A Humanized Mouse Model to Study Hepatitis C Virus Infection, Immune Response, and Liver Disease

MICHAEL L. WASHBURN,*† MOSES T. BILITY,* LIGUO ZHANG,*§ GRIGORIY I. KOVALEV,* ADAM BUNTZMAN,* JEFFERY A. FRELINGER,*‡ WALTER BARRY,* ALEXANDER PLOSS,* CHARLES M. RICE,* and LISHAN SU*†‡§

*Lineberger Comprehensive Cancer Center, †Curriculum in Genetics and Molecular Biology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; ‡Key Laboratory of Immunity and Infection, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China; §Departments of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; and ¶Center for the Study of Hepatitis C, The Rockefeller University, New York, New York

BACKGROUND & AIMS: Studies of hepatitis C virus (HCV) infection, immunopathogenesis, and resulting liver diseases have been hampered by the lack of a small animal model. We developed humanized mice with human immune system and liver tissues to improve the studies of hepatitis C virus pathogenesis and treatment.

METHODS: To promote engraftment of human hematopoietic stem cells, we expressed a fusion protein of the FK506 binding protein (FKBP) and caspase 8 under control of the albumin promoter (AFC8), which induces liver cell death, in Balb/C Rag2−/−γC-null mice. Cotransplantation of human CD34+ human hematopoietic stem cells (HSC) and hepatocyte progenitors into the transgenic mice led to efficient engraftment of human leukocytes and hepatocytes. We then infected these humanized mice (AFC8-hu HSC/Hep) with primary HCV isolates and studied HCV-induced immune responses and liver diseases.

RESULTS: AFC8-hu HSC/Hep mice supported HCV infection in the liver and generated a human immune T-cell response against HCV. HCV infection induced liver inflammation, hepatitis, and fibrosis, which correlated with activation of stellate cells and expression of human fibrogenic genes.

CONCLUSIONS: AFC8-hu HSC/Hep mice are a useful model of HCV infection, the immune response, and liver disease because they contain human immune system and liver cells. These mice become infected with HCV, generate a specific immune response against the virus, and develop liver diseases that include hepatitis and fibrosis. This model might also be used to develop therapeutics for HCV infection.

Keywords: Animal Model of Hepatitis; Human Immunology; Fibrosis; Virology.

Over 175 million people are chronically infected by hepatitis C virus (HCV), often resulting in hepatitis, liver fibrosis, cirrhosis, and development of hepatocellular carcinoma (HCC).1 The liver consists of unique subsets of antigen presenting cells and lymphocytes.2,3 It is postulated that HCV infection in the liver leads to impaired immune response that contributes to its chronic infection in 80% of infected patients. Chronic inflammation and impaired T-cell responses in the liver are proposed to lead to liver disease.4–7 Because of the difficulty of studying HCV infection in human patients, very little is known about how HCV infects patients and evades host immunity to establish chronic infection in the liver.

HCV infection triggers robust CD4 and CD8 cytotoxic T-cell (CTL) responses in infected patients. However, the majority of HCV patients fail to resolve HCV infection and become chronically infected, associated with impaired CD4 and CD8 T-cell functions.4,7 Sustained CD4+ T-helper activity in the blood is a hallmark of infections that are cleared spontaneously in 10%–20% of HCV-infected patients.4,6,8 Whereas chimpanzees that resolve infection have strong CTL response, those with chronic infection show reduced CTL response.8,9 The CTL response in chronically infected HCV patients is also reduced.8,10,11 During chronic HCV infection in humans and chimpanzees, regulatory T cells are also induced to subdue the antiviral immune responses.12,13 Thus, both CD4 and CD8 T cells are involved in HCV immunopathogenesis. However, we understand very little of human immune response to HCV infection because of lack of a robust model to study HCV infection and immunopathogenesis.14

Although chimpanzees have played a critical role in studying HCV infection,15 there are several drawbacks, including low chronic infection rate and lack of liver fibrosis, as well as costs and ethical concerns, that limit

© 2011 by the AGA Institute
0016-5085/$36.00
doi:10.1053/j.gastro.2011.01.001

Abbreviations used in this paper: AFC8, FKBP-Caspase 8 gene driven by the albumin promoter; AFC8-hu HSC/Hep, AFC8 transgenic mouse transplanted with human CD34+ hematopoietic stem cells and hepatocyte progenitor cells; Alb, albumin; ALT, alanine aminotransferase; αSMA, α-smooth muscle actin; CTL, cytotoxic T cell; Fad, fumarate/taacetate hydrolase; FKBP, FK506 binding protein; HCV, hepatitis C virus; Hep, hepatocyte progenitor cells; HIV, human immunodeficiency virus; HSC, hematopoietic stem cells; IFN, interferon; IL, interleukin; LN, lymph nodes; mAb, monoclonal antibodies; PHA, phytohemagglutinin; SCID, severe combined immunodeficiency; TIMP1, tissue inhibitor of metalloproteinases 1; TNF, tumor necrosis factor; uPA, urokinase-type plasminogen activator.
their utility. A mouse engrafted with human liver cells and a functional human immune system will be an excellent model to study the virus. A number of human-mouse chimeric liver models have been developed but allow analyses of only limited aspects of HCV infection and pathogenesis. The albumin (Alb)-urokinase-type plasminogen activator (uPA)/severe combined immunodeficiency (SCID) transgenic mouse contains the uPA transgene under control of an Alb promoter, and homozygous animals are unhealthy and die because of profound hypofibrinogenemia and accelerated hepatocyte death, which can be rescued by transplantation of mouse or human hepatocytes, the Alb-uPA/SCID mouse with efficient human hepatocyte engraftment can be infected with HCV. It was reported recently that fumarylacetoacetate hydrolase (Fah)-Rag2-γC-null mice can also be highly engrafted with human hepatocytes to support HCV infection. However, because of lack of a functional immune system, it is not possible to study HCV immunopathogenesis, and no liver diseases are observed in the Alb-uPA/SCID or Fah-Rag2-γC-null model.

The Balb/C Rag2-γC-null mouse supports development of a functional human immune system after injecting CD34+ human hematopoietic stem cells (HSC). To facilitate engraftment of human liver cells in Balb/C Rag2-γC-null mice, we expressed the active Caspase 8 fused with FK506 binding domain (FKBP) with inducible suicidal activity in mouse liver cells under control of the albumin promoter (AFC8 mice). We cotransplanted human hepatocyte progenitor cells (Hep) and CD34+ HSC into the transgenic mice and treated them with the FKBP dimerizer. Human immune cells and human hepatocytes were both efficiently developed, thereby generating a mouse model containing both a human immune system and human liver cells (AFC8-hu HSC/Hep). AFC8-hu HSC/Hep mice supported HCV infection in the liver and generated human T-cell response to HCV. In addition, HCV infection induced liver inflammation and fibrosis and correlated with activation of stellate cells and expression of human fibrogenic genes.

Generation of Transgenic Mice With FKBP-Caspase 8 Gene Driven by the Alb Promoter

Animal procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee. Balb/C Rag2−/−γC-null mutant female mice were super-ovulated, and the fertilized eggs were isolated to generate transgenic mice by standard procedures in the UNC transgenic core facility. FKBP-Caspase 8 gene driven by the Alb promoter (AFC8) mice were bred and handled under specific pathogen free conditions in the UNC DLAM facility.

Induction of Murine Hepatocyte Death in AFC8 Mice

We injected mice intraperitoneally or intravenously with AP20187 (Ariad Pharmecuticals) or vehicle only (4% ethanol, 10% polyethylene glycol–400, 2% Tween-20 in water) at a dose of 5 μg/g body weight at indicated days post-transplant of human cells. Alanine aminotransferase (ALT) levels were measured in the blood of treated mice.

Human Hepatocyte Progenitor Cells and CD34+ HSC From Human Fetal Liver

Human hepatocyte progenitor and CD34+ cells were isolated from 15- to 18-week-old fetal liver tissue essentially as described. Parenchyma cells (30% EpCAM+ hepatoblasts/progenitors) were separated from the nonparenchyma cells (including CD34 HSC cells). CD34+ cells were isolated by magnetic-activated cell sorting, and the purity was greater than 95%.

Transplantation of AFC8 Mice With Human CD34 HSC and Liver Progenitor Cells

CD34+ HSCs (0.5–1 × 106) and Hep progenitor cells (0.5–1 × 106) from the same donor were coinjected into the liver of 1- to 5-day-old newborn AFC8 and littermate control mice, previously irradiated at 400 rad. We then injected AFC8-hu mice with AP20187 (Ariad Pharmecuticals) at a dose of 5 μg/g body weight as described above.

Blood and Tissue Analysis

AFC8-hu HSC/Hep mice were bled to measure human leukocyte reconstitution at 12–16 weeks post-transplantation. At termination, liver tissue was snap frozen in RNAlater (Qiagen, Valencia, CA) or fixed in 10% formalin. Liver sections were stained with H&E, Sirius Red, and Fast Green or with antibodies: mouse monoclonal anti-human α smooth muscle actin (1A4, Dako, Carpinteria, CA), mouse monoclonal anti-human CD45 (B11 + PD7/26; Dako), and anti-human Alb (Dako). Immunoreactivity was determined by incubation with 3,3′-diaminobenzidine tetrahydrochloride substrate (Pierce, Rockford, IL) or Vulcan red (Dako) and counterstained with hematoxilin.
**Human-Specific Gene Expression**

Human- or mouse-specific quantitative real-time polymerase chain reaction (PCR) primers were designed using National Center for Biotechnology Information primer design program and Blast database (see Supplementary Figures 3 and 6). Gene expression analysis was examined using Thermo Scientific SYBR Green real-time PCR reagents. Values shown are relative gene expression normalized to specific glyceraldehyde-3-phosphate dehydrogenase.

**HCV Infection of AFC8-hu HSC/Hep Mice**

AFC8-hu HSC/Hep mice or control mice were inoculated intravenously with 100 μL of clinical isolates of HCV genotype 1a (1–5 × 10⁷ genome copies/mL) or control human sera. Mouse blood and liver tissues were harvested to prepare RNA for measuring HCV genomic RNA as reported.28

**T-Cell Response Analysis**

Leukocytes from various tissues of AFC8-hu mice were analyzed by fluorescence-activated cell sorter for human surface markers. Total spleens and lymph node cells containing 1 × 10⁵ human CD45⁺ cells were stimulated for 20 hours with 10 μg/mL phytohemagglutinin in Iscove’s Modified Dulbecco’s Medium (IMDM) + 10% fetal bovine serum media (Gibco, Carlsbad, CA). The cells were then stained for human T cells (CD45, CD3, CD4, CD8).

---

**Figure 1.** AFC8 mice support engraftment of human hepatocytes in the liver. (A) Inducible activation of Caspase 8 through dimerization of FKBP-Caspase 8. The chemical dimerizer AP20187 leads to activation of Caspase 8 and apoptosis. (B) Mouse liver injury can be repeatedly induced in AFC8 transgenic mice. AFC8 or littermate control mice (4–6 weeks old) were injected with AP20187 at day 0 and day 6. ALT was measured in serum at -1, 0, 1, 3, 6, and 7 days postdrug treatment. Data are representative of 3 experiments with n = 4 per group. Data represent means ± standard deviation. *P < .05. (C) Liver sections from control mice without transplant (left) and both control (middle) and AFC8 (right) transplanted with human HSC/Hep (AFC8-hu HSC/Hep) were stained with anti-human Alb antibody. Human hepatocytes were present around the veins and dispersed throughout all lobes of the liver. (D) Human Alb positive cells and total nucleated cells in the liver of humanized AFC8 and nontransgenic littermate control mice were counted, and the percentage of Alb⁺ was calculated (n = 16 mice per group, **P < .01).
CD8) and for intracellular human interleukin (IL)-2, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ. To detect HCV-specific human T-cell response, human T cells were expanded by stimulating spleen/lymph node (LN) cells with a pool of 19 HCV core peptides (20mer overlapping by 10) at 10 μg/mL each + 1 μg/mL anti-CD28 monoclonal antibodies (mAb). The cells were then cultured for 8–10 days with expansion medium (IMDM; 10% fetal bovine serum, 10 U/mL human IL-2, and 125 ng/mL IL-7). The cells were restimulated with the same HCV core peptide pool for 18 hours and stained as above.

Statistical Analysis

We used unpaired 2-tailed Student t tests for all comparisons. P < .05 is considered significant. All data are reported as means ± standard deviation or standard error of mean as indicated.

Results

AFC8 Mice Can Be Efficiently Repopulated With Both Human Liver and Immune Cells

We constructed the FKBP-Caspase 8 fusion gene driven by the Alb enhancer/promoter (AFC8 gene). Dimerization of the active Caspase 8 by AP20187 induced apoptosis of cells in which it is expressed but not in bystander cells (Figure 1A and Supplementary Figure 1). We then generated transgenic Balb/C Rag2-γc-null mice with the AFC8 transgene and confirmed the liver-specific expression of the AFC8 transgene (Supplementary Figure 2).

When AFC8+ mice were treated with AP20187, we observed a repeated induction of ALT in AFC8 mice but not in littermate control mice (Figure 1B). To test the ability of the AFC8 mice to engraft human hepatocytes as well as human immune cells, human CD34+ cells (HSC) and Hep from the same human fetal liver tissue were injected into the liver of irradiated newborn AFC8 mice. We observed a significant increase of human Alb+ hepatocytes in the AFC8-hu chimeric liver (Figure 1C). About 15% (n > 10 mice) of total nucleated cells in the chimeric liver expressed human Alb in AFC8-hu HSC/Hep mice from multiple human fetal liver tissues between 5 and 16 weeks post-transplant (Figure 1D and Table 1). Using human gene-specific PCR primers (Supplementary Figure 3A), we also detected expression of human Alb and human hepatocyte genes CYP2E1, CYP2C9, and UGT1A7 in the liver of AFC8-hu HSC/Hep mice (Supplementary Figure 3B). As expected, AFC8-hu HSC/Hep mice also generated human immune cells in all lymphoid organs including the liver (Supplementary Figure 4, and data not shown). Therefore, we established a humanized mouse model with both human immune and human liver cells.

Table 1. Reconstitution of AFC8 Mice With Both Human Blood and Liver Cells

<table>
<thead>
<tr>
<th>Donor</th>
<th>Mouse No.</th>
<th>Sex</th>
<th>AFC8</th>
<th>Cells transplanted</th>
<th>% CD45+ cells</th>
<th>% Albumin+</th>
</tr>
</thead>
<tbody>
<tr>
<td>n/a</td>
<td>1</td>
<td>F</td>
<td>–</td>
<td>None</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>n/a</td>
<td>2</td>
<td>F</td>
<td>–</td>
<td>None</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td>108</td>
<td>M</td>
<td>+</td>
<td>4e5 HSC + 1e6 HPC</td>
<td>1.23</td>
<td>28.5</td>
</tr>
<tr>
<td>1</td>
<td>109</td>
<td>M</td>
<td>+</td>
<td>4e5 HSC + 1e6 HPC</td>
<td>13.14</td>
<td>29.7</td>
</tr>
<tr>
<td>2</td>
<td>112</td>
<td>M</td>
<td>+</td>
<td>4.7e5 HSC + 1.5e6 HPC</td>
<td>–</td>
<td>10.7</td>
</tr>
<tr>
<td>2</td>
<td>116</td>
<td>F</td>
<td>–</td>
<td>4.7e5 HSC + 1.5e6 HPC</td>
<td>11.4</td>
<td>2.3</td>
</tr>
<tr>
<td>2</td>
<td>117</td>
<td>F</td>
<td>+</td>
<td>4.7e5 HSC + 1.5e6 HPC</td>
<td>0.92</td>
<td>9.5</td>
</tr>
<tr>
<td>2</td>
<td>114</td>
<td>M</td>
<td>+</td>
<td>4.7e5 HSC + 1.5e6 HPC</td>
<td>1.7</td>
<td>15.6</td>
</tr>
<tr>
<td>2</td>
<td>115</td>
<td>F</td>
<td>+</td>
<td>4.7e5 HSC + 1.5e6 HPC</td>
<td>1.8</td>
<td>16.7</td>
</tr>
<tr>
<td>3</td>
<td>56×4</td>
<td>F</td>
<td>–</td>
<td>1e5 HSC + 5e5 HPC</td>
<td>1.4</td>
<td>4.5</td>
</tr>
<tr>
<td>3</td>
<td>57×4</td>
<td>F</td>
<td>–</td>
<td>1e5 HSC + 5e5 HPC</td>
<td>0.8</td>
<td>3.8</td>
</tr>
<tr>
<td>3</td>
<td>58×4</td>
<td>F</td>
<td>+</td>
<td>1e5 HSC + 5e5 HPC</td>
<td>0.3</td>
<td>16.0</td>
</tr>
<tr>
<td>3</td>
<td>59×4</td>
<td>M</td>
<td>+</td>
<td>1e5 HSC + 5e5 HPC</td>
<td>–</td>
<td>n/a</td>
</tr>
<tr>
<td>4</td>
<td>98×4</td>
<td>M</td>
<td>–</td>
<td>1e6 HSC + 1e6 HPC</td>
<td>17.9</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>107×4</td>
<td>M</td>
<td>–</td>
<td>1e6 HSC + 1e6 HPC</td>
<td>7.9</td>
<td>n/a</td>
</tr>
<tr>
<td>4</td>
<td>99×4</td>
<td>M</td>
<td>+</td>
<td>1e6 HSC + 1e6 HPC</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>110×4</td>
<td>F</td>
<td>+</td>
<td>1e6 HSC + 1e6 HPC</td>
<td>19.9</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>100×4</td>
<td>M</td>
<td>+</td>
<td>1e6 HSC + 1e6 HPC</td>
<td>24.8</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>92×4</td>
<td>F</td>
<td>+</td>
<td>1e6 HSC + 1e6 HPC</td>
<td>28.3</td>
<td>31</td>
</tr>
<tr>
<td>4</td>
<td>108×4</td>
<td>F</td>
<td>+</td>
<td>1e6 HSC + 1e6 HPC</td>
<td>34.4</td>
<td>26</td>
</tr>
<tr>
<td>5</td>
<td>56</td>
<td>F</td>
<td>+</td>
<td>1e6 HSC + 1e6 HPC</td>
<td>31.1</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>58</td>
<td>M</td>
<td>+</td>
<td>1e6 HSC + 1e6 HPC</td>
<td>32.3</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>54</td>
<td>F</td>
<td>+</td>
<td>1e6 HSC + 1e6 HPC</td>
<td>34.9</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>57</td>
<td>F</td>
<td>+</td>
<td>1e6 HSC + 1e6 HPC</td>
<td>64.9</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>581</td>
<td>F</td>
<td>–</td>
<td>1e6 HSC + 1e6 HPC</td>
<td>10.2</td>
<td>1</td>
</tr>
</tbody>
</table>

*Positive (+), AFC8 transgenic mouse; minus (−), nontransgenic littermates.

*Number of cells transplanted into the mice. HSC = CD34+ hematopoietic stem/progenitor cells. HPC = hepatocyte stem/progenitor cells.

*Percent human CD45+ cells in the blood. Minus (−), < 0.1%.

*Liver sections were stained for human albumin. Human albumin positive cells were counted in more than 4 different fields and averaged. The percentage was calculated relative to total nucleated cells in the liver.
HCV Infection Leads to Liver Infiltration and Hepatitis in AFC8-hu HSC/Hep Mice

We inoculated AFC8-hu mice with patient HCV isolates (genotype 1a) or control human serum (Table 2). We detected HCV genomic RNA in the liver of about 50% HCV-infected AFC8-hu mice (Figure 2A and Table 2) at termination (1–4 months postinfection) but were unable to detect HCV viremia in the blood of infected mice (data not shown). As controls, we did not detect HCV RNA in mock-infected AFC8-hu HSC/Hep mice or in HCV-infected AFC8 mice with no human cell transplant (Figure 2A).

When ALT levels were measured at various times after HCV infection, significant induction of ALT was detected in HCV-infected AFC8-hu mice after 2–4 weeks postinfection but not in control mice (Figure 2B). Consistent with elevated ALT levels, we observed significant leukocyte infiltration into the liver in HCV infected AFC8-hu HSC/Hep mice (Figure 2C). We observed an increase of human CD45+/leukocyte, including CD68+/macrophages and CD3+/T cells, in the liver of HCV-infected mice (Figure 2C, lower panel, and Supplementary Figure 5A). When total intrahepatic leukocytes were analyzed, we detected an increase of multiple human leukocyte subsets including CD3+/CD4+ and CD3+/CD8+ T cells, CD3+/CD56+ natural killer cells, and plasmacytoid dendritic cells (CD123+/CD4+/CD3−) in the liver of HCV-infected mice (Supplementary Figure 5B). Similar infiltrations have been reported in HCV-infected humans.29–31 Thus, AFC8-hu HSC/Hep mice were able to support HCV infection in the liver, leading to liver infiltration and injury.

HCV Infection Induces HCV-Specific Human T-Cell Immune Response

To characterize the human T-cell response in HCV-infected AFC8-hu HSC/Hep mice, we stimulated (phytohemagglutinin for 12 hours) spleen and LN cells from mock- or HCV-infected mice and measured human effector cytokines (IL-2, IFN-γ, and TNF-α) in human CD4+ and CD8+ T cells. We observed a significant increase of all 3 cytokines in both CD4+ (Figure 3A and B) and CD8+ (Figure 3C) T cells from HCV-infected AFC8-hu HSC/Hep mice. Therefore, HCV infection primed human T cells in AFC8-hu HSC/Hep mice.

### Table 2. HCV Infection and Liver Pathology in AFC8-hu HSC/Hep Mice

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>HCV particles inoculated</th>
<th>Days p.i.</th>
<th>Metavir score</th>
<th>Viral load in liver tissue (x10^3 copies/μg RNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PBS</td>
<td>None</td>
<td>80</td>
<td>0 n/d</td>
</tr>
<tr>
<td>2</td>
<td>PBS</td>
<td>None</td>
<td>110</td>
<td>0 n/d</td>
</tr>
<tr>
<td>87</td>
<td>PBS</td>
<td>None</td>
<td>110</td>
<td>1 n/d</td>
</tr>
<tr>
<td>3</td>
<td>HCV patient 1</td>
<td>~9e5</td>
<td>110</td>
<td>1 n/d</td>
</tr>
<tr>
<td>4</td>
<td>HCV patient 1</td>
<td>~9e5</td>
<td>110</td>
<td>1 n/d</td>
</tr>
<tr>
<td>110d</td>
<td>HCV patient 1</td>
<td>~9e5</td>
<td>93</td>
<td>2 viral load</td>
</tr>
<tr>
<td>137d,e</td>
<td>HCV patient 1</td>
<td>~9e5</td>
<td>89</td>
<td>2 viral load</td>
</tr>
<tr>
<td>138</td>
<td>HCV patient 1</td>
<td>~9e5</td>
<td>80</td>
<td>1 viral load</td>
</tr>
<tr>
<td>108</td>
<td>HCV patient 1</td>
<td>~9e5</td>
<td>32</td>
<td>3 viral load</td>
</tr>
<tr>
<td>109</td>
<td>HCV patient 1</td>
<td>~9e5</td>
<td>80</td>
<td>3 viral load</td>
</tr>
<tr>
<td>116</td>
<td>Human sera</td>
<td>None</td>
<td>62</td>
<td>1 n/d</td>
</tr>
<tr>
<td>117</td>
<td>Human sera</td>
<td>None</td>
<td>48</td>
<td>1 n/d</td>
</tr>
<tr>
<td>88</td>
<td>Human sera</td>
<td>None</td>
<td>97</td>
<td>1 n/d</td>
</tr>
<tr>
<td>89</td>
<td>Human sera</td>
<td>None</td>
<td>97</td>
<td>1 n/d</td>
</tr>
<tr>
<td>110*4</td>
<td>Human sera</td>
<td>None</td>
<td>69</td>
<td>1 n/d</td>
</tr>
<tr>
<td>90*4</td>
<td>HCV patient 2</td>
<td>~5e5</td>
<td>34</td>
<td>3 viral load</td>
</tr>
<tr>
<td>91*4</td>
<td>HCV patient 2</td>
<td>~5e5</td>
<td>57</td>
<td>2 viral load</td>
</tr>
<tr>
<td>92*4</td>
<td>HCV patient 2</td>
<td>~5e5</td>
<td>97</td>
<td>4 viral load</td>
</tr>
<tr>
<td>98</td>
<td>HCV patient 2</td>
<td>~5e5</td>
<td>69</td>
<td>1 viral load</td>
</tr>
<tr>
<td>100*4</td>
<td>HCV patient 2</td>
<td>~5e5</td>
<td>69</td>
<td>2 viral load</td>
</tr>
<tr>
<td>108*4</td>
<td>HCV patient 2</td>
<td>~5e5</td>
<td>97</td>
<td>2 viral load</td>
</tr>
</tbody>
</table>

p.i., postinfection.

*Denotes mice killed because of experimental end points or poor health on days post-HCV injection.

*Denotes mice found dead (analyzed between 12 and 24 hours after death) on the indicated days posts inoculation.

*Mouse number 116 is a nontransgenic mouse.
To detect HCV-specific T-cell response, we stimulated the spleen/LN cells from mock and HCV-infected mice with an overlapping peptide pool from the HCV core region in the presence of anti-CD28 mAb. After 8–10 days in culture, we observed a 4- to 5-fold preferential expansion of human CD3+ T cells from HCV-infected mice relative to mock control mice (Figure 4A). When restimulated with HCV core peptides, both human CD4+ (Figure 4B and C) and CD8+ (Figure 4D and E) T cells from HCV-infected mice also responded to produce IFN-γ, IL-2, and TNF-α. Thus, HCV infection in AFC8-hu HSC/Hep mice induced specific human T-cell response to HCV antigens.

**AFC8-hu HSC/Hep Mice Develop Liver Fibrosis After HCV Infection**

We then assessed liver pathology in HCV-infected AFC8-hu HSC/Hep mice. About 50% of HCV-infected AFC8-hu HSC/Hep mice had severe fibrosis throughout the liver parenchyma (Figure 5A, top panels, and Table 2), whereas mock-inoculated AFC8-hu HSC/Hep mice and HCV-inoculated AFC8 mice without human transplants did not develop fibrosis. Infiltrated human CD45+ cells in the liver were detected in the fibrotic regions (Figure 2). In the absence of a functional immune system, uPA/SCID or Fah-Rag-γC-null mice transplanted with only human hepatocytes supported HCV infection but no significant liver fibrosis.17,21 We analyzed HCV infection and liver pathology in 5 AFC8-hu mice transplanted with only human adult hepatocytes and did not detect significant liver fibrosis (Table 2). The 2 AFC8-hu Hep mice with low-grade fibrosis were analyzed 12–24 hours after unexpected death.

In human patients, HCV infection leads to activation of hepatic stellate cells, which contributes to liver fibro-
We stained liver sections with the mouse anti-human α-smooth muscle actin (αSMA) mAb to detect activated stellate cells. We observed activated stellate cells in the liver of HCV-infected AFC8-hu HSC/Hep mice (Figure 5A, bottom panels). Activated hepatic stellate cells also express extracellular matrix protein Collagen 1A1 and tissue inhibitor of matrix metalloproteinases 1 (TIMP1), which inhibits degradation of extracellular matrix and fibrosis resolution. We thus analyzed the expression of these genes in the liver with human-specific or mouse-specific PCR primers (Supplementary Figure 6). Interestingly, we detected specific induction of human TIMP1 (Figure 5B) and human Collagen 1A1 (Figure 5C), but not mouse fibrosis genes, in the liver of some HCV-infected AFC8-hu HSC/Hep mice. These results suggest that HCV infection activated human stellate cells and induced expression of human fibrogenic genes in the chimeric liver of AFC8-hu HSC/Hep mice.

Discussion
We report here a novel mouse model that supports efficient engraftment of both human immune cells and human liver cells. The Balb/C Rag2−/−C-null mouse with the AFC8 transgene enabled us to inducibly deplete murine hepatocytes. In addition to human immune cells

Figure 3. HCV infection induces human T-cell response in AFC8-hu mice. Spleen and lymph node cells from mock- and HCV-infected AFC8-hu mice at 6–8 weeks postinfection were stimulated for 12–16 hours with phytohemagglutinin. Cells were stained with antibodies for human T cells (CD3, CD4, and CD8) and for intracellular human cytokines (IL-2, IFN-γ, and TNF-α). (A) Human CD3+ CD4+ T cells were analyzed for expression of effector cytokines. Representative fluorescence-activated cell sorter plots are shown for each cell population. Numbers represent the percent cytokine+ of CD4 or CD8 T cells. (B) Increase of human CD4 effector T cells in HCV-infected AFC8-hu mice. Summarized data (n = 4 mice/group) are shown. (C) Increase of human CD8 effector T cells in HCV infected AFC8-hu mice. Human CD3+ CD8+ T cells were similarly analyzed for expression of effector cytokines. Data represent mean ± standard error of mean from 2 cohorts of mice with n = 4 per group. *P < .05; **P < .01; ***P < .001.

Figure 4. HCV infection induces HCV-specific human T-cell response in AFC8-hu mice. (A) Preferential expansion of human T cells from HCV-infected AFC8-hu mice after stimulation with HCV Core peptides. Spleen and LN cells from mock and HCV-infected AFC8-hu mice were stimulated with a pool of HCV Core peptides plus anti-CD28 mAb and cultured for 8–10 days. Fold increase of human CD4+ CD8+ T cells from HCV-infected samples (n = 4) was calculated relative to mock samples (n = 3). P < .05. (B–E) The expanded T cells were restimulated with HCV peptides and stained for intracellular human cytokines (IL-2, IFN-γ, and TNF-α). (B) Human CD4+ T cells are analyzed for expression of the cytokines. No stimulation controls show background. (C) Summarized results of individual mice are shown. (D) Human CD8+ T cells are analyzed for expression of the cytokines. Representative fluorescence-activated cell sorter plots are shown for each cell population. Numbers in FACS plots represent the percent cytokine+ of CD4 or CD8 T cells. (E) Shown are summarized data of individual mice from 2 cohorts of mice (n = 4 per group). Lines indicate the mean values. *P < .05; **P < .01; ***P < .001.
in lymphoid and liver organs, AFC8-hu HSC/Hep mice were also efficiently repopulated with human Alb+ liver cells. AFC8-hu HSC/Hep mice supported HCV infection, which induced HCV-specific human immune response, liver infiltration, hepatitis, and fibrosis.

In the absence of a functional immune system, uPA/SCID or Fah-Rag-γC-null mice transplanted with only human hepatocytes supported HCV infection but no significant liver fibrosis. Immunodeficient mice expressing the uPA transgene in the liver or carrying the Fah mutation allow human adult hepatocytes to efficiently repopulate the liver. These mice have poor health including neonatal death and, most importantly, they lack a human immune system. The severe liver injury in these mice may impair the development and function of human liver and immune cells. To overcome these deficiencies, the AFC8 mouse enabled us to inducibly deplete murine hepatocytes through a programmed cell death mechanism without bystander cell killing. In addition, the Rag2-γC-null mouse in Balb/C background permits efficient engraftment of human immune cells. Therefore, AFC8-hu HSC/Hep mice provide the first humanized mouse model with both human immune and liver cells.

We inoculated AFC8-hu HSC/Hep mice with HCV genotype 1a clinical isolates. We were able to detect HCV genomic RNA in the liver of AFC8-hu HSC/Hep mice (Figure 2A and Table 2), but we did not detect significant HCV viremia in the blood of HCV-inoculated AFC8-hu HSC/Hep mice. This may be due to the relative low level of human hepatocyte engraftment (~15%). To detect HCV viremia in the blood, the adult hepatocyte-engrafted uPA or Fah models support >50% engraftment of human hepatocytes. In the future, we will improve the AFC8-hu HSC/Hep model by optimizing the death induction conditions with different doses and times of dimer injections. It is also reported that the NOD-Scid-γC-/- (NSG) mouse is more permissive in accepting human cells. It is likely that the introduction of the AFC8 transgene into NSG mice will create a better host

Figure 5. AFC8-hu HSC/Hep mice develop liver fibrosis after HCV infection. (A) Representative liver sections from AFC8/mock, AFC8-hu/mock, AFC8 (no transplant)/HCV, and 3 AFC8-hu/HCV mice were stained with Sirius Red/Fast Green (top panels) to visualize liver fibrosis. Liver sections were also stained with a mouse monoclonal antibody against human α-smooth muscle actin (αSMA, bottom panels) to visualize activated stellate cells. Human- or murine-specific gene expression of TIMP1 (B) and Collagen 1A1 (COL1A1) (C) in the liver was measured using species-specific PCR primers. Values shown are relative gene expression normalized to human or mouse glyceraldehyde-3-phosphate dehydrogenase, respectively. Data represent mean ± standard error of mean.
for engrafting human immune and liver cells. To get different sources of human HSC and liver progenitor cells, the AFC8-hu mouse model will be useful to study functional differentiation of human embryonic stem or induced pluripotent cells to the human liver lineages. The construction of AFC8-hu HSC/Hep mouse with HSC and liver progenitor cells derived from human embryonic stem or induced pluripotent cells will be an exciting future direction.

HCV infection appeared to induce human immune responses in AFC8-hu HSC/Hep mice. We observed significant infiltration into the liver of HCV-infected AFC8-hu HSC/Hep mice, including human T cells, macrophages, dendritic cells, and natural killer cells. Not only did we observe a preferential expansion of human T cells from HCV-infected AFC8-hu HSC/Hep mice with HCV-derived peptides, but the expanded T cells also expressed IL2, TNF-α, and IFN-γ in response to HCV peptide restimulation (Figure 4). Therefore, HCV infection induced HCV-specific human T-cell responses in AFC8-hu HSC/Hep mice. It will be interesting to test whether depletion of human T cells (CD4 and/or CD8) will affect HCV infection and pathogenesis in AFC-hu HSC/Hep mice. It will also be of interest to characterize and study human T-cell subsets such as regulatory T cells and natural killer T cells in future experiments. Consistent with poor B-cell response reported in all humanized mouse models, we failed to detect HCV-specific human antibodies in HCV-infected AFC8-hu HSC/Hep mice (data not shown).

In human patients, immune responses against HCV are implicated as mediators of liver diseases. Remarkably, HCV-infected AFC8-hu mice developed human leukocyte infiltration, hepatitis, and liver fibrosis throughout the liver parenchyma with bridging septa (Figure 5). Human CD3+ T cells and CD68+ macrophages, as well as human Alb+ hepatocytes and αSMA+ activated stellate cells, were detected in and near the fibrotic region (Supplementary Figure 7), suggesting contribution of human leukocytes to liver fibrosis. We also showed that HCV infection led to increased level of activated human stellate cells in fibrotic livers. Human extracellular matrix protein Collagen 1A1 and inhibitor of matrix degradation TIMP1 were induced in HCV-infected AFC8-hu HSC/Hep mouse livers. It is of interest that the corresponding mouse fibrosis-associated genes were not induced by HCV infection. The lack of liver fibrosis in chronically infected chimpanzees also suggests a species-specific nature of HCV-induced liver fibrosis. The AFC8-hu HSC/Hep mouse will provide an excellent model to elucidate the mechanisms of HCV-induced human liver fibrosis.

Because human immunodeficiency virus (HIV) coinfection occurs in nearly 25% of HCV patients and often leads to accelerated end-stage liver disease, there is a significant need for a model system to study HCV/HIV coinfection. It will be of interest to test how HIV-1 infection will affect HCV infection and pathogenesis in the AFC8-hu HSC/Hep mouse. In addition to HCV infection and immunopathogenesis, AFC8-hu HSC/Hep mice will also be useful to study other liver-tropic pathogens such as hepatitis B virus and malaria.

### Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at [www.gastrojournal.org](http://www.gastrojournal.org), and at doi: 10.1053/j.gastro.2011.01.001.

### References


---

**April 2011**

**HCV PATHOGENESIS IN A HUMANIZED MOUSE MODEL**

1343


Received September 12, 2010. Accepted January 10, 2011.

Reprint requests
Address requests for reprints to: Lishan Su, PhD, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599. e-mail: lusu@med.unc.edu; fax: (919) 966-8212.

Acknowledgments
The authors thank Drs F. Bai and A. Rogers for help with liver pathology analysis; L. Chi, T.A. Curtis, and L. Chen for technical support; Drs M. Fried and J. Darling for providing the patient HCV sera (UNC Division of Gastroenterology and Hepatology); Dr L Arnold for FACS support; and UNC CFAR for virology support.

Conflicts of interest
The authors disclose no conflicts.

Funding
Supported in part by a UNC UCRF innovation grant; grants from NIH (AI076142, AA018009, AI077454 and AA018372 to L.S.), an immunology training grant (T32 AI007273 to M.L.W.) and UNC Lineberger Comprehensive Cancer Center Postdoctoral Training Grant (M.T.B.); and a grant from LCRF (to J.A.F.); from the UNC University Cancer Research Fund innovation grants (to L.S. and J.A.F.); from Ministry of Science and Technology (2009CB522507, 2006CB810901 & KSCX20YW-R-150 to L.Z.); from Ministry of Health (2009ZX10604 to L.Z. and 2008ZX10002-011 to L.Z., L.S.); from the Greenberg Medical Research Institute, the Ellison Medical Foundation, the Starr Foundation, the Ronald A. Shellow Memorial Fund, the Richard Salomon Family Foundation, and by a grant from the Foundation NIH through the Grand Challenges in Global Health initiative (to C.M.R. and A.P.); and a grant from the Center for Clinical and Translational Research (RR024143 to A.P.).