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BAL levels of interleukin-18 do not change before or during acute rejection in lungtransplant recipients

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KEYWORDS
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Summary *Study objective*: Acute rejection (AR) of the allograft is a major clinical problem after lungtransplantation. Repeated episodes of AR increase the risk of developing obliterative bronchiolitis, the main cause of mortality in this patient group. It is believed that AR is caused by T-lymphocytes reacting to donor antigens and in turn activating antigen presenting cells (APC) such as alveolar macrophages. Hypothetically, the interferon-γ inducing cytokine IL-18 released from activated macrophages can play a role in the development of AR by modulating cytotoxic T-lymphocytes.

Design: To determine whether IL-18 may serve as a marker of AR, we retrospectively analysed the concentration of soluble IL-18 protein and inflammatory cells in bronchoalveolar lavage fluid (BAL) from lungtransplant recipients.

Patients: To minimize confounding factors, eight pairs of patients were matched for age, gender, pre-op diagnosis, type of operation, absence of infection and time post transplant.

Methods: BAL levels of IL-18 (ELISA) and BAL cell differentials were analysed before, during and after an episode of AR and compared with the matched control group.

Conclusion: We found no changes in IL-18 concentration in BAL associated with AR. IL-18 in BAL did not correlate with BAL lymphocyte percentage. We conclude that change in soluble IL-18 protein does not constitute a useful marker of acute rejection in lung allograft recipients.

Introduction

Lungtransplantation has become an accepted therapy for end-stage lung disease. Improvements in organ preservation, surgical technique, immunosuppressive treatment and infection control have contributed to increased graft and patient survival[1] but the overall long-term prognosis is still poor. Acute rejection of the transplanted lung is a substantial clinical problem, since it decreases graft survival and predisposes the patient to chronic rejection in the form of obliterative bronchiolitis, the main cause of mortality in this patient group.[2,3]

Acute rejection is characterized by a perivascular lymphocytic infiltration in the parenchyma, with or without concommittant lymphocytic bronchitis/bronchiolitis.[4] The diagnosis is made by a combination of histopathological assessment of transbronchial

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biopsies (TBB) and cytological assessment of bronchoalveolar lavage (BAL).

The cellular mechanisms leading to acute rejection are only partially understood. There is evidence that T-helper cells in cooperation with antigen presenting cells (APC), such as the alveolar macrophage, play a crucial role in the host response to donor antigens.[5,6] Since IL-18 is a cytokine mainly produced by activated macrophages it may be involved in this cellular response.[7] IL-18 is capable of inducing production of the TH-1 cytokine interferon-γ (IFN-γ) in several types of immunocompetent cells, such as Natural Killer-cells (NK), helper T-cells and cytotoxic T-cells, especially in collaboration with interleukin-12).[8–10] IFN-γ in turn decreases the TH2 response and enhances the TH1 response by mobilizing cytotoxic T-cells,[8,11] a phenomenon believed to be of central importance in AR.[12,13]

Hypothetically, IL-18 may therefore serve as a link between activated macrophages and IFN-γ production by cytotoxic T-cells.

In the current study, we examined whether alterations in the concentration of soluble IL-18 protein in the airways is associated with onset of acute allograft rejection in lungtransplant recipients. We also characterized the relationship between airway IL-18 and BAL cell populations in these patients.

Materials and methods

Study design and subjects

In a patient material of 90 lungtransplant recipients, patients with at least one episode of acute rejection degree $\geqslant$A2 were identified. Retrospective analysis of BAL fluid specimens collected before, during and after the episode of acute rejection was performed. The results were compared with BAL sample analysis from a matched control group.

To minimize the influence of confounding factors, eight pairs of patients ($n = 16$) were identified and matched for age, gender, preoperative diagnosis, type of operation, absence of infection and time post transplantation. Patient characteristics are presented in Table 1.

The BAL fluid samples had been obtained for a different study, which had been approved by the ethical committee of the University of Göteborg. All subjects gave their written and oral consent. All organs had been harvested in a similar fashion. Organ donors and recipients were matched for cytomegalovirus serological status. Surgical procedures and immunosuppression therapy was preformed as previously described.[14]

Postoperative follow-up

Transbronchial biopsies (TBB) and bronchoalveolar lavage (BAL) were peformed according to the postoperative follow-up protocol of the lung transplant program of Sahlgrenska University Hospital.[15] Samples were also taken whenever clinical signs of worsening of the patients condition appeared such as dyspnoea, hypoxemia, decline in forced expiratory volume in one second (FEV_1) values, radiographic infiltrate or unexpected fever.

The diagnosis of acute rejection was based on assessment of TBB and BAL samples that were evaluated according to the recommendations of the Lung Rejection Study Group of the International Society for Heart and Lung Transplantation (ISHLT).[4]

Analysis of BAL samples included direct microscopy for CMV inclusion bodies, Pneumocystis carini

Table 1 Patient characteristics.

Acute rejection				No rejection			
Diagnosis	Age	Tx	Gender	Diagnosis	Age	Tx	Gender
COPD	56	SL	Female	COPD	55	SL	Female
COPD	52	SL	Female	COPD	52	SL	Female
COPD	55	SL	Male	COPD	64	SL	Male
PPH	53	BL	Male	PPH	40	BL	Female
PPH	43	BL	Female	Eisenmenger	40	HL	Female
α1-AT	50	SL	Male	α1-AT	51	SL	Male
α1-AT	46	SL	Male	α1-AT	43	SL	Female
Cystic fibrosis	30	BL	Male	Cystic fibrosis	23	BL	Female

COPD: chronic obstructive pulmonary disease, PPH: primary pulmonary hypertension, α1-AT: alfa 1 antitrypsin deficiency.
Type of transplantation (Tx): SL: single lung, BL: bilateral lung, HL: heart-lung.

(PCP), fungi and mycobacteria. In addition immunocytochemistry techniques for PCP, CMV and Legionella pneumophilia were applied. Cultures for bacteria, including legionella and mycobacteria, fungi and viruses were performed and presence of CMV and respiratory syncytial virus (RSV) genome was investigated by polymerase chain reaction (PCR) amplification.

TBB were taken after collection of BAL fluid. Four-to-six macroscopically adequate biopsies were taken under flouroscopic guidance from the lower and middle lobes using alligator forceps. The biopsies were immediately placed in 10% buffered formalin and sent for histological analysis.

Collection of samples

BAL was performed by infusing 7×20 ml warmed sterile phosphate buffered saline (PBS) solution into a segmental middle lobe or lingula bronchus with the bronchoscope in a wedged position. The fluid was aspirated after each 60 ml infusion, collected in a sterile siliconized container and immediately transported on ice to the laboratory. After filtering, cellular components were sedimented by centrifugation at 4°C $200 \times g$ for 10 min, and the supernatant removed. After an additional 10-min centrifugation at $10{,}000 \times g$, the supernatant was frozen at -70°C. Cytocentrifuge slides (Shandon Southern Products Ltd., Runcorn, UK) were made from 100 μl aliquots of the resuspended cell pellet. Slides were immediately fixed in 96% alcohol and stained with May Grunwald Giemsa for later identification of cell types on a morphological basis. Percentages of eosinophil granulocytes (EOS), polymorphonuclear granulocytes (PMN), macrophages (MΔ) and lymphocytes were calculated by counting 200 cells using a standard light microscope. All samples were analysed in a blinded manner.

IL-18 protein in BAL fluid

Soluble IL-18 protein was detected using a commercially available ELISA kit (Quantikine ELISA, R&D systems, Abingdon England) in accordance with the manufacturers instructions. The lower detection limit was 31.2 pg/ml.

Statistical evaluation

Non-parametric analysis of variable differences between different time points and patient groups was performed with Kruskall–Wallis test. If significant, further analysis with Mann–Whitney *U*-test was done. Paired matched samples were analysed with Wilcoxon signed rank test. For correlations Spearman rank correlation test was used. *P* values <0.05 were considered to be statistically significant.

Results

Patients

A close to perfect match for sampling time post Tx within the pairs was obtained, only two sample pairs differed more than 1 month (1.5 and 2 months, respectively). The mean time for the first sample was 4.5 months (baseline), for the second sample 7 months (AR and control, respectively), and for the third sample 12 months (follow up).

Match for gender could not be obtained in three pairs of patients. Match for type of operation and preoperative diagnosis could not be obtained in one pair (Table 1). In one BAL sample we found respiratory syncytial virus and in one candida, otherwise all samples were free of infectious diagnosis.

IL-18 protein in BAL fluid

The mean concentration of IL-18 was not increased before or during an episode of AR, compared with the matched control group (Table 2, Fig. 1).

Correspondingly, the difference in IL-18 between AR and before AR within each subject, did not show

Table 2 Concentration of soluble of IL-18 protein in BAL fluid (ng/ml).

Mean time after Tx (months)	Acute rejection			No rejection		
	4.5	7	12	4.5	7	12
Mean	607	616	510	726	538	543
SD	255	190	277	479	200	193
Median	548	614	399	540	553	529

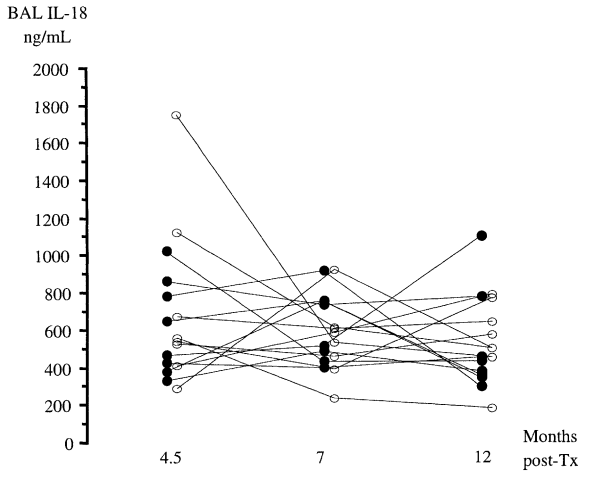

Figure 1 Individual concentration of IL-18 protein in BAL fluid at 4.5, 7 and 12 months time after lung transplantation. Filled dots denote subjects with diagnosis of acute rejection $\geqslant$A2 at the 7-month interval. Open circles denote controls without acute rejection.

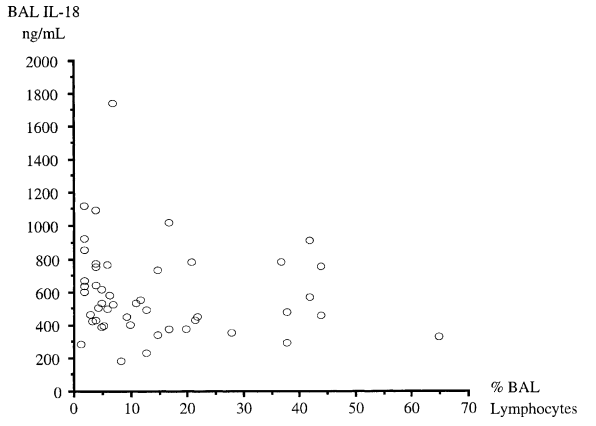

Figure 2 Correlation of individual concentration of IL-18 in BAL with percentage of lymphocytes in BAL.

any difference compared with matched control subjects.

BAL cells and IL-18

No significant correlation was found for the concentration of IL-18 and the percentages of cell subtypes in BAL fluid. Lymphocytosis was seen during acute rejection (mean 21.8% SD 17.3) versus non-rejection (mean 10.3% SD 14.0), but this was not statistically significant. For neutrophils, macrophages and eosinophils, no corresponding differences were found. The relationship between IL-18 and BAL lymphocytes is shown in Fig. 2 ($\rho = -0.23, P > 0.05$). Data for neutrophils, macrophages, eosinophils are not shown.

Discussion

This study on matched samples from lungtransplant recipients could not identify any substantial change in airway IL-18 during an episode of AR. No correlation between IL-18 and different inflammatory celltypes in the airways was found.

Although acute rejection is usually treated sucessfully, it can recur. If immunotherapy is modulated in the early stage of recurring acute rejection, a reduced risk for developing obliterative bronchiolitis could then be achieved.[1,2] Therefore, it is of potential clinical importance to identify predictive biological markers for the onset of acute rejection as well as less invasive methods for the diagnosis of AR other than TBB.

Several pro- and antiinflammatory molecules have been evaluated as candidates for specific markers of lung allograft rejection, but no clinically useful marker has been identified. We expected that IL-18, a key proinflammatory cytokine augmenting the Th1-polarized immune response,[8,11,16] would have a potential for being a marker for AR. Therefore, the current negative findings are somewhat surprising, especially in view of recent data from subjects undergoing bone marrow transplantation. A four-fold increase in IL-18 levels during acute graft versus host disease (aGVHD) was demonstrated after allogen bone marrow transplantation, and high levels of IL-18 strongly correlated with the severity of disease.[17] In our study, however, we were unable to confirm any such pattern and this may be due to the role of IL-18 being more complex. In fact, whilst IL-18 can enhace NK-cell activity[18] and therefore increase the risk of graft rejection, some results indicate it may also protect against AR. In a recent study, depending upon the timepoint when antibodies to IL-18 was administered in a murine bone marrow transplantation model, it accelerated aGVHD related mortality. In contrast, administration of the actual IL-18 protein significantly improved survival suggesting that IL-18 may have a regulatory role in an aggressive systemic alloreaction rather than an amplifier of the cyokine dysregulation that characterizes aGVHD.[19]

The interpretation of the inflammatory reaction during AR in human lungtransplantation is difficult due to the confounding influence of immunosuppressive treatment on inflammatory markers and an increased propensity for infectious complications than do other solid organ transplants. In our study, we therefore used a well-defined patient material where patients with AR$\geqslant$A2 where identified and carefully matched with a control group for age, gender, absence of infection, preoperative

diagnosis, type of operation and time post transplantation. Maintenance immunosuppression did not differ between the two groups. We therefore find it unlikely that any major clinical confounder could have affected our results.

To conclude, this retrospective study does not exclude that IL-18 is involved in the cellular response causing AR in lung transplant recipients but we could not find any evidence that alterations in the airway concentration of IL-18 are useful for early detection of AR.

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