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CD4⁺ T Cell Differentiation in Chronic Viral Infections: The Tfh Perspective

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Abstract

CD4⁺ T cells play a critical role in the response to chronic viral infections during the acute phase and in the partial containment of infections once chronic infection is established. As infection persists, the virus-specific CD4⁺ T cell response begins to shift in phenotype. The predominant change described in both mouse and human studies of chronic viral infection is a decrease in detectable Th1 responses. Some Th1 loss is due to decreased proliferative potential and decreased cytokine production in the setting of chronic antigen exposure. However, recent data suggest that Th1 dysfunction is accompanied by a shift in the differentiation pathway of virus-specific CD4⁺ T cells, with enrichment for cells with a T follicular helper cell (Tfh) phenotype. A Tfh-like program during chronic infection has now been identified in virus-specific CD8⁺ T cells as well. In this review, we discuss what is known about CD4⁺ T cell differentiation in chronic viral infections, with a focus on the emergence of the Tfh program and the implications of this shift with respect to Tfh function and the host-pathogen interaction.

VIRAL INFECTIONS AND THE T CELL RESPONSE

Chronic infections are a ubiquitous part of human history and can lead to dysfunction and neoplastic transformation of infected cells and affected organs¹. Chronic infection can additionally lead to dysfunction of responding immune cells. However, many of these infections contribute to the shape of the normal immune system. For example, laboratory mice have less mature immune systems than mice experimentally infected with multiple pathogens or co-housed with so-called “dirty” or pet store mice^{1–3}. The balance of persisting

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infections, immunity, and clinical disease is dependent on the ongoing interplay between host immune responses and the pathogen. Many studies of CD8⁺ T cells have dissected the mechanisms critical to pathogen containment. However, studies of CD8⁺ T cell responses have also identified CD8⁺ T cell dysfunction when a viral infection is chronic. This phenotype has been described as T cell exhaustion, wherein chronic antigen exposure leads to decreased proliferative potential and effector function of antigen-specific cells (reviewed in ⁴). Mechanistic studies of CD8⁺ T cell exhaustion in chronic infections has identified clinically relevant molecules such as inhibitory receptors (e.g. programmed death-1 (PD-1) and cytotoxic lymphocyte associated protein-4 (CTLA-4)) that, when targeted with blocking reagents, can improve CD8⁺ T cell function^{5,6}. Such checkpoint blockade immunotherapies are now mainstays in the treatment of many cancers⁷, with trials beginning for the study of these immunotherapies in chronic infections^{8,9} (see clinicaltrials.gov). In addition to CD8⁺ T cells, CD4⁺ T cells are also critical in the containment of chronic infections. However, our understanding of the effects of chronic infection on CD4⁺ T cell differentiation and function remains incomplete.

The roles of CD4⁺ T cells in the control of infection are broad. CD4⁺ T cell function is traditionally defined by the provision of help to other effector cells, such as enhancement of the CD8⁺ T cell response, promotion of CD8⁺ T cell memory, enhancement of phagocytic or oxidative burst activities of myeloid cells, and B-cell mediated help. These functions are mediated by CD4⁺ T cells that can be broadly grouped into subsets such as Type 1 helper cells (Th1), Type 2 (Th2), Type 17 (Th17), regulatory T cells (Treg), T follicular helper (Tfh), and cytotoxic CD4⁺ T cells¹⁰. The commitment of CD4⁺ T cells to one of these lineages is influenced by signals received during the initial priming interaction with antigen presenting cells (APC), including cytokines, costimulatory signals, and signals derived from the quality and duration of the T cell receptor (TCR) binding to the major histocompatibility (MHC) II:peptide complex¹¹. Post-priming signals also influence the continued differentiation of CD4⁺ T cell helper lineages. The ways in which persisting antigen and chronic infection affect the initial differentiation and continued function of CD4⁺ T cells are a growing area of research.

The importance of CD4⁺ T cell responses can depend on the nature and duration of an infection. In mouse models of acute viral infection, CD4⁺ T cells are dispensable for viral control, although subsequent memory CD8⁺ T cell responses can be impaired¹²⁻¹⁴. In contrast, containment of infections with persisting viruses is often dependent on CD4⁺ T cell help. The importance of CD4⁺ T cells in chronic viral infection has been demonstrated both in mouse models of chronic viral infection and in human infections with hepatitis B and C viruses ^{12,15-18}. As a result, understanding how the biology of CD4⁺ T cells shifts as an infection persists is important to the overall understanding of how these infections may be better treated or controlled.

In chronic infections, pathogen-specific CD4⁺ T cell differentiation and subset distribution can differ from that observed following acutely cleared infections¹⁹. For example, during many viral infections in humans and mice, CD4⁺ T cells differentiate into antiviral IFN γ -producing Th1 cells,^{10,20} with perhaps a small subset of cytotoxic CD4⁺ T cells that may be related to Th1 cells^{10,21}. In addition, Tfh cells develop during acute infections or

vaccinations, providing essential help to driving B cell responses, germinal center development, antibody affinity maturation, and the development of long-lived plasma cells (Box 1) ^{19,20,22}.

Box 1

Tfh differentiation in acute viral infection

Tfh are a subset of CD4⁺ T cells that provide help to B cells within lymphoid tissues for the generation of affinity-matured antibodies, long-lived plasma cells, and memory B cells²². Tfh express CXCR5, a chemokine receptor that allows trafficking into B cell follicles, and their differentiation requires the transcription factor Bcl6^{23–26}. Most of our knowledge of Tfh development comes from murine models. In acute microbial infections, Tfh development begins in the environment of initial CD4⁺ T cell priming by dendritic cells²⁷. Bcl6 transcription in mice then requires IL-6 signaling through Stat1 in the early phases of Tfh differentiation^{28,29}. A high concentration of IL-2 in the environment inhibits Tfh development, whereas IL-10 has been shown to promote Tfh over Th1 responses^{30,31}. Several other transcription factors (e.g. Lef-1, and Tcf-1) and costimulatory molecules (e.g. ICOS) are also important in the initiation of the mouse Tfh program^{32–36}. While these processes are critical in early Tfh differentiation, nascent Tfh cells require functional B cell interactions to complete Tfh commitment^{37,38}. Finally, the T cell receptor (TCR) of the naïve cell may also shape the differentiation process, given that properties of TCR binding and signaling have been demonstrated to drive Tfh differentiation^{39–44}.

The full scope of events that lead to Tfh differentiation remains to be elucidated. This is especially true for human Tfh, where IL-12 takes place of IL-6, and where mechanistic studies remain limited^{27,45}. Approaches to understanding human Tfh responses, such as the recently performed *in vitro* screen of recombinant proteins in human Tfh differentiation and the evaluation of genotype and phenotype in human primary immunodeficiencies are of particular value to identifying which findings in mice can be potentially translated to humans^{46,47}.

During chronic infections, loss of typical antiviral Th1 responses can occur^{19,48,49}, and there also appears to be a major skewing towards a Tfh-phenotype instead of a more prototypical Th1-like anti-microbial fate^{19,50,51}. More broadly, both virus-specific CD4⁺ T cell responses and unrelated CD4⁺ T cell responses display a pattern of transcription factor and differentiation marker expression that suggest there are altered T helper subset lineage decisions during chronic viral infection in mice^{19,52}. Recent work has also demonstrated that these lineage decisions are plastic, and CD4⁺ T cells can retain the ability to acquire functions of other subsets^{11,53}. To develop therapies and vaccines for chronic infections, it is essential to dissect the functional role of altered CD4⁺ T cell differentiation patterns during such infections, and to identify common pathways that can be exploited for therapeutic gain.

This review discusses several paradigmatic chronic viral infections to examine the changes in CD4⁺ T cells that occur during chronic viral infection. In particular, we explore the emerging data on chronic antigen exposure and chronic inflammation and the corresponding

shift towards a Tfh phenotype. We review lymphocytic choriomeningitis virus (LCMV) mouse infections, as well as human and nonhuman primate infections with HCV, SIV, and HIV; our aim is to promote research that serves to identify the potential mechanisms for Tfh phenotype shifts during chronic viral infections, as well as the ensuing consequences for both host and pathogen.

CD4⁺ T RESPONSES IN MOUSE MODELS OF CHRONIC INFECTION

CD4⁺ T Cell Responses in LCMV Infection

The study of LCMV in the mouse has provided many cellular and mechanistic insights into immune responses to chronic infections. LCMV exists in the form of acutely resolving strains such as Armstrong and chronic strains, such as clone 13 and WE. These viruses differ in a few amino acids that lead to dramatic differences in the ability of the immune system to clear infection⁵⁴. Following infection of immunocompetent mice with acute strains of LCMV, virus is cleared by days 8–10 post infection. In contrast, chronic strains of LCMV maintain viremia and widespread replication for ~2–3 months post infection, and even after viremia resolves, virus persists for extended periods of time in other tissues, such as the brain, salivary gland, and kidney^{55,56}. CD4⁺ T cells are dispensable for the control of acute LCMV infection, but they are critical in the control of chronic infection because CD4⁺ T cell deficiency, even transiently, causes unresolving life-long viremia upon infection with strains such as LCMV clone 13¹².

Several studies have assessed the differentiation and function of CD4⁺ T cells in chronic LCMV clone 13 compared to acute LCMV Armstrong (Arm) infection. Initially, the number and frequency of effector CD4⁺ T cells that produce Th1 cytokines IFN γ and TNF is similar between LCMV Arm and clone 13 infections⁴⁹. However, after the first week of infection, the frequency of Th1-cytokine producing CD4⁺ T cells decreases in clone 13 infection, and LCMV-specific CD4⁺ T cells do not efficiently produce IFN γ or TNF following stimulation *ex vivo*⁴⁹. The defects in CD4⁺ T cell cytokine production persist even after viremia is controlled, and the LCMV-specific CD4⁺ T cells that are present ~3–4 months after clone 13 infection respond poorly and fail to proliferate to *in vivo* re-challenge, compared to those from Arm infection⁴⁹. These features of poor functionality in the setting of chronic antigen exposure are emblematic of T cell exhaustion because defects in effector function are one of the defining features of this state of T cell dysfunction. The causes of CD4⁺ T cell exhaustion or dysfunction in chronic LCMV are not fully understood, but chronic antigen stimulation is unlikely to be the sole cause. Indeed, reduction of signaling by either TGF β or TNF have been recently shown to preserve CD4⁺ T cell function during chronic LCMV infection^{57,58}. To understand whether the shift away from Th1 cytokine production reflects exhaustion alone or whether it reflects a broader change in LCMV-specific CD4⁺ T cells, the transcriptional landscape of all LCMV-specific CD4⁺ T cells during clone 13 infection have been assessed (19). In addition to losses of Th1 cytokine production, LCMV-specific CD4⁺ T cells in chronic infection lose the transcriptional programming associated with a Th1 response (19). Whether this reflects reversible transcriptional changes or instead reflects more permanent epigenetic changes remains to be determined.

A shift towards the Tfh phenotype in chronic LCMV infection

The loss of Th1 frequency in chronic LCMV infection is accompanied by a gain in Tfh. One of the first studies to describe an increase in Tfh frequency during chronic viral infection was published ~10 years after Tfh cells were defined as a separate CD4⁺ T cell lineage, using chemokine receptor CXCR5 surface expression to identify these cells⁵⁰. Both clone 13 and Arm infections led to high numbers of CXCR5⁺ LCMV-specific CD4⁺ T cells in the initial days after infection, comprising 40% of LCMV-specific CD4⁺ T cells in the spleen during Arm infection and 20% in clone 13 infection⁵⁰. As clone 13 infection persisted, however, the frequency of CXCR5⁺ CD4⁺ T cells increased by 3-fold, reaching approximately 2/3 of the total LCMV-specific cell number by day 30⁵⁰. In contrast, the frequency of CXCR5⁺ LCMV-specific cells in the Arm-infected mice remained relatively constant⁵⁰. As a result, Tfh-like CD4⁺ T cells were highly overrepresented in chronic LCMV infection compared to resolved, acute LCMV infection⁵⁰. These Tfh-like CD4⁺ T cells in chronic LCMV infection provided help to B cells *in vitro*, suggesting that there was acquisition of some features of true Tfh cells, such as the ability to help coordinate B cell responses and antibody production⁵⁰.

Confirming this enrichment for Tfh cells more broadly, transcriptional analysis of altered CD4⁺ T cell differentiation in chronic infection identified a loss in Th1 transcriptional profiles during clone 13 infection in addition of enrichment for Tfh-associated transcripts¹⁹. These cells additionally acquired phenotypic (CXCR5) and functional (IL-21 secretion) features of Tfh cells, as observed by other groups^{19,40}. As we work to understand this shift towards Tfh phenotype and function, it is interesting to note that some features of Tfh gained after chronic antigen exposure, including CXCR5 expression, can be partially downregulated with removal of chronic antigen stimulation^{40,50}. Still, other experiments have demonstrated that LCMV-specific Tfh cells could retain transcription factor Bcl6 expression even 2 weeks following their adoptive transfer to uninfected mice⁵⁰. Together, these studies suggest that chronic infection preferentially drives Tfh cell generation and that part of the Tfh program, such as Bcl6 expression, may be a fixed, rather than a transient response to stimulation or inflammation⁵⁰. Thus, chronic viral infections may have long-lasting impacts on the overall pattern of CD4⁺ T cell differentiation, including a skewing towards a Tfh phenotype, even after these infections resolved. Of relevance, there may be implications for viral infections such as chronic HCV, which can now be essentially cured with direct acting antivirals, but nevertheless, where re-exposure/re-activation can occur in high risk patient groups.

Newer themes in follicular programs during chronic LCMV infection

Recent data suggest that Tfh-associated gene networks upregulated during chronic antigen exposure may not be limited to the CD4⁺ T cell lineage only. A population of CD8⁺ T cells expressing the germinal center-driving chemokine receptor CXCR5 emerge during chronic LCMV infection (as well as in SIV and HIV in non-human primates and humans, respectively; see below)⁵⁹⁻⁶¹. This is interesting because only CD4⁺ T cells are thought to play a role in the germinal center reaction. However, the LCMV-specific CD8⁺ T cells appear by day 8 post infection and also express other Tfh-associated proteins including ICOS, Bcl6, and PD-1^{59,61,62}. The development of CXCR5⁺ CD8⁺ T cells in clone 13

infection is dependent on the transcription factor Tcf-1, consistent with its role in CD4⁺ Tfh differentiation (Box 1)^{32–34,59,61}. The functional role of CXCR5⁺ CD8⁺ T cells is incompletely understood, but recent data in LCMV suggest that CXCR5⁺ LCMV-specific CD8⁺ T cells control viremia better than their CXCR5-negative counterparts^{59,62}. The ability to study a follicular program in CD8⁺ T cells occurring mainly during a chronic infection might allow us to dissect certain features of Tfh cells that might be indicative of an acute response, versus those that could represent an adaptation to chronic antigen exposure and inflammation.

One common feature in both CD4⁺ and CD8⁺ T cells with a follicular program during chronic infection is an interferon stimulated gene (ISG) signature^{19,61}. ISGs increase after type I interferon (IFN-I) signaling. Early during infection, detectable circulating IFN levels (and corresponding ISGs) are induced, followed by rapid waning to undetectable levels; these kinetics are similar in conditions of acute or chronic LCMV infection^{63,64}. However, in LCMV-specific CD4⁺ T cells, the ISG signature persists during chronic infection, even after peripherally measurable type I IFN protein levels decline¹⁹. Further, as noted in the CD4⁺ T cell response to chronic LCMV, Tfh-like CD8⁺ T cells also exhibit an increased ISG signature⁶¹. It is unclear why the pathways associated with IFN signaling are maintained in virus-specific T cells despite a decline in detectable IFN-I^{63,64}. Several possibilities exist. First, it is possible that low levels of IFN-I continue to be produced in chronic infection and this residual IFN-I (below the limits of detection) sustains this ISG signature. Newer, more sensitive IFN-I detection assays⁶⁵ may be able to address this question. Second, other cytokines might sustain the ISG signature initially induced by IFN-I. For example, a critical role for cytokine signaling through the IL-6 receptor pathway in the maintenance of a Tfh phenotype during chronic LCMV infection has been shown⁶⁶. Moreover, IL-27, another IL-6 family cytokine, plays a key role in CD4⁺ T cell responses in chronic infection and might also induce ISGs⁶⁷. How these two cytokines relate to the ISG response in Tfh cells, however, remains to be determined. Finally, it may be that subsets of virus-specific CD4⁺ T cells generated during chronic viral infection, such as Tfh, have access to locations where local signals (perhaps local IFN-I) sustain an ISG signature. In the future, it will be important to distinguish these possibilities and dissect the importance of inflammatory signals such as IFN-I and chronic TCR signaling in driving Tfh development and maintenance during chronic infections (Figure 1). An accurate definition of the processes driving increased follicular transcriptional programs -- such as persisting ISG signatures -- should help identify settings of potential Tfh cell dysfunction, or perhaps enable the development of predictions for the effects of immunomodulatory drugs during chronic infections.

Functional Role of Tfh Cells in Chronic LCMV Infection

Does differentiation towards a Tfh program during chronic infections provide a benefit for the host? Possible mechanisms of benefit include (i) reduction of Th1-driven immunopathology, (ii) support of the CD8⁺ T cell response, and (iii) a more canonical role for Tfh cell function, namely, providing help to enable a robust antibody response. A role for reduced immunopathology has been supported by vaccination with an LCMV CD4⁺ T cell epitope driving Th1 responses⁶⁸. Mice that were immunized with the CD4⁺ T cell epitope

experienced marked mortality after challenge with LCMV clone 13 as compared to unimmunized mice⁶⁸. This mortality was associated with an extended duration of the Th1 response, while control mice demonstrated the expected shift towards expressing transcripts associated with Tfh, Treg, and exhausted T cells⁶⁸. A role for Tfh in CD8⁺ T cell support has also been studied using the canonical Tfh cytokine, IL-21^{69–71}. However, it should be noted that the cellular origin of IL-21 is not necessarily limited to Tfh⁷². Compared to wild type controls, LCMV-specific CD8⁺ T cells in IL-21 receptor deficient mice have been shown to be present in lower numbers, and to develop features of more severe exhaustion⁶⁹. This effect has been partially attributed to direct signaling of IL-21 on CD8⁺ T cells, observed when IL-21R deficient T cells were adoptively transferred into wild type hosts⁶⁹. In addition, another study demonstrated that exogenous IL-21 administration improved CD8⁺ T cell cytokine production and decreased viral titers⁷¹. Finally, ablation of the IL-6 family receptor gp130 in CD4⁺ T cells during chronic LCMV infection led to decreased Tfh and CD8⁺ T cell frequencies, decreased IL-21 production, reduced LCMV-specific antibody production, and an inability to contain virus⁷³. These data strongly support the notion that differentiation away from a Th1 phenotype, increasing IL-21 production, and sustaining the Tfh response may have benefits to the host. However, whether Tfh cells generated during chronic infection do function optimally is less clear.

A primary function of Tfh is to support class-switched, high-affinity antibody responses from B cells. However, despite increased Tfh frequencies in chronic LCMV infection, the development of neutralizing antibodies does not occur for many months⁷⁴. Instead, there is a considerable increase in total and non-neutralizing LCMV-specific immunoglobulin^{74,75}, and chronically infected mice can show signs of hypergammaglobulinemia and glomerulonephritis^{75,76}. This high production of poor quality antibodies and delayed production of neutralizing antibodies suggests that there may be dysregulated interactions between Tfh and B cells in chronic LCMV infection, leading to a poor ability of Tfh to help B cells produce optimal antibodies^{74,75}. Supporting the hypothesis of dysregulated Tfh:B cell interactions during chronic LCMV infection, a pair of recent studies have demonstrated a plasmablast response upon deletion of LCMV-specific B cells and a loss of the germinal center B cell response due to early IFN signaling^{77,78}. Together these defects were reported to lead to impaired production of high affinity antibody^{77,78}. While the studies focused on B cell effects of IFN signaling, adoptive transfer experiments demonstrated that the IFN-induced defect was not intrinsic to B cells but exhibited cell-intrinsic effects in monocytes as well as in both CD8⁺ and CD4⁺ T cell populations^{77,78}. These advances only begin to define the abnormalities in Tfh:B cell interactions that arise during chronic infection. Moreover, given that the timely development of neutralizing antibodies is central to viral control⁷⁹, further work is needed to understand which steps of the Tfh:B cell interaction are perturbed by a persisting virus. Consequently, mechanistic studies in chronic LCMV that focus on investigating the changes in B cell dynamics, Tfh quality, and the lymphoid environment will be useful in informing on the characteristics of human immune dysfunction that accompany chronic viral infections.

CD4⁺ T CELLS IN HUMAN AND NON-HUMAN PRIMATE CHRONIC VIRAL INFECTIONS

A major goal of studying chronic infections in mice is to understand, prevent, and treat human disease. Our ability to translate findings from mice to humans has faced several challenges. First, understanding how the immune response changes as a pathogen transitions from an acute to a chronic infection requires the identification of patients in acute stages of infection. Cohorts developed to track high-risk patients have provided invaluable samples and datasets in recent years^{80,81}. However, the difficulty in establishing these cohorts necessarily limits scientific progress. Second, our understanding of the CD4⁺ T cell response to chronic infections in humans is limited by the need for HLA class II-matched tetramers that allow pathogen-specific CD4⁺ T cells to be identified, regardless of whether the cells generate a detectable cytokine or not. Given that a hallmark of immune responses to chronic infection is decreased function, studies of CD4⁺ T cells that rely on cytokine production are likely to fail to capture dysfunctional cells or those that produce low or no cytokines (e.g. Tfh). Human HLA class II diversity is a tremendous challenge in the development of broadly applicable reagents, but recent efforts to develop suites of HLA class II tetramers for chronic infections such as HIV, HCV and tuberculosis have allowed an increased understanding of human CD4⁺ T cell differentiation during chronic infections^{82,83}. However, even tetramers can introduce a bias, given that they can only identify responses to a single epitope (Figure 2). A newer technique that we and others have used is the assessment of activation induced markers (AIM)^{84,85}. Upregulation of AIMs such as CD69, CD200, CD71 and CD40L can be used to identify antigen-specific cells that do not produce high levels of cytokines^{84,85}. While AIMs have been used in particular to identify antigen-specific Tfh, it is also possible that clusters of AIMs are specific for some T helper subsets and not others, which would also introduce bias; this warrants further investigation.

To date, studies of CD4⁺ T cells in human chronic viral infections have shown many similarities with studies and emerging concepts from the mouse model of LCMV infection. In particular, HCV and HIV infections have been associated with i) a loss of Th1-associated cytokine production^{48,83}; ii) a shift towards Tfh differentiation^{51,86}; and iii) evidence of dysfunctional Tfh:B cell interactions⁸⁷⁻⁸⁹. Nevertheless, distinct features of each infection (e.g. direct infection of CD4⁺ T cells, including Tfh, by HIV) require detailed interrogation in humans. Of note, most human immunologic studies to date have been performed on blood, while mouse studies of chronic infection are commonly performed using the spleen. Moving forward, it will be critical to explore the immunology of chronic infection within human tissues such as lymph nodes, spleen, and at other sites of viral infection. Further, clinical studies will greatly benefit from an improved understanding of whether and how Tfh responses detectable in the blood relate to those within lymphoid tissues.

CD4⁺ T Cells in Hepatitis C Virus (HCV) Infection

HCV is a self-limited viral infection in approximately 25% of those who are infected but becomes a chronic infection in the majority of individuals⁹⁰. Untreated HCV infection is associated with the eventual development of liver fibrosis and hepatocellular carcinoma⁹¹. While the difference between acute and chronic LCMV infection in mice is mediated by a

change in the viral genome, differences in acute resolution versus progression to chronic HCV infection in humans are likely mediated by differences in host immunity, including virus-specific CD4⁺ T cell responses⁹². Chimpanzee studies have shown that CD4⁺ T cells are required for protection against disease following immunization or prior to infection⁹³. In humans, viral clearance has been associated with increased peripheral blood HCV-specific CD4⁺ T cell proliferation during acute infection^{83,92–94}. HLA class II tetramers have been used *ex vivo* to measure HCV-specific CD4⁺ T cell responses from HCV-infected patients; these studies reported a lack of proliferating HCV-specific CD4⁺ T cells in individuals who developed chronic HCV infection, mainly due to CD4⁺ T cell deletion or hyporesponsiveness^{95,96}. Specifically, tetramer-positive HCV-specific CD4⁺ T cells were present in individuals with chronic infection, but the frequency was considerably lower than in individuals whose infection had been cleared^{95,96}. In addition, a detailed study of HCV-specific CD4⁺ T cells found that both the initial magnitude of the CD4⁺ T cell response and breadth of targeted epitopes was similar between individuals who developed chronic infection and those who cleared the infection, given that a similar frequency of CD4⁺ T cells from these individuals produced effector cytokines when stimulated, and furthermore, the number of individual peptides from the virus recognized by CD4⁺ T cells was not different⁸³. Together, these data suggest that on the one hand, the initial repertoire of CD4⁺ T cells probably does not alter the ability of the immune system to clear the virus; yet, on the other hand, both the decreased numbers and proliferative potential of HCV-specific CD4⁺ T cells may be associated with HCV persistence^{83,95,96}. The reasons why some individuals develop a more robust CD4⁺ T cell response than others is not well understood and may represent a fruitful area of future investigation.

In addition to the decreased number and proliferation potential, the functional quality of HCV-specific CD4⁺ effector T cells also seems to correlate with protection from reinfection. For instance, in patients spontaneously clearing a primary HCV infection, those clearing a secondary infection upon challenge are more likely to harbor polyfunctional HCV-specific CD4⁺ T cells (producing more than one Th1-associated cytokine) than individuals unable to clear the secondary HCV infection; this suggests, perhaps, an important role for CD4⁺ T cell effector functions in HCV control⁹⁷. Unfortunately, studies of CD4⁺ T cells in HCV infection have been largely limited to an assessment of frequency, proliferation, and Th1 cytokine production; other detailed aspects of CD4⁺ T cell phenotype and differentiation state remain quite limited. Thus, a broader interrogation of the CD4⁺ T cell functional landscape during HCV infection requires further research. The advent of curative antivirals for HCV infection may also provide a rare opportunity to study whether and how the CD4⁺ T cell response corrects after the virus is eliminated.

Tfh Responses in HCV Infection

Most of the analyses concerning CD4⁺ T cell responses in HCV infection have been focused on evaluating Th1-cytokine producing cells, but some data in HCV suggest that this infection behaves similarly to LCMV with respect to Tfh. Early IL-21 production by HCV-specific CD4⁺ T cells has been correlated with individuals who go on to resolve infection⁹⁸, although these cells were not otherwise identified as Tfh. In the chronic phase of infection, increased frequencies of HCV-specific Tfh cells have been described, as observed in

LCMV^{50,86}. Specifically, HCV-specific Tfh cells were enriched in the liver relative to CD4⁺ T cells from the livers of patients with non-viral hepatitis or when compared to the overall frequency of Tfh in peripheral blood⁸⁶. These cells were implicated as Tfh-like because they not only expressed CXCR5 and PD-1 by protein, but they also expressed the Tfh cytokine IL-21 following *in vitro* expansion, further identifying them as probable Tfh. Thus, we posit that these data support a common model of chronic viral infection that leads to a predominant Tfh phenotype, particularly at the site of infection, although further testing is warranted. This shift towards a Tfh phenotype may have disease relevance if the consequence is a loss of more directly antiviral Th1 cells, or, if the excess of Tfh-like cells leads to a dysregulated antibody response.

HCV infection has also been associated with dysregulated immunoglobulin responses. Indeed, a subset of HCV-infected patients has been reported to experience considerable cryoglobulinemia and antibody accumulation, potentially suggesting that amplified B cell responses and/or T cell help might be induced by chronic HCV⁸⁷. Despite increased total antibody, HCV-specific antibody responses are typically poorly effective and fail to control viral replication⁸⁸. These data are consistent with observations in LCMV^{74,75}. In addition, humoral responses against other antigens might be compromised in patients with chronic HCV; specifically, one study noted that antibody responses against hepatitis B vaccination were lower in individuals with chronic HCV than in uninfected individuals⁹⁹. This observation suggested that Tfh:B cell dysfunctional interactions might be due, at least in part, to a putative bystander effect from surrounding inflammation, although this has not been directly tested. Thus, it will be important to understand whether an increased Tfh program in chronic HCV infection is limited to pathogen-specific cells or whether surrounding inflammation might contribute to dysfunctional Tfh:B cell interactions among non-HCV-specific cells. Distinguishing between direct and bystander effects on Tfh:B cell interactions might help shape therapeutics that could target inflammation versus viral replication directly, aiming to improve the production of quality antibody responses to other challenges, including vaccination and respiratory viruses.

CD4⁺ T Cells and Human Immunodeficiency Virus (HIV) and Simian Immunodeficiency VIRUS (SIV) infections

Approximately 37 million people are currently infected with HIV-1 worldwide, and the virus continues to infect ~2 million people each year¹⁰⁰. Because HIV infection is primarily an infection of CD4⁺ T cells, considerable efforts have focused on defining the nature of HIV-1-specific CD4⁺ T cell responses. In acute HIV-1 infection, CD4⁺ T cells produce the Th1 cytokine IFN γ and are directed against several HIV-1 epitopes⁴⁸. Other helper subsets have been described in acute disease and linked to disease progression, including Th1-like cells with cytotoxic potential and Th17 cells based on changes in the frequency of these subsets in relation to viral load, overall CD4 counts and/or progression to AIDS^{101,102,103}. Although acute HIV-1 infection generates robust Th1 CD4⁺ T cell responses, these responses decline within days to weeks, resulting in a decreased frequency of detectable HIV-1-specific IFN γ -producing CD4⁺ T cells and a reduction in the number of antigens to which the Th1 cells respond⁴⁸. The HIV-specific Th1 function does not recover after successful initiation of antiretroviral therapy (ART), although transcriptional signatures of

inhibitory pathways across all CD4⁺ T cells do decrease with effective viral control^{48,58}. It remains unclear whether the loss of HIV-specific Th1 CD4⁺ T cell responses in chronic HIV-1 infection is due to preferential infection of HIV-1-specific CD4⁺ T cells¹⁰⁴ or due to persistent CD4⁺ T cell dysfunction or exhaustion.

To dissect the relationship between the chronic phase of HIV-1 infection and functionality of CD4⁺ T cells, several groups have compared cohorts of patients with differing levels of immunologic or ART-induced viral control^{105,106}. Patients able to sustain immunologic control of virus, termed elite controllers, have a higher frequency of HIV-1-specific CD4⁺ T cells able to produce all three major Th1-associated cytokines (IFN γ , TNF, and IL-2) compared to patients with uncontrolled disease, and on ART, although many of the patients on ART were not shown to be fully suppressed¹⁰⁵. There is also some evidence that early initiation of ART can prevent HIV-specific CD4⁺ T cell dysfunction¹⁰⁶. However, an important remaining question is whether ART suppression initiated later in infection allows CD4⁺ T cells to return to their normal function and subset distribution or whether CD4⁺ T cells continue to remain altered as a result of the initial insult of chronic infection. To understand this question, approaches that profile HIV-specific CD4 T cells beyond measurement of Th1 cytokine production will be important. To date, analyses of broader transcriptional and differentiation programs in these cells have been limited.

Tfh in HIV Infection

HIV is primarily an infection of lymphoid tissues, and like LCMV and HCV, Tfh have been noted to increase at the site of HIV-1-infection^{51,107,108}. As has been observed in LCMV, CXCR5⁺ virus-specific CD8⁺ T cells have also recently been shown to increase in both HIV and in SIV infections^{62,109}, suggesting that the upregulation of a follicular program in both CD4⁺ and CD8⁺ T cells is conserved and that mice may be used to model these processes. Animal modeling in HIV has already helped to describe ways in which the increased Tfh response may be altered by chronic infection. Specifically, data from SIV-infection models suggest that Tfh cells exhibit an altered transcriptional signature compared to Tfh cells from SIV-negative lymph nodes, with ISGs being the most differentially expressed transcripts¹¹⁰. Thus, although, it is not clear whether the mechanisms driving a Tfh-like program in HIV are due to persisting antigen or whether other aspects of the inflammatory environment drive non-HIV specific cells towards a Tfh lineage, there appears to be an alteration in Tfh differentiation and development with HIV-1 infection.

The relationship between HIV and Tfh is further complicated by the fact that Tfh are a major cellular reservoir of replication-competent virus^{51,111–114}. As a result, HIV-1 has been associated with increased frequencies of the very cell type it infects^{51,111}. Moreover, histologic analysis of SIV-infection in non-human primates has suggested that infected Tfh are enriched within the B cell follicles of lymph nodes, which may in turn enable the virus to escape surveillance by cytotoxic CD8⁺ T cells normally excluded from this location¹¹³. Given that the majority of infected Tfh express programmed cell death protein 1 (PD-1), there is additional interest in the potential role of anti-PD1 immunotherapy in modulating the treatment of HIV-1 infected individuals. Indeed, candidate agents targeting PD-1 might be used to deplete this cellular reservoir or provoke viral reactivation from latency (i.e. by

activating these CD4⁺ T cells via PD-1 blockade), thus allowing the virus to be seen by CD8⁺ T cells and eliminated^{8,9,115,116}. Moreover, there are major efforts underway to develop putative prophylactic and therapeutic antibody-based vaccines for HIV-1 infection, and these may heavily depend on effective Tfh responses¹¹⁷, because production of effective antibodies by vaccination requires functional Tfh:B cell interactions. Tfh cells are therefore an important component of adaptive immunity, and a deeper understanding of the perturbed functional role of these cells from HIV infection may improve our understanding of HIV pathogenesis, prevention, and presumably, future cure of HIV infections¹¹⁸. Nevertheless, interrogation of Tfh in lymph nodes and other lymphoid tissues, as well as the location where Tfh:B cell interactions occur, is necessarily limited by access to tissues, although tissue-based research remains a vital goal. Because access to such lymphoid tissues is challenging, the study of HIV and Tfh has also been focused on Tfh-like cells that can be found in peripheral blood: circulating Tfh (box 2). Hence, an important future goal is to understand the relationship(s) between such blood Tfh-like cells and Tfh in lymphoid tissues, both in health, and during chronic infections.

Box 2

Circulating Tfh Cells

To investigate the biology of Tfh in HIV-1 infection in clinical trials and across broad patient groups, we and others have studied the more accessible Tfh-like cells in peripheral blood, termed circulating Tfh (cTfh). The precise ontology of these cells remains unclear. For example, whether cTfh arise from cells that were formerly Tfh in the B cell follicle/germinal center remains to be determined. Nevertheless, cTfh changes have been shown to correlate with immune responses to vaccination against influenza and other viruses^{36,84,119–122}. In HIV-1 infections, cTfh have been correlated with the presence of naturally occurring broadly neutralizing antibodies and with response to prophylactic vaccination^{123,121}. Circulating Tfh have also been found to be infected more frequently than other non-Tfh blood CD4⁺ subsets and to limit HIV-1 specific responses^{112,121}. Of note, cTfh are not all identical in their transcriptional profile or ability to provide B cell help¹²⁴. Circulating Tfh have been described as being polarized towards Th1, Th2, and Th17 cells, while cTfh that express ICOS and other markers of activation have been shown to contain vaccine-specific responses^{36,84,120,124}. Moving forward, it will be critical to define the ways in which cTfh can reflect the ongoing lymph node biology of HIV infection and immune dysfunction. In so doing, particular attention should be given to the identification of cTfh subsets that will be most informative.

Tfh function in HIV

As in LCMV and HCV infections, HIV-1 infection has been associated not only with increased Tfh frequency at the site of infection but also with abnormal B cell responses. For instance, hypergammaglobulinemia has long been a well-documented consequence of untreated HIV-1 infection^{75,89}. This alteration could be postulated to be due to disturbed Tfh:B cell interactions, wherein Tfh cell help is no longer limiting. Consistent with this notion, increased Tfh frequencies in HIV-infected lymph nodes have also shown that

peripheral hypergammaglobulinemia can correlate with Tfh Bcl6 expression⁵¹. However, despite this correlation, *in vitro* studies of lymph node Tfh cells from ART-naïve, HIV-1-infected individuals have indicated that these cells are impaired in their ability to provide B cell help when compared to equal numbers of Tfh from HIV-1-uninfected lymph nodes¹⁰⁸. These functional defects may be modifiable, as cTfh from viremic individuals can improve function when cultured with IL-2 blocking antibody¹²⁵.

Although Tfh are clearly abnormal in ART-naïve HIV-1 infection, less is understood about Tfh differentiation and function following ART control. Of note, lymph node Tfh remain at an elevated frequency, even in patients on ART, although the frequency is lower than that observed in ART-naïve patients⁵¹. Whether long-term ART allows Tfh frequencies to return to normal remains unclear. In addition, a continued deficit in Tfh function during ART suppression has been further suggested by several studies demonstrating that specific antibody responses to influenza and pneumococcal vaccination are impaired during ART-controlled HIV-1 infection^{126–129}. Noteworthy, studies in LCMV and Tfh differentiation have focused on a shift towards the Tfh phenotype in virus-specific cells. In contrast, the increased Tfh frequencies and Tfh dysfunction analyses described with HIV-1 infection have included all Tfh populations, regardless of specificity. Therefore, it is not clear which aspects of the Tfh:B cell dysfunction in HIV are due to persisting antigen (as would be expected for virus-specific cells⁴⁰) or to an inflammatory milieu that affects Tfh:B cell interactions more broadly. Such broad effects might be hypothesized to occur through the direct effects of IFN signaling and cytokine changes during Tfh differentiation, altered B cell functions, and/or through disruption of normal lymphoid architecture¹³⁰. Indeed, altered lymphoid architecture may be especially important during HIV-1 infection, as lymph node fibrosis has been reported, despite immunologic and antiretroviral control; indeed, such lymphoid tissue fibrosis might distort the normal structure of the B cell follicle where Tfh:B cell interactions occur^{131,132}. Studies that define the anatomical and molecular causes of altered Tfh and B cell function in HIV infection will thus help provide insights into candidate therapies for immune restoration. Further, these studies may have the potential to reveal mechanisms that allow the field to target or engineer Tfh cells to help reduce viral reservoirs.

CONCLUDING REMARKS

Chronic infections are a normal part of human immune development that can be contained by, but also alter, CD4⁺ T cell responses^{1–3}. Collectively, LCMV, HCV, SIV and HIV-1 infections indicate a direct relationship between chronic viral infection and Tfh cell biology. The pathways that drive Tfh differentiation are incompletely understood and may include features of both chronic antigen stimulation and of a shifting inflammatory environment. Moreover, while Tfh cells in chronic infection can provide B cell help, it remains unclear how phenotypically and functionally similar Tfh cells generated during chronic infection are to Tfh cells generated during acute infection, even after infection is controlled. Of note, some aspects of these follicular pathways are shared by CD8⁺ T cells during chronic infection, and many of the genes upregulated by these follicular-like CD8⁺ and CD4⁺ T cells are also found to be upregulated by exhausted CD8⁺ T cells in chronic infection and cancer¹⁹. Consequently, Just as studies in CD8⁺ T cell exhaustion have led to therapeutic

advances in cancer, in addition to new trials in chronic infections, understanding CD4⁺ T cell dysfunction in chronic infection has the potential to inform new approaches to subverting immune dysfunction, improve vaccination strategies, and hopefully, contribute to the cure of chronic viral infections (see Outstanding Questions and Box 3).

Box 3

Clinician's corner

- HIV-1 is known to impair immune responses even when CD4⁺ T cell counts are normal, but other chronic infections also impair T and B cell immunity.
- Impaired T cell immunity can be due to loss of immune cells, but it can also be due to a loss or change in cellular function.
- Certain T cell changes (such as exhaustion) noted in chronic viral infections may overlap with those noted during chronic antigen exposure in cancers.
- Altered humoral immunity and decreased vaccine responses have been observed in chronic viral infections, and these defects may be due, in part, to alterations in the follicular helper subset of CD4⁺ T cells (Tfh).
- Understanding changes in CD4⁺ (as well as CD8⁺) T cells during chronic viral infection may inform treatment of these infections and can, by extension, additionally lend insight to further developing cancer immunotherapy strategies.

Outstanding Questions

- What drives increased Tfh lineage differentiation in CD4⁺ and CD8⁺ T cells during chronic viral infections?
- Are Tfh cells derived during chronic viral infections distinct from Tfh cells found after acute viral infections or vaccination? What is the functional significance of these differences and similarities?
- Are there common pathways of differentiation, inflammation or transcriptional control in CD4⁺ T cells that negatively impact humoral immunity in chronic viral infections?
- Is the skewed distribution of pathogen-specific CD4⁺ T cells in chronic viral infections reversible? If so, can this characteristic be exploited for therapeutic purposes?

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Glossary

Activation induced markers

Proteins upregulated on the cell surface following activating signals through the T cell receptor (TCR)

Affinity maturation

Progressive selection of higher affinity B cell-produced antibodies

Anti-PD-1 immunotherapy

A type of checkpoint blockade immunotherapy that binds to PD-1 on the T cell surface and blocks PD-1 inhibitory signaling

Antiretroviral therapy

Combination therapy directed against different stages of the HIV-1 life cycle, usually consisting of two or three medications

B cell follicle

Collection of B cells within the lymphoid tissue, largely consisting of naïve B cells

Bcl6

Transcription factor encoded by the gene *BCL6* that acts as a transcriptional repressor and as an epigenetic modifier in complex with other proteins. Germinal center cells characteristically express high levels of this transcription factor.

Broadly neutralizing antibodies

Class of antibodies able to neutralize many different quasi-species of HIV-1 from different individuals

Bystander effect

Secondary, non-specific effects of a secreted molecule, such as a cytokine on an adjacent cell.

Checkpoint blockade immunotherapies

Clinical therapy with recombinant antibodies that bind inhibitory receptors (e.g. PD-1, CTLA-4). These antibodies prevent inhibitory molecules from exerting a 'check' on the immune response and are used to reinvigorate, for instance, CD8⁺ T cell responses.

Circulating Tfh (cTfh)

CD4⁺ T cells in the peripheral blood that express CXCR5 and PD-1 and are able to provide help to B cells *in vitro*

Class-switch

Process by which B cells switch the production of one antibody isotype to another isotype, i.e. IgM to IgG

Cryoglobulinemia

Medical disorder characterized by the production of immunoglobulins that complex together and clump in cold temperatures, leading to blood vessel inflammation known as vasculitis

Cytotoxic CD4⁺ T cells

A subset of CD4⁺ T cells that express granzyme B and perforin and can exhibit cytotoxic activity.

Effector function

Cellular production of anti-pathogen, anti-tumor or cell communication molecules such as cytokines, chemokines or granzymes, effecting some change (e.g. in the target cell, other immune cells or tissues)

Elite controllers

HIV-1 infected patients who maintain low or undetectable plasma viral loads without receiving antiretroviral therapy

Epigenetic

Regulating gene expression without changes in DNA sequence, often used to refer to processes of DNA methylation, post-translational histone modifications and chromatin accessibility.

Epitope

The sequence of amino acids that is recognized by the TCR when presented in the context of HLA class I or class II molecules or the portion of a molecule that is recognized by the B cell receptor.

Germinal center

A structure within the B cell follicle of lymphoid tissues in which B cells compete for antigen and T cell help to undergo affinity maturation. The germinal center is the site where B cells commit to become memory B cells or plasma cells, with the latter being the source of circulating antibodies.

Glomerulonephritis

Medical disorder characterized by inflammation of the glomeruli in the kidney, thereby affecting the ability of the kidney to properly filter blood. One cause of glomerulonephritis is deposition of immunoglobulin complexes.

HCV infection

HCV is a small, enveloped, positive-sense single-stranded RNA virus responsible for causing chronic infection and long-term liver injury

Humoral responses

Typically refers to the antibody response to an infection but also includes other immunologically-active secreted proteins including complement and anti-microbial peptides

Hypergammaglobulinemia

Medical condition referring to elevated levels of circulating IgG antibodies, usually in response to chronic infection or inflammation

Inhibitory receptors (T cells)

Cell-surface proteins whose expression serves to dampen T cell activation

Interferon-stimulated gene (ISG) signatures

represent a group of hundreds of genes whose expression is induced by the effect of interferons, that may exert antiviral activity. Some scenarios involve characteristic patterns of expression of ISGs, referred to as signatures.

Lymphoid architecture

The physical organization and makeup of the lymphoid tissue

Neoplastic transformation

Transition of a benign tissue lesion into one with invasive, malignant features

Neutralizing antibodies

Antibodies with particular ability to obstruct, hinder, or otherwise block the biological activity of a toxin or pathogen

HLA class II-matched tetramers

A biotinylated multimer consisting of four identical Human Leukocyte Antigen (HLA) proteins loaded with peptide. Biotinylated multimers are labeled using streptavidin linked to a fluorochrome or other molecular reporter. Tetramer technology works when the tetramer is composed of an HLA that matches the HLA type of the individual. HLA Class II tetramers “loaded” with a peptide antigen permit identification of the antigen-specific T cells.

Plasmablast response

Acute increase in circulating frequency of plasmablasts, an early stage of antibody-secreting cells, typically seen following vaccination or infection

Plasma cells

Late stage of B cell differentiation characterized by the ability to produce large amounts of antibodies

Programmed cell death protein 1 (PD-1)

A cell surface inhibitory receptor that belongs to the immunoglobulin superfamily and is predominantly expressed on lymphocytes.

Targeted epitopes

Antigens that are the primary targets of T cell recognition and activity

T cell exhaustion

A state of cellular dysfunction characterized by poor cytokine expression and poor proliferation in response to a stimulus despite presence of high levels of antigen. Exhaustion is commonly associated with increased surface expression of inhibitory molecules.

T cell priming

Binding of the T cell receptor complex with its cognate antigen, leading to differentiation of the T cell.

T helper type 1 cells (Th1)

A subset of CD4⁺ T cells that is driven by the transcription factor T-bet and typically produces the cytokine IFN-gamma. Th1 cells are typically understood to promote antiviral and cell-mediated immunity.

T helper type 2 cells (Th2)

A subset of CD4⁺ T cells that is driven by the transcription factor GATA3 and typically produces the cytokine IL-4. Th2 cells are involved in the response to some extracellular pathogens and have been implicated in the pathogenicity of allergic responses.

T helper type 17 cells (Th17)

A subset of CD4⁺ T cells that is driven by the transcription factor ROR γ T and typically produces the cytokine IL-17. Th17 cells are involved in mucosal immunity and autoimmunity.

T follicular helper cells (Tfh)

A subset of CD4⁺ T cells that is driven by the transcription factor Bcl6 and typically produce the cytokine IL-21. Tfh provide B cell help in the germinal center for antibody affinity maturation and plasma cell development.

Regulatory T cells (Treg)

A subset of T cells that is driven by the transcription factor FoxP3. Regulatory T cells inhibit other T cell responses.

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Trends

- Chronic viral infections can impair CD4⁺ T cell responses, with a loss of Th1 function.
- CD4⁺ T cells are skewed towards a T follicular helper (Tfh) lineage during chronic viral infections in mice and in humans and away from a potentially more antiviral Th1 state
- A Tfh-like program can also occur in some CD8⁺ T cells perhaps giving these cells access to the B cell follicle where they might interact with viral reservoirs.
- Increased Tfh cells may assist CD8⁺ T cells and prevent immune-mediated damage when a viral infection cannot be cleared.
- Tfh cells provide help to B cells for antibody class-switching and affinity maturation, but despite a Tfh bias in chronic viral infections, effective humoral immunity is often impaired.

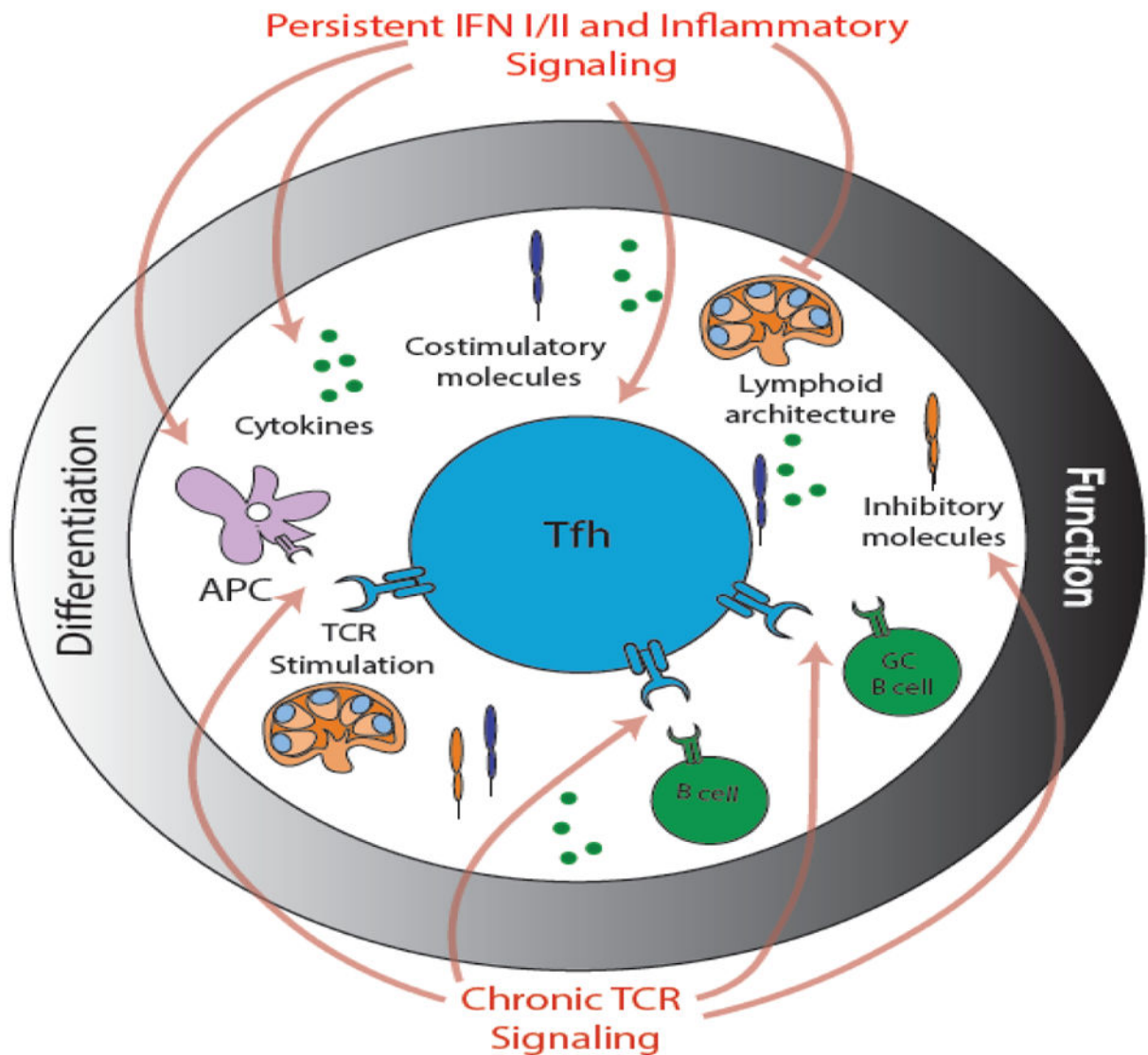


Figure 1. Tfh cell differentiation during acute and chronic viral infections

Commitment to the Tfh lineage after an acute viral infection is a multi-step response requiring both priming by antigen presenting cells (APCs) and additional interactions with B cells. Tfh cell differentiation is further informed by a balance of cytokine, costimulatory, and inhibitory signals that allow the Tfh cell program to mature. Following differentiation, the function of Tfh cells is informed by anatomic location and by the tight coordination of Tfh cell interactions with B cells within the follicle and germinal center. These spatial interactions are guided by the lymphoid architecture. Chronic viral infections are associated with increased Tfh frequencies, increased Tfh-like transcriptional programs in CD8⁺ T cells, as well as by a shift from high quality antibody production, to lower quality, albeit higher quantity of antibody production. The mechanisms that drive this process remain incompletely understood, but continued TCR stimulation and interferon (IFN) signaling due to chronic viral infection presumably affect many components of Tfh cell differentiation and function, including disruption of the lymphoid architecture; changes in the polarization of

the APC; direct effects on B cells and germinal center (GC) B cells; alterations in the cytokine milieu; and changes in costimulatory and inhibitory molecule expression as a result of persistent TCR stimulation.

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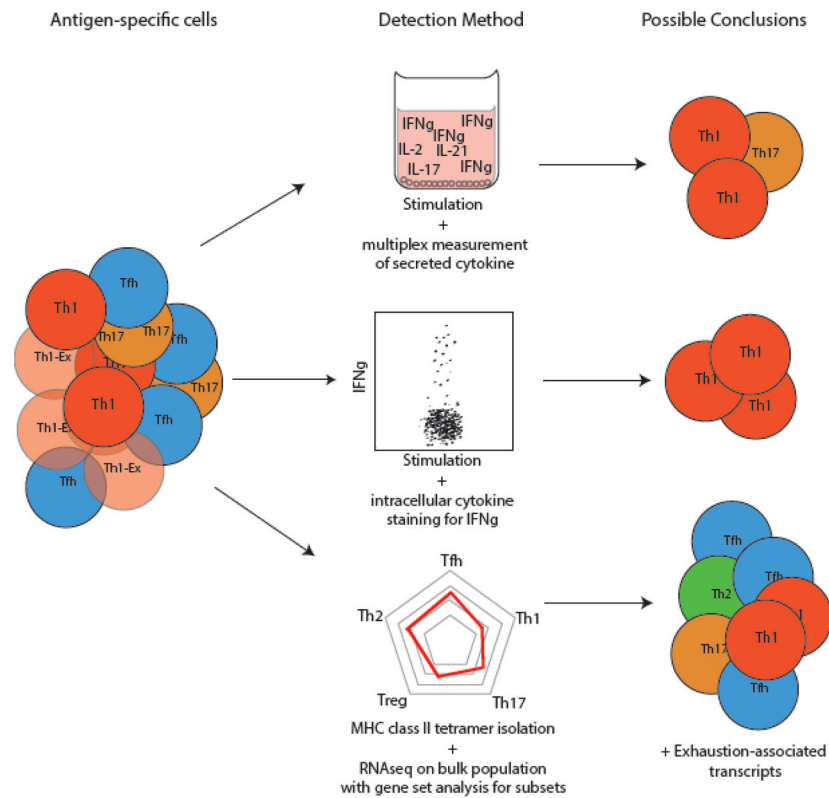


Figure 2. Limitations of the most commonly used assays to characterize CD4⁺ T cell responses in humans

Virus-specific CD4⁺ T cells are heterogeneous, and assays that rely on cytokine production alone will inherently miss the breadth of specific cells. This poor detection is especially true in chronic viral infections, where T cells can exhibit impaired cytokine production (Exhausted Th1 cells, Th1-Ex). Use of *in vitro* stimulation and assessment cytokine production by multiplex immunoassay allows for a diverse capture of secreted cytokines but does not detect cells with minimal cytokine secretion and this approach is unable to identify the sources of expression and co-expression. Flow cytometry after *in vitro* stimulation allows for resolution of individual cells but is limited to cells that produce robust quantities of cytokines. Recent studies have used activation induced markers to identify activated cells that do not produce readily detectable cytokine upon TCR stimulation. HLA class II tetramers allow for the isolation of all CD4⁺ T cells with specificity for a given epitope, regardless of whether the cells can proliferate or produce cytokines. Transcriptional and epigenetic analyses of these cells gives a broad understanding of their phenotypes, although the phenotype of identified cells is likely biased by the epitope. Further, bulk transcriptional analyses of even tetramer-sorted cells are still limited by the inability to resolve whether transcriptional programs are expressed in separate cell populations or whether genes are co-expressed in the same cells. Single cell approaches such as flow cytometry, mass cytometry, and single cell sequencing allow for resolution of such true heterogeneity.