The HLA-A2 Restricted T Cell Epitope HCV Core_{35–44} Stabilizes HLA-E Expression and Inhibits Cytolysis Mediated by Natural Killer Cells

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Impaired activity of natural killer cells has been proposed as a mechanism contributing to viral persistence in hepatitis C virus (HCV) infection. Natural cytotoxicity is regulated by interactions of HLA-E with inhibitory CD94/NKG2A receptors on natural killer (NK) cells. Here, we studied whether HCV core encodes peptides that bind to HLA-E and inhibit natural cytotoxicity. We analyzed 30 HCV core-derived peptides. Peptide-induced stabilization of HLA-E expression was measured flow cytometrically after incubating HLA-E-transfected cells with peptides. NK cell function was studied with a ⁵¹chromium-release-assay. Intrahepatic HLA-E expression was analyzed by an indirect immunoperoxidase technique and flow cytometry of isolated cells using a HLA-E-specific antibody. We identified peptide aa35–44, a well-characterized HLA-A2 restricted T cell epitope, as a peptide stabilizing HLA-E expression and thereby inhibiting NK cell-mediated lysis. Blocking experiments confirmed that this inhibitory effect of peptide aa35–44 on natural cytotoxicity was mediated via interactions between CD94/NKG2A receptors and enhanced HLA-E expression. In line with these *in vitro* data we found enhanced intrahepatic HLA-E expression on antigen-presenting cells in HCV-infected patients. Our data indicate the existence of T cell epitopes that can be recognized by HLA-A2 and HLA-E. This dual recognition may contribute to viral persistence in hepatitis C. (*Am J Pathol* 2005, 166:443–453)

Infection with the hepatitis C virus (HCV), a member of the family of flaviviruses, frequently leads to chronic hepatitis. The mechanisms favoring persistent infection are still poorly understood. Numerous studies suggested an association between an impaired immune response and clinical outcome.^{1–3} However, antibodies as well as T cells specific for epitopes within HCV-proteins/peptides have been shown in chronic HCV-infection, suggesting that mechanisms other than impaired humoral immunity or altered T cell responses might be involved in the development of chronic HCV-infection. Studying peripheral blood mononuclear cells (PBMC) from HCV-infected patients, Corrado and co-workers⁴ proposed suppressed natural killer (NK) cell activity as a possible mechanism. Importantly, Duesberg and colleagues⁶ demonstrated that cytotoxic function of NK cells from HCV-positive individuals was not impaired in general raising the possibility that insufficient NK cell activity in chronic hepatitis C might be caused by HCV antigens interacting specifically with the recognition process of NK cells.

NK cells, a subset of lymphocytes, represent between 5 and 15% of mononuclear cells in the peripheral blood and up to 45% in some organs, such as the liver.⁷ The major functional role of NK cells is the defense against tumor cells and lysis of virus-infected cells.⁷ Depressed NK cell cytotoxic activity was reported in infection with human cytomegalovirus (HCMV), Epstein-Barr virus, herpes simplex virus, and HCV.^{4,5,8,9} Therefore, altered function of NK cells might be a general mechanism by which viruses escape the immune system.¹⁰

Recent studies revealed an important role of the non-classical major histocompatibility (MHC) class I molecule HLA-E in the regulation of NK cell function,^{8,9} as the inhibitory C-type lectin receptor CD94/NKG2A, which is expressed on most NK cells, specifically interacts with HLA-

Supported by a BONFOR grant, grant number O-107.0063, by the Kompetenznetz HIV/AIDS, Foerderkennzeichen "01 KI 0211" and by a grant from the Kompetenznetz Hepatitis.

Accepted for publication October 25, 2004.

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E.¹¹ Interaction of this receptor with HLA-E molecules has been shown to result in depressed NK cell activity.

HLA-E is a non-polymorphic MHC class I molecule, virtually transcribed on all human tissues and cell lines.^{12,13} Cell surface expression of HLA-E depends on binding of nonamer peptides derived from the signal sequence of classical MHC class I molecules. These peptides are processed in the cytosol and then transported into the endoplasmic reticulum (ER) by a transporter associated with antigen presentation (TAP)-dependent mechanism.^{14,15} Inside the ER binding of these peptides stabilizes a complex of HLA-E and β 2-microglobulin, which is then transported to and expressed on the cell surface. Thus, cell surface expression of HLA-E enables NK cells to monitor the expression of a broad panel of polymorphic MHC class I molecules with a single receptor. As shown for the human cytomegalovirus (HCMV), which codes for a peptide (derived from gpUL40) that is a homologue to the signal sequence of the classical MHC I molecule HLA-Cw03 (VMAPRTLIL), viruses can interfere with this mechanism to protect virus-infected cells from lysis by natural killer cells.^{8,9} In this context it is important to note that HLA-E can also bind peptides, which have amino acid sequences different from the signal peptide of MHC class I molecules with several binding motifs predicted.^{14–16} Furthermore, binding to HLA-E has previously been demonstrated for viral peptides corresponding to HLA-A2-restricted epitopes, suggesting that HLA-E might bind, at least in part, a repertoire of antigens comparable to classical HLA molecules.¹⁵ Thus, we speculated that the hepatitis C virus might also have evolved a strategy to interact with HLA-E to evade the immune system. In this context, it is particularly interesting that we previously demonstrated binding of HLA-A2-restricted HCV core-derived peptides to HLA-A2-negative cell lines,¹⁷ raising the possibility that these peptides might interact with non-classical HLA-molecules. Therefore, we tested 30 of the previously analyzed HCV core-derived peptides¹⁷ for their ability to enhance HLA-E expression and to alter NK cell activity, including peptides with high affinity to HLA-A2 and with different putative HLA-E binding motifs.

Materials and Methods

Patients

Seventeen Caucasian individuals were enrolled into this study, including 10 HCV-RNA(+) individuals and seven healthy HCV RNA-negative donors, who donated blood.

Venous blood was drawn from each subject for isolation of PBMC by Ficoll-Paque density gradient centrifugation. In the subjects of each group HLA-E expression was studied on peripheral blood NK cells using a FACScalibur flowcytometer (BD Bioscience, Heidelberg, Germany).

Furthermore, liver specimens from eight HCV RNA(+) patients and eight healthy HCV RNA(–) controls were available for liver immunohistochemistry and flow cytometric analysis of single cell suspensions, respectively.

The study conformed to the guidelines of the Declaration of Helsinki as approved by our ethics committee.

Cells

The MHC class I-deficient cell line K-562 was cultured in RPMI 1640 medium (Gibco, Karlsruhe, Germany) containing 10% fetal calf serum (FCS), 50 U/ml penicillin (PAA, Linz, Austria), 50 μ g/ml streptomycin, and 1 mmol/L sodium pyruvate (PAA, Linz). The HLA-E transfected cell line K-562 HLA-E B5⁸ was cultured in the presence of 0.4 mg/ml G-418 (Calbiochem, Darmstadt, Germany) in complete medium. NKL natural killer cells⁸ were cultured in RPMI 1640 supplemented with 100 U/ml recombinant human IL-2 (PAA, Linz), and 10% FCS at a density of 5×10^5 to 1×10^6 cells per ml.

Peptides and Antibodies

A panel of 30 HCV core-derived synthetic peptides of 8 to 10 amino acids in length was tested for binding to HLA-E (Table 1). The HLA-E-binding peptides Cw03 (VMAPRTLIL) and B*8001 (VMPRTLIL) derived from the leader sequences of HLA-Cw*0304 and HLA-B*8001, respectively, were used as a positive control. The irrelevant peptide IP1 (YLQQNWWTL) was used as a negative control. All peptides were obtained from EMC microcollections (Tübingen, Germany). The MHC class I-specific monoclonal antibody W6/32 also reacts with HLA-E and was used to detect HLA-E expressed on the surface of MHC I-deficient K-562 cells. The HLA-E-specific antibody 3D12 (kindly provided by D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA,¹⁸) was used for detection of HLA-E in liver samples. Anti-CD94 and anti-NKG2A were obtained from BD Bioscience, Heidelberg, Germany. Goat anti-mouse-FITC (BD Bioscience) was used as secondary antibody. Anti-HCV core (aa1–80)-FITC, clone 6A1, was obtained from Acris Antibodies GmbH (Hiddenhausen, Germany). The monoclonal mouse anti-human hepatocyte antibody Hepar1 (clone OCH1E5) was obtained from DakoCytomation (Hamburg, Germany).

Binding Assay

HLA-E in the absence of endogenous high-affinity ligands is only weakly expressed on HLA-E transfected K-562 cells. Cell surface expression is enhanced when the K-562 transfectants are incubated with exogenous HLA-E peptide ligands. To test whether HCV-derived peptides bind to HLA-E and thus stabilize expression of HLA-E, we incubated K-562 or K-562 HLA-E B5 cells at a density of 1×10^6 cells/ml with HCV peptides in 96-well plates according to previously published assays.^{17–20} Synthetic peptides were dissolved in DMSO and then added in a final concentration of 0.1, 1, 10, and 100 μ mol/L, respectively. After an overnight incubation at 26°C cells were harvested, washed with phosphate-buffered saline (PBS), stained with either 3D12 or W6/32 for HLA-E detection, and analyzed by FACS.

FACS Analysis

FACS analysis of HLA-E expression was performed following standard methods. Briefly, cells were preincu-

Table 1. Characteristics of HCV Core-Derived Peptides

Peptide-Sequence										Position	HLA-A2 binding score*
Q	I	V	G	G	V	Y	L	L		aa29–37	27
G	V	Y	L	L	P	R	R	G	P	aa33–42	7
Y	L	L	P	R	R	G	P	R	L	aa35–44	27
G	P	R	L	G	V	R	A	T	R	aa41–50	12
P	R	L	G	V	R	A	T	R		aa42–50	5
L	Y	P	W	P	L	G	N	E	W	aa81–90	9
W	P	L	Y	G	N	E	G	C	G	aa83–92	10
L	Y	G	N	E	G	C	G	W	A	aa85–94	11
E	G	C	G	W	A	G	W	L	L	aa89–98	11
R	N	L	G	K	V	I	D	T	L	aa117–126	26
L	G	K	V	I	D	T	L	T	C	aa119–128	6
I	D	T	L	T	C	G	F	A	D	aa123–132	9
T	L	T	C	G	F	A	D	L	M	aa125–134	23
T	C	G	F	A	D	L	M	G	Y	aa127–136	6
G	F	A	D	L	M	G	Y	I	P	aa129–138	13
A	D	L	M	G	Y	I	P	L	V	aa131–140	28
D	L	M	G	Y	I	P	L	V		aa132–140	28
G	Y	I	P	L	V	G	A	P	L	aa135–144	22
I	P	L	V	G	A	P	L	G	G	aa137–146	11
R	A	L	A	H	G	V	R	V	L	aa149–158	30
A	L	A	H	G	V	R	V	L		aa150–158	30
G	V	N	Y	A	T	G	N	L	P	aa161–170	17
N	A	Y	T	G	N	L	P	G	C	aa163–172	11
A	T	G	N	L	P	G	C	S	F	aa165–174	8
G	N	L	P	G	C	S	F	S	I	aa167–176	22
N	L	P	G	C	S	F	S	I		aa168–176	22
L	P	G	C	S	F	S	I	F	L	aa169–178	12
F	L	L	A	L	L	S	C	L		aa177–185	28
L	L	A	L	L	S	C	L	T		aa178–186	28
L	L	S	C	L	T	V	P	A	S	aa181–190	21

Amino acid (aa) sequence and aa position of HCV core-derived peptides used in the HLA-E binding assay.

* maximal HLA-A2 binding score as predicted based on the algorithm created by Rammensee HG, Bachmann J, Stevanovic S (<http://syfpeithi.bmi-heidelberg.com/Scripts/MHCServer.dll/EpPredict.htm>).

bated with 3D/12 (or W6/32) for 30 minutes at room temperature (RT) followed by washing with PBS and detection of bound primary antibody with a FITC or PE-tagged goat anti-mouse antibody for another 30 minutes at RT. After removing unbound secondary antibody by washing with PBS, samples were analyzed on a FACScalibur using the CellQuest software package (BD Biosciences, Heidelberg, Germany).

To detect intracellular HCV core protein, PBMC from HCV RNA(+) patients were washed with PBS to remove surface bound viral particles, permeabilized with 0.2% saponin, followed by incubation with anti-HCV core-FITC diluted in 0.2% saponin to reveal intracellular HCV core.

Immunostaining Procedures

Liver tissue specimens from chronic hepatitis C ($n = 5$) were obtained during routine diagnostic liver biopsies after informed consent from the patients. Normal liver specimens ($n = 5$) were obtained from unaffected areas of liver resections for secondary hepatic malignancy. Liver tissue was immediately embedded in Tissue Tek OCT compound (Miles Laboratories, Naperville, IL) and snap-frozen in liquid nitrogen. Frozen tissue was kept at -80°C until examined. Sections of 5 to 7 μm were stained by an indirect immunoperoxidase technique. In brief, endogenous peroxidase activity was blocked by 0.03% $\text{H}_2\text{O}_2/\text{NaNO}_3$ (peroxidase blocking reagent, Dako,

Carpinteria, CA). The sections were incubated with the HLA-E-specific antibody 3D12¹⁸ in phosphate-buffered saline (PBS)/1% fetal calf serum (FCS) in a moist chamber at room temperature for 90 minutes. After washing in PBS, peroxidase-coupled goat anti-mouse antibody (Dianova, Hamburg, Germany) was applied for 30 minutes. Any bound antibody was detected with 3-amino-9-ethyl-carbazole (Sigma Chemicals, Munich, Germany). All sections were then counterstained with Mayer's hemalum-eosin. Quantitative analysis was performed by manually counting HLA-E(+) cells in 10 visual fields at $\times 200$ magnification for each liver sample.

Double-Labeling

The identity of the HLA-E(+) cells was analyzed by double-staining with HLA-E- and anti-CD68. After performing the HLA-E-specific immunoperoxidase reaction, we incubated the sections with fluorescein isothiocyanate-conjugated antibodies for 30 minutes. We used the fluorescein isothiocyanate-labeled antibody KP1 (Dako) to identify CD68-positive macrophages/Kupffer cells. Analysis of double-labeling experiments was performed by bright-field and fluorescent photomicrographs on a Leica DMLB fluorescence microscope (Leica, Wetzlar, Germany) with a MPS60 photo camera.

Flow Cytometric Analysis of Cells in the Liver Specimens

Liver biopsy specimens for flow cytometric analysis of intrahepatic cells were obtained from liver biopsies of three HCV RNA(+) patients and three HCV RNA(−) control subjects using 1.5-mm diameter disposable biopsy needles with a length range of 10 to 20 mm. Fresh liver samples were washed twice in fresh medium and shaken gently to avoid blood contamination. Liver specimens were disrupted mechanically into small fragments in RPMI 1640 medium with 10% FCS using a forceps and scalpel. Then the fragments were homogenized on a cell strainer (BD Labware). The resulting cell suspension was washed and resuspended in RPMI 1640 medium. Intrahepatic cells were then flow cytometrically analyzed for expression of HLA-E using the HLA-E-specific mAb 3D12 and co-stained for expression of CD31 (expressed on sinusoidal endothelial cells), CD68 (to identify macrophages/Kupffer cells), CD14 (expressed on monocytes), and CD83 (expressed on dendritic cells), respectively, or with the hepatocyte-specific antibody Hepar1 (clone OCH1E5). Intrahepatic NK cells were analyzed using anti-CD3, anti-CD56, anti-NKG2A, and anti-NKG2C, respectively.

Cytotoxicity Assay

Cytotoxicity assays were performed using K-562 and K-562 HLA-E cells as target (T) cells and NKL cells as effector (E) cells. Before performing the assay 1×10^4 target cells were incubated overnight at 26°C either in the absence or in the presence of the peptides at varying concentrations as indicated. A 51 chromium release assay was performed as described before.⁸ To identify the involved type of NK cell receptor we also pre-incubated NKL cells with either anti-CD94 or anti-NKG2A antibody at 10 μ g/ml before performing the cytotoxicity assay. As a negative control NK cells were incubated with an irrelevant mouse IgG-specific antibody (clone A85-1, BD Biosciences). In some experiments target cells were pre-incubated with W6/32 in 1:100 dilution of ascites or with anti-IgG, respectively. E:T ratios were varied as indicated for the different experiments. Specific lysis was calculated as (release − spontaneous release):(maximum release − spontaneous release) $\times$ 100.

Results

Among the tested panel of 30 HCV core-derived peptides exclusively incubation with peptide HCV core_{35–44} (10 μ mol/L) resulted in stabilized HLA-E surface expression as detected by staining with mAb W6/32 (Figure 1A, left). In contrast, none of the other tested peptides affected expression of HLA-E (Figure 1A, middle, and data not shown). HLA-E expression on K-562 HLA-E B5 cells incubated with HCV core_{35–44} was comparable to the HLA-E expression levels on cells incubated with the control peptides derived from the leader sequence of HLA-Cw0304 (Figure 1A, right) and -B*8001 (data not shown),

Figure 1. HCV core_{35–44} enhances HLA-E surface expression. **A:** K-562 HLA-E B5 cells were incubated overnight at 26°C with the peptides HCV core_{35–44} (left), HCV core_{81–90} (middle), and Cw03 (right) at a concentration of 10 μ mol/L, respectively. Then cells were stained with mAb W6/32 and flow cytometrically analyzed to detect HLA-E expressed on the cell surface. **B:** Cell surface expression of HLA-E after incubation of K-562 HLA-E B5 cells with increasing amounts of peptide HCV core_{35–44} (0.1, 1, and 10 μ mol/L, respectively) as measured by flow cytometry using the HLA-E-specific mAb 3D12. **C:** Staining with mAb 3D12 of K-562 HLA-E B5 incubated with peptide Cw03, which is well known to stabilize HLA-E expression. **D:** Binding of mAb 3D12 to K-562 HLA-E B5 cells incubated with the irrelevant peptide IP1. **E:** As a negative control effects of peptide HCV core_{35–44} on 3D12 binding were studied on HLA-E-negative K-562 cells. The dotted lines refer to the isotype controls, respectively.

which are well known to stabilize the HLA-E/ β 2-microglobulin complex.^{11,21}

HCV core aa35–44-mediated stabilization of HLA-E was confirmed by using the HLA-E-specific antibody 3D12 (Figure 1B, bottom). In titration experiments binding of HCV core_{35–44} to HLA-E was also detected at lower concentrations of 1 μ mol/L and 0.1 μ mol/L, respectively, suggesting a high affinity of this peptide for HLA-E (Figure 1B). Incubation of K-562 HLA-E cells with the irrelevant peptide IP1 did not result in enhanced surface expression of HLA-E (Figure 1D). As a further control we incubated HLA-E-negative K-562 cells with peptide HCV core_{35–44}, which did not result in enhanced 3D12 binding (Figure 1E).

HLA-E/peptide-complexes are recognized by different NK cell receptors mediating either inhibitory or activating signals.^{22,23} Therefore, we studied whether binding of HCV core_{35–44} to HLA-E alters cytotoxic activity of natural killer cells. For this purpose, K-562 HLA-E B5 cells were incubated with the HCV core_{35–44} peptide and a chromium release assay was performed following standard protocols. As indicated in Figure 2A incubation of K-562 HLA-E B5 cells with the HCV core_{35–44} peptide reduced their susceptibility to NKL-mediated cytolysis to a similar extent as was obtained with the Cw03 control peptide, whereas incubation of HLA-E transfected K-562 cells with

Figure 2. HCV core₃₅₋₄₄-mediated up-regulation of HLA-E inhibits cytotoxic NK natural killer cells. **A:** HLA-E transfected K-562 HLA-E B5 cells were incubated with HCV core₃₅₋₄₄ (filled square) or with Cw03 (empty square) at a concentration of 100 μmol/L, respectively, or without peptide (filled triangle). As a negative control, K-562 HLA-E cells were incubated with 100 μmol/L of the irrelevant peptide IP1. After an overnight incubation at 26°C a cytotoxicity assay was performed using NK cells as effectors and K-562 HLA-E B5 cells as targets. *, Mann-Whitney-U-test, lysis of K-562 HLA-E cells incubated with peptide HCV core aa35-44 versus cells incubated without peptide or the irrelevant peptide, respectively. **B:** To validate specificity of HCV core₃₅₋₄₄-mediated impairment of NK cell cytotoxicity we incubated target cells with decreasing amounts of the HCV peptide (filled squares), the irrelevant peptide IP1 (empty triangles) and the control peptide Cw03 (empty squares) and performed a ⁵¹chromium release assay with an E:T ratio of 20:1. **C:** As a further negative control we incubated HLA-E-negative K-562 cells with or without 100 μmol/L of peptide HCV core aa35-44 before performing a ⁵¹chromium release assay using NK as effector cells.

the irrelevant control-peptide IP1 had no effect on cytotoxic function of NK cells.

Of note, dose titration of the HCV-core peptide in direct comparison to a dose titration of the positive control peptide Cw03 confirmed comparable inhibition of NK cell-lysis by both peptides even at low concentrations. Again, incubation of K562 HLA-E cells with the irrelevant peptide IP1 did not affect NK cell lysis (Figure 2B).

The specificity of this effect was furthermore demonstrated by the fact that incubation with HCV core₃₅₋₄₄ did not alter NK cell-mediated cell lysis against HLA-E-negative K-562 cells as target cells (Figure 2C).

To identify the NK cell receptor involved in the recognition of the HLA-E/HCV core₃₅₋₄₄-complex we performed blocking experiments with the monoclonal antibodies anti-CD94 and anti-NKG2A, respectively. As shown in Figure 3A pre-incubation of NK cells with anti-CD94 as well as with anti-NKG2A abolished inhibition of cytolysis, demonstrating that the inhibitory CD94/NKG2A receptor recognized the HLA-E/HCV core₃₅₋₄₄-complex. Killing of K562 HLA-E cells incubated with the irrelevant peptide was not altered in the presence of anti-NKG2A and anti-CD94, excluding the possibility that these antibodies enhance the lytic potential of NK cells in a non-specific manner (Figure 3B).

Likewise, blocking of HLA-E on the surface of K-562 HLA-E B5 target cells with the MHC class I specific antibody W6/32 neutralized the inhibitory effect on natural killer cell activity, which was induced in HLA-E transfected K-562 HLA-E B5 cells by incubation with HCV core₃₅₋₄₄ (data not shown).

To demonstrate the *in vivo* relevance of up-regulated HLA-E expression in chronic hepatitis C, we analyzed the intrahepatic HLA-E expression by an indirect immunoper-

oxidase technique using the HLA-E specific mAb 3D12. We found a significantly enhanced expression of HLA-E in liver samples from HCV-positive patients as compared to those obtained from HCV-negative donors ($P = 0.047$), confirming up-regulation of HLA-E in natural HCV infection (Figure 4A). Cells that were positive for HLA-E showed a macrophage-like morphology (Figure 4A, arrows). Co-staining of liver samples from HCV-infected patients confirmed that a marked proportion of HLA-E(+) cells are CD68-positive macrophage/Kupffer cells (Figure 4B). However, flow cytometric analysis revealed that HLA-E was also expressed on sinusoidal endothelial cells (CD31-positive), on intrahepatic CD83-positive DCs and to a lesser extent on CD14(+) monocytes (Figure 4C). Of note, HLA-E expression was also up-regulated on hepatocytes in livers from HCV RNA(+) patients as compared to hepatocytes derived from the healthy individuals (Figure 4D).

To analyze whether up-regulated intrahepatic expression of HLA-E in chronic hepatitis C was a direct result of infection with HCV or rather a bystander effect, we stained intrahepatic cells from HCV-infected patients intracellularly with a HCV core-specific mAb (clone 6A1) and analyzed HLA-E expression on HCV-core(+) cells in comparison to HCV-core(-) cells. As shown in Figure 4E we found higher surface expression of HLA-E on HCV core-positive cells than on HCV core-negative cells.

HLA-E peptide complexes can also trigger activating signals to NK cells via interacting with NKG2C. Therefore, enhanced HLA-E expression on HCV-infected cells could enhance antiviral immunity if NKG2C dominated over NKG2A in chronic hepatitis C. To address this issue we analyzed surface expression of NKG2A and NKG2C on CD3(-) CD56(+) NK cells. As shown in Figure 5A, we

Figure 3. Involvement of CD94/NKG2A in recognition of the HLA-E/HCV core₃₅₋₄₄ complex. **A:** To analyze whether CD94/NKG2A was involved in impaired cytolytic function of NK cells we blocked this inhibitory NK cell receptor by either a CD94-specific (empty triangles) or a NKG2A-specific monoclonal antibody (filled squares) before performing a ⁵¹chromium release assay. Lysis of K-562 HLA-E cells incubated with HCV aa35-44/+anti-NKG2A versus cells incubated with HCV aa35-44 alone: *P* < 0.05 (Mann-Whitney *U*-test); lysis of K-562 HLA-E cells incubated with HCV aa35-44/+anti-CD94 versus cells incubated with HCV aa35-44 alone: *P* < 0.05 (Mann-Whitney *U*-test). **B:** To ensure specificity of our results and exclude unspecific enhancement of NK cell activity by anti-CD94 or anti-NKG2A we incubated K-562 HLA-E target cells with the irrelevant peptide IP1 instead of peptide HCV core aa35-44. NK cell effector cells were incubated with anti-CD94 or anti-NKG2A, respectively, prior to performing the cytotoxicity assay. Specific lysis was calculated as described in Materials and Methods for the different effector:target (E:T) ratios as indicated in the figure. This figure shows the means of three experiments.

found that NKG2A expression dominates over expression of NKG2C on peripheral NK cells. Moreover, we found a significantly higher expression of the inhibitory NKG2A receptor on peripheral NK cells in chronic hepatitis C than in uninfected individuals, whereas no significant differences were found concerning expression of NKG2C.

Staining of liver infiltrating CD3(-)CD56(+) NK cells for NKG2C and NKG2A confirmed that expression of NKG2A was up-regulated in HCV infection and exceeded expression of NKG2C (Figure 5B).

Next, we analyzed whether HCV core aa35-44-mediated stabilization of HLA-E expression affects lytic affinity of freshly isolated natural killer cells obtained from HCV-infected individuals. Since extrahepatic NK cells showed comparable NKG2-expression patterns as intrahepatic NK cells, which, however, were not available in sufficient amounts to enable functional studies, we used peripheral NK cells for these experiments. In line with the results obtained with NKL cells we found that incubation of HLA-E transfected K-562 cells with peptide HCV core aa35-44 prevented target cell lysis by freshly isolated NK cells (Figure 5C).

Discussion

Natural killer cells importantly contribute to the initial control of viral infections through their rapid and potent cytotoxic activity. They are a pivotal element of the innate immune response, and impaired function of NK cells has been linked with an increasing susceptibility to HIV, EBV, and CMV infection. Moreover, impaired NK cell function has also been reported as potential factor in HCV infection predisposing to the development of chronic hepatitis C.^{4,5,24}

NK cell function is regulated via distinct immunostimulatory or -inhibitory ligand-receptor interactions. Among these, the inhibitory C-type lectin NK cell receptor CD94/NKG2A specifically interacts with HLA-E complexed with hydrophobic peptides derived from the leader peptide of various HLA-class I heavy chains.^{11,21}

Here, we analyzed whether HCV core-derived peptides can interact with HLA-E, thereby altering the function of NK cells.

In our panel, we identified peptide HCV core₃₅₋₄₄ to bind to HLA-E and to stabilize the expression of the HLA-E/ β 2-microglobulin complex. Importantly, HCV core₃₅₋₄₄-mediated stabilization of HLA-E expression on

Figure 4. Intrahepatic expression of HLA-E in HCV. **A:** Representative examples of immunoperoxidase staining with the HLA-E specific mAb 3D12 on liver samples from HCV-negative and HCV-positive donors at a $\times 200$ magnification. In contrast to livers from healthy, uninfected individuals (left) a conspicuous number of HLA-E expressing cells (arrows) were found in HCV-infected livers (right), which showed a macrophage-like morphology. Numerous lymphocytes are also present in this section and their nuclei avidly take up the dark blue counterstain. The inset shows a $\times 400$ magnification of part of the liver section. The diagram on the right gives the number of intrahepatic HLA-E-positive cells (mean and SEM) in liver specimen from five HCV RNA(+) and five HCV RNA(-) individuals. Quantitative analysis was performed by manually counting HLA-E(+) cells in 10 visual fields at $\times 200$ magnification for each liver sample. **B:** To analyze the identity of intrahepatic HLA-E-positive cells liver samples were double-labeled for HLA-E and CD68. All sections were immunostained with the indirect immunoperoxidase method and counterstained with hematoxylin. In double-labeling experiments, we incubated the sections with fluorescein isothiocyanate-conjugated CD68-specific antibodies after performing the HLA-E-specific immunoperoxidase reaction. **C:** Flow cytometric analysis of HLA-E expression of intrahepatic cells from HCV RNA(+) donors. **D:** Expression of HLA-E on hepatocytes from a HCV RNA(+) patient as compared to HLA-E expression on hepatocytes from a HCV RNA(-) individual. First, hepatocytes were identified by staining with mAb Hepar1 (dot plot, left). Hepar1-positive cells (gated) were then stained for expression of HLA-E (histograms, right). **E:** HLA-E-expression in chronic hepatitis C on intrahepatic cells that could be stained with a HCV core-specific mAb (clone 6A1) as compared to cells negative for HCV core antigen.

Figure 5. Surface expression of NKG2A and NKG2C on NK cells in chronic hepatitis C. **A:** Percentage of NKG2A-positive and NKG2C-positive CD3(-) CD56(+) NK cells in the peripheral blood of patients with chronic hepatitis C as compared to healthy individuals. Data are presented as boxplots showing median, upper, and lower quartiles and extreme values. **B:** Surface expression of NKG2A and NKG2C on intrahepatic NK cells from a representative HCV RNA-positive patient and from a healthy control as measured by flow cytometry. **C:** HLA-E transfected K-562 HLA-E B5 cells were incubated with 100 μ mol/L of HCV core₃₅₋₄₄ (filled diamonds) or without peptide (filled squares). After an overnight incubation at 26°C, a cytotoxicity assay was performed using purified natural killer cells of a HCV RNA-positive patient as effectors and K-562 HLA-E B5 cells as targets.

HLA-E transfected K-562 cells resulted in protection against lysis by the human NK cell line NKL. Blocking experiments with monoclonal antibodies confirmed that this resistance of target cells was mediated by interaction of HLA-E with the inhibitory NK cell receptor complex CD94/NKG2A. To our knowledge this is the first report on a direct interaction of a HCV-derived peptide with a non-classical HLA molecule.

The specificity of the stabilization of HLA-E by HCV core₃₅₋₄₄ was demonstrated by the fact that co-incuba-

tion of HLA-E-negative K-562 cells with HCV core₃₅₋₄₄ did not result in enhanced 3D12 staining as measured by FACS analysis. Furthermore, we found that stabilization of HLA-E expression and inhibition of NK cell function were both dose-dependent. To corroborate our results we performed dose titration experiments of the HCV-core peptide in direct comparison to the positive control peptide Cw03 derived from the leader sequence of HLA-Cw03, which is also well-known to stabilize expression of HLA-E. We found that binding of Cw03 enhanced HLA-E expression and impaired NK cell function comparable to HCV core aa35-44. These control experiments support the concept that the HCV core-derived peptide exerts similar interactions with HLA-E as the known HLA leader peptides.

Peptide HCV core aa35-44 is well conserved among the known HCV genotypes (Los Alamos HCV Data Base, <http://hcv.lanl.gov/content/hcv-db/index>). It is particularly noteworthy, that peptide HCV core₃₅₋₄₄ represents a well-characterized HLA-A2-restricted CTL epitope, which has been shown by numerous studies to be generated from the core protein and recognized by circulating cytotoxic T lymphocytes in natural HCV infection.²⁵ Therefore, our observations suggest that the epitope HCV core₃₅₋₄₄ can be presented by both the non-classical MHC class I molecule HLA-E and the classical MHC class I molecule HLA-A2.

Inhibition of NK cell activity due to HLA-E stabilization by an epitope that is capable of inducing CD8+ T cell responses if presented via HLA-A2 raises the question which role this epitope might play in HCV persistence. In this context, it is important to note that cytotoxic T lymphocytes (CTL) also express NKG2A and that inhibitory NKRs such as NKG2A are importantly involved in regulation of CD8+ T cell functionality.²⁶⁻²⁸ Jabri and colleagues²⁷ recently showed that stimulation of NKG2A on CTL leads to inhibition of positive signaling via the T cell receptor (TCR). Thus, it is conceivable that presentation of peptide HCV core aa35-44 via HLA-E in addition to its presentation via HLA-A2 results in impaired TCR-mediated CTL activation due to HLA-E/NKG2A interactions. This hypothesis is further supported by our previous finding of an enhanced expression of NKG2A on CTL in chronic hepatitis C.²⁹

Commonly, expression of HLA-E depends on binding of nonamer peptides derived from the signal sequence of HLA class I molecules.^{15,18,30} However, HLA-E also binds peptides, which differ from the motif in signal sequences of MHC class I molecules with several putative HLA-E epitopes being proposed.^{14,20} Previous studies suggested a methionine at P2 as anchor motif residue^{18,31} whereas other studies have shown that peptides with a threonine at P2 still can bind to HLA-E.³² Using a recombinant random peptide approach Stevens and co-workers¹⁴ predicted the presence of a leucine at positions P4 and P9 and an asparagine at position P7 as anchor motifs and possible additional binding motifs at positions P2, P3, P5, P6, P7, and P8.

Unexpectedly, HCV core₃₅₋₄₄ does not fulfill any of these predicted binding motifs. However, HCV core₃₅₋₄₄ has uncharged, hydrophobic amino acids at most posi-

tions, in accordance with the hypothesis of Stevens et al,¹⁴ that HLA-E has a strong preference for hydrophobic residues. In particular, glycine at position P9 of HCV core₃₅₋₄₄ is an uncharged hydrophobic residue similar to the P9 leucine anchor motif proposed by Stevens and co-workers.¹⁴ Interestingly, Miller et al³³ demonstrated that replacement of methionine with leucine at P2 reduced affinity for HLA-E only slightly in competition-binding experiments, suggesting leucine as a secondary motif at this position. Of note, HCV core₃₅₋₄₄ contains a leucine at P2. Finally, amino acids at non-anchor positions may also influence the selectivity at a given anchor position due to effects on the conformation of the bound peptide or the HLA-E molecule.¹⁴

Binding of HLA-A2-restricted epitopes to HLA-E has been reported previously. Ulbrecht and colleagues¹⁵ showed that HLA-E can bind two peptides corresponding to HLA-A2 restricted epitopes of the EBV BZLF-1 protein and the matrix protein of the influenza A virus, respectively, although these peptides have sequences quite different from classical MHC I leader peptides. These HLA-E complexes, however, were not recognized by CD94/NKG2A. Miller and colleagues³³ showed that an asparagine at P5 is crucial for the interaction of the HLA-E/peptide-complex with CD94/NKG2A. This criterion is met by HCV core₃₅₋₄₄ but not by the EBV- nor by the influenza A virus-derived peptide, which might explain the observed differences in recognition by CD94/NKG2A.

Hepatitis C studies on the innate immune response are hampered by both the usually non-apparent onset of HCV infection and by the lack of a suitable model system. Thus, analysis of the *in vivo* significance of our findings was confined to studying HLA-E expression in chronic hepatitis C. We found a significant up-regulation of HLA-E expression in livers derived from HCV-infected individuals as compared to HCV-negative persons, which support an *in vivo* relevance of our results (Figure 4A). Expression of HLA-E could be detected on a variety of intrahepatic cells in chronic hepatitis C including CD68(+) macrophages/Kupffer cells, CD31(+) sinusoidal endothelial cells as well as CD14(+) and CD83(+) cells.

Due to the fact that hepatocytes only show minimal physiological expression of MHC I molecules³⁴⁻³⁸ and taking into account that HLA-E represents only a minor fraction of total MHC molecules, immunostaining with mAb 3D12 was not sufficiently sensitive to detect HLA-E expression on hepatocytes. However, using a flow cytometric approach enhanced HLA-E expression was also detectable on hepatocytes, the main site of viral replication, confirming the *in vivo* relevance of our data.

Apart from inhibition of NK cells, up-regulation of HLA-E may have further functional consequences. Increased HLA-E expression was particularly seen on hepatic CD68(+) macrophages/Kupffer, which should protect these cells against lysis by NK cells and thus prolong antigen presentation. Indeed, Pietra et al³⁹ demonstrated that autologous antigen presenting cells pulsed with a HLA-E stabilizing peptide (VMAPRTLIL) became less susceptible to lysis mediated by NK cells as compared to unpulsed DCs. Blocking experiments confirmed that this inhibition of NK cell cytotoxicity was a result of interaction

between the inhibitory NK cell receptor NKG2A and HLA-E expressed on dendritic cells. However, it remains unclear whether this mechanism is operative *in vivo*, because it has been shown that unlike NK cells, NK-CTL, which recognize HLA-E via their T cell receptors, acquire *de novo* cytolytic activity against antigen-presenting cells with up-regulated HLA-E expression. Thus, killing of APC by T cells rather than NK cells has been proposed as a feedback mechanism resulting in regulation of immune response.⁴⁰⁻⁴²

Of note, HLA-E surface expression was especially high on cells with intracellular expression of HCV core. This is a further hint to support the hypothesis that peptides such as HCV core aa35-44 might be responsible for enhanced surface expression of HLA-E in HCV-infection.

Recently, IFN-gamma has been shown to stabilize HLA-E expression.⁴³ Thus, we cannot exclude the possibility that IFN-gamma contributes a synergistic effect on peptide-mediated stabilization of HLA-E expression and subsequent impairment of NK cell cytotoxicity in chronic hepatitis C, as has been suggested in CMV infection.⁴³ In addition, IFN-gamma has been shown to protect short-term ovarian carcinoma cell lines from CTL lysis via a CD94/NKG2A-dependent mechanism.⁴⁴ Thus, if IFN-gamma is involved in HLA-E regulation in replicative HCV infection, it is likely to act synergistically on HCV core-mediated inhibition of NK cells lysis rather than to counteract HLA-E signaling.

Inhibition of NK cell activity due to increased expression of HLA-E as a consequence of the binding of peptide HCV core₃₅₋₄₄ resembles similar findings in HCMV infection. The human cytomegalovirus codes for gpUL40, which also provides a ligand for HLA-E. HLA-E complexed with the gpUL40-derived peptide interacts with the inhibitory NK cell receptor CD94/NKG2A resulting in impaired cytotoxicity of natural killer cells. Thus, suppression of NK cell activity due to enhanced expression of HLA-E might be a general mechanism of viruses to escape the immune system. The impact of the HCMV gpUL40-derived HLA-E ligand with regard to NK cell escape of infected cells *in vivo* has not been finally resolved, as conflicting results were obtained with regard to UL40-mediated NK evasion during productive infection. This fact might be explained by variations in the effector cells, as NK cells express a range of activating and inactivating receptors.^{45,46} However, here we showed enhanced expression of the inhibitory NK cell receptor NKG2A on both peripheral and intrahepatic NK cells in comparison to NK cells from healthy HCV RNA-negative individuals. In contrast, expression levels of the activating NKG2C receptor did not differ significantly between healthy and HCV-infected persons, which supports the concept that enhanced HLA-E expression is likely to be functionally relevant concerning down-regulation of NK cell activity during chronic hepatitis C.

In conclusion, our observation of a dual recognition of a major CTL epitope via HLA-A2 and HLA-E is a first hint on a direct interaction of a HCV-derived peptide with a non-classical HLA molecule, which may have important immunomodulatory effects and thus should be further studied to define its role in HCV infection.

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