# Viral reactivations and fungal infections in nonresolving acute respiratory distress syndrome

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**Viral reactivations and fungal infections, in particular by CMV, HSV and *Aspergillus*, occur frequently and form a vicious circle with lung injury in nonresolving ARDS** https://bit.ly/4g0vIrm

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## Abstract

Acute respiratory distress syndrome (ARDS) is a condition affecting 10% of patients requiring admission to the intensive care unit and results from endothelial dysfunction, alveolar epithelial injury and unbalanced inflammation, leading to exudative pulmonary oedema. A significant portion of these patients experience a lung injury that fails to resolve. Persistent or worsening respiratory failure beyond 5 days after the initiation of mechanical ventilation is referred to as nonresolving ARDS. Viral and fungal pathogens can exploit the hyperinflammatory environment and altered immune landscape in ARDS, perpetuating a cycle of ongoing inflammation and lung injury, thereby contributing to the progression towards and persistence of nonresolving ARDS, even in previously immunocompetent patients. This review discusses the significance, pathophysiology, diagnostic challenges and key knowledge gaps concerning various viral and fungal pathogens in nonresolving ARDS, with a particular focus on influenza-associated and COVID-19-associated pulmonary aspergillosis and pulmonary reactivation of *Herpesviridae*, such as cytomegalovirus and herpes simplex virus. Diagnosing these infections is challenging due to their nonspecific clinical presentation and the inability of current tests to distinguish between fungal colonisation or asymptomatic viral shedding and clinically significant infections or reactivations. A deeper understanding of the complex interplay between these pathogens and the host immune system in the context of ARDS, combined with advances in diagnostic and therapeutic strategies, has the potential to enhance the management and prognosis of patients with nonresolving ARDS.

## Introduction

Acute respiratory distress syndrome (ARDS) is the result of endothelial dysfunction, alveolar epithelial injury and dysbalanced inflammation, or a combination thereof, resulting in protein-rich pulmonary oedema [1]. ARDS is common in patients admitted to the intensive care unit (ICU), accounting for approximately 10% of acute ICU admissions [2]. The most common risk factors include pneumonia, sepsis, aspiration, major surgery, trauma, pancreatitis and transfusions. There is no widely accepted pharmacological treatment of ARDS and mortality remains high, at around 30–40%. Lung protective mechanical ventilation and treatment of the underlying cause are the mainstays of ARDS care [3, 4]. There is considerable variation in the duration of mechanical ventilation needed, depending on patient characteristics, the underlying cause of the ARDS and the severity of pulmonary injury.

The trajectory of clinical resolution is informative of patient recovery. Some patients recover from ARDS quickly and these patients have favourable outcomes [5, 6]. On the other hand, an important proportion of patients show persistent or progressive lung injury beyond the first 5 days of mechanical ventilation [7].

This so-called nonresolving ARDS is associated with high morbidity and mortality and caring for these patients has specific challenges. Alveolar hyperinflammation is a likely contributing factor preventing the resolution of lung injury [8]. Reduction of such inflammation with steroids, however, has not provided a clear-cut benefit, especially when administrated after 2 weeks of mechanical ventilation [9].

While nonresolving lung injury is often intrinsic to the initial insult causing ARDS, opportunistic infections have been identified in a considerable proportion of the selected patients in whom open lung biopsies were performed [10]. With the development of culture-independent pathogen identification tools, such as genetic detection using PCR and metabolic detection of cell-wall components, pathogen identification is nowadays more feasible than ever, using less-invasive tools such as bronchoscopy with bronchoalveolar lavage (BAL) [7]. In this narrative review, we will evaluate the currently available evidence linking viral and fungal opportunistic infections to persistent inflammation and lung injury in patients with nonresolving ARDS.

## Methods

To identify relevant studies regarding fungal infections in nonresolving lung injury, we performed a PubMed search using the following the Boolean search string: ("aspergillosis" OR "aspergillus" OR "fungal" OR "mold" OR "mould") AND ("intensive care" OR "ICU" OR "critical care") AND ("ARDS" OR "acute respiratory distress syndrome" OR "lung injury" OR "inflammation" OR "influenza" OR "COVID"). To identify relevant studies on the reactivation of cytomegalovirus (CMV) and herpes simplex virus (HSV) in ARDS patients, we performed a PubMed search using the following Boolean search string: ("acute respiratory distress syndrome" OR "acute lung injury" OR "critically ill" OR "intensive care patients" OR "intensive care unit") AND ("cytomegalovirus reactivation" OR "cytomegalovirus co-infection" OR "herpes simplex virus reactivation" OR "CMV" OR "HSV" OR "herpes simplex virus" OR "cytomegalovirus"). These searches were last updated on 28 June 2024, at which time they yielded 1251 and 1168 results, respectively. Abstracts were screened for relevance to the topic and relevant studies were included in this review. Additional studies were added from reference lists.

## Fungal infections as a source of persistent tissue damage and inflammation

Respiratory fungal disease is widely recognised as a cause of morbidity and mortality in patients with classic host factors for invasive mould disease as defined by the European Organisation for Research and Treatment of Cancer and Mycoses Study Group Education and Research Consortium criteria, such as neutropenia, haematologic malignancy or solid organ or allogeneic stem-cell transplant [11]. However, invasive mould infections can also affect critically ill patients without classic host factors [12]. Fungal pathogens can benefit from the inflammatory environment and altered immune landscape in ARDS and present as a source of persistent tissue damage and lung injury in patients admitted to the ICU with this syndrome (figure 1).

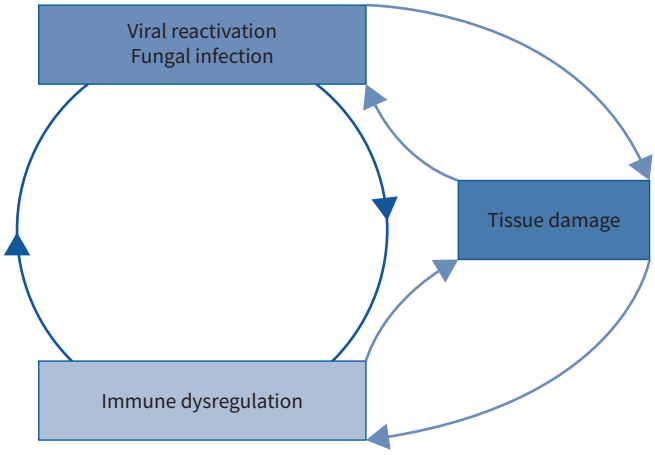


**FIGURE 1** Vicious circle of tissue damage, immune dysregulation and viral or fungal infection. Tissue damage, as seen in (nonresolving) lung injury, provokes immune dysregulation, which on its own may lead to more tissue damage. Immune dysregulation and tissue damage can both enable reactivation of latent viruses (cytomegalovirus (CMV) and herpes simplex virus (HSV)) and growth and germination of fungal spores (especially *Aspergillus*). These pathogens can directly lead to tissue damage or can cause tissue damage by further dysregulating the immune response. Figure created with BioRender.com.

## Clinical aspects of fungal infections in nonresolving ARDS

### Epidemiology and outcome of invasive aspergillosis in ARDS

In the context of nonresolving ARDS, *Aspergillus* species are most frequently causing superinfections and are therefore studied most extensively in this setting. Besides patients with classic host factors, invasive pulmonary aspergillosis (IPA) mainly affects patients with ARDS due to severe viral respiratory infections [11]. Both severe influenza and COVID-19 have been recognised as independent risk factors for developing IPA [13–17]. In thoroughly sampled cohorts (*i.e.* with frequent use of BAL sampling), influenza-associated pulmonary aspergillosis (IAPA) and COVID-19-associated pulmonary aspergillosis (CAPA) can be found in almost 20% of patients admitted to ICU for severe viral pneumonia (table 1) [27]. Patients with IAPA or CAPA exhibit a mortality rate of approximately 50% and therefore exhibit a more than twofold higher all-cause mortality risk compared to patients without *Aspergillus* superinfection [28, 29].

IPA has been reported to a lesser extent in other infections with respiratory viruses such as respiratory syncytial virus, parainfluenza virus and adenovirus, predominantly affecting severely immunocompromised patients, such as organ transplant recipients [30]. Nonviral causes of ARDS may be complicated by IPA as well, for instance bacterial pneumonia [31], drowning [32, 33] or burn inhalation [34]. The exact incidence of IPA in these populations, however, is understudied but appears to be lower than the incidence encountered in severe viral pneumonia [13].

### Diagnosis and treatment of IPA

IPA is hard to diagnose, exemplified by its occurrence in lists of most frequently missed diagnoses found upon autopsy after death in ICU [35]. In non-neutropenic patients with probable or proven IPA, *Aspergillus* galactomannan antigen is positive in serum in only a minority of patients [36]. Therefore, bronchoscopy with BAL sampling for mycological tests is the preferred method for IPA diagnosis [27]. Nonbronchoscopic sampling of the respiratory tract (*e.g. via* nonbronchoscopic lavage or use of tracheal or bronchial aspiration) can be used in case human or material resources for bronchoscopy are lacking. However, mycological techniques in nonbronchoscopic samples often lack validation and show reduced diagnostic accuracy compared to BAL samples [37].

**TABLE 1** Incidence of influenza-associated pulmonary aspergillosis (IAPA) and COVID-19-associated pulmonary aspergillosis (CAPA) in severe influenza and COVID-19

| Study, year | Design | Patient group | Diagnostic criteria | Bronchoscopy or BAL sampling rate[#] | EORTC/MSGERC positivity | Positivity for VAPA |
|---|---|---|---|---|---|---|
| **IAPA** | | | | | | |
| Schauwvlieghe *et al.* [13], 2018 | R | ICU, influenza | mAspICU | 233/432 (54%)[¶] | 117/432 (27%) | 83/432 (19%) |
| Schwartz *et al.* [18], 2020 | R | ICU, influenza | mAspICU | 61/110 (55%)[+] | NA | 8/110 (7%) |
| Waldeck *et al.* [19], 2022 | R | ICU, influenza | (m)AspICU | 51/158 (32%)[¶] | NA | 17/158 (11%) |
| Krifors *et al.* [20], 2023 | P | ICU, influenza | IAPA expert consensus | 24/55 (44%)[¶] | 1/5 (20%) of IAPA cases | 5/55 (9%) |
| Feys *et al.* [21], 2024 | R | ICU, influenza, MV only | IAPA expert consensus | 142/142 (100%)[¶] | 39/142 (27%) | 59/142 (42%) |
| **CAPA** | | | | | | |
| Ergün *et al.* [22], 2021 | P | ICU, COVID-19 | ECMM/ISHAM | 77/219 (35%)[¶] | 21/219 (9%) | 39/219 (18%) |
| Janssen *et al.* [15], 2021 | P | ICU, COVID-19 | ECMM/ISHAM | 301/823 (37%)[¶] | 67/414 (16%)[§] | 63/823 (8%) |
| Ghazanfari *et al.* [23], 2021 | P | ICU, COVID-19, MV only | ECMM/ISHAM, IAPA expert consensus | 105/105 (100%)[¶] | 2/22 (9%) of CAPA cases | 22/105 (21%) |
| Hurt *et al.* [24], 2023 | P | ICU, COVID-19, MV only | ECMM/ISHAM | 130/266 (49%)[¶] | 14/266 (5%) | 29/266 (11%) |

For both IAPA and CAPA, only studies reporting the proportion of patients who had bronchoscopy or bronchoalveolar lavage (BAL) sampling performed during their intensive care unit (ICU) stay were included. For CAPA, only multicentric studies were selected due to the high number of monocentric studies published. [#]: Bronchoscopy or BAL sampling rate represents the proportion of patients who had at least one bronchoscopy or BAL sampling during their ICU stay. [¶]: Rate of BAL sampling (for mycological testing) reported in study. [+]: Bronchoscopy sampling rate reported in study. [§]: Only the number of patients for whom all European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium (EORTC/MSGERC) [11] host factors were assessed are given here. ECMM/ISHAM: European Confederation of Medical Mycology/International Society for Human and Animal Mycology consensus criteria [25]; mAspICU: modified *Aspergillus* in ICU criteria [13]; MV: mechanical ventilation; NA: not applicable; P: prospective; R: retrospective; VAPA: viral-associated pulmonary aspergillosis; IAPA expert consensus: IAPA expert consensus criteria by Verweij *et al.* [26].

Fungal culture, *Aspergillus* antigen and PCR testing are the mycological tools that should be used on BAL samples in the mycological work-up for IPA [17, 37]. Fungal culture should not be used as the sole mycological test, as its sensitivity is only about 50%. ELISA for galactomannan, an *Aspergillus* antigen, is the mycological test with the highest diagnostic yield, with a sensitivity and specificity for IPA exceeding 80% in critically ill patients [38, 39]. Point-of-care lateral flow tests for *Aspergillus* antigens are increasingly used, bypassing the sometimes-long turnaround time for galactomannan ELISA testing (of which most available assays must be performed in batch). Some of these point-of-care-tests have been extensively validated with sensitivity and specificity approaching those of the galactomannan ELISA [40], but many other assays currently lacking clinical validation are coming to the market and should be used with care [37]. Additionally, automated and random-access chemiluminescent assays that allow individual testing with a short turnaround time have been developed [41, 42]. A drawback of the mycological tests mentioned above is that they cannot distinguish between invasive disease and fungal colonisation. To establish a diagnosis of proven aspergillosis, microscopical examination of lung tissue remains necessary [25, 26, 39], which is often only possible to obtain *post mortem* upon autopsy.

Several diagnostic criteria are available for diagnosing IPA in patients admitted to the ICU. While the FUNDICU (Fungal Infections in the Intensive Care Unit) criteria provide recent overarching guidelines for diagnosis of aspergillosis in ICU [43], targeted criteria exist for patients with influenza or COVID-19 specifically [25, 26]. These criteria allow establishing a probable or proven diagnosis of invasive aspergillosis based on the presence of host factors, clinical factors and mycological evidence.

Treatment primarily relies on antifungal agents, with mould-active azoles (voriconazole, isavuconazole or posaconazole) being the first-choice regimen [44, 45]. In case of azole resistance or contraindications to azole usage, liposomal amphotericin B is the alternative drug of choice [45].

#### *Other fungi*

Other fungi are rare causes of superinfection in nonresolving ARDS. Mucorales have been reported to cause rhino-orbital and pulmonary invasive disease in COVID-19, especially in patients with poorly controlled diabetes mellitus, diabetic keto-acidosis, corticosteroid treatment or other immunosuppressive factors [46–48]. Even in India, the country in which COVID-19-associated mucormycosis was described the most, overall incidence in patients admitted to ICU was below 2% [46]. Whereas *Pneumocystis jirovecii* is a well-recognised cause of ARDS in immunocompromised patients, mostly in HIV and prolonged steroid use, it seems to be a rare cause for a second hit in pre-existing ARDS [49]. Lastly, *Candida* species are frequent colonisers in patients with ARDS. In a single-centre prospective cohort study, not a single case of invasive pulmonary candidiasis was detected on histopathology of 135 ICU patients with pneumonia, even though 58% tested positive for *Candida* on respiratory specimens in the preceding 2 weeks [50]. The role of other rare fungal pathogens in nonresolving ARDS is understudied.

### *Pathophysiology of lung injury and invasive fungal infection*

*Aspergillus* species (and other fungi capable of causing invasive mould disease) benefit from a hyperinflammatory environment in lung injury and, at the same time, contribute to the hyperinflammation and lung injury themselves as well (figure 2).

#### *Why* Aspergillus *establishes fungal disease in ARDS*

Many aspects of the hyperinflammation in viral-induced ARDS and nonviral-induced ARDS overlap [51], although the pathogenesis of aspergillosis has only been studied extensively in the former given the relatively higher incidence of IPA in this setting. Therefore, we focus here on research performed in viral-induced ARDS.

Severe influenza and COVID-19 are hallmarked by viral replication in the lungs, leading to a localised hyperinflammatory response through detection of viral particles or damage-associated molecular patterns [52–54]. The inflammation may be further aggravated by disproportionate complement activation [55, 56]. These responses lead to an influx of immune cells, notably pro-inflammatory monocytes and neutrophils. Activated Th1 cells may further worsen inflammation through production of interferon (IFN)-γ, stimulating monocytes and macrophages to produce more pro-inflammatory cytokines [57] and driving the depletion of immunomodulating alveolar macrophages [58].

In this setting of viral-induced hyperinflammation, *Aspergillus* species have been shown to be able to cause IPA. Tissue damage in ARDS may create a nutrient-rich environment for the fungus to grow. Moreover, several layers of antifungal immunity are impaired in patients requiring intensive care for severe influenza or COVID-19 [59]. Epithelial damage, allowing *Aspergillus* to invade the tissue more readily,

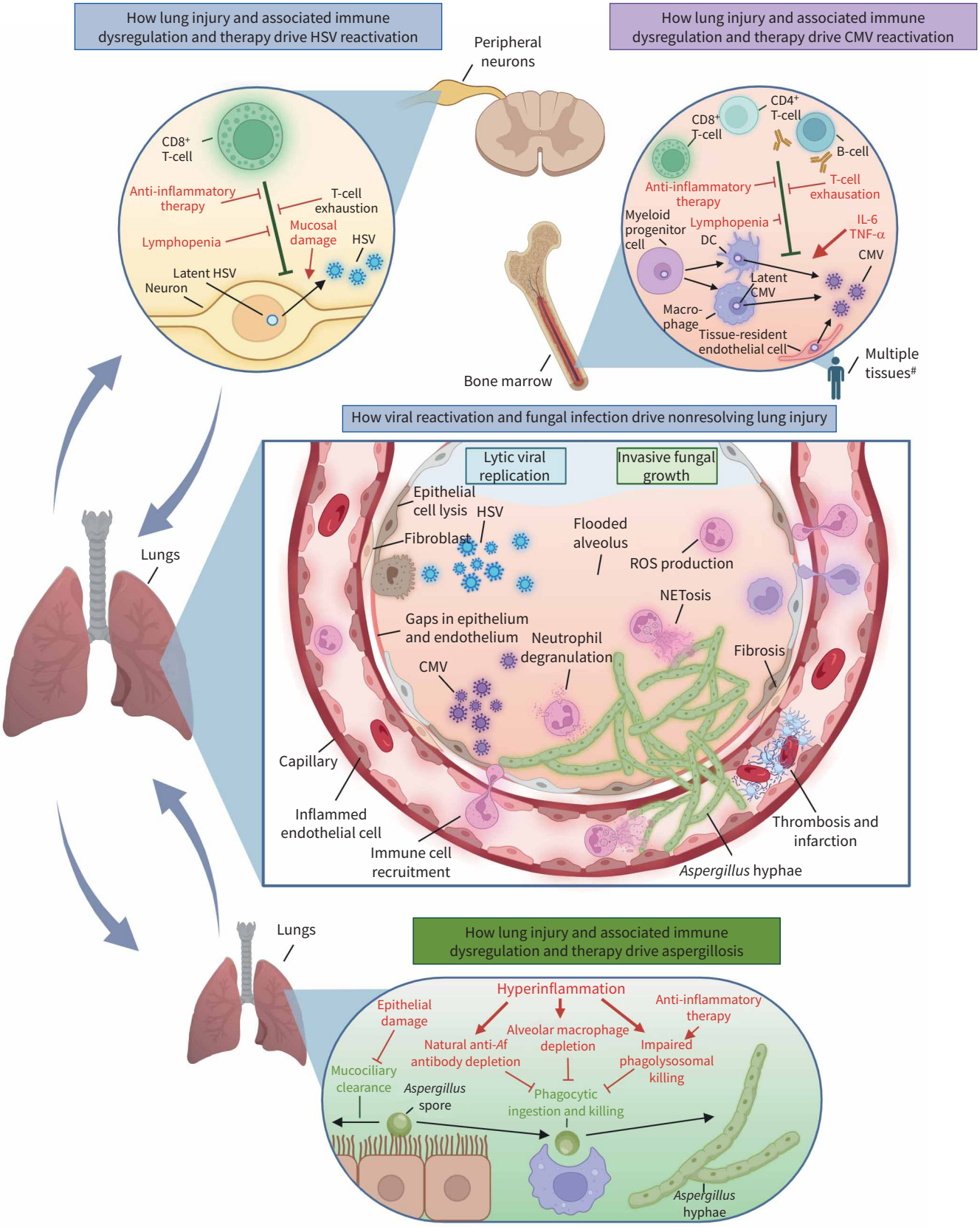


**FIGURE 2** The interplay between lung injury, the immune response, herpes simplex virus (HSV) or cytomegalovirus (CMV) reactivation and *Aspergillus* infection. Lung injury can enable reactivation of latent HSV and CMV and *Aspergillus* growth and germination *via* several mechanisms (depicted in the upper and lower panels in red). These pathogens can further maintain or aggravate lung injury directly or by eliciting an inflammatory response with

collateral damage (depicted in the central panel). Viruses may reactivate in extrapulmonary tissue (notably the spinal cord for HSV and the bone marrow or endothelial cells for CMV) and then spread to the lungs. Anti-inflammatory drugs (notably corticosteroids) can prevent lung injury, but may at the same time impair immunity against these pathogens. #: CMV reactivation can affect a number of tissues beyond the bone marrow and endothelial cells, including, but not limited to, the lungs, gastrointestinal tract and liver. *Af*: *Aspergillus fumigatus*; DC: dendritic cell; IL: interleukin; NETosis: neutrophil extracellular trap formation; ROS: reactive oxygen species; TNF-α: tumour necrosis factor-α. Figure created with BioRender.com.

and the inability of phagocytes to kill *Aspergillus* spores have been shown in animal models and in patients to allow inhaled *Aspergillus* spores to germinate and form hyphae [59–62]. Corticosteroids, which may impair antifungal immunity at several levels [63], are often used in (late-stage) ARDS and may thereby iatrogenically increase the risk for IPA in nonresolving ARDS [29]. Regarding the initial viral infection driving ARDS, influenza seems to impact antifungal immunity more profoundly than COVID-19 [59]. This is exemplified by the earlier occurrence of IAPA (typically within the first 48 h of an ICU stay) compared to CAPA (typically after approximately 1 week in the ICU) [17].

### *How* Aspergillus *drives lung injury in ARDS*

Knowledge of the normal antifungal host response against *Aspergillus* hyphae is necessary to understand how this can drive lung injury. *Aspergillus* hyphae are large, multicellular, three-dimensional structures that cannot be killed *via* phagocytic methods. A concerted action by neutrophils is required to target these structures. For this, neutrophils rely on the production of reactive oxygen species, release of antimicrobial peptides and neutrophil extracellular traps (NETs) [64]. Neutrophil swarming, a phenomenon in which neutrophils form dense clusters, encircling their target in a coordinated way, may prevent fungal escape and further promote clearance [65].

Invasive aspergillosis promotes functional impairment of the gas exchange both when the antifungal host response is hampered and when it is adequate. In cases of an absent antifungal host response, such as in neutropenic patients, massive tissue- and angio-invasion by *Aspergillus* hyphae leads to widespread destruction of the alveolocapillary structures [66], thereby hampering gas exchange. In cases of an adequate or largely adequate host response against *Aspergillus* hyphae, the presence of aspergillosis will induce an inflammatory response with increased cytokine and chemokine production and immune cell recruitment [67], and collateral damage may arise from the mediators of the neutrophil antifungal response. Notably, NETosis has been shown to promote immunothrombosis in severe COVID-19 [68]. In one study that compared the lung immune response in patients with severe COVID-19 with *versus* without aspergillosis, patients with CAPA had significantly higher levels of DNA complexed with citrullinated histone H3, reflecting more NETosis in these patients [69]. COVID-19 patients without aspergillosis with high levels of NETs in BAL fluid were shown to have numerically higher mortality rates. In contrast, higher NET levels indicative of a targeted response against aspergillosis were associated with lower mortality rates in patients with CAPA [69]. This illustrates the thin line between deranged and targeted immune responses (or even targeted responses in a background of deranged immunity), and the requirement of substances with two faces: slowing down the fungus but potentially causing extensive collateral damage to the host.

In most cases of IAPA or CAPA, the antifungal host response is present but inadequate. In these instances, the fungus is able to cause significant invasive disease with tissue damage, whereas the immune system, in a desperate attempt to control the fungus, further impairs the lung functionality. This was showcased in an autopsy study of patients who succumbed due to severe viral pneumonia [39]. In patients with IAPA or CAPA, a neutrophilic necrotising inflammation was the most frequently observed antifungal host response. This response impedes fungal growth, disturbing the characteristic appearance of hyphae, but at the same time hampers gas exchange [39].

## Viral reactivations as a source of persistent tissue damage and inflammation

The majority of the global population is infected with *Herpesviridae* such as CMV and HSV. The rate of CMV seroprevalence increases with age [70] and is estimated to be approximately 60% in high-income countries and 90% in low- and middle-income countries [71]. Approximately 67% of people under 50 years and 13% of people aged 15–49 years are estimated to live with HSV-1 and/or HSV-2 infection, respectively [72].

All *Herpesviridae* establish life-long latency after (often asymptomatic) primary infection and can reactivate from latency with lytic replication. Classical triggers for reactivation include local stress, fever, immunosuppression and damage to tissue innervated by latent infected neurons [73, 74]. Viral reactivations are commonly observed in both immunocompromised and nonimmunocompromised patients admitted to

the ICU [75, 76]. Risk factors for reactivation include mechanical ventilation, sepsis, corticosteroid therapy and prolonged hospitalisations [77]. Most studies into this patient population primarily focused on CMV reactivations, but more recent literature has increasingly highlighted the reactivation of HSV as well [78–81]. The reactivation of these viruses is often associated with ARDS and invasive mechanical ventilation (IMV) [78, 82–85]. In analogy to the fungal infections described above, these viruses not only benefit from lung injury, but also can sustain or further aggravate it (figure 1).

## *Clinical aspects of viral reactivations in nonresolving lung injury*

### *Blood* versus *respiratory tract sampling*

Compartmentalisation plays a crucial role in diagnosing and defining pulmonary viral infection, as herpesviruses can cause local organ disease without systemic reactivation. It has also been suggested that CMV PCR performed on tracheal aspirate is more sensitive compared to blood samples for early diagnosis of pulmonary CMV disease, as lung reactivation observed is earlier compared to blood reactivation (median 14 days *versus* 24 days) [86]. Previous literature indicates a lack of strong concordance between CMV blood reactivation and its manifestation in the lungs [81, 86, 87]. BAL has been shown to be a better surrogate for histologically proven CMV lung infection than plasma sampling [88, 89]. While CMV blood reactivation has been extensively studied in distinct patient populations, such as sepsis or immunocompromised patients, there is ample evidence of pulmonary CMV reactivation in ARDS patients [10, 90]. HSV reactivation in blood is even less studied than CMV. Given that ARDS can have both pulmonary (direct lung injury) and extrapulmonary (indirect lung injury) aetiologies, selecting the appropriate diagnostic method is crucial to align with the expected manifestation. In this review, we focus on pulmonary detection of viral reactivation (lower respiratory tract sampling).

### *Incidence*

The incidence of pulmonary viral reactivation reported in the literature varies widely, largely due to differences in patient selection (including the integration of serostatus for the virus, being at risk for reactivation, in the inclusion criteria), sampling of the upper or lower respiratory tract and the analytical techniques used for virus detection [85, 91–93] (table 2).

**TABLE 2** Incidence of cytomegalovirus (CMV) and herpes simplex virus (HSV) reactivation in several studies presenting lower respiratory tract sampling

| Study | Design | Patient group | Sample type | Analytical technique | Positivity for CMV, n (%) | Positivity for HSV, n (%) |
|---|---|---|---|---|---|---|
| Bruynseels *et al.* [91], 2003 | P | Critically ill patients (ICU >3 days) | BAL | Culture | NA | 58 (16%)[#] |
| Luyt *et al.* [85], 2007 | P | MV (>5 days), immunocompetent | BAL | PCR | NA | 129 (64%)[¶] |
| de Vos *et al.* [94], 2009 | P | Critically ill patients, MV (>2 days), immunocompetent | TA or undiluted BA | PCR | NA | 65 (62%) |
| Chiche *et al.* [80], 2009 | P | MV (>2 days), immunocompetent patients | BAL | Combined assay criteria for diagnosis[+] | 39 (16%) | NA |
| Heininger *et al.* [86], 2011 | P | Sepsis patients, immunocompetent, CMV-seropositive | TA | PCR | 25 (29%)[§] | 46 (53%)[f] |
| Assink-De Jong *et al.* [95], 2013 | P | ICU patients | TA or BAL | PCR | NA | 26 (34%) |
| Hraiech *et al.* [84], 2019 | R | ARDS-ECMO, immunocompetent | BAL | PCR | 21 (35%) | 5 (19%) |
| Boers *et al.* [96], 2024 | P | COVID-19 ARDS, seropositive for CMV or HSV[##] | BAL | PCR | 60 (38%) | 73 (42%) |

[#]: 47+11 (HSV+ in bronchoalveolar lavage (BAL) from both oropharyngeal swab (OPS) HSV+ and OPS HSV− patients), from a total of 361 patients evaluated for HSV. [¶]: 98+31 (HSV+ in BAL from both OPS HSV+ and OPS HSV− patients), from a total of 201 patients evaluated for HSV. [+]: Active CMV infection was defined by at least one of the following criteria: 1) a positive CMV pp65 antigenaemia (⩾1 cell); 2) a positive BAL shell-vial culture; 3) a histologic diagnosis of CMV infection (*i.e.*, open-lung biopsy); and 4) the presence of signs and/or symptoms of pulmonary disease combined with the detection of CMV in BAL fluid or lung tissue samples. [§]: 12+13 (CMV+ in lung and CMV+ in blood and lung) from a total of 86 patients enrolled for data analysis. [f]: 46 (HSV+ in respiratory samples) from a total of 86 patients enrolled for data analysis. [##]: Separately assessed for each virus. ARDS: acute respiratory distress syndrome; BA: bronchial aspirate; ECMO: extracorporeal membrane oxygenation; ICU: intensive care unit; MV: mechanical ventilation; NA: not applicable; P: prospective; R: retrospective; TA: tracheal aspirate.

Based on the collective findings of previous literature regarding frequency, timing and interplay between both viruses, pulmonary HSV reactivations appear more frequently than CMV reactivations and HSV reactivations tend to occur earlier compared to CMV reactivations. Moreover, there is no significant interplay observed between the two viruses [86, 96–99]. However, clinical studies have predominantly concentrated on pulmonary CMV rather than HSV reactivations, suggesting a potential benefit in placing greater emphasis on investigating HSV reactivations in future studies.

### *Quantitative measurements*

Currently, there is no gold standard for defining pulmonary reactivation in blood or respiratory samples from ICU patients. Interpretation of PCR results remains challenging, as clinical features of possible herpesvirus pneumonia overlap with clinical features of ARDS. Therefore, the incidence of CMV and HSV reactivation is dependent on the method of diagnosis [92]. This variability underscores the need for standardised diagnostic criteria to accurately assess the clinical impact of viral reactivations in critically ill patients.

It is crucial to note that viral excretion does not necessarily indicate viral infection. In mechanically ventilated patients, detecting HSV in the lower respiratory tract may simply reflect local excretion or contamination from the mouth or throat [91]. Moreover, although HSV isolation from the respiratory tract of critically ill patients is increasingly reported, its clinical significance remains unclear [100]. The main obstacle is the lack of a diagnostic standard to distinguish between reactivated HSV as an innocent bystander and a clinically significant lung infection [101]. This underscores the need for careful interpretation of viral detection.

It has been previously suggested that real-time PCR, which allows for viral load quantification, should become the gold standard for diagnosing viral infections [102].

### *Clinically relevant cutoff*

A quantitative value would enhance the interpretation of viral significance; however, the literature generally lacks quantitative measurements. Most studies define pulmonary reactivation based on PCR-positivity without reporting viral loads.

Even when considering viral loads, establishing an appropriate cutoff value for clinically significant reactivation is particularly difficult because many critically ill patients experience some degree of viral replication, complicating the determination of what constitutes a clinically relevant cutoff. While a lung biopsy could provide valuable insights, its feasibility in ICU patients is limited.

Many studies have established specific cutoffs in respiratory samples primarily in immunocompromised patients [85, 94, 103–108], but a universally accepted and applicable cutoff for immunocompetent ICU patients remains elusive [109]. Previous literature has suggested various cutoff values, ranging from $>10^3$ copies·mL$^{-1}$ [94] to $>10^4$ copies·mL$^{-1}$ [94, 96], $8\times10^4$ copies per $10^4$ cells [85] and $>10^5$ genome equivalents per millilitre (ge·mL$^{-1}$) [103].

The exponential increase in viral loads observed over time in these studies underscores the utility of a $\log_{10}$ scale in defining diagnostic thresholds [85, 94, 96]. Boers *et al*. [96] further validated the appropriateness of their chosen cutoff of $10^4$ copies·mL$^{-1}$ through a rigorous reassessment within their dataset, aligning with established literature and expert opinion on clinically relevant cutoffs for viral reactivation in critically ill patients. However, additional research is needed to establish a standardised cutoff, which is crucial for guiding treatment decisions.

### *Viral reactivation and mortality*

The association between viral reactivation and mortality is inconsistently reported in previous literature. Several studies identified an association with pulmonary HSV [96, 103, 110], while one study showed an association with CMV reactivation and mortality (which was recently retracted by the journal due to ethical considerations [79]). However, there is also conflicting evidence for both viruses [16]. Several factors may contribute to these discrepancies, including nonquantitative results, nonstandardised viral load estimations, varying cutoff points and inadequate handling of time-dependent biases.

## *Pathophysiology of lung injury and viral reactivation*

*Herpesviridae* such as CMV or HSV establish latent infections once they have infected a host, hiding from the healthy immune response and reactivating in reaction to certain stimuli such as ultraviolet light, stress or fever, or once the immune response is impaired [73, 74, 111]. CMV can infect various cell types, including fibroblasts, epithelial and endothelial cells upon primo-infection, and typically establishes latency

in $CD34^+$ myeloid progenitor cells and endothelial cells [73]. HSV initially infects epithelial cells but establishes latency in the cell body of peripheral neurons [74].

A well-functioning adaptive immune response plays a pivotal role in suppressing HSV reactivation. HSV-specific $CD8^+$ T-cells seem to be the key cell type in this process, inhibiting HSV replication *via* release of granzyme B and IFN-γ without killing the neuron that shows signs of viral reactivation [112, 113]. For CMV, natural killer, $CD4^+$ and $CD8^+$ T-cells and CMV-specific antibodies all perform key functions in curbing CMV reactivation [114] (figure 2).

ARDS may predispose to viral reactivation in several ways (figure 2). Increases in IL-6 and tumour necrosis factor (TNF)-α, as seen in inflammation associated with ARDS, may drive CMV reactivation through induction of expression of the CMV major immediate early gene, the product of which initiates the reactivation process [73]. Hyperinflammation in ARDS may drive immunoparalysis against CMV or HSV through several mechanisms such as apoptosis-mediated depletion of adaptive immune cells and T-cell exhaustion [115–117]. The mucosal damage incurred during intubation and mechanical ventilation is recognised as a significant trigger for HSV reactivation, resulting in active viral shedding in the throat, after which the virus can reach the lung epithelium *via* the airways [91]. Moreover, HSV particles may spread *via* the vagus nerve directly to the lung epithelium [100, 118].

Upon reactivation and viral shedding, CMV and HSV particles may cause lytic infection of several cell types in the lower respiratory tract, leading to lung injury, which may cause a vicious circle (analogous to what happens in fungal infection) of inflammation, viral reactivation and infection, and new lung damage driving more inflammation. These mechanisms underscore the complex interplay between viral reactivation and immune response in critically ill patients.

## Shared issues and knowledge gaps for fungal and viral infections in nonresolving lung injury

### *Causal interference and study methodologies*

Caution is needed in general when interpreting the results of studies into the relationship between fungal infection, viral reactivations and clinical outcomes, due to several methodological challenges.

First, for viral reactivations, the collection of PCR samples from different sites, including the respiratory tract, whole blood, serum and plasma, can introduce heterogeneity. Second, establishing a direct causative link between fungal infection, viral reactivation and clinical outcomes, such as mortality and the duration of IMV, is difficult in observational studies due to various biases. Viral reactivations may merely serve as markers for morbidity and mortality in critically ill patients, amidst a complexity of other risk factors such as sepsis, acute respiratory distress syndrome (ARDS), disease severity, and prolonged IMV [78, 82, 83, 119, 120]. Whereas autopsy studies have pointed out that aspergillosis may cause extensive lung damage, this is not always the case and therefore the attributability of mortality by fungal disease is not always clear [39]. Corticosteroid therapy, another known risk factor for herpesvirus reactivation and aspergillosis (particularly in viral-induced ARDS) [28, 29, 76, 121], further complicates establishing associations between viruses, fungi and outcome, as high-dose steroids are often prescribed to patients with ARDS who have persistent respiratory failure [9]. These factors contribute to the interaction between fungal infection, viral reactivation and outcomes.

Furthermore, the temporal aspect plays a crucial role in this association but is often neglected in previous studies, leading to immortal time bias [122, 123]. Distinguishing confounders from colliders and mediators is particularly challenging, contributing to potential residual confounding in time series analysis [124]. Unfortunately, most studies have employed methodologies insufficient to account for these complexities.

### *Complex interplay between fungal and viral secondary infections*

Another crucial, timing-related issue that requires further investigation is the interaction of viral reactivations with other secondary or opportunistic pulmonary infections, such as fungal and bacterial infections, in ARDS patients. Recent studies suggest an association between reactivation of herpesviruses and an increased risk of ventilator-associated pneumonia [91, 110, 125], and that lung damage caused by HSV reactivation can make patients more susceptible to bacterial superinfections [110]. Moreover, CMV reactivation may further induce immune suppression through complex mechanisms involving TNF-α, interleukin-1β and cellular-mediated responses, increasing the risk for secondary infections [126, 127]. However, persistent uncertainty surrounds the interplay between these infections. In contrast, a recent study showed no clear association between *Aspergillus* and bacterial superinfection occurrence in patients who were mechanically ventilated because of severe influenza or COVID-19 [21].

Fungal disease and viral reactivation may co-occur and interact in ARDS. For instance, preclinical work has shown that *Aspergillus* and CMV may synergise to counter antifungal and antiviral host responses [128]. Moreover, studies pointed to an increased incidence of plasma CMV reactivation in patients with CAPA compared to patients with COVID-19 without CAPA [129] and a worse outcome in CAPA patients with plasma CMV reactivation [130]. Large studies investigating the pathophysiological interplay between *Aspergillus*, CMV and HSV and how this affects the host are however lacking.

The possible pathways and timings of these interactions, including concurrent occurrences and the interplay of variables, add to the complexity. Understanding the roles of these variables in statistical models, whether they act as colliders or mediators, remains inconsistent across studies, posing challenges for making accurate adjustments in the models. Sophisticated analyses with large sample sizes, preferably from multi-centre cohorts, are needed to explore these interactions further.

### *Clinical impact and antiviral and antifungal treatment*

All the above considered, the clinical impact of pulmonary viral reactivations remains unclear. This knowledge gap is reflected by the large debate present in critical care practice regarding the use of antiviral medication. Studies focusing specifically on the pulmonary compartment are notably scarce. For HSV, only a limited number of clinical trials have considered the pulmonary compartment and those studies led to conflicting conclusions. A recent randomised controlled trial concluded that pre-emptive therapy with acyclovir at a prophylactic dose (5 $mg \cdot kg^{-1}$, three times daily) in ventilated patients with an oropharyngeal HSV reactivation did not increase the number of ventilator-free days on day 60 [131]. In contrast, a recent meta-analysis suggested a survival benefit of acyclovir; however, this study was prone to a high risk of bias and contained limited numbers of patients [132]. Intervention studies investigating viral treatment for reactivation in the lower airways have not been conducted. While several randomised controlled trials have been conducted for CMV, none of them focused on preventive strategies, targeting seropositive patients at risk of reactivation [93, 133] or employed a pre-emptive approach based on blood viral loads without confirming pulmonary infection [134]. This is concerning, as CMV can cause compartmentalised disease, such as CMV pneumonia, even in the absence of positive blood viral loads. Therefore, it is crucial that future clinical trials specifically investigate reactivation within the respiratory compartment.

Although the impact of the antiviral treatment on clinical outcomes has not yet been established in the pulmonary compartment, it has been recommended to systematically assess the pulmonary reactivations of CMV and HSV in patients with nonresolving ARDS, as other treatable causes in this cohort of patients are usually lacking [90].

For fungal disease, the clinical impact is less debated than for viral reactivation. Observational data suggests potential benefit of antifungal treatment for IAPA and CAPA. For instance, one multicentre retrospective analysis of 933 patients with CAPA showed 17% lower mortality rates in patients who received systemic antifungals compared to those who did not [135]. Interventional data is however lacking. Currently, the main question for fungal infection in nonresolving lung injury is whether antifungal prophylaxis could have a place in certain patients with high risk for IPA.

## Conclusions

In conclusion, viral and fungal pathogens can exploit the hyperinflammatory environment and altered immune landscape in ARDS, leading to persistent inflammation and lung injury. In nonresolving ARDS, key fungal and viral pathogens complicating disease include *Aspergillus*, CMV and HSV. Diagnosing these infections remains challenging as the clinical presentation is nonspecific and current tests are inadequate at distinguishing between fungal colonisation or asymptomatic viral shedding, and clinically significant infections. Correctly identifying and managing fungal infections likely improves outcomes in patients with nonresolving ARDS. For viral reactivations, more research is necessary to learn to what extent they are clinically relevant. Advances in diagnostic and therapeutic strategies, and a deeper understanding of pathogen–host interactions hold the potential to significantly enhance the management and prognosis of these patients.

**Questions for future research**

- What is the optimal cutoff for CMV and HSV viral load in BAL to identify clinically relevant viral reactivation?
- Do biological and clinical subphenotypes of ARDS affect incidence and outcome of viral and fungal infections in nonresolving lung injury?

- To what extent do viral and fungal infections contribute to lung injury and mortality in the setting of nonresolving lung injury and to what extent can this be mitigated by use of antiviral and antifungal drugs?
- Are there common mechanisms induced by nonresolving lung injury that may predispose to both fungal infection or viral reactivation and can these be targeted by immunomodulatory therapy?
- Can we find biomarkers based on the host immune response to distinguish clinically relevant infection from nonrelevant fungal colonisation?
- Do host genetic profiles predispose to development or poor outcome of viral and fungal infections in nonresolving lung injury and can this be harnessed for disease prediction?
- Could antifungal or antiviral prophylaxis lead to better outcome in selected high-risk patient groups with nonresolving lung injury?

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