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Tissue-Specific Abundance of Regulatory T Cells Correlates with CD8+ T Cell Dysfunction and Chronic Retrovirus Loads

Lara Myers, Ronald J. Messer, Aaron B. Carmody, and Kim J. Hasenkrug

Infection of mice with Friend virus induces the activation of CD4+ regulatory T cells (Tregs) that suppress virus-specific CD8+ T cells. This suppression leads to incomplete virus clearance and the establishment of virus persistence. We now show that Treg-mediated suppression of CD8+ T cells is tissue specific, occurring in the spleen but not the liver. Regardless of infection status, there was a 5-fold lower proportion of Tregs in the liver than in the spleen, much lower absolute cell numbers, and the relatively few Tregs present expressed less CD25. Results indicated that reduced expression of CD25 on liver Tregs was due to microenvironmental factors including low levels of IL-2 production by CD4+ Th cells in that tissue. Low CD25 expression on liver Tregs did not impair their ability to suppress CD8+ T cells in vitro. Correlating with the decreased proportion of Tregs in the liver was a significantly increased proportion of virus-specific CD8+ T cells compared with the spleen. The virus-specific CD8+ T cells from the liver did not appear suppressed given that they produced both IFN-γ and granzyme B, and they also showed evidence of recent cytolytic activity (CD107a+). The functional phenotype of the virus-specific CD8+ T cells correlated with a 10-fold reduction of chronic Friend virus levels in the liver compared with the spleen. Thus, suppression of CD8+ T cells by virus-induced Tregs occurs in a tissue-specific manner and correlates with profound effects on localized levels of chronic infection. The Journal of Immunology, 2009, 183: 1636–1643.

Friend virus (FV) is a naturally occurring retroviral complex that causes diseases ranging from lethal erythrocytosis in susceptible mouse strains to asymptomatic chronic infections in resistant strains (1, 2). Chronic infection of mice with FV is associated with the activation of CD4+ regulatory T cells (Tregs) that suppress CD8+ T cell effector functions such as production of IFN-γ and cytolytic molecules (3, 4). Activated Tregs from mice chronically infected with FV suppress CD8+ T cell function in vitro without any requirement for additional re-stimulation (3). This ability distinguishes them from the natural Tregs in uninfected mice that control autoimmune disease, which are suppressive in vitro only when activated by a stimulus such as anti-CD3. The induction of Tregs is common among many types of infections and is most likely a mechanism to prevent immunopathological damage (5, 6). However, the immunosuppressive properties of Tregs can also permit some viruses to escape eradication by the immune system, thereby allowing the establishment and/or maintenance of virus persistence (4, 7, 8).

It was unclear how widespread Treg-mediated suppression of CD8+ T cell was in the mouse because previous studies were all performed on splenic lymphocytes. We thought that the liver would be interesting to study because it is a nonlymphoid tissue reported to have very low levels of chronic FV infection (4). Hepatocytes are one of the few cell types that lack the receptor for FV (9), but during acute FV infection the liver becomes engorged with infected lymphocytes and monocytes. During chronic infection when the primary reservoir of FV is a small subset of B cells (10), the amount of virus in the liver is extremely low (4). The current studies were performed to determine whether the low infection levels in the liver were related to a lack of Treg-mediated suppression of virus-specific CD8+ T cells in that tissue.

Materials and Methods

Mice, viruses, infection, and tissue harvest

Unless otherwise noted, mice were female (C57BL/10 × A.BY)F1 (H-2b), Fv1b, Rfv3h, Fv2h (abbreviated Y10) between 12 and 24 wk of age at the beginning of the experiments and were bred at the Rocky Mountain Laboratories (Hamilton, MT). The FV stock has been passed in mice for more than three decades and contains three separate viruses: 1) B-tropic Friend murine leukemia helper virus (F-MuLV), which is a replication-competent retrovirus; 2) polycythemia-inducing spleen focus-forming virus, which is a defective retrovirus that is packaged by F-MuLV-encoded virus particles; and 3) lactate dehydrogenase-elevating virus, an endemic murine nodovirus (11). Mice were infected by i.v. injection of 0.5 ml phosphate-buffered balanced salt solution containing 1500 spleen focus-forming units of FV complex. Mice were considered chronically infected at 6 wk postinfection when F-MuLV levels stabilize at ~103 infectious centers per spleen (10). Anesthetized mice were perfused with heparinized PBS to displace blood from the liver and spleen. Hepatocytes were removed from liver homogenates using a 35% Percoll gradient. Mice were treated in accordance with the regulations and guidelines of the Animal Care and Use Committee of the Rocky Mountain Laboratories and the National Institutes of Health (Bethesda, MD).

Surface and intracellular staining Abs and flow cytometry

The Abs used for cell staining were purchased from BD Pharmingen or eBioscience, except where otherwise noted. The Abs used for surface staining were: PerCP-Cy5.5- or allophycocyanin-anti-CD8; FITC-CD107a (1D4B); allophycocyanin- or Alexa700- or PE-Cy7-anti-CD4; FITC-anti-CD103 (M290); PerCP-Cy5.5 or allophycocyanin-anti-CD25 (PC61); PerCP-Cy5.5-anti-CD69; and APC-anti-L-selectin (CD62L). Where applicable, an isotype-matched Ab was used as a control. For tetramer staining, allophycocyanin- or PE-D7-gagL-tetramer (Beckman Coulter) was used. Nonspecific staining was blocked using Fc block before staining. For all

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3 Abbreviations used in this paper: FV, Friend virus; Treg, regulatory T cell; F-MuLV, Friend murine leukemia helper virus; CD62L, L-selectin.
intracellular staining, cells were surfaced stained before fixation and permeabilization. Two protocols were used to stain for intracellular granzyme B. The first method was described by Dr. R. A. Robins on the Sanquin Blood Supply Foundation (Amsterdam, The Netherlands) web site (www.sanquinreagents.com). Briefly, cells were fixed overnight in 0.5% paraformaldehyde-PBS and then permeabilized with 0.1% saponin-PBS containing 0.1% sodium azide, 0.5% BSA, and 50 mM glucose. Cells were then sequentially incubated with goat anti-mouse granzyme B, biotin-anti-goat IgG, and then allopurinol-spectrinavidin. In the second method, cells were fixed for 30 min at 4°C in 2% paraformaldehyde and then permeabilized using the permeabilization buffer described. Cells were then stained with allopurinol-anti-human granzyme B (Caltag). Each method gave comparable results. For intracellular cytokine staining, spleen or liver cells were incubated in complete medium (RPMI or IMDM with 10% FCS) for 5 h at 37°C and 5% CO2 in the presence of brefeldin A (10 μg/ml). Cells were then surface stained with anti-CD8, fixed in 2% paraformaldehyde, permeabilized with 0.1% saponin in PBS, and then intracellularly with PE-anti-IFN-γ or an isotype control Ig. For Bcl-2 staining, cells were fixed for 30 min at 4°C using 2% paraformaldehyde and then permeabilized with 0.1% saponin in PBS. Cells were then stained with FITC-anti-Bcl-2 (BD Pharmingen) following the distributor’s recommendations and then labeled with 5 μM CFSE (Molecular Probes). Tregs were purified using anti-CD8 MACS beads and the Miltenyi MACS system following the manufacturer’s recommendations (R&D Systems).

**IL-2 quantification**

We purified 500,000 CD4+ GFP (Foxp3+) T cells, from either naive or chronically infected Foxp3+ eGFP (Foxp3+/-) mice (12) by FACS sorting and cultured them in IMDM supplemented with 10% FCS. Supernatants from these cultures were harvested after 48 h and assessed for IL-2 levels by ELISA using a mouse IL-2 Quantikine Immunoassay Kit following the manufacturer’s recommendations (R&D Systems).

**In vitro suppression assay**

To assay suppression of both proliferation and function, we used a standard in vitro immunosuppression assay such as described for the suppression of CD4+ T cells (13). CD8+ splenocytes targets from naive Y10 mice were purified using anti-CD8α MACS beads and the Miltenyi MACS system following manufacturer’s recommendations and then labeled with 5 μM CFSE (Molecular Probes). Tregs were purified from the splenic and liver lymphocytes of chronically infected Foxp3+ mice with anti-CD4 and stained with CD4+ GFP+ T cells targets. Purities were ≥90%. Culture were at a 3:1 ratio of Tregs to CD8+ target cells, with 60,000–75,000 Tregs and 20,000–25,000 CD8+ targets. Incubations were for 72 h in IMDM supplemented with 10% FCS. Brefeldin A (10 μg/ml) was added for the last 5 h of culture. The cells were then stained for CD8, fixed, permeabilized, and stained for intracellular IFN-γ and granzyme B as described above.

**Infectious center assay**

Dilutions of lymphocytes were plated onto susceptible Mus musculus mice, incubated for 2–3 days at 37°C and 5% CO2, fixed with 95% ethanol, stained with F-MuLV envelope-specific mAb 720, and then developed with peroxidase-conjugated goat anti-mouse IgG and substrate for foci detection (14, 15).

**T cell-adaptive transfers and blocking Ab administration**

Splenocytes from naive (B6.PL × A.BY)F1 (hereafter called Y6.PL) mice were enriched with CD4 using anti-CD4 paramagnetic beads and the Miltenyi MACS system following the manufacturer’s recommendations. Cells were then stained with PerCP-Cy5.5-anti-CD25 and then sorted on a BD FACSAria into CD3+/CD25+ and CD3+/CD25- populations, with a mean purity of >90%. Between 0.5 and 1.5 × 106 cells in 0.5 ml of phosphate-buffered balanced salt solution containing 2% PBS and 15 U/ml heparin sodium (SoloPak Laboratories) were transferred into naive (B6 × A.BY)F1 (hereafter called Y6) recipients by i.v. injection. For IL-2-blocking studies, naive Y6 mice were injected i.p. on 3 consecutive days with 50 μg each of function-blocking rat anti-J506-1A12 (rat IgG2a) and JES6-5H4 (rat IgG2b) anti-IL-2 mAbs (eBioscience; Ref. 16). Control mice were given 50 μg each of rat IgG2a and rat IgG2b concurrently. Tissues were harvested and analyzed the day after the third and final injection.

**Results**

**Reduced proportion and unique phenotype of Foxp3+ Tregs in the liver**

Chronic FV infection has been shown to induce expression of the early activation marker, CD69, on Tregs in the spleen (3), but it was unknown whether that induction also occurred in the liver. Lymphocytes from the perfused livers and spleens of chronically infected mice were isolated and analyzed by flow cytometry. The percentage of lymphocytes staining positive for CD4 was slightly higher in the liver than in the spleen, but more interestingly, the proportion of CD4+ T cells in the liver that were Tregs (Foxp3+) was almost 4-fold lower than in the spleen (Fig. 1A). Expression of Foxp3, a protein required for the development and function of Tregs (17, 18), was equivalent on Tregs from the spleen and liver (Fig. 1B). The activation status of splenic and liver Tregs was compared by analyzing expression of CD69, a standard marker of early activation. Basal levels of CD69 expression on Tregs from naive mice was higher in the spleen compared with the liver, but chronic FV infection significantly induced CD69 expression on Tregs from both tissues (Fig. 1C, top). Importantly, despite FV-induced CD69 expression, the proportion of activated Tregs (CD69high) was significantly lower in the livers than in the spleens of mice chronically infected with FV (Fig. 1C, bottom). In addition to lower proportions of activated Tregs in the liver compared with the spleen, the absolute number of Tregs, and lymphocytes in general, was much lower in the liver than in the spleen despite the much larger size of the liver (Table I).

As expected, a significantly lower percentage of liver Tregs than splenic Tregs expressed CD62L, the homing receptor for lymphoid tissues, in both naive and chronically infected mice (Fig. 1D, top; Ref. 19). To determine whether Tregs were actively cycling they were examined for Ki-67, a protein that is present during all active phases of the cell cycle but absent from G0 phase resting cells (20, 21). In both naive and chronically infected mice, greater proportions of Tregs from the liver expressed high levels of Ki-67 than Tregs from the liver (Fig. 1D, middle). This result indicated that liver Tregs were actively dividing and that the liver was not simply a repository for inactive or dying cells. The proportion of Tregs expressing Ki-67 was equivalent between naive and chronically infected mice when comparing within the spleen or the liver (Fig. 1D, middle). Thus, chronic FV infection did not significantly enhance the basal levels of Treg proliferation. Tregs were also analyzed for CD25 expression, which is constitutively expressed on natural Tregs (22). CD25 is the α-chain of the IL-2 receptor and imparts the ability to sense low homeostatic concentrations of IL-2, a cytokine required for Tregs survival (23). Although the Foxp3+ Tregs in the livers of chronically infected mice expressed CD25, the proportion of CD25high Tregs was less than one-half that in splenic Tregs from the same mice (Fig. 1D, bottom). Low expression of CD25 on most Tregs in the liver was not related to FV infection as was similarly observed in naive mice (Fig. 1D, bottom).

To determine whether the tissue-specific differences in expression of CD25 on Tregs was an intrinsic property or a response to microenvironmental signals, CD4+ T cells from the spleens of naive Thy1.1+ mice were sorted into CD25low and CD25high populations, with a mean purity of >90%. Between 0.5 and 1.5 × 106 cells in 0.5 ml of phosphate-buffered balanced salt solution containing 2% PBS and 15 U/ml heparin sodium (SoloPak Laboratories) were transferred into naive (B6 × A.BY)F1 (hereafter called Y6) recipients by i.v. injection. For IL-2-blocking studies, naive Y6 mice were injected i.p. on 3 consecutive days with 50 μg each of function-blocking rat anti-J506-1A12 (rat IgG2a) and JES6-5H4 (rat IgG2b) anti-IL-2 mAbs (eBioscience; Ref. 16). Control mice were given 50 μg each of rat IgG2a and rat IgG2b concurrently. Tissues were harvested and analyzed the day after the third and final injection.
Statistical analyses were done by Student’s two-tailed t test. CD4 T cells from spleens and livers of chronically infected mice were analyzed by flow cytometry for the percentage of CD4+ T cells expressing Foxp3. The difference in percentage of that subset expressing Foxp3 was statistically significant (*, p < 0.05; n = 8) and the difference between Foxp3+ CD4+ T cells in the spleen and liver was also statistically significant (*, p < 0.00001, n = 8). Geometric mean fluorescent intensity (MFI) of intracellular Foxp3 staining of CD4+ T cells from the spleens and livers of chronically infected mice was not statistically different (p = 0.9184, n = 6).

C, Overlays of representative histograms of CD69 expression on CD4+Foxp3+ Tregs from the spleens and livers of naive and chronically infected mice. Bars, mean percentage of Tregs that were CD69+ (*, p < 0.05; n = 10–16 per group; Bonferroni correction was used for multiple comparisons). D, Foxp3+CD4+ T cells from the spleens and livers of naive mice and mice chronically infected with FV were analyzed for expression of CD62L, intracellular Ki-67, and CD25 (*, p < 0.0001; n = 5–12). The differences between naive and chronic values within the same tissue were not statistically different.

Next, we assayed production of IL-2 from spleen and liver CD4+ Th cells, the predominant source of IL-2. Foxp3GFP reporter mice (12) were used to obtain >95% pure CD4+ T cells by sorting on CD4+ GFP+ T cells. IL-2 was measured directly ex vivo by ELISA following 48 h of culture with no in vitro stimulation. Helper cells from the spleens of naive mice produced significantly more IL-2 than an equal number of helper cells from the livers of the same mice (Fig. 2E). Chronic FV infection stimulated IL-2 production by CD4+ T cells from both the spleen and the liver. The helper cells in the livers of chronically infected mice produced IL-2 roughly equivalent to amounts in the spleens of naive mice (Fig. 2E), yet CD25 expression on Tregs was not induced (Fig. 1D, bottom). This result indicated that either the relatively low absolute numbers of CD4+ T cells in the liver (Table I) or another microenvironmental factor was important in controlling CD25 expression.

**Tissue-specific Treg localization**

It was previously shown that Tregs from the spleens of chronically infected mice suppressed the antiviral functions of FV-specific CD8+ T cells (4). It was therefore of interest to determine whether the lower proportion and number of Tregs in the liver correlated with improved functional status of the virus-specific CD8+ T cells in that tissue. Tissue-resident CD8+ T cells from perfused livers were analyzed by flow cytometry and compared with those from the spleen to examine their functional properties. On average, more than twice as many CD8+ T cells from the liver stained positive with FV-specific tetramers compared with spleens from

| Table I. Mean absolute numbers of T cell subsets in spleens and livers of naive and chronically infected mice<sup>a</sup> |
|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
|                     | Naive spleen        | Chronic spleen      | Naive liver         | Chronic liver       | 
| CD4<sup>+</sup>     | 1.7 × 10<sup>7</sup> ± 3.2 × 10<sup>6</sup> | 4.8 × 10<sup>7</sup> ± 1.9 × 10<sup>7</sup> | 3.2 × 10<sup>7</sup> ± 4.8 × 10<sup>6</sup> | 3.5 × 10<sup>7</sup> ± 1.6 × 10<sup>6</sup> |  
| CD4<sup>+</sup>Foxp3<sup>+</sup> | 2.9 × 10<sup>6</sup> ± 9.2 × 10<sup>5</sup> | 9.1 × 10<sup>6</sup> ± 4.2 × 10<sup>6</sup> | 9.0 × 10<sup>5</sup> ± 3.4 × 10<sup>4</sup> | 1.6 × 10<sup>6</sup> ± 5.2 × 10<sup>4</sup> |  
| CD8<sup>+</sup>      | 9.7 × 10<sup>6</sup> ± 2.3 × 10<sup>6</sup> | 3.3 × 10<sup>7</sup> ± 1.3 × 10<sup>6</sup> | 7.1 × 10<sup>6</sup> ± 2.2 × 10<sup>5</sup> | 2.8 × 10<sup>6</sup> ± 9.0 × 10<sup>5</sup> |  
| CD8<sup>+</sup>Tet<sup>+</sup> | Background          | 1.2 × 10<sup>7</sup> ± 1.9 × 10<sup>5</sup> | Background          | 2.9 × 10<sup>6</sup> ± 1.6 × 10<sup>5</sup> |  
| Ratio Treg:Tet<sup>+</sup> | n/a                 | −8:1                | n/a                 | −1:1                |  

<sup>a</sup> Data are the mean calculated absolute numbers ± SD for naive mice (n = 5) and mice chronically infected with FV (n = 8). Background, no definable population different than uninfected mice; n/a, not applicable.
the same mice (Fig. 3, A and B). As expected based on previous work (4), the FV-specific CD8+ T cells from the spleen were found to have an activated effector phenotype (CD43+ , CD44+, CD69+, and CD127+ ), and there was no significant difference in expression of these markers on FV-specific CD8+ T cells from the liver (data not shown).

FV-specific CD8+ T cells from the spleen and liver were also analyzed for intracellular expression of Ki-67 to examine recent proliferation. There was a wide distribution in the percentage of tetramer+ CD8+ cells positive for Ki-67 in both tissues but the means were roughly equivalent (Fig. 3C). Thus there was no indication that virus-specific CD8+ T cells proliferated more in the liver than the spleen. As a measure of survival fitness, the intracellular expression of the anti-apoptotic protein Bcl-2 was measured. Bcl-2 expression in the general population of CD8+ T cells (tetramer negative) was equivalent between the spleen and liver (Fig. 3D, left). In contrast, Bcl-2 expression was significantly higher in tetramer+ CD8+ T cells from the liver compared with the spleen (Fig. 3D, right). Taken together, these data suggest that the higher proportion of virus-specific CD8+ T cells in the liver compared with the spleen was due to better survival rather than increased proliferation.

To compare the functional status of FV-specific CD8+ T cells from the spleen and the liver, tetramer+ CD8+ T cells were evaluated directly ex vivo for intracellular expression of the cytotoxic molecule granzyme B and for surface expression of CD107a, a surrogate marker for recent cytolytic activity. As shown previously (27), tetramer+ CD8+ T cells from the spleen expressed relatively
low levels of intracellular granzyme B and essentially stained negative for surface CD107a (Fig. 4, A and B). In striking contrast, tetramer CD8^+ T cells from the liver expressed significantly higher levels of granzyme B and stained positive for surface CD107a, indicating recent cytotoxic activity (Fig. 4, A and B). The cells were also tested for production of IFN-γ, an antiviral cytokine important for control of chronic FV infection (28, 29). CD8^+ T cells from the liver and the spleen were cultured for 5 h in brefeldin A without any in vitro restimulation. Significantly more CD8^+ T cells from the liver than from the spleen stained positive for IFN-γ (Fig. 4C). Thus, FV-specific CD8^+ T cells in the liver were more functionally active than in the spleen and raised the question of whether the Tregs in the liver possessed equivalent suppressive ability compared with those from the spleen.

**Fosp3^+ Tregs from the livers of chronically infected mice suppress CD8^+ T cell targets equivalently to Tregs from the spleen**

To directly compare the suppressive ability of Tregs from the livers with those from the spleen of chronically infected mice, we used a standard in vitro suppression assay to measure suppression of both proliferation and function (13). Because CD25 could not be used to purify Tregs from livers because of low expression levels, we used Fosp3^GFP reporter mice (12) and sorted CD4^+GFP^+ T cells. Liver Tregs cocultured with CD3-stimulated naive CD8^+ T cell targets showed equivalent suppression of CD8^+ T cell proliferation as splenic Tregs (Fig. 5A). Furthermore, they also suppressed IFN-γ and granzyme B production similarly to splenic
Significantly reduced infection in the liver correlates with a reduced ratio of Tregs to virus-specific CD8\(^+\) T cells

Previous in vitro studies on FV-induced regulatory T cells showed that suppression of CD8\(^+\) T cell function required cell-to-cell contact (3). If cell contact is essential in vivo, then the ratio of Tregs to virus-specific CD8\(^+\) T cells could be an important factor in determining the likelihood of Treg-mediated suppression in a given tissue. Based on the absolute numbers of cell subsets in each tissue, the spleen averaged approximately eight Tregs per tetramer\(^+\) CD8\(^+\) T cell whereas the liver averaged less than one Treg per tetramer-positive CD8\(^+\) T cell (Table I). Thus, the increased functional phenotype of virus-specific CD8\(^+\) T cells in the liver compared with the spleen could be based on at least an 8-fold reduced likelihood of contacting a Treg and becoming suppressed.

To determine whether the apparently increased antiviral function of the CD8\(^+\) T cells in the liver was reflected in reduced virus loads, livers were perfused to remove blood cells, and Percoll gradient-purified lymphocytes were tested for virus by an infectious center assay (3). Hepatocytes were excluded because they lack the FV receptor and are not infected by FV (9). The average number of infectious centers per spleen was 18,000, and the average per liver was 41, a 440-fold difference (\(p < 0.05\) but not compared with each other). Mean percentages of CD8\(^+\) T cells staining positively for intracellular IFN-\(\gamma\) (B) and granzyme B (C) showed the same pattern of significance (\(p < 0.05\)).

Decreased virus levels. Taken together, these data indicate relationships among low Treg levels in the liver, increased functionality of FV-specific CD8\(^+\) T cells, and tissue-specific virus control.

Discussion

Results from our analyses of splenic and liver Tregs demonstrate that livers from both naive and chronically infected mice contain a significantly lower proportion of Tregs than spleens. Liver Tregs were maintained at a relatively low frequency despite their apparently high proliferation capacity (Ki-67) and survival fitness (Bcl-2). A likely explanation for these results is that Tregs tend to migrate out of the liver, but direct experiments will have to be done to confirm this. Although natural Tregs are described to express high constitutive levels of CD25, this was not true for most liver Tregs from either naive or chronically infected mice and must be kept in mind when isolating Tregs from this tissue because it makes using anti-CD25 Abs ineffective. We also observed a significant subpopulation of CD25\(^{low}\) Tregs in the spleen as well.

CD25, the \(\alpha\)-chain of the IL-2 receptor, imparts high affinity to the IL-2 receptor, and the expression of CD25 is directly linked to IL-2 signaling (23). In contrast to the spleen, which contains an abundance of helper T cells that are the primary sources of IL-2, the liver is primarily composed of hepatocytes, which are not known to make IL-2 except under extraordinary conditions such as liver transplant rejection (30). To determine whether limited IL-2 concentrations in the liver were the likely cause of low CD25 expression, it was therefore important to assay for IL-2 production by purified CD4\(^+\) T cells, which could produce physiologically relevant levels of IL-2 in microenvironments such as sinusoids and portal tracts where lymphocytes and lymphocyte interactions are concentrated. Conventional (Foxp3\(^-\)) CD4\(^+\) T cells from the livers of naive mice secreted significantly lower levels of IL-2 in vivo cultures than those from with the spleen. However, chronic infection induced CD4\(^+\) Th cells to up-regulate IL-2 production to levels equivalent to that in naive spleens, but the liver Tregs still did not up-regulate CD25 expression. This might be explained by the relatively low absolute numbers of T cells in the liver compared with the spleen, or there might be another microenvironmental factor in the liver that affects CD25 expression levels.

Further evidence of microenvironmental down-regulation of CD25 in the liver came from adoptive transfer experiments showing that CD25\(^{high}\) Tregs that migrated to the liver rapidly adopted
a CD25<sup>low</sup> phenotype. Low expression of CD25 has also been reported on liver Tregs in patients infected with hepatitis B virus, and it was speculated that low CD25 expression might be related to stimulation by cytokines or growth factor deprivation (31). Our results suggest that the lack of CD25 on Tregs from those patients may be more related to tissue specificity than to hepatitis B infection, and that growth factor deprivation, or more specifically IL-2 deprivation, might be one reason. Analyses of samples from normal individuals should clarify this issue.

Despite low CD25 expression, there was no indication that Tregs in the liver were dysfunctional. Liver and spleen Tregs had equivalent levels of Foxp3 expression, were actively cycling based on Ki-67 expression, and expressed proportionately equivalent in decreases in activation (CD69) in response to chronic FV infection. Additionally, the in vitro suppression assays indicated that liver Tregs were equivalent to splenic Tregs in their ability to suppress CD8<sup>+</sup> T cell proliferation and effector function. However, in vivo there were roughly 8-fold fewer Tregs per tetramer CD8<sup>+</sup> T cell in the liver as in the spleen, and if calculated on the basis of the activated Tregs, the ratio increases to 15-fold. Based on these ratios, virus-specific CD8<sup>+</sup> T cells would be approximately an order of magnitude less likely to encounter an activated Treg in the liver than in the spleen. Such encounters are likely important because cell-to-cell contact has been shown to be critical for Treg-mediated suppression, both in the FV model (3) and in other models (32).

Even more interesting than the increased proportions of virus-specific CD8<sup>+</sup> T cells in the liver was that they appeared fully functional or nonsuppressed as demonstrated by expression of granzyme B, IFN-γ, and CD107α. These are the same CD8<sup>+</sup> T cell functions that were found to be suppressed by Tregs both in vitro (3) and in vivo (4, 27). Our results also showed that the presence of functional virus-specific CD8<sup>+</sup> T cells correlated with substantially lower levels of chronic infection in the liver than in the spleen. The most likely explanation for the results as a whole is that in contrast to CD8<sup>+</sup> T cells in the spleens of mice with chronic FV infection, those in the liver are not suppressed by virus-induced Treg and are able to locally contain viral infection. It is still possible that the activity of CD8<sup>+</sup> T cells in the liver may be high simply because the liver preferentially collects activated CD8<sup>+</sup> T cells (33). Even so, fully activated CD8<sup>+</sup> effector T cells have previously been shown to be susceptible to FV-induced Treg-mediated suppression (3); therefore, the lack of Treg-mediated suppression in the liver is likely an important factor in maintaining the functional status of the CD8<sup>+</sup> T cells.

The high functional status of virus-specific CD8<sup>+</sup> T cells in the liver may seem unusual because the liver is considered a tolerogenic organ. For example, allogeneic liver grafts can sometimes be maintained without immunosuppressive drugs (34, 35) and systemic tolerance to allogeneic cells can be induced by injection of the cells into the portal vein (36). However, the liver is also quite capable of sustaining effector T cell responses, such as during human infections with hepatitis A virus (37) and mouse infections with mouse hepatitis virus (38). Thus, the liver is not purely tolerogenic and can adapt responses according to homeostatic signals (39). On the face, our data suggest that the tolerogenic status of the liver is not due to activity by Tregs, which are relatively rare in that tissue. However, it was recently demonstrated that Tregs are at least partially responsible for tolerance in liver allografts (40, 41).

This discrepancy for the role of Tregs in liver tolerance might reflect differences in the type or level of Ag, or the inflammatory status including cytokines and chemokines.

Previous studies have shown that Treg-mediated suppression of CD8<sup>+</sup> T cells can be reversed (42). Thus, rather than the homing of fully activated CD8<sup>+</sup> T cells to the liver, another possibility is that the virus-specific CD8<sup>+</sup> T cells in the liver migrated there as suppressed cells and regained function due to the paucity of Tregs. In addition to the lack of negative regulation in the liver, it is possible that the liver provides positive signals that promote the reversal of suppression. Regardless, the finding of fully active, virus-specific CD8<sup>+</sup> T cells in the liver demonstrates that Treg-mediated suppression is not necessarily a global phenomenon and opens the possibility that therapeutic strategies might be devised to redirect functional CD8<sup>+</sup> T cells to sites of high infection. Such a strategy would also require either the inactivation of regulatory T cells or rendering the effector cells refractory to suppression. For example, the use of immunomodulatory Abs such as anti-glucocorticoid-induced TNFR has previously been shown to induce both of these effects (43) and has already shown some success in reducing chronic FV infections (4). Along that vein, it has also been shown that it is possible to render naive CD8<sup>+</sup> T cells refractory to Treg-mediated suppression by costimulation with anti-CD137 (44). The current findings that low IL-2 levels in the liver appear to regulate CD25 expression and also correlate with decreased Treg proportions have therapeutic implications as well. Understanding the mechanism by which production of IL-2 by CD4<sup>+</sup> T cells is down-regulated in the liver could lead to new ways of manipulating Treg levels in the liver and/or other tissues for the control of autoimmune diseases, immunopathological diseases or the control of chronic infections where Tregs play a role.

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References


