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Systemic FasL Neutralization Increases Eosinophilic Inflammation in a Mouse Model of Asthma

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Abstract

Background—Eosinophils and lymphocytes are pathogenically important in allergic inflammation and sensitive to Fas-mediated apoptosis. Fas Ligand (FasL) activity therefore should play a role in regulating the allergic immune response. We aimed to characterize the role of FasL expression in airway eosinophilia in *Aspergillus fumigatus* (*Af*)-induced sensitization, and to determine if FasL neutralization alters the inflammatory response.

Methods—Sensitized Balb/c mice were sacrificed before (Day 0) and 1, 7 and 10 days after a single intranasal challenge with *Af*. Animals received either neutralizing antibody to FasL (clone

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STATEMENT OF CONTRIBUTION

Satish K. Sharma PhD: Performed the immunohistochemistry, ELISA and qPCR studies, assembled database for the study, analyzed data, participated in manuscript preparation

Francisco A. Almeida MD: Participated in the experimental design and mouse model of allergic airway sensitization

Sonja Kierstein PhD: Participated in experimental design, mouse models of allergic airway sensitization and manuscript preparation

Laszlo Hortobagyi: Performed the splenocyte proliferation studies.

Timothy Lin, MD: Participated in the mouse model of airway sensitization

Allyson Larkin MD Participated in the mouse model of airway sensitization

Jonathan Peterson: Participated in the immunocytochemistry studies and in the mouse model of airway sensitization

Hideo Yagita PhD: Developed the monoclonal anti FasL antibodies used in this study and provided advice and guidance for evaluation of their effects.

James G. Zangrilli MD: Designed the study, analyzed data and prepared the manuscript

Angela Haczku MD PhD: Designed the study, analyzed data and prepared the manuscript

STATEMENT OF CONFLICT OF INTEREST

Satish K. Sharma PhD: NONE

Francisco A. Almeida MD: NONE

Sonja Kierstein PhD: NONE

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Angela Haczku MD PhD: NONE

MFL4) or irrelevant hamster IgG *via* intraperitoneal injection on Days -1 and 5. FasL expression, BAL and tissue inflammatory cell and cytokine profile, and apoptosis were assessed.

Results—Post challenge FasL gene expression in BAL cells and TUNEL positivity in the airways coincided with the height of inflammatory cell influx on Day 1 while soluble FasL protein was released on Day 7, preceding resolution of the inflammatory changes. Although eosinophil numbers showed a negative correlation with soluble FasL levels in the airways, MBP⁺ eosinophils remained TUNEL negative in the submucosal tissue, throughout the 10-day period after *Af* challenge. Systemic FasL neutralization significantly enhanced BAL and tissue eosinophil counts. This effect was associated with increased activation of T cells and release of IL-5, IL-9 and GM-CSF in the BAL fluid of mice indicating an involvement of pro-eosinophilic survival pathways.

Conclusions—FasL activity may play an active role in resolving eosinophilic inflammation through regulating T cells and pro-eosinophilic cytokine release during the allergic airway response.

Keywords

Allergen Challenge; Animal Models; Asthma; Eosinophil; Fas Ligand

INTRODUCTION

Engagement of a Fas-expressing target cell by a Fas Ligand (FasL) bearing effector can result in the programmed cell death of the target. Oligomerization of the Fas receptor by FasL with subsequent recruitment of adapter molecules and activation of the caspase cascade ultimately results in mitochondrial destabilization and destruction of substrates essential for cell survival (1–6). Translation of these *in vitro* observations to human asthma is an emerging area. Airway inflammation in asthma is now well described (7), and its clinical importance is underscored by the fact that regular use of anti-inflammatory agents (particularly corticosteroids) improves symptoms and exacerbations, whereas monotherapy with bronchodilators, does not (8, 9). Many asthmatics have persistent symptoms and residual inflammation despite these drugs, pointing to abnormal activation and/or impaired clearance of inflammatory cells (10). FasL-sensitive macrophages, lymphocytes, eosinophilic and neutrophilic granulocytes figure prominently in the pathogenesis of asthma. The theory that Fas-mediated elimination of inflammatory cells should be an important means of modulating airways inflammation is not new, but has been difficult to prove in the context of a relevant disease process. (11, 12).

There is increasing evidence that Fas/FasL interactions lead to multiple different pathways involved in regulation of immune and inflammatory cell functions. Our study characterizes the time dependent FasL gene and protein expression in the lung in a murine asthma model (13, 14) and provides evidence that systemic neutralization of endogenous FasL activity prolongs airway eosinophilia, supporting a role for Fas/FasL interaction in resolving allergic airway inflammation.

MATERIALS AND METHODS

Aspergillus fumigatus (*Af*) sensitization and challenge model

6–8 weeks old female BALB/c mice (weighing approximately 20g) were sensitized and challenged (Figure 1A) as previously described (13, 14). FasL was neutralized by intraperitoneal injections with anti-FasL (MFL4 provided by Dr. Hideo Yagita, Juntendo University, Tokyo)(15) in PBS (Figure 3A) or irrelevant hamster IgG (Jackson ImmunoResearch, West Grove, PA). Protocols were approved by the Institutional Animal Care and Use Committee.

Bronchoalveolar lavage (BAL) and tissue collection

BAL was obtained and lungs were collected in paraformaldehyde or RNA later as described previously (13, 14, 16). The BAL fluid was processed for total and differential cell counts, and the cell-free supernatant (stored at -80°C) was analyzed for soluble Fas and FasL levels (R&D Systems). Cytokines were measured by the Q-PlexTM mouse inflammation cytokine array (Biolegend; San Diego CA).

Immunohistochemistry

Paraffin-embedded, ethanol fixed tissue sections were studied for presence of apoptotic cells (TUNEL assay; Roche Applied Science, Figure 1E); tissue eosinophils (anti mouse major basic protein [MBP] antibody provided by Dr. Jamie Lee; Mayo Clinic, Scottsdale, AZ; Figure 1E and 4E) and FasL expression (polyclonal anti-FasL N20 antibody, Figure 2C/a–d, or irrelevant rabbit IgG, Santa Cruz Biotechnology; Figure 2C/g).

FasL expression by BAL cells was studied by immunocytochemistry in acetone-methanol fixed cytopins using N20 labeling (Figure 2B) and by indirect immunofluorescence in live cells using biotinylated anti-FasL MFL4 and MFL3 clones (not shown). The specificity of lung FasL staining was confirmed in OCT-embedded (Tissue-Tek®, Fisher Scientific), snap frozen blocks of lung tissue processed as previously described (17) and labeled with anti-FasL MFL3 (not shown) or MFL4 (Figure 2C/f).

TUNEL+ cells were individually quantitated (NIS-elements® image analysis software [Nikon]) by blinded observers. The density threshold was set to trace all positive cells within the subadjacent peribronchial tissue for a given length of airway. TUNEL results were corroborated with a PE conjugated activated Caspase 3 antibody (BD Pharmingen, San Jose, CA; data not shown).

Peribronchial MPB+ eosinophils were assessed the same way (Figure 4E/b) but because eosinophils were occasionally crowded and touching in areas of intense inflammation, and border-finding algorithms for individual cells failed there, we divided the total cellular MPB + area for a given peribronchiolar region by the average area of a single eosinophil ($53\ \mu\text{M}^2$; a constant obtained through direct measurement of 20 individual, well-defined cells). It should be noted that the MBP+ area analyzed was always overtly cellular by direct inspection, and not related to extracellular deposition. Tiny ($< 40\ \mu\text{M}^2$) objects were excluded from this MBP+ area as probable artifacts.

mRNA quantification

First-strand cDNAs were synthesized from 5 μg total RNA using random primers and the High Capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed using the TaqMan® gene expression assay for murine FasL (mRNA from Applied Biosystems) normalized to GAPDH mRNA.

Splenic lymphocyte function

Splenocytes were isolated, washed and cultured at $8 \times 10^6\ \text{cells ml}^{-1}$ in U-bottom 96-well plates (Fisher) in RPMI 1640 medium; ^3H -thymidine uptake was assessed as previously described (16).

Data analysis

Results are expressed as mean $\pm$ standard error of the mean (SEM). Time courses and dose-responses were compared by two-way ANOVA. Individual comparisons were made by the non parametric Mann-Whitney U test. Regression analysis was performed using the

Spearman's correlation test. Statistical significance was set at a p-value less than 0.05. Data were analyzed using Microsoft Excel and GraphPad Prism 5 for Windows.

RESULTS

The peak TUNEL positivity coincided with inflammatory influx in the airways but eosinophils remained TUNEL negative after *Af* challenge

We previously showed that prevention of the inflammatory resolution after allergen inhalation was associated with decreased cell death of MBP⁺ eosinophils (16). To investigate the role of eosinophil apoptosis, here we studied the time dependent relationship between airway eosinophil numbers and TUNEL positivity after *Af* inhalation in sensitized BALB/c mice (Figure 1A). The numbers of inflammatory cells were significantly increased with an eosinophil predominance on Day 1 (Figure 1B) and a subsequent return to near baseline by Day 10 (Figure 1C). Pro-eosinophilic (IL-4, IL-5, GM-CSF and IL-9) cytokine release paralleled the height of inflammation and these cytokines (except IL-9) positively correlated with BAL eosinophil numbers on Day 1 after *Af* challenge (Figure 1D).

TUNEL⁺ apoptotic and aponecrotic cells (green) were frequently present post-allergen challenge (a representative Day 1 photomicrograph is shown, Figure 1E/d). The greatest numbers of TUNEL⁺ cells paralleled the peak of eosinophilia on Day 1 after *Af* challenge (Figure 1F). However, we found only occasional MBP⁺ cell (red) co-localization with TUNEL⁺ nuclei while most MBP⁺ cells remained TUNEL⁻ (Figure 1E/d and 1G). Thus, TUNEL positivity coincided with the height of inflammation but apoptosis was not associated with resolution of the *Af*-induced airway response.

Soluble FasL release into the airways negatively correlated with eosinophil cell numbers

To study the relationship of FasL expression and airway eosinophil numbers, we investigated FasL mRNA and protein levels in BAL cells, supernatant and lung tissue after *Af* challenge of sensitized mice. In the BAL cellular compartment cytoplasmic/membrane protein expression corresponded with increased mRNA activation on Day 1 (Figure 2A–B). Based upon nuclear morphology, the FasL positive cells appeared mononuclear while polymorphonuclear cells and cells with eosinophil-like nuclei remained FasL negative (Figure 2B, a representative Day 1 sample). In the lung tissue, expression of FasL peaked in the proximal airway epithelial cells on Day 7 post challenge (Figure 2C). Although we weren't able to find commensurate increases in lung tissue FasL mRNA by microarray (data not shown), *de novo* FasL synthesis was previously demonstrated by studies in human airway epithelial cells *in vitro* (18) and in Balb/c mice *in vivo* (19) indicating airway epithelium as the source of FasL. Local release (BAL vs. serum) of soluble FasL indeed corresponded with the peak epithelial expression on Day 7 (Figure 2D) and showed a significant negative correlation with the % of BAL eosinophils post *Af* challenge ($r = -0.6$; $p < 0.01$; Figure 2E). Meanwhile levels of soluble Fas in the cell free BAL supernatant increased only on Day 1 (from 247 ± 12 to 407 ± 53 pg/ml) before returning to (and remaining at) near baseline on Day 7 and 10.

Systemic FasL neutralization enhanced lymphocyte activation and cytokine release after *Af* challenge

In the asthmatic airways many factors can regulate survival/death of eosinophils including cytokines released by the highly FasL sensitive T-cells (20–23). To study how systemic blockade of FasL affects T cell function, we treated mice with the neutralizing anti-FasL antibody MFL4 or control hamster IgG by intraperitoneal injection (Figure 3A). MFL4 enhanced proliferation of splenic T cells (Figure 3B–C) harvested on Day 1 or 7, (but not on Day 10 or in non-challenged [Day 0] mice) both at baseline (left panels) and after PHA

stimulation (right panels). Interestingly, dexamethasone responsiveness of the T cells was partially reversed in the MFL4-treated groups (Figure 3C) suggesting that FasL blockade induced heightened activation and a relative T cell glucocorticoid resistance.

MFL4 also enhanced release of the T-cell cytokines IL-5, IL-9 and GM-CSF in the lung in time and dose-dependent manner. Figure 3D shows cytokine levels harvested on Day 1 post *Af* challenge (BAL cytokine release was negligible at other time points). Levels of soluble Fas ligand negatively correlated with IL-5, IL-9 and GM-CSF recovered from the BAL fluid of MFL-treated mice on Day 1 suggesting that systemic FasL neutralization significantly enhances T cell function and local release of pro-eosinophilic (“survival”) cytokines during the *Af*-induced inflammatory response.

FasL blockade enhanced eosinophilic airway inflammation

The BAL and lung tissue inflammatory cell profile was assessed to investigate whether the increased T-cell derived pro-eosinophilic cytokine release was associated with altered eosinophil count after MFL4 treatment. Compared to the PBS-treated mice [0 mg], the greatest, dose-dependent MFL4 effects on the total BAL cell count were seen on Day 7 post *Af* challenge (Figure 4A) with increased numbers of macrophages and eosinophils (Figure 4B & D) and decreased soluble FasL levels (Figure 4C). MBP+ tissue eosinophils (Figure 4E/a) counted in the peribronchial region using density threshold morphometry (Figure 4E/b), showed significantly elevated numbers after *Af* challenge in the MFL4-treated mice compared with the control antibody treated mice ($p < 0.05$, two way ANOVA, Figure 4F). Taken together, blockade of Fas-FasL interaction enhanced airway inflammation and delayed eosinophil clearance after allergen challenge.

DISCUSSION

This study demonstrates that resolution of airway eosinophilia was not directly associated with apoptosis or FasL expression by these cells in allergen exposed mice. Soluble FasL release from the airway epithelium (that peaked 7 days after *Af* challenge) was negatively proportionate with eosinophil counts and pro-eosinophilic cytokine levels in the airways. Systemic FasL blockade increased T cell activation and dose- and time dependently enhanced IL-5, IL-9, GM-CSF and airway eosinophilia. We conclude that FasL mediated pathways are important in attenuating eosinophilic airway inflammation possibly *via* inhibition of survival cytokine production.

The initial interest in Fas/FasL interactions in major asthma effector cell types was piqued in the mid 1990s when ligand-mediated apoptosis was demonstrated in eosinophils (11) and T cells (12). In a model of ozone-induced enhancement of airway eosinophilia we previously found association with diminished cell death and decreased FasL expression in the lung of allergen exposed mice (16). In other studies exogenously administered FasL activity into the airways of mice either by agonist antibody or by viral expression consistently induced a decline in BAL eosinophil numbers post challenge, but had variable effects on tissue infiltration and granulocyte apoptosis (24–27). To investigate the direct relationship between FasL expression, cell death and resolution of airway eosinophilia, in the current study we applied a different approach from previous investigations in that endogenously derived FasL activity was neutralized during the allergen-induced airway inflammatory response (15). Studies using the functional Fas (*lpr*) (28) and FasL (*gld*) (23) deficient mouse models were criticized for their tendency towards immune dysregulation and frank autoimmunity that can confound interpretation of inflammatory endpoints (29). In this respect our extensively characterized anti-FasL approach (15) provides a system free of confounding immune/inflammatory pathologies. The increased airway eosinophilia seen in the allergen challenged mice after FasL neutralization in this model could be due to several possible pathways.

One would intuit that blocking FasL should enhance inflammation by preventing apoptosis of Fas⁺ targets. However, we found no inhibition of apoptosis in MFL4 treated mice. Peak inflammatory changes in the mice were characterized by a major presence of abnormal TUNEL⁺ cells suggesting secondary necrosis (i.e. aponecrosis) (30) and supporting previous observations that inflammatory cells at sites of allergic inflammation may be cleared by means other than classical apoptosis (27, 31). Presence of a negligible number of TUNEL⁺/MBP⁺ cells in the lung tissue of allergen challenged mice also suggested that apoptosis may not have been the main pathway of eosinophil clearance in this model.

After *Af* challenge FasL mRNA activation paralleled inflammatory cell influx in the BAL but overtly positive cell surface expression was rare and showed a mononuclear rather than eosinophil morphology. Based on our results we speculate that the pro-inflammatory MFL4 effects were likely mediated through inhibition of soluble, rather than membrane bound FasL in the lung. These forms have markedly different effects on cell function: While only the membrane bound form can induce apoptosis, soluble FasL has non-apoptotic roles promoting autoimmunity, tumorigenesis and immunosuppression (32). Soluble FasL (thought to be cleaved by matrix metalloproteinases in the BAL supernatant (33)) peaked in the BAL fluid on Day 7 while serum levels remained low throughout after *Af* challenge. Using several anti-FasL reagents we confirmed previous reports (18, 19, 34) showing bronchial epithelial cell expression of FasL immunoreactivity, that was also strongly increased on Day 7. Thus, the predominant source of soluble FasL after allergen challenge is the airway epithelium and a time dependent FasL release coincides with resolution of inflammation.

The increased production of Th2-type cytokines and heightened T cell proliferation from the MFL4-treated mice strongly suggest that FasL acts by limiting the expansion of antigen-specific T cells, a mechanism that would be consistent with known functions of Fas/FasL interactions. Given the strong CD4 T cell dependence of the *Af* sensitization model (35), one way to demonstrate whether FasL inhibition is acting *via* T cells would be through adoptive transfer experiments. Such experiments have been performed in a *S. mansoni* induced asthma model using Fas-deficient (*lpr*) T cells (22) as well as FasL deficient (*gld*) T cells (23). Both studies supported that delayed resolution of eosinophilia is a downstream effect of Fas deficiency on T cells, not eosinophils. Further, our study as well as the models using the *lpr* (22, 28) or the *gld* mice (23) point to the significance of T-cell derived survival cytokines in enhancing allergen-induced airway eosinophilia in the absence of Fas/FasL activity. We proposed in a previous study (16) that proinflammatory cytokines IL-5, GM-CSF, in addition to render eosinophils resistant to Fas/FasL-induced apoptosis may also inhibit FasL expression. This is now refuted in this current study since increased levels of the pro-eosinophilic IL-5, IL-9 and GM-CSF in the BAL fluid of mice coincided with the peak of BAL cell mRNA expression for FasL. The fact that FasL neutralization resulted in significantly increased expression of these cytokines would suggest instead a reverse regulatory pathway in which FasL activation exerts an inhibitory effect on proinflammatory cytokine release during the allergic airway response.

In summary, the enhanced and prolonged airway eosinophilia after systemic FasL neutralization provides mechanistic support that endogenously released FasL participates in resolution of airway inflammation. The inverse relationship between FasL protein expression, T cell activation and pro-eosinophilic cytokine production highlights the importance of these Fas sensitive pathways in keeping allergic airway inflammation at check.

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Figure 1. The peak of apoptotic cell numbers coincided with eosinophil influx into the airways but MBP+ eosinophils lacked TUNEL+ nuclei 1 day after a allergen challenge of sensitized mice (A): BALB/c mice were intraperitoneally (i.p.) sensitized with *Aspergillus fumigatus* (Af) and sacrificed at the time points indicated by the black circles. (B): Absolute cell counts were assessed using Giemsa-stained cytospin preparations on Day 1 after Af challenge (MP: macrophages; NP: neutrophils; EP: eosinophils; LC: lymphocytes). (C): BAL absolute eosinophil count was assessed on Day 0 [prechallenge baseline], and 1, 7 and 10 days post Af challenge. (D): Th2 cytokines in the BAL were analyzed by a multiplex assay (Luminex, top panels). Eosinophil numbers were correlated with IL-4, 5, 9 and GM-CSF levels on Day 1 after Af challenge (Bottom panels; individual data points are shown). (E): Apoptotic cells in the airway were assessed by TUNEL (green) and anti-MBP antibody (red) labeling. (E/a.): Positive (DNase treated) TUNEL control. (E/b.): Negative (no terminal transferase enzyme) control. (E/c.): Representative airway on Day 0 with no apoptotic cells. (E/d.): Representative airway on Day 1 with MBP positive (red) eosinophils and apoptotic cells having TUNEL positive (green) nuclei. (F): Submucosal TUNEL+ cells were quantitated using a digital morphometric technique. Results are expressed as Number of cells/mm basement membrane. (G): An MBP+ eosinophilic granulocyte is indicated by the red arrow. No MBP+ cells showed co-localization with TUNEL+ nuclei (green arrows); Aponecrosis (white arrow); (immersion oil, $\times 1000$). (B–D & F): Mean $\pm$ SEM; n=5 mice/time point.

Figure 2. Eosinophil cell numbers in the airways negatively correlated with soluble FasL release
(A): FasL mRNA expression in the BAL cellular compartment (qPCR, normalized to GAPDH). Cells were harvested from mice sensitized and challenged with *Af* as described. Mean $\pm$ SEM; $n=5$ $p<0.001$ (two-way ANOVA). **(B):** Live BAL cells were stained in suspension with anti-FasL MFL3 or MFL4 clones on gelatinized chamber slides. Samples were obtained on Day 1 after *Af* challenge. **(C):** Formalin fixed, permeabilized tissue was stained for FasL [anti-murine polyclonal anti-FasL (N20)] or irrelevant rabbit IgG. **(C/a-d.):** Representative sections are shown for lungs harvested before (Day 0) and 1, 7 and 10 days after *Af* challenge of sensitized mice. **(C/e.):** Epithelial expression of FasL shown for the day 7 tissues. **(C/f.):** A corresponding frozen section labeled with the anti-FasL MFL4 clone. **(C/g.):** Ab control: irrelevant rabbit Ig. **(D):** Time course of soluble FasL protein expression in the BAL fluid and serum was determined by ELISA. Mean $\pm$ SEM; $n=5$ $p<0.001$ (two-way ANOVA). **(E):** Soluble FasL vs. BAL eosinophils correlation.

Figure 3. Systemic FasL neutralization enhanced lymphocyte activation and cytokine release into the airways after *Af* challenge

(A): Mice were sensitized and challenged as described and received Anti-murine FasL antibody (clone MFL4 or control hamster IgG). (B): Splenocytes were (harvested on Day 7 after *Af* challenge). ³H-thymidine uptake was compared between MFL4 and control antibody-treated mice 48 h later. (cpm): Count per minute in unstimulated (left panel) and in PHA stimulated (right panel) cells. (C): Dexamethasone dose response. n=4 spleens per group, *p<0.05, **p<0.01. (D): BAL was harvested on Day 1 after *Af* challenge. Mean ± SEM of n=16 (E): Correlation of soluble Fas ligand levels with IL-5, 9 and GM-CSF cytokines in the BAL harvested on Day 1 after *Af* challenge of MFL-treated mice.

Figure 4. FasL blockade with MFL4 enhanced eosinophilic airway inflammation

(A): Mice were sensitized and challenged with Af and treated with anti-murine FasL antibody as described. Total BAL cell counts were determined by Coulter counter. (B): Absolute differential cell counts were assessed using the total cell counts and Giemsa-stained cytospin preparations (day 7 after challenge) (MP: macrophages; NP: neutrophils; EP: eosinophils; LC: lymphocytes) (C): Soluble FasL measured in the cell free BAL supernatant harvested on Day 7 after Af challenge (ELISA). (D): Absolute eosinophil count from BAL harvested on Day 7 after Af challenge. (A–D): Mean±SEM pooled from 3 separate experiments with n=14–22 mice per group. *p<0.05, **p<0.01. (E/a.): Tissue eosinophils were labeled by an anti-major basic protein (MBP) monoclonal antibody. (E/b.): Eosinophil numbers were quantitated using a digital morphometric image analysis. (F): The number of submucosal eosinophils was determined by morphometric analysis and expressed as cell number per mm of epithelial basement membrane (BM). Mean±SEM, n=3 mice per group.