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ORIGINAL ARTICLE

Roles of Bcl-2 and caspase-9 and -3 in CD30-induced human eosinophil apoptosis



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Abstract *Background/purpose:* Activation of cell surface CD30 by immobilized anti-CD30 monoclonal antibodies (mAb) induces strong apoptosis in human eosinophils. This anti-CD30 mAb-induced eosinophil apoptosis is inhibited by the addition of inhibitors of p38, ERK1/2 mitogen-activated protein kinases, and phosphatidylinositol 3-kinase. However, there is little data investigating the role of Bcl-2 and caspases in eosinophil apoptosis induced by anti-CD30 mAb. We sought to determine whether anti-CD30 mAb induces human eosinophil apoptosis via Bcl-2 and caspase pathways.

Methods: Peripheral blood was drawn from 37 healthy volunteers. The CD30 expression on eosinophils was measured at various time points. Eosinophils were then cultured in plates pre-coated with anti-CD30 mAb (clone Ber-H8), isotype control immunoglobulin G₁, interleukin (IL)-5, or dexamethasone. Western blot analysis was performed to determine the expression of Bcl-2, procaspase-8, -9, and -3, and caspase-8, -9, and -3 after cross-linking of CD30. Human eosinophils were also cultured in plates precoated with anti-CD30 mAb (clone Ber-H8) in the

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presence or absence of caspase-9 or -3 inhibitors. Eosinophil apoptosis was assessed using flow cytometry.

Results: The addition of anti-CD30 mAb significantly increased eosinophil apoptosis compared with controls. In western blot analysis, the addition of anti-CD30 mAb significantly decreased the expression of Bcl-2 and procaspase-9 and -3 and increased the expression of caspase-9 and -3. The addition of caspase-9 or -3 inhibitors decreased anti-CD30 mAb-induced human eosinophil apoptosis. Procaspase-8 or caspase-8 expression was not changed in response to various stimuli.

Conclusion: Anti-CD30 mAb-induced human eosinophil apoptosis is likely to be mediated through Bcl-2 and caspase-9 and -3.

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Introduction

Eosinophils are resident in various organs such as the respiratory tract, the gastrointestinal tract, mammary glands, and bone marrow. They play an important role in these organs and in maintaining immune homeostasis of these organs.¹ In T-helper 2 (Th2)-type immune response, eosinophils are recruited into inflammation sites, where they produce various kinds of cytokines and chemokines and release toxic granule proteins in response to diverse physiological and artificial stimuli.¹ These molecules may regulate inflammatory immune responses, cause tissue damage, and promote tissue repair.² Eosinophils can also present antigens to naïve and memory T cells and trigger and/or intensify antigen-specific immune responses.¹

Given that eosinophils play essential roles in immune responses, such as allergic diseases, as both effector and modulatory cells, many studies have been conducted to reduce allergic inflammation by selectively inducing apoptosis of eosinophils.^{3–7} We have previously shown that eosinophil survival and apoptosis are regulated by CD9⁸ and FcγRII.⁹ The focus of the previous studies was directed toward the receptors found on the surface of human eosinophils in an attempt to induce eosinophil apoptosis by cross-linking of the surface molecules.^{3–7,10,11} Among them, CD30, a member of the tumor necrosis factor receptor (TNFR) family, has been found to exist on the eosinophil surface.^{3,4,7,12} Although there have been a few studies demonstrating that cross-linking of CD30 on the surface of human eosinophil can induce apoptosis through an intracellular signaling pathway, limited studies have been carried out to determine the mechanism underlying the eosinophil apoptosis after cross-linking of CD30 molecules on the surface of eosinophils.^{3,4,7,12}

Neutrophils, mast cells, T cells, and dendritic cells are important effector cells in allergic diseases. Many proteins are known to play a role in regulating human cell apoptosis. Studies have shown that Bcl-2^{13–17} and caspases^{18–21} play a crucial role in apoptosis in various types of human hematopoietic cells. However, there are few studies in the literature clarifying the possible role of Bcl-2 and caspases in human eosinophil apoptosis after cross-linking of CD30.

In the present *in vitro* study, we sought to determine whether Bcl-2 and caspase-9 and -3 are involved in the signal transduction pathways in human eosinophil apoptosis through cross-linking of CD30.

Methods

Human eosinophil preparation

Eosinophils were purified from the peripheral blood of healthy individuals ($n = 37$) using Percoll-sodium diatrizoate density gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) sedimentation and negative selection with anti-CD16 antibody-coated immunomagnetic beads (Milenyi Biotec, Bergisch Gladbach, Germany) using the FACS Flow Cytometry (Becton Dickinson Co., Mountain View, CA, USA), as previously described.²² Briefly, a total of 90 mL of peripheral blood was obtained from each healthy donor. The mean number of eosinophils isolated from 90 mL was $11.6 \pm 2.3 \times 10^6$ cells. Eosinophils were washed with ice-cold Phosphate-buffered saline (PBS) before lysing them in Radio immune precipitation assay (RIPA) buffer (Sigma–Aldrich, St. Louis, MO, USA) and a cocktail of protease inhibitors (Sigma–Aldrich) at 4°C. Protein was extracted by adding 300 μL of RIPA buffer to 1.2×10^7 cells. The concentrations of total protein varied from 0.82 mg/mL to 2.23 mg/mL. Samples of 30 μg of protein in loading buffer were transferred from polyacrylamide gels onto a nitrocellulose membrane. Both the purity and viability of the eosinophils were confirmed to exceed 98% based on light microscopic examination of cytocentrifuged preparations using Randolph stain and trypan blue dye (Sigma–Aldrich) exclusion, respectively. Written informed consent was obtained from all donors, and the study was approved by the institutional review board of Uijeongbu St. Mary's Hospital. All culture experiments were performed in triplicate.

Human eosinophil culture

Purified eosinophils were suspended at a cell density of 1×10^6 /mL in RPMI 1640 media (Atlanta Biologicals, Norcross, GA, USA) supplemented with 1% or 10% Fetal bovine serum (FBS) (Atlanta Biologicals) and 100 μg/mL streptomycin. CD30 agonistic monoclonal antibodies (mAb), Ber-H8 (BD Pharmingen, San Diego, CA, USA), and control immunoglobulin G₁ (IgG₁; Santa Biotechnology, Santa Cruz, CA, USA) were used to investigate the effect of CD30 activation on eosinophil apoptosis. Ber-H8 and IgG₁ were suspended in PBS at a concentration of 20 μg/mL, and 500 μL/well were transferred to flat bottom 24-well plates (Costar,

Cambridge, MA, USA). After the plates were precoated and immobilized with the mAb at 4°C for 24 hours, the wells were washed three times with PBS containing 1% Bovine serum albumin (BSA) and blocked with 1 mL of 1% BSA at 4°C overnight, and then equal numbers of cells were supplemented. Each experimental group was incubated in the RPMI 1640 supplemented with 10% heat inactivated FBS at different wells in a 37°C humidified atmosphere with 5% CO₂ for 0 hours, 4 hours, 24 hours, 66 hours, or 90 hours. The groups were classified into cells cultured with media only, 10 ng/mL interleukin (IL)-5, 10 μ M/mL dexamethasone (Sigma Chemical Co., St. Louis, MO, USA), 20 μ g/mL Ber-H8, 20 μ g/mL IgG₁, 20 μ g/mL Ber-H8 + 10 ng/mL IL-5, and 20 μ g/mL IgG₁ + 10 ng/mL IL-5. Additionally, some purified eosinophils were also cultured in the presence of caspase-9 inhibitors (Z-LEHD-FMK; BD Pharmingen, San Diego, CA, USA) or caspase-3 inhibitors (Z-DEVD-FMK; BD Pharmingen) for 24 hours and 48 hours and the cells were collected and apoptosis was measured by flow cytometry.

Expression of CD30 on the surface of human eosinophils

The expression of CD30 on eosinophils was examined using indirect immunofluorescence and flow cytometry, as previously described.⁶ Freshly isolated eosinophils were labeled (30 minutes, 4°C) in PBS containing 1% BSA (PBS-BSA; Sigma-Aldrich) and 4 mg/mL human IgG (Sigma-Aldrich) with a saturating concentration of anti-CD30 mAb or an equivalent concentration of irrelevant isotype-matched control mAb. Cells were washed and then incubated (30 minutes, 4°C in PBS-BSA) with appropriate dilutions of fluorescein isothiocyanate (FITC)-conjugated F(ab')₂ goat anti-mouse IgG Ab (BD sciences, Mountain View, CA, USA). After fixation in 1% paraformaldehyde in PBS, fluorescence analysis was conducted in the FACS Flow Cytometry. Fluorescence intensity was determined on 20,000 cells from each sample using logarithmic amplification, which was converted to the linear equivalent using Lysis II software (FACScan; Becton Dickinson Co., Mountain View, CA, USA).

Assessment of human eosinophil apoptosis

The Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit (BD Pharmingen) was used according to the manufacturer's instructions.²³ Briefly, eosinophils were harvested at various time points by gentle pipetting and washed once with PBS. The harvested eosinophils were then suspended in a Ca²⁺-containing buffer and reacted with FITC-conjugated Annexin V for 15 minutes at room temperature. One minute before FACS flow cytometry analysis, the eosinophils were also stained with 10 μ g/mL PI. The FITC-stained cells (Annexin V-positive) and PI-stained cells were assessed using FACS flow cytometry, as previously described.⁴

Assessment of Bcl-2, procaspase-8, -9, and -3, and caspase-8, -9, and -3 expressions in human eosinophils by western blotting

Eosinophils were collected at 24 hours and protein was extracted using RIPA Lysis and Extraction Buffer (Sigma

Aldrich, St. Louis, MO, USA). After centrifugation at 4°C, 14000 g for 20 minutes, protein concentration was measured using the Bradford method and diluted with 5X Sodium dodecyl sulfate (SDS) sample buffer, and then boiled at 100°C for 5 minutes. Protein concentrate of 1 μ g/ μ L was inoculated to 12.5% polyacrylamide gel, transferred to a polyvinylidene fluoride membrane, and separated for 1 hour by 100 mV. The membrane was fixed at room temperature for 1 hour in the solution mixed with Tween-20 Tris-buffered saline dissolved in nonfat dry milk. The primary antibody (Bcl-2, procaspase-8, -9, and -3, and caspase-8, -9, and -3) was combined to the membrane and was reacted at 4°C overnight. The membrane was washed with Tris-buffered saline (TBS)/Tween-20 four times strongly at room temperature and reacted with secondary antibody at room temperature for 2 hours. We assessed Bcl-2, procaspase-8, -9, and -3, and caspase-8, -9, and -3 proteins using western blotting luminal reagent kit (Rochester, NY, USA) and exposed them to Kodak BioMax Light Film and developed them using Kodak GBX Developer and Replenisher (SantaCruz Biotechnology, Santa Cruz, CA, USA).

Statistical analysis

All the data were presented as mean \pm standard error of mean (SEM) unless otherwise indicated. Differences between groups were analyzed using the Mann-Whitney *U* test or the independent *t* test. All analyses were conducted using IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, NY, USA). All statistical tests were two-sided, and *p* < 0.05 represented statistical significance.

Results

Expression of CD30 on human eosinophils cultured under different conditions

Indirect immunofluorescence and flow cytometry were used to examine CD30 expression on eosinophils from 37 healthy donors. Freshly isolated peripheral blood eosinophils were found to express low, but consistently detectable, amounts of CD30. The percentages of eosinophils expressing CD30 in RPMI 1640 media with 1% FBS, 10% FBS, and IL-5 + 10% FBS at 24 hours of culture assessed by flow cytometry were 5.6 \pm 1.1%, 3.5 \pm 1.7%, and 3.5 \pm 1.5%, respectively. The human eosinophils showed lower CD30 expression when cultured with 10% FBS compared with 1% FBS at 24 hours. Addition of IL-5 to eosinophils cultured in RPMI 1640 media with 10% FBS showed no difference when compared with eosinophils cultured in RPMI 1640 media with 10% FBS only.

Expression of CD30 on human eosinophils at various time points

Indirect immunofluorescence and flow cytometry were used to examine CD30 expression on eosinophils incubated in RPMI 1640 media supplemented with 10% FBS at various time points. The percentages of eosinophils expressing CD30 at 0 hours, 24 hours, 66 hours, and 90 hours of culture assessed by flow cytometry were 1.3 \pm 0.4%, 3.5 \pm 1.7%,

$8.3 \pm 0.7\%$, and $17.3 \pm 0.8\%$, respectively ($p < 0.05$). The CD30 expression on human eosinophils increased in a time-dependent manner.

Effects of immobilized anti-CD30 mAb on eosinophil apoptosis

Eosinophils were cultured in 24-well plates with media, IL-5 10 ng/mL, dexamethasone 10 μ M/mL, Ber-H8 (anti-CD30 mAb) 20 μ g/mL, IgG₁ 20 μ g/mL, Ber-H8 20 μ g/mL + IL-5 10 ng/mL, and IgG₁ 20 μ g/mL + IL-5 10 ng/mL, respectively. The eosinophil apoptosis rate was determined after 24 hours and 48 hours. As shown in Figure 1A, eosinophils cultured in the presence of immobilized anti-CD30 mAb, Ber-H8, resulted in increased apoptosis that was significant at 24 hours of culture ($25.2 \pm 5.3\%$), when compared with eosinophils cultured with media, IL-5, dexamethasone, IgG₁, or IgG₁ mAb + IL-5 (Figure 1A). At 48 hours of culture, culture of

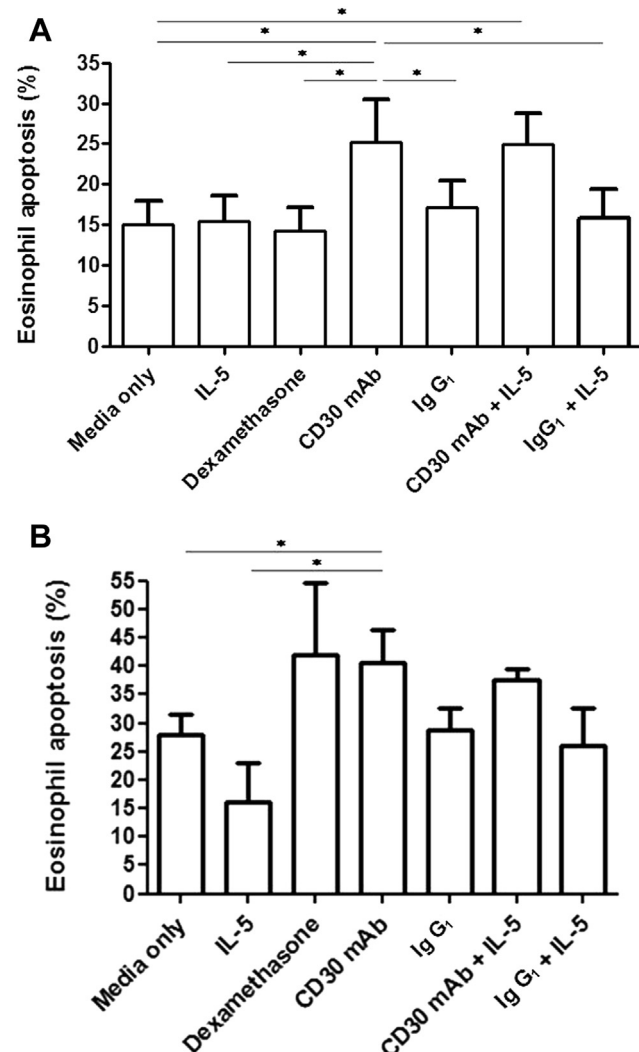


Figure 1. Effects of immobilized anti-CD30 mAb on eosinophil apoptosis measured at (A) 24 hours and (B) 48 hours. * $p < 0.05$, analyzed using Mann-Whitney U test. IgG₁ = immunoglobulin G₁; IL-5 = interleukin-5; mAb = monoclonal antibodies.

eosinophils in the presence of immobilized anti-CD30 mAb, Ber-H8, resulted in increased apoptosis when compared with eosinophils cultured with media only and IL-5 (Figure 1B).

Expression of procaspase-8 and caspase-8 assessed using western blot analysis of human eosinophil extracts after various stimuli

Eosinophils were cultured in IgG₁, IL-5, or anti-CD30 mAb (Ber-H8)-coated plates and the procaspase-8 and caspase-8 expression was assessed at 24 hours using western blotting (Figure 2). Pretreatment of eosinophils with IgG₁ 20 μ g/mL, 10 ng/mL of IL-5, or anti-CD30 mAb (Ber-H8) for 24 hours did not change the procaspase-8 or caspase-8 expression levels (Figure 2).

Expression of Bcl-2 assessed using western blot analysis of human eosinophil extracts after various stimuli

Eosinophils were cultured in IgG₁-coated plates, anti-CD30 mAb (Ber-H8)-coated plates + IL-5, IL-5, or anti-CD30 mAb (Ber-H8)-coated plates and the Bcl-2 expression was assessed at 24 hours using western blotting (Figure 3). Pretreatment of eosinophils with 10 ng/mL of IL-5 for 24 hours significantly increased the Bcl-2 expression, whereas anti-CD30 mAb (Ber-H8) decreased the Bcl-2 expression (Figure 3).

Expression of procaspase-9 and -3 assessed using western blot analysis of human eosinophil extracts after various stimuli

Eosinophils were cultured in IgG₁, IL-5, or anti-CD30 mAb (Ber-H8)-coated plates and the procaspase-9 expression was assessed at 24 hours using western blotting. Pretreatment of eosinophils with 10 ng/mL of IL-5 for 24 hours significantly increased the procaspase-9 expression, whereas anti-CD30 mAb (Ber-H8) significantly decreased the procaspase-9 expression (Figure 4). Pretreatment of eosinophils with 10 ng/mL of IL-5 for 24 hours significantly increased the procaspase-3 expression, whereas anti-CD30 mAb (Ber-H8) significantly decreased the procaspase-3 expression (Figure 5).

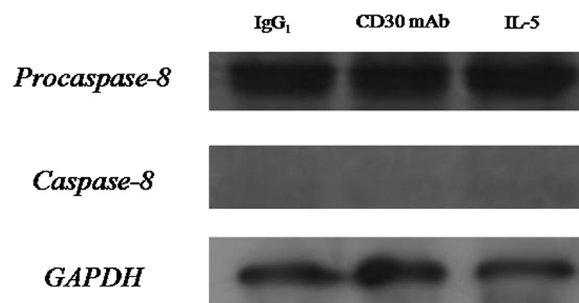


Figure 2. Western blot analysis of procaspase-8 and caspase-8 expression in human eosinophil extracts after various stimuli at 24 hours. IgG₁ = immunoglobulin G₁; IL-5 = interleukin-5; mAb = monoclonal antibodies.

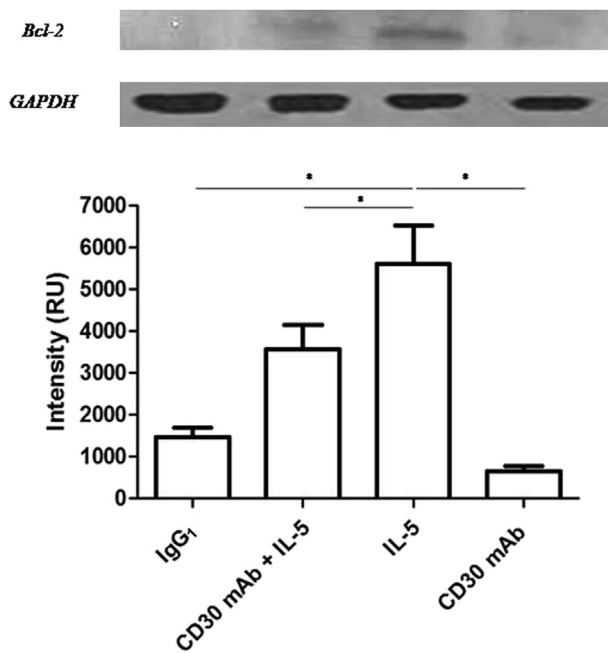


Figure 3. Western blot analysis of Bcl-2 expression in human eosinophil extracts after various stimuli at 24 hours. * $p < 0.05$, analyzed using Mann-Whitney U test. IgG₁ = immunoglobulin G₁; IL-5 = interleukin-5; mAb = monoclonal antibodies.

Expression of caspase-9 and -3 assessed using western blot analysis of human eosinophil extracts after various stimuli when cultured with caspase-9 and -3 inhibitors

Eosinophils were cultured in IgG₁, IL-5, anti-CD30 mAb (Ber-H8)-coated plates + caspase-9 (Z-LEHD-FMK, BD Pharmingen) or caspase-3 inhibitors (Z-DEVD-FMK, BD Pharmingen), or anti-CD30 mAb (Ber-H8)-coated plates and the caspase-9

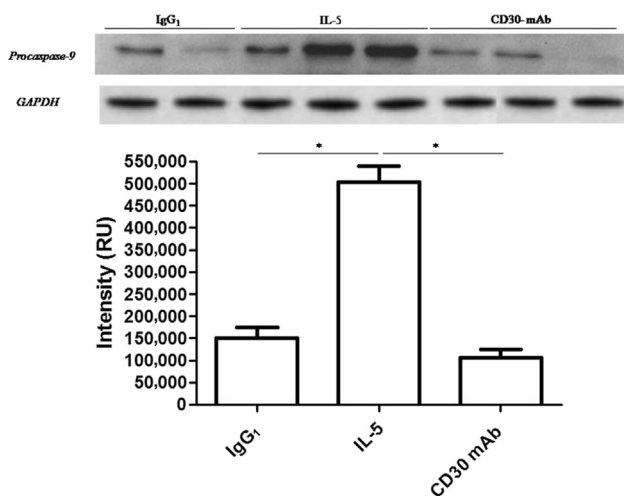


Figure 4. Western blot analysis of procaspase-9 expression in human eosinophil extracts after various stimuli at 24 hours. * $p < 0.05$, analyzed using Mann-Whitney U test. IgG₁ = immunoglobulin G₁; IL-5 = interleukin-5; mAb = monoclonal antibodies.

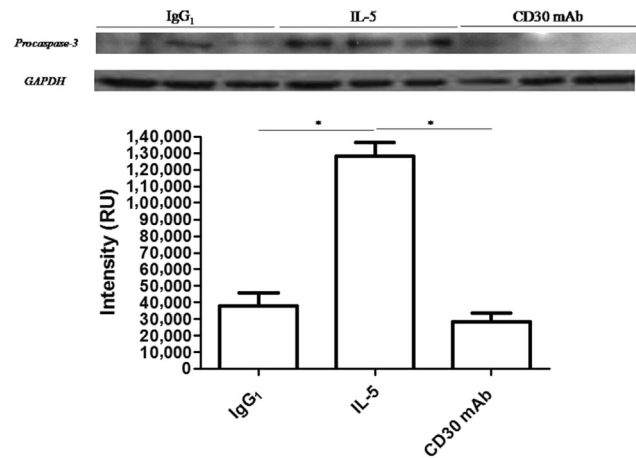


Figure 5. Western blot analysis of procaspase-3 expression in human eosinophil extracts after various stimuli at 24 hours. * $p < 0.05$, analyzed using Mann-Whitney U test. IgG₁ = immunoglobulin G₁; IL-5 = interleukin-5; mAb = monoclonal antibodies.

and -3 expressions were assessed at 24 hours using western blotting. Pretreatment of eosinophils with anti-CD30 mAb (Ber-H8) significantly increased the caspase-9 expression, whereas anti-CD30 mAb (Ber-H8) + caspase-9 inhibitor decreased the caspase-9 expression (Figure 6). Pretreatment of eosinophils with anti-CD30 mAb (Ber-H8) significantly increased the caspase-3 expression, whereas anti-CD30 mAb (Ber-H8) + caspase-3 inhibitor decreased the caspase-3 expression (Figure 7).

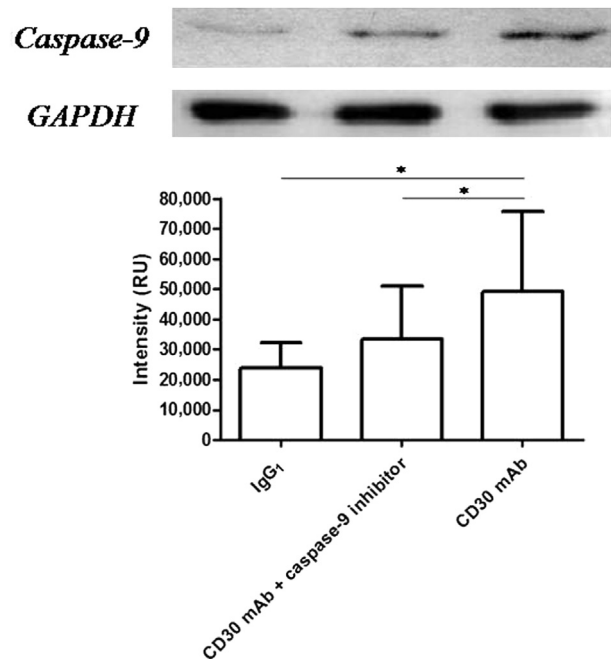


Figure 6. Western blot analysis of caspase-9 expression in human eosinophil extracts after treatment of caspase-9 inhibitor at 24 hours. * $p < 0.05$, analyzed using Mann-Whitney U test. IgG₁ = immunoglobulin G₁; mAb = monoclonal antibodies.

Effects of caspase-9 and -3 inhibitors on immobilized anti-CD30 mAb-induced eosinophil apoptosis

Eosinophils were cultured with media, anti-CD30 mAb (Ber-H8)-coated plates, anti-CD30 mAb (Ber-H8)-coated plates + caspase-3 inhibitor, anti-CD30 mAb (Ber-H8)-coated plates + caspase-9 inhibitor, or IgG₁ for 24 hours and 48 hours, respectively (Figures 8A and 8B). The rate of annexin V positivity was higher in the eosinophils cultured in the anti-CD30 mAb (Ber-H8)-coated plates than in the eosinophils cultured in either media or irrelevant control IgG₁-coated plates at 24 hours and 48 hours, respectively.

In order to determine the involvement of caspase-9 in this apoptosis, caspase-9 inhibitors (Z-LEHD-FMK, BD Pharmingen) were added to anti-CD30 mAb (Ber-H8)-coated plates. Addition of 20 μ M of caspase-9 inhibitors showed a reduction in anti-CD30 mAb (Ber-H8)-induced eosinophil apoptosis at 24 hours and 48 hours, respectively (Figures 8A and 8B). In order to determine the involvement of caspase-3 in this apoptosis, caspase-3 inhibitors (Z-DEVD-FMK, BD Pharmingen) were added to anti-CD30 mAb (Ber-H8)-coated plates. Addition of 20 μ M of caspase-3 inhibitors showed a significant reduction in anti-CD30 mAb (Ber-H8)-induced eosinophil apoptosis at 24 hours and 48 hours, respectively (Figures 8A and 8B).

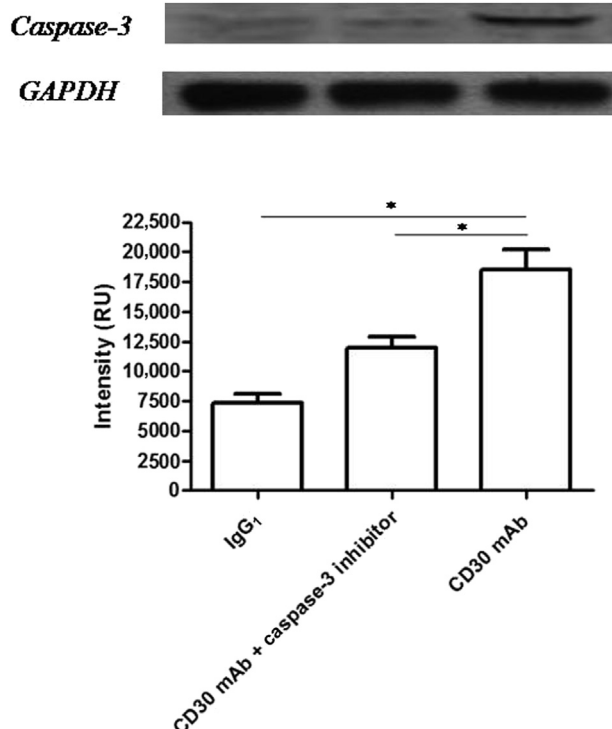


Figure 7. Western blot analysis of caspase-3 expression in human eosinophil extracts after treatment of caspase-3 inhibitor at 24 hours. * $p < 0.05$, analyzed using Mann-Whitney U test. IgG₁ = immunoglobulin G₁; mAb = monoclonal antibodies.

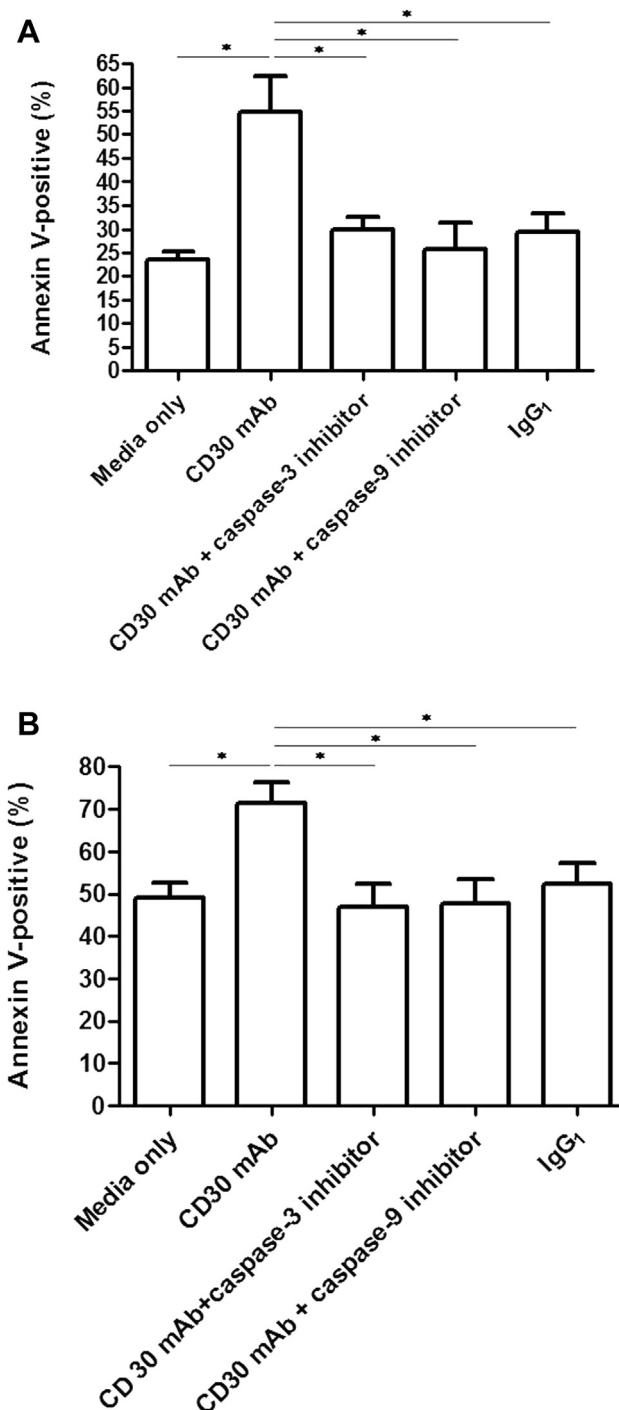


Figure 8. Effects of caspase-3 and -9 inhibitors on immobilized anti-CD30 mAb-induced eosinophil apoptosis at (A) 24 hours and (B) 48 hours. * $p < 0.05$, analyzed using Mann-Whitney U test. IgG₁ = immunoglobulin G₁; mAb = monoclonal antibodies.

Discussion

In the present *in vitro* study, we sought to determine whether Bcl-2 and caspase-9 and -3 are involved in the signal transduction pathways in human eosinophil apoptosis through cross-linking of CD30, and found that they are

critically engaged in the process. To the best of our knowledge, this is the first study to determine that Bcl-2 and caspase-9 and -3 may play a pivotal role in human eosinophil apoptosis through cross-linking of CD30.

Our findings that activation of cell surface CD30 by immobilized anti-CD30 mAb induces intensified apoptosis in human eosinophils *in vitro* are in good agreement with previous studies.^{3,4,7} This effect was observed only when the anti-CD30 mAb was immobilized, not in soluble form, thereby proposing that cross-linking of cell surface molecule CD30 or a second signal may be imperative for the induction of apoptosis.⁴

We investigated whether anti-CD30 mAb (clone Ber-H8) treatment induces human eosinophil apoptosis. As shown in Figure 1, anti-CD30 mAb (clone Ber-H8) induced strong human eosinophil apoptosis when compared with the other stimulators, which correlates well with previous studies.^{3,4,7} An important finding worth mentioning in the present study is that anti-CD30 mAb (clone Ber-H8) induced stronger human eosinophil apoptosis than dexamethasone, a well-known potent eosinophil apoptosis inducer.²⁴ This result suggests that anti-CD30 mAb may well be a promising drug candidate for regulating human eosinophil survival in the treatment of allergic diseases in the future.

We determined whether caspase-8 is involved in the signal transduction pathways in eosinophil apoptosis after anti-CD30 mAb treatment. Procasase-8 and caspase-8 expression levels were not changed when stimulated with IgG₁, anti-CD30 mAb, or IL-5, suggesting that caspase-8 pathway is not involved in the anti-CD30 mAb-induced human eosinophil apoptosis. Previous studies have suggested that several surface molecules such as Fas are capable of inducing apoptosis in human eosinophils through cross-linking.⁶ Fas receptor cross-linking results in death-inducing signaling complex (DISC) assembly initiating within minutes of cross-linking, and localizing initially Fas-associated death domain (FADD) and then caspase-8 to the receptor complex.²⁵ Although we did not analyze the activity of death domain after cross-linking of CD30, it can be inferred that CD30, unlike Fas, lacks an intracellular death domain capable of activating the caspase-8 pathway.

We then examined whether Bcl-2 is engaged in the signal transduction pathways in human eosinophil apoptosis after anti-CD30 mAb treatment. As shown in Figure 3, Bcl-2 protein was highly expressed when stimulated with IL-5, an antiapoptotic protein, and its expression was abolished when stimulated with anti-CD30 mAb, a proapoptotic protein. The Bcl-2 protein was moderately expressed when stimulated with both IL-5 and anti-CD30 mAb. A previous study has reported that when monocytes were stimulated with a member of the tumor necrosis factor superfamily, apoptosis was inhibited, the Bcl-2 protein level increased, and the caspase-3 level decreased compared with unstimulated control cells.²⁶ Another study investigating whether a T cell survives or dies following T cell receptor re-engagement found that caspase-mediated cleavage of antiapoptotic Bcl-2 or Bcl-xL facilitates activation-induced cell death of T cells, suggesting that cleavage of antiapoptotic Bcl-2 and Bcl-xL contributes to the decision between T cell activation and apoptosis following T cell receptor (TCR) re-engagement.¹⁶ Although we did not measure expression of other proteins that may be involved

in the mitochondrial pathway, we speculate that the Bcl-2 protein may be engaged in the signal transduction pathways in human eosinophil apoptosis after anti-CD30 mAb treatment.

Next, we explored whether caspase-9 is engaged in the signal transduction pathways in human eosinophil apoptosis after anti-CD30 mAb treatment. As shown in Figure 4, procaspase-9 was highly expressed when stimulated with IL-5 and its expression was abolished when stimulated with anti-CD30 mAb. In addition, caspase-9 was highly expressed when stimulated with anti-CD30 mAb. The caspase-9 protein was moderately expressed when stimulated with both anti-CD30 mAb and caspase-9 inhibitor. These findings imply that upon stimulation by anti-CD30 mAb, procaspase-9 is converted to caspase-9; thus, it can be inferred that the caspase-9 pathway is involved in the signal transduction pathways in human eosinophil apoptosis after anti-CD30 mAb treatment.

We also determined whether caspase-3 is involved in the signal transduction pathways in human eosinophil apoptosis after anti-CD30 mAb treatment. As demonstrated in Figure 5, procaspase-3 was highly expressed when stimulated with IL-5 and its expression was abrogated when stimulated with anti-CD30 mAb. Furthermore, caspase-3 was highly expressed when stimulated with anti-CD30 mAb. The caspase-3 protein was slightly expressed when stimulated with both anti-CD30 mAb and caspase-3 inhibitor. These results suggest that when stimulated with anti-CD30 mAb, procaspase-3 is converted to caspase-3; thus, we speculate that the caspase-3 pathway is involved in the signal transduction pathways in human eosinophil apoptosis after anti-CD30 mAb treatment.

Finally, we determined whether caspase-9 or -3 inhibitors decrease human eosinophil apoptotic effects of anti-CD30 mAb treatment. As shown in Figure 8, caspase-9 and -3 inhibitors significantly decreased human eosinophil apoptotic effects of anti-CD30 mAb. These data strongly suggest that caspase-9 and -3 are involved in the signal transduction pathways for induction of human eosinophil apoptosis via CD30.

Apoptosis occurs through two main interconnected pathways which are intrinsic and extrinsic pathways.²⁷ The intrinsic pathways, also known as mitochondrial pathways, are triggered by a disparate array of death stress, genomic stress, metabolic stress, presence of unfolded proteins, and other stimuli that induce permeabilization of the outer mitochondrial membrane and release of apoptotic proteins into the cytosol. Several of these proteins, including Bcl-2, initiate caspase activation. Progression through the mitochondrial pathway induces activation of caspase-9, which then cleaves procaspase-3, giving activated caspase-3, which acts as an executioner of eosinophil apoptosis, by cleaving a multiple of other proteins inside the cells. The process of apoptosis by various stimuli is commenced by activating either intrinsic or extrinsic pathways which starts a series of downstream cascades. From our findings that anti-CD30 mAb induced eosinophil apoptosis by decreasing Bcl-2, increasing caspase-9 and -3, and not changing caspase-8, we speculate that anti-CD30 mAb involves the intrinsic pathway.

The present study had some limitations. First, we did not analyze tumor necrosis factor receptor associated factor

(TRAF)-interacting motifs (TIMs) in human eosinophils, which are activated to recruit TRAF family members, and activation of multiple signal transduction pathways that are important in eosinophil apoptosis. Second, we did not measure various molecules in the Bcl-2 family, which are known to regulate mitochondrial dysfunction.

In conclusion, we found that Bcl-2 and caspase-9 and -3 are critically involved in anti-CD30 mAb-induced human eosinophil apoptosis *in vitro*. Downstream signaling molecules of Bcl-2 and caspase-9 and -3 activations in human eosinophils remain to be clarified in future studies.

Conflicts of interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

References

- Kita H. Eosinophils: multifaceted biological properties and roles in health and disease. *Immunol Rev* 2011;242:161–77.
- Spencer LA, Bonjour K, Melo RC, Weller PF. Eosinophil secretion of granule-derived cytokines. *Front Immunol* 2014;5:496.
- Matsumoto K, Terakawa M, Fukuda S, Saito H. Analysis of signal transduction pathways involved in anti-CD30 mAb-induced human eosinophil apoptosis. *Int Arch Allergy Immunol* 2010;152(Suppl. 1):2–8.
- Matsumoto K, Terakawa M, Miura K, Fukuda S, Nakajima T, Saito H. Extremely rapid and intense induction of apoptosis in human eosinophils by anti-CD30 antibody treatment *in vitro*. *J Immunol* 2004;172:2186–93.
- Nutku E, Aizawa H, Hudson SA, Bochner BS. Ligation of Siglec-8: a selective mechanism for induction of human eosinophil apoptosis. *Blood* 2003;101:5014–20.
- Matsumoto K, Schleimer RP, Saito H, Iikura Y, Bochner BS. Induction of apoptosis in human eosinophils by anti-Fas antibody treatment *in vitro*. *Blood* 1995;86:1437–43.
- Berro AI, Perry GA, Agrawal DK. Increased expression and activation of CD30 induce apoptosis in human blood eosinophils. *J Immunol* 2004;173:2174–83.
- Kim JT, Gleich GJ, Kita H. Roles of CD9 molecules in survival and activation of human eosinophils. *J Immunol* 1997;159:926–33.
- Kim JT, Schimming AW, Kita H. Ligation of Fc gamma RII (CD32) pivotally regulates survival of human eosinophils. *J Immunol* 1999;162:4253–9.
- Kiwamoto T, Katoh T, Tiemeyer M, Bochner BS. The role of lung epithelial ligands for Siglec-8 and Siglec-F in eosinophilic inflammation. *Curr Opin Allergy Clin Immunol* 2013;13:106–11.
- Na HJ, Hudson SA, Bochner BS. IL-33 enhances Siglec-8 mediated apoptosis of human eosinophils. *Cytokine* 2012;57:169–74.
- Matsumoto K, Terakawa M, Fukuda S, Saito H. Rapid and strong induction of apoptosis in human eosinophils by anti-CD30 mAb-coated microspheres and phagocytosis by macrophages. *Int Arch Allergy Immunol* 2007;143(Suppl. 1):60–7.
- Luo HR, Loison F. Constitutive neutrophil apoptosis: mechanisms and regulation. *Am J Hematol* 2008;83:288–95.
- Mekori YA, Gilfillan AM, Akin C, Hartmann K, Metcalfe DD. Human mast cell apoptosis is regulated through Bcl-2 and Bcl-XL. *J Clin Immunol* 2001;21:171–4.
- Hacker G, Bauer A, Villunger A. Apoptosis in activated T cells: what are the triggers, and what the signal transducers? *Cell Cycle* 2006;5:2421–4.
- Guerrero AD, Welschhans RL, Chen M, Wang J. Cleavage of anti-apoptotic Bcl-2 family members after TCR stimulation contributes to the decision between T cell activation and apoptosis. *J Immunol* 2013;190:168–73.
- Chen M, Wang J. Regulation of immune responses by spontaneous and T cell-mediated dendritic cell death. *J Clin Cell Immunol* 2011;3.
- Wardle DJ, Burgon J, Sabroe I, Bingle CD, Whyte MK, Renshaw SA. Effective caspase inhibition blocks neutrophil apoptosis and reveals Mcl-1 as both a regulator and a target of neutrophil caspase activation. *PLoS One* 2011;6:e15768.
- Brown MG, Huang YY, Marshall JS, King CA, Hoskin DW, Anderson R. Dramatic caspase-dependent apoptosis in antibody-enhanced dengue virus infection of human mast cells. *J Leukoc Biol* 2009;85:71–80.
- Sekine Y, Yamamoto C, Kakisaka M, Muromoto R, Kon S, Ashitomi D, et al. Signal-transducing adaptor protein-2 modulates Fas-mediated T cell apoptosis by interacting with caspase-8. *J Immunol* 2012;188:6194–204.
- Yen JH, Ganea D. Interferon beta induces mature dendritic cell apoptosis through caspase-11/caspase-3 activation. *Blood* 2009;114:1344–54.
- Hansel TT, De Vries IJ, Iff T, Rihs S, Wandzilak M, Betz S, et al. An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. *J Immunol Methods* 1991;145:105–10.
- Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods* 1995;184:39–51.
- Walsh GM, Sexton DW, Blaylock MG. Corticosteroids, eosinophils and bronchial epithelial cells: new insights into the resolution of inflammation in asthma. *J Endocrinol* 2003;178:37–43.
- Dempsey PW, Doyle SE, He JQ, Cheng G. The signaling adaptors and pathways activated by TNF superfamily. *Cytokine Growth Factor Rev* 2003;14:193–209.
- Kotani H, Masuda K, Tamagawa-Mineoka R, Nomiyama T, Soga F, Nin M, et al. Increased plasma LIGHT levels in patients with atopic dermatitis. *Clin Exp Immunol* 2012;168:318–24.
- Riedl SJ, Salvesen GS. The apoptosome: signalling platform of cell death. *Nat Rev Mol Cell Biol* 2007;8:405–13.