Immune processes in the pathogenesis of chronic lung allograft dysfunction: identifying the missing pieces of the puzzle

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Shareable abstract (@ERSpublications)
CLAD is the end-stage of a disease continuum marked by complex, interacting, innate and adaptive, cellular and humoral, allo- and autoimmune mechanisms, repeated lung injury, tissue remodelling and repair, ultimately leading to allograft dysfunction. https://bit.ly/3Ny3LZz

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Abstract
Lung transplantation is the optimal treatment for selected patients with end-stage chronic lung diseases. However, chronic lung allograft dysfunction remains the leading obstacle to improved long-term outcomes. Traditionally, lung allograft rejection has been considered primarily as a manifestation of cellular immune responses. However, in reality, an array of complex, interacting and multifactorial mechanisms contribute to its emergence. Alloimmune-dependent mechanisms, including T-cell-mediated rejection and antibody-mediated rejection, as well as non-alloimmune injuries, have been implicated. Moreover, a role has emerged for autoimmune responses to lung self-antigens in the development of chronic graft injury. The aim of this review is to summarise the immune processes involved in the pathogenesis of chronic lung allograft dysfunction, with advanced insights into the role of innate immune pathways and crosstalk between innate and adaptive immunity, and to identify gaps in current knowledge.

Introduction
Chronic lung allograft dysfunction (CLAD) remains the major limitation to the long-term success of lung transplantation, occurring in up to 50% of recipients within 5 years post-transplant [1]. CLAD encompasses two distinct but overlapping phenotypes, of which bronchiolitis obliterans syndrome (BOS) is the most prevalent, featuring in ~70% of CLAD patients. The histological hallmark of BOS is small airways fibrosis, known as “obliterative bronchiolitis”, which is clinically characterised by persistent and progressive airflow limitation [2]. Restrictive allograft syndrome (RAS) occurs in 20–30% of CLAD patients and is defined by a restrictive pulmonary function decline and persistent pleuroparenchymal abnormalities on computed tomography [2, 3]. In addition, patients might present with a mixed phenotype or shift from one phenotype to another over time [3]. Different pathophysiological mechanisms have been suggested to be involved in these phenotypes, given the differences in disease course, radiographic imaging, histology, and cytokine, chemokine and growth factor expression. However, it is difficult to clearly categorise the different pathophysiological mechanisms because of the relatively smaller amount of pooled evidence for RAS at present [2, 4].

Unlike other solid organ transplants, the lung allograft is continuously exposed to the external environment and therefore harbours a robust innate immune presence primed to respond to environmental or microbiological challenges and contains more tissue-resident and interstitial immune cells [5]. It is therefore not surprising that various insults to the lung allograft have been identified as important contributing factors to CLAD, and can broadly be described as alloimmune-dependent and -independent (table 1) [4, 6]. Over
In the past decades, we have gained a better understanding of how the immune system contributes to inflammatory responses, airway and parenchymal remodelling, and fibrosis after lung transplantation. However, in order to make therapeutic advances in the prevention and treatment of CLAD, it is critical to develop a full picture of how all the immune processes at play in the lung allograft interact in the pathogenesis of CLAD. Here, we review evidence established to date, with advanced insights into the role of innate immune pathways and crosstalk between innate and adaptive immunity before identifying what is missing from our current understanding of this puzzle.

**Immune processes in CLAD**

**T-cell-mediated immunity**

Cell-mediated immunity is perhaps the best understood alloimmune pathway. It is predominantly driven by T-cells following the presentation of alloantigens by antigen-presenting cells (APCs) via major histocompatibility complex (MHC) molecules, also called human leukocyte antigen (HLA) [7, 8]. HLA genes are highly polymorphic and large interindividual differences in allelic variants are the major immunological barrier to transplantation [9]. Two main modes play a role in this allorecognition. In the direct pathway, allogeneic MHC is presented directly to recipient T-cells by donor APCs. In the indirect pathway, recipient APCs phagocytise and present alloantigens to recipient T-cells as MHC–peptide complexes [8, 10]. MHC classes I and II are, respectively, recognised by CD8+ and CD4+ T-cells [7]. Following allorecognition, T-cells require secondary costimulatory signals, resulting in proliferation and differentiation [11].

Besides cytotoxic CD8+ T-cells, immunological responses are regulated by CD4+ helper T-cells, whose subtypes have different characteristics, ranging from cytolytic activity, activation of innate and other adaptive immune cells, to propagating or dampening inflammation [12, 13]. T-helper 1 (Th1) cells are a key source of interleukin (IL)-2, IL-12, interferon (IFN)-γ and tumour necrosis factor (TNF)-α, which drive a cytotoxic immune response. They are highly effective in activating macrophages, but can also cause direct allograft damage through Fas/Fas ligand-mediated cytotoxicity [6, 12, 14]. Abundant evidence demonstrates the ability of Th1 cells to mediate acute rejection and CLAD [6, 12, 15, 16]. Th2 cells can produce a variety of cytokines (IL-4, -5, -6, -10, -13), some of which downregulate further cytokine production, while others promote humoral immunity [12, 13]. For example, a Th1/Th2 balance in favour of Th2 and IL-10 can reduce rejection rates, and on the other hand, Th2 cells can accelerate rejection by releasing proinflammatory and potent profibrotic mediators such as IL-6 and IL-13 [12, 17]. Next to cytokines, a complex network of chemokines and their receptors, which function to recruit and activate various leukocyte subsets, are involved in the inflammatory processes leading to the development of BOS or RAS (e.g., CCR2/CCL2, CXCR2/ligand, CXCR3/ligand, and CCL5/RANTES interactions) [6, 18, 19] (figure 1).

Two other T-cell subtypes play important roles in the onset of CLAD. Firstly, Th17 cells, which secrete IL-6, IL-17, IL-22 and TNF-α, help to clear pathogens through recruitment and activation of neutrophils and macrophages, but are also associated with autoimmunity in cases of dysregulation or overproduction of IL-6 [13, 14]. Secondly, a unique subset of lymphocytes called regulatory T-cells (Tregs) have an important role in immune homeostasis [13]. Th17 and Tregs both develop from naïve T-cells on stimulation by transforming growth factor (TGF)-β. IL-6, a proinflammatory cytokine, has a pivotal role in regulating the Th17/Treg balance, inducing the generation of IL-17-producing Th17 cells in concert with

| TABLE 1 Risk factors contributing to chronic lung allograft dysfunction onset |
|-----------------|-----------------|-----------------|
| **Alloimmune dependent** | **Alloimmune independent** |
| Acute cellular rejection | Ischaemia-reperfusion injury |
| Lymphocytic bronchiolitis | Allograft infection (bacterial, fungal, viral) |
| Human leukocyte antigen (HLA) mismatching | Gastro-oesophageal reflux |
| Preformed or de novo anti-HLA antibodies, non-HLA antibodies and antibodies to self-antigens | Air pollution |
| Antibody-mediated rejection | Inhaled toxins |
| Donor and recipient genetic variants | |
Inflammatory mediators in CLAD

| Cytokines | Chemokines + receptors | ROS ND |
|-----------|------------------------|--------|
| IL-1, -2, -6, -8 | CCL-2, -3, -5 | CCL2, CXCL2, CXCR2, CXCR3 |
| IFN-γ, IL-2 | CXCL2, -7, -9, -10 |

Migration from the circulation into the allograft

Tissue-resident immune cells (e.g., BALT, interstitial APCs)

Cellular components (proteins, cfDNA)

Allo-antigens

Migration from the allograft into the circulation and lymph nodes

Tissue-resident memory T-cells and/or T-cells

Figure 1: Key elements in the pathogenesis of chronic lung allograft dysfunction (CLAD). Overview of the pathogenesis of CLAD with some of the main immune mechanisms and cytokines involved. Tissue injury by alloimmune-dependent and -independent mechanisms induces the release of...
TGF-β, whilst inhibiting TGF-β-induced Treg differentiation [13, 14]. Both Th17/IL-17 and IL-6 are thought to be involved in the pathogenesis of CLAD, partly through endothelial cell activation and fibroblast activation and proliferation. IL-17 has also been shown to trigger a positive-feedback loop of IL-6 expression [13, 14, 20].

Tregs are essential components of the normal immune system and are responsible for maintaining homeostasis and balance activated immune responses. This is accomplished by the release of immunosuppressive cytokines (TGF-β, IL-10) as well as direct cell–cell interactions (e.g., regulation of dendritic cell maturation and function) [11]. These actions prevent excessive effector T-cell responses [14]. By promoting the differentiation and/or activity of IL-10-secreting T-cells, Tregs also protect against autoimmunity [21]. Tregs have been shown to reduce the onset of CLAD and to establish immune tolerance in animal models [11, 12, 22]. Increased proportions of Tregs, especially in the lung allograft, seemed to stabilise allograft function, while a decline of this cell population has been described in progressive CLAD [11, 23–27].

Apart from classical CD8+ cytotoxic cells, it has recently been shown that other cytotoxic cells with a senescent pattern are thought to be involved in CLAD development and associated with uncontrolled regulation by Tregs or immune checkpoints. For example, increased senescent T- and natural killer T-like lymphocytes with loss of CD28 expression were identified in BOS patients and correlated with increased expression of granzyme B, IFN-γ and TNF-α [28]. Brugier et al. [29] investigated whether the immune checkpoint HLA-G/immunoglobulin-like transcript (ILT)2 expressed by peripheral T-cell subpopulations could predict CLAD and found that an early increase after lung transplantation of cytotoxic CD4+CD57+ILT2+ T-cells, selectively inhibited by HLA-G, may be associated with CLAD onset. The importance of the role of these cells remains to be confirmed in large cohorts, but could open new avenues for targeted therapies.

Little is known about the precise role of other T-cell subsets including T follicular helper cells, Th9 cells and Th22 cells in the lung transplant setting yet, and the exact role of memory T-cells and γδ T-cells in the onset of CLAD remains also unclear [22, 30]. Memory T-lymphocytes are commonly viewed as an important barrier to long-term survival of organ allografts; however, Krupnick et al. [31] demonstrated an unsuspected role in lung allograft tolerance of central memory CD8+ T-cells, characterised by high surface expression of CD62 ligand and CD44, in a murine model. Further research on these T-cell subsets is warranted.

**Humoral immunity**

Traditionally, CLAD was thought to be primarily a manifestation of T-cell-mediated immune responses; however, antibodies and pathological alloreactive B-cells play a significant role in CLAD [7, 32]. HLAs have a crucial role in immune surveillance by presenting peptides to T-cell receptors [8]. T-cells are required for the growth and maturation of antigen-specific B lymphocytes, which produce alloantibodies against mismatched MHC and minor histocompatibility antigens [11, 21]. The presence of donor-specific antibodies (DSAs) is strongly associated with CLAD, through alloimmune responses, complement activation, and complement-independent mechanisms [10]. Moreover, anti-HLA antibodies can induce the release of fibrotic growth factors, including platelet-derived growth factor, insulin-like growth factor-1 and
Similarly, Vandermeulen et al. [32] found higher levels of B-cells in BOS and RAS explant lungs and more lymphoid follicles in RAS tissue. The transformation of intragraft inflammatory infiltrates into tertiary lymphoid tissue, also called lymphoid neogenesis, probably also plays a role in lung allograft dysfunction, as has been reported in several other allograft types [10, 34].

Under some circumstances, humoral immune responses seem to cause little or no damage to the allograft. Accommodation describes a biological state in which the graft function remains stable despite alloantibodies or alloimmune responses, and is probably achieved by graft exposure to low concentrations of DSA or an altered affinity and/or specificity of the immune response [35]. Growing evidence demonstrates that B-cells also play a pivotal role in transplant tolerance [35]. Regulatory B-cells (Bregs) are thought to represent a stage of B-cell development before their differentiation into plasma cells and are potent inhibitors of the immune system, able to suppress allo- and autoimmune responses [33]. Bregs function, at least partly, through the production of IL-10, IL-35 and TGF-β, to suppress antigen presentation and cytokine secretion by APCs, T-cell proliferation, and actions from natural killer (NK) cells, neutrophils and other effector cells. Moreover, Bregs promote T-cell apoptosis and generation of Tregs by directly interacting with T-cell differentiation [35]. In addition to Bregs, other specific B-cell populations may be associated with long-term graft acceptance, such as IL-10 secreting CD9+ transitional B-cells as described by Brosseau et al. [37].

As a result, B-cells are increasingly acknowledged as crucial mediators at the centre of immune regulation with the power to enhance or inhibit allograft immunity.

High incidences of CLAD have been described in patients with previous episodes of AMR, and DSAs are a strong risk factor for acute cellular rejection (ACR), AMR and CLAD [38–42]. Numerous studies have attempted to identify DSA characteristics that correlate with worse outcome. Patients with anti-HLA antibodies prior to transplantation had increased risk post-transplant of developing antibodies to HLA and non-HLA molecules, AMR, CLAD and mortality, although some reports failed to substantiate this. Moreover, there is currently no consensus on the use of peri-operative desensitisation protocols in these patients [43–48]. Post-transplant de novo DSAs are also strongly linked to acute and chronic rejection and graft failure [40, 45–47, 49–54].

Detailed examination of DSA characteristics identified a greater risk for AMR, BOS and allograft loss in patients with DSAs against class II MHC molecules, especially DQ, compared to class I [40, 44, 55, 56]. A link between the number of total HLA mismatches and incidence of BOS has also been described [57]. Furthermore, the impact of circulating DSA depends on its ability to bind complement. Generally, complement-binding DSA was associated with worse CLAD-free and graft survival compared to non-complement-binding DSA [43, 49, 55]. Patients who cleared DSA after therapy had greater freedom from BOS and better survival rates than those who did not, which suggests that ongoing lung injury in the setting of persistent DSA results in accelerated graft dysfunction [46, 55, 58]. Based on these findings, many centres frequently monitor for DSA using highly sensitive immunoassays [10]. However, antibodies detected in the blood do not necessarily represent antibodies acting on the graft [34]. Importantly, it has been recognised that DSA might be absent in serum yet persist in allograft tissue [59].

Few studies have distinguished the effects of DSA or AMR on the development of BOS versus RAS. Until recently, AMR was believed to mainly occur early after transplantation as (hyper)acute rejection. However, AMR is increasingly seen beyond the first year post-transplant, which is likely partly due to increased awareness and implementation of sensitive detection methods. This raises the possibility of chronic AMR as cause for CLAD [60]. Moreover, patients with chronic AMR or persistent DSA seemed to be more prone to develop RAS than BOS [10, 46]. In patients with RAS, the level of tissue-bound DSA in the
allograft seemed higher than in BOS, which might indicate a strong relationship with fibrosis [59]. It is appealing to consider whether RAS is an end-stage of chronic AMR, but definitive data are lacking to date and elevated B-cells, DSA and immunoglobulin G (IgG) were also seen in BOS, implying that chronic (less severe) AMR might also be a driving factor for CLAD phenotype BOS [32, 61].

Autoimmunity

A critical feature of the immune system is to establish effective cell-mediated and humoral responses to foreign antigens while remaining unresponsive to self-antigens. This is checked centrally by negative selection of immature CD4+ T-cells recognising self-antigen and peripherally by anergy, apoptosis, and/or production of Tregs [11]. Mounting evidence has emerged that alloimmunity is not only directed against HLA, but also non-HLA and lung-associated self-antigens, suggesting a role for autoimmunity in the pathogenesis of CLAD [7, 21].

Collagen V (Col-V) and K-alpha 1 tubulin (Kα1T), two prominent self-antigens, are both components of small airways and are normally not exposed to the host immune system [7]. Col-V is found in the skin, lung epithelium and perivascular and peribronchial tissues, and placenta. It is an immunogenic self-protein that normally effectively masks its epitopes from the immune system because it is assembled in the same fibril as collagen I [11]. However, allograft injury (e.g., due to ischaemia-reperfusion injury, infection, DSA) enhances exposure of these antigenic proteins and results in the release of lung-derived autoantigens as soluble antigens, exosomes or apoptotic bodies. These are detected and then presented by APCs leading to the propagation of autoimmune responses through the Th17–IL-17 axis [11, 21]. This is possibly initiated by increased cleavage of Col-V due to upregulation of matrix metalloproteinases (MMP) 2 and 9 [21, 62], alongside loss of peripheral tolerance due to downregulation of Tregs and loss of IL-10 response to self-antigens [10, 62, 63].

Kα1T is a gap junction protein, essential for cytoskeletal structure and normal cellular function [11]. Similar to Col-V, repeated injury of the airway epithelium exposes Kα1T, resulting in expression of transcription and growth factors involved in fibroproliferation, suggesting that antibodies to Kα1T are directly pathogenic [21, 64].

A strong correlation between these antibodies and CLAD has been reported, in some instances in the absence of classic HLA antibodies. Conversely, autoantibody-mediated graft damage can trigger de novo DSA generation [64–66]. Although DSA can be transient, antibodies to self-antigens are often persistent. In patients with antibodies to both DSA and self-antigens, those who cleared DSA but had persistent autoantibodies were significantly more likely to develop BOS [67]. Moreover, patients with pre-existing autoantibodies had increased risk of developing de novo antibodies to DSA and non-HLA, AMR, primary graft dysfunction and CLAD [21, 65, 66]. Large cohort studies revealed that up to 30% of patients undergoing lung transplantation had pre-existing antibodies to lung self-antigens, primarily in patients with idiopathic pulmonary fibrosis and cystic fibrosis [65].

Taken together, both pre-existing and de novo lung self-antigens contribute to acute and chronic lung rejection through an interplay between allo- and autoimmunity, in which allograft immune responses may trigger autoimmune responses, which in turn further activate alloimmune responses. While alloimmunity may have initiated allograft injury, autoimmunity may ultimately contribute to the progression of CLAD [10, 21].

Several other autoantibodies have been described in other solid organ transplant recipients, and data on these autoantibodies are gradually becoming available in lung transplant recipients [68]. Firstly, antibodies to MHC class I-related chain A, expressed on endothelial cells and monocytes, have been associated with increased graft failure after kidney transplantation [68]. Likewise, Lyu et al. [69] and Angaswamy et al. [70] described a correlation between these antibodies and BOS. Secondly, the presence of angiotensin type 1 receptor or endothelin type A receptor antibodies correlated with allograft rejection in kidney and heart transplants [71]. Reinsmoen et al. [71] investigated the impact of these antibodies on graft outcome in lung transplantation and reported a trend toward higher ACR rates and an increased risk of de novo DSA. Follow-up time was not sufficient to observe CLAD outcome.

Innate immunity

It has been increasingly recognised that an advanced interplay between innate and adaptive immunity drives graft injury. Several innate immune pathways facilitate recruitment of inflammatory cells into the allograft and are key elements in the pathogenesis of primary graft dysfunction, acute rejection, and CLAD [72]. Innate immunity encompasses a broad spectrum of immune responses mediated by elements that are
not reliant on gene rearrangement, including polymorphonuclear leukocytes, macrophages, NK cells and the complement system [72]. Innate recognition depends on pathogen- and damage-associated molecular patterns (PAMPs/DAMPs), recognised by pattern recognition receptors such as Toll-like receptors (TLRs), the receptor for advanced glycosylation endproducts and nucleotide-binding oligomerisation domain-like receptors [6]. DAMPs are endogenous molecules released from injured cells, such as high-mobility group box 1, heat-shock protein, hyaluronan, adenosine triphosphate, donor-derived cell-free DNA and mitochondrial DNA [72]. Recognition leads to immediate (sterile) inflammation, characterised by recruitment of mainly neutrophils and macrophages, upregulation of MHC expression and antigen presentation, followed by activation of the adaptive immune system (figure 1) [10]. The exact immune mechanisms in RAS have yet to be elucidated, but DAMPs appeared to be upregulated to a greater extent compared to BOS [73].

TLRs are transmembrane receptors mainly expressed by macrophages and dendritic cells, serving as a bridge between innate and adaptive immunity because of their ability to induce T-cell responses [10]. On the other hand, TLRs might contribute to CLAD directly [10]. For example, TLR4 signalling can induce fibroblast activation together with TGF-β, and in the case of sustained innate immune activation, the process of fibroblast activation might persist, leading to excess repair and fibrotic tissue remodelling [72].

Neutrophils play an important role not only in innate immunity, but also by enhancing antigen presentation and Th1-driven alloimmune responses [7]. Elevated bronchoalveolar lavage (BAL) and allograft neutrophilia have been repeatedly observed in patients with BOS and RAS, and early or persistent BAL neutrophilia correlated with subsequent CLAD occurrence [32, 74–79]. The relevance of neutrophils was further supported by the emergence of neutrophilic reversible allograft dysfunction, characterised by IL-17-mediated airway neutrophilia, in which azithromycin was able to attenuate pulmonary function decline [80]. IL-17 can induce IL-8, a major neutrophil chemo-attractant. Multiple studies demonstrated higher levels of BAL IL-8 in BOS patients with a correlation between neutrophils and IL-8 levels [77, 79]. IL-8 is secreted by alveolar type II epithelial cells, bronchial epithelial cells and macrophages after the release of proinflammatory cytokines [81]. In some patients, neutrophilia was not suppressed or redeveloped despite azithromycin, suggesting a non-IL-17-dependent pathway. Indeed, Vandermelven et al. [80] found worse CLAD-free and overall survival in those patients, possibly driven by increased levels of IL-1β and IL-1β-induced proinflammatory cyto-/chemokines (e.g., IL-6, IL-8, macrophage inflammatory proteins, eosinophil attractants). Suwara et al. [78] also demonstrated an increase in IL-1α and IL-1β in patients with persistent airway neutrophilia. Activated neutrophils have remarkable potential to cause tissue damage through a variety of mechanisms: 1) release of large quantities of reactive oxygen species, 2) release of cytokines, 3) activation of hydrolytic enzymes and proteases, 4) expression of MMP that leads to degradation of collagen matrix [82]. An additional mechanism of neutrophil-mediated injury is the formation of neutrophil extracellular traps (NETs), a process known as NETosis. NETs are extracellular networks of DNA clad with granular proteins that were cast out from neutrophils and are thought to be an effector function of neutrophils [82] (figure 2).

Eosinophils have also been implicated in the pathological process of CLAD. Two decades ago, Scholma et al. [83] had already noted that BAL eosinophilia correlated with increased BOS risk (RAS was not yet identified then). Likewise, a more recent study demonstrated a significant correlation between BAL eosinophilia and the development of CLAD, in particular RAS, and mortality [84]. The same group also found higher eosinophil levels in allograft tissue from RAS patients, and worse CLAD-free survival in patients with high blood eosinophils [32, 85]. Additionally, Darley et al. [86] demonstrated that detection of eosinophils on transbronchial biopsies was independently associated with an increased risk of CLAD and mortality. The actions of eosinophils are thought to be secondary to profibrotic features, by attracting fibroblasts and stimulating TGF-β release, as well as through toxic effects on airway epithelial cells (e.g., increased membrane permeability, ciliary damage) [84, 86]. Conversely, translational data from animal models recently illustrated a role for eosinophils in the downregulation of alloimmunity, potentially by the release of suppressive molecules or interactions with dendritic cells and lymphocytes [87]. These immunosuppressive effects are presumably exerted by a different subtype of eosinophils, such as tissue-resident eosinophils, although this needs to be further elucidated [87].

NK cells act as the first line of defence against infected or transformed cells and can directly respond to alloantigens and non-self cells through an arsenal of effector functions that are vital in innate–adaptive bridging [88, 89]. Increased numbers of activated NK cells were found in the lungs of CLAD patients, with corresponding peripheral blood depletion, suggesting systemic activation and subsequent migration into the allograft tissue [89]. Once activated, NK cells release a wide range of cytolytic proteins, such as granzymes and perforin, and chemotactic cytokines such as IFN-γ and TNF-α, which were found to be

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Repetitive injury to the bronchial epithelium and/or vascular endothelium creates a strong inflammatory cascade, which not only causes direct damage to the allograft, but also results in amplified immune responses, increases the antigenicity of the allograft and risk for development of DSA and tissue-restricted autoimmunity, ultimately contributing to irreversible fibrosis and allograft dysfunction.
upregulated in CLAD [90]. Through the release of these cytokines, NK cells commit T-cells, skew immune responses to Th1, increase MHC class I and II expression, and induce graft infiltration by macrophages, dendritic cells and neutrophils [90]. Moreover, NK cells’ upregulation of Fc-receptors plays an important role in antibody-dependent T-cell-mediated cytotoxicity [88].

There is mounting evidence that NK cells have crucial and sometimes opposing roles in lung allograft rejection, due to either activating or inhibitory actions through different NK receptors [88]. NK cells might enhance CLAD through the above-described cytotoxic and inflammatory effects. On the other hand, it has been postulated that they may promote graft tolerance through depletion of donor APC and alloreactive T-cells via killer immunoglobulin-like receptors or possibly via IL-15–IL-15Ra complex expansion [88, 91]. Nonetheless, the exact mechanisms by which NK cells contribute to CLAD remain to be investigated.

Other innate lymphoid cells (ILC1, ILC2, ILC3) are a recently recognised and understudied group of immune cells, which are difficult to analyse because of their tissue-resident and lineage negative features. However, they can exert different actions such as type 1 immunity with macrophage activation and cytotoxicity, type 2 immunity and the formation of tertiary lymphoid structures by their different subtypes via the release of IFN-γ, granzymes, perforin, TNF-α, IL-13, IL-17, etc. [92, 93]. As such, it does not seem unlikely that they contribute to the pathogenesis of CLAD, and further investigation is warranted [94–96].

The complement system is a complex immune surveillance system made up of a cascade of multiple proteins, crucial in innate defence, and it plays a role in adaptive immunity via cell-mediated and humoral processes [97]. There are three different activation pathways (classical, lectin and alternative), all leading to the formation of a membrane attack complex which induces cell lysis. Furthermore, it stimulates immune complex clearance by opsonisation and, during activation of the complement cascade, signalling components known as anaphylatoxins are released and are capable of summoning various other innate and adaptive immune cells by stimulation of proinflammatory cytokines and chemotaxis [97]. Complement activation plays a role in the pathogenesis of primary graft dysfunction and AMR, which are risk factors for CLAD, but a direct contribution in the pathogenesis of CLAD is also assumed. Deposition of complement factors C1q, C3d and C4d in lung allografts was found to be independently associated with CLAD [97]. Higher levels of complement and IgG deposition were found in RAS compared to BOS patients, pointing to the role of humoral immunity and activation of B-cells in RAS, and the possible overlap between AMR and RAS [98, 99]. Several studies yielded some evidence that mannose-binding lectin, part of the lectin pathway, is involved in CLAD development. Higher levels were found in BOS versus stable patients, and presence of mannose-binding lectin at 3 and 6 months post-transplant correlated with later onset of BOS [97].

In conclusion, innate immune responses provide an early, robust trigger that augment adaptive alloimmunity, ultimately promoting CLAD development.

Exosomes

Recently, exosomes have begun to attract attention as a trigger in CLAD development through activation of cellular and humoral immunity. Exosomes are dual-layer membrane vesicles which can contain HLA and lung self-antigens, adhesion and costimulatory molecules, MHC class II molecules, transcription factors, and 20S-proteasome. They are shed from allograft cells after lung injury and are highly efficient in presenting antigens to the immune system [100–102]. Exosomes have been shown to induce T-cell-mediated immune responses, and the induction and continuous release of exosomes from the allograft may stimulate the process of CLAD [21]. Furthermore, a recent animal study demonstrated the ability of exosomes, derived from lung transplant recipients with respiratory viral infections, to induce epithelial-to-mesenchymal transition (EMT) [103]. The role of exosomes in promoting EMT has been highlighted in cancer research and could be another way by which exosomes might initiate the process leading to CLAD [103]. Several studies demonstrated higher levels of exosomes, which also contained more of the aforementioned factors, in BOS patients [101, 104]. Furthermore, Sharma et al. [102] found that increased levels of circulating exosomes preceded the onset of BOS and could be detected 6–12 months before diagnosis.

Genetic variants associated with CLAD

Several donor- and recipient-related genetic variants may contribute to the development of CLAD [4]. Specific single nucleotide polymorphisms in TLR2, -4 and -9, were associated with a higher incidence of BOS [105]. Other types of polymorphisms in TLR4 correlated with a reduced risk of acute rejection and a trend toward reduced onset of BOS [106]. These findings again reinforce the importance of the link between innate immune responses and alloimmune response in the development of CLAD.
A polymorphism in HLA-G seemed to have a protective role by modulating cytotoxic T-cells and NK cells, while a specific HLA-E allele negatively influenced CLAD onset [107]. There is some evidence that functional polymorphisms in the genes of CD14, dectin-1, IFN-γ, IL-6, IL-17A, killer immunoglobulin-like receptors, mannose-binding lectin, MMP-7 and TGF-β1 are linked to CLAD development [108–110]. Regarding donor-related polymorphisms, gene polymorphisms in surfactant proteins, donor Clara cell secretory proteins, mannose-binding lectin and CD59 correlated with increased CLAD risk [97, 111–114].

In general, these genetic variants affect the innate defence system, altering immune responses to injury, possibly increasing susceptibility for airway inflammation or allograft infection, thereby contributing to the pathogenesis of CLAD [4].

**Repair and regeneration processes**

**Aberrant epithelial repair**

Dysregulated epithelial repair and airway and/or tissue remodelling are cornerstones in the pathogenesis of CLAD [115]. Repetitive or persistent alloreactive, autoreactive, infective or non-specific epithelial injury leads to the loss of epithelial integrity and dysregulated repair [6]. A disbalance between pro- and anti-inflammatory cytokines can induce an excessive fibroblastic response with excessive extracellular matrix remodelling, leading to small airways and/or parenchymal fibrosis [115]. Multiple growth factors are involved in this process and are secreted by epithelial cells, fibroblasts and inflammatory cells [6]. TGF-β1 plays a key role, by inducing fibroblast proliferation and differentiation into myofibroblasts [116]. The process by which the normal epithelium is replaced by fibroblastic scar tissue is believed to be based on TGF-β1-driven EMT, as illustrated in animal models and in vitro [115–117]. This can be further stimulated by activated macrophages via TNF-α [116]. During EMT, epithelial cells lose their epithelial properties and acquire a mesenchymal cell phenotype, including deposition of extracellular matrix and production of MMPs [115]. A similar mechanism has been postulated in RAS. Indeed, *in vitro* treatment of human pleural mesothelial cells with TGF-β1 led to mesothelial-to-mesenchymal transition [118].

MMPs, derived from bronchial/bronchiolar airway epithelium and parenchymal cells, are capable of degrading extracellular matrix proteins, cleaving collagen, and are involved in cell proliferation, migration and apoptosis [119]. Significantly increased MMP levels (MMP-2, -3, -7, -8, -9) were found in BAL and airway epithelial cells from BOS patients, and excess MMP activity may facilitate uncontrolled extracellular matrix turnover, epithelial damage, fibrosis and tissue remodelling [119–121]. In addition to epithelial cells, neutrophils may be another source of MMPs, able to store and release MMPs from their granules [119]. Several studies showed that MMP-8 and -9 levels correlated with BAL neutrophilia in patients with BOS, and along with their role in tissue remodelling, these MMPs may also perpetuate neutrophilic inflammation via a self-sustaining loop [119, 121].

In addition to TGF-β, liver kinase B1 (also known as serine-threonine kinase 11) might also have a role in the process of EMT in CLAD. Liver kinase B1 is a protein kinase that activates adenosine monophosphate-activated protein kinase and many related kinases, and regulates cell growth, cell polarity, cell metabolism and autophagy [122, 123]. Hereby, liver kinase B1 inhibits EMT and tissue fibrosis and Rahman et al. [122] recently demonstrated that liver kinase B1 was significantly downregulated in patients with BOS.

Most studies analysing profibrotic mediators in CLAD focus on (myo)fibroblasts, TGF-β, TNF-α, MMPs and tissue inhibitors of metalloproteinases. With respect to other common fibrotic factors, little is known about the role of connective tissue growth factor (CTGF) in CLAD, though it is an important mediator in several fibrotic diseases, such as idiopathic pulmonary fibrosis. A recent study demonstrated higher levels of tissue CTGF expression in BOS and RAS compared to controls. Interestingly, BAL levels of CTGF were higher in RAS compared to BOS and stable patients, and also elevated at 3 months post-transplant in future RAS patients, perhaps suggesting a more specific role for CTGF in the pathogenesis of RAS [124].

**Angiogenesis and vascular changes**

Besides epithelial injury, damage to the airway microvasculature also seems important. Lung tissue analyses showed that obliterator bronchiolitis was associated with increased angiogenic activity, and vascular remodelling was an important feature of tissue remodelling [6, 125]. Airway inflammation itself appeared to be the main determinant of this angiogenic remodelling, through proinflammatory cyto-/chemokines, with a smaller role for vascular endothelial growth factor [125]. Regardless, the role of angiogenesis in CLAD remains incompletely understood with opposing findings in the literature [126]. Further research on vascular changes in CLAD is needed, especially given a recent study showed that...
nearly half of BOS patients had chronic vascular abnormalities (e.g., pulmonary arteriopathy and venopathy, bronchial arterial vasculopathy) [61].

Alloimmune-dependent risk factors

Acute cellular rejection and lymphocytic bronchiolitis

Alloimmune-dependent factors such as ACR and lymphocytic bronchiolitis (LB) are strongly linked to CLAD [6]. ACR has been studied in more detail, but independent of acute vascular rejection, the onset and severity of LB is also associated with long-term outcomes after lung transplantation and an increased risk of BOS and death [127]. ACR and LB are driven by T-lymphocytes and many actions are similar as described in CLAD such as a predominance of Th1 cells with increased production of IFN-γ, IL-2 and TNF-α, activation of macrophages, direct allograft damage through Fas/Fas ligand-mediated cytotoxicity, and reduced Tregs [16, 25, 122]. There is currently too little evidence, but donor tissue-resident memory T-cells may play a protective role in ACR [22].

Although T-lymphocytes are regarded as the main culprit in ACR, other mechanisms contribute as well. It is well known that increased leukocytes, including lymphocytes and neutrophils, are found in BAL and tissue of patients with ACR, and increasingly more awareness is given to eosinophils and NK cells [84, 128–131]. Not much is currently known about the role of B-cells in acute rejection, but a recent study reported a decrease in the number of Bregs in peripheral blood and BAL during acute rejection and the role of B-cells in local lymphoid follicle formation could also be of importance in LB [16, 132]. It is worth noting that HLA antibodies do not only appear to be involved in the onset of AMR and CLAD, but also in ACR and LB [133, 134]. On the other hand, ACR and LB may predispose to de novo DSA [135].

In LB, there is convincing evidence of an IL-17-mediated pathway, which triggers IL-8-driven neutrophilic airway inflammation [130, 136]. Verleden et al. [136] found that patients with LB had significantly more IL-17+ cells on transbronchial biopsies compared to patients with ACR, and the number of IL-17+ cells correlated with BAL neutrophilia. Not Th17 cells, but CD8+ T-cells were the major source of this IL-17 production, which could be attenuated by azithromycin [137].

Ultimately, the alloreactive T-cell response and IL-17-mediated inflammation generate a profibrotic environment which can contribute to CLAD [130].

Antibody-mediated rejection

AMR results from the recipient’s immune system recognising pre-existing or de novo antibodies to HLA, non-HLA or self-antigens [60]. Pre-existing anti-HLA antibodies may arise after prior sensitizing events such as pregnancy, blood transfusion or organ transplantation [41]. Risk factors for de novo DSA are only beginning to be identified. It is postulated that immunising events (e.g., transfusion, ACR) and lung injury (e.g., ischaemia-reperfusion injury, allograft infection) upregulate the expression of HLA molecules, thereby increasing the graft’s immunogenicity [60]. Antibodies may develop to MHC class I antigens (HLA-A, -B, -C) which are expressed on nearly all nucleated cells, or MHC class II antigens (HLA-DQ, -DR, -DP) on professional APCs [41].

The binding of antibodies to directly accessible allogenic targets expressed by endothelial cells activates the classical complement pathway. This begins with binding of C1q, and eventually leads to membrane attack complex formation and cytotoxicity [34, 41]. Deposition of complement and IgG in lung allograft tissue have both been demonstrated [41, 138]. Activation of endothelial cells leads to the release of adhesion molecules and cytokines that, together with anaphylatoxins C3a and C5a, attract neutrophils, monocytes and NK cells to the allograft, propagating inflammation and graft injury [34, 41]. Complement-mediated allograft injury is a defining pathophysiological characteristic of AMR. However, graft injury can also occur independently of complement pathways through antibody-dependent cell-mediated cytotoxicity [88]. The latter is likely mediated by NK cells, recognising antibodies through their Fc-receptor, CD16, although antibodies can also bind the Fc-receptor of myeloid cells such as macrophages and neutrophils. In this way, antibodies bridge the innate and adaptive arms of the immune system [88].

Both complement-dependent and -independent mechanisms lead to the production of IFN-γ and other proinflammatory cyto-/chemokines, increased MHC expression, recruitment of leukocytes and platelets, amplification of innate and adaptive immunity, and upregulation of adhesion molecules and fibroblast growth factor receptor on endothelial cells. All these mediators contribute to microangiopathy, tissue injury and graft dysfunction [9, 41, 88].
Not all patients with DSA develop AMR, the clinical relevance of DSA may depend on the variable pathogenicity of IgG subclasses. Complement-binding IgG (IgG1, IgG3) seemed more damaging than non-complement-binding IgG (IgG2, IgG4) [43, 55]. Higher rates of early BOS were found in cases of increased C3d and C4d deposition early after transplantation [139]. Similarly, DSA-positive patients with increased C3d deposition had lower graft survival than those without C3d activation [138].

Although AMR might be a reversible cause of acute graft dysfunction, it generally portends a poor prognosis with a high incidence of CLAD amongst survivors and worse long-term survival compared to ACR [60].

**Alloimmune-independent risk factors**

In addition to immune-mediated lung injury, various other factors have been linked to the onset of CLAD, including ischaemia-reperfusion injury, respiratory infections, gastro-oesophageal reflux, air pollution and (inhaled) toxins [4]. Lung allografts are uniquely susceptible to injury from exogenous agents due to their constant exposure to the external environment and, since the oesophagus and trachea are anatomically connected, the lung is at risk of exposure to gastric contents through gastro-oesophageal reflux and (micro) aspiration [7, 72].

In general, it is postulated that these “alloantigen-independent” lung injuries contribute to CLAD by direct damage to the allograft epithelium and/or endothelium as well as upregulating the tissue inflammatory milieu. The induction of a strong inflammatory cascade by epithelial injury directs an alloimmune response via downstream effects, promoting clonal expansion of alloreactive T- and B-cells, upregulation of HLA class II molecules and enhanced antigen presentation. This facilitates allorecognition, thereby increasing the antigenicity of the allograft and risk for development of DSA as well as tissue-restricted autoimmunity. This ultimately predisposes to CLAD through subsequent recruitment of fibroproliferative growth factors, excessive airway/tissue remodelling, and eventually airway/tissue fibrosis and allograft dysfunction [6, 7, 140].

Some of the main mechanisms involved in these non-alloimmune factors are displayed in figure 2.

**Missing pieces of the puzzle**

Based on these findings, we believe future research in the lung transplant setting should focus on:

- The role of Tregs in preventing or slowing down CLAD onset and progression.
- The role of Th9 and Th22 T-cell subsets, memory T-cells, and γδ T-cells in the pathogenesis of CLAD.
- The specifics of B-cell regulation and interactions between B- and T-cells in CLAD pathogenesis, and the possible role of Bregs in immunomodulation and suppression of immune responses in CLAD.
- Subtypes of innate immune cells (e.g., eosinophils, NK cells, macrophages) and their potential to promote or inhibit alloimmune responses in CLAD.
- Description of (chronic) vascular changes in BOS and RAS, and the role of lymphoid neogenesis and angiogenesis in the onset of CLAD.
- How to deal with anti-HLA, non-HLA and autoantibodies prior to and after transplantation.
- Identification of specific immune cells or profibrotic pathways (e.g., EMT) which are targetable for treatment.
- Ways to establish immune tolerance after lung transplantation.

**Conclusion**

Over the last decades, we have gained a better understanding of how the immune system contributes to the development of CLAD, although the exact pathophysiological mechanisms are still not completely understood. Complex and overlapping immune-mediated mechanisms, including cellular, humoral, innate, adaptive and autoimmune processes, have been implicated as the leading causes of CLAD. It is increasingly recognised that non-alloimmune mechanisms have a crucial role due to (repetitive) epithelial injury, creating a privileged immune microenvironment, resulting in amplified immune responses. The central belief is that CLAD is the end-stage of a disease continuum marked by continuous/repeated lung injury, immune activation, tissue remodelling and repair, ultimately leading to irreversible fibrosis and allograft failure.

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