Author/s:
Alexandre, YO;Mueller, SN

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PROFESSOR ROBERT THIMME (Orcid ID : 0000-0003-1417-4135)

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Title: Memory versus memory-like: the different facets of CD8+ T-cell memory in HCV infection

Maike Hofmann*[1], Dominik Wieland*[1], Hanspeter Pircher[2] and Robert Thimme[1]
*equal contribution

[1] Department of Medicine II, University Hospital Freiburg, Faculty of Medicine, University of Freiburg, Germany.
[2] Institute for Immunology, Medical Center, Faculty of Medicine, University of Freiburg, Germany.

Correspondence:
Robert Thimme, Department of Medicine II, University Hospital Freiburg, Hugstetter Str. 55, 79106 Freiburg, Germany
Email: robert.thimme@uniklinik-freiburg.de

Running title: Memory versus memory-like CD8+ T cells in HCV infection

Summary
Memory CD8+ T cells are essential in orchestrating protection from re-infection. Hallmarks of virus-specific memory CD8+ T cells are the capacity to mount recall responses with rapid

induction of effector cell function and antigen-independent survival. Growing evidence reveals that even chronic infection does not preclude virus-specific CD8+ T-cell memory formation. However, whether this kind of CD8+ T-cell memory that is established during chronic infection is indeed functional and provides protection from re-infection is still unclear. Human chronic Hepatitis C Virus infection represents a unique model system to study virus-specific CD8+ T-cell memory formation during and after cessation of persisting antigen stimulation.

Keywords
Memory-like CD8+ T cells, HCV, human viral infection, viral escape, T-cell exhaustion

1. Introduction

Immunological memory is a fundamental property of adaptive immunity and mediates protection from re-infection. Next to humoral responses, memory CD8+ T cells play a central role in protective immunity against viral infections. The key to success of memory CD8+ T cells is their rapid and vigorous recall response with rapid induction of effector cell function. A further hallmark of memory T cells is that they can be maintained independently from viral antigen for decades or even lifelong.[1,2] Of note, virus-specific CD8+ T-cell memory formation has primarily been studied in self-limiting infections. However, there is growing evidence that virus-specific CD8+ T-cell memory is also present during viral persistence. This CD8+ T-cell memory in chronic viral infection, however, seems to be functionally different from conventional CD8+ T cell memory in self-limiting infection. In this article, we focus on Hepatitis C Virus (HCV) infection and recapitulate most recent knowledge about T-cell memory formation during and after chronic viral infection and compare these new findings to T-cell memory in self-limiting viral infection.

2. T-cell memory – lessons from the mouse model

Lymphocytic Choriomeningitis Virus (LCMV) infection provides an elegant model to comparatively study CD8+ T-cell memory in self-limiting versus chronic infection, as the outcome of LCMV infection – viral clearance or persistence – can be regulated by the virus strain and dose.

In self-limiting infection, naïve CD8+ T cells are primed by antigen-presenting cells in secondary lymphoid organs initiating activation, clonal expansion and differentiation of the virus-specific CD8+ T cells. During clonal expansion, CD8+ T-cell progenies are comprised of short-lived effector and memory precursor subsets.[3] These memory precursor CD8+ T cells then build up the memory pool that provides long-term protection. Memory CD8+ T cells consist of

heterogeneous sub-populations; central memory, effector memory, peripheral memory, tissue-resident memory and stem memory T cells. These subsets differ in their proliferative capacity, effector functions and migratory characteristics.[4] Thus, CD8+ T-cell memory is based on the concept of division of labor.

In contrast to self-limiting infections, chronic viral infections are known to trigger T-cell exhaustion, which is characterized by progressive functional impairment of virus-specific CD8+ T cells. T-cell exhaustion represents a distinct differentiation pathway in the context of persistent antigen stimulation and deviates from conventional T-cell differentiation. For a long time, it has been suggested that T-cell exhaustion precludes formation of T-cell memory, as key properties, such as antigen-independent survival and recall responses, could not be observed within exhausted LCMV-specific T cells.[5-7] More recently, however, T-cell subsets with memory-like characteristics could be described within exhausted T-cell populations that co-exist with terminally exhausted T cells. In particular, a LCMV-specific CD8+ T–cell subset could be identified in chronic viral infection that expresses the chemokine receptor CXCR5,[8,9] which mediates lymphocyte migration to B-cell zones in lymph nodes.[10,11] These LCMV-specific CXCR5+ CD8+ T cells are restricted to lymphoid tissue, exhibit proliferative capacities and harbor a transcriptional profile overlapping with CD8+ T-cell memory precursors. Of note, CXCR5+ LCMV-specific CD8+ T cells co-express the transcription factor T-cell factor 1 (TCF1). TCF1 was previously described in self-limiting infections to play a role in CD8+ T-cell memory precursor formation and persistence of conventional memory T cells. Next to this CXCR5+ T-cell subset, another LCMV-specific CD8+ T-cell population was identified that expresses TCF1 but lacks the chemokine receptor CXCR5. This T-cell subset could be found in the periphery and contributes to maintaining the exhausted T-cell pool during chronic viral infection.[12] Remarkably, adoptive T-cell transfer studies even demonstrated that this sub-population of TCF1+ LCMV-specific CD8+ T cells can be maintained in naïve mice independent of virus and even provide recall expansion upon re-infection.[12,13] Interestingly, the LCMV-specific CD8+ T cells that survived antigen-independently still exhibited an exhausted phenotype, like expression of the inhibitory receptor PD-1 and did not reach functionality of conventional memory T cells. Due to the differences to conventional memory T cells, the maintained LCMV-specific CD8+ T cells that established during chronic viral infection need to be distinguished from conventional memory T cells and hence were suggested to be “memory-like” (*Fig. 1*).

Although the LCMV mouse model has been proven highly valuable for the study of T-cell memory formation in self-limiting and chronic viral infection, there are certain limitations of this

model. For example, species differences to human and the artificial housing conditions in abnormally hygienic barrier facilities raise some concerns about data transferability from mice to human.[14,15] Furthermore, the time-span to analyze formation of T-cell memory in chronic viral infection is relatively short when compared to human chronic viral infections. T-cell exhaustion in mice is typically analyzed after several weeks of infection, while exhaustion in humans can develop over years or decades. Also the long-term fate of memory T cells is difficult to address in mice. Consequently, studies in human diseases like HCV infection are crucial to elaborate similarities and differences in the concepts of T-cell memory in chronic viral infection between mice and human.

3. HCV infection – a human model to study different facets of CD8+ T-cell memory

According to the World Health Organization (WHO) an estimated 71 million people are globally infected with HCV and thus HCV infection represents a major global health problem. A majority of 70% of HCV-infected patients fails to control the virus resulting in chronic infection and only in about 30% of patients, HCV infection is self-limiting. HCV is a blood-borne, non-cytopathic virus and thus viral infection per se does not result in direct liver damage. However, the emerging antiviral immune response causes slow but ongoing immunopathology. Long-term consequences of chronic HCV infection are progressive liver damage that can lead to end stage liver disease or cancer rendering HCV infection a leading cause of death worldwide. Currently, there is no preventive vaccine available, however, Direct Acting Antivirals (DAA) are approved for HCV treatment with response rates of over 90%.

Similar to LCMV infection, virus-specific CD8+ T cells are the main effector cells that determine the outcome of HCV infection – viral elimination versus persistence. Indeed, self-limiting HCV infection correlates with polyfunctional CD8+ T-cell responses against multiple HCV epitopes[16-18] whereas in chronic HCV infection, virus-specific CD8+ T-cell responses are typically impaired in their antiviral effector functions. Two major mechanisms underlie HCV-specific CD8+ T-cell failure in chronic infection. First, viral mutations frequently occur in HCV epitopes that are targeted by HCV-specific CD8+ T cells due to the error-prone HCV RNA polymerase.[19] Such viral sequence variations can lead to loss of recognition by epitope-specific CD8+ T cells and thus render the HCV epitope-specific CD8+ T-cell responses ineffective, a process called viral escape. Second, persisting antigen stimulation in chronic HCV infection results in HCV-specific CD8+ T-cell exhaustion.[20-26] Insights into general CD8+ T-cell biology in acute versus chronic infection can therefore be gained by comparing HCV-specific CD8+ T-cell responses in self-limiting HCV infection compared to HCV-specific CD8+ T cells targeting escaped versus non-

escaped epitopes in chronic infection. In addition, clearance of chronic HCV infection by DAA treatment enables longitudinal analyses of HCV-specific CD8+ T-cell responses during and after cessation of persisting antigen stimulation. Hence, HCV infection represents a unique and clinically relevant model to study multi-facetted virus-specific CD8+ T-cell memory formation in chronic compared to self-limiting infection.

4. CD8+ T-cell memory in self-limiting HCV infection

Following spontaneous clearance, HCV-specific CD127+ memory CD8+ T cells are maintained.[26,27] These circulating HCV-specific memory CD8+ T cells are comprised of both effector memory and central memory subsets[28] and exhibit proficient capacities to proliferate and to produce cytokines.[26] Rapid recall responses by HCV-specific memory CD8+ T cells have been shown in the chimpanzee model.[29] Indeed, in blood and liver, HCV-specific CD8+ T-cell responses were detectable earlier after re-infection compared to primary infection. In line with this, duration of HCV re-infection is shorter in chimpanzees compared to primary infection even after re-challenge with different HCV genotypes.[30,31] Thus, these observations demonstrate that stable HCV-specific CD8+ T-cell memory formation occurs in self-limiting HCV infection including the hallmark characteristics of CD8+ T-cell memory, namely antigen-independent maintenance and recall expansion upon antigen re-encounter (Table 1).

HCV vaccination studies provided further evidence that protective HCV memory can be established. For example, it was shown in the chimpanzee model that vaccine-induced HCV-specific CD8+ T cells undergo vigorous recall expansion upon HCV infection.[32] Accordingly, HCV-specific CD8+ T cells peaked earlier and expressed lower levels of PD-1 compared to mock-vaccinated animals indicating early control of viremia. Furthermore, results from a phase I study demonstrated that polyfunctional HCV-specific CD8+ T cells that recognize multiple HCV epitopes of heterologous strains are sustained in humans for a long period of time after vaccination.[33] Hence, HCV-specific CD8+ T-cell memory is not only established after self-limiting infection but can also be efficiently induced by vaccination.

5. Memory potential of CD8+ T cells in chronic HCV infection

5.1 Memory-like T cells in chronic HCV infection

In contrast to self-limiting HCV infection, the establishment of HCV-specific CD8+ T-cell memory in chronic HCV infection has long been controversially discussed. Previously, HCV-specific CD8+ T cells in human chronic viral infections mostly were described as a homogeneously

exhausted T-cell population. To address whether these exhausted T-cell populations harbor a memory-like T-cell subset, we recently performed co-expression analysis of CD127 and PD-1.[12,34] Indeed, we observed the co-existence of a CD127+PD-1+ T-cell sub-population with memory-like characteristics next to a terminally exhausted CD127-PD-1^{hi} HCV-specific CD8+ T-cell population targeting the same epitope (*Fig. 2*). In contrast to the terminally exhausted CD127-PD-1^{hi} subset, the memory-like CD127+PD-1+ subset highly expressed TCF1 and the anti-apoptotic molecule BCL2. Furthermore, this memory-like subset contained the proliferative capacity of the HCV-specific CD8+ T-cell population in chronic HCV infection. Based on this observation, memory-like CD8+ T cells may represent a promising target to re-invigorate exhausted CD8+ T-cell populations in humans. Interestingly, the relative proportions of memory-like versus terminally exhausted T cells varied among the different HCV-specific CD8+ T-cell populations. The factors that determine this ratio of memory-like versus terminally exhausted HCV-specific CD8+ T cells are so far unknown. Moreover, it is important to mention that the analysis of memory-like HCV-specific CD8+ T cells was limited to blood. Thus, further investigations are also required to define heterogeneity of memory-like subsets in tissues, including potential memory stem cells in lymphoid organs. Nevertheless, to our knowledge these data revealed for the first time that a virus-specific CD8+ T-cell population with memory characteristics is present in a human chronic viral infection (Table 1).

5.2 Viral escape: establishment of CD8+ T-cell memory in chronic HCV infection

Viral escape is a central mechanism of CD8+ T-cell failure in HCV clearance and detectable in about 50% of targeted CD8+ T-cell epitopes.[35,36] Interestingly, during chronic HCV infection HCV-specific CD8+ T cells that target an escaped viral epitope are maintained and can easily detectable.[22,27,34] This finding indicates that HCV-specific CD8+ T-cell populations in chronic HCV infection have the capacity to survive independent of viral antigen recognition, a hallmark of T-cell memory. Interestingly, studies from our lab and others also demonstrated that HCV-specific CD8+ T cells homogeneously express CD127 when antigen recognition is lost due to viral escape.[22,27] Recently, we also reported co-expression of PD-1 on these CD127+ HCV-specific CD8+ T cells.[34] Hence, HCV-specific CD8+ T cells after viral escape seem to exhibit a phenotype that closely resembles the memory-like sub-population of HCV-specific CD8+ T cells that still recognizes the viral antigen. Importantly, depending on the time point of viral escape HCV-specific CD8+ T cells that target escaped epitopes may significantly differ. Previous data suggest that viral escape mostly occurs during the acute phase of HCV infection.[35] This acute phase of infection, however, lasts for up to 6 months and thereby already represents a relatively

long-lasting infection. Viral escape occurring very early during the acute phase might prevent a T-cell population from ongoing antigen stimulation that is known to promote the development of T-cell exhaustion. Hence, these HCV-specific CD8+ T cells after early viral escape may display a T-cell differentiation that rather resembles that of conventional memory T cells after spontaneous HCV resolution. In contrast, viral escape occurring later during acute infection may already induce some features of T-cell exhaustion within T-cell populations targeting an escaping viral epitope. The idea about a variable phenotype of HCV-specific CD8+ T cells after viral escape is underlined by previous findings that some (but not all) populations of HCV-specific CD8+ T cells after viral escape harbor a fraction of cells lacking PD-1 expression.[22,27] Such a PD-1-negative T-cell subset is characteristic for conventional memory HCV-specific CD8+ T cells after spontaneous resolution, but cannot be found among memory-like HCV-specific CD8+ T cells in chronically HCV-infected patients.[22,34] Taken together, HCV-specific CD8+ T cells that are present after loss of antigen recognition demonstrate phenotypical features of memory T cells, e.g survival independent of viral antigen recognition. Remarkably, there also is evidence for recall capacities of HCV-specific CD8+ T cells targeting escaped epitopes, as these T cells re-expanded upon therapeutic vaccination of chronically HCV infected patients.[37] The capacity to re-expand upon antigen re-exposure could also be confirmed *in vitro*, as HCV-specific CD8+ T cells targeting escaped epitopes exhibited superior proliferative capacities compared to HCV-specific CD8+ T cells targeting intact viral epitopes.[22] In sum, despite ongoing chronic HCV infection, memory formation occurs within HCV-specific CD8+ T-cell populations targeting escaped viral epitopes (Table 1).

6. CD8+ T-cell memory after resolution of chronic HCV infection

A question of central importance is whether a functional HCV-specific CD8+ T-cell memory is established after therapy-mediated resolution of chronic HCV. Indeed, in human it is an open question whether exhausted CD8+ or memory-like antigen-specific CD8+ T cells can re-differentiate into memory cells as soon as ongoing antigen stimulation disappears and whether these evolving/ remaining cells even confer partial protection from re-infection. The recent approval of DAA therapy provided a unique opportunity to address this important issue in chronically HCV-infected patients. By analyzing HCV-specific CD8+ T-cell responses after DAA-mediated HCV elimination, we could demonstrate that TCF1 expressing CD127+PD1+ memory-like subsets persist after resolution of chronic HCV infection. Thus, memory-like HCV-specific CD8+ T cells are capable of antigen-independent survival, a hallmark of T-cell memory.[34] These memory-like subsets are probably maintained by homeostatic signals via IL-7 and IL-15, as they

express the corresponding receptors for both cytokines (CD127 and CD122, respectively).[34,38] Indeed, IL-7 and IL-15 activate STAT5 that in turn results in pro-survival pathways and up-regulation of anti-apoptotic BCL2.[39] In line with this, high expression of BCL2 was reported in memory-like, but not in terminally exhausted HCV-specific CD8+ T cells.[34] Still, it needs to be clarified whether memory-like T-cell populations are maintained for decades, similar to conventional memory T cells.

Interestingly, while memory-like subsets persist, terminally exhausted T cells are lost after DAA-mediated antigen elimination.[34] This finding shows similarities to the contraction phase after acute viral infection, in which memory T-cell populations are maintained after successful antigen elimination, while terminally differentiated effector T cells disappear. Although low expression of BCL2 and high abundance of active caspase-8 within the HCV-specific terminally exhausted CD8+ T cells indicates removal of this subset by apoptosis, it cannot be excluded that these HCV-specific CD8+ T cells that don't express TCF1 have the potential to re-differentiate to the TCF1+ memory-like T-cell pool. Of note, a recent study in acute LCMV infection demonstrated that TCF1- progeny of LCMV-specific CD8+ T cells represent T cells determined for effector fate that have lost the potential to regain TCF1 expression.[40] This supports the idea that HCV-specific terminally exhausted TCF1- CD8+ T cells cannot re-differentiate to a TCF1 expressing memory-like phenotype and instead are depleted after viral elimination.

A further hallmark of memory T cells is the ability to mount recall responses upon re-exposure to viral antigen. In line with this, we demonstrated an improved *in vitro* proliferative capacity of memory-like HCV-specific CD8+ T cells *in vitro* compared to exhausted HCV-specific CD8+ T cells.[34] Nevertheless, the improved functionality after therapy-mediated HCV resolution did not reach functional capacities of conventional memory T cells. In addition, we also monitored HCV-specific memory-like CD8+ T cells in one patient with HCV viral relapse upon antigen re-exposure and again observed strong recall expansion. Unfortunately, the re-expanded T-cell population did not mediate viral clearance and acquired a predominantly terminally exhausted phenotype (*Fig. 3*). This finding is in agreement with the reported presence of functionally impaired HCV-specific memory CD8+ T cells after viral elimination in the chimpanzee model of HCV infection. In this study, HCV infected chimpanzees that successfully were treated with DAAs re-establish chronic HCV infection upon viral re-exposure.[41] Taken together, these findings indicate that memory-like T cells after resolution of chronic infection are capable to mount recall responses that, however, display a reduced functionality (Table 1). Several factors may contribute to this sustained T-cell dysfunction. For example, viral reservoirs might trigger ongoing low-level stimulation of the memory-like HCV-specific CD8+ T cells and thus delay

functional recovery from chronic infection.[42,43] In addition, recent data from the mouse model indicate that the exhausted phenotype is heavily imprinted on an epigenetic level and may thus be permanent.[44] Hence, it remains an important question whether full functionality of memory-like virus-specific CD8+ T cells can recover after chronic viral infection.

Nevertheless, memory-like T cells with a still reduced functionality may also contribute in sum to a relevant antiviral effect. Hence, memory-like HCV-specific CD8+ T cells that are maintained after chronic viral infection may not necessarily confer full protection from reinfection, but may at least hamper the establishment of a secondary chronic HCV infection.

7. Conclusion and perspectives

The past years provided novel important insights into subset diversity of exhausted CD8+ T cells. They also demonstrated partly overlapping characteristics between specific T-cell sub-populations that emerge during chronic viral infection with conventional memory T cells after self-limiting viral infection. One essential task of memory T cells is to remember a previous infection. In chronic viral infection, this task is fulfilled by memory-like HCV-specific CD8+ T cells that not only survive antigen-independently and respond to secondary infections, but also maintain their unique kind of T-cell differentiation, namely T-cell exhaustion. This imprinting of functional exhaustion, however, may be counterproductive as weak functionality of memory-like HCV-specific CD8+ T cells might prevent pathogen clearance. The discovery of memory-like CD8+ T-cell populations with a reasonable functional responsiveness revealed a promising target for immunotherapy and vaccination strategies. Approaches to boost and functionally recover memory-like T cells might increase the capacity of protection from re-infection. The high costs of HCV therapy and the regular incidence of HCV re-infections represent highly relevant rationales for the development of strategies to protect patients from re-infection. Due to the functional impairment and exhaustive imprinting of memory-like T cells, however, reconstitution of a protective immunity after chronic infection still represents a major difficulty. To fully determine the immunotherapeutic potential of memory-like T cells, future investigations are needed that focus on the transcriptomic and epigenetic regulation that distinguish memory-like T cells from fully functional conventional memory T cells. In sum, recent data about the establishment of T-cell memory in chronic viral infection are highly relevant and render the herein discussed memory-like T cells interesting candidates for immune reconstitution to protect patients from re-infection. However, to what extent this functionally impaired T-cell memory after

chronic viral infection can be re-programmed for full functional recovery or even to adopt the conventional memory T-cell differentiation still needs to be clarified.

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Fig Legends

Fig. 1 Virus-specific CD8+ T-cell subsets in chronic viral infection

In chronic LCMV infection, exhausted virus-specific CD8+ T-cell populations consist of terminally exhausted and memory-like T cells. So far, two different TCF1+ memory-like subsets were described that are discriminated by their CXCR5 expression.

Fig. 2 Exhausted HCV-specific CD8+ T-cell subsets

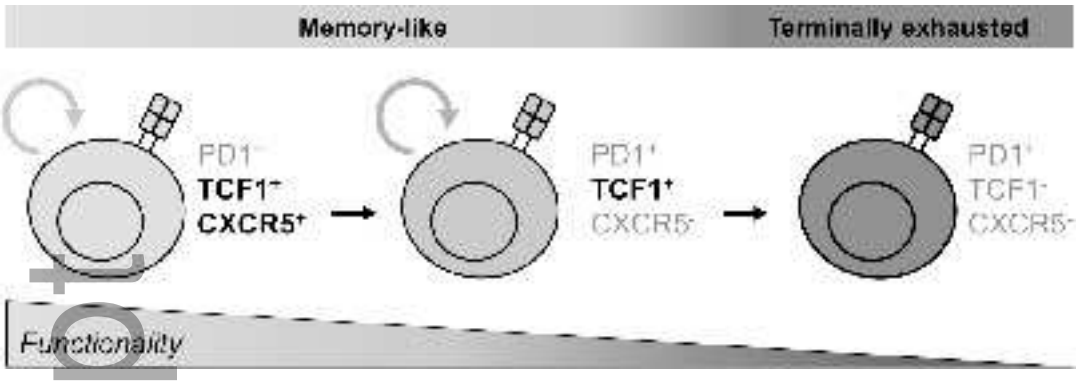
CD127/PD1 co-expression analyses of exhausted HCV-specific CD8+ T cells revealed the existence of different subsets; namely TCF1-CD127-PD-1^{hi} terminally exhausted and TCF1+CD127+PD-1+ memory-like cells.

Fig. 3 Dynamics of HCV-specific CD8+ T-cells after antigen re-exposure

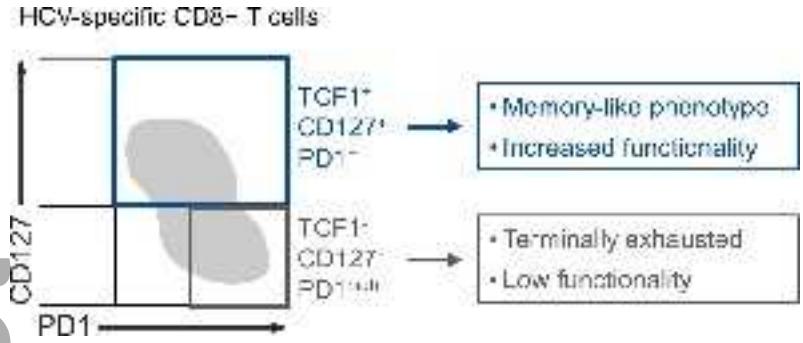
Memory-like HCV-specific CD8+ T cells persist after antigen elimination whereas the terminally exhausted HCV-specific CD8+ T-cell subset disappears. During HCV relapse, terminally exhausted subsets re-appear within the HCV-specific CD8+ T-cell population.

	Self-limiting HCV infection	**During chronic** HCV infection		**After chronic** HCV infection
Inflammation	-	Yes		-
Viral antigen	-	Yes		-
Antigen recognition	-	-	Yes	-
Functionality	++	++/+	+/-	+/- (long-term?)

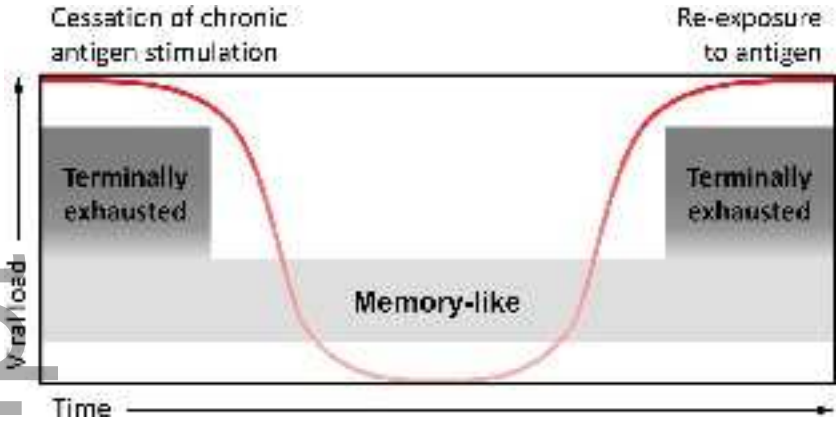
Table 1 Different facets of memory CD8+ T cells in HCV infection



imr_12642_f1.tif



imr_12642_f2.tif



imr_12642_f3.tif