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HLA-B27 subtypes predisposing to ankylosing spondylitis accumulate in endoplasmic reticulum-derived compartment apart from the peptide-loading complex

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ABSTRACT

OBJECTIVE: It was previously shown that HLA-B27 subtypes predisposing to spondyloarthritis (SpA), i.e. B*27:02, B*27:05 and B*27:07, displayed increased propensity to form intra-cellular oligomers and to accumulate to a high density in cytoplasmic vesicles, as compared to the non-SpA-associated HLA-B*07:02 and HLA-B*27:06. The aim of the present study was to characterize the nature and content of HLA-B-containing vesicles and to further examine their relevance to SpA predisposition.

METHOD: Vesicles containing HLA-B proteins were evidenced in transfected HeLa cells and in cells from SpA patients or HLA-B27/human β 2-microglobulin (h β 2m) transgenic rats, by means of microscopy. Nature and content of HLA-B-containing vesicles were determined by co-localisation experiments with appropriate markers.

RESULTS: The SpA-associated HLA-B*27:04 subtype accumulated at higher levels in cytoplasmic vesicles than HLA-B*27:06 from which it differs only by two substitutions, reinforcing the correlation between vesicles formation and SpA predisposition ($P < 10^{-5}$). Co-localisation showed that those vesicles contained misfolded HLA-B heavy chain along with β 2m and endoplasmic reticulum (ER) chaperones (calnexin, calreticulin, BiP, GRP94) and belonged to the ER but were distinct from the peptide-loading complex (PLC). Similar vesicles were observed in immune cells from HLA-B27⁺ SpA patients in greater abundance than in healthy controls ($P < 0.01$), and in dendritic cells from HLA-B27/h β 2m transgenic rats, in correlation with SpA susceptibility.

CONCLUSION: Accumulation of misfolded HLA-B heavy chain along with β 2m and ER chaperones into ER-derived vesicles distinct from the PLC is a characteristic feature of HLA-B27 subtypes predisposing to SpA. This phenomenon could contribute to HLA-B27 pathogenicity, by non-canonical mechanism.

Ankylosing spondylitis (AS), the prototypical form of spondyloarthritis (SpA), is strongly associated with the class I major histocompatibility complex (MHC) molecule HLA-B27 (1). However, the molecular mechanism underlying such association remains unexplained (2,3). Since the original discovery of HLA-B27 association with AS, more than 160 distinct HLA-B27 subtypes have been identified (4). A definite association with AS was observed for nearly all alleles that are sufficiently frequent to allow epidemiological survey, including B*27:02, B*27:04, B*27:05 and B*27:07 (5,6). In contrast, B*27:06 and B*27:09 have been reported as less or not associated with AS (5,6). The case of B*27:09 is particular, since it is extremely rare, except in central Sardinia where it was found not to be associated with AS (7). However, it was detected in SpA/AS patients but not in HLA-B27⁺ healthy controls in other areas, leading to question whether it is truly not associated (2,6). Regarding B*27:06, it has been reproducibly shown not to be associated with AS in several populations of Southeast Asia where it coexists with the strongly associated B*27:04, from which it differs only by two amino-acid substitutions. Thus, comparing the behavior between different HLA-B27 subtypes, in regard of their SpA-association level, may help to clarify distinctive pathogenic features.

In prior studies, we visualized the intracellular trafficking of different AS-associated or non-AS-associated HLA-B alleles using HLA-B^{YFP} fusion proteins and examined the formation and behavior of HLA-B oligomers using Bioluminescence Resonance Energy Transfer (BRET) assays (8,9). In transiently transfected HeLa cells, we evidenced homotypic interactions for all the HLA-B alleles examined, predominating in the ER. Moreover, the strong expression of HLA-B proteins resulted in the formation of large intracellular vesicles, in which conformers of those proteins reactive with HC10 antibody (Ab) that binds unfolded/misfolded class-I MHC heavy chain (HC), accumulated. Most strikingly, the AS-associated B*27:02, B*27:05 and B*27:07 subtypes differed from the non-AS-associated B*27:06 and B*07:02 alleles by an increased abundance of homotypic oligomers and by their higher propensity to accumulate in those intracellular vesicles, in high expression conditions (9). Such distinctive behaviors that could be related to each other were not due to an outbreak of unfolded protein response (UPR) and seemed independent of ER-associated degradation (ERAD) *via* the proteasome.

Here, we wished to further characterize the particular traffic of AS-predisposing HLA-B27 subtypes by identifying the nature and content of the intracellular vesicles in which they accumulate in high expression conditions. We also included in the present study B*27:04, another subtype that is predisposing to SpA and differs only by two substitutions from the non-AS-associated B*27:06, as well as the intermediate mutants to determine the influence of single mutations on those characteristics.

MATERIALS AND METHODS

Plasmids. Plasmid constructs encoding the B*27:02, B*27:05, B*27:06, B*27:07 and B*07:02 proteins fused at their C-terminal to YFP and the control plasmid pcDNA3-YFP were produced as described earlier (9). Site-directed mutagenesis of the HLA-B*27:05 encoding sequence, using a QuikChange site-directed mutagenesis kit (Stratagene), was performed to produce the plasmid constructs encoding the B*27:04 proteins fused at their C-terminal to YFP. The pcDNA3-B*27:06 vector was used as a DNA template to generate the B*27:06-D114H and the B*27:06-Y116D mutants (Fig. 1A), with QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). Primers CCTCCTCCGCGGGTATCACCAGTACG and CGTACTGGTGATACCCGCGGAGGAGG were used for B*2706-D114H, and primers CGGGTATGACCAGGACGCCTACGACGG and CCGTCGTAGGCGTCCTGGTCATACCCG for B*27:06-Y116D. HLA-B constructs were sequenced before use.

HeLa cells transfection and treatments. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 0.1mg/mL streptomycin, at 37 °C in a humidified atmosphere of 5%CO₂. For transient transfections, 15,000 or 7,500 cells were respectively seeded in 8- or 12-wells IBIDI chamber slides and transfected 24 hours later with 0.5µg of each plasmid/dish using DreamFect™ Gold (Ozbiosciences).

Cells were washed 28 hours after transfection, fixed with 2% paraformaldehyde (PFA) and permeabilized with PBS-Triton 0.5 % for immunofluorescence labeling. When indicated, cells were incubated after 3 hours of transfection with 3-Methyladenin (3-MA, 5mM, Sigma-Aldrich) or chloroquine (30µM, Sigma-Aldrich) for 4 hours, to inhibit autophagy, or with the autophagy inducer rapamycin (40µM, Sigma-Aldrich) for 24 hours. Cells were also incubated with nocodazole (0.6 µg/µL, Sigma-Aldrich) for 4 hours to block protein exit from endoplasmic reticulum (ER) or brefeldin A (5µg/µL, Sigma-Aldrich) for 24 hours to block proteins entry into the golgi apparatus, before fixation and permeabilization.

Patients and healthy controls cells. Study patients satisfying the Assessment of SpondyloArthritis international Society classification criteria for axial SpA (10) provided written informed consent and the study was approved by the local ethics committee (St. Germain-en-Laye, France). Controls consisted of HLA-B27-negative healthy blood donors. Peripheral blood mononuclear cells were isolated from freshly drawn blood using Ficoll-Paque PLUS (GE

healthcare Bio-Sciences). A negative selection of CD14⁺ cells was performed using autoMACS (Milteny Biotec) to collect CD14⁺ cells. CD4⁺ T lymphocytes were further purified by positive selection with anti-human CD4 MicroBeads (Miltenyi Biotec) and autoMACS^{TM} Pro.

Lymphoblastoid B cell lines *10151* and *13617* from HLA-B27⁺ AS patients, and *9435* and *9953* from HLA B27-negative healthy controls immortalized with Epstein-Barr virus were previously described (11–13). They were grown in RPMI medium supplemented with 10% FBS, 100 units/mL penicillin and 0,1mg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO₂.

Rat splenic dendritic cells (DCs). The HLAB27/human β 2-microglobulin (h β 2m) transgenic rat line 21-3 and the h β 2m transgenic line 283-2 originally produced at University of Texas Southwestern Medical Center (Dallas, TX) were crossed to obtain the disease-prone [21-3 $\times$ 283-2] F1 rats, bearing 20 copies of HLA-B*27:05 and 50 copies of h β 2m, and the disease-free 21-3 (20 copies of HLA-B*27:05 and 15 copies of h β 2m) and 283-2 (35 copies of h β 2m) lines, all on Lewis background (14). Nontransgenic littermates were used as controls. Age-matched male rats (> 9 months of age) were included in each experiment. Animal procedures were approved by the institutional Animal Experimentation Ethical Committee (CEB-26-2012).

CD103⁺ splenic DCs were obtained as previously described (15), using anti-CD103⁺ (OX-62) MicroBeads (Miltenyi Biotec) and positive selection with autoMACS (Miltenyi Biotec). Cells were cultured overnight in 12-well μ -Slides in Dutch–modified RPMI medium prior to fixation.

Antibodies. The primary Abs are shown in supplementary Table. The secondary Cy5- and HRP-labeled anti-mouse IgG Abs were from KPL and GE Healthcare Life Sciences, respectively. Alexa Fluor® 488-labeled and Alexa Fluor® 594-labeled anti-rabbit or mouse IgG, Alexa Fluor® 488-labeled anti-mouse IgG2a, and Alexa Fluor® 594-labeled anti-mouse IgG1 or IgG2b were from Invitrogen.

Fluorescence labeling and confocal microscopy.

Slides or plates containing cells were washed with PBS 1X and fixed with 2% PFA, permeabilized with PBS-Triton 0.5% and saturated with PBS-Triton 0.3%, BSA 1%. Cells were sequentially incubated with primary and secondary Abs in PBS-Triton 0.3%, BSA 0.1%, for 1 hour each. Lyso-Tracker and ER-Tracker (Invitrogen) were used for visualization of the lysosomes and ER, respectively. For all conditions, negative control conditions consisted of cells incubated in PBS without primary Ab or with irrelevant isotype-control Ab. After washing, cells were mounted in

Vectashield medium (Vector) or Prolong^{TM} Diamond containing 4',6-diamidino-2-phenylindole (DAPI). Observations were performed with a confocal laser-scanning microscope (TCS Sp8 Leica Microsystems, Heidelberg, Germany). Images were analyzed using FIJI software.

Fluorescence quantification: Vesicles fluorescence intensity was determined on confocal microscopy digital images, using area-selection and Region Of Interest (ROI) manager image analysis tools in FIJI software. For quantification, 80-180 labeled vesicles were analyzed per condition and per experiment.

Live-cell imaging. HeLa cells were seeded in 8-well 15 μ -Slides (Ibidi GmbH) and transfected 24h later with 0.15 μ g pcDNA3-HLAB*2705^{YFP}, using X-tremeGENE HP DNA transfection reagent (Roche). Time-lapse acquisitions were done at 37°C using an inverted microscope (Olympus scanR system) equipped with a temperature- and CO₂-controller chamber and a sCMOS ORCA FLASH camera (Hamamatsu Photonics KK, Japan). Acquisition was initiated 7 hours after transfection and images were acquired every 2 minutes using a 40x objective with the scanR Acquisition Software. Time points are indicated in minutes.

Electron microscopy. HeLa cells (2 x 10⁶ per condition) were seeded in 35 mm glass Bottom dishes (MatTek Corporation) and transfected with HLA-B*27:05, HLA-B*07:02 or control pcDNA3. Cultured cells were fixed with 2% glutaraldehyde in 0.1 M Na cacodylate buffer pH 7.2, for 1 hour at room temperature. Samples were then contrasted with Oolong Tea Extract (OTE) 0.5% in cacodylate buffer, post-fixed with 1% osmium tetroxide containing 1.5% potassium cyanoferrate, gradually dehydrated in ethanol (30% to 100%) and substituted gradually in mix of ethanol-epon and embedded in Epon. (Delta microscopie-Labège). Thin sections (70 nm) were collected onto 200 mesh copper grids copper and counterstained with lead citrate.

Cryopreparation and sectioning. For ultrathin cryosectioning and immunogold labeling, cells were fixed with a mixture of 4% PFA and 0.25% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 1 hour and with 4% PFA in 0.1 M phosphate buffer, pH 7.2, for 1 hour. Cells were processed for ultracryomicrotomy as described (16).

Labeling: The grids were washed on 2% gelatine at 37°C to remove the embedding gelatin and remnants of methylcellulose, then quenched with glycine 50mM in phosphate buffer pH7.2 and blocked with buffer containing 1% BSA, 0.1% BSA-c^{TM} (BioValley). Grids were first incubated for 2 hours with HC10, washed twice and then for 1 hour with goat anti-mouse IgG coupled to 6 nm colloidal gold particles (Aurion-Biovalley, 1/40 dilution). Then, we blocked a second time and proceeded to incubation with anti-GRP94 Ab (1/500) and revelation with goat

anti-rabbit IgG coupled to 10 nm colloidal gold particles (Aurion-Biovalley). The grids were again washed, and cryosections were stained with 2% uranyl acetate, and embedded in 2% methylcellulose containing 5% uranyl acetate (4/1). Grids were examined with Hitachi HT7700 electron microscope operated at 80kV (Milexia) and images were acquired with a charge-coupled device camera (AMT). Semi-quantification of gold particles was done as previously described (17).

Western blot. HeLa cells (1.5×10^5) were seeded in P6 plate (10 cm²) and transfected 24 hours later with plasmids encoding HLA-B alleles or control pcDNA3. Twenty-four hours after transfection, culture supernatants were centrifuged (1,400 g; 5 min) and pellets were resuspended in 15μL Laemli Buffer 1X (Biorad). Proteins were separated on Mini-PROTEAN TGX 4-20% gel (Bio-Rad Hercules,) and transferred onto polyvinylidene difluoride membrane by a trans-blot Turbo Transfer pack (Bio-Rad Hercules). Blots were incubated in 5% milk-PBS Tween (PBS 1X, 0.1% Tween-20, Sigma-Aldrich) for 12 hours at 4°C after saturation in 5% milk-PBS Tween, with HC10, anti-GRP94, anti-Lamin C or BBM1 Ab, then washed in PBS-Tween and incubated with HRP-conjugated secondary Ab. Labeled proteins were visualized using enhanced chemiluminescence reagent (GE Healthcare Life Sciences). Chemiluminescence was detected with a ChemiDoc XRS+ system (Bio-Rad) and images acquired and analyzed with the ImageLab software (Bio-Rad).

Statistical analysis. Data are presented as the mean $\pm$ SEM or median and 95% confidence interval (CI). Statistical analysis were performed using two-tailed unpaired t test. *P* values less than or equal to 0.05 were considered significant.

RESULTS

Heightened formation of intracellular vesicles containing HLA-B molecules distinguishes AS-associated B*27:04 from non-AS-associated B*27:06. We previously reported that HeLa cells expressing AS-associated B*27:05^{YFP}, B*27:02^{YFP} and B*27:07^{YFP} subtypes contained higher abundance of vesicles in which HLA-B proteins accumulate mostly as HC10-reactive misfolded conformers, than the non-AS-associated B*27:06^{YFP} and B*07:02^{YFP} alleles (9). Here, we observed that HeLa cells expressing B*27:04^{YFP}, another AS-associated HLA-B27 subtype, displayed also significantly greater frequency of vesicles-bearing cells than those expressing B*27:06^{YFP} (Fig. 1B).

Given that B*27:04 differs only by two substitutions from B*27:06 (Fig. 1A), we examined cells expressing intermediate mutants with single residue substitution, i.e. B*27:06-D114H and B*27:06-Y116D. Interestingly, they behaved comparably to cells expressing B*27:06, indicating that both His-114 and Asp-116 contributed to increased tendency of AS-associated subtype to accumulate in intra-cellular vesicles (Fig. 1B).

Intra-cellular vesicles accumulating misfolded HLA-B proteins belong to the ER. To characterize the nature of those vesicles, we performed co-localization experiments (Fig. 2, S1, S2). In HeLa cells, HLA-B contained in vesicles co-localized with almost all the tested ER markers, including four ER-resident chaperones (i.e. calreticulin, calnexin, BiP and GRP94) and ERp57, an ER oxidoreductase involved in class I MHC loading with peptide (Fig. 2A). They also co-localized with β 2m, despite containing essentially HC10-reactive misfolded HLA-B conformers (Fig. 2A). In contrast, we observed that those HLA-B containing vesicles were neither labeled with early, nor late endosomes, nor golgi apparatus markers (Fig. S1). Neither were they labeled with lysosomes markers using anti-Lamp-1 or Lyso-Tracker, nor with anti-Lman1 (ERGIC-53) ERGIC marker (Fig. S1), nor with autophagosomes anti-LC3 marker (Fig. S2A).

Consistent with those findings, neither treatment of HeLa cells with brefeldin A (Fig. 2B), nor with nocodazole to block protein exit from ER (Fig. 2C), nor with pharmacological inducer (i.e. rapamycin) or inhibitors of autophagy (i.e. chloroquine and 3-MA) (Fig. S2A,B) did affect the formation of HLA-B containing vesicles.

Calreticulin, ERp57, and β 2m, all contribute to the complex that allows HLA-B nascent molecules to be loaded with peptides during their journey in the ER. However, other more specific

peptide-loading complex (PLC) component, such as tapasin, and the closely associated ERAP1 did not co-localize with the HLA-B contained in vesicles (Fig. 2A).

Visualization of intracellular HLA-B-containing vesicles by electron microscopy. We previously described similar HC10-labeled vesicles in cell lines transfected with wild-type HLA-B alleles, showing that vesicle formation was not an artefact due to YFP-tag (9). Thus, HeLa cells transfected with wild-type HLA-B*27:05 were examined by transmission electronic microscopy, confirming the presence of abundant intra-cytoplasmic vesicles of various sizes sometimes containing electron-dense material (Fig. 3A). Immunolabeling with HC10 and anti-GRP94 evidenced the co-localization of HLA-B heavy chain and of GRP94 in the membrane of those vesicles (Fig. 3B). Both markers were more intensely labeled in cells expressing B*27:05 than B*07:02 (Fig. 3B,C).

Quantification of ER proteins in HLA-B-containing vesicles and in cell culture supernatant. We next quantified the intensity of labeling of several proteins of interest (β 2m, GRP94, BiP and ERp57) in the HLA-B-containing vesicles from HeLa cells transfected with various HLA-B^{YFP} alleles, taking B*27:05 as a reference (Fig. 4A). Albeit the content of all those proteins appeared the lowest in HLA-B*07:02-transfected cells, among the different HLA-B27 subtypes, there was no clear correlation between such content and their level of AS-association. Noteworthy, the intensity of staining for all the studied proteins was roughly comparable between the AS associated subtypes and the non-AS-associated B*27:06. This indicates that the number of vesicle-bearing cells rather than the content of those vesicles correlated with AS-predisposition.

Having observed by electron microscopy that some of the intra-cellular vesicles appeared beneath the plasma membrane (Fig. 3A), we suspected that they might release their content in the extra-cellular medium. Thus, we quantified their amount in the supernatant of transfected HeLa cells in culture (Fig. 4B). There was no correlation between the level of HLA-B heavy chain or GRP94 in culture supernatant and AS-association. However the level of β 2m in the supernatant appeared to correlate with AS-association strength.

Cells from AS-prone HLA-B27/h β 2m transgenic rats and from HLA-B27⁺ SpA patients exhibit vesicles containing misfolded HLA-B proteins and ER chaperones as well as h β 2m.

We had previously reported the presence of vesicles containing HC10-reactive HLA-B proteins in splenic DCs from SpA-prone HLA-B27/h β 2m transgenic rat of the 33-3 line (9). Similar HC10-reactive vesicles were readily detected in splenic DCs from [21-3 x 283-2]F1 cross, another AS-

prone transgenic rat line (Fig. 5). Again, misfolded HLA-B proteins co-localized with ER chaperones BiP and GRP94 (Fig. 5B). Moreover, similar anti-h β 2m (BBM1)-reactive vesicles were also detected in DCs from that line (Fig. 5A).

DCs from the 21-3 hemizygous line that remains healthy, due to a lower number of copies of the h β 2m transgene, also contained HC10-reactive vesicles that were marked with anti-BiP but much lesser with GRP94-recognizing Ab than in [21-3 x 283-2]F1 cross (Fig. 5A,B). Moreover, they contained fewer BBM1-reactive vesicles that were smaller than in [21-3 x 283-2]F1 cross (Fig. 5A). Finally, DCs from the 283-2 hemizygous rats that bear solely h β 2m transgene exhibited BiP-containing vesicles as those from nontransgenic rats but only small h β 2m- and virtually no GRP94-containing-vesicles (Fig. 5A,B). Thus, the distinctive feature of DCs from disease-prone HLA-B27 transgenic rats was the large abundance of vesicles containing not only misfolded HLA-B protein, but also h β 2m and BiP along with GRP94.

Finally, we searched if HLA-B-containing vesicles could be detected in cells from SpA patients. Indeed, lymphoblastoid B cell line from HLA-B27⁺ AS patients exhibited HC10-reactive vesicles that were labeled with anti- β 2m, anti-BiP and anti-ERp57 Abs (Fig. 6A). Moreover, similar vesicles accumulating HC10-reactive conformers co-localized with BiP and ERp57 were readily detected in CD4⁺ T cells from HLA-B27⁺ SpA patients (Fig. 6B), showing that such phenomenon also happens in primary cells. Similar vesicles could also be detected in cells from HLA-B27-negative healthy controls but with much lower frequency, indicating SpA-specificity (Fig. 6A,B).

DISCUSSION

Among hypotheses that have been proposed to explain how HLA-B27 confers high susceptibility to SpA, the earliest speculated on the antigen-presenting function of class I MHC molecule to CD8⁺ T cells (18). However, failure to identify an "arthritogenic peptide" and observation that CD4⁺ T cells rather than CD8⁺ T cells were pathogenic in HLA-B27 transgenic rat model of SpA fostered the emergence of alternative theories, based on peculiar biochemical behaviors of the HLA-B27 molecule (3). These include a slow folding rate and a tendency to misfold in the ER which may trigger an UPR in conditions of high expression, and an unusual propensity to form covalently bonded homodimers of HC that are expressed at the cell surface, some of which are recognized by leukocyte immunoglobulin-like receptors (3,19). However, comparative analysis of several HLA-B27 subtypes failed to correlate the level of AS-association with those biochemical behaviors (20). Hence, AS-associated B*27:02, B*27:04 and B*27:05 fold more slowly, bind more optimized peptidomes after tapasin editing, a byproduct of which is increased heavy chain misfolding, and are more stable than the non-AS-associated B*27:06. Yet, B*27:07, which is AS-associated behaves alike B*27:06 regarding the foregoing characteristics. Residue 116 of the HLA-B allele is critical to determine such behavior by controlling the polarity to the F pocket (20). Thus, B*27:02, B*27:04 and B*27:05 having a polar Asp-116 behave differently from B*27:06 and B*27:07 that exhibit a bulky Tyr-116 and hydrophobic F pocket, promoting fast folding with suboptimal peptide repertoire and decreased tapasin dependence (20,21). Thus the most stable AS-associated subtypes, i.e. B*27:02, B*27:04 and B*27:05 were also the less likely to dissociate in endosomes and form homodimers at the cell surface, thereby challenging one of the current hypotheses described above (20).

Thus, the distinctive features of HLA-B27 subtypes that could explain their differential level of association with SpA remain to be identified. We initially examined the propensity of HLA-B alleles to form homotypic dimers and/or oligomers in transfected HeLa cells, using the BRET technology and compared the behavior of AS-associated HLA-B27 subtypes with non-AS-associated alleles (8,9). Interestingly, AS-associated B*27:02, B*27:05 and B*27:07 differed from non-AS-associated B*27:06 and B*07:02 in two aspects that may be linked to one another: a stronger BRET signal in condition of high expression, indicating increased propensity to form oligomers -without inducing an UPR- and increased formation of intracytoplasmic vesicles in which HC10-reactive heavy chains accumulated (9). Here, we further observed enhanced formation of intracytoplasmic vesicles with another AS-associated subtype, B*27:04, as compared

to B*27:06 from which it differs only by two substitutions, Asp-114-His and Tyr-116-Asp. This result reinforced the correlation of such property with SpA-predisposition. Studying intermediate mutants allowed us to determine that both His-114 and Asp-116 were required for heightened accumulation of B*27:04 in intra-cellular vesicles. Thus, the structural features that determine the formation of intracytoplasmic vesicles must be different from those influencing misfolding or tapasin dependence and should account for the behavior of B*27:07 which combines a Tyr-116, similar to B*27:06 with a less polar and more basic Asn at position 114, the combination of which may result in polarity and isoelectric point of the 1114-116 region similar to the other AS-associated subtypes, all sharing His-114 and Asp-116. This could notably influence the strength of association of the MHC class I heavy chain with β 2m, which is dependent on residues situated at the bottom of the peptide-binding groove, in the vicinity of that region (22).

One of the main objectives of the present work was to characterize the nature of accumulating intracytoplasmic vesicles. Confocal microscopy experiments demonstrated that the HLA-B proteins colocalized exclusively with ER-specific markers, including the chaperones calnexin, calreticulin, BiP and GRP94, the oxidoreductase ERp57 and ER-tracker. In contrast, we ruled out that those vesicles belonged to endosome, lysosome, Golgi or ERGIC. Consistently, treating the cells with brefeldin A or nocodazole did not inhibit vesicles formation. Electronic microscopy confirmed the existence of those vesicles containing HC10-reactive HLA-B heavy chain along with GRP94. The labeling of both was more pronounced with HLA-B27:05 than HLA-B*07:02. Interestingly, it was shown that the newly synthesized HLA-B27:05 proteins markedly associate with ER chaperones, including calnexin, BiP and ERp57 and that Asp-116 promoted such associations as well as dimerisation of the nascent heavy chain (23,24). Hence, this phenomenon that occurs before MHC class I association with the PLC could lead to an accumulation of AS-associated HLA-B27 subtypes in ER compartment distinct from the PLC, where neither tapasin, nor ERAP1 co-localized. However, the presence of β 2m and calreticulin in those vesicles suggests that it could take place after the formation of partially folded MHC class I heavy chain/ β 2m heterodimers that may at least partially dissociate in the absence of high-affinity peptide loading (25). Accumulation of those vesicles in conditions of high HLA-B expression level could result from limited availability of the PLC with regard to the load of newly synthesized HLA-B proteins (9). Interestingly, ERAP1 alleles that increase SpA predisposition are associated with heightened ERAP1 activity and it was previously shown that B27 peptides resulting from ERAP1 trimming had lower affinity for B27 (11,26). Thus, SpA-associated ERAP1 allotypes that enhance the

binding of suboptimal peptides by HLA-B27 could favour their dissociation and contribute to the formation of HLA-B-containing vesicles.

We have previously reported that those vesicles accumulated in the absence of UPR induction, one of the mechanisms that may lead to the degradation of misfolded proteins by ERAD (9). Autophagy, is an alternative mechanism for the disposal of misfolded ER proteins that are resistant to proteasomal degradation. It was shown to eliminate misfolded HLA-B27 dimers and oligomers, including BiP-bound HLA-B27 heavy chains and we speculated that those vesicles could correspond to autophagosomes (19). However, neither inducing, nor blocking autophagy affected the formation of those vesicles. Interestingly, dimers of HLA-B27 were shown to be poorly ubiquitinated, which might explain their relative resistance to ERAD and also to autophagy and explain an accumulation of misfolded HLA-B27 in ER compartment, in addition to the lack of UPR induction (27,19,28).

We previously showed that HLA-B-containing vesicles also formed in splenic DCs from SpA-prone HLA-B27 transgenic rats of the 33-3 line (9). Here, we extended this result by showing vesicles containing HC10-reactive HLA-B27 heavy chain along with h β 2m, BiP and GRP94 in DCs from the SpA-prone [21-3 x 283-2] cross. In the 21-3 parental line that differs by a lower number of h β 2m transgene copies and remains healthy, there were fewer vesicles, containing HLA-B heavy chain and BiP, but less h β 2m and GRP94. Smaller h β 2m-containing vesicles were also evidenced in the h β 2m-transgenic 282-2 line. These results establish a correlation between the frequency of HLA-B27 heavy chain-containing vesicles, as well as the content of those vesicles and disease susceptibility in the HLA-B27 transgenic rat model of SpA, pointing out a possible role for h β 2m and GRP94. Interestingly, association of HLA-B*27:05 heavy chain oligomers with BiP was previously shown in splenocytes from the 33-3 line but not in HLA-B*07:02 transgenic rats (29). Moreover, increasing expression of h β 2m by crossing HLA-B27 transgenic rat lines with the 283-2 line promoted arthritis development and contributed to decrease BiP mRNA expression (14). Given that increased expression of h β 2m was associated with a reduction in folded B27 molecules and h β 2m at the cell surface, it can be hypothesized that increasing the level of h β 2m production rather led to its accumulation with misfolded HLA-B27 heavy chain in ER vesicles and contributed to dampen the level of BiP, by dampening UPR. The relevance of such vesicles containing HLA-B27 heavy chain along with BiP, β 2m and ERp57 for human SpA was further supported by showing their presence in EBV-transformed lymphoblastoid

cells and in primary CD4⁺ T cells from SpA patients to a greater extent than in cells from healthy controls.

In conclusion, we have shown that the extent to which SpA-associated HLA-B27 subtypes accumulate in ER-derived vesicles along with chaperones and β 2m correlated well with SpA-susceptibility. However, the mechanism behind such possible link remains hypothetical. We speculated that the content of such vesicles could be released in the extra-cellular milieu. Indeed β 2m was found increased in the supernatant of cells transfected with the SpA-associated subtypes, as compared to non-AS-associated alleles. Albeit such observation would fit into the β 2m amyloid deposition theory of HLA-B27 pathogenicity, such possible consequence remains to be investigated (30). Another possibility is that, upon uptake of apoptotic cells expressing HLA-B27, the aggregation of HLA-B27 heavy chain could foster inappropriate immune response against unusual conformers of HLA-B27 itself, as suggested by others (31). The role of GRP94, as a strong adjuvant of immune response as well as the presence of anti-HLA-B27 autoantibodies in SpA patients could support such hypothesis (32–34). Noteworthy, we recently showed that folded HLA-B27 interacted specifically with activin receptor-like kinase-2 at the cell surface, as a new putative mechanism of HLA-B27 pathogenicity (13). Thus, unfolded HLA-B27 that accumulate in the ER-derived vesicles could possibly reach the cell surface *via* alternative trafficking pathway, in association with β 2m and ectopically acquired peptides, thereby facilitating unusual interaction.

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REFERENCES

1. Taurog JD, Chhabra A, Colbert RA. Ankylosing Spondylitis and Axial Spondyloarthritis. *N Engl J Med* 2016;374:2563–2574.
2. Taurog JD. The mystery of HLA-B27: if it isn't one thing, it's another. *Arthritis Rheum* 2007;56:2478–2481.
3. Bowness P. HLA-B27. *Annu Rev Immunol* 2015;33:29–48.
4. Khan MA. An Update on the Genetic Polymorphism of HLA-B*27 With 213 Alleles Encompassing 160 Subtypes (and Still Counting). *Curr Rheumatol Rep* 2017;19:9.
5. Díaz-Peña R, López-Vázquez A, López-Larrea C. Old and new HLA associations with ankylosing spondylitis: HLA associations with ankylosing spondylitis. *Tissue Antigens* 2012;80:205–213.
6. Breban M, Costantino F, André C, Chiochia G, Garchon H-J. Revisiting MHC genes in spondyloarthritis. *Curr Rheumatol Rep* 2015;17:516.
7. Fiorillo MT, Cauli A, Carcassi C, Bitti PP, Vacca A, Passiu G, et al. Two distinctive HLA haplotypes harbor the B27 alleles negatively or positively associated with ankylosing spondylitis in Sardinia: implications for disease pathogenesis. *Arthritis Rheum* 2003;48:1385–1389.
8. Giquel B, Carmouse S, Denais C, Cherfa A, Chimenti M-S, Fert I, et al. Two HLA-B27 alleles differently associated with spondylarthritis, B*2709 and B*2705, display similar intracellular trafficking and oligomer formation. *Arthritis Rheum* 2007;56:2232–2242.
9. Jeanty C, Sourisce A, Noteuil A, Jah N, Wielgosik A, Fert I, et al. HLA-B27 subtype oligomerization and intracellular accumulation patterns correlate with predisposition to spondyloarthritis. *Arthritis Rheumatol* 2014;66:2113–2123.
10. Rudwaleit M, Heijde D van der, Landewe R, Listing J, Akkoc N, Brandt J, et al. The development of Assessment of SpondyloArthritis international Society classification criteria for axial spondyloarthritis (part II): validation and final selection. *Ann Rheum Dis* 2009;68:777–783.
11. Costantino F, Talpin A, Evnouchidou I, Kadi A, Leboime A, Said-Nahal R, et al. ERAP1 Gene Expression Is Influenced by Nonsynonymous Polymorphisms Associated With

Predisposition to Spondyloarthritis. *Arthritis Rheumatol* 2015;67:1525–1534.

12. Martín-Esteban A, Guasp P, Barnea E, Admon A, López de Castro JA. Functional Interaction of the Ankylosing Spondylitis-Associated Endoplasmic Reticulum Aminopeptidase 2 With the HLA-B*27 Peptidome in Human Cells: ERAP-2 and the HLA-B*27 Peptidome. *Arthritis Rheumatol* 2016;68:2466–2475.
13. Grandon B, Rincheval-Arnold A, Jah N, Corsi J-M, Araujo LM, Glatigny S, et al. HLA-B27 alters BMP/TGF β signalling in *Drosophila*, revealing putative pathogenic mechanism for spondyloarthritis. *Ann Rheum Dis* 2019;78:1653–1662.
14. Tran TM, Dorris ML, Satumtira N, Richardson JA, Hammer RE, Shang J, et al. Additional human β 2-microglobulin curbs HLA-B27 misfolding and promotes arthritis and spondylitis without colitis in male HLA-B27-transgenic rats. *Arthritis Rheum* 2006;54:1317–1327.
15. Glatigny S, Fert I, Blaton MA, Lories RJ, Araujo LM, Chiocchia G, et al. Proinflammatory Th17 cells are expanded and induced by dendritic cells in spondylarthritis-prone HLA-B27-transgenic rats. *Arthritis Rheum* 2012;64:110–120.
16. Tokuyasu KT. A technique for ultracryotomy of cell suspensions and tissues. *J Cell Biol* 1973;57:551–565.
17. Ferguson S, Steyer AM, Mayhew TM, Schwab Y, Lucocq JM. Quantifying Golgi structure using EM: combining volume-SEM and stereology for higher throughput. *Histochem Cell Biol* 2017;147:653–669.
18. Benjamin R, Parham P. Guilt by association: HLA-B27 and ankylosing spondylitis. *Immunol Today* 1990;11:137–142.
19. Navid F, Colbert RA. Causes and consequences of endoplasmic reticulum stress in rheumatic disease. *Nat Rev Rheumatol* 2017;13:25–40.
20. García-Medel N, Sanz-Bravo A, Alvarez-Navarro C, Gómez-Molina P, Barnea E, Marcilla M, et al. Peptide handling by HLA-B27 subtypes influences their biological behavior, association with ankylosing spondylitis and susceptibility to endoplasmic reticulum aminopeptidase 1 (ERAP1). *Mol Cell Proteomics* 2014;13:3367–3380.

21. Galocha B, Castro JAL de. Folding of HLA-B27 subtypes is determined by the global effect of polymorphic residues and shows incomplete correspondence to ankylosing spondylitis. *Arthritis Rheum* 2008;58:401–412.
22. Uchanska-Ziegler B, Ziegler A, Schmieder P. Structural and dynamic features of HLA-B27 subtypes: *Current Opinion in Rheumatology* 2013;25:411–418.
23. Antoniou AN, Ford S, Taurog JD, Butcher GW, Powis SJ. Formation of HLA-B27 Homodimers and Their Relationship to Assembly Kinetics. *Journal of Biological Chemistry* 2004;279:8895–8902.
24. Guiliano DB, Fussell H, Lenart I, Tsao E, Nesbeth D, Fletcher AJ, et al. Endoplasmic Reticulum Degradation-Enhancing α -Mannosidase-like Protein 1 Targets Misfolded HLA-B27 Dimers for Endoplasmic Reticulum-Associated Degradation: MHC Class I Degradation Pathway. *Arthritis & Rheumatology* 2014;66:2976–2988.
25. Antoniou AN, Guiliano DB, Lenart I, Burn G, Powis SJ. The Oxidative Folding and Misfolding of Human Leukocyte Antigen-B27. *Antioxidants & Redox Signaling* 2011;15:669–684.
26. Barnea E, Melamed Kadosh D, Haimovich Y, Satumtira N, Dorris ML, Nguyen MT, et al. The Human Leukocyte Antigen (HLA)-B27 Peptidome in Vivo, in Spondyloarthritis-susceptible HLA-B27 Transgenic Rats and the Effect of Erp1 Deletion. *Mol Cell Proteomics* 2017;16:642–662.
27. Smith M, Wilkinson S. ER homeostasis and autophagy. *Essays In Biochemistry* 2017;61:625–635.
28. Navid F, Layh-Schmitt G, Sikora KA, Cougnoux A, Colbert RA. The Role of Autophagy in the Degradation of Misfolded HLA-B27 Heavy Chains. *Arthritis & Rheumatology* 2018;70:746–755.
29. Tran TM, Satumtira N, Dorris ML, May E, Wang A, Furuta E, et al. HLA-B27 in Transgenic Rats Forms Disulfide-Linked Heavy Chain Oligomers and Multimers That Bind to the Chaperone BiP. *The Journal of Immunology* 2004;172:5110–5119.
30. Uchanska-Ziegler B, Ziegler A. Ankylosing spondylitis: a beta2m-deposition disease? *Trends*

Immunol 2003;24:73–76.

31. Luthra-Guptasarma M, Singh B. HLA-B27 lacking associated beta2-microglobulin rearranges to auto-display or cross-display residues 169-181: a novel molecular mechanism for spondyloarthropathies. *FEBS Lett* 2004;575:1–8.

32. Schwimmbeck PL, Yu DT, Oldstone MB. Autoantibodies to HLA B27 in the sera of HLA B27 patients with ankylosing spondylitis and Reiter's syndrome. Molecular mimicry with *Klebsiella pneumoniae* as potential mechanism of autoimmune disease. *J Exp Med* 1987;166:173–181.

33. Yang Y, Li Z. Roles of heat shock protein gp96 in the ER quality control: redundant or unique function? *Mol Cells* 2005;20:173–182.

34. Messmer MN, Pasmowitz J, Kropp LE, Watkins SC, Binder RJ. Identification of the Cellular Sentinels for Native Immunogenic Heat Shock Proteins In Vivo. *The Journal of Immunology* 2013;191:4456–4465.

FIGURE LEGENDS

Figure 1: High abundance of intra-cellular vesicles containing HLA-B proteins is apparent in HeLa cells transfected with SpA-associated HLA-B*2704 subtype. (A,B) HeLa cells grown in 12-well chamber slides (IBIDI) were transfected with plasmids encoding HLA-B*27:04^{YFP}, B*27:06^{YFP}, the D114H- or the Y116D-HLA-B*27:06^{YFP} mutants. (A) Comparative alignment of HLA-B27 polymorphic residues between both SpA-associated B*27:05 (the ancestral subtype) and B*27:04 subtypes, the non-SpA associated B*27:06 subtype, and intermediate B*27:06/B*27:04 mutants (D114H and Y116D) obtained by site-directed mutagenesis of B*27:06. (B) The transfected cells were fixed with 2% PFA and mounted in Vectashield with DAPI. The relative abundance of transfected cells presenting heavily YFP-labeled vesicles was scored as described in Materials and Methods. At least 6,000 transfected cells per condition were scored in each individual experiment. Values shown are means and SEM of results from 4 individual series of experiments. Statistical analysis was performed using unpaired *t*-test. *: *P* < 0.05; **: *P* < 10⁻⁵; NS: not significant.

Figure 2: HLA-B-accumulating vesicles belong to the ER but do not contain peptide loading complex (PLC) components. (A) Fixed and permeabilized HLA-B^{YFP}-transfected HeLa cells were labeled with anti-calreticulin (Clrt), anti-BiP, anti-calnexin, anti-ERp57, anti-GRP94, anti-tapasin, anti-ERAP1, HC10 (misfolded HLA-B), BBM1 (β2m) Abs or ER-tracker. Secondary Abs were Cy5-labeled (mouse) and Alexa Fluor® 594-labeled anti-rabbit IgG. Cells were mounted in Vectashield Dapi and examined with confocal microscopy using a 63X objective. Results are representative of at least 3 series of experiments done with different subtypes (HLA-B*27:04^{YFP}, B*27:05^{YFP}, B*27:06^{YFP}, B*27:07^{YFP} and B*07:02^{YFP}). Scale bar: 10 μm. (B) HeLa cells grown in 8-well μ-Slides (IBIDI) were transfected with plasmids encoding HLA-B*27:05^{YFP}. After 3 hours, cells were incubated with brefeldin A (5μg/μL) for 24 hours. After treatment, cells were fixed with PFA, and observed with confocal microscopy as above. Results are representative of at least 3 series of experiments performed with HLA- B*27:05^{YFP}, B*27:06^{YFP} and B*07:02^{YFP}. Scale bar: 8 μm. (C) HeLa cells grown in 8-well μ-Slides were transfected with plasmid encoding HLA-B*27:05^{YFP}. After 3 hours, cells were incubated or not for 4 hours with nocodazole (0.6μg/μL). Cells were then washed and visualized for 17 hours with an inverse microscope. Time-lapse videomicroscopy allowed to visualize the appearance of intracellular vesicles. Photography was initiated 7 hours after transfection. The time elapsed between frames is shown as minutes. Images

are representative of 3 experiments including all the different HLA-B alleles. No vesicle was detected in control HeLa cells transfected with pcDNA3-YFP, whether treated or not with brefeldin A or nocodazole (condition not shown).

Figure 3: Characterization of intracellular HLA-B-containing vesicles by electron microscopy. HeLa cells (2×10^6) were seeded in 35 mm glass bottom dishes and transfected with HLA-B*27:05, -B*0702 or pcDNA3 control vector and fixed as described in *Materials and Methods* 24 hours after transfection. **(A)** Thin sections (70 nm) of cells transfected with HLA-B*27:05 were collected onto 200 mesh copper grids copper and counterstained with lead citrate. Grids were examined with Hitachi HT7700 electron microscope operated at 80kV (Milexia – France), and images were acquired with a charge-coupled device camera (AMT). N: Nucleus; m: mitochondria; V: vesicles; er : endoplasmic reticulum; ly: lysosome. Scale bar: 1 μ m. **(B,C)** Cells were labeled with HC10 and anti-GRP94 Abs, followed by goat anti-mouse IgG coupled to 6 nm colloidal gold particles (right column: arrowheads) and goat anti-rabbit IgG coupled to 10 nm colloidal gold particles (right column: arrows), to reveal HC10 and anti-GRP94, respectively. Cells were then analyzed by electron microscopy. **(B)** Representative images are shown. pm: plasma membrane; nm : nuclear membrane; er : endoplasmic reticulum. Left column : scale bar =1 μ m, right column : scale bar = 500nm. Semi-quantitative analysis of the level of positive staining is shown in **(C)**.

Figure 4: Quantification of proteins localized in HLA-B-containing vesicles and released in cell culture supernatant. **(A)** HeLa cells transfected with plasmids encoding HLA-B^{YFP} alleles (B7: B*07:02), were washed and fixed 24 hours after transfection. Cells were labeled with BBM1 (anti- β 2m), anti-GRP94, anti-BiP or anti-ERp57 Ab, then incubated with secondary Cy5-labeled anti-mouse IgG or Alexa Fluor® 594- labeled anti-rabbit IgG Ab. Cells were mounted in Vectashield with Dapi for examination by confocal microscopy. The mean fluorescence intensity (MFI) of the intracellular HLA-B-containing vesicles of transfected cells was measured using ImageJ/FIJI. Measurements were done on all observed vesicles in at least 20 vesicle-bearing cells per condition. In each experiment, data obtained for different HLA-B subtypes were expressed relative to those for B*27:05 (set at 100%). Values are the mean and SEM of results from ≥ 3 sets of experiments. **(B)** Supernatants of HeLa cells transfected with plasmids encoding the different HLA-B^{YFP} were analyzed by Western blot for the presence of β 2m (BBM1), GRP94 and HLA-B heavy chains (HC10). Images were acquired by ImageLab and quantification was obtained by

FIJI. Values are the mean and SEM of results from 4 sets of experiments expressed as arbitrary units (A.U.). *: $P < 0.005$ by unpaired 2-tailed t-test.

Figure 5: Vesicles containing misfolded HLA-B27 proteins are enriched in h β 2m and in BiP and GRP94 chaperones in DCs from disease prone HLA-B*27:05/h β 2m transgenic rats.

Splenic DCs were isolated from 9-11mo-old male Lewis rats belonging to the AS-prone [21-3 x 283-2]F1 cross bearing 20 copies of HLA-B*27:05 and 50 copies of h β 2m, the healthy hemizygous 21-3 line bearing 20 copies of HLA-B*27:05 and 15 copies of h β 2m, the healthy hemizygous 283-2 line bearing 35 copies of h β 2m and nontransgenic rats and seeded in IBIDI 12-well plates, then fixed and permeabilized for **(A)** single-labeling with HC10 (unfolded HLA-B) or BBM1 (h β 2m) Abs followed by Alexa Fluor® 594-labeled anti-mouse IgG or **(B)** double-labeling with HC10 and anti-BiP or anti-GRP94 Abs followed by Alexa Fluor® 594-labeled anti-mouse IgG and Alexa Fluor® 488-labeled anti-rabbit IgG. Cells were mounted in *Vectashield* with Dapi and examined by means of confocal microscopy using a 63X objective. Scale bars: 5 μ m. Results are representative of at least 2 individual series of experiments. Graphs represent the percentage of Ab-reactive vesicles/DC from 2 to 4 rats per condition, with 4-10 cells analyzed/rat (median, 95%CI).

Figure 6: Vesicles containing misfolded HLA-B27 proteins are more frequent in immune cells from HLA-B27⁺ patients than HLA-B27-negative healthy controls. Cells from HLA-B27⁺ SpA patients and HLA-B27-negative healthy controls were fixed and labeled with HC10 for revelation of misfolded HLA-B-containing vesicles, and BBM1 (β 2m) or anti-ERp57 or anti-BiP Abs, or isotype-control Abs. The following secondary Abs were used: Alexa Fluor® 488-labeled anti-mouse IgG2a/Alexa Fluor® 594-labeled anti-mouse IgG2b for HC10/BBM1, Alexa Fluor® 594-labeled anti-mouse IgG/Alexa Fluor® 488-labeled anti-rabbit IgG for HC10/anti- ERp57 or HC10/anti- BiP. Cells were analyzed by confocal microscopy using a 63X objective. Images were analyzed with FIJI. Scale bars: 5 μ m. **(A)** Lymphoblastoid B cell lines from HLA-B27⁺ AS patient and control. This experiment was repeated twice, including different patient/control pairs. The graph shows the number, median and 95% CI of HC10⁺ vesicles/cell, based on 100 cells/sample. **(B)** Fixed and permeabilized human CD4⁺ T lymphocytes from HLA-B27⁺ SpA patient and control. Images are representative from 4 SpA and 3 controls. The graph shows the number, mean and SEM of HC10⁺ vesicles/cell based on 100 cells par sample. *: $P < 0.01$ by unpaired 2-tailed t-test.

Supplementary material

Figure S1: Intracellular vesicles in which HLA-B accumulate do not belong to early or late endosomes, golgi apparatus, ERGIC nor lysosomes. Fixed and permeabilized HLA-B^{YFP}-transfected cells were labeled with anti-EEA1, anti-Rab7, anti-Rab6, anti-Lman1 Abs for visualization of early and late endosomes, golgi apparatus and ERGIC, respectively. Anti-Lamp1 and lyso-tracker were used for visualization of the lysosomes. Cells were incubated with secondary antibodies (Alexa Fluor594®-labeled anti-rabbit IgG or Cy5-labeled anti-mouse IgG) and analyzed by confocal microscopy. Co-localization between HLA-B^{YFP} and various markers was assessed by confocal microscopy. Results are representative of at least 3 individual series of experiments. Scale bar: 10 µm.

Figure S2: Intracellular vesicles in which HLA-B accumulate are not autophagosomes. (A) HeLa Cells grown in 12-well µ-Slides (IBIDI) were transfected with plasmids encoding HLA-B*2705^{YFP}. After 3 hours of transfection cells were incubated with the autophagy inhibitor chloroquine (30µM) or the autophagy inducer rapamycin (40µM) for 24 hours. After treatment, cells were fixed and permeabilized for labeling of autophagosomes with anti-LC3 primary Ab. A594--labeled rabbit anti-IgG was used as secondary Ab and cells were analyzed by confocal microscopy. Confocal microscopy showed that the HLA-B-containing vesicles did not co-localize with LC3 and were not affected by induction or inhibition of autophagy. Results are representative of at least 4 individual series of experiments, including all different HLA-B alleles. Scale bar: 20 µm. **(B)** Time-lapse videomicroscopy (40X objective) was performed to visualize the appearance

of intracellular vesicles in HeLa cells. Cells grown in 8-well μ -Slides were transiently transfected with plasmids encoding for HLA-B*27:05^{YFP} protein. After 3 hours of transfection, cells were incubated with the autophagy inhibitor 3-MA (5mM) for 4 hours. Cells were washed and monitored for HLA-B^{YFP} containing vesicle formation during 17 hours by means of an inverse microscope (ScanR Olympus) equipped with a temperature controller stage using 40X lens. Photography was initiated 7 hours after transfection. The time elapsed between frames is shown as minutes.

A

aa	9	45	59	63	67	69	70	71	74	77	80	81	82	83	94	95	97	103	113	114	116	131	143	152	156	163
B*27:05	His	Glu	Tyr	Glu	Cys	Ala	Lys	Ala	Asp	Thr	Leu	Leu	Arg	Thr	Leu	Asn	Val	Tyr	His	Asp	Ser	Thr	Val	Leu	Glu	
B*27:04	-	-	-	-	-	-	-	-	Ser	-	-	-	-	-	-	-	-	-	His	Asp	-	-	Glu	-	-	
B*27:06	-	-	-	-	-	-	-	-	Ser	-	-	-	-	-	-	-	-	-	Asp	Tyr	-	-	Glu	-	-	
D114H	-	-	-	-	-	-	-	-	Ser	-	-	-	-	-	-	-	-	-	His	Tyr	-	-	Glu	-	-	
Y116D	-	-	-	-	-	-	-	-	Ser	-	-	-	-	-	-	-	-	-	Asp	Asp	-	-	Glu	-	-	

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