology illustrating the severity of the inflammatory infiltrate in ear dermis and epidermis (hematoxylin and eosin, magnification ×200). Inset shows PMN leukocytes (magnification ×1000). G and H, Late-phase PCA was evaluated in c-Kit^{W-sh/W-sh} mice reconstituted (intradermally) with either WT or NK1R^{-/-} BMMCs. Increases in ear thickness (Fig 5, G) or histology of skin sections (Fig 5, H) 24 hours after PCA induction are shown (magnification ×200; inset magnification ×500). *P < .05, **P < .01, and ***P < .001. Ag, Antigen.

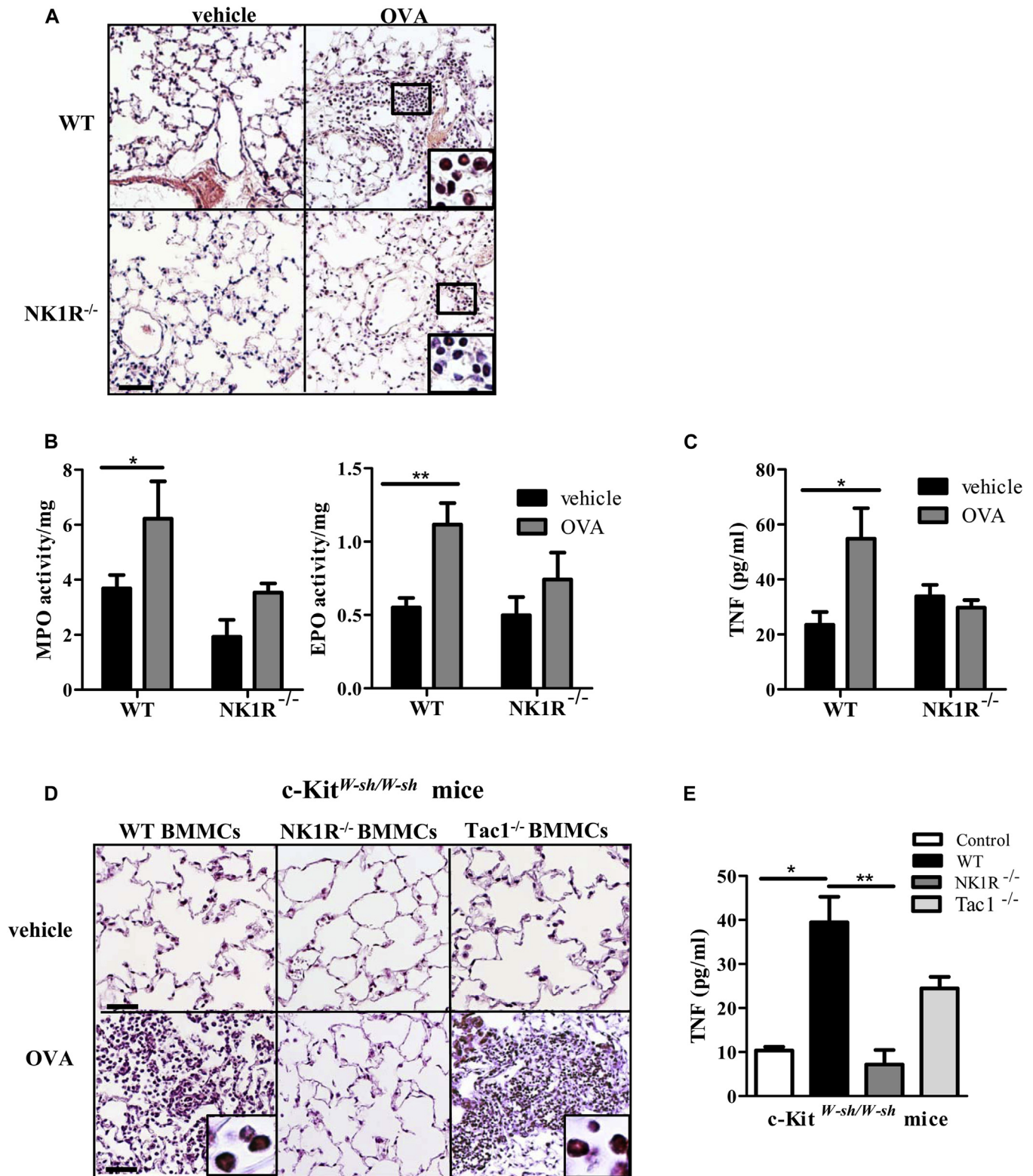


FIG 6. The NK1R is required for MC-dependent airway inflammation. Mice were treated with either vehicle or OVA (10 μ g administered intraperitoneally) on days 0, 2, 4, 6, 8, 10, and 12 and then challenged (200 μ g administered intranasally) on days 40, 43, and 46 to induce MC-dependent airway inflammation. **A**, Inflammatory infiltrate in OVA-sensitized/challenged lungs or lungs of vehicle control mice at day 47 (hematoxylin and eosin, magnification $\times 200$). *Insets* show PMN leukocytes, including eosinophils and neutrophils (magnification $\times 500$). *Bar* = 20 μ m. **B**, Quantification of EPO and MPO in lungs. Means $\pm$ 1 SEM of 5 mice per group are shown. **C**, TNF concentration in BAL fluid from WT or NK1R^{-/-} mice. **D**, Lung histology from Kit^{W-sh/W-sh} mice reconstituted with WT, NK1R^{-/-}, or Tac1^{-/-} BMMCs 8 weeks before induction of airway inflammation (hematoxylin and eosin, magnification $\times 200$). *Inset* shows eosinophils in the lungs of Kit^{W-sh/W-sh} mice reconstituted with WT BMMCs (magnification $\times 1000$). **E**, TNF in BAL fluid of c-Kit^{W-sh/W-sh} mice reconstituted with WT, NK1R^{-/-}, or Tac1^{-/-} BMMCs before induction of experimental airway inflammation. Means $\pm$ SEM of 3 mice per group are shown. * P < .05 and ** P < .01.

there has been an interest in understanding the regulation of MC functions by neuropeptides. Although historical studies addressed the relevance of SP in MC function, these reports were not focused on the activation of MCs by IgE-antigen complexes.¹⁹⁻²¹ Moreover, the use of high concentrations of SP and the technical limitations in these studies preclude the understanding of the physiologic function of the NK1R in MC biology *in vivo*.³⁴ More recently, the description of HK-1 as a potent agonist of the NK1R secreted mainly by leukocytes has changed, in part, the way we regard neuropeptides as relevant regulators of immune function. In the present study we focused on understanding the function of the neuropeptides SP and HK-1 as preferential agonists of the NK1R and their potential endogenous adjuvant effect in the context of MC activation by IgE.

It has been reported that in the absence of FcεRI activation, upregulation of NK1R surface expression by mouse BMMCs requires exogenous IL-4.¹⁶ Similarly, we found dim expression of the functional intracellular form of the NK1R in BMMCs. Expression of the NK1R significantly increased in FcεRI-BMMCs, which was due in part to autocrine IL-4 secretion after FcεRI ligation.

Under our experimental conditions, HK-1, but not SP, favored MC proinflammatory functions. In fact, we demonstrate that after FcεRI ligation and upregulation of the NK1R, MCs significantly increased *Tac4* transcription and HK-1 secretion, whereas *Tac1* mRNA and SP levels remained low. Using NK1R^{-/-} and *Tac1*^{-/-} BMMCs, *Tac4* silencing, and NK1R antagonists, we demonstrate that the HK-1/functional NK1R signaling pathway provides adjuvancy to FcεRI-BMMC proinflammatory functions. Different from SP, which is stored in neuronal bodies and secreted predominantly by sensory nerve fibers, HK-1 is synthesized mainly by leukocytes, indicating that HK-1's proinflammatory function is highly regulated in and by leukocytes. In fact, previous works have demonstrated the roles of HK-1 in leukocyte differentiation, survival, and immune function. Accordingly, HK-1 promotes B- and T-cell differentiation, proliferation, and activation⁹⁻¹² and the proinflammatory function of monocytes.⁶ Here we analyzed the relationship between the NK1R and FcεRI signaling in MCs and demonstrated that autocrine HK-1 amplifies MC inflammatory responses. On the basis of our finding and published works, we could speculate that *in vivo* activation of MCs through FcεRI involves a highly regulated mechanism requiring (1) circulating antigen-specific IgE synthesis by activated plasma cells, (2) IgE-antigen complexes to cross-link FcεRI, and (3) downstream activation of intracellular pathways resulting in upregulation of NK1R and MC synthesis and autocrine secretion of HK-1. In this model HK-1 provides an adjuvant effect and is required for maximal MC activation and efficient development of IgE-mediated atopic disorders. Interestingly, exogenous HK-1 was unable to modify the effects of autocrine HK-1, even when added at high nonphysiologic concentrations. We speculate that this might be caused by autocrine HK-1 binding the NK1R and NK1R internalization, a mechanism previously described as necessary to terminate NK1R signaling, ultimately making the NK1R unavailable.

Certain GPCRs potentiate degranulation,^{18,34,35} potentially through interactions mediating cytoskeletal changes.³⁶ Here we found that fewer NK1R^{-/-} BMMCs degranulated *in vitro* in response to FcεRI cross-linking and that edema associated with FcεRI-mediated degranulation was reduced in NK1R^{-/-} mice. These findings demonstrate a role for NK1R signaling in MC degranulation, as described for other GPCRs.³⁴

We have previously reported in dendritic cells that NK1R signaling activates the PI3K/Akt pathway.⁴ In MCs the PI3K/Akt pathway activates NF-κB. Here we demonstrated that NK1R signaling in FcεRI-BMMCs triggers PI3K/Akt activation downstream of NF-κB nuclear translocation, which is necessary for the synthesis of TNF and IL-6^{27,28} during the late phase of the MC inflammatory response.

Connective tissue- and mucosa-resident MCs are master initiators of acute hypersensitivity reactions and chronic atopic diseases linked to neuromediators. Our data demonstrate that HK-1 signaling through the NK1R of MCs is crucial for the onset and maintenance of these disorders and that HK-1/NK1R interaction is required for every step in the development of PCA and PSA by inflammatory MCs. Previous publications have shown that MC-dependent recruitment of PMN cells in IgE-mediated PCA relies on changes in IL-33³⁷ or IL-6 levels.³⁸ Under our working conditions, the absence of PMN cells infiltrating the skin of NK1R^{-/-} mice during late PCA was completely restored after TNF administration, clearly indicating the importance of TNF in this model.

The adjuvant function of the HK-1/NK1R interaction in MCs was further demonstrated in a model of chronic atopic airway inflammation in which NK1R signaling was necessary for the onset and maintenance of the disease and proved to be critical for T_H2 polarization of the adaptive immune response and recruitment of inflammatory cells to the lungs. By using an MC-independent model of airway hyper-reactivity, a recent study has shown that NK1R antagonists reduced antigen-specific IgE concentrations.³⁹ Under our working conditions, using an MC-dependent model of airway inflammation, abrogation of the NK1R did not affect IgE secretion, confirming that efficient development of the disease requires NK1R signaling that provides adjuvancy to FcεRI-MC mediated disorders.

In summary, we present a comprehensive analysis of NK1R function in MCs both *in vitro* and *in vivo*. To our knowledge, this is the first report addressing the key role that NK1R signaling of FcεRI-MCs has in the pathogenesis of anaphylaxis and T_H2-dependent atopic disorders. Specifically, our findings demonstrate that the HK-1/NK1R interaction is a critical component of FcεRI-MC-mediated inflammatory and immune functions and provide insight into the mechanisms underlying MC-mediated disorders. Our data are immunologic, physiologic, and clinically relevant because current therapeutic strategies targeting MC functions have shown limited efficacy,⁴⁰ in part because of the inability to downregulate TNF and IL-6 secretion initiated through FcεRI. The data presented here support the development of alternative immune therapies based on neutralization of endogenously secreted proinflammatory NK1R agonists.

Key messages

- *In vitro* stimulation of MCs through FcεRI by antigen-IgE complexes initiates the synthesis and autocrine secretion of HK-1, which signals the NK1R to promote secretion of the proinflammatory cytokines TNF and IL-6.
- *In vivo* expression and agonistic signaling of NK1R in MCs is required to initiate local and systemic passive anaphylaxis and to sustain airway inflammation.
- FcεRI signaling of MCs triggers an autocrine/paracrine positive loop mediated by agonistic HK-1/NK1R interaction, which provides endogenous adjuvancy for efficient development of FcεRI-MC-dependent allergic reaction functions.
- The data presented here demonstrate previously unknown aspects of MC-neuropeptide interactions and infer a physiologically oriented therapeutic alternative for IgE-mediated atopic diseases.

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METHODS

Mast cell differentiation and *in vitro* activation

Mast cells were generated from bone marrow progenitors (BMMCs) and cultured in complete RPMI 1640 with IL-3 (30 ng/mL; PeproTech, Rocky Hill, NJ), as previously described,^{E1} for 5 weeks. The purity of FcεRI⁺c-Kit⁺ BMMCs, as determined by flow cytometric analysis, was 95.1% ± 2.9% for WT BMMCs and 96.4% ± 1.8% for NK1R^{-/-} BMMCs (Fig E2).

In vitro BMMCs were activated through the FcεRI, NK1R, or both. Activation through FcεRI (FcεRI-BMMCs) was induced by cross-linking of the receptor by culture with anti-DNP IgE (IgE, clone SPE-7, 1.0 μg/mL, 1 hour, 37°C; Sigma-Aldrich, St Louis, Mo), followed by dinitrophenyl-human serum albumin (DNP-HSA; antigen, 200 ng/mL, 37°C, Sigma-Aldrich). Activation through the NK1R was induced with exogenous mouse HK-1 (Tocris Bioscience, Bristol, United Kingdom) for 18 hours at the indicated concentrations. Blockade of NK1R was performed by means of culture with one of the synthetic nonpeptide antagonists L733,060 or RP 67,580 added with antigen at the indicated doses (37°C, Tocris Bioscience). WT BMMCs were cultured with purified recombinant IL-4 (PeproTech) overnight or WT FcεRI-BMMCs were cultured with anti-IL-4 neutralizing antibody (10 μg/mL, clone 1B11; BD Biosciences, San Jose, Calif) to examine the effects of IL-4 on NK1R induction.

Intracellular flow cytometry

Detection of NK1R protein expression and intracellular signaling pathways were performed by intracellular flow cytometry. FcεRI-BMMCs were fixed with paraformaldehyde (PFA; 2% for 20 minutes at 37°C), permeabilized with methanol (90% for 30 minutes), and incubated with anti-NK1R C-terminus (clone D-11; Santa Cruz Biotechnology, Dallas, Tex) followed by fluorescein isothiocyanate anti-mouse IgG (Sigma-Aldrich) or with one of the following antibodies: pSer473Akt Alexa Fluor 647, IκB-α (Cell Signaling Technology, Danvers, Mass). Data were collected in an LSR II flow cytometer (BD Bioscience) and analyzed with FlowJo version 7.6.4 software (TreeStar, Ashland, Ore).

Semiquantitative RT-PCR

Total RNA was isolated from BMMCs with Trizol (Invitrogen, Carlsbad, Calif) and a polyacryl carrier (Molecular Research Center, Cincinnati, Ohio) and reverse transcribed with iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Hercules, Calif). Expression of *NK1R*, *Tac4*, *Tac1*, or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; primers from SABiosciences, Qiagen, Valencia, Calif) cDNA was determined with Fast SYBR Green MasterMix (Invitrogen) on a StepOnePlus Real Time PCR System (Applied Biosystems, Foster City, Calif). Expression was quantified by means of extrapolation from a standard curve of mouse brain cDNA and normalized to *GAPDH*. In some experiments cells were incubated with 2'-amino-3'-methoxyflavone (PD98059, an ERK inhibitor, 20 μmol/L; Biomol, Farmington, NY), SN-50 (NF-κB inhibitor, 18 μmol/L; Calbiochem, San Diego, Calif), or JNK inhibitor II (10 μmol/L, Calbiochem).^{E2}

Small interfering RNA transfection and luciferase assays

BMMCs were transfected with *Tac4* Stealth RNAi or Silencer Select Negative Control #1 Cy3 (Invitrogen) by using Trans-IT siQUEST Reagent (Mirus Bio, Madison, Wis). For luciferase assays, BMMCs were electroporated (1 pulse, 1600 volts, 20 ms; Neon Transfection System) with a plasmid DNA encoding firefly luciferase under the transcriptional control of the NF-κB responsive element (pNF-κB-Luc; Mercury Systems Vectors, Clontech, Mountain View, Calif), followed by FcεRI activation. Transfection efficiency was 22.5% ± 5.0%. Luciferase activity was measured, as previously described.^{E3}

Assessment of mast cell cytosolic calcium and degranulation

IgE-loaded (1 μg/mL for 1 hour at 37°C) BMMCs were labeled with Fluor-4-AM (2.5 μmol/L for 30 minutes at room temperature; Invitrogen)

and resuspended in BSA-Tyrode buffer.^{E4} Calcium levels were analyzed by flow cytometry for 1100 seconds. First, basal calcium content was analyzed for the initial 100 seconds. Then, calcium levels after antigen stimulation (200 ng/mL) were analyzed for 750 seconds. Finally, maximal responses were achieved with ionomycin (1 μg/mL) for the remaining 250 seconds.

Mast cell degranulation was measured by means of flow cytometry, as previously described.^{E5,E6} In brief, BMMCs were labeled with LysoTracker Blue (500 nmol/L for 30 minutes at 37°C, Invitrogen) and then loaded with IgE (1.0 μg/mL for 1 hour). Cross-linking antigen (200 ng/mL) or compound 48/80 (1.0 μg/mL, Sigma-Aldrich) was added for 90 minutes before Annexin V staining (BD Biosciences).

ELISA

Cytokines, HK-1, SP, or total IgE and anti-OVA IgE concentrations in culture supernatants, sera, or BAL fluid were quantified by means of ELISA (BD Biosciences, eBioscience [San Diego, Calif], Bachem [Torrance, Calif], or Cayman Biochemical [Ann Arbor, Mich]).

Induction of PCA and PSA

For PCA, mice were administered anti-DNP IgE (50 ng/20 μL, PBS, intradermal) or an equal volume of vehicle (control). After 24 hours (time 0), mice were challenged with DNP-HSA (100 μg administered intravenously). In some experiments both ears were sensitized with IgE.

Mouse ear edema was analyzed after antigen cross-linking by measuring the percentage of ear thickness and vascular leakage and were further confirmed by histologic examination. Ear thickness increase was calculated by comparing the thickness of injected ears versus the thickness of untreated ears. Mice were sensitized with IgE and challenged with antigen in 1% Evans Blue solution to quantify vascular leakage. After 1 hour, mice were euthanized, and ears were excised, weighed, and minced. Evans Blue was extracted from tissue by means of overnight incubation in DriSolv (EMD Millipore, Billerica, Mass) and then quantified as absorbance at OD 650 nm. Data show an OD of 650 nm per milligram of tissue. For histologic analysis, ears from euthanized mice were dissected, fixed in 4% PFA, and processed for hematoxylin and eosin. Tissue sections were examined with an Axiostar plus microscope equipped with epifluorescence and a digital camera (AxioCam; Zeiss, Oberkochen, Germany). Images were analyzed with AxioVision (Zeiss) and processed with Adobe Photoshop C4 software. Pathologic assessments were blinded.

Late-phase PCA was assessed by comparing the percentage ear thickness increase and further evaluated by means of histology, as described above. The role of TNF in late-phase PCA was assessed by administration of TNF in the ears of WT or NK1R^{-/-} mice and through detection of TNF content in dermal resident MCs. Mouse recombinant TNF (40 U in 20 μL of PBS [PeproTech] administered intradermally) or an equal volume of vehicle (control) was administered 6 hours after antigen. For quantification of tissue TNF, excised ears were homogenized in T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, Ill) with Protease Inhibitor Cocktail (1×, Sigma-Aldrich). Protein was quantified with the Protein Assay (Bio-Rad Laboratories), and TNF concentrations were determined by ELISA. Results are presented as the percentage of TNF per milligram in the control ear from each mouse. For detection of TNF by immunofluorescence, ear tissue was OCT embedded, cryostat sectioned, and simultaneously stained with biotin-TNF antibody and rat anti-mouse c-Kit antibody (eBioscience), followed by streptavidin-Cy3 (Jackson ImmunoResearch, West Grove, Pa) and anti-rat Alexa Fluor 488 (Invitrogen).

PSA was induced with anti-DNP IgE (20 μg administered intravenously), followed by DNP-HSA (100 μg administered intravenously) 24 hours later. Body temperature was measured with a rectal thermometer (Physitemp, Clifton, NJ) before antigen injection, every 10 minutes after antigen for 60 minutes, and at 90 and 120 minutes. After 120 minutes, mice were killed. Lungs were perfused with PBS-EDTA, fixed in 4% PFA, and processed for hematoxylin and eosin staining. In some experiments mice were challenged with antigen in 0.5% Evans Blue and, Evans blue was extracted from lungs

excised 2 hours later and quantified as described for PCA. Serum cytokine levels were quantified by using an ELISA.

The role of NK1R expression specifically by MCs during PCA and PSA was determined in *in vivo* reconstitution assays. For these experiments, c-Kit^{W-sh/W-sh} mice were reconstituted with either WT or NK1R^{-/-} BMMCs (1×10^6 cells per ear administered intradermally for PCA or 5×10^6 cells administered intravenously for PSA) 8 weeks before induction of PCA or PSA.^{E7,E8}

Induction of airway inflammation

Mice were sensitized with OVA (OVA-Grade V, 10 μ g in 200 μ L administered intraperitoneally, Sigma-Aldrich) or vehicle (PBS) on days 0, 2, 4, 6, 8, 10, and 12; challenged with OVA (200 μ g in 20 μ L administered intranasally) or PBS on days 40, 43, and 46; and euthanized on day 47.^{E9,E10} BAL fluid was obtained by washing lungs with sterile PBS, and cytokine concentrations in the BAL fluid were measured by using ELISA. After lavage, hearts were perfused with PBS-EDTA. One lung lobe was snap frozen for EPO and MPO assays.^{E10} EPO and MPO data are presented as enzyme activity per milligram of protein. The remaining lobes of lungs were fixed in PFA and then processed for hematoxylin and eosin staining. In some experiments BMMCs (5×10^6 administered intravenously) differentiated for 4 weeks *in vitro* were transferred to 12-week-old c-Kit^{W-sh/W-sh} mice, 8 weeks before induction of allergic airway inflammation.^{E7,E8}

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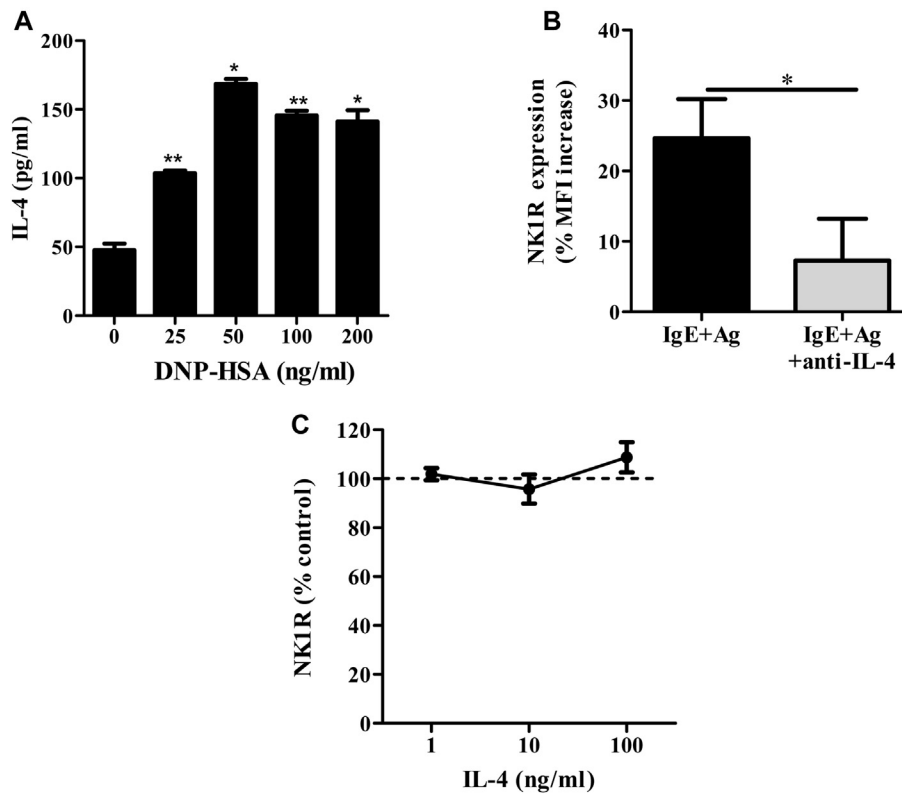


FIG E1. Autocrine IL-4 increases NK1R expression in Fc ϵ RI-BMMCs. **A**, IL-4 secretion by WT Fc ϵ RI-BMMCs detected by means of ELISA in culture supernatants collected 18 hours after Fc ϵ RI stimulation. Cells loaded with IgE in the absence of cross-linking antibody were used as controls. Data are representative of 3 experiments. **B**, NK1R expression in MCs after autocrine IL-4 blockade. WT Fc ϵ RI-BMMCs were cultured with neutralizing anti-IL-4 antibody or isotype control antibody. NK1R expression was quantified by flow cytometry 18 hours later. **C**, Effect of exogenous IL-4 on NK1R expression. WT BMMCs were cultured for 18 hours in the presence or absence of recombinant murine IL-4. NK1R expression was quantified by means of flow cytometry. The graph depicts means $\pm$ 1 SD of the percentage increase over untreated control mice from 3 experiments. * P < .05 and ** P < .01. Ag, Antigen.

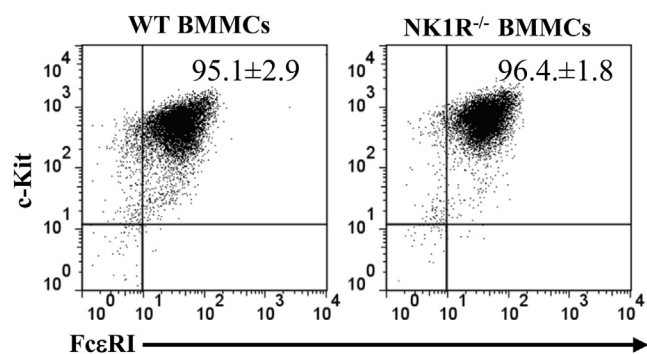


FIG E2. Characterization of BMMCs. BMMCs were generated from WT or NK1R^{-/-} hematopoietic progenitors cultured in the presence of IL-3 and identified by expression of c-Kit and FcεRI. Results are representative of 5 independent experiments. Values denote means $\pm$ SDs of the percentage of cells positive for c-Kit and FcεRI from 5 experiments.

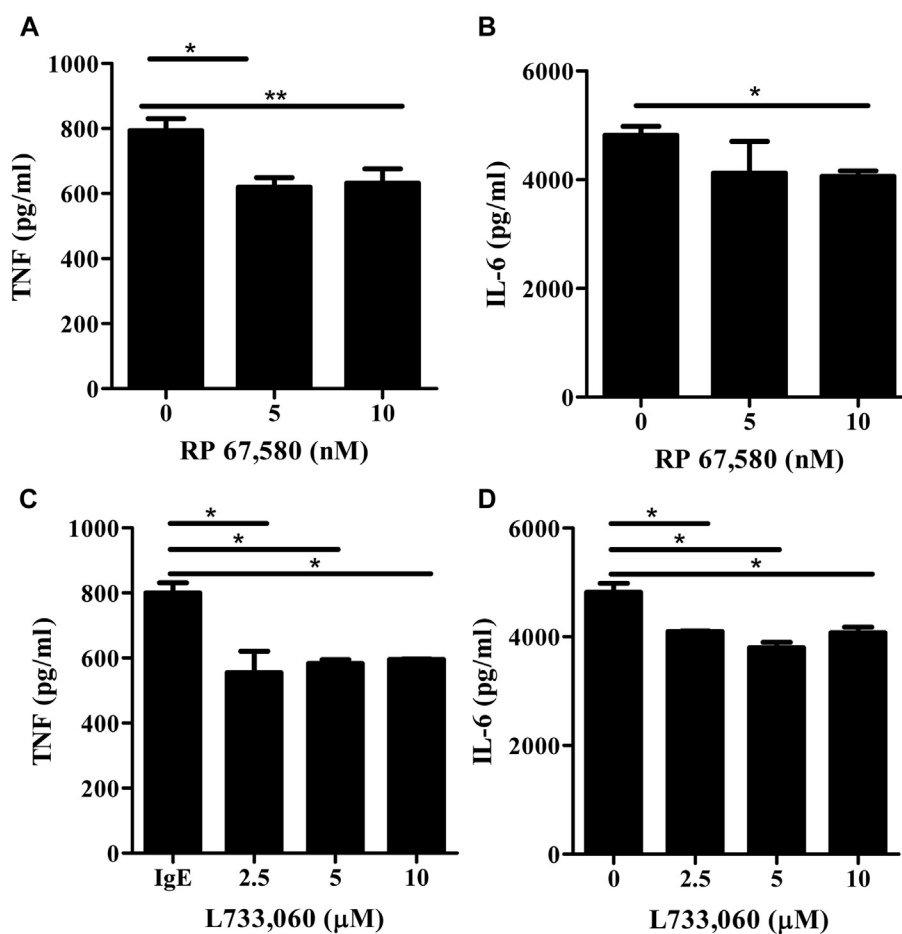


FIG E3. NK1R antagonism reduces TNF and IL-6 secretion by BMMCs. WT FcεRI-BMMCs were activated with antigen in the presence of the NK1R antagonists RP 67,580 (**A** and **B**) or L733,060 (**C** and **D**). Cytokine concentrations were quantified by means of ELISA in BMMC supernatants 18 hours after activation. A representative of 3 experiments is shown. * $P < .05$ and ** $P < .01$.

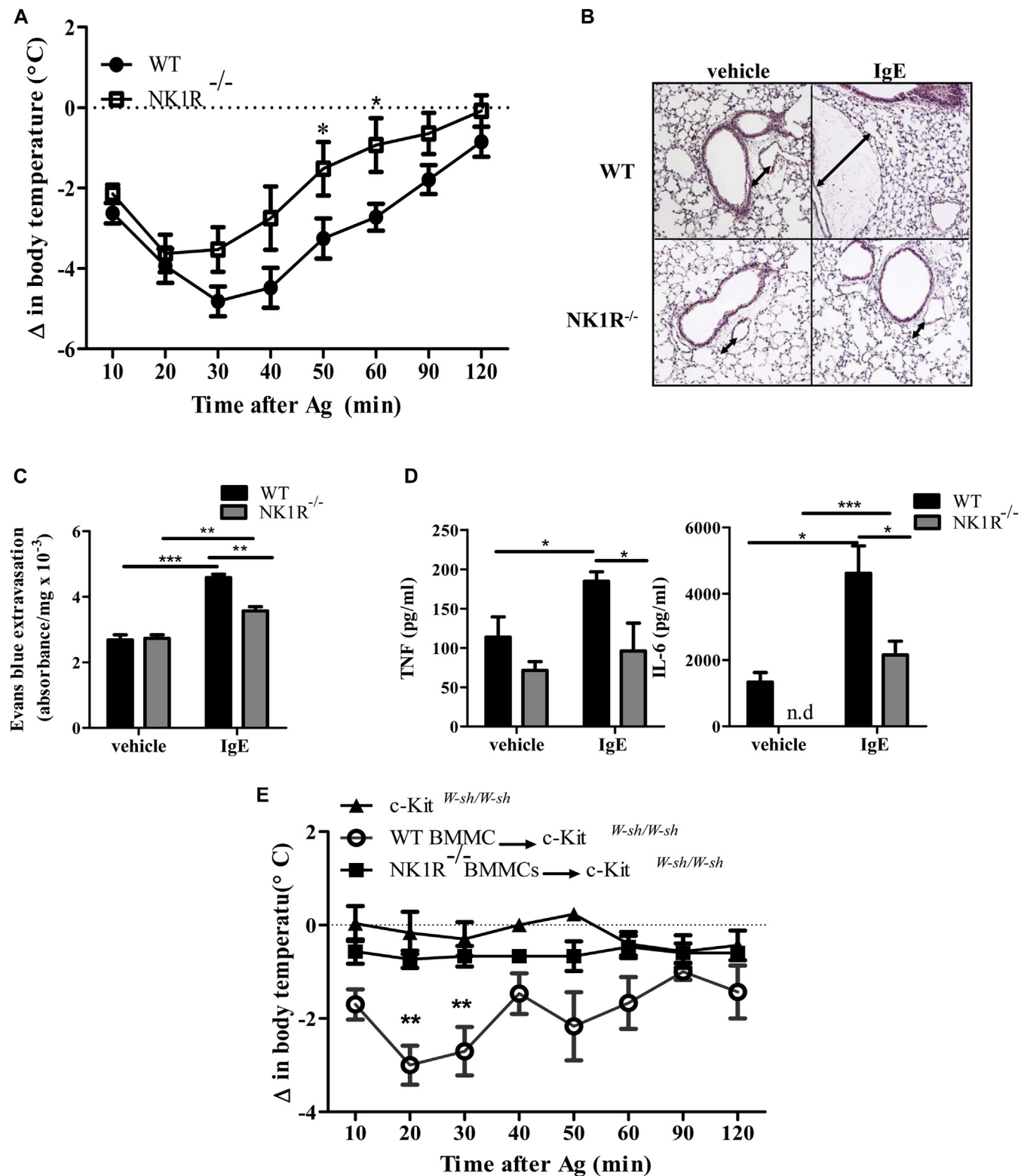


FIG E4. Role of MC-NK1R signaling in PSA. **A**, Temperature analysis in mice undergoing PSA. Mean $\pm$ 1 SEM temperature changes from 3 independent experiments are shown. **B**, Lung histology of mice undergoing PSA. **C**, Evans Blue extravasation in pulmonary tissue. Means $\pm$ 1 SEM of 3 mice per group are shown. **D**, Cytokine detection in mouse serum. Means $\pm$ 1 SEM from 2 independent experiments are shown. **E**, Temperature analysis in c-Kit^{W-sh/W-sh} mice reconstituted with either WT or NK1R^{-/-} BMMCs 8 weeks before induction of PSA. * P < .05, ** P < .01, and *** P < .001. Ag, Antigen.

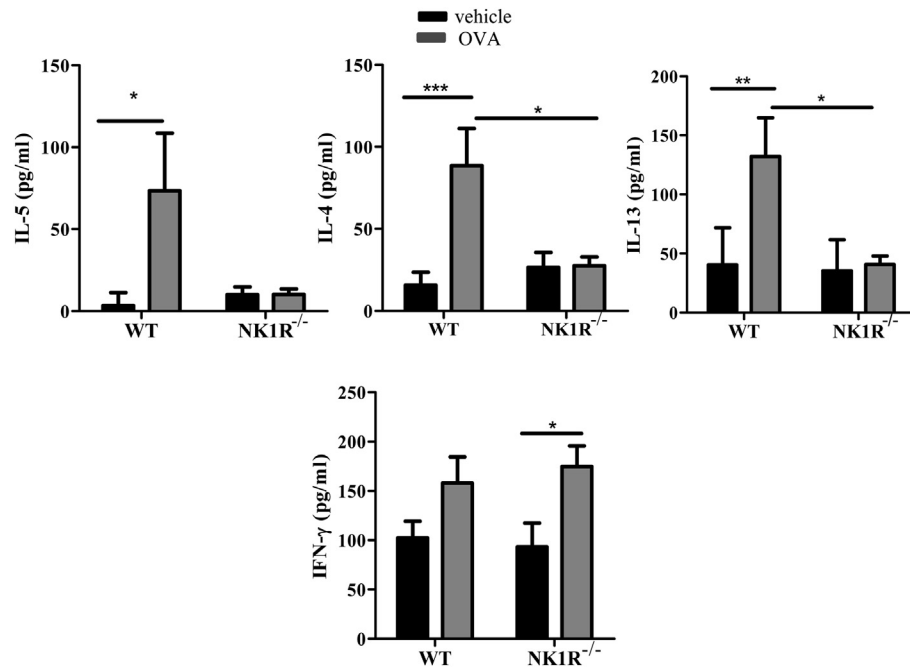


FIG E5. Concentration of cytokines in BAL fluid from mice after induction of airway inflammation. The concentration of cytokines in BAL fluid of WT and NK1R^{-/-} mice 47 days after induction of experimental allergic inflammation, as quantified by using ELISA, is shown. * $P < .05$, ** $P < .01$, and *** $P < .001$.

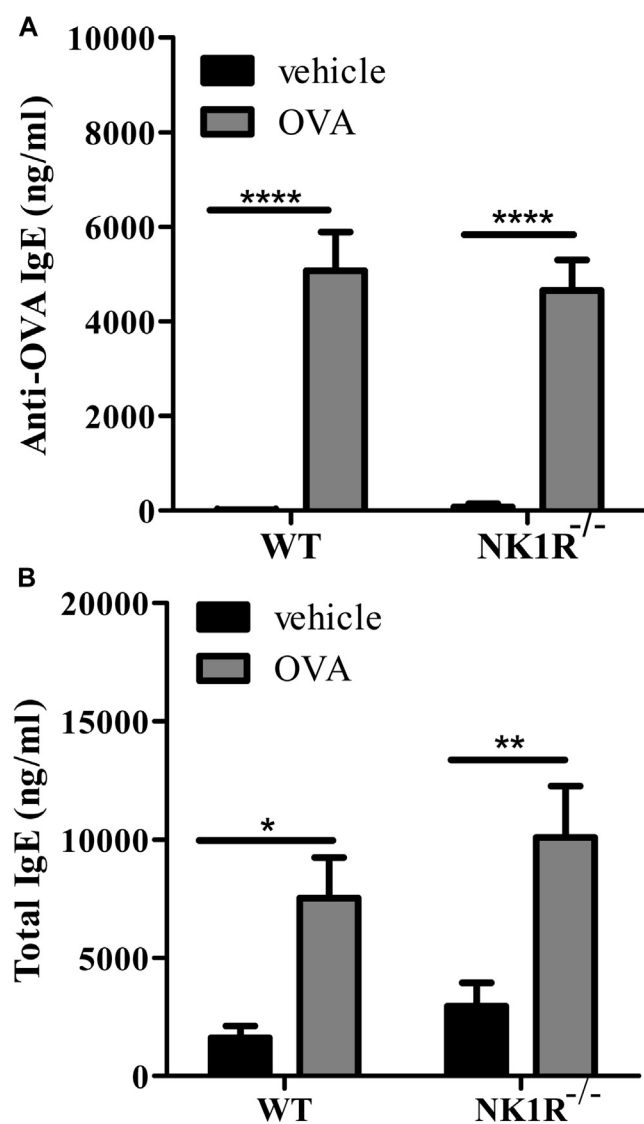


FIG E6. IgE concentrations in mouse serum after induction of airway inflammation. OVA-specific (**A**) and total (**B**) IgE levels were quantified by means of ELISA in the serum of WT or NK1R^{-/-} mice after induction of airway inflammation. * $P < .05$, ** $P < .01$, and **** $P < .0001$.