Generation of T-cell receptors targeting a genetically stable and immunodominant cytotoxic T-lymphocyte epitope within hepatitis C virus non-structural protein 3

Anna Pasetto,1,2 Lars Frelin,2 Anette Brass,2 Anila Yasmeen,1 Sarene Koh,2,3 Volker Lohmann,4 Ralf Bartenschlager,4 Isabelle Magalhaes,5 Markus Maeurer,5 Matti Sällberg2 and Margaret Chen1

Correspondence
Margaret Chen
Margaret.Chen@ki.se

1Department of Dental Medicine, Karolinska Institutet, Stockholm, Sweden
2Department of Laboratory Medicine, Stockholm, Sweden
3Singapore Institute for Clinical Sciences, Agency for Science, Technology and Research (A*STAR), Singapore
4Department of Infectious Diseases, Molecular Virology, University of Heidelberg, Germany
5Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet and Swedish Institute for Infectious Disease Control, Stockholm, Sweden

Hepatitis C virus (HCV) is a major cause of severe liver disease, and one major contributing factor is thought to involve a dysfunction of virus-specific T-cells. T-cell receptor (TCR) gene therapy with HCV-specific TCRs would increase the number of effector T-cells to promote virus clearance. We therefore took advantage of HLA-A2 transgenic mice to generate multiple TCR candidates against HCV using DNA vaccination followed by generation of stable T-cell–BW (T-BW) tumour hybrid cells. Using this approach, large numbers of non-structural protein 3 (NS3)-specific functional T-BW hybrids can be generated efficiently. These predominantly target the genetically stable HCV genotype 1 NS31073–1081 CTL epitope, frequently associated with clearance of HCV in humans. These T-BW hybrid clones recognized the NS31073 peptide with a high avidity. The hybridoma effectively recognized virus variants and targeted cells with low HLA-A2 expression, which has not been reported previously. Importantly, high-avidity murine TCRs effectively redirected human non-HCV-specific T-lymphocytes to recognize human hepatoma cells with HCV RNA replication driven by a subgenomic HCV replicon. Taken together, TCR candidates with a range of functional avidities, which can be used to study immune recognition of HCV-positive targets, have been generated. This has implications for TCR-related immunotherapy against HCV.

INTRODUCTION

It is estimated that 180 million individuals are infected with hepatitis C virus (HCV) worldwide today, of which 130 million are chronic carriers at risk of developing liver cirrhosis and liver cancer. Being a small, enveloped RNA virus, HCV is one of the most persistent viruses in humans, and spontaneous resolution of HCV infection only occurs in a minority of the infected (Alter, 2006). Considering the steady increase of reported incidence in Europe (Rantala & van de Laar, 2008), a reduction of HCV prevalence is not anticipated in the near future.

Analyses of individuals who have resolved their HCV infection have indicated that a successful immunity requires effector T-cells. CD4+ and CD8+ lymphocyte activation early in the infection is associated strongly with eradication of HCV infection (Cucchiariini et al., 2000; Diepolder et al., 1996; Lechner et al., 2000; Pape et al., 1999; Schulze zur Wiesch et al., 2005), and an early development of polyfunctional T-cells further predicts a spontaneous resolution of HCV infection (Badr et al., 2008). However, failure to sustain virus-specific CD8+ lymphocytes is frequently observed in chronically infected patients (Lechner et al., 2000). T-cells from these patients are associated with features such as functional exhaustion (Kantzanou et al., 2003; Radziewicz et al., 2007), developmental arrest (Cox et al.,...
A successful immune response in people infected with HCV is characterized by strong and persistent CTL responses. One well-defined CTL epitope is the HCV non-structural protein 3 (NS3)1073–1081 peptide (NS31073), and responses against this epitope are often found in spontaneous resolution of HCV infection, which are well-maintained after spontaneous recovery of HCV (Wertheimer et al., 2003). Several vaccines that target induction of NS3-specific T-cell immunity are currently being tested in clinical trials (Thompson & McHutchison, 2009). Recently, a novel strategy for engineering new effector T-cells against NS31073 via gene transfer of a T-cell receptor (TCR) in vitro was described (Zhang et al., 2010). Given the observation that the NS31073 epitope can be associated with cross-reactivity with an influenza A virus (IAV) neuraminidase (NA) epitope, and that cross-reactive CD8+ T-cells have been found in fulminant acute hepatitis patients (Urbani et al., 2005), access to T-cells that differ in functional avidity would be desirable for comparison.

To date, only one NS31073-specific human TCR (Zhang et al., 2010) has been identified and no murine alternatives are available. Murine TCRs present several advantages over human TCRs in gene modification of human T-cells, including an enhanced and more sustained level of surface expression and improved anti-tumour activity (Kieback & Uckert, 2010). Moreover, a recent murine TCR gene-therapy trial that targeted tumour antigens demonstrated a therapeutic effect in cancer patients, although some immune responses to murine TCR variable regions were found in a subset of patients (Davis et al., 2010). Given these facts, we therefore chose to explore the HLA-A2 transgenic (HHD) mouse model to establish more TCR gene candidates. Here a large number of functional TCR/CD3 hybrid clones were obtained by fusing the BW5147 cell line and activated splenocytes cells from HHD mice immunized with an electroporation-enhanced HCV NS3 DNA vaccine. The results indicate that the T-BW hybrid clones are highly specific to the NS31073 of HCV genotype 1a and 1b variants. Differences were found regarding their functional avidity and affinity to the NS31073/HLA complex pentamer, as well as their ability to respond to human hepatoma cells harbouring HCV subgenomic RNA.

RESULTS

Generation of HLA-A2-restricted mouse T-BW hybrid clones specific to HCV NS3

We used CD8+ T-cells from HHD (HLA-A2 transgenic) mice (Pascolo et al., 1997) immunized with the electroporation-delivered codon-optimized HCV DNA vaccine coNS3/4A-pVAX1, and performed cell fusion with BW TCRneg cells. Single-cell cloning was carried out under hypoxanthine–aminopterin–thymidine (HAT) selection. T-BW hybridized clones that survived selection were screened for the surface expression of CD3 and positive clones were tested against human T2 cells (a lymphoblastic cell line) loaded with the HLA-A2-restricted NS31073 (CINGVCWTV) or NS31406 (KLVALGVNAV) peptide. Analysis of interleukin-2 (IL-2) and gamma interferon (IFN-γ) release in the supernatant demonstrated that nine of them recognized the NS31073+ loaded T2 cells and none recognized that loaded with NS31406 (Fig. 1a). The reactivity was confirmed by retesting on T2 cells as well as on HHD splenocytes (Fig. 1a–d). TCR/CD3 surface expression for the respective clones was 51% (I8H4), 81% (I8A4), 79% (I4G7), 85% (I4F8), 95% (I2B11), 95% (I4E9), 84% (I6B3), 52% (I7B7) and 89% (I4F9) at the time of the assays.

During the screening process, the hybrid clones were regularly subcloned and checked for CD3 expression to ensure that they maintained a stable CD3 expression and IL-2 activity (Supplementary Fig. S1, available in JGV Online). Although the clone I8A4 demonstrated repetitively low response against NS31073, the percentage of live CD3-expressing cells and the cell-surface CD3 expression of this clone were similar to the others at the time of testing. None of the clones showed CD8 surface expression (data not shown), which is analogous to previous reported BW-derived T-cell clones (Rock et al., 1990); thus, the cytokine release detected here is independent of the CD8 cofactor.

TCR gene usage of NS31073-specific T-BW cell hybrid clones

Examination of the TCR usage of these T-BW hybrid clones was done in order to assess the TCR responsible for recognizing NS31073. RNA was isolated from each of these clones and at least five TCR α and β gene products were sequenced. Sequence analysis showed that six of the nine clones were sister clones (Fig. 2a); AV9 and BV2 gene usage and an identical third complementarity-determining region (CDR3) sequence were found in I4F8, I2B11, I4E9, I6B3, I7B7 and I4F9. The AV9 gene was also used by I4G7, which has a unique CDR3 coding sequence using AJ27. The AV16 and AV2 genes were used by I8H4 and I8A4, respectively, and have their own CDR3 with different AJ genes. The TCR β genes were likewise determined for all clones. Three clones utilized VB13 with various subfamilies BV13-1 (I8H4), BV13-2 (I8A4), and BV13-3 and BV13-1 were found in I4G7 and they all used different BD and BJ genes. The I4F8 and its sister clones all used the BV2 gene and shared identical CDR3 nucleotide sequences.

Here, the CDR3α and CDR3β loops were between 10–13 and 11–14 aa residues, respectively. They exhibit a type II pattern bias (Turner et al., 2006) that is characterized by the preference of the motifs ‘KLTFG’ (CDR3α loops) and ‘GG’ (CDR3β loops). A commonly found motif in the BJ segments here was ‘FGPGTR’, which is reported only in
three out of 13 murine and four out of 14 human TRBJ genes (IMGT Repertoire IG and TR database; http://www.imgt.org/IMGTrepertoire/). Interestingly, the same three motifs also exist in a recently reported human TCR that is specific to the NS3 1073 peptide (Zhang et al., 2010). This result suggests that highly conserved CDR3 motifs are shared by the murine and human TCRs that are specific to the NS3 1073 peptide.

**Affinity to NS3_{1073}/HLA-A2 pentamer**

Since the T-BW hybrid clones lack the CD8 coreceptor, which might be required for the stabilization of the TCR-peptide/HLA complex (Roszkowski et al., 2003), we next asked whether any of these clones could bind the NS3_{1073}/HLA-A2 pentamer. Following incubation with an R-phycocerythrin (R-PE)-labelled NS3_{1073}/HLA-A2 pentamer, the fluorescence intensity of the staining was quantified by flow cytometry. Compared with the negative-control pentamer HBVcore_{18–27}/HLA-A2, it was found that I8H4, one of the high-avidity clones, had an increased fluorescence in NS3_{1073}/HLA-A2 pentamer staining (Fig. 2b).

**Comparison of the functional avidity**

We next asked whether the functional avidity in these T-cell hybrid clones might differ. The clones were regularly subcloned and checked by FACS and were tested when the TCR/CD3 expression was at least 50 % at the time of the experiment. They were tested with T2 target cells pulsed with diluted amounts of NS3_{1073} peptide and the results showed that, while I8A4 required at least 400 ng peptide ml⁻¹ to obtain a half-maximum response of IL-2 release, about 10 ng peptide ml⁻¹ was sufficient for stimulating I8H4, I4G7 and I4F8 for a half-maximum response of IL-2 release (Fig. 3). The TCR/CD3 surface expression was 51 % (I8H4), 82 % (I8A4), 88 % (I4G7) and 93 % (I4F8) at the time of this experiment. The experiment was performed three times with similar results. Based on these data, I8A4 is ranked as a low-avidity T-cell clone, while I8H4, I4G7 and I4F8 resemble moderate/high-avidity T-cells described previously (McKee et al., 2005).

**Cross-genotype reactivity**

We next asked whether these clones cross-reacted with other viral peptides, in particular the naturally occurring genotypes of HCV NS3_{1073} and the IAV NA, Flu-NA_{231}, to which cross-reactivity to HCV NS3_{1073} of genotype 1b strain has been described (Wedemeyer et al., 2001). Shown in Fig. 4, we found that these T-cell clones were activated only when cultured with the genotype 1a and 1b peptide of the HCV NS3_{1073}. None of the other HCV genotype peptide variants tested was sufficiently stimulatory to induce substantial IL-2 production, although all variants displayed significant binding affinity to the HLA-A2 molecule (Fytiti et al., 2008). No IL-2 production was found against the Flu-NA peptide (Fig. 4) or other viral peptides, including the HCV non-structural protein 5 (NS5) peptide NS3_{2221} and NS5_{1992} SPDADLIEANL and VLTDFKTWL, and human cytomegalovirus (HCMV) pp65_{195} NLVPMVATV (data not shown). The TCR/CD3 surface expression was 97 % (I8H4), 70 % (I8A4), 99 % (I4G7) and 95 % (I4F8) at the time of this experiment. The
results suggest that these T-cell clones are highly specific for genotype 1 of HCV NS3.

Relevance of each amino acid position of NS31073 peptide for hybridoma reactivity

Since amino acid positions 2, 7, 9 and positions 3, 4, 5 of NS31073 are key positions in HLA binding and interaction with human TCR (Söderholm et al., 2006), respectively, we next tested whether they have relevance for the four murine T-BW hybrid clones, 18H4, 18A4, 14G7 and 14F8. Alanine-substituted peptide analogues of NS3 1073 loaded on T2 cells in descending concentrations were tested against these T-cell clones. As shown in Fig. 5, alanine substitutions at position 3, 4, 5 and 7 completely abolished the IL-2 release in all four T-cell clones, which is in line with that observed for human T-cells. Complete or partial IL-2 release in response to alanine substitutions at position 1, 2, 6, 8 and 9 was, however, obtained in high-avidity clones, and was also observed at reduced peptide concentrations. This was unfortunately not observed for I8A4 (low avidity), which responded only to alanine substitution at position 1 and none of the other substitutions. The TCR/CD3 surface expression was 51 % (I8H4), 82 % (I8A4), 88 % (I4G7) and 93 % (I4F8) at the time of this experiment.

Recognition of target cells with low HLA-A2 expression

T2 cells are a lymphoblastic cell line commonly used as antigen-presenting cells for loading exogenous peptide and, due to the constitutive HLA-A.2 expression and TAP (transporter associated with antigen processing) deficiency, the density of these target molecules is often saturated. We next asked whether our T-cell hybrid clones recognize target cells that have low HLA-A2 expression. The hepatoblastoma Huh-6 and C1R-A2 cell lines were chosen for this experiment. Cell-surface expression of HLA-A2 assessed by staining with an anti-HLA-A2 mAb (Fig. 6a) after NS3 1073 peptide stabilization showed that both cell lines have lower HLA-A2 expression than T2 cells. Huh-6 cells showed the lowest HLA-A2 expression, as only a
fraction of the cells were positive for staining compared with the negative-control C1R-null cell line (Fig. 6b). We then assessed IL-2 production in the T-cell clones cocultured with these target cells. Shown in Fig. 6(c), NS3_1073 peptide-loaded C1R-A2 cells could stimulate significant IL-2 production in all four T-cell lines, at a similar magnitude to that observed for the T2 target cells. Significant IL-2 release against the peptide-loaded Huh-6 cells was, however,

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**Fig. 4.** Cross-reactivity against other viral peptides that share similarity with the NS3_1073 peptide sequence encoded by the DNA vaccine (genotype 1a). Overnight IL-2 production in T-BW hybrid clones against T2 cells loaded with the indicated viral peptide (10 µg peptide ml⁻¹) was measured for each T-cell clone and is shown as a percentage of IL-2 production by genotype 1a of the NS3_1073 peptide. Amino acids that differ from the genotype 1a are indicated in bold. Mean values and SD of triplicate co-cultures are shown. Flu, Flu-NA_231; gt, genotype.

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**Fig. 5.** Responses to the alanine-substituted peptide analogues of NS3_1073. Overnight IL-2 production in T-BW hybrid clones (I8H4, •; I8A4, ○; I4G7, ■; I4F8, □) co-cultured with T2 cells loaded with descending concentrations of the alanine-substituted peptides of NS3_1073 (substituted from positions 1 to 9). Mean values of duplicate co-cultures are shown.
only detected in the high-avidity clones, especially the I8H4 clone (Fig. 6d). TCR/CD3 surface expression was 90% (I8H4), 80% (I8A4), 89% (I4G7) and 90% (I4F8) at the time of this experiment.

Reactivity against HCV RNA replicon hepatoma cells

We next analysed the ability of the T-cell hybrids to recognize the Huh-7/Lunet HCV replicon cells that harbour the subgenomic HCV replicon. Huh-7/Lunet hepatoma replicon cells maintain constant levels of HCV replication over several years and represent an excellent model of persistent HCV infection (Bartenschlager & Sparacio, 2007). As Huh-7 cells lack HLA-A2 expression, they are stably transduced with lentiviral vectors expressing HLA-A2 and a selectable marker encoding the blasticidin-resistance gene, and transfected with the HCV genotype 1b Con1-ET subgenomic replicon and a selection marker conferring the neomycin-resistance gene by using an approach described previously (Ahlen et al., 2007). As Huh-7 cells lack HLA-A2 expression, they are stably transduced with lentiviral vectors expressing HLA-A2 and a selectable marker encoding the blasticidin-resistance gene, and transfected with the HCV genotype 1b Con1-ET subgenomic replicon and a selection marker conferring the neomycin-resistance gene by using an approach described previously (Ahlen et al., 2007). As shown in Fig. 7(b), antigen-specific IL-2 production by the T-cell clones was detected after co-culture at different ratios with peptide (NS3_{1073} genotype 1a)-loaded HCV replicon cells harbouring both the Con1-ET replicon and HLA-A2 (R-neo/A2) or control cells expressing only the HLA-A2 (A2). In these cultures both IL-2 and IFN-γ were detected, particularly from the high-avidity I8H4, I4F8 and I4G7 T-cell clones, and this indicates that the lentiviral-transferred HLA expression is functional. The TCR/CD3 surface expression was 90% (I8H4), 80% (I8A4), 89% (I4G7) and 90% (I4F8) at the time of the experiment. In the same experiment, we investigated whether these T-cell clones recognized the replicon cells directly without addition of exogenous peptide. Shown in Fig. 7(a), IL-2 production was detected in the co-culture of the I8H4 and HLA-A2-positive HCV replicon cells (R-neo/A2), and this IL-2 production was dependent on the ratio of the added cells. No IFN-γ was detected (data not shown). Stimulation with HCV replicon cells without HLA-A2 expression or cells with HLA-A2 expression only did not stimulated any IL-2 release from the I8H4. This result concurs with previous results that I8H4 is the only clone with affinity to the NS3_{1073}/HLA-A2 pentamer (Fig. 2) and has an increased response to genotype 1b sequence (Fig. 4) and the Huh-6 target cells (Fig. 6).

Transfer of TCR genes into naïve non-HCV specific human T-cells

Although BW hybridomas are a good model to assess TCR functions, they have a limited clinical application in humans. To know whether murine TCRs identified here are functional to redirect non-HCV-specific human
T-lymphocytes against HCV\(^+\) cells, peripheral blood lymphocytes (PBLs) from healthy human donors were thus transduced with retroviral vectors packaged with genes encoding I8H4 TCR and the I4F8 TCR, which reacted strongly against the NS3\(_{1073}\) peptide in previous experiments. Surface expression of the respective TCRs following retroviral transduction was detected following transduction in 10–20 \(\%\) CD3\(^+\) T-cells and not noticeably in mock, untransduced PBL controls (<0.15 \(\%\)) stained with the mouse V\(\beta\) antibodies. Transduced T-cells, here defined as mouseVb\(^+\)humanCD8\(^+\)-expressing cells, were found to produce significant amounts of IFN-\(\gamma\) when stimulated with NS3\(_{1073}\) 1a or 1b peptide-loaded T2 cells (Fig. 8a). Tumour necrosis factor alpha (TNF-\(\alpha\)) was found frequently in this IFN-\(\gamma\)-positive population and a 'triple'-positive population (IL-2\(^+\)TNF-\(\alpha\)- IFN-\(\gamma\)-) was clearly demonstrated (Fig. 8b). The CTL function was studied on luciferase-producing bioluminescent Huh-7/Lunet HCV replicon cells (HLA-A2\(^+\) Luc-ubi-neo Con1\(^+\)) that had been co-incubated with transduced T-cells using a charge-coupled device (CCD) camera. Images were taken at 20 h after co-incubation and analysed by Living Image software (version 4.2; Caliper Life Sciences). The analysis showed an efficient elimination of bioluminescent HCV replicon cells that have been co-incubated with I4F8 and I8H4 TCR-transduced T-cells (Fig. 8c, d), and a significant increase of hepatocellular aspartate transaminase was also detected in these co-culture supernatants when compared with those co-incubated with mock-transduced T-cells (Fig. 8e). In conclusion, our data indicate that murine TCRs identified by the current approach can provide antigen specificity to naïve non-HCV-specific T-cells to generate new effector T-cells that are polyfunctional and can eliminate HCV RNA-replicating hepatoma cells.

**DISCUSSION**

In this study, we describe identification of new HCV-specific T-cell clones in the HLA-A2 transgenic mouse...
model generated by DNA vaccination by electroporation and stable immortalization of these T-cell clones via somatic-cell hybridization with the BW5147 cell line. These T-cell clones recognize the NS31073 epitope, which is one of the most important CTL epitopes in hepatitis C often associated with spontaneous resolution of HCV infection (Wertheimer et al., 2003). As shown here, they are highly specific to NS3 1073 and cross-recognition is restricted within genotype 1a and 1b virus variants and not to other related viral sequences tested. This is interesting because genotypes 1a and 1b viruses are found in up to 70 % of infected patients in the USA and Europe. Moreover, these clones differ from each other with regard to their functional avidity and affinity. This resembles the discrepancy in T-cell avidity observed in humans between donors who have recovered from HCV infection and chronically infected patients (Neveu et al., 2008). In this regard, the high-avidity clones I8H4, I4G7 and I4F8 generated in this study have similar EM50 (effective molarity) values (approx. 10 ng ml$^{-1}$) to those found in CD8$^{+}$ T-cells in individuals who have recovered from HCV infection, while clone I8A4, which has a 10–100-fold-higher EM50 value (400 ng ml$^{-1}$), resembles the T-cells found in chronically infected patients (Neveu et al., 2008). Furthermore, there has been an elegant study by Kasprowicz et al. (2008) in which it was demonstrated that NS31073-specific T-cells that are CD8-independent might represent cells of higher functions, i.e. require substantially less viral peptide as well as fewer cells to achieve the effector function. This might be of relevance for the mouse TCRs reported here, as they were raised in the absence of human CD8 and are capable of recognizing the target cells in a CD8-independent manner.

To our knowledge, this is the first study that has examined the TCR repertoire that is induced by an HCV vaccine. The TCR repertoire induced by natural infection of HCV has so far described the $\beta$ chain (Miles et al., 2011), but it is interesting to find that our murine TCRs not only share similar CDR3 lengths to those found there, but also the same sequence motifs found in a human NS31073 TCR (Zhang et al., 2010). Given the conserved nature of these motifs, it is likely that they play a key role in the interface between TCR and antigen contacts, tickling the T-cell to respond.

Moreover, our report is the first to demonstrate that HCV-specific T-cell hybridomas of different functional avidities towards a human HCV CTL target can be generated rapidly by DNA vaccination of HHD mice. Studies of HCV antigen presentation often require substantial amounts of HCV-specific lymphocytes/primary human T-cells (Accapezzato et al., 2005; Barth et al., 2005, 2008; Lapenta et al., 2006) but, because of the short lifespan and the requirement of repetitive antigen stimulation with primary T-cell lines, an alternative approach to obtaining large amounts of HCV-specific T-cells would be of interest. The T-cell hybrid
clones presented here could be useful reporter T-cells with mouse cytokines as reporter protein; unlike primary T-cell clones, they do not require maintenance through antigen stimulation or cytokine growth factors, but grow vigorously in simple cell-culture medium without special supplements. Their activity does not fluctuate in a cyclical manner, as occurs with some antigen-stimulated T-cell clones. Freezing and recovery are easy and, moreover, good viability and activity are usually observed. Some limitations need to be considered here. One is that avidity is determined in peptide-titration assays and not by the off-rate of pentamers after binding to the TCR. The second is that BW-derived hybrids lack CTL function because CD8 expression is suppressed (Rock et al., 1990).

Despite the fact that only one hybridoma (I8H4) here recognized endogenously processed antigen from human hepatoma cells that replicate HCV RNA, we note that functional properties were further improved when I8H4 and 14F8, the latter also a high-avidity TCR, were genetically transferred and expressed on naïve non-HCV-specific and I4F8, the latter also a high-avidity TCR, were genetically hepatoma cells that replicate HCV RNA, we note that recognized endogenously processed antigen from human despite the fact that only one hybridoma (I8H4) here.

Methods

Animals. Indbred HHD-C57BL/6 (HHD +H-2D b/−/β2m−/−) mice transgenic for HLA-A2.1, and deficient for both H-2D b and murine β2-microglobulin (β2m) (kindly provided by Dr F. Lemonnier, Institut Pasteur, France) (Pascolo et al., 1997) were maintained at the Karolinska Institutet, Division of Comparative Medicine (AKM), Clinical Research Centre, Karolinska University Hospital The regulations of the Ethical Committee for animal research at the Karolinska Institutet were followed.

Human PBMCs. PBMCs from healthy blood donors were collected at the Karolinska University Hospital under informed consent, and isolated using Ficoll-Hypaque density-gradient centrifugation. Ethical permission was obtained from the Regional Ethical Review Board (EPN) of the Karolinska Institutet.

Plasmids and synthetic peptides. The plasmid coNS3/4A-pVAX1, containing the full-length codon-optimized NS3/4A gene of HCV genotype 1a, has been described previously (Frelin et al., 2003, 2004). The HCV NS3 genotype 1a peptides CINGVCWTV (aa 1073–1081) and KLVALGVNAV (aa 1406–1415) are referred to as NS31073 and NS31406 respectively (Brinster et al., 2001; Himoudi et al., 2002). Mutant peptides of NS31073 with indicated alanine (Ala) substitutions were used as indicated. Other viral peptides tested were: Flu-NA CVNGSCFTV (aa 231–239); HCV NS31073 genotype 1a CINGVCWTV, genotype 1b CVNGVCWTV, genotype 2 TISGVLWTV, genotype 3 TIGGVMTVT, genotype 4 AVNGVMWTV, genotype 5 AINGVMWTV; HCV NS5221 and NS51992 SDPADLIEANL (aa 2221–2231) and VLTDKFKTWL (aa 1992–2000); and HCMV pp65 NLVPVMVAT (aa 495–504). All peptides were synthesized (purity >70%) by ChronTech Pharma AB and EZBiolab.

Cell lines. BW5147 alpha-beta-cell line (BW TCR neg cells; kindly provided by Drs J. Kappler and P. Marrack at the National Jewish Medical and Research Center) were grown in complete BW medium [Dulbecco’s modified Eagle’s medium (DMEM) 10% FBS supplemented with non-essential amino acids, l-glutamine and gentamicin], T2, C1R-A2 and C1R-null cells were grown in RPMI 10% FBS with 2 mM l-glutamine and 100 mM HEPES. The hepatoblastoma Huh-6 cell line was cultured in DMEM with 10% FBS. T2 is an HLA A2.1+ cell line, a cloned hybrid between the 721.174 (variant of the LCL 721 B-lymphoblastic cell line) and CEM.R3 (8-azaguanine- and ouabain-resistant clone of the CEM T-lymphoblastic cell line). C1R-A2 and C1R-null are Epstein–Barr virus–transformed B-lymphocyte cell lines. Endogenous peptides bound to HLA-A2 molecules on C1R-A2 cells were removed by mild acid treatment (pH 3.3) and replaced by HCV NS3-specific HLA-A2 peptides (Jäger et al., 1999). All media and supplements were purchased from Invitrogen.

Generation of HCV NS3-specific T-BW hybridoma clones. Splenocytes and lymph node cells were isolated from coNS3/4A-DNA immunized HHD-C57BL/6 mice as described previously (Ahlen et al., 2007). Following 5 days stimulation with HCV NS3 peptides, CD8+ T-cells were purified using CD8 MACS MicroBeads (Miltenyi Biotec) and fused to BW TCR neg cells. Briefly, the CD8+ cells were mixed with BW TCR neg cells in a 1:5 ratio and PEG1450 (Sigma) was added; thereafter, the cells were resuspended in minimal essential medium (MEM) and incubated for 5 min at 37 °C, and cultured for 48 h before selection in HAT (Sigma Aldrich) and HT (HAT without aminopterin; Gibco, Invitrogen) media. Hybridized cell clones were maintained in complete BW medium. Positive clones were restet and regularly subcloned and checked for CD3 expression.

HCV replicon cells. Hepatoma Huh-7-Lunet cells designated Lunet-HlaA2-neoET, Lunet-blr/neo ET or Lunet-HlaA2 (neo) or Lunet-HLA-A2-Luc-ubi-neo Con1 were generated by rotocol similar to that described previously (Ahlen et al., 2007). The Lunet-HlaA2-neoET has ectopic HLA-A2 expression and a selectable HCV subgenomic RNA replicon of genotype 1b, harbouring replication-enhancing mutations in NS3 and NS4B (Con1-ET). This is the same for Lunet-HLA-A2-Luc-ubi-neo
Con1, but it also co-expresses the firefly luciferase gene. The control replicon cell line Lunet-bla/neo ET was transduced with an empty viral vector without the HLA-A2 gene. These cells were maintained in complete DMEM with addition of blasticidin S hydrochloride (3 μg ml⁻¹) and G418 (1 mg ml⁻¹). Lunet-HlaA2, the other control cell line (expressing HLA-A2 under blasticidin selection but without the HCV replicon), was maintained in the same DMEM as above but with 3 μg blasticidin S hydrochloride ml⁻¹ and no G418. For the co-culture experiments, the Lunet cells were washed and reseded 1 day before in antibiotic-free medium. All medium and supplements were purchased from Invitrogen.

Antibodies and flow cytometry. Antibodies against mouse CD3, CD8 and human HLA-A2 (BD Biosciences), and R-PE-labelled HLA-A*0201 Pro5 pentamers refolded with HCV NS31293 or HBV core18–27 (Proimmune) were used. For intracellular multicolour FACS staining, TCR-transduced PBMCs were incubated overnight with indicated stimuli. GolgiPlug (BD Biosciences) was added during the final 12 h. PMA at 50 ng ml⁻¹ and ionomycin at 500 ng ml⁻¹ (Sigma) were used as a positive control. Cells were then washed and stained with: Pacific Blue–anti-human CD3 (Biolegend), allophtococyanin–Cy7–anti-human CD8α (Biolegend), FITC-labelled anti-mouse Vβ 8.3 (BD Biosciences), anti-mouse Vβ8.1-8.2 (BD Biosciences), anti-mouse Vβ6 (BD Biosciences), anti-mouse Vβ4 (BD Biosciences), allophtococyanin–anti-human IL-2 (Biolegend), PE–anti-human IFN-γ (Biolegend) and PE–Cy7–anti-human TNF (BD Biosciences). A BD Cytofix/Cytoperm fixation/permeabilization kit was used. Cells were analysed using a BD LSRFortessa flow cytometer and FlowJo 9.2 (Tree Star) software.

Measurement of cytokine release. Target cells (2 × 10⁵) were cultured for 24 or 48 h with equal numbers or the indicated number of each BW-T cell hybrid clone in duplicates with or without different viral peptides. Concentrations of mouse IL-2 and IFN-γ in the supernatant were measured using mouse IL-2 or IFN-c ELISA (Mabtech) and calculated against standard curves generated with a Mabtech Quantikine kit. All media and supplements were purchased from Invitrogen.

Bioluminescence cell imaging and transaminase measurement. Either 50 000 or 100 000 Lunet-HlaA2-Luc-ubi-neo Con1 cells were co-cultured with transduced or mock-transduced T-cells in a ratio of 2:1. Following 20 h incubation the medium was replaced with luciferin solution prior to imaging with a charge-coupled device camera. Signals from bioluminescent Lunet-HlaA2-Luc-ubi-neo Con1 cells were analysed with the Living Image Software version 4.2 and IVIS Spectrum instrument (Caliper Life Sciences). The AST level in supernatants was quantified by a validated AST assay at the Clinical Chemistry Laboratory at the Karolinska University Hospital.

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