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Local Intrahepatic CD8+ T Cell Activation by a Non-Self-Antigen Results in Full Functional Differentiation

Sherry A. Wuensch, Robert H. Pierce, and I. Nicholas Crispe

The response of T cells to liver Ags sometimes results in immune tolerance. This has been proposed to result from local, intrahepatic priming, while the expression of the same Ag in liver-draining lymph nodes is believed to result in effective immunity. We tested this model, using an exogenous model Ag expressed only in hepatocytes, due to infection with an adeno-associated virus vector. T cell activation was exclusively intrahepatic, yet in contrast to the predictions of the current model, this resulted in clonal expansion, IFN-γ synthesis, and cytotoxic effector function. Local activation of naive CD8+ T cells can therefore cause full CD8+ T cell activation, and hepato cellular presentation cannot be used to explain the failure of CTL effector function against some liver pathogens such as hepatitis C. The Journal of Immunology, 2006, 177: 1689–1697.

The induction of T cell immunity vs tolerance is influenced by the source of Ag, the cell type presenting the Ag, and the environment in which Ag recognition occurs. A well-established model for primary T cell activation that results in immunity is that bone marrow-derived immature dendritic cells (DC) acquire Ag from sites of infection, migrate to lymphoid tissue, differentiate, and engage T cells (1). It is uncertain how far this model applies to the liver, where diverse cell types have the potential to present Ag.

The large liver macrophage population, Kupffer cells (KC), can acquire and present Ag and express the costimulatory CD80 and CD86 molecules (2, 3). A key difference between KC and DC is that the latter acquire Ag in peripheral tissues in an immature differentiation state, migrate to local lymphoid tissue, and must then mature before stimulating naive T cells. In contrast, KC are not known to migrate out of the liver, and could potentially acquire and present Ag locally (4, 5). In experimental models, the hepatocytes themselves can directly present Ag to naive CD8+ T cells (6, 7). This is possible even though MHC I-positive hepatocytes lack CD80 and CD86 because engagement of ICAM-1 may provide an alternative costimulatory signal for CD8+ T cell activation (8–10).

Models based on liver transplantation (11) and models based on transgenic expression of Ag (6, 7, 12) suggest that CD8+ T cells activated by Ag expressed on hepatocytes undergo abortive activation leading to premature apoptosis. It has been proposed that the outcome of priming to liver Ags is determined by the site of Ag encounter (13). Thus, in Alb-Kb transgenic mice where the expression of Ag was limited to hepatocytes, CD8+ T cells were activated abortively; in contrast in Met-Kb transgenic mice, where that Ag was expressed both on hepatocytes and on resident cells in the lymph nodes, an effective immune response was generated, resulting in immune-mediated hepatitis. When primary activation was restricted to the liver, responding CD8+ T cells exhibited defective cytotoxic function and did not mediate hepatitis. These results were consistent with the observation that cultured hepatocytes could activate transgenic T cells but died prematurely (6, 12). If this were generally true, it would suggest that the failure of immunity in some chronic infections of the liver, such as malaria and hepatitis C virus, could be because the priming of T cells on hepatocytes does not provide costimulation sufficient to give a full activation or a survival signal.

Transgenic MHC Ags expressed on hepatocytes are present throughout life and are indifferent to Ag processing and presentation mechanisms. This would be expected to result in immune responses very different from those against Ags expressed de novo as a result of infection. To address the immunobiology of a response to the latter type of Ag, we tested the response to hepatocellular Ags in a model based on the infection of hepatocytes by an adeno-associated virus (AAV)-based vector. The expression of this vector is limited to the liver and causes Ag to be acutely expressed in an immunocompetent animal. The Ag is an intact protein, OVA, from which the antigenic peptide, of sequence SIINFEKL, must be processed. To deliver this Ag, an AAV vector encoding the OVA gene was injected into the liver, resulting in stably transduced hepatocytes.

Our purpose in this study was to test the priming of CD8+ T cells. When CD8+ T cells are present in sufficiently high precursor frequency, they are independent of CD4+ T cell activation and can provide self-help (14). Therefore, we used TCR-transgenic, OVA-specific OT.1 T cells at a cell number that was sufficient to function without CD4+ helper T cells; thus, we could examine the CD8+ T cell response and priming in isolation. Our results show that when acute Ag expression was directed exclusively to hepatocytes using an AAV vector, we obtained full T cell activation leading to proliferation, differentiation, and effector function. Therefore, abortive CD8+ T cell activation by hepatocytes does not explain “liver tolerance.”

Materials and Methods

Mice

Nontransgenic male C57BL/6J mice were purchased from The Jackson Laboratory. Transgenic male C57BL/6-Tg(OT-1)-Rag1tmMom mice were purchased from The Jackson Laboratory. All animal care and procedures were performed in accordance with the guidelines of the American Association for Laboratory Animal Care and the University of Rochester Institutional Animal Care and Use Committee.

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purchased from Taconic Farms. A colony of OT-1 transgenic mice was maintained on a CD45.1 background. Mice were used between 8 and 12 wk of age and housed in a specific pathogen-free environment in compliance with institutional guidelines for animal care.

**AAV vectors**

Serotype 2 AAV vectors containing OVA or enhanced GFP (eGFP) transgene under the control of the CMV promoter were obtained from the Columbus Children’s Research Institute, Viral Vector Core Facility. Briefly, plasmids encoding the AAV/CMV promoters, OVA or eGFP, the rep and cap genes from AAV, and neo/tk selection elements were transfected in HeLa-derived producer cells, which were subsequently infected with wild-type adenovirus type 5. rAAV was purified from the lysed cells, and purified rAAV2 was tested for adenovirus and none was detected (>1 Ad 5 PFU/10^7 rAAV2 DNase-resistant particles (DRP)).

**Liver injection surgery**

Mice at 8 wk of age were anesthetized with an i.p. injection of Avertin, and the central lobe of the liver was exposed through a 2-cm ventral midline incision. Using a 29-gauge insulin syringe, 60 μl of vector suspension was slowly injected into the liver. This contained 7.2 × 10^10 DNase-resistant particles (DRP) diluted in PBS. The peritoneal cavity was sutured with 4-0 PDS. The peritoneal cavity was washed with PBS. Five million OT-1 cells (isolated and depleted of B cells) were depletes of CFSElow splenocytes relative to the unstained population (212.A1 specific for MHC class II molecules, clone 2.4-G2 specific for FcR, clone TIB 146 specific for B220, clone GK1.5 specific for CD4, and clone 212.A1 specific for MHC class II molecules, clone 2.4-G2 specific for FcR, clone TIB 146 specific for B220, clone GK1.5 specific for CD4, and clone HB191 specific for NK1.1 marker) were used to deplete MHC class II-positive DC, B cells, CD4+ T cells, NK cells, and macrophages. Cells coated with primary Abs were removed with magnetic beads coated with the secondary Abs (Biocode goat anti-rat IgG, goat anti-mouse IgG, and goat anti-mouse IgM, Quagen). For in vivo proliferation studies, cells were resuspended in 2.5 ml of PBS with 4 μM CFSE (Molecular Probes) for 10 min at 37°C and washed with PBS. Five million OT-1 cells (>85% pure CD8+ T cells) were injected i.v. into recipient mice that had been injected with AAV vectors 3–4 wk previously.

**Spleen and peripheral lymph nodes**

Spleen and peripheral lymph nodes were harvested and leukocytes isolated. Cytotoxicity was measured as the decrease of SIINFEKL-pulsed CFSEhigh splenocytes relative to the unpulsed CFSElow splenocytes. In vivo cytotoxicity assay

Target cells were created using B6 splenocytes and isolated as described above. The cell suspension was divided into two and stained with either 2 μM (CFSElow cells) or 0.02 μM CFSE (CFSEhi cells). Cells were washed and resuspended in 5 ml of RPMI 1640. SINEFPEL peptide (1 μM) was added to the CFSEhi cell suspension, and both the CFSEhi and CFSElow cells were washed twice with PBS at 37°C, 5% CO2. Cells were washed twice and injected i.v. into mice that had been infected with AAV-OVA or AAV-eGFP, then given OT-1 T cells 5 days previously. After 5 h, peripheral lymph nodes, spleen, and liver were harvested and leukocytes isolated. Cytotoxicity was measured as the decrease of SINEFPEL-pulsed CFSEhi splenocytes relative to the unpulsed CFSElow splenocytes.

**Flow cytometric analysis**

Isolated cells resuspended in staining solution (1% FCS in PBS) and incubated with Fc-block (BD Biosciences) for 5 min before addition of Abs against surface markers. Abs used for flow analysis included anti-CD62L and anti-TIA1.
(PE), anti-CD44 (PE), and anti-CD8 (PerCP), and these were purchased from BD Biosciences. Allophycocyanin-conjugated anti-CD45.1 was obtained from eBioscience. Flow cytometric data were acquired using a FACSCalibur (BD Biosciences) flow cytometer. Analysis was performed using CellQuest software (BD Biosciences) on a Macintosh computer (Apple Computer). Live lymphocytes were identified based on forward and side scatter profiles. The total OT-1 cell number per organ was calculated by multiplication of the total cell number by the percentage of CD45.1^+ CD8^+ cells within the lymphocyte gate.

Calculations and statistical analysis

The equation used to determine percent target cell lysis was percent = (1 − (ratio A/ratio B)) × 100, where ratio A was the percentage of CFSE^high, peptide-pulsed cells divided by percentage of CFSE^low, unpulsed cells after 5 h in the animal, and ratio B was the percentage of CFSE^high, peptide-pulsed cells divided by the percentage of CFSE^low, unpulsed cells before injection. Data in the text and shown in the figures represent the mean and SEM. Student’s t test was used to analyze the results where applicable, and p < 0.05 was considered significant.

Results

Expression of AAV-vector product

We established an in vivo model in which an exogenous, vector-encoded Ag is expressed in hepatocytes (22). We injected directly into the liver an AAV-gene expression vector (serotype 2) expressing either OVA or eGFP cDNA under control of the CMV promoter. To verify that the liver was transduced, liver sections were stained at 4 wk with anti-OVA or anti-GFP Abs. Of 139 cells that stained for OVA, 137 were definitely hepatocytes and the other 2 were probably hepatocytes (Fig 1A). When liver sections from animals injected with the control eGFP vector were stained for anti-eGFP (Fig. 1B), 355 cells were identified among which 348 were definitely hepatocytes, and the others probably hepatocytes. In both cases, absolutely no KC, endothelial cells, stellate cells, or lymphocytes stained for the AAV-encoded product.

To test whether the vector localized to the liver, RT-PCR was used to detect mRNA expression of the vector in a wide array of lymphoid and nonlymphoid tissues. Only in the liver was mRNA for OVA detected (Fig 1C). The same results were seen from tissues isolated from AAV-GFP-treated mice (data not shown). For both AAV vectors, mRNA for OVA and eGFP was detected in two different lobes of the liver (the central lobe, which was the site of the injection, and the right laterocaudal lob, anatomically distant) (data not shown). No message was detected in the other nonlymphoid tissues (muscle, brain, heart, kidney, small intestine, and lung) examined. Nor could the message be detected in the pooled Peyer’s patches, pooled peripheral lymph nodes, mesenteric lymph nodes, or pooled internal lymph nodes (portal vein, renal, lumbar, sacral, sciatic, and mediastinal nodes). These results suggest that expression of the AAV-encoded Ag was localized to the liver.

CD8^+ T cell proliferate when cocultured with hepatocytes

The exclusive detection of OVA or eGFP mRNA in the hepatocytes did not exclude the possibility that Ag was being transported to lymphoid tissues. This is a central issue because the presentation of Ag in lymphoid tissue has been proposed to be essential for effective T cell priming (13). To test whether the AAV-OVA-encoded SIINFEKL Ag was presented in lymphoid tissue and/or liver, single-cell suspensions were prepared from the portal vein lymph nodes, mesenteric lymph nodes, peripheral lymph nodes, spleen, and liver of AAV-transfected animals. Cocultivation conditions were optimized to reflect the difference of cell size and culture conditions between lymphocytes (suspension culture) and hepatocytes (adherent monolayer). These cells were cocultured with transgenic OT.1 CD8^+ T cells that had been stained with CFSE. Fig. 2 shows that OT.1 T cells only proliferated when cultured with an enriched population of hepatocytes from AAV-OVA-treated mice. As a positive control, splenocytes from an untreated mouse were preincubated with SIINFEKL peptide before OT.1 T cells addition. Notably, OT.1 T cells did not proliferate in cocultures with cells from the lymphoid tissues of AAV-OVA- or AAV-eGFP-treated mice. These results argue strongly that Ag presentation was not occurring in lymphoid tissues but that the OVA Ag was presented, as well as expressed, exclusively in the liver.

In vivo proliferation response of CD8^+ T cells to AAV-OVA

We established a model in which CD8^+ T cells would be expected to respond in a helper-independent way so that CD8^+ T cell priming could be studied in isolation. To do this, 5 million OT.1 CD8^+ T cells were injected into mice transfected with AAV-OVA or
AAV-eGFP groups was determined by Student’s t test: *, p < 0.05; ψ, p < 0.01; and ‡, p < 0.001.

AAV-eGFP. A significant increase (p < 0.01) in the percentage of responding transgenic T cells in AAV-OVA-treated animals occurred first in the liver when compared with AAV-eGFP controls on day 3 (Fig. 3A). This was in agreement with the in vitro coculture results, which indicated that Ag was presented in the liver. Following the response of OT.1 CD8+ T cells over time, there was a 10-, 50-, and 37-fold increase in the percentage of transgenic cells from AAV-OVA livers on days 3, 5, and 7, respectively (Fig. 3A). In contrast, no difference in the percentage of transgenic cells from lymph nodes or spleen of these same animals was seen at day 3. Although a small but significant increase was detected at day 5, no difference was detected at day 7. The absolute number of OT.1 T cells from AAV-OVA-transduced liver increased 21-, 221-, and 124-fold over that of transgenic cells isolated from controls at days 3, 5, and 7, respectively. In the spleen of animals treated with AAV-OVA, there was only an increase of 2.8- and 1.8-fold at days 5 and 7 in the number of cells. In the case of OT-1 cells from the lymph nodes, there was a 1.4-fold difference at day 5, and no difference between the groups at days 3 and 7 (Fig. 3B).

The proliferation of OT.1 CD8+ T cells in response to Ag was determined by labeling transgenic cells with CFSE before i.v. injection and measuring the decrease in CFSE intensity as the population expanded. On day 3, only cells isolated from the AAV-OVA liver had expanded (Fig. 4). Over time, transgenic CD8+ T cells continued to expand in the liver to the point where CFSE was not detectable, and only a small nondividing CFSEhigh population remained on days 5 and 7 (Fig. 4). In contrast, the majority of transgenic T cells in AAV-eGFP-treated animals remained CFSE− high in all three compartments. Some expansion of OT.1 CD8+ T cells from the lymphoid tissues of AAV-OVA mice was seen on days 5 and 7. This does not argue for low-level presentation in the lymph nodes because it could occur if a subset of the proliferating cells left the liver and entered the general circulation.

**Differential expression of activation markers on OT-1 T cells**

In parallel with their proliferation, OT-1 T cells change their expression of the activation markers CD62L and CD44. As in the case of proliferation, CD62L down-regulation was first observed on day 3 on OT-1 T cells from AAV-OVA-infected livers and increased over the course of the study (Fig. 5A). Correspondingly, CD44 was uniformly up-regulated on transgenic T cells from AAV-OVA animals vs transgenic T cells from AAV-eGFP controls (Fig. 5B). Cells isolated from the lymphoid tissues of AAV-OVA animals had also down-regulated CD62L and up-regulated CD44 but to a lesser extent. Also, the OT.1 CD8+ T cell response was Ag specific since it was limited to animals transduced with AAV-OVA, whereas OT-1 T cells from AAV-eGFP-treated animals remained undivided, CD62Lhigh and CD44medium.

**Activated OT.1 T cells produce IFN-γ**

Although T cells in the AAV-OVA model proliferated and had surface marker expression consistent with activation, T cells are not necessarily fated to become effectors. A key question in liver tolerance is whether activated T cells in the liver differentiate into functional effector cells. To test function, T cells were assayed for IFN-γ production. Lymphocytes, isolated from their respective tissues on day 5, were cultured with or without SIINFEKL for 6 h in vitro and stained for intracellular IFN-γ. Using flow cytometry, we looked at both the undivided and dividing transgenic CD8+ T cell populations. In the absence of in vitro restimulation with peptide, undivided OT.1 CD8+ T cells did not produce IFN-γ (Fig. 6). Restimulated but undivided lymph node and spleen cells from both AAV-OVA- and AAV-eGFP-treated animals produced IFN-γ, confirming the result of Auphan-Anezin et al. (23), who showed that naive CD8+ T cells can secrete IFN-γ independent of T cell proliferation. We were surprised initially to find that nondividing OT.1 cells from AAV-eGFP-transduced mice also produced IFN-γ, but these cells were not proliferating (Fig. 4), nor were they activated as determined by surface marker expression (Fig. 5). Among the cells showing signs of activation and proliferation, the

![FIGURE 2.](http://www.jimmunol.org/Downloadedfrom) OT.1 T cells proliferate when cocultured with hepatocytes from AAV-OVA mice. CFSE-labeled OT.1 CD8+ T cells were cocultured for 5 days with cells isolated and pooled from three animals from portal vein lymph nodes (PLN), mesenteric lymph nodes (MLN), peripheral lymph nodes (PLN), and spleens (SPL) of animals treated with AAV-OVA (■) or AAV-eGFP (—). Hepatocytes (HEP) were isolated from one liver of either an AAV-OVA or AAV-eGFP animal. Control (CON) wells with splenocytes from untreated mice were pulsed (●) with or without (—) SIINFEKL before OT.1 cell addition. Three wells were set-up for each type of coculture. Histograms are gated on CD8+CD45.1+CFSE− cells, and data represent results from three separate experiments.

![FIGURE 3.](http://www.jimmunol.org/Downloadedfrom) Expansion of OT.1 T cells was first seen in the liver. CFSE-labeled OT.1 T cells were injected into mice 3 wk after AAV vector injection. Average percentages (A) and numbers (B) of OT.1 (CD45.1+CD8+) T cells isolated from lymph nodes (LN), spleen (SPL), and liver (LIV) on days 3, 5, and 7. Plots represent means ± SEM, and data are representative of two independent studies with three mice per group at each time point. At each time point, significance between AAV-OVA and AAV-eGFP groups was determined by Student’s t test: *p < 0.05; ψ, p < 0.01; and ‡, p < 0.001.
synthesis of IFN-γ was found exclusively among OT-1 cells from AAV-OVA-transduced mice. The frequencies of dividing lymph node, spleen, and liver OT-1 T cells synthesizing IFN-γ in this group of mice were 12.6 ± 0.9, 16.6 ± 2.2, and 8.4 ± 1.0%, respectively.

AAV-OVA-activated OT.1 T cells have CTL function

In addition to IFN-γ production, the defining function of a CD8+ effector T cell is cytotoxicity. Thus, an in vivo cytotoxic activity assay was used to evaluate effector function of transgenic T CD8+ cells. Splenocytes differentially labeled with two different concentrations of CFSE were pulsed with SIINFEKL in vitro, or incubated with saline as a control (Fig. 7A), and then a mixture of the labeled and unlabeled target cells was injected (i.v.) into AAV-OVA-transduced animals that had received OT.1 CD8+ T cells 5 days previously. Five hours after splenocyte injection, leukocytes from spleen, lymph nodes, and liver were harvested, and the loss of SIINFEKL-pulsed (M1) vs control (M2) splenocytes was compared. Fig. 7A illustrates that peptide pulsed splenocytes were eliminated preferentially in AAV-OVA-treated animals that received transgenic CD8+ T cells vs the corresponding AAV-eGFP animals. Furthermore, highly significant (p < 0.001) cytolytic activity (58, 74, and 66%) was present in all three tissues, the lymph nodes, spleen, and liver (Fig. 7B). These results suggest that transgenic T CD8+ cells activated in mice injected with AAV-OVA vector differentiated into cytotoxic effectors.

OT.1 T cell activation in Rag-1 gene-deficient mice

The use of TCR transgenic T cells in adoptive transfer is complicated by the issue that allelic exclusion of the endogenous TCR genes is incomplete, particularly for the TCR-β locus. This can result in T cells with dual specificities. These cells are disfavored during thymic selection (24, 25), but nevertheless, some can be

FIGURE 5. Activated OT.1 T cells down-regulate CD62L and up-regulate CD44 over time. A, Adoptively transferred OT.1 (CD8+CD45.1+) T cells from AAV-OVA mice down-regulated CD62L in the liver first, as determined by the percent decrease in cells and (B) correspondingly increase CD44 as determined by mean fluorescence intensity. Histograms represent an individual animal from two independent experiments with three mice per group at each time point.
expressed in the periphery. These cells could be primed by environmental Ags, thus creating bona fide memory cells. To specifically test liver Ags in naive CD8+ T cell priming, we excluded this possibility by using OT-1 T cells that were unable to rearrange endogenous TCR genes due to the lack of Rag-1. In the absence of the Rag-1 gene, OT-1 CD8+ T donor cells proliferated as determined by dilution of CFSE label (Fig. 8A). These results were the same as those seen in Fig. 4 on day 5 for OT-1 CD8+ T cells from Rag-1−/− intact mice. As in the previous experiments, Rag-1−/− OT-1 T cells down-regulated CD62L and increased expression of CD44 (data not shown). Overall percentage (Fig. 8B) and number of cells (Fig. 8A) in the lymph nodes, spleen, and liver in response to Ag were not statistically different from previous studies (Fig. 3). These cells were fully competent to synthesize IFN-γ. Thus, Rag-1−/− OT-1 cells that had divided, isolated from the lymph nodes, spleen, and liver, contained 27.5 ± 4.3, 53.1 ± 6.5, and 31.5 ± 2.0% of IFN-γ-positive cells, respectively (data not shown). These results rule out the possibility that the response of OT-1 T cells to hepatocellular Ag was due to memory cells primed through a second TCR generated by endogenous TCR-α rearrangement.

Discussion

In this study, we used a replication-defective AAV vector to direct the expression of the experimental Ag to hepatocytes. Such AAV vector treatment generally results in a very low level of tissue injury (26–28), and although these vectors have been demonstrated to activate the innate immune system, this is a smaller effect than is seen in other vector systems such as adenovirus (26, 29). The type and magnitude of response to AAV is highly dependent on the animal model, route and site of administration, vector dose, transgene product, and transgene promoter (26, 29). In the case of the innate immune system, when AAV vectors were injected i.m., no localized inflammation was detected (27, 28, 30, 31). However, when AAV was injected via the femoral vein, there was a detectable innate response, with the transient expression of mRNA for cytokines TNF-α, CCL5 (RANTES), CXCL10 (IP-10), CCL4 (MIP-1 β), CCL-2 (MCP-1), and CXCL2 (MIP-2), which returned to baseline within 6 h after AAV administration (26). Because AAV is not completely inert, this may explain why even in the AAV-eGFP-treated mice, nondividing transgenic CD8+ T cells produced background levels of IFN-γ.

Injecting AAV via the portal vein or directly into the liver are both effective routes to infect the liver (22). We chose to inject the liver directly to reduce the potential for systemic induction of the immune system and localize expression of the experimental Ag to the hepatocytes, as seen in Fig. 1. A and B. Direct liver injection also takes advantage of the unique anatomical structure of the liver, a sinusoidal anastomosing network of blood spaces, which results in slow-flowing blood and maximizes the exposure of hepatocytes to an agent introduced into the liver parenchyma. We found...
that mRNA encoding the vector was detected both in the liver lobe that was injected and a lobe distal to the site of injection (data not shown). Of equal importance, this localized injection site limits the potential for others tissues to be exposed to the vector. This is illustrated by the fact that among many tissues sampled, mRNA encoding the vector was detected only in the liver and not in any other lymphoid or nonlymphoid tissues (Fig 1). Of equal importance, this localized injection site limits the potential for others tissues to be exposed to the vector. This is illustrated by the fact that among many tissues sampled, mRNA encoding the vector was detected only in the liver and not in any other lymphoid or nonlymphoid tissues (Fig 1). Of equal importance, this localized injection site limits the potential for others tissues to be exposed to the vector. This is illustrated by the fact that among many tissues sampled, mRNA encoding the vector was detected only in the liver and not in any other lymphoid or nonlymphoid tissues (Fig 1).

The frequency of cells staining positive for AAV-eGFP or AAV-OVA appeared to be low by histology, while it has been reported previously that rAAV vector genomes are found in most hepatocytes nuclei within 24 h of vector infusion. However, only a small subset of cells become stably transduced (32). Efforts to increase the number of stably transduced cells appear to be restricted by the number of transducible hepatocytes and result in transduction of <10% of total hepatocytes (33). In several experimental mouse models that generated therapeutic levels of transgene expression, ~5% of hepatocytes were transduced (33–35), and stable transgene expression occurred between 3 and 4 wk after vector administration (33). Unlike transgenic models where the Ag is expressed potentially on all hepatocytes (6, 7, 12), the low frequency of Ag-expressing hepatocytes in this model is more likely to reflect Ag expression in infection such as viral hepatitis C. The primary objective of this study was to determine whether expression of a hepatocellular Ag under these more physiological conditions activated CD8⁺ T cells. To limit the complicating effect of a host response to the AAV vector, we adjusted our dose of vector to the minimum required to achieve detectable transgene expression and T cell activation. Using 7.2 × 10¹⁰ DRP, we demonstrated expression of vector protein (Fig. 1A) and generated an immune response from adoptively transferred T cells at week 4 (Fig. 3). The CD8⁺ T cell immune response we studied is likely to be independent of CD4⁺ T cell help. The role of CD4⁺ T cell help in CD8⁺ T cell responses is under active investigation, and the need for such help appears to depend on the details of the experimental model (36–38). Although there are models where CTL immunity is dependent on CD4⁺ T cell help (39–42) and on cross-priming via DC (36), recent data suggest that primary activation from naive CD8⁺ T cells can be helper independent, resulting in effector cells (38, 43, 44). Helper independence of CD8⁺ T cells can occur at high precursor frequencies and can still generate a CTL response (45); however, such a helper-independent response may be defective in CD8⁺ T cell memory development (43).

In our model, we were interested in looking for a primary response of the CD8⁺ T cells and bypassed the need for help by using a large number of Ag-specific CD8⁺ T cells, which allowed us to determine whether Ag was being presented to the CD8⁺ T cells locally in the liver or alternatively in lymphoid tissues such as the spleen and lymph nodes. We also found that in vivo proliferation of 5 million CD8⁺ T cells occurs in mice that lack MHC class II (data not shown). In parallel studies, the capacity for CD8⁺ T cells to respond locally was tested in the transplanted liver (46); this study supports our main conclusion that Ags expressed on cells intrinsic to the liver can prime CD8⁺ T cells. The time course study (Fig. 4), where OT1 T cells were first seen to proliferate in the liver, suggests that OVA presentation was localized to the liver and that OVA was not acting as a systemic immunogen. The route of administration may be critical in determining the nature of the AAV response, because when AAV-OVA was injected s.c., i.v., or i.p., a strong OVA-specific CTL response was generated. In contrast, i.m. injections induced only a minimal response (47). We cannot exclude the possibility that there was a host response to AAV or to OVA before OT1 T cell transfer. Such a response to vector Ags would be expected to injure antigenic cells, potentially resulting in Ag dispersal and presentation in peripheral lymph nodes. The data in Fig. 2 argue against such a strong CTL response by the host before adoptive transfer because leukocytes from diverse lymph nodes (the superficial peripheral, mesenteric, and portal vein nodes) and the spleens from AAV-OVA- and AAV-GFP-treated animals were unable to elicit OT1 T cell proliferation, arguing that there was no Ag dispersal. It is true that, in vivo, clonal expansion of OT-1 cells was seen in the lymph nodes and spleen at later time points after AAV-OVA treatment, but due to the time delay and small magnitude of this effect, we argue that the proliferating T cells in the liver may have entered the circulation and seeded peripheral sites. An alternative explanation is that the CTL generated during the OT1 T cell response caused the lysis of vector-transduced cells and the release of Ag that could be processed by APC that migrated to other lymphoid tissue and present...
Ag at those sites. Either way, a local intrahepatic immune response was the primary event.

Both the innate and the adaptive arms of the immune system are optimized to respond to the sudden appearance of foreign molecules. However, in the experiments that provide the strongest evidence for CD8\(^+\) T cell tolerance in response to hepatocellular Ags, Ag expression was driven by constitutively active, tissue-specific transgenes. Such transgene-encoded Ags might be more accurate models of self-Ags, rather than virus-encoded Ags. Several regulatory mechanisms limit the capacity to respond to self-Ags. A central tolerance mechanism in the thymus eliminates endogenous T cells that could potentially respond to self-Ags, but such a mechanism should not affect exogenous T cells delivered by adoptive transfer. However, the expression of AIRE in thymic medullary epithelial cells results in the constitutive synthesis and expression of peripheral tissue-specific Ag (48, 49), including transgenic model self-Ags. This may promote the development of regulatory T cells that could affect the activity of exogenous, non-tolerant precursors cells. Because of this mechanism, a transgenic mouse expressing a tissue-specific Ag may be a more accurately representative model of self-tolerance, rather than of immunity to an infection. Therefore, a T cell response in that context is a better model of autoimmune hepatitis than of antiviral immunity.

Although our experiments exclude the involvement of a circulating population of professional APC that traffic from sites of Ag expression to lymphoid tissue, there are several candidates for local APC. These include the infected hepatocytes themselves, the KC, and the liver sinusoidal endothelial cells (LSEC). T cell activation by the infected hepatocytes would constitute direct presentation; in contrast, both KC and LSEC would need to acquire the Ag through cross-presentation. The mechanism of cross-presentation has been clarified recently; it involves the transfer of Ags as peptides in the presence of molecular chaperones (50). To envisage cross-presentation by either KC or LSEC, we would have to invoke a similar mechanism that acts in these cells but not in circulating APC precursors such as DC. Of these two cell types, KC are not effective APC in vitro and are involved in many forms of liver tolerance (51–54). Isolated LSEC were shown to act as highly effective APC both in vitro and ex vivo (55), but the interpretation of the APC function of isolated LSEC is problematic due to the possibility that other cells copurified from the liver may account for some of their APC activity, and the expression of costimulatory ligands on the isolated cells is controversial (56, 57).

Although we cannot rule out KC or LSEC, our current view is that hepatocytes that have been transduced by AAV are the most likely cells to present Ag in this system. Direct presentation could occur via the classical TAP-dependent MHC class I pathway. Ags encoded by AAV have been shown already to enter this pathway because TAP-2-deficient RMA-S cells infected with AAV-OVA could express OVA but could not stimulate an OVA-specific hybrid in contrast to RMA cells with their Ag-processing mechanisms intact (47). Cultured hepatocytes induce functional activation of naive CD8\(^+\) T cells in the absence of CD28 costimulation (6). However, an alternative costimulation pathway exists for these cells through their expression of ICAM-1. We are currently exploring the role of ICAM-1 in the AAV model because ICAM-1-dependent activation of LFA-1-positive CD8\(^+\) T cells has been documented in a tumor model (58). One difficulty with this interpretation is that, in culture, T cells stimulated by hepatocytes undergo premature apoptosis (6); however, we think that the in vitro results may reveal the deficient APC properties of hepatocytes isolated from their complex tissue milieu. Although we have not yet evaluated the long-term fate of OT-1 T cells in mice transduced with AAV-OVA, the sustained response we see over a 7-day period suggests that the T cells are not prematurely dying. In summary, our study refutes the simple model that local CD8\(^+\) T cell activation in the liver leads to T cell inactivation and premature apoptosis.

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Disclosures

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