Dominance of the CD4+ T helper cell response during acute resolving hepatitis A virus infection

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Hepatitis A virus (HAV) infection typically resolves within 4–7 wk but symptomatic relapse occurs in up to 20% of cases. Immune mechanisms that terminate acute HAV infection, and prevent a relapse of virus replication and liver disease, are unknown. Here, patterns of T cell immunity, virus replication, and hepatocellular injury were studied in two HAV-infected chimpanzees. HAV-specific CD8+ T cells were either not detected in the blood or failed to display effector function until after viremia and hepatitis began to subside. The function of CD8+ T cells improved slowly as the cells acquired a memory phenotype but was largely restricted to production of IFN-γ. In contrast, CD4+ T cells produced multiple cytokines when viremia first declined. Moreover, only CD4+ T cells responded during a transient resurgence of fecal HAV shedding. This helper response then contracted slowly over several months as HAV genomes were eliminated from liver. The findings indicate a dominant role for CD4+ T cells in the termination of HAV infection and, possibly, surveillance of an intrahepatic reservoir of HAV genomes that decays slowly. Rapid contraction or failure to sustain such a CD4+ T cell response after resolution of symptoms could increase the risk of relapsing hepatitis A.

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Abbreviations used: ALT, alanine aminotransferase; CI, confidence interval; HAV, hepatitis A virus; ISG, IFN-stimulated gene; PBMC, peripheral blood mononuclear cell.

Hepatitis A virus (HAV) is a common cause of acute hepatitis and, more rarely, fulminant and potentially fatal liver disease (Martin and Lemon, 2006). A small, nonenveloped RNA virus in the family *Picornaviridae*, it infects millions of people each year and remains a global public health problem despite the availability of an effective vaccine (Van Damme et al., 2003). Improvements in sanitation that accompany economic development have dramatically changed the epidemiology of HAV in some regions of the world where the virus remains endemic and vaccine utilization is low. Under these circumstances, the mean age of HAV infection can shift from the first to the second and third decades of life. Because the severity of liver disease increases with age, the disease burden caused by HAV in these societies has increased (Jacobsen and Wiersma, 2010). The age-related shift in HAV epidemiology and disease severity has highlighted a gap in our understanding of how immune responses contribute to hepatocellular injury and resolution of acute infection. Although HAV does not establish a persistent infection like HCV, disease can relapse in up to 20% of patients after normalization of liver transaminases and control of virus replication (Glikson et al., 1992). Mechanisms of immunity that protect against relapse, and why they occasionally fail, are also unknown.

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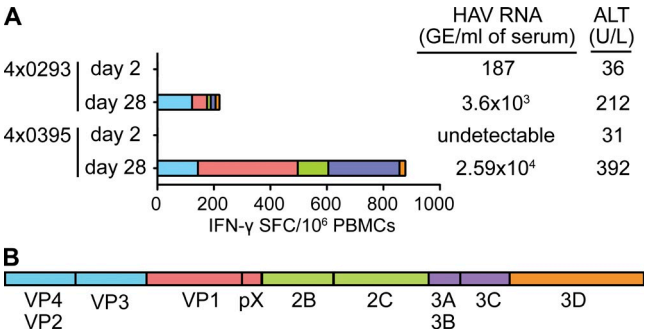

Figure 1. HAV-specific T cell responses at week 4 after infection. Chimpanzees (4X0293 and 4X0395) were inoculated intravenously with HAV on day 0. (A) T cell responses are reported as the number of spot forming colonies (SFCs) per 10^6 PBMCs in the IFN-γ ELISPOT assay. PBMCs were tested against five peptide pools spanning the HAV genome, shown in a schematic presentation with matching colors in B. HAV RNA levels in serum were determined using a real-time quantitative RT-PCR assay as previously described (Lanford et al., 2011) and expressed as genome equivalents (GEs)/ml of serum.

Neutralizing antibodies can protect against HAV infection and prevent liver disease if administered within 14 d of exposure to the virus (Lemon et al., 1997). Because immune control of acute HAV replication usually coincides with the peak in serum alanine aminotransferases (ALT), it has been postulated that resolution of infection depends on a $CD8^+$ T cell response that is cytotoxic for infected hepatocytes (Martin and Lemon, 2006). Evidence supporting this view was first published >20 yr ago, when cytotoxic T cell lines expanded from the blood and liver of jaundiced patients were shown to produce IFN-γ and lyse autologous HAV-infected fibroblasts (Maier et al., 1988; Vallbracht et al., 1989). A more recent study described several HLA class I restricted epitopes targeted by $CD8^+$ T cells from patients with acute and resolved HAV infections (Schulte et al., 2011). $CD8^+$ T cells specific for a dominant VP1 epitope were visualized in the blood of two symptomatic patients using tetrameric class I HLA molecules.

Although these studies point to a role for cellular immunity in resolution of acute HAV infection, a temporal association between a functional $CD8^+$ T cell response and acute liver damage or initial control of viremia has not been established. Moreover, virus-specific $CD4^+$ T cell responses critical to control of other hepatotropic viruses like HBV and HCV have not yet been characterized in HAV infection (Bowen and Walker, 2005; Chisari et al., 2010). We recently described an unexpectedly weak type I IFN-stimulated gene (ISG) response in the liver of HAV-infected chimpanzees (Lanford et al., 2011). Because this innate response can regulate the pace and quality of developing cellular immune responses, we characterized the kinetics and function of virus-specific T cells in these animals. The $CD8^+$ T cell response was not temporally associated with control of virus replication or clearance of infected hepatocytes. $CD8^+$ T cell frequencies declined substantially before HAV replication was fully contained. In contrast, $CD4^+$ T cells that produced multiple cytokines were present in blood at week 4 when HAV replication declined sharply and rebounded during an unexpected resurgence in fecal shedding of the virus 4–6 wk later. Contraction of the $CD4^+$ T cell response was very slow and only stabilized when HAV genomes were cleared from liver several months after infection. These findings document a previously unappreciated role for $CD4^+$ T cells in resolution of acute hepatitis A and, perhaps, in surveillance against relapsing virus replication and liver disease. The study was also designed to provide insight into the function and differentiation of $CD4^+$ T cells elicited by a virus that causes significant disease in humans.

RESULTS

We recently described the pattern of virus replication and hepatocellular damage in chimpanzees challenged with the HM175 strain of HAV (Lanford et al., 2011). In brief, high levels of viremia were detected during the first 3 wk of infection, followed by a sharp decline at week 4 when hepatitis peaked in both animals (Lanford et al., 2011).Viremia and symptoms of hepatitis resolved within 6 (4X0293) or 8 (4X0395) wk

Table 1. HAV class I epitopes

Animal	Class I epitope	HAV protein	Patr class I allele	Peak frequency (LOD = 0.001%)
4X0293	WDGYSGQLV	$2C_{1269}$	A0701	0.007%
4X0293	HPRLAQRILF	$3D_{1875}$	B1301	N.D.
4X0293	FAFMHGYEF	$3D_{2177}$	C0601	N.D.
4X0395	DWLTTHALF	$VP2_{90}$	B3201	N.D.
4X0395	AKLDVVKLL	$VP2_{102}$	B0501	0.011%
4X0395	KVIPVDPYFFQM	$VP3_{322}$	A0901	N.D.
4X0395	RWFFNLFQL	$VP1_{619}$	A0901	0.010%
4X0395	LKYAQEEL	pX_{812}	B0501	0.177%
4X0395	MKDDKIVSL	$2B_{902}$	B0501	0.043%
4X0395	FAFMHGYEF	$3D_{2177}$	C0601	0.158%

Table 2. HAV class II epitopes

Class II epitope	HAV protein
4X0293	
EKFFLIHSADWLTTHALFHE	$VP2_{81}$
LRYHTYARFGIEIQVQINPT	$VP2_{121}$
LLNCNINNVVRIKVPFIYTR	$VP2_{171}$
YHSGRLLFCFVPGNELIDVS	$VP3_{371}$
SDHMSIYKFMGRSHFLCTFT	$VP1_{571}$
DKTDSTFGLVSIQIANYNHS	$VP1_{711}$
ESHIECRKPYKELRLEVGKQ	pX_{791}
RLKYAQEELSNEVLPPPRKM	pX_{811}
LFYTEEHEMMKFSWRGVTAD	$2B_{841}$
CFLLHWLNPKKINLADRMLG	$2B_{951}$
YVDIGCSVISCGKVFSKMLE	$2B_{1051}$
VHLSPLRDCIARVHQKLKNL	$2C_{1191}$
GFINQAMVTRCEPVVCYLHG	$2C_{1211}$
KRGGGKSLTSIALATKICKH	$2C_{1231}$
GCPMRLNMASLEEKGRHFSS	$2C_{1301}$
NPSPKTVYVKEAIDRRLHFK	$2C_{1331}$
GFFQSVTNHKWVAVGAAVGI	$3A_{1451}$
DADPVESQSTLEIAGLVRKN	$3B_{1511}$
GVKDDWLLVPSHAYKFEKDY	$3C_{1551}$
QDVVLMKVPTIPKFRDITEH	$3C_{1601}$
YVHKKNDGTTVDLTVDQAWR	$3C_{1661}$
AGGNSILVAKLVTQEMFQNI	$3C_{1711}$
VVSKTLFRKSPIYHHIDKTM	$3D_{1751}$
LLGVHPRLAQRILFNTVMME	$3D_{1871}$
IDACPLDYTILCRMYWGPAI	$3D_{1921}$
SKIFGKSPVFFCQALKILCY	$3D_{2061}$
NVPQLKPVSELTFLKRSFNL	$3D_{2121}$
LENAQWFAFMHGYEFYQKFY	$3D_{2171}$
4X0395	
SSVHTAEVGSHQVEPLRTSV	$VP2_{51}$
MVPGDQSYGSIASLTVYPHG	$VP2_{151}$
RIKVPFIYTRGAYHFKDPQY	$VP2_{181}$
FRVSTTENVVNLSNYEDARA	$VP3_{251}$
KMSFALDQEDWKSDPSQGGG	$VP3_{271}$
SIPTLAAQFPFNASDSVGQQ	$VP3_{301}$
VFDFQVFPTKYHSGRLLFCF	$VP3_{361}$
PCAVMDITGVQSTLRFRVPW	$VP3_{401}$
VASHVRVNVYLSAINLECFA	$VP3_{461}$
SDHMSIYKFMGRSHFLCTFT	$VP1_{571}$
FNSNNKEYTFPITLSSTSNP	$VP1_{591}$
ATDVDGMAWFTPVGLAVDTP	$VP1_{641}$
DKTDSTFGLVSIQIANYNHS	$VP1_{711}$
ESHIECRKPYKELRLEVGKQ	pX_{791}
LFYTEEHEMMKFSWRGVTAD	$2B_{841}$
TRALRRFGFSLAAGRSVWTL	$2B_{861}$
EMDAGVLTGRLIRLNDEKWT	$2B_{881}$
KINLADRMLGLSGVQEIKEQ	$2B_{961}$
CGKVFSKMLETVFNWQMDSR	$2B_{1061}$
YGVEPEKNIYTKPVASDYWD	$2C_{1251}$
NPSPKTVYVKEAIDRRLHFK	$2C_{1331}$
SQGISDDNDSAVAEFFQSFP	$2C_{1421}$
AVAEFFQSFPSGEPSNSKLS	$3A_{1431}$

Table 2. HAV class II epitopes *(Continued)*

Class II epitope	HAV protein
GFFQSVTNHKWVAVGAAVGI	$3A_{1451}$
GVKDDWLLVPSHAYKFEKDY	$3C_{1551}$
GTYYSISAGNVVIQSLDVGF	$3C_{1581}$
VNGTPMLISEGPLKMEEKAT	$3C_{1641}$
VDLTVDQAWRGKGEGLPGMC	$3C_{1671}$
AGGNSILVAKLVTQEMFQNI	$3C_{1711}$
AINMDSSPGFPYVQEKLTKR	$3D_{1841}$
GDDVLIVFSRDVQIDNLDLI	$3D_{2081}$
LENAQWFAFMHGYEFYQKFY	$3D_{2171}$
YFVQSCLEKEMIEYRLKSYD	$3D_{2191}$
WWRMRFYDQCFICDLS	$3D_{2211}$

of exposure to the virus, a time course typical of most human infections (Lanford et al., 2011).

Hepatitis and initial control of virus replication at week 4 was associated with the appearance of HAV-specific neutralizing antibodies and T cells. Serum IgM and IgG antibodies to HAV were detected within 3–4 wk of infection. The IgG response was sustained through the entire period of follow-up in both animals, as reported in the earlier study of these animals (Lanford et al., 2011). Here, we document that the serum antibodies present at week 4 did neutralize the virus, but titers were relatively weak (unpublished data). This is consistent with low-level neutralization of HAV in humans immediately after seroconversion (Lemon and Binn, 1983). T cells detected in blood at week 4 by IFN-γ ELISpot assay were broadly directed against structural and nonstructural HAV proteins (Fig. 1). To better characterize this response, $CD4^+$ and $CD8^+$ T cell lines generated from the blood and liver of both animals were used to map dominant class I and II epitopes. $CD4^+$ T helper cells accounted for most of the remarkable breadth of the response. For instance, 4X0293 targeted at least 28 distinct class II epitopes but only 3 class I epitopes (Tables 1 and 2). The T cell response in 4X0395 was mapped to 34 class II epitopes and 7 class I epitopes (Tables 1 and 2).

The dominant class I and II epitopes described in Tables 1 and 2 were used to more precisely define the kinetics and function of HAV-specific T cell responses. The $CD4^+$ T cell response was monitored by intracellular staining for four cytokines (IL-2, IL-21, IFN-γ, and TNF) after stimulation of peripheral blood mononuclear cells (PBMCs) with pooled class II epitopes (Fig. 2 B). The timing and magnitude of the helper response, defined by the population of $CD4^+$ T cells that produced at least one of four cytokines, was compared with HAV genome clearance described in the earlier study (Lanford et al., 2011; Fig. 2 A). Week 4 marked the peak of the $CD4^+$ T cell response in animal 4X0293, but it continued to increase in animal 4X0395 through week 6, perhaps because viremia was prolonged in this animal by ∼2 wk. $CD4^+$ T cell frequencies unexpectedly increased again in both animals 4 wk after the initial peak and coincided with resurgence of

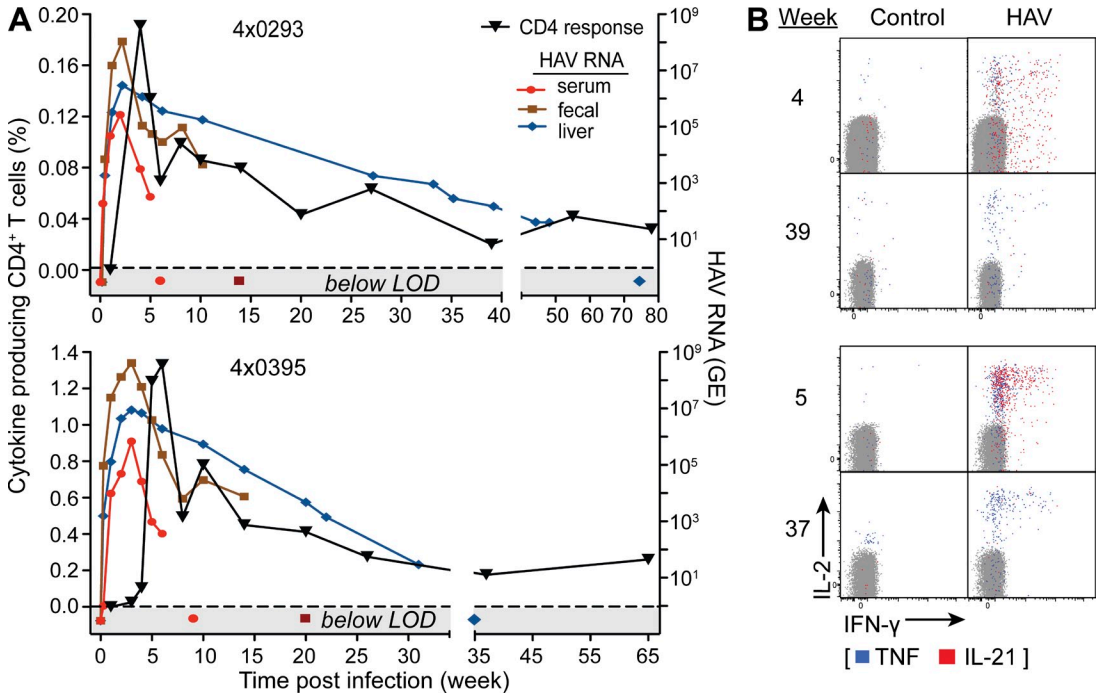

Figure 2. Kinetics of the HAV-specific CD4+ T cell response. (A) The combined percentage of CD4+ T cells expressing at least one cytokine of the four tested (IFN-γ, TNF, IL-2, and IL-21; black ▼) after stimulation with pooled HAV class II epitopes (Table 2). Serum (red ●), fecal (brown ■), and liver (blue ◆) viral titers are shown. The limit of detection (LOD) for viral titers was 10^3 GE/ml of serum, 5×10^3 GE/g of feces, and 10 GE/µg of total liver RNA. The first time point when viral titer dropped below the LOD is indicated in the shaded area. (B) Representative data are shown for the time of peak immune response (4X0293, week 4; 4X0395, week 5) and after the clearance of viremia (4X0293, week 39; 4X0395, week 37). PBMCs were stimulated with pooled HAV class II epitopes (Table 2) and analyzed for IFN-γ, IL-2, and TNF production. Blue dots represent CD4+ T cells that produced TNF with or without IFN-γ/ IL-2 production. Red dots represent CD4+ T cells that produced IL-21 with or without IFN-γ/ IL-2 production.

fecal shedding. Contraction of the CD4+ T cell response was biphasic (Fig. 2 A). For instance, in animal 4X0293 the initial contraction of HAV-specific CD4+ T cells occurred at week 4 as HAV viremia and fecal shedding declined by 3–4 logs. Termination of fecal shedding after week 10 marked a much more gradual phase of contraction that extended over several months as HAV genomes were slowly cleared from liver. The slope of CD4+ T cell decay during the first (−0.0608%/day; 95% confidence interval [CI], −0.0651 to −0.0564%/day) and second (−0.0023%/day; 95% CI, −0.0033 to −0.0012%/day) phases of contraction were substantially different, with 95% CIs that did not overlap. Because initial CD4+ T cell contraction in 4X0395 was delayed until week 6–8 when sampling was less frequent, too few data points were available to establish a 95% CI. Nevertheless, CD4+ T cell decay rates in this animal were also clearly different during the early (−0.418%/day) and late (−0.0195%/day; 95% CI −0.0303 to −0.0086%/day) phases of contraction. CD4+ T cell contraction stopped once HAV RNA genomes were at or below the limit of detection in liver (Fig. 2 A). Circulating memory populations then remained stable at a low frequency (~0.03% for 4X0293 and 0.2% for 4X0395).

An analysis of the quality of the CD4+ T cell response was also consistent with their involvement in control of HAV replication. CD4+ T cells that expanded at week 3 (4X0395) or 4 (4X0293) were polyfunctional, producing IFN-γ, TNF, IL-2, and IL-21 upon stimulation with pooled class II epitopes (Fig. 3 A) and overlapping HAV peptides (not depicted). No production of IL-4, IL-13, IL-17, or IL-22 was detected (unpublished data). At least two of these cytokines (IFN-γ and TNF) have the potential to directly limit HAV replication. Four functional features of the CD4+ T cell response were noted as infection was controlled. First, IL-21 production was lost after virus was cleared from liver (Fig. 3 B, top). Although half of the effector CD4+ T cells expressed IL-21 at the peak of immune response, few if any produced this cytokine at week 39 (4X0293) or 37 (4X0395). Second, the proportion of HAV-specific CD4+ T cells that produced IFN-γ, IL-2, and TNF at week 3 (4X0395) or 4 (4X0293) remained constant during contraction (Fig. 3 B, bottom). Third, when compared with early effectors, more of the HAV-specific memory CD4+ T cells produced multiple cytokines. Finally, HAV-specific memory CD4+ T cells were uniformly capable of producing IL-2 even if they did not simultaneously produce IFN-γ and/or TNF (Fig. 3 B).

We next characterized the CD8+ T cell response to defined class I epitopes targeted by each animal. The three epitopes recognized by chimpanzee 4X0293 (Table 1) were successfully incorporated into class I tetramers as determined by staining of CD8+ T cell lines derived from this animal (not depicted).

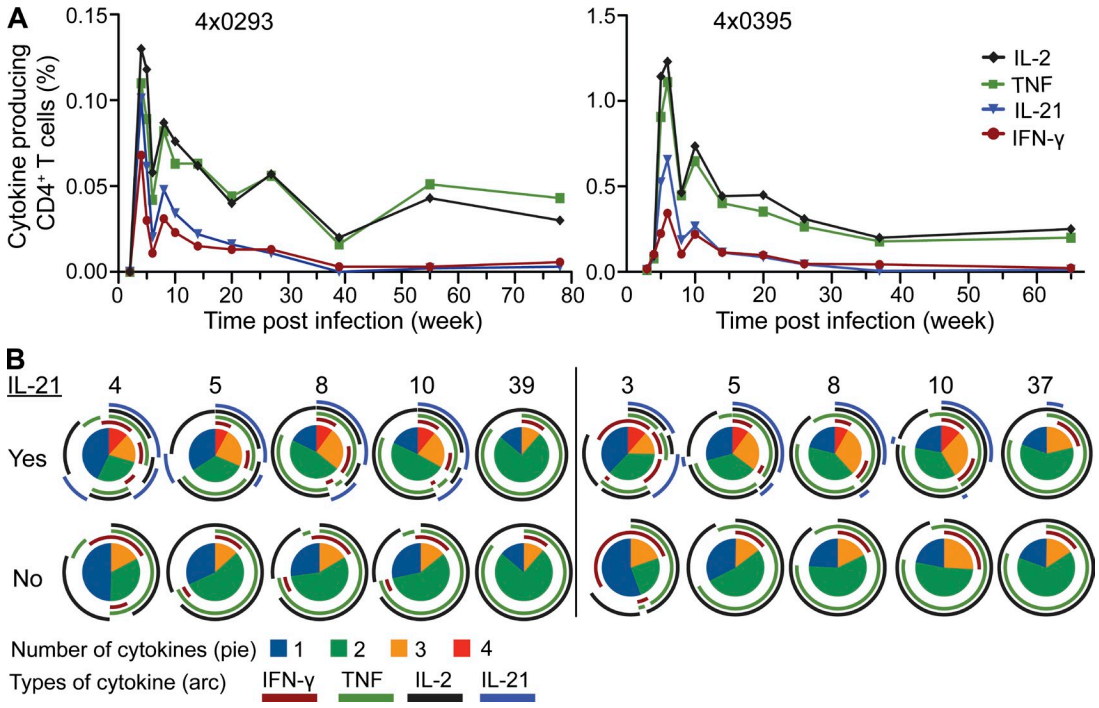

Figure 3. HAV-specific CD4+ T cell cytokine profiles. (A) Time course for production of the indicated cytokines by CD4+ T cells from animals 4X0293 (left) and 4X0395 (right). PBMCs stimulated with pooled HAV class II epitopes as described in Fig. 2 were analyzed for individual cytokine expression. (B) The relative frequency of HAV-specific CD4+ T cells producing a combination of one or more cytokines are presented as pie charts for animal 4X0293 (left) and 4X0395 (right). Arcs represent the proportion of CD4+ T cells that produced the indicated cytokines. In the top row of pie charts, IL-21 expression was included in the analysis. The bottom row excluded IL-21 from the analysis.

CD8+ T cells targeting the Patr-B1301 ($3D_{1875}$) and C0601 ($3D_{2177}$) epitopes were absent from the blood of this animal at all time points during the acute phase of infection (unpublished data). Patr-A0701 restricted $2C_{1269}$-specific CD8+ T cells were visualized in the blood, but only at week 4 and at a peak frequency of 0.007% that barely exceeded the threshold of detection (unpublished data). PBMC collected from animal 4X0293 at week 4 also failed to produce IFN-γ, TNF, or IL-2 after stimulation with the three class I epitopes (unpublished data), as expected because of the failure to visualize cognate CD8+ T cells in the blood with tetramers. Of the seven CD8+ T cell populations that could be tracked in animal 4X0395 with tetramers (not depicted), only three reached measurable frequencies in blood (Fig. 4 A). CD8+ T cells that recognized two Patr-B0501 restricted epitopes (pX_{812} and $2B_{902}$) and one C0601 restricted epitope ($3D_{2177}$) were not visualized in blood until week 4 after infection (Fig. 4 A), 1 wk after the initial substantial increase in serum ALT. They contracted sharply in frequency by week 8 even though viral genomes were still present in liver at high titer. It is notable that the multifunctional and multispecific CD4+ T cell response peaked at this time point when viremia declined ~100-fold (Fig. 3 A). CD8+ T cell function was also evaluated. Stimulation of PBMC from animal 4X0395 with pooled class I epitopes (Table 1) indicated a deficit in CD8+ T cell effector function. At week 5 when HAV-specific CD8+ T cell frequencies peaked as determined by tetramer staining, <15% were positive for expression of IFN-γ or CD107a, a marker of degranulation related to cytotoxic activity (unpublished data). CD8+ T cells targeting these epitopes were still present at high frequency 1 wk later, i.e., week 6. Some IFN-γ production was detected at this time point (Fig. 4 B), but the frequency of functional HAV-specific CD8+ T cells remained 60–70% lower than that measured by tetramer staining (Fig. 4 C, left). Approximately half of the tetramer-positive CD8+ T cells that produced IFN-γ at week 6 also expressed CD107a after antigen stimulation (Fig. 4, B and C, right). Very few coexpressed IL-2 or TNF (Fig. 4, B and C), in sharp contrast to the multifunctional CD4+ T cell response at the same time point. It is also notable that CD8+ T cells did not expand in blood at week 10 when fecal HAV shedding and the frequency of multifunctional CD4+ T cells increased (Fig. 4 A).

To determine if CD8+ T cells visualized in animal 4X0395 shifted to a memory phenotype, expression of CD127 and PD-1 on the pX_{812}- and $3D_{2177}$-specific populations was examined over time. HAV-specific CD8+ T cells shifted rapidly from an effector to memory phenotype despite absent or reduced effector functions during the acute phase of infection (Fig. 5, A and B). PD-1 expression, considered a marker of effector cell activation (Brown et al., 2010), was most intense at week 4 on CD8+ T cells targeting the HAV pX_{812} and $3D_{2177}$ epitopes (Fig. 5, A and B). Expression declined fivefold by week 6 when the animal was still viremic, and >10-fold by week 14 even with continued fecal shedding of HAV.

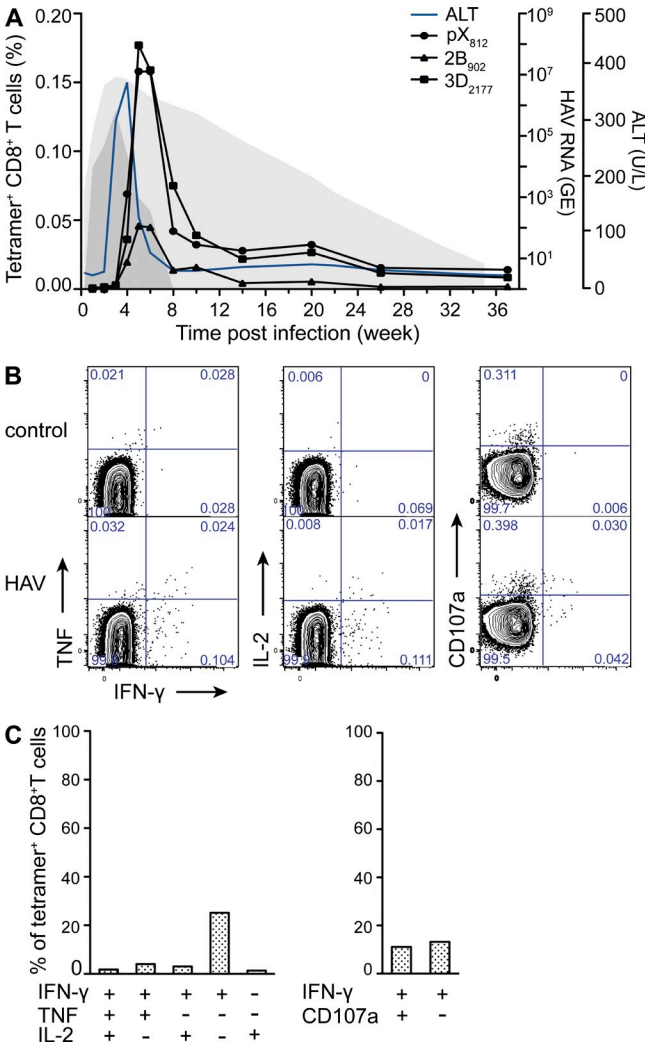

Figure 4. Cytokine production by HAV-specific CD8⁺ T cells. (A) The percentage of circulating CD8⁺ T cells targeting epitopes pX_{812}, $2B_{902}$, and $3D_{2177}$ in 4X0395 was determined by staining with the indicated tetramer. ALT (blue line) and HAV RNA levels in the serum (dark shading) and liver (light shading) are also shown. (B) PBMCs of 4X0395 collected at the peak of the immune response (week 6) were stimulated with pooled HAV class I epitopes (Table 1). Intracellular production of IFN-γ, TNF, IL-2, and surface expression of CD107a was analyzed by flow cytometry. (C) Functional CD8⁺ T cells at week 6, presented as a percentage of those recognized by the pX_{812}, $2B_{902}$, and $3D_{2177}$ class I tetramers at the same time point (see B). Left, CD8⁺ T cells positive for intracellular production of IFN-γ and/or TNF and IL-2, as a percentage of the total number of tetramer-positive cells. Right, CD8⁺ T cells that produced IFN-γ with or without CD107a expression, again as a percentage of the total number of circulating, tetramer-positive cells.

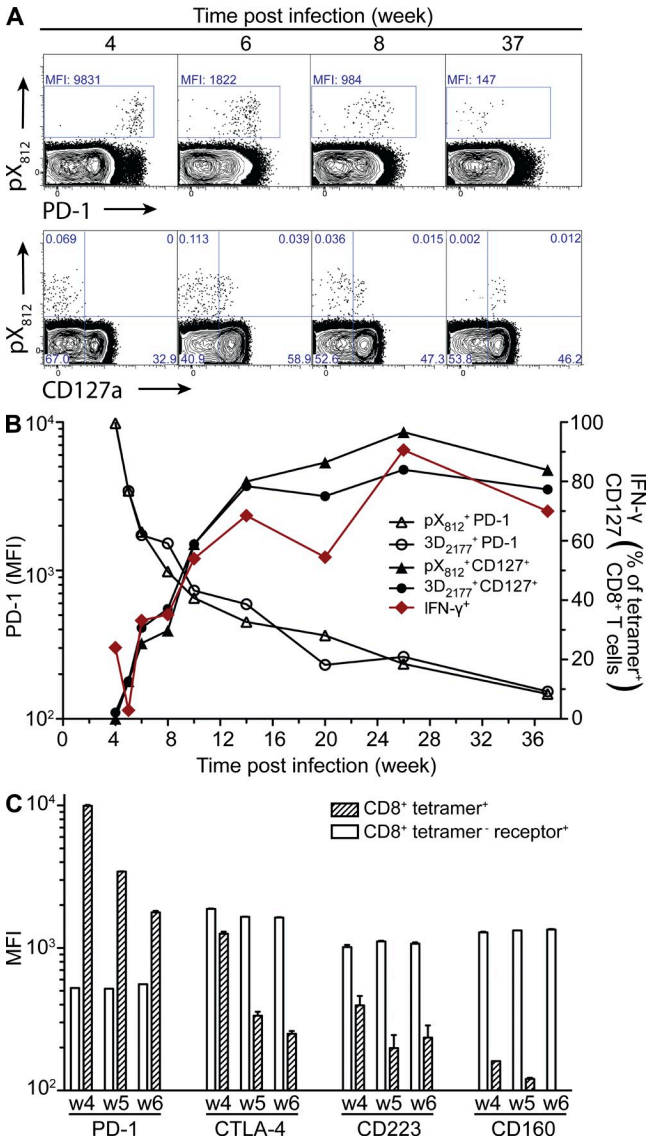

Figure 5. Phenotype of HAV-specific CD8⁺ T cells. (A) Representative data of CD8⁺ T cells from animal 4X0395 stained with pX_{812} tetramer and antibodies against PD-1 and CD127. (B) Mean fluorescence intensity (MFI) of PD-1 on tetramer-specific CD8⁺ T cells and the percentage of tetramer-specific CD8⁺ T cells expressing CD127 were plotted over time. CD8⁺ T cell populations recognized by pX_{812} and $3D_{2177}$ class I tetramers were analyzed for PD-1 and CD127 expression. The proportion of antigen-specific CD8⁺ T cells that produced IFN-γ is also shown (red ◆). It represents the percentage of all tetramer-positive CD8⁺ T cells that produced IFN-γ upon stimulation with pooled class I epitopes. (C) PBMC of 4X0395 collected at indicated times were stained with either the pX_{812} or $3D_{2177}$ class I tetramers and antibodies against CTLA-4, CD223, and CD160. Average MFI for the indicated inhibitory receptor and standard deviation is calculated for CD8⁺ T cells that were tetramer-positive (hatched bars) and those that were tetramer-negative but expressed the indicated receptor (white bars). PD-1 expression data from B is shown for comparison.

At the same time, expression of CD127, a component of the IL-7 receptor required to maintain memory populations (Kaech et al., 2003), steadily increased after week 4 (Fig. 5, A and B). Remarkably, the percentage of tetramer-positive cells that produced IFN-γ upon stimulation with pooled class I epitopes was very closely associated with acquisition of CD127 and loss of PD-1 through 38 wk of follow-up (Fig. 5 B).

Several months after HAV challenge, when viral RNA was still detectable in the liver of 4X0395, the majority of virus-specific CD8⁺ T cells had acquired a typical memory phenotype

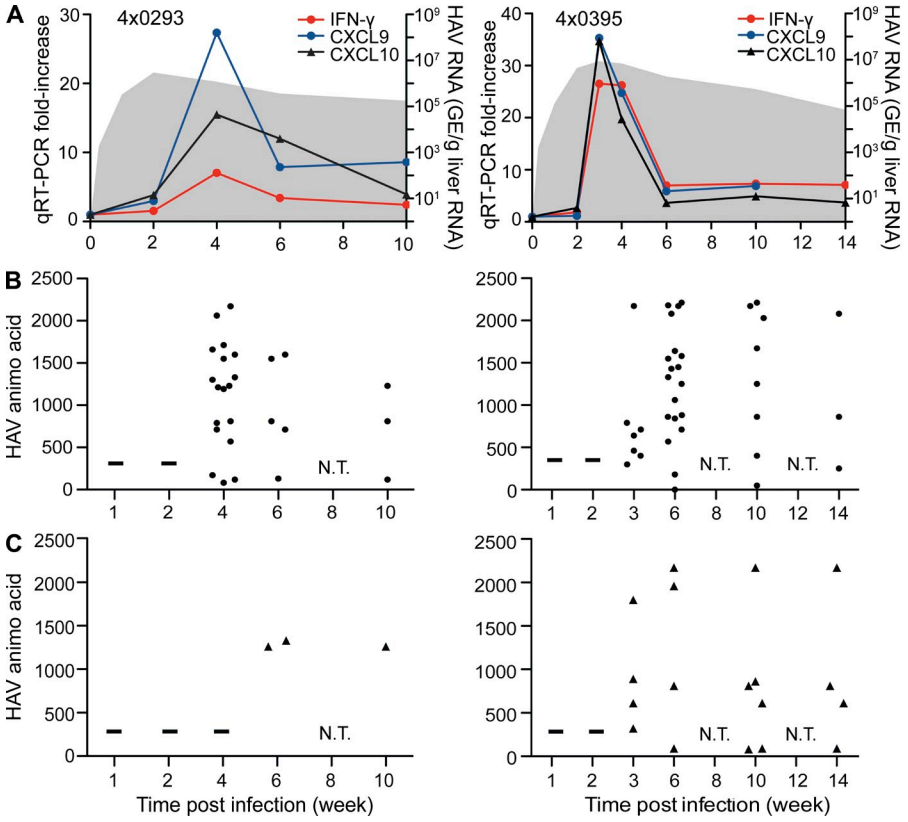

Figure 6. Intrahepatic gene expression and HAV-specific T cell responses. (A) Hepatic expression of IFN-γ, CXCL9, and CXCL10 was measured by TaqMan RT-PCR and is shown as fold change from baseline preinfection values. Liver HAV titer is illustrated by the shaded area. (B) HAV epitopes recognized by intrahepatic CD4+ T cells. CD4+ T cells isolated from liver biopsies at indicated time points were expanded and tested for IFN-γ production upon HAV peptide stimulation. The epitope for each HAV-specific T cell response was mapped down to a single 18-mer peptide by IFN-γ ELISPOT and confirmed by ICS. Each dot in the plot represents a class II epitope recognized by intrahepatic CD4+ T cells. The Y-axis coordinate corresponds to the position of the first amino acid of each epitope. No detectable response against HAV is indicated (-). N.T. indicates no sample taken. (C) HAV epitopes recognized by intrahepatic CD8+ T cells. Intrahepatic CD8+ T cells were expanded and tested for HAV-specific responses as described for CD4+ T cells.

with low PD-1 and high CD127 expression (Fig. 5, A and B). 70–80% of tetramer-positive cells produced IFN-γ, indicating that this long-lived memory population was fully responsive to antigen (Fig. 5 B).

Two features of the early HAV-specific CD8+ T cell response in chimpanzee 4X0395 resembled those commonly observed in persistent viral infections. Specifically, although the animal was viremic, CD8+ T cell effector functions were absent or largely restricted to production of IFN-γ. They also failed to respond to a late rebound in virus replication as measured by fecal HAV shedding. The temporal association between declining PD-1 expression and an increase in the number of CD8+ T cells that produced IFN-γ production prompted us to examine expression of other inhibitory receptors associated with functional exhaustion. CTLA-4, which can also negatively regulate the T cell response during acute and chronic virus infections, was increased on HAV-specific CD8+ T cells at week 4, although not to the same degree as PD-1 (Fig. 5 C). Expression of this inhibitory receptor declined to baseline levels through week 6. CD223 (LAG-3) and CD160 are representative of several inhibitory receptors thought to contribute to functional exhaustion (Wherry, 2011). When measured by mean fluorescence intensity, CD223 and CD160 were not obviously increased on HAV-specific CD8+ T cells during the first 6 wk of infection when compared with other circulating CD8+ T cells that expressed these markers (Fig. 5 C). We next sought to determine if the failure of CD8+ T cells to rebound with the late resurgence of fecal HAV shedding was a result of mutational escape of this RNA virus. HAV genomes were recovered from fecal samples at week 8 (4X0293) and week 10 (4X0395) when the transient increase in viral titer was detected. Sequencing of selected class I epitopes revealed no mutational escape (Fig. S1). For instance, the Patr-B0501 restricted epitopes pX_{812} and $2B_{902}$ targeted by animal 4X0395 were intact at week 2, before the onset of any immune selection pressure, and also at week 10, well after the CD8+ T cells had contracted and gained effector function (Fig. S1). Both animals expressed Patr-C0101. The $3D_{2177}$ epitope presented by that class I molecule also remained intact (Fig. S1). Thus, the two most common mechanisms of immune evasion in persistent virus infections—constitutive expression of multiple co-inhibitory receptors and mutational escape of class I epitopes—did not explain premature contraction, limited function, and poor responsiveness of CD8+ T cells to a rebound in virus replication during acute hepatitis A.

Hepatic expression of IFN-γ and its response genes CXCL9 (MIG) and CXCL10 (IP-10) was measured as a surrogate marker of the cellular immune response at the site of virus replication (Fig. 6 A). Expression of all three genes increased in the liver at week 3 (4X0395) or 4 (4X0293) and then declined to a level slightly above baseline 2 wk later (Fig. 6 A). Peak expression of these genes marked the first time point that HAV-specific T cells were successfully expanded from liver. The breadth of the intrahepatic helper response was greatest at week 4 (4X0293) or week 6 (4X0395) (Fig. 6 B), which matched the time of peak CD4+ T cell breadth and frequency in the blood of these animals (Fig. 2).

The number of class II epitopes targeted by liver-derived $CD4^+$ T cells then declined as viremia and fecal shedding of HAV decreased (Fig. 6 B). HAV-specific $CD4^+$ T cells, but not $CD8^+$ T cells, were expanded from the liver of 4X0293 at week 4 when IFN-γ, CXCL-9, and CXCL-10 expression peaked. Only at week 6, when viremia was no longer detectable and fecal shedding had declined >1,000-fold, were $CD8^+$ T cell lines targeting two epitopes established from liver. This result is consistent with very low to undetectable $CD8^+$ T cell frequencies in the blood of this animal. Intrahepatic $CD8^+$ T cell lines from 4X0395 again targeted more class I epitopes than 4X0293. In both animals, however, far fewer class I (Fig. 6 C) than class II (Fig. 6 B) epitopes were recognized in liver. Thus, in liver, as in blood, the $CD4^+$ T cell response was dominant.

DISCUSSION

We recently observed a very weak type I (IFN-α/β) ISG response in two chimpanzees with acute hepatitis A (Lanford et al., 2011). The present study was undertaken to assess cellular immunity in the setting of weak ISG activity, and to define protective mechanisms that contribute to resolution of acute symptomatic hepatitis A and prevent relapse of liver disease that is observed in some individuals after apparent control of the infection. Our results indicate that control of acute hepatitis A was most closely associated with a $CD4^+$ T cell response that was strong and sustained despite weak ISG activity.

Control of HCV and HBV infections is critically dependent on $CD8^+$ T cells. Studies in humans and chimpanzees have documented a temporal association between the development of a functional $CD8^+$ T cell response and control of liver infection (Rehermann, 2009; Chisari et al., 2010; Walker, 2010). Moreover, infection is prolonged or persists in HCV and HBV-infected chimpanzees after antibody-mediated depletion of $CD8^+$ T cells (Shoukry et al., 2003; Thimme et al., 2003). A dominant role for $CD8^+$ T cells in control of human HAV infections was first postulated over two decades ago when HAV-specific cytotoxic T cell lines were expanded from the liver and blood of jaundiced subjects (Maier et al., 1988; Vallbracht et al., 1989). Cytotoxic cell lines were also successfully established from the infected chimpanzees in this study. Because $CD8^+$ T cells can spontaneously acquire effector functions when expanded in cell culture, we assessed their activity immediately upon isolation from peripheral blood. Measurement of the $CD8^+$ T cell response by direct visualization with class I tetramers or functional assays revealed no obvious temporal association with control of viremia or liver damage. $CD8^+$ T cells were recently visualized in the blood of humans with acute hepatitis A using class I tetramers (Schulte et al., 2011). Whether $CD8^+$ T cells in humans are fully functional at the point that HAV replication is controlled, or are impaired as described in this study of chimpanzees, remains to be determined. Hepatitis was observed in both animals, but transaminase elevations were on the mild end of the spectrum described for symptomatic human infections (Cuthbert, 2001). HAV is not considered directly cytotoxic to infected hepatocytes and hepatitis is thought to result from immunopathology. Even if $CD8^+$ T cells are not necessarily required for clearance of HAV infection, individual variation in the pace or quality of the response could explain a spectrum of liver disease that ranges from mild to severe.

The early functional defect displayed by HAV-specific $CD8^+$ T cells could not be explained by inadequate T cell help. Several cytokines produced by $CD4^+$ T cells, including IFN-γ, IL-2, and IL-21, facilitate optimal development of primary $CD8^+$ T cell responses (Obar and Lefrançois, 2010; Cox et al., 2011). All were produced by HAV-specific $CD4^+$ T cells well before $CD8^+$ T cells expanded or gained effector function. This is quite different from acute hepatitis C where initiation of the $CD4^+$ T cell response was kinetically associated with the onset of $CD8^+$ T cell effector function (Thimme et al., 2001). Regulatory T cell activity might also slow acquisition of function by $CD8^+$ T cells during acute hepatitis A. This is perhaps unlikely, however, as it was recently reported that HAV transiently attenuates the suppressive activity of regulatory T cells in humans with acute hepatitis A by binding to the inhibitory molecule TIM-1 (also designated HAVCR1; Manangeeswaran et al., 2012). Finally, type I IFN is also required for $CD8^+$ T cell expansion (Kolumam et al., 2005; Aichele et al., 2006; Thompson et al., 2006), effector function (Curtsinger et al., 2005; Curtsinger and Mescher, 2010), and development of memory (Kolumam et al., 2005; Ramos et al., 2009; Xiao et al., 2009). The hepatic ISG response was highly attenuated in these animals (Lanford et al., 2011), perhaps because essential adaptor proteins required for the induction of type I IFN via Toll-like receptor 3 and RIG-I–like helicase signaling pathways are cleaved by the HAV protease (Yang et al., 2007). Although this apparent deficit in type I IFN activity might explain suboptimal priming of HAV-specific $CD8^+$ T cells, comparison with HBV and HCV infections suggests otherwise. HBV also induces a weak ISG response (Wieland et al., 2004) but functional $CD8^+$ T cells are clearly required for control of infection. In contrast, the acute phase ISG response to HCV is robust (Bigger et al., 2001; Lanford et al., 2011) even though the HCV and HAV proteases inactivate the same type I IFN signaling intermediates (Foy et al., 2003; Li et al., 2005; Yang et al., 2007). Despite large differences in the vigor of the ISG response to these viruses, $CD8^+$ T cells induced by HAV and HCV (Lechner et al., 2000; Thimme et al., 2001; Shoukry et al., 2003) displayed the same transient impairment of effector functions during acute infection.

There was no obvious association between slow acquisition of effector function by $CD8^+$ T cells and expression of inhibitory receptors. PD-1 and CTLA-4 were elevated during acute infection and decreased as memory populations emerged, but this pattern of expression is similar to that described for successful antiviral $CD8^+$ T cell responses (Ha et al., 2008). Other inhibitory receptors contribute to $CD8^+$ T cell dysfunction during prolonged or persistent viral infection (Klenerman and Thimme, 2011). Two of these molecules,

CD223 (LAG-3) and CD160, were not elevated on HAV-specific $CD8^+$ T cells between weeks 4 and 6 of infection. Because HAV-specific $CD8^+$ T cells rapidly lost expression of PD-1 and CTLA-4, and failed to up-regulate CD223 or CD160, expression of other inhibitory receptors that constitute the exhausted phenotype is unlikely. It is notable that the block on $CD8^+$ T cell effector activity was not alleviated until antigen load was substantially reduced. It is possible that the neutralizing antibody response detected at week 4 contributed to restoration of $CD8^+$ T cell activity by limiting viremia (Lemon and Binn, 1983).

$CD8^+$ T cells contracted rapidly and acquired a memory phenotype before virus was cleared. They also failed to respond to a late resurgence in fecal shedding of virus that increased $CD4^+$ T cell frequencies. These observations suggest a lack of antigen recognition. This could not be explained, however, by mutational escape of MHC class I epitopes. Epitope sequences were intact in HAV genomes recovered from fecal samples of animal 4X0395 at week 10, when shedding transiently increased and $CD8^+$ T cells had contracted by >80%. It is perhaps more likely that HAV-specific $CD8^+$ T cells followed a contraction and differentiation program that is regulated by the early inflammatory response and not levels of antigen production (Badovinac et al., 2002).

The study revealed a previously undefined but potentially significant role for $CD4^+$ T cells in HAV clearance when compared with the $CD8^+$ T cell response. Co-production of IFN-γ, TNF, IL-2, and IL-21 is considered a marker of highly effective $CD4^+$ T cell immunity (Seder et al., 2008). Appearance of multifunctional $CD4^+$ T cells in blood before a decrease in viremia indicated that the broad spectrum of function was a cause and not an effect of viral control (Darrah et al., 2007). Human $CD4^+$ T cell responses to HAV have not yet been described and differences between the species are possible. Chimpanzees and humans express orthologous MHC class II *DP*, *DQ*, and *DR* molecules that present antigenic peptides to $CD4^+$ T cells. Allelic diversity of *DRB* genes is reduced in chimpanzees when compared with humans, and some lineages have been lost, perhaps because of evolutionary pressure resulting from a selective sweep (Bontrop, 2006; de Groot et al., 2009). However, the wide breadth of the response in chimpanzees, with no obvious pattern of dominance by individual HAV epitopes or *Patr-DP*, *-DQ*, or *-DR* molecules, should minimize the impact of these immunogenetic differences between the species. From a practical standpoint, the repertoire of viral peptides presented to $CD4^+$ T cells is influenced by MHC class II supertypes that may substantially overlap between humans and chimpanzees (Shoukry et al., 2004). Cross-species presentation of viral class II epitopes has been observed (Elkington et al., 2004). For these reasons it is likely that the quality and kinetics of the human and chimpanzee $CD4^+$ T cell response to HAV will be more similar than different.

$CD4^+$ T cell differentiation is thought to be a linear, stepwise process characterized by early production of IL-2 and/or TNF, followed later by IFN-γ (Seder et al., 2008). The functional profile of HAV-specific $CD4^+$ T cells as they transitioned from effector to memory populations largely supports this model, with some exceptions. A substantial percentage of $CD4^+$ T cells produced all three cytokines (IFN-γ, IL-2, and TNF) at the time of initial expansion, suggesting that even if differentiation is linear, multiple functions, including IFN-γ production, can be acquired quite early after virus infection. The absence of IL-21 production by antigen-stimulated memory $CD4^+$ T cells was surprising, as up to 50% of effectors produced this cytokine at the peak of the acute phase response. Selective loss of this function may have occurred through silencing of IL-21 gene expression. $CD4^+$ T cells that produced only IFN-γ were lost by week 5 and were not found in the memory pool, consistent with negative regulation of helper cell survival by this cytokine (Wu et al., 2002; Xu et al., 2002). 90% of acute phase HAV-specific $CD4^+$ T cells produced IL-2, and all of those surviving as memory cells retained this function. These observations, combined with early fixation of cytokine production patterns by week 8 after infection, suggests that differentiation of memory $CD4^+$ T cell populations started well before HAV antigen was cleared.

Some features of the $CD4^+$ T cell response elicited by HAV and HCV are similar and may be critical in preventing resurgent replication of these hepatotropic viruses. Findings in this study of HAV infection, and in a recent study of humans with acute hepatitis C (Schulze Zur Wiesch et al., 2012), indicate that the early $CD4^+$ T cell response can be quite broad, targeting multiple class II restricted epitopes. Functional $CD4^+$ T cells remain responsive to fluctuations in HCV replication in those who ultimately resolve the infection (Semmo and Klenerman, 2007). $CD4^+$ T cell contraction observed in this study was similar in magnitude to that described in LCMV- (Homann et al., 2001) or *Listeria monocytogenes* (Pepper et al., 2010)–infected mice but followed an unusual biphasic pattern that was punctuated by brief resurgence of HAV shedding. The first phase of contraction was steep and occurred within 2 wk of peak viremia. The second more gradual phase of $CD4^+$ T cell contraction began after resurgent virus replication was controlled. The rebound in the number of virus-specific $CD4^+$ T cells that occurred with resurgence of fecal HAV shedding was reminiscent of the observations in acute HCV infections that spontaneously resolve. Symptomatic relapse of HAV infection is observed in up to 20% of infected individuals despite high titers of neutralizing antibodies. We propose that $CD4^+$ T cells prevent re-initiation of infection from a pool of HAV genomes that are only slowly cleared from the liver. Noncytolytic control of HBV and HCV infections, presumably by $CD8^+$ T cells, has been described (Guidotti and Chisari, 2001; Chisari et al., 2010). It is possible that $CD4^+$ T cells fulfill this critical function for control of HAV infection. In support of this concept, IFN-γ has been documented to impair HAV replication in cell culture models (Maier et al., 1988). $CD4^+$ T cells might also be responsible for the mild increase in transaminase values through production of TNF.

If $CD4^+$ T cells have an immune surveillance function, those at greatest risk of relapsing liver disease may benefit from vaccination to boost helper activity until HAV is finally cleared from the liver.

METHODS AND MATERIALS

HAV infection, viral titers, and antibody titers. Chimpanzees 4X0293 (female, 24 yr of age) and 4X0395 (male, 25 yr of age) were maintained under standard conditions for humane care at the Texas Biomedical Research Institute. Experimental protocols were approved by the Institutional Animal Care and Use Committee of the Texas Biomedical Research Institute.

Animal 4X0395 was never enrolled in an experimental protocol before this study and had not received commercial HAV or HBV vaccines. Animal 4X0293 was never exposed to HAV or HCV by infection or vaccination. HBV preS1 vaccine was administered in 1991, followed by challenge with HBV. There was apparent protection from infection, as seroconversion to other HBV antigens was not observed and the animal is negative for HBV DNA. Both animals were inoculated intravenously with 5,000 chimpanzee infectious doses of the wild-type HM175 strain of HAV that was recovered from human stool (provided by R. Purcell, National Institute of Allergy and Infectious Diseases [NIAID], National Institutes of Health, Bethesda, MD). Quantitation of viral titers and anti-HAV antibodies in these animals has been previously described (Lanford et al., 2011). HAV titers were measured by quantitative RT-PCR assay. Serum samples were tested for anti-HAV IgM and total Ig using an ELISA assay.

Peptides. 222 peptides (20 amino acids in length overlapping by 10 amino acids) spanning the wild-type HM175 polyprotein sequence were grouped into five pools that spanned the entire HAV polypeptide. Peptides were dissolved in sterile water containing 10% DMSO. The final concentration of each peptide in all assays was 1 μg/ml.

Isolation of lymphocytes from blood and liver. PBMCs were isolated using Ficoll density gradient and cryopreserved for future use. Intrahepatic lymphocytes were isolated from liver obtained by percutaneous biopsy and expanded in culture. Liver tissue in PBS containing 2% FCS was homogenized and T cell subsets were positively isolated with anti-CD4 and anti-CD8 antibodies conjugated to paramagnetic beads (Dynabeads; Invitrogen). Enriched intrahepatic T cells were cultured with 6×10^6 autologous PBMCs that had been irradiated (3,000 R) and pulsed with all HAV peptides in RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin, and 50 U/ml recombinant human IL-2 for 3 wk. These lymphocytes were further expanded with anti-CD3 antibodies and irradiated human PBMCs for an additional 3 wk before they were tested for recognition of HAV peptides.

IFN-γ ELISpot assay. The HAV-specific T cell response was quantified using IFN-γ ELISpot assay (U-Cytech) as previously described with the following modifications (Shoukry et al., 2003). For measurement of responses directly from blood, cryopreserved PBMCs were thawed and seeded at 2×10^5 cells/well in duplicate in 96-well plates precoated with anti–IFN-γ antibodies. 2×10^5 irradiated autologous PBMCs were added to each well. Cells were cultured in the presence of five pools of overlapping HAV peptides, or DMSO and cytomegalovirus peptides (as negative and positive controls, respectively), for 36 h before plates were developed according to the manufacturer's instructions. Background was <10 spot forming colonies per 10^6 PBMCs and was subtracted from responses in the test wells. To detect HAV-specific T cell response after expansion from liver, T cells were plated in AIM-V medium supplemented with 2% human AB serum the day before the assay. Autologous PBMCs or B lymphoblastoid cell lines (B-LCLs) were irradiated and pulsed with HAV peptides. 5×10^4 T lymphocytes were cocultured with 1.5×10^5 irradiated (3,000 R) PBMC or 5×10^3 B-LCL in the IFN-γ ELISpot assay. When a positive response was detected, peptide pools were de-convoluted to identify the targeted peptide.

HAV-specific $CD8^+$ T cell lines. $CD8^+$ T cell lines were derived from peripheral blood or intrahepatic lymphocytes as previously described (Erickson et al., 2001). Cell lines were tested for HAV specificity in the IFN-γ ELISpot assay. Class I MHC restriction of HAV-specific $CD8^+$ T cell lines was determined using 721,222 cells transfected with individual Patr-A, -B, or -C class I MHC molecules. Minimum optimal epitopes were mapped using amino- or carboxyl-truncated peptides in the IFN-γ ELISpot assay.

Cell stimulation and staining. To analyze cytokine production by HAV-specific T cells, cryopreserved PBMCs were thawed and rested in media (RPMI 1640 and 10% FCS) containing 5 U/ml benzonase (EMD) at 37°C for 10 h. Cells were collected and resuspended in media at 10^6/ml. 10^6 cells were incubated in the presence or absence of 1 μg/ml of peptides at 37°C for 16 h. GolgiPlug (BD) was added after the first hour. At the end of incubation, cells were washed with FACS buffer (PBS, 2.5% FCS, and 2% NaN_3) and stained with antibodies recognizing cell surface markers at 4°C for 20 min. After washing with FACS buffer, cells were stained with LIVE/DEAD blue fluorescent reactive dye (Invitrogen) at 4°C for 20 min. Cells were then washed twice with FACS buffer and permeabilized using Cytofix/Cytoperm (BD). Intracellular staining for cytokines was performed in Cytoperm buffer at 4°C for 30 min. Then cells were washed twice with Cytoperm buffer and fixed in 1% paraformaldehyde until analysis.

Class I tetramer staining was performed on cryopreserved PBMCs. Tetramers were functional as assessed by staining of $CD8^+$ T cell lines established from the blood and/or liver. PBMCs collected before HAV infection of the chimpanzees were included as a negative control. PBMCs were rested for 2 h after thawing, washed, and incubated with class I tetramers at 4°C for 30 min. After washing with FACS buffer, cells were stained with antibodies against surface markers and LIVE/DEAD blue fluorescent reactive dye sequentially as mentioned above, and fixed in 1% paraformaldehyde.

Antibodies. Fluorophore-conjugated monoclonal antibodies (CD3-V500, CD8-V500, CD3–Alexa Fluor 700, and TNF-PE-Cy7 from BD; CD14-FITC, CD16-FITC, CD19-FITC, IL-17A-FITC, CD14-PerCP/Cy5.5, CD16-PerCP, CD19-PerCP, IFN-γ–Pacific blue, PD-1–PerCP/Cy5.5, IL-2–APC, and CD4–Alexa Fluor 700 from BioLegend; IL-21–PE, CD127-V450, and IL-22–PerCP-eF710 from Bioscience; and LIVE/DEAD blue fluorescent reactive dye and CD8-Qdot 605 from Invitrogen) were used in these studies.

Flow cytometry. Cells were analyzed by flow cytometry using an LSRII instrument (BD). Data analysis and presentation of distributions was performed using FlowJo (v.9.2, Tree Star) and SPICE version 5.1 (NIAID; Roederer et al., 2011). Dead cells and $CD14^+$/$CD16^+$/$CD19^+$ cells were excluded from the analysis. For cytokine production analysis, between 500,000 and 1,500,000 events were acquired. A response was considered positive when the number of peptide-stimulated cells that produced a cytokine was more than twice that of cells not stimulated with peptide, and the value after background subtraction was at least 0.003%. For tetramer analysis, between 1,000,000 and 2,000,000 events were acquired. Background for tetramer staining was <0.001%.

Quantitation of hepatic gene expression. Preparation and quantification of liver RNA has been described (Lanford et al., 2011). In brief, total RNA was prepared from liver tissue using RNA-Bee extraction, a guanidium HCl-acid phenol based extraction procedure with isopropanol precipitation. For TaqMan analysis of host genes, RT-PCR Assays on Demand (Applied Biosystems) based on human sequences were used.

Mathematical modeling of T cell contraction. Slopes of cytokine-positive $CD4^+$ T cell decay were computed by linear regression. When more than two data points were available, 95% CIs for the decay slope were calculated using SPSS V.18 (SPSS, Inc.) assuming a normal distribution of the residuals.

When comparing two decay slopes, the difference was considered as significant when the CIs of the slopes did not overlap.

Online supplemental material. Fig. S1 shows sequencing traces for selected class I epitopes in HAV genome isolated from the stool before the onset of T cell response (week 2) and after $CD8^+$ T cell response contracted (4X0293, week 8; 4X0395, week 10). Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20111906/DC1.

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