

# Conformational Variants of the Individual HLA-I Antigens on Luminex Single Antigen Beads Used in Monitoring HLA Antibodies: Problems and Solutions

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**Background.** Single antigen beads (SAB) are used for monitoring HLA antibodies in pretransplant and posttransplant patients despite the discrepancy between virtual and actual crossmatch results and transplant outcomes. This discrepancy can be attributed to the presence of conformational variants of HLA-I on SAB, assessment of which would increase the concordance between SAB and flow cytometry crossmatch (FCXM) results, thus enabling improved organ accessibility for the waiting list patients and a better prediction of antibody-mediated rejection. **Methods.** The conformational variants were examined on HLA-I beads, iBeads, acid-/alkali-treated beads, and T cells using HLA-I monoclonal antibodies (W6/32, TFL-006, and heavy chain (HC)-10). **Results.** The affinity of the monoclonal antibodies against HLA-I beads confirmed the presence and heterogeneous density of peptide-associated  $\beta$ 2-microglobulin-associated HLA HC (pepA- $\beta$ 2aHC), peptide-free- $\beta$ 2aHC (pepF- $\beta$ 2aHC), and  $\beta$ 2-free HC ( $\beta$ 2fHC) on every single antigen-coated bead. In contrast, iBeads harbor a high density of pepA- $\beta$ 2aHC, low density of pepF- $\beta$ 2aHC, and are lacking  $\beta$ 2fHC. The FCXM analyses confirmed the prevalence of pepA- $\beta$ 2aHC, but not pepF- $\beta$ 2aHC or  $\beta$ 2fHC on resting T cells. **Conclusions.** The strength of a donor-specific antibody should be assessed with a bead-specific mean fluorescence intensity cutoff based on TFL-006 reactivity against HLA-I beads, and HC-10 against iBeads, where the  $\beta$ 2fHC or pepF- $\beta$ 2aHC normalized donor-specific antibody level would reveal the true anti-pepA- $\beta$ 2aHC reactivity associated with positive FCXM.

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HLA-I and HLA-II antibodies are monitored with a multitude of techniques, including Luminex multiplex HLA-coated single antigen beads (SAB), complement-dependent

cytotoxicity (CDC), and flow cytometry (FC) crossmatching (XM) for preexisting and de novo donor-specific antibodies (DSA) in transplant patients.<sup>1,2</sup> Although increased sensitivity and accuracy of SAB is beneficial for clinical monitoring of HLA antibodies and for performing virtual XMs to improve organ allocation on highly sensitized patients,<sup>3–5</sup> CDCXM are more reliable and have higher specificity in predicting acute antibody-mediated rejection (AMR).<sup>6</sup> The increased sensitivity of SAB has denied transplants to a growing number of sensitized patients on waiting lists, due to the inability to distinguish between clinically relevant and irrelevant HLA antibodies.<sup>7</sup>

HLA-I SAB are coated with both  $\beta$ 2-microglobulin ( $\beta$ 2m)-associated HLA heavy chain ( $\beta$ 2aHC) and  $\beta$ 2m-free HLA heavy chain ( $\beta$ 2fHC).<sup>8,9</sup> Anti- $\beta$ 2fHC HLA-I antibodies were identified in nonalloimmunized men and in cord blood.<sup>8,10</sup> The prevalence of anti- $\beta$ 2fHC antibodies in HLA-I-sensitized patients awaiting a donor kidney was 39% (which accounted for 6% of the HLA-I antibodies detected), these antibodies did not correlate with a positive FCXM<sup>11</sup> because the  $\beta$ 2aHC variant is the most prevalent HLA-I expressed on normal cells, tissues, and donor organs. Anti- $\beta$ 2fHC DSA are found in 20% of renal allograft recipients, but only anti- $\beta$ 2aHC DSAs were predictive of graft failure.<sup>12</sup> iBeads, devoid of  $\beta$ 2fHC, detected mostly anti- $\beta$ 2aHC.<sup>13</sup> Using iBeads in conjunction with HLA-I and acid-treated beads, the prevalence of anti- $\beta$ 2fHC DSA was found to be 12%, which least affected renal allograft survival.<sup>9</sup> In another cohort, 11% of recipients had anti- $\beta$ 2fHC DSA. None of the patients with only anti- $\beta$ 2fHC DSA displayed positive T-cell FCXMs, nor did

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<sup>†</sup> Professor Paul Ichiro Terasaki passed away on January 25, 2016.

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V.J. has no conflicts of interest to disclose. M.H.R. and the late P.I.T., as coinventors, have filed US and European patent applications on HLA-I polyreactive anti-HLA-E mAbs, which include mAb TFL-006.

P.I. initiated this project, discussed its plan and offered advice during the early phases of this investigation. V.J. designed and carried out all the experiments using 3 different monoclonal antibodies and 4 different single-antigen beads, analyzed the data in comparison with previous publications, prepared the tables and figures, wrote the first draft of the article meticulously. M.H.R., based on discussion with the third author, initiated the investigation on the use the 3 monoclonal antibodies on Luminex single antigen beads and on T cells to elucidate variations within a single HLA allele, examined the data, discussed periodically with the first author and contributed to the writing and editing of the article. The late P.I.T. initiated a discussion with the second author about the importance of defining the variations of individual HLA antigens on the Bead using the monoclonal antibody developed at Terasaki Foundation Laboratory (TFL-006) along with mAbs W6/32 and heavy chain (HC)-10. During early phases of data collection, he discussed with the second author highlighting the importance of iBeads for monitoring sera of pre and posttransplant patients' sera.

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they develop acute AMR in the first year or suffer deleterious effects during prolonged graft survival.<sup>14</sup>

Antibodies to both  $\beta$ 2aHC and  $\beta$ 2fHC HLA-I variants are indiscriminately considered unacceptable for organ allocation, despite their differential immunologic risk. Unless the conformational variants for each one of the HLA-I antigens represented in the SAB's panel are characterized and their relative density assessed, neither the DSA "strength" (determined by mean fluorescence intensity [MFI]) nor the MFI cutoff (used to correlate DSA "strength" to AMR) may distinguish the pathogenic DSAs that recognize  $\beta$ 2aHC expressed by the allograft in vivo.

The distribution and density of HLA-I conformational variants which may appear on HLA-I beads, iBeads and acid-treated beads may be revealed by measuring the relative binding of well-characterized anti-HLA-I monoclonal antibodies (mAbs) to these variants. The results of this investigation will determine the reactivity of SAB against anti- $\beta$ 2aHC or  $\beta$ 2fHC DSAs and will validate whether SAB other than conventional HLA-I beads are needed for distinguishing pathogenic from nonpathogenic HLA-I DSA.

## MATERIALS AND METHODS

### Monoclonal Antibodies

W6/32 (IgG2a) reacted with a wide range of cells (except for Daudi Burkitt lymphoma, lacking  $\beta$ 2aHCs). It also immunoprecipitated both a 43kDa HLA heavy chain (HC) and a 12-kDa  $\beta$ 2m.<sup>15</sup> W6/32 formed immune complexes with  $\beta$ 2aHC but not with  $\beta$ 2fHC<sup>16</sup> and bound to both peptide-associated (pepA)  $\beta$ 2aHC and peptide-free (pepF)  $\beta$ 2aHC.<sup>17,18</sup> We obtained W6/32 from One Lambda (Canoga Park, CA).

HC-10, (IgG2a) developed by immunization with the  $\beta$ 2fHC of HLA-B7 and HLA-B40,<sup>19</sup> immunoprecipitated  $\beta$ 2fHC from the cell lysates. The epitope of HC-10 is identified between amino acid positions 57 and 62 of the HLA  $\alpha$ 1 HC; arginine at position 62 (R<sup>62</sup>) is crucial for HC-10 binding.<sup>20</sup> HC-10 repeatedly immunoprecipitated a small fraction of  $\beta$ 2aHC from cell lysate.<sup>21</sup> HC-10 recognized cell surface  $\beta$ 2aHC devoid of a peptide (pepF- $\beta$ 2aHC), whereas the presence of peptide reduced the HC-10 reactivity.<sup>22</sup> HC-10 was purchased from Nordic MUBio (Susteren, Netherlands, Cat MUB2037P).

TFL-006 (IgG2a) was developed by immunizing a properly folded HLA-E  $\beta$ 2fHC.<sup>23-25</sup> TFL-006 bound to  $\beta$ 2fHC of both HLA-E and HLA-Ia and was inhibited by peptides from the amino acid sequences shared by all HLA-I antigen-coated beads and masked by  $\beta$ 2m. TFL-006 was IgG-purified from ascites fluids.

### SAB Luminex-Based Immunoassay

The HLA-I reactivity of the aforementioned mAbs was screened using LabScreen SAB multiplex Luminex Flow cytometry.<sup>2</sup> The SAB used are: (i) HLA-I beads (Cat LS1A04, Lot 8 and Lot 9, One Lambda); (ii) iBeads (One Lambda)<sup>23</sup>; (iii) acid-treated beads, generated by incubating the HLA-I beads in 0.1 M glycine buffer (pH = 2.8) with 1% bovine serum albumin for 30 minutes, then washed thrice with LabScreen Wash Buffer<sup>9,11,14</sup>; (iv) alkali-treated beads, generated by incubating the HLA-I beads in 50 mM Tris-HCl (pH = 8.0) with 6 M guanidium chloride for 30 minutes, then washed thrice as previously described.<sup>26</sup>

All SAB kits covered the same panel of HLA-I molecules (31 HLA-A, 50 HLA-B, and 16 HLA-Cw antigens). The mAbs

were tested on the same day and the same tray for all 4 SAB sets. Although all mAbs were titrated (10, 5, 2.5  $\mu$ g/mL) to assess the optimal concentration saturating the SAB, only the data at 10  $\mu$ g/mL is presented.

The Luminex immunoassay involved incubation of 20  $\mu$ L of mAbs with 2  $\mu$ L of SAB for 30 minutes at room temperature. The SAB were then washed thrice with LabScreen Wash Buffer. The mAbs binding were monitored using phycoerythrin-conjugated Goat Anti-Mouse IgG<sub>2a</sub> ( $\gamma$ <sub>2a</sub> chain specific; Concentration: 0.5 mg/mL; Cat 1080-09; SouthernBiotech, Birmingham, AL), diluted 1 to 100 in wash buffer. The SAB were incubated with 50  $\mu$ L of the second antibody for 30 minutes at room temperature. The SAB were washed thrice as previously described, then resuspended in 1  $\times$  phosphate buffered saline before acquisition. For each sample, at least 100 beads were counted. The IgG reactivity against each HLA-I antigens were recorded as Trimmed MFI. The MFI values were normalized against the negative control bead (bead 1) and sample (1  $\times$  PBS). The MFI cutoff used for a positive reaction was 1000.

### T-Cell FCXM Assay

On the day of the FCXM assay, 40 mL of whole blood from a healthy male (HLA-I typing: A\*11:01/A\*24:02, B\*15:10/B\*44:05, Cw\*02:02/Cw\*07:04, using LABType SSO Typing Test Kit (One Lambda, following manufacturer's instructions) was collected after obtaining consent and institutional review committee approval. T cells were isolated using Dynabeads (Invitrogen by Life Technologies, Carlsbad, CA, Cat 11344D), following manufacturer's negative selection protocol.

The purified T cells were aliquoted ( $2 \times 10^5$  cells) to a U-bottom 96-well tissue culture plate and washed thrice with serum-free AIM-V Medium (Life Technologies-Gibco, Cat 12055-083) with 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH = 7.4). Before staining, the Fc receptors were blocked using Human TruStain FcX (BioLegend, San Diego, CA, Cat 422302). Seventy microliters of primary mAb (W6/32 or TFL-006 or HC-10; concentration, 0.357  $\mu$ g/mL), were added to the T cells and incubated for 30 minutes (4°C, on a shaker). Then, the cells were washed thrice and stained with 50  $\mu$ L of fluorescein isothiocyanate-conjugated goat antimouse IgG (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, Cat. 115-095-003; Concentration, 1.5 mg/mL) diluted 1 to 800. The plates were incubated for 30 minutes (4°C, on a shaker), and after washing, the cells were stained with 50  $\mu$ L of serum-free AIM-V medium with 1% HEPES, containing 2.5  $\mu$ L PE-conjugated antihuman CD4 (BioLegend, Cat 300539) and 2.5  $\mu$ L peridinin-chlorophyll protein-conjugated antihuman CD8 (BioLegend, Cat 344708). After incubating and washing as previously described, the cells were resuspended in 90  $\mu$ L of 1  $\times$  PBS, then acquired with the BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). The cells were analyzed using BD Accuri C6 software, version 1.0.264.21. The median channel shift demarcates the immune-affinity of the mAbs for HLA-I antigens.

### Statistical Analysis

Statistical analyses were performed using STATA 13. All data were tested for normality using the Shapiro-Wilk, and Shapiro-Francia tests. Analysis for significance was performed using the Mann-Whitney *U* and Kruskal-Wallis tests. Two-tailed *P* values less than 0.05 were considered significant.

**RESULTS**

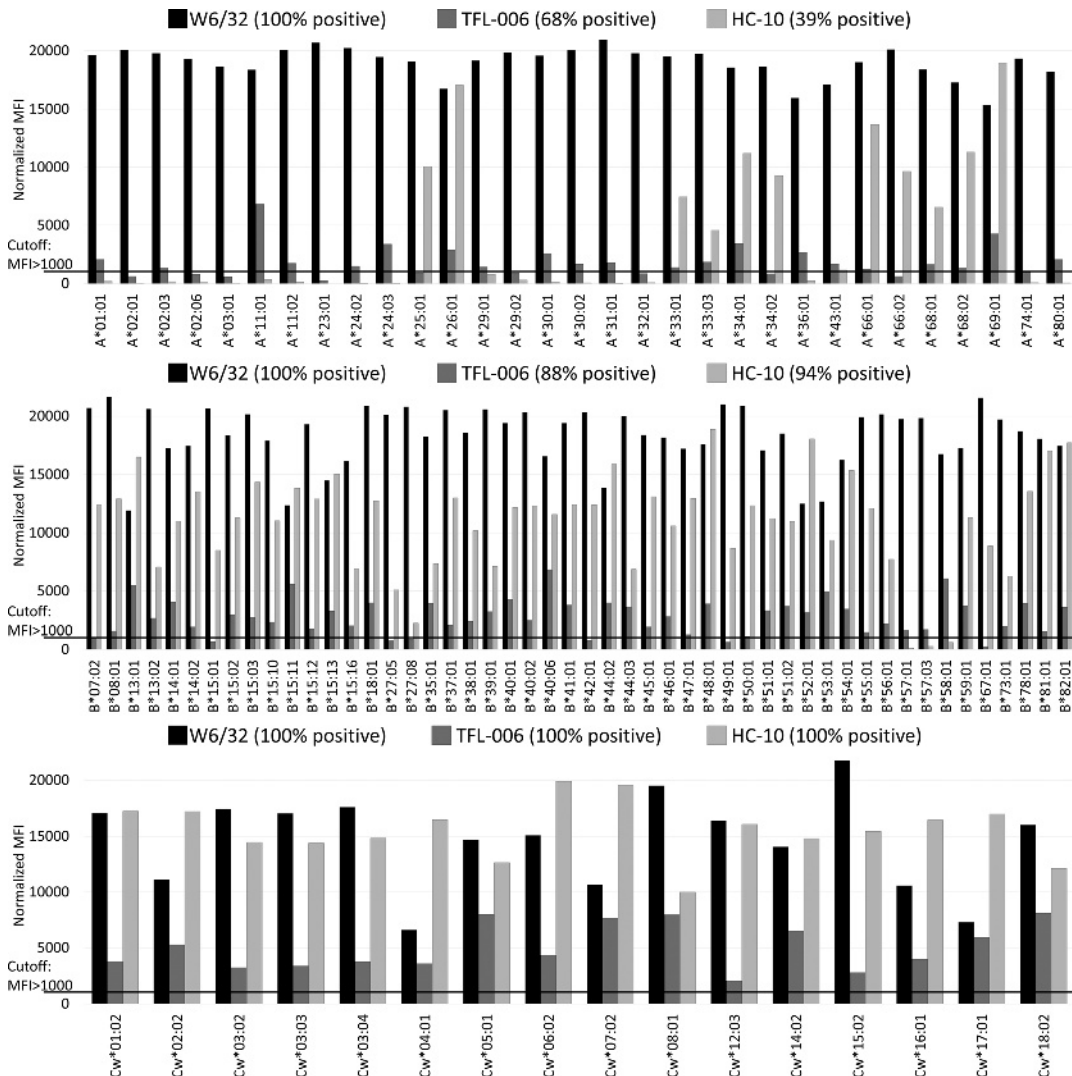
**HLA-I Reactivity of the mAbs Against Different SAB**

On HLA-I beads (Figure 1), W6/32 binds to 100% of HLA-A, HLA-B, and HLA-Cw antigens with MFI ranges: 15 357-20 956, 11 923-21 644, 6609-21 785, respectively, confirming the heterogeneous density of  $\beta$ 2aHC of the different HLA-I antigens. TFL-006 binds to 68% of HLA-A, 88% of HLA-B, and 100% of HLA-Cw antigens with MFI ranges: 283-6811, 255-6843, 2041-8129, respectively, indicating the heterogeneous density of  $\beta$ 2fHC on HLA-I beads. Some HLA-A and -B antigen-coated beads are devoid of  $\beta$ 2fHC. HC-10 binds to 39% of HLA-A, 94% of HLA-B, and 100% of HLA-Cw antigens with MFI ranges: 0-18 962, 114-18 868, 10 002-19 918, respectively, showing the heterogeneous density of  $\beta$ 2fHC on HLA-I beads, whereas higher reactivity of HC-10 compared with TFL-006 suggests the presence pepF- $\beta$ 2aHC.

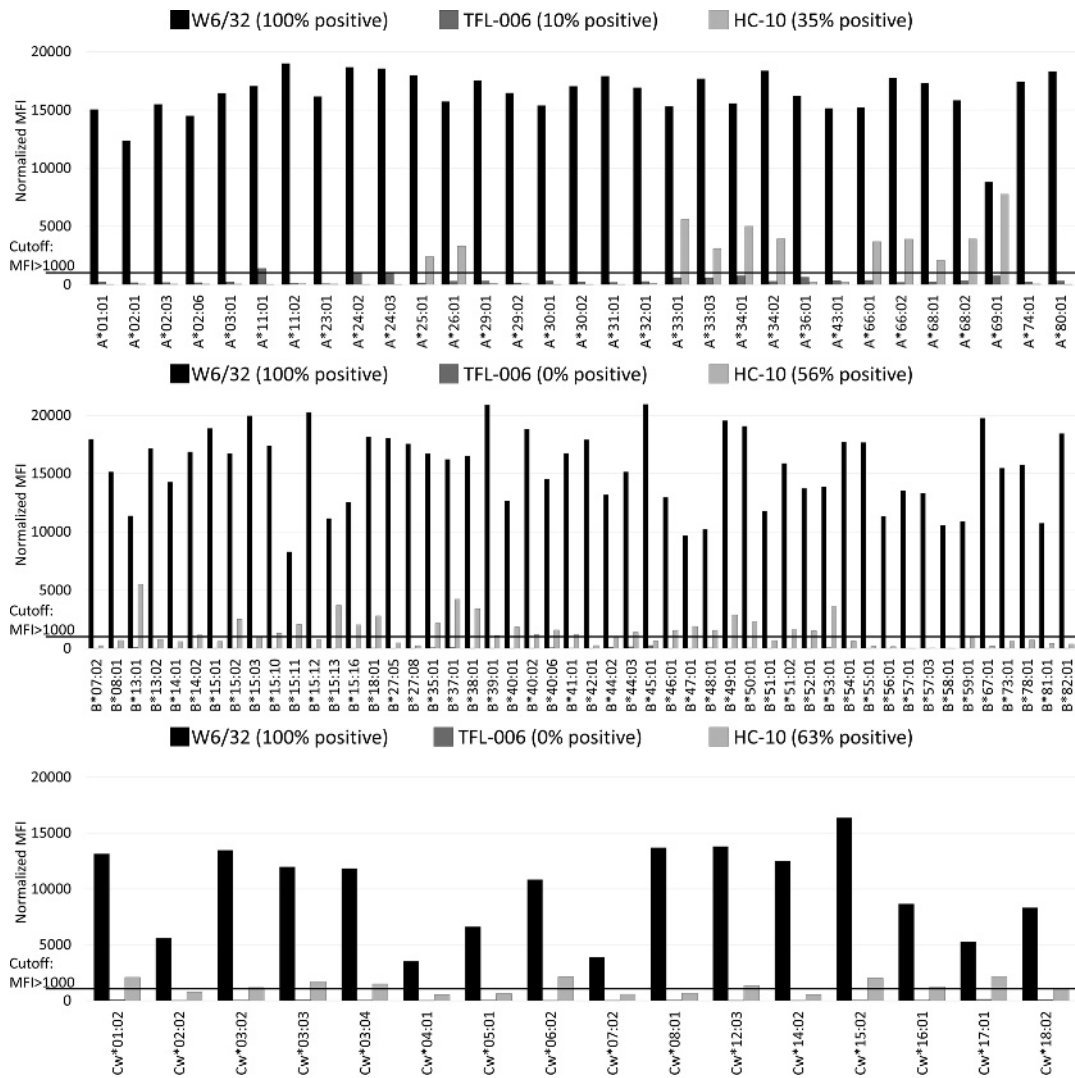
On iBeads (Figure 2), W6/32 binds to 100% of HLA-A, HLA-B, and HLA-Cw antigens with MFI ranges: 8811-19 006,

8314-20 958, 3544-16 377, respectively, confirming the heterogeneous density of  $\beta$ 2aHC of the different HLA-I antigens in iBeads. In contrast, TFL-006 poorly recognized HLA-A (10%; MFI range, 66-1400), and failed to recognize HLA-B and HLA-Cw (0%; MFI range, 3-251, 8-118, respectively) antigens, confirming the absence of  $\beta$ 2fHC on all HLA-B, HLA-Cw and most HLA-A-coated beads. A low density of  $\beta$ 2fHC exists on the following HLA-A beads: A\*11:01, A\*24:02, and A\*24:03 (MFI = 1400, 1005, and 1035, respectively). HC-10 recognized 35% of HLA-A, 56% of HLA-B and 63% of HLA-Cw antigens with MFI ranges: 6-7760, 18-5513, 508-2145, respectively, confirming the heterogeneous density of pepF- $\beta$ 2aHC on iBeads, as HC-10 identifies a variant of  $\beta$ 2aHC that was recognized by W6/32 but not by TFL-006. Thus, HC-10's epitope occurs on both  $\beta$ 2fHC and pepF- $\beta$ 2aHC.

On acid-treated and alkali-treated beads (Figure 3 and 4), W6/32 failed to recognize HLA-A, HLA-B, and HLA-Cw antigens (0%, MFI < 1000), confirming its lack of affinity for  $\beta$ 2fHC. In contrast, TFL-006 exhibited 100% reactivity to



**FIGURE 1.** HLA-I beads. Anti-HLA-I mAbs (W6/32, TFL-006, HC-10) reactivity against all HLA-A (n = 31), HLA-B (n = 50) and HLA-Cw (n = 16) antigens in HLA-I beads. The MFI cutoff used for a positive reaction is 1000. Note that W6/32 binds to 100% of HLA-A, HLA-B, and HLA-Cw antigens and HC-10 binds to 39% of HLA-A, 94% of HLA-B and 100% of HLA-Cw antigens with high MFI. In contrast, TFL-006, though binds to 100% of HLA-A, HLA-B and HLA-Cw antigens, the MFI for the antigens remained lower than that of W6/32 and HC-10.



**FIGURE 2.** iBeads. Anti-HLA-I mAbs (W6/32, TFL-006, HC-10) reactivity against all HLA-A (n = 31), HLA-B (n = 50) and HLA-Cw (n = 16) antigens in iBeads. The MFI cutoff used for a positive reaction is 1000. W6/32 binds to 100% of HLA-A, HLA-B and HLA-Cw antigens with high MFI. TFL-006 poorly recognizes of HLA-A antigens (10%), while failing to recognize HLA-B and -Cw antigens (0%). HC-10 binds to 35% of HLA-A, 56% of HLA-B and 63% of HLA-Cw antigens.

the HLA-A, HLA-B, and HLA-Cw antigens in both acid-treated (100%; MFI range, 3304-7335, 2633-7430, 3164-8255, respectively) and alkali-treated (100%; MFI range: 5731-12 569, 7930-14 817, 9630-13 299, respectively) beads, confirming TFL-006-specific affinity for  $\beta$ 2fHC. Thus, both W6/32 and TFL-006 recognized all HLA-I antigens, but different conformational variants. In contrast to TFL-006, HC-10 binds to 65% of HLA-A (MFI range, 306-22 314) on acid-treated beads and 45% (MFI range, 0-14 572) on alkali-treated beads. HC-10 bound to 100% of HLA-B and -Cw antigens on acid-treated beads with MFI ranges: 1940-22 710, 18 075-22 828, respectively, and to 94% of HLA-B and 100% of HLA-Cw antigens on alkali-treated beads with MFI ranges: 0-15 560, 13 873-15 660, respectively. HLA-I antigens with low MFI (<3000) in acid-treated beads became negative in alkali-treated beads.

Figure 5 shows that the reactivity of the mAbs across the HLA-I antigens represented in the SAB's panels of 2 lots is not consistent. MFI differs between HLA-I beads lot 8 and lot 9 in the 3 different loci (HLA-A, -HLA-B,

and HLA-Cw). The observations point out that lot-to-lot variability exists in the amount of  $\beta$ 2aHC and  $\beta$ 2fHC on HLA-I beads.

### Specific Characteristics of HC-10 Reactivity Against HLA-I Antigens

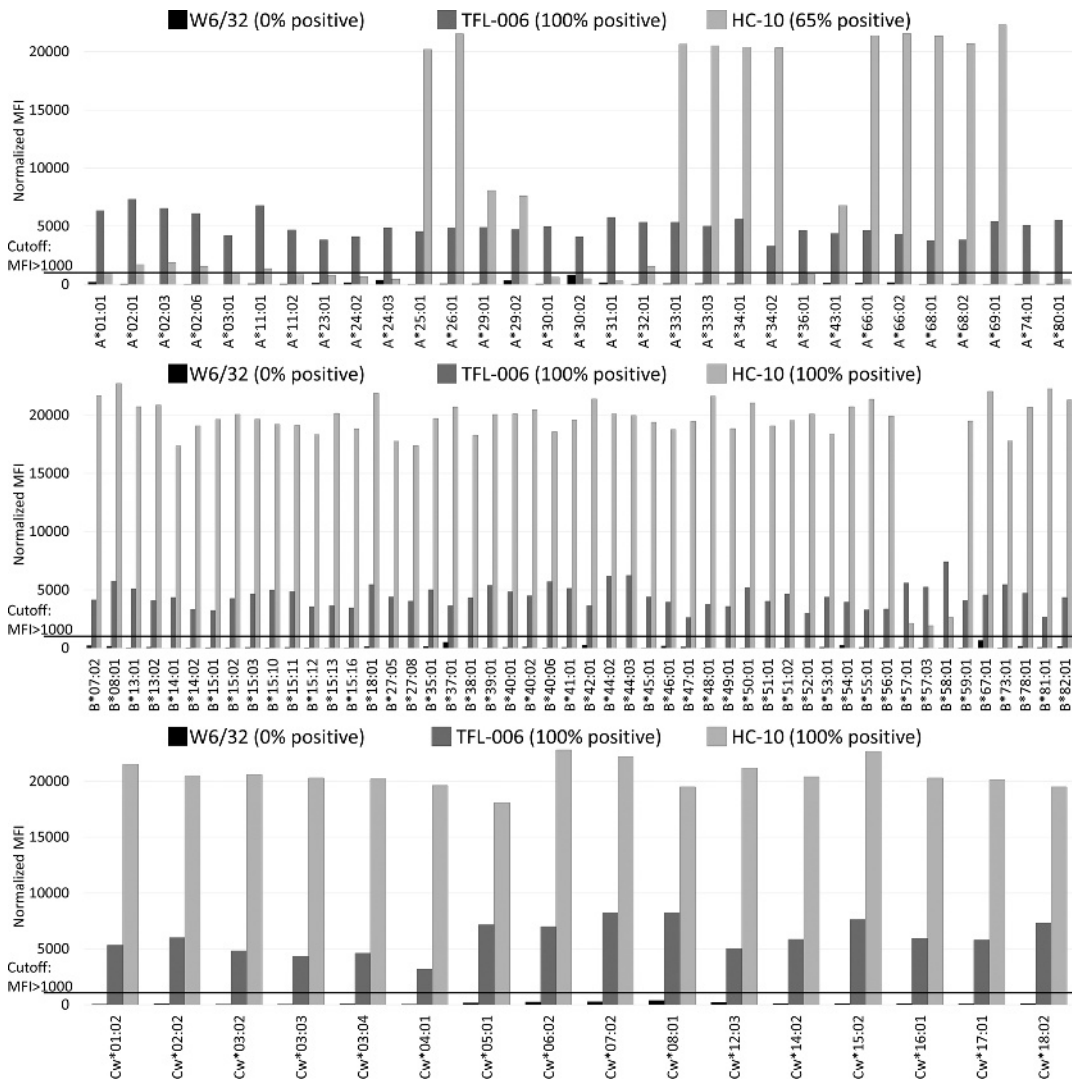
HC-10 reactivity against HLA-I are characterized into 4 different groups (Table 1):

*Group 1. HC-10-reactive antigens on the different SAB:* A shared characteristic of the HC-10-reactive antigens is the presence of the amino acid sequence motif  $^{57}$ PEYWDR $^{62}$  in the  $\alpha$ 1 HC.

*Group 2. HC-10-non-reactive antigens on iBeads only:* HC-10-reactive antigens are found on HLA-I beads and acid-treated beads but not to the same antigens on iBeads. This group also bears the sequence  $^{57}$ PEYWDR $^{62}$ .

*Group 3. HC-10-reactive antigens on acid-treated beads only:* These antigens become reactive to HC-10 upon acid treatment. Interestingly, R $^{62}$  is either replaced by glycine (G), leucine (L), or asparagine (Q) in the peptide sequence





**FIGURE 3.** Acid-treated beads. Anti-HLA-I mAbs (W6/32, TFL-006, HC-10) reactivity against all HLA-A (n = 31), HLA-B (n = 50) and HLA-Cw (n = 16) antigens in acid-treated beads. The MFI cutoff used for a positive reaction is 1000. W6/32 failed to recognize HLA-A, HLA-B and HLA-Cw in acid-treated beads (0%). TFL-006 shows 100% reactivity to the HLA-A, HLA-B and HLA-Cw antigens. HC-10 binds to 65% of HLA-A and 100% of HLA-B and HLA-Cw antigens.

(<sup>57</sup>PEYWDR<sup>62</sup>). Upon alkali treatment, HC-10 failed to recognize the antigens with G<sup>62</sup> and Q<sup>62</sup> (group 3bis).

**Group 4. HC-10 nonreactive antigens on all SAB:** Several antigens are not recognized by HC-10 on all SAB. R<sup>62</sup> is replaced by aspartic acid (E) or Q. Upon alkali-treatment, HC-10 fails to recognize the antigens with E<sup>62</sup> and Q<sup>62</sup>, as well as antigens with G<sup>62</sup> (group 4bis).

HC-10 recognizes, on iBeads, the antigens in group 1 but not in group 2. Because the antigens, in both groups, are recognized by W6/32 but not by TFL-006 on iBeads, HC-10 recognize one of the W6/32-positive variant, namely, pepA-β2aHC or pepF-β2aHC. Because HC-10 binds to the antigens in groups 1 and 2 on acid-treated beads, pepF-β2aHC may be the unique variant recognized by HC-10 on iBeads. Because groups 3 and 4 antigens are not recognized by HC-10 on HLA-I beads and iBeads, they are not included for further characterization of the SAB.

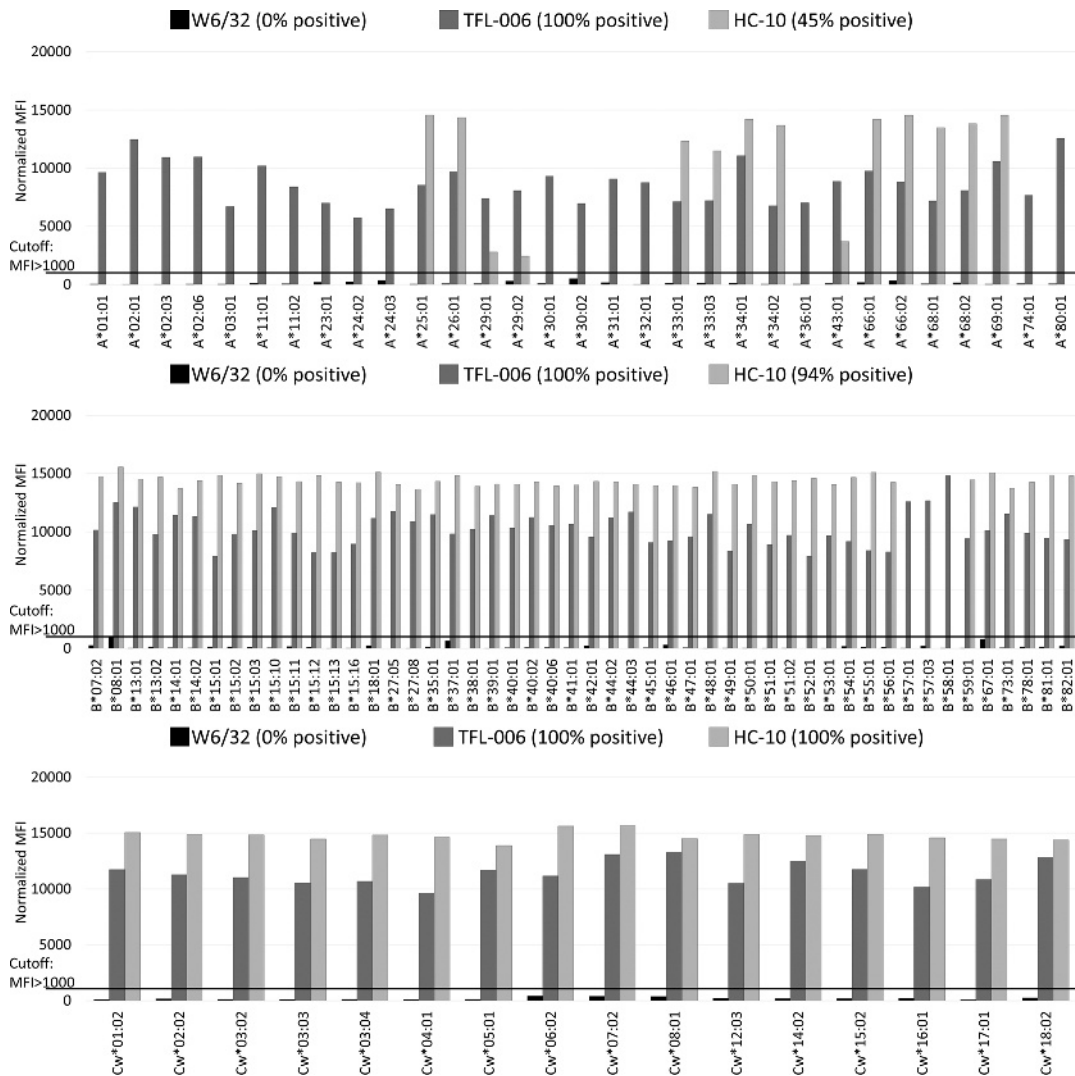
**Density of β2fHC Coated on HLA-I Beads and iBeads**

HLA-I molecules coated on SAB includes: β2aHC, represented by the sum of pepA-β2aHC and pepF-β2aHC,

recognized by W6/32; and β2fHC recognized by TFL-006. Since there is no overlap between the HLA-I variants recognized by these 2 mAbs, the percentage of β2fHC for a specific HLA-I antigen on HLA-I beads and iBeads (Figure 6) is calculated as follows:

$$\% \beta 2 f H C = (T F L - 0 0 6 M F I) / (T F L - 0 0 6 M F I + W 6 / 3 2 M F I)$$

Most HLA-I beads harbor detectable amounts (MFI > 1000) of β2fHC on their surface. However, no detectable amounts (MFI < 1000) of β2fHC are observed on the beads coated with A\*02:01, A\*02:06, A\*03:01, A\*23:01, A\*25:01, A\*29:02, A\*32:01, A\*34:02, A\*66:02, A\*74:01, B15:01, B\*27:05, B\*27:08, B\*42:01, B\*49:01, or B\*67:01 (boxed antigens in Figure 6). HLA-Cw antigens coated on HLA-I beads always had a MFI greater than 1000, representing greater than 10% of β2fHC coated on the beads' surface. In contrast, iBeads mostly harbor β2aHC, with non-detectable (MFI < 1000) β2fHC. Only the following HLA-A beads, A\*11:01, A\*24:02 and A\*24:03 (dotted boxed antigens in Figure 6), harbor detectable amounts of



**FIGURE 4.** Alkali-treated beads. Anti-HLA-I mAbs (W6/32, TFL-006, HC-10) reactivity against all HLA-A ( $n = 31$ ), HLA-B ( $n = 50$ ) and HLA-Cw ( $n = 16$ ) antigens in alkali-treated beads. The MFI cutoff used for a positive reaction is 1000. The binding pattern of W6/32 and TFL-006 on alkali-treated beads are similar to the binding on acid treated beads. HC-10 binds to 45% of HLA-A on alkali treated beads, 94% of HLA-B and 100% of HLA-Cw on alkali-treated beads.

$\beta 2fHC$  ( $1000 < MFI < 1500$ ), with a single exception, A\*69:01, that has nondetectable  $\beta 2fHC$  ( $MFI < 1000$ ) with density of 8%.

Table 2 shows that, in HLA-I beads, the median percentages of  $\beta 2fHC$  in HLA-A, HLA-B, and HLA-Cw loci is  $7\% \pm 5.7\%$ ,  $11.7\% \pm 7.5\%$ , and  $28.2\% \pm 10.6\%$ , respectively, while being lower in iBeads (HLA-A =  $1.5\% \pm 1.9\%$ , HLA-B =  $0.1\% \pm 0.3\%$ , HLA-Cw =  $0.2\% \pm 0.5\%$ ) and higher in acid-treated beads (HLA-A =  $98.3\% \pm 3.2\%$ , HLA-B =  $98.6\% \pm 2.7\%$ , HLA-Cw =  $98.8\% \pm 1.2\%$ ). In HLA-I beads, the density of  $\beta 2fHC$  present on the HLA-A beads is significantly lower compared with HLA-B beads ( $P < 0.005$ ) and HLA-Cw beads ( $P < 0.0001$ ).

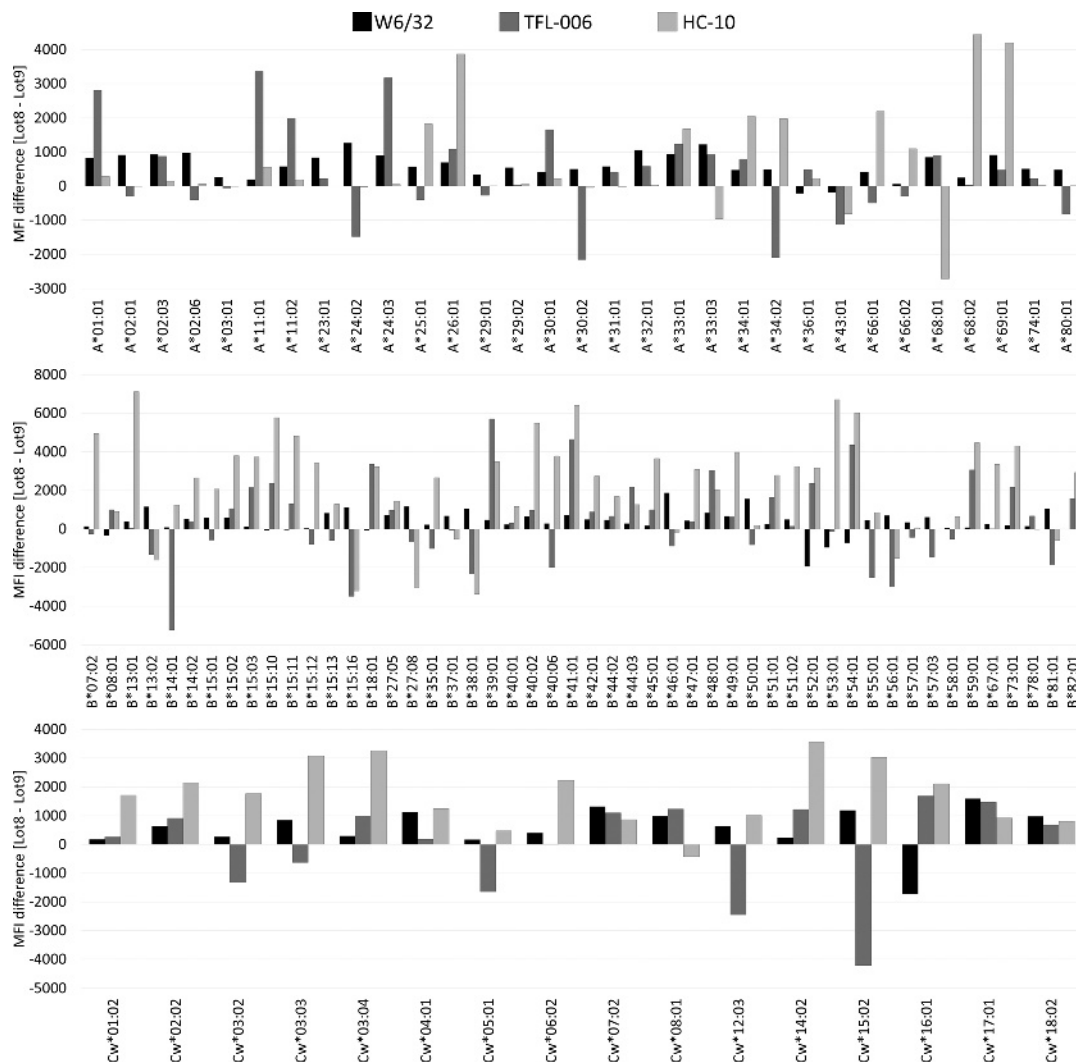
#### Relative Density of pepF- $\beta 2aHC$ on iBeads

W6/32 binds to both pepA- $\beta 2aHC$  and pepF- $\beta 2aHC$  and HC-10 binds to pepF- $\beta 2aHC$  and  $\beta 2fHC$ , suggesting that there is an overlap between the HLA-I conformation recognized by both mAbs. Therefore, the percentage of pepF- $\beta 2aHC$  coated on iBeads is derived only for the HLA-I

antigens expressing the antigenic determinant R<sup>62</sup> recognized by HC-10 (Table 1, groups 1 and 2), because these antigens on iBeads are free of  $\beta 2fHC$  and are coated with HLA-I variants restricted to pepA- $\beta 2aHC$  and pepF- $\beta 2aHC$ . The percentage of pepF- $\beta 2aHC$  for a specific antigen on iBeads (Figure 7) was calculated as follows:

$$\% \text{pepF-}\beta 2aHC = (\text{HC-10MFI}) / (\text{W6/32MFI})$$

Most beads found in the iBeads' panel have detectable ( $MFI > 1000$ ) pepF- $\beta 2aHC$ . Only 19 HLA-B and 6 HLA-Cw have nondetectable ( $MFI < 1000$ ) pepF- $\beta 2aHC$  (boxed-antigens in Figure 7). All HC-10-reactive HLA-A antigens had MFI greater than 1000, demonstrating greater than 10% of  $\beta 2fHC$  coated on the beads. Table 2 shows that the median percentage of pepF- $\beta 2aHC$  on HLA-A, HLA-B, and HLA-Cw loci is  $21.9\% \pm 21\%$ ,  $7.4\% \pm 9.5\%$ , and  $13.2\% \pm 8.1\%$  respectively. HLA-A antigens have significantly higher density of pepF- $\beta 2aHC$  compared with HLA-B ( $P < 0.001$ ) and HLA-Cw ( $P < 0.005$ ).



**FIGURE 5.** HLA-I beads lot-to-lot variability expressed as the difference between anti-HLA-I mAbs (W6/32, TFL-006, HC-10) reactivity against HLA-I beads lot 8 and lot 9 (lot 8 MFI – lot 9 MFI). Positive values illustrate higher reactivity with lot 8, while negative values illustrate higher reactivity with lot 9.

### Ranking of HLA-I Antigen-Coated Beads Based on $\beta$ 2fHC Density in HLA-I Beads

Table 3 shows the ranking of all HLA-I antigens ( $n = 97$ ) in the HLA-I beads' panel, based on TFL-006 reactivity and categorized in 1000 MFI increment (from 1000 to 9000). This stratification points out the heterogeneous density of  $\beta$ 2fHC coated on HLA-I beads and shows how the use of a standard cutoff across all beads may not distinguish anti- $\beta$ 2aHC from anti- $\beta$ 2fHC. Indeed, if 1000 is used, then only 16% of the beads will truly detect anti- $\beta$ 2aHC, the remaining beads may detect both anti- $\beta$ 2aHC and - $\beta$ 2fHC. If 5000 is used, then 88% of the beads will truly detect anti- $\beta$ 2aHC, whereas 72% of the beads may be false negative for anti- $\beta$ 2aHC and 12% may detect both anti- $\beta$ 2aHC and anti- $\beta$ 2fHC. This ranking suggests that each individual bead should have a bead-specific MFI cutoff to critically evaluate and distinguish anti- $\beta$ 2aHC from anti- $\beta$ 2fHC when using HLA-I beads.

### Ranking of HLA-I Antigen-Coated Beads Based on pepF- $\beta$ 2aHC Density in iBeads

Table 4 shows the ranking of all HC-10-reactive antigens ( $n = 74$ ) in the iBeads' panel, based on HC-10 reactivity and

categorized in 1000 MFI increments (from 1000 to 8000). The heterogeneity of pepF- $\beta$ 2aHC density on iBeads is obvious, as the MFI of HC-10 against antigens on iBeads ranged from 175 to 7760. Such a categorization is useful for assessing meaningful cutoffs to evaluate the HLA antibodies against pepA- $\beta$ 2aHC or pepF- $\beta$ 2aHC.

### T-cell FCXM Using the Anti-HLA-I mAbs

Although W6/32 and TFL-006 can bind to all of the donor's HLA-I antigens and HC-10 only to HLA-B and HLA-Cw ones, Table 5 reveals that W6/32 but not TFL-006 and HC-10 showed reactivity to HLA-I expressed on the surface of resting T cell, confirming the prevalence of pepA- $\beta$ 2aHC on the surface of resting T cells (CD4+ or CD8+). Explicitly resting T cells express neither  $\beta$ 2fHC nor pepF- $\beta$ 2aHC.

## DISCUSSION

This investigation reveals the distribution and relative density of the conformational variants of the HLA-I on SAB by comparing the reactivities of mAbs, W6/32, TFL-006, and HC-10 on HLA-I beads, iBeads, acid, and alkali-treated

**TABLE 1.**

**Classification of HLA-I antigens based on HC-10 reactivity against 4 different SAB, (HLA-I beads, iBeads, acid-treated, and alkali-treated beads) coated with 31 antigens of HLA-A, 50 antigens of HLA-B, and 16 antigens of HLA-Cw)**

HC-10 reactive groups	Amino acid position						HLA-I alleles						
	57	58	59	60	61	62							
Group 1 (n = 49): (HLA-A: n = 11; HLA-B: n = 28; HLA-Cw: n = 10) Reactive alleles HLA-I beads: MFI > 1000 iBeads: MFI > 1000 Acid-treated beads: MFI > 1000 Alkali-treated beads: MFI > 1000	P	E	Y	W	D	<b>R</b>	A*25:01 A*66:02 B*13:01 B*15:16 B*40:02 B*48:01 Cw*01:02 Cw*16:01	A*26:01 A*68:01 B*14:02 B*18:01 B*40:06 B*49:01 Cw*03:02 Cw*17:01	A*33:01 A*68:02 B*15:02 B*35:01 B*41:01 B*50:01 Cw*03:03 Cw*18:02	A*33:03 A*69:01 B*15:03 B*37:01 B*44:02 B*51:02 Cw*03:04 Cw*18:02	A*34:01 B*15:10 B*38:01 B*52:01 Cw*06:02	A*34:02 B*15:11 B*39:01 B*53:01 Cw*12:03	A*66:01 B*15:13 B*40:01 B*47:01 B*59:01 Cw*15:02
Group 2 (n = 25): (HLA-A: n = 0; HLA-B: n = 19; HLA-Cw: n = 6) Nonreactive to iBeads alleles only HLA-I beads: MFI > 1000 iBeads: MFI < 1000 Acid-treated beads: MFI > 1000 Alkali treated beads: MFI > 1000	—	—	—	—	—	<b>R</b>	B*07:02 B*27:08 B*67:01 Cw*02:02	B*08:01 B*42:01 B*73:01 Cw*04:01	B*13:02 B*45:01 B*78:01 Cw*05:01	B*14:01 B*51:01 B*81:01 Cw*07:02	B*15:01 B*54:01 B*82:01 Cw*08:01	B*15:12 B*55:01 Cw*14:02	B*27:05 B*56:01
Acid-treated Group 3 (N = 12): (HLA-A: n = 9; HLA-B: n = 3; HLA-Cw: n = 0) Reactive to acid-treated alleles only HLA-I beads: MFI < 1000 iBeads: MFI < 1000 Acid-treated beads: MFI > 1000	—	—	—	—	—	<b>G</b> <b>L</b> <b>Q</b> <b>E</b> <b>Q</b>	A*02:01 A*29:01 A*11:01 A*23:01 A*01:01	A*02:03 A*29:02 A*32:01 A*24:02 A*03:01	A*02:06 A*43:01 A*74:01 A*24:03 A*11:02	B*57:01 B*57:03 A*80:01 A*30:01	B*57:03 B*58:01	A*31:01	A*36:01
Group 4 (N = 11): (HLA-A: n = 11; HLA-B: n = 0; HLA-Cw: n = 0) Nonreactive alleles HLA-I beads: MFI < 1000 iBeads: MFI < 1000 Acid-treated beads: MFI < 1000	—	—	—	—	—								
Alkali-treated Group 3bis (N = 3): (HLA-A: n = 3; HLA-B: n = 0; HLA-Cw: n = 0) Reactive to alkali-treated alleles only HLA-I beads: MFI < 1000 iBeads: MFI < 1000 Alkali-treated beads: MFI > 1000	—	—	—	—	—	<b>L</b> <b>E</b> <b>Q</b> <b>G</b>	A*29:01 A*23:01 A*02:01 A*01:01 A*32:01	A*29:02 A*24:02 A*02:03 A*03:01 A*36:01	A*43:01 A*24:03 A*02:06 A*11:01 A*74:01	A*80:01 B*57:01 A*11:02	B*57:03 B*58:01	A*30:01 A*30:02	A*31:01 A*31:01
Group 4bis (N = 20): (HLA-A: n = 17; HLA-B: n = 3; HLA-Cw: n = 0) Nonreactive alleles HLA-I beads: MFI < 1000 iBeads: MFI < 1000 Alkali-treated beads: MFI < 1000	—	—	—	—	—								

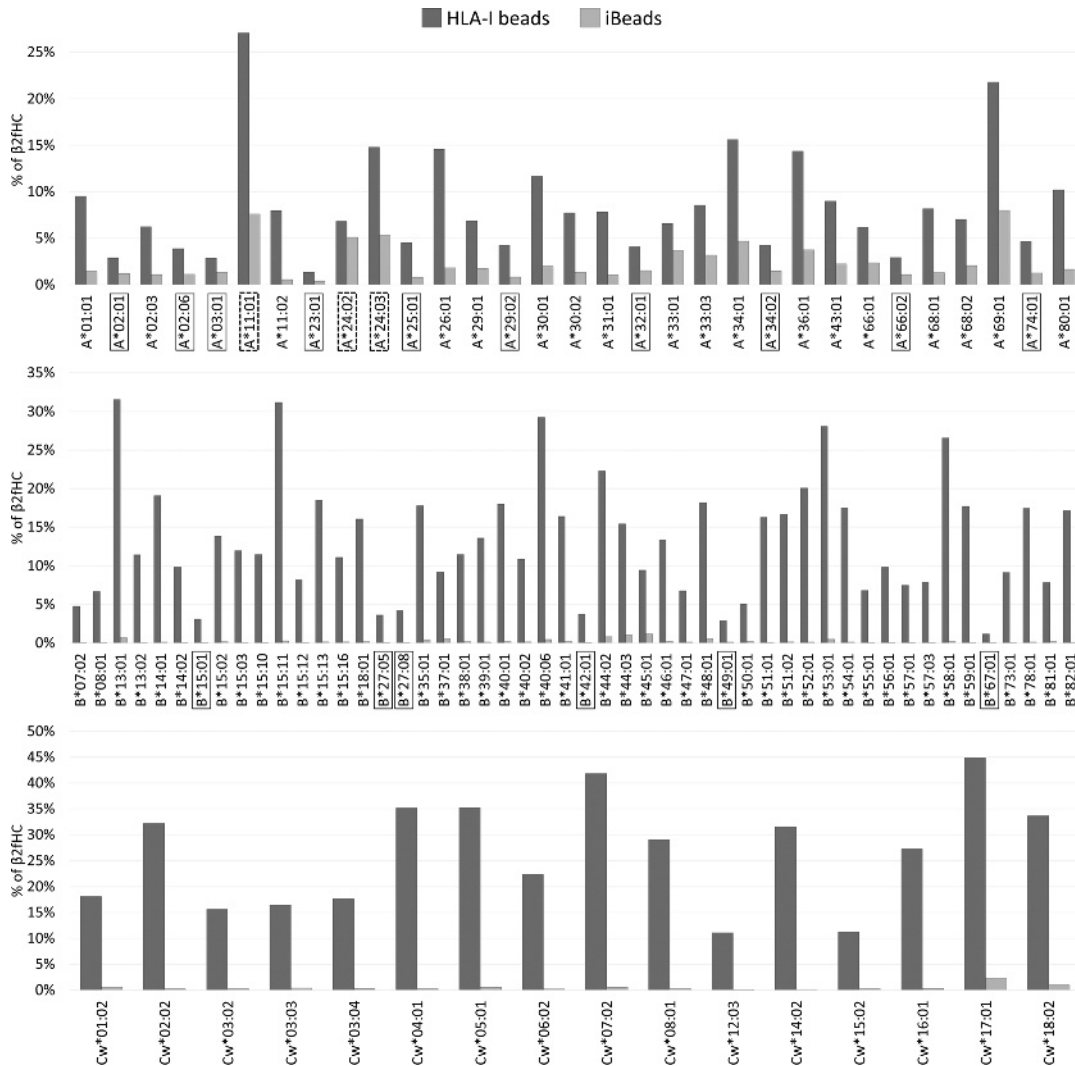
beads. Figure 8 elucidates the specificity of the mAbs used, the relative density of the different conformational variants of HLA-I present on the surface of SAB and the variant that is associated with positive FCXM. The assessment of the intrinsic characteristics of the beads is critical for the reliability of virtual XMs in predicting CDCXM and FCXM outcomes and to increase accessibility of donor organs for highly sensitized patients on the waiting list.

The discrepancy between virtual XMs and actual XMs is influenced by the conventionally used SAB (HLA-I beads) for monitoring HLA-I antibodies. HLA-I antibodies in an allograft recipient can detect different conformations of HLA-I,<sup>9,11,27</sup>

namely,  $\beta$ 2aHC and  $\beta$ 2fHC. Anti- $\beta$ 2aHC IgGs are associated with AMR and/or graft loss,<sup>28,29</sup> whereas anti- $\beta$ 2fHC IgGs may not have a deleterious effect on graft function.<sup>9,12,14</sup> These observations influence the assumption that SAB only detect and identify clinically relevant HLA-I antibodies,<sup>30</sup> without examining the HLA variants (antigen integrity, conformation, and orientation) on the beads.<sup>31</sup>

Anti- $\beta$ 2fHC IgGs were documented in nonalloimmunized men, cord blood, and in CDCXM-negative transplant candidates without prior immunizations.<sup>8,10,32</sup> Similarly, HLA-I-sensitized kidney recipients had anti- $\beta$ 2fHC IgGs without positive FCXM.<sup>11,14</sup> Therefore, HLA-I beads can detect





**FIGURE 6.** Density (%) of  $\beta 2fHC$  coated on HLA-I beads and iBead, as assessed by the following formula:  $\% \beta 2fHC = (TFL-006\ MFI) / (TFL-006\ MFI + W6/32\ MFI)$ . Boxed antigens represent beads coated with non-detectable  $\beta 2fHC$  with MFI < 1000 on HLA-I beads, whereas dotted-boxed antigens represent beads coated with detectable  $\beta 2fHC$  with MFI > 1000 on iBeads.

antibodies that may not be clinically relevant, such as anti- $\beta 2fHC$  not associated with a positive FCXM.<sup>27,30,33,34</sup>

Strategies to minimize the “false SAB reactions” (as implicated by Gombos et al<sup>32</sup>) are critical in maximizing the

specificity of SAB that correlate with positive FCXM results. One approach is to define an MFI cutoff that is associated with transplant outcome, which is challenging to reach.<sup>7,30</sup> The 2 aspects of MFI cutoffs are: a cutoff for a positive

**TABLE 2.**

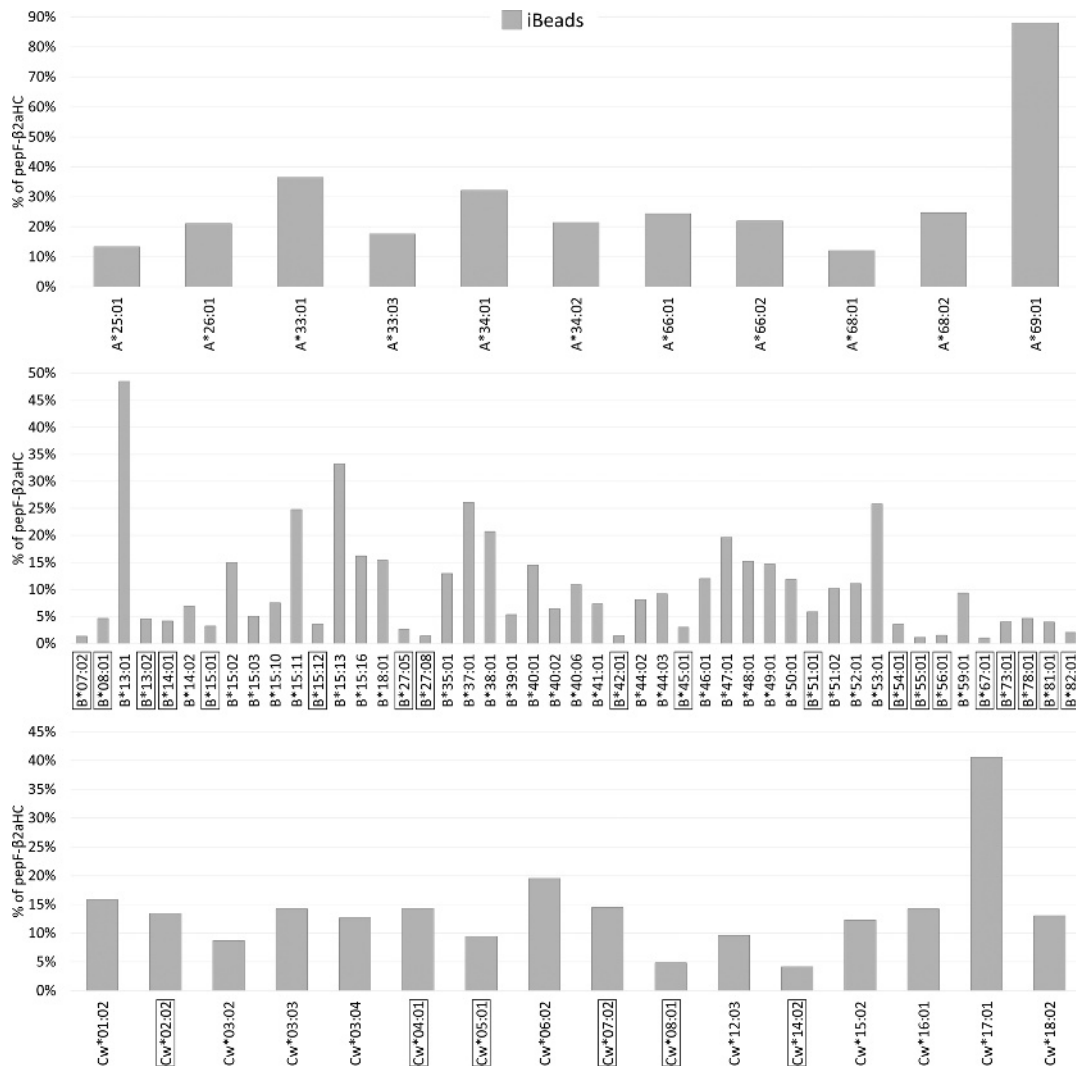
**Density (%) of  $\beta 2fHC$  coated on HLA-A, HLA-B and HLA-Cw antigen-coated beads in HLA-I beads, iBeads and acid-treated beads; and density (%) of pepF- $\beta 2aHC$  on coated on HLA-A, HLA-B, and HLA-Cw antigen-coated beads in iBeads**

	% of $\beta 2fHC$			% of pepF- $\beta 2aHC$ <sup>a</sup>
	HLA-I beads	iBeads	Acid-treated beads	iBeads
HLA-A alleles	7 ± 5.7	1.5 ± 1.9	98.3 ± 3.2	21.9 ± 21
HLA-B alleles	11.7 ± 7.5	0.1 ± 0.3	98.6 ± 2.7	7.4 ± 9.5
HLA-Cw alleles	28.2 ± 10.6	0.2 ± 0.5	98.8 ± 1.2	13.2 ± 8.1
HLA-A vs HLA-B	<i>P</i> < 0.005	<i>P</i> < 0.0001	NS	<i>P</i> < 0.001
HLA-A vs HLA-Cw	<i>P</i> < 0.0001	<i>P</i> < 0.0001	NS	<i>P</i> < 0.005
HLA-B vs HLA-Cw	<i>P</i> < 0.001	NS	NS	<i>P</i> < 0.05
Kruskal-Wallis test	<i>P</i> < 0.0001	<i>P</i> < 0.0001	<i>P</i> < 0.005	<i>P</i> < 0.001

<sup>a</sup>  $\beta 2fHC$  was calculated in HLA-I beads using the formula  $(TFL-006\ MFI) / (TFL-006\ MFI + W6/32\ MFI)$ ; pepF- $\beta 2aHC$  was calculated in iBeads using the formula  $(HC-10\ MFI) / (W6/32\ MFI)$ .

The percentage of pepF- $\beta 2aHC$  cannot be assessed and were not included for these alleles.

<sup>a</sup> HC-10 nonreactive alleles (group 4): A\*01:01, A\*03:01, A\*11:02, A\*23:01, A\*24:02, A\*24:03, A\*30:01, A\*30:02, A\*31:01, A\*36:01, and A\*80:01; and HC-10 acid treated reactive alleles only (group 3): A\*02:01, A\*02:03, A\*02:06, A\*11:01, A\*29:01, A\*29:02, A\*32:01, A\*43:01, A\*74:01, B\*57:01, B\*57:03, and B\*58:01.



**FIGURE 7.** Density (%) of pepF-β2aHC coated on iBeads as assessed by the following formula: %pepF-β2aHC = (HC-10 MFI)/(W6/32 MFI). Boxed antigens represent beads coated with nondetectable pepF-β2aHC with MFI < 1000 on iBeads.

reaction (or sensitivity) and a cutoff for clinical relevance (or specificity); both contributing to the divergence and non-agreement of a specific “standard” MFI cutoff. Indeed, the MFI cutoffs used can range from 300<sup>35</sup> to 6000,<sup>36</sup> while 1000 is most common,<sup>30,32,37-39</sup> particularly when the sera are used neat or minimally diluted, or in the presence of IgM and other interfering serum factors. Both the sensitivity and the specificity of SAB for detecting serum antibodies is related to the antigenic density of the HLA-I proteins displayed on their surface.<sup>40</sup> Our results emphasize that the cutoff should be based on the density of β2fHC, as indicated by the MFI of the mAb TFL-006. Because the MFIs obtained with TFL-006 vary among different antigen-coated beads, a specific cutoff should be attributed for each antigen in the panel of HLA-I beads. Therefore, when an anti-HLA-I DSA is identified, the raw MFI obtained for a DSA should be normalized not only against the negative control bead and a negative control sample, but also against a positive control for DSA reacting to β2fHC, monitored with TFL-006. Only the β2fHC-normalized MFI would represent IgG directed against β2aHC. This approach using a bead-specific MFI cutoff is supported by the findings in literature, where

the levels of anti-β2fHC were documented to vary from low (MFI < 1000) to high (MFI > 9000) levels.<sup>14,32</sup> In the case of low MFI DSA, when TFL-006 MFI is higher than the DSA's MFI, then there is a possibility of underestimating the DSA, if it recognizes an epitope present in both β2aHC and β2fHC, as described earlier.<sup>8,11,14</sup> This limitation is applicable only to HLA-I beads, because this issue is eliminated if iBeads are used because they express predominantly β2aHC.

The other strategy to reduce the amount of “false SAB reactions” is to revive the production of iBeads (by the vendor of HLA-I beads), which eliminates “false positive reactions” caused by the presence of β2fHC on SAB.<sup>13</sup> iBeads are not routinely used in clinical settings; however, promising data suggest that iBeads mainly detect anti-β2aHC<sup>9,41</sup> associated with positive FCXM.<sup>11,14,42</sup> Otten et al<sup>9</sup> has described low levels of “denatured” HLA on iBeads, using mAb HC-10. However, our study shows that iBeads bear an even lower density of β2fHC, as assessed by β2fHC-specific mAb TFL-006. Assessing the reactivities of TFL-006 and HC-10 to iBeads shows evidence that HC-10 reacts to the pepF-β2aHC on iBeads. Visentin et al<sup>11,14,42</sup> have also noted that

**TABLE 3.****Ranking of HLA-I antigens in the HLA-I beads' panel based on their TFL-006 reactivity: bead-specific MFI cutoff of HLA-I beads' panel**

HLA-I Alleles in HLA-I beads	Average TFL-006 MFI (SD)	Theoretical MFI cutoff	% of beads (n = 97)	Cumulative % (n = 97)
A*02:01, A*02:06, A*03:01, A*23:01, A*25:01, A*29:02, A*32:01, A*34:02, A*66:02, A*74:01	702 (209)	MFI > 1000	16%	16%
B*15:01, B*27:05, B*27:08, B*42:01, B*49:01, B*67:01				
A*02:03, A*11:02, A*24:02, A*29:01, A*30:02, A*31:01, A*33:01, A*33:03, A*43:01, A*66:01, A*68:01, A*68:02	1551 (259)	MFI > 2000	25%	41%
B*07:02, B*08:01, B*14:02, B*15:12, B*45:01, B*47:01, B*50:01, B*55:01, B*57:01, B*57:03, B*73:01, B*81:01				
A*01:01, A*26:01, A*30:01, A*36:01, A*80:01	2455 (326)	MFI > 3000	18%	59%
B*13:02, B*15:02, B*15:03, B*15:10, B*15:16, B*37:01, B*38:01, B*40:02, B*46:01, B*56:01				
Cw*12:03, Cw*15:02				
A*24:03, A*34:01	3615 (278)	MFI > 4000	24%	82%
B*15:13, B*18:01, B*35:01, B*39:01, B*41:01, B*44:02, B*44:03, B*48:01, B*51:01, B*51:02, B*52:01, B*54:01, B*59:01, B*78:01, B*82:01				
Cw*01:02, Cw*03:02, Cw*03:03, Cw*03:04, Cw*04:01, Cw*16:01				
A*69:01	4379 (338)	MFI > 5000	5%	88%
B*14:01, B*40:01, B*53:01				
Cw*06:02				
B*13:01, B*15:11	5580 (275)	MFI > 6000	4%	92%
Cw*02:02, Cw*17:01				
A*11:01	6550 (364)	MFI > 7000	4%	96%
B*40:06, B*58:01				
Cw*14:02				
Cw*05:01, Cw*07:02, Cw*08:01	7880 (185)	MFI > 8000	3%	99%
Cw*18:02	8129	MFI > 9000	1%	100%

**TABLE 4.****Ranking of HLA-I antigens in the iBeads' panel based on their HC-10 reactivity: bead-specific MFI cutoff of iBeads' panel**

HLA-I alleles in iBeads	Average HC-10 MFI (SD)	Theoretical MFI cutoff	% of beads (n = 74)	Cumulative % (n = 74)
B*07:02, B*08:01, B*13:02, B*14:01, B*15:01, B*15:12, B*27:05, B*27:08, B*42:01, B*45:01, B*51:01, B*54:01, B*55:01, B*56:01, B*67:01, B*73:01, B*78:01, B*81:01, B*82:01	519 (197)	MFI > 1000	34%	34%
Cw*02:02, Cw*04:01, Cw*05:01, Cw*07:02, Cw*08:01, Cw*14:02				
B*14:02, B*15:03, B*15:10, B*39:01, B*40:01, B*40:02, B*40:06, B*41:01, B*44:02, B*44:03, B*46:01, B*47:01, B*48:01, B*51:02, B*52:01, B*59:01	1371 (264)	MFI > 2000	30%	64%
Cw*03:02, Cw*03:03, Cw*03:04, Cw*12:03, Cw*16:01, Cw*18:02				
A*25:01, A*68:01	2277 (293)	MFI > 3000	18%	81%
B*15:02, B*15:11, B*15:16, B*18:01, B*35:01, B*49:01, B*50:01				
Cw*01:02, Cw*06:02, Cw*15:02, Cw*17:01				
A*26:01, A*33:03, A*34:02, A*66:01, A*66:02, A*68:02	3619 (287)	MFI > 4000	12%	93%
B*15:13, B*38:01, B*53:01				
B*37:01	4236	MFI > 5000	1%	95%
A*33:01, A*34:01	5370 (317)	MFI > 6000	4%	99%
B*13:01				
A*69:01	7760	MFI > 8000	1%	100%

HC-10 nonreactive antigens (groups 3 and 4) are not included.

HC-10 non-reactive alleles (group 4): A\*01:01, A\*03:01, A\*11:02, A\*23:01, A\*24:02, A\*24:03, A\*30:01, A\*30:02, A\*31:01, A\*36:01, and A\*80:01; and HC-10 acid treated reactive alleles only (Group 3): A\*02:01, A\*02:03, A\*02:06, A\*11:01, A\*29:01, A\*29:02, A\*32:01, A\*43:01, A\*74:01, B\*57:01, B\*57:03, and B\*58:01.

The percentage of peptide-free  $\beta$ 2aHC cannot be assessed and were not included for these alleles.

$\beta$ 2fHC is detectable on the following beads: A\*11:01 (MFI = 1400); A\*24:02 (MFI = 1005); A\*24:03 (MFI = 1035).

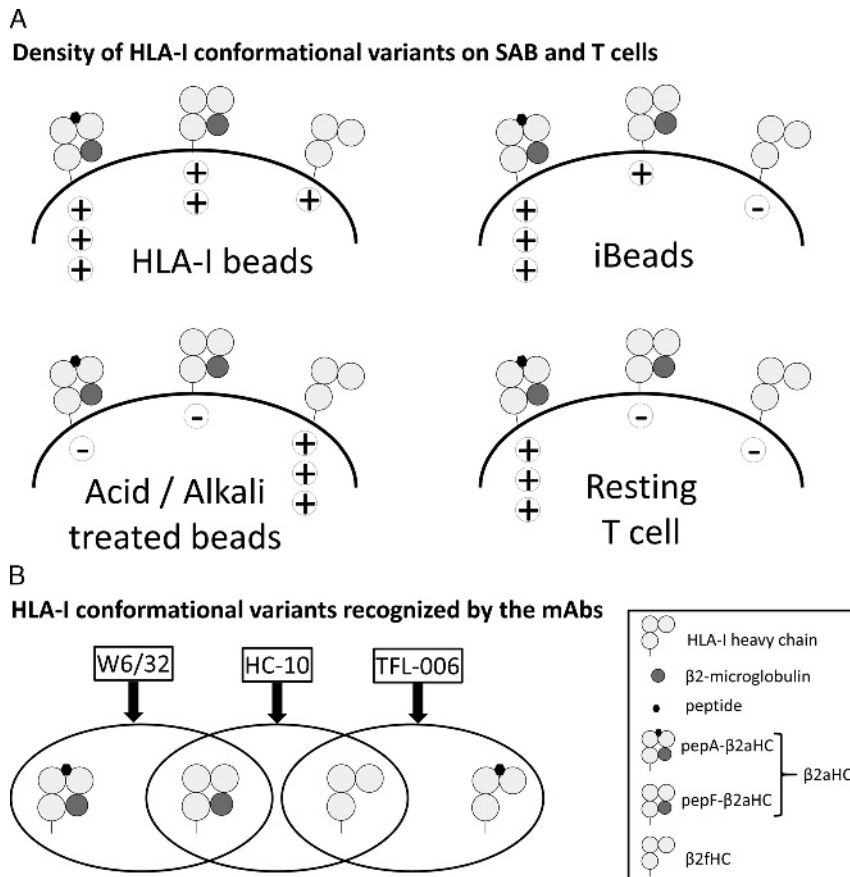
**TABLE 5.****T cell FCXM using anti-HLA-I mAbs W6/32, TFL-006, and HC-10**

T cell FCXM	CD4+ T cells median (SD) channel shift	CD8+ T cells median (SD) channel shift
Mouse isotype (0.357 µg/mL)	151 (19)	119 (12)
W6/32 (0.357 µg/mL)	13 256 (1434)	12 558 (1073)
TFL-006 (0.357 µg/mL)	72 (26)	21 (10)
HC-10 (0.357 µg/mL)	111 (25)	88 (9)

DSA against “denatured” HLA can be iBeads-positive, and such DSAs were not associated with AMR, graft loss, and/or positive FCXM. In this context, our results clarify the DSA detected by Visentin et al may be directed against pepF-β2aHC, equivalent to HC-10-positive DSA on iBeads that are FCXM negative. Therefore, the HC-10 reactivity against specific antigens on iBeads will provide the cutoff for monitoring a DSA reacting to the pepF-β2aHC. Therefore, this study recommends the use of iBeads, when made available, in clinical monitoring of anti-HLA-I DSA, because the DSA detected with iBeads is more reliable than those detected with conventional HLA-I beads. Owing to the work of Visentin et al,<sup>11,14,42</sup> DSA identified with iBeads have a propensity for allograft rejection and positive FCXM, and this DSA can be considered as pathogenic DSA.

Another aspect, emerging from the results, is the impact of a peptide, loaded in the peptide-binding groove of HLA-I HC, on the ability of HC-10 to bind to HLA-I antigens. It is known that R<sup>62</sup> is the antigenic determinant of HC-10<sup>20</sup> which is located in the second helix of the α1 domain.<sup>43</sup> More precisely, R<sup>62</sup> establishes interactions inside the A pocket anchoring the peptide N-terminus (P1), and its orientation could be strongly influenced by the P1 residues of a peptide.<sup>44</sup> Although HC-10 recognizes both β2fHC and pepF-β2aHC, these variants are not expressed on resting T cells. Indeed, the deleterious effect of DSA is confined to the recognition of HLA-I HC loaded with peptides by anti-pepA-β2aHC,<sup>45</sup> which is supported by the flexibility of the peptide-binding groove in the presence or absence of a peptide<sup>26,45,46</sup> and their implication for immune recognition.<sup>46</sup>

In conclusion, while using HLA-I beads for monitoring DSA, it is important to be aware that the DSA detected by the SAB may or may not correlate with positive FCXM and rejection. Two strategies can reduce the discrepancy between SAB and FCXM results while increasing the concordance of virtual and actual XMs. Table 6 illustrates the strategies to follow while seeking “pathogenic” DSA using HLA-I beads or iBeads: a bead-specific MFI cutoff based on either TFL-006 reactivity with HLA-I beads or that based on HC-10 reactivity with iBeads. Essentially, using a cutoff based on TFL-006 and/or HC-10 MFI enables the normalization of DSA “strength”



**FIGURE 8.** A, Density of HLA-I conformational variants on SAB and T-cells. HLA-I beads has highest density of pepA-β2aHC followed by pepF-β2aHC and then low density of β2fHC. iBeads has highest density of pepA-β2aHC and low density pepF-β2aHC and minimal density of β2fHC. Acid/Alkali-treated beads only bear β2fHC. T-cells only bear pepA-β2aHC. B, HLA-I conformational variants recognized by the mAbs. W6/32 recognize both pepA-β2aHC and pepF-β2aHC. HC-10 recognize pepF-β2aHC and pepF-β2fHC. TFL-006 recognize pepF-β2fHC and possibly pepA-β2fHC.



**TABLE 6.****The use of a bead-specific MFI cutoff to increase the concordance between SAB and FCXM results**

SAB assay	Bead-specific MFI cutoff median (range)	HLA Ab MFI	HLA Abs detected	Expected FCXM	HLA-I mAb equivalent
HLA-I beads <sup>a</sup>	HLA-A alleles (n = 31): 1481 (283–6811)	Below bead-specific cutoff	No Abs	Negative	None
	HLA-B alleles (n = 50): 2704 (255–6843)		anti-β2fHC	Negative	TFL-006
	HLA-Cw alleles (n = 16): 4157 (2041–8129)		anti-pepF-β2aHC	Negative	HC-10
iBeads <sup>b</sup>	HLA-A alleles (n = 11): 3886 (2088–7760)	Above bead-specific cutoff	anti-pepA-β2aHC	Positive	W6/32
		Below bead-specific cutoff	anti-pepA-β2aHC	Positive	W6/32
	HLA-B alleles (n = 47): 1171 (175–5513)	Below bead-specific cutoff	No Abs	Negative	None
			anti-β2fHC	Negative	TFL-006
	HLA-Cw alleles (n = 16): 1205 (508–2145)	Below bead-specific cutoff	anti-pepF-β2aHC	Negative	HC-10
			Above bead-specific cutoff	anti-pepA-β2aHC	Positive

<sup>a</sup> MFI cutoff is based on TFL-006 for each HLA-I alleles separately.

<sup>b</sup> MFI cutoff is based on HC-10 for each HLA-I alleles separately, but not including HC-10 non-reactive alleles (Group 4): A\*01:01, A\*03:01, A\*11:02, A\*23:01, A\*24:02, A\*24:03, A\*30:01, A\*30:02, A\*31:01, A\*36:01, and A\*80:01; and HC-10 acid treated reactive alleles only (Group 3): A\*02:01, A\*02:03, A\*02:06, A\*11:01, A\*29:01, A\*29:02, A\*32:01, A\*43:01, A\*74:01, B\*57:01, B\*57:03, and B\*58:01.

against β2aHC by removing “false SAB reactions” triggered by the possible presence of HLA antibodies against β2fHC and/or pepF-β2aHC, which are not associated with positive FCXM.

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