POSITION 97 OF HLA-B, A RESIDUE IMPLICATED IN ANKYLOSING SPONDYLITIS PATHOGENESIS, PLAYS A KEY ROLE IN CELL SURFACE FREE HEAVY CHAIN EXPRESSION

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Objective

Association of position 97 (P97) residue polymorphisms in human leukocyte antigen (HLA)-B, including HLA-B*27, with Ankylosing Spondylitis (AS) has recently been reported. We studied the effect of P97 variations on cell surface expression of the AS-associated HLA-B*27 and HLA-B*51, and the AS-protective HLA-B*7.

Methods

Flow cytometry was used to measure surface expression of HLA-B*27 in C1R/HeLa cells expressing HLA-B*27 (N97) and six mutants at P97 (N97T, N97S, N97V, N97R, N97W and N97D). TAP-deficient T2, Tapasin-deficient 220, β2m-deficient HCT15 and ERAP1 or β2m-CRISPR/Cas9-knockout HeLa cells were used to provide evidence for specific protein interactions. Surface expression of HLA-B*7/HLA-B*51 P97 mutants was also studied.

Results

Mutation of HLA-B*27 P97 to the AS risk residue Threonine increased cell surface free heavy chain (FHC) expression. Protective residues (Serine or Valine) and non AS-associated residues (Arginine or Tryptophan) did not alter FHC expression. The N97D mutation reduced expression of conventional and FHC forms of HLA-B*27. Differences in FHC expression levels between HLA-B*27, HLA-B*27-N97T and HLA-B*27-N97D were dependent on the presence of functional β2m. HLA-B*7, which has an AS-protective Serine at P97, expressed lower levels of FHC than HLA-B*27 or HLA-B*51. Introduction of Asparagine at P97 of both HLA-B*7 and HLA-B*51 increased FHC expression.

Conclusion

The nature of P97 residue affects surface expression of HLA-B*27, B*7 and B*51; with AS-associated residues giving rise to higher FHC expression levels. The association of P97 amino acid polymorphisms with AS could be, at least in part, explained by its effect on HLA-B*27 FHC cell surface expression.

INTRODUCTION

Ankylosing Spondylitis (AS) is a common form of inflammatory arthritis that is strongly associated with possession of the human leukocyte antigen (HLA)-B*27. [1] A recent fine-mapping study of the major histocompatibility complex (MHC) has reported the strong association of AS with carriage of particular amino acids at position 97 (P97) in HLA-B. [2] Indeed, conditional analysis suggested that P97 contributed much of the genetic risk ascribed to HLA-B*27. Six amino acid variants at P97 of HLA-B with differing effects on AS risk were identified and are shown in table 1. Asparagine (N) and Threonine (T) conferred increased risk of AS. Serine (S) and Valine (V) were protective residues, and no association was found for Arginine (R) and Tryptophan (W). Located in the α2 domain of HLA-B*27, P97 lies within a β-sheet that contributes to the C/F peptide-binding pocket, but may also contact β 2-microglobulin (β2m) (figure 1A). [3 4] Notably, the adjacent position 96 (P96) has indeed been shown to contact β2m in molecular-graphics analysis using crystal structures of HLA-A2.1 and HLA-Aw68.1. [5] Mutation of N at P97 to Aspartic acid (D) has been reported to decrease cell surface expression of HLA-B*27:04 free heavy chains (FHCs) and classical complexes. [6]

Table 1 Association of P97 residues of HLA-B with AS

P97 residues	Association with AS	*****Odds ratio	*****P value	Examples of HLA-B allele
N	Risk	16.51	$< 10^{-300}$	***27:05**
T	Risk	1.12	4.5×10^{-3}	***51:01**
				*13:02
R	Non-associated	1	1	*15:01
				*40:01
				*47:01
W	Non-associated	1	0.95	*14:01
S	Protective	0.86	4.81×10^{-8}	***07:02**
				*40:02
V	Protective	0.68	1.41×10^{-8}	*57:01
D	Synthetic	N/A	N/A	N/A

*** Statistics from Cortes A. et al. Nat Commun, 2015, Fisher's exact test used for P value.** The HLA-B allotypes investigated in current study, HLA-B*27:05, HLA-B*51:01 and HLA-B*07:01, are highlighted in bold. Of note, Aspartic acid (D) is not present in P97 of HLA-B.

The mechanism by which HLA-B*27 confers AS disease susceptibility remains unclear. Presentation of arthritogenic peptides by HLA*B27 to CD8+ T cells has been hypothesized.

However, this hypothesis is challenged by the finding that CD8+ T cells are not essential to the pathogenesis of arthritis in the HLA-B*27-trangenic rat model. [7 8] HLA-B*27 has been shown to both misfold in the endoplasmic reticulum (ER), and also to be expressed as FHC forms on the cell surface. [9-13] Cell surface HLA-B*27 FHCs can bind to killer cell immunoglobulin-like receptors including KIR3DL2, which is expressed on natural killer (NK) cells and CD4+ T cells, and can promote Th17 responses in AS patients. [14-16] We hypothesized that variations of residues at P97 might contribute to AS pathogenesis through altering cell surface HLA-B*27 FHC expression.

To test this hypothesis, we studied the effect of P97 residue mutations on HLA-B*27:05 (hereafter referred to as HLA-B*27) FHC expression. CRISPR-Cas9-knockout, siRNA-silenced or naturally-deficient cells were used to study the relative effects of proteins involved in the antigen presentation pathway. Here we show that the nature of the P97 residues affects cell surface expression of both HLA-B*27 FHCs and classical complexes, and we provide evidence that β2m plays an important role. Cell surface expression of HLA-B*7:02 and HLA-B*51:01 (hereafter referred to as HLA-B*7 and HLA-B*51 respectively) is also affected by P97.

MATERIALS AND METHOD

Cell lines

HeLa and C1R cells expressing HLA-B*27 and six mutants at P97 (N97T, N97S, N97V, N97R, N97W and N97D) were generated using lentiviral constructs described previously. [17] HeLa and C1R cells expressing HLA-B*7 and its P97 mutants (S97N, S97T and S97D), HeLa and 221

cells expressing HLA-B*51 and its P97 mutants (T97N and T97D) were also generated using lentiviruses. TAP-deficient T2 and tapasin-deficient LBL721.220 (220) lymphoblastoid cells, and the β2m-deficient HCT15 cancer cell line have been previously described. [18 19] All cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 0.1 mg/ml of streptomycin, and 100 units/ml of penicillin (R10).

Flow cytometry

The HC-10 antibody (mouse IgG2a, specific for HLA class I free heavy chains), was used to stain cell surface expression of HLA-B*27, B*7 and B*51 free heavy chains. [20] The ME-1 antibody (mouse IgG1, specific for classical β2m-associated HLA-B27, B7, B42, B67, B73 and Bw22) was used to measure classical HLA-B*27 and B*7 complexes on the cell surface. W6/32 antibody (mouse IgG2a, specific for classical β2m-associated HLA-A, B or C) was used to stain surface-expressed classical HLA-B*51 complexes in C1R and 221 cells. APC- or PE-conjugated anti-mouse IgG antibody was used as secondary antibody. Dead cells were excluded using LIVE/DEAD® Fixable Violet Dead Cell Stain Kit (Life Technologies). BD LSRFortessa™ and Diva software were used. The latter converts channel value into fluorescence intensity using a logarithmic algorithm, therefore geometric mean fluorescence intensity (GMFI) was used to quantify the intensity of HC-10, ME-1 and W6/32 staining.

Plasmid construction

To generate plasmids expressing P97 mutants, point mutations were delivered into PHR-SIN lentiviral plasmids encoding HLA-B*27, B*7 and B*51 using QuickChange Site-Directed

Mutagenesis Kit (Agilent Technologies). Primers were designed for generation of HLA-B*27 mutants at P97 (TCTCACACCCTCCAGACGATGTATGGCTGC for N97T, TCTCACACCCTCCAGAGCATGTATGGCTGC for N97S, TCTCACACCCTCCAGGTGATGTATGGCTGC for N97V, TCTCACACCCTCCAGAGGATGTATGGCTGC for N97R, TCTCACACCCTCCAGTGGATGTATGGCTGC for N97W, TCTCACACCCTCCAGGATATGTATGGCTGC for N97D). For generation of HLA-B*7 mutants at P97, following primers were used: TCTCACACCCTCCAGAATATGTATGGCTGC for S97N, TCTCACACCCTCCAGACGATGTATGGCTGC for S97T, TCTCACACCCTCCAGGATATGTATGGCTGC for S97D. HLA-B*51 mutants were generated using TCTCACACTTGGCAGAATATGTATGGCTGC (T97N) and TCTCACACTTGGCAGGATATGTATGGCTGC (T97D).

The β2m plasmid was purchased from Origene (SC117632); loss-of-function β2m-C25W plasmid was generated using the same kit described above (primer: CAAATTTCCTGAATTGGTATGTGTCTGGG).

Generation of ERAP1- or β2m -knockout HeLa cells using Cas9 nuclease

A previously described clustered regularly-interspaced short palindromic repeats (CRISPR)-Cas9 plasmid, pSpCas9(BB)-2A-GFP (PX458), was acquired from Addgene. [21] sgRNA for ERAP1 or β2m was designed and cloned into the plasmid (ERAP1: CACCGACCCAGACACATAGCAATTC and AAACGAATTGCTATGTGTCTGGGTC, β2m:

CACCGCTCACTGTGATGGTTATTAG and AAACCTAATAACCATCACAGTGAGC). The PX458-ERAP1 or PX458-β2m plasmid was transfected into HeLa cells using Genejuice (Merck). β2m-knockout HeLa cells were further purified using PE-conjugated W6/32 antibody (eBioscience), anti-PE beads and LD columns (Miltenyi).

Statistics

Results are expressed as mean and standard deviation (figure 1B, 1C, 3B and 4). The statistical significance of differences between means was assessed using an unpaired two-tailed t-test (figure 1B, 1C, 3B and 4). A p value <0.05 was considered significant.

RESULTS

P97 amino acid variants affect HLA-B*27 expression on the cell surface.

We first investigated the effect of position 97 (P97) residue variations on HLA-B*27 expression in two human cell lines. Both Asparagine (N, found in HLA-B*27) and Threonine (T) are associated with increased risk of AS. [2] Figure 1B, C shows that mutation of N at P97 in HLA-B*27 to T increases surface free heavy chain (FHC) expression (measured by the HC-10 antibody) in both C1R and HeLa cells. Notably this occurs without changing the expression level of classical ME-1-reactive HLA-B*27 complexes (see online supplementary figure S1 for FACS plots). In contrast, mutations to protective or un-associated resides do not affect either HC-10 or ME-1 expression (figure 1B, C). Thus N97S, N97R and N97W do not significantly alter levels of HC-10 and ME-1-reactive HLA-B*27. N97V modestly increases HLA-B*27

FHC expression in C1R but not in HeLa cells. The mutation of N to Aspartic acid (D) at P97, which is not found in human HLA-B allotypes, has been reported to abolish the expression of HC-10 and ME1-reactive HLA-B*27:04 molecules.[6] We therefore also studied the N97D HLA-B*27:05 mutant, and found that this mutation significantly reduced expression of both FHC and classical HLA-B27 complexes on the surface of C1R and HeLa cells (figure 1B, C). These results show that the nature of the amino acid at P97 affects cell surface expression of HLA-B*27 FHCs and classical complexes.

We next tried to decipher the mechanisms underlying the effect of P97 on HLA-B*27 expression. Here we focused on comparisons of wild type HLA-B*27 with two mutants that resulted in changes of HLA-B*27 free heavy chain (FHC) expression, N97T and N97D (figure 1B, C). Similar stability of cell surface classical HLA-B*27 between HLA-B*27, N97T and N97D mutants was observed, suggesting that P97 more likely affects maturation or trafficking of HLA-B*27 inside cells (see online supplementary figure S2). Following removal of cell surface HLA-B*27 complexes using activated papain, both N97D FHCs and classical complexes on the cell surface recovered more slowly than HLA-B*27 (see online supplementary figure S3). Interestingly, N97T did not affect the recovery of cell surface classical HLA-B*27 complexes, but promoted the recovery of FHCs at cell surface. We also found that levels of intracellular HLA-B*27 FHCs were highest in the N97D mutant, followed by N97T, and then wild type HLA-B*27 (see online supplementary figure S4).

The effect of N97T and N97D mutations on HLA-B*27 expression is largely dependent on β2m, but not TAP, tapasin or ERAP1.

We then sought to identify the proteins that might interact differentially with different P97 residues to affect cell surface HLA-B*27 expression. Both the transporter associated with antigen processing (TAP) and tapasin are key chaperones for the loading of peptide ligands onto MHC class I molecules, and are important for MHC class I expression.[22] We therefore first tested whether the altered HLA-B*27 FHC expression observed for N97T and N97D mutants was still seen using TAP- and tapasin-deficient cells (figure 2A, B). TAP-deficient T2.B*27 cells did not express HLA-B*27 FHCs and only expressed low level of classical HLA-B*27 complexes when compared to untransfected T2 cells (figure 2A). The N97D mutation nearly abolished expression of classical HLA-B*27 complexes on the cell surface. Significant reduction of HC-10 and ME-1 was found in tapasin-deficient 220 cells expressing HLA-B*27-N97D compared to the wildtype (figure 2B). Similar to its effect in C1R and HeLa cells, the N97T mutation increased HLA-B*27 FHC without affecting classical complexes. Deficiency of TAP and tapasin in T2 and 220 respectively was confirmed by western blot (see online supplementary figure S5A, B). Thus the effect of P97 amino acid on HLA-B*27 expression is unchanged in the absence of TAP or tapasin.

Endoplasmic reticulum aminopeptidase 1 (ERAP1) polymorphisms are strongly associated with ankylosing spondylitis (AS) in patients carrying HLA-B*27, [1] and can promote cell surface expression of HLA-B*27 FHCs. [23] We therefore asked if the absence of ERAP1 changes the effect of the P97 mutation on HLA-B*27 expression. CRISPR-Cas9 was used to knockout

endogenous ERAP1 in HeLa cells (see online supplementary figure S5C for western blot showing efficacy of ERAP1 knockout). Figure 2C shows that, the effect of N97T or N97D mutations on HC-10 or ME-1 staining shown in figure 1C is not affected by the knockout of ERAP1 in HeLa cells. These results strongly suggest that N97T and N97D mutations do not modulate HLA-B*27 FHC expression through interacting with ERAP1.

As shown in figure 1A, P97 locates at the bottom of the HLA-B*27 peptide-binding groove and might contact β2m. Moreover, the adjacent position 96 (P96) has been shown to contact β2m in the crystal structure of HLA-A2.1 and HLA-Aw68.1. [5] We therefore hypothesized that β2m might be important for the effect of P97 mutation on HLA-B*27 expression. Indeed, in β2m-deficient cancer cell line HCT15, no difference in FHC expression between HLA-B*27, HLA-B*27-N97T and HLA-B*27-N97D was observed (figure 2D, see online supplementary figure S5D for western blot and flow cytometry confirming the deficiency of β2m).

Differential surface FHC expression between HLA-B*27, HLA-B*27-N97T and HLA-B*27-N97D is only observed in the presence of β2m.

To confirm the results observed in HCT15 cells, we used CRISPR-Cas9 to generate a β2m-knockout HeLa cell line. We found very similar results (ie N97, N97T and N97D heavy chains are expressed at equal levels) (figure 3A). Absence of β2m protein expression in western blot and abolishment of W6/32-reactive molecules on the cell surface confirmed the successful knockout of β2m in HeLa (see online supplementary figure S6). We also found that, in β2m-knockout HeLa cells, the effect of N97T or N97D mutation on HLA-B*27 expression was

restored by transfection of wildtype-β2m, but not control plasmid or a β2m-C25W loss-of-function mutant (figure 3B). This β2m mutant has been shown to be dysfunctional for HLA class I expression due to misfolding and degradation of β2m. [24] These results strongly suggest that the differential surface expression observed between HLA-B*27, N97T and N97D mutant is dependent on the presence of functional β2m.

P97 amino acid substitutions affect HLA-B*7 and HLA-B*51 expression on the cell surface.

We lastly examined the effect of P97 residue mutations on the expression of two AS-associated non-B*27 HLA-B alleles. HLA-B*7 is protective against AS, carries Serine (S) at P97 and expresses lower FHC levels than HLA-B*27 (see online supplementary figure S7). Mutation of S to Asparagine (N), a residue conferring increased risk of AS, significantly increased surface HLA-B*7 FHC expression in both C1R and HeLa cells (figure 4A, B). Mutation of S to Threonine (T), an AS-predisposing residue, also significantly increased FHC expression in C1R cells (figure 4A). Interestingly, HLA-B*51, which is AS-associated and already carries the AS risk residue Threonine (T) at P97, constitutively expresses FHC at a level as high as that of HLA-B*27 (see online supplementary figure S7). HLA-B*51-T97N further increased the expression of FHC on surface of both C1R and 221 cells (figure 4C, D). As with HLA-B*27, both HLA-B*7 and HLA-B*51 surface expression was significantly reduced by mutation of P97 to Aspartic Acid (D).

DISCUSSION

The amino acid present at position 97 (P97) of the HLA-B heavy chain has recently been shown to be a key determinant of the risk of developing Ankylosing Spondylitis (AS). [2] In the current study we show that the nature of the P97 residue plays a key role in HLA-B*27 free heavy chain (FHC) expression (summarized in online supplementary table S1). Mutation of P97 to the AS risk residue Threonine, but not to the protective residues Serine or Valine, or to the non-associated residues Arginine or Tryptophan, increased cell surface HLA-B*27 FHC. We also studied two additional HLA-B alleles carrying different P97 residues. For HLA-B*7, weakly AS-protective, and HLA-B*51, weakly AS-predisposing, FHC expression on cell surface was also increased by mutation of the P97 residue to Asparagine (N), a residue conferring the highest risk of disease. Our results thus provide a possible explanation for the genetic association of P97 amino acids and Ankylosing Spondylitis (AS) through altered HLA-B FHC expression.

We also show for the first time that the presence of β2m is essential for the effects of the N97T and N97D mutations on HLA-B*27 expression. Differences in FHC expression between HLA-B*27, HLA-B*27-N97T and HLA-B*27-N97D disappeared in the absence of β2m, and were restored by reconstitution of functional β2m. One possible mechanism is that P97 alters the strength of association with β2m and therefore influences FHC expression due to subsequent dissociation of HLA class I complexes, either at the cell surface or inside the cells. Considering the similar stability of cell surface classical HLA-B*27 between HLA-B*27, N97T and N97D mutants, P97 likely affects maturation or trafficking of HLA-B*27 inside cells. The N97D mutant reduced the rate in recovery of cell surface HLA-B*27 FHCs and classical complexes, whereas N97T promoted the recovery of cell surface FHCs without affecting classical complexes.

One possible explanation is that HLA-B*27-N97D heavy chains have such poor affinity for β2m that they rarely pass the quality control in the ER, resulting in low expression levels of both classical complexes and FHCs on the cell surface. By contrast, HLA-B*27-N97T heavy chains likely have weak but sufficient association with β2m to allow complexes to exit the ER, but subsequently dissociate during trafficking to the cell surface leading to elevated FHC on the cell surface. Such a model is shown in cartoon form in figure 5. The hierarchy of HLA-B*27, N97T and N97D in affinity with β2m is supported by our finding that levels of intracellular HLA-B*27 FHCs were highest for the N97D mutant, followed by N97T, and then wild type HLA-B*27.

Notably, mutation of P97 to the protective residues (S and V) did not significantly reduce the expression level of HLA-B*27 FHC on the cell surface (figure 1B, C). One explanation is that the effect of this mutation has been overrided by other inherent features of HLA-B*27 that contribute to its high level of FHC expression. Indeed, formation of HLA-B*27 heavy chain homodimers through cysteine at position 67 and its slow folding kinetics have both been implicated in the generation of HLA-B*27 FHCs. [18 19 25 26]

In addition to HLA-B*27, cell surface expression of HLA-B*7 and HLA-B*51 was also affected by residue variations at P97, suggesting that this may be a more general phenomenon for HLA-B allotypes. However, the effects of different P97 residues may occur in an allotype-specific context. Indeed, the different levels of FHC expression seen between 97N and 97S in HLA-B*7 were not observed in HLA-B*27. Moreover, the mutation of T to N increased FHC expression for HLA-B*51 whereas reverse effect was seen for HLA-B*27.

Of note, cell surface expression levels of classical HLA-B*27 molecules were relatively little affected by the N97T mutation in all cell lines studied, apart from the increase in TAP-deficient T2 cells. The observation that N97D nearly abolishes ME-1 staining in T2 cells suggests that TAP does not interact with amino acids at P97. One possible explanation is that the available peptide pool in the ER of TAP-deficiency T2 cells happens to have higher affinity with the N97T mutant than with HLA-B*27 FHC.

The molecular basis for the association of HLA-B27 with AS remains mysterious. A key pathogenic role for P97 of HLA-B has recently been reported. [2] Our data suggest that a possible functional explanation for this observation lies in the effect of this residue on the assembly of HLA-B with β2m. Further detailed biochemical study of these results needs to be carried out to understand the mechanism leading to differential FHC expression at the cell surface.

Overall, our data show that the P97 residue substantially influences expression levels of HLA-B*27 free heavy chains. The association of P97 amino acid polymorphisms with AS could therefore, at least in part, be explained by its significant effects on HLA-B free heavy chain cell surface expression.

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FIGURE LEGENDS

Figure 1 Location of P97 in HLA-B*27 crystal structure and effects of P97 residue substitutions on HLA-B*27 cell surface expression. Location of P97 in HLA-B*27 structure is shown in side and bottom views (A). C1R (B) or HeLa (C) cells were transduced using lentiviruses to express equal amounts of HLA-B*27 (N97) and six mutants at position 97 (N97T, N97S, N97V, N97R, N97W and N97D). HLA-B*27 free heavy chain (HC-10 antibody) and classical HLA-B*27 complex (ME-1 antibody) cell surface expression is shown. Results are expressed as mean and standard deviation. P value was determined using unpaired two-tailed t-test (** = $P<0.01$, *** = $p<0.001$). All experiments were repeated three times.

Figure 2 Differences in free heavy chain expression levels between HLA-B*27, HLA-B*27-N97T and HLA-B*27-N97D disappear in the absence of β2m, but not TAP, tapasin or ERAP1. TAP-deficient T2 cells (A), tapasin-deficient 220 cells (B), ERAP1-CRISPR-knockout HeLa cells (C) and β2m-deficient HCT15 cells (D) were transduced using lentiviruses to express HLA-B*27(N97), HLA-B*27-N97T and HLA-B*27-N97D mutants. HLA-B*27 free heavy chain (HC-10 antibody) and classical HLA-B*27 complex (ME-1 antibody) cell surface expression is shown. Representative results are shown as FACS plot. All experiments were repeated three times.

Figure 3 Differential expression between HLA-B*27, HLA-B*27-N97T and HLA-B*27-N97D is only observed in the presence of β2m. β2m-CRISPR-knockout HeLa cells (A) were transduced using lentiviruses to express HLA-B*27(N97), HLA-B*27-N97T and HLA-B*27-N97D mutants. β2m-knockout HeLa cells were co-transfected with HLA-B*27/HLA-B*27-

N97T/HLA-B*27-N97D DNA plasmid and β2m/loss-of-function β2m-C25W mutant DNA plasmid (B). HLA-B*27 free heavy chain (HC-10 antibody) and classical HLA-B*27 complex (ME-1 antibody) cell surface expression is shown. Representative results are shown as FACS plots (A) or expressed as mean and standard deviation (B). P value was determined using unpaired two-tailed t-test (** = P<0.01, *** = p<0.001). All experiments were repeated three times.

Figure 4 P97 amino acids affect HLA-B*7 and HLA-B*51 expression on the cell surface. C1R (A) or HeLa (B) cells were transduced using lentiviruses to express HLA-B*7 (S97) and three mutants at position 97 (S97N, S97T and S97D). HLA-B*51 (T97) and its mutants at position 97 (T97N and T97D) were also stably expressed in C1R (C) or 221 (D) cells. HLA-B*7 and HLA-B*51 free heavy chain (HC-10 antibody), classical HLA-B*7 complex (ME-1 antibody) and classical HLA-B*51 complex cell surface expression is shown. Results are expressed as mean and standard deviation. P value was determined using unpaired two-tailed t-test (* = P<0.05, ** = P<0.01, *** = p<0.001). All experiments were repeated three times.

Figure 5 Cartoon showing possible effects of N97T and N97D mutation on HLA-B*27 assembly and cell surface expression. HLA-B*27-N97D heavy chains have such poor affinity for β2m that they rarely pass the quality control in the ER, resulting in low expression levels of both classical complexes and FHCs on the cell surface. By contrast, HLA-B*27-N97T heavy chains likely have weak but sufficient association with β2m to allow complexes to exit the ER, but subsequently dissociate during trafficking to the cell surface leading to elevated FHC on the cell surface.

Table 1 Association of P97 residues of HLA-B with AS

P97 residues	Association with AS	*Odds ratio	*P value	Examples of HLA-B allele
N	Risk	16.51	$< 10^{-300}$	***27:05**
T	Risk	1.12	4.5×10^{-3}	***51:01** *13:02
R	Non-associated	1	1	*15:01 *40:01 *47:01
W	Non-associated	1	0.95	*14:01
S	Protective	0.86	4.81×10^{-8}	***07:02** *40:02
V	Protective	0.68	1.41×10^{-8}	*57:01
D	Synthetic	N/A	N/A	N/A

*** Statistics from Cortes A. et al. Nat Commun, 2015, Fisher's exact test used for P value.** The HLA-B allotypes investigated in current study, HLA-B*27:05, HLA-B*51:01 and HLA-B*07:01, are highlighted in bold. Of note, Aspartic acid (D) is not present in P97 of HLA-B.

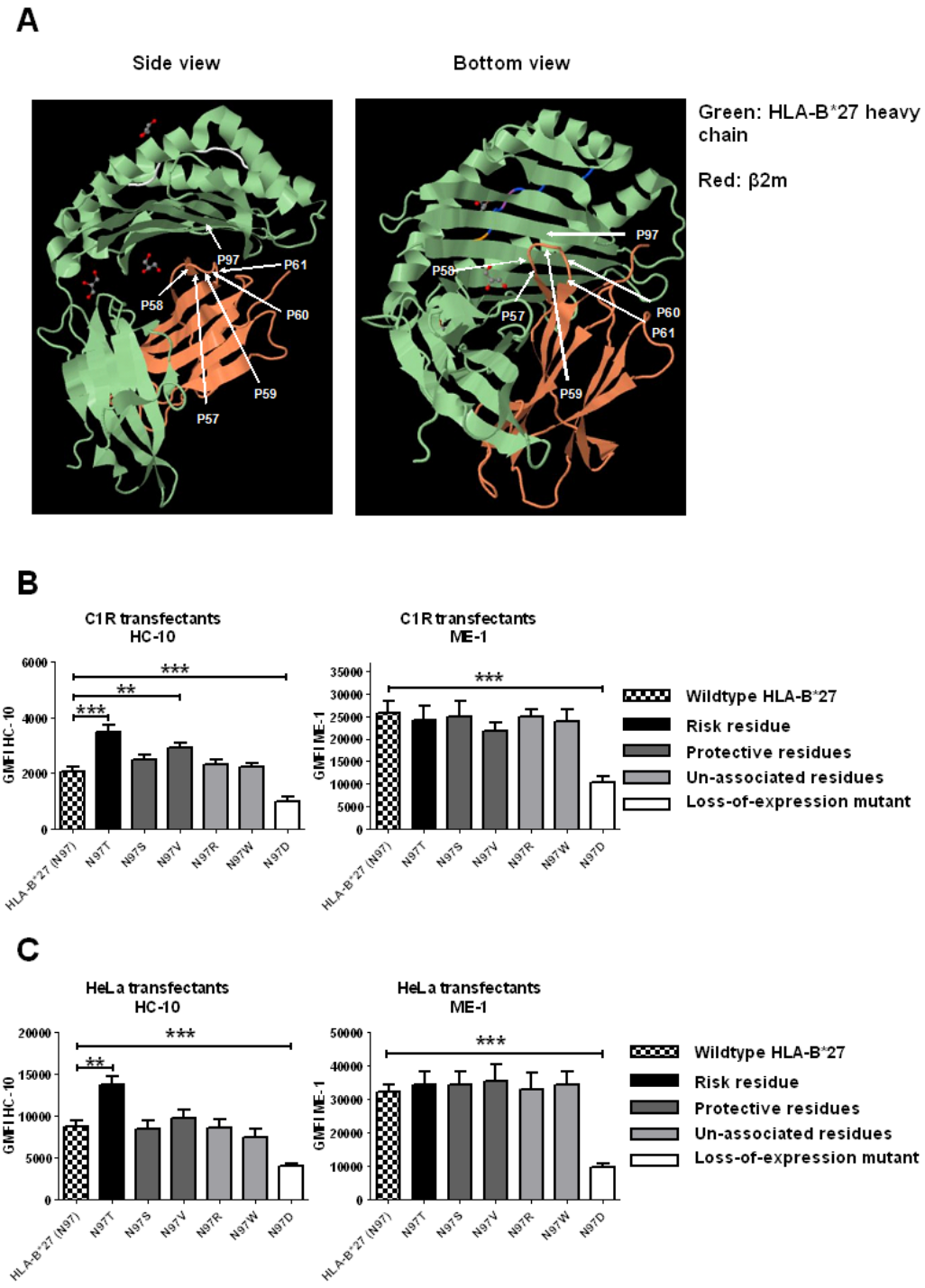


Figure 1 Location of P97 in HLA-B*27 crystal structure and effects of P97 residue substitutions on HLA-B*27 cell surface expression. Location of P97 in HLA-B*27 structure is shown in side and bottom views (A). C1R (B) or HeLa (C) cells were transduced using lentiviruses to express equal amounts of HLA-B*27 (N97) and six mutants at position 97 (N97T, N97S, N97V, N97R, N97W and N97D). HLA-B*27 free heavy chain (HC-10 antibody) and classical HLA-B*27 complex (ME-1 antibody) cell surface expression is shown. Results are expressed as mean and standard deviation. P value was determined using unpaired two-tailed t-test (** = P<0.01, *** = p<0.001). All experiments were repeated three times.

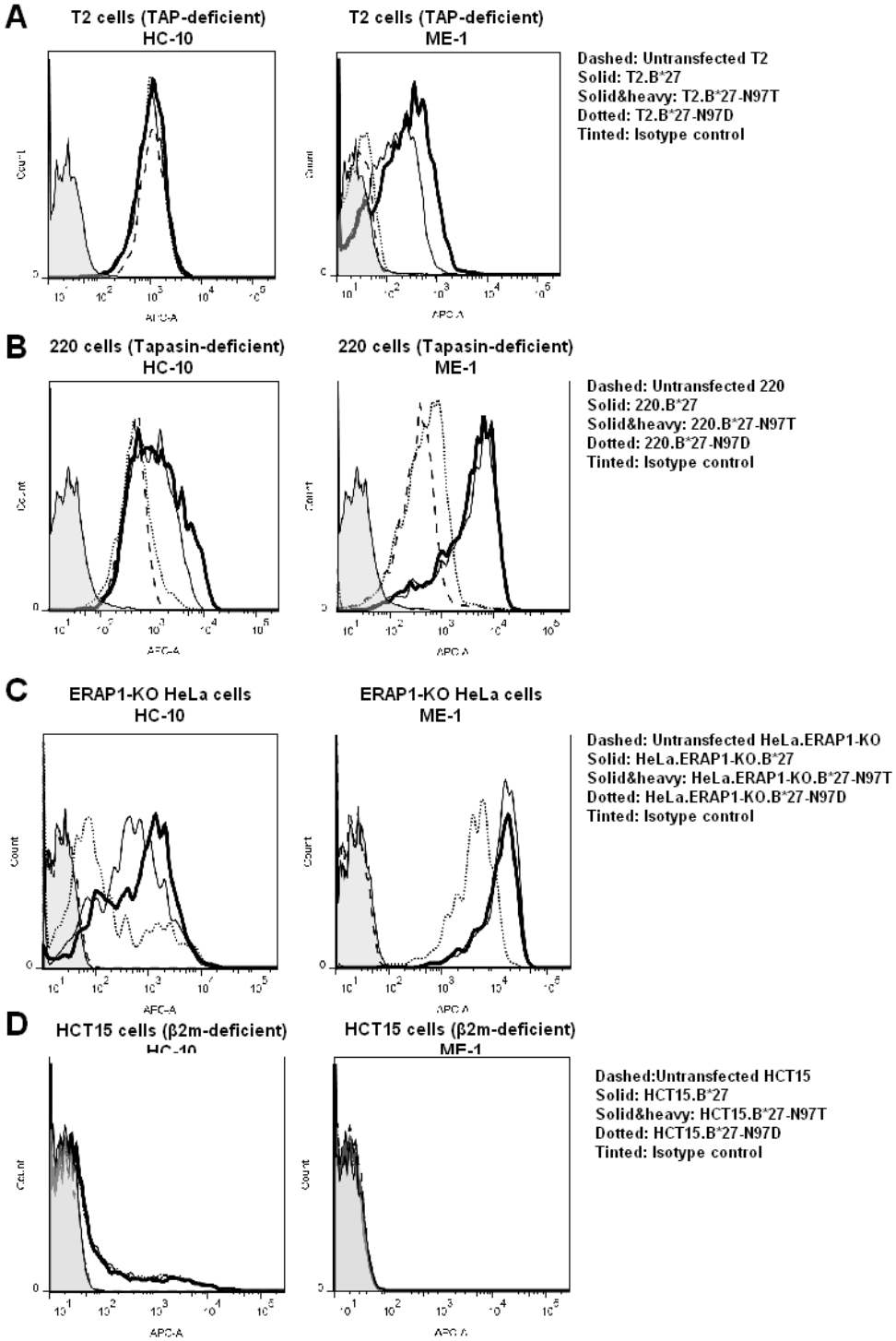


Figure 2 Differences in free heavy chain expression levels between HLA-B*27, HLA-B*27-N97T and HLA-B*27-N97D disappear in the absence of β2m, but not TAP, tapasin or ERAP1. TAP-deficient T2 cells (A), tapasin-deficient 220 cells (B), ERAP1-CRISPR-knockout HeLa cells (C) and β2m-deficient HCT15 cells (D) were transduced using lentiviruses to express HLA-B*27(N97), HLA-B*27-N97T and HLA-B*27-N97D mutants. HLA-B*27 free heavy chain (HC-10 antibody) and classical HLA-B*27 complex (ME-1 antibody) cell surface expression is shown. Representative results are shown as FACS plot. All experiments were repeated three times.

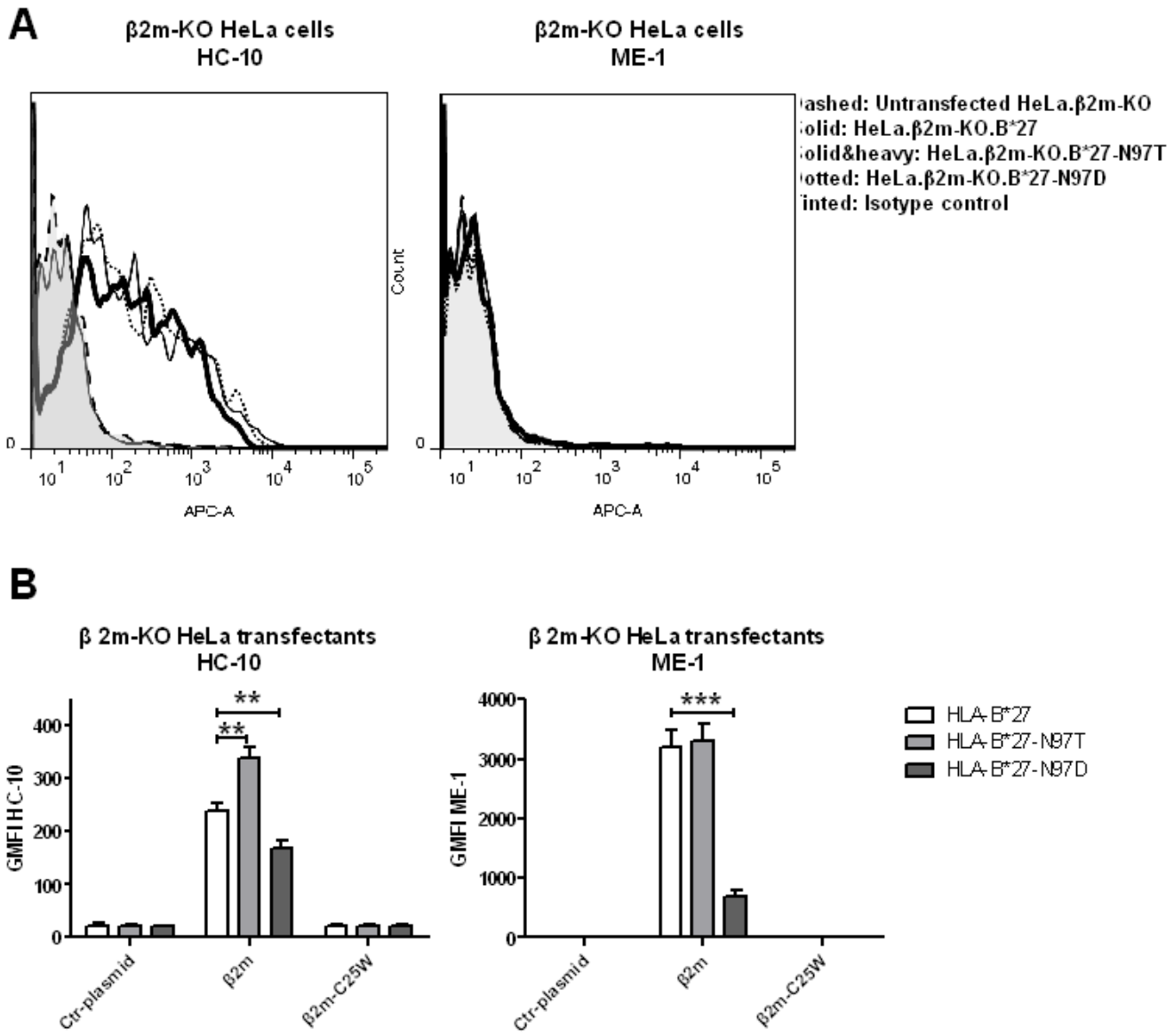


Figure 3 Differential expression between HLA-B*27, HLA-B*27-N97T and HLA-B*27-N97D is only observed in the presence of β2m. β2m-CRISPR-knockout HeLa cells (A) were transduced using lentiviruses to express HLA-B*27(N97), HLA-B*27-N97T and HLA-B*27-N97D mutants. β2m-knockout HeLa cells were co-transfected with HLA-B*27/HLA-B*27-N97T/HLA-B*27-N97D DNA plasmid and β2m/loss-of-function β2m-C25W mutant DNA plasmid (B). HLA-B*27 free heavy chain (HC-10 antibody) and classical HLA-B*27 complex (ME-1 antibody) cell surface expression is shown. Representative results are shown as FACS plots (A) or expressed as mean and standard deviation (B). P value was determined using unpaired two-tailed t-test (** = P<0.01, *** = p<0.001). All experiments were repeated three times.

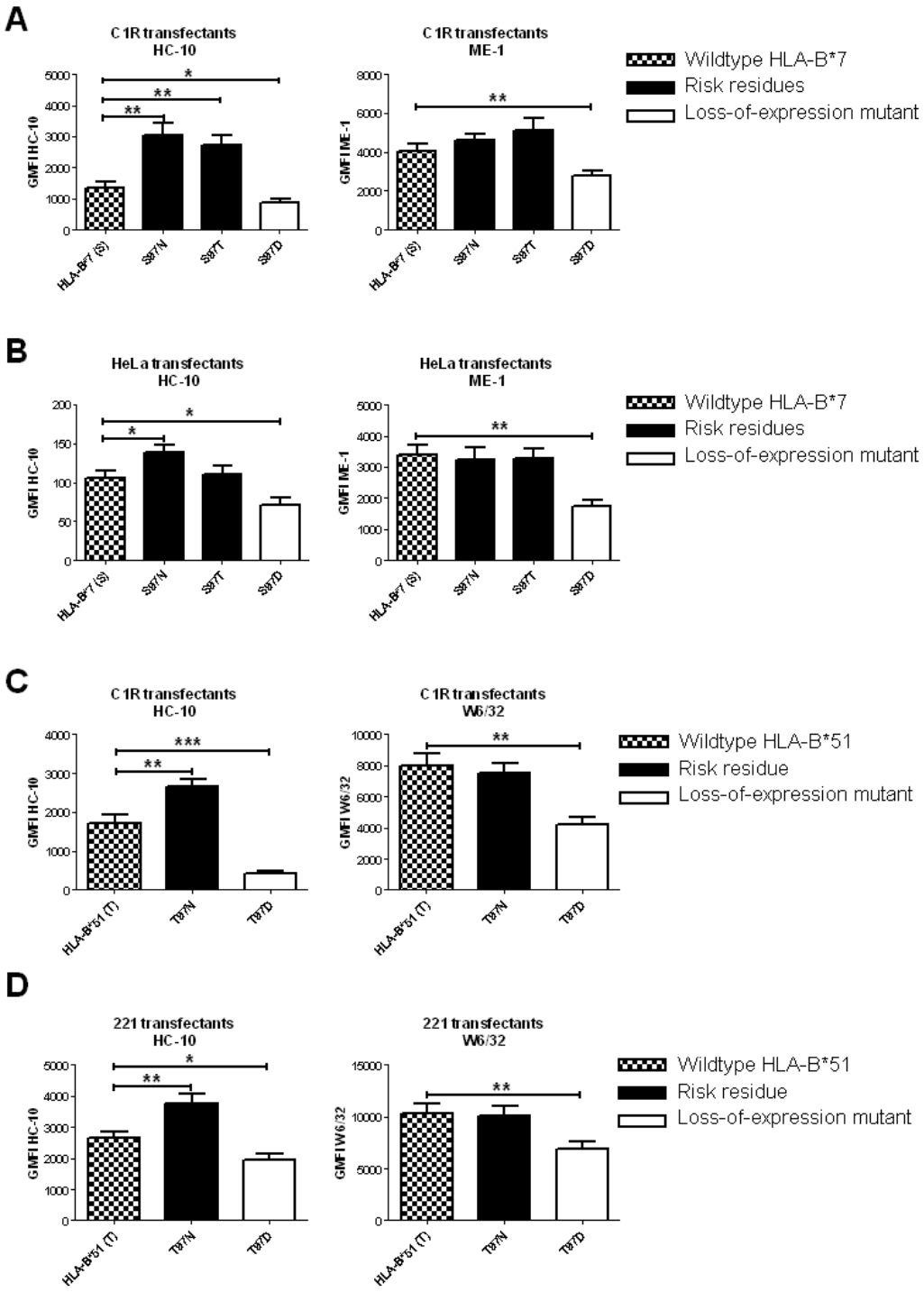


Figure 4 P97 amino acids affect HLA-B*7 and HLA-B*51 expression on the cell surface. C1R (A) or HeLa (B) cells were transduced using lentiviruses to express HLA-B*7 (S97) and three mutants at position 97 (S97N, S97T and S97D). HLA-B*51 (T97) and its mutants at position 97 (T97N and T97D) were also stably expressed in C1R (C) or 221 (D) cells. HLA-B*7 and HLA-B*51 free heavy chain (HC-10 antibody), classical HLA-B*7 complex (ME-1 antibody) and classical HLA-B*51 complex cell surface expression is shown. Results are expressed as mean and standard deviation. P value was determined using unpaired two-tailed t-test (* = $P<0.05$, ** = $P<0.01$, *** = $p<0.001$). All experiments were repeated three times.

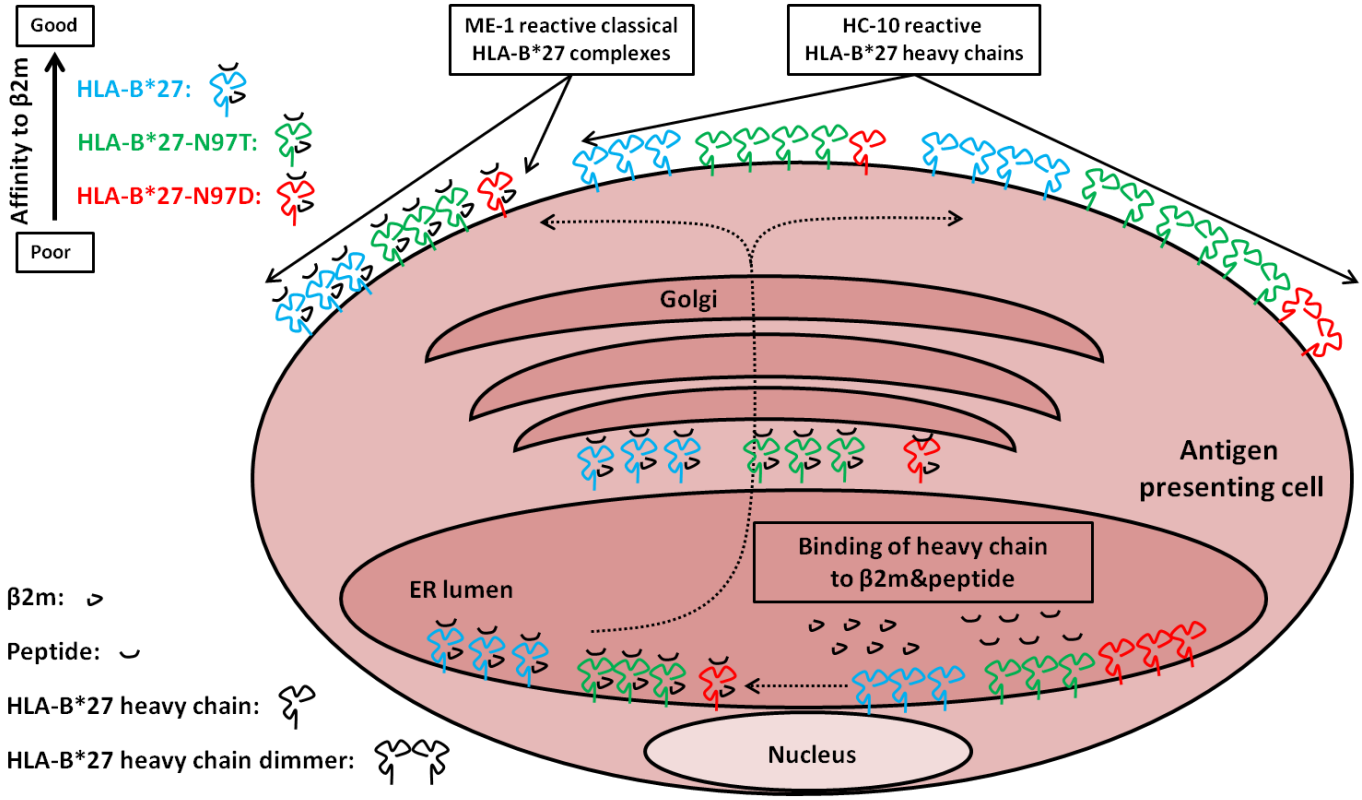


Figure 5 Cartoon showing possible effects of N97T and N97D mutation on HLA-B*27 assembly and cell surface expression. HLA-B*27-N97D heavy chains have such poor affinity for β2m that they rarely pass the quality control in the ER, resulting in low expression levels of both classical complexes and FHCs on the cell surface. By contrast, HLA-B*27-N97T heavy chains likely have weak but sufficient association with β2m to allow complexes to exit the ER, but subsequently dissociate during trafficking to the cell surface leading to elevated FHC on the cell surface.

Table S1 Summary of the effects of P97 residues on HLA-B*27, HLA-B*51 and HLA-B*7 free heavy chain (FHC) expression studied in this paper

HLA-B alleles studied	Association with AS	Baseline FHC	Mutations of P97 residue	Association of residues with AS	Change of FHC
HLA-B*27 (N)	Risk	High	T	Risk	Increase
			S	Protective	No effect
			V	Protective	Mild increase in C1R cells, no effect in HeLa cells
			R	Non-associated	No effect
			W	Non-associated	No effect
HLA-B*51 (T)	Risk	High	N	Risk	Increase
HLA-B*7 (S)	Protective	Low	N	Risk	Increase
			T	Risk	Increase in C1R cells, no effect in HeLa cells

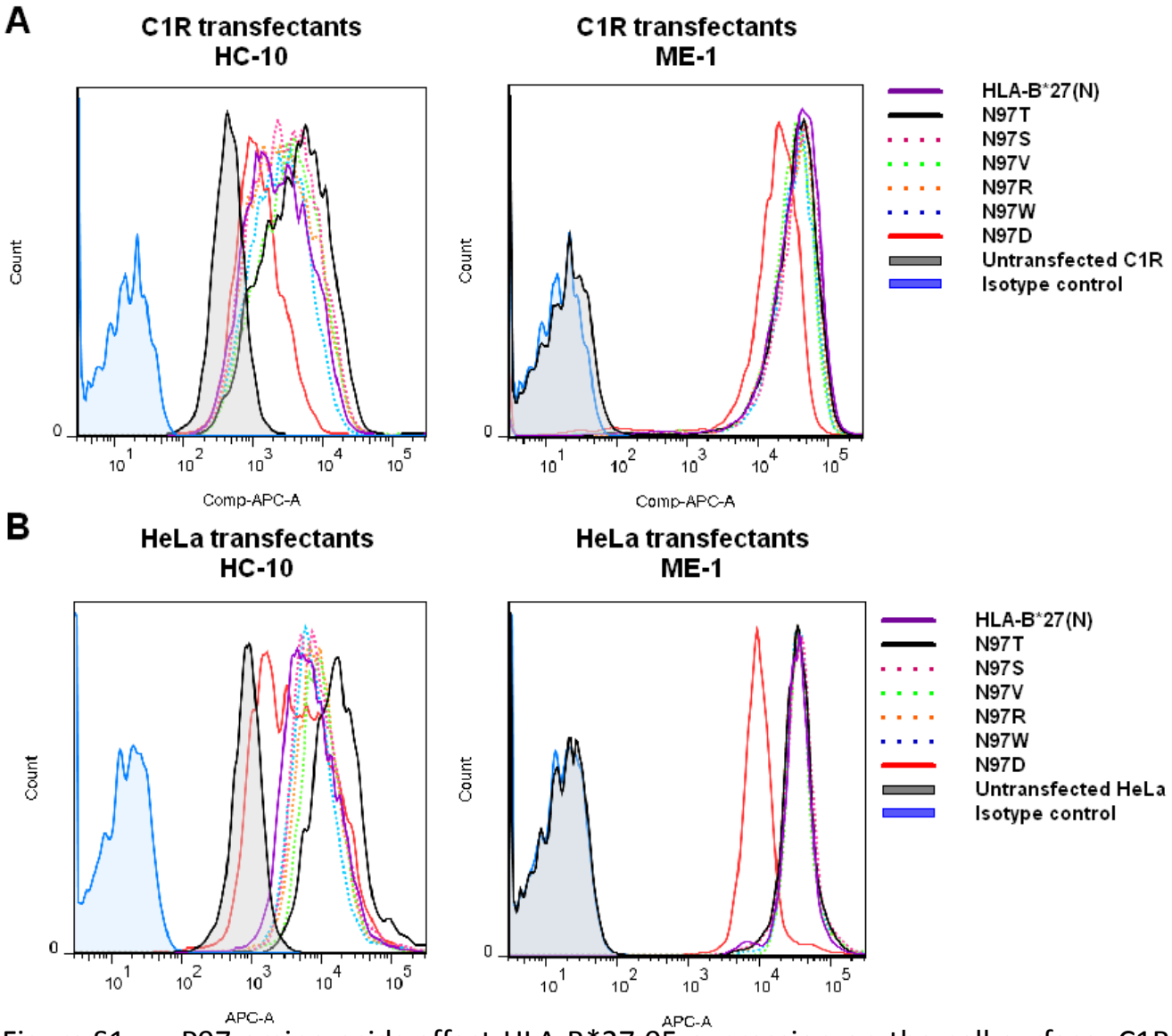


Figure S1 P97 amino acids affect HLA-B*27:05 expression on the cell surface. C1R or HeLa cells were transfected using lentivirus to express HLA-B*27(N) and six mutants at position 97 (N97T, N97S, N97V, N97R, N97W and N97D). HLA-B*27 free heavy chains (HC-10 antibody) and classical HLA-B*27 complexes (ME-1 antibody) cell surface expression by C1R transfectants (A) and HeLa transfectants (B) are shown. Representative of N=3 experiments.

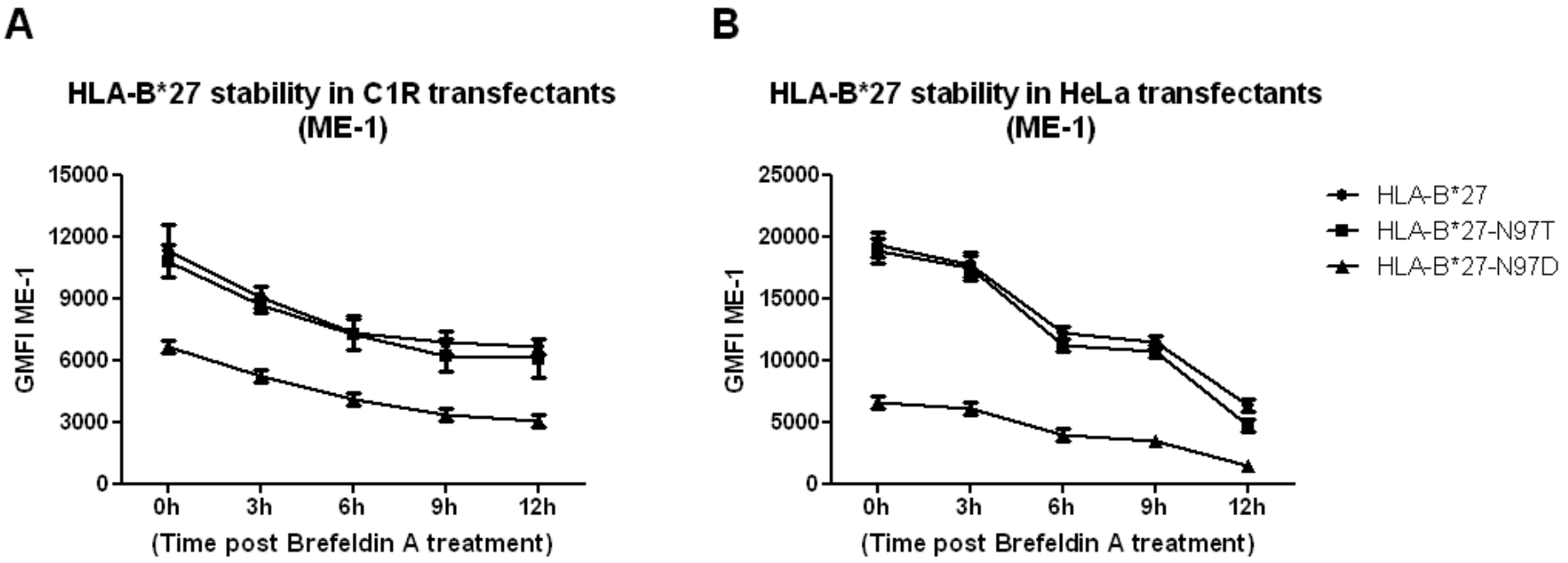


Figure S2. Similar stability of cell surface classical complexes between HLA-B*27, HLA-B*7 and HLA-B*51. C1R (A) or HeLa (B) cells expressing HLA-B*27 (N97), N97T or N97D mutants were treated with 6 μg/ml of Brefeldin A to stop egress of classical HLA-B*27 complexes to cell surface. Cell were then stained using ME-1 for cell surface HLA-B*27 classical complexes at different time points (0h, 3h, 6h, 9h and 12h). The experiment was repeated three times, representative results are shown as mean and standard deviation.

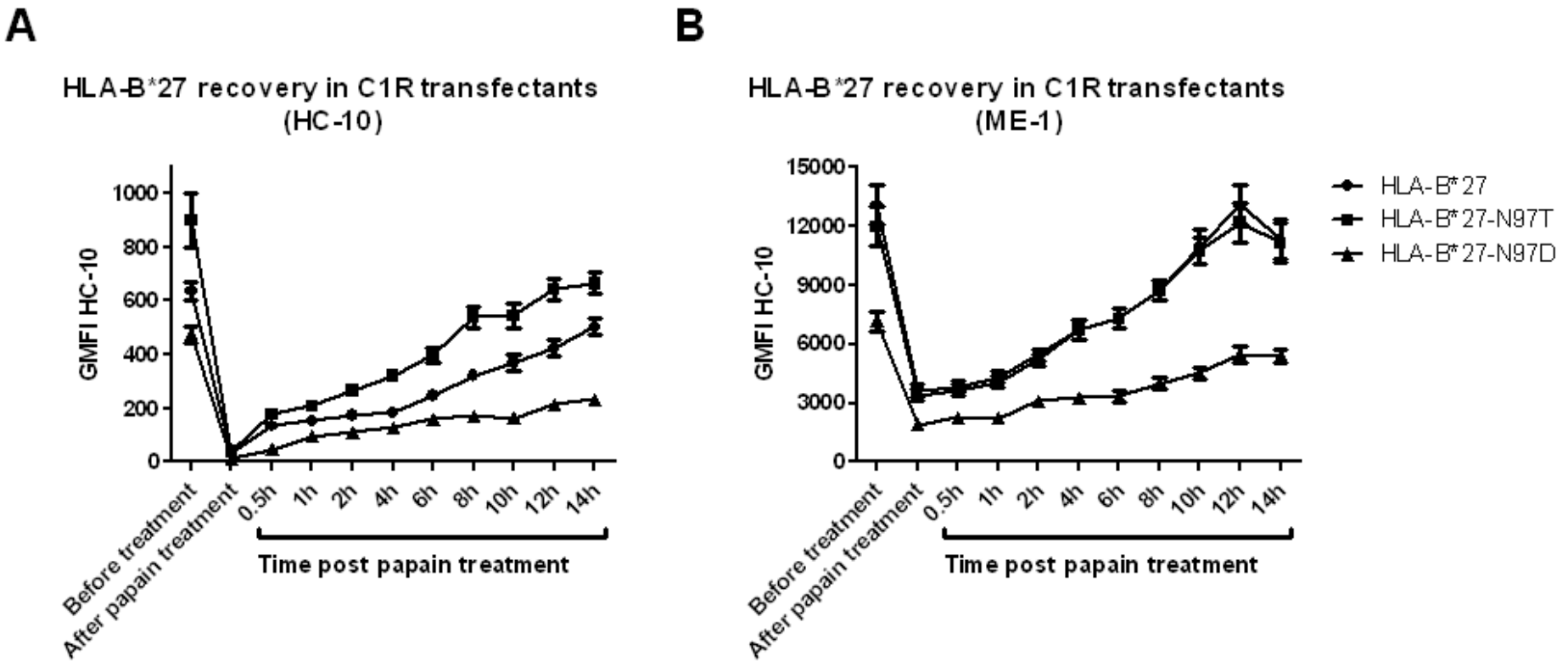


Figure S3. HLA-B*27 N97T mutant free heavy chains are expressed at the cell surface more rapidly than HLA-B*27. C1R cells expressing HLA-B*27, N97T or N97D mutants were treated with 4 mg/ml activated papain (10 mM Cystine, 30 minutes at 37 ^{o}C) for 30 minutes, then recovered in cell culture medium for 14 hours. Cells were stained using HC-10 (A) or ME-1 (B) for cell surface HLA-B*27 FHCs or classical complexes at different time points (before treatment, after papain treatment, 0.5h, 1h, 2h, 4h, 6h, 8h, 10h, 12h and 14h). The experiment was repeated three times, representative results are shown as mean and standard deviation.

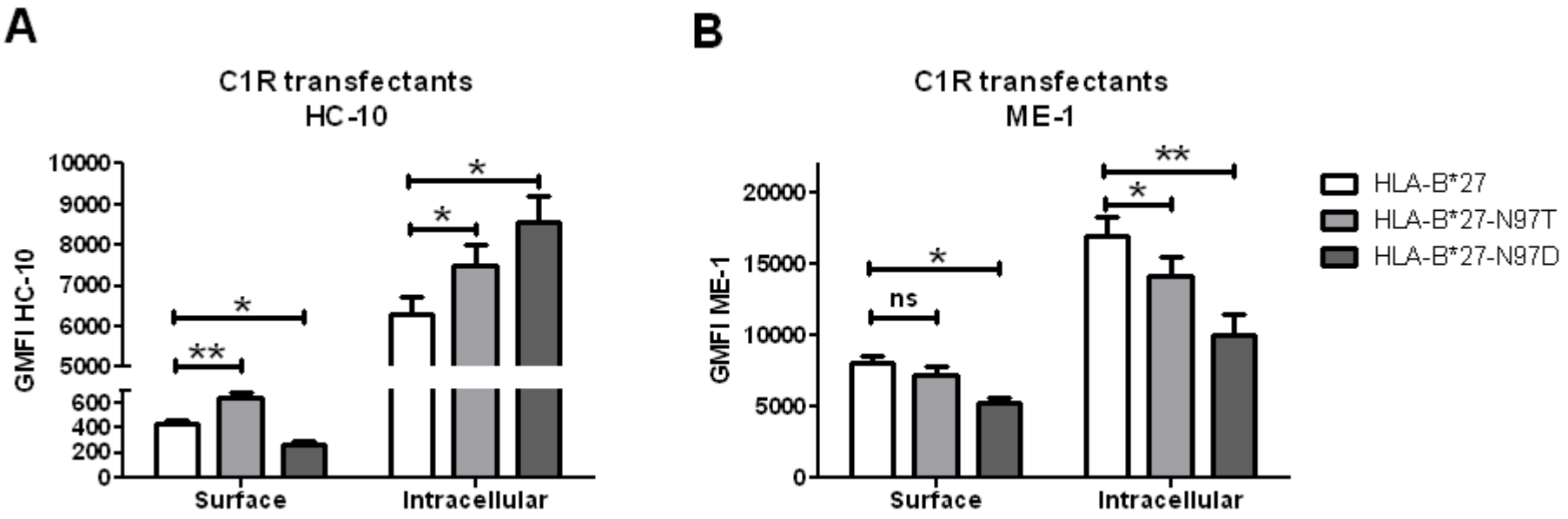


Figure S4. Comparison of intracellular HLA-B*27 expression levels between HLA-B*27, N97T and N97D mutants. C1R cells expressing HLA-B*27, N97T or N97D mutants were either cell surface or intracellularly stained using HC-10 (A) or ME-1 (B) for HLA-B*27 FHCs or classical complexes. The experiment was repeated three times, representative results are shown as mean and standard deviation. P value was determined using unpaired two-tailed t-test (* = $p<0.05$, ** = $p<0.01$). Of note, cell surface HLA-B*27 also contribute to intracellular staining, albeit to a smaller extent than intracellualr HLA-B*27 molecules.

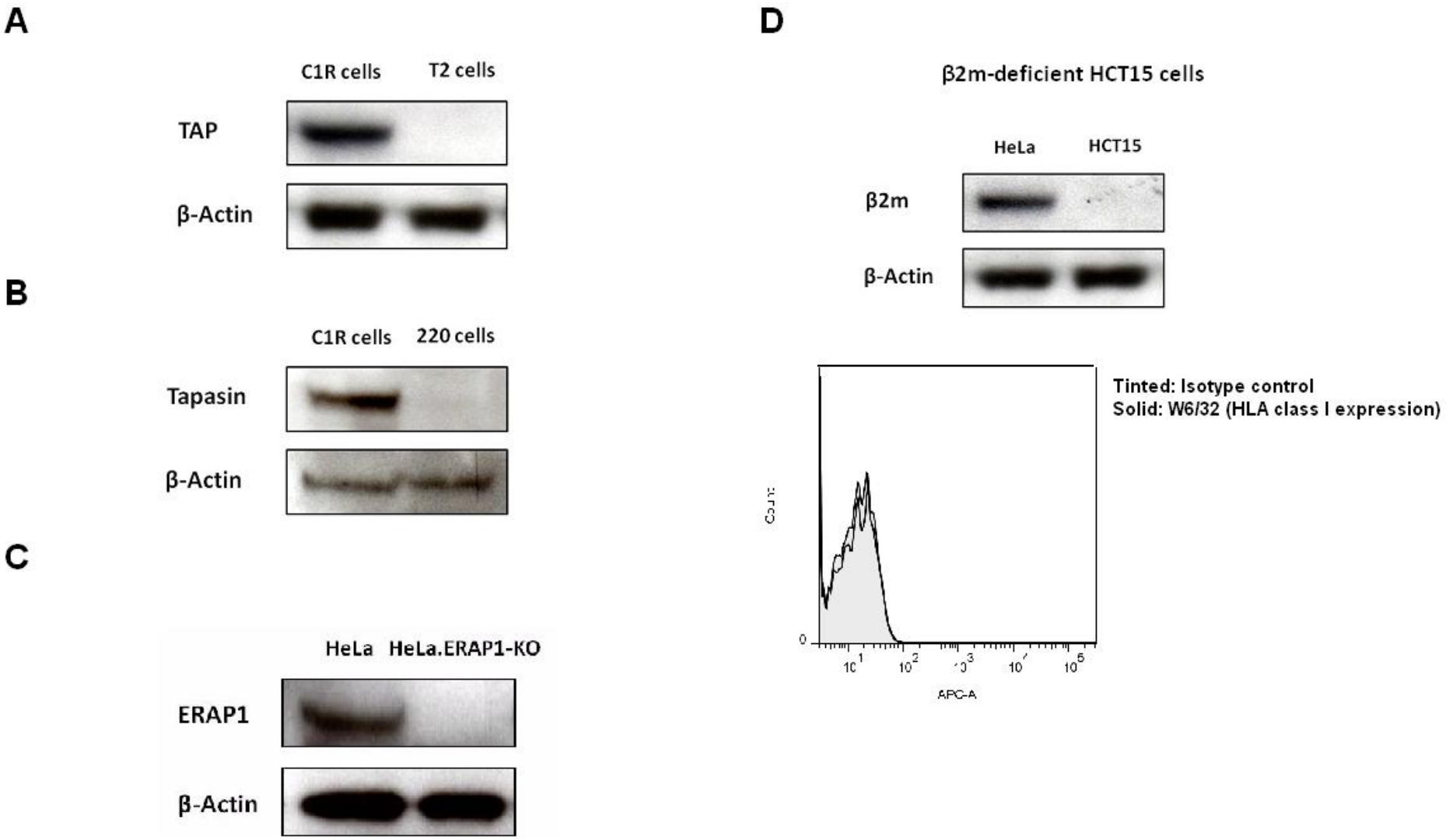


Figure S5. Deficiency of TAP, Tapasin, ERAP1 and β2m in T2, 220, HeLa.ERAP1-KO and HCT15 cells respectively. Expression of TAP (A), Tapasin (B), ERAP1 (C) and β2m (D) is shown by western blot. Absence of HLA class I expression in β2m-deficient HCT15 cells is shown by staining with W6/32 antibody (D).

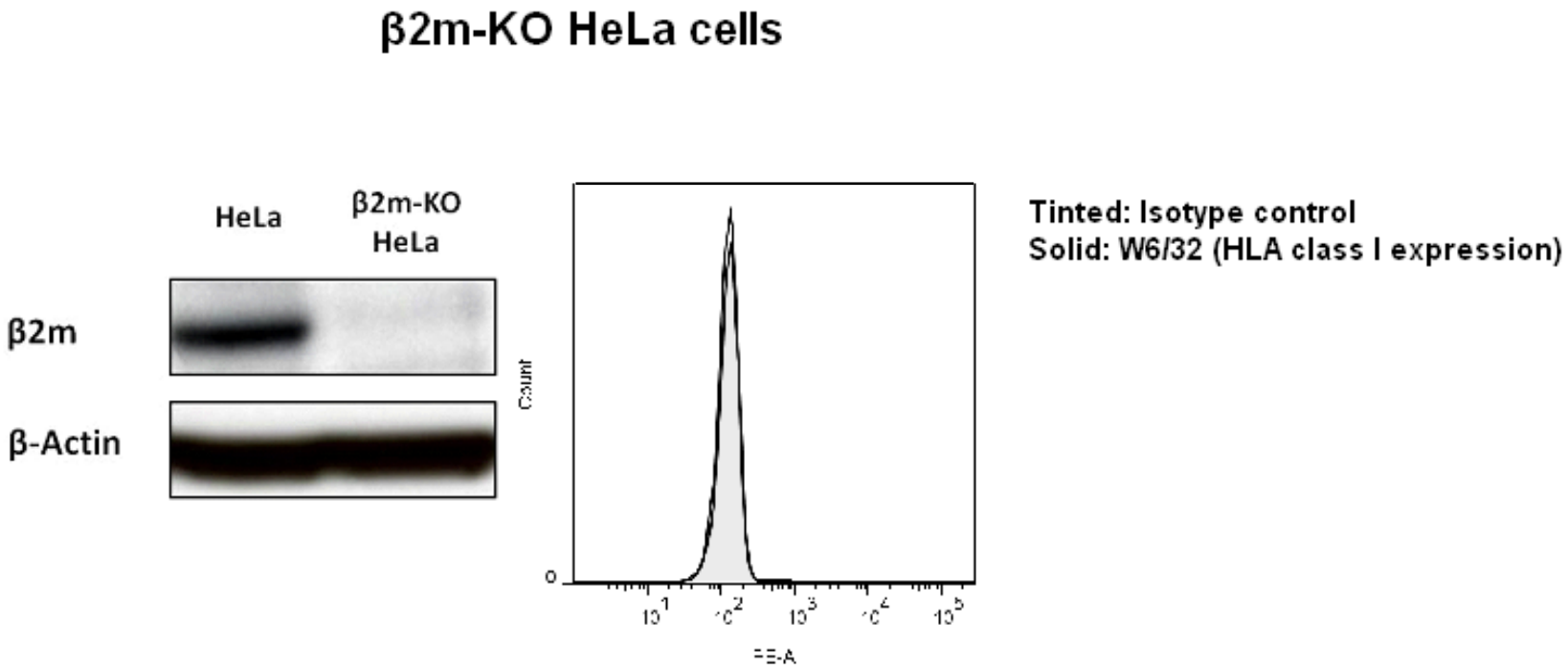


Figure S6. Deficiency of β2m and absence of HLA class I expression in β2m-knockout HeLa cells. β2m expression is shown by western blot, HLA class I expression is shown by staining with W6/32 antibody.

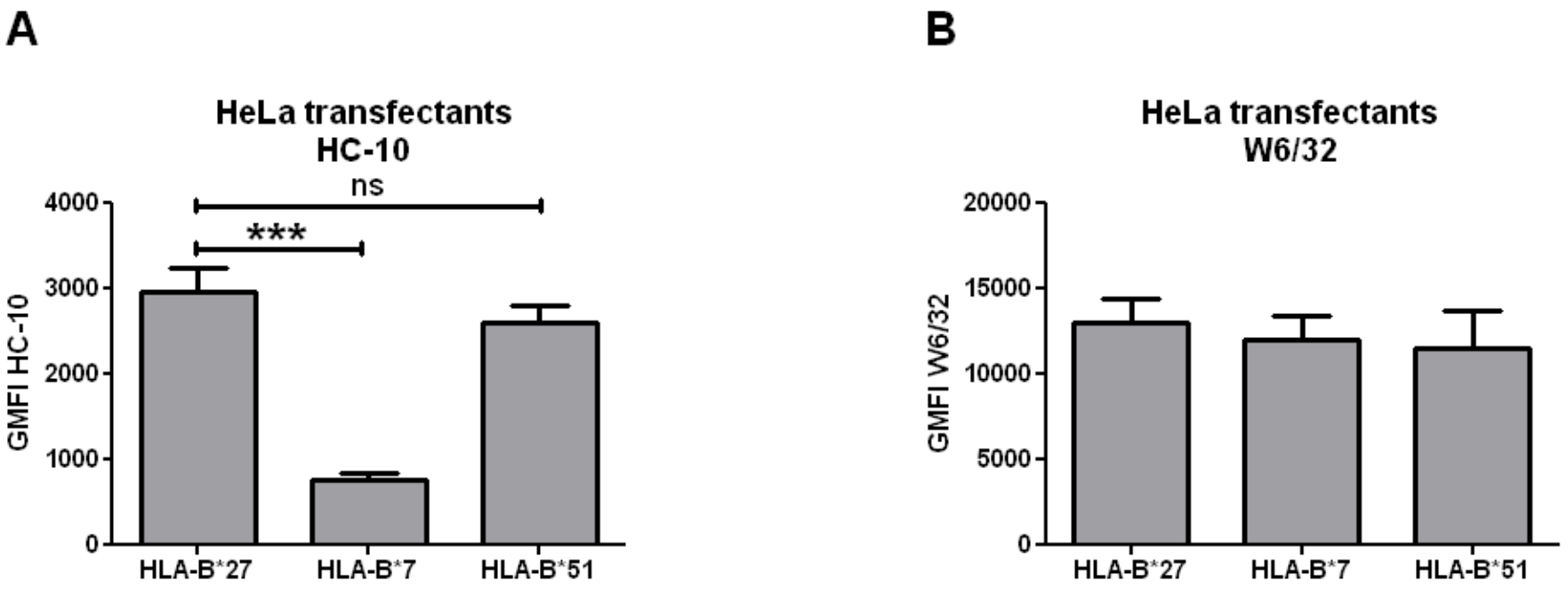


Figure S7. Comparison of HLA-B*27, HLA-B*7 and HLA-B*51 cell surface expression levels. Free heavy chain expression is measured using the HC-10 antibody (A). W6/32 antibody is used to measure classical HLA complexes (B). P value was determined using unpaired two-tailed t-test (*** = $p<0.001$, ns = not significant). The experiment was repeated three times.