Expression of three members of the TNF-R family of receptors (4-1BB, lymphotoxin-β receptor, and Fas) in human lung

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ABSTRACT: Receptors belonging to the tumour necrosis factor receptor (TNF-R) superfamily are implicated in a variety of important biological processes. Although messenger ribonucleic acid coding for many of these receptors has been detected in lung, little is known about their expression in this organ.

In this study, immunohistochemical techniques were used to evaluate the expression of three receptors of this family (4-1BB, lymphotoxin-β receptor (LTβ-R), and Fas) in normal human lung, lung carcinomas and by tuberculous and sarcoid granulomas.

The 4-1BB receptor was uniformly expressed by endothelial cells of small vessels and by basal epithelial cells within pseudostratified bronchial epithelium. LTβ-R expression by parenchymal cells was limited to those of epithelial origin (bronchial and bronchiolar epithelium, hyperplastic alveolar epithelium and lung carcinomas). Fas was present on both fibroblasts and epithelial cells (bronchial and alveolar epithelium and most, but not all, carcinomas), but not on endothelial cells. A subpopulation of T-lymphocytes expressed these receptors, but only Fas was detected on normal alveolar macrophages and epithelioid cells.

Thus, 4-1BB, LTβ-R, and Fas have characteristic and only partially overlapping patterns of expression in the lung. The findings should facilitate further evaluation of their role in lung homeostasis and pathology.
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The tumour necrosis factor receptor (TNF-R) superfamily is comprised of a rapidly growing group of type I membrane-bound receptor molecules. Currently, at least 20 different members expressed by mammalian cells have been identified: TNF-RI (CD120a), TNF-RII (CD120b), nerve growth factor receptor, lymphotoxin-β receptor (LTβ-R), Fas (CD95), 4-1BB (CDw137), CD27, CD30, CD40, OX-40, DR3 (also called TRAMP, Wsl-1, Apo-3), TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR1, TRID), TRAIL-R4, CAR-1, GITR, TR2, RANK and TACI [1–13]. The ligands for these receptors may either be membrane bound (*e.g.* LTβ), soluble molecules (*e.g.* LTα) or exist in both membrane and soluble forms (*e.g.* TNFα and Fas-ligand), thus permitting cell-to-cell, paracrine and endocrine signalling [1–3]. In most cases, these receptors appear to bind a single cognate ligand, although TNF-RI and TNF-RII can bind both TNFα and LTα with equal affinity [3].

Signaling through this family of receptors has been shown to regulate a variety of essential biological processes. Without exception, receptors of the TNF-R superfamily have been found to be expressed by T-lymphocytes, and many are also expressed on B-lymphocytes and antigen-presenting cells. Receptor-ligand interactions involving these molecules have important effects on immune responses, due to the ability of signaling through these receptors to influence the activation, proliferation and differentiation of immune effector cells and to initiate or inhibit programmed cell death (apoptosis) [1–6].

Although the expression of some members of the TNF-R family appears to be restricted to immune/inflammatory cells (*e.g.*, CD27, CD30, OX40) [10, 14], several members of the TNF-R family have also been found to be widely expressed on parenchymal cells in many organs, including the lung (*e.g.*, TNF-RI, TNF-RII, CD40) [3, 6, P. Soler, unpublished data). The evaluation of the effects of signalling through TNF-R superfamily receptors on parenchymal cells has received less attention than that of their role in the course of immune responses. Nevertheless, these receptors have been shown to regulate cell proliferation (*e.g.*, the induction of fibroblast proliferation by CD40), differentiation (*e.g.*, the essential role of LTβ-R in the development of lymph nodes) and activation of parenchymal cells (*e.g.*, the induction of cytokine secretion by signaling through Fas). A subgroup of these receptors can initiate apoptosis in parenchymal cells. In view of these pleiotrophic effects, the TNF-R family of receptors undoubtedly plays a critical role in the homeostasis of normal tissues, including the lung.

In many cases, however, little is known about the expression of these important receptors in lung and other tissues. For example, initial characterization of the TNF-R

superfamily members 4-1BB, LTβ-R and Fas revealed that messenger ribonucleic acid (mRNA) coding for these cytokines was present in normal lung [4, 15–17], but the extent that the corresponding receptor proteins are expressed by human lung cells. and the distribution of such expression has not been hitherto reported. In this study, we have used immunohistochemical techniques to evaluate the expression of these three receptors in normal human lung, lung carcinomas, and by cells composing tuberculous and sarcoid granulomas.

Material and methods

Study populations

Normal lung was obtained from patients (12 males, one female; 58±15 yrs; eight smokers and five nonsmokers) undergoing thoracotomy for localized pulmonary disease (localized carcinoma, n=11; localized bronchiectasis, n=1; bronchial carcinoid, n=1). Grossly normal lung tissue was taken from a site distant from the lesion or from a different lobe. Lung tissue from nonsmokers was normal. Lung tissue from smokers demonstrated the accumulation of pigment-laden macrophages and mild diffuse or focal fibrotic changes in alveolar walls. Cartilaginous bronchi had normal architecture in all cases.

Specimens containing lung carcinoma were obtained from eight patients (seven males, one female; 63±10 yrs; all cigarette smokers). Histological types were: squamous cell carcinoma, n=5; adenocarcinoma, n=3.

Three patients with tuberculosis were studied (two males, one female; 36±6 yrs). The diagnosis was based on the presence of typical granulomas with central necrosis. Serological testing was negative for HIV, and none were receiving antimycobacterial therapy at the time of the study. Three patients with sarcoidosis were studied (one male, 2 females; 40±10 yrs). The diagnosis was based on previously described criteria including clinical and radiological examination and histologic evaluation of the tissue. None were receiving corticosteroids at the time of evaluation. Granulomatous lymph nodes were obtained by mediastinoscopy or open surgery.

A portion of each specimen was fixed in Bouin-Hollande solution, processed by routine techniques and stained with haematoxylin-eosin for histological evaluation. The remaining specimen was snap-frozen and stored in liquid nitrogen.

Immunohistochemical techniques

Monoclonal antibodies recognizing 4-1BB (M127), Fas (M3) and LTβ-R (M12) have previously been described [4, 18, 19]. Anti-CD3 (leu-4) was from Becton Dickinson (Mountain View, CA, USA), and an isotype-matched control antibody (MOPC 21) was obtained from Sigma (St. Louis, MO, USA). For immunohistochemical staining, 5-µm cryostat sections were fixed in acetone and treated with appropriate dilutions of antibody. Positive cells were revealed using the Vectastain ABC Alkaline Phosphatase Kit System (Vector, Burlingame, CA, USA) and the fast red substrate. To test the specificity of the immunohisto-

chemical techniques, antibodies were replaced by an isotype-matched control antibody; under these conditions no positive cells were identified. The intensity of reactivity was graded as follows: 0 (absent), + (weak), ++ (moderate), +++ (intense). Complete agreement was obtained between two independent observers.

Results

Expression of 4-1BB

Expression of 4-1BB in normal lung was extremely limited. In larger airways containing a pseudostratified epithelium, the basal epithelial cells demonstrated very strong expression of 4-1BB (fig. 1A). In contrast, epithelial cells in contact with the bronchial lumen were uniformly negative (table 1). No cells expressing intermediate levels of 4-1BB were observed in pseudostratified epithelia. Bronchiolar and alveolar epithelial cells were negative for 4-1BB, despite the fact that these cells were in direct contact with basement membranes. Similarly, epithelial cells in areas of alveolar epithelial hyperplasia and lung carcinomas did not express 4-1BB (table 2).

Endothelial cells were the only other parenchymal cells found to express 4-1BB. In the normal lung, capillaries and venules were consistently positive. Small calibre vascular structures in the stroma of lung carcinomas and those surrounding granulomas also strongly expressed 4-1BB (compare fig. 1B and C). The endothelial cells of lymphatic vessels, particularly easy to identify in granulomatous lymph nodes, were also positive. In contrast, endothelial cells within arterioles and arteries identifiable by the presence of smooth muscle in the media of the vessels were generally negative. The vasculature of intestine and liver also expressed 4-1BB, demonstrating that expression was not restricted to the pulmonary vessels (data not shown).

A subpopulation of the T-lymphocytes infiltrating normal lung, lung carcinomas and sarcoid and tuberculous granulomas expressed 4-1BB; a majority of the lymphocytes within lymphoid aggregates in the stroma of lung carcinomas (predominantly T-lymphocytes) were also positive. Primary follicles in lymph nodes were also uniformly stained, indicating that B-cells also expressed 4-1BB. In contrast, alveolar macrophages, and epithelioid cells and giant cells within granulomas were uniformly negative.

Expression of LTβ receptors

The expression of LTβ-R in normal lung was restricted to epithelial cells. Within the pseudostratified epithelium of larger bronchioles, prominent staining of the apical portion of ciliated cells in contact with the bronchial lumen was observed (compare fig. 1D and E), although all bronchial and bronchiolar epithelial cells appeared to express LTβ-R. Alveolar epithelium in the normal lung was negative, but hyperplastic alveolar epithelial cells expressed LTβ-R. All lung carcinomas demonstrated weak to moderately intense staining for LTβ-R (fig. 1F). No difference in the intensity of expression was observed comparing adenocarcinomas and squamous cell carcinomas, and within a given tumour staining of the neoplastic cells was homogeneous in all cases.
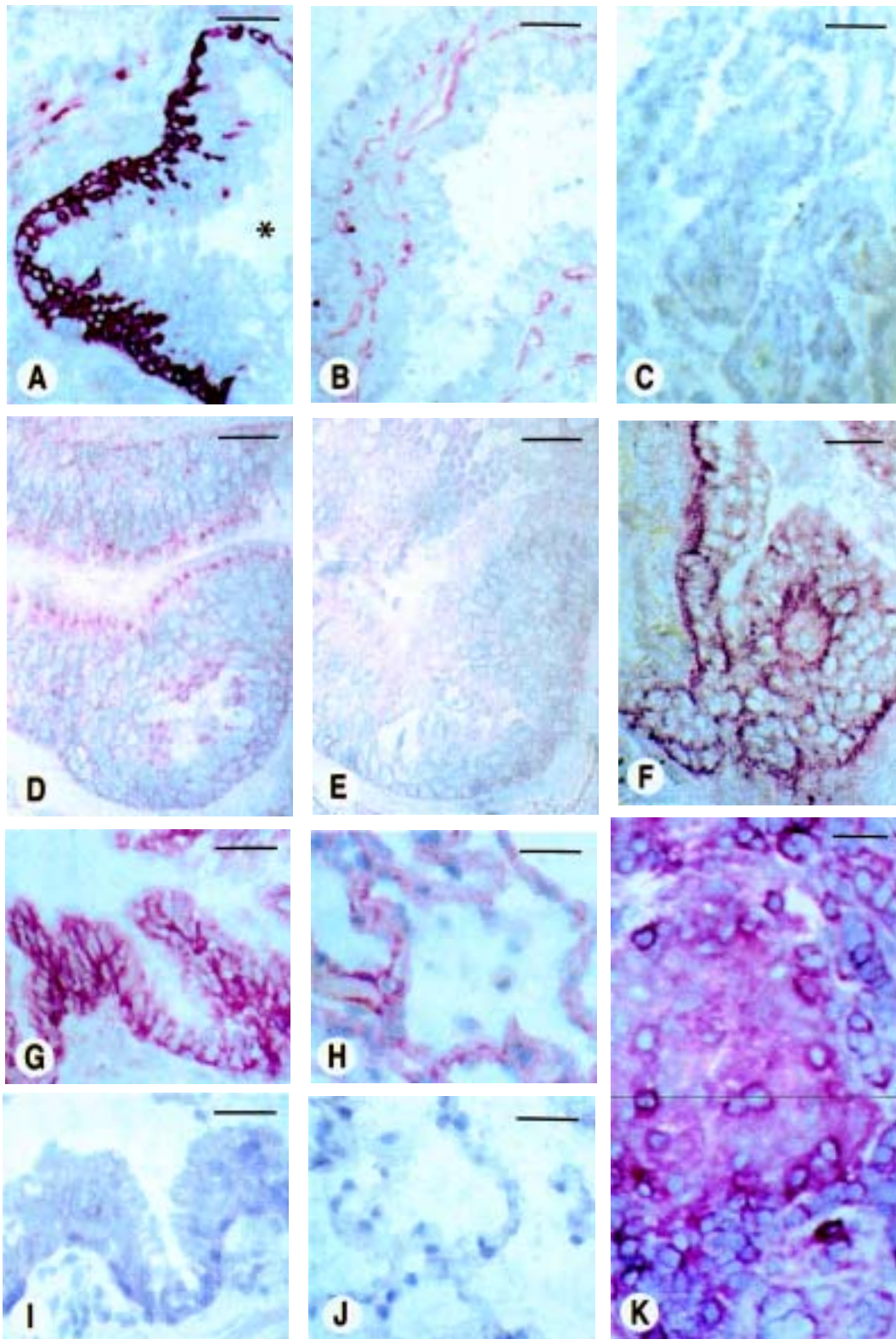
Fig. 1. – Expression of 4-1BB and lymphotoxin-β (LTβ) receptor and Fas in human lung. A) 4-1BB. Basal epithelial cells in a normal bronchiole are intensely stained, whereas the epithelial cells in contact with the lumen (*) are negative. B) 4-1BB. Vessels in the connective tissue stroma surrounding islands of adenocarcinoma are moderately positive, but are not stained with an isotype-matched control antibody (C). D) LTβ-R. Normal bronchial epithelial cells are positive. Expression is especially prominent on the apical portion of the cells. These cells are unreactive with an isotype-matched control antibody (E). F) LTβ-R. Primary adenocarcinoma. Tumour cells are stained moderately. G) Fas. Epithelial cells in a normal human bronchus are uniformly positive. H) Fas. Normal alveolar tissue. Alveolar walls are diffusely stained. Most alveolar macrophages are negative. I) and J) Sections adjacent to those in G and H stained with an isotype-matched control antibody. K) Fas. Sarcoid granuloma. Both epithelioid cells and the T-cells surrounding the granuloma are moderately positive. (Internal scale bars A, D–F=40 μm; B, C=80 μm; G–J=50 μm; K=30 μm.)

Table 1. – Expression of 4-1BB, and Fas by cells in normal lung

Tissue	Reactivity with antibody		
	4-1BB	LTβ receptor	Fas (CD95)
Epithelium			
Bronchial			
Basal cells	+++	+	++
Luminal cells	0	+/++	++
Bronchiolar	0	+	++
Alveolar	0	0	++
Endothelium			
Arteries	0	0	0
Capillaries/venules	++	0	-*
Alveolar interstitium			
Fibroblasts	0	0	+
Smooth muscle#	0	0	0
Immune/inflammatory			
Macrophages	0	0	0/++
Lymphocytes	+	0	+/++

Results are expressed as 0 (absent), + (weak), ++ (moderate), +++ (intense) staining, and summarize findings for specimens from six (4-1BB and LTβ receptor) or seven (Fas) different patients. In cases where staining was not homogenous, the intensities of staining for the most and least positive cells are separated by a slash. *: reactivity could not be defined unambiguously (see text). #: cells in vascular media. LTβ receptor: lymphotoxin-β receptor.

No other lung parenchymal cell types expressed detectable LTβ-R, and immune/inflammatory cells in the normal lung and within granulomatous lesions were negative (tables 2 and 3).

Expression of Fas (CD95)

Fas was widely expressed in normal lung. All bronchial epithelial cells were moderately positive for Fas (fig. 1G). Within the alveolar parenchyma, alveolar epithelial cells, fibroblasts, lymphocytes and a subpopulation of alveolar macrophages were found to express Fas, and resulted in a diffuse staining of the alveolar walls (fig. 1H). None of these normal parenchymal cells was stained with an isotype-matched control antibody (fig. 1I and J). Hyperplastic alveolar epithelium also expressed Fas, as did most, but not all, lung carcinomas (5/6 adenocarcinomas and 2/4 squamous cell carcinomas demonstrated at least weak Fas expression). No correlation was observed between the expression of Fas and LTβ-R by lung carcinomas (data not shown).

Endothelial cells of arteries and arterioles did not express Fas. Because the neighbouring cells in the alveolar walls were diffusely positive, it was difficult to determine whether or not pulmonary capillaries expressed Fas. Capillaries and venules in the connective tissue stroma of lung carcinomas and those surrounding granulomatous lesions were, however, negative for Fas.

Fas is known to be expressed by activated and memory (but not by naive) T-lymphocytes [5]. As expected, lymphocytes infiltrating normal lung parenchyma, lung carcinomas and granulomatous lesions (fig. 1K), known to be predominantly memory or activated T-lymphocytes, were Fas+. B-lymphocytes in primary follicles also expressed Fas. Epithelioid cells, but not giant cells, in granulomatous

Table 2. – Expression of 4-1BB, lymphotoxin-β (LTβ) receptor and Fas in primary lung carcinomas

Tissue	Reactivity with antibody		
	4-1BB	LTβ receptor	Fas (CD95)
Neoplastic cells			
Adenocarcinoma	0	+/++	0/+++
Squamous cell	0	+/++	0/+++
Hyperplastic epithelial cells	0	+	++
Endothelium			
Arteries	0	0	0
Capillaries/venules	+++	0	0
Connective tissue			
Fibroblasts	0	0	+
Smooth muscle*	0	0	0
Immune/inflammatory			
Macrophages	0	0	0/+
Lymphocytes	0/++	0	+/++
Lymphoid follicules	++	0	++

Results are expressed as 0 (absent), + (weak), ++ (moderate), +++ (intense) staining, and summarize findings for specimens from eight different patients (squamous cell carcinoma, n=5; adenocarcinoma, n=3). In cases where staining was not homogenous, the intensities of staining for the most and least positive cells are separated by a slash. *: cells in vascular media.

lesions expressed Fas, but the intensity of expression was less than that observed on T-lymphocytes in these lesions. No differences in reactivity were observed, when comparing sarcoid and tuberculous granulomas.

Discussion

Evaluation of the expression of members of the TNF-R family of receptors in human lung revealed three distinctly different profiles. In lung parenchymal cells, 4-1BB was uniformly expressed by endothelial cells of small vessels and by basal epithelial cells within pseudostratified bronchial epithelium; LTβ-R expression was limited to cells of epithelial origin; Fas was detected on both epithelial

Table 3. – Expression of 4-1BB, lymphotoxin-β (LTβ) receptor and Fas in granulomatous lymph nodes

Tissue	Reactivity with antibody		
	4-1BB	LTβ receptor	Fas (CD95)
Granulomas			
Epitheloid cells	0	0	+
Giant cells	0	0	0
Lymphocytes	0/++	0	++
Surrounding tissue			
Lymphocytes	0/++	0	0/++
Primary follicles	+++	0	++
Connective tissue			
Fibroblasts	0	0	+
Arteries	0	0	0
Capillaries/venules	++	0	0
Lymphatic endothelium	++	0	0

Results are expressed as 0 (absent), + (weak), ++ (moderate), +++ (intense) staining. Specimens from three different patients each with tuberculosis and sarcoidosis were evaluated with each of the antibodies. In cases where staining was not homogenous, the intensities of staining for the most and least positive cells are separated by a slash.

cells and fibroblasts, but was not expressed by endothelial cells. At least a subpopulation of T-lymphocytes expressed all three receptors, but only Fas expression was observed on alveolar macrophages.

4-1BB is known to be expressed by activated (but not resting) T-lymphocytes. The detection of 4-1BB mRNA in activated B-cells, cytokine-treated monocytes and certain carcinoma cell lines has also been reported [20, 21]. The expression of 4-1BB by endothelial cells in lung and the other tissues described here has not been previously noted. Endothelial cells in lung and other tissues also express CD40, another member of the TNF-R family, and signaling through this receptor increases the expression of several adhesion molecules by endothelial cells *in vitro* [22]. We were unable to detect the expression of 4-1BB on human umbilical vein endothelial cells (data not shown), precluding such functional studies. The expression of 4-1BB was particularly strong on vessels within the stroma of lung carcinomas. In this regard, MELERO *et al.* [23] recently reported that the administration of 4-1BB antibody could lead to the eradication of established tumours in mice through a T-cell-dependent mechanism. The possible participation 4-1BB-mediated effects on tumour vessels in this response may merit investigation.

The very strong expression of 4-1BB on basal cells in bronchial epithelium, but not other lung epithelial cells, is also noteworthy. There has been some controversy as to whether basal cells are immature "precursors" of other bronchial epithelial cells or represent a differentiated population with specific functions [24]. The absence of cells expressing intermediate levels of 4-1BB is consistent with the later possibility and suggests that this antibody may be useful in isolating basal cells for functional studies.

LTβ-R expression in the normal lung was restricted to bronchial epithelial cells. Hyperplastic alveolar epithelial cells and all lung carcinomas evaluated also expressed this receptor. Very little prior information is available concerning the LTβ-R expression in normal tissues. Constitutive expression of LTβ-R mRNA by lymphoid tissues and several visceral organs, including lung, has been described [17, 19], but the cell types expressing the receptor have not previously been identified. Expression of LTβ-R on human fibroblasts and human carcinoma cell lines maintained *in vitro* has also been described, and stimulation of these cells through LTβ-R can produce growth stimulation, growth arrest, or cytokine production, depending on the cell type [17, 19, 25]. Cell death induced by an apoptotic mechanism has also been described for a few carcinoma cell lines treated with interferon-γ prior to stimulation of LTβ-R [25]. We could detect LTβ-R on A549 lung carcinoma cells by fluorescence activated cell sorting analysis (FACS) analysis, but crosslinking of the receptor did not induce detectable apoptosis or IL-6 expression (data not shown). Further studies are needed to define the effect of signaling through LTβ-R on normal and transformed airway epithelium. Activated T-lymphocytes appear to be the principal cell type capable of expressing $LT\alpha_1LT\beta_2$, the cognate ligand for LTβ-R [26]. Because expression of LTβ-R was particularly strong on the luminal side of bronchial epithelial cells, T-cells within the bronchial lumen may be particularly important in delivering signals through this receptor.

Numerous lung parenchymal cells were found to express Fas, including both bronchial and alveolar epithe-lial cells, fibroblasts, T-lymphocytes and a subpopulation of pulmonary macrophages. Signalling through Fas is known to induce apoptosis in some cell types. Importantly, however, not all cell types expressing this receptor undergo apoptosis following stimulation of Fas, and Fas-induced co-stimulation of T-lymphocytes and Fas-induced cytokine secretion by carcinoma cells have also been described [5, 27]. The extent that lung cells are sensitive to Fas-induced apoptosis is not well defined. Macrophages, unlike monocytes, appear to be resistant to Fas-induced apoptosis [28]. The finding that intraperitoneal injection of anti-Fas antibodies in mice leads to profound necrosis of hepatocytes, but not lung parenchymal cells, suggests that most lung parenchymal cells are not in a Fas-sensitive state [29]. More recently, intratracheal administration of anti-Fas antibody was shown to induce apoptosis rapidly in bronchiolar epithelial cells, and ultimately to lead to the development of interstitial fibrosis [30]. These results are compatible with the possibility that bronchiolar epithelial cells, which express Fas, are in a Fas-sensitive state, although other mechanisms (*e.g.* Fas-induced production of cytokines [27, 30]) may also be involved. Many, but not all, lung carcinomas also expressed Fas. We found that the A549 lung carcinoma cell line expressed Fas, and was sensitive to Fas-induced apoptosis (data not shown), as has been described for other carcinoma cell lines [25]. Further studies are required to define the effects of Fas-signalling on the different lung cell populations identified here. Both macrophages and activated T-lymphocytes express Fas-ligand and are, therefore, capable of signaling through the Fas receptor [31].

In conclusion, three members of the tumour necrosis factor receptor superfamily of receptors (4-1BB, LTβ-R, and Fas) were found to have characteristic and only partially overlapping patterns of expression in the lung. The identification of the cell types expressing these receptors should facilitate a further evaluation of their role in lung homeostasis and pathology.

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