The site of primary T cell activation is a determinant of the balance between intrahepatic tolerance and immunity

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Hepatic immunobiology is paradoxical: although the liver possesses unusual tolerogenic properties, it is also the site of effective immune responses against multiple pathogens and subject to immune-mediated pathology. The mechanisms underlying this dichotomy remain unclear. Following previous work demonstrating that the liver may act as a site of primary T cell activation, we demonstrate here that the balance between immunity and tolerance in this organ is established by competition for primary activation of $CD8^+$ T cells between the liver and secondary lymphoid tissues, with the immune outcome determined by the initial site of activation. Using a transgenic mouse model in which antigen is expressed within both liver and lymph nodes, we show that while naive $CD8^+$ T cells activated within the lymph nodes were capable of mediating hepatitis, cells undergoing primary activation within the liver exhibited defective cytotoxic function and shortened half-life and did not mediate hepatocellular injury. The implications of these novel findings may pertain not only to the normal maintenance of peripheral tolerance, but also to hepatic allograft tolerance and the immunopathogenesis of chronic viral hepatitis.

Introduction

It is apparent that the liver possesses unique tolerogenic properties (1, 2), yet this organ is susceptible to immune-mediated pathology and is also the site of successful immune responses against a range of pathogens. In transplantation, liver allografts may be spontaneously accepted across complete MHC mismatch in a variety of species (3–5), and acceptance of a liver graft can induce specific tolerance to subsequent transplants of other solid organs, which would otherwise be rejected (6). In addition, it has also been demonstrated in the rat that creation of a mesocaval shunt, whereby portal venous flow is diverted directly into the systemic circulation without initially traversing the liver, impairs the development of oral tolerance (7), which suggests that, at least in certain circumstances, the liver may play a role in this phenomenon. Nevertheless, the liver is the target of a number of relatively rare but clinically important autoimmune conditions (8, 9). Furthermore, liver injury associated with chronic infection by the hepatotropic hepatitis B and C viruses is largely mediated by the resultant immune response (10, 11).

The mechanisms and parameters determining the balance between intrahepatic immunity and tolerance remain unclear. Studies using transgenic mice have provided some insights, as they allow observation of T cells specific for antigens expressed within the liver. Early studies have shown that the liver effectively retains activated T cells (12–15). We have recently extended this observation by showing that antigen-specific retention of naive $CD8^+$ T cells can also occur within the liver, with subsequent activation in situ (16, 17). This property suggests that the liver is an exception to the generally accepted rule of T cell activation and trafficking, which predicts that naive T cells recirculate via the lymph and blood, but do not enter peripheral tissues prior to activation in LNs (18). The ability of the liver to act as a site of primary $CD8^+$ T cell activation may be due to unusual conditions of slow blood flow and the unique structure of the hepatic sinusoid (19), which may favor direct contact between T lymphocytes and liver cells including hepatocytes (20).

Despite the demonstration that naive $CD8^+$ T cells can undergo intrahepatic activation, their function and fate remained uncertain. We have hypothesized that T cell interactions with liver cells result in inappropriate activation and deletion of antigen-specific T cells, a phenomenon that may explain some of the tolerogenic properties of the liver (20). Consistent with this, transgenic T cells activated by hepatocytes in culture became CTLs before dying prematurely (21, 22). In vivo data were conflicting, however, with different transgenic models yielding contradictory results. The adoptive transfer of T cells from the transgenic mouse lineage (Des-TCR) expressing a transgenic T cell receptor (TCR) specific for H-$2K^b$ into Alb-K^b recipient mice, which express the allo-MHC molecule H-$2K^b$ in the liver under the control of the mouse albumin promoter (23), did not result in the development of hepatitis in the absence of prior hepatic inflammation (24). In contrast, the adoptive transfer of Des-TCR T cells into Met-K^b mice, which express the same allo–H-$2K^b$ antigen on hepatocytes under the control of the sheep metallothionein promoter (25), induced a severe but transient hepatitis peaking at day 5–6 and lasting for 4 days (26).

By studying both the Met-K^b and the Alb-K^b transgenic models, we show here that the development of an efficient CTL response resulting in hepatitis was associated with T cell activation and proliferation within the LN, providing, to our knowledge, the first clear demonstration in the intact organism that autoimmunity requires T cell priming in LNs. In contrast, although T cells were activated and proliferated within the liver, primary intrahepatic activation of $CD8^+$ T cells resulted in a defective CTL response

Nonstandard abbreviations used: ALT, alanine aminotransferase; HCV, hepatitis C virus; PI, propidium iodide; TCR, T cell receptor.

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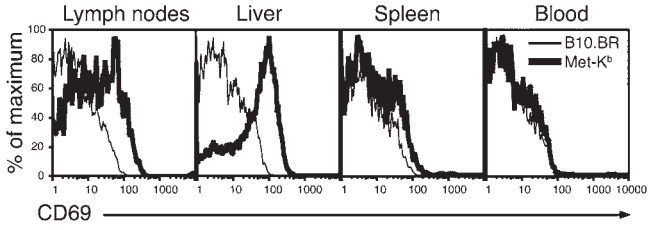

Figure 1
Early activation of naive CD8$^+$ Des-TCR T cells occurred independently within the liver and LNs of Met-K^b mice. CFSE-labeled Des-TCR LN cells were adoptively transferred into Met-K^b and control B10.BR mice, and organs were harvested 2 hours 15 minutes later. Lymphocytes isolated from blood, liver, spleen, and LNs were analyzed by flow cytometry. Representative histograms show CD69 expression by CFSE$^+$ CD8$^+$ CD44^{low} cells within a forward and side scatter gate appropriate for lymphocytes.

insufficient to mediate hepatitis. These results demonstrate, for the first time to our knowledge, that liver and LNs compete for primary activation of CD8$^+$ T cells but commit T cells to distinct pathways of differentiation, which determine the outcome of the immune response. In addition, these data argue against previous claims (24) that prior inflammatory signals are required for CD8$^+$ T cells to access antigen within the liver. This model further explains how effective intrahepatic immune responses are generated, despite the inherent capacity of this organ for the induction of tolerance, and has profound implications for the pathogenesis and treatment of immune-mediated hepatic diseases.

Results

Competition between liver and LNs for primary T cell activation. We have previously examined events following the adoptive transfer of CD8$^+$ Des-TCR T cells, which express an H-2K^b–specific TCR (27), into syngeneic Met-K^b mice on an H-2^k background. These studies demonstrated that TCR transgenic CD8$^+$ T cells were specifically retained in the liver of unmanipulated Met-K^b mice within minutes of adoptive transfer and underwent simultaneous activation within both LNs and liver, but not other organs (16). Consistent with these observations, one of the earliest markers of activation, CD69, was expressed on donor transgenic T cells as early as 2 hours after adoptive transfer (16). As Des-TCR LN cells also contain CD8$^+$ T cells previously activated via a second TCR present because of incomplete allelic exclusion of the endogenous α chain gene (28), we thus investigated whether CD69 was expressed within the naive population of CD8$^+$ Des-TCR T cells, which would not have upregulated CD44 at this very early time point. At 2 hours 15 minutes following adoptive transfer, CD44^{low} T cells expressed CD69 in both the LNs and the liver, but not the blood, of Met-K^b mice; a small population of cells with increased but lower CD69 expression was seen in the spleen (Figure 1). This activation was antigen-specific, as CD69 expression by CD8$^+$ Des-TCR$^+$ T cells was not upregulated in nontransgenic recipients (Figure 1). These experiments demonstrate that CD69 expression occurred within the naive T cell population and confirm that the liver is a site of primary activation for CD8$^+$ T cells. Adoptive transfer of Des-TCR donor T cells deficient for RAG-1, in which there is no recombination of the endogenous α chain and all transferred T cells were naive, confirmed these results (data not shown).

Interestingly, early antigen-specific T cell retention within the liver was associated with reduced numbers of transgenic CD8$^+$ T cells in the LNs (data not shown and ref. 16), which indicated that T cells retained within the liver were initially withdrawn from the circulation, resulting in a lower number of cells gaining access to the LNs. Primary T cell activation within the liver was associated with loss of the LN-homing receptor CD62L (data not shown), making this process irreversible. These results indicated that antigen-expressing liver competes with LNs for retention and primary T cell activation at early time points.

The occurrence of independent synchronous primary activation in the liver and LNs raised the question of whether the site of primary activation could determine differential outcomes in terms of effector function. To examine the relative contribution of intrahepatic versus intranodal primary activation to liver damage, we investigated whether immune-mediated hepatitis could develop when LN entry by naive CD8$^+$ Des-TCR T cells was prevented.

Anti-CD62L mAb prevented LN entry by CD8$^+$ Des-TCR T cells without affecting intrahepatic T cell activation. LN entry by naive T cells is largely mediated by interactions between L-selectin (CD62L) and peripheral LN addressins expressed by the high endothelial venules of LNs (29). Naive T cells express high levels of CD62L, allowing them to preferentially enter LNs. CD62L is rapidly lost from the surface of cells following primary activation via proteolytic cleavage, with a 90% reduction in expression by 4 hours (30). A number of investigators have effectively blocked the majority of LN entry by both naive CD4$^+$ and CD8$^+$ T cells via the administration of anti-CD62L mAb (31–33). To study events occurring in Met-K^b mice in the absence of LN entry by naive CD8$^+$ Des-TCR T cells, this strategy was therefore adopted in this model.

Intraperitoneal administration of a single dose of 250 μg of the anti-CD62L mAb Mel14 to Met-K^b mice 4 hours prior to adoptive transfer of CD8$^+$ Des-TCR LN cells, a similar protocol to that used in previous studies, led to an approximately 90% fall in the number of CD8$^+$ Des-TCR T cells recovered from the LNs 4 hours after transfer in comparison with control recipients (Figure 2A). Consistent with previous data indicating that entry of naive lymphocytes into the spleen is independent of CD62L (32, 34), the number of CD8$^+$ Des-TCR T cells observed in this organ was unchanged. Interestingly, consistent with the competitive model mentioned above, there was no decrease, but rather an increase, in recruitment of CD8$^+$ Des-TCR T cells to the livers of Met-K^b mice. In addition, antibody treatment did not affect the total number of CD8$^+$ Des-TCR T cells recovered from recipients (Figure 2A). Intrahepatic primary activation of CD8$^+$ Des-TCR T cells in Met-K^b mice was not altered by the prior administration of anti-CD62L antibodies, as assessed by CD69 expression at 4 hours after adoptive transfer (Figure 2B). Furthermore, the administration of anti-CD62L mAb had no effect on the recruitment into division or proliferation of adoptively transferred CD8$^+$ Des-TCR T cells within the livers of Met-K^b mice at 2 days following adoptive transfer, as assessed by decreased CFSE content (Figure 2C). Similar results were obtained using splenectomized mice (Figure 2C), demonstrating that the spleen did not significantly contribute to the pool of proliferating CD8$^+$ Des-TCR T cells observed within the liver. It is important to note that, in the absence of anti-CD62L mAb treatment, T cells activated within the liver and LNs were recruited into division and proliferated at very similar rates, irrespective of whether splenectomy was performed (Figure 2D). These results suggest that in contrast to the situation in the LNs, CD62L is not involved

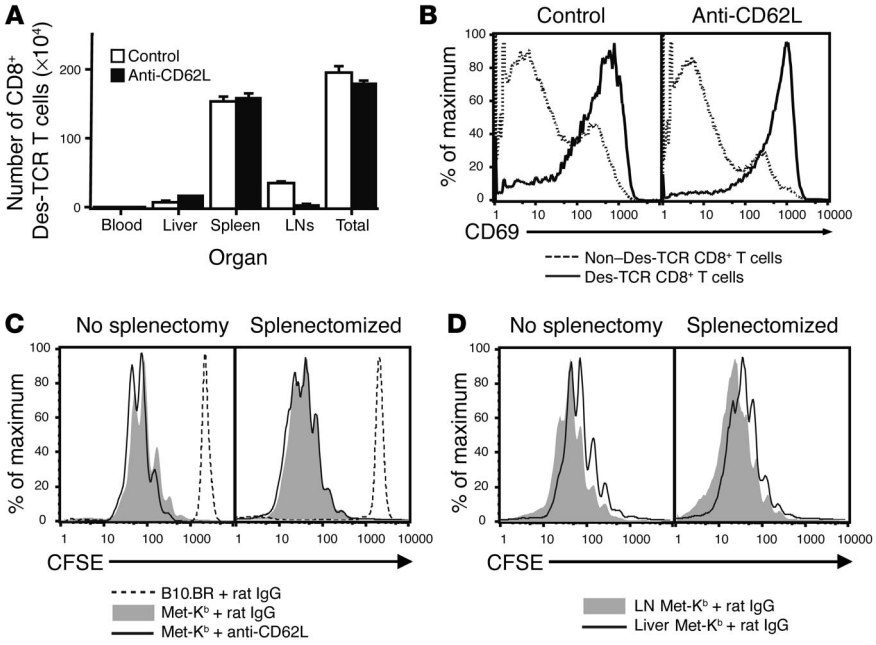

Figure 2

Administration of anti-CD62L antibody reduced LN entry by $CD8^+$ Des-TCR T cells but did not affect their intrahepatic activation or proliferation in Met-K^b mice. (**A**) Total number of Ly5.1$^-$ $CD8^+$ Des-TCR T cells in various organs of Ly5.1$^+$ Met-K^b mice pretreated with anti-CD62L mAb or PBS and injected 4 hours later with Ly5.1$^-$ Des-TCR LN cells. Organs were harvested 4 hours after cell transfer. (**B**) CD69 expression by donor Ly5.1$^-$ $CD8^+$ Des-TCR T cells (solid line) versus recipient Ly5.1$^+$ $CD8^+$ T cells (dotted line) within the livers of Met-K^b mice at 4 hours after adoptive transfer. Representative histograms were gated on forward and side scatter gates appropriate for lymphocytes, and Ly5.1$^-$ $CD8^+$ PI^- cells for $CD8^+$ Des-TCR T cells or Ly5.1$^+$ $CD8^+$ PI^- cells for liver resident non–Des-TCR $CD8^+$ T cells. (**C**) CFSE profiles of donor $CD8^+$ Des-TCR T lymphocytes, at 2.5 days after transfer, isolated from non-splenectomized and splenectomized Met-K^b (shaded plot) and B10.BR (dashed line) mice preadministered control purified rat IgG, or Met-K^b mice preadministered anti-CD62L mAb (solid line). $CD8^+$ Des-TCR T cells did not proliferate in the livers of B10.BR mice administered anti-CD62L (data not shown). (**D**) CFSE profiles of donor $CD8^+$ Des-TCR lymphocytes isolated from the liver (solid line) and LNs (shaded plot) of Met-K^b mice administered control rat IgG illustrate that donor T cells activated at these 2 sites proliferated at a similar rate.

in the retention and subsequent activation and proliferation of naive $CD8^+$ T cells within the liver.

Interference with LN entry by $CD8^+$ Des-TCR T cells prevented development of hepatitis in Met-K^b mice. To determine the effects of blocking the majority of LN entry by naive $CD8^+$ Des-TCR T cells on the development of hepatitis in Met-K^b mice, anti-CD62L mAb was administered to Met-K^b mice 4 hours prior to adoptive transfer of Des-TCR LN cells, and then daily for 5 days. To exclude the possibility that observed effects may be due to intrasplenic primary activation, experiments were carried out in both intact and splenectomized Met-K^b mice. Consistent with previous results, control recipients developed a significant but self-limited hepatitis, peaking at day 6 (Figure 3A). However, treatment with anti-CD62L mAb led to a marked decrease in hepatitis, based on alanine aminotransferase (ALT) levels, in both unmanipulated and splenectomized animals (Figure 3A). Similar results were obtained when anti-CD62L mAb was administered 4 hours before adoptive transfer of Des-TCR LN cells and then once 24 hours after adoptive transfer (Figure 3B), which suggests that the effects of initial mAb blockade were long-lasting and/or irreversible. Consistent with the marked decrease in ALT levels, immunohistochemistry performed on liver sections using an anti-CD8 mAb demonstrated rare and scattered lobular and portal tract infiltrates in the livers of anti-CD62L–treated animals (data not shown). In sharp contrast, significant lobular and portal infiltrates were present in the livers of control Met-K^b animals treated with control Ig (data not shown).

These results indicate that activation of transgenic T cells within the LNs of Met-K^b mice was required for the development of hepatitis. Moreover, despite efficient primary activation and recruitment into proliferation, T cells activated within the liver did not cause hepatitis.

Alb-K^b mice failed to develop hepatitis. To exclude the possibility that inhibition of hepatitis was due to secondary effects of the anti-CD62L mAb, and to confirm the determinant role of intranodal T cell activation in the development of hepatitis, the contribution of intranodal versus intrahepatic primary activation to hepatitis was investigated using Alb-K^b mice. In contrast to Met-K^b mice, previous studies have indicated that Alb-K^b mice do not develop hepatitis following adoptive transfer of $CD8^+$ Des-TCR T cells in the absence of preexisting inflammation (24). It has been argued that discrepancies between these models may be due to different MHC backgrounds used in each model (H-2^k versus H-2^{dk}) and differing levels or sites of transgenic expression (24, 26).

To determine whether the genetic background influenced the response of Des-TCR T cells, Alb-K^b and Met-K^b mice were both bred onto the same H-2^{dk} background and assayed for the development of hepatitis upon transfer of syngeneic Des-TCR (H-2^{dk}) LN cells. Similar to Met-K^b (H-2^k) mice, Met-K^b (H-2^{dk}) mice developed hepatitis following adoptive transfer of Des-TCR (H-2^{dk}) LN cells, suggesting that the H-2^{dk} background did not abrogate the occurrence of hepatitis in Met-K^b mice (Figure 4A). In contrast to Met-K^b mice and consistent with previous results, Alb-K^b mice injected with Des-TCR (H-2^{dk}) LN cells did not develop biochemical hepatitis (Figure 4A). A significant difference between Met-K^b and Alb-K^b mice in the development of hepatitis was also observed upon transfer of Des-TCR RAG-$1^{-/-}$ LN cells (data not shown). These results were confirmed at the histological level on day 6, the biochemical peak of hepatitis in the Met-K^b model (data not shown). While Met-K^b (H-2^{dk}) mice developed hepatitis associated with significant $CD8^+$ T cell lobular and portal infiltrates, only minor $CD8^+$ infiltrates were observed in the livers of Alb-K^b (H-2^{dk}) mice (data not shown).

Differences in these models were not explained by higher levels of H-2K^b expression by Met-K^b hepatocytes: Alb-K^b hepatocytes expressed H-2K^b levels that were comparable to C57BL/6 (H-2^b) WT levels and 2–3 times higher than the H-2K^b levels expressed by Met-K^b hepatocytes (Figure 4B). Furthermore, H-2K^b expres-

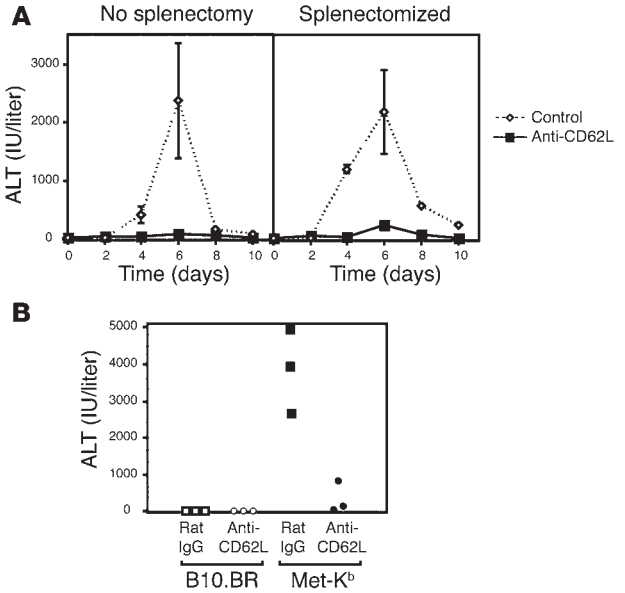

Figure 3
Administration of anti-CD62L mAb abrogated the development of hepatitis in Met-K^b mice following adoptive transfer of CD8$^+$ Des-TCR T cells. (**A**) Non-splenectomized and splenectomized Met-K^b mice were preadministered anti-CD62L mAb (solid line) or PBS alone (dotted line) as described in Figure 2A. Further doses of mAb or PBS alone were administered intraperitoneally to mice at daily intervals until day 5 after adoptive transfer. Hepatocyte damage was assessed by measurement of serum levels of ALT. Data points represent means ± SEM for groups of 3 mice. (**B**) Unmanipulated Met-K^b mice and B10.BR mice were administered anti-CD62L mAb or purified rat IgG. A further dose of antibodies was administered intraperitoneally 24 hours after adoptive transfer. Data points represent serum ALT values from individual mice at day 5.

sion could not be detected on Kupffer cells, liver DCs, or liver sinusoidal endothelial cells from either Alb-K^b or Met-K^b mice by flow cytometry (Supplemental Figure 1A; supplemental material available at http://www.jci.org/cgi/content/full/114/5/701/DC1). Since levels of intrahepatic H-2K^b expression did not explain why hepatitis occurred in Met-K^b but not in Alb-K^b mice, we investigated whether differences between the 2 models correlated with differences in intranodal H-2K^b expression.

Failure to induce hepatitis in Alb-K^b mice was associated with lack of Des-TCR T cell activation within LNs. To establish whether the lack of hepatitis in the Alb-K^b (H-2^{dk}) model was due to failure to induce Des-TCR T cell activation in the LNs, CFSE-labeled Des-TCR (H-2^{dk}) LN cells were adoptively transferred into Alb-K^b (H-2^{dk}), Met-K^b (H-2^{dk}), and control nontransgenic (H-2^{dk}) recipients, and CD69 expression was analyzed 2 hours 15 minutes after transfer. Consistent with results in Met-K^b (H-2^k) mice (Figure 1), CD8$^+$ Des-TCR T cells were found to express CD69 in both the liver and the LNs of Met-K^b (H-2^{dk}) mice (Figure 5A). In common with Met-K^b (H-2^{dk}) mice, CD8$^+$ Des-TCR T cells in the livers of Alb-K^b mice expressed CD69, which suggested they had been activated in situ. However, in contrast to findings in Met-K^b mice, CD69 expression levels of CD8$^+$ Des-TCR T cells in the LNs of Alb-K^b mice were comparable to those in control mice, indicating that in this model, primary activation of Des-TCR T cells did not occur within the LNs (Figure 5A).

It should be noted that some CD8$^+$ Des-TCR T cells in the livers of both Met-K^b and Alb-K^b mice did not upregulate CD69 expression (Figure 5A). These cells probably represented the CD8$^+$ Des-TCR T cells expressing high levels of endogenous TCR α chain, which consequently did not interact with high enough avidity with cells expressing H-2K^b to trigger activation. To explore this possibility, uniformly transgenic TCR-bearing Des-TCR RAG-1$^{-/-}$ LN cells were adoptively transferred into recipients equivalent to those described above. Consistent with results using Des-TCR (H-2^{dk}) donor LN cells, Des-TCR RAG-1$^{-/-}$ T cells were activated in both the livers and the LNs of Met-K^b (H-2^{dk}) mice, whereas an increased proportion of cells upregulating CD69 were identified only in the livers of Alb-K^b (H-2^{dk}) mice (Figure 5A). However, in contrast to Des-TCR (H-2^{dk}) T cells, the majority of CD8$^+$ Des-TCR RAG-1$^{-/-}$ T cells recovered from these compartments expressed CD69, consistent with the hypothesis that intrahepatic activation was restricted to CD8$^+$ T cells capable of high-avidity interaction with intrahepatic antigen-expressing cells.

Proliferation of Des-TCR T cells occurred following activation within the livers of Alb-K^b mice, as assessed by decreasing CFSE content of donor CD8$^+$ cells at 2.5 and 6 days following adoptive transfer (Figure 5, B and C). Fewer dividing cells were present in the LNs or blood, consistent with primary activation occurring within the liver with subsequent recirculation (16). Confirming significant proliferation, the total number of CD8$^+$ Des-TCR T cells upregulating expression of the CD43 activation–associated glycoform, a marker associated with CTL activity (35) and cell division (data not shown), was increased approximately 25-fold within the livers of Alb-K^b recipients over that observed in control antigen-free recipients at day 6 (Figure 5E). Consistent with activation and proliferation of CD8$^+$ Des-TCR T cells being restricted to the livers of Alb-K^b mice, the number of CD43 activation–associated glycoform^{high} CD8$^+$ Des-TCR T cells recovered from other organs of Alb-K^b mice did not differ from background levels in antigen-

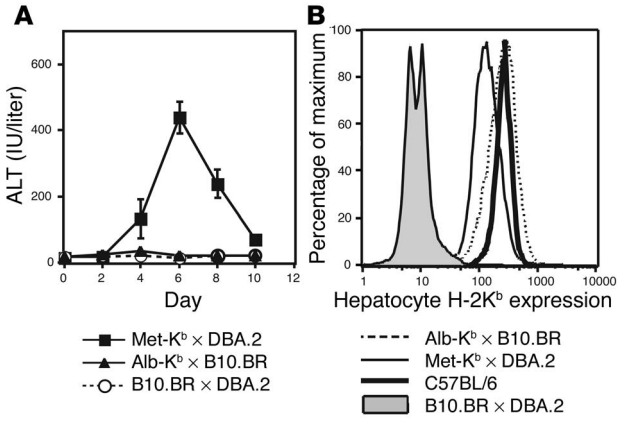

Figure 4
Hepatitis did not occur in Alb-K^b mice on the same genetic background as Met-K^b mice following adoptive transfer of Des-TCR LN cells, despite similar levels of antigen expression by hepatocytes. (**A**) Serum ALT levels of H-2^{dk} recipient Met-K^b, Alb-K^b, and control mice following adoptive transfer of Des-TCR (H-2^{dk}) LN cells. Data points represent means ± SEM for groups of 4 mice. (**B**) H-2K^b expression by viable (PI$^-$) hepatocytes from H-2^{dk} Alb-K^b, Met-K^b, and control mice, analyzed by flow cytometry.

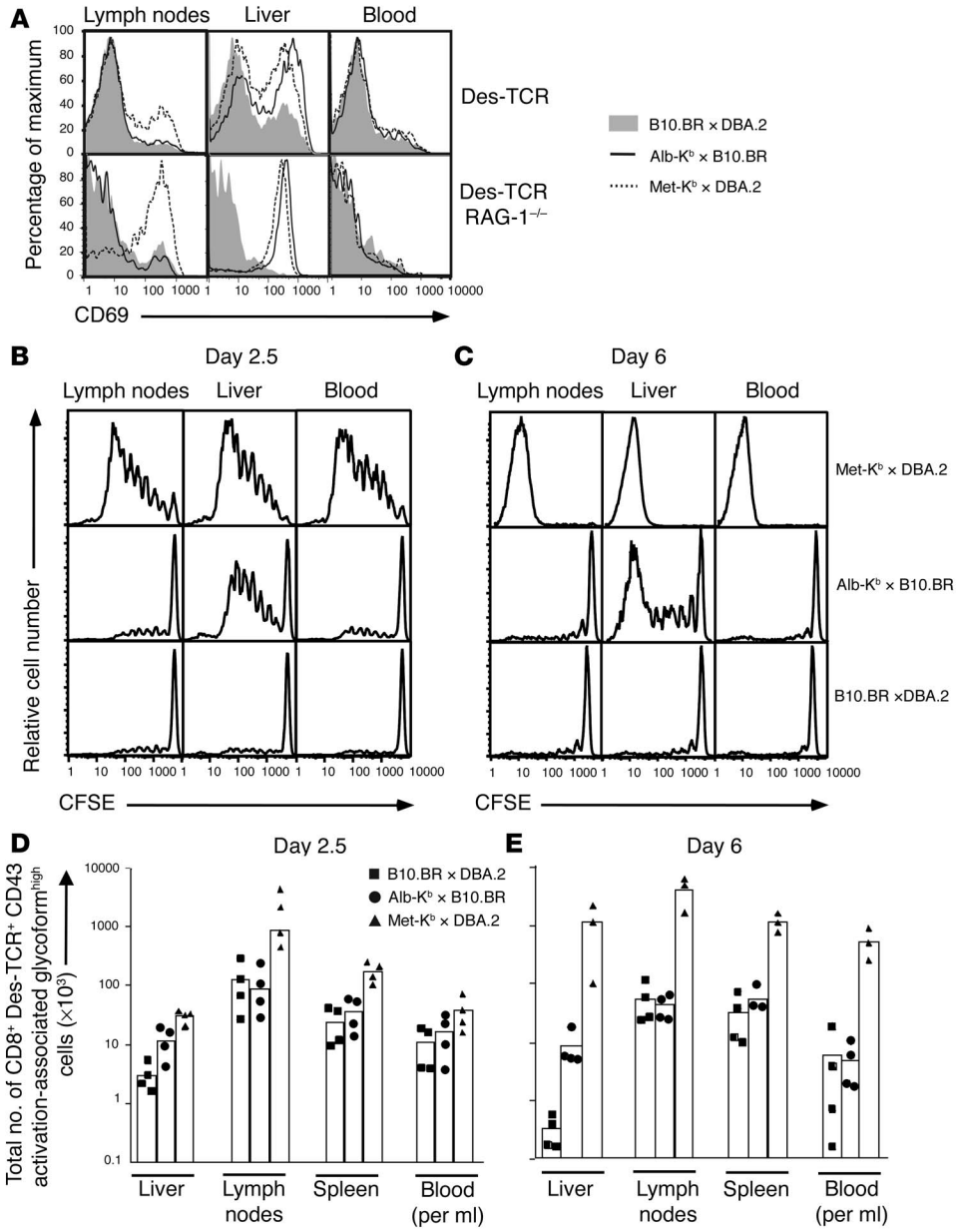

Figure 5
CD8+ Des-TCR T cells were activated within the liver and proliferated following adoptive transfer into H-2^{dk} recipient Alb-K^b and Met-K^b transgenic mice. (**A**) Alb-K^b, Met-K^b, and control mice were injected with CFSE-labeled syngeneic Des-TCR (H-2^{dk}) LN cells (top panels) or Des-TCR RAG-1$^{-/-}$ (H-2^k) LN cells (bottom panels). Lymphocytes were purified from LNs, liver, spleen, and blood of recipient mice at 2 hours 15 minutes following adoptive transfer and analyzed by flow cytometry. Representative histograms show CD69 expression by CFSE+ CD8+ CD44^{low} cells within a forward and side scatter gate appropriate for lymphocytes. (**B** and **C**) Alb-K^b, Met-K^b, and control mice were injected with CFSE-labeled syngeneic Des-TCR (H-2^{dk}) LN cells containing 5 × 10^6 transgenic CD8+ T cells. Lymphocytes were purified from LNs, liver, spleen, and blood of recipient mice at 2.5 days (**B**) or 6 days (**C**) following adoptive transfer and analyzed by flow cytometry. Histograms represent CFSE division profiles of CD8+ Des-TCR+ PI− cells within a forward and side scatter gate appropriate for lymphocytes. (**D** and **E**) Total numbers of Des-TCR+ CD8+ cells expressing upregulated levels of CD43 activation–associated glycoform in various compartments of Alb-K^b, Met-K^b, and B10.BR mice. CFSE-labeled LN cells from Des-TCR mice were adoptively transferred into Alb-K^b, Met-K^b, and B10.BR mice. Organs were harvested at day 2.5 (**D**) or day 6 (**E**), and lymphocytes were isolated and analyzed by flow cytometry. Cell numbers for blood represent Des-TCR CD8+ T cells/ml; plots represent means ± SEM of groups of 3–4 mice.

free control animals at either day 2.5 or day 6 (Figure 5, D and E). Although the total number of effector donor T cells increased significantly in the livers of Alb-K^b mice, it was lower than the number recovered from Met-K^b livers; and while the numbers between the 2 samples differed only 2- to 3-fold at day 2.5 after transfer, this difference increased to 130-fold at day 6. This rising disparity was probably due to the increasing contribution of LN-activated T cells recirculating and accumulating within the livers of Met-K^b mice, whereas, because of the absence of intranodal activation, these cells were absent in Alb-K^b recipients. It is interesting to note that although adoptively transferred CD8+ Des-TCR T cells underwent significant division in both Met-K^b and Alb-K^b mice, a higher proportion of transgenic T cells was recruited into division in Met-K^b liver than in Alb-K^b liver, as judged by the proportion of undivided cells at days 2.5 and 6 (Figure 5, B and C). This difference did vary somewhat from experiment to experiment (compare Figure 5C with Figure 7D) and may be due to higher-avidity interactions resulting from metallothionein promoter–driven transgene upregulation that occurred following infiltration (data not shown). This factor might also contribute to the difference in the total number of effector donor T cells recovered from the livers of Met-K^b and Alb-K^b mice.

Collectively, these experiments indicate that differences in the site of antigen expression and hence the setting of T cell activation are responsible for the disparity in pathology between the Met-K^b and Alb-K^b models. Combined with the results obtained following the administration of anti-CD62L mAb, these results suggest that naive CD8+ T cells activated within the LNs and the liver follow different pathways of differentiation that result in divergent immune responses. To better understand the mechanisms underlying these pathways, we compared the function and survival of intrahepatically and intranodally activated CD8+ T cells.

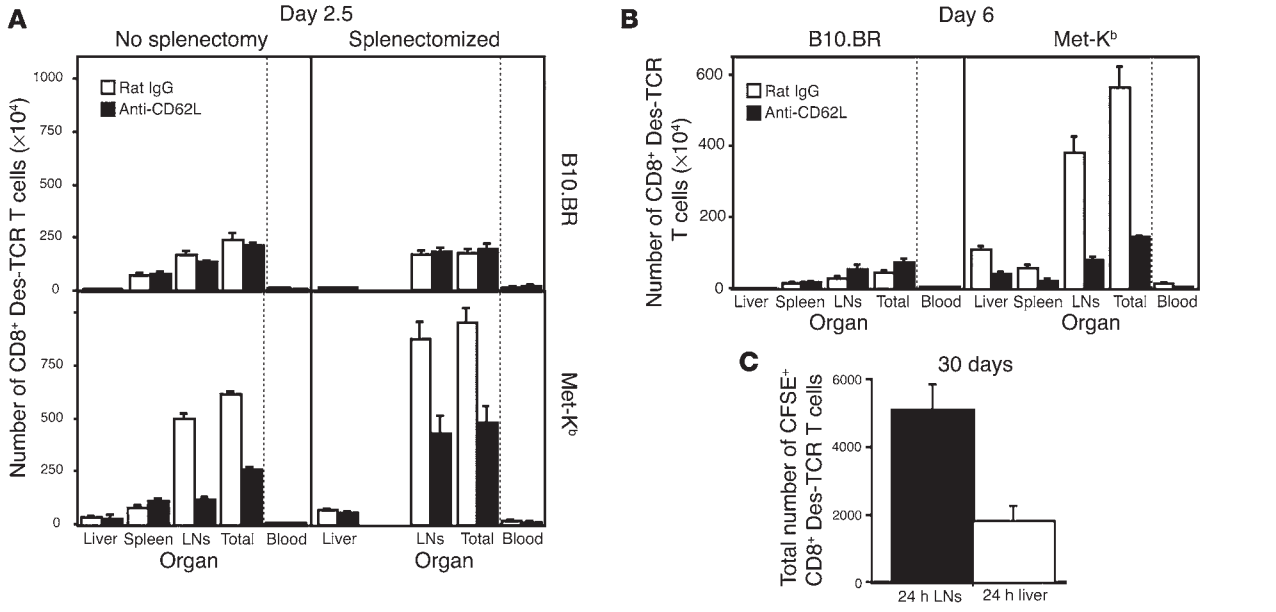

Figure 6

Intrahepatic activation of CD8+ Des-TCR T cells was associated with reduced half-life. (**A** and **B**) Administration of anti-CD62L mAb to Met-K^b mice resulted in reduced CD8+ Des-TCR T cell numbers 2.5 and 6 days after adoptive transfer. Unmanipulated and splenectomized Met-K^b and B10.BR mice were preadministered anti-CD62L mAb or purified rat IgG as described in Methods. A further dose of antibodies was administered intraperitoneally 24 hours after adoptive transfer. Organs were harvested at day 2.5 (**A**) and day 6 (**B**), and lymphocytes were analyzed by flow cytometry. Cell numbers for blood represent CD8+ Des-TCR T cells/ml; cell numbers for other organs represent total CD8+ Des-TCR T cells from the organ. Total cell numbers represent the sum of CD8+ Des-TCR T cells retrieved from these organs. Plots represent means ± SEM for groups of 3 mice. (**C**) T cells activated within the liver are committed to reduced half-life within the first 24 hours following activation. Equal numbers of CFSE-labeled Des-TCR LN cells activated for 24 hours in either liver or LNs of Met-K^b mice were adoptively transferred into a second antigen-free, nontransgenic B10.BR recipient, and 30 days later, lymphocytes from liver, LNs, and spleen were purified as described in Methods. Bars indicate the total number of CD8+ CFSE+ Des-TCR+ cells harvested from the 3 compartments. Plots represent means ± SEM for groups of 3 mice.

CD8+ T cells activated within the liver of Met-K^b mice demonstrated reduced half-life. Results presented above indicated that primary activation of CD8+ Des-TCR T cells within the liver led to an immune response insufficient to cause hepatitis, a phenomenon that may have been due to a quantitative and/or a qualitative defect; that is, due to a deficiency in the number of CTLs generated and/or a defect in CTL function. T cells activated in either Met-K^b or Alb-K^b mice undergo peripheral deletion by 30 days after transfer (16, 36). However, whether T cells activated in the liver and LNs acquire divergent programs of differentiation during this period, leading to variations in half-life, still remained unknown. In order to determine whether T cell activation within the liver or LNs generated different T cell numbers, the number of CD8+ Des-TCR T cells present in various organs of Met-K^b and control B10.BR mice was quantified at 2.5 and 6 days after adoptive transfer, following treatment with anti-CD62L or control Ig. Administration of anti-CD62L mAb to control B10.BR mice did not alter the number of CD8+ Des-TCR T cells recovered from various organs, including LNs (Figure 6, A and B), which indicates that the failure to develop immune-mediated hepatitis in Met-K^b mice was not secondary to anti-CD62L–specific complement-mediated destruction of CD8+ Des-TCR T cells. These results further suggest that despite the initial blocking effect of anti-CD62L mAb at 4 hours after T cell transfer (Figure 2A), naive transgenic T cells were able to again home to the LNs at later time points after mAb treatment; this indicates that the effect of anti-CD62L was not long-lasting when T cells remained naive. In Met-K^b mice, as a result of primary activation at these sites, high numbers of transgenic T cells were recovered from both liver and LNs at 2.5 days after adoptive transfer (Figure 6A). However, in contrast to B10.BR mice, the total number of transgenic T cells recovered from the LNs was reduced in anti-CD62L–treated Met-K^b mice compared with Met-K^b mice treated with control Ig (Figure 6A). This phenomenon was also observed in splenectomized animals (Figure 6A). The apparently long-lasting effects of anti-CD62L mAb treatment in Met-K^b but not B10.BR animals would probably be favored by loss of CD62L expression following activation within the H-2K^b–expressing liver, which would prevent transgenic T cells from homing to the LN.

The reduced number of CD8+ Des-TCR T cells recovered from the LNs of recipient anti-CD62L–treated Met-K^b mice at 2.5 days was not associated with increased numbers of transgenic T cells at extranodal sites, as CD8+ Des-TCR T cell numbers in livers, blood, and spleens of anti-CD62L–treated animals were comparable to those observed in control Ig–treated animals (Figure 6A). Therefore, the total numbers of transgenic T cells recovered were lower in recipient anti-CD62L–treated Met-K^b mice than in mice treated with control Ig (Figure 2D). It could be argued that in the unmanipulated animal, greater numbers of T cells reaching the LNs than the liver would lead to a higher number of cells proliferating within this organ, thus resulting in the generation of lower total T cell numbers in anti-CD62L–treated recipients, in which this mode of activation is blocked. However, this line of reason-

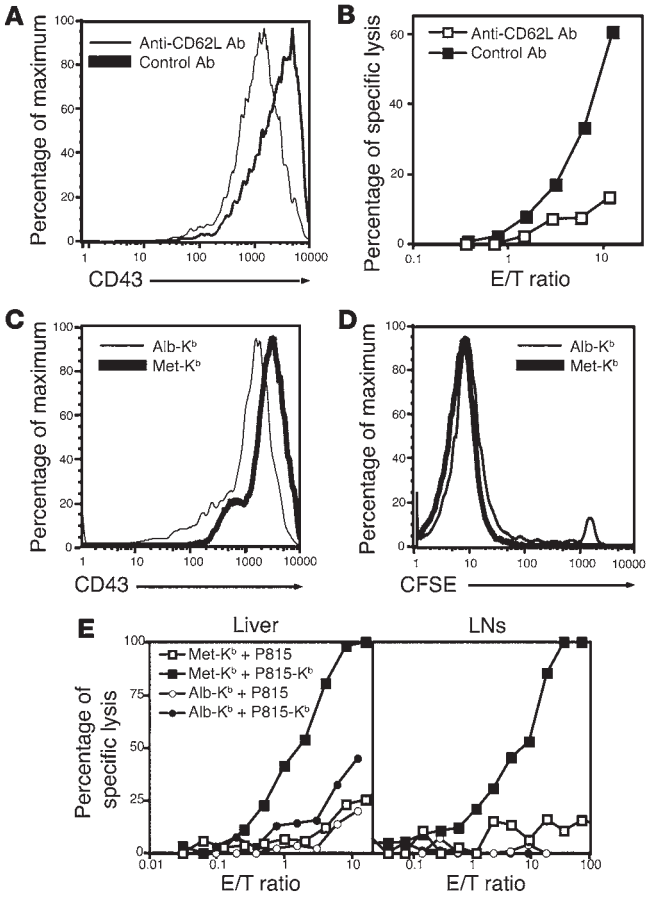

Figure 7
CD8+ Des-TCR T cells activated within the liver exhibited defective CTL function. (**A**) Representative histograms of CD43 activation–associated glycoform expression by CD8+ Des-TCR cells in livers of Met-K^b mice treated with anti-CD62L (thin line) or control IgG (thick line). Organs were harvested 2.5 days after transfer of Des-TCR LN cells. (**B**) CTL activity of CD8+ Des-TCR cells harvested from the livers of Met-K^b mice treated with anti-CD62L (open squares) or control IgG (filled squares) toward P815-K^b target cells at 2.5 days after transfer. The effector/target (E/T) ratio was calculated by determination of the exact number of transgenic T cells present in the well. CTL activity toward P815 control target cells was not detected (data not shown). (**C** and **D**) Representative CD43 activation–associated glycoform histograms (**C**) and CFSE profiles (**D**) of CD8+ Des-TCR cells from the livers of Alb-K^b (H-$2K^{dk}$) (thin line) and Met-K^b (H-$2K^{dk}$) mice (thick line). Organs were harvested 6 days after transfer of Des-TCR (H-$2K^{dk}$) LN cells, and lymphocytes were prepared and analyzed by FACS. All plots were gated on forward and side scatter gates appropriate for lymphocytes and CD8+ Des-TCR+ PI^- cells. (**E**) CTL activity of CD8+ Des-TCR cells harvested from the livers and LNs of Alb-K^b (H-$2K^{dk}$) (round symbols) and Met-K^b (H-$2K^{dk}$) mice (square symbols) incubated with P815 (open symbols) or P815-K^b target cells (filled symbols). Organs were harvested 6 days after transfer of Des-TCR (H-$2K^{dk}$) LN cells. The effector/target ratio was calculated by determination of the exact number of transgenic T cells present in each well.

ing would hold only if, following anti-CD62L treatment, there was loss of cells that would normally enter the LNs, or if recruitment into division or proliferation rates were different in the organs into which cells were diverted and activated. Analysis soon after cell transfer, before division had occurred, demonstrated that there was no early cell loss associated with antibody treatment (Figure 2A). In addition, despite inhibition of T cell entry into LNs, transgenic T cells recovered from anti-CD62L–treated recipient animals both were recruited into division and proliferated at a rate similar to that of transgenic T cells from animals treated with control Ig (Figure 2C). Furthermore, it should be noted that in the absence of anti-CD62L treatment, recruitment into division of transgenic T cells and their proliferation rate were similar within liver and LNs (Figure 2D). These findings were similar irrespective of whether splenectomy was performed. Overall, these results indicate that most transgenic T cells were diverted and activated within the liver of anti-CD62L–treated animals, where recruitment into division and proliferation rates were similar to those in the LNs. However, despite proliferation, these cells did not accumulate in the recipient animal, suggesting that there was progressive loss of transgenic CD8+ T cells following T cell activation and proliferation within the livers of Met-K^b animals. Statistically significant differences in the proportion of apoptotic donor CD8+ T cells were not detected by flow cytometry analysis of annexin V binding by donor cells from anti-CD62L– and control antibody–treated Met-K^b mice at days 1, 2, and 3 (data not shown); this likely indicates that cell loss was progressive and difficult to visualize in a "snapshot."

At 6 days after transfer, the number of CD8+ Des-TCR T cells was reduced in anti-CD62L mAb-treated Met-K^b mice compared with that in control Ig-treated Met-K^b mice in all organs examined, rather than in the LNs alone, in contrast to day 2.5 (Figure 6B). Again consistent with progressive cell loss rather than inhibition of cell division, by this time point, all adoptively transferred CD8+ Des-TCR T cells in both anti-CD62L– and control Ig–treated Met-K^b mice had lost detectable CFSE content (data not shown).

Differing half-lives between CD8+ T cells activated within the liver and those activated within the LNs were further demonstrated by transfer of identical numbers of CD8+ Des-TCR+ T cells that were activated within these compartments for 24 hours. Des-TCR LN cells were first adoptively transferred into Met-K^b hosts, then harvested from liver and LNs at 24 hours, and equal numbers of CD8+ Des-TCR T cells from liver and LNs were transferred into separate, antigen-free B10.BR recipients. Thirty days after transfer, lower numbers of donor-derived CD8+ Des-TCR+ T cells were recovered from recipient animals into which intrahepatically activated T cells were transferred than from those into which LN-activated T cells were injected (Figure 6C). The proportion of donor LN-activated CFSE+ CD8+ T cells recovered 30 days after second adoptive transfer (2.1% of transferred donor CD8+ T cells) was very similar to the proportion of CFSE+ $CD8^-$ cells (mostly CD4+ and B cells) recovered at this time point (2.8% of transferred donor cells), indicating that, unlike intrahepatically activated T cells, intranodally activated T cells were lost at a rate similar to that of nonactivated lymphocytes.

These results indicate that CD8+ T cells activated within the liver had reduced half-life in comparison with T cells activated within LNs, and that this differentiation program was acquired by T cells within the first 24 hours following activation.

Persisting CD8+ T cells activated within the liver exhibited impaired cytolytic function. Although present in reduced numbers, CD8+ transgenic cells were still detectable in Alb-K^b and anti-CD62L–treated Met-K^b mice. We therefore examined the possibility that in addition to a quantitative defect, T cell activation within the liver resulted in a qualitative defect in CTL function. As an indirect correlate of CTL activity, we

first measured the level of CD43 activation–associated glycoform expressed by CD8+ Des-TCR T cells in the livers of anti-CD62L–treated Met-Kb mice at 2.5 days following adoptive transfer. This time point was chosen because previous experiments had demonstrated that, at 48 hours after transfer, CD8+ Des-TCR T cells transferred into Met-Kb mice had divided at least 3 times (Figure 2C), the minimum number of divisions required for their acquisition of specific CTL activity in vitro (37). In addition, since recirculation of dividing cells via the blood was observed at day 2.5 (Figure 5B), cells activated in the LNs could be expected to be present within the livers of non–anti-CD62L–treated Met-Kb mice by this time point. Although upregulation of CD43 activation–associated glycoform was observed, levels expressed by CD8+ Des-TCR+ T cells isolated from the livers of Met-Kb mice treated with anti-CD62L mAb were 2- to 3-fold lower than those expressed by cells from mice treated with control Ig (Figure 7A), which suggested that in mice where the LN contribution was minimized, transgenic T cells activated within the liver exhibited reduced CTL activity. These results were confirmed using a CTL assay in which percentages of specific lysis were corrected for the exact number of transgenic T cells added to the wells. At day 2.5 after transfer, lymphocytes isolated from the liver of Met-Kb mice treated with anti-CD62L displayed lower CTL activity toward H-2Kb–expressing P815 targets than those from the liver of Met-Kb mice treated with control Ig (Figure 7B).

Transgenic T cells adoptively transferred into Alb-Kb mice exhibited a similar phenotype to that observed in Met-Kb mice treated with anti-CD62L mAb. Although upregulation of CD43 activation–associated glycoform was observed, CD8+ Des-TCR+ T cells isolated from the liver of Alb-Kb mice expressed 2- to 3-fold lower levels than transgenic T cells from unmanipulated Met-Kb mice (Figure 7C), consistent with lower CTL activity. This difference in CD43 expression between Alb-Kb and Met-Kb mice was not due to a defect in division-dependent CTL differentiation, as donor T cells exhibited a similar decrease in CFSE content (Figure 7D). To confirm differential CTL activity between unmanipulated Met-Kb and Alb-Kb recipients, we used a classical CTL assay in which percentages of specific lysis were corrected for the exact number of transgenic T cells added to the wells. Organs were harvested at day 6 following T cell transfer, as this was the peak of hepatitis in the Met-Kb model. As shown in Figure 5C, most T cells activated within the LNs of Met-Kb mice recirculated via the blood at day 6 and had already accumulated within the liver, suggesting that most T cells recovered from day 6 Met-Kb livers were of LN origin. In contrast, in the absence of intranodal antigen expression, all activated T cells found in Alb-Kb mice were activated within the liver. Lymphocytes isolated from the livers of Met-Kb mice displayed significantly higher CTL activity toward H-2Kb–expressing P815 targets than those from the livers of Alb-Kb mice, confirming their more potent CTL function (Figure 7E). In contrast to Met-Kb mice, no CTL activity was demonstrated in the LNs of Alb-Kb mice (Figure 7E), a result consistent with lack of transgenic T cell activation and proliferation within this site (Figure 5).

Viewed as a whole, these experiments indicated that the failure to develop a CTL response capable of mediating hepatocellular injury following primary intrahepatic activation is likely to be secondary to a reduction in both CTL activity and CD8+ T cell survival.

Discussion

The data presented above indicate that, although the liver competes with the LNs for primary T cell activation, immune responses generated at these 2 sites differ fundamentally: primary T cell activation within the LNs leads to an efficient response that results in acute hepatitis, whereas primary T cell activation within the liver commits T cells to the development of inefficient CTL function and reduced half-life. Our experiments imply that the outcome of the immune response in the presence of intrahepatically and intranodally expressed antigen will be largely determined by the initial degree of antigen-specific T cell recruitment to these 2 sites. The thesis that the site of primary CD8+ T cell activation thus determines cellular fate, and ultimately the outcome of the immune response, is consistent with recent findings that CD8+ T cells can follow a program of division and differentiation after primary activation, even in the absence of further exposure to antigen (38). Variations in signals imparted during primary activation by different APCs in these organs may hence commit CD8+ T cells to these divergent outcomes.

Our previous experiments have demonstrated that primary activation of CD8+ T cells could occur within the liver in the Met-Kb model, a system in which the cognate antigen was expressed by hepatocytes (16). However, as primary activation of CD8+ T cells also occurred in the LNs of these mice, the outcome of intrahepatic primary activation remained unclear. In particular, hepatitis was demonstrated to occur in this model (16, 26), despite our previous in vitro data suggesting that primary CD8+ T cell activation by hepatocytes led to their premature death due to lack of costimulation (21, 22). The current investigation demonstrated that in the absence of intranodal CD8+ T cell activation, hepatitis did not occur or was mild (Figure 3), and that intrahepatic primary CD8+ T cell activation was associated with an ineffective immune response; hepatitis in the intact Met-Kb model was thus mediated by CD8+ T cells undergoing primary activation within the LNs.

The determinant role of primary T cell activation within the LNs in the development of hepatitis was confirmed using Alb-Kb mice in which H-2Kb expression was effectively restricted to the liver. Previous studies in the Alb-Kb model had failed to elicit hepatitis in unmanipulated animals, despite similar levels of antigen expression on hepatocytes in both models (Figure 4B). The current data indicate that the difference in outcomes between the 2 models was due not to the different backgrounds used in the original studies, but rather to differences in intranodal antigen expression.

The role of LNs in the induction of graft rejection or autoimmunity has been suggested in other studies (39, 40). However, conclusions from these studies remained controversial, since experimental models used to reduce LN contribution, such as surgery or mice with a genetic deficiency (Aly- or lymphotoxin-α–deficient mice), may also have profound unrelated immunological effects (41). To our knowledge, this is the first clear demonstration of the requirement of LNs in the development of autoimmunity in an intact animal.

It has previously been demonstrated that when LN entry by naive T cell cells is blocked, primary activation can occur within the bone marrow (33). However, in contrast to the ineffective immune response occurring after primary CD8+ T cell activation within the liver, observed in the current work, primary activation within the bone marrow has recently been demonstrated to lead to an effective immune response and the generation of memory cells (42). Thus, while the liver is not the sole extralymphoid organ in which primary T cell activation can occur, the tolerogenic outcome seen following primary activation in this organ differs from that observed in the bone marrow.

Because of the requirement for the presence of the intact H-2K^b molecule for activation of Des-TCR CD8$^+$ T cells, cross-presentation is excluded in these models. Primary activation of Des-TCR T cells within the LNs of Met-K^b mice could not therefore be induced by hepatically derived antigen cross-presented within this lymphoid tissue. However, the exact nature of the APCs expressing H-2K^b within the LNs of Met-K^b mice remains to be determined: H-2K^b expression on CD8$^-$ and CD8$^+$ DCs, as well as all other LN cell types, was below that detectable by flow cytometry and immunohistochemistry (refs. 16, 26; and Supplemental Figure 1). However, despite the insensitivity of such techniques in detecting the presence of H-2K^b on LN resident cells, it is clear that biologically significant levels of this antigen are indeed present in the LNs of Met-K^b mice; our previous experiments using bone marrow–chimeric animals have indicated that the APCs responsible for intranodal primary activation of Des-TCR T cells in Met-K^b mice are bone marrow–derived cells, in which "leaky" expression of the H-2K^b transgene occurs (16). In contrast, the lack of activation of Des-TCR T cells in the LNs of Alb-K^b mice is consistent with a lack of antigen expression by any APC at this site.

Especially because the nature of the APCs within the LNs remains undefined, it is unclear why activation within the LNs of healthy Met-K^b mice led to autoimmunity. However, it is conceivable that some DCs might constitutively express low levels of H-2K^b because of "leaky" expression of the transgene and, when activated after an encounter with another, irrelevant antigen, might thus efficiently activate adoptively transferred CD8$^+$ Des-TCR T cells, leading to the development of an autoimmune response to intrahepatically expressed antigen.

It should be noted that, in contrast to intranodal T cell activation, previous work using bone marrow–chimeric animals has indicated that intrahepatic primary T cell activation in the Met-K^b model does not involve bone marrow–derived APCs (16). Consistent with this, recent flow cytometric analysis failed to detect H-2K^b expression by nonhepatocyte cell populations, including F4/80$^+$ MHC II$^+$ liver cells (Kupffer cells and liver DCs) and liver sinusoidal endothelial cells (Supplemental Figure 1). Similar results were obtained in Alb-K^b mice (Supplemental Figure 1). Especially in the presence of significant H-2K^b expression by hepatocytes (Figure 4B), failure to detect antigen expression on other cell types within the liver would be consistent with hepatocytes acting as APCs for naive transgenic T cells in the livers of Met-K^b mice.

Although the fate of Des-TCR T cells in the Met-K^b and Alb-K^b models has been examined in previous studies, conclusions have been contradictory. Early studies indicated that Des-TCR T cells maturing in the Alb-K^b environment became anergic following downregulation of both CD8 and TCR molecules (23). Studies from the same group indicated that antigens expressed by hepatocytes could only be accessed following inflammation in this organ (24). However, this model, in which inflammation determines T cell access to parenchymal cells, failed to explain how T cells could be tolerized within the Alb-K^b liver in the absence of inflammatory signals and why they undergo peripheral deletion (36). This model was also inconsistent with our previous reports demonstrating that Des-TCR T cells induced liver damage and were deleted following recognition of their cognate antigen within the liver in the absence of preexisting inflammation (16, 26). Experiments presented in the current work indicate that these conflicting results were likely due to T cell activation occurring at different sites in the transgenic models studied. The concept that the site of primary T cell activation determines the outcome of the immune response provides a unified model that reconciles disparate data in the literature. Our results indicate that the failure of intrahepatic primary activation to elicit a CTL response sufficient to mediate hepatitis was due not to a defect in CD8$^+$ T cell proliferation, but rather to a combination of reduced half-life and defective CTL function. The mechanisms determining these outcomes remain to be established. Our difficulty in detecting significant differences in the proportion of annexin V$^+$ cells at particular time points suggests that deletion is gradual, a result consistent with other studies (36). Based on in vitro studies showing that hepatocytes induce death by neglect of naive transgenic CD8$^+$ T cells as a result of deficient induction of survival factors such as Bcl-x_L and IL-2 in the absence of costimulation (22), we have recently proposed that T cells activated in the liver undergo passive death, and are currently investigating whether death by neglect does occur in vivo.

Previous studies have indicated that the liver may act as a "trap" for activated CD8$^+$ T cells, and that CD8$^+$ T cells activated in the periphery may accumulate in the liver to undergo apoptosis (13). Such effects appear to be distinct from that described in the present work, where T cell fate is determined by primary activation within the liver itself. It is tempting to speculate that such primary intrahepatic activation may be involved in the induction of peripheral tolerance in the CD8$^+$ T cell compartment. The deletion of high-avidity CD8$^+$ T cells specific for either self-antigen expressed within this organ, or portal-derived substances such as food antigens, may play a role in maintaining the balance within the complex immunological milieu of the liver, and within the organism as a whole.

It should be noted that these models are not intended to mimic a specific human autoimmune liver disease but rather are tools to dissect hepatic-immune interactions. Indeed, it is possible that failure of the intrahepatic mode of activation observed in Met-K^b and Alb-K^b mice can in fact contribute to the pathogenesis of autoimmune liver disease, with autoreactive cells that would otherwise receive tolerogenic signals in the liver receiving more immunogenic signals in the LNs, and perhaps then contributing to the initial events that lead to hepatic autoimmunity. These findings also hold implications for the pathogenesis of other forms of immune-mediated liver injury. In particular, we (16) and others (43) have previously suggested that initial CD8$^+$ T cell interactions within the liver might contribute to the immunopathogenesis of chronic hepatitis C viral infection. The current work indicates that primary activation of high-avidity cells within the liver may lead to impaired CTL function and reduced half-life, especially during early infection, when this is likely the predominant site of antigen presentation; cells more effectively activated in the LNs, possibly later in infection, may thus be of lower avidity, capable of inducing hepatitis but incapable of mediating viral clearance. Consistent with such a hypothesis, recent data suggest that hepatitis C virus–specific (HCV-specific) CD8$^+$ T cells in chronic HCV infection may exhibit impaired effector function, or "stunning" (44, 45). While such defective function may be related to poor HCV-specific CD4$^+$ T cell responses (45, 46), and thereby deficient help, this phenomenon may also be associated with primary activation of high-avidity T cells mediated by hepatocytes or other APCs within the liver.

While it is clear that hepatic allograft tolerance involves multiple complex mechanisms, findings from the current study may also carry implications for hepatic allograft tolerance, particularly early

after transplantation. Acceptance of hepatic allografts has been shown to be associated with the accumulation of apoptotic T cells within the graft (47, 48). It has largely been assumed that these cells have been previously activated in host lymphoid organs, following migration of passenger leukocytes into these tissues (49, 50). However, the current data suggest that at least a portion of the apoptotic cells present within the liver may have undergone activation in situ. Thus, in addition to passenger leukocytes, parenchymal hepatic cells might also be involved in the tolerization of alloreactive $CD8^+$ T cells, thus contributing to the control of acute rejection, with other regulatory mechanisms subsequently supervening.

While hepatitis in the Met-K^b model is dependent on the presence of antigen-specific $CD8^+$ Des-TCR T cells and their activation within the LNs, it remains unclear whether hepatocellular injury is entirely mediated by these cells specific for hepatocyte-expressed antigen, or whether injury is also dependent on other cells recruited to the site of injury. We have previously demonstrated the presence of diverse cell types in hepatic infiltrates at the peak of hepatitis in this model, including $CD4^+$ T cells and macrophages (26), and it is very likely that such cell types play some role in injury. However, the extent to which such cell types contribute to the genesis of the lesion remains to be determined. Similar anti-CD62L antibody treatment in a model such as lymphocytic choriomeningitis virus infection might further clarify the extent to which antigen-specific cells mediate such injury and thus might aid in delineating the role of non–antigen-specific cells, by determining whether, in the absence of entry into LNs by naive T cells, hepatitis can still occur in that model.

The data presented above suggest a model in which the site of primary T cell activation is an important parameter in determining the outcome of $CD8^+$ T cell responses. Activation of $CD8^+$ T cells predominantly within the liver would favor a response leading to tolerance, whereas activation predominantly within lymphoid tissues would generate an effective immune response. Competition between liver and LNs occurs in the initial stages of the response, but T cell recruitment to these 2 sites is governed by different mechanisms. Naive T cell entry into the LNs is independent of antigenic expression, while naive T cell recruitment to the liver is strictly antigen-dependent (16). Furthermore, unlike T cells that have been activated within the LNs, we have shown that intrahepatically activated T cells are not efficiently retained and recirculate as early as 24 hours after activation (16). However, since these T cells lose expression of molecules, such as CD62L (data not shown), required for LN entry, they are unlikely to subsequently enter the LNs and be "reprogrammed" by antigen-expressing professional APCs. Therefore, although competition occurs early in the response, this process is likely to be irreversible and determine the fate of activated T cells. Where antigen is expressed within the liver, but not in the LNs, it is likely that ineffective activation will occur, leading to tolerance. In the setting of antigen expression in both the liver and the LNs, competition between the these organs for primary T cell activation, and thus the balance between intrahepatic tolerance and immunity, might be determined by a range of factors, including the nature of the intrahepatic APCs involved, the levels of antigen expressed, and the avidity of interaction. The novel model suggested by the data presented in this study may provide an explanation for the paradox of the immunopathology observed within the liver and its apparent tolerogenic properties. Such a competitive mechanism would have clear relevance to normal physiology as well as hepatic immunopathology.

Methods

Mice. All mice were maintained in the Centenary Institute of Cancer Medicine and Cell Biology animal facility under specific pathogen–free conditions. B10.BR, DBA.2, and C57BL/6 mice were purchased from the Animal Resources Centre (Perth, Australia). Des-TCR and Met-K^b transgenic mice were maintained on a B10.BR ($H\text{-}2^k$) background. In some experiments, Met-K^b mice bearing the Ly5.1 (CD45.1) allele were used; the generation of these mice has been previously described (16). Des-TCR mice deficient for RAG-1 expression were generated by backcrossing of Des-TCR mice with RAG-$1^{-/-}$ mice crossed onto an $H\text{-}2^k$ background, kindly provided by Barbara Fazekas de St Groth (Centenary Institute of Cancer Medicine and Cell Biology, Sydney, Australia). Met-K^b ($H\text{-}2^{dk}$), Des-TCR ($H\text{-}2^{dk}$), and control nontransgenic ($H\text{-}2^{dk}$) recipient mice were generated as an F_1 cross between DBA.2 mice and Met-K^b, Des-TCR, or B10.BR mice, respectively. No zinc induction was performed on Met-K^b mice. Alb-K^b transgenic mice were a generous gift of Bernd Arnold (Deutsches Krebsforschungszentrum, Heidelberg, Germany) and were maintained within the DBA.2 ($H\text{-}2^d$) background. Alb-K^b ($H\text{-}2^{dk}$) mice were generated as an F_1 cross between Alb-K^b mice and B10.BR mice. All experimental procedures were approved by the University of Sydney Animal Ethics Committee.

Splenectomy. In some experiments, recipient mice were splenectomized following classical procedures for this surgery (51). Experiments were performed at least 2 weeks after surgery.

Adoptive transfer of CFSE-labeled Des-TCR T cells. Single-cell suspensions of pooled LN cells from Des-TCR transgenic mice were labeled with CFSE, as previously described (16). In all experiments, 5×10^6 CFSE-labeled $CD8^+$ T cells expressing Des-TCR were injected into the lateral tail vein of recipient mice. As the proportion of transgenic $CD8^+$ T cells within LNs was 30%, 20%, or 95% depending on whether mice were bred within an $H\text{-}2^k$, an $H\text{-}2^{dk}$, or a RAG-$1^{-/-}$ background, respectively (D. Bowen, unpublished data), the total number of transferred LN cells was adjusted to correct for these differences. Thus, for the transfer of 5×10^6 Des-TCR $CD8^+$ T cells, 1.5×10^7 Des-TCR ($H\text{-}2^k$) LN cells, 2.5×10^7 Des-TCR ($H\text{-}2^{dk}$) LN cells, or 5×10^6 Des-TCR RAG-$1^{-/-}$ LN cells were adoptively transferred into recipient mice.

For experiments in which LN entry by naive T cells was inhibited, mice were administered 250 μg of protein G–purified anti-CD62L mAb (Mel14) or purified rat IgG (ICN Biomedicals Inc.) in 500 μl PBS intraperitoneally 4 hours prior to intravenous adoptive transfer of Des-TCR LN cells. In some experiments, further doses of antibodies were administered intraperitoneally after adoptive transfer.

For the experiment described in Figure 6C, 1.5×10^7 CFSE-labeled Des-TCR ($H\text{-}2^k$) LN cells were adoptively transferred into 10 Met-K^b mice. Twenty-four hours later, lymphocytes from livers and LNs of individual animals were isolated, then pooled into separate fractions, which were purified over Ficoll gradients (HISTOPAQUE; Sigma-Aldrich). The percentages of $CD8^+$ $CFSE^+$ T cells in both cell populations were assessed by flow cytometry. Identical numbers of $CD8^+$ $CFSE^+$ T cells from liver and LN samples (8×10^4 cells per mouse) were then adoptively transferred into separate second antigen-free, nontransgenic B10.BR recipients, and 30 days later, lymphocytes from liver, LNs, and spleen were purified. The total number of $CD8^+$ $CFSE^+$ T cells in each compartment was calculated following flow cytometric analysis.

Isolation of lymphocytes and hepatocytes for flow cytometry. Lymphocytes were isolated from LNs, spleen, blood, and liver as previously described (16). Hepatocytes were isolated as detailed elsewhere (26). Kupffer cells and liver, spleen, and LN DCs were purified as previously described using a Nycodenz gradient (Nycomed Pharma) (16, 17). Liver sinusoidal endothelial cells were identified in the same preparation using a FITC-conjugated ME-9F1 mAb (52).

Antibodies and flow cytometric analysis. Immunostaining for flow cytometry was carried out as described previously (16). Dead cells were excluded from

analysis by propidium iodide (PI) uptake (Molecular Probes Inc.). Antibodies used for flow analysis included the clonotypic anti–Des-TCR mAb "Désiré," anti–H-2K^b (K9.178), and anti-CD44 (IM7.81) purified using a protein G affinity column and conjugated to biotin. Allophycocyanin-, phycoerythrin-, and FITC-conjugated anti-CD8 mAb, phycoerythrin-conjugated anti-CD69, and phycoerythrin-conjugated anti–CD43 activation–associated glycoform were all purchased from Pharmingen. Streptavidin-phycoerythrin was purchased from Caltag Laboratories, and streptavidin–Alexa Fluor 594 and streptavidin-allophycocyanin were purchased from Molecular Probes Inc.

Flow cytometry acquisition was performed using a FACSCalibur (BD) or FACStar Plus (BD) flow cytometer. Analysis was performed using FlowJo software (Tree Star Inc.) on a Macintosh computer (Apple Computer Inc.).

Unless stated otherwise, total cell numbers per organ were calculated by multiplication of the total cell number by the percentage of Des-TCR$^+$ CD8$^+$ cells within the PI$^-$ gate.

Measurement of serum transaminase levels. Serum ALT levels were measured with a Hitachi 917 automatic chemical analyzer (Roche Diagnostics).

Cytotoxicity assays. CTL assays were performed using a classical ^{51}Cr release assay as described elsewhere (22). Briefly, effector cells were isolated from LNs or livers of recipient mice, then further purified over Ficoll gradients and diluted on 96-well U-bottom plates. In some experiments, organs from 5–10 mice were pooled to generate sufficient cell numbers to use in the CTL assay. Target cells (10^6 P815 and P815-K^b cells) were incubated with 50 μCi of ^{51}Cr for 1 hour at 37°C, then added at 3,000 cells per well to the different concentrations of effector cells and incubated for 4 hours at 37°C. Following incubation, 100 μl of supernatant per well was transferred into LumaPlate-96 microplates (PerkinElmer Life Sciences Inc.) and air-dried. Plates were then counted on a Packard TopCount Microplate Scintillation Counter (Packard BioScience). The effector/target ratio was determined by calculation of the exact number of effector transgenic T cells present in each well. This number was derived by multiplication of the total number of lymphocytes harvested from LNs or liver by the percentage of CD8$^+$ Des-TCR$^+$ T cells obtained after flow cytometric analysis.

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1. Bertolino, P., Glimpel, G., and Lemon, S.M. 2000. Hepatic inflammation and immunity: a summary of a conference on the function of the immune system within the liver. *Hepatology.* **31**:1374–1378.
2. Crispe, I.N. 2003. Hepatic T cells and liver tolerance. *Nat. Rev. Immunol.* **3**:51–62.
3. Calne, R.Y., et al. 1969. Induction of immunological tolerance by porcine liver allografts. *Nature.* **223**:472–476.
4. Qian, S., et al. 1994. Murine liver allograft transplantation: tolerance and donor cell chimerism. *Hepatology.* **19**:916–924.
5. Sun, J., McCaughan, G.W., Gallagher, N.D., Sheil, A.G., and Bishop, G.A. 1995. Deletion of spontaneous rat liver allograft acceptance by donor irradiation. *Transplantation.* **60**:233–236.
6. Kamada, N., Brons, G., and Davies, H.S. 1980. Fully allogeneic liver grafting in rats induces a state of systemic nonreactivity to donor transplantation antigens. *Transplantation.* **29**:429–431.
7. Callery, M.P., Kamei, T., and Flye, M.W. 1989. The effect of portacaval shunt on delayed-hypersensitivity responses following antigen feeding. *J. Surg. Res.* **46**:391–394.
8. Kita, H., Mackay, I.R., Van De Water, J., and Gershwin, M.E. 2001. The lymphoid liver: considerations on pathways to autoimmune injury. *Gastroenterology.* **120**:1485–1501.
9. Vierling, J.M. 1992. Immune disorders of the liver and bile duct. *Gastroenterol. Clin. North Am.* **21**:427–449.
10. Chisari, F.V., and Ferrari, C. 1995. Hepatitis B virus immunopathogenesis. *Annu. Rev. Immunol.* **13**:29–60.
11. Cerny, A., and Chisari, F.V. 1999. Pathogenesis of chronic hepatitis C: immunological features of hepatic injury and viral persistence. *Hepatology.* **30**:595–601.
12. Ando, K., Guidotti, L.G., Cerny, A., Ishikawa, T., and Chisari, F.V. 1994. CTL access to tissue antigen is restricted in vivo. *J. Immunol.* **153**:482–488.
13. Huang, L., Soldevila, G., Leeker, M., Flavell, R., and Crispe, I.N. 1994. The liver eliminates T cells undergoing antigen-triggered apoptosis in vivo. *Immunity.* **1**:741–749.
14. Mehal, W.Z., Juedes, A.E., and Crispe, I.N. 1999. Selective retention of activated CD8+ T cells by the normal liver. *J. Immunol.* **163**:3202–3210.
15. Hamann, A., Klugewitz, K., Austrup, F., and Jablonski-Westrich, D. 2000. Activation induces rapid and profound alterations in the trafficking of T cells. *Eur. J. Immunol.* **30**:3207–3218.
16. Bertolino, P., Bowen, D.G., McCaughan, G.W., and Fazekas De St Groth, B. 2001. Antigen-specific primary activation of CD8+ T cells within the liver. *J. Immunol.* **166**:5430–5438.
17. Bowen, D.G., et al. 2002. Cytokine-dependent bystander hepatitis due to intrahepatic murine CD8+ T-cell activation by bone marrow-derived cells. *Gastroenterology.* **123**:1252–1264.
18. Mackay, C.R., Marston, W.L., and Dudler, L. 1990. Naive and memory T cells show distinct pathways of lymphocyte recirculation. *J. Exp. Med.* **171**:801–817.
19. MacSween, R.N.M., and Scothorne, R.J. 1979. Developmental anatomy and normal structure. In *Pathology of the liver.* R.N.M. MacSween, P.P. Anthony, and P.J. Scheuer, editors. Churchill Livingstone. New York, New York, USA. 1–49.
20. Bertolino, P., McCaughan, G.W., and Bowen, D.G. 2002. Role of primary intrahepatic T-cell activation in the 'liver tolerance effect.' *Immunol. Cell Biol.* **80**:84–92.
21. Bertolino, P., Trescol-Biemont, M.C., and Rabourdin-Combe, C. 1998. Hepatocytes induce functional activation of naive CD8+ T lymphocytes but fail to promote survival. *Eur. J. Immunol.* **28**:221–236.
22. Bertolino, P., et al. 1999. Death by neglect as a deletional mechanism of peripheral tolerance. *Int. Immunol.* **11**:1225–1238.
23. Schönrich, G., et al. 1992. Distinct mechanisms of extrathymic T cell tolerance due to differential expression of self antigen. *Int. Immunol.* **4**:581–590.
24. Limmer, A., et al. 1998. Failure to induce organ-specific autoimmunity by breaking of tolerance: importance of the microenvironment. *Eur. J. Immunol.* **28**:2395–2406.
25. Morahan, G., et al. 1989. Expression in transgenic mice of class I histocompatibility antigens controlled by the metallothionein promoter. *Proc. Natl. Acad. Sci. U. S. A.* **86**:3782–3786.
26. Bertolino, P., Heath, W.R., Hardy, C.L., Morahan, G., and Miller, J.F. 1995. Peripheral deletion of autoreactive CD8+ T cells in transgenic mice expressing H-2Kb in the liver. *Eur. J. Immunol.* **25**:1932–1942.
27. Schönrich, G., et al. 1991. Down-regulation of T cell receptors on self-reactive T cells as a novel mechanism for extrathymic tolerance induction. *Cell.* **65**:293–304.
28. Heath, W.R., and Miller, J.F. 1993. Expression of two alpha chains on the surface of T cells in T cell receptor transgenic mice. *J. Exp. Med.* **178**:1807–1811.
29. Girard, J.P., and Springer, T.A. 1995. High endothelial venules (HEVs): specialized endothelium for lymphocyte migration. *Immunol. Today.* **16**:449–457.
30. Chao, C.C., Jensen, R., and Dailey, M.O. 1997. Mechanisms of L-selectin regulation by activated T cells. *J. Immunol.* **159**:1686–1694.
31. Dawson, J., Sedgwick, A.D., Edwards, J.C., and Lees, P. 1992. The monoclonal antibody MEL-14 can block lymphocyte migration into a site of chronic inflammation. *Eur. J. Immunol.* **22**:1647–1650.

32. Lepault, F., Gagnerault, M.C., Faveeuw, C., and Boitard, C. 1994. Recirculation, phenotype and functions of lymphocytes in mice treated with monoclonal antibody MEL-14. *Eur. J. Immunol.* **24**:3106–3112.
33. Tripp, R.A., Topham, D.J., Watson, S.R., and Doherty, P.C. 1997. Bone marrow can function as a lymphoid organ during a primary immune response under conditions of disrupted lymphocyte trafficking. *J. Immunol.* **158**:3716–3720.
34. Hou, S., Hyland, L., Bradley, L.M., Watson, S.R., and Doherty, P.C. 1995. Subverting lymph node trafficking by treatment with the Mel-14 monoclonal antibody to L-selectin does not prevent an effective host response to Sendai virus. *J. Immunol.* **155**:252–258.
35. Harrington, L.E., Galvan, M., Baum, L.G., Altman, J.D., and Ahmed, R. 2000. Differentiating between memory and effector CD8 T cells by altered expression of cell surface O-glycans. *J. Exp. Med.* **191**:1241–1246.
36. Guerder, S., Rincon, M., and Schmitt-Verhulst, A.M. 2001. Regulation of activator protein-1 and NF-kappa B in CD8+ T cells exposed to peripheral self-antigens. *J. Immunol.* **166**:4399–4407.
37. Fazekas de St Groth, B., et al. 1999. Carboxyfluorescein diacetate succinimidyl ester and the virgin lymphocyte: a marriage made in heaven. *Immunol. Cell Biol.* **77**:530–538.
38. Kaech, S.M., and Ahmed, R. 2001. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. *Nat. Immunol.* **2**:415–422.
39. Lakkis, F.G., Arakelov, A., Konieczny, B.T., and Inoue, Y. 2000. Immunologic 'ignorance' of vascularized organ transplants in the absence of secondary lymphoid tissue. *Nat. Med.* **6**:686–688.
40. Gagnerault, M.C., Luan, J.J., Lotton, C., and Lepault, F. 2002. Pancreatic lymph nodes are required for priming of beta cell reactive T cells in NOD mice. *J. Exp. Med.* **196**:369–377.
41. Chin, R., Zhou, P., Alegre, M.L., and Fu, Y.X. 2001. Confounding factors complicate conclusions in aly model. *Nat. Med.* **7**:1165–1166.
42. Feuerer, M., et al. 2003. Bone marrow as a priming site for T-cell responses to blood-borne antigen. *Nat. Med.* **9**:1151–1157.
43. Willberg, C., Barnes, E., and Klenerman, P. 2003. HCV immunology: death and the maiden T cell. *Cell Death Differ.* **10**(Suppl. 1):S39–S47.
44. Gruener, N.H., et al. 2001. Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. *J. Virol.* **75**:5550–5558.
45. Wedemeyer, H., et al. 2002. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. *J. Immunol.* **169**:3447–3458.
46. Missale, G., et al. 1996. Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response. *J. Clin. Invest.* **98**:706–714.
47. Qian, S., et al. 1997. Apoptosis within spontaneously accepted mouse liver allografts: evidence for deletion of cytotoxic T cells and implications for tolerance induction. *J. Immunol.* **158**:4654–4661.
48. Sharland, A., et al. 1999. Evidence that apoptosis of activated T cells occurs in spontaneous tolerance of liver allografts and is blocked by manipulations which break tolerance. *Transplantation.* **68**:1736–1745.
49. Starzl, T.E., et al. 1992. Cell migration, chimerism, and graft acceptance. *Lancet.* **339**:1579–1582.
50. Bishop, G.A., Sun, J., Sheil, R., and McCaughan, G.W. 1997. High dose/activation-associated tolerance. A mechanism for allograft tolerance. *Transplantation.* **64**:1377–1382.
51. Reeves, J.P., Reeves, P.A., and Chin, L.T. 1991. Survival surgery: removal of the spleen or thymus. In *Current protocols in immunology*. J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, editors. John Wiley & Sons Inc. New York, New York, USA. 1.10.11–11.10.11.
52. Klugewitz, K., et al. 2002. Immunomodulatory effects of the liver: deletion of activated CD4(+) effector cells and suppression of IFN-gamma-producing cells after intravenous protein immunization. *J. Immunol.* **169**:2407–2413.