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J Immunol. 2005; 175:1047-1055; doi: 10.4049/jimmunol.175.2.1047
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Hepatocytes Express Abundant Surface Class I MHC and Efficiently Use Transporter Associated with Antigen Processing, Tapasin, and Low Molecular Weight Polypeptide Proteasome Subunit Components of Antigen Processing and Presentation Pathway

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Hepatic expression levels of class I MHC Ags are generally regarded as very low. Because the status of these Ags and their ability to present peptides are important for the understanding of pathogen clearance and tolerogenic properties of the liver, we set out to identify the factors contributing to the reported phenotype. Unexpectedly, we found that the surface densities of Kb and Db on C57BL/6 mouse hepatocytes are nearly as high as on splenocytes, as are the lysate concentrations of mRNA encoding H chain and β2m. In contrast, the components of the peptide-loading pathway are reduced in hepatocytes. Despite the difference in the stoichiometric ratios of H chain/β2m/peptide-loading machineries, both cell types express predominantly thermostable class I and are critically dependent on TAP and tapasin for display of surface Ags. Minor differences in the expression patterns in tapasin−/− background suggest cell specificity in class I assembly. Under immunostimulatory conditions, such as exposure to IFN-γ or Listeria monocytogenes, hepatocytes respond with a vigorous mRNA synthesis of the components of the Ag presentation pathway (up to 10-fold enhancement) but up-regulate H chain and β2m to a lesser degree (<2-fold). This type of response should promote rapid influx of newly generated peptides into the endoplasmic reticulum and preferential presentation of foreign/induced Ag by hepatic class I. The Journal of Immunology, 2005, 175: 1047–1055.

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liver, NK cells, NKT cells, and γδ T cells) express class I MHC-reactive receptors. Expression of class I MHC on parenchymal cells has been reached generally as very low (9, 10), based on a large number of predominantly immunohistochemical studies in mice and humans (11–13). More recent reports (14, 15) have questioned the early conclusions but did not provide quantitative data, nor did they address the question whether hepatically expressed class I H chains are assembled into stable complexes by a classical, peptide-dependent class I Ag presentation pathway.

We have re-examined this long-standing controversy and found that hepatocytes express abundant, thermostable cell surface class I MHC. The assembly of hepatic MHC class I H chain, β2-microglobulin (β2m) and the stabilizing ligands is dependent on the key components of the classical class I presentation pathway, TAP and tapasin (TPN). In healthy adult mice, the stoichiometric ratio of these altered components is elevated in hepatocytes compared with splenocytes and may favor rapid loading of Ags under the conditions associated with IFN-γ up-regulation.

Materials and Methods

Mice

All mice were 8- to 10-wk-old males of C57BL/6 (B6) and H-2<sup>H</sup> haplotype backcrossed to B6 background. B6 and β2m<sup>−/−</sup> mice were purchased from The Jackson Laboratory (K<sup>−/−</sup>, K<sup>−/−</sup>D<sup>−/−</sup>, and TAP1<sup>−/−</sup>/mice were from Dr. J. Forman (University of Texas Southwestern Medical Center, Dallas, TX). These strains and TPN<sup>−/−</sup> mice have been described previously (16–18).

Isolation of primary hepatocytes and splenocytes

Hepatocytes were isolated according to a standard two-step perfusion protocol (19, 20). Briefly, mice were anesthetized with i.p. administration of pentobarbital (50 mg/kg) and the thoracic inferior vena cava was cannulated, and the portal vein was opened for drainage. The liver was sequentially perfused in situ with two solutions. The preperfusion solution was composed of solution A (136 mM NaCl, 5.3 mM KCl, 0.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.4 mM Na<sub>H</sub>P<sub>O</sub><sub>4</sub>, 0.9 mM HEPES, and 4.1 mM NaHCO<sub>3</sub> (pH 7.4)) supplemented with 0.5 mM EGTA and 5 mM β-mercaptoethanol and was administered for 6 min at a flow rate of 5 ml/min. The perfusion solution (solution A supplemented with 0.05% collagenase A (Sigma-Aldrich), 0.004% DNase I (Sigma-Aldrich), 5 mM CaCl<sub>2</sub>, 0.005% trypsin inhibitor (Sigma-Aldrich), and 1% bovine albumin (Sigma-Aldrich)) was administered for 6–8 min at a flow rate of 5 ml/min. Perfused liver tissue was gently dispersed in a washing medium (William’s medium E (Invitrogen Life Technologies) supplemented with 0.05% collagenase A (Sigma-Aldrich), 0.004% DNase I (Sigma-Aldrich), 5 mM CaCl<sub>2</sub>, 0.005% trypsin inhibitor (Sigma-Aldrich), and 1% bovine albumin (Sigma-Aldrich)) and washed by size from PCR products amplified from cDNA samples. Kb-specific ICS-2 and D<sup>α</sup>-specific ICS-3 DNA templates were made by PCR amplification of genomic DNA with K<sup>α</sup>- and D<sup>α</sup>-specific primers derived from exons 2 and exon 3. Both contained intron 2 sequences and differed in size from corresponding K<sup>α</sup> and D<sup>α</sup> cDNA templates.

For competitive RT-PCR, standard titrations of ICSs were prepared by serial dilutions of ICS plasmids. A fixed amount of sample cDNA was coamplified with each of the titrated standards. PCR products were electrophoresed in a 7% Tris-acetate gel (Invitrogen Life Technologies). Measurements of cell dimensions

The average geometric size of hepatocytes and splenocytes was determined using an inverted microscope (Olympus CK40; Olympus). Cell images were recorded with QuantityOne software (Bio-Rad). Perpendicular dimensions of cell width and length were measured on printed images with a caliper meter. Cell diameter (2<sup>π</sup>) was calculated as the mean of perpendicular dimensions. The mean diameters of hepatocytes and splenocytes were estimated as 3.90 and 6.75 ± 0.9 μm, respectively. The fact that hepatocytes and splenocytes are spherical and taking into account the difference in their diameters, it is estimated that a hepatocyte cell surface area (4π<sup>3</sup> r<sup>2</sup>) is 11.2x larger than spleenocyte surface, and hepatocyte volume (4/3 π r<sup>3</sup>) is 37.4x larger than spleenocyte volume.

Cell cultures

Freshly prepared hepatocytes were cultured in flasks precoated with type I collagen (Sigma-Aldrich) in DMEM/Ham’s F-12 (1:1 v/v) supplemented with FBS, insulin, transferrin, dexamethasone, nicotinamide, t-ascorbic acid 2-phosphate, selenium acid, and epidermal growth factor. Splenocytes were resuspended in RPMI 1640 medium supplemented with 10% FBS, 50 μM 2-ME, 1% Glutamax I (Invitrogen Life Technologies) and 10 mM HEPES (complete medium). B3Z T hybridoma cells specific for OVA (SIINFEKL) peptide presented by K<sup>α</sup> were obtained from Dr. S. Ostrand-Rosenberg (University of Maryland, College Park, MD).

RNA isolation, cDNA synthesis, and RT-PCR

Total cellular RNA was isolated with Tri-Reagent (Molecular Research Center). Integrity and standardized loading of RNA were monitored by visualization of ribosomal bands.

For cDNA synthesis, 1 μg of RNA was primed with oligo(dt)<sub>20</sub> primer (Invitrogen Life Technologies), and the first-strand cDNA was synthesized by Omniscript reverse transcriptase (Qiagen) in a reaction mixture of 20 μl. Semiquantitative amplification of cDNA was performed routinely in 25 μl of PCR mixture containing 1–3 μl of template, 0.4 μM concentrations of the appropriate primer (Table I), 200–400 μM concentrations of each dNTP, and 0.5–1.25 U of DNA polymerase. The PCR products were visualized in agarose gels with UV light, and their intensities were recorded using Gel-Doc (Bio-Rad) and quantitated with QuantityOne software. All RNA samples were free of genomic DNA contamination as judged by PCR.

The competitive RT-PCR strategy was designed on the basis of published methodology (22). The internal calibration standard (ICS)-1 DNA template included a DNA linker fragment bordered by 5′- and 3′-primer binding sites specific for class I Ag presentation genes. TAP1, TAP2, TPN, low molecular weight polypeptide proteasome subunit (LMP7), and housekeeping gene GAPDH. The construction of ICS-1 was conducted according to a primer extension method. The sequences of the individual primers are listed in Table I. The primer binding sites were positioned on ICS-1 to ensure that the PCR-amplified fragments would be distinguishable by size from PCR products from cDNA samples. Kb-specific ICS-2 and D<sup>α</sup>-specific ICS-3 DNA templates were made by PCR amplification of genomic DNA with K<sup>α</sup>- and D<sup>α</sup>-specific primers derived from exons 2 and exon 3. Both contained intron 2 sequences and differed in size from corresponding K<sup>α</sup> and D<sup>α</sup> cDNA templates.

For competitive RT-PCR, standard titrations of ICSs were prepared by serial dilutions of ICS plasmids. A fixed amount of sample cDNA was then incubated with each of the titrated standards. PCR products were electrophoresed in a 7% Tris-acetate gel (Invitrogen Life Technologies).
The resolved proteins were transferred to a polycrylamide gel and stained with an unconjugated mAb MC89, followed by secondary recognition with FITC-conjugated goat anti-mouse IgGs (QIFI kit; Immunotech). The resolved proteins were transferred to a polyvinylidene difluoride membrane and detected with the species-appropriate biotinylated secondary Abs (Jackson ImmunoResearch Laboratories) and visualized after exposure to BioMax-MR film (Eastman Kodak). The intensities of the bands were quantitated using NIH Image software.

**ELISA measurements of Kb levels**

The experimental approach was as described previously (28). Purified hepatocytes (2 × 10⁶ cells/ml) and splenocytes (5 × 10⁷ cells/ml) were lysed with ice-cold lysis buffer containing 0.5% Nonidet P-40 (Sigma-Aldrich) and proteinase inhibitors (5 μg/ml pepstatin A, 2 μg/ml leupeptin, 2.5 mM benzamidine, 20 μg/ml soybean trypsin inhibitor, 100 μg/ml PMSF, and 4 mM EDTA) in 0.2 M phosphate buffer (pH 7.0). To measure naturally synthesized, conformed Kb or H chain Kb that can be stabilized by K²-binding peptides, protein-matched aliquots of cell lysates were incubated with saturating amount of relevant mAbs. The bound Abs were detected with the species-appropriate biotinylated secondary Abs (Jackson ImmunoResearch Laboratories) and visualized after incubation with streptavidin-conjugated HRP (Zymed Laboratories). The signals were developed using an ECL system (Amersham Biosciences) and visualized after exposure to BioMax-MR film (Eastman Kodak). The intensities of the bands were quantitated using NIH Image software.

**Quantitative flow cytometry**

One million splenocytes or 3 × 10⁶ hepatocytes were resuspended in 50 μl of FACS buffer (PBS containing 0.1% BSA and 20 mM EDTA), incubated for 30 min on ice with saturating amounts of 2.4G2 Ab to block FcRs, followed by incubation with saturating amount of relevant mAbs. The mAbs to Kb (Y3) and to β₂m (S19.8) were directly conjugated with FITC. D² was stained with an unconjugated mAb MC89, followed by secondary staining with F(ab')₂ of FITC-conjugated goat anti-mouse Igs (QIFI kit; DakoCytomation). Cells were analyzed by FACScan (BD Biosciences). The external calibration standards (QIFI kit; DakoCytomation) were used to establish a linear detection range of fluorescence intensity for both hepatocytes and splenocytes. Data analysis was performed with CellQuest software (BD Biosciences). Viable cells were gated according to their light scatter characteristics and propidium iodide exclusion. Geometric mean of logarithmic fluorescence intensity (GMFI) was recorded. A sample’s specific GMFI (sGMFI) was calculated as: sGMFI = GMFI (specific Ab) – GMFI (isotype control Ab).

**Peptide-induced stabilization of surface Kb**

Hepatocytes were seeded at 1 × 10⁶ cells/cm² in flasks precoated with type I collagen and cultured at 37°C until cells adhered. The cold stabilization always was performed using a simplified procedure of previously described protocols (28). Briefly, hepatocytes and splenocytes (4 × 10⁶ cells/ml) were incubated separately with 1 μg/ml Kb-specific OVA peptides, a control D²-restricted epitope of influenza nucleoprotein NP (ASNEN-METM) or with no peptides for 4 h at 22°C. Cells treated with peptides were incubated for additional 2 h at 37°C to denature unstabilized surface Kb Ags, whereas cells that did not receive peptides were kept at 4°C. After harvesting, cells were stained with Y3-FITC and analyzed by flow cytometry.

**In situ staining of hepatic sinusoidal membrane**

The liver was perfused in situ with the preperfusion solution for 3 min at 37°C, then for 10 min at 4°C. Hepatic hilar vessels were clipped to close the drainage. The liver was perfused for 15 min with 1–2 ml of cold FACS buffer containing FcR blocker (2.4G2), followed by 30 min of incubation with 1–2 ml of cold FACS buffer containing saturating amounts of Y3-biotin or isotype control Abs at 4°C. Drainage was then opened, and standard collagenase perfusions were conducted. After centrifugations and washes, purified hepatocytes were incubated with streptavidin-R-PE (BioSource International) for 30 min on ice, washed three times, and analyzed for surface Kb expression by flow cytometry.

**Induction of MHC class I by IFN-γ and Listeria monocytogenes infection**

For in vitro induction of class I MHC, purified hepatocytes were cultured for 2 days with the hepatocyte culture medium in the presence or absence of 20 U/ml mouse rIFN-γ (Sigma-Aldrich). *L. monocytogenes* infection was performed according to a published protocol (29). *L. monocytogenes* 10403 serotype 1 was obtained from Dr. J. Forman. Bacteria were grown on brain-heart infusion agar plates (Difco). The LD₅₀ for B6 mice is 2 × 10⁴ bacteria (29). B6 mice were injected with 2 × 10⁶ bacteria in 200 μl of PBS or with PBS alone through tail vein. The hepatocytes and splenocytes were isolated and analyzed at days 3 and 5.

**Results**

**Hepatocytes express high levels of class I MHC Ags**

The status of class I MHC expression levels on parenchymal liver cells is controversial, and the extent to which these cells use the...
classical, TAP-dependent class I presentation pathway is not currently known. To address these issues, we set out to compare class I MHC expression of hepatocytes and splenocytes from B6 mice. Because these two cell types differ in size, surface, and biological properties, several independent assays were performed to evaluate the steady-state class I levels in these two backgrounds.

Cell surface expression of classical MHC class I (class Ia) was evaluated first by quantitative flow cytometry using saturating amounts of mAbs recognizing K\(^b\) (Y3) and D\(^b\) (MC89). The cumulative levels of surface-displayed class I Ags (class Ia and the nonclassical class Ib, encoded by Q, T, and M loci of MHC) were evaluated with a \(\beta_2\)m-specific mAb (19.8). To adjust for the differences in cell size and autofluorescence between hepatocytes and splenocytes, commercial calibration standards were included in each experiment and linear fluorescence detection curves were established. Single-cell suspensions of freshly isolated hepatic and splenic cells were analyzed under the same conditions of acquisition, and their fluorescence intensities were measured within the linear detection range (Fig. 1A). Comparison of sGMFI for the observed unimodal populations of splenocytes and hepatocytes demonstrated that, per cell basis, liver parenchymal cells express ~16-fold higher levels of K\(^b\), ~7-fold higher levels of D\(^b\), and ~10-fold higher levels of \(\beta_2\)m-binding class I MHC than splenocytes. The observed relative ratio corresponds to hepatocyte membrane densities being at least 75, 30, and 44% as high as estimated densities of K\(^b\), D\(^b\), and surface \(\beta_2\)m-binding class I, respectively, on splenic small lymphocytes. Assuming that class I Kb density on splenocytes approaches ~5 \times 10^6 molecules/cell (30), the corresponding estimate for Kb density on hepatocytes in B6 mice is ~8 \times 10^5/cell (for conversion of surface/volume parameters see Materials and Methods).

In situ, liver hepatocytes assume a hexahedron-like shape. One-third of their surfaces face into the sinusoidal spaces of Disse, whereas other surfaces contact basolateral and bile canalicular membranes. The sinusoidal membranes are the only surfaces exposed to lymphocytes and molecules circulating in blood. We took advantage of this property to assess whether the sinusoidal surfaces of hepatocytes express class I Ags in situ. Before standard perfusion, the liver was incubated with FcR-blocking Ab followed by biotinylated anti-Kb Y3 mAb. The liver structures were then disrupted by standard collagenase perfusion and hepatocytes were purified. The single-cell suspension of prestained hepatocytes was then exposed to secondary streptavidin-PE conjugate and analyzed by FACS, along with conventionally ex vivo-stained, purified hepatocytes. The results in Fig. 1B are consistent with the interpretation that hepatocytes in situ are highly positive for Kb and that

![FIGURE 1](http://www.jimmunol.org/Downloadedfrom). Class I MHC Ags are abundant on hepatic cells. A, Surface levels of class I measured by quantitative flow cytometry. Top panel, Linear detection range of fluorescent intensities established with external calibration standards (QIFI kit; DakoCytomation). Inset, A linear regression curve plotted by logarithmically transformed specific geometric mean of logarithmic fluorescent intensity sGMFI (sGMFI, x-axis) and specific Ag-binding capacity SABC (SABC, y-axis). Lower panels, Fluorescent intensities of class I on hepatocyte (Hep) and splenocyte (Spl) stained with anti-Kb (Y3), anti-D\(^b\) (MC89), and anti-\(\beta_2\)m (19.8) mAbs, respectively. Filled peaks: specific staining; open peaks: background staining with isotype-matched control Abs. B, Flow cytometry of Kb on sinusoidal membranes stained with Y3 Ab in situ (upper panel) and ex vivo (lower panel). C, Conformed Kb levels in hepatic and splenic cell lysates measured by a Kb-specific sandwich ELISA (28). Data are representative of three independent experiments. Optimal protein loading concentrations were predetermined to allow detection of Kb in the linear range. Hepatic Kb levels are presented relative to B6 splenic Kb levels after matching protein concentrations of both lysates. Error bars denote the SDs of triplicate measurements. D, Hepatic Kb/OVA complexes activate B3Z T hybridomas in a class I- and peptide-specific manner. B3Z T hybridomas was incubated with cells in the presence of titrated amounts of Kb-specific OVA or D\(^b\)-specific NP peptides. Activation of the hybridoma was measured by \(\beta\)-galactosidase assay as described previously (31). Data represent one of three independent experiments. Mean values of triplicate measurements are shown. SDs were <5% of the measured means.
the level of staining of these cells (∼30% of the level observed with ex vivo-stained cells) is proportional to the area predicted to interact with the sinusoidally infused anti-Kb mAb.

The data presented above bear on the levels of class I MHC detectable by specific Ab on the cell surface. Because cells may differ in their ability to transport MHC out of the endoplasmic reticulum (ER)/Golgi, Kb-specific ELISA was performed on total cell lysates, as described previously (28). This two Ab-based, class I capture assay quantitates total conformationally stable, intracellular, and cell surface-associated class I. The results, summarized in Fig. 1C, indicate that the concentration of conformed Kb in hepatocytes is close to 70–80% of the concentration in splenic lysate(s) per protein unit. After adjusting for differences in volume and surface of cells, this estimate indicates that the proportions of surface and intracellular Kb are comparable (within the limits of the technique used) in the two cell types. The specificity of the assay was demonstrated by lack of detectable Kb signal in cell lysates of the control Kb−/−/ Db−/− knockout mice.

To complement these assays, we tested whether hepatoctyes express sufficient levels of class I MHC to present exogenously added Ag-peptide to the OVA-specific B3Z T cell hybridoma (Fig. 1D). In this experimental system, stimulation of the hybridoma is solely dependent on the interaction of the TCR with OVA peptides presented by Kb (31). Graded concentrations of Kb-binding OVA peptide (SINIFKEKL) and Dβ-binding, nonactivating NP peptide (ASNENMETM) were incubated with equal numbers of hepatocytes and splenocytes from B6 and Kb−/− mutant mice. Both B6 hepatocytes and splenocytes stimulated B3Z in a Kb-specific and peptide dose-dependent fashion with hepatocytes appearing to be better stimulators on a per cell basis. The latter observation cannot be interpreted strictly in terms of MHC density as other parameters, distinguishing the surfaces of the presenting cell types, may influence the activation of the hybridoma via TCR.

Transcriptional studies further confirmed that hepatocytes and splenocytes synthesize comparably high concentrations (within a factor of 2) of steady-state levels of total, H chain class Ia/class Ib, and L chain β2m. This is documented by Northern blot analysis (Fig. 2) with a probe specific for β2m and two probes reactive with class Ia/class Ib mRNA: “α3,” hybridizing with the conserved exon 4 of all class I and “Kb,” detecting Kb transcripts, and, to a lesser extent, other homologous class Ia/b transcripts. An independent approach, quantitative RT-PCR (data not shown), gave consistent estimates: the concentrations of Kb-specific transcripts appeared equimolar in the lysates of both cell types per unit of total RNA; the concentration of Dβ mRNA was 2- to 4-fold lower in hepatocytes, depending on the experimental conditions.

The results summarized above provide formal evidence against the notion that hepatocytes are severely deficient in class I MHC.

**Stoichiometry of gene products participating in class I MHC assembly is different in hepatocytes and splenocytes**

Mature class Ia MHC is delivered to the cell surface after its three components: H chain, β2m, and peptide are assembled in the ER (TAP1 and TAP2), stabilization of the class I peptide-loading complex, and class I assembly (TPN) (32). Because the functional properties of class I MHC can be modified in response to changes in the Ag presentation pathway, it is of interest to examine if class I assembly operates similarly in hepatocytes and splenocytes.

The results of Northern hybridization and quantitative RT-PCR presented in Fig. 3 show that the lysate concentrations of the steady-state transcripts of several components of the Ag-processing and presentation pathway differed between hepatocytes and splenocytes: TAP1 and TAP2 were reduced in liver cells ∼10- and ∼5-fold, respectively, TPN 2.9- to 4-fold, LMP2 ∼5.5-fold, LMP7 ∼5.4- to 6.6-fold, LMP10 ∼1.8-fold, and PA28α, PA28β ∼2.5- and ∼4.2-fold, respectively. The relative reduction at the mRNA level was confirmed at the protein level by quantitating steady-state levels of TAP1 and TPN proteins by Western blotting hybridization (Fig. 4). The results demonstrated ∼7.2- and 6.5-fold lower concentration of these two proteins, respectively, in liver cell lysates. The data are consistent, within the limits of the

**FIGURE 2.** MHC class I H and L chains are transcribed in hepatocytes (Hep) at the levels similar to splenocytes (Spl). Northern blot analysis of β2m, α3, and Kb transcripts (upper panel). Ten micrograms of total RNA from hepatocytes and splenocytes were separated by electrophoresis on 1% denaturing agarose gel. RNA blots were hybridized separately with digoxigenin-labeled RNA probes specific for β2m, α3, and Kb (see Materials and Methods). Intensity of hybridization signals was measured by QuantityOne software (Bio-Rad), and the relative intensity estimates are shown under each lane. The control lower panel documents equivalent intensities and intensities of ethidium bromide-stained 28S and 18S RNA bands of sample RNAs.

**FIGURE 3.** Steady-state transcripts of MHC class I Ag-processing and presentation components are severely reduced in hepatocytes. A. Northern hybridizations were performed with digoxigenin-labeled RNA probes specific for LMP2, LMP7, LMP10, TPN, PA28α, and PA28β. The conditions and RNA loading controls were as in Fig. 2. B. Competitive RT-PCR analysis of TAP1, TAP2, TPN, and LMP7 mRNA. One microliter of hepatic or splenic cDNA was coamplified with titrated amounts of ICS-1. The arbitrary titers of ICS-1 are shown on top of each panel, and the titers corresponding to the equivalence points of intensity are indicated in bold. In some cases, estimates were made on the basis of two adjacent titers and are indicated by an asterisk between them.
C
scribed in Fig. 1. Protein-matched lysates were incubated with Kb-bind-
pplied, Kb-binding (OVA) peptides under conditions of a heat shock
splenic lysates are differentially stabilized with exogenously sup-
FIGURE 5. Hepatic (Hep) and splenic (Spl) Kb Ags are thermostable.
The hepatic Kb, just like their splenic counter-
the class I Ag presentation pathway are regulated at the level of
analyses and confirm reports that the levels of the components of
peptides destined for Kb and Db are severely limiting in liver cells.
One consequence of such a scenario would be a scarcity of ther-
complexes and excess of denatured thermolabile class I. Thermolabile, empty class I molecules that
escape degradation in the ER, can be detected in the lysates by
stabilization with exogenous synthetic peptides, followed by stain-
ing with Ab specific for the conformed class I (28). We addressed
this prediction by testing whether Kb from B6 liver lysates and
splenic lysates are differentially stabilized with exogenously sup-
plied, Kb-binding (OVA) peptides under conditions of a heat shock
at 37°C (Fig. 5). The hepatic Kb, just like their splenic counter-
parts, were stable at 37°C and did not respond to the stabilizing
OVA peptide by a detectable enhancement of their expression lev-
els. As a control, we demonstrated that this temperature is suffi-
cient to destabilize empty TAP−/− splenic Kb exposed to nonbind-
ing 2N peptides but is well tolerated by OVA peptide-stabilized Kb
from the same cells. The concentrations of the conformed Kb, de-
tected in the lysates of both cell types, were also similar, in agree-
ment with the data in Fig. 1C. These observations are consistent
with the notion that stable Kb associates intracellularly with en-
dogenous peptide ligands and that this complex formation is sim-
ilarly efficient in both cell types. Similar conclusions were reached
using flow cytometry to probe for conformation of cell surface
expressed Kb (Fig. 6 and data not shown).

Display of thermostable class Ia on hepatocytes could be ex-
plained either by postulating that hepatocytes use the classical
processing and presentation pathway more efficiently than splenocytes or, alternatively, that they rely on a different mecha-
nism for peptide/ligand delivery and for trimer formation. To ad-
dress the latter point, we tested if mice deficient in the components
of the classical pathway, TAP−/− and TPN−/−, display stable class I MHC on liver cells. The results in Fig. 7 indicate that both
mutations lead to the hepatic loss of surface Kb, comparable to the
loss observed in fully class I-negative, β2m-deficient mice. Thus,
we conclude that assembly of class I in hepatocytes is predomi-
nantly TAP and TPN assisted, despite their reduced availability in
normal parenchymal cells.

Hepatocytes use reduced levels of TAP and TPN to assemble
mature, stable class I
Interestingly, the phenotypes of TPN−/− hepatic and splenic cells assessed by flow cytometry (Fig. 7) differ in that the mutant liver cells appear fully negative for Kb (reduced to background level), whereas the mutant splenic cells express Kb levels intermediate between TAP−/− and fully functional B6 lymphocytes. The partial expression of Kb on the surface of TPN−/− cells is thought to result from the assembly and display of this class I loaded with low-
affinity peptides (18). To assess if Kb on TPN−/− hepatocytes is indeed absent at the cell surface or is simply denatured due to the
loss of weakly binding peptides at 37°C, we pulsed hepatocytes and splenocytes with saturating amounts of Kb-binding OVA pep-
tide, predicted “to rescue” cell surface expression of Kb. Indeed,
this approach allowed enhanced detection of conformed Kb on the

FIGURE 5. Hepatic (Hep) and splenic (Spl) Kb Ags are thermostable.
Total conformed Kb levels were determined by a sandwich ELISA as de-
scribed in Fig. 1C. Protein-matched lysates were incubated with Kb-binding
peptide OVA, a nonbinding peptide 2N, or without peptides overnight
on ice. Lysates containing exogenous peptides were then heat-shocked at
37°C for 4 h to denature empty Kb molecules. The peptide-un-treated lys-
ates were kept at 4°C and served as reference control for total Kb. The
mean ODs of triplicate wells were normalized to total Kb (no peptide, 4°C = 100%) and presented as the percentage of the reference sample. The
data were collected at the linear detection range of ELISA.

FIGURE 6. Differential stabilization of surface-expressed Kb by exog-
enous peptides. Hepatocytes (Hep) and splenocytes (Spl) from B6, β2m−/−, TAP−/−, and TPN−/− mutant mice were incubated with or with-
out OVA or NP peptides for 4 h at room temperature. Peptide-treated cells
were heat-shocked for 2 h. Cells were stained with conformation-specific anti-Kb mAb Y3-FTIC and analyzed by flow cytometry. Thermostable Kb
expression levels (sGMFI) are expressed as the percentage of total Kb on
B6 cells.
surface of splenic TAP$^{-/-}$ and TPN$^{-/-}$ mutants, as well as hepatic TAP$^{-/-}$ mutants, but failed to do so with TPN$^{-/-}$ liver cells (Fig. 6). One interpretation of this result is that the altered phenotype of hepatic TPN$^{-/-}$ cells is due to the failure of the residual TAP machinery to deliver a sufficient quantity of peptides into the ER. Because TPN has a dual function as a chaperone in folding and as an enhancer of TAP peptide transport activity (28, 33), its absence may depress already low TAP activity in hepatocytes and produce a phenotype of a severely impaired mutant, such as might be predicted for a double TPN$^{-/-}$-TAP$^{-/-}$ mutation. Taken together, these results indicate that peptide loading in hepatocytes occurs at, or just below, the threshold levels necessary to stabilize the presynthesized class I H chain that eventually reach the cell surface.

What is the impact of infection or inflammation on the components of the class I Ag-processing and presentation pathway? We performed controlled experiments with cultured purified hepatocytes treated with IFN-γ, a cytokine that is known to up-regulate many genes in the class I presentation pathway and is produced at high levels during pathogen exposure. Comparisons of surface Kb and total class I (β,m) on B6 hepatocytes by flow cytometry (Fig. 8A) showed only limited responses (<2-fold enhancement) to IFN-γ. Similar weak responses (<2-fold enhancement) were observed when transcriptional levels of Kb and Dβ H chains were measured by quantitative RT-PCR (data not shown). In contrast, genes in the class I assembly pathway responded by significant elevation of transcriptional activity, as detected by quantitative RT-PCR in Fig. 8B (TAP1, 10-fold; TAP2, 2.5-fold; TPN, 4-fold). A similar gene expression pattern was detected in hepatocytes from mice infected with L. monocytogenes, an intracellular bacterium that promotes strong innate and adaptive immune responses (34). Surface display of Kb (sGMFI) was enhanced upon infection by a factor of 2.1 ± 0.4 (data not shown), whereas transcription of the components of the peptide-loading pathway was generally induced to a greater degree (<8-fold for TAP1, ~3-fold for TAP2, ~4-fold for TPN, and ~6-fold for LMP7; data not shown).

Thus, we conclude that inflammation or infection in the liver promotes a preferential stimulation of the components of Ag-processing and presentation, a change in the preexisting stoichiometry of class I MHC components in the ER and enhanced production/loading of peptides derived from the newly degraded proteins.

Discussion

Although MHC class I expression is frequently described as "ubiquitous," individual cell types differ widely in the density of class I complexes displayed on their cell surfaces. Hemopoietic cells express normally the highest levels of class I, whereas most cells in immunologically privileged sites are nearly devoid of these Ags. Other cell types fall between these two extremes (35, 36). In addition, some cells, particularly Ag-presenting dendritic cells and endothelial cells, regulate surface levels of class I according to their maturation state and in response to various cytokines and/or inflammatory signals (37, 38).

Because the surface levels of class I correlate with the efficiency with which CTLs and NK cells recognize class I/peptide complexes, the cellular MHC status has become one of the criteria for predicting the immunological fate of cells coming into contact with class I-restricted lymphocytes. For example, it has been proposed that viral persistence in MHC-negative neurons is due to the lack of Ag-presenting capacity of target cells and their consequent invisibility to CTLs (39). Accordingly, liver tolerance, among many other hypotheses, has been attributed to the absence of MHC
class I on hepatocytes or, conversely, to tolerogenic effects of hepatic Ag presentation via MHC class I (7, 8). Because of the difficulties in fractionation of hepatocytes, the great majority of the past experiments used immunohistochemical staining of whole liver sections to evaluate MHC levels. In each case when this approach was used, healthy parenchymal cells appeared nearly negative for class I (11, 13, 35).

We had originally undertaken our investigation to identify the molecular mechanisms responsible for low MHC class I on normal hepatic cells. Instead, we found that hepatocytes in situ, or immediately after purification, express abundant and conformationally stable class I complexes approaching in cell surface density the levels of MHC on splenic lymphocytes.

One explanation that could account for the discrepancy between our conclusions and the historical reports is that purified hepatocytes may undergo reprogramming of gene expression immediately after dissociation and acquire the ability to display MHC class I ex vivo. Several lines of evidence argue against this possibility. First, class I transcription declines rather than increases, in cultured hepatic cells (Ref. 7 and our unpublished observations). Second, we examined hepatic sinusoidal membranes in situ and detected high levels of class I by flow cytometry. Third, ELISA protein measurements and transcript measurements performed on lysates from intact unfractionated liver (where hepatocytes constitute >80% of total volume) gave estimates of class I abundance comparable to those seen with purified, dissociated hepatocytes (our unpublished observations). Thus, we propose that immunohistochemical methods used in the past underestimated expression levels of class I on hepatocytes. Our data are in agreement with Skokiewicz et al. (35), who measured concentrations of K\(^{\beta}\) protein in total liver lysates of B10.BR mice by absorption assay, and found similar fractions of thermostable (peptide-loaded) complexes per hepatic cells, MHC class I complexes from both cell types contain

In addition, our estimates of H chain and \(\beta_{2}m\) transcript levels in isolated hepatocytes are consistent with the studies concluding that an intact liver synthesizes abundant class I MHC mRNAs (40, 41).

During the course of our investigation, we noted that hepatic hepatocytes harbor reduced concentrations of transcripts and proteins required for peptide loading, relative to other cells such as splenocytes. Surprisingly, despite the apparent “deficiencies” of TAP1, TAP2, LMP7, LMP10, PA28\(\alpha, \beta\), as well as TPN products in hepatic cells, MHC class I complexes from both cell types contain similar fractions of thermostable (peptide-loaded) complexes per unit of lysate volume. Furthermore, experiments with liver and spleen cells from TAP\(^{-/-}\) and TPN\(^{-/-}\) mice indicated that surface class I display in both cell types is dependent on an intact classical class I Ag presentation pathway. Thus, we hypothesize that the unique architecture and physiology of hepatocytes and their ER compartments helps to compensate for the relative scarcity of peptide delivery machinery.

Because the liver is a site of many viral, bacterial, and parasitic infections, it was of interest to examine how conditions stimulating Ag presentation affect hepatic class I MHC. We found that purified hepatocytes treated with IFN-\(\gamma\) ex vivo or hepatocytes exposed to \(L. monocytogenes\) infection in vivo up-regulated mRNA and surface expression of K\(^{\beta}\) <2-fold, whereas components of the Ag presentation pathway, TAP1, TAP2, TPN, and proteasome subunits, were induced to a greater degree (up to 10-fold). This discordant response would be predicted to selectively enhance production of MHC ligands and their loading into class I grooves. Consequently, immunostimulatory conditions such as those described here or during acute phase response (42) will result in preferential binding of H chain/\(\beta_{2}m\) to newly generated peptides, rather than in elevation of surface MHC class I. This mechanism may be responsible for enhanced CTL recognition of viral epitopes in IFN-\(\gamma\)-treated liver cells (13).

In summary, we have shown here that MHC class I complexes are assembled in hepatocytes in much the same way as in splenocytes. Similarly, high levels of stable class I trimers are displayed on the surface of both cell types. Therefore, hepatocytes should be competent to interact with lymphocytes via class I receptors and may modulate functions of NK and T cells during liver specific interactions (15, 43).
68 with major histocompatibility complex class I molecules with impaired peptide and $\beta_2$-microglobulin assembly. J. Virol. 76: 2796–2803.


