

Molecular Mechanisms of Cytokine and Chemokine Release from Eosinophils Activated by IL-17A, IL-17F, and IL-23: Implication for Th17 Lymphocytes-Mediated Allergic Inflammation

Phyllis F. Y. Cheung,^{1*} Chun K. Wong,^{1*} and Christopher W. K. Lam^{2**†}

IL-17A and IL-17F are members of the IL-17 family that play crucial roles in allergic inflammation. Recent studies reported that IL-17A and IL-17F production from a distinct Th lymphocyte subset, Th17, was specifically induced by IL-23, which was produced by dendritic cells and macrophages in response to microbial stimuli. The IL-23-IL-17 axis might therefore provide a link between infections and allergic diseases. In the present study, we investigated the effects of IL-17A, IL-17F, and IL-23, alone or in combination, on cytokine and chemokine release from eosinophils and the underlying intracellular mechanisms. Human eosinophils were found to constitutively express receptors for IL-17A, IL-17F, and IL-23 at the protein level. IL-17A, IL-17F, and IL-23 could induce the release of chemokines GRO- α /CXCL1, IL-8/CXCL8, and MIP-1 β /CCL4 from eosinophils, while IL-17F and IL-23 could also increase the production of proinflammatory cytokines IL-1 β and IL-6. Synergistic effects were observed in the combined treatment of IL-17F and IL-23 on the release of proinflammatory cytokines, and the effects were dose-dependently enhanced by IL-23, but not IL-17F. Further investigations showed that IL-17A, IL-17F, and IL-23 differentially activated the ERK, p38 MAPK, and NF- κ B pathways. Moreover, inhibition of these pathways using selective inhibitors could significantly abolish the chemokine release induced by IL-17A, IL-17F, and IL-23 and the synergistic increases on IL-1 β and IL-6 production mediated by combined treatment of IL-17F and IL-23. Taken together, our findings provide insight for the Th17 lymphocyte-mediated activation of eosinophils via differential intracellular signaling cascades in allergic inflammation. *The Journal of Immunology*, 2008, 180: 5625–5635.

The IL-17 family is a recently described group of cytokines which are important in inflammation and numerous diseases (1). Functional studies demonstrated that this novel cytokine family was important in mediating the migration and activation of inflammatory cells and collectively perpetuating the chronic airway inflammation that typifies asthma (1, 2). Several IL-17 family members have been identified: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E/IL-25, and IL-17F, in which the highest overall amino acid sequence (50%) is found between IL-17A and IL-17F (2, 3). Recently, increasing interests have focused on IL-17A and IL-17F for their roles in the pathogenesis of allergic asthma (4, 5). Current evidence suggests that both cytokines can investigate pulmonary inflammation by facilitating infiltration of inflammatory cells and amplify the inflammatory responses through the induction of a similar profile of CXC chemokines (4, 6).

IL-23 is a novel member of the IL-12 cytokine family and is composed of a unique p19 subunit and an identical p40 subunit to IL-12. Whereas IL-12 drives the classical Th1 response, IL-23

drives a novel T lymphocyte distinct population, Th17, which is characterized by the production of IL-17-family cytokines IL-17A and IL-17F (7, 8). It is widely accepted that Th17 lymphocytes play a crucial role in autoimmune diseases by promoting chronic inflammatory responses (9, 10). However, increasing evidence has shown that Th17 lymphocytes can also participate in the pathogenesis of allergic diseases by promoting neutrophil recruitment and activation (11, 12). Recently, several groups have suggested that IL-23 was closely associated with infectious diseases. It was reported that IL-23 was produced by dendritic cells and macrophages after exposure to microbial products including LPS, CpG, and peptidoglycan (13–15). Therefore, the ability of IL-23 to induce IL-17A and IL-17F might serve as a link between infections and allergic diseases such as asthma, wherein IL-17A and IL-17F are the principal effector cytokines.

Eosinophils are the most important inflammatory effector cells accumulating at the site of allergic inflammation, e.g., the airway submucosa (16). The severity of clinical symptoms of allergic patients was reported to correlate with the number of eosinophils in the inflamed tissues (16). Activated eosinophils release cytotoxic molecules such as major basic protein, eosinophil peroxidase, eosinophilic cationic protein, and lipid mediators that cause tissue damage and consequently the manifestation of allergic diseases (17, 18). In addition to causing direct tissue damage, eosinophil-derived proinflammatory cytokines and chemokines can perpetuate inflammation, resulting in chronic remodeling in airways (16). Eosinophils are known to produce and release various proinflammatory cytokines such as IL-1 β , IL-6, and TNF- α along with chemokines including IL-8/CXCL8, growth-regulated oncogene (GRO)- α /CXCL1, MIP-1 β /CCL4, MCP-1/CCL2, and RANTES/CCL5 (19).

Transcription factor NF- κ B and MAPKs were found to be involved in the expression of many inflammatory cytokines and

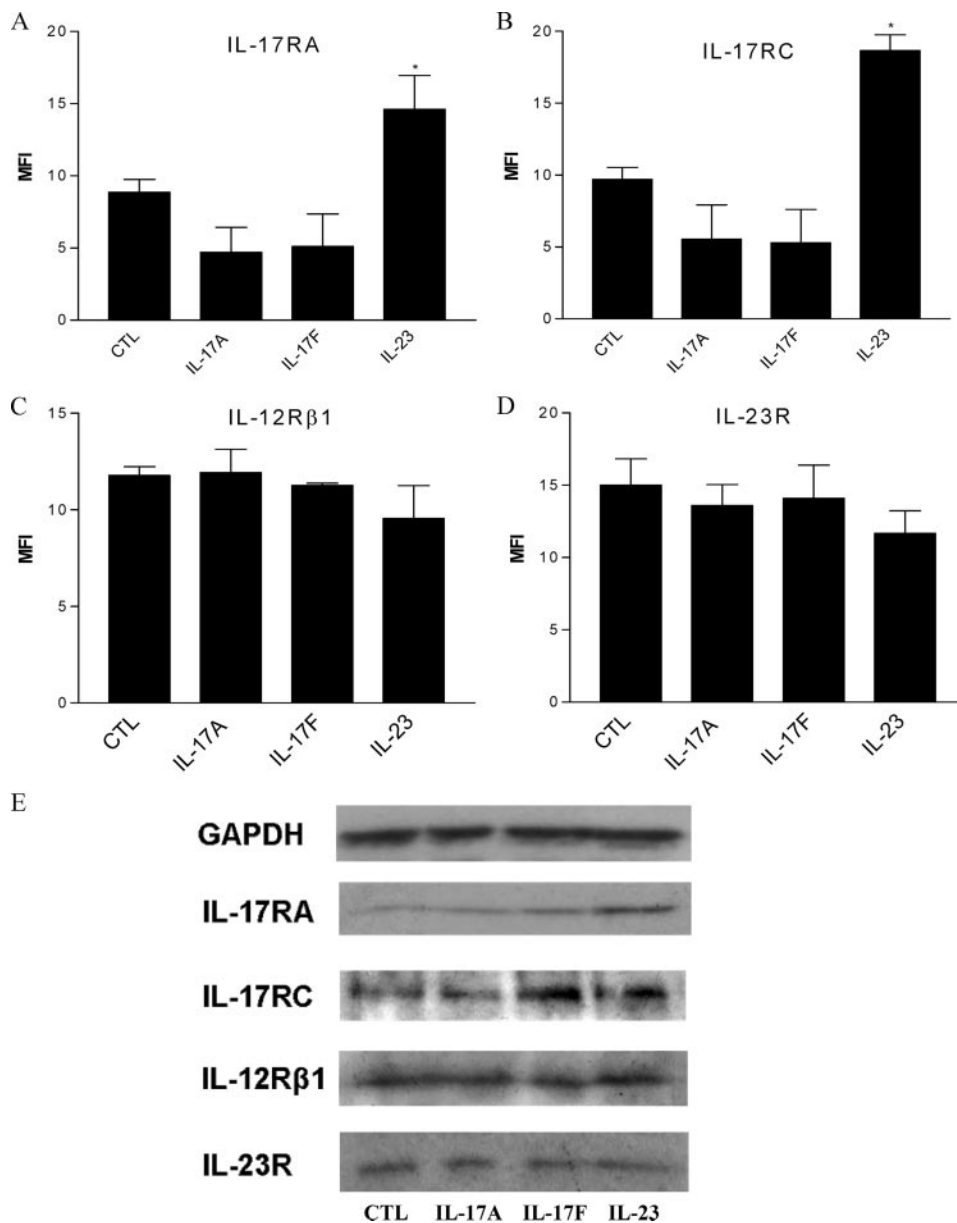
*Department of Chemical Pathology, Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong; and [†]Macau Institute for Applied Research in Medicine and Health, Macau University of Science and Technology Foundation, Macau, China. Received for publication May 29, 2007. Accepted for publication January 31, 2008.

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¹ P.F.Y.C. and C.K.W. share first authorship.

² Address correspondence and reprint requests to Dr. Christopher W. K. Lam, Department of Chemical Pathology, Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, N.T., Hong Kong, China. E-mail address: waikelam@cuhk.edu.hk

FIGURE 1. Protein expression of IL-17RA, IL-17RC, IL-12R β 1, and IL-23R in eosinophils. Eosinophils were treated with or without IL-17A (50 ng/ml), IL-17F (50 ng/ml), and IL-23 (50 ng/ml) for 24 h. Surface expression of IL-17RA (A), IL-17RC (B), IL-12R β 1 (C), and IL-23R (D) on eosinophils (5×10^5 cells) was determined by flow cytometry. The results are shown as MFI subtracting corresponding isotypic control and are expressed as the arithmetic mean $\pm$ SD of three independent experiments. E, Total cellular proteins were extracted from eosinophils (1×10^7 cells) and equal protein amounts were used for the detection of IL-17RA, IL-17RC, IL-12R β 1, and IL-23R proteins by Western blot analysis. GAPDH was used as control to ensure an equal amount of protein was loaded to every treatment. Experiments were performed in three independent replicates with essentially identical results and representative figures are shown. *, $p < 0.05$ when compared with medium control (CTL).



adhesion molecules of eosinophils during allergic inflammation (20). Our previous studies suggested a crucial role of the activation of NF- κ B and p38 MAPK in IL-17E/IL-25-mediated cytokine and chemokine release and the modulation of adhesion molecules of eosinophils (21, 22). However, the intracellular signal transduction for eosinophil activation mediated by IL-17A, IL-17F, and IL-23 has not been studied. In the present study, we hypothesized that Th17 lymphocytes might regulate allergic inflammation by the activation of eosinophils. We investigated the modulation of intracellular NF- κ B and MAPK activities on regulating the release of chemokines and cytokines from eosinophils under the stimulation of IL-17A, IL-17F, and IL-23.

Materials and Methods

Reagents

Recombinant human IL-17A, IL-17F, and IL-23 were purchased from R&D Systems. Cycloheximide and actinomycin D were purchased from Sigma-Aldrich. I κ B- α phosphorylation inhibitor BAY11-7082, MEK1,2 inhibitor U0126, JNK inhibitor SP600125, p38 MAPK inhibitor SB203580, PI3K inhibitor LY294002, and JAK inhibitor AG490 were purchased from Calbiochem. SB203580 and LY294002 were dissolved in wa-

ter while U0126, SP600125, AG490, and BAY11-7082 were dissolved in DMSO. In all studies, the concentration of DMSO was 0.1% (v/v).

Isolation of human blood eosinophils from buffy coat and eosinophil culture

Fresh human buffy coats obtained from the healthy volunteers of the Hong Kong Red Cross Blood Transfusion Service was diluted 1/2 with PBS at 4°C and centrifuged using an isotonic Percoll solution (density, 1.082 g/ml; Amersham Biosciences) for 30 min at 1000 $\times$ g. The eosinophil-rich granulocyte fraction was collected and washed twice with cold PBS containing 2% FBS. The cells were then incubated with anti-CD16 magnetic beads (Miltenyi Biotec) at 4°C for 45 min and CD16-positive cells were depleted by passing through a LS+ column (Miltenyi Biotec) within a magnetic field. With this preparation, the drop-through fraction contained eosinophils with a purity of at least 99% as assessed by Hemacolor rapid blood smear stain (Merck). The isolated eosinophils were cultured in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FBS (Invitrogen Life Technologies) and 20 mM HEPES (Invitrogen Life Technologies).

Endotoxin-free solutions

Cell culture medium was purchased from Invitrogen Life Technologies, free of detectable LPS (<0.1 endotoxin units/ml). All other solutions were prepared using pyrogen-free water and sterile polypropylene plastic ware.

No solution contained detectable LPS, as determined by the *Limulus* amoebocyte lysate assay (sensitivity limit, 12 pg/ml; BioWhittaker).

Protein array analysis of chemokines and cytokines in culture supernatant of eosinophils

The expression profile of 79 different cytokines in culture supernatants of eosinophils was assessed semiquantitatively using Ab-based RayBio human cytokine array V (RayBiotech).

Western blot analysis

Eosinophils were washed with ice-cold PBS and lysed in 0.2 ml of lysis buffer (20 mM Tris-HCl (pH 8.0), 120 mM NaCl, 1% Triton X-100, 10 mM EDTA, 1 mM EGTA, 0.05% 2-ME, and $1\times$ protease inhibitors). Cell debris was removed by centrifugation at $14,000 \times g$ for 15 min, and the supernatant was boiled in Laemmli sample buffer (Bio-Rad) for 5 min. An equal amount of proteins was subjected to SDS-10% PAGE before blotting onto a polyvinylidene fluoride membrane (Amersham Biosciences). The membrane was blocked with 5% skimmed milk in TBS with 0.05% Tween 20 (pH 7.6) for 1 h at room temperature and probed with primary rabbit anti-human GAPDH, IL-17RA, IL-17RC, IL-12R β 1, or IL-23R Abs (R&D Systems) at 4°C overnight. After washing, membrane was incubated with secondary donkey anti-rabbit Ab coupled to HRP (Amersham Biosciences) for 1 h at room temperature. Ab-Ag complexes were then detected using an ECL chemiluminescent detection system (Amersham Biosciences).

Quantitative analysis of IL-1 β , IL-6, CXCL1, CXCL8, and CCL4

Concentrations of proinflammatory cytokine IL-1 β and IL-6 and chemokine CXCL8 in culture supernatants were quantitated using an ELISA kit from BD Pharmingen. Concentrations of chemokines CXCL1 and CCL4 were measured by ELISA kits from R&D Systems and RayBiotech, respectively.

Immunofluorescence staining and flow cytometry

To determine the expression of cytokine receptors on eosinophil surface, the cells were washed and resuspended with cold PBS supplemented with 0.5% BSA after preceding treatments. After blocking with 2% human pooled serum for 20 min at 4°C and washed with PBS supplemented with 0.5% BSA, cells were incubated with FITC-conjugated mouse anti-human IL-17RA, IL-17RC, IL-12R β 1, and IL-23R Abs and mouse IgG1 isotype for 30 min at 4°C in the dark. After washing, the cells were resuspended in 1% paraformaldehyde as fixative and subjected to analysis. To determine the intracellular expression of phosphorylated signaling molecules, eosinophils were fixed with 4% paraformaldehyde for 10 min at 37°C after preceding treatments. After centrifugation, cells were permeabilized in ice-cold methanol for 30 min and then stained with FITC-conjugated mouse anti-human IL-17RA adaptor protein Act1 (Santa Cruz Biotechnology), ERK1/2, p38 MAPK, I κ B- α , or mouse IgG1 Abs (BD Pharmingen) for 30 min at 4°C in the dark. Cells were then washed, resuspended, and subjected to analysis. Expression of cytokine receptors and phosphorylated signaling molecules of 10,000 viable cells was analyzed by flow cytometry (FACSCalibur; BD Biosciences) as mean fluorescence intensity (MFI),³ which includes both the changes of target molecule expression in individual cell and the percentage of cells expressing the target molecules.

Statistical analysis

All data were expressed as mean $\pm$ SD. Differences between groups were assessed by one-way ANOVA analysis. A $p < 0.05$ was considered significantly different. All analyses were performed using the SPSS statistical software for Windows, version 10.1.4.

Results

Protein expression of receptors for IL-17A, IL-17F, and IL-23

Flow cytometric analysis showed that human eosinophils constitutively expressed IL-17RA, IL-17RC, IL-12R β 1, and IL-23R on their surfaces (Fig. 1, A–D). Moreover, IL-23 could up-regulate the surface expression of IL-17RA and IL-17RC, whereas expression of both IL-12R β 1 and IL-23R had no significant change among all of the treatments. However, treatments with IL-17A and IL-17F alone suppressed the surface expression of their cognate receptors

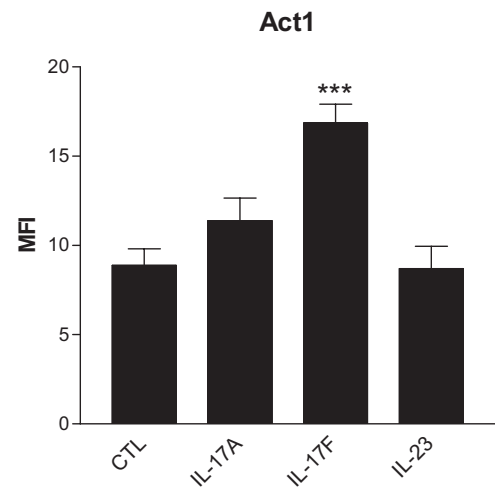


FIGURE 2. Effects of IL-17A, IL-17F, and IL-23 on the protein expression of Act1. Eosinophils (5×10^5 cells) were incubated with or without IL-17A (50 ng/ml), IL-17F (50 ng/ml), and IL-23 (50 ng/ml) for 24 h. The amount of intracellular Act1 in 10,000 permeabilized eosinophils was measured by flow cytometry. Results of Act1 protein expression are shown in MFI subtracting corresponding isotypic control and are expressed as the arithmetic mean $\pm$ SD of three independent experiments. ***, $p < 0.001$ when compared with medium control (CTL).

IL-17RA and IL-17RC, respectively. This might be due to the same epitope recognition by the receptor Abs and receptor ligands IL-17A and IL-17F. Further investigation by Western blot analysis showed that although IL-23 could enhance the protein expression of both IL-17RA and IL-17RC in eosinophils, IL-17F was also found to increase the protein expression of one of its cognate receptors, IL-17RC. For IL-12R β 1 and IL-23R, results concurred with those of flow cytometric analysis that no treatment could exert any effect on their expression (Fig. 1E).

Protein expression of IL-17RA adaptor protein Act1 in eosinophils

Recent reports have provided evidence for the essential involvement of an adaptor protein Act1 in IL-17RA signaling and hence the immune responses elicited by both IL-17A and IL-17F (23, 24). We showed by intracellular staining using flow cytometry that adaptor protein Act1 was constitutively expressed in eosinophils. Besides, we found that IL-17F, but not IL-17A and IL-23, could enhance the protein expression of Act1 (Fig. 2).

Effects of IL-17A, IL-17F, and IL-23 on eosinophil survival

We analyzed the effects of IL-17A, IL-17F, and IL-23 on the viability of eosinophils using an annexin V/propidium iodide (PI) assay by flow cytometry. Results showed that only $42.8 \pm 4.6\%$ of untreated eosinophils remained alive after 24 h of incubation, with the remainder of the cells being either apoptotic or necrotic. However, the percentage of viable cells increased significantly to $75.7 \pm 3.2\%$ and $81.9 \pm 2.9\%$ in the treatment with IL-17F (50 ng/ml) and IL-23 (50 ng/ml), respectively ($p < 0.01$). No significant increase was observed for the treatment with IL-17A (50 ng/ml; data not shown).

Effects of IL-17A, IL-17F, and IL-23 on cytokine and chemokine release

Fig. 3 illustrates the cytokine expression profile using Ab-based human cytokine protein membrane array. All IL-17A, IL-17F, and IL-23 could promote the release of chemokines CXCL1 (*Ij* and

³ Abbreviations used in this paper: MFI, mean fluorescence intensity; PI, propidium iodide.

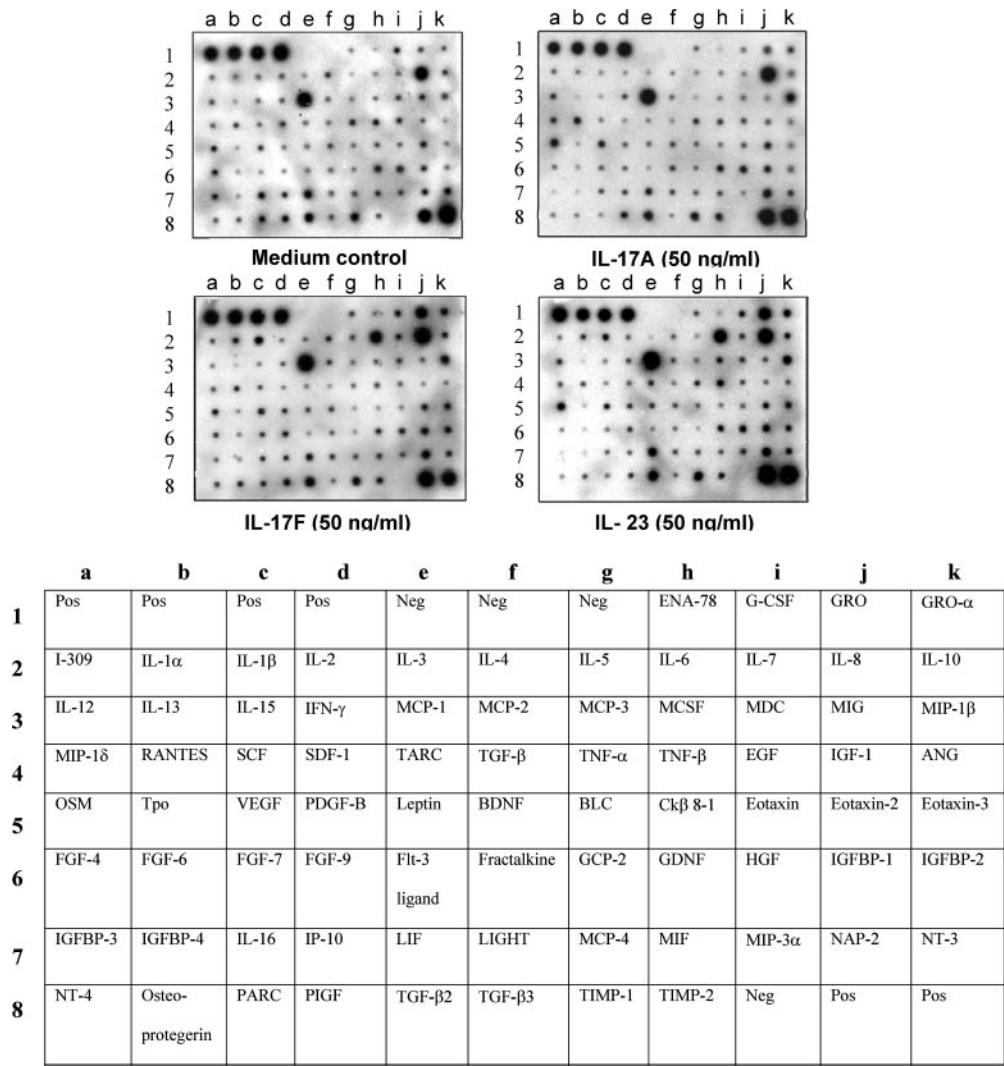


FIGURE 3. Representative profile of the release of cytokines from eosinophils activated by IL-17A, IL-17F, and IL-23. Eosinophils (1×10^6 cells) were cultured with or without IL-17A (50 ng/ml), IL-17F (50 ng/ml), and IL-23 (50 ng/ml) for 24 h. Cell-free culture supernatants were then harvested and 79 different cytokines in culture supernatants were semiquantitated using an Ab-based RayBio human cytokine array V. Positive and negative controls were designated at 1a, 1b, 1c, 1d, 8j, and 8k and 1e, 1f, 1g, and 8i, respectively. Experiments were performed in three independent replicates with essentially identical results and representative results are shown. The table lists the format of Abs on the cytokine membrane array.

1k), CXCL8 (2j), and CCL4 (3k) from eosinophils, whereas IL-17F and IL-23 could also activate eosinophils to increase the production of proinflammatory cytokines IL-1β (2c) and IL-6 (2h). Further investigation using ELISA showed that the production of IL-1β and IL-6 induced by IL-17F and IL-23 and that of CXCL1, CXCL8, and CCL4 induced by IL-17A, IL-17F, and IL-23 could be enhanced dose-dependently (0–100 ng/ml) at 12 and 24 h, in which the levels of cytokine and chemokine release from eosinophils were significantly higher in 24 h than 12 h (Fig. 4).

To verify that the cytokines and chemokines released from eosinophils were newly synthesized upon the stimulation of IL-17A, IL-17F, and IL-23, transcriptional inhibitor actinomycin D and protein synthesis inhibitor cycloheximide were used. The cytotoxicities of actinomycin D and cycloheximide were assessed by an annexin V/PI assay using flow cytometry. Results showed that actinomycin D, but not cycloheximide, exerted significant cytotoxic effects on eosinophils at concentration higher than 10 μM (Fig. 5A). As shown in Fig. 5, B–F, both actinomycin D (1 μM) and cycloheximide (10 μM) alone could significantly suppress the release of cytokines and chemokines induced by IL-17A, IL-17F, and IL-23, respectively. The results therefore indicate that IL-17A,

IL-17F, and IL-23 actually induce the release of newly synthesized IL-1β, IL-6, CXCL1, CXCL8, and CCL4, rather than the pre-formed ones from eosinophils.

Combined effects of IL-17A, IL-17F, and IL-23 on cytokine and chemokine release

Fig. 6 shows that combined treatment of IL-17F (10 ng/ml) and IL-23 (10 ng/ml), but not the other combinations, results in significant synergistic increases in the release of proinflammatory cytokines IL-1β and IL-6. However, only additive effects are found in the release of chemokines CXCL1, CXCL8, and CCL4. To investigate whether the synergistic effects on IL-1β and IL-6 release were dose dependent on IL-17F and/or IL-23, serial concentrations of IL-17F and IL-23 were used in the combined treatments. As shown in Fig. 7, the synergistic effects in both IL-1β and IL-6 could be further enhanced when the IL-23 concentration increased from 5 to 20 ng/ml in combination with a fixed IL-17F concentration (10 ng/ml). In contrast, a fixed concentration of IL-23 (10 ng/ml) with serial concentrations of IL-17F (5–20 ng/ml) showed that the synergistic effect could not be enhanced dose-dependently by IL-17F.

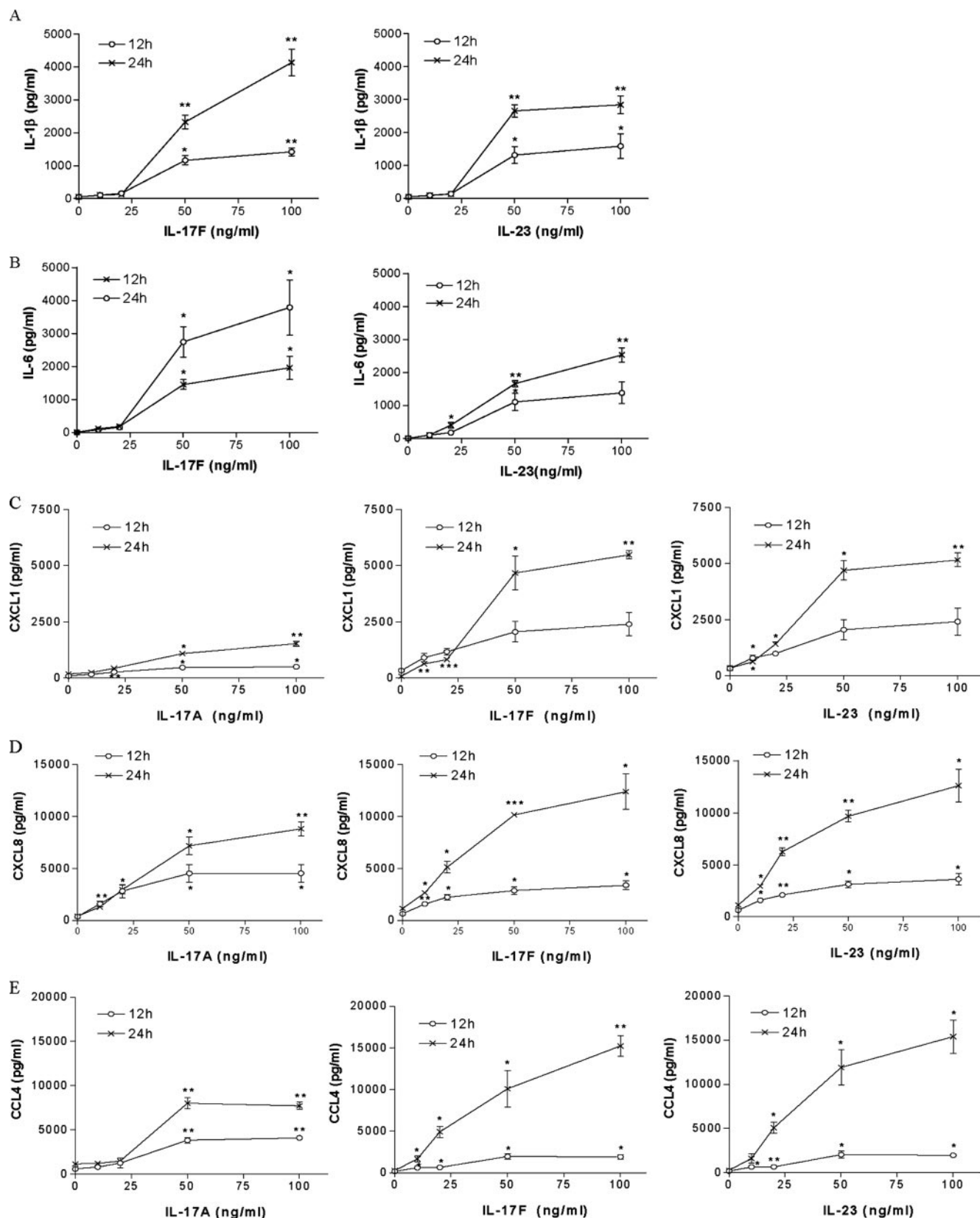


FIGURE 4. Kinetic release of IL-1 β (A), IL-6 (B), CXCL1 (C), CXCL8 (D), and CCL4 (E) from eosinophils activated by IL-17A, IL-17F, and IL-23. Eosinophils (1×10^6 cells) were cultured with or without IL-17A, IL-17F, or IL-23 (0–100 ng/ml) for 12 and 24 h. Cytokines and chemokines released into the culture supernatants were determined 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 the medium control of the same time point.

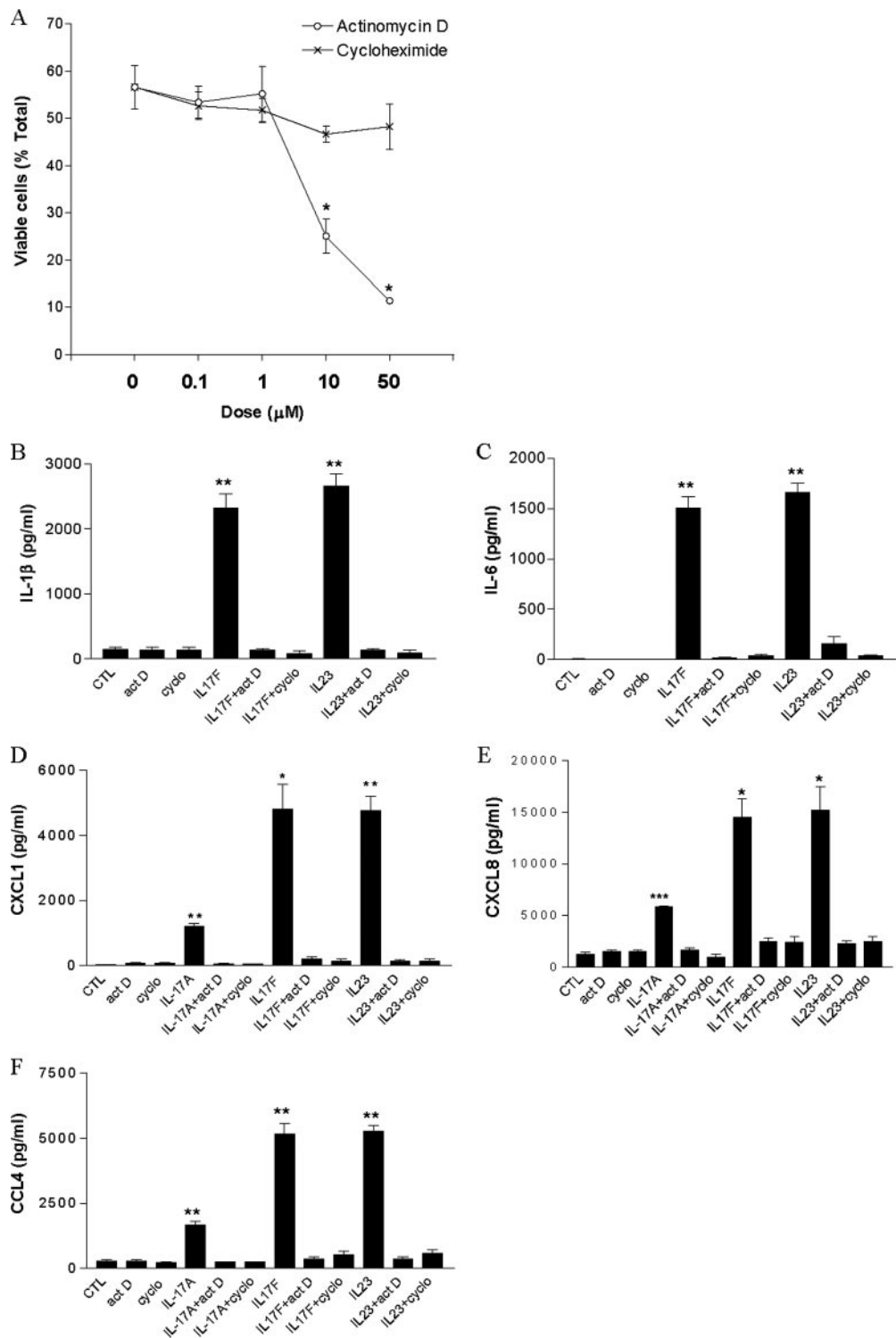


FIGURE 5. A, The cytotoxic effects of actinomycin D (act D) and cycloheximide (cyclo) on the viability of eosinophils. Eosinophils (5×10^5 cells) were treated with actinomycin D or cycloheximide at various concentrations for 24 h. Viability of eosinophils was determined by an annexin V/PI assay using flow cytometry. Viable cells are presented as percentage of gated cells and are expressed as the arithmetic mean $\pm$ SD of three independent experiments. B–F, Effects of actinomycin D and cycloheximide on the release of IL-1 β , IL-6, CXCL1, CXCL8, and CCL4 induced by IL-17A, IL-17F, and IL-23 from eosinophils. Eosinophils (1×10^6 cells) were cultured with or without IL-17A (50 ng/ml), IL-17F (50 ng/ml), or IL-23 (50 ng/ml) in the presence of actinomycin D (1 μ M) or cycloheximide (10 μ M) for 24 h. Cytokines and chemokines released into the culture supernatants were determined by ELISA. Results are expressed as the arithmetic mean $\pm$ SD from three independent experiments. *, $p < 0.05$ and **, $p < 0.01$ when compared with the medium control (CTL).

Effects of signaling inhibitors on cytokine and chemokine release induced by IL-17A, IL-17F, and IL-23

The cytotoxicities of different signaling inhibitors on eosinophils were determined by an annexin V/PI assay using flow

cytometry. We used the optimal concentrations of AG490 (3 μ M), BAY11-7082 (1 μ M), LY294002 (5 μ M), U0126 (10 μ M), SB203580 (7.5 μ M), and SP600125 (3 μ M) with significant inhibitory effects without any cell toxicity. As shown in Fig.

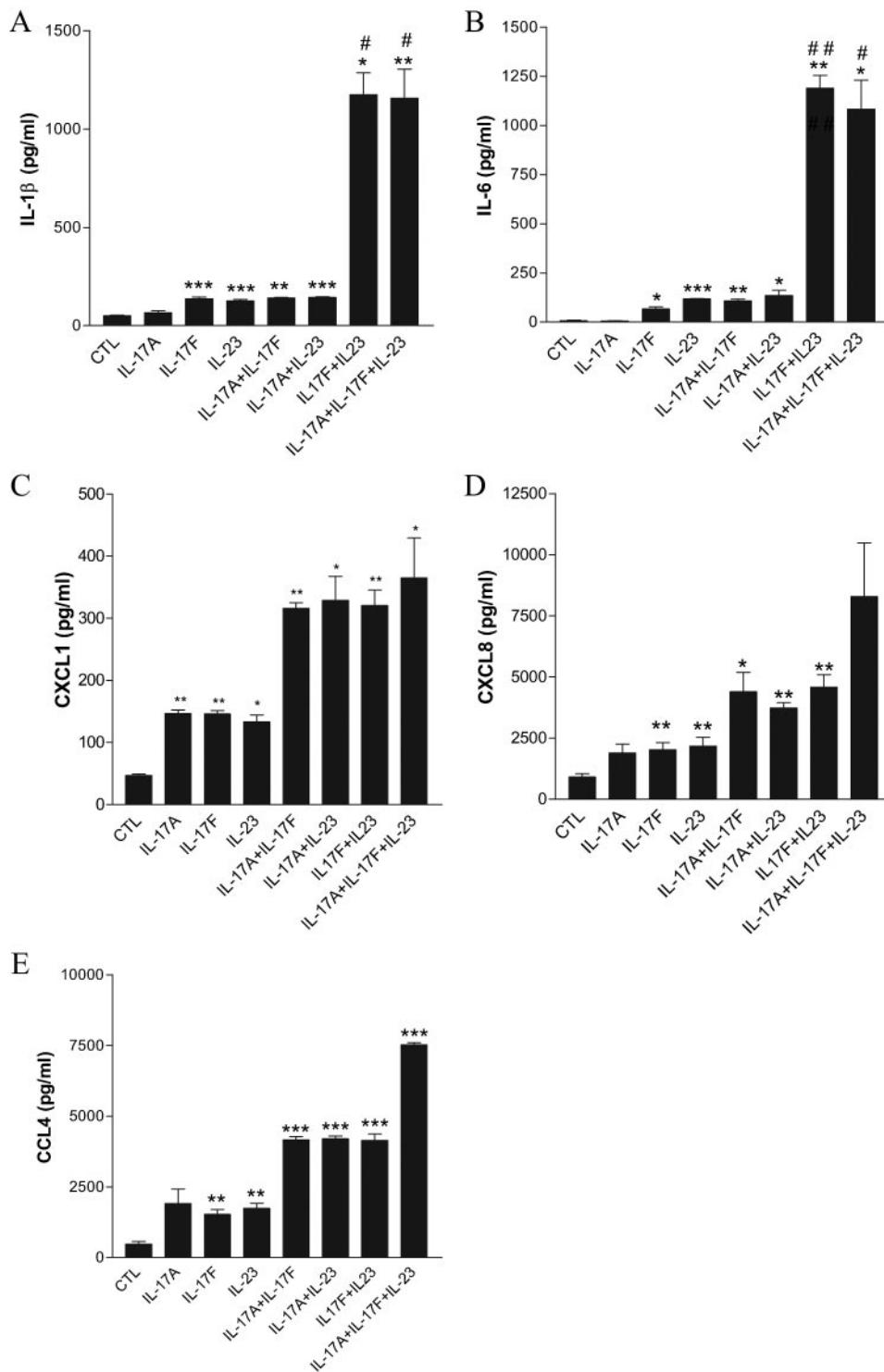


FIGURE 6. Combined effects of IL-17A, IL-17F, and IL-23 on the release of IL-1 β (A), IL-6 (B), CXCL1 (C), CXCL8 (D), and CCL4 (E) from eosinophils. Eosinophils (1×10^6 cells) were cultured with or without IL-17A (10 ng/ml), IL-17F (10 ng/ml), and IL-23 (10 ng/ml) alone or in combination for 24 h. Cytokines and chemokines released into the culture supernatants were determined 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 the medium control (CTL). #, $p < 0.05$ and ##, $p < 0.01$ when compared with the sum of cytokine production in treatment with individual stimulators (IL-17A, IL-17F, or IL-23) added alone.

8, A and B, BAY11-7082 and U0126 could significantly suppress the release of proinflammatory cytokines IL-1 β and IL-6 induced by IL-17F, while the effects of IL-23 could be significantly restrained by BAY11-7082 and SB203580. For the release of chemokines CXCL1, CXCL8, and CCL4, Fig. 8, C–E, show that although SB203580 suppresses the stimulatory effects of IL-17A and IL-23, BAY11-7082 can abrogate those of IL-17F and IL-23. However, AG490, LY294002, and SP600125 do not exert any significant effect on cytokine and chemokine production induced by IL-17A, IL-17F, and IL-23.

Effects of signaling inhibitors on the synergistic release of IL-1 β and IL-6 induced by IL-17F and IL-23

As shown in Fig. 9, the release of IL-1 β and IL-6 induced by IL-17F could be suppressed by BAY11-7082 and U0126, while those induced by IL-23 were suppressed by BAY11-7082 and SB203580. Besides, all BAY11-7082, U0126, and SB203580 could partially but significantly suppress the synergistic effects of IL-17F and IL-23 on IL-1 β and IL-6 release, respectively. However, AG490, LY294002, and SP600125 did not exert any significant effect on the synergistic

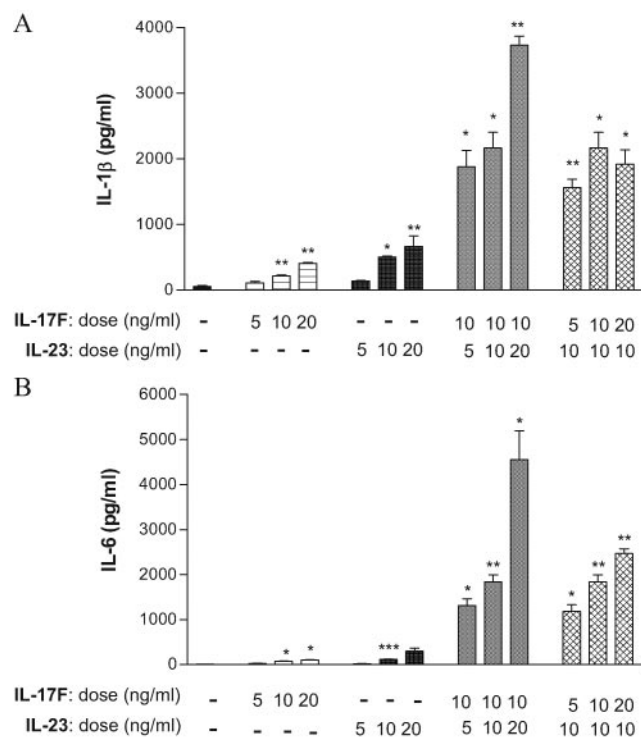


FIGURE 7. Synergistic effects of IL-17F and IL-23 on the release of IL-1 β (A) and IL-6 (B) were IL-23 dependent. Eosinophils (1×10^6 cells) were cultured with varied concentrations of IL-17F and IL-23 in combination for 24 h. Cytokines released into the culture supernatants were determined by ELISA. Results are expressed as the arithmetic mean $\pm$ SD from three independent experiments. *, $p < 0.05$ and **, $p < 0.01$ when compared with the medium control.

release of IL-1 β and IL-6 induced by IL-17F and IL-23 (data not shown).

IL-17A, IL-17F, and IL-23 differentially activated ERK, p38 MAPK, and NF- κ B pathways

Using intracellular fluorescence staining by flow cytometry, we measured the contents of phosphorylated ERK, p38 MAPK, and I κ B- α in permeabilized eosinophils at different time points after the stimulation by IL-17A alone and IL-17F and IL-23 alone and in combination. As shown in Fig. 10A, IL-17A could induce the phosphorylation of p38 MAPK and the activation sustained to 1 h. For IL-17F, it could activate ERK but not the p38 MAPK pathway, in which the phosphorylation of ERK peaked at 30 min and declined afterward (Fig. 10, B and C). For IL-23, it could not induce any activation of ERK, but did cause rapid phosphorylation of p38 MAPK after 5 min of stimulation and the phosphorylation sustained to 1 h (Fig. 10, B and C). For the NF- κ B pathway, both IL-17F and IL-23 could induce significant phosphorylation of I κ B- α within 15 min of stimulation. However, combined treatment of IL-17F and IL-23 could not further increase the phosphorylation of I κ B- α (Fig. 10D). These results confirm that IL-17A, IL-17F, and IL-23 differentially activate the ERK, p38 MAPK, and NF- κ B pathways.

Discussion

Although the studies for physiological roles of IL-17A, IL-17F, and IL-23 are mounting, the responsible cell populations expressing their receptors are not yet fully elucidated in humans (1, 25, 26). The receptor for IL-17F has not been completely identified (26). However, because IL-17F shows a high degree of structural homology with IL-17A and has a similar spectrum of biological

activities, IL-17F may use the same receptors as IL-17A (1). Recent studies identified that IL-17RA and IL-17RC form a heterodimer for the binding of both IL-17A and IL-17F (27); of which IL-17RC binds to both IL-17A and IL-17F with the same affinity, while IL-17RA binds to IL-17F with an ~ 10 -fold lower affinity than to IL-17A (27, 28). Although we cannot rule out the possibility that there may be additional components involved in IL-17F signaling, IL-17RA and IL-17RC at least in part essentially contribute to the recognition and signal transduction of IL-17F. IL-23 is well-documented as binding to the IL-23R complex, composed of IL-23R and IL-12 β 1 (29). In the present study, we have examined the protein expression of IL-17RA, IL-17RC, IL-12 β 1, and IL-23R in human eosinophils, and found that eosinophils constitutively expressed proteins for these receptors on their cell surfaces. Moreover, our results showed that although the protein expression of IL-17RA could be up-regulated by IL-23, IL-17RC expression could be enhanced by its ligand IL-17F, and IL-23. However, the protein expression of both IL-12 β 1 and IL-23R was not affected by IL-17A, IL-17F, or IL-23. Recently, it has been reported that an adaptor protein Act1 was an immediate and essential component of IL-17RA signaling. In this study, we showed by flow cytometry that IL-17F, but not IL-17A and IL-23, could enhance the protein expression of Act1 in human eosinophils. The above findings therefore prompted us to hypothesize that these cytokines might be able to potentiate or enhance the action of each other on human eosinophils.

To date, the most extensively investigated function of IL-17A, IL-17F, and IL-23 is the induction of various cytokines and chemokines that are crucial in regulating inflammatory responses (30–32). Cytokines and chemokines have been shown to play important pathological roles in allergic inflammation (33). In the present study, we used a cytokine protein array to screen for the induction of 79 different cytokines and chemokines from eosinophils induced by IL-17A, IL-17F, and IL-23. All three cytokines could enhance the production of neutrophil chemokines CXCL1, CXCL8, and granulocyte activator CCL4 from eosinophils. Besides, IL-17F and IL-23 could activate eosinophils to increase the release of proinflammatory cytokines IL-1 β and IL-6. Further investigation by ELISA confirmed that IL-17A, IL-17F, and IL-23 could dose-dependently induce a significant in vitro release of IL-1 β and IL-6 and CXCL1, CXCL8, and CCL4. Moreover, both the transcriptional inhibitor actinomycin D and protein synthesis inhibitor cycloheximide could suppress the release of cytokines and chemokines upon IL-17A, IL-17F, and IL-23 stimulation. This demonstrated that IL-17A, IL-17F, and IL-23 could induce the release of newly synthesized, instead of preformed, IL-1 β , IL-6, CXCL1, CXCL8, and CCL4 from eosinophils. The release of IL-1 β could activate macrophages (34), while induction of IL-6 suggested that IL-17F and IL-23 might be involved in mediating inflammatory and Th2 immune responses (35). CCL4 can profoundly activate neutrophils, eosinophils, and basophils and is involved in acute neutrophilic inflammation. In addition, it markedly recruits T lymphocytes from the circulation to lymph nodes during the initiation of primary immune responses (36). Our results also demonstrated that IL-17A, IL-17F, and IL-23 could induce the secretion of neutrophil chemokines CXCL1 and CXCL8 from eosinophils. This provides further explanation for the mechanism of Th17-mediated neutrophil infiltration into the inflammatory sites at bronchial airways (36, 37). A previous study reported that eosinophils in sputum and bronchoalveolar lavage fluids of asthmatic patients expressed IL-17A (38). However, we studied, by real-time PCR, that no mRNA encoding IL-17A, IL-17F, and IL-23 could be detected in peripheral blood eosinophils at both the resting stage and upon stimulation by IL-17A, IL-17F, and IL-23 (data not

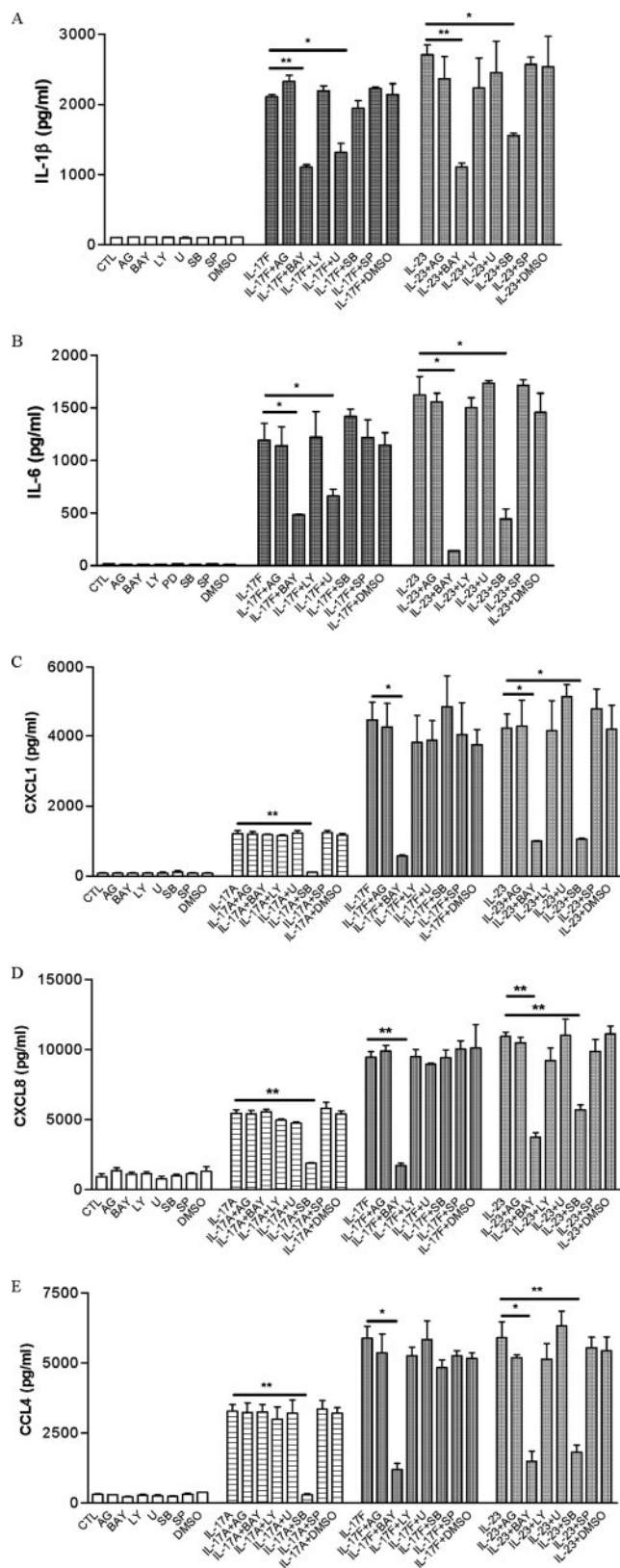


FIGURE 8. Effects of signaling inhibitors on IL-1 β (A), IL-6 (B), CXCL1 (C), CXCL8 (D), and CCL4 (E) release from eosinophils. Eosinophils (1×10^6 cells) were pretreated with AG490 (3 μ M; AG), BAY11-7082 (1 μ M; BAY), LY294002 (5 μ M; LY), U0126 (10 μ M; U), SB203580 (7.5 μ M; SB), and SP600125 (3 μ M; SP) for 45 min, followed by incubation with or without IL-17A (50 ng/ml), IL-17F (50 ng/ml), or IL-23 (50 ng/ml) in the presence of inhibitors for another 24 h. Release of cytokines and chemokines in the culture supernatant was determined by ELISA. Results are expressed as the arithmetic mean $\pm$ SD from three

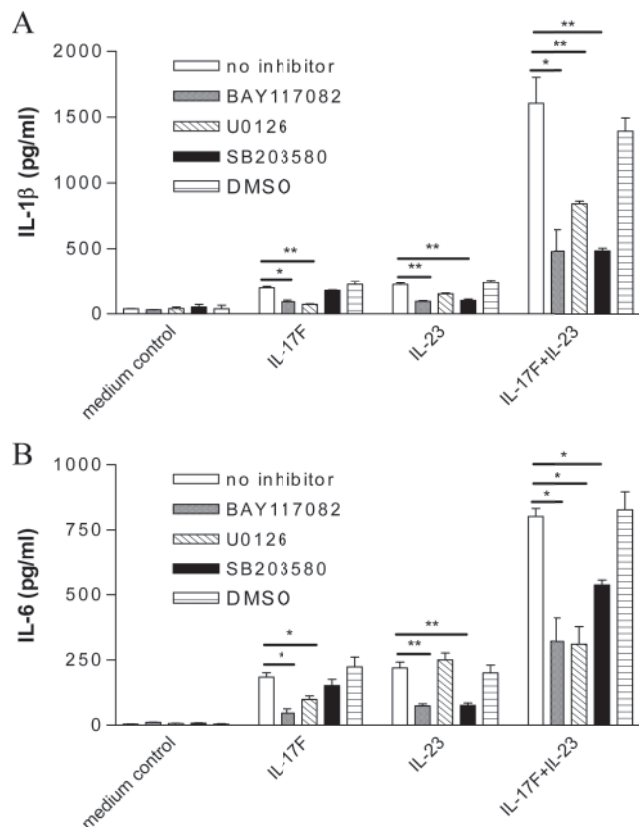


FIGURE 9. Effects of signaling inhibitors on IL-17F- and IL-23-induced synergistic effects on IL-1 β (A) and IL-6 (B) release from eosinophils. Eosinophils (1×10^6 cells) were pretreated with BAY11-7082 (1 μ M), U0126 (10 μ M), and SB203580 (7.5 μ M) for 45 min, followed by incubation with or without IL-17F (10 ng/ml) or IL-23 (10 ng/ml) in the presence of inhibitors for another 24 h. Release of cytokines in the culture supernatants was determined by ELISA. Results are expressed as the arithmetic mean $\pm$ SD from three independent experiments. DMSO (0.1%) was used as the vehicle control. *, $p < 0.05$ and **, $p < 0.01$ when compared between groups denoted by horizontal lines.

shown). Taken together, Th17-mediated eosinophil activation could induce the release of proinflammatory cytokines and chemokines, thereby amplifying inflammatory responses during allergic diseases.

Given that IL-17A, IL-17F, and IL-23 could induce a similar profile of proinflammatory cytokines and chemokines from eosinophils, we further investigated the combined effects of the three cytokines. Synergistic effects were observed in the combined treatment of IL-17F and IL-23 on the release of proinflammatory cytokines IL-1 β and IL-6, while only additive effects were found in the induction of chemokines CXCL1, CXCL8, and CCL4 in all combined treatments. Further study showed that the synergistic effects were dose dependent on IL-23, but not on IL-17F. This might be due to the up-regulation of IL-17RA and IL-17RC expression induced by IL-23, leading to the augmentation of IL-17F-mediated effects on eosinophils. Both IL-1 β and IL-6 are well-recognized as effector cytokines that mediate damage in peripheral tissues (39). Recently, they have been reported as critical inducers for Th17 polarization. Although IL-1 β was sufficient to induce the

independent experiments. DMSO (0.1%) was used as the vehicle control (CTL). *, $p < 0.05$ and **, $p < 0.01$ when compared between groups denoted by horizontal lines.

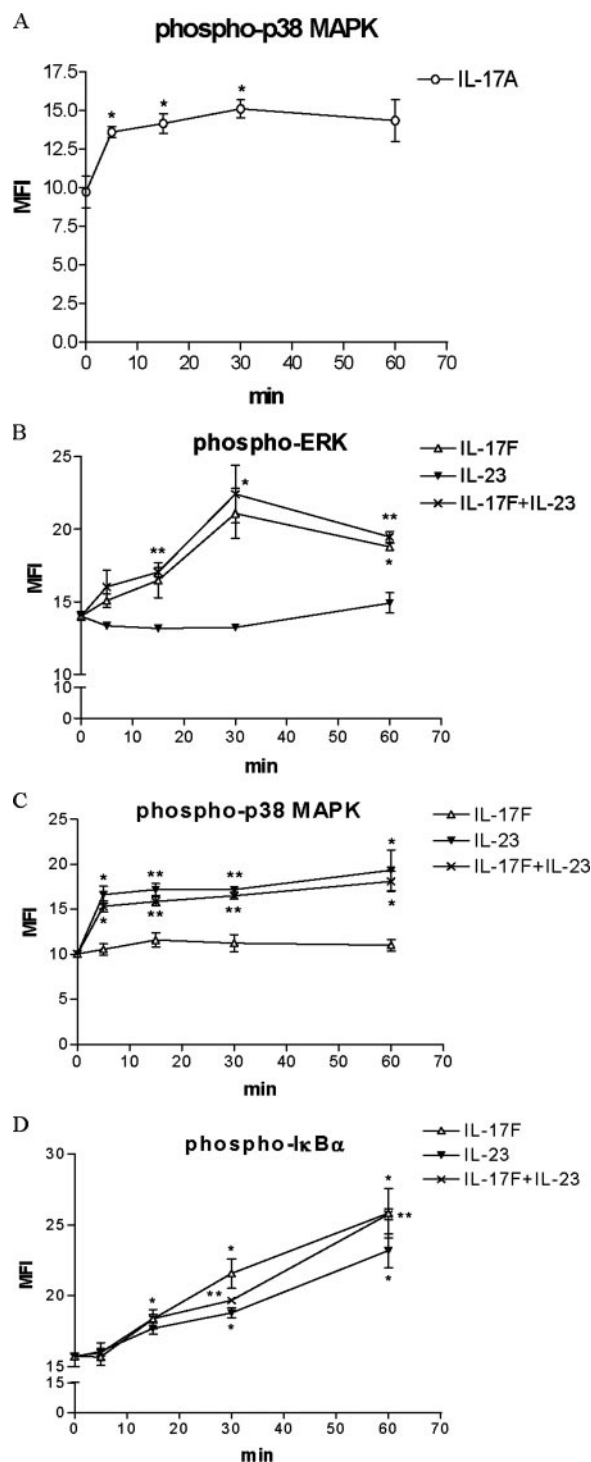


FIGURE 10. Effects of IL-17A, IL-17F, and IL-23 on intracellular ERK, p38 MAPK, and NF- κ B activities. Eosinophils (5×10^5 cells) were incubated with or without IL-17A (50 ng/ml), IL-17F (50 ng/ml), and IL-23 (50 ng/ml), alone or in combination for varied incubation times. The amounts of intracellular phosphorylated signaling molecules in 10,000 permeabilized eosinophils were measured by flow cytometry. Results of phosphorylated p38 MAPK (A and C), phosphorylated ERK (B), and phosphorylated I κ B- α (D) are shown in MFI subtracting corresponding isotopic control and are expressed as the arithmetic mean $\pm$ SD of three independent experiments. *, $p < 0.05$ and **, $p < 0.01$ when compared with medium control.

expression of transcription factor ROR γ t and production of IL-17A, IL-6 could sustain the expression of ROR γ t and promoted the differentiation of Th17 lymphocytes (40). The finding that IL-1 β

and IL-6 are also key inducers of Th17 responses provides an additional rationale for targeting these cytokines in allergic diseases to reduce not only inflammation, but also the priming of inflammatory Th17 lymphocytes in humans. Besides, due to the fact that IL-23 production from dendritic cells and macrophages is induced upon microbial stimuli (13, 15, 41), the ability of IL-23 to enhance IL-17F stimulation on eosinophils might also imply a potential link between microbial infection and allergic diseases. However, further studies are required to support this hypothesis.

For elucidating the molecular mechanism of eosinophil activation induced by IL-17A, IL-17F, and IL-23, we used quantitative intracellular staining by flow cytometry to measure the dynamic phosphorylation levels of signaling molecules in permeabilized eosinophils under the stimulation of the three cytokines. MAPK pathways have been shown to play active roles in a large variety of cellular activities, ranging from cell survival and proliferation to expression of proinflammatory cytokines (42). Our results demonstrated that although both IL-17A and IL-23 could strongly activate the p38 MAPK pathway, IL-17F could induce ERK activation. The observation that IL-17F and IL-23 induced a common panel of cytokines and chemokines suggested that they might transmit overlapping signaling pathways. This postulation was supported by our result that both cytokines alone could significantly induce the phosphorylation of I κ B- α , thereby activating the NF- κ B pathway. NF- κ B is a pivotal regulator of proinflammatory cytokines and chemokines. It is highly active at inflammatory sites in various diseases, including allergic asthma, by enhancing the recruitment of inflammatory cells and production of proinflammatory cytokines and chemokines (20). Since ERK, p38 MAPK, and NF- κ B are common signal transduction molecules by which expression of cytokines and chemokines can be regulated in eosinophils (20), it is logical that they are commonly involved in eosinophil activation mediated by IL-17A, IL-17F, and IL-23.

Our group previously reported the active involvement of NF- κ B, ERK, and p38 MAPK in the cytokine and chemokine release from activated eosinophils upon exposure to diverse stimuli such as IL-5, IL-25, and various microbial products (21, 43, 44). To elucidate the involvement of NF- κ B, ERK, and p38 MAPK in regulating the cytokine and chemokine release induced by IL-17A, IL-17F, and IL-23, selective inhibitors that could suppress the activation of their corresponding signaling pathways were applied. Based on the results of a cytotoxicity assay, we used the optimal concentrations of JAK inhibitor AG490 (3 μ M), NF- κ B inhibitor BAY11-7082 (1 μ M), PI3K inhibitor LY294002 (5 μ M), ERK inhibitor U0126 (10 μ M), p38 MAPK inhibitor SB203580 (7.5 μ M), and JNK inhibitor SP600125 (3 μ M) for significant inhibitory effects without any cell toxicity. It was shown that SB203580 and BAY11-7082 could differentially down-regulate the chemokines CXCL1, CXCL8, and CCL4 induced by IL-17A, IL-17F, and IL-23. For the proinflammatory cytokines IL-1 β and IL-6, although BAY11-7082 could suppress the induction by both IL-17F and IL-23, U0126 and SB203580 could abolish the production of cytokines mediated by IL-17F and IL-23, respectively. Further investigation illustrated that inhibition of the NF- κ B, ERK, and p38 MAPK pathways alone could partially but significantly suppress the synergistic effects on IL-1 β and IL-6 release induced by combined treatment of IL-17F and IL-23. The results therefore suggested that the synergistic effects of combining IL-17F and IL-23 were dependent on the activation of all NF- κ B, ERK, and p38 MAPK pathways.

In conclusion, this is the first report on the activation of eosinophils mediated by IL-17A, IL-17F, and IL-23 for the induction of proinflammatory cytokines and chemokines. An important finding of our study is the synergistic effects of combining IL-17F and

IL-23 on the production of IL-1 β and IL-6, which are critical inducers for Th17 polarization. Along with previous findings on the participation of IL-17E in Th2 responses and airway hyperresponsiveness (45, 46), our results provide further support to the important role of the IL-17 family and Th17 lymphocytes in the amplification of allergic diseases such as allergic asthma. Moreover, the combined effects of IL-23 and IL-17F on eosinophils might provide new insight for linking infection and allergic diseases. In view of recent advances in the application of ERK, p38 MAPK, and NF- κ B inhibitors as potential anti-inflammatory agents in asthma (47, 48), our study of IL-17A, IL-17F, and IL-23 on eosinophil activation should provide new clues on the development of novel treatment for allergic diseases.

Disclosures

The authors have no financial conflict of interest.

References

- Kawaguchi, M., M. Adachi, N. Oda, F. Kokubu, and S. K. Huang. 2004. IL-17 cytokine family. *J. Allergy Clin. Immunol.* 114: 1265–1273.
- Kolls, J. K., and A. Linden. 2004. Interleukin-17 family members and inflammation. *Immunity* 21: 467–476.
- Weaver, C. T., R. D. Hatton, P. R. Mangan, and L. E. Harrington. 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu. Rev. Immunol.* 25: 821–852.
- Molet, S., Q. Hamid, F. Davoine, E. Nutku, R. Taha, N. Page, R. Olivenstein, J. Elias, and J. Chakir. 2001. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J. Allergy Clin. Immunol.* 108: 430–438.
- Kawaguchi, M., L. F. Onuchic, X. D. Li, D. M. Essayan, J. Schroeder, H. Q. Xiao, M. C. Liu, G. Krishnaswamy, G. Germino, and S. K. Huang. 2001. Identification of a novel cytokine, ML-1, and its expression in subjects with asthma. *J. Immunol.* 167: 4430–4435.
- Hizawa, N., M. Kawaguchi, S. K. Huang, and M. Nishimura. 2006. Role of interleukin-17F in chronic inflammatory and allergic lung disease. *Clin. Exp. Allergy* 36: 1109–1114.
- Steinman, L. 2007. A brief history of T_H17, the first major revision in the T_H1/T_H2 hypothesis of T cell-mediated tissue damage. *Nat. Med.* 13: 139–145.
- Hunter, C. A. 2005. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nat. Rev. Immunol.* 5: 521–531.
- McKenzie, B. S., R. A. Kastelein, and D. J. Cua. 2006. Understanding the IL-23-IL-17 immune pathway. *Trends Immunol.* 27: 17–23.
- Bettelli, E., M. Oukka, and V. K. Kuchroo. 2007. T(H)-17 cells in the circle of immunity and autoimmunity. *Nat. Immunol.* 8: 345–350.
- Fujiwara, M., K. Hirose, S. Kagami, H. Takatori, H. Wakashin, T. Tamachi, N. Watanabe, Y. Saito, I. Iwamoto, and H. Nakajima. 2007. T-bet inhibits both TH2 cell-mediated eosinophil recruitment and TH17 cell-mediated neutrophil recruitment into the airways. *J. Allergy Clin. Immunol.* 119: 662–670.
- Bullens, D. M., E. Truyen, L. Coteur, E. Dilissen, P. W. Hellings, L. J. Dupont, and J. L. Ceuppens. 2006. IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respir. Res.* 7: 135.
- Pirhonen, J., S. Matikainen, and I. Julkunen. 2002. Regulation of virus-induced IL-12 and IL-23 expression in human macrophages. *J. Immunol.* 169: 5673–5678.
- Happel, K. I., M. Zheng, E. Young, L. J. Quinton, E. Lockhart, A. J. Ramsay, J. E. Shellito, J. R. Schurr, G. J. Bagby, S. Nelson, and J. K. Kolls. 2003. Cutting edge: roles of Toll-like receptor 4 and IL-23 in IL-17 expression in response to *Klebsiella pneumoniae* infection. *J. Immunol.* 170: 4432–4436.
- Aggarwal, S., N. Ghilardi, M. H. Xie, F. J. De Sauvage, and A. L. Gurney. 2003. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J. Biol. Chem.* 278: 1910–1914.
- Humbles, A. A., C. M. Lloyd, S. J. McMillan, D. S. Friend, G. Xanthou, E. E. McKenna, S. Ghiran, N. P. Gerard, C. Yu, S. H. Orkin, and C. Gerard. 2004. A critical role for eosinophils in allergic airways remodeling. *Science* 305: 1776–1779.
- Walsh, G. M. 2001. Eosinophil granule proteins and their role in disease. *Curr. Opin. Hematol.* 8: 28–33.
- Nelson, H. S. 2007. Advances in upper airway diseases and allergen immunotherapy. *J. Allergy Clin. Immunol.* 119: 872–880.
- Weller, P. F. 1997. Human eosinophils. *J. Allergy Clin. Immunol.* 100: 283–287.
- Wong, C. K., W. K. Ip, and C. W. Lam. 2004. Biochemical assessment of intracellular signal transduction pathways in eosinophils: implications for pharmacotherapy. *Crit. Rev. Clin. Lab. Sci.* 24: 165–186.
- Wong, C. K., P. F. Cheung, W. K. Ip, and C. W. Lam. 2005. Interleukin-25-induced chemokines and interleukin-6 release from eosinophils is mediated by p38 mitogen-activated protein kinase, c-Jun N-terminal kinase, and nuclear factor- κ B. *Am. J. Respir. Cell Mol. Biol.* 33: 186–194.
- Cheung, P. F., C. K. Wong, W. K. Ip, and C. W. Lam. 2006. IL-25 regulates the expression of adhesion molecules on eosinophils: mechanism of eosinophilia in allergic inflammation. *Allergy* 61: 878–885.
- Chang, S. H., H. Park, and C. Dong. 2006. Act1 adaptor protein is an immediate and essential signaling component of interleukin-17 receptor. *J. Biol. Chem.* 281: 35603–35607.
- Qian, Y., C. Liu, J. Hartupce, C. Z. Altuntas, M. F. Gulen, D. Jane-Wit, J. Xiao, Y. Lu, N. Giltaiy, J. Liu, et al. 2007. The adaptor Act1 is required for interleukin 17-dependent signaling associated with autoimmune and inflammatory disease. *Nat. Immunol.* 8: 247–256.
- Lankford, C. S., and D. M. Frucht. 2003. A unique role for IL-23 in promoting cellular immunity. *J. Leukocyte Biol.* 73: 49–56.
- Moseley, T. A., D. R. Haudenschild, L. Rose, and A. H. Reddi. 2003. Interleukin-17 family and IL-17 receptors. *Cytokine Growth Factor Rev.* 14: 155–174.
- Kuestner, R. E., D. W. Taft, A. Haran, C. S. Brandt, T. Brender, K. Lum, B. Harder, S. Okada, C. D. Ostrander, J. L. Kreindler, et al. 2007. Identification of the IL-17 receptor related molecule IL-17RC as the receptor for IL-17F. *J. Immunol.* 179: 5462–5473.
- Hymowitz, S. G., E. H. Filvaroff, J. P. Yin, J. Lee, L. Cai, P. Risser, M. Maruoka, W. Mao, J. Foster, R. F. Kelley, et al. 2001. IL-17s adopt a cysteine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding. *EMBO J.* 20: 5332–5341.
- Parham, C., M. Chirica, J. Timans, E. Vaisberg, M. Travis, J. Cheung, S. Pflanz, R. Zhang, K. P. Singh, F. Vega, et al. 2002. A receptor for the heterodimeric cytokine IL-23 is composed of IL-12R β 1 and a novel cytokine receptor subunit, IL-23R. *J. Immunol.* 168: 5699–5708.
- Jones, C. E., and K. Chan. 2002. Interleukin-17 stimulates the expression of interleukin-8, growth-related oncogene- α , and granulocyte-colony-stimulating factor by human airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 26: 748–753.
- Harnes, T., M. J. Robertson, G. Sledge, S. Kelich, H. Nakshatri, H. E. Broxmeyer, and R. Hromas. 2001. Cutting edge: IL-17F, a novel cytokine selectively expressed in activated T cells and monocytes, regulates angiogenesis and endothelial cell cytokine production. *J. Immunol.* 167: 4137–4140.
- Kleinschek, M. A., U. Muller, S. J. Brodie, W. Stenzel, G. Kohler, W. M. Blumenschein, R. K. Straubinger, T. McClanahan, R. A. Kastelein, and G. Alber. 2006. IL-23 enhances the inflammatory cell response in *Cryptococcus neoformans* infection and induces a cytokine pattern distinct from IL-12. *J. Immunol.* 176: 1098–1106.
- Romagnani, S. 2002. Cytokines and chemoattractants in allergic inflammation. *Mol. Immunol.* 38: 881–885.
- Ferreira, M. A. 2003. Cytokine expression in allergic inflammation: systematic review of in vivo challenge studies. *Mediat. Inflamm.* 12: 259–267.
- Wong, C. K., and C. W. Lam. 2003. Clinical applications of cytokine assays. *Adv. Clin. Chem.* 37: 1–46.
- Lukacs, N. W., and K. K. Tekkanat. 2000. Role of chemokines in asthmatic airway inflammation. *Immunol. Rev.* 177: 21–30.
- Wu, Q., R. J. Martin, J. G. Rino, R. Breed, R. M. Torres, and H. W. Chu. 2007. IL-23-dependent IL-17 production is essential in neutrophil recruitment and activity in mouse lung defense against respiratory *Mycoplasma pneumoniae* infection. *Microbes Infect.* 9: 78–86.
- Molet, S., Q. Hamid, F. Davoine, E. Nutku, R. Taha, N. Pagé, R. Olivenstein, J. Elias, and J. Chakir. 2001. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J. Allergy Clin. Immunol.* 108: 430–438.
- Lacy, P., and R. Moqbel. 2000. Eosinophil cytokines. *Chem. Immunol.* 76: 134–155.
- Acosta-Rodriguez, E. V., G. Napolitani, A. Lanzavecchia, and F. Sallusto. 2007. Interleukins 1 β and 6 but not transforming growth factor- β are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat. Immunol.* 8: 942–949.
- Fedele, G., P. Stefanelli, F. Spensieri, C. Fazio, P. Mastrantonio, and C. M. Aiusello. 2005. *Bordetella pertussis*-infected human monocyte-derived dendritic cells undergo maturation and induce Th1 polarization and interleukin-23 expression. *Infect. Immun.* 73: 1590–1597.
- Underwood, D. C., R. R. Osborn, C. J. Kotzer, J. L. Adams, J. C. Lee, E. F. Webb, D. C. Carpenter, S. Bochnowicz, H. C. Thomas, D. W. Hay, and D. E. Griswold. 2000. SB 239063, a potent p38 MAP kinase inhibitor, reduces inflammatory cytokine production, airways eosinophil infiltration, and persistence. *J. Pharmacol. Exp. Ther.* 293: 281–288.
- Wong, C. K., W. K. Ip, and C. W. Lam. 2003. Interleukin-3, -5, and granulocyte macrophage colony-stimulating factor-induced adhesion molecule expression on eosinophils by p38 mitogen-activated protein kinase and nuclear factor- κ B. *Am. J. Respir. Cell Mol. Biol.* 29: 133–147.
- Wong, C. K., P. F. Cheung, W. K. Ip, and C. W. Lam. 2007. Intracellular signaling mechanisms regulating Toll-like receptor-mediated activation of eosinophils. *Am. J. Respir. Cell Mol. Biol.* 37: 85–96.
- Ballantyne, S. J., J. L. Barlow, H. E. Jolin, P. Nath, A. S. Williams, K. F. Chung, G. Sturton, S. H. Wong, and A. N. McKenzie. 2007. Blocking IL-25 prevents airway hyperresponsiveness in allergic asthma. *J. Allergy Clin. Immunol.* 120: 1324–1331.
- Tamachi, T., Y. Maezawa, K. Ikeda, S. Kagami, M. Hatano, Y. Seto, A. Suto, K. Suzuki, N. Watanabe, Y. Saito, et al. 2006. IL-25 enhances allergic airway inflammation by amplifying a TH2 cell-dependent pathway in mice. *J. Allergy Clin. Immunol.* 118: 606–614.
- Kaminska, B. 2005. MAPK signalling pathways as molecular targets for anti-inflammatory therapy: from molecular mechanisms to therapeutic benefits. *Biochim. Biophys. Acta* 1754: 253–262.
- Henderson, W. R., Jr., E. Y. Chi, J. L. Teo, C. Nguyen, and M. Kahn. 2002. A small molecule inhibitor of redox-regulated NF- κ B and activator protein-1 transcription blocks allergic airway inflammation in a mouse asthma model. *J. Immunol.* 169: 5294–5299.