Published in final edited form as:

Immunol Rev. 2018 July ; 284(1): 91–105. doi:10.1111/imr.12662.

CD4 T cells in protection from influenza virus: Viral antigen specificity and functional potential

DR Andrea J. Sant¹, Anthony T. DiPiazza^{1,3}, Jennifer Nayak^{1,2}, Anuj Rattan¹, and Katherine Richards¹

¹David H. Smith Center for Vaccine Biology and Immunology, Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY, USA

²Department of Pediatrics, Division of Infectious Diseases, University of Rochester Medical Center

³Current address: Viral Pathogenesis Laboratory, Vaccine Research Center, NIAID, NIH

Summary

CD4 T cells convey a number of discrete functions to protective immunity to influenza, a complexity that distinguishes this arm of adaptive immunity from B cells and CD8 T cells. Although the most well recognized function of CD4 T cells is provision of help for antibody production, CD4 T cells are important in many aspects of protective immunity. Our studies have revealed that viral antigen specificity is a key determinant of CD4 T cell function, as illustrated both by mouse models of infection and human vaccine responses, a factor whose importance is due at least in part to events in viral antigen handling. We discuss research that has provided insight into the diverse viral epitope specificity of CD4 T cells elicited after infection, how this primary response is modified as CD4 T cells home to the lung, establish memory, and after challenge with a secondary and distinct influenza virus strain. Our studies in human subjects point out the challenges facing vaccine efforts to facilitate responses to novel and avian strains of influenza, as well as strategies that enhance the ability of CD4 T cells to promote protective antibody responses to both seasonal and potentially pandemic strains of influenza.

Keywords

Influenza; CD4 T cells; antigen; vaccine responses; human immunity; lung; immunodominance; epitopes

Overview: The contributions of CD4 T cells to protection from influenza virus

CD4 T cells convey a number of discrete functions to protective immunity to influenza, a complexity that distinguishes this arm of adaptive immunity from B cells and CD8 T cells

*Corresponding Author: Andrea J.Sant, andrea_sant@urmc.rochester.edu.

Disclosures

All the authors declare no conflicts of interest.

(reviewed in (1–9)). CD4 T cell help for antibody responses is the most generally acknowledged and essential contribution of CD4 T cell responses to protective immunity induced by influenza vaccines and infection. The antigen-specific cognate interactions between CD4 T cells and B cells promotes both extrafollicular responses that generate short-lived, class-switched antibody secreting cells as well as long-lived, affinity-matured antibody secreting cells and memory B cells derived from germinal center reactions (reviewed in (10–17)). CD4 T cells also are important for protective immunity conveyed by CD8 T cells. Studies primarily performed in mice have shown that CD4 T cells enhance recruitment of CD8 T cells to the lymph node (18) and contact with antigen-bearing DC (19, 20). Additionally, CD4 T cells promote priming and expansion of CD8 T cells and are critical for the establishment of long-lived CD8 T cell memory (reviewed in (21–30)). CD4 T cells also promote the ultimate positioning of memory CD8 T cells in the infected respiratory tract (30). Intriguingly, a recent study provided strong evidence that CD4 T cell “help” is critical for development of effector CD8 T cells that have cytotoxic potential, and express molecules critical for homing and extravasation, all key to protective immunity to influenza (31). In addition to potentiating the protective function of other cells in the antigen-specific adaptive response, CD4 T cell memory also likely plays a role in recruitment of early innate effector cells into the lung (32, 33), promoting a complex array of effector mechanisms that can blunt virus infection and replication (reviewed in (34)). A final discreet function of CD4 T cells that has been increasingly validated is direct cytotoxicity (35–42) by a functional subset that is enriched in the lung during infection (43, 44), suggesting that these cells are involved in killing of infected cells, thus diminishing virus replication and spread in the respiratory tract.

In order to understand the important features of CD4 T cells to protective immunity to influenza virus, our laboratory has experimentally pursued a number of key issues. The first critical issue is the diversity and specificity of CD4 T cells elicited by infection, and how this virus-specific repertoire is remodeled over time and by subsequent infections and vaccinations. The second research focus of our laboratory is gaining insight into how the peptide epitope specificity of influenza virus-specific CD4 T cells influences their functional potential. We have also probed characteristics within the influenza-specific CD4 T cell memory repertoire that are most critical in promoting protective antibody responses to influenza. A final area of focus has been evaluation of how the CD4 T cell repertoire generated by seasonal influenza virus infection or vaccination can impact immunity to novel and potentially pandemic strains of influenza.

The specificity of CD4 T cells to influenza virus

The adaptive immune response to influenza virus in humans is particularly complicated, where a long life-span allows multiple encounters with genetically variable influenza viruses and vaccines. In most adults across the globe, the first encounter with influenza is through natural infection in early childhood. Therefore, the original memory compartment of B cells and T cells specific for influenza is generally established through the priming that occurs upon live infection (45–51). After this original contact, individuals encounter influenza virus and influenza-derived proteins periodically, perhaps every 2–3 years, through natural encounter with different subtypes or strains of influenza viruses or, within in more

developed countries, through active vaccination. We hypothesize that these periodic and sequential confrontations with influenza viral proteins, introduced in different formats and with different viral proteins, are likely to remodel both the functionality and specificity of the CD4 T cell repertoire that is maintained in the human host.

In order to understand how the influenza-specific CD4 T cell memory compartment is shaped and revised over time after sequential and intermittent encounter with influenza viruses and vaccines, it is essential to first to understand the diversity and specificity of the primary CD4 T cell response. This is not possible with the sampling possible in infants and young children, who are likely to be the only individuals whose immunological experience with influenza is via a single infection. Our laboratory approached the identification of the epitopes that encompass the primary CD4 T cell response using mouse models of influenza infection utilizing a completely unbiased and comprehensive experimental strategy of epitope discovery. This strategy involves direct *ex vivo* analyses of CD4 T cells isolated from secondary lymphoid tissue of animals infected intranasally with influenza A virus. CD4 T cells from secondary lymphoid tissue (spleen and lung draining mediastinal lymph node) are then surveyed for epitope specificity through the use cytokine EliSpots, where overlapping peptides representing the entire translated sequence of individual virus proteins are used to recall CD4 T cells of each of the viral antigens, allowing complete enumeration of all potential CD4 epitope specificities. For proteins that are relatively large in molecular weight (HA, NP and NA), peptide-pooling matrices are used, based on the matrix strategy described by Tobery and colleagues (52, 53). Here, peptide pools are constructed and then arrayed in intersecting rows and columns with no overlapping peptides in any given row or column. These pools are used to stimulate CD4 T cells isolated from infected mice and the number of responsive cells is quantified in cytokine EliSpot assays. After initial elimination of the peptide pools that are devoid of CD4 T cell epitopes, the remaining candidate peptides are secondarily screened as single peptides. Through this iterative process, CD4 T cell epitopes are identified and finally confirmed. This peptide-pooling strategy has considerable advantages, primarily allowing both minor and major epitopes to be discovered through a relatively efficient process. For smaller proteins such as NS1 and M1, single peptides spanning the entire sequence are tested directly. Most importantly, this direct *ex vivo* approach does not employ biases imposed by pre-selection of epitopes based on particular MHC types expressed in the host, predictive algorithms, nor any *in vitro* expansion of CD4 T cells. In order to derive conclusions that can be extrapolated to other models, we have employed this experimental approach using multiple strains of mice that express distinct allelic forms of class II molecules and CD4 T cells drawn from the polyclonal endogenous repertoire.

Our studies have revealed that the primary CD4 T cell response to live intranasal influenza A infection (H1N1) is highly diverse and represented by CD4 T cells specific for all of the viral proteins tested: HA, NA, NP, M1, NS1 and polymerase proteins. Not surprising, both the breadth of the response and the particular epitopes selected by the host varies dramatically with the MHC class II molecules expressed (**Figure 1**). For example, H-2^b mice (B6 and B10) have an atypically low abundance of CD4 T cells specific for HA (H1), but many CD4 T cells specific for a diverse set epitopes from NP and NA. In contrast, H-2^s

mice elicit CD4 T cells specific for more than a dozen HA (H1)-derived epitopes, and many others specific for NP and M1. After infection, HLA-DR1 transgenic elicit CD4 T cells specific for more than 10 epitopes derived from NS1, 30 from HA, and 10–15 epitopes derived from NA and NP. Early studies by Woodland and colleagues using intracellular cytokine staining to detect and quantify CD4 T cells in the lung noted the preferential reactivity of CD4 T cells in B6 mice toward H3-, NP- and polymerase-derived peptide epitopes (54). These studies illustrated that many epitope-specific CD4 T cells elicited in the primary response to influenza A home to the lung, a conclusion that has been supported by others' work (54–56). If we extrapolate the results established in mouse models of primary infection to humans, who are each likely to express at least 8–10 different HLA-class II molecules due to co-expression of different alleles and isotypes (HLA-DR, DQ and DP), as well as heterozygosity in HLA genotype (57), we anticipate that the CD4 T cell response to the initial influenza infection would encompass more than 100 different epitope specificities. This has not been tested experimentally because of the limited sampling that can take place from the peripheral blood of young children, typically 2–10 ml (58), and the relatively low frequency of CD4 T cells that are specific for single peptides, even in adults who have presumably encountered influenza multiple times (59–64).

Our most recent studies have explored whether the broad immunodominance hierarchies observed in the draining LN are edited as CD4 T cells migrate to the lung after infection (65). Using peptide-specific cytokine EliSpots, the diversity and immunodominance hierarchies of CD4 T cells established in the lung-draining mediastinal lymph node was compared with those CD4 T cells that home to the lung. Our studies revealed that at the peak of the primary response (day 9–10 post infection) CD4 T cells of all viral epitopes specificities that are identified in the draining lymph node are readily detected within the lung. The vast majority most of these lung-homing CD4 T cells are localized within the lung tissue rather than the pulmonary vasculature. Interestingly, despite the maintenance of the established peptide specificity, a striking shift of CD4 T cell functionality takes place, enriching for IFN- γ producing CD4 T cells as cells commit to lung homing effector function, enter the lung vasculature and finally establish their localization in the lung tissue. Surprising, the shifts in cytokine potential as CD4 T cells establish residency in the lung were not associated with any identifiable shifts in the functional avidity of their T cell receptors. We conclude that CD4 T cells of broad viral epitope specificity are recruited into the lung after influenza infection, where they then have the opportunity to encounter diverse infected or antigen-bearing antigen presenting cells (66).

Using mouse models of infection, we have also evaluated whether and how the memory CD4 T cell repertoire changes over time with regard to epitope specificity. In the HLA-DR1 transgenic mouse model of infection, the primary response is exceptionally diverse and includes as many as 70 different peptide specificities, drawn from many different influenza proteins, including HA, NA, NP, and NS1 (67, 68). Our laboratory evaluated whether the immunodominance hierarchy narrows or shifts during contraction of the response and after establishment of memory. Over several time points post-infection (day 8, 15, 30 and 60), more than 50 different CD4 T cell epitope specificities were tracked and quantified, using individual peptides derived from four different influenza proteins. Our studies revealed that the immunodominance hierarchy in the memory CD4 T cell compartment retains an

unexpected degree of diversity, with only a modest perturbation in peptide specificity from that detected in the primary response (69). Similar conclusions were made in other mouse strains. We conclude from this work that almost all CD4 T cell specificities originally generated in the primary response to infection are preserved in the memory compartment, although in dramatically reduced frequency, until the next encounter with vaccines or infection.

We have also evaluated whether and how the CD4 T cell response is potentially altered by pre-existing immunity. During sequential heterosubtypic infections in mice, CD4 T cell responses to the second infecting virus strain can be readily detected. However, strikingly, the specificity of the CD4 T cell response to the second virus is dramatically reshaped compared to the response observed in a primary response of that same virus. On secondary challenge, the elicited CD4 T cells specific for epitopes from conserved internal virion proteins that were shared with the first virus rapidly expanded as detected by enumeration with cytokine EliSpots. This robust CD4 T cell expansion toward conserved viral epitopes was associated with greatly diminished responses to unique epitopes from the novel HA and NA proteins in the second virus (70). With the loss of HA-specific CD4 T cells, compared to those specific for the more highly conserved viral proteins such as NP, a dramatically reduced antibody response to HA occurred. These results suggest that in general, memory CD4 T cells specific for conserved viral proteins will likely have a competitive advantage during sequential infections or vaccination, particularly when confronted with the more limiting abundance of viral antigen that typically occurs upon secondary infection that could lead to a lower viral epitope density presented by APC. Dramatic shifts in the immunodominance hierarchy upon heterosubtypic infection have also been observed for CD8 T cells (71–73). Previously activated CD4 T cells are known to require lower epitope density to become reactivated and also to expand more rapidly than naïve CD4 T cells (74–77). Our later studies showed that the advantage that is afforded to memory CD4 T cells specific for internal virion proteins, at the expense of CD4 T cells specific for HA, could be overcome by an intermediate boost with HA-derived peptides derived from the second virus. This intermediate boosting allowed the CD4 T cell response to the subsequent infection to now include CD4 T cells specific for HA and with them, HA-specific antibody responses for the second virus (78). The quantitative relationship between CD4 T cells recruited into the response and the B cell response is not known at this time. Collectively, our current work suggests that for the goal of producing sterilizing immunity to influenza through production of neutralizing antibodies, vaccines that contain HA, devoid of other viral proteins, may be particularly efficacious. We are currently evaluating this hypothesis.

Human memory CD4 T cells reactive to influenza virus.

For the goal of predicting future responses to infection or vaccination, or development of novel broadly protective influenza vaccines (79–82), knowledge of the existing CD4 T cell repertoire in humans is essential. There is a growing appreciation that vaccine strategies that more fully engage the cellular response can enhance protection. A number of recent studies have sought to quantify and characterize the repertoire of circulating memory CD4 T cells with specificity towards influenza antigens (59, 60, 62, 63, 83–87). Many studies by other groups have concluded that the most prominent influenza-reactive CD4 T cells are those

specific for internal virion proteins, particularly M1 and NP (84–86), as well as HA (63). Using peptides identified by predictive algorithms, combined into larger pools, also showed that CD4 T cells specific for conserved epitopes abundant in internal virion proteins such as M1 and NP are enriched in healthy adults (83, 84). Employing HLA-class II tetramers specific for influenza-derived epitopes, quantifiable CD4 T cells in healthy adults were also identified that were specific for individual epitopes from HA and M1 (60, 61)

Despite the progress in this area, for the purpose of predicting future CD4 T cell response capabilities in humans, it is essential to consider the methods used thus far to assess influenza CD4 T cell specificity and abundance. Whether or not specificity and dominance are determined directly *ex vivo* or after *in vitro* expansion is important. If after expansion, it is critical to note whether all potential epitopes were used to expand the CD4 T cells in culture, or if a subset of peptides or antigens were used. Selective expansion will impact the breadth of the CD4 T cell repertoire later quantified for specificity. Pre-selected epitopes chosen based on predictive algorithms, particular MHC types, and or using CD4 T cells expanded with subsets of antigens may lead to conclusions that, although useful in many respects for the goals of the original study, will contain biases that may lead to under- or over-estimates of particular CD4 T cell epitope or protein specificities circulating in typical adults. It has been our view that direct *ex vivo* stimulation assays on CD4 T cells using peptide libraries from a diverse set of influenza viral proteins will provide the most informative assessment of the total influenza-specific CD4 T cell repertoire. The theoretical potential of CD4 T cells to be recruited into the response to vaccination or infection will be based on these measurements.

Using the preceding approach to analyze PBMC from a large cohort of adult donors, we have evaluated the total abundance of influenza virus protein-specific CD4 T cells. Utilizing overlapping peptide libraries representing all potential CD4 epitopes for each viral protein combined into a single pool, cytokine EliSpots or intracellular cytokine staining assays were employed to quantify influenza virus protein-specific CD4 T cells. A number of discrete conclusions were drawn from this work. Adult subjects have circulating CD4 T cells reactive with influenza-derived peptides, but the degree of total influenza reactivity varies among different individuals. With regard to the antigen specificity of the influenza-reactive CD4 T cells, peptide pools representing the epitopes from many influenza virus proteins (HA, NA, NP, M1, NS1 and polymerase) elicited readily detectable CD4 T cells, based on antigen-dependent cytokine secretion. Interestingly, both in published work from our laboratory (87) and in ongoing work, we have found that the distribution of viral protein specificity varies dramatically among different individuals. The source of the variability is not known, but could reflect several events. First, healthy adults have complex infection histories, likely encountering different strains of influenza (H1N1, H3N2 or influenza B) or drifted strains of the same virus and also could have been exposed to influenza virus proteins through periodic vaccination. Unfortunately, it is extremely difficult to ascertain previous encounters with influenza virus through medical histories, in part, because many adults do not seek medical care with mild respiratory infections and do not record annual vaccinations. HLA class II genotype might also be expected to enrich for some CD4 T cell specificities, although HLA class II molecule diversity should allow presentation of a diverse set of

influenza-derived peptides, diminishing the impact of this variable on overall influenza CD4 T cell abundance.

Our studies revealed that both genetically conserved, internal virion proteins (NP and M1) and genetically variable envelope proteins (HA and NA) contain epitopes recognized by significant numbers of memory CD4 T cells from adult subjects, many of whom have been repeatedly vaccinated. This result indicates that repeat encounters with influenza viruses and vaccines does not lead to exclusive dominance of CD4 T cell reactivity toward conserved internal viral proteins but includes reactivity to the membrane glycoproteins. Substantial numbers of HA- and NA-reactive CD4 T cells, including those specific for influenza A and influenza B, may be promoted by the composition of licensed influenza vaccines that are highly enriched in these proteins. Our studies have shown that CD4 T cell reactivity for HA is enriched for epitopes contained in the highly conserved membrane stalk domain (62). Based on the number of CD4 T cells circulating in peripheral blood that can produce cytokine in response to influenza A derived epitopes, we can estimate that the number of CD4 T cells specific for the influenza A in a typical human is in the range of 175 to 2500 IFN- γ producing CD4 T cells per million CD8 T cell- and NK-depleted PBMC entered into the assay.

The subjects analyzed thus far by our group and other investigators have primarily been drawn from populations in the United States that tend to be regularly vaccinated. It will be of great interest in the future to more fully evaluate CD4 T cell specificity and phenotype among subjects in less industrialized communities who may most regularly contact influenza through infection (88–92). Cohorts for influenza studies are currently being established by several groups, collecting samples after infection or the first vaccination (93–96) and then followed longitudinally. Detailed analyses of these types of cohorts are essential in understanding the major events that establish and remodel the CD4 T cell repertoire. This type of knowledge is essential in the development of new and potentially broadly cross-reactive universal vaccines that have the potential to diminish the global burden and spread of influenza. Such “universal influenza” vaccines may not only protect against the devastating impact of seasonal influenza, particularly among the most vulnerable in our populations, but additionally could protect against the potentially calamitous global emergence of novel pandemic strains of influenza. Rational design of these broadly protective vaccines that recruit more highly cross-reactive effector cells will be enhanced by a more thorough understanding of CD4 T cell repertoire and functional potential in the diverse human populations around the globe.

The role of viral antigen specificity in protective immunity to influenza

The broad protein specificity of CD4 T cells specific for influenza virus discovered in both mouse models of infection and in human circulating CD4 T cells raises the important issue of whether the viral antigen specificity of existing influenza specific CD4 memory populations influences their functional potential or conversely, whether CD4 T cells specific for different viral proteins convey similar functions. Differences in functionality might arise from several different mechanisms. Antigen abundance at priming, where M1>NP>NS1>HA>NA>PA, PB1 and PA (97), could skew functional phenotype, as has

been demonstrated in mouse models of priming (98–103), favoring responses to M1 and NP. Also, periodic boosting of T cells specific for conserved epitopes may also affect functionality, as recent data suggests that each re-stimulation of T cells can progressively alter their transcriptional program (104). Cell type-dependent antigen handling based on interactions with lectin, complement and Fc receptors on different subsets of antigen presenting cells (105–109) might also lead to differences in the specificity or functionality of CD4 T cells specific for cytoplasmic, non-glycosylated internal virion proteins compared to membrane glycoproteins. Also, the presence of viral RNA associated with ribonucleoprotein, acting as TLR ligands (110–112), could change the activation state of the antigen presenting cell and thus the functionality of the CD4 T cells elicited. Finally, whether or not a viral antigen is available only after the highly inflammatory environment of infection (NS1) or is also abundant in vaccines delivered as an intramuscular injection (e.g. HA and NA) can easily be envisioned to influence CD4 T cell function.

Although the impact of each of the factors discussed above is intriguing and subject to investigation within our laboratory, thus far we have seen the most striking restrictions on functionality based on the protein origin in CD4 T cell dependent antibody responses. The data we have accumulated thus far suggests that restrictions on delivery of “help” for antibody response reflects, at least in part, viral antigen handling by virus antigen-specific B cells after infection or vaccination. Using a mouse model of vaccination and infection, we developed a straightforward experimental approach to evaluate the linkage of CD4 T cell help with antibody responses after influenza infection (113). Mice were exclusively populated with CD4 T cells specific for two different viral proteins (NP and HA) through a peptide priming strategy, using previously identified immunodominant peptides. Peptides were combined into the two different pools based on their protein origin and introduced into separate cohorts of mice. After CD4 T cell memory was established, mice from vaccinated or control groups were challenged with influenza virus infection. Secondary lymphoid tissue was examined for the abundance and specificity of the elicited CD4 T cell response, while serum was sampled for quantification of influenza-specific antibodies. These studies revealed that peptide-priming established CD4 T cell memory that could be recalled and rapidly expanded upon influenza infection, resulting in as much as a 2-day advantage in proliferation kinetics. Associated with this rapid boosting of virus-specific CD4 T cells was an accelerated B cell response. Most striking was our finding that CD4 T cell help for antibody responses were selective and obligately linked to the B cell receptor viral antigen specificity. Pre-existing memory HA-specific CD4 T cells accelerated the antibody response to HA, but not NP. Conversely, NP-specific CD4 T cells were able to accelerate the B cell response to NP, but not the HA protein.

The mechanisms that underlie the striking selectivity in CD4 T cell help for antibody responses are unknown, but most likely relate to the form of antigen available to virus-specific B cells after infection. After infection, many cells in the lung become infected (7, 66, 114, 115). Phenotypically distinct subsets of migratory respiratory dendritic cells (cDC1: CD103⁺CD11b⁻, cDC2: CD103⁻CD11b⁺) can become infected or capture viral antigens. Through up-regulation of CCR7, respiratory dendritic cells migrate to the draining lymph node, where T cell priming occurs, either by direct contact with antigen bearing migratory cells or after antigen release and uptake by lymph node resident DC. However, there is little

evidence that the infected migratory DC are able to release intact virions in the lymph node (9), as very few virions and little viral RNA are detected after infection (116–118). Therefore, it is unlikely that there are sufficient levels of influenza virions in the draining lymph node to provide a major source of antigen for influenza-specific B cells. More importantly, B cell uptake of viruses via its immunoglobulin receptor (IgR), although permitting infection and viral protein synthesis, has been shown to lead to rapid B cell death (119), thus eliminating them from the responsive pool that ultimately secretes antibodies and seeds the memory compartment. Accordingly, it seems likely that the major form of virus protein available for B cell receptor-mediated uptake in the draining lymph node may not be free virions, but rather consist of membrane fragments and proteins that are released from dying or infected cells in the lymph node or the lung. The lung-derived antigens might either passively drain to the lymph node or may be actively carried by migratory APC. If this model is correct and the primary available forms of antigen accessible to B cells are isolated viral proteins and membrane fragments, then virus-specific B cells will present a more limited diversity of MHC class II:peptide complexes than would be presented through uptake of virions or through active infection (**Figure 2**), because of the dissociated nature of the antigens. Also, the abundance of viral proteins that access the draining lymph node could vary after release from infected cells. Little is currently known about the nature of antigens that access the lymph node after influenza infection.

A number of studies have provided strong evidence that the only physiological manner in which B cells take up antigen for presentation to antigen-specific CD4 T cells is through their immunoglobulin (Ig) receptor. Not only does antigen engagement through the Ig receptor promote a profoundly increased efficiency of the capture of limiting amounts of antigen (estimated by an early study to be in the range of 1000 to 10,000 fold (120)), but this binding event also signals the B cells. Ig-mediated internalization and signaling leads to many intracellular alterations that are important for antigen processing and presentation of peptide:MHC class II proteins (reviewed in (121, 122)). Engagement of the B cell receptor with antigen-bearing cells such as follicular dendritic cells, DCs (123) and macrophages at the follicle-SCS boundary (124) allows multivalent interactions and sustained B cell receptor-mediated signaling and later B cell differentiation (reviewed in (125–127)). Signaling through the B cell receptor also induces up-regulation of the chemokine receptor CCR7 that promotes migration of the antigen-specific B cells toward the T cell zone of secondary lymphoid tissues, thus allowing contact with and recruitment of CD4 T cell help (128, 129). Thus, antigen uptake by the B cell antigen receptor is essential for the downstream events of B cell antigen processing, presentation and recruitment of CD4 T cell help that is needed for B cell expansion, differentiation and Ig affinity maturation.

The nature of the viral antigen taken up by B cells will determine the repertoire of CD4 T cells that can be recruited to help in the response. In the extreme example, if the HA-specific B cells that have taken up viral antigen through their HA-specific receptor only internalize isolated HA proteins, they will only display peptides from HA and exclusively recruit help from the CD4 T cells specific for HA-derived epitopes. In contrast, by the same restrictions, if NP-specific B cells only take up NP via their Ig receptor, they will only be able to display peptides from NP and therefore will only be able to gain help for antibody production by

NP-specific CD4 T cells. There may be some cases where intermolecular help may occur. For instance, HA is contained in lipid rafts in the plasma membrane of infected cells (130, 131) and it is likely that membrane fragments may also contain NA and M1 and M2. Thus, it is possible that these aggregates are taken up together by antigen-specific B cells. Similarly, NP may be associated with viral RNA and polymerase proteins (132–135) and NP-specific B cells may display peptides derived from all of these and be able to recruit CD4 T cells specific for each of them. Future experiments, empirically examining the peptide repertoire displayed by antigen-specific B cells, as well as the biochemical nature and source of viral antigens reaching the lymph node should collectively help in addressing this intriguing issue.

Provision of CD4 T cell help for B cell responses to seasonal and avian viruses and vaccines

Our studies in human vaccine recipients argue that the same restrictions on recruitment of CD4 T cell help that was identified in animal models of priming and infection (113) also seems to apply to responses to intramuscular split or subunit vaccines. In response to vaccination of human subjects with the novel monovalent influenza (pH1N1) inactivated vaccine produced in eggs, the elicited CD4 T cell responses were directed toward epitopes derived from HA and from the internal virion proteins that often contaminate split and subunit vaccines (136–138). When expansion of CD4 T cells specific for different epitopes was correlated with neutralizing antibody responses, although more robust CD4 T cell responses were observed for cells specific for NP and M1, the most striking correlation with antibody production was observed with expansion of CD4 T cells specific for the genetically conserved epitopes in HA (**see Figure 3, bottom**). Thus, the most effective help for antibody responses elicited by vaccines may occur when the antigen specificity of the CD4 T cells matches that of the B cell antigen receptor or viral proteins that are physically associated with the ligands recognized by the B cell receptor.

Analyses of human responses to avian influenza virus vaccines also support the important role of CD4 T cell specificity in the neutralizing antibody response. Avian influenza vaccines are generally poorly immunogenic in humans, requiring multiple or high doses or addition of adjuvant to elicit protective levels of neutralizing antibody (139–148). As the sequences of avian H5 and its closest seasonal “relative” (H1) have only a limited degree of sequence conservation (**Figure 3, Top**), particularly in the long stretches needed presentation via class II molecules and elicitation of cross-reactive T cells, we hypothesized that in individuals exposed only to seasonal strains, a paucity of H5 HA-specific CD4 T cells might underlie the failure of H5N1 vaccines to elicit neutralizing antibody. If this were true, we speculated that pre-pandemic priming of humans, even with a serologically distinct avian strain, might establish H5-specific CD4 T cell memory that could be recalled upon revaccination. To evaluate this possibility, our laboratory participated in a revaccination study, where individuals who were previously vaccinated years earlier with serologically distinct inactivated H5N1 vaccines were asked to participate in an additional H5N1 vaccine trial. In the participating subjects, we evaluated pre- and post-vaccination CD4 T-cell reactivity and the corresponding antibody responses to H5. A cohort of vaccinees who had

no past exposure to avian viruses or vaccines was used as a control. Using cytokine EliSpot assays with pools of overlapping synthetic peptides as the recall antigen, the accumulation of CD4 T cells specific for H5 in the hosts that had been vaccinated years earlier was evaluated, as was the boosting and expansion of any existing memory H5-specific CD4 T-cell response after the challenge with H5N1-derived vaccine. Our studies revealed that, compared to H5N1-naïve human subjects, those human subjects who were previously immunized with serologically distinct H5N1 vaccine displayed a selective increase in their H5 HA-specific CD4 T-cells prior to revaccination, indicating previous priming and persistence of H5 specific memory CD4 T cells. With this previous H5-specific CD4 memory, the subjects exhibited greatly enhanced H5-specific CD4 T cell responses post vaccination. The robust H5 HA-specific CD4 T-cell response in the previously vaccinated subjects was positively correlated with gains in the H5N1 neutralizing antibody titer. Importantly for the purpose of this discussion, our experiments found no enrichment of CD4 T cells specific for NP in the previously H5N1-vaccinated subjects, nor was there any detectable correlation between CD4 T cell response to NP and the H5-specific antibody response. Together, these findings suggest that HA-specific CD4 T cells have the most potency in delivering help for neutralizing HA-specific antibody responses. Moreover, these studies indicate that a fraction of viral antigen-specific cells in the host (those CD4 T cells specific for NP) have little detectable contribution to the neutralizing HA-specific response. This conclusion supports the model that the nature and complexity of the antigen taken up by antigen-specific B cells will determine the specificity of CD4 T cells that can provide help for expansion and production of high affinity antibodies. The abundance of these CD4 T cell specificities recruited into the response will likely provide the best correlate of protective antibody responses, with other viral antigen specificities either being neutral or negative (149).

The availability of HA-specific CD4 T cells for responses to avian viruses

Because of the importance of HA-specific CD4 T cells in production of neutralizing antibody, and the continued threat of avian influenza, we have explored the availability of peptide epitopes that are conserved within avian and seasonal influenza strains that are available to be utilized for responses to avian vaccines and viruses. The reasoning behind this is that most adult subjects have circulating CD4 T cell memory to HA-derived peptides and we expected that some fraction of these CD4 T cells may be able to recognize the homologous segments in avian HA proteins. This cross-reactive repertoire would theoretically be competent for recruitment into the avian virus response, boosted, expand and provide help to those B cells that have gained avian virus-derived antigen through infection or vaccination.

In our studies, the potential for cross-reactive recognition was first evaluated by a comparison of the amino acid sequence identity between avian and seasonal HA and, secondly, by empirical experimentation. For MHC class II binding and stimulation of CD4 T cells, generally peptides of approximately 11–13 amino acids are sufficient (reviewed in (150–155)). Although conservative substitutions in sequence in peptide epitopes can be tolerated, particularly in amino acids that contribute to peptide binding (156–160), other substitutions, sometimes as limited as a single amino acid, can eliminate CD4 T cell recognition, either through alterations in binding affinity for MHC class II molecules (157,

158, 160, 161) or change in T cell receptor contact sites (162–165). Small substitutions in peptides recognized by CD4 or CD8 T cells can also create partial agonists or altered peptide ligands that can lead to aberrant T cell phenotype in the elicited response (165–172). Therefore, predicting cross reactivity between seasonal strains and avian influenza HA proteins is challenging, particularly because of difficulty in delineating the amino acids that form the critical anchor contacts with the HLA molecules in the host and serve as contact points for T cell receptors.

Using mouse models of infection, where immune history to influenza can be controlled, we identified one major CD4 T cell epitope specific for HA that was located in a highly conserved region of H1 (435–455) that possesses only one amino acid substitution with H5. When examined for the ability of H1N1 primed CD4 T cells to recognize the avian derived peptide, we found that it stimulated more than 85% of the CD4 T cells that were elicited by the H1N1-derived peptide (68). Confirmation that this epitope is recognized in human CD4 T cells elicited by seasonal influenza was provided by Kwok and co-workers using HLA-DR1 tetramers (64). We have also obtained strong evidence that CD4 T cells generated by seasonal influenza can cross react with H7-derived peptides (62). In these studies, 17-mer peptides representing the most highly conserved segments of H7 HA relative to its closest seasonal strain (H3) were identified and tested. A pool of 26 of these H7-derived peptides were evaluated for their ability to activate human CD4 T cells from healthy donors who had never encountered avian vaccines or viruses. Reactivity to NP-derived peptides served as a control. Although, as expected, CD4 T cell reactivity to NP-derived epitopes was much more abundant than those reactive with H7-derived peptides, we found that approximately 75% of the healthy subjects examined had some reactivity to H7 epitopes. When individual peptides from the conserved regions were tested, we found that many individual H7 derived peptides were able to recall CD4 T cells, with frequencies ranging from 5 to >150 reactive CD4 T cells per million tested. Interestingly, when H7N7 vaccine responses were examined in individuals never previously exposed to H7N9, there was expansion of H3-H7 cross-reactive CD4 T cells, arguing that these H7-boosted CD4 T cells can be recruited from the memory population of CD4 T cells originally primed by seasonal influenza, harnessing CD4 T cell memory, while allowing for recruitment of B cells specific for novel epitopes in the head domain of HA.

Based on the development and implementation of epitope algorithms, the H7 protein has been predicted to contain a paucity of T-cell epitopes (173). Our laboratory sought to empirically evaluate this issue using HLA-DR1 and HLA-DR4 transgenic mice. The relative abundance of CD4 T cell epitopes in seasonal vs. avian HA proteins was compared and revealed similar CD4 T cell epitope composition in seasonal and avian HA proteins (174). Thus, this limited sampling did not support the generalized view that H7 is deficient in eliciting CD4 epitopes and suggests that H7N9 avian virus and vaccines will be able to elicit CD4 T cells in human subjects. Experiments testing this are currently ongoing in our laboratory.

Despite the potential for naive CD4 T cells be elicited in response to avian HA proteins during virus infection and vaccination, recalling memory CD4 T cells generated from H3N2 and H1N1 viruses may be critical for a robust, effective and protective CD4 T dependent

antibody response to the first encounter with avian viruses. Recall of memory CD4 T cells have several advantages because they exist at a higher frequency than the naïve CD4 T cell population, expand more rapidly and at lower epitope density than naïve CD4 T cells (74–77, 175, 176). For those healthy adults with a very limited repertoire of CD4 T cells that cross react with avian HA proteins, pre-pandemic priming with H5 or H7 proteins or peptides might be a very useful strategy (147, 148, 177–179). By populating humans with avian HA-epitope-specific CD4 T memory cells that can be recalled, these hosts are more likely to produce neutralizing HA-specific antibody responses and/or to more vigorously respond to avian influenza derived vaccines, if avian influenza viruses gain the ability to transmit across human populations (180–185)

Do T cells compete with each other?

Because of the diversity of epitopes offered to the immune system after both infection and vaccination, the issue of inter-T cell competition is an important consideration for our understanding influenza-specific immune responses and for future vaccine design strategies. For the purposes of this discussion, we are defining competition as any event that leads to selectivity in the specificity of the response due to simultaneous responses of T cells to independent epitopes. Competition among T cells has been described as a consequence of limiting “space” on the presenting APC (186–188), competition for limited resources, such as cytokines in the local environment (189), secretion of antagonistic cytokines (190, 191), or as a consequence of “stripping” of antigen bearing APC of their stimulatory capacity, through removal or blockade of cell surface proteins on the APC by their interaction with dominant T cells (192–195). All of these events might occur among competing T cells during the primary response to infection or vaccination. There is also strong evidence of “immunodomination” by memory cells when they compete with naïve cells. In almost all circumstances of this type, the previously primed memory cells have a significant and competitive advantage over naïve cells (70, 78, 196–199). Most commonly, the negative effects of competition are most profound among T cells when the antigens are delivered and presented by the same APC (187, 192, 196, 200, 201). These findings argue that intimate T cell-APC contact is involved in the negative effects of competition. Interestingly, although intuitively appealing, there is little evidence for competition for binding of antigenic peptide to MHC proteins. This has been addressed by showing that T cells specific for peptides restricted to different MHC isotypes (e.g. I-A vs. I-E (200), or H-2K vs. H-2D (202)) do in fact compete with each other. While most of the studies have examined evidence for competition at the priming or expansion phase, there is also evidence that competition among different specificities can influence selectivity secondarily, as tissue-specific T cell memory is established (203). Our laboratory has also discovered that the negative effects of competition can occur when peptide epitopes are introduced simultaneously during the primary immune response, either through vaccination with complex antigens that contain many epitopes (157, 158, 160) or during simultaneous introduction of antigenic peptides to the host (190, 200, 204). As had been observed in other studies, suppression of the responses of some T cell specificities by simultaneous responses of others is not a systemic event, but instead appears to be a highly localized suppressive event, and requires the co-presentation of competing peptide epitopes on the same APC.

As previously discussed, our laboratory has shown that in the response to influenza virus, memory CD4 T cells specific for internal viral antigens expand at the expense of T cells specific to new epitopes expressed by a secondary virus that shares only a subset of viral proteins with the first. This is a common feature of heterosubtypic influenza infection, because the internal virion proteins are highly conserved in sequence among all strains of influenza. It is clear that memory CD4 T cells have substantial advantages over naïve cells, including higher precursor frequency and lower thresholds of activation (reviewed by (74)). However, there may be other more active means of suppression of naïve T cell responses by memory T cells. We have found that in both competing responses to infection by memory cells and naïve cells (70), and in responses to peptides introduced simultaneously with adjuvant (204), IFN- γ is a prominent cytokine produced by the more dominant T cells. In both situations, the dominant T cells expand first. We suspect that early production of this cytokine by rapidly boosted memory cells suppresses naïve CD4 T cells during secondary challenge. IFN- γ , abundantly expressed by T cells elicited by influenza, is known to have multiple avenues for suppressing CD4 T cell responses (reviewed in (205–207)) that includes direct pro-apoptotic activity on naïve T cells (208). IFN- γ can also act indirectly to down-modulate adaptive T cell immunity through effects on DC, promoting localized production of IDO. IDO is an immunomodulatory enzyme that has multiple effects on immune responses (reviewed in (205, 209–214)).

Independently of the mechanisms that may be in play among competing T cell responses, it is important to consider the consequences of this competition on influenza immunity, where CD4 T cells specific for new epitopes, derived from HA and NA, are always competing with those memory cells specific for conserved epitopes derived from proteins such as NP and M1. Current vaccines are primarily prepared from virions and contain multiple viral proteins in addition to HA, primarily M1 and NP. Both animal models of vaccination (136) and human vaccine responses (178, 215) within our laboratory have shown that epitopes from these proteins recruit CD4 T cells. Therefore, after vaccination with inactivated vaccines, CD4 T cells of multiple protein specificities will be recruited to the response initiated by dendritic cells that are likely to have simultaneously taken up this mixture of viral antigens. CD4 T cell responses to HA, which are in part comprised of novel peptide epitopes within the head due to mutations that permit escape from neutralizing antibodies, may be diminished by the coincident presentation of NP- and M1-derived epitopes on the priming APC. We have shown that the loss in CD4 T cell responses to HA is linked to diminished production of protective antibodies specific for HA (70, 78), suggesting that the consequences of this inter-protein competition may be severe for vaccine-induced protective antibody.

These competitive events in CD4 T cell priming will have an even greater negative consequence when the host is responding to novel potentially pandemic avian strains, where a more limited subset of CD4 T cell epitopes are conserved with the circulating seasonal strains of influenza, which humans have frequent contact with through infection and vaccination. Overcoming the competitive advantages that memory T cells have over naïve cells is an important area to consider in any new vaccine strategies that seek to focus the immune response on new epitopes within influenza virus, on the background T cell reactivity toward highly conserved viral proteins.

Acknowledgments

This project has been funded in whole or in part with Federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under CEIRS Contract No. HHSN272201400006C and Contract No. HHSN272201200005C from the National Institutes of Health to Andrea Sant.

References

1. McKinstry KK , Strutt TM , Swain SL . Hallmarks of CD4 T cell immunity against influenza. *J Intern Med.*2011;269:507–518.21362069
2. Devarajan P , Bautista B , Vong AM , McKinstry KK , Strutt TM , Swain SL . New Insights into the Generation of CD4 Memory May Shape Future Vaccine Strategies for Influenza. *Front Immunol.* 2016;7:136.27148257
3. Bedoui S , Heath WR , Mueller SN . CD4(+) T-cell help amplifies innate signals for primary CD8(+) T-cell immunity. *Immunol Rev.*2016;272:52–64.27319342
4. La Gruta NL , Turner SJ . T cell mediated immunity to influenza: mechanisms of viral control. *Trends Immunol.*2014;35:396–402.25043801
5. Allie SR , Randall TD . Pulmonary immunity to viruses. *Clin Sci (Lond).*2017;131:1737–1762.28667071
6. Zens KD , Farber DL . Memory CD4 T cells in influenza. *Curr Top Microbiol Immunol.* 2015;386:399–421.25005927
7. Yoo JK , Kim TS , Hufford MM , Braciale TJ . Viral infection of the lung: host response and sequelae. *J Allergy Clin Immunol.*2013;132:1263–1276; quiz 1277.23915713
8. Sun J , Braciale TJ . Role of T cell immunity in recovery from influenza virus infection. *Curr Opin Virol.*2013;3:425–429.23721865
9. Hufford MM , Kim TS , Sun J , Braciale TJ . The effector T cell response to influenza infection. *Curr Top Microbiol Immunol.*2015;386:423–455.25033753
10. Butler NS , Kulu DI . The regulation of T follicular helper responses during infection. *Curr Opin Immunol.*2015;34:68–74.25726751
11. Dufaud CR , McHeyzer-Williams LJ , McHeyzer-Williams MG . Deconstructing the germinal center, one cell at a time. *Curr Opin Immunol.*2017;45:112–118.28319730
12. Qi H T follicular helper cells in space-time. *Nat Rev Immunol.*2016;16:612–625.27573485
13. Ueno H Human Circulating T Follicular Helper Cell Subsets in Health and Disease. *J Clin Immunol.*2016;36 Suppl 1:34–39.26984851
14. Vinuesa CG , Linterman MA , Yu D , MacLennan IC . Follicular Helper T Cells. *Annu Rev Immunol.*2016;34:335–368.26907215
15. Sage PT , Sharpe AH . T follicular regulatory cells in the regulation of B cell responses. *Trends Immunol.*2015;36:410–418.26091728
16. Crotty S A brief history of T cell help to B cells. *Nat Rev Immunol.*2015;15:185–189.25677493
17. Crotty S T follicular helper cell differentiation, function, and roles in disease. *Immunity.* 2014;41:529–542.25367570
18. Kumamoto Y , Mattei LM , Sellers S , Payne GW , Iwasaki A . CD4+ T cells support cytotoxic T lymphocyte priming by controlling lymph node input. *Proc Natl Acad Sci U S A.*2011;108:8749–8754.21555577
19. Beuneu H , Garcia Z , Bousso P . Cutting edge: cognate CD4 help promotes recruitment of antigen-specific CD8 T cells around dendritic cells. *J Immunol.*2006;177:1406–1410.16849444
20. Castellino F , Huang AY , Altan-Bonnet G , Stoll S , Scheinecker C , Germain RN . Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. *Nature.*2006;440:890–895.16612374
21. Laidlaw BJ , Craft JE , Kaech SM . The multifaceted role of CD4(+) T cells in CD8(+) T cell memory. *Nat Rev Immunol.*2016;16:102–111.26781939
22. Bevan MJ . Helping the CD8(+) T-cell response. *Nat Rev Immunol.*2004;4:595–602.15286726

23. Rocha B , Tanchot C . Towards a cellular definition of CD8+ T-cell memory: the role of CD4+ T-cell help in CD8+ T-cell responses. *Curr Opin Immunol.*2004;16:259–263.15134772
24. Behrens G , Li M , Smith CM , Helper T cells, dendritic cells and CTL Immunity. *Immunol Cell Biol.*2004;82:84–90.14984599
25. Northrop JK , Shen H . CD8+ T-cell memory: only the good ones last. *Curr Opin Immunol.* 2004;16:451–455.15245738
26. Bourgeois C , Tanchot C . Mini-review CD4 T cells are required for CD8 T cell memory generation. *Eur J Immunol.*2003;33:3225–3231.14635030
27. Khanolkar A , Badovinac VP , Harty JT . CD8 T cell memory development: CD4 T cell help is appreciated. *Immunol Res.*2007;39:94–104.17917058
28. Wiesel M , Oxenius A . From crucial to negligible: functional CD8(+) T-cell responses and their dependence on CD4(+) T-cell help. *Eur J Immunol.*2012;42:1080–1088.22539281
29. Sun JC , Bevan MJ . Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science.*2003;300:339–342.12690202
30. Laidlaw BJ , Zhang N , Marshall HD , CD4+ T cell help guides formation of CD103+ lung-resident memory CD8+ T cells during influenza viral infection. *Immunity.*2014;41:633–645.25308332
31. Ahrends T , Spanjaard A , Pilzecker B , CD4(+) T Cell Help Confers a Cytotoxic T Cell Effector Program Including Coinhibitory Receptor Downregulation and Increased Tissue Invasiveness. *Immunity.*2017;47:848–861 e845.29126798
32. Teijaro JR , Verhoeven D , Page CA , Turner D , Farber DL . Memory CD4 T cells direct protective responses to influenza virus in the lungs through helper-independent mechanisms. *J Virol.* 2010;84:9217–9226.20592069
33. Strutt TM , McKinsty KK , Dibble JP , Memory CD4+ T cells induce innate responses independently of pathogen. *Nat Med.*2010;16:558–564, 551p following 564.20436484
34. Iwasaki A , Pillai PS . Innate immunity to influenza virus infection. *Nat Rev Immunol.* 2014;14:315–328.24762827
35. Brown DM , Lampe AT , Workman AM . The Differentiation and Protective Function of Cytolytic CD4 T Cells in Influenza Infection. *Front Immunol.*2016;7:93.27014272
36. Juno JA , van Bockel D , Kent SJ , Kelleher AD , Zaunders JJ , Munier CM . Cytotoxic CD4 T Cells-Friend or Foe during Viral Infection? *Front Immunol.*2017;8:19.28167943
37. Takeuchi A , Saito T . CD4 CTL, a Cytotoxic Subset of CD4(+) T Cells, Their Differentiation and Function. *Front Immunol.*2017;8:194.28280496
38. van de Berg PJ , van Leeuwen EM , ten Berge IJ , van Lier R . Cytotoxic human CD4(+) T cells. *Curr Opin Immunol.*2008;20:339–343.18440213
39. Brown DM . Cytolytic CD4 cells: Direct mediators in infectious disease and malignancy. *Cell Immunol.*2010;262:89–95.20236628
40. Hua L , Yao S , Pham D , Cytokine-dependent induction of CD4+ T cells with cytotoxic potential during influenza virus infection. *J Virol.*2013;87:11884–11893.23986597
41. Brown DM , Kamperschroer C , Dilzer AM , Roberts DM , Swain SL . IL-2 and antigen dose differentially regulate perforin- and FasL-mediated cytolytic activity in antigen specific CD4+ T cells. *Cell Immunol.*2009;257:69–79.19338979
42. Workman AM , Jacobs AK , Vogel AJ , Condon S , Brown DM . Inflammation enhances IL-2 driven differentiation of cytolytic CD4 T cells. *PLoS One.*2014;9:e89010.24586481
43. Brown DM , Lee S , Garcia-Hernandez Mde L , Swain SL . Multifunctional CD4 cells expressing gamma interferon and perforin mediate protection against lethal influenza virus infection. *J Virol.* 2012;86:6792–6803.22491469
44. Marshall NB , Vong AM , Devarajan P , NKG2C/E Marks the Unique Cytotoxic CD4 T Cell Subset, ThCTL, Generated by Influenza Infection. *J Immunol.*2017;198:1142–1155.28031335
45. Viboud C , Alonso WJ , Simonsen L . Influenza in tropical regions. *PLoS Med.* 2006;3:e89.16509764

46. Chadha MS , Hirve S , Dawood FS , Burden of seasonal and pandemic influenza-associated hospitalization during and after 2009 A(H1N1)pdm09 pandemic in a rural community in India. *PLoS One*.2013;8:e55918.23690913
47. Nair H , Brooks WA , Katz M , Global burden of respiratory infections due to seasonal influenza in young children: a systematic review and meta-analysis. *Lancet*.2011;378:1917–1930.22078723
48. Baggett HC , Chittaganpitch M , Thamthitiwat S , Incidence and epidemiology of hospitalized influenza cases in rural Thailand during the influenza A (H1N1)pdm09 pandemic, 2009–2010. *PLoS One*.2012;7:e48609.23139802
49. Ortiz JR , Englund JA , Neuzil KM . Influenza vaccine for pregnant women in resource-constrained countries: a review of the evidence to inform policy decisions. *Vaccine*.2011;29:4439–4452.21550377
50. McMorrow ML , Emukule GO , Obor D , Maternal influenza vaccine strategies in Kenya: Which approach would have the greatest impact on disease burden in pregnant women and young infants? *PLoS One*.2017;12:e0189623.29283997
51. Alonso WJ , Yu C , Viboud C , A global map of hemispheric influenza vaccine recommendations based on local patterns of viral circulation. *Sci Rep*.2015;5:17214.26621769
52. Tobery TW , Caulfield MJ . Identification of T-cell epitopes using ELISpot and peptide pool arrays. *Methods Mol Med*.2004;94:121–132.14959826
53. Tobery TW , Wang S , Wang XM , A simple and efficient method for the monitoring of antigen-specific T cell responses using peptide pool arrays in a modified ELISpot assay. *J Immunol Methods*.2001;254:59–66.11406153
54. Crowe SR , Miller SC , Brown DM , Uneven distribution of MHC class II epitopes within the influenza virus. *Vaccine*.2006;24:457–467.16140434
55. Knudson CJ , Weiss KA , Hartwig SM , Varga SM . The pulmonary localization of virus-specific T lymphocytes is governed by the tissue tropism of infection. *J Virol*.2014;88:9010–9016.24899187
56. Zens KD , Chen JK , Farber DL . Vaccine-generated lung tissue-resident memory T cells provide heterosubtypic protection to influenza infection. *JCI Insight*.2016;1.
57. Marsh SGE , Parham P , Barber LD . The HLA factsbook San Diego: Academic Press, 2000.
58. Howie SR . Blood sample volumes in child health research: review of safe limits. *Bull World Health Organ*.2011;89:46–53.21346890
59. Uchtenhagen H , Rims C , Blahnik G , Efficient ex vivo analysis of CD4+ T-cell responses using combinatorial HLA class II tetramer staining. *Nat Commun*.2016;7:12614.27571776
60. Yang J , James E , Gates TJ , CD4+ T cells recognize unique and conserved 2009 H1N1 influenza hemagglutinin epitopes after natural infection and vaccination. *Int Immunol*.2013;25:447–457.23524391
61. Yang J , James EA , Huston L , Danke NA , Liu AW , Kwok WW . Multiplex mapping of CD4 T cell epitopes using class II tetramers. *Clin Immunol*.2006;120:21–32.16677863
62. Richards KA , Nayak J , Chaves FA , Seasonal Influenza Can Poise Hosts for CD4 T-Cell Immunity to H7N9 Avian Influenza. *J Infect Dis*.2015;212:86–94.25492919
63. Babon JA , Cruz J , Orphin L , Genome-wide screening of human T-cell epitopes in influenza A virus reveals a broad spectrum of CD4(+) T-cell responses to internal proteins, hemagglutinins, and neuraminidases. *Hum Immunol*.2009;70:711–721.19524006
64. Roti M , Yang J , Berger D , Huston L , James EA , Kwok WW . Healthy human subjects have CD4+ T cells directed against H5N1 influenza virus. *J Immunol*.2008;180:1758–1768.18209073
65. Richards KA DA , Rattan A , Knowlden Z , Yang H , Sant AJ Diverse epitope specificity, immunodominance hierarchy and functional avidity of effector CD4 T cells established during priming is maintained in lung after influenza A infection. *Frontiers in Immunology*.2017;in press.
66. DiPiazza A , Nogales A , Poulton N , Wilson PC , Martinez-Sobrido L , Sant AJ . Pandemic 2009 H1N1 Influenza Venus reporter virus reveals broad diversity of MHC class II-positive antigen-bearing cells following infection in vivo. *Sci Rep*.2017;7:10857.28883436
67. Richards KA , Chaves FA , Krafcik FR , Topham DJ , Lazarski CA , Sant AJ . Direct ex vivo analyses of HLA-DR1 transgenic mice reveal an exceptionally broad pattern of immunodominance in the primary HLA-DR1-restricted CD4 T-cell response to influenza virus hemagglutinin. *J Virol*.2007;81:7608–7619.17507491

68. Richards KA , Chaves FA , Sant AJ . Infection of HLA-DR1 transgenic mice with a human isolate of influenza A virus (H1N1) primes a diverse CD4 T-cell repertoire that includes CD4 T cells with heterosubtypic cross-reactivity to avian (H5N1) influenza virus. *J Virol.*2009;83:6566–6577.19386707
69. Richards KA , Chaves FA , Sant AJ . The memory phase of the CD4 T-cell response to influenza virus infection maintains its diverse antigen specificity. *Immunology.*2011;133:246–256.21517839
70. Nayak JL , Alam S , Sant AJ . Cutting edge: Heterosubtypic influenza infection antagonizes elicitation of immunological reactivity to hemagglutinin. *J Immunol.*2013;191:1001–1005.23794632
71. Belz GT , Xie W , Altman JD , Doherty PC . A previously unrecognized H-2D(b)-restricted peptide prominent in the primary influenza A virus-specific CD8(+) T-cell response is much less apparent following secondary challenge. *J Virol.*2000;74:3486–3493.10729122
72. Duan S , Meliopoulos VA , McClaren JL , Diverse heterologous primary infections radically alter immunodominance hierarchies and clinical outcomes following H7N9 influenza challenge in mice. *PLoS Pathog.*2015;11:e1004642.25668410
73. Chen W , Pang K , Masterman KA , Reversal in the immunodominance hierarchy in secondary CD8+ T cell responses to influenza A virus: roles for cross-presentation and lysis-independent immunodomination. *J Immunol.*2004;173:5021–5027.15470045
74. MacLeod MK , Kappler JW , Marrack P . Memory CD4 T cells: generation, reactivation and re-assignment. *Immunology.*2010;130:10–15.20331469
75. London CA , Lodge MP , Abbas AK . Functional responses and costimulator dependence of memory CD4+ T cells. *J Immunol.*2000;164:265–272.10605020
76. Berard M , Tough DF . Qualitative differences between naive and memory T cells. *Immunology.*2002;106:127–138.12047742
77. Rogers PR , Dubey C , Swain SL . Qualitative changes accompany memory T cell generation: faster, more effective responses at lower doses of antigen. *J Immunol.*2000;164:2338–2346.10679068
78. Alam S , Chan C , Qiu X , Selective pre-priming of HA-specific CD4 T cells restores immunological reactivity to HA on heterosubtypic influenza infection. *PLoS One.*2017;12:e0176407.28493882
79. Paules CI , Marston HD , Eisinger RW , Baltimore D , Fauci AS . The Pathway to a Universal Influenza Vaccine. *Immunity.*2017;47:599–603.29045889
80. Sridhar S Heterosubtypic T-Cell Immunity to Influenza in Humans: Challenges for Universal T-Cell Influenza Vaccines. *Front Immunol.*2016;7:195.27242800
81. Crowe JE . Principles of Broad and Potent Antiviral Human Antibodies: Insights for Vaccine Design. *Cell Host Microbe.*2017;22:193–206.28799905
82. Berlanda Scorza F , Tsvetnitsky V , Donnelly JJ . Universal influenza vaccines: Shifting to better vaccines. *Vaccine.*2016;34:2926–2933.27038130
83. Assarsson E , Bui HH , Sidney J , Immunomic analysis of the repertoire of T-cell specificities for influenza A virus in humans. *J Virol.*2008;82:12241–12251.18842709
84. Savic M , Dembinski JL , Kim Y , Epitope specific T-cell responses against influenza A in a healthy population. *Immunology.*2016;147:165–177.26489873
85. Chen L , Zanker D , Xiao K , Wu C , Zou Q , Chen W . Immunodominant CD4+ T-cell responses to influenza A virus in healthy individuals focus on matrix 1 and nucleoprotein. *J Virol.*2014;88:11760–11773.25078703
86. Hayward AC , Wang L , Goonetilleke N , Natural T Cell-mediated Protection against Seasonal and Pandemic Influenza. Results of the Flu Watch Cohort Study. *Am J Respir Crit Care Med.*2015;191:1422–1431.25844934
87. Richards KA , Topham D , Chaves FA , Sant AJ . Cutting edge: CD4 T cells generated from encounter with seasonal influenza viruses and vaccines have broad protein specificity and can directly recognize naturally generated epitopes derived from the live pandemic H1N1 virus. *J Immunol.*2010;185:4998–5002.20889549
88. Doherty M , Schmidt-Ott R , Santos JI , Vaccination of special populations: Protecting the vulnerable. *Vaccine.*2016;34:6681–6690.27876197

89. Ortiz JR , Perut M , Dumolard L , A global review of national influenza immunization policies: Analysis of the 2014 WHO/UNICEF Joint Reporting Form on immunization. *Vaccine*. 2016;34:5400–5405.27646030
90. Hirve S , Lambach P , Paget J , Vandemaele K , Fitzner J , Zhang W . Seasonal influenza vaccine policy, use and effectiveness in the tropics and subtropics - a systematic literature review. *Influenza Other Respir Viruses*.2016;10:254–267.26842617
91. Fischer WA , Gong M , Bhagwanjee S , Sevransky J . Global burden of influenza as a cause of cardiopulmonary morbidity and mortality. *Glob Heart*.2014;9:325–336.25667184
92. Jennings LC . Influenza vaccines: an Asia-Pacific perspective. *Influenza Other Respir Viruses*. 2013;7 Suppl 3:44–51.
93. Gordon A , Kuan G , Aviles W , The Nicaraguan pediatric influenza cohort study: design, methods, use of technology, and compliance. *BMC Infect Dis*.2015;15:504.26553094
94. Team H-CSP , Consortium H-I . Multicohort analysis reveals baseline transcriptional predictors of influenza vaccination responses. *Sci Immunol*.2017;2.
95. Gordon A , Saborio S , Videe E , Clinical attack rate and presentation of pandemic H1N1 influenza versus seasonal influenza A and B in a pediatric cohort in Nicaragua. *Clin Infect Dis*. 2010;50:1462–1467.20420502
96. Malosh R , Ohmit SE , Petrie JG , Thompson MG , Aiello AE , Monto AS . Factors associated with influenza vaccine receipt in community dwelling adults and their children. *Vaccine*.2014;32:1841–1847.24530926
97. Kummer S , Flottmann M , Schwanhauser B , Alteration of protein levels during influenza virus H1N1 infection in host cells: a proteomic survey of host and virus reveals differential dynamics. *PLoS One*.2014;9:e94257.24718678
98. Constant S , Pfeiffer C , Woodard A , Pasqualini T , Bottomly K . Extent of T cell receptor ligation can determine the functional differentiation of naive CD4+ T cells. *J Exp Med*.1995;182:1591–1596.7595230
99. Hosken NA , Shibuya K , Heath AW , Murphy KM , O'Garra A . The effect of antigen dose on CD4+ T helper cell phenotype development in a T cell receptor-alpha beta-transgenic model. *J Exp Med*.1995;182:1579–1584.7595228
100. Rogers PR , Croft M . Peptide dose, affinity, and time of differentiation can contribute to the Th1/Th2 cytokine balance. *J Immunol*.1999;163:1205–1213.10415015
101. Ise W , Totsuka M , Sogawa Y , Naive CD4+ T cells exhibit distinct expression patterns of cytokines and cell surface molecules on their primary responses to varying doses of antigen. *J Immunol*.2002;168:3242–3250.11907078
102. Keck S , Schmalzer M , Ganter S , Antigen affinity and antigen dose exert distinct influences on CD4 T-cell differentiation. *Proc Natl Acad Sci U S A*.2014;111:14852–14857.25267612
103. Nakajima-Adachi H , Koike E , Totsuka M , Two distinct epitopes on the ovalbumin 323–339 peptide differentiating CD4(+)T cells into the Th2 or Th1 phenotype. *Biosci Biotechnol Biochem*.2012;76:1979–1981.23047087
104. Wirth TC , Xue HH , Rai D , Repetitive antigen stimulation induces stepwise transcriptome diversification but preserves a core signature of memory CD8(+) T cell differentiation. *Immunity*. 2010;33:128–140.20619696
105. Goh BC , Rynkiewicz MJ , Cafarella TR , Molecular mechanisms of inhibition of influenza by surfactant protein D revealed by large-scale molecular dynamics simulation. *Biochemistry*. 2013;52:8527–8538.24224757
106. Wang SF , Huang JC , Lee YM , DC-SIGN mediates avian H5N1 influenza virus infection in cis and in trans. *Biochem Biophys Res Commun*.2008;373:561–566.18593570
107. Reading PC , Tate MD , Pickett DL , Brooks AG . Glycosylation as a target for recognition of influenza viruses by the innate immune system. *Adv Exp Med Biol*.2007;598:279–292.17892219
108. van Dinther D , Stolk DA , van de Ven R , van Kooyk Y , de Gruijl TD , den Haan JMM . Targeting C-type lectin receptors: a high-carbohydrate diet for dendritic cells to improve cancer vaccines. *J Leukoc Biol*.2017;102:1017–1034.28729358
109. Park CG . Vaccine strategies utilizing C-type lectin receptors on dendritic cells in vivo. *Clin Exp Vaccine Res*.2014;3:149–154.25003088

110. Garcia-Sastre A . Induction and evasion of type I interferon responses by influenza viruses. *Virus Res.*2011;162:12–18.22027189
111. Dash P , Thomas PG . Host detection and the stealthy phenotype in influenza virus infection. *Curr Top Microbiol Immunol.*2015;386:121–147.25038940
112. Weber-Gerlach M , Weber F . Standing on three legs: antiviral activities of RIG-I against influenza viruses. *Curr Opin Immunol.*2016;42:71–75.27318973
113. Alam S , Knowlton ZA , Sangster MY , Sant AJ . CD4 T cell help is limiting and selective during the primary B cell response to influenza virus infection. *J Virol.*2014;88:314–324.24155379
114. Neyt K , Lambrecht BN . The role of lung dendritic cell subsets in immunity to respiratory viruses. *Immunol Rev.*2013;255:57–67.23947347
115. Kim TS , Sun J , Braciale TJ . T cell responses during influenza infection: getting and keeping control. *Trends Immunol.*2011;32:225–231.21435950
116. Jelley-Gibbs DM , Brown DM , Dibble JP , Haynes L , Eaton SM , Swain SL . Unexpected prolonged presentation of influenza antigens promotes CD4 T cell memory generation. *J Exp Med.*2005;202:697–706.16147980
117. Zammit DJ , Turner DL , Klonowski KD , Lefrancois L , Cauley LS . Residual antigen presentation after influenza virus infection affects CD8 T cell activation and migration. *Immunity.*2006;24:439–449.16618602
118. Kim TS , Hufford MM , Sun J , Fu YX , Braciale TJ . Antigen persistence and the control of local T cell memory by migrant respiratory dendritic cells after acute virus infection. *J Exp Med.*2010;207:1161–1172.20513748
119. Dougan SK , Ashour J , Karssemeijer RA , Antigen-specific B-cell receptor sensitizes B cells to infection by influenza virus. *Nature.*2013;503:406–409.24141948
120. Lanzavecchia A . Antigen-specific interaction between T and B cells. *Nature.*1985;314:537–539.3157869
121. Avalos AM , Ploegh HL . Early BCR Events and Antigen Capture, Processing, and Loading on MHC Class II on B Cells. *Front Immunol.*2014;5:92.24653721
122. Adler LN , Jiang W , Bhamidipati K , The Other Function: Class II-Restricted Antigen Presentation by B Cells. *Front Immunol.*2017;8:319.28386257
123. Qi H , Egen JG , Huang AY , Germain RN . Extrafollicular activation of lymph node B cells by antigen-bearing dendritic cells. *Science.*2006;312:1672–1676.16778060
124. Carrasco YR , Batista FD . B cells acquire particulate antigen in a macrophage-rich area at the boundary between the follicle and the subcapsular sinus of the lymph node. *Immunity.*2007;27:160–171.17658276
125. Liu W , Sohn HW , Tolar P , Pierce SK . It's all about change: the antigen-driven initiation of B-cell receptor signaling. *Cold Spring Harb Perspect Biol.*2010;2:a002295.20591989
126. Batista FD , Harwood NE . The who, how and where of antigen presentation to B cells. *Nat Rev Immunol.*2009;9:15–27.19079135
127. Heesters BA , van der Poel CE , Das A , Carroll MC . Antigen Presentation to B Cells. *Trends Immunol.*2016;37:844–854.27793570
128. Okada T , Miller MJ , Parker I , Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. *PLoS Biol.*2005;3:e150.15857154
129. Reif K , Ekland EH , Ohl L , Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. *Nature.*2002;416:94–99.11882900
130. Rossman JS , Lamb RA . Influenza virus assembly and budding. *Virology.*2011;411:229–236.21237476
131. Leser GP , Lamb RA . Influenza virus assembly and budding in raft-derived microdomains: a quantitative analysis of the surface distribution of HA, NA and M2 proteins. *Virology.*2005;342:215–227.16249012
132. Noda T , Kawaoka Y . Structure of influenza virus ribonucleoprotein complexes and their packaging into virions. *Rev Med Virol.*2010;20:380–391.20853340
133. Eisfeld AJ , Neumann G , Kawaoka Y . At the centre: influenza A virus ribonucleoproteins. *Nat Rev Microbiol.*2015;13:28–41.25417656

134. Ye Q , Krug RM , Tao YJ . The mechanism by which influenza A virus nucleoprotein forms oligomers and binds RNA. *Nature*.2006;444:1078–1082.17151603
135. Labaronne A , Swale C , Monod A , Schoehn G , Crepin T , Ruigrok RW . Binding of RNA by the Nucleoproteins of Influenza Viruses A and B. *Viruses*.2016;8.
136. Richards KA , Chaves FA , Alam S , Sant AJ . Trivalent inactivated influenza vaccines induce broad immunological reactivity to both internal virion components and influenza surface proteins. *Vaccine*.2012;31:219–225.23099328
137. Garcia-Canas V , Lorbetskie B , Bertrand D , Cyr TD , Girard M . Selective and quantitative detection of influenza virus proteins in commercial vaccines using two-dimensional high-performance liquid chromatography and fluorescence detection. *Anal Chem*.2007;79:3164–3172.17361989
138. Co MD , Orphin L , Cruz J , In vitro evidence that commercial influenza vaccines are not similar in their ability to activate human T cell responses. *Vaccine*.2009;27:319–327.18977404
139. Bart SA , Hohenboken M , Della Cioppa G , Narasimhan V , Dormitzer PR , Kanesa-Thanan N . A cell culture-derived MF59-adjuvanted pandemic A/H7N9 vaccine is immunogenic in adults. *Sci Transl Med*.2014;6:234ra255.
140. Khurana S , Coyle EM , Dimitrova M , Heterologous prime-boost vaccination with MF59-adjuvanted H5 vaccines promotes antibody affinity maturation towards the hemagglutinin HA1 domain and broad H5N1 cross-clade neutralization. *PLoS One*.2014;9:e95496.24755693
141. Khurana S , Verma N , Yewdell JW , MF59 adjuvant enhances diversity and affinity of antibody-mediated immune response to pandemic influenza vaccines. *Sci Transl Med*.2011;3:85ra48.
142. Galli G , Medini D , Borgogni E , Adjuvanted H5N1 vaccine induces early CD4+ T cell response that predicts long-term persistence of protective antibody levels. *Proc Natl Acad Sci U S A*. 2009;106:3877–3882.19237568
143. Jackson LA , Campbell JD , Frey SE , Effect of Varying Doses of a Monovalent H7N9 Influenza Vaccine With and Without AS03 and MF59 Adjuvants on Immune Response: A Randomized Clinical Trial. *JAMA*.2015;314:237–246.26197184
144. Treanor J , Wright PF . Immune correlates of protection against influenza in the human challenge model. *Dev Biol (Basel)*.2003;115:97–104.15088780
145. Treanor JJ , Campbell JD , Zangwill KM , Rowe T , Wolff M . Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. *N Engl J Med*.2006;354:1343–1351.16571878
146. Treanor JJ , Essink B , Hull S , Evaluation of safety and immunogenicity of recombinant influenza hemagglutinin (H5/Indonesia/05/2005) formulated with and without a stable oil-in-water emulsion containing glucopyranosyl-lipid A (SE+GLA) adjuvant. *Vaccine*.2013;31:5760–5765.24075920
147. Zinckgraf JW , Sposato M , Zielinski V , Powell D , Treanor JJ , von Hofe E . Identification of HLA class II H5N1 hemagglutinin epitopes following subvirion influenza A (H5N1) vaccination. *Vaccine*.2009;27:5393–5401.19596415
148. Hoft DF , Lottenbach K , Goll JB , Priming Vaccination With Influenza Virus H5 Hemagglutinin Antigen Significantly Increases the Duration of T cell Responses Induced by a Heterologous H5 Booster Vaccination. *J Infect Dis*.2016;214:1020–1029.27443611
149. DiPiazza A , Richards KA , Knowlden ZA , Nayak JL , Sant AJ . The Role of CD4 T Cell Memory in Generating Protective Immunity to Novel and Potentially Pandemic Strains of Influenza. *Front Immunol*.2016;7:10.26834750
150. Birnbaum ME , Mendoza JL , Sethi DK , Deconstructing the peptide-MHC specificity of T cell recognition. *Cell*.2014;157:1073–1087.24855945
151. Bridgeman JS , Sewell AK , Miles JJ , Price DA , Cole DK . Structural and biophysical determinants of alphabeta T-cell antigen recognition. *Immunology*.2012;135:9–18.22044041
152. Marrack P , Rubtsova K , Scott-Browne J , Kappler JW . T cell receptor specificity for major histocompatibility complex proteins. *Curr Opin Immunol*.2008;20:203–207.18456484
153. Bhati M , Cole DK , McCluskey J , Sewell AK , Rossjohn J . The versatility of the alphabeta T-cell antigen receptor. *Protein Sci*.2014;23:260–272.24375592
154. Rossjohn J , Gras S , Miles JJ , Turner SJ , Godfrey DI , McCluskey J . T cell antigen receptor recognition of antigen-presenting molecules. *Annu Rev Immunol*.2015;33:169–200.25493333

155. Nielsen M , Lund O , Buus S , Lundegaard C . MHC class II epitope predictive algorithms. *Immunology*.2010;130:319–328.20408898
156. Weaver JM , Lazarski CA , Richards KA , Immunodominance of CD4 T cells to foreign antigens is peptide intrinsic and independent of molecular context: implications for vaccine design. *J Immunol*.2008;181:3039–3048.18713974
157. Lazarski CA , Chaves FA , Jenks SA , The kinetic stability of MHC class II:peptide complexes is a key parameter that dictates immunodominance. *Immunity*.2005;23:29–40.16039577
158. Lazarski CA , Chaves FA , Sant AJ . The impact of DM on MHC class II-restricted antigen presentation can be altered by manipulation of MHC-peptide kinetic stability. *J Exp Med*. 2006;203:1319–1328.16682499
159. Robertson JM , Jensen PE , Evavold BD . DO11.10 and OT-II T cells recognize a C-terminal ovalbumin 323–339 epitope. *J Immunol*.2000;164:4706–4712.10779776
160. Sant AJ , Chaves FA , Jenks SA , The relationship between immunodominance, DM editing, and the kinetic stability of MHC class II:peptide complexes. *Immunol Rev*.2005;207:261–278.16181342
161. Ryan KR , McNeil LK , Dao C , Jensen PE , Evavold BD . Modification of peptide interaction with MHC creates TCR partial agonists. *Cell Immunol*.2004;227:70–78.15051516
162. Morris GP , Allen PM . How the TCR balances sensitivity and specificity for the recognition of self and pathogens. *Nat Immunol*.2012;13:121–128.22261968
163. Birnbaum ME , Dong S , Garcia KC . Diversity-oriented approaches for interrogating T-cell receptor repertoire, ligand recognition, and function. *Immunol Rev*.2012;250:82–101.23046124
164. Evavold BD , Sloan-Lancaster J , Allen PM . Tickling the TCR: selective T-cell functions stimulated by altered peptide ligands. *Immunol Today*.1993;14:602–609.8305133
165. Sloan-Lancaster J , Allen PM . Altered peptide ligand-induced partial T cell activation: molecular mechanisms and role in T cell biology. *Annu Rev Immunol*.1996;14:1–27.8717505
166. Grakoui A , Donermeyer DL , Kanagawa O , Murphy KM , Allen PM . TCR-independent pathways mediate the effects of antigen dose and altered peptide ligands on Th cell polarization. *J Immunol*.1999;162:1923–1930.9973460
167. Daniel C , Grakoui A , Allen PM . Inhibition of an in vitro CD4+ T cell alloresponse using altered peptide ligands. *J Immunol*.1998;160:3244–3250.9531280
168. Sloan-Lancaster J , Steinberg TH , Allen PM . Selective activation of the calcium signaling pathway by altered peptide ligands. *J Exp Med*.1996;184:1525–1530.8879224
169. Rabinowitz JD , Beeson C , Wulfig C , Altered T cell receptor ligands trigger a subset of early T cell signals. *Immunity*.1996;5:125–135.8769476
170. Shorter SK , Schnell FJ , McMaster SR , Pinelli DF , Andargachew R , Evavold BD . Viral Escape Mutant Epitope Maintains TCR Affinity for Antigen yet Curtails CD8 T Cell Responses. *PLoS One*.2016;11:e0149582.26915099
171. Sloan-Lancaster J , Evavold BD , Allen PM . Th2 cell clonal anergy as a consequence of partial activation. *J Exp Med*.1994;180:1195–1205.7931057
172. Sloan-Lancaster J , Evavold BD , Allen PM . Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells. *Nature*.1993;363:156–159.8483498
173. De Groot AS , Ardito M , Terry F , Low immunogenicity predicted for emerging avian-origin H7N9: implication for influenza vaccine design. *Hum Vaccin Immunother*.2013;9:950–956.23807079
174. DiPiazza A , Richards K , Poulton N , Sant AJ . Avian and Human Seasonal Influenza Hemagglutinin Proteins Elicit CD4 T Cell Responses That Are Comparable in Epitope Abundance and Diversity. *Clin Vaccine Immunol*.2017;24.
175. Kedl RM , Kappler JW , Marrack P . Epitope dominance, competition and T cell affinity maturation. *Curr Opin Immunol*.2003;15:120–127.12495743
176. Busch DH , Pamer EG . T cell affinity maturation by selective expansion during infection. *J Exp Med*.1999;189:701–710.9989985

177. Goji NA , Nolan C , Hill H , Immune responses of healthy subjects to a single dose of intramuscular inactivated influenza A/Vietnam/1203/2004 (H5N1) vaccine after priming with an antigenic variant. *J Infect Dis.*2008;198:635–641.18694338
178. Nayak JL , Richards KA , Yang H , Treanor JJ , Sant AJ . Effect of influenza A(H5N1) vaccine prepandemic priming on CD4+ T-cell responses. *J Infect Dis.*2015;211:1408–1417.25378637
179. Kamlangdee A , Kingstad-Bakke B , Osorio JE . Mosaic H5 Hemagglutinin Provides Broad Humoral and Cellular Immune Responses against Influenza Viruses. *J Virol.*2016;90:6771–6783.27194759
180. Shi Y , Wu Y , Zhang W , Qi J , Gao GF . Enabling the ‘host jump’: structural determinants of receptor-binding specificity in influenza A viruses. *Nat Rev Microbiol.*2014;12:822–831.25383601
181. Herfst S , Imai M , Kawaoka Y , Fouchier RA . Avian influenza virus transmission to mammals. *Curr Top Microbiol Immunol.*2014;385:137–155.25048542
182. Webster RG , Shortridge KF , Kawaoka Y . Influenza: interspecies transmission and emergence of new pandemics. *FEMS Immunol Med Microbiol.*1997;18:275–279.9348163
183. Yoon SW , Webby RJ , Webster RG . Evolution and ecology of influenza A viruses. *Curr Top Microbiol Immunol.*2014;385:359–375.24990620
184. Naffakh N , Tomoiu A , Rameix-Welti MA , van der Werf S . Host restriction of avian influenza viruses at the level of the ribonucleoproteins. *Annu Rev Microbiol.*2008;62:403–424.18785841
185. Morens DM , Taubenberger JK , Fauci AS . H7N9 avian influenza A virus and the perpetual challenge of potential human pandemicity. *MBio.*2013;4.
186. Garcia Z , Pradelli E , Celli S , Beuneu H , Simon A , Bousso P . Competition for antigen determines the stability of T cell-dendritic cell interactions during clonal expansion. *Proc Natl Acad Sci U S A.*2007;104:4553–4558.17360562
187. Willis RA , Kappler JW , Marrack PC . CD8 T cell competition for dendritic cells in vivo is an early event in activation. *Proc Natl Acad Sci U S A.*2006;103:12063–12068.16880405
188. Kedl RM , Rees WA , Hildeman DA , T cells compete for access to antigen-bearing antigen-presenting cells. *J Exp Med.*2000;192:1105–1113.11034600
189. Ge Q , Bai A , Jones B , Eisen HN , Chen J . Competition for self-peptide-MHC complexes and cytokines between naive and memory CD8+ T cells expressing the same or different T cell receptors. *Proc Natl Acad Sci U S A.*2004;101:3041–3046.14976256
190. Sant AJ , Chaves FA , Leddon SA , Tung J . The control of the specificity of CD4 T cell responses: thresholds, breakpoints, and ceilings. *Front Immunol.*2013;4:340.24167504
191. Chinnadurai R , Copland IB , Patel SR , Galipeau J . IDO-independent suppression of T cell effector function by IFN-gamma-licensed human mesenchymal stromal cells. *J Immunol.* 2014;192:1491–1501.24403533
192. Burchill MA , Tamburini BA , Kedl RM . T cells compete by cleaving cell surface CD27 and blocking access to CD70-bearing APCs. *Eur J Immunol.*2015;45:3140–3149.26179759
193. Hudrisier D , Riond J , Mazarguil H , Gairin JE , Joly E . Cutting edge: CTLs rapidly capture membrane fragments from target cells in a TCR signaling-dependent manner. *J Immunol.* 2001;166:3645–3649.11238601
194. Hwang I , Huang JF , Kishimoto H , T cells can use either T cell receptor or CD28 receptors to absorb and internalize cell surface molecules derived from antigen-presenting cells. *J Exp Med.* 2000;191:1137–1148.10748232
195. Huang JF , Yang Y , Sepulveda H , TCR-Mediated internalization of peptide-MHC complexes acquired by T cells. *Science.*1999;286:952–954.10542149
196. Johnson LR , Weizman OE , Rapp M , Way SS , Sun JC . Epitope-Specific Vaccination Limits Clonal Expansion of Heterologous Naive T Cells during Viral Challenge. *Cell Rep.*2016;17:636–644.27732841
197. Olson MR , Chua BY , Good-Jacobson KL , Doherty PC , Jackson DC , Turner SJ . Competition within the virus-specific CD4 T-cell pool limits the T follicular helper response after influenza infection. *Immunol Cell Biol.*2016;94:729–740.27101922

198. Lambeck AJ , Nijman HW , Hoogeboom BN , Role of T cell competition in the induction of cytotoxic T lymphocyte activity during viral vector-based immunization regimens. *Vaccine*. 2010;28:4275–4282.20434555
199. Gray PM , Reiner SL , Smith DF , Kaye PM , Scott P . Antigen-experienced T cells limit the priming of naive T cells during infection with *Leishmania major*. *J Immunol*.2006;177:925–933.16818747
200. Weaver JM , Chaves FA , Sant AJ . Abortive activation of CD4 T cell responses during competitive priming in vivo. *Proc Natl Acad Sci U S A*.2009;106:8647–8652.19423666
201. Farrington LA , Smith TA , Grey F , Hill AB , Snyder CM . Competition for antigen at the level of the APC is a major determinant of immunodominance during memory inflation in murine cytomegalovirus infection. *J Immunol*.2013;190:3410–3416.23455500
202. Galea I , Stasakova J , Dunscombe MS , Ottensmeier CH , Elliott T , Thirdborough SM . CD8+ T-cell cross-competition is governed by peptide-MHC class I stability. *Eur J Immunol*. 2012;42:256–263.22002320
203. Muschaweckh A , Buchholz VR , Fellenzer A , Antigen-dependent competition shapes the local repertoire of tissue-resident memory CD8+ T cells. *J Exp Med*.2016;213:3075–3086.27899444
204. Tung J , Sant AJ . Orchestration of CD4 T cell epitope preferences after multi-peptide immunization. *J Immunol*.2013;191:764–772.23772029
205. King NJ , Thomas SR . Molecules in focus: indoleamine 2,3-dioxygenase. *Int J Biochem Cell Biol*.2007;39:2167–2172.17320464
206. Schroder K , Hertzog PJ , Ravasi T , Hume DA . Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol*.2004;75:163–189.14525967
207. Regis G , Conti L , Boselli D , Novelli F . IFN γ R2 trafficking tunes IFN γ -STAT1 signaling in T lymphocytes. *Trends Immunol*.2006;27:96–101.16380292
208. Refaelli Y , Van Parijs L , Alexander SI , Abbas AK . Interferon gamma is required for activation-induced death of T lymphocytes. *J Exp Med*.2002;196:999–1005.12370261
209. Fallarino F , Grohmann U . Using an ancient tool for igniting and propagating immune tolerance: IDO as an inducer and amplifier of regulatory T cell functions. *Curr Med Chem*.2011;18:2215–2221.21517758
210. Fallarino F , Grohmann U , Puccetti P . Indoleamine 2,3-dioxygenase: from catalyst to signaling function. *Eur J Immunol*.2012;42:1932–1937.22865044
211. Mellor A . Indoleamine 2,3 dioxygenase and regulation of T cell immunity. *Biochem Biophys Res Commun*.2005;338:20–24.16157293
212. Mellor AL , Munn DH . IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol*.2004;4:762–774.15459668
213. Munn DH . Indoleamine 2,3-dioxygenase, tumor-induced tolerance and counter-regulation. *Curr Opin Immunol*.2006;18:220–225.16460921
214. Mandi Y , Vecsei L . The kynurenine system and immunoregulation. *J Neural Transm (Vienna)*. 2012;119:197–209.21744051
215. Nayak JL , Fitzgerald TF , Richards KA , Yang H , Treanor JJ , Sant AJ . CD4+ T-cell expansion predicts neutralizing antibody responses to monovalent, inactivated 2009 pandemic influenza A(H1N1) virus subtype H1N1 vaccine. *J Infect Dis*.2013;207:297–305.23148285

Figure 1. Patterns of CD4 T cell reactivity to influenza viral proteins in mouse models of primary infection.

IL-2 EliSpot assays were used to enumerate antigen-reactive CD4 T cells from four different mouse strains at the peak of the response to infection. CD4 T cells were isolated from the spleens of C57Bl/6, BALB/c, SJL/J and HLA-DR1 transgenic mice ten days post intranasal infection with A/New Caledonia/20/1999 H1N1 virus. In the top panel the CD4 T cell distribution to each protein is represented in a pie diagram, where the responses to individual peptide epitopes were summed for the indicated proteins and each protein is represented by a different color. In the bottom panel the CD4 T cell distribution for each influenza protein relative to the protein size is indicated to normalize the abundance of epitopes recruited by CD4 T cells based on its molecular weight. Here, the sum of the cytokine EliSpots that were elicited by the epitopes in the indicated viral protein was divided by the number of amino acids in that protein to determine the CD4 T cell reactivity relative to the viral protein size.

Figure 2. Potential routes of antigen handling for provision of help by epitope-specific CD4 T cells to antigen-specific B cells after influenza infection.

Influenza virus replication is largely restricted to the lung, where diverse cell types are infected (66), releasing virions and viral proteins after cell death. Antigen-bearing respiratory dendritic cells (rDC) are mobilized after infection, upregulating CCR7. They traffic to the regional draining lymph node, where contact with T cells occurs. In addition to migratory DC, it is possible that released viral antigens or degraded antigens drain through the afferent lymphatics and are taken up, processed and presented by resident APC (step 1). After priming, a subset of CD4 T cells leaves the draining lymph node (step 2) and migrates to the lung to deliver effector function. A separate subset of CD4 T cells upregulate CXCR5 and localizes to the T-B cell border (step 3a) where they can engage antigen presenting B cells for rapid antibody production via the extrafollicular response or through the germinal center response that permits affinity maturation and establishment of memory B cells and long-lived antibody secreting cells (step 3b). During this same time period, B cells engage viral antigen presented by follicular dendritic cells (step 4) through their Ig receptor (126, 127). Our data indicate restrictions for inter-molecular help, where CD4 T cells specific for NP cannot provide help to HA-specific B cells (113). These data, as well as published work

by others demonstrating B cells that become infected after Ig-mediated uptake of virus do not survive (119), suggests that the major source of antigen available for B cell-mediated antigen presentation is free viral antigen or antigen-laden membrane fragments that drain from the lung or from dying migratory DC within the lymph node. Our data are consistent with the model that Ig-mediated uptake thus separates proteins such as NP from membrane associated HA, shown as different colors in the dotted circle within the Figure. Because of the restricted nature of B cell antigen uptake, viral antigen specific B cells present peptide epitopes limited to those derived from the antigens that are recognized by the B cell receptor. Thus, they recruit help primarily from CD4 T cells specific for the same antigen or antigens that are co-internalized with the Ig receptor (step 5).

Figure 3. Sequence comparisons of influenza HA strains.

The amino acid sequence conservation for influenza HA from seasonal H1N1 A/Brisbane/59/2007, pandemic H1N1 A/California/07/2009 and avian H5N1 A/Vietnam/1203/2004 is shown. For comparison the closer sequence relationship between the novel pH1N1 and the previous seasonal H1N1 is also shown. Yellow bars represent segments of amino acid sequence variation and blue segments denote stretches of sequence identity. Sequence files were downloaded from PubMed and conservation profiles were constructed using CLC Sequence Viewer 7 software.