Induction of Tolerance in CD8+ T Cells to a Transgenic Autoantigen Expressed in the Liver Does Not Require Cross-Presentation

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The clinical responses observed following infusions of T cells into cancer patients (1, 2) have made strategies to augment host T cell responses to tumor Ags with the goal of promoting tumor eradication an increasingly intriguing approach for the treatment of human malignancies (3). However, many obstacles must be overcome to achieve therapeutic success by enhancing T cell responses, as evidenced by the limited efficacy observed in the many human cancer vaccine trials. One major obstacle is the nature of the Ags being targeted—most candidate tumor Ags are normal self-proteins overexpressed by malignant cells, often enhancing oncogenicity by improving survival and proliferative capacity, but the protein can also be detected at lower levels in normal cells. Thus, tumor immunity can be the “flip side” of autoimmunity and generating therapeutic T cell responses to tumor Ags can require overcoming the mechanisms operative to prevent autoimmune injury.

Many T cells, potentially reactive with self-proteins are deleted in the thymus, but some escape because the TCR is of too low affinity to trigger negative selection or the self-Ag is not sufficiently presented in the thymus (4, 5). Therefore, peripheral mechanisms must be operative to delete and/or anergize such self-reactive T cells, and these mechanisms will likely also limit potentially therapeutic responses to tumors.

Several murine models, developed to investigate peripheral CD8\(^+\) T cell tolerance to self-Ags, have highlighted the role of nonactivated, tolerizing APCs in tolerance induction. Studies in chimeric mice in which the male HY Ag was only expressed in male bone marrow (BM)\(^3\) cells demonstrated that persistence of BM-derived APCs has also been demonstrated when the Ag is not directly expressed in APCs. In transgenic (Tg) mice expressing a transgene in pancreatic \(\beta\) cells, BM-derived APCs acquired and cross-presented the Ag to naive Ag-specific CD8\(^+\) T cells in the draining lymph nodes (LN), but not in the pancreas where the transgene is expressed, and this cross-presentation induced a proliferative response that was soon followed by deletion, a phenomenon called cross-tolerance (8–10). However, the level of Ag expression appeared critical, as CD8\(^+\) T cells were not tolerized in Tg mice expressing low levels of the Ag in the pancreas but rather ignored the Ag unless stimulated by fully activated APCs presenting the Ag in vivo— the responding T cells then induced autoimmune pancreatic injury, suggesting expression in parenchymal tissues of levels of Ag sufficient for T cell recognition may be ignored by naive T cells (11).

It remains uncertain whether the rules for tolerance defined with small and specialized organs such as the pancreas apply to all peripheral tissues and tumors. The liver is distinct from other lymphoid and nonlymphoid organs by its unique blood flow, with the majority of blood flow derived from the venous circulation slowly percolating through narrow hepatic sinusoids discontinuously lined with endothelial cells, which can allow naive T cells trafficking through the sinusoids to directly contact parenchymal liver cells (12, 13). Tolerance to Ags expressed by parenchymal cells in

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3 Abbreviations used in this paper: BM, bone marrow; Tg, transgenic; LN, lymph node; LCMV, lymphocytic choriomeningitis virus; LSEC, liver sinusoidal endothelial cell; FMuLV, Friend murine leukemia virus; ALT, alanine aminotransferase; PD, programmed death.

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the liver has been studied in several models. Using mice expressing an allogenic MHC class I Ag (H-2Kb) in the liver but not professional APC as a self-Ag under control of the metallothionein or albumin promoter (12, 14, 15), the encounter of Kb-specific CD8 T cells with liver cells resulted in partial clonal deletion and tolerance with down-regulation of TCR expression in the persisting cells, demonstrating that peripheral T cells do contact liver parenchymal cells and that direct presentation of Ag by liver cells can lead to deletion or peripheral tolerance. However, the use of a class I allele as the “self-Ag,” which is more abundantly expressed than a processed epitope, and of T cells with a high affinity allospecific TCR, makes generalization to responses to more classical self-proteins uncertain. Indeed in Tg mice expressing gp33 from lymphocytic choriomeningitis virus (LCMV) in the liver (16), adoptively transferred gp33-specific CD8 T cells ignored the gp33 Ag in the liver, and this ignorance could be abrogated by virus infection, demonstrating that the ignorance did not reflect absence of Ag expression. Such ignorance has suggested that cross-presentation by APCs in the liver may be essential for tolerance to liver Ags. Liver sinusoidal endothelial cells (LSECs) appear to have specialized functions and have been shown to cross-present exogenous soluble Ags or Ags derived from apoptotic cells, resulting in a proliferative response by naive CD8 T cells that results in loss of the ability to produce cytokines (IL-2 and IFN-γ) and tolerance (17, 18). Cross-presentation by LSECs appears to play a critical role in oral tolerance (19), but its role in tolerance to processed liver Ags remains to be determined.

To examine CD8 T cell tolerance to self-proteins expressed in the liver that represent candidate tumor Ags, we previously developed a Tg mouse model in which a potential target tumor Ag, the Friend murine leukemia virus (FMuLV)-Gag protein, is expressed in hepatocytes under control of the albumin promoter (Alb:Gag-Tg mice) (20). We also created a second Tg mouse strain, TCRGag-Tg mice with CD8 T cells specific for the immunodominant F MuLV-Gag epitope, and crossed these two strains to create double-Tg mice (TCR Gag × Alb:Gag). These mice have Gag-specific CD8 T cells in the periphery, but the cells are tolerant, failing to proliferate or produce IL-2 in response to stimulation with Gag-Ag. However, analysis of tolerance mechanisms in this model revealed promiscuous expression of the Gag transgene in the thymus of Alb:Gag-Tg mice, with deletion of many Gag-specific CD8 T cells during thymic development as shown for many “peripheral” Ags (21), making it unclear if the observed tolerance was induced in the periphery or during thymic development. Therefore, we have now used an adoptive transfer system to investigate if and where in the periphery tolerance is induced. We report that expression of a transgenic Ag in hepatocytes as a self-Ag is sufficient to induce tolerance and deletion of autoreactive CD8 T cells, as has been observed in other tolerance models. However, these events do not require cross-presentation by professional APCs or result from cross-presentation by LSECs, but rather can occur directly by presentation of Ag by hepatocytes. The results imply there must be settings in which naive CD8 T cells can be tolerized in peripheral organs and perhaps tumors by direct encounter with target cells.

Materials and Methods

Mice
All mice used were 6–8 wk old, maintained under specific pathogen-free conditions in our animal facility, and studied under a protocol approved by the Department of Comparative Medicine’s Animal Care Committee. Alb:Gag-Tg and TCR Gag-Tg mice have been previously described (20, 21). TCR Gag-Tg mice were crossed with Thy1.1/+/C57BL/6 (B6) mice for two generations with phenotypic selection to achieve homozygosity for Thy1.1. B6 mice and BALB/c mice were purchased from The Jackson Laboratory.

Cell lines, media, and peptides
FBL, a Friend virus-induced erythroleukemia of B6 (H-2b) origin, expresses FMuLV Eμ- and FMuLV Gag-encoded products and H-2b class I molecules but not class II molecules. The immunodominant H-2Dd-restricted FMuLV Gag epitope in FBL in B6 mice CCLCLTVFBL has been described previously (22) and a modified more soluble peptide with the three cysteine residues replaced with amino-butyric acids that mimics the immunogenicity was synthesized by SynPep (23). LCMV-gp33 peptide KAVYNFATM was also purchased from SynPep. All cell cultures were performed in RPMI 1640 supplemented with antibiotics, 2-ME, and 10% FBS.

Adoptive transfer and immunization
Recipient B6 or Alb:Gag-Tg mice were injected i.v. with 2 × 106 purified naive Gag-specific CD8 Thy1.1+ T cells from splenocytes of Thy1.1 TCR Gag-Tg mice. At 16 days after cell transfer, selected mice were immunized i.p. with 2 × 107 irradiated FBL (10,000 rad). In some experiments, the purified naive TCR Gag-CD8 Thy1.1+ T cells were labeled with 1 μg/ml CFSE (Invitrogen Life Technologies) for 30 min at 37°C before injection.

Isolation of lymphocytes from liver
Hepatic lymphocytes were isolated as previously described (12). Briefly, liver was first perfused by injecting PBS containing heparin (10 U/ml), mincing, and then gentle pressing through a stainless steel mesh. The cells were centrifuged at 1200 rpm for 10 min and the pellet was resuspended in Percoll solution (36% isotonic Percoll in PBS). The suspension was centrifuged at 2000 rpm for 20 min to remove liver parenchymal cells. The pellet was then treated with an RBC lysis solution and washed with PBS three times before flow cytometric analysis.

Isolation of Kupffer cells and LSECs
Liver nonparenchymal cells containing Kupffer cells and LSECs were separated from whole liver cells by low-speed centrifugation (24). To isolate the Kupffer cells, liver nonparenchymal cells were preincubated with FcyR-blocking Ab, then incubated with anti-mouse CD11b and CD54 mAbs, and sorted for CD11b/CD54+ cells with a FACS Vantage (BD Biosciences). To isolate LSECs, the cells were preincubated with FcyR-blocking Ab, then incubated with anti-mouse CD105 and CD54 mAbs, and sorted for CD105+ CD54+ cells with a FACS Vantage (BD Biosciences).

Cell culture
For in vitro proliferation assays, 2 × 106 splenocytes from recipient mice injected with naive TCR Gag-CD8 Thy1.1+ T cells were labeled with CFSE for 30 min at 37°C, incubated for 4 days at 37°C in 96-well plates in a total volume of 200 μl of medium with 2 μg/ml Gag or gp33 peptide, or with medium alone, and CFSE dilution of Thy1.1+ cells was assessed by flow cytometry. In some experiments, 1 × 106 splenocytes from chimeric recipients injected with naive TCR Gag-CD8 Thy1.1+ T cells were labeled with CFSE for 30 min at 37°C, incubated for 4 days at 37°C in 96-well plates in a total volume of 200 μl of medium with 1 × 106 B6 splenocytes and 2 μg/ml Gag or gp33 peptide, or with medium alone, and CFSE dilution of Thy1.1+ cells was assessed by flow cytometry. To assess Ag presentation by LSECs, freshly isolated LSECs were either used directly or pulsed with 2 μg/ml Gag peptide for 3 h at 37°C and extensively washed, and then 3 × 105 LSECs were cocultured with CFSE-labeled 1.5 × 105 naive TCR Gag-CD8 Thy1.1+ T cells for 4 days at 37°C in collagen-coated 24-well plates, with CFSE dilution of Thy1.1+ cells assessed by flow cytometry. In some experiments, isolated Kupffer cells were used either directly or pulsed with 2 μg/ml Gag peptide for 1 h at 37°C and extensively washed, and then 4 × 104 Kupffer cells were cocultured with CFSE-labeled 4 × 104 naive TCR Gag-CD8 Thy1.1+ T cells for 3 days at 37°C in 96-well plates, with CFSE dilution of Thy1.1+ cells assessed by flow cytometry. Ag presentation by LSECs and Kupffer cells was also assessed by measuring CD107a expression on FMuLV Eμ-specific effector CTLs from degranulation. Freshly isolated LSECs used either directly or pulsed with Gag peptide for 3 h at 37°C were washed, and 3 × 105 LSECs were then cultured with 1 × 106 FMuLV Eμ-specific CTLs for 5.5 h at 37°C in collagen-coated 24-well plates. Freshly isolated Kupffer cells used either directly or pulsed with Gag peptide for 1 h at 37°C were washed and 4 × 105 LSECs were then cultured with 2 × 105 FMuLV Eμ-specific CTLs for 5.5 h at 37°C in 96-well plates. FMuLV Eμ-specific CTLs were generated in vitro as previously described (21) and effector CTLs were used 7 days after in vitro stimulation.
Recipient mice were lethally irradiated (1000 rad) and then injected i.v. with 5 \times 10^6 BM cells depleted of CD4 T cells and CD8 T cells using Dynal magnetic beads according to the manufacturer’s protocol. For infection prophylaxis, 1 mg/ml sulfadoxin and 0.2 mg/ml trimethoprim was added to the drinking water during the first 2 wk. Chimeras were used in experiments 6 mo after transplantation. Before use in experiments, peripheral blood from all chimeras, and spleen and liver from one mouse from each chimeric group, were examined by flow cytometry to verify complete donor chimerism.

**Flow cytometric analysis**

Anti-mouse CD8 (53-6.7), Thy1.1 (OX-7), CD44 (IM7), CD25 (PC61), CD69 (H1.2F3), CD54 (3E2), CD11b (M1/70), CD11c (HL3), CD107a (1D11B), Vα6.5 (RR3-16), Vβ12 (MR11-1), H-2D^d (K1B5), and H-2D^d (34-212) were all commercially obtained (BD Pharmingen). Anti-mouse CD105 (MJ7/18) was obtained from eBioscience. Cells were evaluated with a FACScan flow cytometer (BD Biosciences) and analyzed with CellQuest software (BD Biosciences).

**Analysis of liver injury**

Blood was obtained by retro-orbital eye bleeding at noted time points after transfer of naive TCRGagCD8 T cells and serum alanine aminotransferase (ALT) levels were measured with a standard clinical automated analyzer.

**Statistical analysis**

Statistical comparison between groups was performed with a two-tailed unpaired Student’s t test. Data were considered statistically significant when the p value was <0.05.

**Results**

The majority of naive Gag-specific CD8^+ T cells encountering Gag as a self-Ag in the periphery of Alb:Gag-Tg mice are deleted

The outcome for naive T cells that initially encounter the Gag Ag as a self-protein in the periphery was assessed by transferring naive TCRGagCD8^+Thy1.1 T cells that developed in normal TCRgag-Tg mice into Alb:Gag-Tg mice. At 4 and 16 days after cell transfer, the spleen, liver, hepatic LN as a draining LN, and inguinal LN as a nondraining LN, were obtained, and the presence of transferred TCRgagCD8^+Thy1.1 ^+ T cells assessed. On day 4, TCRgagCD8^+ T cells were detectable in the spleen, liver, hepatic LN, and the inguinal LN of Alb:Gag-Tg recipients, but the proportion of TCRgagCD8^+ T cells among the total CD8^+ T cells and lymphocytes in the spleen, liver, and hepatic LN was decreased ~2-fold compared with cells recovered following transfer into normal B6 recipients not expressing Gag (Fig. 1A). At 16 days after transfer, TCRgagCD8^+ T cells persisted in B6 recipients, but only small numbers could be recovered from Alb:Gag-Tg recipients (Fig. 1A). The TCRgagCD8^+ T cells persisting in Alb:Gag-Tg recipients, in distinction to B6 recipients, were almost entirely CD44^high (Fig. 1B), consistent with having encountered Ag in vivo. Thus, the predominant fate of naive Gag-specific CD8^+ T cells that encounter Gag-Ag for the first time as a self-protein in the periphery appears to be deletion. This fate was increasingly evident at 14 wk after cell transfer, at which time it was difficult to recover TCRgagCD8^+ T cells from liver or lymphoid tissues in Alb:Gag-Tg recipients, although the cells remained readily detectable in B6 recipients (Fig. 1C).

**Initial Ag encounter in the periphery induces proliferation and activation in naive Gag-specific CD8^+ T cells without causing hepatocellular damage**

Studies in other models of peripheral tolerance have also demonstrated that the initial exposure to self-Ag in the periphery induces a proliferative response that expands the reactive cells but then is soon followed by deletion. This Ag-induced proliferation generally

**FIGURE 1.** The predominant fate of Gag-specific CD8^+ T cells following encounter with Gag Ag in the periphery is clonal deletion. A, B6 and Alb: Gag-Tg recipients were injected with 2 \times 10^6 naive Gag-specific CD8^+Thy1.1 T cells i.v. At 4 and 16 days after cell transfer, the spleen, liver, hepatic LN, and the inguinal LN were removed and percentage of Gag-specific CD8^+Thy1.1 ^+ T cells within the total CD8^+ T cells (upper) and within the total lymphocytes (lower) analyzed by flow cytometry (n = 4/group). Data are presented as the mean ± SEM. Numbers represent the average percentages. B, At 16 days after cell transfer, the spleen, liver, hepatic LN, and the inguinal LN were removed and CD44 expression of Gag-specific CD8^+Thy1.1 ^+ T cells was determined by flow cytometry. Plots are gated on CD8^+Thy1.1 ^+ T cells. Numbers represent the percentage of CD44^high cells within the Gag-specific CD8^+Thy1.1 ^+ T cell populations. Data are representative of three independent experiments. C, At 14 wk after cell transfer, the spleen, liver, hepatic LN, and the inguinal LN were removed and the presence of Gag-specific CD8^+Thy1.1 ^+ T cells analyzed by flow cytometry. Numbers represent the percentage of Gag-specific CD8^+Thy1.1 ^+ T cells within the total CD8^+ T cell populations. Data are representative of three independent experiments.
does not occur in the tissue expressing the Ag, but rather in the LN draining the organ as a result of stimulation by quiescent BM-derived professional APCs cross-presenting the Ag in a tolerizing context (8–10, 25). The results with “self-Ags” expressed in the liver have been less clear, with liver cells appearing capable of direct presentation if expressing an allo-class I molecule or following in vivo peptide-pulsing (12, 26), whereas a self-protein requiring classical processing and presentation was ignored (16).

As our data clearly indicated that the Gag-Ag was not being ignored, we assessed proliferation in distinct sites by labeling naive TCR\textsubscript{Gag}CD\textsuperscript{8}\textsubscript{+}/H11001 Thy1.1\textsubscript{+}/H11001 T cells with CFSE, transferring the cells into Alb:Gag-Tg recipients, and sacrificing the mice 4 days later. More than 80% of TCR\textsubscript{Gag}CD\textsuperscript{8}\textsubscript{+}/H11001 T cells had divided many times in Alb:Gag-Tg recipients by 4 days, and cells that had divided extensively were detectable not only in the draining hepatic LN but also in the spleen, nondraining inguinal LN, and the liver (Fig. 2A).

In contrast, CFSE-labeled naive TCR\textsubscript{Gag}CD\textsuperscript{8}\textsubscript{+}/H11001 T cells did not divide after transfer into B6 recipients (Fig. 2A), affirming that cell division in Alb:Gag-Tg recipients was triggered by Ag recognition.

At 4 days after transfer, the TCR\textsubscript{Gag}CD\textsuperscript{8}\textsubscript{+}/H11001 T cells recovered from the spleen, liver, hepatic LN, and the inguinal LN of B6 recipients retained their naive phenotype of CD44\textsuperscript{low}, CD69\textsuperscript{low}, and CD25\textsuperscript{low} (Fig. 2B). TCR\textsubscript{Gag}CD\textsuperscript{8}\textsubscript{+} T cells recovered from the spleen, hepatic LN, and the inguinal LN of Alb:Gag-Tg recipients were CD44\textsuperscript{high}, consistent with having encountered Ag, but did not exhibit up-regulation of CD69 or CD25 (Fig. 2B), suggesting this encounter might have been brief or not completely productive. The TCR\textsubscript{Gag}CD\textsuperscript{8}\textsubscript{+} T cells isolated from the liver of Alb:Gag-Tg recipients were also CD44\textsuperscript{high}, CD69\textsuperscript{low} but showed a significant shift in CD25 expression, with >40% clearly positive (Fig. 2B), suggesting distinct activation events might be occurring in the liver. Despite the large numbers of proliferating and activated T cells in the liver, there was no evidence of hepatocellular damage in Alb:Gag-Tg recipients after transfer of naive TCR\textsubscript{Gag}CD\textsuperscript{8}\textsubscript{+} T cells, as reflected by no increase in ALT levels (Fig. 3).

Anergy is induced in surviving Gag-specific CD\textsuperscript{8}\textsuperscript{+} T cells that have encountered Gag in the periphery of Alb:Gag-Tg mice

Peripheral deletion of potentially autoreactive T cells in other autoimmunity/tolerance models appears either incomplete or requires an extended time to complete, requiring additional mechanisms to be operative to assure tolerization of the remaining T cells specific for self-Ags (6–8, 27). The failure of the TCR\textsubscript{Gag}CD\textsuperscript{8}\textsubscript{+} T cells remaining after the initial Ag encounter to induce liver injury suggests such tolerizing mechanisms might be operative in Alb:
Gag-Tg mice. Down-regulation of expression of TCRs and the accessory CD8 molecules following encounter with self-Ags has been demonstrated to reduce responsiveness in other models (14, 28, 29). Therefore, we examined TCR<sub>Gag</sub>CD8<sup>+</sup> T cells following encounter with their self-Ag. At 4 days after transfer into Alb:Gag-Tg and B6 recipients, Thy1.1 cells from the spleen and the liver were stained with Vα3, Vβ12, and CD8. Reduced expression of both TCR chains and CD8 molecules was detected on TCR<sub>Gag</sub>CD8<sup>+</sup> T cells recovered from the liver of Alb:Gag-Tg recipients, but was not different from controls in cells recovered from the spleen (Fig. 4).

We previously reported that the Gag-specific CD8<sup>+</sup> T cells persisting in the periphery of TCR<sub>Gag</sub> × Alb:Gag mice are anergic, failing to proliferate or produce IL-2 in response to stimulation with FBL or the Gag-peptide in vitro, but still retain effector func-

tion—the ability of FMuLVGag-specific effector CTLs to expel lytic tumor and assessing the proportion of TCR<sub>Gag</sub>CD8<sup>+</sup> T cells in the spleen, liver, hepatic LN, and the inguinal LN at day 16 and at 3 days after immunization. The persisting TCR<sub>Gag</sub>CD8<sup>+</sup> T cells in the spleen, liver, hepatic LN, and the inguinal LN of B6 recipients demonstrated a robust proliferative response to FBL (Fig. 5B), whereas TCR<sub>Gag</sub>CD8<sup>+</sup> T cells in Alb:Gag-Tg recipients failed to expand significantly at any site analyzed (Fig. 5B). Thus, shortly following the proliferative response induced by a first encounter with the Gag-Ag in the periphery, Gag-specific CD8<sup>+</sup> T cells acquire a proliferative block, losing the capacity to continue to expand in response to Ag, even if the Ag is presented in a more immunogenic context.

**Initial activation of Gag-specific CD8<sup>+</sup> T cells is induced by hepatocytes**

The distinct CD25<sup>+</sup> phenotype of TCR<sub>Gag</sub>CD8<sup>+</sup> T cells residing in the liver of Alb:Gag-Tg recipients suggests that Ag recognition could be occurring in the liver. To address whether activated TCR<sub>Gag</sub>CD8<sup>+</sup> T cells detected within the liver of Alb:Gag-Tg recipients had previously been activated in the draining hepatic LN and then recirculated to the liver, the expression of CD69 by TCR<sub>Gag</sub>CD8<sup>+</sup> T cells was analyzed at 1.5 h posttransfer (Fig. 6Aa). Up-regulation of CD69 expression by TCR<sub>Gag</sub>CD8<sup>+</sup> T cells was already evident in cells in the liver, but not in the spleen, hepatic LN, inguinal LN, or blood of Alb:Gag-Tg recipients. This activation in the liver was Ag specific because CD69 up-regulation was not detected in B6 recipients. Moreover, TCR<sub>Gag</sub>CD8<sup>+</sup> T cells were specifically retained in the liver of Alb:Gag-Tg recipients at 1.5 h posttransfer, as reflected by the higher proportion of TCR<sub>Gag</sub>CD8<sup>+</sup> T cells in the total CD8<sup>+</sup> T cells in the liver of Alb:Gag-Tg recipients compared with the liver of B6 recipients. The proportion of TCR<sub>Gag</sub>CD8<sup>+</sup> T cells in the total CD8<sup>+</sup> T cells in the spleen was similar between B6 and Alb:Gag-Tg recipients, but the proportion of TCR<sub>Gag</sub>CD8<sup>+</sup> T cells in total CD8<sup>+</sup> T cells in the hepatic and inguinal LN of Alb:Gag-Tg recipients was lower than in B6 recipients, presumably due to the selective retention of the cells in the liver (Fig. 6Ab). These data suggest that the liver is a site of primary activation for naive TCR<sub>Gag</sub>CD8<sup>+</sup> T cells. To determine whether LSECs cross-presenting the hepatic Ag might be the cells responsible for triggering the response of naive TCR<sub>Gag</sub>CD8<sup>+</sup> T cells in the liver, LSECs from Alb:Gag-Tg mice were purified for use as in vitro stimulators of CFSE-labeled naive TCR<sub>Gag</sub>CD8<sup>+</sup>Thy1.1<sup>+</sup> T cells, with CFSE dilution analyzed 4 days later. CD105 (endoglin) is a protein highly expressed by LSECs commonly used for LSEC isolation (30, 31) and the CD105<sup>+</sup> cells purified from liver nonparenchymal cells and used for stimulation increased CD54, CD11b, and CD86, but not CD11c (Fig. 6B), consistent with the reported phenotype of LSECs (30, 32). When naive TCR<sub>Gag</sub>CD8<sup>+</sup> T cells were cultured with freshly isolated LSECs from B6 mice, no proliferating cells were detected (Fig. 6C), whereas the majority of cells proliferated if the LSECs from B6 mice were first pulsed with the Gag peptide (Fig. 6C). However, LSECs isolated from Alb:Gag-Tg mice also failed to stimulate TCR<sub>Gag</sub>CD8<sup>+</sup> T cells (Fig. 6C), suggesting insufficient cross-presentation to induce a response. The potential for cross-presentation by LSECs from Alb:Gag-Tg mice was further examined using a more sensitive assay for T cell recogni-

**FIGURE 4.** Down-regulation of TCR and CD8 expression on Gag-specific CD8<sup>+</sup> T cells isolated from the liver of Alb:Gag-Tg mice. B6 and Alb:Gag-Tg recipients were injected with 2 × 10<sup>6</sup> naive Gag-specific CD8<sup>+</sup>Thy1.1<sup>+</sup> cells. At 4 days after cell transfer, spleens and livers were removed and expression of TCR (Vα3 and Vβ12) and CD8 molecules on Thy1.1<sup>+</sup> cells was analyzed by flow cytometry. Shaded histograms represent unstained control. All histograms are gated on Thy1.1<sup>+</sup> cells.
activation of primed CD8<sup>+</sup> T effector cells than other functions such as production of cytokines (34). Degranulation by FMuLV<sub>Gag</sub>-specific effector CTLs was evident following culture with LSECs from B6 mice that were pulsed with Gag peptide but not with LSECs not pulsed with peptide. Such degranulation of effector CTLs was detectable following culture with LSECs from B6 mice pulsed with a low concentration of Gag-peptide (20 ng/ml), a peptide concentration incapable of inducing proliferation of naive Gag-specific CD8<sup>+</sup> T cells even if pulsed on professional APCs (Fig. 6E). In contrast, CTLs cultured with LSECs from Alb:Gag-Tg mice failed to degranulate, suggesting that in this tolerance model cross-presentation by LSECs is not significantly contributing to activation of TCR<sub>Gag</sub>CD8<sup>+</sup> T cells.

These results suggested that hepatocytes might be capable of primary presentation of processed Ag, as has been observed for allogeneic T cells with class I molecules (12, 35). However, alternative BM-derived APC, such as Kupffer cells, might also be capable of cross-presenting the Ag in vivo. To address this issue, BM chimeric mice were established by injecting T cell-depleted BALB/c BM cells into lethally irradiated (1000 rad) Alb:Gag-Tg recipients (BALB/c→Alb:Gag-Tg). In these chimeric mice, only hepatocytes should be able to present the Gag-Ag to H-2<sup>b</sup>-restricted TCR<sub>Gag</sub>CD8<sup>+</sup> T cells, because all BM-derived APCs express only H-2<sup>d</sup> molecules. At 6 mo after hemopoietic transplant to assure full reconstitution, one mouse from each group of experimental BALB/c→B6 or BALB/c→Alb:Gag-Tg and control B6→Alb:Gag-Tg chimeras was sacrificed, and splenocytes and Kupffer cells (CD11b<sup>+</sup>CD54<sup>+</sup> nonparenchymal liver cells) were analyzed to confirm donor chimerism. The irradiated groups that received BALB/c BM cells demonstrated conversion of splenocytes and Kupffer cells to H-2<sup>d</sup>, although a small population of Kupffer cells expressing H-2<sup>b</sup> was still evident compared with unmanipulated BALB/c mice, and the group that received B6 BM cells remained H-2<sup>b</sup>-restricted TCR<sub>Gag</sub>CD8<sup>+</sup> T cells, because all BM-derived APCs express only H-2<sup>d</sup> molecules. At 6 mo after hemopoietic transplant to assure full reconstitution, one mouse from each group of experimental BALB/c→B6 or BALB/c→Alb:Gag-Tg and control B6→Alb:Gag-Tg chimeras was sacrificed, and splenocytes and Kupffer cells (CD11b<sup>+</sup>CD54<sup>+</sup> nonparenchymal liver cells) were analyzed to confirm donor chimerism. The irradiated groups that received BALB/c BM cells demonstrated conversion of splenocytes and Kupffer cells to H-2<sup>d</sup>, although a small population of Kupffer cells expressing H-2<sup>b</sup> was still evident compared with unmanipulated BALB/c mice, and the group that received B6 BM cells remained H-2<sup>b</sup>-restricted TCR<sub>Gag</sub>CD8<sup>+</sup> T cells, because all BM-derived APCs express only H-2<sup>d</sup> molecules.

FIGURE 5. Surviving Gag-specific CD8<sup>+</sup> T cells fail to respond to Gag Ag stimulation. A, At 4 and 16 days after cell transfer, splenocytes were labeled with CFSE and then stimulated with Gag or irrelevant gp33 peptide, or medium alone in vitro. After 4 days, CFSE dilution in Thy1.1<sup>+</sup> cells was analyzed by flow cytometry. Numbers represent the percentage of dividing cells within the Gag-specific CD8<sup>+</sup> Thy1.1<sup>+</sup> T cell populations. Data are representative of three independent experiments with two mice per group. B, At 16 days after cell transfer, some mice were sacrificed to analyze the proportion of Gag-specific CD8<sup>+</sup> Thy1.1<sup>+</sup> T cells, and some mice were immunized with 1×10<sup>7</sup> irradiated FBL. At 3 days after this immunization, the spleen, liver, hepatic LN, and the inguinal LN were removed and the proportion of Gag-specific CD8<sup>+</sup> Thy1.1<sup>+</sup> T cells analyzed by flow cytometry. Numbers represent the percentage of Gag-specific CD8<sup>+</sup> Thy1.1<sup>+</sup> cells within the total CD8<sup>+</sup> T cell populations. Data are representative of three independent experiments.
of Gag Ag. However, these cells were capable of presenting Ag and inducing proliferation, as revealed if the Kupffer cells were pulsed with Gag peptide before being used to stimulate naive TCRGagCD8^+/H11001 T cells (Fig. 7D). In contrast, Kupffer cells isolated from Alb:Gag-Tg mice did not induce TCRGagCD8^+/H11001 T cells to proliferate (Fig. 7D). Moreover, Kupffer cells from Alb:Gag-Tg mice were unable to induce degranulation by FMuLVGag-specific effector CTLs (Fig. 7E), suggesting that the Kupffer cells were not likely responsible for cross-presentation of the Ag in vivo.

The recovery and distribution of proliferated TCRGagCD8^+ T cells in secondary lymphoid organs was also similar in B6→Alb:Gag-Tg and BALB/c→Alb:Gag-Tg chimeras (Fig. 7C), despite the absence of professional APCs that can present Ag in these organs in BALB/c→Alb:Gag-Tg chimeric mice, suggesting that the T cells induced to respond in the liver can rapidly migrate to other sites following Ag encounter with hepatocytes. This presumably reflects the unique anatomy and blood flow in the liver as compared with a secondary lymphoid organ, with the T cells encountering
hepatocyte-presented Ags through fenestrations in the sinusoids but still remaining in the peripheral circulation (12, 13, 36).

Gag-specific CD8⁺ T cell tolerance is induced by hepatocytes

The ability of hepatocytes to directly present Ag and induce proliferation of naive CD8⁺ T cells would be problematic if, unlike presentation by nonactivated cross-presenting APCs, this led to a productive rather than tolerized response. Therefore, we evaluated the persisting TCRGag⁺CD8⁺ T cells in the chimeric recipients at 16 days after transfer. In BALB/c→Alb:Gag-Tg chimeras, the proportion of TCRGag⁺CD8⁺ T cells recovered from the spleen, liver, hepatic LN, and the inguinal LN was

FIGURE 7. Hepatocytes induce a proliferative response in naive CD8⁺ T cells. Six months after reconstitution, splenocytes (A) and Kupffer cells (CD11b⁺CD54⁻ nonparenchymal liver cells) (B) were stained for the expression of H-2b and H-2d to confirm donor chimerism. C, BM chimeric mice were injected with 2 × 10⁶ CFSE-labeled naive Gag-specific CD8⁺ Thy1.1⁺ T cells i.v. At 4 days after cell transfer, the spleen, liver, hepatic LN, and inguinal LN were removed and CFSE dilution in Thy1.1⁺ T cells analyzed by flow cytometry. Data are representative of two independent experiments. D, CFSE-labeled naive Gag-specific CD8⁺ Thy1.1⁺ T cells were cultured with freshly isolated Kupffer cells from B6 or Alb:Gag-Tg mice or Gag peptide (2 μg/ml) pulsed Kupffer cells from B6 mice. After 3 days, CFSE dilution of Thy1.1⁺ cells was analyzed by flow cytometry. Plots are gated on Thy1.1⁺ cells. Numbers represent the percentage of dividing cells within the Thy1.1⁺ cells. Data are representative of three independent experiments. E, FMuLVGag-specific Thy1.1⁺ in vitro-generated effector CTLs were cultured with freshly isolated Kupffer cells from B6 or Alb:Gag-Tg mice or Gag-peptide pulsed Kupffer cells from B6 mice at the noted concentrations. After 5.5 h, CD107a expression on Thy1.1⁺ cells was analyzed by flow cytometry. Plots are gated on Thy1.1⁺ cells. Numbers represent the percentage of CD107a-positive cells within the Thy1.1⁺ population. Data are representative of two independent experiments.
Thy1.1 BM cells were labeled with CFSE and stimulated with B6 splenocytes after cell transfer, splenocytes from chimeric mice that received BALB/c Data are representative of two independent experiments.

Hepatocytes induce clonal deletion and anergy in responding CD8+ T cells. A, BM chimeric mice were injected with 2 × 10⁶ naive Gag-specific CD8+Thy1.1+ T cells i.v. At 16 days after cell transfer, the spleen, liver, hepatic LN, and inguinal LN were removed and percentage of Gag-specific CD8+Thy1.1+ T cells within the total CD8+ T cells (left) and total lymphocytes (right) were analyzed by flow cytometry. Data are representative of two independent experiments. Numbers represent the averages of percentage. B, At 16 days after cell transfer, the spleen, liver, hepatic LN, and inguinal LN were removed and CD44 expression of Gag-specific CD8+Thy1.1+ T cells was determined by flow cytometry. Plots are gated on CD8+Thy1.1+ T cells. Numbers represent the percentage of CD44high cells within the Gag-specific CD8+Thy1.1+ T cell populations. Data are representative of two independent experiments. C, At 16 days after cell transfer, splenocytes from chimeric mice that received BALB/c BM cells were labeled with CFSE and stimulated with B6 splenocytes pulsed with Gag or gp33 peptide in vitro. After 4 days, CFSE dilution in Thy1.1+ cells was analyzed by flow cytometry. Data are representative of two independent experiments.

A fraction of transferred TCRGagCD8+ T cells did remain detectable in the liver and secondary lymphoid tissues at 16 days after transfer, providing an opportunity to determine whether primary stimulation by hepatocytes programs these cells for subsequent responses or anergy. Therefore, cells recovered from chimeric recipients at 16 days after cell transfer were labeled with CFSE and stimulated with Gag peptide. The persisting TCRGagCD8+ T cells from BALB/c→B6 chimeras proliferated in response to stimulation with Gag peptide, but not irrelevant gp33 peptide (Fig. 8C). The cells recovered from BALB/c→Alb:Gag-Tg chimeras showed no evidence of a proliferating population, and, as previously observed, most cells failed to survive in the in vitro environment (Fig. 8C). Thus, parenchymal liver cells expressing a self-protein are capable of directly presenting Ag to naive CD8+ T cells without a requirement for cross-presentation, but most of the T cells triggered by recognition of hepatocyte-presented self-Ag are rapidly deleted and the remaining cells acquire a proliferative block that prevents further expansion in response to the Ag.

**Discussion**

Ag-specific tolerance of T cells reactive with candidate tumor Ags that are expressed at detectable levels in normal tissues represents one of major obstacles to the development of T cell immunotherapy for human cancers. Tolerance is also often observed to even unique Ags expressed by tumors and the mechanisms responsible for tolerance to such tumor Ags first encountered in the periphery by T cells that have completed thymic development can resemble those responsible for inducing/maintaining peripheral tolerance to “normal” self-Ags (37–39). Thus, understanding the events responsible for tolerance to self-Ags expressed in the periphery is not only important for mitigating autoimmunity, but for elucidating strategies for more effective immunotherapy. In the current study, we have examined the fate of naive CD8+ T cells, specific for a candidate tumor Ag that has been shown to be an effective therapeutic target (20), that first encounter the Ag as a normal self-Ag expressed by hepatocytes at lower levels than in the tumor. Our results demonstrate the potential importance of direct presentation of Ag by peripheral parenchymal tissues for inducing deletion and tolerization of such Ag-specific CD8+ T cells.

Shortly following adoptive transfer of naive Gag-specific CD8+ T cells into Alb:Gag-Tg recipients, dividing Gag-specific CD8+ T cells were detected both in secondary lymphoid tissues and the liver. This could reflect activation in the lymphoid tissues by BM-derived APCs cross-presenting Ag derived from hepatocytes followed by recirculation to the liver, which both expresses the Ag and can function as a nonspecific reservoir for activated cells (40, 41), and/or activation in the liver followed by systemic recirculation to secondary lymphoid tissues. Our data indicate that activated Gag-specific CD8+ T cells were not likely being activated in the lymphoid tissues and then recirculated to the liver, because CD69 up-regulation by Gag-specific CD8+ T cells was first observed in the liver rather than in the lymphoid tissues or blood. By contrast, the presence of proliferating cells in distant lymphoid organs would appear to reflect activation in the liver and export through the hepatic circulation, because APCs purified from the spleen...
and inguinal LN, as well as the draining hepatic LN, of Alb:Gag-Tg mice failed to present sufficient Ag to stimulate responses by TCRGagCD8+ T cells in vitro (data not shown). Naive CD8+ T cells have been demonstrated to be capable of directly interacting with the surface of hepatocytes without leaving the hepatic circulation through fenestrations in LSECs (13) and the cells responsible for the activation/tolerization of Gag-specific CD8+ T cells in our study appear to be hepatocytes. The Gag protein under the control of the albumin promoter has been shown to be preferentially expressed by hepatocytes and not other peripheral tissues (20) and neither LSECs nor Kupffer cells from Alb:Gag-Tg mice were capable of inducing either activation of naive Gag-specific CD8+ T cells or cross-presenting sufficient Ag to be recognized in a sensitive degranulation assay by effector CD8+ T cells.

A large fraction of Gag-specific CD8+ T cells recovered from the liver of Alb:Gag-Tg recipients exhibited up-regulated expression of the high-affinity IL-2R (CD25), which was not evident in cells isolated from lymphoid tissues, suggesting more profound and continuous TCR triggering is likely occurring directly in the liver. In settings in which cross-presentation by tolerizing BM-derived APCs is known to occur, the signal often appears too weak to induce detectable CD25 up-regulation (7, 8). Continuous TCR triggering in the liver is also suggested by the observed down-regulation of TCR and CD8 molecules by Gag-specific CD8+ T cells recovered from the liver, but not the spleen, of Alb:Gag-Tg recipients. If the liver is the only or dominant site of Ag recognition and activation, one issue is why activated cells were readily detected within a few days at distant sites. This may reflect the nature of the blood flow in the liver, which can permit T cells trafficking through sinusoids that have endothelial cells lacking tight junctions (12, 13) to be engaged by Ag presented on microvilli of hepatocytes penetrating the fenestrations, followed by rapid access to the systemic circulation. Additionally, the nature of the tolerizing activation event may promote egress from rather than retention in the liver. Although CD69 up-regulation by transferred TCRGagCD8+ T cells was detectable in the liver of Alb:Gag-Tg recipients at 1.5 h after transfer, these cells failed to sustain CD69 expression, because TCRGagCD8+ T cells recovered from the liver of Alb:Gag-Tg recipients at 2 days after transfer—a time when most cells have started diluting CFSE—no longer expressed CD69 (data not shown), potentially reflecting down-regulation as a consequence of subsequent divisions (8). Although the functional consequences of up-regulation CD69 are still being resolved, CD69 expression can decrease egress from lymphoid organs by negatively regulating the sphingosine 1-phosphate receptor-1 (42); thus, failure to sustain CD69 up-regulation may facilitate lymphocyte movement from the liver.

The fate of the majority of Gag-specific CD8+ T cells following triggering by hepatocytes was deletion. This presumably begins occurring shortly after triggering, because no accumulation of Gag-specific CD8+ T cells was detectable within the liver or at other sites at 4 days after adoptive transfer despite evidence of extensive proliferation, suggesting Gag-specific CD8+ T cells were both proliferating and dying. The deletion likely reflects characteristics of the interaction with hepatocytes as APCs. Hepatocytes do not express the costimulatory molecules CD80 and CD86 (43), which can deliver signals to T cells via CD28 to induce bcl-xL expression and promote survival (44). Analysis of in vitro responses of naive alloreactive CD8+ T cells stimulated by hepatocytes have also demonstrated an initial proliferative response, but poor induction of bcl-xL and IL-2, and the response was followed by premature T cell death. A critical role for the lack of CD28 signaling has been suggested, because cross-linking CD28 during stimulation with hepatocytes resulted in up-regulation of bcl-xL and production of IL-2, preventing the premature T cell death (35, 43).

It has been proposed that the liver can promote Ag-specific immunity as well as tolerance, as evidenced by effector CD8+ T cell responses to hepatocytes expressing hepatitis B virus Ags or Listeria monocytogenes Ags following infection. However, the reported responses to hepatocytes expressing these pathogen-derived Ags resulted from transfer of primed CD8+ T cells that had initially encountered Ag in a different context (45–47), and the site where primary T cell activation occurred may determine the balance between intrahepatic tolerance and immunity, with the liver inducing tolerance and professional APCs in lymphoid tissues potentially inducing immunity (48, 49).

We previously demonstrated that Gag-specific CD8+ T cells in the periphery of TCRGag × Alb:Gag mice, despite exhibiting tolerance as manifested by an inability to proliferate in response to Gag Ag, have acquired effector functions as a consequence of their experience with the self-Ag, and are capable of producing IFN-γ and killing Gag+ target cells without further immunization (21, 50). This appears similar to the reported effector functions acquired by naive CD8+ T cells that encounter hepatic Ags in a transplantation model, although the long-term fate of cells in that model is unclear (26). The presence of such effector functions in the peripheral Gag-specific CD8+ T cells in TCRGag × Alb:Gag mice might be anticipated to induce chronic liver injury, but no liver injury has been observed previously (21). Similarly, no evidence of liver damage was detected in the current study after adoptive transfer of large numbers of naive Gag-specific CD8+ T cells into Alb:Gag-Tg mice, despite evidence of activation, proliferation, and differentiation in the liver, including increased expression of granymes and acquisition of the ability to produce IFN-γ (data not shown). One potential mechanism proposed to protect hepatocytes from damage by effector CD8+ T cells has been signaling through the immunoinhibitory receptor—programmed death 1 (PD-1)—on the T cells (46), because the mRNAs for both PD-1 ligand 1 (PD-L1) and PD-1 ligand 2 (PD-L2), the two ligands for PD-1, have been detected in the liver (51, 52). This mechanism, recently shown to mitigate responses to chronic viral infections (53, 54), may also be operative in our model, as PD-1 expression was detectable on the proliferating Gag-specific CD8+ T cells isolated from the liver (data not shown), but the relevance of this finding requires further study.

It should be noted that the fate of potentially autoreactive CD8+ T cells observed in our model (tolerance) differs from what has been observed in a model with LCMV-gp33 as a transgene in the liver (ignorance). In the latter model, the gp33 Ag expressed in the liver failed to induce cell division, peripheral deletion, or unresponsiveness of transferred autoreactive P14 TCR-Tg CD8+ T cells. However, activation of these functional naive autoreactive CD8+ T cells by LCMV infection resulted in recognition of gp33 Ag in the liver and consequent liver injury (16), consistent with the ability of such T cells to sample hepatocyte Ags. Similar ignorance of a gp33 transgene in the pancreas but induction of injury to β islet cells following LCMV infection has been reported in RIP-gp33 Tg mice (11). In our model, the Gag Ag is sufficiently expressed in the liver to be recognized by naive Gag-specific CD8+ T cells, resulting in proliferation and tolerization. However, immunization of transferred naive T cells with irradiated Gag−FBL tumor to induce T cell effector function, or transfer of in vivo activated Gag-specific effector CTL (and 4 days of 10,000 U IL-2 supplementation in vivo) failed to induce liver injury (Ref. 20 and data not shown). Thus, for a particular autoreactive T cell and normal tissue target a combination of the affinity of the TCR, the Ag expression level, and the inflammatory state induced during Ag
exposure may determine the outcome of tolerance vs ignorance and the potential for target injury. In summary, our results demonstrate that a transgenic Ag expressed in hepatocytes as a self-Ag and endogenously processed and presented in the context of Class I molecules induces tolerance in autoreactive CD8+ T cells than immunity. The relative efficiencies of endogenous presentation and cross-presentation of an Ag is in part dependent on the nature of the protein (55) and natural characteristics of the Gag protein may be contributing to our ability to detect evidence for in vivo presentation by a parenchymal cell type. In part, this may be due to deletion and anergy induction. It seems likely that such tolerizing events in the periphery by parenchymal cells need not be entirely limited to hepatocytes, and that the operative mechanisms may well be co-opted by growing tumors with abnormal vasculature as a strategy to evade immune responses. Thus, future studies defining the responsible molecular and cellular events resulting from interactions with these nonprofessional APCs presenting an epitope from an endogenously processed protein may provide insights relevant to both autoimmunity and tumor immunity.

Disclosures

The authors have no financial conflict of interest.

References


