TLR-4 Regulates CD8+ T Cell Trapping in the Liver

Beena John and Ian Nicholas Crispe

*J Immunol* 2005; 175:1643-1650; doi: 10.4049/jimmunol.175.3.1643
http://www.jimmunol.org/content/175/3/1643

References

This article cites 33 articles, 10 of which you can access for free at:
http://www.jimmunol.org/content/175/3/1643.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
TLR-4 Regulates CD8+ T Cell Trapping in the Liver

Beena John and Ian Nicholas Crispe

Mammalian TLRs are understood primarily as an activating system for innate and adaptive immunity, but have also been implicated in sensing cellular damage and in promoting intestinal integrity. In this study we show that TLR-4 also controls the in vivo distribution of activated CD8+ T cells. The liver is a site for trapping and apoptosis of activated CD8+ T cells during systemic immune responses, but the reason for this is unknown. In this study we tested the hypothesis that the liver’s constant exposure to endotoxin, derived from commensal bacteria in the gut, acts via TLR-4 to promote activated T cell adhesion. In the absence of TLR-4, the liver was compromised in its ability to sequester activated CD8+ T cells, and there was an inverse correlation between the frequency of activated CD8+ T cells trapped in the liver and their frequency in the circulating pool. Thus, in the absence of any inflammation, TLR-4 ligands play a significant role in the ability of the liver to trap activated CD8+ T cells. This provides a new perspective on the regulation of immune responses by TLR-4 under basal conditions. The Journal of Immunology, 2005, 175: 1643–1650.

A
tivated CD8+ T cells, primed in response to an antigenic challenge, enter the blood and circulate widely through the tissues. These T cells undergo diverse fates. A subset of the cells undergoes apoptosis, whereas others enter the memory pool. Among the cells that undergo apoptosis, an unusually large proportion is trapped in the liver due to the expression of ICAM-1 and VCAM-1 on hepatic sinusoidal endothelium (1). Such trapping and intrahepatic apoptosis of activated CD8+ T cells are seen in mouse models driven by antigenic peptide, in SIV (LSECs) are the main scavengers for LPS, although hepato-

Copyright © 2005 by The American Association of Immunologists, Inc.
response during the accumulation of activated CD8⁺ T cells in the liver. To explain how exposure to pathogen-associated molecular patterns in the liver is compatible with immune tolerance, we proposed the hypothesis that TLR-4 signaling promotes the trapping of activated CD8⁺ T cells in the liver, leading to their apoptosis and the induction of unresponsiveness due to peripheral clonal deletion. To test this hypothesis, we have used two different LPS-unresponsive mouse strains: TLR-4 gene-targeted mice, which lack TLR-4 molecules, and TLR-4 mutant mice, which are defective in signaling upon surface TLR-4 ligation. CD8⁺ T cells were activated either by Ag-specific TCR ligation or using cells expressing a superantigen, and we determined the localization of the responding CD8⁺ T cells in TLR-4-nonresponsive mice. In this study we show that TLR-4 plays an important part in the ability of the liver to trap activated CD8⁺ T cells.

Materials and Methods

Mice

TLR-4-deficient mice (C57BL/10 ScN), their wild-type (wt) counterparts (C57BL/10 SnJ), TLR-4 mutant mice (C3H/HeJ), and their wt counterparts (C3H/HeOuJ), and the AKR/J strains of mice were purchased from The Jackson Laboratory and housed in a specific pathogen-free environment in compliance with institutional guidelines for animal care. A colony of OT1 transgenic mice (originally on a C57BL/6J background) was extensively backcrossed with B6.SJL mice to obtain CD45.1 homozygous OT1 transgenic mice. A second colony of OT1 transgenic mice was maintained on a CD45.1/CD45.2 heterozygous background by crossing CD45.1/⁻/⁻ OT1 transgenics with C57BL/6J (CD45.2/⁺/⁺) mice.

CD8⁺ T cells for localization experiments

Lymphocytes were isolated from the spleen and peripheral lymph nodes of OT1 TCR transgenic mice, which were on a CD45.1 homozygous background. They were activated in vitro for 72 h with 1 μM SIINFEKL peptide in the presence of spleen APCs. This was used as a source of activated CD8⁺ T cells. Lymphocytes isolated from spleens and peripheral lymph nodes of OT1 TCR transgenic mice, on a CD45.1/⁻/CD45.2 heterozygous background, were used as a source of the naive CD8⁺ T cells. Equal numbers of activated and naive T cells (1 0 0 0 0 0 of each) were injected into either wt or TLR-4-deficient mice i.v. The recipient mice were either wt (C57BL/10SnJ) or TLR-4⁻/⁻ (C57BL/10ScN) and were all on a CD45.2 background. Two hours later, we analyzed the homing of the two different cell types to various compartments. The activated, naive, and host cells were distinguished from one another based on their expression of the alloantigenic markers, CD45.1, CD45.2, or both.

Isolation of splenic dendritic cells (DCs)

DCs were enriched from the spleen using the technique established by Livingstone et al. (27). Briefly, spleens were digested in an enzyme mixture containing 2.4 mg/ml collagenase IV (Sigma-Aldrich) and 1 mg/ml DNase (Sigma-Aldrich) for 30 min at 37°C. The spleen cell digest was made into a single-cell suspension with a syringe and needle, followed by two washes with HBSS. The cell pellet was then resuspended in 60% Percoll (2 ml/spleen). This was overlaid with 2 ml of HBSS and centrifuged at 2000 rpm for 20 min. The interface was harvested, and the cells were washed twice. They were then resuspended in RPMI 1640 (with 10% FCS), transferred to large petri dishes, and incubated for 90 min at 37°C. The nonadherent cells were removed, and the adherent cells were cultured overnight (~18 h) with 1 ng/ml GM-CSF and 1 μM SIINFEKL peptide. The nonadherent cells were harvested the next day by gentle pipetting, and the cells were washed. B cell contaminants in this population were removed using goat anti-mouse IgM and goat anti-mouse IgG magnetic beads (Qiagen). The peptide-loaded, DC-rich cell preparation was then injected i.p. into mice (1 0 0 0 0 0 cells/mouse). On the average, 60–65% of the cells stained positively for markers characteristic of DCs: CD11c, MHC class II, CD80, and CD86.

Adoptive transfer and in vivo activation

Single-cell suspensions were made from the spleen and peripheral lymph nodes of OT1 transgenic mice by mechanical homogenization. B cells were removed by density gradient centrifugation (Lympholyte-M; Cedarlane Laboratories). CD8⁺ T cells were purified by depletion of MHC class II-positive DCs, B cells, and macrophages using an Ab mixture (clone 212.A1 specific for MHC class II molecules, clone 2.4-4G2 specific for FeRs, clone TIB 146 specific for B220, clone GK1.5 specific for CD4, and clone HB191 specific for NK1.1 marker). Magnetic beads coated with the secondary Ab were used to remove cells coated with primary Abs. Five million OT1 T cells (>90% pure CD8) were injected i.v. into recipient mice. The mice were activated with peptide-loaded APCs injected i.p. 24 h after injection of OT1 cells.

Intracellular staining

Lymphocytes were isolated from the spleen, lymph nodes, and livers of the different groups of mice, and ~2 0 10⁶ lymphocytes were either unstimulated or restimulated with 1 μM SIINFEKL peptide in complete medium with 50 U/ml mouse rIL-2 (Endogen) and 1 μM Golgi Plug (BD Pharmingen) in 96-well plates. After 6 h of culture, the cells were washed and stained for surface markers. They were then fixed and permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen), and intracellular staining was performed according to the manufacturer’s instructions.

In vivo cytotoxicity assay

Splenocytes were isolated from wt C57BL/10SnJ mice and subjected to a Lympholyte gradient to eliminate RBCs. Half of the cells were labeled with 2 μM CFSE (CFSElow), and the other half were labeled with 0.2 μM CFSE (CFSEhigh) for 10 min, followed by two washes with PBS. The CFSEhigh cells were pulsed with 1 μM SIINFEKL peptide for 1 h at 37°C, whereas the CFSElow cells were left unpulsed. The cells were washed extensively and counted, and equal numbers of the two different populations were mixed together and injected i.v. into the mice. Approximately 10 0 0 0 0 cells from each of the target groups were injected per mouse. The mice were killed 5 h later, and the various organs were harvested.

Viral superantigen (vSAG)-mediated activation

Splenocytes were isolated from AKR/J strains of mice and subjected to a Lympholyte gradient. AKR/J splenocytes (10 0 0 0 0) were injected into either C3H/HeJ (TLR-4 mutant) or C3H/HeOuJ (wt) mice. The AKR/J splenocytes express the vSAG-7 protein and can activate Vβ6⁺ T cells in the host. Spleen, lymph nodes, and liver lymphocytes were isolated from the C3H/HeJ or C3H/HeOuJ strain of mice at various time points after transfer of AKR/J splenocytes.

Cell isolation, staining, and flow cytometric analysis

Peripheral lymph node and spleens were isolated from the mice on days 3, 5, and 7 after injection of pulsed or unpulsed DCs. Single-cell suspensions were obtained by mechanical homogenization using frosted glass slides. The livers were perfused before they were harvested, and intrahepatic lymphocytes were isolated using a standard protocol. Briefly, the livers were homogenized and treated with collagenase (0.05%) and DNase (0.002%) for 45 min at 37°C. The hepatocytes were removed by low-speed centrifugation (3 0 0 0 g for 5 min), and the remaining cell suspension was washed and subjected to an Optiprep gradient (Accurate Chemicals). Optiprep was used at a final concentration of 22% mixed with the cell suspension. This was overlaid with 2 ml of serum-free medium and centrifuged at 1500 g for 20 min at 4°C. The cells in the interface were isolated, washed, and counted as intrahepatic lymphocytes.

Statistical analysis

The statistical significance of the differences between groups of mice was tested using Student’s t test. A value of p < 0.05 was considered significant.

Results

In the absence of TLR-4, the liver is defective in the acute trapping of activated CD8⁺ T cells

To test the role of TLR-4 in the accumulation of activated CD8⁺ T cells in the liver, we developed a simple competitive trapping assay, designed to minimize any confounding effects of TLR-4 deficiency on CD8⁺ T cell activation or survival. In this assay, a mixture of activated and resting, OT1 TCR transgenic CD8⁺ T cells was injected i.v. into either wt (C57BL/10SnJ) or TLR-4-deficient mice (C57BL/10ScN) and located by FACS 2 h later. The activated and resting cells were identified based on their expression of distinct allotypes of CD45. Fig. 1A shows the expression of activation markers on the two cell populations. The activated cells...
CD45.1 cells (CD45.1 × CD45.2) seen in the upper right quadrant, expressed higher levels of CD44, CD69, and CD25 than the resting cells (CD45.1 × CD45.2) seen in the lower right quadrant.

Two hours after T cell injection into normal mice (wt host), resting CD8⁺ T cells localized preferentially to the spleen and lymph nodes, whereas activated CD8⁺ T cells were overrepresented in the liver (Fig. 1B, left panel). Injection of the same cell mixture into TLR-4-deficient mice (TLR-4⁻/⁻ host) resulted in a different distribution of the cells (Fig. 1B, right panel). In TLR-4-deficient mice, the preferential accumulation of activated CD8⁺ T cells in the liver was lost; conversely, such cells increased in the blood. Fig. 1C shows the ratio of activated to naive CD8⁺ T cells in the spleen, lymph nodes, peripheral blood, and liver of wt and TLR-4⁻/⁻ mice (n = 5/group). The ratio of activated to naive cells in the livers of TLR-4⁻/⁻ mice was significantly lower than that in wt mice. This decrease in the liver was compensated by an increase in this ratio in the peripheral blood of TLR-4⁻/⁻ mice. We conclude that TLR-4 promotes the removal of activated CD8⁺ T cells from the circulation and their preferential localization in the liver.

In vivo activation

Testing the role of TLR-4 in the intrahepatic accumulation of CD8⁺ T cells activated in situ raises the problem that the TLR-4-deficient mice could be compromised in their ability to mount a normal immune response. Therefore, we developed a model in which normal OT1 TCR transgenic CD8⁺ T cells were transferred into either wt or TLR-4-deficient mice, and then primed in vivo using adoptively transferred, spleen-derived DCs from wt mice that had been pulsed in vitro with the specific antigenic peptide (SIINFEKL). This model depends on direct priming, and the endogenous TLR-4-deficient APCs are not involved (28). Using this model, we observed equivalent clonal expansion of OT1 T cells in the spleen and lymph nodes, but reduced accumulation of OT1 T cells in the liver on day 5 of the response (Fig. 2). Fig. 2A shows individual examples of the frequencies of activated OT1 CD8⁺ T cells in different organs on day 5; the frequencies were similar in the lymph nodes of wt and TLR-4-deficient mice, but there were 8-fold fewer OT1 T cells in the livers of TLR-4-deficient mice. The analysis of groups of mice (n = 6) on day 3 showed that there were no significant differences in the percentages (Fig. 2B, top panel) and numbers (Fig. 2C, top panel) of OT1 cells in the spleen, lymph nodes, or livers of wt and TLR-4-deficient mice on day 3 (top panel), suggesting that the priming and clonal expansion of OT1 cells were comparable between the two groups of mice. However, on day 5, there was a significant reduction in the percentage (Fig. 2B, lower panel) and numbers of OT1 cells (Fig. 2C, lower panel) in the livers of TLR-4-deficient mice. The reduced accumulation of the activated OT1 cells in the livers of TLR-4-deficient mice was accompanied by an increase in their percentages in the spleen (also seen in the representative example in Fig. 2A), suggesting that the cells that were not trapped in the liver were migrating to the spleen. We conclude that the intrahepatic accumulation of activated CD8⁺ T cells during an in situ immune response is promoted by TLR-4.

Activation of the OT1 cells is similar in wt and TLR-4-deficient mice

To address the issue of whether the reduction in the OT1 cell numbers seen in the TLR-4-deficient livers was a result of differential activation, the responses in the two groups of mice was examined more closely. Fig. 3 shows that 3 days after Ag exposure, the OT-1 T cells were activated normally in TLR-4-deficient mice. Thus, the cells showed equivalent clonal expansion (for example, in the spleen from 0.60 to 1.69% of all lymphocytes in B6 mice and from 0.55 to 1.44% in TLR-4-deficient mice), and this was also true in lymph nodes and the liver. The down-regulation of CD26L and up-regulation of CD44 also occurred identically in the B6 and the TLR-4-deficient hosts (Fig. 3). This was not surprising, because both the wt and TLR-4⁻/⁻ mice were activated with DCs from wt mice. Such equivalent activation was important in allowing us to interpret the observed differences in the TLR-4⁻/⁻ livers.

Activated OT1 cells in wt and TLR-4-deficient mice can both produce IFN-γ and kill Ag-loaded targets in vivo

To validate our conclusion that OT1 cells were activated normally by wt DCs in TLR-4-deficient hosts, we examined the ability of the...
OT1 cells activated in normal and TLR-4-deficient mice to synthesize the effector cytokine IFN-γ and to kill target cells in vivo. In wt mice, OT1 T cells that were sham primed with PBS-pulsed APCs, did not divide, and only a few cells (4.63% in lymph nodes and 1.56% in spleen) were competent to make IFN-γ upon restimulation in culture with the antigenic peptide (Fig. 4). OT1 cells in wt mice that were primed with peptide-pulsed APCs had gone through at least six divisions by day 3, and they were capable of synthesizing IFN-γ upon restimulation with SIINFEKL peptide. The IFN-γ production was Ag specific, because it was seen only when the cells were restimulated with the antigenic peptide in the 6-h in vitro assay. A comparison of the dilution of CFSE in the OT1 population from the wt and TLR-4-deficient mice revealed no significant differences in the number of cell divisions that occurred through at least six divisions by day 3, and they were capable of synthesizing IFN-γ upon restimulation with SIINFEKL peptide. The IFN-γ production was Ag specific, because it was seen only when the cells were restimulated with the antigenic peptide in the 6-h in vitro assay. A comparison of the dilution of CFSE in the OT1 population from the wt and TLR-4-deficient mice revealed no significant differences in the number of cell divisions that occurred.

FIGURE 2. TLR-4/−/− mice retain fewer activated OT1 cells in their livers compared with wt mice in an in situ immune response. A. Percentage of OT1 cells (CD45.1 Vα2+) on day 5 in spleen, lymph nodes, and liver of wt or TLR-4/−/− mice that received OT1 cells and were activated with splenic DCs pulsed with SIINFEKL peptide. The average OT1 percentage (B) and cell numbers (C) in spleen (■), lymph nodes (□), and liver (■) of wt or TLR-4/−/− mice 3 days (top panels) and 5 days (bottom panels) after immunization are also depicted.

OT1 cells activated in normal and TLR-4-deficient mice to synthesize the effector cytokine IFN-γ and to kill target cells in vivo. In wt mice, OT1 T cells that were sham primed with PBS-pulsed APCs, did not divide, and only a few cells (4.63% in lymph nodes and 1.56% in spleen) were competent to make IFN-γ upon restimulation in culture with the antigenic peptide (Fig. 4). OT1 cells in wt mice that were primed with peptide-pulsed APCs had gone through at least six divisions by day 3, and they were capable of synthesizing IFN-γ upon restimulation with SIINFEKL peptide. The IFN-γ production was Ag specific, because it was seen only when the cells were restimulated with the antigenic peptide in the 6-h in vitro assay. A comparison of the dilution of CFSE in the OT1 population from the wt and TLR-4-deficient mice revealed no significant differences in the number of cell divisions that occurred through at least six divisions by day 3, and they were capable of synthesizing IFN-γ upon restimulation with SIINFEKL peptide. The IFN-γ production was Ag specific, because it was seen only when the cells were restimulated with the antigenic peptide in the 6-h in vitro assay. A comparison of the dilution of CFSE in the OT1 population from the wt and TLR-4-deficient mice revealed no significant differences in the number of cell divisions that occurred.

FIGURE 3. Activation of adoptively transferred OT1 T cells is comparable between wt and TLR-4/−/− mice: The data show the percentage of OT1 cells (CD45.1+Vα2+) in spleen, lymph nodes, and liver of wt and TLR-4/−/− mice 3 days after they were given either unpulsed APCs or SIINFEKL peptide-pulsed APCs. The figure also shows the down-regulation of CD62L and the up-regulation of CD44 upon activation of OT1 cells in wt and TLR-4/−/− mice.
in the two recipients. There was also no significant difference between the frequency of IFN-γ-producing OT1 cells, which were activated in wt or TLR-4−/− mice. The data shown in the figure are representative of six mice in each group.

The hallmark of a fully functional effector CD8+ T cell is its ability to kill target cells expressing specific Ags; hence, we tested the cytotoxic capability of OT1 T cells that were activated in TLR-4-deficient mice using an in vivo cytotoxicity assay. Specific targets (loaded with SIINFEKL peptide) and nonspecific target cells (not loaded with peptide) were labeled with two different concentrations of CFSE so that they could be tracked in the various organs 5 h later. Fig. 5A shows that in normal mice that received OT1 cells, but were sham primed with PBS-pulsed APCs, the ratio of specific (CFSEhigh) to nonspecific (CFSELow) targets in lymph nodes was comparable to the same ratio before injection. In contrast, mice in which OT1 cells were primed with peptide-pulsed APCs showed a reduction in the percentage of CFSEhigh targets. This shows that the activated OT1 cells were cytotoxic and specifically killed the SIINFEKL peptide-loaded targets. In TLR-4−/− mice, which were immunized with peptide-pulsed APCs, there was a similar specific loss in the peptide-loaded target cell population (Fig. 5A, bottom panel). Fig. 5B shows the percentage of specific target cell lysis in spleen, lymph nodes, and livers of wt and TLR-4−/− mice (n = 6/group). The extent of loss of specific target cells was similar in wt and TLR-4−/− mice in all three organs tested, suggesting that there was no difference in the cytotoxic activity of OT1 cells activated in the two recipients.

The identical activation and function of OT1 cells in wt and TLR-4−/− mice suggested that the reduced accumulation of these cells in livers of TLR-4-deficient mice later in the immune response was the result of a local effect of TLR-4 in the liver on trapping, rather than a systemic effect on priming.

FIGURE 4. The wt and TLR-4-deficient mice are comparable in their ability to proliferate and synthesize IFN-γ. The data show the dilution of CFSE as a function of IFN-γ synthesis on the gated CD45.1+Vα2+ cells (OT1 cells) from spleen, lymph nodes, and liver of wt or TLR-4−/− mice 3 days after they were given unpulsed or peptide-pulsed APCs. The cells were restimulated in culture for 6 h with or without the specific antigenic peptide, SIINFEKL. The data are representative of three separate experiments with three mice per group in each experiment.

FIGURE 5. The OT1 cells activated in both wt and TLR-4-deficient mice are equally cytotoxic. A. CFSE levels in unpulsed and SIINFEKL peptide-pulsed targets before transfer and percentage of OT1 cells (CD45.1) and the two target cell populations (CFSEhigh and CFSELFow) in lymph nodes of wt and TLR-4−/− mice. B. Average percentage of target cell lyses in the lymph nodes (LN), spleen (SP), and liver (L) of wt and TLR-4−/− mice (n = 5/group). The percentage of target cell lyses was calculated using the formula (1 - (% of peptide pulsed target/% of unpulsed targets)) × 100.

The reduced percentage of OT1 cells in the TLR-4−/− liver on day 5 could be attributed to an increased death rate of these cells. To test this possibility, we estimated the percentage of dying cells by measuring caspase-3 activity. Caspase-3 is downstream of both the active (death receptor-mediated) and passive (mitochondrial)
death pathways and hence is an indication of the total cell death regardless of the mechanism. Our experiments showed that there was no difference in the percentage of caspase-3-positive OT1 cells on day 3 or 5 between wt and TLR-4−/− mice (data not shown). This led us to conclude that the decrease in the percentage and numbers of OT1 cells in TLR-4−/− mice was not the result of a higher rate of apoptosis of these cells in TLR-4-deficient mice.

**TLR-4 mutant mice show similar lack of trapping of vSAG-7-activated cells**

Our data suggest that in the absence of TLR-4, the liver loses its ability to trap activated CD8+ T cells efficiently. To test whether this was dependent on the capacity of TLR-4 to engage downstream signaling pathways, we used the TLR-4 mutant strain, C3H/HeJ. Polyclonal activation of Vβ6+ T cells was induced in two strains of C3H mice: the C3H/HeJ (TLR-4 mutant) strain and the C3H/HeOuJ (normal TLR-4) strain. In both cases, Vβ6 T cells were activated with an injection of AKR/J spleen cells, which express the endogenous retrovirus Mtv-7, encoding the superantigen vSAG-7. This procedure causes activation, followed by deletion of both CD4+ and CD8+ T cells expressing Vβ6 as part of the TCR (29). Fig. 6A shows the representative frequencies of Vβ6 CD8+ T cells in the lymph nodes and livers of wt and TLR-4 mutant strains mice on day 0 (preimmunization) and day 8 postimmunization. On day 0, the percentage of Vβ6 CD8+ T cells was comparable in both lymph nodes and livers of the two strains of mice. However, on day 8, fewer activated Vβ6 CD8+ T cells were seen in the livers of TLR-4 mutant mice. Fig. 6B shows the average percentage of Vβ6 CD8+ T cells (as a percentage of the total CD8+ T cells) over time in lymph nodes and livers of TLR-4 mutant and wt mice (n = 6 at each time point for each of the groups). Both C3H/HeJ and C3H/HeOuJ strains of mice showed a comparable clonal expansion in their Vβ6 CD8+ T cell population, followed by deletion in the lymph nodes (Fig. 6B) and spleen (data not shown) over a period of 15 days (top panel). This deletion from the periphery was accompanied by the accumulation of cells in the liver. TLR-4 mutant mice showed a lower accumulation of Vβ6 CD8+ T cells in the liver compared with wt mice, which was more apparent and significant on days 8 and 12. Vβ6 CD4+ T cells also went through activation and deletion, but few of these cells accumulated in the liver, and no significant difference was seen between the two strains of mice (data not shown).

These data argue that TLR-4 does not affect vSAG-induced T cell activation in the lymphoid organs, but is involved in promoting the accumulation of activated CD8+ T cells in the liver. These data support the short-term trapping experiments and the in vivo priming experiments in TLR-4-deficient mice. In all three experimental models we found that TLR-4 promotes the trapping of activated CD8+ T cells in liver.

**Discussion**

The extensive literature on TLRs emphasizes their role in augmenting and initiating innate immune responses. Thus, TLRs are involved in the maturation of specialized APCs, such as DC, the induction of costimulatory molecules, the production of cytokines and chemokines by cells of the innate immune system, and the induction of costimulatory molecules, the production of cytokines and chemokines by cells of the innate immune system, and the resistance of DCs to regulatory T cells (14, 15). However, in recent years, several other aspects of TLR biology have emerged. In the liver, Ag presentation is strongly influenced by LPS, but in an unexpected way; endotoxin down-regulates T cell activation by LSECs, and the CD4+ and CD8+ T cells that are activated by LSECs show a tolerant phenotype (30, 31). Thus, in this context, TLR engagement is immunosuppressive. Similarly, LPS acting on Kupffer cells and LSECs lead to the secretion of the immunosuppressive mediators such as IL-10 and TGF-β (32).

More recently, the recognition of commensally derived products by TLRs has been shown to play an important role in normal intestinal epithelial homeostasis (33). Our data indicate a different function for TLR-4 under noninflammatory conditions; TLR-4 ligands, possibly from the normal enteric flora, have a direct effect on the ability of the liver to trap activated CD8+ T cells.

The central issue in experiments designed to test ideas concerning the influence of TLR-4 on the distribution of circulating CD8+ T cells is the concern that TLR-4-deficient mice might have defects in priming outside the liver, which might have secondary consequences for intrahepatic trapping of cells. To address this concern, we adopted a very simple, direct, short-term, in vivo localization assay in which a mixture of activated and naive CD8+ T cells was i.v. transferred into either TLR-4−/− or normal mice and located by FACS at 2 h. This leads to differential partitioning in the various tissues of a normal mouse. Naïve CD8+ T cells were preferentially localized in spleen and lymph nodes, whereas activated cells were predominantly in liver. In the absence of TLR-4, fewer activated CD8+ T cells were extracted from peripheral
blood into liver, indicating that TLR-4 signaling promotes the localization of circulating activated CD8⁺ T cells to the liver. The diagnostic feature of the liver-specific effect in this assay is the change in the abundance of activated vs resting CD8⁺ T cells in the liver and a reciprocal effect in the blood. It is quite possible that in the absence of TLR-4, the adhesion mechanisms in the periphery are also defective. However, the fact that there were no differences in the small fraction of activated CD8⁺ T cells that had migrated into the spleen and lymph nodes of wt and TLR-4⁻/⁻ mice suggests otherwise. In this experimental model, recipient mice only interact with the input cells for 2 h of the assay, which emphasizes effects on T cell localization over considerations such as priming and survival.

To interpret the consequences of the lack of trapping of activated CD8⁺ T cells in the liver, it was imperative to test this effect in an in situ immune response. However, it was also important to control for the known and unknown defects in priming in TLR-4-deficient mice. To achieve this, we adoptively transferred OT1 cells into either wt or TLR-4-deficient mice and primed using wt, peptide-pulsed APCs. The clonal expansion and proliferation of OT1 cells that were activated in either wt or TLR-4-deficient mice were comparable on day 3. However, at 5 days, fewer activated CD8⁺ T cells were retained in the livers of TLR-4-deficient mice. We tested the hypothesis that there was greater apoptosis of OT1 cells in the absence of TLR-4 in the liver. The lack of any significant difference in the percentage of caspase-3-positive OT1 cells in liver, spleen, or lymph nodes of wt and TLR-4-deficient mice (data not shown) indicated that the TLR-4 effect could not be attributed to differential apoptosis of OT1 cells. We also examined the functional competence of OT1 cells that were activated in either wt or TLR-4-deficient mice and found that they were identical in terms of their ability to produce IFN-γ and cytotoxicity. All of this suggested that the lower numbers of OT1 cells seen in the liver in the absence of TLR-4 are, in fact, due to a difference in the trapping and retention in the liver, rather than to differential priming or survival of these cells elsewhere. The compensatory increase in the percentage of OT1 cells in the spleens of TLR-4⁻/⁻ mice is further evidence of this.

In our adoptive transfer experiments, we transferred OT1 transgenic T cells, which are on a C57BL/6 background, into C57BL/10 congenic recipients. The strains 6 and 10 of C57BL mice (C57BL/6 and C57BL/10) differ at a few minor histocompatibility Ag loci. However, we noticed no difference in the survival (up to 10 wk) or activation status of OT1 transgenic cells in the absence of any stimulation when transferred into either C57BL/6J or C57BL/10SnJ mice. Hence, we concluded that the use of C57BL/6 T cells in C57BL/10 congenic hosts did not compromise the experiments.

To test whether the observed effect was due to signaling downstream of TLR-4 in a normal liver, we used the TLR-4 mutant mouse strain (C3H/HeJ), which can bind LPS, but cannot signal through it. Using a different model of activation (superantigen encoded by an endogenous retrovirus), the TLR-4 mutant mice still accumulated fewer activated cells compared with the wt mice. In both V$_\alpha$SAG-7-mediated activation of Vβ6 CD8⁺ T cells and activation of OT1 cells by SIINFEKL-pulsed APCs, the difference in accumulation of activated CD8⁺ T cells in TLR-4 mutant or -deficient livers was seen at the later phases of the response. In TLR-4 mutant mice, up to day 8, the accumulation of Vβ6 T cells in the liver was comparable to that in control mice. It was when the response began to fade in the periphery that the difference in accumulation in the liver was more apparent.

Our current model to explain these observations is 1) commensally derived products from the gut engage TLR4 in the liver; 2) TLR-4 signaling promotes the expression of adhesion molecules; 3) activated CD8⁺ T cells are retained in the hepatic sinusoids due to these adhesion mechanisms; and 4) such sequestration removes them from the circulating pool. The next task is to determine the molecular mechanisms through which TLR-4 signaling controls intrahepatic T cell localization.

Based on this model we predict that germfree mice, which lack the constant supply of TLR-4 ligands entering the liver through the gut, would show a similar defect in the ability of the liver to trap activated CD8⁺ T cells.

**Disclosures**

The authors have no financial conflict of interest.

**References**