



# The inflammatory response triggered by Influenza virus: a two edged sword

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**Abstract** Influenza A virus (IAV) is a relevant respiratory tract pathogen leading to a great number of deaths and hospitalizations worldwide. Secondary bacterial infections are a very common cause of IAV associated morbidity and mortality. The robust inflammatory response that follows infection is important for the control of virus proliferation but is also associated with lung damage, morbidity and death. The role of the different components of immune response underlying protection or disease during IAV infection is not completely elucidated. Overall, in the context of IAV infection, inflammation is a ‘double edge sword’ necessary to control infection but causing disease. Therefore, a growing number of studies suggest that immunomodulatory strategies may improve disease outcome without affecting the ability of the host to deal with infection. This review summarizes recent aspects of the inflammatory responses triggered by IAV that are preferentially involved in causing severe pulmonary disease and the anti-inflammatory strategies that have been suggested to treat influenza induced immunopathology.

**Keywords** Inflammation · Influenza · Immunopathology · Secondary infection

## Introduction

Influenza virus infection is one of the leading causes of mortality and morbidity worldwide. Seasonal influenza infections causes three to five million cases of severe illness every year, and approximately 250,000–500,000 deaths worldwide [1]. Furthermore, due to major genetic changes in influenza virus, the so-called antigenic shift, when whole segments of different virus strains are interchanged in the same host, the virus can become highly pathogenic and cause global pandemics with great number of deaths [2].

Influenza A is the best studied member of the *Orthomyxoviridae* family, comprised of negative-sense single-stranded RNA (ssRNA) enveloped virus. It contains a genome composed of eight segments of ssRNA enclosed by nucleoprotein forming the so-called ribonucleoprotein (RNP) complexes that carry also their own polymerases PA, PB1 and PB2 [3]. The subtypes of the virus are defined by the differences in viral glycoproteins hemagglutinin (HA) and neuraminidase (NA) and virtually all the various virus subtypes can be found in migratory birds—the natural reservoir of influenza A virus [4]. Different subtypes can infect and cause disease in a diverse number of species including birds, pigs and humans [5]. In humans, influenza virus infection is in most cases confined to the respiratory tract [6], and most commonly caused by influenza A H1N1, influenza A H3N2, influenza B Victoria and influenza B Yamagata [7]. The virus reaches the human respiratory epithelium and attaches to the N-acetylneuraminic acid (also called sialic acid) of epithelial cells. HA is the protein needed for virus binding to the host cell and differences in structure and conformation of this viral glycoprotein determines the receptor specificity of influenza A virus [8]. After infecting epithelial cells the virus can spread and infect immune or non-immune cells in the respiratory tract.

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The lung is in constant contact with the environment and in consequence with different potential pathogens. Therefore, there are several barriers that can limit the growth and establishment of a microorganism in the respiratory surface, including the mucus and the immune system [9]. The inflammatory response is an important defense mechanism against influenza A virus infection (IAV) preventing the replication and spread of the virus. However, an uncontrolled and exacerbated response to the virus may be associated with intense lung injury and death [10–12]. Indeed, there are several studies which have suggested a tight association between inflammation and the most severe cases of IAV infection [13–15]. Therefore, the inflammatory response triggered by IAV infections can be described as a double edge sword—it is necessary to protect against viral infection but may also cause severe pulmonary injury (Fig. 1). It is our hypothesis that there are mediators of the inflammatory response that are preferentially associated with severe disease but not necessary for protection against the virus. Based on this tenet we might be able to develop novel therapeutic targets to treat the severe manifestations of the disease. This review will focus on features of the inflammatory innate responses against influenza A virus that are preferentially involved in causing severe pulmonary disease.

### Influenza A virus recognition

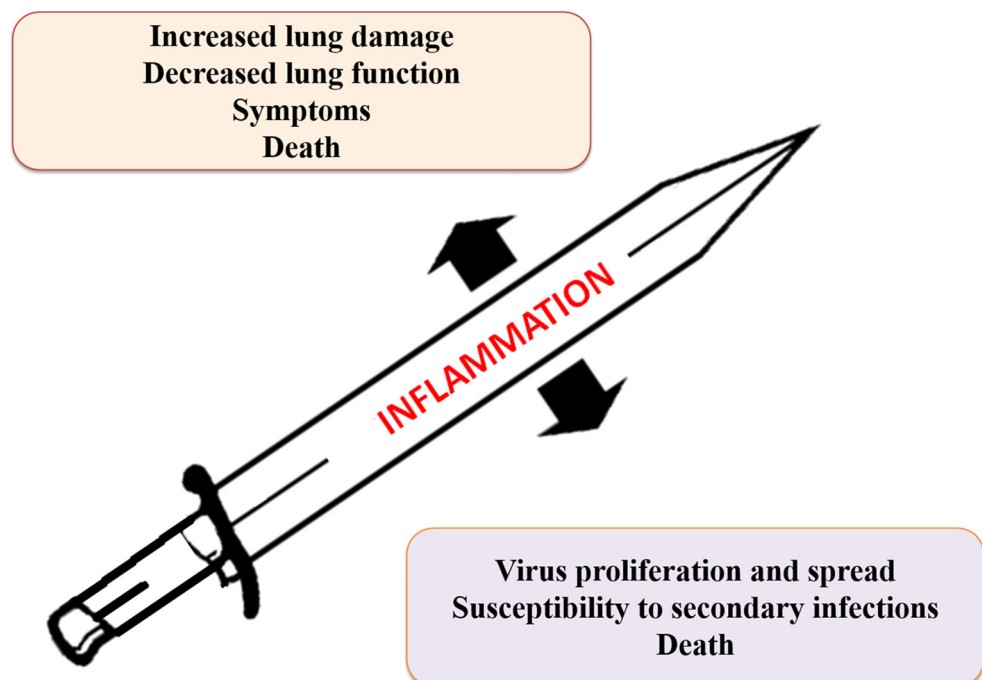
Once inside the cells of the respiratory tract, IAV is recognized by the innate immune system during the steps of virus infection and replication because of the presence of

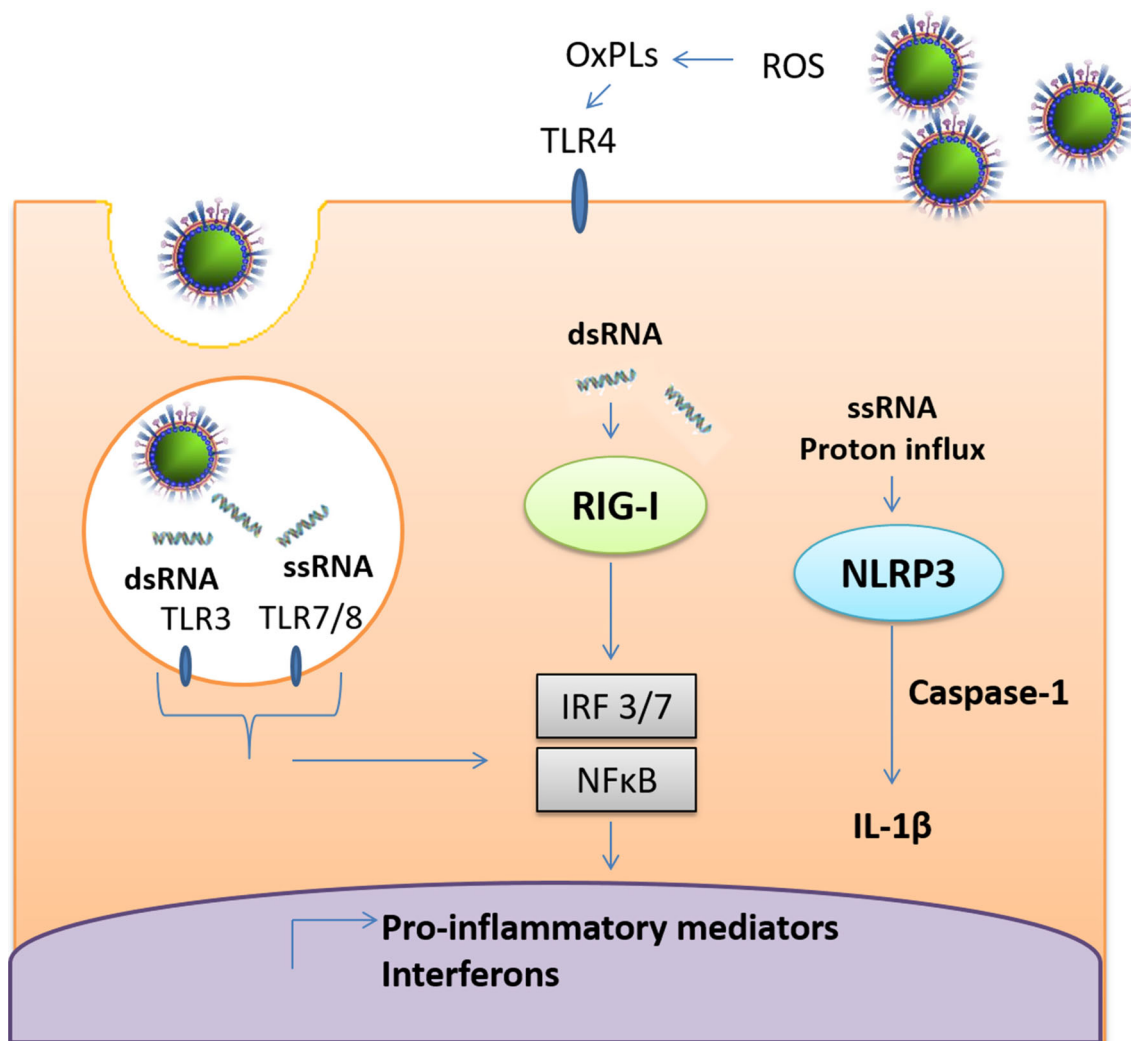
the pattern recognition receptors (PRRs). These receptors can sense the so-called pathogen-associated molecular patterns (PAMPs) that are common molecules to different types of microorganisms, including IAV (Fig. 2). There are three classes of PRRs that can recognize and signal the sensing of IAV infection: toll-like receptors (TLRs), nod-like receptors (NLRs) and Retinoic acid Induced Gene 1 like receptor (RIG-I). A myriad of these receptors is activated during the different steps of IAV infection and replication and act synergistically to activate transcription factors, including NF- $\kappa$ B and IRF3/7 resulting in a pro-inflammatory response and local antiviral state [2, 16, 17].

Toll-like receptors (TLRs) are the most well-studied and characterized class of PRRs [18]. Based on their cellular localization, TLRs can sense different types of virus-derived or induced molecules. The endosome-localized TLRs (TLR 3, 7, 8 and 9) play an important role in IAV recognition. Due to the intracellular localization of these receptors, they can sense and signal the presence of IAV nucleic acids. On the other hand, the role of surface localized TLRs (TLR 1, 2, 4, 5 and 6) in IAV infection recognition is indirect. This type of TLRs can contribute to the IAV infection response due to the recognition of damage associated molecular patterns (DAMPs) that are produced in response to infection [19].

Respiratory tract epithelial cells constitutively express TLR3 which can be activated during IAV infection by double-strain RNA produced during virus replication [20]. The activation of TLR3 contributes to the production of pro-inflammatory chemokines and cytokines and despite

**Fig. 1** Inflammation as a double edge sword during Influenza A infection. The low response leads to an insufficient control of the virus and can also predisposes to secondary bacterial infections. On the other hand, an excessive uncontrolled inflammatory response leads to increased immunopathology, morbidity and mortality





**Fig. 2** Schematic representation of IAV initial recognition by pattern recognition receptors (PRRs). The endosome-localized TLR3 and TLR7/8 are activated by virus RNA upon infection. TLR4 is activated by oxidized phospholipids (OxPLs) produced during the oxidative burst triggered by IAV infection. The cytoplasmic NLRP3 can sense and signal modifications of protons concentrations due to the ion

channel M2 viral protein and also is activated by ssRNA. RIG-I, also expressed in cytoplasm, recognizes viral RNA due to its 5'-triphosphate. The activation of PRRs initiates an antiviral and pro-inflammatory response with transcription of pro-inflammatory mediators and IFN

generating an antiviral response it also contributes to the increased immunopathology and mortality in mice [20–22]. TLR7 signaling is also important for the development of inflammatory responses during IAV infection. Plasmacytoid dendritic cells (pDCs) can sense viral ssRNA through TLR7 leading to maturation of these cells that are an important source of type I interferons [23]. ssRNA is also an agonist of TLR8. However, the specific role of this receptor is not well defined during IAV infection.

TLR10 was recently shown to be important for the development of innate immune responses during IAV infection. During influenza infection the expression of TLR10 is increased and the activation of this receptor induces secretion of pro-inflammatory cytokines and

interferons. Therefore, these receptors may also contribute to the viral clearance and immunopathology associated with IAV infection [24].

The second group of PRRs that can recognize IAV infection are the NLRs. NLRs, together with the adaptor ASC and pro-caspase-1, compose a multiprotein platform called inflammasome. This complex is responsible for the cleavage of pro-caspase-1 in its active form which cleaves pro-IL1β and pro-IL-18 into IL-1β and IL-18 that contribute to the inflammatory response against IAV infection [25]. NLRP3 is highly expressed during IAV infection in the lungs and immune cells recruited to the airways and can be activated by ssRNA, proton influx through the influenza virus-encoded matrix 2 (M2) ion channel [26] and the

virulence protein PB1-F2 [27]. Activation of NLRP3 inflammasome is important for virus clearance, but may also contribute to the intense inflammation associated with severe cases of IAV infection [26, 27].

Lastly, the other group of PRRs that sense IAV infection are the inducible cytoplasm sensors called retinoic acid inducible gene-I receptors (RIG-I) that recognize the 5'-triphosphate viral ssRNA that is produced during viral replication [28]. These receptors are expressed in several cell types such as alveolar macrophages, conventional dendritic cells and lung epithelial cells. Its activation and interaction with adaptor protein mitochondrial antiviral signalling (MAVS/IPS-1) and virus induced signaling adaptor (cardif/VISA) leads to translocation of NF $\kappa$ B and/or IRF3/7 to the nucleus and production of pro-inflammatory cytokines and type I and III interferon. Therefore, RIG-I is very important for the development of an antiviral response by regulation of production of IFNs and mediating the activation of dendritic cells and T cells responses [29, 30].

## Inflammatory mediators

The signaling pathways activated by all classes of PRRs lead to the transcription of pro-inflammatory genes and production of large amounts of cytokines and chemokines

that orchestrate the inflammation in the lungs during infection. The equilibrium of the inflammatory response in the lungs is determinant for the outcome of IAV infection. Pro-inflammatory cytokines, such as interferons, interleukins, chemokines and tumor necrosis factor are the main molecules controlling the lung environment during the infection [31]. These molecules are responsible for the communication between immune or non-immune cells and can drive several important response steps against IAV, including epithelium activation, leukocyte recruitment, cell proliferation and differentiation and development of adaptive immunity [31]. Thus, cytokines control the immune events during infection and are associated not only with the protective responses and viral clearance but also with the pathology associated with inflammation during IAV infection. Most of the effects of some inflammatory mediators in immunity or disease during IAV infection are summarized in Table 1.

Pro-inflammatory cytokines and chemokines activate and recruit leukocytes into the lungs and airways, and these cells can produce large amounts of these molecules in a positive feedback loop. If this cycle is not controlled, it can lead to the exacerbation of the inflammatory response. The systemic presence and large levels of these signaling molecules lead to an event known as “cytokine storm” which is one of the causes of increased mortality during severe IAV infections [32–34]. “Cytokine storm” is a term

**Table 1** Mediators of inflammation: role in immunity and pathogenesis

Inflammatory mediators	Role in immunity	Role in pathogenesis
Type II IFN	Increases TCD4 <sup>+</sup> , TCD8 <sup>+</sup> and antibodies responses in mice [58–60]	Immunopathology during IAV infection could be related with an overproduction of IFN- $\gamma$ in a murine model [61–63]
Type I and III IFN	Increase antiviral responses in epithelial cells, enhances viral clearance and regulate neutrophil trafficking to the lungs in mice [65–68]	Increase susceptibility to pneumococcal secondary infections in mice [170]
IL-1 $\beta$	Increases immunity against the virus in mice (recruitment of leukocytes and activation of effector cells) [69–73]. Decreases susceptibility to secondary bacterial infections [74, 75]	Increased levels are correlated with increased lung injury in mice and symptoms in humans during IAV infection [72, 76]
TNF- $\alpha$	No or minor contribution to viral clearance or immunity [78]. Important to control bacteria outgrowth during IAV and pneumococcal coinfection in mice [119]	Associated with increased lung injury, inflammation and symptoms in humans and mice [78, 80]
IL-17	Increases neutrophil and B cells influx into the lungs, protects mice from lethality [81–83]	Increased production is associated with severity in IAV infected patients and contributes to immunopathology due to overwhelming inflammation in mice [84, 85]
CXCL2	Recruitment of leukocytes into the lungs, controls pathogen replication and protects mice during IAV and pneumococcal coinfection [56]	Increased lung damage, weight loss and death in murine models of IAV infection [121–123]
CCL2	Recruitment of NK cells and macrophages, important for viral control in murine models of IAV infection [115, 119, 120]	Increased levels was associated with a worse disease outcome and lung injury and recruitment of inflammatory cells in mice [116, 118]
Lipid mediators (PAF and LTB <sub>4</sub> )	LTB <sub>4</sub> production increases viral clearance by the induction of antimicrobial peptides in the lungs of infected mice [102, 103]	PAF production is associated with overwhelming inflammation and increased lung injury in mice [96, 97]

largely used to characterize the intense cytokine response against distinct subtypes of influenza, such as the pandemic avian A (H5N1) and A(H1N1)pdm09 [12, 13, 35, 36]. The cytokine storm is correlated with the emergence of severe clinical symptoms, including alveolar hemorrhage, acute pneumonia, extensive pulmonary edema, acute respiratory distress syndrome and death [13]. The higher amounts of chemokines and cytokines produced after IAV infection leads to an excessive recruitment and activation of neutrophils, macrophages and T lymphocytes resulting in an intense production of proteases, oxygen and nitrogen reactive species that can directly contribute to tissue damage [37, 38]. It is observed an increase in cells apoptosis and necrosis and increased tissue permeability which results in lung edema [39, 40]. In addition, the excessive production of cytokines can lead to a rare event called haemophagocytosis which is associated with the more severe cases of H5N1 IAV infection [41]. This disorder characterized by an intense efferocytosis of leukocytes, erythrocytes and platelets and the proliferation of histiocytes and is correlated with the multiple organ failure observed during the severe cases of pandemic avian flu [42]. Although the inflammatory responses triggered by seasonal and less severe types of IAV is much less intense than that triggered by the pandemic subtypes, this response is also correlated with the symptoms and morbidity of patients [43, 44].

During infection, there appears to be different cytokine waves as infection progresses. The first cytokines to be produced in the lungs after IAV infection are the chemokines CCL5/RANTES, CCL2/MCP-1 and CXCL8/IL-8 and the main antiviral cytokines IFN- $\alpha$ , IFN- $\beta$  and IFN- $\lambda$  [20, 45, 46]. When the virus reaches the lower respiratory tract, it can infect alveolar macrophages that are activated and secrete even greater levels of pro-inflammatory and antiviral cytokines including IL-1 $\alpha/\beta$ , IL-6, TNF- $\alpha$ , IL-18, IFN type I, CCL2/MCP-1, CCL3/MIP-1 $\alpha$ , CCL4/MIP-1 $\beta$ , CCL5/RANTES, CCL7/MCP-3, CCL20/MIP-3 $\alpha$ , CXCL10/IP-10 and CXCL8/IL-8 [47].

Much knowledge about IAV induced pathogenesis comes from murine models. In mice, as in humans, levels of pro-inflammatory cytokines are directly associated with the poor prognosis. H5N1 virus infection evokes a more robust and acute inflammatory response than inflammation triggered by seasonal IAV strains. H5N1 infection resulted in higher levels of IL-6, TNF- $\alpha$ , IFN- $\gamma$  and chemokines in serum of patients, particularly in the fatal cases [12, 48]. The 2009 H1N1 pandemic virus was also associated with the development of a stronger and more sustained inflammation than the disease observed during seasonal IAV infections [49].

Interferons (IFNs) compose a family of cytokines that have a key antiviral role during the innate immune

response against viral infections [50]. There are three major types of interferons: type I (IFN- $\alpha$  and IFN- $\beta$ ), type II (IFN- $\gamma$ ) and type III (IFN- $\lambda$ ) [51]. Type I IFN plays an important role in protection during IAV infection [52–54]. Some studies have suggested an essential role of type I IFNs in the development of antiviral responses [55], whereas other studies have also suggested that type I IFNs play an inhibitory role on CXCL1 and CXCL2 chemokine production and thus control neutrophil influx during IAV infection protecting against lung injury [56]. However, a recent study showed that an excess of release of these mediators are correlated with overwhelming inflammation and severity during IAV infection [54]. Indeed, another recent study demonstrated that type I IFN is necessary for neutrophil and Ly6C<sup>high</sup> inflammatory monocyte recruitment to the IAV infected lungs and clearance of virus but is also important to prevent the exaggerated inflammatory response caused by IFN  $\gamma$  [57].

IFN- $\gamma$  is known to play an important role in the response against several viral infections [58, 59]. This cytokine exerts a stimulatory effect on macrophage function, upregulates the major histocompatibility complex molecules (MHC) on antigen-presenting cells and also is important to regulate the cytokine production and CD4<sup>+</sup> T cells activation during infection [60, 61]. However, IFN- $\gamma$  seems to have a minor role in mounting the immune response and mediating viral clearance during IAV infection [52]. Despite the inability to compromise the response against IAV, the absence of this cytokine during IAV infection leads to an unregulated specific CD8<sup>+</sup> T cell trafficking to the lungs [62]. Furthermore, during adoptive transfer of CD8<sup>+</sup> T cells to mice with alveolar epithelial cells that express an influenza antigen, the resulted immunopathology was associated with IFN- $\gamma$  production [63]. Accordingly, in vivo blockage of IFN- $\gamma$  in mice led to a significant reduction in the number of infiltrating leukocytes into the lungs after IAV infection [64]. The study from Stifter and colleagues also demonstrated that type I and III IFN inhibit neutrophil trafficking during IAV infection in a synergic way [57]. Type III interferons have an important role in inducing antiviral state in respiratory epithelial cells [65] and were shown to contribute to the innate immunity against IAV [66]. Type I and III IFN stimulate the transcription of over 300 interferon induced genes (ISGs) that are responsible for the production of several antiviral proteins (including MxA, ISG15, OAS and PKR) that interfere with different steps of viral replication [67, 68].

Interleukins are another important family of mediators of inflammation. They are a group of cytokines with several biological functions and can drive pro-inflammatory or anti-inflammatory responses [31]. IL-1 $\alpha$  and IL-1 $\beta$  are pro-inflammatory interleukins produced after the activation of

NLRP3 inflammasome and cleavage of the pro-IL-1 form. These cytokines are important for the development of immunity against viral infections by inducing a cascade of inflammatory events (recruitment of leukocytes and induction of production of other cytokines) and also lead to T cell activation [69–71]. Indeed, the absence of IL-1 signaling is associated with increased mortality during IAV infection in mice and genetic variants of the genes for IL-1 $\alpha$  and IL-1 $\beta$  are correlated with IAV infection susceptibility in human patients [72, 73]. It has also been shown that reduced levels of IL-1 $\beta$  can contribute to the increased risk of secondary bacterial infections after IAV infection [74, 75]. However, other studies have shown that these molecules also have a role in driving lung injury and their levels in the lung fluid correlate with the severity of symptoms during IAV infection. These studies are consistent with a dual role of IL-1 during infection [72, 76].

Interleukin-6 (IL-6), together with tumor necrosis factor alpha (TNF- $\alpha$ ), are pro-inflammatory cytokines that are also involved in the onset of inflammation during IAV infection [31]. These cytokines contribute to endothelium activation leading to expression of P-selectin, E-selectin, and integrins that are essential for leukocyte recruitment to the lungs [77]. The production of these mediators during IAV infection is correlated with symptoms in human patients [42, 43] and the blockade of IL-6 or TNF- $\alpha$  protects against the severity of IAV infection in mice [78, 79]. Indeed, TNF receptor 1-deficient mice show significantly reduced morbidity following IAV H5N1 infection compared with wild-type mice, despite no difference in viral titers [80]. Therefore, pro-inflammatory cytokines, such as TNF- $\alpha$ , contribute to the exacerbation of inflammation during IAV infection with no or a minor contribution to the defense against the virus. In this regard, targeting TNF may have potential therapeutic benefits in patients with IAV infection.

The participation of IL-17 during the immune responses against IAV infection has also been reported. IL-17A was shown to protect mice from lethal challenge with IAV H1N1 and H3N2 and it was related with an increased and early neutrophil influx into the lungs of animals [81, 82]. Another study showed that IL-17 can play a critical role in mediating the recruitment of B cells to the lungs during a H5N1 infection in mice [83]. However, its role in protecting against IAV infection is controversial. Another study indicated that IL-17 rather than acting to improve viral clearance, contributed to the immunopathology associated with IAV infection by augmenting inflammatory responses [84]. In agreement with the latter finding, elevated levels of IL-17 were found in the serum of patients with severe pandemic H1N1 infection possibly contributing to overwhelming inflammation, morbidity and poor prognosis [85].

IL-27 and IL-22, two regulatory cytokines, also have been suggested to play a significant role during IAV infection by controlling the inflammatory responses and the return to lung homeostasis [86]. IL-27 was shown to increase the levels of IFN- $\gamma$  in T CD8<sup>+</sup> cells during infection [87], and an antiviral activity of this cytokine was also reported during an *in vitro* study [88]. IL-22 was shown to have an important protection role during sub-lethal IAV infection as the production of this cytokine reduced lung injury and inflammation and decreased mice lethality during secondary bacterial infections [89]. In addition, this cytokine is important for lung repair responses after IAV infection contributing to restoration of lung function [90].

Another family of inflammatory mediators that deserves attention in the context of IAV infection is the family of lipid mediators, including platelet-activation factor (PAF) and leukotriene B4 (LTB4). PAF is an inflammatory phospholipid that acts through a G protein-coupled receptor (PAFR) inducing several inflammatory events, including lung edema and recruitment and activation of leukocytes [91–93]. Immune cells, such as monocytes, macrophages, neutrophils and lymphocytes can produce, by hydrolyzing membrane phospholipids, and be activated by PAF [94, 95]. During IAV infection, the expression of the enzyme responsible for PAF synthesis in inflammatory conditions (LPAFAT/LPAFAT2) is increased after 1 day of infection in the lungs [96] and it coincides with the first wave of leukocyte recruitment [97] revealing the possible role of PAF during the inflammatory response. Our group showed that absence of PAFR (as seen in PAFR-deficient mice) or administration of a PAFR antagonist prevented the intense inflammatory response, lung injury and death associated with infection by IAV without affecting viral titers in the lungs [96]. Of interest, the PAFR antagonist was effective even when drug treatment was started 3 days after infection, suggesting there was a therapeutic window for administration of PAFR antagonists. Our studies clearly reinforce the pivotal role of inflammation at inducing mortality and morbidity during IAV infection and suggest immunomodulation as a strategy to control the symptoms and death related to infection.

LTB4 is another member of the family of lipid mediators that are produced mainly by leukocytes of the myeloid-lineage [98]. It acts through two G protein-coupled receptors: a high affinity receptor called BLT1R (predominantly expressed in leukocytes) [99] and a low-affinity receptor named BLT2R (expressed in different cell types) [100, 101]. On contrary to PAF, LTB4 has a protective role in the context of IAV infection. It was shown that administration of LTB4 increased the expression of antimicrobial peptides in the lungs and improved viral clearance [102]. In agreement with that, another study showed that LTB4-

treated human neutrophils increased levels of myeloperoxidase and the antimicrobial peptide  $\alpha$ -defensin enhancing the antiviral state of the cells against IAV infection [103].

The complement system is an important component of innate responses against pathogen infections [104]. Its activation cascade is a multistep cleavage process leading to opsonization or/and lysis of the target cell and can also produce the anaphylatoxins C3a and C5a that are worth to mention in this section [105–107]. C5a is a potent neutrophil chemoattractant and activates neutrophils to generate reactive oxygen species and release of enzymes contributing to the inflammatory response during IAV pneumonia [108, 109]. Indeed, extensive deposition of cleavage products of the complement cascade was found in the lungs of fatal cases of IAV pandemic strains, 2009 H1N1 and also 1957 H2N2 [110]. Therefore, C5a plays a detrimental role during IAV infection leading to increased lung injury and death. Our group and others showed that during IAV infection, the levels of C3a and C5a are increased in bronchoalveolar lavage fluid (BALF) of mice and it is associated with intense inflammation and lung damage [109, 111]. Furthermore, the inhibition of C5 cleavage or use of anti-C5a during IAV infection in mice or green monkeys could prevent lung damage and decreased several inflammatory parameters that are associated with poor outcome from disease [109, 112].

Lastly but not less important are the chemokines. These molecules are chemotactic cytokines that control the migration and homing of immune cells during homeostasis or disease [77]. During infections, the recruitment of leukocytes is essential for proper setting of the immune response, pathogen clearance and subsequent reestablishment of tissue homeostasis. Chemokines are a large family of 50 small molecular weight proteins (8–12 kDa) that are subdivided into four groups based on the positioning of their N-terminal cysteine residues [77]. The C–X–C group is defined by the presence of a variable amino acid separating the first two cysteines; C–C chemokines are characterized by the presence of two cysteines adjacent to each other; the C group contains only a single cysteine residue in the conserved position and in the CX3C group the last two cysteine residues are separated by three amino acids. The CXC and CC groups are the most studied type of chemokines and are produced and act on a variety of immune cells [113].

During IAV infection there is marked and early production of chemokines and consequently recruitment of macrophages and neutrophils to the lungs and airways [114]. The production of chemokines such as CCL2 induces the recruitment of natural killer cells and inflammatory monocytes that are important for the early control of virus replication [115]. As discussed for the other cytokines in

this section, the chemokine production must be finely tuned to generate effective adaptive immune responses and viral control but to avoid inflammatory injury. In fact, several studies showed that the increased production of CCL2 and consequent recruitment of inflammatory monocytes can increase the morbidity due to influenza infection and the absence of CCL2 receptor (CCR2) also decreased mice susceptibility to a bacterial secondary infection [116–118]. On the other hand, CCL2 seems to be important for an efficient immune response during IAV infection as CCL2 knockout mice presented increased weight loss, viral loads and levels of pro-inflammatory cytokines regardless of the reduced number of macrophages and neutrophils in the lungs [119], in addition, treatment with neutralizing CCL2 antibodies enhanced epithelial damage following IAV infection in mice [120, 121]. The differences presented by the distinct studies might be explained by the strategies used and the affected cells. CCL2 is considered protective when knockout mice or depleting antibodies are used against the chemokine, which causes a great reduction in lymphocytes and thus turns the immunity down [119]. On the other hand, a detrimental role is associated to CCR2, when receptor knockout or blockage is used, resulting in reduced lung inflammatory monocytes.

The neutrophils-associated chemokines such as CXCL1 and CXCL2 are mostly associated with a harmful role during IAV infection. The activation of receptor for both chemokines, CXCR2, was shown to be important for the intense recruitment of neutrophils and contributes to the lung injury and mortality of mice during IAV infection, without affecting viral clearance [122]. In fact, production of CXCL2 by the pulmonary epithelial cells is associated with an increased recruitment of inflammatory cells and is crucial for the acute lung damage during flu [123]. Moreover, the overproduction of CXCL2 is associated with increased lung damage, weight loss and death of mice in a model of IAV and bacteria coinfection [56].

In summary, different pro-inflammatory cytokines can contribute to the generation of an effective antiviral response during IAV infection, but can also be harmful as they can exacerbate the inflammatory response and contribute to lung injury. Therefore, understanding the dynamics of these molecules can be helpful in the development of new therapeutic targets in the context of IAV infection.

## Leukocytes and influenza

After IAV infection, resident cells orchestrate the sequential recruitment of different leukocyte populations to the lung parenchyma and airway space. The recruitment of these cells is dependent on virus sensing and release of

inflammatory mediators by the infected cells. Innate and adaptive immune cells play an important role in the clearance of the virus but, similar to cytokines production, the activation of leukocytes has to be finely controlled to insure effective killing of virus, minimal lung damage and restoration to organ homeostasis.

Alveolar macrophages (AMs) are resident lung cells that present variable susceptibility to influenza virus infection depending on the viral strain; strains that do not infect AMs, like PR8, are more virulent to mice [124]. Upon infection with different IAV strains, as shown for strains ST169 (H1N1), ST602 (H3N2) and HKG9 (H9N2), AMs polarize early into the M1 phenotype, with high expression of pro-inflammatory cytokines and iNOS, enhanced endocytic functions and enhanced ability to kill intracellular pathogens. Later, macrophages became M2b activated, with reduced IL-6 and IL-12 expression, but increased IL-10 and STAT1 expression. AMs become immunosuppressed with downregulation of most markers of immune response. This polarization occurs via the PI3 K/Akt signaling pathway [125]. AMs are very important to keep lung homeostasis and gas exchange function upon IAV infection. Two recent studies in which depletion of AMs was achieved by different genetic approaches showed increased morbidity and pathology following IAV infection and this was associated with increased respiratory failure with loss of gas exchange. The depletion of AMs also results in increased viral loads in early time points after infection, but no changes in cellular CD8<sup>+</sup> T cell responses [126]. A previous study has shown that AM depletion before, but not after infection with recombinant Influenza virus bearing HA and NA from the 1918 IAV, resulted in increased lethality and virus levels coincident with reduced IFN- $\alpha$ , IFN- $\gamma$ , TNF- $\alpha$  and CCL3 [127].

Contrasting conclusions about the role of alveolar macrophages in alveolar epithelial damage have been published. As mentioned before, the protective role of alveolar macrophages was shown to be mediated by the preservation of lung tissue and the epithelial barrier [126]. On the other hand, an *ex vivo* study pointed that the production of IFN- $\beta$  by AM during IAV infection might contribute to apoptosis of alveolar epithelial cell and lung injury [128]. The differences in the studies may be due to the different experimental strategies and the more complex system during *in vivo* conditions. Another important feature of AMs biology during influenza infection is their ability to phagocyte, together with neutrophils, apoptotic infected cells, probably via TLR4 activation contributing to cross-presentation and an adequate adaptive response [129]. Therefore, AM are essential for the development of an effective innate response against the virus and might contribute to both epithelial injury and maintenance of lung homeostasis. The conflicting results highlight the need of

more kinetic studies for understanding the contribution of AM and the various macrophage subtypes during infection with different influenza subtypes and strains.

Besides alveolar macrophage polarization upon influenza infection, inflammatory macrophages (Ly6C<sup>high</sup>) are recruited early to lungs and airways during lethal IAV infection and accumulation is observed until late time points [130]. CD11b<sup>+</sup> cells upregulate CD11c and MHCII and mature to exudate macrophages or monocyte-derived DC and contribute to tissue damage and mortality via NOS2 expression, CCR2 activation and production of inflammatory cytokines during IAV infection in mice [130].

Neutrophils are recruited early to the lungs after influenza infection and can be infected and produce viable virus progeny, at least for the A(H1N1)pdm09 strain [131]. These cells have an important role in killing the virus [132], especially by phagocytosing infected apoptotic cells [129]. In addition to their role in innate control of the virus, neutrophils are associated with immunopathology. A direct correlation between neutrophil recruitment and severity of lung disease associated to highly pathogenic avian influenza has been shown [133]. Aiming to investigate the role of neutrophils during influenza infection, different studies have performed neutrophil depletion protocols in experimental infection in mice. If neutrophils are depleted before infection using the antibody RB6, the disease is more severe and lethality is enhanced [134]. If these cells are depleted after 3 or 5 days of infection, there was no change in disease severity and lethality [127]. Using different schedules and doses of the antibody 1A8, it has been shown that limiting antibody concentrations protected mice, without changes in virus levels. In contrast, high doses of the antibody, and therefore neutrophil depletion, enhanced disease severity [130]. Although the distinct strategies of neutrophil depletion differs in specificity—RB6 depletes both Gr1<sup>+</sup> neutrophils and non-neutrophils Gr1<sup>+</sup> cells like monocytes, whereas 1A8 depletes only neutrophils [135]—the two strategies showed that a finely tuned equilibrium in the number of neutrophils is important to control infection and prevent lung damage and lethality.

Distinct mechanisms of neutrophil activation have also been investigated in the context of influenza infection. Release of myeloperoxidase and neutrophil extracellular traps (NETs) by activated neutrophils has been shown to contribute to disease severity [136]. Reactive oxygen species released by activated neutrophils and macrophages might be a potential target to limit disease severity. Indeed, antioxidant agents such as catalase, N-acetyl-L-cysteine or Nox2 inhibitor result in reduced disease severity and also virus levels in murine models of influenza infection [137–140].



NK cells, a group 1 innate lymphoid cell (ILC) population, are normally present in the airways in homeostatic states. During influenza infection, more NK cells are recruited and become activated by type I IFN to produce IFN- $\gamma$ , granzyme B and IL-12 [141]. The involvement of NK cells in virus control or influenza-related pathology is not completely understood. It has been shown that in the absence of NCR1 (NKp46), the main NK cell receptor, influenza infection becomes more lethal in two mouse strains [142]. Indeed, the human NK receptors NKp46 and NKp44 are capable of recognizing the IAV hemagglutinin and neuraminidase and lead to the death of the infected cell and control of virus proliferation [143]. Together with the direct recognition of infected cells, NK cells were recently shown to be capable of mediating antibody-dependent cellular cytotoxicity in IAV infected patients [144]. Furthermore, another study showed a correlation between high NK activation and low pathogenic avian influenza, whereas highly pathogenic avian influenza induced weak NK activation [145]. However, NK cells have also a contrasting role during IAV infection: it can mediate part of the injury observed after infection. It was shown that depletion of NK cells using anti-GM1 or anti-NK1.1 reduced lethality in influenza infected mice and this deleterious role of NK cells was observed only in high inoculum of influenza infection [146]. In addition, the absence of IL-15, the cytokine responsible for the proliferation and maintenance of NK cells, or depletion of these cells protected mice from lethality and morbidity caused by influenza infection. It was associated with a reduction of neutrophils and mononuclear cells recruitment as well as reduced IL-6 and IL-12 levels and increased IL-10 levels, but no changes in viral control [147].

Innate lymphoid cells 2 (ILC2) have been reported to play distinct roles during influenza A infection. This cell population is activated by IL-33 production and is a source of IL-5 and IL-13, important for eosinophil recruitment and epithelial proliferation. During experimental murine infection with H3N1 [148] or H1N1—PR8 [149, 150] or pdmH1N1 [151]—increased lung ILC2 populations as well as higher IL-33, IL-5 and IL-13 levels were found. Experimental H3N1 infection causes an intense airway hyper-reactivity (AHR) and inflammation. The AHR induced by H3N1 infection was shown to be an innate and acute mechanism, dependent on ST2 (IL-33 receptor), stimulated by the induction of IL-33 production, mainly by AM, which, in turn, results in greater ILC2 abundance in the lungs, and therefore, intense IL-13 production [148]. Using a different virus strain, PR8, Monticelli and colleagues demonstrated that this IAV also induces ILC2 population. On the contrary to the H3N1 infection, PR8 induces intense epithelial lung injury and do not induce AHR; in this scenario, ILC2 induction promotes the

activation of genes involved in wound healing and amphiregulin production, which restores lung function epithelial integrity and airway remodeling [150]. Later, another study also investigated another role of ILC2 during influenza infection. Using PR8 mouse infection, it was shown that NKT cells, together with AM, are also an important source of IL-33 that will result in greater infiltration of ILC2 that in turns produce IL-5 and stimulate eosinophils recruitment in the recovery phase of the infection [149]. Lastly, a recent study showed that among children presenting acute asthmatic symptoms after pandemic H1N1 virus infection, most of them (81 %) did not report previous history of asthma. Mice infected with the pandemic virus presented AHR manifestations associated with ILC2, IL-4, IL-5 and IL-33 presence [151]. To sum up, ILC2 induction during IAV infection might induce acute AHR (pathogenic), or late tissue epithelial repair (protective), depending on the virus strain and the microenvironment [152].

Lipid reactive lymphocytes with reduced variability and expression of NK cell markers, the so-called invariant NKT cells, are important for innate responses to influenza infection. The activation of NKT favors virus clearance and reduces disease severity [153], whereas NKT depletion using CD1d KO or J $\alpha$ 18 KO mice succumb to influenza infection [154]. iNKT activation is also important to control airway inflammation and to stimulate CD8<sup>+</sup> T cell response [155]. Also, the role of iNKT cells mediating the protection during a secondary pneumococcal infection was recently demonstrated [156].

Adaptive immune responses during influenza infection are performed by CD4<sup>+</sup> and CD8<sup>+</sup> T cells and B cells, that become activated, expand clonally and migrate to the lungs around seven days after infection. The latter cells, respectively, produce cytokines, lyses virally infected cells or produce great amounts of antibodies that can directly neutralize the virus (IgM) or confer protection to a secondary homosubtypic influenza infection (IgA and IgG) (reviewed in [157]). CD4<sup>+</sup> and CD8<sup>+</sup> T cells have also been related to influenza immunopathology (reviewed by [158]). One example is that, in the absence of the immunoadaptor DAP12 expressed in innate immune cells, there is marked infiltration of CD4<sup>+</sup> T cells presenting enhanced FasL expression and cytotoxicity that may cause uncontrolled lung injury and death [159]. Antigen-specific CD8<sup>+</sup> T cells may also contribute to immunopathology by producing TNF- $\alpha$  after recognizing IAV antigen in infected alveolar cells [160]. Indeed, classical CD8<sup>+</sup> T cells (“Tc1”) can produce large amounts of IFN- $\gamma$ , TNF- $\alpha$  and IL-2, activating themselves and other cell types during IAV infection [161]. Transferring IAV-specific CD8<sup>+</sup> T cells to a transgenic mice with expression of HA in alveolar epithelial cells leads to intense lung injury and progressive

weight loss, reinforcing the harmful role of overactivation of those cells during IAV infection [162]. The balanced response and activation of CD8<sup>+</sup> T cells present several regulation mechanisms including inhibitory and costimulatory signals, cytokine production and signaling and also cell–cell interactions to assure effective viral clearance and less cytotoxic damage. CD4<sup>+</sup> regulatory T cells (Treg) are induced after influenza infection and are important in limiting antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell activation [163]. During avian H5N1 influenza infection CD8<sup>+</sup>, but not CD4<sup>+</sup>, Tregs are induced and suppress CD8<sup>+</sup> T cell response by IL-10 production, and therefore, limit control of virus infection [164].

## Secondary bacterial infections

It is estimated that one quarter of deaths during IAV epidemics [165] and 50–95 % of deaths during IAV pandemics [166, 167] are caused by secondary bacterial pneumonia. This lethal synergism between IAV and bacteria such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Streptococcus pyogenes* or *Haemophilus influenzae* is only partially understood. The increased susceptibility to bacterial infections during or shortly after IAV infection is multifactorial but there are two main theories. Enhanced susceptibility could be due to the intrinsic virulence of the virus, i.e., IAV infection directly causes lung damage and, thus, facilitates adherence and spread of bacteria [168]. Indeed, data from autopsy studies from patients of IAV pandemics showed increased adherence of bacteria on damaged epithelium [169]. Therefore, the cytotoxic potential of the virus, which may cause tissue damage, is important for the increase in secondary bacterial infection susceptibility. As an example, the protein PB1-F2, expressed by some influenza virus, is a cytotoxin known to increase cell death and inflammation thus contributing for the severity of pneumonia during coinfection [170]. Natural barriers such as the mucus layer and the tracheal mucociliary epithelium are important for host protection against respiratory pathogens. The neuraminidase activity of IAV can expose attachment sites for bacteria by the cleavage of sialic acids from the pulmonary mucus layer [171, 172] and provide sialylated substrates as nutrients for the proliferation of bacteria [173]. In addition, IAV infection decreases the mucociliary velocity and, as a consequence, also decreases the clearance of bacteria during co-infection [174].

The other theory for the increased susceptibility to secondary bacterial infection after IAV infection is the impairment of host defense mechanisms. It is suggested that IAV infection might compromise the local immunity of ears or lungs then predisposing the host to secondary

infections. It has been shown that the production of type I IFNs after IAV infection in mice can impair the effective response against bacteria by decreasing the Th17 responses which are important to bacteria clearance [56, 175]. IFN- $\gamma$  production during IAV infection is also associated with an impairment of phagocytosis [176]. Moreover, IAV infection can decrease intracellular production of oxygen reactive species and phagocytosis in neutrophils [177] and increase the production of the anti-inflammatory mediator IL-10 [178]. In addition, some studies have shown that IAV infection can lead to increased apoptosis of macrophages and neutrophils and that the resulting leukopenia could predispose the host to a sequential bacterial infection [172, 179, 180]. However, a large number of studies demonstrate that in most cases of secondary bacterial infection leukocytosis rather than leukopenia occurs [181].

Regardless of the mechanisms explaining enhanced susceptibility to secondary infection, it is clear that the synergistic effects of viral and bacterial infection in stimulating inflammation contribute to the severity of pneumonia [172]. Indeed, the intense inflammatory response observed in co-infected cases is associated with increased mortality as the persistent activation of macrophages and neutrophils in the lungs contributes to intense tissue damage [172]. In a murine model of secondary bacterial infection post-Influenza, there is massive influx of neutrophils and macrophages into the lungs and higher levels of pro-inflammatory cytokines in the lungs and blood [182]. It was also reported that human dendritic cells exposed to IAV and then infected with *Streptococcus pneumoniae* change their phenotypes and become more pro-inflammatory, producing higher levels of TNF- $\alpha$ , IL-6 and IL-12 [183, 184]. These data in conjunction with postmortem findings of patients that died with secondary bacterial infection after IAV infection [185] support the idea of tissue damage due to inflammation as an important contributor to the severity of pneumonia and morbidity.

Anti-inflammatory cytokines, such as IL-10, are also increased in the lungs after IAV and during bacteria co-infection [178, 182]. IL-10 has been suggested to play a role in the impairment of host defenses that follows IAV infection because treatment with anti-IL10 during a model of secondary bacterial infection restored the host ability to deal with infection [178]. Therefore, despite the increased expression of pro-inflammatory cytokines and striking influx of leukocytes into the lungs, the host defenses are impaired during post-influenza bacterial infections in part because of the markedly increased levels of IL-10. Another cytokine group that may contribute to the hyporesponsiveness of the immune system after an IAV infection is the type I and II interferons. Type I interferons may be important for the antiviral response during IAV infection but can also suppress IL-17 production by  $\gamma\delta$  T cells [175].

The inhibition of the Th17 pathway attenuates the production of antimicrobial peptides necessary for bacterial clearance in the lung [186], thus contributing to the increased susceptibility to secondary bacterial infection. Type II interferon (IFN- $\gamma$ ) produced after viral infections leads to a defect in alveolar macrophage-mediated phagocytosis of bacteria during a secondary infection and neutrophil dysfunction has also been observed after IAV infection [176, 177, 187, 188]. Associated with the dysfunction of phagocytes, it has been suggested that impairment of NK cells responses after IAV infection increases susceptibility to secondary *S. aureus* infection.

IAV infection can also lead to downregulation and desensitization of receptors such as the class A scavenger receptor MARCO and TLRs in alveolar macrophages thus impairing detection, phagocytosis and killing of bacteria [176, 189]. The desensitization of TLRs can persist for weeks or months after IAV infection contributing to the sustained susceptibility to secondary bacterial infections [189]. In addition, the increased expression of the receptor for the negative regulatory ligand CD200 during the recovery phase of IAV infection augments the threshold for innate immune activation enabling bacterial outgrowth [190].

Lastly, the increased severity of pneumonia observed during a co-infection can also be related with the impairment of the lung repair responses after IAV infection. A murine model of infection with a pandemic strain of IAV showed that the expression of epithelium cell proliferation and lung repair genes were decreased after virus infection and pro-inflammatory genes were strongly increased during the secondary pneumococcal infection [191]. In agreement with that, a most recent study showed that during co-infection with IAV and *Legionella pneumophila* genes involved in tissue protection and repair were specifically downregulated and it was determinant for the high morbidity and mortality observed [192]. Therefore, the lack of appropriate lung repair responses after IAV infection can also play a role in enhancing disease severity during bacterial co-infection.

The intense inflammatory response in the lungs that is caused by IAV infection is followed by a resolution state characterized by enhanced susceptibility to bacterial infection. A few molecular players of enhanced susceptibility have been found but there is much more research to be conducted to understand how IAV predisposes to subsequent infection.

### Anti-inflammatory therapies

As discussed above, inflammatory responses are essential for the clearance of the virus and return to lung homeostasis during IAV infection. However, excessive, altered

(different mediators) or misplaced (systemic) activation of inflammatory responses is clearly associated with intense lung damage and death. In principle, it is possible that we may develop immunomodulatory therapies aiming to modifying the unwanted inflammatory response triggered by IAV infection without interfering with the inflammatory response necessary to clear the virus [193]. The idea is that administration of such anti-inflammatory drugs during severe cases of flu may reduce patient symptoms related with the increased levels of pro-inflammatory mediators [43] and potentially modify favorably the prognosis of the infection. In this respect, one would expect that anti-inflammatory drugs decreased the number of hospitalizations and complications associated with IAV infection. Additional benefits of this kind of therapy are that therapeutic strategies aiming at host responses would be expected to decrease inflammatory responses regardless of the virus strain [194] and that they may be synergic with antiviral drugs.

Table 2 summarizes some of the immunomodulatory strategies that have been investigated for IAV infection treatment. Four important aspects deserve consideration in the context of IAV infection. First, timing of administration should be such that any anti-inflammatory drug would need to be effective even after infection had initiated and symptoms were present (in infected patients) or to prevent occurrence of severe disease in patients with contact with other infected patients. In this regard, pre-clinical studies do need to take into account this delayed administration of drugs and test potential anti-inflammatory compound in a more therapeutic setting: i.e., treatment should start and be effective several days after the infection of experimental animals. Second, anti-inflammatory therapies against IAV should work in association with and provide additional benefit to currently used antiviral drugs. Third, anti-inflammatory therapies should preferentially decrease the risk and/or severity of secondary bacterial infections that follow IAV infection. Fourth and most importantly, anti-inflammatory therapies should not interfere with the ability of the human host to deal with infection, even mild infection, i.e., the generation of adaptive immune responses should not be altered by anti-inflammatory drugs. An example of detrimental effects of anti-inflammatory/immunomodulatory drugs during IAV infection was observed during the 2009 IAV pandemic. Systemic treatment with corticosteroids during early or mild stages of pH1N1 infection increased the risk of secondary bacterial or fungal infections, critical illness and death [195, 196]. However, a Japanese study found no association between corticosteroid therapy during IAV infection and worse outcome [197]. Regardless of the conflicting available evidence, the data do reflect the necessary caution for the use of systemic corticosteroids to treat acute infections and put a word of

**Table 2** List of anti-inflammatory strategies tested in the context of IAV infection

Immunomodulatory strategy	Targets	Administration effects
Statins	Neutrophil recruitment and activation	Reduction of H1N1 infected patients mortality [201] Decreases lung injury in H5N1 and H1N1 infected mice [204]
	Secretion of pro-inflammatory cytokines	Inhibition of virus replication [205]
COX-2 inhibitors	Host pro-inflammatory mediators production	Combination of COX-2 and neuraminidase inhibitors improves survival of H5N1 infected mice [209] Prevents immunopathology in H1N1 and H3N2 infected mice [210]
PAFR antagonist	PAF binding and signaling	Reduction of lethality of H1N1 infected mice [96] Decreases the inflammatory responses and prevents lung injury in H1N1 infected mice [96]
CCR2 antagonist	CCL-2 binding and mononuclear cells recruitment	Prophylactic treatment reduced pathology, morbidity, and mortality during IAV infection [217]
PPAR agonists	Activation and signaling of PPA- $\alpha$ and PPA- $\gamma$ receptors	Decreases lethality of H2N2 and H1N1 infected mice [213, 214] Reduces pro-inflammatory cytokines production [212, 214]
Inhibition of complement activation	Inhibition of the anaphylotoxins C5a or C3a pro-inflammatory effects	Reduction of overwhelming inflammation [109, 112] Prevents lung injury during H5N1 and H1N1 infection in mice [109, 112]
Sphingosine-1-phosphate receptor agonist	Activation and signaling of S1P receptor	Decreases lethality of H1N1 infected mice Prevents lung injury during H1N1 infection in mice Inhibition of pro-inflammatory T cell responses [32, 220]
Protectin D1	Increase the levels of this pro-resolving mediator	Decreases lethality of H5N1 infected mice [216] Inhibition of virus replication [216]
Macrolides (azithromycin, clindamycin, erythromycin, clarithromycin)	Antibiotics that also have anti-inflammatory effects	Erythromycin: prevents lethality of H2N2 infected mice [222] Clindamycin and azithromycin: improves outcomes of secondary bacterial pneumonia post-IAV infection [223]
	Decreases host pro-inflammatory cytokine production	Clarithromycin: reduction of fever in patients infected with seasonal or pandemic H1N1 IAV [224]
	Decreases leukocyte recruitment to tissue sites	
PDE4 inhibitors	Inhibition of phosphodiesterase 4 Increases cAMP levels	Prevents lethality and immunopathology of H1N1 infected mice, specially when combined with antivirals [227]
Apocynin	Inhibition of Nox2 (catalytic subunit of NADPH oxidase)	Pre-treatment decreased levels of superoxide, viral titers and infiltration of leukocytes into the airways of mice infected with IAV H3N2 and H1N1 [138]
Catalase	Antioxidant Inhibition of ROS	Decreases lethality, reduced the inflammatory response and pulmonary injury and lowered the viral load in the lungs of mice infected with H1N1 IAV [139]
N-acetyl-L-cysteine	Antioxidant Inhibition of ROS	Reduces mortality and inflammation in mice infected with H9N2 swine, H5N1 and H1N1 IAV infection [137, 140], specially when combined with antivirals
A1-adenosine receptor (A1-AdoR) antagonist	Inhibition of A1-AdoR	Reduced hypoxemia, pulmonary edema, and leukocyte recruitment to the airways of mice infected with H1N1 IAV [226]
Signaling pathways inhibitors	Inhibition of Raf/MEK/ER K and NFK-B pathways	Reduction of pro-inflammatory cytokines levels, lung immunopathology and inhibition of steps of the virus cycle [225]

caution to any potential anti-inflammatory strategy against severe IAV infection.

Statins are an example of drugs used as anti-inflammatory and immunomodulatory in the context of IAV infection. Statins are drugs used to lower lipids levels in blood, but are known also to decrease several features of inflammation, including neutrophil recruitment and

secretion of pro-inflammatory cytokines [198]. There are a few studies that suggest that statins are beneficial in the context of IAV infection [199, 200]. A relevant study of 3043 patients hospitalized with IAV infection showed that administration of statins prior to or during the hospitalization period could reduce mortality by 41 % [201]. However, other groups have not observed the same effects

of statin administration during IAV infection [202]. Studies in murine models of IAV infection are also controversial. Kumaki et al. in 2012 showed that oral or systemic administration of different statins could not improve the outcome of mice after infection with the highly pathogenic avian influenza H5N1, seasonal or H1N1pdm09 virus [203]. On the other hand, Liu et al. 2009 showed that statin treatment ameliorated lung damage and inhibited viral replication of influenza H5N1, H3N2 or H1N1, especially when given before infection [204]. A more recent study, also demonstrated that administration of simvastatin *in vitro* could decrease the levels of pro-inflammatory cytokines and reduce virus replication by inhibiting proteins related to cytoskeleton function [205]. Therefore, given the safety profile of statins in the context of IAV infection, the use of these drugs may be useful patients at high risk of severe disease secondary to IAV infection, a tenet that deserves further investigation.

Another class of anti-inflammatory drugs that have been suggested to improve patient outcome during IAV infection is the cyclooxygenase-2inhibitors. Cyclooxygenase-2 (COX-2) are enzymes that catalyze the conversion of arachidonic acid in prostaglandins and, thus, may contribute to inflammation [206]. COX inhibitors are largely used as analgesic, anti-inflammatory and anti-pyretic drugs and have been suggested to be of therapeutic benefit during IAV infection [207]. Cyclooxygenase-2 is up-regulated during infection with the pandemic H5N1 influenza virus *in vitro* and increased expression of this enzyme is also observed in lung tissue samples obtained during autopsy of patients who died of H5N1 disease [208]. Inhibition of COX-2 during a lethal H5N1 virus challenge was demonstrated to be beneficial when associated with a neuraminidase inhibitor, even when given as a delayed treatment [209]. In addition, inhibition of COX-2 by paracetamol or a selective inhibitor (celecoxib) during H1N1 and H3N2 infection prevented lung immunopathology without affecting virus clearance in mice [210]. Associated with these findings, COX-2 deficiency is protective in a murine model of IAV H3N2 infection and it is associated with a decrease of the inflammatory responses [211]. Therefore, animal studies suggest that the use of COX-2 inhibitors during IAV infection could be beneficial. However, clinical studies with human patients are still lacking to reinforce the proof of concept of using this class of drugs during IAV infection. Currently, a clinical trial is recruiting patients to test the efficacy of celecoxib during influenza infection (ClinicalTrials.gov Identifier: NCT02108366).

Several anti-inflammatory strategies have been studied in the context of experimental IAV infection (see Table 2). Many of these have been investigated in some detail and will be described below. Peroxisome proliferator-activated

receptor (PPAR) agonists provide beneficial effects in treatment of metabolic disorders, such as diabetes, and may also have anti-inflammatory effects [194]. PPAR activation leads to down regulation of NF- $\kappa$ B, AP1 and STAT signaling resulting in reduction of several pro-inflammatory cytokines [212]. In murine models of IAV infection, administration of Gemfibrozil, a synthetic PPAR- $\alpha$  agonist, was shown to protect mice from severe H2N2 infection possible due to its anti-inflammatory effect [213]. The same protection could not be seen in a model of H5N1 infection [209]. PPAR- $\gamma$  agonists such as pioglitazone and rosiglitazone were also suggested as immunomodulatory drugs to treat IAV infection. Interestingly, prophylactic treatment with both of these drugs reduced weight loss and lethality induced in a murine model of seasonal H1N1 infection. In addition, rosiglitazone pre-treatment could also partially prevent lethality of animals infected with the 2009 pandemic H1N1 strain [214].

Pro-resolving mediators are also listed as a potential strategy for controlling the overwhelming inflammation during IAV infection. Lipoxins and protectins are lipid mediators that have anti-inflammatory and pro-resolving effects and are shown to be protective during several models of infection [215]. During IAV H5N1 infection, there was down regulation of genes related to lipoxin pro-resolving effects and up regulation of pro-inflammatory cytokines genes and these changes associated with dissemination of the virus to other organs. Therefore, it seems that lipoxin may have protective roles during IAV infection, acting as a modulator of the inflammatory responses. Levels of another endogenous resolution-associated molecule, protectin D1, was decreased during a model of severe IAV H5N1 infection and correlated inversely with immunopathology [216]. Associated with this, protectin D1 treatment improved survival of infected mice and could also inhibit IAV replication [216].

We have previously shown that blockade of the PAFR could improve survival of IAV infected mice as it decreased several parameters of the inflammatory response [96]. Effects of PAFR antagonists were observed even when the drug was initiated 3 days after infection. Importantly, association of PAFR antagonist and oseltamivir (Tamiflu<sup>®</sup>) provided cooperative inhibition of lethality in infected mice (World Intellectual Property Organization, International publication number: WO 2009/05938 A1).

CCR2 is chemokine receptor expressed on a variety of leukocyte and has been shown to contribute to recruitment of mononuclear phagocytes [217]. Monocyte-derived mononuclear cells are thought to contribute to the immunopathology observed during IAV infection [118]. Indeed, pharmacological blockage of CCR2 reduced pathology, morbidity, and mortality during IAV infection

when given as a prophylactic treatment without affecting the virus clearance or aggravating secondary bacterial infections [218].

Another receptor that has been described as a therapeutic target during IAV infection is the sphingosine-1-phosphate receptor (S1P). Sphingosine 1-phosphate is a metabolite of sphingolipid that binds to five G protein-coupled receptors (S1P<sub>1-5</sub>) and controls different processes, including the immune response [219]. Administration of S1P ligands reduces the levels of cytokines and chemokines, and the tissue injury associated with infection with a human IAV strain [32] and a mouse-adapted influenza virus [220]. This effect was mostly due to the binding of the S1P agonist on S1P<sub>1</sub> expressed by lung endothelium, suggesting that endothelial cells have an important role in inflammation induction after IAV infection [221]. Of note, specific S1P<sub>1</sub> agonists did not impair the generation of neutralizing antibodies and did not alter viral clearance [220].

In summary, different studies have been suggested modulation of inflammatory response as a potential strategy for a better outcome during severe cases of flu [222–227].

## Concluding remarks

There is now no doubt that severe influenza cases are associated with intense lung inflammation and injury. However, the inflammatory response is also necessary to control infection acutely and drive adaptive immune responses that will ultimately control viral replication. As described above, several molecules or cell types which are necessary for protective immune responses may also contribute to tissue injury and death when present in large quantities. In addition, there are molecules and pathways which are preferentially associated with tissue injury and death rather than protection. Defining pathways of disease in clinical samples and pre-clinical studies is crucial if we are to identify novel targets for IAV infection. There are several pre-clinical studies which have suggested potential targets and these clearly provided the necessary proof of concept that it is possible to develop anti-inflammatory drugs for influenza. The difficult task ahead will be to translate these pre-clinical findings into therapies for humans. A great advantage of anti-inflammatory drugs is that, on the contrary of antivirals, they are not susceptible to virus resistance. Therefore, it is very important to consider combined treatment of antiviral and anti-inflammatory drugs to reduce virus burden and disease severity.

Ideal anti-inflammatory drugs should be effective when administered after symptoms onset, be used in combination with approved antivirals such as oseltamivir or zanamivir,

be able to reduce the risk of secondary bacterial infection and not to interfere with virus clearance mechanisms. Novel targets are needed and it is central that pre-clinical experiments model all of these parameters during drug development. Clinical studies with statins have provided mixed results and available murine studies with COX-2 inhibitors suggest these drugs may have potential benefit to patients.

The principle that infectious diseases are amenable to treatment with anti-inflammatory drugs appears to be a valid one. Ahead, we need to move into clinical development potential anti-inflammatory strategies that will decrease the suffering and death caused by severe IAV infection and secondary bacterial infections.

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## Compliance with ethical standards

**Conflict of interests** The authors declare that there is no conflict of interests regarding the publication of this paper.

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