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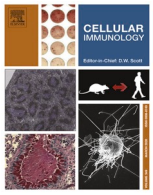

Analysis of the different subpeptidomes presented by the HLA class I molecules of the B7 supertype

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ABSTRACT

MHC-I molecules of the HLA-B7 supertype preferentially bind peptides with proline at position 2. HLA-B*51:01 and B*51:08 present two predominant subpeptidomes, one with Pro2 and hydrophobic residues at *P*1, and another with Ala2 and Asp enriched at position 1. Here, we present a *meta*-analysis of the peptidomes presented by molecules of the B7 supertype to investigate the presence of subpeptidomes across different allotypes. Several allotypes presented subpeptidomes differing in the presence of Pro or another residue at *P*2. The Ala2 subpeptidomes preferred Asp1 except in HLA-B*54:01, where ligands with Ala2 contained Glu1. Sequence alignment and the analysis of crystal structures allowed us to propose positions 45 and 67 of the MHC heavy chain as relevant for the presence of subpeptidomes. Deciphering the principles behind the presence of subpeptidomes could improve our understanding of antigen presentation in other MHC-I molecules.

Running title: HLA-B7 supertype subpeptidomes.

1. Introduction

Human leukocytes antigens class I molecules (HLA-I) bind peptides derived from cytosolic or nuclear proteins and present them to cytotoxic T lymphocytes (CTL), which are able to discriminate between "self" and "non-self" peptides and subsequently kill infected or transformed cells. HLA-I are heterodimers composed of a heavy and variable α chain encoded in the major histocompatibility complex (MHC) region and a light and constant chain, beta-2-microglobulin (β2m), encoded outside the MHC. The distal extracellular α1 and α2 domains of the heavy chain form the peptide binding site, composed by two α helices and a β sheet of 8 anti-parallel β strands, forming six different pockets that accommodate the side chains of some residues of the peptide [1]. In HLA-I, the most restrictive pockets are those that bind the side chains of the second and last peptide positions (*P*2 and PΩ, respectively) [2]. Three *loci* encode the heavy chain of the classical HLA-I molecules: *HLA-A*, *HLA-B* and *HLA-C*. *HLA* genes are the most polymorphic genes in the genome, and most of their polymorphisms are located in the peptide binding site, making different HLA molecules bind different peptidomes with specific anchor motifs [2]. The interaction of HLA-I with specific residues of the peptide restricts the number of potential ligands. Thus, the correct identification of the anchor motifs from the different HLA-I allotypes is crucial to properly predict which peptides from a given protein have the potential to bind to each HLA molecule and be presented to CTLs.

HLA-B molecules have been grouped in 6 different supertypes based on the anchor motifs of their peptide ligands, called B7, B8, B27, B44, B58 and B62 supertypes [3–7]. HLA-I molecules belonging to the same supertype bind partially overlapping peptidomes. The B7 supertype is composed of HLA-B molecules that bind Pro at the *P*2 peptide position and aromatic, aliphatic and hydrophobic residues at their PΩ [3].

HLA-B*51:01 and -B*51:08, two members of the B7 supertype, bind peptides with Pro2 or Ala2 and hydrophobic residues at PΩ [8–11].

Abbreviations: HLA, human leukocyte antigens; CTL, cytotoxic T lymphocytes; MHC, major histocompatibility complex; β2m, Beta-2 microglobulin; IEDB, Immuno Epitope Database and Analysis Resource; PRBAM, Peptide Repertoire-Based Anchor Motif; PDB, Protein Data Bank.

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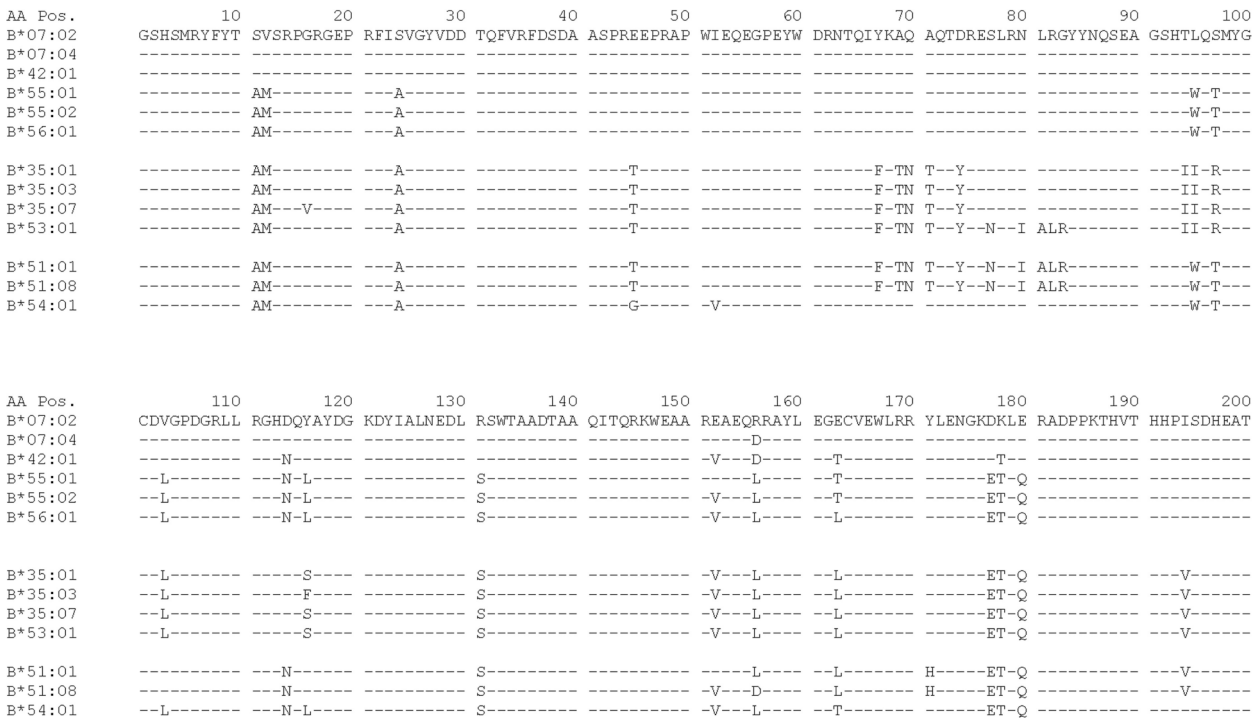

Fig. 1. Sequence alignment of the of 13 B7 supertype allotypes. Primary sequences of the HLA-I molecules analyzed in this work were aligned and variable residues with regard to HLA-B*07:02 are indicated.

Table 1
General features of the B7 supertype peptidomes.

HLA-B*	Total	Peptides by number of residues (%)						AAs average
		8	9	10	11	12	13	
07:02	12,033	7.83	49.49	21.94	12.29	5.68	2.78	9.67
07:04	1480	5.74	61.22	20.00	10.34	1.89	0.81	9.44
35:01	1954	5.27	57.37	17.14	11.67	5.53	3.02	9.64
35:03	1527	4.78	76.36	10.35	6.09	1.83	0.59	9.26
35:07	1933	3.31	68.39	14.49	9.67	2.69	1.45	9.44
42:01	3428	5.86	59.42	17.53	12.95	2.92	1.31	9.52
51:01	5157	27.38	55.85	8.45	4.07	2.39	1.86	9.04
51:08	624	13.62	77.88	4.17	3.04	1.28	0.00	9.00
53:01	2204	4.54	63.43	17.56	9.62	3.58	1.27	9.48
54:01	1441	11.24	52.26	16.52	12.28	4.44	3.26	9.56
55:01	1548	11.30	55.81	19.77	9.04	2.39	1.68	9.40
55:02	1549	3.42	54.36	22.66	13.43	4.13	2.00	9.66
56:01	2113	4.92	51.49	24.33	13.54	3.74	1.99	9.66

Previous work reported that the peptide repertoires bound to these allotypes are composed by two different subpeptidomes: one with Pro2 and a preference for hydrophobic residues at *P*1, and one containing Ala2 with preference for Asp1 [12], and subsequently confirmed [13]. More recently, it was described the presence of subpeptidomes in other HLA-I peptide pools [13,14], showing that it is not unique to the -B*51 subtypes.

The presence or absence of subpeptidomes and the different features that can show these subpeptidomes among close related HLA-I molecules may explain the presentation or not of putative relevant T cell epitopes from pathogens or self-antigens. In addition, the knowledge of the structural features explaining the presence of subpeptidomes may be useful to predict which allotypes are able to bind specific peptides.

Here, we present a *meta*-analysis of the peptidomes of 13 HLA-I molecules belonging to the -B7 supertype: we analyzed the presence or absence of subpeptidomes and the effect of the amino acid variation at the anchor position 2 on the residue at position 1. The presence of subpeptidomes varied among the HLA-B7 allotypes. Sequence alignment of the HLA-I molecules strongly suggests that residues Thr45 and Phe67 are important for the presence of subpeptidomes in the B7 supertype molecules. Positions 45 and 67 are part of the Pocket B [15] and have been described to alter peptide presentation [16]. Finally, the analysis of multiple crystal structures of -B*07:01, -B*35:01 and -B*51:01 showed that the orientation of residue Asn63 varied depending on the presence of Glu45-Tyr67 (in -B*07:01) or Thr45-Phe67 (in -B*35:01 and -B*51:01).

2. Materials and methods

2.1. HLA-B peptidomes

Peptidomes from Immuno Epitope Database and Analysis Resource (IEDB) were extracted at 02–15-2022. Duplicated peptides in any HLA-B peptidome were eliminated and considered only once. Peptides with or without post-translational modifications were treated as unique non-modified peptides. Only peptidomes from HLA-B molecules with more

Table 2
Immunopeptidome overlap.

Peptide Number		12033	1480	1954	1527	1933	3428	5157	624	2204	1441	1548	1549	2113
	HLA allotype	**B*07:02**	**B*07:04**	**B*35:01**	**B*35:03**	**B*35:07**	**B*42:01**	**B*51:01**	**B*51:08**	**B*53:01**	**B*54:01**	**B*55:01**	**B*55:02**	**B*56:01**
12033	**B*07:02**		822	228	314	199	1693	203	33	181	82	286	230	271
1480	**B*07:04**	55.5 \ 6.8		25	59	41	878	32	9	36	19	120	80	85
1954	**B*35:01**	11.7 \ 1.9	1.3 \ 1.7		246	865	115	153	3	452	68	9	22	25
1527	**B*35:03**	20.6 \ 2.6	3.9 \ 4.0	16.1 \ 12.6		371	237	93	12	322	45	34	39	55
1933	**B*35:07**	10.3 \ 1.7	2.1 \ 2.8	44.7 \ 44.3	19.2 \ 24.3		168	60	3	613	28	21	31	42
3428	**B*42:01**	49.4 \ 14.1	25.6 \ 59.3	3.4 \ 5.9	6.9 \ 15.5	4.9 \ 8.7		129	49	149	66	238	191	209
5157	**B*51:01**	3.9 \ 1.7	0.6 \ 2.2	3.0 \ 7.8	1.8 \ 6.1	1.2 \ 3.1	2.5 \ 3.8		337	178	215	100	99	130
624	**B*51:08**	5.3 \ 0.3	1.4 \ 0.6	0.5 \ 0.2	1.9 \ 0.8	0.5 \ 0.2	7.9 \ 1.4	54.0 \ 6.5		34	30	23	25	25
2204	**B*53:01**	8.2 \ 1.5	1.6 \ 2.4	20.5 \ 23.1	14.6 \ 21.1	27.8 \ 31.7	6.8 \ 4.3	8.1 \ 3.5	1.5 \ 5.4		37	32	36	41
1441	**B*54:01**	5.7 \ 0.7	1.3 \ 1.3	4.7 \ 3.5	3.1 \ 2.9	1.9 \ 1.4	4.6 \ 1.9	14.9 \ 4.2	2.1 \ 4.8	2.6 \ 1.7		226	329	425
1548	**B*55:01**	18.5 \ 2.4	7.8 \ 8.1	0.6 \ 0.5	2.2 \ 2.2	1.4 \ 1.1	15.4 \6.9	6.5 \ 1.9	1.5 \ 3.7	2.1 \ 1.5	14.6 \ 15.7		710	777
1549	**B*55:02**	14.8 \ 1.9	5.2 \ 5.4	1.4 \ 1.1	2.5 \ 2.6	2.0 \ 1.6	12.3 \ 5.6	6.4 \ 1.9	1.6 \ 4.0	2.3 \ 1.6	21.2 \ 22.8	45.8 \ 45.9		1207
2113	**B*56:01**	12.8 \	4.0 \	1.2 \	2.6 \	2.0 \	9.9 \	6.2 \	1.2 \	1.9 \	20.1 \	36.8 \	57.1 \	
		2.3	5.7	1.3	3.6	2.2	6.1	2.5	4.0	1.9	29.5	50.2	77.9	

Note: Above the diagonal, absolute numbers of shared peptides are indicated. Under the diagonal, the percentage of shared peptides among each peptidome are indicated (the number before backslash correspond to the allotype indicated in the left, and the number after blacklash corresponds to the allotype indicated above). Note: Light grey shows that shared peptides represent more than 10% of the total peptidome of at least one of the pair. Dark shows that shared peptides represent more than 20% of the total peptidome of at least one of the pair.

than 500 peptides were included in this study.

2.2. Identification of the HLA-I anchor motifs

To determine the HLA-I anchor motifs, peptidomes were analyzed with the Peptide Repertoire-Based Anchor Motif (PRBAM) program [17]. In order to simplify the output, only nonamers were analyzed to define the anchor motifs of these allotypes.

2.3. MHC binding affinities of HLA-I ligands.

The theoretical affinity of the peptides for HLA–I molecules was calculated using the NetMHC4.0 1.1 Server (https://www.cbs.dtu.dk/services/NetMHC/) [18,19].

2.4. Statistical analysis.

Differences in residue frequencies were analyzed by chi-square test with Bonferroni correction as previously described [12].

Table 3
Amino acid presence at the peptide position 2 among the peptidomes of the B7 supertype molecules.

	Total	*P*2	A2	Others
HLA-B*07:02	12,033	9956 (82.7%)	338 (2.8%)	1739 (14.5%)
HLA-B*07:04	1480	1233 (83.3%)	58 (3.9%)	189 (12.8%)
HLA-B*35:01	1954	1322 (67.7%)	297 (15.2%)	335 (17.1%)
HLA-B*35:03	1527	1251 (81.9%)	177 (11.6%)	99 (6.5%)
HLA-B*35:07	1933	1373 (71.0%)	399 (20.6%)	161 (8.3%)
HLA-B*42:01	3428	2987 (87.1%)	100 (2.9%)	341 (9.9%)
HLA-B*51:01	5157	2566 (49.8%)	1552 (30.1%)	1039 (20.1%)
HLA-B*51:08	624	285 (45.7%)	339 (54.3%)	0 (0.0%)
HLA-B*53:01	2204	1600 (72.6%)	436 (19.8%)	168 (7.6%)
HLA-B*54:01	1441	1038 (72.0%)	145 (10.1%)	258 (17.9%)
HLA-B*55:01	1548	1378 (89.0%)	42 (2.7%)	128 (8.3%)
HLA-B*55:02	1549	1407 (90.8%)	40 (2.6%)	102 (6.6%)
HLA-B*56:01	2113	1873 (88.6%)	54 (2.6%)	186 (8.8%)

Differences in MHC binding affinities were analyzed by the Mann-Whitney *U* test. Different P values were considered.

2.5. Analysis of crystal structures

Crystal structures of different HLA molecules were previously reported and deposited on the Protein Data Bank (PDB, rcsb.org) [20]. To perform the analysis, three crystals structures of HLA-B*51:01: 1E27, 1E28, 4MJI [21,22], twelve of HLA-B*07:02: 3VCL [23], 5EO, 5EO1 [24], 5WMN, 5WMO, 5WMP [25], 6AT5 [26], 6UJ7, 6UJ8 [27], 7LG0, 7LGD, 7LGT [28] and eighteen of HLA-B*35:01: 1A1N [29], 1A9B, 1A9E [30], 1XH3 [31], 1ZSD [32], 2AXG [33], 2CIK [34], 2FYY, 2FZ3 [35], 2H6P [36], 3LKN, 3LKO, 3LKP, 3LKQ, 3LKR [37], 4PR5, 4PRA, 4PRN [38] were chosen. Sequences were aligned and analyzed using the PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.

3. Results

3.1. Features of the B7 supertype peptidomes

We obtained the peptide repertoires of different HLA-B molecules belonging to the B7 supertype from IEDB [39]. Peptidomes with more than 500 peptides ranging from 8 to 13 residues long were considered. Following this criterium, we selected 13 peptidomes, derived from HLA-B*07:02, -B*07:04, -B*35:01, -B*35:03, -B*35:07, -B*42:01, -B*51:01, -B*51:08, -B*53:01, -B*54:01, -B*55:01, -B*55:02 and -B*56:01. Fig. 1 shows the sequence alignment of these proteins. The number of reported peptides from each molecule ranged from 624 to 12,033 peptides (Table 1). As expected, nonamers comprised the majority of each peptidome (from 49.5% to 77.9%), with the percentage of octamers and peptides 10–13 residues long varying among different allotypes. HLA-B*51:01 and -B*51:08 contained the highest proportion of octamers (27.4% and 13.6%, respectively) and the lowest number of peptides of 10 or more residues. In contrast, HLA-B*07:02, -B*07:04, -B*55:02 and –B*56:01 bound more than 20% of 10-mer and more than 10% of 11-mer peptides. The average number of residues in the analyzed peptidomes was similar (ranging from 9.3 to 9.7), except for the two B*51 alleles, with an average number of 9.0 residues.

The B7 supertype is characterized by the presence of Pro at *P*2 and aromatic, aliphatic and hydrophobic residues at PΩ [3]. We analyzed the degree of overlap between the peptidomes of the different members of the B7 supertype. The 13 peptide pools were composed of a total of 27,039 peptides, of which 6134 (22.7%) were shared by at least two allotypes and 2445 (9.0%) by at least three HLA-I molecules. The degree of overlap was analyzed by pairs of HLA-B molecules (Table 2). In general, peptidomes from different subtypes of the same allele group (HLA-B*07:02 with -B*07:04; -B*35:01 with -B*35:03 and -B*35:07; -B*51:01 with -B*51:08; and -B*55:01 with -B*55:02) shared a higher number of peptides than with other molecules. Nevertheless, in this dataset, the highest overlap was found between HLA-B*55:02 and -B*56:01, sharing 1207 peptides (77.9% and 57.1% of the -B*55:02 and -B*56:01 peptide repertoires, respectively) (Table 2).

3.2. Anchor motifs of the B7 supertype HLA molecules

All 13 molecules bind preferentially peptides with Pro2, while peptides with Ala2 are tolerated to different degree, ranging from 2.6% of the peptidome in B*55:02 and B*56:01 to 54.3% in B*51:08 (Table 3). To further characterize the anchor motifs of these molecules, their peptidomes were analyzed with the PRBAM software. This software identifies the anchor motifs of HLA-I and HLA-DR molecules based on the amino acid abundance at each position. The program returns a deviation from the mean in the proteome (DMP) value for each amino acid in each peptide position. The resulting anchor motifs for nonamers are shown in Supplementary Fig. 1. Seven allotypes presented peptidomes with favored Ala2 (the detected abundance of Ala at *P*2 is higher than its abundance in the human proteome): HLA-B*35:01, -B*35:03, -B*35:07, -B*51:01, -B*51:08, -B*53:01 and -B*54:01 (Supplementary Fig. 1).

3.3. HLA-B*54:01 presents, as B*51:01 and B*51:08, two subpeptidomes with favored acidic residues in P1 in the subpeptidome containing Ala2, but with Glu1 preferred to Asp1

The peptide repertoire of HLA-B*51:01 was previously described based on the identification of 1620 eluted peptides, and two different subpeptidomes were identified based on the presence of Pro or Ala at *P*2. Peptides with Pro2 contained mostly aliphatic and aromatic residues at *P*1, while peptides with Ala2 showed preferentially Asp at *P*1 [12]. We expanded the analysis with 5157 ligands from the IEDB obtaining similar results, with peptides with Pro2 presenting aliphatic or aromatic residues at *P*1 and peptides with Ala1 containing mainly Asp1 (Fig. 2).

The HLA-B*51:08 peptidome, containing 624 unique peptides, was analyzed previously [40]. As shown in Fig. 2, the HLA-B*51:08 peptide repertoire is also composed by two subpeptidomes with the same features than those of HLA-B*51:01.

The IEDB peptidome of HLA-B*54:01 consisted of 1441 peptides, from which 1038 (72.0%) contained Pro2, 145 (10.1%) Ala2 and 258 (17.9%) other amino acids at *P*2. As in HLA-B*51:01 and -B*51:08, the Ala2 subpeptidome from HLA-B*54:01 showed an increase in acidic residues at *P*1. Interestingly, peptides contained more Glu1 than Asp1. Peptides with Pro2 presented aliphatic or aromatic residues in *P*1.

Thus, the existence of two subpeptidomes, one with Pro2 associated to a prevalence of hydrophobic residues at *P*1 and a second one with Ala2 and acidic residues at *P*1, is present in at least other molecule of the B7 supertype different to the B*51 subtypes.

3.4. HLA-B35 subtypes and HLA-B*53:01 show two subpeptidomes with polar and acidic residues favored at P1 in the subpeptidome containing Ala2

The HLA-B*35:01, -B*35:03, -B*35:07 and -B*53:01 peptidomes also presented, in addition to Pro2, Ala increased at *P*2 (Table 3 and Supplementary Fig. 1). The analysis of these peptidomes indicated that the subpeptidomes containing Pro2 presented a preference for aliphatic or aromatic residues at *P*1, while the subpeptidome with Ala2 preferred for polar and acidic residues. In this case, polar residues (Ser, Thr, Asn and Gln) were preferred at *P*1 to Asp and Glu (Fig. 3). In conclusion, HLA-B*35:01, -B*35:03, -B*35:07 and -B*53:01 present two subpeptidomes: one with Pro2 and preference for hydrophobic residues at *P*1, and another one presenting Ala2 and polar and acidic residues favored at *P*1.

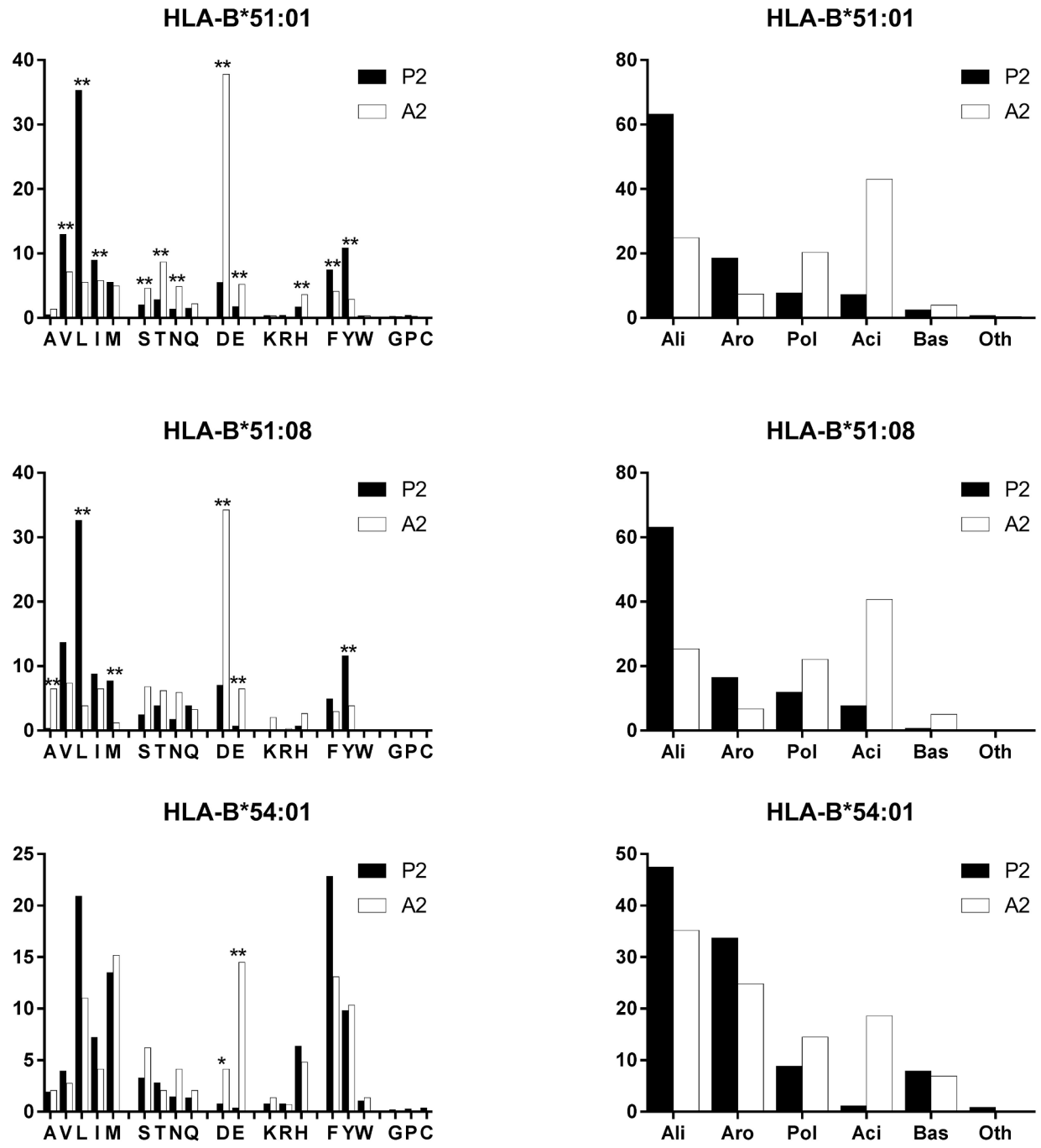

Fig. 2. Differential residue usage at the *N*-terminal position (*P*1) between HLA-B*51:01, -B*51:08 and -B*54:01 ligands with Pro at position 2 (*P*2) or Ala at position 2 (A2). The single-letter code for amino acids is used. Left panels, Position 1 residue frequencies among peptides with Pro2 (*P*2, black) or Ala2 (A2, white). Residues are sorted according to their chemical features. Statistically significant differences, as determined by the chi-square test with Bonferroni correction were indicated as * < 0.05 and ** < 0.01. Right panels, frequency of residues at *P*1 grouped by their chemical nature.

3.5. Other HLA-B molecules of the B7 supertype do not present apparent subpeptidomes

The peptide repertoires of the other HLA-B molecules of the B7 supertype obtained from the IEDB (HLA-B*07:02, -B*07:04, -B*42:01, -B*55:01, -B*55:02 and -B*56:01) only had Pro favored at *P*2. Although peptides with Ala2 were not favored in these peptidomes, some ligands of these molecules contained Ala at *P*2 (with an abundance ranging from 2.6% to 3.9% of the total peptides). We analyzed the residue usage at *P*1 of these peptides and compared it to that from Pro2 peptides. As shown in Fig. 4, we were not able to detect significant differences at *P*1, contrary to what was observed in other members of the B7 supertype, although Pro and Gly were increased in the peptides containing Ala2 and Ser and Arg tended to be increased in the peptides with Pro2.

3.6. Peptidomes containing different amino acids than pro or Ala at P2 show similar features to the Ala2 subpeptidome

A considerable number of peptides contained different residues than Pro or Ala at *P*2 (Table 3). To evaluate whether these peptides are similar to the Pro2 or Ala2 subpeptidomes, or instead form a third subpeptidome on their own, we compared this peptide pool with those

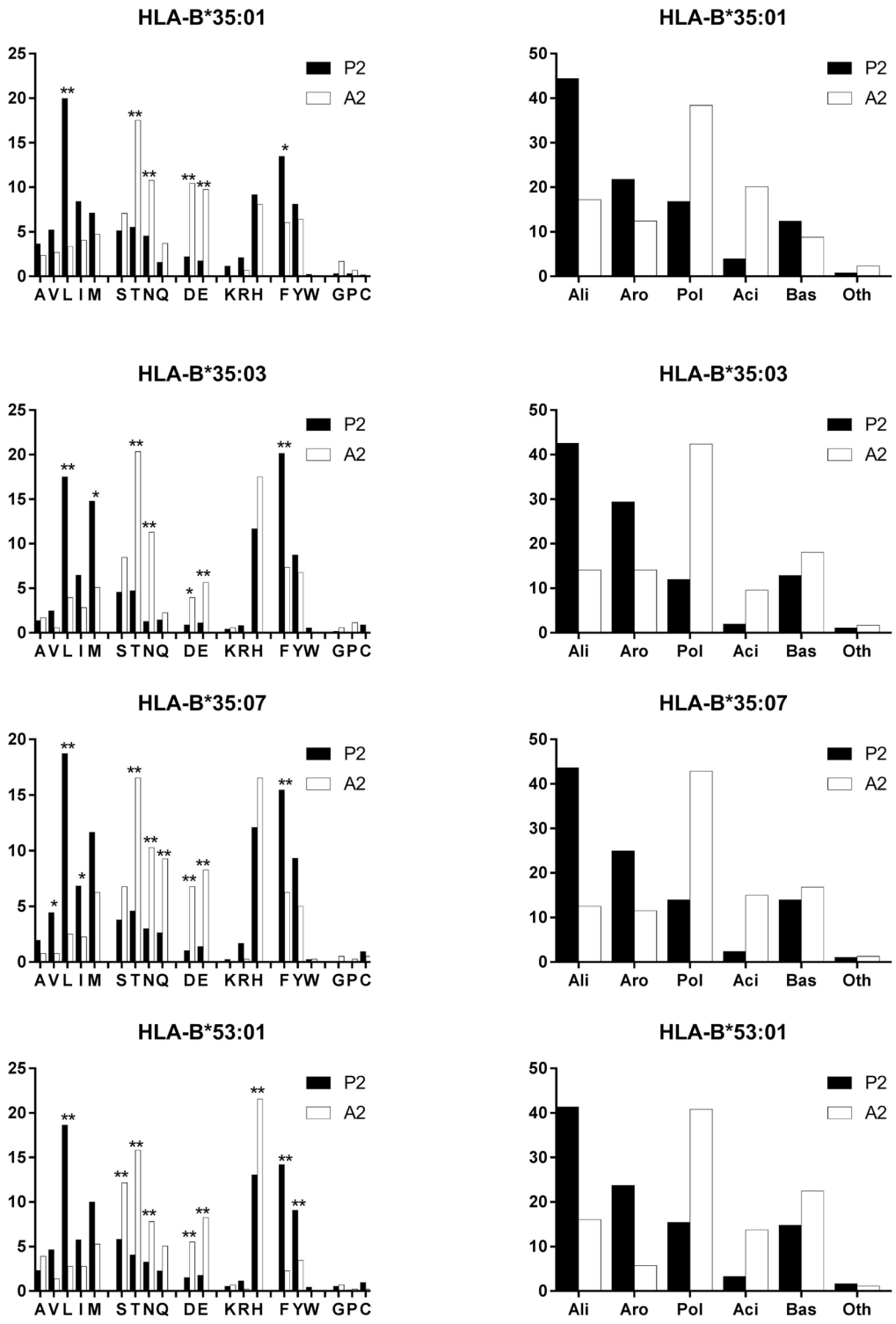

Fig. 3. Differential residue usage at the *N*-terminal position (*P*1) between HLA-B*35:01, -B*35:03, -B*35:07 and -B*53:01 ligands with Pro at position 2 (*P*2) or Ala at position 2 (A2). The single-letter code for amino acids is used. Left panels, Position 1 residue frequencies among peptides with Pro2 (*P*2, black) or Ala2 (A2, white). Residues are sorted according to their chemical features. Statistically significant differences, as determined by the chi-square test with Bonferroni correction were indicated as * < 0.05 and ** < 0.01. Right panels, frequency of residues at *P*1 grouped by their chemical nature.

containing Pro2 or Ala2. In general, this peptide pool was similar to those with the subpeptidome of Ala2 in reference to the *P*1 composition (Supplementary Fig. 2 and Supplementary Fig. 3).

3.7. The presence of Thr45 and Phe67 instead of Glu45 and Tyr67 is associated with the presence of subpeptidomes in the B7 supertype molecules

The α chain sequence alignment of the members of the B7 supertype indicated that molecules that do not present subpeptidomes contain Glu45, while the alleles that present subpeptidomes contain Thr45, except for HLA-B*54:01 which has Gly45 (Table 4). In addition, Phe67 is present in the alleles with subpeptidomes, except for HLA-B*54:01 which, like the allotypes without apparent subpeptidomes, contains Tyr67.

The analysis of multiple crystal structures of HLA-B*07:02 showed that Glu45 directly interacts with Tyr67 and Asn63 (a non-polymorphic residue present in all the allotypes analyzed) (Fig. 5A and Supplementary Fig. 4. This interaction does not exist when Thr and Phe are present at positions 45 and 67, respectively, as is the case of -B*35:01 and

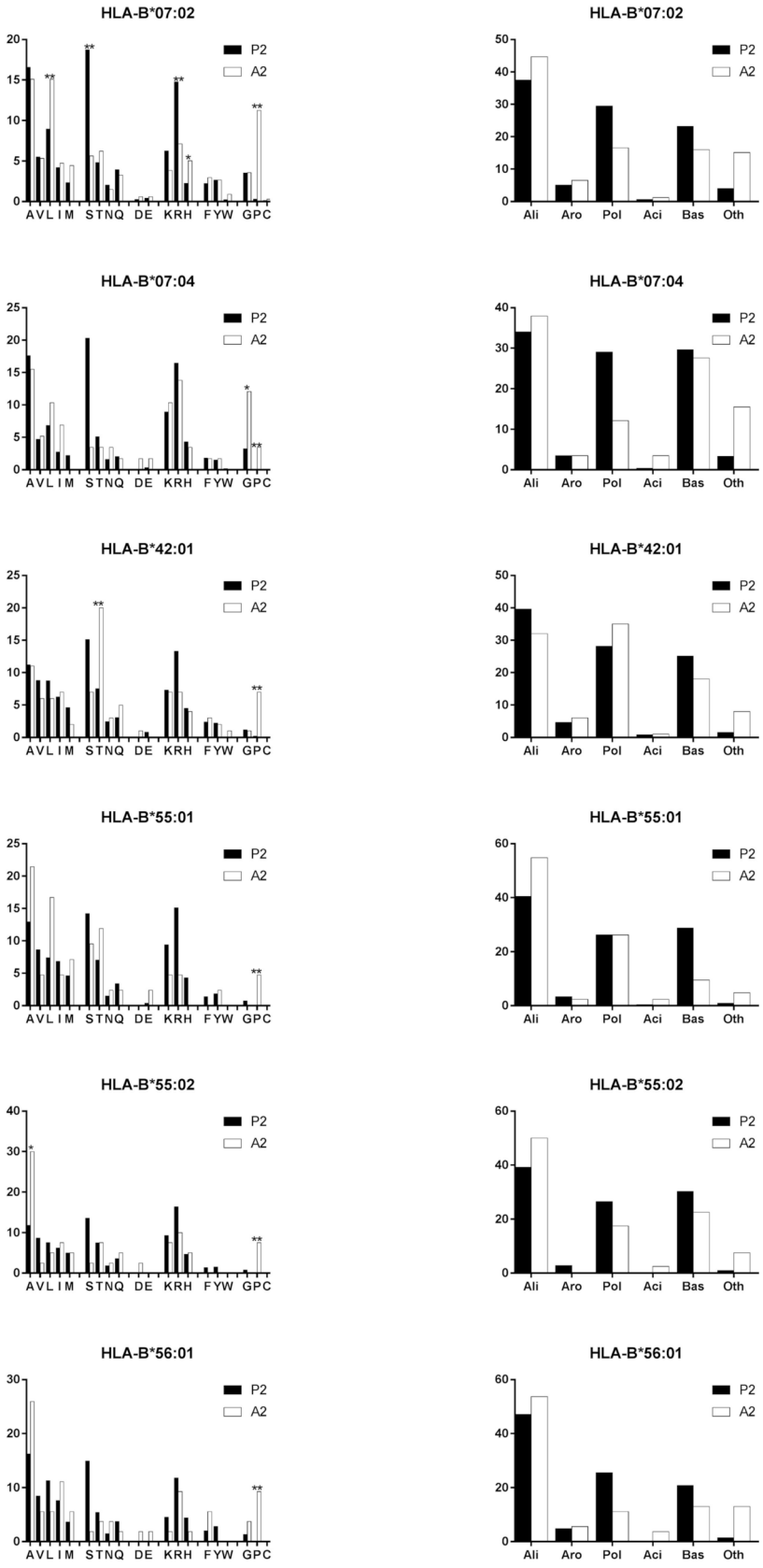

Fig. 4. Differential residue usage at the *N*-terminal position (*P*1) between HLA-B*07:02, -B*07:04, -B*42:01, -B*55:01, -B*55:02 and -B*56:01 ligands with Pro at position 2 (*P*2) or Ala at position 2 (A2). The single-letter code for amino acids is used. Left panels, Position 1 residue frequencies among peptides with Pro2 (*P*2, black) or Ala2 (A2, white). Residues are sorted according to their chemical features. Statistically significant differences, as determined by the chi-square test with Bonferroni correction were indicated as * < 0.05 and ** < 0.01. Right panels, frequency of residues at *P*1 grouped by their chemical nature.

Table 4
Residues at positions 45, 67 and 116 in the HLA-I molecules analyzed.

HLA MOLECULE	POSITION			MAIN PEPTIDE RESIDUE AT PΩ
	45	67	116	
B*07:02	E	Y	Y	Leu or Met
B*07:04	–	–	–	Leu or Met
B*42:01	–	–	–	Leu or Met
B*55:01	–	–	L	Ala or Val
B*55:02	–	–	L	Ala or Val
B*56:01	–	–	L	Ala or Val
B*35:01	T	F	S	Aromatic
B*35:03	T	F	F	Leu or Met
B*35:07	T	F	S	Aromatic
B*53:01	T	F	S	Aromatic
B*51:01	T	F	–	Leu or Val
B*51:08	T	F	–	Leu or Val
B*54:01	G	–	L	Ala or Val

-B*51:01 (Fig. 5B and 5C and supplementary Fig. 4). The interaction of Glu45 with Asn63 changes the orientation of the latter compared to the orientation of this residue in the allotypes that contain different subpeptidomes (Fig. 5 and supplementary Fig. 4).

3.8. Residue 116 defines different residue preferences at the peptide C-terminal position

We can identify three main groups based on the peptide C-terminal residue usage: 1) HLA-B*35:01, -B*35:07 and -B*53:01 present mainly aromatic residues at PΩ; 2) HLA-B*54:01, -B*55:01, -B*55:02 and -B*56:01 bind peptides with Ala or Val at PΩ; 3) the rest of the analyzed allotypes bind peptides with Leu or Met at PΩ (HLA-B*07:02, -B*07:04, -B*35:03 and -B*42:01) or Leu or Val (HLA-B*51:01 and -B*51:08) at PΩ (Supplementary Fig. 1). The analysis of the HLA residues that interact with the F pocket revealed that the presence of different residues at position 116 is associated with the preference for specific amino acids at PΩ. Thus, the allotypes that bind peptides with aromatic residues at the C-terminus have Ser116, the ones that bind Ala or Val contain Leu116, and the molecules binding peptides with Leu, Met or Val at C-terminus with as the most abundant amino acids have Tyr116, except for HLA-B*35:03 that has Phe116 (both aromatic residues) (Table 4).

3.9. Theoretical binding affinity of ligands to HLA molecules of the B7 supertype

The theoretical binding affinity of the peptides bound to different members of the B7 supertype was evaluated with NetMHC 4.1, a powerful tool that calculates the affinity of peptides to HLA-I molecules. We analyzed all the molecules included in this analysis that were available to study in NetMHC4.1. These molecules were: HLA-B*07:02, -B*35:01, -B*35:03, -B*51:01, -B*53:01 and -B*54:01. The predicted affinity of the peptides with Pro2 to their corresponding HLA-B molecule was consistently higher than those with Ala2 (Fig. 6). Interestingly, in the case of -B*07:02, the subpeptidome containing Ala2 showed a lower theoretical affinity that the total immunopeptidome. In the other HLA-I molecules, which present preferences by acidic or polar residues in *P*1, the theoretical affinity was similar or higher than that of the total peptidome (Fig. 6).

4. Discussion

It has been described that some HLA alleles show multiple specificities for their ligands [13]. In this study, we analyzed the peptidomes presented by 13 allotypes belonging to the HLA-B7 supertype. We found that, although all the B7 molecules can bind Pro and Ala (in addition to other less abundant residues) at *P*2, several members of the B7 supertype, but not all, present two subpeptidomes in which these amino acids are favored at *P*2, a feature previously described in HLA-B*51:01 and -B*51:08 [12,40]. We analyzed the effect of the presence of these amino acids at *P*2 on the residues favored at *P*1 and analyzed the structural features involved in this effect. Although the presence of subpeptidomes have been previously reported in different HLA-I molecules [13,14], to our knowledge, this is the first study that systematically characterizes the presence of subpeptidomes across different members of the B7

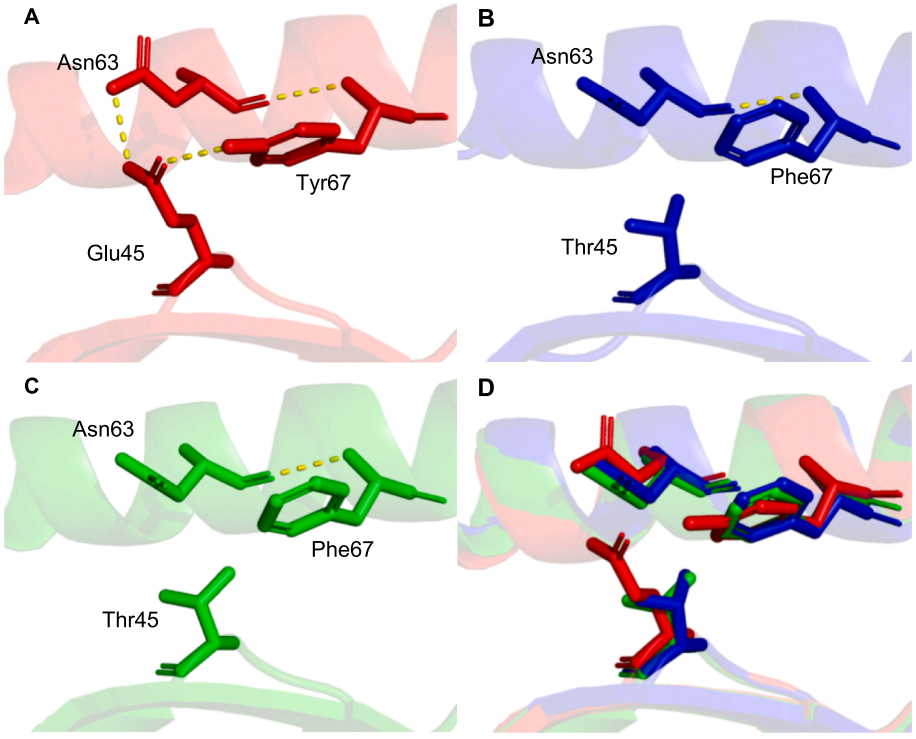

Fig. 5. A-C. Representation of residues 45, 63 and 67 in HLA-B*07:02, -B*35:01 and -B*51:01. HLA in HLA-B*07:02 (A), -B*35:01 (B) and -B*51:01 (C). D. Superposition of HLA-B*07:02 (red), -B*35:01 (blue) and B*51:01 (green). Residues 46, 63 and 67 are represented as sticks. Polar contacts are represented as dotted yellow lines. PDB entries: 1E27, 1A1N and 6UJ7. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

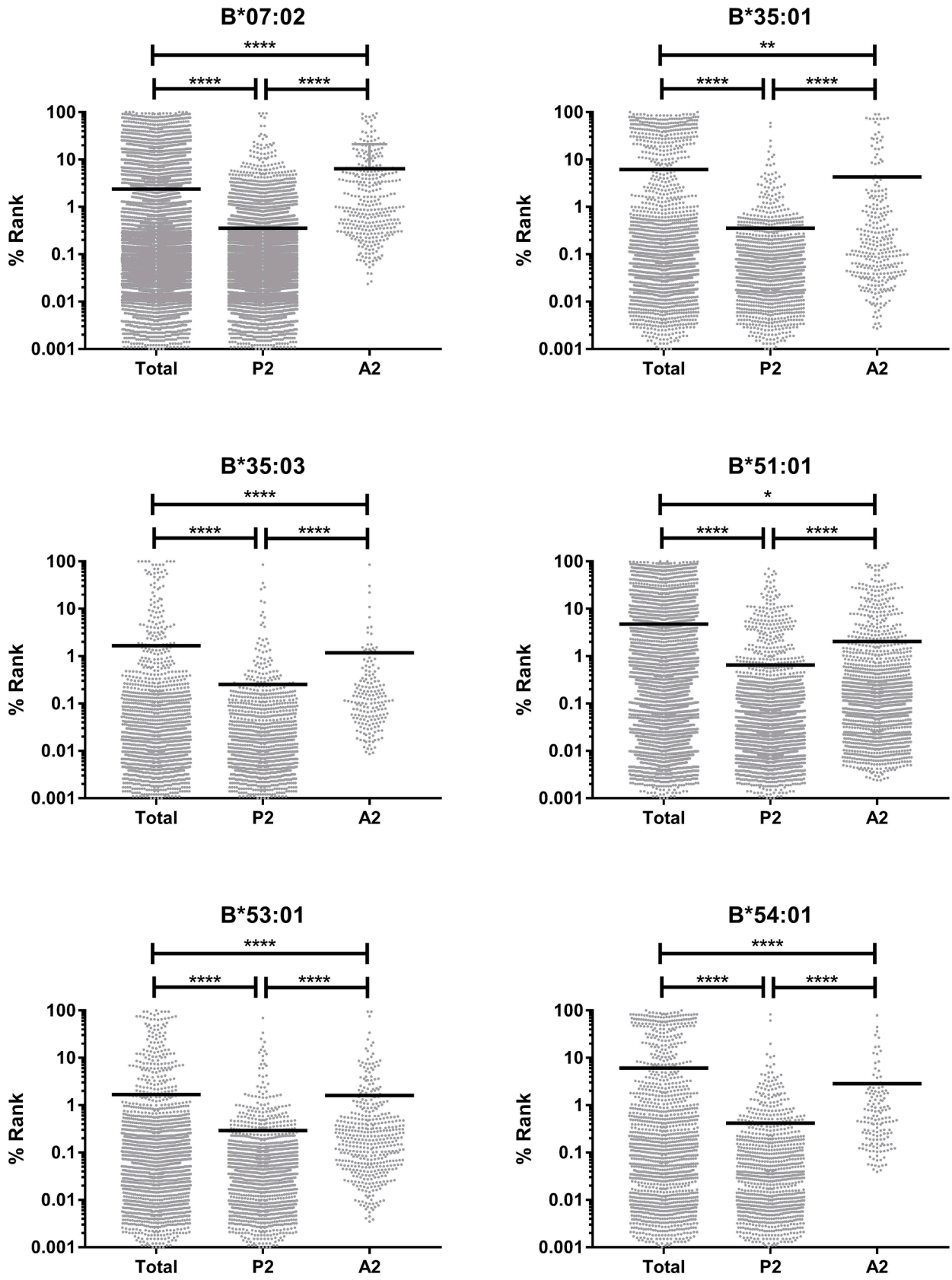

Fig. 6. Theoretical binding affinity to HLA of the subpeptidomes of the members of the B7 supertype. The %Rank of the peptides belonging to the whole petidome (total), the Pro2 subpeptdiome (*P2*) and the Ala2 subpeptidome (A2) to their respective HLA molecule is indicated. Symbols represent individual peptides; horizontal lines represent the median. * = 0.01 < P < 0.1; ** = 0.001 < P < 0.01; *** = 0.001 < P < 0.01; **** = P < 0.0001 by Mann-Whitney *U* test.

supertype and provides a structural explanation for the existence of such subpeptidomes.

The peptidomes presented by the analyzed members of the B7 supertype showed different degrees of overlap, being in general higher among the subtypes of the same allele group. However, the most similar peptidomes were those from HLA-B*55:02 and -B*56:01. Both alleles contain Val152, a position described to affect peptide selection at the internal positions [40,41], which could explain the lower overlap with the peptidome presented by HLA-B*55:01, which carries Glu152. HLA-B*55 and -B*56, together with HLA-B*54, compose the broad serotype B22. Nevertheless, the peptidome presented by HLA-B*54:01 shows a relatively low overlap with those of HLA-B*55:01, -B*55:02 and -B*56:01. This could be explained by the polymorphisms Glu45Gly and Ile52Val in HLA-B*54:01 compared to -B*55 and -B*56.

The analyzed peptides come both from transfected cells expressing only one HLA-B allotype and from cells expressing different HLA-I molecules. In the last case the HLA-B molecule assignation was made based on algorithms. We cannot discard that the use of algorithms could introduce a bias in the assignation. Similarly, although the peptides were obtained by similar methodology, the large differences in the size of the peptidomes may have introduced some bias in the anchor motif, as it is expected that the peptide repertoires with a lower number of peptides are composed of the more abundant and with a higher affinity for the HLA-B molecule.

The presence of subpeptidomes among the members of the B7 supertype correlates with their ability to bind other residues than Pro2 at *P*2. By performing sequence alignments we were able to identify polymorphisms Glu45Thr and Tyr67Phe as important residues for the presence of subpeptidomes, except for HLA-B*54:01, that carry Gly45 and Tyr67. The crystal structures of HLA-B*07:02 show that Glu45 interacts with both Tyr67 and Asn63, a residue conserved among all the analyzed alleles. On the other hand, Thr45, present in the alleles that bind subpeptidomes, is not able to interact with Asn63 and Phe67, as shown by the structures of HLA-B*51:01 and –B*35:01. We propose that the interactions mediated by Glu45 reorient Asn63 and, overall, change the structure of the Pocket B, making it more rigid, limiting its ability to bind residues different than Pro. Additionally, peptide bonds involving Pro are rigid and can exist in the *cis*-configuration due to the cycled nature of this amino acid. This characteristic could modify the interactions of the *P*1 residue with the Pocket A when followed by Pro, altering the HLA specificities when binding ligands with Pro at *P*2 compared to other residues at that position. As described elsewhere [13], the crystal structures of HLA-B*51:01 bound to a ligand with Pro2 or with Ala2 showed that, when Ala is present at *P*2, the *P*1 residue can interact with Arg62 favoring the presence of acidic and polar residues at that position, while in ligands with Pro2 this interaction is impeded, favoring the presence of non-polar residues at *P*1. Previously, it was described that Endoplasmic Reticulum Aminopeptidase 1 (ERAP1) is responsible for the differential residue usage at *P*1 between the two subpeptidomes of HLA-B*51:01 [12]. ERAP1 trims peptides in the endoplasmic reticulum (ER) to the correct length so that they can be loaded onto the HLA molecules [42,43]. This process occurs with different efficiency depending on the nature of the *P*1 residue; moreover, ERAP1 is unable to trim peptide bonds involving Pro [44]. Thus, peptides that carry an ERAP1-susceptible residue at *P*1 followed by an amino acid other than Pro would be degraded by the enzyme. On the other hand peptides with ERAP1-resistant *P*1 residues, such as Asp, a residue predominant at *P*1 in the Ala2 subpeptidome, will remain in the ER long enough to bind the HLA molecule and be presented on the cell surface. Since ERAP1 cannot degrade the X-Pro bond, even peptides with ERAP1-susceptible residues at *P*1, such as aliphatic residues, will not be degraded by the enzyme when followed by Pro and will bind HLA-B*51:01. The peptidomes presented by cells carrying ERAP1 variants with different activity [40] and by cells lacking ERAP1 [45] support this mechanism but do not fully explain the different usage at *P*1 between subpeptidomes, suggesting that the HLA molecules themselves exert different binding selections among the subpeptidomes. The effects of ERAP1 activity on the peptidomes presented by the different members of the B7 supertype has not yet been characterized, but its role on shaping the different subpeptidomes of these alleles could be similar to the one described for HLA-B*51:01.

The predictive affinity to HLA of the Pro2 subpeptidome was systemically higher than that of the Ala2 subpeptidome across the different members of the B7 supertype. These data are in agreement with previous data on HLA-B51 allotypes [12]. In the case of HLA-B*07:02, the theoretical affinity of the total peptidome presented a higher affinity that the Ala2-containing subpeptidome. The other 5 allotypes analyzed, which presented subpeptidomes with acid or polar residues favored in *P*1 did not present this lower affinity in the Ala2-containing subpeptidome. Thus, although -B*07:02 can bind peptides with Ala2, the absence of polar or acidic residues at *P*1 may produce a lower affinity. We cannot discard that the presence of two subpeptidomes, one of them with more peptides than the other, may result in the underestimation of the affinity of peptides belonging to the less abundant subpeptidome.

In conclusion, this work indicates that the study of the peptidomes presented by an HLA-I molecules is complex, and that a thorough analysis is required, at least among the members of the B7 supertype, to consider differences between subpeptidomes. A detailed analysis of these subpeptidomes is necessary to understand the rules underlying HLA ligand selection and, consequently, to correctly predict which peptides have the potential to bind to and be presented by the different HLA alleles.

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CRediT authorship contribution statement

Adrián Tirado-Herranz: Conceptualization, Methodology, Data curation. **Pablo Guasp:** Conceptualization, Methodology, Data curation. **Alba Pastor-Moreno:** Methodology, Data curation. **María Area-Navarro:** Methodology, Data curation. **Iñaki Alvarez:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Validation, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2023.104707.

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