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FIGURE LEGENDS

Fig. 1. Establishment of stable macrophage cell lines expressing HCV proteins. (A) Cell lysates were prepared from macrophage cell lines expressing each of HCV proteins (4×10^6 cells) and immunoblotted with antibodies against HCV proteins or β-actin. (B) Total RNA was extracted from the macrophage cell lines expressing NS5A or control and expression of mRNA of TLRs was determined by real-time PCR. (C) The subcellular localization of NS5A was examined by confocal microscopy. Cells were fixed with 4% paraformaldehyde-PBS, permeabilized with 0.5% Triton X-100, and stained with specific antibodies. Cells expressing NS5A or control cells were extracted into cytosol (C), membrane-organelle (M) and nuclear (N) fractions. Each fraction was concentrated and subjected to immunoblotting with specific antibodies. PA28α, calregulin and histone H1 were used as markers for cytosol, membrane-organelle, and nuclear fractions, respectively.

Fig. 2. Expression of HCV nonstructural proteins modulates IL-6 production and MAPK cascades through the TLR dependent signaling pathway in macrophage cell lines. (A) Cells were seeded into 96-well plates (1×10^5 cells/well) and stimulated with the indicated amounts of mCpG, R-837, LPS or PGN. After 24 h of stimulation, IL-6 production in the culture supernatants was determined by sandwich ELISA. Data are shown as mean±SD. (B) Cells (2×10^6 cells/well) were stimulated with 10 μg/ml of R-837 for the times indicated and ERK1/2 phosphorylation was determined by immunoblotting with antibodies to ERK and phosphorylated ERK (p-ERK). Asterisks indicate non-specific bands. (C) Cells (2×10^6 cells/well) were stimulated with 10 μg/ml of mCpG, 25 ng/ml of LPS, or 10 μg/ml of PGN for the times indicated and ERK1/2 phosphorylation was determined by immunoblotting.

Fig. 3. Effect of NS5A expression on the production of cytokines and chemokines in response to the TLR ligands in macrophage cell lines. Cells (3×10^6 cells/well) were stimulated with 10 μg/ml of mCpG, 10 μg/ml of R-837, 25 ng/ml of LPS, and 10 μg/ml of PGN for the times indicated. Total RNA was extracted from the macrophage cell lines expressing NS5A (gray bars) or control (white bars) and the expression of mRNA of IL-1α and Ccl2 (A), and IFN-β and IL-6 (B) were determined by real-time PCR.

Fig. 4. TLR-dependent and -independent immune activation of the macrophage cells expressing NS3/4A or NS5A protein by RNA virus and dsRNA (A) Myc-His-mTRIF was co-expressed with Flag-NS3, -NS3/4A, or -NS3/4A(S139A) in 293T cells and immunoblotted with antibodies against His and Flag. (B) Alignment of the franking sequence of NS3 protease cleavage sites of NS4A/4B, NS4B/5A, TRIF and IPS-I of human and murine origins and cleavage site was indicated by arrow. (C) Flag-mIPS-I and a mutant replaced Cys^{508} to Ala (C508A) were co-expressed with Flag-NS3, -NS3/4A, or -NS3/4A(S139A) in 293T cells and immunoblotted with antibodies against mIPS-I and NS3. (D) Processing of the endogenous mIPS-I. Cell lysates of the macrophage cell lines expressing NS3, NS3/4A, NS3/4A(S139A) were immunoblotted with antibodies against mIPS-I, NS3 and β-actin. The cleavage product of mIPS-I was indicated at mIPS-I*. (E) Cells (3×10^6 cells/well) were stimulated with 2×10^5 pfu/ml of VSV or 50 μg/ml of poly:I-C for the times indicated. Total RNA was extracted from the macrophage cell lines expressing NS3/4A (black bars), NS5A (gray bars) or control (white bars) and the expression of mRNA of IFN-β, IL-1α, IFN-α1, and IFN-α4 were determined by real-time PCR.

Fig. 5. NS5A interacts with MyD88. (A) MyD88-His was co-expressed with Flag-Core/E1/E2 or -NS5A, or (B) Flag-NS3, -NS3/4A, -NS4B, -NS5A or -NS5B in 293T cells, immunoprecipitated with anti-Flag, E1, or E2 antibody and immunoblotted with anti-His antibody.

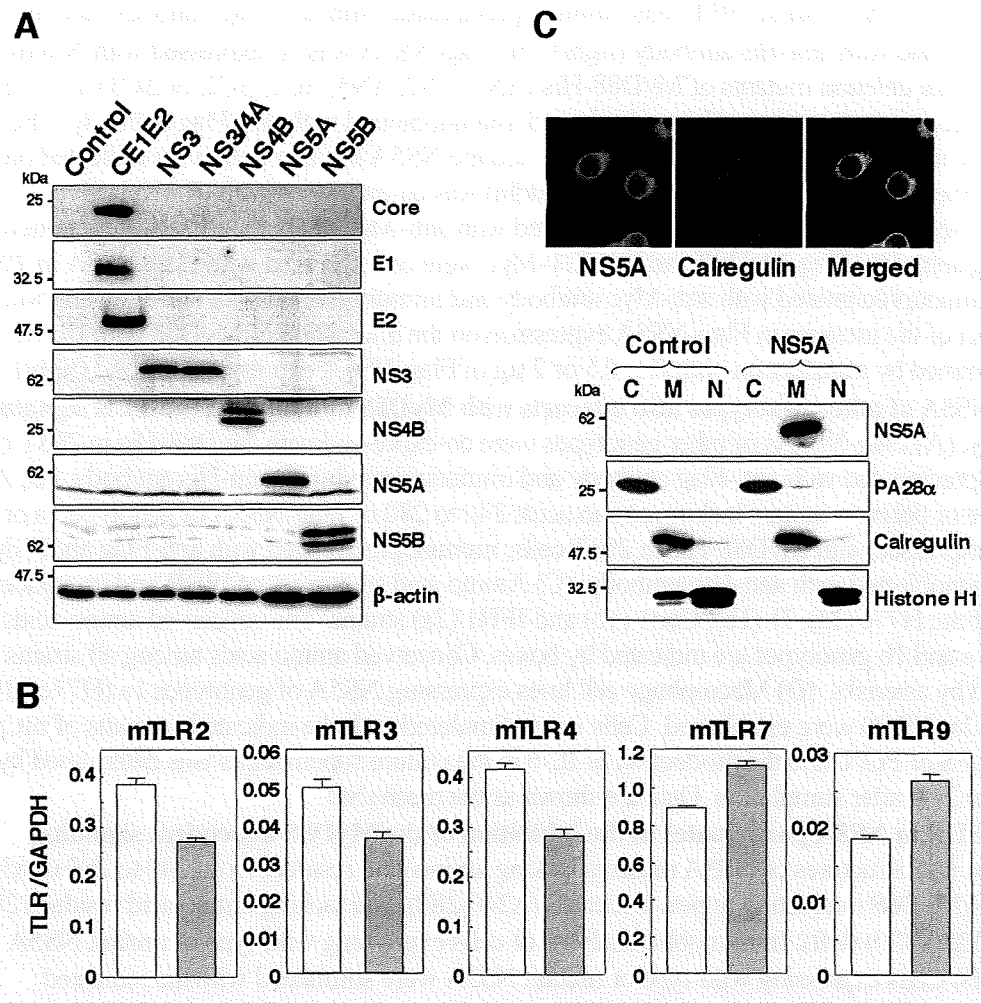
(C) Flag-NS5A was co-expressed with MyD88-His, TRAM-His, TIRAP-His or TRIF-HA in 293T cells, and immunoprecipitated with anti-His or -HA antibody. The immunoprecipitates were immunoblotted with anti-Flag antibody. Asterisks indicate non-specific bands.

Fig. 6. NS5A interacts with the death-domain of MyD88 through the ISDR and inhibits recruitment of IRAK to MyD88. (A) The structure of NS5A and the MyD88 binding region are indicated on the top. MyD88-His was co-expressed with C-terminal deletion mutants of Flag-NS5A in 293T cells, immunoprecipitated with anti-His antibody, and immunoblotted with anti-Flag antibody (left). MyD88-His was co-expressed with Flag-NS5A deletion mutants (Δ240-280 or Δ280-300) in 293T cells, immunoprecipitated with anti-Flag antibody, and then immunoblotted with anti-His antibody (right). (B) Flag-NS5A was co-expressed with N-terminal or C-terminal deletion mutants of MyD88-His (ΔN1, ΔN2, ΔN3, ΔC1, ΔC2, or ΔC3) in 293T cells, immunoprecipitated with anti-His antibody and immunoblotted with anti-Flag antibody. The structures of MyD88 and the deletion mutants, and the NS5A binding region are indicated on the left. (C) Flag-MyD88 (left) or Flag-NS5A (middle) was co-expressed with IRAK-1-Myc or IRAK-4-Myc in 293T cells, immunoprecipitated with anti-Myc antibody and immunoblotted with anti-Flag antibody. Flag-MyD88 and IRAK-1-Myc were co-expressed with Flag-NS5A in 293T cells, immunoprecipitated with anti-Myc antibody and immunoblotted with anti-Flag antibody. The effect of the increase in Flag-NS5A expression on the interaction of MyD88 with IRAK-1 was examined by transfection with 0.1, 0.5 or 2 μg of Flag-NS5A expression plasmid (right).

Fig. 7. NS5A of other genotypes also interacts with MyD88 and inhibits the TLR signaling pathway. (A) Flag-NS5As of other genotypes were co-expressed with MyD88-His in 293T cells, immunoprecipitated with anti-Flag antibody and immunoblotted with anti-His antibody. (B) A wild-type or deletion mutant lacking amino acids 240 to 280 of Flag-NS5A of genotype 1a or 2a was co-expressed with MyD88-His in 293T cells, immunoprecipitated with anti-Flag antibody and immunoblotted with anti-His antibody. (C) Amino acid sequences of ISDR and its adjacent region of the H77c (1a), J1 (1b), Con1 (1b) and JFH1 (2a) strains. The conserved amino acids among 1a and 1b genotypes are indicated by boxes. Conserved amino acids among all strains are indicated by asterisks. (D) Macrophage cell lines expressing NS5A of genotypes 1a (H77c), 1b (J1) and 2a (JFH1) were established. Cells were stimulated with the indicated amounts of mCpG, R-837, LPS or PGN, and the production of IL-6 in the culture supernatants was determined by ELISA at 24 h after stimulation. Data are shown as the mean±SD.

Fig. 8. ISDR in NS5A participates in the inhibition of the MyD88-dependent signaling pathway. (A) Structures of NS5A mutants lacking amino acid residues from 240 to 280 in which the ISDR/MyD88 interacting region is located (Δ240-280), and lacking amino acid residues 280 to 300 (Δ280-300) (left). Immunoblot analyses of cells expressing wild-type or mutant NS5A (right). (B) Cells expressing wild-type or mutant NS5A were stimulated with the indicated amounts of mCpG, R-837, LPS or PGN, and the production of IL-6 in the culture supernatants was determined by ELISA at 24 h after stimulation. Data are shown as the mean±SD. (C) Phosphorylation of STAT1 or PKR in response to treatment with murine IFN-α or infection with VSV. The cell lines were stimulated with two doses of murine IFN-α (2×10^3 and 2×10^2 units/ml) or VSV (2×10^7 and 2×10^6 pfu/ml). After 24 h of stimulation, cell extracts were immunoblotted with specific antibodies. Phosphorylated STAT1 and PKR and the total amounts of STAT1 and β-actin were determined. Asterisk indicates non-specific bands.

Figure 1 Abe T. *et al.*



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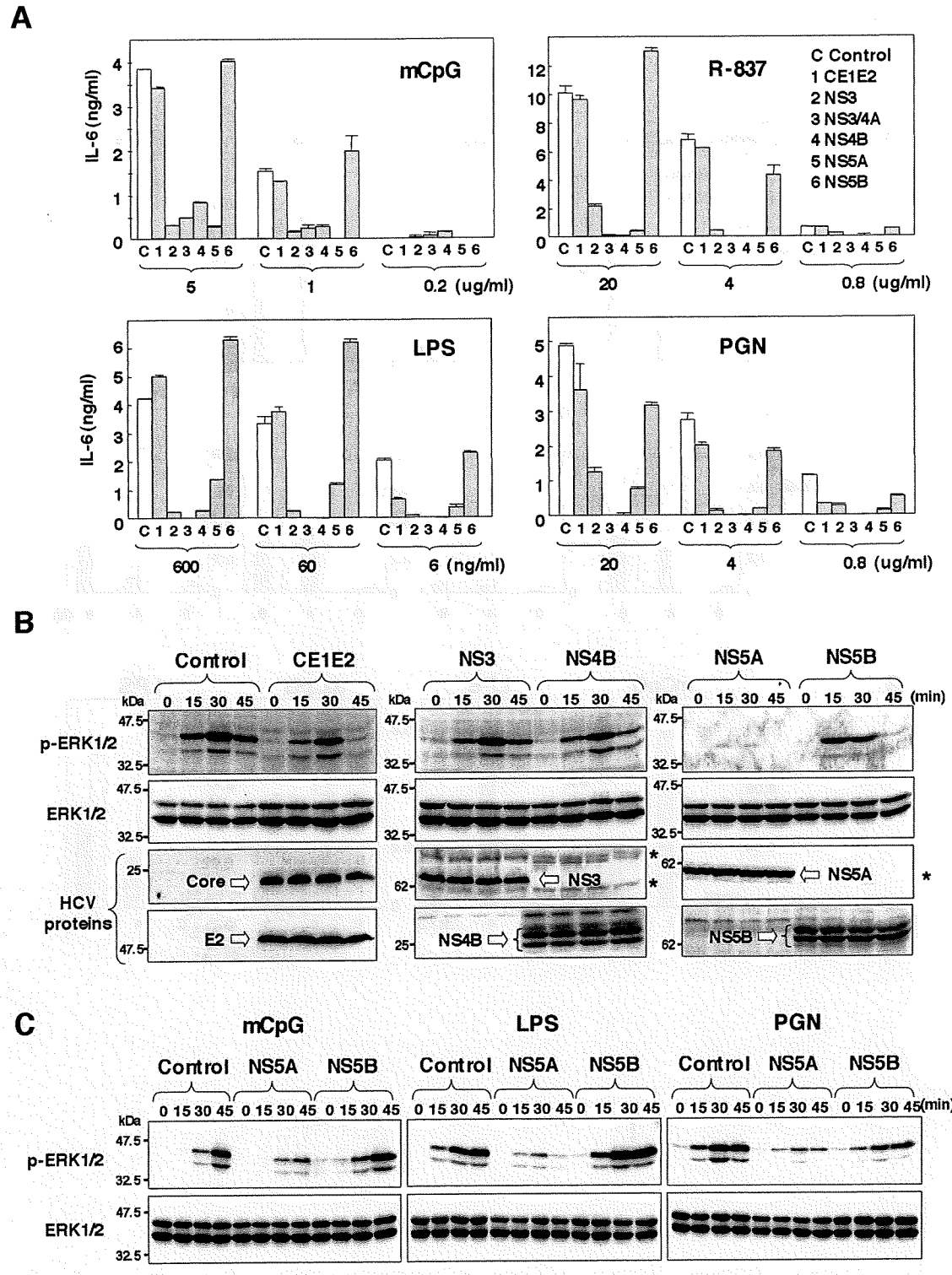

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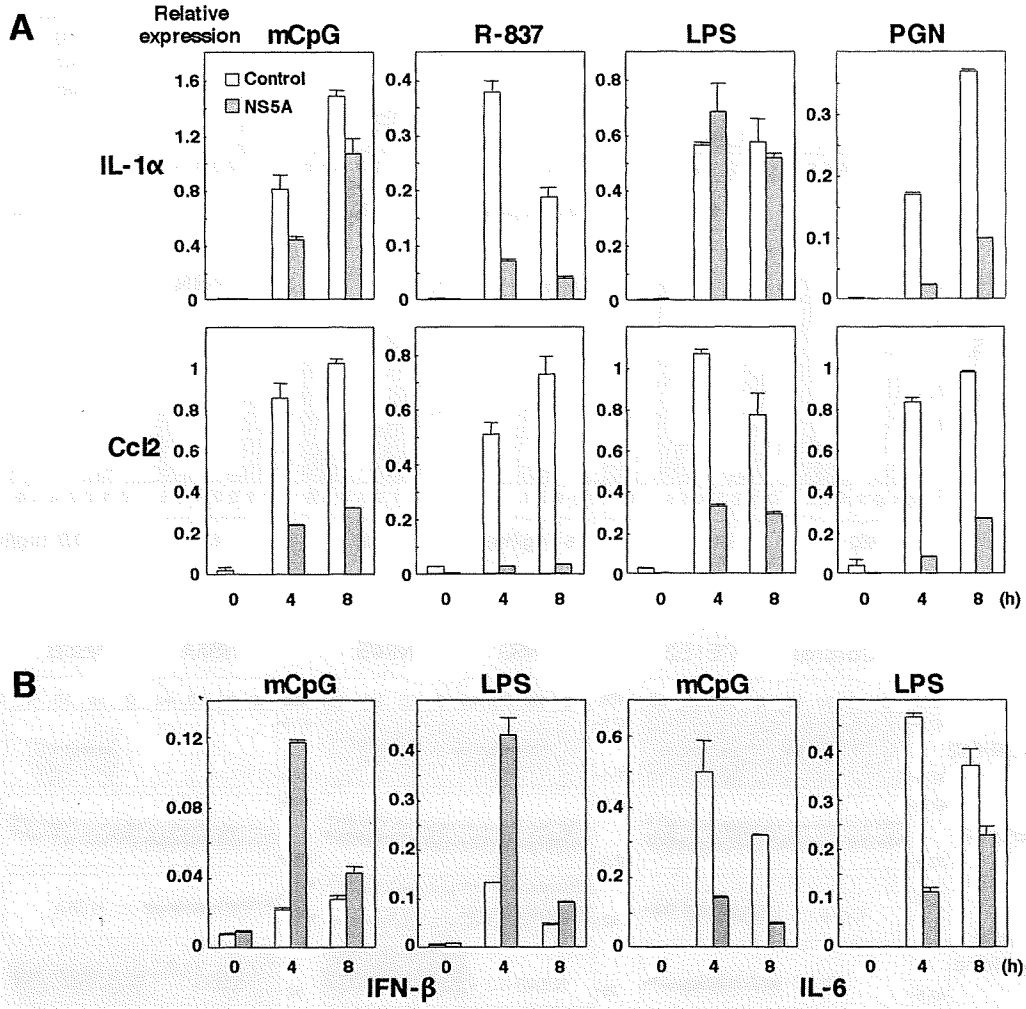

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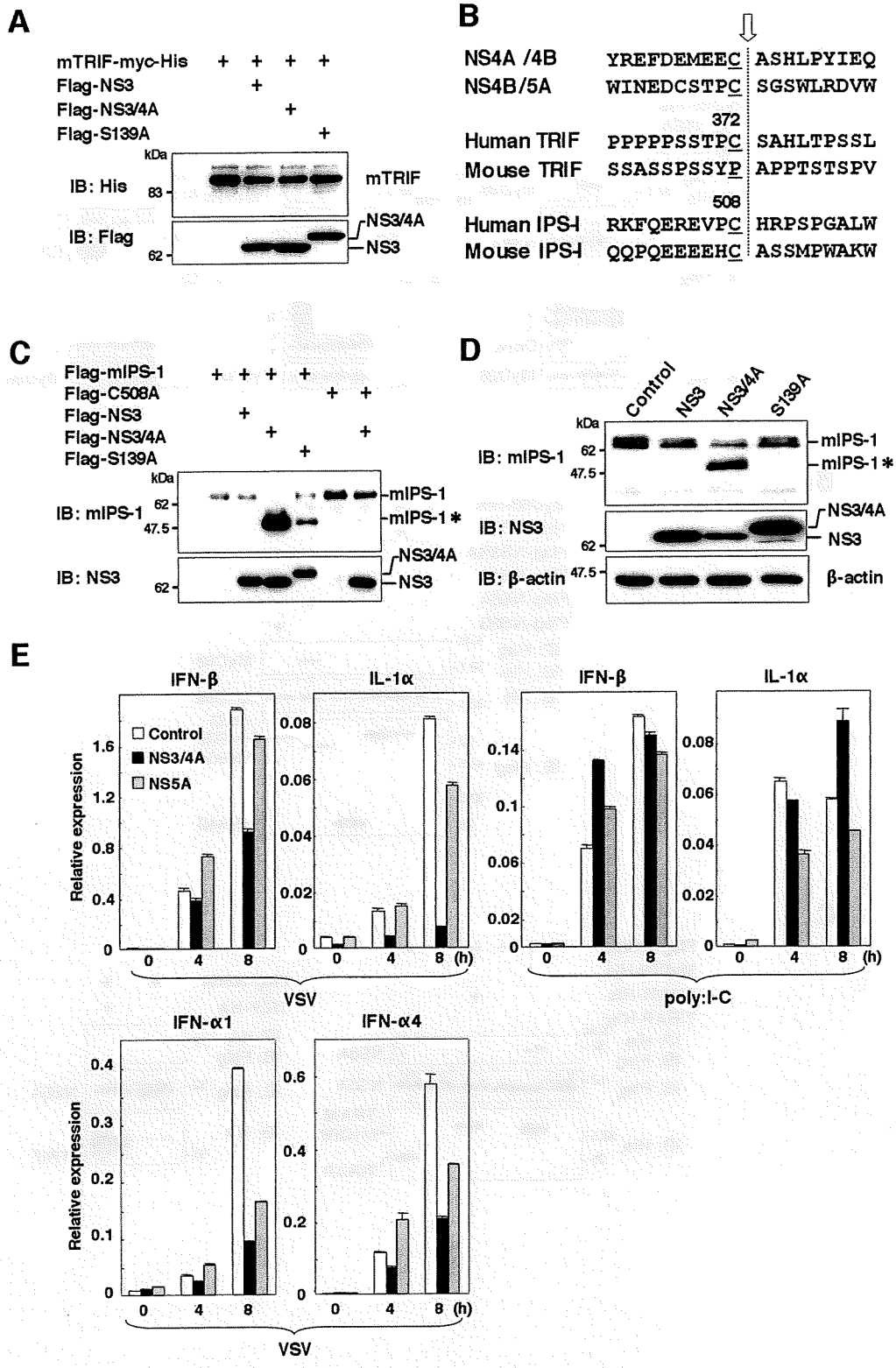

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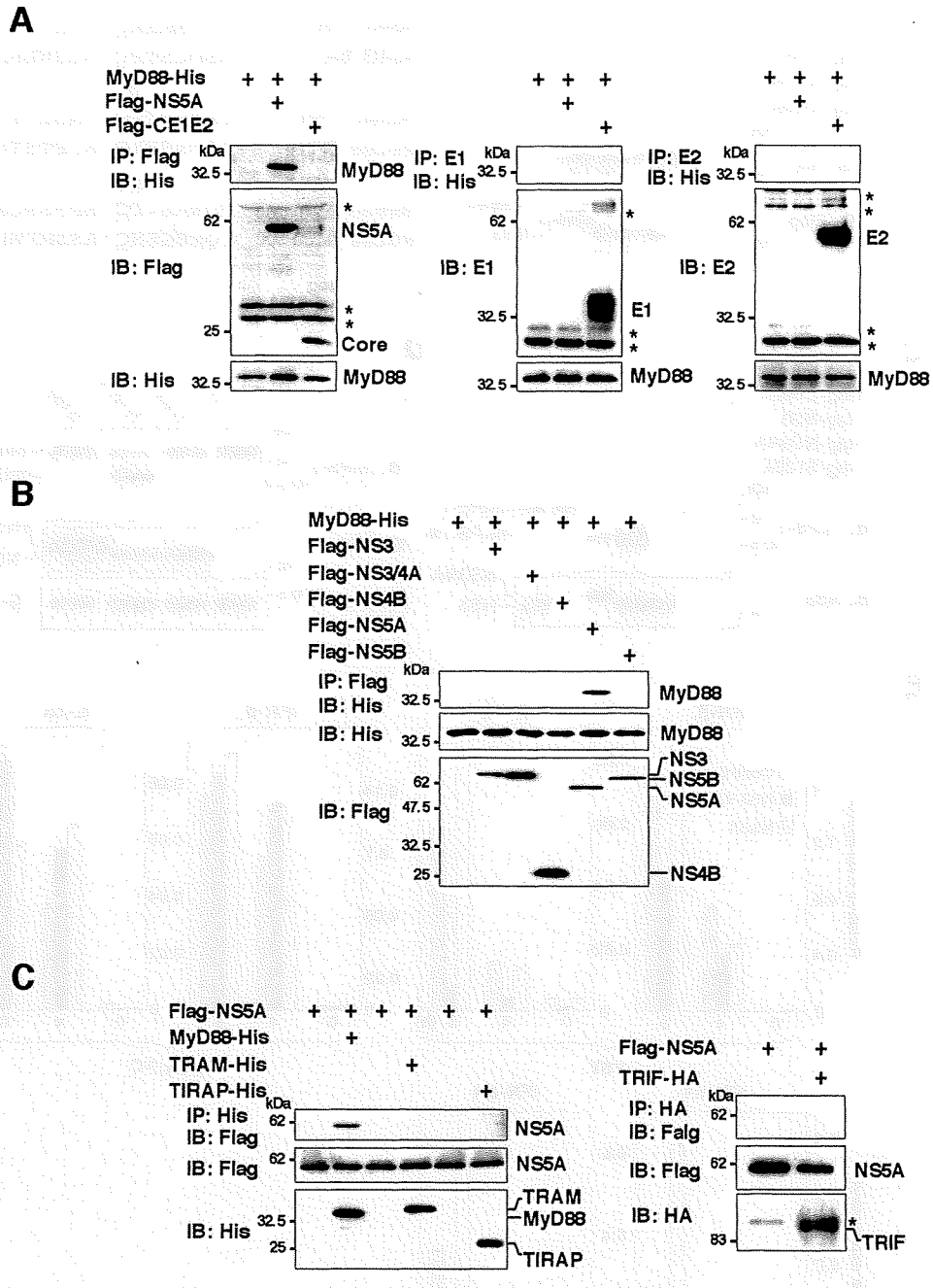

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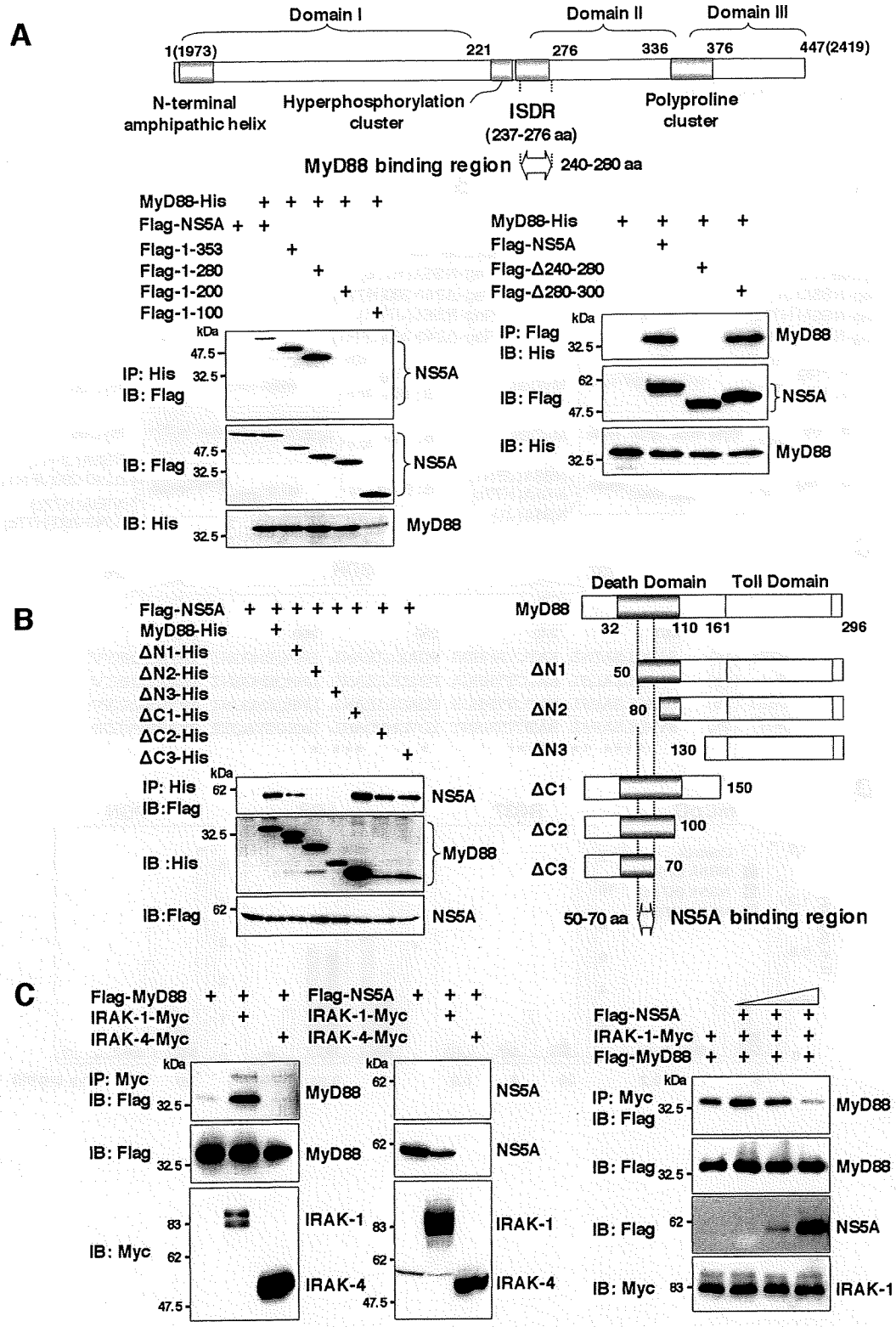

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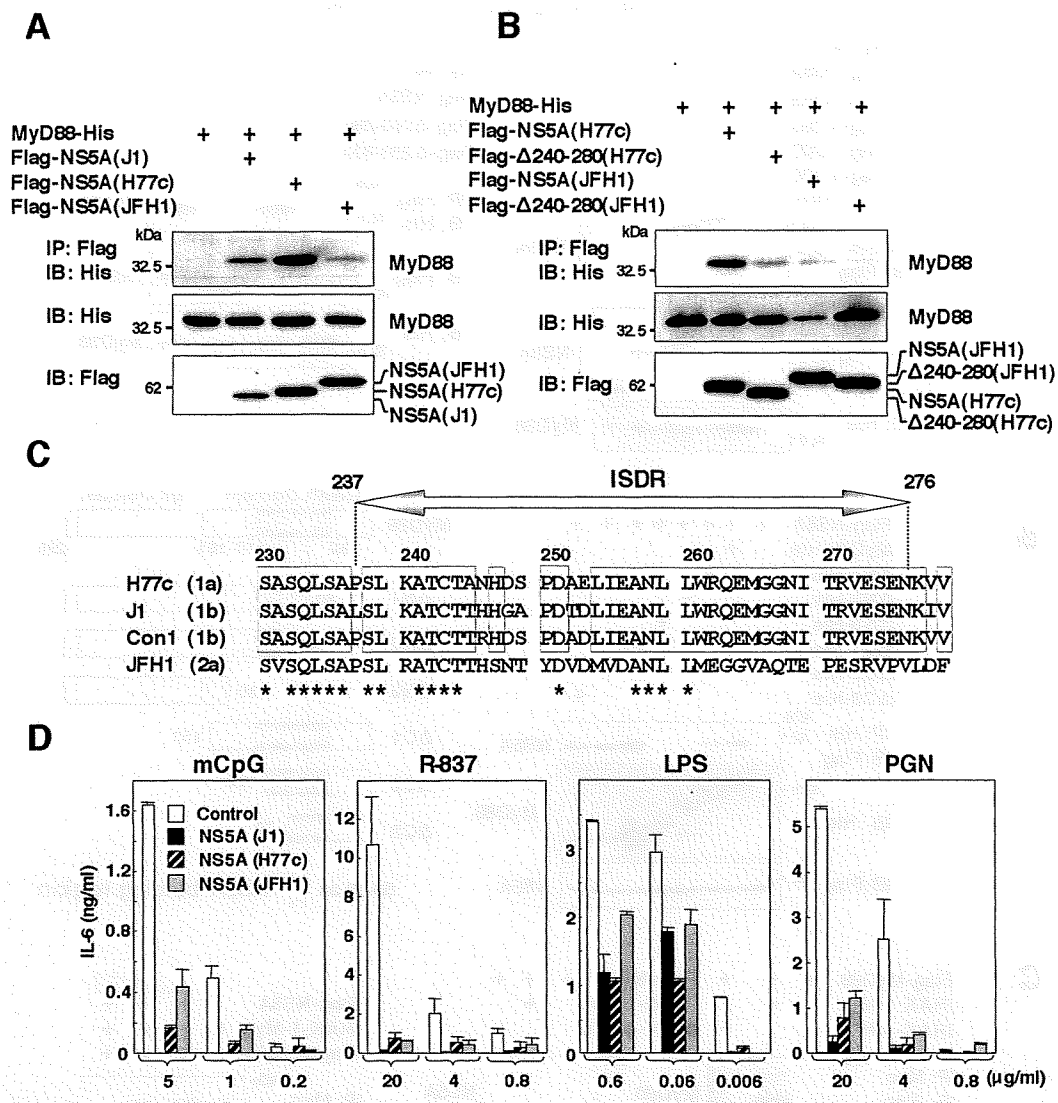

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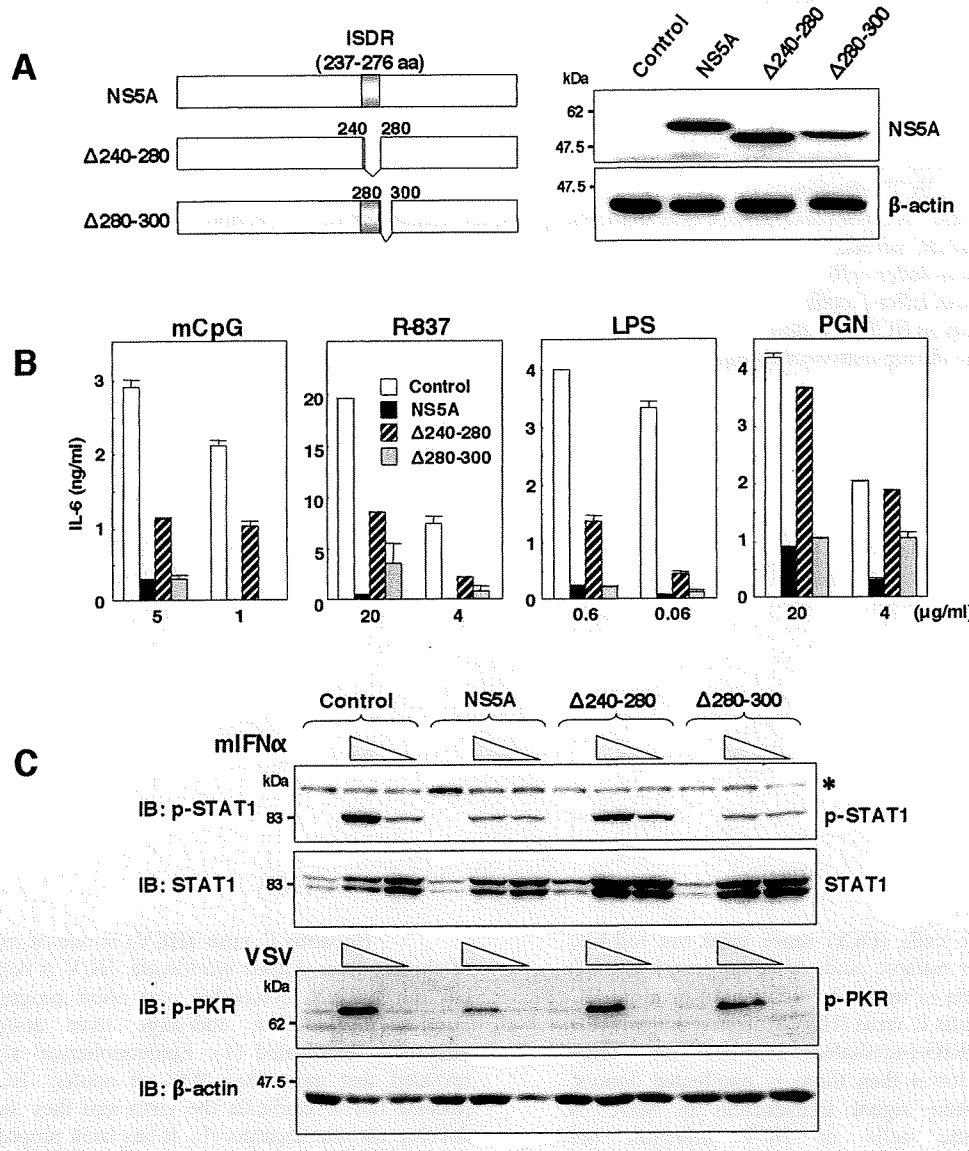

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Virus associated innate immunity in liver

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1. ABSTRACT

Dendritic cells (DCs) sense virus via toll-like receptors (TLR) or retinoic acid inducible gene-I (RIG-I) and evoke a cascade of immune reactions. In myeloid DC (MDC) from hepatitis C virus (HCV)-infected patients, the levels of TLR/RIG-I-mediated IFN-beta or TNF-alfa induction are lower than those in uninfected donors, suggesting that their signal transduction in MDC is impaired. Dendritic cells in HCV infection are unresponsive to interferon (IFN)-alfa, thus failing to enhance MHC class-I related chain A/B and subsequent NK cell activation. Alternatively, NK cells from the patients down-regulate DC in the presence of HLA-E-expressing hepatocytes by secreting IL-10 and TGF-beta1. Such functional alteration of NK cells in HCV infection is ascribed to the enhanced expression of NKG2A/CD94. Activated NKT cells from the patients produce higher levels of IL-13 but comparable IFN-gamma with those from controls, showing their bias to Th2-type. In pegylated IFN-alfa/ribavirin therapy for chronic hepatitis C, improved DC function is related with successful HCV eradication. In conclusion, cross-talks among DCs and innate lymphocytes are critical in shaping immune response against HCV, either spontaneously or therapeutically.

2. INTRODUCTION

Hepatitis C virus (HCV) is one of major causes of chronic liver disease worldwide. HCV is hepatotrophic, but not directly cytopathic and elicit progressive liver injuries resulting in end-stage liver disease unless effectively eradicated (1). Epidemiological studies have revealed that more than 80% of acutely HCV-infected patients fail to eradicate the virus and they subsequently develop chronic hepatitis (1). It has been proposed that the ability of infected hosts to mount vigorous and sustained cellular immune reactions to HCV is necessary for control in primary infection (2). Once HCV survives the initial interaction with the host immune system, it uses several means to nullify the selective immunological pressure during the later phases of infection. First, the virus alters its antigenic epitopes recognized by T cells and neutralizing antibodies to escape immune surveillance. Second, HCV also subverts immune functions in an antigen-specific manner, from innate to adaptive immunity (3).

Cumulative reports have shown that innate immune system dictates the direction and magnitude of subsequent adaptive immune response. It is generally accepted that HCV-specific $CD8^+$ T cells are responsible

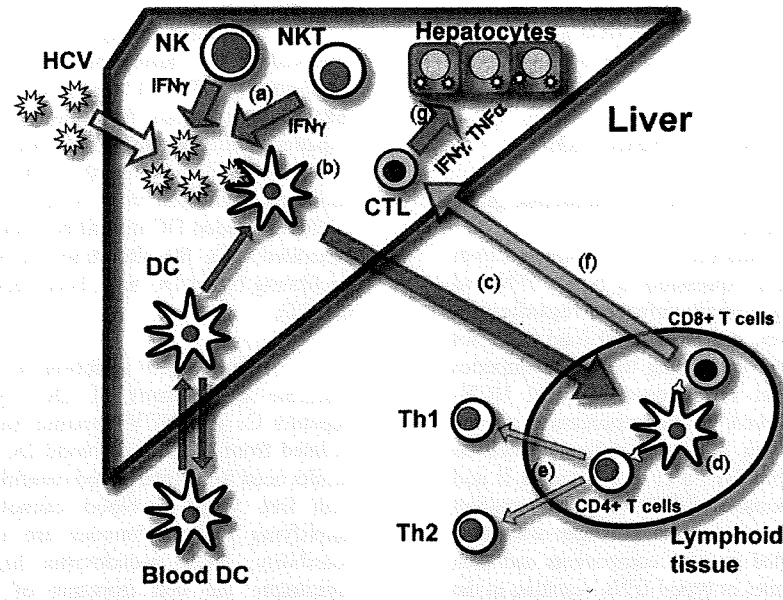

Figure 1. Key players in immune reactions in viral hepatitis. CTL, cytotoxic T lymphocyte; DC, dendritic cell; HCV, hepatitis C virus; NK, natural killer cell; Th, helper T cell. (a)- (g), see text.

for HCV elimination by inducing hepatocyte apoptosis (2). Innate immune cells, including NK cells and NKT cells, may contribute to HCV eradication after primary infection; however, their roles in chronically-infected state remain elusive. Since dendritic cells (DCs) orchestrate anti-HCV immune response by linking innate and adaptive arms of immune system (4), functional impairment of DC leads to failure of NK cells, NKT cells, $CD4^+$ and $CD8^+$ T cells. Infiltration of disabled $CD8^+$ T cells to the infected liver may result in weak liver inflammation that is not sufficient for HCV eradication (5).

In this paper, we discuss the current understandings of the roles of innate immunity in the pathogenesis of HCV infection as well as efficacy of anti-HCV therapy, especially focused on interferons (IFN), DCs, NK cells and NKT cells.

3. KEY PLAYERS IN IMMUNE RESPONSES TO VIRAL HEPATITIS

After HCV infects the liver, viral replication continues and viral particles are continuously released into the circulation. The first lines of defense are provided by NK and NKT cells, of which populations are relatively increased in the liver compared to the periphery These cells are activated in the liver, where expression of IFN-alfa and IFN-inducible genes are extremely high during the early phase of hepatitis virus infection (6). Activated NK and NKT cells secrete IFN-gamma, which inhibits replication of HCV through a non-cytolytic mechanism (Figure 1-a) (7).

Dendritic cells (DCs) or resident macrophages in the liver are capable of taking up viral antigens, and processing and presenting them to other immune cells (Figure 1-b) (4). Since DCs express distinct sets of toll-like receptors (TLRs) (8), it is likely that some viral components stimulate DCs through cytosolic ligation of TLRs. DCs develop a mature phenotype and migrate to lymphoid tissues (Figure 1-c), where they stimulate effectors, including T cells and B cells (Figure 1-d). Following the encounter of DCs with other cells, DCs secrete various cytokines (IL-12, TNF-alfa, IFN-alfa and IL-10) instructing or regulating the functions of the adjacent cells (4). In addition to these cytokines, DCs express various co-stimulatory molecules and ligands to enhance or limit the functions of immune and infected cells. The existence of functionally and ontogenetically distinct DC subsets has been reported: i.e., myeloid DC (MDC) and plasmacytoid DC (PDC) (9). MDC predominantly produce IL-12 or TNF-alfa following pro-inflammatory stimuli, while PDC release a considerable amount of IFN-alfa upon virus infection depending on the immune stimulus; both cytokines in actuality can be made by both cells. Helper T cells have an immunoregulatory function mediated by the secretion of cytokines that support either cytotoxic T lymphocyte (CTLs) generation (Th1 with secretion of IL-2, IFN-gamma and TNF-alfa) or B cell function and antibody production (Th2 with secretion of IL-4, IL-5, IL-10 and IL-13) (Figure 1-e). DC ontogeny and DC-derived cytokines are crucially associated with the polarization of helper T cell subsets.

It is generally accepted that adaptive immunity performs a critical role during the clinical courses of hepatitis. The involvement of antigen-specific $CD4^+$ T cells in HCV eradication has been well described during both acute or chronic infection (10). However, there is little evidence that $CD4^+$ T cells mediate direct liver cell injury in HCV infection. Thus, it is likely that $CD4^+$ T cells play a critical role in facilitating other antiviral immune

mechanisms, such as enhancing $CD8^+$ effector function. The antigen-primed CTLs recruit to the liver (Figure 1-f) and constitute the critical element in the eradication of virus-infected cells (Figure 1-g).

4. INNATE IMMUNITY IN HCV INFECTION

4.1. Toll-like receptors and retinoic acid inducible gene-I as sensors for virus infection

Gene expression analyses in HCV-infected liver revealed that HCV triggers expression of type I IFN and IFN-induced genes during primary infection regardless of the outcomes (6). However, the HCV viral load does not decrease in the early phase, suggesting that HCV impedes the execution of anti-viral machineries. Several HCV-derived proteins are involved in the suppression on the signaling pathways inducing anti-viral proteins, such as interferon regulatory factor (IRF)-3 (11), NF-kappa B and RNA-dependent protein kinases (PKR) (12). Mammalian toll-like receptors (TLRs) sense some pathogen-associated molecular patterns embedded in virus components and then induce inflammatory cytokines or type-I IFNs, resulting in the augmentation of anti-virus immune reactions (8). Retinoic acid inducible gene-I (RIG-I) is a cytosolic molecule that senses dsRNA of virus replicative intermediate, which subsequently activates IRF-3 and NF-kappa B pathways (13). By using HCV subgenomic replicon system, it has been demonstrated that HCV NS3/4A protein influences on the functions of adaptor molecules mediating TLR-dependent and RIG-I-dependent pathways, resulting in an impairment of the induction of IFN-beta as well as subsequent interferon-stimulated genes (14, 15). However, it is yet to be proven whether the results obtained from HCV replicon are applicable or not for HCV-infected individuals.

Large-scale cohort study on US veterans revealed that the prevalence of various infectious diseases, including virus, bacteria and parasites, in HCV-infected individuals is significantly higher than those in uninfected controls (16). These observations suggest that first-line defense against pathogens, of which system is initiated by TLR/RIG-I stimulation, is functionally impaired in HCV infection. To investigate the roles of TLR/RIG-I in HCV infection, we compared their expressions and the functions in MDC and PDC between the patients and donors. In MDC from HCV-infected patients, TLR2, TLR4 and RIG-I expression were significantly higher than those in healthy counterparts. Of particular interest, regardless of the higher expressions, specific agonists for these sensors stimulated patients MDC to induce lesser amount of IFN-beta and TNF-alfa compared to donor MDC (unpublished data). These results show that the signal transduction via these receptors is strongly impeded in HCV infection. Inconsistent with the findings of MDC, we previously reported that TLR2 expression on monocyte-derived DCs (MoDCs) in chronic hepatitis C is lower than those in healthy donors (17). Since MoDC is an in vitro-generated DC mimic, the opposite results of TLR2 in HCV infection might be explained by impaired ability of MoDC to mature in response to cytokines, as reported elsewhere (18). Further investigation is needed to clarify which TLR or RIG-I is predominantly utilized by HCV to evoke immune reactions.

4.2. Blood DC subsets

Impaired antigen presentation by DC might be involved in the failure of the maintenance of sustained HCV-specific T cell response. Monocyte-derived DCs (MoDCs) generated from hepatitis C patients have an impaired ability to stimulate allogeneic $CD4^+$ T cells (19, 20). Functional impairment of DC diminished when HCV had been eradicated from patients, revealing the evidence of HCV-induced DC disability (19). In addition to in vitro-generated DCs, the alterations in number and function of circulating blood DC have been reported in HCV infection (21, 22).

Direct HCV infection of DCs might be one of the plausible mechanisms of DC dysfunction in chronic hepatitis C. The HCV genome has been reported to be isolated from MoDCs or blood DCs (19). However, these results need to be interpreted carefully, since contamination with free virus in blood cannot be ruled out when amplifying PCR techniques are used. To exclude this possibility, HCV pseudovirus has been developed to investigate the cell tropisms of HCV as well as to determine putative HCV entry receptors to cells. By using this, MDC, but not PDC, displayed susceptibility to HCV pseudovirus possessing chimeric HCV E1/E2 proteins (23).

Several criticisms have been raised recently about DC dysfunction in the setting of chronic HCV infection (24), failing to demonstrate any DC defects which may have to do with differences in the populations studied. Cohort studies on chimpanzees following HCV infection showed that functional impairment of DCs was observed in some cases but was not a prerequisite of persistent infection (25). Further study needs to be done to clarify whether DCs are indeed disabled in the setting of human chronic hepatitis C and furthermore whether this contributes to the development of HCV persistence or it is simply a consequence of active HCV infection.

4.3. Natural killer cells

Natural killer cells express various functional receptors; the one group that transduces inhibitory signals (Killer Inhibitory Receptors/KIRs, CD94, NKG2A) and the other does activating signals (NKG2D). The function of NK cells is dynamically regulated in vivo by the balance between expressions of counteracting receptors and their association with relevant ligands (26). First, we compared the expressions of NK cell receptor between HCV-infected patients and healthy donors. As for inhibitory receptors, KIR expressions are not different between the groups; however, CD94 and NKG2A expressions are higher in patients than controls (27). In contrast, activating receptor NKG2D expression is comparable between the groups (Figure 2). It is yet to be determined how the expression of NK cell receptor is regulated. In our hands, HCV pseudovirus did not enter purified NK cells, suggesting that NK cells are not susceptible to direct HCV infection (unpublished data). Thus, some soluble factors and/or direct binding of HCV particles to NK cells might be the cause of NK receptor dysregulation.

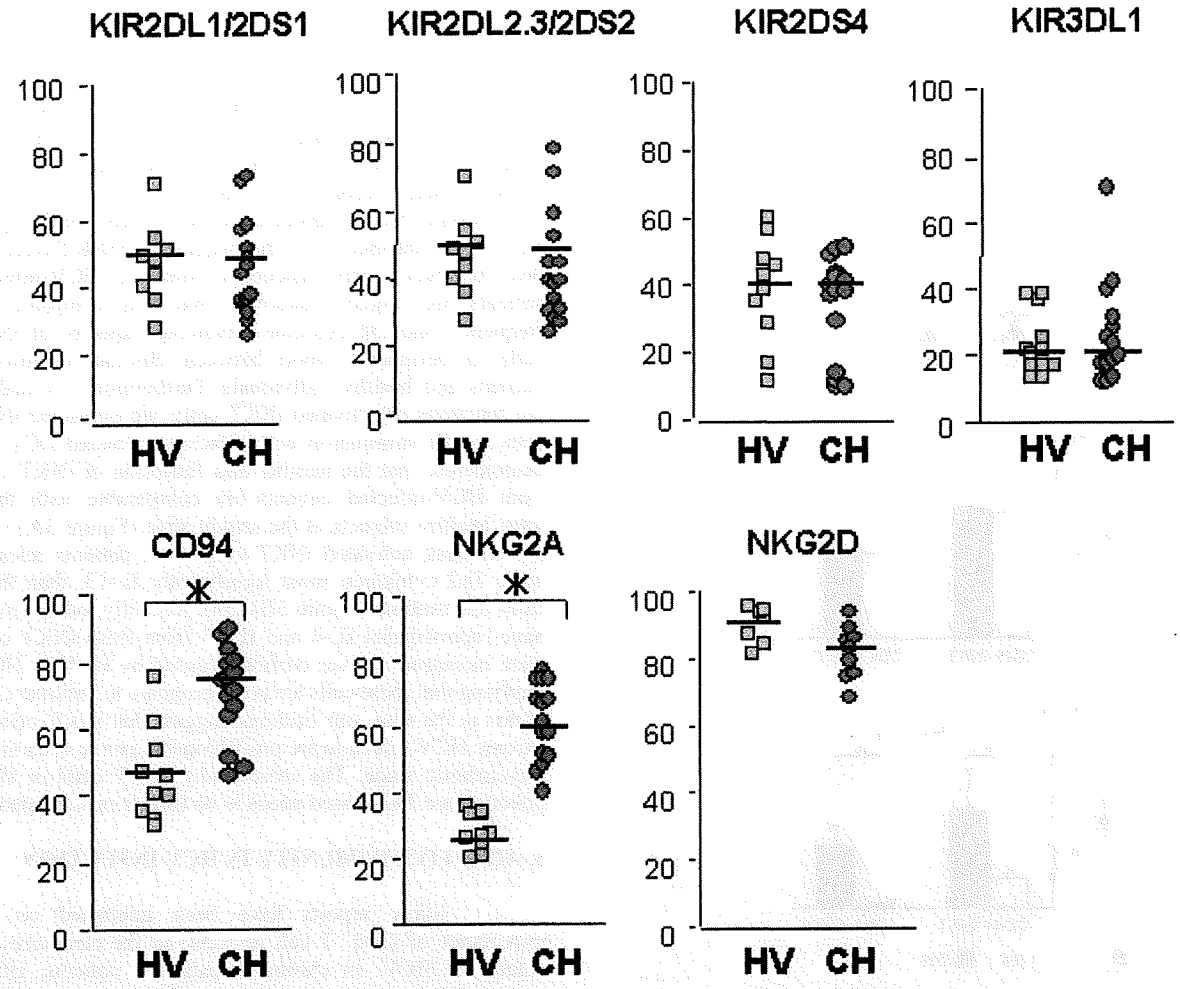

Figure 2. The expressions of NK receptors on NK cells from chronic hepatitis C patients and healthy subjects. The percentages of NK cell that express various NK receptors were determined by flow cytometry. HV, healthy volunteers; CH, chronic hepatitis C patients. Horizontal bars represent the median. *$P < 0.05$ by Mann-Whitney U test.

DCs play a decisive role in shaping innate immunity by interacting with NK cells. DCs have two means to stimulate NK cells via the production of cytokines (IL-12, IL-18 or IFN-alfa) and through the expression of NK-activating ligands. In response to IFN-alfa, DCs are able to express MHC class-I related chain A/B (MICA/B) and activate NK cells following ligation of the NK receptor, NKG2D (28). Interestingly, DCs from HCV-infected patients are unresponsive to exogenous IFN-alfa to enhance MICA/B expression and fail to activate NK cells (28). It is tempting to speculate that the impairment of DCs in NK cell activation is responsible for the failure of HCV control in the early phase of primary HCV infection, where HCV continues to replicate in spite of high-level IFN-alfa expression in the liver. Alternatively, NK cells from HCV-infected patients down-regulate DC functions in the presence of hepatocytes by secreting suppressive cytokines, IL-10 and TGF-beta1 (27). Such functional alteration of NK cells in HCV infection was ascribed to the enhanced expression of inhibitory receptor NKG2A/CD94 compared to the healthy counterparts (27). Further study is necessary to determine if the NK-mediated DC suppression is instrumental or not in acute HCV infection.

4.4. Natural killer T cells

Natural killer T (NKT) cells are a unique lymphocyte subset co-expressing T-cell receptor (TCR) and NK cell markers (29). The NKT cell population is highly heterogeneous according to the differences in types and tissue distribution; invariant (or classical) NKT (iNKT) cells express an invariant TCR, composed of Valfa24-JalfaQ preferentially paired with Vbeta11 in humans (29), whereas non-invariant NKT cells express diverse TCR. Invariant NKT cells recognize glycolipid antigens presented on CD1d expressed by DCs (29). Although endogenous ligands of iNKT cells are little known, alfa-galactosyl-ceramide (alfaGalCer) has been used as a surrogate for natural ligands. In contrast, non-invariant NKT cells are activated by CD1d-dependent manner but are not reactive to alfaGalCer. Baron *et al.* reported that, in hepatitis B virus-transgenic mice, non-invariant NKT cells are critically involved in acute liver injury (30). As for a human counterpart, Exley *et al.* observed that CD1d

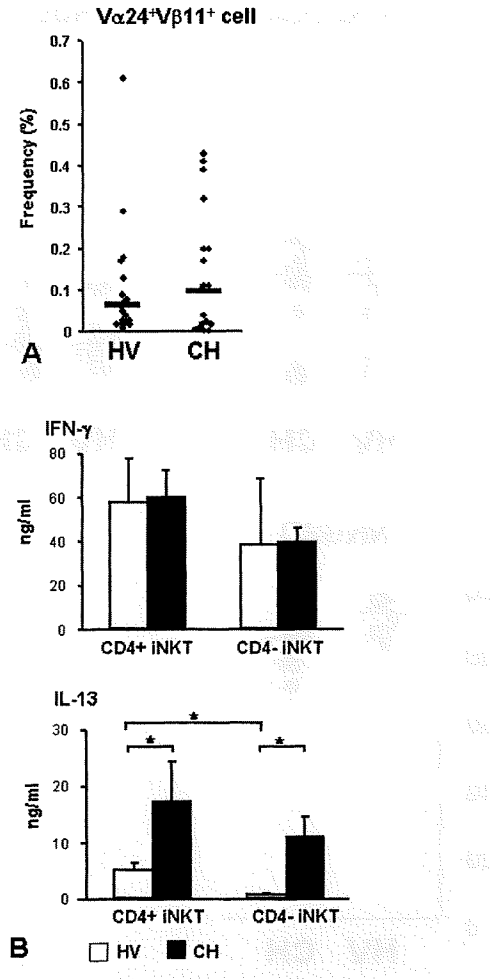

Figure 3. Frequency and cytokine production of invariant NKT cell subsets in healthy subjects and chronic hepatitis C patients. (A) The frequencies of total invariant NKT (iNKT) cells (Valfa24$^+$Vbeta11$^+$ cells) in PBMCs were determined by flow cytometry HV, CH; See Fig 2. Horizontal bars represent the median. (B) Invariant NKT (iNKT) cells were expanded by culture with alfaGalCer-pulsed autologous monocyte-derived DCs (MoDCs) and CD4$^+$ and CD4$^-$ iNKT cells were collected by subsequent cell sorting. The activated iNKT cells were stimulated with alfaGalCer-pulsed allogeneic MoDCs for 24 h and the supernatants were collected for cytokine ELISA. The bars represent mean ± SE of 5 different subjects. HV, CH; See Figure 2. *$P < 0.05$ by Mann-Whitney U test.

restricted non-invariant NKT cells infiltrate in HCV-infected liver, where they presumably exert their promoting role in liver inflammation (31). Hepatic inflammatory cells or biliary cells up-regulate CD1d which subsequently supports NKT cell activation (32). In addition, hepatic stellate cells are capable of activating NKT cells via surface CD1d and secretion of IL-15 (33).

Although iNKT cells comprise a small portion of hemotopoietic cells, they regulate various immune responses by secreting Th1 as well as Th2 cytokines in clinical settings. It has been demonstrated that phenotypic as well as functional subsets exist for iNKT cells, which are CD4$^+$, CD4$^-$CD8$^-$ double negative (DN) and CD8$^+$ ones. The CD4$^+$ and DN iNKT cells produce both Th1 (IFN-gamma) and Th2 cytokines (IL-4, IL-5, IL-13). The CD4$^+$ iNKT cells secrete more Th2 cytokines than DN, while CD8$^+$ subsets predominantly secrete Th1 cytokines (34). For chronic HCV infection, some controversial reports have been published about the frequency of iNKT cells (35, 36), however, their functional roles in HCV-infected patients are largely unknown. We thus compared the frequency and the cytokine producing capacity of iNKT cells in peripheral blood between chronic hepatitis C patients and healthy individuals. Furthermore, to analyze the functions of activated iNKT cells, we expanded iNKT cells by the stimulation with alfaGalCer-loaded DCs. We demonstrate that the number and functions of iNKT cells from HCV-infected patients are comparable with those from healthy subjects at the steady state (Figure 3A) (37). By contrast, activated iNKT cells from patients released more Th2 cytokines, most significantly IL-13, than those from the controls (Figure 3B) (37). Recently, other groups have reported that IL-4 and IL-13 from fresh iNKT cells were increased in liver cirrhosis caused by HBV or HCV, implying that these cells are pro-fibrogenic to the liver (38). If this is the case, our findings suggest that iNKT cells in chronic HCV infection are pro-fibrogenic per se even in the pre-cirrhotic stage. The reason why iNKT cells in HCV infection are Th2-biased needs to be further investigated.

5. ADAPTIVE IMMUNITY IN HCV INFECTION

Many reports have been published on the importance of CD4$^+$ T cell response in the clearance and control of HCV. In chronic hepatitis C patients, HCV-specific CD4$^+$ T cells were functionally impaired and their activity was not sustained (39), which was in clear contrast with resolved cases. Inoculation studies of infectious HCV to recovered chimpanzees demonstrated that CD4$^+$ T cell help was indispensable for the development of effective CD8$^+$ T cell response to protect from HCV persistence (40).

With regard to HCV-specific CD8$^+$ T cells observed during the chronic stages of disease, conflicting results have been reported for their roles in HCV replication and liver inflammation. Several investigators have shown that the HCV-specific CTL response is inversely correlated with viral load, suggesting its inhibitory capacity on HCV replication (41). However, others did not find a significant relationship between these parameters (42). HCV-specific CD8$^+$ T cells in chronic hepatitis C patients possess lesser capacity to proliferate and produce less IFN-gamma in response to HCV antigens. Since CD8$^+$ T cells are reported to be involved in HCV-induced liver inflammation, inefficient CD8$^+$ T cells may evoke only milder hepatocyte injury, which level is not sufficient for HCV eradication (5).

Several plausible mechanisms have been proposed for T cell functional failure observed in chronic HCV infection (3): 1) HCV escape mutation, 2) primary T

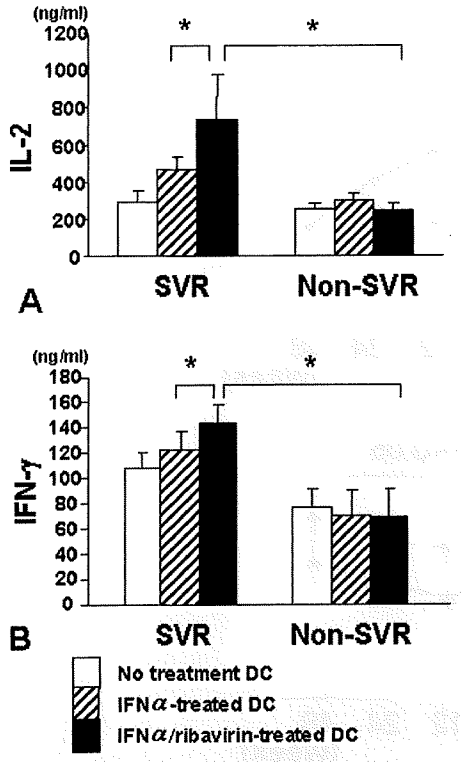

Figure 4. Improvement of Th1-inducing ability of dendritic cells by IFN-alfa and ribavirin from chronic hepatitis C patients with sustained virological response in combination therapy Monocyte-derived dendritic cells (DC) were generated with GM-CSF and IL-4 in the presence or absence of IFN-alfa and ribavirin and were cultured with allogeneic naïve $CD4^+$ T cells for 6 days. On day 4 of the culture, half of the supernatants were collected for the assessment of IL-2. After 6 days, the cultured cells were stimulated with phorbol myristate acetate and ionomycin for 24 hours. The results of IFN-gamma (A) and IL-2 (B) determined by ELISA were compared among them in the SVR and non-SVR group. The results are expressed as mean ± SE from five SVR and nine non-SVR patients. SVR, sustained virological responder in 24 weeks of IFN-α and ribavirin therapy $*P < 0.05$ by Mann-Whitney U-test.

cell failure or T cell exhaustion, 3) impaired antigen presentation, 4) suppression by HCV proteins, 5) impaired T cell maturation, 6) suppression by regulatory T cells and 7) tolerogenic environment in the liver.

6. IMMUNE RESPONSE DURING ANTI-VIRAL THERAPY

Anti-viral agents, pegylated (PEG) IFN-alfa and ribavirin, have been widely used for the treatment of chronic HCV infection in order to prevent the development to liver cirrhosis and hepatocellular carcinoma (1). In addition to providing direct inhibition of viral replication, these agents modulate antiviral immune responses, which greatly contribute to the successful therapeutic response. Earlier studies reported that HCV-specific $CD8^+$ T cell response, as examined by CTL precursor frequency, was not enhanced after IFN-alfa monotherapy (43). Furthermore, analyses of MHC class-I tetramer-positive cells in patients who underwent IFN-alfa and ribavirin therapy revealed that $CD8^+$ T cells did not increase following treatment and they were not associated with outcome (44). Combination therapy of IFN-alfa and ribavirin increases antigen-specific $CD4^+$ T cell proliferation and IFN-gamma production by $CD4^+$ T cells (45, 46). The "vigor" of the $CD4^+$ T cell response to HCV eradication is reported to be variable, something which is considered quite controversial (44).

Currently, no data is available for the involvement of innate immunity in the efficacy of IFN-alfa-based anti-HCV therapy We thus examined whether IFN-alfa and ribavirin give a positive impact on DC capacity to induce $CD4^+$ T cell (Th1) response. By using in vitro culture system, monocyte-derived DC from chronic hepatitis C patients were impaired in the ability to drive Th1 in response to IFN-alfa. When we compared such DC capacity between patients who cleared HCV (sustained virological responders, SVR) by IFN-alfa/ribavirin therapy and those who failed to do so, impaired DC function was restored in response to IFN-alfa/ribavirin in SVR patients but not in non-SVR ones (Figure 4) (47). These results imply that DC responsiveness to anti-viral agents is restored in patients who potentially gain favorable outcomes in IFN-alfa/ribavirin therapy

Next, we aimed to elucidate if the frequency or function of DC and innate lymphocytes is related to the outcome of pegylated IFN-alfa and ribavirin therapy In comparison with SVR patients, non-SVR ones and transient responders (TR) showed a decline of PDC frequency from weeks 1-12 and impaired DC function at the end of treatment (Figure 5A) (48). The frequency of NK cells, as defined as $CD3^-CD56^+$ cells, in SVR patients was lower than those in TR ones (Figure 5B). In contrast, the frequency of invariant NKT cells ($Valfa24^+Vbeta11^+$ cells) did not differ between the groups in the course of the treatment (data not shown). These results show that restoration of DC function is critically involved in favorable response in pegylated IFN-alfa/ribavirin therapy. In other words, DC system could be a target of therapeutic immune modulation.

The questions remain unsolved are if impaired immune system in chronic HCV infection is restored or not by the successful HCV eradication after anti-viral therapy Controversial results have been reported about the durability of treatment-induced recovery in HCV-specific immune response (49, 50), which seems to be clearly distinct from that observed in spontaneous HCV resolvers.

7. PERSPECTIVE

Protease inhibitors against HCV NS3/4A are now ready to use in clinics (51). Since they possess potent ability to suppress HCV replication, they are quite promising as an alternative approach for non-responders in PEG-IFNalfa/ribavirin therapy. In addition to that, it is anticipated that protease inhibitors are able to restore innate

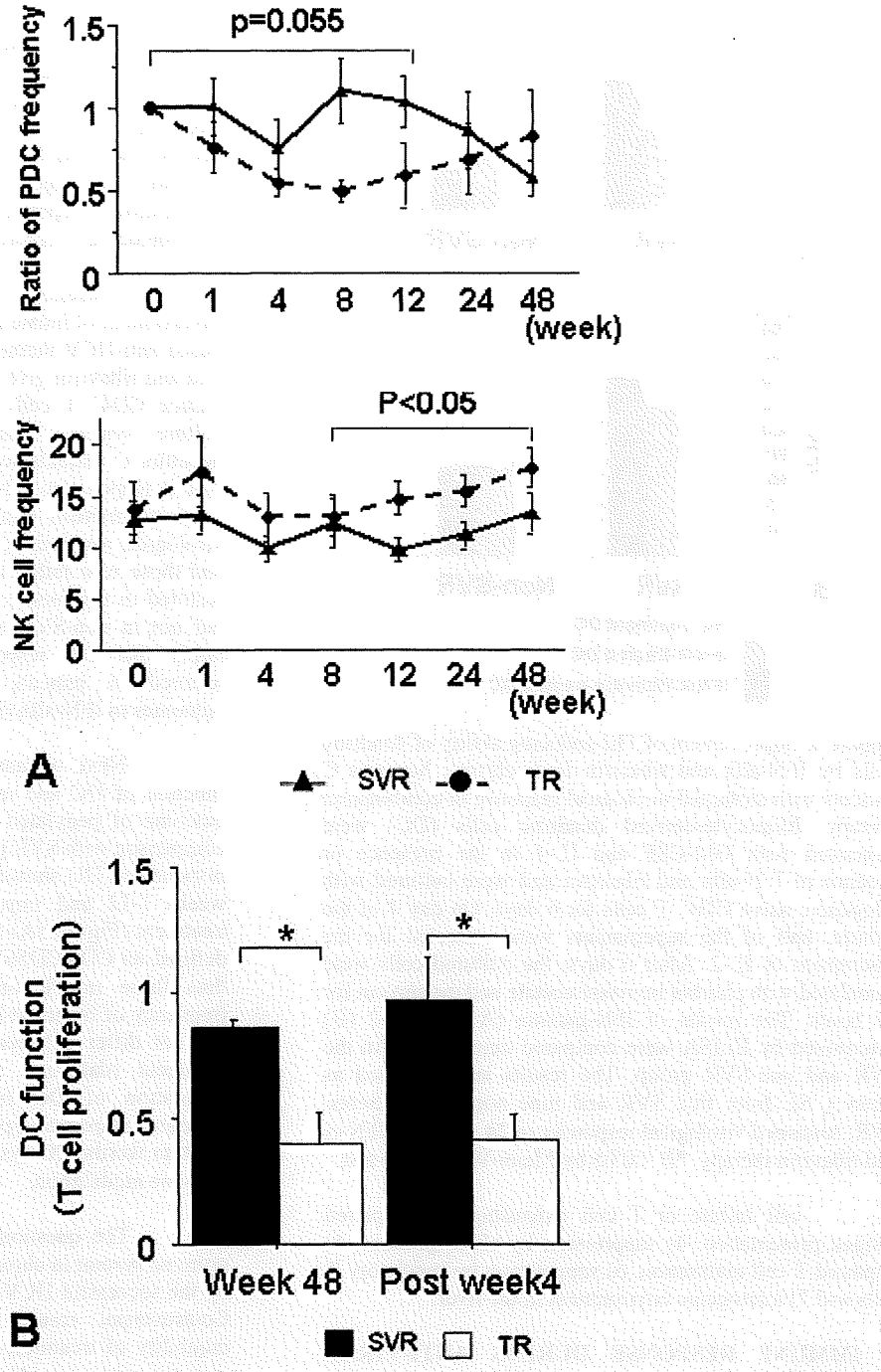

Figure 5. Early phase decline pf plasmacytoid dendritic cell frequency and sustained impairment of dendritic cell ability are related to transient response in 48-week pegylated IFN-alfa and ribavirin therapy Frequencies and their ratios of plasmacytoid dendritic cells (PDC) and NK cells in the patients during the pegylated IFN-alfa and ribavirin therapy were determined by flow cytometric analysis. PDC were defined as Lineage-negative, HLA-DR^{+}, CD11c^{-} and CD123^{high} cells and NK cells were as CD3-negative and CD56^{+} cells, respectively. The results are expressed as mean $\pm$ SE. *$P < 0.05$ by ANOVA. At the end of treatment (Week 48) and at Week 4 after the completion of therapy, monocyte-derived DC were generated from the patients or healthy donors and their allostimulatory capacity was evaluated by mixed lymphocyte reaction (MLR). The MLR ratio between patients and controls was determined from the counts per minute of ^{3}H-thymidine incorporated into CD4^{+} T cells at T cell/DC ratio of 10/1. The results are expressed as the mean $\pm$ SE of 11 SVR and 11 transient responders. SVR and TR, sustained virological responders and transient responders in 48 weeks of pegylated IFN-alfa and ribavirin therapy *$P < 0.05$ by Mann-Whitney U test.

immunity by disarming NS3/4A-mediated suppression on TLR/RIG-I-dependent or -independent pathways. Therefore, extensive immunological studies on the patients treated with protease inhibitors are needed to elucidate if the therapeutic modulation of innate immunity could shape HCV-specific adaptive immunity or not. The next steps in evolving innovative approaches to establish HCV-specific immunotherapy are to determine the means to, direct the magnitude, breadth, quality and duration of antigen-specific immune responses in a desired way Active modulation of innate immunity may be one of the strategies to gain access to the goal.

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