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Full length article

**Increased expression of TNF-related apoptosis inducing ligand receptor 3 on
eosinophils in Churg-Strauss syndrome**

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Abbreviations: CSS: Churg-Strauss syndrome, TRAIL: TNF-related apoptosis-
inducing ligand.

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Abstract

Objective: Prolonged eosinophil survival plays an important role in the pathogenesis of Churg-Strauss syndrome (CSS); however, its detailed molecular mechanism is still unclear. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and its receptors are expressed on a variety of cells including eosinophils. In this study, we examined TRAIL receptor expression on eosinophils in CSS. **Methods:** We assessed TRAIL receptor expression on eosinophils in healthy volunteers and in patients suffering from CSS, asthma, and hypereosinophilia due to parasitic infection. In addition, we compared TRAIL-induced eosinophil apoptosis between CSS and asthma patients. We suppressed TRAIL receptor 3 expression through RNA interference and investigated the resultant effect on the TRAIL-induced apoptosis of eosinophils from CSS patients. **Results:** The expression level of TRAIL receptor 3, a decoy receptor that acts as an antiapoptotic receptor, on eosinophils from CSS patients was significantly higher than that in the other subjects. In CSS, serum TRAIL receptor 3 levels revealed significant positive correlations with peripheral eosinophil counts. Expression of TRAIL receptor 3 by tissue-infiltrating eosinophils was observed immunohistochemically in the CSS patients. Peripheral T cells expressed TRAIL on their surface. In comparison with asthma, the percentage of recombinant TRAIL and autologous T cell-induced eosinophil apoptosis and the active eosinophil caspase-3 level in CSS were significantly lower. Suppression of TRAIL receptor 3 expression through

RNA interference significantly increased the recombinant TRAIL-induced eosinophil apoptosis in the CSS patients. **Conclusion:** We observed increased TRAIL receptor 3 expression on eosinophils in the CSS patients. Increased TRAIL receptor 3 expression on eosinophils might be involved in the molecular pathogenesis of CSS eosinophilia.

Introduction

Churg-Strauss syndrome (CSS) is a multiorgan disease with eosinophilia in tissues and blood (1). Eosinophils can secrete a number of cytokines (1-3), such as eosinophil major basic protein (MBP) and eosinophil-derived neurotoxin (EDN) (4), these cytokines play a pivotal role in the pathogenesis of disorders with eosinophilia. They are thought to cause tissue damage due to their cytotoxic activities (5;6). Thus, marked eosinophilia in tissues and blood due to prolonged eosinophil survival (7) seems to be implicated in the pathogenesis of CSS (1;2). In this regard, impaired apoptosis of eosinophils has been reported (8-10) ; however, the detailed mechanism of eosinophilia in CSS has not been fully elucidated.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily (11). TRAIL can interact with 2 death receptors (DRs), namely, TRAIL receptor 1 (DR4) (12) and TRAIL receptor2(DR5) (13), and 2 decoy receptors (DcRs), namely, TRAIL receptor 3 (DcR1) (12;14;15) and TRAIL receptor4(DcR2) (16;17). TRAIL receptors 1 and 2 contain an intracellular motif called the “death domain” that subsequently activates cysteinyl aspartic acid protease (caspase)-8 (12;13). Active caspase-8 transmits its proapoptotic activity to executioner caspases such as caspase-3 or caspase-7 (12;13;18;19). In contrast, TRAIL receptors 3 and 4 have either truncated or missing intracellular domains and are unable to transduce the death

signal (13;15;17); this suggests that these receptors may compete for ligand-binding and act as antiapoptotic receptors (20). These receptors have become known as decoy receptors and are thought to be critical to the regulation of TRAIL signaling by competing for TRAIL binding sites (21).

TRAIL is known to induce apoptosis not only in many tumor cells (11) but also in non-malignant cells such as neutrophils in systemic lupus erythematosus (22) and hepatic cells in hepatitis virus type C infection (23). TRAIL receptors are expressed on eosinophils (24;25). However, the role of TRAIL in CSS remains unknown. In this study, we investigated the TRAIL receptors on eosinophils and the apoptosis caused by TRAIL-induced eosinophils using samples from patients suffering from CSS and asthma. A difference in the expression pattern of TRAIL receptors between CSS patients and asthma patients has been discovered. In addition, we have observed that eosinophil apoptosis in CSS patients induced by recombinant TRAIL and autologous T cells that express TRAIL on the surface was attenuated by the increased expression of TRAIL receptor 3 on the eosinophils. Glucocorticoids are capable of modulating the expressions of TRAIL receptor 3 in eosinophils. It is suggested that TRAIL is potentially involved in the pathogenesis of eosinophilia in CSS.

Materials and Methods

Patients

This study was reviewed and approved by the Kagoshima University Faculty of Medicine Committee on Human Research. We investigated 34 patients (males, 14; females, 20) with CSS who were admitted to the Division of Respiratory Medicine, Respiratory and Stress Care Center, Kagoshima University Hospital from 1995 to 2005 for the diagnosis and treatment of CSS. The mean age of the patients was 56.3 ± 19.2 years (mean \pm standard deviation). All patients were initially diagnosed with CSS because of the presence of asthma and eosinophilia; the diagnosis of CSS was confirmed by observing the existence of eosinophilic vasculitis in skin or nerve biopsy. Clinical features of the CSS patients upon admission are shown in Table 1. For comparison, we also investigated 29 patients (male, 13; female, 16; mean age, 58.9 ± 15.3 years) with asthma. All asthma patients were fresh cases and did not receive any medication. We examined 11 patients (male, 5; female, 6; mean age, 57.3 ± 23.1 years) with hypereosinophilia (HES) due to parasitic infection before the start of therapy. Out of these, 3 patients were infected with *Ascaris suum*, 2 with *Strongyloides stercoralis*, 4 with *Paragonimus westermani*, and 2 with *Dirofilaria immitis*. We also recruited 14 healthy volunteers (male, 7; female, 7; mean age, 58.9 ± 13.1 years). All participants provided written consent to participate in the study. The classification of CSS was made according to the 1990 edition of CSS published by the American College of Rheumatology (26) ; all patients with CSS fulfilled more than 5 criteria outlined in the publication. We excluded patients with rheumatoid arthritis, diabetes mellitus,

acute or chronic liver disease, and immunological abnormalities that predispose to opportunistic infection.

Preparation of eosinophils and T cells

Human eosinophils were obtained from heparinized venous blood of the CSS and asthma patients before they underwent therapy. Heparinized venous blood was mixed with a quarter volume of 2% dextran solution (Sigma-Aldrich Corp., St Louis, MO, USA) to precipitate red blood cells. After incubation for 30 min at room temperature, the leukocyte-rich plasma was laid onto Histopaque (Sigma-Aldrich Corp., St Louis, MO, USA) and centrifuged at $800 \times g$ for 20 min at room temperature. Granulocytes were separated from erythrocytes by lysis in 0.2% NaCl and washed in phosphate-buffered saline (PBS) 3 times at 4°C; next, eosinophils were isolated by negative selection using magnetic beads (Eosinophil Isolation Kit; Miltenyi Biotec GmbH, Bergisch, Germany) according to the manufacturer's protocol. Eosinophils were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal calf serum (FCS) and streptomycin/penicillin (the complete medium). The purity of eosinophils was more than 99% by morphological examination after staining with Diff-Quick (Wako, Tokyo, Japan).

Moreover, peripheral blood mononuclear cells (PBMCs) were separated from heparinized venous blood of CSS and asthma patients by Histopaque gradient

centrifugation as described previously (22). T cells were isolated by negative selection using the abovementioned magnetic beads according to the manufacturer's protocol. The purity of T cells was observed to be more than 95% by fluorescence-activated cell sorting (FACS) analysis.

Flow cytometry analysis

To detect the expression of TRAIL receptors on eosinophils, 5×10^5 cells were collected after incubation in various states. The cells were washed with PBS 3 times and then incubated with human serum (pooled sample from healthy volunteers) for 10 min; subsequently, they were further incubated with biotinylated goat antihuman TRAIL receptor 1, 2, 3, and 4 antibodies (R&D Systems, Minneapolis, MN, USA) for 20 min at 4°C. After washing 3 times with PBS, the cells were incubated with streptavidin-fluorescein isothiocyanate (FITC) (PharMingen, San Diego, CA, USA) for 15 min at 4°C. After washing 3 times with PBS, flow cytometry analysis was performed by a FACS scan using the CellQuest software (PharMingen).

Measurement of soluble TRAIL and TRAIL receptor 3 in sera

We measured the serum levels of TRAIL and TRAIL receptor 3 in CSS, asthma, and HES patients and in healthy volunteers before they underwent therapy. In 11 patients with CSS, serum TRAIL and TRAIL receptor 3 levels were determined before therapy

and 3 months after the start of therapy. TRAIL and TRAIL receptor 3 concentrations in sera were measured in duplicate for each sample using a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according to the manufacturer's protocols.

Immunohistochemical staining for TRAIL receptor 3

Eleven CSS patients and 8 patients with Wegener's granulomatosis (male, 2; female, 6; mean age, 60.1 ± 13.1 years) were randomly selected. The preliminary diagnosis of Wegener's granulomatosis was made from clinical symptoms and confirmed by the existence of vasculitis in tissue biopsy. The biopsied tissues obtained from these patients were examined by immunohistochemical staining for TRAIL receptor 3 using a goat anti-TRAIL receptor 3 polyclonal antibody (R&D Systems) and visualized by employing the diaminobenzidine (DAB) method as described previously (27). Briefly, 4-mm thick sections were mounted on poly-L-lysine-coated slides, dewaxed, and washed in Tris-buffered saline (pH 7.4) for 10 min. For optimal antigen retrieval, sections were pressure cooked in 0.01 M citrate buffer (pH 6.0) for 90 s. Endogenous peroxidase activity was blocked using a 3% hydrogen peroxide solution in methanol for 10 min. Following 2 washes in (PBS) with 1% saponin, the blocking reaction was performed. The sections were incubated with the primary antibody solution for 2 h at room temperature using a 1:50 dilution of the antibody. Negative control slides were

incubated with goat immunoglobulin G (IgG) (R&D Systems). Secondary biotinylated anti-immunoglobulin antibody (R&D Systems) was added, and the mixture was incubated for 30 min at room temperature. Following washing, the sections were incubated with streptavidin conjugated to horseradish peroxidase (Amersham) and then rinsed with deionized water. DAB substrate solution was added and the mixture was incubated for 10 min. A brown color reaction represented a positive result.

Assay for apoptosis

After incubation in various states, 1×10^6 eosinophils were collected and the exposure of phosphatidylserine was detected by flow cytometry analysis using the Annexin V-FITC Apoptosis Detection kit (PharMingen), as described in the previous section. In all experiments, apoptotic data were confirmed by terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenin uridine triphosphate (dUTP) nick-end labeling (TUNEL) assay using a commercially available kit (TUNEL Label Mix; Roche Diagnostics), according to the manufacturer's instructions.

Measurement of active caspase-3

Incubation of 1×10^7 eosinophils was done with various concentrations of TRAIL in the complete medium. After the incubation, eosinophils were collected and active

caspase-3 levels were measured using the ELISA kit that recognizes caspase-3 zymogen and other active caspases, according to the manufacturer's instructions (R&D Systems).

⁵¹Cr release assay

To investigate T-cell-mediated neutrophil apoptosis, we performed ⁵¹Cr release assay as previously described (22). Briefly, 10 million eosinophils (target cells) were incubated with 100 µCi (3.7 MBq) [⁵¹Cr] sodium chromate/10⁶ cells for 1 h and washed 5 times with PBS. The labeled eosinophils (5 × 10³ cells/well) were incubated with autologous T cells (effector cells). Preliminarily, we performed coincubation for various durations of time (8, 12, 24, 36, and 48 h) and observed that 24 h of coincubation was optimal. After 24 h of incubation, supernatants of each well were collected and radioactivity was measured with a gamma counter (1480 WIZARTM 3; PerkinElmer, Downers Grove, IL, USA). Specific lysis was calculated by the formula: specific ⁵¹Cr-release (%) = [(mean experimental cpm – mean spontaneous cpm) / (mean maximum cpm – mean spontaneous cpm)] × 100 in which spontaneous release represents counts per min (cpm) in supernatants from wells containing target cells with the medium only, and maximum release represents cpm in supernatants from wells containing target cells in the medium with 2% Triton X-100. The data obtained from 8 of the subjects are presented as the mean ± standard deviation.

Western blotting analysis

To determine the effect of glucocorticoids on TRAIL receptor 3, 1×10^7 eosinophils were incubated with glucocorticoids (500 nM) (Sigma-Aldrich Corp.) for various time durations. After incubation of eosinophils, western blot analysis was performed as previously described (28;29). Briefly, 1×10^7 eosinophils were collected and lysed on ice for 20 min in 1 mL of lysis buffer containing 50 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), 150 mM NaCl, 1% Triton X-100, 10% glycerol, and a cocktail of protease inhibitors (Roche, Indianapolis, IN, USA). The lysates were spun, and the 20- μ L supernatants were collected and the same volume, i.e., 20 μ L of double-strength sample buffer (20% glycerol, 6% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol) was added. The samples were boiled for 10 min. Proteins were analyzed on 10% polyacrylamide gels by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to nitrocellulose membranes at 150 mA for 1 h by using a semidry system. The membranes were incubated with mouse monoclonal antihuman TRAIL receptor 3 antibody (Clone 90903; R&D Systems), mouse monoclonal anti-human TRAIL receptor 1 antibody (Clone 69036; R&D Systems), or mouse antihuman actin monoclonal antibody (Santa-Cruz Biotechnology Inc., Santa-Cruz, CA USA) followed by a sheep antimouse IgG coupled with horseradish peroxidase. Peroxidase activity was visualized by the Enhanced Chemiluminescence detection system (GE Healthcare, Little Chalfont, Bucks, UK).

Development of short interfering RNA (siRNA) vector against TRAIL receptor 3

The pSUPER-retro-GFP vector was purchased from OligoEngine Japan (Tokyo, Japan), and TRAIL receptor 3 knockdowns were constructed in accordance with the manufacturer's protocol. The oligonucleotides were designed as follows: sense, 5'-CAGACTCAACGCTTCCAACAATGAACC-3'; anti-sense, 5'-GGTTCATTGTTGGAAGCGTTGAGTCTG-3'. These were ligated into BglII and BamHI sites of the vector. These vectors were transfected to packaging cells (PG-13; American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. At 48 h after transfection, the culture supernatants were collected and used for further transfection. Transfection of 1×10^6 cells/ml eosinophils in the complete medium was achieved with TRAIL receptor 3 siRNA according to the manufacturer's protocol. After 48 h of incubation, the cells were rinsed with PBS and used for further analysis as described above.

Statistical Analysis

We used one-way factorial analysis of variance (ANOVA) with the Bonferroni-Dunn test and the Mann-Whitney test. For statistical analysis, StatView and MedCalc software were used. Statistical significance was inferred at a value of $P < 0.05$. Most values were expressed as mean \pm standard deviation (SD)

Results

Expression of TRAIL receptor on eosinophils

For flow-cytometry analysis, we used eosinophils from 14 CSS patients (eosinophil count = $3893.4 \pm 2317.5/\mu\text{L}$), 11 asthma patients (eosinophil count = $610.3 \pm 345.2/\mu\text{L}$), 7 HES patients (eosinophil count = $3652.4 \pm 2432.9/\mu\text{L}$), and 8 healthy volunteers (eosinophil count = $422.3 \pm 267.2/\mu\text{L}$). As shown in Fig. 1, eosinophils from the CSS, asthma, and HES patients, and the healthy volunteers expressed all the TRAIL receptors on their surface. With regard to TRAIL receptor 3, a decoy receptor of TRAIL, the TUNEL positive cell percentage was significantly higher in CSS patients compared to asthma and HES patients and to healthy volunteers (Fig. 1B). The expression level of TRAIL receptor 4, the other decoy receptor of TRAIL, in healthy volunteers was significantly lower than those in the other subjects. Contrarily, the expression level of TRAIL receptor 2 was significantly higher in the healthy volunteers compared to that in the other participants. The expression level of TRAIL receptor 1 was higher in the healthy volunteers compared to those in the others; however, the difference was not statistically significant (Fig. 1B).

Serum TRAIL receptor 3 levels

The serum TRAIL receptor 3 levels in the CSS patients were significantly higher than those in the asthma and HES patients and the healthy volunteers (CSS, mean \pm SD, 1155.2 ± 962.1 pg/ml; asthma, 782.4 ± 337.8 pg/ml; HES, 851.3 ± 417.7 pg/ml; healthy volunteers, 589.4 ± 155.5 pg/ml; Fig. 2A). In terms of distinguishing CSS from asthma or HES using serum TRAIL receptor 3 level, the sensitivity and specificity were 60.9% and 94.8%, respectively (cutoff serum TRAIL receptor 3 value = 841.9 pg/ml). The serum TRAIL receptor 3 levels of 11 CSS patients significantly decreased 3 months after the start of therapy (before, mean \pm SD, 2374.0 ± 1669.9 pg/ml; after, 986.1 ± 604.7 pg/ml; Fig. 2B). The clinical symptoms of CSS patients improved with the use of glucocorticoids. Serum TRAIL receptor 3 levels before the start of therapy exhibited a significant positive correlation with peripheral eosinophil counts ($P < 0.05$, $r = 0.412$), but not with neutrophil counts ($P = 0.912$, $r = -0.112$) and lymphocyte counts ($P = 0.829$, $r = 0.109$).

Immunohistochemical staining for TRAIL receptor 3

The skin biopsy specimens of CSS patients demonstrated massive eosinophil infiltration following hematoxylin-eosin staining (Fig. 3A, 3E). In immunohistochemical analysis for TRAIL receptor 3, infiltrating inflammatory cells in the lesions stained intensely positive for TRAIL receptor 3 (Fig. 3B, 3F). On the contrary, the infiltrating

inflammatory cells observed in the renal lesions in Wegener's granulomatosis patients did not stain positive for the receptor (Fig. 3D, 3H).

Serum TRAIL levels

To evaluate the influence of TRAIL, we investigated the serum TRAIL levels. There was no significant difference in serum TRAIL levels among the 4 groups (CSS patients, 80.7 ± 45.9 pg/ml; asthma patients, 81.6 ± 50.3 pg/ml; HES patients, 79.9 ± 44.5 pg/ml; healthy volunteers, 84.2 ± 40.5 pg/ml). Moreover, no significant difference was observed between the serum TRAIL levels before and after therapy in the 11 CSS patients (before therapy, 89.6 ± 40.4 pg/ml; after therapy, 89.6 ± 40.2 pg/ml). The serum TRAIL level did not reveal any significant correlation with peripheral eosinophil counts ($P = 0.16$, $r = -0.242$) and neutrophil counts ($P = 0.06$, $r = 0.312$); however, it showed significant positive correlation with lymphocyte counts ($P < 0.01$, $r = 0.521$).

Comparison of the effect of recombinant TRAIL on eosinophil apoptosis in CSS and asthma patients

To compare the effect of TRAIL on eosinophil apoptosis, we incubated eosinophils with various amounts of recombinant TRAIL for 12 h and investigated the apoptotic cell percentage. Preliminarily, we performed incubation for various time durations (4, 8, 12, and 18 h) or various concentrations of TRAIL (50 ng/ml, 100 ng/ml, 500 ng/ml, 1

µg/ml, 10 µg/ml, and 50 µg/ml); we observed that 12 h of incubation and 10 µg/ml of concentration are optimal. The concentration of 10 µg/ml recombinant TRAIL induced apoptosis in eosinophils (Fig. 4A), and apoptotic cell percentage induced by TRAIL (10 µg/ml) was significantly lower in CSS patients than in asthma patients (Fig. 4B). The addition of a smaller amount of recombinant TRAIL (500 ng/ml, 100 ng/ml, and 50 ng/ml) did not induce the apoptosis of eosinophils in both groups (data not shown). All apoptotic data were confirmed by TUNEL staining (data not shown).

Additionally, we measured the level of active caspase-3, which is an intracellular protease in the apoptosis-induced signaling pathway activated by TRAIL. The active caspase-3 concentration induced in the 1 µg/ml and 10 µg/ml concentrations of TRAIL was significantly lower in CSS patients than in asthma patients (Fig. 4C).

Expression of TRAIL on lymphocytes and lymphocyte cytotoxicity against eosinophils

Peripheral blood T cells are known to express TRAIL on their surface in atopic dermatitis (30) and in some autoimmune diseases such as systemic lupus erythematosus (22). Moreover, in our study, we observed significant positive correlation between the serum TRAIL level and peripheral lymphocyte count. Therefore, we examined TRAIL expression on T cells and evaluated the cytotoxicity of T cells against eosinophils. T cells obtained from the CSS and asthma patients expressed TRAIL on their surface (Fig. 5A). There was no significant difference in the percentage of

TRAIL expression between CSS patients and asthma patients (Fig. 5B). The result of autologous T-lymphocyte coincubation with eosinophils is shown in Fig. 5C. For incubation with a high quantity of T cells (effector cell:target cell, 40:1 and 80:1), the specific ^{51}Cr release was significantly lower in CSS patients compared to asthma patients.

Influence of glucocorticoids on TRAIL receptor 3 expression by eosinophils

To investigate the effect of glucocorticoids on the TRAIL receptor 3 expression by eosinophil in CSS, we incubated eosinophils from the CSS patients with glucocorticoids. Preliminarily, we performed incubation under various concentrations of glucocorticoids (10, 50, 100, 500, and 1000 nM) and observed that 500 nM is the optimal concentration. Glucocorticoids downregulated TRAIL receptor 3 expression by eosinophils from the CSS patients (Fig. 6A).

Influence of TRAIL receptor 3 suppression in CSS eosinophils on TRAIL-induced apoptosis

To evaluate the transfection efficiency, we measured the green fluorescence protein (GFP) positive cells in the transfected eosinophils. As shown in Fig. 6B, transfection efficiency against eosinophils from the CSS patients was about 20%. The transfection efficiency against eosinophils from the asthma patients was almost same as that of the

CSS patients. TRAIL receptor 3 siRNA significantly inhibited the endogenous TRAIL receptor 3 expression on the eosinophils obtained from the CSS patients while showing no effect on TRAIL receptor 1 and actin expression by the eosinophils (Fig. 6C). The suppression of TRAIL receptor 3 expression by siRNA significantly increased the TRAIL-induced apoptosis of eosinophils from the CSS patients (Fig. 6D, 6E, $P < 0.05$). The TRAIL receptor 3 suppression of eosinophils from asthma patients did not display any significant effect on the eosinophil apoptosis.

Discussion

To our knowledge, this is the first report illustrating the increased expression of TRAIL receptor 3, a decoy receptor of TRAIL, on eosinophils in CSS. A previous study suggested that the impairment of CD95 (Apo1/Fas)-mediated apoptosis induced prolonged survival of eosinophils in CSS (10), however, the detailed mechanism of tissue and blood eosinophilia is still unclear. With regard to TRAIL and eosinophil survival, it was suggested that purified eosinophils from atopic patients revealed the expressions of TRAIL receptor 1, 3, and 4 surface proteins (24). In addition, Robertson et al (25) reported that, in the airways after exposure to allergens, enhanced expression of TRAIL and TRAIL receptor 4 and decreased expression of the DRs (TRAIL receptors 1 and 2) on eosinophils might contribute to prolonged survival of the eosinophils. In our study, the expression pattern of TRAIL receptor on eosinophils in

the asthma patients and healthy volunteers were almost the same as the results obtained by Robertson et al (25). The expression level of TRAIL receptor 3 was significantly higher in the CSS patients than in the asthma and HES patients. The TRAIL concentration that induced eosinophil apoptosis in asthma patients was higher than that reported by Robertson et al (25). The number of autologous T-cells required to induce eosinophil apoptosis was high (T cell:eosinophil, 40:1). Peripheral blood T cells are known to express TRAIL on their surfaces in atopic dermatitis (30) and in some autoimmune diseases such as systemic lupus erythematosus (22;31), which can induce the apoptosis of leukocytes (22). In asthmatic patients, TRAIL is expressed in the airways by inflammatory cells infiltrating the bronchial mucosa, as well as by structural cells of the airway wall including fibroblasts and epithelial, endothelial, and smooth muscle cells (32). In our study, peripheral T-cells expressed TRAIL on their surfaces and serum TRAIL receptor 3 levels in the CSS patients demonstrated a significant positive correlation with peripheral eosinophil counts; however, no such correlation was observed with lymphocyte and neutrophil counts and the serum levels of the receptor decreased in parallel with the improvement in the disease. Immunohistochemically, tissue infiltrating eosinophils expressed TRAIL receptor 3 in case of CSS; however, the inflammatory cells infiltrating the tissues in case of the renal lesions of Wegener's granulomatosis did not stain positively for TRAIL receptor 3. Although the transfection effect was about 20%, the suppression of TRAIL receptor 3 expression

using siRNA significantly increased the TRAIL-induced apoptotic eosinophils in the CSS patients. Taken together, we think that the increased expression of TRAIL receptor 3 might protect eosinophils from TRAIL induced apoptosis in peripheral blood and tissue microenvironments in case of CSS.

Glucocorticoids are the cornerstone of CSS treatment (1;2;8). In fact, in our study, the clinical symptoms improved and eosinophil count decreased with the use of glucocorticoids. There are several reports concerning glucocorticoid associated eosinophil apoptosis. Glucocorticoids can induce apoptosis of eosinophils but not neutrophils (33;34) ; they can do so via the interaction with its receptor on eosinophil membrane. The existence of IL-5 affects the apoptotic effect of glucocorticoids (35). On the other hand, glucocorticoids enhance the recognition and engulfment of apoptotic eosinophils by macrophages or bronchial epithelial cells (36). Glucocorticoids can also affect the chemokine production and adhesion molecules expression by eosinophils. Thus, glucocorticoids have multiple effects on eosinophil function. In this study, we found that glucocorticoids could affect TRAIL receptor 3 expression by eosinophils from CSS patients. This is a newly identified effect of glucocorticoids on the eosinophil molecule and might become regarded as one of the reasons for glucocorticoid-induced eosinophil apoptosis.

Successful treatment of CSS entails the differentiation of this syndrome from asthma and appropriate immunosuppressive therapy (1;2;8). We found that the sensitivity and specificity of distinguishing CSS from asthma or HES using serum TRAIL receptor 3 levels were 60.9% and 94.8%, respectively (cutoff serum TRAIL receptor 3 value, 841.9 pg/ml). Abnormal laboratory findings in patients with CSS include an increased peripheral blood eosinophil count and a raised erythrocyte sedimentation rate (ESR) (1;37). However, it is sometimes difficult to distinguish CSS from asthma using these markers because of following reasons: 1) in rare cases of CSS, eosinophilia is absent and wide-ranging and rapid changes in eosinophil counts take place (38); 2) use of corticosteroids to treat asthma may result in a failure to detect eosinophilia in patients with undiagnosed CSS; 3) increase in ESR occurs in other disorders such as infection. Other serological markers such as antineutrophil cytoplasmic antibodies are present in approximately 40% of CSS patients with the most common pattern being their perinuclear distribution (39;40). In addition, antineutrophil cytoplasmic antibody-positivity needs to be confirmed by the demonstration of myeloperoxidase in the serum (2). In this study, the specificity of serum TRAIL receptor 3 was relatively high. A positive result for the serum TRAIL receptor 3 might become one of the distinguishing markers of CSS from asthma; however, a negative result cannot preclude the existence of CSS because the sensitivity of the test is not so high. In addition, all the CSS patients investigated in this study were in a vasculitic phase, and it is not clear

whether the asthma patients investigated in this study would be developing CSS in the future. Little is known about the serum marker that can distinguish between asthma and CSS (41). Our study was relatively small in terms of the study population and was of a short duration; hence, definitive conclusions cannot be drawn from the results obtained. Therefore, we propose that clinical studies of longer durations and larger sample sizes addressing this point are necessary to judge the true diagnostic value of the serum TRAIL receptor 3 level.

Some caspases have an important role in inducing TRAIL receptor-mediated apoptotic signaling. TRAIL interacting with activated DR4 or DR5 can induce active caspase-8 that initiates proapoptotic signaling cascades leading to the cleavage of cellular factors and transmits its proapoptotic activity to executioner caspases such as caspase-3 and -7, thereby inducing apoptosis (12;16;18;19;21). Thus, caspases are key intracellular molecules in the regulation of apoptosis, and defects in caspase-induced apoptosis can cause hypereosinophilia (36). In our study, the caspase-3 activity induced by recombinant TRAIL was significantly lower in the CSS patients compared to the asthma patients. Impaired TRAIL-induced apoptosis signaling might have contributed to the eosinophilia observed in the CSS patients.

Prominent eosinophilia is one of the defining features of CSS (38). Its magnitude commonly reflects the clinical disease activity and, in many situations, eosinophil

suppression results in clinical improvement (38;42;43). CSS can affect virtually any organ system in the body (44;45), and, therefore, systemic symptoms are prominent in the case of CSS (1;2). Analysis of tissue biopsy specimens from patients with CSS reveals an eosinophil-rich inflammatory cell infiltrate with granuloma formation in connective tissues and blood-vessel walls (1;2). Eosinophils can cause tissue injury by releasing an array of toxic products such as eosinophil cationic protein (1;2;8). Thus, eosinophils play an important role in the pathogenesis of CSS and, therefore, the elucidation of the mechanism of eosinophil survival in tissue microenvironment is necessary. In this regard, our study provides a new insight; therefore, we think that further clinical studies addressing this point are necessary to clarify the pathogenesis of CSS.

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Figure legends

FIGURE 1. Comparison of TNF-related apoptosis-inducing ligand (TRAIL) receptor expression on eosinophils of healthy volunteers and patients suffering from asthma, hypereosinophilia (HES), and Churg-Strauss syndrome (CSS). Eosinophils obtained from each group of patients expressed all TRAIL receptors investigated in this study (representative data, Fig. 1A). TRAIL receptor 3 expression level was significantly higher in the CSS patients compared to the others. In the healthy volunteers, the expression level of TRAIL receptor 4 was significantly lower than those in the others, while that of TRAIL receptor 2 was significantly higher. The expression level of TRAIL receptor 1 was higher in the healthy volunteers compared to the others; however, the difference was not statistically significant (Fig. 1B, $*P < 0.01$, $**P < 0.05$, Bonferroni/Dunn with a one-way factorial ANOVA).

FIGURE 2. Serum level of TNF-related apoptosis-inducing ligand (TRAIL) receptor 3. Serum TRAIL receptor 3 levels in Churg-Strauss syndrome (CSS) patients were significantly higher than those in asthma and hypereosinophilia (HES) patients and in healthy volunteers. All patients were studied prior to the start of therapy. Bars indicate mean values in each group (Fig. 2A, $*P < 0.01$, $**P < 0.05$, Bonferroni/Dunn

with one-way factorial ANOVA; s-TRAIL-R3, serum TRAIL receptor 3). Serum TRAIL receptor 3 in 11 CSS patients significantly decreased 3 months after the start of therapy. Bars indicate mean values in each group (Fig. 2B, $**P < 0.05$, Bonferroni/Dunn with one-way factorial ANOVA; s-TRAIL-R3, serum TRAIL receptor 3).

FIGURE 3. Immunohistochemical analysis of TNF-related apoptosis-inducing ligand (TRAIL) receptor 3. Skin biopsy specimen of Churg-Strauss syndrome (CSS) patients demonstrated massive infiltration of eosinophils in the lesion (hematoxylin-eosin staining, $\times 150$ Fig. 3A, $\times 400$ Fig. 3E). Infiltrating inflammatory cells, which are considered to be eosinophils, are stained positive for TRAIL receptor 3 ($\times 150$ Fig. 3B and $\times 400$ Fig. 3F). Negative control for the first antibody is shown in Fig. 3C ($\times 150$) and Fig. 3G ($\times 400$). The infiltrating inflammatory cells in the renal lesions in Wegener's granulomatosis patients were not stained positive for TRAIL receptor 3 ($\times 150$ Fig. 3D, $\times 400$ Fig. 3H).

FIGURE 4. Effect of recombinant TNF-related apoptosis-inducing ligand (TRAIL) on eosinophil apoptosis. Addition of 10 $\mu\text{g/ml}$ recombinant TRAIL induced apoptosis

in eosinophils obtained from asthma patients but did not induce apoptosis in eosinophils acquired from Churg-Strauss syndrome (CSS) patients (Fig 4A, representative data). After incubation with 10 µg/ml recombinant TRAIL, the annexin V positive percentage was significantly higher in the asthma patients compared to the CSS patients (Fig. 4B, $*P < 0.01$, Bonferroni/Dunn with one-way factorial ANOVA). Active caspase-3 levels after the incubation with 1 µg/ml and 10 µg/ml recombinant TRAIL was significantly higher in the asthma patients compared to the CSS patients ($**P < 0.05$, $*P < 0.01$, Bonferroni/Dunn with one-way factorial ANOVA).

FIGURE 5. TNF-related apoptosis-inducing ligand (TRAIL) expression on T cells and coincubation of eosinophils with autologous T cells. Peripheral T lymphocytes from Churg-Strauss syndrome (CSS) patients expressed TRAIL on their surfaces (Fig. 5A, representative data). There was no significant difference in TRAIL expression level between CSS patients and those suffering from asthma (Fig. 5B). Upon incubation with a high quantity of T cells (effector cell:target cell = 40:1 and 80:1), the specific ^{51}Cr release was significantly lower in the CSS patients than in the asthma patients ($**P < 0.05$, $*P < 0.01$, Bonferroni/Dunn with one-way factorial ANOVA).

FIGURE 6. Influence of glucocorticoids and siRNA against TNF-related

apoptosis-inducing ligand (TRAIL) receptor 3. Incubation with 500 nM glucocorticoids downregulated TRAIL receptor 3 expression in eosinophils from Churg-Strauss syndrome (CSS) patients (Fig. 6A, representative data from 5 individual experiments using 5 different CSS patients; negative control was incubated with PBS). The transfection efficiency of siRNA against TRAIL receptor 3 was almost 20% (Fig. 6B, representative data from 5 individual experiments using 5 different CSS patients). The siRNA decreased endogenous TRAIL receptor 3 expression of eosinophils from the CSS patients; however, it did not reveal any effect on the expressions of TRAIL receptor 1 and actin from eosinophils (Fig. 6C, representative data). Suppression of TRAIL receptor 3 expression by siRNA significantly increased the TRAIL-induced apoptosis of eosinophils from the CSS patients (Fig. 6D; representative data, 6E; $*P < 0.05$, $n = 8$, Bonferroni/Dunn with one-way factorial ANOVA). The TRAIL receptor 3 siRNA did not show any significant effect against the apoptosis of eosinophils from asthma patients (Fig. 6D, 6E).

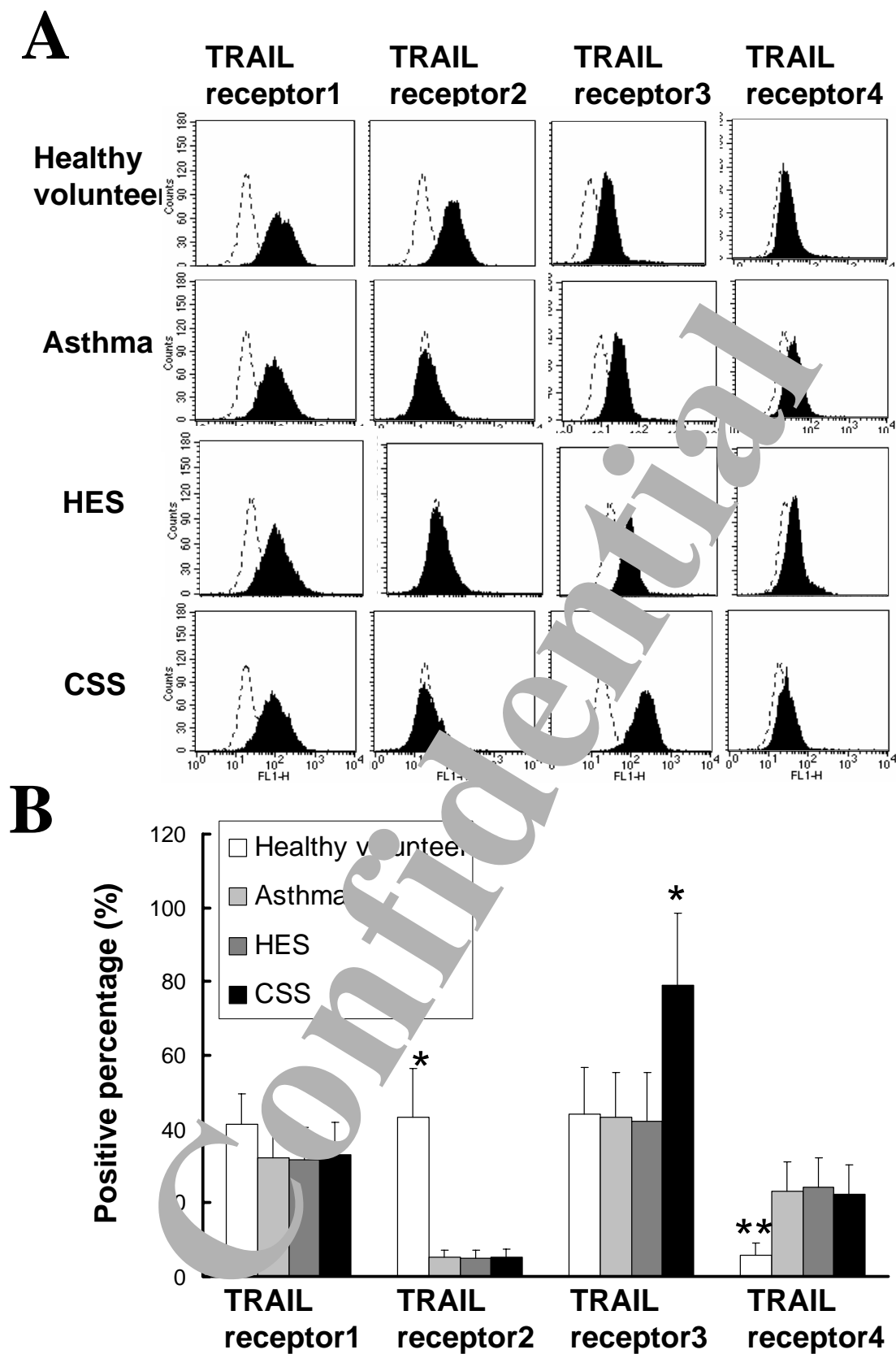
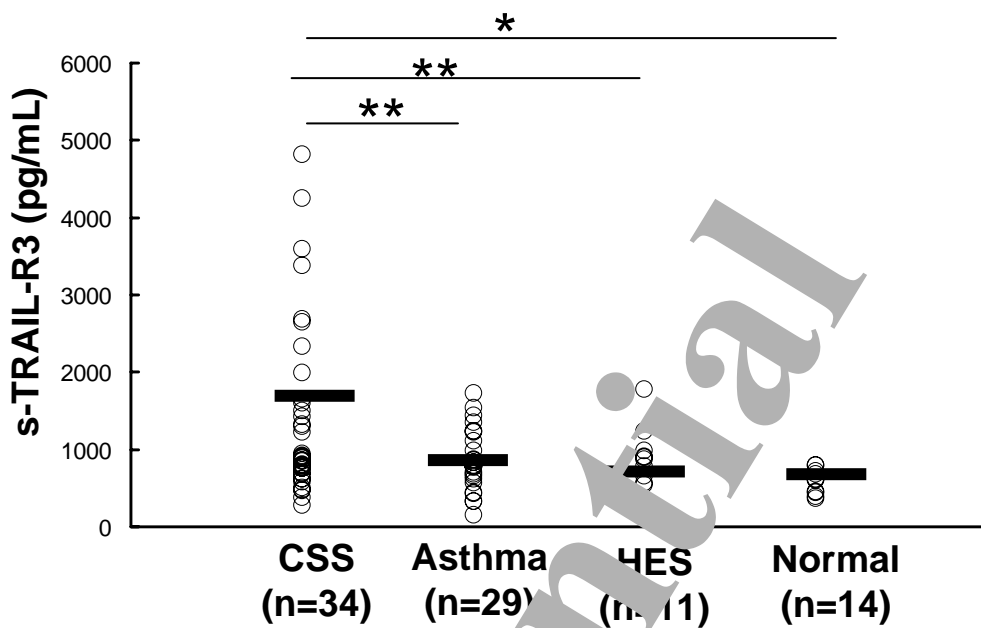
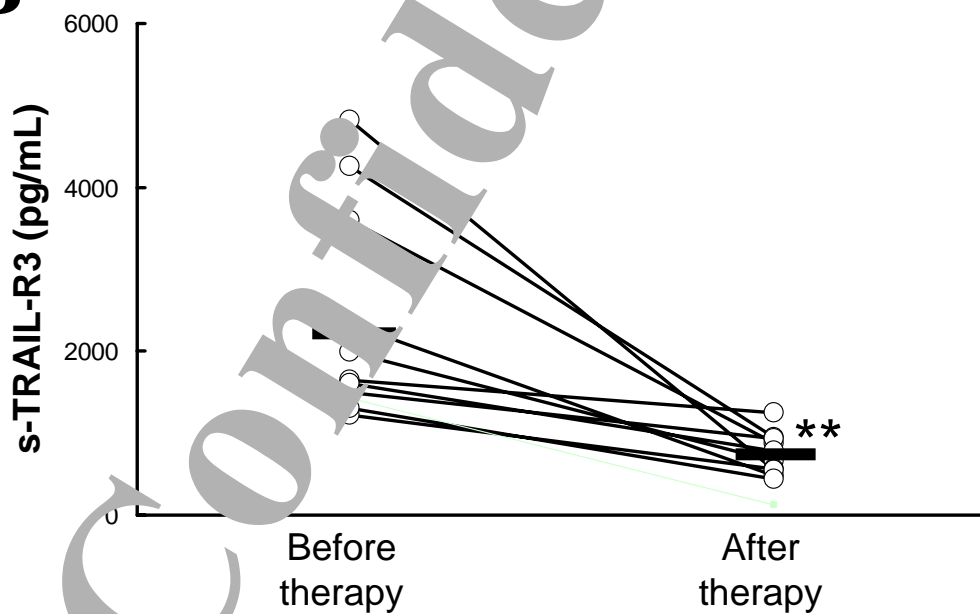


Figure 1

A**B****Figure 2**

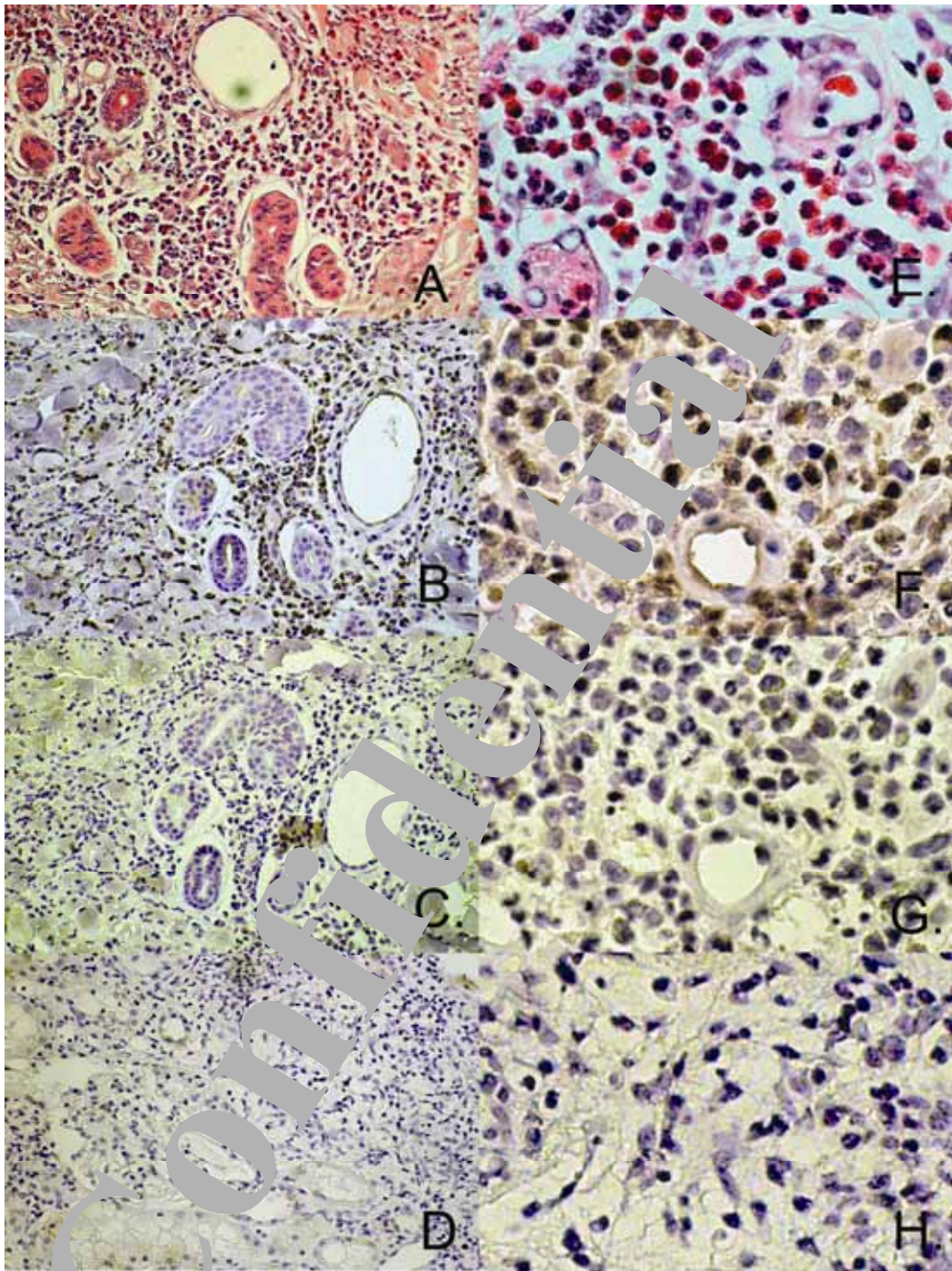


Figure 3

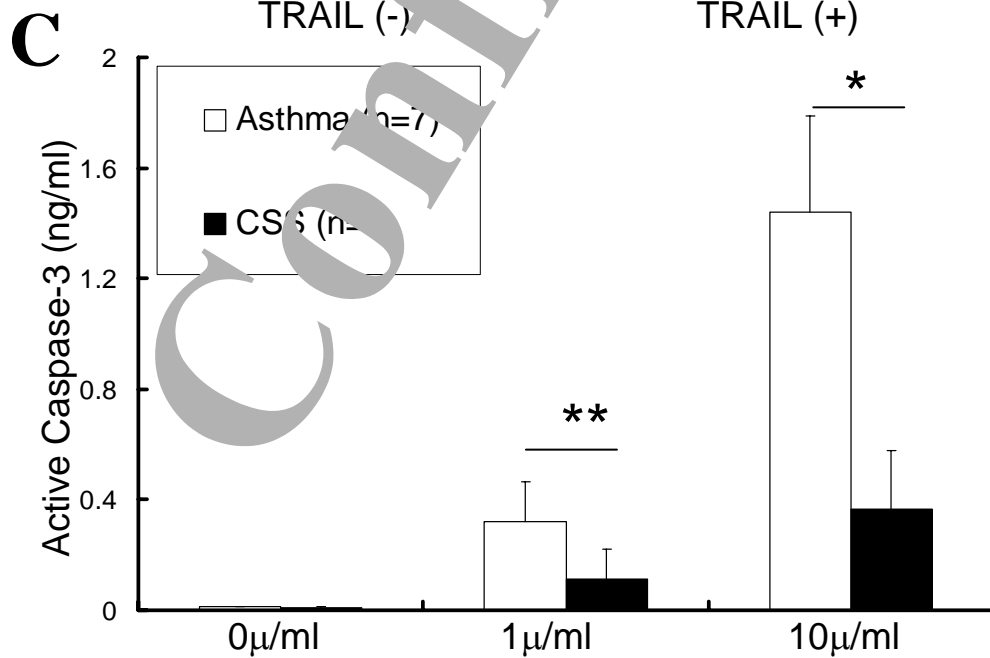
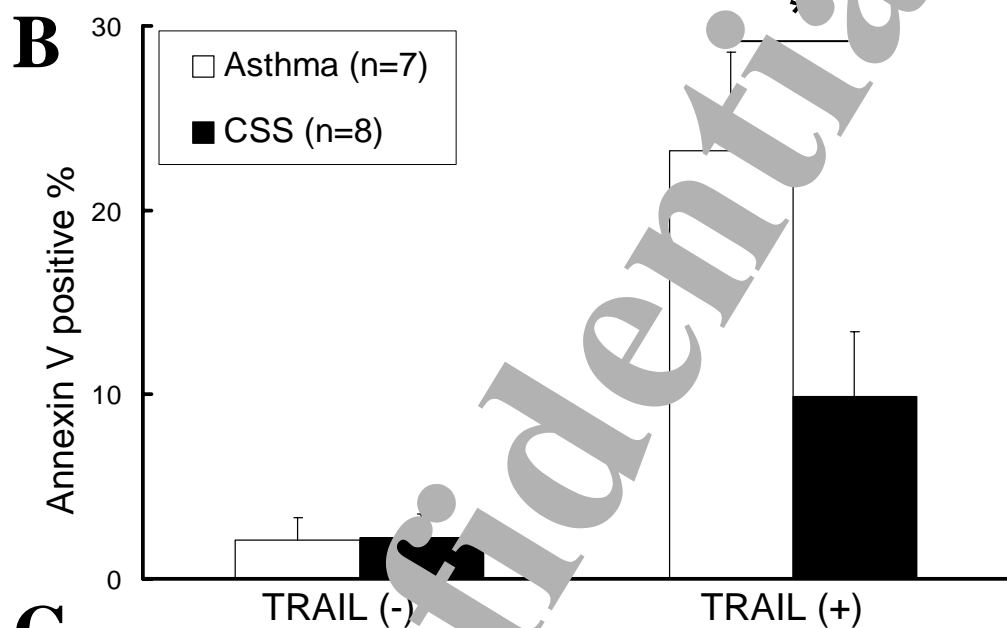
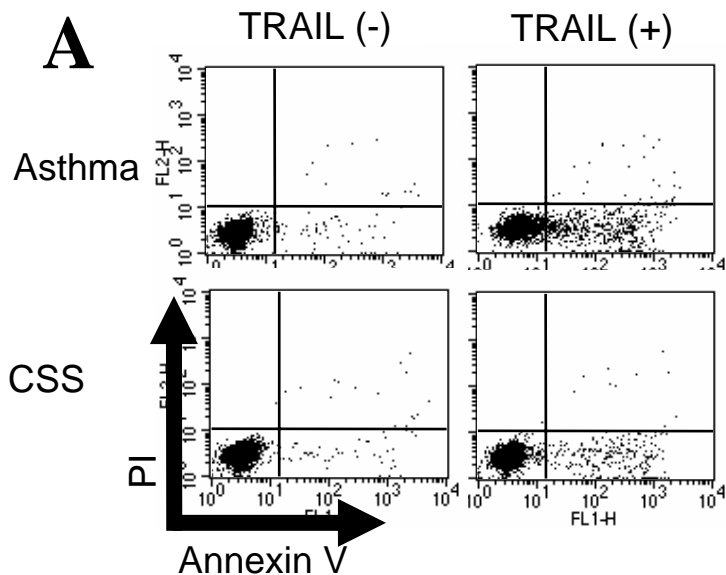
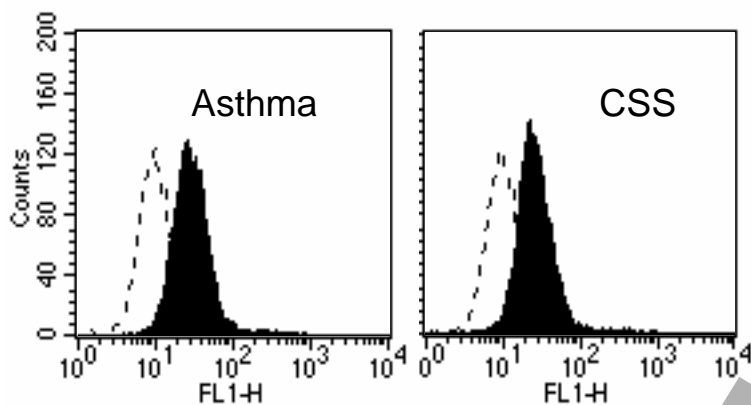
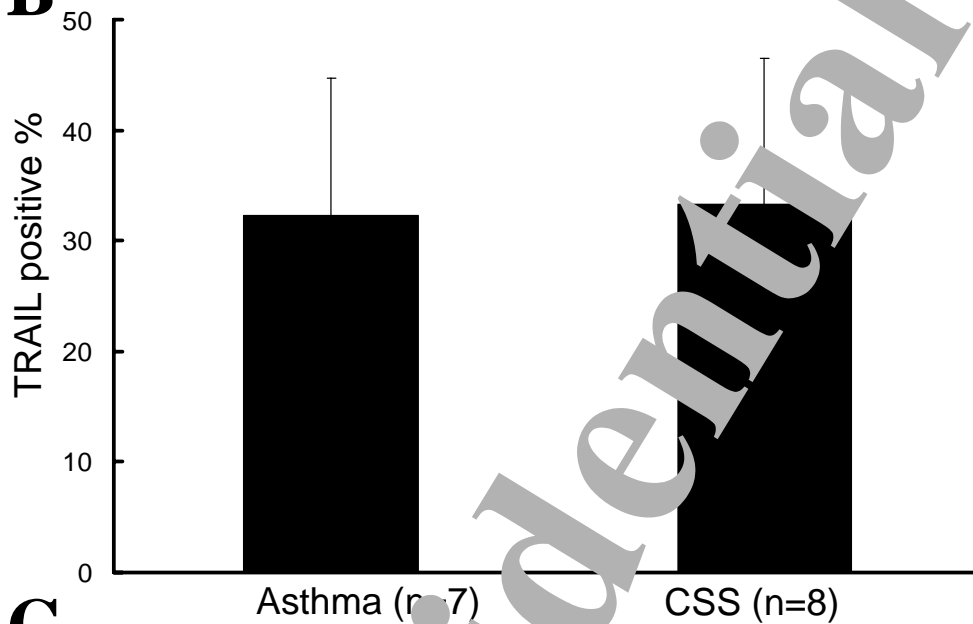
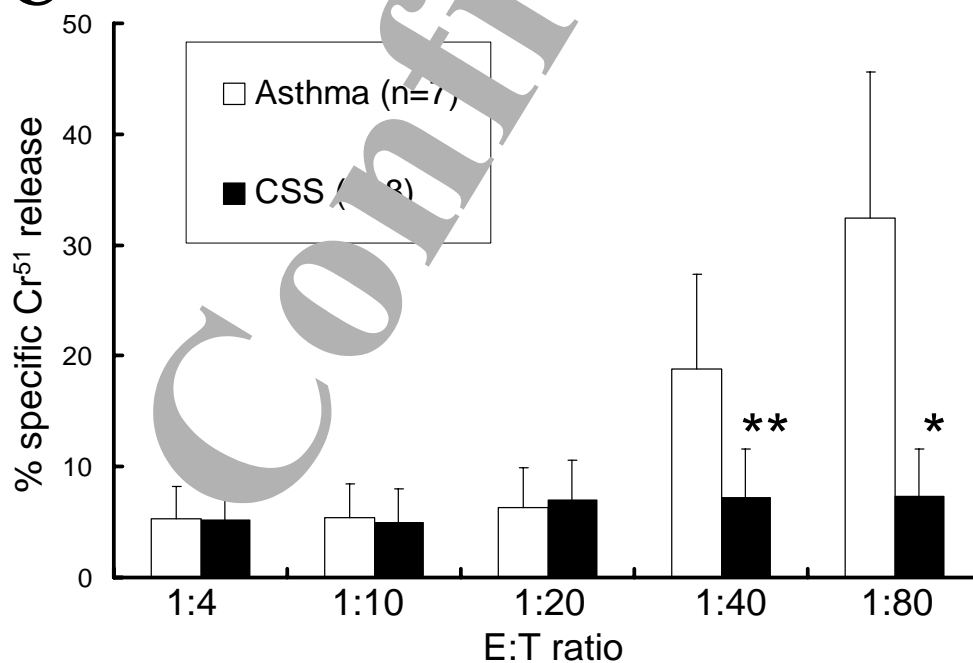
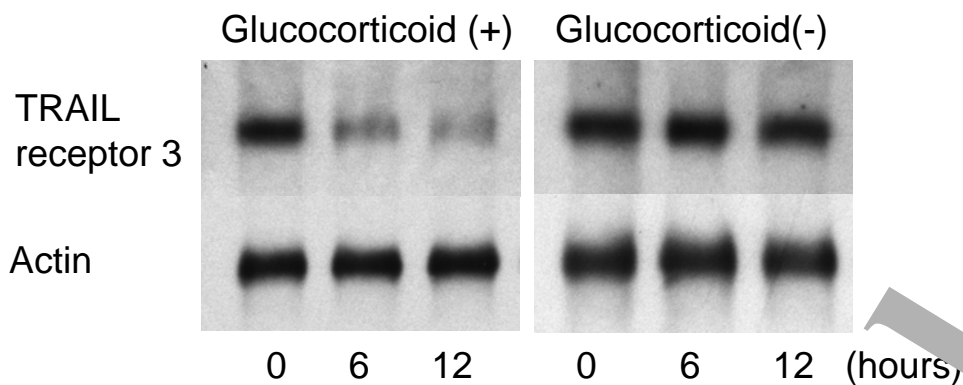
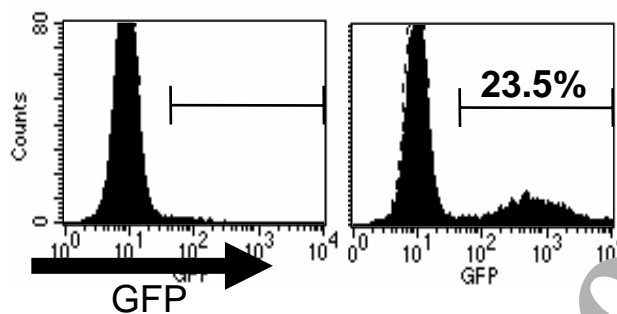
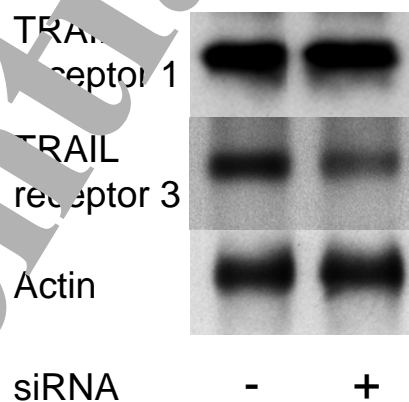
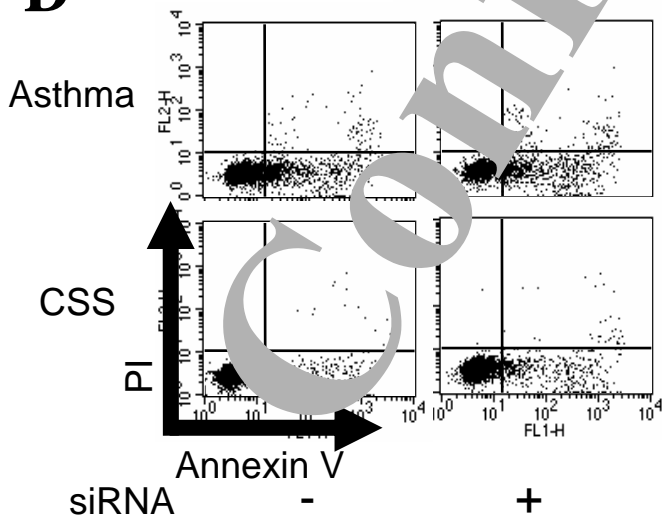
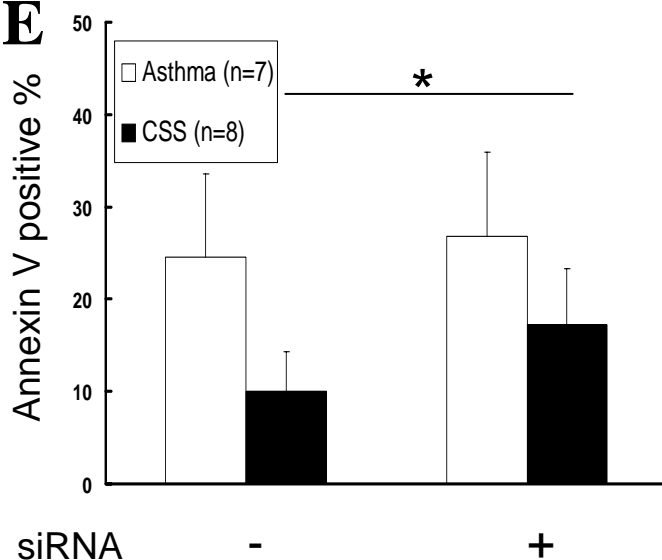


Figure 4

A**B****C****Figure 5**

A**B****C****D****E****Figure 6**