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Influenza A virus-specific CD8⁺ T-cell responses: from induction to function

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Abstract

Seasonal influenza virus infection is a leading cause of illness and mortality in young children and the elderly each year. Current influenza vaccines generate protective antibody responses; however, these must be given annually to provide protection against serologically distinct viruses. By contrast, CD8⁺ T cells are capable of recognizing conserved antigenic determinants within the influenza virion and, as such, may provide protection against a number of variant strains of the virus. CD8⁺ T cells play a critical key role in controlling and resolving influenza virus infections via the production of cytokines and cytolytic mediators. This article focuses on the induction of the influenza-specific CD8⁺ T-cell response and how these cells acquire and maintain effector function after induction. Moreover, we discuss how cytotoxic T-lymphocyte function correlates with protection following vaccination.

Keywords

cytotoxic T lymphocytes; dendritic cells; influenza; vaccination; virus

Worldwide, seasonal influenza virus infection is a major cause of morbidity and mortality. Protection from infection requires annual vaccination since influenza viruses rapidly mutate – a process known as ‘antigenic drift’. Current vaccines are very effective and act predominantly through the generation of strain-specific antibodies. However, this form of vaccination does not provide protection from infection with serologically distinct strains of

influenza A virus (IAV). CD8⁺ T cells or cytotoxic T lymphocytes (CTLs) play an important role in the control of virus infections and tumors. CTLs recognize short, pathogen-encoded peptides on the surface of infected cells presented on MHC class I molecules. These sequences are normally associated with conserved regions of internal viral proteins and, as such, are likely to be conserved between different strains of virus (including pandemic strains). Although there is evidence to suggest that immune pressure encourages mutations in the IAV genome that result in the loss of CTL recognition [1,2], a number of dominant CTL epitopes have remained unchanged throughout the evolution of the virus. Therefore, the generation of a broadly crossreactive CTL-based vaccine remains a viable vaccine option for the prevention/amelioration of future pandemics. However, we must first gain a better understanding of how CTL responses are induced and how their function is maintained in order to produce vaccines that induce potent and highly functional CTL responses.

In previous years, we have extensively reviewed CD8⁺ T-cell immunodominance hierarchies, CD8⁺ T-cell memory, T-cell response repertoire and IAV-induced immunopathology [3–5]. This article focuses on recent studies investigating the induction and acquisition of CTL function following IAV infection. We will also touch on how the function of the IAV-specific CTL response correlates to protection following vaccination.

Dendritic cell migration & induction of the influenza-specific CD8⁺ T-cell response

Virus infection in nature largely occurs via the skin or at mucosal surfaces. At these sites of infection, virus and/or viral antigens must be transported to secondary lymphoid organs (SLO) for the initiation of the adaptive immune response. Interestingly, both the role of transporting viral antigens from the site of infection and presentation of antigenic determinants to naive CD8⁺ T cells in the SLO is largely carried out by dendritic cells (DCs). DCs exist as two general populations, tissue-resident (e.g., migratory) or SLO-resident (e.g., nonmigratory) DCs. Tissue-resident DCs continually sample their environment for the presence of invading pathogens. Upon recognition of invading pathogens, these cells undergo a developmental program that involves maturation, the upregulation of costimulatory molecules and trafficking to SLOs. For example, skin-resident DCs or Langerhan cells carry herpes simplex virus (HSV) antigens to the skin-draining lymph nodes (LNs) after infection of the skin. These cells act as a courier, delivering an antigen from the site of infection to the draining LNs, where it is presented to a naive HSV-specific CD8⁺ T cells [6]. This section of the article will focus on recent studies examining the trafficking of DCs from the lung to the lung-draining LNs (i.e., mediastinal LNs [MedLNs]) after influenza infection. Furthermore, we will examine the importance of migratory and nonmigratory DCs in the generation of the IAV-specific CTL responses.

DC migration following intranasal infection with IAV

Previous studies have indicated that lung-resident DCs traffic from the lung to the lung-draining LNs (MedLN) following intranasal fungal infection [7]. Legge *et al.* demonstrated that intranasal administration of carboxyfluorescein succinimidyl ester (CFSE) fluorescently tagged lung-resident DCs, or respiratory (r) DCs, which could be followed after subsequent IAV infection [8]. These experiments demonstrated that rDCs migrated to the MedLNs rapidly after IAV infection, peaking in number at approximately 18 h postinfection. Interestingly, this migration was transient and dissipated by 48 h, despite the continued presence of virus in the lungs [8]. Further studies by this group have indicated that the rate of migration also depends on the strain of IAV used for infection. Yoon *et al.* demonstrated that both the rate and quantity of migrating rDCs differed between IAV/Japan and IAV/PR8

strains of the virus – IAV/PR8 infection resulted in delayed and decreased trafficking of rDCs to the LNs [9]. This difference in rDC migratory behavior also corresponded with differences in antigen presentation in the MedLNs [9]. Taken together, these data suggest that DC migration directly correlates with antigen presentation in the LNs. However, these data are controversial as Belz *et al.* [10] and Lay *et al.* [11] have demonstrated that antigen presentation peaks at approximately 3 days post-IAV infection in the MedLN and persists for an additional 1–2 weeks. Therefore, a discrepancy exists between rDC migration and presentation of antigen in the MedLNs, which is unlikely to be accounted for by differences in mouse and IAV strains. These data indicate that the rDCs trafficking to the LNs may not be the same cells that present the antigen to naive IAV-specific CTLs. For example, in HSV infection, Langerhan cells that transport viral antigen from the skin to the skin-draining LNs play little to no role in presenting this antigen to naive CTLs [6]. Instead, these cells merely ferry antigens to SLO-resident CD8 α^+ DCs, which in turn, present these antigenic determinants to naive CTLs [6]. Similarly, intranasal infection with IAV results in robust replication of the virus at a peripheral site of infection – the lung. Therefore, it is critically important to examine and identify DC subsets that play a role in transporting antigens to the SLO, and in antigen presentation to naive IAV-specific CTLs in the lung-draining LNs.

Identification of DC subsets that are required for inducing the IAV-specific CTL response

The data presented previously suggest that migratory/lung-resident DCs play a role in transporting viral antigen from the site of infection to the SLO, where this antigen is ‘passed off’ to SLO-resident DCs for presentation to naive CD8 $^+$ T cells. However, recent evidence suggests that migratory rDCs are not merely antigen ferries. In fact, rDCs appear to play a direct role in the induction of IAV-specific CTL responses. By depletion of SLO-resident CD8 α^+ DCs, Belz *et al.* demonstrated that these cells were only responsible for approximately 50% of antigen presentation to IAV nucleoprotein (NP)₃₆₆-specific T-cell hybridomas [10]. Moreover, these studies demonstrated that CD11b $^+$ DCs that migrated from the lung to the MedLNs following IAV infection could also present antigens to naive CTLs [10]. More recent studies have lent support to this idea. Geurts van Kessel *et al.* demonstrated that migratory DCs not only present antigen to naive IAV-specific CTL *in vitro*, but also play a critical role in generating CTL responses [12]. In these studies, CD11b $^+$ migratory DCs were depleted using a novel gene knock in approach, making use of the diphtheria toxin receptor under control of the langerin promoter. Thus, mice treated with diphtheria toxin exhibited depletion of langerin $^+$ /CD11b $^+$ migratory DCs. IAV infection of diphtheria toxin-treated mice revealed a profound systemic disease, as measured by enhanced weight loss, reduced IAV-specific CTL responses and dramatically delayed viral clearance [12]. These data suggest that migratory DCs play a significant role in the generation of the IAV-specific CTL response; however, it is difficult to determine from these studies whether this phenotype is due to a reduced direct antigen presentation by migratory DCs to naive CTLs or a result of reduced antigen trafficking to the MedLNs.

Although activated CD11b $^+$ DCs were not originally thought to migrate to the MedLNs upon activation [13], they have recently been shown to accumulate there following IAV infection [14]. These cells contain high levels of IAV antigen and express costimulatory molecules [14]; therefore, it is reasonable to assume that they may play a role in generating the IAV-specific CTL response. Concurrent depletion of both langerin $^+$ and CD11b $^+$ DCs resulted in a reduction in the total number of IAV-specific CTLs in the MedLNs [14]. These data suggest, but do not conclusively demonstrate, that CD11b $^+$ migratory DCs might also play a role in the generation of IAV-specific CTL responses.

Plasmacytoid DCs reside in the naive lung and LNs, and are characterized by expression of B220 and mPDCA-1, and intermediate expression of CD11c [15]. Functionally, these cells are renowned for their ability to produce type I interferons in response to virus infection [16,17]. Interestingly, although recent evidence suggests that plasmacytoid DCs are also able to present antigens to naive CTLs [18,19], they do not appear to play a role in the generation of the IAV-specific CTL response after intranasal IAV infection [12,20].

Role of DCs in the accumulation of IAV-specific CTLs in the lung

Both lung-resident and SLO-resident DCs appear to play roles in the induction of T-cell immunity to influenza; however, recent data suggest that DCs may also play a role in the maintenance and accumulation of IAV-specific CTLs in the lung and lung airways. McGill *et al.* demonstrated that, following the induction of the CTL response, depletion of airway DCs (aDCs) decreased the number of IAV-specific CTLs in the lung, delayed viral clearance and, ultimately, resulted in the death of aDC-depleted mice [21]. Furthermore, the role of DCs in maintaining the CTL response in the lung was dependent on cell–cell contact, MHC I and viral antigen. Together, these data indicate that aDCs maintain CTLs in the lung by delivering direct signals through the T-cell receptor and providing other unique DC-mediated interactions.

It has recently been demonstrated that TNF/inducible nitric oxide synthase-producing DCs (tip-DCs) are critical for the accumulation of IAV-specific CTL responses in the lungs, and that accumulation is dependant on CC chemokine receptor (CCR)2. In these studies, CCR2-deficient mice exhibit a profound absence of tip-DCs in the lungs following IAV infection, correlating with a dramatic decrease in the number of IAV-specific CTLs in the airways [22]. Moreover, IAV-specific CTL responses were restored in CCR2-deficient mice by adoptive transfer of tip-DCs from wild-type IAV-infected donors. Taken together, these data suggest that DCs are not only important for induction of the CTL response in the lung-draining LNs, but also for the maintenance and effector function of these cells in infected tissues.

In summary, migratory and SLO-resident DCs are required for the generation and maintenance of the IAV-specific CTL response. Each plays a role in inducing the CTL response; however, it is the DCs that accumulate in the lung that are critical for maintaining the response once the CTLs emigrate from the LN to the site of infection. Although multiple DC populations can induce naive IAV-specific CTLs, important questions that remain unanswered include:

- Are CTLs induced by different DCs functionally equivalent?
- Do they acquire effector functions (i.e., cytokine production and cytolytic activity) with different kinetics?
- Do they have a similar ability to clear infected host cells from the lung?
- Do they establish equivalently persistent long-lived memory populations?

The answers to these questions will give us a better understanding of how the initial antigenic encounter can influence the functional capacity of influenza-specific CTL responses.

Acquisition of CTL effector function in response to influenza infection

In response to the presentation of virally derived peptides by DCs, naive virus antigen-specific CD8⁺ T cells undergo a program of division and differentiation that results in the acquisition of effector functions and facilitates viral clearance [4]. Acquired functions

include the production of cytotoxic molecules of the granzyme (Grz) family of serine proteases and the pore-forming protein perforin (Pfp) [23]. Pfp and Grz are packaged in granules and delivered to infected cells by exocytosis, where they act synergistically to trigger caspase-dependent and -independent apoptotic pathways. Activated or effector CTLs also secrete proinflammatory cytokines that, in the context of an IAV infection, act to limit virus replication and aid in the resolution of infection [24]. After successful elimination of the invading pathogen, effector CTLs decrease in number and form a stable memory pool. Interestingly, pathogen-specific memory CTLs maintain the capacity to rapidly produce cytokines and cytolytic molecules after secondary exposure to their cognate antigen. This section focuses on how influenza-specific CTLs acquire effector function after infection and the potential mechanisms involved to maintain the functional capacity of these cells into memory.

In the well-characterized C57BL/6 (B6) mouse model of IAV infection, CTL proliferation peaks at approximately 10 days postinfection, coinciding with the timing of viral clearance [4]. Studies of the CTL response to IAV infection have focused mainly on the immunodominant H-2D^b epitopes NP₃₆₆₋₃₇₄ and acid polymerase (PA)₂₂₄₋₂₃₃. Interestingly, NP₃₆₆₋₃₇₄ and PA₂₂₄₋₂₃₃ CTLs are codominant following primary IAV infection, whereas NP₃₆₆₋₃₇₄ CTL numbers are five- to ten-fold higher following secondary infection [25–27]. Importantly, stimulation with the cognate peptides results in a higher proportion of PA₂₂₄₋₂₃₃ CTLs secreting multiple effector molecules (i.e., IFN- γ , TNF- α and IL-2) [28,29]. However, these differences do not alter the overall cytolytic activity of each population: *in vitro* and *in vivo* killing is equivalent following primary and secondary infection [25,30]. It is currently unclear what governs the differential acquisition of effector function in these cells. This section of the article focuses on how CTLs progress from quiescent, naive precursors and acquire and maintain effector CTL function following activation.

The protein perforin, GrzA and B are the most abundant cytotoxic molecules found in CTL granules, suggesting their importance in the cytolytic functions of CTLs [31]. However, the role of GrzA and B in clearance of IAV infections is far from clear. While the extent of GrzB production is a good correlate of immunity against IAV infection in humans [32,33], *grzA* and *B* double-deficient mice clear IAV infection with the same kinetics as wild-type mice. Furthermore, CTLs from *grzA* and *B* double-deficient mice show only a slightly reduced ability to kill virus-infected target cells *in vitro* [34]. Taken together, these data suggest that other molecules are able to functionally substitute for GrzA and B, at least in the context of IAV infection. Jenkins *et al.* demonstrate that *grzK* mRNA is produced rapidly after IAV infection, having similar kinetics to the transcription of *grzB* [34]. On this basis, the authors suggest that GrzK might facilitate killing in the absence of GrzA and B. Although Pfps and GrzB are important effector molecules produced by activated CTLs, other reports have also identified that TNF-related apoptosis inducing ligand (TRAIL)–TRAIL receptor and Fas–Fas ligand interactions may also play a role in CTL immunity to influenza virus [35,36]. However, it is currently unclear how expression of these effector molecules is regulated and how their expression is maintained in IAV-specific CTLs. Thus, it is important to further our understanding regarding how the kinetics of cytolytic molecule expression correlates with the ability of these cells to kill infected target cells.

Naive CD8⁺ T cells begin producing Grz and Pfp mRNA as early as 12 h post-IAV infection and protein as early as 2–3 days postinfection, suggesting that protein expression is controlled at the level of translation [37]. Single-cell reverse-transcriptase PCR experiments demonstrate that, following IAV infection, mRNAs encoding GrzB and K are expressed more strongly than those encoding Pfp and GrzA [34]. These findings were also interesting in that they showed that individual CTLs within a single population can display very

different effector expression profiles, despite having the same antigen-specificity (CTLs were isolated on their ability to recognize the ovalbumin SIINFEKL peptide) – each cell typically produced mRNAs encoding multiple effector molecules, with the exact combination being highly variable from cell to cell. The reasons for this heterogeneity are unclear, perhaps reflecting stochastic effects or the different experiences of individual cells, but what is clear from these data is that when we think about effector CTLs, we must bear in mind that we are not talking about a strictly homogenous population.

It is also clear from the literature that the differentiation of CTLs leading to the acquisition of some effector functions is dependent on cell division. Evidence for this was provided by Gett and Hodgkin, who used CFSE to demonstrate that the number of cell divisions was a strong predictor of cytokine expression profiles following *in vitro* stimulation of CTLs with anti-CD3 and in the presence of IL-4 [38]. This notion was supported by the work of Jenkins *et al.*, who combined CFSE staining with single-cell reverse-transcription PCR to demonstrate a positive correlation between the number of cell divisions and *grz* and *pfp* expression. Importantly, the increase in mRNA expression correlated with increased *ex vivo* cytotoxicity, as determined by chromium release assays using peptide-pulsed EL4 cells as targets, and this correlation held for eight cell divisions [39]. However, the acquisition of some effector functions is independent of cell division. Lawrence *et al.* demonstrated that while IAV-specific CTLs required cell division for production of GrzB, greater than 40% of nondivided IAV-specific CTLs could produce IFN- γ and TNF- α after stimulation with their cognate peptide [40]. This idea is supported by Brehm *et al.* who demonstrated that naive lymphocytic choriomeningitis virus (LCMV)-specific CD8⁺ T cells can also produce TNF- α and IFN- γ prior to division (i.e., as early as 1 h following *in vitro* stimulation) [41]. These studies demonstrated that TNF- α is produced by naive CD44^{low} CD8⁺ T cells, whereas IFN- γ is produced only by naive CD44^{hi} CD8⁺ T cells [41]. Haluszczak *et al.* demonstrated that these naive CD44^{hi} CD8⁺ T cells arise from homeostatic proliferation, not previous antigen encounter, and exhibit an enhanced ability to produce cytokines after stimulation relative to CD44^{low} counterparts [42]. Taken together, these data suggest that although cell division is required for the acquisition of some effector functions (i.e., GrzB production), it is not absolutely required for the production of antiviral cytokines. This regulation of cytokine expression and cytolytic molecule expression may serve to protect DCs from cytolytic killing by activated CTLs, while early cytokine production may play a role in modulating activation and antigen presentation by DCs.

Long-lived memory virus-specific CTLs retain the capacity for the rapid production of cytokines and cytolytic molecules long after the virus is cleared from the infected host [43–45]. The maintenance of this enhanced functional capacity appears to be controlled, at least in part, by epigenetic mechanisms. For example, Kersh *et al.* demonstrated that memory CTLs more rapidly demethylated the IFN- γ gene locus as compared with their naive counterparts [46]. Furthermore, mice deficient in methyl-CpG-binding domain protein (MBD)2, a protein that binds methylated DNA and represses transcription, exhibit defective effector CTLs after viral infection and a reduced capacity to generate a long-lived memory pool [47]. In agreement with these data, MBD2-deficient mice display higher viral loads following secondary LCMV infection as compared with wild-type mice [47]. More recent studies have also demonstrated that epigenetic regulation of gene expression can be governed by the presence of chemically modified histones in the promoter regions of CTL effector genes. Araki *et al.* demonstrated that human memory CTLs exhibit an enrichment of histone 3 acetylated at lysine 9 – an epigenetic mark that correlates with enhanced transcriptional activity – in the *gzmB* and *pfp* promoter regions as compared with naive CD8⁺ T cells [48]. These data suggest that while these effector gene promoter regions are inaccessible to necessary transcriptional factors in naive CD8⁺ T cells, these genes are in a ‘poised’ or ‘primed’ state in memory CTLs. It is currently unclear what mechanisms control

the expression of effector genes in IAV-specific CTLs following infection. At present, very little work exists that examines the role of epigenetic gene regulation in memory IAV-specific CTLs. The majority of the work discussed previously is derived from *in vitro* stimulation of naive CD8⁺ T cells or following infection with the model virus LCMV. How these findings relate to the acquisition and function of IAV-specific CTLs will be critical for understanding how these cells maintain their function into memory and how highly reactive memory cells can be elicited after vaccination.

CTL vaccines & IAV

Highly effective seasonal influenza vaccines are known to stimulate strong neutralizing antibody responses that target antigenically matched hemagglutinin and neuraminidase proteins. Antigenic drift leads to an accumulation of mutations on these surface glycoproteins over time, which results in a loss of antibody recognition as a result of immune pressure. As such, the composition of seasonal influenza vaccines is reviewed annually and modified by the WHO when appropriate, taking into account currently circulating viruses. Unfortunately, to the best of our knowledge, seasonal inactivated influenza vaccines will be largely ineffective in the event of a pandemic. Through a process known as ‘antigenic shift’, novel gene segments are introduced into viruses by reassortment, resulting in the emergence of viruses that are not recognized by circulating antibodies in the population (as seen recently with the emergence of swine-origin influenza H1N1 09). However, CTLs target conserved internal viral proteins found in heterologous virus strains. Work in the late 1970s from the McMichael group followed the re-emergence of the A/Munich/1/79 (H1N1) subtype, and demonstrated that large numbers of CTLs in individuals experimentally infected with H1N1 decreased the amount of virus shedding when type-specific antibody was absent [49]. More recent work has demonstrated that there is a high degree of conservation of human CTL-recognized epitopes between the seasonal H1N1 as compared with the pandemic H1N1 strain. This large degree of conservation may play an important role in reducing the impact of pandemic H1N1 in elderly individuals [50]. Therefore, development of a universal CTL-based vaccine may be a way of preventing the devastating morbidity and mortality in the event of future pandemics. Since both the virus-specific humoral response and the CTL response are commonly maintained for life in vaccinated individuals [51,52] and are both important for protection against secondary influenza infection, the development of novel influenza vaccines should induce both robust influenza-specific antibody and CTL responses for maximal protection.

Previous studies have demonstrated a clear link between the development of polyfunctional T-cell responses and effective clearance of the invading pathogen. For instance, CD4⁺ T cells that produce multiple cytokines in response to *Leishmania* infection are superior in their protective capacity compared with CD4⁺ T cells that produce only one or two cytokines [53]. Furthermore, there is a positive correlation in the generation of HIV-specific polycytokine-producing CTLs and improved clinical outcome in HIV-1-infected individuals [54,55]. Therefore, we need a greater understanding of how these polyfunctional CTLs are generated and how to best elicit these cells following IAV vaccination.

Several vaccine strategies have been developed to stimulate effective CTL immunity. Live viral vectors, including vaccinia virus [56], sindbis virus [57,58] and Semliki Forest virus [59], have all been used to generate robust CTL responses that were shown to be protective. Moreover, experiments by Epstein and colleagues have been able to use adenovirus serotype 5 expressing nucleoprotein derived from the H1N1 PR8 influenza virus to generate cross-protective CTL responses against the H5N1 avian influenza virus [60]. Our own laboratory has vaccinated mice with PA₂₂₄-specific lipopeptides (PA₂₂₄₋₂₃₃ T-cell epitope conjugated to a lipid moieties), which induced a highly functional IAV-specific CTL response that

produced IFN- γ , TNF- α and IL-2 [61]. Although this vaccination strategy induced fewer IAV-specific CTLs than intra peritoneal infection with IAV/PR8, both IAV/PR8 immunized and PA₂₂₄-lipopeptides cleared a subsequent IAV/HK $\times$ 31 challenge with similar kinetics. We have also used a natural killer T-cell activating adjuvant, α -galactosylceramide (α -GalCer), in combination with inactivated PR8 virus to generate a highly effective CTL-based vaccine. In these studies, α -Gal-Cer and inactivated IAV/PR8-immuized mice developed a more polyfunctional IAV-specific CTL response as compared with inactivated IAV/PR8-immunized controls. This vaccination strategy also enhanced prosurvival gene expression in IAV-specific CTLs and, thus enhanced CTL survival into memory. Importantly, α -GalCer-treated mice exhibited accelerated viral clearance following heterologous secondary challenge [62]. However, more studies must be carried out in order to identify the exact mechanisms by which natural killer T cells enhance CTL function and survival into memory. Taken together, these studies highlight how the context in which a naive CTL 'sees' its cognate antigen can affect the functional capacity of the resulting CTL response.

Conclusion

The IAV-specific CTL response is induced in the lung-draining LNs. However, the mechanisms by which this occurs, and the particular subsets of DCs that are involved, are not well understood. It is clear, however, that viral antigen is transported to the LN via DCs, and it is possible that these antigenic determinants are presented to naive CTLs by both migratory and LN-resident DCs. Regardless of the exact manner in which this occurs, naive CTLs are activated, begin dividing and start to undergo the differentiation process that involves the acquisition of effector function even before they leave the draining LN. It is currently unclear which of these effector functions are absolutely necessary for the clearance of virus once virus-specific CTLs traffic to the site of infection. However, it is apparent that there is a great deal of redundancy in this system, as influenza-specific CTLs can effectively reduce pulmonary viral titers when lacking several of their key effector molecules. One of the key issues in CTL immunology is how to properly harness the abilities of these cells to generate robust and highly functional CTL responses following vaccination, without causing enhanced immunopathology after natural infection with the target pathogen. In murine models of vaccination, CTL-based vaccines appear to be efficacious. However, there are no effective human vaccines in current use that rely on CTL immunity. One potential reason for the lack of human CTL-based vaccines may be due to our lack of understanding on how highly functional CTLs can best be induced via vaccination and how to maintain long-lived memory populations that are capable of mounting robust secondary responses following infection.

Future perspective

These are very exciting times in the field of CTL immunology. We sit at the dawn of the epigenetic age. In just a few short years, and with access to deep sequencing technology, we will be able to characterize and describe complex epigenetic regulatory networks involved in the activation and suppression of effector gene function. It may in fact be possible at some stage to perform single-cell transcriptomics. This unprecedented and detailed level of understanding will almost certainly provide researchers with the tools required to develop new vaccines and therapies. It may even result in the development of the 'Holy Grail' influenza vaccine – a cross-protective CTL-based vaccine that provides protection against future pandemic IAV strains.

Executive summary

Dendritic cell migration after intranasal infection with influenza A virus

- Dendritic cells (DCs) migrate rapidly from the site of infection – the lung – to the lung-draining lymph nodes after influenza A virus (IAV) infection.
- There is a discrepancy between the kinetics of DC migration and the duration of antigen presentation in the draining lymph nodes.

Identification of DC subsets that are required for inducing the IAV-specific cytotoxic T lymphocyte response

- Both migratory DCs and secondary lymphoid organ-resident DCs appear to have a capacity to induce IAV-specific cytotoxic T lymphocyte (CTL) responses.
- Plasmacytoid DCs are not involved in the generation of the IAV-specific CTL response.

Role of DCs in the accumulation of IAV-specific CTLs in the lung

- Depletion of respiratory DCs following the induction of the IAV-specific CTL response results in reduced numbers of pulmonary CTLs and a delayed clearance of virus from the lungs.
- TNF/inducible nitric oxide synthase-producing DCs contribute to the survival of IAV-specific CTLs in the lung following infection and enhance their effector function.

Acquisition of CTL effector function in response to influenza infection

- Acquisition of cytolytic molecule expression by IAV-specific CTLs is cell division dependent, whereas the ability to produce inflammatory cytokines (i.e., IFN- γ and TNF- α) is independent of cell division.
- Patterns of effector gene expression are highly variable, even within populations of CTLs that have the same antigen specificity.
- Patterns of effector gene expression vary between populations of CTLs with different antigen specificities.

CTL vaccines & IAV

- Influenza virus antigenic drift and shift can enable the virus to evade antibody-mediated immunity by mutating or switching their surface glycoproteins.
- IAV-specific CTLs are capable of recognizing antigenic determinants from highly conserved viral proteins.
- Several CTL-based vaccines have resulted in an accelerated viral clearance following immunization in murine models of IAV infection.

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Bibliography

Papers of special note have been highlighted as:

■ of interest

■ ■ of considerable interest

1. Price GE, Ou R, Jiang H, Huang L, Moskophidis D. Viral escape by selection of cytotoxic T cell-resistant variants in influenza A virus pneumonia. *J. Exp. Med.* 2000; 191(11):1853–1867. [PubMed: 10839802]
2. Rimmelzwaan GF, Kreijtz JH, Bodewes R, Fouchier RA, Osterhaus AD. Influenza virus CTL epitopes, remarkably conserved and remarkably variable. *Vaccine.* 2009; 27(45):6363–6365. [PubMed: 19840674]
3. La Gruta NL, Kedzierska K, Stambas J, Doherty PC. A question of self-preservation: immunopathology in influenza virus infection. *Immunol. Cell Biol.* 2007; 85(2):85–92. [PubMed: 17213831]
4. Stambas J, Guillonneau C, Kedzierska K, Mintern JD, Doherty PC, La Gruta NL. Killer T cells in influenza. *Pharmacol. Ther.* 2008; 120(2):186–196. [PubMed: 18801385]
5. Turner SJ, La Gruta NL, Kedzierska K, Thomas PG, Doherty PC. Functional implications of T cell receptor diversity. *Curr. Opin. Immunol.* 2009; 21(3):286–290. [PubMed: 19524428]
6. Allan RS, Smith CM, Belz GT, et al. Epidermal viral immunity induced by CD8 α ⁺ dendritic cells but not by Langerhans cells. *Science.* 2003; 301(5641):1925–1928. [PubMed: 14512632]
7. Bozza S, Gaziano R, Spreca A, et al. Dendritic cells transport conidia and hyphae of *Aspergillus fumigatus* from the airways to the draining lymph nodes and initiate disparate Th responses to the fungus. *J. Immunol.* 2002; 168(3):1362–1371. [PubMed: 11801677]
- 8 ■ ■. Legge KL, Braciale TJ. Accelerated migration of respiratory dendritic cells to the regional lymph nodes is limited to the early phase of pulmonary infection. *Immunity.* 2003; 18(2):265–277. [PubMed: 12594953] [One of the first studies examining dendritic cell (DC) migration from the lung to the lung-draining lymph nodes following influenza infection. These studies demonstrate that DC migration following influenza A virus (IAV) infection is rapid and refractory to further inflammatory stimulus]
9. Yoon H, Legge KL, Sung SS, Braciale TJ. Sequential activation of CD8⁺ T cells in the draining lymph nodes in response to pulmonary virus infection. *J. Immunol.* 2007; 179(1):391–399. [PubMed: 17579060]
- 10 ■. Belz GT, Smith CM, Kleinert L, et al. Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus. *Proc. Natl Acad. Sci. USA.* 2004; 101(23):8670–8675. [PubMed: 15163797] [Opens up the possibility that both migratory and nonmigratory DCs may play a role in the presentation of IAV antigens to naive cytotoxic T lymphocytes (CTLs)]
11. Lay MD, Zhang L, Ribeiro RM, Mueller SN, Belz GT, Davenport MP. Kinetics of major histocompatibility class I antigen presentation in acute infection. *J. Immunol.* 2009; 182(2):902–911. [PubMed: 19124733]
12. Geurtsvankessel CH, Willart MA, van Rijt LS, et al. Clearance of influenza virus from the lung depends on migratory langerin⁺CD11b⁺ but not plasmacytoid dendritic cells. *J. Exp. Med.* 2008; 205(7):1621–1634. [PubMed: 18591406]
13. Belz GT, Smith CM, Eichner D, et al. Cutting edge: conventional CD8 α ⁺ dendritic cells are generally involved in priming CTL immunity to viruses. *J. Immunol.* 2004; 172(4):1996–2000. [PubMed: 14764661]
14. Kim TS, Braciale TJ. Respiratory dendritic cell subsets differ in their capacity to support the induction of virus-specific cytotoxic CD8⁺ T cell responses. *PLoS One.* 2009; 4(1):e4204. [PubMed: 19145246]

15. de Heer HJ, Hammad H, Soullie T, et al. Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. *J. Exp. Med.* 2004; 200(1):89–98. [PubMed: 15238608]
16. Asselin-Paturel C, Boonstra A, Dalod M, et al. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat. Immunol.* 2001; 2(12):1144–1150. [PubMed: 11713464]
17. Cella M, Facchetti F, Lanzavecchia A, Colonna M. Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent Th1 polarization. *Nat. Immunol.* 2000; 1(4):305–310. [PubMed: 11017101]
18. Soumelis V, Liu YJ. From plasmacytoid to dendritic cell: morphological and functional switches during plasmacytoid pre-dendritic cell differentiation. *Eur. J. Immunol.* 2006; 36(9):2286–2292. [PubMed: 16892183]
19. Villadangos JA, Young L. Antigen-presentation properties of plasmacytoid dendritic cells. *Immunity.* 2008; 29(3):352–361. [PubMed: 18799143]
20. Wolf AI, Buehler D, Hensley SE, et al. Plasmacytoid dendritic cells are dispensable during primary influenza virus infection. *J. Immunol.* 2009; 182(2):871–879. [PubMed: 19124730]
21. ■■. McGill J, Van Rooijen N, Legge KL. Protective influenza-specific CD8 T cell responses require interactions with dendritic cells in the lungs. *J. Exp. Med.* 2008; 205(7):1635–1646. [PubMed: 18591411] [Among the first studies to identify a role for pulmonary DCs in maintaining the IAV-specific CTL response once they traffic to the site of infection]
22. Aldridge JR Jr, Moseley CE, Boltz DA, et al. TNF/iNOS-producing dendritic cells are the necessary evil of lethal influenza virus infection. *Proc. Natl Acad. Sci. USA.* 2009; 106(13):5306–5311. [PubMed: 19279209]
23. Trapani JA, Davis J, Sutton VR, Smyth MJ. Proapoptotic functions of cytotoxic lymphocyte granule constituents *in vitro* and *in vivo*. *Curr. Opin. Immunol.* 2000; 12:323–329. [PubMed: 10781403]
24. Julkunen I, Sareneva T, Pirhonen J, Ronni T, Melen K, Matikainen S. Molecular pathogenesis of influenza A virus infection and virus-induced regulation of cytokine gene expression. *Cytokine Growth Factor Rev.* 2001; 12:171–180. [PubMed: 11325600]
25. 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(8):3486–3493. [PubMed: 10729122]
26. Belz GT, Xie W, Doherty PC. Diversity of epitope and cytokine profiles for primary and secondary influenza A virus-specific CD8⁺ T cell responses. *J. Immunol.* 2001; 166(7):4627–4633. [PubMed: 11254721]
27. Flynn KJ, Belz GT, Altman JD, Ahmed R, Woodland DL, Doherty PC. Virus-specific CD8⁺ T cells in primary and secondary influenza pneumonia. *Immunity.* 1998; 8(6):683–691. [PubMed: 9655482]
28. La Gruta NL, Kedzierska K, Pang K, et al. A virus-specific CD8⁺ T cell immunodominance hierarchy determined by antigen dose and precursor frequencies. *Proc. Natl Acad. Sci. USA.* 2006; 103(4):994–999. [PubMed: 16418289]
29. La Gruta NL, Turner SJ, Doherty PC. Hierarchies in cytokine expression profiles for acute and resolving influenza virus-specific CD8⁺ T cell responses: correlation of cytokine profile and TCR avidity. *J. Immunol.* 2004; 172(9):5553–5560. [PubMed: 15100298]
30. Stambas J, Doherty PC, Turner SJ. An *in vivo* cytotoxicity threshold for influenza A virus-specific effector and memory CD8⁺ T cells. *J. Immunol.* 2007; 178(3):1285–1292. [PubMed: 17237374]
31. Smyth MJ, Kelly JM, Sutton VR, et al. Unlocking the secrets of cytotoxic granule proteins. *J. Leukocyte Biol.* 2001; 70:18–29. [PubMed: 11435481]
32. McElhaney JE, Gravenstein S, Upshaw CM, et al. Granzyme B: a marker of risk for influenza in institutionalized older adults. *Vaccine.* 2001; 19:3744–3751. [PubMed: 11395209]
33. McElhaney JE, Ewen C, Zhou X, et al. Granzyme B: correlates with protection and enhanced CTL response to Influenza vaccination in older adults. *Vaccine.* 2009; 27:2418–2425. [PubMed: 19368783]

34. Jenkins MR, Trapani JA, Doherty PC, Turner SJ. Granzyme K expressing cytotoxic T lymphocytes protects against influenza virus in granzyme AB^{-/-} mice. *Viral immunol.* 2008; 21(3):341–346.
35. Brincks EL, Katewa A, Kucaba TA, Griffith TS, Legge KL. CD8 T cells utilize TRAIL to control influenza virus infection. *J. Immunol.* 2008; 181(7):4918–4925. [PubMed: 18802095]
36. Topham DJ, Tripp RA, Doherty PC. CD8⁺ T cells clear influenza virus by perforin or Fas-dependent processes. *J. Immunol.* 1997; 159(11):5197–5200. [PubMed: 9548456]
37. Chiu C, Heaps AG, Cerundolo V, McMichael AJ, Bangham CR, Callan MF. Early acquisition of cytolytic function and transcriptional changes in a primary CD8⁺ T-cell response *in vivo*. *Blood.* 2007; 109(3):1086–1094. [PubMed: 16990607]
- 38 ■. Gett AV, Hodgkin PD. Cell division regulates the T cell cytokine repertoire, revealing a mechanism underlying immune class regulation. *Proc. Natl Acad. Sci. USA.* 1988; 95:9488–9493. [PubMed: 9689107] [Provides the first definitive evidence that cell division correlates with cytokine production]
39. Jenkins MR, Mintern J, La Gruta NL, Kedzierska K, Doherty PC, Turner SJ. Cell cycle-related acquisition of cytotoxic mediators defines the progressive differentiation to effector status for virus-specific CD8⁺ T cells. *J. Immunol.* 2008; 181(6):3818–3822. [PubMed: 18768835]
40. Lawrence CW, Braciale TJ. Activation, differentiation, and migration of naive virus-specific CD8⁺ T cells during pulmonary influenza virus infection. *J. Immunol.* 2004; 173(2):1209–1218. [PubMed: 15240712]
41. Brehm MA, Daniels KA, Welsh RM. Rapid production of TNF- α following TCR engagement of naive CD8 T cells. *J. Immunol.* 2005; 175(8):5043–5049. [PubMed: 16210607]
42. Haluszczak C, Akue AD, Hamilton SE, et al. The antigen-specific CD8⁺ T cell repertoire in unimmunized mice includes memory phenotype cells bearing markers of homeostatic expansion. *J. Exp. Med.* 2009; 206(2):435–448. [PubMed: 19188498]
43. Bachmann MF, Barner M, Viola A, Kopf M. Distinct kinetics of cytokine production and cytotoxicity in effector and memory T cells after viral infection. *Eur. J. Immunol.* 1999; 29(1):291–299. [PubMed: 9933111]
44. Grayson JM, Murali-Krishna K, Altman JD, Ahmed R. Gene expression in antigen-specific CD8⁺ T cells during viral infection. *J. Immunol.* 2001; 166(2):795–799. [PubMed: 11145652]
45. Veiga-Fernandes H, Walter U, Bourgeois C, McLean A, Rocha B. Response of naive and memory CD8⁺ T cells to antigen stimulation *in vivo*. *Nat. Immunol.* 2000; 1(1):47–53. [PubMed: 10881174]
46. Kersh EN, Fitzpatrick DR, Murali-Krishna K, et al. Rapid demethylation of the IFN- γ gene occurs in memory but not naive CD8 T cells. *J. Immunol.* 2006; 176(7):4083–4093. [PubMed: 16547244]
47. Kersh EN. Impaired memory CD8 T cell development in the absence of methyl-CpG-binding domain protein 2. *J. Immunol.* 2006; 177(6):3821–3826. [PubMed: 16951344]
- 48 ■. Araki Y, Fann M, Wersto R, Weng NP. Histone acetylation facilitates rapid and robust memory CD8 T cell response through differential expression of effector molecules (eomesodermin and its targets: perforin and granzyme B). *J. Immunol.* 2008; 180(12):8102–8108. [PubMed: 18523274] [Among the first studies to identify that chemically modified histones may play a role in the accelerated re-acquisition effector function of memory CTLs]
49. McMichael AJ, Gotch FM, Noble GR, Beare PA. Cytotoxic T-cell immunity to influenza. *N. Eng. J. Med.* 1983; 309(1):13–17.
50. Greenbaum JA, Kotturi MF, Kim Y, et al. Pre-existing immunity against swine-origin H1N1 influenza viruses in the general human population. *Proc. Natl Acad. Sci. USA.* 2009; 106(48):20365–20370. [PubMed: 19918065]
51. Amanna IJ, Carlson NE, Slifka MK. Duration of humoral immunity to common viral and vaccine antigens. *N. Eng. J. Med.* 2007; 357(19):1903–1915.
52. Amanna IJ, Slifka MK, Crotty S. Immunity and immunological memory following smallpox vaccination. *Immunol. Rev.* 2006; 211:320–337. [PubMed: 16824139]
53. Darrah PA, Patel DT, De Luca PM, et al. Multifunctional Th1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. *Nat. Med.* 2007; 13(7):843–850. [PubMed: 17558415]

54. Betts MR, Nason MC, West SM, et al. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8⁺ T cells. *Blood*. 2006; 107(12):4781–4789. [PubMed: 16467198]
55. Lichterfeld M, Yu XG, Waring MT, et al. HIV-1-specific cytotoxicity is preferentially mediated by a subset of CD8⁺ T cells producing both interferon- γ and tumor necrosis factor- α . *Blood*. 2004; 104(2):487–494. [PubMed: 15059848]
56. Tamura M, Saikh KU, Kurane I, Ennis FA. Immunization with the N-terminal region of nonstructural protein NS1 promotes survival after challenge with lethal influenza A virus dose. *Viral Immunol*. 1998; 11(3):131–135. [PubMed: 9918404]
57. Miller A, Center RJ, Stambas J, et al. Sindbis virus vectors elicit hemagglutinin-specific humoral and cellular immune responses and offer a dose-sparing strategy for vaccination. *Vaccine*. 2008; 26(44):5641–5648. [PubMed: 18761047]
58. Tsuji M, Bergmann CC, Takita-Sonoda Y, et al. Recombinant Sindbis viruses expressing a cytotoxic T-lymphocyte epitope of a malaria parasite or of influenza virus elicit protection against the corresponding pathogen in mice. *J. Virol*. 1998; 72(8):6907–6910. [PubMed: 9658144]
59. Huckriede A, Bungener L, Holtrop M, et al. Induction of cytotoxic T lymphocyte activity by immunization with recombinant Semliki Forest virus: indications for cross-priming. *Vaccine*. 2004; 22(9–10):1104–1113. [PubMed: 15003637]
60. Epstein SL, Kong WP, Mispion JA, et al. Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein. *Vaccine*. 2005; 23(46–47):5404–5410. [PubMed: 16011865]
61. Day EB, Zeng W, Doherty PC, Jackson DC, Kedzierska K, Turner SJ. The context of epitope presentation can influence functional quality of recalled influenza A virus-specific memory CD8⁺ T cells. *J. Immunol*. 2007; 179(4):2187–2194. [PubMed: 17675478]
- 62 ■■. Guillonnet C, Mintern JD, Hubert FX, et al. Combined NKT cell activation and influenza virus vaccination boosts memory CTL generation and protective immunity. *Proc. Natl Acad. Sci. USA*. 2009; 106(9):3330–3335. [PubMed: 19211791] [Demonstrates that immunization coupled with α -galactosylceramide treatment, which activates natural killer T cells, greatly enhances IAV-specific CTL function and maintenance into a long-lived memory population]