

**Fig. 5.** PA28 $\gamma$  is required for HCV core-dependent activation of the *srebp-1c* promoter. (A) Effect of PA28 $\gamma$  knockdown on the LXR $\alpha$ /RXR $\alpha$ -DNA complex. FLAG-LXR $\alpha$  and HA-RXR $\alpha$  were expressed in FLC4 (control) or PA28 $\gamma$ -knockdown (PA28 $\gamma$  KD) cells together with or without HCV core protein. Cells were harvested at 48 h posttransfection, and nuclear extracts were mixed with the reaction buffer for EMSA. (Upper) The resulting mixtures were subjected to PAGE and blotted with horseradish peroxidase-streptavidin. The mobility shift of the LXRE probe is indicated by an arrow. (Lower) Expression of HCV core, HA-RXR $\alpha$ , FLAG-LXR $\alpha$ , and PA28 $\gamma$  in cells was detected by immunoblotting. (B) Effect of PA28 $\gamma$  knockout on the LXR $\alpha$ /RXR $\alpha$ -DNA complex in the mouse liver. (Upper) Nuclear extracts were prepared from the livers of 2-month-old PA28 $\gamma$ <sup>-/-</sup>, PA28 $\gamma$ <sup>+/+</sup>CoreTg, PA28 $\gamma$ <sup>-/-</sup>CoreTg, and PA28 $\gamma$ <sup>-/-</sup> mice and subjected to EMSA. The mobility shift of the LXRE probe is indicated by an arrow. (Lower) The expression of HCV core, PA28 $\gamma$ , and  $\beta$ -actin in the livers of the mice was detected by immunoblotting. (C) Effect of HCV core protein on *srebp-1* promoter activity in PA28 $\gamma$ -knockout fibroblasts. A plasmid encoding firefly luciferase under the control of the *srebp-1c* promoter was transfected into MEFs prepared from PA28 $\gamma$ <sup>+/+</sup> (Left) or PA28 $\gamma$ <sup>-/-</sup> (Right) mice together with a plasmid encoding a *Renilla* luciferase. An empty plasmid or plasmids encoding mouse RXR $\alpha$  or LXR $\alpha$  were also cotransfected into the cells together with (gray bars) or without (white bars) a plasmid encoding HCV core protein. Luciferase activity under the control of the *srebp-1c* promoter was determined, and it is expressed as the fold increase in relative luciferase activity after standardization with the activity of *Renilla* luciferase.

tiates *srebp-1c* promoter activity in an LXR $\alpha$ /RXR $\alpha$ -dependent manner.

**HCV Core Protein Activates the *srebp-1c* Promoter in an LXR $\alpha$ /RXR $\alpha$ - and PA28 $\gamma$ -Dependent Manner.** To examine whether PA28 $\gamma$  is required for HCV core-induced enhancement of *srebp-1c* promoter activity in human liver cells, a PA28 $\gamma$ -knockdown human hepatoma cell line (FLC4 KD) was prepared. Enhancement of binding of the LXRE probe to LXR $\alpha$ /RXR $\alpha$  by coexpression of HCV core protein and LXR $\alpha$ /RXR $\alpha$  in FLC4 cells was diminished by knockdown of the PA28 $\gamma$  gene (Fig. 5A). Furthermore, formation of the LXR $\alpha$ /RXR $\alpha$ -LXRE complex was enhanced in the livers of PA28 $\gamma$ <sup>+/+</sup>CoreTg mice but not in those of PA28 $\gamma$ <sup>-/-</sup>, PA28 $\gamma$ <sup>+/+</sup>, or PA28 $\gamma$ <sup>-/-</sup>CoreTg mice (Fig. 5B). The expression of the HCV core protein in the mouse embryonic fibroblasts (MEFs) of PA28 $\gamma$ <sup>+/+</sup> mice induced the activation of the mouse *srebp-1c* promoter through the endogenous expression of LXR $\alpha$  and RXR $\alpha$  (Fig. 5C Left). Further enhancement of the activation of the *srebp-1c* promoter by HCV core protein in PA28 $\gamma$ <sup>+/+</sup> MEFs was achieved by the exogenous expression of both LXR $\alpha$  and RXR $\alpha$ . However, no enhancing effect of HCV core protein on *srebp-1c* promoter activity was observed in PA28 $\gamma$ <sup>-/-</sup> MEFs (Fig. 5C Right). These results support the notion that HCV core protein enhances the activity of the *srebp-1c* promoter in an LXR $\alpha$ /RXR $\alpha$ - and PA28 $\gamma$ -dependent manner.

**Table 1.** HCC in mice at 16–18 months of age

Mouse and sex	Total no. of mice	No. of mice developing HCC	Incidence, %
PA28 $\gamma$ <sup>+/+</sup> CoreTg			
Male	17	5	29.4
Female	28	3	10.7
PA28 $\gamma$ <sup>-/-</sup>			
Male	16	0	0
Female	4	0	0
PA28 $\gamma$ <sup>-/-</sup> CoreTg			
Male	23	0	0
Female	13	0	0
PA28 $\gamma$ <sup>-/-</sup> CoreTg			
Male	15	0	0
Female	21	0	0

**PA28 $\gamma$  Plays a Crucial Role in the Development of HCC in PA28 $\gamma$ <sup>+/+</sup>CoreTg Mice.** The incidence of hepatic tumors in male PA28 $\gamma$ <sup>+/+</sup>CoreTg mice older than 16 months was significantly higher than that in age-matched female PA28 $\gamma$ <sup>+/+</sup>CoreTg mice (6). We reconfirmed here that the incidence of HCC in male and female PA28 $\gamma$ <sup>+/+</sup>CoreTg mice at 16–18 months of age was 29.4% (5 of 17 mice) and 10.7% (3 of 28 mice), respectively. To our surprise, however, no HCC developed in PA28 $\gamma$ <sup>-/-</sup>CoreTg mice (males, 15; females, 21), although, as expected, no HCC was observed in PA28 $\gamma$ <sup>+/+</sup> (males, 16; females, 4) and PA28 $\gamma$ <sup>-/-</sup> mice (males, 23; females, 13) (Table 1). These results clearly indicate that PA28 $\gamma$  plays an indispensable role in the development of HCC induced by HCV core protein.

### Discussion

HCV core protein is detected in the cytoplasm and partially in the nucleus and mitochondria of culture cells and hepatocytes of transgenic mice and hepatitis C patients (6, 23, 24, 26). Degradation of HCV core protein was enhanced by deletion of the C-terminal transmembrane region through a ubiquitin/proteasome-dependent pathway (27). We previously reported (18) that PA28 $\gamma$  binds directly to HCV core protein and then enhances degradation of HCV core protein in the nucleus through a proteasome-dependent pathway because HCV core protein was accumulated in nucleus of human cell line by treatment with proteasome inhibitor MG132. In this work, accumulation of HCV core protein was observed in nucleus of hepatocytes of PA28 $\gamma$ <sup>-/-</sup>CoreTg mice (Fig. 1D). This result directly demonstrates that HCV core protein migrates into the nucleus and is degraded through a PA28 $\gamma$ -dependent pathway. However, HCV core protein accumulated in the nucleus because knockout of PA28 $\gamma$  gene abrogated the ability to cause liver pathology, suggesting that interaction of HCV core protein with PA28 $\gamma$  in the nucleus is prerequisite for the liver pathology induced by HCV core protein. We have previously shown (18) that HCV core protein is degraded through a PA28 $\gamma$ -dependent pathway, and Minami *et al.* (28) reported that PA28 $\gamma$  has a cochaperone activity with Hsp90. Therefore, degradation products of HCV core protein by means of PA28 $\gamma$ -dependent processing or correct folding of HCV core protein through cochaperone activity of PA28 $\gamma$  might be involved in the development of liver pathology. We do not know the reason why knockout of the PA28 $\gamma$  gene does not affect the total amount of HCV core protein in the liver of the transgenic mice. PA28 $\gamma$ -dependent degradation of HCV core protein may be independent of ubiquitination, as shown in SRC-3 (21), whereas knockdown of PA28 $\gamma$  in a human hepatoma cell line enhanced the ubiquitination of HCV core protein [supporting information (SI) Fig. 6], suggesting that lack of PA28 $\gamma$  suppresses a ubiquitin-independent degradation but enhances a ubiquitin-dependent degradation of HCV core protein. Therefore, the total amount of HCV

core protein in the liver of the mice may be unaffected by the knockout of the PA28 $\gamma$  gene.

Our results suggest that the interaction of HCV core protein with PA28 $\gamma$  leads to the activation of the *srebp-1c* promoter along an LXR $\alpha$ /RXR $\alpha$ -dependent pathway and the development of liver steatosis and HCC. HCV core protein was not included in the LXR $\alpha$ /RXR $\alpha$ -LXRE complex (Fig. 3A), suggesting that HCV core protein indirectly activates the *srebp-1c* promoter. Cytoplasmic HCV core protein was shown to interact with Sp110b, which is a transcriptional corepressor of RAR $\alpha$ -dependent transcription, and this interaction leads to the sequestering of Sp110b in the cytoplasm, resulting in the activation of RAR $\alpha$ -dependent transcription (29). The sequestration of an unidentified corepressor of the LXR $\alpha$ /RXR $\alpha$  heterodimer in the cytoplasm by HCV core protein may also contribute to the activation of the *srebp-1c* promoter. Although the precise physiological function of PA28 $\gamma$ -proteasome activity in the nucleus is not known, PA28 $\gamma$  has previously been shown (21) to regulate nuclear hormone receptors by means of the degradation of its coactivator SRC-3 and to participate in the fully Hsp90-dependent protein refolding (28). It appears reasonable to speculate that degradation or refolding of HCV core protein in a PA28 $\gamma$ -dependent pathway might be involved in the modulation of transcriptional regulators of various promoters, including the *srebp-1c* promoter. Saturated or monounsaturated fatty acids have been shown to enhance HCV RNA replication in Huh7 cells containing the full-length HCV replicon (7). The up-regulation of fatty acid biosynthesis by HCV core protein may also contribute to the efficient replication of HCV and to the progression of HCV pathogenesis.

Expression of HCV core protein was reported to enhance production of reactive oxygen species (ROS) (30), which leads to carbonylation of intracellular proteins (31). Enhancement of ROS production may trigger double-stranded DNA breaks and result in the development of HCC (30, 32, 33). HCV core protein could enhance the protein carbonylation in the liver of the transgenic mice in the presence but not in the absence of PA28 $\gamma$  (SI Fig. 7), suggesting that PA28 $\gamma$  is required for ROS production induced by HCV core protein. Development of HCC was observed in PA28 $\gamma^{+/+}$ CoreTg mice but not in PA28 $\gamma^{-/-}$ CoreTg mice (Table 1). Enhancement of ROS production by HCV core protein in the presence of PA28 $\gamma$  might be involved in the development of HCC in PA28 $\gamma^{+/+}$ CoreTg mice.

It is well known that resistant viruses readily emerge during the treatment with antiviral drugs targeting the viral protease or replicase, especially in the case of infection with RNA viruses. Therefore, antivirals targeting the host factors that are indispensable for the propagation of viruses might be an ideal target for the development of antiviral agents because of a lower rate of mutation than that of viral genome, if they have no side effects to patients. Importantly, the amino acid sequence of PA28 $\gamma$  of mice is identical to that of human, and mouse PA28 $\gamma$  is dispensable because PA28 $\gamma$  knockout mice exhibit no abnormal phenotype except for mild growth retardation. Therefore, PA28 $\gamma$  might be a promising target for an antiviral treatment of chronic hepatitis C with negligible side effects.

In summary, we observed that a knockout of the PA28 $\gamma$  gene from PA28 $\gamma^{+/+}$ CoreTg mice induced the accumulation of HCV core protein in the nucleus and disrupted the development of both steatosis and HCC. Activation of the *srebp-1c* promoter was up-regulated by HCV core protein both *in vitro* and *in vivo* through a PA28 $\gamma$ -dependent pathway, suggesting that PA28 $\gamma$  plays a crucial role in the development of liver pathology induced by HCV infection.

## Materials and Methods

Histology and immunohistochemistry, real-time PCR, and detection of proteins modified by ROS are discussed in *SI Materials and Methods*.

**Plasmids and Reagents.** Human PA28 $\gamma$  cDNA was isolated from a human fetal brain library (18). The gene encoding HCV core protein was amplified from HCV strain J1 (genotype 1b) (34) and cloned into pCAG-GS (35). Mouse cDNAs of RXR $\alpha$  and LXR $\alpha$  were amplified by PCR from the total cDNAs of the mouse liver. The RXR $\alpha$  and LXR $\alpha$  genes were introduced into pEF-FLAGSpGBK (36) and pcDNA3.1 (Invitrogen, Carlsbad, CA), respectively. The targeting fragment for human PA28 $\gamma$  knockdown (GGATCCGGTGGATCAGGAAGTGAAGTTCAAGAGACTTCACTTCTGATCCACCTTTTTTGGAAAAGCTT) was introduced into the BamHI and HindIII sites of pSilencer 4.1 U6 hygro vector (Ambion, Austin, TX). Mouse anti-FLAG (M2) and mouse anti- $\beta$ -actin antibodies were purchased from Sigma (St. Louis, MO). Rabbit polyclonal antibody against synthetic peptides corresponding to amino acids 70–85 of PA28 $\gamma$  was obtained from AFFINITI (Exeter, U.K.). Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgGs were purchased from ICN Pharmaceuticals (Aurora, OH). Rabbit anti-HCV core protein was prepared by immunization with recombinant HCV core protein (amino acids 1–71), as described in ref. 24. Mouse monoclonal antibody to HCV core protein was kindly provided by S. Yagi (37). The plasmid for expression of HA-tagged ubiquitin was described in ref. 27.

**Preparation of PA28 $\gamma$ -Knockout HCV CoreTg Mice.** The generation of C57BL/6 mice carrying the gene encoding HCV core protein genotype 1b line C49 and that of PA28 $\gamma^{-/-}$  mice have been reported previously (22, 25). Both strains were crossbred with each other to create PA28 $\gamma^{-/-}$ CoreTg mice. PA28 $\gamma^{-/-}$ CoreTg mice were identified by PCR targeted at the PA28 $\gamma$  or HCV core gene (22, 25). Using 1  $\mu$ g of genomic DNA obtained from the mouse tail, the PA28 $\gamma$  gene was amplified by PCR with the following primers: sense, PA28-3 (AGGTGGATCAGGAAGTGAAGCTCAA); and antisense, PA28 $\gamma$ -5cr (CACCTACTTGTGATCCGCTCTTGAAAGAATCAACC). The targeted sequence for the PA28 $\gamma$ -knockout mouse was detected by PCR using the PA28-3 primer and the PAKO-4 primer (TGCAGTTCATTCAGGGCACCAGGACAG). The transgene encoding HCV core protein was detected by PCR as described in ref. 25. The expression of PA28 $\gamma$  and HCV core protein in the livers of 6-month-old mice was confirmed by Western blotting with mouse monoclonal antibody to HCV core protein, clone 11-10, and rabbit antibody to PA28 $\gamma$ . Mice were cared for according to the institutional guidelines. The mice were given ordinary feed, CRF-1 (Charles River Laboratories, Yokohama, Japan), and they were maintained under specific pathogen-free conditions.

All animal experiments conformed to the Guidelines for the Care and Use of Laboratory Animals, and they were approved by the Institutional Committee of Laboratory Animal Experimentation (Research Institute for Microbial Diseases, Osaka University).

**Preparation of Mouse Embryonic Fibroblasts.** MEFs were prepared as described in ref. 22. MEFs were cultured at 37°C under an atmosphere of 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% FBS, penicillin, streptomycin, sodium pyruvate, and nonessential amino acids.

**Transfection and Immunoblotting.** Plasmid vectors were transfected into the MEFs and 293T cells by liposome-mediated transfection by using Lipofectamine 2000 (Invitrogen). The amount of HCV core protein in the liver tissues was determined by an ELISA as described in ref. 37. The cell lysates were subjected to SDS/PAGE (12.5% gel), and they were then transferred onto PVDF membranes. Proteins on the membranes were treated with specific antibody and Super Signal Femto (Pierce, Rockford, IL). The results were then visualized by using an LAS3000 imaging system (Fuji Photo Film, Tokyo, Japan). The method of immunoprecipitation test is described in ref. 18.

**Reporter Assay for *srebp-1c* Promoter Activity.** The genomic DNA fragment encoding the *srebp-1c* promoter region (located from residues -410 to +24) was amplified from a mouse genome. The fragment was introduced into the KpnI and HindIII sites of pGL3-Basic (Promega, Madison, WI), and it was designated as pGL3-*srebp-1c*Pro. The plasmids encoding RXR $\alpha$  and LXR $\alpha$  were transfected into MEFs together with pGL3-*srebp-1c*Pro and a control plasmid encoding *Renilla* luciferase (Promega). The total DNA for transfection was normalized by the addition of empty plasmids. Cells were harvested at 24 h posttransfection. The ligand of RXR $\alpha$ , 9-*cis*-retinoic acid (Sigma), and that of LXR $\alpha$ , 22(R)-hydroxycholesterol (Sigma) were added at a final concentration of 5  $\mu$ M each to the culture medium of 293T cells transfected with pGL3-*srebp-1c*Pro together with expression plasmids encoding RXR $\alpha$ , LXR $\alpha$ , and HCV core protein at 24 h posttransfection. Cells were harvested 24 h after treatment. Luciferase activity was measured by using the dual-luciferase reporter assay system (Promega). Firefly luciferase activity was standardized with that of *Renilla* luciferase, and the results are expressed as the fold increase in relative luciferase units.

**Electrophoresis Mobility Shift Assay (EMSA).** EMSA was carried out by using a LightShift Chemiluminescent EMSA kit (Pierce) according to the manufacturer's protocol. Nuclear extract of the cell lines and liver tissue was prepared with an NE-PER nuclear

and cytoplasmic extraction reagent kit (Pierce). Briefly, double-stranded oligonucleotides for EMSA were prepared by annealing both strands of each LXRE of the *srebp-1c* promoter (5'-GGACGCCCCGCTAGTAACCCCGGC-3') (16). Both strands were labeled at the 5' ends with biotin. The annealed probe was incubated for 20 min on ice with nuclear extract (3  $\mu$ g of protein) in a reaction buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 0.05  $\mu$ g/ $\mu$ l poly(dI-dC), 2.5% glycerol, 0.05% Nonidet P-40, and 0.1 nM labeled probe, with or without 1 mM nonlabeled probe. The resulting mixture was subjected to PAGE (5% gel) at 120 V for 30 min in 0.5 $\times$  TBE. The DNA-protein complex was transferred to a Hybond N+ membrane (Amersham, Piscataway, NJ), incubated with horseradish peroxidase-conjugated streptavidin, and visualized by using an LAS3000 imaging system.

**Statistical Analysis.** The results are expressed as the mean  $\pm$  SD. The significance of differences in the means was determined by Student's *t* test.

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- Wasley A, Alter MJ (2000) *Semin Liver Dis* 20:1-16.
- Bach N, Thung SN, Schaffner F (1992) *Hepatology* 15:572-577.
- Lefkowitz JH, Schiff ER, Davis GL, Perrillo RP, Lindsay K, Bodenheimer HC, Jr., Balart LA, Ortigo TJ, Payne J, Dienstag JL, et al. (1993) *Gastroenterology* 104:595-603.
- Barba G, Harper F, Harada T, Kohara M, Goulinet S, Matsuura Y, Eder G, Schaff Z, Chapman MJ, Miyamura T, Brecht C (1997) *Proc Natl Acad Sci USA* 94:1200-1205.
- Hope RG, McLauchlan J (2000) *J Gen Virol* 81:1913-1925.
- Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, Matsuura Y, Kimura S, Miyamura T, Koike K (1998) *Nat Med* 4:1065-1067.
- Kapadia SB, Chisari FV (2005) *Proc Natl Acad Sci USA* 102:2561-2566.
- Su A, Pezacki JP, Wodicka L, Brideau AD, Supekova L, Thimme R, Wieland S, Bukh J, Purcell RH, Schultz PG, Chisari FV (2002) *Proc Natl Acad Sci USA* 99:15669-15674.
- Wang C, Gale M, Jr, Keller BC, Huang H, Brown MS, Goldstein JL, Ye J (2005) *Mol Cell* 18:425-434.
- Horton JD, Shimomura I, Brown MS, Hammer RE, Goldstein JL, Shimano H (1998) *J Clin Invest* 101:2331-2339.
- Pai JT, Guryev O, Brown MS, Goldstein JL (1998) *J Biol Chem* 273:26138-26148.
- Shimano H, Horton JD, Hammer RE, Shimomura I, Brown MS, Goldstein JL (1996) *J Clin Invest* 98:1575-1584.
- Shimano H, Horton JD, Shimomura I, Hammer RE, Brown MS, Goldstein JL (1997) *J Clin Invest* 99:846-854.
- Shimano H, Shimomura I, Hammer RE, Herz J, Goldstein JL, Brown MS, Horton JD (1997) *J Clin Invest* 100:2115-2124.
- Repa JJ, Liang G, Ou J, Bashmakov Y, Lobaccaro JM, Shimomura I, Shan B, Brown MS, Goldstein JL, Mangelsdorf DJ (2000) *Genes Dev* 14:2819-2830.
- Yoshikawa T, Shimano H, Amemiya-Kudo M, Yahagi N, Hasty AH, Matsuoka T, Okazaki H, Tamura Y, Iizuka Y, Ohashi K, et al. (2001) *Mol Cell Biol* 21:2991-3000.
- Tsutsumi T, Suzuki T, Shimoike T, Suzuki R, Moriya K, Shintani Y, Fujie H, Matsuura Y, Koike K, Miyamura T (2002) *Hepatology* 35:937-946.
- Moriishi K, Okabayashi T, Nakai K, Moriya K, Koike K, Murata S, Chiba T, Tanaka K, Suzuki R, Suzuki T, et al. (2003) *J Virol* 77:10237-10249.
- Masson P, Andersson O, Petersen UM, Young P (2001) *J Biol Chem* 276:1383-1390.
- Li J, Rechsteiner M (2001) *Biochimie* 83:373-383.
- Li X, Lonard D, Jung SY, Malovannaya A, Feng Q, Qin J, Tsai SY, Tsai M, O'Malley BW (2006) *Cell* 124:381-392.
- Murata S, Kawahara H, Tohma S, Yamamoto K, Kasahara M, Nabeshima Y, Tanaka K, Chiba T (1999) *J Biol Chem* 274:38211-38215.
- Falcon V, Acosta-Rivero N, Chinea G, Gavilondo J, de la Rosa MC, Mendendez I, Duenas-Carrera S, Vina A, Garcia W, Gra B, et al. (2003) *Biochem Biophys Res Commun* 305:1085-1090.
- Suzuki R, Sakamoto S, Tsutsumi T, Rikimaru A, Tanaka K, Shimoike T, Moriishi K, Iwasaki T, Mizumoto K, Matsuura Y, et al. (2005) *J Virol* 79:1271-1281.
- Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, Miyamura T, Koike K (1997) *J Gen Virol* 78:1527-1531.
- Yasui K, Wakita T, Tsukiyama-Kohara K, Funahashi SI, Ichikawa M, Kajita T, Moradpour D, Wands JR, Kohara M (1998) *J Virol* 72:6048-6055.
- Suzuki R, Tamura K, Li J, Ishii K, Matsuura Y, Miyamura T, Suzuki T (2001) *Virology* 280:301-309.
- Minami Y, Kawasaki H, Minami M, Tanahashi N, Tanaka K, Yahara I (2000) *J Biol Chem* 275:9055-9061.
- Watashi K, Hijikata M, Tagawa A, Doi T, Marusawa H, Shimotohno K (2003) *Mol Cell Biol* 23:7498-7509.
- Machida K, Cheng KT, Lai CK, Jeng KS, Sung VM, Lai MM (2006) *J Virol* 80:7199-7207.
- Nystrom T (2005) *EMBO J* 24:1311-1317.
- Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE, Jr (1999) *Cell* 98:295-303.
- Carballo M, Conde M, El Bekay R, Martin-Nieto J, Camacho MJ, Monteseirin J, Conde J, Bedoya FJ, Sobrino F (1999) *J Biol Chem* 274:17580-17586.
- Aizaki H, Aoki Y, Harada T, Ishii K, Suzuki T, Nagamori S, Toda G, Matsuura Y, Miyamura T (1998) *Hepatology* 27:621-627.
- Niwa H, Yamamura K, Miyazaki J (1991) *Gene* 108:193-199.
- Huang DC, Cory S, Strasser A (1997) *Oncogene* 14:405-414.
- Aoyagi K, Ohue C, Iida K, Kimura T, Tanaka E, Kiyosawa K, Yagi S (1999) *J Clin Microbiol* 37:1802-1808.

## Involvement of the PA28 $\gamma$ -Dependent Pathway in Insulin Resistance Induced by Hepatitis C Virus Core Protein<sup>∇</sup>

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The hepatitis C virus (HCV) core protein is a component of nucleocapsids and a pathogenic factor for hepatitis C. Several epidemiological and experimental studies have suggested that HCV infection is associated with insulin resistance, leading to type 2 diabetes. We have previously reported that HCV core gene-transgenic (PA28 $\gamma^{+/+}$ CoreTg) mice develop marked insulin resistance and that the HCV core protein is degraded in the nucleus through a PA28 $\gamma$ -dependent pathway. In this study, we examined whether PA28 $\gamma$  is required for HCV core-induced insulin resistance *in vivo*. HCV core gene-transgenic mice lacking the PA28 $\gamma$  gene (PA28 $\gamma^{-/-}$ CoreTg) were prepared by mating of PA28 $\gamma^{+/+}$ CoreTg with PA28 $\gamma$ -knockout mice. Although there was no significant difference in the glucose tolerance test results among the mice, the insulin sensitivity in PA28 $\gamma^{-/-}$ CoreTg mice was recovered to a normal level in the insulin tolerance test. Tyrosine phosphorylation of insulin receptor substrate 1 (IRS1), production of IRS2, and phosphorylation of Akt were suppressed in the livers of PA28 $\gamma^{+/+}$ CoreTg mice in response to insulin stimulation, whereas they were restored in the livers of PA28 $\gamma^{-/-}$ CoreTg mice. Furthermore, activation of the tumor necrosis factor alpha promoter in human liver cell lines or mice by the HCV core protein was suppressed by the knockdown or knockout of the PA28 $\gamma$  gene. These results suggest that the HCV core protein suppresses insulin signaling through a PA28 $\gamma$ -dependent pathway.

Hepatitis C virus (HCV) is the causative agent in most cases of acute and chronic non-A, non-B hepatitis (15). Over one-half of patients with the acute infection evolve into a persistent carrier state (24). Chronic infection with HCV frequently induces hepatic steatosis, cirrhosis, and eventually hepatocellular carcinoma (22) and is known to be associated with diseases of extrahepatic organs, including an essential mixed cryoglobulinemia, porphyria cutanea tarda, membranoproliferative glomerulonephritis, and type 2 diabetes (13).

HCV is classified into the genus *Hepacivirus* of the family *Flaviviridae* and possesses a viral genome consisting of a single positive-strand RNA with a nucleotide length of about 9.5 kb. This viral genome encodes a single polyprotein composed of approximately 3,000 amino acids (9). The polyprotein is post-translationally cleaved by host cellular peptidases and viral proteases, resulting in 10 viral proteins (6, 10, 12). The HCV core protein is known to interact with viral-sense RNA of HCV to form the viral nucleocapsid (44). The HCV core protein is cleaved off at residue 191 by the host signal peptidase to release it from the E1 envelope protein and then by the host signal peptide peptidase at around amino acid residues 177 to 179 within the C-terminal transmembrane region (30, 39, 40). The mature core protein is retained mainly on the endoplasmic reticulum, although a portion moves to the nucleus and mitochondria (11, 51).

Recent epidemiological studies have indicated that type 2

diabetes is an HCV-associated disease (7, 29). However, it remains unclear how insulin resistance is induced in patients chronically infected with HCV, since there is no suitable model for investigating HCV pathogenesis. Type 2 diabetes is a complex, multisystemic disease with pathophysiology that includes a high level of hepatic glucose production and insulin resistance, which contribute to the development of hyperglycemia (8, 18). Although the precise mechanism by which these factors contribute to the induction of insulin resistance is difficult to understand, a high level of insulin production by pancreatic  $\beta$  cells under a state of insulin resistance is common in the development of type 2 diabetes. The hyperinsulinemia in the fasting state that is observed relatively early in type 2 diabetes is considered to be a secondary response that compensates for the insulin resistance (8, 18).

The HCV core protein is also known as a pathogenic factor that induces steatosis and hepatocellular carcinoma in mice (33, 35). Previously, we reported that insulin resistance occurs in HCV core gene-transgenic mice due at least partly to an increase in tumor necrosis factor alpha (TNF- $\alpha$ ) secretion (47) and that the HCV core protein is degraded through a PA28 $\gamma$ /REG $\gamma$  (11S regulator)-dependent pathway in the nucleus (32). It is well known that PA28 $\gamma$  enhances latent proteasome activity, although the biological significance of PA28 $\gamma$  is largely unknown, with the exception that PA28 $\gamma$  is known to regulate steroid receptor coactivator 3 (28). Although several reports suggested that the degradation of insulin receptor substrate (IRS) proteins by a ubiquitin-dependent proteasome activity contributes to insulin resistance (43, 50), the involvement of the HCV core protein in cooperation with PA28 $\gamma$  in the stability of IRS proteins and in the development of insulin resis-

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tance is not known. In this study, we examined the involvement of PA28 $\gamma$  in the induction of insulin resistance by the HCV core protein in vivo.

#### MATERIALS AND METHODS

**Preparation of PA28 $\gamma$ -knockout HCV core gene-transgenic mice.** C57BL/6 mice carrying the gene encoding HCV core protein genotype 1b (PA28 $\gamma^{+/+$  CoreTg) line C49 and PA28 $\gamma^{-/-}$  mice have been described previously (35, 36). These two genotypes were crossbred to create PA28 $\gamma^{+/+$  CoreTg mice. PA28 $\gamma^{-/-}$  CoreTg mice were bred to generate PA28 $\gamma^{-/-}$  CoreTg mice (35, 36). The HCV core gene and the target sequence to knock out the PA28 $\gamma$  gene were identified by PCR. The mice were given ordinary feed (CRF-1; Charles River Laboratories, Yokohama, Japan) and were maintained under specific-pathogen-free conditions.

**Glucose tolerance test.** The mice were fasted for more than 16 h before glucose administration. D-Glucose (1 g/kg body weight) was intraperitoneally administered to the mice. Blood samples were taken from the orbital sinus at the indicated time points. The plasma glucose concentration was measured by means of a MEDI-SAFE Mini blood glucose monitor (TERUMO, Tokyo, Japan). The serum insulin level was determined by a Mercodia (Uppsala, Sweden) ultrasensitive mouse insulin enzyme-linked immunosorbent assay (ELISA).

**Insulin tolerance test.** The mice were fed freely and then fasted during the study period. Human insulin (2 U/kg body weight) (Humulin; Eli Lilly, Indianapolis, IN) was intraperitoneally administered to the mice. The plasma glucose concentration was measured at the indicated time and was normalized based on the glucose concentration at the time just before insulin administration.

**Histological analysis of pancreatic islets.** Pancreas tissues were fixed with paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The relative islet area and islet number were determined with Image-Pro PLUS image analyzing software (NIPPON ROPER, Tokyo, Japan).

**Estimation of tumor necrosis factor alpha and HCV core protein.** Mouse TNF- $\alpha$  was measured by using a mouse TNF- $\alpha$  ELISA kit (Pierce, Rockford, IL) and normalized based on the amount of total protein in each sample. The protein concentration was estimated by using a BCA protein assay kit (Pierce). The amount of HCV core protein in the liver tissues was determined by using an ELISA system as described previously (4).

**In vivo insulin stimulation and immunoblot analysis.** Mice were fasted for more than 16 h before insulin stimulation and then anesthetized with ketamine and xylazine. Five units of insulin were injected into the mice via the interior vena cava. Livers of the mice were collected 5 min after the insulin injection and frozen in liquid nitrogen. Immunoblot analyses of the HCV core protein, PA28 $\gamma$ , and each of the insulin-signaling molecules were carried out with the liver tissue homogenates prepared in the homogenizing buffer containing 25 mM Tris-HCl (pH 7.4), 10 mM Na<sub>2</sub>VO<sub>4</sub>, 100 mM NaF, 50 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 10 mM EGTA, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P40 supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) (53). Tissue lysates were subjected to sodium dodecyl sulfate-2% to 15% gradient polyacrylamide gel electrophoresis (PAG Mini DAIICHI 2/15 13W; Daiichi Diagnostics, Tokyo, Japan) and electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). The protein transferred onto the membrane was reacted with rabbit anti-HCV core (32), rabbit anti-Akt (Cell Signaling, Danvers, MA), rabbit anti-phospho-Ser473-Akt (Cell Signaling), rabbit anti-IRS1 (Upstate, Lake Placid, NY), rabbit anti-phospho-Tyr608 mouse insulin receptor substrate 1 (Sigma, St. Louis, MO), or rabbit anti-IRS2 (Upstate) polyclonal antibody and then incubated with horseradish peroxidase-conjugated anti-rabbit antibody. Blotted protein was visualized using Super Signal Femto (Pierce) and an LAS3000 imaging system (Fuji Photo Film, Tokyo, Japan).

**Quantitative reverse transcription-PCR (RT-PCR).** Total RNA was isolated from mouse liver using an RNeasy kit (QIAGEN, Valencia, CA). The RNA preparation was treated with a TURBO DNA-free kit (Ambion, Austin, TX) to remove DNA contamination in the samples. The first-strand cDNAs were synthesized by a first-strand cDNA synthesis kit (Amersham Biosciences, Franklin Lakes, NJ). The targeted cDNA was estimated by using Platinum SYBR Green qPCR Super Mix UDC (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The fluorescent signal was measured by using an ABI Prism 7000 (Applied Biosystems, Foster City, CA). The genes encoding mouse TNF- $\alpha$ , IRS1, IRS2, and hypoxanthine phosphoribosyl transferase were amplified with the following primer pairs: 5'-GGTACAACCCATCGGCTGGCA-3' (forward) and 5'-GCGACGTGGAAGTGGCAGAAG-3' (reverse) for TNF- $\alpha$ , 5'-ATAG

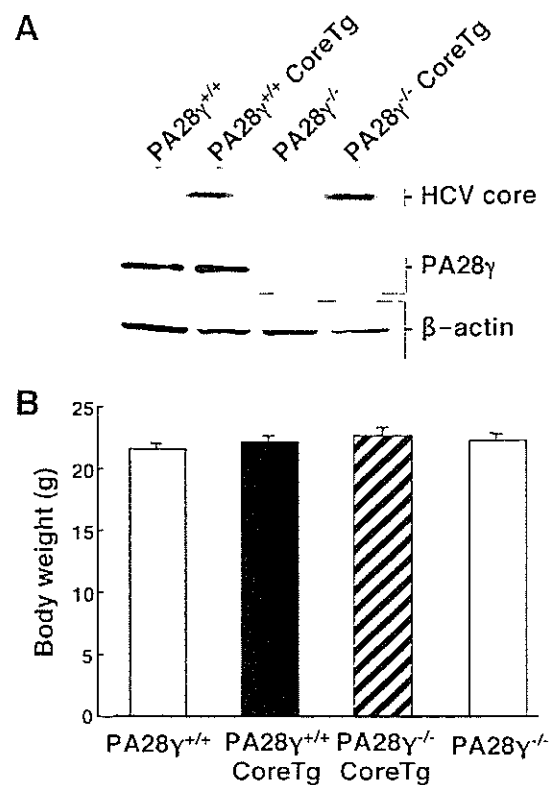


FIG. 1. Characterization of HCV core gene-transgenic mice deficient in the PA28 $\gamma$  gene. (A) Expression of the HCV core protein and PA28 $\gamma$  in the livers of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{+/+}$  CoreTg, PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{-/-}$  CoreTg mice. Lysates obtained from liver tissues of the mice (100  $\mu$ g protein/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting using antibodies to the HCV core protein, PA28 $\gamma$ , and  $\beta$ -actin. (B) Body weights of the mice. Body weights of 2-month-old mice were measured ( $n = 7$  in each group). There were no statistically significant differences in body weights among the mice ( $P > 0.05$ ).

CTCTGAGACCTTCTCAGCACCTAC-3' (forward) and 5'-GGAGTTGCCCTCAITGCTGCCTAA-3' (reverse) for IRS1, 5'-AGCCTGGGGATAATGGTGACTATACCGA-3' (forward) and 5'-TTGTGGGCAAAGGATGGGGACACT-3' (reverse) for IRS2, and 5'-CCAGCAAGCTTGAACCTTAACCA-3' (forward) and 5'-GTAATGATCAGTCAACGGGGGAC-3' (reverse) for hypoxanthine phosphoribosyl transferase. Each PCR product was found as a single band with the correct size by agarose gel electrophoresis (data not shown).

**Reporter assay for TNF- $\alpha$  promoter activity.** The promoter region of the TNF- $\alpha$  gene (located from residues -1260 to +140) was amplified from mouse genomic DNA and was then introduced into the KpnI and BglII sites of pGL3-Basic (Promega, Madison, WI) (25). The resulting plasmid was designated as pGL3-tnf- $\alpha$ Pro. The gene encoding the HCV core protein was amplified from HCV strain J1 (genotype 1b) and cloned into pCAG-GS (1, 38). To avoid contamination with endotoxin from *Escherichia coli*, the plasmid DNA was purified by using an EndoFree Plasmid Maxi kit (QIAGEN). The total amount of transfected DNA was normalized by the addition of empty plasmids. Plasmid vector was transfected into hepatoma cell lines by lipofection using Lipofectamine 2000 (Invitrogen). Cells were harvested at 24 h posttransfection. Luciferase activity was determined by using the Dual-Luciferase Reporter Assay system (Promega). Firefly luciferase activity was normalized to coexpressed *Renilla* luciferase activity. The amount of firefly luciferase activity was presented as the increase ( $n$ -fold) relative to the value for the sample lacking the HCV core protein, which was taken to be 1.0. PA28 $\gamma$ -knockdown cell lines were established by using pSilencer 2.1 U6 Hygro (Ambion) according to the manufacturer's protocol.

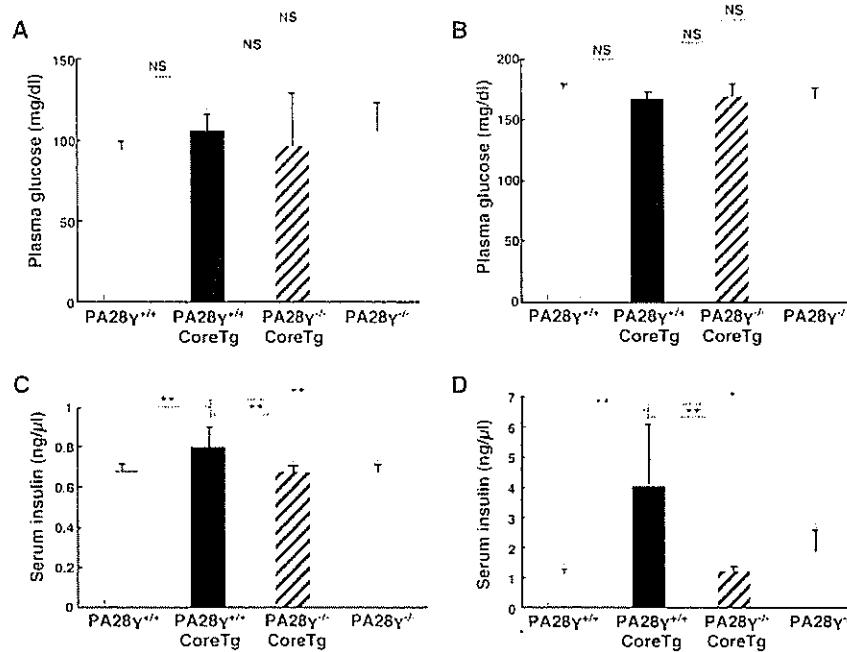


FIG. 2. Knockout of the PA28 $\gamma$  gene inhibited the hyperinsulinemia induced by HCV core protein. Plasma glucose levels of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{+/+}$  CoreTg, PA28 $\gamma^{-/-}$  CoreTg, and PA28 $\gamma^{-/-}$  mice under fasting (A) or fed (B) conditions ( $n = 7$  in each group) are shown. Serum insulin levels in fasting (C) or fed (D) mice ( $n = 7$  in each group) are also shown. Values are represented as means  $\pm$  standard deviations. \* $P < 0.05$ ; \*\* $P < 0.01$ . NS, not statistically significant.

Statistical analysis. The results are presented as means  $\pm$  standard deviations. The significance of the differences was determined by Student's  $t$  test.  $P$  values of  $<0.05$  were considered statistically significant.

RESULTS

HCV core gene-transgenic mice deficient in the PA28 $\gamma$  gene. To investigate the role of PA28 $\gamma$  in the development of insulin resistance in HCV core gene-transgenic (PA28 $\gamma^{+/+}$  CoreTg)

mice, we generated HCV core gene-transgenic mice deficient in the PA28 $\gamma$  gene (PA28 $\gamma^{-/-}$  CoreTg). A PA28 $\gamma^{+/+}$  CoreTg mouse expressing an amount of PA28 $\gamma$  equal to that of its normal littermates (Fig. 1A) was crossed with a PA28 $\gamma^{-/-}$  mouse to generate a PA28 $\gamma^{+/+}$  CoreTg mouse. PA28 $\gamma^{+/+}$  CoreTg mice were bred with each other, and a PA28 $\gamma^{-/-}$  CoreTg mouse was selected by PCR. The HCV core protein was expressed in PA28 $\gamma^{+/+}$  CoreTg and PA28 $\gamma^{-/-}$  CoreTg

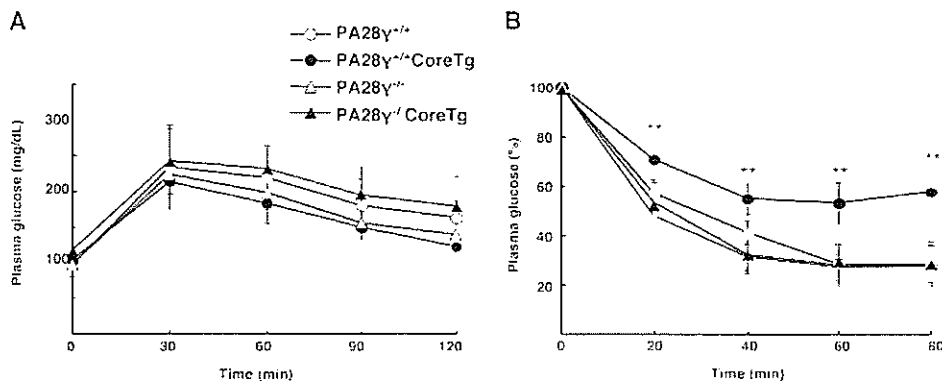


FIG. 3. Knockout of the PA28 $\gamma$  gene inhibits the insulin resistance induced by the HCV core protein. (A) Glucose tolerance test. D-Glucose was intraperitoneally administered to mice fasted for more than 16 h at 1 g/kg of body weight. Plasma glucose levels were estimated at the indicated times ( $n = 5$  in each group). There were no significant differences in glucose levels among the mice ( $P > 0.05$ ). (B) Insulin tolerance test. Human insulin (2 units/kg body weight) was intraperitoneally administered to the mice, and the plasma glucose levels were estimated at the indicated times. Values were normalized to the baseline glucose concentration at the time of insulin administration ( $n = 5$  in each group). The values for the PA28 $\gamma^{+/+}$  (open circles), PA28 $\gamma^{-/-}$  CoreTg (closed circles), PA28 $\gamma^{-/-}$  (open triangles), and PA28 $\gamma^{-/-}$  CoreTg (closed triangles) mice are represented as means  $\pm$  standard deviations. Significant differences in insulin sensitivity ( $P < 0.01$ ) in PA28 $\gamma^{+/+}$  CoreTg mice compared to that in PA28 $\gamma^{+/+}$ , PA28 $\gamma^{-/-}$ , or PA28 $\gamma^{-/-}$  CoreTg mice are indicated by double asterisks (\*\*). There were no significant differences among PA28 $\gamma^{+/+}$ , PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{-/-}$  CoreTg mice ( $P > 0.05$ ).

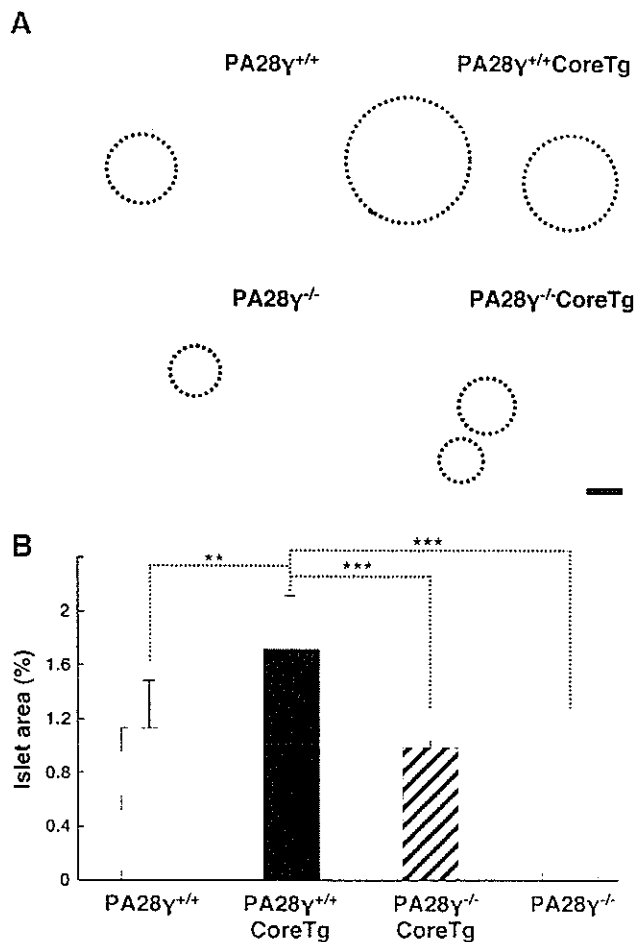


FIG. 4. PA28 $\gamma$  participated in the enlargement of pancreatic islets induced by the HCV core protein. (A) Histological sections prepared from pancreas tissues of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{-/-}$ CoreTg mice were stained with hematoxylin and eosin. Dotted circles indicate pancreatic islets. (B) The area occupied by pancreatic islets was measured by computer software in three different fields of every six randomly selected sections of 10 mice per genotype and is represented as a percentage of the total pancreatic area. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . The scale bar indicates 100  $\mu$ m.

mice but not in PA28 $\gamma^{+/+}$  (normal littermates) or PA28 $\gamma^{-/-}$  mice. PA28 $\gamma$  was found at a similar level in PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{+/+}$  mice but was not present in either PA28 $\gamma^{-/-}$  or PA28 $\gamma^{-/-}$ CoreTg mice (Fig. 1A). The expression of the HCV core protein in the livers of 2-month-old male mice was slightly higher in PA28 $\gamma^{-/-}$ CoreTg ( $1.36 \pm 0.44$  ng/mg of total protein;  $n = 7$ ) than in PA28 $\gamma^{+/+}$ CoreTg ( $1.23 \pm 0.22$  ng/mg of total protein;  $n = 7$ ) mice, but these values were not significantly different ( $P > 0.05$ ). Insulin sensitivity is dependent on several conditions such as body weight, obesity, and liver steatosis (26). PA28 $\gamma^{-/-}$  mice were slightly smaller than their normal littermates (PA28 $\gamma^{+/+}$ ) at more than 3 months old, as described previously (36), but this was not significantly different in 2-month-old mice (Fig. 1B). PA28 $\gamma^{+/+}$ CoreTg mice exhibited severe hepatic steatosis from 4 months of age (35). To avoid the influence of hepatic steatosis and body weight on the examination of insulin resistance, 2-month-old mice were

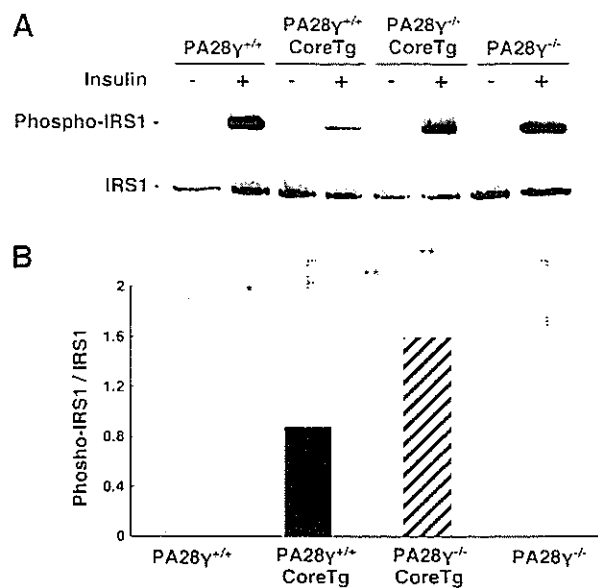


FIG. 5. PA28 $\gamma$  participated in the inhibition of the tyrosine phosphorylation of IRS1 induced by the HCV core protein. Liver tissues from PA28 $\gamma^{+/+}$ , PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{-/-}$ CoreTg mice were prepared after administration of insulin (+) or phosphate-buffered saline (-). The samples (100  $\mu$ g of total protein) were examined by immunoblotting with antibodies against IRS1 and phospho-Tyr608 of mouse IRS1 (A). Phosphorylated IRS1 was estimated from the density on the immunoblotted membrane by using computer software (B) ( $n = 5$  in each group). The data presented are representative of three independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ .

used in this study. Figure 1B shows the body weights of 2-month-old mice. There were no significant differences in body weight among PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ CoreTg, PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{+/+}$  mice. Steatosis was not detected in the livers of the 2-month-old mice (data not shown).

PA28 $\gamma$  is involved in the development of hyperinsulinemia and insulin resistance in PA28 $\gamma^{+/+}$ CoreTg mice. In our previous study, we found a significant difference in serum insulin levels, but not in plasma glucose levels, between PA28 $\gamma^{+/+}$ CoreTg mice and normal littermates (47). To determine the involvement of PA28 $\gamma$  in the development of insulin resistance in PA28 $\gamma^{+/+}$ CoreTg mice, we examined here the plasma glucose and insulin levels in the mice under fasting and fed conditions. Although no significant difference in plasma glucose levels was observed in the mice under either fasting (Fig. 2A) or fed (Fig. 2B) conditions, serum insulin levels were significantly higher in PA28 $\gamma^{+/+}$ CoreTg mice than in PA28 $\gamma^{+/+}$  mice under both conditions (Fig. 2C and D), as described previously (47). In contrast, the serum insulin concentration in PA28 $\gamma^{-/-}$ CoreTg mice was recovered to a normal level similar to that of PA28 $\gamma^{+/+}$  and PA28 $\gamma^{-/-}$  mice under either fasting (Fig. 2C) or fed (Fig. 2D) conditions.

To determine the glucose intolerance among the mice, glucose was administered to the mice after fasting, and the plasma glucose level was then determined. There was no significant difference among the genotypes at any time point in the glucose tolerance test (Fig. 3A), suggesting that the volume of glucose was maintained at a normal level by the higher concentration of insulin in PA28 $\gamma^{+/+}$ CoreTg mice. In our previ-

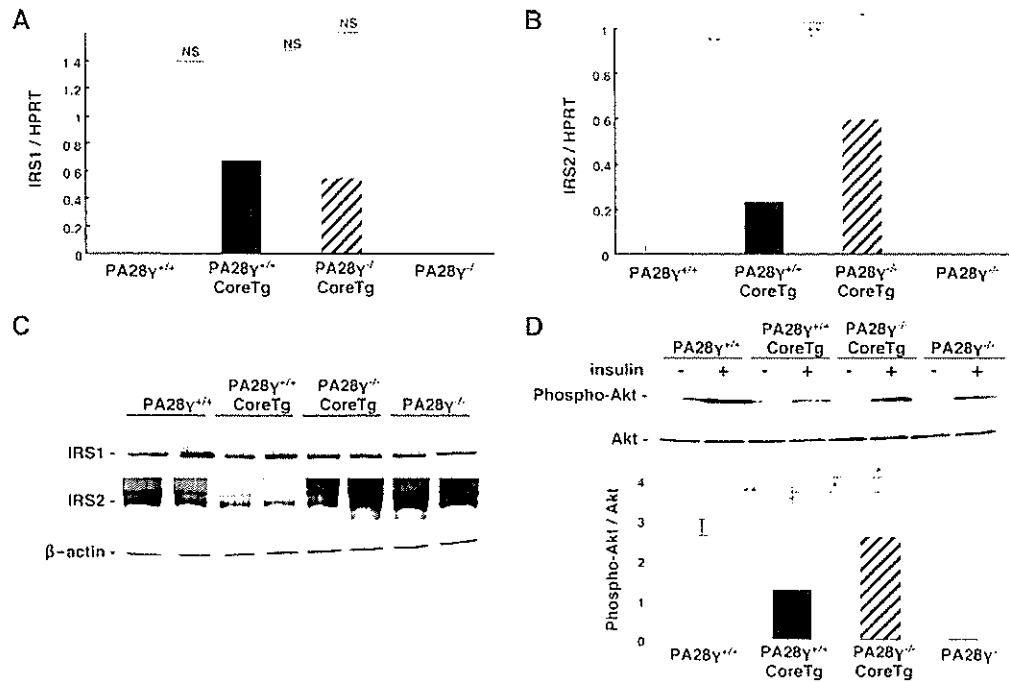


FIG. 6. PA28 $\gamma$  participated in the inhibition of the IRS2 expression and Akt phosphorylation induced by HCV core protein. The transcription of IRS1 (A) and IRS2 (B) was estimated by quantitative RT-PCR ( $n = 5$  in each group). (C) The expression levels of IRS1 and IRS2 in the livers of the mice were determined by immunoblotting with specific antibodies. (D) Phosphorylation of Akt in the livers of the mice was examined by immunoblotting with antibodies against Akt and phosphorylated Akt. The ratio of Akt phosphorylation was determined by computer software based on the densities of phosphorylated Akt and a total amount of Akt ( $n = 3$  in each group). The data presented are representative of three independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ . NS, not statistically significant; HPRT, hypoxanthine phosphoribosyl transferase.

ous study, the reduction in the plasma glucose concentration after insulin administration was impaired in PA28 $\gamma^{+/+}$  CoreTg mice (47). In this study, PA28 $\gamma^{-/-}$  CoreTg mice exhibited a normal insulin level comparable to those of PA28 $\gamma^{+/+}$  and PA28 $\gamma^{-/-}$  mice by an insulin tolerance test, in contrast to PA28 $\gamma^{+/+}$  CoreTg mice, in which a high concentration of plasma glucose was detected at all time points, as previously reported (Fig. 3B). These data suggest that hyperinsulinemia was induced in PA28 $\gamma^{+/+}$  CoreTg mice to compensate for insulin resistance and retain a physiological level of plasma glucose and that PA28 $\gamma$  participates in the development of hyperinsulinemia and insulin resistance in PA28 $\gamma^{+/+}$  CoreTg mice.

**Morphology of pancreatic islets.** Hyperinsulinemia and insulin resistance are expected to enlarge the pancreatic islet mass due to the overexpression of insulin. Our previous report showed the enlargement of the pancreatic islets in PA28 $\gamma^{-/-}$  CoreTg mice. To clarify whether a knockout of the PA28 $\gamma$  gene restores the enlarged pancreatic islets to their normal size, the morphology of the pancreatic islets of the mice was evaluated by histologic examination (Fig. 4A). The relative islet area in the pancreatic cells of the PA28 $\gamma^{-/-}$  CoreTg mice was smaller than that of PA28 $\gamma^{-/-}$  CoreTg mice and comparable to that of PA28 $\gamma^{+/+}$  and PA28 $\gamma^{-/-}$  mice (Fig. 4B). Infiltration of inflammatory cells within or surrounding the islets was not found in all genotypes of mice. These results suggest that PA28 $\gamma$  also participates in the enlargement of pancreatic islets induced in PA28 $\gamma^{+/+}$  CoreTg mice.

PA28 $\gamma$  impairs the insulin-signaling pathway through the suppression of both tyrosine phosphorylation of IRS1 and expression of IRS2. Insulin binds to insulin receptors, resulting in the activation of downstream signaling (26). The activated insulin receptors phosphorylate themselves, IRS1, and IRS2. Phosphorylated IRS1 and IRS2 can activate phosphatidylinositol 3 (PI3)-kinase signaling, leading to the activation of glucose metabolism and cell growth. Our previous report showed that tyrosine phosphorylation of IRS1 is suppressed in the livers of PA28 $\gamma^{+/+}$  CoreTg mice and that the administration of anti-TNF- $\alpha$  antibody restores insulin sensitivity (47). We examined whether a knockout of the PA28 $\gamma$  gene could restore the tyrosine phosphorylation of IRS1. Tyrosine phosphorylation of IRS1 was suppressed in the livers of PA28 $\gamma^{+/+}$  CoreTg mice in response to insulin stimulation, whereas it was recovered in PA28 $\gamma^{-/-}$  CoreTg mice to levels comparable to those in PA28 $\gamma^{-/-}$  and PA28 $\gamma^{-/-}$  mice (Fig. 5).

Chronic hyperinsulinemia downregulates the expression of IRS2, which is one of the essential components of the insulin-signaling pathway in the liver (46). However, in our previous study, we showed that there was no significant difference in the phosphorylation of IRS2 between PA28 $\gamma^{+/+}$  CoreTg mice and their normal littermates (47). To gain more insight into the mechanisms of regulation of IRS expression, we determined the transcription and translation of IRS1 and IRS2 in the livers of the mice by real-time PCR and Western blotting, respectively. Although there was no significant difference in IRS1 expression at either the transcriptional or translational level among the mice



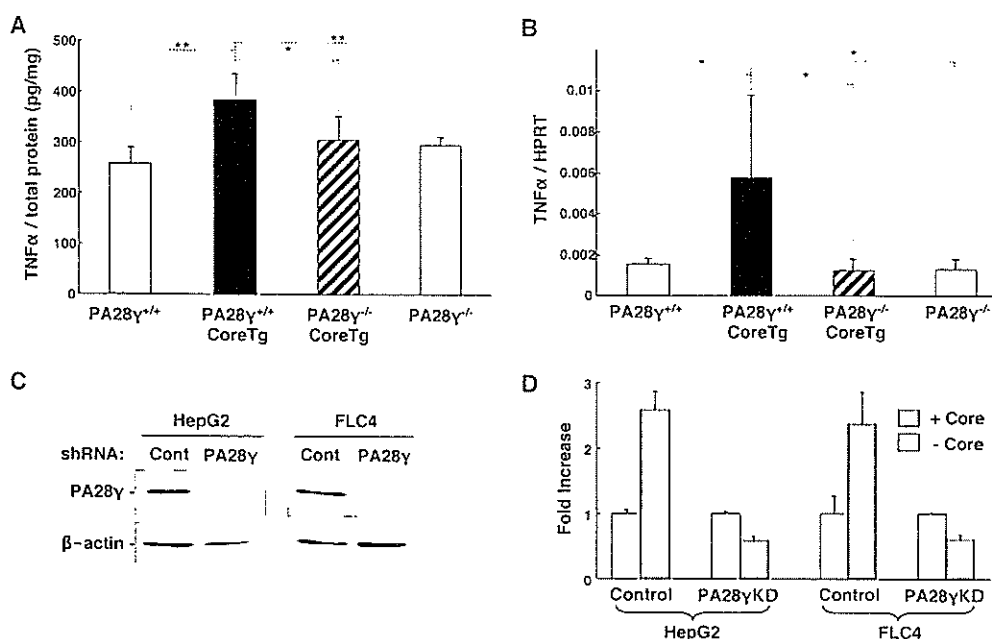


FIG. 7. PA28 $\gamma$  was required for activation of the TNF- $\alpha$  promoter by the HCV core protein. (A) Expression of TNF- $\alpha$  in the livers of mice was determined by ELISA ( $n = 5$  in each group). (B) TNF- $\alpha$  mRNA in the livers of mice was examined by quantitative RT-PCR ( $n = 5$  in each group). (C) Knockdown of the expression of PA28 $\gamma$  in the HepG2 and FLC-4 cell lines by the introduction of a plasmid encoding a short hairpin RNA (shRNA) targeted to the PA28 $\gamma$  gene. The expression levels of PA28 $\gamma$  and  $\beta$ -actin were determined by immunoblotting with specific antibodies. (D) Promoter activity of TNF- $\alpha$  in the presence or absence of the HCV core protein was determined by luciferase assay in the PA28 $\gamma$ -knockdown and control cell lines. The data presented are representative of three independent experiments. HPRT, hypoxanthine phosphoribosyl transferase.

(Fig. 6A and C), the expression of IRS2 was clearly impaired in PA28 $\gamma^{+/+}$ CoreTg mice at both the transcriptional and translational levels compared with that in other mice (Fig. 6B and C). The serine/threonine protein kinase Akt is phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) under the activated condition of IRS family proteins (26). The insulin-induced phosphorylation of Akt was suppressed in the livers of PA28 $\gamma^{+/+}$ CoreTg mice but not in those of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{-/-}$ , or PA28 $\gamma^{-/-}$ CoreTg mice (Fig. 6D). These results suggest that the expression of the HCV core protein in the livers of mice in the presence of PA28 $\gamma$  impairs the insulin-signaling pathway through the suppression of both the tyrosine phosphorylation of IRS1 and the expression of IRS2.

**PA28 $\gamma$  is required for activation of the TNF- $\alpha$  promoter by HCV core protein.** TNF- $\alpha$  is an adipokine (54) and suppresses the signaling pathway of IRS1 and IRS2 (14, 42). Several reports suggested that the serum TNF- $\alpha$  level is higher in HCV patients than in healthy individuals (19, 37). Elevations of TNF- $\alpha$  levels have also been demonstrated in the livers of PA28 $\gamma^{+/+}$ CoreTg mice (47). To determine the involvement of PA28 $\gamma$  in the enhancement of TNF- $\alpha$  expression, the expression of TNF- $\alpha$  in the livers of each genotype was determined by ELISA and real-time PCR (Fig. 7A and B). Transcription and translation of TNF- $\alpha$  were increased in the livers of PA28 $\gamma^{+/+}$ CoreTg mice but were restored in the livers of PA28 $\gamma^{-/-}$ CoreTg mice to levels comparable to those of PA28 $\gamma^{+/+}$  and PA28 $\gamma^{-/-}$  mice. To determine the effect of PA28 $\gamma$  expression on the promoter activity of TNF- $\alpha$  in human liver cells, PA28 $\gamma$ -knockdown human hepatoma cell lines HepG2 and FLC4 were

established by the introduction of a plasmid encoding a short hairpin RNA targeting the PA28 $\gamma$  gene in the cell lines. The expression of PA28 $\gamma$  was clearly suppressed in the cell lines (Fig. 7C). The expression of HCV core protein in the hepatoma cell lines potentiated TNF- $\alpha$  promoter activity, whereas the promoter activation by the HCV core protein was suppressed in the PA28 $\gamma$ -knockdown cell lines (Fig. 7D). These results suggest that PA28 $\gamma$  is required for the activation of the TNF- $\alpha$  promoter induced by the expression of the HCV core protein in human hepatoma cell lines.

## DISCUSSION

HCV infection has a close association with type 2 diabetes, which is a polygenic disease with a pathophysiology that includes a defect in insulin secretion, increased hepatic glucose production, and resistance to the action of insulin (2, 8, 18). Insulin binds to insulin receptors, which exhibit tyrosine kinase activity, leading to the autophosphorylation and phosphorylation of IRS (56). Tyrosine phosphorylation in IRS proteins leads to the interaction between IRS proteins and the regulatory subunit p85 of PI3-kinase, which enhances glucose uptake and inhibits lipolysis (21). Activated PI3-kinase phosphorylates phosphatidylinositol 4,5-bisphosphate to produce phosphatidylinositol 3,4,5-trisphosphate, which contributes to the activation of PDK1 (55). Activated PDK1 phosphorylates downstream substrates including Akt and other kinases (55). A diabetic phenotype that included insulin resistance was found in IRS2-knockout mice with normal growth (57), although a

knockout of the IRS1 gene has been shown to lead to growth retardation and insulin resistance but not overt diabetes (5, 52). The double knockdown of IRS1 and IRS2 genes in the liver induces hyperinsulinemia and insulin resistance in mice (53). The reduction of both IRS1 and IRS2 under conditions of insulin resistance and hyperinsulinemia (3) and in the livers of *ob/ob* mice, an obese diabetic mouse model (20), has been reported previously. In the present study, the expression of the HCV core protein reduced the phosphorylation of tyrosine on IRS1 and the production of IRS2 in the livers of mice but did not completely abolish the activities of these genes, suggesting that residual activities of IRS transfer a faint signal to the downstream region of IRS. Therefore, PA28 $\gamma^{+/+}$  CoreTg mice may exhibit a milder phenotype than IRS1- and/or IRS2-knockout mice. In this study, knockout of the PA28 $\gamma$  gene restored the insulin sensitivity and signaling of IRS1 and IRS2 in PA28 $\gamma^{+/+}$  CoreTg mice, suggesting that the expression of the HCV core protein leads to the dysfunction of both IRS1 and IRS2 through a PA28 $\gamma$ -dependent pathway.

Our previous study suggested that the induction of TNF- $\alpha$  by the HCV core protein plays a role in insulin resistance (47). An increase in TNF- $\alpha$  levels has been correlated with obesity and insulin resistance in animal models and humans (14, 42). However, the mechanism by which TNF- $\alpha$  induces insulin resistance is not completely known. The expression of TNF- $\alpha$  has been shown to be increased in PA28 $\gamma^{+/+}$  CoreTg mice, resulting in the suppression of phosphorylation of IRS1, and insulin sensitivity in PA28 $\gamma^{+/+}$  CoreTg was improved by the administration of an anti-TNF- $\alpha$  antibody (47). In the present study, the expression level of TNF- $\alpha$  in PA28 $\gamma^{-/-}$  CoreTg mice was similar to that in PA28 $\gamma^{-/-}$  mice or their normal littermates. The expression of the HCV core protein enhanced the promoter activity of the TNF- $\alpha$  gene in human liver cell lines but not in those with a knockdown of the PA28 $\gamma$  gene by RNA interference (Fig. 7D). These data suggest that PA28 $\gamma$  plays a crucial role in HCV core-induced expression of TNF- $\alpha$ . Sterol regulatory element-binding proteins (SREBPs) were shown to be increased at the stage of viremia in HCV-infected chimpanzees (49). SREBPs are known to regulate not only the biosynthesis of lipid but also the transcription of IRS2 and TNF- $\alpha$  (17, 45). Therefore, it might be feasible to speculate that the HCV core protein may cooperate with PA28 $\gamma$  to regulate the expression of SREBPs.

Houstis et al. previously reported that reactive oxygen species (ROS) are increased in both cellular and mouse models of insulin resistance induced by treatment with TNF- $\alpha$  or dexamethasone and that insulin sensitivity was restored by treatment with small antioxidant molecules (16). The HCV core protein potentiates ROS production in hepatoma cells and HCV core gene-transgenic mice (23, 34, 41). Accelerated production of ROS results in mitochondrion dysfunction, which contributes to a decrease in fatty acid oxidation. Defects in mitochondrial fatty acid oxidation enhance the production of intracellular fatty acyl coenzyme A (CoA) and diacylglycerol (48, 58). Mitochondrion dysfunction and accumulation of lipid droplets in mice expressing the HCV core or the full-length HCV polyprotein have been reported (27, 34). An increase in lipid droplets also leads to the accumulation of fatty acid CoA and diacylglycerol (48, 58). Fatty acyl CoA and diacylglycerol nonspecifically activate the Ser/Thr kinase cascade, leading to the enhancement of the serine phosphorylation of IRS1 (26). Serine phosphorylation on IRS1 blocks the tyrosine

phosphorylation of IRS1 by insulin receptors (26). In the present study, however, serine phosphorylation of IRS1 in PA28 $\gamma^{+/+}$  CoreTg mice was similar to that in PA28 $\gamma^{-/-}$  CoreTg mice (data not shown). TNF- $\alpha$  signaling pathways other than the accumulation of ROS and fatty acid intermediates may also participate in the inhibition of tyrosine phosphorylation on IRS1 in PA28 $\gamma^{+/+}$  CoreTg mice.

How does the HCV core protein induce TNF- $\alpha$  production? Our previous report suggests that the HCV core protein is degraded through a PA28 $\gamma$ -dependent pathway (32). Recently, PA28 $\gamma$  has been shown to participate in the proteasome-dependent degradation of steroid receptor coactivator 3 (28). Degradation products of the HCV core protein via the PA28 $\gamma$ -dependent pathway may regulate the promoter activity of the TNF- $\alpha$  gene. PA28 proteins are necessary and sufficient to fully reconstitute Hsp90-initiated refolding together with Hsc70 and Hsp40 (31). Therefore, it might also be feasible to speculate that the HCV core protein refolded by an Hsp90/PA28 $\gamma$ -dependent pathway activates the promoter of the TNF- $\alpha$  gene together with an unknown transcription factor(s) or regulator(s).

In conclusion, the data obtained in this study suggest that the expression of the HCV core protein enhances the production of TNF- $\alpha$  and suppresses the phosphorylation of tyrosine on IRS1 and the production of IRS2 through a PA28 $\gamma$ -dependent pathway, thereby leading to insulin resistance. PA28 $\gamma$  may be a novel target for the treatment of HCV-induced diabetes.

#### ACKNOWLEDGMENTS

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#### REFERENCES

- Aizaki, H., Y. Aoki, T. Harada, K. Ishii, T. Suzuki, S. Nagamori, G. Toda, Y. Matsuura, and T. Miyamura. 1998. Full-length complementary DNA of hepatitis C virus genome from an infectious blood sample. *Hepatology* 27: 621-627.
- Allison, M. E., T. Wreghitt, C. R. Palmer, and G. J. Alexander. 1994. Evidence for a link between hepatitis C virus infection and diabetes mellitus in a cirrhotic population. *J. Hepatol.* 21:1135-1139.
- Anai, M., M. Funaki, T. Ogiwara, J. Terasaki, K. Inukai, H. Katagiri, Y. Fukushima, Y. Yazaki, M. Kikuchi, Y. Oka, and T. Asano. 1998. Altered expression levels and impaired steps in the pathway to phosphatidylinositol 3-kinase activation via insulin receptor substrates 1 and 2 in Zucker fatty rats. *Diabetes* 47:13-23.
- Aoyagi, K., C. Ohue, K. Iida, T. Kimura, E. Tanaka, K. Kiyosawa, and S. Yagi. 1999. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. *J. Clin. Microbiol.* 37:1802-1808.
- Araki, E., M. A. Lipes, M. E. Patti, J. C. Brunning, B. Haag III, R. S. Johnson, and C. R. Kahn. 1994. Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. *Nature* 372:186-190.
- Bukh, J., R. H. Purcell, and R. H. Miller. 1994. Sequence analysis of the core gene of 14 hepatitis C virus genotypes. *Proc. Natl. Acad. Sci. USA* 91:8239-8243.
- Caronia, S., K. Taylor, L. Pagliaro, C. Carr, U. Palazzo, J. Petrik, S. O'Rahilly, S. Shore, B. D. Tom, and G. J. Alexander. 1999. Further evidence for an association between non-insulin-dependent diabetes mellitus and chronic hepatitis C virus infection. *Hepatology* 30:1059-1063.
- Cavaghan, M. K., D. A. Ehrmann, and K. S. Polonsky. 2000. Interactions between insulin resistance and insulin secretion in the development of glucose intolerance. *J. Clin. Investig.* 106:329-333.
- Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 244:359-362.

10. Choo, Q. L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, R. Medina-Selby, P. J. Barr, et al. 1991. Genetic organization and diversity of the hepatitis C virus. *Proc. Natl. Acad. Sci. USA* 88:2451-2455.
11. Falcon, V., N. Acosta-Rivero, G. China, J. Gaviñondo, M. C. de la Rosa, I. Menendez, S. Ducenas-Carrera, A. Vina, W. Garcia, B. Gra, M. Noa, E. Reytor, M. T. Barcelo, F. Alvarez, and J. Morales-Grillo. 2003. Ultrastructural evidences of HCV infection in hepatocytes of chronically HCV-infected patients. *Biochem. Biophys. Res. Commun.* 305:1085-1090.
12. Grakoui, A., D. W. McCourt, C. Wychowski, S. M. Feinstone, and C. M. Rice. 1993. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polypeptide cleavage sites. *J. Virol.* 67:2832-2843.
13. Gumber, S. C., and S. Chopra. 1995. Hepatitis C: a multifaceted disease. Review of extrahepatic manifestations. *Ann. Intern. Med.* 123:615-620.
14. Hotamisligil, G. S. 1999. The role of TNF $\alpha$  and TNF receptors in obesity and insulin resistance. *J. Intern. Med.* 245:621-625.
15. Houghton, M., A. Weiner, J. Han, G. Kuo, and Q. L. Choo. 1991. Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. *Hepatology* 14:381-388.
16. Houstis, N., E. D. Rosen, and E. S. Lander. 2006. Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature* 440:944-948.
17. Ide, T., H. Shimano, N. Yahagi, T. Matsuzaka, M. Nakakuki, T. Yamamoto, Y. Nakagawa, A. Takahashi, H. Suzuki, H. Sone, H. Toyoshima, A. Fukamizu, and N. Yamada. 2004. SREBPs suppress IRS-2-mediated insulin signalling in the liver. *Nat. Cell Biol.* 6:351-357.
18. Kahn, B. B. 1998. Type 2 diabetes: when insulin secretion fails to compensate for insulin resistance. *Cell* 92:593-596.
19. Kallinowski, B., K. Haseröth, G. Marinos, C. Hanck, W. Stremmel, L. Theilmann, M. V. Singer, and S. Rossol. 1998. Induction of tumour necrosis factor (TNF) receptor type p55 and p75 in patients with chronic hepatitis C virus (HCV) infection. *Clin. Exp. Immunol.* 111:269-277.
20. Kerouz, N. J., D. Horsch, S. Pons, and C. R. Kahn. 1997. Differential regulation of insulin receptor substrates-1 and -2 (IRS-1 and IRS-2) and phosphatidylinositol 3-kinase isoforms in liver and muscle of the obese diabetic (ob/ob) mouse. *J. Clin. Investig.* 100:3164-3172.
21. Kido, Y., J. Nakae, and D. Accili. 2001. Clinical review 125: the insulin receptor and its cellular targets. *J. Clin. Endocrinol. Metab.* 86:972-979.
22. Kiyosawa, K., T. Sodeyama, E. Tanaka, Y. Gibo, K. Yoshizawa, Y. Nakano, S. Furuta, Y. Akahane, K. Nishioka, R. H. Purcell, et al. 1990. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 12:671-675.
23. Korenaga, M., T. Wang, Y. Li, L. A. Showalter, T. Chan, J. Sun, and S. A. Weinman. 2005. Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. *J. Biol. Chem.* 280:37481-37488.
24. Kuo, G., Q. L. Choo, H. J. Alter, G. L. Gitnick, A. G. Redeker, R. H. Purcell, T. Miyamura, J. L. Dienstag, M. J. Alter, C. E. Stevens, et al. 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science* 244:362-364.
25. Kuprash, D. V., I. A. Udalova, R. L. Turetskaya, D. Kwiatkowski, N. R. Rice, and S. A. Nedospasov. 1999. Similarities and differences between human and murine TNF promoters in their response to lipopolysaccharide. *J. Immunol.* 162:4045-4052.
26. Lazar, D. F., and A. R. Saltiel. 2006. Lipid phosphatases as drug discovery targets for type 2 diabetes. *Nat. Rev. Drug Discov.* 5:333-342.
27. Lerat, H., M. Honda, M. R. Beard, K. Loesch, J. Sun, Y. Yang, M. Okuda, R. Gosert, S. Y. Xiao, S. A. Weinman, and S. M. Lemon. 2002. Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. *Gastroenterology* 122:352-365.
28. Li, X., D. M. Leonard, S. Y. Jung, A. Malovannaya, Q. Feng, J. Qin, S. Y. Tsai, M. J. Tsai, and B. W. O'Malley. 2006. The SRC-3/AIB1 coactivator is degraded in a ubiquitin- and ATP-independent manner by the REG $\gamma$  proteasome. *Cell* 124:381-392.
29. Mason, A. L., J. Y. Lau, N. Hoang, K. Qian, G. J. Alexander, L. Xu, L. Guo, S. Jacob, F. G. Regenstein, R. Zimmerman, J. E. Everhart, C. Wasserfall, N. K. Maclaren, and R. P. Perrillo. 1999. Association of diabetes mellitus and chronic hepatitis C virus infection. *Hepatology* 29:328-333.
30. McLauchlan, J., M. K. Lemberg, G. Hope, and B. Martoglio. 2002. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. *EMBO J.* 21:3980-3988.
31. Minami, Y., H. Kawasaki, M. Minami, N. Tanahashi, K. Tanaka, and I. Yahara. 2000. A critical role for the proteasome activator PA28 in the Hsp90-dependent protein refolding. *J. Biol. Chem.* 275:9055-9061.
32. Moriishi, K., T. Okabayashi, K. Nakai, K. Moriya, K. Koike, S. Murata, T. Chiba, K. Tanaka, R. Suzuki, T. Suzuki, T. Miyamura, and Y. Matsuura. 2003. Proteasome activator PA28 $\gamma$ -dependent nuclear retention and degradation of hepatitis C virus core protein. *J. Virol.* 77:10237-10249.
33. Moriya, K., H. Fujie, Y. Shintani, H. Yotsuyanagi, T. Tsutsumi, K. Ishibashi, Y. Matsuura, S. Kimura, T. Miyamura, and K. Koike. 1998. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat. Med.* 4:1065-1067.
34. Moriya, K., K. Nakagawa, T. Santa, Y. Shintani, H. Fujie, H. Miyoshi, T. Tsutsumi, T. Miyazawa, K. Ishibashi, T. Horie, K. Imai, T. Todoroki, S. Kimura, and K. Koike. 2001. Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. *Cancer Res.* 61:4365-4370.
35. Moriya, K., H. Yotsuyanagi, Y. Shintani, H. Fujie, K. Ishibashi, Y. Matsuura, T. Miyamura, and K. Koike. 1997. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J. Gen. Virol.* 78:1527-1531.
36. Murata, S., H. Kawahara, S. Tohma, K. Yamamoto, M. Kasahara, Y. Nabeshima, K. Tanaka, and T. Chiba. 1999. Growth retardation in mice lacking the proteasome activator PA28 $\gamma$ . *J. Biol. Chem.* 274:38211-38215.
37. Nelson, D. R., H. L. Lim, C. G. Marousis, J. W. Fang, G. L. Davis, L. Shen, M. S. Urdea, J. A. Kolberg, and J. Y. Lau. 1997. Activation of tumor necrosis factor- $\alpha$  system in chronic hepatitis C virus infection. *Dig. Dis. Sci.* 42:2487-2494.
38. Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108:193-199.
39. Ogino, T., H. Fukuda, S. Imajoh-Ohmi, M. Kohara, and A. Nomoto. 2004. Membrane binding properties and terminal residues of the mature hepatitis C virus capsid protein in insect cells. *J. Virol.* 78:11766-11777.
40. Okamoto, K., K. Moriishi, T. Miyamura, and Y. Matsuura. 2004. Intramembrane proteolysis and endoplasmic reticulum retention of hepatitis C virus core protein. *J. Virol.* 78:6370-6380.
41. Okuda, M., K. Li, M. R. Beard, L. A. Showalter, F. Scholle, S. M. Lemon, and S. A. Weinman. 2002. Mitochondrial injury, oxidative stress, and anti-oxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* 122:366-375.
42. Ozes, O. N., H. Aka, L. D. Mayo, J. A. Gustin, T. Maehama, J. E. Dixon, and D. B. Donner. 2001. A phosphatidylinositol 3-kinase/Akt/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling through insulin receptor substrate-1. *Proc. Natl. Acad. Sci. USA* 98:4640-4645.
43. Rui, L., T. L. Fisher, J. Thomas, and M. F. White. 2001. Regulation of insulin/insulin-like growth factor-1 signaling by proteasome-mediated degradation of insulin receptor substrate-2. *J. Biol. Chem.* 276:40362-40367.
44. Shimoike, T., S. Mimori, H. Tani, Y. Matsuura, and T. Miyamura. 1999. Interaction of hepatitis C virus core protein with viral sense RNA and suppression of its translation. *J. Virol.* 73:9718-9725.
45. Shimomura, I., R. E. Hammer, J. A. Richardson, S. Ikemoto, Y. Bashmakov, J. L. Goldstein, and M. S. Brown. 1998. Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. *Genes Dev.* 12:3182-3194.
46. Shimomura, I., M. Matsuda, R. E. Hammer, Y. Bashmakov, M. S. Brown, and J. L. Goldstein. 2000. Decreased IRS-2 and increased SREBP-1c lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and ob/ob mice. *Mol. Cell* 6:77-86.
47. Shintani, Y., H. Fujie, H. Miyoshi, T. Tsutsumi, K. Tsukamoto, S. Kimura, K. Moriya, and K. Koike. 2004. Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology* 126:840-848.
48. Shulman, G. I. 2000. Cellular mechanisms of insulin resistance. *J. Clin. Investig.* 106:171-176.
49. Su, A. J., J. P. Pezacki, L. Wodicka, A. D. Briden, L. Supekova, R. Thimme, S. Wieland, J. Bukh, R. H. Purcell, P. G. Schultz, and F. V. Chisari. 2002. Genomic analysis of the host response to hepatitis C virus infection. *Proc. Natl. Acad. Sci. USA* 99:15669-15674.
50. Sun, X. J., J. L. Goldberg, L. Y. Qiao, and J. J. Mitchell. 1999. Insulin-induced insulin receptor substrate-1 degradation is mediated by the proteasome degradation pathway. *Diabetes* 48:1359-1364.
51. Suzuki, R., S. Sakamoto, T. Tsutsumi, A. Rikinaru, K. Tanaka, T. Shimoike, K. Moriishi, T. Iwasaki, K. Mizumoto, Y. Matsuura, T. Miyamura, and T. Suzuki. 2005. Molecular determinants for subcellular localization of hepatitis C virus core protein. *J. Virol.* 79:1271-1281.
52. Tamemoto, H., T. Kadowaki, K. Tobe, T. Yagi, H. Sakura, T. Hayakawa, Y. Terauchi, K. Ueki, Y. Kaburagi, S. Satoh, et al. 1994. Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. *Nature* 372:182-186.
53. Taniguchi, C. M., K. Ueki, and R. Kahn. 2005. Complementary roles of IRS-1 and IRS-2 in the hepatic regulation of metabolism. *J. Clin. Investig.* 115:718-727.
54. Uysal, K. T., S. M. Wiesbrock, M. W. Marino, and G. S. Hotamisligil. 1997. Protection from obesity-induced insulin resistance in mice lacking TNF- $\alpha$  function. *Nature* 389:610-614.
55. Vanhaesebroeck, B., and D. R. Alessi. 2000. The PI3K-PDK1 connection: more than just a road to PKB. *Biochem. J.* 346:561-576.

56. White, M. F. 1998. The IRS-signalling system: a network of docking proteins that mediate insulin action. *Mol. Cell. Biochem.* 182:3-11.
57. Withers, D. J., J. S. Gutierrez, H. Towery, D. J. Burks, J. M. Ren, S. Previs, Y. Zhang, D. Bernal, S. Pons, G. I. Shulman, S. Bonner-Weir, and M. F. White. 1998. Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* 391:900-904.
58. Yu, C., Y. Chen, G. W. Cline, D. Zhang, H. Zong, Y. Wang, R. Bergeron, J. K. Kim, S. W. Cushman, G. J. Cooney, B. Atcheson, M. F. White, E. W. Kraegen, and G. I. Shulman. 2002. Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J. Biol. Chem.* 277: 50230-50236.

# Natural killer cell and hepatic cell interaction via NKG2A leads to dendritic cell-mediated induction of CD4<sup>+</sup> CD25<sup>+</sup> T cells with PD-1-dependent regulatory activities

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## Summary

Natural killer (NK) cells have the ability to control dendritic cell (DC)-mediated T cell responses. However, the precise mechanisms by which NK receptor-mediated regulation of NK cells determines the magnitude and direction of DC-mediated T cell responses remain unclear. In the present study, we applied an *in vitro* co-culture system to examine the impact of NK cells cultured with hepatic cells on DC induction of regulatory T cells. We found that interaction of NK cells and non-transformed hepatocytes (which express HLA-E) via the NKG2A inhibitory receptor resulted in priming of DCs to induce CD4<sup>+</sup> CD25<sup>+</sup> T cells with regulatory properties. NKG2A triggering led to characteristic changes of the cytokine milieu of co-cultured cells; an increase in the transforming growth factor (TGF)- $\beta$  involved in the generation of this specific type of DC, and a decrease in the tumour necrosis factor- $\alpha$  capable of antagonizing the effect of TGF- $\beta$ . The regulatory cells induced by NK cell-primed DCs exert their suppressive actions through a negative costimulator programmed death-1 (PD-1) mediated pathway, which differs from freshly isolated CD4<sup>+</sup> CD25<sup>+</sup> T cells. These findings provide new insight into the role of NK receptor signals in the DC-mediated induction of regulatory T cells.

**Keywords:** NK receptor; regulatory T cell; HLA-E; liver; HCV

## Introduction

CD4<sup>+</sup> CD25<sup>+</sup> regulatory T (Treg) cells have been identified as the main suppressors of immune responses.<sup>1-5</sup> Although the mechanisms by which CD4<sup>+</sup> CD25<sup>+</sup> Treg cells exert their suppressive actions have not been fully elucidated, negative costimulatory signals via cytotoxic T lymphocyte antigen-4 (CTLA-4) or inducible costimulator (ICOS)-mediated signals, have been suggested to play a key role in the activation of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells.<sup>6,7</sup> Programmed death-1 (PD-1), another molecule identified as a negative costimulatory receptor, has also serves as a negative regulator for effector immune responses.<sup>8</sup> Recent reports have demonstrated that PD-1 is expressed in CD4<sup>+</sup> CD25<sup>+</sup> Treg cells, suggesting its potential roles in the regulation of T cell tolerance.<sup>9</sup> However, the precise

roles of PD-1 in CD4<sup>+</sup> CD25<sup>+</sup> Treg cell functions remain elusive.

The mechanisms by which CD4<sup>+</sup> CD25<sup>+</sup> Treg cells are generated have been extensively investigated. Dendritic cells (DCs), the sentinels between innate and adaptive immunity, have recently emerged as candidate cells involved in the differentiation and/or activation of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells.<sup>10</sup> Various kinds of factors have been identified as involved in DC induction of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells. Mouse immature DC promotes the differentiation of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells through the DEC 205-mediated targeting of self-antigen in the steady state.<sup>10,11</sup> The immune regulatory cytokines interleukin (IL)-10/transforming growth factor (TGF)- $\beta$  have also been reported to play important roles in DC generation and activation of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells.<sup>12-14</sup>

Abbreviations: CTLA-4, cytotoxic T lymphocyte antigen-4; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; GITR, glucocorticoid-induced TNF receptor; HCV, hepatitis C virus; HLA, human leucocyte antigen; NH, human non-transformed hepatocyte; NK, natural killer; PD-1, programmed death-1; PDL-1, programmed death ligand 1; PBMC, peripheral blood mononuclear cell; Treg, regulatory T.

Several lines of evidence have revealed that natural killer (NK) cell-mediated innate immunity regulates DC functions to determine the direction and magnitude of adaptive T cell immunity.<sup>15–18</sup> It has also been established that NK cell function is regulated by positive and negative signals through their receptor and ligand interactions.<sup>19</sup> We previously reported that, upon exposure to non-transformed hepatocytes (NHs), IL-2-primed NK cells negatively regulated DC functions, which appeared to be dependent on NKG2A inhibitory signals during co-culture of NK cells and NHs. Immunosuppressive cytokines such as IL-10 and TGF- $\beta$ , but not direct NK–DC contact, were responsible for this action.<sup>20</sup> However, it remains unclear whether these NK/hepatocyte co-cultures can also influence the induction as well as activation of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells.

In the present study, we investigated whether DCs stimulated with the co-culture supernatant of IL-2-prestimulated NK cells and NHs can modulate Treg cell functions. We found that TGF- $\beta$  produced from NK cell/hepatocyte co-culture via NKG2A activation is responsible for modulating DCs to induce and maintain regulatory phenotypes and functions of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells. Furthermore, the generated CD4<sup>+</sup> CD25<sup>+</sup> Treg cells suppressed T cell activation via interaction between PD-1 and programmed death ligand 1 (PDL-1). These findings represent new evidence that NK receptor-mediated modulation of NK cells may dictate DC-induced adaptive immunity toward an immunogenic or tolerogenic status via induction of Treg cells.

## Materials and methods

### Antibodies

Anti-NKG2A monoclonal antibody (mAb) (Z199), PC5-labelled CD25 mAb or isotype-matched control IgG1 and IgG2a mAb were purchased from Beckmann-Coulter (Fullerton, CA). Anti-IL-10, anti-TGF- $\beta$ , anti-CTLA-4, anti-GITR (glucocorticoid-induced TNF receptor) and anti-PD-1 polyclonal Abs were purchased from R & D Systems (Minneapolis, MN) and phycoerythrin (PE)-labelled mAb CTLA-4 from BD Biosciences (San Jose, CA). Anti-HLA-E mAb 3D12 was kindly provided by Dr E. Geraghty (Fred Hutchinson Cancer Research Institute, Seattle, WA) and used as reported previously.<sup>21</sup> Anti-MIC mAb 6D4, anti-ULBP1 mAb 3F1 and anti-ULBP2 mAb DH1 were kindly provided by Drs T. Spies and V. Groh (Fred Hutchinson Cancer Research Institute) and used as reported previously.<sup>22</sup>

### Human hepatic cells

Human non-transformed hepatocytes (NHs) derived from mixed heterogeneous donors were purchased from the

Applied Cell Biology Research Institute (Kirkland, WA) and cultured in CS-C complete medium according to the manufacturer's instructions.

### Isolation of peripheral blood lymphocyte populations

Resting NK cells (CD56<sup>+</sup> CD3<sup>+</sup>), naive CD4<sup>+</sup> T cells (CD45RA<sup>+</sup> RO<sup>+</sup>) or CD8<sup>+</sup> T cells were isolated from peripheral blood mononuclear cells (PBMCs) with a positive cell isolation kit according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4<sup>+</sup> CD25<sup>+</sup> T cells were further separated from naive CD4<sup>+</sup> T cells using anti-CD25 microbeads (Miltenyi Biotec). Their purity was >90% by flow cytometry. Informed consent was obtained from all blood donors.

### Generation of monocyte-derived DC

Monocytes were isolated by plastic adherence from PBMCs and cultured in RPMI-1640 supplemented with granulocyte–macrophage colony stimulating factor (GM-CSF) (PeproTech, London, UK) and IL-4 (PeproTech): At day 6, they were stimulated with or without the co-culture supernatant of NK cells and hepatic cells. At day 7, non-adherent cells were harvested and used as described below.

### Stimulation of DCs by co-culture supernatants of NK cells and hepatic cells

Freshly isolated NK cells were cultured with or without IL-2 for 24 hr. IL-2-prestimulated or non-stimulated NK cells were seeded in 24-well plates and then co-cultured for 24 hr with NHs ( $1 \times 10^5$  cells/well), respectively. Monocyte-derived DCs were cultured for 24 hr with 1 ml of the co-culture supernatant of IL-2-prestimulated NK cells and NHs (NH/IL-2 NK-primed DC). In some experiments, anti-NKG2A mAb (Z199) or isotype-matched control Ab was added during the co-cultures of NK cells and hepatic cells. Z199 mAb was previously confirmed to block the NKG2A-mediated signal.<sup>23</sup> In some experiments, the supernatant of NK/hepatic cell co-cultures was also treated with anti-IL-10 or anti-TGF- $\beta$  neutralizing Ab and used for DC stimulation for 24 hr. In some experiments, tumour necrosis factor (TNF)- $\alpha$ , TGF- $\beta$  or both were used for DC stimulation for 24 hr.

### Isolation of CD4<sup>+</sup> CD25<sup>+</sup> T cells

DCs ( $1 \times 10^5$ ) were stimulated for 24 hr with the supernatant obtained from the co-cultured medium. After washing three times, DCs were cultured with allogeneic CD4<sup>+</sup> T cells for 48 hr; CD4<sup>+</sup> CD25<sup>+</sup> fractions were isolated from DC and CD4<sup>+</sup> co-culture and subjected to further analysis. CD4<sup>+</sup> CD25<sup>+</sup> fractions were also isolated

from PBMCs and cultured with 1 µg/ml plate-bound anti-CD3 mAb (UCHT1; Beckmann-Coulter) for 24 hr to efficiently induce their suppressive properties as described previously.<sup>3</sup> These cells are referred to as natural CD4<sup>+</sup> CD25<sup>+</sup> T cells.

#### Flow cytometry

The expression of NK inhibitory ligands (human leucocyte antigen, HLA, class I, HLA-E) was examined on NHs by using w6/32 or 3D12, respectively. MIC, ULBP1 or ULBP2 expression on hepatocytes was also evaluated by mAb 6D4, 3F1 or DH1, respectively. For CD4<sup>+</sup> CD25<sup>+</sup> T cell staining, the cells were costained with PC5-labelled CD25 mAb with PE-labelled mAb of CTLA-4, GITR or PD-1 polyclonal Ab. The cells were analysed by flow cytometry using a fluorescence-activated cell sorter (FACScan) system, and data analysis was performed using CELLQUEST software.

#### Measurements of cytokine production in culture supernatant

The culture supernatants of interferon (IFN)-γ, TNF-α, IL-10 and TGF-β were examined using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions (IFN-γ, TNF-α and IL-10, Endogen, Tokyo, Japan; TGF-β, R & D Systems).

#### Analysis of Foxp3 mRNA expression

Polymerase chain reaction (PCR) analysis was performed to determine Foxp3 mRNA expression of CD4<sup>+</sup> T cells using a commercial PCR panel according to the manufacturer's instructions (Gibco BRL, Rockville, MD). The following primers were used: 5'-CCCACTTACAGGCACTCTC-3' (forward) and 5'-CTTCTCCTTCTCCAGCACCA-3' (reverse).<sup>24</sup> Amplification was carried out for 35 cycles of 20 seconds at 95°, 20 seconds at 58° and 30 seconds at 72°. As a control for the integrity of mRNA, primers specific for GAPDH (glyceraldehyde 3-phosphate dehydrogenase) were used as follows: 5'-GCCACCCAGAAGACTGTGGATGGC-3' (forward) and 5'-CATGTAGGCCATGAGGTCCACCAC-3' (reverse). The PCR products were analysed by ethidium bromide-stained 1.5% agarose gel electrophoresis.

#### Analysis of CD4<sup>+</sup> CD25<sup>+</sup> T cell suppressor functions

DCs (5 × 10<sup>4</sup>/well) were cultured with allogeneic CD4<sup>+</sup> T cells (5 × 10<sup>5</sup>/well) for 48 hr, after which CD4<sup>+</sup> CD25<sup>+</sup> T cells were isolated from the co-cultured cells. CD4<sup>+</sup> CD25<sup>-</sup> T cells were freshly isolated from the same donors and activated with 1 µg/ml plate-bound anti-CD3 mAb in the presence or absence of autologous

CD4<sup>+</sup> CD25<sup>+</sup> T cells for 48 hr. The ability of CD4<sup>+</sup> CD25<sup>+</sup> T cells to suppress proliferation and IFN-γ production of activated CD4<sup>+</sup> CD25<sup>-</sup> T cells was determined by [<sup>3</sup>H]thymidine incorporation and ELISA assay, respectively. To further examine the mechanisms of CD4<sup>+</sup> CD25<sup>+</sup> T cell suppressive actions, neutralizing Ab of IL-10 or TGF-β, anti-CTLA-4, anti-GITR or anti-PD-1 was added at the beginning of CD4<sup>+</sup> CD25<sup>+</sup> T cell and CD4<sup>+</sup> CD25<sup>-</sup> T cell co-cultures.

#### Statistical analysis

Comparisons between groups were analysed by *t*-test with Welch's correction or ANOVA for experiments with more than two subgroups. Differences were considered significant when the *P*-value was < 0.05.

## Results

#### IL-2-primed NK cells upon exposure to NH-modulated DCs on the induction of regulatory CD4<sup>+</sup> CD25<sup>+</sup> T cells

Natural CD4<sup>+</sup> CD25<sup>+</sup> T cells from human peripheral blood lymphocytes (PBLs) expressed CTLA-4 and GITR, both of which have been identified as regulatory markers,<sup>6,25</sup> but did not express PD-1 (Fig. 1a). To examine whether DCs can modulate the expression of these regulatory markers on CD4<sup>+</sup> CD25<sup>+</sup> T cells, we stimulated monocyte-derived DCs for 24 hr, either by the culture supernatant of IL-2-stimulated NK cells (IL-2 NK) or by the co-culture supernatant of NH/IL-2 NK. After washing, the resulting DCs were cultured for 48 hr with CD4<sup>+</sup> T cells isolated from allogeneic donors. CD4<sup>+</sup> CD25<sup>+</sup> T cells were isolated from the DC and CD4<sup>+</sup> T cell co-culture and subjected to analysis for regulatory markers. The expression levels of CTLA-4 and GITR decreased on CD4<sup>+</sup> CD25<sup>+</sup> T cells after stimulation of IL-2 NK-primed DCs (Fig. 1b). By contrast, CD4<sup>+</sup> CD25<sup>+</sup> T cells stimulated with NH/IL-2 NK-primed DCs remained positive for CTLA-4 and GITR on their surface. Of note is the finding that PD-1 was induced on these cells, showing their phenotypic properties to differ from natural CD4<sup>+</sup> CD25<sup>+</sup> T cells (Fig. 1b, c). The induction of PD-1 on CD4<sup>+</sup> CD25<sup>+</sup> T cells was further confirmed when IL-2NK/NH-primed DCs from different donors were used as stimulators (Fig. 1d). The supernatant of NH without NK cells had little effect on phenotypic changes of CD4<sup>+</sup> CD25<sup>+</sup> T cells by DCs (data not shown).

The forkhead transcription factor Foxp3 has been recently identified as a master gene for defining Treg cells.<sup>26</sup> We therefore performed reverse transcription-PCR (RT-PCR) analysis of CD4<sup>+</sup> T cells to evaluate the mRNA expression of Foxp3. Foxp3 expression was detected in natural CD4<sup>+</sup> CD25<sup>+</sup> T cells. When CD4<sup>+</sup> T cells were

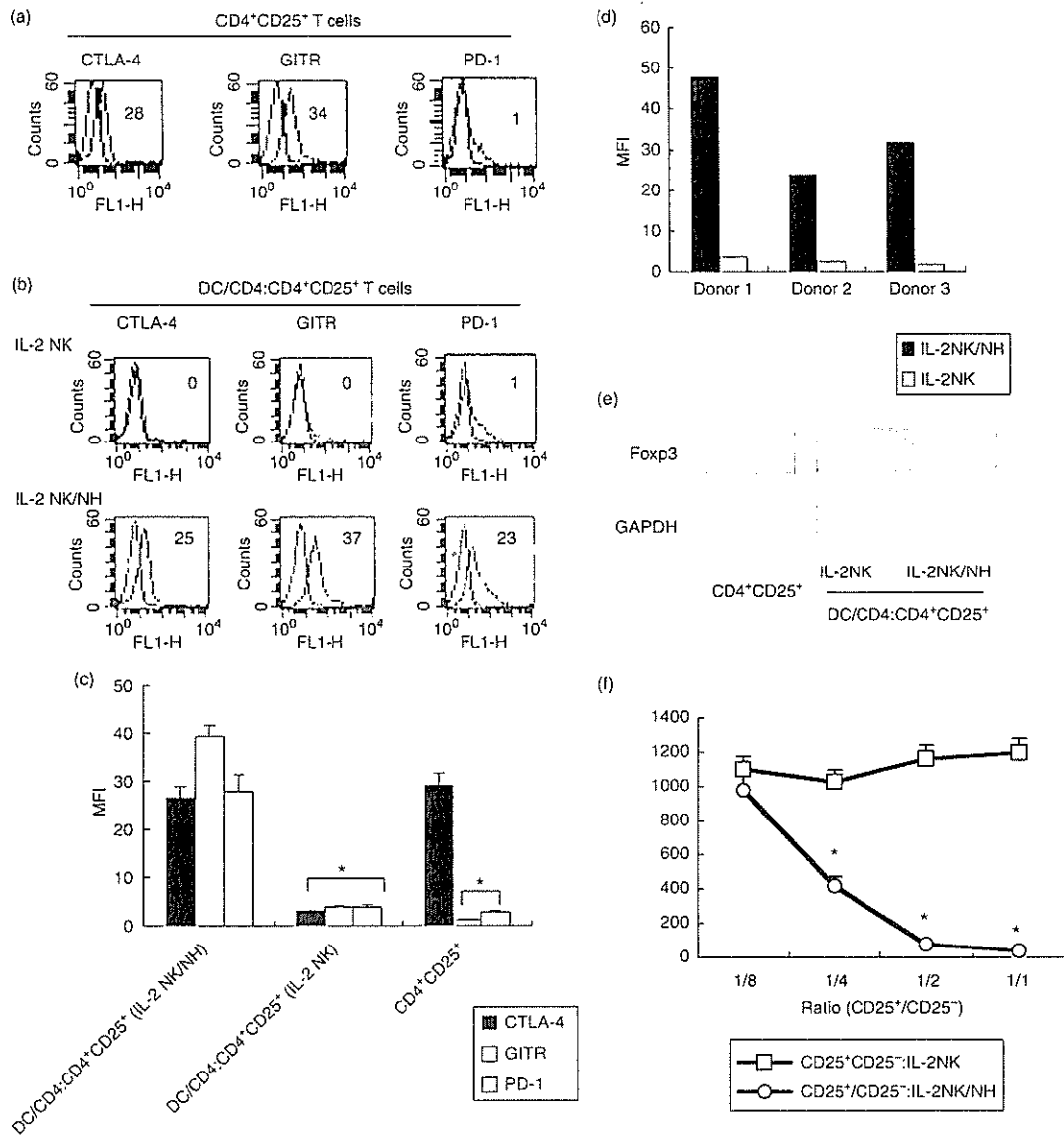


Figure 1. Human non-transformed hepatocyte (NH) modulation of activated natural killer (NK) cells endows dendritic cells (DCs) with the ability to induce CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells. (a) Freshly isolated CD4<sup>+</sup> CD25<sup>+</sup> T cells were cultured in the presence of plate-bound anti-CD3 antibody (Ab) for 24 hr, and then subjected to flow cytometry to examine their expression of cytotoxic T lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced TNF receptor (GITR) and programmed death-1 (PD-1) (closed histograms). Open histograms represent the staining of control Ab. Numbers on the upper right indicate the mean fluorescence intensity (MFI) of each type of stained cells. (b) NK cells were preactivated with 50 ng/ml interleukin (IL)-2, and co-cultured in the absence (IL-2 NK) or presence (IL-2 NK/NH) of NHs at a ratio of 1 : 1 for 24 hr. DCs (1 × 10<sup>5</sup>) were stimulated for 24 hr with the supernatant obtained from the co-cultured medium. After washing three times, DCs were cultured with allogeneic CD4<sup>+</sup> T cells for 48 hr. CD4<sup>+</sup> CD25<sup>+</sup> fractions were isolated from the DC/CD4<sup>+</sup> T cell co-culture and subjected to flow cytometry for expression of CTLA-4, GITR or PD-1 (closed histograms). Open histograms show isotype control staining. Numbers on the upper right indicate the MFI of each type of stained cell. (c) All experiments in (a) and (b) were performed three times and the composite results with statistical analysis are shown as the MFI of the staining cells. \*P < 0.05 vs. responses of IL-2 NK/NH group. The experiment was performed with a different set of donors and similar results were obtained. (d) PD-1 expression on CD4<sup>+</sup> CD25<sup>+</sup> T cells stimulated with allogeneic DCs from three different donors, shown as the MFI. (e) CD4<sup>+</sup> CD25<sup>+</sup> T cells were prepared as described above. The mRNA expression of Foxp3 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was examined by reverse transcription-polymerase chain reaction (RT-PCR). (f) CD4<sup>+</sup> CD25<sup>+</sup> fractions were isolated from DC/CD4<sup>+</sup> T cell co-cultures. Different numbers of these CD4<sup>+</sup> CD25<sup>+</sup> T cells were co-cultured with freshly isolated autologous CD4<sup>+</sup> CD25<sup>-</sup> T cells (1 × 10<sup>5</sup>/well) in the presence of plate-bound anti-CD3 Ab (CD4<sup>+</sup> CD25<sup>+</sup>/CD4<sup>+</sup> CD25<sup>-</sup>). The anti-CD3 Ab-activated CD4<sup>+</sup> CD25<sup>-</sup> T cells alone were used as a positive control (CD4<sup>+</sup> CD25<sup>-</sup>). IFN-γ was measured for each supernatant obtained after 48 hr of co-culture by enzyme-linked immunosorbent assay. \*P < 0.05.



stimulated with IL-2 NK-primed DCs for 24 hr, Foxp3 was not expressed on CD4<sup>+</sup> CD25<sup>+</sup> T cells. By contrast, they dominantly transcribed Foxp3 at levels comparable with those of natural CD4<sup>+</sup> CD25<sup>+</sup> T cells when stimulated with NH/IL-2 NK-primed DCs (Fig. 1e). Taken together, CD4<sup>+</sup> CD25<sup>+</sup> T cells, when stimulated by NH/IL-2 NK-primed DCs, maintained regulatory phenotypes such as CTLA-4, GITR and Foxp3, and properties distinct from those of natural CD4<sup>+</sup> CD25<sup>+</sup> Treg cells in terms of PD-1 expression.

#### CD4<sup>+</sup> CD25<sup>+</sup> T cells on stimulation of NH/IL-2 NK-primed DC suppressed effector cell functions

We next analysed the functions of CD4<sup>+</sup> CD25<sup>+</sup> T cells stimulated by NH/IL-2 NK-primed DC. CD4<sup>+</sup> CD25<sup>+</sup> T cells were co-cultured for 72 hr with CD4<sup>+</sup> CD25<sup>+</sup> T cells freshly isolated from the same donors. During the co-cultures, CD4<sup>+</sup> CD25<sup>+</sup> T cells were stimulated with plate-bound anti-CD3 Ab. The CD4<sup>+</sup> CD25<sup>+</sup> T cells induced by NH/IL-2 NK-primed DCs dose-dependently suppressed the proliferation of co-cultured cells, whereas those induced by IL-2 NK-primed DC did not (data not shown). CD4<sup>+</sup> CD25<sup>+</sup> T cells induced by NH/IL-2 NK-primed DCs also dose-dependently inhibited IFN- $\gamma$  production of the co-cultured cells, by contrast with those induced by IL-2 NK-primed DCs (Fig. 1f). The suppressive activities of these CD4<sup>+</sup> CD25<sup>+</sup> Treg cells were similar to those of natural CD4<sup>+</sup> CD25<sup>+</sup> Treg cells (data not shown). These results demonstrate that CD4<sup>+</sup> CD25<sup>+</sup> T cells induced by NH/IL-2 NK-primed DCs exert suppressive actions to effector cell functions, consistent with their expression of regulatory markers. Taken together, these results indicated that NK cell modulation of DCs leads to the CD4<sup>+</sup> CD25<sup>+</sup> Treg cell-mediated suppression of effector cell responses when NK cells encounter hepatocytes.

#### NKG2A signal of NK cells is responsible for the modulation of DCs to activate CD4<sup>+</sup> CD25<sup>+</sup> Treg cells

We examined the expression of various ligands for NK cell receptors on NHs. NHs expressed HLA-E, the ligand of NKG2A, but did not express NKG2D receptor ligands, MIC and ULBP1-2 (Fig. 2a). Given our previous findings that NHs negatively regulated IL-2 NK-mediated modulation of DC functions through the interaction of the NKG2A inhibitory receptor and its ligand HLA-E,<sup>20</sup> we evaluated the role of these receptor signals in the induction of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells by DCs. When anti-NKG2A Ab was added during the co-culture of NH and IL-2 NK and DCs were stimulated with the resultant supernatant, the expression of CTLA-4, GITR and PD-1 was diminished on CD4<sup>+</sup> CD25<sup>+</sup> T cells (Fig. 2b, c).

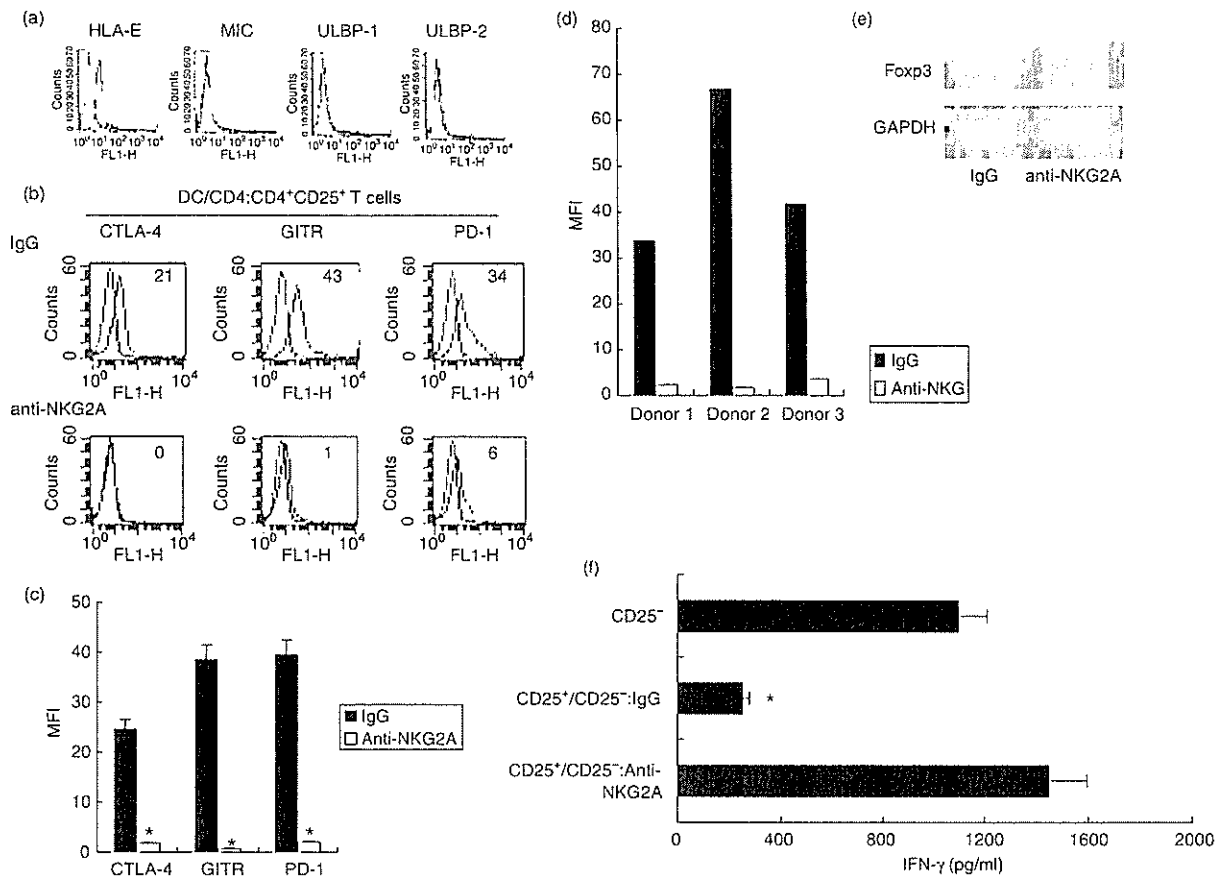
NKG2A blockade also suppressed PD-1 expression on CD4<sup>+</sup> CD25<sup>+</sup> T cells stimulated with IL-2/NH-primed DCs from three different donors (Fig. 2d). The anti-NKG2A neutralizing Ab treatment also abrogated Foxp3 expression in CD4<sup>+</sup> CD25<sup>+</sup> Treg cells (Fig. 2e). Moreover, the blockade of NKG2A signals during NH and IL-2 NK co-cultures resulted in inhibition of the DC ability to induce CD4<sup>+</sup> CD25<sup>+</sup> T cells with regulatory functions; these CD4<sup>+</sup> CD25<sup>+</sup> T cells did not suppress proliferation or IFN- $\gamma$  production (Fig. 2f and data not shown) of CD4<sup>+</sup> CD25<sup>+</sup> T cells. Altogether, the activation of NKG2A inhibitory signals during NK cell and hepatocyte interaction was required for the DC induction of CD4<sup>+</sup> CD25<sup>+</sup> T cells with regulatory phenotypes and functions.

#### Change of cytokine milieu, triggered by NKG2A signals, plays a critical role in DC-mediated induction of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells

TNF- $\alpha$  has been well known as a critical factor for NK cell-mediated maturation of DCs.<sup>27</sup> By contrast, IL-10 and TGF- $\beta$  are known to act as suppressive factors of effector immune responses, and their roles in modulating DCs for Treg cell induction has recently been validated.<sup>12-14</sup> These findings led us to evaluate the change in cytokine production patterns in NH and IL-2 NK co-cultures in the presence or absence of anti-NKG2A Ab. ELISA data showed that the production of IFN- $\gamma$  and TNF- $\alpha$  from NH and IL-2 NK co-cultures were substantially increased in the presence of anti-NKG2A Ab. By contrast, the addition of NKG2A masking Ab during the co-culture resulted in the marked reduction of IL-10 and TGF- $\beta$  from co-cultured cells (Fig. 3a).

We next examined whether these changes of cytokine profiles were responsible for the DC induction of the CD4<sup>+</sup> CD25<sup>+</sup> Treg cells. For this purpose, the NH and IL-2 NK co-culture supernatant was treated with neutralizing Ab of IL-10 or TGF- $\beta$  before DC stimulation, and suppressive activity was evaluated by analysing CD4<sup>+</sup> CD25<sup>+</sup> T cells obtained from CD4<sup>+</sup> and DC mixtures. The neutralization of IL-10 did not reverse the suppressive actions of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells, but the blockade of TGF- $\beta$  led to reversal of CD4<sup>+</sup> CD25<sup>+</sup> Treg cell activities (Fig. 3b).

We directly examined the effect of TGF- $\beta$  on the modulation of DC ability to induce CD4<sup>+</sup> CD25<sup>+</sup> Treg cells. TGF- $\beta$  endowed DCs with the ability to induce CD4<sup>+</sup> CD25<sup>+</sup> Treg cells. TNF- $\alpha$  inhibited TGF- $\beta$ -mediated DC induction of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells (Fig. 3c). By contrast, IFN- $\gamma$  had little effect on the modulation of DC by TGF- $\beta$  (data not shown). Taken together, these results strongly suggest that increased TGF- $\beta$  and decreased TNF- $\alpha$  production, the change of cytokine profiles mediated by the NKG2A signals, are involved in DC-mediated CD4<sup>+</sup> CD25<sup>+</sup> Treg cell induction.



**Figure 2.** NKG2A signals of natural killer (NK) cells are required for the dendritic cell (DC) induction of CD4<sup>+</sup> CD25<sup>+</sup> T cells with the regulatory phenotype. (a) Surface expression of the ligands of NKG2A (HLA-E) as well as NKG2D (MIC, ULBP1 and ULBP2) in human non-transformed hepatocytes (NHs) were assessed by flow cytometry (closed histograms). Open histograms show isotype control staining. (b, c) Interleukin (IL)-2-primed NK cells were co-cultured with NHs in the presence of 30  $\mu$ g/ml of anti-NKG2A neutralizing antibody (Ab) (anti-NKG2A) or control IgG. DCs ( $1 \times 10^5$ ) were then stimulated with the supernatant obtained from the co-cultured medium for 24 hr. After washing three times, DCs were cultured with allogeneic CD4<sup>+</sup> T cells for 48 hr. CD4<sup>+</sup> CD25<sup>+</sup> cells isolated from the co-culture were subjected to FCM for their surface expression of cytotoxic T lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced TNF receptor (GITR) and programmed death-1 (PD-1) (closed histograms). Open histograms show isotype control staining. Numbers on the upper right indicate the mean fluorescence intensity (MFI) of each type of stained cell. All experiments were performed three times. Representative data (b) and composite results with statistical analysis (c) are shown as the MFI of the staining cells. \* $P < 0.05$  vs. responses of IgG group. The experiment was performed in different set of donors and similar results were obtained. (d) The inhibitory effect of anti-NKG2A Ab on PD-1 expression of CD4<sup>+</sup> CD25<sup>+</sup> T cells stimulated with allogeneic DCs from three different donors. Data are shown as MFI. (e) CD4<sup>+</sup> CD25<sup>+</sup> T cells were stimulated and purified as described above. The mRNA expression of Foxp3 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was examined by reverse transcription-polymerase chain reaction (RT-PCR). (f) CD4<sup>+</sup> CD25<sup>+</sup> T cells ( $1 \times 10^5$ /well) isolated from DC and CD4<sup>+</sup> T cell co-cultures were cultured with freshly isolated autologous CD4<sup>+</sup> CD25<sup>-</sup> T cells at a ratio of 1 : 1 in the presence of plate-bound anti-CD3 Ab (CD25<sup>+</sup>/CD25<sup>-</sup>). The anti-CD3 Ab-activated CD4<sup>+</sup> CD25<sup>-</sup> T cells alone were used as a positive control (CD25<sup>-</sup>). Interferon (IFN)- $\gamma$  was measured for each supernatant obtained after 48 hr of co-culture by enzyme-linked immunosorbent assay (ELISA). \* $P < 0.05$ . All experiments were performed three times; representative results are shown.

**Suppressive actions of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells, induced by NH/IL-2 NK-primed DCs, depends on PD-1-mediated negative costimulatory signals**

The suppressive activities of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells reportedly depend on various kinds of mediators, such as CTLA-4, IL-10 and/or TGF- $\beta$ , but the exact mechanisms of the actions have not been fully elucidated.<sup>1,6,12-14</sup>

PD-1, recently identified as a negative costimulatory receptor of the B-7 family, is expressed in CD4<sup>+</sup> CD25<sup>+</sup> Treg cells, indicating that PD-1-mediated negative signals may be involved in the regulatory functions of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells.<sup>9</sup> Thus, we evaluated the involvement of these molecules in the suppressive activities of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells. For this purpose, the blocking Ab of CTLA-4, GITR, PD-1, TGF- $\beta$  or IL-10 was added

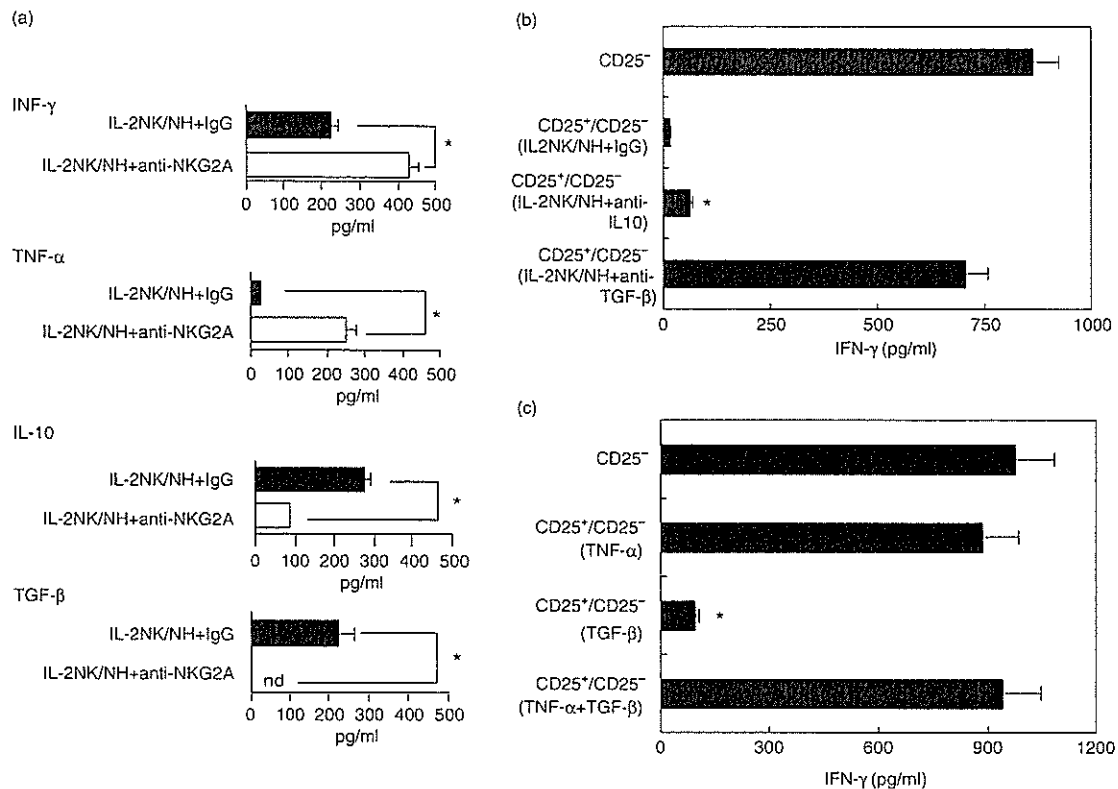


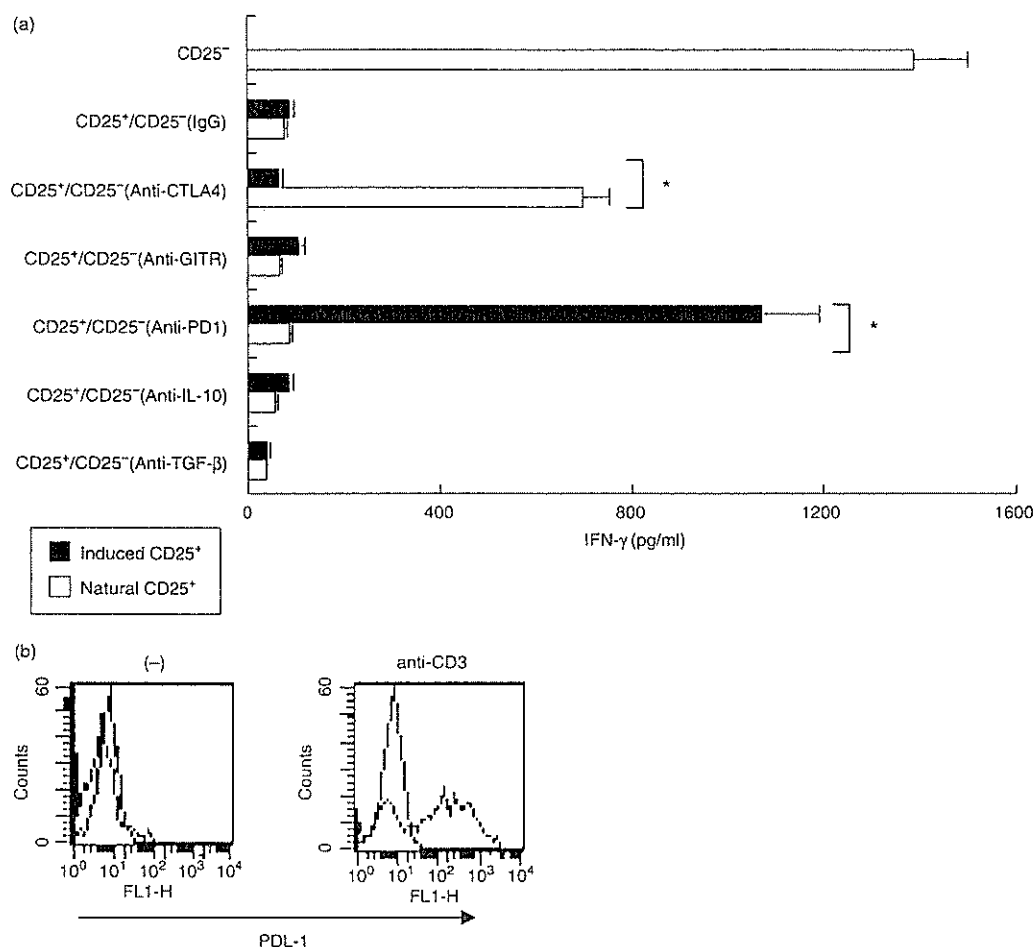
Figure 3. Change of cytokine production pattern of natural killer (NK) cells through NKG2A signals is responsible for the dendritic cell (DC) induction of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells. (a) NK cells prestimulated with interleukin (IL)-2 were cultured with human non-transformed hepatocytes (NHs) in the presence of masking antibodies (Abs) of NKG2A (IL-2 NK/NH + anti-NKG2A) or isotype control IgG (IL-2 NK/NH + IgG) for 24 hr. \**P* < 0.05. (b) IL-2 activated NK cells were co-cultured with NHs (IL-2 NK/NH). DCs ( $1 \times 10^5$ ) were stimulated with the culture supernatant in the presence of anti-IL-10, anti-transforming growth factor (TGF)-β neutralizing Ab or control IgG for 24 hr. DCs were washed thoroughly and co-cultured with allogeneic CD4<sup>+</sup> T cells for 48 hr. Next, the isolated CD4<sup>+</sup> CD25<sup>+</sup> T cells ( $1 \times 10^5$ /well) were co-cultured with autologous CD4<sup>+</sup> CD25<sup>-</sup> T cells in the presence of plate-bound anti-CD3 Ab at a ratio of 1 : 1. Interferon (IFN)-γ production from the culture supernatant was examined by enzyme-linked immunosorbent assay. \**P* < 0.05 vs. responses of anti-CD3 Ab-stimulated CD4<sup>+</sup> CD25<sup>-</sup> T cells. (c) DCs ( $1 \times 10^5$ ) were stimulated with 50 ng/ml TNF-α, 100 ng/ml TGF-β or both for 24 hr. After thorough washing, they were co-cultured with allogeneic CD4<sup>+</sup> T cells for 48 hr. CD4<sup>+</sup> CD25<sup>+</sup> T cells ( $1 \times 10^5$ /well) were isolated from the DC and CD4<sup>+</sup> co-cultures and cultured with freshly isolated autologous CD4<sup>+</sup> CD25<sup>-</sup> T cells at a ratio of 1 : 1 in the presence of plate-bound anti-CD3 Ab. IFN-γ production was examined as described above. \**P* < 0.05 vs. responses of anti-CD3 Ab-stimulated CD4<sup>+</sup> CD25<sup>-</sup> T cells.

during co-cultures of CD4<sup>+</sup> CD25<sup>+</sup>/CD4<sup>+</sup> CD25<sup>-</sup> T cells in the presence of anti-CD3 Ab. In case of natural CD4<sup>+</sup> CD25<sup>+</sup> T cells, their suppressive action was partially reversed on addition of anti-CTLA-4 Ab. By contrast, they preserved their suppressive capacity even in the presence of the blocking Ab of GITR, PD-1, TGF-β or IL-10 (Fig. 4a). When CD4<sup>+</sup> CD25<sup>+</sup> Treg cells induced by NH/IL-2 NK-primed DCs were used instead of natural CD4<sup>+</sup> CD25<sup>+</sup> T cells, their suppressive activity was markedly reduced on addition of the blocking Ab of PD-1 but not CTLA-4, IL-10, TGF-β or GITR (Fig. 4a). The regulatory functions of these Treg cells were required for direct cell-to-cell contact because separation of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells and CD4<sup>+</sup> CD25<sup>-</sup> T cells in transwell chambers virtually abolished their suppressive effects (data not shown). We also confirmed the presence of PDL-1

expression on CD4<sup>+</sup> CD25<sup>-</sup> T cells when they were activated with anti-CD3 Ab (Fig. 4b), suggesting that effector cells themselves induce suppressive activities of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells. Taken together, these results further reinforced the hypothesis that CD4<sup>+</sup> CD25<sup>+</sup> Treg cells induced by NH/IL-2 NK-primed DCs were different from natural CD4<sup>+</sup> CD25<sup>+</sup> Treg cells in their PD-1-dependent suppressive functions.

## Discussion

Recent studies have revealed that activated NK cells positively regulate DC activation and maturation either through direct contact via NK cell receptors (NKP30, NKG2D, etc.) or in co-ordination with various kinds of cytokines (IFN-γ, TNF-α, etc.).<sup>15-18</sup> However, the issue of



**Figure 4.** CD4<sup>+</sup> CD25<sup>+</sup> Treg cells induced by interleukin (IL)-2 natural killer (NK)/human non-transformed hepatocytes (NH)-treated dendritic cell (DC) suppressed T cell activation through programmed death-1 (PD-1)/programmed death ligand-1 (PDL-1) interactions. (a) DCs ( $1 \times 10^5$ ) were stimulated with the IL-2 NK/NH supernatant for 24 hr, and then cultured with allogeneic CD4<sup>+</sup> T cells for 48 hr. CD4<sup>+</sup> CD25<sup>+</sup> fractions were isolated from the DC/CD4<sup>+</sup> T cell mixtures. Freshly isolated CD4<sup>+</sup> CD25<sup>+</sup> T cells (natural CD25<sup>+</sup>) or CD4<sup>+</sup> CD25<sup>+</sup> T cells induced by NK/NH-primed DCs (induced CD25<sup>+</sup>) were co-cultured with freshly isolated autologous CD4<sup>+</sup> CD25<sup>-</sup> T cells at a ratio of 1 : 1 upon stimulation of plate-bound anti-CD3 antibody (Ab). Anti-CTLA-4 (cytotoxic T lymphocyte antigen-4) Ab, anti-GITR (glucocorticoid-induced TNF receptor) Ab, anti-PD-1 Ab, anti-IL-10 Ab, anti-TGF-β Ab or isotype control IgG (20 μg/ml for each) were incubated during CD4<sup>+</sup> CD25<sup>+</sup>/CD4<sup>+</sup> CD25<sup>-</sup> T cell co-cultures. Interferon (IFN)-γ was measured for each supernatant obtained after 72 hr of co-culture by enzyme-linked immunosorbent assay. \**P* < 0.05 vs. responses of anti-CD3 Ab-stimulated CD4<sup>+</sup> CD25<sup>-</sup> T cells. (b) Freshly isolated CD4<sup>+</sup> CD25<sup>-</sup> T cells were incubated with (anti-CD3) or without (-) plate-bound anti-CD3 Ab for 24 hr. PDL-1 expression was assessed by flow cytometry (closed histograms). Open histograms show isotype control staining.

whether NK cells are involved in DC-mediated Treg cell induction has not been resolved. In the present study, we report that the expression of regulatory markers and functions was markedly decreased on CD4<sup>+</sup> CD25<sup>+</sup> T cells upon exposure to IL-2 NK-primed DCs. By contrast, the interaction of activated NK cells and NH through the NKG2A inhibitory receptor led to DC induction of CD4<sup>+</sup> CD25<sup>+</sup> T cells with regulatory properties. Furthermore, NKG2A-mediated increase in TGF-β as well as decrease in TNF-α in an NH and NK cell mixture contributed to DC induction of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells. This is consistent with previous reports showing that TGF-β

plays a role in generating the specific DC that activates CD4<sup>+</sup> CD25<sup>+</sup> Treg cells.<sup>10,11</sup> The findings that TNF-α suppressed TGF-β-mediated priming of DCs to induce Treg cells also extended the previously identified role of TNF-α as a positive regulator of DC activation. In line with our findings, previous reports showed that impairment of CD4<sup>+</sup> CD25<sup>+</sup> Treg cell activities restored their suppressive functions after blocking TNF-α signals in non-obese diabetic (NOD) mice or in patients with Crohn's disease.<sup>28,29</sup> To our knowledge, the present study is the first description of modulation of NK cells and human hepatocytes through NKG2A-mediated inhibitory