Adult Human Liver Contains CD8^{pos} T Cells with Naive Phenotype, but Is Not a Site for Conventional $\alpha\beta$ T Cell Development[1]

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Normal adult human liver (AHL) contains populations of unconventional lymphocytes that have been shown in the mouse to mature locally. The presence of lymphoid progenitors together with IL-7, recombinase-activating gene, and pre-TCR-α expression in AHL suggests similar local T cell development activity in humans. Flow cytometry was used to characterize potentially naive hepatic $\alpha\beta$-T cells. We looked for evidence of TCR-$\alpha\beta$ cell development in AHL by quantifying δ deletion TCR excision circles (TRECs) in CD3^{pos} populations isolated from the liver and matched blood of eight individuals. Phenotypic analysis of hepatic T cells suggests the presence of Ag-inexperienced populations. TRECs were detected in all blood samples (mean, 164.10 TRECs/μg DNA), whereas only two hepatic samples were positive at low levels (59.40 and 1.92). The relatively high level of CD8^{pos} T cells in these livers with a naive phenotype suggests that in addition to its role as a graveyard for Ag-specific activated CD8^{pos} T cells, naive CD8^{pos} T cells may enter the liver without prior activation. The almost complete absence of TRECs suggests that normal AHL is not a site for the development of conventional $\alpha\beta$ T cells. *The Journal of Immunology,* **2004, 172: 5980–5985.**

The lymphocyte composition of normal liver in both mice and humans includes large numbers of cells with powerful innate and adaptive potential that can be targeted against infectious or malignant stimuli (1–3). The presence of lymphocytes in disease-free liver may be due solely to inflammatory processes induced by infection, ischemia, or drug damage (4). Indeed, the liver has been postulated to be a site for the elimination of activated peripheral T cells by apoptosis; thus, lymphocytes in disease-free liver may simply be an artifact secondary to its role in peripheral clearance of Ag-experienced CTLs (5). However, studies in mice have established that hepatic lymphocytes play an important role in organ-specific immunity, and that some of these lymphocytes, including NKT cells, can differentiate locally in the liver (3, 6, 7). Rearrangement of the murine preferred NKT TCR (Vα14-Jα281) occurs in the liver at 2.5 times the frequency of that in the thymus; another rearrangement Vα1.1-Jα281 was undetectable in the liver, although it was abundant in the thymus, suggesting an extrathymic origin for NKT cells. However, the development origins of NKT cells remain controversial, as both intrathymic and extrathymic pathways of NKT cell differentiation have been proposed (7, 8).

Evidence supports the existence of similar T cell development activity in normal adult human liver (AHL).[4] Human hepatic T lymphocytes include a large number of NKT-like cells whose phenotypic and functional characteristics resemble those of murine hepatic-derived innate T cells (2, 9). Recombinase-activating gene 1 (RAG1) and RAG2, the molecular machinery required for T cell development, and pTα, a chaperone involved in early $\alpha\beta$-T cell development, are expressed in lymphoid populations derived from normal AHL (10). The liver is also a rich source of IL-7 (11), which has been shown to be critical for normal T cell development (12). In addition, extramedullary erythropoiesis in the liver and reconstitution of multilineage hemopoiesis by donor-derived cells in patients with normal bone marrow function have been reported after liver transplantation (13). Finally, normal AHL contains functional stem cells (14–16), >50% of which express markers of activation and up to 70% of which are lymphoid progenitors (16). Taken together, these studies argue for an active lymphopoietic role for normal AHL, although direct evidence of ongoing T cell development in the liver is presently lacking.

The cell-specific event that identifies developing T cells is the generation of a TCR. The genes that code for the TCR are not contiguous in the germline, but exist as a number of nonfunctional V, J, and, in some cases, D gene segments. Each unique Ag receptor is generated by random rearrangement of these gene segments, a process known as V(D)J recombination (17). Recent studies using a technique that targets DNA by-products (TCR excision circles (TRECs)) produced as a result of ongoing V(D)J recombination in $\alpha\beta$ T cells have provided direct evidence that adult humans can produce T cells de novo (18–20) despite the reduced amount of functional thymus tissue. The TCR-δ locus lies within the α locus; thus, the initiating events for rearrangement at the TCR-α locus involve deletion of the TCR-δ locus. In 70% of conventional $\alpha\beta$ T cells, the same noncoding sequences (δRec and ψJα) combine to effect δ deletion (21). The intervening DNA is

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[4] Abbreviations used in this paper: AHL, adult human liver; HMNC, hepatic mononuclear cell; RAG, recombinase-activating gene; TREC, TCR excision circle.

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spliced out and circularized. The stable, nonreplicating, circular excisional DNA products, termed TRECS, generated by δ deletion can be measured in a single quantitative PCR (18). As TRECs do not replicate on cell division, they are found only in thymocytes and naive T cells, thus δ deletion TRECS represent a direct index of de novo conventional αβ-T cell generation (22).

In this study flow cytometry was used to characterize potentially naive αβ-T cells isolated from normal donor liver and matched peripheral blood samples, and quantification of δ deletion TRECs was used to obtain evidence of recent rearrangements at the TCR-α locus. We show that normal AHL contains significant populations of CD8^{pos} αβ-T cells that display a naive phenotype. However, quantification of δ deletion TRECs provides evidence that normal AHL is not a site of ongoing conventional αβ T cell development.

Materials and Methods

Tissue samples

Normal liver wedge biopsies ($n = 8$; mean age, 47 years; range, 20–47 years; five men and three women) were obtained from donor organs before liver transplantation. Donor organs were extensively perfused with University of Wisconsin solution before obtaining the biopsy. In all cases, 10 ml of matched venous blood was collected in lithium-heparin tubes. The ethics and medical research committee at St. Vincent's University Hospital (Dublin, Ireland) granted approval for the study.

Isolation of hepatic and blood mononuclear cells

Hepatic mononuclear cells (HMNCs) were prepared from liver biopsy samples using mechanical and enzymatic disruption as described previously (23). PBMC were prepared over Lymphoprep (Nycomed, Norway). Cell yields and viability were assessed microscopically by ethidium bromide/acridine orange staining. Cell suspensions were diluted to 1×10^6 cells/ml in RPMI 1640 (Life Technologies, Paisley, Scotland). One million cells (1 ml) were used for flow cytometric analysis, and a second 1-ml fraction was used for CD3 enrichment and subsequent TREC analysis.

Flow cytometric analysis

A panel of mAbs (all supplied by BD Biosciences, Oxford, U.K.) was used to characterize hepatic and matched peripheral blood T lymphocytes. Cell suspensions were stained with a range of fluorescence-labeled mAbs that included anti-CD3-PerCP (clone SK7), anti-TCR-αβ-FITC (clone WT31), anti-TCR-γδ-PE (clone 11F2), anti-CD4 (PE/PerCP; clone SK3), anti-CD8-PerCP (clone SK1), and anti-CD45-RO-PE (clone UCHL-1). HMNC and PBMC preparations were stained for three-color flow cytometric analysis as described previously (23). All samples were also stained with the appropriate isotype-matched control Abs. Acquisition and analysis were conducted using a FACScan flow cytometer and CellQuest software (BD Biosciences). CD3 or TCR-αβ-positive cells were gated using FL3 (PerCP, CD3^{pos}) or FL1 (FITC, TCR-αβ^{pos}) and side scatter (granularity) parameters. Ten thousand CD3^{pos}/TCR-αβ^{pos} events were acquired for each stain. The levels of staining for the other two fluorescence markers (FITC and PE or PE and PerCP) above those observed with isotype-matched control Abs were analyzed within the CD3^{pos} or TCR-αβ^{pos} population. Further characterization of the activation status of hepatic CD8^{pos} population ($n = 6$) was conducted by gating on CD8^{pos}CD45RO$^{neg/pos}$ populations, and histogram analysis was used to determine the expression of CD3, CD27 (FITC, clone L128), HLA-DR (FITC, clone L243), and CD69 (FITC, clone L78).

Enrichment of CD3^{pos} cells

CD3^{pos} cells were positively selected from HMNC and PBMC fractions using the MiniMacs CD3-MicroBead system according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were stained before (whole fraction) and after (positive and negative fractions) separation with anti-CD3-FITC and IgG-FITC (negative control), and the purity of the separated fractions was assessed by flow cytometry. Cell yields were assessed by ethidium bromide/acridine orange staining. Mean yields were 256,250 and 531,250 CD3^{pos} cells from a starting number of 1 million HMNCs and PBMCs, respectively. The lower yields from liver were due to tissue debris in the samples, which necessitated two additional wash steps. The purity of CD3^{pos} cells separated from liver and matched blood was >80% in all cases, as assessed by flow cytometry (Fig. 1).

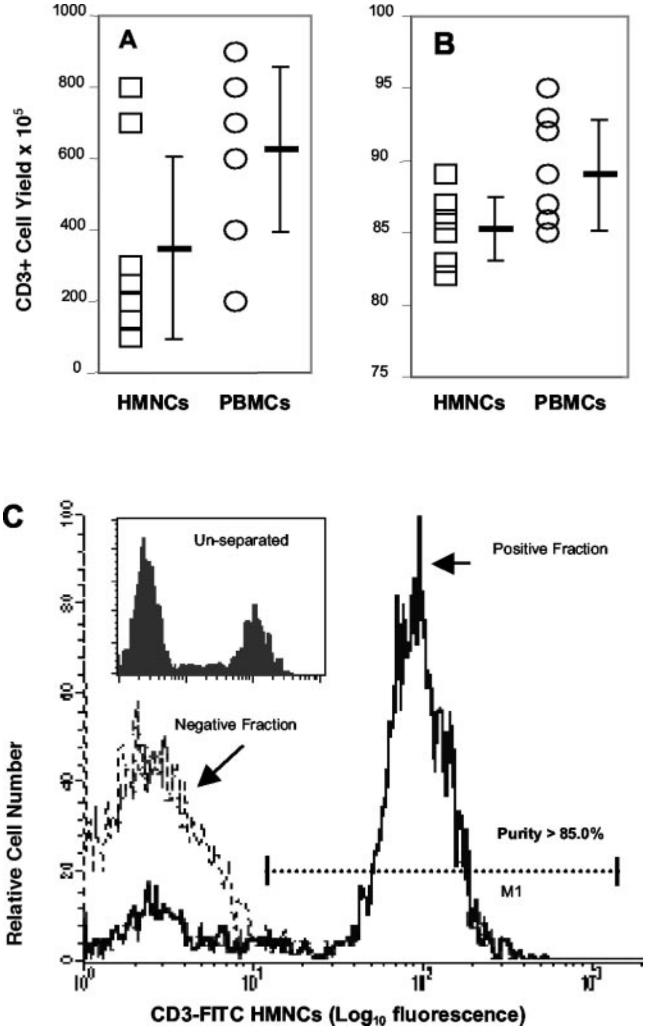

FIGURE 1. Purity and yields of hepatic and blood CD3-enriched cells. *A*, Cell yields after CD3 enrichment. *B*, The viability of CD3-enriched cells as a percentage of the total isolated cells. The error bars show the mean ± SD. *C*, The histogram shows one hepatic sample before enrichment for CD3 (*inset*) and the positive (solid black line) and negative fractions (dotted black line) after enrichment. In the whole HMNC fraction, before separation 40.30% of cells are CD3^{pos}. After enrichment, in the positive fraction the percentage of CD3^{pos} cells increased to 85.99%, whereas 9.73% of the negative fraction expressed low levels of CD3.

TREC analysis of CD3-enriched cells

A quantitative real-time PCR assay was used to quantify the number of δ deletion (signal joint) TRECS in the CD3-enriched cell populations isolated from HMNC and PBMC samples as described previously (18, 20). One microgram of human genomic DNA is equivalent to 150,000 cells.

Statistical analysis

Differences between groups were assessed using two-tailed paired Student's *t* test; $p < 0.05$ was taken as significant. Spearman rank was used for correlation analysis.

Results

Phenotypic analysis of hepatic and matched blood TCR and coreceptor expression

Flow cytometric analysis was used to characterize T cells derived from liver and matched blood samples with respect to TCR-αβ/γδ gene expression. A significantly higher proportion of hepatic T cells express TCR-γδ than matched peripheral blood T cells (mean, 11.12 vs 5.64%; $n = 8$; $p < 0.02$). The TCR-$\alpha\beta^{\mathrm{pos}}$ populations were further characterized with respect to CD8 and CD4 coreceptor expression. CD8$^{\mathrm{pos}}$ T cells occur more frequently in hepatic TCR-$\alpha\beta^{\mathrm{pos}}$ cell populations than in peripheral blood (mean, 68.37 vs 33.27%; $n = 8$; $p < 0.0004$). Double negatives (T cells that express neither CD8 nor CD4 coreceptors) were also significantly increased in the liver-derived TCR-$\alpha\beta^{\mathrm{pos}}$ populations (mean, 5.49 vs 1.25%; $n = 8$; $p < 0.02$). However, no statistically significant difference was observed in the double-positive (CD8$^{\mathrm{pos}}$CD4$^{\mathrm{pos}}$) TCR-$\alpha\beta^{\mathrm{pos}}$ populations (Fig. 2).

Naive $\alpha\beta$ T cell populations

Memory or activated T cells often coexpress the CD45RA and CD45RO isoforms of the pan-leukocyte cell surface Ag. We therefore used the complete absence of CD45RO as a more reliable marker of naive T cells. In this study the naive (CD45RO$^{\mathrm{neg}}$) or activated/memory (CD45RO$^{\mathrm{pos}}$) status of TCR-$\alpha\beta^{\mathrm{pos}}$ subpopulations was determined. The majority of CD45RO$^{\mathrm{neg}}$ $\alpha\beta$ T cells isolated from liver samples coexpressed the CD8 molecule (mean, 27.07%; range, 13.26–41.73%). Circulating CD8$^{\mathrm{pos}}$CD45RO$^{\mathrm{neg}}$ $\alpha\beta$ T cells were detected at a significantly lower level in matched samples (mean, 19.44%; range, 7.28–33.59%; $p < 0.05$). Naive TCR-$\alpha\beta^{\mathrm{pos}}$CD4$^{\mathrm{pos}}$ cells were found in small proportions in hepatic preparations (mean, 4.39%; range, 2.05–10.74%), suggesting that the majority of the CD4$^{\mathrm{pos}}$ TCR-$\alpha\beta$ cells in the liver had previously encountered Ag. In contrast, a significantly higher proportion of CD4$^{\mathrm{pos}}$ $\alpha\beta$ T cells in matched blood samples was negative

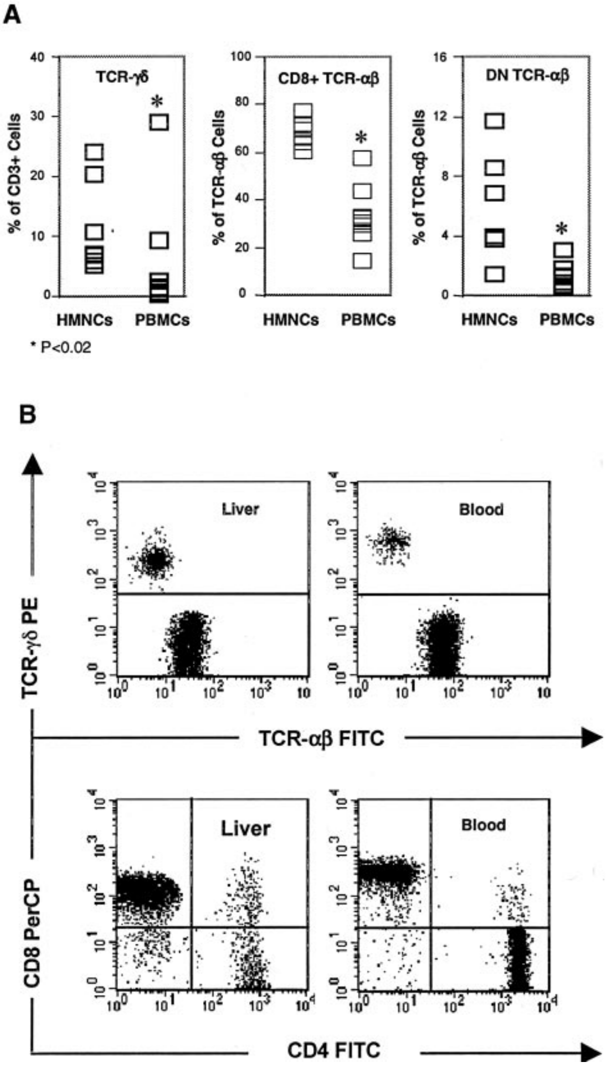

FIGURE 2. TCR-$\gamma\delta$ T cells and CD8$^{\mathrm{pos}}$ and CD4CD8 double-negative $\alpha\beta$ T cells occur more frequently in hepatic T cell populations. *A*, □, individual samples. A significantly higher proportion of hepatic T cells (CD3$^{\mathrm{pos}}$) expresses the $\gamma\delta$ isoform of the TCR. A significantly higher proportion of hepatic $\alpha\beta$-T cells expresses the CD8 coreceptor or is double negative (DN; CD8$^{\mathrm{neg}}$CD4$^{\mathrm{neg}}$). *B*, Flow cytometry dot plots of CD3$^{\mathrm{pos}}$ hepatic and matched peripheral blood T cells showing the higher frequency of TCR-$\gamma\delta^{\mathrm{pos}}$ T cells in hepatic populations. Flow cytometry dot plots of TCR-$\alpha\beta^{\mathrm{pos}}$ hepatic and matched peripheral blood T cells showing the higher frequency of CD8$^{\mathrm{pos}}$ and DN T cells in hepatic populations are also shown.

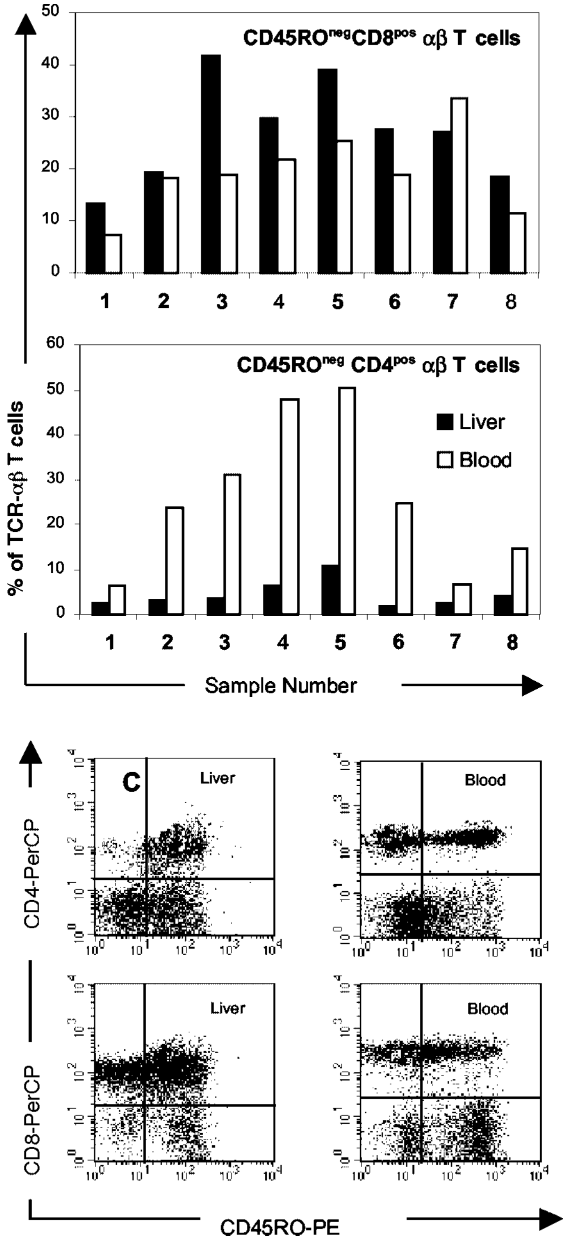

FIGURE 3. Naive TCR-$\alpha\beta^{\mathrm{pos}}$ cells in hepatic and matched peripheral blood. TCR-$\alpha\beta^{\mathrm{pos}}$ cells were gated, and the levels of CD4/CD8 (PerCP) and CD45RO (PE) staining within the TCR-$\alpha\beta^{\mathrm{pos}}$ populations were analyzed. Bar charts show CD8$^{\mathrm{pos}}$ TCR-$\alpha\beta$ (*A*), CD4$^{\mathrm{pos}}$ TCR-$\alpha\beta$ (*B*), cells with naive phenotype (CD45RO$^{\mathrm{neg}}$) in hepatic and matched blood samples. In contrast to those in blood, the majority of hepatic naive $\alpha\beta$ T cells coexpress CD8. *C*, Representative flow cytometry dot plots of hepatic and matched blood $\alpha\beta$ T cells showing CD4$^{\mathrm{pos}}$ and CD8$^{\mathrm{pos}}$ CD45RO$^{\mathrm{neg}}$ cells in the *upper left quadrant*.

for the CD45RO Ag (mean, 25.75%; range, 6.34–50.53%; $p < 0.005$; Fig. 3). CD45RO$^{+/-}$ levels did not correlate with age, but levels in the periphery correlated directly with those found in the liver.

Characterization of hepatic CD8^{pos} T cells

As it could be argued that the hepatic CD8$^{\text{pos}}$CD45RO$^{\text{neg}}$ population may represent a recently activated effector cell population, rather than a naive population (24), we further characterized this hepatic population isolated from six livers for expression of the CD27 coreceptor and markers of activation (HLA-DR, CD95, and CD69). The vast majority of CD8$^{\text{pos}}$ cells were T cells, as evidenced by the coexpression of CD3 (mean, 96.46%; range, 90.48–100%). Primed CD8$^{\text{pos}}$ T cells that express CD45RA and may be negative for CD45RO are characterized by the absence of the costimulatory molecule CD27 (25). For all hepatic CD8$^{\text{pos}}$CD45RO$^{\text{neg}}$ populations tested ($n = 6$), a significant CD27-coexpressing subset was detected, although there was wide intrasample variation (mean, 49.76%; range 35.64–87.16%). This CD45RO$^{\text{neg}}$ population expressed CD95/Fas (mean, 15.41%) at a significantly lower level than the matched CD8$^{\text{pos}}$CD45RO$^{\text{pos}}$ population (65.09%; $p < 0.01$). Markers of activation CD69 and HLA-DR were also significantly reduced in the CD8$^{\text{pos}}$CD45RO$^{\text{neg}}$ hepatic population compared with their corresponding CD8$^{\text{pos}}$CD45RO$^{\text{pos}}$ counterparts (48.23 vs 91.62% ($p < 0.0001$) 35.09 vs 80.09% ($p < 0.001$), respectively), suggesting that the CD8$^{\text{pos}}$CD45RO$^{\text{neg}}$ hepatic cells contain Ag-inexperienced cell populations (Fig. 4).

TREC levels

DNA was isolated from CD3-enriched populations of the eight matched hepatic and blood samples. One microgram of DNA from each of the samples was used to determine the number of TRECS. TRECs were detected in all blood samples at a mean level of 164.10 (range, 22.92–579). Only two of the liver samples (no. 3 and 6) were positive at levels of 59.4 and 1.92 (Fig. 5).

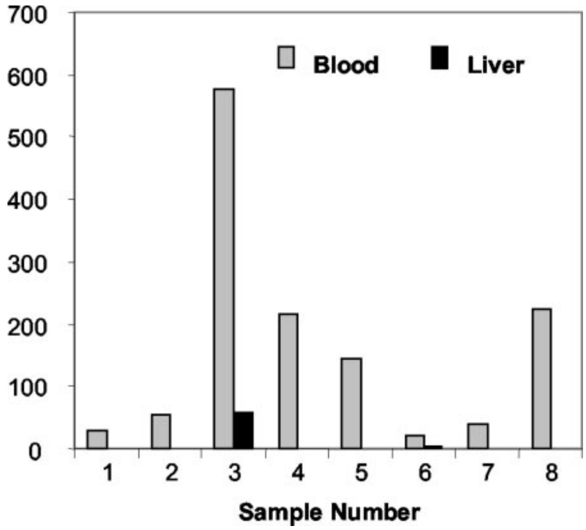

FIGURE 5. Levels of TRECs detected in naive hepatic and blood T cell populations. TRECs were detected in all peripheral blood samples at varying levels. Only two matched hepatic lymphocyte populations were positive for TRECS at low levels.

Discussion

In this study we focused on the hepatic $\alpha\beta$ T cells found in perfused donor organs. The hepatic $\alpha\beta$-TCR$^{\text{pos}}$ population contains more than twice as many CD8$^{\text{pos}}$ cells and 4 times as many double-negative cells as the corresponding population in matched peripheral blood. As evidenced by the absence of CD45RO (26), almost half of the peripheral blood and one-third of hepatic $\alpha\beta$ T cells have a naive-like phenotype. The majority of $\alpha\beta^{\text{pos}}$CD45RO$^{\text{neg}}$ cells in the periphery coexpress CD4, whereas <5% of $\alpha\beta^{\text{pos}}$CD45RO$^{\text{neg}}$ hepatic cells coexpress CD4. This suggests that naive CD4 expressing $\alpha\beta$ T cells are rare in the liver and abundant

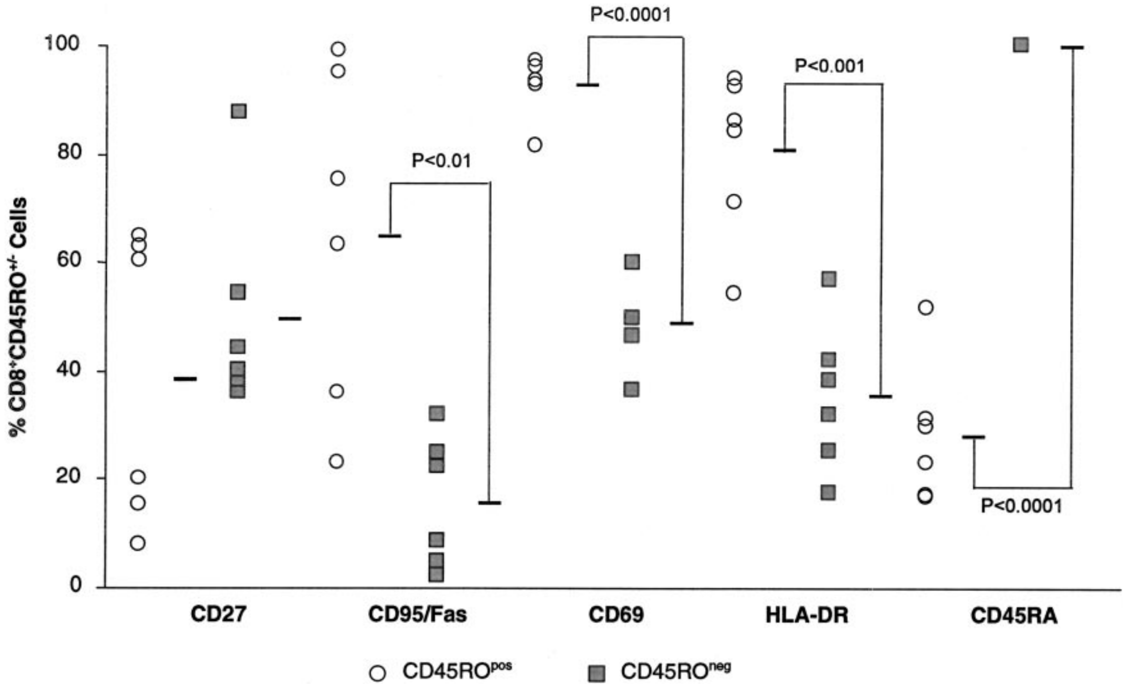

FIGURE 4. Comparison of CD8pos CD45RO$^{\text{pos/neg}}$ hepatic populations. Hepatic CD45RO$^{\text{neg}}$ populations expressed CD95/Fas at a significantly lower level than matched CD8$^{\text{pos}}$CD45RO$^{\text{pos}}$ population. Markers of activation CD69 and HLA-DR were also significantly reduced in the CD8$^{\text{pos}}$CD45RO$^{\text{neg}}$ hepatic population compared with their corresponding CD8$^{\text{pos}}$CD45RO$^{\text{pos}}$ counterparts.

in the periphery. In contrast, CD8 coexpressing $\alpha\beta^{pos}$CD45RO^{neg} cells are more abundant in the liver than in matched blood. This suggests that the normal liver may contain a significant population of naive $\alpha\beta^{pos}$CD8^{pos} cells.

However, although CD45RO defines a memory population, up to 20% of CD8^{pos}CD45RO^{neg} T cells in the periphery represent a recently activated effector population (25). The liver has been postulated to be a site for the elimination of activated CD8^{pos} T cells by apoptosis after activation in the periphery (5). The high proportion of CD8^{pos} T cells in normal liver could be a reflection of this scavenging function. However, accumulation of activated CD45RO^{pos}CD8^{pos} T cells only occurs in circumstances where there is disruption of an apoptotic pathway; due to rapid elimination, this is not usually observed in normal liver (27). The high levels of CD45RO^{neg} cells within the CD8^{pos} hepatic T cell populations observed in this study may thus represent recently activated cells that have exited from the periphery and are retained in the liver for disposal. However, the donor organs used in this study were extensively perfused, which would remove loosely held cells, and it is unlikely that cells actively undergoing apoptosis would survive the extraction process to which the liver tissue is subjected. CD8^{pos}CD45RO^{neg} effector populations are characterized by the absence of CD27 and high expression of activation markers such as Fas (CD95) and CD69 (25). We further characterized hepatic $\alpha\beta$-CD8^{pos}CD45RO$^{pos/neg}$ T cell populations with respect to the expression of CD27 and a range of activation markers to assess the presence or the absence of naive cells. Almost half the CD8^{pos}CD45RO^{neg} population coexpressed CD27, suggesting that they are not a recently activated population, and they express much lower levels of activation markers than their memory counterparts, which argues for the existence of a local pool of naive CD8^{pos} T cells. In support of this, a recent study by Bertolino et al. (28) has demonstrated that naive CD8^{pos} T cells are retained and undergo primary Ag-specific activation in the liver of adult mice.

TRECS were detected in all blood samples; the wide variation in the blood samples probably reflects different individual thymic activities (18). To control for intraindividual variation, matched liver samples were used in this study. Surprisingly, no TRECS were detectable in six of the eight liver samples tested. Of note, even blood in which relatively high levels of TRECS were detected were negative for the matched liver sample. In fact, the blood sample in which the lowest level of TRECS was detected was one of the two samples that were positive in matched liver; therefore, failure to detect TRECs in the liver is unlikely to be due to low levels in the periphery. The absence of TRECs in liver could be due to a number of factors. Naive cells developing locally or entering from the circulation may rapidly undergo extensive proliferation in the liver or are not retained if not activated and thus are absent from perfused liver. However, even if this were the case, one would expect to detect TRECS in all samples, albeit at a very low level. The presence of only experienced $\alpha\beta$-T cells in the liver would account for the absence of TRECS; however, the presence of a significant population of CD8^{pos}CD45RO^{neg}CD27^{pos} population in all livers tested suggests the presence of naive $\alpha\beta$-T cells in these livers.

Investigation of a limited number of TRECs in murine hepatic populations demonstrated that Vα14-Jα281 rearrangements occur at 2.5 times the frequency in thymus; another rearrangement Vα1.1-Jα281 was undetectable in the liver, although it was abundant in thymus (7). It is difficult to make a direct comparison between the mouse TREC study and ours because they targeted specific recombinations and we used deletion of the δ locus as an index of $\alpha\beta$-T cell development. Also, they did not use peripheral blood, but thymus, as a control, and they detected the Vα14 TRECs in thymus, albeit at a lower frequency than in liver, which could be a reflection of preferential homing of a naive Vα14 population to the liver rather than local development. Indeed, the development origin of murine NKT cells remains controversial, and another study suggests a solely intrathymic origin (8). Our study is easier to interpret because the absence of TRECs is more conclusive than finding them at reduced or increased frequency.

The assay used in this study detects TRECS in 70% of $\alpha\beta$-T cells and may well be specific only for conventional $\alpha\beta$-T cells found in the circulation. As the expression of pTα has been detected in hepatic lymphoid populations, suggesting that some of the T cells developing locally express TCR-$\alpha\beta$, hepatic TCR-α rearrangement may give rise to TRECs not involving the δRec and ψJα recombination event. These alternative TRECS may be generated during the development of unconventional T cells, such as Vα24 invariant NKT cells. Failure to detect TRECs in the liver in our study does not exclude the possibility that local $\gamma\delta$-T cell development is ongoing; indeed, the expression of RAG1 and RAG2 by hepatic $\gamma\delta$-T cells supports this possibility (29).

The almost complete absence of TRECs in normal human liver provides strong evidence that the normal AHL is not a site for the development of conventional $\alpha\beta$ T cells.

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