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Intrahepatic murine CD8 T cell activation associates with a distinct phenotype leading to Bim-dependent death

Lauren E Holz¹, Volker Benseler^{1,4}, David G Bowen¹, Philippe Bouillet², Andreas Strasser², Lorraine O'Reilly², William d'Avigdor¹, Alex G Bishop³, Geoffrey W McCaughan¹, and Patrick Bertolino¹

¹Centenary Institute, AW Morrow Gastroenterology and Liver Centre, Royal Prince Alfred Hospital and Faculty of Medicine, University of Sydney, Camperdown, NSW, 2050, Australia

²The Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, 3050, Australia

³Collaborative Transplant Laboratory, Blackburn Building, University of Sydney, NSW, 2006, Australia

⁴Department of Surgery, University of Regensburg, Bavaria, 93053, Germany

Abstract

Background and aims—Chronic infections by hepatotropic viruses such as hepatitis B and C are generally associated with an impaired CD8 T cell immune response that is unable to clear the virus. The liver is increasingly recognised as an alternative site where primary activation of CD8 T cells takes place, a property that might explain its role in inducing tolerance. However, the molecular mechanism by which intra-hepatically activated T cells are tolerized is unknown. Here we investigated the phenotype and fate of naïve CD8 T cells activated by hepatocytes *in vivo*.

Methods—Transgenic mouse models in which the antigen is expressed in lymph nodes and/or in the liver were adoptively transferred with naïve CD8+ T cells specific for the hepatic antigen.

Results—Liver-activated CD8 T cells displayed poor effector functions and a unique CD25^{low} CD54^{low} phenotype. This phenotype was associated with increased expression of the proapoptotic protein Bim, and caspase-3, demonstrating that these cells are programmed to die following intrahepatic activation. Importantly, we show that T cells deficient for Bim survived following intrahepatic activation.

Conclusions—This study identifies Bim for the first time as a critical initiator of T cell death in the liver. Thus, strategies inhibiting the upregulation of this molecule could potentially be used to rescue CD8 T cells, clear the virus and reverse the outcome of viral chronic infections affecting the liver.

Introduction

In recent years it has emerged that despite being a non-lymphoid organ, the liver displays immunological properties distinct from other solid organs and is associated with the induction of T cell tolerance¹. This property has been demonstrated in several clinical settings including transplantation², and hepatotropic viral infections, such as those induced by HBV and HCV³.

Correspondence: Dr Patrick Bertolino; Phone: +61 2 95656186; p.bertolino@centenary.usyd.edu.au.

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Many models have been proposed to explain the “liver tolerance effect”, but the molecular and cellular mechanism/s mediating this phenomenon remain unknown. We have demonstrated that the liver is an alternative site of primary activation for CD8 T cells⁴. This finding, recently confirmed by other groups^{5–8}, has opened the way to possible new explanations for liver-induced immunological tolerance.

Many liver cells have been shown to act as antigen presenting cells (APCs) for naïve T cells⁹. Our study focuses on activation of naïve T cells by hepatocytes, a type of activation that might play a major role during the early stages of HCV infection and/or peripheral tolerance to hepatocyte-specific antigens. Three recent pieces of evidence suggest that hepatocytes can activate naïve T cells: firstly, hepatocytes can act as highly efficient APCs for naïve CD8 T cells *in vitro*¹⁰. Secondly, in the absence of basal membrane separating hepatocytes and liver sinusoidal endothelial cells (LSEC), pseudopods of circulating lymphocytes have been shown to extend through the fenestrations in LSEC and contact hepatocytes¹¹. Finally, naïve T cell receptor (TCR) transgenic T cells adoptively transferred into transgenic mice expressing antigen exclusively on hepatocytes were activated in the liver, demonstrating that primary T cell activation occurred *in situ*¹².

Our previous *in vitro* studies suggested that in contrast to T cells activated in lymph nodes (LN), T cells activated in the liver exhibit poor effector function and are short-lived, thus leading to tolerance rather than effector function^{12, 13}, however, the molecular mechanisms regulating this process *in vivo* have not been elucidated.

In this study, we used transgenic mouse models in which T cells were activated in the liver by hepatocytes and/or in the LN by professional APCs to investigate the phenotype and fate of CD8 T cells activated at these two sites, and to identify the mechanism of T cell death following intra-hepatic activation.

Methods

Mice

Des-TCR¹⁴, BimDes, Met-K^{b15}, Alb-K^{b16} and 178.3¹⁷ mice were maintained at the Centenary Institute under pathogen free conditions (see Suppl. data 1 for strain details).

Generation of chimeric mice

B10.BR mice were irradiated with 9.5Gy and reconstituted with 5×10^6 – 10^7 bm leukocytes from either B10.BR or Met-K^b mice. Chimeric mice were left for 6 weeks before experiments were performed.

Adoptive transfer experiments

Single cell suspensions of pooled LN cells were labeled with CFSE (Molecular Probes, Eugene, OR, USA) as previously described¹². 2×10^6 CFSE-labeled DesRAG lymphocytes, or 1.5×10^7 Des-TCR or BimDes lymphocytes were injected into the tail veins of recipient mice. Serum ALT levels were measured at the indicated time points.

Immunofluorescent staining and flow cytometric analysis

Liver, spleen, blood and LN leukocytes were prepared as previously described^{12, 18}. Monoclonal antibodies were purchased from Becton Dickinson (Franklin Lakes, NJ, USA) or Cell Signaling Technology (Danvers, MA, USA). Antibodies specific for the clonotypic transgenic TCR Desiré were generated in-house. 3C5, which recognizes all isoforms of Bim19 was purchased from Alexis (Lausen, Switzerland). Annexin V, surface and intracellular staining followed by flow cytometric analysis were carried out as previously described^{4, 20}.

For cytokine assays, lymphocytes were stimulated *in vitro* for 6 h with C57BL/6 splenocytes and Brefeldin A added after 4 h (Sigma Aldrich, Australia)²¹ before staining.

CTL assays

In vitro CTL assays were performed as previously described¹². For *in vivo* assays, recipient mice were injected with 10^6 DesRAG cells. CTL activity was tested 4 days later by injecting recipient mice with a 1:1 mix of 178.3 (target expressing H-2K^b) and control B10.BR (H-2K^b⁻) splenocytes (10^7 cells total), each previously labelled with 3 μ M or 0.5 μ M CFSE respectively. The percentages of CFSE^{low} propidium iodide (PI)- cells and CFSE^{hi} PI- that survived in the liver and spleen of recipient animals were detected by FACS analysis 24 h post-splenocyte transfer and % CFSE^{low}/ % CFSE^{high} ratios were calculated for each liver and spleen samples. These ratios were used to determine percentage of specific killing according to the equation below: % Specific killing = $[1 - \text{ratio B10.BR} / \text{ratio Met or Alb-K}^b] \times 100$.

Results

Most donor CD8 T cells isolated from the liver and LN at day 2 post-adoptive transfer do not recirculate and have been activated *in situ*

To analyze the phenotype of CD8 T cells activated in the liver and compare them to T cells activated in LN, we used a well characterized Tg mouse line (Met-K^b mice) in which the allo-major histocompatibility (MHC) class I molecule H-2K^b is expressed in both the LN on bm-derived cells and in the liver on hepatocytes⁴. Previous studies have shown that in Met-K^b mice, activation of adoptively transferred naïve Des-TCR CD8 T cells specific for H-2K^b occurs simultaneously in both the liver and LN⁴. As the Des-TCR cells recognize the intact H-2K^b molecule, cross-presentation is not possible in this model. To ensure that donor T cells were naïve, we used Des-TCR Tg mice bred onto a RAG-1^{-/-} background (DesRAG mice) and tracked the recirculation of these cells following activation in either the liver or LNs to identify a suitable time to phenotype these cells.

To follow the recirculation of DesRAG cells activated in the LN alone we took advantage of the leaky expression of the transgene on bm-derived cells in Met-K^b Tg mice⁴. Bone marrow cells from Met-K^b mice were used to reconstitute lethally irradiated B10.BR mice, which do not express this antigen, in order to generate Met-K^b bm→B10.BR (M-B) chimeras. These mice expressed H-2K^b on bm-derived cells in lymphoid tissues but detectable levels of the transgene were not found on resident hepatic bm-derived cells⁴. B10.BR bm→B10.BR (B-B) chimeras served as controls. Two months after hematopoietic reconstitution, CFSE-labeled DesRAG cells were transferred into M-B and B-B chimeric mice. Two days after transfer, most DesRAG cells isolated from the LN of M-B mice were activated, which is consistent with antigen expression at this site, while the donor-derived CD8 T cells remained naïve in B-B mice (Fig. 1A). Conversely, very few activated DesRAG T cells were found in the blood, livers or spleens of M-B mice at this time point. At day 3 however, significant numbers of LN-activated CD8 cells were found in the liver (Suppl. Fig. 1A), indicating that the majority of LN-activated CD8 cells did not begin to recirculate until after 2 days of activation.

To follow the recirculation of donor CD8 T cells activated solely in the liver, we used Alb-K^b recipient mice, which only express H-2K^b on hepatocytes^{12, 16}. Activation of DesRAG cells transferred into these mice was restricted to the liver, and observed as early as day 1. The majority of donor T cells migrate to lymphoid tissues (Fig. 1B and Supp. Fig. 1B) where they maintain a naïve phenotype however there is a small but significant antigen-specific retention of donor T cells in the livers of Alb-K^b mice. Thus over time in Alb-K^b mice all donor T cells are eventually activated in the liver (Holz et al. manuscript in preparation). Small numbers of activated donor cells could be detected in the blood and spleen of Alb-K^b mice at day 2 post-

transfer but not in the LN, indicating that although hepatocyte-activated T cells did recirculate early after activation, they were unable to enter LN on day 2 (Fig.1B), a finding consistent with their down-regulation of CD62L (Fig.2B).

From these experiments we conclude that most activated T cells found in the liver and LN at day 2 after antigen encounter represented cells genuinely activated in these compartments rather than recirculating cells. Thus, we chose this time point to study the phenotype of hepatocyte- and LN-activated CD8 T cells.

CD25 and CD54 but not other activation markers discriminate hepatocyte-from LN-activated CD8 T cells

To address the question whether hepatocyte-activated T cells display a distinct phenotype from those activated in the LN, CFSE-labeled DesRAG cells were injected into Met-K^b and B10.BR mice and analyzed two days later for 'classical activation markers' (CD69, CD44, CD25, CD62L), adhesion molecules (CD54, LFA-1, $\beta_7\beta_1$ and α_{IEL} integrins, CD11c) and chemokine and cytokine receptors (CCR5, CD25, CD122, CD132). As assessed by dilution of their CFSE content, donor CD8 T cells isolated from the liver and LNs of Met-K^b mice divided at a comparable rate, whereas cells transferred into B10.BR mice did not proliferate and displayed a naïve phenotype (Fig.2A). The majority of markers were either upregulated or downregulated on donor CD8 cells to a similar extent regardless of the site of activation. Two notable differences were CD25, the alpha chain of the interleukin-2 receptor (IL-2R), and CD54 or intercellular adhesion molecule-1 (ICAM-1). Both of these markers were expressed at significantly higher levels on those donor T cells activated in the LN of Met-K^b mice than those from the liver (Fig.2A). Interestingly, the beta (CD122) and gamma (CD132) chains of the IL-2R were not differentially expressed between liver- versus LN-activated CD8 T cells (Fig.2A). The phenotype of donor T cells isolated from the liver of Alb-K^b mice was analogous to that seen in the liver of Met-K^b mice (Figs. 2A and B) suggesting that it is a general phenotype that characterizes T cell activation by hepatocytes. As expected, in the Alb-K^b model, activation of donor cells did not occur in the LN thus DesRAG T cells displayed a naïve phenotype, similar to CD8 T cells isolated from B10.BR control animals (Fig.2B).

A detailed time course study of CD25 and CD54 expression on blood-, liver-, LN- and spleen-isolated cells from as early as 2 h post-transfer up to day 5 into Met-K^b or B10.BR mice revealed that both LN- and liver-activated DesRAG T cells began to express CD25 and CD54 as soon as 14 h post-transfer and continued to express these marker at this increased level for up to 24 h. However, in contrast to T cells activated in the LN, expression of these markers was not maintained on liver-activated T cells after 24 h (Suppl. Fig.2).

Collectively, these results demonstrate that hepatocytes are capable of activating naïve CD8 T cells, but imprint a distinct programme of differentiation, leading to unsustained expression of CD25 and CD54.

Hepatocyte-activated CD8 T cells exhibit impaired effector function in vivo

The hallmark of fully activated CD8 T cells is the ability to exert effector functions, such as cytokine production and cytolytic activity. To assess the ability of hepatocyte- and LN-activated CD8 T cells to kill target cells, we performed an *in vivo* CTL assay (see details in the methods). Donor T cells activated in Met-K^b mice were effective CTL with an approximately 75% killing rate, whereas those activated in the livers of Alb-K^b mice displayed only a 5% or lower killing rate (Fig.3A). As expression of H-2K^b in Alb-K^b and Met-K^b hepatocytes is analogous resulting in the same level of activation¹², and the mice differ only in activation of donor T cells in the LNs, we concluded that LN-activated T cells became efficient CTLs, whereas those activated in the liver displayed poor cytotoxic function. Previous *in vitro* studies

using the same number of CTL per well have demonstrated that poor effector function observed in Alb-K^b mice was not only due to a difference in CTL frequency but also a consequence of a CTL-intrinsic defect¹² (see also Fig.5A).

The ability of liver- and LN-activated T cells to produce IFN- γ and IL-2 was also assessed following activation in Met-K^b mice. Within 14 h of activation, LN-activated T cells produced approximately twice the copies of IFN- γ mRNA than liver-activated T cells (Suppl. Fig.3A). Similarly, IL-2 mRNA expression was greater early after activation in those cells activated in the LN (Suppl. Fig.3B).

This difference was confirmed at the protein level using flow cytometry (Fig.3B and C). A similar defect was observed in T cells activated in the livers of Alb-K^b mice (Suppl. Fig.4).

These data suggest that, consistent with their inability to maintain CD25 and CD54 expression, naïve CD8 T cells activated by hepatocytes were defective in producing IL-2 and IFN- γ and developing cytotoxic effector function.

Hepatocyte-activated T cells die by neglect *in vivo*

Co-stimulation and secretion of IL-2 during primary activation has long been shown to influence the survival of T cells²². Our previous *in vitro* findings²¹ combined with the low expression of IL-2 and CD25 on liver-activated CD8 T cells observed in this study are also consistent with the notion that these T cells suffer a defect in survival. To determine whether hepatocyte-activated CD8 T cells also underwent premature death *in vivo*, we analyzed expression of pro- and anti-apoptotic members of the Bcl-2 family by Real Time RT-PCR at 14 and 24 h post-transfer. Expression of the anti-apoptotic member Bcl-x_L was comparable between liver- and LN-activated T cells at both time points (data not shown). However, significant differences were found in the expression of the proapoptotic Bcl-2 family member Bim, such that 24 h after activation liver-activated T cells expressed between 2- to 3-fold more mRNA copies (Suppl. Fig.5). As the balance between anti- and pro-apoptotic Bcl-2 family members regulates cell death, these results suggest that liver-activated T cells are more prone to death than those activated in LNs. These observations were confirmed at the protein level by intracellular staining for Bcl-2, Bcl-x_L and Bim followed by flow cytometric analysis (Fig. 4A). Bcl-2 and Bcl-x_L protein expression strictly correlated with primary T cell activation: there was a strong increase in both of these proteins in donor CD8 T cells activated in the liver and LN of Met-K^b mice but no increase was seen in control, non-activated CD8 cells from the LNs of B10.BR mice. Importantly, there was no difference in expression levels of Bcl-2 and Bcl-x_L between CD8 T cells activated in the liver versus LN, suggesting that these proteins did not discriminate between cells activated at these two sites (Fig.4A). In contrast to Bcl-2 and Bcl-x_L, Bim expression in donor CD8 cells remained low following activation in Met-K^b LNs but was strongly up-regulated in donor CD8 T cells activated in both Met-K^b and Alb-K^b livers (Fig.4A and data not shown). That hepatocyte-activated T cells were undergoing apoptosis was confirmed by the detection of activated caspase 3 and cell surface annexin V at day 3 post-transfer in liver-activated CD8 cells in both Met-K^b and Alb-K^b mice (Fig.4B and 4C). DesRAG T cells in the LN of both lines displayed low levels of Annexin V and active form of caspase 3. These results provide strong evidence that hepatocyte-activated T cells express high levels of Bim at day 2 and started to die as soon as day 3 *in vivo*.

Death of hepatocyte-activated T cells is Bim-dependent

To demonstrate more directly the role of Bim in T cell death following intrahepatic activation, we generated Des-TCR mice bred onto a Bim deficient background²³ (BimDes). Loss of Bim did not affect the phenotype and effector function of Des-TCR T cells activated in Met-K^b and Alb-K^b mice. Using both *in vitro* and *in vivo* CTL assays (Fig.5A and Suppl. Fig.6B and C)

and cytokine expression (Fig.5B), T cells isolated from Met-K^b mice displayed good effector function in contrast to activated T cells isolated from Alb-K^b mice, regardless of Bim expression. Furthermore BimDes T cells activated by hepatocytes were also CD25^{low} CD54^{low} (Suppl. Fig.6A and data not shown).

To determine whether the survival of liver-activated T cells was Bim-dependent, naïve BimDes and wildtype Des-TCR cells were transferred into Alb-K^b or control B10.BR mice. Consistent with the role of Bim in protecting T cells from death caused by cytokine deprivation, significantly greater numbers of donor CD8 T cells could be harvested from Alb-K^b recipient mice when donor T cells lacked the Bim gene at late time points (day 15) (Fig.5C). Interestingly, “rescued” hepatocyte-activated BimDes cells did not accumulate in the liver, but rather migrated to lymphoid tissues following intrahepatic activation (Fig.5C). There was no enhanced survival of BimDes cells in B10.BR mice; thus, the effect seen in Alb-K^b mice was activation and antigen-specific. Despite increased numbers of BimDes CD8 cells, Alb-K^b mice did not develop overt hepatitis at day 5, as determined by measurement of ALT levels in serum (Fig.5D). This is consistent with lack of CTL activity detected at the peak of hepatitis (Fig. 5A). In contrast, Met-K^b mice injected with Des-TCR or BimDes T cells developed similar hepatitis at day 5 post-transfer, as previously reported (Fig.5D)¹². These results suggest that effector function and survival due to loss of Bim are independent parameters: CTL activity is not generated or restored in Bim-deficient T cells surviving intrahepatic activation in the Alb-K^b model and is not altered in T cells that have acquired this function following activation in the LNs of Met-K^b mice.

This may indicate that the BimDes CD8 cells that persist abnormally upon transfer into Alb-K^b mice do not cause immunopathology because they are anergic. To investigate this possibility, we tested the ability of these lymphocytes to proliferate following *in vitro* restimulation. A significant number of BimDes CD8 cells recovered from Alb-K^b mice at day 15 divided efficiently when cultured with allogeneic splenocytes but not when cultured with B10.BR splenocytes (Fig.5E), indicating that these cells were not anergic. These results demonstrate that Bim is essential for the premature death of CD8 T cells activated by hepatocyte-derived antigens in the liver and they also indicate that preventing the death of these T cells on its own does not lead to immunopathology.

Discussion

Although primary activation of CD8 T cells in the liver and subsequent tolerance has now been demonstrated by several independent studies^{4, 6–8, 24}, the molecular mechanisms responsible for this process remain unknown. In this study, we focused on T cell activation by hepatocytes in the absence of inflammation, a situation that is relevant to very early stages of HCV infection and/or peripheral tolerance to hepatocyte-expressed antigens. We provide evidence that following intrahepatic primary activation CD8 T cells acquire a tolerant phenotype, characterized by poor effector function and early Bim-dependent apoptotic death.

Primary T cell activation in the liver occurs under a very different context compared to the confined environment of the LN. Despite this difference, T cells activated by hepatocytes or LN-APCs both upregulated several classical ‘activation markers’ in a similar fashion, at least initially. Amongst the markers examined, CD25 and CD54 were the only two molecules that were differentially expressed between LN- versus hepatocyte-activated T cells. Detailed kinetic analysis suggested that this phenotype was imprinted very early during the activation process, as striking differences in CD54 expression were evident at day one, even before the T cells had undergone their first division. It is tempting to speculate that the differences in expression of CD25 and CD54 between liver- versus LN-activated T cells is caused by differences in co-stimulatory signals received by the T cells during primary activation in these

two locations. CD25 transcription in T cells that have not received B7:CD28 co-stimulation has been reported to be much lower than that achieved in T cells activated with optimal co-stimulation²⁵.

Unlike professional APCs, in the absence of inflammation hepatocytes do not express costimulatory molecules, such as CD80 and CD86 (Supp. Fig.7) and thus cannot provide the full signal required to produce efficient levels of IL-2 and CD25. As CD25 expression is amplified by IL-2:IL-2R stimulation²⁵, this mechanism might explain why hepatocyte-activated T cells initiated but failed to maintain high expression of CD25. Alternatively, or in addition, the confined environment of the LNs might allow IL-2 to accumulate and thereby increase CD25 expression, while the cytokine is rapidly diluted when T cells produce it within the liver. The lower expression of IL-2 detected within T cells isolated from the liver compared to those activated in LNs supports the first model.

Due to their low expression of CD25 and low IL-2 production, it would be expected that hepatocyte-activated T cells are more prone to death. Developmentally programmed and stress-induced apoptotic cell death is controlled by pro- (Bim, Bak, Bax, Puma) and anti-apoptotic (Bcl-2, Bcl-x_L) members from the Bcl-2 family of proteins²⁶. In healthy cells Bax and Bak are kept in check by the anti-apoptotic Bcl-2 family members. Apoptotic stimuli, such as cytokine withdrawal, cause activation of pro-apoptotic proteins (e.g. Bim), induction of the caspase cascade and apoptosis.

We found that the expression of Bcl-2 and Bcl-x_L was upregulated following T cell activation and their levels were similar between those cells activated in the liver or LNs. Interestingly, levels of Bim increased in T cells activated in the liver but not in those activated in the LN. The induction of Bim, the expression of Annexin V at the cell surface and the presence of active caspase-3 in hepatocyte-activated T cells indicates that these cells are programmed to die, and this is consistent with studies on T cells activated by hepatocytes *in vitro*¹⁰. Des-TCR CD8 T cells lacking Bim accumulated to considerably greater numbers upon injection into Alb-K^b mice than control Des-TCR CD8 cells. To our knowledge this is the first report showing that T cells activated by hepatocytes *in situ* die after 3 days of activation and that Bim is essential for this process. Given the defects in IL-2 and IL-2R α chain production seen in liver-activated CD8 T cells, it appears likely that Bim is induced as a consequence of insufficient IL-2/IL-2R signaling. Consistent with this notion, loss of Bim was shown to render T cells resistant to IL-2 deprivation-induced apoptosis²³.

In accordance with previous studies²⁷, “rescued” Bim-deficient T cells did not accumulate at the site of antigen expression, but were rather found to accumulate in lymphoid tissues. However not all liver-activated BimDes cells persisted indefinitely in lymphoid tissues of Alb-K^b mice. At day 30 following transfer, fewer BimDes T cells were recovered from these animals (data not shown), suggesting that other Bim-independent mechanisms regulate T cell death in the longer term.

Despite their increased survival *in vivo* and their ability to exert full effector function, BimDes cells were unable to cause hepatitis in Alb-K^b mice. These results are consistent with those obtained in a model of T cell tolerance to an islet β cell antigen²⁸, providing further evidence that preventing death of autoreactive T cells alone is not sufficient to induce autoimmunity.

Functional and deletional tolerance following intrahepatic primary T cell activation seems to contradict recent studies showing that naïve T cells activated in a mouse liver allograft acquired full effector function⁶. The reasons for discrepancies between the two models remain unclear, but might be due to the very different intrahepatic environment in which the T cells were primed. In our model, naïve T cells are activated in the liver solely by hepatocytes and in the absence of inflammation or tissue damage. In contrast, in the allograft transplant model all

hepatic cells, not only hepatocytes but also Kupffer cells and LSEC, participate in the activation of naïve T cells and notably, this occurs in the context of significant surgery-induced damage/inflammation.

Although tolerogenic mechanisms involved in liver transplantation remain to be addressed, those mediated by hepatocytes in the absence of inflammation are equally important. This type of antigen presentation might play an important role in peripheral tolerance. It is possible that some T cells specific for antigens presented exclusively by hepatocytes escape thymic censorship and meet their cognate antigen in the liver. We propose that in this context, naïve T cells will undergo limited proliferation without acquisition of effector functions, rapidly followed by Bim-dependent apoptosis.

Our findings may also be relevant to early HBV and HCV infection. Both HBV and HCV are hepatotropic and non-cytopathic, suggesting that it might take several days before cross-presentation of viral antigens is initiated in LNs. During this time, T cell deletion following intrahepatic activation might create a hole in the repertoire of high affinity virus-specific T cells thereby compromising subsequent cross-presentation induced immune responses in lymphoid tissues. We have hypothesized^{13, 29} that this mechanism contributes to the ability of HBV and HCV to persist in the host and establish chronic infections. The role of the innate immune response in this process remains unclear and need to be investigated. Recent studies indicate that HBV induces little expression of pro-inflammatory cytokines during the incubation time, suggesting that it acts as a “stealth virus” that circumnavigates the activation of innate immunity.³⁰ Our model would predict that antigen presentation in the liver under these conditions would favour tolerance rather than immunity. Interestingly, using an unbiased approach of gene expression profiling, a recent study³³ has demonstrated that Bim is one of the main molecule upregulated on HBV-specific CD8 T cells in patients with chronic compared to resolved infection. Our work suggests that this pro-apoptotic phenotype is induced by hepatocyte priming and contributes to the failure of viral control in these patients. In contrast to HBV, HCV infection appears to activate innate immunity.³⁰ If this activation is important to the triggering of effective adaptive immunity, it is tempting to speculate that HCV has evolved other strategies to take advantage of intrahepatic activation and counteract this early immune defence. Several studies,^{31, 32} suggest that HCV spreads very quickly. This might allow the virus to induce tolerance in a short period of time before full activation of the innate immunity. Other inhibitory mechanisms, such as PD-1 engagement, regulatory T cells and HCV-inhibitory effects on dendritic cells, might operate at a later phase of the infection to maintain immunological tolerance.²⁹

In conclusion, this study provides the first evidence that T cells activated by hepatocytes acquire a phenotype distinct from those activated in LN, characterized by abnormally low expression of CD25 and CD54, poor effector function, and high expression of pro-apoptotic proteins, a phenotype consistent with death caused by cytokine deprivation. Identification of Bim as a critical regulator of this death pathway provides a new target for novel strategies to reverse or promote this process to modulate immune responses in the liver.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALT	serum alanine aminotransferase
APC	antigen presenting cell
Bcl-2	B cell lymphoma 2
BFA	Brefeldin A
bm	bone marrow
CFSE	CarboxyFluorescein Succinimidyl Ester
HCV	hepatitis C virus
IL-2R	Interleukin-2 receptor
LSEC	Liver sinusoidal endothelial cell
LN	Lymph node
M.F.I	Mean fluorescence intensity
PI	Propidium iodide

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Fig. 1.

Most liver- and LN-activated cells do not recirculate during the first two days of activation.

A) Chimeric mice were created in which cognate antigen for the TCR Tg CD8 T cells was restricted to the LN (Met-K^b bm→B10.BR, M-B) or absent (B10.BR bm→B10.BR, B-B). 2×10^6 CFSE-labeled DesRAG T cells were adoptively transferred into these mice and the blood, livers, LNs and spleens were examined 2 days later for the presence of activated donor CD8 T cells by staining with antibodies to CD8 and CD69 followed by FACS analysis. Histograms displayed are derived from CFSE+CD8+PI- T cells within samples and are representative of 2 independent experiments (n=6). **B)** CFSE-labeled DesRAG T cells were adoptively transferred into B10.BR or Alb-K^b mice and analyzed as described in Fig.1A (n=12).

Fig. 2.

Hepatocyte-activated CD8 T cells display a unique CD25^{low} CD54^{low} phenotype. 2×10^6 CFSE-labeled DesRAG T cells were adoptively transferred into B10.BR, Met-K^b or Alb-K^b mice and analyzed as for Fig.1A. Histograms displayed are derived from CFSE+CD8+PI- cells in all samples.

Fig. 3.

Hepatocyte-activated CD8 T cells exhibit defects in cytotoxicity and cytokine production. **A)** 10^6 DesRAG T cells were adoptively transferred into Met-K^b, Alb-K^b or B10.BR mice and an *in vivo* CTL assay was performed at day 4. Histograms (left panel) displayed are gated on CFSE +PI- lymphocytes within samples. Results displayed in the right panel represent the means of triplicate samples $\pm$ S.E.M in 3 independent experiments. **B)** and **C)** CFSE-labeled DesRAG T cells were injected into the tail veins of B10.BR or Met-K^b mice. After 2 days, livers and LN were harvested, lymphocytes isolated, stimulated *ex vivo*, stained and analyzed for intracellular IFN- γ (**B**) or intracellular IL-2 (**C**) by flow cytometry. Histograms are representative of 3 individual experiments performed in triplicate and gated on CFSE+CD8+ cells in all samples.

Fig. 4.

Hepatocyte-activated CD8 cells express excess Bim and are abnormally prone to apoptosis. CFSE-labeled DesRAG T cells were adoptively transferred into Met-K^b, Alb-K^b or B10.BR mice. Livers and LN were harvested after 2 (**A**) or 3 days (**B** and **C**) and lymphocytes isolated. Cells were stained with anti-CD8 and Desiré antibodies and CFSE+CD8+ Des-TCR+ cells were gated and analyzed by FACS to detect intracellular Bcl-x_L, Bcl-2, Bim (**A**), the activated form of caspase-3 (**B**) or cell surface Annexin V (**C**). Except for C), histograms displayed are representative of 3 individual experiments performed in triplicate.

Fig. 5.

Loss of Bim promotes accumulation of Des-TCR CD8 T cells activated in the liver but does not enhance immunopathology. For all experiments 1.5×10^7 Des-TCR or BimDes T cells were injected into the tail veins of B10.BR or Alb-K^b mice. Met-K^b mice were used as positive controls in some experiments. (A) Livers of recipient mice were harvested at day 6 and CTL activity was measured *in vitro*. (B) Organs were harvested after 2 days and assessed for cytokine expression as for Figure 3B. (C) Organs were harvested after 15 days, leukocytes isolated, counted and stained with anti-CD8 and Desiré antibodies. The percentages of Des+CD8+PI-cells was determined by FACS and the mean $\pm$ S.E.M of total numbers of CD8+Des+ T cells from 2 independent experiments are shown (n=8). (D) Serum ALT levels were measured and plotted against time for Alb-K^b and B10.BR recipient mice. Day 5 results for all recipient lines are shown as histograms. The mean $\pm$ S.E.M from 2 independent experiments is shown (n=8). (E) LN and spleens from recipient mice in Fig.5C were harvested at day 15. Lymphocytes were isolated, CFSE labeled and cultured for 2 days with irradiated C57BL/6 or B10.BR splenocytes. CFSE profiles were analyzed by FACS 48 h later and representative histograms from 2 independent experiments are shown.