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Comprehensive lung injury pathology induced by mTOR inhibitors

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Abstract
Interstitial lung disease is a rare side effect of temsirolimus treatment in renal cancer patients. Pulmonary fibrosis is characterised by the accumulation of extracellular matrix collagen, fibroblast proliferation and migration, and loss of alveolar gas exchange units. Previous studies of pulmonary fibrosis have mainly focused on the fibro-proliferative process in the lungs. However, the molecular mechanism by which sirolimus promotes lung fibrosis remains elusive. Here, we propose an overall cascade hypothesis of interstitial lung diseases that represents a common, partly underlying synergism among them as well as the lung pathogenesis side effects of mammalian target of rapamycin inhibitors.

Keywords
Interstitial lung disease; Pulmonary fibrosis; Sirolimus; ROS; Rapamycin

The mammalian target of rapamycin (mTOR)

Rapamycin and its derivatives are immunosuppressor macrolides that block mammalian target of rapamycin (mTOR) functions and yield anti-proliferative activity in a variety of malignancies. Rapamycin analogues currently selected for clinical development are CCI-779 (Temsirolimus), RAD001 (Everolimus) and AP23573 (Fig. 1).

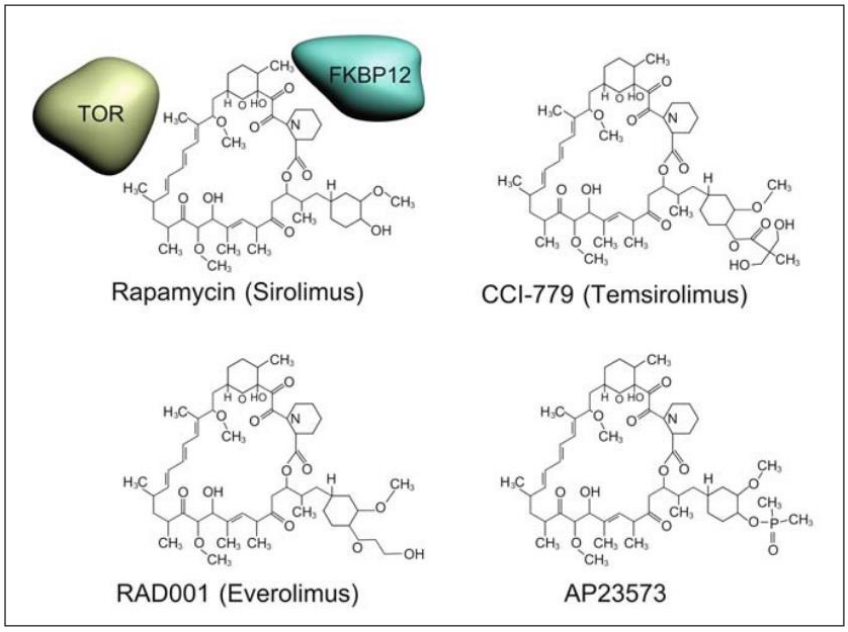

Fig. 1. Molecular structure of rapamycin and some of its analogs currently selected for clinical development. For the rapamycin, it is schematized the mode of binding to TOR (target of rapamycin) and FKBP12, its targets

mTOR was identified as the kinase target linked to the cellular protein FKBP12 (FK506-binding protein). It was therefore also named FKBP-RAP associated protein (FRAP), RAP FKBP12 target (RAFT1) and RAP target (RAPT1). In the mammalian related proteins, mTOR are required for signalling translational initiation and therefore cell cycle progression from the G0/G1 to S phase. In humans, mTOR primarily appears to be a nutrient-sensing protein: mTOR is constitutively activated in the presence of growth factor and nutrients and acts as a master switch of cellular catabolism and anabolism.

The TOR proteins are members of the phosphoinositide 3-kinase-related kinase (PIKK) family, whose members (ATM, ATR, DNA-PK, hSMG1, mTOR and TRAPP in mammalian cells) transmit signals related to cell growth, proliferative and stress response [1]. Like the other PIKKs, mTOR bears a catalytic domain with significant homology to the lipid kinase phosphatidylinositol 3-kinase (PI3K); however, in spite of the sequence homology to lipid kinases, mTORs function exclusively as protein serine-threonine kinases.

The clinically active rapalogues inhibit mTOR kinase through the immunophilin FKBP-12 (FK506 binding protein-12), forming a FKBP12-rapamycin complex that binds to mTOR through the FRB domain [2]. The FRB domain is found only in the TOR proteins. The selectivity of these compounds for mTOR is remarkable: the drug directly attacks only one subpopulation of mTOR proteins residing in multiprotein complexes, termed mTORC1. Additional complexes, mTORC2, hold mTOR in a form that cannot be recognised or inhibited by FKBP12-rapamycin [3, 4].

Thereby, the rapamycin-FKBP12 complex can inhibit mTOR [5], preventing further phosphorylation of P70S6K, 4E-BP1 and, indirectly, other proteins involved in transcription and translation and cell cycle control. In addition to its immunosuppressive properties, sirolimus has been shown to exert anticancer effects through different mechanisms.

Sirolimus and its derivatives constitute a family of anti-neoplastic drugs that possess acceptable toxicity profiles.

The main toxicity observed with rapamycin (mTOR) inhibitors has been dose-dependent and consists primarily of hypercholesterolaemia, hypertriglyceridaemia and thrombocytopenia. Associated pulmonary toxicity in the form of diffuse interstitial pneumonia has been described with sirolimus and temsirolimus [6–11]. While extensive data have been published on sirolimus-induced lung toxicity, limited information about the pulmonary effects of temsirolimus exists in the literature [12, 13]. No pathogenic mechanisms have been studied as potential causes of this visceral toxicity with temsirolimus.

Therefore, it is important to uncover the mechanistic role of factors that may induce pulmonary toxicity in sirolimus treatment. A cell-mediated autoimmune response has been suggested [8, 12], as well as a T-cell-mediated delayed type hypersensitivity mechanism [8, 10], through immunogenicity of sirolimus when it is binding to plasma proteins. Sirolimus becomes immunogenic, implicating T-cell recognition of the processed antigen complex, release of cytokines, and recruitment and activation of macrophages and other inflammatory cells.

Usually, in the immunocompetent host, interstitial lung disease can be recognised as a heterogeneous group, as a syndrome with the following common clinical features: exertional dyspnoea; bilateral diffuse infiltrates on chest radiographs; physiological abnormalities with a restrictive lung defect, decreased diffusing capacity (DLCO) and abnormal alveolar-arterial oxygen gradient (PaO_2–PaO_2) at rest or with exertion; absence of pulmonary infection and neoplasm; and histopathology with varying degrees of fibrosis and inflammation, with or without evidence of granulomatous or secondary vascular changes in the pulmonary parenchyma.

Temsirolimus-induced lung toxicity was first communicated after a randomised phase II study [6]. Out of 111 patients, 6% were reported to have possible non-specific pneumonitis. Additional evidence of possible temsirolimus-associated lung toxicity has been reported in 3 phase II studies [14–16].

The rare occurrences of sirolimus-associated pulmonary toxicity are in the form of diffuse interstitial pneumonitis. In contrast, patients that develop pulmonary abnormalities are more frequent, and these abnormalities were categorised into two different radiological patterns: ground glass opacities (with or without diffuse interstitial disease) and lung parenchymal consolidation [7].

Bronchoalveolar fluid analysis and lung biopsy, in selected case reports, revealed several distinct histologic features, including lymphocytic alveolitis, lymphocytic interstitial pneumonitis, bronchoalveolar obliterans organising pneumonia, focal fibrosis, pulmonary alveolar haemorrhage or a combination of these [10].

Causes of interstitial lung disease

Among different aetiologies, several drugs are well known to cause interstitial pulmonary disease. These include chemotherapeutic and cytotoxic agents [17–22]: methotrexate, bleomycin, mitomycin, nitrosureas, azathioprine, nitrogen mustard, procarbazine, busulfan, cyclophosphamide, melphalan, chlorambucil, non-steroidal anti-inflammatory agents, antibiotics, narcotic analgesics, antiarrhythmics and tricyclic antidepressants.

The list continues to increase as new antineoplastic agents and regimens are constantly being incorporated in treatments for cancer patients (Table 1). These novel agents have new mechanisms of action such as tyrosine kinase inhibitors or old agents with new indications like thalidomide.

Table 1. Pulmonary complications generated by different chemotherapeutic agents

Chemotherapeutic agents	Pulmonary complications
Erlotinib	Acute pneumonitis, ARDS
Etoposide	Acute pneumonitis, diffuse alveolar damage
Everolimus	Acute pneumonitis
Gefitinib	Acute pneumonitis, diffuse alveolar damage, diffuse alveolar haemorrhage, pulmonary fibrosis
Gemcitabine	Capillary lead syndrome/pulmonary oedema, ARDS, diffuse alveolar damage and haemorrhage, bronchospasm
Ifosfamide	Interstitial pneumonitis
Imatinib	Acute pneumonitis, pleural effusion, fluid retention and pulmonary oedema
Oxiplatin	Pulmonary fibrosis, respiratory failure, eosinophilic pneumonia
Taxanes	Acute pneumonitis, pleural effusion
Temozolamide	Acute pneumonitis
Temsirolimus	Acute pneumonitis
Thalidomide	Pulmonary embolism, organising pneumonia, acute pneumonitis, pleural effusion
Trastuzumab	Acute lung injury, acute pneumonitis, organising pneumonia, bronchospasm

Histological features

Interstitial lung disease is sometimes also known as interstitial pulmonary fibrosis. The terms interstitial lung disease, pulmonary fibrosis and interstitial pulmonary fibrosis are often used to describe the same condition.

Lung fibrosis is the common endpoint of a heterogeneous group of pathological entities termed *interstitial lung diseases*, which are characterised by chronic inflammation and progressive fibrosis of the pulmonary interstitium.

Interstitial lung disease is a generic term that includes a variety of chronic lung disorders such as *idiopathic lung fibrosis* (IPF), *desquamative interstitial pneumonia* (DIP), *respiratory bronchiolitis interstitial lung disease* (RB), *lymphoid interstitial pneumonia* (LIP), *cryptogenic organising pneumonia* (COP), *diffuse alveolar damage* (DAD) or *acute interstitial pneumonia* (AIP), and *non-specific interstitial pneumonia* (NSIP). The lung is affected in three ways: first, the lung tissue is damaged in some known or unknown way; second, the walls of the air sacs in the lung became inflamed; finally, scarring or fibrosis begins in the interstitium (or tissue between the air sacs) and the lung becomes stiff.

The term interstitium refers to the microscopic anatomic space bounded by the basement membranes of epithelial and endothelial cells. Within this interstitial space, fibroblast-like cells (mesenchymal and connective tissue cells) and extracellular matrix components (interstitial collagens, elastin, proteoglycans) are present. It is clear that the disease in not restricted to the interstitium, as it involves epithelial, endothelial and mesenchymal cells, macrophages and recruited inflammatory cells, secreted proteins and an aberration of matrix components within the alveolar walls. In addition, the disease process can be extended into the alveolar space, acini, bronchiolar lumen and bronchioles.

The bronchoalveolar lavage shows neutrophilic alveolitis. Alveolitis is the initial abnormality in the development of interstitial fibrosis: the accumulation of macrophages, neutrophils and lymphocytes within the lower respiratory tract damages alveolar structures.

The pathologic features of acute interstitial pneumonia include interstitial and intra-alveolar oedema, hyaline membrane formation and interstitial inflammation. Initially, the inrterstitium is expanded by oedema and inflammatory cells; this change is followed by loss of both type I pneumocyte epithelial cells and capillary endothelial cells. After the onset of the illness fibrosis becomes prominent, and hyperplastic type II pneumocyte cells line residual air spaces and the end-stage honeycomb lung are found. The type II pneumocyte cells proliferate to resurface the denuded epithelial basal lamina and they cover the collapsed portions, rather than relining an intact and expanded alveolus [23].

The fibrosis is considered to result mainly in the proliferation of fibroblasts within the interstitium, along with an imbalance in collagen synthesis and breakdown. Fibrosis develops when alveolar injury is sufficiently severe that repair by epithelial cells is impossible and is thought to result mainly from the accumulation of fibroblasts and collagen within the interstitium [24, 25].

Cytotoxic-induced lung injury begins with an explosive inflammatory response in the alveolar wall. In the after-math of tissue destruction, a fi bro-proliferative response ensues, leading to extensive intra-alveolar granulation tissue comprised mainly of fibroblasts and their connective tissue products. As inflammation resolves, a fibro-proliferative process dominates in which fibroblasts move through the damaged epithelium and begin to lay down connective tissue products that results in dense intra-alveolar granulation tissue and impaired gas exchange. In these cases thickening of alveolar septa results from combinations of spindle cells, lymphocytes and plasma cells within an oedematous stroma. Type II pneumocytes often exhibiting considerable cytologic abnormality, lining the residual air spaces, and hyaline membranes, lining the alveolar septa, are present. Also small clusters of eosinophilic material, likely representing remnants of hyaline membranes, are found within the interstitium. Obliteration of lung architecture by fibroblast proliferation is observed and it becomes difficult to distinguish air spaces from interstitium.

Pathogenesis

The pathogenesis of lung fibrosis is complex and is thought to involve a number of processes that lead to an altered alveolar environment and to an abnormal repair process with accumulated fibrosis [23, 26, 27] (Table 2, Fig. 2).

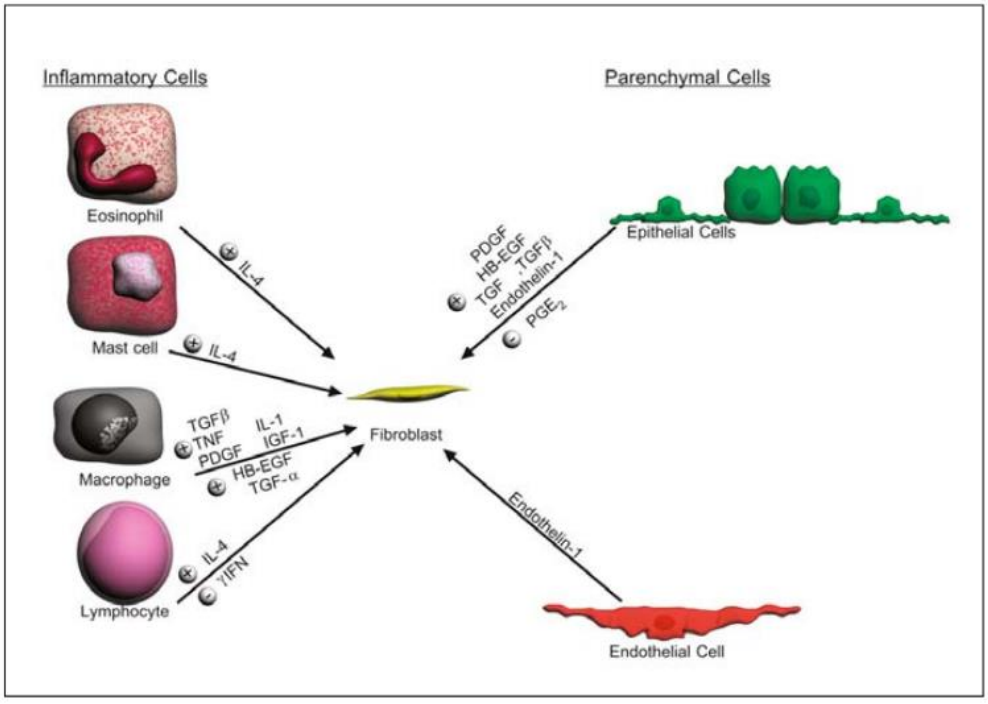

Fig. 2. Pathogenesis of pulmonary fibrosis involving the interaction of inflammatory cascade mediators (cytokine and growth factors) with parenchymal lung cells (epithelial and endothelial cells). Signals coming from inflammatory cells include stimulatory signals such as IL-4 (interleukin 4), IL-1 (interleukin 1), TGF-β and -α (tumor growth factor beta and alpha), TNF (tumor necrosis factor), PDGF (plateled-derived growth factor), IGF-1 (insulin-like growth factor 1), and HB-EGF (Heparin-binding EGF-like growth factor), and inhibitory signals such as IFN-γ (interferon-gamma). Signals from epithelial cells include positive signals such as PDGF, HB-EGF, TGF, and endothelin-1, and inhibitory signals such as PGE_2 (prostaglandin E2). From endothelial cells, it is sent the signal of endothelin 1

Table 2. Pathogenesis of the interstitial lung thickening

Partial or complete collapse of alveoli and permanent apposition of their walls
Influence with normal re-epithelisation
Incorporation of intra-alveolar exudates into the alveolar walls
Increased apposition of fibroblast, macrophages, lymphocytes and plasma cells in the interstitium
Greater deposition numbers of collagen and elastic fibres than normal

A large part of research in the area has focused on the pathogenesis of interstitial pulmonary disease. Characteristic features include fibrotic lesions with inflammatory cell infiltrates.

Inflammation can originate from processes of acute/chronic injury or irritation. The inflammatory response leads to lung infiltration by neutrophils (myeloperoxidase activity in the bronchoalveolar lavage total and differential count) and recruitment of mast cells and leukocytes to the site of the damage with a subsequent release of free radicals, including reactive oxygen species (ROS), lung myeloperoxidases, transforming growth factor (TGF)-β, inducible nitric oxide synthase (iNOS), nitrotyrosine and poly (ADP-ribose) polymerase (PARP). These free radicals are known to damage macromolecules, including lipids and DNA [28, 29]. In addition to the cellular and genomic damage that occurs as part of free-radical activity and other by-products, the increase of eicosanoid 5-lipoxygenase (5-LO) and decrease of prostaglandin E_2 (PGE2) enhance fibroblast proliferation. The rate and magnitude of these processes are regulated by profibrotic cytokine diffusible factors such as platelet-derived growth factor (PDGF), IGF-1 and TGF-β, and by alveolar type II epithelial cell migration and proliferation, which cover the repaired basement membrane, closing the lung fibrosis process.

Interstitial pulmonary fibrosis results from three super-imposed processes of immune inflammation, tissue injury and attempted repair/fibrosis (Fig. 3).

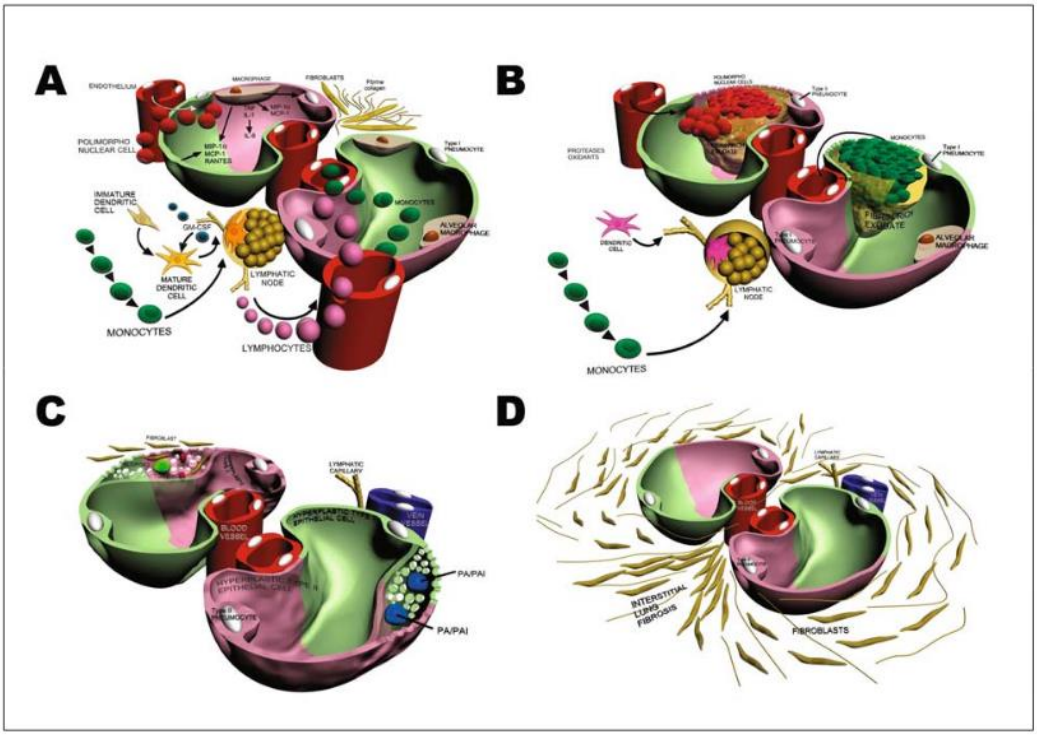

Fig. 3. Pathogenesis of idiopathic lung fibrosis results from the superimposed processes of immune inflammation, tissue injury, and attempted repair. **A**. Immune response. **B**. Epithelial cell injury. **C**. Repair of the damaged alveoli. **D**. Reepithelialization

Immune/inflammatory

The aetiologic agent is presumed to interact with resident pulmonary immune cells (T lymphocytes) to generate immune and inflammatory responses. The specific immune responses are initiated following migration of antigen-presenting cells and antigen-specific immune responses are likely to be generated early in the course of lung injury.

T-lymphocytes may play a dual role by contributing to both lung injury and the modulation of disease progression. he generation of specific immune response within the pulmonary interstitium is important in inducing the recruitment of inflammatory cells (i.e., monocytes, lymphocytes, polymorphonuclears, fibroblast, alveolar macrophages and dendritic cells).

Injury

Epithelial (type I and type II epithelium) cell injury is a hallmark of lung fibrosis; the alveolar basal lamina may also be destroyed. Recruited inflammatory cells cause epithelial cell injury, epithelial cell loss and basal lamina destruction.

Repair/fibrosis

Damaged alveoli require replacement of injured alveolar cells and the restoration of damaged extracellular matrix through fibrotic growth factors (TGF-β, TNF-α, PDGF, IGF-I), fibronectin, thrombin, fibrinopeptides and arachidonic acid metabolites converting the fibrin-rich exudate into scar. Alveolar repair is accomplished by hyperplastic type II epithelial cells that proliferate and resurface the repaired basement membrane.

NFκB/IKK-β: a master regulator of inflammation

The nuclear factor (NF)-kappaB family is composed of homodimers and heterodimers of proteins: NFκB1 (p50), NFκB2 (p52), RelA (p65), RelB and c-Rel (Rel). NFκB (p50/p65) is a ubiquitous, constitutive and inducible heterodimer. The general NFκB traditionally refers to the p50/p65 (p50/RelA) heterodimer, which is an anti-apoptotic gene regulator; the p65 (RelA) subunit provides the gene regulatory function [30, 31].

NFκB (Fig. 4), a sequence-specific transcription factor, is a primary regulator of inflammatory responses [32]. NFκB is a group of related homodimeric and heterodimeric transcription factors that are likely to activate distinct sets of target genes.

Their transcriptional actions play a central part in responses to inflammatory signalling not only through Toll-like receptors, but also through tumour necrosis factor (TNF) receptors and the IL-1 receptor, as well as in the diverse responses to other signals operating through the TNF receptor superfamily. They are also essential for responses to signalling through the variable antigen receptors of lymphocytes.

The classical pathway of NFκB activation operates through a heterodimer of p50 and p65. NFκB dimers are held in the active state by a family of inhibitors called IκB. Receptor signalling leads to activation of a multisubunit IκB kinase (IKK) complex, which phosphorylates IκB. Phosphorylation of IκB marks it for degradation by the ubiquitin pathway, so that the NFκB dimer is liberated to translocate to the nucleus, bind DNA and active transcription. There are three main members of the IκB family: IκB-βand IκBe, and IκBa [33–36].

IκK-β is required for its activation during acute inflammation [37] and for innate adaptative immunity [38]. The major function of IκK-β is activation of NFκB through phosphorylation of IκBs [38, 39]. After activation, NFκB mediates induction of proinflammatory cytokines, such as IL-1, IL-6 and TNF-α [37, 39]. As IL-1 and TNF-α activate myeloid cells to induce production of more proinflammatory cytokines, it is possible that myeloid cells may propagate an initial inflammatory signal.

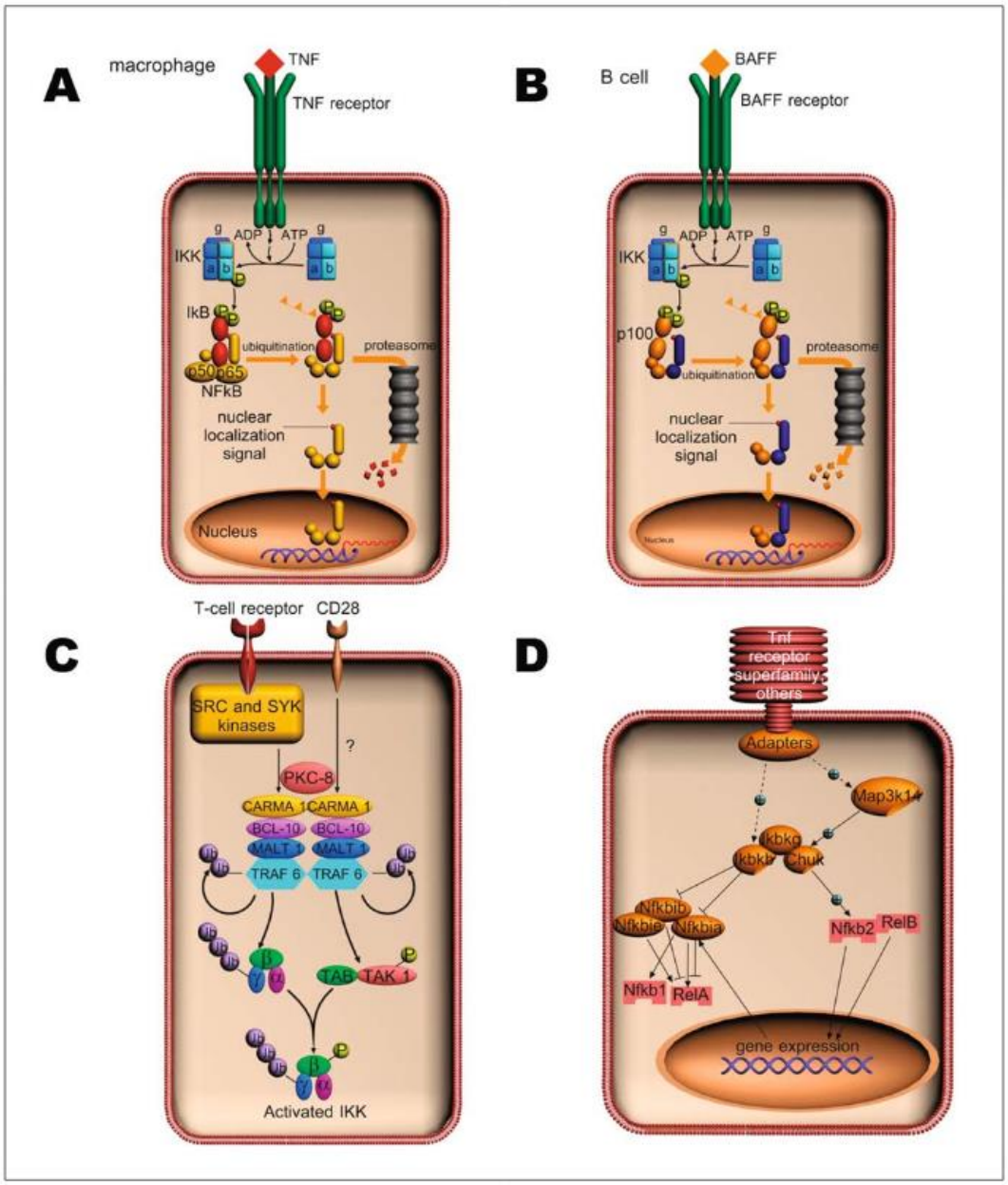

Fig. 4. NF-kB/Ikk-β pathway signalling. Nuclear factor kB (NF-kB) is a primary regulator of inflammatory response; Ikk-β is required for its activation during acute inflammation and for innate adaptive immunity

Macrophages and neutrophils are more important participants in systemic inflammatory responses [33], probably through IκK-β delivery; notably, one of the many functions of macrophages is clearance of oxidised lipid deposits through scavenger receptors [40].

Although it is not clear (demonstrated) how temsirolimus causes activation of NFκB and its target genes in alveolar/interstitial cells, it is possible that excessive fatty acid oxidation in mitochondria generates peroxidation products [41] that may initiate a signalling cascade that culminates in NFκB activation.

This pathway suggests that IκK-β/NFκB can be involved in the pathogenesis of interstitial pulmonary disease induced by rapamycin (mTOR) inhibitors.

Reactive oxygen species (ROS)

Recent developments in cell biology have led to the perception that ROS, including superoxide (O_2^-), nitric oxide (NO) and hydrogen peroxide (H_2O_2), may be employed not only as cellular tools of destruction, but also to serve as mediators of cellular signal transduction (Fig. 5a). NO is synthesised from L-argine by the action of nitric oxide synthase (NOS) in a two-step oxidation process (Fig. 5b).

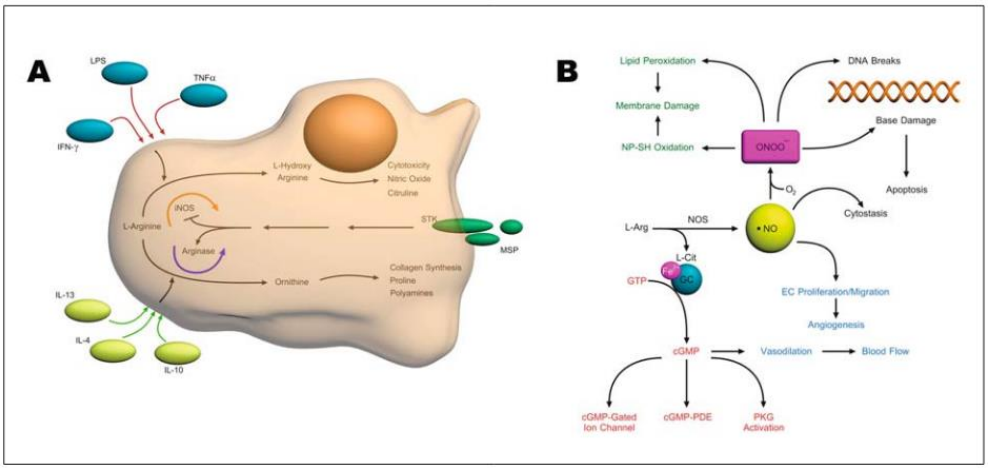

Fig. 5. Regulation of iNOS at the cellular levels. **A**. Cytokines controlling the iNOS induction in macrophages IFN-γ, produced mainly by NK cells, stimulates iNOS production. This IFN-γ-induced production of iNOS can be inhibited by IL. Upon stimulation of macrophages they produce TNF which synergizes with the IFN-γ -induced pathways and inhibits the inhibitory signals of IL. **B**. Reactive oxygen species (ROS) including superoxide (O_2^-), nitric oxide (NO) and hycrogen peroxide (H_2O_2) may play as mediators of cellular signal transduction. Nitric oxide (NO) plays an important role in the regulation of a wide range of physiological processes (i.e. broncho-dilatation; vasodilatation and blood flow; proliferation; angiogenesis) and pathologic events (i.e. DNA damage; pulmonary fibrosis). Abbreviations: IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IFN, interferon; iNOS, inducible nitric oxide synthase; MSP, macrophage stimulating 1 (hepatocyte growth factor-like); Arg, arginine; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate; PKG, protein kinase G; PDE, phosphodiesterase; L-cit, L-citrulline; GC, guanylate cyclase; EC, endothelial cell, $ONOO^-$, reactive oxygen compound

Previous studies on pulmonary fi brosis have mainly focused on the fibroproliferative process in the lung. There has been increasing interest in ROS generation in lung fi brosis [42, 43]. ROS, such as superoxide anions, hydrogen peroxides and hydroxyl radicals, have been demonstrated to be an important mediator in cytostatic-induced lung fi brosis [44, 45]. Excessive production of ROS is known to induce tissue damage or cell death, which could lead to several physiological and pathological processes.

ROS overproduction results in tissue injury, with activation of several intracellular signalling pathways leading to the production of pro-inflammatory cytokines [46]. DNA is a target for ROS activity as well. Radical oxygen species production, by determination of DNA damage, in turn activates PARP. This largely expressed nuclear protein contributes to the maintenance of genomic stability and to the repair of oxidative DNA damage [47]. Although PARP activity promotes cell survival, PARP activation depletes NAD+ and decreases ATP levels, thus leading to cell death after extensive DNA strand breaks [48]. Therefore, ROS produced in response to oxidative stress can contribute by multiple pathways to the pathogenesis of temsirolimus-induced lung injury.

Although the exact role of PARP in human lung fibrosis has not been investigated, it has been shown that PARP is implicated in experimental fibrosis and that PARP inhibition confers protection from inflammation and fibrosis in different animal models [49].

On the other hand, ROS can mediate tissue injury by an increased susceptibility to temsirolimus in patients lacking extracellular superoxide dismutase (SOD), which indicates that superoxide anion radicals play a main role in experimental fibrosis [50]. Superoxide reacts with nitric oxide to generate highly reactive metabolites such as peroxynitrite. This compound is able to oxidise proteins, resulting in direct nitration of tyrosine residues. Protein structure and function can be subsequently altered and enzymatic activity affected. Proteins containing nitrotyrosine residues have been detected in different pathologies associated with enhanced oxidative stress and increased levels of peroxynitrite [51, 52].

Oxidative stress is known to contribute to pulmonary fibrosis (Fig. 6), which occurs in two distinct phases. First, there is an acute phase characterised by an influx of inflammatory cells (macrophages and polymorphonuclear leukocytes). Secondly, this is followed by a chronic stage characterised by extracellular matrix remodelling and collagen deposition [53, 54]. An important feature of this model is that activated phagocytes release large amounts of ROS. The ROS generated include the superoxide anion, hydroxyl radicals, nitric oxide and hydrogen peroxide [55–57].

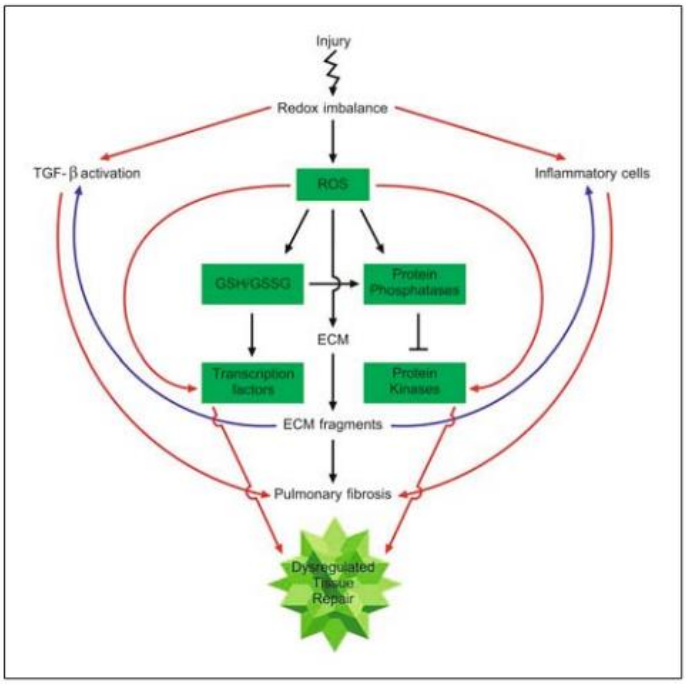

Fig. 6. Roles of reactive oxygen species (ROS) in the pathogenesis of pulmonary fibrosis. Some factors create a redox imbalance, causing a production of ROS. ROS degrade some components of the extracellular matrix (ECM), leading to ECM remodelling. The ECM fragments generated can cause inflammatory cell recruitment to the site and increase ROS production. Continuous inflammation leads to the fibrogenic process. ROS and ROS-induced ECM fragmentation products activate fibrotic cytokines/growth factors (i.e. TGF-β), thus favouring this process. EC-SOD can descend the oxidative stress in the lung parenchyma in human lung

NO, a highly reactive, diffusible and unstable radical, plays an important role in the regulation of a wide range of physiological processes. NO mediates vaso- and broncho- dilatation, which are involved in the physiological regulation of airway function [58].

NO is endogenously produced by NOS, a family of enzymes that currently include three different isoenzymes in mammals [51]: a soluble constitutively expressed enzyme found in high concentrations in the brain (bNOS, nNOS or NOS-1), a constitutively expressed endothelial membrane bound enzyme (eNOS, NOS-3) and an inducible enzyme (iNOS or NOS-2) that is associated with the cytotoxic function of macrophages. These three isoforms exhibit similarities in

their structure and mechanisms of action. Calmodulin is required for the activity of all three isoforms.

iNOS is an inducible enzyme now known to be expressed in a wide variety of cell types. This isoenzyme may be distinguished from endothelial (eNOS) and neuronal NOS (nNOS), the other family members, by at least two criteria: fi rst, iNOS is not constitutively expressed in cells and regulation of this isoenzyme, in contrast to eNOS or nNOS, is widely considered to occur at the transcriptional level only; second, only iNOS produces micromolar NO concentrations, amounts that are high by comparison with the picomolar to nanomolar concentrations resulting from Ca^{2+} -controlled NO production by the eNOS or nNOS [59]. Due to this property, iNOS expression and activity have also been linked to a number of human pathologies and particularly inflammation.

iNOS generates much larger quantities of nitric oxide than the constitutive isoforms and it is directly involved in host defence and in various models of inflammation [60, 61].

Exogenous nitric oxide is able to stimulate *in vitro* fibroblast proliferation [62] whereas iNOS up-regulation in lung fi broblasts is associated with the early proliferative response to cytokine stimulation [63].

Similarly, iNOS, which is the main source of NO during inflammation, and nitrotyrosine, a by-product of peroxynitrite activity, are up-regulated in pulmonary fibrosis [64].

Fibrogenic cytokines

A number of cytokines have been shown to stimulate fibrotic events and include TGF-β, TNF-α, PDGF, granulocyte-macrophage colony-stimulating factor (GM-CSF), endothelin and interleukins (IL-1β, IL-10, IL-13) [65].

TGF-β is a multifunctional mediator capable of regulating cell proliferation and differentiation as well as synthesis of many components of the extracellular matrix [66–72]. Virtually all human cell types are able to produce TGF-β which and they have a number of effects (Table 3) on cellular responses including modulating cell growth, migration, differentiation and apoptosis.

Table 3. TGF-β and oxidative stress/cellular effects

Modulating cell growth, migration, differentiation and apoptosis [67]
Inducing expression of both catalase and mitochondrial SOD2 [68]
Inducing myofibroblast differentiation [69]
Inducing extracellular matrix [69]
Inhibiting ECM breakdown [69]
Decreasing cellular GSH levels [70]
Inducing mitochondrial dysfunction [71]
Inducing ROS production [72]

TGF-β1 plays a central role in fibrotic disorders in different organs, including fibrosis of the lung (Fig. 7). In fact, it stimulates collagen and fibronectin production in fibroblasts [67]. TGF-β was discovered as a factor that induces anchorage-independent growth of fibroblasts [68, 69], and thus, initially, it was thought to stimulate cell proliferation. On the other hand, it can suppress the production of proteases that degrade the extracellular matrix [66]. Morphogenic responses to TGF-β include cell migration and epithelial/endothelial–mesenchymal transition (EMTs), which are crucial during embryogenesis, and fibrotic diseases [70, 71]. TGF-β is a strong promoter of the epithelial-to-mesenchymal transition (EMT) in cooperation with the RAS-MAPK pathway [73]; also, in certain cases, TGF-β induces EMT through the up-regulation of Snail, a transcriptional repressor of E-cadherin [74].

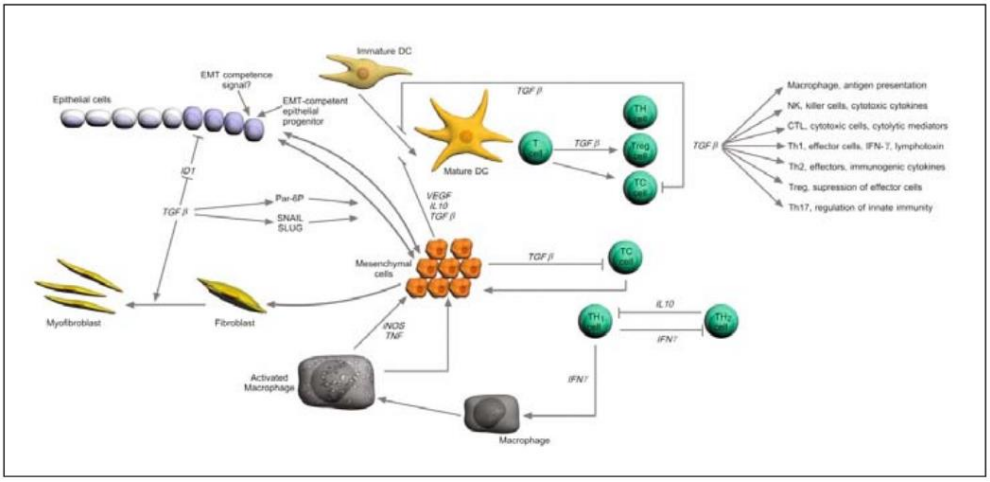

Fig. 7. TGF-β is a pivotal protein in the inflammation and lung fibrosis dependent on the interaction of the alveolar macrophages activation, mesenchymal and dendritic cells (DC) and T-lymphocytes

The EMT is a characteristic change in polarised epithelial cells in which the cell–cell and cell–matrix adhesion is disrupted, the surrounding matrix is degraded and the cell phenotype is changed by rearranging its actin cytoskeleton to become more motile and invasive.

TGF-β_1 has been shown to be increased in bleomycininduced lung fibrosis in the alveolar inflammatory infiltrate [75]. The increase of TGF-β1 mRNA precedes the biosynthesis of type I and type II procollagen in lung fibrosis [75].

The source of TGF-β protein may be a result of increased gene expression by eosinophils and macrophages (Fig. 7) (active TGF-β_1 by alveolar macrophages is augmented after toxic drug administration) whereas later TGF-β_1 secretion remains elevated due to lung mesenchymal cells (myofibroblast and/or fibroblast-like cells, which are characterised by a high expression of α-smooth muscle actin and collagen [76, 77].

TGF-β is a key suppressor of destructive immune and inflammatory reactions [78, 79], and impaired immune regulation with excessive expansion of T-cell populations. As an immunosuppressive cytokine, TGF-β inhibits the development, proliferation and function of both the innate and the adaptative arcus of the immune system. Targets of TGF-β include CD4+ effector T-cells (Th1 and Th2), CD8+ cytotoxic T-cells (CTLs), dendritic cells, NK cells and macrophages (Fig. 7). Additionally, TGF-β stimulates the generation of regulatory T-cells (Treg), which inhibit effector T-cell function, and IL17-producing Th17-cells, which regulate NK and macrophages. By curtailing the activities of macrophages, natural killer cells and effector T-cells, TGF-β suppresses inflammation to promote immune tolerance.

Tolerance can be particularly important in the airway mucosa, where reactions to opportunistic flora and polluting antigens must be restrained. Malfunctions in TGF-β signalling are suspected to be a root cause of these conditions.

It is probable that the extent of inflammation and fibrosis in this model depends on the amount of active TGF-β available [80]. Thus, TGF-β may be associated with alterations in the phenotype of resident fibroblast found in human idiopathic pulmonary fibrosis [81].

Conclusions

One critical issue in the development of new cancer therapeutic agents is elucidating their biomarker, but an issue that could limit application of any drug is toxicity, so risk/benefit ratios must continually be assessed for patients receiving chemotherapy.

For example, rapamycin and its analogues cause specific antagonistic action on the function of the PI3 kinase-Akt-mTOR signalling pathway, induce G1-S cell cycle delay and apoptosis, and inhibit not only lymphocyte proliferation but also growth factor-induced smooth-muscle cell activation, which can explain their immunosuppressive properties and probably their lung side effects, too. This interstitial pneumonitis is a severe and infrequent side effect of sirolimus therapy. But even then, it is probable that a higher incidence of lung injury than that previously reported occurs when we use these drugs in poor-risk renal cancer and in other solid tumours, so the physicians must remain vigilant regarding its potential pulmonary complications, clinical features and management.

Nevertheless, the most important way to avoid this specific toxicity is try to discover those pathogenic mechanisms, biomolecular pathways, and signals of which is known or is thought that are crucial in the development of lung injury. The characterisation of mechanisms involved in the pathogenesis requires deeper investigation about rapamycin analogs, their target genes, and complex pathways activated in the alveolar and interstitial environment of damaged lung. Multiple cytokines and growth factors are able to modulate this lung injury and positive regulators would include the NFκB family, ROS and fibrogenic cytokines (TGF, TNF, GM-CSF, PDGF, endothelin and interleukins). Other proposed mechanisms include the recruitment of inflammatory cells, and changes in vascularity and the extracellular matrix.

Nowadays, management should start with the identification of factors that favour its appearance and of the fist and most common symptoms, dyspnoea and dry cough. After that, for diagnostic confirmation we need to be sure that there are not other causes of lung damage like infection, metastases and cardiac fault, which explain the symptoms and imaging results. The bronchoalveolar lavage can help us because no biological tests or markers have been validated.

Additionally, if we successfully make a precocious diagnosis of lung injury, the best treatment is the discontinuation of the rapamycin analogue because no drugs have demonstrated stoppage of the development of the damage beneficial enough for patients. In the absence of effective alternative therapies, pharmacological treatment of this toxicity is based on antioxidants, corticoids, carnosine, superoxide dismutase and amifostine. These drugs exert an antiinflammatory effect, attenuate the oxidative stress, and stop the redox imbalance to reduce the progressive lung damage. However, these are not real therapeutic advances so other pathways and novel therapeutic strategies may be found to block the molecular mechanism of fibrogenesis, to play major roles in stopping the progression of lung disease and, most of all, to improve the clinical management of patients.

To our knowledge, the present review is the first published of sirolimus-associated pneumonitis, providing new information about the disease development and the pathways implied. Encouragingly, this could be the way to investigate new treatments to resolve or to prevent this lung injury.

To summarise, this entity is likely to increase with wider use of rapamycin analogues in the treatment of solid tumours, so our knowledge of its pathogenesis, biomolecular pathways, and how to avoid and treat it is important.

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Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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