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Intrahepatic Activation of Naive CD4+ T Cells by Liver-Resident Phagocytic Cells

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Naive T cell activation is normally restricted to the lymphoid organs, in part because of their limited ability to migrate into the parenchyma of peripheral tissues. The liver vasculature is unique, however, and circulating leukocytes within the hepatic sinusoids have direct access to liver-resident cells, which include an abundant population of Kupffer cells. It is well accepted that recognition of cognate Ag within the liver leads to naive CD8+ T cell activation in situ, but it is unclear whether the liver also supports naive CD4+ T cell activation. In this study, we show that naive CD4+ T cells can be activated to proliferate in the liver when cognate Ag expression is induced in hepatocytes by recombinant adeno-associated viral vectors. Ag-specific retention and activation of naive CD4+ T cells within the liver are independent of lymphoid tissues but dependent on a clodronate liposome-sensitive population of liver-resident phagocytic cells. To our knowledge, this study provides the first unequivocal evidence that naive CD4+ T cells can be activated in a nonlymphoid organ. It also logical responses during hepatotropic infections and autoimmune liver disease. The Journal of Immunology, 2014, 193: 2087–2095.
studies in which OVA-specific CD4+ T cells (from TCR-transgenic [Tg] OT-II and D0.10.11 mice) were adoptively transferred into mice expressing OVA in the liver, intrahepatic activation or retention of donor CD4+ T cells was not observed (13–16). These studies led to the conclusion that naive CD4+ T cells cannot undergo primary activation within the liver, and this was consistent with the paradigm of naive CD4+ T cell homing and activation being restricted to lymphoid organs. Based on these experiments, it was hypothesized that lack of provision of CD4 help in the liver contributes to failure to develop optimal CD8+ T cell function (13–16).

Using recombinant adenovirus-associated virus (rAAV) vectors expressing an epitope from the mycobacteria Ag85B in hepatocytes, we demonstrate in this study that the ability of the liver to induce activation of naive CD4+ T cells is epitope dependent. We show that naive Tg Tg+ T cells specific for the Ag85B epitope can be selectively recruited to the liver and undergo activation and proliferation following recognition of their cognate Ag within this organ. Both retention and intrahepatic activation are dependent on a population of liver-resident phagocytic cells, suggesting that hepatocyte-expressed Ag could be captured and presented by these cells to naive CD4+ T cells in the liver. To our knowledge, this is the first report showing that naive CD4+ T cells can be activated in a nonlymphoid organ.

Materials and Methods

rAAV vectors and Ags expressed

Ags expressed in this study are detailed in Supplemental Table I. The membrane-bound OVA (mOVA) sequence used to generate rAAV encoding mOVA (rAAV.mOVA) was kindly provided by Dr. Frank Carbone (University of Melbourne), and the generation of this construct was described elsewhere (17). To transduce mice from which Kcs were isolated for in vitro assays, the rAAV vector rAAV.GFP-KVTI expressing GFP and a control self-peptide recognized by the Tg Des TCR (17) was used. mRNA-OMA-Ag85B and GFP-2A-OVA-Ag85B were partially synthesized by GenArt Gene Synthesis (Invitrogen, Mulgrave, VIC, Australia) and subcloned into the pAM2AA expression vector that incorporates a liver-specific human α-1-antitrypsin promoter and two apoE hepatic control regions, flanked by AAV2 inverted terminal repeats (18). rAAV was packaged in HEK293D cells using the pXx6 helper plasmid (courtesy of Dr. R. Jude Samulski, University of North Carolina at Chapel Hill, Chapel Hill, NC) and pseudo-serotyped to type 8 capsid (courtesy of Dr. James Wilson, University of Pennsylvania, Philadelphia, PA). rAAV was purified on cesium chloride gradients, and viral genome copies were determined by quantitative PCR (19). rAAV was administered i.v. i.v. via the tail vein. Each rAAV batch was titrated in vivo, and the dose required to achieve 100% hepatocyte transduction was selected and used in all experiments.

Animals

C57BL/6 (CD45.2+) and B6.SJL-PtprcPep3bBoyJ (CD45.1+) mice were purchased from the Animal Resources Centre (Perth, WA, Australia). P25 mice that express a Tg TCR specific for FQDAYNAAGHHNAVF (Ag85B320-324) presented by I-A (20) were generated by Dr. K. Takatsu (University of Tokyo, Tokyo, Japan) and were a kind gift of Dr. James Triccas (University of Sydney, Sydney, NSW, Australia). OT-II and OT-I T cells express a TCR specific for ISQAVHAAHINEAGR (OVA323–331) presented by I-A (21) and for SIINFEKL (OVA257–264) presented by H-2Kb (22), respectively. P25, OT-II, and OT-I mice were backcrossed to B6.SJL-PtprcPep3bBoyJ mice to obtain CD45.1+ donor cells for some experiments. P25 mice were also crossed to C57BL/6-Tg(UBC-GFP) to achieve 100% hepatocyte transduction was selected and used in all experiments.

Cell harvest for flow cytometric analysis

Livers, spleen, individual LNs, and blood were harvested at the indicated times after adoptive transfer and stained for flow cytometric analysis, as described (4). Abs were from BD Biosciences, unless otherwise stated, and included CD45.1 Horizon V450 (A20), CD4-PE (RM4-5), CD69-PE (H1.2F3), CD62L-PE (MEL-14), CD4-PerCP/Cy5.5 (RM4-5), CD69-PerCP/Cy5.5 (H1.2F3), CD25-allophycocyanin (PC61), CD69-allophycocyanin (H1.2F3), and CD44-allophycocyanin-Cy7 (IM7; BioLegend). Cells were stained with 0.1 μg/ml DAPI (Invitrogen) before acquisition on a BD LSRFortessa flow cytometer (BD Biosciences) and analyzed using FlowJo v9.4.11 software (TreeStar, Ashland, OR). Cell numbers were enumerated using Spherotech AccuCount beads.

Immunofluorescence and confocal microscopy of frozen and thick liver sections

Livers were perfused in situ with PBS, followed by 10% formalin, for 1 h. Mice were treated with a high dose (1 × 1011 viral genome copies) of rAAV.GFP-OVA-Ag85 or control rAAV.GFP-KVTI, which does not express OVA or Ag85 epitopes, as previously described (17). After 2 wk, the livers were excised, chopped into fine pieces, and digested with 1 mg/ml collagenase IV (Sigma-Aldrich) and 280 U/ml DNase I (Roche) at room temperature for 40 min. Cells were filtered (40-µm pore size) through a 4-0 Deknatel silk suture (Teleflex Medical, Stuttgart, Germany), and the skin incision was closed using 4-0 Deknatel silk suture (Teleflex Medical, Stuttgart, Germany), and the skin incision was closed using sutures. Mice were placed on a heating pad until recovery and treated s.c. with buprenorphine (0.03 mg/kg) every 8 h thereafter up to P25 T cell adoptive transfer 1–2 d after surgery.

KC isolation and in vitro T cell activation assay

Mice were treated with a high dose (1 × 1011 viral genome copies) of rAAV.GFP-OVA-Ag85 or control rAAV.GFP-KVTI, which does not express OVA or Ag85 epitopes, as previously described (17). After 2 wk, the livers were excised, chopped into fine pieces, and digested with 1 mg/ml collagenase IV (Sigma-Aldrich) and 280 U/ml DNase I (Roche) at room temperature for 40 min. Cells were filtered (40-µm pore size) through a 4-0 Deknatel silk suture (Teleflex Medical, Stuttgart, Germany), and the skin incision was closed using sutures. Mice were placed on a heating pad until recovery and treated s.c. with buprenorphine (0.03 mg/kg) every 8 h thereafter up to P25 T cell adoptive transfer 1–2 d after surgery.

Purification and adoptive transfer of donor cells

Single-cell suspensions of LN cells from OT-II, P25, or OT-I mice were isolated and labeled with 5 µM CFSE, as described (4), for i.v. injection into the tail vein. Recipient mice were adoptively transferred with 2–8 million donor LN cells from Tg TCR mice. In some experiments, CD4+ T cells from P25 × UBC-GFP mice were purified by incubating LN cells with a mixture of Abs specific for CD4 (YTS169; hybridoma purified at The Centenary Institute), CD11b (M1/70; hybridoma), F4/80 (CLEA-1; hybridoma), CD19 (1D3; BD Pharmingen), and NK1.1 (PK136; BD Pharmingen), followed by negative selection with anti-mouse IgG Dynabeads and anti-rat IgG Dynabeads (Invitrogen) on LD columns using a MidMACS magnet (Milteny Biotec, Macquarie Park, NSW, Australia). CD4+ T cell purity was > 90%.

Cell harvest for flow cytometric analysis

Splenectomy and lymphadenectomy

Mice were treated i.p. with buprenorphine (0.03 mg/kg) and placed under general anesthesia by isoflurane/oxygen delivered via a nose cone. Under a surgical microscope, a midline incision was made to expose the abdominal cavity. Gauze was used to protect the organs, and the left liver lobes were carefully manipulated with cotton buds to expose the portal vein and the underlying portal organ, celiac LN, and first mesenteric LN (24). The vessels supplying these LNs were cauterized using a fine-tip Accu-Temp Cautery (Medtronic Australia, North Ryde, NSW, Australia), and these LNs were excised before replacing the liver lobes. The spleen was carefully exposed by removing the incision, the skin incision was closed, and the spleen was removed. The peritoneum was closed using a 4-0 Deknatel silk suture (Teleflex Medical, Stuttgart, Germany), and the skin incision was closed using staples. Mice were placed on a heating pad until recovery and treated s.c. with buprenorphine (0.03 mg/kg) every 8 h thereafter up to P25 T cell adoptive transfer 1–2 d after surgery.
peptide was added to some cultures. After 4 d of incubation, cells were stained with CD45.1-PE (A20), CD4-Horizon V500, CD69-PerCP-Cy5.5, CD25-allophycocyanin, CD44-allophycocyanin-Cy7, and DAPI. Proliferation of P25 CD4+ T cells (CD45.1+) was analyzed via flow cytometry.

**Results**

**High levels of OVA expression by hepatocytes did not lead to OT-II CD4+ T cell activation**

Previous studies showed that OT-II and D0.10.11 TCR-Tg CD4+ T cells failed to be activated in mice that expressed OVA by transgenesis or by rAAV-mediated transduction (13, 15, 16), suggesting that the liver cannot support primary activation of naive CD4+ T cells. To exclude the possibilities that these findings were either due to tolerance to OVA caused by Ag expression from birth or the low-transduction efficacy of type 2 adenovirus (AAV) vectors resulting in a limited amount of Ag available for presentation, we used rAAV vectors pseudotyped to type 8 capsid that were shown to mediate efficient transduction of 100% of hepatocytes in adult mice (17–19). Hepatocyte-specific expression was achieved using the human α1-anti-trypsin promoter and two ApoE hepatic control regions. Consistent with previous studies (17–19), we achieved high levels of Ag expression in the liver in mice injected i.v. with rAAV encoding for GFP (rAAV.GFP) (Supplemental Table I). We also showed that transgene expression mediated by these rAAV vectors was restricted to the liver (18, 19), whereas all leukocytes, including DCs and macrophages, did not express detectable levels of the transgene (18, 19). Within the liver, GFP was expressed by all hepatocytes but not by F4/80+ KCs, CD31+ liver endothelial cells (mostly liver sinusoidal endothelial cells) or GFAP+ hepatic stellate cells (Fig. 1A) (17).

To express OVA in hepatocytes, C57Bl/6 mice were injected i.v. with rAAV.mOVA (Supplemental Table I). Similar to rAAV.GFP, this vector induced efficient expression of OVA restricted to all hepatocytes but not in F4/80+ KCs and CD31+ endothelial cells (Fig. 1B) (17). Despite abundant OVA expression in the livers of rAAV.mOVA-treated mice (Fig. 1B), OT-II T cells failed to undergo activation when transferred into these animals. OT-II T cells did not upregulate expression of the activation marker CD69 at 3 h after transfer (Fig. 1C), nor did they proliferate in the liver, spleen, or liver-draining celiac LNs by day 3 (Fig. 1D) or day 6 (data not shown) after transfer. There also were no significant differences observed in the accumulation of OT-II T cells in OVA-expressing mice compared with controls (Fig. 1E). It should be noted that, although OT-II T cells were never activated in the spleen, LNs, or liver of most recipient mice treated with the rAAV-expressing vectors, upregulation of CD69 and marginal proliferation (one division of a few cells at day 3) was occasionally detected in some OT-II T cells in the liver-draining LNs. This weak activation was never observed in spleen, non-draining LNs, or liver. This suggests that, although the correct OVA epitope was expressed by hepatocytes, it was not able to drive efficient OT-II activation, even when presented by professional APCs in LNs. To further exclude the possibility that poor OT-II activation was due to a limited amount of epitopes generated by rAAV transduction, we increased the amount of epitopes generated by rAAV transduction, we increased the possibility that poor OT-II activation was due to a limited expression of OVA-Ag85B/mCherry fusion protein (rAAV.mChry-OVA-Ag85) or for a bicistronic sequence encoding for both an OVA-Ag85B fusion protein and GFP (rAAV.GFP-OVA-Ag85) (Supplemental Table I). A standard dose of rAAV.GFP-OVA-Ag85 induced GFP expression on >80–85% of hepatocytes in vivo (Fig. 2A), whereas the mCherry-OVA-Ag85 fusion protein fluoresced in vitro in the packaging cell line but not in hepatocytes in vivo (data not shown). Consistent with our observations that OT-II T cells were not activated in mice treated with rAAV.mOVA (Fig. 1B–D), OT-II T cells did not proliferate when transferred into mice treated with rAAV.GFP-OVA-Ag85 (Fig. 2B, 2C) or rAAV.mChry-OVA-Ag85 (Fig. 2D), despite the high transduction efficiencies. In contrast, P25 CD4+ T cells underwent several divisions by days 2 and 3 in mice treated with either rAAV vector (Fig. 2B–D). The activation of P25 T cells was Ag85B specific, because they were not activated in mice treated with rAAV.mOVA, which encoded mOVA without the Ag85B epitope (Figs. 3, 4). These differences between OT-II and P25 T cells were due to different compositions of OT-II and P25 cells pretransfection: LN cells isolated from OT-II and P25 mice contained similar percentages of CD4+ CD25high T cells and NKT cells and the same ratio of naive/activated T cells (Supplemental Fig. 1).

Proliferation of P25 CD4+ T cells in mice expressing OVA-Ag85 demonstrates that a cognate Ag expressed by hepatocytes can be presented to naive CD4+ T cells and is able to elicit efficient activation and proliferation.

**Naive P25 T cells recognizing cognate Ag expressed by hepatocytes were retained and activated in the liver**

To determine the initial site of naive CD4+ T cell activation, we investigated the kinetics of primary CD4+ T cell activation. P25 T cells were transferred into mice treated with rAAV.mChry-OVA-Ag85, and their activation status in the blood, liver, LNs, and spleen was assessed between 1 and 3 h posttransfer. P25 T cells were found in all of these compartments, but recently activated CD69high P25 T cells were detected only in the liver and in one or two of the main liver-draining LNs: the portal and celiac LNs (24) (Fig. 3A). The absence of recently activated P25 T cells in the blood suggested that CD69high P25 T cells identified within the liver were not derived from a recirculating population of extrahepatically activated cells. There also were increased numbers of P25 T cells in Ag85B-expressing livers compared with control livers (Fig. 3B, 3C), indicating selective
FIGURE 1. OT-II T cells failed to undergo activation and proliferated in mice expressing high levels of OVA on hepatocytes. (A) GFP expression in C57BL/6 livers after treatment with rAAV.GFP (7 d prior). Immunostaining of GFP-expressing liver sections with rat anti-F4/80, rat anti-CD31, and rabbit anti-GFAP Abs and Alexa Fluor 594–anti-rabbit IgG or Alexa Fluor 647–anti-rabbit IgG demonstrated that GFP was highly expressed in hepatocytes but was not expressed in KCs, liver sinusoidal endothelial cells (LSEC), or hepatic stellate cells (HSC). Mice treated with PBS did not display any fluorescence (data not shown). Images are representative of two mice/treatment group. Low magnification (left panels) and high magnification (right panels) are shown. Scale bars, 50 μm. (B) mOVA expression in C57BL/6 livers after treatment with rAAV.mOVA or control rAAV.Kb (7 d prior) was detected by immunostaining with anti-F4/80 Ab and Alexa Fluor 594–anti-rat IgG and with rabbit-anti-OVA (clone 1670) and Alexa Fluor 488–conjugated anti-rabbit IgG (green). Images are representative of three mice/treatment group. Scale bars, 100 μm. (C) LN cells from OT-II mice were transferred into C57BL/6 recipients administered rAAV.mOVA 7 d prior. At 3 h posttransfer, organs were harvested, and CD69 expression was measured on gated CD45.1+CD4+ OT-II T cells. Graphs are representative of six rAAV.mOVA-treated mice (black line) and four untreated C57BL/6 controls (shaded) in two independent experiments. (D) CFSE-labeled OT-II LN cells were transferred into C57BL/6 mice administered rAAV.mOVA or rAAV.YmII (7–14 d prior), 200 μg of OVA (30 min later), or untreated controls. Three days posttransfer, CFSE profiles of OT-II cells (rectangle; CFSE+CD4+ cells; values indicate percentage of DAPI events) were analyzed. Plots represent five mice/group from two independent experiments. (E) Percentages of OT-II T cells (CD45.1+CD4+) recovered from the liver 3 d after transfer of OT-II LN cells into C57BL/6 mice treated with rAAV.mOVA (7–21 d prior) or untreated controls. (F) CFSE-labeled OT-I LN cells were transferred into C57BL/6 mice administered rAAV.mOVA or rAAV.YmII (7–14 d prior) or untreated controls. Three days posttransfer, CFSE profiles of OT-I cells (rectangle; CFSE+CD8+ cells; values indicate percentage of DAPI events) were analyzed. Representative plots of three mice are shown.
retention of CD4+ T cells following cognate Ag recognition. In contrast, P25 T cell numbers within spleens and pooled LNs did not differ significantly (Fig. 3B). In the first 3 h posttransfer, the proportion of intrahepatic P25 T cells expressing CD69 progressively increased (Fig. 3D). This increase occurred in parallel in both the liver and the celiac LN (Fig. 3D), suggesting that activation occurred independently in both liver and draining LNs. We did not detect specific upregulation of a range of adhesion molecules and chemokine receptors on P25 T cells from the liver and draining LNs as a consequence of Ag encounter (Supplemental Fig. 2). However, T cells in the draining LNs expressed higher levels of some chemokine receptors (CXCR3, CXCR5, CCR7) and integrins (CD49e, CD49f) compared with P25 T cells in the liver. Again, the expression of these receptors was not Ag specific (Supplemental Fig. 2).

To formally exclude the possibility that the intrahepatic CD69high P25 T cells originated from the pool of T cells primed by Ag encounter within the liver-draining LNs, we surgically removed the liver-draining LNs and spleens from rAAV-treated mice 1 or 2 d before adoptive transfer of P25 T cells. Intrahepatic P25 T cell activation and proliferation were still detected in the absence of the spleen and the liver-draining LNs (Fig. 3E).

Collectively, these findings indicate that the liver is able to support Ag-specific activation of naive CD4+ T cells independently of lymphoid tissues.

Intrahepatic P25 activation was mediated by liver phagocytic cells

Liver phagocytic cells (mostly KCs) make up the largest proportion of intravascular MHC class II+ cells in the liver and were shown...
FIGURE 3. Primary activation of P25 CD4+ T cells occurs in the liver and liver-draining LNs. (A) Expression of CD69 on P25 CD4+ T cells (CD4+CD45.1+Vb11+) isolated from individual LNs and organs at 1.5 h after transfer of P25 LN cells into C57BL/6 mice treated 7 d prior with rAAV.mChry-OVA-Ag85 (black line) compared with untreated controls (shaded graphs). Representative plots of three mice are shown. (B) Absolute numbers of P25 T cells (CD45.1+CD4+CD44low) in the liver 1.5 h after transfer of unlabeled P25 LN cells into C57BL/6 mice treated with rAAV.mChry-OVA-Ag85 (28 d prior) or rAAV-mOV A (28 d prior). Data are mean ± SD of three mice/group. (C) Higher frequencies of GFP+ P25 T cells were seen in the livers of rAAV.mChry-OVA-Ag85–treated mice compared with controls. Scale bars, 150 μm. (D) Kinetics of CD69 upregulation on P25 T cells (CD45.1+CD4+CD44low) from the liver or celiac LN at the indicated times after 3–7 × 10^6 unlabeled P25 LN cells were transferred into C57BL/6 mice treated with rAAV.GFP-OVA.Ag85 (5 d prior). Percentages of CD69high cells among CD44lowCD45.1+CD4+ P25 T cells are shown. Data were pooled from five different experiments and are expressed as mean ± SD of three to five mice/time point. (E) Intrahepatic activation of P25 T cells was measured in C57BL/6 mice treated with rAAV.mChry-OVA-Ag85 (14–34 d prior) that had undergone surgical removal of spleen and three liver-draining LNs (celiac, portal, first mesenteric; Splx/LNx) 2–3 d before T cell transfer. CFSE-labeled P25 LN cells were transferred into mice, and livers were harvested at 3 h (upper right panel) or 44 h (lower left panel) after T cell transfer. Graph of CD69 expression on P25 T cells (CD44lowCD45.1+CD4+) (upper left panel) and mean ± SD of percentages of CD69high cells (upper right panel). Data are from n = 2 mice/group (rAAV treated, no surgery) or n = 3 mice/group (untreated, rAAV+Splx/LNx). CFSE and CD69 profiles of P25 T cells (CD44lowCD45.1+CD4+) (lower left panel). The percentages of P25 T cells that were CFSEhighCD69high (activated but not yet divided) and the percentages of CFSElow (divided) cells were summed. Data are mean ± SD of three mice per group. *p < 0.05; ns, not significant by ANOVA and Bonferroni post hoc analysis. MLN, mesenteric LNs.
FIGURE 4. Clodronate liposome treatment inhibited the retention and activation of P25 T cells in Ag-expressing livers. (A) P25 T cells (in green and indicated by arrows) interacted with F4/80+ KCs (in red) in Ag85B epitope-expressing livers. Purified CD4+ T cells from LN of P25 × Ubiquitin-eGFP mice were transferred into C57BL/6 recipients treated with rAAV.mChry-OVA-Ag85 (21 d prior). Three hours after T cell transfer, livers were harvested; formalin-fixed sections were stained with rat anti-F4/80 and Alexa Fluor 594–anti-rat IgG. Most P25 CD4+ T cells were seen interacting with F4/80+ cells. Scale bar, 20 μm. (B) Effect of clodronate liposome treatment (200 μl i.v. daily for 2 d) on C57BL/6 mice treated with rAAV.GFP (21 d prior) compared with treatment with PBS liposomes. Formalin-fixed liver sections were stained with Abs against F4/80 and MHC class II and detected with Alexa Fluor 594–anti-rat IgG. Scale bars, 100 μm. (C) P25 T cell retention 3 h after transfer of CFSE-labeled P25 LN cells into C57BL/6 mice treated with rAAV.GFP-OVA-Ag85 or rAAV.mOVA (7 d prior) and that received daily doses of either clodronate liposomes or PBS liposomes (200 μl i.v.) for 2 d before P25 transfer. Absolute numbers of P25 T cells (CD45.1+CD4+CD44low) in the liver at 3 h; data are mean ± SD from three mice/group. (D) P25 activation was measured by assessing CD69 expression on P25 T cells (CD45.1+CD4+CD44low) 3 h after transfer of CFSE-labeled P25 LN cells into C57BL/6 mice treated with rAAV.GFP-OVA-Ag85 or rAAV.mOVA (7 d prior) and that received daily doses of either clodronate liposomes or PBS liposomes (200 μl i.v.) for 2 d before P25 transfer. Graphs represent one of three mice/group (left panel); the three liver-draining LNs were pooled for this experiment. The percentage of CD69highCD45.1+CD4+CD44low P25 T cells in the liver and pooled celiac and portal LNs (right panel). Data are mean ± SD from three mice/group. ***p < 0.001, *p < 0.05 ANOVA and Bonferroni post hoc analysis.
to induce tolerance after portal venous administration of alloantigen (26), whereas mouse hepatocytes do not normally express MHC class II. Therefore, we hypothesized that KCs were important for naïve CD4+ T cell activation in the liver, especially considering their relatively homogeneous distribution throughout the liver and the high efficiency of P25 T cell activation (Fig. 3C). Indeed, direct visualization of intrahepatic P25 T cells following their adoptive transfer indicated that these cells established intimate contacts with F4/80-expressing cells with a morphology consistent with KCs (Fig. 4A). To test whether KCs were important for inducing P25 T cell activation, we injected rAAV-treated mice with clodronate liposomes before T cell transfer at an i.v. dose that depletes KCs while sparing hepatic CD11c<sup>high</sup> DCs (28). This treatment resulted in efficient specific depletion of F4/80<sup>+</sup> cells (Fig. 4B). Ag-specific retention of P25 T cells in Ag85B-expressing livers was significantly reduced after clodronate liposome treatment compared with controls treated with PBS liposomes (Fig. 4C). The early activation of P25 T cells in the liver also was significantly reduced after clodronate liposome treatment (Fig. 4D). In contrast, clodronate liposome treatment did not affect P25 activation in the liver-draining celiac LN (Fig. 4D). To demonstrate that phagocytic cells could capture and present hepatocyte-expressed Ag to CD4+ T cells, cells that expressed a phenotype consistent with KCs (F4/80<sup>+</sup> CD11c<sup>−/−</sup> CD11b<sup>+</sup>) were sorted by flow cytometry from the livers of rAAV-treated mice and tested for their ability to activate P25 CD4+ T cells in vitro. KCs from rAAV-GFP-OVA.Ag85–treated mice expressed MHC class II and induced efficient activation of P25 T cells (Fig. 4E), suggesting that Ag85-derived peptide was presented appropriately on MHC class II for CD4+ T cell activation. Interestingly, strong P25 T cell activation was observed when all KCs were loaded with exogenous P25 peptide, whereas T cell activation by KCs was not potentiated by the addition of exogenous IL-2 (Fig. 4E). These results suggest that the frequency of intrahepatic phagocytic cells expressing hepatocyte-derived epitopes via MHC class II could be low in vivo.

Collectively, these results suggest that KCs have an important role in naïve CD4+ T cell activation in the liver.

**Discussion**

This study demonstrates that an Ag expressed by hepatocytes can be captured by phagocytic cells (most likely KC), degraded into peptides able to bind MHC class II molecules, and form a complex that promotes efficient activation of circulating naïve CD4+ T cells.

Naïve T cells have been detected in the lung, liver, brain, testes, and pancreas (29–31), suggesting that primary CD4+ T cell activation could potentially occur outside of lymphoid tissues. Although some studies suggested that naïve CD4+ T cells occur in the lung in response to allergen challenge (32), as well as in the gut after bacterial infection (33), none of these studies demonstrated primary activation in these organs without ambiguity. To our knowledge, our study is the first demonstration of primary CD4+ T cell activation in a nonlymphoid organ. This study challenges the current paradigm, which holds that naïve CD4+ T cells have restricted access to nonlymphoid organs and can only undergo primary activation in secondary lymphoid organs (2, 3, 34–36). This is not totally surprising, because the liver is home to a large repertoire of immune cells, and its unique architecture permits direct contact of circulating T cells with liver-resident cells (reviewed in Refs. 4, 12).

That activated CD4+ T cells detected in the liver had been activated in situ, rather than derived from cells that recirculated to the liver after activation in lymphoid tissues, was demonstrated in mice in which the spleen and celiac and portal LNs were surgically removed. P25 T cell activation in the liver remained unaffected by the surgery, whereas no activation was detected in any of the remaining mesenteric LNs or more distant LNs (data not shown). In addition to being activated in the liver, P25 CD4+ T cells were activated concomitantly in the liver-draining LNs (Fig. 3D), suggesting intranodal activation rather than recirculation from the liver. This was also demonstrated by the ability of draining LN cells from transduced animals to elicit in vitro activation of naïve CD4 P25 T cells (Fig. 4E).

Previous studies investigating the ability of the liver to support primary activation of CD4+ T cells concluded that naïve CD4+ T cells failed to undergo intrahepatic activation. We propose that the findings were confounded by the use of OT-II TCR-Tg CD4+ T cells that, as shown in the current study, often were unable to be activated in situ. Why OT-II and DO11.10 CD4+ T cells failed to undergo intrahepatic activation is not entirely clear. One possibility is that hepatocytes fail to generate a functional OT-II epitope as the result of proteolytic cleavage of the peptide recognized by OT-II T cells. This might explain why DO11.10-Tg T cells that recognize the same nine-residue peptide as OT-II T cells (37) also were not activated in the liver of mice treated with rAAV-OVA (16). However, the occasional activation of OT-II T cells that we detected in the liver-draining LNs of a few of our rAAV-treated mice argue against this possibility, at least in the case of vectors used in the current study. Based on studies showing that recruitment of CD4+ T cells into proliferation and differentiation in the periphery requires TCR:pMHC-II interactions of sufficient avidity (38, 39), we favor the hypothesis that the different responses of OT-II and P25 T cells were related to different TCR affinities. The affinities of OT-II and P25 for their ligands have never been compared, although the OT-II TCR is generally thought to be a low-affinity receptor based on the low efficiency of positive selection of OT-II T cells in tyrosine-kinase Itk-deficient–mice, compared with other CD4+ TCR-Tg clones assessed (40). Consistent with the hypothesis that the divergent outcomes observed were due to differing affinities, P25-Tg CD4 T cells underwent more cell divisions than did OT-II–Tg CD4 T cells when equimolar amounts of relevant peptide were injected 3 d prior (data not shown).

Our results indicate that intrahepatic activation and retention of CD4+ T cells were less efficient than those observed for CD8+ T cells (17) (data not shown) and that CD4+ T cells were activated more efficiently in the draining LNs compared with the liver. This is likely due to the requirements for CD4+ T cells to overcome the strong shear force of blood flow in the sinusoids and to be activated by interspersed APCs that do not necessarily express the full plethora of adhesion and costimulatory molecules required for naïve T cell activation. The liver is also a large organ compared with LN, and it is unclear what percentage of KCs or phagocytic cells in the liver had captured and processed hepatocyte-derived Ag. In contrast, LNs are specialized compartments of CD4+ T cell activation in which T cells do not have to overcome the shear force of blood flow, and T cells migrate to specialized regions for efficient activation by professional APCs that express the requisite set of adhesion and costimulatory molecules for optimal naïve T cell activation. This would result in the recruitment of a higher number of T cells into division and might explain the difference between liver and LNs observed in this study.

The primary role, function, and fate of CD4+ T cells activated in the liver are unknown, and future studies are needed to clarify whether these cells are involved in immune surveillance or tolerance induction or have other influences on the quality of liver immune responses. However, this study raises the possibility that systemic tolerance observed in animal models in association with the targeting of Ag expression to hepatocytes (41–44) may relate,
at least in part, to direct activation of naive CD4+ T cells within the liver. To our knowledge, this model is the first in which naive CD4+ T cell responses are elicited in the liver in response to cognate Ag and, thus, represents a valuable tool for further studies to gain mechanistic insight into how CD4+ T cells specific for Ag expressed in the liver participate in protective or pathological responses, such as those occurring during hepatotropic infections and autoimmune liver disease.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References

The authors have no financial conflicts of interest.

References

**Acronym:** Details of construct.

<table>
<thead>
<tr>
<th><strong>GFP:</strong></th>
<th>GFP gene cloned into pAM2AA.</th>
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<tr>
<td><strong>mOVA:</strong></td>
<td>First 118 amino acids of the human transferrin receptor trans-membrane domain linked to OVA139-385, digested by HindIII from pBlueRIP-OVA (Kurts C et al, J Exp Med 1997, 186:239-245) into pAM2AA.</td>
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<td><strong>YmII:</strong></td>
<td>cDNA sequence comprising ten ISQAVHAHAHEINEAGR (OVA323-339) epitopes and one each of SIINFEKL (OVA257-264), SSIEFARL HSV gB498-505 and ASNENMDAM HA MHC-I epitopes fused to yellow fluorescent protein amplified by 5’ and 3’ primers incorporating EcoRI and HindIII from the pBlueRIP-EYFP-YMII plasmid (Carbone, Heath) and cloned into pAM2AA.</td>
</tr>
<tr>
<td><strong>mChry-OVA-Ag85:</strong></td>
<td>murine Ig κ-chain leader sequence (residues 8-28), HA tag (YPYDVPDYA) from human influenza haemagglutinin HA98-106 (39-37), OVA250-344 (38-140), Ag85B224-325 (141-242), Myc tag EQKLISEEDL (243-253), mCherry (254-472) Myc tag (471-482), platelet-derived growth factor receptor trans-membrane domain (483-532).</td>
</tr>
<tr>
<td><strong>GFP-OVA-Ag85:</strong></td>
<td>Enhanced GFP with stop codon removed (1-241), GGSG linker (242-245), F2A sequence (246-267), TG linker (268-269), MARCKS membrane localisation sequence MGCCFSKT (270-277), GSG linker (278-280), HA tag YPYDVPDYA (281-289), OVA239-344 (290-395), Ag85B224-325 (396-497).</td>
</tr>
<tr>
<td><strong>K</strong>:</td>
<td>MHC class I H-2Kb gene into pAM2AA.</td>
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**Supplementary Table I:** Details of different rAAV constructs used in the study.
Supplementary Figure 1: Phenotypic analysis of donor cells isolated from the LNs of OT-II and P25 Tg lines used for T cell transfer. Lymphocytes from the LNs of OT-II and P25 Tg mice were purified and stained with antibodies specific for CD3, CD4, NK1.1, CD8, CD11b, CD44, CD25, Valpha2 and CD127. Expression of these different markers was compared and revealed no major difference between the two lines.
**Supplementary Figure 2:** Expression of different adhesion molecules by T cells isolated from the LNs and livers of mice treated with rAAV vectors. B6 mice were injected i.v. with $1 \times 10^{11}$ vgc of rAAV.GFP-OVA-Ag85 or rAAV.GFP-KVIT. 14 days later, $5 \times 10^6$ P25 LN cells were adoptive transferred into mice i.v. Three hours later, leukocytes were isolated from the livers and the liver-draining LNs and stained with CD45.1-Horizon V450, CD4-Horizon V500, CD44-APC-Cy7, CXCR5-biotin (2G8, BD Pharmingen), CCR7-biotin (4B12, eBioScience), CD49f-biotin (eBioGoH3, eBioScience), beta7-FITC (M293, BD), CD49d-FITC (R1-2, eBioScience), CD49b-FITC (DX5, BD), CXCR3-PE (CXCR3-173, eBioScience), CD49e.PE (5H10-27 (MFR5), Biolegend), CD29-PE (HMb-1, eBioScience), CD11a-APC (M17/4, eBioScience), BTLA-APC (8F4, eBioScience), CXCR6-APC (FAB2145A, R&D), and avidin–PerCP–Cy5.5. Adhesion molecule and chemokine receptor expression was determined on CD45.1+CD4+CD44lo naïve or recently-activated P25 T cells.