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ALLELE-DEPENDENT PROCESSING PATHWAYS GENERATE THE ENDOGENOUS HUMAN LEUKOCYTE ANTIGEN (HLA) CLASS I PEPTIDE REPERTOIRE IN TAP-DEFICIENT CELLS[*]

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Running head: Antigen processing pathways in TAP-deficient cells

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The transporters associated with antigen processing (TAP) allow the supply of peptides derived from the cytosol to translocate to the endoplasmic reticulum, where they complex with nascent human leukocyte antigen (HLA) class I molecules. However, infected and tumor cells with TAP molecules blocked or individuals with non-functional TAP complexes are able to present HLA class I ligands generated by TAP-independent processing pathways. These peptides are detected by the CD8$^+$ lymphocyte cellular response. Herein, the generation of the overall peptide repertoire associated with four different HLA class I molecules in TAP-deficient cells was studied. Using different protease inhibitors, four different proteolytic specificities were identified. These data demonstrate the different allele-dependent complex processing pathways involved in the generation of the HLA class I peptide repertoire in TAP-deficient cells.

The newly synthesized proteome is sampled continuously by CD8$^+$ lymphocytes as short peptides presented by human leukocyte antigen (HLA) class I molecules at the cell surface. The majority of the peptides presented by HLA class I molecules are produced from proteolysis by the proteasome and other cytosolic proteases, such as tripeptidyl peptidase II (1-3), puromycin-sensitive aminopeptidase (4), insulin degrading enzyme (5), thimet oligopeptidase (6), and caspases (7;8). These peptides are transported into the endoplasmic reticulum (ER) by TAP transporters, with subsequent N-terminal trimming by the metallo-aminoproteases ERAP1 and 2 frequently being required (9;10). Peptide binding to nascent HLA class I molecules generates stable peptide/HLA complexes that are exported to the cell membrane where are they exposed to cytotoxic CD8$^+$ T lymphocyte recognition (reviewed in (11)).

TAP$^{-/-}$ humans (12) and mice (13) have a reduced functional CD8$^+$ population but do not appear to have an increased susceptibility to neoplasms or viral infections. Thus, the TAP-independent pathways may be sufficient to control these diseases and allow individuals with this HLA class I deficiency to live normal life spans with only a limited susceptibility to chronic respiratory bacterial infections. In addition, evidence for TAP-independent pathways of antigen presentation by MHC class I molecules of particular but diverse pathogenic epitopes was previously reported (reviewed in (14-16)). The identified proteases involved in the generation of specific ligands in TAP-deficient cells include ER signal peptidase (SPase) (17;18), ER signal peptide peptidase (SPPase) (19;20), trans-Golgi network furin (21;22), and lysosomal cathepsins (23). However, systematic studies of TAP-independent pathways involved in the generation of the overall peptide repertoire associated with different HLA class I molecules have not been reported. Studying the re-expression of newly synthesized complexes of different HLA class I molecules in the presence of

diverse protease inhibitors allowed the determination of several allele-dependent processing pathways in TAP-deficient cells.

Experimental Procedures

Cell lines- T2 is a TAP-deficient human cell line that express HLA-A2, -B51, and -Cw1 class I molecules on the cell surface (24). T2-B27 cells were generated by transfection of T2 cells with HLA-B27 (a gift from Dr. David Yu, University of California, Los Angeles, CA). T2 and T2-B27 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum.

Chemicals - Brefeldin A (BFA), chloroquine, and all protease inhibitors were from Sigma-Aldrich except leupeptin (Amersham-UBS), pepstatin (Boehringer Mannheim), butabindide (Tocris), decanoyl-Arg-Val-Lys-Arg-CMK (dec-RVKR) (Bachem), (z-LL)$_2$ ketone (Merck), and lactacystin (Dr. E. J. Corey, Harvard University). The general specificity and activity of all inhibitors used in this study are summarized in Table 1. At the concentrations indicated, no inhibitors were toxic as demonstrates the normal HLA class I re-expression of at least one allele for each inhibitor (see below).

Acid stripping and HLA class I re-expression - Cells were washed with RPMI in the absence of serum and incubated for 90 s with ice-cool acid-stripping medium (0.3 M glycine-HCl and 1% BSA in water, pH 2.4) as previously reported (25). Culture medium was added to neutralize the pH. Cells were washed three times, resuspended in assay medium (RPMI 1640 with 1% BSA) at 10^6/ml, and incubated at 37ºC for 6 h in the presence or absence of inhibitors at the indicated concentrations (Table 1). Serum-free conditions were used throughout.

Flow cytometry - HLA expression levels were measured using the monoclonal antibodies (Abs) ME1 (anti-HLA-B27) (26) in T2-B27 cells; polyclonal H00003106-B01P (specific for HLA-B class I molecules; Abnova) in T2 cells; and monoclonal PA2.1 (anti-HLA-A2) (27) and

polyclonal SC-19438 (specific for HLA-C class I molecules; Santa Cruz Biotechnology) in T2 and T2-B27 cells simultaneously as previously described (28). Samples (10^4 cells) were run on a FACSCanto flow cytometer (BD Biosciences) and analyzed using CellQuest Pro 2.0 software (BD Bioscience). Percentage of inhibition of HLA re-expression obtained by the addition of the indicated inhibitors (Table 1) was calculated utilizing the following equation:

$$\text{\% Inhibition} = 100 - \frac{\text{MFI}_{+\text{Inhibitor}} - \text{MFI}_{+2^{nd}\text{Ab}}}{\text{MFI}_{\text{without Inhibitor}} - \text{MFI}_{+2^{nd}\text{Ab}}} \times 100$$

Statistical analysis **-** To analyze statistical significance, an unpaired Student t test was used. P values < 0.01 were considered to be significant.

RESULTS

Endogenous processing of TAP-independent HLA ligands.

To examine the generation of the TAP-independent HLA-bound peptide repertoire, TAP-deficient T2 and T2-B27 cells, treated with acid to remove surface class I peptide complexes, were allowed to re-express newly synthesized complexes for 6 h. The T2-B27 cell line was selected because it expresses one each of the endogenous HLA-A, -B, and –C alleles and an additional HLA-B class I molecule, thus mimicking a partial heterozygous haplotype. This expression pattern allows four different class I molecules to be studied. As previously reported for some MHC class I alleles (29), different re-expression levels were found in each of the four HLA class I molecules studied (Fig. 1, upper panel). To test if TAP-independent ligands require endogenous processing, HLA re-expression was analyzed in the presence of BFA. This drug blocks class I export beyond the cis-Golgi compartment (30;31), preventing the surface expression of newly assembled class I-peptide complexes from endogenous origin. The re-expression level of all four HLA class I alleles expressed by the TAP-deficient cells

used was significantly decreased: 84% ± 10% for HLA-A2, 87% ± 8% for HLA-B27, 80% ± 7% for HLA-B51 and 63% ± 8% for HLA-Cw1 (Fig. 1). These results demonstrated that the generation of the TAP-independent HLA-ligands was generated primarily from proteins endogenously processed in TAP-deficient cells.

Proteasome inhibitors differentially affect the TAP-independent expression of distinct HLA class I molecules.

To study the involvement of different proteolytic activities in the generation of TAP-independent ligands presented by HLA class I molecules, the surface re-expression of different HLA class I molecules after acid stripping in T2-B27 TAP-deficient cells was performed in the presence or absence of different inhibitors. Previously, partial block (~ 50% of inhibition) of HLA-A2 re-expression in TAP-deficient cells caused by the addition of lactacystin (LC), a *Streptomyces* metabolite (32-34) (Table 2), demonstrated a role for proteasomes in HLA class I processing of TAP-independent HLA-A2 ligands (25). The involvement of multicatalytic complex proteasome in the processing of ligands presented by other HLA class I was studied. Both LC and epoxomicin (35;36), another proteasome inhibitor, partially block both HLA-A2 and –B51 re-expression (Fig. 2), implicating the proteasomes in the generation of HLA ligands presented by these alleles By contrast, in the same experiment, both proteasome inhibitors have no effect on the re-expression of HLA-B27 and -Cw1 class I molecules. Thus, these data indicate that the proteasome activity is not absolutely required to generate ligands bound to HLA-B27 and –Cw1 class I molecules.

Metallo-aminopeptidases inhibitor specifically blocks the TAP-independent expression of peptide-HLA-A and –B but not -C complexes.

To characterize proteases distinct from proteasomes that may contribute to processing of HLA ligands, experiments with several specific protease inhibitors were performed. Leupeptin (LEU) (37),

pepstatin (PEPST) (37;38), E64 (39), and 1,10-phenanthroline (PHE) (38;40) inhibitors were initially tested because they are specific for different protease families (Table 1) and cover a wide range of protease classes. These three inhibitors had no effect on the HLA re-expression of any of the four alleles studied (Fig. 3). Thus, the enzymes inhibited by these drugs cannot be formally involved in the generation of TAP-independent ligands.

In addition, as the activity of ERAP, an enzyme previously involved in antigen processing (41;42), is not fully blocked by PHE at the concentration used in this study, the inhibitor leucinethiol (LeuSH) (Table 2) (43) was used. A partial inhibition of surface re-expression of HLA-A2 (38% ± 4%), -B27 (36% ± 10%), and –B51 (48% ± 8%) but not HLA-Cw1 (0% ± 5%) in TAP-deficient cells treated with LeuSH was found (Fig. 3). These inhibitions are similar to those previously reported in TAP-sufficient cells, where the surface quantities of the murine MHC class I molecules, K^k and L^d, decreased between 20 and 40% by specific ERAP inhibition (43). Thus, these data implicate ERAP, or other metallo-aminopeptidases, in the generation of a subset of TAP-independent ligands presented by three of four HLA class I molecules examined.

SPPase is involved in the generation of TAP-independent HLA-A2, and –B51 ligands

Different proteases such as tripeptidyl peptidase II (1;2), puromycin-sensitive aminopeptidase (4), and furin (21;22) were previously implicated in antigen processing as being able to generate pathogen-derived peptides. The possible role of these enzymes in endogenous presentation of TAP-independent ligands was studied using available specific inhibitors (Table 1). Inhibition of HLA re-expression was not detected with any of these drugs (Fig. 4) in all HLA class I alleles studied, and thus, the enzymatic activity of these peptidases is not absolutely required in the generation of TAP-independent ligands.

Different endogenous TAP-independent HLA-A2 class I ligands are derived by cleavage of the respective signal sequences generated by the SPase complex (16;44). A specific inhibitor of this enzymatic activity is unavailable; thus, direct involvement of SPase complex in the generation of TAP-independent HLA-A2 ligands could not be studied. However, two SPase-processed peptides need further cleavage by SPPase (19;20), allowing the hypothetical involvement of SPPase to be examined by treating TAP-deficient cells with the SPPase-specific inhibitor (z-LL)$_2$ ketone (19;20). This drug specifically inhibits TAP-independent HLA re-expression of HLA-A2 (82% ± 10%), and –B51 (48% ± 10%), but not –B27 (10% ± 7%) or -Cw1 (1% ± 12%). These results demonstrate the role of SPPase in the generation of TAP-independent ligands for some HLA class I molecules.

TAP-independent expression of peptide-HLA-B27 complexes is chloroquine (CQ)-sensitive.

Previously, three epitopes processed in an endosomal/lysosomal antigen processing pathway for murine MHC class I presentation in TAP-deficient cells were blocked in cells treated with CQ (23;45;46). Thus, the contribution of the CQ-sensitive (47;48) processing pathway to the generation of overall TAP-independent ligands was evaluated. In our experiments, specific reduction (41% ± 8%) of the HLA-B27 surface levels in presence of CQ was detected (Fig. 4). In contrast, the re-expression of HLA-A2, -B51, and –Cw1 class I molecules was not altered by this drug (Fig. 4). These data indicate that the endosomal/lysosomal antigen-processing pathway is HLA class I allele-dependent.

Summary of inhibitions of HLA class I re-expression.

With the drugs used in this study (Table 1), three different inhibition patterns of HLA class I expression were found (summarized in Table 2). The inhibition obtained in all HLA-class I alleles examined in presence of BFA indicates that most of the TAP-independent HLA-bound peptides were endogenously processed. The surface levels of HLA-A2 and –B51 class I were dependent on proteasome, metallo-aminopeptidases (probably ERAP), and SPPase activities. Metallo-aminopeptidases and CQ-sensitive processing are relevant for generating HLA-B27 ligands. By contrast, none of the compounds used in this study decreased HLA-Cw1 surface expression.

DISCUSSION

This study was undertaken to compare the generation of the peptide repertoire associated with four different HLA class I molecules in TAP-deficient cells. Previously, the expression of various HLA class I molecules were differentially affected by proteasome inhibitors in TAP-sufficient cells (25). These inhibitors blocked the re-expression of HLA-A2 (60%) and -B51 (80%) class I molecules. In contrast, HLA-B27 was largely insensitive to proteasome inhibitors (only 30% of inhibition of re-expression) (25). In the same study, the role of the proteasome in processing TAP-independent HLA-A2 ligands was reported (25). We found that a different MHC class I molecule, HLA-B51, is also proteasome-dependent in the generation of the HLA peptide repertoire in TAP-deficient cells. In addition, the proteasome inhibitors have no effect on the expression of TAP-independent peptide/HLA-B27 or –Cw1 repertoires. Thus, differential involvement of the proteasome in the generation of ligands bound to HLA class I molecules was found in this study. There was a direct correlation of the role of proteasomes in the processing of HLA peptide repertoires of TAP-dependent and –independent ligands between TAP-sufficient and deficient cells.

The role of SPase in the processing of TAP-independent presented HLA-A2 peptides has been found previously (17;18;44). Moreover, SPPase catalyzes intra-membrane proteolysis of two signal peptides after they have been cleaved from a pre-protein as the signal sequence-derived HLA-E peptides (19;20). Thus, sequential cleavage by SPase and SPPase was involved in the processing for some

peptides. We found that the specific inhibitor for SPPase activity significantly decreased the HLA expression of some alleles in TAP-deficient cells involving these sequential enzymatic activities in the processing of TAP-independent ligands bound to HLA-A2 and –B51 class I molecules.

It is well documented that trimming of ligand precursors in the endoplasmic reticulum (ER) is important for the generation of appropriate peptides for HLA class I binding and that ER-resident aminopeptidase activity has an important impact on the repertoire of ligands presented in TAP-sufficient cells (reviewed in (49)). The present study demonstrates that metallo-aminoprotease-sensitive trimming is also required to generate TAP-independent ligands presented by different HLA class I molecules.

HLA-A2 and –B51 class I molecules demonstrated a similar HLA-expression inhibition pattern involving the proteasome, SPPase, and metallo-aminopeptidase enzymatic activities in the generation of TAP-independent ligands. Thus, the most likely explanation is summarized via the following model. Multiple endogenous proteins are proteolyzed by the proteasome in the cytosol. Some of the generated peptides are released into the ER as indicated by the presentation in some instances of cytosolic proteins in cells lacking TAP (50;51). This presentation of peptides could occur by passive diffusion (52), hydrophobic peptides with specific ability to traverse membranes (53), or unidentified transport. In parallel, SPase releases peptides with signal sequence into the ER. Lastly, a fraction of total ER-peptides could be processed by SPPase activity and/or ERAP trimming prior to their binding to HLA-A2 or –B51 molecules.

The presentation pathway for extracellular antigens requires endocytosis and degradation in endolysosomal compartments. HLA class II molecules bind the peptides generated and these complexes are transported to the cell surface (54). Reagents that prevent endosomal acidification block this type of processing, inhibiting the protein cleavage by endosomal enzymes, such as the acidophilic amine CQ. Previously CQ-sensitive endosomal processing of internalized endogenous transmembrane proteins (23) or viral particles (45) for the generation of murine MHC class I-binding peptides was reported. The current study shows the formation of the overall TAP-independent peptide/HLA-B27 complexes by a proteasome-independent but BFA- and CQ-sensitive pathway. These data imply that class I molecules on the cell surface are internalized to an endolysosomal compartment where they intersect with peptides either supplied by the lysosomal polypeptide transporter TAPL (reviewed in (55)) or those directly processed by lysosomal proteases. In addition, some of these HLA-B27-restricted peptides need further trimming by metallo-aminopeptidases sensitive to LeuSH.

In addition, inhibition of the HLA-Cw1 surface expression was not detected with the chemicals used in this study. Thus, the identification of the different peptidase(s) of the antigen-processing pathway involved in the generation of HLA-Cw1 ligand awaits further molecular and cellular biology studies.

In summary, different and complex processing pathways involving at least four diverse proteolytic specificities in miscellaneous subcellular locations are required to generate the HLA class I peptide repertoire in TAP-deficient cells.

REFERENCES

1. Seifert, U., Marañón, C., Shmueli, A., Desoutter, J. F., Wesoloski, L., Janek, K., Henklein, P., Diescher, S., Andrieu, M., de la Salle, H., Weinschenk, T., Schild, H., Laderach, D., Galy, A., Haas, G., Kloetzel, P. M., Reiss, Y., and Hosmalin, A. (2003) *Nat.Immunol.* **4,** 375-379

2. Guil, S., Rodríguez-Castro, M., Aguilar, F., Villasevil, E. M., Antón, L. C., and Del Val, M. (2006) *J.Biol.Chem.* **281,** 39925-39934

3. York, I. A., Bhutani, N., Zendzian, S., Goldberg, A. L., and Rock, K. L. (2006) *J.Immunol.* **177,** 1434-1443

4. Stoltze, L., Schirle, M., Schwarz, G., Schröter, C., Thompson, M. W., Hersh, L. B., Kalbacher, H., Stevanovic, S., Rammensee, H. G., and Schild, H. (2000) *Nat.Immunol.* **1,** 413-418

5. Parmentier, N., Stroobant, V., Colau, D., de Diesbach, P., Morel, S., Chapiro, J., van Endert, P., and Van den Eynde, B. J. (2010) *Nat.Immunol.* **11,** 449-454

6. Kessler, J. H., Khan, S., Seifert, U., Le Gall, S., Chow, K. M., Paschen, A., Bres-Vloemans, S. A., de Ru, A., van Montfoort, N., Franken, K. L., Benckhuijsen, W. E., Brooks, J. M., van Hall, T., Ray, K., Mulder, A., Doxiadis, I. I., van Swieten, P. F., Overkleeft, H. S., Prat, A., Tomkinson, B., Neefjes, J., Kloetzel, P. M., Rodgers, D. W., Hersh, L. B., Drijfhout, J. W., van Veelen, P. A., Ossendorp, F., and Melief, C. J. (2011) *Nat.Immunol.* **12,** 45-53

7. López, D., Garcia-Calvo, M., Smith, G., and Del Val, M. (2010) *J.Immunol.* **184,** 5193-5199

8. López, D., Jiménez, M., García-Calvo, M., and Del Val, M. (2011) *J Biol.Chem.* **286,** 16910-16913

9. Rock, K. L., York, I. A., and Goldberg, A. L. (2004) *Nat.Immunol.* **5,** 670-677

10. Saveanu, L., Carroll, O., Lindo, V., Del Val, M., López, D., Lepelletier, Y., Greer, F., Schomburg, L., Fruci, D., Niedermann, G., and van Endert, P. M. (2005) *Nat.Immunol.* **6,** 689-697

11. Jensen, P. E. (2007) *Nat.Immunol.* **8,** 1041-1048

12. Cerundolo, V. and de la Salle, H. (2006) *Semin.Immunol.* **18,** 330-336

13. van Kaer, L., Ashton Rickardt, P. G., Ploegh, H. L., and Tonegawa, S. (1992) *Cell* **71,** 1205-1214

14. Del Val, M. and López, D. (2002) *Mol.Immunol.* **39,** 235-247

15. Johnstone, C. and Del Val, M. (2007) *Traffic* **8,** 1486-1494

16. Larsen, M. V., Nielsen, M., Weinzierl, A., and , L. O. (2006) *Current Immunology Reviews* **2,** 233-245

17. Wei, M. L. and Cresswell, P. (1992) *Nature* **356,** 443-446

18. Henderson, R. A., Michel, H., Sakaguchi, K., Shabanowitz, J., Appella, E., Hunt, D. F., and Engelhard, V. H. (1992) *Science* **255,** 1264-1266

19. Weihofen, A., Lemberg, M. K., Ploegh, H. L., Bogyo, M., and Martoglio, B. (2000) *J.Biol.Chem.* **275,** 30951-30956

20. Weihofen, A., Binns, K., Lemberg, M. K., Ashman, K., and Martoglio, B. (2002) *Science* **296,** 2215-2218

21. Gil-Torregrosa, B. C., Castaño, A. R., López, D., and Del Val, M. (2000) *Traffic* **1,** 641-651

22. Gil-Torregrosa, B. C., Castaño, A. R., and Del Val, M. (1998) *J.Exp.Med.* **188,** 1105-1116

23. Tiwari, N., Garbi, N., Reinheckel, T., Moldenhauer, G., Hammerling, G. J., and Momburg, F. (2007) *J.Immunol.* **178,** 7932-7942

24. Salter, R. D. and Cresswell, P. (1986) *EMBO J.* **5,** 943-949

25. Luckey, C. J., Marto, J. A., Partridge, M., Hall, E., White, F. M., Lippolis, J. D., Shabanowitz, J., Hunt, D. F., and Engelhard, V. H. (2001) *J.Immunol.* **167,** 1212-1221

26. Ellis, S. A., Taylor, C., and McMichael, A. (1982) *Hum.Immunol.* **5,** 49-59

27. Parham, P. and Bodmer, W. F. (1978) *Nature* **276,** 397-399

28. López, D., Samino, Y., Koszinowski, U. H., and Del Val, M. (2001) *J.Immunol.* **167,** 4238-4244

29. Anderson, K. S., Alexander, J., Wei, M., and Cresswell, P. (1993) *J Immunol.* **151,** 3407-3419

30. Yewdell, J. W. and Bennink, J. R. (1989) *Science* **244,** 1072-1075

31. Nuchtern, J. G., Bonifacino, J. S., Biddison, W. E., and Klausner, R. D. (1989) *Nature* **339,** 223-226

32. Fenteany, G., Standaert, R. F., Lane, W. S., Choi, S., Corey, E. J., and Schreiber, S. L. (1995) *Science* **268,** 726-731

33. Omura, S., Fujimoto, T., Otoguro, K., Matsuzaki, K., Moriguchi, R., Tanaka, H., and Sasaki, Y. (1991) *J.Antibiot.(Tokyo)* **44,** 113-116

34. Lee, D. H. and Goldberg, A. L. (1998) *Trends.Cell Biol.* **8,** 397-403

35. Schwarz, K., de Giuli, R., Schmidtke, G., Kostka, S., van den, B. M., Kim, K. B., Crews, C. M., Kraft, R., and Groettrup, M. (2000) *J.Immunol.* **164,** 6147-6157

36. Hanada, M., Sugawara, K., Kaneta, K., Toda, S., Nishiyama, Y., Tomita, K., Yamamoto, H., Konishi, M., and Oki, T. (1992) *J Antibiot.(Tokyo)* **45,** 1746-1752

37. Umezawa, H. (1976) *Methods Enzymol.* **45,** 678-695

38. Kozlowski, S., Corr, M., Shirai, M., Boyd, L. F., Pendleton, C. D., Berzofsky, J. A., and Margulies, D. H. (1993) *J.Immunol.* **151,** 4033-4044

39. Hanada, K., Tamai, M., Yamagishi, M., Ohmura, S., Sawada, J., and Tanaka, I. Isolation and characterization of E-64, a new thiol protease inhibitor. Agric Biol Chem **42**, 523-528. 1978.

40. Thornberry, N. A. (1994) *Methods Enzymol.* **244:615-31,** 615-631

41. Saric, T., Chang, S. C., Hattori, A., York, I. A., Markant, S., Rock, K. L., Tsujimoto, M., and Goldberg, A. L. (2002) *Nat.Immunol.* **3,** 1169-1176

42. York, I. A., Chang, S. C., Saric, T., Keys, J. A., Favreau, J. M., Goldberg, A. L., and Rock, K. L. (2002) *Nat.Immunol.* **3,** 1177-1184

43. Serwold, T., González, F., Kim, J., Jacob, R., and Shastri, N. (2002) *Nature* **419,** 480-483

44. Weinzierl, A. O., Rudolf, D., Hillen, N., Tenzer, S., van Endert, P., Schild, H., Rammensee, H. G., and Stevanovic, S. (2008) *Eur J Immunol.*

45. Schirmbeck, R., Melber, K., and Reimann, J. (1995) *Eur.J Immunol.* **25,** 1063-1070

46. Fromm, S. V., Duady-Ben Yaakov, S., Schechter, C., and Ehrlich, R. (2002) *Cell Immunol.* **215,** 207-218

47. Ziegler, H. K. and Unanue, E. R. (1982) *Proc.Natl Acad.Sci U.S.A* **79,** 175-178

48. Chesnut, R. W., Colon, S. M., and Grey, H. M. (1982) *J Immunol.* **129,** 2382-2388

49. van Endert, P. (2011) *Cell Mol.Life Sci* **68,** 1553-1567

50. Johnstone, C., Guil, S., García-Barreno, B., López, D., Melero, J. A., and Del Val, M. (2008) *J.Gen.Virol.* **89,** 2194-2203

51. Neumeister, C., Nanan, R., Cornu, T. I., Lüder, C. G. K., ter Meulen, V., Naim, H., and Niewiesk, S. (2001) *J.Gen.Virol.* **82,** 441-447

52. Lautscham, G., Mayrhofer, S., Taylor, G., Haigh, T., Leese, A., Rickinson, A., and Blake, N. (2001) *J.Exp.Med.* **194,** 1053-1068

53. Lautscham, G., Rickinson, A., and Blake, N. (2003) *Microbes.Infect.* **5,** 291-299

54. van den, H. T., Paul, P., Jongsma, M. L., and Neefjes, J. (2011) *Curr.Opin.Immunol.* **23,** 88-95

55. Bangert, I., Tumulka, F., and Abele, R. (2011) *Biol.Chem.* **392,** 61-66

56. Rose, C., Vargas, F., Facchinetti, P., Bourgeat, P., Bambal, R. B., Bishop, P. B., Chan, S. M., Moore, A. N., Ganellin, C. R., and Schwartz, J. C. (1996) *Nature* **380,** 403-409

57. McDonald, J. K., Reilly, T. J., Zeitman, B. B., and Ellis, S. (1968) *J Biol.Chem.* **243,** 2028-2037

58. Contin, C., Pitard, V., Itai, T., Nagata, S., Moreau, J. F., and Dechanet-Merville, J. (2003) *J Biol.Chem.* **278,** 32801-32809

The abbreviations used are: CQ, chloroquine; ER, endoplasmic reticulum; SPase, ER signal peptidase; SPPase, ER signal peptide peptidase; HLA, human leukocyte antigen; LC, lactacystin; LeuSH, leucinethiol; LEU, leupeptin; PEPST, pepstatin; PHE, 1,10-phenanthroline; TAP, transporters associated with antigen processing.

FIGURE LEGENDS

Figure 1.

Surface re-expression of HLA class I molecules after acid stripping in the presence of BFA.

T2 and T2-B27 cells were untreated (filled bars) or incubated with 5 μg/ml BFA for 6 h (thick bars) after acid washing. Stability at the cell surface of HLA-A2, -B27, -B51, and –Cw1 class I molecules of the TAP-deficient cells was measured by flow cytometry using monoclonal Ab ME1 (anti-HLA-B27) in T2-B27 cells; polyclonal H00003106-B01P (specific for HLA-B class I molecules) in T2 cells; and monoclonal Abs PA2.1 (anti-HLA-A2) and polyclonal SC-19438 (specific for HLA-C class I molecules) in T2 and T2-B27 cells simultaneously. The data are expressed as MFI $\pm$ SD (upper panel) or percentage of inhibition $\pm$ SD (lower panel) of HLA surface re-expression in presence of BFA and are the means of three-four different experiments.

Figure 2.

Effect of several proteasome inhibitors on surface re-expression of HLA class I molecules after acid washing.

T2 and T2-B27 cells as in Figure 1 were incubated with LC (open bars) or EPOX (filled bars) at the indicated concentrations (Table 1) as in Figure 1. The data are expressed as percentage of inhibition $\pm$ SD as in Figure 1 and are the means of four-five different experiments.

Figure 3.

Surface re-expression of HLA class I molecules after acid stripping in the presence of several protease classes inhibitors.

T2 and T2-B27 cells as in Figure 1 were incubated with the indicated inhibitors at the concentrations summarized in Table 1. HLA-A2 (filled bars), -B27 (open bars), -B51 (right thick bars), and –Cw1 (left thick bars) surface re-expression was measured. The data are expressed as percentage of inhibition $\pm$ SD as in Figure 1 and are the means of three-six different experiments. A representative experiment with T2 cells stained with anti-HLA-A2 Ab was depicted in the bottom panel. The coded used as in follows: second Ab alone (negative control, shaded histogram), no inhibitor (thin line) and 30 μM LeuSH (thick line).

Figure 4.

Outcome of different protease-specific inhibitors or a lysosomotropic agent on surface re-expression of HLA class I molecules after acid washing.

T2 and T2-B27 cells as in Figure 1 were incubated with the indicated drugs at the concentrations summarized in Table 1. HLA-A2 (filled bars), -B27 (open bars), -B51 (right thick bars), and –Cw1 (left thick bars) surface re-expression was calculated. The data are

expressed as percentage of inhibition ± SD as in Figure 1 and are the means of three-six different experiments. A representative experiment with T2 cells stained with anti-HLA-A2 Ab was depicted in the bottom panel. The coded used as in follows: second Ab alone (shaded histogram), no inhibitor (thin line) and 100 μM z-LL$_2$ (thick line).

Table 1

General specificity of inhibitors used in this study

Inhibitor	Abbreviation	Specificity	Reference	Concentration
Brefeldin A	BFA	Vesicle transport	(30;31)	5 μg/ml
Lactacystin	LC	Proteasome chymotryptic and tryptic activities	(32-34)	10 μM
Epoxomicin	EPOX	Proteasome chymotrypsin-like activity	(35;36)	1 μM
E64	E64	Cysteine proteases C1	(39)	100 μM
Leupeptin	LEU	Trypsin-like proteases and cysteine proteases	(37)	100 μM
Pepstatin	PEPST	Aspartic proteases	(37;38)	100 μM
1-10 Phenanthroline	PHE	Metalloproteases and caspase-1	(38;40)	50 μM
Leucinthiol	Leu-SH	Metallo-aminopeptidases including ERAAP	(43)	30 μM
Butabindide	BUT	Tripeptidyl peptidase II	(56)	100 μM
Puromycin	PUR	Dipeptidyl-peptidase II and puromycin-sensitive aminopeptidase	(57)	0.5 μg/ml
Decanoyl-Arg-Val-Lys-Arg-CMK	dec-RVKR	Furin and other members of the SPC family	(58)	100 μM
(z-LL)$_2$ ketone	z-LL$_2$	Signal peptide peptidase	(19;20)	100 μM
Chloroquine	CQ	Lysosomotropic agent	(47;48)	50 μM

Table 2

Summary of inhibition of HLA re-expression

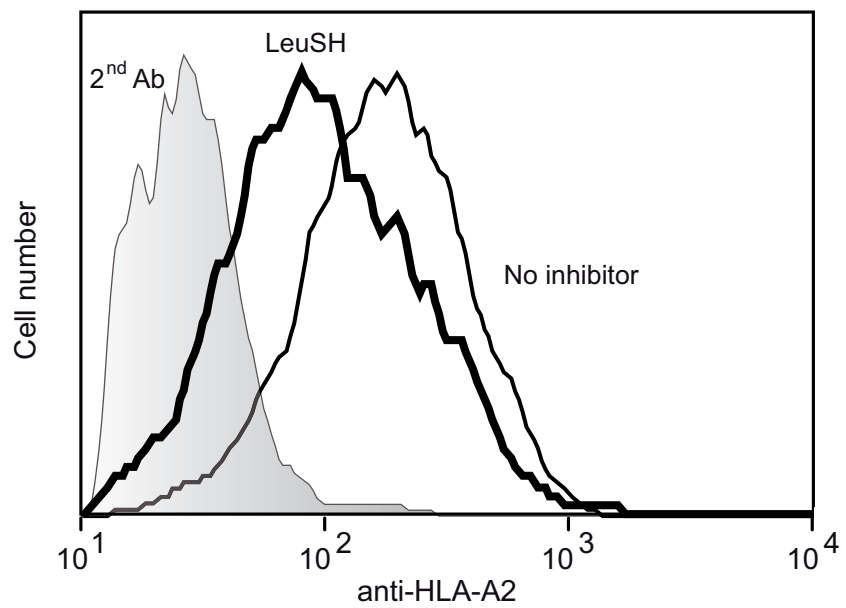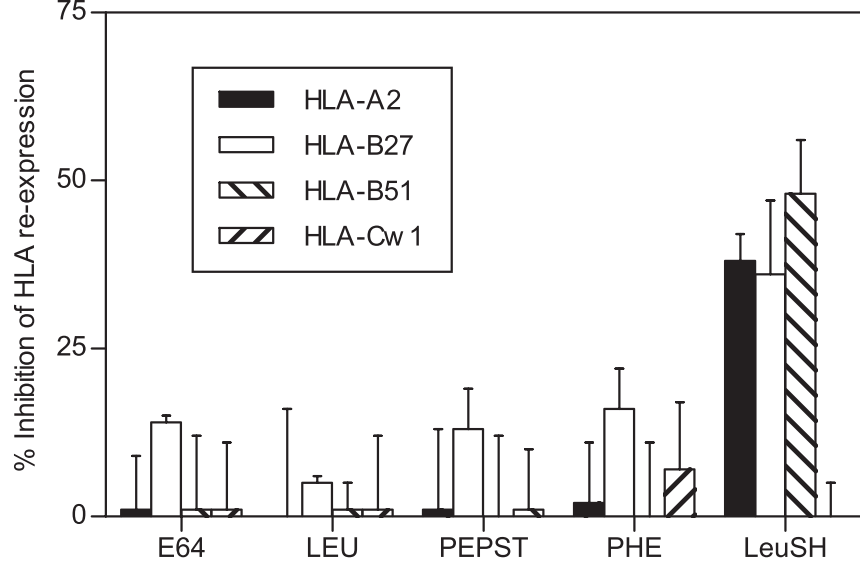| Alelle	BFA [a]	LC/EPOX	LeuSH	z-LL$_2$	CQ
HLA-A2	+[b]	+	+	+	-
HLA-B27	+	-	+	-	+
HLA-B51	+	+	+	+	-
HLA-Cw1	+	-	-	-	-

[a] For specificity of different inhibitors see Table 1.

[b] + and - indicate % inhibition > 35% and < 10%, respectively. All + inhibitions show significant P values (P < 0.01) versus controls without an inhibitor.
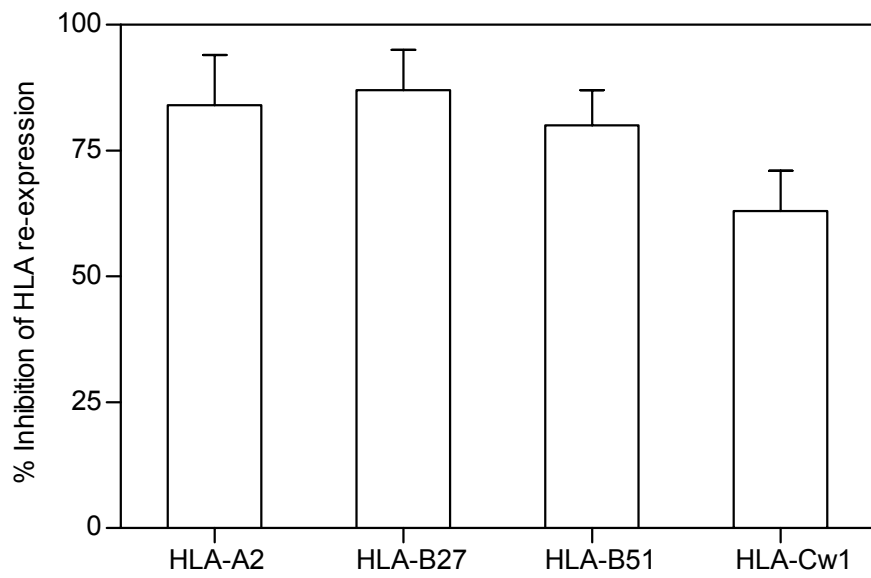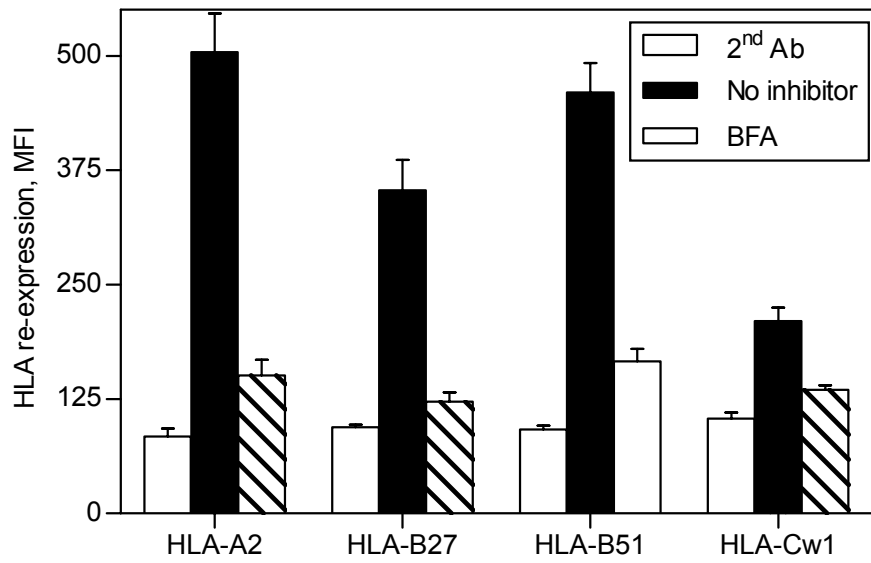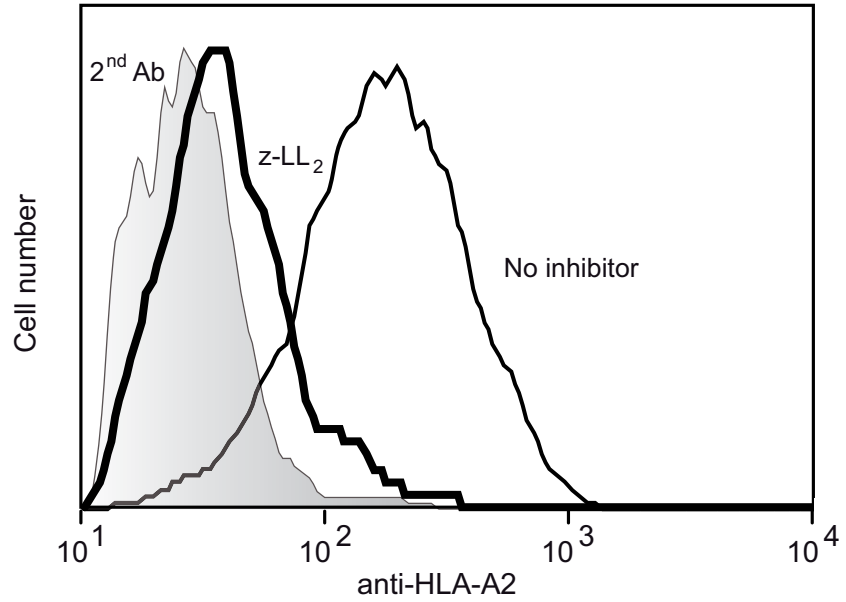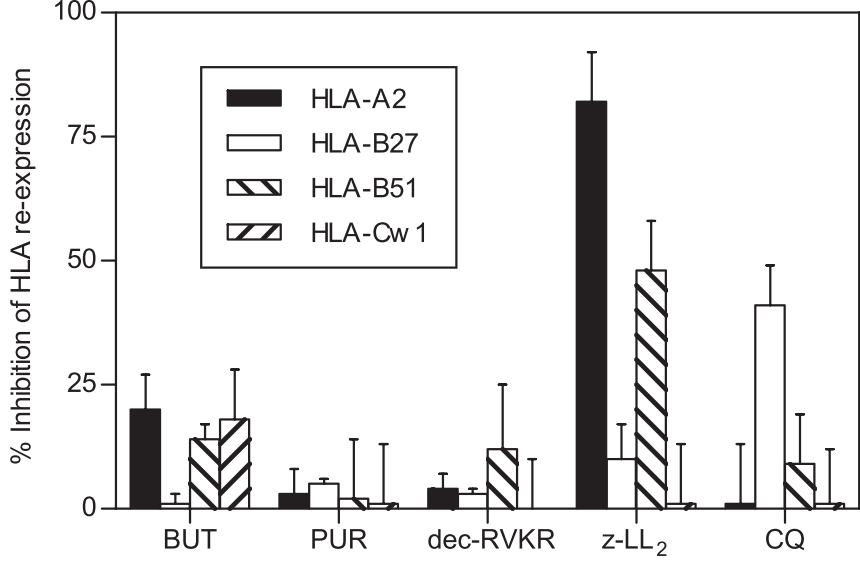
Lorente et al Figure 1
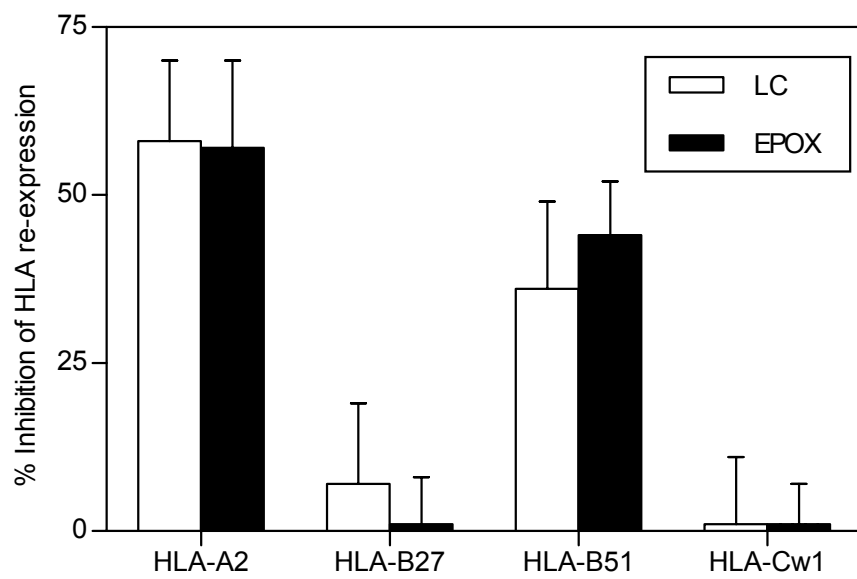
Lorente et al Figure 2

Lorente et al Figure 3

Lorente et al Figure 4