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### Research Article

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# Activation of the Fas Receptor on Lung Eosinophils Leads to Apoptosis and the Resolution of Eosinophilic Inflammation of the Airways

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## Abstract

While considerable progress has been made in understanding the events by which eosinophils accumulate in various pathophysiological conditions, the mechanisms controlling the resolution of eosinophilic inflammation are poorly understood. In the present study, we demonstrate that lung eosinophils obtained by bronchoalveolar lavage (BAL) after aerosol allergen provocation of immunized mice expressed the Fas receptor. Stimulation of purified eosinophils *in vitro* with a monoclonal anti-Fas mAb (1 ng–1 µg/ml) induced a dose/time dependent loss of cell viability from 24–72 h. Measurement of DNA fragmentation with propidium iodide, confirmed that anti-Fas induced eosinophil death by apoptosis. While incubation with IL-3, IL-5, or GM-CSF prevented spontaneous apoptosis, these factors failed to prevent anti-Fas induced apoptosis. Administration of anti-Fas mAb to the lungs after the induction of a lung eosinophilia increased the number of peroxidase positive macrophages in BAL fluid 4–12 h later which was followed by a marked reduction in the number of eosinophils in the airways. Importantly, Fas-mediated resolution of eosinophilic inflammation occurred in the absence of any overt secondary inflammatory changes in the lungs. We speculate that defects in this pathway may at least in part explain the chronic eosinophilic inflammation often observed in the lungs of asthmatic individuals. (*J. Clin. Invest.* 1995; 96:2924–2931.)

**Key words:** Fas • eosinophil • apoptosis • lung • asthma

## Introduction

The accumulation and activation of eosinophils, with the subsequent release of highly toxic cationic proteins, is believed to be important in the pathogenesis of bronchial asthma (1, 2). Recent studies performed in experimental animals have suggested that eosinophil infiltration into the airways is dependent on IL-5 derived from the Th2 subset of CD4+ T cells (3, 4). However, there is little information concerning the events that govern

the resolution of the eosinophilic inflammatory response. An appreciation of these processes is central in understanding whether an inflammatory response is either acute and self-resolving or becomes chronic and self-perpetuating. *In vitro*, eosinophil survival is maintained by the eosinophil haematopoietins, IL-3, IL-5 and GM-CSF, which prevent programmed cell death or apoptosis (5, 6), a process accompanied by condensation of the cytoplasm, segmentation of the nucleus and extensive degradation of chromosomal DNA. However, it is unclear if eosinophil death only occurs passively following withdrawal/absence of the eosinophil survival factors, or whether mechanisms exist to actively induce eosinophil apoptosis.

The cell surface molecule Fas/Apo-1 (CD95) is a 45 kD protein belonging to the TNF/NGF receptor family (7). Cross-linking of the Fas receptor with monoclonal antibodies or its recently identified ligand, Fas Ligand (FasL) leads to the induction of apoptosis in lymphocytes (8–11) and provides a mechanism for the removal of antigen activated T cells (12–14) and autoreactive B cells (15). The importance of this pathway in the regulation of lymphocyte apoptosis is illustrated in mice homozygous for either *lpr* (7, 9) or *gld* (11) mutations, which lack functional Fas receptor and FasL respectively and develop similar auto immune responses and accumulate a large number of non malignant CD4<sup>+</sup>CD8<sup>-</sup> T cells in the spleen.

There is now increasing evidence to suggest that cell death mediated by apoptosis is also of major importance in the resolution of inflammatory responses (16). Resolution of inflammation would require swift recognition of effete cells to insure against the detrimental consequences to the surrounding tissue that would follow if cell disintegration or necrosis occurred (16). In view of the relationship between apoptosis and the resolution of inflammatory responses, we have investigated whether murine lung eosinophils express the Fas receptor and undergo apoptosis following receptor crosslinking. Moreover, we have determined whether administration of anti-Fas mAb *in vivo* after the establishment of an airway eosinophilic response would lead to the resolution of lung eosinophilic inflammation.

## Methods

**Induction and purification of lung eosinophils.** To induce a lung eosinophilia, Sv 129 mice (20–25 grams) were immunized *i.p.* with 10 µg of ovalbumin (OA), (Grade V; Sigma Chemical Co., St. Louis, MO) in 0.2 ml of alum (Serva, Heidelberg, Germany) on day 0 and day 14. 7–10 d after the last immunization, animals were challenged with an aerosol of OA (50 mg/ml) for 20 min as described previously (4). 72 h after antigen inhalation, mice were anesthetized with urethane (Sigma Chemical Co.), the trachea cannulated and a bronchoalveolar lavage (BAL) performed by 10 repeated lavages with 0.5 ml of ice cold DMEM (containing 5% FCS, mercaptoethanol [50 µM], L-glutamine [2 mM], sodium pyruvate [1 mM], Hepes [10 mM] and gentamycin [50 µg/ml]) injected into the lungs via the trachea. The cells were washed and macrophages removed from the cell suspension by adherence to plastic

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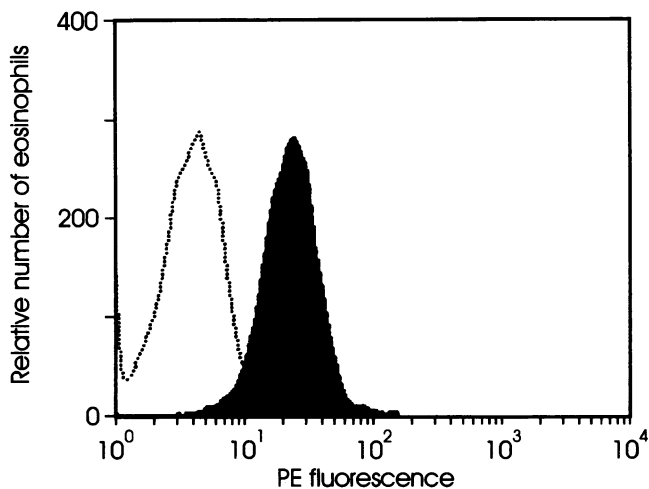
1. Abbreviations used in this paper: BAL, bronchoalveolar lavage; DAB, diaminobenzidine; FasL, Fas ligand; PI, propidium iodide.

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**Figure 1.** Representative figure demonstrating expression of the Fas receptor on the eosinophil as assessed by flow cytometry. Eosinophils were purified (92–98%) from the BAL of 6–8 mice and stained with either matched isotype control antibody (*open histogram*) or PE-labeled hamster anti-mouse Fas receptor (*closed histogram*). This figure is representative of three other experiments

and by centrifugation (100 rpm for 20 min) at 4°C. The 72-h time point was chosen as this induces a selective eosinophil infiltration, with < 2% neutrophils or lymphocytes (4), thus allowing eosinophils to be purified. In contrast, earlier time points are associated with a mixed granulocyte infiltration, which in the absence of any murine eosinophil specific surface markers would confound the interpretation of our data. Eosinophil purity was determined by staining cytospin preparations (Shandon Scientific Ltd, Cheshire, UK) with Diff-Quik® (Baxter Diagnostics, Düringen, Switzerland). In all experiments eosinophil purity was between 92–98%.

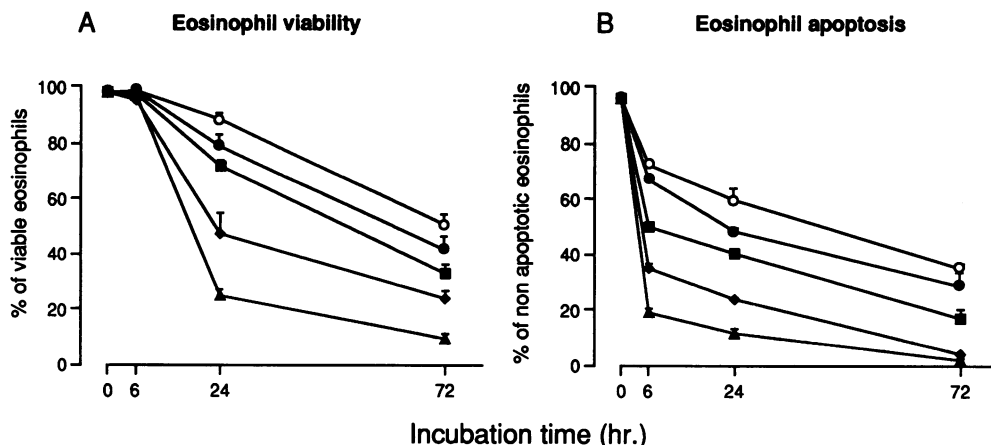
**Induction of eosinophil apoptosis *in vitro* by anti-Fas mAb.** After purification, eosinophils ( $2 \times 10^5$ /well) were cultured in 96-well plates in either medium alone or in the presence of 1 ng/ml of either murine recombinant IL-3, IL-5, or GM-CSF (Pharmingen, Palo Alto, CA). Cells were stimulated with anti-Fas mAb (1 ng–1  $\mu$ g/ml), an isotype matched control mAb (1  $\mu$ g/ml) or were untreated and viability determined by trypan blue exclusion 6, 24, and 72 h later. To determine whether anti-Fas induced cell death by apoptosis, DNA fragmentation was analyzed by flow cytometry following staining with propidium iodide (PI) using the method of Nicoletti and colleagues (17), which has been demonstrated to be as sensitive as performing DNA agarose gel electrophoresis with the added advantage, that quantification of the degree of DNA fragmentation can be readily performed.

**Flow cytometry.** For FACS® analysis, eosinophils were obtained from the pooled lavage fluid of 6–8 mice. Purified eosinophils were incubated for 30 min at 4°C with PE-labeled anti-Fas receptor (Clone Jo2; Pharmingen, Palo Alto, CA) or irrelevant isotype matched control mAb (Hamster IgG; Pharmingen, Palo Alto, CA). The cells were then washed and Fas receptor expression determined by flow cytometry (FACStar®; Beckton Dickinson, Immunocytometry systems, San Jose, CA) using Consort 30 software (10,000 events acquired). Fluorescence analysis refers to all scatter-defined living cells. To collect all cell fragments of propidium iodide (PI) single-stained samples (acquisition of 20,000 events) the forward scatter (FSC) threshold was set on 18 (range, 0–1000). The fluorescence analysis refers to all acquired fragments.

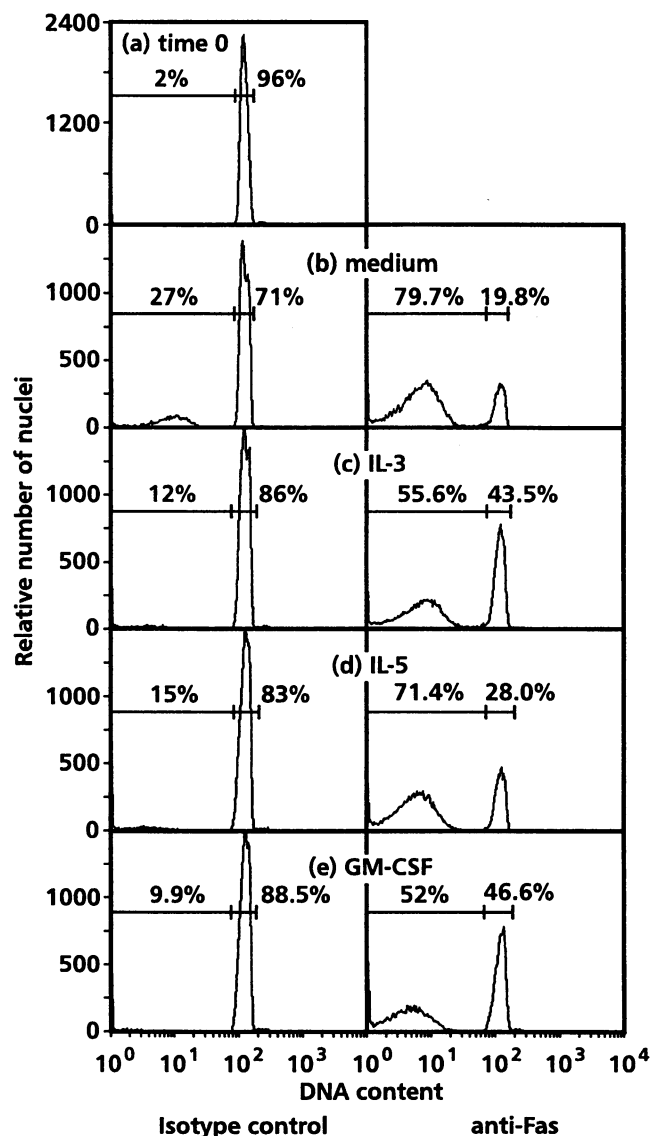
**Resolution of eosinophilic inflammation by anti-Fas.** Preliminary experiments demonstrated that airway antigen challenge of immunized mice results in a selective recruitment of eosinophils into the BAL, which reached a plateau 72 h later and was sustained for a further 48 h. Therefore, we administered anti-Fas mAb 72 h after allergen provocation to determine whether activation of the Fas receptor would lead to the resolution of the lung eosinophilic inflammatory response. This was performed by anesthetizing mice with a mixture of Hypnorm® (2 mg/L/kg; Janssen Pharmaceuticals, Beerse, Belgium) and Dormicon® (10 mg/kg; Roche Pharmaceuticals, Basel, Switzerland) and administering 5, 20, and 50  $\mu$ g of anti-Fas mAb to the lungs via the intranasal route. Control mice received 50  $\mu$ g of the hamster IgG isotype control mAb. All antibodies were applied in 50  $\mu$ l PBS. 48 h later, a BAL was performed and the cellular composition determined. Blood was also taken from the tail vein at each of these time points, total leukocyte counts determined (Sysmex; Toa Medical Electronics Co., Ltd., Kobe, Japan) and blood smears prepared. Slides were stained with Dif-Quik® (Baxter Diagnostics, Düringen, Switzerland) and a total of 500 cells counted differential using standard histological criteria.

**Lung Histology.** 48 h after lung application of either anti-Fas mAb (50  $\mu$ g) or control isotype matched mAb in immunized, antigen challenged mice, the lungs were prepared for histological examination. Mice were anesthetized with urethane, the trachea cannulated, the chest opened and the lungs perfused via the trachea with 4% buffered formaldehyde in PBS (pH 7.0, formal saline) at exactly 15 cm H<sub>2</sub>O maintained by a pressure seal for 60 min. The fixed lungs were trimmed of the heart and large blood vessels and removed en bloc into formal saline and processed into paraffin. 15- $\mu$ m sections were cut and stained with hematoxylin and eosin. Stained and coded slides were examined under low power ( $\times 100$ ) to study gross histopathological changes and regions of interest further studied and photographed using a Zeiss Axiophoto photomicroscope at higher magnification.

**Ex vivo macrophage recognition assay.** To understand more fully how anti-Fas mAb leads to the resolution of the lung eosinophilic inflammatory response, we administered a submaximal dose (20  $\mu$ g) of anti-Fas mAb or the isotype matched control (20  $\mu$ g) and performed a BAL, 4, 12, 24, and 48 h later and the number of eosinophils quantified



**Figure 2.** Eosinophils ( $2 \times 10^5$ /ml) were cultured in medium and (A) cell viability determined by trypan blue exclusion or (B) Eosinophil apoptosis determined by DNA fragmentation following PI staining, 6–72 h later after stimulation with either isotype control (1  $\mu$ g hamster IgG,  $\circ$ ) or anti-Fas (1  $\mu$ g [●], 10 [■], 100 ng [◆], or 1 [▲]  $\mu$ g). Data are shown as the mean  $\pm$  SEM of the percentage of (A) viable or (B) non-apoptotic cells for  $n = 3$  experiments.



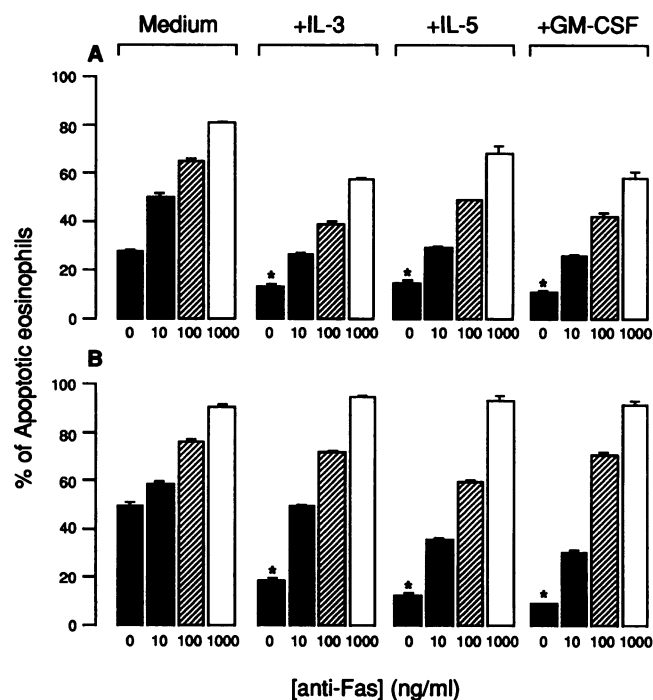
**Figure 3.** Representative FACS<sup>®</sup> analysis of anti-Fas induced eosinophil apoptosis. Apoptosis was measured following staining with PI and analyzed by flow cytometry for 20,000 nongated events. DNA fragmentation was measured either (a) before culture or 6 h after culture in (b) medium alone, (c) IL-3, (d) IL-5 or (e) GM-CSF and stimulated with either anti-Fas (1  $\mu$ g/ml) (right panel) or IgG isotype matched control (1  $\mu$ g/ml) (left panel). The percentage of non-apoptotic and apoptotic cells are indicated in each panel.

as described. Cytospin preparations were also stained for endogenous peroxidase activity by washing the cytospin slides twice with 50 mM Tris-HCl buffer (pH 7.6) and freshly made diaminobenzidine (DAB) solution (18) (0.5 mg/ml DAB, hydrogen peroxide (1%), in 50 mM Tris-HCl buffer, pH 7.6) was added at room temperature for 10 min. The reaction was stopped by washing in Tris-HCl buffer. A total of 200 macrophages were counted and the percentage of peroxidase positive macrophages determined.

**Statistical analysis.** To determine statistical significance, a students *t* test for nonpaired data was performed and a value of  $P < 0.05$  considered significant.

## Results

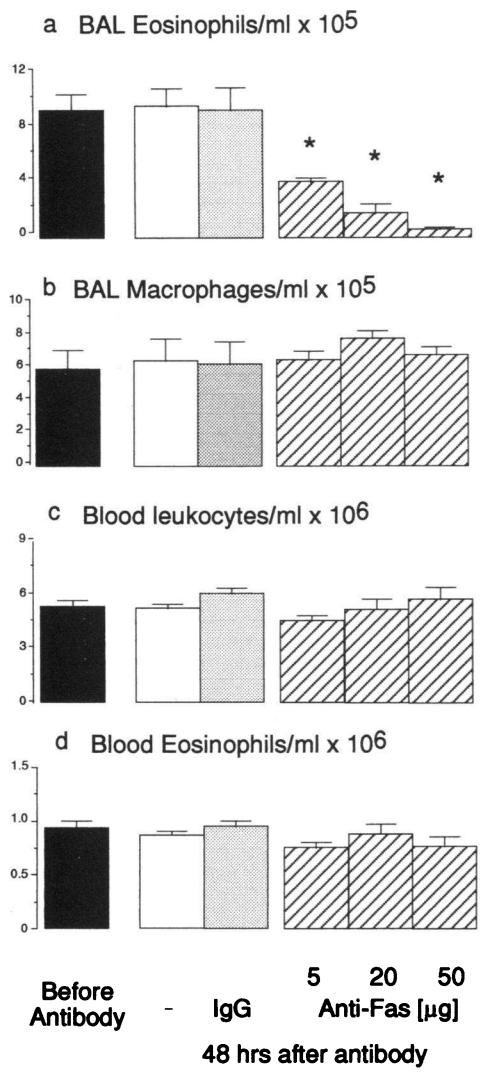
**Lung eosinophils express the Fas receptor.** Antigen provocation of OA immunized mice resulted in a selective eosinophil infil-



**Figure 4.** Eosinophils ( $2 \times 10^5$ /ml) were cultured for (A) 6 h or (B) 72 h in medium alone or with 1 ng/ml of murine recombinant IL-3, IL-5, or GM-CSF and stimulated with either anti-Fas (10–1000 ng/ml) or isotype control (1  $\mu$ g/ml) (closed columns). Eosinophil apoptosis was measured following staining with PI and analyzed by flow cytometry. Data are shown as the mean  $\pm$  SEM of the percentage apoptotic cells for  $n = 4$  experiments. Significance ( $*P < 0.05$ ) for the effect of IL-3, IL-5, and GM-CSF compared to medium alone was determined by a Students *t* test.

tration into the lungs 72 h later, constituting  $> 70\%$  of the total cell population. Removal of macrophages by adherence increased the eosinophil purity to 92–98% in each experiment. Staining with PE-labeled anti-Fas mAb revealed that almost all the lung eosinophils expressed the Fas receptor (mean channel fluorescence for isotype control, 3.9 and PE-labeled anti-Fas mAb, 27.4). Fig. 1 is a representative FACS<sup>®</sup> analysis of eosinophils purified from the BAL fluid of 6–8 mice. A similar profile was observed in three other separate experiments.

**Induction of eosinophil death via apoptosis following Fas receptor activation.** After purification,  $> 98\%$  of the eosinophils were viable as determined by trypan blue exclusion. Over the next 72 h, eosinophils cultured in medium alone resulted in a spontaneous loss of cell viability (72 h:  $48.3 \pm 4.2\%$  nonviable). Incubation with the matched isotype control had no effect of the loss of cell viability (72 h:  $49.7 \pm 4.6\%$  nonviable). Incubation with anti-Fas induced a time and dose dependent increase in cell death which became apparent 24 h after culture (Fig. 2 A). To determine whether anti-Fas induced eosinophil death via apoptosis, eosinophils were stained with PI to measure the degree of DNA fragmentation. After 72 h incubation in medium alone, the percentage of apoptotic cells increased from  $< 2\%$  after purification to  $65.5 \pm 4.4\%$ ,  $n = 4$ ,  $P < 0.01$ . Incubation with the isotype matched control mAb did not have any significant effect on eosinophil apoptosis ( $68.2 \pm 4.6\%$  apoptotic cells,  $n = 4$ ). Stimulation with anti-Fas induced a time and dose dependent increase in the percentage of apoptotic cells. Importantly, DNA fragmentation preceded the loss of cell viability



**Figure 5.** Effect of anti-Fas (5, 20, and 50  $\mu\text{g}$ ) (hatched columns) on (a) BAL eosinophils (b) BAL macrophages and (c) total peripheral blood leukocytes, (d) peripheral blood eosinophils, 48 h later (day 5). Untreated mice (open columns) or animals treated with control IgG isotype matched antibody (shaded columns) are shown for comparison. The closed columns represent cell number 3 d after allergen provocation, before antibody administration. Data are shown as the mean  $\pm$  SEM of cells for  $n = 3-6$  mice. Significance ( $*P < 0.05$ ) was determined by a Student's  $t$  test.

indicating that anti-Fas induced cell death via apoptosis (Fig. 2 B).

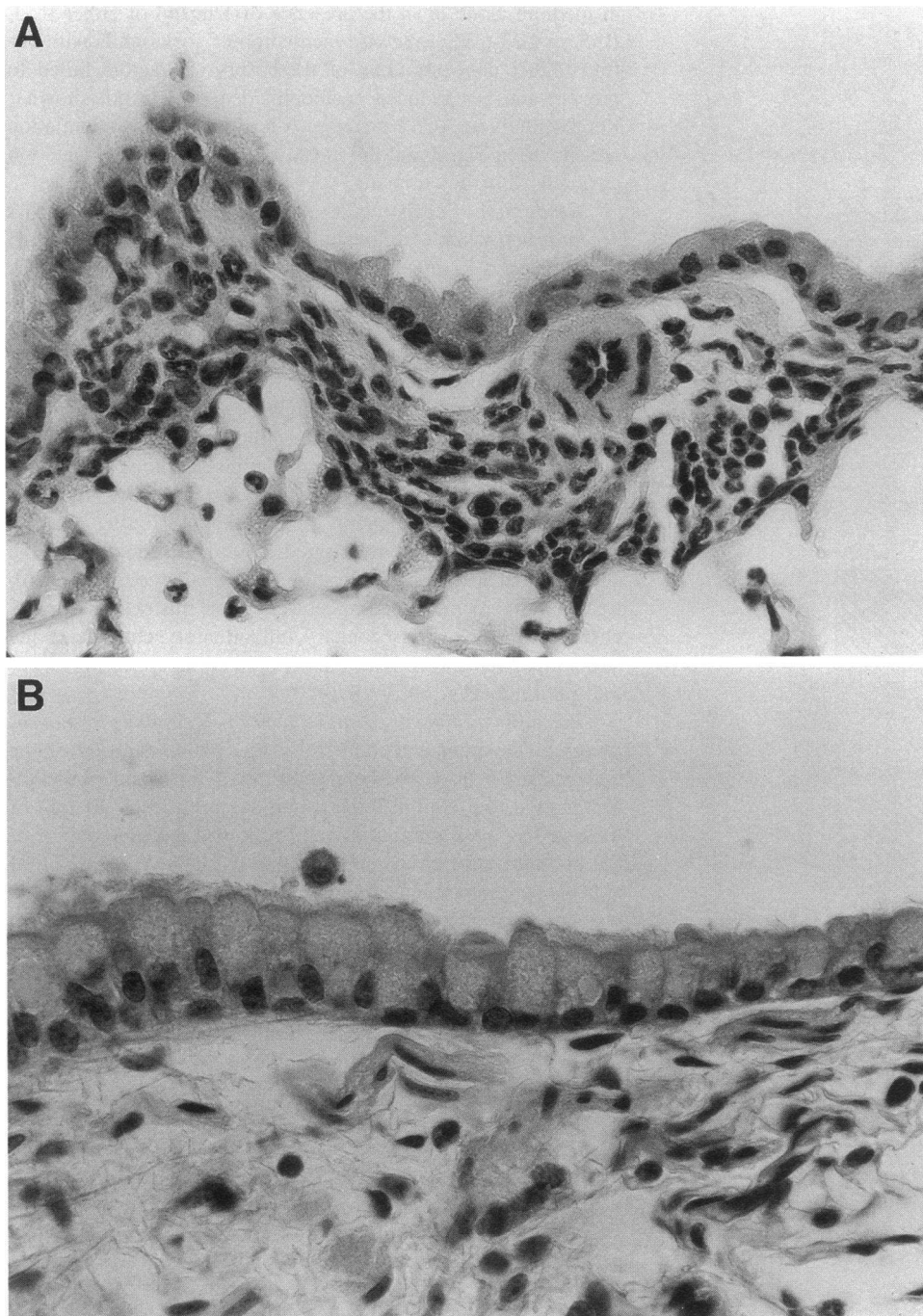
**Effect of IL-3, IL-5, and GM-CSF on anti-Fas induced eosinophil apoptosis.** 6 h after incubation in medium alone,  $27.4 \pm 0.7\%$  of the eosinophils were apoptotic. Incubation with 1 ng/ml of either IL-3, IL-5, or GM-CSF prevented spontaneously occurring apoptosis at 6 hrs (IL-3,  $13.2 \pm 0.8\%$ ,  $n = 4$ ,  $P < 0.05$ ; IL-5,  $14.7 \pm 1.0\%$ ,  $n = 4$ ,  $P < 0.05$ ; GM-CSF,  $11.2 \pm 0.2\%$ ,  $n = 4$ ,  $P < 0.05$ ). This difference was more profound after 72 h with  $49.6 \pm 1.3\%$  of the cells being apoptotic when incubated in medium alone and  $18.6 \pm 1.0\%$  with IL-3 ( $n = 4$ ,  $P < 0.05$ ),  $12.0 \pm 1.0\%$  with IL-5 ( $n = 4$ ,  $P < 0.05$ ) and  $8.6 \pm 0.2\%$  with GM-CSF ( $n = 4$ ,  $P < 0.05$ ).

Anti-Fas stimulation induced a comparable proportional increase in DNA fragmentation, when cells were either incubated

in medium alone or in the presence of 1 ng/ml of either IL-3, IL-5, or GM-CSF. Likewise incubation of eosinophils with 10-fold higher concentrations of these survival factors failed to prevent anti-Fas induced eosinophil death (data not shown). Representative FACS<sup>®</sup> analyses 6 h after anti-Fas stimulation are shown in Fig. 3 and the mean data for DNA fragmentation at 6 and 72 h shown in Fig. 4, A and B, respectively.

**Resolution of eosinophilic inflammation of the lung by Fas receptor activation.** Administration of anti-Fas mAb (5–50  $\mu\text{g}$ ) to the airways 72 h (after antigen challenge resulted in a dose dependent resolution of the eosinophilic inflammation 48 hrs later (Fig. 5a). There was no change in the number of eosinophils in the lung after the administration of the matched isotype control mAb, compared with before mAb administration. Importantly, the resolution of eosinophilic inflammation occurred without any significant change in either the BAL macrophage number (Fig. 5 b), the number of peripheral blood leukocytes (Fig. 5 c) or peripheral blood eosinophils (Fig. 5 d). Additionally, histological examination of the lungs 48 h after anti-Fas mAb administration demonstrated a marked reduction in the subepithelial eosinophil infiltration as compared to isotype mAb treated antigen challenged mice. Moreover, this occurred in the absence of any overt secondary inflammatory changes in the structure of airways with the airway epithelium appearing essentially normal (Fig. 6, a–b).

**Fas activation leads to engulfment of eosinophils by macrophages.** To understand more fully how administration of the anti-Fas mAb leads to the resolution of the eosinophilic inflammation, we administered a submaximal dose (20  $\mu\text{g}$ ) of the anti-Fas mAb intranasally and followed the time course of the disappearance of eosinophils from the lung. Within 12 h of anti-Fas administration, the number of eosinophils in the BAL was greatly reduced as compared to the matched isotype mAb treated mice and this suppression was maintained through the next 48 h (Fig. 7). To determine the fate of the eosinophils upon Fas receptor stimulation, we then stained cytospin preparations with DAB-peroxidase. Preliminary experiments demonstrated that macrophages from immunized, non antigen challenged mice failed to stain positive for peroxidase, while 100% of the eosinophils, 72 h after antigen challenge were peroxidase positive. However, 4 and 12 h after anti-Fas administration, the number of peroxidase positive macrophages in the BAL fluid increased from  $0.8 \pm 0.4\%$  before mAb administration to  $11.9 \pm 4.5\%$  at 4 h and  $15.1 \pm 5.1\%$  12 h later, as compared to isotype mAb treated mice (4 h:  $0.5 \pm 0.2\%$ ,  $n = 4$ ,  $P < 0.05$ ; 12 h:  $4.3 \pm 1.0\%$ ,  $n = 4$ ,  $P < 0.05$ ), indicative that macrophages had phagocytosed the eosinophils. This is unlikely to be related to a recruitment of immature peroxidase positive macrophages, as neither bone marrow derived or blood monocytes stained positive with DAB peroxidase. Moreover, it is highly unlikely that this increase reflects neutrophil engulfment, as neutrophils at any time point were  $< 1\%$  of the total BAL population and were also DAB-peroxidase negative. 24–48 h after administration of anti-Fas the percentage of peroxidase positive macrophages in the anti-Fas treated group was not significantly different from isotype mAb treated mice (Fig. 8). There was however, a significant increase in the number of peroxidase positive macrophages 24 and 48 h after isotype mAb treatment  $4.1 \pm 0.95\%$   $P < 0.05$  and  $6.0 \pm 1.5\%$ ,  $n = 4$ ,  $P < 0.05$ ) as compared with time 0 ( $0.8 \pm 0.4\%$ ). Representative photomicrographs of BAL cells 12 h after mAb administration are shown in Fig. 9. The increase in the number of peroxidase positive macrophages following administration of anti-Fas was tempo-



**Figure 6.** Representative lung histology 48 h after treatment with either (A) isotype control ( $\times 600$ ) or (B) anti-Fas ( $\times 1000$ ) in immunized antigen challenged mice. In mice treated with the isotype control there is a marked subepithelial eosinophil infiltration in the bronchi, which was absent in the anti-Fas treated lungs. Importantly, there is no overt secondary inflammatory changes in the lungs and the airway epithelium appears to be essentially normal.

rally associated with the decrease in eosinophils in the BAL from 12–48 h.

### Discussion

Apoptosis induced-cell death is believed to play a major role in the deletion of unwanted T cells in the thymus after their encounter with self-antigens and in the periphery, where mature antigen-activated lymphocytes are deleted by activation induced cell death (AICD). Activation of cell surface Fas receptor by cognate interactions with cells expressing FasL, transmits a death signal to the target cell and has been shown to play a central role in AICD, although not in T cell thymic apoptosis, as mice homozygous for the *lpr* mutation, show no impairment

in their ability to delete autoreactive lymphocytes (19). There is now also increasing evidence to suggest that death by apoptosis is of widespread importance in various inflammatory processes and provides a mechanism whereby effete effector cells can be removed from the site of inflammation without damage to the surrounding host tissue (12). It is in this context, we report that lung eosinophils express the Fas receptor, which upon ligation with an anti-Fas mAb, transmits a death signal to the eosinophil, leading to DNA fragmentation followed by loss of cell viability. While eosinophils derived from the airways expressed the Fas receptor, it is unclear whether Fas is also expressed on peripheral blood eosinophils. However, due to the technical limitations in purifying murine peripheral blood eosinophils in the absence of any eosinophil specific surface

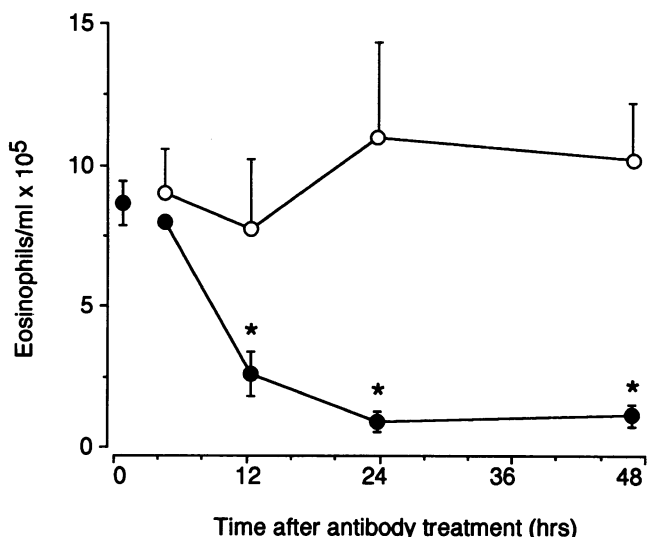


Figure 7. Time course for the disappearance of eosinophils from the lung as assessed by BAL either before antibody treatment (shaded circle) or after either the isotype control (open circles) or anti-Fas antibody (closed circles). Results are expressed as mean  $\pm$  SEM of  $n = 5-7$  animals. Significance was determined by a Student's  $t$  test and a  $P$  value (\*) of  $< 0.05$  was considered significant.

markers, we are at present unable to address this question. Thus it remains to be studied whether normal peripheral blood eosinophils are also positive for the Fas receptor or whether expression is induced/increased as a consequence of eosinophil activation and migration into the airway lumen.

In agreement with previous studies on human peripheral blood eosinophils (5, 6), incubation of murine lung eosinophils with murine recombinant IL-3, IL-5, and GM-CSF, prevented spontaneously occurring apoptosis. However, Fas receptor stim-

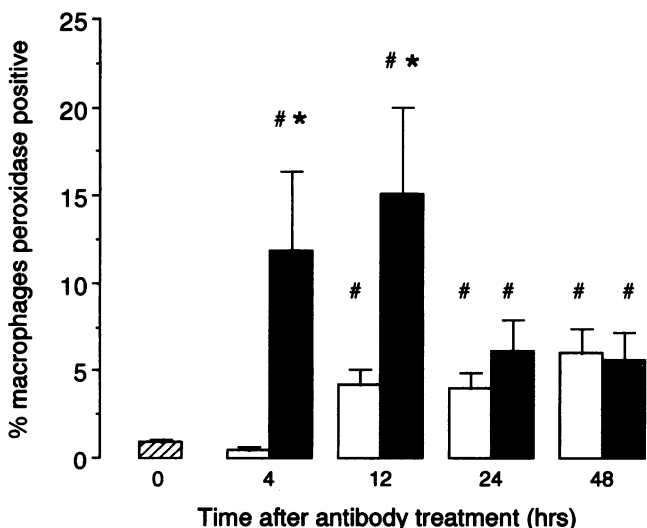


Figure 8. The percentage of peroxidase positive macrophages in the BAL before (shaded columns) and 4, 12, 24, and 48 h after administration of either the isotype control (open columns) or anti-Fas antibody (closed columns). Results are expressed as mean  $\pm$  SEM of  $n = 3-5$  animals. Significance was determined by a Student's  $t$  test and a  $P$  value (\*) of  $< 0.05$  considered significant compared with isotype treated mice or \*  $P < 0.01$ , compared with time 0, prior to antibody administration.

ulation not only abrogated the prolonged survival mediated by these factors, but induced a comparable degree of DNA fragmentation as to that observed when cells were incubated in medium alone. Our results therefore demonstrate that eosinophil death does not only occur passively after the withdrawal or absence of these eosinophil survival factors, but can be actively induced by ligation of the Fas receptor, even in the presence of the eosinophil haematopoietins.

As death by apoptosis has been suggested to be a central mechanism by which inflammatory responses are terminated (16), it then became important to investigate whether administration of anti-Fas mAb would lead to the resolution of a sustained lung eosinophilic inflammation induced by allergen exposure. Administration of the anti-Fas mAb resulted in a marked reduction in the number of eosinophils in the lung 48 h later, as assessed both histologically and by an analysis of the composition of the BAL fluid. It is important to note that the resolution of eosinophilic inflammation occurred in the absence of any change in either macrophage or neutrophil number in the lavage fluid or in the total number of leukocytes or eosinophils in the peripheral blood. Histological examination of the lung tissue after anti-Fas administration also revealed a marked reduction in the number of eosinophils in the airways, as compared to animals treated with the matched isotype control mAb. Furthermore, the resolution of eosinophilic inflammation occurred in the absence of any overt inflammatory changes in the airways, with the epithelium appearing essentially normal. These observations following direct intra-airway application of the anti-Fas mAb contrasts with i.p. administration, where a dose equivalent to that used in the present study was reported to induce a marked leukopenia, fulminant hepatitis and death within 6 h (20). This difference is most likely related to a poor systemic absorbance of the mAb following airway administration of the anti-Fas mAb.

Recent in vitro studies have demonstrated that apoptotic cells are rapidly recognized by macrophages and phagocytosed without eicosanoid or cytokine secretion (21-24). Our observations support this hypothesis and demonstrate that macrophage mediated phagocytosis of eosinophils, as revealed by an increase in the number of peroxidase positive macrophages, also occurred in vivo after treatment with the anti-Fas mAb and was temporally associated with the resolution of the eosinophilic inflammation. The increase in the number of peroxidase positive macrophages was however rather transient in that by 24 h after administration of the anti-Fas mAb, there was no difference in the number of peroxidase positive macrophages as compared with isotype Ab treated animals. These findings are supported by in vitro observations demonstrating that when neutrophils are ingested by macrophages, they are degraded within 30 mins and no longer recognizable (21). 24-48 h after isotype mAb treatment, the number of peroxidase positive macrophages increased as compared to time 0 (i.e., 72 h after antigen challenge). This suggests that under control conditions, eosinophils undergo apoptosis and are engulfed by macrophages as the "normal surveillance" mechanism to remove unwanted apoptotic cells. This hypothesis is supported by the recent demonstration that macrophage mediated engulfment of apoptotic neutrophils after lipopolysaccharide infection is required for the resolution of acute neutrophilic inflammation (26). Eosinophil apoptosis and their engulfment by macrophages would therefore provide a highly efficient mechanism for the removal of unwanted cells in vivo. Moreover, as other workers have demonstrated that apoptotic cells are engulfed as intact cells (21, 26,

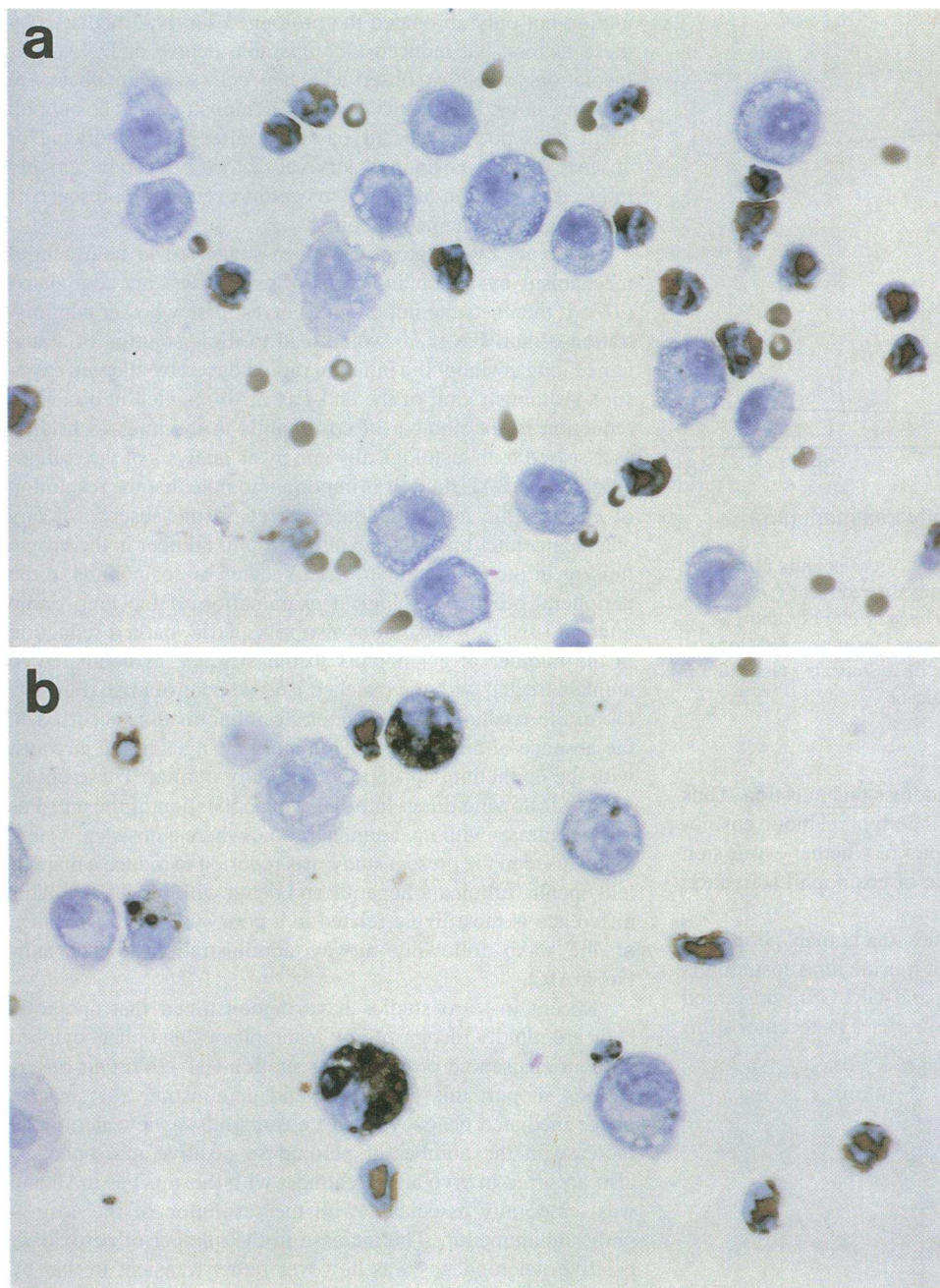


Figure 9. Photomicrograph showing BAL cells stained with peroxidase 12 h after administration of (a) isotype control or (b) anti-Fas antibody. In the isotype control treated mice, only the eosinophils stain for peroxidase, the macrophages being peroxidase negative. In contrast, after anti-Fas treatment, there is an increase in the number of peroxidase positive macrophages indicating that these cells had engulfed the eosinophils.  $\times 600$ .

27), this would prevent incidental damage to the surrounding tissue if eosinophil degranulation occurred with the subsequent release of their highly toxic granular proteins, such as MBP (28). Further studies are however required to determine whether Fas mediated resolution of lung eosinophilic inflammation is also associated with a reversal of airway dysfunction.

A major role of the  $\alpha_v\beta_3$  integrin/CD36/thrombospondin receptor system on the surface of the macrophage in the recognition of apoptotic lymphocytes and neutrophils has been suggested (22, 24). While mAbs against the  $\alpha_v\beta_3$  integrin inhibited the recognition of apoptotic cells by macrophages, other murine monoclonal antibodies of various isotypes and differing specificities failed to promote phagocytosis of freshly isolated cells, suggesting that opsonization of neutrophils or lymphocytes by macrophages could be excluded as a possible recognition mechanism for apoptotic cells (22, 24). In the present study however,

in the absence of any hamster anti-murine mAbs that binds to the eosinophils with a different specificity from the Fas receptor, we are unable to rule out the possibility that hamster mAbs in vivo might opsonize eosinophils for macrophages and hence contribute to the resolution of eosinophilic inflammation. Whatever the precise mechanism involved, it is evident that this process is highly efficient, in that 24 h after anti-Fas stimulation, almost 90% of the eosinophils in the lung had been removed.

While our study demonstrates that eosinophilic inflammation can be resolved after Fas receptor activation, the question of whether cognate interactions between eosinophils and other cells types (including eosinophils themselves) expressing FasL actually occur in vivo as a pathophysiological mechanism to terminate eosinophilic inflammation still remains to be determined. Antigen activated T lymphocytes have been reported to express FasL and provide one possible killing mechanism (12–



14). Additionally, recent studies have shown that FasL can be secreted from the cell surface and therefore may transmit a "death signal" at sites distal to the effector cell (12). However, FasL appears to be differentially expressed on lymphocyte subsets in that FasL is present on only the Th1 subset, but not Th2 subset of CD4+ T cells. (29, 30). As we have previously reported that lung CD4+ T cells are switched to the Th2 phenotype after allergen provocation (4), it is unlikely that these cells can directly induce eosinophil apoptosis by way of Fas L/Fas interactions. Thus, it is interesting to speculate that not only is the switch of CD4+ T cells to the Th2 phenotype deleterious to the asthmatic state by providing IL-4 to mediate B cell isotype switch to IgE and IL-5 to recruit eosinophils to the lungs, but once present in the airways, Th2 cells would no longer have the capacity to kill eosinophils via FasL/Fas interactions.

In conclusion, we have demonstrated that murine lung eosinophils express the Fas receptor and can undergo apoptosis following receptor activation, abrogating the prolonged survival mediated by IL-3, IL-5, and GM-CSF. Likewise, Fas receptor activation in vivo leads to the recognition of eosinophils by lung macrophages resulting in a resolution of eosinophilic inflammation, which occurs in the absence of any overt damage to the surrounding tissue. Studies into the interactions between Fas receptor and cells bearing FasL may shed light into the pathogenesis of chronic eosinophilic inflammation in the lungs of individuals with bronchial asthma.

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