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Multi-modal Binding of a 'Self' Peptide by HLA-B*27:04 and B*27:05 Allelic Variants, but not B*27:09 or B*27:06 Variants: Fresh Support for Some Theories Explaining Differential Disease Association

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Abstract A self-derived-peptide with the same amino acid sequence (N-RRYLENGKETLQR-C) as residues 169–181 of the human leukocyte antigen (HLA) B27 heavy chain is known to bind to MHC Class I complexes containing the HLA-B27 heavy chain. This observation has been invoked previously in at least two different (but related) molecular explanations for the disease-association of the HLA-B27 allele. Here, we use a combination of fluorescence polarization, competitive inhibition and gel filtration chromatographic studies to show that a fluorescently-labeled peptide of the above sequence binds to two disease-associated subtypes of HLA-B27 (namely HLA-B*27:04 and HLA-B*27:05) but not to non-disease-associated subtypes (HLA-B*27:06 or HLA-B*27:09). This differential binding behavior is seen both in (a) peptide binding to complexes of heavy chain (HLA-B27) and light chain (β_2 microglobulin), and in (b) peptide binding to β_2 microglobulin-free heavy chains in the aggregated state. Such subtype-specific differences are not seen with two other control peptides known to bind to HLA-B27. Our results support the likelihood of differential peptide binding holding at least one of the keys to HLA-B27's disease association.

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Abbreviations

HLA	Human leukocyte antigen
HMW	High molecular weight
HC	Heavy chain
β_2m/beta-2m	β_2 Microglobulin

1 Introduction

The MHC class I molecule, HLA-B27 (human leukocyte antigen), is present on the surfaces of nucleated cells that express the protein as a trimolecular (or ternary) complex consisting of a heavy chain (the HLA-B27 HC itself), a light chain (known as β_2-microglobulin, or β_2m) and a small peptide which is generally 8–11 amino acids in length. The residues on the heavy chain comprising the lining of the peptide-binding pockets of the MHC class I complex are important determinants of the nature(s) of the peptides which bind to these pockets and become presented to cytotoxic T lymphocytes (CTLs) [1].

HLA-B27 is strongly associated with diseases characterized by inflammatory and auto-immune features (reviewed in [2]). Several of the current hypotheses attempting to explain the disease-association of HLA-B27 focus on misfolding of the heavy chain in the absence of β_2m. In particular, attention tends to get paid to heavy chain dimerization [3], or multimerization [2, 4], in the absence of β_2m.

In this paper, we explore differential binding of peptides by various forms of different allelic variants, or subtypes, of the HLA-B27 chain in complex with β_2m and in the

absence of β_2m. Our focus is primarily on two subtypes of HLA-B27, namely HLA-B*27:05 and B*27:09, which differ at a single residue. At residue position 116 in the heavy chain, Asp (D) in B*27:05 is replaced by His (H) in B*27:09. While B*27:05 is associated with disease, B*27:09 is largely non-disease-associated [5]. Our focus is also on two other related subtypes, HLA-B*27:04 and B*27:06, which differ in only two residue positions. His (H) at position 114 in B*27:04 is replaced by Asp (D) in B*27:06, and Asp (D) at position 116 in B*27:04 is replaced by Tyr (Y) in B*27:06. Once again, while HLA-B*27:04 is disease associated, HLA-B*27:06 is not. Thus, with the minor sequence differences outlined above, two subtypes are disease-associated and two are not.

Differences in disease association amongst HLA allelic variants may result from their binding to different peptides, or even to a specific peptide. We point out this possibility because peptide elution/sequencing experiments show that a peptide with the sequence 'RRYLENGKETLQR' is eluted from HLA-B*27:05 but not from HLA-B*27:09 or HLA-B*27:06 [6, 7]. The sequence of this eluted free peptide is identical to residues 169–181 of the HLA-B27 heavy chain itself, in the subtype, HLA-B*27:05. The same sequence is also present at comparable sequence locations in all of the three other subtypes being discussed here.

Since the peptide is eluted from two subtypes and not from the other two subtypes, reasonably it appears that either this peptide stretch is not released through natural proteolytic digestion of all four variants of HLA-B27 (to become available for binding), or that it is released through proteolysis but somehow able to bind only to two HLA-B27 variants, and not to the other two variants. It is important to establish whether the peptide, RRYLENGKETLQR, actually binds differentially to different subtypes, through actual binding experiments in vitro involving synthetic peptide and recombinant forms of chains of the different subtypes associated into ternary complexes and other associated forms (such as aggregates).

There are two proposed molecular explanations for disease-association which invoke the binding of the said self-derived (i.e., HLA-B27-derived) peptide stretch to HLA-B27-containing MHC Class I complexes (Fig. 1). One explanation is based on the finding mentioned already, i.e., that free peptides bearing the sequence 'N-RRYLENGKETLQR-C' have been purified from MHC Class I complexes present on the surfaces of some HLA-B27 expressing cells [6]. The second explanation is based on the proposal that within beta-2-microglobulin-free (β_2m-free) HLA-B27 chains which are retained on the cell surface after shedding of β_2m, there is survival of some native-like

structure, aided by intermolecular heavy-chain associations into soluble, or insoluble, aggregated forms [4]. It has been proposed that this survival of some internal structure following shedding of β_2m allows 'distorted' peptide-binding pockets to exist and remain capable of binding to residues 169–181 of either the same chain (through 'auto' self-binding and -display), or of other chains (through 'cross' self-binding and display). It may be noted that in the latter proposal, there is no proteolytic excision of residues 169–181 and rebinding of the same stretch of residues as an antigenic (free) peptide; instead, the binding actually causes chains to remain associated through 'cross' self-display involving binding of a section of each chain by the pocket of a neighbouring chain. This arrangement has also been proposed to help in the proteolytic survival of the resultant aggregate.

To reconcile the two models, i.e., (1) involving free peptide bound to MHC Class I pockets in regular (ternary) complexes, and (2) involving 'cross' display of chain sections bearing the same sequence as the free peptide, in soluble or insoluble aggregates, we have tried to find a common ground between them in this paper. We examined whether any of the four HLA-B27 subtypes bind to the peptide, 'N-RRYLENGKETLQR-C', with any sign of differential binding behavior. Our results suggest both the 'notional' possibility of cross self-display involving binding of the residue stretch RRYLENGKETLQR within aggregates of β_2m-free HLA-B27 chains, as well as the 'actual demonstration' of binding of the free peptide, 'N-RRYLENGKETLQR-C' to β_2m-bound HLA-B27 chains or β_2m-free aggregates.

We suggest, therefore, that it is not necessary for every β_2m-free chain within an aggregate to engage in cross-display. Since protein aggregation occurs through the occurrence of many different kinds of interactions, it is not necessary that every polypeptide chain exists in the same structural format. Thus, some chains could become incorporated within the aggregate in native-like structural format, with peptide binding pockets capable of binding to free peptides like N-RRYLENGKETLQR-C. At the same time, other chains in the aggregate could engage in self-display or cross-display of the sequence, RRYLENGKETLQR. At the same time, we suggest that it is not necessary for every β_2m-free chain to escape proteolysis, for the free peptide must be generated in vivo through such proteolysis. Therefore, some chains could generate the HLA-B27-derived peptide, N-RRYLENGKETLQR-C, making it available for binding to other chains, both within heavy-chain aggregates or dimeric states reported to form through misfolding and disulfide bonding as well as to normal β_2m-bound HLA-B27 chains with empty peptide-binding pockets [8, 9].
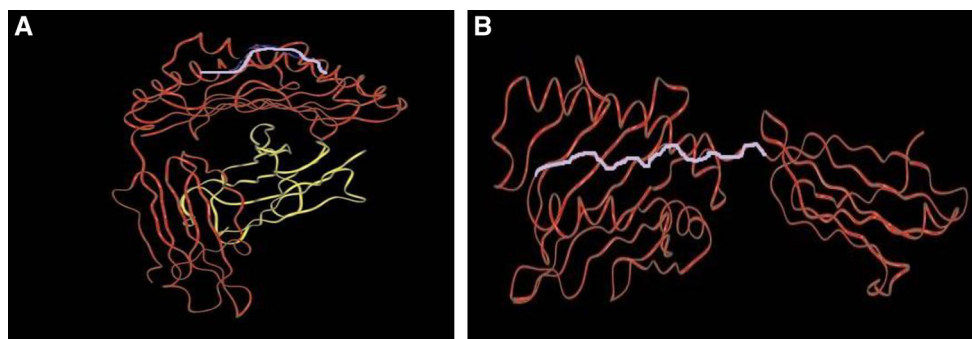
Fig. 1 Schematic diagrams illustrating the two molecular explanations for HLA-B27 disease association, invoking the binding of the self peptide (residues 169–181). **a** Represents the finding that free peptides bearing the sequence 'N-RRYLENGKETLQR-C' have been eluted and purified from MHC Class I complexes present on the surfaces of some HLA-B27 expressing cells. The figure shows the trimolecular complex of HLA heavy chain (*red*), β_2m (*yellow*) and the self peptide (*purple*). **b** Represents HLA-B27 chains which are retained on the cell surface after shedding of β_2m. These chains are proposed to contain 'distorted' peptide-binding pockets, capable of binding to residues 169–181 of either the same chain (through 'auto' display), or of other chains (through 'cross' display). In this panel, the bigger mass on the left represents the combined α1 and α2 domains, and the smaller mass on the right represents the α3 domain; the stretch of residues 169–181 is in *purple*, and shown to occupy the 'distorted' but native-like peptide-binding cleft (Color figure online)

2 Materials and Methods

2.1 Protein Expression

Plasmids pLM1-HLA-B*27:05 and pQE30-β_2m encoding the extracellular domain of HLA-B*27:05 (having 6X His tag at N-terminus) and β_2m respectively, described earlier [9], were used for the current work. HLA-B*27:04 was prepared by site directed mutagenesis using the primer sets: forward primers 5′-CCGAGAGAGCCTGCGGA CCC-3′, 5′-CGGCCCGTGAGGCGGAGCAG-3′, 5′-CTACCCTG GGGAGATCACAC-3′ and reverse primers 5′-GGGTCCG CAGGCTCTCTCGG-3′, 5′-CTGCTCCGCCTCACGGGC CG-3′, 5′-GTGTGATCTCCCCAGGGT AG-3′. HLA-B* 27:09 was generated using forward primer 5′-CCACCAG CACGCCTACGAC-3′ and reverse primer 5′-GTCGTAGG CGTGCTGGTGG-3′. HLA-B*27:06 was prepared from HLA-B*27:04 using forward primer 5′-CGCGGGTATGA CCAGTACGCCTACGACG-3′ and reverse primer 5′-CGT CGTAGGCGTACTGGTCATACC CGCG-3′. Proteins were expressed and purified as described [9].

2.2 Labeling of Peptides with Fluorescamine Dye

The labeling of peptides was done following the procedure of Dhaunta et al. [10], which ensures that labeling occurs only at the alpha amino group at the N-terminus and not at any of the epsilon amino groups present on the side chains of e.g., the residue lysine, through carrying-out of the labelling reaction at a pH of 6.0. The peptides were dissolved (2 mg/ml) in water. The stock solution of fluorescamine (1 mg/ml) was prepared in acetoniltrile (ACN). For peptide reactions with fluorescamine to occur at pH 6.0, the peptide solution was mixed with sodium citrate buffer (40 mM) in 1:1 (v/v) ratio. The peptides were mixed with fluorescamine dye in 1:50 molar ratio, at room temperature for 3 h. The conjugation was confirmed by MALDI-MS.

2.3 Formation of Complexes of HLA (B*27:04, B*27:05, B*27:06 and B*27:09) with β_2m and Peptide

The B27 HC, β_2m and fluorescent peptides were taken in concentrations of 1 μM, 3 μM and 50 nM, concentrations, respectively, in refolding buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA, 0.1 mM CHAPS, 5.0 mM oxidized glutathione, 0.5 mM reduced glutathione, 0.5 mM PMSF, pH 8.0) and incubated for 16–18 h at 25 °C with constant stirring as described earlier [11]. Three peptides (fluorescamine conjugated) were used for studies of complex formation: (1) HIV-1 gag peptide (KRWIIMGLNK), (2) m9 peptide (GRFAAAIAK) and (3) self-peptide (RRYLENGKETLQR).

2.4 Gel Filtration Chromatography

Gel filtration was performed on an AKTA protein purification system (GE Healthcare system). Superdex-200 column (bed volume: 24 ml) was equilibrated with 20 mM Tris, 150 mM NaCl (pH 8.0) at a flow rate of 0.7 ml/min. 100 μl of sample was loaded onto the column and the elution volumes were transformed into molecular weight data, based on the elution profiles of the molecular weight standards run on the same column. The fluorescence of eluted fractions was monitored at a wavelength of 465 nm, with an excitation wavelength of 390 nm.

2.5 Fluorescence Polarization Studies

The association of peptides with B*27:04, B*27:05, B*27:06 and B*27:09 subtypes (in solution) in the presence of β_2m, was determined by measuring fluorescence polarization or anisotropy of the emission using excitation and the emission wavelength of 390, and 465 nm, respectively, using slit widths of 5 nm, at 25 °C, on a HORIBA Fluoromax spectrofluorimeter fitted with a fluorimetric monochromator attachment. Polarization measurements were made at different time points (up to 12 h), following the incubation of the proteins with the peptide [11].

2.6 Peptide Binding Competition Experiments

For competition experiments, B27 HC, β_2m and fluorescently labeled M9 peptide were incubated in concentrations of 1, 2 μM and 50 nM, respectively, in the presence of varying concentrations of unlabeled self-peptide, gag peptide or M9 peptide (in the range of 10 nM–250 μM) in refolding buffer, for 24 h at 10 °C, with continuous shaking. Fluorescence polarization (FP) was measured as described above, in triplicate. Data was analyzed using ORIGIN 8.0 software; non-linear regression analysis was done, using the dose response model.

3 Results

The recombinant heavy chains (HC) of the extracellular domains of B*27:04, B*27:05, B*27:06, B*27:09 (1 μM), β_2m polypeptide (3 μM), and fluorescamine-labeled peptides (50 nM) were mixed in the refolding buffer to generate trimeric complexes. Fluorescamine, an amino group-reactive fluorescent dye (excitation wavelength maximum of 390 nm; emission wavelength maximum of 465 nm)

was used to monitor the binding of peptides to the complex by two methods: fluorescence polarization and gel filtration chromatography, combined with competition experiments.

3.1 Binding of Peptides Monitored by Fluorescence Polarization

The formation of the ternary complex of HC, β_2m and fluorescent peptide was monitored by recording the fluorescence polarization of the dye conjugated to the peptide at different time points (up to 12 h), with excitation involving 390 nm light, using a slit width of 5 nm, at 25 °C. We monitored the binding of three peptides, gag peptide, m9 peptide and self-peptide, independently, to four different HLA-B27 subtypes B*27:04, B*27:05, B*27:06 and B*27:09. The gag peptide (N-KRWIIMGLNK-C) and m9 peptide (N-GRFAAAIAK-C) served as controls, since their binding to these B27 subtypes is well-documented [12, 13]. The gag peptide (N-KRWIIMGLNK-C) showed a significant increase in fluorescence polarization, with time, in the case of B*27:04, B*27:05 and B*27:06, as shown in Fig. 2a, suggesting that it forms a complex with β_2m-bound forms of these heavy-chain HLA-B27 subtypes. However, the increase in fluorescence polarization was significantly lower for B*27:09, suggesting that the gag peptide binds poorly to this B27 subtype.

The m9 peptide (N-GRFAAAIAK-C) was found to bind equally well to all four subtypes, with significant increase in fluorescence polarization, suggesting efficient formation of trimeric complexes of HC-β_2m-m9 peptide with all four subtypes (Fig. 2b).

In the case of the self-peptide, N-RRYLENGKETLQR-C, as seen in Fig. 2c, there was a significant increase in fluorescence polarization with time, in the case of only the B*27:04 and B*27:05 subtypes, suggesting complex formation of the peptide with HC and β_2m for these subtypes;
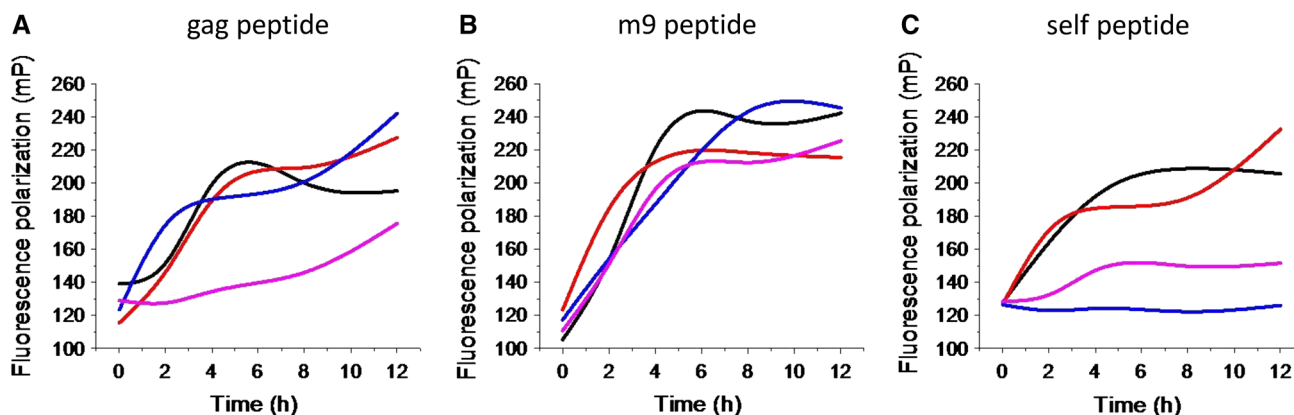

Fig. 2 Fluorescence polarization studies to evaluate binding of labeled peptides to HLA-B*27:04 (*black*), B*27:05 (*red*), B*27:06 (*blue*) and B*27:09 (*magenta*) subtypes in the presence of β_2m. **a** gag peptide (KRWIIMGLNK), **b** m9 peptide (GRFAAAIAK), **c** self-peptide (RRYLENGKETLQR) (Color figure online)

Fig. 3 Dose response curves for competitive binding of labeled M9 peptide versus unlabeled M9 peptide (*red, filled circles*), gag peptide (*blue, filled triangles*) and self peptide (*black, filled squares*), measured by recording fluorescence polarization values. The experiment was done in triplicate; representative image is shown. *mP* indicates millipolarization (Color figure online)
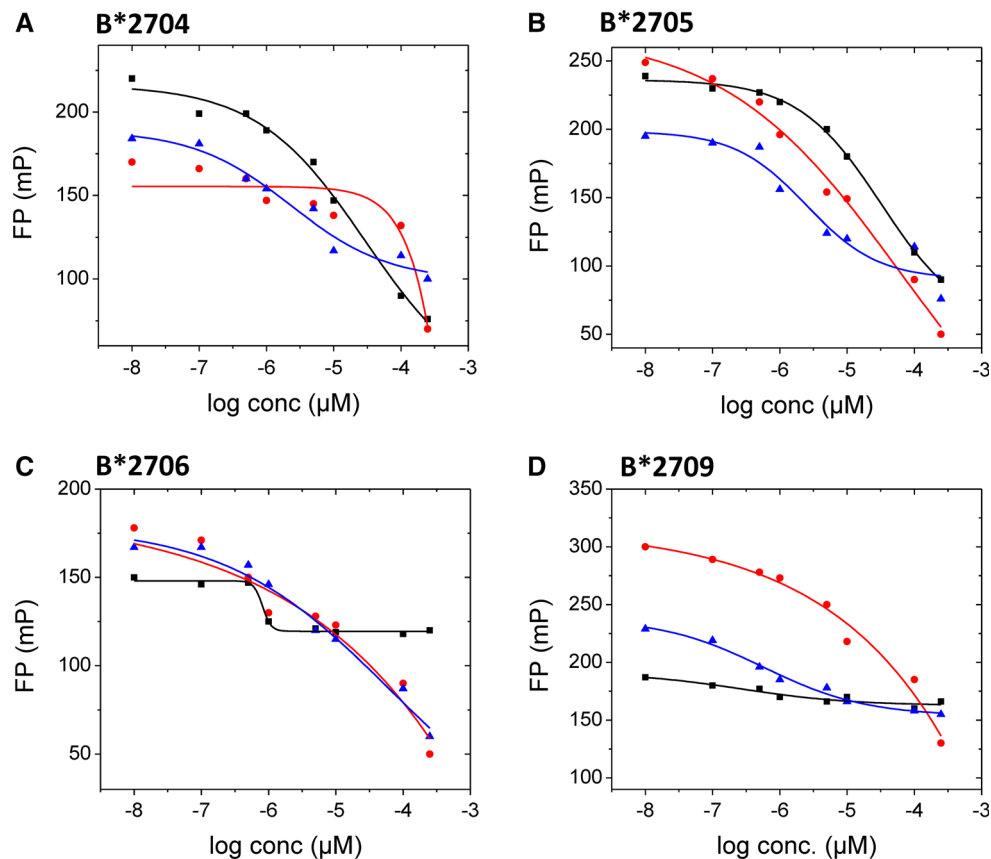
in contrast, in the case of the B*27:09 and B*27:06 subtypes, there was very negligible increase in fluorescence polarization, suggesting that the self-peptide binds very poorly to B*27:09 and B*27:06.

For determining the specificity of peptide binding, fixed concentrations of heavy chain, β_2m and labeled peptide (M9) were incubated in the presence of the unlabeled (competitor peptides) for all four subtypes, followed by determination of FP values after 24 h. Figure 3 shows the plot of FP values against total concentration of competitor. The dose-dependent decrease in FP value with competitor concentration is observed for all peptides binding to all four subtypes, except in the case of binding of unlabeled self-peptide to B*27:06 and B*27:09. This reflects the specificity of the nature of binding, and further reconfirms the fact that the self peptide does not bind to B*27:06 and B*27:09 subtypes of HLA-B27.

3.2 Binding of Peptides Monitored by Gel Filtration Chromatography

For each of the four subtypes, refolding of the heavy chain (HC), β_2m and peptide was carried out together to form ternary complexes. Gel filtration chromatography was performed on a Superdex-200 gel filtration column, to examine

the status of formation of complexes. As seen in Supplementary Fig. 1A, in which protein elution is signified by an increase in absorbance at 280 nm, the high molecular weight or HMW aggregates/oligomers corresponding to molecular weights of 670 and 180 kDa, elute at 8 and 11 ml, respectively. The ternary complex of HC, β_2m and peptide (48 kDa) elutes at a volume of 15.5 ml, while the monomeric HC (34 kDa) population which has escaped aggregation and HMW formation, elutes at 16.4 ml. The protein eluting as HMW species was further examined through denaturing SDS-PAGE gel electrophoresis under different sample-loading conditions, followed by western blotting with HC-10 (an antibody that recognizes β_2m-free heavy chains). When only SDS is present in the sample-loading buffer (SLB) lacking β-mercaptoethanol (BME), increased amounts of HMW species are evident (Supplementary Fig. 1B), which resolve to give rise to monomeric species alone, upon inclusion of BME in the SLB (Supplementary Fig. 1C). When SDS is lacking from both the SLB and the tank buffer used for electrophoresis, but when BME is present in the SLB, however, the HMW species do not enter the gel at all (data not shown). These results show that although disulfide-bonded species exist within HMW aggregates, non-covalent forces are also important in the formation of the HMW aggregates.

Now, we describe gel filtration chromatographic data obtained using fluorescamine-labeled peptides (with elution of the labeled peptide being monitored through emission readings at 465 nm, based on absorption/excitation at 390 nm). Figure 4a shows the gel filtration data for complex formation of gag peptide with B*27:04, B*27:05, B*27:06 and B*27:09 subtypes. This data may be viewed in light of the gel filtration and gel electrophoresis data already presented in the supplementary data, and described in the paragraphs above. The fraction collected at 15.5 ml, corresponding to the molecular weight of 48 kDa, showed significant increase in fluorescence intensity, suggesting the formation of ternary complexes of B*27:04, B*27:05 and B*27:06 subtypes with the gag peptide. In the case of B*27:09, no ternary complex appeared to have formed in the presence of the gag peptide. The HMW aggregates/oligomeric forms of HC of B*27:09 showed binding to gag peptide (with increase in fluorescence intensity in the fractions collected between 8 and 10 ml), suggesting that the peptide-binding pocket of the β_2m-bound form is not able to accommodate the gag peptide in B*27:09, although the peptide-binding pocket of misfolded/oligomeric form can accommodate the gag peptide.

Figure 4b shows gel filtration data for complex formation of the m9 peptide with β_2m and HC of each of the four subtypes. Increase in fluorescence intensity was observed

in the fractions collected at 15.5 ml in all the four subtypes of HLA-B27 used in this study, suggesting that B*27:04, B*27:05, B*27:06 and B*27:09 are all able to form the ternary complex with m9 peptide. In addition to this, all the four subtypes also showed an increase in fluorescence intensity in the fractions collected in the range of 10–11 ml, corresponding to a molecular weight of 310–420 kDa (approximating the sum of molecular weight of between 9 and 12 HC molecules) suggesting that chains within the HMW aggregates/oligomeric forms of the HC, retain to some extent the native conformation of the peptide binding pocket, thereby allowing the HC to accommodate the m9 peptide in all four subtypes.

Figure 4c shows gel filtration data for complex formation by the self-peptide used in this study, i.e., residues 169–181 of the HLA-B27 chain (N-RRYLENGKETLQR-C). The self-peptide also formed ternary complexes with β_2m and HC of B*27:04 and B*27:05 subtypes, with elution at 15.5 ml showing significant increase in fluorescence intensity. An increase in fluorescence intensity was also observed in the fraction collected at $\sim$11–12 ml, corresponding to molecular weight of 200–320 kDa in B*27:05. With B*27:09 as well as B*27:06, no increase in fluorescence intensity was observed in the fraction collected at 15.5 ml, suggesting that these subtypes were not able to form ternary complexes with the self-peptide. The HMW

Fig. 4 Gel filtration chromatography using Superdex 200 column, to assess the formation of hetero-trimeric complex/oligomers/high molecular weight (HMW) species formed by HLA-B*27:04 (black), B*27:05 (red), B*27:06 (blue) and B*27:09 (magenta) subtypes in the presence of β_2m and fluorescamine-labeled, a gag peptide (KRWIIMGLNK), b m9 peptide (GRFAAAIAK), c self-peptide (RRYLENGKETLQR). The fluorescence intensity of eluted fractions was plotted as a function of elution volume (excitation 390 nm, emission 465 nm). d elution of peaks 1, 2, 3 and 4 represent elution of thyroglobulin, gamma globulin, ovalbumin and myoglobin respectively, monitored by absorbance at 280 nm (Color figure online)
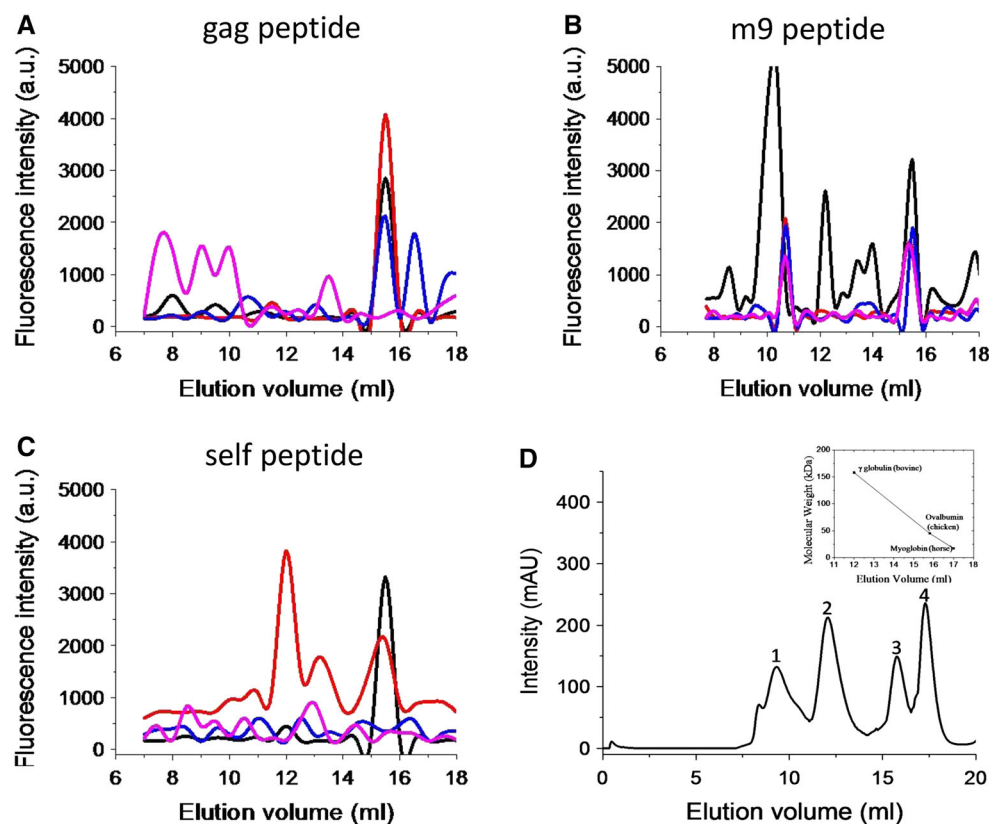
aggregates/oligomeric forms of the HC of these two sub-types also showed very negligible binding to the self-peptide, suggesting that the peptide-binding pocket of the native form, as well as of the misfolded/oligomeric form, is not able to accommodate the self-peptide in these two subtypes of HLA-B27.

4 Discussion

Peptide presentation by HLA with autoimmune consequences is known for non-HLA-B27 Class I risk modifiers, including HLA-DQ in the context of myasthenia gravis, where the peptide presented is similar to a section of the acetylcholine receptor, AChR [14], and HLA-B60 in the context of spondyloarthropathies [2, 15, 16]. In the case of HLA-B27, there are several hypotheses proposed to explain its strong association with spondyloarthropathies [17]. These include hypothesis concerning (1) arthritogenic peptides [18], (2) the slow folding kinetics associated with misfolding/aggregation tendency of HLA-B27, leading to the unfolded protein response [19, 20], (3) the ability of HLA-B27 to form disulfide bonded homodimers [8, 21–23], (4) the possibility of amyloid formation by surface-shed β_2m [24], (5) a role played by a specific arthritogenic peptide (involving a self-peptide) [6], and (6) the possibility of misfolding of the heavy chains (resulting in the formation of HMW species) involving the binding of a stretch of self-peptide [4].

A quick review of the above hypotheses reveals that three of them (hypotheses 1, 5 and 6) hold related, distinct, non-mutually exclusive points of view involving the binding of free peptides, or sections of polypeptide chain(s), by native, or misfolded, HLA-B27. Two of them, i.e., hypotheses 5 and 6, involve the exact same peptide sequence, RRYLENGKETLQR; as a free proteolytically-derived peptide in hypothesis 5, and as a part of the HLA-B27 chain in hypothesis 6. In this paper, we use this peptide and two additional control peptides known to bind to HLA-B27, as well as recombinant heavy-chains of four HLA-B27 subtypes in the β_2m-free and β_2m-bound states. We show that various peptides bind with apparently different efficiencies to HLA-B27 chains in different forms, with some peptides capable of competing out other peptides, and yet other peptides displaying no binding at all to certain HLA-B27 subtypes. In particular, the association of the self-peptide with the sequence, RRYLENGKETLQR, is seen with subtypes B*27:04 and B*27:05 but not with subtypes B*27:06 and B*27:09, in both β_2m-free and β_2m-bound states. This provides some insight into disease-association and the possibly important role played by this peptide. It has been previously noted that most HLA heavy chains are unstable in the absence of bound β_2m and

become degraded [25]; B*27:05 has been suggested to be an exception, escaping such degradation and retaining some native-like structure and stability after β_2m shedding [9]. Therefore, in B*27:06 and B*27:09, the difference between subtypes could involve non-survival of the heavy chain, survival of the heavy chain without formation of a peptide-binding pocket capable of binding to RRY-LENGKETLQR, or formation of a pocket incapable of binding of the self-derived peptide. We cannot distinguish between these possibilities. In B*27:04 and B*27:05, the binding of the self-peptide stretch could explain how this elicits autoimmune mechanisms. Before concluding, it is imperative that we also point out the potential caveats and flaws in this work. We have naively assumed that the binding of the fluorescently-labeled peptides occurs only to the peptide-binding pockets whereas it remains possible that at least some binding involves non-specific adsorption. Also, we do not specifically examine whether there is only a small 'subset' of normally structured heavy chains in the aggregates which engages in the binding of the gag, m9 and self-peptides.

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