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Activation of eosinophils by IL-12 family cytokine IL-27: Implications of the pleiotropic roles of IL-27 in allergic responses

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ABSTRACT

Interleukin (IL)-27 is a novel IL-12 family cytokine and its immunomodulatory effects on T-lymphocytes, B-lymphocytes and mast cells were extensively studied. IL-27 could suppress Th2-mediated allergic diseases in mouse models. However, the role of IL-27 on eosinophils, the principal effector cells in allergic diseases, remains unexplored. Our present study revealed that eosinophils constitutively express functional IL-27 receptor heterodimer, gp130 and WSX-1. IL-27 could prolong eosinophil survival by reducing apoptosis, modulate the expression of adhesion molecules to facilitate eosinophil adhesion and accumulation, and induce the release of proinflammatory cytokines IL-6, tumor necrosis factor- α , IL-1 β and chemokines CCL2, CXCL8 and CXCL1. The stimulation effects of IL-27 on eosinophils could not be abrogated by Th2 cytokine IL-25, hematopoietic cytokine granulocyte macrophage colony-stimulating factor, and toll-like receptor 4 ligand lipopolysaccharide (LPS). These findings are different from the effects of IL-27 and LPS on monocytes. Intracellular signaling mechanistic studies showed that IL-27-mediated eosinophil activation was differentially regulated by the activation of extracellular signal-regulated kinase, c-Jun N-terminal kinase and p38 mitogen-activated protein kinase, as well as nuclear factor- κ B. Based on the above results, IL-27 could play crucial roles in allergic diseases by the activation of eosinophils via differential intracellular signaling cascades. According to the present findings of its activating effects on human eosinophils, IL-27 may play pleiotropic roles in human allergic responses.

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Introduction

Eosinophils are a distinct subset of bone marrow generated granulocytes that circulate in peripheral blood, migrate into and ordinarily reside in different tissues (Hogan et al. 2008). As one of the most important innate immune cells, increased circulating number and infiltration of eosinophils at local tissues have been associated with allergic diseases such as allergic asthma and atopic dermatitis (Simon et al. 2004). The prominent detrimental effects of

eosinophils are involved in allergic inflammation by the release of toxic granule proteins, lipid mediators, cytokines and chemokines (Hogan et al. 2008).

Interleukin (IL)-27, a new member of the IL-12 family cytokines, is mainly produced by antigen-presenting cells (APC). IL-27 is composed of two subunits, IL-12 p35-related protein p28 and IL-12 p40-analogous protein EBV-induced gene 3 (EBI-3) (Pflanz et al. 2002). The IL-27 receptor is a heterodimer, consisting of orphan cytokine receptor WSX-1 (also called T cell cytokine receptor, TCCR) and gp130 subunit (Pflanz et al. 2004). It is simultaneously expressed on dendritic cells (DC), CD4⁺ and CD8⁺ T cells, natural killer (NK) cells, NKT cells, B cells, monocytes, macrophages, mast cells and keratinocytes. In parallel with the receptor expression profile, IL-27 acts directly on the above cells to induce the tyrosine phosphorylation of signal transducers and activators of transcription (STAT) family (Batten and Ghilardi 2007; Goriely et al. 2009; Hölscher et al. 2005; Kalliolias and Ivashkiv 2008; Kanda and Watanabe 2008; Kastelein et al. 2007; Larousserie et al. 2006; Owaki et al. 2006; Takeda et al. 2003).

Previous studies of IL-27 have revealed its proinflammatory effects by promoting early stage for the activation of naive CD4⁺ T cells and T helper (Th)1 differentiation (Owaki et al. 2005). IL-

Abbreviations: CBA, cytometric bead array; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte macrophage colony-stimulating factor; ICAM-1, intercellular adhesion molecule-1; JNK, C-Jun NH2-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MFI, mean fluorescence intensity; OVA, ovalbumin; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; PI, propidium iodide; STAT, signal transducers and activators of transcription; TLR, Toll-like receptor.

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27 receptor deficient (WSX-1^{-/-}) mice showed defects in Th1 response when infected with *Listeria monocytogenes* or *Leishmania major* (Chen et al. 2000; Yoshida et al. 2001). IL-27 could also induce proinflammatory cytokines from human monocytes and mast cells (Kalliolias and Ivashkiv 2008; Pflanz et al. 2004). Besides, IL-27 has a potential role for Th2 cytokine production. Upon stimulation by α -galactosylceramide, a synthetic glycolipid agonist of NKT cells, EBI3^{-/-} mice showed decrease in Th2 cytokine IL-4 production and were resistant to oxazolone-induced colitis which were primarily mediated by Th2 cytokines from inducible NKT cells (Nieuwenhuis et al. 2002). In addition, IL-27 enhanced IL-1 β -induced human β -defensin-2 production by keratinocytes and showed antitumor activity with antiangiogenic effect (Kanda and Watanabe 2008; Shimizu et al. 2006).

On the other hand, recent work has emphasized the immunosuppressive roles for IL-27, such as its inhibition of the development of Th1, Th2 and Th17 lymphocyte subsets (Kastelein et al. 2007). WSX-1^{-/-} mice produced high levels of IFN- γ when infected with *Toxoplasma gondii* or *Trypanosoma cruzi* and large amounts of proinflammatory cytokines in the concanavalin A-induced hepatitis model subsets (Hamano et al. 2003; Villarino et al. 2003; Yamanaka et al. 2004). In ovalbumin (OVA)-induced allergic asthma model, WSX-1^{-/-} mice demonstrated hyperproduction of various cytokines and exhibited progressive asthmatic symptoms. IL-27 was found to inhibit Th2 cell development as well as Th2 cytokines production from polarized Th2 cells by down-regulation of transcription factor GATA-3 but up-regulation of T-bet expression simultaneously, thereby suppressing allergic responses (Artis et al. 2004; Fujita et al. 2009; Miyazaki et al. 2005; Yoshimoto et al. 2007). In addition, IL-27 repressed the development of Th17 cells and induced anti-inflammatory cytokine IL-10 to suppress IL-17-mediated inflammation (Amadi-Obi et al. 2007; Awasthi et al. 2007; Batten et al. 2006; Fitzgerald et al. 2007; Ilarregui et al. 2009; Murugaiyan et al. 2009; Stumhofer et al. 2006, 2007; Yang et al. 2008). Moreover, IL-27 could inhibit the function of murine DCs, mast cells and macrophages (Artis et al. 2004; Yoshimoto et al. 2007). Therefore, the inhibitory effects of IL-27 on murine innate immune cells are in contrast to the activation effects of IL-27 for human studies (Kalliolias and Ivashkiv 2008; Pflanz et al. 2004).

Though IL-27 has been shown to exhibit multiple effects on various immune cells, little information is known about the effects of IL-27 on eosinophils. In addition, the other two IL-12 family cytokines IL-12 and IL-23 seemed to exert opposite effects on eosinophils (Cheung et al. 2008; Nutku et al. 2001). In an attempt to further elaborate the role of IL-12 family members in allergic responses, we investigated the biological effects of IL-27 on human peripheral blood eosinophils and the underlying mechanisms.

Materials and methods

Reagents

Recombinant human IL-27, IL-25, granulocyte macrophage colony-stimulating factor (GM-CSF), mouse anti-TCCR/WXS-1 and anti-gp130 mAb, and FITC-anti-L-selectin were purchased from R&D Systems (MN, USA). Rabbit anti-gp130 antibody was purchased from Millipore Corp., MA, USA. Fluorescein isothiocyanate (FITC)-anti-CD18 and mouse IgG1, phycoerythrin (PE)-anti-intercellular adhesion molecule (ICAM)-1 and mouse IgG1, mouse anti-phospho-extracellular signal-regulated kinase (ERK) (T202/Y204), anti-phospho-C-Jun NH2-terminal kinase (JNK) (pT183/pY185), anti-phospho-p38 mitogen-activated protein kinase (MAPK) (T180/Y182), anti-phospho-inhibitor (I)- κ B α , anti-Stat1 (Tyr 701), anti-human I κ B α , anti-p38 α , anti-ERK, anti-JNK and anti-Stat1 were purchased from BD Biosciences (CA, USA).

Rabbit anti-STAT3, STAT5, phospho-STAT3 (Tyr 705), phospho-STAT5 (Tyr 694) and GAPDH antibodies were purchased from Cell Signaling Technology (MA, USA). I κ B α phosphorylation inhibitor BAY11-7082, ERK inhibitor U0126, JNK inhibitor SP600125, and p38MAPK inhibitor SB203580 were purchased from Calbiochem (CA, USA). SB203580 was dissolved in water while other inhibitors were dissolved in DMSO. In all studies, the concentration of DMSO was 0.1% (v/v).

Isolation of human eosinophils and neutrophils

All standard protocols were performed as previously described (Cheung et al. 2008). Fresh human buffy coat obtained from healthy volunteers of Hong Kong Red Cross Blood Transfusion Service was diluted 1:2 with PBS and centrifuged using an isotonic Percoll solution. Peripheral blood mononuclear cells (PBMC) fraction was obtained from the thin middle layer and granulocytes-enriched fraction was precipitated in the bottom. Eosinophils were isolated by negative immunomagnetic selection using anti-CD16 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD16⁺ neutrophils were collected in the positive fraction. The purity of eosinophils was found to be at least 98% assessed by Hemacolor rapid blood smear stain (E Merck Diagnostica, Darmstadt, Germany).

Apoptosis assay

Eosinophils were cultured for 48 h with indicated treatment. Apoptosis was assessed by TACSTM Annexin V-FITC assay (Trevigen Inc., MD, USA) using flow cytometry (FACSCalibur, BD Biosciences). The population of viable cells was characterized by low mean fluorescence intensity (MFI) of both FITC-Annexin V and propidium iodide (PI).

Quantitation of IL-6, IL-1 β , TNF- α , IL-8, CXCL1 and CCL2

Concentrations of proinflammatory cytokine IL-6, IL-1 β , TNF- α and chemokine CXCL8 in culture supernatant were measured by human inflammation cytokine cytometric bead array (CBA) using flow cytometry (BD Biosciences). Concentrations of CXCL1 and CCL2 were determined with ELISA reagent from R&D Systems and BD Pharmingen, respectively.

Western blot analysis

Cells were washed, lysed, and equal amount of proteins was subjected to SDS-PAGE and then blotted onto PVDF membrane (GE Health Care Corp, NJ, USA). The membrane was blocked with 5% skimmed milk and probed with primary antibody at 4 °C overnight. After washing, the membrane was incubated with secondary antibody coupled to horseradish peroxidase (GE Healthcare) for 1 h at room temperature. Antibody-antigen complexes were then detected using an ECL chemiluminescent detection system (GE Healthcare).

RT-PCR analysis

Total RNA was extracted using TRIzol (Invitrogen, CA, USA) and cDNA was prepared using oligo (dT) primer. PCR was performed using Taq DNA polymerase (Applied Biosystems). The following primers were used: human WSX-1 forward, 5'-TGGACTTTTCCGAGGATGAC-3', and reverse, 5'-GGAGCAGCAGCAGGTAATTC-3', yielding a 451 bp product; human gp130 forward, 5'-TGCTGATTGCAAAGCAAAAC-3', and reverse, 5'-CCCACTTGCTTCTTCACTCC-3', yielding a 452 bp product; β -actin, forward, 5'-CTTCTTCTCTGGCATGGAG-3', and reverse,

5'-TGGAGGGGCGGACTCGTCA-3', yielding a 300 bp product. After the amplification using PTC 100 thermal cycler (MJ Research Inc), PCR products were electrophoresed on 2% agarose gel in TAE buffer and stained with ethidium bromide. The electrophoretic bands were documented with Gene Genius Gel Documentation System (Syngene Inc., Cambridge, UK).

Flow cytometric analysis of cell surface molecules

The immunofluorescence staining was performed as previously described (Cheung et al. 2008). Eosinophils were washed and blocked with human pooled serum and incubated with either fluorescence dye-conjugated primary mouse anti-human IgG antibody against L-selectin, CD18 or ICAM-1. Unconjugated mouse anti-human gp130 followed by secondary FITC-conjugated anti-mouse

IgG antibodies were used for the staining of IL-27 receptor. FITC-or PE-mouse IgG isotypic antibody (BD Pharmingen Corp., CA, USA) was used as isotypic control. Expression of gp130 and adhesion molecules on 10,000 viable gated cells was analysed by FACSCalibur flow cytometer using CellQuestPro software (BD Biosciences).

Adhesion assay

The adhesion assay was performed as previously described with slight modification (Cheung et al. 2006). Eosinophils (1×10^6 cells/ml) with different treatments were cultured in 24-well plates coated with fibronectin (BD Bioscience) and incubated at 37 °C, in a 5% CO₂, humidified atmosphere for 16 h. After incubation, non-adherent cells were removed and the plates were washed three times with 37 °C PBS. Eosinophil adherence in each well was

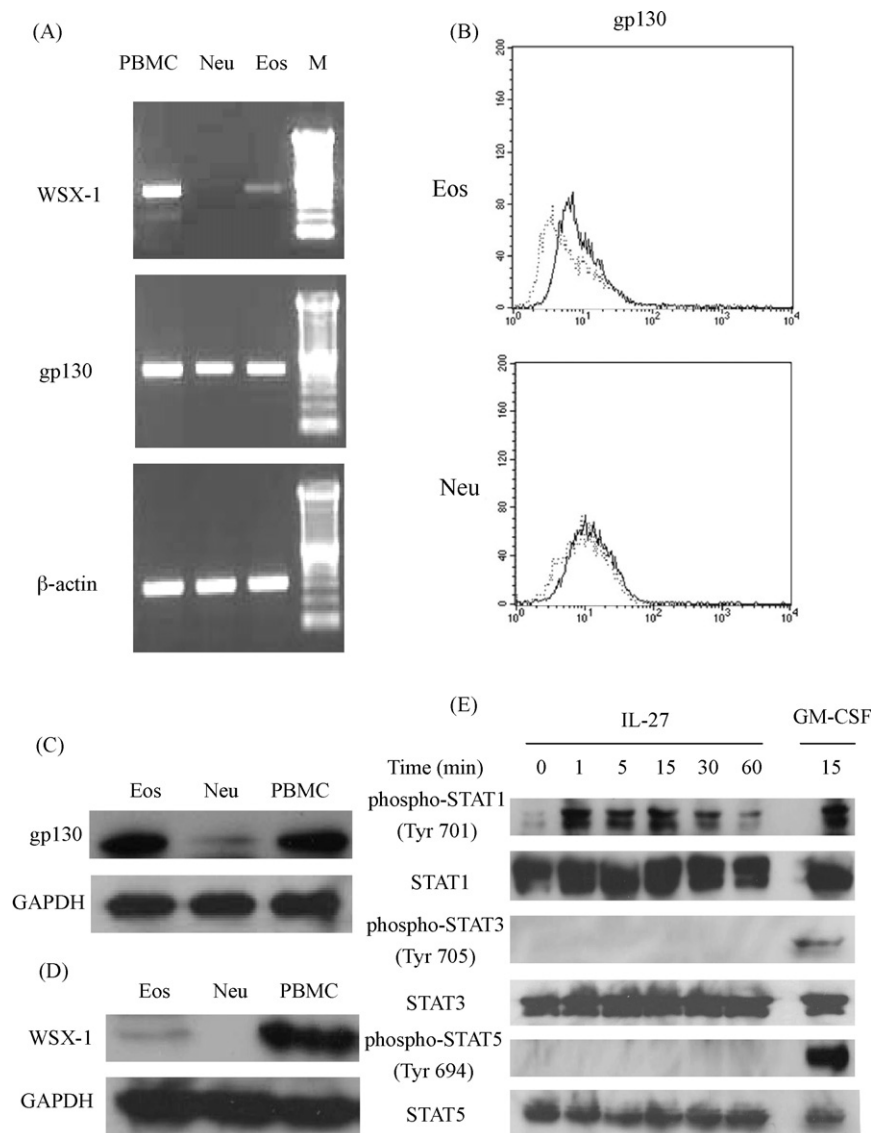


Fig. 1. Expression of functional IL-27 heterodimeric receptor on eosinophils. (A) Total RNA was extracted from eosinophils, neutrophils and PBMC, followed by RT-PCR analysis for WSX-1, gp130 and β-actin (house keeping gene) expression. (B) Representative histograms of cell surface expression of gp130 on eosinophils and neutrophils determined by flow cytometry. Dotted line: isotypic control; solid line: anti-human gp130. (C) Representative Western blot analysis of gp130 protein expression of human eosinophils, neutrophils and PBMC. GAPDH was used as protein control to ensure an equal amount of loaded protein. (D) Representative Western blot analysis of WSX-1 protein expression of human eosinophils, neutrophils and PBMC. GAPDH was used as protein control to ensure an equal amount of loaded protein. (E) Eosinophils were stimulated with IL-27 (50 ng/ml) or GM-CSF (10 ng/ml) for indicated incubation time. Total cellular proteins were extracted and STAT1, STAT3 and STAT5 tyrosine phosphorylation were detected by Western blot. All the experiments were performed in three independent replicates with essentially identical results. M: 100 base-pair molecular size marker; Eos: eosinophils; Neu: neutrophils; PBMC: peripheral blood mononuclear cells.

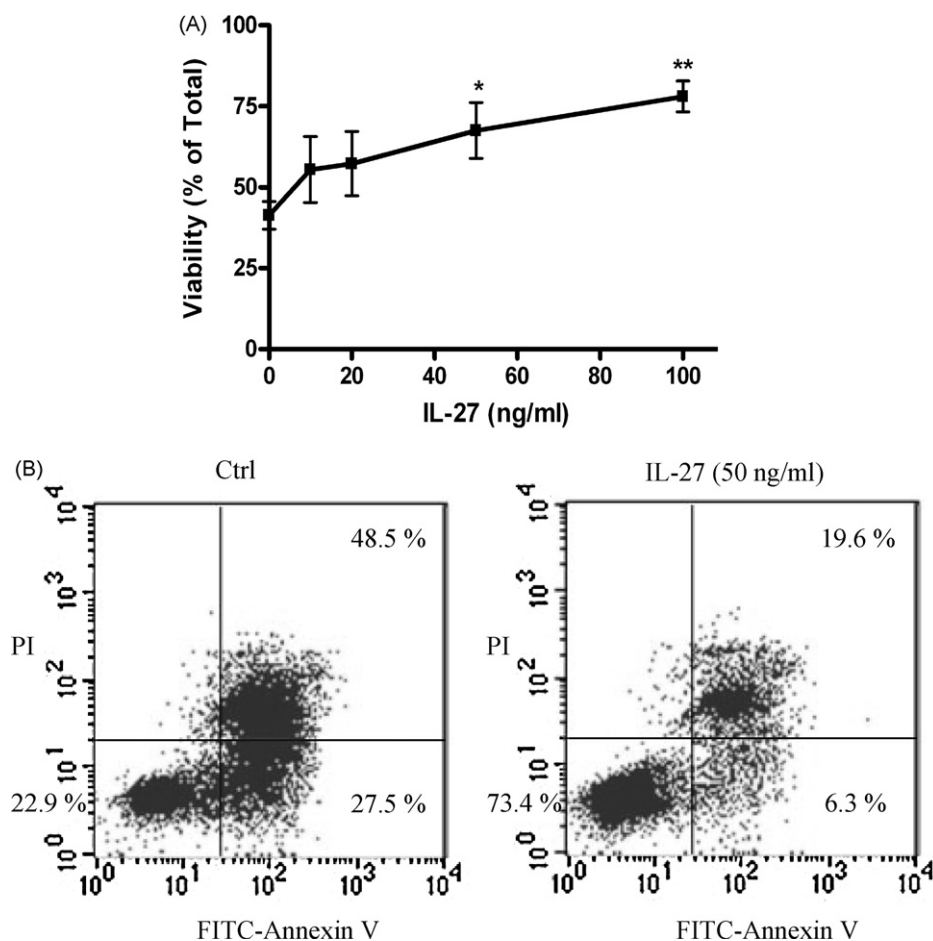


Fig. 2. Effect of IL-27 on the survival of eosinophils. Eosinophils were incubated with IL-27 (0–100 ng/ml) for 48 h and then were analysed by flow cytometry using annexin V-FITC and PI staining. (A) Results of % viability are expressed as the arithmetic mean $\pm$ SD from triplicate experiments. (B) Representative dot plots showed early apoptotic cells in the lower right quadrant (annexin V-FITC-positive), late apoptotic (necrotic) cells in the upper right quadrant (annexin V-FITC-positive and PI-positive), and viable cells in the lower left quadrant (double negative). * $p < 0.05$ and ** $p < 0.01$ when compared with medium control. Ctrl: control.

assessed by counting the number of eosinophils adhered onto the fibronectin-coated well in four high-power fields (magnification: 100 $\times$) per well under inverted microscope.

Electrophoretic mobility shift assay (EMSA)

EMSA were used to assess the activation of NF- κ B in eosinophils upon IL-27 stimulation. Nuclear extracts were prepared using Nuclear Extraction Kit (Panomics Inc., Fremont, CA). Equal amounts of nuclear protein extracts were subjected to a test for NF- κ B protein/DNA binding using NF- κ B EMSA kit (Panomics) according to the manufacturer's instruction. The consensus NF- κ B oligonucleotides provided in the kit was 5'-AGTTGAGGGGACTTCCAGGC-3'.

Statistical analysis

The statistical significance of differences was determined by one-way ANOVA or Student's *t*-test. The values were expressed as means $\pm$ SD from three independent experiments. Any difference with *p* values less than 0.05 was considered significant. When ANOVA indicated a significant difference, Bonferroni post hoc test was then used to assess the difference between groups. All analyses were performed using Graphpad PRISM software (version 5.01), GraphPad Software, Inc., CA, USA.

Results

Human eosinophils express functional IL-27 receptor

We first examined the expression of IL-27 receptor complex, WSX-1 and gp130, on freshly isolated human peripheral blood eosinophils, while neutrophils and PBMC were used as cell control. RT-PCR analysis showed that gp130 mRNA was highly expressed on eosinophils, neutrophils and PBMC (Fig. 1A). For the subunit WSX-1, though PBMC showed high expression, expression level was low for eosinophils and undetectable for neutrophils (Fig. 1A). In consistent with mRNA level, flow cytometric analysis showed that gp130 constitutively expressed on the surface of eosinophils (Fig. 1B). Western blot analysis also confirmed that eosinophils expressed higher protein level of gp130 compared to neutrophils which showed relatively low level of protein expression. PBMC was used as positive control (Fig. 1C). Because of the lack of commercial available anti-human WSX-1 antibody for flow cytometry, we confirmed the expression of WSX-1 on eosinophils and PBMC but not on neutrophils using Western blot (Fig. 1D).

IL-27 stimulation could lead to receptor-mediated tyrosine phosphorylation of STAT family (Kastelein et al. 2007). To verify whether IL-27 receptor expressed on eosinophils was functional, activation of STATs upon IL-27 stimulation was examined. Incubation with IL-27 resulted in potent STAT1 tyrosine phosphorylation within 1 min and maintained for over 30 min in eosinophils (Fig. 1E). Hematopoietic cytokine GM-CSF stimulation was used as

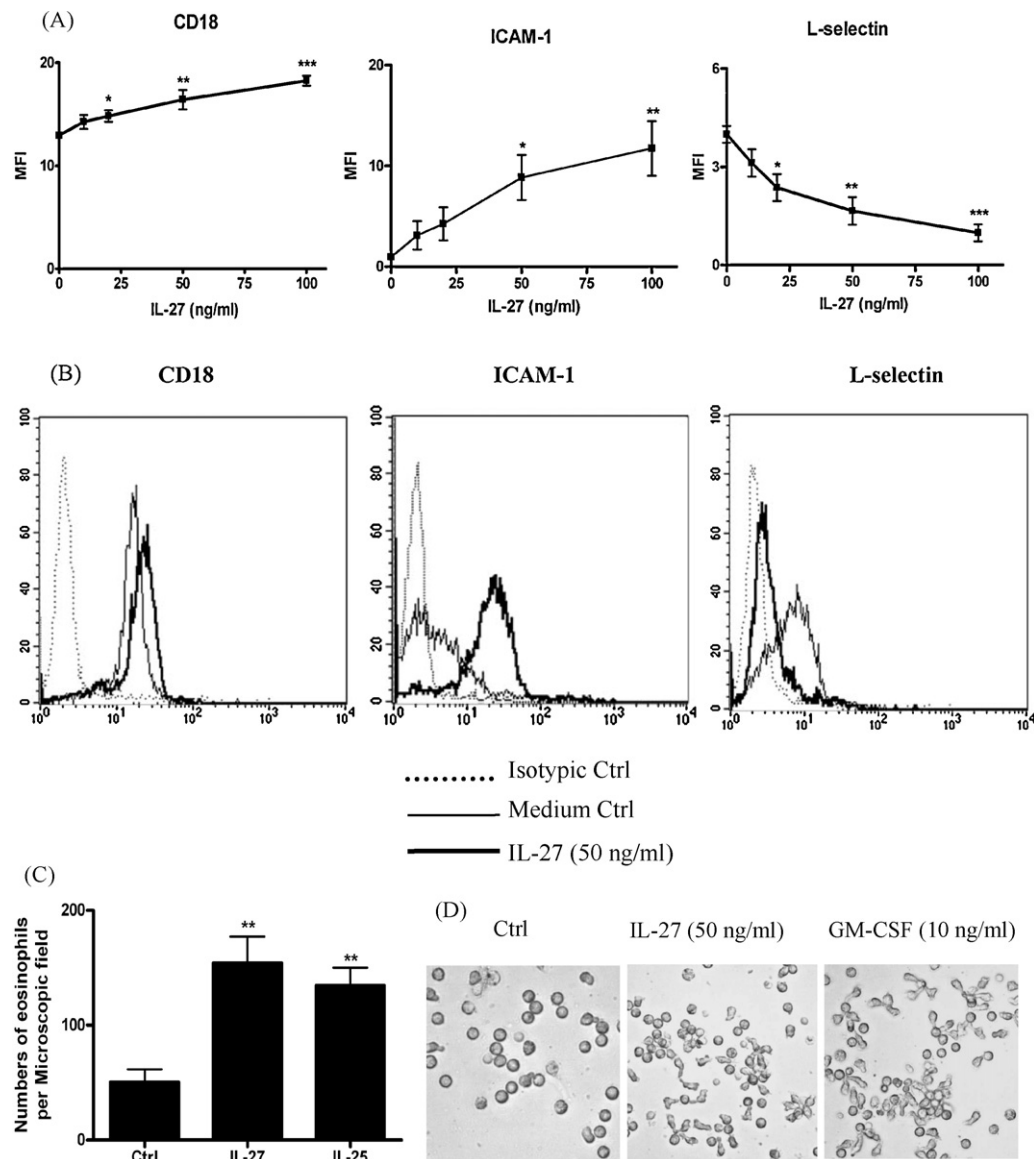


Fig. 3. Effect of IL-27 on the cell surface expression of adhesion molecules. Eosinophils were cultured with IL-27 (0–100 ng/ml) for 16 h and then the surface expression of adhesion molecules was analysed by flow cytometry. (A) The modulation of surface expression of adhesion molecules are shown as MFI. Results have been normalized by subtracting appropriate isotypic control and are expressed as the arithmetic mean $\pm$ SD of three independent experiments. (B) Representative histograms of cell surface expression of CD18, ICAM-1 and L-selectin on eosinophils are shown. Dotted line: isotypic control; grey line: medium control; black line: IL-27 treatment. (C) Eosinophils were stimulated with IL-27 (50 ng/ml) and IL-25 (50 ng/ml) for 16 h in fibronectin-coated wells. Results are expressed as the arithmetic mean of the number of cells adhered onto fibronectin-coated wells in four random 100 $\times$ field plus SD of quadruplicate experiments. (D) Eosinophils were incubated with medium, IL-27 (50 ng/ml) or GM-CSF (10 ng/ml) for 16 h. Photomicrographs show the morphology of eosinophils with 400 $\times$ magnification. * p < 0.05, ** p < 0.01 and *** p < 0.001 when compared with medium control.

positive control. However, the phosphorylation of STAT3 and STAT5 was not detected, up to 1 h incubation with IL-27 (Fig. 1E).

IL-27 can enhance eosinophil survival

Eosinophils have a very short life-span and delayed eosinophil apoptosis is a key mechanism for eosinophilia (Simon et al. 2004). Less than 40% of untreated eosinophils remained viable after 48 h incubation (Fig. 2). After incubation with IL-27, the percentage of viable eosinophils significantly enhanced and the effect was concentration-dependent (about 75% of viable eosinophils at 50 ng/ml IL-27, all p < 0.05), while Annexin V positive population was significantly reduced, which indicated that IL-27 maintained eosinophil survival through the suppression of apoptosis (Fig. 2).

IL-27 can modulate the cell surface expression of adhesion molecules on human eosinophils

Eosinophil migration and recruitment into specific tissues is finely regulated by the interaction between adhesion molecules and their counter ligands (Hogan et al. 2008). Eosinophils maintain basal level expression of ICAM-1 and L-selectin, and moderate expression of CD18 (Cheung et al. 2006, 2008; Wong et al. 2007). As shown in Fig. 3A and B, IL-27 significantly up-regulated the surface expression of CD18 and ICAM-1, but down-regulated the expression of L-selectin in a dose-dependent manner at 16 h incubation time. In addition, IL-27 could significantly increase the number of eosinophils adhered to fibronectin-coated wells, with IL-25 treatment serving as the positive control (Fig. 3C) (Cheung et al. 2006). Morphological analysis showed that untreated eosinophils maintained the round shape, while IL-27-treated eosinophils processed

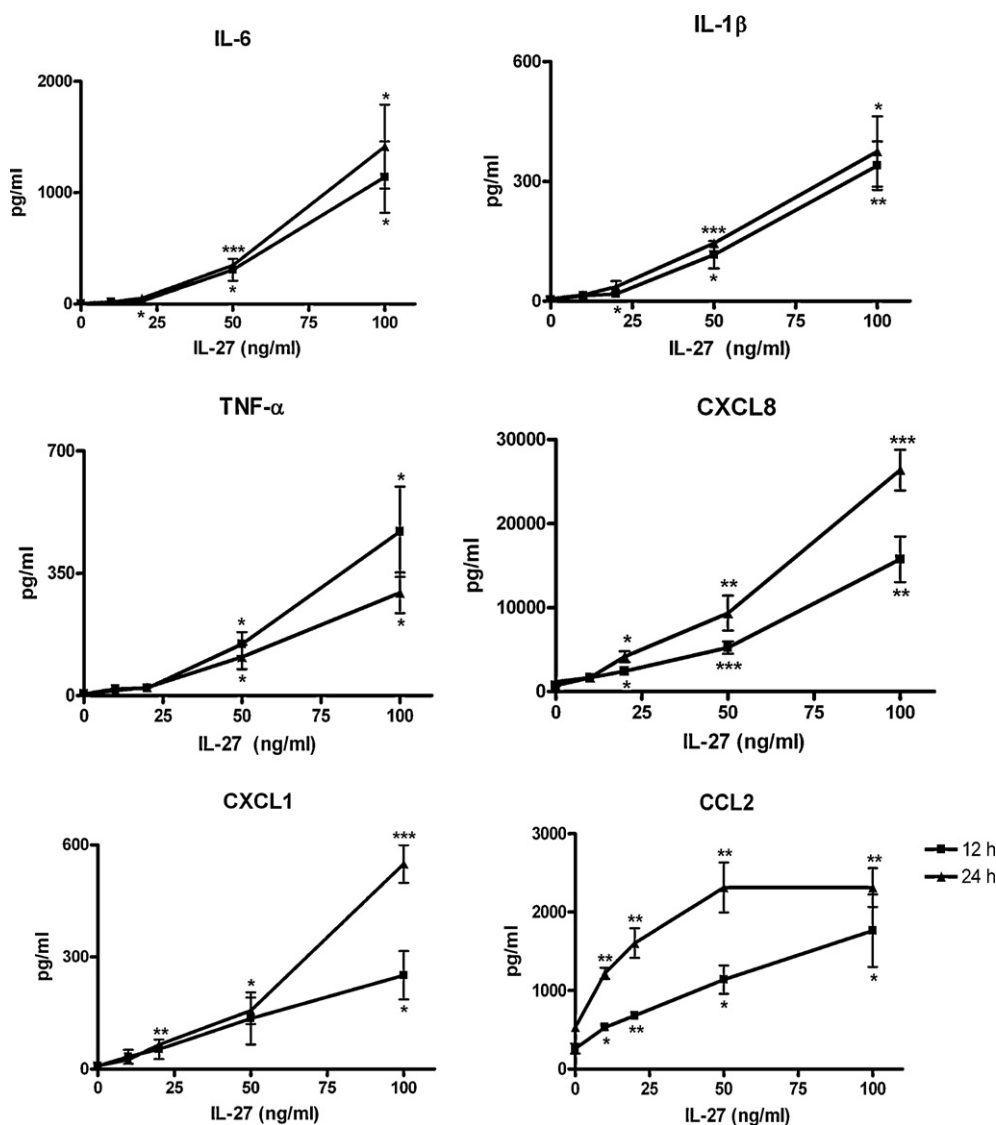


Fig. 4. Effects of IL-27 on cytokine and chemokine release from eosinophils. Eosinophils were cultured with IL-27 (0–100 ng/ml) for 12 or 24 h and the release of IL-6, TNF- α , IL-1 β and CXCL8 in culture supernatant were determined by CBA while CXCL1 and CCL2 were measured by ELISA. Results are expressed as the arithmetic mean $\pm$ SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared with medium control.

elongated shape and aggregated together which is similar to that of GM-CSF-treated eosinophils (Fig. 3D) (Takashi et al. 2001).

IL-27 can potently induce the release of cytokines and chemokines from human eosinophils

Eosinophils produce a variety of cytokines and chemokines aggravating immune responses (Hogan et al. 2008). IL-27 treatment significantly induced the release of inflammatory cytokines IL-6, TNF- α , IL-1 β and chemokines CCL2, CXCL8 and CXCL1 at 12 and 24 h dose-dependently. The release of chemokines CCL2, CXCL8 and CXCL1 at 12 h was much lower than that at 24 h (Fig. 4). However, the induction of inflammatory cytokines IL-6 and IL-1 β at 12 h and 24 h were similar and the amount of early response cytokine TNF- α was higher at 12 h than that at 24 h (Fig. 4).

Since IL-27 (50 ng/ml) significantly enhanced eosinophil survival, modulated adhesion molecules and induced cytokine and chemokine release, the optimal concentration (50 ng/ml) was used in the following mechanistic study.

IL-27 activates intracellular MAPKs and NF- κ B signaling pathway

MAPKs and NF- κ B signaling pathways play important roles in eosinophil activation (Cheung et al. 2006, 2008; Wong et al. 2007). Upon IL-27 stimulation, all three MAPK members, ERK, JNK and p38MAPK were phosphorylated in eosinophils (Fig. 5A). The rapid activation of the transcription factor NF- κ B is due to the rapid degradation of I κ B α . In resting state, NF- κ B is present in the cytosol interacting with inhibitory I κ B α proteins. Upon activation, I κ B α was phosphorylated and degraded by proteasome-mediated proteolysis, resulting in the release and nuclear translocation of active NF- κ B (Baldwin 1996). We therefore examined I κ B α phosphorylation as the indicator for NF- κ B activation. Similar to MAPK activation, NF- κ B was also activated as demonstrated by I κ B α phosphorylation (Fig. 5A). The kinetic studies showed that all the signaling activation appeared within 1 min, peaked at 5 or 15 min, maintained the activation status for 30 min and then down-regulated to the basal level within 60 min. However, PI3K/Akt activation in IL-27-treated eosinophils could not be detected (data not shown).

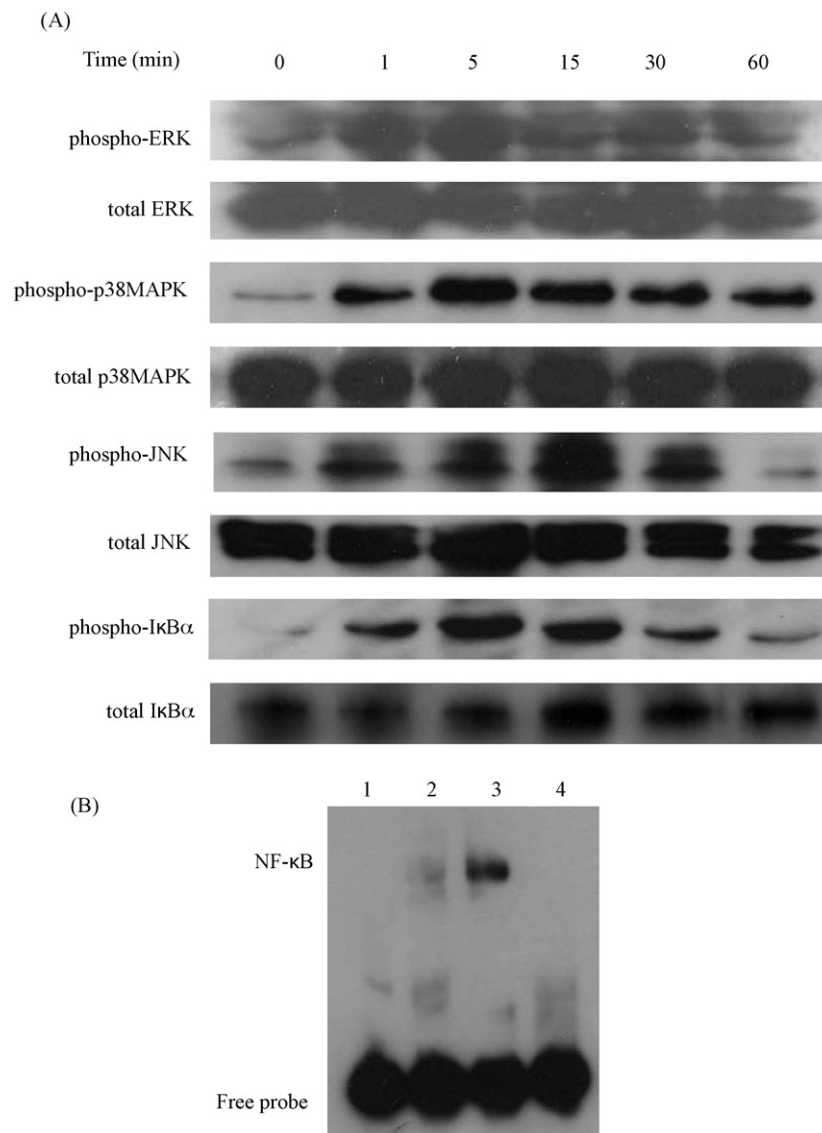


Fig. 5. Effects of IL-27 on intracellular ERK, JNK, p38MAPK and NF-κB activation. (A) Eosinophils were stimulated with IL-27 (50 ng/ml) for indicated incubation time. Total cellular proteins were extracted. Total and phospho-ERK, total and phospho-JNK, total and phospho-p38MAPK and total and phospho-IκBα were assessed by Western blot. Total protein was used to ensure an equal amount of loaded protein. (B) Eosinophils were stimulated with IL-27 (50 ng/ml) for 2 h. Nuclear proteins were extracted and then subjected to EMSA. Lane 1: labeled probe only without sample; Lane 2: labeled probe with untreated eosinophil nuclear proteins; Lane 3: labeled probe with IL-27-treated eosinophil nuclear proteins; Lane 4: IL-27-treated eosinophil nuclear proteins with excessive cold and labeled probes. Experiments were performed in three independent replicates with essentially identical results and representative results are shown.

To further confirm the nuclear translocation of active NF-κB by IL-27 stimulation, EMSA was performed with nuclear extracts from untreated and IL-27-treated eosinophils. Untreated eosinophils showed a very faint shifted band (Fig. 5B, Lane 2), while eosinophils treated with IL-27 (50 ng/ml) for 2 h showed a significant increase in band shift (Fig. 5B, Lane 3), indicating that IL-27 could potentially induce the activation of NF-κB-DNA binding activity. Competitive control using excessive unlabeled NF-κB binding DNA totally suppressed the IL-27-induced band shift, which confirmed the specificity of NF-κB-DNA interaction (Fig. 5B, Lane 4). Collectively, the above results demonstrated that IL-27 could stimulate NF-κB and MAPK activation in eosinophils.

Roles of MAPK and NF-κB pathways in IL-27-mediated eosinophil activation

To further address the relation between intracellular signaling pathways and IL-27-induced eosinophil activation, eosinophils

were pre-treated with specific signaling molecule inhibitors and then stimulated with IL-27. The optimal concentration of these inhibitors was tested and used as previously reported with the highest inhibition without any cytotoxicity (Cheung et al. 2008). IκBα kinase inhibitor BAY11-7082 and p38MAPK inhibitor SB203580 could significantly suppress IL-27-enhanced eosinophil survival (Fig. 6A). Pretreatment of eosinophils with ERK inhibitor U0126, p38MAPK inhibitor SB203580 and JNK inhibitor SP600125 could significantly suppress IL-27-induced up-regulation of CD18 and ICAM-1 but restore IL-27-induced down-regulation of L-selectin (Fig. 6B). In addition, IκBα kinase inhibitor BAY11-7082 could also reverse IL-27-induced up-regulation of CD18 and ICAM-1 but not for down-regulation of L-selectin (Fig. 6B). For cytokine and chemokine release, IL-27-induced release of IL-6, TNF-α and CXCL8 was partially abolished by inhibitor BAY11-7082, U0126 and SB203580, but not by SP600125. The release of IL-1β and CXCL1 was partially suppressed by inhibitor BAY11-7082 and SB203580, while CCL2 was only inhibited by BAY11-7082 and U0126 (Fig. 6C). Col-

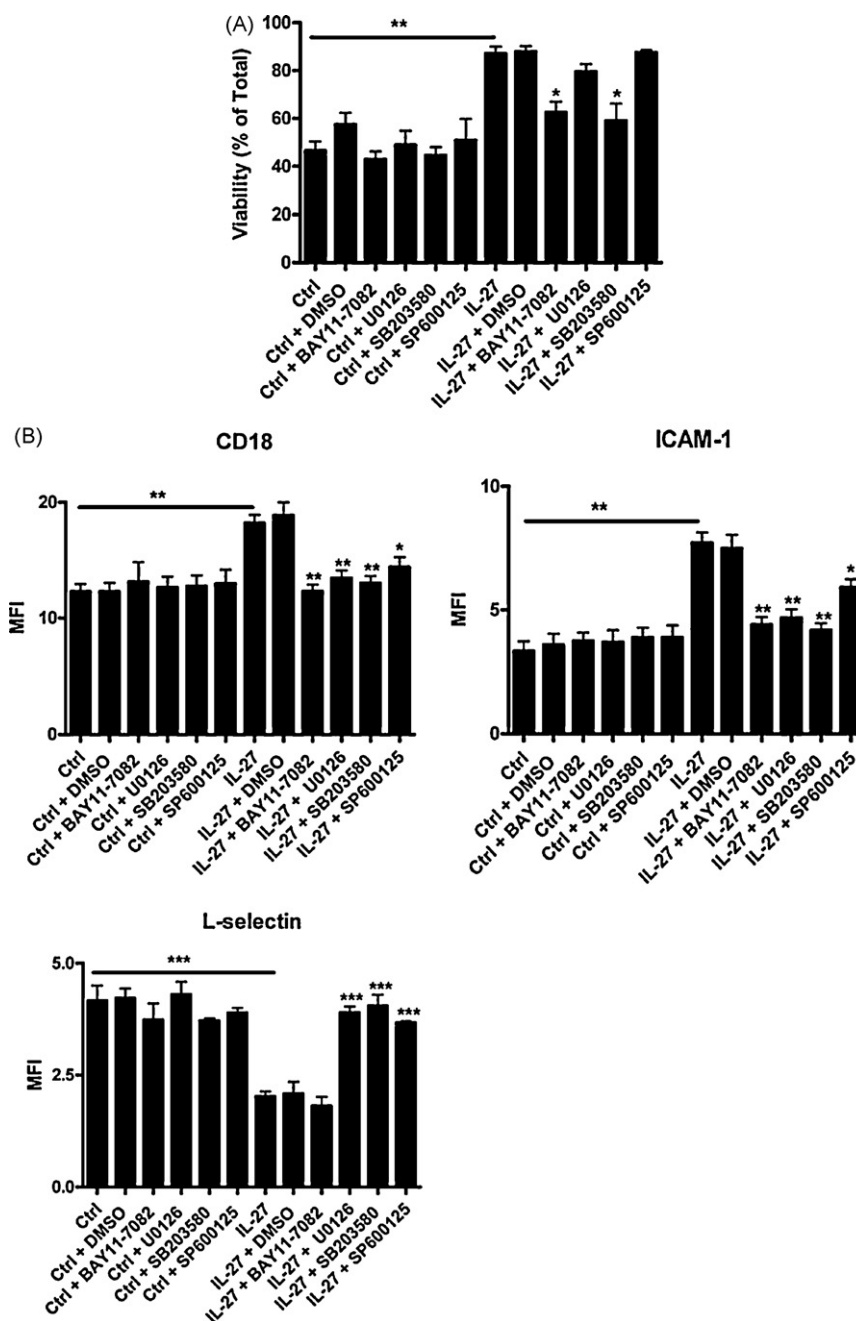


Fig. 6. Effects of signaling molecule inhibitors on viability, adhesion molecule expression, cytokine and chemokine release from eosinophils. Eosinophils were pre-treated with BAY11-7082 (1 μ M), U0126 (2.5 μ M), SB203580 (2.5 μ M) and SP600125 (2 μ M) for 1 h, followed by incubation with or without IL-27 (50 ng/ml) in the presence of inhibitors for further (A) 48 h for viability assay, (B) 16 h for adhesion molecule expression and (C) 24 h for cytokine and chemokine release. Results are expressed as the arithmetic mean plus SD from three independent experiments. DMSO (0.1%) was used as the vehicle control. * p < 0.05, ** p < 0.01 and *** p < 0.001 when compared between groups denoted by horizontal lines or groups of IL-27 with or without inhibitor treatment.

lectively, the results demonstrated that NF- κ B and MAPK pathways were differentially involved in IL-27-mediated diverse activation on eosinophils.

GM-CSF, IL-25 and lipopolysaccharide (LPS) could not abolish IL-27-mediated eosinophil activation, and vice versa

IL-27 or toll-like receptor (TLR) ligands alone could potentially activate human monocytes, however, the inflammatory functions of IL-27 for monocytes could be abrogated by TLR4 ligand LPS (Kalliolias and Ivashkiv 2008). Similar to monocytes, IL-27, GM-CSF, IL-25 or LPS alone could potentially activate eosinophils by increasing the viability and release of CXCL8 (Fig. 7). However, dif-

ferent from monocytes, all of these stimulations (GM-CSF, IL-25 and LPS) could not abrogate IL-27-mediated eosinophil activation, as demonstrated by the unaltered levels of eosinophil survival and even exhibit additive effect for the secretion of chemokine CXCL8. In addition, IL-27 pretreatment also could not affect GM-CSF, IL-25 or LPS-enhanced eosinophil survival and secretion of CXCL8 (data not shown).

Discussion

IL-12 family member IL-23 can enhance survival and activation of eosinophils in Th17 lymphocyte-mediated allergic inflammation, while another member IL-12 can induce eosinophil apoptosis

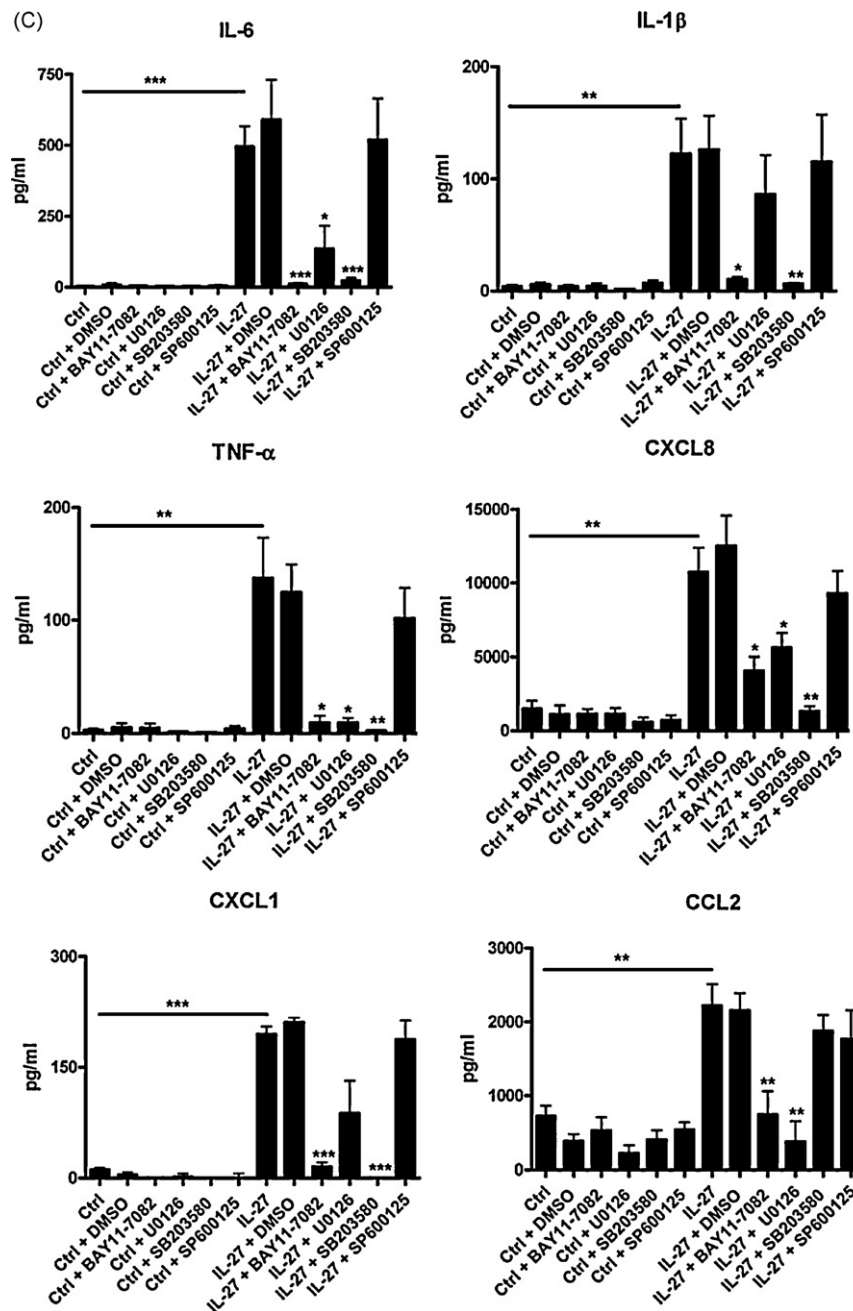


Fig. 6. (Continued)

and decrease subsequent tissue eosinophilia (Cheung et al. 2008; Nutku et al. 2001). Although IL-27, the new member of IL-12 family, could play both pro- and anti-inflammatory function (Kastelein et al. 2007), the present study demonstrated the *in vitro* stimulation effects of IL-27 on human eosinophils. Human eosinophils constitutively expressed functional IL-27 receptor complex WSX-1 and gp130, and STAT1 but not STAT3 was phosphorylated upon IL-27 stimulation. IL-27-mediated STAT1 phosphorylation is mainly required for cell activation (Kastelein et al. 2007). For example, IL-27 could activate STAT1 to induce IL-12Rβ2 expression in naïve CD4⁺ T cells and contribute to Th1 differentiation, whereas STAT1 activation was decreased in IL-27-activated CD4⁺ T cells (Batten and Ghilardi 2007; Takeda et al. 2003). IL-27-induced STAT1 activation was associated with proinflammatory effects in human monocytes and keratinocytes (Kalliolias and Ivashkiv 2008; Kanda and Watanabe 2008). To further explore the effects of IL-27 on

eosinophils, we performed functional and underlying mechanistic studies.

IL-27 induced proliferation of human naïve CD4⁺ T cell and activated naïve B cells (Kastelein et al. 2007; Larousse et al. 2006). However, as the terminally differentiated cells, eosinophils would not proliferate but undergo apoptosis instead. IL-27 indeed enhanced eosinophil survival through reducing apoptosis. Belonging to IL-12 family cytokines, IL-23 and IL-27 enhanced eosinophil survival. On the other hand, IL-12 increased *in vitro* human eosinophil apoptosis (Cheung et al. 2008; Nutku et al. 2001). This discrepancy of the effects of IL-12 family members was also observed in T cell differentiation (Kastelein et al. 2007). IL-12 drives Th1 differentiation but IL-23 is mainly responsible for Th17 differentiation, while IL-27 involves in early steps of Th1 differentiation and the subsequent suppression of Th1, Th2 and Th17 differentiation (Kastelein et al. 2007). The reasons for the diverse effects

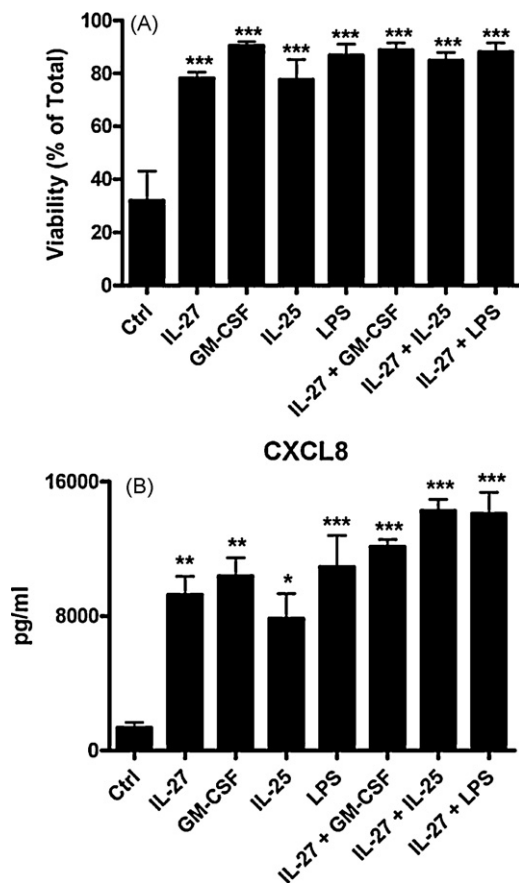


Fig. 7. Effects of GM-CSF, IL-25 and LPS on IL-27-mediated activation of eosinophils. Eosinophils were cultured in the presence or absence of GM-CSF (10 ng/ml), IL-25 (50 ng/ml) or LPS (100 μ g/ml) for 30 min and then stimulated with IL-27 (50 ng/ml) for (A) 48 h for viability assay and (B) 24 h for chemokine CXCL8 release. Results are expressed as the arithmetic mean plus SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared with medium control.

of IL-12 family members may be due to the different intracellular signaling mechanisms which require further investigation.

In the present study, IL-27-induced expression profile of adhesion molecules is similar to that of other eosinophil stimulator such as Th2 cytokine IL-25 and adipokine leptin (Cheung et al. 2006; Wong et al. 2007). The modulated expression profile of adhesion molecules can facilitate eosinophil adhesion and accumulation (Cheung et al. 2006). Actually, IL-27 has been shown to up-regulate ICAM-1 expression on CD4⁺ T cells and anti-Ig-stimulated B cells (Kastelein et al. 2007; Larousserie et al. 2006). The induction of ICAM-1 expression indicated that ICAM-1 might be a target gene regulated by IL-27 and IL-27-mediated STAT1 activation was demonstrated to be essential for the up-regulation of ICAM-1 expression (Owaki et al. 2005). In addition, it was found that ICAM-1 promoter contained a large number of binding sites for inducible transcription factors, including NF- κ B and STAT1 which were also activated by IL-27 in eosinophils (Owaki et al. 2005; Roebuck and Finnegan 1999). As a ligand of β 2-integrin, ICAM-1 might facilitate the adhesion of leukocytes because microscopic examination found that IL-27-activated eosinophils aggregated together, which was similar to that of GM-CSF stimulation (Takashi et al. 2001).

The activation effects of IL-27 on eosinophils were further demonstrated by the induction of inflammatory cytokines and chemokines. Similar to human mast cells and monocytes, early response proinflammatory cytokines TNF- α , IL-1 β and IL-6 were promptly induced by IL-27 and reached plateau within 12 h. Chemokines CCL2, CXCL8 and CXCL1 for chemotactic migration

of macrophages and neutrophils were also induced in a dose- and time-dependent manner. These chemokines have proinflammatory effects such as the up-regulation of adhesion systems, regulation of vascular permeability, mucus secretion and smooth muscle constriction (Hogan et al. 2008). Eosinophils can release IL-10 upon IL-12 stimulation and IL-27 has been shown to induce IL-10 from CD4⁺ T cells, however, the induction of IL-10 by IL-27 from eosinophils could not be detected by cytometric bead assay (CBA) using flow cytometry (detection limit: 3.3 pg/ml), which further confirmed the activation but not suppressive effects of IL-27 for eosinophils (Awasthi et al. 2007; Fitzgerald et al. 2007; Ilarregui et al. 2009; Murugaiyan et al. 2009; Spencer et al. 2009; Stumhofer et al. 2007). The induction of IL-10 from eosinophils by IL-12 but not IL-27 also supported the diverse effects of IL-12 family members.

The intracellular mechanistic study indicated that IL-27 activated all the three MAPK members, ERK, JNK and p38MAPK as well as NF- κ B. These pathways are crucial cascades for regulating multiple cellular responses, including growth, proliferation, survival, and expression of proinflammatory cytokines and chemokines (Ho et al. 2008; Cheung et al. 2006). In addition, previous studies showed that IL-25, GM-CSF and LPS could also activate MAPK and NF- κ B in eosinophils (Cheung et al. 2006; Plötz et al. 2001; Wong et al. 2003). The similar activation profile of intracellular signaling transduction of IL-27, IL-25, GM-CSF and LPS may be the underlying mechanism by which IL-25, GM-CSF and LPS could not abrogate IL-27-mediated eosinophil activation (Fig. 7). The effect was different from monocytes in which inflammatory activation of IL-27 could be abrogated by LPS (Kalliolias and Ivashkiv 2008). Another reason for the discrepancy between IL-27 and LPS for the activation of monocytes and eosinophils may be due to the fact that eosinophils are terminally differentiated cells and can only maintain for short life-span without proliferation. Our results also indicated that IL-27-mediated intracellular signaling transduction was different between eosinophils and other cell types such as T cells, macrophages and keratinocytes, because JNK and NF- κ B pathways activated by IL-27 have not been previously reported (Batten and Ghilardi 2007; Kastelein et al. 2007).

However, there are still questions we need to address. Eosinophils generally involved in Th2-related diseases such as allergic diseases, while IL-27 was reported to suppress Th2 responses in animal studies (Kastelein et al. 2007). Our findings regarding *in vitro* stimulation effects of IL-27 on human eosinophils seemed to be contradictory to IL-27-suppressed Th2 immunity. One reason for the contradiction may due to the differences between human and mouse studies. IL-27 leads to the production of proinflammatory cytokines from human mast cells and monocytes (Kalliolias and Ivashkiv 2008; Pflanz et al. 2004), however, IL-27 negatively regulate murine mast cells and activated macrophages (Artis et al. 2004; Hölscher et al. 2005). The contradiction may also be due to the *in vitro* and *in vivo* assay designs. *In vivo* studies are complicated and involve multi-factorial interaction, while *in vitro* studies simplify the direct effects of IL-27 on targeted cells. IL-27 inhibition of Th2-related diseases, such as *Trichuris muris* infection and OVA-induced asthma were all derived from *in vivo* mouse models (Artis et al. 2004; Fujita et al. 2009; Miyazaki et al. 2005). However, no *in vivo* human studies of IL-27 have been reported and the stimulation effects of IL-27 on human eosinophils, monocytes and mast cells have all been derived from *in vitro* studies (Kalliolias and Ivashkiv 2008; Pflanz et al. 2004). The third reason may be due to the different signal transduction pathways. In human eosinophils, IL-27 could not activate STAT3 which is an important transcription factor for IL-27-mediated suppression (Kastelein et al. 2007), while MAPKs were involved in IL-27-mediated activation effects. Moreover, IL-27 inhibits acquired immune responses mediated by Th1, Th2 and Th17 cells, but not innate immune responses, while eosinophils are considered to be involved in innate immu-

nity (Hogan et al. 2008; Kalliolias and Ivashkiv 2008). The acquired immune system initiated by innate immune responses can be subsequently activated. We postulated that IL-27 may be served as an immunomodulatory cytokine in allergic diseases. Human IL-27 could activate innate immune responses through the stimulation of mast cells and eosinophils upon allergen stimulation, and then IL-27 inhibited Th2-acquired immune responses. This regulatory mechanism may be helpful to react with foreign stimuli and limit the allergic inflammation.

In conclusion, our studies have demonstrated that APC-released IL-27 can stimulate eosinophils, thereby extending the novel IL-27 activation effects on human innate immune system and further elaborating the roles of IL-12 family cytokines on eosinophils in allergic inflammation. Since single nucleotide polymorphisms of IL-27p28 were related to human asthma (Chae et al. 2007), the pleiotropic roles of IL-27 in human allergic responses need further exploration.

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