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Synergism of Tapasin and Human Leukocyte Antigens in Resolving Hepatitis C Virus Infection

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CD8+ T-cell responses to hepatitis C virus (HCV) are important in generating a successful immune response and spontaneously clearing infection. Human leukocyte antigen (HLA) class I presents viral peptides to CD8+ T cells to permit detection of infected cells, and tapasin is an important component of the peptide loading complex for HLA class I. We sought to determine if tapasin polymorphisms affected the outcome of HCV infection. Patients with resolved or chronic HCV infection were genotyped for the known G/C coding polymorphism in exon 4 of the tapasin gene. In a European, but not a US, Caucasian population, the tapasin G allele was significantly associated with the outcome of HCV infection, being found in 82.5% of resolvers versus 71.3% of persistently infected individuals ($P = 0.02$, odds ratio [OR] = 1.90 95% confidence interval [CI] = 1.11-3.23). This was more marked at the HLA-B locus at which heterozygosity of both tapasin and HLA-B was protective ($P < 0.03$). Individuals with an HLA-B allele with an aspartate at residue 114 and the tapasin G allele were more likely to spontaneously resolve HCV infection ($P < 0.00003$, OR = 3.2 95% CI = 1.6-6.6). Additionally, individuals with chronic HCV and the combination of an HLA-B allele with an aspartate at residue 114 and the tapasin G allele also had stronger CD8+ T-cell responses ($P = 0.02$, OR = 2.58, 95% CI-1.05-6.5). *Conclusion*: Tapasin alleles contribute to the outcome of HCV infection by synergizing with polymorphisms at HLA-B in a population-specific manner. This polymorphism may be relevant for peptide vaccination strategies against HCV infection. (HEPATOLOGY 2013;58:881-889)

Hepatitis C virus (HCV) is a common chronic viral infection with between 50% and 80% of individuals exposed to HCV becoming chronically infected. Understanding the immunological determinants of resolution of HCV infection is important in order to develop vaccines and also immunologically based therapeutics. A broad and multispecific CD8+ T-cell response may be important in resolving HCV infection successfully. The strength of this CD8+ response is dependent on a number of factors. These include antigen processing and presentation, an appropriate cytokine microenvironment, and the presence of CD4+ T-cell help. In addition to these cellular factors, host genetic factors may also play an important role. Specific human leukocyte antigen (HLA) class I alleles are associated with the outcome of HCV infection, and certain HLA class I alleles, including HLA-B*27 and B*57, are associated with a strong CD8+ response and hence viral clearance.[1-8]

The selection of HCV peptides for presentation is determined by the allelic diversity of HLA and also the supply of HCV peptides to the endoplasmic reticulum. Additionally, peptide loading of HLA class I is complex. It involves several proteins including tapasin, TAP,

Abbreviations: HCV, hepatitis C virus; HLA, human leukocyte antigen; MHC, major histocompatability complex; PBMC, peripheral blood mononuclear cell; PLC, peptide loading complex; SNP, single nucleotide polymorphism.

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ERp57, and calreticulin that together form a peptide loading complex (PLC) (Fig. 1).[9] Overall, these proteins act in a collaborative way to properly fold HLA class I with β2-microglobulin and endogenous or virally derived peptides. ERp57 and calreticulin promote folding and disulphide bond formation of HLA class I, while tapasin acts as a "bridge" between the components of the PLC.[10] In doing so it is thought to promote the binding of high-affinity peptides to HLA class I for presentation on the cell surface[11,12] (Fig. 1). In the presence or absence of tapasin the hierarchy of peptides presented by HLA class I can be substantially different.[13,14] However, only certain HLA class I alleles have been shown to be tapasin-dependent in biochemical assays. These include HLA-A*3001, HLA-B*2705, HLA-B*3501, HLA-B*4402, HLA-B*5001, and HLA-B*5701.[15-17] This dependence is governed by residues 114, 116, and 156 of the HLA class I molecule, in the floor of the peptide binding groove.[11,16,18,19] Thus, tapasin can have a direct effect on peptide binding to HLA class I, and as the tapasin gene lies at the centromeric end of the major histocompatability complex (MHC), but is beyond a recombination hotspot, it is not thought to be in tight linkage with HLA class I.[20,21] The tapasin gene has a number of single nucleotide polymorphisms (SNPs).[21-24] However, of the exonic SNPs only one is nonsynonymous and both alleles are commonly found in the Caucasian population.[24] This SNP is in exon 4 and codes for a nonconservative arginine to threonine substitution at position 260 of the tapasin protein. The aim of this study was to determine whether polymorphisms in tapasin and HLA class I may interact to determine the outcome of HCV infection.

Patients and Methods

Patients

All patients provided written informed consent and participated in the study in agreement with the relevant local Ethics Committee's approval and the Declaration of Helsinki.

UK Cohort 1. These individuals were from the previously described UK cohort of HCV-exposed individuals,[25] with the addition of 71 chronically infected individuals. Patients were recruited from hepatology clinics at King's College Hospital, London; Addenbrooke's Hospital NHS Trust, Cambridge; and Southampton General Hospital, Southampton, UK. There were 120 (37 female, 83 male, mean age 41.5 years) individuals with resolved infection. All had a self-reported Caucasian ethnicity and had at least two negative polymerase chain reaction (PCR) reactions (HCV COBAS Amplicor; Roche Diagnostics, Pleasanton, CA) at least 6 months apart. The chronic HCV population consisted of 300 chronically infected individuals (81 female, 219 male, mean age 43.0 years). Two hundred and eighty-six (95.3%) had a self-reported Caucasian ethnicity.

UK Cohort 2. These consisted of a cohort of 79 (64 male) Caucasian individuals with an extensive history of injection drug use, but repeated negative testing for HCV antibody, as previously described.[26] This group has been termed HCV-exposed seronegative aviremic. The median duration of injection drug use was 8.62 years (range, 0.3-24) with a median number of injections of 4,927 (range, 36-41,620). Their median age was 28 years. As a control group 79 additional individuals with chronic HCV infection were recruited from the outpatient hepatology clinic at Southampton General Hospital. Fifty-seven were male and 75 (94.9%) were of Caucasian origin. The mean age at diagnosis was 48.4.

German Cohort. Forty-two patients (17 female, 25 male, mean age 48.6 years) with chronic HCV from the University Hospital Freiburg were included. Thirteen had genotype 1a and 29 genotype 1b infection. All patients had a Caucasian self-reported ethnicity. Sixteen of the patients were positive for HLA-B*18, 17 for HLA-B*35, six for HLA-B*57, and six for HLA-B*58 (three of the patients were positive for two of these HLA alleles). Peripheral blood mononuclear cells (PBMCs) were isolated from patient blood by gradient centrifugation, as described.[27]

US Cohort. These individuals were from the previously described USA cohort of HCV-exposed individuals limited to those of Caucasian ethnicity.[8] Briefly, patients were recruited from the infectious diseases or hepatology clinics at Massachusetts General Hospital with local Ethics Committee approval. There were 53

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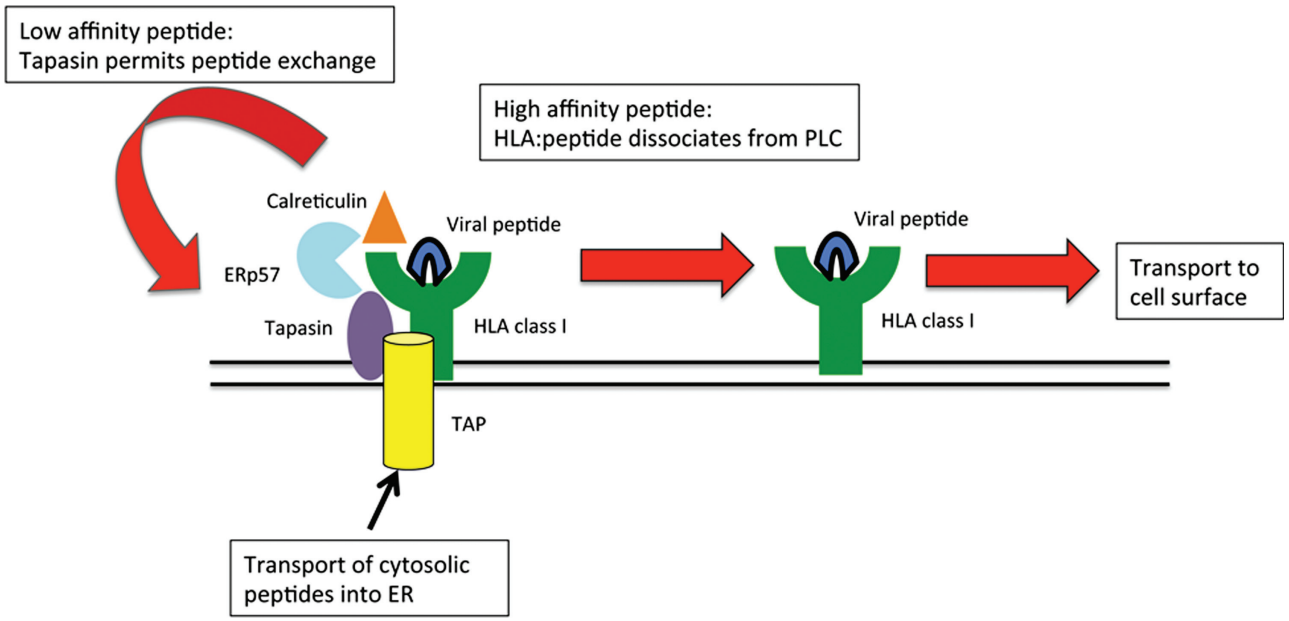

Fig. 1. Schematic showing the role of tapasin in peptide loading. Tapasin along with the transporter associated with antigen processing (TAP), ERp57, and calreticulin form the peptide loading complex (PLC), which loads viral peptides onto HLA class I. Tapasin binds to HLA class I and is thought to assist in the dissociation of peptides that bind with low affinity. These dissociated peptides can be replaced by high-affinity peptides. When a high-affinity peptide binds HLA class I the HLA class I and peptide are released from tapasin and the PLC and egress to the cell surface.

(27 female, 26 male, mean age 39.2 years) individuals with resolved infection. Each had at least two negative PCR reactions (HCV COBAS Amplicor; Roche Diagnostics) at least 6 months apart, and 196 chronically infected individuals (69 female, 127 male, mean age 41.4 years).

Genotyping

Tapasin genotyping was performed on genomic DNA extracted using a QiaAmp DNA Blood mini kit (Qiagen, Crawley, UK). A 51 basepair region of the tapasin gene flanking the polymorphism was amplified using the primers 5′-GACCTTCTGGCTGCCTAC-3′ (G allele) and 5′-GACCTTCTGGCTGCCTAG-3′ (C allele) and 5′-GC CAGATAGGTGCCCTCCTG-3′ (reverse). PCR reactions were detected using the Quantitect SYBR Green PCR kit (Qiagen) in standard 20 μL PCR reactions. Thermal cycling conditions were: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 58°C for 20 seconds. HLA genotyping was performed by PCR-SSP as described[28] or at NCI Frederick (Dr. Mary Carrington) by the PCR-SSOP (sequence-specific oligonucleotide probing) typing protocol and PCR-SBT (sequence based typing) as recommended by the 13th International Histocompatibility Workshop (http://www.ihwg.org).

Generation of Peptide-Specific T-Cell Lines

Peptides (Table 1) were synthesized by Genaxxon BioScience (Ulm, Germany) and have a purity of at least 70%. Peptide-specific T-cell lines were generated as described.[29] 4×10^6 PBMC were resuspended in 1 mL medium and stimulated with peptide at a final concentration of 10 μg/mL and anti-CD28 (BD PharMingen) at a final concentration of 0.5 μg/mL. On days 3 and 10, 1 mL culture medium and recombinant IL-2 (Hoffmann-La Roche, Basel, Switzerland) at a final concentration of 20 U/mL was added to each well. On day 7, the cultures were restimulated with the corresponding peptide (10 μg/mL) and 10^6 irradiated autologous feeder cells. After 2 weeks, PBMC were tested for peptide-specific interferon-gamma

Table 1. CD8+ T-Cell Epitopes Analyzed

HLA Allele	Protein	Position	Epitope
B*18	NS5A	2143-2151	DEVSFRVGL(gt 1a)/ DEVTFQVGL(gt 1b)
	NS3	1581-1589	DNFPYLVAY(gt 1b only)
B*35	E1	234-242	NASCRCWVAV(gt 1a)/ NSSCRCWVAL(gt 1b)
	NS3	1359-1367	HPNIEEVAL
	NS4A	1695-1702	IPDREVLY
	NS5A	2163-2171	EPEPDVAVL
B*57/B*58	E2	521-529	RSGAPTYSW
	E2	541-550	NTRPPLGNWF
	E2	708-716	SIASWAIKW
	NS3	1596-1604	RAQAPPPSW
	NS4	1968-1972	CTTPCSGSW
	NS5B	1801-1809	LTTSQTLLF
	NS5B	2629-2637	KSKKTPMGF
	NS5B	2912-2921	LGVPPLRAWR

Table 2. Association of Tapasin Alleles With Outcome in the Different Cohorts

a) UK: Spontaneous Resolving Cohort

Tapasin Allele	Resolver (n = 120)	Chronic (n = 300)	*P* Value	OR	95% CI
C	85 (70.8%)	223 (74.3%)	0.47	0.84	0.52-1.3
G	99 (82.5%)	214 (71.3%)	0.02	1.90	1.11-3.23

b) USA: Spontaneous Resolving Cohort

Tapasin Allele	Resolver (n = 53)	Chronic (n = 196)	*P* Value
C	38 (71.7%)	145 (74.0%)	>0.1
G	43 (81.1%)	160 (81.7%)	>0.1

c) UK: Exposed Uninfected Cohort

Tapasin Allele	Exposed Uninfected (n = 75)	Chronic (n = 79)	*P* Value
C	54 (72.0%)	60 (75.9%)	>0.1
G	63 (84.0%)	60 (75.9%)	>0.1

(IFN-γ) production by intracellular cytokine staining after 5 hours of restimulation with the respective peptide (10 μg/mL) as described.[29] Peptides were matched to the HLA-B alleles of the respective patients.

Statistical Analysis

This was performed using SPSS v. 17.0 and using the Bonferroni correction where necessary. Specifically for the residue analysis the correction factor was n = 34, which is the number of different residues at the analyzed positions (114, 116, and 156) at all three loci.

Results

The Tapasin G Allele Is Protective Against Chronic HCV Infection. We typed 120 individuals with resolved and 300 individuals with chronic HCV infection for the G/C SNP in exon 4 of tapasin. Ninety-nine (82.5%) out of 120 individuals with resolved infection versus 214 (71.3%) out of 300 individuals with chronic infection had the G allele, which encodes for the arginine variant ($P = 0.019$, odds ratio [OR] = 1.90 95% confidence interval [CI] = 1.11-3.23) (Table 2a). Similar frequencies of individuals with the tapasin C allele had resolved as compared to chronic HCV infection (70.8% versus 74.3%, $P > 0.1$). Thus, the tapasin G allele is associated with protection against chronic HCV infection in this cohort.

Tapasin Advantage Is Related Mainly to HLA-B Alleles. Tapasin helps to optimize the MHC class I peptide repertoire and some MHC class I alleles are more dependent on tapasin for optimization of their peptide repertoire than others. We therefore explored how this property was manifest in a more in-depth immunogenetic analysis. We hypothesized that heterozygosity of MHC class I and also of tapasin could be predicted to increase the number of peptides presented to CD8+ T cells and hence augment the immune response to HCV. We therefore determined the association of heterozygosity for tapasin with the outcome of infection. In this analysis heterozygosity at HLA class I was defined as alleles with different HLA types as determined by four-digit typing, which correlates with differences at the single amino acid level. Sixty-four (53.3%) of resolvers and 137 (45.7%) of chronically infected individuals were heterozygous for tapasin ($P > 0.1$). Thus, for both spontaneous resolvers and chronically infected individuals the individual gene frequencies were in Hardy-Weinberg equilibrium. However, when individual HLA class I loci were considered there was a trend for the combined association of heterozygosity of tapasin with HLA-B heterozygosity with outcome ($P = 0.028$, $P_c = 0.084$ (χ^2 trend test)), but not for tapasin and heterozygosity at the other loci (Fig. 2).

Although some HLA class I alleles are more tapasin-dependent than others, we found only relatively weak associations of outcome and specific HLA class I alleles with the tapasin G and C alleles. These associations include resolution of HCV and the tapasin G allele in

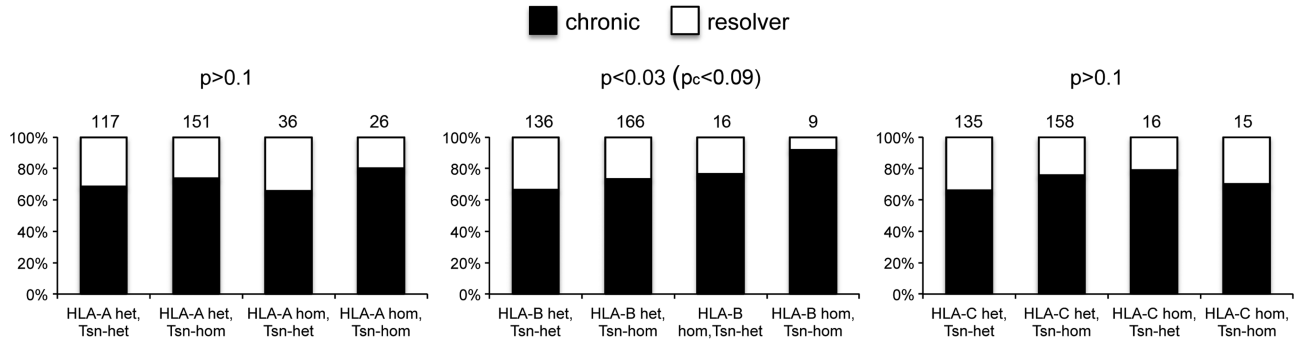

Fig. 2. Heterozygosity of tapasin and HLA-B are protective against chronic HCV infection. Analysis of the combination of tapasin heterozygosity with HLA heterozygosity at HLA-A, HLA-B, and HLA-C. Heterozygosity was determined at the four-digit level. Numbers in each group are indicated above the relevant bars. *P* Values were calculated using a chi-square test for trend and the Bonferroni correction applied (P_c).

Table 3. Association of the Tapasin (Tsn) G Allele With Specific Amino Acid Residues in the HLA Class I Heavy Chain

a) HLA-A

		Resolve		Chronic				
Residue	N	Tsn G+	Tsn G−	Tsn G+	Tsn G−	*P* Value	OR	95% CI
114								
Glu	11	1	1	7	2			
His	317	86	16	153	62	0.012	2.18	1.18-4.01
Gln	99	16	5	57	21			
Arg	399	91	20	203	85	0.022	1.91	1.1-3.29
116								
Asp	498	107	25	260	106	0.028	1.75	1.07-2.85
His	11	1	1	7	2			
Tyr	317	86	16	153	62	0.012	2.18	1.18-4.01
156								
Leu	481	114	19	262	86	0.014	1.97	1.14-3.39
Gln	120	24	7	60	29			
Arg	148	43	8	61	36	0.008	3.17	1.34-7.5
Try	77	13	8	37	19			

b) HLA-B

		Resolve		Chronic					
Residue	N	Tsn G+	Tsn G−	Tsn G+	Tsn G−	*P* Value	P_c Value	OR	95% CI
114									
Asp	473	127	16	234	96	<0.00003	0.0009	3.26	1.84-5.77
His	32	9	4	18	1				
Asn	317	62	20	160	75				
116									
Asp	185	40	8	102	35				
Phe	65	15	5	27	18				
Leu	66	9	4	40	13				
Ser	200	64	8	93	35	0.007		3.01	1.3-6.91
Try	306	70	15	150	71	0.011		2.21	1.18-4.13
156									
Asp	217	46	7	108	56	0.003		3.41	1.45-8.04
Leu	438	100	25	226	87				
Arg	110	32	4	51	23	0.032		3.61	1.14-11.4
Try	57	20	4	27	6				

c) HLA-C

		Resolve		Chronic					
Residue	N	Tsn G+	Tsn G−	Tsn G+	Tsn G−	*P* Value	P_c Value	OR	95% CI
114									
Asp	583	144	33	286	120	0.006		1.83	1.19-2.83
Asn	231	46	9	124	52				
116									
Phe	247	52	10	132	53				
Leu	21	1	3	13	4				
Ser	424	100	20	202	102	0.0005	0.015	2.53	1.48-4.32
Try	122	37	9	63	13				
156									
Asp	15	6	1	7	1				
Leu	371	94	23	179	75				
Gln	38	6	2	18	12				
Arg	258	51	10	139	58	0.047		2.13	1.01-4.48
Try	132	33	6	67	26				

Shown are the number of HLA alleles with each specific residue at positions 114, 116 and 156 in the tapasin G-positive and G-negative groups in individuals that resolved HCV infection or remained chronically infected. Only *P* values, or Bonferroni corrected *P* values <0.05 are shown.

individuals who were positive for HLA-A*0101 ($P = 0.008$, OR = 3.17, 95% CI 1.4-7.5), B*0702 ($P = 0.01$, OR = 4.84, 95% CI 1.3-17.6), and Cw*0701 ($P = 0.04$, OR = 2.69, 95% CI 1.07-6.77). Conversely, the tapasin C allele was associated with chronic infection in individuals who had the B*0801 allele ($P = 0.008$, OR = 0.23, 95% CI 0.08-0.66) and the B*3501 allele ($P = 0.01$, OR = 0.16, 95% CI 0.04-0.65).

The Protective Effect of Tapasin G Alleles Is Determined by Specific HLA Class I Residues. Biochemical studies have indicated that specific HLA-class I residues may be relevant for their interaction with tapasin. In particular, residues 114, 116, and 156 of the MHC class I heavy chain determine its interaction with tapasin, with the effect of position 114 being dominant over 116.[14-16,30] We therefore investigated whether these residues determined the association of the tapasin G allele with the outcome of HCV infection. As the three class I loci have distinct sequences we subdivided the analysis by locus, so that in each analysis 2n alleles were considered (Table 3). One hundred and twenty-seven out of 143 (89%) of HLA-B alleles with aspartate at residue 114 (Asp114) were associated with the tapasin G allele in individuals who resolved infection, as compared to 234 out of 330 (71%) of HLA-B alleles from those with chronic infection ($P < 0.00003$ ($P_c = 0.0009$), OR = 3.26, 95% CI = 1.84-5.77) (Table 3). Overall, 105 resolvers had at least one HLA-B allele with Asp114, and 93 (89%) of these had a tapasin G allele, as compared to 174 of 246 (71%) individuals with chronic infection ($P = 0.0002$, OR = 3.50, 95% CI = 1.77-6.93). However, the presence of an HLA-B allele with Asp114 *per se* was not significantly associated with resolution ($P > 0.1$).

A similar, but weaker, association of the tapasin G allele with Asp114 was seen at HLA-C, as this combination was found in 144 out of 177 alleles (81%) in resolvers but only 286 out of 406 (70%) in chronically infected individuals ($P = 0.006$ ($P_c > 0.05$), OR = 1.83, 95% CI = 1.19-2.83) (Table 3). The only other positive association to remain after Bonferroni correction (n = 34) was for serine at residue 116 of an HLA-C allele. One hundred out of 120 HLA-C alleles with Ser116 were associated with tapasin G (83%) in resolvers versus 202 out of 304 (66%) in chronically infected individuals ($P = 0.0005$ ($P_c = 0.015$), OR = 2.53, 95% CI = 1.48-4.32). This association was also shared with HLA-B alleles, in which Ser116 was found in 64 out of 72 (89%) of resolvers versus 93 out of 128 (73%) chronically infected individuals

Table 4. Logistic Regression Analysis of Tapasin G With Specific Amino Acid Residues at the Three Different HLA Class I Loci and the Outcome of Infection

a) HLA-A

	P	OR	95% CI
Tapasin G	0.001	2.29	1.42-3.70
Tapasin G+ Arg 156	P>0.1		
Arg 156	P>0.1		

b) HLA-B

	P	OR	95% CI
Tapasin G	0.001	2.72	1.75-5.04
Tapasin G+ Asp114	0.027	3.00	1.13-7.93
Tapasin G+ Asp156	0.048	2.79	1.01-7.68
Asp156	0.053	0.61	0.37-1.01
Asp114	0.079	0.65	0.40-1.05
Tapasin G+ Ser116	>0.1		
Ser116	>0.1		

c) HLA-C

	P	OR	95% CI
Tapasin G	<0.001	2.33	1.45-3.74
Asp114	0.002	2.43	1.39-4.25
Ser116	0.006	0.51	0.31-0.82
Tapasin G+ Ser116	0.011	3.48	1.32-9.13
Tapasin G+ Asp114	0.058	0.34	0.11-1.04

An OR >1 indicates a positive association with resolution.

($P = 0.007$ ($P_c > 0.1$), OR = 3.01, 95% CI = 1.3-6.9). Of note is that neither Asp114 nor Ser116 are present in any of the HLA-A alleles present in our population. Conversely, Arg156 is present in alleles at all three loci and this was associated with resolution in combination with tapasin G at: HLA-A in 43 out of 51 (84%) versus 61 out of 97 (63%) ($P = 0.008$ ($P_c > 0.1$), OR = 3.17, 95% CI = 1.3-7.5); HLA-B 32 out of 36 (89%) versus 51 out of 74 (69%) ($P = 0.03$ ($P_c > 0.1$), OR = 3.61, 95% CI = 1.1-11.4) and HLA-C 51 out of 61 (84%) versus 139 out of 197 (71%) ($P = 0.05$ ($P_c > 0.1$), OR = 2.13, 95% CI = 1.0-4.5) in resolvers versus chronically infected individuals, respectively. Thus, we found a consistent trend for this residue to be significantly associated with tapasin G and the outcome of HCV infection.

In order to determine whether the effects we noted were due to the association of specific HLA class I residues with a tapasin G allele or were independent, we performed logistic regression analysis using the most significantly associated residues at each locus (those with $P < 0.01$) and tapasin G as the variables (Table 4a-c). In these three analyses tapasin G remained significant throughout. However, for HLA-A alleles no significant associations remained. For HLA-B alleles Asp114 ($P = 0.027$) and Asp156 ($P = 0.048$) remained significantly associated with resolution in combination with the tapasin G allele, with a trend towards chronicity in the absence of tapasin G (Table 4b). However, at HLA-C the strongest effects were for Asp114 with resolution ($P = 0.002$) and Ser116 with chronicity ($P = 0.006$) in the absence of an effect of tapasin, and a weaker protective effect of Ser116 and tapasin G ($P = 0.011$). Thus, consistent with our observations on heterozygosity at the MHC, the effect of tapasin is strongest in combination with HLA-B, rather than HLA-A or HLA-C, alleles. This epistatic association is unlikely to be due to linkage between tapasin and specific HLA-B alleles, as we found that no specific HLA-B alleles with aspartate at position 114 were significantly ($P < 0.05$) associated with the tapasin G allele (Supporting Table 1).

The Association of Tapasin G With HCV Is Population-Specific. To determine if the protective effect of tapasin was universal, we tested a second cohort of Caucasian individuals from the USA. Fifty-three individuals had resolved infection and 196 were chronically infected. Similar frequencies of tapasin alleles were found in both groups. The tapasin G allele was found in 43 (81.1%) resolvers versus 160 (81.7%) chronically infected and the tapasin C allele was found in 71.7% of resolvers and 74.0% of chronically infected individuals (all $P > 0.1$) (Table 2b). Thus, the effect of tapasin was confined to European Caucasians. Comparison of the different HLA-class I alleles from the two populations revealed slight but significant differences between the HLA-A, HLA-B, and HLA-C alleles in both groups, which may be relevant to the lack of association in this cohort (Supporting Table 2). To confirm that this effect was indeed population-specific, we typed a further UK cohort of 75 individuals at high risk of HCV infection due to multiple episodes of intravenous drug use but who remained seronegative and aviremic. These individuals have detectable T-cell responses, a protective KIR:HLA-C type, and distinct serum cytokine profile, consistent with exposure to HCV infection.[26,31,32] As a comparator group we typed a further 79 individuals from the UK with chronic HCV infection. The tapasin G allele was present in 63 (84%) individuals in the exposed seronegative aviremic group, similar to that found in the UK spontaneous resolvers, but this was not significantly different from the 60 (76%) with the tapasin G allele in the second UK chronically infected population (Table 2c). However, there was an increased frequency of HLA-B Asp114 in combination with tapasin G in the

Table 5. Association of Tapasin (Tsn) Alleles With HLA-B Alleles With Asp 114 in the Exposed Seronegative Aviremic and Second UK Chronic Populations

	Exposed Seronegative (2n = 104)	Chronic (2n = 102)	*P* Value	OR (95% CI)
HLA-B Asp114+ Tsn G	57 (54.8%)	41 (40.2%)	0.036	1.8 (1.04-3.14)
HLA-B Asp114+ Tsn C	50 (48.1%)	43 (42.1%)	0.4	
HLA-B Asp114+ Tsn GG	17 (16.4%)	7 (6.9%)	0.034	2.65 (1.05-6.70)

exposed seronegative aviremic individuals as compared to the chronically infected (54.8% versus 40.2%; $P < 0.04$, OR = 1.8 95%, CI = 1.04-3.14) (Table 5). Furthermore, they also had an increased frequency of HLA-B Asp114 in combination with two tapasin G alleles (16.4% versus 6.9%; $P < 0.04$, OR = 2.7, 95%, CI = 1.05-6.70). Thus, tapasin G and HLA-B appear to have a similar protective effect in this cohort.

Tapasin G Is Associated With Stronger IFN-γ CD8+ T-Cell Responses in Chronic HCV Infection. To date the functional role of tapasin polymorphism on CD8+ responses has not been described in viral infection. Tapasin optimizes peptide:MHC class I interactions, likely impacting priming and induction of CD8+ T cells. In order to determine whether there is an effect of the tapasin polymorphism on antiviral CD8+ responses, we determined peptide-specific CD8+ responses from 42 patients with chronic HCV genotype 1 infection who had HLA-B alleles with aspartate at residue 114, as this was our most significant association with the tapasin polymorphism in the genetic studies. These alleles included B*18, B*35, B*57, and B*58. PBMC were stimulated for 2 weeks, using specific HLA-B restricted HCV-derived peptides (Table 1), matched to the HLA-B allele of the patient and analyzed for IFN-γ secretion by flow cytometry. In order to avoid confounding effects through HCV genotype mismatches between patients and epitope peptides, we restricted our analysis to patients infected with HCV genotype 1, using only peptides identified from this genotype. One hundred and ninety-one assays using 16 different peptides were performed in these individuals (145 assays in the 35 patients with the tapasin G allele [median 4, range 1-8] versus 46 assays in the 10 patients without the tapasin G allele [median 3, range 2-8]; $P = 0.9$). In patients with these HLA-B alleles and also a tapasin G allele measurable responses were made in 51 out of 145 (35.1%) assays as compared to 8 out of 46 (17.3%) in patients without a tapasin G allele ($P = 0.02$, OR = 2.58, 95% CI = 1.05-6.5). Next, the magnitude of the response to these peptides was compared. Individuals carrying a tapasin G allele had a greater overall frequency of CD8+ T-cell responses compared to individuals without the tapasin G allele ($P = 0.037$) (Fig. 3). Taken together, our data suggest that the tapasin G allele and specific HLA class I alleles may synergize to generate stronger CD8+ T-cell responses. As discussed below, future studies performed in acute infection are required to extend our observations from chronically infected patients.

Discussion

The association of the tapasin G allele with outcome in our cohort is consistent with the role of the CD8+ T-cell response in determining the outcome of acute HCV infection. Furthermore, the functional data in an unrelated cohort indicates that this polymorphism has a downstream effect on the magnitude of the CD8+ response. Thus, individuals with a protective tapasin allele have greater CD8+ responses than those without. The increased frequency of IFN-γ-positive CD8+ T cells following peptide stimulation in these individuals may be related either to a larger pool of memory CD8 T cells or alternatively memory T cells that proliferate more efficiently. It is important to note, however, that our functional CD8+ T-cell data

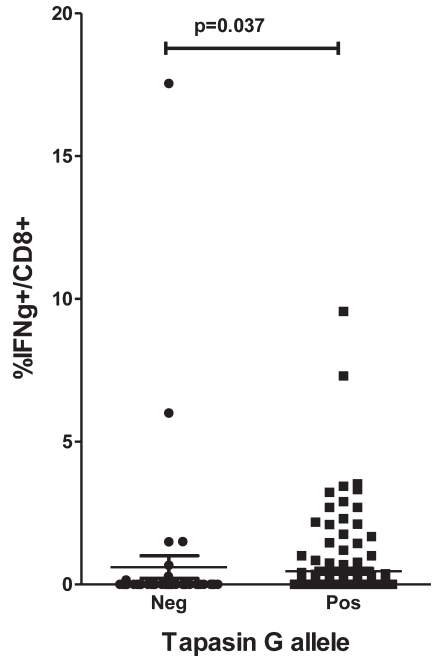

Fig. 3. The tapasin G allele is associated with stronger IFN-γ-producing T-cell responses in individuals with an HLA-B allele with Asp114. The results of intracellular cytokine staining for IFN-γ on CD8+ T cells stimulated with various HCV-derived HLA restricted peptides. All patients tested had an HLA-B allele with Asp114 and peptides were matched to the HLA-B allele of the individual. Data are plotted for individuals with and without a tapasin G allele.

have several limitations. First, the strength of HCV-specific CD8+ T-cell memory responses may be confounded by viral sequence variations in the respective CD8+ T-cell epitopes. We thus analyzed the autologous viral sequences in a subset of patients. Importantly, viral sequence mutations occurred at very similar frequencies in patients irrespective of tapasin genotypes (CC: 9/18 epitopes, 50.0%; CG: 12/22, 54.6%; GG: 5/11, 45.45%; $P > 0.1$), indicating that viral sequence variations did not substantial confound our results. Second, the CD8+ T-cell response in the chronic phase of infection may not necessarily correlate with the CD8+ T-cell response in the acute phase of infection, when the outcome is determined. Longitudinal analyses of patients with acute-persistent infection as well as similar immunodominance of CD8+ T-cell responses in acute and chronic infection, however, argue against this notion.[8,29,33] Third, the rather small number of patients per individual HLA allele may influence our results. Thus, further studies in the acute phase of infection are required to confirm our finding that tapasin polymorphisms and specific HLA class I alleles synergize to generate stronger antiviral CD8+ responses.

In the tapasin knockout mouse there is an alteration in peptide presentation as compared to the wildtype mouse.[34] In these experiments the presence of tapasin favors CD8+ T-cell responses to peptides with slow off-rates from MHC class I and its absence is associated with CD8+ responses to peptides with a fast off-rate. How this may be affected by a polymorphism in tapasin is not clear. The R260T polymorphism is present in one of the Ig-like domains of tapasin. In the crystal structure it is in a loop, close to the site of interaction with the oxido-reductase ERp57, which is another key component of the peptide loading complex involved in determining the peptide repertoire expressed on the cell surface.[35] Thus, the effect of this polymorphism on the interaction with HLA class I is not likely to be a direct effect on tapasin binding to HLA class I. It may be a remote effect on HLA class I binding, or alternatively an indirect effect mediated via the interaction with ERp57.

The finding of an absence of tapasin association in a second unrelated cohort is not unexpected, as these are individuals who have a discrete ancestry and are exposed to different viral populations.[36,37] These differences are especially noticeable at the MHC, which is characterized by substantial population diversity. In analyses of HLA class I different protective HLA class I alleles are found in European as opposed to USA cohorts.[3-5] However, despite these population differences, the levels of homozygosity at the MHC were similar between the US and UK populations. For instance, at HLA-B 9.2% of the US and 7.1% of the UK populations were homozygous using four-digit HLA typing.

Another source of confounding genetic effects include resolution through different genetic pathways. These include KIR and HLA-C, and also potentially interleukin (IL)-28B. Subanalysis of the US cohort by KIR ligand (HLA-C) type was not associated with any significant findings (Supporting Table 3) and IL-28B is thought to be protective in both US and UK Caucasian populations.[38] Thus, these known protective factors are unlikely to account for our observed population differences. Additionally, there is diversity in the prevalent HCV-infecting genotypes between the USA and the UK,[36,37] which may also impact the mechanism of resolution of infection.

Our genetic data correlate well with the biochemistry of tapasin, with key residues being 114 and 156.[16] Thus, for HLA-B*4402, which has Asp114 and Asp156, the presence of a functional tapasin protein alters the peptide repertoire.[39] Thus, not only can polymorphisms at these residues impact their interaction with tapasin, but the specific residues that are most significant in our study have been previously identified by biochemical experiments. Overall, tapasin is thought to be under purifying selection, being well conserved across species with 84% amino acid identity between human and sheep.[40] This implies that there is selection pressure to maintain the sequence of tapasin and the coding SNP is likely to be the most relevant due to its potential for functional interaction with HLA or ERp57. Tapasin is thus likely to be important in generating optimal HLA class I peptide repertoires in the antiviral immune response. Consideration of this polymorphism may be important for the implementation of peptide vaccination strategies for HCV.

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