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Review

Strategies for the measurements of expression levels and half-lives of HLA class I allotypes

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ABSTRACT

HLA class I molecules are highly polymorphic cell surface proteins that trigger immune responses by CD8$^+$ T cells and natural killer (NK) cells. Most humans express six different HLA class I proteins encoded by the HLA-A, HLA-B and HLA-C genes. HLA class I molecules bind to peptide antigens and present these antigens to T cell receptors (TCR) of CD8$^+$ T cells. HLA class I expression levels also regulate NK cell activation. The presence of individual HLA class I genes is linked to many different disease, transplantation and therapy outcomes. An understanding of HLA class I expression and stability patterns is fundamentally important towards a better understanding of the associations of HLA class I genes with disease and treatment outcomes, and towards HLA class I targeting for vaccine development. Quantitative flow cytometry allows for assessments of variations in expression levels of HLA class I molecules in cells from a single blood donor over time, as well as averaged measurements across donors for the same allotype. Since all HLA class I molecules are structurally-related, cellular measurements of the HLA class I expression levels and stabilities of individual variants in human cells require careful choices of donors and antibodies, which are discussed here.

1. Introduction

HLA class I molecules are heterotrimeric proteins comprising a polymorphic heavy chain, an invariant light chain (β2-microglobulin; β2m) and a short peptide. HLA class I molecules control two different arms of the immune response, those mediated by CD8$^+$ T cells, and those mediated by NK cells. These molecules bind to TCR of CD8$^+$ T cells or to various antigen receptors of NK cells to induce CD8$^+$ T cell responses and regulate NK cell responses (reviewed in [1,2]). The HLA class I locus is polygenic and polymorphic (reviewed in [3]). The high degree of polymorphisms in the antigen binding sites of the HLA class I leads to a diversity of HLA antigens that are presented to the immune system (reviewed in [4]). Allelic sequence variations affect the specificities of peptide antigen binding by HLA class I proteins and determine histocompatibility during transplantation.

The expression densities of HLA class I -peptide complexes as well as their longevities on the cell surface are expected to be important determinants of CD8$^+$ T cell and NK cell responses, based on prior studies. While the well-known variations in the peptide-binding characteristics of HLA class I molecules can influence specific immune responses, differences in HLA class I expression levels can also influence immune outcomes. Indeed, previous studies point to important influences of HLA-A and HLA-C mRNA levels on immune control in HIV infections [5–7]. Multiple polymorphic sites are suggested to determine the expression level of a given HLA-A allele [7]. A variant upstream of HLA-C (rs9264942; termed −35) associates with HLA-C mRNA levels [6]. The −35 variant marks another polymorphism in the 3′ untranslated region of HLA-C, which determines micro RNA (mIR-148) binding and HLA-C mRNA levels [5]. In turn, the variations affect HLA-C protein levels, control of HIV viral loads and AIDS progression [8]. Additionally, high HLA-C expression was also identified as a risk factor for Crohn's disease [8]. Recent studies also show that regulatory polymorphisms influence HLA class II expression levels [9]. In systemic lupus erythematosus (SLE), changes in regulatory sequences led to changes in cell surface expression levels of HLA class II genes as well as to toll-like receptor ligand-induced changes to HLA class II expression. Increased HLA class II expression is suggested lead to the hyperactive immune response in lupus [9].

The inhibitory functions of HLA class I molecules in NK cell biology [10] imply that high HLA class I levels could also result in less effective

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pathogen-specific immunity. Indeed, recent studies point to the immune inhibitory influences of high HLA-A expression levels [7]. In HIV infections, high HLA-A levels associate with high viral loads and reduced CD4^{+} T cell counts. HLA class I signal sequence-derived peptides are major ligands for the assembly and cell-surface expression of HLA-E [11,12], a non-classical HLA class I molecule that engages the inhibitory NK cell receptor NKG2A (reviewed in [2]). All HLA-A's and HLA-C's and some HLA-B's have signal sequences containing an appropriate motif for HLA-E binding [11,12]. In the context of HLA-B's with HLA-E-compatible signal sequences, high HLA-A expression levels were correlated with high HLA-E expression levels, and in turn, diminished activation of NKG2A^{+} NK cells, in response to antibody-coated target cells [7].

Thus, a number of prior studies point to important allele-dependent differences in expression levels of HLA class I and HLA class II proteins, which can enhance immunity and autoimmunity or in some cases, suppress immunity. Further understanding the specifics of such differences is important for better elucidating the molecular basis of HLA influences on disease outcomes, and generating better targeted interventions in disease. The HLA-B genes are the most polymorphic among HLA class I genes and individual alleles exert strong genetic influences upon infectious diseases such as HIV [13,14], inflammatory diseases such as ankylosing spondylitis [15] and Bechet's disease [16] as well as in cancers [17]. While prior studies point to the absence of differences in mRNA expression among several HLA-B alleles [18], recent studies indicate that there are post-transcriptional effects that determine HLA-B protein levels at the cell surface [19–21]. HLA class I molecules have a complex intracellular folding and assembly pathway [22,23]. Peptides that bind HLA class I molecules typically derive from the cytosol, are transported into the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP). In the ER, peptides assemble with HLA class I heterodimers with the assistance of tapasin and the chaperone/oxidoreductase complex, calreticulin and ERp57 (reviewed in [22,23]). HLA-B allotypes are known to display varying stabilities and dependencies on assembly factors tapasin and TAP [24–28]. Such variations can influence the selection of high affinity binding peptides, resulting in variable cell surface stabilities and steady state expression levels of HLA class I complexes. Recent studies suggest HLA-B cell surface expression differences can arise due to the due to variations in their peptide repertoires [20], modes of peptide binding (which confer great stability to some peptide-HLA allotypes) [21], or specificity mismatches between the HLA class I and the transporter associated with antigen processing (TAP), which limits assembly and cell surface stability of some HLA-B allotypes in a cell-type-dependent manner [21]. In this article, we review methods for measurements of expression levels and stabilities of HLA class I allotypes, to assess the prevalence of donor, cell and allele-dependent variations.

2. HLA class I expression measurements: Choice of antibodies

In undertaking HLA class I protein expression measurements, there are important points to consider in the use of antibodies as tools. The HLA class I loci share a high degree of similarity because of the evolutionary history of the loci and gene duplication [29]. Alleles have extensive sequence conservation and thousands of variants are present within human populations. The HLA-A, HLA-B and HLA-C genes all encode HLA class I proteins. The high polymorphisms result in heterozygosity at each locus, and the presence of six different HLA class I proteins at the cell surface, for most individuals. The high degree of sequence conservation at the amino acid level between HLA class I proteins makes antibody cross-reactivity a critical issue to be considered in characterizing the properties of individual HLA class I variants in human cells.

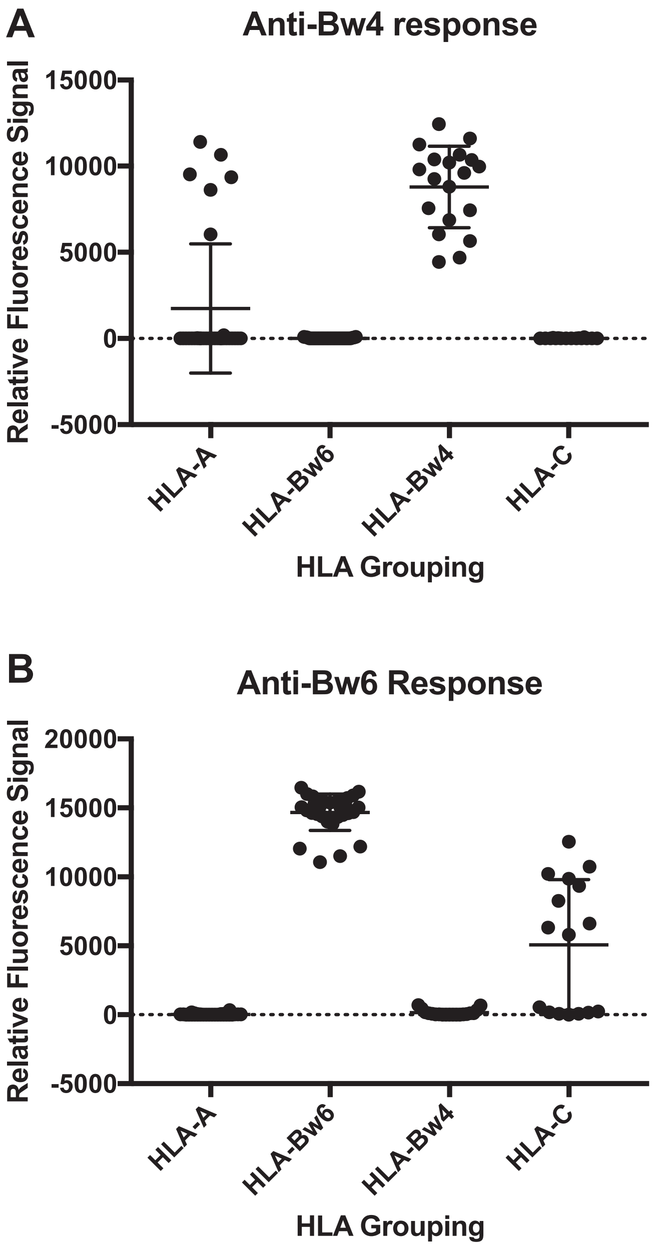


Fig. 1. Luminex-based assays to detect patient anti-HLA antibody specificities for transplantation matching. A) The patient serum shown in the top panel displays pre-existing antibodies to the "Bw4 epitopes", contained within many HLA-B allotypes and a few HLA-A allotypes. B) The patient serum shown in the lower panel displays pre-existing antibodies to the "Bw6 epitopes", contained within many HLA-B allotypes and some HLA-C allotypes.

The specificities of different HLA class I antibodies can be assessed in a high throughput manner using the LABScreen™ Single Antigen HLA Class I assay from (One Lambda, Thermo Fisher) that utilizes the Luminex® Technology. Purified HLA class I proteins from cell lines expressing a single recombinant HLA class I are coupled to the surface of a single ID Luminex® proprietary microsphere. Internally, these beads are stained with a variable ratio of fluorochromes. The unique fluorescent identity of these microspheres allows multiplexed detection of multiple analytes in a single reaction. In a classical immunoassay format, bead binding by different antibodies followed by a secondary fluorophore-conjugated antibody can be measured, to determine antibody binding patterns. Representative Luminex data are shown in Fig. 1. In these analyses, patient serum samples were analyzed for the presence of anti-HLA antibodies. In the absence of transplantation, or prior to transplantation, such antibodies can be induced by factors such as pregnancies, that expose the immune system to a different set of HLA molecules than those that are endogenously expressed. HLA-B alleles

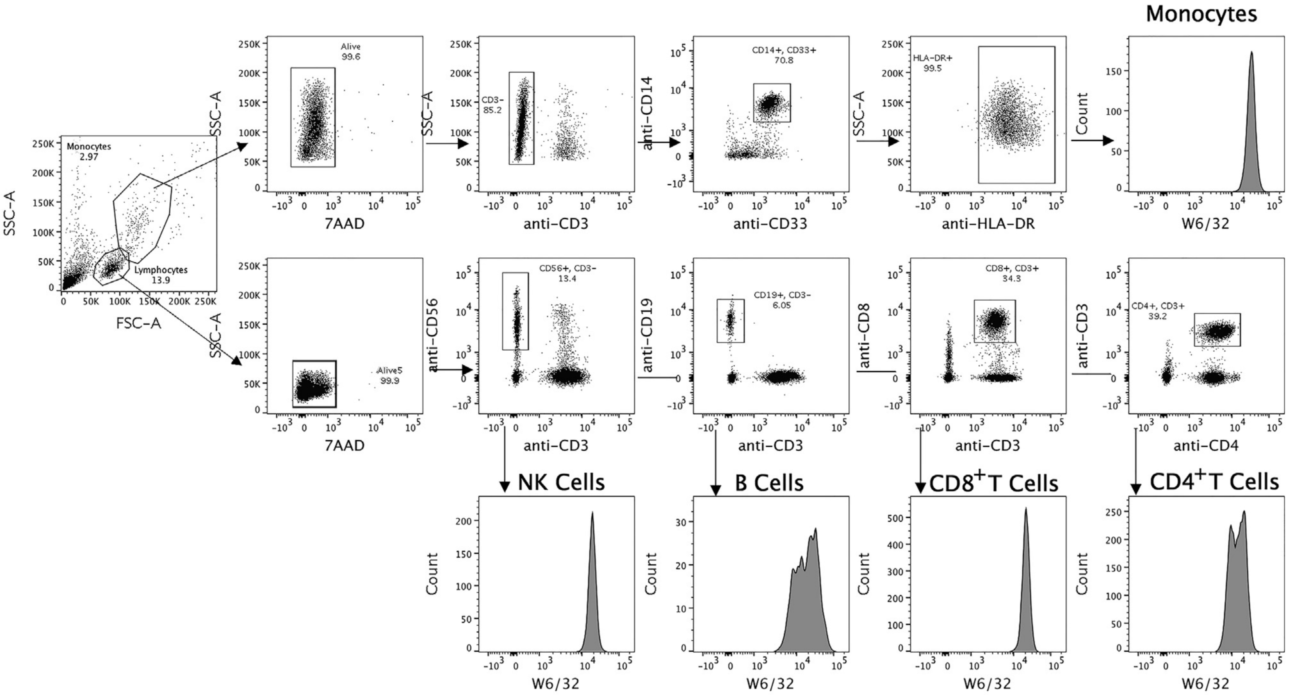


Fig. 2. Representative Leukocyte Gating Strategy as described in reference [21]. Top row: monocytes are gated based first on the forward and side scatter profiles. In general, freshly isolated PBMCs do not have many dead cells (< 5%). Cells that are alive are gated (7-AAD-negative), followed by a negative anti-CD3 gate. Generally anti-CD14 can be used in conjunction with anti-HLA-DR to obtain a double-positive population that is composed of monocytes, but here we add a third antibody (anti-CD33) to confirm the monocyte population. The histogram depicts a representative set of analyses for monocytes HLA class using the W6/32 antibody. Middle and bottom rows: Lymphocytes are gated based first on the forward and side scatter profiles. Live cells are gated (7-AAD negative) next. Final gating is based on cell-specific markers. NK cells are CD3-negative, but CD56-positive. B cells are CD19-positive, but CD3-negative. $CD8^+$ T cells are CD8 and CD3 double-positive. $CD4^+$ T cells are CD4 and CD3 double-positive. Further gating can be performed for subsets of cells within each cell type. The histogram depicts a representative W6/32 (total HLA class I) signal for NK cells, B cells, $CD8^+$ T cells, and $CD4^+$ T cells.

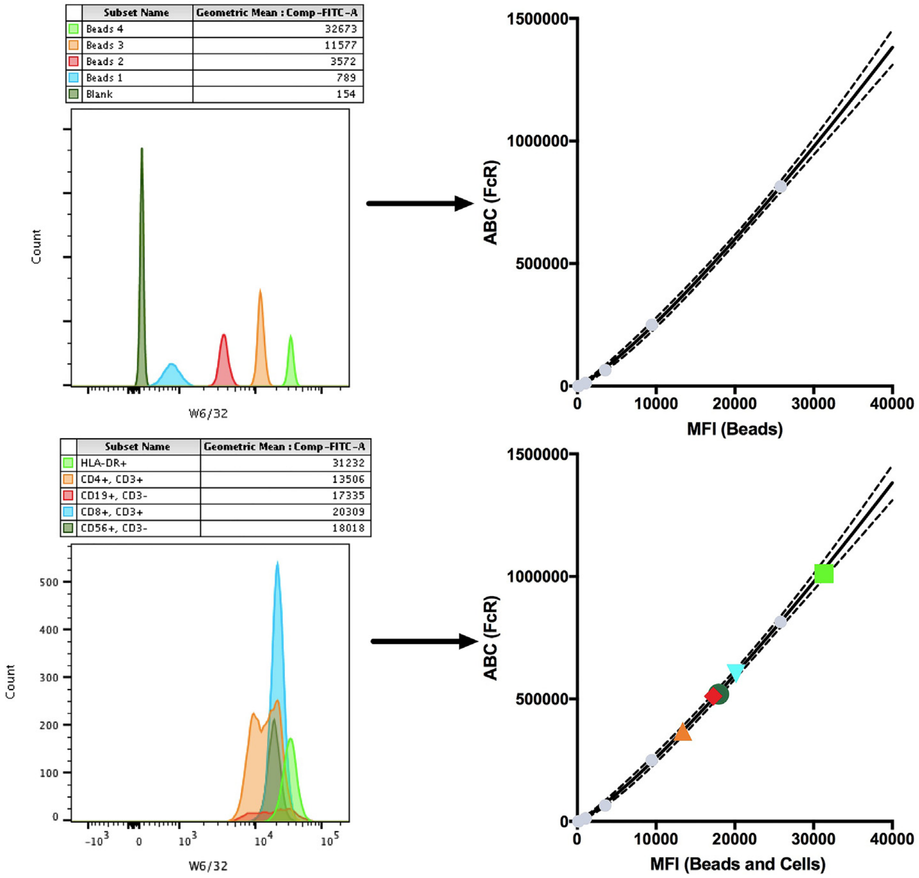
are grouped into either Bw4 or Bw6 serotypes, based on the sequences within residues 77–83 of the heavy chain [30]. The Bw6 sequence is also present in some HLA-C alleles, and the Bw4 sequence is also present in a few HLA-A alleles [21]. Fig. 1A is an example of a classical anti-Bw4 response, wherein several HLA-B allotypes with the Bw4 motif are recognized, and few HLA-A allotypes that contain the Bw4 motif are also recognized. Fig. 1B is an example of a classical anti-Bw6 response, wherein several HLA-B allotypes and some HLA-C allotypes are recognized. For single HLA-B protein expression measurements, we can ideally use the anti-Bw4 and anti-Bw6 antibodies with donors who are HLA-Bw4/Bw6 heterozygous, and lacking cross-reactive HLA-A's and HLA-C's. For such an approach, it would be important to know the full HLA class I genotypes of the blood donors, to exclude donors with cross-reactive HLA-A (for Bw4 measurements) or cross-reactive HLA-C (for Bw6 expression measurements).

As noted below, specificity of detection is necessary but not sufficient for the use of antibodies to measure relative expression levels of individual HLA class I allotypes in human cells. For allotypes whose expression levels are being compared, it is also important that relevant antibody binding affinities are in a similar range.

3. HLA class I expression measurements: Knowledge of full HLA genotypes of donor cells

High resolution typing of a large number of subjects can help screen and identify donor samples which will be useful for HLA class I expression assessments, in conjunction with particular antibodies. For characterizing the HLA class I variants at the sequence level, the samples can be typed with traditional typing methodologies that use hybridization to sequence specific oligos (SSO), PCR with Sequence Specific Primers (SSP) or Sanger sequence-based typing (SBT) or newer technologies such as Next Generation Sequencing (NGS) [31]. The traditional typing methods interrogate the exons that code for peptide binding regions. Genotype ambiguities and cis/trans ambiguities make unambiguous allele typing via these methods quite challenging. The use of traditional HLA typing combined with resources available for imputation, can allow for higher confidence in the HLA typing obtained by these technologies [32]. The advent of NGS [33] allows for simultaneous sequencing of all exons and introns to provide the complete HLA type of a blood donor. NGS can provide the sequence of the entire locus, including introns and exons, and of 5′ and 3 UTR regions that can influence HLA expression levels, via, for example, microRNA binding [5]. The additional information obtained from NGS is critical for further elucidating the molecular basis for HLA class I expression differences and for future predictions of such differences. This is particularly important for clinical laboratories, to better understand and predict transplantation, immunotherapy and vaccination outcomes.

For HLA-B expression measurements with anti-Bw4 and anti-Bw6, HLA genotyping by NGS allowed identification of Bw4/Bw6 heterogyzous donors and assessments of the presence or absence of HLA-A and HLA-C that are cross-reactive with anti-Bw4 and anti-Bw6 [21]. Since a large number of HLA-C's cross-react with anti-Bw6 (Fig. 1), there were few donors in the HLA-Bw4/Bw6 heterozygous group who also did not express an HLA-C that was cross-reactive with the anti-Bw6 [21]. However, since HLA-C expression levels are low compared to HLA-B (for example, see [34]), donors with a single cross-reactive HLA-C were included in the study [21], with the expectation that anti-Bw6-based expression measurements would yield values that are predominantly driven by the relevant HLA-B. Thus, a knowledge of the full HLA class I genotypes allows for exclusion or minimization of cross-reactive alleles in cells used for individual HLA class I expression measurements.



	Subset Name	Geometric Mean : Comp-FITC-A
🟩	Beads 4	32673
🟧	Beads 3	11577
🟥	Beads 2	3572
🟦	Beads 1	789
🟩	Blank	154

	Subset Name	Geometric Mean : Comp-FITC-A
🟩	HLA-DR+	31232
🟧	CD4+, CD3+	13506
🟥	CD19+, CD3-	17335
🟦	CD8+, CD3+	20309
🟩	CD56+, CD3-	18018

Fig. 3. Antibody Binding Capacity Calculations. Top panel: The geometric MFI values of the antibody florescence of the bead sets and the FcR quantifications from the manufacturer are used generate a standard curve (Log/Log). Bottom panel: The Geometric MFI values of the antibody florescence of the cell subsets is placed on the standard curve and used to generate ABC values. Colored points represent geometric mean W6/32 values for the listed cell subsets.

4. HLA class I expression measurements: Quantitative flow cytometry

For examining HLA class I surface expression on human peripheral blood mononuclear cells (PBMC) subsets by flow cytometry, cells can be stained with fluorescently-labeled antibodies to allow for gating on specific cell types of interest. Anti-CD3, anti-CD14, anti-CD33 and anti-HLA-DR allow gating on monocytes (Fig. 2: Top row), whereas anti-CD3, anti-CD4, anti-CD8, anti-CD19, and anti-CD56 can be used to identify specific lymphocytes populations (Fig. 2: Bottom row). Lymphocytes are further divided into populations of NK cells (anti-CD3$^-$ anti-CD56$^+$), B cells (anti-CD3$^-$ anti-CD19$^+$), CD4$^+$ T cells (anti-CD3$^+$ anti-CD4$^+$), and CD8$^+$ T cells (anti-CD3$^+$ anti-CD8$^+$).

Once a broad spectrum or selective antibody specificity has been identified using the Luminex assay (Fig. 1), flow cytometry assays can be used to quantify expression of HLA class I on the cell surface and generate a *globally normalized* value called the antibody binding capacity (ABC) (Fig. 3) [35]. ABC values standardize antibody-based measurements across multiple time-separated experiments within a donor and across multiple donors, for example, those expressing a given HLA class I allele. Cell surface mean fluorescence (MFI) intensities obtained with a particular antibody are plotted against MFI values obtained from parallel staining (with the same antibody) of beads (procured from commercial sources such as Bangs Laboratory) displaying varying known amounts of Fc receptors (FcR) (quantified by the manufacturer). Both cells and beads are incubated in parallel with saturating amounts of antibodies. The log MFI values obtained from the beads are plotted against the log of the FcR content of the beads to generate a standard curve (Fig. 3, top panel). The geometric MFI values obtained from the cell subsets are input into the standard curve, which allows for quantification of the number of antibody binding sites on each cell subset, termed ABC, which is proportional to the number of molecules (detected by the antibody) per cell [36] (Fig. 3, lower panel). MFI values are relative fluorescence values which cannot be compared for repeat time-disparate measurements of the same donors or multiple donors. On the other hand, ABC values are globally normalized representations of expression levels, which can be compared across samples and time.

Importantly, expression levels can only be compared for measurements with a single antibody, as different antibodies can have different affinities for their ligands. For allotypes that bind to a given antibody (for example those within the Bw4 or Bw6 groups), the Luminex assay can be used to determine relative binding specificities as described in Fig. 1, but also the relative binding preferences. For example, among allotypes that bind anti-Bw4 or anti-Bw6 (Fig. 1), the assay can also provide quantitative information relating to binding preference differences, in addition to specificity differences. This can be done by normalizing the binding signal relative to that obtained with an antibody such as W6/32 [37], an HLA class I antibody that has similar binding preferences for a majority of tested HLA class alleles (Fig. 4, left panel). Such normalization can control for any differences in amounts of HLA class I coupled to the Luminex system beads.

Based on its broad and relatively equivalent recognition of the majority of HLA class I allotypes, W6/32 is a good antibody to use for assessments of total HLA class I expression variations (all alleles) among healthy donors. A representative set of HLA class I expression measurements with the W6/32 antibody is shown in Fig. 4. The studies were performed on fresh PBMCs derived from healthy human donors in the Ann Arbor, MI area. There are significant donor to donor variations in total HLA class I expression between individuals, and additional expression differences between cell types (Fig. 4 right panel). Monocytes have the highest cell surface HLA class I expression of all the cell types examined, with individual lymphocyte subsets falling into lower ranges.

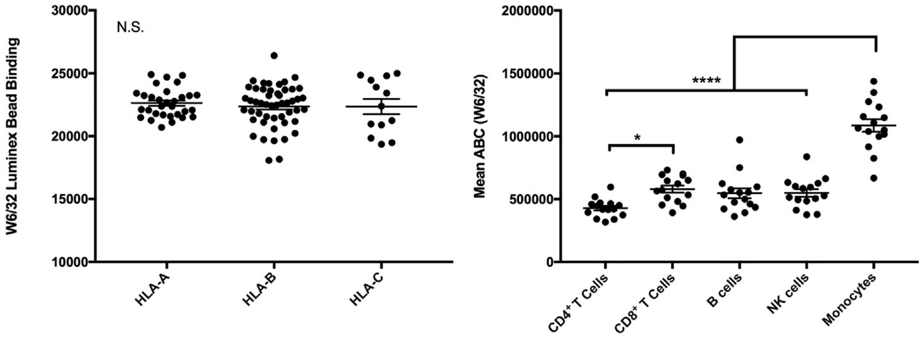


Fig. 4. W6/32 Luminex Beads and ABC Values for W6/32-based measurements from healthy donor PBMC. Left panel: W6/32 binds similarly to a majority of individual HLA class I alleles, based on the Luminex assay. Right panel: Mean W6/32 ABC values for 15 donors from an Ann Arbor, MI cohort of various cells identified within PBMC. Each dot represents the mean of four separate measurements on individual donors, and significance is based on a one-way ANOVA analysis. Measurements are shown from a subset of donors described in reference [21].

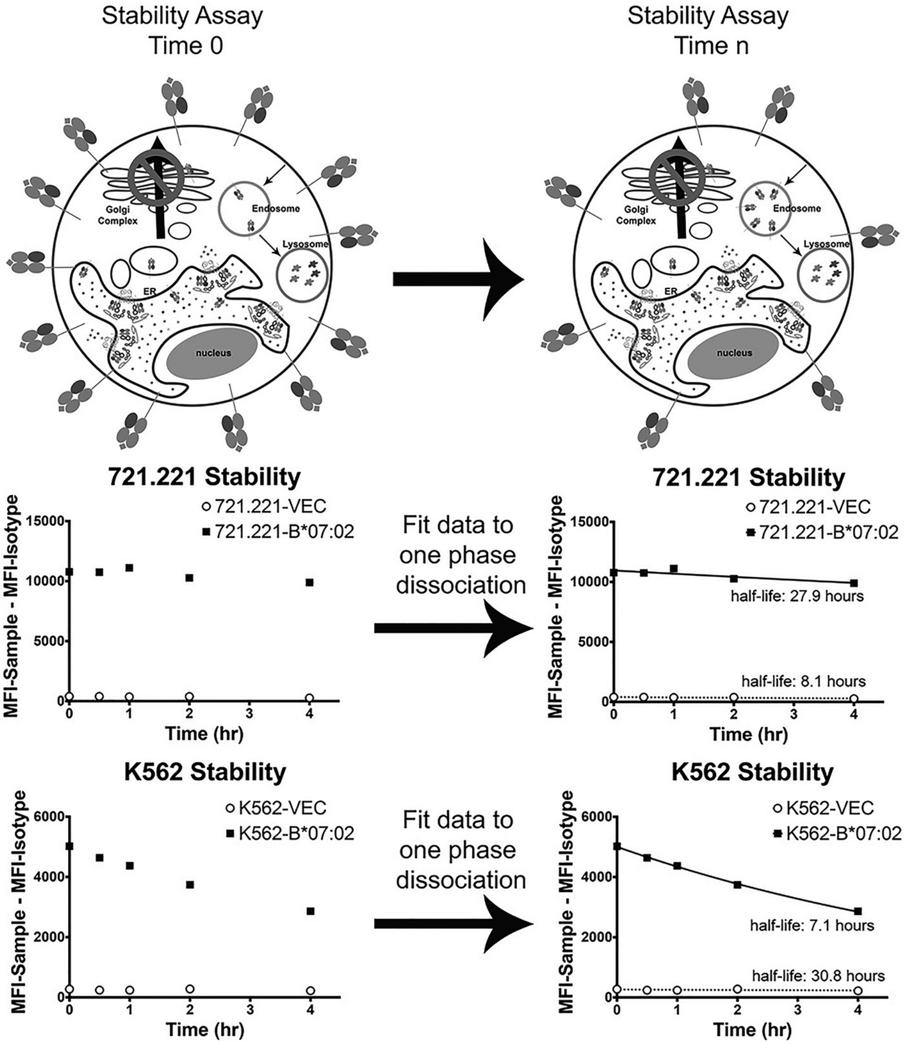


Fig. 5. HLA class I cell surface stability measurements. Top panel: A schematic representation of the cell surface HLA class I at time 0 and time n. BFA has prevented trafficking of new HLA class I from the ER to the cell surface and the amount of remaining HLA class I on the cell surface is diminished at time-point n relative to the starting value at time point 0. Middle and Lower panels: Representative data depicting primary data points of W6/32 MFI signal and the signal decay (over a 4-hour period) from the surface of BFA-treated HLA-null 721.221 (a B cell line; middle panel) or K562 (a chronic myelogenous leukemia cell line; lower panels) cells that were retrovirally transduced with a virus encoding HLA-B*07:02 (solid black) or a virus lacking HLA-B (open circles). The data are fit to a one-phase decay model and the half-lives calculated. Different decay rates are obtained for the same HLA-B allotype when expressed in the different cells.

Similar methods as those shown with W6/32 (Fig. 4) can be used to examine expression levels of individual HLA class I allotypes. Monoclonal antibodies against the Bw4 and Bw6 epitopes of HLA-B alleles are commercially available (One Lambda and Miltenyi). When used with donors who are heterozygous for HLA-Bw4 and HLA-Bw6, these monoclonals can be used for single HLA-B expression measurements [21], after ensuring (i) the HLA class I cross-reactivity patterns are individually assessed, and donors chosen to avoid or minimize cross-reactivity and (ii) antibody binding preference differences within the Bw4 and Bw6 groups are accounted for and (iii) the data are normalized relative to the total HLA class I (W6/32) signals, to account for allele-independent donor–donor variations that influence expression of all HLA class I alleles. Such measurements have indicated allele-dependent and cell-dependent variations in HLA-B expression levels [21].

5. HLA class I stability (half-lives) measurements

In addition to the amounts of HLA class I proteins that are expressed on the cell surface, it is also important to understand the cell surface longevities (half-lives) of these proteins. A given cell has thousands of different peptides bound to each HLA class I on the cell surface [38]. For HLA class I proteins, the cell surface stability can be measured following cell incubation with Brefeldin A (BFA), a protein that blocks trafficking from the Golgi apparatus to the cell surface [39]. Such experiments are performed by incubating cells in complete media with a minimal amount of BFA (to minimize cell toxicity) for specific time points. As BFA blocks trafficking of newly synthesized HLA class I molecules to the cell surface, changes in HLA class I expression over time under these conditions reflect the loss of cell surface HLA class I, typically via internalization and degradation (Fig. 5). The primary data can be fit to the exponential decay model and a half-life value can be extracted. This type of method has previously been used to determine how tapasin, an ER chaperone, influences the peptide repertoire of particular HLA class I allotypes [40]. Similar methods can be applied to determine intrinsic half-life differences for the same HLA class I allotype from different cells, as well as between different HLA class I allotypes in a given cell type. Half-life values for a given HLA class I allotype in healthy *vs*. infected cells can also be compared, to indirectly determine changes in peptidome content. For stability assessments of individual HLA class I allotypes on primary cells, the choices of donor and antibody have to be made similarly to those described for the expression measurements. The cell surface half-life of an individual HLA class I allotype is a measure of the averaged stability of all the corresponding HLA-peptides complexes on the cell surface. Our studies indicate correlations between steady-state HLA-B cell surface expression and half-lives [21].

6. Summary and conclusions

HLA class I expression and stability differences are expected to influence the strength of TCR-mediated signals as well as those of NK cell receptors. This review highlights important considerations for accurate assessments of expression and stabilities of individual HLA class I allotypes. Over the past roughly 3 to 4-year period, we have undertaken measurements of expression levels and stabilities of individual HLA-B allotypes, based on the methods described above, and using the anti-Bw4 and anti-Bw6 antibodies. We observe significant allele and cell-dependent differences in HLA-B expression and half-life. The picture that is emerging points to important differences in the antigen acquisition pathways of HLA-B molecules that influence expression and stability in a cell-type-dependent manner [21]. These, and other findings highlighted above [5–7,9,19–21], that indicate variations in HLA class I and class II expression levels, have broad implementation for clinical research settings where HLA expression levels could be crucial determinants of the outcomes of immunization, immunotherapy trials and transplantation.

Acknowledgements

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