

ARTICLE

IL-27 signaling negatively regulates FcεRI-mediated mast cell activation and allergic response

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Abstract

IL-27 is a member of the IL-12 family, exerting both anti- and pro-inflammatory activity in a cell-dependent and disease context-specific manner. Antigen-mediated cross-linking of IgE on mast cells triggers a signaling cascade that results in mast cell degranulation and proinflammatory cytokine production, which are key effectors in allergic reactions. Here, we show that the activation of mast cells is negatively regulated by IL-27 signaling. We found that mice lacking IL-27Rα (WSX-1) displayed increased sensitivity to IgE-mediated skin allergic response and chronic airway inflammation. The bone marrow-derived mast cells (BMMCs) of IL-27Rα-deficient mouse showed greater high-affinity receptor FcεRI-mediated activation with significantly enhanced degranulation and cytokine production. Mechanistically, the dysregulated signaling in IL-27Rα^{-/-} mast cells is associated with increased activation of Grb2-PLC-γ1-SLP-76, PI3K/Akt/IκBα signaling and decreased phosphorylation level of SH2 domain-containing protein phosphatase1 (SHP1). Furthermore, IL-27 treatment could inhibit mast cell activation directly, and retrovirus-based IL-27 expression in lung attenuated the airway inflammation in mice. Collectively, our findings reveal that IL-27 signaling negatively regulates mast cell activation and its mediated allergic response.

KEYWORDS

allergic response, chronic airway inflammation, FcεRI signaling, IL-27, mast cells

1 | INTRODUCTION

Mast cells are tissue-resident hematopoietic cells and are widely found in mucosal and epithelial tissues throughout the body. Mast cells participate in a variety of immune responses, including allergic reaction, innate immunity, and autoimmunity.^{1–3} High-affinity IgE FcεRI receptor expresses on the mast cells surface, composed of an IgE-

binding α subunit, a signal-modulating β subunit, and 2 disulfide-linked signal-generating γ subunit.⁴ Engagement of IgE-bound FcεRI by multivalent antigens triggers mast cell activation, leading to degranulation and releasing preformed pro-inflammatory chemical mediators such as histamine and proteases. In addition, cytokines and chemokines are secreted at late stage.⁵ These released molecules play pivotal roles in the inflammatory reactions observed in patients with allergy and autoimmune diseases.^{6–8} When IgE-FcεRI-mediated signaling is started, FcεRI β chain-associated Src family kinase (SFK), such as Lyn and Fyn, is activated initially, which phosphorylates immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic domains of β and γ chains of FcεRI and Syk tyrosine kinase.⁹ This results in the

Abbreviations: BALF, bronchoalveolar lavage fluid; BMMCs, bone marrow derived mast cells; DNP, dinitrophenyl; EBV3, Epstein-Barr virus-induced gene 3; FcεRI, high-affinity receptor FcεRI; gp130, glycoprotein 130; ITAM, immunoreceptor tyrosine-based activation motif; KO, knockout; OVA, ovalbumin; PAS, periodic acid-Schiff; PCA, Passive cutaneous anaphylaxis; PSA, Passive systemic anaphylaxis; SFK, Src family kinase; SHP1, SH2 domain-containing protein phosphatase1; WT, wild-type.

phosphorylation of linker for activation of T cells (LAT) and the formation of tyrosine-phosphorylated ITAMs signalsomes which further activate positive signaling mediators, such as Grb2, PLC- γ 1 and SLP-76, leading to the activation of mitogen-activated protein kinases, protein kinase C pathways and calcium flux.^{10,11} Besides Lyn, Fc ϵ RI aggregation also activates Fyn, which phosphorylates Gab2 to activate the PI3K pathway to play a complementary role in mast cell activation.¹²

IgE-mediated immune response is involved in protecting against environmental substances and parasite infection. However, excessive response can also cause harmful symptoms such as itching, inflammation, or tissue injury.¹³ Therefore, several negative signaling molecules have been implied in regulation of Fc ϵ RI-dependent mast cells activation such as SHIP1 and 2, and SHP1.^{14,15}

IL-27 is a member of IL-6/IL-12 family, composed of Epstein-Barr virus-induced gene 3 (EBI3) and p28 subunit and mainly produced by activated APCs that predominantly activates JAK-STAT signaling.¹⁶ IL-27 receptors consist of 2 chains, glycoprotein 130(gp130) and IL-27R α (WSX-1), expressed on naïve T cells, activated T cells, dendritic cells (DCs), NK cells, activated mast cells, and so on.¹⁷ Early studies showed that it was a proinflammatory cytokine that promotes a Th1 immune response during infection with intracellular pathogen *Listeria monocytogenes* or *Leishmania major* whose clearance mainly depends on Th1 response and IFN- γ production.^{18,19} In contrast, exacerbated Th1 responses were observed in IL-27R $\alpha^{-/-}$ mice infected with *Trypanosoma cruzi*, *Plasmodium berghei*, and *Leishmania donovani*, which revealed that IL-27 was not required for the production of IFN- γ but instead was critical to limit immunopathology.^{20–22} IL-27 is also a key inhibitor of Th2 response, which is described by several independent groups. Early studies showed that IL-27R $\alpha^{-/-}$ mice challenged with *L. major* produce increased Th2 response.²³ Based on Th2 response caused by parasite infection, IL-27-deficient mice produce increased IL-4, IL-5, and IL-13 cytokine, and can enhance protective immunity to helminth and *T. cruzi* infection.^{21,24} In the mouse model of asthma mediated by Th2 cells, IL-27 also limits Th2 response and IL-27R $\alpha^{-/-}$ mice exhibit severe pathologic character accordingly.^{25–27} Thus, we are now understanding that the biologic function of IL-27 signaling may be stimulus specific, immune-cell type dependent, and influenced by the microenvironment.

IL-27 receptors also express on mast cell surface, and the function of IL-27 to mast cell has not been described extensively. The study by Artis et al.²⁴ showed IL-27R α -deficient mice display higher mast cell activities in mast cell-dependent passive cutaneous anaphylaxis (PCA). Moreover, Traum et al.²⁸ indicated that mouse BMMCs and peritoneal mast cells are unresponsive to IL-27, due to constitutively express SOCS3, a major negative regulator of gp130 signaling, whereas IL-10 can increase gp130 expression, which resulted in a functional gp130 receptor on the BMMCs.

In the present study, we found that the mice lacking IL-27R α displayed increased sensitivity to IgE-mediated skin allergic responses and chronic airway inflammation. Loss of IL-27R α in BMMCs led to the hyper-responsiveness to the stimulation via Fc ϵ RI. The dysregulated signaling in IL-27R $\alpha^{-/-}$ mast cells is associated with increased

activation of “Grb2-PLC- γ 1-SLP-76” and “PI3K/Akt/I κ B α ” signaling, also decreased phosphorylated SHP1. Therefore, our findings reveal that IL-27 negatively regulates mast cell activation and allergic response.

2 | METHODS

2.1 | Mice

IL-27R α (WSX-1) knockout (KO) mice on the C57BL/6 background were purchased from The Jackson Laboratory (Stock No: 018078). PCR strategy was used to genotype the animals. PCR primers for genotyping are as follows: WT, 5'-CAAGACCTTGTGTGCAGGTG-3' and 5'-GTCACCATCTTGAGCCCACT-3'; IL-27R α KO mutant, 5'-CTTGGGTGGAGAGGCTATTC-3' and 5'-AGGTGAGATGACAGGAGATC-3'.

Eight to ten weeks IL-27R α KO mice and their wild-type (WT) male littermates were used for experiments. All mice were maintained in specific pathogen-free conditions. Experiments and animal care were performed according to protocols approved by the Zhejiang University Institutional Animal Care and Use Committee.

2.2 | Antibodies

FITC-conjugated antibody to mouse CD117 (c-kit) and PE-conjugated antibody to mouse Fc ϵ RI were from BioLegend. Anti-Erk1-Erk2 (9102), antibody to Erk1-Erk2 phosphorylated at Thr 202 and Tyr 204 (9101), anti-Jnk1-Jnk2 (56G8), antibody to Jnk1-Jnk2 phosphorylated at Thr183 and Tyr185 (9251), anti-p38 (9212), antibody to p38 phosphorylated at Thr 180 and Tyr 182 (9211), anti-Akt (C67E7), antibody to Akt phosphorylated at Ser473 (D9E), at anti-I κ B (44D4), antibody to I κ B phosphorylated at Ser32 and Ser36 (5A5), anti-Lyn (2732), anti-SHP1 (C14H6), antibody to stat3, antibody to stat3 phosphorylated at Ser727, and antibody to SHP1 phosphorylated at Tyr564 (D11G5) were purchased from Cell Signaling Technology. Anti-Grb2 (Y237), anti-PLC- γ 1 (EP1898-7Y), antibody to PLC- γ 1 phosphorylated at Tyr 783 (EP1898Y), antibody to Lyn phosphorylated at Tyr507 (EP504Y), and antibody to LAT phosphorylated at Tyr191 (E225) were from Epitomics.

2.3 | Cell culture

For BMMCs, mouse bone marrow cells were isolated from femurs and cultured in RPMI medium plus 10% FBS (Gibco) with 10 ng/ml IL-3 (PeproTech) and 10 ng/ml stem cell factor (SCF) (PeproTech). Half of the medium was replaced with fresh medium with cytokines every 4–5 days during culture. After 4–6 weeks in culture, BMMCs were stained to confirm the surface expression of Fc ϵ RI and c-Kit. Cells with purity >95% were used for subsequent experiments.

2.4 | Alcian blue/safranin staining

Cells were cytocentrifuged, air-dried, incubated for 20 min with 0.5% Alcian blue in 0.3% acetic acid, rinsed in water, and incubated for 20 min with 0.1% safranin in 1% acetic acid. Cells were examined under a microscope (IX71; Olympus).

2.5 | Real-time PCR analysis

Total RNA was extracted from BMMCs or tissue with TRIZOL according to the manufacturer's instructions. Total RNA was used to generate cDNA. IL-4, IL-5, IL-13, IL-6, TNF α , GM-CSF, and β -actin were quantified by real-time PCR method using gene-specific primers. Results were normalized to β -actin expression.

2.6 | ELISA assay

BMMCs were sensitized overnight at 37°C with 1 μ g/ml IgE anti-dinitrophenyl (DNP) in 1 ml culture medium and then stimulated for 24 h at 37°C with 10 ng/ml DNP-BSA. The amount of cytokine in the supernatant was determined by ELISA (eBioscience) according to the manufacturer's instructions. Histamine release in the supernatant and bronchoalveolar lavage fluid (BALF) was measured by ELISA (Invivo-gen) according to the manufacturer's instructions.

2.7 | Histamine release assays

1×10^6 BMMCs were sensitized with IgE anti-DNP and challenged with 10–50 ng/ml DNP-BSA. Supernatants were collected 1 h after challenge. Histamine was measured in cells and supernatants using fluorometric assay.²⁹ The percentage of histamine release was quantified by the following equation: histamine in supernatant/(histamine in supernatant + histamine in pellet) $\times$ 100.

2.8 | Measurement of cell-electrode impedance

The xCELLigence system (Roche), also known as the Real-Time Cell Electronic Sensing System (ACEA Biosciences), has been described previously.³⁰ The detailed procedure was that the wells were coated with 100 μ g/ml fibrinogen in PBS for 2 h at 37°C and blocked with 0.5% BSA for 30 min at 37°C, and then washed 3 times with PBS. For time-dependent cell response profile measurements, 50 μ l of medium was added to 16-well E-plates to obtain background readings, followed by the addition of 100 μ l mast cell suspension (6×10^4 cells/well). The E-plates containing the indicated cells were incubated at room temperature for 30 min before being placed onto the reader in the incubator. 1 μ g/ml anti-DNP IgE was added to the medium for sensitization; 5 h later, cells were challenged with 100 ng/ml DNP-BSA for 16 h. Impedance as reflected by the CI was recorded continuously.

2.9 | Passive systemic anaphylaxis(PSA)

WT and WSX-1-deficient mice were sensitized by i.v. injection of 10 μ g mouse IgE anti-DNP (SPE-7; Sigma-Aldrich) for 24 h and challenged i.v. with 100 μ g DNP-BSA. After antigen challenge, rectal temperature was measured and recorded every 5 min for 100 min with a digital thermometer (Omron). Histamine in serum of these mice was measured by ELISA.

2.10 | Passive cutaneous anaphylaxis

Experimental PCA was performed as previously described.³¹ Mice were injected intradermally with 1 μ g (20 μ l) IgE anti-DNP in the right ear or the back skin and 20 μ l saline in the left ear or in the back skin of control mice. The next day, the mice were challenged by i.v. injection of 100 μ g DNP-BSA in 100 μ l saline containing Evans blue dye (1%). Back skin extravasation biopsies were incubated at 63°C overnight in 300 μ l formamide. Formamide extracts were analyzed by measuring the absorbance of Evans blue at 620 nm. Ear-swelling responses were assessed by measurement of ear thickness with a digital thickness gauge (G-1A). Net ear swelling was calculated as the difference in thickness of the right and left ears.

2.11 | Airway inflammation

WT and IL-27R α -deficient mice were intraperitoneally sensitized with 10 μ g (100 μ l) ovalbumin (OVA) (Sigma-Aldrich) on day 0, 7, 14, 21, 28, and 35. Then, the mice were intranasally challenged with 20 μ g (20 μ l) OVA or PBS on day 40, 43, and 46. Mice were sacrificed 24 h after the last challenge. BALF was collected. Lung tissues were fixed with 10% formaldehyde and embedded in paraffin. Sections were stained with H&E and periodic acid-Schiff (PAS).

2.12 | Ca²⁺ measurement

1×10^7 BMMCs were sensitized with 1 μ g/ml IgE anti-DNP in 1 ml medium for 8 h. Cells were washed twice with PBS and then loaded with 4 μ M Fluo-4 AM (Molecular Probes) for 45 min. Cells were washed again and further incubated in PBS for 30 min at room temperature. 10 ng/ml DNP-BSA was then used to induce calcium flux, which was measured using a flow cytometer (FACSCalibur; BD) to monitor the fluorescent emission.

2.13 | Immunoblot analysis

Cells were lysed in SDS sample buffer by the addition of 1/4 volume of 5 $\times$ SDS sample buffer directly into the cell suspensions. Samples were then boiled for 5 min and separated on 10% SDS-PAGE. Immunoblot analyses were done as described.

2.14 | Retrovirus packaging

Two IL-27 subunits, EBI3 and p28, were coexpressed with a 4 repeated linker GGCGGCGGCAGC, and then retrovirus-expressing EBI3-linker-p28 (IL-27) was packaged using pMXs-IRES-GFP Retroviral Vector and Platinum-E retroviral packaging cell line.

2.15 | Statistical analysis

Statistical analysis was performed with GraphPad Prism software. All data are expressed as the mean $\pm$ SEM or SD. Statistical significance between 2 groups was determined using an unpaired, 2-tailed Student's *t*-test. Three or more groups were compared with ANOVA. Statistical significance was determined as $p < 0.05$.

3 | RESULTS

3.1 | Mast cell development was not influenced in IL-27R α KO mice

To evaluate the role of IL-27 signaling in the development of mast cells, we first prepared different tissues including back skin, stomach, and lung from WT and IL-27R α ^{-/-} (KO) mice stained with toluidine blue. WT and KO mice displayed the comparable numbers of mast cells in these tissues (Figure 1(A)). Similarly, the percentages of mast cells marked with Fc ϵ RI and c-Kit from peritoneal fluid showed no difference between WT and KO mice, and BMMCs proliferated equally well in vitro in media containing IL-3 (Figure 1(B)). Moreover, Alcian blue-Safranin O staining and electron microscopy showed that KO BMMCs did not differ morphologically from WT BMMCs (Figure 1(D)). Collectively, these data demonstrate that the development of mast cells in vivo and vitro is not impaired by IL-27R α deficiency. We also determined whether IL-27R α deficiency could affect basophils as mast cells and basophils have similar characteristics in function and development. As shown in Figure 1(C), the portions of basophils in the spleen and bone marrow were comparable between WT and KO mice (Figure 1(C)). We next followed the expression of 2 IL-27 receptors, gp130 and IL-27R α on mast cells, and found that both expressions were low in resting BMMCs but increased significantly in Fc ϵ RI-activated BMMCs (Figure 1(E)). In addition, this flow cytometry results confirmed the IL-27R α deficiency at the protein level in IL-27R α KO mice (Figure 1(E)).

3.2 | Enhanced response of PSA and PCA in IL-27R α ^{-/-} mice

To investigate the role of IL-27 signaling in mast cells activation in vivo, 2 types of mast cell-dependent immune response models were constructed. The first model was PSA, a type I immediate hypersensitivity

reaction, characterized by histamine release in serum and body temperature drop.³¹ WT and KO mice were systemically sensitized with anti-DNP IgE antibody for 24 h and then i.v. injected with DNP-BSA the next day to trigger a PSA response. Rectal temperature was assessed every 5 min, and the results showed DNP-BSA injection induced significant body temperature drops in both WT and KO mice, whereas KO mice exhibited more significant body temperature drops and enhanced hypersensitivity reaction compared with WT mice (Figure 2(A)). Consistently, histamine release in serum of KO mice was elevated more dramatically (Figure 2(B)).

The second was a PCA model, which depends largely on histamine and serotonin secreted by mast cells in the acute phase, whereas depends on mast cell-derived cytokines in the late phase.¹⁷ In PCA experiment, ear or back skin were sensitized with anti-DNP IgE for 24 h, then DNP-BSA containing 1% Evans blue dye was i.v. injected into WT and KO mice the next day. Immunohistochemistry results showed a significant increase of IL-27 in the back skin biopsies of IgE-Fc ϵ RI-activated mice including epidermis and dermis, suggesting IL-27 may play a role in the PCA reaction (Figure 2(C)). Evans blue dye extravasation visualized and quantified in the reaction site of back skin were significantly enhanced in KO mice (Figures 2(D) and 2(E)). Consistently, the ear swelling from inflammatory edema in KO mice was more severe than that in WT mice (Figure 2(G)). In conclusion, these data suggest that IL-27R α deficiency promotes IgE-Fc ϵ RI-mediated mast cell activation and therefore enhances anaphylactic responses.

3.3 | Enhanced chronic airway inflammatory response in IL-27R α ^{-/-} mice

To further illuminate the function of IL-27 signaling in other allergic diseases, mast cell-dependent chronic airway inflammation was induced. According to the published protocol, WT and KO mice were sensitized with OVA for 24 h followed by intranasal OVA challenges.^{32,33} Hallmark of this model was accumulation of eosinophils, goblet cells hyperplasia, Th2, and inflammatory-related cytokines. We first detected the IL-27 expression by immunohistochemistry, and the results showed that IL-27 abundantly expressed in lung tissues accompanied by infiltration of inflammatory cells in both WT and KO mice (Figure 3(A)). Increased infiltration of inflammatory cells and enhanced hyperplasia of mucus-secreting goblet cells (PAS⁺) in large bronchioles were observed in KO mice, which was assessed by H&E and PAS staining respectively (Figure 3(B)). In addition, protein concentration in BALF from challenged KO mice was significantly higher than that from WT mice measured by BCA method (Figure 3(C)). Similarly, total cells, eosinophils, and lymphocytes accumulation in BALF were also increased in KO mice (Figure 3(D)). Consistently, KO mice produced higher levels of Th2-related cytokines like IL-4, IL-5, and IL-13, and inflammatory cytokines as IL-6, TNF- α , and GM-CSF in BALF (Figures 3(E) and 3(F)). Together, our results demonstrate that IL-27R α deficiency exacerbated chronic airway inflammatory response in vivo.

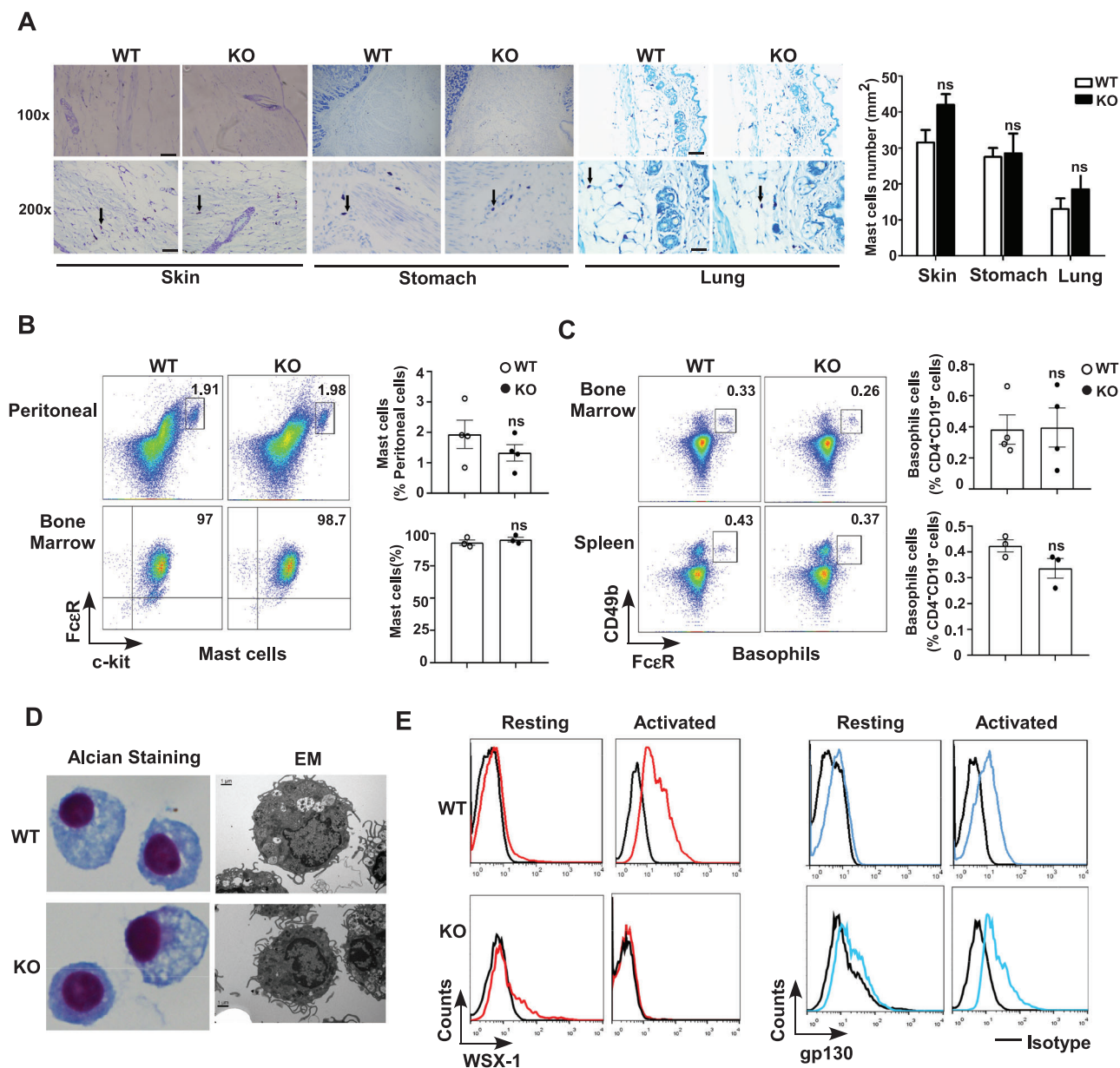


FIGURE 1 Mast cell development was not influenced in IL-27R α KO mice. (A) Absolute numbers of mast cells per mm² skin, stomach, and lung tissue (mean $\pm$ SD). Scale bars, 100 $\times$: 100 μ m; 200 $\times$: 50 μ m. (B) Flow cytometry analysis of the surface marker Fc ϵ RI and c-Kit expression in WT and IL-27R α KO peritoneal mast cells and BMMCs. (C) Flow cytometry analysis of Basophils in bone marrow and spleen from WT and KO mouse. (D) Alcian blue-Safranin O staining of BMMCs from WT and KO mice. Original magnification $\times$ 1000. Electronic microscopy analysis of WT and KO BMMC. Scale bars, 1 μ m. (E) Flow cytometry analysis of the expression of IL-27R α (WSX-1) and gp130 in resting and activated BMMCs. Statistical significance was assessed by unpaired 2-tailed Student's t-test; n.s., not significant

3.4 | Elevated Fc ϵ RI-mediated activation in IL-27R α ^{-/-} BMMCs

To further confirm whether the excessive allergic response in IL-27R α ^{-/-} mice is dependent on mast cell activation, WT BMMCs were transferred to KO mice. The results showed that this adoptive transfer reversed the elevated airway inflammation in KO mice, as evident from H&E and PAS staining (Figure 4(A)). Consistently, WT BMMC transferred KO mice secreted less cytokines (Figure 4(B)). Thus, these

results indicated that IL-27 effects on mast cells played an essential role during chronic allergic inflammation.

Next, to study the role of IL-27 signaling in regulation of IgE-Fc ϵ RI-mediated mast cell activation in vitro, we cultured BMMCs from WT and KO mice in the presence of IL-3 and SCF. Six weeks later, more than 95% pure population of mast cells was generated as shown in Figure 1(B). IgE-Fc ϵ RI-mediated mast cell activation includes rapid cell degranulation and secretion of newly synthesized metabolites, cytokines, and chemokines, such as histamine, IL-4, IL-5, and

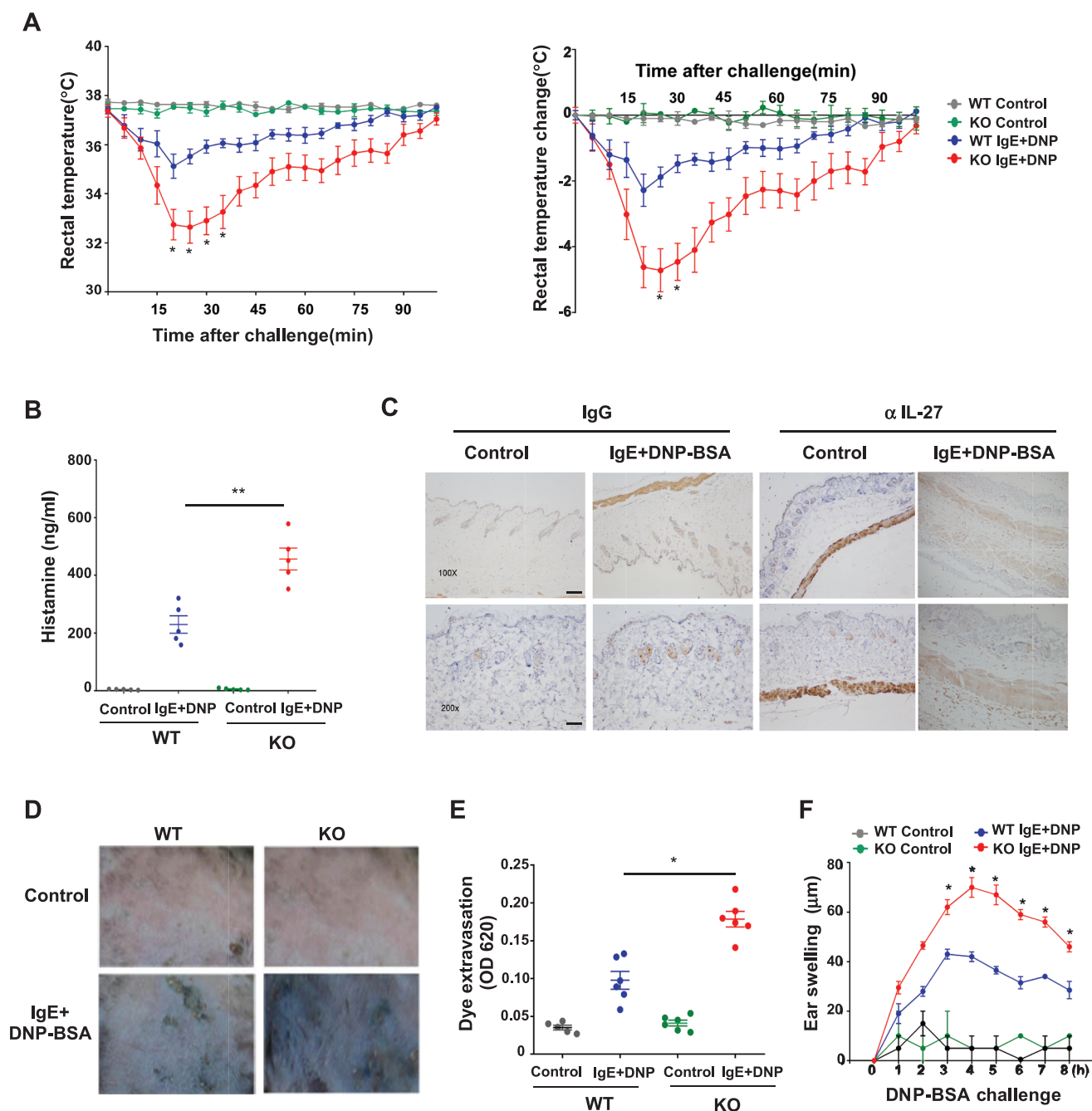


FIGURE 2 IL-27R α deficiency aggravates PSA and PCA in vivo. (A) Mice were sensitized i.v. with anti-DNP IgE overnight, and then challenged with PBS or DNP-BSA next day. Rectal temperatures were assessed every 5 min. (WT control, KO control: $n = 3$; WT IgE+DNP-BSA, KO IgE+DNP-BSA: $n = 5$). Statistical significance was assessed by 2-way ANOVA. (B) Serum levels of histamine at 100 min after PBS or DNP-BSA challenge. Statistical significance was assessed by unpaired 2-tailed Student's t -test. (C) Immunohistochemical staining of the back skin of DNP-BSA-injected mice treated with IgE by anti-IL-27 antibody. Scale bars, upper panel 100 $\times$: 100 μ m; lower panel 200 $\times$: 50 μ m. (D) WT and IL-27 KO mice were sensitized intradermally with anti-DNP IgE or PBS, then challenged with DNP-BSA in PBS/Evans blue 24 h later, and the skin was examined visually ($n = 6$ per genotype). (E) Spectrophotometry of Evans blue extracted in formaldehyde from the reaction sites in D and quantified as absorbance at 620 nm. Statistical significance was assessed by unpaired 2-tailed Student's t -test. (F) WT and KO mice were sensitized by intradermal injection of anti-DNP IgE (left ear) or PBS (right ear), then challenged with DNP-BSA in PBS/Evans blue 24 h later. Ear swelling was calculated as the difference between the thickness of the right and left ear ($n = 4$ per genotype). Statistical significance was assessed by 2-way ANOVA. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. Data are representative of 3 independent experiments. Data represent mean and SD

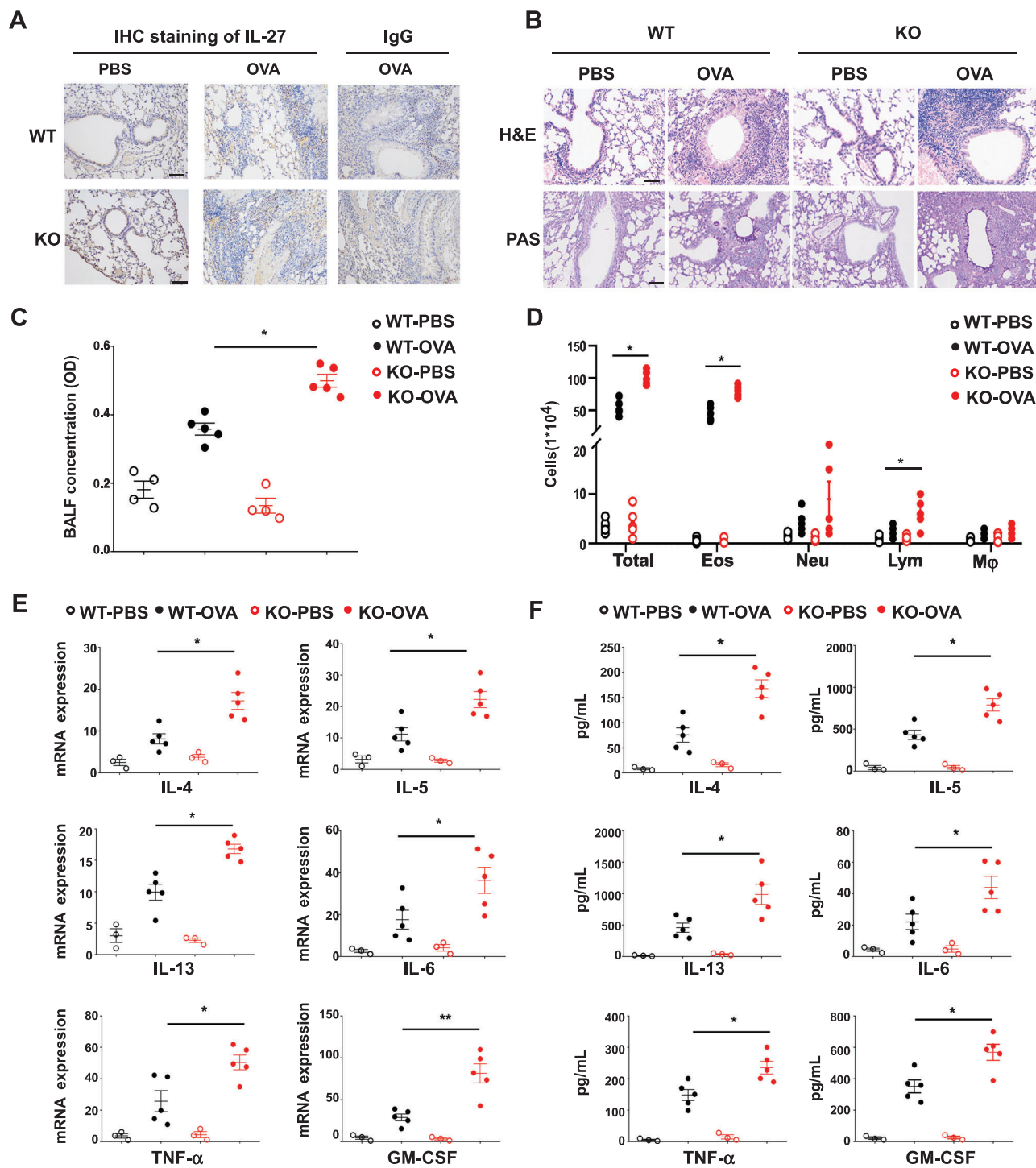


FIGURE 3 IL-27R α deficiency aggravates OVA-induced chronic airway inflammation. (A) Immunohistochemistry staining of the lung of DNP-BSA-injected mice treated with IgE by anti-IL-27 antibody. Scale bars, 100 μ m. (B) Lung tissue sections were stained with H&E and PAS. Scale bars, 50 μ m. (C) Protein concentration in BALF was detected by BCA assay. (D) Cell counts in BALF (n = 5 per group). Eos, eosinophils; Neu, neutrophils; Lym, lymphocytes; M ϕ , macrophages or monocytes. (E) Real-time PCR analysis of IL-4, IL-5, IL-13, IL-6, TNF- α , and GM-CSF mRNA expression in lung from WT and IL-27R α ^{-/-} mice. (F) Secretion of cytokines in BALF from WT and IL-27 KO mice was determined by ELISA. Statistical significance was assessed by unpaired 2-tailed Student's t-test. * p < 0.05. Data are representative of 3 independent experiments. Data represent the mean and SD

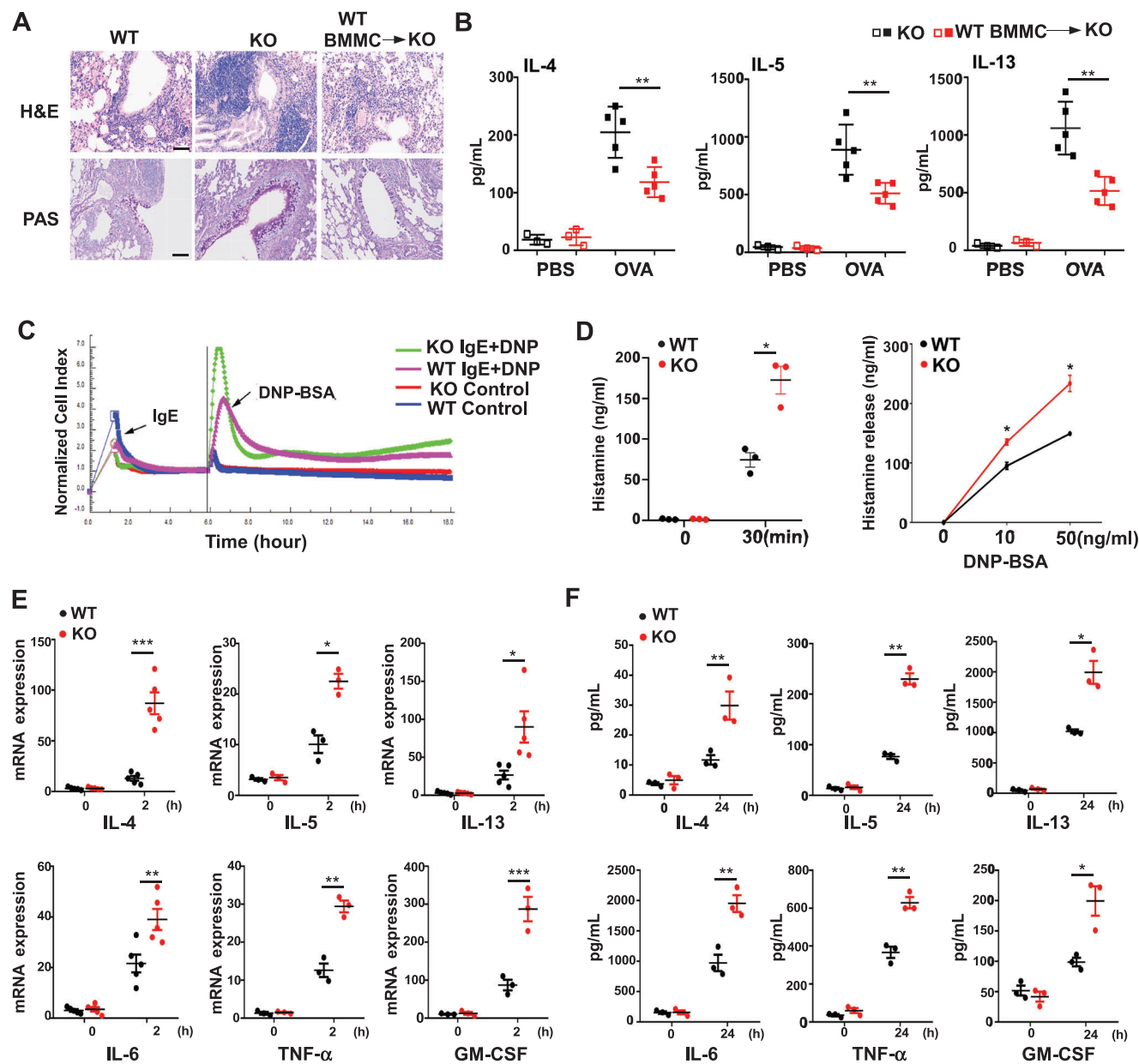


FIGURE 4 IL-27 α deficiency promotes IgE–Fc ϵ RI-mediated degranulation and cytokines production in mast cells. (A) 8×10^6 WT BMMCs were transferred to KO mice 12 h before OVA challenged. Lung tissue sections were stained with H&E and PAS. Scale bars, 50 μ m. (B) Secretion of cytokines in BALF was determined by ELISA. (C) WT and KO BMMCs were seeded onto the fibrinogen-coated surface of microelectronic cell sensor arrays integrated into the bottom of the microtiter plates and the impedance was continuously measured every 5 min and analyzed by xCelligence (real-time cell analyzer). (D) Secretion of histamine from anti-DNP IgE-sensitized WT and KO BMMCs stimulated with DNP-BSA was measured by ELISA. (E) Real-time PCR analysis of mRNA expressions of various cytokines IL-4, IL-5, IL-13, IL-6, TNF- α , and GM-CSF in anti-DNP IgE-sensitized WT and KO BMMCs stimulated with DNP-BSA for 2 h. (F) ELISA assay of cytokines secreted by anti-DNP IgE-sensitized WT and KO BMMCs stimulated with DNP-BSA for 24 h. Statistical significance was assessed by unpaired 2-tailed Student's *t*-test. **p* < 0.05, ***p* < 0.01. Data are representative of 3 independent experiments. Data represent the mean and SD

so on. Degranulation was accompanied by cytoskeletal rearrangement, which can be monitored by xCELLigence after IgE-sensitized BMMCs challenged with allergen, based on a microelectronic cell sensor technology.³⁴ We found that loss of IL-27 signaling significantly increased the normalized cell index of BMMCs, which correlated well with enhanced degranulation (Figure 4(C)). Meanwhile, the release of histamine upon IgE–Fc ϵ RI activation was higher in the KO BMMCs

treated with triton x-100 (Figure 4(D)). Next, we quantified Th2-related and inflammatory-related cytokines in WT and KO BMMCs at mRNA and protein levels. As shown in Figure 4(E), real-time PCR analysis showed IL-4, IL-5, IL-13, IL-6, TNF- α , and GM-CSF mRNA expressions were significantly up-regulated in IgE-sensitized KO BMMCs upon DNP-BSA stimulation, and protein levels of these cytokines in supernatant from cultured KO BMMCs were also higher (Figure 4(F)).

Together, these data indicate that IL-27 signaling negatively regulates FcεRI-mediated BMMCs rapid degranulation and cytokine release in late phase.

3.5 | Altered signaling in FcεRI-mediated activation of IL-27Rα^{-/-} BMMCs

The data above demonstrate that lack of IL-27 signaling promotes either mast cell degranulation in early phase or cytokine production in late phase both in vivo and in vitro. Next, we detected the change of FcεRI signaling pathway. WT and KO BMMCs incubated with recombinant IL-27 were sensitized with anti-DNP IgE for 24 h, followed by DNP-BSA stimulation. Upon DNP-BSA stimulation, the signaling cascades are initiated by activation of the SFKs Lyn and Fyn, further promoting LATs-dependent Grb2/PLC-γ1/SLP-76 signaling.³⁰ Meanwhile, Fyn can directly phosphorylate another adaptor, Gab2, and activating "Gab-2/PI3K/Akt/IκBα" signaling pathway. As shown in the Figure 5(A), significantly enhanced activation of Lyn was detected in KO BMMCs compared with WT BMMCs after stimulation, suggesting that the up-regulated response in KO BMMCs may due to the upstream molecules, at least Lyn. In addition, higher levels of phosphorylated LAT, PLCγ1, and phosphorylated AKT, IκBα were also up-regulated in KO BMMCs after stimulation (Figure 5(A)). Several research groups demonstrated that activation of mitogen-activated protein kinases ERK, JNK, and p38 is critical for the FcεRI-dependent cytokine and chemokine production by mast cells.^{10,35} As expected, IL-27 signaling deficiency in KO BMMCs resulted in significant enhancement of Jnk, and p38 phosphorylation, which was consistent with up-regulated cytokine production in KO BMMCs after stimulation (Figure 5(A)). Moreover, phosphorylated SHP1, a negative regulator involved in mast cell activation, was down-regulated in KO BMMCs after activation (Figure 5(A)). Intracellular calcium flux is a hallmark of the degranulation in mast cells after allergen stimulation, and both the principle and complementary axis could influence the calcium influx during mast cell activation.^{36,37} As shown in Figure 5(B), higher calcium flux signal was detected by flow cytometry in KO BMMCs challenged with DNP-BSA. Taken together, these data indicated that FcεRI-induced principal axis (Grb2/PLCγ1/SLP-76), the complementary axis (Gab-2/PI3K/Akt), and MAPK signaling pathway were all amplified in IL-27 signaling deficient BMMCs.

IL-27 mainly activates JAK/STAT signaling, and we found that more phosphorylated STAT3 was detected in WT BMMCs incubated with recombinant IL-27 (Figure 5(C)). So we tried to explore the correlation between STAT3 signaling and phosphorylated SHP1 or positive regulator, phosphorylated Lyn. JAK2 inhibitors, AG490 and Ruxolitinib, dramatically decreased the level of phosphorylated STAT3 in WT BMMCs (Figure 5(D)). When treated by AG490 or Ruxolitinib, phosphorylated SHP1 was enhanced and phosphorylated Lyn was decreased in KO BMMCs, which was comparable to that in WT BMMCs after stimulation (Figure 5(E)). Together, these data suggested that the inhibitory function of IL-27 signaling on FcεRI-mediated mast cell activation is related to the increased "Grb2-PLC-γ1-SLP-76," "PI3K/Akt/IκBα" sig-

naling, and decreased phosphorylated SHP1, which is dependent on JAK2/STAT3 signaling.

3.6 | IL-27 attenuated mast cells activation in vitro and in vivo

Considering the elevated FcεRI-mediated mast cell activation and the increased allergic response in IL-27Rα^{-/-} mice, we further investigated whether IL-27 could inhibit mast cell activation directly and be a potential immunotherapeutic for chronic airway inflammation. Thus, we first detected the degranulation and cytokine production in IgE-FcεRI-mediated WT mast cells with or without IL-27 pretreatment. The results were in accordance with that from IL-27Rα^{-/-} BMMCs. ELISA assay showed decreased histamine released into supernatant from IgE-sensitized WT BMMCs incubated with recombinant murine IL-27 (Figure 6(A)). CD107A up-regulation is a marker of degranulation in mast cells, and the flow cytometry showed that IL-27 significantly decreased the expression of CD107A (Figure 6(B)). In accordance with these results, BMMCs incubated with recombinant IL-27 greatly decreased the expression of inflammatory cytokines (Figure 6(C)). Collectively, these data indicate that IL-27 could suppress the mast cell activation including degranulation and cytokine production directly.

To further confirm the function of IL-27 in vivo, retrovirus-expressing EB13-linker-p28 (IL-27) was packaged and proved to strongly induce IL-27 (Figure 6(D)). We injected retrovirus expressing IL-27 twice into lung before OVA challenge in chronic airway inflammation model. High IL-27 level was detected in BALF by ELISA and in lung by immunohistochemistry (Figures 6(E) and 6(F)). The infiltration of total cells and eosinophils in BALF was also significantly decreased in WT mice with retrovirus mediated IL-27 overexpression in lung (Figure 6(G)). In addition, PAS staining suggested retrovirus-mediated expression IL-27 in the lung resulted in decreased inflammatory response (Figure 6(H)). Consistently, cytokines IL-4, IL-5, and IL-13 in BALF were decreased by IL-27 overexpression in lung (Figure 6(I)). Taken together, these results suggested IL-27 overexpression in lung could efficiently inhibit the mast cell-mediated allergic airway inflammation.

4 | DISCUSSION

Initial studies indicated that IL-27 was involved in proinflammatory response in the Th1 responses. However, subsequent works using pathogen-induced and autoimmune inflammation models have indicated that IL-27 has immunosuppressive function by inhibition on Th1, Th2, and Th17 subsets of T cells as well as the expansion of inducible regulatory T cells.³⁸⁻⁴¹ In addition, IL-27 also acted on other types of immune cells. IL-27 has an impact on B cell differentiation and Ig production, but the effect varies depending on the mode of B cell activation. IL-27 could induce T-bet expression in naive and memory B cells stimulated by CD40 or surface Ig engagement, also increase proliferation of anti-Ig-activated naive B cells and of anti-CD40-lated naive

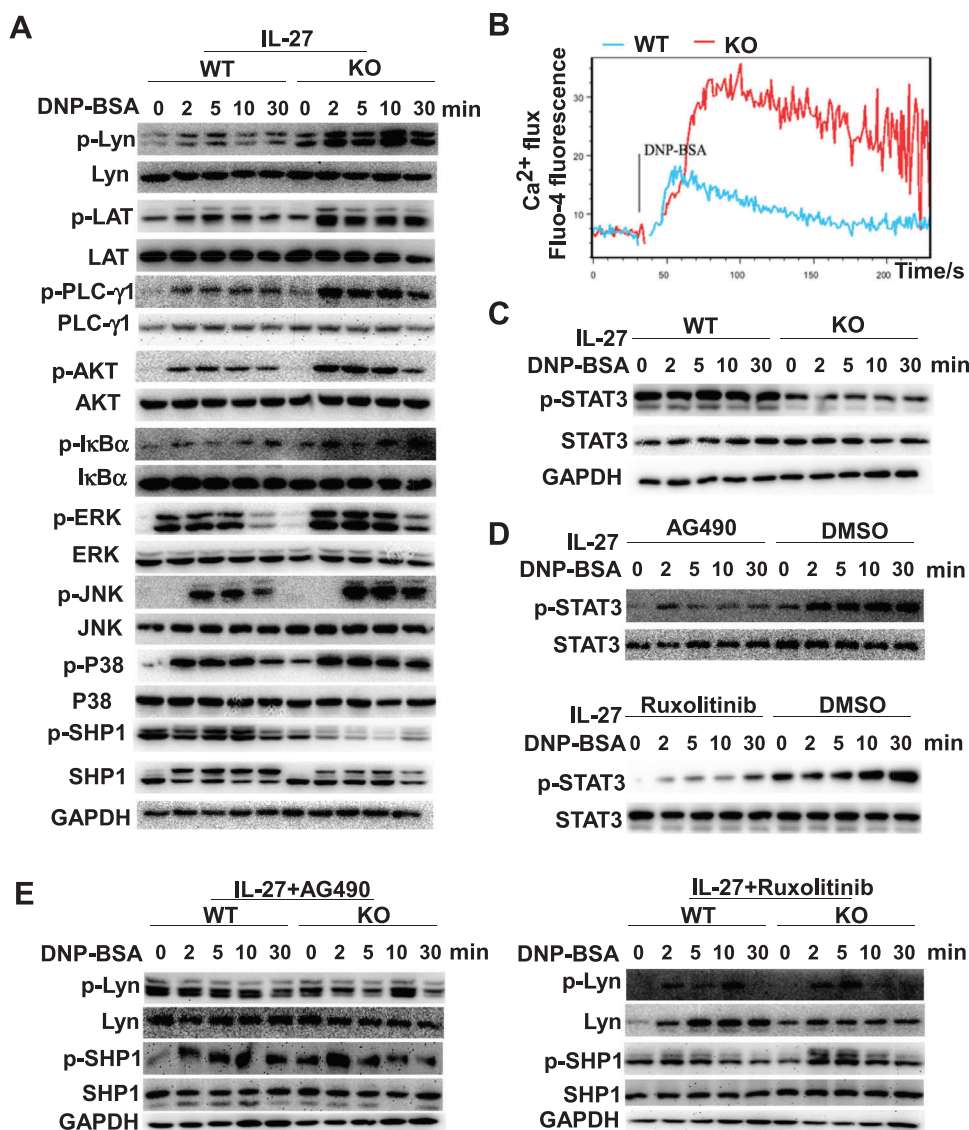


FIGURE 5 Altered signaling of IgE-Fc ϵ RI-mediated activation in WT and KO BMMCs. WT and KO BMMCs were sensitized with anti-DNP IgE overnight, incubated with IL-27 for 4 h before challenged with DNP-BSA. (A) Immunoblot analysis in lysates of the WT and KO BMMCs stimulated by IgE-Fc ϵ RI-mediated activation for the indicated times. (B) Calcium flux in anti-DNP-BSA IgE-sensitized WT and KO BMMCs after stimulation with DNP-BSA. (C) Total and phosphorylated STAT3 in WT and KO BMMCs stimulated with IL-27 for 3 h before DNP-BSA. (D) Total and phosphorylated STAT3 in activated WT BMMCs treated with AG490 or Ruxolitinib for 3 h. (E) Phosphorylated Lyn and SHP1 in WT and KO BMMCs treated with AG490 or Ruxolitinib for 3 h before DNP-BSA stimulation

and germinal center B cells.⁴² And IL-27 is reported to be involved in augmenting of NK cell activity in IFN γ -dependent and IFN γ -independent way.^{43–45} Effects of IL-27 on macrophages/monocytes and DCs are complicated too. IL-27 may enhance immune responses by inducing MHC class I/II expression, costimulatory molecules CD80 and CD86, which is essential for the antigen presenting activity of THP-1 cells.⁴⁶ While Mascanfroni et al.⁴⁷ indicated that IL-27 induces expression of CD39 on DCs and blocks the development of Th1 and Th17 cells to inhibit EAE induction. However, few studies about the role of IL-27 signaling in regulating mast cells were reported. We found that IL-27 receptors, WSX-1 and gp130, express on mast cells surface confirmed by flow cytometry, and their expression was up-regulated after Fc ϵ RI-dependent BMMC activation, suggesting that IL-

27 might be involved in mast cells-mediated immune response. Rapid degranulation and production of cytokines in late phase of Fc ϵ RI-dependent mast cell activation was regulated by different mechanisms. Here PSA, PCA, and chronic airway inflammation model were used to evaluate the role of IL-27 to regulate the degranulation and production of cytokines of activated mast cells. We found that mice lacking IL-27R α displayed increased sensitivity to IgE-mediated passive allergic response and chronic airway inflammation. Moreover, loss of WSX-1 in mouse BMMCs led the cells to be hyperresponsive to the stimulation via Fc ϵ RI, indicating that IL-27 signaling negatively regulates Fc ϵ RI-mediated BMMC rapid degranulation and release of cytokines IL-4, IL-5, IL-13, IL-6, TNF- α , and GM-CSF in late phase.

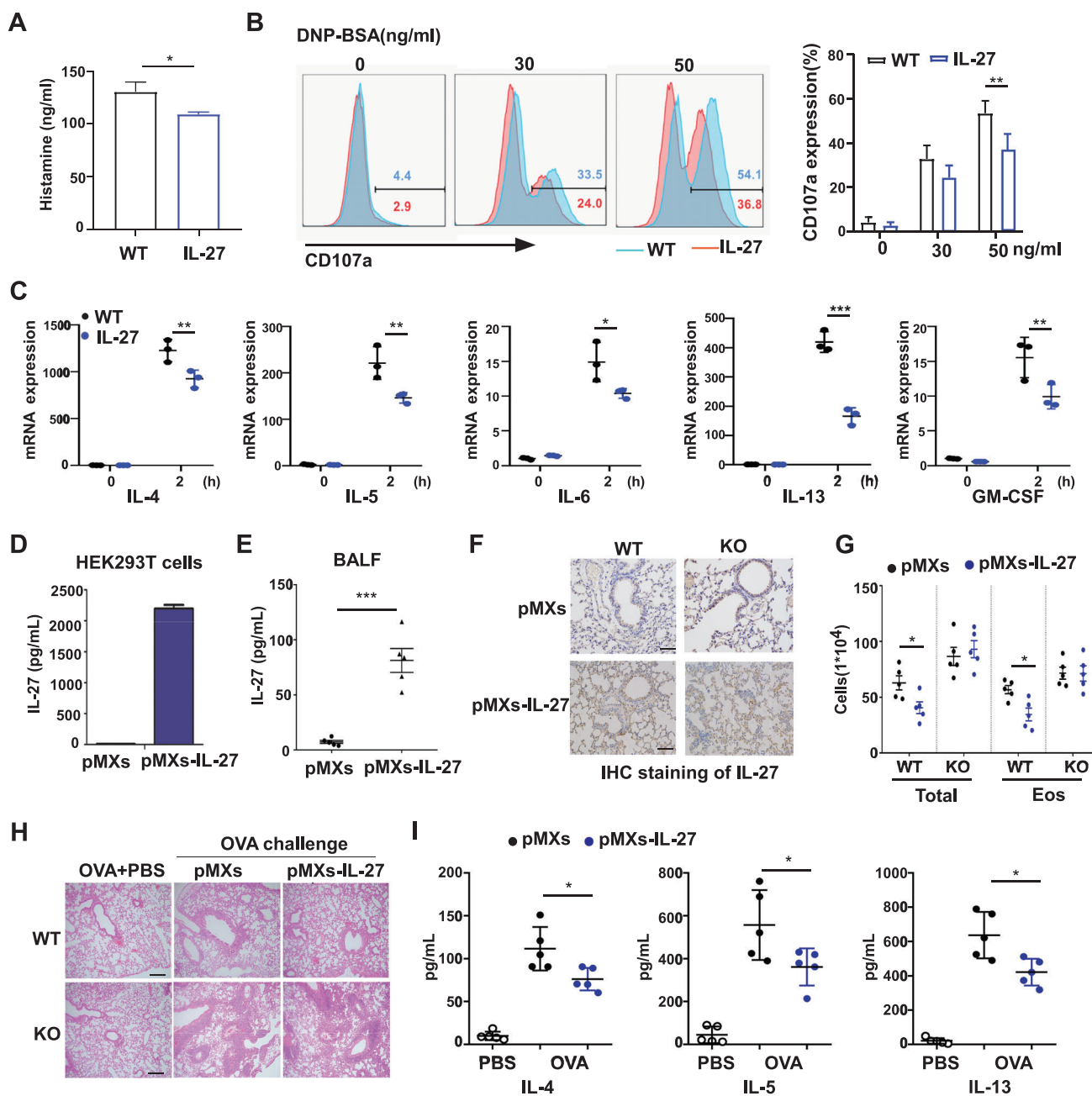


FIGURE 6 IL-27 attenuated mast cells activation in vitro and in vivo. (A–C) WT BMMCs were sensitized with anti-DNP IgE overnight, incubated with IL-27 for 8 h before challenged with DNP-BSA. (A) Secretion of histamine was measured in supernatant of cultured BMMCs stimulated with 10 ng/ml DNP-BSA for 0.5 h. (B) Degranulation was assessed by surface CD107a expression on BMMCs. (C) Real-time PCR analysis of mRNA expressions of various cytokines IL-4, IL-5, IL-6, IL-13, and GM-CSF in BMMCs stimulated with DNP-BSA for 2 h. (D–I) WT and KO mice were injected 6 times with OVA intraperitoneally, and IL-27-expressing retrovirus and control retrovirus were injected into trachea for 3 days before challenged initially, and second injection at the day of first challenge. (D) EB13-lingker-p28 (IL-27) expression in HEK293T cells. (E) IL-27 production in BALF from mice treated by retrovirus-based IL-27 overexpression in OVA-induced airway inflammation. (F) Immunohistochemistry of IL-27 expression in lung from WT and KO mice with retrovirus-mediated IL-27 overexpression. Scale bars, 50 μ m. (G) Cell counts in BALF from WT and KO mice with IL-27 overexpressed in lung. $n = 5$ per group. Eos, eosinophils. (H) Lung tissue sections were stained by PAS in mice with IL-27 overexpression. Scale bars, 50 μ m. (I) Protein levels of IL-4, IL-5, and IL-13 in BALF were detected by ELISA. Statistical significance was assessed by unpaired 2-tailed Student's *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are representative of at least 3 independent experiments. Data represent the mean and SD

Our results indicated that the dysregulated signaling in KO mast cells is associated with increased activation of "Grb2-PLC- γ 1-SLP-76" signaling and "PI3K/Akt/I κ B α " signaling. These results suggest that IL-27 acts as an inhibitory signal in mast cells activation. Up on Fc ϵ RI stimulation, Lyn, a SFK, was activated and phosphorylates tyrosine residues within the ITAMs in the cytoplasmic domains of the β and γ chains, which is critical for transducing activation signaling. Our results showed that Fc ϵ RI-proximal tyrosine kinases Lyn is activated strongly in KO BMMCs after stimulation. Meanwhile, SHP1, a negative regulator involved in mast cell activation was elevated in WT BMMCs compared with KO BMMCs. Previous study showed that in CML cells from blast crisis patients, Jak2 inhibition deactivates Lyn kinase through the SET-PP2A-SHP1 pathway,⁴⁸ which provided some clues that IL-27 may promote expression of phosphorylated SHP1 to regulate the activation of Lyn. SHP1 is mainly considered as a negative regulator of mast cell activation, however, it also has been reported that SHP1-deficient mast cells showed elevated cytokine production, but reduced calcium mobilization and degranulation, indicating that SHP1 may have different roles in controlling activation of mast cells under certain condition.^{49,50} IL-27 mainly utilizes JAK/STAT pathway to transduce downstream signals, and we found that WT BMMCs exhibited increased phosphorylated STAT3 compared with KO BMMCs after stimulation with recombinant IL-27. Moreover, 2 kinds of JAK2 inhibitor could suppress STAT3 and SHP1 levels in BMMCs, which was associated with decreased phosphorylation of Lyn in KO BMMCs. Collectively, our findings reveal that IL-27 might negatively regulate mast cells mediated immune response via JAK2/STAT3/SHP1/Lyn signaling pathway. One important question is whether IL-27 activated JAK2/STAT3 signaling inhibits Lyn activation directly or indirectly, and whether other molecules are involved in IL-27 signaling. These questions are still needed to be illuminated in further study.

IL-27 has the capability to induce IL-10 production in a wide range of cell types, including Th1, Th2, Th17, and Treg cells.⁵¹ IL-10 is a well-known anti-inflammatory cytokine secreted by various immune cells including mast cells. In addition, mast cells are also responsive to IL-10. Previous studies have showed IL-10 inhibits Fc ϵ RI-mediated TNF production in a Stat3-dependent manner in mast cells,⁵² but enhances IgE-mediated mast cell activation in a Stat3-dependent manner through the induction of miR-155.²⁸ Therefore, whether IL-27-IL-10 signaling axis participates in mast cell activation needs to be further investigated.

Cytokines are critical mediators in hypersensitivity and autoimmunity. Cytokine-based therapies that modify the inflammatory response and restore normal immune response hold the promise of finding a factual treatment. A variety of therapeutic strategies has been developed to down-regulate the inflammation and prevents autoimmune pathology. The main quandary to be clarified before the IL-27 signal as a promising target of immune therapy is the dissection of the pro- and anti-inflammatory signal pathways downstream of the IL-27R.⁵³ Extension or blocking of IL-27 signaling has been demonstrated widely in various disease models.⁵⁴ For instance, blockade of IL-27 signaling mitigates the severity of GVHD by augmenting Treg reconstitution.⁵⁵ Injection of IL-27 significantly suppressed eosinophilia and the produc-

tion of type 2 cytokines in the lungs in a *Alternaria alternata*-induced asthma model.⁵⁶ IL-27 p28 might be a capable therapeutic agent that limits inflammation and following disease by inhibition of gp130 signaling.

Since IL-27 has the inhibitive effect on mast cell activation, IgE-sensitized KO mast cells exhibited elevated response upon DNP-BSA stimulation. So we tried to evaluate the effect of IL-27 on chronic airway inflammation in mice. When retrovirus-based IL-27 was over-expressed in lung of WT mice, total and eosinophils cell infiltrations and inflammatory response were significantly decreased in BALF, suggesting that IL-27 might have potential application for the intervention of mast cell mediated inflammation and autoimmunity. Although the role of IL-27 in human immune system is very complicated, IL-27 or its signaling network still might act as an acceptable immunologic intervention target.

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AUTHORSHIP

Q. W., L. L., Y. Z., and X. W. designed the research. X. W., Y. Z., Q. T., M. Y., J. H., J. W., H. T., Y. S., P. X., Y. L., L. L., and X. C. performed the experiments and analyzed the data. X. W., L. L., and Q. W. wrote the paper. X. W., Y. Z., and H. T. contributed equally to this work.

DISCLOSURES

The authors declare no conflicts of interest.

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