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Review

HCV immunology – Death and the maiden T cell

C Willberg,[1] E Barnes[1] and P Klenerman*,[1]

[1] Nuffield Department of Medicine, Peter Medawar Building for Pathogen Research, University of Oxford, South Parks Road, Oxford OX1 3SY
* Corresponding author: P Klenerman; Tel: 01865 281885; Fax: 01865 281236; E-mail: Klener@molbiol.ox.ac.uk

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Abstract

Cellular immune responses play an important role in the control of hepatitis C virus (HCV), although in the majority of cases they ultimately fail. We examine the mechanisms by which virus-specific T cells may interact with a cell that is infected with HCV and how this interaction may explain the success and failure of the immune response. As an infected cell presenting foreign antigen, the hepatocyte will interact with a large number of lymphocytes, both by direct cell to cell contact and by indirect means through the secretion of cytokines and chemokines. These interactions may lead on the one hand to the death of infected hepatocytes or suppression of viral replication and on the other hand to the death of T lymphocytes or down regulation of their function. We suggest that activation of lymphocytes in lymphoid organs leads to generation of effector T cells (positive loop), while at the same time presentation of antigen in the liver either on hepatocytes or other specialised antigen presenting cells depresses these responses (negative loop). This model helps to explain both the specific phenotype and low frequencies of HCV specific CTL in chronic infection, through early elimination of cells before expansion and maturation can occur. The outcome of HCV infection is likely to result from the early balance between these two simultaneous loops.
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Keywords: hepatitis C virus; $CD8^+$ T lymphocytes; $CD4^+$ T lymphocytes; hepatocytes; immune escape; hepatocyte

Abbreviations: APC, antigen presenting cell; CMV, cytomegalovirus; CTL, cytotoxic T lymphocytes; EBV, Epstein–Barr virus; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; IP-10, IFN-γ induced protein 10; LCMV, lymphocytic choriomeningitis virus; LSEC, liver sinusoidal epithelial cells; MHC, major histocompatibility complex; MIG, monokine induced by IFN-γ; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction

Introduction

Hepatitis C virus affects an estimated 170 million people worldwide.[1] Unlike other common hepatitis viruses (particularly A and B), it sets up persistent infection in the majority of those infected. Hepatitis B virus (HBV) causes severe acute hepatitis and can become persistent, particularly if acquired in early life, but in contrast to HCV, it is normally controlled if acquired in adult life. Because it so readily becomes persistent, and no vaccine exists, HCV has become a major public health problem in many countries. The problem is particularly acute in some, such as Egypt, where iatrogenic spread was common and where the prevalence may be up to 30% in some populations.[2]

Once HCV persistence is established – the case in about 85% of those infected – patients fall broadly into two groups. A minority of patients develop progressive liver inflammation, fibrosis, and ultimately cirrhosis with liver failure or liver cancer. The progression is generally slow in immunocompetent hosts (over decades), although can progress more rapidly in the immunosuppressed. The majority however, remain persistently viraemic, but develop much lower levels of inflammation and fibrosis and may remain asymptomatic lifelong.[3]

Immune responses to HCV

Immune responses to HCV may play an important role at various stages of infection: there is emerging evidence that during acute infection, cellular immune responses are important in the initial control of viral replication in those patients who subsequently control infection. In the chronic phase of infection, immune responses potentially determine the rate of progression of disease both by limiting viral replication and by contributing to immunopathology. Finally, during combination therapy, cellular immune responses may contribute to the success or failure of treatment.

Components of the immune response

The various components contributing to an effective antiviral immune response against a hepatotropic virus in model systems such as lymphocytic choriomeningitis virus have been reviewed recently elsewhere.[4]

In summary, it is essential that such a response comprises: (a) Effective innate immune effector mechanisms such as Type I interferons;[5] (b) Anatomically intact lymphoid organs in which to generate efficient antiviral B and T cell responses;[6] (c) An antibody response, which has some degree of neutralising capacity (even weak neutralising capacity may be important in certain animal models);[7,8] (d) Intact antigen presentation pathways; (e) A strong and sustained CD4+ T helper response; [9] (f) A strong and sustained CD8+ T lymphocyte (CTL) response.[8]

These components must act in concert in order to gain long term control of viruses. The use of gene targeted knock-outs and depletion experiments in animal models have convincingly shown the interdependence of the various immune mediators.[8,10] For the purposes of this discussion, however, we are concentrating on conventional T cell subsets (CD4+ T helper cells and CD8+ cytotoxic T lymphocytes (CTL)). This is firstly because most data exists in this area and these data suggest a critical role for these cells. It is also because we hypothesise that the molecular interactions at the interface between an infected hepatocyte and an antigen-specific T cell play a crucial role in determining the outcome of HCV infection.

CTL responses (successful)

CD8+ T lymphocytes (CTL) recognise virally infected cells through the presentation of viral peptides in the groove of host MHC Class I molecules. In certain uninfected antigen presenting cells cross presentation may also occur – i.e. presentation of exogenous proteins through the Class I pathway. CTL, when they recognise their targets, typically release interferon gamma, chemokines such as RANTES and MIP-1 α, and cause death of their target cell. These mechanisms serve to eliminate virus infected cells or suppress viral replication in neighbouring cells. Because CTL are very sensitive to low levels of viral peptide, this recognition and effector function can potentially occur at an early stage in viral replication.

Until recently, CTL were identified through functional assays such as ELISpot (release of interferon-gamma) or cytolysis, but more recently, the use of Class I peptide tetramers has allowed direct *ex vivo* detection independent of function. This technology has allowed also detailed phenotyping of the surface expression of a range of molecules on antigen specific CTL (reviewed in[11]).

In acute disease, where this has been studied, using such techniques, vigorous HCV-specific CTL responses have been observed.[12–15] These have been particularly associated with control of viremia, but substantial responses have also been seen in those who fail to control virus.[13] Multiple epitopes may be targeted simultaneously.[12] The response shows dynamics typical of many other acute infections, with highly activated CTL seen at the time of acute disease in frequencies of up to 7%, and rapid contraction of these populations thereafter. In those who control virus, memory populations are maintained long term.[12] Although not conclusive, these data suggest that CTL play an important role in the initial resolution of HCV infection.

CD4+ T helper responses (successful)

Antigen-specific CD4+ T cells can recognise only cells expressing MHC Class II molecules – in other words professional antigen presenting cells or cells which have upregulated Class II as a result of inflammatory stimuli. Although, from first principles and from animal models such as LCMV it would be expected that CTL would play the most important role in controlling a pathogen that was replicating within hepatocytes, in fact there is considerable data supporting an important role for CD4+ T cell responses. Most striking of these is a genetic association between Class II genotype and spontaneous clearance of virus. Numerous studies have shown a link between possession of HLA DRB1*1101/DQ0301 (the two genes are in close linkage disequilibrium) and spontaneous viral control (these patients are recognised because they are HCV antibody positive but PCR negative).[16] Additionally such patients possess stronger HCV specific CD4+ T cell responses than non DQ0301+ controls.[17] As is the case for CTL, in acute disease strong and multispecific CD4+ T cell responses can be seen at the time of resolution of infection.[18] Thus it is likely that early T helper responses play a significant role in determining the initial outcome of infection, although whether this is as direct effector cells or through maintenance of CTL responses is still unclear.

Unsuccessful T cell responses

Most patients fail to clear infection and here the picture becomes more confusing. Initial CTL and CD4+ T cell responses may still be observed[18] and it remains to be determined what the differences between a successful and unsuccessful response are at this stage of infection. Once persistence is established an important difference becomes clear – *ex vivo* responses in chronically infected individuals are markedly attenuated.[12,19] Possible reasons for this include viral variation and escape, T cell compartmentalisation to the liver,[19] and the use of assays which rely on function which underestimate cell frequencies.[20] However, overall the frequencies of virus-specific CTL are much lower than during chronic infection with HIV.[21] With careful screening techniques, a CTL response can be obtained in PBMC from about half the patients studied,[22] and similar results have been obtained in liver derived lymphocytes.[23] For CD4+ T helper responses it is relatively unusual to find strong and sustained responses in chronically infected individuals while this is common in spontaneous clearers.[17] Treatment with interferon alpha has been shown to induce recovery of some T helper responses, indicating that these T cells are not deleted completely.[24]

Paradoxically then, hepatic inflammation arises during persistent HCV infection where antigen specific T cells are usually weak or undetectable. There are two explanations for this. Firstly, levels of antigen specific T cells might be higher in the liver than in blood. However, while this discrepancy may be about one order of magnitude, specific responses probably constitute only a minority of the infiltrate.[25] Alternatively the large proportion of the infiltrate, which may be responsible for tissue damage, is non antigen specific. A similar situation has been observed in inflamed joints.[26]

Mechanisms behind immunological failure

A few potential mechanisms might explain both the relative lack of effectiveness of T cell responses overall (reflected in

the capacity of the virus to persist) and the relative weakness of T cell responses seen in chronic infection.

Viral variation

Since HCV has a huge capacity for variation, generation of immune escape mutants is likely to play an important role in viral persistence. In chimpanzee models this has been very clearly shown – and appears to occur mainly during the initial months of infection.[27] However, to date, the evidence from human studies has been relatively limited.[28] Interestingly, studies of E2 protein in the putative region targeted by antibodies, has also shown evidence of immune mediated selection during acute infection.[29]

T cell attenuation or modulation

In murine models, at high viral loads of particular strains, T cells go through a process of exhaustion leading ultimately to clonal deletion.[30,31] The mechanism behind this is not clear, but it is accelerated in the absence of functional CD4+ T cells, neutralising antibodies or cytokines such as interferons.[32] Interestingly, in some situations, prior to deletion, a transient loss of the ability to secrete interferon gamma is observed[33] – a situation which has been also noted in HCV (stunned phenotype).[12,14] Some have suggested that HCV derived proteins themselves may play a role in attenuating T cell responses. There is some *in vivo* evidence for an immunomodulatory role for HCV core as expressed in a murine model.[34]

Another potential explanation is that CTL may not achieve appropriate effector function, as has been suggested for HIV specific CTL.[35] The phenotype of CTL identified in HCV is indeed distinct from that of CTL specific for EBV, HIV and CMV being high in CD27 and CD28[36] (Figure 1) although this may simply reflect their different lifespans, rather than skewed maturation. However, although these results are intriguing, it is too early to conclude anything about *in vivo* efficacy.

The liver

A variation on the theme of T cell modulation is the liver effect. It is well known that the liver environment itself is a suitable one for promoting T cell tolerance. This evidence comes largely from early transplantation studies,[37] where engraftment in certain species occurred across MHC barriers and where tolerance to other grafted organs may be generated. Important evidence from murine models suggests that there is an important role for the hepatocyte in antigen presentation and tolerance induction. Specific T cell proliferation of naive T cells, induced by hepatocytes, was comparable in magnitude to that seen in response to dendritic cells and was independent of CD4+ T cell help or bystander professional APC co-stimulation. However, thereafter, T cells exposed to hepatocytes underwent apoptosis.[38]

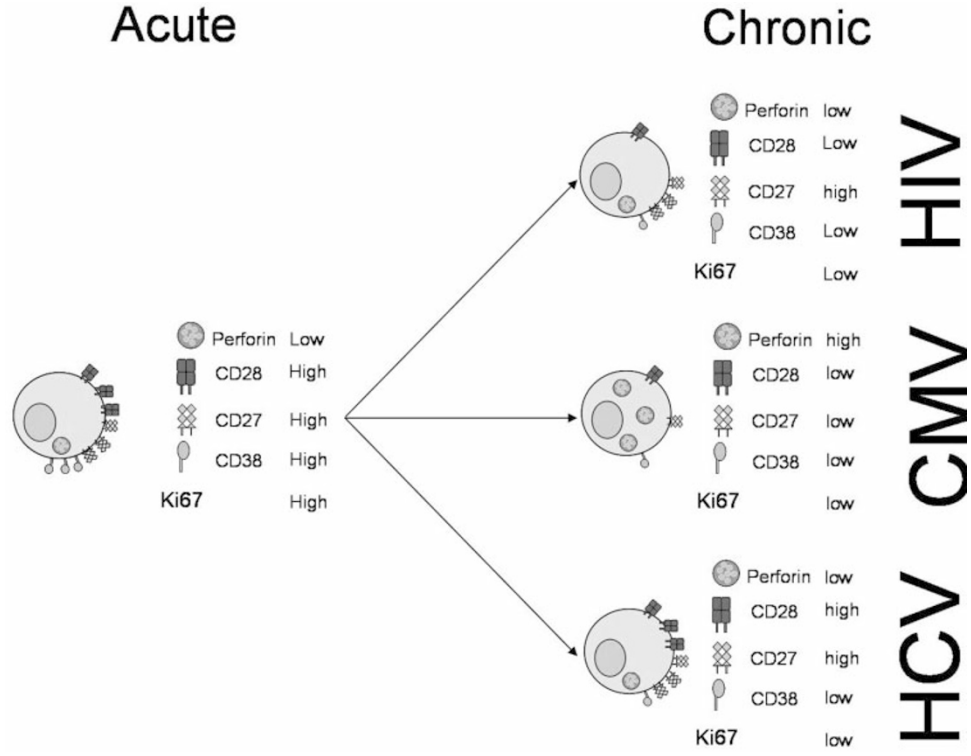

Figure 1 Comparison of CD8+ T cells surface markers in three different acute and chronic viral infections, HIV, CMV, and HCV. During the acute stage T cells show similar phenotypes, however this is not so during chronic stages of infection. CD28 is a co receptor for antigen induced stimulation. CD27 is a member of the TNF receptor family which is downregulated upon maturation of T cells. CD38 is a bifunctional surface enzyme, and is a marker associated with sustained activation of T cells

Apoptosis of activated T cells is also seen following contact with hepatocytes.[39]

As yet the cellular and molecular basis of this tolerance induction is not fully understood. Part of it may arise from the particular anatomy of the liver, in which a rich blood supply (about a litre per min) is greatly slowed in the capacious liver sinusoids. Here the lining cells (liver sinusoidal endothelial cells – LSECs), are thought to present a relatively loose barrier, having no basement membrane. It is possible that naive lymphocytes within the liver may contact hepatocytes directly – a situation which would be unique to the liver as naive T cells normally only recirculate to lymphoid tissue.

Additionally the slow transit time within the sinusoid is thought to allow ready contact with liver resident Kuppfer cells (liver macophages) and LSECs (Figure 2). It has been suggested that specific functions of Kuppfer cells, liver sinusoidal endothelial cells, as well as hepatocytes themselves may play a role in generating tolerance (via clonal deletion or induction of anergy).[40] Teleologically, there may be important reasons for the liver to possess this capacity since many foreign antigens, which are absorbed through the gut, are present in this environment carried there by the hepatic portal vein. In the case of HCV infection, the virus may therefore have exploited this T cell downregulatory effect to establish persistence. Since the main target of HCV is the hepatocyte, we will now explore in detail the hepatocyte/T cell interaction, and address what the potential consequences are for both cell types and what the influence of the virus might be.

Hepatocyte – T cell interactions

Virally infected hepatocytes will at some point during the infection interact with T cells via a number of different surface molecules, several of which have the potential to affect T cell activation.

MHC class I

Like all nucleated cells within the body hepatocytes express these human leukocyte antigens (HLAs). Their expression, which is generally low on hepatocytes, can be increaed in the presence of IFN-γ and IFN-α.[41] A number of stealth viruses such as CMV are able to interfere with antigen presentation and thus hide from T-cell surveillance.[42] So far only one report has looked at antigen presentation on cell lines expressing HCV proteins showing that there is no impairment.[43]

Other aspects of hepatocyte surface phenotype

One of the restrictions facing the study of hepatocytes is the difficulty of working with normal primary hepatocytes. Acquiring, isolating, and growing primary hepatocytes are all difficult and cultures only last short term. Hepatocellular carcinoma lines (e.g. HepG2 and Huh7) present a more convenient model due to the ease by which they can be grown. However, like all tumours, hepatocellular carcinoma lines have undergone transformation and possibly have an altered surface phenotype. Table 1 summarises the current

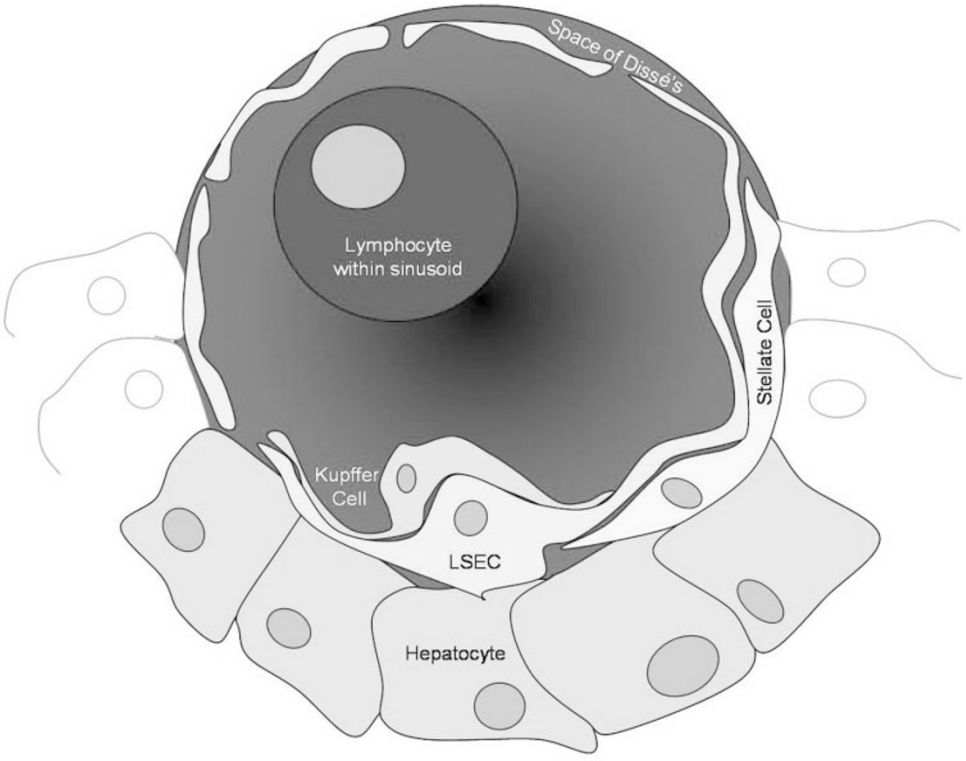

Figure 2 Cartoon of the liver sinusoidal micro-environment. T cell access to hepatocytes could occur via fenestrations in the liver sinusoidal epithelial cells (LSEC). Interactions with LSEC may also be important

Table 1 Comparison of surface molecules between primary hepatocytes and hepatocarimona lines

	HepG2	Huh7	Normal hepatocytes	References
MHC class I	✔	✔	✔	61–64
MHC class II	✖	✖	✔/✖ (mixed reports)	61, 65
ICAM-1 (CD54)	✔	?	✔	61, 66, 67
LFA-3 (CD58)	✔	?	✔	61
EGF receptor	✔	✔	✔	61
Fas/Apo-1 (CD95)	✔	✔	✔	61, 68, 69
FASL (CD95L)	✔	✖	✔*	47, 61, 70, 71
CD40	✖/✔ (mixed reports)	?	✔	61, 72
CD44	✖	?	✔	61, 73, 74
CD81	✖	?	✔	75
CD1d	?	?	✔	76
DR4	?	✔	?	77
DR5	?	✔	?	77
CD80	?	?	✔*	47, 78
CD86	?	?	✔*	78

*Upregulated in chronic hepatitis

known phenotype for HepG2 and Huh7 hepatoma lines, and primary hepatocytes.

Functional consequences of hepatocyte-T cell interactions

Perforin and granzyme induced cell death is an important mechanism in the clearance of infected cells by CTLs. However, hepatocytes in mice have been shown to be relatively poorly susceptible to perforin-dependent pathways.[44] Perforin-independent IFN-γ-dependent mechanisms within the liver are thought to be particularly important[45] in antiviral control. Nevertheless perforin deficient mice still fail to clear non-cytopathic LCMV from the liver,[46] although potentially Kuppfer cells rather than hepatocytes still harbour virus in this case.[45]

Both human and murine primary hepatocytes have been shown to be sensitive to CD95L induced apoptosis. Fas has been shown to be more frequently up-regulated in HCV patients on hepatocytes surrounding CTL infiltrates.[47] Fas-FasL interaction may also be involved in bystander killing accounting for some of the pathology seen during HCV infections.[48] Normal healthy hepatocytes do not express FasL. However, Galle *et al*.[49] showed that FasL mRNA was expressed in hepatocytes from livers with ongoing damage and in livers with Hepatitis B virus infection FasL expression was seen in areas of lymphocyte infiltration, potentially leading to killing of the antiviral T-cells.

Cytokines and chemokines

T cells within the liver, as elsewhere, will be responsive to a variety of cytokines, but much less is known about the effects of such cytokines on hepatocytes, particularly the effects that might influence hepatocyte-T cell interactions.

Table 2 Cytokines potentially present during HCV infection and their effects on hepatocytes

	Hepatocytes
IFN-γ	• Increased expression of MHC class I and ICAM-1 (CD54)[41] • In primary hepatocytes p53 is induced followed by cell cycle arrest and apoptosis (independent of p53)[79]
TNF-α	• Proliferation[80] • Apoptosis under hepatotoxic conditions[81–83] • Increased MHC class I surface expression and ICAM-1[62,84]
IL-4	• Induction of interleukin-1 receptor antagonists[85] • Up-regulation of Cytochrome P4502E1 (CYP2E1)[86]
IL-6	• Up-regulation of acute phase proteins
IL-10	• Inhibition of antiviral action by IFN-α by inducing SOCS2, SOCS3, and CIS expression[51]
IL-12	• Production of chemokines, monokine induced by IFN-γ (MIG) and inducible protein 10, (IP-10)[87]

While many of the cytokines may be derived from T cells and resident Kuppfer cells. It has been suggested that hepatocytes themselves may also make cytokines.[50]

The responses of hepatocytes to cytokines are outlined in Table 2. With regards to the Th1 type cytokines (IFN-γ and IL-12) their effects overall appear to be to improve the ability of the hepatocytes to present antigen, to promote influx of activated lymphocytes through chemokine secretion, and to prime hepatocytes for death through apoptosis. Much less is known about a Th2 type cytokine effect on the liver. Interestingly IL-10 appears to inhibit the action of the antiviral cytokine IFN-α.[51]

Hepatocyte derived chemokines, notably monokine induced by IFN-γ (MIG) and IFN-γ induced protein 10 (IP-10), which have been shown to play a role in murine models of HBV and are elevated in chronic HCV are likely to be important in regulating lymphocyte recruitment to HCV infected livers.[52–54]

Hypothesis – two competing loops for lymphocytes

In acute infection with HCV, antigen presentation is likely to occur in two environments – the liver and within lymph nodes. Antigen will be presented in lymph nodes either through cross-presentation or via presentation on infected dendritic cells.[55] Expansion of virus specific T cell clones accompanied by activation and expression of appropriate homing and chemokine receptors will provide a set of virus specific T cells ready for their maiden voyage into the liver (maiden T cells). We currently lack evidence that this phase of the immune response is impaired, since virus-specific T cells can be readily detected during acute infection,[12,13] although they do take several weeks to emerge.[14]

T cells, both naive and maiden (i.e. on their first voyage into the liver) which enter the liver during acute infection will contact infected hepatocytes bearing peptide MHC complexes on their cell surface, as well as LSECs and Kupffer cells. We hypothesize that at this point, the cellular and molecular features outlined above lead to downregulation of T cell function and attenuation of the functional response. At the same time, effector functions of these T cells will

lead to downregulation of virus replication through secretion of antiviral cytokines, and apoptosis and death of the infected cells, and potentially bystander cells.[39]

The outcome of this process will depend on the dynamics involved. If cross-presentation is relatively inefficient, as has been suggested,[56] T cell responses will emerge relatively late, at a time point when a substantial number of hepatocytes are infected. (There are 10^8 hepatocytes per gram of liver tissue and approximately 1500 grams of liver in an average human, thus 1.5×10^{11} total hepatocytes). The rate of elimination of virus infected cells may thus be slower than the rate of infection of new hepatocytes and so persistence will ensue.

Beyond the simple dynamics, the other key factor determining outcome is the relative efficiency of control of viral replication. Immune responses to hepatitis B virus are subject to the same basic constraints, with late generation of T cell responses in the face of a very large number of infected hepatocytes – yet in adults this virus is largely controlled. A number of factors might explain the difference – the capacity to generate viral variants, the inability to generate neutralising antibodies, and – potentially – differential sensitivity to antiviral cytokines. Many of these factors may compound each other – thus attenuation of T cell responses at the surface of the hepatocyte might lead to maintenance of high viral loads and therefore promote immune escape. Further persistence might then lead to downregulation of CD4+ T cell responses and therefore impairment of CTL responses.[57]

Two loop theory

We have proposed the two loop theory, whereby presentation of HCV antigens within the liver environment promotes attenuation of T cell responses (or T cell dysfunction) and viral persistence, while simultaneously presentation in lymphoid organs promotes effective T cell responses and control of viremia.[58] During persistence the negative loop is dominant, and in resolved disease the positive loop dominant. This pattern is seen in both chronic HBV and HCV infection and differs markedly from other viruses, which are less strictly hepatotopic (EBV, CMV and HIV) and where relatively strong T cell responses are maintained (Figure 3).

This theory helps to explain the conundrum of apparently weak T cell responses but persistent intrahepatic inflammation. Since T cells are continuously activated, they will transit through the blood and enter the liver, where they receive signals leading to death or downregulation of function.[39] At the hepatocyte surface, they can induce apoptosis of target cells and secrete inflammatory cytokines, but then will be

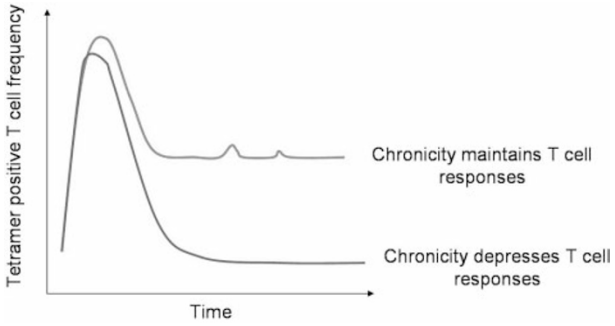

Figure 3 Patterns of virus specific T cells found in blood in different types of persisting viral infections. Red indicates the tetramer positive T cell response (frequency) to EBV or CMV or HIV. The blue line indicates tetramer positive responses to chronic HCV or HBV. Acutely, a response of 1 – 10% of CD8 T cells per epitope would be typical, although these may occasionally be much higher in EBV. In the chronic phase a response of $<0.1\%$ of CD8+ T cells would be typical for chronic HBV and HCV, while responses of 1 – 10% may be sustained in the other infections

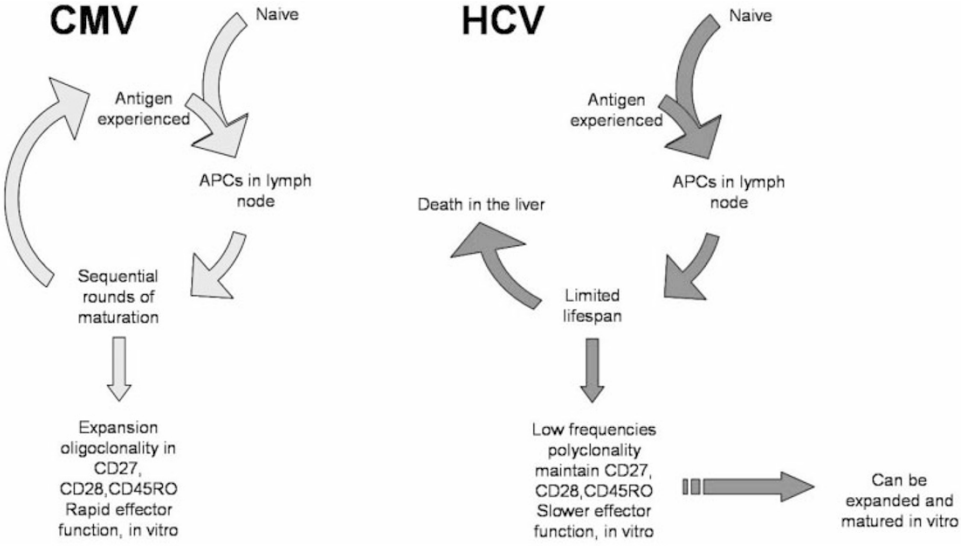

Figure 4 Hypothesis: Death and the maiden T cell. CTL specific for CMV go through multiple rounds of reactivation over time leading to a mature or effector phenotype. Although death may occur in the liver, since little CMV antigen is presented there the effect is not significant overall. In contrast, HCV specific populations will undergo death when they encounter antigen in the liver and not go through multiple rounds of restimulation *in vivo*. This explains the lower frequency in blood, immature phenotype, and weaker effector activity * – but their intact ability to proliferate in response to antigen *in vitro*

eliminated. Thus, overall, fairly low numbers of T cells will be maintained, even though the generation of such cells may remain intact. If this equilibrium is disturbed – e.g. through treatment – a reduction in the death of cells within the liver will lead to an apparent re-emergence of previously undetectable immune responses.

This theory also helps explain an apparent discrepancy in the literature. Early papers, which used *in vitro* restimulation protocols to detect CTL, often described multispecific responses in chronically infected patients,[59,60] while current *ex vivo* assessments often find very low T cell numbers.[12] If CTLs are indeed present but the frequency is low due to liver-mediated downregulation, they may easily be missed *ex vivo*, since such tests may pick up about 0.02% of CD8+ T cells. However, rapid expansion *in vivo* is readily demonstrated[22] indicating that such cells do exist and that they have the capacity to respond to antigen. *In vitro* restimulation simply accentuates the positive loop and eliminates the negative loop.

Finally, this theory helps explain the distinctive phenotype of HCV specific CTL. Sequential encounters with antigen will lead to a phenotype which is low in CD28, CD27, CD45RO, high in perforin and shows rapid IFN-γ secretion, as exemplified by CMV. HCV specific CTL are at the opposite end of this spectrum. One simple reason may be that they do not undergo multiple rounds of antigen re-stimulation, but rather die in the liver before expansion and maturation can occur. Thus the phenotype does not represent 'skewing' but merely 'inexperience' of the T cell population (see Figure 4).

If this is the case, *in vivo* immune modulation to promote the T cell responses and aid resolution of chronic infection could potentially be successful – as long as the negative loop is at least temporarily reduced. Similarly T cell vaccines, which accelerate the initiation of and maintenance of the positive loop, may also be approached with some confidence.

Conclusions

Although a reasonable amount of information is available concerning circulating HCV specific T cells at various stages of disease, we are still very much in the dark about the important events that occur at the surface of an infected hepatocyte. We have reviewed the data on the phenotype and immunological function of hepatocytes, but it is clearly limited. More *in vitro* data is required using non transformed human cells and other liver derived cells such as LSECs. Also, more *in vivo* data is needed in model situations which are relevant to human chronic viral infections. This is an important area, both in terms of normal immunobiology and pathology. Until we understand it at a molecular level, watching T cell populations wax and wane in infected patients will remain a mysterious dark art.

Acknowledgements

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