Diploma thesis

The cGAS-STING pathway drives inflammation in Usual Interstitial Pneumonia, phagocytosis could prevent inflammation but is inhibited by the don’t eat me signal CD47 (2024)

submitted by

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Graz, 11.11.2024

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Zusammenfassung

Hintergrund: Die gewöhnliche interstitielle Pneumonie (UIP), eine fibrosierende Lungenentzündung, ist mit idiopathischer Lungenfibrose, chronischer Autoimmunerkrankung (AID) oder Überempfindlichkeitspneumonie assoziiert. Sauerstoffradikale aus Tabakrauch können DNA schädigen und könnten PARP1 hochregulieren. Zytosolische DNA aus sterbenden Pneumozyten aktiviert den zytosolischen GMP-AMP-Synthase-Stimulator von Interferon-Genen (cGAS-STING)-Signalweg und TREX1. Eine anhaltende Entzündung führt zu Zellseneszenz, die durch Phagozytose gehemmt werden könnte. Wir wollten die Aktivierung des cGAS-STING-TREX1-Signalwegs bei UIP untersuchen und herausfinden, ob Phagozytose und Anti-Phagozytose der Entzündung entgegenwirken könnten.

Methoden: 44 Fälle von UIP mit IPF oder AID wurden auf die Expression von cGAS, pSTING, TREX1 und PARP1 untersucht. Die Expression von LAMP1 und Rab7 diente als Phagozytosemarker. CD47, das vor Phagozytose schützt, und p16 zur Identifizierung seneszenter Zellen wurden ebenfalls untersucht.

Ergebnisse: Epithelzellen in umgebauten Arealen und Makrophagen exprimierten cGAS-pSTING, TREX1; Epithelien, aber nicht Makrophagen, wurden für PARP1 gefärbt. Myofibroblasten, Endothelien und bronchiale/bronchioläre Epithelzellen waren alle negativ, mit Ausnahme früher myofibroblastischer Herde, die cGAS exprimierten. Typ-II-Pneumozyten exprimierten cGAS und PARP1, aber weniger pSTING. TREX1 wurde zwar exprimiert, war aber nicht aktiviert. Makrophagen und viele regenerierende Epithelzellen exprimierten LAMP1 und Rab7. CD47, das „Friss-mich-nicht-Signal“, wurde von Makrophagen und Epithelzellen, einschließlich seneszenten Zellen, innerhalb der umgestalteten Bereiche exprimiert.

Schlussfolgerungen: Der cGAS-STING-Signalweg wird in Makrophagen und Epithelzellen in umgestalteten Bereichen aktiviert. Wahrscheinlich kann TREX1, da es nicht aktiviert ist, DNA-Fragmente nicht ausreichend abbauen. Die Aktivierung von PARP1 deutet auf die durch Rauchen verursachte Freisetzung von Sauerstoffradikalen hin, die die Entzündung verlängert und zu Fibrose führt. Durch die Expression von CD47 schützen sich Epithelzellen in umgestalteten Bereichen vor der Eliminierung durch Phagozytose.

Abstract

Background: Usual Interstitial Pneumonia (UIP) a fibrosing pneumonia is associated with idiopathic pulmonary fibrosis, chronic autoimmune disease (AID), or hypersensitivity pneumonia. Oxygen radicals, due to tobacco smoke, can damage DNA and might upregulate PARP1. Cytosolic DNA from dying pneumocytes activate cytosolic GMP-AMP-synthase–stimulator of interferon genes (cGAS-STING) pathway and TREX1. Prolonged inflammation induces senescence, which might be inhibited by phagocytosis, eliminating nuclear debris. We aimed to evaluate activation of cGAS-STING-TREX1 pathway in UIP, and if phagocytosis and anti-phagocytosis might counteract inflammation.
Methods: 44 cases of UIP with IPF or AID were studied for the expression of cGAS, pSTING, TREX1 and PARP1. LAMP1 and Rab7 expression served as phagocytosis markers. CD47 protecting phagocytosis and p16 to identify senescent cells were also studied.
Results: Epithelial cells in remodelled areas and macrophages expressed cGAS-pSTING, TREX1; epithelia but not macrophages stained for PARP1. Myofibroblasts, endothelia, and bronchial/bronchiolar epithelial cells were all negative except early myofibroblastic foci expressing cGAS. Type II pneumocytes expressed cGAS and PARP1, but less pSTING. TREX1 although expressed was not activated. Macrophages and many regenerating epithelial cells expressed LAMP1 and Rab7. CD47, the ’don’t-eat-me-signal‘, was expressed by macrophages and epithelial cells including senescence cells within the remodelled areas.
Conclusions: The cGAS-STING pathway is activated in macrophages and epithelial cells within remodeled areas. Likely TREX1 because not activated cannot sufficiently degrade DNA fragments. PARP1 activation points to smoking-induced oxygen radical release, prolonging inflammation and leading to fibrosis. By expressing CD47 epithelial cells within remodelled areas protect themselves from being eliminated by phagocytosis.

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The cGAS-STING pathway drives inflammation in Usual Interstitial Pneumonia, phagocytosis could prevent inflammation but is inhibited by the don't eat me signal CD47

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ABSTRACT

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Results: Epithelial cells in remodeled areas and macrophages expressed cGAS-pSTING, TREX1; epithelia but not macrophages stained for PARP1. Myofibroblasts, endothelia, and bronchial/bronchiolar epithelial cells were all negative except early myofibroblastic foci expressing cGAS. Type II pneumocytes expressed cGAS and PARP1, but less pSTING. TREX1 although expressed was not activated. Macrophages and many regenerating epithelial cells expressed LAMP1 and Rab7. CD47, the 'don't-eat-me-signal', was expressed by macrophages and epithelial cells including senescence cells within the remodeled areas.

Conclusions: The cGAS-STING pathway is activated in macrophages and epithelial cells within remodeled areas. LikelyTREX1 because not activated cannot sufficiently degrade DNA fragments. PARP1 activation points to smoking-induced oxygen radical release, prolonging inflammation and leading to fibrosis. By expressing CD47 epithelial cells within remodeled areas protect themselves from being eliminated by phagocytosis.

1. Introduction

Usual Interstitial Pneumonia (UIP) is one of the deadliest fibrosing pneumonias, when clinically associated with idiopathic pulmonary fibrosis (IPF). UIP can be associated with chronic autoimmune diseases, or hypersensitivity pneumonia, for which the prognosis is not as clear. As a treatment antifibrotic drugs are recommended in all of them. In immune diseases, corticosteroids or immunosuppressive drugs are added. The underlying mechanisms of how UIP is started are not completely understood. Telomere shortening in pneumocytes due to mutations in the telomerase complex has been shown acting in UIP [12], but also mutations in the homeostasis maintaining surfactant apoprotein genes [10,20,32]. Finally, genetic changes in the MUC5B promoter have been associated with IPF [14]. Besides idiopathic pulmonary fibrosis, chronicity in autoimmune diseases (AID) and hypersensitivity pneumonia (HP) can also result in an UIP pattern. If an autoimmune reaction against pneumocytes is the starting point in these diseases, has not been evaluated. All of the above genetic changes have been found in UIP

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associated with AID and HP too [16]. In autoimmune diseases cytosolic DNA might play a role in the induction of UIP. If cytosolic DNA or double-stranded RNA is encountered, the cytosolic GMP-AMP and synthase–stimulator of the interferon genes (cGAS, STING) pathway is activated. Normally this pathway is part of the defense system against DNA viruses, but also acts, when RNA viruses form RNA double strands [29]. cGAS-STING signals downstream into the interferon α/β system, but also can activate interleukins such as IL6 and 8 [9]. Activation of the cGAS-STING pathway can be confirmed, when phosphorylated STING can be proven in histological sections. If this mechanism underlies the proinflammatory action of senescence cells, is not clear and one of the questions in this investigation. Here we use cGAS-pSTING when referring to the activation, and cGAS-STING pathway when discussing the function of the pathway.

Cytosolic DNA fragments also cause an upregulation of TREX1 (three prime repair exonuclease 1). This exonuclease normally degrades or repairs DNA. TREX1 has a proofreading function and plays a role in eliminating damaged or aberrant DNA (double or single strands) and in apoptosis. If there is a loss of function of TREX1, for example by mutation, it can activate the cGAS-STING pathway [1,34]. Defects in TREX1 and/or STING have been found in some autoimmune diseases. For its function TREX1 need cooperating partners DNAse 2 and PARP1 for its translocation to the nuclear envelope. Therefore, nuclear staining is a sign of activation. TREX1 is supposed to prevent the activation of cGAS-STING.

The activation of the cGAS-STING cascade as well as TREX1 induces inflammasomes and creates an inflammatory microenvironment, usually as long as DNA and dsRNA are present [11]. If STING expression is induced experimentally, this results in a morphologic phenotype with vasculitis [28]. Case reports on the autoinflammatory disease SAVI (STING-associated Vasculopathy with Onset in Infancy) have been reported, which showed vasculitis in skin and lung, and lung fibrosis [25]. Mutations of the transmembrane protein 173 (TMEM173, alias STING) were identified in patients with SLE, primary antiphospholipid syndrome, systemic sclerosis, dermatomyositis, and vasculitis [5].

Due to the shortening of telomeres and/or an imbalance of the homeostasis by mutations in the surfactant apoprotein genes, cellular debris including DNA fragments from dying pneumocytes accumulates in the alveolar spaces. This accumulation of debris can prolong inflammation and might act as a factor for the induction of senescence [13]. Most patients with UIP are cigarette smokers, therefore their lung is constantly exposed to toxins, which contribute to the formation of oxygen radicals. Oxygen radicals activate PARP1 (Poly[ADP-Ribose] Polymerase 1), and this induces upregulation of the TGFβ signaling pathway, which also results in fibrosis [22]. Fibrosis by itself leads to DNA damage with double strand breaks (derived from pneumocytes), increases oxygen radical formation, and thus drives a vicious circle. In IPF upregulation of PARP1 was shown [35]. During prolonged inflammation, senescence cells develop, which secrete inflammatory cytokines and thus maintain inflammation and proliferation of myofibroblasts [13]. If the cGAS-STING pathway is involved, has not been investigated.

The accumulation of cellular debris, including DNA fragments resulting in inflammation, might be counteracted and limited by phagocytosis. Macrophages are commonly seen in UIP, accumulating not only in areas of cystic remodeling, but also adjacent to myofibroblastic foci. Macrophages might phagocytose and digest cellular debris and also attack senescence cells, thus limiting the inflammation. Phagocytosis cannot be proven in tissue sections, but surrogate markers for active phagocytosis can provide an answer. LAMP1 (lysosome associated membrane protein 1) and Rab7 (Ras-related protein 7), are expressed in macrophages when phagocytosis is activated [27]. As pinocytosis, which is seen in epithelial cells, is based on a similar process as phagocytosis, the expression of LAMP1 and Rab7 might be demonstrated in epithelial cells as well. LAMP1 is present at lysosomal membranes and plays a role in phagocytosis and autophagy. Rab7 is a small GTPase which regulates vesicle trafficking and plays a role in autophagosome maturation [4]. When macrophages have ingested foreign material, they need to activate LAMP1 and Rab7 for lysosomal digestion. The expression of both molecules can therefore be interpreted that the cells are able to phagocytose. Macrophages also need to protect themselves from phagocytosis by other macrophages; this is facilitated by the expression of CD47, the so-called "don't-eat-me-signal" [23]. CD47 expressed on the surface of cells inhibits the attack of macrophages and natural killer cells [7], in normal cells it helps to discriminate viable healthy cells from abnormal cells [18].

In a previous study senescence cells were identified in cells replacing the original alveolar surface (remodeled area) in fibrosing pneumonias [13]. In the present investigation we raise the question, i) if the proinflammatory cGAS-STING-TREX1-Interferon pathway was activated in senescence cells. Another question is, if PARP1 is expressed. As a counter-regulation activated macrophages might remove debris and also attack senescence cells, leading to the second question, ii) if phagocytosis is defective, and if senescence cells might protect themselves from being phagocytosed. To see if pro- and anti-inflammatory mechanisms are different in UIP/IPF and UIP/AID two groups of patient derived tissues were compared.

2. Material and method

Forty-four cases of UIP were selected from the consultation files of one author. For two cases biopsies from two different lobes were available, which accounts for 46 sections to be evaluated. Most cases were derived from cryobiopsies. They all were sent for consultation from the Institute of Pathology, Clinics München-Bogenhausen. In all these cases, the underlying etiology was known due to clinical investigations and follow-up data (provided by Dr. Rampp). There were 35 male and 9 female patients. The mean age for patients with autoimmune diseases was 59 for IPF patients 68 years. Within the group of autoimmune diseases there were 9 patients with systemic sclerosis, 8 with rheumatoid arthritis, 1 with dermatomyositis, and 8 patients with undefined AID. Eighteen patients were diagnosed as having IPF. All IPF patients were current smokers, whereas 12 patients in the AID group were smokers, the others former smokers. For the UIP pattern the following features were required: Peripheral accentuation of the lesions, myofibroblastic foci present, temporal heterogeneity, which means early lesions (myofibroblastic foci) and late lesions (fibrosis and scars), and normal uninvolved peripheral lung. From formalin-fixed and paraffin embedded blocks 4 μm tissue sections were cut and stained by hematoxylin-eosin (H&E). The cases were grouped into IPF and chronic AID. No cases of UIP in chronic HP were included, because the number of cases was too small to get a conclusive result.

Another set of sections were cut and used for immunohistochemical investigations. All cases were investigated for the immunohistochemical expression of cGAS, pSTING (phosphorylated STING), TREX1, PARP1, the phagocytosis markers LAMP1 and Rab7, and the phagocytosis-protection marker CD47. In addition, to better characterize those cells expressing CD47, a double immunohistochemical staining was done using p16 as a marker for senescent cells (details of the antibodies used and the immunohistochemical methodology are given in Table 1). Control sections for the validity of the antibodies were used as recommended by the manufacturers. The study was approved by the ethical committee of the Medical University (24–135 ex 11/12). Informed consent from the patients was not required, as no sensitive patient data were included.

3. Results

All regenerating cells within the remodeled areas were positive for cGAS, pSTING, TREX1 (only cytoplasmic), and PARP1 (Fig. 1ABC). Most alveolar macrophages expressed cGAS (43/46), pSTING and TREX1 (46/46), but were negative for PARP1. Myofibroblasts expressed cGAS in early lesions, but were negative in more advanced lesions with more

Table 1
Details for the antibodies used in this investigation.

Antibody, company	clone	dilution	pretreatment	visualization
cGAS abcam	polyclonal	1:1000	MW9,0	Dako Envision K5007
pSTING invitrogen	polyclonal	1:50	MW9,0	Dako Envision K5007
TREX1 abcam	EPR14985	1:50	CC1 (32 min)	Ventana ultraViewDAB
LAMP1 abcam	polyclonal	1:200	MW6,0	Dako Envision K5007
Rab7 abcam	EPR7589	1:100	MW6,0	Dako Envision K5007
CD47 abcam	polyclonal	1:100	MW9,0	Dako Envision K5007
p16 Enzo	P16INK4A	1:1000	CC1 (64 min)	Ventana optiViewDAB
PARP1 abcam	EPR18461, monoclonal	1:1000	MW Tris/ EDTA, pH9	Dako Envision K5007

mature collagen deposits. They were negative for pSTING except in one case, showed TREX1 in three cases, and were all negative for PARP1. Many pneumocytes in normally structured lung were positive for cGAS (44/46), pSTING (14/42), TREX1 (3/39) and PARP1 (30/46) – in some slides normal structured lung was lost in consecutive sections and therefore could not be evaluated. Lymphocytes, when present were negative for cGAS, pSTING (with one exception), but positive for TREX1 and PARP1. Expression of TREX1 was cytoplasmic in all cases, which can be interpreted that TREX1 was not activated (translocated into nuclei).

Endothelia and smooth muscle cells were negative for cGAS, pSTING, and PARP1, but in 5 cases endothelia expressed TREX1. In bronchial and bronchiolar epithelia, expression for cGAS was negative in all cases, but an expression of pSTING, TREX1, and PARP1 was seen in 21, 33, and 46 cases respectively, again cytoplasmic for TREX1. The expression of PARP1 was specifically confined to the basal cells (Fig. 2). Neutrophils, if present, were usually negative for all four markers (pSTING in one case positive) (Suppl.Table 1). Positive and negative reactions for cGAS-pSTING, TREX1 and PARP1 were seen in both groups of UIP without a significant difference between IPF and AID.

Phagocytosis-associated molecules (LAMP1, Rab7) were expressed in all macrophages (Fig. 3), but also in a considerable proportion of regenerating cells within the remodeled areas (46/46, and 30/46, respectively). Endothelia, smooth muscle cells, and bronchial/bronchiolar epithelial cells were negative for all markers tested in this study. Expression of LAMP1 and Rab7 was sometimes seen focally in type II pneumocytes within normally structured lung (46/46 and 24/45 cases, respectively). Myofibroblasts were negative for Rab7, but in 3/46 cases a focal positivity was seen for LAMP1. Lymphocytes were negative for both markers. CD47 was expressed by macrophages in all cases. An expression was seen in most regenerating epithelial cells within the remodeled peripheral lung (36/38; Fig.4AB), but less intense compared to the macrophages (Suppl.Table 1).

In some slides the remodeled areas were lost during sectioning, resulting that not all 46 sections could be evaluated. A double stain for CD47 and p16 as a marker for senescence cells showed a positive expression for both (Fig. 4C).

As the staining intensities and percentages for the above markers were similarly seen in UIP/IPF and UIP/AID no semiquantitative or subgroup analysis was done.

4. Discussion

Usual interstitial pneumonia is a progressive fibrosing disease, for which no curative treatment is available [17,31]. Fibrosis can be ameliorated by antifibrotic drugs, but the patients will ultimately die.

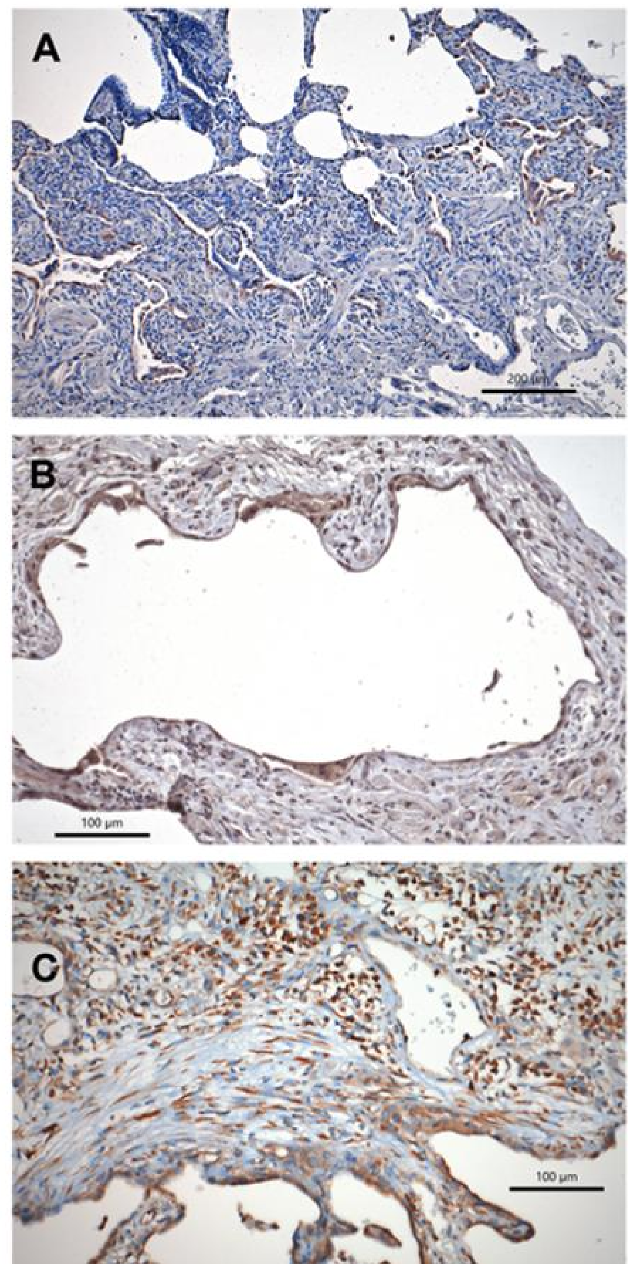

Fig. 1. A) Regenerating epithelia within the remodeled area stained positively for cGAS (cytoplasmic staining), whereas myofibroblasts were negative. Note also positive alveolar macrophages within the cystic lumina. B) Positive nuclear and cytoplasmic reaction for pSTING in regenerating epithelia; few macrophages within the lumina are also positively stained. C) Expression of TREX1 in regenerating epithelia, some myofibroblasts, and macrophages within the interstitium. Immunohistochemical reactions, bars 200 and 100 µm.

An UIP pattern is seen in idiopathic pulmonary fibrosis (IPF), but also in chronic hypersensitivity pneumonia and autoimmune diseases [26]. The underlying etiology in both UIP/IPF and its variants remains unknown. Mutations in the telomere and surfactant apoprotein genes have been detected, but it is not clear if they are ultimately responsible for UIP. With respect to the pathogenesis we know a bit more about the sequence of events: Pneumocytes type II (PC-II) die prematurely and are not replaced by peripheral precursor cells [6]. Tobacco smoke toxins might contribute to PC-II apoptosis. The denuded surface induces a repair mechanism, by which fibroblasts are transformed into myofibroblasts

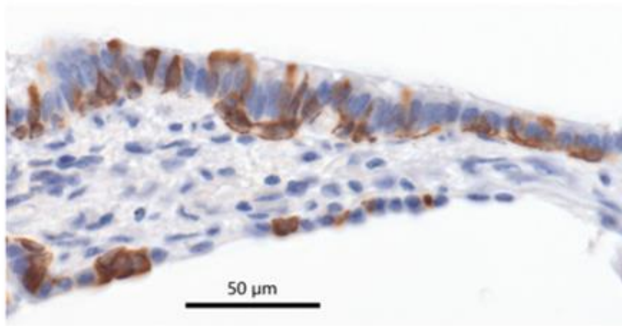

Fig. 2. PARP1 was expressed in bronchiolar epithelia, specifically basal cells. Immunohistochemical reactions, bar 50 µm.

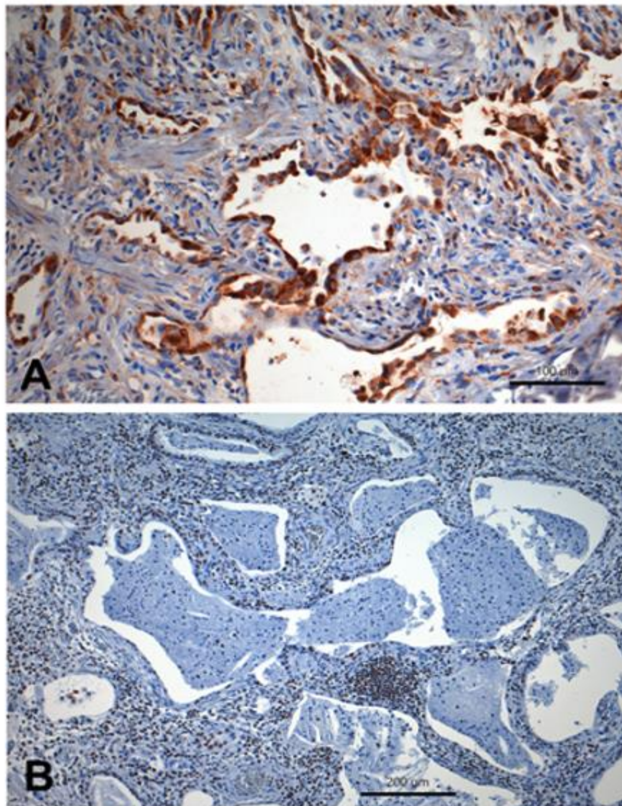

Fig. 3. A) Positive reaction for LAMP1 (cytoplasmic) and B) Rab7 (nuclear) in regenerating epithelia within the remodeled area. Also, macrophages express these phagocytosis-associated markers, whereas myofibroblasts were negative. Immunohistochemical reactions, bars 100 and 200 µm.

[24]. As normal regeneration is impaired, no PC-II will grow over the denuded surface. Therefore, the recovery of the alveolar surface is facilitated by cells moving in from basal bronchiolar epithelial cells (keratin 5 positive) [13]. Nuclear debris and likely fragments of nucleic acids accumulate in the alveolar lumina. Some cells within the remodeled cystic areas evolve into senescence cells, which stimulate inflammation by secreting inflammasome-associated molecules [13].

In this study, the activation of the cGAS-STING pathway in regenerating cells within the cystic remodeled peripheral lung has been identified. In addition, macrophages also expressed this pathway. Myofibroblasts, not unexpectedly, were negative, except for those in very young foci. This might be due to an ongoing transformation process, whereby fibroblasts and immature mesenchymal cells are transformed into myofibroblasts [33]. The cGAS-STING pathway was activated in cases of UIP/IPF as well as in those with underlying chronic autoimmune disease (UIP/AID). This pathway is well known to be activated by cytosolic DNA or dsRNA [21]. Moreover, TREX1 can also be activated by damaged DNA (single or double-stranded). In both UIP/AID and UIP/IPF, TREX1 could be proven, but was not activated (no nuclear translocation). Normally, TREX1 would eliminate aberrant self-DNA to prevent autoimmunity. As TREX1 was not activated in UIP, it therefore was not able to repair or eliminate damaged DNA fragments [19]. The fact that TREX1 is expressed but not activated in all regenerating cells within the remodeled cystic area, including senescent cells, raises the question about the missing activation of TREX1. Some reports and our own findings show that TREX1 is downregulated or inactive in senescence cells and this in turn activates the cGAS-STING pathway [30]. For its activation/translocation to the nuclear envelope TREX1 needs the cooperation of DNAse 2 and PARP1 [3]. Further investigation for DNAse 2 is warranted to elucidate the role of TREX1 as PARP1 was expressed in the epithelia.

PARP1 expression is most often induced by oxygen radicals (ROS), and therefore seen in the airway epithelia of cigarette smokers [15]. PARP1 expression seen here in normal pneumocytes and bronchiolar cells as well as in cells within remodeled areas might be a response to tobacco smoke exposure, which is common in patients with UIP pattern. PARP1 expression in former smokers (UIP/AID group) might be due to passive smoking or exposure to environmentally inhaled substances. Furthermore, as PARP1 might contribute to fibrosis by upregulating TGFβ, several features present in UIP can be explained [22]. Again, this aligns well with the increase of damaged DNA.

Some aspects of cGAS expression in normal cells need to be discussed. The finding that more cells express cGAS than STING, might be explained as follows: Cytosolic DNA fragments will stimulate cGAS, but the second step of phosphorylation of STING does not occur in many cells, therefore only a minority of these cells activate the cGAS-STING pathway. The upregulation of the cGAS-STING pathway in macrophages makes sense, as these cells are involved in the removal of debris and DNA fragments. The negative reaction for PARP1 is not unexpected, because macrophages use other mechanisms to react to oxygen radicals, such as the glutathione system [8].

Under normal conditions, macrophages would remove cellular debris as well as DNA fragments and digest it. This could result in regeneration and restitution of the peripheral lung, limit the activation of the inflammatory cascade, and might even prevent senescence. Phagocytosis might be impaired in UIP, however, our results show that macrophages regularly express phagocytosis-associated surrogate markers LAMP1 and Rab7. Therefore, phagocytosis seems to be intact. This raises the question if another mechanism exists which inhibits the prolonged inflammation. The expression of phagocytosis markers in normal epithelia outside the cystic areas cannot be fully explained. But the expression of these markers might be associated with pinocytosis. LAMP1 and Rab7 are associated with endosomal structures and lysosomes, which also play a role in pinocytosis and therefore the expression might be related to this phenomenon [2].

Epithelial cells within the remodeled area express keratin 5, which is a marker in basal cells of the bronchi and bronchioles. These cells might have migrated into the denuded alveoli as no pneumocyte precursor cells can be seen. The cuboidal morphology, also called bronchiolar metaplasia, is an indication of such a process. These cells, especially those which underwent senescence, are not part of the normal alveolar epithelium. Therefore, they might be attacked by cells of the innate immune system. Such an action would normally limit inflammation. In our investigation a protective mechanism is demonstrated: These cells express CD47, the 'don`t-eat-me-signal`. However, not only senescent cells (p16-positive), but also other epithelial cells within the remodeled area express CD47. It can be speculated that all regenerating cells need this protection, or that these cells are just transforming into senescent

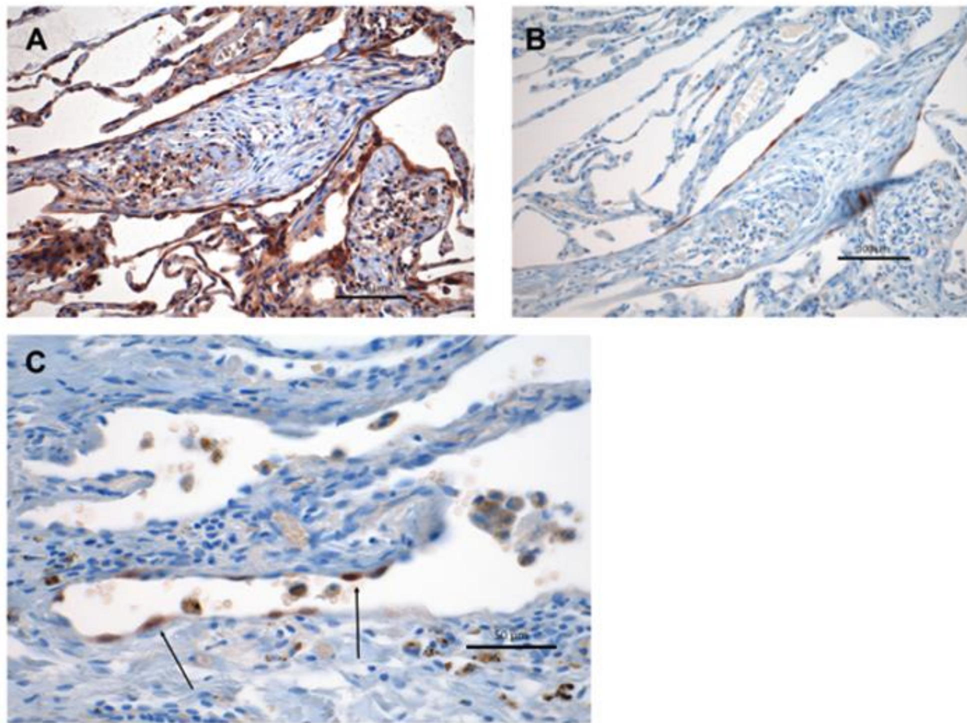

Fig. 4. Cytoplasmic expression of CD47 (A) and the nuclear expression of p16 in consecutive sections (B). All regenerating epithelia are positive for CD47, whereas only few express p16 in the nuclei. In C) a double staining for CD47 and p16 is shown: more cells are positive for CD47 (brown cytoplasmic stain), whereas only two of them also show p16 (red nuclear stain), assigning these cell as senescent ones. Immunohistochemical reactions, bars 100 and 50 µm.

cells. The expression of CD47 in macrophages, again, is logical, as they also need this protection from not being phagocytosed by other macrophages.

Contributions

AG and MN evaluated the morphology and the immunostains, SE performed immunohistochemistry and evaluated the antibodies, AN contributed the cases and evaluated the histopathology, HP designed the study, supervised the work of AG and MN by evaluating the morphology and the immunostains, and wrote the draft. All authors worked on the final manuscript

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Author statement

Dear Prof. Rössner: I have revised our manuscript and responded to the reviewers point by point. The revised manuscript has track changes activated, so the reviewers can see what has been changed in response to their comments and critics. I hope the revised manuscript might be accepted in the journal.

CRediT authorship contribution statement

Andreas Nerlich: Writing – review & editing, Resources, Data curation. **Helmut Popper:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Investigation, Data curation, Conceptualization. **Alissa Gruenwald:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Margarete Neururer:** Writing – review & editing, Validation, Investigation, Formal analysis. **Sylvia Eidenhammer:** Writing – review & editing, Methodology.

Declaration of Competing Interest

No conflict of interest has to be declared by all five authors.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prp.2024.155432.

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