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Immunity to seasonal and pandemic influenza A viruses

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Abstract

The introduction of a new influenza strain into human circulation leads to rapid global spread. This review summarizes innate and adaptive immunity to influenza viruses, with an emphasis on T-cell responses that provide cross-protection between distinct subtypes and strains. We discuss antigenic variation within T-cell immunogenic peptides and our understanding of pre-existing immunity towards the pandemic A(H1N1) 2009 strain.

Keywords

influenza virus; adaptive immunity; innate immunity; H1N1 2009; antigenic variation

1. Introduction

Influenza is a rapidly spreading acute respiratory disease that causes profound morbidity and mortality. Annual seasonal influenza epidemics result in ~500,000 deaths worldwide and cause huge losses to global economies. Influenza viruses grow rapidly in the human respiratory mucosa, with respiratory epithelial cells being the primary target, producing large amounts of virus that then infects alveolar macrophages and local dendritic cell (DC) populations. The first line of defence towards influenza infection is mediated by the innate immune system [1]. Infiltration of neutrophils and monocytes/macrophages into the lung is needed for host protection during the initial stages of infection as well as the recruitment of the adaptive arm of immunity, influenza-specific B and T cells.

Immune B cells secrete antibodies to effectively prevent infection by neutralising the virus and are involved in the resolution of the disease process. Antibody-based vaccines towards the variable surface glycoproteins, the hemagglutinin (HA) and neuraminidase (NA) are the most effective way to combat seasonal infections. However, vaccines need to be updated annually due to antigenic changes within the HA and NA. Importantly, antibody-based

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vaccines fail in the event of an influenza pandemic caused by the emergence of a novel influenza strain or when the vaccine strain does not match the circulating strain, while T cells elicit broader immunity against several influenza strains (both seasonal and pandemic) as they recognise more conserved internal components of the virus. Pre-existing CD8⁺ T cells directed towards conserved viral regions promote more rapid recovery via the production of pro-inflammatory cytokines and the direct killing of virus-infected cells [2]. Such CD8⁺ T cell-mediated cross-protection might, in the face of a pandemic, function to decrease disease severity and lead to better clinical outcomes, though the development of any T cell-based vaccination strategy would need to be approached with caution to rule out the possibility of immunopathology. Also, CD8⁺ T cell responses can exert selective immune pressure, leading to escape mutations in the viral peptide (p) component of the antigenic pMHC complex. To date, the extent of viral escape within influenza T cell epitopes (mutations occur in 71.4% of human T cell peptides across 23 influenza strains tested [3]) has not received much attention due to the acute nature of influenza infection.

This review summarizes innate and adaptive immunity to influenza A virus infection, with a particular emphasis on CD8⁺ T cell responses that (at least in animal models) can provide a substantial level of cross-protection between distinct influenza subtypes and strains. We discuss the antigenic variation within T cell immunogenic peptides and review our current knowledge on pre-existing immunity towards the newly emerged pandemic A(H1N1)-2009 strain.

2. Innate immune responses to influenza infection

Our understanding of innate immune involvement during influenza virus infection has greatly expanded in the last several years [4]. Influenza virus infection triggers mechanisms mediated via all three major families of innate receptors, namely the Toll like receptors (TLRs), Nod-like receptors (NLRs) and RIG-I like receptors (RLRs). TLR7 recognizes influenza ssRNA, activating a transcriptional program that leads to the induction of Type I IFN, IL-12, and IL-6 [5]. The NOD-like receptor NLRP3 is reported to become activated in response to influenza RNA [6-8], in a process that requires the viral M2 protein ion channel [8]. After NLRP3 activation, the cytoplasmic signalling platform called the inflammasome forms, generating active caspase-1, which can then cleave pro-IL-1 β , IL-18 and other reported ligands. In a different class of NLRs, NOD2 has been shown to mediate recognition of viral ssRNA in RSV and influenza virus model systems, leading to Type I interferon production [9]. Finally, the prototypical RLR, RIG-I, recognizes influenza virus RNA, a process which appears to induce significant IFN- α production by the infected cell. Knockouts of RIG-I (or a downstream pathway, MAVS), TLR7 or NLRP3 all lead to increased mortality, with RIG-I/MAVS and TLR7 being somewhat redundant for generating a Type I IFN response. A double knockout mouse deficient in both pathways experiences dramatically increased lethality after infection. These results highlight the central importance of the Type I IFN response in early influenza protection [10]. NLRP3-deficiency also results in reduced survival, though from increased early lung damage rather than major defects in Type I IFN production.

These early responses are generally mediated by resident alveolar macrophages, DCs and respiratory epithelial cells. A key feature of any immediate inflammatory responses is the secretion of chemokines to recruit additional populations of potential effector cells [11]. Resident leukocytes and infiltrating cell populations are activated and involved in multiple protective functions, though this area still remains relatively unexplored. Inflammatory monocytes populations have been described using various nomenclatures and phenotypes, but there is general agreement that a CCR2⁺, bone marrow-derived CD11b⁺ monocyte is a key constituent of the innate response during the first week to ten days of infection [7,

12-14]. The proposed functions of these cells include modulating inflammatory microenvironments, direct killing of infected epithelial cells via TRAIL, and regulation of CD8 T cell proliferation and survival. Similarly, migrating dendritic cells from the lungs and recruited inflammatory DCs from the blood play critical roles in lymph node antigen presentation. While many of these DCs originate in the lung, they generally appear to be phylogenetically distinct from the set that extravasated during the course of infection [15-17]. Neutrophils also invade the lung in the early stages of infection, but their function remains ambiguous, with evidence of both disease control and exacerbation being reported variously for different infection protocols [18, 19].

Defining the specific antiviral effector mechanisms of the innate immune response and their contribution to lung pathology and remodelling remains an important area of investigation. The rapid onset of morbidity and even death in some individuals argues strongly for early clinical intervention (either “professional” cells or the host epithelium), but any therapy must not limit the protective effects resulting from the activation of the innate response, including the determining influence on the subsequent generation of adaptive immunity. Clearly, we need a better understanding of the early phase of influenza virus infection.

3. The antibody response to influenza virus

Antibody molecules recognize conformational epitopes in the context of whole protein, both on free virus particles and on the surface of infected cells. Following primary influenza virus infection, B cells in the draining mediastinal lymph node (MLN) encounter antigens transported from the site of infection by presenting DCs by day 3 after infection [20]. Specific recognition of viral antigens and co-stimulation signals such as CD40-CD40L from “licensed” DCs results in the rapid division and production of antibody by B cells. Such B cell responses can be either T_H-dependent or independent, though any T_H-independent responses are likely to be small in magnitude and of reduced longevity. Extra-follicular T_H-independent short-lived plasma B cells are generated at the edge of T/B cell zones [21], whereas intra-follicular CD4 T_H-B cell interactions occur within the germinal centres. The T_H-dependent germinal centre reaction induces long-lived humoral immunity [22]. Both intra- and extra-follicular B cell responses have been shown for influenza virus infection [20].

A striking feature of influenza virus infection is the speed with which the virus replicates once established in the respiratory epithelium. Preformed virus-specific antibodies in the serum or, preferably, at the airway mucosal surface, can block virus entry and the subsequent establishment of infection [23]. As a result, all licensed, inactivated influenza vaccines to date are designed primarily to generate an antibody response [24] against the major hemagglutinin (HA) and neuraminidase (NA) virus surface molecules. A single infection with any strain of influenza viruses elicits lifelong antibody-mediated protection against the exact same virus strain [25], as evidenced by neutralizing antibodies found in donors infected with the 1918 “Spanish” influenza virus 90 years previously [26]. This protection is primarily directed at the HA glycoprotein. Vaccination or infection-induced memory against a particular strain of influenza A virus may, however, generally ineffectual with other strains, or even slightly “drifted” variants of the same strain [24, 25]. Such drifted strains can be generated within a single influenza season, a result of the persistent immune pressure driving frequent genetic and, consequently, antigenic variation that allows the virus to escape antibody neutralisation. This process of immune escape as a consequence of antigenic drift is thought to reflect both amino acid mutations that alter antigenicity, particularly within the HA, and other mutations in the globular head of the HA that adjust receptor binding affinity [27]. Thus, despite annual influenza vaccination programs, subsequent seasonal influenza infections cause considerable morbidity and mortality.

A further, and different source of variation occurs when two influenza A viruses co-infect the same host cell, producing “re-assorted” progeny virions with HA molecules that have not been introduced widely into the human population (e.g. avian or swine HA), a phenomenon termed “antigenic shift”. A third potential viral immune evasion mechanism is the recall of an HA molecule that may have circulated in people decades earlier, then disappeared into the wildlife or domestic animal reservoir so that younger humans are effectively naïve. In either of these scenarios, if the newly emergent viruses acquire the ability to transmit readily among humans, an influenza pandemic is almost inevitable [28]. This is what happened with the 2009-H1N1 virus, with the usual profile of influenza being more severe in the elderly modified so that those alive before 1950 were relatively protected. Other viral surface glycoproteins that are targets of the antibody response are the NA and the matrix 2 (M2) proteins. We now focus on the challenges and recent advances in generating protective antibody responses to the influenza A viruses.

(i) The hemagglutinin (HA)

The HA protein mediates binding to lung epithelial cells, leading to virus entry via receptor-mediated endocytosis [29]. In addition, HA also fuses with the endosomal membrane, releasing viral genomic materials into the cytosol [29]. This “invasion” role makes HA the primary target of antibody protection. If present in sufficiently high titers, virus-specific neutralising immunoglobulins (Ig) can prevent virus binding and the invasion of airway epithelial cells. Such sterilizing immunity can be mediated via at least five, defined neutralizing antibody recognition sites that surround the receptor binding site on the surface of the HA molecule [29]. Although HA-specific Ig targeting any one or more of these sites can efficiently block infectivity, antigenic variation at all sites thwarts the development of long-term, protective influenza vaccines. Thus, the challenge for generating broadly protective anti-HA Ig response is two-fold: first the capacity of such antibodies to cross-react with different HA subtypes and, second, the extent of residual recognition for antibody escape variants. Some have suggested that such broad specificities are unlikely to exist in nature, or repeated infection would have selected for them. However, several recent studies have described antibodies targeting structurally conserved regions in the HA stem region that have broad neutralization potential across many subtypes [30-32]. Additionally, the conservation of this region among different HA subtypes indicates that there are structural restrictions that can potentially function to limit antigenic escape. This suggests that the immunogenicity and protective potential of antibodies targeting the HA stem merits further analysis. Indeed, a recent study used a vaccination approach of plasmid DNA encoding H1N1 influenza hemagglutinin (HA) and found broadly neutralizing influenza antibodies directed to the constant stalk region of HA upon boosting with seasonal vaccines [33]. Further promising development is the oil/water emulsion MF59 based adjuvant that is combined with human seasonal influenza vaccines in Europe (Fluad®) [34]. Based on *in vitro* assays, the antibody response generated with MF59-adjuvanted influenza subunit vaccines looks to be broadened [35].

(ii) The neuraminidase (NA)

As an abundant virus surface protein, the neuraminidase (NA) molecule is also a potential antibody target. Unlike anti-HA Ig, NA-specific antibodies do not block the initiation of infection to give sterilizing immunity, but operate to reduce morbidity and mortality as they inhibit virus release [23]. Similar to HA, NA can show a high degree of antigenic variability in response to immune pressure, rendering anti-NA-mediated protection somewhat inconsistent. Even so, antibodies elicited by human NA1 can confer a measure of protection against virulent H5N1 infection [36].

(iii) The matrix protein 2 (M2)

A third antibody target is the low abundance M2 ion channel protein found on surface of the virion. Early mouse studies showed that anti-M2 antibodies provide partial protection [37], fuelling speculation that the highly conserved M2 (versus HA and NA) might be the long sought target for a universal influenza vaccine. However, M2-specific antibodies are not produced after natural influenza virus infection. Also, disappointingly, the anti-M2-mediated protection shown in mice has been less dramatic in other, more relevant animal models such as ferrets [38].

4. Virus-specific CD8⁺ T cells and their differentiation during influenza infection

Immune CD8⁺ T cells (cytotoxic T cells, CTL) play a key part in the effective clearance of influenza A virus infection. Influenza-specific CD8⁺ CTLs are thought to promote the elimination of the virus and host recovery via the production of pro-inflammatory cytokines and the direct killing of virus-infected cells. Once infectious virus is cleared, the CTL population contracts, leaving a pool of long-lived, antigen specific memory CD8⁺ T cells capable of rapid recall to effector function. This enhanced secondary response forms the basis for vaccination strategies based on priming CD8⁺ T cell memory to promote early virus clearance and decreased morbidity.

Although our virology colleagues regard ferrets as the “gold standard” for influenza infection, the lack of immune reagents limits their utility for the analysis of host response. On the other hand, the well-developed influenza mouse model (though less faithfully reproducing the human disease) provides a robust and strong and informative system for studying CD8⁺ T cell responses. Recovery from influenza can be mediated by the T_H-dependant antibody response or by CD8⁺ T cells but, in the absence of the CTLs, virus clearance is delayed and illness is augmented. The other half of the equation in mice and (perhaps) people is that CD8⁺ T cells can induce significant immunopathology [1, 39]. In the most severe cases, following infection with highly pathogenic avian influenza viruses, there are indicators that CTL-mediated immunopathology can be fatal [40, 41].

During primary influenza virus infection of C57BL/6 (B6) mice, the cellular immune response peaks around day 10 after infection, compared to day 8 following secondary virus challenge. Multiple H-2^b-restricted CD8⁺ T cell epitopes have been described, allowing in-depth studies of the CTL immunodominance hierarchies that characterise primary and secondary responses. The mechanisms underlying these hierarchies have been probed by looking for compensation in the absence of immunodominant epitopes, and by the identification of “hidden” epitopes that emerge in the absence of other dominant responses [42]. During primary infection, D^bPA224-233 epitope-specific T cells emerge first in significant numbers around day 5 followed quickly by the D^bNP366-374-specific set, with the two populations reaching generally comparable numbers by the time that the virus is cleared [43]. In the secondary response, D^bNP366-374 is clearly the dominant epitope, with this profile being maintained in the long-term for the total memory population [42, 44].

After infection, antigen-presenting DCs stimulate CD8⁺ T cells to differentiate and proliferate in the draining lymph nodes. The clonally expanded CTL effectors then migrate to the site of infection in the respiratory epithelium. Subsequent to TCR ligation, granule exocytosis then leads to lysis of the virus-infected cells [45]. Although FasL-mediated killing is typically reserved for maintenance of homeostasis, there is some evidence that CD8⁺ T cells can (in the absence of perforin) also use this pathway to clear influenza-infected epithelium [45]. In addition to mediating direct cell lysis, CD8⁺ T cells are also thought to control influenza infection via the secretion of antiviral cytokines. Antigen-

stimulated influenza-specific CD8⁺ T cells first produce IFN- γ , followed sequentially by TNF and IL-2 as the CTL effectors become more extensively activated [46].

Influenza-specific CD4⁺ T cells have received less attention, but they still play an important part in controlling this infection. Primary CD8⁺ T cell responses do not appear to be substantially affected by the absence of CD4⁺ T cell help in the lymph node [47]. However, the subsequent influenza-specific CD8⁺ T cell memory sets are compromised following T_H-independent priming [48], and both the magnitude and recall of CD8⁺ T cell responses is reduced with a consequent delay in virus clearance. Protection induced by vaccination with live-attenuated virus was also severely compromised following influenza-specific CD4⁺ T cells depletion [49]. This CD4⁺ T cell help requirement for maximally efficient influenza-specific CD8⁺ T cell memory is also described for other systems, and may be (nearly) a universal phenomenon [50]. Such help may be supplied through direct T cell-T cell interactions, or via the intermediary of the antigen-presenting cells (APC), requiring signalling through CD40-CD40L interactions.

Insofar as cytolytic potential is concerned, it has been suggested that CD4⁺ T cells can engage in direct cell lysis during influenza virus infection [51]. More recently, TCR transgenic CD4⁺ T cells generated *in vitro* were transferred into CD8- or B cell-deficient mice that had been challenged with a lethal dose of influenza virus [52]. Secondary CD4⁺ effectors protected against lethal challenge, but primary effectors were unable to clear virus. Subsequent experiments established that low antigen load and signalling via IL-2 drove CD4⁺ T cells toward perforin-mediated cytolytic activity [53]. Further analysis showed that Th1- and Th17-polarized memory CD4⁺ T cells also controlled viral replication through the upregulation of multiple, innate inflammatory cytokines and chemokines [54]. This protection was independent of memory CD4⁺ T cell production of IFN- γ and TNF in response to heterosubtypic virus challenge.

The development and establishment of CD8⁺ T cell memory is a subject of intense study, burgeoning with new data and possible potential for therapeutic application in, particularly, cancer models. The naïve TCRs of CD8⁺ T cells recognize epitopes comprising a complex of “non-self” peptide (p) and “self” class I MHC glycoprotein (pMHC-I) after which they are activated on DCs. Then additional (to the TCR/pMHC-I interaction) signalling via various co-stimulatory molecules and cytokine-mediated mechanisms is followed by rapid, exponential proliferation, culminating finally after antigen elimination in a massive contraction that spares only a very small percentage (<10%) that either transit to, or has pre-determined [55], memory status.

Much of the analysis of memory over the past decade or more has focused on the idea that the memory population can be classified into two subtypes [56]. Central memory CD8⁺ T cells (T_{cm}) that express CD62L and CCR7 migrate to the LN, but do not express immediate effector function. In contrast, effector memory CD8⁺ T cells (T_{em}) are CD62L^{lo}/CCR7⁻, but secrete IFN- γ and home to inflamed tissue. It was later proposed that development of the T_{cm} population followed T_{em} in a linear differentiation model [57], with the T_{em} → T_{cm} conversion being programmed during the initial priming event. Indeed, there is emerging evidence that influenza-specific memory formation and differentiation are established very early in the primary response, suggesting that “predestination” may be at work [43, 55, 58]. Of the many proposals to explain T cell differentiation, the most interesting is the asymmetric division model [59]. The idea is that, activated T cells divide asymmetrically, with the resultant, uneven distribution of intracellular material having differential consequences for the development of each daughter cell. In terms of simple phenotypic classification, however, later studies that focused on early time points after infection with lymphocytic choriomeningitis virus (LCMV) or *Listeria monocytogenes* showed that

memory cells could be identified by expression of CD127 (IL-7R α) and KLRG1, a marker associated with senescence and poor proliferation. The idea has been that memory precursor effectors (MPEC) are KLRG1-/CD127+, while short-lived effectors are (SLEC) KLRG1+/CD127- [60, 61]. The SLEC population declines from a week after virus challenge, yielding to the MPECs, which assume dominance within CD8⁺ T cell memory population by 5 months after infection. Furthermore, KLRG1 has been shown to correlate inversely with CD127 and CD62L expression [62], demonstrating a strong relationship between the Tem/Tcm and MPEC/SLEC paradigms.

While the MPEC/SLEC classification may, perhaps, be useful for the LCMV and *Listeria* model systems, the results have not necessarily translated to other virus infections. Clear delineations of the MPEC and SLEC populations are not found following primary exposure to the influenza A viruses (JAR and PGT; KK, unpublished). Studies with both the influenza [43] and Sendai [63] models of respiratory virus infection showed long-term survival of both CD62L^{lo} and CD62L^{hi} memory subpopulations, though improved proliferation was more prominent in the CD62L^{hi} set [Roberts, 2005 #334]. Again for both Sendai and influenza, it was shown that CD43 and CD27 expression is both highly indicative of memory CD8⁺ T cell differentiation and that activity is unrelated to the Tcm and Tem classification [64] (JAR and PGT; KK, unpublished). While the specifics are likely to be dependent on the particular experimental system, it seems likely that memory precursor populations can be identified phenotypically very early during infection, suggesting that some pathogens induce “pre-programmed” memory response during the early stages of the initial infection [55].

While phenotypic analysis based on monoclonal antibody staining profiles and flow cytometry has given useful insights into the nature of memory, taking the next step depends inevitably on defining the molecular events within differentiating (and differentiated) immune CD8⁺ T cells. The SLEC population has been shown to have increased activity for the *T-bet* transcription factor, which correlates with increased KLRG1 and reduced CD127 levels [61]. Induced by IL-12, T-bet was originally shown to control IFN- γ expression and to induce Th1 development *in vitro* [65] and its expression was shown to determine formation of the SLEC population. In the context of influenza infection, the T-bet activated IFN- γ response requires signaling through the IL-27R [66]. More recently, human CD8⁺ Tem cells were shown to upregulate perforin and T-bet, but this was not seen for the Tcm set [67]. In addition, T-bet has been shown to suppress IL-2 [65, 68], though IL-2 is necessary for both the development of fully armed effector CTLs and the programming of memory CD8⁺ T cells in LCMV infection [69]. Other have shown that only the memory precursors are capable of producing IL-2 [62].

Another T box transcription factor, eomesodermin (eomes), displays high redundancy with T-bet [70]. Both these transcription factors enhance the expression of CD122, the IL-2/IL-15 β receptor. Consequently, in the absence of both T-bet and eomes, memory CD8⁺ T cell development is devastated. Despite the similarities in function, these transcription factors are inversely regulated by IL-12. During *Listeria* infection [71], IL-12 induces T-bet and SLEC development, whereas IL-12 deficiency promotes the expression of eomes and the generation of memory cells [61, 71]. Other transcription factors that have recently received attention as players in memory CD8⁺ T cell development include Blimp1, Bcl-6, and Id2. As with T-bet and eomes, there is a paucity of work investigating the roles of these factors in the context of influenza virus infection. Blimp1 deficiency evidently has no effect on the development of influenza-specific CD8⁺ memory T cells, though it does seem to play a part in the recall response following secondary challenge [72]. Similarly, Bcl-6^{-/-} mice showed significant reductions in the quantity of D^bNP366-374-specific CD8⁺ T cells following influenza A virus infection [73].

5. Human influenza-specific CD8⁺ T cell responses

All adults from at least 15 years of age have circulating influenza-specific memory CD8⁺ T cells [74], with there being indications that the persistence of these memory populations can provide a measure of protection against subsequent virus exposure [39]. Evidence from human studies [75-77] indeed suggests a role for CD8⁺ T-cell-mediated protection following heterologous prime/challenge between H1N1, H2N2, H3N2 and H5N1 viruses. Such cross-reactive CD8⁺ T-cell-mediated immunity has, in the face of an emerging influenza pandemic, the potential to ameliorate the disease by promoting enhanced recovery and better clinical outcomes.

Human CD8⁺ T cells recognise viral peptides predominantly in the context of MHC class I glycoproteins encoded at two loci, HLA-A and HLA-B. Apart from the Ig and TCR V genes, HLA is the most polymorphic region in the human genome with over 700 HLA types and alleles [78]. As presentation of the viral peptide by the MHC-I relies on binding to hydrophobic pockets within the MHC-I cleft, HLA types can be grouped into ‘superfamilies’ based on the specific characteristics of the binding pockets [79]. The possibility of generating a universal CTL-based anti-influenza vaccine depends upon defining conserved influenza peptides presented by HLA superfamilies that elicit effective immune responses [3, 80]. Similar to influenza-specific CTLs in the mouse model, human CD8⁺ T cells typically recognise peptides derived from internal components of the virus [81], which can be conserved across strains and serologically distinct subtypes of influenza [3, 77, 82]. Thus, CTL-based immunity could potentially provide broader coverage of possible influenza virus strains and subtypes in comparison to strain-specific antibodies to the viral HA and NA glycoproteins.

Effective, heterologous CTL immunity depends on the extent of sequence homology for the immunogenic peptides of different influenza strains [3], with there being some cross-protection directed at non-conserved peptides. The idea is that the structural homology of some pMHC-I complexes allows CD8⁺ T cells to provide inter-epitope cross-reactive immunity [83], allowing TCRs of a given specificity to recognize one or more different epitope variants [84].

Using human PBMC and CTL clones, pre-existing CD4⁺ and CD8⁺ T cell responses have been recalled against serologically distinct strains of influenza, such as the pathogenic H5N1 avian virus [3, 77, 82]. A great example of a highly conserved immunogenic T cell peptide is the M158-66 presented by HLA-A*0201 [85]. The M1₅₈ peptide has been constant since 1918 for all seasonal and pandemic subtypes and strains, including the H5N1 and current pandemic A(H1N1)-2009 influenza viruses. Such conservation is most likely due to structural and/or functional constraints, as viruses with mutations in M1₅₈ generated by the reverse genetics technology are of lower fitness [86, 87]. The conserved, immunodominant M1₅₈ peptide [88] would thus seem to be an ideal vaccine candidate for providing a measure of broad protection to the 25-30% of the world’s population that is HLA-A*0201, providing it generates effective *in vivo* CTL immunity. What constitutes optimal CTL immunity is still being debated, though we do know that a diverse TCR repertoire give perforin, granzyme and cytokine-producing effectors with a spectrum of “sufficient” TCR/pMHC-I avidities is likely to be optimal for virus control. Most research to date has focused on the A2⁺M1₅₈⁺CD8⁺ T cell responses of *in vitro* cultured cell line or clones, with a particular emphasis on defining TCR-pMHC-I interactions [74, 85, 89, 90]. Direct *ex vivo* evidence is needed to determine the functional efficacy of immunodominant M1₅₈⁺CD8⁺ T cells and other anti-influenza CTL responses if we are to understand their direct role in clearance and protection.

6. Antigenic variation within human T cell peptides

Immune CD8⁺ T cells exert selective pressure, leading to escape mutations in the viral peptide component of the antigenic pMHC-I complex. Virus escape mutants constitute a major problem for CD8⁺ T cell-mediated control and vaccine design, with the effect being well documented for persistent infections (HIV, HCV, SIV). With regard to the influenza viruses, the extent of mutational escape from CTL recognition has received relatively little attention due to the acute nature of the disease, and the focus on antibody-driven antigenic drift. However, developing a better understanding of the consequences of mutational changes that influence pMHC-I antigenicity may be highly relevant when it comes to dealing with current and future influenza pandemic threats posed by newly emerging strains.

The absence of pre-existing neutralising antibody means that any protection afforded by previous, natural encounters with other influenza A viruses is likely to be mediated by established, cross-protective CTL memory. It is also possible that novel vaccination strategies focused on developing antibodies that recognize M2 and/or conserved “stem” regions of the viral HA [33] may ultimately provide a measure of cross-protection. Even so, it may be worthwhile to think in terms of a broadly reactive “belt and braces” vaccine that promotes both humoral and CTL immune memory, with each component providing a functionally different, but likely incomplete, protective mechanism. In general, breaking through this barrier of “doing better than nature” is the challenge for developing vaccines against HIV, HCV and influenza.

The cross-reactive recall of existing CTL responses to different influenza strains depends on the sequence, or structural homology of the immunogenic peptide within the pMHC-I context. A single amino acid mutation within the antigenic peptide can lead to escape from TCR recognition by existing CD8⁺ T cells specific for the wild-type epitope. Up to 71% of influenza-derived T cell peptides differ between “seasonal” influenza strains [3, 80], and there is only 50% T cell peptide similarity between A(H1N1)-09 and preceding seasonal influenza sequences [84, 91]. Variations within influenza-specific T cell peptides are undoubtedly driven by immune selection pressure as more amino acid changes can be found within the CTL peptide regions of influenza viruses versus non-epitope regions [86]. Further evidence stems from a mouse model of influenza infection, in which escape variants emerged in TCR transgenic mice specific for the D^bNP₃₆₆ epitope, where mutations occurred predominantly at TCR contact sites [92]. Due to the acute nature of influenza virus infection and seasonal epidemics, selection is driven at the population level. The emergence of any *within epitope* variation depends on both the prevalence of the particular HLA allele in the population and the “fitness” of the resultant mutant virus. In seasonal influenza infection, escape from established CD8⁺ T cell immunity is relevant to the persistence of the virus within the population (longevity and/or severity of influenza season). In the face of a novel pandemic, mutations can lead to a lack of CD8⁺ T cell cross-protection in individuals with specific HLA alleles. Therefore, it is important to determine the factors that contribute to epitope variation and to understand the determinants of cross-reactive CD8⁺ T cell responses to “escape” variants.

Mutations in T cell immunogenic peptides can manifest in defects in processing, presentation or TCR recognition. Previously, influenza ‘escape’ variants have been found with mutations at either an MHC anchor residue, resulting in a loss of MHC-I binding, or at a TCR contact site to prevent/decrease T cell recognition [93]. Changes that alter anchor-binding residues render a peptide no longer immunogenic as exemplified by the HLA-B*2705 and HLA-B*0801 presented NP₃₈₃/NP₃₈₀ peptides which (for both H3N2 and H1N1) mutated in the mid 1990’s at a critical anchor residue, R₃₈₄K/G. As the R₃₈₄K/G mutation is detrimental to the viral fitness, the virus acquired compensatory co-mutations in

flanking regions to overcome this fitness cost [94]. The R₃₈₄K/G mutation, together with compensatory flanking co-mutations, became fixed in the circulation within one influenza season, leading to the loss of two immunogenic peptides (NP₃₈₃ and NP₃₈₀) [87].

Although mutations that disrupt pMHC-I binding provide an effective escape from CD8⁺ T cell immunity, mutations at TCR contact sites are more common [92, 95]. The NP₄₁₈₋₄₂₆ peptide consistently and at high levels presented by the HLA-B*7 superfamily (including HLA-B*3501, B*3503, B*0702) has sequentially mutated at 4 different TCR contact positions, generating over 20 different peptide sequences over the past 90 years [84, 96, 97]. Depending on mutations occurring at the most solvent exposed sites [84], CTL responses show distinct profiles of cross-reactivity towards seasonal influenza-derived NP₄₁₈ peptides. Further dissection of immunogenicity and TCR usage for variable NP₄₁₈⁺ CD8⁺ cell responses has the potential to provide insights into possible cross-reactive CTL immunity that could limit the emergence of at least some virus escape variants. Additional variations have been recently identified for two other immunogenic peptides derived from the NP protein, NP₂₅₁₋₂₅₉ (HLA-B*4002) and NP₁₀₃₋₁₁₁ (HLA-B*1503) [98], confirming the importance of NP-derived peptides as dominant CTL targets. Such TCR contact mutants are still presented by the MHC-I, raising the possibility that the peptide is potentially immunogenic and can generate unique endogenous responses [99] or recall pre-existing, cross-reactive CD8⁺ sets [84, 100]. As mentioned above, mutations within CTL peptide regions can impose functional constraints resulting in fitness costs [86, 101], thereby limiting the number of possible variants. As a consequence, it may be feasible to prime against commonly occurring mutants and generate broadly protective CTL immunity against variable epitopes. Recent experiments, however, provide a cautionary tale for planning vaccine strategies that include immunization against commonly selected cross-reactive variants with mutations at partially-solvent exposed residues [100]. Priming T cells specific for such variants (eg D^bNP₃₆₆-N3A in B6 mice) may lead to a marked decrease in the magnitude and quality of CD8⁺ T cells elicited after heterologous challenge, and consequently delayed virus clearance from the infected lung.

7. Adaptive immunity to the pandemic A(H1N1)-2009 influenza virus

An outbreak of novel influenza virus infection was reported in April 2009, with evidence that cases had been occurring in Veracruz, Mexico for months before the problem was formally recognised. This A(H1N1)-2009 influenza virus was soon shown to be derived from well known “swine” components (HA, NP and NS), and to contain elements from Eurasian (NA and M) and North American (PA, PB1, and PB2) swine lineage reassortants [102]. On 11 June 2009, reflecting the rapid, global spread of the virus, the WHO declared A(H1N1)-2009 influenza as the first influenza pandemic of the 21st century [103]. Pre-existing antibody levels were clearly minimal. Classical, swine-lineage H1N1 influenza viruses have been maintained in pigs since ~1918, while seasonal H1N1 viruses evolved in the human population over the past 90 years. Similar to the catastrophic 1918 H1N1 influenza pandemic, which started as a mild infection but then resulted in >40M deaths, the 2009 H1N1 pandemic has been particularly apparent in children and young adults. The infection rate decreases with age, with the lowest incidence being in individuals born before 1950 [104, 105], suggesting that there is some level of pre-existing antibody immunity to the re-emerged virus.

As of May 2010, the WHO Regional Offices reported over 18,097 deaths associated with pandemic A(H1N1)-2009 influenza worldwide, with this virus accounting for 80.9% of all influenza A viruses subtyped globally by the WHO Collaborating Centres through that interval (www.health.gov.au). Although A(H1N1)-2009 influenza virus causes a generally milder disease [106] and lower mortality (0.03%) than recent seasonal viruses (<0.1%), fatal

cases in otherwise healthy children and young adults have been reported. Furthermore, women in the third trimester, the very obese, the immunocompromised, diabetics and people living in communities with poor general health and nutrition seem to be at an increased risk. Of concern, is the possibility that A(H1N1)-2009 may mutate to increased virulence. Continuing research aimed at understanding the extent of pre-existing immunity towards the pandemic A(H1N1)-2009 strain generated by exposure to successive seasonal influenza epidemics is thus worthwhile.

(i) Cross-reactive immunity between A(H1N1-2009) and seasonal influenza viruses

Recent studies suggest that individuals who were alive before 1950 have some level of pre-existing neutralising antibodies directed towards A(H1N1)-2009 [104, 105]. Thus, exposure to the influenza viruses that circulated 60 years ago conferred some level of protection, while recent, seasonal influenza A viruses fail to do so. Indeed, sequence analysis of all the known influenza-specific B cell epitopes listed in the Immune Epitope Database (IEDB; www.immuneepitope.org) showed that only 31% of 26 B cell epitopes were conserved between A(H1N1)-2009 and recent, seasonal H1N1 viruses [91]. Most importantly, only one B cell epitope was conserved out of the main HA and NA immunogenic peptides, providing the scientific evidence for the minimal antibody-based immunity observed at the population level.

Conservation between A(H1N1)-2009 and recent seasonal influenza strains was higher for T cell peptides (51%; 111 out of 217 T cell peptides) than for B cell epitopes. This presumably reflects that the immunogenic T cell peptides are derived predominantly from internal, and generally less variable, viral proteins [91]. Peptides that target CD8⁺ T cells had a greater level of sequence similarity (69%) compared to the corresponding CD4⁺ determinants (41%). Further experimental approaches using PBMCs from normal healthy donors without any prior exposure to A(H1N1)-2009 established that some level of pre-existing T cell immunity towards the pandemic virus was conferred by recent exposure to the H1N1 seasonal influenza A strains [91]. When PBMCs were cultured with peptides that target CD4⁺ or CD8⁺ T cells, the pre-existing memory sets produced IFN- γ (assessed by *ex vivo* ELISPOT) following exposure to conserved or non-conserved seasonal H1N1 and pandemic A(H1N1)-2009-derived epitopes. As CTL responses minimise the severity of the disease, such established T cell memory could contribute, at least in part, to the mild outcome of influenza A(H1N1)-2009 infection.

Pre-existing T cell immunity to A(H1N1)-2009 was further confirmed and characterised for both CD8⁺ [107] and CD4⁺ T cells [108]. Bulk CTL populations in PBMCs from healthy individuals who had been exposed to seasonal viruses had the potential to kill autologous monocyte-derived macrophages (MDMs) infected with A(H1N1)-2009 virus and to produce IFN- γ and TNF- α [107]. This potential for protection was exemplified by M158⁺ CD8⁺ T cells from healthy HLA-A2-positive individuals that could lyse both M158 peptide-pulsed as well as A(H1N1)-2009 virus-infected HLA-A2 targets. These findings are not entirely surprising as M158-66 is one of the most conserved (since 1918) influenza-derived T cell peptides [109], and is 100% identical for the seasonal strains and A(H1N1)-2009. These data further suggest that the ~30% of the world's population that expresses the HLA-A2 allele and experienced previous encounters with influenza, should have protective CD8⁺ T cell immunity directed at M158 derived from any seasonal or pandemic influenza strains.

Similarly, pre-existing CD4⁺ T cell immunity has been found towards the pandemic A(H1N1)-2009 virus within HLA-DR-restricted T cell epitopes [108]. Ge *et al.* showed CD4⁺ T cell cross-reactivity by performing elegant experiments utilising dual pMHC-II tetramers corresponding to either seasonal or A(H1N1)-2009 derived peptides. The levels of staining with the different fluorochromes used were higher for the epitopes derived from

seasonal H1N1 viruses. The combination of a dual-tetramer approach together with IFN- γ ELISPOT showed that A(H1N1)-2009 derived peptides require higher concentrations to induce cross-reactivity, indicating that there is a lower A(H1N1)-2009 TCR-pMHC avidity profile for pre-existing memory that have been primed by exposure to seasonal viruses, or alternatively fewer T cells specific for the new epitopes. Partial protection against A(H1N1)-2009 after prior infection with seasonal H1N1 or H3N2 strains has also been shown using the ferret [110] and guinea pig [111] models. Such cross-reactive protection reflects pre-existing T cell immunity rather than antibody-mediated responses [110].

Our studies [84] concentrated on non-conserved T cell peptides within the pandemic A(H1N1)-2009 virus, since effective T cell responses can be directed at variable T cell peptides [93, 96, 97]. Thus, it is possible that the 5-% of individuals with common HLA types that present variable T cell peptides [84, 91, 107] could have some T-cell immunity to the pandemic A(H1N1)-2009 strain. We asked whether exposure to successive, seasonal influenza epidemics generated at least some memory T cells that can recognize non-conserved peptides derived from the A(H1N1)-2009 virus. Our experimental approach used influenza sequence analysis, immunological data and pMHC-I structural definition. We focused on the variable (but highly cross-reactive) immunodominant NP₄₁₈-426 peptide that binds to a B7 allelic family (HLA-B*3501/HLA-B*3503/HLA-B*0702)[93, 97], which is widely found (28% Caucasians, 35% Irish, 16% African American) within the human population (www.allelefreqencies.net). To assess the level of cross-reactivity between the pandemic A(H1N1)-2009 and seasonal NP₄₁₈ variants, PBMCs were cultured with either a pool of different NP₄₁₈ peptides (or individual peptides) for 10-days and subsequently re-stimulated with the variants in an IFN- γ ICS assay (Fig. 1A). Our data confirmed a high degree of cross-reactivity for the NP₄₁₈ seasonal variants, particularly those displaying a “DK” motif (Fig. 1B). However, analysis of PBMCs from healthy HLA-B*3501, HLA-B*3503 and HLA-B*0702 donors with no prior history of A(H1N1)-2009 infection showed no pre-existing CD8⁺ T cell memory towards 2009-NP₄₁₈-LPFERATVM (Fig 1B). Conversely, strong PBMC responses to A(H1N1)-2009-NP₄₁₈ were found in HLA-B*0702-positive patients who had been hospitalized with severe A(H1N1)-2009 infection and subsequently recovered (Fig. 1C, 2). This provided evidence that exposure to A(H1N1)-2009 can indeed prime CD8⁺ T-cell memory, but these NP₄₁₈⁺ CD8⁺ responses are not cross-reactive with the variants derived from earlier seasonal strains. Our immunological data were confirmed by the structural analysis of HLA-B*3501 complexed to variant NP₄₁₈ peptides, showing that the central, prominent residues in the NP₄₁₈ mutants are at positions (P) 4 and 5 [84]. Thus, while the seasonal NP₄₁₈ variants are characterised by P4-D and P5-K, the P4-E/P5-R peptide found in A(H1N1)-2009-NP₄₁₈ bulges-out of the binding cleft, exposing its side chains for potential TCR contact. Variation at these most exposed residues possibly explains the mutually exclusive recognition of A(H1N1)-2009-NP₄₁₈ and the seasonal NP₄₁₈ variants.

Taken together, although some level of pre-existing T cell immunity exists towards both the conserved (e.g. M1₅₈) and variable peptides derived from the pandemic A(H1N1)-2009 [91, 107, 108], the newly emerged virus manages to escape some of the memory T cell responses directed at immunodominant peptides such as NP₄₁₈ [84]. As NP₄₁₈ is one of the only two influenza-derived peptides consistently presented for CTL recognition by the large B7 allelic family [81], it appears that a considerable proportion of the human population did not have pre-existing, prominent memory CD8⁺ T cell sets to combat a newly encountered pandemic A(H1N1)-2009 infection.

(ii) Cross-reactive immunity between the pandemic A(H1N1)-2009 and A(H1N1)-1918 viruses

As both the A(H1N1)-2009 and A(H1N1)-1918 influenza A viruses infected pigs, cross-reactive immunity between those two pandemic H1N1 viruses is of obvious interest. Sequence analysis of influenza viruses within the main T-cell immunogenic proteins, especially NP, showed that several of the A(H1N1)-2009-derived variable peptides highly resemble those found in the pandemic 1918-H1N1 viruses [84]. Thus, as one might expect, PBMCs derived from healthy individuals showed no reactivity to the 1918-NP₄₁₈-LPFERATIM variant. Interestingly, natural H1N1-2009 influenza infection induced strong CD8⁺ T cell responses toward the NP₄₁₈-2009 peptide that cross-reacted with the NP₄₁₈-1918 variant (Fig.1C). Similarly to 2009-NP₄₁₈, the 1918-NP₄₁₈ variant displays the characteristic ER motif at the most solvent exposed P4-P5 region of the peptide, which may explain how the same TCRs respond to variants derived from both pandemic viruses but not to seasonal peptides.

In accordance with our findings for CTLs, a structural basis for cross-reactivity between the pandemic 2009-H1N1 and 1918-H1N1 antibody responses has also been recently demonstrated [112]. Comparison of the antibody antigenic sites within the viral HA shows high antigenic conservation between the H1N1-2009- and H1N1-1918-derived Sa, Sb and Cb epitopes. As suggested by the previous sequence analysis of B cell epitopes [91], the antibody antigenic sites within the H1N1-2009 pandemic strain showed only 50% conservation in comparison to the currently circulating seasonal strains. Thus, the structural and immunological data for both T cell immunity [84] and antibodies [112] show high conservation between the A(H1N1)-2009 and A(H1N1)-1918 pandemic strains, which may explain the low infection rate with the A(H1N1)-2009 influenza strain in the elderly.

8. Summary

Having to deal with both the 2009 “swine” H1N1 influenza pandemic and the occasional, severe human cases associated with the continuing H5N1 bird influenza flu epizootic, has emphasized the need for continued investigation of the pathogenic, immunogenic and virological determinants of influenza virus infection. The introduction of a newly reassorted A(H1N1)-2009 influenza strain into human circulation led to the rapid spread of this virus across the continents, though there were fewer deaths than expected and overall morbidity was reduced. The lack of pre-existing antibody immunity in those under 60 no doubt facilitated the dissemination of this novel 2009 virus, though the reasons for the generally mild pathogenicity are still unclear. As evidenced by recent studies, pre-existing CD8⁺ T cell-mediated immunity directed towards conserved viral regions can provide a degree of protection against newly emerged influenza A viruses and promote more rapid host recovery. For the future, a universal influenza vaccine that confers a measure of resistance to all potential seasonal and pandemic influenza A viruses would need to trigger cross-strain T cell immunity while retaining the capacity to induce antibodies, hopefully to shared determinants. From the CD8⁺ T cell aspect, we need to develop a much better understanding of peptide immunogenicity, TCR recognition and the key determinants of memory recall and effector function if such an immunogen is to emerge.

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Fig 1. Infection with A(H1N1)-2009 elicits CD8⁺ T-cell responses towards pandemic NP₄₁₈2009 and NP₄₁₈1918 but not seasonal variants

(A) PBMCs from donors belonging to the large B7 allelic family were cultured *in vitro* for 10 days with a pool of 12 variant NP₄₁₈ peptides (or alternatively with individual peptides), then tested for CD8⁺ T-cell specificity by IFN- γ production in an ICS assay. The final 7-hr stimulation was with CIR-B35 cells pulsed with individual peptides (or a pool of peptides as a control). Representative dotplots show CD8⁺ T cell responses towards an NP₄₁₈ pool, NP₄₁₈ seasonal, NP₄₁₈2009 or NP₄₁₈1918 variants for (B) HLA-B*3503 donor previously unexposed to A(H1N1)-2009 and (C) HLA-B*0702⁺ donor hospitalised with A(H1N1)-2009 who subsequently recovered.

Fig 2. NP₄₁₈-2009⁺CD8⁺ T cell immunity occurs only after infection with the pandemic A(H1N1)-2009 influenza A virus

CD8⁺ T cell reactivity against the NP₄₁₈ pool (clear bars) or the A(H1N1)-2009 NP₄₁₈ variant (black bars) is shown for single-peptide PBMC cultures obtained from two HLA-B*0702 A(H1N1)-2009 patients and four HLA-B*0702/B*3501/B*3503 donors previously unexposed to A(H1N1)-2009. Results are represented as % of maximum IFN γ production minus no peptide controls.