Characterization of a Proteasome and TAP-independent Presentation of Intracellular Epitopes by HLA-B27 Molecules*

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Background: HLA-B*2705-Asp116 and HLA-B*2709-His-116 associate differently with AS. Both possess an unpaired Cys-67 in the B pocket.

Results: The two molecules cross-present antigens through a proteasome-independent route. Cys-67, although not endorsing this alternative pathway, contributes variably to peptide stabilization.

Conclusion: B27 molecules can load peptides in post-ER compartments. Asp-116/His-116 influences the need of an intact B-pocket.

Significance: Identifying B27 distinctive features might contribute to the understanding of AS pathogenesis.

Because of the strong association with Spondyloarthropathies, HLA-B27 family of alleles is one of the most investigated HLA molecules. Accordingly, much is known about their structure (1), function (2, 3), geographical distribution (4, 5), and disease association, in particular with Ankylosing Spondylitis (AS), a chronic rheumatic inflammatory disease. More recently, the interest toward these molecules has been boosted by the observation that they have a protective role in some major viral infections such as AIDS or Hepatitis C (6–11). HLA-B27 includes a number of alleles that differ from each other in one or a few amino acids, and some of them, located in the peptide binding groove, have been proven to be functionally relevant. Noteworthy, some of these alleles such as the B*2709 in Sardinia (12, 13) and the B*2706 in Asia (14), are not associated with AS since they have not been reported, except anecdotically, in patients, and therefore have become a powerful tool to gain insights into disease association. Functional differences between AS-associated and non-AS-associated B27 subtypes (15), B27 transgenic animal models (16) and a recent report showing an association between AS and ERAP1 (17), an ER aminopeptidase trimming the peptides presented by the HLA-class I molecules, strongly suggest a direct role for the HLA-B27 molecules in disease pathogenesis, possibly presenting tissue-specific peptides (18).

It has been recently demonstrated that in TAP-defective cells, HLA-class I molecules can exchange peptides in post-ER vesicles such as in Trans Golgi network (TGN) (19). We have also shown that an HLA-B27-restricted epitope from Influenza virus can be cross-presented to CD8 T cells when exogenously carried to the TGN by chimeric proteins. In this case the presentation was proteasome and TAP-independent. TAP-competent cells such as EBV-B lymphocytes and HeLa cells (19) were also capable of such cross-presentation, allowing to hypothesize...
size that HLA-B27 molecules can routinely exploit this pathway.

It has been demonstrated that B27 molecules can form unstable complexes with peptides longer than nonamers (21). Furthermore, under some circumstances, the presence on the cell surface of misfolded HLA-B27 molecules as well as of B27 oligomers has been documented (22, 23). This suggests that a portion of B27 molecules reach the TGN in partially destabilized three-party complexes and are therefore prone to exchange peptides along their way to the cell surface. To further investigate these aspects, we used here TAT-driven carrier proteins in which the original HLA-B27-restricted epitope from the nucleoprotein of Influenza virus, SRYWAIRTR aa 383–391, was replaced with other HLA-B27 or HLA-A2 restricted viral epitopes. These proteins are delivered to the TGN by specific sequences in the C terminus of the influenza virus nucleoprotein (20). We found that cross-presentation by the HLA-A2 molecules required specialized Antigen-Presenting Cells (APC) and was both proteasome and TAP-dependent. Conversely, both B*2705 and B*2709, different from each other in a single amino acid (H116D) that affects binding specificity and T-cell presentation (24–27), used the proteasome and TAP-independent route. Since a hallmark of HLA-B27 molecules is the presence of a highly reactive cysteine at position 67, which influences the shaping of the B pocket and allows homodimer formation (28), this position was mutated in both HLA-B27 (C67S) and in the HLA-A2 molecules (V67C). Expression and antigen presentation were not altered in the case of HLA-A2 while they were dramatically affected in the case of HLA-B27 with striking differences between B*2705 and B*2709 molecules. These findings are further evidence of the functional relevance of the micropolymorphism at amino acid 116 distinguishing the HLA-B27 subtypes differentially associated with AS.

**EXPERIMENTAL PROCEDURES**

*Plasmids*—To produce genetic in-frame TAT fusion proteins, DNA sequence encoding for the amino acid region 301–498 of the influenza A virus nucleoprotein (Np) was inserted into the expression vector pTAT-HA (29) as already described (20). The hybrid constructs were generated by double digestion with Xhol and Eco0109I (Fermentas, Burlington, Ontario, Canada) located, respectively, upstream and downstream the NpFlu epitope, to insert the mutated sequences (29 aa–391). The truncated mutants were obtained by digestion with Eco0109I and re-ligation of the plasmid. The sequence of all recombinant constructs was confirmed by using BigDye Terminator cycle sequencing kit (Applied Biosystems, Carlsbad, CA) and ABI 377 DNA sequencer (Applied Biosystems).

DNA constructs HLA-B*2705, B*2709 and A*0201 were cloned in the pcDNA3 vector (Invitrogen). The mutated constructs HLA-B*2705Y320F (a gift from S. J. Powis), B*2705C67S, B*2709C67S, and A2V67C were generated through PCR-based mutagenesis of the wild-type cDNAs. To produce the B*2705C67S and B*2709C67S mutants, B*2705 and B*2709 respectively were subjected to site-directed mutagenesis to replace Cys-67 by Ser (C67S), using the primer 5′-GGAGACACACAGATCGCAAGCCAAGGCC and its respective reverse complement primer. To produce the A2V67C mutant, A2 was subjected to site-directed mutagenesis to replace Val-67 by Cys (V67C), with the primer 5′-GACACGGAAATGCAAGCCACTGCT and its respective reverse complement primer. All sequences were confirmed by DNA sequencing analysis.

**Production, Purification, and Detection of TAT Fusion Proteins**—TAT fusion proteins were produced as reported (20), according to Nagahara et al. (29). To purify the TAT recombinant proteins, Ni-nitrotriacetic acid (Ni-NTA) resin was used according to the protocol provided by Qiagen (Qiagen spa, Milan, Italy). After 24 h of dialysis with 1× phosphate-buffered saline (PBS), the samples were concentrated with an Ultrafree-CL 10 or 30 system (Millipore, Billerica, MA). The proteins were loaded on a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and detected by Coomassie Blue staining. For Western blotting analysis, after SDS-PAGE, samples were transferred to nitrocellulose filter and marked with mouse monoclonal immunoglobulin G (IgG) anti-HA (probe F7; Santa Cruz Biotechnology) diluted 1:200 in 1× PBS-0.1% Tween 20–5% dry milk for 1 h and revealed by a goat anti-mouse IgG conjugated to horseradish peroxidase (Pierce).

**Synthetic Peptides**—The synthetic peptides, RRIYDLIEL (pEBNA3C aa 258–266), RRRWRLTV (pLM2P aa 236–244) (PRIMM GmbH, Dubendorf, Zuerich, Switzerland), CVN-GVCWTV (pNS3-1 aa 1073–1081) and KLVALGINAV (pNS3-2 aa 1406–1415) (Epimmune Inc., San Diego, CA), were dissolved in dimethyl sulfoxide (DMSO). Concentrations were assayed by BCA assay according to the manufacturer’s protocol (Pierce).

**Cell Lines**—The following cell lines were used in this study: Epstein-Barr virus-transformed B lymphoblastoid cell lines (B-LCLs) (20), TAP-defective CEM 174.T2 cells (ATCC number: CRL-1992™) and HeLa cells (ATCC number: CCL2™) stably transfected with cDNA encoding B*2705, B*2709 (T2 and HeLa), A*0201 wt, and B*2705Y320F, B*2705C67S, B*2709C67S, and A2V67C mutants (HeLa).

B-LCLs were cultured in heat-inactivated 10% fetal calf serum (FCS)-RPMI 1640 medium (Lonza, Basel Switzerland), supplemented with 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin. PBMCs fromuffy-coats of anony-
mous healthy donors were isolated on a gradient of Lymphoprep (Cedarlane Laboratories limited, Hornby, Ontario, Canada). DCs were differentiated from monocytes isolated from PBMCs using Macs Microbeads CD14+ (Miltenyi Biotec, Bergisch Gladbach, Germany) and maintained in 10% FCS-RPMI 1640 medium supplemented with 20 ng/ml human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF; Immuno Tools, Friesoythe, Germany) and 2 ng/ml IL-4 (Eurobio, Les Ulis, France).

T2 B*2705 and B*2709 cells were generated by transfection of the CEM 174.T2 cell line with pcDNA3 vector (Invitrogen) in which cDNA encoding for B*2705 and B*2709 had been cloned. Stable transfectants were cultured in complete medium (10% FCS-RPMI 1640 RPMI with 2 mM l-glutamine, 10 units/ml penicillin, and 100 μg/ml streptomycin) supplemented with G418 (800 μg/ml).

HeLa cells transfection was performed with Lipofectamine 2000 Reagent, according to the protocol provided by Invitrogen. After transfection, cells were cultured in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 10 units/ml penicillin, and 100 μg/ml streptomycin and selected with G418 (800 μg/ml). NPFLU (aa 383–391), EBNA3C (aa 258–266), and LMP2 (aa 236–244), specific CTLs and NS3–2 (aa 1406–1415) and NS3–1 (aa 1073–1081) specific CTLs were obtained as previously described (20, 30).

Cytotoxicity Assays—Cytotoxic activity of CD8+ T cell lines was assayed by standard 51Cr release assay. CTL were mixed in 96-well U-bottom plates with 51Cr-labeled target cells for 4 h at 37 °C. The effector/target cell ratio was 20:1. Target cells had been previously incubated overnight with synthetic peptides or recombinant proteins or in medium alone. In the inhibition assay, target cells were preincubated with lactacystin (80 μM, Sigma-Aldrich), 45 min before adding the recombinant protein or the synthetic peptides. The percentage of specific lysis was calculated as 

\[ \text{Spontaneous cpm}/(\text{experimental cpm} - \text{spontaneous cpm})/ \]

(maximum cpm – spontaneous cpm).

**IFN-γ Production Assay and FACS Analysis**—CD8+ T cell clones specific for NS3–2 (KLVALGINAV aa 1406–1415) or NS3–1 (CVNGVCWTVA aa 1073–1081) or for the HLA-B27 binding peptide EBNA3C were stimulated with DCs preincubated with the relevant peptide or the recombinant protein in U-bottom microculture wells at 2 × 10^3 DC/3 × 10^3 CD8+ T cell/well in 0.2 ml of RPMI 1640 medium supplemented with 10% fetal calf serum (RPMI 1640 –10%). DCs loaded with either the peptide or the recombinant protein for 18 h at 37 °C were then washed with RPMI 1640 –10% and added to the culture at the DC/T cell ratio of 1:1.5. After 2 h, the cells were further treated with brefredin-A (10 mg/ml; Sigma-Aldrich) at 37 °C for 18 h. Cells were washed and stained with PerCP-conjugated anti-CD8 (Becton Dickinson, San Jose, CA) for 20 min at 4 °C, fixed, permeabilized using Cytofix/Cytoperm solution (BD Pharmingen) at 4 °C for 20 min, washed with Perm Wash Buffer (BD Pharmingen), intracellularly stained with FITC-conjugated anti-IFN-γ antibody (BD Pharmingen) for 30 min at 4 °C, washed, and finally analyzed by the FACSCalibur flow cytometer (Becton Dickinson), using CellQuest software (Becton Dickinson). An isotype-matched mAb was used as negative control.

For the surface expression of wt and mutated HLA molecules, cells were harvested and washed twice with PBS. Staining was performed at 4 °C for 1 h. Cells were first labeled with the nonconjugated primary mAbs: conformational-dependent ME1 specific for HLA-B27 and BB7.2 for HLA-A2, conformational-independent HC10 for HLA-B and HLA-C molecules and HCA2 for HLA-A2, followed by F(ab')2 of rabbit antimouse IgG-FITC (Jackson ImmunoResearch Europe Ltd., Suffolk, UK). Isotype-matched mouse Igs were used as negative controls. The analysis was performed by FACSCalibur (BD Biosciences). For each sample, 10,000 events were acquired using forward/side light scatter characteristics and analyzed using Cell Quest software (Becton Dickinson).

**Confocal Microscopy**—For Confocal Laser Scanning Microscopy (CLSM) analyses, 1 × 10^5 HeLa cells were grown on cover glasses (diameter 12 mm) for 24 h and then incubated with 0.4 mM TAT fusion proteins. At established times, cells were washed in 1% PBS, fixed by paraformaldehyde 2% in 1 × PBS for 20 min at room temperature, and then permeabilized with 0.1% Triton X-100 in PBS containing 1% BSA. For double-staining experiments the cells were incubated in the primary antibody at room temperature for 1 h with the following antibodies: TAT proteins were stained using anti-HA probe-FITC Ab (F7; Santa Cruz Biotechnology; sc7392 FITC) (1:75 in PBS); Furin was stained using rabbit anti-furin Ab (H-220; Santa Cruz Biotechnology; sc20801) (1:25 in PBS). As the fluorescence-labeled secondary antibody, Texas Red-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology; sc2780) (1:200 in PBS) was used. Confocal observations were performed using a Leica DMI8 apparatus (Leica Microsystems, Heidelberg, Germany) equipped with an argon-krypton laser, double-dichroic splitters (488/568 nm) and using a 63 × o immersion lens. Signal from different fluorescent probes were taken in sequential scan settings to prevent cross-talk between the two signals, and co-localization was detected in yellow (pseudo-color). Image acquisition and processing were achieved using the Leica Confocal Software (LCS) (Leica Lasertechnik, Heidelberg, Germany) and Adobe Photoshop software programs (Adobe System, Mountain View, CA). At least 50 cells for each condition, randomly taken from three independent experiments, were analyzed.

**Bioinformatics**—HotPOINT bioinformatics tool was used to calculate computational hot spots based on conservation, solvent accessibility, and statistical pairwise residue potentials in protein interfaces. TpairPotential feature calculated by HotPOINT algorithm is a statistically significant parameter useful to discriminate hot spots and non-hot-spots (p value: 5.4 × 10^-6) and the threshold value (equal to 18.0) was determined by using residues deposited in the Alamine Scanning Energetics database (ASEdb) (31). The modeled complex was analyzed with the LigPit tool (32) to identify the peptide-protein interactions.

**RESULTS**

HLA-B27, but Not HLA-A2 Molecules, Exploit a Non-canonical Pathway of Processing and Presentation for CTL Epitopes—Chimeric proteins driven by TAT transduction domain and the nucleoprotein (Np) of influenza A virus (TAT-NpFlu) were used to deliver CTL epitopes restricted for HLA-B27 (20). Here, these proteins were exploited to introduce either HLA-B27- or
HLA-A2-restricted viral epitopes. To this purpose, TAT-NpFlu was modified to produce hybrid proteins, in which the HLA-B*2705 NpFlu (SRYWAI RT, aa 383–391) was replaced with other HLA-B27 restricted epitopes from EBV such as EBNA3C (258–266) or pLMP2 (236–244) from EBV, or by the HLA-A2-restricted pNS3-2 (1406–1415) or pNS3-1 (1073–1081) from the NS3 protein of HCV. The constructs containing or not the C terminus of the nucleoprotein of influenza virus (end of Np) are indicated. The motif Arg/Ser (RS) is included at both the N or the C termini of the epitopes in the hybrid proteins.

The Effect of Cys-67 on Antigen Presentation—One of the features distinguishing the HLA-B27 from most of the other HLA-class I molecules is the presence of an unpaired and highly reactive cysteine at position 67. The substitution of this residue in the case of the EBV-B and HeLa cells (Fig. 4, lower panels) has been reported to influence, although not dramatically, the process of antigen presentation. In some experiments, we also noticed a partial inhibition of the production of IFN-γ by CTLs when the exogenous peptides were presented, probably due to the toxic effect of the drug, however the presentation of both NS3–2 and NS3–1 epitopes was virtually suppressed by lactacystin. On the contrary, B*2705 positive DC, although able to cross-present the B27-restricted epitope carried by the TATNPena3C protein, were only partially inhibited by lactacystin, suggesting that the DC exploit the proteasome-independent additional route described in the case of the EBV-B and HeLa cells (Fig. 4).

The HLA-B*2705 Y320F Mutant Affecting Recycling Was as Efficient as Wild Type in Presenting the Chimeric Proteins—Once established that the antigen presentation pathway described here was accessible to HLA-B27 but not to HLA-A2 molecules, we asked whether the HLA-B27 molecules that load the excised epitopes could be molecules recycling from the cell membrane. To verify this hypothesis, HLA-B27 cDNA carrying the mutation Y320F which has been shown to impair the endocytosis of HLA-class I molecules (33), was stably transfected into HeLa cells (Fig. 5A) which, like B-LCL, were able to cross-present the epitopes inserted into the chimeric proteins. Cross-presentation experiments were performed as above at different incubation times since the shedding of the molecules from the cell membrane is increased by this mutation. The HLA-B*2705 Y320F mutant was capable to cross-present pLMP2 inserted into the chimeric protein as efficiently as the wt molecules at each time point (4 h or ON) (Fig. 5B).

The HLA-B*2705 Y320F mutant was expressed at levels comparable to those of the wt molecule. As expected, wt T2 cells were able to present the epitope insertions into the hybrid proteins. In contrast, T2-B*2705 and T2-B*2709 could present both pEBNA3C and pLMP2 B27-restricted epitopes when delivered by the chimeric proteins. The failure to present the HLA-A2-restricted NS3 epitopes was not due to an impairment to cross the cell membrane since the recombinant protein containing the HLA-A2-restricted NS3–2 epitope, like that containing the EBNA3C epitope, colocalizes with furin in the TGN showing that the two proteins follow the same route (Fig. 3). We therefore asked whether the HLA-A2-restricted presentation could be performed by professional APC. Accordingly, Dendritic Cells (DCs) from three different HLA-A2 positive donors were generated, incubated with the chimeric proteins, and used as APC in cross-presentation experiments. In this cellular context, cross-presentation of the same chimeric proteins to the specific CTLs, was successful (Fig. 4). However, the processing was almost completely inhibited by lactacystin, demonstrating that the HLA-A2-restricted epitopes could be excised and processed by specialized APC in a proteasome-dependent pathway. In some experiments, we also noticed a partial inhibition of the production of IFN-γ by CTLs when the exogenous peptides were presented, probably due to the toxic effect of the drug, however the presentation of both NS3–2 and NS3–1 epitopes was virtually suppressed by lactacystin. On the contrary, B*2705 positive DC, although able to cross-present the B27-restricted epitope carried by the TATNPena3C protein, were only partially inhibited by lactacystin, suggesting that the DC exploit the proteasome-independent additional route described in the case of the EBV-B and HeLa cells (Fig. 4, lower panels).

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The Effect of Cys-67 on Antigen Presentation—One of the features distinguishing the HLA-B27 from most of the other HLA-class I molecules is the presence of an unpaired and highly reactive cysteine at position 67. The substitution of this residue has been reported to influence, although not dramatically, the peptide repertoire of the HLA-B*2705 molecules (34). We therefore asked whether the presence of Cys-67 was required to cross-predict epitopes embedded into the chimeric proteins. The two subtypes, HLA-B*2705 (Asp-116) and B*2709 (His-116), were mutated at position 67 (C67S) and stably transfected into HeLa cells (Fig. 5A) which, like B-LCL, were able to cross-present the epitopes inserted into the chimeric proteins. Cross-presentation experiments were performed as above at different incubation times since the shedding of the molecules from the cell membrane is increased by this mutation. The HLA-B*2705 Y320F mutant was capable to cross-present pLMP2 inserted into the chimeric protein as efficiently as the wt molecules at each time point (4 h or ON) (Fig. 5B).
expression of the mutant was similar to that of the wild type as monitored by the specific antibody BB7.2 with a small increase of the HCA2 (linear epitope) reactivity (Fig. 6C). The experiments were repeated with a different set of transfectants and the results were comparable (not shown). The transfectants were then used to present the exogenous peptides or the epitopes embedded into the chimeric proteins to specific CD8<sup>+</sup>/H<sub>11001</sub>T cells. The results show that, out of the three HLA-B27-restricted epitopes (pEBNA3C and pLMP2 for both B*2705 and B*2709 and pNpFlu for B*2705, pEBNA3C was the only one to be presented by both mutants (Fig. 7). V67C HLA-A2 mutant was able to present the pNS3–2 peptide as well as wt indicating that this mutation is well tolerated by the HLA-A2 molecules, but it failed, however, to make the HeLa transfectants competent to cross-present the epitope when delivered by the protein (Fig. 7).

To gain insight into the presentation of the EBNA3C epitope by the two HLA-B27 mutants, a dose-response curve was performed using pEBNA3C peptide or the chimeric protein in the two B27 contexts (Fig. 8). The results highlighted an extremely
different effect of the mutation C67S in the two B27 contexts: while the C67S B*2705 mutant could present the pEBNA3C at the highest concentration only, the C67S B*2709 mutant was more efficient than wt. Accordingly, the presentation of the chimeric epitopes showed the same trend with a very poor and a very efficient antigen presentation by the C67S B*2705 and the C67S B*2709 mutants respectively thus suggesting that the newly generated epitopes encountering the HLA-B27 molecules in the TGN were likely to be the same as those added as soluble peptides.

We postulated that such differences in presenting pEBNA3C were due to the only amino acid difference between B*2705 and B*2709 and therefore to the interaction between L9 and Asp-116 or His-116. To better investigate this, the complex between B*2709 protein and the peptide pEBNA3C was built initially using a template-based approach, based on the available structure of the B*2705:pEBNA3C complex (PDB ID: 2BST). The main chain atoms of the B*2709 comparative model and pEBNA3C peptide were optimally superimposed with the main chains of B*2705 and pEBNA3C in the complex. We refined the B*2709:pEBNA3C model using Rosetta FlexPepDock (35), a high resolution of the peptide-protein docking protocol to refine the starting structure of a peptide-protein complex (Fig. 9A). Next, we looked at the interactions and total contact potentials between L9 in the B*2709:pEBNA3C structure by using both LigPlot (32) and HotPOINT algorithm (31). HotPOINT labels His-116 as a hot spot residue at the protein-peptide binding interface, detecting a tpairPotential equal to 26.35. L9 and His-116 are also involved in hydrophobic interactions identified by LigPlot (Fig. 9B); on the opposite HotPOINT predicts Asp-116 in B*2705:pEBNA3C complex as a non-hot spot residue predicting a tpairPotential much below the threshold value and no interactions are detected between L9 and Asp-116 (Fig. 9D). This indicates that the L9 peptide residue is more stably accommodated in the F pocket of the B*2709 than on that of the B*2705 molecules.

DISCUSSION

The association of Ankylosing Spondylitis with HLA-B27 has been a conundrum for the last 40 years. One of the first hypothesis postulates the existence of HLA-B27-restricted "arthritogenic" peptides as triggers of the disease. This view has now been strengthened by the finding in the HLA-B27-positive but not in the B27 negative patients of a genetic association with ERAP1, an aminopeptidase involved in trimming the HLA-class I epitopes (17). Much is known about the rules governing the HLA-B27 binding properties and it has been recently reported that many peptides derived from cartilage/bone-related proteins are possible ligands for HLA-B27 molecules (18). We have previously shown that HLA-B27 molecules can exploit a proteasome- and TAP-independent pathway for antigen presentation when presenting the HLA-B27-restricted epitope of the nucleoprotein of Influenza virus driven into the cells by TAT transduction domain of HIV. We asked here whether this pathway was accessible to other HLA molecules.
Accordingly, the NpFlu 383–391 epitope (R/SRYWAIRTR/S) was replaced with other HLA-B27 or HLA-A2 restricted viral epitopes within the chimeric proteins used as carriers. The results indicated that this route was accessible to both the HLA-B27 alleles analyzed, B*2705 and B*2709, but not to HLA-A2, which however, likewise HLA-B27, co-localizes with the TAT-driven proteins in the TGN. Noteworthy, the embedded HLA-A2-restricted epitopes are successfully processed and presented by professional DC in a canonical, proteasome-dependent pathway indicating these chimeric proteins as good carriers to induce cross-priming through the classical pathway of antigen presentation exploited by DC. The TAT-driven proteins carrying the HLA-A2 or the HLA-B27-restricted epitopes differ only at the antigenic peptide sequence and one of the two HLA-A2-specific epitopes, NS3–2 (RS/KLVALGINAV/RS), possesses N- and a C terminus sequences (RS/RRRWRRLTV/RS), similar to pLMP2 with a Val at P9 and a conservative Lys instead of an Arg at P1. Although the HLA-B27-restricted epitope NpFlu (R/SRYWAIRTR/S) does not possess a canonical furin recognition sequence (RX(K/R)R) at its N terminus, we attempted to inhibit its presentation by using the inhibitor Dec-RVKR-CMK specific for furin or proprotein convertase 7 (PC7). We also used a siRNA strategy to block the expression of the ubiquitous nardylisin (36) also targeting cationic-rich peptide sequences, but we could not observe any inhibition (data not shown). However, although the HLA-B27 epitopes are enriched of Arg at their N terminus, the cut by furin-like pro-proteases would yield too short peptides deprived of the N-terminal anchor. This is most unlikely since the pattern of recognition of both the soluble and the chimeric epitopes by the CD8 T

**FIGURE 6.** Expression of C67S HLA-B27 and V67C HLA-A2 mutants. Expression of wt and C67S B*2705 molecules in A and wt and C67S B*2709 molecules in B) on the cell surface of HeLa cells was evaluated with the mAb ME1 that recognizes conformational molecules (left panels) and mAb HC10 that recognizes unfolded molecules (β2m-free heavy chains) (right panels). C, expression of wt HLA-A2 molecules and V67C mutants was evaluated on HeLa transfectants with BB7.2 mAb that recognizes conformational molecules (left panel), and HCA2 mAb that recognizes unfolded molecules (β2m-free heavy chains) (right panel). One representative staining of three-five independent flow cytometry analysis is shown here.

**FIGURE 7.** Effect on antigen presentation of C67S and V67C mutations in respectively HLA-B27 and HLA-A2 molecules. A, antigen-specific CTLs were tested in standard 51Cr-release assays using as target the HeLa cells expressing wt molecules or C67S mutants in the context of B*2705 (upper panels) and B*2709 (bottom panels) and pulsed with the indicated peptides (50 μM) or in medium alone (med). B, HeLa cells stably transfected with HLA-A2 wt or V67C mutant were incubated overnight with TAT-NpNS3–2/RSend recombinant protein (1.4 μM), pNS3–2 synthetic peptide (50 μM), or with medium alone (med) and used as targets for NS3–2 specific CTLs. Data are the mean percentage of lysis ± S.D. of three independent experiments.

**FIGURE 8.** Cysteine 67 is crucial for presentation of pEBNA3C by the HLA-B*2705 molecules. Dose-response curves in which HeLa B*2705 and B*2709 transfectants and the corresponding C67S mutants are used as targets in a standard 51Cr-Chromium-release assay at the indicated concentration of pEBNA3C or TATpEBNA3C/RSend recombinant protein. Data show the mean percentage of specific lysis ± S.D. of three independent experiments.
cells is virtually identical. This suggests that other, unknown proteases are involved in the excision of the embedded epitopes, probably targeting the RS epitope-flanking sequences. Given these considerations, we favor the hypothesis that the HLA-B27 molecules, or at least a fraction of them carrying longer or loosely bound peptides, are particularly prone to exchange them in the TGN. This might be favored by the lower pH (5.9) characterizing the Golgi vesicles compared with the ER (pH 7.1) (37, 38) as described for the HLA-B51 molecules in TAP-defective cells (19). Interestingly, both HLA-B51 and HLA-B27 are associated with autoimmune/autoinflammatory diseases, an association rarely found for other HLA-class I molecules. HLA-B*2705 and HLA-B*2709 molecules differentially associated with AS, were both able to present the chimeric epitopes. A feature of the HLA-B27 molecules is the presence of an unpaired, highly reactive cysteine at position 67. This amino acid has been shown to contribute to the shaping and stability of the B pocket where the primary anchor of the bound peptides, almost invariably an Arg, sits and its substitution promotes faster dissociation and modifies the repertoire of the bound peptides (39, 40). Analyzing the C67S B*2705 and B*2709 mutants, we observed a stronger requirement for C67 to stabilize the complexes in the case of B*2705 molecules which, compared with B*2709, display a significant higher ratio between the HC10-reactive (unfolded) and the ME1-reactive (folded) forms on the cell membrane. Accordingly, when we analyzed the antigen presenting competence of the two mutants, we found that neither of them could present pNP-Flu and pLMP2 as soluble peptides or as chimeric proteins, while the presentation of pEBNA3C was dramatically different: very poor by the B*2705 mutant and highly efficient by the C67S B*2709 mutant. A possible explanation for this observation is that B*2705-pEBNA3C and B*2709-pEBNA3C could display distinct conformations whose stability is influenced in different ways by the B pocket. The structure of the pEBNA3C: B*2705 complexes has been solved (41). Therefore, we built a model for this complex and analyzed the interactions and total contact potentials between L9 of pEBNA3C and His-116 or Asp-116
respectively in the B*2709:pEBNA3C three-dimensional model and B*2705 pEBNA3C complex. His-116 was labeled as hot spot residue at the protein-peptide binding interface and we observed hydrophobic interactions between His-116 and L9. On the opposite Asp-116 was predicted as a non-hot spot residue, suggesting that the strong interaction between L9 and His-116 makes the B pocket dispensable for peptide stabilization by the B*2709 but not by the B*2705 molecules. In any case, these results indicate the existence of a subset of peptides differently dependent on B pocket for their stability when bound by the two B27 subtypes. This is also pointed out by the increase of not correctly folded C67S B*2705 molecules on the cell surface. Whether this can be related to disease susceptibility, it cannot be said by this set of experiments. However, the data, once again, show subtle differences between B27 alleles carrying or not the Asp-116 which might bind the same peptides but display different conformations (2, 42). The structural features of this residue clearly influence the strength of binding of some peptides, made here more evident by the structural subversion of the B pocket. This could make the two molecules differently prone to exchange peptides in the TGN where they can encounter a set of tissue-specific peptides different from those generated by the classical proteasomal machinery. Ultimately, the rate of peptide exchange will be influenced by the repertoire of peptides generated in the ER, which in turn depends on the trimming activity of ERAP1 that changes according to the allelic variants, some of which are strongly associated with AS.

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