Material and Methods

Cell lines. The EBV-negative Hodgkin’s lymphoma cell line L428 (1) and the lymphoblastoid cell lines LG2 (2), LCL721.221 (3), BM-LCL (a gift from Dr. Rajiv Khanna, Brisbane, Australia), ML-LCL, PJ-LCL, SL-LCL and the EBV+ Burkitt’s lymphoma cell line Ag876 (a gift from Dr. Rajiv Khanna, Brisbane, Australia) were cultured in RPMI-1640 + 10% FCS + 2 mM glutamine + 2 µg/ml Gentamycin. ML-LCL, PJ-LCL and SL-LCL were generated by culturing PBMCs of healthy donors with supernatant of the marmoset cell line B95-8 (4) with RPMI-1640 + 20% FCS + glutamine + gentamycin + 1 µg/ml Cyclosporin A. The stable EBNA1-transfectant L428E1PC5 (5) was derived from the EBV-negative Hodgkin cell line L428 and was maintained in RPMI-1640 + 10% FCS + glutamine + Gentamycin + 1 µg/ml Puromycin (Sigma-Aldrich, St. Louis, MO).

T cell clones. The EBNA1-specific CD4+ T cell clones A4.E116 and RJD.79 were generated as previously described in (6) and (7). Briefly, EBNA1-specific CD4+ T cell lines were obtained after stimulation of MACS-purified CD4+ T cells with irradiated autologous vvEBNA1ΔGA-infected or EBNA1514-527 peptide-pulsed autologous DCs for one week and one week restimulation with irradiated autologous rEBNA1-loaded DCs. The MS.B11 T cell clone was derived from an EBNA3A-specific T cell line, which was obtained from an HLA-B8+ donor by two weeks of stimulation with the autologous LCL, followed by magnetic separation with EBNA3A325-333/HLA-B8 tetramer-PE and αPE-MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany). T cells were cloned by limiting dilution at 10, 1, or 0.3 T cells/well and expanded in RPMI-1640 + 8% PHS +
150 U/ml rhIL-2 (Chiron, Emeryville, CA) + 1µg/ml PHA-L (Sigma-Aldrich, St. Louis, MO) + glutamine + gentamycin. 10^5 irradiated PBMCs/well and 2 x 10^3 - 2 x 10^4 irradiated LCLs/well were added as feeder cells. After 14 days, expanded cells were tested in split-well IFNγ-ELISPOT assays for peptide specificity and MHC-restriction and specific clones were expanded under the same conditions. The EBNA1-specific CD4+ T cell clone P3-B7 was a gift from Drs. Kui Shin Voo and Rong-Fu Wang, Houston, TX (8). A4.E116 recognizes the peptide EBNA1514-527 restricted by HLA-DR1. RJD.79 specifically recognizes peptide EBNA1481-501 in the context of HLA-DQ2/3. P3-B7 specifically recognizes peptide EBNA1519-532 in the context of HLA-DP3. Finally the control CD8+ T cell clone MS.B11 detects the peptide EBNA3A325-333 bound to HLA-B8 and the QIMR-WIL EBV strain transformed HLA-B8+ LCL BM-LCL. Peptides were pulsed on target cells at 1-10µM and 37°C for 1h in RPMI, followed by two washes.

Inhibitors, synthetic peptides and recombinant proteins. Ammonium chloride, chloroquine and 3-Methyladenine were purchased from Sigma, St. Louis, MO. Lactacystin was from Calbiochem, San Diego, CA. Peptides EBNA1481-501, EBNA1514-527 and EBNA3A325-333 were synthesized by the Fmoc solid phase method on a peptide synthesizer (model 432A, Applied Biosystems, Foster City, CA). Peptide purity and identity were confirmed by reverse phase HPLC (System Gold, Beckman, Palo Alto, CA) and mass spectrometry (LD-TOF G2025A, Hewlett-Packard, Palo Alto, CA). All peptides were a kind gift from Dr. Stefan Stevanovic, Tübingen, Germany. Lyophilized peptides were dissolved in 1% DMSO to a concentration of 1 mM.

Deconvolution microscopy. Cells were washed in RPMI, left to sediment on poly-lysine treated Carlson Scientific slides for 30 minutes in RPMI at 37°C and fixed in
4% paraformaldehyde/PBS/0.1% saponin (Sigma Aldrich) for 20 minutes at 4°C. The cells were then washed three times with blocking buffer from the TSA amplification kit (NEN Life Sciences, Boston, MA). For EBNA1 detection, the cells were blocked for 30 minutes at room temperature with blocking buffer and incubated with the 1H4 (9) or 5F12 (10) monoclonal antibodies in RPMI containing 0.1% saponin and 5% normal goat serum for two hours at 4°C. After three blocking buffer washes, the cells were incubated with HRP-conjugated goat anti-rat or goat anti-mouse antibody (Amersham Pharmacia, Uppsala, Sweden) in RPMI containing 0.1% saponin, for two hours at 4°C. The Cyanin-3 TSA amplification kit (NEN Life Sciences, Boston, MA) was used according to the manufacturer’s instructions. LAMP1 was detected with the mouse monoclonal antibody H4A3 (Southern Biotech, Birmingham, AL) and the 20S proteasome α/β subunits with polyclonal sheep IgG (Affiniti, Exeter, UK) followed by suitable secondary antibodies. Monodansylcadaverine was purchased from Sigma, St. Louis, MO, and staining was performed as described in (11). After labeling, all cells were washed three times and incubated for one minute with DAPI (Sigma-Aldrich). After three washes the cells were mounted using Aqua Polymount (Polysciences, Warrington, PA). The slides were analyzed using an Olympus deconvolution microscope. Pictures were taken with an Olympus digital camera and pictures were processed with Metamorph software (Universal Imaging Corporation).

Subcellular fractionation and immunoblotting. Subcellular fractionation was performed as previously described (12). 1-3x 10^7 L428E1PC5 or 10^8 LG2 cells were washed three times in cold PBS before resuspension in 10ml of ice cold homogenization buffer (10mM Tris-HCl pH 7.4, 250mM sucrose, 1mM DTT, 1mM EDTA, 30µg/ml
DNase, 0.1mM PMSF, 1µg/ml Leupeptin, 1µg/ml Pepstatin A) and homogenization in a 15ml Dounce homogenizer. Whole cells, cell debris, and nuclei were pelleted by two low speed spins (3,000g, 10min). Microsomes and mitochondria were pelleted by a subsequent high speed spin (100,000g, 1h). Pellets were resuspended in 1ml homogenization buffer and were loaded on top of a two-step sucrose gradient (2M, 0.5 M). After centrifugation at 100,000g for 1h, microsomes were collected from the interphase. Aliquots were taken from all fractions and were analyzed by SDS-PAGE / immunoblotting with the following antibodies: The mouse monoclonal antibody 5F12 (10), the anti-EBNA3A polyclonal rabbit antibody from Oncogene, San Diego, CA and the mouse monoclonal α-LAMP1 antibody from Southern Biotech, Birmingham, AL. Proteins were visualized with HRP-coupled secondary antibody and the ECLplus detection system (Amersham Biosciences, Buckinghamshire, UK).

Electron microscopy. L428 Hodgkin’s lymphoma cells, transfected with full-length EBNA1, were used for these studies. The cells were first synchronized with medium containing 100nM colchicine for 48 hours. After washing, lysosomal acidification was blocked in 50µM chloroquine-containing medium or medium alone, for another 48 hours. Then the cells were prepared for electron microscopy: the cells were plated on Alcian Blue coated petri dishes, Fixed in PLP fixative (periodate-lysine-paraformaldehyde fixative (13)), and permeabilized in 0.01% Saponin/0.1% BSA/PBS (Buffer A). For the EBNA1 and isotype control staining, the 5F12 anti-EBNA1 antibody or the isotype control antibody was diluted 1/50, goat anti-mouse-biotin (Hercules, CA) 1/300, and neutravidin-HRP (Molecular Probes, Leiden, Holland), in buffer A. After another fixation step in 0.5% glutaraldehyde, stable DAB was applied for 10 minutes, and
the cells were postfixed and stained in reduced Osmium tetroxide [1% OsO₄, 1%
K₄Fe(CN)₆]. Then the cells were dehydrated in a graded series of ethanol washes (70%,
95%, 100%), and removed from the petri dishes by adding propylene oxide and gently
pipeting. After rinsing the cells several times in propylene oxide to remove dissolved
plastic from the culture dishes, the cells were resuspended in Embed (Electron
Microscopy Sciences), microfuged into a pellet in the resin and allowed to polymerize in
a 60°C oven for 48 hours. Thin sections were cut in an Ultracut E and viewed and
photographed at 80kV in a JEOL100CXII electron microscope. The morphology studies
were carried out as follows: Cells were fixed in 2.5% Glutaraldehyde fixative and
microfuged into a pellet. After postfixing in 1% OsO₄ on ice and block staining in 0.5%
uranyl acetate, cells were dehydrated and processed into Embed as stated above. Sections
were post-stained with uranyl acetate and lead before viewing.

*IFNγ ELISPOT assay.* Enzyme-linked immunospot (ELISPOT) assays for IFNγ-
secreting cells were performed as described previously (14). Briefly, MAHA S45 plates
(Millipore) were coated with anti-IFNγ-antibody 1-D1K (Mabtech) overnight at 4°C.
Plates were blocked with RPMI + 5% pooled human serum. Afterwards, 10⁵ clonal T
cells and 5x10⁴ stimulator B cells were added per well and incubated overnight. Then
plates were incubated with biotinylated anti-IFNγ-antibody 7-B6-1 (Mabtech, Nacka,
Sweden). Afterwards, preassembled avidin-peroxidase complexes (Vectastain ABC kit,
Vecor Laboratories, Burlingame, CA) were added. Spots were developed by addition of
stable DAB (Research Genetics, Huntsville, AL). Plates were washed three times with
water and air-dried. SFC (spot forming cells) were counted using a stereomicroscope
(mean counts of duplicates).
**SiRNA-mediated gene silencing.** The following 21-nt siRNA oligos were used:

- **Atg12.1** sense: 5’-GUGGGCAGUAGAGCGAACAUdT, Atg12.1 antisense: 5’-UCAUGUAGUAGCAAGUUGA UdT (nt. 466-484 of gene bank entry NM_004707);
- **Atg12.2** sense: 5’-UCAA CUUGCUACUACUA CAUGA UdT; Atg12.2 antisense: 5’-UCAUGUAGUAGCAAGUUGA UdT (nt. 687-705 of NM_004707);
- **GFP** sense: 5’-CUUGAA GAAGUCGUGCUGCUGCUd T; GFP antisense: 5’-GCAGCACGACUUCUUC AAGUd T.

For delivery of siRNA duplexes into B-LCL cell lines, cells were washed in serum-free Opti-MEM medium and 5x10^6 cells in 300 µl Opti-MEM in a 2mm gene pulser cuvette were electroporated with 10 µM siRNA at 300 V and 150 µF using a Biorad gene pulser II.

**RT-PCR.** After 2-4 days total RNA was isolated using the RNeasy mini kit (Qiagen, Chatsworth, CA) and reverse transcription and PCR was carried out by using the OneStep RT-PCR kit from Qiagen. Different cycle numbers were used to amplify the PCR product and the lowest cycle number for which a band was detectable on an agarose gel was used to quantify the mRNA level.

**IFN\(\gamma\) ELISA assays.** The \(\alpha\)-IFN\(\gamma\) antibody 1-D1K (Mabtech Inc, Mariemont, OH) was coated overnight onto a 96-well Nunc-Immuno\textsuperscript{TM} MaxiSorp plates (Nalge Nunc Intl., Rochester, NY). For detection of secreted IFN\(\gamma\), culture supernatants were plated directly onto coated ELISA plates and IFN\(\gamma\) was detected with the biotinylated \(\alpha\)-IFN\(\gamma\) antibody 7-B6-1 (Mabtech) and Streptavidin-HRP, using the peroxidase substrate TMB (Sigma, St. Louis, MO). Recombinant human IFN\(\gamma\) diluted in culture medium (10,000 to 30pg/ml) was used as a standard.
*Flow cytometry.* HLA-DR surface levels were determined by staining with the FITC-conjugated αHLA-DR specific antibody Tü36 and its isotype IgG2b control (BD Pharmingen, San Diego, CA). Samples were analyzed on a FACScalibur instrument (Becton-Dickinson).

*Statistics.* Paired student T test statistics were performed where indicated.

**Supplemental references**

Supplemental figure legends:

**Supplemental figure 1.** EBNA1 containing cytosolic vesicles are degraded with fast kinetics. PJ-LCL were treated for two days with 20mM NH₄Cl. Inhibitors were removed and cells were fixed at the indicated timepoints. A: Cells were stained for EBNA1 and DNA content (DAPI). B: Percentage of cells containing cytosolic EBNA1 was quantified at the indicated timepoints by evaluating nuclear versus cytosolic EBNA1 localization in 100 cells per condition. One of two experiments is shown.

**Supplemental figure 2.** Upon inhibition of lysosomal acidification, EBNA1 colocalizes with an autophagy substrate, the proteasome. The lymphoblastoid cell line SL-LCL was stained for the 20S proteasome, DNA content (DAPI) and EBNA1 in the absence (-CQ) or presence (+CQ) of 20-100µM chloroquine. One of three experiments is shown.
Supplemental Figure 1
Supplemental Figure 2
Supplemental Table I: Natural MHC class I and II ligand protein sources differ in their half-lifes. The three most frequent cytosolic/nuclear MHC class II natural ligand sources (HSC70, HSP70 and GAPDH) are compared to the most-frequent cytosolic/nuclear MHC class I ligand source (cyclins). This analysis demonstrates that cytosol or nucleus derived, natural MHC class II ligands are most frequently derived from long-lived proteins, while cytosol or nucleus derived, natural MHC class I ligands are most frequently derived from short-lived proteins (data were compiled from the SYFPEITHI database of MHC ligands: www.syfpeithi.com).

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Supplemental references for table 1