

Accepted Manuscript

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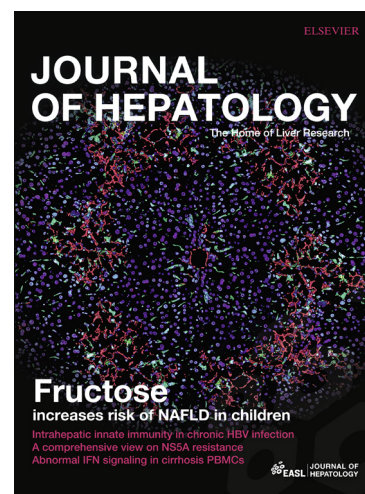
PII: S0168-8278(19)30111-4
DOI: <https://doi.org/10.1016/j.jhep.2019.01.034>
Reference: JHEPAT 7259

To appear in: *Journal of Hepatology*

Received Date: 25 June 2018
Revised Date: 30 January 2019
Accepted Date: 31 January 2019

Please cite this article as: Kemming, J., Reeves, E., Nitschke, K., Widmeier, V., Emmerich, F., Hermle, T., Gostick, E., Walker, A., Timm, J., Price, D.A., Hofmann, M., Thimme, R., James, E., Neumann-Haefelin, C., ERAP1 allotypes impact the epitope repertoire of virus-specific CD8⁺ T cell responses in acute hepatitis C virus infection, *Journal of Hepatology* (2019), doi: <https://doi.org/10.1016/j.jhep.2019.01.034>

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ERAP1 allotypes impact the epitope repertoire of virus-specific CD8⁺ T cell responses in acute hepatitis C virus infection

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Keywords: epitope repertoire, ERAP1, hepatitis C virus, HLA-B*27, T cells.

Electronic word count: 5,995 (including abstract, references and figure legends).

Number of figures and tables: 5/2.

Conflict of interest statement:

Nothing to declare.

Financial support statement:

This study was funded in part by the Deutsche Forschungsgemeinschaft (SFB1160 “Immune-mediated pathology as a consequence of impaired immune reactions [IMPATH]”, projects 8 and 10 awarded to R.T. and C.N.H.) and Cancer Research UK (Programme Grant A16997 awarded to E.J.). D.A.P. is a Wellcome Trust Senior Investigator (100326/Z/12/Z).

Author contributions:

J.K., E.R., T.H., D.A.P., M.H., R.T., E.J., and C.N.H. developed the concept and designed the study; J.K., E.R., K.N., V.W., F.E., E.G., and A.W. performed experiments and procedures; J.K., E.R., K.N., J.T., D.A.P., M.H., R.T., E.J., and C.N.H. wrote the manuscript.

Abstract

Background & Aims: Endoplasmic reticulum aminopeptidase 1 (ERAP1) polymorphisms are linked with human leukocyte antigen (HLA) class I-associated autoinflammatory disorders, including ankylosing spondylitis and Behçet's disease. Disease-associated ERAP1 allotypes exhibit distinct functional properties, but it remains unclear how differential peptide trimming *in vivo* affects the repertoire of epitopes presented to CD8⁺ T cells. The aim of this study was to determine the impact of ERAP1 allotypes on the virus-specific CD8⁺ T cell epitope repertoire in an HLA-B*27:05⁺ individual with acute hepatitis C virus (HCV) infection.

Methods: We performed genetic and functional analyses of ERAP1 allotypes and characterized the HCV-specific CD8⁺ T cell repertoire at the level of fine epitope specificity and HLA class I restriction.

Results: Two hypoactive allotypic variants of ERAP1 were identified in an individual with acute HCV infection. The associated repertoire of virus-derived epitopes recognized by CD8⁺ T cells was uncommon in two respects. First, reactivity was directed away from classically immunodominant epitopes, preferentially targeting either novel or subdominant epitopes. Second, reactivity was biased towards longer epitopes (10–11mers). Despite the presence of favorable prognostic indicators, these atypical immune responses failed to clear HCV.

Conclusions: ERAP1 allotypes modify the virus-specific CD8⁺ T cell epitope repertoire *in vivo*, leading to altered immunodominance patterns that may contribute to the failure of antiviral immunity after infection with HCV.

(218 words)

Lay summary

Endoplasmic reticulum aminopeptidase 1 (ERAP1) plays a key role in antigen presentation. Genetic variants of ERAP1 are linked with human leukocyte antigen (HLA) class I-associated autoinflammatory disorders, such as ankylosing spondylitis and Behçet's disease, but it remains unclear how these functionally distinct allotypes influence the repertoire of epitopes presented to CD8⁺ T cells. We found that ERAP1 allotypes can modify the virus-specific CD8⁺ T cell epitope repertoire *in vivo*, leading to altered immunodominance patterns that may contribute to the failure of antiviral immunity after infection with hepatitis C virus (HCV).

Introduction

Endoplasmic reticulum aminopeptidase 1 (ERAP1) trims peptides to an optimal length (usually 8 or 9 amino acids) for presentation in the context of human leukocyte antigen (HLA) class I molecules [1, 2]. Genome-wide association studies have identified single nucleotide polymorphisms (SNPs) in ERAP1 as important risk factors in several HLA class I-associated autoinflammatory disorders [3], including ankylosing spondylitis [4], especially in conjunction with HLA-B*27 [5], and Behçet's disease, which is strongly linked with HLA-B*51 [6]. In addition, SNPs in ERAP1 can combine to encode discrete allotypes with composite functional properties that further increase the risk of disease [7]. Biochemical analyses have revealed differential peptide trimming among ERAP1 allotypes, with hypoactive forms typically generating longer fragments (10–12mers), and hyperactive forms typically generating shorter fragments (7–8mers) [8-10]. However, the impact of specific ERAP1 allotypes on the naturally presented repertoire of peptide epitopes is currently unclear [11].

Hepatitis C virus (HCV) has infected approximately 71 million people worldwide. Persistent infection ensues in 50–80% of cases, with a high risk of liver disease, which may progress to cirrhosis, liver failure, and hepatocellular carcinoma [12]. Virus-specific CD8⁺ T cells are thought to play a key role in immune protection against HCV [13]. This consensus is predicated on associative studies that have linked viral clearance with potent and broadly directed CD8⁺ T cell responses and the presence of certain HLA class I allotypes [14]. In particular, up to 80% of HLA-B*27⁺ individuals clear HCV spontaneously, contrasting with only 20–50% of individuals in the general population [15]. We previously attributed this protective effect to the immunodominant HLA-B*27-restricted CD8⁺ T cell epitope NS5B₂₈₄₁₋₂₈₄₉ (ARMILMTHF), which is targeted in almost all HLA-B*27⁺ individuals during acute infection with HCV [16, 17].

In this study, we performed a comprehensive analysis of ERAP1 allotypes and virus-specific CD8⁺ T cell responses in an HLA-B*27:05⁺ individual with acute HCV infection. Despite a symptomatic presentation, expression of HLA-B*27, and a favorable interleukin 28B

(IL28B)/interferon lambda 4 (IFNL4) genotype, all of which predict viral clearance, this individual progressed to chronic infection, with low level viremia (mostly <100 IU/mL) throughout a follow-up period of 20 months before the initiation of antiviral therapy. In-depth genetic, biochemical, and immunological analyses revealed the presence of ERAP1 allotypes with hypoactive trimming functions, which shaped the epitope repertoire to elicit atypical HCV-specific CD8⁺ T cell responses.

Patients and methods

Samples. A donor (MM) with acute HCV genotype 1a infection acquired via needle-stick injury was recruited after providing written informed consent in line with federal guidelines and the Declaration of Helsinki. Ethical approval for this study was obtained from the Ethik-Kommission der Albert-Ludwigs-Universität Freiburg (#524/14). Venous blood samples (50 mL per draw) were collected in ethylene diamine tetraacetic acid (EDTA)-anticoagulated tubes at longitudinal time points. Peripheral blood mononuclear cells (PBMCs) were isolated using lymphocyte separation medium density gradients (PAA Laboratories, Austria) and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum, 1% streptomycin/penicillin, and 1.5% HEPES buffer 1M (complete medium; all additives from Thermo Fisher Scientific, Germany).

Peptides, antibodies, and multimers. Overlapping peptides (mostly 15 amino acids each, overlapping by 11 amino acids) spanning the entire polyprotein of HCV strain H77 (genotype 1a) were obtained from BEI Resources (Manassas, VA, USA) via the National Institute of Allergy and Infectious Diseases (Bethesda, MD, USA). Additional peptides were synthesized with a free amine NH_2 terminus and a free acid COOH terminus using standard Fmoc chemistry (Genaxxon Bioscience, Germany). The viability dye 7-aminoactinomycin D (7-AAD) and the directly conjugated monoclonal antibodies (mAbs) anti-CD8-PE (clone RPA-T8), anti-CD8-APC (clone SK1), anti-CD8-BV421 (clone RPA-T8), and anti-IFN- γ -FITC (clone 25723.11) were obtained from BD Biosciences (Germany). Multimeric complexes of HLA-B*27:05/NS5B₂₉₃₆₋₂₉₄₄ (GRAAICGKY) and HLA-B*27:05/NS5B₂₉₃₆₋₂₉₄₆ (GRAAICGKYLK) were generated as described previously [18].

CD8 selection and polyclonal expansion of PBMCs. Procedures were carried out as described previously [19]. More details may be found in the supplementary information.

Peptide-specific T cell lines. PBMCs were activated with peptides as described previously [17]. More details may be found in the supplementary information.

Enzyme-linked immunospot assay. An IFN- γ enzyme-linked immunospot (ELISpot) kit (Diaclone, France) was used to quantify peptide-specific T cells as described previously [17]. Peptides were distributed across 92 pools in a matrix format ($n = 10$ peptides per pool). Each peptide was present in two different pools, allowing identification of specific responses to individual peptides. Tests with peptide pools were performed in duplicate. In each assay, negative control wells ($n = 4$) contained medium alone, and positive control wells ($n = 4$) contained medium supplemented with phytohemagglutinin or phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma-Aldrich, Germany). Responses were considered positive if the mean number of spots per test well was at least three times as high as the mean number of spots per negative control well. Positive responses were confirmed by intracellular IFN- γ staining with single peptides.

Intracellular IFN- γ staining. Procedures were carried out as described previously [20]. More details may be found in the supplementary information.

HLA/peptide multimer staining. Procedures were carried out as described previously [21]. More details may be found in the supplementary information.

Isolation and cloning of ERAP1 allotypes. ERAP1 was amplified from donor cDNA as described previously [7, 10]. The amplicon was cloned into pcDNA3.1 (Life Technologies, UK) and sequenced to identify both chromosomal allotypes of ERAP1. Sequences from donor MM were compared with the prototype (PT) ERAP1 using DNADynamo alignment software (Blue Tractor Software, UK).

DNA constructs. The pcDNA3.1 minigenes (ES)-SHL8 and (ES)-X-SHL8 were engineered to encode an ER targeting signal sequence [10]. The pcDNA3.1-(ES)-SRG-SHL8 construct was generated via the incorporation of an additional nucleotide sequence encoding serine, arginine, and glycine (SRG) in pcDNA3.1-(ES)-SHL8, engineered using the primers 5'-CTTGCGGCAGTCTGCAGCGCGAGCCGTGGCAGCATCATCAACTTCGAGCAC-3' and 5'-GTGCTCGAAGTTGATGATGCTGCCACGGCTCGCGCTGCAGACTGCCGCAAG-3'. Three

HCV epitope-specific constructs were generated via the insertion of oligonucleotides into the EcoRI/XbaI sites of pcDNA3.1, encoding an ER translocation signal sequence followed by the amino acid sequences GRAAICGKY, SRGGRAAICGKY, or GRAAICGKYLF. Plasmid constructs encoding PT ERAP1, E320A nonfunctional ERAP1, and HLA-B*27:05 were described previously [7]. All constructs were sequence-verified using DNADynamo alignment software (Blue Tractor Software, UK).

Cell lines and transfections. ERAP1 knock-out 293T (293T E1KO) cells were transfected using the FuGENE 6 Transfection Reagent (Promega, UK) [7]. For assessment of ERAP1 trimming activity, 293T E1KO cells were transfected with 1 µg total DNA, consisting of 0.5 µg PT ERAP1, 0.5 µg E320A nonfunctional ERAP1, or 0.25 µg of each MM allotype-encoded ERAP1, together with 0.25 µg H2-K^b and 0.25 µg SHL8 or the extended precursor SRG-SHL8. For assessment of ERAP1 amino acid specificity, 293T E1KO cells were transfected with 0.1 µg total DNA, consisting of 0.05 µg MM allotype-encoded ERAP1, 0.025 µg H2-K^b, and 0.025 µg X-SHL8. All transfected cells were incubated for 24 hours at 37 °C. Presentation of trimmed SHL8 and activation of the LacZ-inducible B3Z T cell hybridoma were assessed by measurement of intracellular LacZ activity using the substrate chlorophenol red-β-d-galactopyranoside (Roche, UK) [10].

Activation of donor MM T cells. 293T E1KO cells were transfected as above with 1 µg total DNA, consisting of 0.5 µg ERAP1, 0.25 µg HLA-B*27:05, and 0.25 µg GRAAICGKY, SRGGRAAICGKY, or GRAAICGKYLF. Transfected cells were incubated for 24 hours, harvested, and cultured for a further 24 hours with donor MM T cells at an effector-to-target ratio of 3:1. Activation of donor MM T cells was determined by IFN-γ staining as above.

Viral sequence analyses. Details may be found in the supplementary information.

Statistical analysis. Differences among multiple groups were evaluated for significance using a one-way ANOVA with Dunnett's posttest implemented in GraphPad Prism (www.graphpad.com).

Results

Persistent low-level viremia after infection with HCV despite multiple predictors of spontaneous viral clearance

Donor MM experienced an accidental needle-stick injury from an HCV genotype 1a-infected patient and developed symptomatic HCV infection over a period of 20 months (Figure 1). Seven weeks after the needle-stick injury, alanine aminotransferase peaked at 1,307 U/L, viremia peaked at 1.15×10^7 IU/mL, HCV-specific antibodies became detectable in the serum, and the donor suffered from fatigue, nausea, and moderate jaundice (bilirubin peaked at 5.3 mg/dL). Further virological analyses confirmed infection with HCV genotype 1a. Genetic analyses revealed that donor MM had the favorable IFNL4 (previously named IL28B) genotype CC (SNP rs12979860) and expressed the HLA class I molecules A*01:01, A*26:01, B*08:01, and B*27:05. Three positive predictors of spontaneous viral clearance were therefore present in this individual, namely symptomatic infection [22], IFNL4 genotype CC [23], and HLA-B*27 [15]. However, donor MM continued to display moderately elevated liver enzymes and remained positive for HCV RNA, albeit below the lower level of quantification (LLOQ, 12 IU/mL). In addition, donor MM suffered from oscillating complaints, including subfebrile temperatures and flu-like symptoms. After 20 months, treatment was initiated with direct-acting antivirals (DAAs), leading to normalization of liver enzymes, clearance of the virus (negative HCV RNA 12 and 24 weeks after discontinuation of DAAs) and resolution of symptoms.

Lack of viral clearance associated with altered HCV-specific CD8⁺ T cell responses

To determine the immunological correlates of persistent infection in donor MM, we performed a comprehensive analysis of HCV-specific CD8⁺ T cell responses, which are thought to play a key role in viral clearance [13]. We used 460 overlapping peptides (olps) spanning the complete HCV genotype 1a polyprotein and performed IFN- γ ELISpot assays in a matrix

format [24, 25]. Positive responses were confirmed individually by intracellular staining for IFN- γ . Using this approach, we identified six HCV-specific CD8⁺ T cell responses in donor MM (olp-77, olp-203, olp-209/210, olp-211, olp-323/324, and olp-450/451; Figure 2 and Table 1).

The responses elicited by olp-209/210, olp-211, and olp-450/451 potentially targeted previously described epitopes restricted by HLA-A*26:01 (NS3₁₃₈₃₋₁₃₉₁, EVIKGGRHL) [26], HLA-B*08:01 (NS3₁₃₉₅₋₁₄₀₃, HSKKKCDEL) [27, 28], and HLA-B*27:05 (NS5B₂₉₃₆₋₂₉₄₄, GRAAICGKY) [17, 29]. We therefore tested these optimal 9mer epitopes in peptide-specific cell lines. Unexpectedly, we only observed a response to NS3₁₃₉₅₋₁₄₀₃ (HSKKKCDEL), corresponding to olp-211 (Figure 2). Responses to NS3₁₃₈₃₋₁₃₉₁ (EVIKGGRHL) and NS5B₂₉₃₆₋₂₉₄₄ (GRAAICGKY) were either absent or very weak (Figure 2). Using EBV-immortalized B cell lines partially matched for HLA class I, we confirmed that CD8⁺ T cells targeting olp-209/210 were restricted by HLA-A*26:01 (Figure 3A and 3B), and that CD8⁺ T cells targeting olp-450/451 were restricted by HLA-B*27:05 (Figure 3C). These findings suggested that CD8⁺ T cells from donor MM were specific for length variants of NS3₁₃₈₃₋₁₃₉₁ (EVIKGGRHL) and NS5B₂₉₃₆₋₂₉₄₄ (GRAAICGKY). We therefore performed *in vitro* analyses using peptides of different lengths. For both reactivities, the cognate epitopes were defined by 11mer peptides (Figure 3D and 3E; Table 2). The immunodominant response to the extended HLA-B*27:05-restricted 11mer peptide GRAAICGKYLF was further confirmed using HLA-B*27:05/peptide multimers. In dual staining experiments, the HLA-B*27:05/GRAAICGKYLF multimer identified substantially higher frequencies of cognate CD8⁺ T cells among PBMCs from donor MM at week 10 and week 32 post-infection compared with the HLA-B*27:05/GRAAICGKY multimer (Figure 3F, upper panel), whereas the HLA-B*27:05/GRAAICGKY multimer identified substantially higher frequencies of cognate CD8⁺ T cells among PBMCs from two HLA-B*27:05⁺ donors with resolved HCV infection compared with the HLA-B*27:05/GRAAICGKYLF multimer (Figure 3F, lower panel).

The responses elicited by o1p-77, o1p-203, and o1p-323/324 did not correspond with previously described HCV-specific CD8⁺ T cell epitopes. *In silico* and *in vitro* analyses revealed three novel reactivities: (i) o1p-77 – HLA-A*01:01-restricted 10mer epitope (E2₅₁₈₋₅₂₇, TTDRSGAPTY; Suppl. Figure S1A and S1B); (ii) o1p-203 – HLA-A*26:01-restricted 11mer epitope (NS3₁₃₃₇₋₁₃₄₇, ETAGARLVVLA; Suppl. Figure S1C and S1D); and (iii) o1p-323/324 – HLA-B*27:05-restricted 11mer epitope (NS5A₂₁₃₁₋₂₁₄₁, HRFAPPCKPLL; Suppl. Figure S1E and S1F; Table 2).

Surprisingly, we did not observe HCV-specific CD8⁺ T cell responses to overlapping peptides incorporating the previously defined immunodominant epitopes NS3₁₄₃₆₋₁₄₄₄ (ATDALMTGY, restricted by HLA-A*01:01), NS5A/B₂₄₁₆₋₂₄₂₄ (DVVCCSMSY, restricted by HLA-A*26:01), or NS5B₂₈₄₁₋₂₈₄₉ (ARMILMTHF, restricted by HLA-B*27:05) [17, 26, 27]. To confirm the absence of these epitope-specific responses, we tested the optimal 9mer peptides in intracellular IFN- γ staining assays. A weak response was detected against ARMILMTHF (Figure 2), but no responses were detected against ATDALMTGY or DVVCCSMSY (Suppl. Figure S2).

Our detailed analysis of HCV-specific CD8⁺ T cell responses in donor MM therefore revealed that: (i) reactivity was directed away from typically immunodominant epitopes, preferentially targeting either novel or subdominant epitopes; and (ii) reactivity was biased towards longer epitopes (10–11mers) (Table 2).

Altered immunodominance associated with hypoactive variants of ERAP1

The immunoreactivity patterns identified in donor MM suggested either that the previously described optimal epitopes were not presented or that the longer targeted epitopes were more abundant on the surface of infected cells. We therefore hypothesized a determinative role for hypoactive allotypes of ERAP1, which allow surface expression of C-terminally extended peptides in the context of HLA class I [8]. Molecular cloning revealed the presence of two ERAP1 allotypes in this donor (Figure 4A): (i) ERAP1*001 (P127, V349, R528, N575,

Q725, E730), a known hypoactive variant [7]; and (ii) a previously described allotype (Hap7) [30], designated here as ERAP1*014 (K56, P127, R528, E730). To determine the trimming function of these two ERAP1 allotypes individually and in combination, we used the well characterized SIINFEHL (SHL8) murine model system, in which an ER-targeted N-terminally extended precursor of SHL8 was transfected into 293T E1KO cells alongside each or both allotypes of ERAP1. The expression of trimmed SHL8 presented by H2-K^b on the cell surface was measured via stimulation of the SHL8-specific LacZ reporter T cell hybridoma B3Z. We further related these experiments to the poor immunogenicity of GRAAICGKY in donor MM by using an N-terminally extended version of SHL8 (SRG-SHL8) corresponding to the predicted precursor peptide generated via proteasomal cleavage of the parent protein NS5B (SRG-GRAAICGKY). As shown in Figure 4B and 4C, both ERAP1 allotypes expressed by donor MM were hypoactive, with trimming activities between those of the negative control (a nonfunctional ERAP1 variant known as E320A, which carries a mutation in the active-site GAMEN motif) and the prototype (PT) control (ERAP1*002). Similar trimming efficiencies were observed with both allotypes in combination (Figure 4B and 4C). To analyze the effect of these allotypes on trimming in the context of other HCV-derived epitopes, we quantified the generation of SHL8 from precursors with different N-terminal extensions (X-SHL8). The combined ERAP1 allotypes of donor MM again performed suboptimally in most cases (Figure 4D), displaying marked hierarchical differences compared with the trimming activity of ERAP1*002 [10]. The altered immunodominance pattern in donor MM therefore correlated with the expression of two hypoactive variants of ERAP1.

Differential generation of the GRAAICGKYLF epitope by allotypic variants of ERAP1

To extend these findings, we transfected 293T E1KO cells with: (i) HLA-B*27:05; (ii) minigenes expressing the 9mer GRAAICGKY, the 11mer GRAAICGKYLF, or an N-terminally extended 12mer precursor of the 9mer epitope (SRG-GRAAICGKY); and (iii) the nonfunctional ERAP1 variant (E320A), the active PT control ERAP1*002, or the donor MM

allotypes ERAP1*001 and ERAP1*014. Presentation of the 11mer epitope by HLA-B*27:05 on the surface of transfected cells was measured using a GRAAICGKYL F-specific CD8⁺ T cell line from donor MM. We first confirmed that CD8⁺ T cells from donor MM failed to recognize the 9mer epitope, irrespective of the N-terminal sequence and the expressed ERAP1 allotype (Figure 5A, 5C, and 5D). As expected, transfection of the 11mer peptide minigene with inactive ERAP1 generated a strong response (Figure 5B, left panel), whereas cells expressing the PT control ERAP1*002 elicited a significantly weaker response, consistent with overtrimming and destruction of GRAAICGKYL F (Figure 5B, middle panel). Interestingly, cells expressing the ERAP1 allotypes from donor MM also elicited a strong response, indicating preservation of GRAAICGKYL F (Figure 5B, right panel). The immunodominant 9mer epitope was therefore generated from an N-terminally extended precursor, but not from a C-terminally extended precursor, in the presence of functional ERAP1. In the presence of hypoactive ERAP1, however, the N-terminally extended precursor was not trimmed to the optimal length, hence the absence of the usual 9mer peptide, whereas the C-terminally extended 11mer version escaped destruction to prime and activate virus-specific CD8⁺ T cells.

Additional factors that may impact immunodominance patterns

Immunodominance patterns can be influenced by factors other than antigen presentation [31]. Donor MM was infected with a viral strain that contained nonsynonymous mutations in four of the previously described epitopes restricted by HLA-A*01:01, HLA-A*26:01, HLA-B*08:01, or HLA-B*27:05 (supplementary Table S1). However, the typically immunodominant HLA-B*27:05-restricted epitope NS5B₂₈₄₁₋₂₈₄₉ (ARMILMTHF) incorporated a conservative isoleucine to valine switch at position 4 (ARMVLMTHF), which did not affect immune recognition (Suppl. Figure S3), consistent with a previous report [16]. In contrast, the typically immunodominant HLA-A*01:01-restricted epitope NS3₁₄₃₆₋₁₄₄₄ (ATDALMTGY) incorporated a tyrosine to phenylalanine switch at the C-terminus (ATDALMTGF), which has been shown

previously to escape immune recognition [32]. The lack of ATDALMTGY-specific CD8⁺ T cells in donor MM therefore likely reflected sequence variation in the infecting viral strain rather than SNPs in ERAP1. Competition among epitopes and cognate CD8⁺ T cell avidity can also affect immunodominance patterns. In donor MM, epitopes restricted by HLA-B*08:01, an HLA allele that usually restricts dominant CD8⁺ T cell responses in HCV infection, were only weakly targeted. The immunodominant HLA-B*27:05-restricted epitope NS5B₂₉₃₆₋₂₉₄₆ (GRAAICGKYLF), however, was targeted with relatively high avidity (supplementary Figure S4), but it remains to be determined if this attribute contributed to the numerical dominance of GRAAICGKYLF-specific CD8⁺ T cells. It is also important to point out, however, that the virus-specific CD8⁺ T cell responses in donor MM decreased in strength over time, while their immunodominance pattern remained unchanged even at week 68 after infection (supplementary Figure S5).

Discussion

ERAP1 has garnered interest as a consequence of genetic associations with several autoinflammatory disorders, such as ankylosing spondylitis, birdshot chorioretinopathy, psoriasis, and Behçet's disease [3]. Epistatic interactions between variants of ERAP1 and the corresponding disease-linked HLA alleles further implicate peptide handling in the immunopathology in these disorders [5, 6]. Much effort has therefore been dedicated to understanding the impact of ERAP1 polymorphisms on the naturally presented epitope repertoire [8, 9, 33]. However, previous studies have not addressed the physiological relevance of these effects on the immune response to exogenous antigens, for example in the context of viral infections. We identified two hypoactive ERAP1 allotypes in a donor with acute HCV infection. Donor MM expressed four distinct HLA class I allotypes (A*01:01, A*26:01, B*08:01, and B*27:05), all of which have been shown to present HCV-derived epitopes that reproducibly elicit hierarchical CD8⁺ T cell responses [17, 26, 28, 32]. In contrast to these classical immune profiles, CD8⁺ T cells from donor MM targeted either longer versions of the typically immunodominant 9mer epitopes or novel 10mer or 11mer epitopes, characterized here for the first time. These results suggested that the relatively subtle effects of ERAP1 polymorphisms *in vitro* profoundly influenced the antigen-specific CD8⁺ T cell repertoire *in vivo*.

Presentation of the B*27:05-restricted 11mer epitope GRAAICGKYLF was reduced only slightly in 293T E1KO cells harboring active prototype control ERAP1*002 relative to 293T E1KO cells expressing the hypoactive donor MM allotypes ERAP1*001 and ERAP1*014. Nonetheless, GRAAICGKYLF-specific CD8⁺ T cells vastly outnumbered GRAAICGKY-specific CD8⁺ T cells in donor MM. These divergent *in vitro* and *in vivo* observations suggested a degree of leakiness in the ERAP1 system, enabling the presentation of epitopes spanning different lengths, which subsequently elicited a highly focused CD8⁺ T cell response. Minor differences in antigen presentation were therefore sufficient to invert the archetypal immunodominance hierarchy in donor MM. In clinical terms, donor MM had no

history of severe autoinflammatory disorders, but suffered from protracted viral infections during childhood and developed mild psoriasis on completion of this study. It therefore remains to be determined how the corresponding ERAP1 allotypes impact adaptive immunity beyond the context of HCV.

Despite the presence of several favorable prognostic indicators (symptomatic course of infection, IL28B/IFNL4 genotype CC, and expression of HLA-B*27), donor MM failed to clear the virus and developed persistent low-level infection with HCV. Although it is tempting to link this outcome with the hypoactive ERAP1 allotypes and the skewed repertoire of HCV-specific CD8⁺ T cells, there was no direct evidence for a causal association between these variables. Moreover, longitudinal sequence variations emerged in two of the three NS3-derived epitopes targeted in donor MM, consistent with mutational escape: K1386R in the HLA-A*26:01-restricted 11mer epitope NS3₁₃₈₃₋₁₃₉₃ and L1403F in the HLA-B*08:01-restricted 9mer epitope NS3₁₃₉₅₋₁₄₀₃. This observation suggested a potential role for viral adaptation in the outcome of infection, but equivalent data were not available for targeted NS5B-derived epitopes, in part due to the persistent low-level viremia in donor MM. However, previous studies have demonstrated an association between ERAP2 polymorphisms and resistance to HIV-1 [34, 35]. In addition, the ERAP1 polymorphism I276M (rs26618 T>C) was found to be enriched in individuals with chronic HCV infection relative to healthy controls [36]. The non-risk residue was present at this position in both ERAP1 allotypes expressed by donor MM. It is also notable that CD8⁺ T cell responses to the 11mer epitopes HRFAPPCKPLL and GRAAICGKYLK were not detected in a cohort of ten HLA-B*27⁺ patients with chronic HCV genotype 1 infection (data not shown). Rare combinations of ERAP1 allotypes may therefore shape the repertoire of CD8⁺ T cell epitopes more distinctly than common variants of ERAP1 [7].

The peptide trimming function of ERAP1 can be profoundly altered by the allotype expressed [37, 38]. These allotypic differences lead to a significantly altered peptide repertoire presented at the cell surface, which as observed for patient MM, may affect the presentation

of immunodominant HCV epitopes, and thus, CD8⁺ T cell responses. In addition to peptide trimming, ERAP1 has been suggested to function in the shedding of the cytokine receptors IL-6R, TNFR1, and IL-1RII [39]. These cytokines (IL-6, TNF, IL-1) are important in inflammatory responses to viral infection. It is not currently known how ERAP1 works to facilitate cytokine receptor shedding, as it does not possess any ectodomain cleavage activity [39]. However, ERAP1 binds to the receptors and may therefore act as an important bridge to promote cytokine receptor cleavage through a third party. The cytokine receptor cleavage activity was not examined in this study, although it is tempting to speculate that allotype variants may have differential functional capacities in this pathway. This may alter levels of cytokine receptor shedding and levels of these receptors at the cell surface and in serum as previously observed for ERAP1 with reduced function, which resulted in higher levels of IL-1 β , TNF and IL-6 [40], modulating the inflammatory environment in infection.

In conclusion, we have demonstrated that hypoactive ERAP1 allotypes can impact the repertoire of epitopes targeted by antigen-specific CD8⁺ T cells in a relevant human viral infection, thereby supporting the general concept that peptide handling underlies the immunopathogenesis of HLA class I-associated autoinflammatory disorders. Further studies are therefore warranted to explore similar phenomena in other clinical settings, especially where disease susceptibility has been genetically linked with functional polymorphisms in ERAP1.

Acknowledgements

We thank all individuals who donated blood for our studies. We also thank BEI Resources and the National Institute of Allergy and Infectious Diseases for providing overlapping peptides spanning the complete polyprotein of HCV.

Figure Legends

Figure 1: Clinical course of HCV infection in donor MM. The needle-stick injury was set as day 0. LLOQ, lower level of quantification (12 IU/mL). ULN, upper limit of normal (50 U/L).

Figure 2: Comprehensive analysis of HCV-specific CD8⁺ T cell responses in donor MM.

Overlapping peptides (olps) spanning the complete HCV proteome (n = 460) were tested in IFN- γ ELISpot assays using a matrix format. Individual responses were confirmed via intracellular IFN- γ staining. Previously described HCV-derived epitopes restricted by HLA-A*01:01, HLA-A*26:01, HLA-B*08:01, or HLA-B*27:05 were tested individually. Only positive responses are displayed. Medium alone served as the negative control; medium supplemented with PMA and ionomycin served as the positive control.

Figure 3: HLA class I restriction and fine mapping of HCV-derived CD8⁺ T cell epitopes in donor MM.

(A) Representative HLA class I restriction analysis for CD8⁺ T cells targeting olp-209/210. A CD8⁺ T cell line specific for olp-210 was tested for IFN- γ production after incubation with EBV-immortalized B cell lines partially matched for the indicated HLA class I alleles, either unloaded (top row) or loaded with olp-210 (bottom row). The incorporated peptide was restricted by HLA-A*26:01. (B) Representative HLA class I restriction analysis for CD8⁺ T cells targeting olp-209/210 (as in panel A). White bars: unloaded; black bars: loaded with olp-210. (C) Representative HLA class I restriction analysis for CD8⁺ T cells targeting olp-450/451. (D, E) Fine mapping experiments for CD8⁺ T cell epitopes in olp-209/210 (D) and olp-450/451 (E). CD8⁺ T cell lines specific for each olp were incubated with the indicated 8–11mer peptides and assessed for production of IFN- γ . (F) *Ex vivo* staining with HLA-B*27:05/GRAAICGKY and HLA-B*27:05/GRAAICGKYLFL multimers labeled with distinct fluorochromes. Upper panel: donor MM (week 10 and week 32 post-infection). Lower

panel: control HLA-B*27:05⁺ donors with spontaneous resolution or treatment-induced resolution of HCV infection.

Figure 4: Hypoactive trimming function of ERAP1 allotypes expressed in donor MM.

(A) ERAP1 allotypes expressed in donor MM *versus* the PT control allotype ERAP1*002 and their frequency in the central European (CEU) population [30]. (B, C) The three amino acid N-terminally extended epitope precursor SRG-SIINFEHL (SRG-SHL8) was transfected into 293T E1KO cells together with the ERAP1 allotypes ERAP1*002 (PT), ERAP1*001 (expressed in donor MM), ERAP1*014 (expressed in donor MM), both donor ERAP1 allotypes (labelled MM), or E320A (a nonfunctional active-site mutant of ERAP1). The expression of trimmed SHL8 presented by H2-K^b on the cell surface was measured via stimulation of the SHL8-specific T cell hybridoma B3Z. (B) A representative titration curve showing activation of B3Z with increasing numbers of target cells. (C) Results normalized to ERAP1*002 activity. Bars show results pooled from three independent experiments \pm SEM. One-way ANOVA with Dunnett's posttest was performed. *** $p < 0.0003$, ** $p < 0.001$. (D) Ability of combined donor MM allotypes to generate the optimal SHL8 peptide from precursors with different N-terminal extensions (X-SHL8). Results are normalized to the response observed with SHL8, which does not require trimming by ERAP1. Bars show results pooled from three independent experiments \pm SEM.

Figure 5: The 11mer GRAAICGKYLF epitope is not destroyed by ERAP1 allotypes expressed in donor MM.

293T E1KO cells were transfected with: (i) HLA-B*27:05; (ii) minigenes expressing the 9mer GRAAICGKY (A), the 11mer GRAAICGKYLF (B), or the N-terminally extended 12mer precursor of the 9mer epitope SRG-GRAAICGKY (C); and (iii) a nonfunctional ERAP1 mutant (E320A, left column), the PT allotype ERAP1*002 (middle column), or the ERAP1 allotype combination from donor MM (right column). Presentation of

the 11mer peptide by HLA-B*27:05 was assessed by measuring IFN- γ production after stimulation of a GRAAICGKYL F-specific CD8⁺ T cell line established from donor MM. (A–C) Representative dot plots. (D) Summary of three independent experiments \pm SEM. One-way ANOVA with Dunnett's posttest was performed. **, $p < 0.0039$, * $p < 0.05$. NS, not significant.

ACCEPTED MANUSCRIPT

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Table 1: Summary of responses to olps and previously described HCV-specific epitopes in donor MM. Optimal epitopes are indicated in bold.

Peptide	HCV location	Peptide aa sequence	HLA restriction
OLP77	E2 ₅₁₁₋₅₂₇	PSPVVVGTTDRSGAPTY	A*01:01
OLP203	NS3 ₁₃₃₆₋₁₃₅₃	AETAGARLVVLATATPPG	A*26:01
OLP209	NS3 ₁₃₇₆₋₁₃₉₃	YGKAIPLEVIK GGRHLIF	A*26:01
OLP210	NS3 ₁₃₈₃₋₁₃₉₉	EVIKGGRHLIFCHSKKK	
predescribed	NS3 ₁₃₈₃₋₁₃₉₁	EVIKGGRHL	
OLP211	NS3 ₁₃₈₉₋₁₄₀₆	RHLIFCHSKKK CDELAKK	B*08:01
predescribed	NS3 ₁₃₉₅₋₁₄₀₃	HSKKKCDEL	
OLP323	NS5A ₂₁₂₄₋₂₁₄₁	ELDGVRLHRFAPP CKPLL	B*27:05
OLP324	NS5A ₂₁₃₁₋₂₁₄₈	HRFAPPCKPLLREEVSFR	
predescribed	NS5B ₂₈₄₁₋₂₈₄₉	ARMILMTHF	B*27:05
OLP450	NS5B ₂₉₃₀₋₂₉₄₆	RLLSRG G RAAIC GKYL F	B*27:05
OLP451	NS5B ₂₉₃₆₋₂₉₅₃	G RAAIC GKYL FNWAV RTK	
predescribed	NS5B ₂₉₃₆₋₂₉₄₄	G RAAIC KGY	

HLA allele	Described HCV-specific epitopes (immunodominant epitopes in bold)			Epitopes targeted in donor MM (dominant responses in bold)		
A*01:01	NS3 ₁₄₃₆₋₁₄₄₄	ATDALMTGY	9-mer	E2 ₅₁₈₋₅₂₇	TDRSGAPTY	10-mer
A*26:01	NS3 ₁₃₈₃₋₁₃₉₁	EVIKGRHL	9-mer	NS3 ₁₃₈₃₋₁₃₉₃	EVIKGRHL-IF	11-mer
	NS3 ₁₅₈₂₋₁₅₉₀	ENLPYLVA	9-mer			
	NS3/4A ₁₆₅₄₋₁₆₆₂	EVVTSTWVL	9-mer			
	NS5A/B ₂₄₁₆₋₂₄₂₄	DVCCSMSY	9-mer	NS3 ₁₃₃₇₋₁₃₄₇	ETAGARLVLA	11-mer
B*08:01	NS3 ₁₃₉₅₋₁₄₀₃	HSKKKDEL	9-mer	NS3 ₁₃₉₅₋₁₄₀₃	HSKKKDEL	9-mer
	NS3 ₁₄₀₂₋₁₄₁₀	ELAAKLVAL	9-mer			
	NS3 ₁₆₁₁₋₁₆₁₈	LIRLKPTL	8-mer			
B*27:05	P7 ₇₈₀₋₇₈₈	GRWVPGAAY	9-mer			
	NS3 ₁₄₉₂₋₁₅₀₁	GRGKPGIYRF	10-mer			
	NS5B ₂₈₄₁₋₂₈₄₉	ARMILMTHF	9-mer	NS5B ₂₈₄₁₋₂₈₄₉	ARMILMTHF	9-mer
	NS5B ₂₉₃₆₋₂₉₄₄	GRAAICGKY	9-mer	NS5B ₂₉₃₆₋₂₉₄₆	GRAAICGKY-LF	11-mer
				NS5A ₂₁₃₁₋₂₁₄₁	HRFAPPCKP-LL	11-mer

Table 2: Epitopes targeted in donor MM and previously described HCV-specific CD8+ T cell epitopes restricted by the HLA class I alleles present in donor MM

Highlights

- Endoplasmic reticulum aminopeptidase 1 (ERAP1) polymorphisms are strongly linked with HLA class I-associated autoinflammatory disorders, such as ankylosing spondylitis and Behçet's disease.
- We identified two hypoactive ERAP1 allotypes in an HLA-B*27:05⁺ individual with acute hepatitis C virus (HCV) infection.
- These ERAP1 allotypes modified the HCV-specific CD8⁺ T cell epitope repertoire *in vivo*, leading to altered immunodominance patterns that potentially contributed to the failure of antiviral immunity.

anti-HCV	-	-	+						
Bilirubin [mg/dl]	-	-	3.8	5.3	-	-	-	-	-

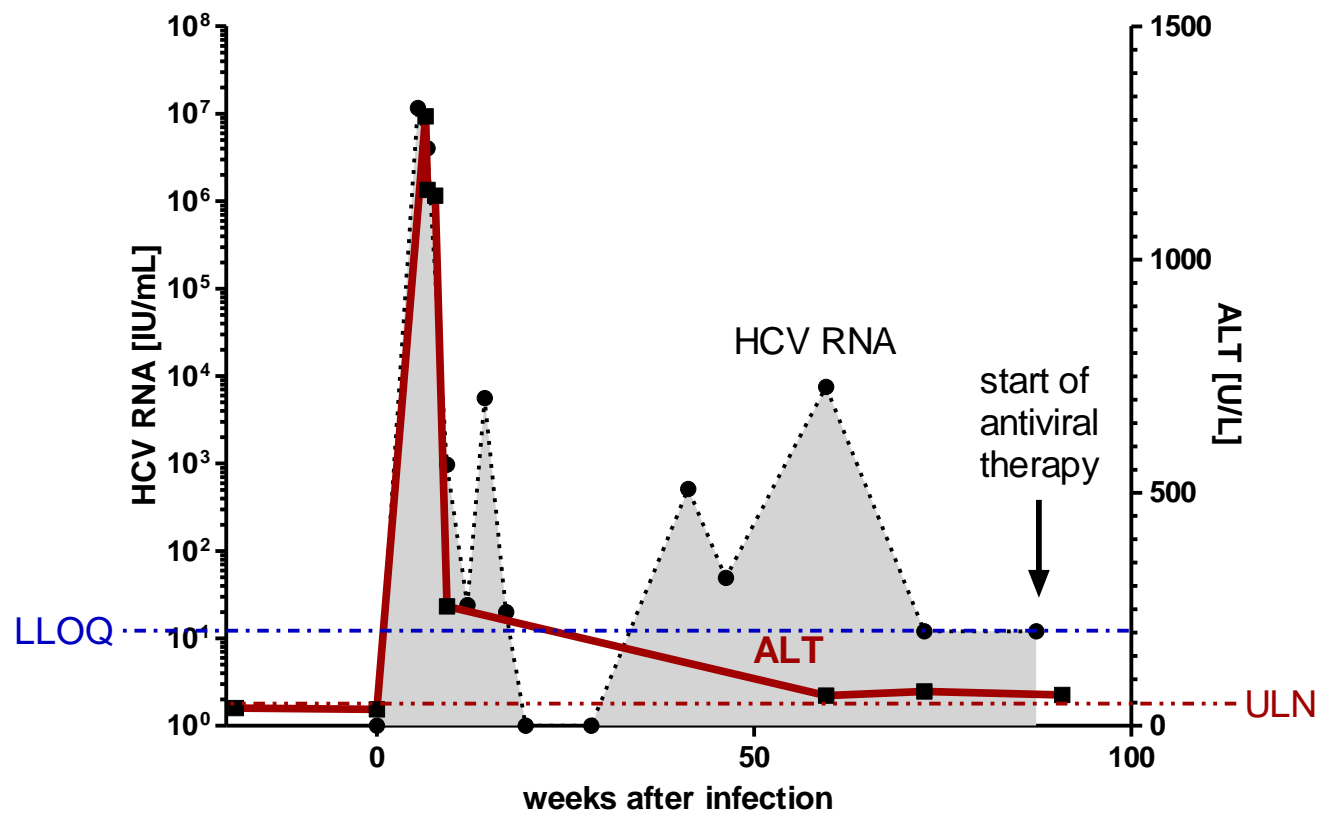


Figure 1

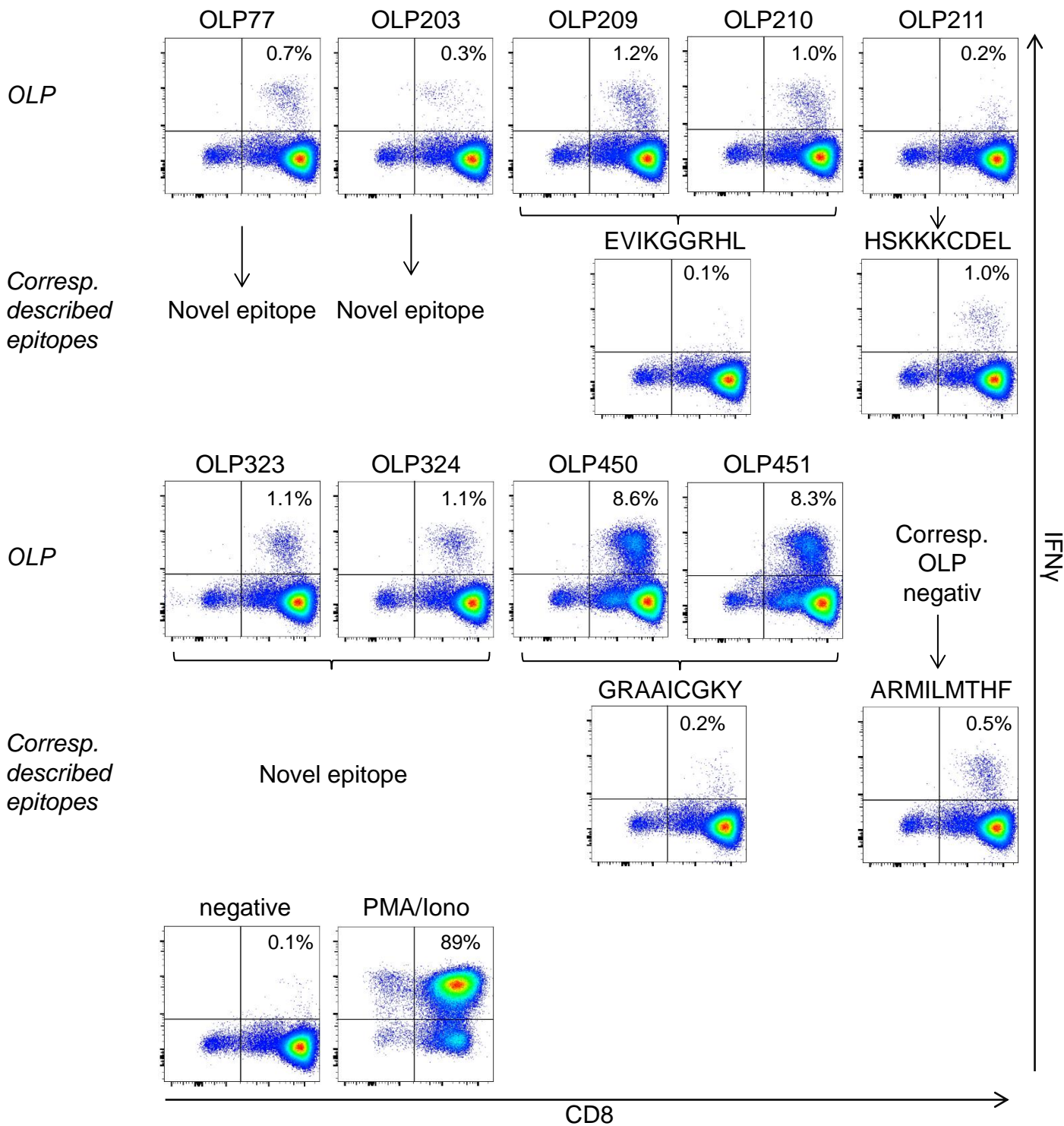


Figure 2

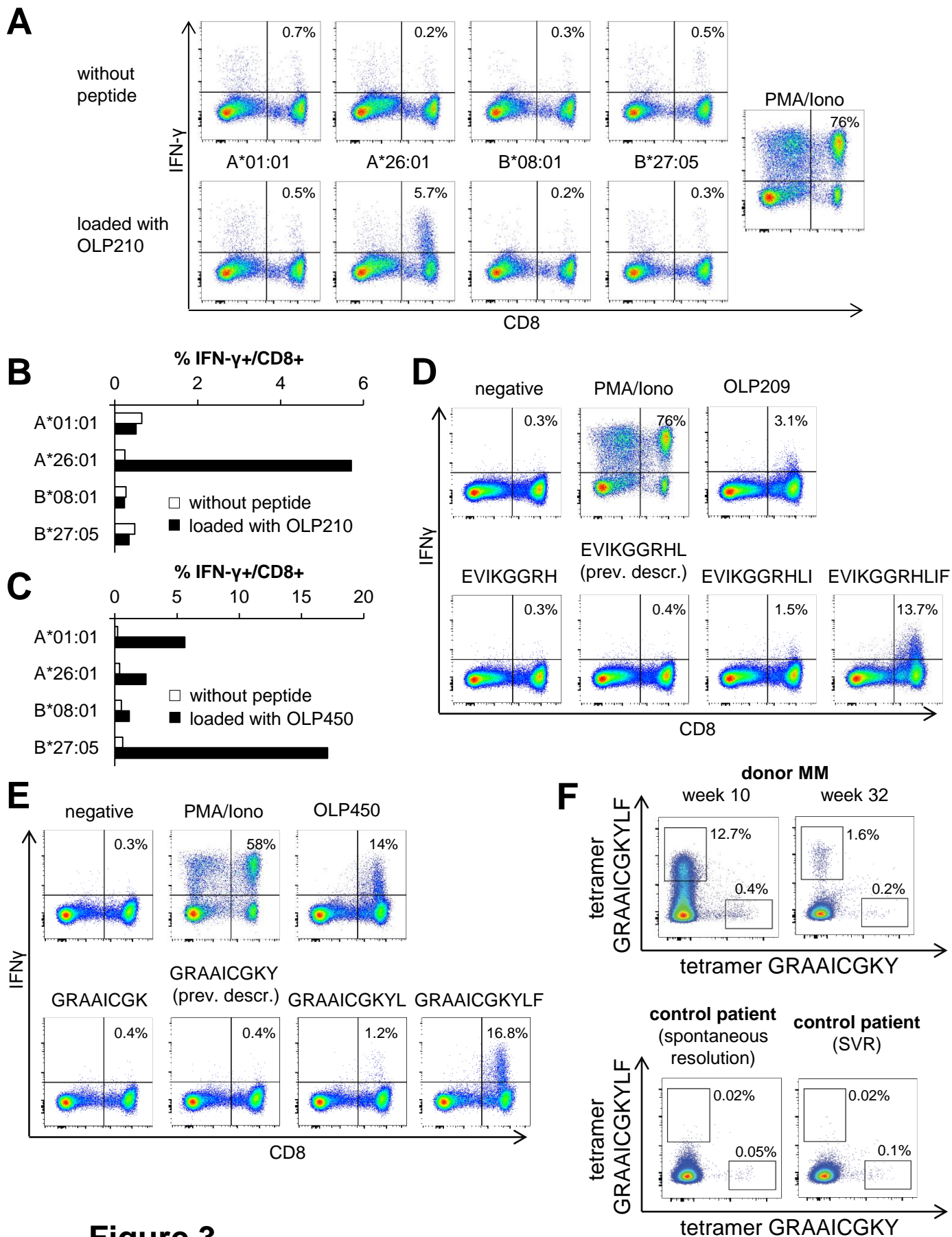


Figure 3

A

	ERAP1 allotypes	Amino acid at indicated position									Frequency in CEU population	ERAP1 trimming function
		56	127	276	346	349	528	575	725	730		
Prototype	*002	E	R	I	G	M	K	D	R	Q	13.7%	Efficient
Donor MM	*001	E	P	I	G	V	R	N	Q	E	26.2%	Undertrimmer
Donor MM	*014	K	P	I	G	M	R	D	R	E	2.5%	Undertrimmer

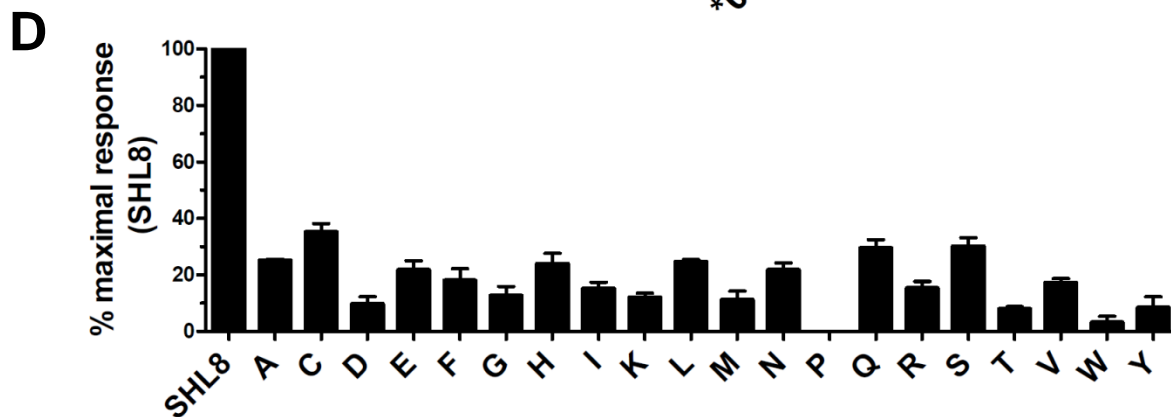
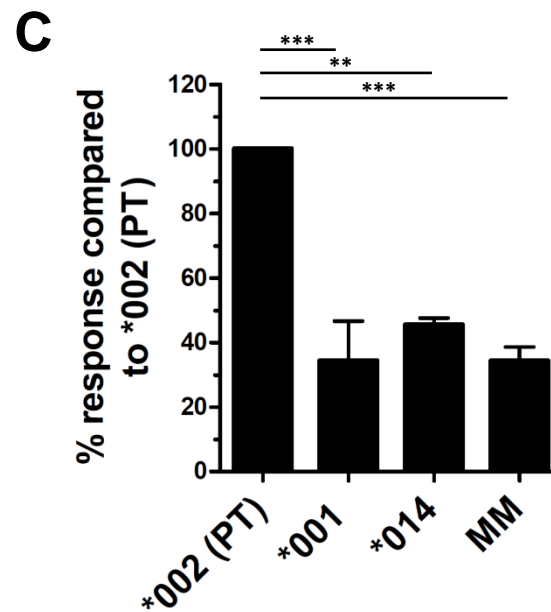
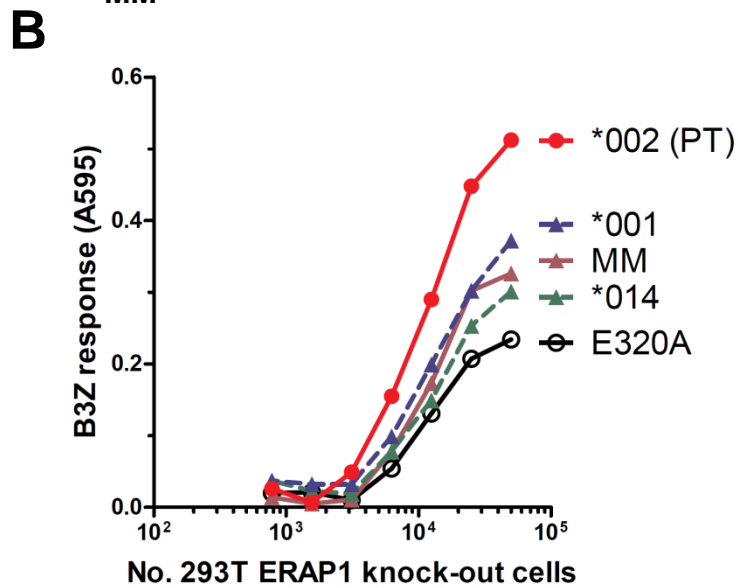


Figure 4

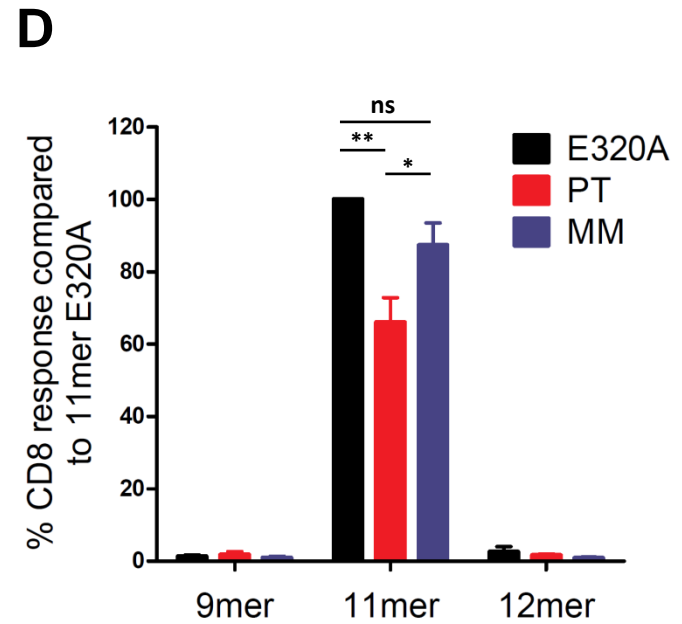
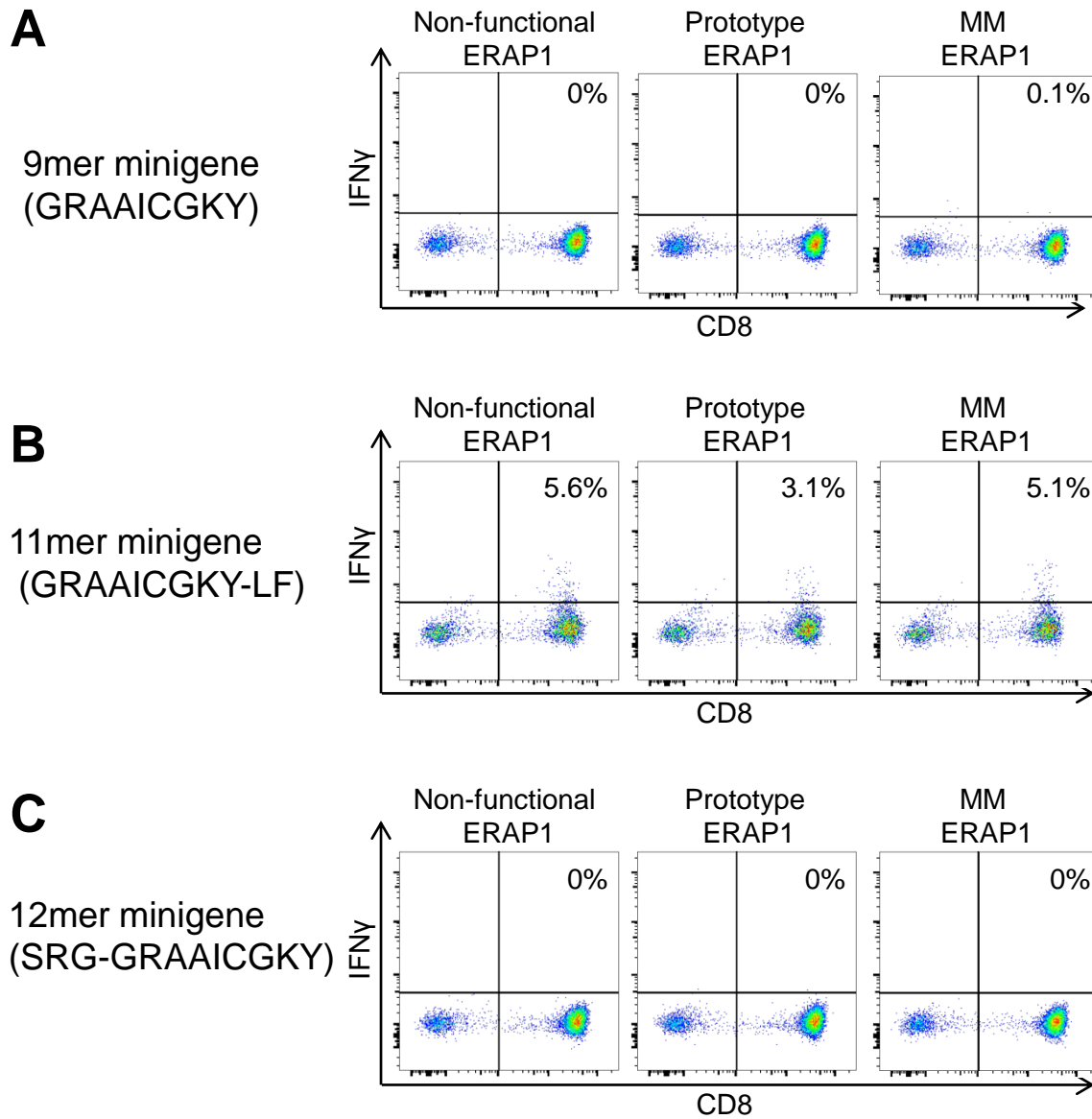


Figure 5

Epitope trimming
by ERAP1



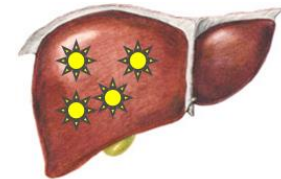
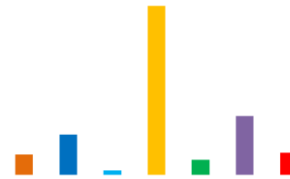
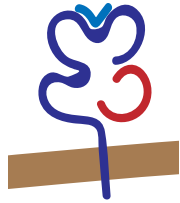
Cell surface presentation



Epitope repertoire



Outcome of HCV
infection in HLA-
B*27+ patients



Efficient

9-mer epitopes

Conserved
immunodominance

80% spontaneous
resolution

Hypoactive

10-/11-mer epitopes

Skewed
immunodominance

Low-viremic
persistent course

Graphical abstract