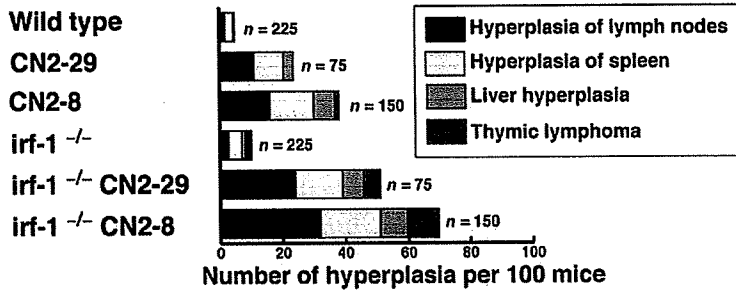


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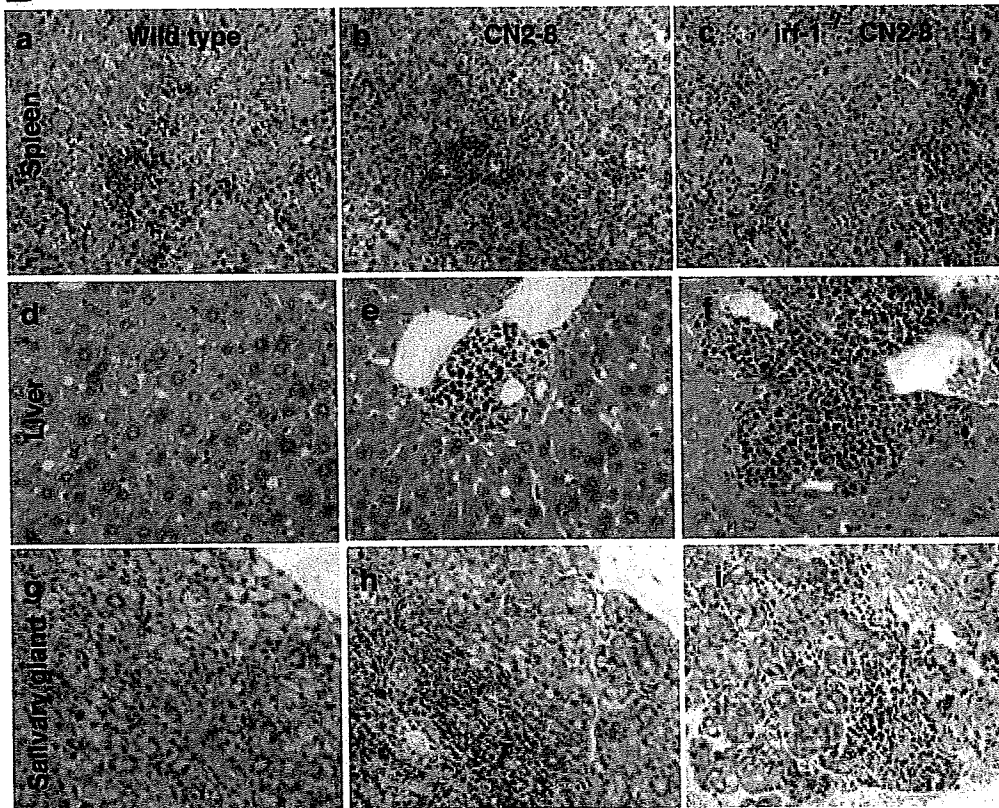


Figure 2. Disruption of *irf-1* aggravates lymphocyte infiltration in combination with HCV transgene expression. (A) Histologic analysis of spontaneous proliferative disturbances in the CN2 transgenic mice. Of the 900 mice injected with AxCANCre, 25 of 75 (33%) CN2-29, 47 of 150 (31%) CN2-8, 29 of 75 (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 1F. (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.

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mouse were significantly increased by the ablation of *irf-1* (Figure 1F). 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 1D and 1F), 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 2A). In contrast, hepatocytes with abundant expression of HCV proteins rarely developed into hepatocellular carcinomas. H&E staining of splenomegaly revealed extensive hyperplasia of the white pulp zones, in which the cortical zones contained lym-

phoid follicles and scattered germinal centers, although mitotic figures were rarely observed (Figure 2B 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

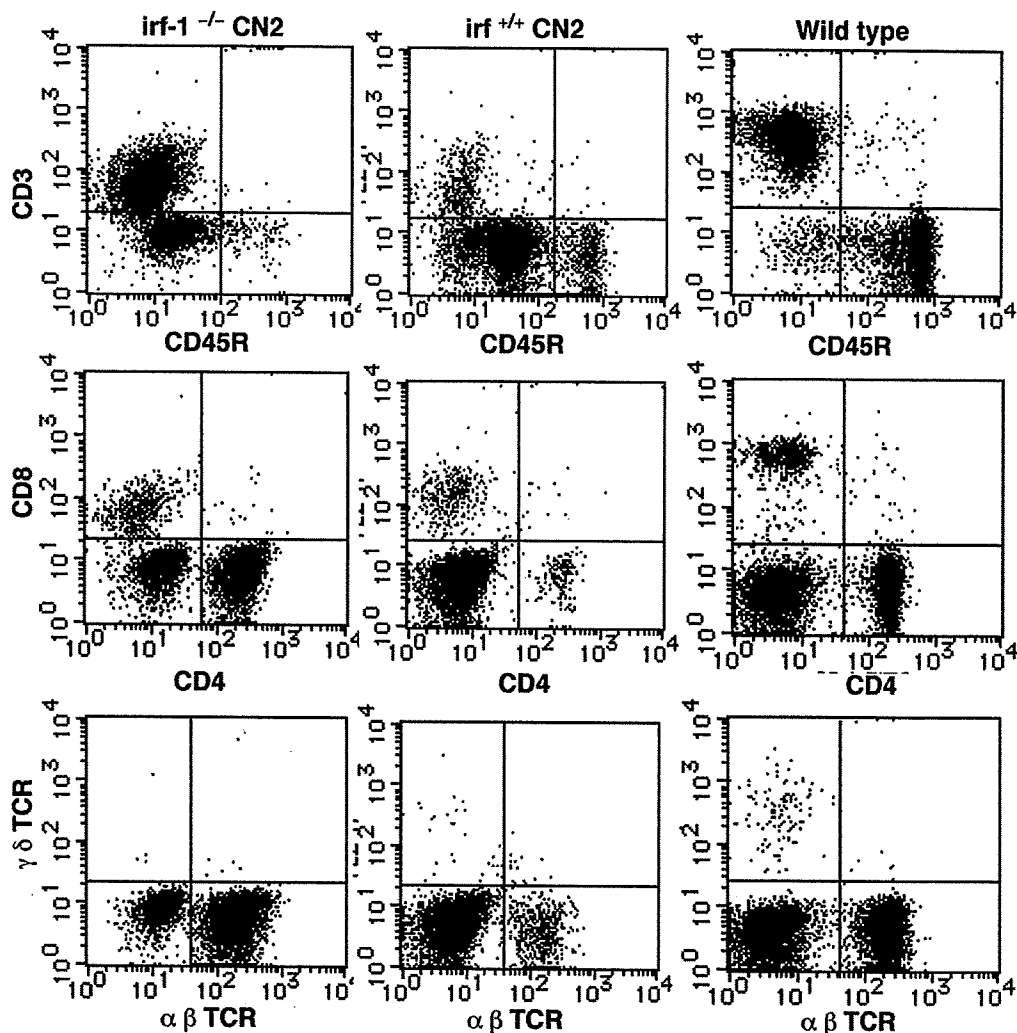


Figure 3. HCV expression and *irf-1* ablation affect the lymphocyte population. T-cell and B-cell proliferation in *irf-1*^{+/+} CN2 mice, *irf-1*^{-/-} CN2 mice, and WT mice. CD3⁺, CD45R⁺, CD4⁺, CD8⁺, and T-cell receptor-positive cells from age-matched *irf-1*^{-/-} CN2, *irf-1*^{+/+} CN2, and WT mice 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 (α , β , γ , and δ 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 4A). 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

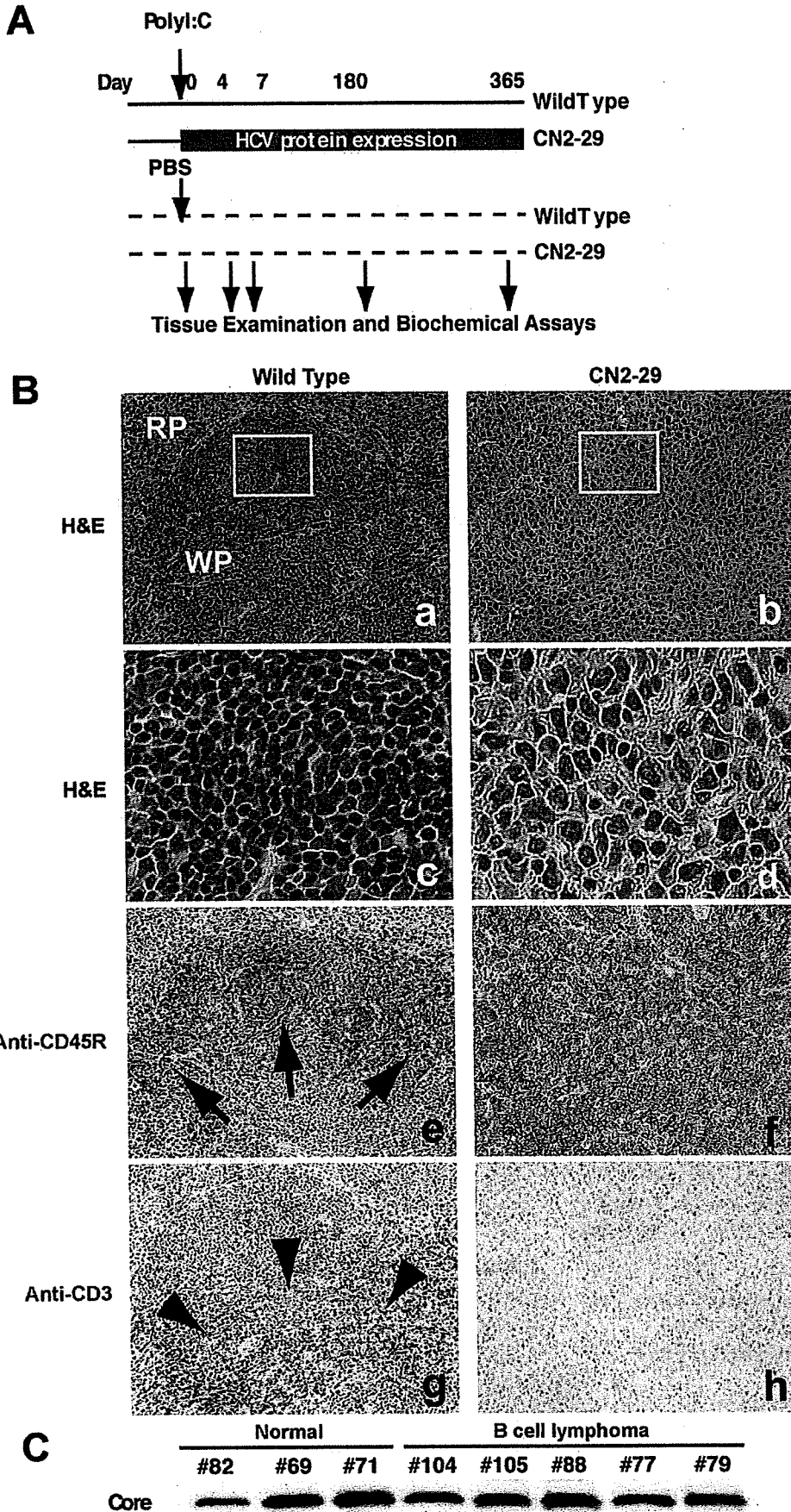
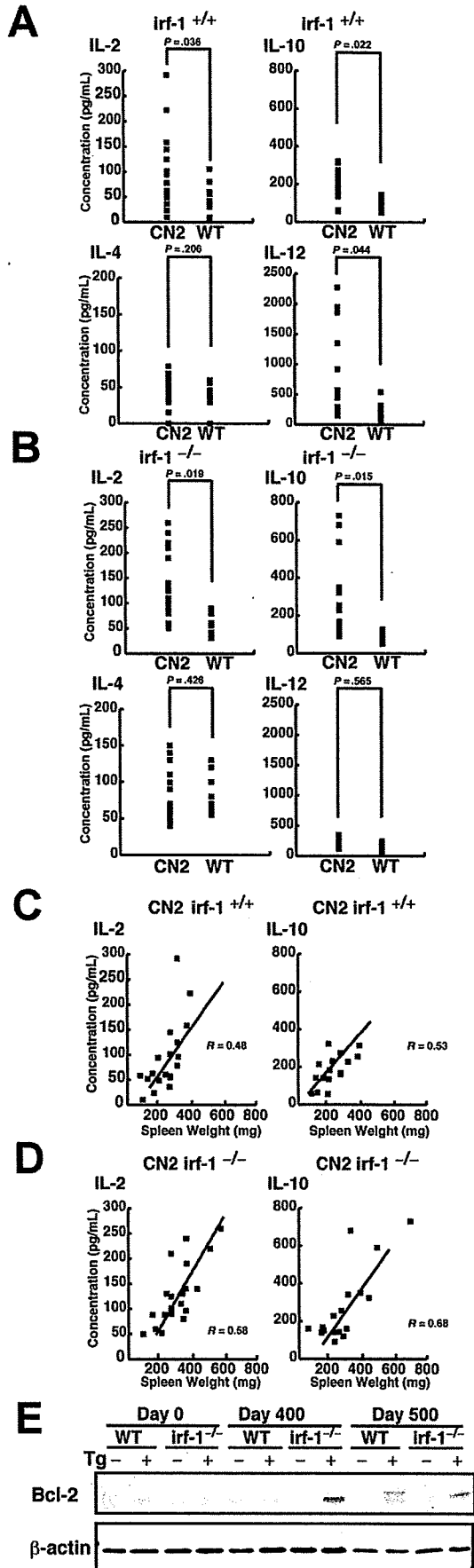


Figure 4. Stable expression of HCV viral proteins induces lymphoproliferative diseases. (A) Switching of the expression of HCV proteins was conducted using the Mx promoter-driven cre recombinase with poly(I:C) induction. The Mx promoter is active in hepatocytes as well as in hematopoietic cells. We crossed 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.

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(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 4B). 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 4C), 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 4B). 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 5A). 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 5B). 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) CN2*irf-1*^{+/+} or (D) CN2*irf-1*^{-/-} 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.

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in the induction of the lymphoproliferative phenotype in *irf1*^{-/-} 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 5C and 5D). 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 *irf1*^{-/-} background ($R = 0.58$, $P < .05$, and $R = 0.68$, $P < .05$, respectively) (Figure 5D). 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 *irf1*^{+/+} background ($R = 0.43$, $P < .05$, and $R = 0.53$, $P < .05$, respectively) (Figure 5C). 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 lymphoproliferation²³ 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 5E). Lymph nodes collected from *irf1*^{-/-} 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 6A). Colony counting was performed at postinfection day 28 (Figure 6B). Combined treatment with IL-2 and IL-10 greatly enhanced colony formation, especially in the splenocytes of HCV transgenic mice (CN2-29, *irf1*^{-/-} CN2-29). The addition of IL-12 suppressed colony formation induced by combined treatment with IL-2 and IL-10. In the *irf1*^{-/-} 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 6C). Because IL-2 enhances T-lympho-

cyte 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 *irf1*, 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 6D and 6E). IL-10 treatment in the presence of IL-2 greatly inhibited Fas-induced apoptosis. Furthermore, *irf1* 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 6C and 6F). 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 7A). 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 7B). 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-

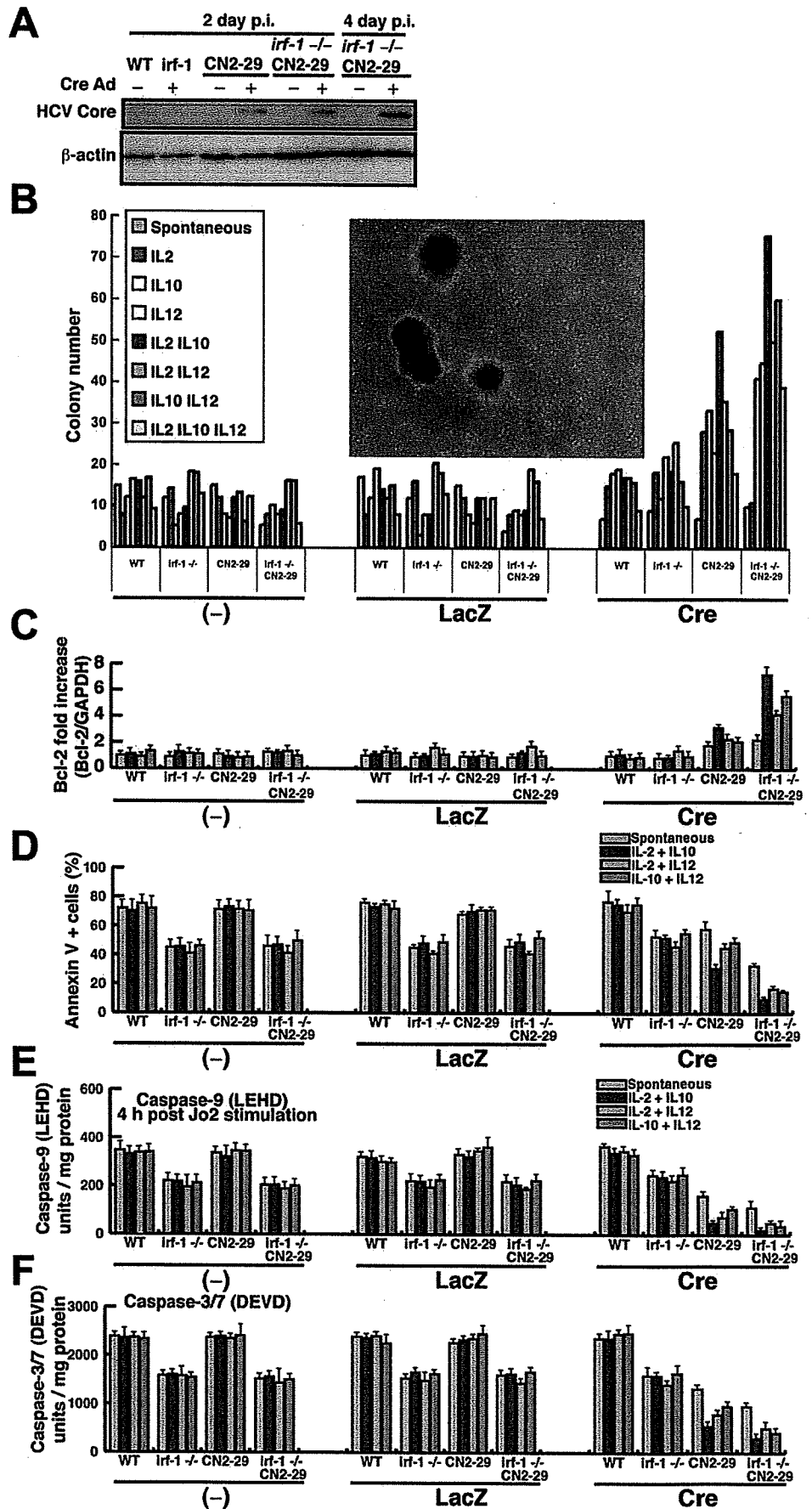


Figure 6. Lymphocyte transformation by aberrant cytokines and inhibition of apoptotic signaling. (A) Expression of the HCV core protein (21 kilodaltons) in *irf-1*^{+/+} (WT) and *irf-1*^{-/-} transgenic (CN2-29) and WT mice 2 or 4 days postinfection (p.i.) with AxCANCre (multiplicity of infection, 1.0). 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 the 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.

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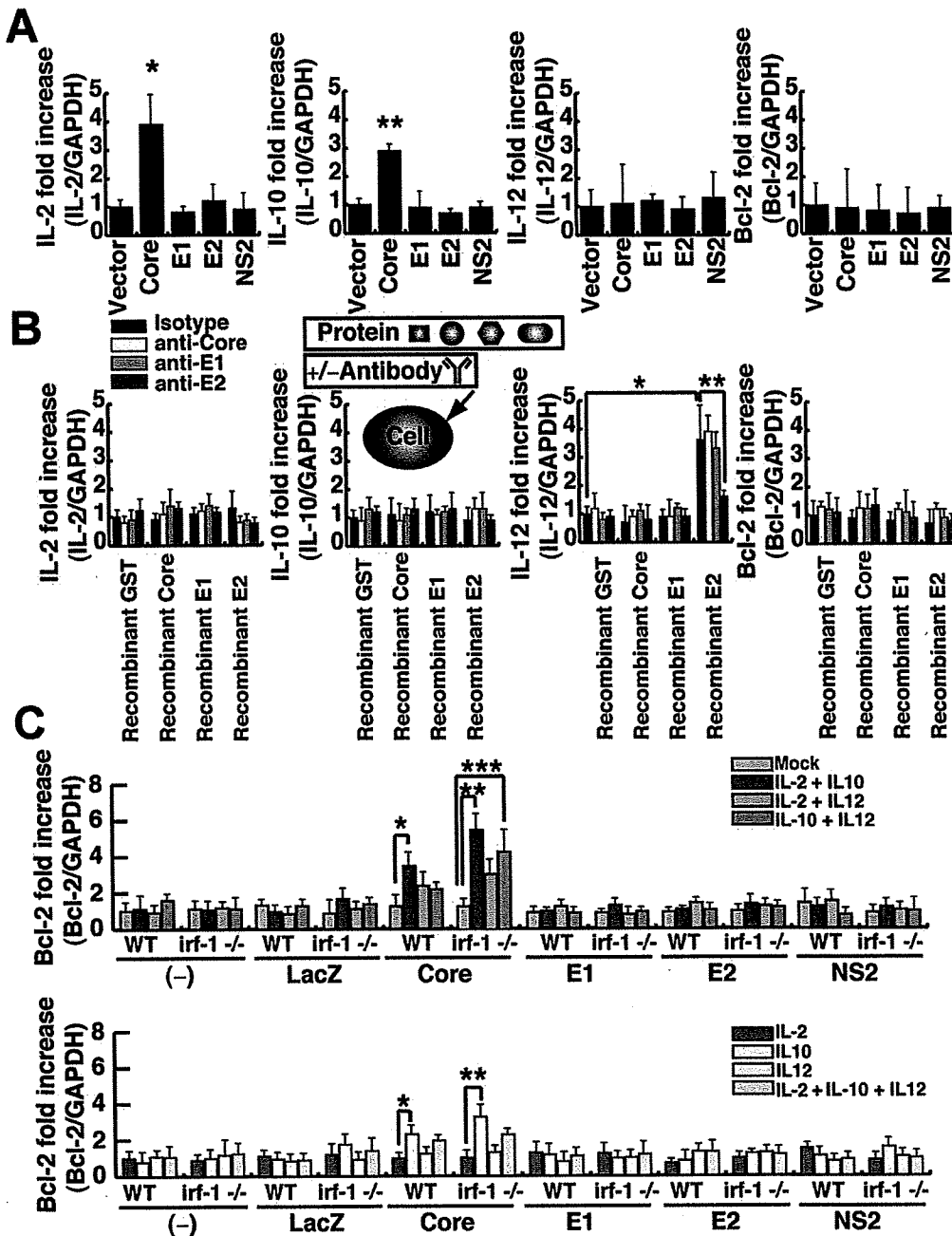


Figure 7. Induction of IL-2 and IL-10 by HCV core and IL-12 by E2 and of Bcl-2 by HCV core plus cytokines. (A) Individual viral proteins were stably expressed in splenocytes using recombinant lentiviruses that expressed the HCV core, E1, E2, NS2, and lacZ. Each gene expression profile was determined 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 mRNA in 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.

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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 *irf1*

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 *irf1* 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.

References

- Saito I, Miyamura T, Ohbayashi A, et al. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci U S A* 1990;87:6547-6549.
- Simonetti RG, Camma C, Fiorello F, et al. Hepatitis C virus infection as a risk factor for hepatocellular carcinoma in patients with cirrhosis. A case-control study. *Ann Intern Med* 1992;116:97-102.
- Ferri C, Monti M, La Civita L, et al. Infection of peripheral blood mononuclear cells by hepatitis C virus in mixed cryoglobulinemia. *Blood* 1993;82:3701-3704.
- Silvestri F, Pipan C, Barillari G, et al. Prevalence of hepatitis C virus infection in patients with lymphoproliferative disorders. *Blood* 1996;87:4296-4301.
- Rui L, Goodnow CC. Lymphoma and the control of B cell growth and differentiation. *Curr Mol Med* 2006;6:291-308.
- Dietrich CF, Lee JH, Herrmann G, et al. Enlargement of perihepatic lymph nodes in relation to liver histology and viremia in patients with chronic hepatitis C. *Hepatology* 1997;26:467-472.
- Ascoli V, Lo Coco F, Artini M, et al. Extranodal lymphomas associated with hepatitis C virus infection. *Am J Clin Pathol* 1998;109:600-609.
- De Vita S, De Re V, Sansonno D, et al. Gastric mucosa as an additional extrahepatic localization of hepatitis C virus: viral detection in gastric low-grade lymphoma associated with autoimmune disease and in chronic gastritis. *Hepatology* 2000;31:182-189.
- Mele A, Pulsoni A, Bianco E, et al. Hepatitis C virus and B-cell non-Hodgkin lymphomas: an Italian multicenter case-control study. *Blood* 2003;102:996-999.
- Cohen SB, Crawley JB, Kahan MC, et al. Interleukin-10 rescues T cells from apoptotic cell death: association with an upregulation of Bcl-2. *Immunology* 1997;92:1-5.
- Pawlotsky JM. The nature of interferon-alpha resistance in hepatitis C virus infection. *Curr Opin Infect Dis* 2003;16:587-592.
- Levine AM, Shimodaira S, Lai MM. Treatment of HCV-related mantle-cell lymphoma with ribavirin and pegylated interferon alfa. *N Engl J Med* 2003;349:2078-2079.
- Moriya K, Fujie H, Shintani Y, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 1998;4:1065-1067.
- Lerat H, Honda M, Beard MR, et al. Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. *Gastroenterology* 2002;122:352-365.
- Wakita T, Taya C, Katsume A, et al. Efficient conditional transgene expression in hepatitis C virus cDNA transgenic mice mediated by the Cre/loxP system. *J Biol Chem* 1998;273:9001-9006.
- Machida K, Tsukiyama-Kohara K, Seike E, et al. Inhibition of cytochrome c release in Fas-mediated signaling pathway in transgenic mice induced to express hepatitis C viral proteins. *J Biol Chem* 2001;276:12140-12146.
- Yokota T, Oritani K, Takahashi I, et al. Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood* 2000;96:1723-1732.
- Taki S, Sato T, Ogasawara K, et al. Multistage regulation of Th1-type immune responses by the transcription factor IRF-1. *Immunity* 1997;6:673-679.
- Yanagi M, Purcell RH, Emerson SU, et al. Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *Proc Natl Acad Sci U S A* 1997;94:8738-8743.
- Yanagi M, St Claire M, Shapiro M, et al. Transcripts of a chimeric cDNA clone of hepatitis C virus genotype 1b are infectious in vivo. *Virology* 1998;244:161-172.
- Machida K, Cheng KT, Pavio N, et al. Hepatitis C virus E2-CD81 interaction induces hypermutation of the immunoglobulin gene in B cells. *J Virol* 2005;79:8079-8089.

22. Kondo Y, Sung VM, Machida K, et al. Hepatitis C virus infects T cells and affects interferon-gamma signaling in T cell lines. *Virology* 2007;361:161–173.
23. McDonnell TJ, Deane N, Platt FM, et al. bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell* 1989;57:79–88.
24. Lacronique V, Mignon A, Fabre M, et al. Bcl-2 protects from lethal hepatic apoptosis induced by an anti-Fas antibody in mice. *Nat Med* 1996;2:80–86.
25. Ito T, Deng X, Carr B, et al. Bcl-2 phosphorylation required for anti-apoptosis function. *J Biol Chem* 1997;272:11671–11673.
26. Stern JB, Smith KA. Interleukin-2 induction of T-cell G1 progression and c-myc expression. *Science* 1986;233:203–206.
27. Levy Y, Brouet JC. Interleukin-10 prevents spontaneous death of germinal center B cells by induction of the bcl-2 protein. *J Clin Invest* 1994;93:424–428.
28. Taniguchi T, Ogasawara K, Takaoka A, et al. IRF family of transcription factors as regulators of host defense. *Annu Rev Immunol* 2001;19:623–655.
29. Penninger JM, Sirard C, Mittrucker HW, et al. The interferon regulatory transcription factor IRF-1 controls positive and negative selection of CD8+ thymocytes. *Immunity* 1997;7:243–254.
30. Lohoff M, Ferrick D, Mittrucker HW, et al. Interferon regulatory factor-1 is required for a T helper 1 immune response in vivo. *Immunity* 1997;6:681–689.
31. Machida K, Cheng KT, Sung VM, et al. Hepatitis C virus induces a mutator phenotype: enhanced mutations of immunoglobulin and protooncogenes. *Proc Natl Acad Sci U S A* 2004;101:4262–4267.
32. Machida K, Kondo Y, Huang JY, et al. Hepatitis C virus (HCV)-induced immunoglobulin hypermutation reduces the affinity and neutralizing activities of antibodies against HCV envelope protein. *J Virol* 2008;82:6711–6720.
33. Dolganiuc A, Paek E, Kodys K, et al. Myeloid dendritic cells of patients with chronic HCV infection induce proliferation of regulatory T lymphocytes. *Gastroenterology* 2008;135:2119–2127.

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

Reprint requests

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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.

Supplementary Materials and Methods

Animal Experiments

CN2, *irf-1*^{-/-}, and *irf-9*^{-/-} mice were maintained in conventional animal housing under specific pathogen-free conditions. AxCANCre and AxCAw1, which are replication-deficient recombinant adenoviruses that lack the E1A, E1B, and E3 regions, were obtained from Dr Izumu Saito (University of Tokyo).¹ AxCANCre expresses the Cre recombinase, whereas AxCAw1, which was used as a control, lacks the inserted *cre* gene. The Cre-expressing recombinant adenovirus (AxCANCre) and AxCAw1 were expanded in HEK293 cells (kindly gifted by Dr Izumu Saito) and purified by sucrose gradient centrifugation.¹ AxCANCre or AxCAw1 (2×10^9 plaque-forming units) was injected intravenously into CN2 or nontransgenic female mice aged 9–13 weeks. The mice were killed at 4, 7, 10, 14, 21, 60, 90, 180, 400, 500, or 600 days after injection ($n = 15$ mice/group; total of 900 mice). Mouse behavior and phenotypes were assessed weekly, and the phenotypes were confirmed at necropsy. At necropsy, the proportion of mice with enlarged lymph nodes, according to weight, was larger in the CN2 group than in the WT group ($P < .05$). To elicit Fas-induced liver damage, adult mice were injected intravenously with 10 μ g of an anti-mouse Fas monoclonal antibody that was purified from a hamster (clone Jo2; Pharmingen) in 200 μ L phosphate-buffered saline (without Ca^{2+} or Mg^{2+}). Animals were observed over a 24-hour period for survival or killed at matched time points; livers were collected for histology or biochemical studies. All the animal experiments were performed according to the guidelines of the Tokyo Metropolitan Institute of Medical Science Subcommittee for Laboratory Animal Care. The protocol was approved by an institutional review board.

For CN2-29 Mx1-Cre mice, Cre expression in the liver was induced by intraperitoneal injection of poly(I:C) (GE Healthcare UK Ltd, Buckinghamshire, England). A total of 300 μ L of poly(I:C) solution (1 mg/mL in phosphate-buffered saline) was injected 3 times at 48-hour intervals.

Southern Blot Analysis

Mouse livers were digested overnight at 55°C in lysis buffer (50 mmol/L Tris-HCl [pH 7.6], 100 mmol/L NaCl, 20 mmol/L EDTA, 1% sodium dodecyl sulfate) that contained 1 mg/mL proteinase K (Boehringer Mannheim, Mannheim, Germany). DNA was extracted with phenol-chloroform and precipitated with ethanol.¹ The DNA pellet was washed with 70% ethanol, dried, and resuspended in 10 mmol/L Tris (pH 7.5). Restriction endonuclease digestion with XbaI, agarose gel electrophoresis, and Southern blotting were performed using the DIG DNA Labeling and Detection System (Boehringer Mannheim). XbaI-mediated DNA fragmentation was used to examine the rate of recombination of the HCV transgene construct.¹ Recombination of the trans-

gene (CN2) was confirmed by Southern blotting. Southern blotting of DNA samples extracted from mouse livers was performed using the DIG DNA Labeling and Detection System (Boehringer Mannheim).

Quantification of HCV Core Protein in Mouse Livers

Mouse livers were homogenized in 1 mL RIPA buffer (1% sodium dodecyl sulfate, 0.5% [vol/vol] Nonidet P40, 10 mmol/L Tris [pH 7.4], 0.15 mol/L NaCl). The HCV core protein concentrations in the liver lysates were quantified using a fluorescent enzyme immunoassay with 2 high-affinity monoclonal antibodies against the HCV core protein.² The HCV core protein concentration in each liver was normalized by the total protein concentration, as measured using the DC protein assay (Bio-Rad Laboratories, Hercules, CA).

Collection of Sera and Enzyme-Linked Immunosorbent Assay

To assess Fas-induced liver failure, blood samples were collected from the supraorbital veins at 0, 1, 2, 3, 4, 5, and 6 hours after administration of the Fas monoclonal antibody or by heart puncture of killed mice. Blood samples were centrifuged at 10,000g for 15 minutes at 4°C to isolate serum. The serum levels of IL-2, IL-4, IL-10, and IL-12 were determined using enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. Serum alanine aminotransferase (ALT) activity was determined using a commercially available kit (Nissui, Tokyo, Japan).

Histology and Immunohistochemical Staining

Livers were frozen in Tissue-Tek OCT compound (SAKURA Finetek, Tokyo, Japan) in dry ice/ethanol, thawed, and fixed in 1:1 (vol/vol) acetone/methanol at -20°C for 10 minutes. Immunohistochemical staining was completed using tyramide signal amplification (NEN Life Science Products, Boston, MA). Nuclei were stained with 0.5 μ g/mL Hoechst 33342 (Molecular Probes, Carlsbad, CA) for 1 minute at room temperature. Sections were observed under a fluorescence microscope (510 Meta; Carl Zeiss, Oberkochen, Germany). Liver samples for histology were cut into small (<1.0 cm) pieces, fixed overnight in 10% paraformaldehyde, dehydrated with ethanol, blocked in paraffin, sectioned at 10- μ m thickness, stained with H&E, and viewed under a microscope (510 Meta; Carl Zeiss). For HCV core protein detection, immunohistochemistry was performed using PFA-fixed or OCT frozen sections. Tissue sections were fixed in 4% PFA and permeabilized in 0.3% H_2O_2 and methanol for 10 minutes. Antigen retrieval was achieved by autoclaving at 121°C for 5 minutes or 10 minutes or by treatment with proteinase K at 37°C for 10 minutes or 30 minutes. To detect core antigen, a mouse anti-core antibody (BT 515) and Alexa 488-conjugated streptavidin were used.

Staining was detected by the DAB substrate reaction. For CD3 and CD45R protein detection, immunohistochemistry was performed on PFA-fixed sections. Tissue sections were fixed with 4% PFA and permeabilized by treating with 0.3% H₂O₂ and methanol for 10 minutes. Antigen retrieval was achieved by autoclaving at 121°C for 10 minutes. To detect the CD3 and CD45R antigens, mouse anti-CD3 and anti-CD45R antibodies (AbD Serotec MorphoSys UK Ltd, Oxford, England) were used. Staining was detected by the addition of 3,3'-diaminobenzidine tetroxide (Dojin Kagaku, Kumamoto, Japan).

Flow Cytometry

Lymphocytes were stained with appropriate dilutions of the following antibodies: PE-conjugated anti-CD3, fluorescein isothiocyanate (FITC)-conjugated anti-B220, FITC-conjugated anti-CD4, PE-conjugated anti-CD8, FITC-conjugated anti-TCR- $\alpha\beta$, and PE-conjugated anti-TCR- $\gamma\delta$. Flow cytometry was performed using the FACSCalibur (Becton Dickinson, San Jose, CA).

Western Blot Analysis

Minced livers, lymph nodes, spleens, and splenocytes were homogenized with a Dounce glass homogenizer at 4°C in lysis buffer (1% bovine serum albumin, 1 mmol/L ethylene glycol-bis[2-aminoethylether]-N,N,N',N'-tetraacetic acid, 300 mmol/L sucrose, 5 mmol/L 3-[N-morpholino]propanesulfonic acid, 5 mmol/L KH₂PO₄ [pH 7.4]) that contained complete protease inhibitor cocktail (Boehringer Mannheim). After centrifugation at 27,000g for 15 min, 50 μ g of supernatant protein were loaded in each lane of a 10% or 15% polyacrylamide gel, separated by electrophoresis in Tris-Tricine buffer (Daiichi Pure Chemicals), and transferred to polyvinylidene difluoride membranes (Amersham Life Science, Tokyo, Japan). The membranes were then incubated for 2 hours at room temperature in TBS-T (20 mmol/L Tris-HCl, 500 mmol/L NaCl, 0.1% [vol/vol] Tween 20) that contained 1% bovine serum albumin, followed by incubation with a primary antibody directed against Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA), protein kinase R (a kind gift of Dr H. Taira, Iwate University, Japan), mouse IRF-1 (Santa Cruz Biotechnology), mouse Fas (Wako), or HCV core (31-2).³ The membranes were washed with TBS-T, incubated with horseradish peroxidase-conjugated secondary antibodies, visualized using a chemiluminescence reagent (Amersham International, Tokyo, Japan), and detected using Hyperfilm-MP (Amersham International).

Detection of Apoptotic Cells by DNA Fragmentation

Small pieces of liver (50–80 mg in weight) were homogenized and digested overnight with proteinase K (Boehringer Mannheim) at 55°C in lysis buffer (50 mmol/L Tris-HCl [pH 7.6], 100 mmol/L NaCl, 20

mmol/L EDTA, 1% sodium dodecyl sulfate). Lysates were extracted with phenol-chloroform-isoamyl alcohol, treated with ribonuclease, precipitated with ethanol, and subjected to 2% agarose gel electrophoresis. Breaks in the hepatocyte DNA were detected using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay kit (R&D Systems) according to the manufacturer's instructions.

Ribonuclease Protection Assay

The caspase transcript levels in the livers from *irf-1*^{-/-} and *irf-1*^{+/+} mice were determined in a ribonuclease protection assay that used mouse apoptosis multi-probe template sets (Pharmingen). Briefly, total RNA was extracted from the livers and assayed with the RiboQuant Multi-Probe RNase Protection Assay System mAPO-1 (Becton Dickinson). Multiple ³²P-labeled caspase probes and the control glyceraldehyde-3-phosphate dehydrogenase probe were synthesized and hybridized overnight to the target RNA, treated with ribonuclease, and separated by electrophoresis on a denaturing polyacrylamide gel. The radioactive bands representing undigested RNAs were then detected and quantified using the BAS 2000 phosphoimager (Fuji Photo Film, Tokyo, Japan). Band intensities were normalized to the glyceraldehyde-3-phosphate dehydrogenase mRNA band in the same lane.

RNA Isolation and Reverse-Transcription PCR

The mRNA species from mouse splenocytes and peripheral blood lymphocytes were isolated using Isogen (Nippon Gene, Shizouka, Japan). The core, E1, E2, NS2, and lacZ mRNA species were detected by reverse-transcription PCR using the following primers: for core-621S, 5'-gggcaggatggctcctgtca-3'; for core-831A, 5'-ttcacgccgctccacaac-3'; for E1-991S, 5'-agcggacatgatcatgata-3'; for E1-1314A, 5'-atcatcatgtcccaagccat-3'; for E2-1601S, 5'-tggcacatcaacaggactgc-3'; for E2-1860A, 5'-actggaccacacaccttga-3'; for NS2-2807S, 5'-gtgtttgtaggctgtact-3'; for NS2-2925A, 5'-atccacactgacgggtgcgc-3'; for lacZ 30S, 5'-aacctggcgttaccact-3'; and for lacZ 430A, 5'-gaacgcgagttaacgcca-3'.

Quantitative Real-Time PCR Amplification of Cytokine and Bcl-2 mRNA Species

The RNA from 10,000 cells was used for art-PCR in the ABI Prism 7900-HT Sequence Detection System with the Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and the RT2 qPCR Primer sets for murine IL-2, IL-4, IL-10, IL-12, *bcl-2*, and glyceraldehyde-3-phosphate dehydrogenase (SABioscience Inc, Frederick, MD; and Applied Biosystems), as described previously.⁴ The quantitative real-time PCR results are presented as the average values from at least 3 independent experiments with duplicate PCR analyses.

Measurements of Caspase Activities Through Cleavage of Fluorogenic Substrates

The cytosolic fractions of splenocytes were isolated as described previously,⁵ with some modifications. Briefly, splenocytes were homogenized in a Dounce glass homogenizer (loose type) with lysis buffer (0.3 mol/L mannitol, 5 mmol/L 3-[*N*-morpholino]propanesulfonic acid, 1 mmol/L ethylene glycol-bis[β -aminoethyl ether]-*N,N,N',N'*-tetraacetic acid, 4 mmol/L KH_2PO_4 , 20 $\mu\text{g}/\text{mL}$ leupeptin, 10 $\mu\text{g}/\text{mL}$ pepstatin A, 10 $\mu\text{g}/\text{mL}$ aprotinin, 2 mmol/L phenylmethylsulfonyl fluoride) and fractionated into pellet, heavy membrane, light membrane, and cytosolic fractions. The lysates were centrifuged at 600g for 10 minutes at 4°C to remove debris or fibers. The supernatants were centrifuged at 10,000g for 15 minutes at 4°C, and the pellets were collected as the heavy membrane fractions. The supernatants were then centrifuged at 100,000g for 45 minutes at 4°C and collected as the cytosolic fraction. The activities of caspase-9 and caspases-3/7 were measured using Ac-LEHD-AFC or Ac-DEVD-AFC (Enzyme Systems Products, Livermore, CA) as substrates, respectively. The substrates were preincubated for 10 minutes at 37°C in reaction buffer.⁶ Thereafter, 100 μg of lysate was added to 1.25 mL of the substrate reaction buffer and incubated for 60 minutes at 37°C. The fluorescence of the released 7-amino-4-trifluoromethylcoumarin was detected using a fluorescence spectrometer (μQUANT ; Bio-tech Instruments, Winooski, VT), with excitation at 400 nm and emission at 505 nm. One unit of caspase activity corresponds to the activity required to cleave 1 pmol of the substrate in 60 minutes at 37°C.

Colony Formation Assay

The colony formation assay was performed by the methylcellulose membrane method, as described previously.^{7,8} Briefly, splenocytes were isolated from mice, plated at 4×10^5 cells/mL in 1% methylcellulose culture medium (Methocult 3430; StemCell Technology, Vancouver, Canada) that contained 100 μL of Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA) with 25 $\mu\text{g}/\text{mL}$ of stem cell factor (R&D Systems, Minneapolis, MN) and hemin (Sigma-Aldrich, St. Louis, MO). Cells were infected with adenovirus that expressed the cre DNA recombinase (AxCANCre) or LacZ (AxCALacZ) gene and treated with cytokines (IL-2, IL-10, IL-12, and combinations thereof). Erythroid burst-forming units, as well as granulocyte, erythrocyte, macrophage, and megakaryocyte colony-forming units, and granulocyte/macrophage colony-forming units were measured.^{7,8} To purify the $\text{CD}4^+$, $\text{CD}8^+$, and $\text{CD}45\text{R}/\text{B}220^+$ cell populations, peritoneal lymph node cells were isolated using immunomagnetic beads conjugated to the corresponding antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany), as described previously.⁹ In our series, >95% of the purified cells expressed the indicated phenotypes, and

viability was >98%.¹⁰ Colony numbers were counted at 7, 14, and 28 days posttreatment. Colonies that contained more than 30 cells were counted under sealed codes, which obscured the identities of the samples.

Antibody Coating and Cell Stimulation

The cell stimulation experiments adhered to the published protocols.¹¹ The following purified monoclonal antibodies were used in the cell stimulation experiments: anti-core, anti-E1, anti-E2 (Biodesign International, Saco, MN), and anti-His6 (Qiagen, Hilden, Germany). Ninety-six-well plates were coated with the various antibodies according to published methods.¹¹ For the experiments involving the blocking anti-core, E1, and E2 antibodies, the recombinant proteins were first treated with the blocking antibodies at 37°C for 30 minutes. The cells were then added to the core-, E1-, E2-, or antibody-coated plates, as previously described.¹²

Statistical Analysis

Data are presented as mean \pm SD. Differences were compared using the Student *t* test and considered statistically significant at $P < .05$.

Supplementary Results

Inhibition of Fas-Induced Apoptosis by 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.¹³ Therefore, we determined the sensitivity of *irf-1*^{-/-} hepatocytes to Fas-induced apoptosis. The *irf-1*^{-/-} mice and WT littermates were injected intravenously with an anti-Fas monoclonal antibody. All 10 *irf-1*^{-/-} mice survived, whereas 4 of 10 WT littermates died within 24 hours due to acute liver failure associated with massive hepatic apoptosis and hemorrhagic necrosis ($P < .05$; Supplementary Figure 2A). Acute and severe liver injury was confirmed by measurement of the serum ALT levels, which were increased by up to 200-fold in WT mice within 3 hours of administration of the anti-Fas monoclonal antibody (Supplementary Figure 2B). In contrast, delayed and less prominent increases in serum ALT levels were observed in the *irf-1*^{-/-} mice 3 hours and 6 hours after injection of the anti-Fas monoclonal antibody (Supplementary Figure 2B). The WT mice had severe lesions of the liver, and staining with 4',6-diamidino-2-phenylindole revealed morphologic changes that were characteristic of apoptosis (Supplementary Figure 2C). Massive panlobular and multilobular apoptotic necrosis (>90%) and peliosis were observed in the *irf-1*^{-/-} CN2 mice in conjunction with 20% mortality (Supplementary Figure 2C [a and b]). Staining using TUNEL showed significantly lower levels of Fas-induced DNA fragmentation in the *irf-1*^{-/-} hepatocytes than in the WT hepatocytes

(compare Supplementary Figure 2C [i and j]), and the onset of DNA fragmentation in *irf-1*^{-/-} hepatocytes was delayed compared with that in WT hepatocytes (data not shown). There were no significant injuries to other organs, such as the thymus and kidneys. Thus, the WT mice showed severe, acute Fas-induced hepatic cytolysis, whereas the *irf-1*^{-/-} mice had significantly delayed serum ALT production and lower serum ALT levels, along with delayed and less severe apoptosis of liver cells, indicating that disruption of *irf-1* inhibits the Fas-induced apoptosis of hepatocytes in vivo.

Several caspases, especially caspase-7 and caspase-3, are activated to cleave cytoplasmic and cytoskeletal proteins during Fas-induced apoptosis of murine hepatocytes.^{14,15} To determine the mechanism by which disruption of *irf-1* inhibits Fas-induced apoptosis of hepatocytes, we examined caspase mRNA expression in ribonuclease protection assays. The levels of caspase-1 mRNA in *irf-1*^{-/-} hepatocytes were significantly lower than those in WT hepatocytes, which is in agreement with previously reported results.¹⁶ Interestingly, the caspase-6 and caspase-7 mRNA levels were also lower in the *irf-1*^{-/-} mice than in the WT mice (Supplementary Figure 2D and E). The disruption of *irf-1* did not affect the levels of Fas or protein kinase R protein (data not shown). Furthermore, the anti-Fas monoclonal antibody induced the release of comparable levels of cytochrome *c* from the mitochondria to the cytoplasm in the WT and *irf-1*^{-/-} hepatocytes (data not shown). These results suggest that the reduced expression of effector caspases may delay Fas-mediated apoptosis in *irf-1*^{-/-} mice and abrogate the elimination of HCV-expressing cells in vivo.

Supplementary Discussion

HCV-infected patients have elevated levels of serum IL-10 and IL-12.¹⁷ As shown in the present study, altered cytokine production may enhance HCV-induced lymphoproliferation. IL-10-transgenic mice develop Fas ligand-mediated exocrinopathy that resembles Sjögren's syndrome.¹⁸ This exocrinopathy was also observed in some of the HCV protein-expressing mice in the current study. Therefore, the induction of cytokine expression in HCV transgenic mice may trigger lymphomagenesis. The induction of the gene that encodes the p40 subunit of IL-12, which is essential for Th1-type differentiation of the immune system, is entirely dependent on IRF-1,^{19,20} which indicates that *irf-1* disruption affects the production of IL-12. In addition, HCV core protein reportedly activates the promoter for IL-2,²¹ which is a growth factor for antigen-stimulated T lymphocytes that is responsible for antigen-induced clonal expansion of T cells. The high serum concentrations of IL-2 and IL-10 may also accelerate the differentiation of memory B cells.²² Moreover, the induction of splenocyte proliferation by treatment with IL-2 plus IL-10 was suppressed by IL-12 in *irf-1*^{-/-} CN2 mice. This suggests a potential application of IL-12

therapy for lymphomas associated with HCV infection. Long-term HCV infection may result in clonal B-cell expansion of immunoglobulin-secreting lymphocytes; approximately 35% of HCV-infected patients are reported to develop B-cell lymphoma.²³ HCV infection is associated not only with non-Hodgkin's B-cell lymphoma, but also with other lymphoid and myeloid malignancies, including non-Hodgkin's T-cell lymphomas; this association is consistent with our findings, especially in the *irf-1*^{-/-} HCV transgenic mouse model (Figures 3 and 6).²⁴⁻²⁷ It is also worth clarifying whether and at what frequency polyclonal lymphoid hyperplasia precedes lymphomagenesis and whether progression from hyperplasia to lymphoma is heralded by a more striking impairment of cytokine (ie, IL-2, IL-10, or IL-12) production.

We found that Fas-induced apoptosis was significantly suppressed in *irf-1*^{-/-} hepatocytes. This suppression may be related to the essential role that IRF-1 plays in the induction of caspase expression.^{16,28} Almost all of the *irf-1*^{-/-} CN2 mice persistently expressed higher levels of HCV core protein in the liver, as compared with the *irf-1*^{+/+} CN2 mice, which suggests wither deficient immunity or inefficient induction of apoptosis in *irf-1*^{-/-} lymphocytes and hepatocytes. Immune surveillance in *irf-1*^{-/-} mice may not eliminate efficiently the HCV protein-expressing cells due to reductions in the numbers of natural killer¹⁹ and CD8⁺ T cells.⁹ Furthermore, IRF-1 is an essential mediator of IFN- γ -induced cell cycle arrest and apoptosis.²⁹ Because activated lymphocytes are normally eliminated by activation-induced cell death, we speculate that the inhibition of apoptosis of *irf-1*^{-/-} lymphocytes results in the accumulation of these cells in the lymph nodes and spleen. HCV core protein also modulates the activities of IFN-induced transacting factors in the Jak/Stat signaling pathway.³⁰ In addition, a protein kinase R-binding domain polypeptide of the HCV NS5A protein relieves the blockade of the IRF-1 pathway, resulting in the induction of IRF-1-dependent antiviral effector genes.³¹ HCV NS5A alone is sufficient to block both the activation of IRF-1 and the induction of an IRF-1-dependent cellular promoter by double-stranded RNA.³¹ Therefore, blockade of the IRF-1 pathway by HCV infection may facilitate the persistent expression of HCV proteins in hepatocytes or lymphocytes, resulting in the inhibition of Fas-mediated apoptosis in *irf-1*^{-/-} hepatocytes. Indeed, genetic abnormalities in the Fas pathway in humans or *lpr* mice cause an autoimmune lymphoproliferative syndrome.³² Differential modulation of apoptosis sensitivity in CD95 type I and type II cells (hepatocytes and some T lymphocytes) has been established.^{33,34} It is noteworthy that both T lymphocytes and hepatocytes have CD95 type II signaling. It is interesting that lymphomagenesis is dominant over liver tumor development. The molecular mechanism underlying this dominance remains unknown. Further molecular analysis will

enlighten the differential signaling pathways between hepatocytes and lymphocytes.

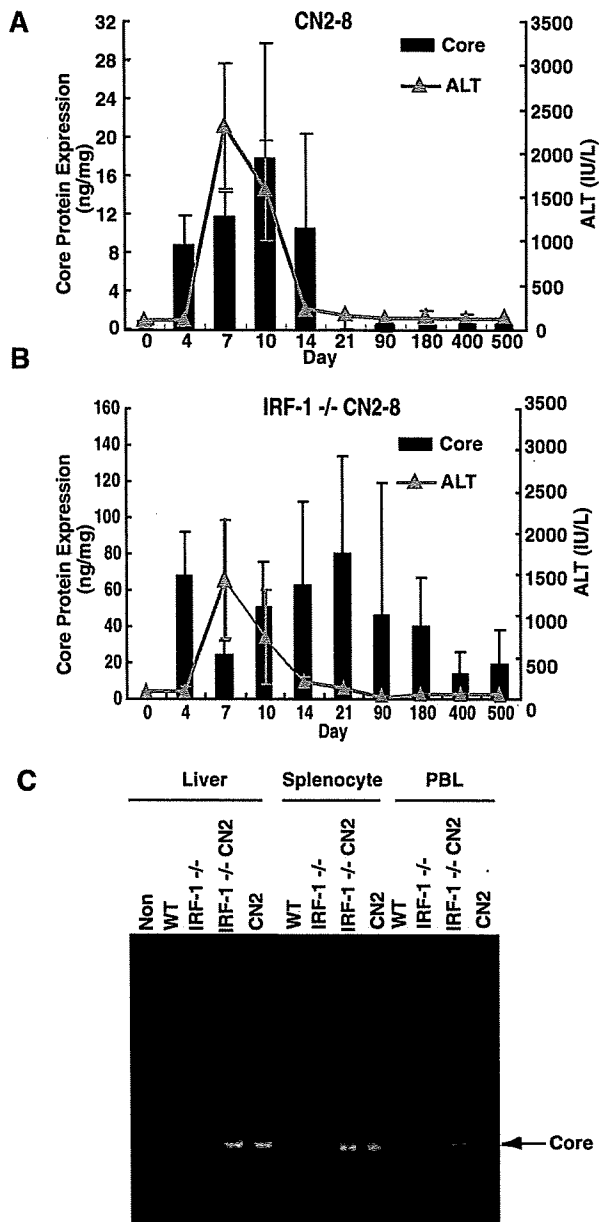
Overexpression of *bcl-2* in B-lymphoid cells causes autoimmune disease,³⁵ and *bcl-2* immunoglobulin-transgenic mice develop follicular lymphoproliferation.³⁶ Therefore, the aberrant expression of *bcl-2* in CN2 lymphocytes may be another factor that inhibits activation-induced cell death, leading to a predisposition for lymphoproliferative disorders. In support of this notion, HCV-infected patients express elevated levels of Bcl-2³⁷ and frequently carry the t(14;18) chromosomal translocation.³⁸ Suppression of cell death by full-length Bcl-2 requires phosphorylation of Ser70^{39,40}; in line with this, our Western blot analysis revealed 2 Bcl-2 species in the enlarged lymph nodes of the HCV protein-expressing mice. Bcl-2 and Fas/APO-1 regulate distinct pathways related to lymphocyte apoptosis.³⁵ Thus, both mitochondrion-dependent and mitochondrion-independent pathways exist for the induction of apoptosis. The dominant pathway differs depending on the tissue type.^{13,35} Therefore, Bcl-2 expression and *irf-1* disruption may synergistically inhibit the apoptotic response in lymphocytes. Indeed, the expression of HCV proteins, chronic inflammation, and predisposition to lymphoproliferative disorders were all enhanced in CN2 mice upon *irf-1* disruption. Collectively, these results suggest that HCV stimulates oncogenesis by multiple mechanisms. Although the loss of the *irf-1* alleles does not result in spontaneous tumor development in mice, it enhances dramatically the risk of tumors caused by the *c-Ha-ras* transgene or by *p53* nullizygosity,⁴¹ suggesting that the loss of *irf-1* confers susceptibility to tumor formation.

It has been proposed that HCV-infected lymphocytes induce t(18;14) translocation, which may result in the overexpression of Bcl-2, and a second mutation (in the *myc* oncogene), which leads to the development of lymphoma.^{42,43} Because the promoter region of Bcl-2 includes SP1, Cre, nuclear factor κ B,⁴⁴ and Akt/protein kinase B,⁴⁵ the combination of core expression and IL-10 and/or IL-2 may up-regulate these transcription factors by integrating several different pathways.

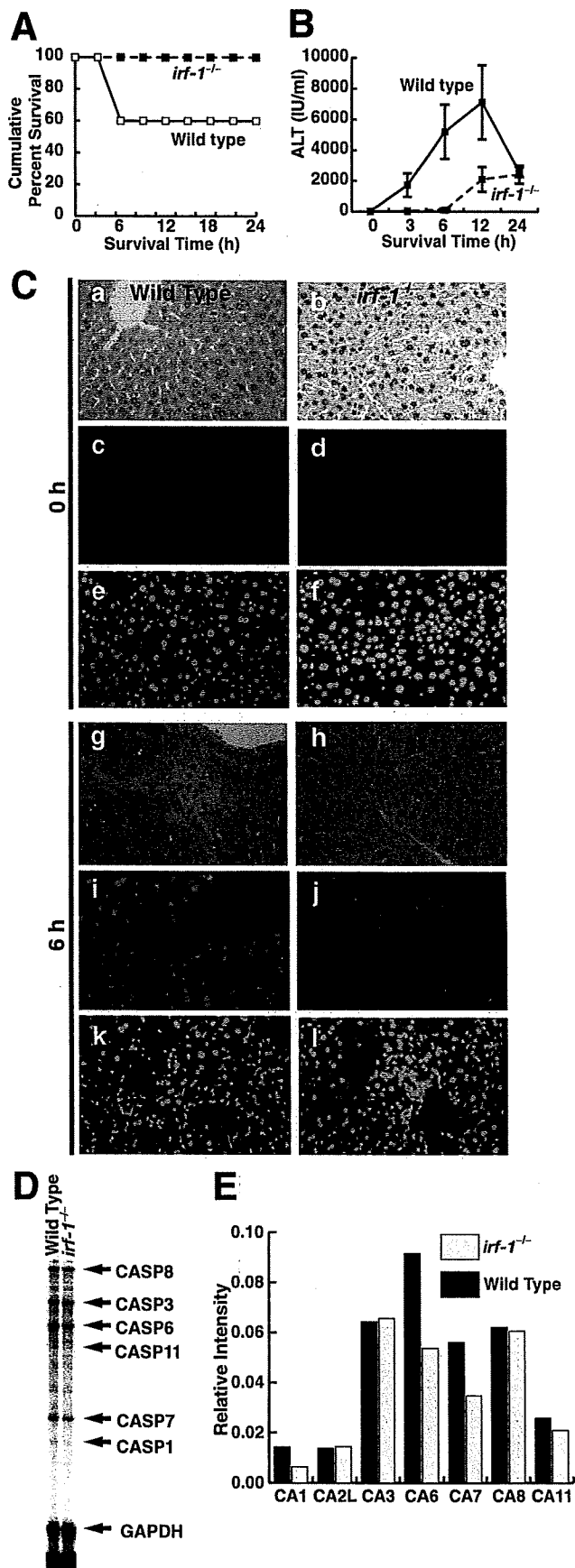
Supplementary References

- Wakita T, Taya C, Katsume A, et al. Efficient conditional transgene expression in hepatitis C virus cDNA transgenic mice mediated by the Cre/loxP system. *J Biol Chem* 1998;273:9001–9006.
- Kashiwakuma T, Hasegawa A, Kajita T, et al. Detection of hepatitis C virus specific core protein in serum of patients by a sensitive fluorescence enzyme immunoassay (FEIA). *J Immunol Methods* 1996;190:79–89.
- Miyazari Y, Atsuzawa K, Usuda N, et al. The lipid droplet is an important organelle for hepatitis C virus production. *Nat Cell Biol* 2007;9:1089–1097.
- Duan H, Heckman CA, Boxer LM. Histone deacetylase inhibitors down-regulate *bcl-2* expression and induce apoptosis in t(14;18) lymphomas. *Mol Cell Biol* 2005;25:1608–1619.
- Machida K, Tsukiyama-Kohara K, Seike E, et al. Inhibition of cytochrome c release in Fas-mediated signaling pathway in transgenic mice induced to express hepatitis C viral proteins. *J Biol Chem* 2001;276:12140–12146.
- MacFarlane M, Cain K, Sun XM, et al. Processing/activation of at least four interleukin-1beta converting enzyme-like proteases occurs during the execution phase of apoptosis in human monocytic tumor cells. *J Cell Biol* 1997;137:469–479.
- Gao JL, Wynn TA, Chang Y, et al. Impaired host defense, hematopoiesis, granulomatous inflammation and type 1-type 2 cytokine balance in mice lacking CC chemokine receptor 1. *J Exp Med* 1997;185:1959–1968.
- Cotterell SE, Engwerda CR, Kaye PM. Enhanced hematopoietic activity accompanies parasite expansion in the spleen and bone marrow of mice infected with *Leishmania donovani*. *Infect Immun* 2000;68:1840–1848.
- Yokota T, Oritani K, Takahashi I, et al. Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood* 2000;96:1723–1732.
- Oritani K, Hirota S, Nakagawa T, et al. T lymphocytes constitutively produce an interferonlike cytokine limitin characterized as a heat- and acid-stable and heparin-binding glycoprotein. *Blood* 2003;101:178–185.
- Crotta S, Stilla A, Wack A, et al. Inhibition of natural killer cells through engagement of CD81 by the major hepatitis C virus envelope protein. *J Exp Med* 2002;195:35–41.
- Machida K, Cheng KT, Pavo N, et al. Hepatitis C virus E2-CD81 interaction induces hypermutation of the immunoglobulin gene in B cells. *J Virol* 2005;79:8079–8089.
- Kondo Y, Sung VM, Machida K, et al. Hepatitis C virus infects T cells and affects interferon-gamma signaling in T cell lines. *Virology* 2007;361:161–173.
- Jones TA, Jones SM. Short latency compound action potentials from mammalian gravity receptor organs. *Hear Res* 1999;136:75–85.
- Collins CE, Quaggiotto P, Wood L, et al. Elevated plasma levels of F2 alpha isoprostane in cystic fibrosis. *Lipids* 1999;34:551–556.
- Kim EJ, Lee JM, Namkoong SE, et al. Interferon regulatory factor-1 mediates interferon-gamma-induced apoptosis in ovarian carcinoma cells. *J Cell Biochem* 2002;85:369–380.
- Kakumu S, Okumura A, Ishikawa T, et al. Production of interleukins 10 and 12 by peripheral blood mononuclear cells (PBMC) in chronic hepatitis C virus (HCV) infection. *Clin Exp Immunol* 1997;108:138–143.
- Saito I, Haruta K, Shimuta M, et al. Fas ligand-mediated exocrinopathy resembling Sjogren's syndrome in mice transgenic for IL-10. *J Immunol* 1999;162:2488–2494.
- Taki S, Sato T, Ogasawara K, et al. Multistage regulation of Th1-type immune responses by the transcription factor IRF-1. *Immunity* 1997;6:673–679.
- Lohoff M, Ferrick D, Mittrucker HW, et al. Interferon regulatory factor-1 is required for a T helper 1 immune response in vivo. *Immunity* 1997;6:681–689.
- Bergqvist A, Rice CM. Transcriptional activation of the interleukin-2 promoter by hepatitis C virus core protein. *J Virol* 2001;75:772–781.
- Choi YS. Differentiation and apoptosis of human germinal center B-lymphocytes. *Immunol Res* 1997;16:161–174.
- Galossi A, Guarisco R, Bellis L, et al. Extrahepatic manifestations of chronic HCV infection. *J Gastrointest Liver Dis* 2007;16:65–73.
- Mele A, Pulsoni A, Bianco E, et al. Hepatitis C virus and B-cell non-Hodgkin lymphomas: an Italian multicenter case-control study. *Blood* 2003;102:996–999.

25. Dietrich CF, Lee JH, Herrmann G, et al. Enlargement of perihepatic lymph nodes in relation to liver histology and viremia in patients with chronic hepatitis C. *Hepatology* 1997;26:467-472.
26. Ascoli V, Lo Coco F, Artini M, et al. Extranodal lymphomas associated with hepatitis C virus infection. *Am J Clin Pathol* 1998; 109:600-609.
27. Silvestri F, Pipan C, Barillari G, et al. Prevalence of hepatitis C virus infection in patients with lymphoproliferative disorders. *Blood* 1996;87:4296-4301.
28. Liu ZX, Govindarajan S, Okamoto S, et al. Fas- and tumor necrosis factor receptor 1-dependent but not perforin-dependent pathways cause injury in livers infected with an adenovirus construct in mice. *Hepatology* 2000;31:665-673.
29. Kano A, Haruyama T, Akaike T, et al. IRF-1 is an essential mediator in IFN-gamma-induced cell cycle arrest and apoptosis of primary cultured hepatocytes. *Biochem Biophys Res Commun* 1999;257:672-677.
30. Potapova O, Basu S, Mercola D, et al. Protective role for c-Jun in the cellular response to DNA damage. *J Biol Chem* 2001;276: 28546-28553.
31. Pflugheber J, Fredericksen B, Sumpter R Jr, et al. Regulation of PKR and IRF-1 during hepatitis C virus RNA replication. *Proc Natl Acad Sci U S A* 2002;99:4650-4655.
32. Sobel ES, Kakkanaiah VN, Kakkanaiah M, et al. Co-infusion of normal bone marrow partially corrects the gld T-cell defect. Evidence for an intrinsic and extrinsic role for Fas ligand. *J Immunol* 1995;154:459-464.
33. Scaffidi C, Fulda S, Srinivasan A, et al. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J* 1998;17:1675-1687.
34. Scaffidi C, Schmitz I, Zha J, et al. Differential modulation of apoptosis sensitivity in CD95 type I and type II cells. *J Biol Chem* 1999;274:22532-22538.
35. Strasser A, Harris AW, Bath ML, et al. Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. *Nature* 1990;348:331-333.
36. McDonnell TJ, Deane N, Platt FM, et al. bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell* 1989;57:79-88.
37. Casato M, Mecucci C, Agnello V, et al. Regression of lymphoproliferative disorder after treatment for hepatitis C virus infection in a patient with partial trisomy 3, Bcl-2 overexpression, and type II cryoglobulinemia. *Blood* 2002;99:2259-2261.
38. Kitay-Cohen Y, Amiel A, Hilzenrat N, et al. Bcl-2 rearrangement in patients with chronic hepatitis C associated with essential mixed cryoglobulinemia type II. *Blood* 2000;96:2910-2912.
39. Ito T, Deng X, Carr B, et al. Bcl-2 phosphorylation required for anti-apoptosis function. *J Biol Chem* 1997;272:11671-11673.
40. Kuwata H, Watanabe Y, Miyoshi H, et al. IL-10-inducible Bcl-3 negatively regulates LPS-induced TNF-alpha production in macrophages. *Blood* 2003;102:4123-4129.
41. Nozawa H, Oda E, Nakao K, et al. Loss of transcription factor IRF-1 affects tumor susceptibility in mice carrying the Ha-ras transgene or nullizygosity for p53. *Genes Dev* 1999;13:1240-1245.
42. Zignego AL, Giannelli F, Marocchi ME, et al. T(14;18) translocation in chronic hepatitis C virus infection. *Hepatology* 2000;31: 474-479.
43. Ellis M, Rathaus M, Amiel A, et al. Monoclonal lymphocyte proliferation and bcl-2 rearrangement in essential mixed cryoglobulinemia. *Eur J Clin Invest* 1995;25:833-837.
44. Ma Q, Li X, Vale-Cruz D, Brown ML, et al. Activating transcription factor 2 controls Bcl-2 promoter activity in growth plate chondrocytes. *J Cell Biochem* 2007;101:477-487.
45. Pugazhenti S, Nesterova A, Sable C, et al. Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. *J Biol Chem* 2000;275:10761-10766.
46. Taniguchi T, Ogasawara K, Takaoka A, et al. IRF family of transcription factors as regulators of host defense. *Annu Rev Immunol* 2001;19:623-655.
47. Penninger JM, Sirard C, Mittrucker HW, et al. The interferon regulatory transcription factor IRF-1 controls positive and negative selection of CD8+ thymocytes. *Immunity* 1997;7:243-254.

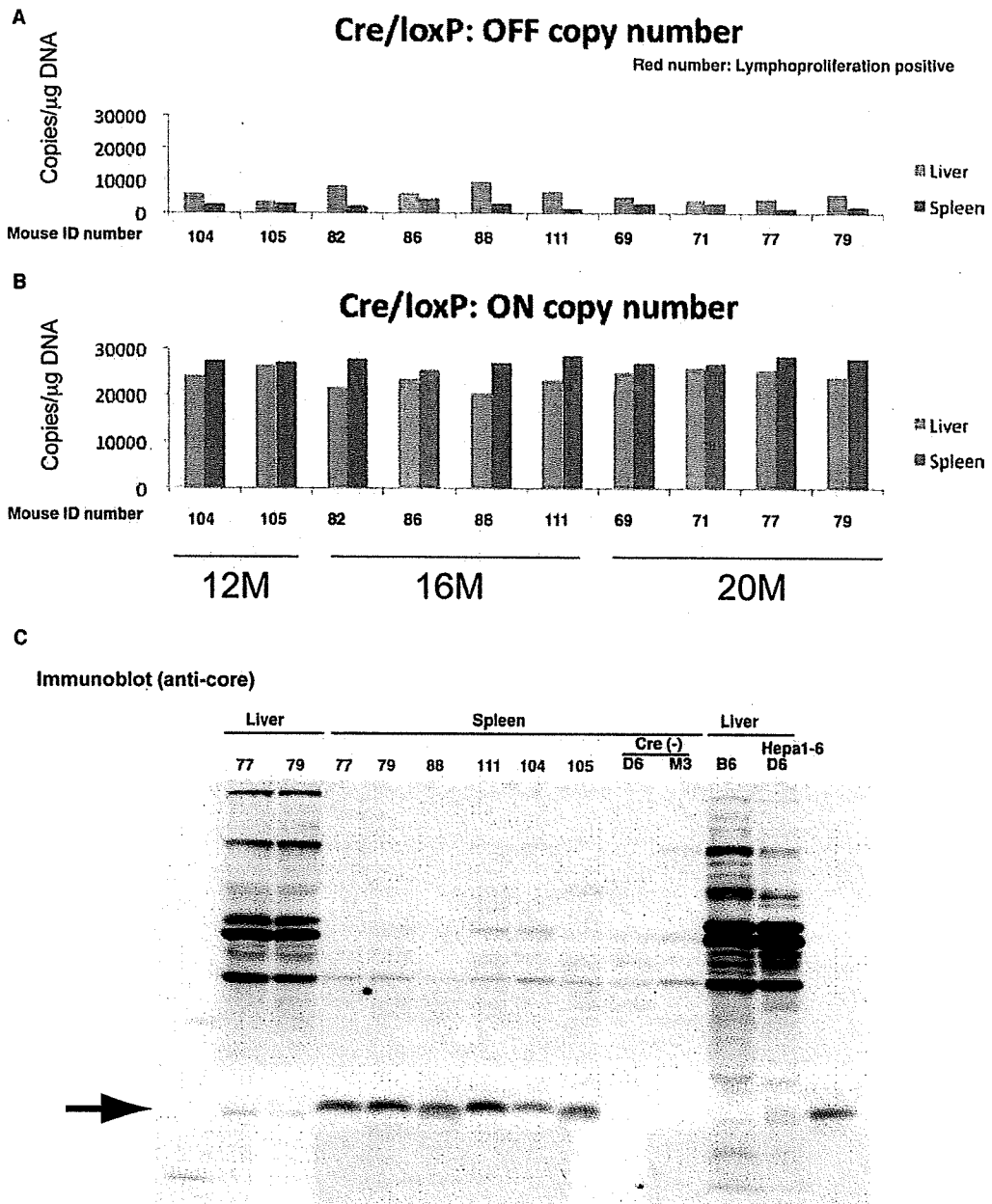


Supplementary Figure 1. (A and B) Levels of serum ALT and core protein expression in the hepatocytes of (A) CN2-8 and (B) *irf-1*^{-/-} CN2-8 mice following the administration of AxCANCre (n = 225 for *irf-1*^{-/-}; n = 75 for *irf-1*^{-/-} CN2-29; n = 150 for *irf-1*^{-/-} CN2-8; n = 225 for WT; n = 75 for CN2-29; and n = 150 for CN2-8; total of 900 mice). (C) Expression levels of the HCV core gene in the liver, splenocytes, and peripheral blood lymphocytes of the WT, *irf-1*^{-/-}, *irf-1*^{-/-} CN2, and CN2 mice, as determined by PCR.

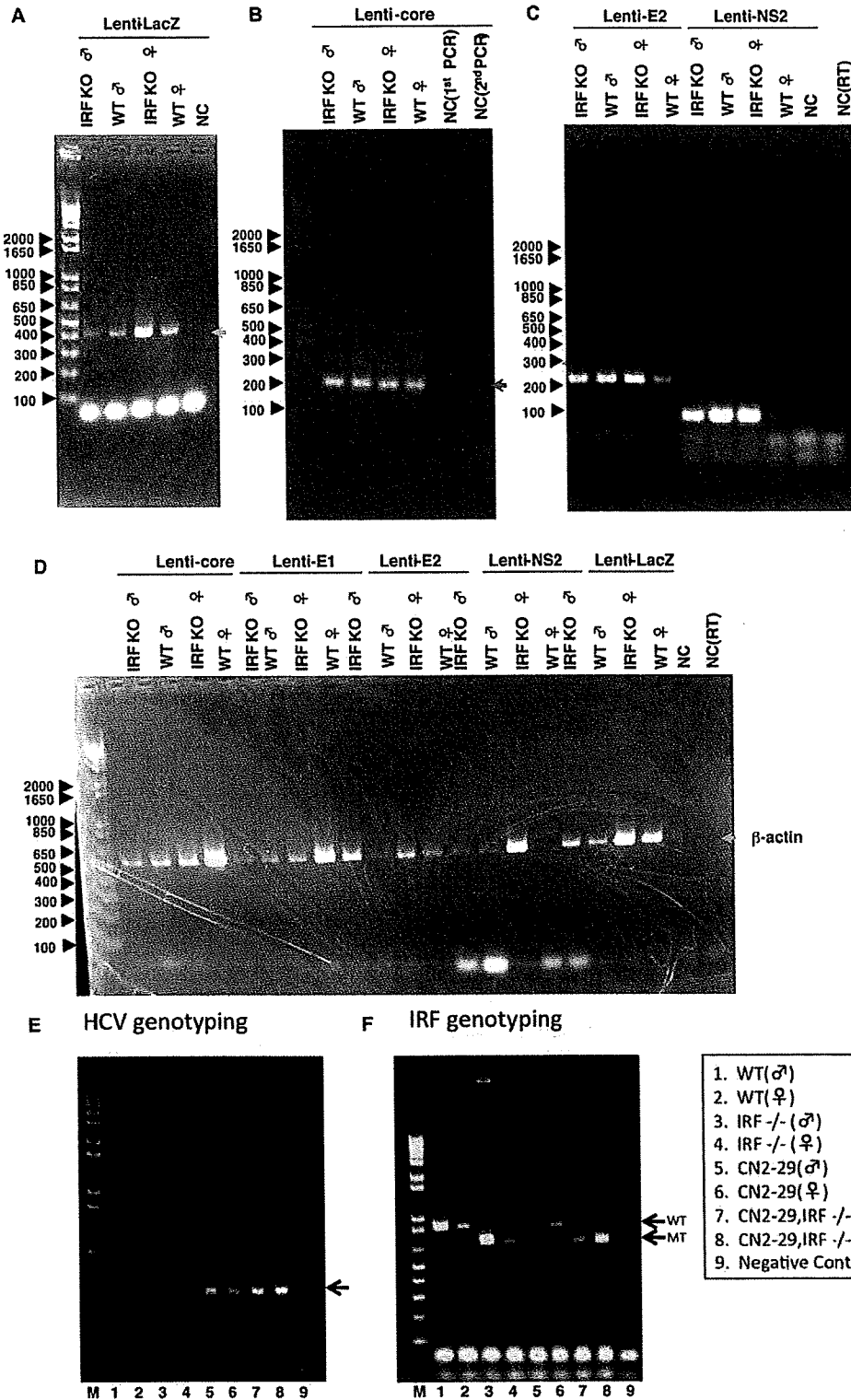


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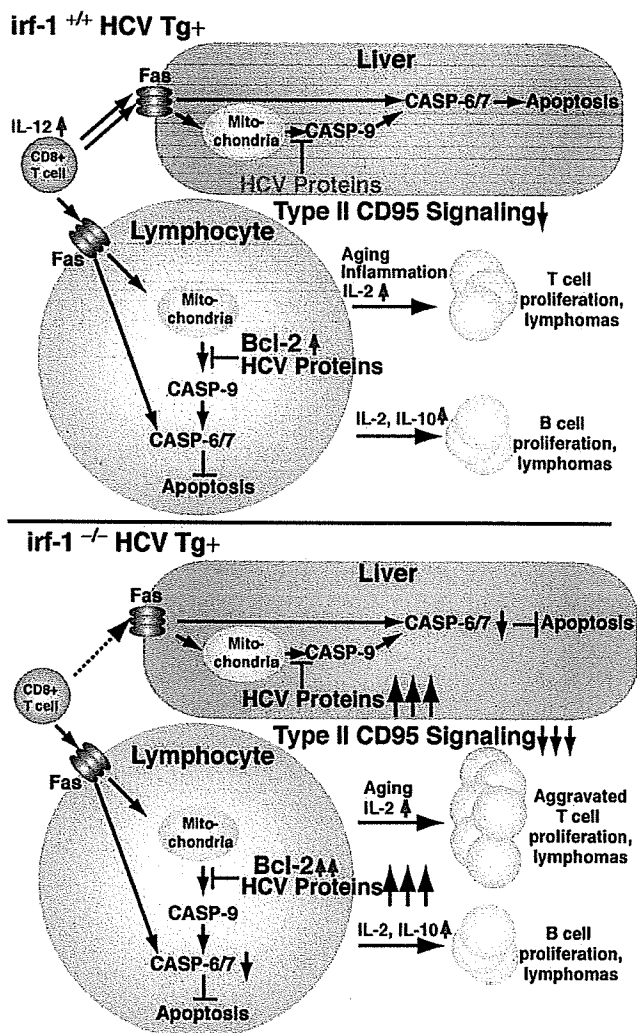
Supplementary Figure 2. Disruption of *irf-1* allows persistent expression of HCV proteins and inhibits Fas-induced apoptosis. The percentages of survivors 24 hours after injection of the anti-Fas monoclonal antibody into *irf-1*^{+/+} and *irf-1*^{-/-} mice. Serum ALT levels in *irf-1*^{-/-} and *irf-1*^{+/+} mice following anti-Fas monoclonal antibody injection. Histology of livers from *irf-1*^{-/-} CN2 and *irf-1*^{+/+} (WT) mice following injection of the anti-Fas monoclonal antibody. (a, b, g, and h) Liver sections stained with H&E 6 hours after injection of the anti-Fas monoclonal antibody (original magnification 40×). (c, d, i, and j) Apoptotic cells in the liver tissues identified by TUNEL staining 6 hours after injection of the anti-Fas monoclonal antibody. (e, f, k, and l) Nuclear staining with 4',6-diamidino-2-phenylindole of liver sections from WT and *irf-1*^{-/-} mice. The levels of expression of caspases -1, -2A, -3, -6, -7, -8, and -11 and control (glyceraldehyde-3-phosphate dehydrogenase) transcripts were determined in an ribonuclease protection assay using hepatocyte RNA species from WT and *irf-1*^{-/-} mice. Relative intensities of caspase mRNA expression in the hepatocytes of the WT and *irf-1*^{-/-} mice, with normalization for the expression of *GAPDH*.



Supplementary Figure 3. Switching efficiency for the expression of the HCV transgene using quantitative real-time PCR. (A and B) Viral gene expression levels were examined by quantitative real-time PCR before and after poly(I:C) injection. (C) Representative immunoblot for the detection of HCV core protein expression in murine livers and spleens.



Supplementary Figure 4. Real-time PCR for HCV transgene expression. (A–D) The HCV gene expression levels in splenocytes transfected with lentiviral vectors were examined by real-time PCR. Lentiviruses that expressed (A) LacZ, (B) core, and (C) E2 and NS2 were used to infect the splenocytes. The mRNA expression levels were detected by real-time PCR. (D) The real-time PCR of β -actin serves as a control. (E) Representative agarose gel images for the genotyping of HCV transgenes and *irf-1*.¹⁷



Supplementary Figure 5. Hypothetical model for the triggering of oncogenic potential by disruption of *irf-1* in synergy with HCV transgene expression. The levels of Bcl-2 and cytokines, as well as aging and inflammation, may enhance the development of lymphoproliferative disorders caused by HCV proteins. Disruption of *irf-1* enables the persistent expression of HCV proteins, leading to lymphoproliferative disease due to reduced apoptosis (ie, lower expression of caspase-1, -6, and -7). HCV CN2 transgenic (Tg+) mice are resistant to Fas-induced apoptosis due to inhibition of cytochrome *c* release from the mitochondria.⁵ Mice with disruption of the *irf-1* gene have several defects of the innate and adaptive immunity systems, such as lineage-specific defects in thymocyte development, whereby immature T cells can develop into mature CD4⁺ cells but not into CD8⁺ T cells.^{19,46} IRF-1 controls the positive and negative selection of CD8⁺ thymocytes.⁴⁷ 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.^{19,20} The number of natural killer cells is dramatically reduced in the *irf-1*^{-/-} mice.¹⁹ Expression of the IL-12 p40 subunit is defective in *irf-1*^{-/-} mice.¹⁹ In the present study, mice with persistent expression of HCV proteins had elevated serum levels of IL-2, IL-10, and IL-12 and higher levels of Bcl-2 protein in enlarged lymph nodes, and these phenomena were more pronounced in the absence of IRF-1.

Supplementary Table 1. Mouse Strains and Viral Protein Functions

Viral protein	Function	CN2-8 mouse strain		CN2-29 mouse strain	
Core	Nucleocapsid	Cre/loxP	Expressed	Cre/loxP	Expressed
E1	Envelope	Switching	Expressed	Switching	Expressed
E2	Envelope	On/off	Expressed	On/off	Expressed
NS2	Proteinase	Regulation	Expressed	Regulation	Expressed