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Expression of Human CD81 in Transgenic Mice Does Not Confer Susceptibility to Hepatitis C Virus Infection

Francesca Masciopinto,* Giulia Freer,† Vito L. Burgio,‡ Shoshana Levy,§ Luisa Galli-Stampino,* Mauro Bendinelli,† Michael Houghton,¶ Sergio Abrignani,*,¶ and Yasushi Uematsu*,[1]

*Department of Immunology, IRIS, Chiron S.p.A., I-53100, Siena, Italy; †Centro Retrovirus and Dipartimento di Biomedicina, University of Pisa, I-56127, Pisa, Italy; ‡Fondazione "A. Cesalpino," Dipartimento Medicina Interna, Università La Sapienza, Policlinico Umberto I, I-00161 Rome, Italy; §Division of Oncology, Department of Medicine, Stanford University Medical Center, Stanford, California 94305; and ¶Chiron Corporation, Emeryville, California 94068

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We previously demonstrated that hepatitis C virus (HCV) binds to human CD81 through the E2 glycoprotein. Therefore, expression of the human CD81 molecule in transgenic mice was expected to provide a new tool to study HCV infection *in vivo*, as the chimpanzee is the only species currently available as a laboratory animal model that can be infected with HCV. We produced transgenic mice expressing the human CD81 protein in a wide variety of tissues. We confirmed binding of recombinant E2 glycoprotein to the liver tissue as well as to thymocytes and splenic lymphocytes in the transgenic mice. We inoculated chimpanzee plasma infected with HCV into these animals. None of these transgenic animals showed evidence of viral replication. Furthermore, human CD81 transgenic mice that lack expression of endogenous mouse CD81 were also resistant to HCV infection. We conclude that expression of human CD81 alone is insufficient to confer susceptibility to HCV infection in the mouse. The presence of additional possible factors for HCV infection is discussed.

Key Words: hepatitis C virus; CD81; transgenic mice; infection; animal model.

INTRODUCTION

The host range of virus infection is determined by several factors including surface receptors, cellular machinery for replication, translation and assembly of viral proteins, and budding of infectious particles. It remains unclear which of these factors is crucial for the restricted host range of hepatitis C virus (HCV). Among various species of laboratory animals, only the chimpanzee (*Pan troglodytes*) is known to be susceptible to HCV infection. Establishment of a new animal model for HCV infection would therefore be extremely useful not only to study pathogenesis of hepatitis C but also for the development of vaccines and therapeutic drugs against this disease. We previously demonstrated that chimpanzees protected from HCV infection by immunization with recombinant structural proteins of HCV produce neutralizing antibodies which block binding between the cell surface and E2, the major glycoprotein of HCV (Rosa *et al.,* 1996). This suggests that interaction between the E2 protein and a cellular receptor for E2 may be crucial for virus particles to enter into the target cell. Furthermore, we reported that E2 binds to CD81 in a species-specific manner. We also demonstrated that a soluble form of the large extracellular loop (LEL) of human CD81 enriched HCV genomic RNA *in vitro*, suggesting that "bona fide" HCV particles bind to LEL of the CD81 molecule. Moreover, chimpanzee sera with neutralizing activity could block binding of HCV to the soluble human CD81 molecule in a dose-dependent manner (Pileri *et al.,* 1998). These findings strongly suggest that the human CD81 molecule may act as a cellular receptor for HCV. However, there is no direct proof that attachment of HCV particles to the CD81 protein induces internalization of the virus followed by replication of the viral genome. Although cultured human cells express the CD81 molecule on their cell surface, the lack of a robust *in vitro* culture system for HCV hampers finding an answer to this question. There may be several explanations of why human cells can support replication of HCV *in vitro* only weakly and transiently, if at all: (1) Culture conditions downregulate a cellular factor indispensable for HCV replication machinery; (2) Culture media lack important molecules that support efficient replication of HCV; (3) Replication of HCV requires a different cell type or an appropriate stage of differentiation of the target cell that is not represented in the cell lines tested.

Transgenesis is then a possible *in vivo* strategy to test whether the human CD81 molecule is sufficient to confer susceptibility to HCV infection to a naturally refractory species such as the mouse.

[1] To whom correspondence and reprint request should be addressed at Department of Immunology, IRIS, Chiron S.p.A., Via Fiorentina, 1, I-53100, Siena, Italy. Fax: +39-0577-243564. E-mail: yasushi_uematsu@chiron.it.

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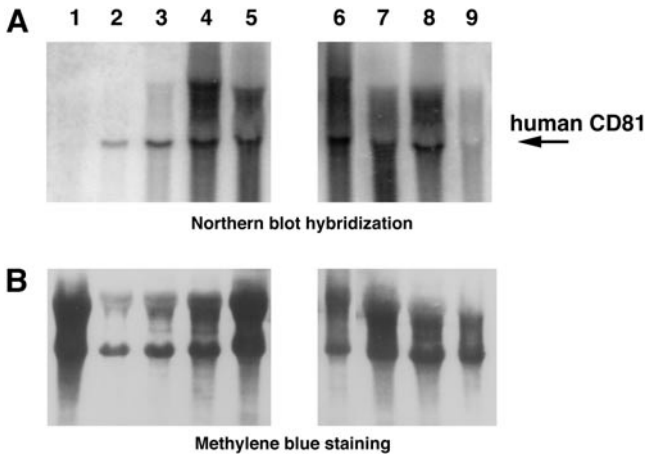

FIG. 1. Expression of the human CD81 transcript in organs of the transgenic mice. (A) Hybridization with a human CD81 specific probe. Ten to thirty micrograms of total RNA from a nontransgenic littermate (Lane 1) and the human CD81 transgenic mouse (Lanes 2–9) were separated on a formaldehyde–agarose gel and transferred on a nylon membrane. Lane 1: liver; Lane 2: thymus; Lane 3: lung; Lane 4: heart; Lane 5: spleen; Lane 6: kidney; Lane 7: brain; Lane 8: liver; Lane 9: testis. The arrow indicates the position of the human CD81-specific transcript. (B) Methylene blue staining to detect transferred RNA on the hybridization filter.

RESULTS

Transgene expression in human CD81 transgenic mice

Human CD81 transgenic mice were produced under the control of the CAG regulatory elements (Niwa *et al.,* 1991) to allow ubiquitous expression of the transgene. In these mice, the transgene transcript was detected by Northern blot hybridization (Fig. 1A). Judging from the total amount of RNA detected by methylene blue staining (Fig. 1B), although the spleen, brain, and testis expressed human CD81-specific RNA at relatively low levels, the transgene transcript was detected in all organs including the thymus, liver, heart, lung, and kidney.

We also tested transgene expression at the protein level. In cryostat preparations of liver specimens, anti-mouse immunoglobulin developed deep blue color on the vascular surface of the hepatocyte due to residual host immunoglobulin molecules (Fig. 2A). When we added JS81, a monoclonal antibody against human CD81, the transgenic liver preparation yielded red signals on the biliary surface clearly distinguishable from the non-transgenic liver preparation (Figs. 2B and 2C), indicating that the human CD81 protein was expressed on the cell surface of the transgenic hepatocytes. Although there are other cell types such as endothelial cells, Kupfer cells, and lipocytes in the liver, these cells are located in the sinusoidal area. Our results therefore exclude the possibility that the transgene is expressed only in these nonhepatocytic lineage cells in the liver. We then tested transgene expression in lymphoid organs by cytofluorometric analysis. Although the specific RNA was produced at a much lower level, transgenic splenocytes and thymocytes successfully expressed the human CD81 protein (Fig. 3A, a and b) at comparable levels. We also detected the human CD81 protein on peripheral blood lymphocytes (PBL) (Fig. 3B, b). Polymorphonuclear cells (PMN) expressed the transgene at particularly high levels (Fig. 3B, c). Altogether, the human CD81 transgenic mice expressed the transgene ubiquitously. Transgene expression did not affect the number of lymphocytes as well as the B/T ratio, suggesting no gross alteration in lymphocyte development (data not shown).

Binding of the recombinant HCV-E2 glycoprotein to transgenic cells

We examined the binding of the recombinant HCV-E2 (I-$E2_{715}$) protein to transgenic liver cells by immunohistochemistry. I-$E2_{715}$ bound specifically to transgenic liver cells (Fig. 4B) but not to liver cells from nontransgenic mice (Fig. 4A). The E2 binding on the transgenic liver specimen was competed by preincubation with JS81 (Fig. 4C), indicating that I-$E2_{715}$ bound directly to the human CD81 molecule. We also checked for the binding of I-$E2_{715}$ to lymphocytes by cytofluorometry. The recombinant I-$E2_{715}$ protein also bound to the transgenic splenocytes (Fig. 5A, a) and the thymocytes (Fig. 5A, b) as expected. Intriguingly, although PBL from transgenic mice failed to bind the I-$E2_{715}$ protein (Fig. 5B, b), PMN in the peripheral blood successfully bound to the recombinant E2 glycoprotein (Fig. 5B, c).

Inoculation of human CD81 transgenic mice with plasma infected with HCV

We prepared four series of intravenous inoculations (20–2000 units of 50% chimp infectious dose, CID_{50}) to test susceptibility of human CD81 transgenic mice to HCV infection *in vivo*. Each group contained three to four animals, 3–6 months old at the time of inoculation; nontransgenic littermates were also grouped and inoculated with the same infectious plasma in the same manner as negative controls. Sera of inoculated animals were collected at regular intervals for up to 6 months to examine the presence of HCV-specific RNA. No viral-specific sequence was amplified from sera of human CD81 transgenic mice or of normal littermates. Since the human CD81 transgenic mice express high levels of the transgene product in PMN (Fig. 3B, c), intravenously injected virions might have been captured by PMN before they reached the liver. To avoid this potential problem, we also inoculated the infectious plasma intrahepatically. Furthermore, we produced transgenic mice with the human CD81 gene under control of the albumin promoter, which express the transgene in a liver-specific manner (data not shown). These mice were also inoculated with

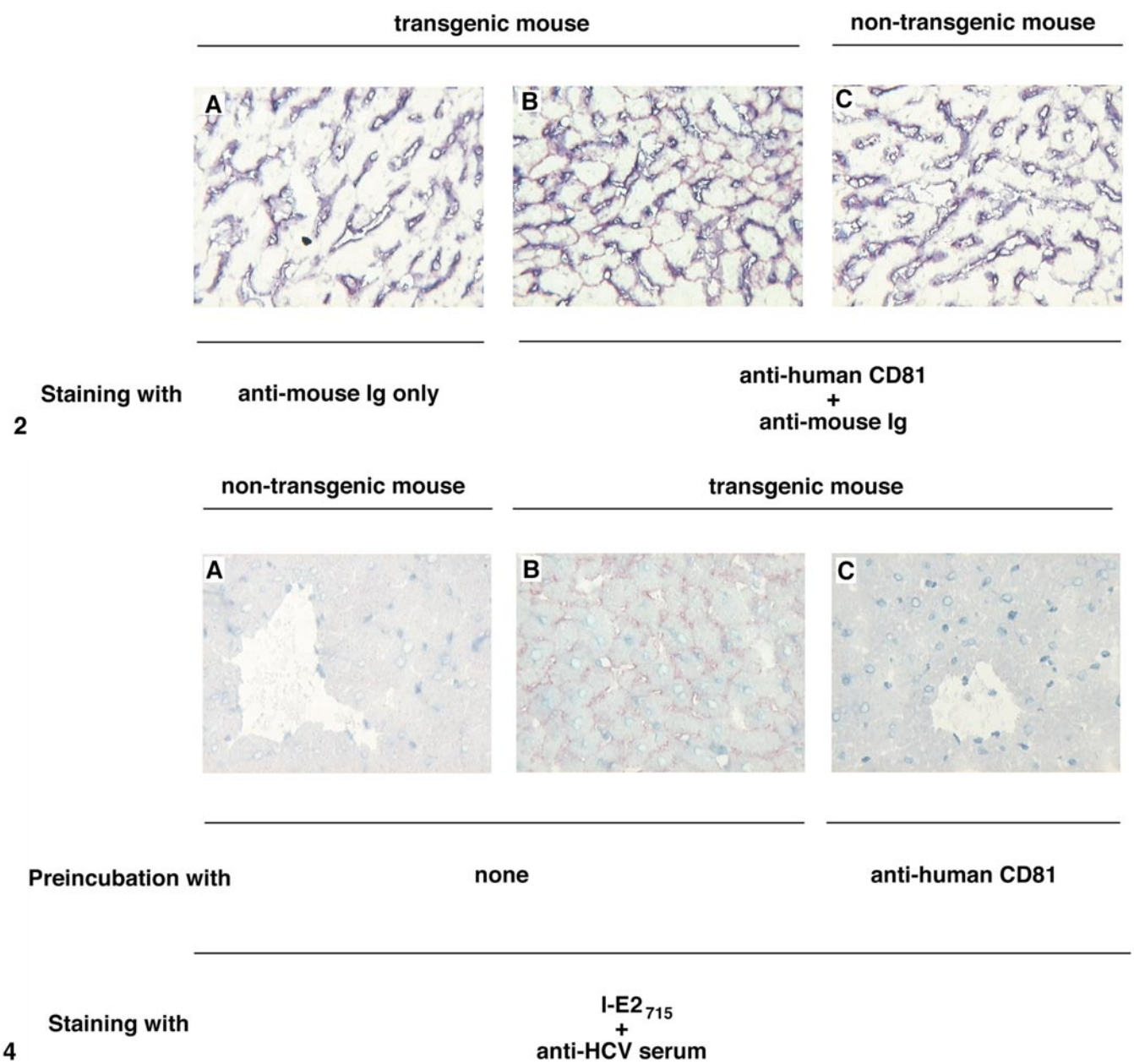

FIG. 2. Expression of the human CD81 protein in the liver of transgenic mice. Cryostat specimens were prepared from human CD81 transgenic (panels a and b) nontransgenic mice (panel c). The specimens were pretreated with F(ab)′ of goat anti-mouse serum. Remaining unblocked mouse immunoglobulins were visualized as blue color. The specimens were further stained with anti-mouse immunoglobulin only (panel a) or anti-human CD81 plus anti-mouse immunoglobulin (panels b and c). The positive binding was visualized as red color. Original magnification, ×400.

FIG. 4. Binding of I-E2$_{715}$ to the liver of transgenic mice. Cryostat specimens were prepared from a nontransgenic (panel a) and human CD81 transgenic mice (panels b and c). These specimens were incubated with I-E2$_{715}$ followed by anti-HCV serum from immunized chimpanzee. One specimen was preincubated with anti-human CD81 prior to addition of I-E2$_{715}$ (panel c). The positive binding was visualized as red color. The specimens were also counterstained with hematoxylin. Original magnification, ×400.

the same infectious plasma. These experiments again yielded no evidence of viral replication.

We also asked whether these mice had built an immune response against HCV, although they had not given rise to whole virions. We performed enzyme-linked immunosorbent assay (ELISA) and Western blot analysis to detect antibodies in plasma of inoculated animals against recombinant structural (core) and nonstructural (NS3, NS4, and NS5) proteins of HCV. None of the inoculated animals showed humoral immune responses (i.e., production of specific antibodies) to any of these viral proteins encoded by HCV.

It is intriguing that I-E2$_{715}$ does not bind to peripheral blood cells except PMN, which lack endogenous CD81 expression (Tedder *et al.,* 1995). Although mouse CD81 does not bind HCV (Pileri *et al.,* 1998), we could not

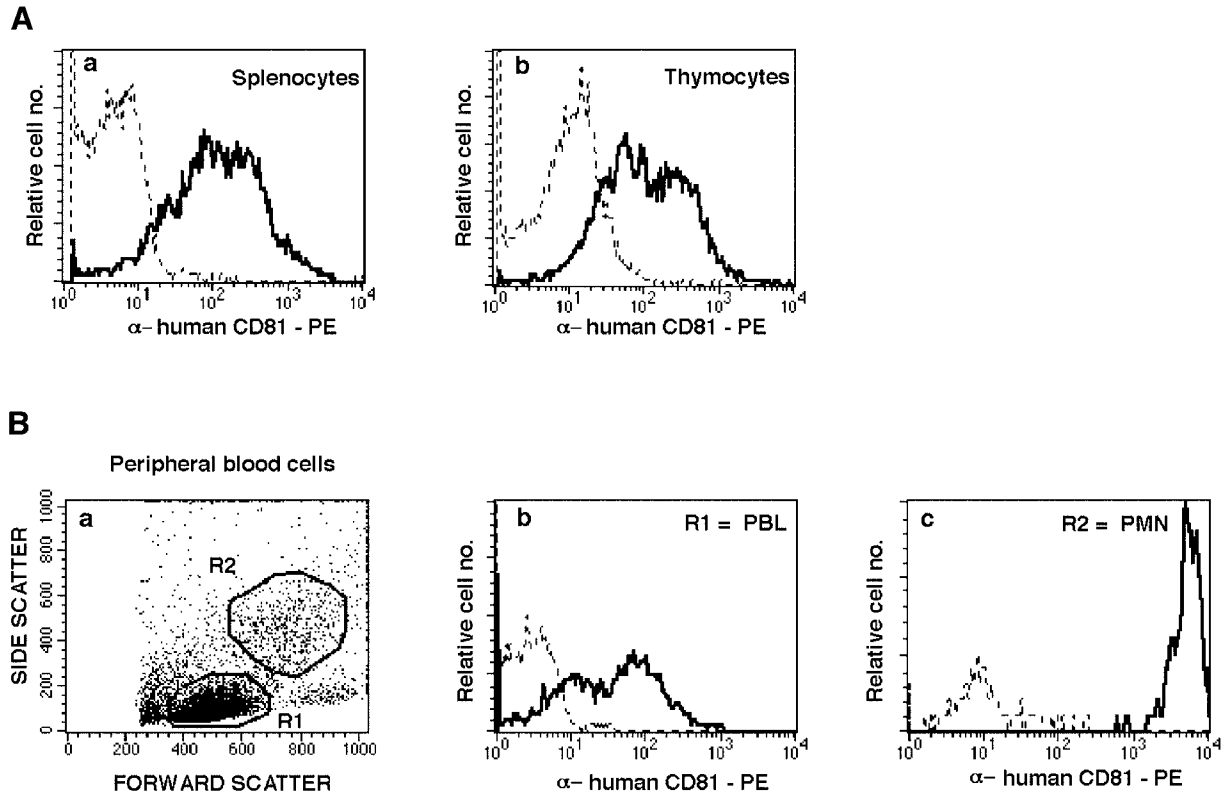

FIG. 3. Human CD81 expression in lymphoid tissues of transgenic mice. (A) Histogram showing binding of anti-human CD81 antibody on splenocytes (panel a) and thymocytes (panel b). The *x*- and *y*-axes indicate the fluorescent antibody molecules bound to the cell surface and the relative cell number, respectively. Patterns of the antibody binding to the cells from transgenic and nontransgenic mice are indicated as solid and broken lines, respectively. (B) The two-dimensional dot-plot with forward and side scatters is used to distinguish between lymphocytes (small cell-size = small forward scatter, round-shaped nucleus = small side scatter) (R1) and polymorphonuclear cells (PMN) (large cell-size = large forward scatter, irregular-shaped nucleus = large side scatter) (R2) of mouse peripheral blood cells (panel a). The antibody binding on PBL and PMN is drawn as histograms (panels b and c) by selecting cells within R1 and R2 (panel a), respectively. Patterns of the antibody binding to the cells from transgenic and nontransgenic mice are indicated as solid and broken lines, respectively.

exclude the possibility that endogenous CD81 might compete with human CD81 to form a molecular complex that allows the HCV-E2 glycoprotein to bind and function as a receptor for HCV on PBL. To test this hypothesis, we bred the human CD81 transgenic mice with CD81-deficient ($CD81^{-/-}$) mice (Maecker and Levy, 1997) and inoculated these animals too with HCV infectious plasma. Again, there was no evidence of replication of HCV in the $CD81^{-/-}$ mice expressing human CD81. After this experiment, we analyzed spleen cells from the inoculated mice to confirm that the animals lacked endogenous CD81 expression (Fig. 6A).

We also checked the phenotype of these animals. PBL from the mice still failed to bind I-$E2_{715}$ (Fig. 6B, a and b), while thymocytes from the same animals could bind I-$E2_{715}$ (Fig. 6B, c). These observations indicated that coexpression of endogenous CD81 is not responsible for failure of HCV to infect human CD81 transgenic mice.

DISCUSSION

We have proposed that human CD81 is a putative HCV receptor because we demonstrated that human CD81 could bind bona fide HCV particles (Pileri *et al.,* 1998). Other groups have suggested the low-density lipoprotein receptor (LDLR) as another candidate for the HCV receptor (Agnello *et al.,* 1999; Monazahian *et al.,* 1999; Wunschmann *et al.,* 2000), although there is contradictory evidence that a patient with familial hypercholesterolemia, who had a deficiency of LDLR, was infected with HCV (Marson *et al.,* 1999), showing that if LDLR is a receptor for HCV, it is not the only one. To date, the importance of CD81 cannot be assessed by such an epidemiologic survey because no individual with CD81 deficiency has been reported in the human population. Lack of CD81 expression in humans may not bring about recognizable clinical symptoms, similarly to mice, where CD81 deficiency only leads to mild impairment of immune responses (Maecker and Levy, 1997; Miyazaki *et al.,* 1997; Tsitsikov *et al.,* 1997).

Although it has been reported that a number of cell lines support HCV infection *in vitro* (Bertolini *et al.,* 1993; Cribier *et al.,* 1995; Dash *et al.,* 1997; Fournier *et al.,* 1998; Iacovacci *et al.,* 1997; Ikeda *et al.,* 1998; Kato *et al.,* 1998; Lanford *et al.,* 1994; Morrica *et al.,* 1999; Seipp *et al.,*

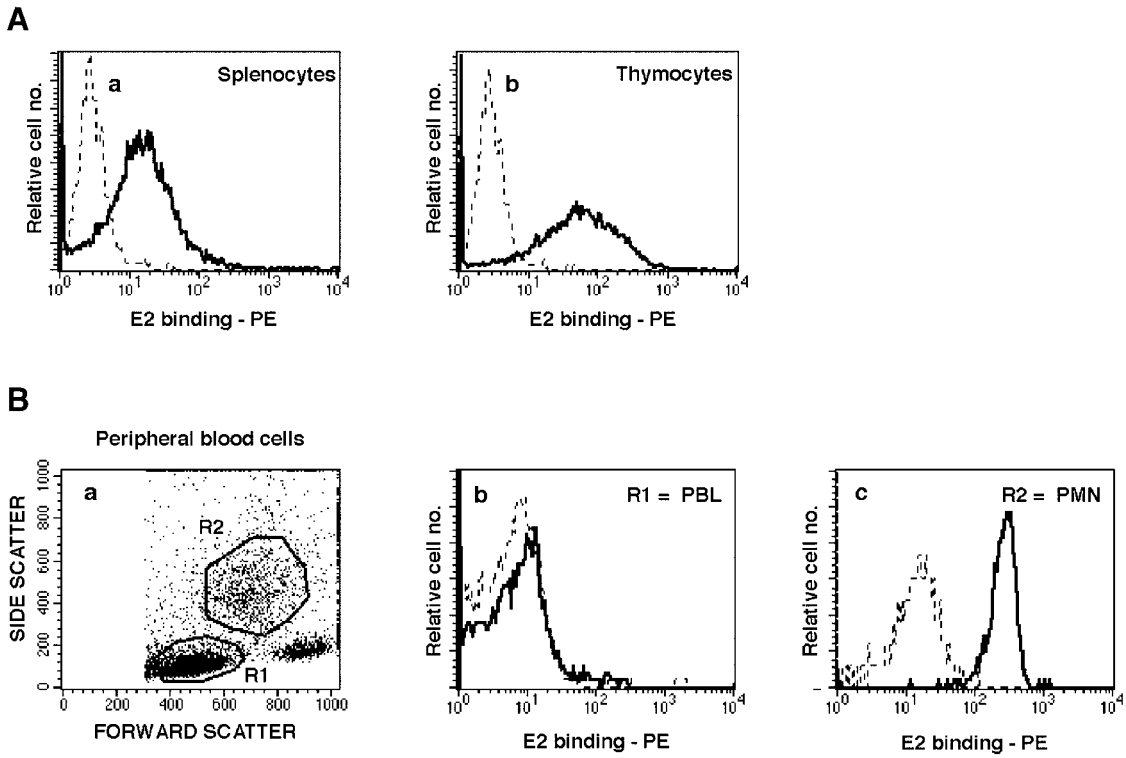

FIG. 5. E2 binding to lymphocytes of transgenic mice. (A) Histogram showing binding of I-$E2_{715}$ on splenocytes (panel a) and thymocytes (panel b). The *x*- and *y*-axes indicate the fluorescent protein bound to the cell surface and the relative cell number, respectively. Patterns of the I-$E2_{715}$ binding to the cells from transgenic and nontransgenic mice are indicated as solid and broken lines, respectively. (B) The two-dimensional dot-plot with forward and side scatters is used to distinguish between lymphocytes (small cell-size = small forward scatter, round-shaped nucleus = small side scatter) (R1) and polymorphonuclear cells (PMN) (large cell-size = large forward scatter, irregular-shaped nucleus = large side scatter) (R2) of mouse peripheral blood cells (panel a). The I-$E2_{715}$ binding on PBL and PMN is drawn as histograms (panels b and c) by selecting cells within R1 and R2 (panel a), respectively. Patterns of the I-$E2_{715}$ binding to the cells from transgenic and nontransgenic mice are indicated as solid and broken lines, respectively.

1997; Shimizu *et al.,* 1998; Shimizu and Yoshikura, 1994; Tagawa *et al.,* 1995; Valli *et al.,* 1995), none of them have been used to examine the importance of human CD81 in HCV infection, probably because of technical difficulties. Mice transgenic for human CD81 were considered to offer a possibility to study the involvement of the putative receptor during HCV infection *in vivo*. We addressed the question of whether expression of human CD81 in transgenic mice confers susceptibility to HCV infection *in vivo*. We produced transgenic mice expressing human CD81 ubiquitously. Expression of human CD81 did not cause gross differences in mouse development, including lymphopoiesis. We inoculated HCV into these transgenic mice intravenously or intrahepatically. Even though we used high doses (20–2000 CID_{50}) of infectious plasma, no animals showed evidence of viral replication.

Because CD81, similar to other tetraspanin molecules, forms molecular complexes with different molecules on various type of cells (reviewed in Levy *et al.,* 1998; Maecker *et al.,* 1997), the presence of the mouse CD81 molecule might reduce the chance for human CD81 to form a "functional" receptor together with appropriate molecules. We addressed the question of whether a possible competition between the human CD81 molecule and the mouse counterpart might account for the failure of HCV to infect transgenic mice. Although the endogenous mouse CD81 molecule does not bind HCV (Pileri *et al.,* 1998), it might compete with human CD81 to form a functional receptor complex for internalization of the virus. In addition, in our previous work, we observed that the crystallized LEL of CD81 formed homodimers (Kitadokoro *et al.,* 2001) and such a homodimer may also form in the cell surface as a receptor. If this were the case, it would be likely that a human–mouse chimeric CD81 dimer complex is formed on the transgenic cells. Although we do not know the stoichiometry of CD81-E2 interaction, such a chimeric dimer may be inactive as a receptor. To test this hypothesis, we also transferred the transgene to CD81-deficient mice (Maecker and Levy, 1997). These mice, however, were also refractory to HCV infection.

Taken together, these data show that expression of the human CD81 molecule alone in mice is insufficient for conferring susceptibility to HCV infection. There are several possible reasons expression of human CD81 in mice failed to confer susceptibility to HCV infection: (1) A

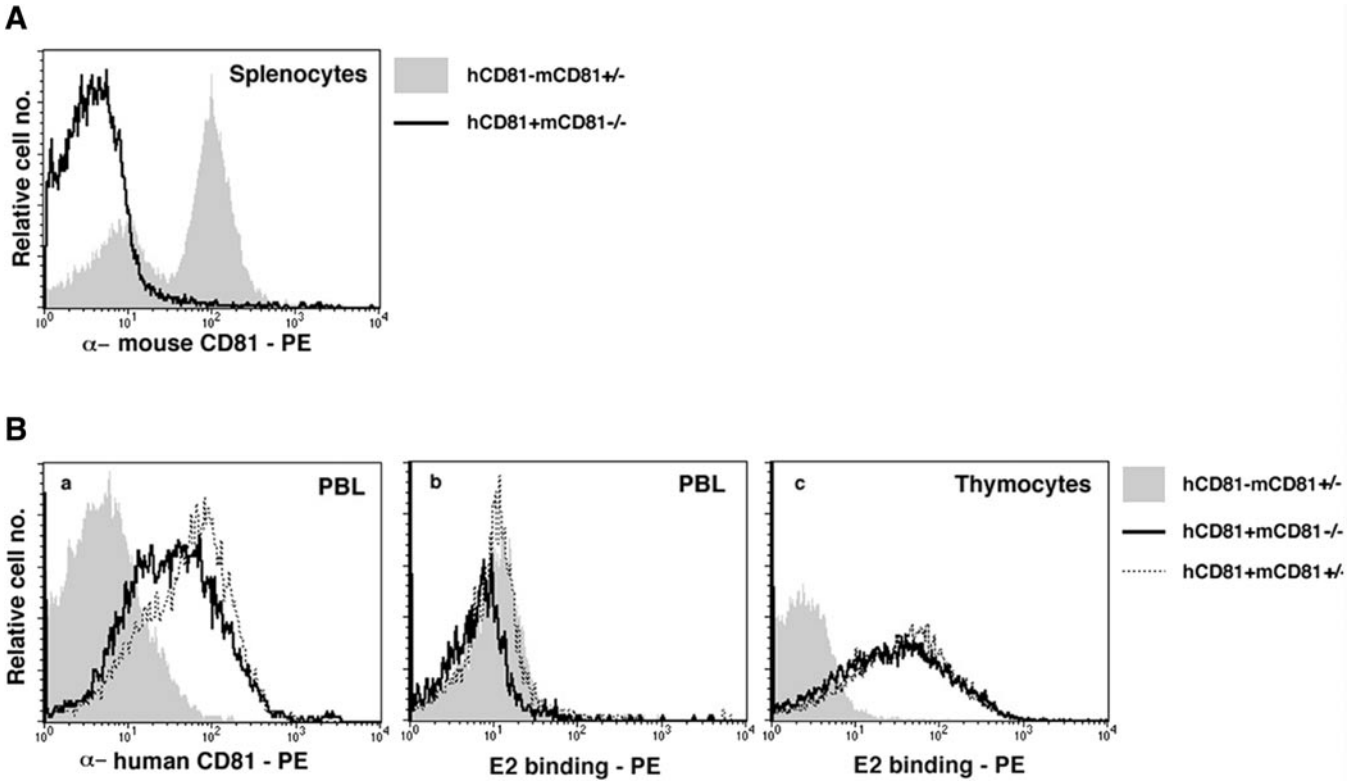

FIG. 6. Expression of CD81 and E2 binding in hCD81^{+}mCD81$^{-/-}$ mice. (A) Lack of endogenous CD81 expression in hCD81^{+}mCD81$^{-/-}$ mice was confirmed on splenocytes with the Eat2 monoclonal antibody. The *x*- and *y*-axes indicate the fluorescent antibody molecules bound to the cell surface and the relative cell number, respectively. (B) Expression of human CD81 transgene expression (panel a), E2 binding (panel b) on PBL, and E2 binding on thymocytes (panel c). The *x*- and *y*-axes indicate the fluorescent antibody molecules (panel a)/the recombinant proteins (panels b and c) bound to the cell surface, and the relative cell number, respectively. Histograms of hCD81^{+}mCD81$^{-/-}$ and hCD81^{+}mCD81$^{\pm}$ animals are indicated as solid and broken lines, respectively. The histograms of a negative control animal (hCD81^{-}mCD81$^{+/+}$) are shaded.

second receptor is required. It has been reported that CD81 from the cotton-top tamarin (*Saguinus oedipus*) binds the E2 protein of HCV even better than human CD81 (Allander *et al.,* 2000; Meola *et al.,* 2000), although this primate is refractory to HCV infection (Garson *et al.,* 1997). In addition, we reported that cross-linking of CD81 on the human cells induces only minimal internalization of the CD81 protein (Petracca *et al.,* 2000). These findings suggest that an additional molecule may be required for entry of the virus particle into the target cell. (2) A certain organ or a cell type (e.g., PBL) that fails to bind HCV-E2 in the transgenic mice is a crucial site for HCV replication. Unexpectedly, we observed that the E2 glycoprotein of HCV, which may serve as a ligand for human CD81, did not bind to lymphocytes in peripheral blood, although the transgene product was detected on these cells by a specific antibody to human CD81. This suggests that mouse molecules forming complexes with the human CD81 protein mask the binding site of the E2 glycoprotein on the surface of certain cell types. (3) The life cycle of HCV requires some species-specific intracellular factor(s). To date, it is difficult to determine which of the circumstances outlined above is true without a robust *in vitro* culture system.

Finally, it is of interest to consider the existence of molecules protecting from viral infection. For example, the interferon-inducible Mx1 protein is known to protect wild mouse strains from influenza virus infection (reviewed in Arnheiter and Meier, 1990). The presence of a resistance gene against replication of HCV in *Mus musculus* would on the one hand preclude using the transgenic mice system for HCV infection. On the other hand, identification of such gene products would be of great value not only for fundamental understanding of HCV biology but also for development of preventive or therapeutic strategies for HCV infection.

MATERIALS AND METHODS

Construction of human CD81 transgene and production of transgenic mice

A 1-kb fragment of human CD81 cDNA (Pileri *et al.,* 1998) was inserted between the 0.64-kb *Bam*HI-*Eco*RI fragment containing the second intron and the 0.56-kb *Eco*RI-*Pvu*II fragment containing the third exon including the polyadenylation signal of the rabbit β-globin gene (Fig. 7A). This transcription unit of the transgene was joined with the 1.6-kb regulatory unit of pCAGGS (Niwa *et*

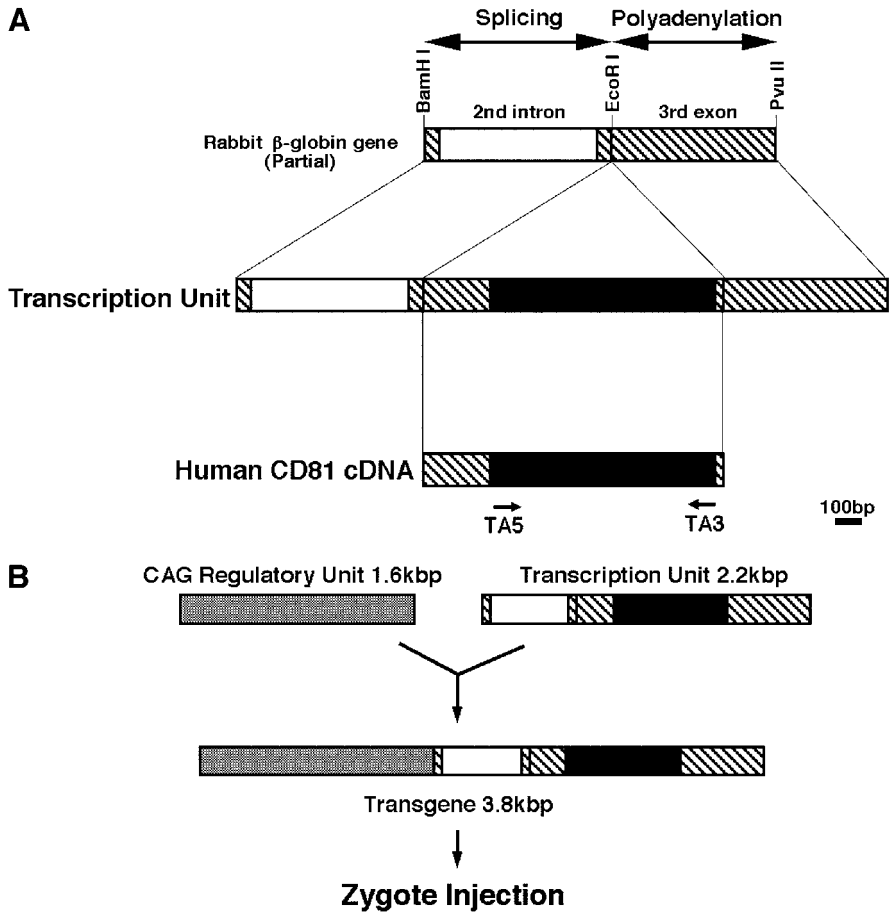

FIG. 7. Strategy to construct the human CD81 transgene. (A) Transcription unit of human CD81. The partial rabbit β-globin gene containing the 2nd intron and the 3rd exon was split and used for splicing and polyadenylation sites to ensure efficient transcription of the transgene. The intron is indicated as an open bar. The nontranslated exon and the human CD81-coding region are indicated as hatched and closed bars, respectively. The PCR primers to detect transgene integration in the mouse genome (TA5 and TA3) are indicated as small arrows (not drawn to scale). (B) Construction of the transgene. The CAG regulatory unit (indicated as a shaded bar) was joined at the 5′ end of the transcription unit.

al., 1991) (Fig. 7B). The 3.8-kb fragment including the CAG regulatory unit and human CD81 transcription unit purified from this plasmid was used for zygote pronuclear microinjection. The transgenes were microinjected into zygotes of the FVB mouse by a commercial transgenic animal production service (Eurogentech Bel S.A., Seraing, Belgium).

Transgenic animals were identified by PCR to detect transgene integration in genomic DNA from tail biopsies. The tail DNA was prepared using DNeasy Tissue Extraction Kit (Qiagen). An aliquot of extracted DNA was submitted to a 20-μl PCR. The reaction mixture contained 1× PCR buffer (67 mM Tris–HCl pH 8.8, 16.6 mM ammonium sulfate, 2 mM $MgCl_2$, 0.2 mM deoxyribonucleotide mix, 0.1 mg/ml bovine serum albumin), 0.1 units *Taq* platinum polymerase (Life Technologies), and 5 pmol each of the TA5 and TA3 primers (Table 1, Fig. 7A).

The reaction mixture was incubated at 94°C for 10 min followed by 35 cycles of a one-step PCR (30 s/94°C, 1 min/60°C, and 1 min/72°C) using a GeneAmp PCR System 9700 (Perkin–Elmer Applied Biosystems). The reaction was further chased by incubation at 72°C for 15 min. Ten microliters of the reaction mixture was analyzed by agarose gel electrophoresis.

Positive founder animals were further crossed with C57Bl/6 mice. Offspring positive for the transgene segregation was monitored by PCR as described above.

TABLE 1

PCR Primers Used in This Study

Primer	Sequence	Position[a]
TA5	5′-ACCTGCTCTTCGTCTTCAATTTCG	273–296
TA3	5′-GTACACGGAGCTGTTCCGGATGCC	923–900
162AS	5′-GATGCACGGTCTACGAGACCTC	341–320
163S	5′-AACTACTGTCTTCACGCAGAA	53–73
164AS	5′-GCGACCCAACACTACTCGGCT	272–252
165S	5′-ATGGCGTTAGTATGAGTG	85–102

[a] The positions of the human CD81 cDNA (for primers TA5 and TA3) and the HCV genomic RNA (for primers 162SA, 163S, 164AS, and 165S) are adapted from the GenBank nucleotide database Accession Nos. NM_004356 and D45172, respectively.

Two lines were also crossed with CD81 knockout mice (Maecker and Levy, 1997). Homozygosity of the CD81-deficient allele was checked by Southern blot hybridization (Sambrook *et al.,* 1989) using a mouse CD81-specific DNA probe.

Northern blot hybridization

Total RNA from various organs of mice was isolated using Trizol Reagent (Life Technologies). Ten to thirty micrograms of RNA were separated on a formaldehyde-agarose gel. RNA was transferred to a Hybond membrane (Amersham) and hybridized with a ^{32}P-labeled human CD81 cDNA probe using a standard protocol (Sambrook *et al.,* 1989). Hybridized radioactivity on the membrane was visualized on a PhosphorImager (Molecular Dynamics).

Immunohistochemistry

Cryostat liver sections of 3 μm thickness were fixed in acetone at room temperature for 10 min. Before staining with JS81, the mouse tissue was blocked by preincubation with F(ab′)$_2$ fragments derived from goat anti-mouse immunoglobulin (Cappel) (1/10,000 dilution in 50 mM Tris-buffered saline, TBS) for 30 min. After rinsing with TBS for 10 min, the sections were incubated with rabbit anti-mouse serum (Dako, Z259) (1/30 dilution in TBS) for 30 min, followed by alkaline phosphatase anti-alkaline phosphatase (APAAP) immunocomplex (Dako) (1/50 dilution in TBS) for 30 min. The residual unblocked mouse tissue Ig was then developed in blue using naphthol-AS-BI phosphate (Aldrich) as a substrate and Fast Blue BB salt (Aldrich) as a chromogen. After this treatment, the liver section was incubated with JS81 (0.5 mg/ml) (1/50 to 1/400 dilution in TBS) at room temperature for 1 h. The specimen was again incubated with the rabbit anti-mouse serum and treated with APAAP immunocomplex as described above. The positive staining was developed in red color with naphthol-AS-BI phosphate as a substrate, and New Fuchsin (Dako) as a chromogen.

To detect E2 binding, the procedure to develop blue color was omitted because no antibody against mouse immunoglobulins was used. The preincubated specimen was incubated with 30 μg/ml I-E2$_{715}$ and subsequently with anti-HCV chimpanzee serum (20 μg/ml as protein concentration) and with alkaline phosphatase conjugated goat anti-human immunoglobulins (Cappel) (1/200 dilution in TBS). Each incubation step was performed at room temperature for 30 min, followed by a 10-min rinsing step with TBS. E2 binding was developed in red color as described above. In some experiments, an incubation step with JS81 (1/50 dilution in TBS) was inserted before I-E2$_{715}$ incubation for specific blocking. The specimens for E2 binding were also counterstained with hematoxylin.

Cytofluorometric analysis

Splenocytes, thymocytes, and peripheral blood leukocytes were washed once with cold washing buffer (phosphate-buffered saline supplemented with 5% fetal calf serum). For detecting transgene expression, the cells were incubated with phycoerythrin (PE)-conjugated JS81 (Pharmingen), a monoclonal antibody specific for human CD81. Mouse CD81 was detected by incubating lymphocytes with biotinylated Eat2 (Pharmingen), a monoclonal antibody specific for mouse CD81 (Maecker *et al.,* 2000), and PE-conjugated streptavidin (Johnson Immuno-Research Laboratories). For E2 binding assay, the cells were treated with the recombinant I-E2$_{715}$ protein (Heile *et al.,* 2000), biotinylated 291A2 (a monoclonal antibody specific for E2) (Rosa *et al.,* 1996), and PE-conjugated streptavidin. CD81 expression and I-E2$_{715}$ binding were detected on FACScan (Becton–Dickinson) or FACS Caliber (Becton–Dickinson). The acquired data were analyzed with the CellQuest program (Becton–Dickinson).

Infection assay

All animals engaged in infection experiments were housed in the infectious disease isolation facility under European Community law conditions at the Department of Biomedicine, University of Pisa. Transgenic mice and normal littermates were submitted to infection experiments. Each type of mice was divided into four groups of three or four animals. Each group was challenged either intravenously or intrahepatically with diluted infectious chimpanzee plasma containing various doses (20–2000 CID$_{50}$) of HCV virions. Every month after inoculation, plasma was taken from animals to extract RNA using High Pure Viral Nucleic Acid (Boehringer Mannheim). The purified RNA was submitted to HCV-specific nested PCR. Human serum infected with HCV was used as a positive control for detection of the HCV-specific sequence. Five microliters of extracted RNA was reverse-transcribed at 42°C for 60 min in a 15 μl of reaction cocktail with the 162AS primer (Table 1) in the presence of 20 units of RNase inhibitor (Boehringer Mannheim), 2 mM deoxyribonucleic acid mixture, 3 units of avian myeloblastosis virus (AMV) reverse transcriptase (Promega) with its accompanying buffer. The reverse-transcription reaction was terminated by incubating at 94°C for 5 min. The cDNA was then directly amplified in a 40 μl reaction mix containing the appropriate PCR buffer with the 163S primer (Table 1) and *Taq* DNA polymerase (1.25 units) under the following conditions: five cycles of 94°C 60 s, 55°C 60 s, 72°C 90 s, followed by 30 cycles of 94°C 45 s, 55°C 30 s, 72°C 60 s. Five microliters of the first-step amplification reaction was submitted to the second PCR with the 164AS and 165S primers (Table 1) and freshly added *Taq* DNA polymerase (1.25 units) in a 50 μl reaction volume under the following conditions: five cycles of 94°C 60 s, 55°C 45 s, 72°C 90 s, followed by 20 cycles

of 94°C 30 s, 55°C 30 s, 72°C 60 s. A chase step (72°C 15 min) followed at the end of the PCR. Ten microliters of the reaction mixture was analyzed by agarose gel electrophoresis.

Anti-HCV antibody assay

HCV-specific antibodies were measured by ELISA and Western blot analysis. For these experiments, 5 months postinfection mouse plasma was used. For ELISA, plasma diluted to 1:20 was submitted to Ortho HCV 3.0 ELISA Test System (Ortho-Clinical Diagnostics GmbH). A human HCV-positive serum and normal mouse plasma were used as positive and negative controls, respectively. For Western blot, 50–75 μg of human superoxide dismutase (SOD)-conjugated recombinant HCV proteins (C22-3, C33C, C100-3, NS5) and recombinant human SOD (negative control) were electrophoresed on a 12% polyacrylamide gel in denaturing and reducing conditions. After the separated antigens in the gel were transferred onto a nitrocellulose membrane as described (Freer *et al.,* 1998), the membrane was pretreated and incubated with diluted plasma (1/20 dilution in TBS supplemented with 5% bovine serum albumin) to detect the presence of HCV-specific antibodies. The presence of HCV-specific antibodies was visualized with horseradish peroxidase labeled goat anti-mouse IgG (or anti-human for the positive control) and diaminobenzidine.

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