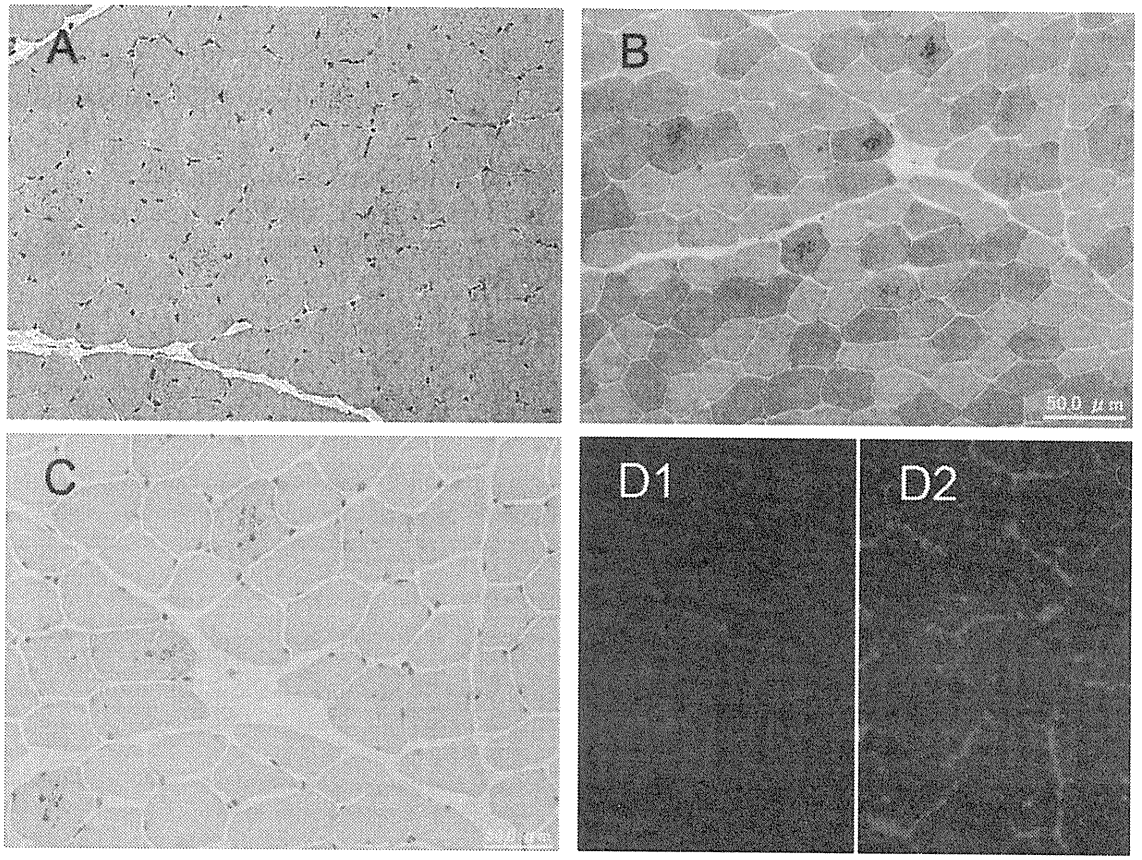

FIGURE 1. Skeletal muscle biopsy. Many scattered muscle fibers with basophilic granules and vacuoles (hematoxylin and eosin staining) **(A)**. Vacuolar membranes show nonspecific esterase **(B)**, and acetylcholinesterase activities **(C)**. Immunohistochemistry shows complete absence of LAMP-2 expression in muscles **(D1)**, compared with the normal control **(D2)**. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

activities (image not shown), demonstrating the features of autophagic vacuoles with unique sarcolemmal features (AVSF). Immunohistochemistry showed complete absence of LAMP-2 compared with the normal control sample (Fig. 1D). Neither necrotic nor regenerating fibers were observed.

Western Blot Analysis. We performed Western blot analysis with the LAMP-2 antibody in muscle extracts from normal controls (male and female) and the patient. LAMP-2 was present in normal controls but was totally absent in the patient's muscle (Fig. 2A).

Mutation Analysis. Sequence analysis revealed a novel heterozygote single nucleotide deletion of one of the four guanines in the region 238–241 (c.241delG; p.D81MfsX7) of the *LAMP2* gene, which causes substitution of amino acid 81 (aspartic acid) with a methionine, frameshift, and stop codon 7 residues downstream. This mutation was not found in her parents, brother, or sister, and it was also absent in 100 alleles of normal controls (Fig. 2B).

DISCUSSION

Female patients with Danon disease have a relatively mild clinical phenotype, and their mean age at presentation of the disease is reported to be 38 ± 12 years.[5] They usually manifest with symptoms of dilated cardiomyopathy, congestive heart failure, and arrhythmia. Most of the female patients are diagnosed when male probands with Danon disease are evaluated. Herein we diagnosed a manifesting female patient with proximal muscle weakness and hypertrophic cardiomyopathy at the age of 13 years.

Factors determining the phenotype of Danon disease in a heterozygotic female patient could originate either from skewed X inactivation or haploinsufficiency.[6] There are limited data regarding LAMP-2 expression in tissues of female patients. Fanin et al. reported a 56-year-old female patient who had weakness, cardiomyopathy, and easy fatigability since she was 26 years of age. She had Wolff–Parkinson–White syndrome, nonspecific hepatitis, and progressive heart failure. After cardiac transplantation at age 52, her cardiac problem was resolved, but weakness and myalgia persisted. LAMP-2 expression in her muscles was of similar intensity to control samples.[6] Sugie et al. reported a 47-year-old female patient

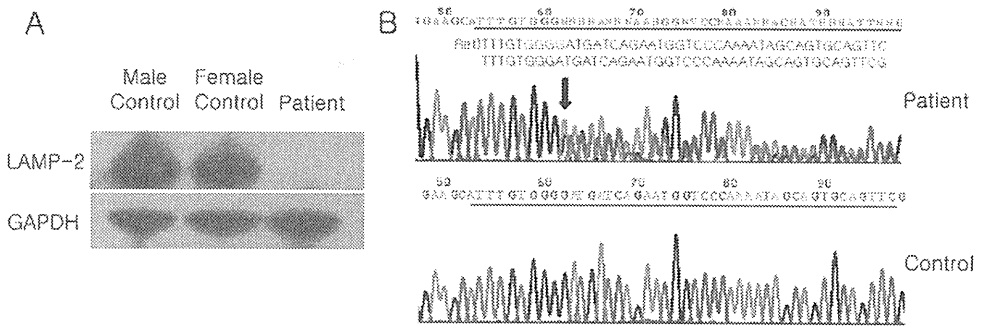

FIGURE 2. Western blot analysis of LAMP-2 and sequence analysis of *LAMP2* gene. Skeletal muscle extracts from male, female normal controls, and the patient were blotted and labeled with anti–LAMP-2 antibody and GAPDH. LAMP-2 expression is present in both male and female normal controls, but it is absent in the patient's muscles **(A)**. Sequence analysis reveals a heterozygote single nucleotide deletion (arrow) in the *LAMP2* gene (c.241delG; p.D81MfsX7) **(B)**. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

with cardiomyopathy. She had cardiomyopathy for which she had been treated for several years, but she had no weakness or cognitive decline. Immunohistochemistry of LAMP-2 was not absent, but it was weaker than normal controls.[6] In contrast, our patient had complete absence of LAMP-2 expression in her muscle. It can be assumed that the complete absence of LAMP-2 could have resulted in early manifestation and overt clinical myopathy. The mechanism underlying the complete absence of LAMP-2 in a heterozygoous female carrier needs further investigation in our patient.

Sarcolemma-specific proteins, including dystrophin, sarcoglycan, dystroglycan, and laminin, are present in autophagic vacuoles of patients with Danon disease.[7] Along with the acetylcholinesterase activity, the presence of sarcolemma-specific proteins indicates that the vacuolar membranes have the feature of sarcolemma, and the pathologic hallmark of Danon disease is autophagic vacuoles with unique sarcolemmal features.[2] Skeletal muscle findings in female patients with Danon disease have been reported in only 4 cases. One of them was muscle from an autopsy specimen that showed no remarkable findings,[8] and the other was from a 32-year-old female patient with mild left ventricular enlargement and atrial fibrillation that showed variable fiber size and acid phosphatase–positive inclusions in some fibers.[9] Reports from Sugie et al. and Fanin et al. both showed size variations without vacuoles (their clinical features have already been described).[5,6] In serial biopsies from a male patient at age 20 months and 16 years, Sugie et al. suggested that accumulation of autophagic vacuoles correlated with disease progression.[6] Fanin et al. later confirmed that the extent of these vacuolar changes was related to the degree of clinical muscle involvement.[5]

The muscle biopsy in our patient showed many scattered fibers with intracytoplasmic vacuoles, membranes of which had the unique features of sarcolemma. The prominent vacuolar change in muscle fibers in our patient may explain the clinically overt weakness and early presentation. Gathering all these findings, we assume that the complete absence of LAMP-2 led to increased numbers of autophagic vacuoles in muscle fibers and produced clinically overt skeletal myopathy and hypertrophic cardiomyopathy beginning in adolescence. To our knowledge, this is the first report of a female patient with this condition showing characteristic skeletal muscle pathology. This case supports evidence that the expression of clinically overt myopathy in Danon disease is related to the accumulation of autophagic vacuoles correlated with the complete absence of LAMP-2 expression, even in female patients.

There are few reports of hepatic involvement in Danon disease. There was one report that assessed 4 genetically confirmed Danon disease patients (3 males and 1 female).[5] In that study, the 20-year-old male index patient had a tender and enlarged liver with ascites. Hepatosplenomegaly was detected by ultrasonography. Patient 2 was a 9-year-old boy with jaundice and laboratory abnormality. A liver biopsy showed chronic hepatitis. Patient 3 was a 12-year-old boy with hepatomegaly, and a liver biopsy showed no abnormal findings. Patient 4 (her clinical features were described earlier) had nonspecific hepatitis with ultrasound evidence for hepatosplenomegaly. Tuñon et al. reported an 18-year-old boy with hypertrophic cardiomyopathy and elevated transminases. A liver biopsy showed slight portal fibrosis and unicellular hepatocyte necrosis.[10] In a mouse with LAMP-2 deficiency, characteristic autophagic vacuoles were found in hepatocytes.[11] In our patient, laboratory abnormalities were the only evidence of hepatic involvement. It is uncertain whether this patient had subclinical involvement of the liver or whether it was related to a different etiology. Our patient first manifested with elevations in liver enzymes and was diagnosed and treated for autoimmune hepatitis. After checking the muscle enzyme levels, she was referred to the neurology clinic, and we reached the final

diagnosis. It is common for a patient with myopathy to show liver enzyme abnormalities. Therefore, it is important to check muscle enzymes in patients when the diagnosis and cause of abnormal liver enzymes is uncertain.

In conclusion, we have demonstrated the absence of LAMP-2 expression in skeletal muscles from a female patient with early-onset overt proximal weakness, many vacuolated fibers, and a de novo novel mutation in the *LAMP2* gene. We strongly suggest that the pathogenesis of proximal weakness is more related to autophagic vacuoles than primary LAMP-2 expression. We also believe that there may be other factors that cause cardiomyopathy in Danon disease.

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SENSORY ATAXIC NEUROPATHY WITH DYSARTHRIA AND OPHTHALMOPARESIS (SANDO) IN LATE LIFE DUE TO COMPOUND HETEROZYGOUS *POLG* MUTATIONS

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ABSTRACT: Missense mutations in the gene for polymerase γ 1 (*POLG1*) cause a number of phenotypically heterogeneous mitochondrial diseases, most commonly progressive external ophthalmoplegia, and are characterized by the accumulation of multiple, large-scale deletions of mitochondrial DNA. The triad of sensory ataxic neuropathy, dysarthria, and ophthalmoparesis (SANDO) has been demonstrated in a small subset of patients with *POLG1* mutations. We report a sporadic case of an 80-year-old compound heterozygote man who presented with SANDO and was found to have three known pathogenic mutations in the *POLG1* gene (p.T251I/p.P587L/p.G848S). To our knowledge, none of these mutations have been demonstrated previously in SANDO. This patient's late presentation illustrates that a mitochondrial disorder should be considered regardless of age if the clinical symptoms warrant.

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Abbreviations: ANA, antinuclear antibodies; *ANT1*, adenine nucleotide translocator 1; COX, cytochrome *c* oxidase; EMG, electromyography; PEO, progressive external ophthalmoplegia; MIRAS, mitochondrial ataxic syndrome without ophthalmoplegia; mtDNA, mitochondrial DNA; *POLG1*, acetylcholine receptor; SANDO, sensory ataxic neuropathy, dysarthria, and ophthalmoparesis; SNAP, sensory nerve action potential

Key words: dysarthria; polymerase γ; mitochondrial myopathy; progressive external ophthalmoplegia; sensory ataxic neuropathy

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Mutations in polymerase γ 1 (*POLG1*) lead to a number of mitochondrial disease phenotypes associated with multiple mitochondrial DNA deletions. Such mutations present clinically in a heterogeneous manner and include both autosomal dominant and recessive forms of progressive external ophthalmoplegia (PEO); mitochondrial ataxic syndrome without opthalmoplegia (MIRAS); and the clinical triad of sensory ataxic neuropathy, dysarthria, and ophthalmoparesis (SANDO).[1,2] There is no specific treatment for diseases related to *POLG1* mutations, although valproic acid is contraindicated, as it may precipate fulminant liver disease. Only a few cases of SANDO associated with *POLG1* mutations have been reported.[3–8] We report a case of SANDO associated with known pathogenic *POLG1* mutations that presented in late life in a compound heterozygote male.

CASE REPORT

An 80-year-old-man presented with a 7-year history of progressively droopy eyelids, a 4-year history of double vision, and 3 years of an increasingly nasal

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IMMUNOLOGY ORIGINAL ARTICLE

LAMP-2-deficient human B cells exhibit altered MHC class II presentation of exogenous antigens

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Summary

Major histocompatibility complex (MHC) class II molecules present antigenic peptides derived from engulfed exogenous proteins to $CD4^+$ T cells. Exogenous antigens are processed in mature endosomes and lysosomes where acidic proteases reside and peptide-binding to class II alleles is favoured. Hence, maintenance of the microenvironment within these organelles is probably central to efficient MHC class II-mediated antigen presentation. Lysosome-associated membrane proteins such as LAMP-2 reside in mature endosomes and lysosomes, yet their role in exogenous antigen presentation pathways remains untested. In this study, human B cells lacking LAMP-2 were examined for changes in MHC class II-restricted antigen presentation. MHC class II presentation of exogenous antigen and peptides to $CD4^+$ T cells was impaired in the LAMP-2-deficient B cells. Peptide-binding to MHC class II on LAMP-2-deficient B cells was reduced at physiological pH compared with wild-type cells. However, peptide-binding and class II-restricted antigen presentation were restored by incubation of LAMP-2-negative B cells at acidic pH, suggesting that efficient loading of exogenous epitopes by MHC class II molecules is dependent upon LAMP-2 expression in B cells. Interestingly, class II presentation of an epitope derived from an endogenous transmembrane protein was detected using LAMP-2-deficient B cells. Consequently, LAMP-2 may control the repertoire of peptides displayed by MHC class II molecules on B cells and influence the balance between endogenous and exogenous antigen presentation.

Keywords: exogenous antigens; human B cells; MHC class II presentation

Introduction

Major histocompatibility complex (MHC) class II molecules present antigenic peptides derived from exogenous proteins to $CD4^+$ T cells.[1] These MHC class II proteins are constitutively expressed on the surface of a number of professional antigen-presenting cells (APC) such as dendritic cells, B cells and macrophages. The MHC class II complexes consist of α and β subunits which are first assembled in the endoplasmic reticulum with the chaperone molecule invariant chain (Ii).[2,3] The cytoplasmic tail of Ii contains a motif that targets the Ii–MHC class II complexes to endosomal/lysosomal compartments. Here, acidic proteases degrade Ii to a small fragment known as class II-associated invariant chain peptide (CLIP), which remains associated with the MHC class II peptide-binding groove.[4,5] Antigens delivered into the endosomal/lysosomal network via receptor-mediated or fluid-phase endocytosis are also exposed to proteases and denaturing reactions, yielding peptide ligands for class II molecules.[6] CLIP removal and the capture of antigenic peptides by MHC class II proteins is catalysed by the MHC-encoded molecule HLA-DM[7–9] and occurs in mature endosomes or pre-lysosomes known as MIIC.[10] The resulting peptide–MHC class II complexes are ultimately trafficked to the cell surface for immune surveillance by $CD4^+$ T cells.

Abbreviations: B-LCL, B-lymphoblastoid cell line; CHS, Chediak–Higashi syndrome; CLIP, class II-associated invariant chain peptide; GAD, glutamate decarboxylase; HSA, human serum albumin; LAMP, lysosome-associated membrane protein; LYST, lysosomal trafficking regulator; MFI, mean fluorescence intensity.

Mature endosomes and lysosomes play critical roles in routine intracellular processes such as protein degradation as well as more specialized functions related to antigen presentation by MHC class II molecules.[10,11] These morphologically heterogeneous organelles are distinguishable from other intracellular compartments in most cells by the presence of mature acid-dependent hydrolases and lysosome-associated membrane proteins such as LAMP-1 and LAMP-2.[12] LAMP-1 and LAMP-2 are members of a family of highly glycosylated transmembrane proteins primarily located in mature endosomes and lysosomes.[13] A deficiency in LAMP-2 is linked with the development of an X-linked lysosomal storage disorder known as Danon disease;[14] genetic analysis of patients with this disorder demonstrated several mutations in the LAMP-2 gene causing protein truncations and an absence of protein expression in patient tissues.[15] Danon disease patients display an accumulation of dense and translucent vacuoles, possibly autophagosomes, in the cells of multiple tissues.[15] Additionally, studies with LAMP-2 knockout mice reveal an accumulation of autophagic vacuoles in many tissues possibly because of impaired lysosomal trafficking.[16,17]

The LAMP-2 gene encoded on the X-chromosome gives rise to several alternative transcripts encoding protein isoforms that differ primarily in their cytoplasmic tail domains.[18] Among these isoforms, LAMP-2A and -2B proteins are ubiquitously expressed in most tissues including lymphocytes.[19] LAMP-2A serves as the lysosomal receptor for chaperone-mediated autophagy, a pathway promoting the transport of specific cytosolic proteins into lysosomes via a molecular chaperone/receptor complex.[20–22] Overexpression of LAMP-2A or hsc70, a chaperone protein that co-operates with LAMP-2A in chaperone-mediated autophagy, enhanced the MHC class II-restricted presentation of two cytoplasmic autoantigens in human B cells, hence establishing a role for LAMP-2 in cytoplasmic antigen presentation.[19] Remarkably, a partial decrease in total LAMP-2 expression in human B cells reduced not only cytoplasmic antigen presentation but also exogenous antigen presentation by MHC class II molecules.[19] Studies here address how the complete loss of LAMP-2 in human B cells modulates epitope selection and display in the context of MHC class II. In the absence of LAMP-2, human B cells displayed a reduced capacity for MHC class II-restricted presentation of exogenous antigen and peptides but maintained the presentation of epitopes from an endogenous transmembrane protein.

Materials and methods

Cell lines

The human B lymphoblastoid cell lines (B-LCL) Priess [(homozygous DR4 (DRβ1*0401)] and Frev [DR1(DRβ1*0101), DR4(DRβ1*0401)] were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat inactivated calf serum. The human B-LCL 7C3.DR4 was retrovirally transduced to express HLA-DR4[23] and cultured in IMDM supplemented with 5% heat inactivated calf serum. A B-LCL from a Danon disease patient (Danon B-LCL) [DR14(DRβ1*1401), DR15(DRβ1*1502)] was cultured in IMDM supplemented with 10% heat inactivated calf serum. In these cells, a 2-base-pair deletion in exon 3 of the LAMP-2 gene in the single X-chromosome-encoded copy disrupts LAMP-2 gene expression. Priess and 7C3.DR4 cells express endogenous immunoglobulin G (IgG) κ light chain while Frev and Danon B-LCL are negative for κ light chain expression by Western blot analysis and instead, express IgG λ light chain. Danon B-LCL were transduced with DRβ1*0401 complementary DNA along with the mammalian selection marker histidinol using the retroviral cell line PA317hddw4c1 obtained from Dr William Kwok (Benaroya Research Institute at Virginia Mason, Seattle, WA). HLA-DR4$^+$ Danon B-LCL clones (DB.DR4) were selected by their growth in IMDM supplemented with 10% heat inactivated calf serum and 8 mM histidinol (Sigma-Aldrich, St Louis, MO). HLA-DR4 expression in the DB.DR4 transfectants was evaluated by flow cytometry using the HLA-DR4-specific antibody 3.5.9-13F10. The murine B-cell CH27 was retrovirally transduced with DRα and DR4β to express HLA-DR4 and cultured in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal bovine serum and 0·1% β-mercaptoethanol. The T-cell hybridoma 17.9 is specific for the HSA_{64-76} epitope from human serum albumin (HSA).[24] The T-cell hybridomas 2.18 and 1.21 are specific for the $\kappa I_{188-203}$ and $\kappa II_{145-159}$ epitopes from the human IgG κ light chain, respectively.[25] The T-cell hybridoma 33.4 is specific for the HLA-A_{52-70} epitope from the α chain of HLA-A.[26] All T-cell hybridomas were generated in the DR4(DRβ1*0401) transgenic mice[27] and were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 0·1% β-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Peptides and antigens

Human $GAD_{273-285}$ (IAFTSEHSHFSLK), HSA_{64-76} (VKLVNEVTEFAKT), human IgG immunodominant $\kappa I_{188-203}$ (KHKVYACEVTHQGLSS), biotinylated $\kappa I_{188-203}$ (biotin-KHKVYACEVTHQGLSS), human IgG subdominant $\kappa II_{145-159}$ (KVQWKVDNALQSGNS) and human HLA-A_{52-70} (VDDTQFVRFDSDAASQRME) peptides were synthesized, purified to > 90% purity by reverse-phase high-performance liquid chromatography, and the sequences were confirmed by mass spectral analysis in conjunction with Quality Controlled Biochemicals (QCB;

Hopkinton, MA). The HSA and human IgG antigens were purchased from Sigma-Aldrich.

Antibodies

The mouse monoclonal antibodies (mAb) specific for either human LAMP-1 (H4A3) or human LAMP-2 (H4B4) were purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA) for use in Western blots. The mouse mAb specific for human LAMP-1 and conjugated with AlexaFluor647 for use in immunofluorescence was purchased from eBioscience (San Diego, CA). The rat antibody 3.5.9-13F10[28] was used to detect surface HLA-DR4β chains while the mouse mAb L243[29] was used to detect intracellular and surface HLA-DR$\alpha\beta$ dimers by flow cytometry. L243 conjugated with fluorescein isothiocyanate (FITC) was purchased from BD Biosciences (San Jose, CA) and used to detect HLA-DR$\alpha\beta$ dimers in immunofluorescence. The mouse mAb W6/32 was used to detect intracellular MHC class I molecules. The mouse mAb MaP.DM1 was a gift from Dr Peter Cresswell (Yale University, New Haven, CT) and was used to detect intracellular HLA-DM molecules. A mouse mAb used to detect intracellular HLA-DO molecules by flow cytometry was purchased from BD Biosciences. The mouse mAb DA6.147 was used to detect intracellular HLA-DR$\alpha\beta$ dimers by Western blotting.[30] The mouse mAb specific for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Chemicon (Temecula, CA). For immunoblotting, the polyclonal anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP) was purchased from Jackson Laboratories (West Grove, PA). For flow cytometry, the FITC-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG and the Cy2-conjugated $F(ab')_2$ fragment of donkey anti-rat IgG were purchased from Jackson Laboratories. The phycoerythrin (PE) -conjugated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulin was purchased from Dako (Carpinteria, CA).

Western blotting

Danon or Frev B-LCL were lysed on ice for 20 min in buffer containing 10 mM Tris–HCl, pH 7·2, 150 mM NaCl, 1% Triton X-100, and the following protease inhibitors: 4-(2-aminoethyl)benzenesulphonyl fluoride hydrochloride, pepstatin A, E-64, bestatin, leupeptin and aprotinin (Sigma-Aldrich). Total protein concentration of the cell lysates was determined using the Bio-Rad Protein Assay reagent (BioRad Laboratories, Inc., Hercules, CA). Between 50 and 100 μg of protein/sample were resolved on 8% sodium dodecyl sulphate (SDS) –polyacrylamide gel electrophoresis gels, transferred onto nitrocellulose membranes (BioRad), and immunoblotted using antibody specific for LAMP-1 or LAMP-2 followed by incubation with a polyclonal anti-mouse-HRP-conjugated secondary antibody. To detect HLA-DR$\alpha\beta$ dimers, samples were prepared in non-reducing, non-boiled conditions. Blots were visualized with enhanced chemiluminescence (Pierce, Rockford, IL). The membranes were stripped in buffer containing Tris–HCl, SDS, and β-mercaptoethanol and reprobed for GAPDH as a control for protein loading among samples.

Quantitative real-time polymerase chain reaction analysis

Total RNA was prepared from wild-type or LAMP-2-deficient B-LCL using Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH). Reverse transcription was performed using an Advantage RT-for-PCR kit (Clontech Laboratories Inc., Palo Alto, CA) according to the manufacturer's instructions. The 5′ primer for HLA-DRα chain was 5′-CAAAGAAGGAGACGGTCTGG-3′ and the 3′ primer was 5′-AGCATCAAACTCCCAGTGCT-3′. GAPDH primers were used as a control. The correct sizes of the amplification products using these primers in reverse transcription–polymerase chain reaction (RT-PCR) were confirmed by ethidium bromide staining and UV transillumination before their use in quantitative RT-PCR. For quantitative RT-PCR, SYBR® GREEN PCR Master Mix (Applied Biosystems, Foster City, CA) was used for all amplifications, which were performed in a 7500 Real-Time PCR thermal cycler (Applied Biosystems) using the following parameters: 95° for 15 seconds, then 60° for 60 seconds for 40 cycles. GAPDH was used as the endogenous reference while Priess messenger RNA (mRNA) was used as the calibrator. Quantification of gene expression was determined using the relative standard curve method developed by Applied Biosystems. Briefly, a standard curve is generated with gene-specific oligonucleotide primers and cellular mRNA from the calibrator sample (Priess), and this curve is used to determine the quantity of specific mRNA in the unknown samples. All samples are normalized to the endogenous reference mRNA (GAPDH) and are then divided by the normalized calibrator value. The normalized calibrator therefore has a value of 1, and the normalized unknown samples are expressed as an n-fold difference relative to the calibrator.

Flow cytometric analysis

Wild-type or LAMP-2-deficient B-LCL were incubated with the rat 3.5.9-13F10 antibody or the mouse L243 mAb for 60 min on ice to detect surface HLA-DR4β or HLA-DR dimers, respectively. After washing with phosphate-buffered saline (PBS) + 1% bovine serum albumin (BSA) + 0·1% NaN_3, cells were incubated with the FITC-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG or the Cy2-conjugated $F(ab')_2$ fragment of donkey anti-rat IgG secondary antibody for 30 min on ice. Cells were

washed again and fixed in 1% paraformaldehyde. Additionally, wild-type or LAMP-2-deficient B-LCL were fixed with 1% paraformaldehyde, permeabilized with 0·1% saponin, blocked with goat serum in PBS + 1% BSA + 0·1% NaN_3, and incubated for 60 min on ice with the mouse mAb W6/32 or L243 to detect intracellular MHC class I molecules and HLA-DR dimers, respectively or with the mouse mAb MaP.DM1 or a mouse mAb for HLA-DO to detect intracellular HLA-DM or HLA-DO, respectively. After washing with PBS + 1% BSA + 0·1% NaN_3, cells were incubated with the PE-conjugated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulin for 30 min on ice. Cells were washed again before analysis. Flow cytometry was performed on a FACScan™, and the data were analysed with CELLQUEST™ software (BD Biosciences).

Endocytosis assay

Wild-type 7C3.DR4 and LAMP-2-deficient DB.DR4 B-LCL were washed with cold Hanks' balanced salt solution (HBSS) + 3% BSA and incubated with 5 mg/ml FITC-albumin (Sigma-Aldrich) for 0 and 120 min at 37°. At each time-point, cells were again washed with cold HBSS + 3% BSA and fixed with 1% paraformaldehyde. Uptake of FITC-albumin was determined using flow cytometry performed on a FACScan™, and the data were analysed with CELLQUEST™ software (BD Biosciences).

Indirect immunofluorescent microscopy

Wild-type Frev or LAMP-2-deficient DB.DR4 B-LCL were incubated with 200 nM LysoTracker Red (Invitrogen, Carlsbad, CA) for 18 hr at 37°. Cells were washed and plated on poly-*L*-lysine-treated (Sigma-Aldrich) coverslips. The cells were then fixed with 4% paraformaldehyde, permeabilized with 0·1% saponin, blocked with PBS + 2% BSA, and incubated for 60 min at room temperature with FITC-conjugated L243 to detect HLA-DR dimers. Additionally, unlabelled Frev or DB.DR4 cells were plated on poly-*L*-lysine-treated coverslips, fixed with 4% paraformaldehyde, and permeabilized with 0·1% saponin. After blocking with PBS + 2% BSA, cells were incubated for 60 min at room temperature with FITC-conjugated L243 to detect HLA-DR dimers and with AlexaFluor647-conjugated-anti-LAMP-1 antibody to detect LAMP-1. All samples were washed again before analysis. Cells were viewed using a Perkin Elmer Spinning Disk Confocal Microscope, and a single plane through the cell is depicted. Images were processed using NIH IMAGE J software.

Antigen presentation assays

To measure exogenous antigen presentation, DB.DR4, Frev, Priess, or 7C3.DR4 cells (APC) were incubated with various concentrations of purified antigen for 16 hr at 37° or synthetic peptides for 4 or 16 hr at 37°. Samples were washed and then fixed with 0·5% paraformaldehyde for 10 min at room temperature. Then, 4×10^4 APC were incubated with 2×10^4 epitope-specific T cells for 24 hr at 37°. For endogenous antigen presentation, variable numbers of APC were incubated with 2×10^4 epitope-specific T cells for 24 hr at 37°. To measure the effect of pH on exogenous peptide presentation, APC were incubated with peptide in either cell culture medium (pH 7) or 150 mM Na_2HPO_4 buffer adjusted to pH 5·5 with citric acid for 4 hr at 37°. To strip surface MHC class II, APC were first treated with 160 mM NaCl adjusted to pH 4 with citric acid, three treatments for 30 min each on ice. Cells were washed and fixed as described above before incubation with exogenous peptide and co-culture with epitope-specific T cells. An interleukin-2-dependent cell line, HT-2, was used to measure interleukin-2 production following T-cell activation, and HT-2 proliferation was quantified using [^{3}H]thymidine incorporation. Data are expressed as the average counts per minute (c.p.m.) of triplicate samples for each assay.

Capture enzyme-linked immunosorbent assay (ELISA)

DB.DR4 or 7C3.DR4 cells were first fixed with paraformaldehyde and then incubated overnight at 37° with 100 μM biotinylated $\kappa I_{188-203}$ peptide. Lysates were prepared and added to plates coated with an anti-HLA-DR4 antibody to capture HLA-DR4 molecules in the lysates. The binding of biotinylated $\kappa I_{188-203}$ peptide to the captured HLA-DR4 was measured using europium-strepavidin.[25]

Results

Defects in exogenous antigen presentation in Danon B-LCL

A hallmark characteristic of Danon disease in humans is the absence of LAMP-2 protein expression in multiple tissues, particularly cardiac and skeletal muscle, because of mutations in the LAMP-2 gene.[15] We evaluated the expression of the LAMP-2 protein in the B-LCL derived from a patient with Danon disease (Danon B-LCL) by Western blotting. As observed in fibroblasts from patients with Danon disease (data not shown), Danon B-LCL did not express any detectable LAMP-2 protein (Fig. 1a). Interestingly, the levels of another lysosomal transmembrane protein LAMP-1 were equivalent in both Danon and wild-type Frev B-LCL (Fig. 1a).

The importance of lysosomal proteases and thiol reductases in MHC class II-mediated antigen presentation was established using pharmacological inhibitors and gene-deficient APC.[6,31–33] Yet far less is known about the role

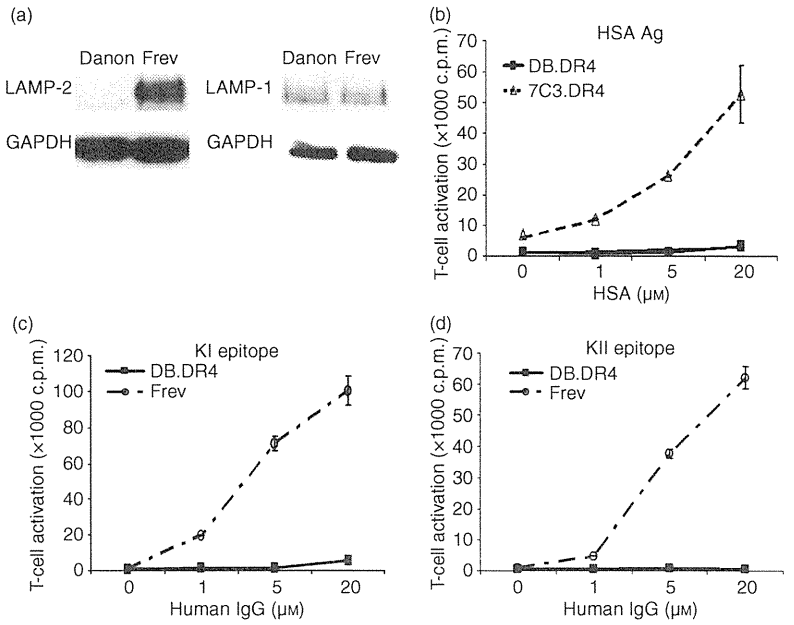
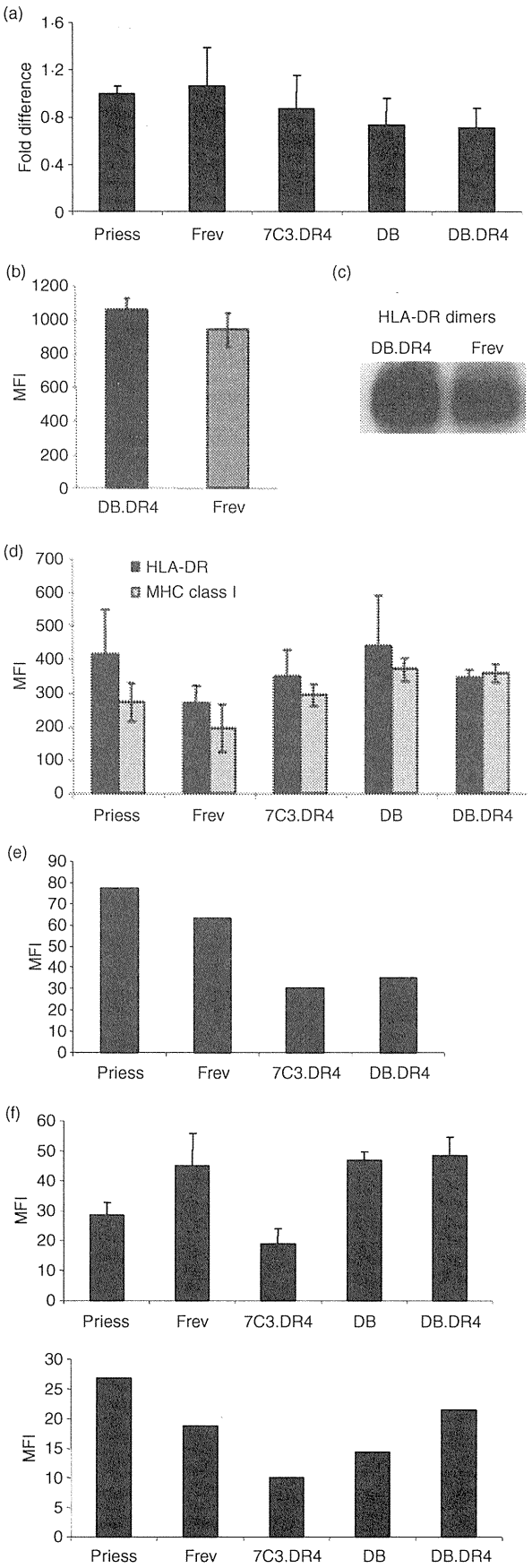


Figure 1. Exogenous antigen presentation is defective in LAMP-2-deficient Danon B-LCL. (a) Cell lysates were prepared from Danon or wild-type Frev B-LCL, the proteins were resolved by gel electrophoresis, and immunoblotted with an antibody to LAMP-2 or LAMP-1. The blot was stripped and reprobed with an antibody to GAPDH. Data are representative of at least two independent experiments. (b) LAMP-2-deficient DB.DR4 or wild-type 7C3.DR4 cells were incubated with various concentrations of human serum albumin antigen overnight and then cultured with HSA_{64-76}-specific T cells to measure MHC class II presentation. (c, d) DB.DR4 or wild-type Frev cells were incubated with various concentrations of human IgG antigen overnight and then cultured with either $\kappa I_{188-203}$-specific T cells (c) or $\kappa II_{145-159}$-specific T cells (d) to measure MHC class II presentation. Neither DB.DR4 nor Frev expressed endogenous IgG κ light chain. Data are representative of three independent experiments, and the error bars indicate the mean T-cell activation ± the standard deviation. Note that the size of the symbols at some points masks the error bars.

of lysosomal transmembrane proteins in modulating MHC class II function and antigen recognition. Hence, studies were conducted to address whether the absence of LAMP-2 expression observed in Danon B-LCL altered exogenous antigen presentation. Wild-type 7C3.DR4 and LAMP-2-deficient DB.DR4 were incubated with various concentrations of exogenous HSA antigen and then co-cultured with an HLA-DR4-restricted T-cell hybridoma specific for the HSA_{64-76} epitope.[24] Even at high concentrations of HSA (20 μM) after an overnight incubation, the LAMP-2-deficient DB.DR4 were unable to activate HSA-specific T cells (Fig. 1b). The ability of DB.DR4 to present a second exogenous antigen, human IgG κ light chain, was also evaluated. 7C3.DR4 cells express endogenous IgG κ while DB.DR4 and the wild-type Frev B-LCL are negative for endogenous IgG κ by Western blotting and instead, express IgG λ light chain (data not shown). DB.DR4 or Frev cells were incubated with IgG and then co-cultured with HLA-DR4-restricted T-cell hybridomas specific for either of two epitopes from IgG, $\kappa I_{188-203}$ or $\kappa II_{145-159}$.[25] Again, even at high concentrations of human IgG (20 μM), the LAMP-2-deficient DB.DR4 cells were unable to present either $\kappa I_{188-203}$ or $\kappa II_{145-159}$ epitopes to activate the κI- or κII-specific T cells (Fig. 1c,d). Together these results suggest that the absence of LAMP-2 expression in human B cells disrupts exogenous MHC class II-mediated antigen presentation.

Comparable expression of surface and intracellular MHC class II in Danon and wild-type B-LCL

We next examined whether the absence of LAMP-2 in Danon B-LCL influenced the expression of MHC class II molecules as a potential explanation for the observed defects in exogenous antigen presentation. First, the levels of HLA-DRα chain mRNA in a panel of wild-type and Danon B-LCL were determined using quantitative RT-PCR. Both wild-type and Danon B-LCL express very similar amounts of HLA-DRα mRNA (Fig. 2a). In addition, the levels of surface and intracellular HLA-DR$\alpha\beta$ dimers were also determined for these cells using flow cytometry. Although surface expression of HLA-DR$\alpha\beta$ was slightly increased in LAMP-2-deficient DB.DR4 compared with wild-type Frev B-LCL (Fig. 2b) as detected using an antibody that recognizes MHC class II $\alpha\beta$ dimers, we were able to detect similar levels of HLA-DR$\alpha\beta$ dimers upon Western blotting cell lysates of DB.DR4 and Frev (Fig. 2c). No significant difference in the total levels of cell surface and intracellular expression of HLA-DR or MHC class I proteins was observed in Danon versus



wild-type B-LCL after permeabilization (Fig. 2d). Retroviral transduction of Danon or a wild-type B-LCL (7C3) with a vector encoding the complementary DNA for the DRβ1*0401 β chain resulted in efficient pairing with endogenous HLA-DRα and similar surface expression of this DR4β chain (Fig. 2e). No staining for surface HLA-DR4 was observed in untransduced Danon B cells (data not shown). The similar HLA-DR4 surface expression on DB.DR4 and 7C3.DR4 cells was by comparison approximately twofold lower than that detected on B-LCL expressing endogenous HLA-DR4. Yet as demonstrated in Fig. 1, only DB.DR4 cells displayed a deficiency in exoge-

Figure 2. Comparable expression of MHC class II messenger RNA and protein in Danon and wild-type B-LCL. (a) Total RNA was extracted from various wild-type or Danon B-LCL, complementary DNA was synthesized, and quantitative reverse transcription–polymerase chain reaction analysis performed using primers specific for HLA-DRα chain or for GAPDH as a control. Data are representative of the fold difference in messenger RNA expression observed in three independent experiments. (b) Wild-type Frev or LAMP-2-deficient DB.DR4 cells were incubated with L243 antibody to detect total surface HLA-DR and then stained with a fluorescein isothiocyanate-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG secondary antibody. The mean fluorescence intensity (MFI) as measured by flow cytometry indicates the level of surface HLA-DR, and data are the average MFI of three independent experiments. (c) Cell lysates were prepared from LAMP-2-deficient DB.DR4 or wild-type Frev B-LCL, the proteins resolved by gel electrophoresis under non-reducing conditions to preserve MHC class II dimers, and immunoblotted with an antibody to HLA-DRα chain. Data are representative of at least five independent experiments. The ratios of HLA-DR$\alpha\beta$ dimers to GAPDH as a loading control were 1·5 and 1·3 for DB.DR4 and Frev, respectively. (d) Various wild-type or Danon B-LCL were first permeabilized and then incubated with L243 or W6/32 antibody to detect total intracellular HLA-DR or MHC class I molecules, respectively. Cells were then stained with a phycoerythrin-conjugated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulin secondary antibody. The MFI as measured by flow cytometry indicates the levels of total surface or intracellular MHC class I or class II molecules. Data are the average MFI of three independent experiments. (e) Wild-type Priess and Frev expressing endogenous HLA-DR4 and wild-type 7C3.DR4 or LAMP-2-deficient DB.DR4 transfectants were incubated with 3.5.9-13F10 antibody to detect surface HLA-DR4β chains and then stained with a Cy2-conjugated $F(ab')_2$ fragment of donkey anti-rat IgG secondary antibody. The MFI as measured by flow cytometry indicates the level of surface HLA-DR4, and data are representative of more than three independent experiments. (f) Various wild-type or Danon B-LCL were first permeabilized and then incubated with MaP.DM1 (top panel) or a monoclonal antibody to HLA-DO (bottom panel) to detect total intracellular HLA-DM or HLA-DO molecules, respectively. Cells were then stained with a phycoerythrin-conjugated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulin secondary antibody. The MFI as measured by flow cytometry indicates the levels of total intracellular HLA-DM or HLA-DO molecules. Data for HLA-DM staining are the average MFI of three independent experiments while the data for HLA-DO staining are a representative experiment.

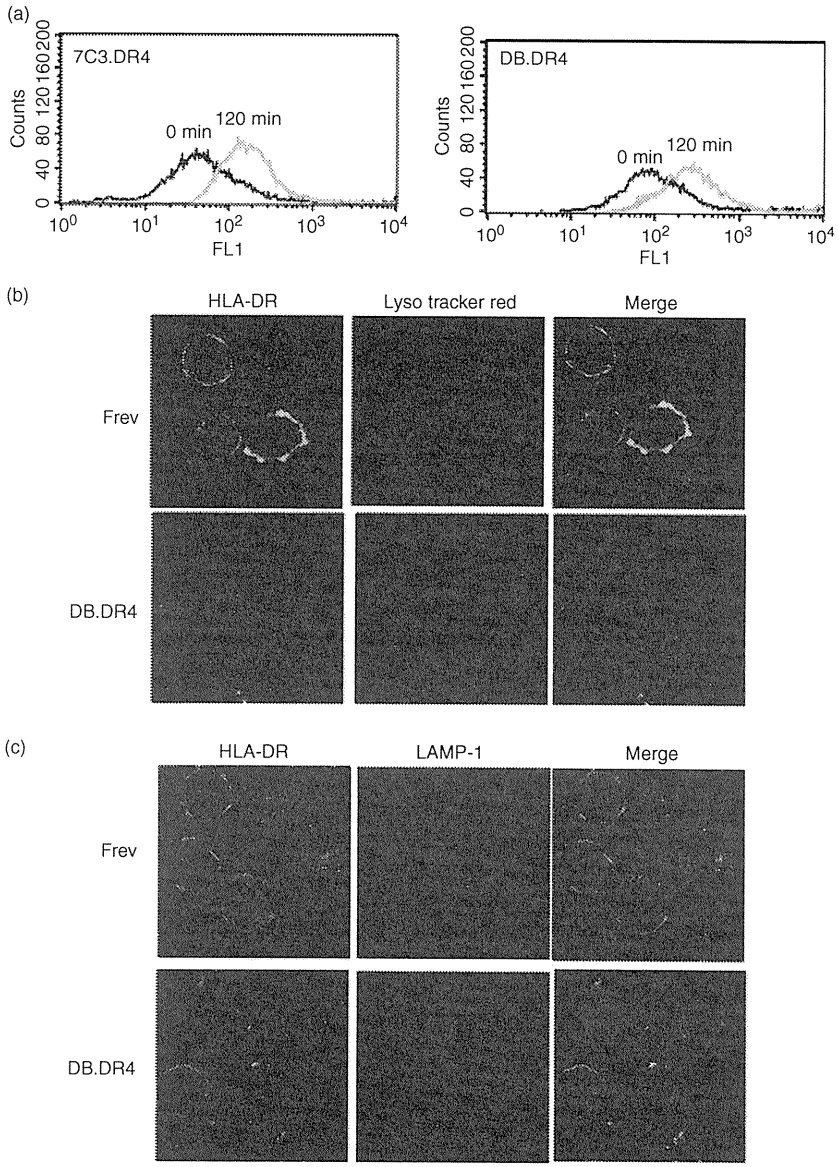


Figure 3. Endocytosis and MHC class II distribution in Danon and wild-type B-LCL. (a) Wild-type 7C3.DR4 or LAMP-2-deficient DB.DR4 B-LCL were incubated with fluorescein isothiocyanate (FITC) -albumin for 0 or 120 min at 37° and uptake was measured by flow cytometry. Data are representative of two independent experiments. (b) Wild-type Frev or LAMP-2-deficient DB.DR4 B-LCL were incubated with LysoTracker Red at 37° overnight to label acidic organelles, permeabilized, and then incubated with FITC-conjugated L423 antibody to detect HLA-DR. The results are representative of three independent experiments. (c) Wild-type Frev or LAMP-2-deficient DB.DR4 B-LCL were first permeabilized and then incubated with FITC-conjugated L423 antibody to detect HLA-DR and AlexaFluor647-conjugated-LAMP-1 Ab to detect LAMP-1. The results are representative of two independent experiments.

nous antigen presentation. Lastly, we examined whether the expression of two other MHC-encoded gene products, HLA-DM and HLA-DO, was altered in the LAMP-2-deficient Danon B-LCL. HLA-DM facilitates the removal of CLIP and the capture of antigenic peptides by MHC class II proteins[7–9] whereas HLA-DO associates with HLA-DM and serves as a negative regulator of this complex.[34] The levels of intracellular HLA-DM and HLA-DO were determined in a panel of wild-type and Danon B-LCL after permeabilization using flow cytometry. Both LAMP-2-deficient cell lines DB and DB.DR4 express equivalent levels of HLA-DM as compared with Frev (Fig. 2f, top) even though human B cells have been shown to express varying levels of HLA-DM.[35,36] Variation in the intracellular levels of HLA-DO was also evident in the panel of wild-type and Danon B-LCL although the expression of HLA-DO in the LAMP-2-deficient and wild-type cells was almost equivalent (Fig. 2f, bottom). Taken together, these results suggest that the absence of LAMP-2 in the Danon B-LCL did not substantially alter the levels of intracellular

MHC class II HLA-DR dimers, HLA-DM, and HLA-DO nor the steady-state levels of MHC class II complexes that ultimately reach the cell surface.

Endocytosis and distribution of MHC class II in Danon and wild-type B-LCL

While LAMP-2 deficiency in the Danon B-LCL did not affect the overall expression of MHC class II, we sought to determine if differences in endocytosis or the distribution of class II within the endocytic network might account for the defects in exogenous antigen presentation observed in the LAMP-2-deficient B-LCL. We first examined the ability of the LAMP-2-deficient DB.DR4 and wild-type 7C3.DR4 to endocytose a model exogenous antigen, FITC-albumin and observed that uptake of the FITC-albumin after 120 min was not substantially different between DB.DR4 and 7C3.DR4 cells (Fig. 3a). In data not shown, we also observed the persistence of the FITC-albumin at 8 hr in both DB.DR4 and 7C3.DR4 cells while a small amount of this labelled protein was detected in some of the LAMP-2-deficient DB.DR4 cells even at 24 hr, suggesting a slight reduction in the degradation of this molecule in some LAMP-2-negative cells. These results suggest that the absence of LAMP-2 in the Danon B-LCL does not substantially affect the internalization of exogenous proteins or their trafficking along the endocytic pathway.

We next asked whether MHC class II molecules were similarly distributed within the endosomal/lysosomal network of wild-type and LAMP-2-deficient B-LCL. After assembly in the endoplasmic reticulum, MHC class II molecules are targeted to endosomal/lysosomal compartments for peptide loading. Antigenic peptides bind to MHC class II molecules in the MIIC, an acidic compartment resembling a mature endosome or prelysosome. Using LysoTracker Red to mark acidic organelles such as late endosomes and lysosomes, these compartments were detected in both LAMP-2-deficient DB.DR4 and wild-type Frev cells (Fig. 3b). While the majority of MHC class II molecules localized to the cell surface in both DB.DR4 and Frev, greater co-localization of intracellular class II proteins in the LysoTracker Red$^+$ compartments was observed in the LAMP-2-deficient DB.DR4 cells compared with Frev (Fig. 3b). These findings suggest a potential difference in the intracellular distribution of class II molecules in the absence of LAMP-2. We detected MHC class II in late endosomes/lysosomes in both DB.DR4 or Frev cells as measured by LAMP-1 staining (Fig. 3c); yet there appeared to be slightly more class II in larger LAMP-1$^+$ vesicles in DB.DR4 cells. In wild-type Frev cells, intracellular class II was co-localized with LAMP-2 as well as LAMP-1 (data not shown). MHC class II molecules were not abundant in early endosomes in either wild-type or LAMP-2-deficient cells as detected by staining for co-localization with the early endosome antigen, EEA-1 (data not shown). These results suggest that in LAMP-2-deficient cells, a greater number of MHC class II molecules may transit through or be retained in a mature endosome or lysosome-like compartment compared with wild-type B cells.

Efficient presentation of an endogenous antigen by Danon B-LCL

Biochemical analysis of MHC class II ligands from human B-LCL revealed the presentation of epitopes from endogenous membrane antigens as well as exogenous protein antigens.[37] Presentation of these endogenous antigens requires proteolytic processing to yield peptides that efficiently bind to MHC class II molecules within the endosomal/lysosomal compartments of APC. The presence of HLA-DR$\alpha\beta$ dimers at the cell surface of Danon B-LCL suggested these class II molecules may acquire peptides from a source other than exogenous antigen. The ability of the LAMP-2-deficient DB.DR4 to present antigenic peptides derived from an endogenous transmembrane protein was evaluated using an HLA-DR4-restricted T-cell hybridoma that recognizes an epitope from MHC class I HLA-A alleles.[26] DB.DR4 cells were capable of efficiently activating the HLA-A-specific T cells to an extent slightly greater than the wild-type 7C3.DR4 cells (Fig. 4). A murine B cell CH27 transfected with HLA-DR4 (CH27.DR4) was only recognized by the HLA-A-specific T cells when pulsed with the HLA-A_{52-70} pep-

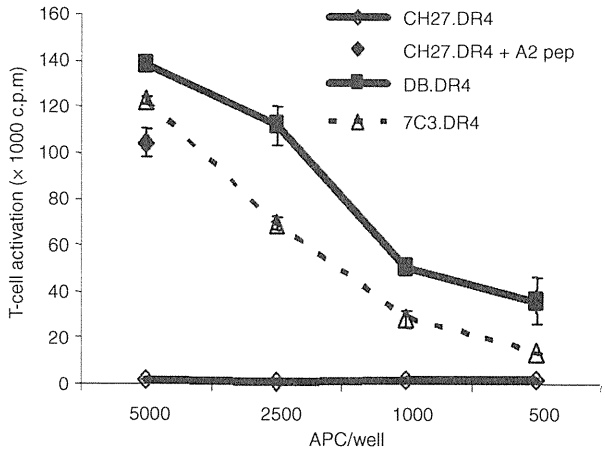


Figure 4. Efficient presentation of an endogenous antigen by Danon B-LCL. Variable numbers of DB.DR4 or 7C3.DR4 cells were cultured with HLA-A_{52-70}-specific, HLA-DR4-restricted T cells to measure MHC class II presentation of endogenous HLA-A. As a control, the HLA-DR4 transfected murine B cell CH27.DR4 was left untreated or incubated with 10 μM HLA-A_{52-70} peptide and then cultured with HLA-A_{52-70}-specific, HLA-DR4-restricted T cells. Data are representative of three independent experiments, and the error bars indicate the mean T-cell activation ± the standard deviation.

tide before the addition of T cells (Fig. 4), confirming the specificity of these T cells for the HLA-A epitope. These results suggest that while MHC class II-restricted exogenous antigen presentation was impaired in the absence of LAMP-2 in the DB.DR4 cells, the presentation of an endogenous transmembrane protein in the context of class II could be readily detected.

Reduction in MHC class II-peptide binding in Danon B-LCL

The ability of the LAMP-2-deficient DB.DR4 cells to functionally present exogenously added synthetic peptides was determined using HLA-DR4-restricted T cells. In contrast to wild-type B-LCL, DB.DR4 cells failed to efficiently present to T cells a variety of high-affinity and low-affinity peptides,[24,25,38] including an epitope from the autoantigen glutamate decarboxylase $GAD_{273-285}$[39] (Fig. 5a), HSA_{64-76} (Fig. 5b), $\kappa I_{188-203}$ (Fig. 5c), or $\kappa II_{145-159}$ (Fig. 5d). However, incubation of DB.DR4 cells with either very high concentrations of synthetic peptide (100 μM instead of 10 μM) or with peptides for prolonged periods of time (16 hr instead of 4 hr) before co-culture with epitope-specific T cells resulted in reduced but detectable MHC class II-restricted peptide presentation (Fig. 5 and data not shown). T-cell activation in response to exogenous peptides and DB.DR4 cells was reduced consistently when compared with MHC class II presentation by wild-type B-LCL. These results were in stark contrast to the efficient activation of T cells recognizing the endogenous HLA-A_{52-70} epitope (Fig. 4) using DB.DR4 cells as the APC, suggesting that in the absence of LAMP-2, a different repertoire of peptides is selected for display by MHC class II molecules.

To determine whether LAMP-2-deficient DB.DR4 cells differentially bind exogenous peptides, a capture ELISA was used to biochemically measure the amount of peptide bound to HLA-DR4 on DB.DR4 cells compared with wild-type 7C3.DR4 cells. DB.DR4 and 7C3.DR4 express equivalent levels of HLA-DR4 (Fig. 2c), and the expression of endogenous IgG κ in 7C3.DR4 does not interfere with the measurement of the binding of the biotinylated $\kappa I_{188-203}$ peptide to HLA-DR4. At physiological pH, the binding of 100 μM biotinylated $\kappa I_{188-203}$ peptide to HLA-DR4 from DB.DR4 cells was reduced approximately two-fold compared with 7C3.DR4 (Fig. 6a). Relatively similar differences in peptide-binding to HLA-DR4 were also detected at lower peptide concentrations (data not shown). As antigenic peptides bind to MHC class II molecules in acidic compartments such as mature endosomes and lysosomes,[10] the binding of biotinylated $\kappa I_{188-203}$ to HLA-DR4 on DB.DR4 and 7C3.DR4 cells at pH 5·5 was

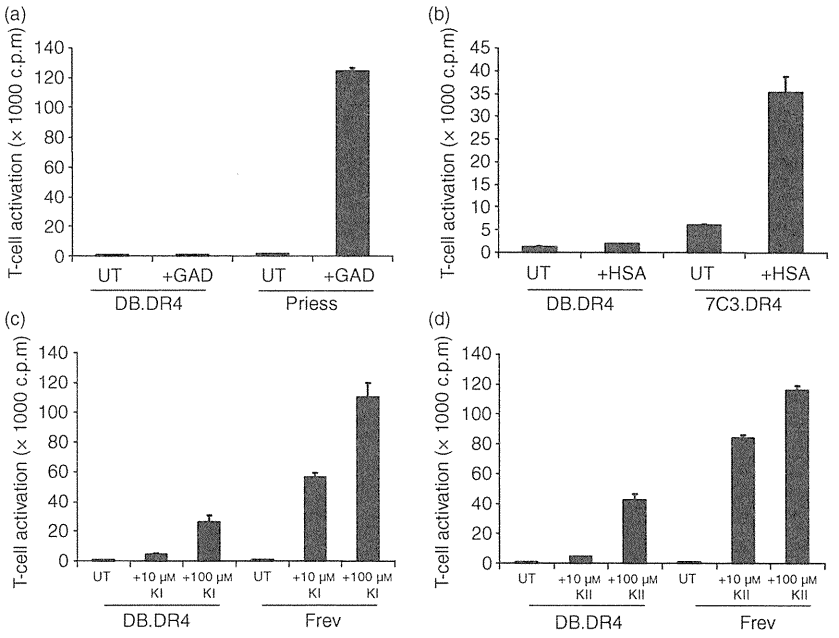


Figure 5. LAMP-2-deficient DB.DR4 cells fail to efficiently present exogenous peptides to $CD4^+$ T cells. (a) LAMP-2-deficient DB.DR4 or wild-type Priess cells were left untreated (UT) or incubated with 10 μM exogenous $GAD_{273-285}$ peptide for 4 hr and then cultured with $GAD_{273-285}$-specific T cells to measure MHC class II presentation. (b) DB.DR4 or wild-type 7C3.DR4 cells were left untreated (UT) or incubated with 10 μM exogenous HSA_{64-76} peptide for 4 hr and then cultured with HSA_{64-76}-specific T cells to measure MHC class II presentation. (c, d) DB.DR4 or wild-type Frev cells were left untreated (UT) or incubated with 10 μM or 100 μM exogenous $\kappa I_{188-203}$ peptide (c) or $\kappa II_{145-159}$ peptide (d) for 4 hr and then cultured with $\kappa I_{188-203}$-specific or $\kappa II_{145-159}$-specific T cells, respectively, to measure MHC class II presentation. Data in (a–d) are representative of three independent experiments, and the error bars indicate the mean T-cell activation ± the standard deviation.

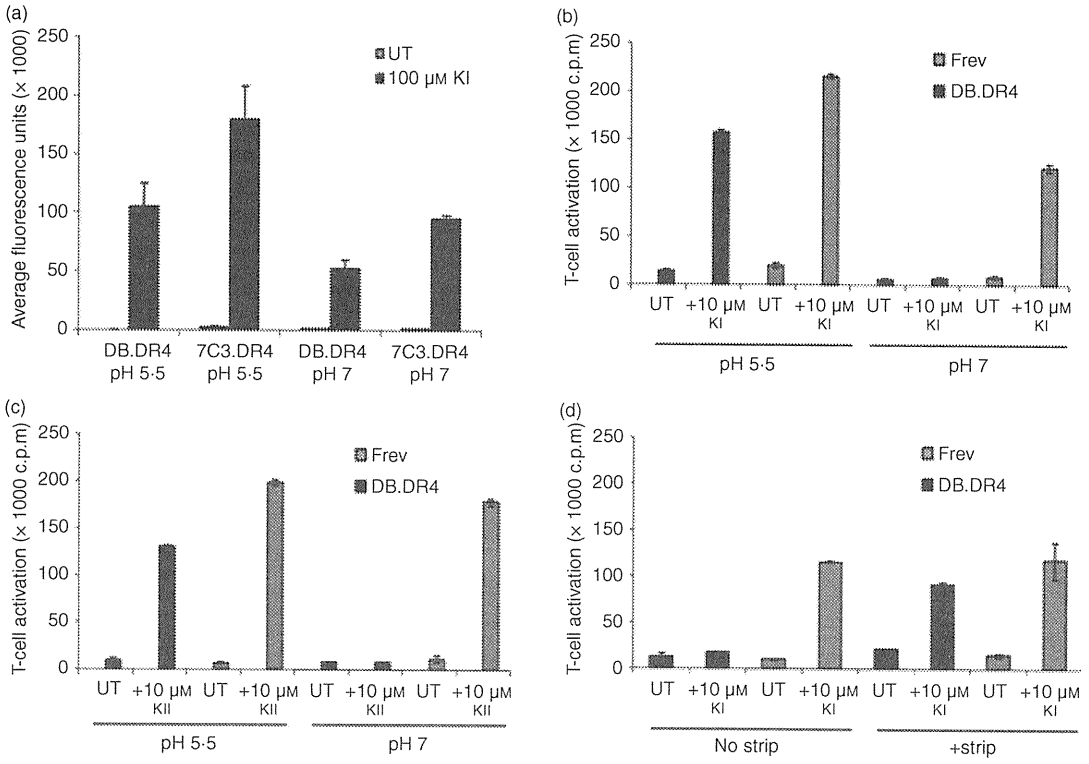


Figure 6. Acidic pH restores exogenous peptide presentation by LAMP-2-deficient DB.DR4. (a) LAMP-2-deficient DB.DR4 or wild-type 7C3.DR4 cells were left untreated (UT) or incubated overnight with 100 μM biotinylated $\kappa I_{188-203}$ peptide. Lysates were prepared and added to plates coated with an anti-HLA-DR4 antibody to capture HLA-DR4 molecules in the lysates. The binding of biotinylated $\kappa I_{188-203}$ peptide to captured HLA-DR4 was measured using europium-strepavidin. The results are the average of three independent experiments, and the error bars indicate the standard deviation ± between the experiments. (b, c) DB.DR4 or wild-type Frev cells were left untreated (UT) or incubated with 10 μM exogenous $\kappa I_{188-203}$ peptide (b) or 10 μM exogenous $\kappa II_{145-159}$ peptide (c) in either cell culture medium (pH 7) or in sodium phosphate/citric acid buffer (pH 5·5) for 4 hr and then cultured with $\kappa I_{188-203}$-specific or $\kappa II_{145-159}$-specific T cells, respectively, to measure MHC class II presentation. Data in (b, c) are representative of three independent experiments, and the error bars indicate the mean T-cell activation ± the standard deviation. (d) DB.DR4 or Frev cells were left untreated (UT) or treated with a NaCl/citric acid buffer to strip surface MHC class II before incubation with 10 μM exogenous $\kappa I_{188-203}$ peptide for 4 hr. Cells were then cultured with $\kappa I_{188-203}$-specific T cells to measure MHC class II presentation. Data are representative of two independent experiments, and the error bars indicate the mean T-cell activation ± the standard deviation.

also evaluated in this assay. Overnight incubation of the cells at low pH improved the binding of 100 μM biotinylated $\kappa I_{188-203}$ to HLA-DR4 from both DB.DR4 and 7C3.DR4, but peptide-binding to DB.DR4 remained approximately two-fold less compared with 7C3.DR4 (Fig. 6a). The binding of peptides to DB.DR4 cells was also evaluated using strepavidin-HRP in Western blots to detect the formation of biotinylated $\kappa I_{188-203}$ peptide–HLA-DR4 complexes at pH 5·5 in DB.DR4 cells compared with 7C3.DR4 cells. Biotinylated $\kappa I_{188-203}$ peptide-HLA-DR4 complexes were detected in DB.DR4 cells after 4 hr of peptide-incubation only at pH 5·5, and the numbers of these complexes were reduced compared with wild-type 7C3.DR4 cells (data not shown). Overall, these results suggest that in cells lacking LAMP-2, class II protein binding to exogenously added peptides was impaired or limited particularly at neutral pH. Peptide binding to these class II molecules could be restored in part by exposure to low pH.

Acidic pH restores exogenous peptide presentation by Danon B-LCL

Since incubating LAMP-2-deficient DB.DR4 at pH 5·5 improved the binding of biotinylated $\kappa I_{188-203}$ to HLA-DR4 on these cells, studies were designed to test whether low pH would also facilitate class II-mediated presentation of exogenous $\kappa I_{188-203}$ and $\kappa II_{145-159}$ peptides to epitope-specific T cells. DB.DR4 cells or wild-type Frev B-LCL, neither of which express endogenous IgG κ, were incubated with 10 μM $\kappa I_{188-203}$ or $\kappa II_{145-159}$ peptides at pH 5·5 for 4 hr and then co-cultured with HLA-DR4-restricted, epitope-specific T cells at physiological pH 7·2. Incubating DB.DR4 cells at acidic pH in the presence of $\kappa I_{188-203}$ or $\kappa II_{145-159}$ peptides partially restored exogenous peptide presentation such that activation of epitope-specific T cells was only minimally reduced compared with wild-type Frev cells (Fig. 6b,c). To determine whether exposure to low pH was necessary to alter class II accessibility to peptides or to

directly enhance peptide-binding, additional studies were performed. Acid stripping has been used to dissociate receptor–ligand complexes including releasing endogenous ligands from the groove of MHC class I and class II molecules.[36,40,41] Here, LAMP-2-deficient DB.DR4 and wild-type Frev cells were briefly exposed to acid stripping buffer before incubating with 10 μM $\kappa I_{188-203}$ or $\kappa II_{145-159}$ peptide at neutral pH for 4 hr. Following acid-stripping, both $\kappa I_{188-203}$ and $\kappa II_{145-159}$ peptides were more efficiently presented in the context of HLA-DR4 on the surface of DB.DR4 to epitope-specific T cells (Fig. 6d and data not shown). Notably, the activation of κI-specific T cells by acid-stripped DB.DR4 cells was still slightly reduced relative to levels of peptide presentation observed with untreated or acid-stripped wild-type Frev cells (Fig. 6d). These results demonstrate that the incubation of peptides with APC at low pH partially rescued class II-mediated presentation of exogenous peptides in the LAMP-2-deficient DB.DR4 cells.

Discussion

In this study, a novel mutant B-cell line from a patient with Danon disease lacking expression of the lysosomal membrane protein LAMP-2 was used to investigate the role of LAMP-2 in MHC class II-mediated antigen presentation. In the absence of LAMP-2, MHC class II presentation of exogenous antigens and peptides to $CD4^+$ T cells was significantly impaired. This was not because of alterations in the levels of cell surface or total MHC class II molecules in LAMP-2-deficient Danon B-LCL. In wild-type and LAMP-2-deficient cells, the majority of class II molecules were expressed at the cell surface, yet some class II proteins were observed in intracellular punctuate vesicles, probably mature endosomes or pre-lysosomes. The co-localization of class II molecules in $LAMP\text{-}1^+$ vesicles appeared greater in the LAMP-2-deficient cells. Biochemical analysis of class II molecules from Danon B-LCL revealed a reduced capacity for peptide-binding compared with class II complexes isolated from wild-type cells. Peptide-binding to class II molecules from these LAMP-2-deficient cells could be partially restored upon incubation of cells with peptides at acidic pH. Incubation of Danon B-LCL at low pH for even a brief period before the addition of peptide also partially restored T-cell recognition of the resulting peptide–MHC class II complexes on these cells. Interestingly, class II presentation of an epitope from an endogenous transmembrane protein was similarly detected in wild-type or LAMP-2-deficient Danon B-LCL. Overall, these results suggest that the absence of LAMP-2 within the endosomal/lysosomal network selectively altered class II acquisition and presentation of peptide ligands to T cells.

Danon disease is a rare, X-linked lysosomal disorder characterized by the accumulation of dense, translucent vacuoles in the cytoplasm of skeletal and cardiac muscle cells as the result of the absence of LAMP-2 protein expression.[15] Preliminary electron microscopy studies have revealed the presence of vesicles with inclusions in both fibroblasts and B cells from patients with Danon disease (unpublished observations). Intracellular immunofluorescence revealed greater co-localization of class II molecules with the late endosome/lysosome marker LAMP-1 in DB.DR4 cells from a patient with Danon disease compared with wild-type cells. These vesicles appeared slightly larger and more clustered than the $LAMP\text{-}1^+$ vesicles in wild-type cells, and stained more brightly for LysoTracker Red. Proteins associated with early endosomes (EEA1) or autophagosomes (LC3) were not detected co-localizing with these class II compartments, again suggesting that this compartment is more closely related to mature endosomes or lysosomes (data not shown). Enlarged $LAMP\text{-}1^+$ vesicles were also detected clustered in the cytoplasm of LAMP-2-deficient neutrophils.[42] Defects in phagocytosis, an important component of the innate immune response to intracellular pathogens, were observed in these neutrophils that lacked LAMP-2.

The current study is the first report of a deficiency in exogenous antigen presentation in human B cells lacking LAMP-2 expression. Treatment of a wild-type B-cell line Priess transfected with antisense complementary DNA for LAMP-2, partially reduced cellular LAMP-2 expression.[19] While exogenous antigen presentation was partially diminished in these cells, class II presentation of an exogenous peptide was comparable with cells with normal LAMP-2 levels. In the current study, the complete absence of LAMP-2 protein in Danon B-LCL had a more profound effect, abolishing exogenous antigen presentation and greatly reducing exogenous peptide presentation by these cells. These results also differ significantly from a previous study involving B cells from patients with another rare hereditary immunodeficiency Chediak–Higashi syndrome (CHS).[43] This syndrome results from mutations in a single gene encoding a large cytosolic protein, termed lysosomal trafficking regulator (LYST).[44–46] Similar to LAMP-2-deficient Danon B cells, CHS B cells display reduced MHC class II-mediated presentation of exogenous antigen. However, in contrast to Danon B cells, addition of exogenous peptide to CHS B cells restored class II presentation to the levels observed with wild-type B cells.[43] These results not only support the importance of the lysosomal network in MHC class II-mediated antigen presentation, but they also suggest that alterations in different components of the lysosomal pathway may reveal novel regulatory events in antigen presentation.

The absence of LAMP-2 did not alter the cell surface levels of MHC class II molecules, suggesting that the egress of peptide–MHC class II complexes from the

endosomal network to the plasma membrane is maintained. However, MHC class II molecules from LAMP-2-deficient Danon B-LCL displayed a reduced capacity for peptide-binding at the cell surface. Binding of exogenous peptides to class II could be restored upon incubation of these cells with peptides at acidic pH. Furthermore, incubation of Danon B-LCL at low pH before the addition of peptide also partially restored T-cell recognition of the resulting peptide–MHC class II complexes on these cells. Restoration of MHC class II function in Danon B-LCL treated with a low pH buffer may facilitate the removal of some endogenous ligands from the peptide-binding groove of class II molecules. Alternatively, this low pH treatment may stabilize class II molecules in a conformation more receptive to peptide loading. These studies therefore suggest that LAMP-2 influences the repertoire of peptides binding MHC class II molecules in human B cells.

Despite deficiencies in exogenous antigen and peptide presentation, Danon B-LCL were capable of presenting an epitope from an endogenous transmembrane protein, the MHC class I molecule HLA-A, to epitope-specific $CD4^+$ T cells. Incubation of Danon B-LCL at low pH did not enhance T-cell recognition of the HLA-A epitope and HLA-DR4 at the cell surface. Yet, endogenous peptides such as the epitope from HLA-A may bind tightly to class II molecules in the acidic $LAMP\text{-}1^+$ vesicles detected in LAMP-2-deficient cells, and facilitate the export of these class II molecules to the cell surface. In contrast to our previous observation that LAMP-2 facilitated the MHC class II-mediated presentation of the cytoplasmic GAD antigen, the absence of LAMP-2 in Danon B-LCL did not hinder the presentation of the endogenous HLA-A epitope. The HLA-A epitope is one of the most abundant epitopes detected bound to HLA-DR4 as measured by peptide-elution studies and mass spectrometry and is probably formed during the turnover of class I A alleles in lysosomes.[47] GAD is found only in the cytosol and relies on chaperone-mediated autophagy and LAMP-2A to access class II for presentation.[19]

In conclusion, our data support a role for LAMP-2 in the MHC class II-mediated presentation of exogenous antigens and peptides in human B cells. Peptide-binding to MHC class II on LAMP-2-deficient B cells was reduced at the cell surface yet could be restored by incubation at acidic pH. Restoration of MHC class II function in Danon B-LCL upon incubation at low pH buffer may facilitate the removal of endogenous ligands from the peptide-binding groove of MHC class II molecules or stabilize class II molecules in a conformation more receptive to peptide loading. Efficient loading of exogenous epitopes by MHC class II molecules is therefore dependent upon LAMP-2 expression in B cells. LAMP-2-deficient B cells displayed slightly enhanced presentation of an epitope derived from an endogenous transmembrane protein suggesting that LAMP-2 may control the overall repertoire of peptides displayed by MHC class II molecules on B cells and subsequently, $CD4^+$ T-cell activation.

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Disclosures

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6 筋疾患とオートファジー

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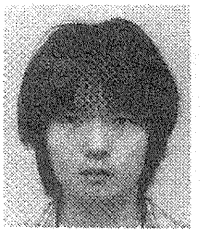
疾病研究第一部



本田 真也
2008年3月東京理科大学院基礎工学研究科生物工学専攻卒業，同年4月国立精神・神経センター神経研究所疾病研究第一部流動研究員。研究テーマは，ダノン病，XMEA治療法開発に向けた分子病態の解明。趣味は，サッカー。

Key words：ミオパチー，オートファジー，自己貪食空胞，遺伝性筋疾患

Abstract
筋細胞におけるオートファジーの役割，およびリソソームの機能は長らく不明であった。しかし，自己貪食空胞が蓄積する筋疾患の存在から，筋細胞におけるリソソームとオートファジーの重要性が注目されるようになっている。オートファジーの異常を示す筋疾患は，その原因遺伝子と病理観察から，①オートファジー/リソソーム系の異常によるものと，②オートファジーそのものの異常ではなく，2次的にオートファジーが惹起されるものの2つに分類することができる。

1. 骨格筋・心筋におけるオートファジー研究

正常な骨格筋・心筋においては，形態学的観察でリソソームや自己貪食空胞が認められることは少なく，骨格筋・心筋におけるオートファジーの役割，およびリソソームの機能は長らく不明のままであった。しかしながら，自己貪食空胞が蓄積する筋疾患の存在が明らかになり，筋肉におけるリソソームとオートファジーの重要性が重視されるようになってきた。我々はこれまでにオートファジーの機能異常により筋線維内に自己貪食空胞が蓄積する自己貪食空胞性ミオパチー(Autophagic vacuolar myopathy：AVM)という一群の筋疾患を提唱し，広く世界に受け入れられている。

原因遺伝子の明らかになっているAVMとして，ダノン病・X-linked myopathy with excessive autophagy (XMEA)・酸マルターゼ欠損症・縁取り空胞型遠位型ミオパチー(distal myopathy with rimmed vacuoles：DMRV)があげられる。それぞれの原因遺伝子はLAMP-2 (lysosome-associated membrane protein-2)・Vma21・酸マルターゼ・GNE（ウリジン二リン酸-N-アセチルグルコサミン2-エピメラーゼ／N-アセチルマンノサミンキナーゼ）である。LAMP-2はリソソーム膜タンパク質，Vma21はV-ATPaseの複合体形成因子，酸マルターゼはグルコース分解を行うリソソーム酵素，GNEはシアル酸合成に必須な2つの酵素をコードしている。

このようにAVMは原因遺伝子から，①オートファジー / リソソーム系そのものの機能異常により病態を示すもの，②オートファジー / リソソーム系の機能異常が本質的な疾患原因ではなく，2次的にオートファジーの働きが惹起し病態を示すものの2つに分類される。前者ではダノン病・XMEA・酸マルターゼ欠損症，後者ではDMRVが代表的な筋疾患として分類

Autophagic vacuolar myopathy: Shinya Honda and Ichizo Nishino, Department of Neuromuscular Research, National Institute of Neuroscience, NCNP

される。以下，それぞれの筋疾患に対しての病理症状およびこれまでの研究成果について記す。

2. 自己貪食空胞性ミオパチー（AVM）

1）ダノン病

ダノン病は，X連鎖優性遺伝のまれな疾患で，全世界で数十例の報告があるにすぎない。ダノン病は，X染色体上にコードされているリソソームの膜タンパク質の1つLAMP-2が欠損することにより起こる遺伝性疾患であり，精神遅滞・ミオパチー・肥大型心筋症を3徴とする。ダノン病は症状が潜行性のため早期の発見が難しいが，すべての患者で進行性の心筋症がみられる。この心筋症はしばしば不静脈を伴い突然死にいたることがあり，現在のところ心臓移植以外に効果的な治療法は報告されていない。患者の筋組織をみると，筋線維内に多数の小胞が蓄積しており，電子顕微鏡観察から，それらが自己貪食空胞であることが分かる。また，これら蓄積空胞の周囲には，それを取り囲むように筋鞘膜様の特徴をもつ膜 (autophagic vacuoles with sarcolemmal features : AVSF)が観察される（図1-A)。AVSFでは空胞膜にほぼすべての筋鞘膜タンパク質が発現しており，アセチルコリンエステラーゼ活性を有している。これら特徴からAVSF形成は，細胞が自身の内部に細胞外環境を作り出しているかのような状況であるといえる。しかしながら，現在のところ，このAVSFがどのようにして形成されるのか？病態への関与は何なのか？などといった疑問は解明されておらず，そもそもAVSF形成が，生体防御的に働いているのか，あるいは病態悪化要因であるのかさえ不明である。

LAMP-2は，その常染色体上ホモログであるLAMP-1と共に，リソソーム膜の約50%を構成するリソソーム膜主要構成タンパク質であり，ノックアウトマウスを用いた研究からリソソームの移動・オートファゴソームとの融合に関与していることが明らかになっている。LAMP-2は正常では心臓・骨格筋・脳において強く発現しており，ダノン病では，これらの領域において，恒常的におこっているオートファジーがその最終段階（オートファゴソームとリソソームの融合段階）でストップしており，症状を呈するようになると考えられる。

2） X-linked myopathy with excessive autophagy (XMEA)

XMEAはX染色体劣性遺伝形質をとる遺伝性筋疾患であり，女性保因者は発症しない。この疾患は臨床的にはダノン病よりも軽く，心筋障害もきたさないが，筋組織所見は極めて類似している。近位筋優位の進行性の筋力低下と委縮が認められるが，程度は軽く，60歳を過ぎても歩行可能な例が多い。

XMEAは長らくその原因遺伝子が不明のままであったが，2009年についにその原因遺伝子がVMA21であることが明らかになった。VMA21はV-ATPaseの複合体構成因子であり，その欠損はV-ATPaseの活性を低下させる。V-

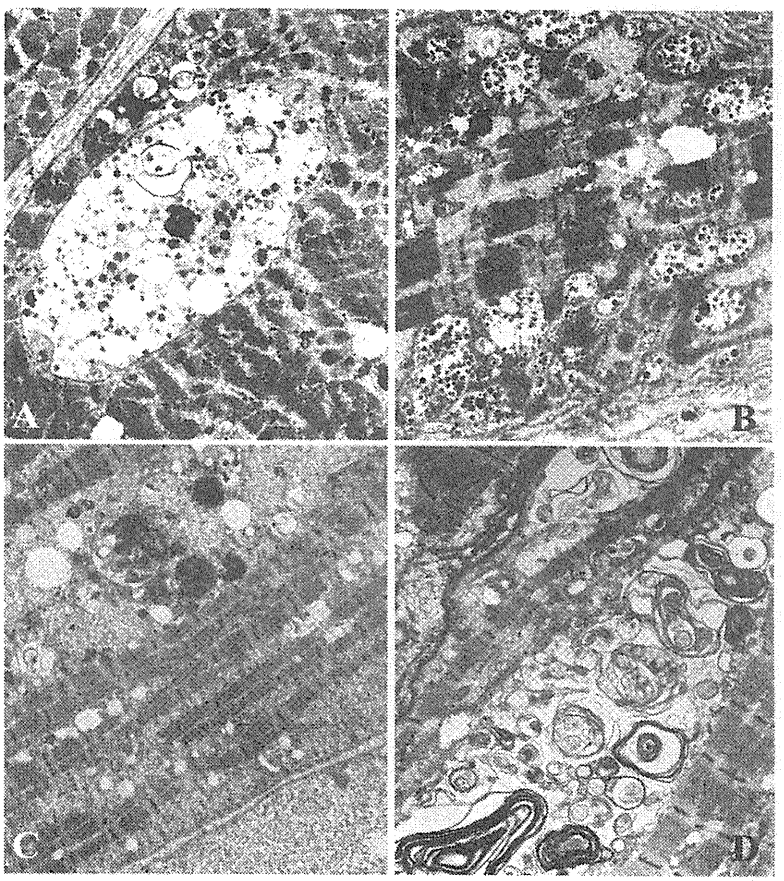


図1　AVM骨格筋の電子顕微鏡写真
A：ダノン病，B：XMEA，C：酸マルターゼ欠損症，D：DMRV

ATPaseはリソソーム内を酸性に保つためのプロトンポンプとして働いており，その機能低下によりリソソーム内のpHが上昇し，タンパク質の分解が低下する。つまり，XMEAではVMA21の変異により，リソソームの分解能が低下することで，オートファジーの機能異常がおこり病状を呈する。

XMEAの筋病理ではダノン病と同様に筋線維内にAVSFを認める。通常AVMの電子顕微鏡観察では，蓄積した自己貪食空胞内には分解前の細胞内小器官，分解された不定形の分解物のどちらかを認めるが，XMEAにおいては空胞内に電子密度の高い円い顆粒状の内容物が観察される（図1-B）。さらに，蓄積された空胞があたかもエキソサイトーシスにより細胞外に放出されているような像も観察される。また筋鞘膜部の基底膜が肥厚化し，自己貪食空胞内に見られたものと同様の細胞質分解産物の蓄積が観察される。これらの現象の意義はいまだ明らかになっていないが，この現象は，細胞が分解できずに蓄積した空胞を，エキソサイトーシスの経路に乗せかえること

により，細胞外に放出している可能性を推測させる。

3）酸マルターゼ欠損症

酸マルターゼ欠損症は糖原病II型として分類される常染色体劣性遺伝性の疾患である。酸マルターゼはグリコーゲン代謝に必須のリソソーム酵素であり，その機能不全によりリソソーム内外に未分解のグリコーゲンが異常に蓄積する（図1-C）。

酸マルターゼ欠損症は，臨床的に乳児型・小児型・成人型の3つに分類される。臨床的に最も重篤な症状を示す乳児型は，出生時より筋力の低下，筋緊張の低下と肥大型心筋症を呈し，心肺機能不全により2歳以前に死亡する。小児型は幼児期あるいは幼年期に呼吸筋の筋力低下として発症するが，乳児型とは異なり，通常，心臓の障害は認められない。また成人型は主として20歳以降に発症し，肢体筋の筋力低下・萎縮を伴う。小児型と同様に呼吸筋の低下が見受けられ，呼吸不全を初期症状とする。小児型と成人型はまとめて遅発型と呼ばれることもある。酸マルターゼ欠損による筋障害発症機構は明らかにはなっていないが，現在のところ,その機能不全によりリソソーム内さらには2次的に細胞質にグリコーゲンが蓄積し，それによりオートファジーが盛んにおこることで,筋線維を破壊し，筋障害にいたるのではないかと考えられている。

4）縁取り空胞型遠位型ミオパチー(DMRV)

DMRVは，第9染色体にある*GNE*遺伝子の主にミスセンス変異を原因とする常染色体劣性遺伝性疾患である。*GNE*はシアル酸生合成経路の律速酵素であり，また，高等生物ではこれが唯一のシアル酸合成経路であるため，*Gne*欠損マウスは胎生致死を示す。

DMRVは1981年に埜中らにより報告されたもので，遺伝性封入体ミオパチーもしくは，埜中ミオパチーとも呼ばれている。筋生検をすると，筋線維の中に細かい顆粒状の物質で縁取られた空胞(縁取り空胞：rimmed vacuole)が検出されるのが特徴的である。患者は10代後半～30代後半に掛けて発症し，主に，遠位筋の筋力低下と筋萎縮をきたし，10年程度で急速に歩行不能となる進行性の筋疾患である。先にも述べたように，筋病理所見として縁取り空胞を認めるが，この縁取り空胞は実際には染色操作の過程でできる人工産物である。電子顕微鏡観察では，筋線維内の縁取り空胞部分には，堆積したもろい自己貪食空胞とミエロイド小体が蓄積していることが確認される（図1-D）。

DMRVは*GNE*の機能喪失型変異により発症する。これまでの研究から，低シアル酸状態が各種の病理学的変化を引き起こしていることが明らかとなっている。また，本疾患では，縁取り空胞内部あるいは周辺部にβ-アミロイドが蓄積することが明らかになっている。これらの結果から，何らかの理由により，本来は分解されて低レベルでしか存在しないはずの異常タンパク質や基質が蓄積し，結果的にオートファジーの機能が惹起しているものと推測される。