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Quick method of multimeric protein production for biologically active substances such as human GM-CSF (hGM-CSF)

Eiji Shinya [a,*], Atsuko Owaki [a], Yoshihiko Norose [a], Shigeru Sato [b], Hidemi Takahashi [a]

[a] Department of Microbiology and Immunology, Nippon Medical School, Tokyo 113-8602, Japan
[b] Central Institute for Electron Microscopic Research, Nippon Medical School, Tokyo 113-8602, Japan

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ABSTRACT

The C-terminal fragment of C4b-binding protein (C4BP)-based multimerizing system was applied to hGM-CSF to induce dendritic cells (DCs) from peripheral blood monocytes (PBMCs), to see whether the C4BP could stimulate immature DCs, since DCs, equipped with pattern recognition receptors such as toll-like receptors (TLRs), are hypersensitive to various immunologically active molecules like LPS. *hGM-CSF* gene was merged to the 3′-terminal region of the *C4BPα-chain* gene, and the transfected human 293FT cells produced sufficient amount of octameric hGM-CSF, which resulted in iDCs with the same phenotype and the same response to a TRL4 ligand, LPS and a TLR3 ligand, poly I:C, as those induced with authentic monomeric hGM-CSF. These results suggest that the C4BP-based multimerizing system could facilitate the design of self-associating multimeric recombinant proteins without stimulating iDCs, which might be seen with the other multimerizing systems such as that using Fc fragment of IgM.

Introduction

Recombinant proteins are generally produced as single chain molecules. In contrast, natural biological ligand–receptor systems are often multimeric, assembling different molecules into complexes and bringing together different functions. Moreover, it is frequently observed that monomeric ligand–receptor interactions at the cell surface are not able to trigger signal transduction or cellular activation. There have only been a few attempts to produce recombinant multimeric molecules mimicking natural biological system, which were usually unsatisfactory mainly due to the undesirable effects of these multimerizing systems such as the immunoglobulin Fc fragment-based multimerizing system that interacts with cell surface receptors and activates complement [1,2]. There are also multimerizing systems based on leucine zippers [3], chemical polyethylene glycol linkage [4], diabodies [5], streptavidin [6], and protein A [7], which use intracellular or foreign proteins. Therefore, they are likely being immunogenic. Chemical linkages using polyethylene glycol are not stable enough *in vivo* and do not mimic natural biological complexes [4].

C4b-binding protein (C4BP) is a spider-like molecule [8,9] involved in the regulation of the complement cluster family and consists of short consensus repeat units (Fig. 1). Five to seven α-chains, that bind C4b and one β chain, which binds protein S, or eight α-chains only are covalently associated together at their C-terminal portion [10]. A multimerizing system was designed based on the C-terminal portion of the *C4BPα chain gene* [11], and the *hGM-CSF* gene was selected as an example of a multimeric recombinant protein to see whether iDCs, which are among the most sensitive cells to immunological stimulation, could be induced from peripheral blood mononuclear cells to confirm that the C4BPα-multimerizing system does not show immunostimulation.

Materials and methods

Cells and medium. 293FT cells (Invitrogen) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich) supplemented with 10% FCS (Moregate, Queensland, Australia), penicillin (50 U/ml), and streptomycin (50 U/ml) (Invitrogen). HepG2 cells were obtained from ATCC (Manassas, VA, USA).

Antibodies for cell staining. The mouse mAbs HI149 (anti-human CD1a), M-T101 (anti-human CD1b), FITC conjugated mouse anti-human mAbs, G46-2.6 (anti-HLA-abc) and G46-6 (anti-HLA-DR), and phycoerythrin (PE)-conjugated mouse mAb HB15e (anti-CD83) and IT2.2 (anti-CD86) were all purchased from BD Pharmingen (San Diego, CA, USA). PE-conjugated goat $F(ab')_2$ antibody to mouse IgG (IM0855) was from Beckman Coulter (Fullerton, CA, USA).

* Corresponding author. Address: Department of Microbiology and Immunology, Nippon Medical School, Graduate School 2D03, 1-1-5 Sendagi, Bunkyo City, Tokyo 113-8602, Japan. Fax: +81 3 3827 3381.
E-mail address: eiji@nms.ac.jp (E. Shinya).

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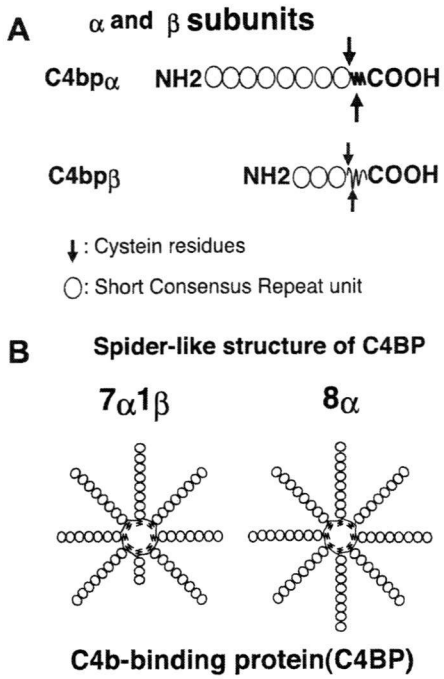

Fig. 1. Spider-like structure of the C4b-binding protein (C4BP). (A) There are two subunits of C4BP: C4BPα consists of eight short consensus repeat (SCR) units and a C-terminal sequence. C4BPβ consists of three SCR units and a C-terminal sequence. (B) C4BP is a high molecular mass (570 kDa) glycoprotein in plasma. The major form is usually composed of seven C4Bpα subunits and one C4BPβ subunit (7α1β) or eight C4Bpα subunits (8α) linked by disulfide bridges. In electron microscope-derived images, C4BP appears as a spider-like structure [9].

DNA constructs. The human *GM-CSF* gene was amplified from the plasmid pCM-hGM (RIKEN DNA Bank, Japan) [12] by PCR using the following primers:

5′-CGCGGATCCATGTGGCTGCAGAGCCTGCT-3′
5′-GGAATTCCTCCTGGACTGGCTCCCAGC-3′

Amplified DNA was digested with BamHI and EcoRI. The digested DNA was subcloned between BamHI and EcoRI sites of plasmid pEF-1a/Myc/6× His/A (Invitrogen) and designated as hGM-CSF/EF1/Myc/His.

Total RNA was extracted from human HepG2 cells using an RNeasy kit (Qiagen, Hilden Germany) and the C-terminal region of the human *C4BPα* gene was cloned by RT-PCR methods using the following primers:

A-1: 5′-CGGAATTCTTCGGGACAGGTCCTGC-3′
A-2: 5′-GCTCTAGATAGTTCTTTATCCAAAG-3′

The amplified C-terminal region of the human *C4BPα* gene was digested with EcoRI and XbaI, subcloned 3′ to the *hGM-CSF* gene in hGM-CSF/EF1/Myc/His between the EcoRI and XbaI sites, and designated as hGM-CSF-C4BPαEF1/Myc/His.

Production of recombinant multimeric GM-CSF. Transfection of 293FT cells with hGM-CSF/EF1/Myc/His or hGM-CSF-C4BPα/EF1/Myc/His was performed using polyethyleneimine as described previously [13,14], and G418-resistant clones were selected. The conditioned culture media of the selected clones were collected, sterilized with a 0.2 μm filter, and quantified using hGM-CSF ELISA kit (R&D Systems, Minneapolis, MN, USA) for the further experiments. As a reference, recombinant hGM-CSF purchased from Peprotech EC (London, Great Britain) was used (see Fig. 2).

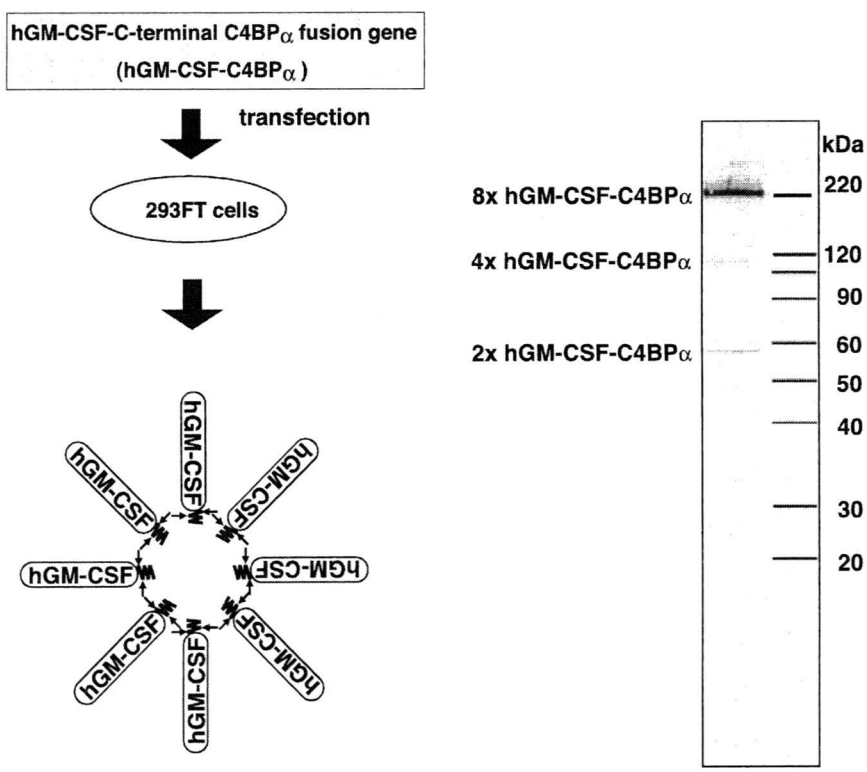

Fig. 2. Production of recombinant octameric hGM-CSF. The hGM-CSF-C4BPα fusion gene was transfected into 293T cells. An immunoblot analysis with anti-myc-epitope antibody revealed a molecular mass of 224 kDa, indicating an octamer of hGM-CSF, as well as small amounts of 112 kDa (tetramer) and 56 kDa (dimer) proteins.

PBMC-derived immature DCs. Immature DCs were obtained from PBMCs as described previously [13]. Briefly, PBMCs were freshly isolated with Ficoll-paque (Amersham-Pharmacia, Uppsala, Sweden) from the peripheral blood of healthy volunteers, and $CD14^+$ monocytes were separated immediately by magnetic depletion using a monocyte isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) containing hapten-conjugated antibodies to CD3, CD7, CD19, CD45RA, CD56, and anti-IgE Abs, and a magnetic cell separator (MACS, Miltenyi Biotec) in accordance with the manufacturer's instructions, routinely resulting in >90% purity of $CD14^+$ cells. The cells were cultured in 24-well culture plates for 6–7 days in complete medium supplemented with 20 ng/ml IL-4 (Biosource Intl., Camarillo, CA, USA) and 50 ng/ml of hGM-CSF obtained from PeproTech EC, or from the conditioned culture medium of 293FT cells transfected with *hGM-CSF* gene or *hGM-CSF-C4BPα* gene, in order to obtain iDCs. After 4–6 days of incubation, fluorescent activated cell sorter (FACS) analysis was performed to analyze the phenotype of the cells.

Immunoblotting. The cells were lysed in triple-detergent lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 0.1% SDS, 100 g/ml phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1% Nonidet P-40, and 0.5% sodium deoxycholate). The obtained samples were run on a 4–12% NuPAGE Bis–Tris gel (Invitrogen) using MES (morpholine ethanesulfonic acid), SDS (sodium dodecyl sulfate) buffer (1000 mM MES, 1000 mM Tris, 70 mM SDS, 20 mM EDTA) under non-reducing conditions and transferred to a PVDF (polyvinylidene difluoride) membrane (ATTO, Tokyo, Japan). The membrane was incubated with rabbit anti-myc serum (Invitrogen) and immunoblotting was carried out using horseradish peroxidase conjugated-goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) and a 3,3′,5,5′-tetramethylbenzidine (TMB) substrate kit for peroxidase (VECTOR Laboratory, Burlingame, CA).

Electron microscopy. The conditioned culture medium containing hGM-CSF-C4BPα gene-derived multimeric hGM-CSF was dialyzed against 0.1 M NH_4OAc/0.05 M NH_4HCO_3, pH 7.35, were adsorbed to thin carbon films and were negatively stained with 4.0% uranyl acetate. The photographs were taken at a primary magnification of 40,000 in a Hitachi H-7500 transmission electron microscope, operating at 80 kV.

Results

hGM-CSF produced by 293FT cells could induce iDCs

The first question was whether 293FT cells could produce enough amount of hGM-CSF to prepare iDCs from PBMCs. Human embryonic 293FT cells were transfected with the *hGM-CSF* gene and the hGM-CSF concentration was analyzed by ELISA. As a result, the hGM-CSF concentration in the conditioned medium was always high enough for dendritic cell preparation; between 150 and 200 ng/ml after transient transfection, or with cloned 293FT cells producing hGM-CSF.

The second question was whether the conditioned medium of the *hGM-CSF*-transfected 293FT cells could induce iDCs together with IL-4, because dendritic cells are equipped with pattern recognition receptors such as toll-like receptors that enable DCs to respond to very scarce amounts of stimulants such as LPS or nucleotides. Flow cytometric analysis of DCs prepared with the conditioned culture medium of *hGM-CSF*-transfected 293FT and commercial IL-4 showed the typical phenotype of iDCs derived from PBMCs, suggesting that the conditioned medium of 293FT did not contain any stimulants that induced the maturation of DCs (Fig. 3, right panels).

Production of multimeric hGM-CSF

Next, it was attempted to produce multimeric hGM-CSF using the *hGM-CSF-C4BPα* gene to see whether it could induce iDCs from

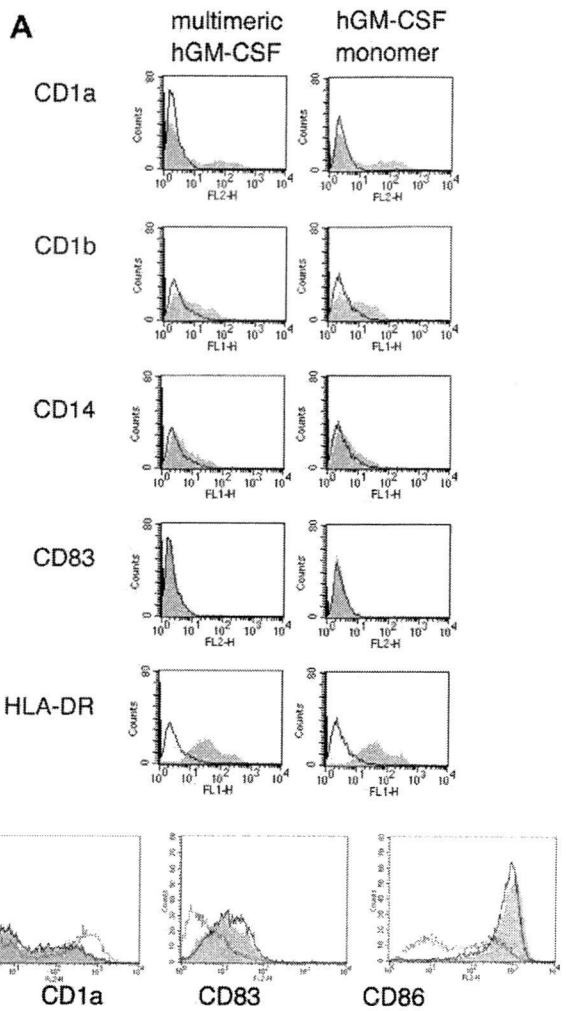

Fig. 3. FACS analysis of the immature dendritic cells. (A) The hGM-CSF gene or the hGM-CSF-C4BPα fusion gene was transfected into 293FT cells and conditioned medium containing hGM-CSF or multimeric GM-CSF was used in combination with IL-4 to induce dendritic cells from peripheral blood monocytes. The resultant DCs either with hGM-CSF (right panels) or multimeric GM-CSF (left panels) showed an identical phenotype each other. The results from the hGM-CSF gene and the hGM-CSF-C4BPα fusion gene are shown as grey lines and negative control data are shown as black lines. (B) The immature DCs induced with multimeric hGM-CSF showed the same response to LPS and polyI:C as that of the iDCs induced with hGM-CSF. Multimeric hGM-CSF-induced iDCs were incubated with LPS (200 ng/ml) (solid line) or poly I:C (100 μg/ml) (filled line) for 48 h and FACS analysis was performed. The iDCs without LPS or poly I:C is shown as dotted line.

PBMCs as well as authentic monomeric hGM-CSF. A chimeric gene of *hGM-CSF* fused to *C4BPα* was transfected into 293FT cells and the conditioned culture medium was analyzed by ELISA for hGM-CSF. Immunoblotting was also performed to analyze the *hGM-CSF-C4BPα* chimeric gene product under non-reducing conditions, which was observed as a band with a molecular mass of 220 kDa as well as faint bands with molecular masses of 56 and 112 kDa (Fig. 3). The predicted molecular weight of the monomeric *hGM-CSF-C4BPα* gene product is 28 kDa (Table 1), indicating that the major product of the *hGM-CSF-C4BPα* gene was an octameric protein, but dimer and tetramer proteins were also produced.

Visualization of multimeric hGM-CSF

The purified multimeric hGM-CSF was examined in the electron microscope and a typical field view is shown in Fig. 4A. Several hGM-CSF-C4BPα molecules were examined and the molecules

Table 1
Characteristics of hGM-CSF-C4Bpα monomer with myc and 6× histidine tag.

	Value*
Length	245 amino acids
Molecular weight	27961.0 Da
Isoelectric point	5.07
Net charge at pH 7	−12.3

* The values were calculated using Gene Inspector 1.6 software (Textco BioSoftware, West Lebanon, NH, USA).

exhibited a morphology that resembles the aggregation of globular molecules (Fig. 4B). Human GM-CSF is a flattened globular molecule that is about 4.0 and 2.4 nm thick [15], the size of which corresponds with the electron micrographs of multimeric hGM-CSF.

Multimeric hGM-CSF produced by 293FT cells could induce iDCs

Finally, the induction of iDCs from PBMC using recombinant IL4 and multimeric hGM-CSF was attempted. The conditioned culture medium of 293FT cells transfected with *hGM-CSF-C4BPα* gene and recombinant IL4 were used to make iDCs from PBMC. FACS analysis showed the typical phenotype of iDCs (Fig. 3, left panels). The obtained iDCs were also incubated with either LPS (200 ng/ml for 48 h) or poly I:C (100 μg/ml for 48 h) and the typical phenotypic responses of iDCs to LPS and poly I:C were observed, such as the down-regulation of CD1a surface expression and up-regulation of CD83 and CD86 surface expression (Fig. 3B).

Discussion

For the protein expression, there are several methods such as those using *Escherichia coli*, insect cells, or mammalian cells, but each has their own advantages and disadvantages [16]. First, *E. coli* expression techniques are probably the most popular. The techniques necessary to express sufficient amounts of protein are relatively simple and the amount of time necessary to generate an over-expressing strain is very short. Therefore, *E. coli* is widely used for the expression of commercially important proteins. However, protein expression in *E. coli* does have some disadvantages. Recombinant eukaryotic proteins produced in *E. coli* are not properly modified and often precipitate into insoluble aggregates called "inclusion bodies." The recombinant protein could only be recovered in an active form by solubilization in denaturing agents followed by careful renaturation. Moreover, it is relatively difficult

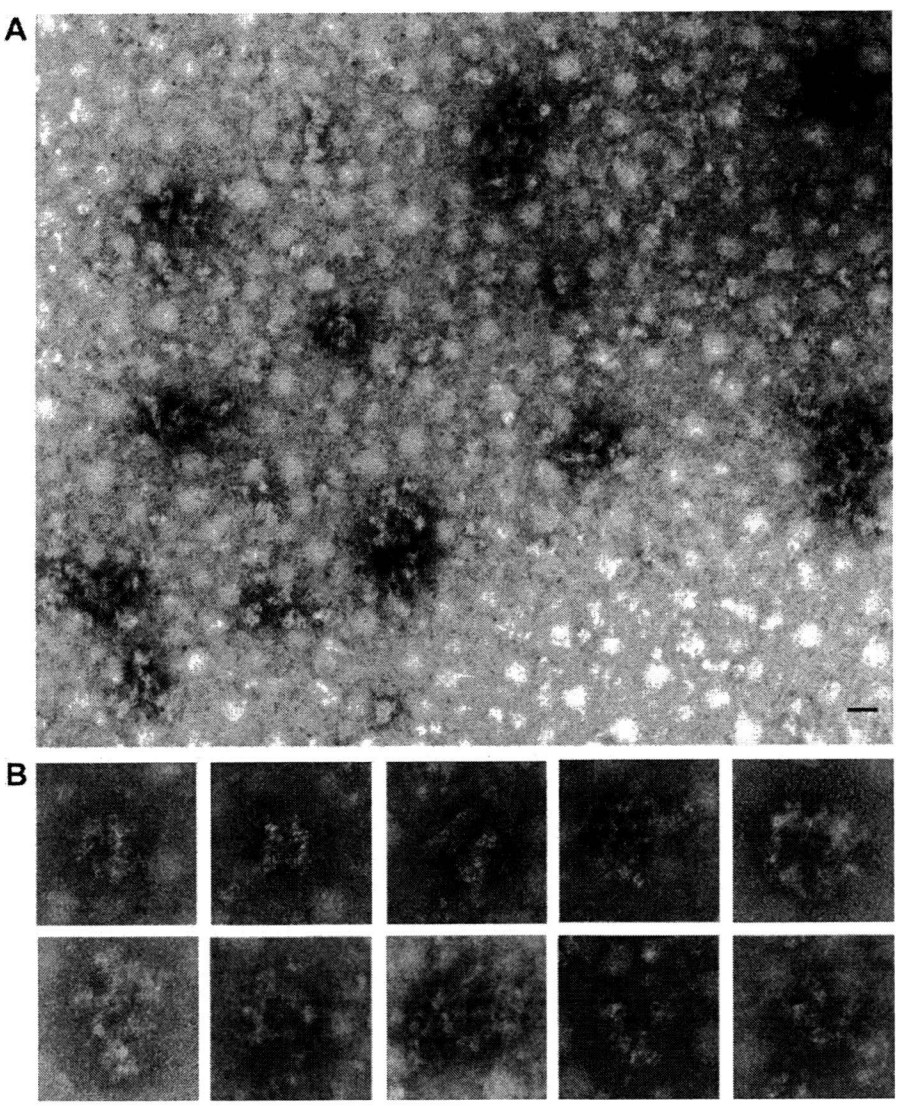

Fig. 4. Electron micrographs of multimeric hGM-CSF. (A) Field view of multimeric hGM-CSF. (B) Selected images demonstrating the aggregation of globular molecules of hGM-CSF. The scale bar in (A) represents 60 nm.

to arrange the secretion of the expressed proteins from *E. coli* if the amount is large. In addition, if the desired recombinant protein was hGM-CSF for DC preparation, the hGM-CSF produced should absolutely be without any trace of LPS, but it is very difficult to obtain recombinant proteins expressed in *E. coli* that are free of LPS. The baculoviral expression system using insect cells also has a number of advantages. With this system, proteins can be expressed at high levels and usually in the proper cellular compartment. For example, membrane proteins are usually localized to the membrane and nuclear proteins to the nucleus in insect cells as well as in mammalian cells, although the proteins expressed in insect cells are not always properly modified. Compared to the above two systems, mammalian expression techniques have certain advantages, especially for the expression of higher eukaryotic proteins. The expressed proteins are usually properly modified and they accumulate in the correct cellular compartment, but it is difficult to perform large-scale expression experiments. To obtain large amounts of recombinant protein using eukaryotic cells, CHO cells are often used because of the high amounts of the desired protein that can be obtained via dihydrofolate reductase (DHFR) [17] based amplification of recombinant genes using increasing concentrations of methotrexate (MTX) [18]. However, the DHFR amplification process is lengthy and may require several months to isolate and characterize a stable, amplified line of CHO cells. Moreover, even after the long amplification period, the amount of product is not always enough for some experimental uses.

This study utilized 293FT cells instead of CHO cells, which were transfected with the *hGM-CSF* gene to see if they could produce sufficient amounts of hGM-CSF to induce iDCs in combination with IL-4 in a laboratory setting. All the procedures took no more than one month from the cloning of the gene, which included subcloning into the vector plasmid, transfection into 293FT cells, production of sufficient amounts of hGM-CSF, and analysis of the quality of the hGM-CSF obtained for the preparation the iDCs. The resultant iDCs showed the same phenotype as iDCs incubated with commercial hGM-CSF and IL-4 as well as the same response to a TLR-4 ligand, LPS, and to a TLR-3 ligand, poly I:C.

Recently, many biologically active substances, such as cytokines and chemokines, have been identified, some of which are often needed for use in further experiments immediately. Therefore, there is a clear need for methods to obtain newly identified biologically substances immediately and without difficulty. The eukaryotic expression system using 293FT cells is a promising candidate because this system can obtain biologically active substances of good quality within a few weeks, and the amount of these substances is usually enough for further experiments because these substances have very high biological activity.

Furthermore, using a C-terminal fragment of the *C4BPα* gene fused to the 3′ end of the *hGM-CSF* gene, the recombinant multimeric hGM-CSF was obtained, which was successfully secreted into the culture medium of the cells despite of its high M_W of 200 kDa, and it was shown that this recombinant hGM-CSF could be used for iDCs preparation from PBMCs, whose phenotype was exactly the same as those iDCs incubated with authentic monomeric hGM-CSF and IL-4. These iDCs, prepared with multimeric hGM-CSF and IL-4, responded to LPS and poly I:C in exactly the same manner as those prepared with authentic monomeric hGM-CSF, which supported the notion that the C4BPα-multimerizing system can be used without the potential risk of immunological stimulation, because iDCs are among the cells that are most sensitive to immunological stimulation. Furthermore, it took no more than one month to obtain multimeric hGM-CSF with the C4BPα-multimerizing system using 293FT cells, which was enough to obtain iDCs from PBMCs.

In conclusion, the present C4BP-based multimerizing system combined with 293FT cells was shown to be the promising quick method to produce sufficient amounts of recombinant multimeric protein with biological activity without any untoward stimulation of the immune system.

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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.

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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

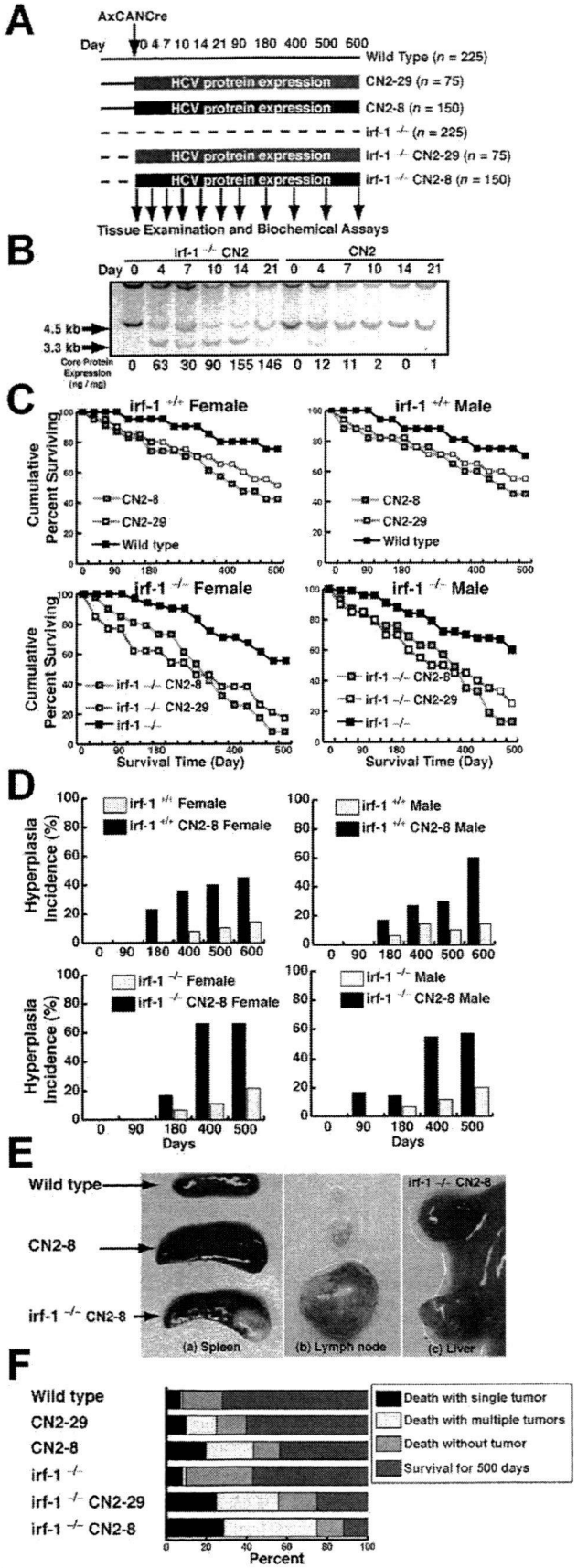
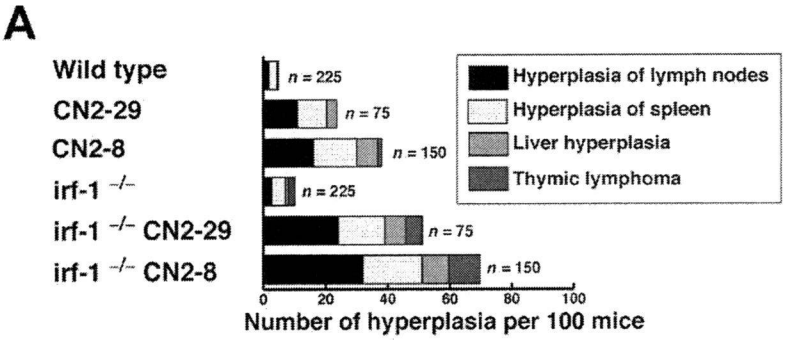


Figure 1. Disruption of *irf-1* enhances oncogenic potential in combination with HCV transgene expression. (*A*) Experimental design for the animal model. Transgenic mice and their nontransgenic littermates (10–14 weeks of age) were administered the *Cre*-expressing adenovirus (AxCANCre) and killed after 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.



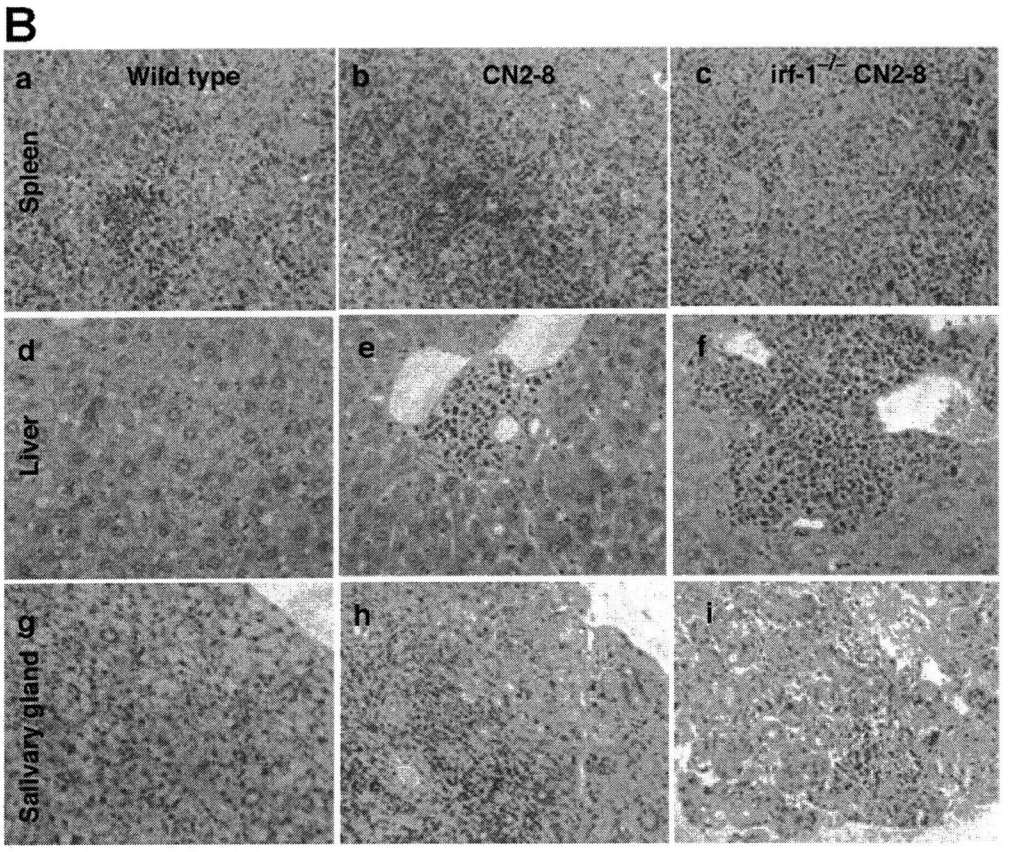


Figure 2. Disruption of *irf-1* aggravates lymphocyte infiltration in combination with HCV transgene expression. (*A*) Histologic analysis of spontaneous proliferative disturbances in the CN2 transgenic mice. Of the 900 mice injected with AxCANCre, 25 of 75 (33%) CN2-29, 47 of 150 (31%) CN2-8, 29 of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

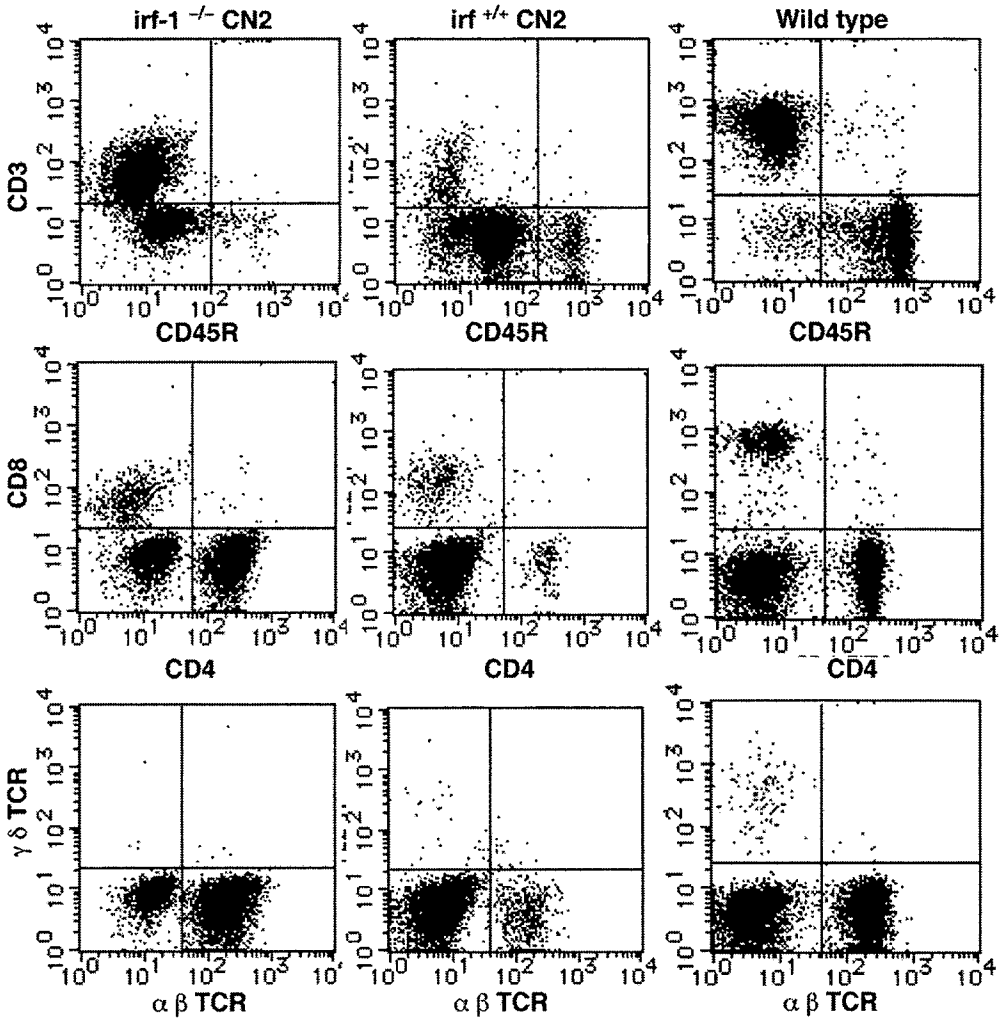


Figure 3. HCV expression and *irf-1* ablation affect the lymphocyte population. T-cell and B-cell proliferation in *irf-1*$^{+/+}$ CN2 mice, *irf-1*$^{-/-}$ CN2 mice, and WT mice. CD3$^+$, CD45R$^+$, CD4$^+$, CD8$^+$, and T-cell receptor–positive cells from age-matched *irf-1*$^{-/-}$ CN2, *irf-1*$^{+/+}$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

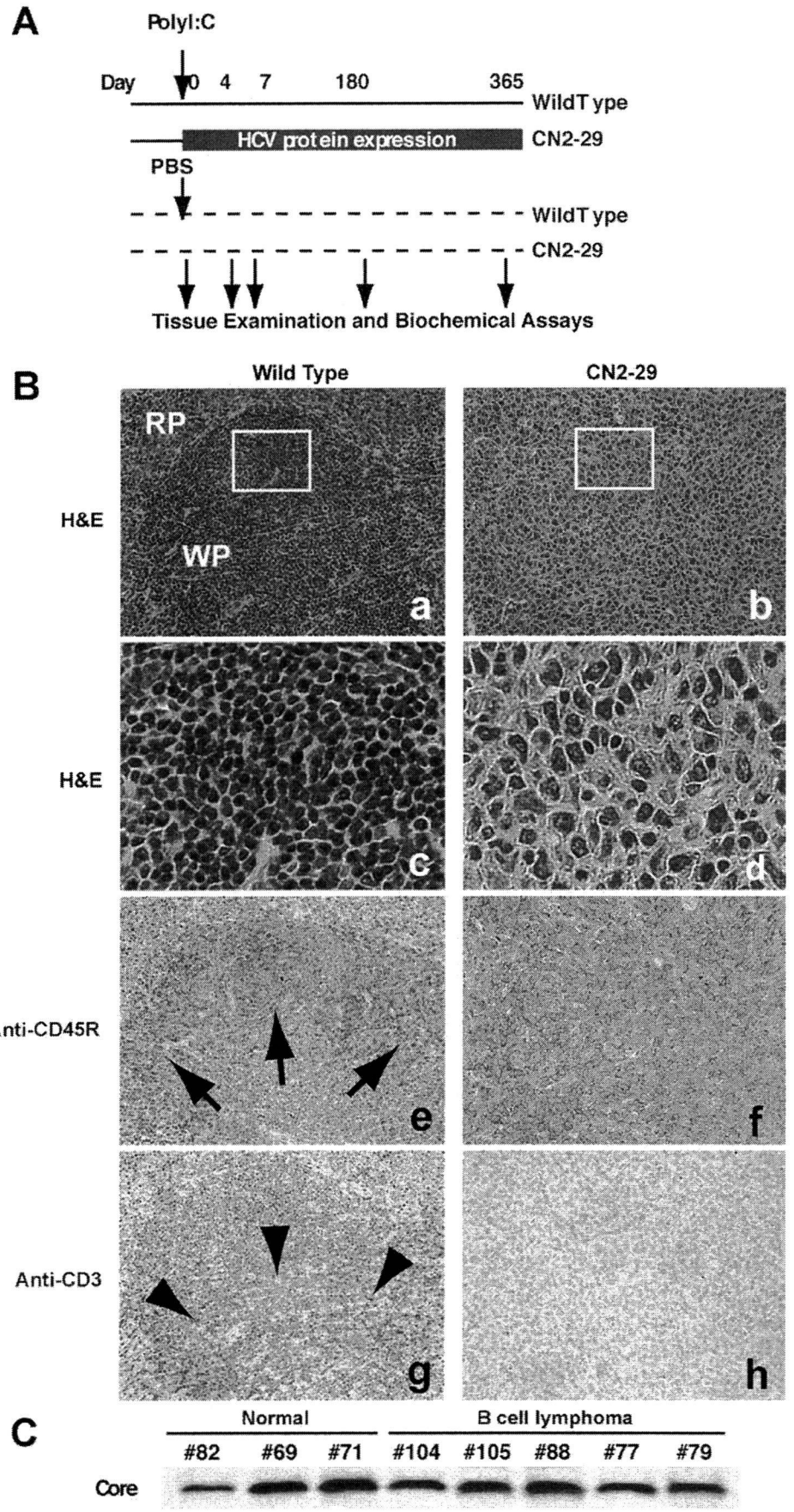
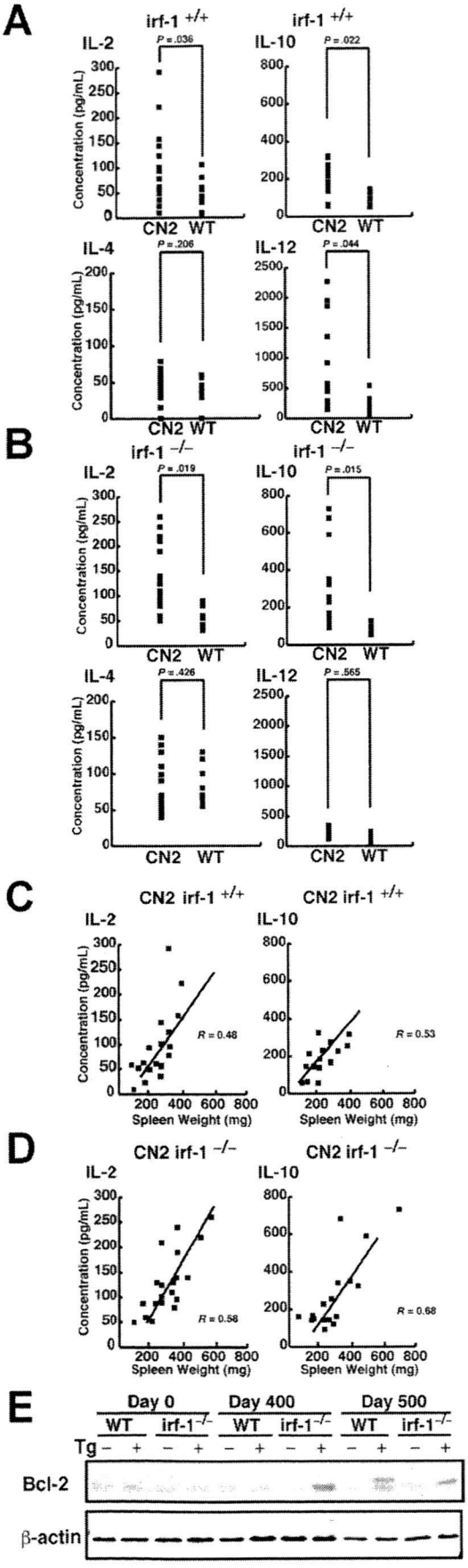


Figure 4. Stable expression of HCV viral proteins induces lymphoproliferative diseases. (*A*) Switching of the expression of HCV proteins was conducted using the Mx promoter–driven cre recombinase with poly(I:C) induction. The Mx promoter is active in hepatocytes as well as in hematopoietic cells. We 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 6C 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-

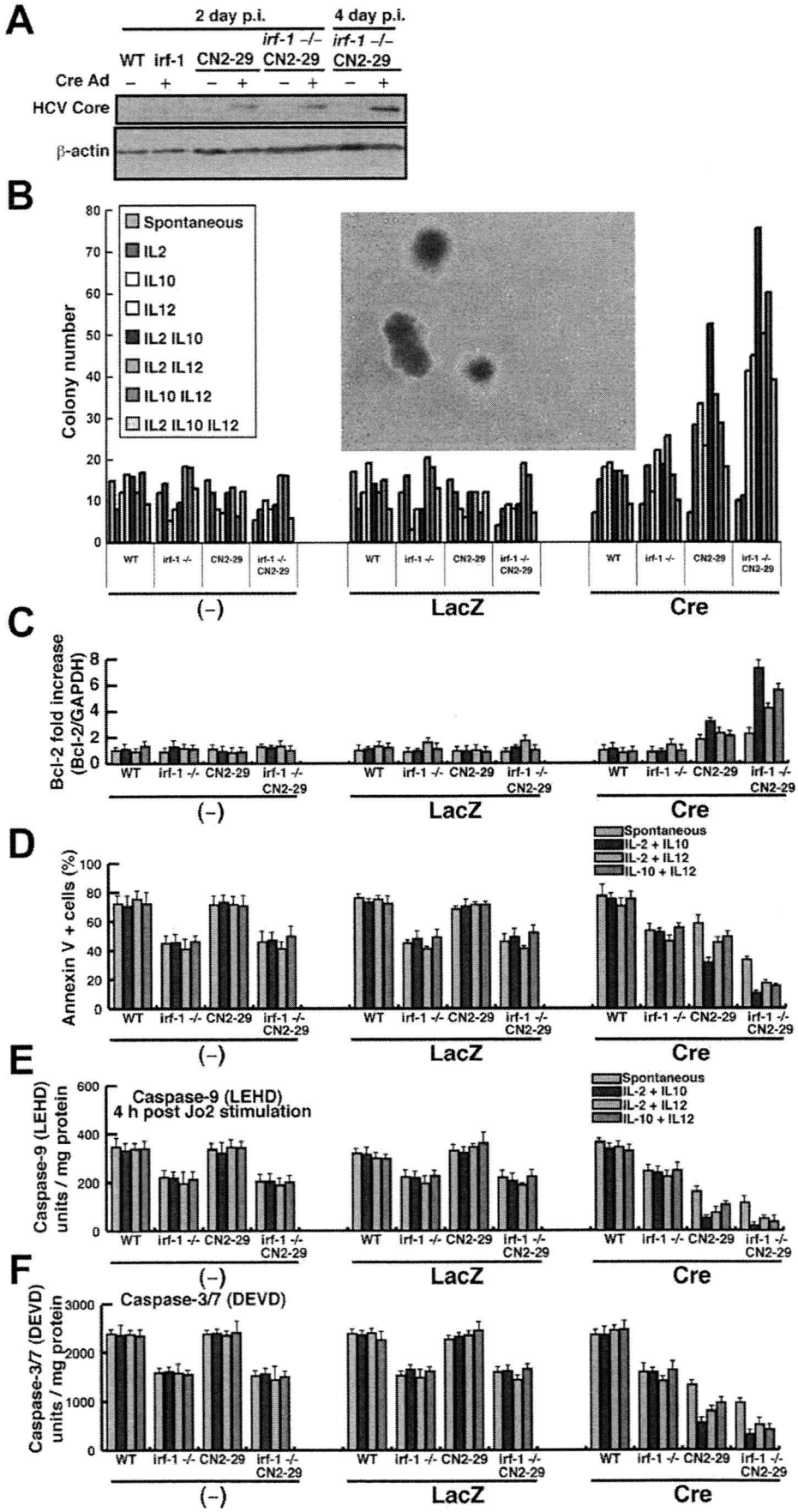


Figure 6. Lymphocyte transformation by aberrant cytokines and inhibition of apoptotic signaling. (*A*) Expression of the HCV core protein (21 kilodaltons) in *irf-1*$^{+/+}$ (WT) and *irf-1*$^{-/-}$ transgenic (CN2-29) and WT mice 2 or 4 days postinfection (p.i.) with AxCANCre (multiplicity of infection,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

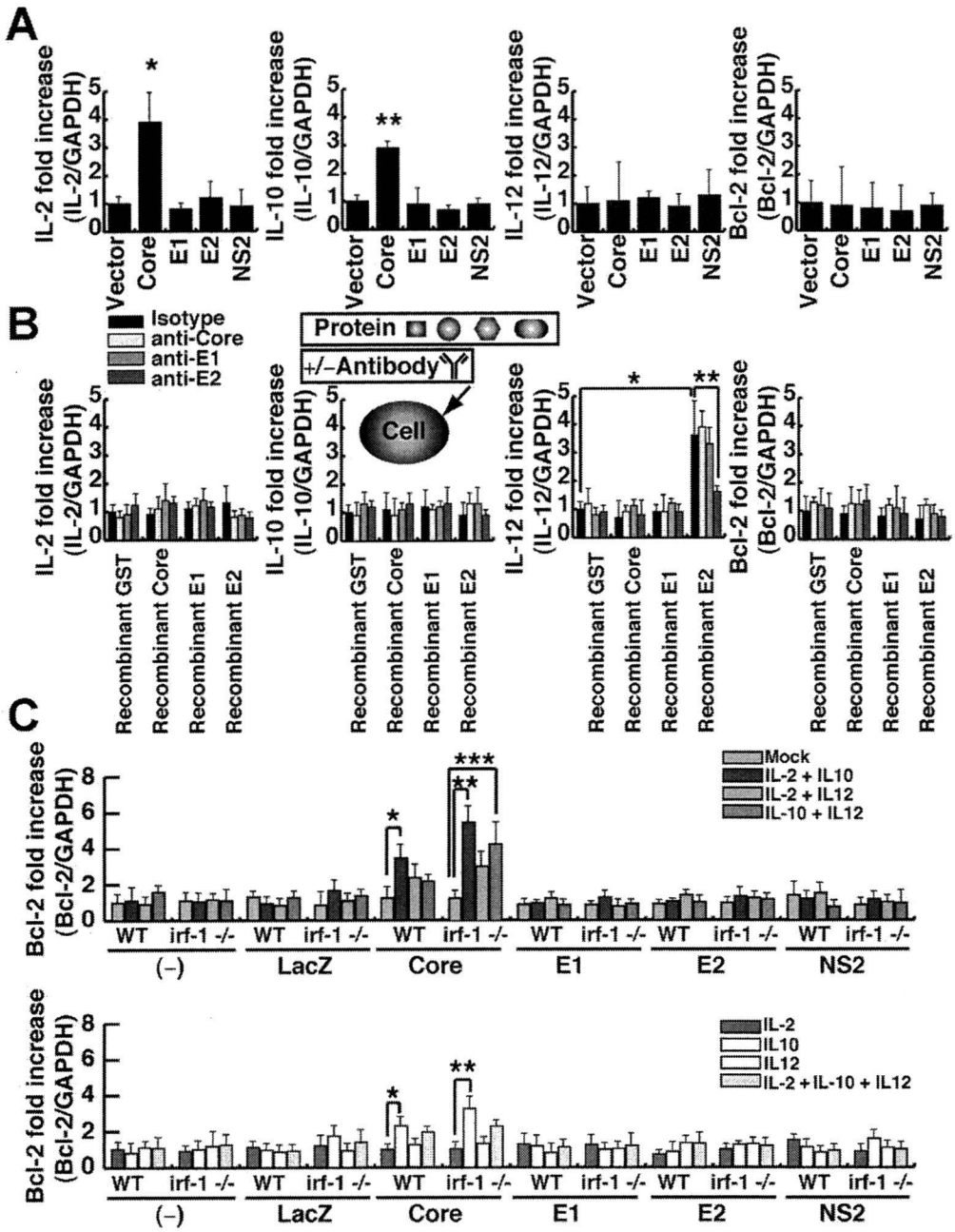


Figure 7. Induction of IL-2 and IL-10 by HCV core and IL-12 by E2 and of Bcl-2 by HCV core plus cytokines. (*A*) Individual viral proteins were stably expressed in splenocytes using recombinant lentiviruses that expressed the HCV core, E1, E2, NS2, and lacZ. Each gene expression profile was determined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

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Conflicts of interest

The authors disclose no conflicts.

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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

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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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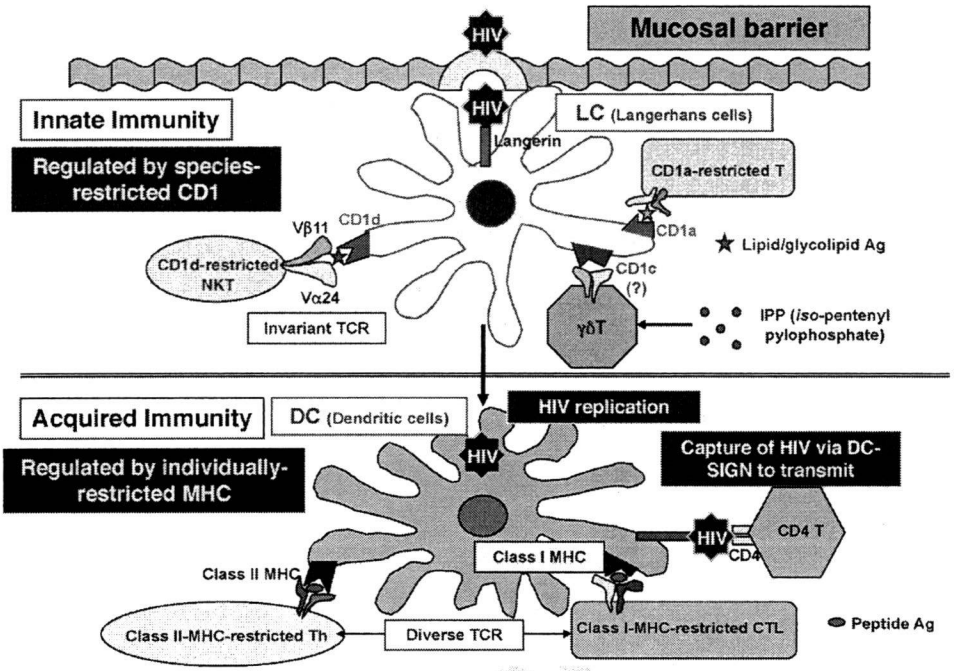

Fig. 1. Innate immunity and acquired immunity. Our internal defense system is composed of two distinct elements. One is innate immunity composed of γδT cells and NKT cells as effectors expressing fixed invariant receptors controlled mainly by species-restricted CD1 molecules on Langerhans cells and (LC) 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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