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Expression of tumour necrosis factor receptors by bronchoalveolar cells in hypersensitivity pneumonitis

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ABSTRACT: Tumour necrosis factor receptors (TNFR) and the Fas receptor (FasR) have been implicated in the pathogenesis of interstitial lung diseases. The current authors examined the expression of TNFR-1, TNFR-2 and FasR by bronchoalveolar cells in hypersensitivity pneumonitis (HP).

Cell surface receptor expression on bronchoalveolar lavage cells was analysed by immunocytochemistry in 11 HP patients, 11 idiopathic pulmonary fibrosis (IPF) patients and 10 controls.

TNFR-1, TNFR-2 and FasR were expressed on a higher percentage of alveolar macrophages (AM) in HP compared with controls and IPF patients. TNFR-2 and FasR expression on lymphocytes was also higher in HP than in controls and in IPF. TNFR-1, TNFR-2 and FasR expression correlated positively with the percentage of lymphocytes, and negatively with the percentage of AM in HP. Expression of TNFR-1 on AM and TNFR-2 on lymphocytes correlated with the percentage of neutrophils in HP.

In conclusion, this study shows evidence of altered expression of tumour necrosis factor superfamily receptors in hypersensitivity pneumonitis.

KEYWORDS: Alveolar lymphocytes, alveolar macrophages, Fas receptors, hypersensitivity pneumonitis, tumour necrosis factor receptors 1 and 2

Tumour necrosis factor (TNF)-α is a pro-inflammatory cytokine, produced mainly by activated monocytes/macrophages, and exerts its function by binding to its cognate tumour necrosis factor receptor (TNFR)-1 and TNFR-2 on the cell surface. This has been implicated in the pathogenesis of a number of inflammatory lung diseases [1, 2]. The biological effects elicited by TNF-α include cytotoxicity, a role in endotoxic shock, and inflammatory, immunoregulatory, proliferative and antiviral responses [3].

The TNFR superfamily is a group of cell-surface receptors crucially involved in the maintenance of homeostasis of the immune system. These receptors can lead to either apoptosis or survival of immune cells when ligated by their corresponding ligands [4]. Some studies have shown the involvement of apoptotic receptors in interstitial lung diseases [5, 6].

The TNF receptors, TNFR-1 (p55/60, CD120a) and TNFR-2 (p75/80, CD120b), can exist as membrane-associated or soluble proteins. The soluble receptors possess a TNF-neutralising ability [1]. TNFR-1 shares homology with Fas and contains a death domain in the cytoplasmic tail capable of transducing a death signal. TNFR-2 does not contain a death domain in its cytoplasmic tail; its role during TNF-mediated signal transduction is considered to be indirect in that it appears to aid in the recruitment of TNF to the cell membrane and passes the signal to TNFR-1 [7], or regulates the amount of TNF that is accessible to TNFR-1 [8]. The TNF receptors can lead to either apoptosis or survival. TNF and Fas-mediated apoptosis is induced *via* the activation of caspases. TNF-mediated anti-apoptosis involves TNFR-associated factor-2, which indirectly binds to TNFR-1 through the TNF receptor-associated death domain and the receptor-interacting protein or directly binds to TNFR-2. This activates the transcription nuclear factor-κB, which triggers inflammation and also plays an important role in regulating apoptosis [9, 10].

The Fas receptor (FasR; Apo1/CD95) may mediate apoptosis in susceptible target cells when bound to the Fas ligand (FasL) or to agonistic anti-Fas antibodies [11]. Soluble isoforms of the Fas molecule have been identified, which can block apoptosis induced by FasL *in vitro* [12].

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Dysregulation of the Fas system, leading to accelerated Fas-mediated death, has been implicated in diseases. In contrast, deficient Fas-mediated apoptosis, *e.g.* by genetic alteration of the receptor or the ligand, has been shown to lead to a syndrome of lymphoproliferation and auto-immunity in mouse strains and in patients [13, 14].

The expression of TNFR-2 and FasR was found to be increased on bronchoalveolar lavage (BAL) lymphocytes in sarcoidosis and hypersensitivity pneumonitis (HP), and also on circulating T-cells from the peripheral blood in both diseases [5]. In contrast, expression of TNFR-1 was not observed on BAL lymphocytes in both diseases, and only minor expression was reported on blood lymphocytes in HP [5]. The expression of TNFR-1 and FasR on BAL macrophages was noted to be increased in sarcoidosis [6]. These two receptors were found to be upregulated on synovial tissue cells in rheumatoid arthritis compared with peripheral blood mononuclear cells (PBMC) [15]. Upregulated expression of FasR was described on peripheral T-cells in patients with systemic lupus erythematosus (SLE) [16]. Thus, it has been suggested that the expression of these three receptors is involved in the inflammatory immune response.

In the pathogenesis of HP, alveolitis has been linked to numerous endogenous mediators, especially TNF-α. To test whether increased expression of TNFR-1, TNFR-2 and FasR is present in the inflammatory response of HP, the current authors evaluated the expression of these three receptors by alveolar cells in patients with HP, in comparison with patients with idiopathic pulmonary fibrosis (IPF) and control subjects.

MATERIALS AND METHODS

Study population

In total, 11 consecutive patients with HP (mean$\pm$SEM age 51.4$\pm$3.4 yrs) were investigated (table 1). All were untreated at the time of BAL. They were lavaged 0–4 days after the last antigen exposure. Seven were budgerigar fanciers, two bird fanciers and two had humidifier's lung. Of these, three presented with the acute disease, and eight with the chronic form of insidious outset. All patients fulfilled the following diagnostic criteria: 1) a history of exposure to organic antigens; 2) clinical signs and symptoms consistent with HP; 3) radiological features and/or functional abnormalities characteristic of interstitial lung disease; 4) evidence of serum precipitins against one or more organic antigens; and 5) BAL fluid with increased lymphocytes. The manifestations on high resolution computed tomography (HRCT) of the eight patients with chronic form showed widespread, dominant ground-glass densities, with only minor reticular shadowing and no honeycombing. Late-stage cases with extensive fibrosis were not investigated in this study.

TABLE 1 Demographics of the patients

	HP	IPF	Controls
Female/male	4/7	2/9	4/6
Age yrs	51.4$\pm$3.4#	70.7$\pm$1.5¶	51.3$\pm$5.4
Nonsmokers	11	3	4
Current smokers	0	0	2
Ex-smokers	0	8	4
VC % pred	68.3$\pm$5.1	73.2$\pm$5.4	86.2$\pm$3.9
TLC % pred	75.8$\pm$6.0	66.7$\pm$2.5¶	91.8$\pm$3.5
FEV1/VC %	80.1$\pm$3.2	80.9$\pm$3.0	73.7$\pm$3.2
P_{a,O_2} mmHg	78.0$\pm$3.8	69.3$\pm$1.5	77.4$\pm$3.8
P_{A-a,O_2} mmHg	22.0$\pm$3.0	31.3$\pm$2.0	27.1$\pm$4.5

Data are presented as n and mean$\pm$SEM. HP: hypersensitivity pneumonitis; IPF: idiopathic pulmonary fibrosis; VC: vital capacity; TLC: total lung capacity; FEV1: forced expiratory volume in one second; P_{a,O_2}: arterial oxygen tension; P_{A-a,O_2}: alveolar–arterial oxygen tension difference. #: $p<0.01$ (HP *versus* IPF); ¶: $p<0.01$ (HP or IPF *versus* controls). kPa=mmHg$\times$0.133.

In total, 11 consecutive patients with IPF (aged 70.7$\pm$1.5 yrs) were included. They all fulfilled the recently published American Thoracic Society/European Respiratory Society criteria, including the HRCT characteristics for the diagnosis of IPF [17]. None had left ventricular cardiac failure or a history of chronic pulmonary infections.

The control subjects consisted of 10 patients (aged 51.3$\pm$5.4 yrs). They had no evidence of interstitial lung disease and no history of exposure to antigens known to cause HP. They underwent diagnostic bronchoscopy for various reasons (mediastinal abnormalities, pleural disease, suspicion of recurrent tuberculosis that was not confirmed, exclusion of sarcoidosis or other interstitial lung disease) and had a normal BAL cytology.

Written, informed consent was obtained from the patients according to institutional guidelines.

Bronchoalveolar lavage

BAL was performed by instilling a total volume of 200 mL of sterile isotonic saline in 10$\times$20-mL aliquots into the right middle or left lingular lobe *via* a fibreoptic bronchoscope with immediate aspiration by gentle suction after each aliquot. A volume of >50% was retrieved. The recovered BAL fluid was filtered through two layers of sterile gauze and subsequently centrifuged at 500$\times g$ for 10 min at 4℃. The cells were counted in a haemocytometer. Cell viability was assessed by Trypan blue dye exclusion and the cell number was counted by a standard haemocytometer. Cell differentials were made on smears stained with May-Grünwald-Giemsa by counting 600 cells.

Immunocytochemical analysis

The monoclonal antibodies (MAb) used in this analysis included CD3, CD4 and CD8 (Dako, Copenhagen, Denmark), FasR (Upstate Biotechnology Incorporated, Lake Placid, NY, USA), TNFR-1 (Bender MedSystems, Vienna, Austria), and TNFR-2 (Serotec Ltd, Oxford, UK). The peroxidase–antiperoxidase (PAP) method was applied to identify membrane antigens on the freshly recovered BAL cells, as previously described [18]. Briefly, 10 mL cell suspension (5×10^6 cell·mL^{-1}) was added to the reaction areas of adhesion slides (Bio-Rad, Munich, Germany) for 10 min. After the cells had settled on the glass surface, they were incubated with MAb for 15 min and fixed with 0.05% glutaraldehyde for 5 min, then washed three times with HEPES-buffered washing solution containing NaCl and KCl. Cells were then incubated with a

gelatine-containing medium supplemented with 10% swine serum and 0.2% bovine serum albumin for 15 min to prevent nonspecific binding of immunoglobulin (Ig) to glass and cells. Subsequently, the cells were incubated with rabbit anti-mouse IgG, then with swine anti-rabbit IgG and, finally, with the rabbit PAP immunocomplex, each incubation lasting for 5 min (all reagents from Dako). Diaminobenzidine was used as substrate to visualise the reaction, and OsO_4 for post-fixation. Specificity of the immunostaining was determined by omitting the primary MAb on a reaction area and using mouse IgG instead. No cross-reaction was observed. To evaluate the percentage of positive cells, $\geqslant$200 macrophages or lymphocytes were counted under a light microscope.

Statistical analysis

All data were expressed as mean±SEM. The differences between HP and controls or IPF were compared using a Mann-Whitney Rank Sum test (for nonparametric data) or an unpaired t-test (for parametric data). The correlation of different parameters was analysed by Spearman rank order correlation coefficients. A level of $p<0.05$ was accepted as statistically significant.

RESULTS

Bronchoalveolar lavage fluid cell findings

As shown in table 2, patients with HP had significant increases in the total cell number, the percentage of lymphocytes and mast cells, a significant decrease in the percentage of macrophages compared with controls and IPF, and significant increases in neutrophils and eosinophils only in comparison with controls. There were significant differences in the proportions of macrophages, eosinophils and neutrophils between IPF and controls.

Expression of TNFR-1, TNFR-2 and FasR on BAL cells

The percentages of TNFR-1+ alveolar macrophage (AM), TNFR-2+ AM and FasR+ AM were much higher in HP than in controls and IPF patients (all $p<0.01$; fig 1a). The percentage of TNFR-2+ AM was lower in IPF than in controls ($p<0.05$). TNFR-1 was not expressed on BAL lymphocytes. The percentages of TNFR-2+ and FasR+ BAL lymphocytes were markedly elevated in HP in comparison with controls and IPF (fig 1b). In IPF, the FasR was expressed by a significantly higher percentage of BAL lymphocytes than in controls.

TABLE 2 Profile of bronchoalveolar lavage from patients with hypersensitivity pneumonitis (HP), idiopathic pulmonary fibrosis (IPF) and control subjects

	HP	IPF	Controls
Subjects n	11	11	10
Total cells $\times 10^6$	61.4±9.4**,#	30.4±5.3	19.5±3.7
Macrophages %	15±2.9**,##	80±3.1*	90.4±1.4
Lymphocytes %	77±3**,##	14.1±3.0	8±1
Neutrophils %	4±1*	3.5±0.8*	1.5±0.4
Eosinophils %	3±0.7**	2.5±0.6**	0.08±0.04
Mast cells %	1.1±0.3**,#	0.23±0.08	0.04±0.03

Data are presented as n and mean±SEM. *: $p<0.05$; **: $p<0.01$ (HP or IPF *versus* control); #: $p<0.05$; ##: $p<0.01$ (HP *versus* IPF).

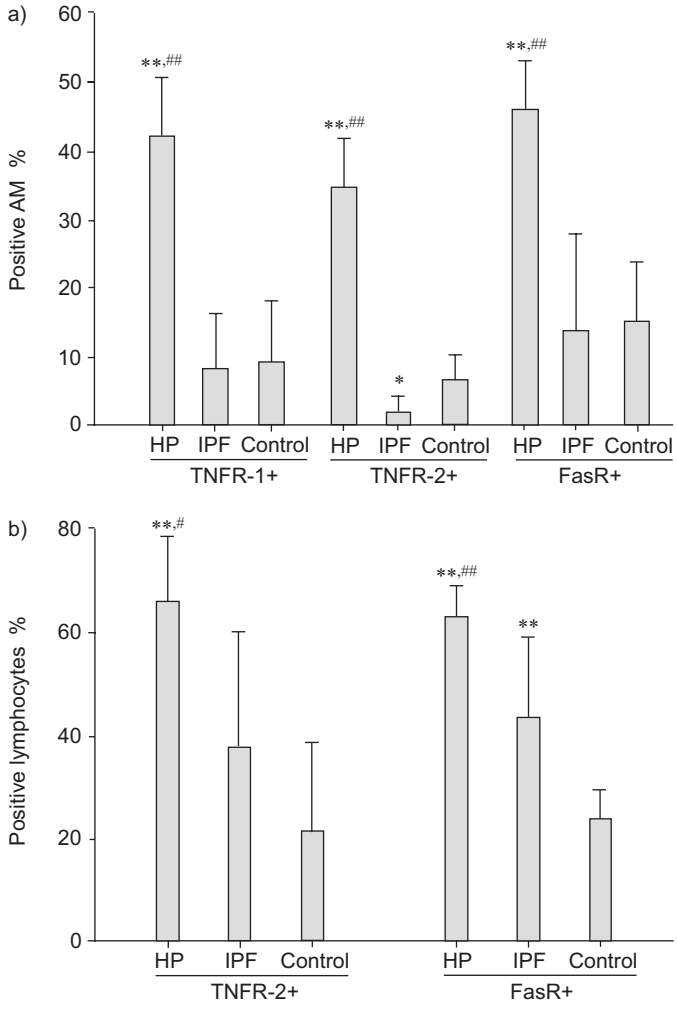

FIGURE 1. a) Expression of tumour necrosis factor receptor (TNFR)-1, TNFR-2 and Fas receptor (FasR) on alveolar macrophages (AM) in hypersensitivity pneumonitis (HP), idiopathic pulmonary fibrosis (IPF) and controls. b) Expression of TNFR-2 and FasR on bronchoalveolar lavage lymphocytes in HP, IPF and controls. Data are expressed as mean±SEM. *: $p<0.05$; **: $p<0.01$ (HP or IPF *versus* control); #: $p<0.05$; ##: $p<0.01$ (HP *versus* IPF).

Correlation between TNFR-1+, TNFR-2+ and FasR+ BAL cells and other BAL parameters

As shown in table 3, close correlations were found between the proportion of FasR+ AM and the proportion of TNFR-1+ AM and TNFR-2+ AM, as well as between TNFR-1+ AM and TNFR-2+ AM ($r=0.84$, $p<0.001$; $r=0.7$, $p=0.015$; $r=0.6$, $p=0.047$, respectively). The expression of TNFR-1, TNFR-2 and FasR correlated positively with the percentage of lymphocytes ($r=0.72$, $p=0.01$; $r=0.8$, $p=0.001$; $r=0.87$, $p<0.001$, respectively), and negatively with the percentage of macrophages in BAL of HP patients ($r=-0.83$, $p<0.001$; $r=-0.76$, $p=0.006$; $r=-0.83$, $p<0.001$, respectively). The proportion of neutrophils in BAL of HP correlated with the expression of TNFR-1 on AM and TNFR-2 on BAL lymphocytes ($r=0.61$, $p=0.04$; $r=0.68$, $p=0.02$, respectively).

TABLE 3 Correlations between tumour necrosis factor receptor (TNFR)-1+, TNFR-2+ and Fas receptor (FasR)+ alveolar cells and other bronchoalveolar lavage parameters in hypersensitivity pneumonitis

	TNFR-2+ AM		FasR+ AM		AM %		Lymphocytes %		Neutrophils %	
	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value
TNFR-1+ AM	0.60	0.047	0.84	<0.001	-0.83	<0.001	0.72	0.01	0.61	0.04
TNFR-2+ AM			0.70	0.015	-0.76	0.006	0.8	0.001		
FasR+ AM					-0.83	<0.001	0.87	<0.001		
TNFR-2+ AL									0.68	0.02

AM: alveolar macrophages; AL: alveolar lymphocytes.

DISCUSSION

In this study, the current authors showed that the percentages of TNFR-1+ AM, TNFR-2+ AM and FasR+ AM, and TNFR-2+ and FasR+ BAL lymphocytes were markedly higher in HP patients in comparison with controls and IPF patients. There were positive correlations between the expression of TNFR-1 and TNFR-2 on AM in HP.

Recently, it has been shown that HP is characterised by a T-helper (Th)-1 type cytokine response, including increased release of interleukin (IL)-12 and IL-18 by AM [19, 20]. Numerous other proinflammatory cytokines, in particular TNF-α produced by macrophages, play a role in the inflammatory response of HP [2]. The role of TNF receptors in the pathogenesis of this disease is still poorly defined.

A previous study from the current authors revealed an increased expression of TNFR-1 and FasR on BAL macrophages of patients with sarcoidosis, which is another granulomatous lung disease [6]. The expression of TNFR-2 and FasR has been reported to be increased on BAL lymphocytes in sarcoidosis and HP [5]. In agreement with these data, GAEDE *et al*. [21] showed in a group of patients with various pulmonary disorders that TNFR-1 is predominantly expressed on AM and PBMC, and TNFR-2 was mainly found on AM, PBMC and BAL lymphocytes.

In a mouse model, TNFR-2 has been shown to trigger apoptosis of activated T-cells, and more than two-thirds of the apoptotic cells were found to be CD8+ T-cells [22]. TNFR-2-deficient CD8+ T-cells are resistant to Fas/FasL-induced cell death [23]. TNF mediates the death of most CD8+ T-cells, whereas FasL mediates the death of most CD4+ T-cells [24, 25]. TNFR-2 can also signal lymphocyte activation, as shown for human thymocytes and peripheral T-cells stimulated by concavalin A [3]. In the current study, an increased expression of TNFR-2 on BAL lymphocytes in HP was found, which is characterised by intra-alveolar accumulation of CD8+ T-cells. TNFR-2 expression on such CD8+ T-cells in HP may trigger these cells to undergo apoptosis. Previously, it was shown that soluble TNFR-2 levels are increased in the BAL of HP [26], and it was suggested that this might serve as a negative feedback mechanism for the elimination and downregulation of activated T-cells within the alveolar space in HP in order to suppress the intense pulmonary inflammation.

Increased FasR expression on peripheral T-cells and PBMC has been found in sarcoidosis and HP, and on peripheral T-cells for SLE. This was discussed as a possible mechanism for the lymphopenia observed in SLE [5, 16]. FasR can also be expressed on AM in sarcoidosis [6] and on human blood eosinophils [11]. The present study revealed that the FasR expression on AM and BAL lymphocytes is enhanced in HP. The FasR expression on AM correlated positively with the expression of TNFR-1, TNFR-2 and the percentage of lymphocytes, and negatively with the percentage of AM in HP. A recent study demonstrated that circulating monocytes and tissue macrophages release TNF-α and IL-8 following Fas ligation [27]. This indicates that Fas ligation can promote proinflammatory activity. It is conceivable that the increased expression of FasR plus TNFR-1 and TNFR-2 on AM may be indicators of the activation of AM and their proinflammatory activity, while the increased expression of FasR and TNFR-2 on BAL lymphocytes may be relevant to the occurrence of apoptosis of activated lymphocytes in the pulmonary microenvironment of HP.

In the present study, it was demonstrated that the expression of TNFR-1 closely correlated with the expression of TNFR-2 on AM, and that both receptors correlated with the percentage of lymphocytes, and inversely with the percentage of macrophages in the BAL of HP patients. The increased expression of TNFR-1 and TNFR-2 on AM may reflect the local inflammatory activity of the lung. The current authors further found that the expression of TNFR-2 on BAL lymphocytes and of TNFR-1 on AM correlated with the percentage of neutrophils in the BAL of HP patients. Along these lines, a recent study showed that TNFR-1 facilitates neutrophil recruitment in BAL fluid after inhalation of lipopolysaccharide in mice [28]. Thus, TNFR-1 and TNFR-2 may have a function to recruit neutrophils to the local inflammatory site of the lung.

IPF is a chronic fibroproliferative disease, and suggested to be a Th2-mediated response. One study proposed that apoptosis of epithelial cells by Fas–FasL cross-linking is causally related to fibrosis [29]. The percentage of BAL lymphocytes expressing Fas were found to be higher in IPF than in controls, but lower than in HP. There were no differences in the expression of the other receptors on alveolar cells between IPF and controls, except for a lower proportion of TNFR-2+ AM in IPF than in controls.

Taken together, this study demonstrates evidence for an increased expression of tumour necrosis factor receptor-1, tumour necrosis factor receptor-2 and Fas receptor on bronchoalveolar lavage cells in hypersensitivity pneumonitis in comparison with controls and idiopathic pulmonary fibrosis. The increased expression of these three receptors may be important in the pathogenesis of hypersensitivity pneumonitis.

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