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TOPIC HIGHLIGHT

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Memory CD8+ T cell differentiation in viral infection: A cell for all seasons

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Abstract

Chronic viral infections such as hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) are major global health problems affecting more than 500 million people worldwide. Virus-specific CD8+ T cells play an important role in the course and outcome of these viral infections and it is hypothesized that altered or impaired differentiation of virus-specific CD8+ T cells contributes to the development of persistence and/or disease progression. A deeper understanding of the mechanisms responsible for functional differentiation of CD8+ T cells is essential for the generation of successful therapies aiming to strengthen the adaptive component of the immune system.

Key words: Viral infection; Hepatitis C virus; Memory T cell phenotype; Differentiation

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INTRODUCTION

The adaptive immune response is characterized by the ability to respond specifically and quickly to antigens that the host has encountered previously. Virus-specific CD8+ T cells critical in this response can be divided into naïve, effector and memory CD8+ T cells. In the strictest sense, the memory response should be maintained in the absence of antigen, poised to respond quickly, specifically, and with sufficient amplitude to protect the host from repeated infection by a previously encountered pathogen[1,2]. The ability to survive in the absence of antigen differentiates memory T cells from effector cells that exist at the peak of the immune response, while antigen is present. However, in the context of viral infection, differentiation from effector T cells into memory cells may differ depending on the nature of the pathogen.

Many viral infections are acutely cleared by the immune response, whereas others result in persistent infection and are associated with altered differentiation of host T cells. For example, antigen-specific CD8+ T cells isolated from persons with resolved infections such as influenza (Flu) or respiratory syncytial virus (RSV) clearly represent functional memory. With "latent" infections such as Epstein-Barr virus (EBV) or cytomegalovirus (CMV) where low level of virus may still be intermittently present, a strict definition of memory may not be appropriate for these antigen-specific CD8+ T cells[2]. In fact, van Leeuwen *et al*[3] proposed to classify these cells as "resting vigilant effector cells" due to their ability to continuously control the latent virus. Currently, neither the frequency nor mechanism of re-encounter with antigen after resolved, primary EBV or CMV infection is well understood. For chronic viral infections such as HIV, hepatitis B virus (HBV) and hepatitis C virus (HCV), isolated antigen-specific CD8+ T cells also may not represent true memory T cells, but rather effector-type cells, or perhaps a population comprising effector and memory cells.

The criteria used to define the differentiation of virus-specific T cells are complicated. In mouse models of viral infection, time after experimental infection is often used to delineate effector T cells from memory T cells. For example, after experimental LCMV infection and clearance, d 40+ has been set as a time at which stable memory CD8+ T cells can be isolated, with many memory qualities being acquired between d 8 and 21 post infection[4,5]. In addition to time-after-infection, a number of surface antigen markers have been used to differentiate effector CD8+ T cells from memory CD8+ T cells, and to differentiate subsets of memory cells that possess varied levels of differentiation and function. Activation markers

such as CD38 and HLA-DR are often used to identify effector cells which up-regulate these markers during an immune response and which are generally not expressed on quiescent memory cells. Memory cells that re-encounter antigen re-express these activation markers[6]. Surface expression of the IL-7 receptor-alpha (CD127) is also used to differentiate effector from memory cells. Naïve and memory cells require an ability to survive and proliferate in the absence of antigen, a process called "homeostatic proliferation". Cytokines, such as IL-7, and signaling through CD127 are critical in this process[7]. Naïve cells exposed to antigen decrease expression of CD127 on their cell surface (effector phenotype). Once antigen is cleared, CD127 is re-expressed on memory cells and enables their maintenance. This re-expression characterizes them as memory cells. However, CD127 expression may not be a fool-proof marker of memory, since some subsets of CD8+ T cells identified as memory CD8+ T cells express only low levels of CD127, particularly in latent/chronic viral infections. Whether these cells represent true memory in the strict sense of the definition, or rather represent a population of effectors amidst memory cells is not yet fully elucidated. Finally, immediate cytolytic activity may be the best way to differentiate effector from memory T cells, though some memory cell types also possess immediate cytolytic activity albeit at lower levels[8].

Improved understanding of functional memory CD8+ T cell development and the identification of unique phenotypic markers of memory CD8+ T cells could be helpful in vaccine development for viral infections where adaptive immune responses play an important role in control and/or clearance (HIV, HBV, HCV). In theory, if efficient memory T cell responses could be induced by vaccination, protective immunity could be achieved. Additionally, further enlightenment into the transition from effector CD8+ T cell into memory CD8+ T cell could aid in the discovery and use of immune modulating therapies that might heighten the response to vaccination. As an example, adoptive transfer studies in mice of antigen-specific CD8+ CD127+ T cells taken during the effector phase of the immune response to LCMV infection have identified CD127 as a marker of CD8+ T cells able to control virus upon re-infection[5,9]. Therefore, it can be hypothesized that vaccine induction of CD127 expression on CD8+ T cells may be beneficial and should be a goal of any effective vaccine for chronic viral infections. In this review, we will summarize current knowledge of antiviral CD8+ T cell differentiation with a focus on persistent infections such as HCV.

MODELS OF MEMORY CD8+ T CELL DIFFERENTIATION

Several models have been proposed to describe the differentiation of CD8+ T cells from naïve cells to memory cells[10,11]. In the "linear" or "progressive" model, naïve cells undergo an effector T cell phase prior to developing into memory cells, and all memory T cells are direct descendents of effector cells. This model posits that memory T cells do not develop until antigen is markedly reduced or eliminated. In the "divergent model", a stimulated naïve T cell gives rise to either an effector or memory T cell. In this model, naïve T cells can directly give rise to memory T cells without going first through an effector phase. The "decreasing-potential" model accounts for scenarios where antigen persists after primary infection and posits that naïve T cells differentiate into effector cells first. If antigen is cleared early after infection, functional memory T cells develop. If antigen persists, the function of the effectors is sequentially impaired and memory CD8+ T cell development is compromised. Eventually, persistent antigen leads to a non-functional effector cell and eventual cell death by apoptosis. As in the linear model, functional memory cells do not develop until antigen is cleared.

Subsets within the memory CD8+ T cell compartment have been segregated based on markers other than CD127. Sallusto *et al*[12] utilized the expression of the lymph node homing receptor, CCR7, and a transmembrane phosphatase involved in T cell signaling, CD45RA, to distinguish central memory (CCR7+ CD45RA-) and effector memory (CCR7-CD45RA- and CCR7-CD45RA+) CD8+ T cell populations[12]. Central memory cells were characterized by rapid proliferation after antigenic stimulation, while effector memory cells were more capable of immediate effector functions[12]. In theory, central memory cells are most capable of surveying lymph nodes and responding to antigen with enhanced proliferative capacity, while effector memory cells are more capable of migrating into tissues and exacting immediate effector functions. Appay *et al*[13] have proposed another model of CD8+ T cell differentiation during chronic or persistent infection. Studying antigen-specific CD8+ T cells during several different viral infections, they hypothesized that there is a progressive memory differentiation based on differential expression of CD27 and CD28 co-stimulatory molecules[13]. They defined early (CD27+, CD28+), intermediate (CD27+, CD28-) and late memory CD8+ T cell subsets of virus-specific cells[13]. The early subset had the greatest proliferative capacity while the intermediate and late subsets had progressively greater cytotoxic potential[13]. Furthermore, the late subset also expressed CD57, a marker of replicative senescence[13].

Recently, Romero *et al*[14] combined the phenotypic markers used by Sallusto *et al*[12] and Appay *et al*[13] to further dissect the memory CD8+ T cell pool. They identified four subsets within the effector memory (CD45RA-, CCR7-) pool based on differential staining of CD27 and CD28. Interestingly, the different subgroups differed not only phenotypically, but showed a progressive reduction in telomere length coinciding with a progressive increase in cytotoxic molecules (granzyme B, perforin). Their model lends support to the idea that there is a progressive up-regulation of cytolytic activity and a stepwise loss of CCR7, CD28 and CD27 during the differentiation process. They showed that CD8+ T cells specific for a resolved infection (Flu) consisted of both a central memory (CCR7+, CD45RA-, CD27+ and CD28+) population and a sub-population of effector memory cells termed EM1 (CCR7-, CD45RA-, CD27+ and CD28+).

They hypothesized that this effector memory population, which has only a low expression of the lymphocyte homing receptor, CCR7, confers memory functions and provides surveillance in peripheral tissues[14].

ACUTE VIRAL INFECTION

Effector phase

In the classic understanding of an adaptive T cell immune response, there is an initial massive expansion of antigen-specific T cells, followed by a period of marked contraction as the pathogen is cleared. This period of expansion and contraction can be referred to as the "acute" or "effector" phase of the immune response. Exceptions to this paradigm likely exist following chronic or latent infections, for example, in CMV infection of humans, where the contraction phase may be more limited[3]. During this process of expansion and contraction, functional memory cells are formed and persist to protect the host from future infection. Upon infection of mice with LCMV, rare naïve T cells specific for cognate antigen increase exponentially within secondary lymphoid tissues[15,16]. The responding T cells in this "clonal burst" clear the infection *via* dissemination to non-lymphoid tissues (common sites of infection), the secretion of anti-microbial cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), and direct lysis of infected host cells[17-19]. Interestingly, no antigenic stimulation is needed after the initial clonal stimulation, meaning that daughter-cell expansion and differentiation into a memory population occurs in an antigen-independent manner[20-22]. However, mediation of effector functions, such as cytokine production and killing, is dependent upon contact with antigen bearing targets. In situations where virus is successfully eliminated, > 90% of effector T-cells undergo apoptosis (contraction), and a small population of CD127+ surviving cells undergo further differentiation from an activated phenotype to a resting phenotype to generate a long-lived memory pool that is ready to respond more rapidly upon secondary infection[5,10]. This pool is maintained in the absence of antigen, and is characterized by IL-7 and IL-15-dependent homeostatic proliferation resulting in relatively constant numbers of CD8+ T cells[23-25]. During the progression from naïve to effector to memory cells, the homing potential of these cells changes. Upon differentiation to effectors, CD8+ T cells down-regulate lymphoid homing molecules such as CD62L and CCR7, and begin to migrate to nonlymphoid effector sites. Eventually, these lymphoid homing molecules are gradually up-regulated, giving cells the ability to home to lymphoid tissues. This dichotomy of homing potential has prompted researchers to further define memory cells into effector memory or central memory subsets, with the latter having lymphoid homing potential[26,27].

Much of what we know about memory T cell differentiation is from murine models of infection since identifying humans in the acute phase of viral infection is often difficult, and time from acquisition of infection is rarely precisely known (exceptions described below for accidental infection). Additionally, for some viral infections the acute phase of infection may be relatively short in duration, while for other infections the "acute" phase may last for weeks to months (HCV). Nevertheless, studying the phenotype of antigen-specific CD8+ T cells during different human acute viral infections has shown that there is a remarkable similarity in terms of surface marker expression and function. During acute infectious mononucleosis, EBV-specific CD8+ T cells show massive expansion (up to 44% of total CD8+ T cells in peripheral blood), and the majority express the activation markers, HLA-DR and CD38, and the memory marker CD45RO (also CD45RA low), but have down-regulated the lymphocyte homing molecule CD62L[28]. CD28 expression on EBV-specific cells has been shown to range from 9% to 86% depending on the donor, with CD57 expression ranging from 2% to 37%. Appay *et al*[13] have also shown that during acute infection, some EBV- specific CD8+ T cells express the proliferation marker Ki67. Additionally, these cells were prone to apoptosis since they expressed minimal levels of the anti-apoptotic factor Bcl-2[13].

In an individual with acute CMV infection, Carmichael *et al*[29] showed that at the peak of clinical symptoms (3 wk after symptom onset), 80% of CMV-specific CD8+ T cells were CD45RO high, CD28 negative, and CCR7 negative. Studying primary CMV infection after kidney transplantation in humans, Gamadia *et al*[30] showed that CMV-specific CD8+ T cells evaluated at wk 31 after first positive CMV PCR, were nearly all CD27 positive, but with mixed expression of CD28 (54%). Nearly all expressed CD45RO (minimal CD45RA), and most were CCR7 negative (91%)[30]. They expressed significant levels of Ki67 (78%), granzyme B (93%) and perforin (100%)[30]. Few of these acute CMV-specific CD8+ T cells expressed CD127 (1%)[30].

Other viruses known to cause more persistent infections including HIV, HBV, and HCV, still show characteristic effector T cell development in the early stages after acute infection. For example, in primary HIV infection, nearly all HIV-specific CD8+ T cells expressed CD38 and approximately 11%-41% were Ki67 positive[13,31]. There was minimal Bcl-2 expression, which correlated inversely with CD38 expression[13]. Very early after HIV infection, the majority of HIV-specific CD8+ T cells also expressed CD27 and approximately 40% expressed CD28[31].

Evaluation of 5 persons with acute HBV infection showed that the majority of HBV-specific CD8+ T cells were HLA-DR positive (92%-98%) and CD45RO positive (95%-100%), and most were CCR7 and CD45RA negative, again consistent with an effector phenotype[32]. The majority expressed CD27, and similar to HIV, 40%-50% expressed CD28[32]. The frequency of CD127 expression on these antigen-specific cells was also very low[33].

The phenotype of HCV-specific CD8+ T cells from the peripheral blood of patients evaluated during the acute phase of HCV infection also showed the characteristic expression patterns of effector T cells[13,34]. Studying 9 patients with acute HCV infection, Lechner *et al*[34] demonstrated that during acute infection, the activation marker CD38 was up-regulated on HCV specific CD8+ lymphocytes from all patients irrespective of their clinical outcome. By wk 20 after the acute phase, there was a loss

of expression of this activation marker on HCV-specific CD8+ T cells[34]. Similarly, HLA class Ⅱ was elevated early during infection and decreased over time[34]. Studying five health care workers exposed to HCV via accidental needle stick, Thimme *et al*[35] demonstrated that HCV-specific CD8+ T cells detectable from a patient with spontaneous viral clearance expressed CD38 on wk 8 and 10 after infection, but by wk 12 and thereafter they were CD38 negative[35]. CD38 expression correlated with hepatitis, as measured by ALT level[35]. Interestingly, these activated cells were unable to produce IFN-γ when stimulated by cognate peptide *in vitro*, and the appearance of HCV-specific, IFN-γ producing CD8+ T cells coincided with the disappearance of CD38 expression[35]. Lechner *et al*[36] studied acute infection in one patient, and noted that in addition to increased expression of CD38 and HLA class Ⅱ on HCV-specific CD8+ T cells early during infection, CCR5 expression was also maximal during the first 20 wk. During the acute phase, CD127 expression was minimal on HCV-specific CD8+ T cells[37,38].

In summary, for the majority of these viral infections, during the acute phase, there is an increase in expression on antigen-specific CD8+ T cells of activation and proliferation markers, and a decrease in lymph node homing molecules and CD127 expression. Cytolytic molecules are increased but anti-apoptotic factors are decreased. In general, CD27 expression remains present and there are intermediate levels of CD28 expression.

VIRAL PERSISTENCE OR CLEARANCE

Memory phase

While acute models of infection in mice have provided much phenotypic and functional insight into memory CD8+ T cell generation, chronic models of infection have shed light on the inadequacies of cellular responses, and how the resulting persistence of antigen load can affect the differentiation and function of these antigen-specific cells. Indeed, the course of memory CD8+ T cell differentiation during chronic infections can vary greatly from that which is seen in acute infections, including unique tissue distribution of antigen-specific T cells, dominance of T cell populations that normally have subdominant specificities, and even gradual exhaustion or deletion of entire T cell populations from the repertoire[25]. The hallmark of differentiation in chronic infection is a stepwise loss of T cell effector functions that becomes more severe as time progresses, as opposed to the gain of effector functions that is seen in acute infections[39]. This "exhaustion" can be broken into several categories, corresponding to the severity of impairment of effector function and proliferative potential. Initial antigen stimulation leads to CD8+ T cells that are functionally competent in that they can produce IFN-γ, TNF-α, IL-2, are cytolytic, and have robust proliferative capacity. However, if virus persists, these cells become partially exhausted, losing their ability to lyse target cells and produce IL-2 first, followed by decreased TNF-α production[40,41]. Interestingly, cells that are partially exhausted may still have the ability to proliferate and produce IFN-γ, albeit with reduced efficiency. As antigen persists, cells may become fully exhausted, completely losing both effector functions and the ability to proliferate[39,41,42]. It has also been shown in chronic LCMV infection that deletion of antigen-specific CD8+ T cells can occur if antigen load is both extremely high and persistent[41,43]. Additionally, CD4+ T cells play an important role in the chronic exhaustion of CD8+ T cell responses, both throughout infection and in the priming of cells during the acute phase of infection. Unlike antigen load, CD4+ T cell help is directly related to the functionality of the CD8+ T cell effector response: the absence of this help leads to a more rapid and severe progression to the exhausted phenotype[25].

Unlike the similarities in the range of phenotype of antigen-specific cells seen during the acute phase of different viral infections in humans, the phenotype of antigen-specific cells isolated during different latent/chronic viral infections is more diverse. In the chronic phase of HCV infection, Lechner *et al*[36] were unable to detect the activation markers CD38 or HLA class Ⅱ expression on any HCV-specific CD8+ T cells. This is different from HIV infection, where during chronic infection a proportion of HIV-specific CD8+ T cells in the blood expressed CD38 and HLA-DR[44]. Appay *et al*[13] compared HIV, CMV, EBV and HCV-antigen specific CD8+ T cells taken from blood during the latent/chronic stage of infection. Though the majority of antigen-specific CD8+ T cells for these viral infections had all lost evidence for activation (minimal CD38 expression) and proliferation (minimal Ki67 expression), and had up-regulated the survival factor, Bcl-2, the expression of CD27 and CD28 differed[13]. The majority of HCV-specific CD8+ T cells expressed both CD27 (90%) and CD28 (90%), while EBV-specific CD8+ T cells had comparable levels of CD27 expression but lower CD28 expression (60%). HIV-specific CD8+ T cells had relatively high levels of CD27 expression (80%), but very low levels of CD28 expression (10%). Finally, the majority of CMV-specific CD8+ T cells had relatively low expression of both CD27 (30%) and CD28 (20%). This comparison of differentiation in the chronic/latent phase of multiple infections prompted the authors to label EBV- and HCV-specific CD8+ T cells "early", HIV-specific CD8+ T cells "intermediate" and CMV-specific CD8+ T cells as "late" differentiated. CCR7 expression was low on HIV-, CMV- and EBV-specific CD8+ T cells[13], whereas others have shown that peripheral HCV-specific CD8+ T cells are largely CCR7+[45,46]. Others have shown that EBV-specific CD8+ T cells may be better represented as a mixture of effector memory and central memory cells (or early and late differentiation states) as delineated by differential staining of CCR7 and CD45RA[47].

For cleared viral infections such as influenza and RSV, antigen-specific CD8+ T cells resembled these HCV and EBV-specific cells in that they were mostly CD27+ and CD28+[48,49]. RSV-specific CD8+ T cells were mostly CCR7 negative (92%)[49] in contrast with influenza, where a greater frequency of specific CD8+ T cells were CCR7 positive[49]. The range of CCR7 expression on both RSV and Flu, however, were broad among different patients in this study (0%-71% for RSV and 0%-57% for Flu)[49].

Similar to the mouse, CD127 expression appears to be

Table 1 Phenotype of antigen-specific CD8+ T cells found in blood and tissue during different viral infections

Virus	Peripheral blood phenotype	Tissue	Tissue phenotype
Flu	CD27+ CD28+ CCR7+ CD127+	Lung	CD27- CD28-
RSV	CD27+ CD28+ CCR7- CD127+	Lung	CD27- CD28-
EBV	CD27+ CD28+ CCR7- CD127-	Tonsil	↑ CD38 ↑ CCR7 ↑ CD127
CMV	CD27- CD28- CCR7- CD127- CD45RA+	Tonsil	↑ CD127
HIV	CD27+ CD28- CCR7- CD127-	Rectum	CCR7- CD127- ↓ perforin
HCV	CD27+ CD28+ CCR7+ CD127+	Liver	↑ CD69 CCR7- CD127-
HBV	CCR7+ CD127+	Liver	↑ HLA-DR

Arrows indicate increase or decrease relative to expression in blood.

associated with memory phenotype and viral clearance of some infections in humans. CD8+ T cells isolated from persons with resolved viral infections such as influenza or RSV expressed high levels of CD127[50]. Similarly, longitudinal analysis of 6 patients with acute resolving HBV infection showed that after viral clearance, CD127 expression increased markedly and correlated with the loss of CD38 and PD-1 expression, acquisition of CCR7 expression and enhanced proliferative capacity[33]. In contrast, for latent infections (EBV, CMV) and persistent viral infections (HIV) low levels of CD127 have been noted on virus-specific CD8+ T cells[50-52]. However, in EBV infection CD127 expression was higher on cells specific for latent epitopes compared with lytic epitopes[47]. When we evaluated CD127 expression on peripheral HCV-specific CD8+ T cells from patients with chronic HCV infection, we were surprised to find that in the majority of patients, nearly all expressed high CD127 expression despite the high level of antigen present[53]. This phenotype is reminiscent of resolved infection such as influenza. However, Bengsch *et al*[37] identified two subsets of patients with chronic HCV: One with HCV-specific CD8+ T cells predominantly expressing low levels of CD127 and the other expressing higher levels of CD127. Interestingly, the CD127 low group also had higher level of CD38+ frequencies and lower level of CCR7 expression hinting that in this group, re-activation of these cells may have induced the down-regulation of CD127.

Table 1 summarizes the phenotype of antigen-specific cells from the peripheral blood of persons with resolved, latent and chronic viral infections. There is substantial heterogeneity between the different viral infections, as noted in the table.

MECHANISMS OF VARIED MEMORY DIFFERENTIATION IN PERSISTENT OR LATENT INFECTION

Currently, the explanation for the variation in differentiation phenotype seen in the setting of different viral infections is not completely understood, but a number of hypotheses exist. Clonal expansion, effector functions and memory formation require three signals during the immune response: antigen (signal 1), co-stimulation (signal 2) and cytokine (signal 3)[54]. Alterations in these signals could contribute to alterations in CD8+ T cell phenotype and function. Optimal expansion and function of naïve CD8+ T cells required antigen and co-stimulation to be present for approximately 36 h and cytokine (IL-12) present from about h 12 to h 60[54,55]. These signals launch a complex program of proliferation and differentiation. Given this "autopilot" response[56], the quality and context of the original signal may have a critical impact on subsequent T cell differentiation[57]. Differing numbers of naïve precursor cells, antigen loads, cytokine milieu and primary location of infection (lymph node, gut, lung, liver) seen with the different viral infections would offer additional possible explanations for the diversity of these CD8+ T cells. In line with this hypothesis, Marzo *et al*[58] have shown that initial precursor frequency is critical in determining effector and central memory CD8+ T cell differentiation. Increasing the input number of antigen-specific CD8+ T cells during the primary immune response resulted in increasingly larger populations of central memory cells. Furthermore, effector memory CD8+ T cells generated from high or low numbers were fundamentally different, in that cells generated from low initial naïve T cell precursor frequency were unable to interconvert and re-express CD62L[58].

Even after establishment of persistent viral infection, initial events in naïve T cell proliferation and differentiation likely continue to play a critical role in the varied CD8+ T cell differentiation phenotypes that are seen in different viral infections. Vezys *et al*[59] have recently shown that during persistent viral infection, there is a continuous recruitment of naïve T cells that contributes to the heterogeneity of antiviral CD8+ T cells. In their model, antigen-specific memory T cells were not maintained in the presence of antigen without replenishment from thymic emigrants. By induction of a partial hematopoietic chimerism in persistently infected mice using busulfan and congenic bone marrow, they showed that there were variations in the expression of CD27, CD62L, CD127 and bcl-2 between cell populations primed at different times[59], and that heterogeneity in the memory population was related to this. Their study certainly complicates the current models of T cell differentiation described above, and highlights the dynamic nature of chronic viral infections, even despite relatively stable levels of viral load measured in the peripheral blood of patients with chronic HBV, HCV or HIV.

After initial viral infection and programming of naïve CD8+ T cells, downstream events may also impact on CD8+ T cell differentiation. Wherry *et al*[60] have recently shown that in the setting of chronic infection, it is viral antigen and extensive division of virus-specific CD8+ T cells that maintains cell numbers, in marked contrast to the slow turnover seen during homeostatic proliferation of memory T cells from cleared viral infection. These cells, in the context of persistent infection, would be expected to display a different phenotype from those isolated from cleared infection, given differences in cell turnover. After

naïve CD8+ T cells are activated and become effector cells, they shortly lose the ability to produce IL-2 upon re-encounter with antigen and co-stimulation, a condition referred to as activation induced non-responsiveness (AINR)[54]. IL-2 (provided by CD4+ T cells) is able to reverse this state. Co-stimulation via molecules other than CD28, such as OX40 (CD134), and 4-1BB (CD137) may also be critical in providing a stimulation for continued expansion once AINR develops[54]. Interestingly, we have shown in chronic HCV infection that the co-inhibitory molecule, PD-1, is highly expressed on HCV-specific CD8+ T cells and on total CD8+ T cells at the site of infection in the liver[53]. It is possible, that a lack of adequate co-stimulation and/or vigorous co-inhibitory signals prevent reversion of AINR and explains the exhaustion and CD8+ T cell deletion seen during this chronic viral infection. In addition to loss of IL-2 production, chronic antigen stimulation eventually also leads to characteristic progressive loss of TNF-α and finally IFN-γ production by cytotoxic CD8+ T cells[39,57,61]. Since cytokines alone have been shown to induce T cell differentiation[62] a lack of available cytokine during persistent infection may also explain altered differentiation patterns seen with different viral infections. In a transgenic mouse model, HBV-specific CD8+ T cells are rapidly induced to produce IFN-γ when they enter the liver, but are then rapidly suppressed despite continued antigen[63]. Suppression of cytokine production was mediated by the co-inhibitor, PD-1, since blockade of PD-1 led to a delay in the suppression of IFN-γ producing cells[63].

The potency of the pathogen and the antigen load may also influence memory differentiation. In a mouse model of infection, reducing the stimulation of CD8+ T cells by using an attenuated pathogen led to primarily a central memory subset, while infection with a more virulent pathogen led to effector cell development[64]. Tussey *et al*[65] compared the phenotype of HIV-specific CD8+ T cells in the setting of viral control (using anti-retroviral medication) and in the setting of uncontrolled viremia. They showed that the phenotype of these antigen-specific cells differed based on the level of viremia, and hypothesized that the level of antigen burden determined the differentiation state. Similarly, Papagno *et al*[66] showed that in HIV infection excessive levels of antigen stimulation as determined by level of HIV disease progression lead to a progressive differentiation toward a state of replicative senescence. Given these studies, the importance of the quantity and duration of persistent antigen would seem to be very important factors leading to varied differentiation programs. However, in chronic HCV infection, antigen burden is large, with viral loads often on the order of 10^6-10^7, yet as previously described, differentiation of HCV-specific CD8+ T cells is more limited and these cells have been termed "early". Viral escape is one possibility for the lack of progressive differentiation, yet when we sequenced HCV isolated from the peripheral blood at the epitope specific for the tetramer used to identify these "early" differentiated HCV-specific CD8+ T cells, no mutations were seen[53]. Another possibility to explain variation in differentiation phenotype is differences in innate signals among different infections, and differences in these signals occurring at early and late time-points during persistent infection[67]. This could be particularly relevant for HCV infection, since it has been shown that the NS3-4A serine protease of HCV degrades the adapter molecule, Cardif[68], and thereby interferes with the RIG-I mediated process of innate recognition of dsRNA. The "early" differentiated phenotype of HCV-specific CD8+ T cells during chronic infection could be a reflection of this impairment in innate signaling. Pulendran *et al*[67] has also speculated that during the early stages of an immune response, highly stimulatory DC subsets might deliver strong TCR signals favoring effector T cell differentiation, while at later stages, a milder form of T cell stimulation by less stimulatory DC subsets could favor the development of central memory T cells.

Location, location, location

Finally, a perhaps somewhat overlooked cause for differences in memory phenotype in different viral infections may be related to the anatomic location of the different viral infections, and to discrepancies between the active site of infection (liver, lung, *etc.*) and the site from which cells were obtained for study. Mice infected with vesicular stomatitis virus (VSV) developed memory cells with functional differences in cytolytic activity based on their location in either tissue (lung, liver, small intestine) or secondary lymphoid organs, highlighting the importance of anatomic location in type of isolated memory cell subset[69]. Masopust *et al*[70] have also shown that virus specific intraepithelial lymphocytes in the gut resemble neither central nor effector memory CD8+ T cells isolated from spleen or blood by almost all properties examined, including effector function, differentiation, homing receptors and cell cycle. In fact, memory CD8+ T cells changed phenotype upon change of location[70].

As noted above, we were surprised to find a phenotype of HCV-specific CD8+ T cells that resembled the phenotype of a resolved infection (Flu) in terms of high expression of CD127, CCR7, CD28 and CD27. However, analysis of antigen-specific cells at the site of active infection, the liver, revealed that nearly all of the antigen-specific CD8+ T cells displayed a different phenotype, with low CD127 expression[37,53]. Similarly, Accapezzato *et al*[71] have shown that HCV-specific CD8+ T cells isolated from liver express markedly lower CCR7 expression in comparison with peripheral blood. HCV-specific CD8+ T cells in the liver also displayed an activated phenotype with elevation of expression of the early activation marker CD69[72]. We hypothesize that a lack of exposure to antigen by the peripheral CD8+ T cells enabled up-regulation of CD127 and CD62L and memory formation, since exposure to cognate peptide *in vitro* induced a down-regulation of CD127 on these same cells. The effector-like cells isolated from the liver were likely actively involved in the immune response occurring at the site of infection. Similar to our findings with chronic HCV infection, peripheral blood HBV-specific CD8+ T cells expressed high levels of CD127 during chronic HBV infection despite high levels of antigen load[73]. Analysis of HBV-specific CD8+ T cells in the liver of patients with resolving HBV infection showed that a greater frequency

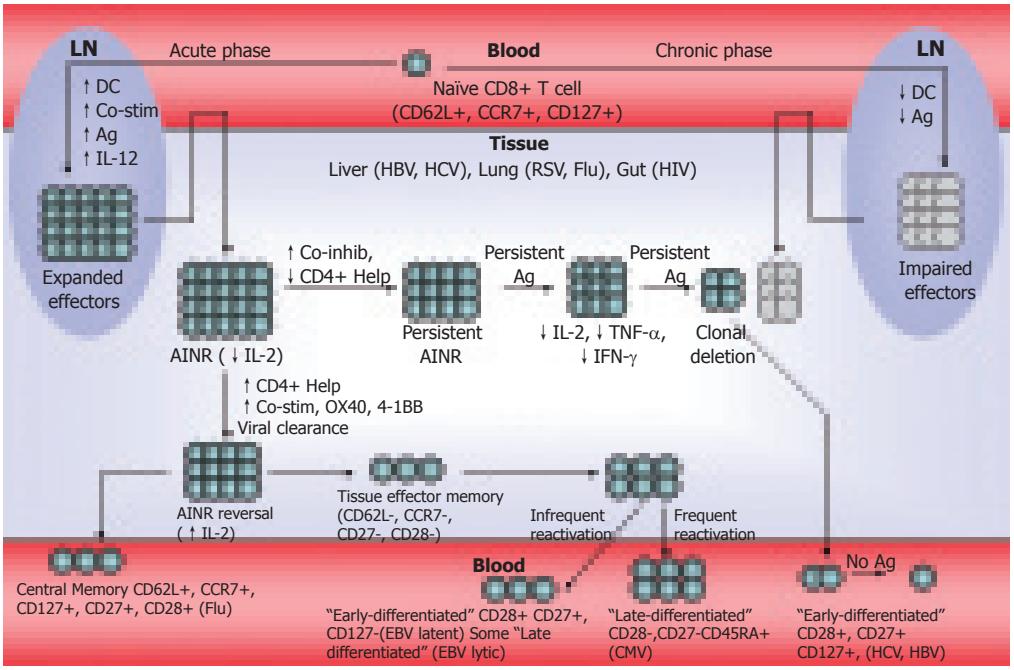

Figure 1 Tissue-specific model of antigen-specific memory CD8+ T cell differentiation.

were activated, as demonstrated by HLA-DR expression, in comparison with the peripheral blood[74]. It will be interesting to further analyze the phenotype of these HBV-specific cells at the site of infection.

In addition to the liver, human memory CD8+ T cells at other locations are clearly influenced by the anatomic site where they reside. De Bree *et al*[75] have compared Flu and RSV-specific CD8+ T cells in the lungs and peripheral blood. They found that the lung contained markedly higher frequencies of Flu-and RSV-specific cells compared with the peripheral blood[75]. A substantial percentage of these lung residing antigen-specific CD8+ T cells had progressed to a relatively late differentiation phenotype with low expression of CD28 and CD27[75]. EBV-specific CD8+ T cells isolated from the tonsils of long-term carriers were more likely to express the activation marker CD38 and CD103, an integrin induced by epithelium-derived cytokine TGF-β[76]. Both EBV (lytic)- and CMV-specific CD8+ T cells in the tonsils were shown to have increased CD127 expression in comparison with peripheral blood[47]. In HIV infection, rectal HIV-specific CD8+ T cells expressed a similar effector memory phenotype as in the peripheral blood (CCR7-), but expressed minimal perforin, unlike in the peripheral blood where as many as 23% of Gag-specific CD8+ T cells expressed perforin[77]. Table 1 summarizes the phenotype differences of viral-specific CD8+ T cells between peripheral blood and tissue (liver, lung, tonsil, gut). Clearly, there is much to be learned about memory T cell differentiation by further evaluation of T cells residing in tissues other than peripheral blood.

A MODEL OF TISSUE DEPENDENT MEMORY DIFFERENTIATION

So, how might the variation in differentiation phenotypes of antigen-specific CD8+ T cells from different viral infections and from different anatomic locations look? A schematic is shown in Figure 1. During the acute phase of infection, naïve CD8+ T cells surveying the lymph node (LN) may encounter antigen presented by activated dendritic cells (↑ DC) in the context of high antigen concentration (↑ Ag), significant co-stimulatory signal (↑ Co-stim), and cytokine (IL-12 or type I interferon) (↑ IL-12). This leads to an expansion of competent effectors that have decreased lymph node homing receptors (CD62L, CCR7) and migrate to sites of infection (e.g. liver, lung, gut). These cells lose the ability to produce IL-2 (activation induced non-responsiveness, AINR)[54]. With proper CD4+ T cell help (↑ CD4+ Help) or other co-stimulatory signals, such as via 4-1BB (↑ Co-stim, OX40, 4-1BB), these cells maintain function and succeed in clearing virus. One population of cells forms central memory cells (CD62L+, CCR7+, CD127+, CD45RA-) that have up-regulated lymph node homing molecules and are easily detected in the peripheral blood. A second population of effector memory cells is maintained in the tissue (perhaps by homeostatic mechanisms) (CD62L-, CCR7-, CD27-, CD28-). For the latent viruses, EBV and CMV, we hypothesize that differences in the frequency and/or location of re-activation offers an explanation for the "early" *vs* "late" phenotype. Perhaps CMV reactivation or even low-level persistence occurs at peripheral sites, and sampling of antigen-specific CD8+ T cells from the peripheral blood reflects this (late differentiation). EBV reactivation may be less frequent (and not persistent) and, therefore, a phenotype similar to a resolved infection such as Flu or RSV is sometimes seen (though CD127 expression is diminished). EBV reactivation may also lead to a population of more differentiated cells specific for the lytic epitope. If AINR cannot be reversed, as might be the case with a lack of CD4+ T cell help (↓ CD4+ Help) or via enhanced co-inhibitory signals (↓ Co-inhib), there is a progressive loss of function of virus-specific CD8+ T cells (↓ IL-2, ↓ TNF-α, ↓ IFN-γ) eventually leading to clonal deletion. For HCV and HBV infections,

perhaps at the site of infection in the liver, where antigen is present and concentrated, persistent antigen maintains a population of effector-like cells. As Wherry *et al*[78] have described in chronic LCMV infection, this cell population would not be maintained without antigen present. These effector cells would be expected to express low levels of CD127 and lymph node homing molecules. Additionally, naïve cells encounter antigen during the chronic phase; however in this setting, DCs are less stimulatory (↓ DC) and antigen is lower (↓ Ag) than what is seen during acute infection. The resulting impaired effectors contribute to the pool of antigen-specific cells. In the periphery, HCV- and HBV-specific CD8+ T cells are not maintained by persistent antigen, but rather proceed to form functional memory T cells and are maintained via homeostatic signals. We and others have found that peripheral blood HCV-specific CD8+ T cells expressing CD127 have a capacity for proliferation upon ex vivo antigen encounter similar to other functional memory T cells. For HIV infection (and persistent LCMV infection), where antigen is located in the periphery, these cells are maintained only by the presence of antigen and thereby, display the phenotype of an effector-like cell in the periphery.

CONCLUSION

The phenotype of antigen-specific CD8+ T cells persisting during different viral infections is quite varied. This variation may be related to a number of factors including level of antigen persistence, strength of antigen presenting cell interactions, balance of co-stimulatory/co-inhibitory signals, and the influence of the anatomic location of infection. The requirement of continued antigen in the maintenance of virus-specific CD8+ T cells during chronic viral infection and continued recruitment of naïve CD8+ T cells into the population of antigen-specific cells highlight the dynamic nature of these infections and the cells responding to them. Improved understanding of the relative contribution of each of these factors in the formation of functional memory cells may aid in the development of virus-specific treatments to enhance the immune response to infection or vaccines.

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