Increased expression of granzymes A and B in fatal asthma

To the Editor:

Inflammatory mediators other than the classic T-helper cell type 2 (Th2)-related pathway play a role in asthma, especially in severe asthma and in exacerbations [1]. Recent studies have suggested a role for cytotoxic CD8 T-cells in lung function impairment [2], lung function decline [3] and smoking-associated asthma [4].

The granules of effector cytotoxic CD8 cells and natural killer (NK) cells contain a family of serine proteases called granzymes (granule-secreted enzymes) [5]. Granzymes are stored in the cytotoxic granules and require perforin to be delivered into the target cells to exert their cytotoxic function [5, 6]. Granzymes can also be released to the extracellular space and cleave extracellular matrix components, contributing to remodelling in chronic inflammation [5, 6]. Granzyme (Gzm)A, the most abundant granzyme, can activate macrophages to secrete cytokines [6].

CD8+ T-cells with increased perforin expression have been identified in the lungs of patients who died of asthma [7]. Several other cell types also express granzymes, and the role of granzymes expressed by these cells other than cytotoxic T-cells and NK cells remains unclear. After allergen challenge, asthmatics present increased levels of GzmB in the bronchoalveolar lavage (BAL), blood and sputum, and increased GzmA in the BAL and sputum [8–10]. Because fatal asthma is associated with exposure to triggers such as viruses, allergens and air pollution, we hypothesised that during a fatal exacerbation in asthmatics there would be an increase in granzymes in the different lung compartments. Therefore, we studied the presence of granzymes in different anatomical areas of the human lung in subjects that died of asthma.

Post mortem lung tissues from subjects with fatal asthma or from a nonpulmonary cause of death (control) were retrieved from the Dept of Pathology of São Paulo University (São Paulo, Brazil) [11]. A detailed clinical and demographic description of this population has been previously published by MAUAD et al. [12].

Random samples from the peripheral parenchyma, including distal airways and one or two samples from central airways, embedded in paraffin, were processed as previously described [11]. For the GzmA, GzmB and CD8 T-cell immunohistochemical stainings, we used the following primary antibodies: anti-GzmA (Sanquin, Amsterdam, the Netherlands), anti-GzmB and anti-CD8 (both Dako, Glostrup, Denmark). Negative controls and isotype-matched antibody controls were performed.

The stained slides were digitised by a 3DHistech Slide Panoramic Scanner (3DHistech, Budapest, Hungary) and cell counting data were analysed using Image-Pro® Plus 4.1 for Windows' software (Media Cybernetics, Silver Spring, MD, USA). One or two large airways, all transversally cut small airways and at least 10 peribronchiolar and distal alveolar septa were analysed per subject. In the large and small airways, the cells were quantified and presented as the number of positive cells in the lamina propria (inner layer) and the adventitia (outer layer) per millimetre basement membrane (cells-mm⁻¹) and as the number of positive cells per area (cells-mm⁻²) in the airway smooth muscle. In the peribronchiolar and distal alveolar septa, the results were expressed as number of positive cells per millimetre septum length (cells-mm⁻¹).

To quantify the percentage of GzmA⁺ and GzmB⁺ cells that were CD8 T-cells, we stained 2-μm serial sections of large and small airways of four asthmatics with the higher granzyme expression and four controls with GzmA, CD8T and GzmB antibodies.

Comparisons between groups were performed using unpaired t-tests or Mann–Whitney U-tests, depending on the data distribution. Differences at a p-value of ≤0.05 were considered statistically significant. Statistical analysis was performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA).

21 asthmatics (median age 37 years (interquartile range (IQR) 23 years), 11 female, 11 smokers (mean±SD 34±25.3 pack-years)) and 14 nonsmoker nonasthmatic controls (median age 54 years (IQR 16 years), seven female) were included (p<0.001 for the comparison of age between the two groups). 28% of the asthmatic patients used corticosteroids (systemic or inhaled) and 95% used short-acting bronchodilators. The
The median duration of the disease was 26 years (IQR 18 years) in the fatal asthma smokers and 23 years (IQR 20 years) in fatal asthma nonsmokers.

We found a higher number of GzmA+ and GzmB+ cells in the inner and outer layers of the airways of the fatal asthma cases compared with the controls (fig. 1). No differences in granzyme expression were observed in the smooth muscle area (data not shown). There was a higher number of GzmA+ and GzmB+ cells in the peribronchial septa of fatal asthma cases compared with the controls, but no differences were observed in the distal alveolar septa (fig. 1).

There were no differences in GzmA+ and GzmB+ cells between asthmatics who were smokers and nonsmokers.

For most of the lung compartments, both in asthmatics and controls, around 50% of the Gzm+ cells were CD8+ T-cells, except for a lower percentage of GzmA+ cells present in CD8+ T-cells in large airways of controls (25%; \( p=0.04 \)). Although we have not quantified the percentage of granzymes expressed by other cell types, the increase in granzymes in fatal asthma can be partially explained by a total increase of inflammatory cells expressing granzymes in the lungs, rather than expression per cell or cell type.

Our results show that, in fatal asthma cases, GzmA and GzmB expression is increased in the large and small airways as well as in the peribronchiolar septa, showing that both granzymes may be involved in the mechanisms preceding asthma deaths. The increased expression of granzymes compared with controls without pulmonary disease could be related to the triggers leading to the severe exacerbation of disease, such as viruses and allergens. Granzymes are critically involved in virus-induced cytotoxicity \[13\], and viruses

FIGURE 1 Cellular distribution in the fatal asthma group and the controls of a, c, e) granzyme (Gzm)A and b, d, f) GzmB in the a, b) large airways (LA), c, d) small airways (SA) and e, f) peribronchial septa (PS) and distal septa (DS). BM: basement membrane. \(* * * \): \( p<0.001 \); \(* \): \( p=0.002 \); \( \# \): \( p=0.01 \); \(+ \): \( p=0.12 \).
have been previously identified in the lungs of fatal asthma patients [7, 14]. In addition, allergen exposure in asthmatics has been shown to cause increased GzmB expression in BAL, blood and sputum [8–10]. Conversely, oxidative stress and increased inflammatory mediators in the airways may contribute to the expression and release of effector molecules, such as granzymes, that can promote local tissue damage by inducing cell death, inflammation and/or by cleaving extracellular substrates. Thus, granzymes may be the effector molecules that serve as agents in promoting a positive feedback cycle that may be common to many chronic inflammatory disorders. It is likely that granzymes are not only involved in exacerbations but also contribute to chronic inflammation in stable asthma. A positive correlation between asthma severity and perforin/granzyme expression in CD4+ invariant NK T-cells has been previously shown [15]. The presence of increased granzymes in the peribronchiolar septa observed in our study could be associated with the marked remodelling changes that have been described at this level in fatal asthma [16].

The density of cells expressing GzmA was higher than that of cells expressing GzmB. This is in line with the notion that GzmA is the most abundant granzyme in lymphocytes and NK cells, although it has less cytotoxic capacity. In asthma cases, only the percentage of CD8 T-cells in the large airway that expressed GzmA was significantly increased. It is possible that the increase of granzymes observed in the lungs is also due to their presence in other cell types, since CD4+ T-cells, B-cells, monocytes, granulocytes and dendritic cells might express granzymes [5, 17, 18]. A limitation of our study is that we did not investigate other cell types expressing granzymes.

Unfortunately, because many of these individuals had no regular medical follow-up, no patient data were available to identify possible triggers, such as viral infection, that could have contributed to a fatal exacerbation. Additionally, to better clarify the putative differential role of granzymes in exacerbations versus chronic inflammation, it may have been desirable to have a group of matched asthmatic individuals who did not die from asthma. In summary, the number of GzmA and GzmB expressing cells in the airways and lung parenchyma was higher in subjects who died from asthma. Understanding the mechanisms related to severe, fatal exacerbations is important for the development of new treatment strategies for asthma control.

Increased numbers of GzmA+ and GzmB+ cells in the airways and lung parenchyma of subjects who died from asthma http://ow.ly/lcKV7

@ERSPublications

Raquel Annoni1,4, Luiz Fernando F. Silva1,4, Yvonne Nussbaumer-Ochsner2, Annemarie van Schadewijk2, Thais Mauad1, Pieter S. Hiemstra3 and Klaus F. Rabe5,6

1Dept of Pathology, São Paulo University Medical School, São Paulo, Brazil. 2Dept of Pulmonology, Leiden University Medical Centre (LUMC), Leiden, The Netherlands. 3LungenClinic Grosshansdorf, Member of the German Center for Lung Research (DZL), Grosshansdorf, Germany. 4Both authors contributed equally.

Correspondence: Raquel Annoni, São Paulo University Medical School, Av. Dr. Arnaldo, 455, room 1155, 01246-903, São Paulo, SP, Brazil. E-mail: rqannoni@usp.br

Received: Aug 20 2014 | Accepted after revision: Jan 11 2015

Support statement: The study was funded by an unrestricted grant from AstraZeneca Germany and the Brazilian National Research Council (CNPq). Funding information for this article has been deposited with FundRef.

Conflict of interest: Disclosures can be found alongside the online version of this article at erj.ersjournals.com

Acknowledgements: We would like to thank G.M. Möller (Ziekenhuis Rivierenland Tiel, The Netherlands) for helpful suggestions during the study.

References
2 Dakhama A, Collins ML, Ohnishi H, et al. IL-13-producing BLT1-positive CD8 cells are increased in asthma and are associated with airway obstruction. Allergy 2013; 68: 666–673.