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Increased levels of T cell granzyme b in bronchiolitis obliterans syndrome are not suppressed adequately by current immunosuppressive regimens

S. Hodge,* G. Hodge,* J. Ahern,* C.-L. Liew,*† P. Hopkins,‡ D. C. Chambers,‡ P. N. Reynolds* and M. Holmes*†

*Lung Research Laboratory, Hanson Institute, †South Australian Lung Transplant Service, Royal Adelaide Hospital, Adelaide, SA, and ‡Queensland Centre for Pulmonary Transplantation and Vascular Disease, The Prince Charles Hospital, Brisbane, Qld, Australia

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Correspondence: S. Hodge, Lung Research, Hanson Institute, Frome Road, Adelaide, SA 5001, Australia.
E-mail: sandy.hodge@imvs.sa.gov.au

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Summary

Bronchiolitis obliterans syndrome (BOS) is characterized by persistent alloreactive, infective and non-specific epithelial injury, loss of epithelial integrity and dysregulated repair. We have reported increased apoptosis of epithelial cells collected from the large airway in lung transplant recipients. As part of the alloreactive response, T cells induce apoptosis of target epithelial cells by secreting granzyme b. We hypothesized that granzyme b would be increased in lung transplant patients with acute rejection and BOS and that commonly used immunosuppressive agents would fail to suppress this serine protease adequately. We investigated intracellular T cell granzyme b in blood, bronchoalveolar lavage (BAL) and large airway brushing (23 controls, 29 stable transplant, 23 BOS, 28 acute rejection, 31 infection) using flow cytometry and assessed the effect of clinically relevant concentrations of cyclosporin A, tacrolimus, methylprednisolone and a protease inhibitor, gabexate mesilate, on *in vitro* granzyme b production. Granzyme b was increased significantly in all compartments of all transplant groups compared to controls. Surprisingly, granzyme b was even higher in patients with BOS than in patients with acute rejection. In longitudinal analysis in three patients, blood granzyme b increased prior to or at the onset of BOS. *In vitro*, methylprednisolone and gabexate mesilate had no effect and cyclosporin A and tacrolimus only a moderate effect on production of granzyme b by $CD8^+$ T cells. Increased T cell granzyme b production may contribute to BOS pathogenesis and is not curtailed by current immunosuppressants. Longitudinal investigation of granzyme b in blood may provide an adjunctive non-invasive method for predicting BOS/OB.

Keywords: BOS, flow cytometry, granzyme b, immunosuppressive therapy, T cell

Introduction

Long-term survival following human lung transplantation is limited by progressive deterioration in lung function due to obliterative bronchiolitis (OB) and its clinical manifestation, bronchiolitis obliterans syndrome (BOS). OB is an essentially irreversible process, characterized by persistent alloreactive, infective and non-specific epithelial injury and dysregulated repair. These processes culminate in remodelling and fibrotic obstruction of small airways, leading directly to patient morbidity and mortality [1,2]. There has been no substantial improvement in the reported incidence of OB over the last 10 years, despite improvements in immunosuppression and patient management [1,2]. The strongest risk factor for OB is acute rejection, with repeated episodes of even minimal acute rejection increasing the risk of subsequent OB significantly [3]. This association suggests that immune mechanisms may in part underlie OB and have thus led to the hypothesis that the disorder is a form of chronic rejection.

As part of the alloreactive response T cells secrete granzyme b, which can induce apoptosis in target epithelial cells by processing and activating members of the caspase family. Elevated levels of granzyme b have been noted in acute rejection of kidney and heart allografts and granzyme b mRNA expression has been shown previously to correlate with acute lung transplant rejection [4–6]. Further, our group has shown previously an increase in apoptosis of airway epithelial cells in transplanted lungs [7], and a significant correlation between

Table 1. Demographic characteristics of the population studied.

	Age	*n*	Gender (m/f)	FEV_1 (% pred)	FVC (% pred)	Time post-transplant (months)
Healthy controls	49·7 ± 18	23	13/10	97·7 ± 14	101·4 ± 13	n.a.
Transplant: stable	50·8 ± 11	29	14/15	77·9 ± 23	79·9 ± 18	11 ± 19
Transplant: BOS	54·3 ± 10	23	13/10	57·7 ± 21	64·3 ± 17	20 ± 21
Transplant: acute rejection	49·9 ± 11	28	15/13	71·9 ± 29	70·4 ± 24	10 ± 8
Transplant: infection	42·5 ± 13	31	17/14	69·6 ± 22	71·3 ± 19	15 ± 11

Data presented as mean ± standard error of the mean. BOS, bronchiolitis obliterans syndrome; FEV_1, forced expiratory volume in 1 s; n.a., not applicable.

the percentage of T cells expressing granzyme b and apoptosis of bronchial epithelial cells in the airway [8]. Granzymes may also be released extracellularly, particularly during cytotoxic T cell degranulation [9], and it has been reported that the secreted granzyme b can contribute to tissue destruction by degrading various extracellular proteins [10].

The aim of the present study was to evaluate the utility of measuring granzyme b as a predictor of BOS/OB following lung transplantation.

Intracellular granzyme b was measured in T cells from large airway brushing, peripheral blood and bronchoalveolar lavage (BAL), collected from stable transplant patients and those with evidence of acute rejection, BOS or infection. Released granzyme b was measured in BAL.

The most effective transplantation immunosuppressive strategies are based on interruption of interleukin (IL)-2 signalling by calcineurin inhibitors, cyclosporin A and tacrolimus. However, intensification of immunosuppressive therapies has not led to any consistent improvement in BOS [1,2]. We therefore also investigated the ability of these commonly used immunosuppressive agents, and a novel protease inhibitor, gabexate mesilate, to inhibit T cell granzyme b production *in vitro*.

Materials and methods

Immunological reagents

CD45 and CD3 [PC-5] (Immunotech/Coulter, Marseille, France) were used to identify leucocyte contamination and identify T cells in BAL and airway brushing. The following were also employed: CD8 [fluorescein isothiocyanate (FITC)] (BD Biosciences, San Jose, CA, USA), granzyme b [phycoerythrin (PE)] (Serotec, Oxford, UK), IgG1/IgG1 negative control (FITC/PE) (BD Biosciences), red blood cell lysing agent [fluorescence activated cell sorter (FACS)lyse], cell membrane permeabilizing agent [FACSperm] (BD Biosciences), methylprednisolone (David Bull Laboratories, Melbourne, Australia), cyclosporin A (Novartis Pharmaceuticals, North Ryde, NSW, Australia), tacrolimus (Janssen-Cilag, North Ryde, NSW, Australia) and gabexate mesilate (Sigma, St Louis, MO, USA).

Subject population

Currently, our lung transplant patients undergo frequent bronchoscopic evaluation, either in the context of surveillance to detect preclinical histological signs of rejection or in the context of acute deteriorations, where samples are required for a specific diagnosis. These routine procedures have provided an ideal opportunity to evaluate the role of key mediators in both stable subjects (including longitudinally in the same subject) and those with BOS, and to assess less invasive approaches to diagnosis and surveillance. Transplant subjects undergoing routine surveillance bronchoscopy (according to international guidelines) as described previously [11] were recruited from three centres: the Royal Adelaide Hospital, South Australia, the Royal Perth Hospital, Western Australia and the Prince Charles Hospital, Brisbane, Australia. Patient rejection status was categorized both clinically and histologically on transbronchial biopsies according to standard criteria [12]. Histological assessment was performed by pathologists who were blinded to the results of flow cytometric testing. A further group of healthy never-smoker volunteers with no history of lung disease were recruited as controls. Ethics approval was obtained from all institutions and informed consent obtained. Patient demographic details are presented in Table 1. Patients were maintained on standard immunosuppressive therapy (cyclosporin or tacrolimus, mycophenolate or azathioprine and prednisolone). Treatment for acute rejection consisted of therapy with prednisolone (10 mg/kg daily for 3 days followed by a PO taper). BAL, large airway brushing and peripheral blood was obtained from 23 healthy controls, 29 stable transplant subjects, 23 patients with evidence of BOS, 28 with acute rejection and 31 patients with proven infection. All subjects underwent spirometry as part of their routine clinical assessment.

Bronchoscopy procedure and preparation of samples

BAL and large airway brushings (to obtain intraepithelial T cells) was obtained via flexible bronchoscopy as described previously [7,8]. Samples were processed within 20 h. We have shown previously that granzyme b levels are unchanged

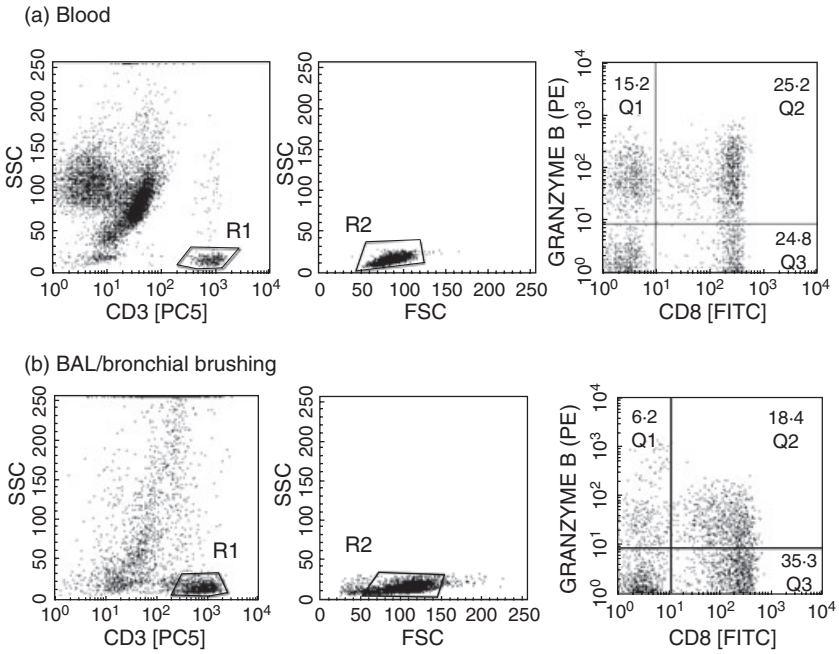

Fig. 1. Representative flow cytometric dot plots of granzyme b expression by T cells from (a) blood, (b) bronchoalveolar lavage (BAL) or intraepithelial compartment. T cells were identified in R1 as staining brightly with CD3 [PC5]. The T cell gate was purified further by back-gating cells from R1 on forward (FSC)-*versus* side (SSC)-scatter characteristics. Subsequent analysis was carried out on cells from R1 + R2. T cells expressing granzyme b were identified by bright staining with monoclonal antibodies to granzyme b [PE]. Data are expressed as a percentage of total CD3 T cells (Q1 + Q2) and as a percentage of $CD8^+$ T cells (Q2/Q2 + Q3).

during this time period (unpublished observations). For each collection from an individual patient the first BAL aliquot was processed for microbiology and the second and third aliquots were pooled, kept on ice and processed within 24 h of collection. BAL-derived cells were washed and resuspended in RPMI-1640 + 10% fetal calf serum (GIBCO, BRL, Germany) and 1% weight per volume penicillin/streptomycin (GIBCO) (culture medium) as described previously [7,8] at a concentration of 4×10^5/ml. Total cell counts in BAL were performed using a modified Neubauer haemocytometer.

Venous blood was collected into 10 U/ml preservative free sodium heparin (DBL, Sydney, Australia). Blood films were stained by May–Grunwald–Giemsa using an automated staining machine (Shandon Veristat Southern Products, Astmore, UK) and differential cell counts were performed using a CELL DYN 4000 (Abbott Diagnostics, Sydney, Australia).

Detection of intracellular granzyme b by flow cytometry

Two hundred ml aliquots of prepared BAL or large airway brushing were added to FACS tubes. For blood, FACSlyse was added for 10 min. All cells were washed with a buffer containing 0·5% BSA in Isoflow, centrifuged at 500 ***g*** for 90 s, and the supernatant discarded. Cell membranes were permeabilized with FACSperm for 10 min, then washed. Cells were then incubated for 20 min with directly conjugated monoclonal antibody (mAb) to granzyme b. Data were acquired using a FACScalibur flow cytometer and analysed using CellQuest software. Representative plots are presented in Fig. 1.

Enzyme-linked immunosorbent assay (ELISA) measurement of released granzyme b

BAL and peripheral blood were prepared as described above from 10 patients in each group. BAL supernatant and plasma were frozen at −80°C. Thawed samples were activated through the addition at 1:5 of 0·1 ml 1 N HCl and neutralized with 1:5 1·2 N NaOH/0·5 M HEPES. BAL supernatants were then concentrated using Amicon Ultra-4 10 kDa centrifugal filter devices (Millipore, Billerica, MA, USA). Granzyme b levels were quantified by ELISA (Diaclone, Besançon, France), according to the manufacturer's instructions. The range for detection using this kit is 31·25–1000 pg/ml. A quality control sample of BAL from one of the lung transplant patients was run on each ELISA plate with the interassay coefficient of variation for the plates remaining within ± 17%.

The effects of immunosuppressive agents on production of granzyme b

To evaluate the effects of commonly applied immunosuppressive agents on granzyme b production, a novel *in vitro* assay was utilized. Mononuclear cells were isolated from peripheral blood of healthy human donors using Lymphoprep (Axis-Shield, Oslo, Norway), according to the manufacturer's instructions. The mononuclear cell layer was washed in culture medium at a concentration of 4×10^5 cells/ml. Mononuclear cells were incubated with gabexate mesilate (0·1–250 mg/ml) or various physiologically relevant concentrations of 10^{-6} M methylprednisolone, 5 ng/ml cyclosporin A or 25 ng/ml tacrolimus (diluted with RPMI-1640), or RPMI-1640 as a control, for 24 h at 37°C in an atmospheric pressure of 5% CO_2. Cells were then stimulated for a further

48 h with Dynabeads CD3/CD28 T cell expander (Dynal Biotech, Oslo, Norway). The pellet containing mononuclear cells was then resuspended and flow cytometry staining for granzyme b performed immediately. Apoptosis was assessed using 7-aminoactinomycin D (7AAD) as described previously [13].

Statistical analysis

Statistical analysis was performed using the non-parametric Kruskall–Wallis, Mann–Whitney and Spearman's correlation tests. SPSS software and statistically significant differences between groups of $P < 0{\cdot}05$ were applied.

Results

Intracellular granzyme b

The percentage of $CD3^+$ T cells expressing granzyme b was increased in blood, BAL and large airway (intraepithelial compartment) of all transplant groups compared to healthy controls, although statistically significant only in the BOS group (Fig. 2a). The increase was significant in $CD8^+$ T cells from the BOS group and in $CD4^+$ T cells from all transplant groups (see Fig. 2b). Granzyme b expression by $CD3^+$ T cells was increased significantly in blood, intraepithelial compartment and BAL in patients with BOS *versus* those with stable graft function, acute rejection or infection (all $P < 0{\cdot}031$) (Fig. 2a). Similar differences in granzyme b expression were noted for $CD4^+$ and $CD8^+$ T cells, although the differences between BOS and acute rejection or infection did not reach statistical significance (blood data presented in Fig. 2b).

Soluble granzyme b

Levels of soluble granzyme b were below the limits of detection in plasma. BAL from patients with BOS or infection contained more soluble granzyme than BAL from patients with stable graft function or acute rejection (BOS: mean ~900 ± 102; infected mean 664 ± 210; stable; 157 ± 102; acute rejection 349 ± 197). The differences did not reach statistical significance (Fig. 2c).

Longitudinal analyses of intracellular and soluble granzyme b

For one patient, blood levels of intracellular granzyme b were consistently high over a 12-month period prior to diagnosis of BOS by histopathological examination of biopsy (Fig. 3a). A further two patients demonstrated increased production of granzyme b by blood T cells coincident with a decrease in lung function and diagnosis of BOS (Fig. 3b and c).

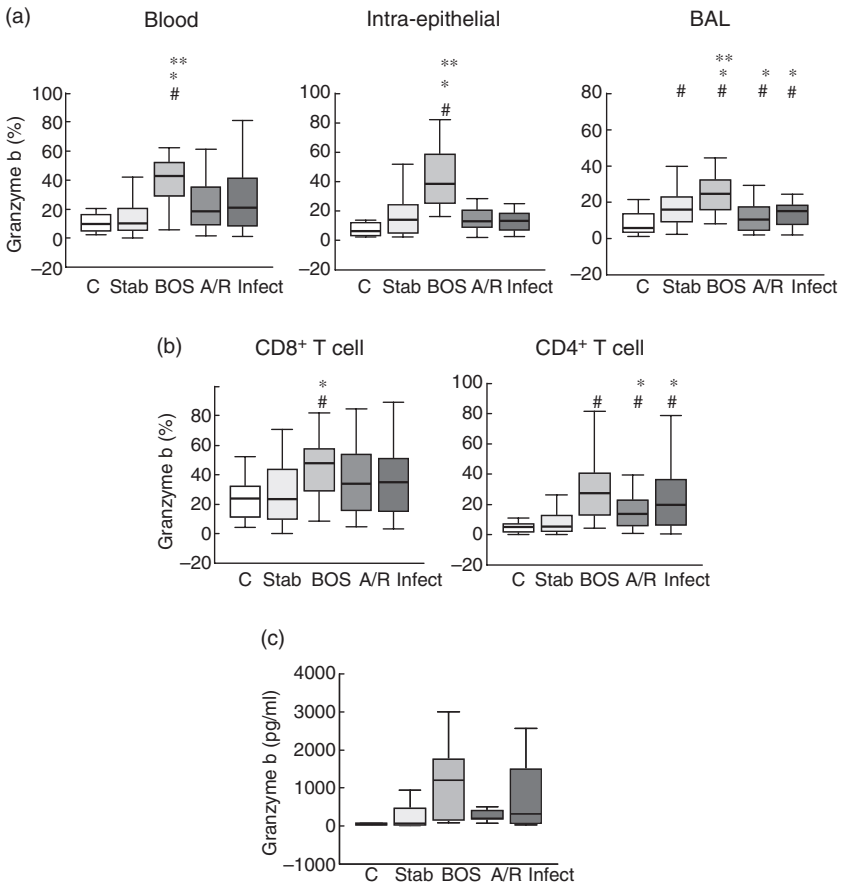

Fig. 2. Intracellular granzyme b expression by (a) $CD3^+$ T cells in blood, intraepithelial compartment and bronchoalveolar lavage (BAL); (b) $CD8^+$ and $CD4^+$ T cell subsets in blood; (c) soluble granzyme b in BAL. Intracellular granzyme b was measured by flow cytometry and soluble granzyme b by enzyme-linked immunosorbent assay. Box plots present median ± 25th and 27th percentiles (solid box) with the 10th and 90th percentiles shown by whiskers outside the box. C, healthy controls; Stab, stable lung function; BOS, bronchiolitis obliterans syndrome; A/R, acute rejection; Infect, stable lung function with proven infection. #Significant increase compared to controls; **significant increase compared to patients with acute rejection or infection; *significant increase compared to transplant patients with stable function. Note non-significant trend for increased soluble granzyme b in BAL from patients with BOS compared to other groups.

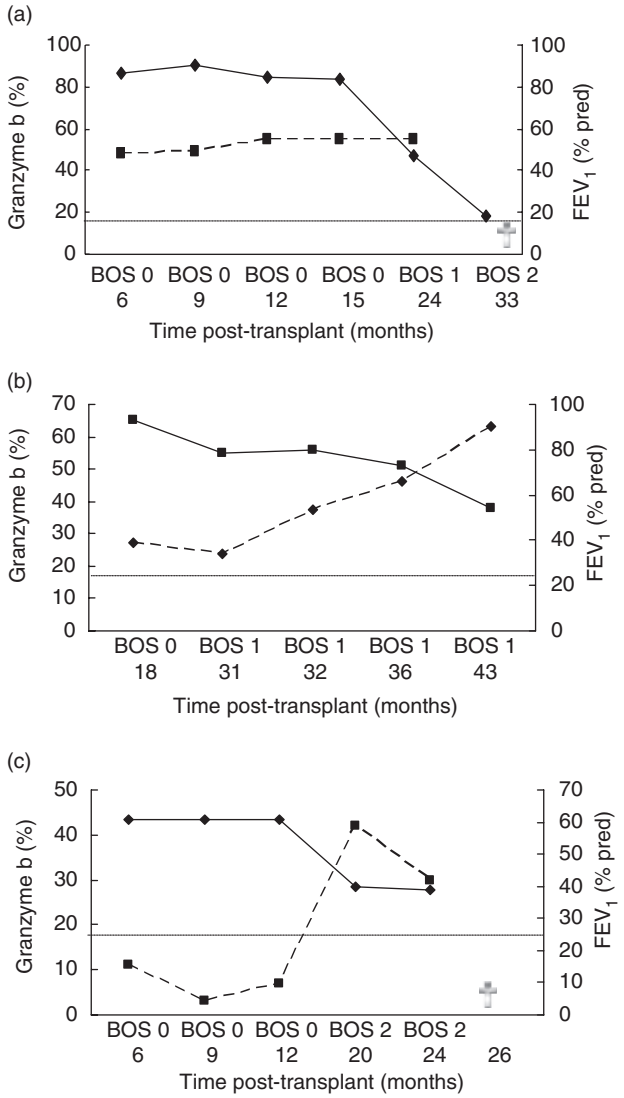

Fig. 3. Longitudinal measurements of granzyme b in blood T cells from three lung transplant patients. Bronchiolitis obliterans syndrome (BOS) category and time post-transplant is indicated on the *x*-axis. Note increased granzyme b for 15 months prior to diagnosis of BOS in patient A, and increase in granzyme b concurrent with fall in forced expiratory volume in 1 s (FEV_1) in patients B and C. — FEV_1 (% predicted), —— granzyme b (% blood $CD3^+$ T cells), ····· mean % granzyme b in blood T cells from stable transplant group.

The effects of immunosuppressive agents on production of granzyme b

The production of granzyme b was investigated in T cells exposed to physiologically relevant concentrations of methylprednisolone, tacrolimus, cyclosporin A and gabexate mesilate in an *in vitro* model of T cell activation. Fewer than 5% of unstimulated T cells produced granzyme b. The effectiveness of the model was confirmed by a significant increase in granzyme b expression following stimulation with CD3/CD28 T cell expander compared to unstimulated cells (Fig. 4). None of the agents tested reduced granzyme b production by more than 48% over the experimental timeframe. Cyclosporin A (5 ng/ml) and tacrolimus (25 ng/ml) were the most effective for reducing granzyme b production, and this reduction was significant for both $CD4^+$ and $CD8^+$ T cells (Fig. 4). Methylprednisolone (10^{-6} M) effectively reduced overall T cell production of granzyme b; however, this drug had no significant effect on granzyme b production by $CD8^+$ T cells. Dose–response experiments were not possible for methylprednisolone or cyclosporin due to increased non-specific cytotoxicity of T cells with increasing concentration as determined with 7AAD staining (data not shown). Dose–response experiments showed that tacrolimus exerted a dose-dependent reduction in granzyme b production (the maximum dose tested was 25 ng/ml, which is slightly higher than the therapeutic range of 5–20 ng/ml) (Fig. 5).

Gabexate mesilate, a protease inhibitor, decreased granzyme b production by both $CD4^+$ and $CD8^+$ T cells in a dose-dependent manner (Fig. 6). The decrease was significant for $CD4^+$ T cells at a concentration of 25 mg/ml, and for $CD4^+$ and $CD8^+$ T cells at higher concentrations of 50 and 250 mg/ml. High levels of non-specific T cell cytotoxicity (>40% 7AAD positive apoptotic cells) were noted in the presence of 250 mg/ml gabexate mesilate.

Discussion

Lung transplantation is being accepted and utilized increasingly as a therapy for end-stage lung disease. Chronic graft failure, due mainly to BOS and its pathological correlate OB, is high, and the 5-year survival following transplant is less than 50% [1,2]. BOS is characterized by loss of airway epithelial integrity and dysregulated repair. The causes are not completely understood, but may include persistent alloreactive, infective and non-specific epithelial injury resulting in increased epithelial cell apoptosis [7]. As part of the alloreactive response, T cells induce apoptosis of target epithelial cells by secreting granzyme b.

We noted a significantly increased proportion of T cells expressing intracellular granzyme b protein in BAL, large airway brushings and peripheral blood and increased released granzyme b in BAL from all transplant groups compared to healthy controls. Surprisingly, granzyme b was even higher in patients with BOS than in patients with acute rejection. Our data suggest that increased T cell-induced apoptosis leading to dysregulated epithelial repair may have a strong immunological role in BOS, and that measurement of granzyme b may have potential as an adjunct investigational tool for accurate prediction of the onset of the disease. The 'gold standard' for the diagnosis of acute rejection is the histological evaluation and grading of transbronchial biopsy according to the International Society for Heart and Lung Transplant guidelines [12]. To this end, the practice of surveillance bronchoscopy has been adopted widely in an effort to detect important changes prior to signs of physiological dysfunction so that

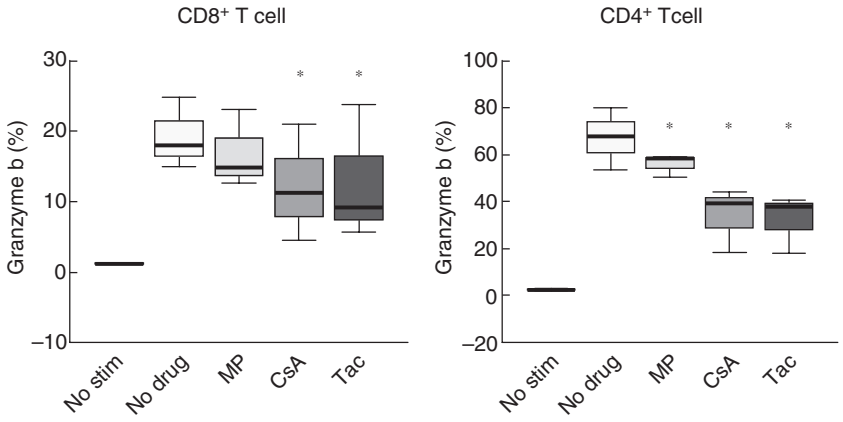

Fig. 4. The effects of immunosuppressive agents on production of granzyme b. Intracellular granzyme b was investigated by flow cytometry in $CD8^+$ or $CD4^+$ T cells stimulated with CD3/CD28 T cell expander and exposed to physiologically relevant concentrations of methylprednisolone (MP) (10^{-6} M), cyclosporin A (CsA) (5 ng/ml) and tacrolimus (Tac) (25 ng/mL). *Significantly decreased granzyme b compared to stimulated cells with no drug. Data presented as box plots as described in Fig. 2 of three separate experiments performed in triplicate.

early therapeutic intervention can be instigated. However, the inadequacy of bronchoscopy and the difficulty in applying this approach to clinical management is evident by the wide range of acute rejection reports (22–73%) that may result from sampling errors and low sensitivity [14,15]. A recent study showed that acute lung transplantation rejection can be predicted with a sensitivity of 94% and specificity of 67% using a cut-off value of 3·1 fg of granzyme b RNA per microgram of total RNA (derived from cells obtained by BAL) [6]. However, reliable quantitative measurement of RNA is difficult in routine practice due to rapid RNA degradation. Further, RNA measurement is only at best a guide to the levels of active protein present. Measurement of granzyme b protein may relate better to clinical disease and be more applicable to routine practice. Interestingly, levels of intracellular granzyme b protein were higher in peripheral blood than in BAL, possibly reflecting the increased release of granzyme b by cytotoxic T cells in this compartment. The lack of significance in our measurements of soluble granzyme b in BAL may reflect the small numbers tested ($n = 10$ in each group) compared to the relatively large numbers included in our analyses of intracellular granzyme b.

We performed preliminary evaluation of the potential diagnostic value of serial monitoring of granzyme b in peripheral blood. Importantly, we were able to detect the onset of BOS/OB in three patients prior to histological confirmation. This finding supports the potential utility of monitoring blood levels of granzyme b as a relatively non-invasive tool for prediction of BOS in lung transplant recipients, although microbiological and clinicopathological correlation is warranted for accurate interpretation of data.

Commonly used immunosuppressive agents fail to halt the progression of BOS/OB. To investigate the effectiveness of these agents for controlling granzyme b we applied physiologically relevant concentrations of methylprednisolone, tacrolimus and cyclosporin in an *in vitro* model of T cell activation. None of the agents tested reduced granzyme b production by more than 48% over the experimental time-

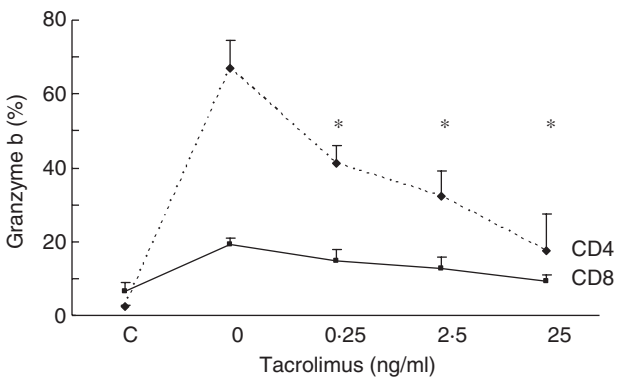

Fig. 5. The effects of varying concentrations of tacrolimus on production of granzyme b. Intracellular granzyme b was investigated by flow cytometry in $CD4^+$ or $CD8^+$ T cells stimulated with CD3/CD28 T cell expander and exposed to physiologically relevant concentrations of tacrolimus (TAC). Data show mean ± standard error of the mean of three separate experiments performed in triplicate. *Significant (dose-dependent) reduction in granzyme b with increasing dose (the maximum dose tested was 25 ng/ml, which is slightly higher than the therapeutic range of 5–20 ng/ml).

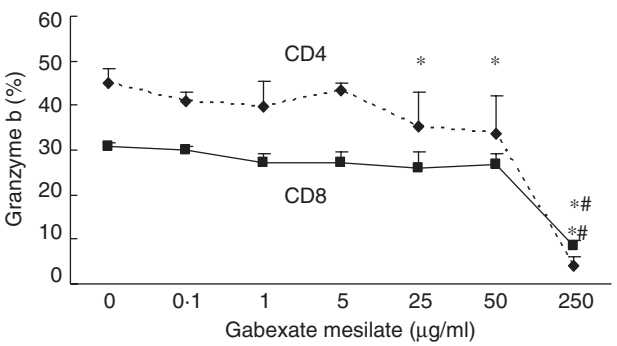

Fig. 6. The effects of gabexate mesilate on production of granzyme b. Intracellular granzyme b was investigated by flow cytometry in $CD4^+$ or $CD8^+$ T cells stimulated with CD3/CD28 T cell expander and exposed to 0–250 μg/ml gabexate mesilate. *Significantly decreased granzyme b compared to unstimulated cells (no drug); #high levels of apoptosis (>40%) assessed by 7-aminoactinomycin D (7AAD) staining. Data show mean ± standard error of the mean of three separate experiments performed in triplicate.

frame. Cyclosporin A and tacrolimus were the most effective for reducing granzyme b production by both $CD4^+$ and $CD8^+$ T cells. Methylprednisolone (10^{-6} M) reduced overall T cell production of granzyme b effectively; however, this drug had no significant effect on granzyme b production by $CD8^+$ T cells. These results are consistent with our previous findings that current immunosuppression protocols have a limited effect on $CD8^+$ T cell production of IFN-γ in the peripheral blood [16] and T helper type 1 (Th1) proinflammatory cytokines in BAL from lung transplant patients [17]. Taken together, our findings indicate that currently used immunosuppressive agents may not be sufficient to suppress high $CD8^+$ T cell production of the proinflammatory Th1 cytokines and the cytotoxic mediator granzyme b in lung transplant recipients. Of particular concern was the inability of methylprednisolone, an agent used commonly for treatment for acute rejection, to reduce granzyme b production by $CD8^+$ T cells.

We thus investigated further a novel protease inhibitor, gabexate mesilate. This agent is administered commonly for the treatment of acute pancreatitis [18]. It has also been shown to reduce ischaemia/reperfusion-induced hepatic injury and prevent the ischaemia/reperfusion-induced decrease in bile flow in rats [19]. Further, gabexate mesilate significantly lowered neutrophil infiltration, lung injury score and wet/dry ratio BAL protein and improved gas exchange in an animal model of lung transplantation [20]. In the present study, gabexate mesilate was very effective at reducing granzyme b production by $CD4^+$ T cells; however, significant suppression of this mediator by $CD8^+$ T cells was achievable only at a concentration that also caused increased non-specific cytotoxicity of T cells. The relatively high doses required for significant inhibitory effects and the lack of effect on $CD8^+$ T cells may thus preclude gabexate mesilate from clinical use in the context of lung transplantation; however, further studies are warranted.

Taken together, our results indicate that increased granzyme b production by T cells may contribute to the pathogenesis of BOS/OB by contributing to epithelial cell apoptosis and abnormal airway repair and that currently used immunosuppressive agents may not be adequate to completely control granzyme b production in these patients. Longitudinal investigation of granzyme b in peripheral blood T cells may provide a novel and non-invasive method for predicting BOS following lung transplantation.

Disclosure

The authors have no conflict of interest to declare in relation to this manuscript.

References

1 Boehler A, Estenne M. Post-transplant bronchiolitis obliterans. Eur Respir J 2003; **22**:1007–18.

2 Trulock EP, Christie JD, Edwards LB *et al.* Registry of the International Society for Heart and Lung Transplantation: twenty-fourth official adult lung and heart-lung transplantation report-2007. J Heart Lung Transplant 2007; **26**:782–95.

3 Hopkins PM, Aboyoun CL, Chhajed PN *et al.* Association of minimal rejection in lung transplant recipients with obliterative bronchiolitis. Am J Respir Crit Care Med 2004; **170**:1022–6.

4 Sharma VK, Bologa RM, Li B *et al.* Molecular executors of cell death-differential intrarenal expression of Fas ligand, Fas, granzyme B, and perforin during acute and/or chronic rejection of human renal allografts. Transplantation 1996; **62**:1860–6.

5 Shulzhenko N, Morgun A, Zheng XX *et al.* Intragraft activation of genes encoding cytotoxic T lymphocyte effector molecules precedes the histological evidence of rejection in human cardiac transplantation. Transplantation 2001; **72**:1705–8.

6 Shi R, Yang J, Jaramillo A *et al.* Correlation between interleukin-15 and granzyme B expression and acute lung allograft rejection. Transpl Immunol 2004; **12**:103–8.

7 Hodge S, Hodge G, Reynolds PN, Holmes M. Differential rates of apoptosis in BAL and blood of lung transplant patients. J Heart Lung Transplant 2005; **24**:1305–14.

8 Hodge S, Hodge G, Nairn J, Holmes M, Reynolds PN. Increased airway granzyme b and perforin in current and ex-smoking COPD subjects. COPD 2006; **3**:1–9.

9 Takayama H, Sitkovsky MV. Antigen receptor-regulated exocytosis in cytotoxic T lymphocytes. J Exp Med 1987; **166**:725–43.

10 Froelich CJ, Zhang X, Turbov J *et al.* Human granzyme B degrades aggrecan proteoglycan in matrix synthesized by chondrocytes. J Immunol 1993; **151**:7161–71.

11 Estenne M, Maurer JR, Boehler A *et al.* Bronchiolitis obliterans syndrome 2001: an update of the diagnostic criteria. J Heart Lung Transplant 2002; **21**:297–310.

12 Stewart S, Fishbein MC, Snell GI *et al.* Revision of the 1996 working formulation for the standardization of nomenclature in the diagnosis of lung rejection. J Heart Lung Transplant 2007; **26**:1229–42.

13 Hodge S, Hodge G, Holmes M, Reynolds PN. Increased apoptosis in the airways in COPD persists after smoking cessation. Eur Respir J 2005; **25**:447–54.

14 Topalidis T, Warnecke H, Müller J, Hetzer R. Endomyocardial biopsies for diagnosis of rejection – the potential margin of error. Transplant Proc 1990; **22**:1443.

15 Sibley RK, Berry GJ, Tazelaar HD *et al.* The role of transbronchial biopsies in the management of lung transplant recipients. J Heart Lung Transplant 1993; **12**:308–24.

16 Hodge G, Hodge S, Reynolds P, Holmes M. Intracellular cytokines in blood T cells in lung transplant patients – a more relevant indicator of immunosuppression than drug levels. Clin Exp Immunol 2005; **139**:159–64.

17 Hodge G, Hodge S, Reynolds P, Holmes M. Increased intracellular pro- and anti-inflammatory cytokines in bronchoalveolar lavage T cells of stable lung transplant patients. Transplantation 2005; **80**:1040–5.

18 Fantini L, Tomassetti P, Pezzilli R. Management of acute pancreatitis: current knowledge and future perspectives. World J Emerg Surg 2006; **23**:1–16.

19 Harada N, Okajima K, Kushimoto S. Gabexate mesilate, a synthetic protease inhibitor, reduces ischemia/reperfusion injury of rat liver by inhibiting leukocyte activation. Crit Care Med 1999; **27**:2062–3.

20 Luh SP, Tsai CC, Shau WY *et al.* The effects of inhaled nitric oxide, gabexate mesilate, and retrograde flush in the lung graft from non-heart beating minipig donors. Transplantation 2000; **69**:2019–27.