GASTROENTEROLOGY 2009;137:285–296

# BASIC—LIVER, PANCREAS, AND BILIARY TRACT

## Hepatitis C Virus and Disrupted Interferon Signaling Promote Lymphoproliferation via Type II CD95 and Interleukins

KEIGO MACHIDA,[*,‡,§] KYOKO TSUKIYAMA-KOHARA,[*,‖] SATOSHI SEKIGUCH,[*] EIJI SEIKE,[¶] SHIGENOBU TÓNE,[#] YUKIKO HAYASHI,[**] YOSHIMI TOBITA,[*] YURI KASAMA,[‖] MASUMI SHIMIZU,[‡‡] HIDEMI TAKAHASHI,[‡‡] CHYOJI TAYA,[§§] HIROMICHI YONEKAWA,[§§] NOBUYUKI TANAKA,[‡,‖‖] and MICHINORI KOHARA[*]

*[*]Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan; [‡]Department of Immunology, Graduate School of Medicine and Faculty of Medicine, University of Tokyo, Tokyo, Japan; [§]Department of Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California, Los Angeles, California; [‖]Department of Experimental Phylaxiology, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan; [¶]Department of Internal Medicine, Self-Defense Forces Central Hospital, Tokyo, Japan; [#]Department of Biochemistry, Kawasaki Medical School, Okayama, Japan; [**]Department of Pathology, Tokyo Metropolitan Komagome Hospital, Tokyo, Japan; [‡‡]Department of Microbiology and Immunology, Nippon Medical School, Tokyo, Japan; [§§]Laboratory of Animal Science, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan; and [‖‖]Department of Molecular Oncology, Institute of Gerontology, Nippon Medical School, Kanagawa, Japan*

**BACKGROUND & AIMS:** The molecular mechanisms of lymphoproliferation associated with the disruption of interferon (IFN) signaling and chronic hepatitis C virus (HCV) infection are poorly understood. Lymphomas are extrahepatic manifestations of HCV infection; we sought to clarify the molecular mechanisms of these processes. **METHODS:** We established interferon regulatory factor-1-null (irf-$1^{-/-}$) mice with inducible and persistent expression of HCV structural proteins (irf-1/CN2 mice). All the mice (n = 900) were observed for at least 600 days after Cre/loxP switching. Histologic analyses, as well as analyses of lymphoproliferation, sensitivity to Fas-induced apoptosis, colony formation, and cytokine production, were performed. Proteins associated with these processes were also assessed. **RESULTS:** *Irf-1*/CN2 mice had extremely high incidences of lymphomas and lymphoproliferative disorders and displayed increased mortality. Disruption of *irf-1* reduced the sensitivity to Fas-induced apoptosis and decreased the levels of caspases-3/7 and caspase-9 messenger RNA species and enzymatic activities. Furthermore, the *irf-1*/CN2 mice showed decreased activation of caspases-3/7 and caspase-9 and increased levels of interleukin (IL)-2, IL-10, and Bcl-2, as well as increased Bcl-2 expression, which promoted oncogenic transformation of lymphocytes. IL-2 and IL-10 were induced by the HCV core protein in splenocytes. **CONCLUSIONS: Disruption of IFN signaling resulted in development of lymphoma, indicating that differential signaling occurs in lymphocytes compared with liver. This mouse model, in which HCV expression and disruption of IFN signaling synergize to promote lymphoproliferation, will be an important tool for the development of therapeutic agents that target the lymphoproliferative pathway.**

More than 175 million people worldwide are infected with hepatitis C virus (HCV), which is a positive-strand RNA virus that infects both hepatocytes and peripheral blood mononuclear cells.[1–4] Chronic hepatitis infection can lead to hepatitis, cirrhosis, hepatocellular carcinoma, and lymphoproliferative diseases, such as B-cell non-Hodgkin's lymphomas and mixed cryoglobulinemia.[5–10] The current therapy for chronic HCV infection involves treatment with type I interferon (IFN) and derivatives of IFN, such as pegylated IFN.[11] Treatment with type I IFN is associated with regression of lymphoma in patients with hepatitis C.[12] However, more than 50% of HCV-infected individuals are resistant to treatment, which indicates that the inhibition of IFN signal transduction facilitates the persistent expression of HCV proteins by hepatocytes.

Transgenic mice that express the HCV core protein have been established using a promoter derived from hepatitis B virus,[13] whereas mice that express structural or complete viral proteins have been established using promoters derived from the albumin gene.[14] These mice are immunotolerant to the transgene and do not develop hepatic inflammation, although they do develop age-related hepatic steatosis and hepatocellular carcinomas. We also developed a transgenic mouse model in which the HCV complementary DNA, including viral genes that encode the core, E1, E2, and NS2 proteins, was conditionally expressed by the Cre/*lox*P system (CN2 mice).[15]

*Abbreviations used in this paper:* IFN, interferon; IL, interleukin; IRF, interferon-regulatory factor; PCR, polymerase chain reaction; WT, wild-type.


0016-5085/09/$36.00
doi:10.1053/j.gastro.2009.03.061

The conditional expression of HCV proteins protected mice from Fas-mediated lethal acute liver failure by inhibiting cytochrome *c* release from the mitochondria.[16] However, the expression of HCV in these mice was usually lost after 21 days. Therefore, an animal model of persistent HCV protein expression is required to examine the effects of chronic HCV infection in vivo.

IFN signaling mediates tumor suppressor effects and antiviral responses and is regulated by key transcription factors of the interferon-regulatory factor (IRF) protein family, including Irf-1, -2, -3, -7, and -9. Targeted disruption of *irf-1* results in aberrant lymphocyte development and a marked reduction in the number of $CD8^+$ T cells in the peripheral blood, spleen, and lymph nodes.[17] In addition, natural killer cell development is impaired in *irf-1*$^{-/-}$ mice.[18] The mechanisms by which HCV infection induces IFN resistance and influences the development of lymphomas are poorly understood. Therefore, in the present study, we established an *irf-1*$^{-/-}$ CN2 mouse model of persistent HCV expression, which allows investigation of the effects of HCV on lymphatic tissue tumor development.

## Materials and Methods

### *Animal Experiments*

Wild-type (WT), CN2, *irf-1*$^{-/-}$, and *Mx1-cre* mice were maintained in conventional animal housing under specific pathogen-free conditions. AxCANCre and AxCAw1 were obtained from Dr Izumu Saito (University of Tokyo).[15] To elicit Fas-induced liver damage, adult mice were injected intravenously with 10 μg of purified hamster monoclonal antibody against mouse Fas (clone Jo2; BD Biosciences, San Diego, CA) in 200 μL of phosphate-buffered saline. All animal experiments were performed according to the guidelines of the Tokyo Metropolitan Institute of Medical Science or Kumamoto University Subcommittee for Laboratory Animal Care. The protocol was approved by an institutional review board. Detailed procedures, including induction of the HCV transgene by poly(I:C) in CN2-29 Mx1-Cre mice, are described in Supplementary Materials and Methods.

### *Measurements of Caspase Activities*

The cytosolic splenocyte fractions were isolated as described,[16] and the detailed procedures are described in the Supplementary Material and Methods.

### *Lentiviral Vectors and Infection*

Isolated splenocytes from WT or *irf-1*$^{-/-}$ mice (total of $10^7$ cells) were infected with recombinant lentiviruses that express HCV core, E1, E2, NS2, *lacZ*, and empty vector, respectively. One day after infection, cells were selected with puromycin (final concentration of 1 μg/mL). After 5 days of puromycin selection, viable cells were examined.

### *Baculovirus Expression and Purification of HCV Core, E1, and E2 Proteins*

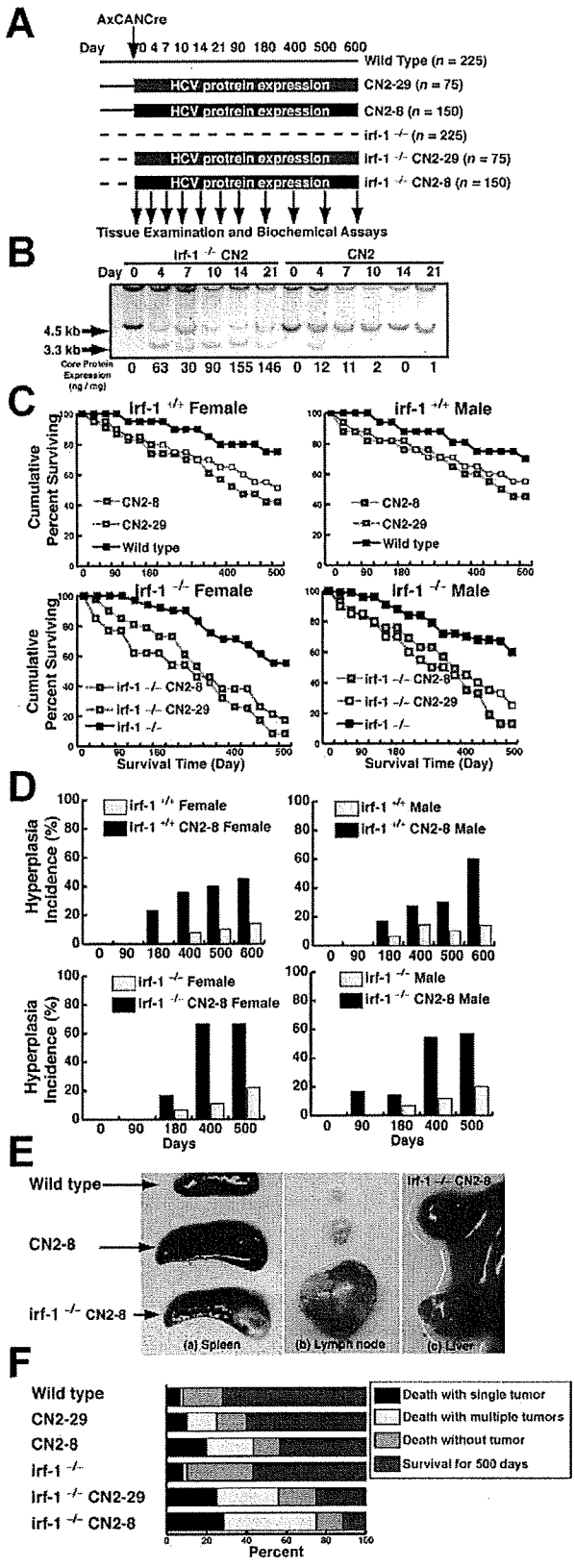
The E1 and E2 sequences from a genotype 1a isolate (strain H77)[19] and a genotype 1b isolate (strain HC-J4),[20] without the C-terminal transmembrane domains but containing the $His_6$ tag at the C terminus, were cloned into a transfer vector (pBlueBacHis2; Invitrogen, Carlsbad, CA). The expression of recombinant core, E1, and E2 proteins in insect cells and their purification have been described previously.[21]

## Results

### *Viral Protein Expression and Disruption of* irf-1 *Synergistically Increase the Development of Lymphoproliferative Disorders*

To clarify the in vivo effects of HCV protein expression, we examined the survival of mice that carry the CN2 transgene (CN2-8, CN2-29).[15] The experimental design is shown in Figure 1*A* (total number of mice, 900). Without Cre/*lox*P switching, the animals that carry the HCV transgene (CN2-8 and CN2-29: core, E1, E2, and NS2 proteins) appeared healthy and developed normally.[15] All of the transgene carriers were observed for at least 600 days after Cre/*lox*P switching (Figure 1*A*). Administration of a recombinant adenovirus that expresses *cre* (AxCANCre) induced the efficient recombination of CN2 transgenes in the hepatocytes from CN2 and *irf-1*$^{-/-}$ CN2 mice (Figure 1*B*). Recombination produced the floxed CN2 transgene (3.3 kilobases) and was completed within 4–7 days; it diminished before day 21 in CN2 mice but persisted in *irf-1*$^{-/-}$ CN2 mice. The expression of core protein in the hepatocytes of CN2 mice peaked on day 7 and decreased to an undetectable level by day 21 (Supplementary Figure 1*A*). The expression of core protein in hepatocytes coincided with a high level of inflammation, as determined by measurements of serum alanine aminotransferase activity (Supplementary Figure 1*A* and data not shown). The HCV core protein was detected in CN2-8 mice 4–14 days after the administration of AxCANCre, and disruption of *irf-1* ensured core protein expression for more than 500 days (Supplementary Figure 1*A* and 1*B*). Therefore, *irf-1* disruption allowed efficient and persistent expression of HCV proteins. HCV core protein gene expression was confirmed by reverse-transcription polymerase chain reaction (PCR) of livers, splenocytes, and peripheral blood monocytes (Supplementary Figure 1*C*). AxCANCre administration to the transgenic mouse induced the efficient expression of HCV transgenes in lymphocytes and splenocytes (Supplementary Figure 1*C*).

The survival rate of WT mice injected with the *cre*-adenovirus (AxCANCre) (Figure 1*C*) or control adenovirus (AxCAw1) (data not shown) was higher than that of the transgenic mice (CN2-8 and CN2-29), which excludes the possibility that the recombinant adenovirus affec-

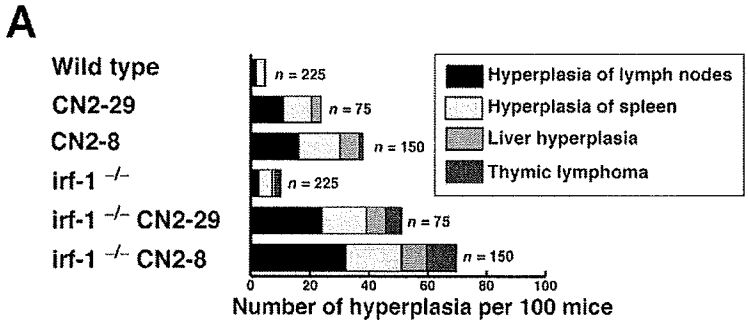



ted the results. More than 75% of the WT mice injected with AxCANCre survived to day 500, whereas the HCV-expressing mice had lower survival rates. The *irf-1*$^{-/-}$ CN2-8 and *irf-1*$^{-/-}$ CN2-29 strains had even lower survival rates, indicating that persistent HCV protein expression in combination with *irf-1* disruption significantly decreases survival (Figure 1C).

### *Lymphoproliferative Disorders Are Accelerated With Age and Level of Viral Protein Expression*

To determine the mechanism underlying the increased mortality caused by persistent HCV protein expression in *irf-1*$^{-/-}$ CN2 mice, we examined the kinetics of dysplasia (Figure 1*D*). Strikingly, 67% of the female *irf-1*$^{-/-}$ CN2 mice and 70% of the male *irf-1*$^{-/-}$ CN2 mice developed tumors 400 days after the administration of AxCANCre. Some of the *irf-1*$^{-/-}$ CN2 mice developed hyperplasia of the lymph nodes, and these tumors developed much earlier than the tumors in their *irf-1*$^{+/+}$ or CN2 counterparts (Figure 1*D*). Aberrant cell proliferation developed randomly among the male and female carrier animals between day 180 and day 600. On day 400 after Cre/*lox*P switching, the average weights of the spleens of the WT, CN2, and *irf-1*$^{-/-}$ CN2 mice were 90, 160, and 310 mg, respectively. The disruption of *irf-1* aggravated the HCV-induced spontaneous proliferative disturbances in lymphatic tissues. The number of CN2 mice that died with at least one tumor and the number of tumors per

**Figure 1.** Disruption of *irf-1* enhances oncogenic potential in combination with HCV transgene expression. (*A*) Experimental design for the animal model. Transgenic mice and their nontransgenic littermates (10–14 weeks of age) were administered the *Cre*-expressing adenovirus (AxCANCre) and killed after 4, 7, 10, 14, 21, 90, 120, 400, 500, or 600 days. (*B*) Southern blot analysis of hepatocyte DNA from mice derived by crossing *irf-1*$^{-/-}$ and HCV-transgenic (CN2) mice. Genomic DNA samples from WT (+/+) and CN2 mouse hepatocytes were digested with *Xba*I and subjected to Southern blot analysis using a radiolabeled genomic flanking probe to determine the rate of recombination of the HCV transgene construct (3.3-kilobase fragment). Disruption of *irf-1* allows persistent expression of HCV proteins. The effects of HCV protein expression on the survival rates of male and female *irf-1*$^{-/-}$ and *irf-1*$^{+/+}$ CN2 mice are shown. (*C*) Kaplan–Meier survival curves for WT mice, *irf-1*$^{-/-}$ mice, CN2 transgenic mouse strains 8 and 29, and *irf-1*$^{-/-}$ CN2-8 and CN-29 mice following infection with a recombinant adenovirus that expresses *cre* (AxCANCre). (*D*) HCV protein expression enhances hyperplasia in male and female CN2 and *irf-1*$^{-/-}$ CN2 mice. The occurrence of hyperplasia was monitored every 7 days for 600 days following the administration of AxCANCre. (*E*) Spleens (*a*) and lymph nodes (*b*) from age-matched WT, CN2, and *irf-1*$^{-/-}$ CN2 mice 500 days after the administration of AxCANCre. (*c*) Liver from the same *irf-1*$^{-/-}$ CN2 mouse (developing severe lymphadenopathy and splenomegaly) following the administration of AxCANCre. (*F*) The cause of death in CN2 transgenic mice with hyperplasias. Mice of each genotype (n = 150) were monitored up to day 600 after the administration of AxCANCre, and necropsies were performed to determine the number of tumors. Tumors included thymomas, splenomas, lymphomas, and hepatocellular carcinomas.



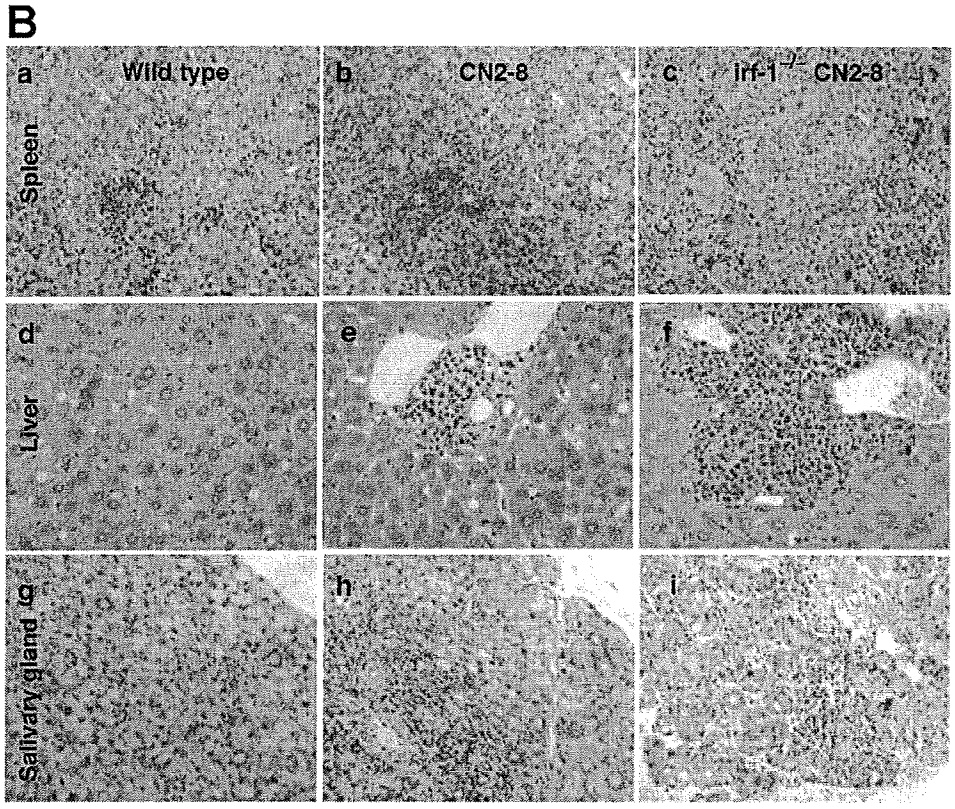




**Figure 2.** Disruption of *irf-1* aggravates lymphocyte infiltration in combination with HCV transgene expression. (*A*) Histologic analysis of spontaneous proliferative disturbances in the CN2 transgenic mice. Of the 900 mice injected with AxCANCre, 25 of 75 (33%) CN2-29, 47 of 150 (31%) CN2-8, 29 of 75 (39%) *irf-1*$^{-/-}$ CN2-29, and 62 of 150 (41%) *irf-1*$^{-/-}$ CN2-8 mice developed proliferative disturbances. Data shown are from the same cohort of mice analyzed in Figure 1*F*. (*B*) H&E-stained tissue sections of (*a–c*) spleens, (*d–f*) livers, and (*g–i*) salivary glands from age-matched WT, CN2, and *irf-1*$^{-/-}$ CN2 mice after the administration of AxCANCre.



mouse were significantly increased by the ablation of *irf-1* (Figure 1*F*). Although the type of hyperplasia did not differ significantly between the *irf-1*$^{-/-}$ CN2 mice and their *irf-1*$^{+/+}$ CN2 siblings, the time to onset of tumorigenesis differed dramatically (Figure 1*D* and 1*F*), indicating that age is a significant factor in the promotion of lymphomagenesis by HCV proteins.

A significant percentage of the mice that expressed the HCV core protein (*irf-1*$^{-/-}$ CN2 mice) developed polyclonal lymphoid growth disturbances, including splenomegaly, expanded lymph nodes, adenocarcinoma in the abdomen or leg, and lymphoma of the liver or Peyer's patches (Figure 2*A*). In contrast, hepatocytes with abundant expression of HCV proteins rarely developed into hepatocellular carcinomas. H&E staining of splenomegalic tissue revealed extensive hyperplasia of the white pulp zones, in which the cortical zones contained lymphoid follicles and scattered germinal centers, although mitotic figures were rarely observed (Figure 2*B* and data not shown). These results indicate that persistent expression of HCV proteins frequently induces lymphoproliferative disorders in addition to liver hyperplasia, which is consistent with the phenotype of patients with hepatocellular carcinoma.[3,4,9]

### *Abnormal T-Cell and B-Cell Proliferation in HCV Transgenic Mice*

To characterize the disruption of lymphocyte proliferation due to HCV protein expression in the transgenic mice, we used flow cytometry to determine the ratio of T cells to B cells by staining with antibodies directed against CD3, CD45R, CD4, CD8, and the T-cell receptor. The average ratio of T cells to B cells in the lymph nodes and spleens of CN2 mice was significantly higher than

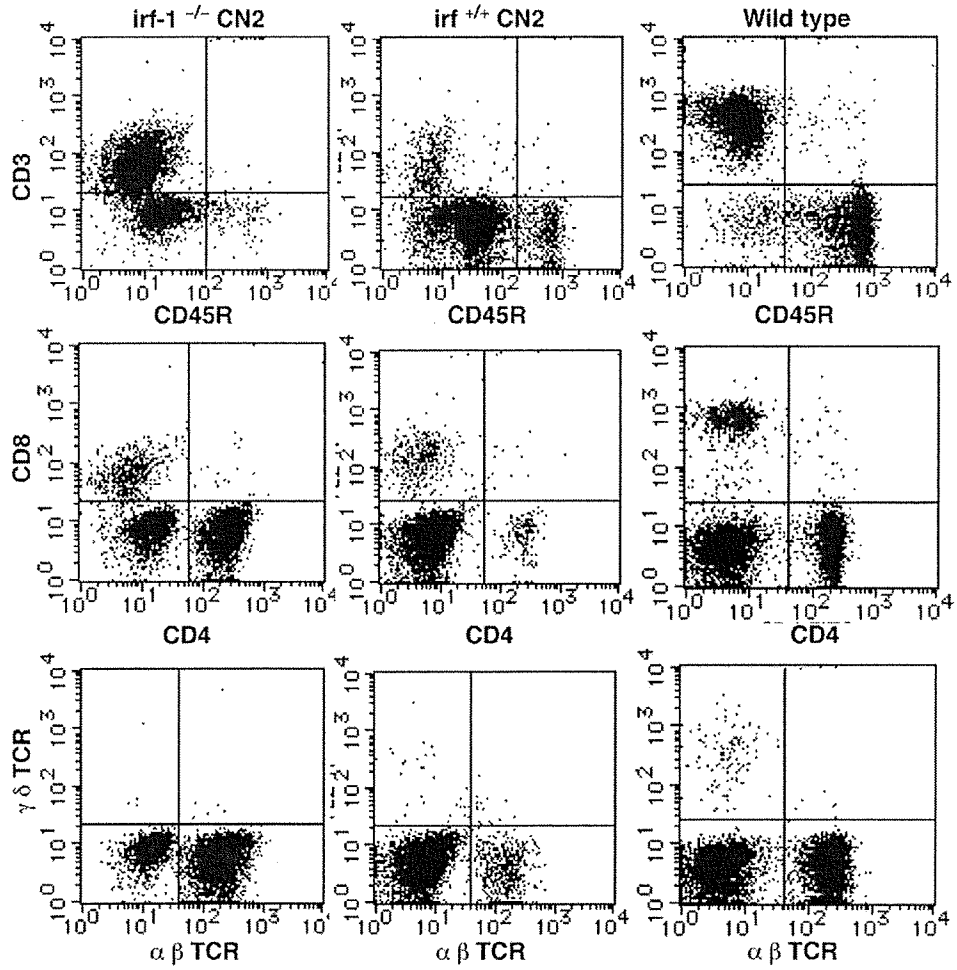



**Figure 3.** HCV expression and *irf-1* ablation affect the lymphocyte population. T-cell and B-cell proliferation in *irf-1*$^{+/+}$ CN2 mice, *irf-1*$^{-/-}$ CN2 mice, and WT mice. CD3$^+$, CD45R$^+$, CD4$^+$, CD8$^+$, and T-cell receptor–positive cells from age-matched *irf-1*$^{-/-}$ CN2, *irf-1*$^{+/+}$ CN2, and WT mice with hyperplasia were analyzed by fluorescence-activated cell sorting. Lymphocytes were prepared from CN2-8 and WT littermates at the age of 16 months, after administration of AxCANCre for 400 days.

that in the WT mice. The majority of the CD3$^+$ lymphocytes and a few CD8$^+$ lymphocytes expressed CD4 on their surfaces. The proliferating cells were mainly CD4$^+$ T cells, although some were CD45R$^+$B cells (Figure 3 and data not shown). The *irf-1*$^{-/-}$ CN2 mice also developed B-cell lymphomas (data not shown). These results confirm that HCV protein expression induces lymphoproliferative disorders that involve excessive expansion of both T and B cells. In *irf-1*$^{-/-}$ CN2 mice, the cell population that was negative for T-cell receptor ($\alpha$, $\beta$, $\gamma$, and $\delta$ isoforms) staining was smaller than that in the other mice.

### *Inhibition of Fas-Induced Apoptosis Owing to Disruption of* irf-1 *Leads to Persistent Expression of HCV in Transgenic Mouse Livers*

The Fas ligand is essential for the development of hepatitis via cytotoxic T-lymphocyte-mediated cell killing.[22] Therefore, we determined the sensitivities of *irf-1*$^{-/-}$ hepatocytes to Fas-induced apoptosis. The *irf-1*$^{-/-}$ mice and WT littermates were injected intravenously with a monoclonal antibody against Fas. The disruption of *irf-1* inhibited Fas-induced apoptosis, presumably by decreasing the levels of caspase-6 and -7 messenger RNA (mRNA; Supplementary Figure 2). These results suggest that the reduced expression of effector caspases delays Fas-mediated apoptosis in *irf-1*$^{-/-}$ mice and abrogates the elimination of HCV-expressing cells in vivo.

### *Stable Expression of HCV Proteins Induces Lymphoproliferative Diseases*

To confirm that HCV proteins induce lymphoproliferation without the adenoviral vector system, switching of the expression of HCV proteins was conducted using the Mx promoter-driven cre recombinase with poly(I:C) induction (Figure 4*A*). The Mx promoter is active in hepatocytes as well as in hematopoietic cells. We crossed CN2 mice with Mx1-Cre transgenic mice; CRE recombinase was expressed from the IFN-inducible *Mx1* promoter. Injection of the Mx1-Cre/CN2-29 mice with poly (I:C) induced IFN production and efficiently induced the generation of CN2 gene products in hematopoietic cells

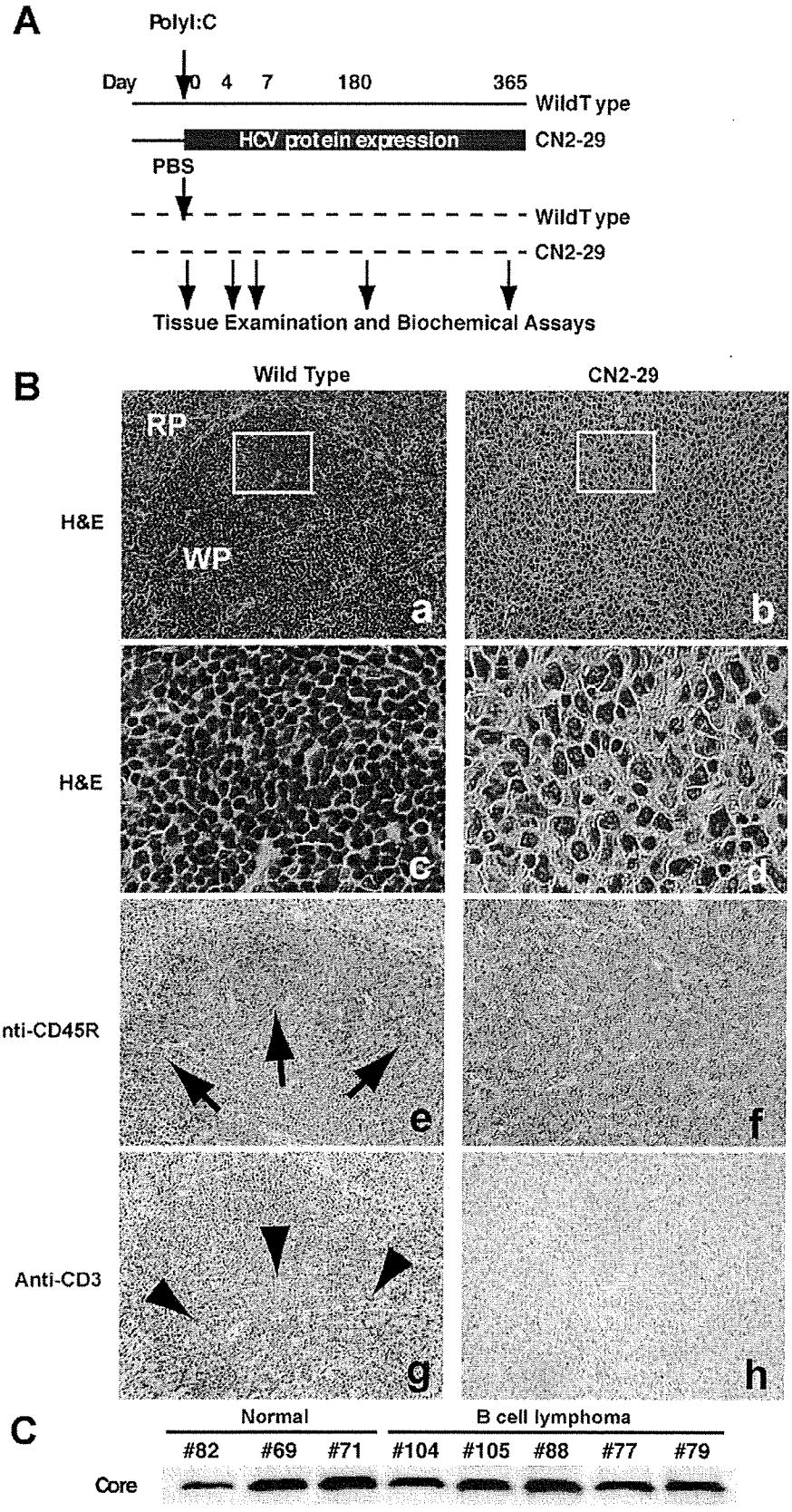
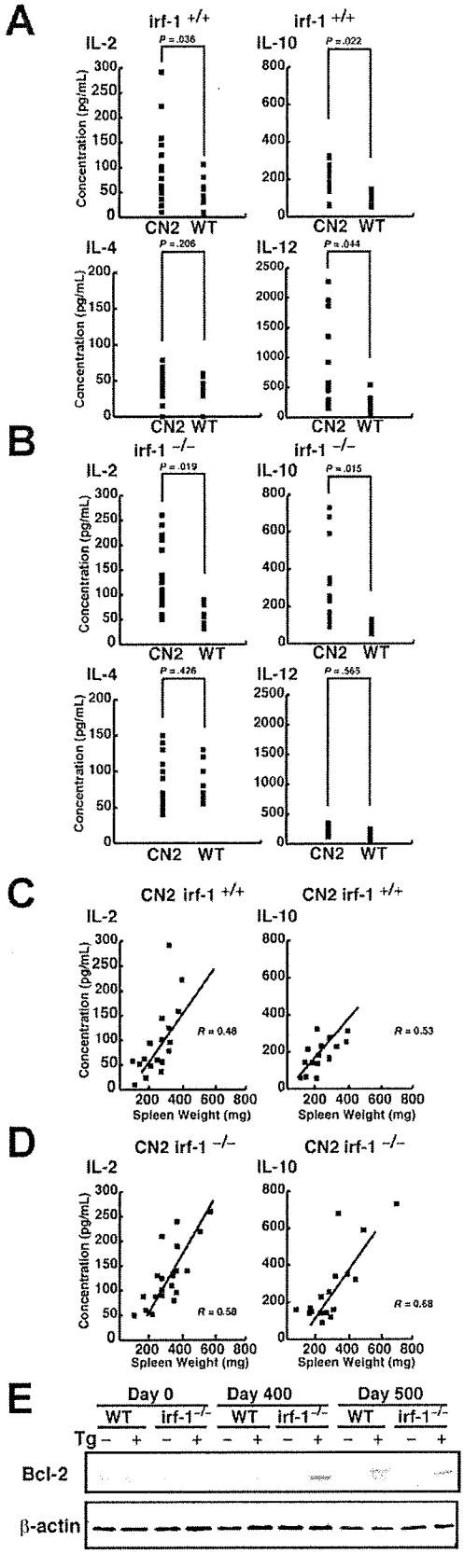



**Figure 4.** Stable expression of HCV viral proteins induces lymphoproliferative diseases. (*A*) Switching of the expression of HCV proteins was conducted using the Mx promoter–driven cre recombinase with poly(I:C) induction. The Mx promoter is active in hepatocytes as well as in hematopoietic cells. We crossed CN2 mice with Mx1-Cre transgenic mice; Cre recombinase was expressed from the IFN-inducible *Mx1* promoter. Injection of Mx1-Cre/CN2-29 mice with poly(I:C) induces IFN production and efficiently induces the expression of CN2 gene products in hematopoietic cells (mainly in Kupffer cells and lymphocytes), livers, and spleens but not in most other tissues. (*B*) The white pulp (WP) and red pulp (RP) comprise the components of the spleen in WT mice. The neoplastic cells replace the normal structures, such as the white pulp and red pulp. (*c* and *d*) The neoplastic cells are larger than lymphocytes (*c*), and the nuclei are irregular, round, oval, elongated, and polygonal (*d*). (*e* and *g*) The white pulp in WT mice consists of both a B-cell–rich area (*arrows*, *e*) and T-cell–rich area (*arrowheads*, *g*). (*f* and *h*) The neoplastic cells show staining for the B-cell marker CD45R, thereby supporting the diagnosis of B-cell lymphoma (*f*), while they do not show staining for the T-cell marker CD3 (*h*). *Frames c* and *d* are higher-magnification views of the white box areas in *a* and *b*, respectively. (*C*) Core protein expression was confirmed by immunoblotting.




(mainly in Kupffer cells and lymphocytes), liver, and spleen but not in most other tissues. At 7 days after induction of viral proteins, HCV core proteins were detected in both hepatocytes and hematopoietic cells (data not shown). After 180 days, almost 40% of the CN2(-29) mice developed lymphomas, whereas the WT mice did not (Figure 4*B*). The neoplastic cells were larger than lymphocytes, and their nuclei were irregular, round, oval, elongated, and polygonal. HCV core protein expression was confirmed by immunoblotting (Figure 4*C*), and increases in the levels of interleukin (IL)-2, IL-10, and IL-12 were observed (data not shown). The hematopoietic marker CD45R was detected in the lymphoproliferative regions and spleens (Figure 4*B*). The efficiency of expression switching was confirmed by both the HCV transgene copy numbers and protein expression using quantitative PCR and immunoblotting, respectively (Supplementary Figure 3). These results further validate that sustained expression of HCV proteins induces lymphoproliferation.

### *Increased IL-2, IL-10, and IL-12 Levels in HCV Transgenic Mice*

To study the mechanisms of HCV-induced lymphoproliferative diseases, we measured the serum IL-2, IL-4, IL-10, and IL-12 levels in the CN2 transgenic mice and their WT littermates (Figure 5*A*). The serum IL-4 concentration did not differ significantly between the CN2 and WT mice following injection with AxCANCre. However, the CN2 mice had significantly increased levels of serum IL-2, IL-10, and IL-12. Notably, the CN2 mice with proliferative disturbances in the lymph nodes and spleen had dramatically elevated levels of these cytokines, suggesting that altered cytokine production is involved in aberrant lymphocyte proliferation or differentiation in CN2 mice. In contrast, the *irf-1*$^{-/-}$ CN2 mice did not show elevated levels of serum IL-12 but had significantly higher levels of serum IL-2 and IL-10 compared with *irf-1*$^{-/-}$ mice (Figure 5*B*). Thus, the disruption of *irf-1* abrogates the increase in IL-12 level but augments the increases in the levels of IL-2 and IL-10 in CN2 mice. These results indicate that IL-2 and IL-10 play key roles

**Figure 5.** HCV protein expression alters the cytokine profile. (*A*) The serum IL-2, IL-4, IL-10, and IL-12 levels in *irf-1*$^{+/+}$ CN2 (Tg+) and *irf-1*$^{+/+}$ WT mice were measured by enzyme-linked immunosorbent assay. (*B*) The serum IL-2, IL-4, IL-10, and IL-12 levels in *irf-1*$^{-/-}$ CN2 (Tg+) and *irf-1*$^{-/-}$ WT mice were measured by enzyme-linked immunosorbent assay. The *P* values are based on comparisons of the mean cytokine concentrations. (*C* and *D*) Relationship between the IL-2 or IL-10 concentration in the serum and the spleen weights of (*C*) CN2irf-1$^{+/+}$ or (*D*) CN2irf1$^{-/-}$ mice with progressive lymphoproliferation. The numbers of points in the graphs correspond to the numbers of tested animals. (*E*) Bcl-2 protein levels in the lymph nodes of *irf-1*$^{+/+}$ (WT) and *irf-1*$^{-/-}$ transgenic (CN2) (Tg+) and WT mice on days 0, 400, and 500 after the administration of AxCANCre. Bcl-2 migrates at 26 kilodaltons. β-Actin was used as a loading control.

in the induction of the lymphoproliferative phenotype in *irf-1*$^{-/-}$ CN2 mice.

To verify the relationship between the weights of the lymph organs and the cytokine levels, the correlation coefficients were calculated according to Pearson (Figure 5*C* and 5*D*). Whereas spleen weight did not markedly influence the increase in IL-4 level (data not shown), a significant positive correlation was found between spleen weight and increased IL-2 and IL-10 levels in CN2 gene-expressing mice on the *irf-1*$^{-/-}$ background ($R = 0.58$, $P < .05$, and $R = 0.68$, $P < .05$, respectively) (Figure 5*D*). With respect to the serum levels of IL-2 and IL-10, a less intensive but significant positive correlation was found between the cytokine levels and spleen weights of CN2 gene-expressing mice on the *irf-1*$^{+/+}$ background ($R = 0.43$, $P < .05$, and $R = 0.53$, $P < .05$, respectively) (Figure 5*C*). These results indicate that IL-2 and IL-10 are involved in lymphoproliferation in viral protein-expressing mice.

### *Aberrant Expression of Bcl-2 in Expanded Lymph Nodes of CN2 Mice*

Bcl-2 immunoglobulin transgenic mice develop follicular lymphoproliferation[23] due to the inability of various stimuli to induce apoptosis in these mice.[24] Therefore, to examine whether HCV causes dysregulation of Bcl-2 in lymphoid tissues, we examined the expression of Bcl-2 (Figure 5*E*). Lymph nodes collected from *irf-1*$^{-/-}$ CN2 mice 400 days after the administration of AxCANCre showed elevated levels of Bcl-2. Immunoblot analysis revealed that a doublet for Bcl-2 (26 and 28 kilodaltons) appeared in some samples 500 days after AxCANCre administration, suggesting the presence of phosphorylated and nonphosphorylated Bcl-2.[25]

### *Combination Cytokine Treatment Enhances Splenocyte Colony Formation in Synergy With Viral Protein Expression*

To determine whether aberrant cytokine profiles contribute to lymphocyte transformation, a colony formation assay was performed using the methylcellulose method. Mouse splenocytes were infected with adenoviruses that expressed the *cre* DNA recombinase or *lacZ* control. Expression of HCV core proteins was induced by cre-adenovirus infection of the splenocytes (Figure 6*A*). Colony counting was performed at postinfection day 28 (Figure 6*B*). Combined treatment with IL-2 and IL-10 greatly enhanced colony formation, especially in the splenocytes of HCV transgenic mice (CN2-29, *irf-1*$^{-/-}$ CN2-29). The addition of IL-12 suppressed colony formation induced by combined treatment with IL-2 and IL-10. In the *irf-1*$^{-/-}$ background, treatment with IL-2 plus IL-10 or IL-2 plus IL-12 greatly enhanced colony formation. To determine whether enhanced colony formation correlated with cytokine-induced Bcl-2 expression, the Bcl-2 mRNA levels in the splenocytes were quantified (Figure 6*C*). Because IL-2 enhances T-lymphocyte proliferation and transformation,[26] it is of particular interest that treatment with IL-2 plus IL-10 resulted in marked increases in both lymphocyte transformation and the Bcl-2 mRNA levels upon HCV transgene expression. These results indicate that dysregulated cytokine expression, disruption of *irf-1*, and HCV transgene expression synergistically enhance splenocyte transformation.

### *Cytokine Treatment and HCV Transgene Expression Synergistically Inhibit Fas-Mediated Apoptosis*

To determine whether cytokines inhibit Fas-induced apoptosis, we treated the splenocytes from transgenic and WT mice with cytokines and then measured Fas-induced apoptosis by Annexin V staining and fluorescence-activated cell sorting, and we also assayed caspase enzymatic activity (Figure 6*D* and 6*E*). IL-10 treatment in the presence of IL-2 greatly inhibited Fas-induced apoptosis. Furthermore, *irf-1* disruption made the splenocytes resistant to Fas-induced apoptosis in the presence of IL-2, IL-10, and/or IL-12. In particular, IL-2 plus IL-10 treatment produced the strongest inhibition of Fas-induced apoptosis. These cytokines also up-regulated the Bcl-2 mRNA levels in splenocytes, which indicates that IL-2, IL-10, and/or IL-12 up-regulate *bcl-2* expression, which subsequently inhibits Fas-induced apoptosis. This result is consistent with reports that IL-10 and/or IL-2 treatment induce *bcl-2* in B or T lymphocytes.[10,27] Caspase-3/7 activity was correlated with the level of *bcl-2* expression (Figure 6*C* and 6*F*). These results indicate that aberrant cytokine expression and disruption of IFN signaling affect *bcl-2* expression, which is associated with the inhibition of caspase expression.

### *HCV Core and E2 Proteins Mediate IL-2, IL-10, and IL-12 Expression*

To determine which viral protein is responsible for cytokine expression, individual viral proteins were stably expressed in splenocytes using recombinant lentiviruses that express the HCV core, E1, E2, NS2, and lacZ. Each gene expression profile was confirmed by reverse-transcription PCR (Supplementary Figure 4). Only the HCV core protein induced IL-2 and IL-10 (Figure 7*A*). To determine whether extracellular viral proteins trigger cytokine expression, recombinant viral proteins were added to the cells. Only the viral envelope protein E2 induced IL-12 (Figure 7*B*). These results indicate that the HCV core and E2 proteins are responsible for IL-2, IL-10, and IL-12 expression.

### *HCV Core and IL-10 Induce Bcl-2 Expression*

To determine whether viral protein expression and cytokine stimulation synergistically induce Bcl-2 expression, individual viral proteins were stably expressed using lentiviral vectors, and the cells were tested for Bcl-2 expression. Core protein expression and IL-10 stimula-

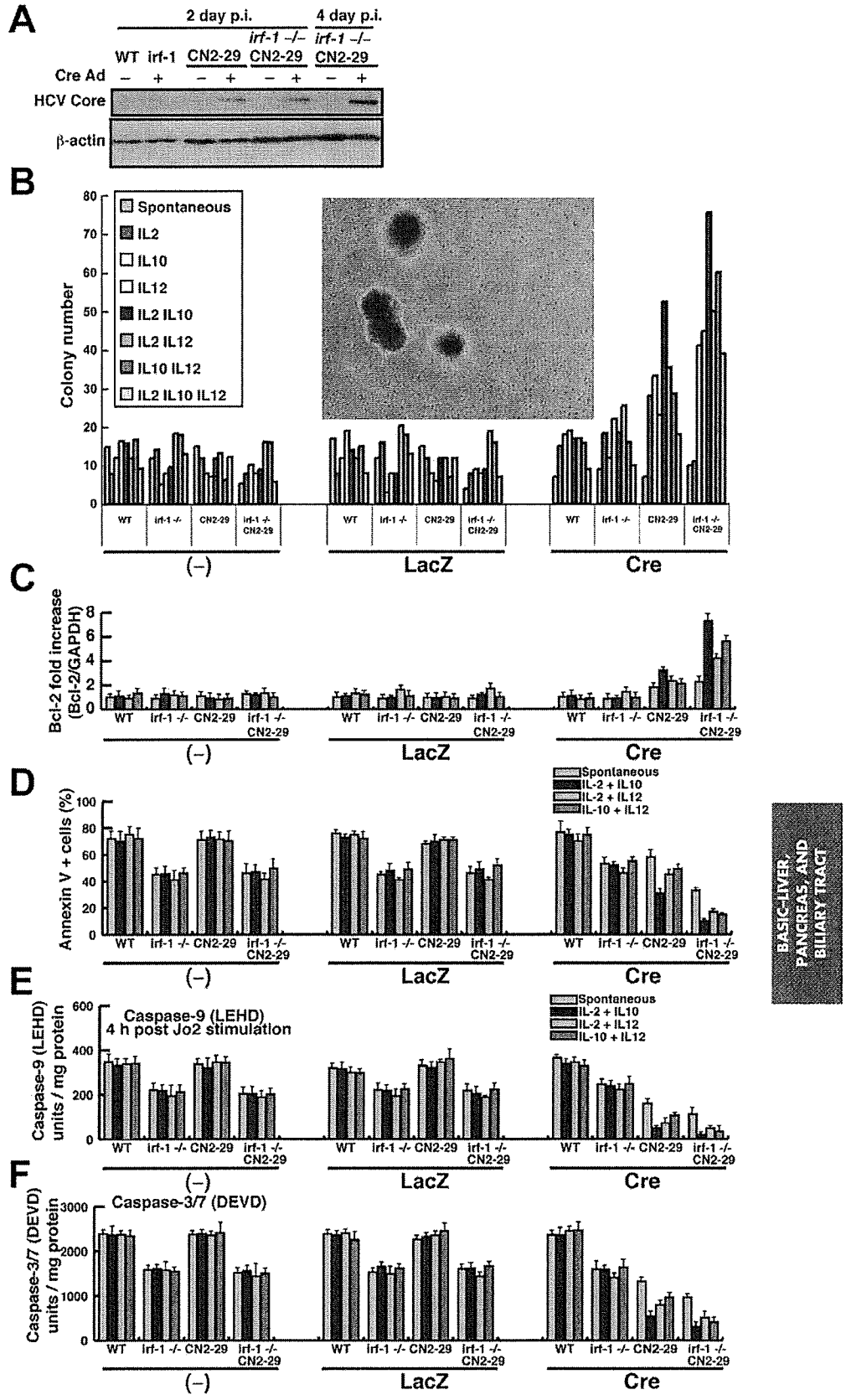



**Figure 6.** Lymphocyte transformation by aberrant cytokines and inhibition of apoptotic signaling. (*A*) Expression of the HCV core protein (21 kilodaltons) in *irf-1*$^{+/+}$ (WT) and *irf-1*$^{-/-}$ transgenic (CN2-29) and WT mice 2 or 4 days postinfection (p.i.) with AxCANCre (multiplicity of infection, 1.0). β-Actin was used as a loading control. (*B*) Colony formation assay for splenocytes from *irf-1*$^{+/+}$ (WT) and *irf-1*$^{-/-}$ WT or transgenic (CN2-29) mice in the absence or presence of the indicated cytokine and infected with mock, LacZ, and Cre adenoviruses. The *inset* shows an image of the colonies generated from the *irf-1*$^{-/-}$ CN2 splenocytes (original magnification 10×). (*C*) Quantification, by quantitative reverse-transcription PCR of Bcl-2 mRNA relative to control glyceraldehyde-3-phosphate dehydrogenase mRNA in the splenocytes of *irf-1*$^{+/+}$ (WT) and *irf-1*$^{-/-}$ or transgenic (CN2-29) mice treated with the indicated cytokines and infected with mock, LacZ, and cre adenoviruses. (*D*) Apoptosis measured by Annexin V fluorescence-activated cell sorting analysis of splenocytes from *irf-1*$^{+/+}$ (WT) and *irf-1*$^{-/-}$ or transgenic (CN2-29) mice treated with the indicated cytokines and infected with the mock, LacZ, and cre adenoviruses. (*E* and *F*) The caspase-9 and caspase-3/7 enzymatic activities in splenocytes from *irf-1*$^{+/+}$ (WT) and *irf-1*$^{-/-}$ or transgenic (CN2-29) mice treated with the indicated cytokines were measured using a substrate cleavage assay after infection with the mock, LacZ, and Cre adenoviruses. Caspase-9 activity was measured 4 hours after injection of the anti-Fas monoclonal antibody (Jo2). LEHD, substrate for caspase-9; DEVD, substrate for caspase-3/7. Vertical bars are SD and were determined using the Student *t* test.

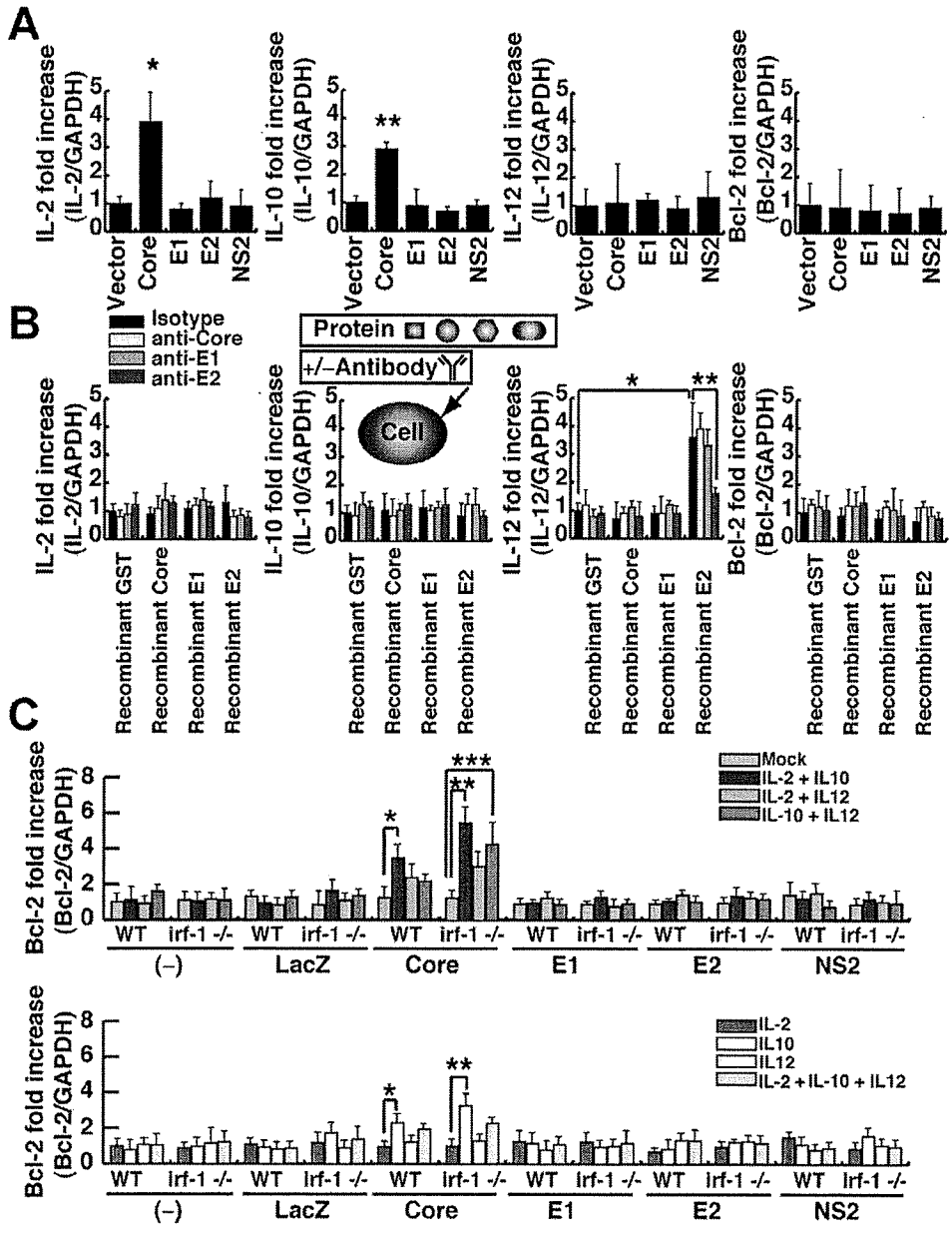



**Figure 7.** Induction of IL-2 and IL-10 by HCV core and IL-12 by E2 and of Bcl-2 by HCV core plus cytokines. (*A*) Individual viral proteins were stably expressed in splenocytes using recombinant lentiviruses that expressed the HCV core, E1, E2, NS2, and lacZ. Each gene expression profile was determined by quantitative reverse-transcription PCR. (*B*) E2 binding induces IL-12 in Raji cells, as determined by quantitative reverse-transcription PCR. Cells were treated with HCV core, E1, E2 (genotypes 1a and 1b), or glutathione *S*-transferase proteins, and the cytokine and bcl2 cellular RNA levels were examined using quantitative reverse-transcription PCR. (*C*) Quantification by quantitative reverse-transcription PCR of Bcl-2 mRNA relative to control glyceraldehyde-3-phosphate dehydrogenase mRNAin splenocytes from *irf-1*$^{+/+}$ (WT) and *irf-1*$^{-/-}$ WT or irf-1$^{-/-}$ mice treated with the indicated cytokines and infected with lentiviruses that express mock, core, E1, E2, NS2, and LacZ. Individual viral proteins were stably expressed using lentiviral vectors, and the cells were tested for Bcl-2 expression.

tion induced Bcl-2, while the other proteins did not (Figure 7C). Interestingly, the combination of IL-2 and IL-12 only induced Bcl-2 in the *irf-1*$^{-/-}$ background, while triple stimulation (IL-2, IL-10, and IL-12) did not induce Bcl-2 (Figure 7C). These results indicate that complex signaling networks induce Bcl-2 in the presence of viral nucleocapsid proteins.

## Discussion

The present study shows that Bcl-2 levels, cytokine levels, aging, and inflammation enhance the development of lymphoproliferative disorders caused by HCV proteins (Supplementary Figure 5). Disruption of *irf-1* enables the persistent expression of HCV protein, leading to lymphoproliferative diseases owing to reduced apoptosis (ie, lower levels of caspase-1, -6, and -7 expression). HCV CN2 transgenic (Tg+) mice are resistant to Fas-induced apoptosis due to the inhibition of cytochrome *c* release from mitochondria.[16] Mice with disruption of *irf-1* have several defects of their innate and adaptive immunity, such as lineage-specific defects in thymocyte development; immature T cells can develop into mature CD4$^+$ cells but not into CD8$^+$ T cells.[18,28] IRF-1 controls the positive and negative selection of CD8$^+$ thymocytes.[29] IRF-1 is required for the development of the Th1-type immune response, and

its absence leads to the induction of the Th2-type immune response.[18,30] Because the number of natural killer cells is dramatically reduced in *irf-1*$^{-/-}$ mice,[18] this defect may cause the marked increase in viral protein expression and the inhibition of tumor surveillance mechanisms, leading to the development of non-Hodgkin's lymphoma. Expression of the IL-12 p40 subunit is defective in *irf-1*$^{-/-}$ mice.[18]

Lymphomagenesis may require the additional genetic instability provided by HCV-induced hypermutation (2-hit model). Important questions are raised regarding the lymphoproliferative mechanisms of lymphomas in HCV-infected patients (B-cell malignancies predominate). Hypermutation of the immunoglobulin genes in B cells induced by HCV infection is the cause of the lymphomagenesis seen in HCV infection,[21,31] and this model may provide more direct insights into lymphoma production, because HCV-induced hypermutation causes genetic instability and causes chromosomal aberrations, possibly resulting in neoplastic transformation.[32] In addition, the antiapoptotic phenotype generated by sustained viral protein expression may enhance the survival of lymphocytes and inhibit activation-induced cell death to turn off the activated lymphocytes. The dysregulated cytokine profiles and sustained lymphocyte survival may alter the fates of regulatory T cells and dendritic cells.[33]

In conclusion, the present study shows that the conditional expression of HCV proteins induces inflammation and lymphoproliferative disorders, which are enhanced by *irf-1* disruption. Therefore, IRF-1–inducible genes probably play essential roles in suppressing HCV-induced lymphoma and in eliminating HCV protein-expressing cells. Our transgenic mice provide evidence that the overexpression of apoptosis-related proteins, including Bcl-2, and/or aberrant cytokine production are primary events in HCV-induced lymphoproliferation. It is interesting to note that lymphoproliferation was dominant over liver tumor development in the present study. Approximately 40% of the CN2-29Mx1Cre mice developed B-cell lymphomas, while 5% of the mice developed liver tumors. Further molecular analyses will enlighten the differential signaling pathways between hepatocytes and lymphocytes and increase our understanding of the differences between lymphomagenesis and liver tumor development caused by HCV.

## Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.03.061.

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Received June 25, 2008. Accepted March 31, 2009.

*Reprint requests*

Address requests for reprints to: Michinori Kohara, PhD, Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan. e-mail: mkohara@rinshoken.or.jp; fax: (81) 3-3828-8945.

*Acknowledgments*

The authors thank Prof Tadatsugu Taniguchi for his scholarly support of this study; Kazuaki Inoue and Kentaro Tomita for their advice on histology; Yutaka Amako, Isao Maruyama, and Kohsuke Tanaka for technical assistance; and Mitsugu Takahashi for breeding the transgenic mice.

*Conflicts of interest*

The authors disclose no conflicts.

*Funding*

Supported in part by a research fellowship from the Japan Society for the Promotion of Science; a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan; a grant from the Ministry of Health, Labour and Welfare of Japan; and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation of Japan. This project was also supported by National Institutes of Health research grants P50AA11999, 5P30DK048522-13, and CA108302.

Vaccine xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

# Species-specific CD1-restricted innate immunity for the development of HIV vaccine

Hidemi Takahashi*

*Department of Microbiology and Immunology, Nippon Medical School, Tokyo 113-8602, 1-1-5 Sendagi, Bunkyo-ku, Japan*

ARTICLE INFO

*Article history:*
Received 12 May 2009
Received in revised form 14 July 2009
Accepted 24 July 2009
Available online xxx

*Keywords:*
HIV-1
Innate immunity
Acquired immunity
Vaccine development
DC

ABSTRACT

The human immune defense system is composed of two distinct elements: innate immunity located primarily at body surfaces restricted by species-specific CD1 molecules and acquired immunity found mainly in internal compartments associated with individually restricted MHC molecules. Historically, effective vaccines have focused on eliciting pathogen epitope-specific acquired immune responses to protect against infectious diseases; however, such traditional approaches to developing HIV vaccines have been unsuccessful. This review addresses the importance of activating host species-restricted innate immunity to enhance the virus epitope-specific acquired immunity that is needed for HIV vaccines.



## 1. Introduction

Our internal defense system is composed of two distinct elements. One is local innate immunity principally arranged on surface areas, such as skin or mucous membrane to establish barriers against various pathogens, and the other is systemic acquired immunity, mainly found in systemic components, for example, circulating blood or lymphoid organs, such as lymph nodes and spleen, to survey and control internal damage and disorders. The former innate arm is chiefly regulated via species-restricted CD1 antigen-presenting molecules and the latter acquired arm is orchestrated by individually restricted MHC molecules (Fig. 1).

In vaccine development for both the prevention of pathogen intrusion and suppression of its expansion as well as tumor growth, we have been focusing on the induction of acquired immune responses composed of MHC molecule-restricted peptide epitope-specific T cells and antibodies that bind specifically to the particular epitopes on pathogens or tumors through their definite receptors created by gene re-arrangements. Thus, the main work to advance vaccine development has been focusing on the identification of epitopes and the establishment of a powerful and non-toxic adjuvant for the induction of epitope-specific immunity. However, because pathogen- or tumor-derived epitopes vary among diverse MHC molecules, the analysis and discovery of cross-reactive immuno-dominant epitope(s) should be considered to overcome MHC diversity [1,2].

Under these conditions, a lack of correlation between acquired virus-specific immunity and resistance to infection with simian immunodeficiency virus (SIV) in rhesus monkeys has been reported recently [3]. Also, most exposed, uninfected commercial sex workers eventually became infected after quitting their jobs to limit mucosal human immunodeficiency virus type-1 (HIV-1) exposure, although virus-specific cell-mediated immunity and immunoglobulin A (IgA) antibody responses had been confirmed [4,5], suggesting that continuous mucosal virus stimulation may be required to maintain protective acquired immunity against persistently infected pathogens. Moreover, the reservoir for HIV-1 in persistently infected patients with no free virus particles in the circulating blood after highly active anti-retroviral treatment (HAART) has been identified as innate CD4-positive dendritic cells (DC) or natural killer T (NKT) cells in the small intestine (J.M. and H.T.; unpublished observation). In the present review, based on our recent progress, the importance of activating species-restricted local innate immunity to develop and HIV-1 vaccine rather than individually restricted systemic acquired immunity will be addressed.

## 2. Species-specific antigen-presenting molecule CD1s

Species-specific CD1 molecules are further divided into two sub-classes, group I CD1 (CD1a–CD1c) and group II CD1 (CD1d) [6]. These CD1s have been found to present lipid/glycolipid antigens to the appropriate T cells bearing relatively invariant

* Tel.: +81 3 3822 2131x5381; fax: +81 3 3316 1904.
*E-mail address:* htkuhkai@nms.ac.jp.

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doi:10.1016/j.vaccine.2009.07.086

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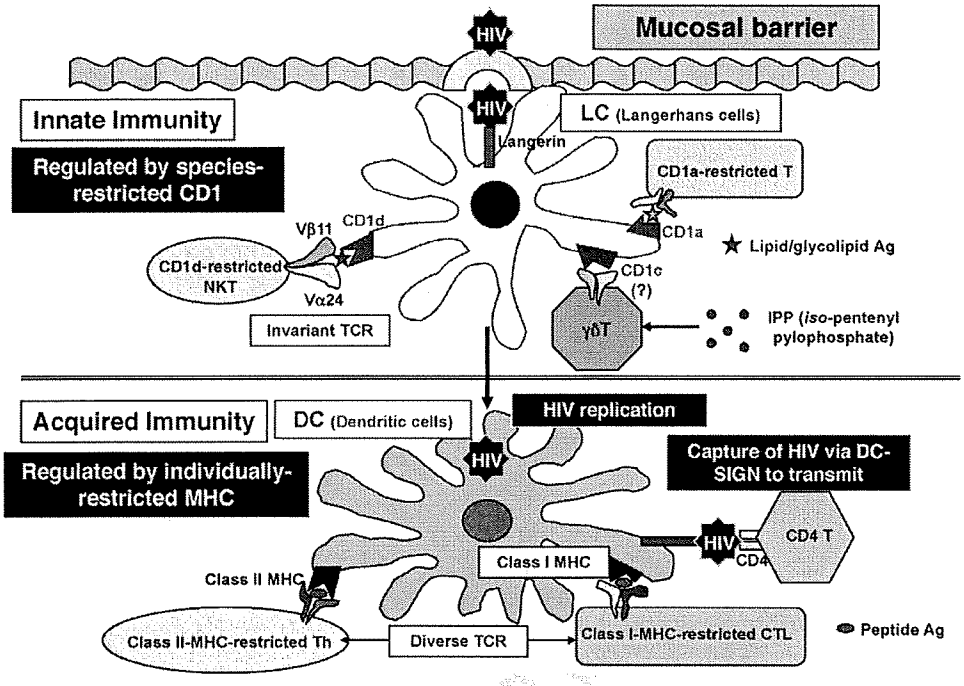


**Fig. 1.** Innate immunity and acquired immunity. Our internal defense system is composed of two distinct elements. One is innate immunity composed of γδT cells and NKT cells as effectors expressing fixed invariant receptors controlled mainly by species-restricted CD1 molecules on Langerhans cells and (LC) dendritic cells (DC), and the other is systemic acquired immunity composed of helper T cells (Th), cytotoxic T lymphocytes (CTL), and antibodies bearing diverse receptors from re-arranged genes orchestrated by individually restricted MHC molecules.

αβT-cell receptors (TCR), most of which are conserved among species; for example, highly conserved CD1d molecules present α-galactosyl ceramide (α-GalCer) to natural killer T (NKT) cells of their own species. Indeed, human NKT cells generally express unique combinations of TCRs that consist of an invariant Vα24 chain preferentially paired with a Vβ11 [7], while murine α-GalCer-reactive CD1d-restricted NKT cells express invariant Vα14 paired with various Vβ combinations [8].

The structures of CD1 molecules are similar to those of class I MHCs bearing non-covalently bound β2-microglobulin that may regulate the antigen-binding capacity of the presenting molecules; however, CD1s show limited polymorphism and do not map to MHC genes [9]. Also, CD1-encoding genes are highly conserved and their structures are shared among species [10]. We have confirmed recently that the genetic structure of CD1d molecules is very tightly conserved among species, such as rhesus macaques, African green monkeys (AGMs), and chimpanzees, and would not be affected by long-term environmental stimulation [11]. It is important to note that, unlike rhesus macaques and AGMs, both α1 and α2 domains of the CD1d of chimpanzees were identical to those of humans, although 4 amino acids in α3 domain differed [11]. Since the α2 domain of CD1d molecules with a hydrophobic ligand-binding pocket critical for antigen presentation [12], changes of amino acids in the α2 domain may alter the capacity of the presented glycolipid/lipid antigens to effector NKT cells. It is widely known that both humans and chimpanzees are susceptible to HIV-1 [13,14] but very weakly to SIV, to which rhesus macaques and AGMs are susceptible. These findings suggest an evolutionary relationship between species-specific CD1d molecules and retrovirus susceptibility through the activation of innate effector NKT cells.

## 3. Individually restricted antigen-presenting molecule MHC

In contrast, both class I and class II MHC molecules are extremely diverse among species with self-restricted elements that can present internally processed peptide antigens only to the same MHC molecule-bearing cells. Such individually restricted peptide epitopes will be recognized by highly diverse αβTCRs established via suitable intracellular gene rearrangements that create antigen-specificity. In general, CD8αβ-positive T cells recognize the processed epitope peptide presented by class I MHC molecules, whereas CD4-positive T cells recognize epitope peptide in association with class II MHC [15]. Both class I and class II MHC molecule-restricted T cells can be elicited by individual class I and class II MHC molecule-expressing DC that capture antigenic proteins and select to present specific epitopes with their MHC molecules; therefore, the epitope-specific rearranged αβTCR-expressing T cells in the acquired arm seem to be controlled by individual antigen-captured DC *in vivo*.

In viral infection, various viral proteins and genetic components are disseminated throughout the body. The former viral proteins may be captured by immature DC (iDC) and the latter genetic components may stimulate antigen-loading iDC to mature via Toll-like receptors (TLR), inducing virus-specific cellular immunity, in particular, cytotoxic T lymphocytes (CTL) that eliminate virus-infected cells. Double-strand RNA, polyriboinosinic polyribocytidylic acid (poly(I:C)), which reflects a natural genetic product from a variety of viruses during replication, has recently been identified as one of the critical stimuli of TLR3 [16]. We and others have shown that iDC could present processed antigen from captured purified protein in association with class I MHC molecules via a cross-presentation mechanism when iDC were stimulated with poly(I:C) [17,18]. Also, such cross-presentation of externally added purified proteins can be achieved by a saponin-associated adjuvant like ISCOMs [19] or cholera toxin (CT) [20]. Taken together, virus-specific acquired immunity restricted by individual MHCs can be spontaneously elicited by the appropriate activation of innate iDC that capture viral antigenic molecules during the course of infection.

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## 4. Interaction between HIV-1 and DC

DC at the mucosal site appear to be a natural reservoir for HIV-1, whose Nef protein is known as a key factor in disease progression. Indeed, nef-deficient HIV-1 as well as SIV markedly slowed the clinical manifestation of AIDS [21]. We and others have recently reported that the surface expression of CD1a and CD1d was selectively down-regulated among CD1 molecules as well as class I MHC on HIV-1 infected iDC by Nef [22,23], indicating that iDC may lose their function to present virus-associated antigens to both innate and acquired effectors, which may cause disease progression. Thus, stimulation of iDC or deletion of the Nef effect in HIV-1 infected DC may alter the immunological state of HIV-1-infected individuals.

Two distinct types of innate DC are observed at the local mucosal site. One is sentinel Langerhans cells (LC) that are present in the surface epithelial compartment to survey antigens, including viruses, via the LC-specific sampling receptor, Langerin; langerin-bound antigens are internalized into Birbeck granules and degraded. Recently de Witte et al. [24] proposed that LCs provide a barrier to HIV-1 infection by demonstrating that HIV-exposed skin-derived LCs captured HIV via Langerin and degraded the virus. However, evidence contradicting the capacity of LCs to protect against HIV was obtained using human vaginal explant cultures, a more direct, biologically relevant model of HIV-sexual transmission [25]. Hladik et al. showed that after HIV-1 exposure of vaginal explants, LCs were rapidly virus-penetrated primarily by endocytosis via multiple receptors and that endocytosed virions could persist in the cytoplasm. Also, DC-SIGN (CD209) and langerin (CD207) apparently had a negligible role in mediating endocytotic HIV infection by vaginal LCs. Thus, depending on the tissue source of LCs and the precise experimental conditions, human LCs are capable of degrading HIV-1 or being infected and harboring HIV-1. It is important to note that Haldik et al.'s results support prior observations of rapid vaginal LC infection in macaques after experimental SIV vaginal inoculation [26]. We have reported that DC-SIGN-positive macrophages in the early colostrum (breast milk macrophages: BrMMØ) and their DC-SIGN expression were markedly enhanced by externally added interleukin (IL)-4 [27]. IL-4-treated BrMMØ showed strong capacity to transmit HIV-1 to $CD4^+$ cells via DC-SIGN [28]. Therefore, evidence from different human tissue culture model systems indicates that both LCs and DC-sign-positive DCs can be reservoirs for HIV-1.

## 5. Selective activation of innate DC lineage cells for the induction of HIV-1-specific acquired immunity

The two major distinct subsets of DC are arranged to regulate immune responses *in vivo*; 33D1-positive and DEC-205-positive DC. Using anti-33D1-specific monoclonal antibody (mAb), 33D1-positive DC were successfully depleted from C57BL/6 mice *in vivo*. When the remaining DEC-$205^+$ DC in 33D1$^+$ DC-depleted mice were stimulated with LPS, serum IL-12 but not IL-10 secretion was markedly enhanced, which may induce Th1 dominancy upon TLR signaling. After implanting various syngeneic tumor cells into the dermis of 33D1$^+$ DC-depleted mice, subcutaneous injection with LPS resulted in significant suppression of tumor growth *in vivo*. Moreover, apparent proliferation of class I MHC molecule-restricted epitope-specific $CD8^+$ CTL among tumor infiltrating lymphocytes (TIL) against already established syngeneic tumors was observed in the LPS-stimulated 33D1$^+$ DC-deleted mice administered intraperitoneally with very small unaffected amount of melphalan (L-phenylalanine mustard; L-PAM) (K.M., A.W., and H.T.; unpublished observation).

These findings indicate the importance and effectiveness of selective targeting of a specific subset of innate DC, such as DEC-205$^+$ DC alone or with very small amount of anti-cancer drugs to activate functional acquired epitope-specific $CD8^+$ CTL without externally added antigen stimulation *in vivo*. This may be true for HIV-1 intrusion in the local mucosal area, in which selective activation of suitable DC with or without small amount of anti-HIV-1 drugs will induce effective acquired immunity specific for the pathogen.

## 6. Concluding remarks

Most vaccine work to block pathogens has focused on how to artificially elicit acquired individual MHC molecule-restricted effectors specific for pathogens or pathogen-infected cells before pathogen invasion by using attenuated pathogens or pathogen-derived immunogenic molecules containing epitopes with a strong adjuvant. However, to establish the most suitable pathogen-specific acquired immunity before intrusion in individuals may be very difficult and sometimes harmful since some deteriorate products, such as CTL and antibodies, may spread in the blood before pathogen intrusion. Thus, the acquired products circulating throughout the body may attack or bind the pathogens, their destructive products, or pathogen-infected cells, having negative effects on the body.

As indicated above, it should be considered that pathogens will attack species but not individuals like HIV-1, in humans and chimps, while SIV in monkeys. Humans have survived battled against various pathogens for a long period probably as a result of species-specific CD1-restricted innate immunity rather than individually restricted acquired immunity. Species-restricted innate cells are mainly arranged on the surface area where pathogens invade from outside. Here, an innovative vaccination strategy against various pathogens or tumors is suggested. Namely, similar to tumors, selective activation of innate immunity with slight damage of the pathogens using a potent drug or an antibody will spontaneously achieve to establish most favorable acquired immunity in each individual.

However, innate immune cells, such as DC, do not usually keep long, persistent memories, and thus have to be stimulated constantly to maintain their activities. Under the conditions in which selective and constant activation of DC is performed, attenuated or killed pathogens or their components, or even live infectious pathogens themselves, should be addressed to establish immunity. Such a procedure may help to generate the most suitable acquired immunity to control pathogens spontaneously. Also, as suggested in exposed, uninfected commercial sex workers, constant activation of innate DC is required to maintain the most favorable acquired immunity [29].

Intravesical bacillus Calmette-Guerin (BCG) therapy against human bladder carcinoma is considered the most successful immunotherapy against solid tumors [30]. During the course of determining the actual effector cells activated by intravesical BCG therapy to inhibit the growth of bladder carcinoma, we found that innate alert cells, such as $V\gamma2V\delta2$ T cells and NKT cells derived from peripheral mononuclear cells (PBMC) activated by live BCG-pretreated DC, appear to inhibit the proliferation of T24 tumor cells as well as eliminate them [31]. These findings strongly suggest that some products in live BCG or live BCG itself must stimulate suitable DC for tumor surveillance and such DC will help to induce and maintain most effective acquired effectors against the tumor. We are currently searching for the substances from live BCG.

Collectively, to develop an ideal vaccine, the activation of species-restricted innate immunity located at the surface compartment should be the focus to establish more favorable individual-restricted acquired immunity against external pathogens, such as HIV-1, or internal tumors. The fact that one can

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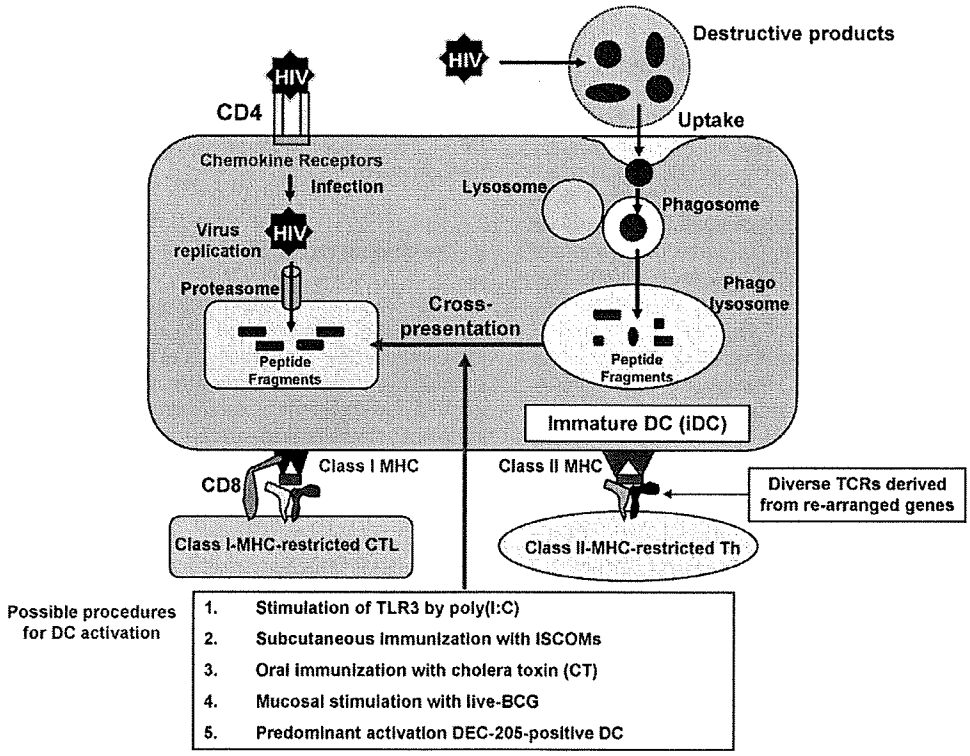


**Fig. 2.** Antigen-presentation by class I and class II MHC molecules. Intracellular antigens are degraded by proteasomes into peptides that are loaded into class I MHC and displayed on the cell surface to CD8-positive CTL. In contrast, extracellular antigens are taken up by phagosomes fused with lysosome containing various enzymes and processed into peptides that bind to class II MHC molecules to activate CD4-positive Th. Such extracellular peptide antigens can be displayed in association with class I MHC molecules to prime CD8-CTL when antigen-presenting DC are treated with various stimuli.

usually obtain a suitable protective acquired immunity including MHC molecule-restricted CTL after a pathogen infection indicates that some factors that activate innate DC to generate suitable acquired immunity via cross-presentation seem to be hidden in the pathogen itself (Fig. 2).

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 11, pp. 6841–6846, March 13, 2009


# Heat-shock Protein 90 Is Essential for Stabilization of the Hepatitis C Virus Nonstructural Protein NS3*

Received for publication, August 20, 2008, and in revised form, December 22, 2008 Published, JBC Papers in Press, January 16, 2009, DOI 10.1074/jbc.M806452200

**Saneyuki Ujino[‡], Saori Yamaguchi[‡], Kunitada Shimotohno[§¶], and Hiroshi Takaku[‡‖1]**

*From the [‡]Department of Life and Environmental Sciences, [‖]High Technology Research Center, and [§]Research Institute, Chiba Institute of Technology, 2-17-1 Tsudanuma, Narashino, Chiba 275-0016, Japan and the [¶]Center for Integrated Medical Research, School of Medicine, Keio University, Shinanomachi, Tokyo 160-8582, Japan*

**The hepatitis C virus (HCV) is a major cause of chronic liver disease. Here, we report a new and effective strategy for inhibiting HCV replication using 17-allylaminogeldanamycin (17-AAG), an inhibitor of heat-shock protein 90 (Hsp90). Hsp90 is a molecular chaperone with a key role in stabilizing the conformation of many oncogenic signaling proteins. We examined the inhibitory effects of 17-AAG on HCV replication in an HCV replicon cell culture system. In HCV replicon cells treated with 17-AAG, we found that HCV RNA replication was suppressed in a dose-dependent manner, and interestingly, the only HCV protein degraded in these cells was NS3 (nonstructural protein 3). Immunoprecipitation experiments showed that NS3 directly interacted with Hsp90, as did proteins expressed from ΔNS3 protease expression vectors. These results suggest that the suppression of HCV RNA replication is due to the destabilization of NS3 in disruption of the Hsp90 chaperone complex by 17-AAG.**

Infection by the hepatitis C virus (HCV)[2] is a major public health problem, with 170 million chronically infected people worldwide (1, 2). The current treatment by combined interferon-ribavirin therapy fails to cure the infection in 30–50% of cases (3, 4), particularly those with HCV genotypes 1 and 2. Chronic infection with HCV results in liver cirrhosis and can lead to hepatocellular carcinoma (5, 6). Although an effective combined interferon-α-ribavirin therapy is available for about 50% of the patients with HCV, better therapies are needed, and preventative vaccines have not yet been developed.

HCV is a member of the *Flaviviridae* family and has a positive strand RNA genome (7, 8) that encodes a large precursor polyprotein, which is cleaved by host and viral proteases to generate at least 10 functional viral proteins: core, E1 (envelope 1), E2, p7, NS2 (nonstructural protein 2), NS3, NS4A, NS4B, NS5A, and NS5B (9, 10). NS2 and the amino terminus of NS3 comprise the NS2-3 protease responsible for cleavage between NS2 and NS3 (9, 11), whereas NS3 is a multifunctional protein consisting of an amino-terminal protease domain required for processing NS3 to NS5B (12, 13). NS4A is a cofactor that activates the NS3 protease function by forming a heterodimer (14–17), and the hydrophobic protein NS4B induces the formation of a cytoplasmic vesicular structure, designated the membranous web, which is likely to contain the replication complex of HCV (18, 19). NS5A is a phosphoprotein that appears to play an important role in viral replication (20–23), and NS5B is the RNA-dependent RNA polymerase of HCV (24, 25). The 3′-untranslated region consists of a short variable sequence, a poly(U)-poly(UC) tract, and a highly conserved X region and is critical for HCV RNA replication and HCV infection (26–29).

Hsp90 (heat-shock protein 90) is a molecular chaperone that plays a key role in the conformational maturation of many cellular proteins. Hsp90 normally functions in association with other co-chaperone proteins, which together play an important role in folding newly synthesized proteins and stabilizing and refolding denatured proteins in cells subjected to stress (30–34). Its expression is induced by cellular stress and is also associated with many types of tumor. Hsp90 inhibitors are currently showing great promise as novel pharmacological agents for anticancer therapy.

Hsp90 inhibitors have two major modes of action as preferential clients for protein degradation or as Hsp70 inducers. The benzoquinone ansamycin antibiotic geldanamycin and its less toxic analogue 17-allylamino-17-demethoxygeldanamycin (17-AAG) directly bind to the ATP/ADP binding pocket of Hsp90 (34–36) and thus prevent ATP binding and the completion of client protein refolding. Recently, Waxman *et al.* (37) demonstrated a role for Hsp90 in promoting the cleavage of HCV NS2/3 protease, using NS2/3 translated by rabbit reticulocyte lysate. Nakagawa *et al.* (38) also reported that inhibition of Hsp90 is highly effective in suppressing HCV genome replication. Hsp90 may directly or indirectly interact with any of the proteins NS3 through NS5B to regulate replication of the HCV replicon. More recently, Okamoto *et al.* (39) reported that Hsp90 could bind to FKBP8 (FK506-binding protein 8) and form a complex with NS5A. The interaction with FKBP8 has also been shown to be the mechanism by which Hsp90 regulates HCV RNA replication, a process in which Hsp90 clearly plays an important role.

In this study, we have demonstrated that NS3 also forms a complex with Hsp90, which is critical for HCV replication. On the basis of the findings that treating HCV replicon cells with

* This work was supported by a grant-in-aid for HCV research from the Ministry of Health, Labor, and Welfare of Japan and by a grant-in-aid for high technology research from the Ministry of Education, Science, Sports, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: Dept. of Life and Environmental Science and High Technology Research Center, Chiba Institute of Technology, 2-17-1 Tsudanuma, Narashino-shi, Chiba 275-0016, Japan. Tel.: 81-47-478-0407; Fax: 81-47-471-8764; E-mail: hiroshi.takaku@it-chiba.ac.jp.

[2] The abbreviations used are: HCV, hepatitis C virus; 17-AAG, 17-allylamino-17-demethoxygeldanamycin.

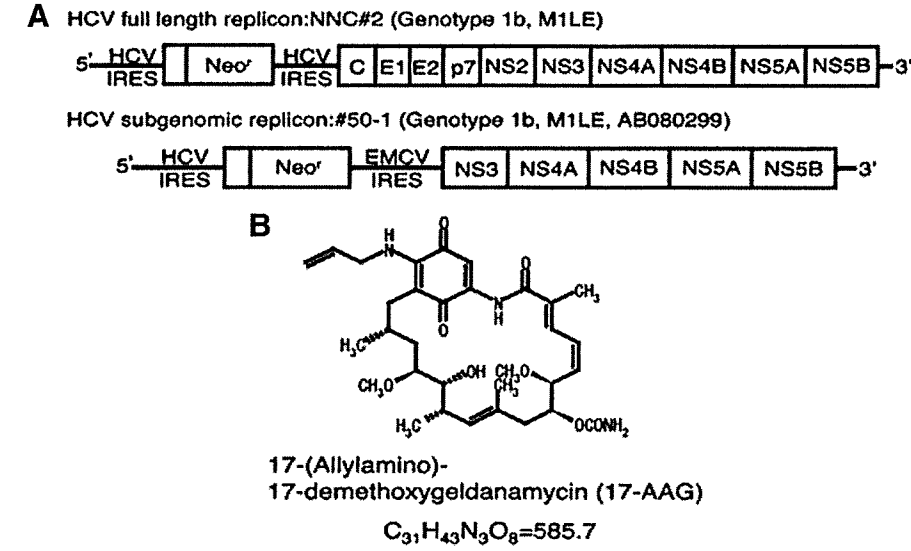


FIGURE 1. **Schematic representation of HCV replicon and structure of 17-AAG.** *A*, structure of the HCV replicon RNAs, comprising the HCV 5′-untranslated region, including the HCV internal ribosome entry site (*IRES*), the neomycin phosphotransferase gene (*Neo'*), the encephalomyocarditis virus (*EMCV*) IRES or HCV IRES, and the coding region for HCV proteins NS3 to NS5B (in the HCV subgenomic replicon) or core to NS5B (in the HCV full-length replicon). *B*, structures of the Hsp90 inhibitor, 17-AAG.

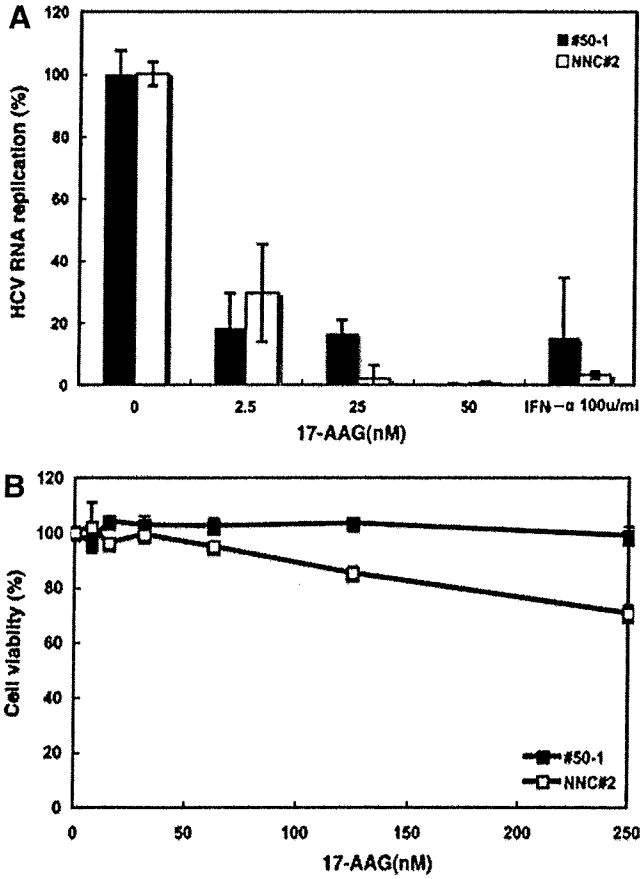


FIGURE 2. **Hsp90 inhibits HCV RNA replication in HCV replicon cells.** *A*, inhibition of HCV replication by 17-AAG in NNC#2 (*white squares*) and #50-1 cells (*black squares*) measured by real time reverse transcription-PCR after 72 h. Interferon-α was used as a positive control. The data are means ± S.D. from triplicate experiments. *B*, cytotoxic effects of 17-AAG in NNC#2 (*white squares*) and #50-1 (*black squares*), shown as the percentage reduction in viable cell numbers in an [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (inner salt)] assay. The data are means ± S.D. from triplicate experiments.

the Hsp90 inhibitor, 17-AAG, suppressed HCV RNA replication, and that the only HCV protein degraded in these cells was NS3, we suggest a crucial role for Hsp90-NS3 protein complexes in the HCV life cycle.

## EXPERIMENTAL PROCEDURES

*Cell Culture and Reagents*—The HCV replicon cell lines #50-1 (NN/1b/SG) (40), which carries a subgenomic replicon, and NNC#2 (NN/1b/FL) (41), which carries a full genome replicon, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, nonessential amino acids, L-glutamine, penicillin/streptomycin, and 300–1,000 μg/ml G418 (Invitrogen) at 37 °C in 5% $CO_2$. The human embryonic kidney-derived cell line 293T was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. 17-AAG was purchased from Sigma.

*Measuring HCV RNA by Real Time PCR*—HCV replicon cells were seeded at $1.5 \times 10^5$ cells in 24-well plates and cultured for 72 h. Total RNA was then isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. HCV RNA was quantified by real time reverse transcription-PCR using an ABI 7700 sequence detector (PerkinElmer Life Sciences) and the following primers and TaqMan probes located in the 5′-untranslated region: forward primer (nucleotides 130–146), 5′-CGGGAGAGCCATAGTGG-3′; reverse primer (nucleotides 272–290), 5′-AGTACCACAAGGCCTTTCG-3′; and TaqMan probe (nucleotides 148–168), 5′-CTGCGGAACCG-GTGAGTACAC-3′ (all purchased from Applied Biosystems). The probe sequence was labeled with the reporter dye, 6-carboxyflurorescein, at the 5′-end and with the quencher dye TAMRA at the 3′-end (42).

*Western Blotting and Immunoprecipitation Analyses*—Cells were lysed in 1× CAT enzyme-linked immunosorbent assay buffer (Roche Applied Sciences). Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes, and these were blocked with 5% skimmed milk. The primary antibodies used were monoclonal or polyclonal antibody against FLAG-M5 (Sigma), Hsp70 (Sigma), Hsp90 (Cell Signaling Technologies, Danvers, MA), Hsp90α (Calbiochem), Hsp90β (Calbiochem), and Hsf-1 (Calbiochem). Core, NS4A, and NS4B were a gift from Dr. M. Kohara (Tokyo Metropolitan Institute of Medical Science). E1, E2, NS3, NS5A, and NS5B were a gift from Prof. Y. Matsuura (Osaka University, Japan). Immunoprecipitation from cell lysates was carried out using anti-FLAG M5 antibody (Sigma) and the Protein G immunoprecipitation kit (Sigma), according to the manufacturer's instructions, and the immunoprecipitates were analyzed by Western blotting.

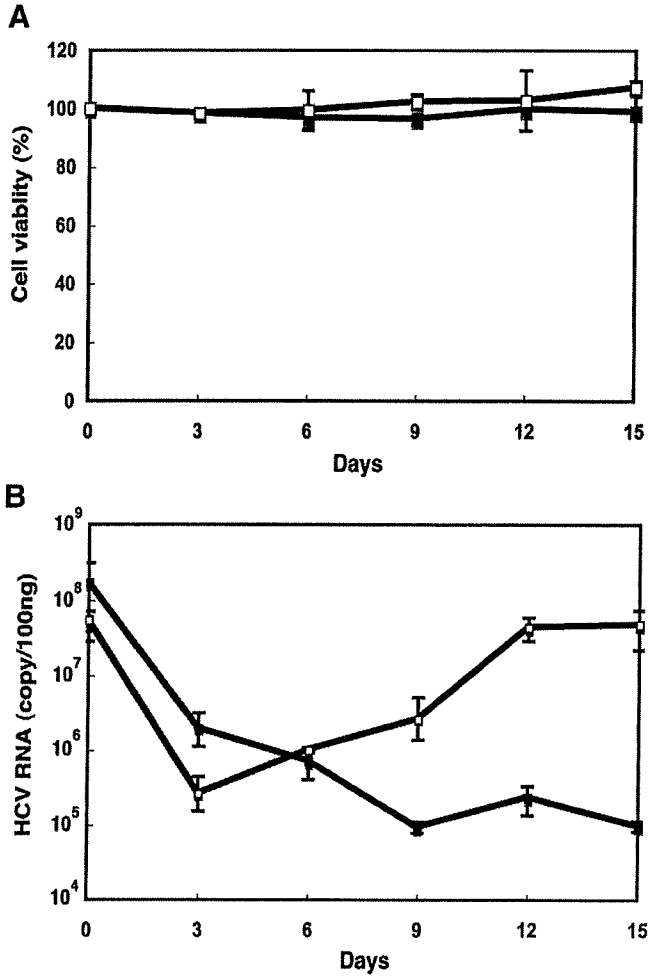


FIGURE 3. **Long term inhibition of HCV replication in NNC#2 cells.** *A*, cytotoxic effect of 17-AAG in NNC#2 cells, shown as the percentage reduction of viable cell numbers assessed by trypan blue staining. NNC#2 cells were treated with 50 nM 17-AAG on day 0 only (*white squares*) or at 3-day intervals for 15 days (*black squares*). The data are means ± S.D. from triplicate experiments. *B*, measurement of HCV replication by real time reverse transcription-PCR. Inhibition of HCV RNA replication in NNC#2 cells treated with 50 nM 17-AAG on day 0 only (*white squares*) or at 3-day intervals for 15 days (*black squares*). *Day 0*, mock. The data are means ± S.D. from triplicate experiments.

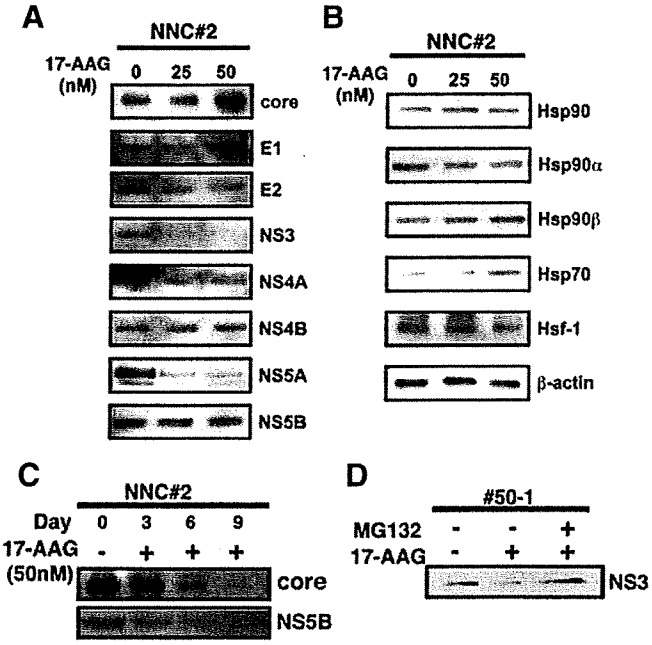


FIGURE 4. **Effect of 17-AAG on HCV NS3 protein levels.** *A*, Western blot analysis of HCV protein expression in NNC#2 or #50-1 cells treated with 17-AAG. NNC#2 or #50-1 cells were treated with 25 and 50 nM 17-AAG for 3 days. Cell lysates were separated by SDS-PAGE, immunoblotted, and probed with antibodies specific for HCV core, E1, E2, NS3, NS4A, NS4B, NS5A, and NS5B. *B*, Western blot analysis of Hsp90, Hsp70, and other chaperone expression in NNC#2 cells treated with 17-AAG (25 and 50 nM, as indicated) for 3 days. *C*, expression of HCV core and NS5B protein in cells treated with 50 nM 17-AAG for 9 days. *D*, effect of 50 nM 17-AAG on NS3 expression in #50-1 cells simultaneously treated with 100 nM MG132.

*[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt Assay*—HCV replicon cells were seeded in 96-well plates at $3 \times 10^4$ cells/well in a final culture volume of 100 μl for 72 h before the addition of increasing concentrations of 17-AAG. After incubation for 3 days, viable cell numbers were determined using the Celltiter 96 Aqueous nonradioactive cell proliferation assay (Promega Corp., Madison, WI). The value of the background absorbance at 490 nm ($A_{490}$) of wells without cells was subtracted. The percentages of viable cells were then calculated using the formula, ($A_{490}$ of 17-AAG-treated sample/$A_{490}$ of untreated cells) × 100.

*Plasmids and Transfection*—The pFLAG-CMV-NS3 vector was constructed by subcloning a DNA fragment encoding full-length NS3, Δhelicase, Δprotease, ΔPH 1, ΔPH 2, and ΔH 1 into the EcoRI and XbaI sites of the pFLAG-CMV™-2 expression vector (Sigma), so that the amino-terminal FLAG epitope was fused in frame with NS3. The core expression vector was a gift from Dr. M. Kohara. The vector was transfected into 293T cells using the FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer's instructions.

## RESULTS

*Hsp90 Inhibitor 17-AAG Suppresses HCV RNA Replication*—To investigate the effect of 17-AAG on HCV replication, cells containing a full HCV genome replicon (NNC#2) or a subgenomic replicon (#50-1) were treated with 17-AAG (Fig. 1, *A* and *B*). Both of the HCV replicon cell lines were treated for 72 h with different concentrations of 17-AAG or with DMSO as a control. In cells treated with 50 nM 17-AAG, HCV RNA replication was suppressed by 99% in both of the HCV replicon cell lines, and the inhibition of RNA replication occurred in a dose-dependent manner (Fig. 2*A*). The half-maximal inhibitory concentration ($IC_{50}$) values of 17-AAG for HCV replication were 0.3 nM in NNC#2 cells and 0.1 nM in #50-1 cells. Furthermore, we used a tetrazolium-based [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay to determine the viability of NNC#2 and #50-1 cells in the presence of 17-AAG. 17-AAG showed no toxicity to NNC#2 and #50-1 cells at 50 nM, (Fig. 2*B*). These results suggested that 17-AAG had a greater inhibitory effect on HCV RNA replication than 100 units/ml interferon-α.

*Long Term Suppression of HCV RNA Replication*—We next examined the effect of 17-AAG on HCV replication over time. When NNC#2 cells were cultured with 50 nM 17-AAG only on day 0 (*white squares*), the level of HCV RNA was reduced by 2 log on day 3 but had increased to control levels by day 12 (Fig.