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Distinct and complementary roles of CD4 T cells in protective immunity to influenza virus

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CD4 T cells play a multiplicity of roles in protective immunity to influenza. Included in these functions are help for high affinity antibody production, enhancement of CD8 T cell expansion, function and memory, acceleration of the early innate response to infection and direct cytotoxicity. The influenza-specific CD4 T cell repertoire in humans established through exposures to infection and vaccination has been found to be highly variable in abundance, specificity and functionality. Deficits in particular subsets of CD4 T cells recruited into the response result in diminished antibody responses and protection from infection. Therefore, improved strategies for vaccination should include better methods to identify deficiencies in the circulating CD4 T cell repertoire, and vaccine constructs that increase the representation of CD4 T cells of the correct specificity and functionality.

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Key issues and persistent challenges in understanding the contributions of CD4 T cells in protective immunity to influenza

Understanding the precise role of CD4 T cell immunity to influenza infection and vaccination is achievable but challenging. A key distinction between CD4 T cells and other cells in the adaptive immune response, such as B cells and CD8 T cells, is the multiplicity of functions that they contribute to protective influenza immunity. In order to potentiate CD4 T cell immunity for influenza, it is important to factor in three considerations: the diversity, specificity and abundance of influenza-specific CD4 T cells in human populations, the discreet functional activities that CD4 T cells convey in response to infection or vaccination, and finally, the CD4 T cell functions that are limiting factors in the efficacy of protection from influenza infection or responses to vaccination. In this review, we will discuss each of these issues, focusing whenever possible on the most recent studies and those derived from analyses of humans.

Abundance and specificity of human influenza-specific CD4 T cells

Recent studies have sought to quantify and characterize the repertoire of circulating CD4 T cells from human subjects with specificity towards influenza antigens [1–8]. In general, these studies have found that in most healthy adults, there are detectable influenza-specific CD4 T cells, but that the abundance is highly variable [1,3,5,7,9]. With the increased interest in the design of universal influenza vaccines [10,11], there has been particular interest in candidate epitopes that would elicit broadly cross-reactive CD4 T cells that are genetically conserved across viral strains [12–14]. In healthy donors, there is prominent reactivity in influenza CD4 T cells specific for the internal virion proteins M1, NP [5–7,9], and polymerase [1,4]. HA-reactive CD4 T cells have also been reported to be abundant in the memory compartment of many individuals [2–4,9,15], and these may be particularly important for provision of help for neutralizing antibody responses [16,17•,18•,19,20]. In humans, reactivity to HA is enriched for specificities in the more highly conserved HA2 domain [3], allowing candidate epitopes in this region to be utilized in universal vaccine efforts. CD4 T cells specific for M1, NP, polymerase proteins and highly conserved segments of HA are likely to be the major specificities elicited in response to infection with heterologous or novel potentially pandemic strains [2,21–27] and may contribute to attenuating the course of infection. The degree of cross reactivity in CD4 T cells elicited in response to the novel 2009 potentially pandemic strain is likely to have contributed to the mild course of disease observed in many subjects, despite a lack of cross-reactive neutralizing antibody.

The method used to assess CD4 T cell specificity and abundance is important to consider in interpretation of any studies that quantify influenza reactivity. First, it is essential to note whether or not specificity and dominance are determined directly *ex vivo* [1,3–5,7,8,15] or after expansion [6,9]. If after expansion, whether all potential

epitopes or a subset of peptides or antigens were used is also important. Complex or uneven mixtures of antigens [6] or pre-selected epitopes based on predictive algorithms [1,7], or particular MHC types [1,6,8] will bias the generality of the conclusions. In addition, short term *in vitro* activation of T cells may lead to expansion of selective specificities or functions [28]. Direct *ex vivo* studies with unbiased and overlapping peptide sets, coupled with intracellular cytokine staining or cytokine EliSpots allows the most specificities to be quantified. These methods do have the caveat that quantification will be limited to CD4 T cells that produce known secreted mediators. Other cytokine-independent approaches, such as use of peptide-MHC multimers [8] overcome this deficiency, but this method only samples a fraction of the repertoire based on MHC restriction, and detects only those CD4 T cells with relatively high avidity for their ligand [29].

Functional contribution of CD4 T cells to protective immunity to influenza

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 future infection [30]. The antigen-specific cognate interactions between CD4 T cells and B cells promotes both the rapid extrafollicular and the later-evolving germinal center response that drives immunoglobulin affinity maturation and long lived B cell immunity (reviewed in [31,32]). Recent data has shown a close correlation between the elicited antibody response to vaccination and the emergence of cells in peripheral blood with markers reminiscent of follicular helper CD4 T cells (reviewed in [33–35]), arguing that CD4 T cell help for vaccine responses can be tracked soon after vaccination [36].

CD4 T cells also play key functions distinct from delivery of B cell help. They are important for protective immunity conveyed by CD8 T cells, enhancing priming, expansion and establishment of long-lived memory [37]. Intriguingly, a recent study [38•] provided strong evidence that CD4 T cell help is critical for development of effector CD8 T cells that have cytotoxic potential, and express molecules important for homing and extravasation, all features required for protective immunity to influenza. CD4 T cell help for CD8 T cells during priming may involve sequential contact with discreet subsets of antigen presenting cells that culminates in localized delivery of help [39]. At the site of infection, CD4 T cells also promote the positioning of memory CD8 T cells in the infected respiratory tract [40]. Another discreet function of CD4 T cells that has been increasingly validated is cytotoxicity, which has the potential to directly eliminate infected cells (reviewed in [41–43]). In addition to enhancing adaptive immunity, a potentially critical function of influenza-specific CD4 T cell memory is the ability to accelerate recruitment of innate effectors to the lung. The role of the early innate response to infection is well documented (reviewed in [44]) and recent studies have shown influenza-specific CD4 T memory promotes this early response, and blunts virus infection [45,46]. A final activity of influenza specific CD4 T cells, identified primarily in animal models, is regulatory and repair function. These activities are contributed by diverse cell types including conventional Treg, as well as IL-10-producing or amphiregulin-producing cells. Such CD4 T cells can diminish the damage associated with the profound inflammatory responses [47–50] that occur upon infection with highly pathogenic strains of influenza. In general, although influenza-specific CD4 T cells are enriched in IFN-γ production and thus can be considered 'Th1-like', cytokine production in humans is tremendously complex [27,51], relative to what is typically observed in mice, which have more limited frequencies of exposure to influenza antigens. Therefore, assigning functional activities of influenza-specific human CD4 T cells to single cytokines is quite difficult. Below, we detail recent studies on two of the important and distinct functional subsets of influenza-specific CD4 T cells: those with cytolytic function and those important for enhancing high affinity antibody responses.

Cytolytic CD4 T cells

There has been increasing appreciation of a distinct subset of CD4 T cells with cytolytic function (reviewed in [41–43]). Influenza-specific CD4 T cells with cytotoxic potential, first identified after *in vitro* culture, have now been detected directly *ex vivo*. In many respects, cytotoxic CD4 T cells resemble CD8 T cells both in terms of key transcriptional regulators [52•], efficiency of killing [53] and in primary cytotoxic mechanisms involving perforin and granzymes [41,43]. Both IL-2 and inflammatory signals, abundant in responses to viral infections, are thought to be central elements that promote the cytolytic potential of CD4 T cells [54,55]. Recent studies in human peripheral blood CD4 T cells have identified the transcription factor HOBIT ('Homolog of Blimp-1 in T cells') as a unique identifier of cells expressing granzymes, perforin and other markers linked to cytolytic function [56]. Until recently, CD4 T cells with cytotoxic function have been quantified by expression of stored cytolytic mediators, such as granzyme, requiring that cells be permeabilized prior to analyses. However, recently, cell surface markers, such as CRTAM (class I — restricted T cell — associated molecule) [52•] and the natural killer cell marker NKG2C/E [57•] have been defined that have the potential to quantify and permit isolation of intact cells with cytotoxic potential. The role of cytolytic CD4 T cells in influenza immunity has been supported by the prominence of this phenotype in the infected lung [41,54,57•] and has been found as a correlate of protection in human challenge studies [5].

One intriguing unresolved question is whether cytolytic CD4 T cells perform a unique function distinct from CD8 T cells. Because of their similarity in function and development, why does the host need the apparent redundant mechanism for cell mediated cytotoxicity? Two possible non-mutually exclusive possibilities can be envisioned. Cytotoxic CD4 T cells may serve as a complementary mechanism, eliminating infected cells at selected sites within the lung, perhaps controlled by a unique array of lung positioning molecules [58,59]. Recent studies have revealed the diversity of infected and antigen bearing cells detectable in the lung early after infection [60] that may be located at distinct sites in the respiratory tract. Alternatively or additionally, CD4 T cells may be serve as a failsafe mechanism when CD8 T cell epitopes are lost from circulating influenza strains [61], which may be less likely to occur in CD4 T cells due to their broader epitope specificity.

Follicular helper cells

Because of the importance of neutralizing antibody in protection against influenza [62–64], there has been increasing emphasis on the subset of CD4 T cells termed T follicular helper cells (Tfh) that promote the generation and maintenance of the germinal center reaction and the production of high affinity, class-switched antibody. Tfh are characterized by the expression of the chemokine receptor CXCR5, promoting localization to the B cell zone of secondary lymphoid organs. Here, they also express high levels of ICOS and PD-1 ([65,66] and reviewed in [35,67,68]). The transcriptional repressor Bcl-6 directs lineage commitment [69,70]. Cytokines, including IL-6 and IL-21 [71], and T cell receptor (TcR) signal strength [72–74,75•] have all been shown to influence Tfh differentiation. Interestingly, beyond TcR signal strength and cytokine milieu, particular dendritic cell subsets may also be critical in priming of Tfh. Lung CD11b+ migratory dendritic cells (cDC2) have been implicated in priming following intranasal immunization [76•], and late appearing APCs were identified in the Tfh response following influenza virus infection [77].

The relationship between circulating CXCR5+ cells and the Tfh within secondary lymphoid organs was uncertain until relatively recently. In circulation, CXCR5+ cells have lower expression of Bcl-6 as well as ICOS and PD-1 [78,79•,80]. However, despite these phenotypic differences, circulating CXCR5+ CD4 T cells demonstrate a superior capacity to help B cells upon activation [78,80,81]. Circulating CXCR5+ cells can be further distinguished by expression of CCR6, CXCR3, ICOS and PD-1, with helper activity concentrated within the $CXCR3^{-}$ cells that are PD1+ and/or ICOS+[36,78,81,82,83•].

There has been much progress in understanding the human Tfh response following influenza vaccination. Increases in Tfh expressing activation markers (ICOS+ or ICOS+ PD1+) correlates with the magnitude [36,82,83•,84] and avidity [85•] of influenza-specific antibody. Circulating Tfh cells identified after vaccination appear to be clonally related to those within the germinal center and transition to a more quiescent phenotype at memory [79•,86]. While circulating influenza-specific Tfh have reactivity to multiple influenza proteins [85•], they are relatively enriched within cells specific for the HA compared to the NP protein [15]. Although the mechanisms underlying this disparity are unknown, studies to characterize whether there are differences in Tfh frequency between epitopes within the more conserved and the more divergent portions of HA could help to clarify whether repeated boosting by vaccination or infection influences Tfh abundance.

The role of antigen specificity in CD4 T cell help for antibody response is a critical parameter in predicting and boosting the capacity of the human host to produce protective antibody. Recent studies in both animal models of infection [18•,20,87] and human vaccination studies [16,17•] suggest that the most effective help for antibody responses occurs when the antigen specificity of the CD4 T cells matches that of the B cell. This obligate linkage likely reflects the nature of the antigen taken up by the immunoglobulin receptor, and the resulting MHC class II: peptide complexes that are displayed at the cell surface of the antigen specific B cells that recruit CD4 T cell help in secondary lymphoid tissue. This constraint likely limits the CD4 T cell help available for antibody responses to novel avian strains of influenza.

The challenge: identification of the CD4 T cells subset(s) that are a limiting factor for human protective immunity to influenza virus

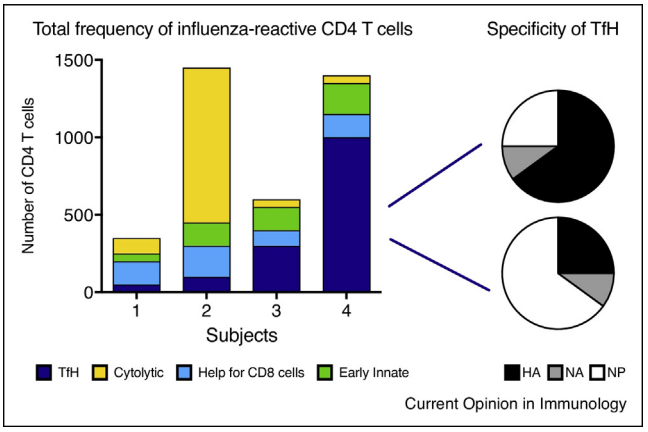
It is clear that the presence of neutralizing antibody to influenza is the best correlate of protection. However, the ability of influenza virus to mutate and to deviate from serum-mediated protection and the resulting high burden of seasonal influenza infection indicates that current strategies to induce sterilizing immunity are inadequate. Thus, there is a need to enhance protective immunity by more effectively engaging the cellular arm of the immune response. Despite this need, a significant challenge is definition of the CD4 T cells whose representation is deficient and needs to be boosted in humans by vaccination. In studies of human immunity to influenza, one of the most challenging issues is identification of cells that will be drawn into and participate in the response to infection or vaccination. For example, the vast majority of studies that have identified correlates of influenza-specific CD4 T cells with the elicited antibody responses have done so only through examination of cells that have increased their representation after vaccination [16,17•,36,82,84,88,89]. Very few correlates have been identified within the circulating CD4 T cell repertoire prior to vaccination or infection. One human influenza challenge study did correlate

levels of pre-existing cytotoxic CD4 T cells with protection, but this subset was identified after *in vitro* expansion [5]. More sensitive detection of additional markers such as chemokine receptors expressed by influenza-specific CD4 T cells, coupled with early sampling times (e.g. 1–3 days post vaccination or infection) and human challenge studies [90,91,92•,93] would likely provide insight into the circulating memory CD4 T cell subsets that are recruited into the response and whose presence in peripheral blood of humans either positively or negatively correlates with protective immunity to influenza. As knowledge is gained in these issues, we will have more of the needed insight into the most critical deficiencies in the influenza-specific CD4 T cell repertoire and with this, the needed framework for improved vaccine strategies.

Many of the *in vivo* studies performed to date that have implicated particular effector functions of CD4 T cells have utilized animal models where the contribution of CD4 T cells to immunity has been demonstrated either using adoptive transfer strategies to introduce a CD4 T cell with dominant function into a naïve host or where functionality of the CD4 T cells can be eliminated or promoted by genetic means [94–96]. Extrapolating these types of studies to humans is difficult because of complex pre-existing immunity that accumulates over a lifetime of exposure. To be able to exploit the contribution of CD4 T cells toward protective immunity or more robust responses to vaccination, it is essential to quantify the functional activities of CD4 T cells that are limiting in protective immunity to influenza (see Figure 1). Do most people need more cytotoxic CD4 T cells or is more tissue resident memory needed? Is CD4 T cell help for rapid antibody responses to infection limiting for most people? Do only a small number of influenza-specific cells in humans have the capacity to be recruited to the lung early after infection and quickly mobilize the innate response? The unique and opposing transcription factor expression in cytolytic CD4 T cells and Tfh cells indicates that these subsets are reciprocally regulated [43,97•] and vaccine strategies may need to focus the elicited response on one subset or the other. Understanding these issues will help direct the priorities in design and composition of more effective vaccines that most effectively establish protective immunity.

From the studies of human responses to vaccination and animal models of infection, it is clear that CD4 T cells of the correct functional subset [82,83•,84,85•] and epitope specificity [16,18•,20,87,98] can be a limiting factor in eliciting neutralizing antibody. Evidence from animal models has supported the possibility that more abundant CD4 T cells of the correct specificity can enhance antibody production during the course of infection [18•]. Such early antibody responses, even of non-neutralizing HA or NA specificities, could blunt the course of infection, through cellular and Fc-receptor-based mechanisms [98–100,101•,102•]. Vaccine

Figure 1



The range of abundance and functional capacity of influenza-specific human CD4 T cells within different subjects. Existing data from PBMC from healthy donors indicates a broad range in frequency of influenza-specific CD4 T cells. Shown here for illustration are the differences depicted as bar heights, reflecting the total abundance of influenza specific CD4 T cells. The frequency of each functionally distinct subset of cells are indicated by different colored segments within each bar. This theoretical representation reflects the extreme view that there are non-overlapping subsets of CD4 T cells that convey each of the indicated functions. Thus far, only the strict dichotomy between cytolytic cells and Tfh cells is supported by their unique and opposing transcription factors [97•]. In the example shown, subject 2 and subject 4 have similar frequencies of influenza-specific CD4 T cells, but may be poised differently for protective immunity. Subject 2, having more abundant cytolytic cells might be best protected from infection by eliminating infected cells, while subject 4, with high frequency of Tfh cells, would exhibit the more robust antibody response. The right pie diagrams illustrate that in provision of help for B cell responses, in subject 4, the antigen specificity of the CD4 T cells is critically important in providing help [16,17•,18•]. The neutralizing antibody response to vaccination would likely vary, depending on the abundance of HA-specific Tfh cells drawn into the response, with different outcomes depending on whether subject 4 had high (top) or low (bottom) levels of HA-specific CD4 T cells. It is not yet known whether other functions of CD4 T cells, such as recruitment of innate effectors to the lung, or cytotoxicity, track with their antigen specificity.

strategies that populate the host with CD4 T cells that are poised to become Tfh cells of the desired protein specificity, matching the B cell specificity, may thus lead to antibody responses that can be lead to either sterilizing immunity and/or attenuated disease. Greater peripheral Tfh following adjuvanted vaccination [103], use of high dose influenza vaccination in the elderly [104•], and strategies that target protein vaccines to cell surface proteins such as Clec9a [105], or particular dendritic cell subsets [106] suggest that it may be possible to use alternative vaccination strategies to optimize Tfh priming, which may foster stable Tfh memory that can promote future protective antibody responses.

The possibility of enhancing protection from influenza infection through amplification of T cell responses has

been supported by recent vaccine and challenge studies in humans [90,91]. Use of virus vectors to expand T cells specific for M1 and NP, without elicitation of antibodies, was associated with a lower infection frequency, virus shedding and diminished symptomatology when a challenge virus was administered [90]. Consistent findings were observed when multi-epitope vaccines were similarly tested [91]. It is likely that these types of experimental challenge models [92•,93], coupled with strategies to selectively promote expansion of CD4 T cells with the desired functionality and specificity could provide critical new insight into the most limiting protective functions of CD4 T cells.

The roles of specific viral antigens as targets of vaccination, different innate activators, and antigen persistence are increasingly better understood [107–110,111•]. C-lectin receptors are becoming increasingly used to both target antigens to APC and activate intracellular signaling that collectively leads to initiation of particular effector functions (reviewed in [112–114]). For example, coupling antigens to antibodies specific for Clec9a can promote establishment of Tfh cells [105], whereas an antigen conjugate directing uptake Dectin 1 can enhance recruitment of Th17 [115]. Also, because virus infection, known to induce long-term protective memory, has been shown to lead to long-lived reservoirs of viral antigen [116–120], vaccine strategies that can establish these depots may be particularly efficacious. Finally, the increasing recognition of the importance of local immunity in the respiratory tract suggests that efforts to promote resident CD4 T cells in the lung, through the use of intranasal attenuated viruses or synthetic vaccines, may promote establishment of memory cells with enhanced ability to home back to the lung after infection. Priming of CD4 T cells with lung-derived dendritic cells, either in isolation [121] or through infection or administration of attenuated vaccines [30,108] is known to enhance homing potential of CD4 T cells to the lung, support this view.

Collectively, recent advances have provided much of the insight needed to initiate cross protective immunity at the systemic level as well as the site of infection. The control of CD4 T cell fate decisions that regulate generation and expansion of discreet functional subsets of CD4 T cells are complex and multilayered. The regulatory events likely involve distinct sets of APC, T cell receptor-derived signals, cytokines and innate activators. The major challenge will be to design the appropriate vaccination and challenge studies to reveal and ultimately correct the most critical deficits in CD4 T cell functions in human populations.

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- •• of outstanding interest

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