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Direct binding to antigen-coated beads refines the specificity and cross-reactivity of four monoclonal antibodies that recognize polymorphic epitopes of HLA class I molecules

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Abstract

Monoclonal antibodies with specificity for HLA class I determinants of HLA were originally characterized using serological assays in which the targets were cells expressing 3-6 HLA class I variants. Because of this complexity, the specificities of the antibodies were defined indirectly by correlation. Here we use a direct binding assay, in which the targets are synthetic beads coated with one of 111 HLA class I variants, representing the full range of HLA-A, -B and -C variation. We studied one monoclonal antibody with monomorphic specificity (W6/32) and four with polymorphic specificity (MA2.1, PA2.1, BB7.2 and BB7.1) and compared the results with those obtained previously. W6/32 reacted with all HLA class I variants. MA2.1 exhibits high specificity for HLA-A*02, -B*57 and -B*58, but also exhibited cross-reactivity with HLA-A*11 and -B*15:16. At low concentration (1μg/ml) PA2.1 and BB7.2 were both specific for HLA-A*02 and -A*69, and at high concentration (50μg/ml) exhibited significant cross-reactions with HLA-A*68, -A*23, and -A*24. BB7.1 exhibits specificity for HLA-B*07 and -B*42, as previously described, but reacts equally well with HLA-B*81, a rare allotype defined some 16 years after the description of BB7.1. The results obtained with cell-based and bead-based assays are consistent and, in combination with amino acid sequence comparison, increase understanding of the polymorphic epitopes recognized by the MA2.1, PA2.1, BB7.2 and BB7.1 antibodies. Comparison of two overlapping but distinctive bead sets from two sources gave similar results, but the overall levels of binding were significantly different. Several weaker reactions were observed with only one of the bead sets.

Keywords

HLA class I; monoclonal antibodies; epitope; polymorphism

Introduction

Since first being reported in 1978[1], monoclonal antibodies with specificity for HLA class I molecules have been invaluable tools for both basic and clinical research in human immunology. These antibodies can be divided into two groups according to the types of epitope they recognize[2]. Monomorphic antibodies, such as W6/32, the antibody described by Barnstable et al (1), recognize monomorphic determinants that are common to all HLA class I variants, whereas polymorphic antibodies recognize determinants carried by a subset

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of such variants. Well-studied examples of polymorphic antibodies are PA2.1, BB7.1, BB7.2 and MA2.1. Originally, PA2.1 and BB7.2 were seen to be specific for HLA-A2[2–4], but with more extensive characterization they were also shown to recognize and define HLA-A*69, a variant that is a recombinant of HLA-A*02 and HLA-A*68[5]. In a similar fashion, BB7.1 was originally seen to be specific for HLA-B*07[2] but was subsequently shown to recognize HLA-B*42[6], a recombinant of HLA-B*07 and HLA-B*08[7] that is characteristic of African populations[8]. MA2.1, which was originally described as recognizing HLA-A2 and HLA-B17 antigens[9], has been shown to react with both the B*57 and B*58 components of the HLA-B17[10], but no additional reactivities have been reported.

In large part, the HLA class I specificity of monoclonal antibodies has been determined using panels of cells each of which minimally expresses one HLA-A, one HLA-B and one HLA-C allotype and more commonly express two allotypes for HLA-A, -B and -C. This complexity means that binding reactions cannot be directly attributed to particular HLA class I variants but must be inferred through various types of correlation. As a consequence, there are limitations in the extent to which data can be interpreted and thus in the resolution and accuracy of the data. An initial approach to address these limitations was the use of mutant cell lines that lacked endogenous HLA class I expression and could be transfected to express a single HLA class I allotype of choice[11]. A more recent approach has been to replace cells as the target antigen with synthetic beads each of which is coated with a single HLA class I allotype[12,13]. Elimination of cells from the assay facilitated the commercial development of panels of >90 different beads that provide a representation of the range of HLA-A, B and C diversity. In using such beads to determine the HLA class I specificities of Fc-fusion proteins made from killer-cell immunoglobulin-like receptors (KIR), we have achieved results of higher resolution that are more reproducible and insightful than was possible with cell-based assays[14,15]. Here we have reexamined the HLA class I specificities of the MA2.1, PA2.1, BB7.2 and BB7.1 monoclonal antibodies using two panels of beads coated with HLA class I molecules.

Materials and Methods

Binding of MA2.1, PA2.1, BB7.2 and BB7.1 antibodies to beads, each coated with a representative range of HLA-A, HLA-B and HLA-C allotypes was assessed in a multiplex assay on the Luminex platform (Austin, TX). The bead panels tested were (a) LabScreen single-antigen beads (One Lambda, Canoga Park, CA) and (b) LifeCodes single-antigen beads (Gen-Probe, San Diego, CA). Antibodies (1μg/ml and 50μg/ml) were incubated with each set of beads for 60 mins at 4°C, washed four times and then labeled with anti-mouse Fc antibody conjugated with phycoerythrin and incubated for a further 60 mins at 4°C. The fluorescent intensity and identification of individual labels of the beads were visualized on a Luminex 100 reader. In each experiment data was collected from a minimum of 100 antigen-coated beads for each combination of bead and monoclonal antibody. The results presented are the mean fluorescence relative to the fluorescence intensity obtained with the W6/32 antibody obtained from three replicate experiments performed for each monoclonal antibody.

Results

Figure 1 provides a distillation of the results obtained from previous work using cell-based assays to study the HLA class I specificities of the MA2.1, PA2.1, BB7.2 and BB7.1 mouse monoclonal antibodies and the epitopes of HLA class I molecules that these antibodies recognize. In this study, these four antibodies were tested for binding to beads coated with single HLA-A, -B or -C allotypes. Two sets of commercially available beads (from One Lambda and Gen-Probe) were tested and compared. Together the two bead sets allowed us

to test a total of 111 HLA class I allotypes (33 HLA-A, 66 HLA-B and 21 HLA-C allotypes). The HLA class I allotypes represented in each bead set are shown in Figure 2A.

The assay used to measure the binding of the monoclonal antibodies is essentially the same as the one that we designed and have used for measuring the binding of KIR-Fc fusion products to beads coated with HLA class I[14]. In this assay the binding of the antibodies recognizing polymorphic epitopes was normalized to that of the W6/32 monoclonal antibody that recognizes an epitope common to all HLA class I epitopes[1,16]. This normalization corrects for differences in the absolute amount of HLA class I on the various beads. As measured by the binding of W6/32, the amount of HLA class I on the beads varied with allotypes, but a more general property was that the One Lambda Labscreen beads consistently bound more W6/32 than the Gen-Probe LifeCodes beads (Figure 2B). Gen-Probe beads showed a mean reduction in W6/32 binding of 54% (range 35–69%), 52% (range 20–71%) and 50% (range 23–75%) for HLA-A, -B and -C allotypes respectively as compared to One Lambda beads.

Specificity of the MA2.1 monoclonal antibody

The MA2.1 antibody bound strongly to the six HLA-A*02 subtypes tested (A*02:01, :02, :03, :05 and :06) and also to HLA-B*57:01, B*57:03 and B*58:01 (corresponding to the serological HLA-B17 antigen) (Figure 3A). These results are consistent with the previously defined specificity of MA2.1 for the HLA-A2 and -B17 antigens[9]. In addition, we observed a weak reactivity of MA2.1 with HLA-B*15:16 and an even weaker one with HLA-A*11. These weak cross-reactions were detected only with the One Lambda beads.

Results from several groups of investigators are consistent with the GETR motif at residues 62–65 in the helix of the α_1 domains of HLA-A*02, B*57 and B*58 being critical for forming the epitope recognized by MA2.1 (Figure 1). Within this motif, glycine 62 is the only residue that is not found in any of the other 89 HLA-A, -B and -C allotypes tested. HLA-B*15:16 and HLA-A*11 differ only at position 62, having the RETR and QETR motifs respectively, which correlates with them having some avidity for MA2.1. The replacement of glycine 62 with arginine in B*15:16 is seen to be more favorable for binding MA2.1 than the glutamine residue at this position in A*11 (Figure 3B). Other HLA class I allotypes having the QETR motif (e.g HLA-A*03, A*30 and A*36) are not recognized by MA2.1, suggesting that further residues, away from this motif in the α_1 domain also influence recognition of HLA class I by MA2.1.

In addition to the GETR motif at residues 62–65 in the α_1 domain, mutation at several positions in the α_2 domain (149, 152, 170, 174, 175 and 181) has been shown to influence MA2.1 binding[17–19], as has the residue at position 8 in the peptide bound to HLA-A2[20] (Figure 3C). These influences could involve direct contact with the antibody or be a consequence of indirect effects that alter the conformation of residues 62–65.

Specificity of the PA2.1 and BB7.2 monoclonal antibodies

When used at a concentration of 1μg/ml the PA2.1 and BB7.2 antibodies gave similarly strong reactions with five subtypes of HLA-A*02 and with HLA-A*69:01 (Figure 4A). For each of the these reactions the Gen-Probe beads gave weaker reactions than the One Lambda beads, the difference being greatest for HLA-A*02:01, as was also the case for MA2.1 (Figure 3A). These results are consistent with those obtained with the W6/32 antibody (Figure 2B).

When a much higher concentration of antibody was used (50μg/ml), cross-reactions of the PA2.1 and BB7.2 antibodies were observed with HLA-A*23:01, A*24:02, A*24:03, A*68:01, A*68:02 and A*69:01 (Figure 4B). These cross-reactions correspond well with the

cross-reactivity first described in the 1960s[21] between the serological A2, A28 (A*68 and A*69) and A9 (A*23 and A*24) antigens and more recently described for cell-binding assays with the I-145 monoclonal antibody[22]. These cross-reactions were stronger for BB7.2 than PA2.1, a feature observed for both sets of HLA class I coated beads. The cross-reactivity was much stronger for A*24:03 than either A*24:02 or A*23:01. This must be due to the two substitutions that distinguish A*24:03 from both A*24:02 and A*23:01[23] and which are at positions 166 and 167 in the α_2 domain (Figure 3C). Consistent with the relative binding to PA2.1 and BB7.2, HLA-A*24:03 shares the glutamate 166, tryptophan 167 motif with HLA-A*02, whereas HLA-A*24:02 and A*23:01 share the aspartate 166, glycine 167 motif with HLA-A*01:01, which binds neither PA2.1 nor BB7.2.

The combination of sequence comparisons[2,4,5,10] and site-directed mutagenesis[24–26] has shown that tryptophan 107 in the α_2 domain is a critical factor in forming the epitope recognized by PA2.1 and BB7.2 (Figure 4B, Figure 1). In addition, mutations at positions 161, 163, 166, 167 and 169 can lead to loss of binding by PA2.1 and BB7.2, showing that these residues also contribute to forming the epitope[19,26] (Figure 4C).

Specificity of the BB7.1 monoclonal antibody

As previously reported, we find that BB7.1 reacts with HLA-B*07:02 and HLA-B*42:01 (Figure 5A). In addition we found that HLA-B*81:01, an African allotype[27,28], also reacts strongly with BB7.1, which fits with both HLA-B*81:01, B*42:01 and B*0701 having α_1 domains with identical sequence (Figure 5B) and residues 63–70 of the α_1 domain being critical for the BB7.1 epitope[2,6,29–34] (Figure 1). Comparably strong reactions for HLA-B*07:02, B*42:01 and B*81:01 were observed with the One Lambda beads, but the binding of BB7.1 to the Gen-Probe B*42:01 bead was relatively weak. Even weaker was the binding to the B*07:03 bead, which may reflect an inherent difference between B*07:02 and B*07:03 in their avidities for BB7.1. That B*07:03 differs from B*07:02 by non-conservative substitutions at positions 69, 70 and 71 is also evidence that favors this interpretation[35].

Both HLA-B*56:01 and B*82:01 have α_1 domains with identical sequence to that of HLA-B*07:02, B*42:01 and B*81:01 but these allotypes are not bound by BB7.1 suggesting that residues in the α_2 domain abrogate their recognition. HLA-B*07:02, B*42:01 and B*81:01 encode arginine at position 131 whereas B*56:01 and B*82:01 encode serine at this position. Similarly, both B*56:01 and B*82:01 encode leucine at position 163 whereas HLA-B*07:02 and B*81:01 both encode glutamic acid. Interestingly, HLA-B*42:01 encodes a threonine at position 131 which may explain its weaker recognition by BB7.1 as detected on both the One Lambda and Gen-Probe beads. That BB7.1 interacts with residues in the α_2 domain is further supported by the observation that mutation at positions 166 and 169 of HLA-B*07 eliminates the BB7.1 epitope[36].

Therefore, whilst the α_1 domain residues between positions 63–71 likely form the dominant epitope for recognition by BB7.1, binding is influenced by polymorphism in the α_2 domain, suggesting that this antibody binds both the α_1 and α_2 regions of HLA class I. Given that this footprint would span the peptide binding cleft, peptide variability is likely to influence BB7.1 recognition of HLA class I.

In summary, we find that the patterns of antibody reactivity observed here are entirely consistent with those obtained previously, and extend those results by being able to detect and distinguish reactions and cross-reactions in a quantitative manner. All the observed reactions are with HLA-A and -B variants, with no cross-reactivity for HLA-C further emphasizing the distinctive properties of HLA-C[37].

Discussion

Consistent with previous studies we showed that MA2.1 bound to HLA-A*02 subtypes and to HLA-B*57 and HLA-B*58 allotypes[9,26]. Experimental data and sequence analysis suggest that the α_1 residues GETR at positions 62–65 are critical in forming the epitope recognized by MA2.1[26,38]. In this study we showed weak interactions with MA2.1 are also formed when glycine at position 62 is substituted for arginine, as in HLA-B*15:16 and glutamine as in HLA-A*11:02. This finding contrasts with results of a cellular assay in which substitution of glycine for arginine at position 62 abrogated recognition of HLA-A*02 by MA2.1[26]. HLA-B*15:16 has a high degree of sequence homology with HLA-B*57 which appears sufficient to confer reactivity with MA2.1 despite the introduction of a residue apparently less favorable for recognition by MA2.1 at position 62. That glutamine at position 62 results in weak binding to HLA-A*11 and no detectable binding to other HLA-A allotypes with glutamine at this position (e.g HLA-A*03, A*30 and A*36) would suggest that this residue is less favorable for binding than both glycine and arginine. An alternative explanation is that the range of peptides presented by either HLA-B*15:16 or HLA-A*11 collectively form an epitope with a high affinity for MA2.1, sufficient to overcome the reduction in affinity incurred by the presence of either arginine or glutamine at position 62.

Several lines of evidence suggest that peptide variability influences recognition of HLA class I by MA2.1. Although the residues critical for formation of the epitope recognized by MA2.1 are located in the α_1 domain, mutational experiments indicate that the binding footprint extends to residues in the α_2 domain of the HLA class I molecule[17,18]. This is supported by the finding that MA2.1 recognition of HLA-A2 is prevented in a competitive binding assay with PA2.1[10], an antibody whose epitope is formed exclusively by residues in the α_2 domain. Together these studies suggest that MA2.1 binds to an area spanning the peptide binding groove on the face of HLA class I molecule. Direct evidence of the effect of peptide variability is provided from the observation that MA2.1 recognizes HLA-A2 complexed with the HIV-1 p17 epitope (SLYNTVATL) at least 30 times more strongly than all other complexes studied and this enhanced reactivity was sensitive to point mutation of threonine to alanine at position 8 in the peptide[20]. Therefore there exist two possible explanations for the ability of the bound peptide to influence MA2.1 reactivity. Either MA2.1 is sensitive to peptide induced conformational changes of the helices, or it directly contacts certain peptides in the groove of HLA class I. The identity of the bound peptides presented by HLA class I on beads manufactured by One Lambda and Gen-Probe is unknown but presumed to be highly heterogeneous.

The weaker interactions between MA2.1 and HLA-B*15:16 and A*11:02 were evident only in the One Lambda LabScreen beadset. Similarly, weak interactions between BB7.1 and HLA-B*48:01 and B*67:01 were identified on the One Lambda beads but these interactions were not observed in the Gen-Probe beadset. Our data and that of others suggests that the residues critical for formation of the epitope recognized by BB7.1 are found in the α_1 and α_2 domains suggesting that, like MA2.1, the identity of the peptide might play a role in the recognition of HLA class I by this antibody. Therefore, the weak interactions observed with BB7.1 might reflect the different peptide pools found in the One Lambda and Gen-Probe beadsets with those from One Lambda collectively having higher binding affinity than those found in the Gen-Probe beadset. Such weak interactions were not evident for either PA2.1 or BB7.2, antibodies thought to recognize an epitope formed exclusively by residues found in the α_2 domain and as such they are unlikely to be subject to the same changes in peptide pool that we have hypothesized to influence recognition of MA2.1 and BB7.1.

An alternative explanation for the lower affinity of MA2.1 and BB7.1 for HLA class I allotypes presented on the Gen-Probe beadset is the lower antigen density present on these

beads. We have shown that the antigen density present on the Gen-Probe beadset is approximately 50% of that present on the One Lambda beadset as evidenced by binding of the monomorphic HLA class I antibody W6/32. As a result the weak interactions noted on the One Lambda beadset might not reach the lower level of detection of the assay with the Gen-Probe beadset.

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	Result	Reference		
MA2.1 PA2.1 BB7.2	PA2.1 is specific for HLA-A*02 and does not fix complement	1978	Parham and Bodmer	(3)
	BB7.2 has the same specificity as PA2.1 and does not fix complement	1979	Brodsky *et al.*	(2)
	MA2.1 is specific for an epitope shared by HLA-A*02 and B17 (HLA-B*57 and B*58) and does not fix complement	1980	McMichael *et al.*	(9)
	BB7.2 and PA2.1 recognize HLA-A*69 as well as HLA-A*02	1981	Parham and Brodsky	(4)
	The epitopes recognized by MA2.1 and PA2.1/BB7.2 overlap	1983	Ways and Parham	(10)
	Mutation at position 161 abrogates the epitope recognized by PA2.1 and BB7.2	1983	Taketani *et al.*	(19)
	MA2.1 binds HLA-A*02:01, A*02:02, A*02:03, B*57:01 and B*58:01 with similar affinity, which is 100-fold higher than the low affinities of PA2.1 and BB7.2	1983	Ways and Parham	(10)
	The HLA-A*69:01 sequence combines the α_1 domain of A*68:01 with the α_2 domain of A*02 and locates the PA2.1/BB7.2 epitope to the α_2 domain	1985	Holmes and Parham	(5)
	The HLA-B*58:01 sequence locates the MA2.1 epitope to residues 62-65 of the α_1 domain	1986	Ways *et al.*	(38)
	Substituting tryptophan at position 107 in HLA-A*02:01 eliminates the epitope recognized by PA2.1 and BB7.2	1987	Salter *et al.*	(24)
	Substituting glycine for tryptophan at position 107 in HLA-A*02:01 eliminates the PA2.1 & BB7.2 epitope, mutations at positions 62, 63, 65 & 66 eliminates the MA2.1 epitope	1988	Santos-Aguando *et al.*	(25)
	Mutations at positions 149 or 152 in HLA-A*02:01 reduce MA2.1 binding	1989	Hogan *et al.*	(17)
	Correlation between the specificities of mouse monoclonal antibodies and human alloantibodies reacting with HLA-A*02	1990	Fuller *et al.*	(34)
	Mutations at positions 170, 174, 175 & 181 of HLA-A*02 reduce MA2.1 binding	1991	Lombardi *et al.*	(18)
	Mutation at position 62 of HLA-A*02 eliminates the MA2.1 epitope, mutations at positions 107, 162 and 169 eliminates the PA2.1/BB7.2 epitope	1992	Hogan and Brown	(26)
	The peptides bound by HLA-A*02 can alter its avidity for MA2.1 by ~30 fold. This enhanced avidity is sensitive to the residue at peptide position 8	1995	Barouch *et al.*	(20)
BB7.1	BB7.1 is specific for HLA-B*07	1979	Brodsky *et al.*	(2)
	The epitope recognized by BB7.1 does not overlap with the Bw6 epitope	1983	Parham	(31)
	Sequence comparison of Bw4+ HLA-A and -B allotypes locates the Bw6 and Bw4 epitopes to positions 79-83 in the α_1 domain	1986	Wan *et al.*	(33)
	BB7.1 also recognizes the characteristically African allotype HLA-B*42	1986	Parham *et al.*	(6)
	The HLA-B*42:01 sequence combines the α_1 domain of HLA-B*07 with the α_2 domain of HLA-B*08 and locates the BB7.1 epitope to the α_1 domain	1988	Parham *et al.*	(7)
	Recombination mutants between B7 and B27 map the BB7.1 epitope to positions 63, 67 & 70 and the Bw6 epitope to positions 82 & 83	1988	Toubert *et al.*	(32)
	Correlation between the specificities of mouse monoclonal antibodies and human alloantibodies reacting with HLA-B*07	1990	Fuller *et al.*	(34)
	Replacing residues 65-80 of HLA-A*02 with the the HLA-B*07 counterparts does not introduce the BB7.1 epitope	1991	Domenech *et al.*	(30)
	Mutation at positions 166 and 169 in the α_2 domain of HLA-B*07 eliminates the BB7.1 epitope	1992	McCutcheon and Lutz	(36)
	Natural and mutant recombinants show tyrosine 67 in HLA-B*07 is critical in forming the BB7.1 epitope	1993	McCutcheon *et al.*	(29)

Figure 1.

Summary of studies investigating the monomorphic antibodies MA2.1, PA2.1, BB7.2 (top panel) and BB7.1 (bottom panel). Listed is the main finding from each study, the study's author and year of publication. The specific reference as listed in the current publication is noted to the right.

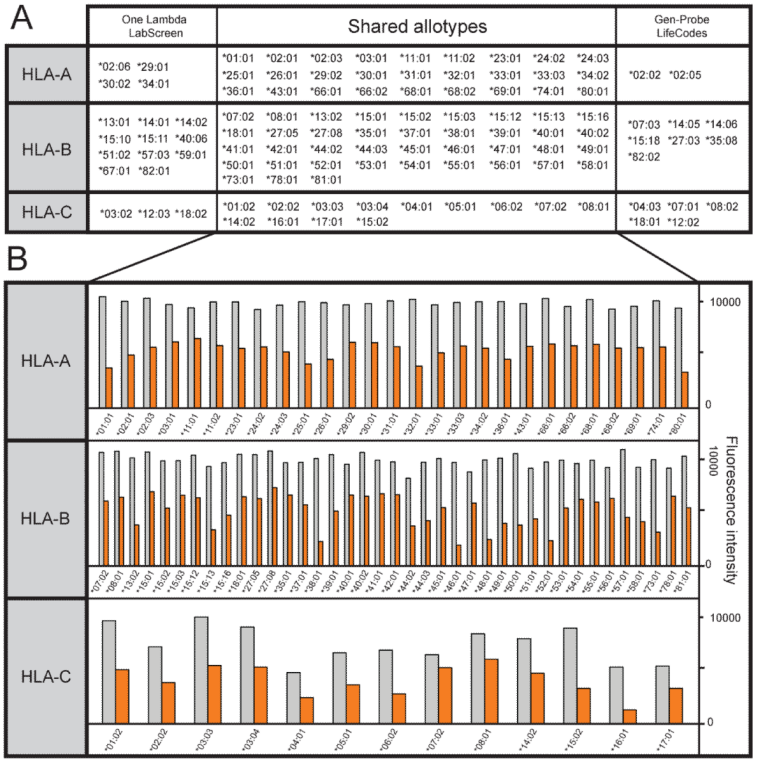

Figure 2.

(A) HLA class I allotypes represented by the One Lambda Labscreen and Gen-Probe LifeCodes beadsets. (B) Binding of the monomorphic HLA class I antibody W6/32 to beads from One Lambda LabScreen (grey bars) and Gen-Probe LifeCodes (orange bars). The allotypes shown are those common to both beadsets.

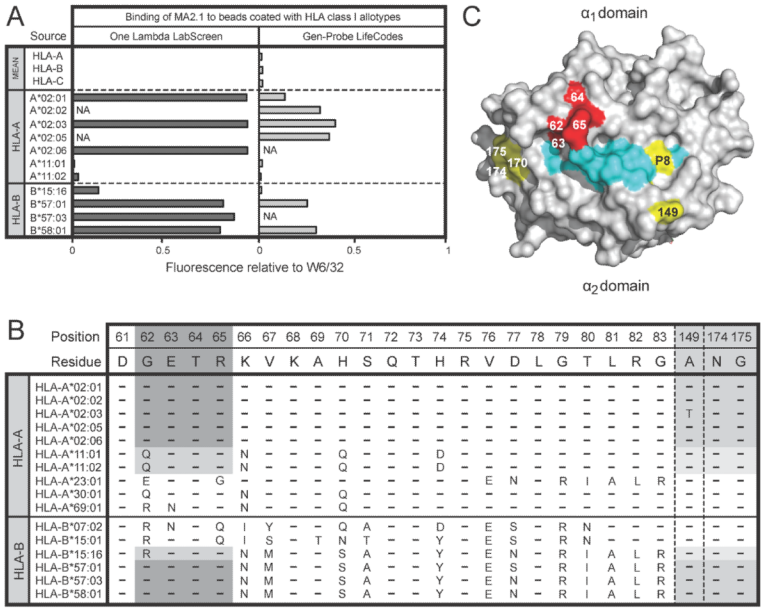

Figure 3.

(A) Binding of MA2.1 (1μg/ml) to beads coated with HLA class I allotypes from the One Lambda LabScreen (left panel) and Gen-Probe LifeCodes (right panel) beadsets. (B) Alignment of HLA class I allotypes showing selected residues in the α1 and α2 domains. Residues from allotypes that form the epitope recognized by MA2.1 are shaded in grey. (C) Space-filling model of the binding surface of HLA-A*02 (grey) with associated peptide (cyan). Residues highlighted in yellow fall within the footprint recognized by MA2.1. Residues 62–65 are critical for formation of the epitope recognized by MA2.1 and are highlighted in red.

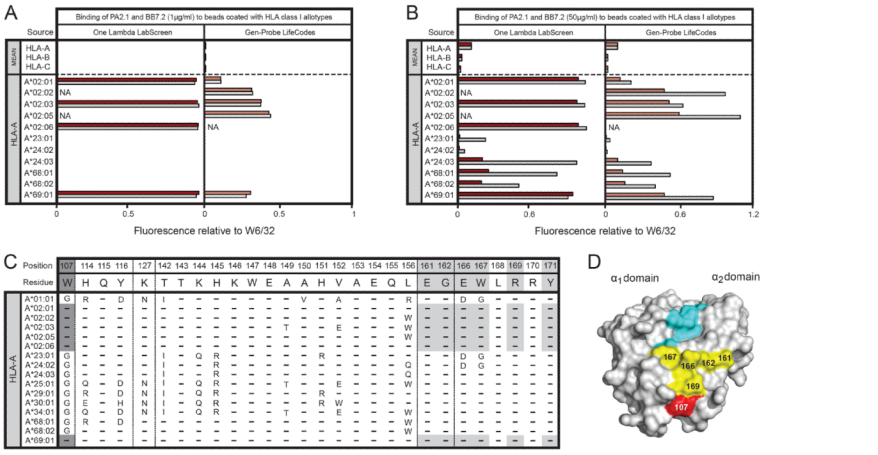

Figure 4.

(A) Binding of PA2.1 (1μg/ml) and BB7.2 (1μg/ml) to beads coated with HLA class I allotypes from the One Lambda LabScreen (left panel) and Gen-Probe LifeCodes (right panel) beadsets. (B) Binding of PA2.1 (50μg/ml) and BB7.2 (50μg/ml) to beads coated with HLA class I allotypes from the One Lambda LabScreen (left panel) and Gen-Probe LifeCodes (right panel) beadsets. (C) Alignment of HLA class I allotypes showing selected residues in the α2 domain. Residues from allotypes that form the epitope recognized by PA2.1 and BB7.2 are shaded in grey. (D) Space-filling model of HLA-A*02 (grey) with associated peptide (cyan). Residues highlighted in yellow fall within the footprint recognized by PA2.1 and BB7.2. Tryptophan at position 107 is considered critical for formation of the epitope recognized by PA2.1 and BB7.2 and is highlighted in red.

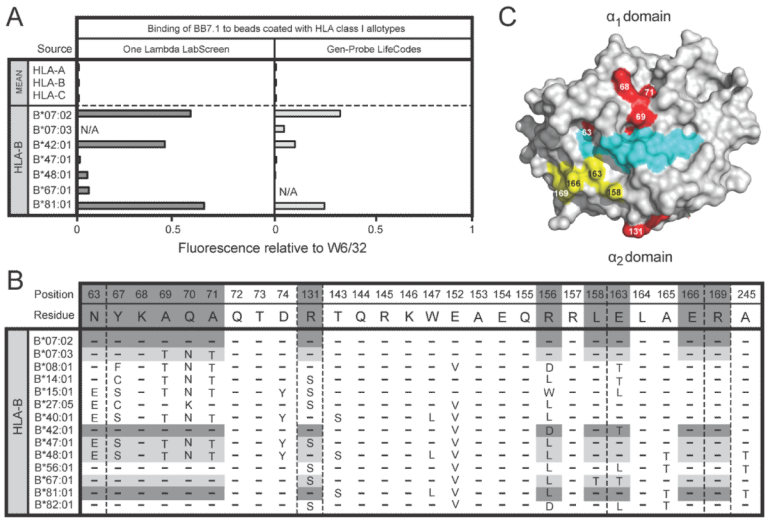

Figure 5.

(A) Binding of BB7.1 (1μg/ml) to beads coated with HLA class I allotypes from the One Lambda LabScreen (left panel) and Gen-Probe LifeCodes (right panel) beadsets. (B) Alignment of HLA class I allotypes showing selected residues in the α1 and α2 domains. Residues from allotypes that form the epitope recognized by BB7.1 are shaded in grey. (C) Space-filling model of the binding surface of HLA-B*07 (grey) with associated peptide (cyan). Residues highlighted in yellow fall within the footprint recognized by BB7.1. Residues 63–71 in the a1 domain and position 131 in the α2 domain are critical for formation of the epitope recognized by BB7.1 and are highlighted in red.