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Apoptosis Review Series

The central role of Fas–ligand cell signaling in inflammatory lung diseases

G. A. DosReis [a] *, Valeria M. Borges [b], W. A. Zin [a]

[a] Instituto de Biofísica Carlos Chagas Filho, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil
[b] Centro de Pesquisas Gonçalo Moniz, FIOCRUZ, Salvador, BA, Brazil

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Abstract

Following inflammation and injury in the lung, loss of epithelial cell precursors could determine the balance between tissue regeneration and fibrosis. This review discusses evidence that proapoptotic Fas-Fas ligand (FasL) signaling plays a central role in pulmonary inflammation, injury and fibrosis. FasL signaling induces inflammatory apoptosis in epithelial cells and alveolar macrophages, with concomitant IL-1β and chemokine release, leading to neutrophil infiltration. FasL signaling plays a critical role in models of acute lung injury, idiopathic pulmonary fibrosis and silicosis; blockade of Fas-FasL interactions either prevents or attenuates pulmonary inflammation and fibrosis. Serologic and immunohistochemical studies in patients support a major pathogenic role of Fas and FasL molecules in inflammatory lung diseases. Identification of the pathogenic role of FasL could facilitate the discovery of more effective treatments for currently untreatable inflammatory lung diseases.

Keywords: Fas ligand - apoptosis - inflammation - neutrophil - macrophage - phagocytosis - fibrosis - lung - injury - silicosis

* Correspondence to: George A. DOSREIS, M.D.
Instituto de Biofísica Carlos Chagas Filho,
Universidade Federal do Rio de Janeiro, CCS, Bloco G
Ilha do Fundão, Rio de Janeiro, RJ, 21949-900, Brasil.
Tel: +55-21-2562-6522, Fax: +55-21-2280-8193
E-mail: gdosreis@biof.ufrj.br

Introduction

The lung consists of a series of branching tubules ending as highly vascularized alveolar sacs. The alveolar epithelium is a flat cell monolayer in contact with particles and microbes from the environment. Integrity of the alveolar epithelium depends on multiple defense mechanisms, including: mucociliary clearance, secretion of antimicrobial molecules, and alveolar macrophages. When stimulated, phagocytic and epithelial cells secrete chemokines and cytokines that recruit neutrophils and lymphocytes. The combined effect of these mechanims is to recognize and remove insulting agents. Removal of particles and microorganisms is usually accomplished without compromising tissue architecture. On the other hand, extensive tissue injury can lead to defective tissue repair and remodeling [1]. Apoptosis is a tightly regulated form of programmed cell death, essential to embryogenesis, tissue renewal, and immune regulation. However, occurrence of either exacerbated or deficient apoptosis is associated with disease [2]. In the lung, apoptosis is a common cellular reaction to insult that can result in epithelial cell loss and fibrosis. The Fas death receptor triggers apoptosis when engaged by Fas ligand (FasL) [3]. Both Fas and FasL are expressed by lymphoid and non-lymphoid tissues [3], including adult lung [4]. Recent studies have demonstrated that Fas-FasL interactions play a central role in apoptosis in the lung, leading to pulmonary inflammation, injury and fibrosis [5]. FasL-mediated apoptosis could provide new targets for treatment of pulmonary diseases.

Proinflammatory gene expression induced by FasL

Fas (CD95) is a 48-kDa, type I transmembrane protein belonging to the TNF Receptor family [3]. FasL (CD178) is a 40-kDa, type II homotrimeric transmembrane protein that belongs to the TNF gene family [3]. FasL undergoes metalloproteinase mediated cleavage generating a soluble form (sFasL). Fas is constitutively expressed in several tissues, including brochiolar and alveolar epithelial cells. On the other hand, constitutive expression of FasL is restricted to immuneprivileged sites, such as the eyes and testis [6]. Immune privilege is, at least in part, caused by induction of apoptosis on recruited Fas-expressing inflammatory cells [6]. In addition, FasL expression can be induced in a tightly controlled manner in lymphoid and non-lymphoid cells [7]. FasL expression is induced by several transcription factors, such as NFAT, NF-kB, AP-1, SP-1, Egr, IRFs, and forkhead transcriptional regulator [7]. FasL plays a central role in apoptosis induced by a variety of chemical and physical insults. Activation of NF-kB and AP-1 transcription factors are crucially involved in FasL expression induced by DNA-damaging agents, such as genotoxic drugs and ultraviolet (UV) radiation [8]. Cell death induced by UV radiation can be blocked by a FasL neutralizing antibody [9]. Furthermore, cell death induced by reactive oxygen intermediates (ROI) depends on FasL expression mediated by redox sensitive activation of NF-kB [10]. Exposure to silica particles causes persistent inflammation and fibrosis in the lung. Generation of oxidant ROI and reactive nitrogen species by silica have been implicated in induction of cell death and inflammation in the lung, through NF-kB activation and expression of FasL [11]. Mechanical stress induced by blood flow on exposed vascular smooth muscle cells (VSMC) leads to FasL expression and apoptosis mediated by FasL [12].

Apoptosis is initiated when Fas is engaged by FasL trimers (Fig. 1). The adaptor protein Fas-associated death domain (FADD) associates with multimerized Fas, and recruits procaspase-8 units to form the death-inducing signaling complex (DISC) [13]. Procaspase-8 units activate each other, and active caspase-8 induces activation of effector caspases -3, -6, and -7. Effector caspases, in turn, induce cleavage of structural and regulatory cellular proteins, and DNA fragmentation, characteristic of apoptosis (Fig. 1). It was generally believed that apoptosis does not induce an inflammatory response. In fact, Fas-mediated apoptosis of effector T lymphocytes terminates immune responses and is antiinflammatory [3, 6]. However, recent studies demonstrated that Fas-mediated apoptosis can result in inflammation [14, 15]. Signaling through the Fas pathway is regulated by the caspase-8 homolog cellular FLICE-like inhibitory protein (cFLIP). There are two isoforms of cFLIP: long ($cFLIP_L$) and short ($cFLIP_S$). Binding of $cFLIP_S$ to the DISC inactivates caspase-8 [13]. However,

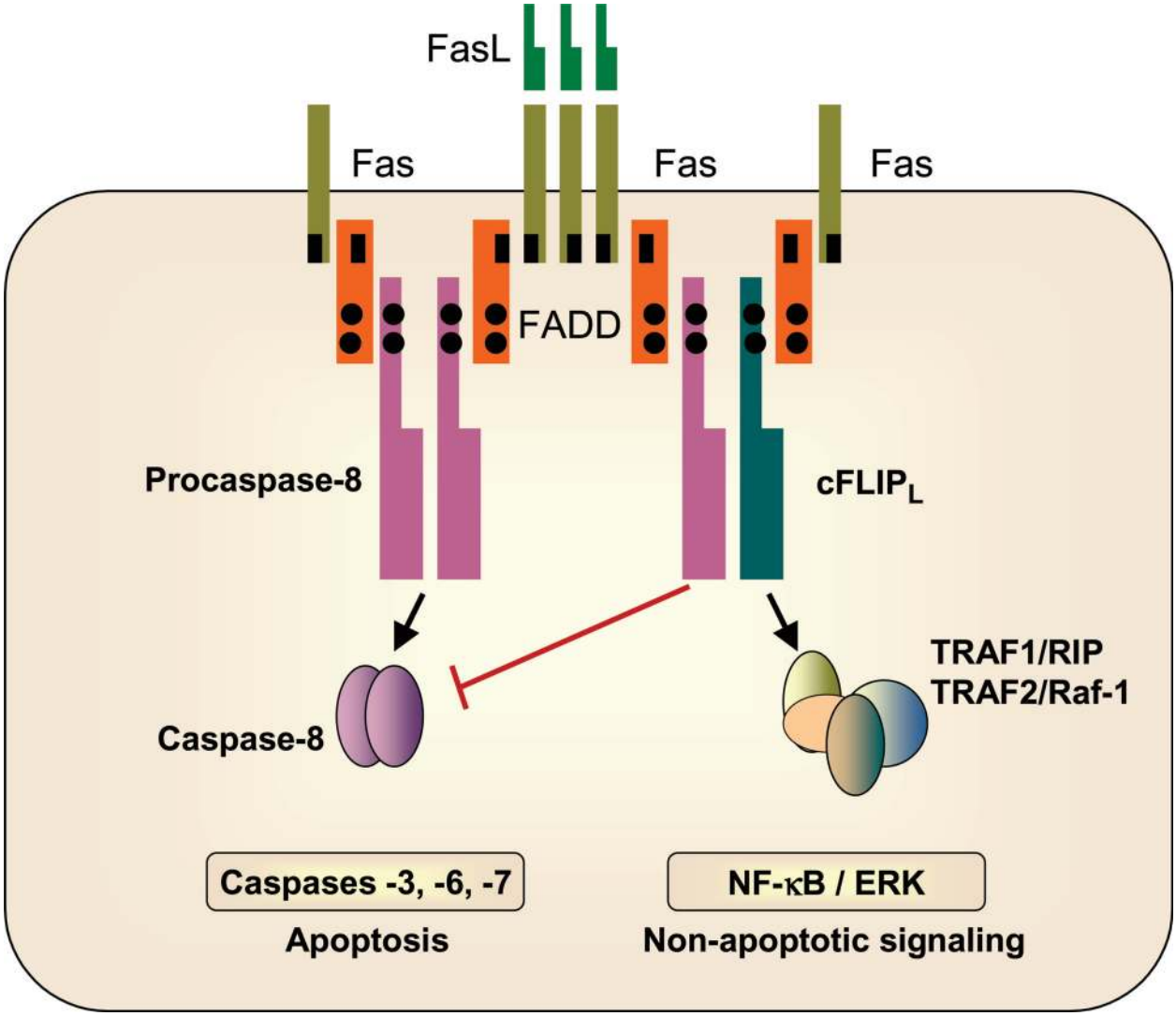

Fig. 1 Regulation of Fas death-receptor gene expression by FLIP. Black boxes indicate Death Domain homology pairing; black dots indicate DED homology pairing.

binding of cFLIP$_L$ to the DISC plays an additional role by shifting the Fas pathway to non-apoptotic signaling [13]. As shown in Figure 1, cFLIP$_L$ associates with TRAF1 and TRAF2, and with the kinases RIP and Raf-1, resulting in activation of ERK pathway and NF-kB [13]. Non-apoptotic signaling pathways induced by cFLIP$_L$ could potentially explain proinflammatory effects of Fas engagement (Fig. 1). Instead of inducing apoptosis, sFasL increases proliferation of activated human T cells [16]. Under these conditions, FasL recruits cFLIP to the DISC, and activates NF-kB and AP-1 transcription factors [16]. FLIP interacts with TRAF1/RIP and with TRAF2/Raf-1, leading to NF-kB and ERK/AP-1 activation, respectively. Both pathways are required for increased IL-2 production and T-cell proliferation [16]. Fas ligation also induces IL-8 secretion associated with NF-kB activation in bronchiolar epithelial cells [17]. Moreover, Fas ligation induces TNF-α and IL-8 secretion associated with nuclear translocation of NF-kB in human macrophages, in the absence of cell death [18]. Injection of low doses of anti-Fas antibody *in vivo* leads to hepatic inflammation due to secretion of chemokines KC and MIP-2 [19]. Interestingly, AP-1, and not NF-kB translocation was detected in liver

[19]. Soluble FasL activates ERK1/2 and NF-kB in serum-starved fibroblasts, leading to IL-6 and IL-8 secretion in the complete absence of cell death [20]. Serum starvation reduced caspase-8, but not cFLIP expression, and treatment with an antisense caspase-8 construct increased NF-kB activation induced by sFasL. This study suggests that a change in the ratio of cFLIP and caspase-8 shifts intracellular signaling to the Fas-activated NF-kB pathway [20]. However, molecular details underlying proinflammatory signaling induced by FasL are not completely understood.

In general, FasL induced inflammation is characterized by neutrophil infiltration. Forced FasL expression on tumor cell lines induced their rejection by infiltrating neutrophils [14]. Intraperitoneal injection of FasL vesicles induced apoptosis in Fas-expressing, Mac-1^{hi} resident macrophages, resulting in secretion of IL-1β, chemokines MIP-1α, MIP-1β and MIP-2, followed by neutrophil extravasation [15]. Fas engagement also induced a proinflammatory response on VSMC, resulting in IL-1, IL-8 and MCP-1 secretion [21]. However, the response elicited by stimulated VSMC was massive monocyte, and not neutrophil infiltration into rat carotid arteries, a phenomenon potentially relevant for pathogenesis of atherosclerotic lesions [21]. The biochemical pathways involved in chemokine production are being characterized.

Fas-mediated apoptosis in acute lung injury

Recent work indicated that Fas-FasL death pathway is involved in pathogenesis of inflammatory lung conditions of clinical and epidemiological relevance [5]. One such condition is acute lung injury (ALI) / acute respiratory distress syndrome (ARDS). ALI is characterized by hypoxemia due to noncardiogenic pulmonary edema, and it can progress to ARDS, a life-threatening condition with 40% mortality that complicates sepsis, trauma, fat embolism, multiple transfusions, and coronavirus infection (SARS, for severe acute respiratory syndrome) [22]. In the lung, inflammation followed by loss of epithelial precursors beyond a safeguard threshold, leads to increased mesenchymal repair and remodeling [1]. Bronchiolar and alveolar epithelial cells express Fas. Administration of inhaled agonist anti-Fas antibody induced alveolar cell apoptosis, increased production of the fibrogenic cytokine TGF-β, and fibrosis after 1-2 weeks in mice [23]. Anti-Fas induced epithelial cell apoptosis, an increase in alveolar protein content, neutrophil extravasation and cytokine/chemokine secretion in the lung, as early as 24 hours after treatment [24]. In these studies [23, 24], apoptosis was identified *in situ* by cytochemical detection of nick-end labeling of fragmented DNA (TUNEL assay, for Terminal deoxynucleotidyl dUTP nick-end labeling). Electron microscopy analysis of lung sections demonstrated apoptosis of type II pneumocytes, alveolar septal thickening, hemorrhage, and neutrophilic infiltrates [23, 24]. These results indicate that Fas engagement in airway or alveolar epithelial cells triggers pulmonary injury and neutrophilic inflammation. The major gram-negative bacterial endotoxin lipopolysaccharide (LPS) plays a central role in ARDS secondary to sepsis [22]. Injection of LPS reproduces major features of ARDS in experimental models [25]. Furthermore, inhalation of *E. coli* LPS induces rapid alveolar leakage of albumin, and an inflammatory response characterized by neutrophil extravasation [25]. Instillation of an antagonist anti-Fas antibody attenuated apoptosis of alveolar epithelial cells and neutrophils, as measured by TUNEL assay, and reduced inflammation in this model [26]. Mice carrying the *lpr* (for lymphoproliferation) and *gld* (for generalized lymphoproliferative disease) mutations are defective in the function of Fas and FasL molecules, respectively [3]. Due to a defect in Fas-mediated lymphocyte apoptosis, these mice express a lymphoproliferative disease and a lupus-like autoimmune syndrome characterized by production of autoantibodies against DNA and nuclear antigens, and glomerulonephritis caused by deposition of immune complexes [3]. Mutant *lpr* and *gld* mice showed an attenuated neutrophilic inflammation in the lung following LPS administration [27]. These results confirmed an important proinflammatory role for Fas-FasL interactions in ALI. However, it should be pointed out that the initial events leading to Fas-mediated apoptosis of epithelial cells and neutrophil extravasation remain unclear. The findings above correlated well with data obtained from human patients with ALI/ARDS. Compared with patients without pulmonary disease, patients with

ALI/ARDS had an increase in levels of soluble Fas and FasL in pulmonary edema fluid, and an increase in cellular expression of Fas and FasL in epithelial alveolar and in inflammatory cells in the air spaces, as measured by immunofluorescence staining [28]. Using *in situ* detection of apoptosis by TUNEL assay and immunohistochemistry, it was demonstrated that patients with ALI/ARDS showed increased apoptosis of alveolar epithelial and inflammatory cells, which colocalized with apoptosis markers Bax, p53 and activated caspase-3 [28].

Fas signaling in idiopathic pulmonary fibrosis

Idiopathic Pulmonary Fibrosis (IPF) is a progressive and fatal interstitial pneumonitis of unknown cause. It is characterized by sequential episodes of ALI with subsequent scarring, leading ultimately to death from respiratory failure. Currently, there is intense research focusing possible pathogenic mechanisms of IPF. Using immunofluorescence staining and TUNEL assay, increased expression of Fas and FasL on bronchiolar and alveolar cells, as well as on infiltrating neutrophils and lymphocytes were demonstrated in patients with IPF, and expression of these markers correlated with increased apoptosis [29]. The cytotoxic drug bleomycin induces DNA damage and apoptosis and, when applied intratracheally, it induces acute pulmonary toxicity that progresses to fibrosis. Despite some limitations, bleomycin-induced lung inflammation is regarded as an experimental model for IPF. Intratracheal administration of either an antagonist anti-Fas antibody, or an antagonist Fas construct reduced fibrosis *in vivo* in this model [30]. Furthermore, Fas-deficient *lpr* and FasL-deficient *gld* mice were resistant to induction of fibrosis [30]. These results suggest that exacerbated and sustained Fas-FasL interactions are involved in epithelial cell loss and pulmonary remodeling. The pathogenic role of apoptosis in this model was confirmed by the finding that *in vivo* treatment with the pan-caspase inhibitor zVAD-fmk decreased apoptosis, as measured by TUNEL assay, and reduced inflammation and lung fibrosis in bleomycin treated mice [31]. Recently, an experimental model was created that allowed conditional ablation of Fas on T cells, but not on non-lymphoid cells, leading to overexpression of FasL on activated T cells [32]. These mice developed a fatal wasting syndrome caused by massive leukocyte infiltration in the lungs, and pulmonary fibrosis that strikingly resembled IPF [32], where an important component of T-lymphocyte infiltration exists [30]. These results support a central pathogenic role of FasL and activated T cells in IPF. However, the mechanisms leading to both priming and activation of T cells, and to the loss of Fas-dependent regulation of effector T cells remain unknown.

FasL triggers pulmonary silicosis

Occupational inhalation of silica particles, as happens with workers of coal mining, sandblasting and quarrying, leads to silicosis, a granulomatous interstitial pneumonitis that causes respiratory failure due to a progressive fibrotic process. It is generally believed that macrophages activated by silica particles attract neutrophils, which in turn, promote alveolitis and tissue destruction [33]. Interestingly, ingestion of silica particles by alveolar macrophages induces macrophage apoptosis [34] and generation of ROI, which are directly involved in inflammation and fibrosis [11]. The roles of alveolar macrophages and FasL signaling were investigated in experimental silicosis [35]. Compared with controls, FasL-deficient *gld* mice were completely resistant to development of silicosis [35]. Experiments with bone marrow radiation chimeras showed that hematopoietic, and not epithelial cells were required for silicosis induction. FasL-deficient mice were unable to recruit neutrophils to the lungs, although they attracted large numbers of macrophages. This finding suggests that macrophages initiate, but are not the effectors of disease. In fact, local adoptive transfer of FasL-sufficient alveolar macrophages attracted neutrophils to the lungs and restored silicosis in *gld* mice [35]. Exposure to silica induced FasL expression and apoptosis in wild-type, but not in *gld* macrophages, and FasL expression required generation of ROI by silica [35]. These results suggest that resistance of *gld* mice is due to the failure in macrophage apoptosis, which is required to recruit neutrophils.

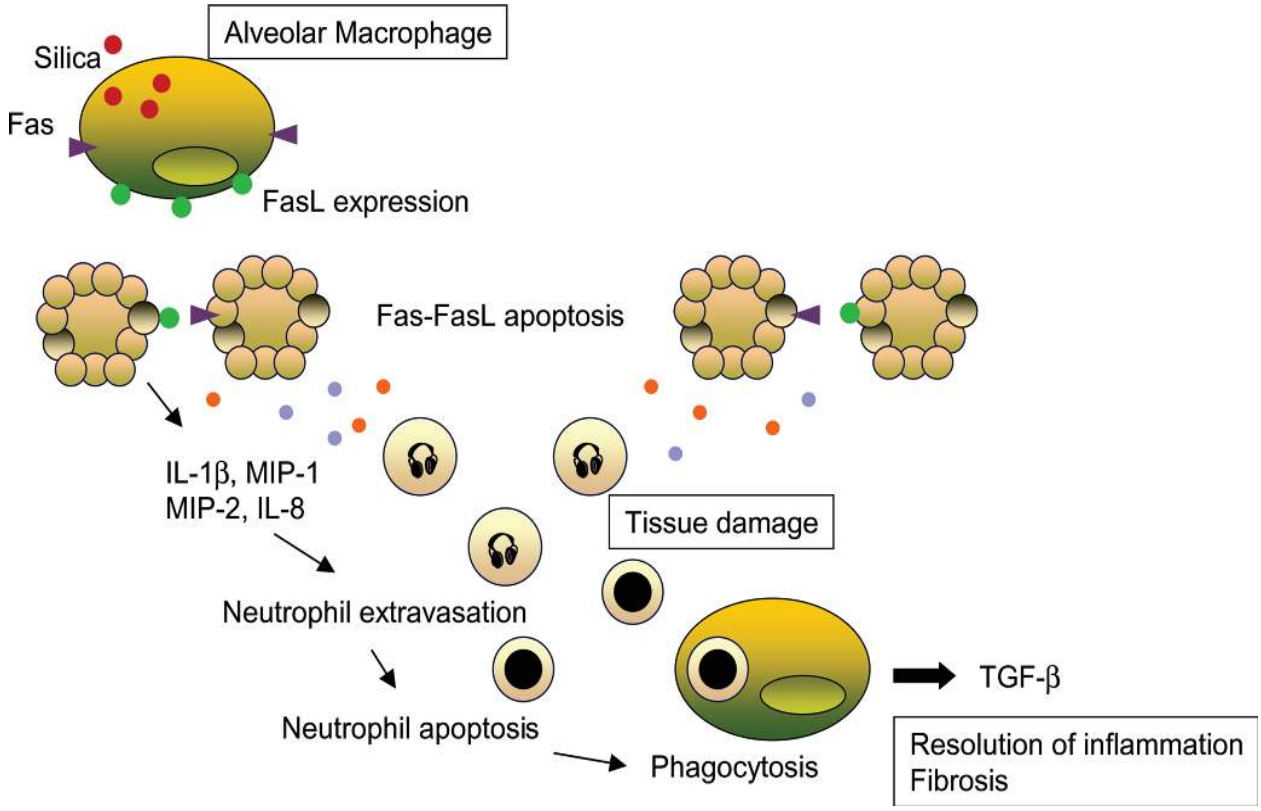

Fig. 2 Molecular and cellular mechanisms of pulmonary inflammation induced by silica.

Treatment with an antagonist anti-FasL antibody completely prevented TNF-α production and development of silicosis *in vivo* [35]. By immunohistochemistry and TUNEL assay, there was increased apoptosis of inflammatory cells that colocalized with increased expression of FasL in silicotic lung [35, 36]. Apoptosis played an important role in silicosis, since treatment with the pancaspase inhibitor zVAD-fmk attenuated neutrophil inflammation and lung fibrosis [36]. These results demonstrated that FasL expression in macrophages induces apoptosis and attracts neutrophils to the lung parenchyma in the course of acute silicosis (Fig. 2). Neutrophilic inflammation can be explained by the proinflammatory role of FasL mediated macrophage apoptosis. Release of cytokines and chemokines attracts neutrophils, initiating tissue inflammation and injury through neutrophil release of toxic proteases, ROI and cytokines (Fig. 2). The major neutrophil chemotactic factor released by macrophages is unknown. However, studies in mice deficient in IL-1 receptor demonstrated that IL-1 is required for neutrophil infiltration elicited by FasL-expressing cells [37]. Therefore, IL-1β is a likely candidate. In agreement with a central role of IL-1β, pulmonary silicosis is attenuated in animals deficient in IL-1β [38]. There was increased T-lymphocyte apoptosis associated with silica-ladden macrophages in thoracic lymph nodes draining silicotic lungs [36]. This finding might be relevant for pathogenesis. Patients with silicosis have increased incidence of autoimmune diseases [39] and pulmonary tuberculosis [40]. Recent evidence indicates that phagocytic clearance of apoptotic cells primes the immune system against autoantigens, and plays an immunoregulatory role [41]. Macrophages that have ingested apoptotic lymphocytes produce TGF-β and become inactivated, allowing exacerbated growth of intracellular pathogens [42]. Therefore, lymph nodes draining silica might constitute a site for priming against self antigens derived from apoptotic cells, and a permissive environment for establishment of microbial infection.

Resolution of inflammation and clearance of neutrophils

In the lung, the extent of loss of epithelial precursors could determine the balance between tissue regeneration and fibrosis. The pathogenic role of FasL can be explained by the finding that FasL signaling leads to apoptosis, IL-1β release, and neutrophil infiltration (Fig. 2). The importance of IL-1β is underscored by the finding that transient transgenic expression of IL-1β leads to ALI, chronic repair and remodeling [43]. The common finding of fibrosis following inflammation could be explained by the roles TGF-β plays in regulation of pulmonary repair and remodeling. Under inflammatory conditions, epithelial cells activate latent TGF-β through the epithelial integrin $\alpha_V\beta_6$ [44]. However, TGF-β plays a dual role in the lung parenchyma, both reducing inflammation and promoting fibrogenesis. This notion was confirmed in animals deficient in $\alpha_V\beta_6$. Following treatment with bleomycin, animals showed increased pulmonary inflammation, but dramatically reduced fibrosis, compared to non-deficient animals [44]. Resolution of inflammation also depends on turnover of tissue-infiltrating neutrophils (Fig. 2). Neutrophils die by apoptosis and are ingested by macrophages, leading to TGF-β secretion [45]. The consequence is that phagocytic removal of senescent neutrophils reduces inflammation and promotes fibrogenesis through TGF-β secretion (Fig. 2). However, recent studies suggest that phagocytic clearance of neutrophils could be more complex than previously anticipated. Compared to other macrophage types, alveolar macrophages are deficient in removing apoptotic cells [46]. Therefore, a proportion of lung-infiltrating neutrophils could undergo secondary necrosis before being ingested. In agreement, neutrophils undergoing necrosis have been described at late stages of ALI [47]. Since necrotic neutrophils release large amounts of toxic enzymes and other inflammatory mediators, deficient clearance by lung macrophages could explain the extreme toxicity of neutrophils for the lung parenchyma. Another study demonstrated that phagocytic clearance of tissue-infiltrating neutrophils leads to either TGF-β or TNF-α production by macrophages, depending on host genotype [48]. Release of neutrophil elastase appears to be required for macrophage activation and TNF-α production [48]. These results suggest that innate genetic differences between individuals regulate the functional outcome of neutrophil clearance in the tissues.

Summary and conclusion

This review provided evidence that the Fas-FasL molecular pathway of cell death plays a central role in pulmonary diseases characterized by inflammation, abnormal tissue repair and fibrosis. While Fas is constitutively expressed in the adult lung, FasL expression can be induced in epithelial cells and alveolar macrophages by a variety of chemical and infectious insults, leading to apoptosis. Although molecular mechanisms of signaling are not completely understood, apoptosis of both lung epithelial cells and macrophages is proinflammatory. Besides triggering caspase-8 activation and apoptosis, Fas engagement also induces secretion of IL-1β, MIP-1α, MIP-1β, MIP-2 and IL-8, resulting in neutrophil extravasation. An alternative Fas signaling cascade involves activation of c-$FLIP_L$, TRAF1, TRAF2, and kinases RIP and Raf-1, and results in NF-kB and ERK/AP-1 activation (Fig. 1). This alternative signaling pathway appears to be involved in non-apoptotic, proinflammatory FasL signaling. FasL signaling is induced by ingestion of silica particles by alveolar macrophages, leading to macrophage apoptosis, recruitment of neutrophils and acute silicosis (Fig. 2). A similar proinflammatory pathway appears to be induced following apoptosis of alveolar epithelial cells. Identification of the pathogenic roles of FasL offers new therapeutic opportunities for largely untreatable lung diseases, such as ALI/ARDS, IPF and silicosis. For example, inflammation and fibrosis induced by bleomycin and silica in mice, were attenuated by treatment with the tripeptide caspase inhibitor zVAD-fmk [31, 36]. These results indicate that apoptosis generates inflammation in the lung, and suggest that caspases are promising targets for drug development. In addition, cDNA expression microarrays could help identifying new genes involved in common pulmonary diseases [44]. Identification of target genes could facilitate the development of more effective drugs for treating lung diseases.

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