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Review

Host Immunity against Influenza Virus Infection

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Abstract

Influenza is a major global health problem, causing infections of the respiratory tract, often leading to acute pneumonia, life-threatening complications and even death. During natural infection, viral replication is initially controlled by innate immunity before adaptive immune responses achieve viral clearance and host recovery. Adaptive T and B cells maintain immunological memory and provide protection against subsequent infections with related influenza viruses. Recent studies have also shed light on the role of innate T cells (MAIT cells, γδ T cells, and NKT cells) in controlling influenza and linking innate and adaptive immune mechanisms. Here, we review the various arms of the immune response to influenza virus infections and discuss how influenza viruses can escape immunity. These findings have helped delineate the interactions between influenza viruses and the host immune system, which will facilitate the development of novel treatment strategies and vaccines with enhanced efficacy.

Key words: Host Immunity, Influenza Virus Infection, Acute Pneumonia, Innate T Cells.

1. Introduction

Influenza viruses are enveloped negative-sense RNA viruses with segmented genomes from the family *Orthomyxoviridae* that cause a highly contagious and rapidly spreading acute respiratory infection (1). Three types of influenza viruses, classified as A, B and C, infect humans (2). While influenza A and B viruses co-circulate annually during seasonal epidemics, influenza C viruses generally cause mild infections in humans. Influenza virus infections present a significant global health problem, causing up to 650,000 fatal cases annually (3). Infections with influenza virus evoke host

immune responses, which ultimately result in the termination of virus replication. In addition, immunological memory is induced, which may protect against subsequent influenza virus infections. Currently influenza A viruses of the H1N1 and H3N2 subtypes and influenza B viruses are responsible for seasonal outbreaks of influenza. In 1918, influenza A viruses of the H1N1 subtype were introduced into the human population, causing the 'Spanish Flu' outbreak. These viruses circulated in humans until 1957 when influenza H2N2 viruses caused the 'Asian Flu' outbreak and completely replaced viruses of the H1N1 subtype. Within a decade, viruses of the H2N2 subtype were replaced by viruses of the influenza H3N2 subtype, which caused the 1968 'Hong Kong' pandemic. In 1977, influenza A viruses of the H1N1 subtype were reintroduced without causing a major pandemic. In 2009, a new influenza H1N1 virus of swine-origin caused the first influenza pandemic of the 21st century (4). Sporadically, a new influenza virus emerges from an animal host and rapidly spreads throughout the susceptible population, resulting in a pandemic often associated with significant morbidity and mortality (5). The pathogenesis of highly pathogenic avian influenza (HPAI) viruses in humans differs from that of seasonal influenza A viruses of the H1N1 and H3N2 subtype (6).

Influenza virus belongs to the *Orthomyxoviridae* family (1). Its genome is comprised of eight single-stranded viral RNA segments (vRNA) of negative polarity. Influenza viruses are divided into subtypes based on the structure of virus surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). Currently, there are 18 known subtypes of HA (H1-18) and 11 of NA (N1-11) (7). The replication cycle of influenza virus begins when the HA bind to sialic acids on the surface of epithelial cells of the respiratory tract, dendritic cells, type II pneumocytes, alveolar macrophages, or retinal epithelial cells (8). Viruses are internalized by endocytosis and then transported to late endosomes (9). The acidic environment in the late endosomes facilitates HA-mediated fusion of the viral and endosomal membrane, followed by degradation of M1 and release of vRNPs in the cytoplasm (10). The vRNPs enter the nucleus and in the nucleus, negative-sense vRNA is transcribed into positive-sense mRNA using viral polymerase (11). The viral proteins are translated from mRNA in the cytoplasm by ribosomes in a cap-dependent manner. Some viral proteins are imported into the nucleus to replicate vRNA.

Immunity to influenza virus infection has been a research topic for more than 70 years (12). Although many original questions in this field have been answered, many outstanding issues need to be addressed. In this review, we will discuss some important aspects and new insights of innate and adaptive immune responses against influenza virus infection.

2. Innate immunity

The innate immune system forms the first line of defence against influenza virus infection. This system consists of components (e.g., mucus and collectins) aimed to prevent the infection of respiratory epithelial cells. Rapid innate cellular immune responses are induced to regulate virus replication. Innate immunity to influenza viruses also involves several innate T cell subsets, such as mucosal-associated invariant (MAIT), natural killer (NKT) and γδ T cells, which have been the focus of recent studies.

2.1 Intracellular innate sensing of influenza virus infection

Influenza A virus infection is sensed by infected cells via pattern-recognition receptors (PRRs) that recognize viral RNA, the main pathogen-associated marker pattern (PAMP) of influenza A viruses. The PRRs include toll-like receptors (TLRs), retinoic acid inducible gene-I (RIG-I) and the NOD-like receptor family pryin domain-containing 3 (NLRP3) protein (13).

TLR7 binds single-stranded viral RNA (especially in plasmacytoid dendritic cells), and TLR3 and RIG-

I bind double-stranded viral RNA (in most other infected cells). The signalling of these receptors leads to the production of proinflammatory cytokines and type I interferons (IFN-I) (14, 15). IFN-β is produced and regulated via positive feedback by interferon regulatory factor (IRF) 7, stimulating the expression of both IFN-α and IFN-β (16). These interferons exert strong antiviral activity, inhibiting protein synthesis in host cells and limiting virus replication. IFN-I also induces interferon-stimulated genes (ISGs) via the JAK/STAT signalling pathway (17). One of the ISGs is a myxovirus (MX) gene, which encodes the MxA protein, a GTPase with strong antiviral activity that can inhibit influenza virus replication (18). IFN-I also stimulates dendritic cells (DCs), resulting in the enhancement of antigen presentation to $CD4^+$ and $CD8^+$ T cells, thus contributing to the initiation of adaptive immune responses (19). NLRP3 is part of the NLRP3 inflammasome, a cytoplasmic complex associated with immunity against influenza virus. The receptor is activated by influenza virus infection and M2 ion channel activity, leading to the conversion of pro-IL-1β into IL-1β, a cytokine that is involved in the induction of Th17 cells, and the expansion of antigen-specific $CD4^+$ T cells (19, 20).

Over the past ten years, it has been established that non-coding RNAs (ncRNAs), including small non-coding RNAs (e.g., microRNAs (miRNAs), small interfering RNAs (siRNAs)) and long non-coding RNAs (lncRNAs), act as an important class of regulators involved in virus–host interaction, especially in the antiviral immune response. After sensing the invading virus via PRRs, host cells trigger the activation and nuclear translocation of transcription factors and produce robust IFNs and various cytokines. In turn, the IFNs and cytokines initiate the receptor-associated signaling pathways that lead to the production of antiviral proteins (e.g., ISGs) to defend against viral invasion. Many functional miRNAs and lncRNAs, including nuclear enriched abundant transcript 1 (NEAT1), bone marrow stromal antigen 2 (BST2) IFN-stimulated positive regulator (BISPR), negative regulator of antiviral response (NRAV) and lncRNA cytidine/uridine monophosphate kinase 2 (lncRNA-CMPK2), are evidenced to be involved in these signalling pathways by regulating different steps (21). Some miRNAs have also been identified to play important roles in host innate antiviral response to influenza virus infection. They appeared to inhibit the viral infection (e.g., miR-4276 (22) and miR-650 (23)) or to facilitate virus replication (e.g., miR-451 (24) and miR-548an (25)). Interestingly, some of these ncRNAs modulate the viral gene expression directly. On the other hand, some ncRNAs are hijacked by the virus for use for infection and replication. Taken together, these findings have substantially indicated that ncRNAs act as key regulators of host–influenza virus interaction and are critically involved in influenza virus pathogenesis.

2.2 The role of alveolar macrophages in influenza virus infection

Upon infection of the alveoli, alveolar macrophages become activated and phagocytose influenza virus-infected cells, thus limiting viral spread (26). However, once macrophages become activated in the lungs during influenza virus infections, these cells produce nitric oxide synthase 2 (NOS2) and tumour necrosis factor alpha (TNF-α), contributing to influenza virus-induced pathology (27). These two distinct and competing functions of alveolar macrophages emphasize the importance of a balanced immune response against influenza virus infection. Infections with HPAI virus are more prone to cause immunopathology, and these viruses can infect blood-derived and alveolar macrophages (28). Blood-derived macrophages infected with influenza H5N1 virus produce large amounts of proinflammatory cytokines. In contrast, H5N1-infected alveolar macrophages do not show effective virus production and produce only low levels of pro-inflammatory cytokines (29). Furthermore, alveolar macrophages regulate immune responses, particularly the development of antigen specific T cell immunity (30). Influenza viruses (e.g., H5N1) that infect alveolar macrophages are potentially able

to undermine the immunosuppressive functions of these cells. It has been reported that alveolar macrophage and macrophages cultured from monocytes are phenotypically different cells, where alveolar macrophage have a alternatively activated phenotype, whereas monocytes macrophages have a classical activated phenotype (31). Furthermore, it has been reported that influenza virus infection of human alveolar and blood-derived macrophages is different in accessory cell function and interferon production (32). More research is needed to define the role of alveolar macrophages and their interactions in the immune responses against influenza virus infection.

2.3 Dendritic cells and their role in influenza virus infection

DCs are considered professional antigen-presenting cells in influenza virus infections. The conventional DCs (cDCs), situated underneath the airway epithelium barrier and above the basal membrane, monitor the airway lumen via dendrites extended through the tight junctions between airway epithelial cells. The cDCs can not only detect and neutralize virions and apoptotic bodies from infected cells but also become infected. Upon entry of the virion into the cell, the cDC migrates, depending on CCR7, via the afferent lymphatic system to the draining lymph node. In the lymph node, the cDCs present influenza virus-derived antigens to and activate T cells (33). The cDCs degrade viral proteins and subsequently the epitopes are presented by MHC class I or class II molecules. For MHC class I presentation, the influenza virus-derived peptides are liberated in the cytosol by proteasomes and subsequently transported to the endoplasmic reticulum where they associate with MHC class I molecules. Then, the MHC class I/peptide complexes are transported via the Golgi complex to the cell membrane for recognition by specific $CD8^+$ cytotoxic T cells (CTL). For MHC class II presentation, viral proteins are degraded in endosomes/lysosomes, and the resulting peptides associate with MHC class II molecules. These complexes are then transported to the cell membrane for recognition by $CD4^+$ T helper (Th) cells. Among the various other activities of DCs in influenza virus infection, these cells can also exert cytolytic activity (interferon killer DC) and contribute to the formation of bronchus-associated lymphoid tissue (iBALT) ($CD11^+$ DC) (34).

2.4 Innate T cells

2.4.1 MAIT cells

MAIT cells constitute up to 5% of the total T cell pool in humans and are characterized by the expression of the semi-invariant T cell receptor (TCR) Vα7.2, which recognizes bacterial-derived vitamin B2 derivatives presented by MHC-like protein 1 (MR1) (35). Upon activation, MAIT cells produce proinflammatory cytokines and upregulate cytotoxic granzymes and perforin (36). Although MAIT cells are predominantly associated with the control of bacterial infections, there is increasing evidence of their clinical importance in viral infections, autoimmune diseases, and cancers (37). With respect to human influenza infection, van Wilgenburg et al. showed that the number of human MAIT cells in the peripheral blood of severely ill individuals was reduced by nearly half and these cells were highly activated, as shown by the upregulation of granzyme B (GrB) (37). Furthermore, Loh et al. showed that patients who died from severe H7N9 infection had 3- to 5-fold fewer MAIT cells in the blood, compared to patients who recovered from severe H7N9 infection or were healthy (38). These studies suggest a loss and/or efflux of MAIT cells from the blood to sites of infection during severe influenza disease. Further, both studies showed that the MAIT cell activation in influenza infection was MR1-TCR independent and mediated by IL-18 (37), which is mainly produced by $CD14^+$ monocytes. A reduction in MAIT cell numbers during acute influenza infection could impair protective anti-bacterial immunity, increasing the risk of bacterial co-infection, which would enhance disease severity and mortality (39).

2.4.2 NKT cells

Natural killer T cells are innate T lymphocytes restricted by the glycolipid α-galactosylceramide (αGC) presented on MHC class I-like CD1d molecules. Similar to conventional T cells, NKT cells express αβ-TCRs as well as NK cell markers, including CD3, CD56 (humans) or NK1.1 (mice). NKT cells constitute approximately 0.1-0.2% of lymphocytes in human PBMCs and are broadly classified into 2 groups: type I invariant NKT (iNKT) cells, expressing the invariant Vα14-Jα18 TCRα chain paired with either Vβ2, Vβ7 or Vβ8 in mice and Vα24-Jα18/Vβ11 in humans, and type II diverse NKT cells, which display diverse αβ-TCR gene pairings (40). In animals, NKT cells protect against viral infections and cancer (40) by regulating innate and adaptive immune responses via the rapid secretion of cytokines (41), the upregulation of CD40, and the activation of DCs (42).

In a mouse model of influenza infection, the intraperitoneal (i.p.) administration of αGC along with the intranasal (i.n.) administration of an influenza virus (H1N1/H3N2 recombinant) activates iNKT cells (without affecting the adaptive T cell responses), consequently reducing body weight loss and lung virus titres (43). Conversely, $CD1d^{-/-}$ mice lacking the CD1d molecule (and therefore, lacking CD1d-mediated stimulation of NKT cells) developed more severe disease following infection with a sublethal dose of influenza (PR8-H1N1) virus, as evidenced by lower body weight, higher lung virus titres, decreased IFN-γ production in bronchoalveolar lavage (BAL), and impaired NK and T cell activities, compared to wild-type (WT) mice [35]. Similarly, $Jα18^{-/-}$ mice (lacking NKT cells expressing Jα18) infected with the virulent influenza PR8-H1N1 virus displayed increased lung injury and mortality, associated with increased infiltration of $Ly6C^{hi}Ly6G^{-}$ inflammatory monocytes in the lungs, compared to $Jα18^{-/-}$ mice receiving αGC-boosted iNKT cells. Moreover, αGC-boosted iNKT cells reduced MCP-1 levels by lysing influenza-infected monocytes in a CD1d-dependent manner (44). These findings were consistent with those of another study, in which a rapid reduction in body weight and 100% mortality by 11 days post-infection (d.p.i.) was observed in $Jα18^{-/-}$ mice. The enhanced pathology in influenza-infected $Jα18^{-/-}$ mice was associated with a 4-fold decrease in $D^{b}PA_{224-233}$- specific $CD8^{+}$ T cells in lungs at 4 and 7 d.p.i. and the differential accumulation and maturation of $CD103^{+}$ DCs (45). Moreover, the absence of iNKT cells led to the suppression of influenza virus responses due to the increased expression of the arginase and nitric oxide system, and this effect was mitigated by the adoptive transfer of iNKT cells that restored antiviral immunity in a CD1d- and CD40-dependent manner (46). Furthermore, the use of αGC as a vaccine adjuvant improved the long-term survival of $CD8^{+}$ cytotoxic T lymphocytes and provided heterologous protection in a mouse model of influenza infection (47). Similarly, αGC analogues, KBC-007 and KBC-009, administered with inactivated influenza A/PR/8/34 virus boosted humoural and cellular immune responses in mucosal and systemic compartments post-immunization and induced protective immune responses when challenged with PR8 virus (48). The intramuscular administration of inactivated A/California/04/2009 pandemic H1N1 virus with αGC to piglets increased NKT-cell numbers, induced high titres of anti- hemagglutinin (HA) mAbs, and boosted influenza virus-specific $CD8^{+}$ T cells post-vaccination. Virus challenge in piglets vaccinated with αGC/inactivated virus resulted in reduced viral shedding compared to that in piglets vaccinated with whole inactive virus alone (49). Intranasal immunization with inactivated influenza A/PR/8/34 virus with αGC in BALB/c mice induced IgG and IgA antibodies for up to 3 months and boosted cellular immunity compared to that in mice immunized with inactivated PR8 virus alone (50). Collectively, these animal studies demonstrate the role of NKT cells in modulating innate and adaptive immune responses during influenza virus infection. Additionally, αGC as a vaccine adjuvant boosted NKT numbers and improved protection, further supporting the role of

NKT cells in ensuring optimal influenza-specific T and B cell responses in mucosal and systemic compartments. Despite the large number of animal studies investigating the role of NKT cells during influenza infection, there is a paucity of data supporting their protective role in human influenza, warranting further investigation.

2.4.3 γδ T cells

Notably, γδ T cells (approximately 1%-10% of peripheral blood T cells in humans) are important components of both innate and adaptive immunity, displaying a vast array of effector functions that make them attractive targets for therapeutic vaccines. The γδ T cells are characterized by a diverse range of TCRs, with specific Vγ-Vδ pairings found across different anatomical sites. The antigen specificity of γδ T cells remains largely unclear. Butyrophilin (BTN) protein family molecules can regulate the immunosurveillance of γδ T cells (51), while phosphoantigens (pAg, recognized via a mechanism involving BTN3A1), human MutS homologue 2 and F1-ATPase along with apolipoprotein can activate Vγ9Vδ2 T cells, and CD1d or MICA-lipid complexes and endothelial protein C receptor can stimulate non-Vδ2 T cells. Indeed, γδ T cells can directly kill virus-infected, cancerous, or stressed cells by secreting perforin, GrB, and granulysin and can promote inflammation and wound healing, as well as assist antigen-presenting cells in generating optimal, antigen-specific T cell responses (52).

Vγ9Vδ2 T cells might contribute to the early stage of immune protection through at least three possible mechanisms. (1) Direct recognition of tumour or infected target cell. Following TCR engagement, Vγ9Vδ2 T cells release rapidly large amounts of inflammatory cytokines (TNF-α and IFN-γ) and lytic mediators (perforin, granzyme B and granulysin), leading to the destruction of target cells and internalized pathogens such as mycobacteria. (2) Priming of other immune effectors. Vγ9Vδ2 T cells are activated by pathogen-infected DCs and promote accelerated and complete DC maturation through strong release of TNF-α and CD40 engagement. Maturing DCs release significant levels of IL-12, which amplifies IFN-γ secretion by activated T lymphocytes. Released cytokines, as well as fully matured DCs, can then contribute to the priming of innate and adaptive immune effectors subsequently involved in the clearance of pathogens. (3) APC functions. Upon short-term antigenic stimulation, Vγ9Vδ2 T cells acquire several attributes of APCs (CD40, MHC I and II, and costimulatory molecules) and can promote the priming of naive conventional αβ T cells. B cells might be required to get optimal induction of those APC features.

Murine studies of influenza infection showed that 15-30% of the lymphocytes in the BAL between 10 and 15 d.p.i. were γδ T cells, compared to the few lymphocytes observed at 5-7 d.p.i. This finding suggests that γδ T cells may play a pivotal role in recovery from influenza infection (53). Moreover, in a secondary influenza infection model of mice challenged with the H1N1 virus one month following H3N2 virus infection, γδ T cells isolated from the BAL acquired an NK1.1 phenotype and displayed potent cytotoxic functions, as shown by the killing of H3N2 virus-infected target cells in the presence of PHA (54). Moreover, γδ T cell hybridomas, derived from TCRβ$^{-/-}$ mouse, efficiently cleared influenza A and B virus infections via Hsp60 upregulation in infected cells and not by any viral proteins (55). In humans, ex-vivo γδ T cells rapidly produced IFN-γ and upregulated CD25 and CD69 following influenza virus infection. The activation of γδ T cells is dependent on the mevalonate pathway, as shown by a potent reduction in antiviral responses upon the addition of mevastatin, a mevalonate pathway inhibitor (56). Furthermore, *in vitro* studies with human peripheral blood mononuclear cells (PBMCs) showed that pAg-activated γδ T cells were capable of killing macrophages and lung alveolar epithelial cells infected with both human (H1N1) and avian (H5N1) influenza viruses (57). Moreover, pAg isopentenyl pyrophosphate (IPP)-expanded human Vγ9Vδ2 T cells displayed cytolytic activity (as measured by % target cell death), increased production of IFN-γ against pandemic H1N1 virus-

infected monocyte-derived macrophages, and expressed the inflammatory chemokine receptors CCR1, CCR5, and CXCR5 (57). pAg-reactive Vγ9Vδ2 T subsets harbour a semi-invariant Vγ9 pairing with diverse Vδ2 TCRs (58). Vγ9Vδ2 T cells can be stimulated prior to exposure with environmental antigens, as exemplified by foetal blood-derived Vγ9Vδ2 T cells and their potent activation following pAg exposure (59). Overall, these human studies indicate that the pAg-reactive Vγ9Vδ2 T subsets harbour innate-like activity towards influenza infection. These findings highlight the anti-viral potential of γδ T cells during influenza infection and suggest that the enhancement of γδ T cell responses might be beneficial for protection against influenza viruses.

3. Adaptive immunity

The adaptive immune system forms the second line of defence against influenza virus infection. This system consists of humoural and cellular immunity mediated by virus-specific antibodies and T cells, respectively.

3.1 Humoural immunity

Influenza virus infection induces virus-specific antibody responses (60). Particularly, antibodies specific for the two surface glycoproteins HA and NA are of importance, since the presence of antibodies recognizing these proteins is correlated with protective immunity (61). The HA-specific antibodies predominantly bind to the trimeric globular head of HA and inhibit viral attachment and entry into host cells. HA-specific antibodies thus can neutralize the virus. Furthermore, these antibodies facilitate the phagocytosis of virus particles by Fc receptor-expressing cells. Furthermore, binding to HA, expressed on infected cells, mediates antibody-dependent cell-mediated cytotoxicity (ADCC). HA-specific antibodies are a solid correlate of protection provided that these molecules match the virus causing the infection (62). In a recent study, Whittle et al. describes a human antibody that is directed against the HA receptor-binding pocket and can neutralize antigenically diverse influenza viruses of the same subtype (63). In contrast to the relatively variable HA globular head, the HA stem region is highly conserved because this part of the glycoprotein is physically masked for the immune system. Antibodies are formed against the stem region during influenza virus infection, although titres are low. Interestingly, some of these antibodies recognize and bind HA molecules from different subtypes and have broad neutralizing capacities (64).

Additionally, antibodies to NA have protective potential. The enzymatic activity of the NA protein results in the cleavage of sialic acid residues on the cell surface to facilitate the release and spread of newly formed virus particles. By binding NA, antibodies, thus, do not directly neutralize the virus but by inhibiting enzymatic activity these molecules indirectly limit virus spread. Furthermore, NA-specific antibodies also facilitate ADCC and may also contribute to the clearance of virus-infected cells (65). Matrix protein 2 (M2) is a third viral membrane protein. M2 forms tetramers that function as ion channels responsible for both the acidification of the virion and the neutralization of the pH in the Golgi network during early and late virus replication. The protein is highly conserved among influenza viruses of different subtypes and immunity against M2 was first demonstrated in mice by using a therapeutic monoclonal antibody raised against the protein (66). The protein itself is present at low concentrations in infected cells, and thus, M2-specific antibodies are raised after natural infection to a limited extent.

Nucleoprotein (NP) is an important target for protective T cells. However, NP-specific antibodies may also contribute to protection against influenza virus infection (67). Although the precise mechanism of protection remains unknown, these antibodies can induce the complement-mediated cell lysis of infected cells (68).

The main antibody isotypes in influenza-specific humoural immune responses are IgA, IgM, and IgG. Mucosal or secretory IgA antibodies are locally

produced and transported along the mucus of the respiratory tract by transepithelial transport and can afford local protection from the infection of airway epithelial cells. These antibodies also neutralize influenza viruses intracellularly (69). Serum IgAs are rapidly produced after influenza virus infection, and the presence of these antibodies is indicative of a recent influenza virus infection (70). Serum antibodies of the IgG subtype predominantly transudate into the respiratory tract and afford long-lived protection (71). IgM antibodies initiate the complement-mediated neutralization of influenza virus and are a hallmark of primary infection (72).

3.2 Cellular immunity

Upon infection with influenza virus $CD4^+$ T cells, $CD8^+$ T cells and regulatory T cells (Tregs) are induced. $CD4^+$ T cells are activated after recognizing virus-derived MHC class II-associated peptides on APCs, which also express co-stimulatory molecules. Some $CD4^+$ T cells display cytolytic activity towards infected cells (73). However, the most important phenotype of these cells is that of T helper (Th) cells. Different subsets of Th cells are distinguished based on their cytokine expression profiles. Th2 cells produce IL-4 and IL-13 and are considered to predominantly promote B cell responses (74), whereas Th1 cells produce IFN-γ and IL-2 and are involved mainly in cellular immune responses. In addition, regulatory T cells (Tregs) and T helper 17 (Th17) cells that regulate the cellular immune response to influenza virus infection have been identified. The first subset controls both the T helper cell and the $CD8^+$ T cell response after infection. Moreover, after influenza vaccination, Tregs are induced that do not alter the B cell response but suppress the T helper response induced by vaccination (75). In contrast, in an inflammatory environment, Th17 cells improve T helper responses by producing IL-6, which inhibits Treg function (76).

The main function of virus-specific $CD8^+$ T cells is that of cytotoxic T lymphocytes (CTL). Upon influenza virus infection, these cells are activated in lymphoid tissues and recruited to the site of infection. There, CTLs recognize and eliminate influenza virus-infected cells and thus prevent the production of progeny virus. Their lytic activity is mediated by the release of perforin and granzymes (e.g., GrA and GrB). Perforin permeabilizes the membranes of the infected cells and subsequently granzymes enter the cell and induce apoptosis. A recent study demonstrated that even in the absence of GrA and GrB, influenza virus-specific CTLs were able to lyse target cells *in vivo* (77). GrA is associated with the induction of proinflammatory cytokines and display noncytotoxic activities (78). These noncytotoxic activities focus on inhibition of virus replication via the cleavage of viral proteins and host cell proteins that are involved in protein synthesis. CTLs also induce the apoptosis of infected cells through Fas/FasL interactions. Furthermore, these cells produce cytokines that improve antigen presentation by stimulating MHC expression and display antiviral activity.

Post-infection virus-specific CTLs are found in lymphoid organs and in circulation. These cells also reside at sites of infection in iBALT structures, which are formed and maintained under the control of T cell-produced IL-17 and dendritic cells (34, 79). These memory CTL populations can act upon subsequent influenza virus infections. The reactivity and affinity of memory CTLs during a secondary infection depends on the co-stimulation these cells receive during their initial differentiation phase (80). Human CTLs induced by influenza virus infection are mainly directed against NP, M1 and PA proteins (81). These proteins are highly conserved, and therefore, CTL responses display a high degree of cross-reactivity, even between different subtypes of influenza A virus.

4. How influenza viruses escape immunity

Influenza virus proteins bind and inhibit various components of the innate immune system. The immune pressure in the human population and the high mutation rate of the influenza viral genome give rise to antigenically distinct influenza viruses that are

only partially or not at all recognized by the existing adaptive humoural and cellular immune repertoire.

4.1 Escape from innate immunity

Influenza virus NS1 protein can bind viral RNA with its RNA binding domain and mask it from recognition by TLRs and RIG-I, which would induce IFN-I production upon binding viral RNA (82). NS1 proteins can also bind tripartite motif-containing protein 25 (TRIM25) to inhibit the activation of RIG-I or form a complex with RNA-dependent protein kinase (PKR) and inhibit its function (82). Normally, PKR is activated upon the detection of double-stranded viral RNA, leading to translation arrest and the inhibition of viral protein synthesis (83). Normally PKR activity is regulated by the cellular inhibitor P58IPK, which forms a complex with heatshock protein 40 (hsp40). A recent study demonstrated that influenza virus NP interacts with this complex, leading to the dissociation and activation of P58IPK. This factor then inactivates PKR and inhibits the antiviral state of the cell to enable viral protein synthesis and virus replication (84). In addition, influenza virus M2 protein inhibits P58IPK by associating with the inhibitor and hsp40. This association results in the inhibition of protein synthesis, induction of cell apoptosis, and facilitates the release of newly formed virus particles (85).

Influenza PB1-F2 with a serine at position 66 can inhibit IFN-I production by binding and inactivating the mitochondrial antiviral signalling protein (MAVS) (86). The 1918 influenza H1N1 virus and HPAI H5N1 viruses have a PB1-F2 polymorphism associated with increased virulence (87). Interestingly, the introduction of this polymorphism in the new 2009 pandemic H1N1 virus resulted in higher levels of proinflammatory cytokines induced by infection, whereas the impact on virulence was minimal (88). The PB1-F2 protein is also associated with the induction of apoptosis and has a synergistic effect on the function of influenza virus polymerases PA and PB2 (87). The latter can inhibit IFN-I production by associating with MAVS, similar to PB1-F2 (89). The association of PB2 with MAVS depends on a single amino acid polymorphism in the mitochondrial localization signal. Interestingly this polymorphism is found in seasonal influenza viruses and not in HPAI viruses, implying that the latter are not optimized to counteract the human innate immune response. PB2 can also bind and inhibit interferon promoter stimulator 1 (IPS-1), which typically promotes IFN-β production (90).

4.2 Escape from humoral immunity

Influenza virus HA is the main target for the humoral response and immune pressure mediated by antibodies induced by infections of the human population, resulting in escape variants of this protein. Since influenza viruses lack proofreading, random point mutations occur in the viral genome during replication. From the quasi species, viruses are positively selected with mutations in regions encoding the antibody binding sites in the HA (91). These mutations result in changes in the antigenic regions of the HA protein, a phenomenon called antigenic drift. Thus far, five antigenic sites have been identified in the globular head of influenza virus HA, and these sites determine the antigenic properties of this virus. Changes in these sites alter antigenicity or increase the receptor binding affinity of the HA (92).

In contrast to antigenic drift, antigenic shift is a more dramatic change in virus antigenicity. Either a new influenza virus subtype is introduced in the human population (e.g., H5N1, H7N9, and H9N2) or a human influenza A virus reassorts with an animal influenza A virus of a novel subtype. The latter can occur when the two viruses infect a “mixing vessel” (e.g., humans or pigs), and a new influenza virus emerges with gene segments form both parent viruses. Antibodies raised against seasonal influenza A virus poorly recognize influenza A viruses of novel subtypes and will afford limited protection.

4.3 Escape from cellular immunity

Epitopes recognized by virus-specific CTL are also under selective pressure. The ratio between synonymous and non-synonymous (Ds/Dn)

mutations in the NP gene is lower in CTL epitope sequences than in the other proteins (93). Indeed, a number of amino acid substitutions observed in CTL epitopes during the evolution of influenza H3N2 viruses were associated with escape from recognition by virus-specific CTLs (94). Substitutions in the HLA anchor residue of the epitope as well as in the T cell receptor contact residues can affect influenza virus-specific T cell responses significantly (95). Interestingly, a R384G substitution in NP epitopes affected viral fitness, and two compensatory extra-epitopic mutations were necessary to restore viral fitness. The virus thus has the capacity to overcome functional constraints to evade T cell immunity. The rapid fixation of the R384G substitution was explained by strong bottleneck and founder effects at the population level in a theoretical model. Other epitopes remain fully conserved, including the immunodominant M158-66 epitope, which is restricted by HLA-A*0201 and has a high prevalence in most countries. Furthermore, functional constraints may limit influenza viruses to efficiently escape from recognition by CTLs specific for highly conserved epitopes (96).

5. Conclusions

Although many of the questions raised 72 years ago have been answered, there are still major gaps in the current understanding of the various components of the immune system and their viral targets. Future studies should be aimed at elucidating the immune mechanisms of protection against influenza viruses to facilitate the design of novel drugs or vaccines. Collectively these efforts will improve an arsenal of possibilities to combat influenza virus infections in the future.

Declarations

1) **Consent to publication**
We declare that all authors agreed to publish the manuscript at this journal based on the signed Copyright Transfer Agreement and followed publication ethics.
2) **Ethical approval and consent to participants**
Not applicable.
3) **Disclosure of conflict of interests**
We declare that no conflict of interest exists.
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5) **Availability of data and material**
We declare that the data supporting the results reported in the article are available in the published article.
6) **Authors' contributions**
Authors contributed to this paper with the design (ZDX), literature search (LLX), drafting (LLX), revision (LLX), editing (ZDX) and final approval (ZDX).
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