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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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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 sequestering 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 nonreactive reactivity 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 topicalically 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 installed 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 cytopsins stained with Wright-Giemsa stain. Cytosips 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 Ca 2+ or Mg 2+ ) 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 trypsin blue exclusion. Eosinophil purity is assessed by Wright-Giemsa stained cytopsin. 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 (Milteny 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 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 2 hours at 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/ml; 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).

Table II. BAL cells

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 9</th>
<th>Day 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosinophils (%)</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>0.38 ± 0.16</td>
<td>0.5 ± 0.3</td>
<td>0.6 ± 0.167</td>
<td>0.4 ± 0.175</td>
</tr>
<tr>
<td>Controls (± SD)</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>Eosinophils (×10⁹/ml)</td>
<td>0.06 ± 0.03</td>
<td>0.1 ± 0.03</td>
<td>0.03 ± 0.02</td>
<td>0.06 ± 0.04</td>
</tr>
<tr>
<td>Asthmatic (± SEM)</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>Controls (± SEM)</td>
<td>92.2 ± 1.42</td>
<td>86.3 ± 9.52</td>
<td>84.3 ± 6.95</td>
<td>90.8 ± 1.5</td>
</tr>
<tr>
<td>Macrophages (%)</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 (± SD)</td>
<td>14.2 ± 1.47</td>
<td>16.3 ± 1.45</td>
<td>12.2 ± 1.98</td>
<td>12.1 ± 0.84</td>
</tr>
<tr>
<td>Controls (± SEM)</td>
<td>14.2 ± 1.47</td>
<td>16.3 ± 1.45</td>
<td>12.2 ± 1.98</td>
<td>12.1 ± 0.84</td>
</tr>
</tbody>
</table>
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.

**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.
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 intensity was held constant. Background density was subtracted from each

### 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 are 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 (72.0 ± 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 subG1 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.
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.
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), 1 wk (day 9), and 2 wk (day 16) after SAC \( (p < 0.05) \). No signal was observed with a control non-specific polyclonal Ab. Smaller cells having bilobar nuclei (eosinophils) are marked with black arrowheads, and alveolar macrophages are marked with green arrowheads. All micrographs were printed at the same magnification. No signal was observed with an isotype-matched control mAb. B and C, Quantitation of TRAIL staining intensity of BAL eosinophils and alveolar macrophages according to leukocyte size. Analysis was performed using Phase 3 image analysis. Results are expressed as mean density. Values are means ± SE. Statistical analysis was performed using two-way ANOVA and then the Student-Newman-Keuls method. For alveolar macrophages, there was a significant difference between clinical groups \( (p < 0.05) \) at day 2. When individual days were considered, there was a significant difference for BAL eosinophils in asthmatics at days 2 and 9 vs day 1 \((*, p < 0.05)\). For alveolar macrophages, a significant difference was observed at day 2 vs day 1 \((*, p < 0.05)\) in the asthmatic group and at days 2 and 9 vs day 1 \(\#(p < 0.05)\) in the control group.

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 control nonasthmatic subjects, DR4 levels in alveolar macrophages increased 24 h post-SAC and remained elevated relative to basal levels 1 wk post-SAC. The difference in DR4 stain intensity in alveolar macrophages between asthmatic and control groups showed a trend toward significance (\(p = 0.067\)).

As shown in Fig. 8, DR5 expression in BAL eosinophils was reduced following SAC with a trend toward significance observed at 24 h (day 2), 1 wk (day 9), and 2 wk (day 16) after SAC. Within the asthmatic subjects, DR5 expression in alveolar macrophages was reduced 24 h (day 2), 1 wk (day 9), and 2 wk (day 16) after SAC were stained with polyclonal anti-human DR5. Smaller cells having bilobar nuclei (eosinophils) are marked with black arrowheads, and alveolar macrophages are marked with green arrowheads. All micrographs were printed at the same magnification. B and C, Quantitation of DR5 staining intensity of BAL eosinophils and alveolar macrophages according to leukocyte size. Analysis was performed using Phase 3 image analysis. Results are expressed as mean density. Values are means ± SE. For alveolar macrophages, a trend toward significance was observed between asthmatic and control groups (\(p = 0.067\)).

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 (\(p = 0.1\)) and 2 wk (\(p = 0.08\)) post-SAC. In the control nonasthmatic subjects, DR4 stain intensity was significantly reduced 2 wk (\(p < 0.05\)) post-SAC. In contrast, DR4 levels in alveolar macrophages of control nonasthmatic subjects increased 24 h post-SAC and remained elevated throughout the 2 wk post-SAC.

As shown in Fig. 8, DR5 expression in BAL eosinophils was reduced following SAC with a trend toward significance observed at 24 h post-SAC. Within the asthmatic subjects, DR5 expression in alveolar macrophages was reduced 24 h and 2 wk post-SAC, whereas, in control nonasthmatic subjects, DR5 levels increased 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
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.

**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 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
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 eosinophilic presence in the airway. Immunohistochemical data suggest that Ag challenge resulted in increased eosinophil survival and prolonging injury in allergic asthmatic subjects after Ag exposure. These data suggest that TRAIL may act either as an integral membrane protein via direct cell-to-cell contact or as a soluble effector, to prolong survival of BAL eosinophils in the asthmatic airway. The balance between receptors, the physiological functions of these receptors, and the elucidation of the molecular mechanisms of the TRAIL signaling pathways await further investigation.

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