

Raf kinase inhibitor protein negatively regulates FcεRI-mediated mast cell activation and allergic response

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Edited by Stephen J. Galli, Stanford University School of Medicine, Stanford, CA, and accepted by Editorial Board Member Ruslan Medzhitov September 10, 2018 (received for review April 7, 2018)

The signaling cascades triggered by the cross-linkage of immunoglobulin E (IgE) with its high-affinity receptor (FcεRI) on mast cells contribute to multiple allergic disorders, such as asthma, rhinitis, and atopic dermatitis. Restraint of intracellular signals for mast cell activation is essential to restore homeostasis. In this study, we found that Raf kinase inhibitor protein (RKIP) negatively regulated mast cell activation. RKIP-deficient mast cells showed greater IgE–FcεRI-mediated activation than wild-type mast cells. Consistently, RKIP deficiency in mast cells rendered mice more sensitive to IgE–FcεRI-mediated allergic responses and ovalbumin-induced airway inflammation. Mechanistically, RKIP interacts with the p85 subunit of PI3K, prevents it from binding to GRB2-associated binding protein 2 (Gab2), and eventually inhibits the activation of the PI3K/Akt/NF-κB complex and its downstream signaling. Furthermore, the expression of RKIP was significantly down-regulated in the peripheral blood of asthma patients and in the IgE–FcεRI-stimulated mast cells. Collectively, our findings not only suggest that RKIP plays an important role in controlling mast cell-mediated allergic responses but also provide insight into therapeutic targets for mast cell-related allergic diseases.

RKIP | mast cell | allergic response | asthma | FcεRI signaling

Allergic diseases are characterized by increased serum levels of total immunoglobulin E (IgE) and specific IgE against common allergens (1–3). Mast cells are key mediators of IgE-mediated allergic diseases, including asthma, rhinitis, and atopic dermatitis (4). Studies of intracellular regulatory mechanisms in the context of mast cell activation could elucidate the contribution of this mechanism and lead to developments in therapeutic approaches against asthma, rhinitis, etc.

Derived from pluripotent hematopoietic stem cells, mast cells are predominantly localized in the respiratory mucosa, gastrointestinal tract, and connective tissues (5). The aggregation of the high-affinity IgE receptor (FcεRI) on the mast cell plasma membrane, when specific antigens cross-link FcεRI-bound IgE, activates a complex intracellular signaling pathway, resulting in degranulation, cytokine or chemokine production, and eicosanoid release (3, 6). Mast cell degranulation releases preformed proinflammatory chemical mediators, such as histamine and proteases, regulating the early-phase response, or type I immediate hypersensitivity reaction (7). Cytokines and chemokines, as well as some newly synthesized proinflammatory lipid mediators, are secreted in the late stage, mediating a more delayed proinflammatory response (6). The intracellular signaling cascades elicited by the IgE–FcεRI aggregation in mast cells have been studied extensively (5, 8, 9). Briefly, cross-linkage of the IgE–FcεRI complex with an allergen activates a variety of Src family kinases, such as Lyn and Fyn, which are essential for initiating IgE–FcεRI-mediated activation of mast cells. Phosphorylated linker for activation of T cells (LAT)/immunoreceptor tyrosine-based activation motif (ITAM) signalingosomes initiate the principal signaling through the growth-factor receptor-bound protein 2 (Grb2)/phospholipase C-γ1 (PLC-γ1)/SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) axis, leading to the activation of mitogen-activated protein kinases (MAPKs), protein kinase C (PKC) pathways, and calcium flux (Ca²⁺) (5, 10, 11).

Meanwhile, Fyn can directly facilitate a complementary axis, which also helps with lipid mediator production, calcium release, and cytokine production (12). This complementary signaling pathway leads to the phosphorylation of the cytosolic adaptor molecule GRB2-associated binding protein 2 (Gab2) and phosphatidylinositol 3-kinase (PI3K), resulting in the recruitment of serine/threonine kinase 3-phosphoinositide-dependent protein kinase 1 to the plasma membrane, where it subsequently phosphorylates and activates another serine/threonine kinase, Akt. Phosphorylated Akt positively regulates the function of the transcription factor NF-κB and mediates cytokine production (11, 13).

The IgE–FcεRI-mediated activation and subsequent inhibition in mast cells consist of highly ordered, sequential molecular events. An excessive allergic response by mast cells would be harmful to the host. Therefore, the activated signaling is counterbalanced by certain negative regulatory mediators that keep mast cells in a controllable, tightly restricted state and return them to their basal status after activation. Therefore, understanding negative regulatory mechanisms is a prerequisite for acquiring comprehensive knowledge of FcεRI signaling and thereby helps reveal strategies for the treatment of allergic diseases.

Raf kinase inhibitor protein (RKIP) is widely expressed in mammalian tissues such as brain, liver, stomach, spleen, colon, and muscle (14). RKIP is known to act as a tumor suppressor (14). We recently demonstrated that RKIP plays a crucial role in mediating human and mouse colitis by promoting intestinal epithelial cell (IEC) apoptosis (15). Furthermore, we discovered that RKIP contributes to antiviral immune responses (16) and promotes TLR3-induced inflammation

Significance

Mast cell activation contributes to multiple allergic disorders, such as asthma, rhinitis, and atopic dermatitis. Here, we demonstrate that the Raf kinase inhibitor protein (RKIP) functions as an inhibitor of mast cell activation. RKIP negatively regulates the pathogenesis of the mast cell-mediated anaphylactic response and allergic asthma in vivo. Furthermore, the expression of RKIP was significantly down-regulated in peripheral blood from asthma patients. Collectively, our findings not only suggest that RKIP plays an important role in controlling mast cell-mediated allergic responses but also provide insight into therapeutic targets for mast cell-related allergic diseases.

Author contributions: X.W. designed research; W.L., F.S., R.G., and N.W. performed research; Y.Z. contributed new reagents/analytic tools; W.L., F.S., R.G., and X.W. analyzed data; and W.L. and X.W. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. S.J.G. is a guest editor invited by the Editorial Board.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1805474115/-DCSupplemental.

(17). Therefore, RKIP functions as a physiological and pathological regulator more than as a tumor suppressor. Here, we have found that RKIP acts as an inhibitory modulator of IgE–FcεRI-mediated mast cell activation by targeting the PI3K p85 subunit. RKIP deficiency significantly exacerbates mast cell-mediated anaphylactic responses and allergic asthma in mice.

Results

RKIP Expression Is Down-Regulated in IgE–FcεRI-Stimulated Mast Cells and in Peripheral Blood from Asthma Patients. Mast cells have been thought to play a central role in the pathological process of IgE-mediated allergic diseases (5). The gene expression of RKIP in the IgE–FcεRI-activated mast cell was first analyzed. As shown in *SI Appendix, Fig. S1A*, the mRNA expression of *RKIP* was significantly reduced in mouse bone marrow-derived mast cells (BMMCs) on stimulation with IgE–FcεRI (after presensitization by anti-dinitrophenyl (DNP)-IgE and activation by DNP-BSA), similar to its expression profile, as shown in the BioGPS gene expression atlas database (*SI Appendix, Fig. S1B*). Human CD34⁺-derived mast cells were used to explore whether IgE–FcεRI stimulation affects the expression of *RKIP* in the human mast cell. As shown in *SI Appendix, Fig. S1C*, IgE–FcεRI stimulation significantly inhibited the mRNA expression of *RKIP* in human mast cells. Collectively, these data suggest that RKIP might be involved in IgE–FcεRI-mediated mast cell function.

Next, we measured the expression levels of *RKIP* in peripheral blood from asthma patients and healthy control individuals by real-time PCR. The mRNA level of *RKIP* was significantly down-regulated in the asthma patients' peripheral blood ($n = 52$) compared with the samples from healthy control individuals ($n = 56$) (Fig. 1*A*). Furthermore, mRNA expression of *RKIP* was significantly lower in the severe group than in the mild/moderate group (Fig. 1*B*). GEO data (GEO accession no. GSE16032) suggest that the expression of *RKIP* is much higher in the pe-

ripheral blood of convalescent asthma patients than in that of patients with exacerbated asthma (*SI Appendix, Fig. S1D*). We collected peripheral blood samples from patients with asthma at the onset and remission stages of asthma signs and symptoms to further determine the kinetics of *RKIP* expression during the processing of asthma. As shown in Fig. 1*C*, *RKIP* mRNA expression was much higher in the same patient at the remission of asthma symptoms than that at the onset of asthma, indicating that *RKIP* expression was linked to the occurrence of signs and symptoms in patients with asthma.

Previous studies have reported that the enhancer of zeste homolog 2 (EZH2) binds to the proximal E boxes of the RKIP promoter and inhibits RKIP transcription in breast and prostate cell lines (18). We recently found that the enhancer of zeste homolog 1 (EZH1) negatively regulated the expression of RKIP in the IECs (15). Interestingly, IgE–FcεRI treatment enhanced the mRNA and protein expression of EZH2 in mouse BMMCs and human mast cells (Fig. 1*D* and *SI Appendix, Fig. S1E and F*) but inhibited the expression of RKIP (Fig. 1*D* and *SI Appendix, Fig. S1A and C*). However, IgE–FcεRI did not induce any significant effect on EZH1 expression in mouse BMMCs and human mast cells (Fig. 1*D* and *SI Appendix, Fig. S1G and H*). Furthermore, knocking down the expression of EZH2 reversed the down-regulation of RKIP protein levels by IgE–FcεRI stimulation in the BMMCs (Fig. 1*E* and *SI Appendix, Fig. S1I*). Consistently, the mRNA level of *EZH2*, but not *EZH1*, significantly increased in the peripheral blood of individuals with asthma compared with that of healthy controls (Fig. 1*F* and *SI Appendix, Fig. S1J*). Moreover, linear regression analysis showed that the mRNA level of *EZH2* was negatively correlated with the mRNA level of *RKIP* in the peripheral blood of individuals with asthma (Fig. 1*G*).

We next used a specific antibody against EZH2 to ascertain whether EZH2 can interact with the proximal E boxes of the RKIP promoter in BMMCs as in breast and prostate cell lines

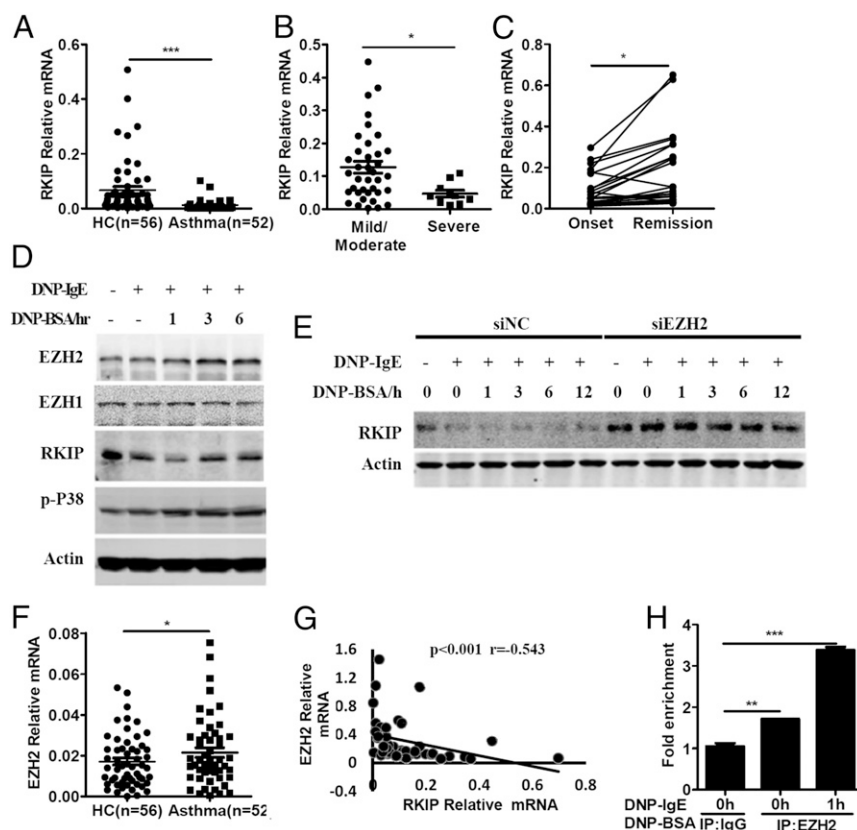


Fig. 1. RKIP expression is down-regulated in FcεRI-stimulated mast cells and peripheral blood from asthma patients. (A) Real-time PCR analysis of *RKIP* mRNA expression in peripheral blood from asthma patients ($n = 52$) or healthy control individuals ($n = 56$). (B) Statistical analysis of the correlation between *RKIP* expression and severity of asthma in patients: mild/moderate ($n = 37$) or severe ($n = 10$). (C) Real-time PCR analysis of *RKIP* mRNA expression in peripheral blood from children with asthma ($n = 26$) at the onset and remission of asthma signs and symptoms and statistical analysis of the kinetics of *RKIP* expression relative to the onset and remission of asthma. (D) Western blot analysis of EZH2, EZH1, and RKIP expression in the IgE–FcεRI-activated BMMCs with indicated antibodies. (E) Western blot analysis of RKIP expression in the EZH2-specific siRNA (siEZH2)- or negative siRNA (siNC)-transfected BMMCs with IgE–FcεRI-mediated stimulation. (F) Real-time PCR analysis of *EZH2* mRNA expression in peripheral blood from asthma patients ($n = 52$) and healthy controls ($n = 56$). (G) Statistical analysis of the correlation between the *RKIP* expression and *EZH2* expression in peripheral blood from asthma patients ($n = 52$). (H) Anti-EZH2 antibody was subjected to cell lysate of the IgE–FcεRI-activated WT BMMCs, and the enrichment of EZH2 to proximal E boxes of RKIP promoter was analyzed by real-time PCR. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Data are representative of the three experiments. Data represent the mean and SD.

(18). As shown in Fig. 1H, enrichment of EZH2 at the proximal E boxes of the RKIP promoter in BMMCs was significantly enhanced compared with an anti-IgG control, and IgE–FcεRI stimulation promoted the binding, indicating that EZH2 may interact with the RKIP promoter and suppress the expression of RKIP in IgE–FcεRI-treated mast cells.

Elevated IgE–FcεRI-Mediated Activation in RKIP-Deficient BMMCs. IgE–FcεRI-mediated mast cell activation triggers allergic responses by inducing rapid cell degranulation and secretion of newly synthesized metabolites, cytokines, and chemokines (2, 19). To determine whether RKIP plays a role in the IgE–FcεRI-mediated BMMC response, we measured the gene expression of cytokines and chemokines in RKIP knockout (KO) and wild-type (WT) BMMCs with IgE–FcεRI stimulation. As shown in Fig. 2A, RKIP deficiency greatly increased the expression of cytokines and chemokines on stimulation with IgE–FcεRI. Furthermore, RKIP KO BMMCs exhibited much higher IgE–FcεRI-mediated secretion of the cytokines IL-4, IL-5, IL-13, IL-6, TNF-α, and granulocyte macrophage colony-stimulating factor (GM-CSF) than did the WT BMMCs (Fig. 2B). Meanwhile, RKIP silencing enhanced the IgE–FcεRI-induced expression of cytokines and chemokines (Fig. 2C). Consistently, IgE–FcεRI-mediated mRNA expression of cytokines and chemokines was significantly inhibited in Flag-RKIP-overexpressed RBL-2H3 cells (rat-derived basophils cell line) compared with that in control-transfected cells (SI Appendix, Fig. S24). The overexpression of RKIP in RBL-2H3 cells was confirmed by Western blot (SI Appendix, Fig. S2B). We further explored whether the incubation of mast cells (BMMCs) with recombinant RKIP protein purified from *Escherichia coli* BL21 strain would affect mast cell activation. To our surprise, the incubation of BMMCs with recombinant RKIP protein significantly inhibited the IgE–FcεRI-mediated proinflammatory cytokine expression in the BMMCs (SI Appendix, Fig. S2C). Western blot assay confirmed the entry of exogenous RKIP protein into the BMMCs (SI Appendix, Fig. S2D). The efficiency of RKIP silencing in BMMCs is shown in SI Appendix, Fig. S2E and F.

In addition, the loss of RKIP significantly promoted BMMC degranulation, as assessed by surface expression of the degranulation marker CD107a (Fig. 3A). The release of histamine upon IgE–FcεRI activation with suboptimal doses of allergen was higher in the RKIP KO BMMCs than in the WT BMMCs (Fig. 3B). In accordance with these results, RKIP deficiency in mast cells resulted in elevated IgE–FcεRI-elicited release of preformed β-hexosaminidase, leukotriene C4 (LTC4), and prostaglandin D2 (PGD2) (Fig. 3C–E). Since mast cell degranulation is coupled with cytoskeletal rearrangement, we employed the xCELLigence system (Roche), a microelectronic cell sensor-based technology, for real-time monitoring of the electrical impedance of mast cells, which reflects cytoskeletal rearrangement and degranulation after activation by an allergen (19). As shown in Fig. 3F, with anti-DNP-IgE and DNP-BSA stimulation, both the WT and RKIP KO BMMCs exhibited robust increases in electrical impedance as reflected by the normalized cell index, whereas RKIP deficiency significantly enhanced the normalized cell index, which correlated well with the enhanced degranulation in the RKIP KO BMMCs. Moreover, IgE–FcεRI-elicited Ca²⁺ flux was significant higher in the RKIP KO BMMCs than that in the WT BMMCs (Fig. 3G). Collectively, these data suggest that RKIP negatively regulates IgE–FcεRI-mediated mast cell activation in vitro.

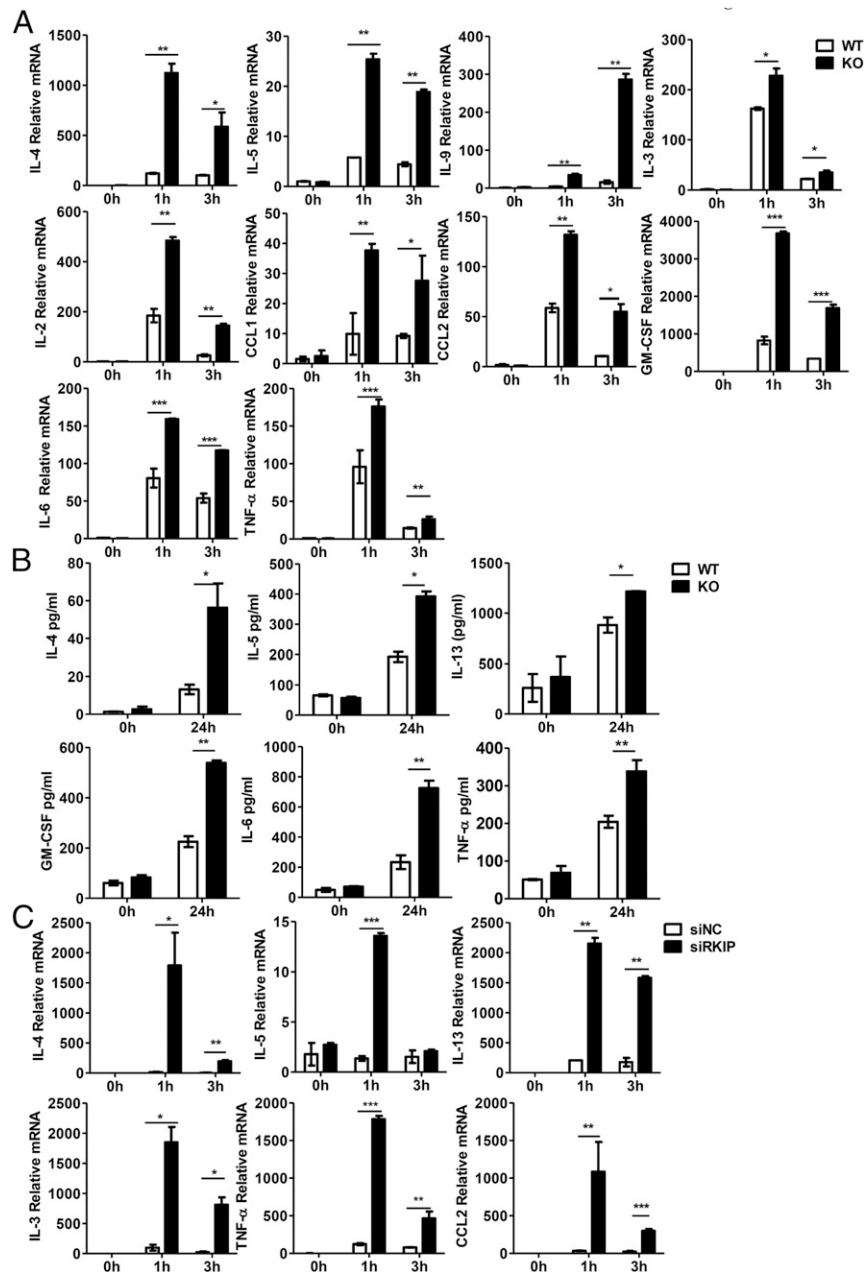
RKIP Deficiency Aggravates Passive Systemic Anaphylaxis and Passive Cutaneous Anaphylaxis in Vivo. To investigate the physiological role of RKIP in mast cell activation in vivo, we used mast cell-dependent animal models of passive systemic anaphylaxis (PSA) and passive cutaneous anaphylaxis (PCA) induced by anti-DNP-IgE/DNP-BSA to assess WT and RKIP KO mice (6, 19). Reportedly, no immune defects occur in the RKIP KO mice (14),

and no differences occur in the mast cell development between WT and RKIP KO mice (SI Appendix, Fig. S3A and B). The absolute numbers of mast cells in back skin was comparable in the WT and RKIP KO mice (SI Appendix, Fig. S3A). No significant differences occurred in mast cell morphology or cell surface expression of the c-Kit and FcεRI markers between the WT and RKIP KO BMMCs, as assessed by electron microscopy and flow cytometry (FACS) (SI Appendix, Fig. S3C and D). Additionally, the gene expression of mouse mast cell proteases 1, 2, 4, 5, and 6 and the sensitivity of mast cells to IL-3–induced signaling (assessed by cellular levels of p-STAT5 and p-Erk1/2) were comparable in the WT and RKIP KO BMMCs (SI Appendix, Fig. S3E and F). These results suggest that RKIP deficiency has no effect on the development of mast cells.

WT and RKIP KO mice were systemically presensitized with anti-DNP-IgE and then i.v.-injected with DNP-BSA to trigger a PSA response. DNP-BSA injection induced significant body temperature drops in both WT and RKIP KO mice, whereas RKIP KO mice showed much greater magnitude and duration of body temperature drops than that shown by the WT mice (Fig. 4A). Consistently, in association with the anaphylactic response, RKIP KO mice had much higher serum levels of histamine, LTC4, and PGD2 at 100 min after antigen challenge than exhibited by the WT mice (Fig. 4B–D).

Next, we elicited IgE–FcεRI-mediated PCA reactions to demonstrate the functional role of RKIP in both immediate and late-phase anaphylactic responses. Early-phase PCA and PSA reactions depend largely on the histamine and serotonin secreted by mast cells (20), whereas late-phase PCA reactions are associated with mast cell-derived cytokines in mouse. The ear swelling from inflammatory edema was greater in KO mice than that in WT mice for the entire observation period (Fig. 4E). Consistent with the ear swelling, the degree of inflammatory cell infiltration (Fig. 4F and G) and the concentration of Evans blue dye (Fig. 4H) in response to IgE–FcεRI-mediated activation were higher in the ears of the KO mice than in the ears of the WT mice. We quantified mast cell degranulation in the ear skin from the KO or WT mice as previously reported (21). Diffuse toluidine blue-positive cells with no clearly defined cell membrane indicate degranulated mast cells. As shown in Fig. 4I and J, the IgE–FcεRI-mediated mast cell degranulation in the ear of the RKIP KO mice was significantly higher than that in the ear of WT mice. To investigate the role of RKIP in early-phase PCA, 2 d after intradermal injection of anti-DNP-IgE or saline, we injected WT and KO mice with DNP-BSA dissolved in saline containing Evans blue. In saline-presensitized WT and KO mice, dye extravasation at 1 h after the antigen stimulus was comparable in WT and KO skin biopsies. However, Evans blue dye extravasation visualized and quantified in the back skin biopsies of IgE–FcεRI-activated mice was significantly enhanced compared with that of saline-presensitized mice, and the RKIP KO mice exhibited more Evans blue dye extravasation than the WT mice showed after IgE–FcεRI-mediated activation (Fig. 4K and L). Consistently, the RKIP KO mice showed higher mast cell degranulation in the back skin than the WT mice showed upon IgE–FcεRI stimulation (Fig. 4M and N). Generally, these data suggest that RKIP deficiency promotes IgE–FcεRI-mediated mast cell activation associated pathology in vivo and therefore enhances anaphylactic responses.

RKIP Deficiency Aggravates IgE–FcεRI-Mediated Allergic Responses in a Mast Cell-Dependent Manner. To further confirm whether the in vivo role of RKIP in allergic response is dependent on mast cell activation, we applied a mouse model of PSA in genetically mast cell-deficient Kit^{W-sh/W-sh} C57BL/6 mice, reconstituted with WT or KO BMMCs. No differences were observed in the mast cell engraftment between WT BMMC- and KO BMMC-reconstituted mice, as assessed by enumeration of the cells positive for toluidine blue staining in a mesenteric window (Fig. 5A and B). As



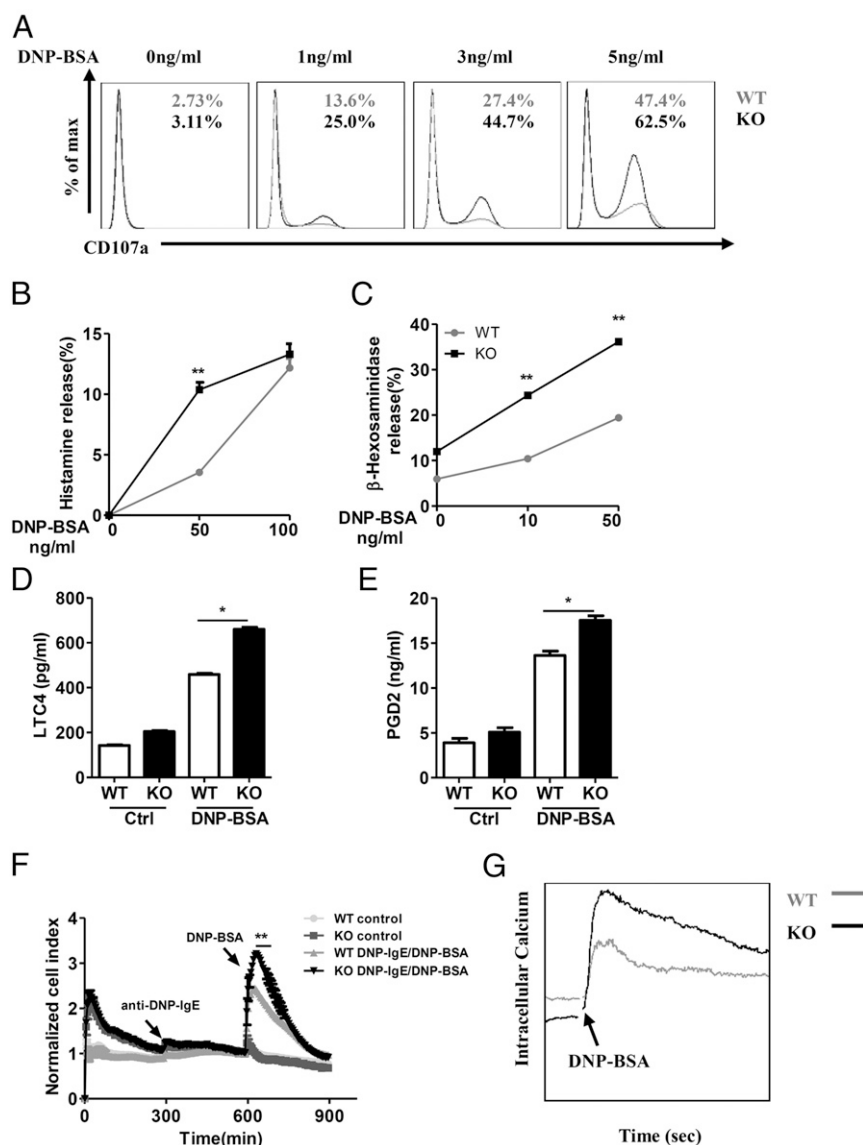


Fig. 3. RKIP deficiency promotes IgE–Fc ϵ RI-mediated degranulation in mast cells. (A) The WT and RKIP KO BMMCs were presensitized with 1 μ g/mL of anti-DNP-IgE overnight and activated with the indicated concentrations of DNP-BSA for 10 min. The BMMCs were incubated with anti-mouse CD107a, c-Kit, or Fc ϵ RI antibodies and then subjected to FACS analysis. C-Kit and Fc ϵ RI double-positive cells were gated out as BMMCs, and degranulation was assessed by CD107a expression levels on BMMCs. The numbers in the graphs indicate the percentages of CD107a⁺ cells. (B) WT and RKIP KO BMMCs were presensitized with 1 μ g/mL of anti-DNP-IgE overnight and activated with the indicated concentrations of DNP-BSA for 1 h, and the secretion of histamine was determined by ELISA. Degranulation was assessed by histamine release (presented as percentages of total histamine content). (C) BMMCs were presensitized with 1 μ g/mL of anti-DNP-IgE overnight and activated with the indicated concentrations of DNP-BSA for 30 min; degranulation was assessed by measuring β -hexosaminidase levels. (D and E) The WT and RKIP KO BMMCs were presensitized with 1 μ g/mL of anti-DNP-IgE overnight and activated with 100 ng/mL of DNP-BSA for 1 h; the release of the (D) LTC4 and (E) PGD2 was measured by competitive ELISA (Cayman Chemicals). (F) Measurement of IgE–Fc ϵ RI-mediated cell electrical impedance. The WT and RKIP KO BMMCs were seeded onto the fibrinogen-coated surfaces of microelectronic cell sensor arrays integrated into the bottoms of microtiter plates and presensitized with 1 μ g/mL of anti-DNP-IgE for 5 h. Subsequently, the cells were stimulated with 100 ng/mL of DNP-BSA, with the impedance being measured every 5 min and then analyzed by xCELLigence (a real-time cell analyzer). (G) Calcium flux was measured at the indicated times after 1 μ g/mL of anti-DNP-IgE presensitization and DNP-BSA stimulation in the WT and RKIP KO BMMCs. * P < 0.05; ** P < 0.01. Data are representative of the three experiments. Data represent mean and SD.

further promote LATs (including LAT1 and LAT2)-dependent Grb2/PLC- γ 1/SLP-76/MAPKs/PKC δ signaling. However, Fyn can directly phosphorylate another adaptor, Gab2, and further recruits PI3K to phosphatidylinositol 4,5-bisphosphate to activate the PI3K/Akt/IKK/NF- κ B pathway (10, 22). WT and RKIP KO BMMCs were presensitized with anti-DNP-IgE and stimulated with DNP-BSA for the lengths of time indicated in Fig. 6A. As shown in the figure, the activation of the Fc ϵ RI-proximal tyrosine kinases Lyn, Fyn, Syk, and PLC- γ 1 were comparable in the WT and KO

BMMCs. IgE–Fc ϵ RI-mediated phosphorylation of the MAPKs, such as p38 and ERK1/2, was not altered in the absence of RKIP. However, RKIP deficiency or silencing resulted in a more striking enhancement in the activation of distal signaling, including the phosphorylation of PI3K, Akt, the NF- κ B p65 subunit, and I κ B α (Fig. 6A and *SI Appendix, Fig. S4A*). Incubation with recombinant RKIP protein significantly inhibited the IgE–Fc ϵ RI-mediated signaling transduction, including phosphorylation of Akt and the NF- κ B p65 subunit, whereas it had no effect on the phosphorylation

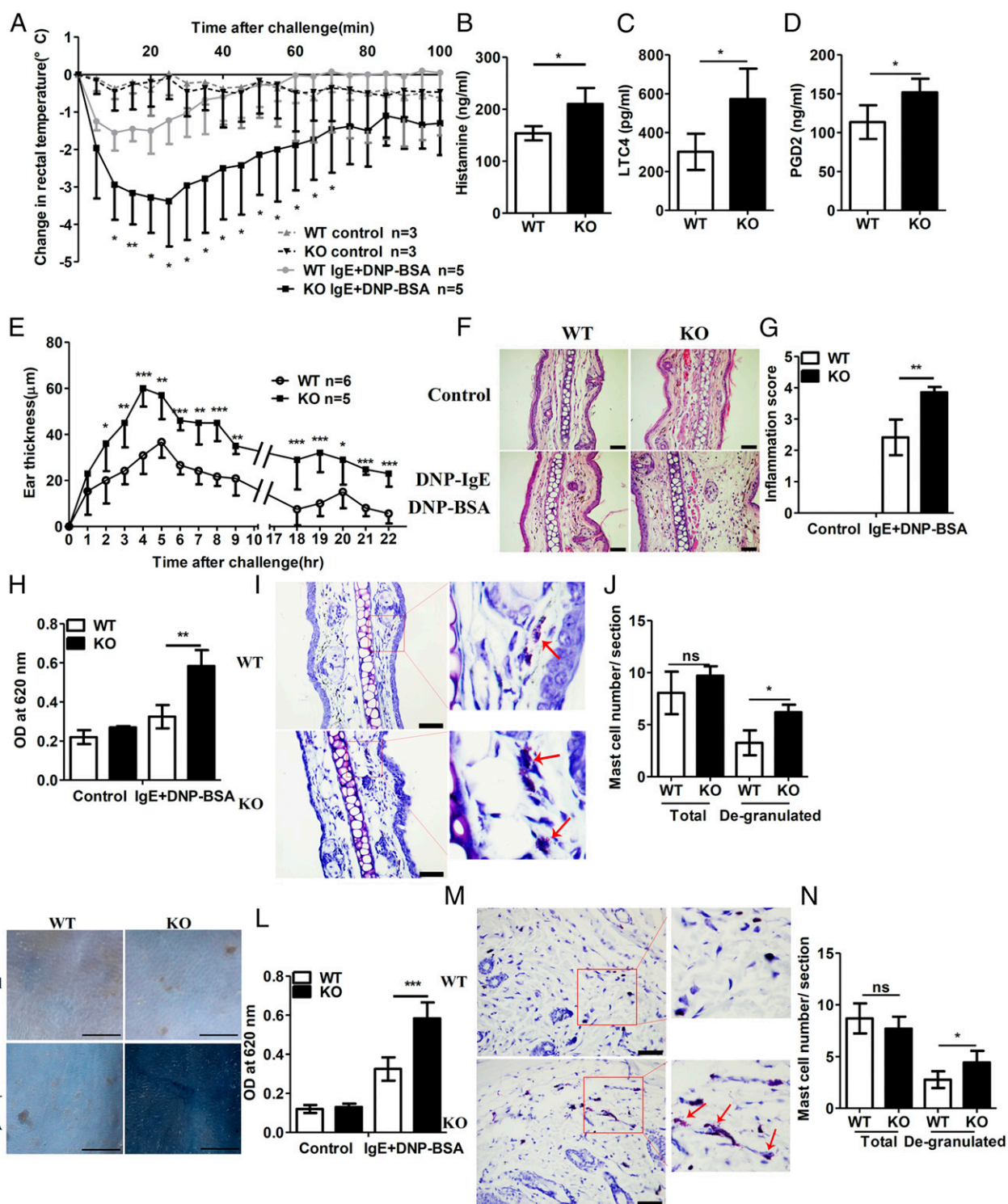


Fig. 4. RKIP deficiency aggravates PSA and PCA in vivo. (A) Mice were injected i.v. with 10 μ g of anti-DNP-IgE per mouse and challenged 24 h later with PBS or 100 μ g of DNP-BSA per mouse. Rectal temperatures were assessed every 5 min. (B–D) Releases of (B) histamine, (C) LTC₄, and (D) PGD₂ in the serum were measured at 100 min after DNP-BSA activation. (E) Mice were presensitized by intradermal injection of 3 μ g of anti-DNP-IgE per ear (left ear) or an identical volume of PBS (right ear), and, 24 h later, the mice were challenged i.v. with 100 μ g of DNP-BSA per mouse in PBS/Evans blue. Ear swelling was calculated as the difference between the thickness of the right and left ears (WT $n = 6$, KO $n = 5$). (F) H&E staining and (G) inflammatory cell infiltration scores of the histological sections of the right (control) and left ears (DNP-BSA) from mice treated as in E. (Scale bars in F, 50 μ m.) (H) Evans blue dye was extracted in formaldehyde from the reaction sites in E and quantified according to the absorbance at 620 nm. (I) Toluidine blue staining and (J) quantification (blind analysis) of mast cells per section of the left ears from mice treated as shown in E. Red arrows indicate degranulated mast cells. (Scale bars in I, 50 μ m.) (K) Mice were presensitized intradermally with anti-DNP-IgE or PBS; 2 d later, the mice were challenged with DNP-BSA in PBS/Evans blue, and the skin was examined visually ($n = 6$). (Scale bars, 10 mm.) (L) Evans blue dye was extracted in formaldehyde from the reaction sites in K and quantified as absorbance at 620 nm. (M) Toluidine blue staining and (N) quantification (blind analysis) of mast cells per section of back skin (DNP-BSA) from mice treated as in K. Red arrows indicate degranulated mast cells. (Scale bars in M, 50 μ m.) ns, no significant difference; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Data are representative of three experiments. Data represent mean and SD.

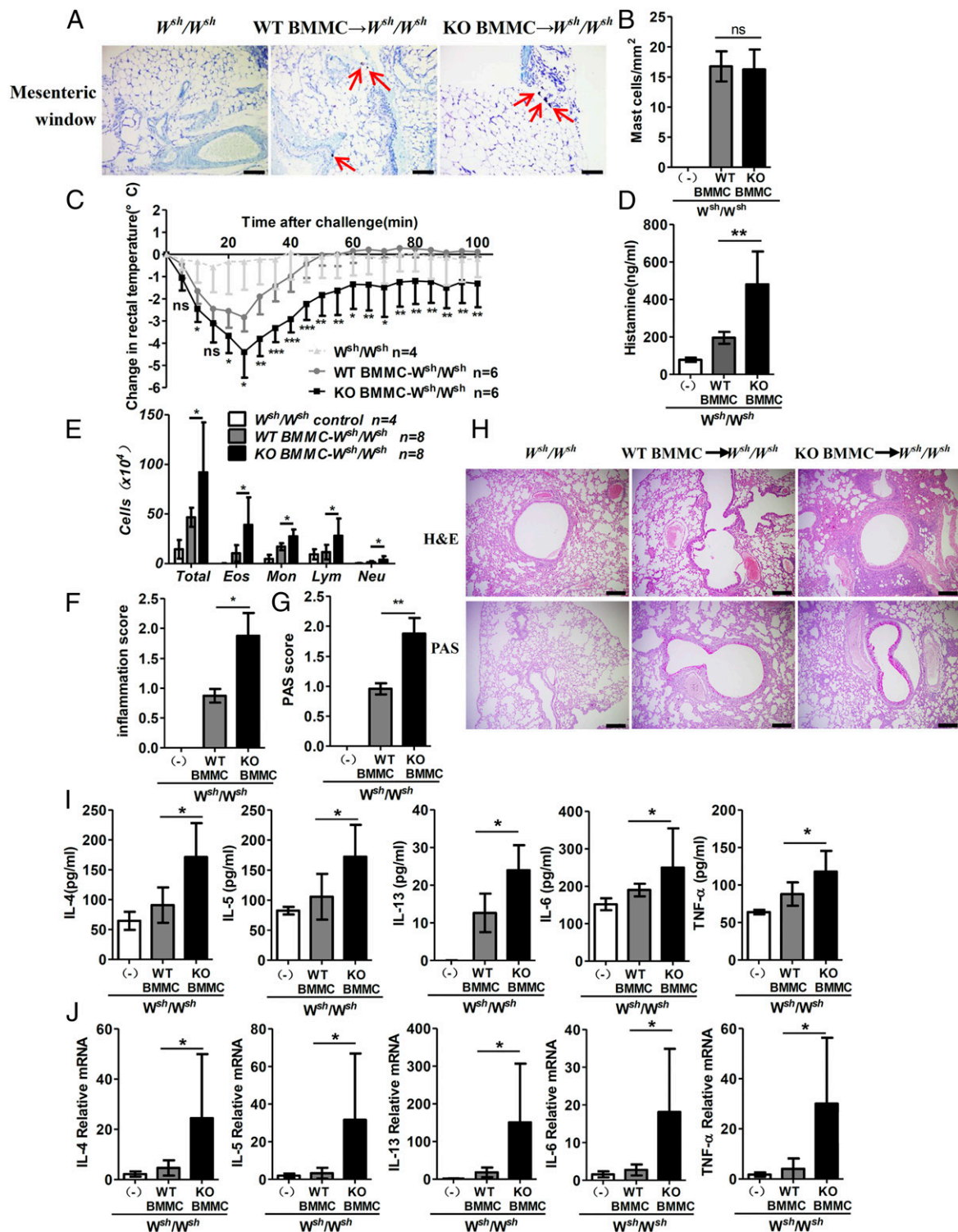


Fig. 5. RKIP deficiency in mast cells aggravates IgE-mediated allergic response. Mast cell-deficient mice were given no BMMCs (W^{sh}/W^{sh} control; $n = 4$) or were reconstituted with WT BMMCs (WT BMMC → W^{sh}/W^{sh} ; $n = 6$) or RKIP KO BMMCs (KO BMMC → W^{sh}/W^{sh} ; $n = 6$). (A) Four months later, tissue mast cells in a mesenteric window were assessed in the indicated chimeric mice by toluidine blue staining. Red arrows indicate mast cells. (Scale bars, 50 μ m.) (B) Absolute numbers of mast cells per square millimeter in W^{sh}/W^{sh} control mice ($n = 4$) or chimeric mice ($n = 6$ per group). Reconstituted mice were presensitized with anti-DNP-IgE overnight and challenged 24 h later with 100 μ g of DNP-BSA or PBS in Evans blue dye. (C) Rectal temperatures were assessed every 5 min. (D) Histamine release in serum was measured at 100 min after PBS or DNP-BSA challenge. W^{sh}/W^{sh} control ($n = 5$), WT BMMC → W^{sh}/W^{sh} mice ($n = 8$), and KO BMMC → W^{sh}/W^{sh} mice ($n = 8$) were challenged with OVA or PBS in the airway inflammation model and analyzed 24 h after the final challenge. (E) Numbers of total cells (total), eosinophils (Eos), neutrophils (Neu), lymphocytes (Lym), and macrophages (Mac) in the BALF were assessed after Wright–Giemsa staining. (F) Inflammation scores and (G) PAS scores were analyzed as described in *Materials and Methods*. (H) Sections of lung tissues were stained with H&E and PAS staining. (Scale bars, 200 μ m.) (I) The mRNA expression of IL-4, IL-5, IL-13, IL-6, and TNF- α in lung tissue was assessed by real-time PCR ($n = 6$). (J) Secretion of IL-4, IL-5, IL-13, IL-6, and TNF- α in BALF as measured by ELISA ($n = 6$). ns, no significant difference; * $P < 0.05$; ** $P < 0.01$. Data are representative of three experiments. Data represent the mean and SD.

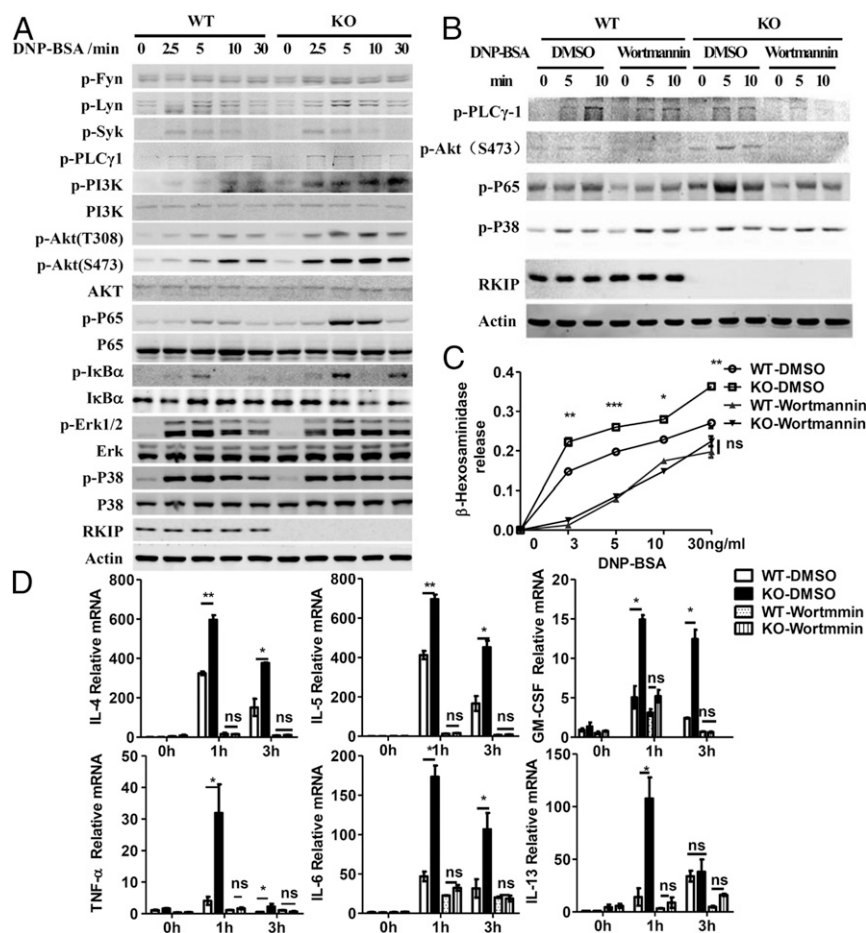


Fig. 6. RKIP deficiency increases FcεRI-mediated PI3K/Akt/NF-κB axis signaling activation in mast cells. (A) Immunoblot analysis in lysates of the WT and RKIP KO BMMCs stimulated by IgE–FcεRI-mediated activation for the indicated times. (B–D) WT and RKIP KO BMMCs were presensitized with 1 μg/mL of anti-DNP-IgE overnight, pretreated with wortmannin (PI3K inhibitor) for 2 h, and then stimulated with 100 ng/mL of DNP-BSA for the indicated times. (B) Immunoblot analysis was performed to identify the total or phosphorylated proteins in lysates using indicated antibodies. (C) The β-hexosaminidase was measured as described in *Materials and Methods*. (D) The mRNA expression levels of cytokines and chemokines were measured by real-time PCR. ns, no significant difference; **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Data are representative of three experiments. Data represent mean and SD.

of the MAPKs, such as p38 and ERK1/2, in the mast cells (*SI Appendix, Fig. S4B*). To further examine whether RKIP KO-mediated mast cell activation depends on the enhancement of the PI3K/Akt pathway, we pretreated the RKIP WT and KO BMMCs with wortmannin, a specific inhibitor of PI3K. As shown in Fig. 6B, the specific PI3K inhibitor not only inhibited Akt activation but also abolished the increased NF-κB p65 phosphorylation in the RKIP KO BMMCs. Furthermore, on suppression of PI3K activity by wortmannin, IgE–FcεRI-mediated degranulation (measured by β-hexosaminidase) (Fig. 6C) and the production of cytokines by KO BMMCs reverted to levels comparable to those in the WT BMMCs (Fig. 6D). Moreover, the PI3K-silenced RKIP KO BMMCs showed signaling activation and cytokine expression comparable to the PI3K-silenced WT BMMCs (*SI Appendix, Fig. S4 C and D*). Overall, these data suggest that RKIP inhibits mast cell activation by targeting PI3K activation.

RKIP Competes with Gab2 to Interact with the PI3K p85 Subunit and Suppresses the Activation of PI3K on IgE–FcεRI Stimulation. PI3K consists of the p85 regulatory subunit and the p110 catalytic subunit, and both are involved in mast cell activation signaling (23). During FcεRI-mediated mast cell activation, the adapter protein Gab2 binds to the PI3K p85 regulatory subunit and recruits it to the cell membrane, resulting in activation of p85 by Fyn or Syk (5). As shown in Fig. 7A, Myc-RKIP specifically

interacted with the PI3K p85 subunit but not with Lyn, Fyn, PLC-γ1, or Akt. In addition, RKIP did not bind to Gab2 (*SI Appendix, Fig. S5B*) and the PI3K p110 subunit (*SI Appendix, Fig. S5C*). The Flag-p85 or Flag-EGFP proteins were purified from overexpressed HEK293T cells and incubated with recombinant GST-RKIP protein. As shown in Fig. 7B, RKIP directly interacted with p85. RKIP physically associated with p85 in the mast cells, and IgE–FcεRI stimulation promoted this association in a time-dependent manner, as the interaction peaked at 5 min after DNP-BSA treatment in the BMMCs (Fig. 7C). All PI3K p85 isoforms have an N-terminal Src homology 3 (SH3) domain; two Pro-rich regions near the N terminus; a BCR homology domain, which binds to phosphorylated tyrosine; and a PI3K p110 binding domain located between two Src homology 2 domains, which form the PI3K p85 core domain (*SI Appendix, Fig. S5C*) (23). Domain-mapping experiments showed that RKIP interacted with p85 in a manner dependent on the domain of the p85 core (aa 332 to 724) but not on the SH3 domain and P-BH-P domain (aa 1 to 331) (*SI Appendix, Fig. S5C*). Considering that RKIP has no effect on IgE–FcεRI-mediated Syk or Fyn phosphorylation but affects the PI3K activation, we next explored whether RKIP could inhibit the association of Gab2 with the PI3K p85 regulatory subunit, which is essential for the activation of PI3K. As shown in Fig. 7D, overexpression of RKIP in HEK293T cells inhibited the interaction of p85 with Gab2. Next,

suppress expression of RKIP under IgE–FcεRI-mediated activation. Interestingly, GEO data and our findings show that the expression of *RKIP* in peripheral blood is negatively correlated with the severity of human asthma. We further determined that *RKIP* expression was negatively related to the occurrence of signs and symptoms in patients with asthma. Since mature mast cells are not present in blood, reduced *RKIP* expression in the blood of asthmatic patients suggests *RKIP* in cells other than mast cells could contribute to human asthma, which needs to be further investigated in the future.

The signal transduction that mediates mast cell activation by IgE–FcεRI is accomplished mainly by phosphorylation of two similar linker proteins, LAT1 and NTAL (LAT2). LATs activate mainly the major activation signaling pathway (Grb2/PLC-γ1/SLP-76/MAPK), whereas a complementary signaling pathway (Gab2/PI3K/Akt/NF-κB) is directly activated by phosphorylated Fyn. *RKIP* deficiency does not affect the activation of the tyrosine kinases Lyn, Fyn, and Syk or the MAPKs but significantly enhances the phosphorylation of PI3K/Akt and p65. The promoting effect of the *RKIP* KO on IgE–FcεRI-induced signaling activation, the downstream secretion of cytokines and chemokines, and the release of β-hexosaminidase disappears when specific inhibitors are used to block PI3K activity, indicating that *RKIP* KO increases the IgE–FcεRI-induced mast cell activation by promoting PI3K activation. Coimmunoprecipitation experiments show that *RKIP* specifically interacts with the PI3K regulatory subunit p85. We further demonstrate that *RKIP* competes with Gab2 to interact with p85 and interferes with the association of p85 PI3K and Gab2, dampening the PI3K/Akt/NF-κB signaling activation and the release of downstream mediators.

RKIP was originally identified as a Raf-1 binding protein that inhibits MEK/ERK1/2 phosphorylation (26). However, the activation of ERK1/2 did not differ between the WT and *RKIP*-deficient BMMCs subjected to the IgE–FcεRI-mediated activation. PKC switches *RKIP* from a Raf-1 kinase inhibitor to a G protein-coupled receptor regulator by phosphorylating the *RKIP* at Ser153 (27). We recently demonstrated that *RKIP* is phosphorylated at Ser109 on infection with virus; this phosphorylation is critical to *RKIP*'s ability to promote TBK1 activation and antiviral response. Although we have found down-regulated *RKIP* expression in IgE–FcεRI-activated mast cells, further investigation is needed to determine whether the IgE–FcεRI induced posttranslational modification of *RKIP*, causing *RKIP* to function as a PI3K inhibitor instead of a Raf kinase inhibitor.

Together, our findings reveal an important negative regulatory function of *RKIP* in mast cell activation. *RKIP* deletion considerably enhances the immunopathological phenotype of mice with mast cell-mediated PSA, PCA, and allergic asthma. Moreover, *RKIP* expression is down-regulated in asthma patients' peripheral blood and is negatively correlated with the process of asthma, indicating that the *RKIP* peptide-mimicking drugs might provide a viable approach to the treatment of allergic asthma.

Materials and Methods

Mice. *RKIP* KO (*rkip*^{−/−}) mice were kindly provided by John Sedivy of Brown University. *RkIP*^{−/−} mice and their littermates with a C57BL/6 background were used in this study. The progeny of the *rkIP*^{+/−} intercrosses were genotyped by PCR analysis of DNA isolated from the tail. The following three primers were used for the PCR: *RKIP* KO sense (5'-GAGCCCTGGCCGTCTCCTTGTCCCAACTTT-3'), *RKIP* WT antisense (5'-CACAAAACCAATCTTAAAGACCA-3'), and *RKIP* KO antisense (5'-CCAAAAGGCTTTGAGCACCAGAGGACATCCG-3'). Kit^{W-sh/W-sh} mice (B6.Cg-KitW-sh/HNhrJaeBsmGllJ, Stock No. 012861 | sash) were purchased from The Jackson Laboratory. All of the mice were kept in specific pathogen-free conditions. All animal experiments were performed in accordance with protocols approved by the Scientific Investigation Board of Zhejiang University.

Human Peripheral Blood Samples. Peripheral blood samples were obtained from healthy controls (*n* = 56) or children with asthma (*n* = 52) on admission from Children's Hospital, Zhejiang University School of Medicine. Peripheral blood samples analyzed in Fig. 1C were obtained from children with asthma (*n* = 26) at the onset and remission of asthma signs and symptoms on admission from Children's Hospital, Zhejiang University School of Medicine. The diagnosis and evaluation of asthma severity were made according to the Expert consensus document on diagnosis and treatment of asthma. Detailed patients' information is listed in [Datasets S1](#) and [S2](#). The study was approved by the ethics committee of the Children's Hospital, Zhejiang University School of Medicine. Written informed consent was obtained from at least one guardian of each patient before enrollment. The data from the patients were analyzed anonymously.

Statistical Analysis. Statistical significance of the differences between the two experimental groups was assessed using unpaired two-tailed Student's *t* test. The level of statistically significant difference was defined as *P* < 0.05.

ACKNOWLEDGMENTS. This work is supported by the National Key Basic Research Program of China Grant 2014CB542101; National Natural Science Foundation of China Grants 31570864, 31700765, and 81871264; and National Postdoctoral Science Foundation of China Grants 2017M612006 and 2017T100433.

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