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Characterization of Human Antiviral Adaptive Immune Responses during Hepatotropic Virus Infection in HLA-Transgenic Human Immune System Mice

Eva Billerbeck,* Joshua A. Horwitz,* Rachael N. Labitt,* Bridget M. Donovan,* Kevin Vega,* William C. Budell,* Gloria C. Koo,† Charles M. Rice,* and Alexander Ploss*

Humanized mice have emerged as a promising model to study human immunity in vivo. Although they are susceptible to many pathogens exhibiting an almost exclusive human tropism, human immune responses to infection remain functionally impaired. It has recently been demonstrated that the expression of HLA molecules improves human immunity to lymphotropic virus infections in humanized mice. However, little is known about the extent of functional human immune responses in nonlymphoid tissues, such as in the liver, and the role of HLA expression in this context. Therefore, we analyzed human antiviral immunity in humanized mice during a hepatotropic adenovirus infection. We compared immune responses of conventional humanized NOD SCID IL-2Rγ-deficient (NSG) mice to those of a novel NOD SCID IL-2Rγ-deficient strain transgenic for both HLA-A*0201 and a chimeric HLA-DR*0101 molecule. Using a firefly luciferase-expressing adenovirus and in vivo bioluminescence imaging, we demonstrate a human T cell–dependent partial clearance of adenovirus-infected cells from the liver of HLA-transgenic humanized mice. This correlated with liver infiltration and activation of T cells, as well as the detection of Ag-specific humoral and cellular immune responses. When infected with a hepatitis C virus NS3–expressing adenovirus, HLA-transgenic humanized mice mounted an HLA-A*0201–restricted hepatitis C virus NS3-specific CD8+ T cell response. In conclusion, our study provides evidence for the generation of partial functional antiviral immune responses against a hepatotropic pathogen in humanized HLA-transgenic mice. The adenovirus reporter system used in our study may serve as simple in vivo method to evaluate future strategies for improving human intrahepatic immune responses in humanized mice. The Journal of Immunology, 2013, 191: 000–000.

C hronic human hepatotropic infections remain major medical problems. At least 500 million individuals are infected with hepatitis B and hepatitis C virus (HCV) (1, 2), and an estimated 250 million cases of malaria result in ~1 million deaths each year (3). These pathogens exhibit an almost unique human tropism, and the lack of amenable small animal models has slowed our understanding of pathogenesis and stalled the search for drugs and vaccines. HCV, for example, establishes persistence in ~70% of infections, yet the immunological mechanisms that determine viral clearance versus persistence are not fully understood (4).

Over the past two decades, humanized mice, which are animals engrafted with human tissue and/or engineered to express human genes, have emerged as powerful systems to study species-restricted pathogens and human immunity in vivo (5, 6). Efficient immune system engraftment can be achieved by transplantation of human CD34+ hematopoietic stem cells (HSC) into highly immunocompromised xenorecipients (e.g., the NOD, SCID IL-2Rγ-deficient [IL-2Rγnull, NSG] strain) (7, 8). Such human immune system (HIS) mice can generate Ag-specific human immune responses when infected with lymphotropic viruses like EBV or HIV (6, 9). Similarly, the successful generation of human liver chimeric mouse models has been reported (10, 11). These animals can be infected with HCV and mount virus-specific immune responses when dually reconstituted with human liver and HIS compartments (12).

Despite these advances, current HIS models still suffer from significant functional deficiencies (5). For example, development of human myeloid and NK cell lineages is impaired, possibly due to the limited cross-reactivity of critical hematopoietic cytokines between mice and humans (13). In addition, the development of functional adaptive immune responses is limited by the lack of HLA gene expression. It has recently been suggested that the expression of a human MHC class II molecule, HLA-DR4, partially improves the development of functional human T and B cells (14, 15). Furthermore, transgenic expression of a human MHC class I molecule, HLA-A2, has been shown to significantly in-
crea antiviral HLA-restricted human T cell responses in HIS mice infected with the human lymphotropic pathogens EBV or dengue virus (16–18). It needs to be determined, however, whether these T cell responses can contribute to clearance of the virus infection in humanized mice.

The lack of HLA expression also limits recognition of pathogen-derived Ag presented on infected nonhematopoietic tissue. Very little is known about immunity to pathogens of tissues such as the liver, however, because most studies in humanized mice to date have focused on lymphotropic pathogens. In this study, we used hepatotropic adeno-viruses expressing either firefly luciferase (Fluc) or HCV nonstructural proteins to analyze the extent of functional intrahepatic immune responses in humanized mice, specifically focusing on the effect of HLA expression.

Materials and Methods

Mice

NOD.Cg-Prkdcscid Il2rgtmlWjl/Sz (NSG) mice, BALB/c mice, and C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The NSG-DRB*0101 (NOD.Cg-Prkdcscid Il2rgtmlWjl/Sz; Tg[HLA-DRB*0101]; NSG-A2*0201 (NOD.Cg-Prkdcscid Il2rgtmlWjl/Sz; Tg[HLA-A2.1];Eng/Sz) (17, 19–21) were originally crossed with the support of Dr. Richard O'Reilly (Memorial Sloan-Kettering Cancer Center, New York, NY). Specifically, to obtain NSG-DRB*0101 mice, previously described NOD/scid- DRB*0101 mice (19, 20) were backcrossed to the NSG background exceeding a total of 10× crosses. Microsatellite analysis was performed to confirm sufficient backcrossing on the NSG background. A total of 128 microsatellites were tested, and 127 were identical with NSG, confirming the strain background. Subsequently, NSG-A*0201 and -DRB*0101 were intercrossed until homozygous expression of both transgenes on the NSG background was established.

Peptides and tetramer

The HLA-A2-restricted HCV1 peptides HCV-NS3 1073–1081 (ALYDVVTKL) and the AdV5-hexon overlapping peptide library were generated at the Rockefeller University Proteomics Resource Center. HLA-A2 tetramers corresponding to the HCV peptides were obtained until the National Institutes of Health Tetramer Core Facility (Emory University, Atlanta, GA).

Production of adenoviruses

Adenoviral constructs encoding Fluc or HCV proteins were created using the AdEasy Adenoviral Vector System (Agilent Technologies, Santa Clara, CA) according to the manufacturer’s instructions. Briefly, Fluc, HCV1-NS3, and -SSB genes were PCR-amplified from Jc1FLAG2(2pFluc2A) (23) or HCV1/SF (kindly provided by Dr. Robert Lanford) plasmids, respectively, and inserted into the pShuttle-CMV using KpnI/Xhol sites (Sall/EcoRV) for NS5B. Recombinant pShuttle-CMV plasmids were linearized with Pmel and ligated to pAdEasy by homologous recombination followed by electroporation into BJ5183 cells (Agilent Technologies). Recombinant pShuttle-pAdEasy constructs were identified by PacI restriction analysis. All plasmid constructs were verified by DNA sequencing. For the production of virus stocks, adenoviral constructs were transfected into HEK293 cells using the calcium phosphate method. Transfected cultures were maintained until cells exhibited full cytopathic effect, then harvested and freeze-thawed. Supernatants were serially passaged two more times with harvest at full cytopathic effect and freeze-thaw. For virus purification, cell pellets were resuspended in 0.01 M sodium phosphate buffer (pH 7.2) and lysed in 5% sodium deoxycholate, followed by DNAse I digestion. Lysates were centrifuged, supernatant were layered onto a 1.2–1.46 g/ml CsCl gradient, and the virus was separated by ultracentrifugation. Adenovirus bands were isolated and further purified on a second CsCl gradient. Resulting purified adenoviral bands were isolated and twicedialyzed against 4% sucrose. Adenovirus concentrations were measured at 1013 times the OD260. Adenovirus stocks were stored at −80°C. All mice were infected i.v. with 5×105 or 1010 particles recombinant adeno-virus serotype 5 (Ad5V).

In vivo imaging

For in vivo imaging, mice were anesthetized and injected i.p. with 200 μg (1.5 mg/ml) luciferin (Caliper Life Sciences). Bioluminescence was analyzed 5 min after luciferin injection for a period of 1 min using an IVIS Lumina II system (Caliper Life Sciences).

Cell isolation

Cells were isolated exactly as described previously (22). Briefly, blood-derived leukocytes were isolated by Ficoll density gradient (Cellgro) centrifugation (20 min, 2000 rpm); spleen- and liver-derived leukocytes were isolated by a collagenase IV digestion (HBSS, 0.1% collagenase type IV, 40 mM HEPES, 2 M CaCl2, and 2 U/ml DNAse I) (30 min, 37°C) of the minced liver and spleen tissue followed by Ficoll density gradient centrifugation. Isolated leukocytes were washed twice in PBS and directly resuspended in PBS.

Flow cytometry

For the analysis of the adenovirus-infected T cells, the thymus was homogenized through a cell strainer (100 μm; BD Biosciences) to obtain a single-cell suspension. For the isolation of hepatic leukocytes, mice were anesthetized (100 mg/kg ketamine/xylazine), and the liver was perfused through the vena cava by a collagenase solution (4.8 mM CaCl2, 100 U/ml collagenase type IV, and 0.05 M HEPES [pH 7.3] in Ca/Mg-free HBSS). The purified liver tissue was subsequently passed through a cell strainer (100 μm; BD Biosciences), washed twice in HBSS, and fixed in 4% paraformaldehyde.

Abs

The following anti-human Abs were used: CD45–Pacific Orange, CD4–Alexa Fluor 700, CD16–Pacific Blue, CD3–PE-Texas Red, CD8–PE-Cy7, CD127–Alexa Fluor 700 (Invitrogen); CD8–APC-Cy7, CD4–PE-Cy7, and CD3–PerCP-Cy5.5, 7–PE, HLA-DR–allophycocyanin, Granzyme B–Alexa Fluor 700, HLA-A2–FITC (BD Biosciences); CD1c–Pacific Blue, CD3–allophycocyanin–eFlour780, CD8–allophycocyanin–eFlour780, CD45RO–PerCP–eFlour710, CD27–PECy7, CD68–PE, CD127–PECy7, programmed death-1 (PD-1)–PerCP–eFlour710, IFN-γ–PECy7, TNF-α–FITC, and IL-4–PE (eBioscience); and CD4–Pacific Blue, CD36–FITC, CD26–Kansas (Invitrogen); CD8–PerCP-Cy7, Alexa Fluor 647, and PerCP-Cy7, Amoebo-Cy7, Alexa Fluor 647 (BioLegend). The following anti-mouse Abs were used: CD45–PECy7, epithelial cell adhesion molecule–PECy7, F4/80–PE, CD49b–Alexa Fluor 488, CD80–Pacific Blue, CD86–PerCP-Cy5.5 (Bio-Legend); CD3–allophycocyanin–Cy7, H2Kd–PE, (BD Biosciences); B220–Alexa Fluor 700 (Invitrogen); and CD11b–PECy7 (eBioscience). Appropriate isotype controls were also purchased from each company.

Flow cytometry

Ab staining, MHC tetramer staining, and FACS analysis was performed exactly as described previously (22, 24) using an LSRII Flow Cytometer (BD Biosciences).
In vivo T cell depletion

Human T cells were depleted from humanized mice (12 wk post–HSC transplantation) by i.p. injection of 100 μg Okt-4 and Okt-8 (BioXCell) on 3 consecutive d. T cell depletion was confirmed by FACS analysis. To maintain T cell depletion for the duration of the experiment (20 d), Okt-4 and Okt-8 treatment was repeated 10 d later.

Generation of bone marrow–derived macrophages

Bone marrow cells were isolated from femur and tibia of NSG and NSG-A2/DR1 mice. L929 cells were used as a source of GM-CSF. For the generation of bone marrow–derived macrophages (BMDM), bone marrow cells were cultured in L929 cell-conditioned medium (DMEM; Life Technologies, Invitrogen) with 20% FBS, 30% supernatant from confluent L929 cells, and 1% penicillin/streptomycin. After 7–10 d of culture, 90% of cells stained positive for the macrophage marker F4/80 and CD11b.

Macrophage–T cell cocultures

NSG- and NSG-A2/DR1-derived BMDM were infected with 5 × 10^3 particles AdV5 expressing Fluc (AdV5-Fluc)/cell. Infection of BMDM was verified by testing cell lysates for Fluc activity 48 h postinfection (Luciferase assay systems; Promega). Human T cells were isolated from AdV5-infected (day 20 postinfection) and control HIS and HIS-A2/DR1 mice by using a human CD3 T cell MACS selection kit (Miltenyi Biotec). T cell purity postselection as determined by FACS analysis was ≥97%. T cells were cocultured with AdV5-infected or control BMDM (5:1 T/BMDM ratio) for 5 d in complete medium (RPMI; Life Technologies, Invitrogen) with 10% FBS, 1.5% HEPES, and 1% penicillin/streptomycin. Supernatants were collected and analyzed for concentration of human IFN-γ and TNF-α (cytometric bead array; BD Biosciences).

Peptide-specific T cell stimulation

Leukocytes were isolated from blood, spleen, and liver of AdV5-Fluc, AdV5-HCV-NS3, or AdV5-HCV-NS5B–infected HIS and HIS-A2/DR1 mice and control groups 14 or 20 d postinfection. Pooled blood-, spleen-, and liver-derived leukocytes were plated on a 96-well plate and stimulated with 10 μg/ml HCV-specific peptides, 10 μg/ml AdV5-hexon peptide pools, or PMA/ionomycin (positive control) in the presence of GolgiPlug (BD Biosciences). After 5 h of incubation at 37°C, cells were analyzed for intracellular production of IFN-γ and TNF-α by flow cytometry. Cells were gated on human CD45, CD3, and CD4 or CD8 expression.

Neutralization assays

Serum from AdV5-infected (day 20–30 postinfection) and control BALB/c, HIS, and HIS-A2/DR1 mice was diluted 1:2, 1:10, 1:100, and 1:1000 in DMEM and incubated with 5 × 10^5 particles AdV5 for 1 h at 37°C. Serum–virus mixtures were subsequently added to 10^5 HeLa cells. After 30 min of incubation, HeLa cells were washed twice with DMEM and cultured in fresh medium. Twenty-four hours later, Fluc activity in cell lysates was analyzed (Luciferase assay systems; Promega). Percentage of neutralization was calculated using the following equation: 100 – (average number of sample/average number of control × 100).

FIGURE 1.  HIS reconstitution in HLA class I and class II double-transgenic mice. The tissue-specific expression of HLA-A2 and HLA-DR1 on murine thymic epithelial cells (epithelial cell adhesion molecule [epCAM]), splenocytes (H2-Kd+), and hepatocytes (albumin+) derived from NSG and NSG-A2/DR1 mice was determined by flow cytometry. Representative FACS plots are shown in (A). HIS mice were generated by transplantation of human CD34+ HSC into newborn NSG mice and NSG-A2/DR1 mice. Twelve weeks posttransplantation, blood, spleen, and liver were analyzed for HIS reconstitution. (B) Total human CD45+ leukocyte, CD3+ T cell, and CD19+ B cell numbers in spleen and liver of HIS and HIS-A2/DR1 mice. (C) Representative FACS plots showing the peripheral frequency of human leukocytes (human [h]CD45+) within the total leukocyte population (top left panel) and the frequencies of CD3+ T cells, CD19+ B cells (top right panel), CD56+CD16+/− NK cells (bottom left panel), and CD33+CD14+/− myeloid cells (bottom right panel) within the human CD45+ cell population of HIS-A2/DR1 mice. (D) Group data showing the frequencies of B cells, T cells, NK cells, and myeloid cells in blood, spleen, and liver of HIS versus HIS-A2/DR1 mice. Summarized data of n = 8 HIS and n = 10 HIS-A2/DR1 mice are shown. Data represent mean ± SEM. Statistically significant differences were analyzed using unpaired Student t test. mCD45, murine CD45.
Statistics
An unpaired Student t test was used to evaluate statistically significant differences (GraphPad Prism; GraphPad).

Results
HIS reconstitution in HLA-transgenic mice
The expression of HLA improves human adaptive immune responses during lymphotropic virus infection in humanized mice (16, 17). In this study, we aimed to analyze the extent of functional human immune responses during a hepatotropic virus infection and the role of HLA expression in this context. First, we performed a comparative analysis of HIS reconstitution efficiency in conventional NSG mice and a novel NSG mouse strain transgenic for HLA-A*0201 (HLA-A2) and a chimeric human HLA-DR*0101 (HLA-DR1), termed NSG-A2/DR1. In these mice, HLA-A2 is ubiquitously expressed, whereas HLA-DR1 is under the control of a murine MHC class II promotor, ensuring physiological expression only on lymphoid tissue. We confirmed the tissue-specific expression of the human transgenes in thymus, spleen, and liver of NSG-A2/DR1 mice by flow cytometry (Fig. 1A). Transplantation of human fetal liver-derived CD34+ HSC into sublethally irradiated NSG and NSG-A2/DR1 neonates resulted in comparable reconstitution of a HIS in both mouse strains 12 wk later (Fig. 1). Numbers and frequencies of human leukocytes, T cells, B cells, myeloid cells, and NK cells in blood, spleen, and liver (Fig. 1B–D) reflected what has been described previously for the NSG strain (16, 22). We refer to NSG mice engrafted with a HIS as HIS mice and reconstituted NSG-A2/DR1 mice as HIS-A2/DR1 mice. All NSG-A2/DR1 mice received HLA-A2+ HSC, but only some fully matched HLA-A2+/HLA-DR1+ HSC due to limited availability. Figs. 1 and 2 show com-

FIGURE 2. Tissue distribution of human T cell subsets in HIS and HIS-A2/DR1 mice. Twelve weeks after human CD34+ HSC transplantation, the phenotype and function of human T cells present in HIS and HIS-A2/DR1 mice was analyzed by flow cytometry. Frequencies of human CD4+ and CD8+ T cells in blood, spleen, and liver of HIS and HLA-transgenic HIS mice are shown in (A). To determine the percentage of effector T cells, CD4+ and CD8+ T cells were stained for the expression of CD45RO, CCR7, CD62L, and HLA-DR. (B) Frequencies of human CD45RO+CCR7+CD62L−HLA-DR+ effector CD4+ (left panel) and CD8+ (right panel) T cells. (C) Representative FACS plot showing CD45RO expression on CD4+ T cells (left panel) and CD62L and CD45RO expression on gated CD8+ T cells (right panel) from HIS-A2/DR1 mice. (D) Histograms comparing the expression of CCR7 (left panel) and HLA-DR (right panel) on CD45RO−CD8+ effector T cells (black line) and CD45RO+CD8+ naive T cells (gray solid). To determine the ability of human CD4+ and CD8+ T cells to produce effector cytokines and molecules, cells were stimulated with PMA/ionomycin and analyzed for the intracellular production of IFN-γ, TNF-α, granzyme B, perforin, IL-17, and IL-4. (E) Group data showing the frequency of cytokine-producing CD8+ T cells (top panels) and CD4+ T cells (bottom panels) in blood, spleen, and liver of HIS and HIS-A2/DR1 mice. (F) FACS plots showing granzyme B and IFN-γ expression of TNF-α+CD8+ T cells from blood and liver of HIS-A2/DR1 mice. Summarized data of n = 8 HIS and n = 10 HIS-A2/DR1 mice are shown. Data represent mean ± SEM.
bined data of HIS-A2/DR1 mice reconstituted with either HLA-A2+ or HLA-A2+/HLA-DR1+ HSC. As shown in Supplemental Fig. 1A–C, we did not detect statistically significant differences in the reconstitution of human immune cells between mice that re-received HLA-A2+ or HLA-A2+/HLA-DR1+ HSC. Table I further shows the human CD45+ cell reconstitution of groups of mice reconstituted with individual human HSC donors.

**Tissue distribution of human T cell subsets in HIS and HIS-A2/DR1 mice**

The presence of functional T cells at the site of infection is essential for viral clearance. Little is known about the tissue distribution of different T cell subsets in humanized mice. Thus, we characterized human T cells present in blood, spleen, and liver of HIS and HIS-A2/DR1 mice 12 wk after human CD34+ HSC transplantation. Within the CD3+ population, CD4+ T cells comprised ∼60–70% and CD8+ T cells ∼30–40%; there was no significant difference in this ratio between different tissue compartments and the two mouse strains (Fig. 2A). About 15–20% of CD4+ and CD8+ T cells displayed a CD45RO+CD62L2CCR72HLADR+ effector T cell phenotype (Fig. 2B–D).

We further analyzed the ability of T cells to produce IFN-γ, TNF-α, IL-4, IL-17, granzyme B, and perforin. After nonspecific stimulation with PMA/ionomycin, an average of 10–20% of

![Image](http://www.jimmunol.org/)

**FIGURE 3.** Improved in vivo clearance of a hepatotropic adenovirus in HIS-A2/DR1 mice. BALB/c, MIS-NSG, HIS, and HIS-A2/DR1 mice (12 wk post–HSC transplantation) were infected i.v. with 10^{10} particles of a hepatotropic replication-deficient E1/E2-deleted AdV5-Fluc. Fluc expression was longitudinally monitored using an in vivo imaging system and quantified in photons per second. (A) Representative images showing the hepatic Fluc expression in BALB/c, MIS-NSG, HIS, and HIS-A2/DR1 mice at days 1, 10, and 20 postinfection. (B) Intrahepatic AdV5-Fluc clearance in groups of BALB/c (n = 6), MIS-NSG (n = 4), HIS (n = 10), and HIS-A2/DR1 (n = 10) mice quantified in photons per second. (C) Comparison of Fluc expression in HIS, HIS-A/DR1, and nonreconstituted NSG mice at days 10 and 20 postinfection. (D) Comparison of Fluc expression in HIS, HIS-A/DR1, and nonreconstituted NSG mice at days 20 and 40 postinfection with 5 × 10^{10} AdV5-Fluc particles. (E) Fluc expression in T cell–depleted HIS (n = 5) and HIS-A2/DR1 (n = 5) mice compared with control HIS (n = 5) and HIS-A2/DR1 (n = 5) mice at day 10 post-AdV5 infection. Data represent mean ± SEM. Unpaired Student t test: *p ≤ 0.05.

### Table I. Overview of human CD34+ HSC donors used for transplantation of NSG and NSG-A2/DR1 mice

<table>
<thead>
<tr>
<th>HSC donor</th>
<th>HLA Type</th>
<th>Type and No. of Mice Transplanted</th>
<th>Level of Human Cell Reconstitution: Percent human CD45 (mean ± SEM)</th>
<th>Level of Antiviral Immunity: Photons/s at Day 10 Postinfection (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A2+DR1+</td>
<td>NSG</td>
<td>0</td>
<td>41.3 ± 4.6</td>
</tr>
<tr>
<td>2</td>
<td>A2+DR1−</td>
<td>NSG-A2/DR1</td>
<td>4</td>
<td>52.2 ± 6.2</td>
</tr>
<tr>
<td>3</td>
<td>A2-DR1−</td>
<td>NSG</td>
<td>3</td>
<td>53.8 ± 1.7</td>
</tr>
<tr>
<td>4</td>
<td>A2+DR1+</td>
<td>NSG</td>
<td>5</td>
<td>61.2 ± 2.2</td>
</tr>
<tr>
<td>5</td>
<td>A2-DR1−</td>
<td>NSG</td>
<td>6</td>
<td>60.1 ± 5.3</td>
</tr>
<tr>
<td>6</td>
<td>A2+DR1+</td>
<td>NSG-A2/DR1</td>
<td>0</td>
<td>54.1 ± 4.4</td>
</tr>
</tbody>
</table>
blood-, spleen-, and liver-derived CD8\(^+\) T cells produced IFN-\(\gamma\) and granzyme B, whereas perforin production was almost undetectable (Fig. 2E). The most abundant cytokine produced by CD8\(^+\) T cells was TNF-\(\alpha\), specifically in blood (mean HIS: 30%; mean HIS-A2/DR1: 47%; \(p = 0.02\)) and spleen (mean HIS and HIS-A2/DR1: 30%) (Fig. 2E). A large fraction of TNF-\(\alpha\)CD8\(^+\) T cells also produced IFN-\(\gamma\) and granzyme B, highlighting the polyfunctionality of CD8\(^+\) T cells (Fig. 2F). About 20% of CD4\(^+\) T cells in blood, spleen, and liver produced IL-4, whereas <10% produced IFN-\(\gamma\). IL-17–producing cells were detectable only at low frequencies (Fig. 2E). Similar to the CD8\(^+\) T cell population, TNF-\(\alpha\) was the most abundant cytokine produced by blood- and spleen-derived CD4\(^+\) T cells (Fig. 2E). In summary, these data show that different functional human T cell subsets are detectable in the blood, spleen, and liver of HIS and HIS-A2/DR1 mice.

**Improved in vivo clearance of a hepatotropic adenovirus in HIS-A2/DR1 mice**

To analyze the extent of functional intrahepatic human immune responses in HIS and HIS-A2/DR1 mice, we established a model of a hepatotropic virus infection based on a replication-incompetent AdV5-Fluc. Adenoviral vectors efficiently target the liver and induce strong innate and adaptive immune responses in immunocompetent mice, which result in clearance of the virus and loss of transgene expression (25–27). Intrahepatic infection kinetics could be readily monitored longitudinally by bioluminescent imaging (Fig. 3A). The luciferase reporter signal, which was readily detectable in the liver following infection of immunocompetent BALB/c mice with \(10^{10}\) AdV5-Fluc particles i.v., peaked shortly postinfection and dropped below the limit of detection (10\(^5\) photons/s) within 10 d (Fig. 3A, 3B). When HIS and HIS-A2/DR1 mice were infected with AdV5-Fluc 12 wk post–CD34\(^+\) HSC transplantation, substantially higher levels of luciferase were expressed in the liver at days 10 and 20 postinfection as compared with the BALB/c cohort (Fig. 3A, 3B). Together, this suggests that the HIS does not efficiently control adenoviral infection in the liver of humanized mice.

To test whether the humanization procedure itself (irradiation and intrahepatic injection of neonates) interferes with the ability of chimeric animals to clear virus-infected cells from the liver, we transplanted NSG mice with MHC-mismatched C57BL/6 bone marrow cells. Twelve weeks posttransplantation, NSG recipients were engrafted to high levels with a heterologous mouse immune system (MIS), including NK, B, and T cells (data not shown). We found that MIS-NSG mice were able to clear AdV5-Fluc–infected cells in the liver, albeit with slightly slower kinetics than BALB/c mice (Fig. 3A, 3B). The delay may be attributable to the MHC mismatch.

Interestingly, the photon flux in HIS-A2/DR1 mice decreased significantly between days 10 and 20, whereas it remained stable in HIS mice (mean HIS day 20: \(1 \times 10^8\); mean HIS-A2/DR1 day 20: \(1.6 \times 10^7\) ) (Fig. 3B). Further, compared with HIS mice and the nonreconstituted NSG background strain, HIS-A2/DR1 mice showed significantly reduced photon flux at days 10 and 20 post-

![FIGURE 4. Increase of intrahepatic human B cells and macrophages during adenovirus infection. Ten days postinfection of HIS and HIS-A2/DR1 mice with \(10^{10}\) particles AdV5-Fluc, the infiltration of human B cells, macrophages, and NK cells into the liver was examined by flow cytometry. (A) Percentages of human CD68\(^+\) macrophages in the liver of virus-infected HIS (\(n = 5\)), HIS-A2/DR1 (\(n = 7\)) mice, and controls (\(n = 3\) each). (B) Representative FACS plots showing intrahepatic CD68\(^+\) macrophages of HIS-A2/DR1 mice. (C) Frequencies of human CD3\(^+\)CD56\(^+\) NK cells in blood, spleen, and liver of HIS-A2/DR1 mice. (D) Frequencies of human CD19\(^+\) B cells in blood, spleen, and liver of AdV5-infected HIS (\(n = 6\)) and HIS-A2/DR1 (\(n = 7\)) mice as compared with the control groups (\(n = 8\) each). (E) Representative FACS plot showing the frequency of CD19\(^+\) B cells in the liver of AdV5-infected HIS-A2/D1 mice and control.](http://www.jimmunol.org/DownloadedFrom)
infection (Fig. 3C). In separate groups of mice, we analyzed luciferase expression up to day 40 postinfection (infectious dose: $5 \times 10^5$ particles) and detected a further significant decrease in HIS-A2/DR1 mice (Fig. 3D). However, at this time point, a reduction in luciferase expression even in nonreconstituted NSG mice was observed. Also not statistically significant in these mice (Fig. 3D), this observation might indicate a general loss of the viral vector at time points beyond day 40, hence we stopped the kinetic study at day 40.

In sum, these results suggest a beneficial effect of HLA expression on the ability to clear the adenovirus from the liver of humanized mice. To determine whether this effect is directly mediated by T cells, we depleted human CD4$^+$ and CD8$^+$ T cells in HIS and HIS-A2/DR1 mice prior to AdV5-Fluc expression. In HIS-A2/DR1 mice, T cell depletion abrogated the observed reduction of luciferase expression at day 10 (Fig. 3E) and day 20 (data not shown) postinfection, whereas in HIS mice, T cell depletion had no significant effect (Fig 3E). These data clearly indicate that the partial clearance of an adenovirus infection from the liver of HIS-A2/DR1 mice is mediated mostly by human T cells.

Data presented in Fig. 3 summarize the combined results of HIS-A2/DR1 mice reconstituted with either HLA-A2$^+$ or HLA-A2$^+$/HLA-DR1$^+$ HSC. As shown in Supplemental Fig. 2A, we could not detect statistically significant differences between mice that received HLA-A2$^+$ or HLA-A2$^+$/HLA-DR1$^+$ HSC. Table I further shows the average photon flux (day 10 postinfection) of groups of mice reconstituted with individual human HSC donors. Together, these findings suggest that HLA-DR1 expression does not play an essential role in the partial in vivo adenovirus clearance in HLA-transgenic mice. Unless stated otherwise, all of the following figures also show combined data of HIS-A2/DR1 mice reconstituted with either HLA-A2$^+$ or HLA-A2$^+$/HLA-DR1$^+$ HSC. Significant differences were not observed between both groups.

**Induction of functional human humoral immune responses during adenovirus infection**

To further characterize the antiviral human immune responses during adenovirus infection on a cellular level, we analyzed human leukocytes isolated from blood, spleen, and liver in HIS and HIS-A2/DR1 mice at day 10 following infection. In accordance with previously published data (13), innate human immune responses were limited in humanized mice (Fig. 1D). Nevertheless, we observed a significant increase in intrahepatic human CD68$^+$ macrophages (from ~5–11%) in both HIS and HIS-A2/DR1 mice during infection (Fig. 4A, 4B). NK cell frequencies in blood, spleen, and liver, however, did not change upon infection in HIS (data not shown) or HIS-A2/DR1 mice (Fig. 4C).

The frequency of B cells significantly increased in the liver of HIS-A2/DR1 mice (mean control: 47%; mean AdV5: 57%; $p = 0.003$) at day 10 postinfection, but decreased in the blood (mean control: 43%; mean AdV5: 32%; $p = 0.01$) (Fig. 4D, 4E). These findings suggest that human B cells are recruited into the infected liver of HIS-A2/DR1 mice. We also determined human IgM and IgG serum concentrations in infected mice and control groups at days 10 and 20 postinfection. Serum IgM levels remained constant (~50 $\mu g/ml$) in both HIS and HIS-A2/DR1 mice during the course of infection (Fig. 5A). Although overall human IgG levels were very low in both strains (Fig. 5B), they were significantly higher in uninfected HIS-A2/DR1 mice as compared with HIS mice (HIS mean: 0.07 $\mu g/ml$; HIS-A2/DR1 mean: 0.7 $\mu g/ml$; $p = 0.01$). IgG concentrations further increased at day 20 post–AdV5 infection (mean: 4 $\mu g/ml$) (Fig. 5B), indicating improved production of human IgG in HIS-A2/DR1 mice. Thereby, reconstitution with HLA-A2$^+$/HLA-DR1$^+$ HSC resulted in slightly elevated IgG levels, but this trend did not reach statistical significance (Supplemental Fig. 2B).

To test whether HIS and HIS-A2/DR1 mice produce virus-specific Abs, we tested sera from AdV5-Fluc–infected mice (20–30 d postinfection) and control groups for the capacity to neutralize AdV5 infection in vitro. As expected, serum of AdV5-Fluc immune but not naive BALB/c mice efficiently neutralized the virus at dilution of 1:2–1:1000 (Fig. 5C). The serum of ~50% of HIS and HIS-A2/DR1 mice tested had a similar capacity to neutralize AdV5-Fluc at 1:2 and 1:10 dilutions (Fig. 5C). These results indicate that both humanized mouse strains are able to generate Ag-specific human Abs that partially neutralize AdV5-Fluc in an in vitro assay.

**FIGURE 5.** Increase of total human IgG and generation of neutralizing Abs during adenovirus infection. The concentration of human IgM (A) and IgG (B) in sera of AdV5-infected HIS ($n = 5$) and HIS-A2/DR1 mice ($n = 8$) (days 10 and 20 postinfection) and control groups ($n = 5$ each) was quantified by cytometric bead array. (C) The presence of AdV5-Fluc–specific neutralizing Abs in the serum of AdV5-infected BALB/c ($n = 3$), HIS ($n = 4$), and HIS-A2/DR1 ($n = 4$) mice (20–30 d postinfection) and control mice was determined by incubation of virus with serum dilutions (1:2–1:1000) and subsequent infection of HeLa cells. At 24 h postinfection, Fluc activity in HeLa cell lysates was determined, and neutralization efficiency was calculated. Data represent mean ± SEM.
Adenovirus infection induces liver infiltration and activation of human T cells

We next characterized the induction of human T cell responses during AdV5 infection. Although frequencies and total numbers of intrahepatic CD3+ T cells were only slightly elevated HIS mice at 10 d postinfection (mean control: 30%; mean AdV5: 39%; \( p = \text{NS} \)), those values were significantly increased in HIS-A2/DR1 mice (mean control: 27%; mean AdV5: 45%; \( p = 0.0005 \)) (Fig. 6A, 6B). The ratio CD4+ to CD8+ T cells remained unchanged (data not shown). Within the blood- and liver-derived CD4+ and the CD8+ populations from HIS-A2/DR1 mice, the percentage of CD45RO+ effector T cells was elevated at day 10 postinfection; this was most pronounced in the intrahepatic CD8+ T cell population (\( p = 0.01 \)) (Fig. 6C, 6D). In HIS mice, we also observed an increase in CD45RO+ T cells after AdV5 infection, although this did not reach statistical significance (data not shown). CD4+ and CD8+ effector T cells, particularly in the livers of infected HIS-A2/DR1 mice (Fig. 6E), were highly activated, as indicated by the expression of CD38, HLA-DR or both on >90% of these subsets. Significant increase in human Rantes/CCL5 serum concentration at days 3 and 10 postinfection provides additional evidence for T cell activation (HIS-A2/DR1 mean control: 26 pg/ml; mean day 3: 96 pg/ml; mean day 10: 85 pg/ml; \( p = 0.01 \)) (Fig. 6F). CCL5 is expressed by activated T cells and plays an important role in the recruitment of leukocytes to the site of infection. Of note, we did not detect changes in the serum concentration of IFN-\( \alpha \), IFN-\( \gamma \), IL-12, TNF-\( \alpha \), IL-10, IL-6, IL-1b, IL-8, CXCL9, CCL2, or CXCL10 (data not shown).

We then focused on the expression profiles of CD127 and PD-1 to phenotype human T cells in humanized mice during AdV infection. CD127, the IL-7R \( \alpha \)-chain, is highly expressed on naive T cells and long-lived memory T cells but downregulated on effector cells after Ag recognition (28). PD-1 is an inhibitory receptor that is transiently expressed on activated effector T cells during acute infection and highly upregulated on exhausted T cells during chronic infection (29). We detected significant changes in...
CD127 and PD-1 expression within the peripheral T cell populations of HIS and HIS-A2/DR1 mice (Fig. 7A, 7B, 7D). In naive mice, the majority of CD8⁺ and CD4⁺ T cells displayed a CD127⁺ PD-1⁻ phenotype (~75–80% in both mouse strains). During virus infection (day 10 and day 20), CD127 expression was downregulated, and large subsets of CD127⁺PD-1⁻, CD127⁻PD-1⁺, and CD127⁻PD-1⁻ T cells emerged (Fig. 7A, 7B). These phenotypic changes were most significant in the CD8⁺ T cell population of HIS-A2/DR1 mice. Similar to blood-derived T cells, we detected changes in CD127 and PD-1 expression patterns on intrahepatic T cells at day 20 postinfection (HIS-A2/DR1: Fig. 7C, 7D; HIS: data not shown). Taken together, these data provide further evidence for T cell Ag recognition and development of activated effector cells during AdV5 infection.

**Generation of Ag-specific and HLA-restricted T cells during adenovirus infection**

Finally, we analyzed whether the humanized mice could mount Ag-specific T cell responses during adenovirus infection. To assess the ability of human T cells to acquire Ag-specific effector functions when exposed to a virally infected mouse cells, we cocultured T cells isolated from HIS and HIS-A2/DR1 mice (20 d postinfection) or control mice with AdV5-infected or control autologous BMDM of NSG or NSG-A2/DR1 mice. We detected elevated concentrations of human TNF-α, but not IFN-γ, in the supernatants of T cells derived from AdV5-infected HIS-A2/DR1 mice and cocultured with AdV5-infected BMDM (Fig. 8A). Of note, TNF-α levels were not increased in cocultures of T cells derived from control mice and AdV5-infected BMDM (Fig. 8A). These results suggest Ag-specific cytokine secretion by in vivo primed human T cells when encountering an infected mouse cell. Next, we determined whether humanized mice generate Ag-specific T cell responses directed against Ags of the adenoviral vector. Specifically, we stimulated isolated leukocytes of AdV5-infected HIS-A2/DR1 mice for 5 h with peptide pools specific for the AdV5 hexon protein followed by intracellular cytokine staining. Hexon-specific T cell responses were readily detectable in immunocompetent AdV5-infected mice and also in adenovirus-exposed humans (30, 31). In HIS and HIS-A2/DR1 mice, we detected TNF-α production by CD4⁺ T cells, but not by CD8⁺ T cells, in response to several hexon peptide pools (Supplemental Fig. 3A, 3B and data not shown), indicating the priming of Ag-specific CD4⁺ T cells directed against viral proteins in both mouse strains.

To analyze the presence of HLA-A2–restricted CD8⁺ T cells, we infected HIS and HIS-A2/DR1 mice with adenoviruses expressing the HCV-NS3 or HCV-NS5B protein. NS3 and NS5B contain several well-described immunodominant HLA-A2–restricted epitopes (32, 33), and CD8⁺ T cells specific for these epitopes are readily detectable in HCV-infected patients. We stained isolated pooled blood-, spleen-, and liver-derived leukocytes of uninfected and AdV5-infected HIS and HIS-A2/DR1 mice directly ex vivo with HLA-A2 tetramers specific for NS31073–1081, NS31406–1415, and NS5B2594–2602. CD8⁺ T cells specific for NS31073–1081, but not the two other epitopes, were detectable in ~50% of infected HIS-A2/DR1 mice (Fig. 8B, 8D). Tetramer⁺CD8⁺ T cells were not detectable in nontransgenic (Fig. 8D) and uninfected mice (data not shown). To verify the positive tetramer results, we stimulated leukocytes from the same mice for 5 h with the cognate Ag followed by intracellular cytokine staining. CD8⁺ T cells from mice positive for the NS31073–1081 tetramer also produced TNF-α in response to the same Ag (Fig. 8C, 8D). These results indicate that hepatotropic virus infection in HLA-transgenic humanized mice
can induce HLA-restricted T cell responses toward epitopes that have been described to be immunodominant in humans.

**Discussion**

Mice engrafted with an HIS are becoming more widely used to study human infectious diseases. However, due to significant shortcomings in the function of the engrafted HIS, a major topic in current humanized mouse research is the improvement of the model systems (5, 13). Transgenic HLA expression, for example, has been shown to improve human antiviral HLA-restricted T cell responses during human lymphotropic virus infections, such as EBV or dengue virus infection (17, 18). However, little is known about human immune responses during hepatotropic virus infection. One recent study demonstrated the generation of HCV-specific T cell responses during hepatotropic virus infection. One recent study demonstrated the generation of HCV-specific T cell responses in HCV-permissive mice reconstituted with both a human liver and an HIS (12). However, in this study, the functional capacity of the engrafted HIS was not evaluated, and it remains unclear whether the observed immune responses could contribute to clearance of HCV infection in humanized mice (12).

In our study, we analyzed the extent of functional antiviral intrahepatic human immune responses in humanized mice, specifically focusing on the impact of ectopic HLA expression. To simplify a functional readout, we devised a hepatotropic virus infection system based on a replication-incompetent recombinant AdV5-Fluc. AdV5 is an attractive model virus because it efficiently infects the murine liver, and infection elicits significant innate and adaptive immune responses without being lethal or causing severe disease (23, 26).

Under steady-state conditions, we detected significant numbers of human immune cells in the liver, in particular macrophages, B cells, and polyfunctional T cells, suggesting homing of these immune cell subsets to the mouse liver. Of note, NK cells, which are abundant in the normal mouse and human liver (34), were detectable only at low frequencies in the liver of humanized mice. This finding is in line with previous reports about general defects in human NK cell development in these mice (13).

During virus infection, we observed an improved capacity of humanized HLA-transgenic mice to clear AdV5 from the liver as compared with nontransgenic humanized mice. Indeed, these mice showed a significant loss of in vivo Fluc expression over time that correlated with an intrahepatic accumulation of human macrophages, B cells, and T cells, the induction of human T cell acti-
vation, and the generation of Ag-specific B and T cell responses. T cell depletion abrogated the loss of in vivo Fluc expression in HLA-transgenic mice, clearly demonstrating the direct role of human T cells in mediating virus clearance in these mice.

Although antiviral immune responses were also detectable in non–HLA-transgenic humanized mice, they did not correlate with in vivo virus clearance.

These findings indicate that HLA expression in humanized mice enables the generation of partially functional adaptive human immune responses during virus infection of the mouse liver. HLA expression also enabled the generation of HLA-A2–restricted virus-specific T cells that were detectable by tetramer staining ex vivo.

Despite partial virus clearance and the generation of antiviral adaptive immune responses during adenovirus infection, HLA-transgenic humanized mice failed to completely eliminate the virus from the liver, as indicated by a detectable Fluc signal even at days 20–40 postinfection with a low infectious dose of 5 × 10⁹ particles. Remaining deficits in humanized immune system function may be responsible for this observation: human NK cells are present only at very low frequency; possibly impaired homing and recruitment of human immune cells through murine tissue; and limited interspecies cross-reactivity of chemokines and cytokines produced during the infection. The Fluc adenovirus model system should serve as a useful and simple platform to evaluate future improvement strategies, such as human cytokine or chemokine expression.

To analyze immune responses and pathogenesis of clinically relevant hepatotropic pathogens, it is necessary to dually engraft donor matched human liver cells and human immune cells. Proof-of-concept for this approach has recently been established (12), but dually reconstituted animals are difficult to generate and limited in numbers. It is also important to note that the liver sinusoidal endothelium, even in human liver chimeric mice, is of mouse origin. Liver sinusoidal endothelial cells play an important role in shaping intrahepatic immune responses by mediating Ag presentation and immune cell homing into the liver (35, 36). Thus, insights into human immune cell migration through the liver endothelium and possible modes of Ag presentation of murine liver sinusoidal endothelial cells to human T cells will be of great importance to evaluate the utility of dually engrafted mice for the study of human hepatotropic pathogens.

In conclusion, our study provides a detailed characterization of the extent of functional human antiviral immune responses during a hepatotropic virus infection in humanized mice. We show that the transgenic expression of HLA improves human adaptive immune responses during virus infection of the mouse liver and enables the detection of HLA-A2–restricted virus-specific CD⁸⁺ T cells. The experimental system used in our study should help to evaluate future strategies for the improvement of intrahepatic immune responses in humanized mice. These observations might guide improvements in dually reconstituted hepatitis B– or HCV-permissive mice harboring both human liver and immune system and contribute to the generation of a functional and reliable model for the preclinical evaluation of drug and vaccine candidates for human hepatotropic pathogens.

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