Differential Expression of TRAIL and TRAIL Receptors in Allergic Asthmatics Following Segmental Antigen Challenge: Evidence for a Role of TRAIL in Eosinophil Survival

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Asthma is a chronic lung disease exhibiting airway obstruction, hyperresponsiveness, and inflammation, characterized by the infiltration of eosinophils into the airways and the underlying tissue. Prolonged eosinophilic inflammation depends on the balance between the cell’s inherent tendency to undergo apoptosis and the local eosinophil-viability enhancing activity. TRAIL, a member of the TNF family, induces apoptosis in most transformed cells; however, its role in health and disease remains unknown. To test the hypothesis that Ag-induced inflammation is associated with TRAIL/TRAIL-R interactions, we used a segmental Ag challenge (SAC) model in ragweed-allergic asthmatics and nonasthmatic patients and analyzed bronchoalveolar lavage (BAL) material for 2 wk. In asthmatic patients, the level of TRAIL in BAL fluid dramatically increased 24 h after SAC, which significantly correlated with BAL eosinophil counts. Immunohistochemical analysis of bronchial biopsies from asthmatic patients demonstrated that TRAIL staining was increased in epithelial, airway smooth muscle, and vascular smooth muscle cells and throughout the interstitial tissue after SAC. This was confirmed by quantitative immunocytochemical image analysis of BAL eosinophils and alveolar macrophages, which demonstrated that expression levels of TRAIL and DcR2 increased, whereas expression levels of the TRAIL-Rs DR4 and DR5 decreased in asthmatic subjects after SAC. We also determined that TRAIL prolongs eosinophil survival ex vivo. These data provide the first in vivo evidence that TRAIL expression is increased in asthmatics following Ag provocation and suggest that modulation of TRAIL and TRAIL-R interactions may play a crucial role in promoting eosinophil survival in asthma. The Journal of Immunology, 2002, 169: 5986–5996.

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Asthma is characterized by reversible airway obstruction, inflammation, and structural changes in the bronchi, including epithelial cell denudation, mucous gland hyperplasia, smooth muscle hypertrophy and hyperplasia, and excess collagen deposition (1–7). Cytokines play a key role in the coordination and persistence of the chronic inflammatory process in asthma. The airway inflammation that occurs after repeated exposure to allergens or during the late-phase reaction results from a complex network of interactions between inflammatory cells (mast cells, eosinophils, macrophages, and activated T and B cells) and cells comprising the lung structure (endothelial cells, fibroblasts, bronchial epithelial cells, and smooth muscle cells) (6, 7). The proinflammatory role of eosinophils has been revealed in bronchial biopsies, bronchoalveolar lavage (BAL) fluid, and peripheral blood of patients with mild to moderate asthma, and the degree of blood eosinophilia has been correlated with disease severity (1, 8). Eosinophils mediate some of their unique cytotoxic and inflammatory functions by the production, storage, and regulated release of their granule proteins, contributing to tissue damage on bronchial epithelium and secretion of various cytokines (2, 3). Thus, there is great interest in defining the mechanisms by which the selective accumulation, activation, and maintenance of eosinophils at the site of inflammation in allergic-type responses are regulated. Known mechanisms of this complex series of events include selective adhesion pathways, specific chemotactic factors, and enhanced survival by certain cytokines, principally the T cell-derived eosinopoietins, IL-5, IL-3, and GM-CSF (9–11). In addition, eosinophils secrete cytokines and chemokines, including TGF-α and -β, TNF-α and -β, macrophage inflammatory protein 1α, IL-1, IL-3, IL-5, IL-6, IL-8, and GM-CSF. The secretion of these factors may continue eosinophil participation in the inflammatory response, in part, by promoting their survival.

The members of the TNF family play critical roles as prominent mediators of immune regulation and the inflammatory response (12–18). Members of this family act either as integral membrane proteins through direct cell-to-cell contact or as soluble effectors capable of diffusing to more distant targets to act in an autocrine or paracrine manner (12). Binding to their cognate receptors may lead to the activation of various signaling pathways, including the NF-κB family of transcription factors, c-Jun N-terminal protein

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4 Abbreviations used in this paper: BAL, bronchoalveolar lavage; SAC, segmental Ag challenge; PI, propidium iodide; FEV1, forced expiratory volume in 1 s.
kinases, extracellular signal-regulated kinases, and the caspase cascade (19–24).

A TNF-related ligand, TRAIL, is a member of the TNF superfamily of cytokines and is capable of inducing apoptosis in a variety of transformed cells in vitro (25). Early studies with TRAIL indicated that most normal cells were resistant to its cytotoxic effects. TRAIL exhibits the highest homology to Fas ligand, which has been implicated in T cell cytotoxicity and immune regulation (25). In contrast to the expression of Fas ligand, which is restricted mainly to activated T cells, NK cells and immune-privileged sites, TRAIL mRNA is constitutively expressed in a variety of tissues and cells (25). Resting peripheral blood T cells are resistant to the cytotoxic effects of TRAIL; however, T cells stimulated with IL-2 acquire sensitivity. This suggests a role for this ligand in peripheral deletion. In vivo, a role for TRAIL in type-I IFN-mediated enhancement of cytolytic T cell function, and modulation of tumoral activity of NK cells, dendritic cells, and monocytes has been described recently (26–29). Importantly, the regulation of TRAIL expression and its physiological role remain to be determined.

TRAIL interacts with five distinct receptors: DR4, DR5, DcR1, DcR2, and osteoprotegerin (15–17, 30–36). DR4 and DR5 each contain a canonical “death domain” that is required for apoptosis induced by these receptors. DcR1 lacks an intracytoplasmic domain, thereby quelling TRAIL and abolishing its ability to transmit apoptotic signaling through its death receptors (15, 33, 35). DcR2 exhibits high sequence homology to the extracellular domains of DR4, DR5, and DcR1; however, its cytoplasmic domain contains a truncated death domain. Unlike DcR1, DcR2 has a functional intracellular signaling domain and therefore protects cells from apoptosis by either acting as a decoy receptor or transmitting antiapoptotic signals. Stimulation of DcR2 by TRAIL activates the transcription factor NF-κB and thereby prevents apoptosis (34). It has been proposed that the expression of TRAIL-Rs may regulate a cell’s sensitivity to TRAIL. Specifically, the presence or absence of TRAIL decoy receptors may determine whether a cell is resistant or sensitive to TRAIL stimulation.

The present study investigates the role of TRAIL in asthma. We propose that enhanced expression of TRAIL and DcR2, its decoy receptor, and decreased expression of death receptors DR4 and DR5 may contribute to prolonged survival of eosinophils in the airways after allergen challenge. To test this hypothesis, we investigated TRAIL expression in bronchial biopsies and BAL cells and fluid. In addition, TRAIL-R expression in BAL cells and the effects of TRAIL on peripheral blood eosinophil viability were examined. The immune responses of asthmatic and nonasthmatic subjects were compared to determine how TRAIL functions in vivo following Ag challenge.

Materials and Methods

Subject characterization

Eight non-ragweed-allergic healthy volunteers and eight ragweed-allergic asthmatic volunteers who met standard criteria for the diagnosis of asthma by the National Institutes of Health/National Heart, Lung, and Blood Institute expert panel gave informed consent and were enrolled in this study, which was approved by the Jefferson Medical College Institutional Review Board. Asthmatics took no long-term medication; rather, they were treated only with β-agonist as needed. Subject characterization and intradermal ragweed testing (0.001–10 U/ml short ragweed Ag E (Amb a 1); Greer Laboratories, Lenoir, NC) in normal saline solution with 0.03% human serum albumin), methacholine, and whole-lung ragweed challenge were performed as described previously. Spirometry to measure forced expiratory volume in 1 sec (FEV1) and forced vital capacity was performed on all subjects. Airway reactivity to ragweed allergen and nonspecific reactive to methacholine were determined by whole-lung challenges. Reactivity, PC20, is the provocative concentration of methacholine producing a 20% fall in FEV1 from post-saline baseline. Subject demographic and physiologic parameters are detailed in Table I.

Bronchoscopy, BAL, and segmental Ag challenge (SAC)

All subjects underwent bronchoscopic lavage, biopsy, and challenge with ragweed as described previously (37, 38). All subjects were premedicated with albuterol (four puffs by metered-dose inhaler) and inhaled and received topicaly applied lidocaine in the airways. Subjects underwent BAL with 150 ml of 37°C saline in three aliquots after the bronchoscope was wedged in one of the left lower lobe segments, followed by endobronchial forces biopsies (three to five samples/session). After these day-1 samples were obtained, the subjects were challenged with ragweed in a segment of the right middle lobe of the lung with 5 ml of ragweed solution in a concentration 100-fold greater than that producing a positive intradermal skin reaction. The solution was instilled in a “semiwedged” position with efforts made to accomplish instillation across two subsegmental bronchi to optimize future bronchoscopic sampling. The Ag-challenged segment was sampled (BAL and endobronchial forces biopsies) 24 h later (day 2), at which time a second Ag-challenge procedure was performed in the lingula. This second challenged segment was sampled 7 days later (day 9). Finally, the initial Ag-challenged segment was resampled at 2 wk (day 16). The entire protocol of four bronchoscopies included a total of two SACs and four BALs and endobronchial forces biopsies (up to five were taken after the SAC) per subject.

Cell and fluid handling

BAL fluid was filtered through two thicknesses of gauze to remove large mucus particles, and the cells were pelleted by centrifugation. The total number of cells was determined by counting Turk’s stained cells with a hemocytometer and a differential cell count was taken on cytospins stained with Wright-Giemsa stain. Cytospins of the total BAL cells were fixed in paraformaldehyde and stored at −20°C. The resultant BAL fluid was concentrated 120-fold for use in experiments and stored at −80°C. The BAL pellet was then resuspended in HBSS (without Ca2+ or Mg2+) with 0.1% BSA, layered over Percoll (adjusted to 1.079 g/ml with saline), and centrifuged for 20 min at 1200 × g. The granulocyte layer was washed once, and a cell count and viability were determined by trypan blue exclusion. Eosinophil purity is assessed by Wright-Giemsa stained cytospins. The purity of BAL eosinophils used was >95%.

Blood leukocytes were separated by sedimenting five parts of whole blood with one part of 6% dextran in PBS for 50 min. The cells were pelleted and washed with HBSS, and the granulocytes were fractionated over 1.085 g/ml Percoll in saline for 20 min at 1200 × g. Neutrophils were separated from the eosinophils by incubation with anti-human-CD16 immunomagnetic beads (Miltenyi Biotech, Auburn, CA) for 30 min at 4°C and passage through a magnetic column. The cells in the eluate were pelleted, resuspended in HBSS, and subjected to a final count. Viability was determined by trypan blue exclusion, and purity was assessed by Wright’s stain. Average purity of eosinophils was >98%.

ELISA for TRAIL

After thawing, BAL samples were assayed for TRAIL protein using a solid-phase ELISA (Pharmacia, Peapack, NJ) according to the manufacturer’s procedures.

In vitro eosinophil viability and cell cycle assay

Eosinophils were cultured in 24-well plates at a concentration of 1 × 106 cells per ml. The cells were exposed to TRAIL (10–1000 ng/ml). Survival was assessed throughout the time course by trypan blue exclusion and

Table I. Subject Demographics*

<table>
<thead>
<tr>
<th></th>
<th>Asthmatic</th>
<th>Nonasthmatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (gender)</td>
<td>8 (3 females/5 males)</td>
<td>8 (4 females/4 males)</td>
</tr>
<tr>
<td>Age (mean ± SEM)</td>
<td>28.8 ± 1.5</td>
<td>29 ± 2.4</td>
</tr>
<tr>
<td>FEV1</td>
<td>3.67 ± 0.24</td>
<td>3.71 ± 0.28</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>80.56 ± 1.92</td>
<td>83.13 ± 2.79</td>
</tr>
<tr>
<td>PC20</td>
<td>2.66 ± 1.02</td>
<td>&gt;32</td>
</tr>
</tbody>
</table>

Abbreviations used: FVC, forced vital capacity; and PC20, concentration of methacholine producing a 20% fall in FEV1.
4′,6′-diamidino-2-phenylindole staining. Cells were pretreated with either 50 μM zVAD-fmk or an equivalent volume of inhibitor vehicle (MeSO₄) for 37°C, before the addition of TRAIL. Peripheral blood eosinophils were treated for 48 h with BAL fluid obtained at baseline (day 1) and 24 h (day 2), 1 wk (day 9), and 2 wk (day 16) after SAC. Addition of neutralizing Ab to TRAIL (5 μg/ml) or nonspecific IgG was added at time 0. Two hundred cells per slide were evaluated in duplicate for each time point. Cells processed for flow cytometry were rinsed twice in chilled PBS, pelleted, and resuspended in 70% ethanol. The cells were kept on ice for 10 min, pelleted, and then treated with RNase A (1.8 μg; Roche Applied Science, Indianapolis, IN) for 30 min at room temperature. Propidium iodide (PI) was added to a final concentration of 2 μg/ml for an additional 15 min at room temperature. Cell cycle analysis was then performed on a Coulter Epics XL Flow Cytometer (Beckman Coulter, Miami, FL).

**FIGURE 1.** TRAIL prolongs survival of peripheral blood eosinophils. A, Survival curves based on trypan blue exclusion assay were generated for peripheral blood eosinophils or BAL eosinophils (obtained 24 h post-SAC), cultured for a 72-h time course with either recombinant TRAIL (500 ng/ml) or the pancaspase inhibitor zVAD-fmk (50 μM). Data are mean ± SD, n = 4. B, Cell cycle analysis of a representative PI staining of peripheral blood eosinophils following a 72-h time course with either TRAIL (500 ng/ml) or control cultures. The cells in the subG₁ region (indicated in c and e) document the significant cell death of peripheral blood eosinophils. C, TUNEL stains of peripheral blood eosinophils cultured with recombinant TRAIL (500 ng/ml) were evaluated by TUNEL analysis. In the TUNEL assay (FITC), all the cells were visualized by PI stain. D, Western blot analysis for pro-caspase-3 (p32), active caspase-3 (p17), and β-actin was performed in whole-cell lysates from peripheral blood eosinophils cultured for a 72-h time course with or without TRAIL (500 ng/ml).

<table>
<thead>
<tr>
<th>BAL cells</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 9</th>
<th>Day 16</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Eosinophils (%)</strong></td>
<td>0.44 ± 0.22</td>
<td>59.8 ± 5.08</td>
<td>42.2 ± 8.7</td>
<td>24.23 ± 5.19</td>
</tr>
<tr>
<td>Asthmatic (± SD)</td>
<td>Controls (± SD)</td>
<td>0.38 ± 0.16</td>
<td>0.5 ± 0.3</td>
<td>0.6 ± 0.167</td>
</tr>
<tr>
<td><strong>Eosinophils (×10⁴/ml)</strong></td>
<td>0.07 ± 0.059</td>
<td>73.3 ± 23.30</td>
<td>62.0 ± 38.66</td>
<td>16.04 ± 8.69</td>
</tr>
<tr>
<td>Asthmatic (± SEM)</td>
<td>Controls (± SEM)</td>
<td>0.06 ± 0.03</td>
<td>0.1 ± 0.03</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td><strong>Macrophages (%)</strong></td>
<td>86.7 ± 3.12</td>
<td>33.0 ± 4.5</td>
<td>51.6 ± 8.7</td>
<td>62.4 ± 5.96</td>
</tr>
<tr>
<td>Asthmatic (± SD)</td>
<td>Controls (± SD)</td>
<td>92.2 ± 1.42</td>
<td>86.3 ± 9.52</td>
<td>84.3 ± 6.95</td>
</tr>
<tr>
<td><strong>Macrophages (×10⁴/ml)</strong></td>
<td>20.9 ± 7.04</td>
<td>28.6 ± 4.42</td>
<td>27.5 ± 7.99</td>
<td>23.63 ± 8.52</td>
</tr>
<tr>
<td>Asthmatic (± SEM)</td>
<td>Controls (± SEM)</td>
<td>14.2 ± 1.47</td>
<td>16.3 ± 1.45</td>
<td>12.2 ± 1.98</td>
</tr>
</tbody>
</table>
TUNEL assay

Cells were fixed with 4.0% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min at room temperature and permeabilized for 2 min at 0°C with 0.1% Triton X-100 in 0.1% sodium citrate. The slides were incubated with the TUNEL reagent (Roche, Basel, Switzerland) for 1 h at 37°C and counterstained with 0.05 mg/ml PI in PBS. The cells were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and examined using confocal laser-scanning microscopy.

SDS-PAGE and Western blotting

Cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose. The membranes were blocked overnight at 4°C in 10% nonfat milk (NFM)/PBS/0.1% Tween 20, and incubated for one hour with either anti-TRAIL mAb (1:1000) (Pharmacia) or with an anti-active-caspase-3 rabbit polyclonal Ab (1:1000) (BD Biosciences, Mountain View, CA) or anti-pro-caspase-3 Ab (1:1000) (Cell Signaling Technology, Beverly, MA), diluted in 5% NFM/PBS/0.1% Tween 20. The membranes were then incubated for 1 h with either HRP-labeled goat anti-mouse or anti-rabbit Ab (diluted 1:2500 in 5% NFM/PBS/0.1% Tween 20). Proteins were detected using ECL.

Immunohistochemistry

Cytospin slides were washed once with PBS and blocked with 100% goat serum for 30 min. The slides were washed three times with PBS/0.05% Tween 20 (PBST), blocked 15 min with Avidin D (Vector Laboratories), washed again, and blocked 15 min with biotin solution. The slides were washed three times with PBST and incubated with 1 µg/ml primary Ab diluted in 10% goat serum overnight at 4°C. After washing three times with PBST, the slides were incubated with 5 µg/ml of the appropriate biotinylated secondary Ab for 1.5 h at room temperature. The slides were incubated with streptavidin-alkaline phosphatase reagent for 30 min, and then washed and incubated with an alkaline phosphatase substrate (5-bromo-4-chloro-3-indoxyl phosphate/nitroblue tetrazolium) supplemented with 1 mM Levamisole (DAKO, Carpinteria, CA), to inhibit endogenous alkaline phosphatase activity for 30 min. Finally, slides were washed and mounted.

The 5-µm biopsy specimens were deparaffinized in Microclear (Micron Environmental Industries, Fairfax, VA), rehydrated, and permeabilized with proteinase K (6 µg/ml; Roche Applied Science) for 15 min at 55°C. Sections were blocked with 10% normal goat serum for 4 h, washed with PBS and incubated with primary Ab overnight at 4°C. The sections were washed in PBS/0.1% Tween 20 and incubated with biotinylated anti-mouse secondary Ab for 1 h at room temperature. The sections were washed with PBS/0.1% Tween 20 and incubated with streptavidin-alkaline phosphatase reagent for 1 h at room temperature, and then washed and incubated with the alkaline phosphatase substrate (5-bromo-4-chloro-3-indoxyl phosphate/nitroblue tetrazolium) supplemented with 1 mM Levamisole for 2 h at room temperature. Sections were counterstained with saponin (0.0025%) for 30 s, rinsed in H2O, and mounted in Glycergel (DAKO).

Image analysis procedure

Immunocytochemically stained slides from each time point for eight asthmatics and eight normal controls were assessed for stain density (Phase 3 image analysis software; Image Pro Plus/Cybernetics, Silver Spring, MD). Stained cells could typically be identified morphologically in most cases; however, cell area was used in conjunction with the differential cell count from Wright (Accustain; Sigma-Aldrich, St. Louis, MO)-stained slides, to infer cell type as described previously (38). Eosinophils, neutrophils, lymphocytes, and macrophages were measured for area (50 cells in each category), and mean ± SD was obtained. Based on the mean cell area, lymphocytes were found to group within 0–125 µm² (2), granulocytes were within 126–250 µm² (2), and macrophages were >250 µm² (2). All cells in randomly selected frames were measured until ~200 cells were assessed.

\[
\text{Mean density} = \frac{\text{Number of cells}}{\text{Area of image}}
\]

\[
\text{Day 1} \quad \text{Day 2} \quad \text{Day 9} \quad \text{Day 16}
\]

\[
\text{Airway Epithelium} \quad \text{Airway Smooth Muscle}
\]

**FIGURE 2.** TRAIL expression is increased in endobronchial biopsies of asthmatic subjects. A, Biopsies obtained during the SAC protocol were paraffin embedded and analyzed by immunohistochemistry using an anti-TRAIL mAb. Representative staining for an asthmatic and nonasthmatic subject is shown. The epithelium is indicated with a thick arrowhead, and the smooth muscle is indicated with a thin arrowhead. No signal was observed with an isotype-matched control mAb. B and C, Quantitation of stain density for TRAIL in epithelium and smooth muscle (n = 5). Analysis was performed using Phase 3 image analysis. Results are expressed as mean density (normalized for area). Values are means ± SE. A significant difference in TRAIL stain intensity was observed in the asthmatic subjects 24 h after SAC (day 2) compared with that of the control group (p < 0.05) for airway epithelium and airway smooth muscle.
for area and stain density on each immunostained slide. Microscope light intensity was held constant. Background density was subtracted from each slide. Measurements for cells were sorted according to cell area based on intensity was held constant. Background density was subtracted from each immunostained slide. Microscope light windows (version 2.03) statistical packages. SigmaStat tests data for normality.

Statistical analysis

Data were analyzed using Microsoft Excel 2000 and SigmaStat for Windows (version 2.03) statistical packages. SigmaStat tests data for normality as well as equal variance. Group data are expressed as mean ± SEM. Data were assessed for significance by Student’s t test or two-way repeated measure ANOVA, as appropriate. When ANOVA indicated a significant difference, pairs were examined using the Student-Newman-Keuls method or the Tukey test to determine at which time points the difference existed. A p value of <0.05 was interpreted as significant.

Results

TRAIL promotes peripheral blood eosinophil survival

Airway eosinophilia is a well-described feature of the inflammatory infiltrate in asthma. BAL eosinophils were obtained from allergic, asymptomatic asthmatics after SAC with ragweed. To determine differences in the viability characteristics between BAL and peripheral blood eosinophils, purified cells were cultured in RPMI 1640 with 15% FBS without the addition of exogenous cytokines. In Fig. 1A, the mean number of surviving BAL and peripheral blood eosinophils were compared at the different time points. Significant differences in the number of viable cells were seen beginning at 48 h (98.0 ± 2.0 vs 30 ± 1.6, respectively; p < 0.05), and this difference was maintained at 72 h (97 ± 7.6 vs 3.0 ± 1.4; p < 0.05). We next conducted kinetic studies to examine the effects of TRAIL on cell viability in primary cultures of peripheral blood eosinophils and BAL eosinophils. Significant differences in survival of peripheral blood eosinophils treated with TRAIL compared with untreated cells were observed beginning at 48 h (95.0 ± 2.2 vs 30 ± 1.6, respectively; p < 0.05), and this difference was maintained at 72 h (69 ± 7.6 vs 3.0 ± 1.4; p < 0.05). BAL eosinophils exhibit ~98% viability in the presence or absence of TRAIL. Because caspase activation is required for apoptosis, we examined the ability of the cell-permeable pancaspase inhibitor zVAD-fmk to prevent apoptosis. Cells cultured with zVAD-fmk demonstrated increased cell survival compared with that of untreated cells at 48 h (72 ± 2.2 vs 30 ± 1.6, respectively; p < 0.005) and 72 h (69 ± 7.6 vs 3.0 ± 1.4; p < 0.05).

Cell cycle analysis further confirmed that peripheral blood eosinophils cultured in the presence of TRAIL were resistant to apoptosis. In the absence of TRAIL, peripheral blood eosinophils exhibit significant cell death documented by the shift of cells to the subG 1 region of the cell cycle profile, as shown in Fig. 1B. c and e, whereas incubation with TRAIL prevents cells from undergoing apoptosis (d and f). We also evaluated the effect of TRAIL on peripheral blood eosinophils by the TUNEL method (Fig. 1C). In the absence of exogenous TRAIL, peripheral blood eosinophils exhibited the typical morphologic changes associated with apoptosis, such as cell shrinkage, nuclear blebbing, and the formation of apoptotic bodies. In addition, the nuclei exhibited fluorescence indicating the presence of labeled 3'-OH ends characteristic of apoptotic cells. In contrast, in the presence of TRAIL, these morphologic alterations of peripheral blood eosinophils were inhibited. To assess whether caspases were inhibited by TRAIL to prevent eosinophil apoptosis, peripheral blood eosinophils were treated, and cell extracts were examined by Western blot analysis. As shown in Fig. 1D, peripheral blood eosinophils cultured in the absence of exogenous TRAIL exhibited cleavage of the effector caspase-3 (p32) to the enzymatically cleaved product (p17), which indicates conversion to the mature or activated caspase. In contrast, in cells cultured with TRAIL, the proenzyme form of caspase-3 remains intact and unactivated. These data suggest that apoptosis of peripheral blood eosinophils induced by growth factor withdrawal involves activation of the caspase cascade and is inhibited in the presence of either TRAIL or the pancaspase inhibitor zVAD-fmk.

TRAIL expression after allergen challenge in vivo

To determine whether TRAIL plays a role in asthma, we used a SAC model in eight ragweed-allergic asthmatics and eight nonasthmatic subjects (Table I). The SAC of the lung protocol provided an opportunity to obtain cells infiltrating into the airways, including alveolar macrophages/monocytes, and eosinophils. BAL was performed at baseline (day 1) and 24 h (day 2), 1 wk (day 9),
and 2 wk (day 16) following SAC. As reported previously, SAC resulted in increased cell influx into the challenged segment of asthmatics but not into that of control subjects (Table II).

TRAIL was initially assessed in endobronchial biopsies in allergic asthmatic vs nonasthmatic subjects after Ag challenge. As shown in Fig. 2, immunohistochemical staining of biopsies of asthmatic subjects showed the following: 1) faint, distinct staining of epithelial cells and moderate staining of airway smooth muscle for TRAIL from a biopsy obtained on day 1 before SAC, 2) a marked increase in staining for TRAIL in both epithelial cells and airway smooth muscle and vascular smooth muscle cells, from biopsies obtained at days 2, 9, and 16 after SAC. Increasing levels of TRAIL expression were also observed throughout the interstitial tissue after SAC. In contrast, the biopsies of nonasthmatic subjects exhibited low levels of TRAIL staining of epithelial cell and airway smooth muscle following Ag challenge. No staining was observed when sections were stained with an isotype-matched control mAb. A significant difference in TRAIL stain intensity was observed in the asthmatic subjects 24 h after SAC (day 2) compared with that of the control group \((p < 0.05)\) for airway epithelium and airway smooth muscle.

### Appearance of soluble TRAIL following SAC

To assess TRAIL release into the airway, BAL fluid at various time points was analyzed by Western analysis, as shown in Fig. 3, A and B. We observed that the allergic asthmatic subjects had a significant increase in TRAIL compared with that of controls, \((p < 0.05)\). Within asthmatic subjects, TRAIL expression was found to be significantly increased day 2 post-SAC compared with that of the control group \((p < 0.05)\), with levels returning to baseline at or close to 2 wk. In allergic asthmatic subjects, increases in total cells and eosinophils were marked on day 2 and persisted through day 9 (Table II). As shown in Fig. 3C, we observed a significant correlation \((r = 0.83, p < 0.05)\) between the increase in eosinophils and the level of TRAIL following SAC on day 2.

#### Effect of neutralizing Ab to TRAIL on BAL-mediated survival of peripheral blood eosinophils

Next we sought to quantify TRAIL protein concentration in BAL fluid in the asthmatic and nonasthmatic subjects (Fig. 4A). We observed that, in the asthmatic subjects, TRAIL expression was significantly increased at day 2 and day 9 post-SAC compared with that of controls \((p < 0.05)\). To determine whether the TRAIL present in the BAL fluid promotes survival of eosinophils, peripheral blood eosinophils were treated with BAL fluid obtained from asthmatic subjects at each time point and their viability was assessed. As shown in Fig. 4B, addition of BAL fluid to the culture medium of peripheral blood eosinophils prolonged eosinophil viability. The bioactivity from BAL obtained on days 2 and 9 was partly neutralized by anti-TRAIL Ab. These studies suggest that TRAIL can indeed play an important role in the survival of eosinophils in the atopic asthmatic response.

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**FIGURE 4.** Quantitation of TRAIL and effects of neutralizing Ab to TRAIL on BAL-mediated survival of peripheral blood eosinophils. A, TRAIL was measured in BAL fluid obtained from nonallergic, nonasthmatic control subjects \((n = 8)\) and asthmatic subjects allergic to ragweed \((n = 8)\) following SAC. TRAIL levels were reported as picomoles per milliliter of BAL fluid. There was no difference in the volumes of BAL fluid recovered from asthmatic and control subjects (data not shown). Data are expressed as mean ± SE. Statistical analysis was performed using two-way ANOVA and then multiple comparison test. *\(p < 0.05\). B, Survival-enhancing effects of TRAIL are blocked by neutralizing Ab. Peripheral blood eosinophils were treated for 48 h with BAL fluid obtained at baseline (day 1, \(\sim 25 \text{ pg/ml}\)) and 24 h (day 2, \(\sim 75 \text{ pg/ml}\)), 1 wk (day 9, \(50 \text{ pg/ml}\)), and 2 wk (day 16, \(10 \text{ pg/ml}\)) after SAC. Neutralizing Ab to TRAIL (5 \(\mu\text{g/ml}\)) or nonspecific IgG was added at time 0. Cell survival at 48 h was determined. Data are the mean ± SD of three representative experiments (\(^*\), \(p < 0.05\)).
Expression and quantitation of TRAIL and TRAIL-Rs in BAL cells following SAC

We examined whether eosinophils that have infiltrated to the airway lumen expressed receptors for TRAIL and whether they and other infiltrating leukocytes express TRAIL. As shown in Figs. 5-8, BAL cells migrating into the airway of an allergic asthmatic and control subjects post-SAC were examined by immunocytochemistry for the expression of TRAIL, DR4, DR5, DcR1, and DcR2. As shown in Fig. 5, TRAIL expression in BAL eosinophils and alveolar macrophages was found to be significantly increased day 2 post-SAC compared with the basal level of expression \((p < 0.05)\). Therefore, both increased cell numbers and increased staining intensity contributed to the TRAIL levels in BAL fluid in asthmatics.

Interestingly, an increase in stain intensity for TRAIL expression in alveolar macrophages within control subjects over the basal level was observed 24 h (day 2) and 1 wk (day 9) after SAC \((p < 0.05)\). In addition, we evaluated BAL cells for expression of the decoy receptors DcR1 and DcR2. We were unable to detect DcR1 using commercially available anti-DcR1 Ab (data not shown). As shown in Fig. 6, DcR2 expression in BAL eosinophils was significantly increased at 24 h, 1 wk, and 2 wk post-SAC \((p < 0.001)\). In alveolar macrophages, DcR2 expression was significantly increased within asthmatic subjects 24 h post-SAC \((p < 0.05)\); however, there was not a statistically significant difference between the asthmatic and control groups.
We next evaluated the BAL cells for expression of the death receptors DR4 and DR5. As illustrated in Fig. 7, within asthmatic subjects, the expression level of DR4 in BAL eosinophils was significantly reduced 24 h (p < 0.05) with a trend toward significance at 1 wk (p = 0.08) and 2 wk (p = 0.08) post-SAC. In the alveolar macrophages of asthmatic subjects, DR4 levels in alveolar macrophages were reduced 24 h post-SAC and remained elevated relative to basal levels 1 wk post-SAC. The difference in DR5 stain intensity in alveolar macrophages between asthmatic and control groups showed a trend toward significance (p = 0.067). These data are summarized and
alveolar macrophages from asthmatic patients express higher levels of TRAIL than BAL cells from normal subjects. This suggests that the sources of TRAIL are likely to be local production by both airway smooth muscle and epithelial cells, with additional contribution from infiltrating inflammatory cells. Thus, this is the first study to demonstrate allergen-induced in vivo production of TRAIL in association with cellular inflammation in the airways of human allergic asthmatic subjects.

Presently, the in vivo role of TRAIL remains unknown. We show that peripheral blood eosinophils cultured in the presence of exogenous TRAIL exhibit prolonged viability ex vivo. Thus, TRAIL appears to inhibit the spontaneous apoptosis of peripheral blood eosinophils induced by growth factor withdrawal. Similarly, we found that BAL fluid concentrates supported prolonged peripheral blood eosinophil viability and that this enhanced viability was reduced by anti-TRAIL Ab.

The strong survival signal provided by TRAIL is a novel and interesting observation. Once an eosinophil has exited the circulation, its continued presence in the tissue space as a viable effector depends on the balance between the cell’s natural tendency to undergo apoptosis and the local eosinophil-viability enhancing activity. This accumulation of eosinophils is regulated by the generation of survival and activation factors (i.e., the type-I hematopoietic cytokines, IL-3, IL-5, and GM-CSF, and several members of the IL-2 family of cytokines, IL-9, IL-13, and IL-15 (39–40)). Using microarray technology, Temple et al. (41) identified candidate genes involved in eosinophil survival and apoptosis in peripheral blood eosinophils and IL-5-dependent TF1.8 cells. Interestingly, following IL-5 withdrawal, TRAIL expression was down-regulated >2-fold in TF1.8 cells, supporting a role for TRAIL in survival.

We found increased levels of TRAIL in airway smooth muscle and epithelium of asthmatics compared with those of normal control subjects after SAC, suggesting a role in inflammation. TRAIL expression in both airway epithelial cells and airway smooth muscle was reported to be induced >2-fold by IL-13, using microarray analysis (42). These results suggest that TRAIL can perturb airway function through direct effects on resident airway cells and cells migrating into the airway following Ag exposure; however, the mechanisms responsible for this remain to be defined.

BAL cells migrating into the airway of allergic asthmatic subjects post-challenge were examined by immunocytochemistry for the expression of TRAIL and TRAIL-Rs and compared with those of control nonasthmatic subjects. In the asthmatic subjects, at baseline, both TRAIL and DcR2 were expressed by alveolar macrophages/monocytes, the predominant cell type present. Twenty-four hours after Ag challenge, eosinophils migrate into the asthmatic airway, and the expression levels of both TRAIL and DcR2 on eosinophils and alveolar macrophages were significantly increased. We also observed that BAL cells from nonasthmatic subjects express TRAIL and DcR2 in alveolar macrophages, which suggests a role in the innate immune response. Both DR4 and DR5, which were expressed on day-1 alveolar macrophages/monocytes in asthmatic subjects, were decreased following Ag challenge. This contrasts sharply with our observations of DR4 and DR5 in nonasthmatic subjects, in whom the expression of DR4 and DR5 was relatively high at baseline and remained high after challenge. BAL cells obtained from either the asthmatic or nonasthmatic subjects did not express detectable levels of DcR1. These data suggest that the differential expression of TRAIL and TRAIL-R(s) and their interactions in the asthmatic airway may play a role in modulating eosinophil survival, thereby prolonging the inflammatory response.

A possible mechanism for the resistance of normal cells to TRAIL is believed to be the existence of naturally occurring antagonistic or decoy receptors, DcR1 and DcR2. DcR2 exhibits high

FIGURE 9. Graph summarizes immunostaining data for the expression levels of TRAIL, DcR2, DR4, and DR5 in BAL cells following SAC (Figs. 5–8). Data are expressed as the mean of the difference relative to the basal level stain intensity. *, p < 0.05); †, p < 0.001.

Discussion
The maintenance of hematopoietic cell homeostasis by apoptosis is a critical regulatory mechanism in the normal immune response. Disruption of this physiological balance is clinically manifested in several disease states including asthma. How cells regulate the apoptotic pathway remains poorly understood. Members of the TNF family, including TRAIL, may play an important role in regulating these apoptotic signals.

In this study, we observed that, in the allergic asthmatic subjects, there was an increased level of TRAIL in BAL fluid after SAC, with levels dramatically increasing at 24 h and, for the most part, returning to baseline at 2 wk. In allergic asthmatic subjects, increases in total cells and eosinophils were marked on day 2 and persisted through day 9. TRAIL concentrations were higher in BAL from the allergen-challenged asthmatic subjects and strongly correlated with eosinophil influx, particularly on day 2 after challenge. Bronchial biopsies obtained throughout the SAC protocol further revealed that both airway smooth muscle and epithelial cells of asthmatics express increased levels of TRAIL. Both BAL eosinophils and

presented as the change from baseline expression in Fig. 9 and suggest a differential expression of TRAIL and TRAIL-R in the asthmatic vs the control subjects.
sequence homology to the extracellular domains of DR4, DR5, and DcR1; however, DcR2 contains a truncated cytoplasmic death domain (34). Despite similar affinity for TRAIL, DcR2 does not induce apoptosis and appears to protect against TRAIL-mediated apoptosis via active induction of genes whose products provide resistance to apoptosis, suggesting the involvement of secondary signaling mechanisms (34). Although the ratios of death-inducing and decoy receptors for TRAIL may or may not play an important role in determining cell susceptibility to TRAIL-mediated apoptosis, intracellular signaling mechanisms may be crucial.

In conclusion, TRAIL concentrations significantly increased in allergic asthmatic subjects after Ag challenge, and these values correlated with an increased expresion of TRAIL in the asthma airway. The balance between receptors, the physiological functions of these receptors, and the elucidation of the molecular mechanisms of the TRAIL signaling pathways awaits further investigation.

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