

Activation of human eosinophils and epidermal keratinocytes by T_H2 cytokine IL-31: implication for the immunopathogenesis of atopic dermatitis

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Abstract

IL-31 is a novel T_H type 2 cytokine that can induce pruritus and dermatitis in mice resembling human atopic dermatitis (AD). Eosinophil infiltration in skin lesions is a predominant pathological feature of AD. In the present study, we investigated the effects of IL-31 on the activation of human eosinophils and epidermal keratinocytes. Eosinophils and keratinocytes were cultured either together or separately in the presence or absence of IL-31 stimulation. IL-31 could significantly induce the release of pro-inflammatory cytokines IL-1 β , IL-6 and AD-related chemokines CXCL1, CXCL8, CCL2 and CCL18 from eosinophils, via functional cell surface IL-31 receptor. Such induction was further enhanced upon the co-culture of eosinophils and keratinocytes, in which eosinophils were the main source for releasing pro-inflammatory cytokines and chemokines. The presence of transwell inserts in co-culture system demonstrated that the direct interaction between eosinophils and keratinocytes was required for IL-31-induced cytokine and chemokine release. Cell surface expression of adhesion molecule CD18 on eosinophils and intercellular adhesion molecule-1 on keratinocytes was up-regulated in the co-culture, and levels were further enhanced upon IL-31 stimulation. The interaction between eosinophils and keratinocytes under IL-31 stimulation was differentially mediated through intracellular mitogen-activated protein kinases, nuclear factor- κ B and phosphatidylinositol 3-kinase–Akt pathways. The above findings suggest a crucial immunopathological role of IL-31 in AD through activation of eosinophils–keratinocytes system.

Keywords: adhesion molecules, chemokines, eosinophils, IL-31, keratinocytes, mitogen-activated protein kinases, signaling molecules

Introduction

IL-31 is a novel T_H type 2 cytokine that is produced mainly by the CD45RO⁺ cutaneous lymphocyte antigen (CLA)⁺ T lymphocytes (1). When over-expressed in transgenic mice, IL-31 could induce pruritus and skin dermatitis resembling human atopic dermatitis (AD) (2, 3). Anti-IL-31 antibody could ameliorate the scratching behavior in murine model of AD (4). AD is a pruritic and chronically relapsing inflammatory skin disease with increasing prevalence (5, 6). Previous studies have reported that plasma IL-31 concentration was significantly elevated in AD patients compared with healthy individuals (7), and correlated positively with disease severity (8). In the inflammatory infiltrate of AD patients, CD45RO⁺ CLA⁺

lymphocytes were found to express high levels of IL-31 messenger RNA (7, 9, 10). IL-31 signaled via a heterodimeric receptor composed of IL-31RA and oncostatin M receptor β (OSMR β) (11, 12), which were expressed constitutively on epithelial cells and keratinocytes (2, 13). Comparison of skin from AD patients with healthy individuals showed higher expression levels of IL-31RA on epidermal keratinocytes in AD samples (1). Infiltrating cells such as macrophages, which were found to be more numerous in skin from patients with AD, also expressed IL-31RA (1). These cells responded to IL-31 stimulation and were likely to be involved in the dermatitis and pruritus of transgenic mice over-expressing IL-31.

Keratinocytes are known to play a crucial role in local inflammatory response by producing a panel of pro-inflammatory cytokines and chemokines that not only induce cellular infiltration but can also influence antigen-presenting cell-mediated skewing of the T-lymphocyte cytokine profile (14). Dysregulated production of these pro-inflammatory mediators is thought to be a contributing factor to the development of AD (15, 16). In AD patients, keratinocytes express higher levels of T_H2 chemokines including CCL17, CCL22, CCL24 and CCL26 than normal subjects (15, 16). Epidermal keratinocytes from AD patients were found to produce significantly higher level of CCL5, a potent eosinophil chemoattractant, following stimulation with tumor necrosis factor- α (17). All these chemokines have been implicated in the recruitment of T_H2 lymphocytes and eosinophils into the skin of patients with AD, eventually contributing to the inflammatory processes associated with the pathogenesis of AD (16–20).

The histology of AD is characterized by a dermal and epidermal inflammatory infiltrate including eosinophils (6, 21). There has been clear evidence for eosinophil infiltration and activation in AD skin lesions (22–24). Both tissue and blood eosinophilia are features in acute and chronic stages of AD and they were found to positively correlate with disease severity (25, 26). Moreover, eosinophilic granular protein deposition has been found in nearly all biopsies of AD lesions (27, 28). However, it was reported that eosinophils were not involved in the pathogenesis of non-intrinsic AD (29). Although the above studies have shown that eosinophils play crucial pathologic role in AD, their exact actions remain to be determined.

Interaction between eosinophils and keratinocytes represents a crucial mechanism of initiating local inflammatory response in AD (15). In this study, we investigated the effects of IL-31 on human eosinophils and epidermal keratinocytes upon their interaction, in terms of the release of cytokines and chemokines and expression of adhesion molecules. Although mitogen-activated protein kinases (MAPK) and nuclear factor kappa B (NF- κ B) have been extensively shown to play crucial roles in cytokine release and the modulation of adhesion molecules of eosinophils (30, 31), the signal transduction mechanism mediating the effects upon eosinophil–keratinocyte interaction has not been elucidated. Therefore, we also investigated the intracellular signaling pathway(s) involved in the above cellular events.

Methods

Materials

Recombinant human IL-31 was purchased from R&D Systems, Minneapolis, MN, USA. I κ B- α phosphorylation inhibitor BAY11-7082, extracellular signal-regulated kinase (ERK) inhibitor PD98059, JNK inhibitor SP600125, p38 MAPK inhibitor SB203580, phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 and JAK inhibitor AG490 were purchased from Calbiochem Corp., San Diego, CA, USA. SB203580 was dissolved in water while PD98059, LY294002, SP600125, AG490 and BAY11-7082 were dissolved in dimethyl sulfoxide (DMSO). In all studies, the concentration of DMSO was 0.1 % (vol/vol).

Endotoxin-free solutions

Cell culture medium was purchased from Gibco Invitrogen Corp., Grand Island, CA, USA, free of detectable LPS (<0.1 EU ml^{-1}). 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 lyase assay (sensitivity limit 12 pg ml^{-1} ; Biowhittaker Inc., Walkersville, MD, USA).

Isolation of human blood eosinophils from buffy coat and eosinophil culture

Fresh human buffy coat obtained from the healthy volunteers of 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^{-1} ; Amersham and Pharmacia Biotech, Uppsala, Sweden) for 30 min at 1000 $\times g$. The eosinophil-rich granulocyte fraction was collected and washed twice with cold PBS containing 2% fetal bovine serum (FBS) (Gibco). The cells were then incubated with anti-CD16 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) 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 (E Merck Diagnostica, Darmstadt, Germany). The isolated eosinophils were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS and 20 mM HEPES (Gibco).

Co-culture of eosinophils and keratinocytes

The human primary epidermal keratinocytes was purchased from ScienCell Research Laboratories, Carlsbad, CA, USA. Keratinocytes were grown in keratinocyte medium (ScienCell) in poly-L-lysine coated flasks or cell culture plates at 37°C with 5% CO_2 , 95% humidified air until confluence to cell monolayer. The medium was then replaced with RPMI 1640 medium supplemented with 10% FBS with or without eosinophils.

Co-culture of fixed eosinophils and keratinocytes

Confluent keratinocytes or eosinophils were treated with 1% paraformaldehyde in PBS on ice for 1 h to prevent the release of mediators from cells while preserving the cell membrane integrity to maintain intercellular interaction. After fixation, cells were washed at least 10 times with PBS containing 2% FBS, and fixed or unfixed eosinophils or keratinocytes were co-cultured in RPMI 1640 supplemented with 10% FBS.

Co-culture of eosinophils and keratinocytes in the presence of transwell inserts

To prevent direct interaction between eosinophils and keratinocytes in the co-culture, transwell inserts (pore size: 0.4 μM) (BD Biosciences Corp., San Jose, CA, USA) were used to separate the cells into two compartments. Confluent keratinocytes and eosinophils were cultured together in the presence of transwell inserts, in which eosinophils were placed in the upper compartment and keratinocytes were in the lower one.

Apoptosis assay

Apoptosis of eosinophils was assessed by the TACS™ Annexin V-FITC assay (Trevigen Inc., Gaithersburg, MD, USA) using flow cytometry (FACSCalibur, BD Biosciences Corp.) on eosinophils gated on the basis of their forward and side light scatter with any cell debris excluded from analysis. The population of viable cells was characterized by low mean fluorescence intensity (MFI) of both Annexin V-FITC and propidium iodide.

Quantitative analysis of IL-1 β , IL-6, CXCL1, CXCL8, CCL2 and CCL18

Concentrations of pro-inflammatory cytokines IL-1 β and IL-6 and chemokines CXCL8 and CCL2 in culture supernatant were quantitated with ELISA kits from BD Pharmingen Corp., San Diego, CA, USA. Concentrations of chemokines CXCL1 and CCL18 were measured with ELISA kits from R&D Systems.

Immunofluorescence staining and flow cytometry

To determine the expression of IL-31 receptor and adhesion molecules on the cell surface, the cells were washed and resuspended with cold PBS after preceding treatments. After blocking with 2% human pooled serum for 20 min at 4°C and washing with cold PBS, cells were incubated with mouse anti-human intercellular adhesion molecule (ICAM)-1, mouse anti-human CD18, mouse IgG $_{1\kappa}$ isotype (BD Pharmingen), mouse anti-human OSMR β (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rat anti-human CCR3, rat anti-human IgG2A isotypes, goat anti-human IL-31RA or goat IgG isotype antibodies (R&D Systems) 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, cells 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 phosphorylated Akt, phosphorylated ERK1/2, phosphorylated p38 MAPK, phosphorylated I κ B- α or mouse IgG $_1$ antibodies (BD Pharmingen) for 30 min at 4°C in dark. Cells were then washed, resuspended and subjected to analysis. Expression of surface adhesion molecules and intracellular phosphorylated signaling molecules of 10 000 viable cells was analyzed by flow cytometry (FACSCalibur) as MFI, which includes both the changes of target molecule expression in individual cell and the percentage of cells expressing the target molecules.

Assay of intracellular phosphorylated STAT3

Briefly, eosinophils were solubilized by lysis buffer (20 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1% Triton X-100, 10 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 0.05% 2-mercaptoethanol, 1 $\times$ protease inhibitors). Cell debris was removed by centrifugation at 14 000 $\times g$ for 15 min to obtain the soluble protein lysate. Equal amounts of extracted protein were subjected for the analysis of the level of the intracellular phosphorylated signal transducers and activator of transcription (STAT3) (Tyr 705) using MILLIPLEX MAP phospho-STAT3 (Tyr705) MAPmate kit with Luminex 200 system (Millipore Corp., Billerica, CA, USA).

Statistical analysis

All data were expressed as mean $\pm$ SD. Differences between groups were assessed by one-way analysis of variance. A probability $P < 0.05$ was considered to be significantly different. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) statistical software for Windows, version 10.1.4 (SPSS Inc., Chicago, IL, USA).

Results

Surface expression of IL-31 receptor on human eosinophils and epidermal keratinocytes

IL-31 signals through a heterodimeric receptor composed of IL-31RA and OSMR β (11, 12). We examined the surface expression of IL-31RA and OSMR β on eosinophils and keratinocytes. As shown in Fig. 1, IL-31RA and OSMR β were constitutively expressed on the surface of human eosinophils (Fig. 1A) and keratinocytes (Fig. 1B). Previous studies have demonstrated that the binding of IL-31 to its receptor complex could induce the phosphorylation of STAT3, which exerted a dominant function in the entire receptor complex (11, 32). In order to show that the IL-31 receptors on both cell types are functionally active, phosphorylation of STAT3 in eosinophils and keratinocytes upon IL-31 stimulation was examined. Figure 1(C) illustrates that upon IL-31 addition, phosphorylation of STAT3 was significantly increased in both eosinophils and keratinocytes, indicating that the IL-31 receptor expressed on both cell types were functionally active.

Effect of IL-31 on the apoptosis rate of eosinophils and keratinocytes

After 24 h incubation, the apoptosis rate was significantly reduced for IL-31 (50 ng ml $^{-1}$)-treated eosinophils (19.3 $\pm$ 4.0%) compared with that without IL-31 treatment (61.2 $\pm$ 7.1%) ($P < 0.001$, Fig. 2). The viability of eosinophils with or without IL-31 treatment was 80.7 $\pm$ 4.0 and 38.8 $\pm$ 7.1%, respectively ($P < 0.001$). However, IL-31 (50 ng ml $^{-1}$) did not exhibit any significant effect on the apoptosis rate and viability of keratinocytes after 24 h culture (both $P > 0.05$, data not shown).

Cytokine and chemokine release upon the interaction of eosinophils and keratinocytes activated by IL-31

Figure 3 shows the cytokine and chemokine release when eosinophils and keratinocytes were cultured either together or separately with or without IL-31 treatment. IL-31 (50 ng ml $^{-1}$) could significantly promote the release of pro-inflammatory cytokines IL-1 β (Fig. 3A), IL-6 (Fig. 3B), chemokines CXCL1 (Fig. 3C), CXCL8 (Fig. 3D), CCL2 (Fig. 3E) and CCL18 (Fig. 3F) from eosinophils, but no effect was observed on keratinocytes. Upon co-culture, the levels of all pro-inflammatory cytokines and chemokines were found to be higher than those of eosinophils alone or keratinocytes alone. In addition, levels of these cytokines and chemokines were found to be significantly higher in co-culture than in eosinophils alone under the stimulation of IL-31.

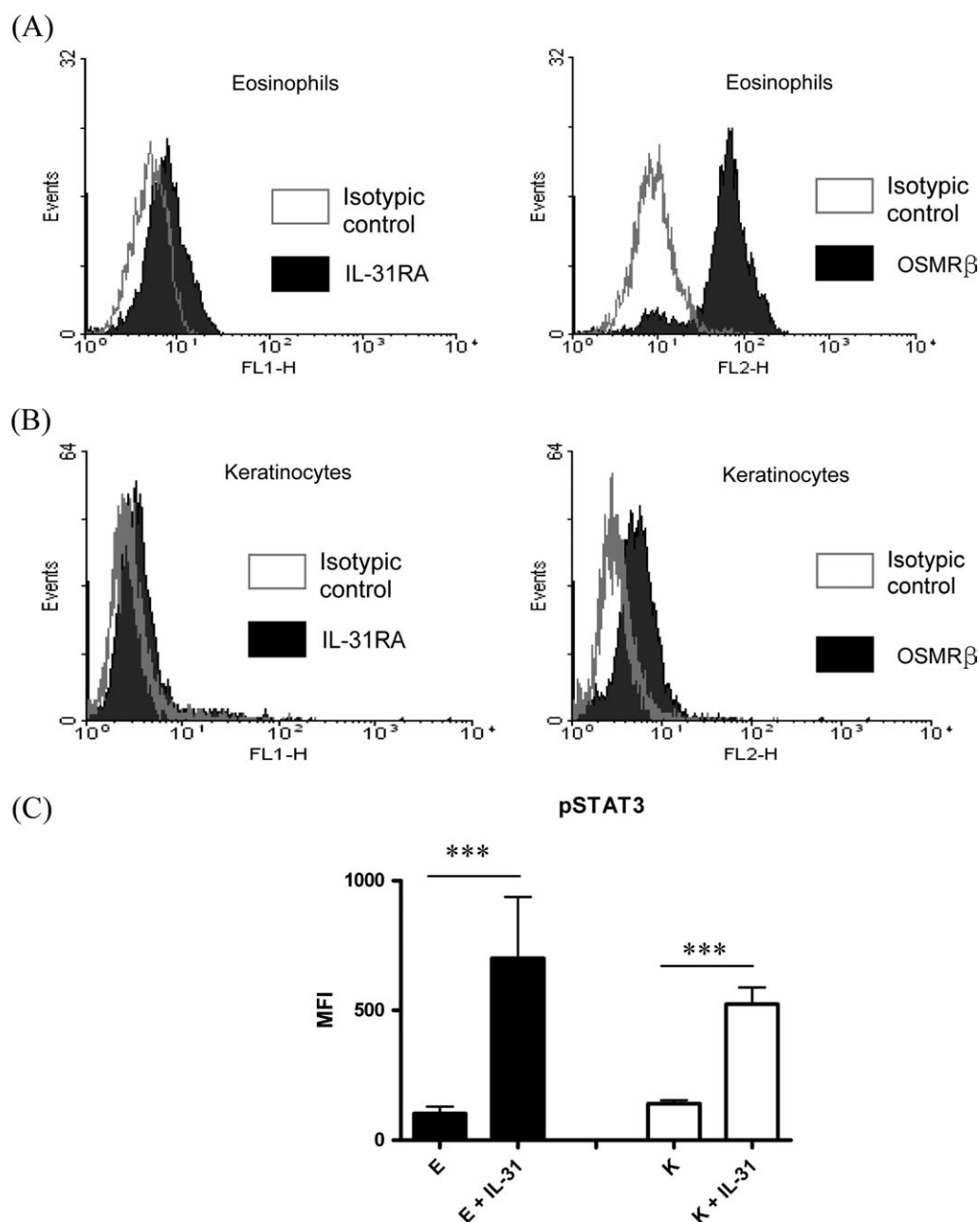


Fig. 1. Surface expression and activity of IL-31 receptor on human eosinophils and keratinocytes. Surface expression of IL-31RA and OSMRβ on (A) eosinophils (5×10^5 cells) and (B) keratinocytes (5×10^5 cells) was determined by flow cytometry. Results are expressed as histograms of relative cell counts with MFI. Triplicate experiments using eosinophils from three healthy blood donors were performed with essentially identical results and representative figures are shown. (C) Functional activity of the receptor complex for IL-31. IL-31 (50 ng ml^{-1}) was added to eosinophils and keratinocytes for 10 min. Phosphorylation of STAT3 (pSTAT3) in eosinophils and keratinocytes was quantitated by Luminex xMAP technology. Results are shown in MFI and expressed as the arithmetic mean $\pm$ SD of three independent experiments using eosinophils from three healthy blood donors. E: eosinophils; K: keratinocytes and *** $P < 0.001$ when compared between groups denoted by horizontal lines.

Source of the release of cytokines and chemokines in the co-culture system upon IL-31 stimulation

To investigate the source of cytokines and chemokines released in the co-culture supernatant, 1% paraformaldehyde was used to fix eosinophils or keratinocytes to prevent cytokine and chemokine release while preserving the cell membrane integrity to maintain intercellular interaction. We

compared the cytokine and chemokine levels in the co-culture of normal cells with the cells fixed with 1% paraformaldehyde (Fig. 4). In the co-culture of fixed keratinocytes and unfixed eosinophils, the stimulatory effects of co-culture on the release of IL-1β, IL-6, CXCL1, CXCL8, CCL2 and CCL18 were greatly suppressed, while the IL-31-induced stimulation was preserved (Fig. 4). On the contrary, fixation of eosinophils alone could almost completely abolish the

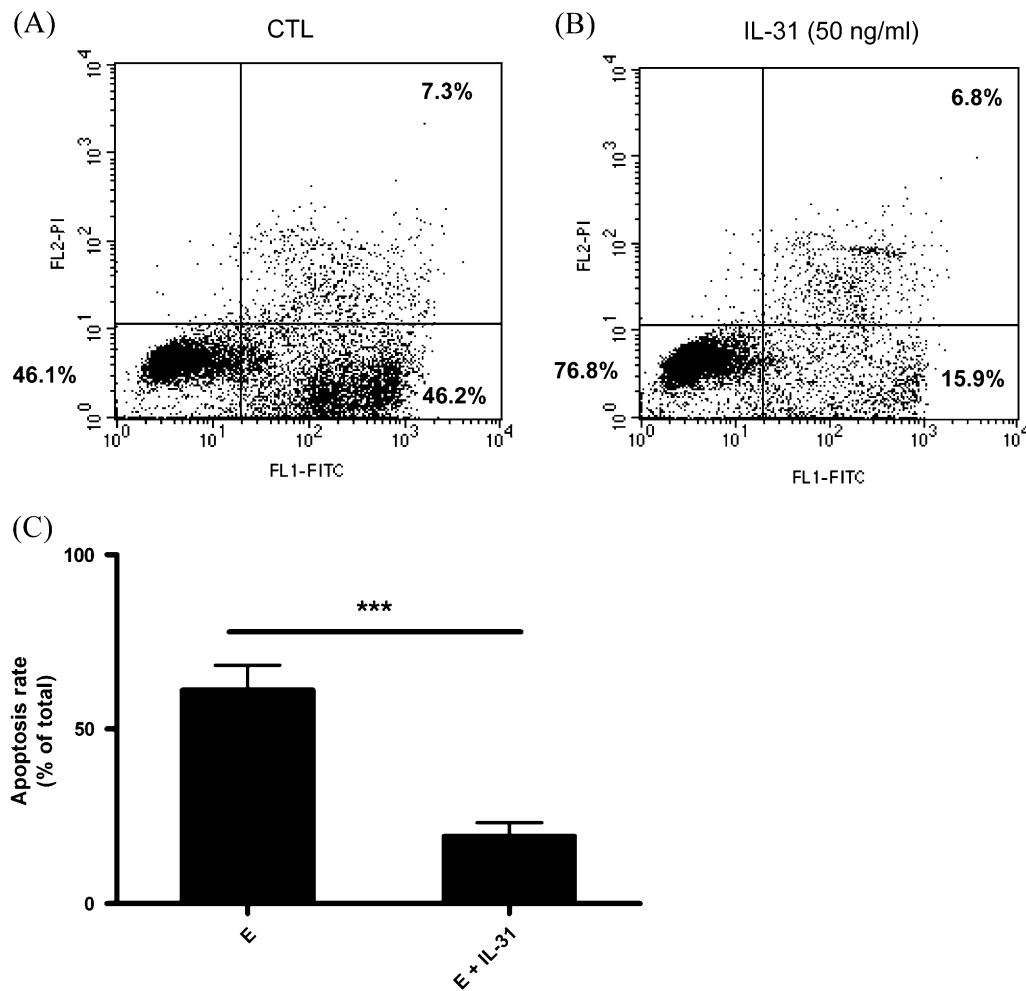


Fig. 2. Effects of IL-31 on viability of eosinophils. Eosinophils (5×10^5 cells) were cultured with or without IL-31 (50 ng ml⁻¹) for 24 h in a 24-well plate. Flow cytometric analysis was performed on gated cells on the basis of their forward and side light scatter with any cell debris excluded. The population of viable cells was characterized by low MFI of both FITC–Annexin V and propidium iodide (PI) (lower left quadrant), while that of early apoptotic cells was shown by high MFI of FITC–Annexin V but low PI (lower right quadrant), and late apoptotic cells represented by high MFI of both FITC–Annexin V and PI (upper right quadrant). Representative dot plots of (A) medium control (CTL) and (B) IL-31-treated eosinophils were shown from triplicate experiments using eosinophils from three healthy blood donors. (C) Bar chart of the apoptotic rate (mean + SD) of medium and IL-31-treated eosinophils with triplicate independent experiments was shown. E: eosinophils and *** $P < 0.001$.

secretion of cytokines and chemokines in co-culture with or without IL-31 stimulation, indicating that eosinophils were the main source for releasing IL-1 β , IL-6, CXCL8, CXCL1, CCL2 and CCL18 in co-culture upon IL-31 stimulation (Fig. 4). However, increases in IL-6 and CCL18 levels in co-culture were still observed when only eosinophils were fixed, indicating that keratinocytes contributed at least partially to the production of IL-6 in the co-culture system.

Direct interaction between eosinophils and keratinocytes is required for cytokine and chemokine release in IL-31-treated co-culture

To examine whether direct interaction was essential for IL-1 β , IL-6, CXCL8, CXCL1, CCL2 and CCL18 release in the co-culture upon IL-31 stimulation, transwell inserts (pore size: 0.4 μ M) were used to separate eosinophils and keratinocytes into two compartments in the co-culture system. Figure 5 shows that the presence of transwell inserts signifi-

cantly suppress the IL-31-induced secretion of IL-1 β , IL-6, CXCL1, CXCL8, CCL2 and CCL18 in co-culture. Without IL-31 stimulation, induction of IL-6, CXCL1, CXCL8, CCL2 and CCL18 release, but not IL-1 β , in co-culture was also significantly abolished in the presence of transwell insert (Fig. 5), implying that the release of these cytokines and chemokines in co-culture might depend on direct interaction between eosinophils and keratinocytes.

Effect of IL-31 on adhesion molecule expression on eosinophils and keratinocytes in co-culture system

As shown in Fig. 6, IL-31 could subtly but significantly up-regulate the surface expression of CD18, the integrin family member lymphocyte function-associated antigen-1 (LFA-1) (CD11a/CD18), on eosinophils alone (Fig. 6A) and ICAM-1 on keratinocytes alone (Fig. 6B). Upon co-culture, CD18 expression on eosinophils and ICAM-1 expression on keratinocytes were also subtly but significantly increased; while the

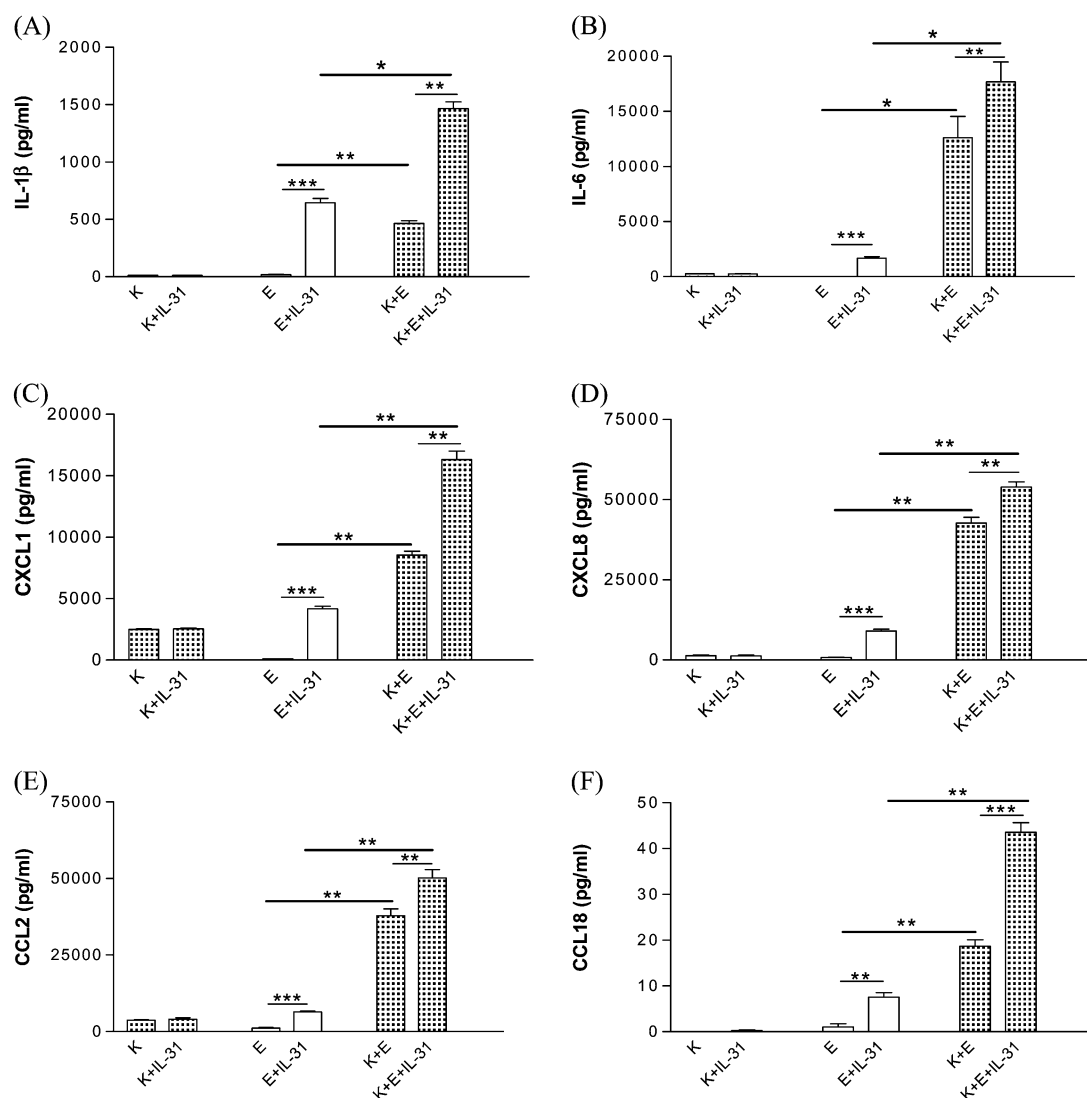


Fig. 3. Effect of IL-31 on the induction of (A) IL-1 β , (B) IL-6, (C) CXCL1, (D) CXCL8, (E) CCL2 and (F) CCL18 upon the interaction of eosinophils and keratinocytes. Eosinophils (5×10^5 cells) and confluent keratinocytes (1×10^5 cells) were cultured either together or separately with or without IL-31 (50 ng ml^{-1}) for 24 h. Cytokines and chemokines released in culture supernatant were determined by ELISA. Results are expressed as the arithmetic mean $\pm$ SD of three independent experiments using eosinophils from three healthy blood donors. E: eosinophils; K: keratinocytes and * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared between groups denoted by horizontal lines.

levels were further enhanced in the presence of IL-31. However, surface expression of ICAM-1 on eosinophils and that of CD18 on keratinocytes remained unchanged upon co-culture and the addition of IL-31 (Fig. 6A and B). Results in Fig. 6(C and D) indicated that anti-IL-31RA antibody but not control goat IgG could suppress the IL-31-induced cell surface expression of CD18 on eosinophils and ICAM-1 on keratinocytes. Results therefore verified the specific effect of IL-31 through IL-31 receptor for the activation of eosinophils and keratinocytes.

Intracellular signaling pathways involved in the interaction of eosinophils and keratinocytes under IL-31 stimulation

To investigate the underlying signaling mechanism(s), intracellular staining by flow cytometry was employed. After fixation and permeabilization, eosinophils and keratinocytes formed discrete populations and were gated on the basis of their forward and side light scatter (Fig. 7A). In order to dis-

tinguish the two populations, the cells were stained with anti-human CCR3 antibody because CCR3 is an exclusive chemokine receptor found on eosinophils, but not on keratinocytes. Figure 7(A) indicates that the gated population with lower forward side scatter (FSC), R1, showing positive CCR3 expression is eosinophils; while the one with higher FSC, R2, showing no CCR3 expression is keratinocytes. Figure 7(B–E) shows that upon stimulation of IL-31 and co-culture, ERK, p38 MAPK, Akt and I κ B- α were phosphorylated in eosinophils, while activation of p38 MAPK, Akt and I κ B- α was found in keratinocytes.

To verify the involvement of the above signaling pathways in the interaction between eosinophils and keratinocytes upon IL-31 stimulation, various signaling molecule inhibitors were used. As shown in Figs. 8 and 9, PI3K inhibitor LY294002 ($5 \mu\text{M}$), ERK inhibitor PD98059 ($10 \mu\text{M}$), p38 MAPK inhibitor SB203580 ($7.5 \mu\text{M}$) and NF- κ B inhibitor

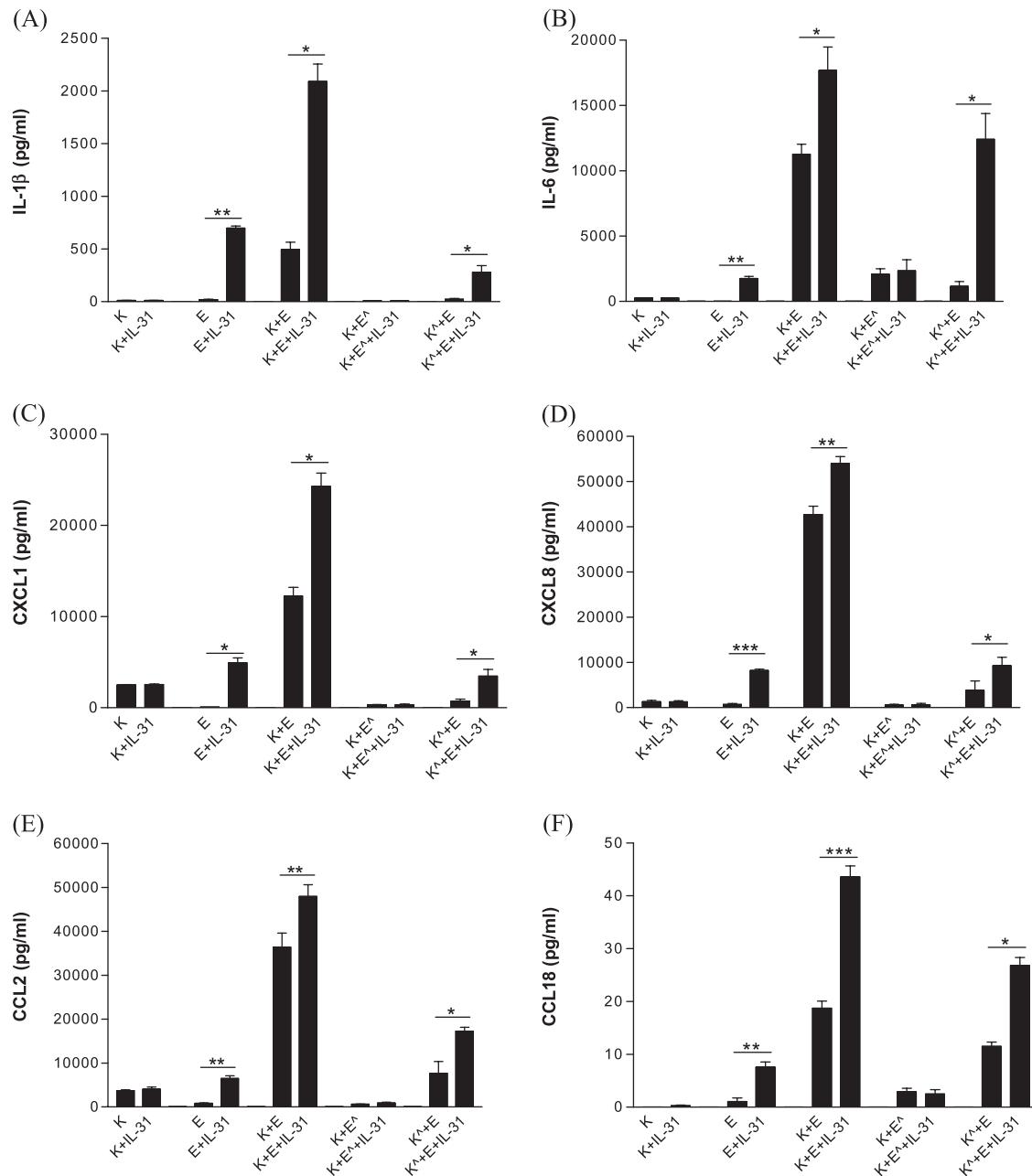


Fig. 4. Source of (A) IL-1 β , (B) IL-6, (C) CXCL1, (D) CXCL8, (E) CCL2 and (F) CCL18 in co-culture of eosinophils and keratinocytes under IL-31 stimulation. Eosinophils (5×10^5 cells) and confluent keratinocytes (1×10^5 cells) were treated with or without 1% paraformaldehyde for 1 h on ice prior to being cultured together with or without IL-31 (50 ng ml^{-1}) for 24 h. Cytokines and chemokines released in culture supernatant were determined by ELISA. Results are expressed as the arithmetic mean $\pm$ SD of three independent experiments using eosinophils from three healthy blood donors. E: unfixed eosinophils; E $^{\wedge}$: fixed eosinophils; K: unfixed keratinocytes; K $^{\wedge}$: fixed keratinocytes and * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared between groups denoted by horizontal lines.

BAY11-7082 ($1 \mu\text{M}$) could significantly suppress the IL-31-induced release of cytokines and chemokines, and also the expression of adhesion molecules of eosinophils and keratinocytes.

Discussion

Although the presence of eosinophils in the inflammatory infiltrate of AD has long been recognized (21–26), their pathogenic role in the development of local inflammation of the

disease is not well understood. Epidermal keratinocytes are known to play crucial role in AD by recruiting inflammatory cells into skin lesions of AD patients (15). Investigation of the interaction between eosinophils and keratinocytes may therefore help to elucidate the mechanism of initiating local inflammatory response in AD. In this study, we developed an *in vitro* co-culture system to elucidate the mechanism by which eosinophils interact with keratinocytes for inflammatory responses.

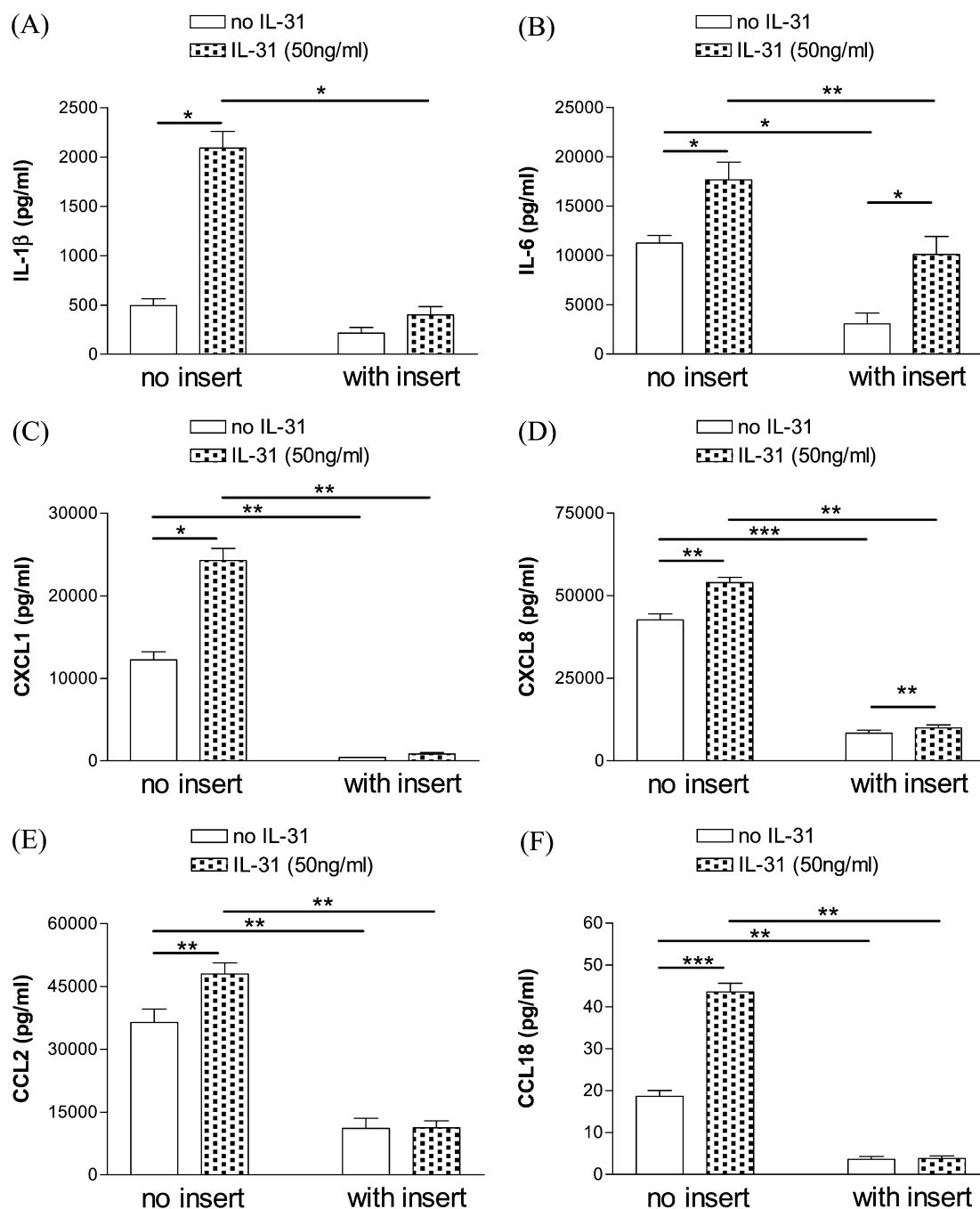


Fig. 5. Effect of transwell inserts on the induction of (A) IL-1 β , (B) IL-6, (C) CXCL1, (D) CXCL8, (E) CCL2 and (F) CCL18 in co-culture of eosinophils and keratinocytes under IL-31 stimulation. Eosinophils (5×10^5 cells) and confluent keratinocytes (1×10^5 cells) were cultured together with or without IL-31 (50 ng ml^{-1}) in the presence or absence of transwell inserts for 24 h. Cytokines and chemokines released in culture supernatant were determined by ELISA. Results are expressed as the arithmetic mean + SD of three independent experiments using eosinophils from three healthy blood donors. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared between groups denoted by horizontal lines.

Recently, there has been mounting evidence suggesting an important role of IL-31 in the pathogenesis of AD (1, 2, 7). IL-31 receptor was reported to be expressed on epithelial cells, keratinocytes, infiltrating macrophages in skin biopsy specimens from patients with AD (2, 13). In this study, we showed that primary human eosinophils and keratinocytes constitutively expressed functionally active receptor

complex for IL-31. This prompted us to further investigate the action(s) of IL-31 on the co-culture of eosinophils and keratinocytes.

We demonstrated that IL-31 could delay apoptosis of eosinophils, and significantly stimulate eosinophils, but not keratinocytes, to secrete higher levels of pro-inflammatory cytokines IL-1 β , IL-6, chemokines CXCL1, CXCL8, CCL2

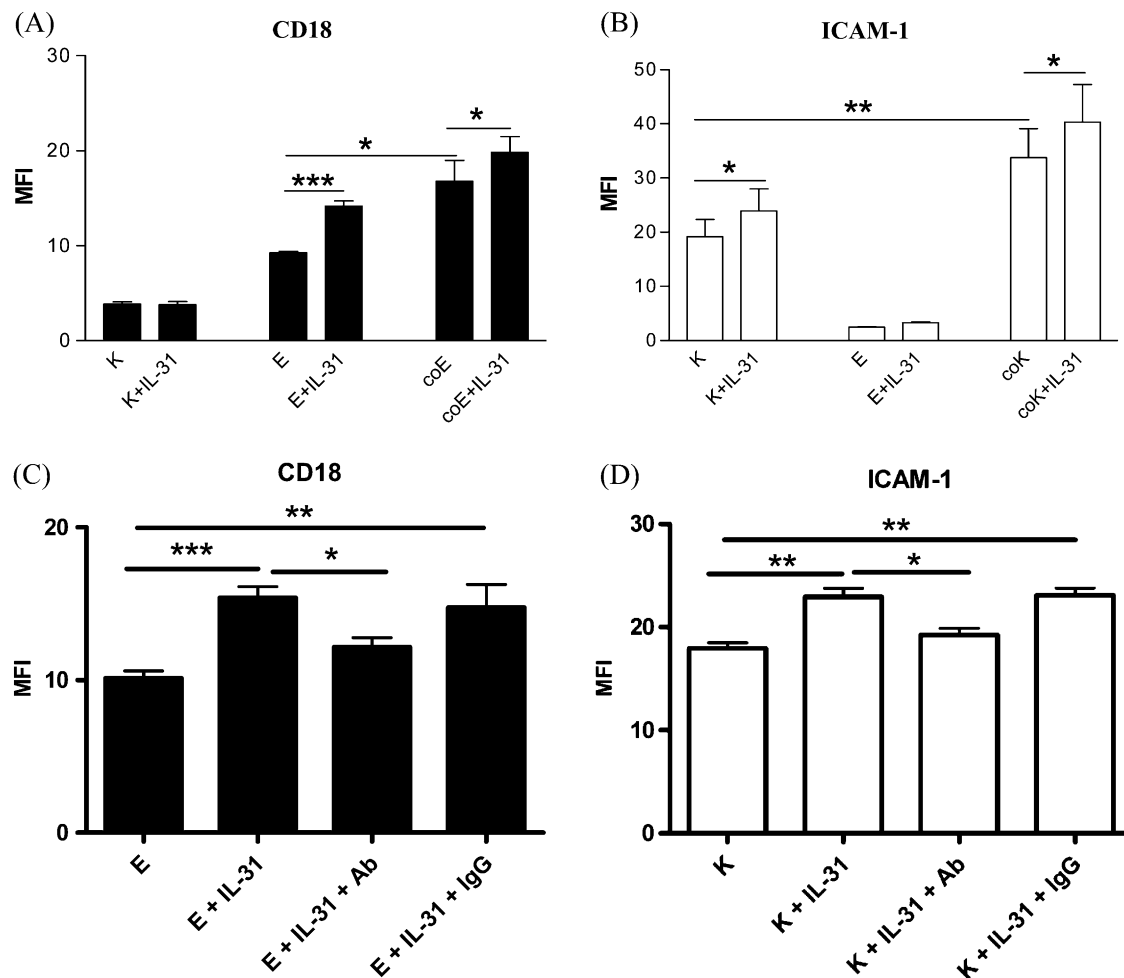


Fig. 6. Effect of IL-31 on the surface expression of (A) CD18 on eosinophils and (B) ICAM-1 on keratinocytes in the co-culture. Eosinophils (5×10^5 cells) and confluent keratinocytes (1×10^5 cells) were cultured either together or separately with or without IL-31 (50 ng ml^{-1}) for 16 h. Surface expression of (A) CD18 and (B) ICAM-1 on 10 000 cells was analyzed by flow cytometry as MFI. (C, D) Eosinophils ($5 \times 10^5/\text{well}$) were cultured with or without anti-IL-31RA antibody or negative control goat IgG ($10 \mu\text{g ml}^{-1}$) together with IL-31 (50 ng ml^{-1}) for 16 h. Surface expression of CD18 and ICAM-1 on 10 000 eosinophils and keratinocytes, respectively, was analyzed by flow cytometry as MFI. Results have been normalized by subtracting appropriate isotypic control and are expressed as the arithmetic mean + SD of three independent experiments using eosinophils from three healthy blood donors. E: eosinophils only; coE: eosinophils in co-culture; K: keratinocytes only; coK: keratinocytes in co-culture; Ab: anti-IL-31RA antibody; IgG: control goat IgG and $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ when compared between groups denoted by horizontal lines.

and CCL18. Co-culture of eosinophils and keratinocytes could also stimulate the secretion of these pro-inflammatory cytokines and chemokines, whose levels were found to be further up-regulated by IL-31. Although the prolonged survival of eosinophils might also contribute to the higher cytokine and chemokine levels, the induction of cytokines and chemokines should be due to the result of IL-31 stimulation on eosinophils because the anti-apoptotic effect of IL-31 on eosinophils was <1 -fold, while the cytokine and chemokine levels could be up to 1000-fold under IL-31 stimulation. In order to investigate the source of cytokines and chemokines released in the co-culture system, we compared the cytokine and chemokine levels in co-culture of normal cells with the cells fixed with 1% paraformaldehyde. Our findings suggested that eosinophils, instead of keratinocytes, were the main source for releasing these cytokines and chemokines in co-culture upon IL-31 stimulation.

It is widely accepted that both pro-inflammatory cytokines IL-1 β and IL-6 can activate T_H lymphocytes and induce the T_H2 immune responses. IL-1 β activates macrophages and facilitates local accumulation of neutrophils, while IL-6 induces the synthesis of acute-phase proteins and mediates various inflammatory responses (33, 34). The induction of IL-1 β and IL-6 in the co-culture of eosinophils and keratinocytes upon IL-31 stimulation might therefore contribute to the development of local inflammatory responses found in AD. For chemokines, CCL2 and CCL18 are known to be AD-associated chemokines (16). CCL18 was found to be the most highly expressed specific chemokine in epidermis of patients with AD, but not in other inflammatory skin or autoimmune diseases such as psoriasis and cutaneous lupus erythematosus (16, 35, 36). CCL18 has been shown to be secreted by eosinophils and keratinocytes during allergic inflammation (37, 38). CCL18 initiates and amplifies AD by

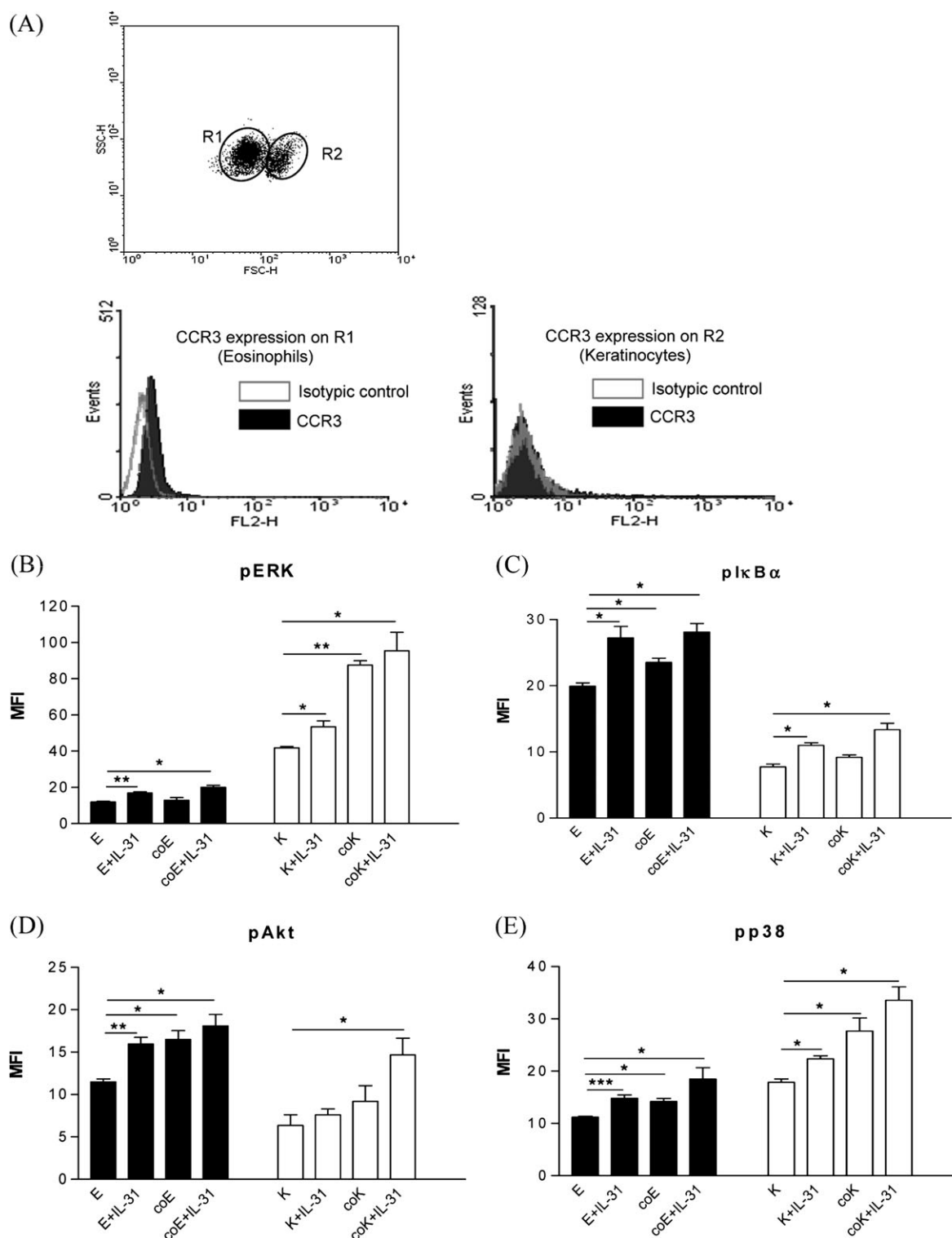


Fig. 7. Activation of ERK, p38 MAPK, Akt and I κ B- α in co-culture of eosinophils and keratinocytes under IL-31 stimulation. Eosinophils (5×10^5 cells) and confluent keratinocytes (1×10^5 cells) were cultured either together or separately with or without IL-31 (50 ng ml^{-1}) stimulation for 10 min. (A) After fixation and permeabilization, eosinophils (5×10^5 cells) and keratinocytes (1×10^5 cells) formed discrete populations and were gated based on forward and side light scatter with CCR3 staining to distinguish eosinophils (R1) and keratinocytes (R2). The intracellular contents of phosphorylated (B) ERK, (C) p38 MAPK, (D) Akt and (E) I κ B- α of permeabilized eosinophils and keratinocytes were measured by intracellular immunofluorescence staining using flow cytometry. Results are shown in MFI subtracting corresponding isotypic control and are expressed as the arithmetic mean $\pm$ SD of three independent experiments using eosinophils from three healthy blood donors. E: eosinophils only; coE: eosinophils in co-culture; K: keratinocytes only; coK: keratinocytes in co-culture and * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared between groups denoted by horizontal lines.

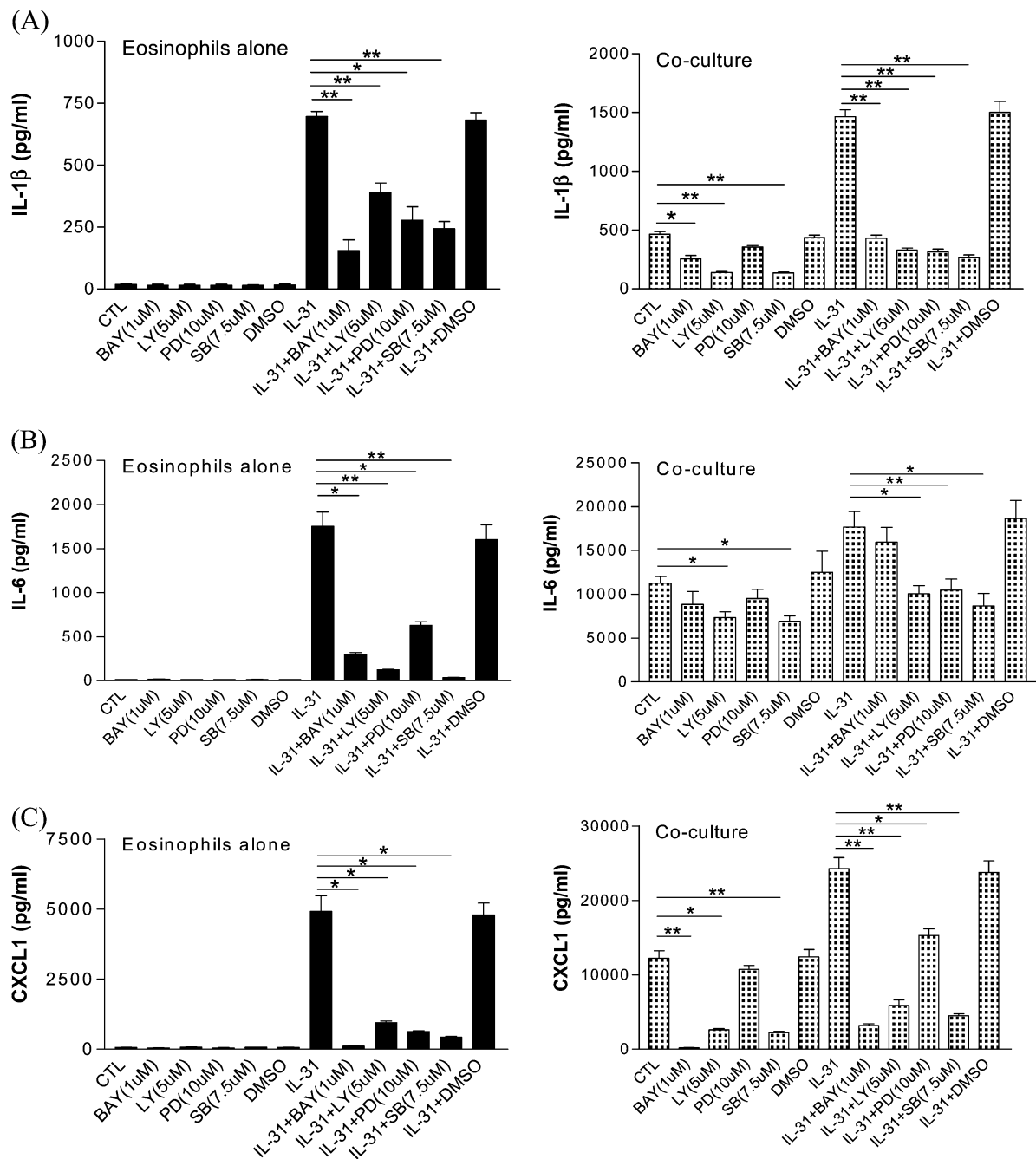


Fig. 8. Effects of signaling molecule inhibitors on the release of (A) IL-1 β , (B) IL-6, (C) CXCL1, (D) CXCL8, (E) CCL2 and (F) CCL18 from eosinophils alone and co-culture with or without IL-31 stimulation. Eosinophils (1×10^6 cells) cultured alone or together with confluent keratinocytes (2×10^5 cells) were pretreated with BAY11-7082, LY294002, PD98059 or SB203580 for 45 min, followed by incubation with or without IL-31 (50 ng ml^{-1}) in the presence of inhibitors for further 24 h. Released cytokines and chemokines in culture supernatant were determined by ELISA. Results are expressed as the arithmetic mean $\pm$ SD of three independent experiments using eosinophils from three healthy blood donors. DMSO (0.1%) was used as the vehicle control. BAY: BAY11-7082; LY: LY294002; PD: PD98059; SB: SB203580 and $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ when compared between groups denoted by horizontal lines.

interacting with CLA $^{+}$ memory T lymphocytes in peripheral blood, leading to the recruitment of T lymphocytes to the sites of atopic skin inflammation (36). CCL2 is known to recruit dendritic cell precursors from circulation to the sites of atopic skin inflammation, while both CXCL8 and CXCL1 are potent chemoattractants for neutrophils and basophils

(39–41). The induction of these chemokines might amplify the inflammatory responses in AD by recruiting various inflammatory cells into the skin.

In an attempt to verify whether direct contact between eosinophils and keratinocytes was required for the induction of cytokines and chemokines in co-culture system under

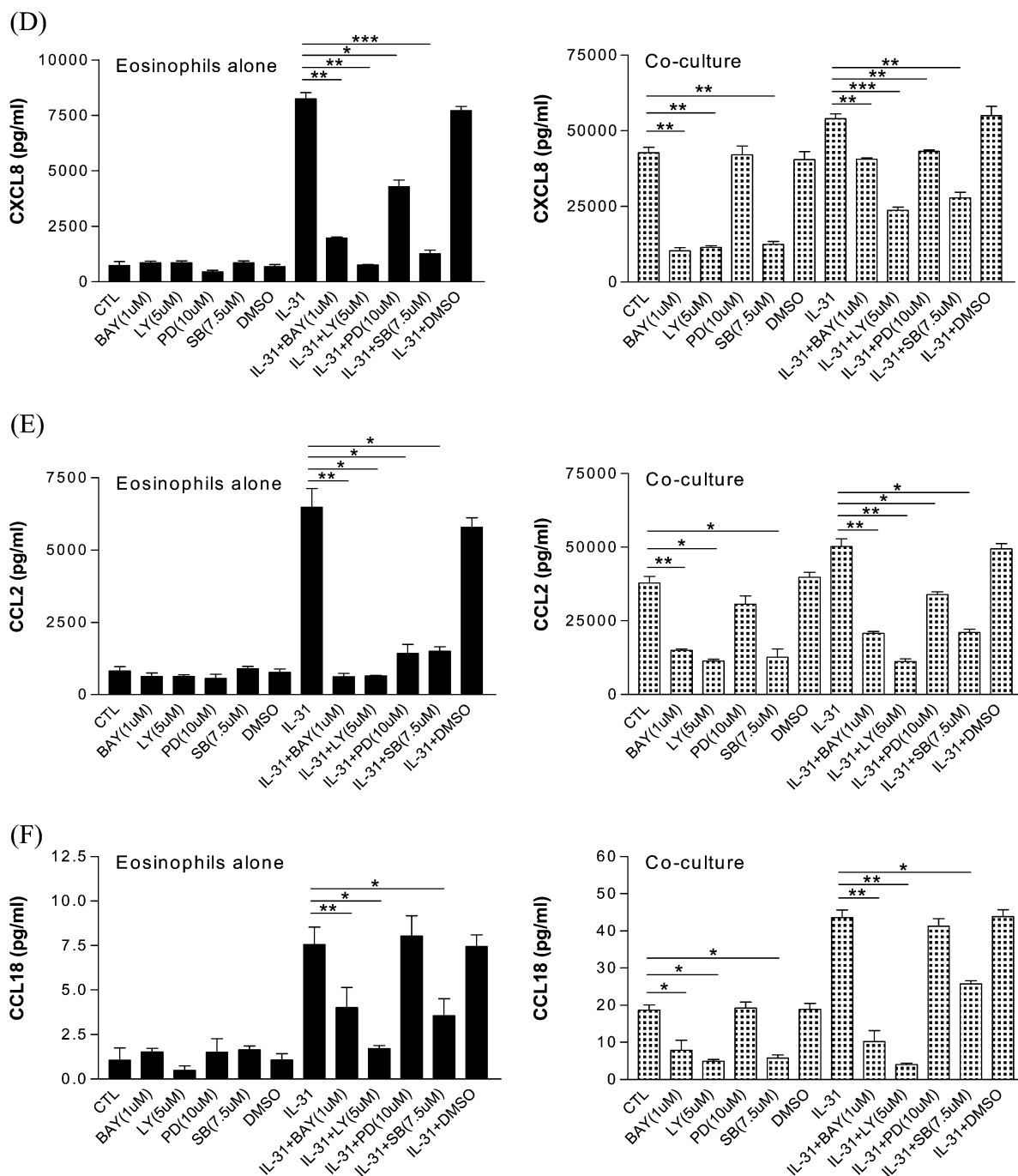


Fig. 8. Continued.

IL-31 stimulation, permeable transwell barriers were applied. Results showed that the presence of transwell inserts could significantly suppress the IL-31-induced secretion of IL-1 β , CXCL1, CXCL8, CCL2 and CCL18 in co-culture. Although we cannot rule out the possibility that there exist other mechanisms regulating the release of these cytokines and chemokines in co-culture upon IL-31 stimulation, direct interaction between eosinophils and keratinocytes should at least partially contribute to the stimulation of the production of these

cytokines and chemokines. One possible mechanism for the stimulatory effect on the induction of cytokines and chemokines might be the direct activation of cells mediated through the interaction between their surface adhesion molecules. Therefore, we further examined the expression of adhesion molecules on eosinophils and keratinocytes in co-culture upon IL-31 stimulation.

Recruitment of eosinophils to the sites of cutaneous inflammation is of critical importance for the pathogenesis of

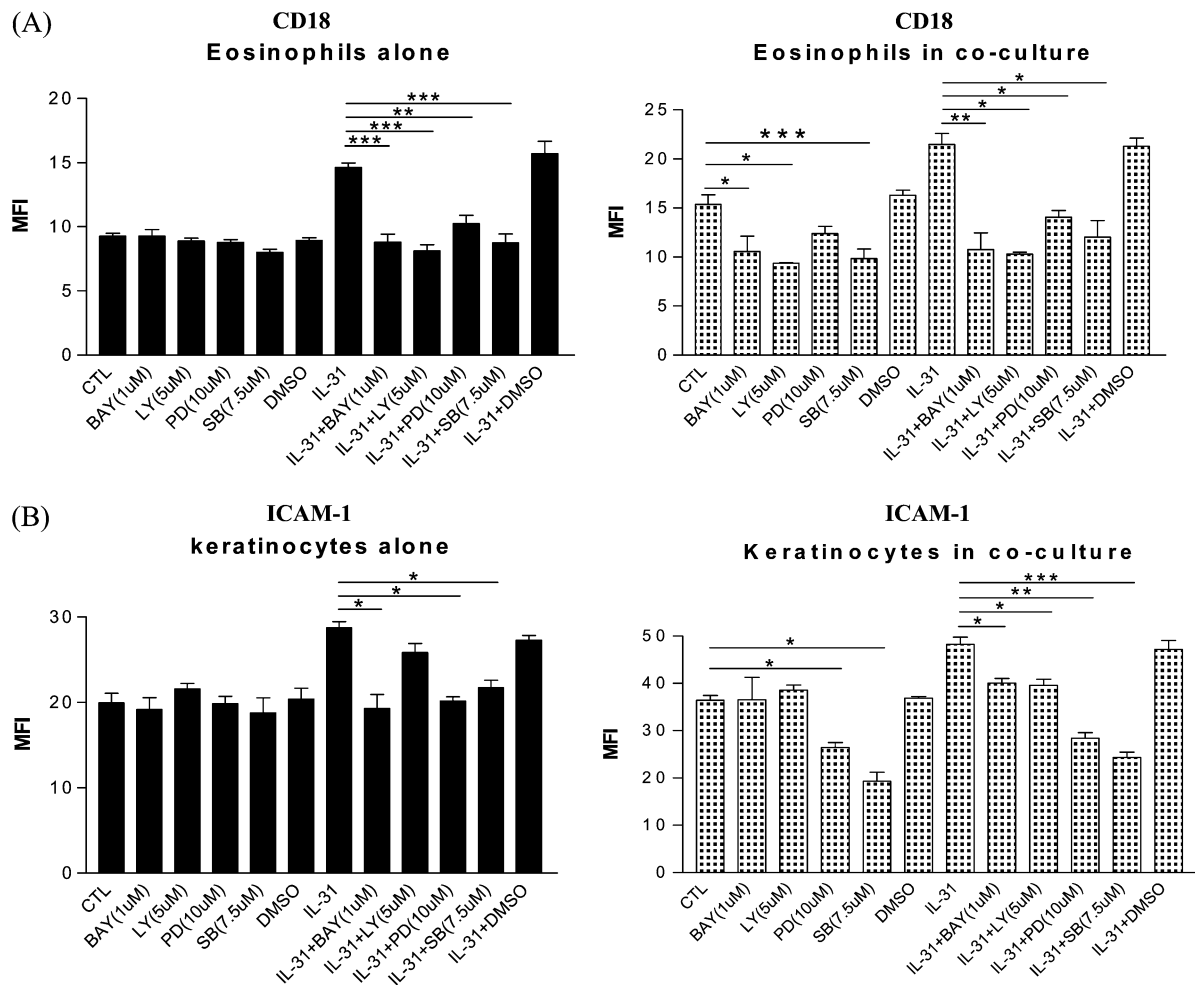


Fig. 9. Effects of signaling molecule inhibitors on the cell surface expression of (A) CD18 on eosinophils and (B) ICAM-1 on keratinocytes in the co-culture. Eosinophils (1×10^6 cells) and confluent keratinocytes (2×10^5 cells), either cultured separately or together, were pretreated with BAY11-7082, LY294002, PD98059 or SB203580 for 45 min, followed by incubation with or without IL-31 (50 ng ml^{-1}) in the presence of inhibitors for further 16 h. Surface expression of CD18 and ICAM-1 on 10 000 cells was analyzed by flow cytometry as MFI. Results have been normalized by subtracting appropriate isotypic control and are expressed as the arithmetic mean $\pm$ SD of three independent experiments using eosinophils from three healthy blood donors. DMSO (0.1%) was used as the vehicle control. BAY: BAY11-7082; LY: LY294002; PD: PD98059; SB: SB203580 and $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ when compared between groups denoted by horizontal lines.

AD (28). Following the endothelium-regulated processes of rolling, tethering, adhesion and extravasation, eosinophils are attracted to the keratinocytes by chemoattractants and maintained by adhesion molecules (42). ICAM-1 is a crucial adhesion molecule present on keratinocytes and plays essential role in cell adherence. ICAM-1 interacts with the highest affinity to the integrin family member LFA-1 (CD11a/CD18) (43, 44). We found that both ICAM-1 on keratinocytes and CD18 on eosinophils were significantly enhanced in co-culture. Besides, further up-regulation of the adhesion molecule levels was found upon addition of IL-31. Such ICAM-1/CD18 interaction might provide a potential stimulation on the release of cytokines and chemokines in the co-culture system activated by IL-31.

Regarding the signal transduction mechanism, we used a rapid and quantitative method, intracellular staining using flow cytometry, to investigate the phosphorylation levels of signaling molecules in permeabilized eosinophils and kerati-

nocytes in co-culture under IL-31 stimulation. IL-31 could activate ERK in eosinophils, it may therefore account for the delayed apoptosis of eosinophils. Our results demonstrated that in the co-culture system, PI3K-Akt, p38 MAPK and NF- κ B pathways were activated in eosinophils, while only ERK and p38 MAPK were phosphorylated in keratinocytes. NF- κ B is known to play a pivotal role in the transcription of a variety of pro-inflammatory cytokines and chemokines (45). Activation of NF- κ B pathway in eosinophils, but not keratinocytes, might explain our observed phenomenon that eosinophils are the main source for the pro-inflammatory cytokines and chemokines released in the co-culture system instead of keratinocytes.

Using a pharmacological approach, we further elucidated the involvement of above signaling pathways in the interaction of eosinophils and keratinocytes in co-culture under stimulation of IL-31. We found that the production of cytokines and chemokines, and adhesion molecule expression

of eosinophils and keratinocytes induced by IL-31 could be significantly suppressed by specific inhibitors of PI3K–Akt, ERK, p38 MAPK and NF- κ B pathways. The results from these inhibition experiments were therefore consistent with that of intracellular staining by flow cytometry.

Our group has previously demonstrated the involvement of PI3K–Akt, ERK, p38 MAPK and NF- κ B pathways in regulating adhesion molecule expression, cytokine and chemokine release of activated eosinophils upon exposure to different stimuli such as leptin, T_H17 cytokines and various microbial products (30, 46, 47). Besides, we also reported the involvement of p38 MAPK and NF- κ B in activated eosinophils in co-culture with epithelial cells upon stimulation by Der p1 (48). Therefore, it is logical that the above pathways are commonly involved in IL-31-induced effects on eosinophils cultured with keratinocytes. Moreover, our findings on signaling mechanism in keratinocytes concurred with some previous studies that showed the involvement of PI3K–Akt, MAPKs and NF- κ B pathways in regulating cytokine and chemokine release from keratinocytes (49–51). Similar to that of eosinophils, MAPKs and NF- κ B are also the central cascades for regulating cytokine and chemokine release and adhesion molecule expression of human epidermal keratinocytes.

In conclusion, this is the first report on the role of IL-31 in the interaction between human eosinophils and keratinocytes. Our results demonstrated a stimulatory effect induced by co-culture of eosinophils and keratinocytes on cytokine and chemokine release and adhesion molecule expression. An important finding is that the above stimulatory effects could be significantly enhanced by the novel AD-related T_H2 cytokine IL-31. Together with some previous reports on the participation of IL-31 in pruritus and dermatitis in animal models (2, 3), our results provide further support to the facilitating role of IL-31 in the pathogenesis of AD. Since the interaction of eosinophils and keratinocytes under IL-31 stimulation was found to be mediated through PI3K–Akt, ERK, p38 MAPK and NF- κ B pathways, our study should also provide new clues on the development of novel treatment for AD. In order to further elaborate the immunopathological roles of IL-31 in AD, future study of the IL-31 activating effects on skin-resident eosinophils obtained from AD patients is needed.

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Conflict of Interest

None declared.

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